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Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

O envolvimento de microRNAs no diabetes mellitus tipo 1 e na doença renal do diabetes

Taís Silveira Assmann

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Orientadora: Profa. Dra. Daisy Crispim Moreira

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“Seja lá o que for que você faça, empregue toda tua energia e todo teu espírito nesta tarefa. Ninguém conquista um sonho sem persegui-lo, ninguém anda uma milha sem dar o primeiro passo. Se ao fim da estrada alguma sombra de arrependimento te atacar, ainda assim, levante a cabeça, orgulhe-se por ter tentado, por ter buscado, por ter empregado todas as tuas forças até o último instante. Tanto pior e sempre pior é arrepender-se daquilo que você não fez”. *Augusto Branco*

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Esta tese de doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição da Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de uma breve introdução sobre o assunto, seguida dos manuscritos originais sobre o tema da tese.

- **Artigo 1:** "MicroRNA expression profile and type 1 diabetes mellitus: systematic review and bioinformatic analysis"

- **Artigo 2:** "MicroRNA expression profile in plasma of patients with type 1 diabetes: a case-control study and bioinformatic analysis"

- **Artigo 3:** "Polymorphisms in genes encoding miR-155 and miR-146a are associated with protection to type 1 diabetes mellitus"

- **Artigo 4:** "MicroRNA expression profile and diabetic kidney disease: systematic review and bioinformatic analysis"

- **Artigo 5:** "Circulating miRNAs in diabetic kidney disease: case-control study and *in silico* analyses"

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ÍNDICE DE ABREVIATURAS

1. Introdução

3'UTR	Região 3' não traduzida
CKD-EPI	*Chronic Kidney Disease Epidemiology Collaboration*
CTLA4	Antígeno 4 de linfócitos T citotóxico
DM	Diabetes mellitus
DM1	Diabetes mellitus tipo 1
DRC	Doença renal crônica
DRD	Doença renal do diabetes
DRCT	Doença renal crônica terminal
GAD65	Anticorpos para a descarboxilase do ácido glutâmico
HLA	Antígeno leucocitário humano
IA-2	Anticorpos para a fosfatase de tirosina 2
IA-2β	Anticorpos para a fosfatase de tirosina 2β
IAAs	Anticorpos para insulina
ICAs	Anticorpos para as células das ilhotas de Langerhans
IL-10	Interleucina-10
IL-1β	Interleucina-1β
KDIGO	*Kidney Disease Improving Global Outcomes*
MDRD	*Modification of Diet for Renal Disease*
MIN6	Linhagem celular de insulinoma de camundongo
miRNAs	microRNAs
miRSNP	Polimorfismo de troca única de nucleotídeo em genes de miRNAs
Pré-miRNA	microRNA precursor
Pri-miRNA	microRNA primário
RISC	Complexo de silenciamento induzido por RNA
RNAm	RNA mensageiro
SNP	Polimorfismo de troca única de nucleotídeo
TFG	Taxa de filtração glomerular
TFGe	Taxa de filtração glomerular estimada

TGF-β1	Fator de transformação do crescimento beta 1
TNF	Fator de necrose tumoral
ZnT8	Anticorpos para o transportador de zinco específico de células beta

2. Artigos

A549	Lung cancer cell line
AP-1	Activator protein 1
BCL2	B-cell lymphoma 2
BCL2l11	Bcl-2-like protein 11
BMI	Body mass index
cAMP-PKA	Cyclic adenosine monophosphate-protein kinase A
CBL	Casitas B-lineage lymphoma
cMyc	MYC proto-oncogene
DKD	Diabetic kidney disease
DM	Diabetes mellitus
DNMT1	DNA methyltransferase 1
DPP-4	Dipeptidyl peptidase-4
DUSP5	Dual specificity phosphatase 5
DUSP6	Dual specificity phosphatase 6
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
Erk	Extracellular regulated MAP kinase
ESRD	End-stage renal disease
EVs	Extracellular vesicles
FDR	False discovery rate
FoxO	Forkhead box O
FYN	Proto-oncogene tyrosine-protein kinase Fyn
GADD45α	Growth arrest and DNA-damage-inducible protein GADD45 alpha
GFR	Glomerular filtration rate
GO	Gene Ontology
HbA1c	Glycated hemoglobina
HLA	Human leukocyte antigen

HWE	Hardy-Weiberg equilibrium
IFNLR1	Interferon lambda receptor 1
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IGF2BP	Insulin-like growth factor 2 mRNA-binding proteins
IKKs	IκB kinases
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL2RB	Interleukin 2 receptor subunit beta
IL6R	Interleukin 6 receptor
IRAK-1	Interleukin-1 receptor-associated kinase 1
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
JAK2	Janus kinase 2
JC	Jaccard similarity coeficient
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LIFR	Leukemia inhibitory factor receptor
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCF-7	Human breast adenocarcinoma cell line
MCL1	Induced myeloid leukemia cell differentiation protein
MCs	Mouse mesenchymal cells
MDRD	Modification of Diet in Renal Disease
MeSH	Medical subject headings
MIAME	Minimum Information About a Microarray Experiment
MIN-6	Mouse insulinoma cell line
MIQE	Minimum Information for publication of Quantitative real-time PCR Experiments
miRNAs	microRNAs
mTOR	Mechanistic target of rapamycin
NF-κB	Nuclear factor kappa B
NOD	Non-obese diabetes mice

OR	Odds ratio
PAK4	p21 (RAC1) activated kinase 4
PBMCs	Peripheral blood mononuclear cells
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIAS	Protein inhibitor of activated STAT
PIK3R2	Phosphoinositide-3-kinase regulatory subunit 2
PTCs	Human proximal tubular cells
PTEN	Phosphatase and tensin homolog
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
qPCR	quantitative PCR
QUADAS-2	The Quality Assessment of Diagnostic Accuracy Studies-2
RA	Rheumatoid arthritis
Ras	Resistance to audiogenic seizures
RasGRP1	RAS guanyl releasing protein 1
sCR	Serum creatinine
SHP2	K-box region and MADS-box transcription factor family protein
SLE	Systemic lupus erythematosus
Smad	Mothers against dpp
Smad7	SMAD family member 7
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
SOCS1	Suppressor of cytokine signaling 1
SOS	Son of sevenless
SPRED1	Sprouty related EVH1 domain containing 1
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
STREGA	Strengthening the reporting of genetic association studies
STROBE	StrengThening the Reporting of OBservational studies in Epidemiology
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCR	T cell receptor
TECs	Tubular epithelial cells

TGF-β1	Transforming growth factor beta 1
Th1	T helper cells 1
Th2	T helper cells 2
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRAF-6	TNF receptor associated factor 6
Tregs	Regulatory T cells
UACR	Urinary albumin to creatinine rate
UAE	Urinary albumin excretion
UUO	Unilateral ureteral obstruction
VAMP2	Vesicle associated membrane protein 2
VCAM-1	Vascular cell adhesion molecule 1
VEGFA	Vascular endothelial growth factor A
Zeb1	Zinc Finger E-Box Binding Homeobox 1
Zeb2	Zinc Finger E-Box Binding Homeobox 2

RESUMO

Os microRNAs (miRNAs) são pequenas moléculas de RNA que regulam a expressão gênica e, por isso, estão envolvidos em vários processos biológicos e patológicos. Evidências sugerem que os miRNAs possuem um papel tanto no sistema imune quanto na proliferação, metabolismo e morte das células beta pancreáticas, os quais são processos envolvidos na patogênese do diabetes mellitus tipo 1 (DM1). Diversos estudos investigaram se perfis de expressão de miRNAs estão associados ao DM1; no entanto, seus resultados são ainda inconclusivos. Sendo assim, realizou-se uma revisão sistemática e um estudo caso-controle, seguidos de análises de bioinformática, com o objetivo de se identificar um perfil de expressão de miRNAs em pacientes com DM1.

A revisão sistemática incluiu 33 estudos que investigaram a expressão de miRNAs em indivíduos com e sem DM1 e em modelos animais dessa doença. Em geral, 11 miRNAs circulantes no sangue estavam consistentemente desregulados em pacientes com DM1 comparado aos indivíduos controles. Desses 11 miRNAs, miR-146a-5p, miR-150-5p, miR-342-3p e miR-1275 estavam menos expressos em pacientes com DM1 comparado aos controles; enquanto miR-21-5p, miR-24-3p, miR-100-5p, miR-148a-3p, miR-181a-5p, miR-210-3p e miR-375 estavam mais expressos em pacientes com DM1. Além disso, análises *in silico* demonstraram que esses 11 miRNAs estão envolvidos em vias relacionadas a funções do sistema imune, apoptoso e biossíntese de insulina, sugerindo que esses miRNAs podem ter um papel importante na patogênese do DM1.

No estudo caso-controle, a expressão de 48 miRNAs foi analisada no plasma de 33 pacientes com DM1 (casos) e de 26 indivíduos não-diabéticos (controles),

utilizando-se a técnica de *macroarray*. Em seguida, cinco miRNAs diferencialmente expressos entre casos e controles foram selecionados para a análise de validação dos resultados de expressão, em uma amostra independente (27 casos e 14 controles), utilizando-se RT-qPCR. Como resultado da análise de *macroarray*, nove miRNAs foram diferencialmente expressos em pacientes com diagnóstico recente de DM1 (<5 anos de diagnóstico) comparado com pacientes diabéticos com ≥5 anos de diagnóstico e indivíduos controles. Nenhuma diferença foi observada entre pacientes com DM1 com ≥5 anos de diagnóstico e o grupo controle. Além disso, a expressão aumentada dos miR-103a-3p, miR-155-5p, miR-200a-3p e miR-210-3p, bem como a expressão diminuída do miR-146a-5p nos pacientes com DM1 de diagnóstico recente comparado aos outros dois grupos foram confirmadas utilizando-se RT-qPCR. As análises de bioinformática evidenciaram que esses cinco miRNAs regulam genes de vias associadas ao DM1, como sistema imune inato, MAPK, apoptose e insulina.

Além dos estudos de expressão de miRNAs, evidências recentes demonstraram que polimorfismos em genes codificantes de miRNAs podem alterar a biogênese dos miRNAs, bem como suas ligações aos RNAm alvos, conferindo, assim, suscetibilidade para algumas doenças. Sendo assim, as frequências dos polimorfismos rs2910164 no miR-146a, rs767649 no miR-155 e rs6715345 no miR-375 foram investigadas em 490 pacientes com DM1 e em 469 indivíduos não-diabéticos. Os alelos mais raros dos polimorfismos rs2910164 no miR-146a e rs767649 no miR-155 foram menos frequentes em pacientes com DM1 do que nos controles. A associação desses alelos com proteção para o DM1 foi confirmada no modelo de herança dominante [polimorfismo rs2910164: Razão de chances (RC) = 0,56 (IC 95% 0,36 – 0,87); polimorfismo rs767649: RC = 0,51 (IC 95% 0,26 – 0,97)], ajustando-se para idade, etnia e haplótipo HLA DR/DQ de alto risco para o DM1. As frequências alélicas e

genotípicas do polimorfismo rs6715345 no miR-375 não diferiram entre casos e controles.

Além dos miRNAs estarem envolvidos na patogênese do DM1, diversos estudos demonstraram que a expressão desregulada de miRNAs também pode estar envolvida nas complicações crônicas do DM1, como a doença renal do diabetes (DRD); entretanto, os resultados em relação à DRD também são inconclusivos. A fim de proporcionar um melhor entendimento sobre o papel dos miRNAs na DRD, realizamos uma revisão sistemática e um estudo caso-controle, seguidos de análises *in silico*, visando a identificação de um perfil de expressão de miRNAs associados com essa complicação.

A revisão sistemática incluiu 27 estudos que investigaram a expressão de miRNAs em pacientes com DRD e em indivíduos controles. Como resultado, seis miRNAs estavam consistentemente desregulados em pacientes com diferentes graus de DRD comparados aos controles (pacientes com DM1 sem DRD e/ou indivíduos saudáveis). Desses seis miRNAs, miR-21-5p, miR-29a-3p, miR-126-3p, miR-214-3p e miR-342-3p estavam aumentados, enquanto o miR-192-5p estava diminuído em pacientes com DRD comparado aos controles. A análise de bioinformática evidenciou que esses miRNAs regulam genes que participam de vias como apoptose, fibrose e acúmulo de proteínas na matriz extracelular, demonstrando que eles podem ter um papel importante na patogênese da DRD.

No estudo caso-controle, investigamos a expressão de 48 miRNAs no plasma de 23 pacientes >10 anos de DM1 (controles DM1), 35 pacientes com diferentes graus de DRD (casos) e 10 indivíduos saudáveis, através de análise de *macroarray*. Posteriormente, cinco miRNAs desregulados nos casos foram escolhidos para validação em uma amostra independente (10 controles com DM1, 19 casos e 10 indivíduos

saudáveis), utilizando-se RT-qPCR. Como resultado do *macroarray*, nove miRNAs foram diferencialmente expressos em pacientes com DRD comparados aos controles DM1. A expressão aumentada dos miR-21-3p e miR-378a-5p e a expressão diminuída dos miR-16-5p e miR-29a-3p nos pacientes com DRD comparado aos controles DM1 foi confirmada na amostra de validação. Não foi confirmada a associação do miR-503-5p com DM1. A análise *in silico* demonstrou que os cinco miRNAs selecionados para a validação regulam genes das vias PI3K/Akt, longevidade, TGF-β1 e relaxina, o que indica que esses miRNAs podem ter um papel importante na patogênese da DRD.

ABSTRACT

MicroRNAs (miRNAs) are small RNA molecules that regulate gene expression; consequently they are involved in several biological and pathological processes. Growing evidence suggests that miRNAs play a key role in immune system functions as well as in pancreatic beta-cell metabolism, proliferation and death, which are processes involved in type 1 diabetes mellitus (T1DM) pathogenesis. Several studies have investigated if miRNA expression profiles are associated with T1DM; however, their results are still inconclusive. Thus, we performed a systematic review and a case-control study, both followed by bioinformatic analysis, aiming to identify a miRNA expression profile associated with T1DM.

The systematic review included 33 studies that analyzed miRNA expressions in T1DM patients (cases) and nondiabetic subjects (controls) or in murine models of this disease. In general, 11 circulating miRNAs were consistently dysregulated in cases compared to controls. Among these 11 miRNAs, miR-146a-5p, miR-150-5p, miR-342-3p e miR-1275 were downregulated in T1DM patients compared to controls; while, miR-21-5p, miR-24-3p, miR-100-5p, miR-148a-3p, miR-181a-5p, miR-210-3p e miR-375 were upregulated in T1DM patients. Moreover, *in silico* analysis showed that these 11 miRNAs are involved in pathways related to the imune system, apoptosis and insulin biosynthesis, suggesting that these miRNAs could be involved in T1DM pathogenesis.

In the case-control study, expressions of 48 miRNAs were analyzed in plasma of 33 T1DM patients (cases) and 26 age- and gender-matched nondiabetic subjects (controls), using a macroarray technique. After that, five differently expressed miRNAs between cases and controls were selected for validation of their results in an independente sample (27 cases and 14 controls), using RT-qPCR. As result of the

macroarray analysis, nine miRNAs were diferently expressed in plasma of recently-diagnosed T1DM patients (< 5 years of diagnosis) compared to T1DM patients with ≥ 5 years of diagnosis and controls. No differences in miRNA expressions were detected between controls and T1DM patients with ≥ 5 years of diagnosis. Moreover, miR-103a-3p, miR-155-5p, miR-200a-3p, and miR-210-3p were confirmed as being upregulated in recently-diagnosed T1DM patients compared to the other two groups, using RT-qPCR. In the same way, miR-146a-5p was confirmed as being downregulated in recently-diagnosed T1DM patients compared to the other groups. Bioinformatic analysis demonstrated that these five miRNAs regulate several genes from pathways associated with T1DM, such as innate immune system-, MAPK-, apoptosis-, -and insulin pathways.

In addition to the miRNA expression studies, recent evidence has shown that polymorphisms in genes codifying miRNAs may alter their biogenesis as well as their binding to the corresponding mRNAs; thus, confering susceptibility for a given disease. Therefore, frequencies of miR-146a rs2910164, miR-155 rs767649 and miR-375 rs6715345 polymorphisms were analyzed in 490 T1DM patients and 469 nondiabetic subjects. Frequencies of the minor alleles of miR-146a rs2910164 and miR-155 rs767649 polymorphisms were lower in T1DM patients compared to nondiabetic subjects. Their association with T1DM protection was confirmed for the dominant model of inheritance [rs2910164 polymorphism: Odds ratio (OR) = 0.557, 95% CI 0.355 – 0.874; rs767649 polymorphism: OR = 0.508, 95% CI 0.265 – 0.973], after adjustment for age, ethnicity, and T1DM high-risk HLA DR/DQ haplotypes. MiR-375 rs6715345 allele and genotype frequencies did not differ between cases and controls.

In addition to the miRNAs involved in T1DM pathogenesis, a number of studies have shown that dysregulated miRNAs expressions may also be involved in chronic

complications of T1DM, such as diabetic kidney disease (DKD); however, the results in relation to DKD are also inconclusive. In order to provide a better understanding about the relantionship between miRNAs and DKD, we performed a systematic review and a case-control study, both followed by in silico analysis, aiming to identify a miRNA expression profile associated with this chronic complication.

The systematic review included 27 studies that compared miRNA expressions between patients with DKD (cases) and control subjects. As result, six miRNAs were consistently dysregulated in patients with different stages of DKD compared to controls (T1DM patients without DKD or healthy individuals). Among these six miRNAs, miR-21-5p, miR-29a-3p, miR-126-3p, miR-214-3p, and miR-342-3p were upregulated, while, miR-192-5p was downregulated in DKD cases compared to controls. Bioinformatic analysis indicated that these six miRNAs regulate genes involved in pathways related to DKD pathogenesis, such as apoptosis, fibrosis, and extracellular matrix accumulation.

In the case-control study, we analyzed expressions of 48 miRNAs in plasma of 23 patients with > 10 years of T1DM diagnosis (T1DM controls), 35 patients with diferent stages of DKD and 10 healthy subjects using macroarray analysis. Five dysregulated miRNAs in T1DM cases were then selected for validation in an independent sample (10 T1DM controls, 19 DKD cases, and 10 healthy subjects), using RT-qPCR. As result, nine miRNAs were differentially expressed between DKD cases and T1DM controls. After validation, miR-21-3p and miR-378-3p were confirmed as being upregulated, while miR-16-5p and miR-29a-3p were confirmed as being downregulated in DKD cases compared to T1DM controls. The association between miR-503-5p expression and T1DM was not confirmed. The in silico analysis indicated that the five miRNAs choosen for validation regulate genes from PI3K/Akt, longevity,

TGF-β1, and relaxin signaling pathways, reinforcing that these miRNAs have a role in DKD pathogenesis.

1. INTRODUÇÃO

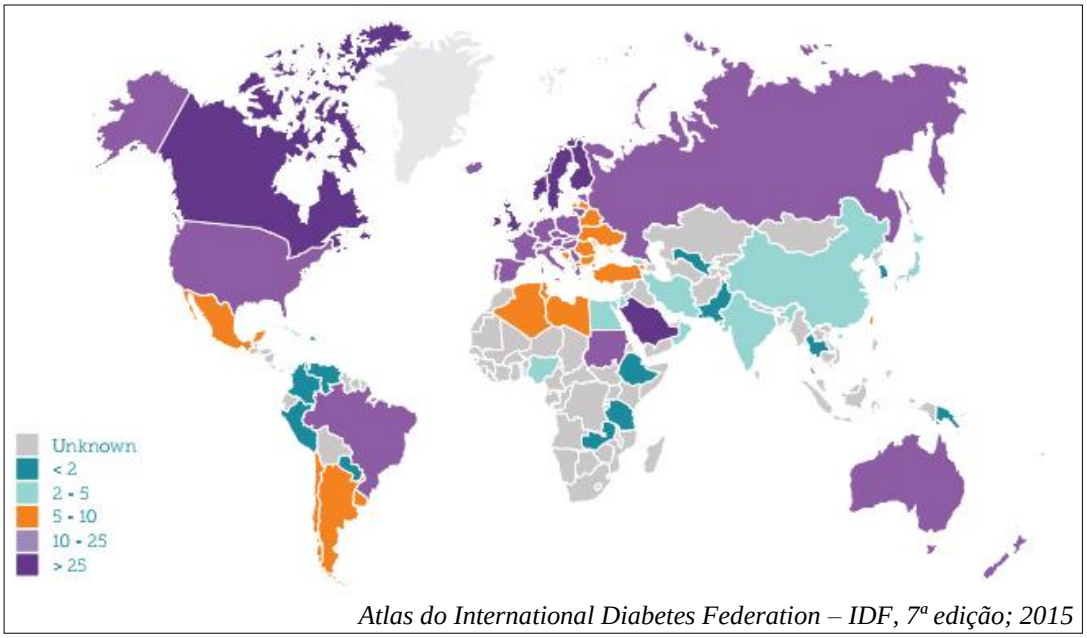
1.1. Diabetes mellitus tipo 1

O diabetes mellitus tipo 1 (DM1) é uma doença crônica caracterizada pela destruição autoimune das células beta das ilhotas de Langerhans, resultando na incapacidade progressiva de produzir insulina (1). A destruição das células beta pode levar meses ou anos, mas, em geral, o DM1 é clinicamente detectado após a destruição de mais de 80% dessas células nas ilhotas pancreáticas (2). Inúmeros fatores genéticos, epigenéticos e ambientais contribuem para a ativação imunológica que desencadeia a destruição das células beta (3, 4).

A natureza autoimune do DM1 é evidenciada pela presença de células T autorreativas e autoanticorpos para a insulina (IAAs), para as células das ilhotas de Langerhans (ICAs), para a descarboxilase do ácido glutâmico (GAD65), para o transportador de zinco específico de células beta (ZnT8) e para as fosfatases de tirosina (IA-2 e IA-2β) (5, 6). Estima-se que entre 85 e 90% dos pacientes com DM1 apresentam um ou mais desses anticorpos no momento do diagnóstico (5). Além disso, esses anticorpos podem estar presentes em alguns indivíduos por décadas antes do desenvolvimento da doença (5, 6). Porém, o conhecimento a respeito dos mecanismos que desencadeiam a inflamação e morte das células beta ainda é limitado. Algumas evidências indicam que as células imunes infiltradas nas ilhotas de pacientes recém-diagnosticados são, predominantemente, linfócitos CD8, células T CD4, células B e macrófagos, implicando na ativação tanto da resposta imune inata quanto da adaptativa, o que culmina na morte das células beta (3, 5, 6).

A incidência de DM1 está aumentando na maioria dos países, especialmente em crianças com idade inferior a 15 anos (**Figura 1**). Por mais que exista grande diferença

na incidência de acordo com a região geográfica, o crescimento anual do DM1 em todo o mundo é em torno de 3%. O país com maior número absoluto de crianças com DM1 é o Estados Unidos, com 84.100 crianças afetadas, seguido pela Índia com 70.200 crianças. O Brasil encontra-se em terceiro lugar nesse ranking com 30.900 crianças diagnosticadas com DM1 no ano de 2015 (7).



Atlas do International Diabetes Federation – IDF, 7ª edição; 2015

Figura 1. **Número de novos casos de DM1 (por 100.000 crianças de 0 a 14 anos, por ano).** Dados da *International Diabetes Federation*, 2015.

1.2. Doença renal do diabetes

A hiperglicemia crônica pode provocar lesões estruturais no endotélio vascular e no tecido nervoso que causam danos, disfunções e falhas de diversos órgãos e tecidos, levando ao aparecimento das complicações crônicas do DM (3). Essas complicações podem ser categorizadas em microvasculares [retinopatia diabética (RD), doença renal do diabetes (DRD) e neuropatia periférica (NP)] ou macrovasculares (infarto agudo do miocárdio, acidente vascular cerebral e gangrena) (3). Dentre as complicações microvasculares, a DRD é a causa mais frequente de falência e necessidade de transplante de rins em vários países (8).

Embora a melhora do controle glicêmico reduza o desenvolvimento das complicações microvasculares do diabetes, como a DRD, a incidência dessas complicações continua crescendo no mundo todo (9). Atualmente, a DRD é a principal causa de doença renal crônica (DRC) e doença renal crônica terminal (DRCT), sendo um grande preditor de mortalidade em pacientes diabéticos (10, 11). Ainda, a DRD é a principal responsável por transplantes renais em diversos países (10). Nos Estados Unidos, cerca de 200.000 pacientes são tratados para DRCT por apresentarem DRD, sendo que, a cada ano, 50.000 novos pacientes iniciam diálise (11). Pacientes com DRD que apresentam DRCT se deparam com uma taxa de mortalidade de 20% após o primeiro ano de diálise, o que é maior que a taxa de mortalidade conhecida para muitos cânceres de órgãos sólidos, como os cânceres de próstata e de mama (11).

A DRD é definida como anormalidades estruturais ou funcionais dos rins, por pelo menos três meses, manifestada por dano no tecido renal ou por taxa de filtração glomerular (TFG) diminuída (< 60 ml/min/1,73m²). O dano renal pode ser expresso por proteinúria, dano tubular, alterações na imagem ou necessidade de transplante. Segundo o *Kidney Disease Improving Global Outcomes* (KDIGO), a classificação dos estágios da DRC deve se basear na excreção urinária de albumina (EUA) e TFG (**Figura 2**).

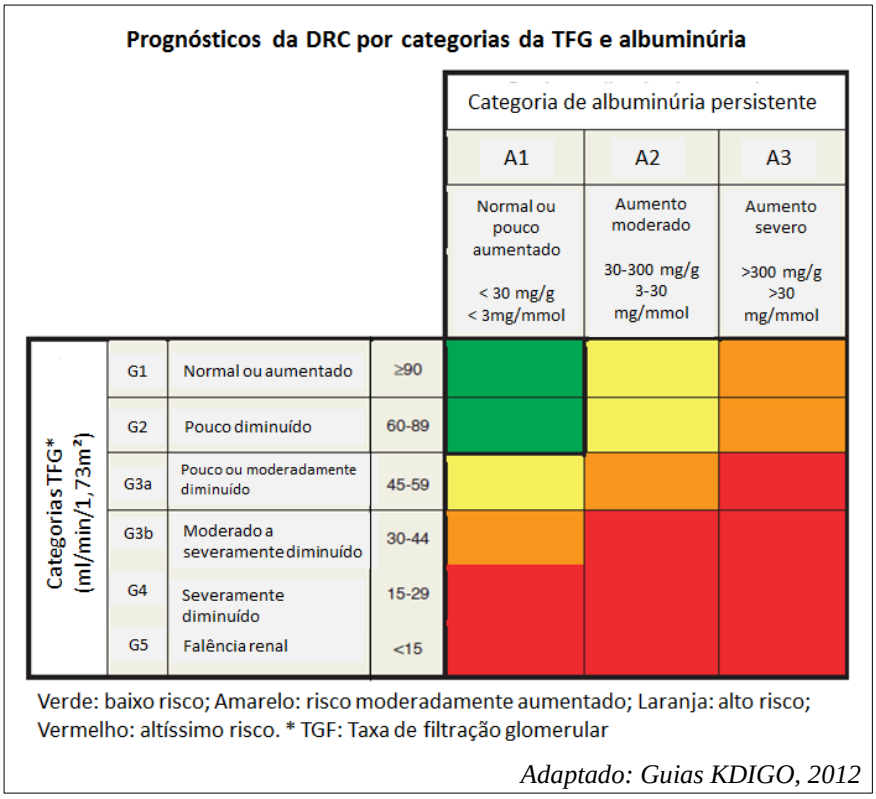


Figura 2. Classificação da Doença Renal Crônica segundo a KDIGO, 2012.

A TFG estimada (TFGe) é amplamente utilizada para indicar a função renal (11, 12). Existem diversas maneiras de estimarmos a TFG, no entanto, atualmente duas fórmulas matemáticas são as mais utilizadas: 1) fórmula MDRD (*Modification of Diet for Renal Disease*) (13) e 2) fórmula CKD-EPI (*Chronic Kidney Disease Epidemiology Collaboration*) (14).

A equação MDRD (13) para estimativa da TFG foi originalmente desenvolvida em pacientes com DRC e não incluía indivíduos saudáveis. A TFG calculada pela equação do MDRD e a TFG real são muito próximas para resultados < 60 mL/min/1,73 m^2, enquanto a TFG excede a taxa estimada por um valor pequeno quando a TFG é ≥ 60 mL/min/1,73 m^2. A equação MDRD utiliza como parâmetros na fórmula: níveis de creatinina sérica, gênero, idade e etnia.

A equação CKD-EPI (14) utiliza as mesmas quatro variáveis que a equação do MDRD; no entanto, foi validada em uma coorte que compreendia indivíduos saudáveis

e indivíduos com DRC, e, por isso, apresenta melhor desempenho e previsão de desfechos adversos. As observações de menor viés e maior acurácia da equação CKD-EPI em comparação à equação MDRD, particularmente nas faixas de TFG ≥ 60 mL/min/1,73 m^2, constituem o racional para preconizar o seu uso clínico em substituição às equações de estimativa da TFG até então utilizadas (15).

Entretanto, a TFGe indica apenas função renal, mas não dano renal. O diagnóstico histopatológico é considerado o padrão-ouro; entretanto, é pouco utilizado na prática clínica. As principais alterações fisiopatológicas da DRD estão demonstradas na **Figura 3**. A DRD inicia com espessamento da membrana basal glomerular e aumento da deposição de colágeno por células mesangiais na matriz extracelular, obliteração de pedicelos secundários e diminuição da quantidade de podócitos. Esses fatores contribuem para que ocorra excreção de proteínas de alto peso molecular, como a albumina. Além disso, outras regiões do rim são afetadas, como, por exemplo, no túbulo proximal ocorre atrofia do epitélio tubular com perda de microvilosidades, diminuição da quantidade de capilares e infiltração inflamatória.

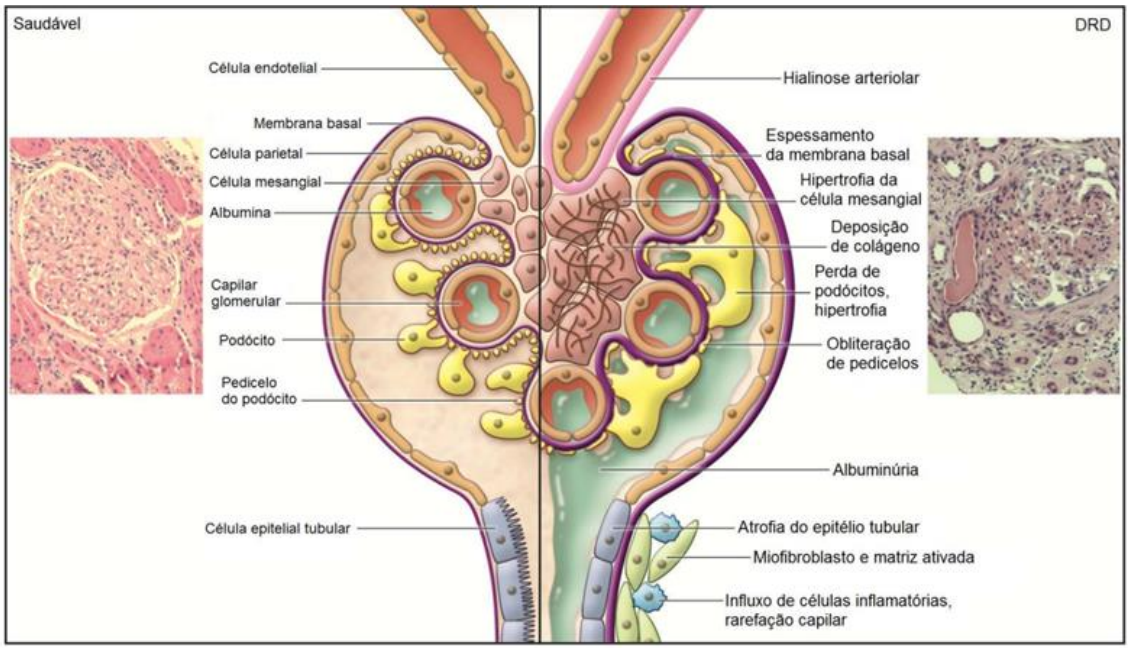


Figura 3. Lesões histopatológicas da Doença Renal do Diabetes. O glomérulo de um paciente saudável inclui arteríola aferente, capilares glomerulares, células endoteliais, membrana basal, podócitos, células epiteliais parietais, células túbulo-epiteliais e é impermeável à albumina. Em contraste, o glomérulo de um paciente com diabetes apresenta hialinose arteriolar, expansão mesangial, deposição de colágeno, espessamento da membrana basal, perda e hipertrofia de podócitos, albuminúria, atrofia do epitélio tubular, acúmulo de matriz e miofibroblastos ativados, influxo de células inflamatórias e rarefação de capilares. Também é mostrado tecido renal de glomérulo saudável e de paciente com DRD (corado com ácido periódico de Schiff). Adaptado de Reidy e colaboradores (11).

1.3. MicroRNAs

Os microRNAs (miRNAs) são uma classe de pequenos RNAs de 19–25 nucleotídeos, não codificadores de proteínas, que agem como potentes reguladores pós-transcricionais da expressão gênica em plantas e animais (16). Lin4 (do inglês *lineage-deficient-4*) foi descoberto em 1993 como o primeiro miRNA, sendo nesta época associado à regulação do desenvolvimento larval em *Caenorhabditis elegans* (17).

Os miRNAs exercem seus efeitos regulatórios ligando-se principalmente à região 3’ não traduzida (3’UTR) de RNAs mensageiros (RNAm) alvos, levando à

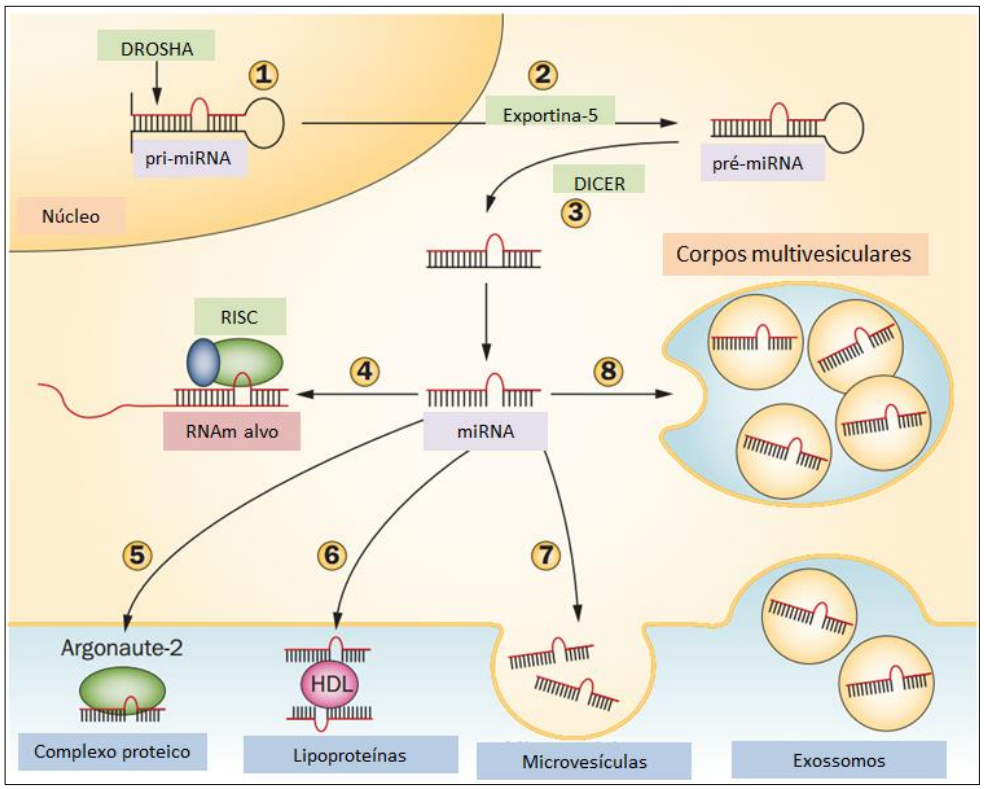
repressão da tradução e ou à degradação do RNAm (18, 19). Além disso, estima-se que os miRNAs regulam a expressão de 60% dos genes codificantes de proteínas, consequentemente controlando diversos processos biológicos e patológicos (18, 20). Interessantemente, alguns estudos demonstraram que os miRNAs podem ser transportados de célula para célula, bem como circular de forma estável nos fluidos biológicos; coordenando a expressão gênica em células vizinhas ou em células distantes. A transferência dos miRNAs entre células distantes pode constituir uma nova forma de comunicação célula-célula (20). Além disso, a expressão dos miRNAs nos fluidos biológicos geralmente reflete um dano tecidual específico, fazendo deles candidatos ideais a biomarcadores circulantes (21).

Há evidência de que a especificidade do miRNA está ligada principalmente a uma pequena sequência de nucleotídeos, de aproximadamente 8 pares de bases, chamada de *seed sequence*. A variação significativa no grau de complementariedade dessas sequências permite que um único miRNA se ligue a diversos RNAm, e, da mesma forma, cada RNAm pode ser regulado por vários miRNAs (16, 22).

Mais de 2.400 miRNAs já foram descritos em humanos (23). Os estudos de associação entre a expressão de miRNAs e patologias humanas são mais frequentes na área de oncologia (24, 25), enquanto o padrão de expressão de miRNAs em outras doenças, especialmente no diabetes e suas complicações crônicas, começaram a ser objeto de estudo no últimos anos (26-28).

Os miRNAs são transcritos primeiramente pela RNA polimerase II em miRNAs primários (pri-miRs) no núcleo. Os pri-miRs são relativamente longos e podem conter uma ou mais estruturas em forma de grampo. Um complexo formado pela ribonuclease DROSHA associada à proteína DGCR8 processa o pri-miR em miRNA precursor (pré-miRNA), que possui uma estrutura secundária em forma de grampo de

aproximadamente 70 nucleotídeos. Posteriormente, esse pré-miR é exportado para o citoplasma, pela enzima exportina-5, onde é reconhecido e clivado pela enzima DICER e seus cofatores, gerando a molécula madura de miRNA, composta de cerca de 22 nucleotídeos. Após, o complexo de silenciamento induzido por RNA (RISC) direciona o miRNA maduro até o RNAm alvo (**Figura 4**). O reconhecimento do alvo pelo miRNA ocorre na região 3' do RNAm alvo, devido à complementariedade com a *seed sequence* do miRNA. Dependendo do grau de complementariedade, o RNAm alvo é clivado ou sua tradução é inibida, ou ainda, é direcionado para a degradação nos corpúsculos P (16, 20, 22).



<u>Figura 4</u>. Biogênese e liberação dos microRNAs. No núcleo, os microRNAs primários (pri-miRNAs) são clivados pela enzima ribonuclease DROSHA, gerando os pré-miRNAs (1). Os pré-miRNAs são transportados para o citoplasma pela enzima exportina-5 (2) e clivados pela enzima DICER, formando um miRNA duplex (3). Uma das fitas do duplex de miRNA maduro irá se associar com o complexo RISC e guiar a repressão da tradução de RNAm alvos (4) ou será liberado pela célula. Quando o miRNA maduro é liberado pela célula, ele pode se ligar a proteínas, como a argonauta-2

(5), ou a lipoproteínas, como a HDL (6). Alternativamente, os miRNAs podem ser liberados em microvesículas formadas pela membrana plasmática (7) ou em exossomos que são liberados no espaço extracelular por meio de exocitose ou fusão de corpos multivesiculares com a membrana plasmática (8). Adaptado de Guay e colaboradores (20).

Considerando que os miRNAs podem modular processos fisiológicos e a patofisiologia de diversas doenças, é reconhecido que existe a necessidade de se identificar os miRNAs (e seus alvos) associados com o DM1 e suas complicações crônicas, pois isto propiciaria a identificação de novos marcadores para o diagnóstico e prognóstico, bem como poderia sugerir novos alvos terapêuticos (20, 26-28). Além da sua função intracelular, estudos recentes demonstraram que os miRNAs podem ser exportados ou liberados pelas células na circulação em uma forma estável. A descoberta dos miRNAs circulantes sugeriu a possibilidade de utilização dos padrões de expressão destas moléculas como biomarcadores, avaliados de forma não invasiva, para a detecção precoce do DM1 e suas complicações crônicas, o que poderia auxiliar no manejo clínico dos desfechos de longo prazo (20, 27, 29, 30).

1.3.1 MicroRNAs e diabetes mellitus tipo 1

Evidências sugerem que os miRNAs possuem um papel tanto no sistema imune quanto na proliferação, metabolismo e morte das células beta, os quais são processos envolvidos na patogênese do DM1 (19, 20, 31). Ainda citocinas pró-inflamatórias como IL-1β e TNF podem induzir a expressão de diversos miRNAs na linhagem celular MIN6 e em ilhotas pancreáticas humanas (32), sugerindo que esses miRNAs podem estar envolvidos na destruição das células beta mediada por citocinas.

Um estudo indicou que o miR-342 e o miR-191 são menos expressos, enquanto o miR-510 é mais expresso em células Treg de pacientes com DM1 (33). Nielsen e

colaboradores investigaram os níveis de expressão dos miRNAs no soro de crianças e adolescentes com DM1 recém-diagnosticado e demonstraram que 12 miRNAs estavam diferencialmente expressos nesses pacientes: miR-24, miR-25, miR-26a, miR-27a, miR-27b, miR-29a, miR-30a-5p, miR-148a, miR-152, miR-181a, miR-200a e miR-210. Osipova e colaboradores relataram que o miR-21 e 0 miR-210 estavam aumentados tanto no plasma quanto na urina de crianças com DM1, enquanto que o miR-126 estava diminuído apenas na urina dos pacientes quando comparado com crianças saudáveis (34). Diversos outros estudos investigaram perfis de expressão em pacientes com DM1; entretanto, os resultados são ainda inconclusivos (21, 35-41).

Além disso, alguns miRNAs parecem modular a expressão dos autoantígenos associados ao DM1 (38, 42) ou predizer a função residual das células beta após o diagnóstico do DM1 (43). A expressão do miR-24-3p, miR-146a-5p, miR-194-5p, miR-197-3p, miR-301a-3p e miR-375 foi correlacionada com a função residual da célula beta após 3 meses de diagnóstico do DM1. Além disso, os níveis de peptídeo C estimulado em 12 meses após o diagnóstico foram preditos pela expressão do miR-197-3p no terceiro mês após o diagnóstico (37). Snowhite e colaboradores identificaram quinze miRNAs diferencialmente expressos em irmãos com autoanticorpos positivos comparados com irmãos com autoanticorpos negativos. Adicionalmente, sete miRNAs foram diferencialmente expressos entre pacientes com autoanticorpos positivos de acordo com a progressão da doença, identificando um perfil de miRNAs associados com o desenvolvimento do DM1 (36).

1.2.2 MicroRNAs e a doença renal do diabetes

Atualmente, os marcadores laboratoriais da DRD não são capazes de prever quais os pacientes com DM possuem risco de desenvolver essa complicação antes do aparecimento do dano renal. Por isso, a descoberta de novos biomarcadores se faz

necessária para identificar pacientes com maior risco de desenvolver a DRD. Um tratamento precoce nesse grupo de pacientes poderia ajudar a retardar ou prevenir o início dessa complicação, bem como a progressão para DRCT (44). Nesse contexto, estudos recentes têm demonstrado que os miRNAs são mediadores chave na patogênese da DRD, sugerindo que miRNAs circulantes podem ser utilizados como biomarcadores para prever o desenvolvimento e prognóstico da DRD (44-47). A detecção de miRNAs circulantes oferecem um método fácil, rápido e potencialmente automatizado para o diagnóstico da DRD em comparação à biópsia renal.

Além disso, os miRNAs podem ser induzidos em células renais *in vivo* e *in vitro* em condições hiperglicêmicas e podem promover a acumulação de proteínas de matriz extracelular relacionadas à fibrose e à disfunção glomerular (46). De acordo com o papel dos miRNAs na fibrose renal, vários miRNAs circulantes no sangue ou na urina foram relatados como desregulados em estádios específicos da DRD (30, 48-55) (28, 49, 50, 53, 57-61); entretanto, os resultados são ainda inconclusivos visto que a maioria dos resultados não foi reproduzida em outras populações.

1.4. Polimorfismos em genes codificantes de microRNAs

A variação genética responsável pela predisposição para o DM1 ainda é desconhecida para a maioria dos *loci* gênicos associados com a patologia e suas complicações crônicas. Até o momento, mais de 50 genes foram associados com o DM1, sendo o *HLA* classe II DR/DQ o que possui maior impacto na suscetibilidade à doença (56). Outros *loci* possuem menor impacto na suscetibilidade ao DM1; no entanto, a combinação dos haplótipos *HLA* e polimorfismos não-HLA tem melhorado a predição do DM1 (57).

A maior parte dos estudos de associação genética tem avaliado a ocorrência de polimorfismos nas regiões codificadoras dos genes; porém, evidências sugerem que polimorfismos nas regiões não codificadoras dos genes, incluindo a região promotora, os acentuadores (*enhancers*) e as regiões alvo dos miRNAs, podem ter um efeito significativo na patogênese de diversas doenças (58). Análises *in silico* demonstraram que variações em regiões alvo de miRNAs podem alterar a expressão de genes já associados com o DM1. Um estudo identificou que polimorfismos na região 3'UTR de genes candidatos para o DM1, como o *CTLA4* e *IL-10*, afetam a capacidade dos miR-302a e miR-523 se ligarem aos RNAm desses genes, respectivamente, alterando suas expressões (59).

Por outro lado, polimorfismos também podem acontecer nos genes que codificam miRNAs. Polimorfismos de troca única (SNPs - do inglês *Single Nucleotide Polymorphisms*) em genes codificantes de miRNAs (miRSNPs) podem afetar o processamento dos pri-miRNAs, pré-miRNAs e/ou dos miRNAs maduros e ainda modificar a ligação aos RNAm alvo (60). Mais especificamente, os miRSNPs podem ser divididos em dois grupos: 1) polimorfismos na sequência dos pri-miRNAs e pré-miRNAs que podem afetar a biogênese dos miRNAs e assim alterar os níveis de expressão dos miRNAs maduros; e 2) polimorfismos que alteram a habilidade dos miRNAs maduros de reconhecer a sequência dos RNAm alvos ou que influenciam a ligação do miRNA com seu RNAm alvo, pois a ligação miRNA-RNAm é feita por complementariedade de pares de base (61). Até o momento, mais de 240 miRSNPs foram descritos em pri-miRNAs, pré-miRNAs ou miRNAs maduros e alguns desses miRSNPs foram associados com diversas doenças humanas, como doenças autoimunes, Alzheimer, Parkinson, doenças cardíacas e vários tipos de câncer (62-67).

Polimorfismos na estrutura de pré-miRNAs foram associados com apoptose das células beta (rs72631823 no *pré-miR-34a* e rs41274239 no *pré-miR-96*). O polimorfismo rs72631823 foi associado com um aumento da expressão do *pré-miR-34a*, ocasionando um aumento na apoptose das células beta (68). Até o momento, o polimorfismo rs2910164 no miR-146a é um dos mais estudados, tendo sido associado com diferentes patologias, como diversos tipos de câncer (69-71), doenças autoimunes (72, 73), polineuropatia diabética e neuropatia cardiovascular autonômica em pacientes com DM tipo 2 (74) e DRD em pacientes com DM1 (75).

O polimorfismo rs2910164 está localizado na sequência *seed* do *pré-miR-146a-3p* (76) e o alelo mais raro desse polimorfismo (C) foi associado com alterações na estrutura do *pré-miR-146a*, modificando a interação do miR-146a maduro com seus alvos (69, 77). Jazdzewski e colaboradores (69) demonstraram que a presença do alelo C altera a eficácia de processamento do pré-miRNA em miRNA maduro, bem como diminui a afinidade do miRNA pelos seus RNAm alvos. Por outro lado, um estudo identificou uma maior expressão do miR-146a associada ao genótipo C/C em células mononucleares de pacientes com doença arterial coronariana (78). Park e colaboradores (73) realizaram uma meta-análise com o objetivo de investigar a associação entre três polimorfismos (rs2910164, rs57095329, rs2431697) no miR-146a e doenças autoimunes. O alelo C do polimorfismo rs2910164 foi associado com risco para esclerose múltipla e com proteção para psoríase, doença de Behcet, asma e uveíte. Por outro lado, os alelos mais raros dos polimorfismos rs2431697 (C/T) e rs57095329 (A/G) foram associados com risco para lúpus eritematoso sistêmico. Além disso, polimorfismos no miR-155 e miR-375 foram associados com outras patologias, como diversos tipos de câncer (79-81) e doenças cardiovasculares (82). Porém, polimorfismos no miR-155 e miR-375 ainda não foram estudados no DM1.

Dessa forma, percebemos que variações genéticas que alteram a especificidade dos miRNAs por seu alvo ou que alteram os níveis de expressão fisiológicos desses miRNAs podem ter consequências importantes na expressão proteica dos genes alvos e, assim, contribuir para o desenvolvimento de diversas patologias.

REFERÊNCIAS DA INTRODUÇÃO

1. Roden M. Diabetes mellitus: definition, classification and diagnosis. Wiener klinische Wochenschrift. 2016;128 Suppl 2:S37-40.
2. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiological reviews. 2011;91(1):79-118.
3. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care, 2013. 36 Suppl: p.S67-74.
4. Pirot P, Cardozo AK, Eizirik DL. Mediators and mechanisms of pancreatic beta-cell death in type 1 diabetes. Arq Bras Endocrinol Metabol. 2008;52(2):156-65.
5. Ziegler AG, Nepom GT. Prediction and pathogenesis in type 1 diabetes. Immunity. 2010;32(4):468-78.
6. Zipris D. Epidemiology of type 1 diabetes and what animal models teach us about the role of viruses in disease mechanisms. Clin Immunol. 2009;131(1):11-23.
7. Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, et al. IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Res Clin Pract. 2017;128:40-50.
8. Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T. Diabetic nephropathy: diagnosis, prevention, and treatment. Diabetes Care. 2005;28(1):164-76.
9. Nadkarni GN, Yacoub R, Coca SG. Update on glycemic control for the treatment of diabetic kidney disease. Curr Diab Rep. 2015;15(7):42.
10. Carpena MP, Rados DV, Sortica DA, Souza BM, Reis AF, Canani LH, et al. Genetics of diabetic nephropathy. Arq Bras Endocrinol Metabol. 2010;54(3):253-61.
11. Reidy K, Kang HM, Hostetter T, Susztak K. Molecular mechanisms of diabetic kidney disease. J Clin Invest. 2014;124(6):2333-40.
12. Tuttle KR, Bakris GL, Bilous RW, Chiang JL, de Boer IH, Goldstein-Fuchs J, et al. Diabetic kidney disease: a report from an ADA Consensus Conference. Am J Kidney Dis. 2014;64(4):510-33.
13. Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med. 1999;130(6):461-70.
14. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150(9):604-12.
15. Matsushita K, Selvin E, Bash LD, Astor BC, Coresh J. Risk implications of the new CKD Epidemiology Collaboration (CKD-EPI) equation compared with the MDRD Study equation for estimated GFR: the Atherosclerosis Risk in Communities (ARIC) Study. Am J Kidney Dis. 2010;55(4):648-59.
16. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol. 2005;6(5):376-85.
17. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843-54.
18. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet. 2011;12(12):861-74.

19. Butz H, Kinga N, Racz K, Patocs A. Circulating miRNAs as biomarkers for endocrine disorders. J Endocrinol Invest. 2016;39(1):1-10.
20. Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. Nature reviews Endocrinology. 2013;9(9):513-21.
21. Seyhan AA, Nunez Lopez YO, Xie H, Yi F, Mathews C, Pasarica M, et al. Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study. Scientific reports. 2016;6:31479.
22. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008;9(2):102-14.
23. Beuvink I, Kolb FA, Budach W, Garnier A, Lange J, Natt F, et al. A novel microarray approach reveals new tissue-specific signatures of known and predicted mammalian microRNAs. Nucleic Acids Res. 2007;35(7):e52.
24. Srivastava K, Srivastava A. Comprehensive review of genetic association studies and meta-analyses on miRNA polymorphisms and cancer risk. PLoS One. 2012;7(11):e50966.
25. Baer C, Claus R, Plass C. Genome-wide epigenetic regulation of miRNAs in cancer. Cancer Res. 2013;73(2):473-7.
26. Natarajan R, Putta S, Kato M. MicroRNAs and diabetic complications. J Cardiovasc Transl Res. 2012;5(4):413-22.
27. Lorenzen J, Kumarswamy R, Dangwal S, Thum T. MicroRNAs in diabetes and diabetes-associated complications. RNA Biol. 2012;9(6):820-7.
28. Kantharidis P, Wang B, Carew RM, Lan HY. Diabetes complications: the microRNA perspective. Diabetes. 2011;60(7):1832-7.
29. Martino F, Lorenzen J, Schmidt J, Schmidt M, Broll M, Gorzig Y, et al. Circulating microRNAs are not eliminated by hemodialysis. PloS one. 2012;7(6):e38269.
30. Barutta F, Tricarico M, Corbelli A, Annaratone L, Pinach S, Grimaldi S, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. PloS one. 2013;8(11):e73798.
31. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. Nat Immunol. 2008;9(8):839-45.
32. Roggli E, Britan A, Gattesco S, Lin-Marq N, Abderrahmani A, Meda P, et al. Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. Diabetes. 2010;59(4):978-86.
33. Hezova R, Slaby O, Faltejskova P, Mikulkova Z, Buresova I, Raja KR, et al. microRNA-342, microRNA-191 and microRNA-510 are differentially expressed in T regulatory cells of type 1 diabetic patients. Cellular immunology. 2010;260(2):70-4.
34. Osipova J, Fischer DC, Dangwal S, Volkmann I, Widera C, Schwarz K, et al. Diabetes-associated microRNAs in pediatric patients with type 1 diabetes mellitus: a cross-sectional cohort study. The Journal of clinical endocrinology and metabolism. 2014;99(9):E1661-5.
35. Nabih ES, Andrawes NG. The Association Between Circulating Levels of miRNA-181a and Pancreatic Beta Cells Dysfunction via SMAD7 in Type 1 Diabetic Children and Adolescents. Journal of clinical laboratory analysis. 2016;30(5):727-31.
36. Snowhite IV, Allende G, Sosenko J, Pastori RL, Messinger Cayetano S, Pugliese A. Association of serum microRNAs with islet autoimmunity, disease progression and metabolic impairment in relatives at risk of type 1 diabetes. Diabetologia. 2017;60(8):1409-22.

37. Samandari N, Mirza AH, Nielsen LB, Kaur S, Hougaard P, Fredheim S, et al. Circulating microRNA levels predict residual beta cell function and glycaemic control in children with type 1 diabetes mellitus. Diabetologia. 2017;60(2):354-63.
38. Yang M, Ye L, Wang B, Gao J, Liu R, Hong J, et al. Decreased miR-146 expression in peripheral blood mononuclear cells is correlated with ongoing islet autoimmunity in type 1 diabetes patients 1miR-146. J Diabetes. 2015;7(2):158-65.
39. Perez-Bravo F, Matthews DR, Haahr HL, Syed F. Differential apoptosis in lymphocytes of patients with type 1 diabetes associated with relative expression of microRNA-146a. Diabetologia. 2014.
40. Salas-Perez F, Codner E, Valencia E, Pizarro C, Carrasco E, Perez-Bravo F. MicroRNAs miR-21a and miR-93 are down regulated in peripheral blood mononuclear cells (PBMCs) from patients with type 1 diabetes. Immunobiology. 2013;218(5):733-7.
41. Erener S, Marwaha A, Tan R, Panagiotopoulos C, Kieffer TJ. Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. JCI insight. 2017;2(4):e89656.
42. Abuhatzira L, Xu H, Tahhan G, Boulougoura A, Schaffer AA, Notkins AL. Multiple microRNAs within the 14q32 cluster target the mRNAs of major type 1 diabetes autoantigens IA-2, IA-2beta, and GAD65. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2015;29(10):4374-83.
43. Samandari N, Mirza AH, Nielsen LB, Kaur S, Hougaard P, Fredheim S, et al. Circulating microRNA levels predict residual beta cell function and glycaemic control in children with type 1 diabetes mellitus. Diabetologia. 2016.
44. DiStefano JK, Taila M, Alvarez ML. Emerging roles for miRNAs in the development, diagnosis, and treatment of diabetic nephropathy. Curr Diab Rep. 2013;13(4):582-91.
45. Chung AC, Yu X, Lan HY. MicroRNA and nephropathy: emerging concepts. International journal of nephrology and renovascular disease. 2013;6:169-79.
46. Kato M, Natarajan R. MicroRNAs in diabetic nephropathy: functions, biomarkers, and therapeutic targets. Annals of the New York Academy of Sciences. 2015;1353:72-88.
47. Simpson K, Wonnacott A, Fraser DJ, Bowen T. MicroRNAs in Diabetic Nephropathy: From Biomarkers to Therapy. Curr Diab Rep. 2016;16(3):35.
48. Pezzolesi MG, Satake E, McDonnell KP, Major M, Smiles AM, Krolewski AS. Circulating TGF-beta1-Regulated miRNAs and the Risk of Rapid Progression to ESRD in Type 1 Diabetes. Diabetes. 2015;64(9):3285-93.
49. Eissa S, Matboli M, Bekhet MM. Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis. Biomed Pharmacother. 2016;83:92-9.
50. Kato M, Dang V, Wang M, Park JT, Deshpande S, Kadam S, et al. TGF-beta induces acetylation of chromatin and of Ets-1 to alleviate repression of miR-192 in diabetic nephropathy. Science signaling. 2013;6(278):ra43.
51. Argyropoulos C, Wang K, McClarty S, Huang D, Bernardo J, Ellis D, et al. Urinary microRNA profiling in the nephropathy of type 1 diabetes. PloS one. 2013;8(1):e54662.
52. Argyropoulos C, Wang K, Bernardo J, Ellis D, Orchard T, Galas D, et al. Urinary MicroRNA Profiling Predicts the Development of Microalbuminuria in Patients with Type 1 Diabetes. Journal of clinical medicine. 2015;4(7):1498-517.

53. Baker MA, Davis SJ, Liu P, Pan X, Williams AM, Iczkowski KA, et al. Tissue-Specific MicroRNA Expression Patterns in Four Types of Kidney Disease. Journal of the American Society of Nephrology : JASN. 2017.
54. Cardenas-Gonzalez M, Srivastava A, Pavkovic M, Bijol V, Rennke HG, Stillman IE, et al. Identification, Confirmation, and Replication of Novel Urinary MicroRNA Biomarkers in Lupus Nephritis and Diabetic Nephropathy. Clinical chemistry. 2017.
55. Jia Y, Guan M, Zheng Z, Zhang Q, Tang C, Xu W, et al. miRNAs in Urine Extracellular Vesicles as Predictors of Early-Stage Diabetic Nephropathy. Journal of diabetes research. 2016;2016:7932765.
56. Nguyen C, Varney MD, Harrison LC, Morahan G. Definition of high-risk type 1 diabetes HLA-DR and HLA-DQ types using only three single nucleotide polymorphisms. Diabetes. 2013;62(6):2135-40.
57. Pociot F, Lernmark A. Genetic risk factors for type 1 diabetes. Lancet. 2016;387(10035):2331-9.
58. Wu D, Yang G, Zhang L, Xue J, Wen Z, Li M. Genome-wide association study combined with biological context can reveal more disease-related SNPs altering microRNA target seed sites. BMC genomics. 2014;15:669.
59. de Jong VM, Zaldumbide A, van der Slik AR, Persengiev SP, Roep BO, Koeleman BP. Post-transcriptional control of candidate risk genes for type 1 diabetes by rare genetic variants. Genes Immun. 2013;14(1):58-61.
60. Hinds DA, Stuve LL, Nilsen GB, Halperin E, Eskin E, Ballinger DG, et al. Whole-genome patterns of common DNA variation in three human populations. Science. 2005;307(5712):1072-9.
61. Kroliczewski J, Sobolewska A, Lejnowski D, Collawn JF, Bartoszewski R. microRNA single polynucleotide polymorphism influences on microRNA biogenesis and mRNA target specificity. Gene. 2017.
62. Sethupathy P, Collins FS. MicroRNA target site polymorphisms and human disease. Trends in genetics : TIG. 2008;24(10):489-97.
63. Ghanbari M, Darweesh SK, de Looper HW, van Luijn MM, Hofman A, Ikram MA, et al. Genetic Variants in MicroRNAs and Their Binding Sites Are Associated with the Risk of Parkinson Disease. Human mutation. 2016;37(3):292-300.
64. Ghanbari M, Ikram MA, de Looper HW, Hofman A, Erkeland SJ, Franco OH, et al. Genome-wide identification of microRNA-related variants associated with risk of Alzheimer's disease. Scientific reports. 2016;6:28387.
65. Kim J, Choi GH, Ko KH, Kim JO, Oh SH, Park YS, et al. Association of the Single Nucleotide Polymorphisms in microRNAs 130b, 200b, and 495 with Ischemic Stroke Susceptibility and Post-Stroke Mortality. PloS one. 2016;11(9):e0162519.
66. Mullany LE, Herrick JS, Wolff RK, Buas MF, Slattery ML. Impact of polymorphisms in microRNA biogenesis genes on colon cancer risk and microRNA expression levels: a population-based, case-control study. BMC medical genomics. 2016;9(1):21.
67. Moszynska A, Gebert M, Collawn JF, Bartoszewski R. SNPs in microRNA target sites and their potential role in human disease. Open biology. 2017;7(4).
68. Locke JM, Lango Allen H, Harries LW. A rare SNP in pre-miR-34a is associated with increased levels of miR-34a in pancreatic beta cells. Acta Diabetol. 2014;51(2):325-9.
69. Jazdzewski K, Murray EL, Franssila K, Jarzab B, Schoenberg DR, de la Chapelle A. Common SNP in pre-miR-146a decreases mature miR expression and

predisposes to papillary thyroid carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(20):7269-74.
70. Xu W, Xu J, Liu S, Chen B, Wang X, Li Y, et al. Effects of common polymorphisms rs11614913 in miR-196a2 and rs2910164 in miR-146a on cancer susceptibility: a meta-analysis. PloS one. 2011;6(5):e20471.
71. Tian T, Xu Y, Dai J, Wu J, Shen H, Hu Z. Functional polymorphisms in two pre-microRNAs and cancer risk: a meta-analysis. International journal of molecular epidemiology and genetics. 2010;1(4):358-66.
72. Li C, Fu W, Zhang Y, Zhou L, Mao Z, Lv W, et al. Meta-analysis of microRNA-146a rs2910164 G>C polymorphism association with autoimmune diseases susceptibility, an update based on 24 studies. PloS one. 2015;10(4):e0121918.
73. Park R, Lee WJ, Ji JD. Association between the three functional miR-146a single-nucleotide polymorphisms, rs2910164, rs57095329, and rs2431697, and autoimmune disease susceptibility: A meta-analysis. Autoimmunity. 2016:1-8.
74. Ciccacci C, Morganti R, Di Fusco D, D'Amato C, Cacciotti L, Greco C, et al. Common polymorphisms in MIR146a, MIR128a and MIR27a genes contribute to neuropathy susceptibility in type 2 diabetes. Acta diabetologica. 2014;51(4):663-71.
75. Kaidonis G, Gillies MC, Abhary S, Liu E, Essex RW, Chang JH, et al. A single-nucleotide polymorphism in the MicroRNA-146a gene is associated with diabetic nephropathy and sight-threatening diabetic retinopathy in Caucasian patients. Acta diabetologica. 2016.
76. Liu C, Zhang F, Li T, Lu M, Wang L, Yue W, et al. MirSNP, a database of polymorphisms altering miRNA target sites, identifies miRNA-related SNPs in GWAS SNPs and eQTLs. BMC genomics. 2012;13:661.
77. Cammaerts S, Strazisar M, De Rijk P, Del Favero J. Genetic variants in microRNA genes: impact on microRNA expression, function, and disease. Frontiers in genetics. 2015;6:186.
78. Ramkaran P, Khan S, Phulukdaree A, Moodley D, Chuturgoon AA. miR-146a polymorphism influences levels of miR-146a, IRAK-1, and TRAF-6 in young patients with coronary artery disease. Cell biochemistry and biophysics. 2014;68(2):259-66.
79. Xie K, Ma H, Liang C, Wang C, Qin N, Shen W, et al. A functional variant in miR-155 regulation region contributes to lung cancer risk and survival. Oncotarget. 2015;6(40):42781-92.
80. Wang S, Cao X, Ding B, Chen J, Cui M, Xu Y, et al. The rs767649 polymorphism in the promoter of miR-155 contributes to the decreased risk for cervical cancer in a Chinese population. Gene. 2016;595(1):109-14.
81. Shaker OG, Mohammed SR, Mohammed AM, Mahmoud Z. Impact of microRNA-375 and its target gene SMAD-7 polymorphism on susceptibility of colorectal cancer. Journal of clinical laboratory analysis. 2017.
82. Choi GH, Ko KH, Kim JO, Kim J, Oh SH, Han IB, et al. Association of miR-34a, miR-130a, miR-150 and miR-155 polymorphisms with the risk of ischemic stroke. International journal of molecular medicine. 2016;38(1):345-56.

2. JUSTIFICATIVA

Estatísticas mostram que o número de indivíduos com DM1 está aumentando e providências se fazem necessárias para modificar a trajetória dessa doença que tem um tratamento de elevado custo. O DM1 é geralmente diagnosticado quando >80% das células beta são destruídas pelo sistema imune. Esta destruição das células beta é lenta, fornecendo uma janela de tempo para se identificar indivíduos em risco e talvez prevenir o DM1. Atualmente, o desenvolvimento do DM1 pode ser predito pela presença de autoanticorpos contra as ilhotas pancreáticas; entretanto, esses anticorpos aparecem aumentados apenas tardiamente no curso da doença, não sendo biomarcadores ideais da destruição das células beta. Dessa forma, a identificação de novos biomarcadores poderá melhorar a identificação de indivíduos em risco para o DM1 em um período em que medidas preventivas podem ainda ser eficazes.

A DRD é uma complicação microvascular que afeta cerca de 40% dos pacientes com DM1 e está associada com elevada morbidade e mortalidade em indivíduos jovens em idade produtiva. A dosagem de albumina na urina tem sido utilizada como um marcador laboratorial para predizer essa complicação; entretanto, estudos recentes mostram que há pacientes que desenvolvem a DRD antes que um aumento na albuminúria seja detectado. Assim, miRNAs circulantes tanto na urina quanto no sangue periférico representam uma alternativa viável para o monitoramento da DRD em pacientes com DM1.

MiRNAs são moléculas de RNA pequenos não-codificantes que regulam negativamente a expressão gênica. Mudanças na expressão de miRNAs foram observadas em diversas situações patológicas, incluindo doenças autoimunes. Recentemente, mudanças no perfil de expressão de miRNAs também foram relatadas

em pacientes com DM1, com ou sem complicações crônicas, quando comparados a indivíduos controles. Dentre os miRNAs diferencialmente expressos no DM1 ou na DRD, os miRNAs circulantes são de especial interesse como biomarcadores, pois podem ser coletados facilmente, são estáveis sob diferentes condições de estocagem e também podem ser medidos usando-se técnicas específicas. Todavia, muitos estudos ainda são necessários para que um perfil de expressão de miRNAs possa ser usado como biomarcador para o desenvolvimento do DM1 ou de suas complicações crônicas, como a DRD. Além disso, pouco se sabe sobre a associação entre polimorfismos em genes codificadores de miRNAs e o desenvolvimento do DM1 ou de suas complicações crônicas.

A identificação de perfis de expressão de miRNAs que possam ser usados como biomarcadores da destruição inicial das células beta no DM1 ou para o surgimento ou prognóstico da DRD poderá contribuir para a busca da prevenção do DM1 ou diminuição da progressão da DRD. Além disso, estudos que identifiquem o papel dos miRNAs na modulação do sistema imune, função das células beta ou fisiopatogênese da DRD podem contribuir para a elucidação de importantes mecanismos regulatórios, gerando potenciais alvos terapêuticos.

3. OBJETIVO

3.1. Objetivos gerais

- Realizar uma revisão sistemática dos estudos que investigaram a expressão de miRNAs em pacientes com DM1 e indivíduos não-diabéticos.

- Investigar um perfil de expressão de miRNAs no DM1.

- Avaliar a associação entre os polimorfismos rs6715345 (C/G) no miR-375, rs2910164 (C/G) no miR-146a e rs767649 (A/C) no miR-155 e o DM1.

- Realizar uma revisão sistemática dos estudos que investigaram a expressão de miRNAs em pacientes com DRD e em pacientes sem essa complicação crônica do DM.

- Investigar um perfil de expressão de miRNAs na DRD em pacientes com DM1.

3.2 Objetivos específicos

- Realizar uma revisão sistemática de todos os estudos de expressão de miRNAs em pacientes com DM1, visando identificar um perfil de miRNAs associado a esta doença.

- Avaliar a expressão de 48 miRNAs circulantes no plasma de pacientes com DM1 (casos) e indivíduos não-diabéticos (controles), através de PCR em tempo real

usando-se *TaqMan Low Density Array cards* (TLDA), os quais permitem a quantificação relativa de todos estes miRNAs em um único experimento (análise de *macroarray*).

- Validar os resultados obtidos pela técnica de *macroarray* em uma segunda amostra de pacientes com DM1 e indivíduos sem DM, utilizando a técnica de RT-qPCR.

- Avaliar as distribuições dos polimorfismos rs6715345 (C/G) no miR-375, rs2910164 (C/G) no miR-146a e rs767649 (A/C) no miR-155 em pacientes com DM1 (casos) e indivíduos não-diabéticos (controles).

- Realizar uma revisão sistemática de todos os estudos de expressão de miRNAs em pacientes com DRD, visando identificar um perfil de miRNAs associado com os diferentes graus dessa complicação.

- Avaliar a expressão de outros 48 miRNAs circulantes no plasma de pacientes com DM1 sem DRD (controles) ou com diferentes graus de DRD (casos) através de *macroarray* TLDA.

- Validar os resultados obtidos pela técnica de *macroarray* em uma segunda amostra de pacientes com DM1 com e sem DRD, utilizando a técnica de RT-qPCR.

Capítulo 1

“MicroRNAs e diabetes mellitus tipo 1”

ARTIGO 1

MicroRNA expression profiles and type 1 diabetes mellitus: systematic review and bioinformatic analysis

MicroRNA expression profiles and type 1 diabetes mellitus: systematic review and bioinformatic analysis

Taís S Assmann[1,2], Mariana Recamonde-Mendoza[3], Bianca M De Souza[1,2] and **Daisy Crispim[1,2]**

[1]Endocrine Division, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil
[2]Postgraduation Program in Medical Sciences: Endocrinology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[3]Institute of Informatics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

Correspondence should be addressed to D Crispim
Email
dcmoreira@hcpa.edu.br

Abstract

Growing evidence indicates that microRNAs (miRNAs) have a key role in processes involved in type 1 diabetes mellitus (T1DM) pathogenesis, including immune system functions and beta-cell metabolism and death. Although dysregulated miRNA profiles have been identified in T1DM patients, results are inconclusive; with only few miRNAs being consistently dysregulated among studies. Thus, we performed a systematic review of the literature on the subject, followed by bioinformatic analysis, to point out which miRNAs are dysregulated in T1DM-related tissues and in which pathways they act. PubMed and EMBASE were searched to identify all studies that compared miRNA expressions between T1DM patients and non-diabetic controls. Search was completed in August, 2017. Those miRNAs consistently dysregulated in T1DM-related tissues were submitted to bioinformatic analysis, using six databases of miRNA–target gene interactions to retrieve their putative targets and identify potentially affected pathways under their regulation. Thirty-three studies were included in the systematic review: 19 of them reported miRNA expressions in human samples, 13 in murine models and one in both human and murine samples. Among 278 dysregulated miRNAs reported in these studies, 25.9% were reported in at least 2 studies; however, only 48 of them were analyzed in tissues directly related to T1DM pathogenesis (serum/plasma, pancreas and peripheral blood mononuclear cells (PBMCs)). Regarding circulating miRNAs, 11 were consistently dysregulated in T1DM patients compared to controls: miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275. The bioinformatic analysis retrieved a total of 5867 validated and 2979 predicted miRNA–target interactions for human miRNAs. In functional enrichment analysis of miRNA target genes, 77 KEGG terms were enriched for more than one miRNA. These miRNAs are involved in pathways related to immune system function, cell survival, cell proliferation and insulin biosynthesis and secretion. In conclusion, eleven circulating miRNAs seem to be dysregulated in T1DM patients in different studies, being potential circulating biomarkers of this disease.

Key Words
- systematic review
- microRNA
- type 1 diabetes mellitus
- bioinformatic analysis

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Introduction

Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of pancreatic beta-cells by T lymphocytes and macrophages (1). The disease is usually diagnosed when over 80–90% of beta-cells have been destructed by the infiltrating immune system. T1DM development is slow, providing a potentially long window of time in which it is possible to identify and theoretically treat individuals at risk (2, 3).

The first sign of autoimmunity against beta-cells, frequently detectable a few months/years before the appearance of clinical symptoms, is the occurrence of antibodies against beta-cell antigens (4). These autoantibodies are used as biomarkers of T1DM risk and are directed against insulin, glutamic acid decarboxylase, zinc cation efflux transporter and tyrosine phosphatases-2 and -2β (4). The presence of more than two of these autoantibodies indicates high risk for T1DM development (5, 6). However, the use of islet autoantibodies as biomarkers of T1DM progression has some limitations, especially because a subset of children with new-onset T1DM is negative for islet autoantibodies (6), and many autoantibody-positive subjects will never develop T1DM (2, 7). Moreover, autoantibodies cannot be used as markers to initiate a potential treatment at earlier stages of the disease when many beta-cells are still present (2, 7). Thus, new biomarkers of T1DM are necessary to complement the information obtained from the presence of autoantibodies together with genetic and environmental risk factors (8).

In this context, several microRNAs (miRNAs) are released in the circulation and might be used as biomarkers to evaluate health status and disease progression (2). miRNAs are a class of small noncoding RNAs that negatively regulate gene expression by partially pairing to the 3′, 5′ untranslated regions of their target mRNAs, leading to translation repression and/or transcript degradation (9, 10, 11). They have recognized roles in the regulation of various processes, such as cellular differentiation, proliferation, metabolism, aging and apoptosis (10, 12). miRNAs are estimated to regulate the expression of more than 60% of protein-coding genes (9); consequently, changes in their expressions have been linked to many diseases, including cancer, endocrine disorders and autoimmune diseases (13, 14, 15).

Growing evidence suggests that miRNAs also play a key role in immune system functions as well as in beta-cell metabolism, proliferation and death, which are processes involved in T1DM pathogenesis (2, 10, 16, 17). Indeed, IL-1β and TNF inflammatory cytokines were reported to induce miR-21-5p, miR-30b-3p, miR-34, miR-101a and miR-146a-5p expressions in MIN6 cells and human pancreatic islets (18, 19), suggesting that these miRNAs may have a role in cytokine-mediated beta-cell destruction. miRNA-specific profiles were observed in PBMCs or serum from T1DM patients (20, 21, 22, 23, 24), and some miRNAs seem to modulate mRNA expressions of the major T1DM autoantigens (24, 25).

Several studies identified a large number of miRNAs as being differentially expressed in T1DM samples (2, 10). These studies were performed in cultured cells, body fluids or solid tissue samples from T1DM patients or murine models of the disease, using different techniques to quantify gene expression. Consequently, findings are inconsistent among studies; with only few miRNAs actually being important signatures of T1DM. Therefore, to further investigate which miRNAs may be used as new potential biomarkers of T1DM, we performed a systematic review of the literature on the subject. Additionally, bioinformatic analyses were performed to investigate the regulatory and functional roles of miRNAs in T1DM. For this, six databases of miRNA–target gene interactions were queried, including experimentally validated and computationally predicted miRNA–target gene interactions. The functional enrichment analysis of miRNAs target genes was performed using pathways annotation from the KEGG Pathway Database.

Methods

Search strategies and eligibility of relevant studies

This systematic literature search was designed and described in accordance with current guidelines (26, 27). PubMed and EMBASE repositories were searched to identify all studies that evaluated miRNA expressions in T1DM samples. The following medical subject headings (MeSH) were used: (‘diabetes mellitus’ OR ‘type 1, diabetes mellitus’) AND (‘microRNA’ OR ‘RNA, small untranslated’). The search was restricted to English, Portuguese or Spanish language papers and was completed on August, 2017. We also manually checked the reference lists of all articles retrieved to identify other important citations. To ensure that relevant studies were not overlooked, searches in Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) and Array Express (www.ebi.ac.uk/arrayexpress) databases were also performed.

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We included original reports that analyzed miRNA expressions in T1DM patients (cases) and non-diabetic subjects (controls) or in murine model of this disease. Studies that did not have a control group or studies performed in cell lines were excluded. Two investigators (T S A and B M S) independently reviewed titles and abstracts of articles retrieved in order to evaluate whether the studies were eligible for inclusion in this review.

Data extraction and quality assessment of each individual study

Data were independently extracted by two investigators (B M S and T S A) using a standardized abstraction form (26), and consensus was sought in all extracted items. Information extracted from each study in humans were as follows: (1) characteristics of studies and samples; (2) information regarding miRNA expression (method used for quantification, tissue analyzed, number of miRNAs analyzed) and (3) miRNA expression in groups. For those studies performed in mice/rats, we also collected information about the murine model analyzed. All miRNA names were standardized based on miRBase v21 prior to analysis.

Two investigators (T S A and B M S) assessed the quality of each eligible study using The Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) (28). This tool comprises 4 key domains (patient selection, index test, reference standard and flow/timing) supported by 7 questions to aid judgment on risk of bias, rating risk of bias and concerns about applicability of studies. Each question can be answered with 'yes', 'no' or 'unclear'. Then, a score of 1 is given for each 'yes' (low risk/high concern), a score of 0.5 for each 'unclear' and a score of 0 for each 'no' (high risk/low concern). Quality scores range from 0 to 7, with studies being classified as having 'good quality' (scores 6–7), 'fair' (scores 4–5) and 'poor quality' (scores <3).

Additionally, we checked if the articles were performed in accordance to Minimum Information about a Microarray Experiment (MIAME) guideline, version 2.0 (29) or Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guideline (30). Only articles in accordance with these guidelines were included in the systematic review.

Bioinformatic prediction and analysis of miRNA's target genes

To investigate in greater depth the functional involvement of miRNAs in T1DM, we selected those miRNAs that were consistently dysregulated in T1DM-related tissues (PBMCs, serum/plasma and pancreas) and performed bioinformatic analysis to retrieve their putative targets and identify potentially affected biological pathways under their regulation (Supplementary Fig. 1, see section on supplementary data given at the end of this article). For this, we queried the databases miRTarbase release 6.1 (31) and starBase, v2.0 (32) of experimentally validated data concerning miRNA–target interactions, restricting the search for interactions classified as functional in miRTarBase (33), and interactions predicted by two or more software with at least one supporting experiment in starBase. Moreover, we obtained the complete collection of validated targets provided by miRecords. The union of all interactions retrieved from the 3 queried sources was considered as the set of validated miRNA–target gene interactions in our study.

To complement the information derived from experimental validation and search for additional miRNA targets, we also applied *in silico* target prediction algorithms for selected miRNA sequences using web-based tools TargetScan, v7.1 (34), Diana MicroT-CDS (35) and miRanda-mirSVR (August 2010 Release) (36, 37). To control for false-positive rates, we adopted the following filtering criteria: (1) for TargetScan, v7.1, we considered interactions involving conserved miRNA sites and with context++ scores <−0.1; (2) for Diana MicroT-CDS, we kept interactions with prediction scores ≥0.7; (3) for miRanda-mirSVR, we selected interactions involving conserved miRNAs and with scores <−0.1; (4) the compilation of miRNA–target interactions gathered from *in silico* analysis was built based on target genes predicted by at least 2 adopted computational tools. The combination of validated and predicted miRNA–target interactions was used for further analyses. miRNAs and gene identifiers were mapped to miRBase, v21 and Human Gene Nomenclature Committee (38, 39) or Mouse Genome Information nomenclature (40, 41).

Next, we implemented functional enrichment analysis of miRNAs target genes using pathways annotation from the KEGG Pathway Database (42, 43) and the clusterProfiler package in R/Bioconductor environment (44). This investigation was performed for targets of each individual miRNA as well as for targets of miRNAs grouped by tissue (PBMCs, serum/plasma or pancreas). Significance for KEGG pathways enrichment was estimated with a hypergeometric test and adjusted to account for multiple hypotheses using the false discovery rate (FDR) procedure implemented in the q-value R package (45). Pathways with a q-value <0.05 were considered strongly enriched for the genes targeted by selected miRNAs.

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Results

Literature search, characteristics of the eligible studies and quality assessment

The flow diagram showing the strategy used to identify and select studies for inclusion in this systematic review is depicted in Fig. 1. According to the search criteria, a total of 1738 publications were retrieved from databases; however, after full text analysis, only 33 articles (20, 22, 23, 24, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74) fulfilled the eligibility criteria and were included in the review. The main characteristics of these 33 articles are shown in Table 1. Among these studies, 19 reported miRNA expression profiles in human, 13 focused on miRNA profiles in murine models, and only one analyzed both human and murine samples (46). Sample sizes ranged from 10 to 162 in studies that analyzed human samples and from 6 to 60 in studies with murine models. The number of miRNAs analyzed ranged from 1 to 847, with the number of miRNAs differentially expressed between groups varying from 1 to 136 (Table 1).

Regarding tissues analyzed, 24.1% of the studies evaluated miRNA expression in serum/plasma samples, 20.7% in PBMCs/T cells, and 6.9% in pancreas tissue. The remaining studies evaluated other tissues related to T1DM chronic complications, such as urine, kidney, heart and retina (Table 1). Two articles analyzed different tissues (46, 52) and were considered separately, totalizing 36 studies.

Quality of each study included in this review was assessed using QUADAS-2, as reported in the Methods section. Overall, most studies were considered as having a good quality since 62.5% of studies received QUADAS-2 scores between 6 and 7 (Table 1). No study scored less than 5.0.

Dysregulated miRNAs in T1DM-related tissues

Out of 278 dysregulated miRNAs reported in 36 studies that compared T1DM patients and controls, 72 miRNAs (25.9%) were reported in at least two studies (Supplementary Table 1). However, only 48 of them were analyzed in tissues directly related to T1DM pathogenesis (PBMCs, serum/plasma and pancreas). Hence, these 48 miRNAs were chosen for further evaluation (Table 2).

Eight miRNAs were consistently downregulated in T1DM-related tissues from patients compared to controls (miR-100-5p, miR-1275, miR-150-5p, miR-151-3p, miR-146a-5p, miR-151-3p, miR-574-3p and miR-720), while 10 miRNAs were upregulated in cases (miR-21-5p, miR-24-3p, miR-25-3p, miR-27b-3p, miR-148a-3p, miR-181a-5p, miR-210-5p, miR-375, miR-450a-2-3p and miR-454-3p) (Fig. 2A and Table 2). Thirty miRNAs were reported as being downregulated in cases from one study and upregulated in cases from another study, possibly due to the different tissues or species that were analyzed (Table 2).

miRNA expression profiles according to species

In subgroup analysis of species, 19 studies reported expressions of 139 miRNAs in different tissues from T1DM patients and controls, with 36 of these miRNAs being

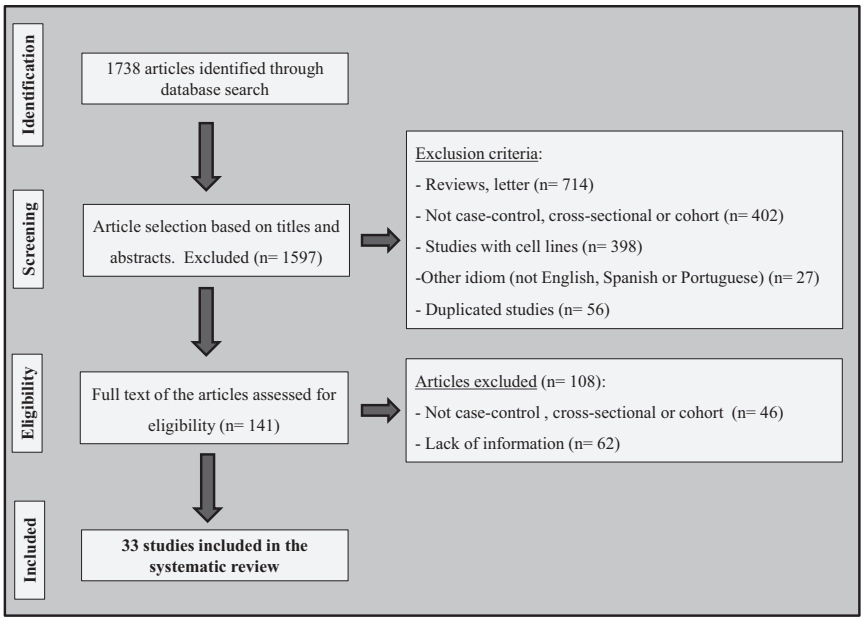

Figure 1
Flowchart illustrating the search strategy used to identify association studies of miRNAs expression with type 1 diabetes for inclusion in the systematic review.

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Table 1 Characteristics of studies included in the systematic review.

		T1DM				Differentially expressed microRNAs				
First author, year (Ref)	Country	Diabetic sample	Tissue	Sample size Case/Control	Method	Cut-off criteria	Total	Increased	Decreased	Quality/ QUADAS-2
Alipour *et al.* 2013 (58)	Iran	Male Sprague-Dawley rats induced with STZ	Kidney	6/6	RT-PCR	0.05	1	1	0	5.5
Bacon *et al.* 2015 (59)	Ireland	T1DM patients	Urine	44/26	RT-PCR	N/A	2	2	0	6.0
Barutta *et al.* 2013 (46)	Italy	Normoalbuminuric T1DM patients	Urinary Exossome	12/12	RT-PCR	2-fold	2	2	0	6.0
Barutta *et al.* 2013 (46)	Italy	Male C57BL6/J mice induced with STZ	Glomeruli	30/30	RT-PCR	2-fold	1	1	0	5.5
Barutta *et al.* 2013 (46)	Italy	Male C57BL6/J mice induced with STZ	Exossome	30/30	RT-PCR	2-fold	1	1	0	5.5
Diao *et al.* 2011 (47)	China	Male C57BL/6 mice induced with STZ	Heart	15/10	Microarray analysis	0.05	16	10	6	6.0
Emadi *et al.* 2014 (48)	Iran	Male Wistar rats	Aorta	6/6	RT-PCR	0.05	1	0	1	6.0
Erener *et al.* 2013 (63)	Canada	C57BL/6 mice induced with STZ and NOD mice	Plasma	6/6	RT-PCR	0.05	1	1	0	5.5
Erener *et al.* 2017 (71)	Canada	Recent-onset T1DM patients	Serum	10/7	RT-PCR	N/A	35	27	8	6.0
Estrella *et al.* 2016 (65)	Chile	T1DM patients	PBMCs	20/20	RT-PCR	N/A	2	1	1	6.0
Garcia de la Torre *et al.* 2015 (64)	Spain	T1DM patients without DR	EPC	76/38	RT-PCR	2-fold	1	1	0	6.0
Garcia-Contreras *et al.* 2017 (72)	USA	T1DM patients	Plasma-derived exosome	36/36	Microarray and RT-PCR	N/A	7	1	6	5.5
Hezova *et al.* 2010 (49)	Czech Republic	T1DM patients	T cells	5/5	TLDA	0.05	3	1	2	5.5
Kato *et al.* 2010 (50)	USA	C57BL/6 mice induced with STZ	Kidney	3/3	RT-PCR	0.05	1	1	0	6.0
Kovacs *et al.* 2011 (51)	USA	Male Sprague-Dawley rats induced with STZ	Retina	3/3	Microarray	0.05	17	14	3	5.5
Li *et al.* 2009 (67)	China	C57BL/6 mice induced wit STZ	Liver	8/8	Microarray	0.05	2	1	1	5.5
Ma *et al.* 2016 (61)	China	NOD mice	Pancreas	6/6	RT-PCR	N/A	1	0	1	6.0
Marchand *et al.* 2016 (66)	France	T1DM patients recently diagnosed	Serum	22/10	RT-PCR	0.05	1	1	0	6.0
Nabih *et al.* 2016 (62)	Egypt	Children and adolescents with T1DM	Serum	40/40	RT-PCR	N/A	1	1	0	6.0
Nielsen *et al.* 2012 (22)	Denmark	Children with newly diagnosed T1DM	Serum	108/54	Solexa sequencing/ RT-PCR	2-fold	24	24	0	6.5
Osipova *et al.* 2014 (52)	Germany	T1DM pediatric patients	Serum	68/79	RT-PCR	0.05	2	2	0	6.0
Osipova *et al.* 2014 (52)	Germany	T1DM pediatric patients	Urine	68/79	RT-PCR	0.05	3	2	1	6.0
*Perez-Bravo *et al.* 2014 (69)	Chile	T1DM patients	PBMCs	5/5	RT-PCR	N/A	1	0	1	–
Qing *et al.* 2014 (56)	China	T1DM patients	Retina	90/20	TLDA	N/A	3	3	0	5.0
Salas-Perez *et al.* 2013 (20)	Chile	T1DM pediatric patients	PBMCs	20/20	RT-PCR	0.05	2	0	2	5.0
*Sebastiani *et al.* 2012 (68)	Italy	T1DM patients	Serum	20/20	TLDA	0.05	64	21	43	–

(Continued)

Table 1 Continued.

		T1DM				Differentially expressed microRNAs				
First author, year (Ref)	Country	Diabetic sample	Tissue	Sample size Case/Control	Method	Cut-off criteria	Total	Increased	Decreased	Quality/ QUADAS-2
Sebastiani *et al.* 2017 (74)	Italy	T1DM patients	T-cells from pancreatic lymphnodes		Megaplex RT-stem-loop microRNA Pool A v2.1	N/A	1	1	0	6.0
Seyhan *et al.* 2016 (70)	USA	T1DM patients	Plasma	16/27	RT-PCR	0.05	4	4	0	6.5
Silva *et al.* 2011 (53)	Brazil	Male Wistar rats, induced with STZ	Retina	3/3	RT-PCR	0.05	1	1	0	6.5
Takahashi *et al.* 2014 (23)	Brazil	T1DM patients	PBMCs	6/6	Microarray	N/A	44	35	9	6.0
Tian *et al.* 2015 (60)	China	Male NIH mice induced with STZ	Pancreas	3/3	Microarray	2-fold	136	64	72	6.0
Wang *et al.* 2014 (54)	USA	Male Long Evans rats induced with STZ	Retina	3/3	RT-PCR	N/A	1	1	0	5.5
Wang *et al.* 2017 (73)	China	T1DM patients	PBMCs	78/56	RT-PCR	N/A	3	0	3	6.0
Xiong *et al.* 2014 (57)	China	Rat induced with STZ	Retina	6/6	RT-PCR	0.05	17	15	2	6.0
Yang *et al.* 2015 (24)	China	Newly diagnosed T1DM patients	PBMCs	12/10	Microarray/ RT-PCR	0.05	24	5	19	6.5
Yousefzadeh *et al.* 2015 (55)	Iran	Male Sprague-Dawley rats induced with STZ	Sciatic nerve	6/6	RT-PCR	0.05	1	1	0	6.0

DR, diabetic retinopathy; EPC, Endothelial Progenitor cells; N/A, not available; QUADAS-2, Quality Assessment of Diagnostic Accuracy Studies 2; RT-PCR, Reverse transcription polymerase chain reaction; STZ, streptozotocin; T1DM, Type 1 diabetes mellitus; TLDA, TaqMan Low Density Array. *Abstract from Congress.

reported by at least two studies (Supplementary Table 1). One study analyzed both human and murine samples. Additionally, 13 miRNA profile studies were performed in murine models of T1DM, identifying 173 dysregulated miRNAs in different tissues, with only 45 of them being reported by at least two studies (Supplementary Table 1).

Considering only the 48 miRNAs expressed in serum/plasma, PBMCs or pancreas, 12 miRNAs were dysregulated exclusively in human samples, with 4 miRNAs (miR-100-5p, miR-146a-5p miR-150-5p and miR-1275) being downregulated and 8 upregulated (miR-10a-5p, miR-21-5p, miR-24-3p miR-26b-5p, miR-27b-3p, miR-148a-3p, miR-181a-5p and miR-210-5p) in T1DM patients compared to controls. Only one miRNA (mmu-miR-26a-5p) was consistently downregulated in pancreas from murine models of T1DM. Four miRNAs (miR-151-3p, miR-324-5p, let-7a-5p and let-7c-5p) were shown to be downregulated in tissues from both human and mice with diabetes, and only miR-375 was upregulated in tissues from human and mice (Fig. 2B and Table 2).

Dysregulated miRNAs as circulating and tissue biomarkers of T1DM

Several miRNAs are released into the bloodstream or expressed in blood cells and might be used as circulating biomarkers of T1DM (2). Among miRNAs that were analyzed in more than one study, 21 (miR-15b, miR-20b-5p, miR-21-5p, miR-22-3p, miR-24-3p, miR-25-3p, miR-26b-5p, miR-27b-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-200c-3p, miR-210-5p, miR-335-5p, miR-342-3p, miR-375, miR-1275, let-7f-5p and let-7g-5p) were expressed in serum/plasma or PBMCs/T cells and, therefore, have the potential to be circulating biomarkers of T1DM (Table 2). Nevertheless, only 11 of them were consistently dysregulated, being analyzed in the same tissue by at least two studies: miR-146a-5p, miR-150-5p, miR-342-3p and miR-1275 were downregulated in PBMCs from T1DM cases compared to controls, while miR-21-5p, miR-24-3p, miR-100-5p, miR-148a-3p, miR-181a-5p, miR-210-5p and miR-375 were upregulated in serum/plasma from cases (Fig. 2C and Table 2).

Two studies evaluated miRNA expression profiles in pancreas from murine models of T1DM (60, 61) and showed that miR-26a-5p expression was downregulated in pancreas from NOD or streptozotocin (STZ)-induced diabetes mice compared to control mice (Fig. 2C and Table 2). No study has evaluated miRNA expressions in human pancreas from T1DM patients and non-diabetic controls.

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Table 2 miRNAs differently expressed in tissues related to T1DM analyzed in at least two studies.

miRNA ID	First author (ref.)	Species	Sample type	Change of expression
let-7a-5p	Tian *et al*. (60)	Mice	Pancreas	Down
	Yang *et al*. (24)	Human	Pancreas	Down
let-7c-5p	Tian *et al*. (60)	Mice	Pancreas	Down
	Yang *et al*. (24)	Human	Pancreas	Down
let-7f-5p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Yang *et al*. (24)	Human	PBMCs	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
let-7g-5p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Erener *et al*. (71)	Human	Serum	Up
	Yang *et al*. (24)	Human	PBMCs	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-10a-5p	Nielsen *et al*. (22)	Human	Serum	Up
	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-100-5p	Hezova *et al*. (49)	Human	T cells	Down
	Erener *et al*. (71)	Human	Serum	Down
miR-126-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-1275	Takahashi *et al*. (23)	Human	PBMCs	Down
	Yang *et al*. (24)	Human	PBMCs	Down
miR-146a-5p	Yang *et al*. (24)	Human	PBMCs	Down
	Hezova *et al*. (49)	Human	T cells	Up
	Sebastiani *et al*. (68)	Human	Serum	Down
	Perez-Bravo *et al*. (69)	Human	PBMCs	Down
	Wang *et al*. 2017 (73)	Human	PBMCs	Down
miR-148a-3p	Nielsen *et al*. (22)	Human	Serum	Up
	Takahashi *et al*. (23)	Human	PBMCs	Up
	Seyhan *et al*. (70)	Human	Plasma	Up
miR-148b-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-150-5p	Estrella *et al*. (65)	Human	PBMCs	Down
	Wang *et al*. 2017 (73)	Human	PBMCs	Down
miR-151-3p	Hezova *et al*. (49)	Human	T cells	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-154-3p	Tian *et al*. (60)	Mice	Pancreas	Up
	Erener *et al*. (71)	Human	Serum	Down
miR-15b	Takahashi *et al*. (23)	Human	PBMCs	Up
	Yang *et al*. (24)	Human	PBMCs	Down
miR-16-5p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Garcia-Contreras *et al*. (72)	Human	Plasma-derived exosome	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-181a-5p	Nielsen *et al*. (22)	Human	Serum	Up
	Nabih *et al*. (62)	Human	Serum	Up
miR-199a-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Sebastiani *et al*. (68)	Human	Serum	Down
miR-19a-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-200c-3p	Nielsen *et al*. (22)	Human	Serum	Up
	Yang *et al*. (24)	Human	PBMCs	Down
miR-20b-5p	Hezova *et al*. (49)	Human	T cells	Down
	Takahashi *et al*. (23)	Human	PBMCs	Up
miR-210-5p	Nielsen *et al*. (22)	Human	Serum	Up
	Osipova a *et al*. (52)	Human	Serum	Up
miR-21-5p	Seyhan *et al*. (70)	Human	Plasma	Up
	Nielsen *et al*. (22)	Human	Serum	Up
	Osipova *et al*. (52)	Human	Serum	Up
	Takahashi *et al*. (23)	Human	PBMCs	Up
miR-221-3p	Erener *et al*. 2017 (71)	Human	Serum	Up
	Yang *et al*. (24)	Human	PBMCs	Down

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Table 2 Continued.

miRNA ID	First author (ref.)	Species	Sample type	Change of expression
miR-22-3p	Yang *et al.* (24)	Human	PBMCs	Down
	Estrella *et al.* (65)	Human	PBMCs	Up
miR-24-3p	Seyhan *et al.* (70)	Human	Plasma	Up
	Erener *et al.* 2017 (71)	Human	Serum	Up
	Nielsen *et al.* (22)	Human	Serum	Up
miR-25-3p	Nielsen *et al.* (22)	Human	Serum	Up
	Garcia-Contreras *et al.* (72)	Human	Plasma-derived exosome	Up
	Erener *et al.* 2017 (71)	Human	Serum	Up
	Yang *et al.* (24)	Human	PBMCs	Down
miR-26a-5p	Nielsen *et al.* (22)	Human	Serum	Up
	Ma *et al.* (61)	Mice	Pancreas	Down
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-26b-5p	Nielsen *et al.* (22)	Human	Serum	Up
	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-27a-3p	Nielsen *et al.* (22)	Human	Serum	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-27b-3p	Nielsen *et al.* (22)	Human	Serum	Up
	Takahashi *et al.* (23)	Human	PBMCs	Up
miR-30b-3p	Tian *et al.* (60)	Mice	Pancreas	Up
	Yang *et al.* (24)	Human	PBMCs	Down
miR-324-3p	Tian *et al.* (60)	Mice	Pancreas	Down
	Erener *et al.* 2017 (71)	Human	Serum	Up
miR-324-5p	Takahashi *et al.* (23)	Human	PBMCs	Down
	Erener *et al.* 2017 (71)	Human	Serum	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-32-5p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-335-5p	Hezova *et al.* (49)	Human	T cells	Down
	Takahashi *et al.* (23)	Human	PBMCs	Up
miR-342-3p	Takahashi *et al.* (23)	Human	PBMCs	Down
	Sebastiani *et al.* (68)	Human	Serum	Up
	Yang *et al.* (24)	Human	PBMCs	Down
miR-375	Erener *et al.* (63)	Mice	Plasma	Up
	Marchand *et al.* (66)	Human	Serum	Up
	Sebastiani *et al.* (68)	Human	Serum	Down
	Seyhan *et al.* (70)	Human	Plasma	Up
miR-377-3p	Sebastiani *et al.* (68)	Human	Serum	Down
	Erener *et al.* 2017 (71)	Human	Serum	Up
miR-378	Erener *et al.* (63)	Mice	Plasma	Up
	Garcia-Contreras *et al.* (72)	Human	Plasma-derived exosome	Down
miR-424-5p	Wang *et al.* 2017 (73)	Human	PBMCs	Down
	Takahashi *et al.* (23)	Human	PBMCs	Up
miR-450a-2-3p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Up
miR-454-3p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Erener *et al.* 2017 (71)	Human	Serum	Up
miR-490-5p	Tian *et al.* (60)	Mice	Pancreas	Up
	Erener *et al.* 2017 (71)	Human	Serum	Down
miR-574-3p	Garcia-Contreras *et al.* (72)	Human	Plasma-derived exosome	Down
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-720	Takahashi *et al.* (23)	Human	PBMCs	Down
	Erener *et al.* 2017 (71)	Human	Serum	Down
miR-9-3p	Tian *et al.* (60)	Mice	Pancreas	Up
	Sebastiani *et al.* (68)	Human	Serum	Down
miR-98-5p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Down

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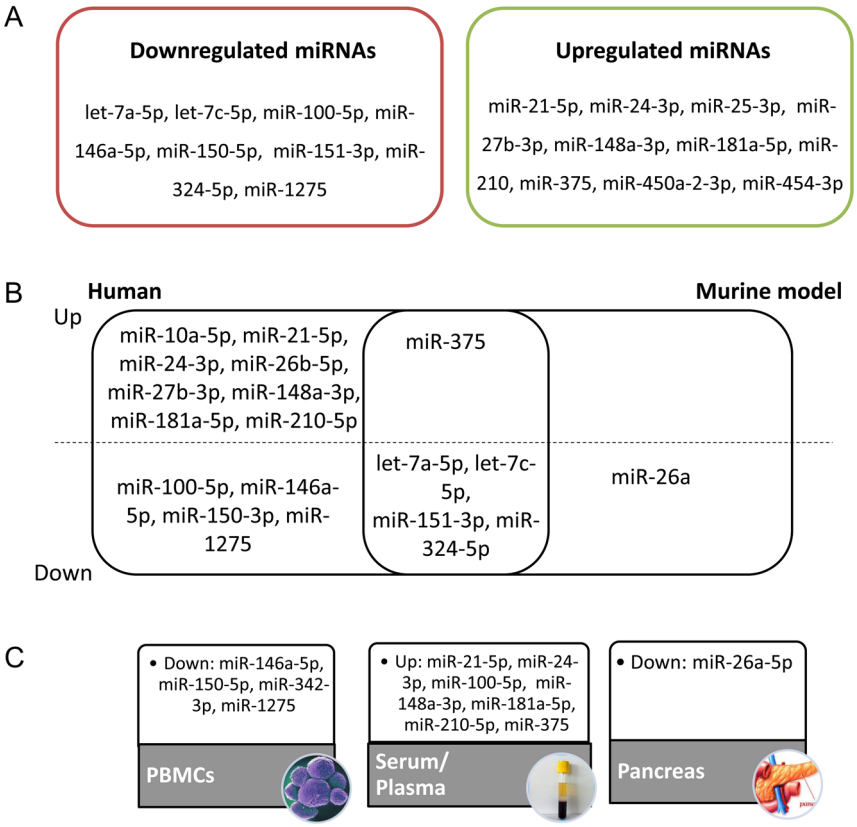

Figure 2
miRNA expression analyzed in at least two studies considering their expression profile in all tissues analyzed independently of the species (A). miRNA expression profile between the species (B). miRNA expression profile in tissues related to T1DM pathogenesis (C).

Moreover, 3 miRNAs (miR-151-3p, let-7a-5p and let-7c-5p) were downregulated in pancreas from diabetic mice as well as PBMCs/T cells from T1DM patients compared to the respective control groups. Inversely, several miRNAs were downregulated in pancreas from diabetic mice but upregulated in PBMCs or serum/plasma from T1DM patients (Table 2), which might reflect differential expression in tissues and/or species.

Perturbed pathways in type 1 diabetes mellitus

Bioinformatic analyses were performed to retrieve putative targets and pathways potentially modulated by 12 miRNAs (hsa-miR-21-5p, hsa-miR-24-3p, mmu-miR-26a-5p, hsa-miR-100-5p, hsa-miR-146a-5p, hsa-miR-148a-3p, has-miR-150-5p, hsa-miR-181a-5p, hsa-miR-210-5p, hsa-miR-342-3p, hsa-miR-375 and hsa-miR-1275) consistently dysregulated in T1DM-related tissues. Species prefixes were used in miRNA identifiers to clearly designate the species under consideration while reporting these results. First, we searched for targets of these miRNAs using 6 distinct resources, including experimentally validated databases and prediction programs (Supplementary Fig. 1). A total of 5867 validated and 2979 predicted miRNA–target interactions were retrieved for human miRNAs, while 573 validated and 453 predicted interactions were retrieved for the mmu-miR-26a-5p (Table 3; Supplementary Table 2).

After target prediction, we performed functional enrichment analysis of miRNA target genes using pathway maps from the KEGG Pathway Database, aiming to better understand the biological pathways affected by the selected miRNAs. Out of 518 pathways annotated in KEGG Database (accessed in August 2017), a total of 127 pathways were significantly overrepresented (q-value <0.05) in the putative target lists analyzed, and 77 KEGG terms were enriched for more than one miRNA. Targets of hsa-miR-21-5p, hsa-miR-24-3p, mmu-miR-26a-5p, hsa-miR-100-5p, hsa-miR-146a-5p, hsa-miR-148a-3p, hsa-miR-150-5p hsa-miR-181a-5p, hsa-miR-342-5p and hsa-miR-375 are involved in several pathways (Supplementary Table 3), many of them having a recognized role in T1DM pathogenesis, such as TNF, MAPK, Jak-STAT, PI3K-Akt, apoptosis, insulin, toll-like receptors (TLRs) and T cell receptor (TCR) signaling pathways (Supplementary Table 3). No significantly enriched KEGG terms were found for hsa-miR-210-5p and hsa-miR-1275, probably due to the small number of retrieved targets (40 and 121, respectively), as well as for hsa-miR-150-5p despite its broad regulatory action.

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Table 3 Number of miRNA-target interactions for each analyzed miRNA considered individually and grouped by tissue related to T1DM.

	miRNA/tissue	Validated interactions	Predicted interactions
Analysis by miRNA	hsa-miR-1275	121	–
	has-miR-100-5p	279	14
	hsa-miR-146a-5p	300	409
	hsa-miR-148a-3p	621	375
	has-miR-150-5p	637	433
	hsa-miR-181a-5p	1159	361
	hsa-miR-21-5p	725	198
	has-miR-24-3p	1052	450
	hsa-miR-210-5p	40	–
	hsa-miR-342-3p	433	436
	hsa-miR-375	500	303
	mmu-miR-26a-5p	573	453
Analysis by tissue related to T1DM	PBMCs	1491	1278
	Serum/Plasma	4376	1701
	Pancreas	573	453

PBMCs, peripheral blood mononuclear cells.

Considering different T1DM-related tissues, results indicated 2539 targets in PBMCs, 4665 targets in serum/plasma and 1026 targets in pancreas for the selected miRNAs (Table 3), where numbers reflect the size of the non-redundant set of target genes found for the group of miRNAs differentially expressed in each T1DM-related tissue. Forty-five significant KEGG pathways were found for targets of miRNAs dysregulated in PBMCs, which included NF-KB, apoptosis and neurotrophin signaling pathways. Similarly, 17 KEGG terms were found in pancreas, including Wnt and phosphatidylinositol signaling pathways. For serum and plasma, 94 significant KEGG terms were found, comprising signaling pathways by TNF, Jak-STAT, MAPK, TCR and insulin as well as pathways associated to protein processing in endoplasmic reticulum and apoptosis, which have key roles in T1DM pathogenesis (Supplementary Table 4; Fig. 3).

Next, we searched for KEGG terms linked to T1DM pathogenesis regardless of the functional enrichment analysis of miRNA targets and found 5 significant signaling pathways associated with this disease: type 1 diabetes (KEGG hsa04949), TCR (KEGG hsa04660), cytokine–cytokine receptor interaction (KEGG hsa04060), Jak-STAT (KEGG hsa04630) and neurotrophin (KEGG hsa04722). Then, TCR and Jak-STAT pathways were selected for further detailed analysis since they are targeted by most of the miRNAs in the list of interest (11 miRNAs each).

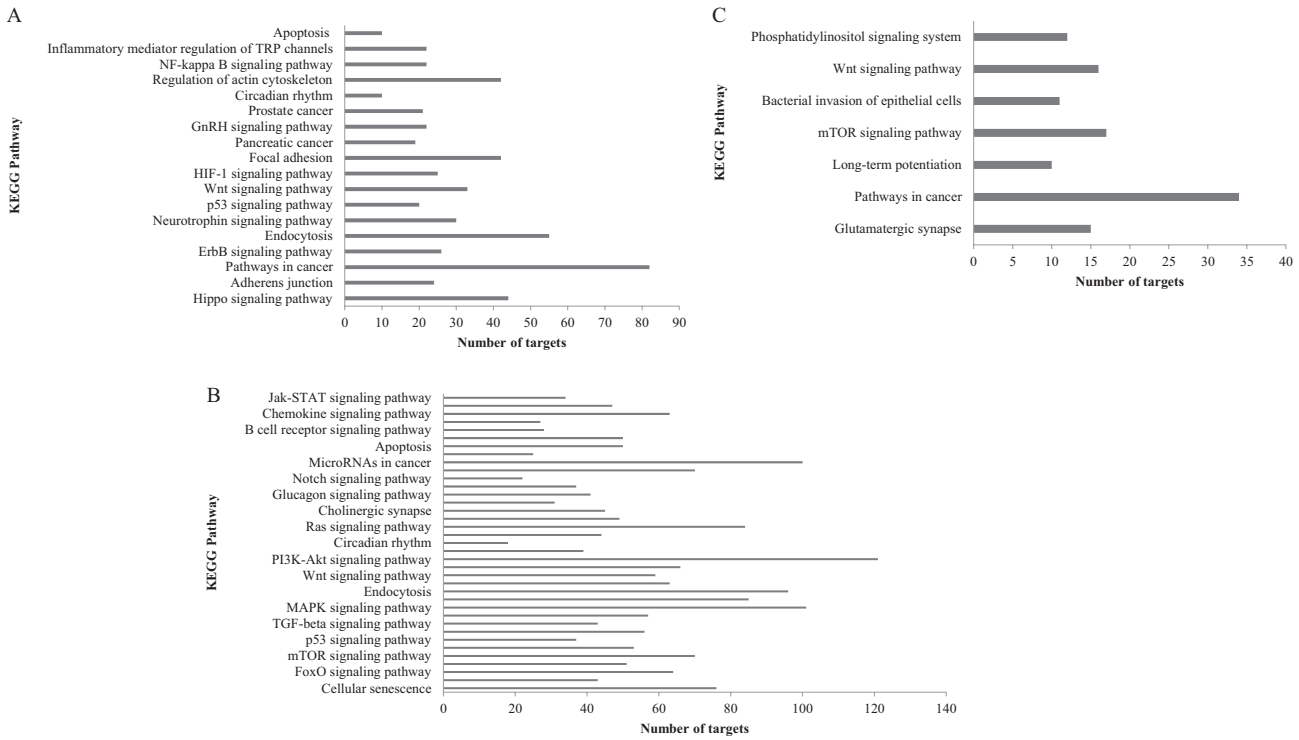

Figure 3
KEGG pathway functional annotation of the differentially miRNAs expressed in PBMCs (A), serum/plasma (B) and pancreas (C). Enrichment scores corresponding to each pathway are displayed as number of targets.

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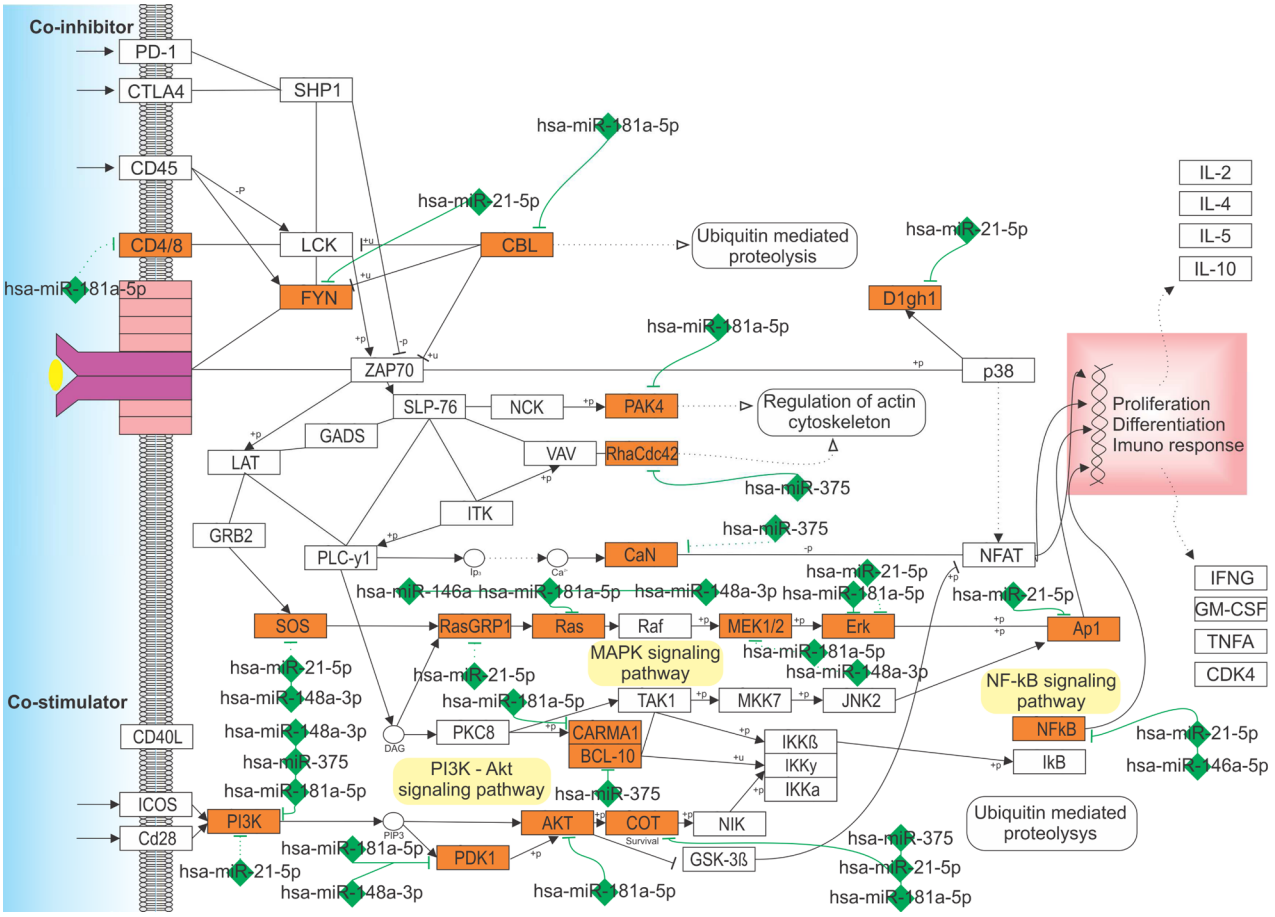

Figure 4
Schematic diagram of the selected miRNA-mRNA interaction networks involved in T cell receptor signaling pathway. The network was built based on KEGG pathway map (KEGG: hsa04660). The miRNAs are indicated by rhombus. The predicted miRNA-mRNA interactions are indicated by dotted lines and the thick lines indicate validated miRNA-mRNA interactions.

In the TCR signaling pathway (Fig. 4), miR-181a-5p directly targets mRNAs for CD4⁺ and CD8⁺ cell receptors. Moreover, this miRNA post-transcriptionally regulates genes associated with PI3K-Akt (*PI3K*, *Akt*, *COT*), actin cytoskeleton (*PAK4*) and ubiquitin mediated proteolysis (*CBL*) pathways, which are triggered after activation of T cell and co-stimulatory receptors. miR-21-5p targets mRNAs from MAPK (*Ras*, *ErkSOS* and *RasGRP1*) and ubiquitin-mediated proteolysis (*FYN*) pathways. Furthermore, miR-146a-5p targets mRNAs from MAPK (*Ras*) and NF-κB signaling pathways. miR-148a-5p, miR-100-5p, miR-24-3p and miR-150-5p target mRNAs from ubiquitin-mediated proteolysis (*CBL*), PI3K-Akt, NF-κB and MAPK pathways (Fig. 4).

In the Jak-STAT signaling pathway (Fig. 5), miR-21-5p, miR-24-3p, miR-181a-5p and miR-210-5p target mRNAs for different cytokine and hormone receptors, such as IL6R, LIFR, IL2RB and IFNLR1. miR-375 targets *JAK2* mRNA while miR-181a-5p and miR-21-5p bind to *STAT3* mRNA. In addition, miR-375, miR-181a-5p, miR-146a-5p, miR-148a-3p, miR-100-5p, miR-150-5p and miR-21-5p target different mRNAs codifying proteins related to apoptosis (BCL2, SOCS, PIAS and MCL1), cell cycle progression (cMyc), cell cycle inhibition (p21), proliferation and differentiation (SHP2, SOS and Ras) and cell survival (PI3K and Akt) (Fig. 5).

Discussion

Since the exact origin of T1DM remains uncertain, the discovery of novel biomarkers and their implications in T1DM pathogenesis may contribute to a better understanding of the mechanisms involved in this disease. Clinically, new biomarkers might enable an earlier T1DM diagnosis as well as a more adequate treatment of T1DM

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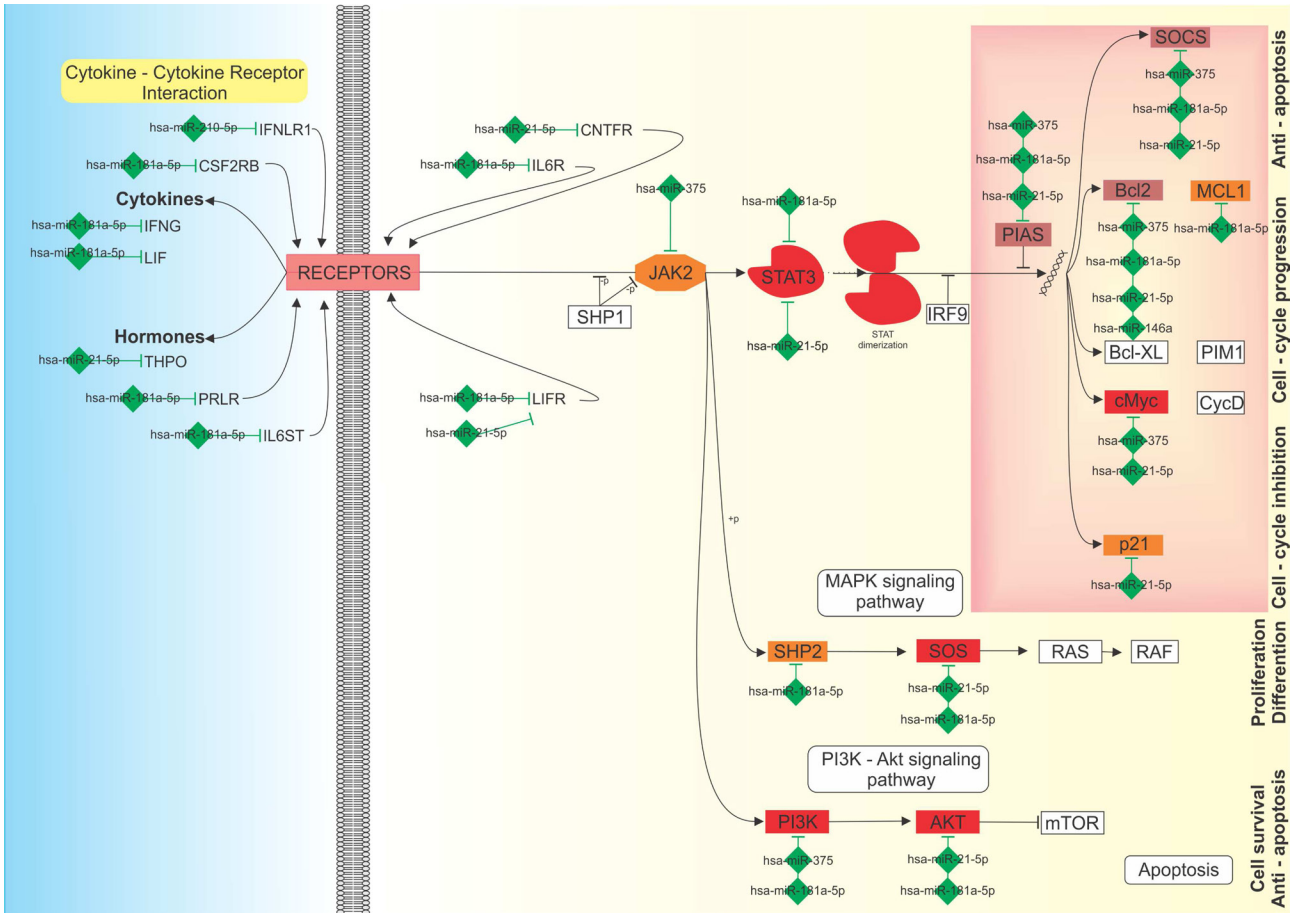

Figure 5
Schematic diagram of the selected miRNA-mRNA interaction networks involved in Jak-STAT signaling pathway. The network was built based on KEGG pathway map (KEGG: hsa04630). The miRNAs are indicated by rhombus. The predicted miRNA-mRNA interactions are indicated by dotted lines and the thick lines indicate validated miRNA-mRNA interactions.

patients, improving their quality of life (23). Circulating miRNAs are ideal biomarkers because they are stable and resistant to degradation by ribonucleases or repeated freezing/thawing cycles and can be easily detected in body fluids by highly sensitive and specific quantitative RT-PCR (2). Thus, as part of the ongoing effort to identify a profile of circulating miRNAs as biomarkers of T1DM, we performed a systematic review of studies that evaluated miRNA expressions in tissues from T1DM patients and non-diabetic controls. Eleven circulating miRNAs (miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275) were consistently dysregulated in T1DM patients, suggesting that they may be potential minimally invasive biomarkers of this disease.

miR-21-5p, miR-24-3p, miR-148a-3p, miR-181a-5p, miR-210-5p and miR-375 seem to be upregulated in serum/plasma or PBMCs from T1DM compared to controls (Table 2). Emerging studies have indicated diverse roles of miR-21-5p in immunity (75). Particularly, this miRNA acts in TCR signaling transduction, augmenting T cell proliferation (76); regulates Th1 vs Th2 responses (77) and Treg development (78) and is a key mediator of the anti-inflammatory response in macrophages (79). miR-21-5p also appears to have anti-inflammatory and anti-apoptotic effects since it inhibits the proinflammatory tumor suppressor programed cell death protein 4 (PDCD4), which promotes the activation of NF-KB and suppresses IL-10 (80). Several studies have reported increased miR-21-5p expression in diseases characterized by impaired immune responses, including asthma, cancer, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), chronic bacterial and viral infections (14, 79, 81, 82) and T1DM (reviewed here).

The role of miR-21-5p in beta-cells has not yet been clearly elucidated, but its function also seems to rely on the

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effect of cytokines via NF-KB (83). IL-1β and TNF strongly induce miR-21-5p expression in both insulin-secreting MIN6 cells and human islets (18). Moreover, miR-21-5p expression was increased during the development of pre-diabetic insulitis in islets from NOD mice, possibly as a protective response since miR-21-5p knockdown in MIN-6 cells promoted apoptosis (18). Accordingly, Ruan and coworkers (84) reported that NF-KB activated miR-21-5p in beta-cells, decreasing PDCD4 levels, inhibiting NF-KB activity in a negative regulatory loop, thus rendering beta-cells resistant to death. In contrast, Backe and coworkers (85) showed that miR-21-5p overexpression potentiated cell death after exposure to proinflammatory cytokines, leading to a reduced beta-cell number. Also, overexpression of miR-21-5p led to impaired glucose-stimulated insulin secretion through decreased *VAMP2* expression, a secretory granule protein that is essential for insulin exocytosis (18).

miR-148a-3p is a potent regulator of B cell tolerance and autoimmunity through suppression of *GADD45a*, *PTEN* and *BCL2l11* expressions (86). In agreement with this study, Pan and coworkers (81) showed elevated miR-148a-3p and miR-21-5p expression in $CD4^+$ T cells of SLE patients, which contributed to DNA hypomethylation by targeting the DNA methyltransferase 1 (DNMT1). Melkman-Zehavi and coworkers (87) reported that knockdown of miR-148a-3p in primary islets from mice or cultured beta-cells decreased insulin promoter activity and insulin mRNA levels.

miR-181a-5p also has a recognized role in the immune system (17, 88). This miRNA increases $CD19^+$ B populations and regulates T cell function (17, 89, 90, 91). miR-181a-5p seems to 'tune' TCR signal strength by targeting tyrosine phosphatases *SHP-2*, *PTPN22* and *DUSP5/6*, which enhances the basal activation of the TCR signaling molecules LcK and Erk, thus having an important role in thymic positive and negative selection (17, 88, 90). Xie and coworkers (92) reported that LPS and STZ strongly induced miR-181a-5p in macrophages from mice. Moreover, this miRNA was upregulated in patients with RA, which correlated with levels of proinflammatory cytokines (92). In this context, miR-181a-5p seems to have an anti-inflammatory function since it inhibited the increase of IL-1β, IL-6 and TNF in macrophages treated with LPS (93).

miR-210-5p is currently considered as a 'master miRNA' of hypoxic response as it was found upregulated by hypoxia in several cell types analyzed (94, 95). Consequently, miR-210-5p has been linked to various cancers and cardiovascular diseases (95, 96, 97, 98). Targets of this miRNA are involved in mitochondrial metabolism, angiogenesis, DNA repair and cell survival (95). Given that miR-210-5p targets many mitochondrial components, it is not surprising that manipulation of this miRNA leads to mitochondrial dysfunction and oxidative stress (94).

miR-375 is the most abundant miRNA detected in islets and is important for the development and maintenance of normal alpha- and beta-cell mass and insulin synthesis and secretion (16, 99, 100). Consequently, this miRNA has been proposed as a biomarker to detect beta-cell death and to predict the development of T1DM (2, 63, 66). Accordingly, massive beta-cell loss elicited by administration of STZ in C57BL/6 mice caused a dramatic increase in circulating levels of miR-375 (63). Moreover, plasma levels of this miRNA were increased in NOD mice 2 weeks before the onset of T1DM (63).

miR-24-3p seems to mark better preserved beta-cell function and/or insulin sensitivity 12 months after diagnosis (101). Moreover, this miRNA was elevated in serum of T1DM children (22), and its overexpression has been shown to inhibit beta-cell proliferation and insulin secretion (102).

miR-146a-5p, miR-150-5p, miR-342-3p, miR-1275 and miR-100-5p seem to be downregulated in serum/plasma or PBMCs from T1DM patients (Table 2). miR-146a-5p regulates Treg-mediated suppression of IFNγ-dependent Th1 responses and associated autoimmunity by directly targeting *STAT-1* (103), which was confirmed in our bioinformatic analysis (Supplementary Table 3). This miRNA has also a recognized role in innate immunity by negatively regulating the inflammatory response after recognition of bacterial components by TLRs on monocytes and macrophages (104). Upon activation by TNF and IL-1β (18), this miRNA downregulates *TRAF-6* and *IRAK-1* expressions, decreasing NF-KB activity, which seems to be a fine-tuning mechanism that prevents the overstimulation of the TLR pathway (104, 105). In disagreement with the downregulation of miR-146-5p in serum/PBMCs from T1DM patients, Roggli and coworkers (18) reported that this miRNA was increased in islets from NOD mice during development of insulitis. Blocking miR-146a-5p protected MIN6 cells from cytokine-induced apoptosis and also prevented the reduction in glucose-stimulated insulin secretion observed after IL-1β exposure (18).

Although literature on miR-100 is sparse, it has been implicated in some types of cancer (106, 107, 108) and tissue differentiation (109, 110, 111). Expression of circulating miR-100-5p was significantly decreased

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in obese normoglycemic subjects and subjects with type 2 diabetes mellitus (T2DM) compared to healthy, lean individuals. Moreover, in visceral adipose tissue, expression of miR-100 was lower in obese subjects with T2DM compared to obese subjects without T2DM (112).

miR-342-3p has been found to be dysregulated in different cancers (113, 114, 115), SLE (14), obesity (116, 117), diabetic kidney disease (118), T2DM and gestational diabetes (119). It is involved in beta-cell differentiation and maturation by targeting *FOXA2* and *MAFB* (120). Furthermore, miR-342-3p was downregulated in human leukocytes in response to LPS (121) and in Tregs from T1DM patients (49), suggesting that this miRNA may be involved in the development of autoimmunity and inflammation in T1DM patients.

Only a few studies have analyzed miR-1275 expression in different diseases. Due to its downregulation in certain cancers, miR-1275 has been referred to as a tumor suppressor (122, 123, 124, 125). In a HuH-7 hepatocarcinome cell line, miR-1275 overexpression suppressed *IGF2BP* expression, effectively impairing tumor cell proliferation, migration, viability and colony formation (124). The role of this miRNA in autoimmunity and beta-cell function is unknown.

Bezman and coworkers (126) showed that miR-150 plays a critical role in the innate immune system, and decreased expression level of miR-150-5p was negatively associated with GADA autoantibody titers, independently of hyperglycemia and disease duration (73).

Our bioinformatic analysis suggest that miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275 significantly regulate several mRNAs from pathways related to immune system and T1DM pathogenesis, such as MAPK, Jak-STAT, NF-KB, PI3K-Akt, apoptosis, TNF, TLRs, insulin and TCR signaling pathways (Figs 3, and). This might help to raise hypothesis about genes and pathways under influence of these circulating miRNAs.

Regarding the pathway analysis methods, there are many tools in literature that provide support for this type of investigation. Nonetheless, they are very similar as they all calculate the enrichment *P* values of pathways for a user pre-selected list of genes using a statistical test and a database with functional annotation that links genes to biological pathways. In addition, the hypergeometric test, which we adopted in our paper, has been widely applied in literature and previous studies discuss that when identifying significant pathways, the differences among the statistical methods will not be dramatic (127). The functional annotation has been traditionally performed in literature using annotations derived either from Gene Ontology (GO) or KEGG Pathway. KEGG Pathway Database has less annotated terms compared to GO, but it covers a wide range of molecular mechanisms and diseases, providing a graphical description of pathways, which is an important resource in the interpretation of results. Thus, bioinformatic tools used in this study provide robust data, which might be very similar to those generated using different tools.

In conclusion, this systematic review and bioinformatic analysis suggest that 11 circulating miRNAs (miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275) are consistently dysregulated in T1DM patients. Further studies aiming at clarifying the specific role of these 11 miRNAs in pancreatic islets and islet-infiltrating immune cells are needed to shed light if they are biomarkers of T1DM and which are their specific roles in beta-cell function.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EC-17-0248.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

1 American Diabetes Association. Classification and diagnosis of diabetes. *Diabetes Care* 2015 **38** S8–S16. (doi:10.2337/dc15-S005)
2 Guay C & Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. *Nature Reviews Endocrinology* 2013 **9** 513–521. (doi:10.1038/nrendo.2013.86)
3 Atkinson MA & Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 2001 **358** 221–229. (doi:10.1016/S0140-6736(01)05415-0)
4 Pirot P, Cardozo AK & Eizirik DL. Mediators and mechanisms of pancreatic beta-cell death in type 1 diabetes. *Arquivos Brasileiros De Endocrinologia e Metabologia* 2008 **52** 156–165. (doi:10.1590/S0004-27302008000200003)

5 Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, Winkler C, Ilonen J, Veijola R, Knip M, *et al*. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. *JAMA* 2013 **309** 2473–2479. (doi:10.1001/jama.2013.6285)

6 Eisenbarth GS & Jeffrey J. The natural history of type 1A diabetes. *Arquivos Brasileiros De Endocrinologia e Metabologia* 2008 **52** 146–155. (doi:10.1590/S0004-27302008000200002)

7 Purohit S & She JX. Biomarkers for type 1 diabetes. *International Journal of Clinical and Experimental Medicine* 2008 **1** 98–116.

8 Bonifacio E. Predicting type 1 diabetes using biomarkers. *Diabetes Care* 2015 **38** 989–996. (doi:10.2337/dc15-0101)

9 Esteller M. Non-coding RNAs in human disease. *Nature Reviews Genetics* 2011 **12** 861–874. (doi:10.1038/nrg3074)

10 Butz H, Kinga N, Racz K & Patocs A. Circulating miRNAs as biomarkers for endocrine disorders. *Journal of Endocrinological Investigation* 2016 **39** 1–10. (doi:10.1007/s40618-015-0316-5)

11 Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009 **136** 215–233. (doi:10.1016/j.cell.2009.01.002)

12 Carrington JC & Ambros V. Role of microRNAs in plant and animal development. *Science* 2003 **301** 336–338. (doi:10.1126/science.1085242)

13 Lin S & Gregory RI. MicroRNA biogenesis pathways in cancer. *Nature Reviews Cancer* 2015 **15** 321–333. (doi:10.1038/nrc3932)

14 Pauley KM, Cha S & Chan EK. MicroRNA in autoimmunity and autoimmune diseases. *Journal of Autoimmunity* 2009 **32** 189–194. (doi:10.1016/j.jaut.2009.02.012)

15 Zalts H & Shomron N. The impact of microRNAs on endocrinology. *Pediatric Endocrinology Reviews* 2011 **8** 354–362.

16 Guay C, Roggli E, Nesca V, Jacovetti C & Regazzi R. Diabetes mellitus, a microRNA-related disease? *Translational Research* 2011 **157** 253–264. (doi:10.1016/j.trsl.2011.01.009)

17 Baltimore D, Boldin MP, O'Connell RM, Rao DS & Taganov KD. MicroRNAs: new regulators of immune cell development and function. *Nature Immunology* 2008 **9** 839–845. (doi:10.1038/ni.f.209)

18 Roggli E, Britan A, Gattesco S, Lin-Marq N, Abderrahmani A, Meda P & Regazzi R. Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. *Diabetes* 2010 **59** 978–986. (doi:10.2337/db09-0881)

19 Zheng Y, Wang Z, Tu Y, Shen H, Dai Z, Lin J & Zhou Z. miR-101a and miR-30b contribute to inflammatory cytokine-mediated beta-cell dysfunction. *Laboratory Investigation* 2015 **95** 1387–1397. (doi:10.1038/labinvest.2015.112)

20 Salas-Perez F, Codner E, Valencia E, Pizarro C, Carrasco E & Perez-Bravo F. MicroRNAs miR-21a and miR-93 are down regulated in peripheral blood mononuclear cells (PBMCs) from patients with type 1 diabetes. *Immunobiology* 2013 **218** 733–737. (doi:10.1016/j.imbio.2012.08.276)

21 Sebastiani G, Grieco FA, Spagnuolo I, Galleri L, Cataldo D & Dotta F. Increased expression of microRNA miR-326 in type 1 diabetic patients with ongoing islet autoimmunity. *Diabetes/Metabolism Research and Reviews* 2011 **27** 862–866. (doi:10.1002/dmrr.1262)

22 Nielsen LB, Wang C, Sorensen K, Bang-Berthelsen CH, Hansen L, Andersen ML, Hougaard P, Juul A, Zhang CY, Pociot F, *et al*. Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. *Experimental Diabetes Research* 2012 **2012** 896362. (doi:10.1155/2012/672865)

23 Takahashi P, Xavier DJ, Evangelista AF, Manoel-Caetano FS, Macedo C, Collares CV, Foss-Freitas MC, Foss MC, Rassi DM, Donadi EA, *et al*. MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus. *Gene* 2014 **539** 213–223. (doi:10.1016/j.gene.2014.01.075)

24 Yang M, Ye L, Wang B, Gao J, Liu R, Hong J, Wang W, Gu W & Ning G. Decreased miR-146 expression in peripheral blood mononuclear cells is correlated with ongoing islet autoimmunity in type 1 diabetes patients. *Journal of Diabetes* 2015 **7** 158–165. (doi:10.1111/1753-0407.12163)

25 Abuhatzira L, Xu H, Tahhan G, Boulougoura A, Schaffer AA & Notkins AL. Multiple microRNAs within the 14q32 cluster target the mRNAs of major type 1 diabetes autoantigens IA-2, IA-2beta, and GAD65. *FASEB Journal* 2015 **29** 4374–4383. (doi:10.1096/fj.15-273649)

26 Moher D, Liberati A, Tetzlaff J, Altman DG & Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Journal of Clinical Epidemiology* 2009 **62** 1006–1012. (doi:10.1016/j.jclinepi.2009.06.005)

27 Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA & Thacker SB. Meta-analysis of observational studies in epidemiology: a proposal for reporting Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 2000 **283** 2008–2012. (doi:10.1001/jama.283.15.2008)

28 Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, Leeflang MM, Sterne JA, Bossuyt PM & Group Q-. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Annals of Internal Medicine* 2011 **155** 529–536. (doi:10.7326/0003-4819-155-8-201110180-00009)

29 Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, *et al*. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nature Genetics* 2001 **29** 365–371. (doi:10.1038/ng1201-365)

30 Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, *et al*. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* 2009 **55** 611–622. (doi:10.1373/clinchem.2008.112797)

31 Chou CH, Chang NW, Shrestha S, Hsu SD, Lin YL, Lee WH, Yang CD, Hong HC, Wei TY, Tu SJ, *et al*. miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. *Nucleic Acids Research* 2016 **44** D239–D247. (doi:10.1093/nar/gkv1258)

32 Li JH, Liu S, Zhou H, Qu LH & Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Research* 2014 **42** D92–D97. (doi:10.1093/nar/gkt1248)

33 Xiao F, Zuo Z, Cai G, Kang S, Gao X & Li T. miRecords: an integrated resource for microRNA-target interactions. *Nucleic Acids Research* 2009 **37** D105–D110. (doi:10.1093/nar/gkn851)

34 Agarwal V, Bell GW, Nam JW & Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *eLife* 2015 **4** e05005. (doi:10.7554/elife.05005)

35 Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, Filippidis C, Dalamagas T & Hatzigeorgiou AG. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. *Nucleic Acids Research* 2013 **41** W169–W173. (doi:10.1093/nar/gkt393)

36 Betel D, Koppal A, Agius P, Sander C & Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biology* 2010 **11** R90. (doi:10.1186/gb-2010-11-8-r90)

37 Betel D, Wilson M, Gabow A, Marks DS & Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Research* 2008 **36** D149–D153. (doi:10.1093/nar/gkm995)

38 Wain HM, Bruford EA, Lovering RC, Lush MJ, Wright MW & Povey S. Guidelines for human gene nomenclature. *Genomics* 2002 **79** 464–470. (doi:10.1006/geno.2002.6748)

39 Gray KA, Yates B, Seal RL, Wright MW & Bruford EA. Genenames.org: the HGNC resources in 2015. *Nucleic Acids Research* 2015 **43** D1079–D1085. (doi:10.1093/nar/gku1071)

40 Bult CJ, Eppig JT, Blake JA, Kadin JA, Richardson JE & Mouse Genome Database G. Mouse genome database 2016. *Nucleic Acids Research* 2016 **44** D840–D847. (doi:10.1093/nar/gkv1211)

http://www.endocrineconnections.org
DOI: 10.1530/EC-17-0248

41 Eppig JT, Blake JA, Bult CJ, Kadin JA, Richardson JE & Mouse Genome Database G. The Mouse Genome Database (MGD): facilitating mouse as a model for human biology and disease. *Nucleic Acids Research* 2015 **43** D726–D736. (doi:10.1093/nar/gku967)
42 Kanehisa M, Sato Y, Kawashima M, Furumichi M & Tanabe M. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Research* 2016 **44** D457–D462. (doi:10.1093/nar/gkv1070)
43 Kanehisa M & Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* 2000 **28** 27–30. (doi:10.1093/nar/28.1.27)
44 Yu G, Wang LG, Han Y & He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 2012 **16** 284–287. (doi:10.1089/omi.2011.0118)
45 The R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2013.
46 Barutta F, Tricarico M, Corbelli A, Annaratone L, Pinach S, Grimaldi S, Bruno G, Cimino D, Taverna D, Deregibus MC, *et al*. Urinary exosomal microRNAs in incipient diabetic nephropathy. *PLoS ONE* 2013 **8** e73798. (doi:10.1371/journal.pone.0073798)
47 Diao X, Shen E, Wang X & Hu B. Differentially expressed microRNAs and their target genes in the hearts of streptozotocin-induced diabetic mice. *Molecular Medicine Reports* 2011 **4** 633–640.
48 Emadi SS, Soufi FG, Khamaneh AM & Alipour MR. MicroRNA-146a expression and its intervention in NF-small ka, CyrillicB signaling pathway in diabetic rat aorta. *Endocrine Regulations* 2014 **48** 103–108. (doi:10.4149/endo_2014_02_103)
49 Hezova R, Slaby O, Faltejskova P, Mikulkova Z, Buresova I, Raja KR, Hodek J, Ovesna J & Michalek J. microRNA-342, microRNA-191 and microRNA-510 are differentially expressed in T regulatory cells of type 1 diabetic patients. *Cellular Immunology* 2010 **260** 70–74. (doi:10.1016/j.cellimm.2009.10.012)
50 Kato M, Wang L, Putta S, Wang M, Yuan H, Sun G, Lanting L, Todorov I, Rossi JJ & Natarajan R. Post-transcriptional up-regulation of Tsc-22 by Ybx1, a target of miR-216a, mediates TGF-beta-induced collagen expression in kidney cells. *Journal of Biological Chemistry* 2010 **285** 34004–34015. (doi:10.1074/jbc.M110.165027)
51 Kovacs B, Lumayag S, Cowan C & Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. *Investigative Ophthalmology and Visual Science* 2011 **52** 4402–4409. (doi:10.1167/iovs.10-6879)
52 Osipova J, Fischer DC, Dangwal S, Volkmann I, Widera C, Schwarz K, Lorenzen JM, Schreiver C, Jacoby U, Heimhalt M, *et al*. Diabetes-associated microRNAs in pediatric patients with type 1 diabetes mellitus: a cross-sectional cohort study. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** E1661–E1665. (doi:10.1210/jc.2013-3868)
53 Silva VA, Polesskaya A, Sousa TA, Correa VM, Andre ND, Reis RI, Kettelhut IC, Harel-Bellan A & De Lucca FL. Expression and cellular localization of microRNA-29b and RAX, an activator of the RNA-dependent protein kinase (PKR), in the retina of streptozotocin-induced diabetic rats. *Molecular Vision* 2011 **17** 2228–2240.
54 Wang Q, Bozack SN, Yan Y, Boulton ME, Grant MB & Busik JV. Regulation of retinal inflammation by rhythmic expression of MiR-146a in diabetic retina. *Investigative Ophthalmology and Visual Science* 2014 **55** 3986–3994. (doi:10.1167/iovs.13-13076)
55 Yousefzadeh N, Alipour MR & Soufi FG. Deregulation of NF-small ka, CyrillicB-miR-146a negative feedback loop may be involved in the pathogenesis of diabetic neuropathy. *Journal of Physiology and Biochemistry* 2015 **71** 51–58. (doi:10.1007/s13105-014-0378-4)
56 Qing S, Yuan S, Yun C, Hui H, Mao P, Wen F, Ding Y & Liu Q. Serum miRNA biomarkers serve as a fingerprint for proliferative diabetic retinopathy. *Cellular Physiology and Biochemistry* 2014 **34** 1733–1740. (doi:10.1159/000366374)
57 Xiong F, Du X, Hu J, Li T, Du S & Wu Q. Altered retinal microRNA expression profiles in early diabetic retinopathy: an in silico analysis. *Current Eye Research* 2014 **39** 720–729. (doi:10.3109/02713683.2013.872280)
58 Alipour MR, Khamaneh AM, Yousefzadeh N, Mohammad-nejad D & Soufi FG. Upregulation of microRNA-146a was not accompanied by downregulation of pro-inflammatory markers in diabetic kidney. *Molecular Biology Reports* 2013 **40** 6477–6483. (doi:10.1007/s11033-013-2763-4)
59 Bacon S, Engelbrecht B, Schmid J, Pfeiffer S, Gallagher R, McCarthy A, Burke M, Concannon C, Prehn JH & Byrne MM. MicroRNA-224 is readily detectable in urine of individuals with diabetes mellitus and is a potential indicator of beta-cell demise. *Genes* 2015 **6** 399–416. (doi:10.3390/genes6020399)
60 Tian C, Ouyang X, Lv Q, Zhang Y & Xie W. Cross-talks between microRNAs and mRNAs in pancreatic tissues of streptozotocin-induced type 1 diabetic mice. *Biomedical Reports* 2015 **3** 333–342.
61 Ma H, Zhang S, Shi D, Mao Y & Cui J. MicroRNA-26a promotes regulatory T cells and suppresses autoimmune diabetes in mice. *Inflammation* 2016 **39** 1–9. (doi:10.1007/s10753-015-0215-0)
62 Nabih ES & Andrawes NG. The association between circulating levels of miRNA-181a and pancreatic beta cells dysfunction via SMAD7 in type 1 diabetic children and adolescents. *Journal of Clinical Laboratory Analysis* 2016 **30** 727–731. (doi:10.1002/jcla.21928)
63 Erener S, Mojibian M, Fox JK, Denroche HC & Kieffer TJ. Circulating miR-375 as a biomarker of beta-cell death and diabetes in mice. *Endocrinology* 2013 **154** 603–608. (doi:10.1210/en.2012-1744)
64 Garcia de la Torre N, Fernandez-Durango R, Gomez R, Fuentes M, Roldan-Pallares M, Donate J, Barabash A, Alonso B, Runkle I, Duran A, *et al*. Expression of angiogenic microRNAs in endothelial progenitor cells from type 1 diabetic patients with and without diabetic retinopathy. *Investigative Ophthalmology and Visual Science* 2015 **56** 4090–4098. (doi:10.1167/iovs.15-16498)
65 Estrella S, Garcia-Diaz DF, Codner E, Camacho-Guillen P & Perez-Bravo F. Expression of miR-22 and miR-150 in type 1 diabetes mellitus: possible relationship with autoimmunity and clinical characteristics. *Medicina Clinica* 2016 **147** 245–247. (doi:10.1016/j.medcli.2016.05.016)
66 Marchand L, Jalabert A, Meugnier E, Van den Hende K, Fabien N, Nicolino M, Madec AM, Thivolet C & Rome S. miRNA-375 a sensor of glucotoxicity is altered in the serum of children with newly diagnosed type 1 diabetes. *Journal of Diabetes Research* 2016 **2016** 1869082.
67 Li S, Chen X, Zhang H, Liang X, Xiang Y, Yu C, Zen K, Li Y & Zhang CY. Differential expression of microRNAs in mouse liver under aberrant energy metabolic status. *Journal of Lipid Research* 2009 **50** 1756–1765. (doi:10.1194/jlr.M800509-JLR200)
68 Sebastiani G, Spagnuolo I, Patti A, Grieco FA, Cataldo D, Ferretti E, Tiberti C & Dotta F. MicroRNA expression fingerprint in serum of type 1 diabetic patients. *Diabetologia* 2012 **55** S48.
69 Perez-Bravo F, Matthews DR, Haahr HL & Syed F. Differential apoptosis in lymphocytes of patients with type 1 diabetes associated with relative expression of microRNA-146a. *Diabetologia* 2014 **57** (Supplement 1) S144 abstract 332. (doi:10.1007/s00125-014-3355-0)
70 Seyhan AA, Nunez Lopez YO, Xie H, Yi F, Mathews C, Pasarica M & Pratley RE. Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study. *Scientific Reports* 2016 **6** 31479.
71 Erener S, Marwaha A, Tan R, Panagiotopoulos C & Kieffer TJ. Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. *JCI Insight* 2017 **2** e89656.
72 Garcia-Contreras M, Shah SH, Tamayo A, Robbins PD, Golberg RB, Mendez AJ & Ricordi C. Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes. *Scientific Reports* 2017 **7** 5998. (doi:10.1038/s41598-017-05787-y)
73 Wang G, Gu Y, Xu N, Zhang M & Yang T. Decreased expression of miR-150, miR146a and miR424 in type 1 diabetic patients:

http://www.endocrineconnections.org
DOI: 10.1530/EC-17-0248

association with ongoing islet autoimmunity. *Biochemical and Biophysical Research Communications* 2017 [in press]. (doi:10.1016/j.bbrc.2017.06.196)
74 Sebastiani G, Ventriglia G, Stabilini A, Socci C, Morsiani C, Laurenzi A, Nigi L, Formichi C, Mfarrej B, Petrelli A, *et al*. Regulatory T-cells from pancreatic lymphnodes of patients with type-1 diabetes express increased levels of microRNA miR-125a-5p that limits CCR2 expression. *Scientific Reports* 2017 **7** 6897. (doi:10.1038/s41598-017-07172-1)
75 Simpson LJ & Ansel KM. MicroRNA regulation of lymphocyte tolerance and autoimmunity. *Journal of Clinical Investigation* 2015 **125** 2242–2249. (doi:10.1172/JCI78090)
76 Wang L, He L, Zhang R, Liu X, Ren Y, Liu Z, Zhang X, Cheng W & Hua ZC. Regulation of T lymphocyte activation by microRNA-21. *Molecular Immunology* 2014 **59** 163–171. (doi:10.1016/j.molimm.2014.02.004)
77 Lu TX, Hartner J, Lim EJ, Fabry V, Mingler MK, Cole ET, Orkin SH, Aronow BJ & Rothenberg ME. MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-gamma pathway, Th1 polarization, and the severity of delayed-type hypersensitivity. *Journal of Immunology* 2011 **187** 3362–3373. (doi:10.4049/jimmunol.1101235)
78 Rouas R, Fayyad-Kazan H, El Zein N, Lewalle P, Rothe F, Simion A, Akl H, Mourtada M, El Rifai M, Burny A, *et al*. Human natural Treg microRNA signature: role of microRNA-31 and microRNA-21 in FOXP3 expression. *European Journal of Immunology* 2009 **39** 1608–1618. (doi:10.1002/eji.200838509)
79 Sheedy FJ. Turning 21: induction of miR-21 as a key switch in the inflammatory response. *Frontiers in Immunology* 2015 **6** 19.
80 Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, Johnson DS, Chen Y & O'Neill LA. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nature Immunology* 2010 **11** 141–147. (doi:10.1038/ni.1828)
81 Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, Li J, Zhou H, Tang Y & Shen N. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. *Open Journal of Immunology* 2010 **184** 6773–6781. (doi:10.4049/jimmunol.0904060)
82 Churov AV, Oleinik EK & Knip M. MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. *Autoimmunity Reviews* 2015 **14** 1029–1037. (doi:10.1016/j.autrev.2015.07.005)
83 Ventriglia G, Nigi L, Sebastiani G & Dotta F. MicroRNAs: novel players in the dialogue between pancreatic islets and immune system in autoimmune diabetes. *BioMed Research International* 2015 **2015** 749734.
84 Ruan Q, Wang T, Kameswaran V, Wei Q, Johnson DS, Matschinsky F, Shi W & Chen YH. The microRNA-21-PDCD4 axis prevents type 1 diabetes by blocking pancreatic beta cell death. *PNAS* 2011 **108** 12030–12035. (doi:10.1073/pnas.1101450108)
85 Backe MB, Novotny GW, Christensen DP, Grunnet LG & Mandrup-Poulsen T. Altering beta-cell number through stable alteration of miR-21 and miR-34a expression. *Islets* 2014 **6** e27754. (doi:10.4161/isl.27754)
86 Gonzalez-Martin A, Adams BD, Lai M, Shepherd J, Salvador-Bernaldez M, Salvador JM, Lu J, Nemazee D & Xiao C. The microRNA miR-148a functions as a critical regulator of B cell tolerance and autoimmunity. *Nature Immunology* 2016 **17** 433–440. (doi:10.1038/ni.3385)
87 Melkman-Zehavi T, Oren R, Kredo-Russo S, Shapira T, Mandelbaum AD, Rivkin N, Nir T, Lennox KA, Behlke MA, Dor Y, *et al*. miRNAs control insulin content in pancreatic beta-cells via downregulation of transcriptional repressors. *EMBO Journal* 2011 **30** 835–845. (doi:10.1038/emboj.2010.361)
88 Seoudi AM, Lashine YA & Abdelaziz AI. MicroRNA-181a - a tale of discrepancies. *Expert Reviews in Molecular Medicine* 2012 **14** e5. (doi:10.1017/S1462399411002122)
89 Cichocki F, Felices M, McCullar V, Presnell SR, Al-Attar A, Lutz CT & Miller JS. Cutting edge: microRNA-181 promotes human NK cell development by regulating Notch signaling. *Journal of Immunology* 2011 **187** 6171–6175. (doi:10.4049/jimmunol.1100835)
90 Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, Manoharan M, Soutschek J, Skare P, *et al*. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 2007 **129** 147–161. (doi:10.1016/j.cell.2007.03.008)
91 Zhang J, Jima DD, Jacobs C, Fischer R, Gottwein E, Huang G, Lugar PL, Lagoo AS, Rizzieri DA, Friedman DR, *et al*. Patterns of microRNA expression characterize stages of human B-cell differentiation. *Blood* 2009 **113** 4586–4594. (doi:10.1182/blood-2008-09-178186)
92 Xie W, Li Z, Li M, Xu N & Zhang Y. miR-181a and inflammation: miRNA homeostasis response to inflammatory stimuli in vivo. *Biochemical and Biophysical Research Communications* 2013 **430** 647–652. (doi:10.1016/j.bbrc.2012.11.097)
93 Xie W, Li M, Xu N, Lv Q, Huang N, He J & Zhang Y. MiR-181a regulates inflammation responses in monocytes and macrophages. *PLoS ONE* 2013 **8** e58639. (doi:10.1371/journal.pone.0058639)
94 Magenta A, Greco S, Gaetano C & Martelli F. Oxidative stress and microRNAs in vascular diseases. *International Journal of Molecular Sciences* 2013 **14** 17319–17346. (doi:10.3390/ijms140917319)
95 Devlin C, Greco S, Martelli F & Ivan M. miR-210: more than a silent player in hypoxia. *IUBMB Life* 2011 **63** 94–100.
96 Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, Davuluri R, Liu CG, Croce CM, Negrini M, *et al*. A microRNA signature of hypoxia. *Molecular and Cellular Biology* 2007 **27** 1859–1867. (doi:10.1128/MCB.01395-06)
97 Greco S, Gaetano C & Martelli F. HypoxamiR regulation and function in ischemic cardiovascular diseases. *Antioxidants and Redox Signaling* 2014 **21** 1202–1219. (doi:10.1089/ars.2013.5403)
98 Lu J, Xie F, Geng L, Shen W, Sui C & Yang J. Potential role of microRNA-210 as biomarker in human cancers detection: a meta-analysis. *BioMed Research International* 2015 **2015** 303987.
99 Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M & Stoffel M. miR-375 maintains normal pancreatic alpha- and beta-cell mass. *PNAS* 2009 **106** 5813–5818. (doi:10.1073/pnas.0810550106)
100 Li X. MiR-375, a microRNA related to diabetes. *Gene* 2014 **533** 1–4. (doi:10.1016/j.gene.2013.09.105)
101 Samandari N, Mirza AH, Nielsen LB, Kaur S, Hougaard P, Fredheim S, Mortensen HB & Pociot F. Circulating microRNA levels predict residual beta cell function and glycaemic control in children with type 1 diabetes mellitus. *Diabetologia* 2017 **60** 354–363. (doi:10.1007/s00125-016-4156-4)
102 Zhu Y, You W, Wang H, Li Y, Qiao N, Shi Y, Zhang C, Bleich D & Han X. MicroRNA-24/MODY gene regulatory pathway mediates pancreatic beta-cell dysfunction. *Diabetes* 2013 **62** 3194–3206. (doi:10.2337/db13-0151)
103 Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, Yoshimura A, Baltimore D & Rudensky AY. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. *Cell* 2010 **142** 914–929. (doi:10.1016/j.cell.2010.08.012)
104 Rusca N & Monticelli S. MiR-146a in immunity and disease. *Molecular Biology International* 2011 **2011** 437301.
105 Taganov KD, Boldin MP, Chang KJ & Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *PNAS* 2006 **103** 12481–12486. (doi:10.1073/pnas.0605298103)
106 Motawi TK, Rizk SM, Ibrahim TM & Ibrahim IA. Circulating microRNAs, miR-92a, miR-100 and miR-143, as non-invasive biomarkers for bladder cancer diagnosis. *Cell Biochemistry and Function* 2016 **34** 142–148. (doi:10.1002/cbf.3171)
107 Wang M, Ren D, Guo W, Wang Z, Huang S, Du H, Song L & Peng X. Loss of miR-100 enhances migration, invasion, epithelial-

http://www.endocrineconnections.org
DOI: 10.1530/EC-17-0248

mesenchymal transition and stemness properties in prostate cancer cells through targeting Argonaute 2. *International Journal of Oncology* 2014 **45** 362–372. (doi:10.3892/ijo.2014.2413)
108 Gong Y, He T, Yang L, Yang G, Chen Y & Zhang X. The role of miR-100 in regulating apoptosis of breast cancer cells. *Scientific Reports* 2015 **5** 11650. (doi:10.1038/srep11650)
109 Sylvius N, Bonne G, Straatman K, Reddy T, Gant TW & Shackleton S. MicroRNA expression profiling in patients with lamin A/C-associated muscular dystrophy. *FASEB Journal* 2011 **25** 3966–3978. (doi:10.1096/fj.11-182915)
110 Wang L, Su W, Du W, Xu Y, Wang L, Kong D, Han Z, Zheng G & Li Z. Gene and microRNA profiling of human induced pluripotent stem cell-derived endothelial cells. *Stem Cell Reviews* 2015 **11** 219–227. (doi:10.1007/s12015-014-9582-4)
111 Ortega FJ, Moreno-Navarrete JM, Pardo G, Sabater M, Hummel M, Ferrer A, Rodriguez-Hermosa JI, Ruiz B, Ricart W, Peral B, *et al*. MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. *PLoS ONE* 2010 **5** e9022. (doi:10.1371/journal.pone.0009022)
112 Pek SL, Sum CF, Lin MX, Cheng AK, Wong MT, Lim SC & Tavintharan S. Circulating and visceral adipose miR-100 is down-regulated in patients with obesity and Type 2 diabetes. *Molecular and Cellular Endocrinology* 2016 **427** 112–123. (doi:10.1016/j.mce.2016.03.010)
113 Fayyad-Kazan H, Bitar N, Najar M, Lewalle P, Fayyad-Kazan M, Badran R, Hamade E, Daher A, Hussein N, ElDirani R, *et al*. Circulating miR-150 and miR-342 in plasma are novel potential biomarkers for acute myeloid leukemia. *Journal of Translational Medicine* 2013 **11** 31. (doi:10.1186/1479-5876-11-31)
114 Van der Auwera I, Limame R, van Dam P, Vermeulen PB, Dirix LY & Van Laere SJ. Integrated miRNA and mRNA expression profiling of the inflammatory breast cancer subtype. *British Journal of Cancer* 2010 **103** 532–541. (doi:10.1038/sj.bjc.6605787)
115 Ronchetti D, Lionetti M, Mosca L, Agnelli L, Andronache A, Fabris S, Deliliers GL & Neri A. An integrative genomic approach reveals coordinated expression of intronic miR-335, miR-342, and miR-561 with deregulated host genes in multiple myeloma. *BMC Medical Genomics* 2008 **1** 37.
116 Oger F, Gheeraert C, Mogilenko D, Benomar Y, Molendi-Coste O, Bouchaert E, Caron S, Dombrowicz D, Pattou F, Duez H, *et al*. Cell-specific dysregulation of microRNA expression in obese white adipose tissue. *Journal of Clinical Endocrinology and Metabolism* 2014 **99** 2821–2833. (doi:10.1210/jc.2013-4259)
117 Wang L, Xu L, Xu M, Liu G, Xing J, Sun C & Ding H. Obesity-associated miR-342-3p promotes adipogenesis of mesenchymal stem cells by suppressing ctbp2 and releasing C/EBPalpha from CtBP2 binding. *Cellular Physiology and Biochemistry* 2015 **35** 2285–2298. (doi:10.1159/000374032)
118 Eissa S, Matboli M & Bekhet MM. Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis. *Biomedicine and Pharmacotherapy* 2016 **83** 92–99. (doi:10.1016/j.biopha.2016.06.018)
119 Collares CV, Evangelista AF, Xavier DJ, Rassi DM, Arns T, Foss-Freitas MC, Foss MC, Puthier D, Sakamoto-Hojo ET, Passos GA, *et al*. Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients. *BMC Research Notes* 2013 **6** 491.
120 Kaviani M, Azarpira N, Karimi MH & Al-Abdullah I. The role of microRNAs in islet beta-cell development. *Cell Biology International* 2016 **40** 1248–1255. (doi:10.1002/cbin.10691)
121 Schmidt WM, Spiel AO, Jilma B, Wolzt M & Muller M. In vivo profile of the human leukocyte microRNA response to endotoxemia. *Biochemical and Biophysical Research Communications* 2009 **380** 437–441. (doi:10.1016/j.bbrc.2008.12.190)
122 Debernardi S, Massat NJ, Radon TP, Sangaralingam A, Banissi A, Ennis DP, Dowe T, Chelala C, Pereira SP, Kocher HM, *et al*. Noninvasive urinary miRNA biomarkers for early detection of pancreatic adenocarcinoma. *American Journal of Cancer Research* 2015 **5** 3455–3466.
123 Yan B & Wang Z. Long noncoding RNA: its physiological and pathological roles. *DNA and Cell Biology* 2012 **31** (Supplement 1) S34–S41.
124 Fawzy IO, Hamza MT, Hosny KA, Esmat G, El Tayebi HM & Abdelaziz AI. miR-1275: a single microRNA that targets the three IGF2-mRNA-binding proteins hindering tumor growth in hepatocellular carcinoma. *FEBS Letters* 2015 **589** 2257–2265. (doi:10.1016/j.febslet.2015.06.038)
125 Pena-Chilet M, Martinez MT, Perez-Fidalgo JA, Peiro-Chova L, Oltra SS, Tormo E, Alonso-Yuste E, Martinez-Delgado B, Eroles P, Climent J, *et al*. MicroRNA profile in very young women with breast cancer. *BMC Cancer* 2014 **14** 529.
126 Bezman NA, Chakraborty T, Bender T & Lanier LL. miR-150 regulates the development of NK and iNKT cells. *Journal of Experimental Medicine* 2011 **208** 2717–2731. (doi:10.1084/jem.20111386)
127 Hong G, Zhang W, Li H, Shen X & Guo Z. Separate enrichment analysis of pathways for up- and downregulated genes. *Journal of the Royal Society Interface* 2014 **11** 20130950.

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SUPPLEMENTAL MATERIAL LIST

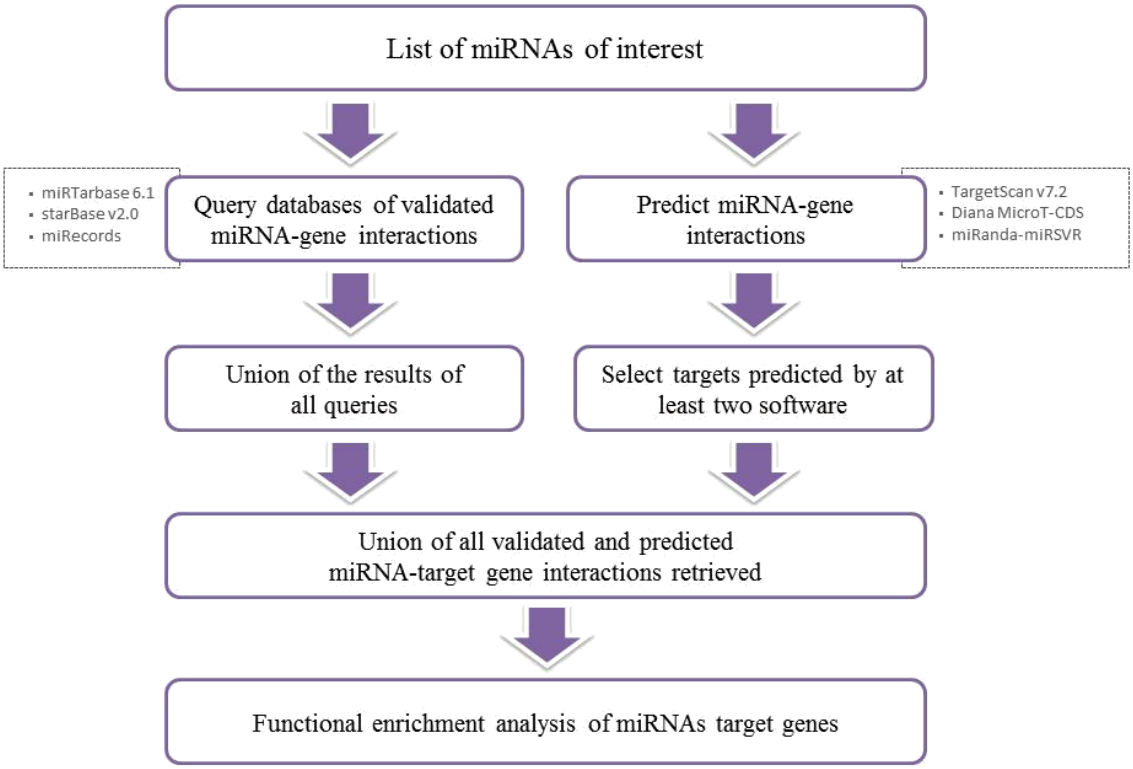
Supplementary Figure 1. Flowchart illustrating the search strategy used to perform the bioinformatics prediction and analysis of miRNA target genes.

Supplementary Table 1. MiRNAs analyzed in all studies included in the systematic review.

Supplementary Table 2. MiRNA-target gene interactions for the miRNAs found differently expressed in at least two studies in tissues related to T1DM. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 3. Significant KEGG biological pathways enriched for each differentially expressed miRNA. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 4. Significant KEGG biological pathways enriched for miRNAs expressed in tissues related to T1DM (PBMCs, serum/plasma and pancreas) (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).



Supplementary Figure 1. Flowchart illustrating the search strategy used to perform the bioinformatics prediction and analysis of miRNA target genes.

Supplementary Table 1. miRNAs analyzed in all studies included in the systematic review

miRNA ID	First author	Specie	Sample type	Change of expression
let-7a-5p	Tian et al.	Mice	Pancreas	Down
	Yang et al.	Human	Pancreas	Down
let-7c-5p	Tian et al.	Mice	Pancreas	Down
	Yang et al.	Human	Pancreas	Down
let-7d-5p	Tian et al.	Mice	Pancreas	Down
let-7e-5p	Erener et al.	Human	Serum	Up
let-7f-5p	Takahashi et al.	Human	PBMCs	Up
	Yang et al.	Human	PBMCs	Down
	Tian et al.	Mice	Pancreas	Down
let-7g-5p	Takahashi et al.	Human	PBMCs	Up
	Erener et al.	Human	Serum	Up
	Yang et al.	Human	PBMCs	Down
	Tian et al.	Mice	Pancreas	Down
let-7i-5p	Yang et al.	Human	PBMCs	Down
miR-10a-5p	Nielsen et al.	Human	Serum	Up
	Takahashi et al.	Human	PBMCs	Up
	Tian et al.	Mice	Pancreas	Down
miR-10b-5p	Tian et al.	Mice	Pancreas	Down
miR-100-5p	Hezova et al.	Human	T cells	Down
	Erener et al.	Human	Serum	Down
miR-101-3p	Takahashi et al.	Human	PBMCs	Up
miR-101a-3p	Tian et al.	Mice	Pancreas	Down
miR-101b-3p	Tian et al.	Mice	Pancreas	Down
miR-103a-3p	Nielsen et al.	Human	Serum	Up
	Bacon et al.	Human	Urine	Up
miR-103a-2-5p	Erener et al.	Human	Serum	Up
miR-107	Sebastiani et al.	Human	Serum	Down
miR-1179	Qing et al.	Human	Retina	Up
miR-1187	Tian et al.	Mice	Pancreas	Down
miR-122-5p	Li et al.	Mice	Liver	Down
miR-1231-3p	Tian et al.	Mice	Pancreas	Up
miR-1247-5p	Tian et al.	Mice	Pancreas	Up
miR-1249-3p	Tian et al.	Mice	Pancreas	Up
miR-1255b	Qing et al.	Human	Retina	Up
miR-125a-3p	Erener et al.	Human	Serum	Up
miR-125a-5p	Sebastiani et al. 2017	Human	T cells	Up
miR-125b	Hezova et al.	Human	T cells	Down
miR-126-3p	Takahashi et al.	Human	PBMCs	Up
	Garcia de la Torre et al.	Human	EPC	Up
	Tian et al.	Mice	Pancreas	Down
	Osipova et al.	Human	Urine	Down
miR-126-5p	Takahashi et al.	Human	PBMCs	Up
miR-1275	Takahashi et al.	Human	PBMCs	Down
	Yang et al.	Human	PBMCs	Down
miR-1276	Yang et al.	Human	PBMCs	Down

miR-1301	Yang et al.	Human	PBMCs	Up
miR-130a-3p	Barutta et al.	Human	Urine	Up
miR-132-5p	Tian et al.	Mice	Pancreas	Up
miR-133a-3p	Tian et al.	Mice	Pancreas	Up
miR-134	Nielsen et al.	Human	Serum	Up
miR-137-5p	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Up
miR-139-5p	Tian et al.	Mice	Pancreas	Up
miR-140-3p	Takahashi et al.	Human	PBMCs	Down
miR-140-5p	Erener et al.	Human	Serum	Up
miR-142-3p	Diao et al.	Mice	Heart	Up
	Tian et al.	Mice	Pancreas	Down
miR-143-3p	Diao et al.	Mice	Heart	Down
	Tian et al.	Mice	Pancreas	Down
miR-144-5p	Erener et al.	Human	Serum	Up
miR-145-5p	Barutta et al.	Human	Urine	Up
	Barutta et al.	Mice	Glomeruli	Up
	Barutta et al.	Mice	Exossome	Up
miR-146a-5p	Yang et al.	Human	PBMCs	Down
	Emadi et al.	Rat	Aorta	Down
	Hezova et al.	Human	T cells	Up
	Wang et al.	Rat	Retina	Up
	Yousefzadeh et al.	Rat	Sciatic nerve	Up
	Alipour et al.	Rat	Kidney	Up
	Sebastiani et al.	Human	Serum	Down
	Perez-Bravo et al.	Human	PBMCs	Down
	Wang et al. 2017	Human	PBMCs	Down
miR-1468	Erener et al.	Human	Serum	Up
miR-148a-3p	Nielsen et al.	Human	Serum	Up
	Takahashi et al.	Human	PBMCs	Up
	Seyhan et al.	Human	Plasma	Up
	Qing et al.	Human	Retina	Up
miR-148b-3p	Takahashi et al.	Human	PBMCs	Up
	Tian et al.	Mice	Pancreas	Down
miR-150-5p	Estrella et al.	Human	PBMCs	Down
	Wang et al. 2017	Human	PBMCs	Down
miR-151-3p	Hezova et al.	Human	T cells	Down
	Tian et al.	Mice	Pancreas	Down
miR-152	Nielsen et al.	Human	Serum	Up
miR-154-3p	Tian et al.	Mice	Pancreas	Up
	Erener et al.	Human	Serum	Down
miR-155-5p	Barutta et al.	Human	Urine	Down
	Sebastiani et al.	Human	Serum	Down
miR-15a-5p	Tian et al.	Mice	Pancreas	Down
miR-15b	Takahashi et al.	Human	PBMCs	Up
	Yang et al.	Human	PBMCs	Down
miR-16-5p	Takahashi et al.	Human	PBMCs	Up

	Garcia-Contreras et al.	Human	Exossome plasmatic	Down
	Tian et al.	Mice	Pancreas	Down
miR-17-5p	Tian et al.	Mice	Pancreas	Down
miR-18a-5p	Erener et al.	Human	Serum	Up
miR-181a-5p	Nielsen et al.	Human	Serum	Up
	Nabish et al.	Human	Serum	Up
miR-181c-5p	Qing et al.	Human	Retina	Up
miR-182-5p	Erener et al.	Human	Serum	Up
miR-183a-5p	Erener et al.	Human	Serum	Up
miR-184	Kovacs et al.	Rat	Retina	Up
miR-185	Tian et al.	Mice	Pancreas	Down
miR-186-5p	Takahashi et al.	Human	PBMCs	Up
miR-18b-5p	Takahashi et al.	Human	PBMCs	Up
miR-190a-5p	Sebastiani et al.	Human	Serum	Up
miR-191-5p	Hezova et al.	Human	T cells	Down
miR-192-5p	Erener et al.	Human	Serum	Up
miR-194-2-3p	Tian et al.	Mice	Pancreas	Up
miR-1948-5p	Tian et al.	Mice	Pancreas	Down
miR-1953	Tian et al.	Mice	Pancreas	Up
miR-195-5p	Diao et al.	Mice	Heart	Up
	Takahashi et al.	Human	PBMCs	Up
miR-1956	Tian et al.	Mice	Pancreas	Up
miR-199a-3p	Diao et al.	Mice	Heart	Up
	Kovacs et al.	Rat	Retina	Up
	Takahashi et al.	Human	PBMCs	Up
	Sebastiani et al.	Human	Serum	Down
miR-199a-5p	Nielsen et al.	Human	Serum	Up
miR-19a-3p	Takahashi et al.	Human	PBMCs	Up
	Tian et al.	Mice	Pancreas	Down
miR-19b-3p	Yang et al.	Human	PBMCs	Down
miR-1a-3p	Diao et al.	Mice	Heart	Down
miR-200a-3p	Kovacs et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Down
miR-200b-3p	Kovacs et al.	Rat	Retina	Up
miR-200c-3p	Nielsen et al.	Human	Serum	Up
	Yang et al.	Human	PBMCs	Down
miR-201-3p	Tian et al.	Mice	Pancreas	Up
miR-203a-3p	Xiong et al.	Rat	Retina	Up
miR-205-5p	Kovacs et al.	Rat	Retina	Up
miR-208a-3p	Diao et al.	Mice	Heart	Up
miR-20a-5p	Diao et al.	Mice	Heart	Down
	Takahashi et al.	Human	PBMCs	Up
miR-20b-5p	Hezova et al.	Human	T cells	Down
	Kovacs et al.	Rat	Retina	Down
	Takahashi et al.	Human	PBMCs	Up
miR-210-5p	Nielsen et al.	Human	Serum	Up
	Osipova b et al.	Human	Urine	Up

	Osipova a et al.	Human	Serum	Up
miR-212-3p	Xiong et al.	Rat	Retina	Up
miR-21-5p	Diao et al.	Mice	Heart	Up
	Nielsen et al.	Human	Serum	Up
	Osipova et al.	Human	Serum	Up
	Takahashi et al.	Human	PBMCs	Up
	Qing et al.	Human	Retina	Up
	Seyhan et al.	Human	Plasma	Up
	Osipova et al.	Human	Urine	Up
miR-214-5p	Erener et al.	Human	Serum	Up
miR-216a-5p	Kato et al.	Mice	Kidney	Up
	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Down
miR-219-1-3p	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Up
miR-21a	Salas-Perez et al.	Human	PBMCs	Down
miR-220b	Diao et al.	Mice	Heart	Down
miR-221-3p	Diao et al.	Mice	Heart	Up
	Yang et al.	Human	PBMCs	Down
	Erener et al. 2017	Human	Serum	Up
	Garcia de la Torre et al.	Human	EPC	Up
miR-222-3p	Erener et al. 2017	Human	Serum	Up
miR-223-3p	Kovacs et al.	Rat	Retina	Up
miR-22-3p	Yang et al.	Human	PBMCs	Down
	Estrella et al.	Human	PBMCs	Up
miR-224-5p	Bacon et al.	Human	Urine	Up
miR-23a-3p	Yang et al.	Human	PBMCs	Down
miR-23b-3p	Erener et al.	Human	Serum	Up
miR-24-1-5p	Qing et al.	Mice	Retina	Up
miR-24-3p	Diao et al.	Mice	Heart	Up
	Erener et al. 2017	Human	Serum	Up
	Seyhan et al.	Human	Plasma	Up
miR-25-3p	Nielsen et al.	Human	Serum	Up
	Garcia-Contreras et al.	Human	Plasmatic-exosome	Up
	Erener et al. 2017	Human	Serum	Up
	Yang et al.	Human	PBMCs	Down
miR-26a-5p	Nielsen et al.	Human	Serum	Up
	Ma et al.	Mice	Pancreas	Down
	Tian et al.	Mice	Pancreas	Down
miR-26b-5p	Nielsen et al.	Human	Serum	Up
	Takahashi et al.	Human	PBMCs	Up
	Tian et al.	Mice	Pancreas	Down
miR-27a-3p	Nielsen et al.	Human	Serum	Up
	Tian et al.	Mice	Pancreas	Down
miR-27b-3p	Nielsen et al.	Human	Serum	Up
	Takahashi et al.	Human	PBMCs	Up
miR-28-5p	Yang et al.	Human	PBMCs	Down

miR-28a-5p	Tian et al.	Mice	Pancreas	Down
miR-292-5p	Tian et al.	Mice	Pancreas	Up
miR-296-3p	Tian et al.	Mice	Pancreas	Up
miR-29a-3p	Diao et al.	Mice	Heart	Down
	Yang et al.	Human	PBMCs	Down
miR-29b-3p	Nielsen et al.	Human	Serum	Up
	Silva et al.	Rat	Retina	Up
miR-301a-3p	Takahashi et al.	Human	PBMCs	Up
miR-302a-3p	Tian et al.	Mice	Pancreas	Up
miR-302b-5p	Tian et al.	Mice	Pancreas	Up
miR-302d	Garcia-Contreras et al.	Human	Exossome plasmatic	Down
miR-3058-3p	Tian et al.	Mice	Pancreas	Up
miR-3070a-5p	Tian et al.	Mice	Pancreas	Up
miR-3078-3p	Tian et al.	Mice	Pancreas	Up
miR-3081-3p	Tian et al.	Mice	Pancreas	Up
miR-3082-5p	Tian et al.	Mice	Pancreas	Down
miR-3086-3p	Tian et al.	Mice	Pancreas	Down
miR-3090-5p	Tian et al.	Mice	Pancreas	Up
miR-3095-5p	Tian et al.	Mice	Pancreas	Up
miR-30b-3p	Tian et al.	Mice	Pancreas	Up
	Yang et al.	Human	PBMCs	Down
miR-30b-5p	Tian et al.	Mice	Pancreas	Down
miR-30c-5p	Yang et al.	Human	PBMCs	Down
miR-30e-3p	Takahashi et al.	Human	PBMCs	Up
miR-30e-5p	Erener et al. 2017	Human	Serum	Up
miR-3102-3p	Tian et al.	Mice	Pancreas	Up
miR-3105-3p	Tian et al.	Mice	Pancreas	Up
miR-3105-5p	Tian et al.	Mice	Pancreas	Up
miR-3109-5p	Tian et al.	Mice	Pancreas	Up
miR-31-3p	Kovacs et al.	Rat	Retina	Up
miR-31-5p	Hezova et al.	Human	T cells	Down
	Kovacs et al.	Rat	Retina	Up
miR-320a	Nielsen et al.	Human	Serum	Up
miR-320b	Yang et al.	Human	PBMCs	Up
miR-323-3p	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Up
miR-324-3p	Tian et al.	Mice	Pancreas	Down
	Erener et al. 2017	Human	Serum	Up
miR-324-5p	Takahashi et al.	Human	PBMCs	Down
	Erener et al. 2017	Human	Serum	Up
	Tian et al.	Mice	Pancreas	Down
miR-325-5p	Tian et al.	Mice	Pancreas	Up
miR-32-5p	Takahashi et al.	Human	PBMCs	Up
	Tian et al.	Mice	Pancreas	Down
miR-331-3p	Erener et al. 2017	Human	Serum	Up
miR-335-3p	Kovacs et al.	Rat	Retina	Up
miR-335-5p	Hezova et al.	Human	T cells	Down

	Takahashi et al.	Human	PBMCs	Up
miR-337-3p	Tian et al.	Mice	Pancreas	Up
miR-338-3p	Takahashi et al.	Human	PBMCs	Up
miR-33a-5p	Takahashi et al.	Human	PBMCs	Up
miR-340-3p	Takahashi et al.	Human	PBMCs	Up
miR-340-5p	Nielsen et al.	Human	Serum	Up
miR-342-3p	Takahashi et al.	Human	PBMCs	Down
	Sebastiani et al.	Human	Serum	Up
	Yang et al.	Human	PBMCs	Down
miR-345-5p	Erener et al. 2017	Human	Serum	Up
miR-346-5p	Tian et al.	Mice	Pancreas	Up
miR-34a-5p	Sebastiani et al.	Human	Serum	Up
miR-34b-3p	Kovacs et al.	Rat	Retina	Up
miR-34c-5p	Kovacs et al.	Rat	Retina	Up
	Xiong et al.	Rat	Retina	Up
miR-350-3p	Xiong et al.	Rat	Retina	Up
miR-351-5p	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Up
miR-361-3p	Tian et al.	Mice	Pancreas	Up
miR-363-5p	Qing et al.	Mice	Retina	Up
	Tian et al.	Mice	Pancreas	Up
miR-365a-3p	Hezova et al.	Human	T cells	Down
miR-369-5p	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Up
miR-372	Sebastiani et al.	Human	Serum	Down
miR-373-3p	Diao et al.	Mice	Heart	Down
	Sebastiani et al.	Human	Serum	Up
miR-374a-5p	Qing et al.	Human	Retina	Up
miR-375	Xiong et al.	Rat	Retina	Up
	Marchand et al.	Human	Serum	Up
	Sebastiani et al.	Human	Serum	Down
	Seyhan et al.	Human	Plasma	Up
	Erener et al.	Mice	Plasma	Up
miR-376	Marchand et al.	Human	Serum	Up
miR-377-3p	Sebastiani et al.	Human	Serum	Down
	Erener et al. 2017	Human	Serum	Up
miR-378	Erener et al.	Mice	Plasma	Up
	Garcia-Contreras et al.	Human	Exossome plasmatic	Down
miR-378a-5p	Kovacs et al.	Rat	Retina	Up
miR-410-3p	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Down
miR-423-5p	Takahashi et al.	Human	PBMCs	Down
	Qing et al.	Human	Retina	Up
miR-424-5p	Barutta et al.	Human	Urine	Down
	Wang et al. 2017	Human	PBMCs	Down
	Takahashi et al.	Human	PBMCs	Up
miR-450a-2-3p	Takahashi et al.	Human	PBMCs	Up

	Tian et al.	Mice	Pancreas	Up
miR-451a	Tian et al.	Mice	Pancreas	Down
miR-454-3p	Takahashi et al.	Human	PBMCs	Up
	Erener et al. 2017	Human	Serum	Up
miR-466a-5p	Tian et al.	Mice	Pancreas	Down
miR-466b-5p	Tian et al.	Mice	Pancreas	Down
miR-466c-5p	Tian et al.	Mice	Pancreas	Down
miR-466e-5p	Tian et al.	Mice	Pancreas	Down
miR-466f-3p	Tian et al.	Mice	Pancreas	Down
miR-466f-5p	Tian et al.	Mice	Pancreas	Down
miR-466i-3p	Tian et al.	Mice	Pancreas	Down
miR-466i-5p	Tian et al.	Mice	Pancreas	Down
miR-466q	Tian et al.	Mice	Pancreas	Down
miR-467c-3p	Tian et al.	Mice	Pancreas	Down
miR-467f-3p	Tian et al.	Mice	Pancreas	Down
miR-467g	Tian et al.	Mice	Pancreas	Down
miR-467h	Tian et al.	Mice	Pancreas	Down
miR-486-5p	Yang et al.	Human	PBMCs	Up
miR-488	Kovacs et al.	Rat	Retina	Up
miR-490-5p	Tian et al.	Mice	Pancreas	Up
	Erener et al. 2017	Human	Serum	Down
miR-491-5p	Tian et al.	Mice	Pancreas	Up
miR-493-5p	Tian et al.	Mice	Pancreas	Down
miR-494-3p	Qing et al.	Human	Retina	Up
miR-499-3p	Diao et al.	Mice	Heart	Up
miR-499-5p	Kovacs et al.	Rat	Retina	Down
miR-500a-5p	Erener et al. 2017	Human	Serum	Up
miR-502-3p	Erener et al. 2017	Human	Serum	Up
miR-505-3p	Qing et al.	Human	Retina	Up
miR-510	Hezova et al.	Human	T cells	Up
miR-5110	Tian et al.	Mice	Pancreas	Up
miR-5114	Tian et al.	Mice	Pancreas	Up
miR-5115	Tian et al.	Mice	Pancreas	Up
miR-5119	Tian et al.	Mice	Pancreas	Up
miR-5120	Tian et al.	Mice	Pancreas	Up
miR-5130	Tian et al.	Mice	Pancreas	Up
miR-5132-5p	Tian et al.	Mice	Pancreas	Up
miR-520d-3p	Qing et al.	Human	Retina	Up
miR-520e	Sebastiani et al.	Human	Serum	Up
miR-5-3p	Tian et al.	Mice	Pancreas	Up
miR-542-3p	Takahashi et al.	Human	PBMCs	Up
miR-548c-3p	Takahashi et al.	Human	PBMCs	Up
miR-548d-3p	Qing et al.	Human	Retina	Up
miR-5616-3p	Tian et al.	Mice	Pancreas	Up
miR-5617	Tian et al.	Mice	Pancreas	Up
miR-5622-3p	Tian et al.	Mice	Pancreas	Up
miR-5624-3p	Tian et al.	Mice	Pancreas	Down

miR-5625-5p	Tian et al.	Mice	Pancreas	Down
miR-570	Garcia-Contreras et al.	Human	Exossome plasmatic	Down
miR-574-3p	Kovacs et al.	Rat	Retina	Up
	Garcia-Contreras et al.	Human	Exossome plasmatic	Down
	Tian et al.	Mice	Pancreas	Down
miR-576-3p	Qing et al.	Human	Retina	Up
miR-579	Garcia-Contreras et al.	Human	Exossome plasmatic	Down
miR-592-5p	Xiong et al.	Rat	Retina	Up
miR-601	Qing et al.	Human	Retina	Up
miR-606	Qing et al.	Human	Retina	Up
miR-627	Qing et al.	Human	Retina	Up
miR-630	Erener et al. 2017	Human	Serum	Down
miR-636	Erener et al. 2017	Human	Serum	Down
miR-639	Erener et al. 2017	Human	Serum	Down
miR-652-3p	Yang et al.	Human	PBMCs	Up
miR-653-3p	Tian et al.	Mice	Pancreas	Up
miR-668-3p	Tian et al.	Mice	Pancreas	Up
miR-6691-5p	Tian et al.	Mice	Pancreas	Down
miR-669-3p	Tian et al.	Mice	Pancreas	Up
miR-669a-3-3p	Tian et al.	Mice	Pancreas	Down
miR-669a-3p	Tian et al.	Mice	Pancreas	Down
miR-669b-3p	Tian et al.	Mice	Pancreas	Down
miR-669c-3p	Tian et al.	Mice	Pancreas	Down
miR-669d-2-3p	Tian et al.	Mice	Pancreas	Down
miR-669d-5p	Tian et al.	Mice	Pancreas	Down
miR-669e-3p	Tian et al.	Mice	Pancreas	Down
miR-669e-5p	Tian et al.	Mice	Pancreas	Down
miR-669f-3p	Tian et al.	Mice	Pancreas	Down
miR-669f-5p	Tian et al.	Mice	Pancreas	Down
miR-669k-5p	Tian et al.	Mice	Pancreas	Down
miR-669p-3p	Tian et al.	Mice	Pancreas	Down
miR-670-5p	Tian et al.	Mice	Pancreas	Up
miR-675-3p	Erener et al. 2017	Human	Serum	Down
miR-690	Kovacs et al.	Rat	Retina	Down
miR-697	Tian et al.	Mice	Pancreas	Up
miR-700-3p	Diao et al.	Mice	Heart	Up
	Tian et al.	Mice	Pancreas	Up
miR-705	Diao et al.	Mice	Heart	Up
miR-706	Tian et al.	Mice	Pancreas	Up
miR-712-3p	Tian et al.	Mice	Pancreas	Up
miR-720	Takahashi et al.	Human	PBMCs	Down
	Erener et al. 2017	Human	Serum	Down
miR-744-5p	Tian et al.	Mice	Pancreas	Up
miR-758-3p	Xiong et al.	Rat	Retina	Up
	Tian et al.	Mice	Pancreas	Down
miR-758-5p	Tian et al.	Mice	Pancreas	Down
miR-7-5p	Takahashi et al.	Human	PBMCs	Up

miR-766	Takahashi et al.	Human	PBMCs	Down
miR-7a-5p	Tian et al.	Mice	Pancreas	Down
miR-7b-3p	Tian et al.	Mice	Pancreas	Up
miR-7b-5p	Tian et al.	Mice	Pancreas	Down
miR-802-5p	Tian et al.	Mice	Pancreas	Down
miR-881-3p	Tian et al.	Mice	Pancreas	Up
miR-88-1-5p	Tian et al.	Mice	Pancreas	Up
miR-886-5p	Qing et al.	Human	Retina	Up
miR-92a-1-5p	Xiong et al.	Rat	Retina	Up
miR-935	Xiong et al.	Rat	Retina	Up
miR-93-5p	Salas-Perez et al.	Human	PBMCs	Down
miR-9-3p	Tian et al.	Mice	Pancreas	Up
	Sebastiani et al.	Human	Serum	Down
miR-940	Takahashi et al.	Human	PBMCs	Down
miR-98-5p	Takahashi et al.	Human	PBMCs	Up
	Tian et al.	Mice	Pancreas	Down
miR-99a-5p	Hezova et al.	Human	T cells	Down

ARTIGO 2

MicroRNA expression profile in plasma from type 1 diabetic patients: case-control study and bioinformatic analysis

MicroRNA expression profile in plasma from type 1 diabetic patients: case-control study and bioinformatic analysis

Taís S. Assmann[1,2], Mariana Recamonde-Mendoza[3], Márcia Puñales[4], Balduíno Tschiedel[4], Luís H. Canani[1,2] and Daisy Crispim*[1,2].

1- Endocrine Division, Hospital de Clínicas de Porto Alegre. Porto Alegre, Rio Grande do Sul, Brazil.

2- Postgraduation Program in Medical Sciences: Endocrinology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

3- Institute of Informatics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

4- Instituto da Criança com Diabetes, Hospital Nossa Senhora da Conceição, Porto Alegre, Rio Grande do Sul, Brazil.

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* Correspondence to: Dr. Daisy Crispim. Ramiro Barcelos Street 2350; building 12; 4° floor. Zip Code 90035-003. Porto Alegre, Rio Grande do Sul, Brazil. E-mail: dcmoreira@hcpa.edu.br

ABSTRACT

Context: MicroRNAs (miRNAs) are small noncoding RNAs that negatively regulate gene expression. A number of miRNAs circulate in the bloodstream, usually reflecting tissue-specific injuries, which makes them ideal candidates as biomarkers of disease progression. In this context, changes in miRNA expression have been described in several pathologies, including type 1 diabetes mellitus (T1DM).

Objective: To investigate a miRNA expression profile in plasma from T1DM patients and control subjects and analyze the pathways involved.

Design: Expressions of 48 miRNAs were analyzed in plasma from 33 T1DM patients and 26 age-and-gender-matched controls using Stem-loop RT-PreAmp PCR and TaqMan Low Density Arrays (Thermo Fisher Scientific). Five dysregulated miRNAs were then chosen for validation in an independent sample of 27 T1DM patients and 14 controls, using RT-qPCR. Bioinformatic analyses were performed to determine in which pathways these miRNAs were involved.

Results: Nine miRNAs were differentially expressed between recently-diagnosed T1DM patients (<5 years of diagnosis) and controls. No differences were observed between patients with ≥5 years of diagnosis and controls. After validation in an independent sample of T1DM patients, miR-103a-3p, miR-155-5p, miR-200a-3p, and miR-210-3p were confirmed to be upregulated in recently-diagnosed T1DM patients compared with controls or patients with ≥5 years of diagnosis. Moreover, miR-146a-5p was downregulated in recently-diagnosed T1DM patients compared with the other groups. These five miRNAs regulate several genes from the innate immune system-, MAPK-, apoptosis-, insulin- and cancer-related pathways.

Conclusions: Our study demonstrates that five miRNAs are dysregulated in recently-diagnosed T1DM patients, providing information about the biological pathways in which they are involved.

Keywords: microRNAs, type 1 diabetes, epigenetics, bioinformatics

1. INTRODUCTION

Type 1 diabetes mellitus (T1DM) is characterized by severe autoimmune destruction of pancreatic beta-cells by T lymphocytes and macrophages, rendering patients insulin-dependent for life [1]. This disease is generally diagnosed when over 80-90% of beta-cells have been destroyed by the immune system [2]. Shortly after the initial insulin treatment, many children show a period of increased endogenous insulin residual secretion followed by a reduced requirement for exogenous insulin, called the remission phase [3]. The partial regenerative capacity that occurs during this period offers a unique opportunity for an interventional treatment to maintain insulin secretion [4]. In this context, potential new biomarkers could help predict the destruction or regeneration of residual beta-cell mass and, thus, help monitor T1DM progression.

MicroRNAs (miRNAs) are a class of small noncoding RNAs of 21-23 nucleotides that negatively regulate gene expression by partially pairing to the 3', 5' untranslated regions of their target mRNAs, leading to translation repression and/or transcript degradation [5,6]. MiRNAs are estimated to regulate the expression of more than 60% of protein-coding genes, consequently controlling a number of biological and pathological processes [5,2]. Interestingly, recent studies have demonstrated that miRNAs can be transported from cell-to-cell and can circulate as stable molecules in body fluids; thereby, coordinating gene expression in neighboring and/or distant cells. Hence, miRNA transfer between distant cells could potentially be a new mode of cell-to-cell communication [2]. Moreover, miRNA levels in body fluids usually reflect tissue-specific injuries, making them ideal candidates as potential circulating biomarkers [7,8].

Deviation from homeostatic miRNA levels, whether upregulation or downregulation, has been linked to many diseases, including cancer, endocrine disorders and autoimmune diseases [9-12]. Growing evidence suggests that miRNAs also play a role in immune system functions, as well as in beta-cell metabolism, proliferation and death, which are processes involved in T1DM pathogenesis [2,6,13]. Indeed, IL-1β and TNF inflammatory cytokines were reported to induce miR-21, miR-30b, miR-34, miR-101a and miR-146a expressions in MIN6 cells and human pancreatic islets [14], suggesting that these miRNAs may play a role in cytokine-mediated beta-cell destruction. Moreover, miRNA specific profiles have been observed in peripheral blood mononuclear cells (PBMCs) or plasma/serum from T1DM patients [4,15-18], and some miRNAs seem to modulate mRNA expressions of the major T1DM autoantigens [16,19] or predict residual beta-cell function after T1DM diagnosis [20]. Indeed, serum miRNA levels were associated with disease progression and conferred an additional risk of T1DM development in young autoantibody-positive relatives of T1DM patients [21].

Therefore, considering that miRNAs might be potential T1DM biomarkers, the aim of this study was to investigate a circulating miRNA expression profile in the plasma of T1DM patients compared with non-diabetic subjects. We also carried out bioinformatic analyses to investigate the pathways in which these miRNAs are involved.

2. METHODS

2.1 Study population

This case-control study was designed in accordance with STROBE guidelines for reporting of association studies [22] and included two independent samples of T1DM

patients and non-diabetic subjects (controls), called the "Screening sample set" and the "Validation sample set". The "Screening sample set" included 33 T1DM patients [16 patients with less than 5 years of diagnosis (recently-diagnosed group) and 17 patients with 5 or more years of diagnosis] and 26 controls. The "Validation sample set" included 27 T1DM patients (13 patients with <5 years of diagnosis and 14 with ≥5 years of diagnosis) and 14 controls. T1DM patients and non-diabetic controls were age- and gender-matched in both samples.

All T1DM patients were recruited from the outpatient clinic at the Hospital de Clínicas de Porto Alegre and the Instituto da Criança com Diabetes of the Grupo Hospitalar Conceição (Rio Grande do Sul, Brazil) between August 2014 and September 2016. Patients were diagnosed with T1DM according to American Diabetes Association guidelines [1]. T1DM patients presenting recent episodes of ketoacidosis, chronic inflammatory diseases, chronic rheumatic disease, hepatitis, HIV-positivity, glucocorticoid treatment or any diabetic complications were excluded from the study.

The control groups consisted of healthy blood donors recruited from the Hospital de Clínicas de Porto Alegre between August 2014 and September 2016. Only subjects with glycated hemoglobin (HbA1c) < 5.7% were considered for inclusion as controls [1]. Exclusion criteria for the control groups were: alcoholism, obesity, smoking habits, infections, inflammatory diseases or a family history of diabetes.

A standard questionnaire was used to collect information on current age, age at T1DM diagnosis, T1DM duration and drug treatment. All patients underwent physical and laboratory evaluations, as described elsewhere [23]. Ethnicity was defined according to self-classification, and only patients of European descendant were included in the study. Since HLA class II (DR and DQ) haplotypes may influence the association between non-HLA genes and T1DM [24], frequencies of HLA haplotypes associated

with high risk of T1DM were assessed in case and control subjects of both samples, as described elsewhere [25]. The study received appropriate ethics committee approval from Institutional Review Boards of the Hospital de Clínicas de Porto Alegre and the Grupo Hospitalar Conceição and was conducted according to the Declaration of Helsinki. Subjects and/or their parents gave assent and written informed consent prior to participation.

2.2 RNA extraction

Peripheral blood samples from all subjects were collected in EDTA-coated tubes. Sample collection was performed in the morning, at least 8 hours after the last meal. Immediately after collection, blood samples were checked for hemolysis. Non-hemolyzed samples were centrifuged at 3500 rpm for 15 min at 4°C, and plasma aliquots were stored at -80°C until miRNA expressions were quantified.

Total RNA was isolated from 450 µl of plasma using the MiRVana PARIS miRNA Isolation Kit (Ambion, Thermo Fisher Scientific, DE, USA), according to the manufacturer's instructions. The purity and concentration of RNA samples were measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Only RNA samples that achieved adequate purity ratios (A260/A280 = 1.9 - 2.1) were used for subsequent analyses [26]. RNA integrity was also checked on agarose gels containing GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

2.3 Quantification of miRNA expressions using Custom TaqMan Array MicroRNA cards

Relative expressions of 48 miRNAs were investigated in plasma from all subjects of the "Screening sample set" using Custom TaqMan Array MicroRNA cards (Thermo Fisher

Scientific), which contain probes for 45 target miRNAs and for three reference genes [*RNU44*, *RNU48* and *small nuclear RNA U6* (*U6snRNA*)] (**Supplementary Table 1**). Selection of these miRNAs was based on the available literature [4,15,2] and by searching in the miRWalk 2.0 database [27] those miRNAs potentially associated with T1DM in humans.

MiRNA expressions were performed in 3 separate reactions. First, the total RNA (10 ng) was reverse-transcribed into specific miRNAs using TaqMan miRNA Reverse-Transcription (RT) kit and Multiplex RT Pool Set (Thermo Fisher Scientific), in which stem-loop RT primers bind specifically to their corresponding miRNAs and begin reverse-transcription. Second, specific cDNAs for the 48 miRNAs analyzed were pre-amplified using TaqMan PreAmp Master Mix 2x kit and Custom PreAmp Primer Pool (Thermo Fisher Scientific), following manufacturer's suggestions. The PreAmp product was diluted with 175 µl of 0.1x TE buffer, pH 8.0.

RT-qPCR experiments were then performed in a Vii™ 7 Fast Real-Time PCR System (Thermo Fisher Scientific), using the TaqMan Low Density Array (TLDA) block. RT-qPCR reactions were performed using 1.13 µl of the diluted PreAmp product and 56.25 µl of the TaqMan Universal PCR Master Mix II no UNG 2x (Thermo Fisher Scientific), in a total volume of 112.5 µl dispensed into the 384 micro-wells of the array plates by centrifugation. The cycling conditions were as follows: 95°C for 10 min, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. Relative expressions were calculated using the $2^{-\Delta\Delta Cq}$ method [28]. For analysis, only the *U6snRNA* was used as a reference gene since it showed the lowest variation among the samples. Data are shown as n-fold changes in relation to a calibrator sample.

2.4 Validation of Custom TaqMan Array MicroRNA results using individual RT-qPCR

Five miRNAs were chosen for confirmation of their expressions in the "Validation sample set" using individual RT-qPCR assays, according to MIQE guidelines [29]. The criteria for selecting those miRNAs were: 1) miRNAs more differentially expressed between T1DM patients and controls from the "Screening sample set"; 2) evidence in the literature showing that they were associated with T1DM or with immune system functions [4,15,16]; and/or 3) the number of miRNA targets involved in pathways related to T1DM pathogenesis, which was assessed by bioinformatic analysis.

RT-qPCR was performed in two separate reactions: first, the total RNA was reverse-transcribed into cDNA and, second, the cDNA was amplified by RT-qPCR. Reverse transcription of 2 ng/μl of RNA into cDNA was carried out using specific TaqMan miRNA RT kits (Thermo Fisher Scientific) for each miRNA of interest.

RT-qPCR experiments were carried out in a Vii™ 7 Fast Real-Time PCR System. PCR reactions were performed using 0.5 μl TaqMan miRNA Assays 20x (Thermo Fisher Scientific; **Supplementary Table 1**) for target miRNAs or *U6snRNA*, 5 μl TaqMan Universal PCR Master Mix II no UNG 2x, and 1 μl of cDNA (10ng/μl), in a total volume of 10 μl. Each sample was assayed in triplicate and a negative control was included in each experiment. Cycling conditions for these genes were an initial cycle of 95°C for 10min, followed by 50 cycles of 95°C for 15s and 60°C for 90s. Quantification of the five target miRNAs was performed using the $2^{-\Delta\Delta Cq}$ method and the *U6snRNA* gene as the reference [26] and are shown as n-fold changes in relation to the calibrator sample.

2.5 Bioinformatic analyses

To further investigate the functional involvement of the nine miRNAs dysregulated in the plasma of T1DM patients from the "Screening sample set", we performed bioinformatic analyses to retrieve their putative targets. Targets of these miRNAs were investigated using a combination of experimentally validated data and computational prediction tools. To accomplish this, experimentally validated miRNA-target gene interactions were retrieved from miRTarbase v.6.1 [30], starBase v.2.0 [31], miRecords (accessed July 2016) [32] and TarBase v.5.0 [33] databases. The complete collection of validated targets provided by miRecords and TarBase were collected for analysis. For miRTarBase, the search was restricted to miRNA-target gene interactions classified as "Functional MTI", while for starBase, the data was filtered to include only interactions supported by at least 2 experiments ("medium stringency") and predicted by at least four out of five prediction programs implemented in the starBase interface.

Additionally, to complement the information derived from experimental validation and search for additional miRNA targets, we also applied *in silico* target prediction algorithms for selected miRNA sequences using the web-based tools TargetScan v.7.1 [34] and Diana MicroT v.4.0 [35]. To control for false positive rates and obtain a high-confidence list of candidate miRNA-target gene interactions, we required evidence of site conservation for TargetScan predictions and also applied score thresholds for both TargetScan (score < -0.2) and Diana MicroT (score > 0.5) sources. The final list of putative targets of the nine dysregulated miRNAs was then obtained by integrating the results from the aforementioned six sources, based on a union operation. Prior to the analyses, miRNA and gene identifiers were mapped to miRBase v.21.0 and HUGO Gene Nomenclature Committee standards [36] to allow consistent data integration across sources.

We then selected only the five miRNAs chosen for validation in the "Validation sample set" to search for potential biological pathways under their regulation. To accomplish this, we performed a functional enrichment analysis of experimentally and computationally predicted targets of the five miRNAs using pathways annotation from the KEGG database [37]. A hypergeometric test was used to calculate the statistical significance of the enriched pathways, and P values were corrected for multiple tests using the Benjamini-Hochberg procedure, which provides a False Discovery Rate (FDR) adjusted-P value (q-value). KEGG pathways associated with a q-value < 0.05 were considered significantly enriched for genes targeted by the five selected miRNAs. Moreover, to search for a consensus of pathways among different bioinformatic tools, the results obtained in the above-described analysis (based on miRTarBase, starBase, miRecords, TarBase, TargetScan, and Diana MicroT databases, and pathway annotations from KEGG) were compared with pathways obtained using mirPath, a tool from the DIANA suite [38], and the Enrichr web server [39,40]. For a detailed description of this analysis, please see **Supplementary Material 1.**

All bioinformatic analyses were conducted in the R/Bioconductor environment using the clusterProfiler [41], org.Hs.eg.db [42], and plyr [43] packages. Cytoscape v.3.4.0 was used for visualizing miRNA-target gene interactions.

2.6 Statistical analysis

Normal distribution was checked using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Variables with normal distribution are presented as mean ± SD. Variables with skewed distribution were log-transformed before analyses and are presented as median (25^{th}-75^{th} percentiles). Categorical data are shown as percentages. Clinical and laboratory characteristics and miRNA expressions were compared among groups using

Student's t-test, One-way ANOVA or χ^2 tests, as appropriate. Correlations between quantitative variables were assessed using Pearson's correlation tests. To investigate the discriminatory power of the five differentially expressed miRNAs to distinguish between T1DM patients and non-diabetic subjects, receiver-operating characteristic (ROC) curves were generated and the areas under the curves (AUCs) were calculated. All statistical analyses were performed using the SPSS statistical package (v.18.0) for Windows (SPSS Inc, Chicago, IL), and P values < 0.05 were considered statistically significant.

3. RESULTS

3.1 Clinical and laboratory characteristics of T1DM patients and non-diabetic subjects included in the study

Clinical and laboratory characteristics of T1DM cases and non-diabetic controls included in the "Screening" and "Validation" sample sets are shown in **Table 1**. There were no differences between cases and controls from both samples regarding age, gender, and mean body mass index (BMI) ($P > 0.05$). As expected, mean HbA1c levels and prevalence of hypothyroidism were higher in T1DM cases than in the respective control groups ($P < 0.05$). Moreover, T1DM high-risk HLA haplotypes (DR4/DQ8, DR3/DR4, DR3/DR3) showed higher frequencies in T1DM patients compared with controls ($P = 0.001$). Of note, the distributions of clinical, biochemical and genetic (HLA haplotypes) characteristics were similar between subjects from the "Screening" and "Validation" samples.

3.2 Expression profile of 45 miRNAs in subjects from the "Screening sample set"

Expressions of 45 circulating miRNAs were evaluated in plasma from T1DM patients and non-diabetic subjects of the "Screening sample set". Of these 45 miRNAs, 33 (73.3%) had detectable expressions in the plasma. However, only 26 miRNAs were expressed in at least 10% of the samples, allowing reliable statistical analyses (**Figure 1** and **Table 2**). Of these 26 miRNAs, only 9 were differentially expressed between non-diabetic subjects and recently-diagnosed T1DM patients (**Table 2**). MiR-21-5p, miR-101-3p, miR-103a-3p, miR-148b-3p, miR-155-5p, miR-200a-3p, miR-210-3p, and miR-1275 were upregulated in recently-diagnosed T1DM patients compared with controls. In contrast, miR-146a-5p was downregulated in recently-diagnosed T1DM patients. Interestingly, no differences in miRNA expressions were detected between controls and T1DM patients ≥5 years of diagnosis **(Table 2)**.

3.3 Validation of the five selected miRNAs by RT-qPCR

Of the nine miRNAs differently expressed among the analyzed groups of the "Screening sample set", we chose five miRNAs to validate by RT-qPCR in the "Validation sample set": miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p and miR-210-3p. These five miRNAs were selected based on the criteria described in the Methods section. As shown in **Figure 2** (**A** and **B**), miR-103a-3p and miR-200a-3p were upregulated in recently-diagnosed T1DM patients compared with both the control group and T1DM patients with ≥5 years of diagnosis (all $P < 0.05$). MiR-155-5p also seems to be upregulated in recently-diagnosed T1DM patients compared with the other groups, although the comparison with the control group did not reach formal statistical significance ($P = 0.06$; **Figure 2C**). Similarly, miR-210-3p seems to be upregulated in recently-diagnosed T1DM patients compared with the other two groups, although the comparison with the T1DM group with ≥5 years of diagnosis did not reach statistical

significance (P = 0.06; **Figure 2D**). MiR-146a-5p was downregulated in recently-diagnosed T1DM patients compared with the other two groups (P < 0.0001; **Figure 2E**). In accordance with the array data, these five miRNAs had similar expressions between controls and T1DM patients with ≥5 years of diagnosis (P > 0.05; **Figure 2**).

3.4 Association between the five validated miRNAs and T1DM clinical and laboratory characteristics

We evaluated possible correlations among the five validated miRNAs (miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p and miR-210-3p) dysregulated in T1DM patients and T1DM clinical and laboratory characteristics. For these analyses, both the "Screening" and "Validation" samples were analyzed together to increase the statistical power. MiR-200a-3p expression showed a significant negative correlation with insulin doses (r = -0.424, P = 0.005). Moreover, miR-155-5p and miR-200a-3p expressions were negatively correlated with HbA1c levels (r = -0.377, P = 0.009; and r = -0.331, P = 0.026; respectively) in the T1DM group. MiR-210-3p expression was negatively correlated with TSH levels (r = -0.430, P = 0.040) in T1DM patients. None of the five validated miRNAs was significantly correlated with total cholesterol, HDL-cholesterol, triglycerides, creatinine or albuminuria levels (all P > 0.05).

Notably, miR-103a-3p expression was positively correlated with miR-155-5p (r = 0.538, P = 0.0001), miR-200a-3p (r = 0.586, P = 0.0001) and mir-210-3p (r = 0.645, P = 0.0001) expressions in the whole sample. MiR-155-5p was also correlated with miR-200a-3p (r = 0.662, P = 0.0001) and miR-210-3p (r = 0.810, P = 0.0001) expressions. MiR-200a-3p was also correlated with miR-210-3p expression (r = 0.793, P = 0.0001). MiR-146a-5p expression did not correlate with the other four validated miRNAs.

3.5 ROC curve analyses for miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p and miR-210-3p

We next calculated the sensitivity and specificity of the five validated miRNAs for detecting T1DM using ROC curve analyses (**Supplementary Table 2**). For miR-200a-3p, miR-103a-3p and miR-210-3p, we found that the AUC was 0.800, 0.788 and 0.775 with a cutoff of -0.660, -0.201 and -0.288, resulting in a sensitivity of 0.903, 0.889 and 0.867, and specificity of 0.700, 0.706 and 0.700, respectively, for recently-diagnosed T1DM patients, adjusting for high-risk HLA haplotypes. MiR-146a-5p and miR-155-5p showed lower sensitivity and specificity for recently-diagnosed T1DM patients compared with the other three miRNAs (**Supplementary Table 2**). Furthermore, the AUC for pooled expressions of all five validated miRNAs in recently-diagnosed T1DM patients compared with controls was 0.896 (95% CI 0.807 – 0.985; $P < 0.000001$).

3.6 Target prediction for dysregulated miRNAs and perturbed pathways under their regulation

Bioinformatic analyses were performed to retrieve putative targets and pathways possibly regulated by the five validated miRNAs (hsa-miR-103a-3p, hsa-miR-146a-5p, hsa-miR-155-5p, hsa-miR-200a-3p and hsa-miR-210-3p) dysregulated in plasma from recently-diagnosed T1DM patients. First, we searched for targets of these five miRNAs using distinct resources, including experimentally validated data and computational target prediction tools. Of the total 1982 genes identified as potential targets of these miRNAs, only 234 were found to be modulated by two or more miRNAs (**Supplementary Table 3 and Supplementary Figure 1**). Individually, 877 target genes were found for miR-103a-3p, 208 for miR-146a-5p, 501 for miR-155-5p, 590 for miR-200a-3p, and 68 for miR-210-3p (**Supplementary Table 4**).

Following target identification, we carried out functional enrichment analysis of miRNA targets using pathway maps from the KEGG Database, aiming to explore biological pathways possibly affected by the five validated miRNAs (miR-103-3p, miR-146a-5p, miR-155-5p, miR-200a-3p and miR-210-3p). A total of 108 pathways were significantly enriched (q-values < 0.05) for these miRNAs (**Supplementary Table 5**). Many of these pathways consist of genes known to be related to T1DM pathogenesis, including cancer, MAPK, apoptosis, insulin and immune system (Toll-like receptor, NOD-like receptor, RIG-I-like receptor, and B cell receptor) signaling pathways (**Figure 3**).

Subsequently, insulin, apoptosis and innate immune system signaling pathways were selected for analysis in greater depth, since they are key pathways related to T1DM pathogenesis (**Figure 4**). MiR-146a-5p regulates several target genes from the innate immune system and apoptosis pathways, as well as *NRAS*, a gene common to both insulin and apoptosis pathways. MiR-155-5p and miR-103a-3p target genes from the three pathways. MiR-200a-3p regulates several genes from the innate immune system and insulin pathways and one gene from the apoptosis pathway (*TP53*), while miR-210-3p only targets *PTPN1*, which participates in the insulin signaling pathway.

As demonstrated in **Figure 4**, some genes are regulated by more than one miRNA: *FADD*, for example, is regulated by miR-146a-5p and miR-155-5p, *IRS2* is regulated by miR-103a-3p and miR-200a-3p, and *CXCL8*/*IL8* is regulated by miR-146a-5p and miR-155-5p. Moreover, *PIK3R1* (targeted by mir-103a-3p and miR-155-5p), *PIK3CB* (targeted by miR-146a-5p), and *MAPK8*/*JNK*, *AKT3* and *IKBKB*/*IKKB* (targeted by miR-103a-3p) are important genes shared among the three pathways.

Target genes and significant pathways for the four non-validated miRNAs (hsa-miR-21-5p, hsa-miR-101-3p, hsa-miR-148b-3p, and hsa-miR-1275), which could be

dysregulated in recently-diagnosed T1DM patients from the "Screening sample set" are shown in **Supplementary Table 6**. No significant pathways were found for miR-1275.

The results of the consensus analysis are summarized in **Supplementary Figure 2**. Our original bioinformatic results (based on the miRTarBase, starBase, miRecords, TarBase, TargetScan, and Diana MicroT databases, as well as on pathway annotations from KEGG database) were compared with pathways obtained from the mirPath and Enrichr tools. **Supplementary Figure 2A** provides a visual representation of a "binary matrix" showing a total of 163 unique KEGG pathways found in these tools, which are also shown in **Supplementary Table 7**. The highest overlap observed among tools was concentrated in the upper portion of the matrix. In general, 75% of the enriched terms shared some degree of consensus among the tools, being supported by more of two databases. This overlap of KEGG terms among the tools is also represented in Venn diagrams for the "Pathways Union" (**Supplementary Figure 2B**) and "Genes Union" (**Supplementary Figure 2C**) options of mirPath.

Finally, pairwise Jaccard Similarity Coefficients (JC) among lists of enriched terms are depicted in **Supplementary Figure 2D**. Interestingly, the overlap shared by results obtained with distinct Union options (Pathways/Genes), but same list of targets [MicroT (MT) or TarBase (TB)] using mirPath, is moderated, ranging from 0.534 (for TB) to 0.574 (for MT). However, this overlap lessened when we compared enriched terms for distinct lists of targets provided by mirPath, MT and TB, across Union options, ranging from 0.252 (MT-GeneUnion *vs*. TB-PathUnion) to 0.343 (MT-PathUnion *vs*. TB-GeneUnion). These values were comparable to JCs between our original results and miRPath enrichment analysis, which ranged from 0.336 to 0.450. Importantly, when we compared the enriched terms from the Enrichr analysis and our

own results, both based on the same list of target genes, we observed a high degree of overlap (JC = 0.904), indicating that our results are robust.

4. DISCUSSION

The first sign of autoimmunity against beta-cells, usually detectable a few years or months before the onset of clinical symptoms, is the appearance of antibodies against beta-cell antigens [44]. These circulating autoantibodies are acknowledged T1DM biomarkers since the presence of more than two of them indicates a high risk of disease development [45]. However, their use as routine biomarkers of T1DM progression has certain limitations, mainly because some children with new-onset T1DM are negative for islet autoantibodies [45], and many autoantibody-positive subjects never develop T1DM [2]. Therefore, new T1DM biomarkers together with genetic risk factors, such as HLA-high risk haplotypes, may present an improvement over information derived from islet autoantibodies alone [46].

A number of miRNAs circulate in the bloodstream and are ideal biomarkers of health status and disease progression due to their stability and resistance to degradation by ribonucleases or repeated freezing/thawing cycles, as well as to their easy detection in body fluids using highly sensitive and specific quantitative RT-qPCR [2]. Indeed, circulating miRNAs have being suggested as reproducible and non-invasive biomarkers for detecting and monitoring certain cancers [47,9]. Specific miRNA profiles have also been observed in PBMCs or plasma/serum from T1DM patients [4,15-18].

Thus, considering that miRNAs are potential T1DM biomarkers, we investigated whether a profile of 45 circulating miRNAs was dysregulated in T1DM patients. In the "Screening sample set", we found that nine miRNAs were differently expressed in the

plasma of recently-diagnosed T1DM patients compared with controls or T1DM patients with ≥5 years of diagnosis. Five of these miRNAs (miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p, and miR-210-3p) were chosen for validation in an independent sample, and the expression trends were similar to those observed in the "Screening sample set".

MiR-103a-3p, miR-155-5p, miR-200a-3p and miR-210-3p were upregulated in recently-diagnosed T1DM patients compared with the other groups. MiR-103a-3p has been shown to be upregulated in liver of type 2 diabetes mellitus (T2DM) patients [48], as well as liver [49] and adipocytes [50] of obese mice, acting in the insulin signaling pathway by primarily targeting caveolin-1, an insulin receptor regulator [49]. Hence, silencing this miRNA in mice improved glucose homeostasis and insulin sensitivity [49]. In agreement with our data, miR-103a-3p was also upregulated in PBMCs of T1DM patients from another Brazilian population [51]. Besides its action in regulating the insulin signaling pathway, miR-103a-3p was also found to be involved in cellular proliferation/apoptosis in cancer cell lines [52]. Moreover, some of the effects of miR-103a-3p might be mediated through other miRNAs since it strongly inhibits the miRNA-processing enzyme Dicer [53].

MiR-155-5p is abundantly expressed in macrophages and activated B and T cells [54], being induced by proinflammatory cytokines and bacterial- and viral-derived TLR ligands [55,56]. Because miR-155-5p induction involves both NF-κB and JNK pathways, it fits the profile of a typical immune-response molecule, regulating genes in both innate and adaptive immunity [13,54,55]. During normal conditions, NF-κB upregulates miR-155-5p, which, in turn, downregulates IκB kinases (IKKs), thus reducing NF-κB activation [54,55]. Moreover, miR-155-5p positively regulates host antiviral immune response by promoting type I IFN signaling through targeting *SOCS1*,

a negative regulator of the type I IFN pathway [56]. Consequently, miR-155-5p dysregulation has been associated with different cancers, cardiovascular diseases, viral infections, rheumatoid arthritis, and chronic diabetic complications [54,55,57]. In contrast with our results, this miRNA was reported as being downregulated in serum from newly-diagnosed T1DM patients from Italy [58], as well as in urinary exosomes from T1DM patients with albuminuria [59]. These results might be explained by differences in the study populations, tissue types (plasma *vs*. urine), and the criteria used to define newly-diagnosed T1DM.

Heegaard *et al*. [60] reported that circulating miR-103a-3p and miR-155-5p showed rhythmic fluctuations in healthy individuals during a 24-hour period. While miR-103a-3p was more expressed during the day, with peak of expression at 2:44 pm, miR-155-5p was more expressed at night, with peak of expression at 4:25 am [60]. Thus, additional studies are necessary to evaluate the influence of day time on the expressions of the five miRNAs validated in this study.

MiR-200a-3p plays a key role in the development of different types of cancer by downregulating epithelial to mesenchymal transition as well as the proliferation, motility and migration of tumor cells, including pancreatic cancer stem cells [5,61,62]. In beta-cells, members of the miR-200 family negatively regulate an anti-apoptotic and stress-resistance network that includes the chaperone *DnaJc3* and the caspase inhibitor *Xiap* [63]. Moreover, miR-200 positively controls the activation of the tumor suppressor *Tp53*; thereby, creating a pro-apoptotic genetic expression signature found in islets of diabetic mice [63]. MiR-200a-3p seems to be involved in insulin secretion since its knockdown reduced glucose-stimulated insulin secretion (GSIS) in MIN6 cells [64]. To date, no study has evaluated the expression of miR-200a-3p in serum/plasma from T1DM patients, although Nielsen *et al*. [4] observed that miR-200c-3p, another member

of the miR-200 family, was upregulated in the serum of newly-diagnosed T1DM patients from Europe, which agrees with our data.

MiR-210-3p is expressed in Tregs and negatively regulates *FOXP3* expression, which is known to be mandatory for Treg function [65]. Moreover, this miRNA is considered a "master miRNA" of hypoxic response, being induced by hypoxia in several cell types [66]. Targets of miR-210-3p are involved in mitochondrial metabolism, angiogenesis, DNA repair, and cell survival [66]. Consequently, this miRNA has being involved in various cancers and cardiovascular diseases [47,66]. It has also been reported as being upregulated in plasma/serum and/or urine from pediatric T1DM patients [4,67], in accordance with our results.

We found that miR-146a-5p was downregulated in recently-diagnosed T1DM patients compared with the other groups. This miRNA regulates Treg-mediated suppression of IFNγ-dependent Th1 responses and associated autoimmunity by directly targeting the transcription factor *STAT-1*, which contributes to a severe failure of immune tolerance [68]. Moreover, miR-146a-5p plays a recognized role in innate immunity by negatively regulating the inflammatory response after recognition of bacterial components by TLRs on monocytes and macrophages [69]. Following activation by TNF and IL-1β [14], miR-146a-5p downregulates *TRAF-6* and *IRAK-1*, thus decreasing NF-κB activity, which constitute a fine-tuning mechanism to prevent overstimulation of the TLR pathway [13]. Consistent with its role in the immune system, miR-146a-5p has been reported as being downregulated in serum and PBMCs of newly-diagnosed T1DM patients compared with healthy control subjects [16,58]. Interestingly, decreased miR-146a-5p expression was associated with high serum glutamic acid decarboxylase antibody titers [16]. These data are in line with our finding of decreased miR-146a-5p expression in T1DM patients with <5 years of diagnosis. In

contrast, Roggli *et al*. [14] reported that this miRNA was increased in islets of NOD mice during insulitis development. Blocking miR-146a-5p protected MIN6 cells from cytokine-induced apoptosis and also prevented GSIS reduction after IL-1β exposure [14].

Although not validated, miR-21-5p, miR-101-3p, miR-148b-3p and miR-1275 also seem to be upregulated in recently-diagnosed T1DM patients from the "Screening sample set", and should be further investigated. Among these miRNAs, miR-21-5p plays several recognized roles in immunity, including regulation of T cell proliferation and Treg development [70]. Moreover, this miRNA seems to regulate the effect of proinflammatory cytokines on beta-cells via NF-κB [71], being consistently upregulated in T1DM patients from different populations [4,7,15,67]. The role of miR-101-3p, miR-148b-3p and miR-1275 in beta-cells is still unknown, despite being previously reported as dysregulated in PBMCs of T1DM patients [15,16].

Besides indicating a group of miRNAs dysregulated in recently-diagnosed T1DM patients, the present study also provides new insights into the complex molecular mechanisms involved in T1DM by revealing pathways that may be regulated by miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p, and miR-210-3p. These miRNAs potentially regulate genes from 108 significant KEGG pathways, including cancer, MAPK, apoptosis, insulin and immune system (Toll-like receptor, NOD-like receptor, RIG-I-like receptor, and B cell receptor) signaling pathways (**Figure 3**), which have been previously associated with T1DM.

All of the T1DM patients included in this study had poor glycemic control according to their HbA1c levels. Thus, there is a possibility that our results could be partially due to an association with hyperglycemia *per se* and not with autoimmunity and beta-cell destruction. To investigate this in great depth, we performed correlation

analyses between the five validated miRNAs and HbA1c levels. Indeed, miR-155-5p and miR-200a-3p expressions were negatively correlated with HbA1c levels in the whole T1DM group, suggesting that these two miRNAs could be associated with hyperglycemia. However, these two miRNAs also participate in pathways associated with the immune system and apoptosis, which are related to beta-cell destruction (**Figure 4**). Similar to our results, Erener *et al.* [17] found a set of miRNAs that were correlated with hyperglycemia in serum from children in the early stages of T1DM. Expression levels of miR-146a-5p, miR-103a-3p, and miR-210-3p were not correlated with HbA1c levels, suggesting their specificity for autoimmunity and beta-cell destruction rather than hyperglycemia *per se*. The fact that all five validated miRNAs were associated with T1DM only in the recently-diagnosed group further indicates that the associations reported here are not only due to an association with hyperglycemia since glycemic control was similar for all T1DM patients, regardless the time since diagnosis. Accordingly, Yang *et al.* [16] reported that miR-146a was downregulated in newly-diagnosed T1DM patients compared to non-diabetic individuals independently of glycemic control.

As a further step, we compared our miRNA expression data with a dataset (GSE26168) available in the Gene Expression Omnibus (GEO) database (**Supplementary Table 8**). This dataset was chosen because it was the only one that contained expression data for the miRNAs of interest in plasma from patients with type 2 diabetes mellitus (T2DM), pre-diabetes, and non-diabetic controls [72]. None of the five validated miRNAs was differently expressed between T2DM patients *vs*. controls, T2DM *vs*. pre-diabetes patients, or pre-diabetes *vs*. control groups, showing that in this population from GEO these specific miRNAs are not associated with T2DM or pre-diabetes. **Supplementary Table 8** also shows a comparison of the GSE26168 dataset

with our data, demonstrating that the greatest differences in n-fold changes were between recently-diagnosed T1DM patients and controls (present results).

Some limitations and strengths of our study should be considered. As limitation, we could not validate all differently expressed miRNAs, and of these validated miRNAs, two miRNAs showed a negative correlation with hyperglycemia, not being specific for T1DM. As strengths, we use a well-defined cohort of T1DM patients, and we found three miRNAs that were not correlated with HbA1c levels, indicating specificity of these miRNAs to T1DM rather than hyperglycemia itself. Additionally, we performed a robust bioinformatic analyses to investigate the pathways these miRNAs are participating, explaining the association with T1DM.

Briefly, the present study identified a set of nine dysregulated miRNAs in T1DM patients with <5 years of diagnosis. Five of these were validated in an independent sample, confirming that miR-103a-3p, miR-155-5p, miR-200a-3p, and miR-210-3p are upregulated in recently-diagnosed T1DM patients and miR-146a-5p is downregulated in this group. Since these miRNAs regulate genes from the innate immune system-, MAPK-, apoptosis-, insulin- and cancer-related pathways, they are potential T1DM biomarkers, which require further investigation in different populations, as well as evaluation of their functional impacts on putative target genes and pathways.

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Declaration of interest

All authors declare no conflict of interest.

Author Contributions

TSA designed the study, acquired and analyzed the data, and drafted the manuscript. MRM performed the bioinformatics analysis and reviewed the manuscript. MP, BT and LHC interpreted the data and reviewed the manuscript. DC supervised the study, analyzed the data and drafted the manuscript. All authors approved the final version.

REFERENCES

1. American Diabetes Association. Classification and diagnosis of diabetes. Diabetes Care **38 Suppl**, S8-S16 (2015). doi:10.2337/dc15-S005
2. Guay, C., Regazzi, R.: Circulating microRNAs as novel biomarkers for diabetes mellitus. Nat Rev Endocrinol **9**(9), 513-521 (2013). doi:10.1038/nrendo.2013.86
3. Aly, H., Gottlieb, P.: The honeymoon phase: intersection of metabolism and immunology. Curr Opin Endocrinol Diabetes Obes **16**(4), 286-292 (2009). doi:10.1097/MED.0b013e32832e0693
4. Nielsen, L.B., Wang, C., Sorensen, K., Bang-Berthelsen, C.H., Hansen, L., Andersen, M.L., Hougaard, P., Juul, A., Zhang, C.Y., Pociot, F., Mortensen, H.B.: Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. **Exp Diabetes Res 2012**, 896362 (2012). doi:10.1155/2012/896362
5. Esteller, M.: Non-coding RNAs in human disease. Nat Rev Genet **12**(12), 861-874 (2011). doi:10.1038/nrg3074
6. Butz, H., Kinga, N., Racz, K., Patocs, A.: Circulating miRNAs as biomarkers for endocrine disorders. **J Endocrinol Invest 39**(1), 1-10 (2016). doi:10.1007/s40618-015-0316-5
7. Seyhan, A.A., Nunez Lopez, Y.O., Xie, H., Yi, F., Mathews, C., Pasarica, M., Pratley, R.E.: Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study. Sci Rep **6**, 31479 (2016). doi:10.1038/srep31479
8. Higuchi, C., Nakatsuka, A., Eguchi, J., Teshigawara, S., Kanzaki, M., Katayama, A., Yamaguchi, S., Takahashi, N., Murakami, K., Ogawa, D., Sasaki, S., Makino, H., Wada, J.: Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes. Metabolism: clinical and experimental **64**(4), 489-497 (2015). doi:10.1016/j.metabol.2014.12.003
9. Lin, S., Gregory, R.I.: MicroRNA biogenesis pathways in cancer. Nature reviews. Cancer **15**(6), 321-333 (2015). doi:10.1038/nrc3932
10. Pauley, K.M., Cha, S., Chan, E.K.: MicroRNA in autoimmunity and autoimmune diseases. Journal of autoimmunity **32**(3-4), 189-194 (2009). doi:10.1016/j.jaut.2009.02.012
11. Zalts, H., Shomron, N.: The impact of microRNAs on endocrinology. Pediatric endocrinology reviews : PER **8**(4), 354-362; quiz 362-353 (2011).
12. Flowers, E., Aouizerat, B.E., Abbasi, F., Lamendola, C., Grove, K.M., Fukuoka, Y., Reaven, G.M.: Circulating microRNA-320a and microRNA-486 predict thiazolidinedione response: Moving towards precision health for diabetes prevention. Metabolism: clinical and experimental **64**(9), 1051-1059 (2015). doi:10.1016/j.metabol.2015.05.013
13. Baltimore, D., Boldin, M.P., O'Connell, R.M., Rao, D.S., Taganov, K.D.: MicroRNAs: new regulators of immune cell development and function. Nature immunology **9**(8), 839-845 (2008). doi:10.1038/ni.f.209
14. Roggli, E., Britan, A., Gattesco, S., Lin-Marq, N., Abderrahmani, A., Meda, P., Regazzi, R.: Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. Diabetes **59**(4), 978-986 (2010). doi:10.2337/db09-0881
15. Takahashi, P., Xavier, D.J., Evangelista, A.F., Manoel-Caetano, F.S., Macedo, C., Collares, C.V., Foss-Freitas, M.C., Foss, M.C., Rassi, D.M., Donadi, E.A.,

Passos, G.A., Sakamoto-Hojo, E.T.: MicroRNA expression profiling and functional annotation analysis of their targets in patients with type 1 diabetes mellitus. Gene **539**(2), 213-223 (2014). doi:10.1016/j.gene.2014.01.075
16. Yang, M., Ye, L., Wang, B., Gao, J., Liu, R., Hong, J., Wang, W., Gu, W., Ning, G.: Decreased miR-146 expression in peripheral blood mononuclear cells is correlated with ongoing islet autoimmunity in type 1 diabetes patients 1miR-146. Journal of diabetes **7**(2), 158-165 (2015). doi:10.1111/1753-0407.12163
17. Erener, S., Marwaha, A., Tan, R., Panagiotopoulos, C., Kieffer, T.J.: Profiling of circulating microRNAs in children with recent onset of type 1 diabetes. JCI insight **2**(4), e89656 (2017). doi:10.1172/jci.insight.89656
18. Garcia-Contreras, M., Shah, S.H., Tamayo, A., Robbins, P.D., Golberg, R.B., Mendez, A.J., Ricordi, C.: Plasma-derived exosome characterization reveals a distinct microRNA signature in long duration Type 1 diabetes. Sci Rep **7**(1), 5998 (2017). doi:10.1038/s41598-017-05787-y
19. Abuhatzira, L., Xu, H., Tahhan, G., Boulougoura, A., Schaffer, A.A., Notkins, A.L.: Multiple microRNAs within the 14q32 cluster target the mRNAs of major type 1 diabetes autoantigens IA-2, IA-2beta, and GAD65. FASEB journal : official publication of the Federation of American Societies for Experimental Biology **29**(10), 4374-4383 (2015). doi:10.1096/fj.15-273649
20. Samandari, N., Mirza, A.H., Nielsen, L.B., Kaur, S., Hougaard, P., Fredheim, S., Mortensen, H.B., Pociot, F.: Circulating microRNA levels predict residual beta cell function and glycaemic control in children with type 1 diabetes mellitus. Diabetologia (2016). doi:10.1007/s00125-016-4156-4
21. Snowhite, I.V., Allende, G., Sosenko, J., Pastori, R.L., Messinger Cayetano, S., Pugliese, A.: Association of serum microRNAs with islet autoimmunity, disease progression and metabolic impairment in relatives at risk of type 1 diabetes. Diabetologia **60**(8), 1409-1422 (2017). doi:10.1007/s00125-017-4294-3
22. von Elm, E., Altman, D.G., Egger, M., Pocock, S.J., Gotzsche, P.C., Vandenbroucke, J.P., Initiative, S.: The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. Journal of clinical epidemiology **61**(4), 344-349 (2008). doi:10.1016/j.jclinepi.2007.11.008
23. Assmann, T.S., Brondani Lde, A., Bauer, A.C., Canani, L.H., Crispim, D.: Polymorphisms in the TLR3 gene are associated with risk for type 1 diabetes mellitus. European journal of endocrinology / European Federation of Endocrine Societies **170**(4), 519-527 (2014). doi:10.1530/EJE-13-0963
24. Nguyen, C., Varney, M.D., Harrison, L.C., Morahan, G.: Definition of high-risk type 1 diabetes HLA-DR and HLA-DQ types using only three single nucleotide polymorphisms. Diabetes **62**(6), 2135-2140 (2013). doi:10.2337/db12-1398
25. Assmann, T.S., Duarte, G.C., Brondani, L.A., de Freitas, P.H., Martins, E.M., Canani, L.H., Crispim, D.: Polymorphisms in genes encoding miR-155 and miR-146a are associated with protection to type 1 diabetes mellitus. Acta diabetologica (2017). doi:10.1007/s00592-016-0961-y
26. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T.: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem **55**(4), 611-622 (2009). doi:clinchem.2008.112797 [pii]
10.1373/clinchem.2008.112797

27. Dweep, H., Gretz, N.: miRWalk2.0: a comprehensive atlas of microRNA-target interactions. Nature methods **12**(8), 697 (2015). doi:10.1038/nmeth.3485
28. Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**(4), 402-408 (2001). doi:10.1006/meth.2001.1262
29. Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C., Gaasterland, T., Glenisson, P., Holstege, F.C., Kim, I.F., Markowitz, V., Matese, J.C., Parkinson, H., Robinson, A., Sarkans, U., Schulze-Kremer, S., Stewart, J., Taylor, R., Vilo, J., Vingron, M.: Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nature genetics **29**(4), 365-371 (2001). doi:10.1038/ng1201-365
30. Chou, C.H., Chang, N.W., Shrestha, S., Hsu, S.D., Lin, Y.L., Lee, W.H., Yang, C.D., Hong, H.C., Wei, T.Y., Tu, S.J., Tsai, T.R., Ho, S.Y., Jian, T.Y., Wu, H.Y., Chen, P.R., Lin, N.C., Huang, H.T., Yang, T.L., Pai, C.Y., Tai, C.S., Chen, W.L., Huang, C.Y., Liu, C.C., Weng, S.L., Liao, K.W., Hsu, W.L., Huang, H.D.: miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. Nucleic acids research **44**(D1), D239-247 (2016). doi:10.1093/nar/gkv1258
31. Li, J.H., Liu, S., Zhou, H., Qu, L.H., Yang, J.H.: starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic acids research **42**(Database issue), D92-97 (2014). doi:10.1093/nar/gkt1248
32. Xiao, F., Zuo, Z., Cai, G., Kang, S., Gao, X., Li, T.: miRecords: an integrated resource for microRNA-target interactions. Nucleic acids research **37**(Database issue), D105-110 (2009). doi:10.1093/nar/gkn851
33. Paraskevopoulou, M.D., Georgakilas, G., Kostoulas, N., Vlachos, I.S., Vergoulis, T., Reczko, M., Filippidis, C., Dalamagas, T., Hatzigeorgiou, A.G.: DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic acids research **41**(Web Server issue), W169-173 (2013). doi:10.1093/nar/gkt393
34. Agarwal, V., Bell, G.W., Nam, J.W., Bartel, D.P.: Predicting effective microRNA target sites in mammalian mRNAs. eLife **4** (2015). doi:10.7554/eLife.05005
35. Maragkakis, M., Vergoulis, T., Alexiou, P., Reczko, M., Plomaritou, K., Gousis, M., Kourtis, K., Koziris, N., Dalamagas, T., Hatzigeorgiou, A.G.: DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association. Nucleic acids research **39**(Web Server issue), W145-148 (2011). doi:10.1093/nar/gkr294
36. Wain, H.M., Bruford, E.A., Lovering, R.C., Lush, M.J., Wright, M.W., Povey, S.: Guidelines for human gene nomenclature. Genomics **79**(4), 464-470 (2002). doi:10.1006/geno.2002.6748
37. Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M.: KEGG as a reference resource for gene and protein annotation. Nucleic acids research **44**(D1), D457-462 (2016). doi:10.1093/nar/gkv1070
38. Vlachos, I.S., Zagganas, K., Paraskevopoulou, M.D., Georgakilas, G., Karagkouni, D., Vergoulis, T., Dalamagas, T., Hatzigeorgiou, A.G.: DIANA-miRPath v3.0: deciphering microRNA function with experimental support. Nucleic acids research **43**(W1), W460-466 (2015). doi:10.1093/nar/gkv403
39. Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., Ma'ayan, A.: Enrichr: interactive and collaborative HTML5 gene list enrichment

analysis tool. BMC bioinformatics **14**, 128 (2013). doi:10.1186/1471-2105-14-128
40. Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G., Monteiro, C.D., Gundersen, G.W., Ma'ayan, A.: Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic acids research **44**(W1), W90-97 (2016). doi:10.1093/nar/gkw377
41. Yu, G., Wang, L.G., Han, Y., He, Q.Y.: clusterProfiler: an R package for comparing biological themes among gene clusters. Omics : a journal of integrative biology **16**(5), 284-287 (2012). doi:10.1089/omi.2011.0118
42. Carlson, M.R., Pages, H., Arora, S., Obenchain, V., Morgan, M.: Genomic Annotation Resources in R/Bioconductor. Methods in molecular biology **1418**, 67-90 (2016). doi:10.1007/978-1-4939-3578-9_4
43. Wickham, H.: The Split-Apply-Combine Strategy for Data Analysis. Journal of Statistical Software **40**(1), 1-29 (2011).
44. Pirot, P., Cardozo, A.K., Eizirik, D.L.: Mediators and mechanisms of pancreatic beta-cell death in type 1 diabetes. Arquivos brasileiros de endocrinologia e metabologia **52**(2), 156-165 (2008).
45. Eisenbarth, G.S., Jeffrey, J.: The natural history of type 1A diabetes. Arq Bras Endocrinol Metabol **52**(2), 146-155 (2008).
46. Bonifacio, E.: Predicting type 1 diabetes using biomarkers. Diabetes care **38**(6), 989-996 (2015). doi:10.2337/dc15-0101
47. Lu, J., Xie, F., Geng, L., Shen, W., Sui, C., Yang, J.: Potential Role of MicroRNA-210 as Biomarker in Human Cancers Detection: A Meta-Analysis. BioMed research international **2015**, 303987 (2015). doi:10.1155/2015/303987
48. Zhu, H., Leung, S.W.: Identification of microRNA biomarkers in type 2 diabetes: a meta-analysis of controlled profiling studies. Diabetologia **58**(5), 900-911 (2015). doi:10.1007/s00125-015-3510-2
49. Trajkovski, M., Hausser, J., Soutschek, J., Bhat, B., Akin, A., Zavolan, M., Heim, M.H., Stoffel, M.: MicroRNAs 103 and 107 regulate insulin sensitivity. Nature **474**(7353), 649-653 (2011). doi:10.1038/nature10112
50. Lovis, P., Roggli, E., Laybutt, D.R., Gattesco, S., Yang, J.Y., Widmann, C., Abderrahmani, A., Regazzi, R.: Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction. Diabetes **57**(10), 2728-2736 (2008). doi:10.2337/db07-1252
51. Collares, C.V., Evangelista, A.F., Xavier, D.J., Rassi, D.M., Arns, T., Foss-Freitas, M.C., Foss, M.C., Puthier, D., Sakamoto-Hojo, E.T., Passos, G.A., Donadi, E.A.: Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients. BMC research notes **6**, 491 (2013). doi:10.1186/1756-0500-6-491
52. Kfir-Erenfeld, S., Haggiag, N., Biton, M., Stepensky, P., Assayag-Asherie, N., Yefenof, E.: miR-103 inhibits proliferation and sensitizes hemopoietic tumor cells for glucocorticoid-induced apoptosis. Oncotarget **8**(1), 472-489 (2017). doi:10.18632/oncotarget.13447
53. Rottiers, V., Naar, A.M.: MicroRNAs in metabolism and metabolic disorders. Nature reviews. Molecular cell biology **13**(4), 239-250 (2012). doi:10.1038/nrm3313
54. Faraoni, I., Antonetti, F.R., Cardone, J., Bonmassar, E.: miR-155 gene: a typical multifunctional microRNA. Biochimica et biophysica acta **1792**(6), 497-505 (2009). doi:10.1016/j.bbadis.2009.02.013

55. Ma, X., Becker Buscaglia, L.E., Barker, J.R., Li, Y.: MicroRNAs in NF-kappaB signaling. Journal of molecular cell biology **3**(3), 159-166 (2011). doi:10.1093/jmcb/mjr007
56. Wang, P., Hou, J., Lin, L., Wang, C., Liu, X., Li, D., Ma, F., Wang, Z., Cao, X.: Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. Journal of immunology **185**(10), 6226-6233 (2010). doi:10.4049/jimmunol.1000491
57. Khamaneh, A.M., Alipour, M.R., Sheikhzadeh Hesari, F., Ghadiri Soufi, F.: A signature of microRNA-155 in the pathogenesis of diabetic complications. Journal of physiology and biochemistry **71**(2), 301-309 (2015). doi:10.1007/s13105-015-0413-0
58. Sebastiani, G.: MicroRNA expression fingerprint in serum of type 1 diabetic patients. Diabetologia **55**, S48 (2012).
59. Barutta, F., Tricarico, M., Corbelli, A., Annaratone, L., Pinach, S., Grimaldi, S., Bruno, G., Cimino, D., Taverna, D., Deregibus, M.C., Rastaldi, M.P., Perin, P.C., Gruden, G.: Urinary exosomal microRNAs in incipient diabetic nephropathy. PloS one **8**(11), e73798 (2013). doi:10.1371/journal.pone.0073798
60. Heegaard, N.H., Carlsen, A.L., Lilje, B., Ng, K.L., Ronne, M.E., Jorgensen, H.L., Sennels, H., Fahrenkrug, J.: Diurnal Variations of Human Circulating Cell-Free Micro-RNA. PloS one **11**(8), e0160577 (2016). doi:10.1371/journal.pone.0160577
61. Xu, Y.F., Hannafon, B.N., Ding, W.Q.: microRNA regulation of human pancreatic cancer stem cells. Stem cell investigation **4**, 5 (2017). doi:10.21037/sci.2017.01.01
62. Humphries, B., Yang, C.: The microRNA-200 family: small molecules with novel roles in cancer development, progression and therapy. Oncotarget **6**(9), 6472-6498 (2015). doi:10.18632/oncotarget.3052
63. Belgardt, B.F., Ahmed, K., Spranger, M., Latreille, M., Denzler, R., Kondratiuk, N., von Meyenn, F., Villena, F.N., Herrmanns, K., Bosco, D., Kerr-Conte, J., Pattou, F., Rulicke, T., Stoffel, M.: The microRNA-200 family regulates pancreatic beta cell survival in type 2 diabetes. Nature medicine **21**(6), 619-627 (2015). doi:10.1038/nm.3862
64. Hennessy, E., Clynes, M., Jeppesen, P.B., O'Driscoll, L.: Identification of microRNAs with a role in glucose stimulated insulin secretion by expression profiling of MIN6 cells. Biochemical and biophysical research communications **396**(2), 457-462 (2010). doi:10.1016/j.bbrc.2010.04.116
65. Fayyad-Kazan, H., Rouas, R., Fayyad-Kazan, M., Badran, R., El Zein, N., Lewalle, P., Najar, M., Hamade, E., Jebbawi, F., Merimi, M., Romero, P., Burny, A., Badran, B., Martiat, P.: MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed microRNAs on expression of two genes essential to their function. The Journal of biological chemistry **287**(13), 9910-9922 (2012). doi:10.1074/jbc.M111.337154
66. Devlin, C., Greco, S., Martelli, F., Ivan, M.: miR-210: More than a silent player in hypoxia. IUBMB life **63**(2), 94-100 (2011). doi:10.1002/iub.427
67. Osipova, J., Fischer, D.C., Dangwal, S., Volkmann, I., Widera, C., Schwarz, K., Lorenzen, J.M., Schreiver, C., Jacoby, U., Heimhalt, M., Thum, T., Haffner, D.: Diabetes-associated microRNAs in pediatric patients with type 1 diabetes mellitus: a cross-sectional cohort study. The Journal of clinical endocrinology and metabolism **99**(9), E1661-1665 (2014). doi:10.1210/jc.2013-3868

68. Lu, L.F., Boldin, M.P., Chaudhry, A., Lin, L.L., Taganov, K.D., Hanada, T., Yoshimura, A., Baltimore, D., Rudensky, A.Y.: Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. Cell **142**(6), 914-929 (2010). doi:10.1016/j.cell.2010.08.012
69. Saba, R., Sorensen, D.L., Booth, S.A.: MicroRNA-146a: A Dominant, Negative Regulator of the Innate Immune Response. Frontiers in immunology **5**, 578 (2014). doi:10.3389/fimmu.2014.00578
70. Simpson, L.J., Ansel, K.M.: MicroRNA regulation of lymphocyte tolerance and autoimmunity. The Journal of clinical investigation **125**(6), 2242-2249 (2015). doi:10.1172/JCI78090
71. Ventriglia, G., Nigi, L., Sebastiani, G., Dotta, F.: MicroRNAs: Novel Players in the Dialogue between Pancreatic Islets and Immune System in Autoimmune Diabetes. BioMed research international **2015**, 749734 (2015). doi:10.1155/2015/749734
72. Karolina, D.S., Armugam, A., Tavintharan, S., Wong, M.T., Lim, S.C., Sum, C.F., Jeyaseelan, K.: MicroRNA 144 impairs insulin signaling by inhibiting the expression of insulin receptor substrate 1 in type 2 diabetes mellitus. PloS one **6**(8), e22839 (2011). doi:10.1371/journal.pone.0022839

Table 1. Clinical and laboratory characteristics of subjects included in the study.

Characteristic	Screening sample set				Validation sample set			
	Controls (n = 26)	T1DM <5 years (n = 16)	T1DM ≥5 years (n = 17)	P*	Controls (n = 14)	T1DM <5 years (n = 13)	T1DM ≥5 years (n = 14)	P*
Age (years)	21.5±2.7	19.2±6.4	19.9±4.6	0.267	20.6±1.7	21.0±6.2	22.1±4.5	0.663
Sex (% male)	54.9	51.9	55.3	0.955	53.9	54.1	52.6	0.934
BMI (kg/m²)	24.5±3.2	23.1±3.4	23.4±3.1	0.379	23.4±2.5	24.0±2.6	23.9±2.3	0.840
HbA1c (%)	5.3±0.3[a]	8.9±1.6[b]	9.4±1.7[b]	0.0001	5.3±0.2[a]	8.6±1.4[b]	8.5±1.6[b]	0.0001
Age at diagnosis (years)	-	17.0 (16.2 – 23.7)	9 (1 – 9.75)	0.0001	-	20 (15 – 23.7)	10 (7 – 13.5)	0.001
Duration of diabetes (years)	-	2.5 (1 - 4)	12.5 (7 - 24)	0.0001	-	2.5 (1 - 4)	14.0 (6 - 19)	0.0001
Insulin dose (unit/kg·day)	-	0.86±0.33	0.96±0.35	0.439	-	0.75±0.23	0.83±0.36	0.524
Triglycerides (mg/dl)	-	85 (61.2 – 159.7)	55 (46.7 – 112.5)	0.289	-	84 (63 – 117.5)	77 (54 – 104.7)	0.594

Total-CT (mg/dl)	-	158.3±40.8	168.8±33.1	0.362	-	160.3±23.2	165.8±44.1	0.785
HDL-CT (mg/dl)	-	43.8±17.1	45.7±10.5	0.810	-	48.2±19.6	52.8±12.8	0.597
Creatinine (μg/dl)	-	0.7 (0.6 – 0.8)	0.8 (0.7 – 0.9)	0.509	-	0.7 (0.6 – 0.9)	0.7 (0.6 – 0.9)	0.353
TSH (μUl/ml)	-	1.5±0.7	3.0±1.6	0.166	-	1.7±1.2	1.3±0.9	0.694
Hypothyroidism (%)	0.0[a]	13.3[a]	29.4[b]	0.001	0.0[a]	13.3[b]	19.8[b]	0.016
High-risk HLA haplotypes (%) [#]	12.5[a]	61.5[b]	56.3[b]	<0.001	7.1[a]	44.4[b]	50.0[b]	0.001

Quantitative variables with normal distribution are presented as mean ± SD, while quantitative variables with skewed distribution were log-transformed before analyses and are presented as median (25^{th}-75^{th} percentiles). Categorical data are shown as %. * P values were computed using χ^2 or ANOVA, followed by post-hoc tests (residual analysis or Tukey's tests, respectively), as appropriate. Analyses with significant differences are indicated as follows: means, medians or % indicated by different superscript letters differed from the other groups at $P < 0.05$. [#] High-risk HLA haplotypes: DR4/DQ8 or DR3/DR4-DQ8 or DR3/DR3. BMI = body mass index; HbA1c = glycated hemoglobin; CT = cholesterol.

Table 2. MiRNA expressions in plasma from "screening sample" subjects obtained using Custom TaqMan Array MicroRNA cards.

miRNA	**Controls (n= 26)**	**T1DM <5 years (n= 16)**	**T1DM ≥5 years (n= 17)**	**P***
miR-15b-5p	1.77 (0.47 – 44.36)	4.72 (0.39 – 91.08)	1.13 (0.22 – 31.33)	0.654
miR-21-5p	0.87 (0.32 – 4.05)[a]	6.74 (0.56 – 31.98)[b]	0.56 (0.17 – 2.58)[a]	**0.022**
miR-21-3p	0.13 (0.02 – 0.50)	0.46 (0.002 – 33.92)	0.18 (0.004 – 15.49)	0.489
miR-25-3p	1.25 (0.49 – 3.28)	2.43 (0.47 – 14.17)	1.03 (0.33 – 4.92)	0.625
miR-26b-5p	40.27 (9.25 – 50.17)	ND	48.66 (10.06 – 162.85)	0.418
miR-29a-3p	0.66 (0.17 – 1.20)	1.42 (0.31 – 3.59)	0.40 (0.09 – 0.70)	0.211
miR-29b-3p	0.75 (0.27 – 2.7)	0.24 (0.03 – 75.7)	0.96 (0.04 – 4.85)	0.533
miR-101-3p	1.58 (0.20 – 5.15)[a]	24.97 (3.98 – 241.18)[b]	1.41 (0.69 – 36.90)[a]	**0.024**
miR-103a-3p	0.72 (0.06 – 1.72)[a]	121.0 (0.79 – 905.79)[b]	0.27 (0.08 – 36.33)[a]	**0.023**
miR-125b-5p	0.48 (0.03 – 17.69)	0.33 (0.03 – NO[#])	0.38 (0.07 – 156.22)	0.666
miR-126-3p	0.34 (0.07 – 1.06)	0.42 (0.28 – 1.3)	0.08 (0.02 – 0.66)	0.077
miR-133a-5p	0.30 (0.08 – 0.88)	0.95 (0.05 – 5.83)	0.31 (0.04 – 0.76)	0.466
miR-146a-5p	1.64 (0.74 – 3.57)[a]	0.29 (0.05 – 1.03)[b]	3.01 (1.13 – 5.47)[a]	**0.0001**

miR-148a-3p	3.76 (0.79 – 6.16)	56.03 (0.38 – 214.86)	2.36 (1.01 – 48.22)	0.933
miR-148b-3p	0.06 (0.03 – 0.36)[a]	60.44 (0.16 – 2483.72)[b]	1.13 (0.05 – 33.50)[a]	**0.030**
miR-155-5p	0.45 (0.08 – 1.20)[a]	1.88 (0.38 – 7.68)[b]	0.26 (0.05 – 0.64)[a]	**0.012**
miR-181a-5p	1.31 (0.23 – 8.15)	ND	0.07 (0.01 – 497.70)	0.486
miR-200a-3p	1.06 (0.18 – 1.73)[a]	59.90 (2.90 – 80.08)[b]	0.78 (0.17 – 1.18)[a]	**0.001**
miR-200c-3p	0.30 (0.07 – 1.12)	1.49 (0.02– 5.26)	0.05 (0.02 – 1.05)	0.165
miR-210-3p	0.86 (0.19 – 1.99)[a]	4.79 (1.25 – 42.77)[b]	0.45 (0.25 – 3.22)[a]	**0.014**
miR-222-3p	1.99 (0.46 – 4.38)	2.87 (1.18 – NO[#])	0.98 (0.07 – 1.52)	0.318
miR-320	0.52 (0.22 – 1.96)	1.87 (0.37 – 6.88)	0.42 (0.12 – 2.10)	0.096
miR-338-3p	1.49 (0.20 – 2.57)	0.77 (0.02 – NO[#])	7.82 (1.62 – 127.49)	0.180
miR-340-5p	0.46 (0.22 – 1.49)	ND	1.56 (0.51 – 2.70)	0.285
miR-342	0.04 (0.005 – 0.41)	1.97 (0.001 – NO[#])	0.07 (0.007 – 2.53)	0.425
miR-1275	0.94 (0.02 – 124.99)[a]	489.80 (6.47– 911.68)[b]	9.91 (0.04 – 164.54)[a,b]	**0.039**

Data are shown as median (25^{th} – 75^{th} percentiles) of n-fold change values since the distribution of this variable was skewed. *P values were obtained from One-way ANOVA followed by Tukey post-hoc tests using the log-transformed variable. Values with significant differences are

indicated by different superscript letters ($P < 0.05$), while statistically similar values are indicated by the same superscript letters. ND (not detected) = no detectable miRNA expression was observed for this group. In this case, Student's t-tests were used for comparisons between the two remaining groups. # NO (non-observed) = no miRNA expression higher than the 50^{th} percentile was observed for any subject. The other 19 miRNAs included in the array cards had no detectable expression or were expressed in less than 10% of the samples.

FIGURES

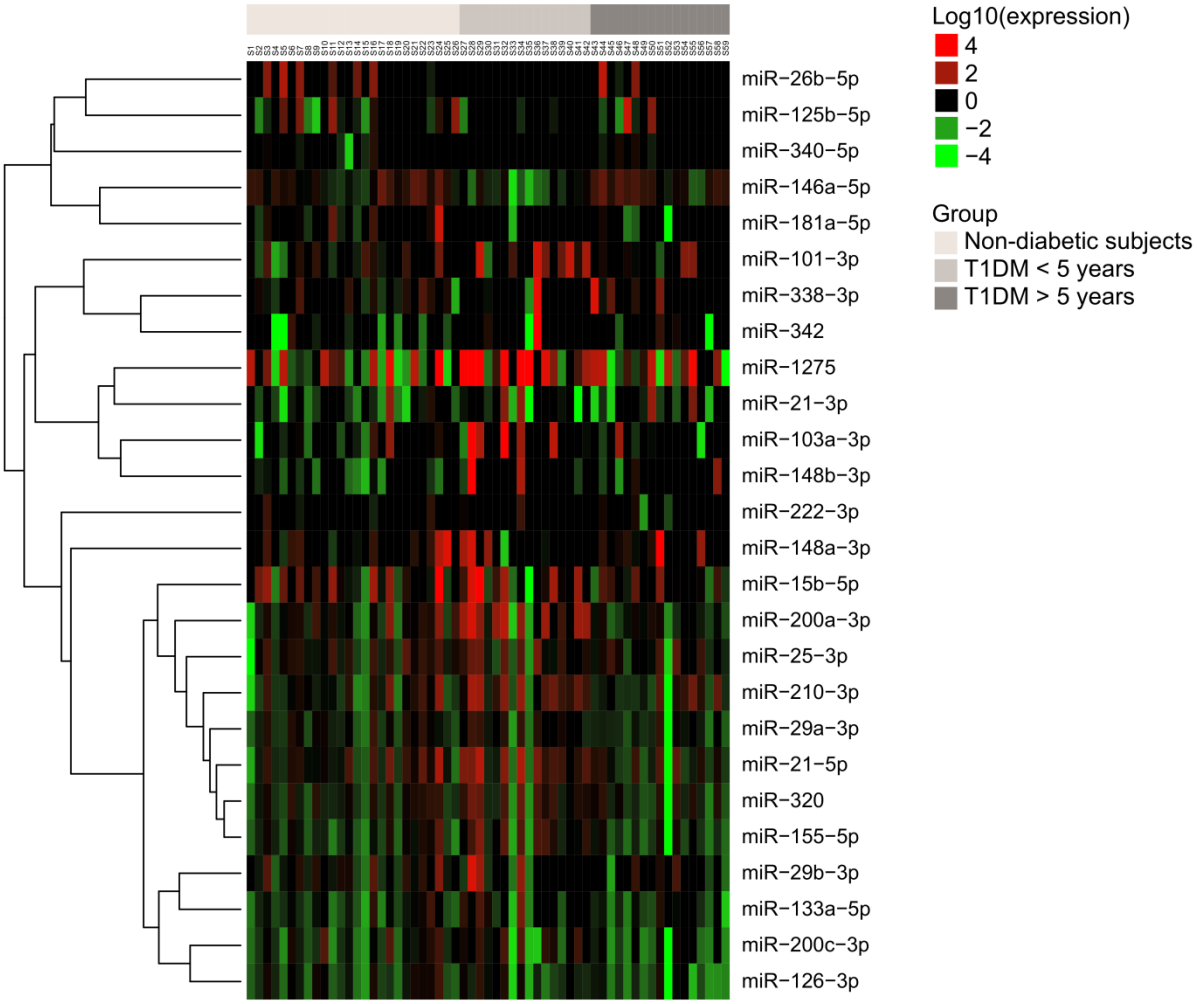
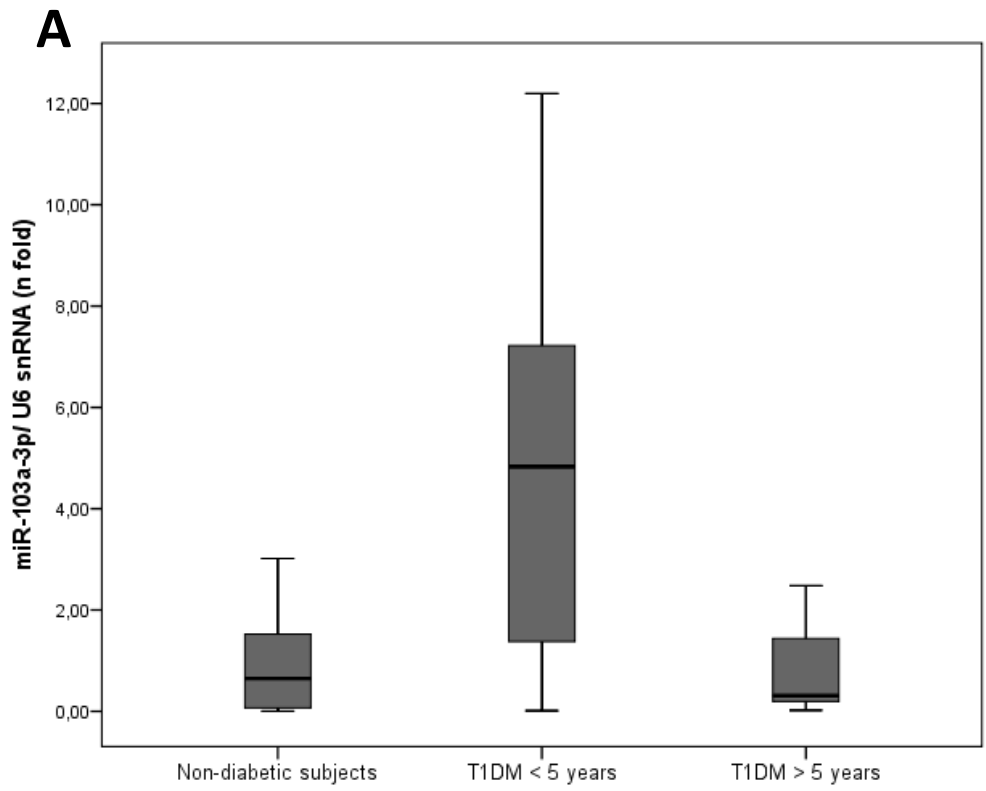
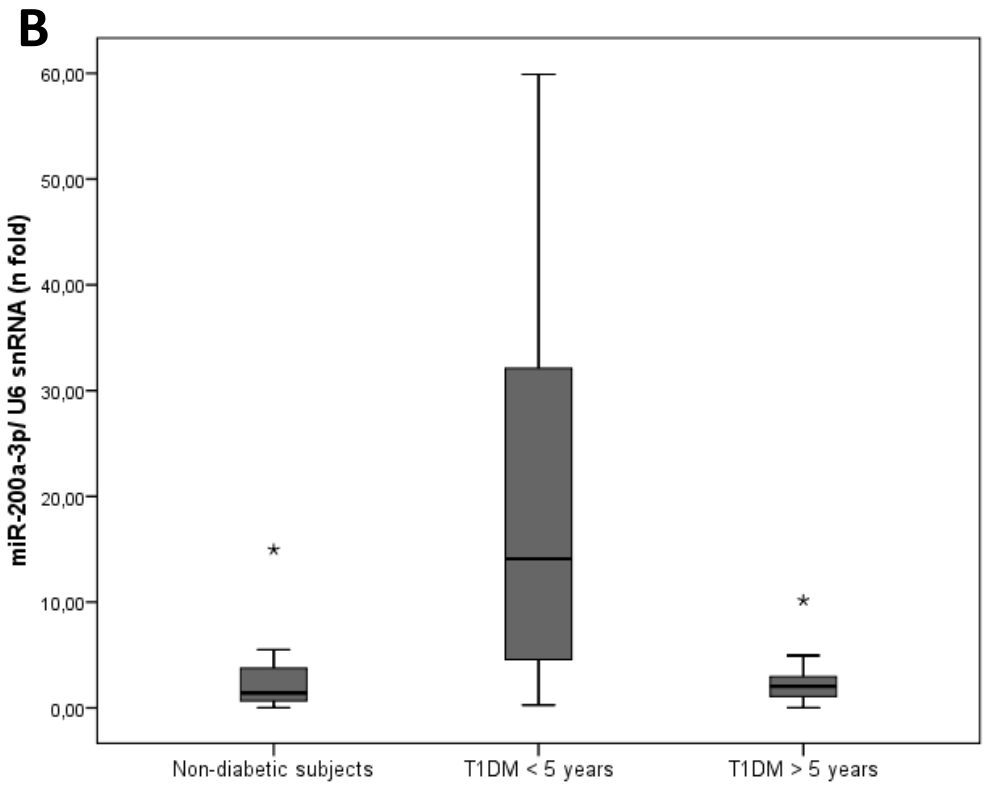
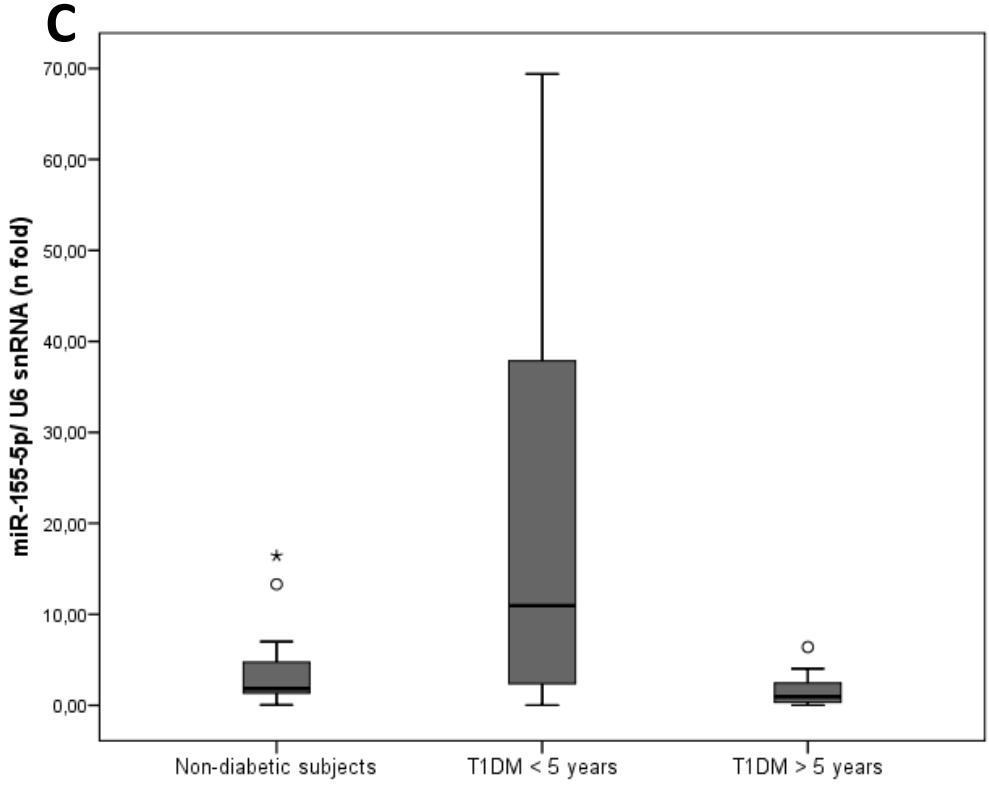


Figure 1. Distinct miRNA expression patterns in T1DM patients grouped according to T1DM duration. Heatmap of the 26 miRNAs expressed in the analyzed samples. The miRNAs were clustered using the Pearson uncentered distance metric with average linkage. Each column represents the samples in each group and each row represents an individual miRNA. Expression levels of miRNAs are shown in red (upregulated) and green (downregulated), with brighter shades indicating higher fold differences (log_{10} n-fold change values) in relation to the calibrator sample. Lack of difference in expression levels is represented in black.

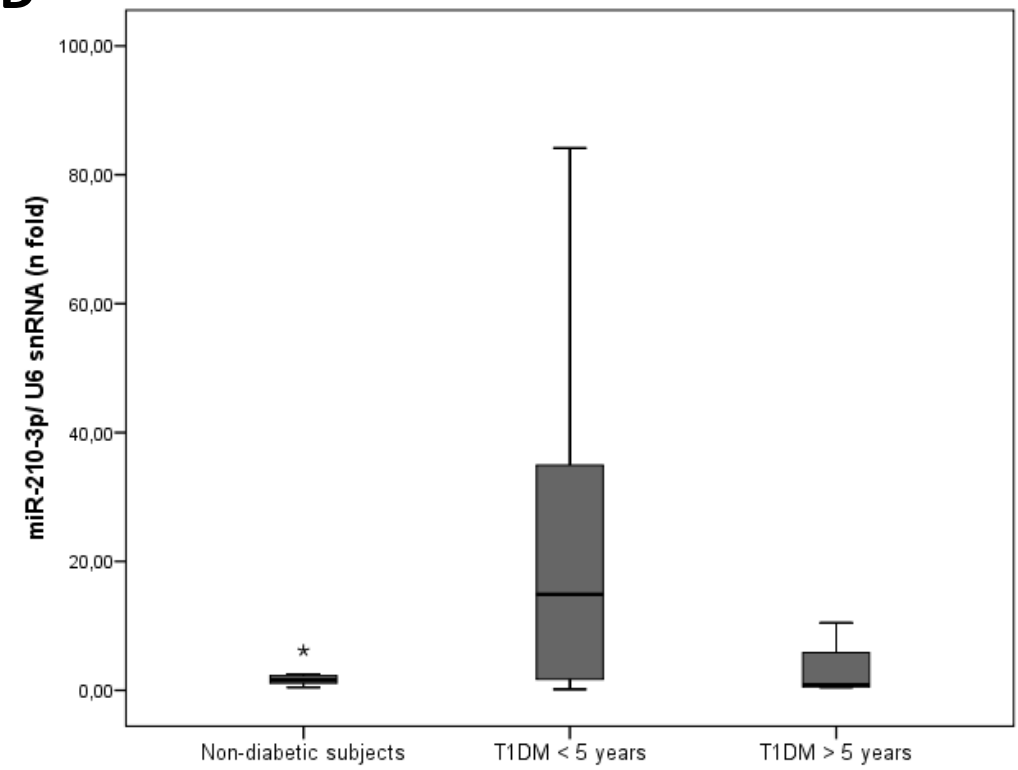

A
12,00
10,00
8,00
6,00
4,00
2,00
0,00
miR-103a-3p/ U6 snRNA (n fold)
Non-diabetic subjects
T1DM < 5 years
T1DM > 5 years


B
60,00
50,00
40,00
30,00
20,00
10,00
0,00
miR-200a-3p/ U6 snRNA (n fold)
Non-diabetic subjects
T1DM < 5 years
T1DM > 5 years

C


miR-155-5p/ U6 snRNA (n fold)
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
Non-diabetic subjects
T1DM < 5 years
T1DM > 5 years

D


miR-210-3p/ U6 snRNA (n fold)
100,00
80,00
60,00
40,00
20,00
0,00
Non-diabetic subjects
T1DM < 5 years
T1DM > 5 years

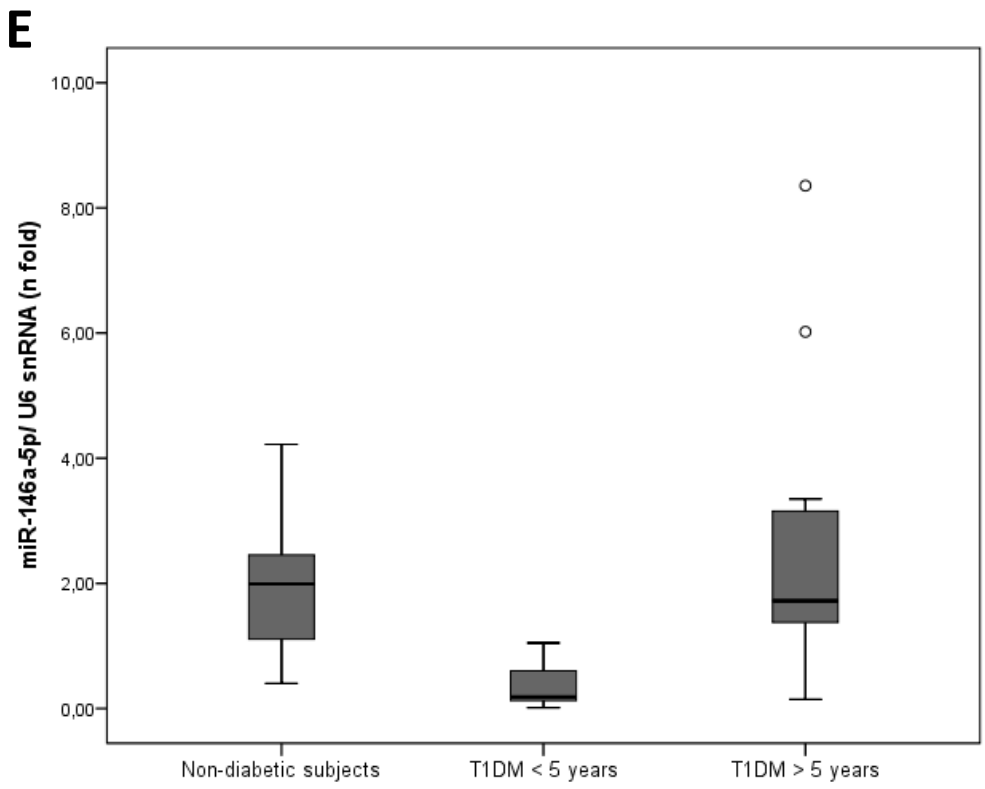


Figure 2. Differently expressed miRNAs in plasma from T1DM patients (with <5 years or ≥ 5 years of diagnosis) and non-diabetic subjects from the "Validation sample set". Relative expressions of (**A**) miR-103a-3p, (**B**) miR-200a-3p, (**C**) miR-155-5p, (**D**) miR-210-3p and (**E**) miR-146a-5p were assessed by quantitative RT-qPCR. Results are expressed as n-fold changes in relation to the calibrator sample ($\Delta\Delta C_q$ method), using *U6snRNA* as the reference gene, and are shown as median (25^{th} – 75^{th} percentiles). P-values were obtained using one-way ANOVA tests with Tukey's post-hoc tests, using the log-transformed variable. * P-value ≤ 0.05; ** P-value ≤ 0.01

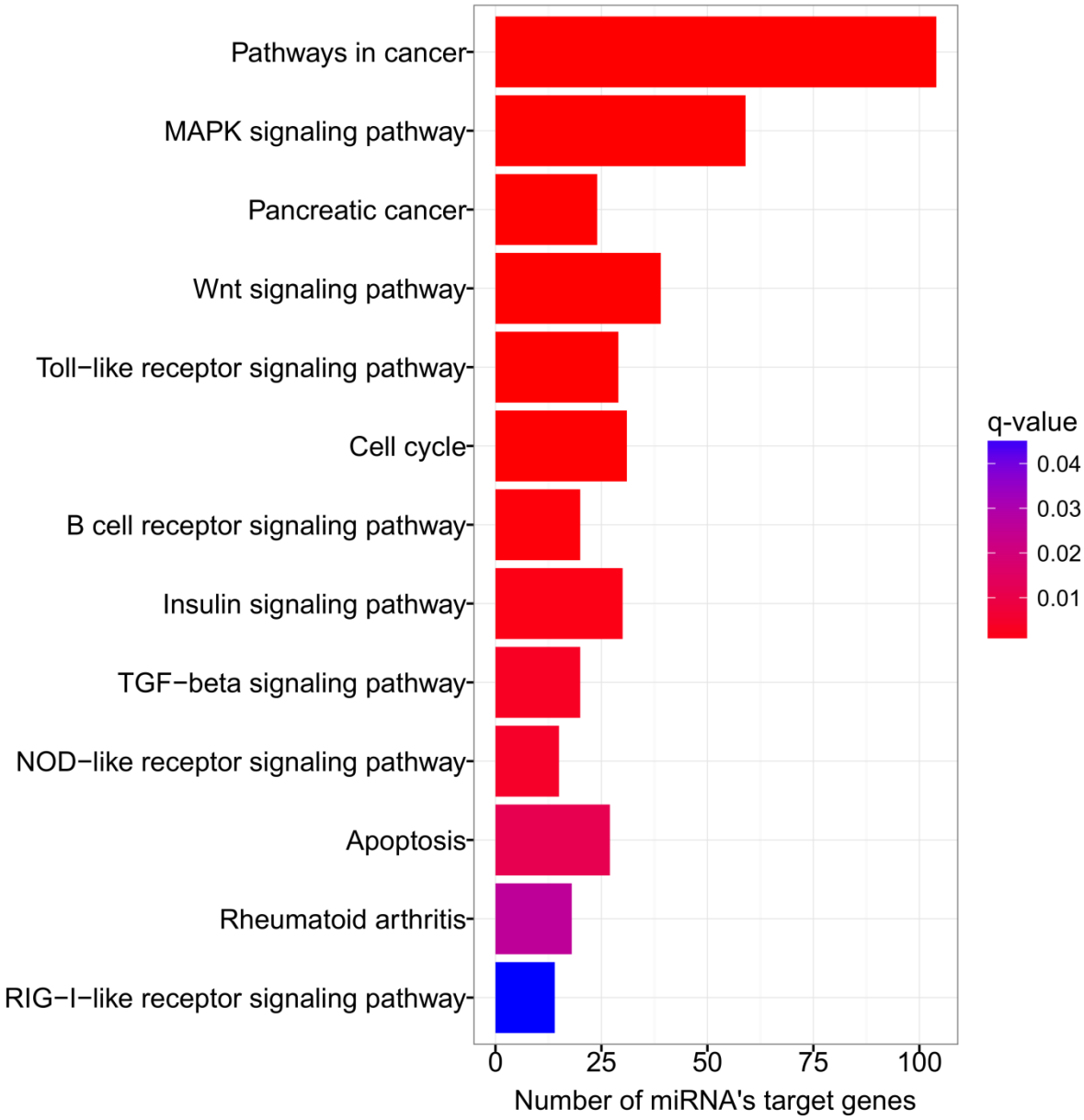


Figure 3. Significant KEGG pathways related to T1DM and regulated by the five dysregulated miRNAs (miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p and miR-210-3p) in recently-diagnosed T1DM patients. Raw data used for this analysis were retrieved from KEGG database [37]. Q-values: P values corrected for multiple tests using the Benjamini-Hochberg procedure, which provides a False Discovery Rate (FDR) adjusted-P value.

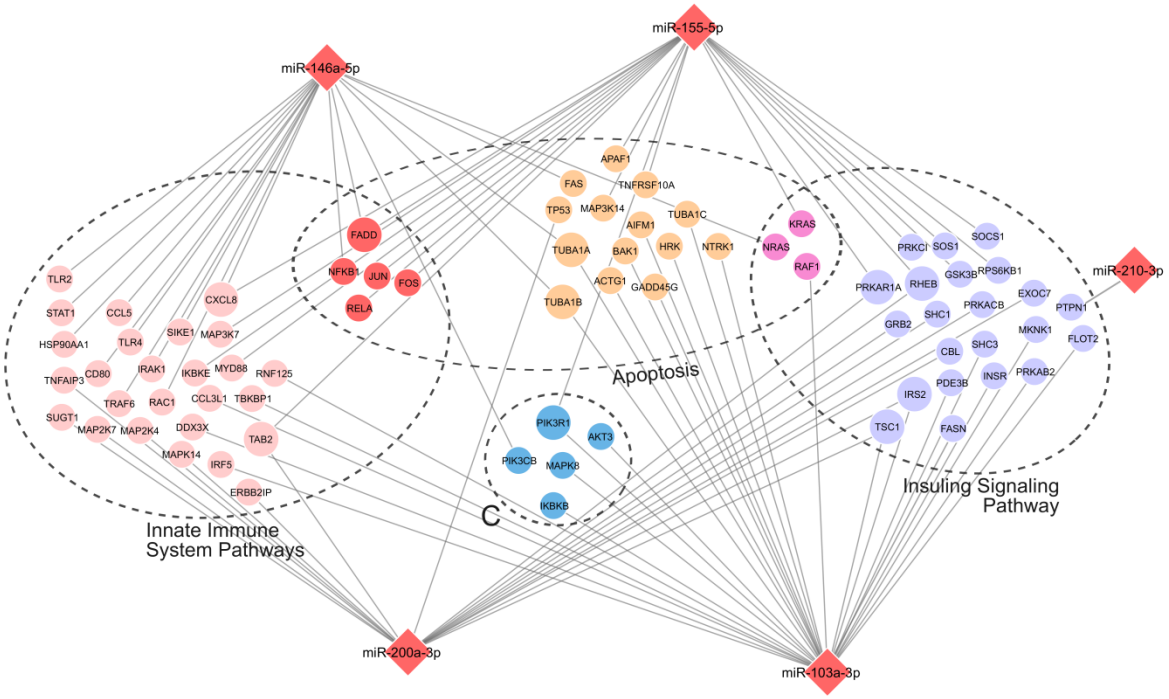


Figure 4. Interactions among miRNAs and their target genes. Rhombuses represent miRNAs and the circles represent their target genes. C= common target genes among all the three pathways. Raw data used for this analysis were retrieved from KEGG database [37].

SUPPLEMENTARY MATERIAL LIST

Supplementary Material 1. Detailed methods used for the "Bioinformatic Consensus Analysis".

Supplementary Figure 1. Venn diagram demonstrating the number of target genes shared among the five dysregulated miRNAs validated in the "Validation sample set". The targets of these miRNAs were investigated using a combination of validated experimental data and computational prediction tools, as described in the Methods section.

Supplementary Figure 2. Consensus analysis of the bioinformatic tools used to identify pathways. **A)** "Binary matrix" indicating whether a given pathway (rows) is enriched (black) or not (white) in the results of a given functional enrichment tool (columns). The greatest overlap among tools was concentrated in the upper portion of the matrix, which in rows 1-108 represents the enriched terms of our original results, ordered by decreasing level of significance. **B)** Venn diagrams for the "Pathways Union", and **C)** for the "Genes Union" options of mirPath, showing the number of pathways/genes shared among tools. **D)** Pairwise Jaccard Similarity Coefficients. Values closer to 1 (depicted in dark blue): indicate that two sets are more similar in terms of their elements. Detailed information about these analyses can be found in the Methods section and Supplementary Material 1.

Supplementary Table 1. Assay reference numbers for the 48 analyzed miRNAs included in the TaqMan Low Density Array cards**.**

Supplementary Table 2. Receiver operating curves (ROC) for miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p and miR-210-3p expressions in plasma from T1DM patients with less than 5 years of diagnosis and non-diabetic subjects.

Supplementary Table 3. MiRNA-target gene interactions for the five miRNAs validated in the study (hsa-miR-103a-3p, hsa-miR-200a-3p, hsa-miR-155-5p, hsa-miR-210-3p and hsa-miR-146a-5p). (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 4. Number of miRNA-target interactions for each of the five miRNAs validated in the study (hsa-miR-103a-3p, hsa-miR-200a-3p, hsa-miR-155-5p, hsa-miR-210-3p and hsa-miR-146a-5p).

Supplementary Table 5. Significant KEGG pathways for the five dysregulated miRNAs validated in the study (hsa-miR-103a-3p, hsa-miR-200a-3p, hsa-miR-155-5p, hsa-miR-210-3p and hsa-miR-146a-5p), imputed together. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 6. Results of target analyses for four possibly dysregulated miRNAs not validated in the study (hsa-miR-21-5p, hsa-miR-101-3p, hsa-miR-148b-3p, and hsa-miR-1275). (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 7. Consensus analysis of pathways obtained using different bioinformatic tools (miRTarBase, starBase, miRecords, TargetScan, and DIANA MicroT) compared with pathways obtained from mirPath (a DIANA suit tool) and the Enrichr web server. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 8. Differential expression between our results and those from a dataset available in the GEO database (GSE26168).

Supplementary Material 1. Detailed methods used for the "Bioinformatic Consensus Analysis".

Material and method – "Bioinformatics analysis"

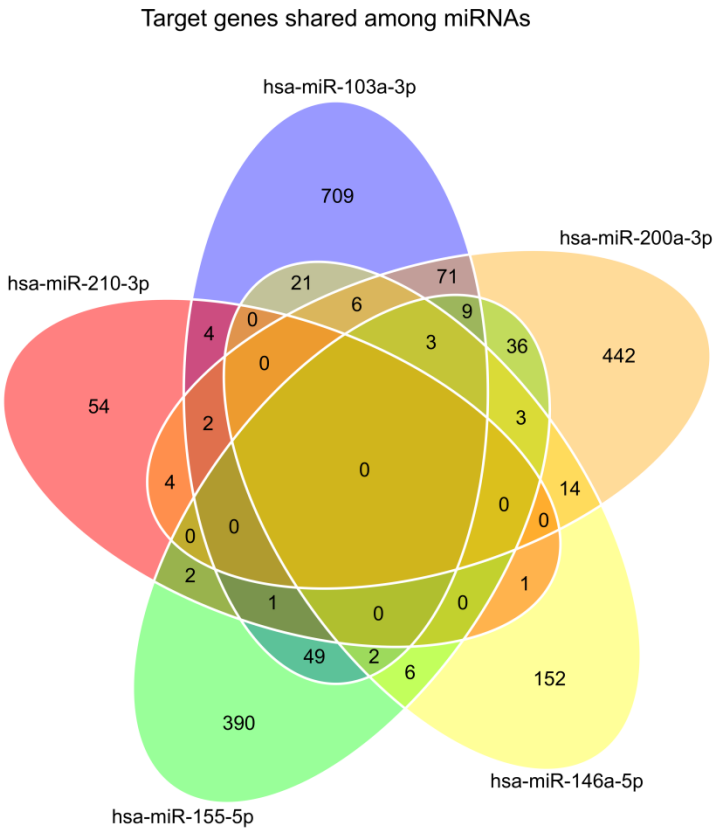
To investigate a consensus of pathways, we compared the data between the databases we choose first, as described in the material and method section in the text, with a tool of DIANA suite, namely the mirPath, and with Enrichr web server (http://amp.pharm.mssm.edu/Enrichr/).

We used the **mirPath** web tool from DIANA suite to investigate enriched pathways for our set of 5 differentially expressed miRNAs. One important thing to consider is that when submitting a list of miRNAs, miRPath allows two options for recovering the set of target genes to be analyzed, either validated targets from TarBase or predicted targets from MicroT. Therefore, mirPath does not allows the joint analysis of validated and predicted targets for functional enrichment.

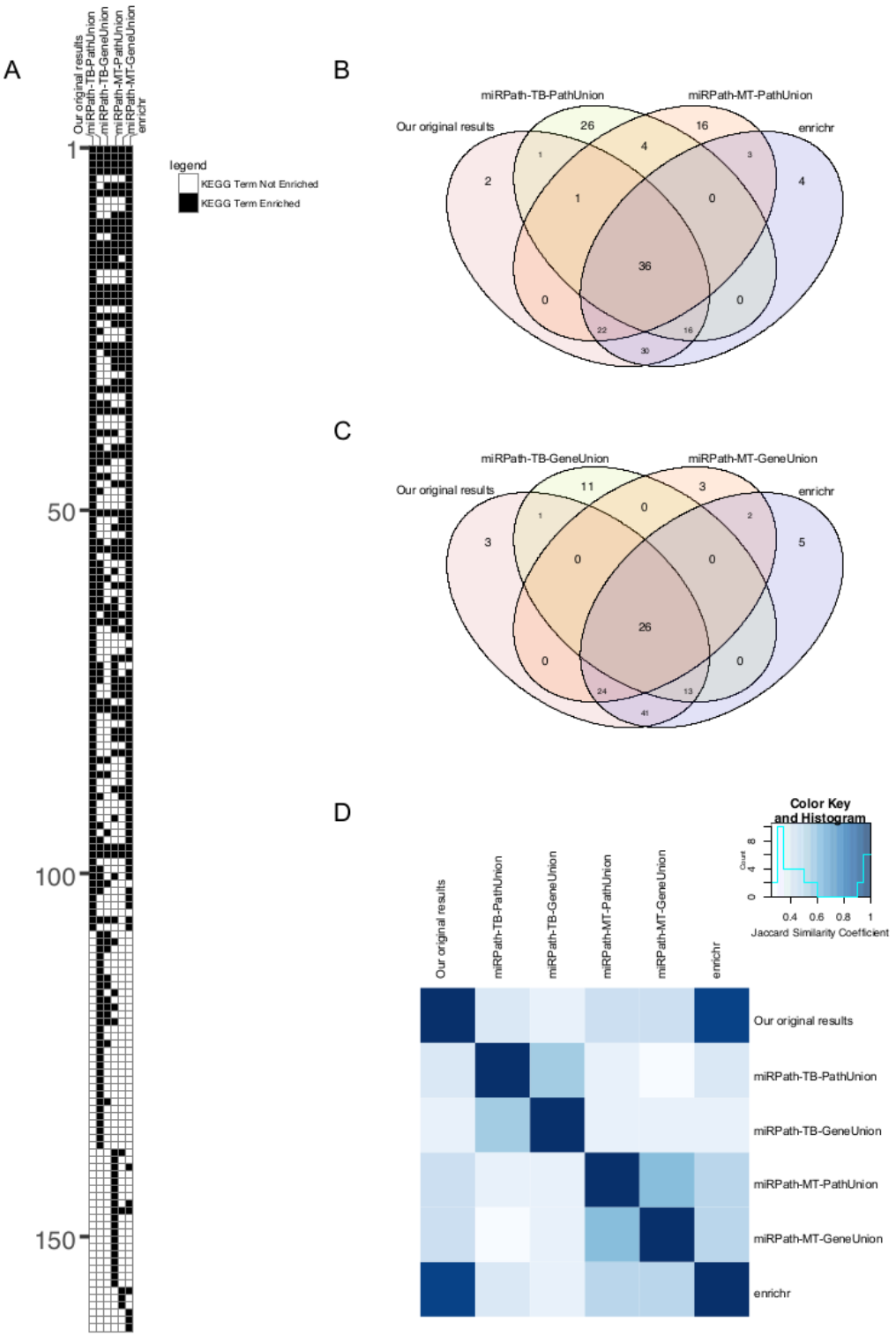
In addition, mirPath allows the definition of enriched pathways based on four approaches, i.e., union of targets ("Genes Union"), intersection of targets ("Genes Intersection"), union of individually enriched pathways ("Pathways Union"), and intersection of individually enriched pathways ("Pathways Intersection"), among which "Genes Union" is the default type of analysis. Since we are interested in investigating the systemic and combinatorial effect of the dysregulation of these miRNAs, we considered the union-based option more suitable for our study. Therefore, we carried out four functional enrichment analysis with miRPath: i) analysis of targets from TarBase (TB) based on Genes Union, ii) analysis of targets from TB based on Pathways

Union, iii) analysis of targets from microT (MT) based on Genes Union, and finally, iv) analysis of targets from MT based on Pathways Union.

In addition, we performed functional enrichment analysis with **Enrichr**. Although there is a plethora of other data that we could use to analyze the biological function of the differentially expressed miRNAs with Enrichr, we decided to maintain the focus on KEGG Pathways given that this information give us a broad picture of functional role and molecular interactions for target genes, as annotated in the pathways maps from the database. Nonetheless, Enrichr does not support miRNAs identity (ids) as input, such that the list of target genes retrieved by our approach (described in Section "Materials and Methods - Bioinformatics analysis") was used as input for this tool.



Supplementary Figure 1. Venn diagram demonstrating the number of target genes shared among the five dysregulated miRNAs validated in the "Validation sample set". The targets of these miRNAs were investigated using a combination of validated experimental data and computational prediction tools, as described in the Methods section.


A
Our original results
miRPath-TB-PathUnion
miRPath-TB-GeneUnion
miRPath-MT-PathUnion
miRPath-MT-GeneUnion
enrichr
1
50
100
150
legend
KEGG Term Not Enriched
KEGG Term Enriched
B
miRPath-TB-PathUnion
miRPath-MT-PathUnion
Our original results
enrichr
2
26
16
4
1
4
1
3
0
36
0
0
22
16
30
C
miRPath-TB-GeneUnion
miRPath-MT-GeneUnion
Our original results
enrichr
3
11
3
1
0
2
5
0
0
0
26
0
0
24
13
41
D
Color Key and Histogram
Count
0
4
8
0.4
0.6
0.8
1
Jaccard Similarity Coefficient
Our original results
miRPath-TB-PathUnion
miRPath-TB-GeneUnion
miRPath-MT-PathUnion
miRPath-MT-GeneUnion
enrichr

Supplementary Figure 2. Consensus analysis of the bioinformatic tools used to identify pathways. **A)** "Binary matrix" indicating whether a given pathway (rows) is enriched (black) or not (white) in the results of a given functional enrichment tool (columns). The greatest overlap among tools was concentrated in the upper portion of the matrix, which in rows 1-108 represents the enriched terms of our original results, ordered by decreasing level of significance. **B)** Venn diagrams for the "Pathways Union", and **C)** for the "Genes Union" options of mirPath, showing the number of pathways/genes shared among tools. **D)** Pairwise Jaccard Similarity Coefficients. Values closer to 1 (depicted in dark blue): indicate that two sets are more similar in terms of their elements. Detailed information about these analyses can be found in the Methods section and Supplementary Material 1.

Supplementary Table 1. Assay reference numbers for the 48 analyzed miRNAs included in the TaqMan Low Density Array cards**.**

miRNA ID	Assay reference number*
hsa-miR-103a-3p	000439
hsa-miR-10a-3p	002288
hsa-miR-124-5p	002197
hsa-miR-125b-5p	000449
hsa-miR-126-3p	002228
hsa-miR-133a-3p	002246
hsa-miR-146a-5p	000468
hsa-miR-148a-3p	000470
hsa-miR-148b-3p	000471
hsa-miR-152-3p	000475
hsa-miR-155-5p	002623
hsa-miR-181a-5p	000480
hsa-miR-191-5p	002299
hsa-miR-199a-5p	000498
hsa-miR-200a-3p	000502
hsa-miR-200c-3p	002300
hsa-miR-210-3p	000512
hsa-miR-21-3p	002438
hsa-miR-222-3p	002276
hsa-miR-24-3p	000402
hsa-miR-25-3p	000403
hsa-miR-26a-5p	000405
hsa-miR-26b-5p	000407
hsa-miR-27b-3p	000409
hsa-miR-29a-3p	002112
hsa-miR-29b-3p	000413
hsa-miR-31-5p	002279
hsa-miR-320a	002277
hsa-miR-326	000542
hsa-miR-340-5p	002258

hsa-miR-342-5p	002147
hsa-miR-34a-5p	000426
hsa-miR-375	000564
hsa-miR-510-5p	002241
hsa-miR-93-3p	002139
hsa-let-7f-5p	000382
hsa-miR-101-3p	002253
hsa-miR-1275	002840
hsa-miR-15b-5p	000390
hsa-miR-195-5p	000494
hsa-miR-32-5p	002109
hsa-miR-338-3p	002252
hsa-miR-424-5p	000604
hsa-miR-21-5p	000397
U6 snRNA	001973
RNU44	001094
RNU48	001006

* Assay numbers for the miRNA catalogue of Thermo Fisher Scientific (DE, USA) from where the assays were acquired. ID = miRNA identity in miRBase. hsa = homo sapiens.

Supplementary Table 2. Receiver operating curves (ROC) for miR-103a-3p, miR-146a-5p, miR-155-5p, miR-200a-3p, and miR-210-3p expressions in plasma from T1DM patients with less than 5 years of diagnosis and non-diabetic subjects.

T1DM patients < 5 years of diagnosis *vs* non-diabetic subjects	**AUC**	**95% CI**	**P value**	**Cut off**	**Sensitivity**	**Specificity**
miR-103a-3p unadjusted	0.759	0.576-0.942	0.011			
miR-103a-3p adjusted*	0.778	0.610-0.946	0.005	-0.201	0.889	0.706
miR-146a-5p unadjusted	0.845	0.742-0.949	0.0001			
miR-146a-5p adjusted*	0.811	0.695-0.927	0.0001	-0.387	0.781	0.636
miR-155-5p unadjusted	0.688	0.511-0.864	0.029			
miR-155-5p adjusted*	0.699	0.529-0.869	0.018	-0.706	0.656	0.737
miR-200a-3p unadjusted	0.781	0.618-0.945	0.001			
miR-200a-3p adjusted*	0.800	0.650-0.950	0.0001	-0.660	0.903	0.700
miR-210-3p unadjusted	0.744	0.625-0.92	0.001			
miR-210-3p adjusted*	0.775	0.632-0.918	0.001	-0.288	0.867	0.700

AUC = area under the curve; CI = confidence interval. AUC, Cut-off, sensibility and specificity values are provided by ROC curve analysis, as described in the Methods Section. *ROC curve analyses after adjustment for T1DM high-risk HLA haplotypes.

Supplementary Table 4. Number of miRNA-target interactions for each of the 5 miRNAs validated in the study.

miRNA	Experimental + predicted evidences (non-redundant)	Experimental evidences only	Predicted evidences only
hsa-miR-103a-3p	877	142	857
hsa-miR-146a-5p	208	75	140
hsa-miR-155-5p	501	264	333
hsa-miR-200a-3p	590	143	527
hsa-miR-210-3p	68	45	33

Numbers of targets were obtained using distinct bioinformatic databases, and include both experimentally validated and computational predicted targets, as described in the Methods Section. hsa = homo sapiens.

Supplementary Table 8. Differential expression between our results and the results from a dataset available at GEO database (GSE26168).

miRNA	Our results		GSE26168 (avg. fold change among probes)		
	T1DM < 5 years *vs.* non-diabetic	**T1DM < 5 years *vs.* T1DM ≥ 5 years**	**T2DM *vs.* non-diabetic**	**T2DM *vs.* pre-diabetes**	**Pre-diabetes *vs.* non-diabetic**
hsa-miR-103a-3p	2.130*	2.139*	-1.491	0.010	-1.501
hsa-miR-200a-3p	2.022*	1.781*	0.001	-0.006	0.007
hsa-miR-155-5p	1.269*	1.114*	-0.021	0.013	-0.033
hsa-miR-210-3p	1.558*	1.693*	-0.015	-0.031	0.016
hsa-miR-146a-5p	-1.014*	-0.837*	-0.044	-0.019	-0.025

Values are shown as $\log_{10}$(fold change) in relation to the comparative group. *P <0.05 (obtained from t-tests using the log-transformed variable). Log_{10}(fold changes) showed for miRNAs expressed in the samples from GEO were provided in the original publication reported in GSE26168. Moreover, P values for the comparisons among groups from GSE26168 were retrieved from the original publication (all P values >0.05).

Capítulo 2

“Polimorfismos em genes codificantes de microRNAs e o diabetes mellitus tipo 1”

ARTIGO 3

Polymorphisms in genes encoding miR-155 and miR-146a are associated with protection to type 1 diabetes mellitus

Acta Diabetol
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ORIGINAL ARTICLE

Polymorphisms in genes encoding miR-155 and miR-146a are associated with protection to type 1 diabetes mellitus

Taís S. Assmann[1,2] · Guilherme C. K. Duarte[1,2] · Letícia A. Brondani[1] · Pedro H. O. de Freitas[1] · Égina M. Martins[1] · Luís H. Canani[1,2] · Daisy Crispim[1,2]

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Abstract
Aims Type 1 diabetes mellitus (T1DM) is characterized by severe autoimmune destruction of pancreatic beta-cells. The triggering of autoimmunity against beta-cells is probably caused by a combination of environmental and genetic risk factors. Even though much is known about the genetic of T1DM, more information is needed to completely unravel this tangled disease. MicroRNAs (miRNAs) are a class of small noncoding RNAs molecules that negatively regulate gene expression by inducing target mRNA cleavage or by inhibiting protein translation. Abnormal miRNA expressions have been described in autoimmune diseases and T1DM. Polymorphisms in genes codifying miRNAs may alter the expression of the corresponding miRNA and, thus, confer susceptibility for a given disease. Therefore, the aim of this study was to investigate whether polymorphisms in genes encoding miR-155, miR-146a, and miR-375 are associated with T1DM.
Methods Frequencies of the *miRNA-146a* rs2910164, *miRNA-155* rs767649 and *miRNA-375* rs6715345 polymorphisms were analyzed in 490 T1DM patients and in 469 nondiabetic subjects.
Results The *miR-146a* rs2910164 and *miR-155* rs767649 polymorphisms were associated with protection for T1DM, and the strongest association was observed for the dominant model [odds ratio (OR) = 0.557 95% CI 0.355–0.874 and OR = 0.508, 95% CI 0.265–0.973, respectively, after adjustment for age, ethnicity, and risk *HLA loci*]. However, *miR-375* rs6715345 frequencies did not differ between cases and controls.
Conclusion *MiR-146a* rs2910164 and *miR-155* rs767649 polymorphisms were associated with protection for T1DM.

Keywords Type 1 diabetes mellitus · Polymorphisms · MicroRNA

Introduction

Type 1 diabetes mellitus (T1DM) results from a cellular-mediated autoimmune destruction of pancreatic beta-cells, leaving patients insulin-dependent for life [1]. The triggering of autoimmunity against beta-cells arises from a multifaceted interaction between multiple genetic and environmental risk factors [2]. More than 50 genes have been identified to influence the risk of T1DM, with *HLA* class II genes having the greatest impact on susceptibility [3]. Other loci have minor impact on the risk for T1DM; however, the combination of HLA haplotypes and non-HLA polymorphisms has been shown to aid disease prediction [4]. Hence, the discovery of new polymorphisms associated with T1DM may improve the prediction of this disease.

MicroRNAs (miRNAs) are small noncoding RNAs that negatively regulate gene expression by partially pairing to the 3′ untranslated region of their target mRNAs, leading to translation repression and/or transcript degradation [5, 6].

Managed by Antonio Secchi.

✉ Daisy Crispim
dcmoreira@hcpa.edu.br

[1] Endocrine Division, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos 2350, prédio 12, 4° andar, Porto Alegre, Rio Grande do Sul 90035-003, Brazil

[2] Postgraduation Program in Medical Sciences: Endocrinology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

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Their roles have been demonstrated in the regulation of various physiological and pathophysiological functions of immune system as well as beta-cell development and death, which are mechanisms involved in T1DM pathogenesis [7, 8]. In this context, *miR-146a* and *miR-155* expressions have being described as dysregulated in T1DM [9, 10] and other autoimmune diseases [11, 12]. These miRNAs were linked with inflammatory responses due to their upregulation in multiple cell lineages by proinflammatory cytokines and pathogen-derived ligands of toll-like receptors (TLRs) [13, 14]. Moreover, because induction of miR-146a and miR-155 is dependent of the transcription factor NF-κB, they were suggested as immune-response molecules for both adaptive and innate immune systems [15–17]. miRNA-375 is highly expressed in pancreatic islets and is important for insulin secretion and beta-cell development and maintenance [18, 19]. Consequently, this miRNA has been proposed as a biomarker to detect beta-cell death and to predict the development of T1DM [6, 20, 21].

Although the research on miRNAs and T1DM has focused on gene expression studies [6, 22, 23], polymorphisms in *miR-146a*, *miR-155* and *miR-375* genes might be involved in T1DM development since they could alter both the expression and functionality of these miRNAs; possible resulting in dysregulation of their target genes [24]. To date, only few studies reported associations between polymorphisms in the *miR-146a* gene and autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis, asthma, and Crohn's disease [25–27]. No study has evaluated the association between polymorphisms in *miR-146a*, *miR-155* and *miR-375* genes and T1DM development. Therefore, the aim of this study was to investigate the association of *miR-146a* rs2910164, *miR-155* rs767649 and *miR-375* rs6715345 polymorphisms with T1DM in a Brazilian population.

Subjects and methods

Subjects, phenotype measurements, and laboratory analyses

This case–control study was designed in accordance with STROBE and STREGA guidelines for reporting of genetic association studies [28, 29]. The case sample comprised 490 T1DM patients recruited from the outpatient clinic at Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil) between January 2005 and December 2013. Patients were diagnosed as having T1DM according to American Diabetes Association guidelines [1]. The nondiabetic group (controls) comprised 469 healthy blood donors recruited from the same hospital, who did not have diabetes or family history of this disease. Moreover, only subjects with glycated hemoglobin (HbA1c) <5.7% were included in the control group [1]. The ethnic group was defined based on self-classification, and the ethnic distribution between samples was as follows: 1.0% of black patients in the T1DM group and 7.3% of black subjects in the control group ($P = 0.0001$). All subjects gave assent and written informed consent prior to participation.

A standard questionnaire was used to collect information on age, age at DM diagnosis, T1DM duration and drug treatment, and all patients underwent physical and laboratory evaluations, as previous described [30]. Serum and plasma samples were taken after 12 h of fasting for laboratory analyses [30].

Genotyping

DNA was extracted from peripheral blood leukocytes by a standardized salting-out procedure. *MiR-146a* rs2910164 (C/G) and *miR-155* rs767649 (T/A) polymorphisms were genotyped using TaqMan SNP Genotyping Assays 20x (Thermo Fisher Scientific, Foster City, CA, USA), while the *miR-375* rs6715345 (C/G) polymorphism was genotyped using the Custom TaqMan Genotyping Assay 20x (Thermo Fisher Scientific). Reactions were conducted in 384-well plates, in a total of 5 µl volume, using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Thermo Fisher Scientific) and TaqMan Genotyping Assay 1x. Plates were then positioned in a real-time PCR thermal cycler (ViiA7 Real-Time PCR System; Thermo Fisher Scientific) and heated for 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 62 °C for 1 min. Polymorphisms of interest in *miR-146a*, *miR-155* and *miR-375* genes were selected using the miRNASNP 2.0 website (http://www.bioguo.org/miRNASNP/) [31] and miRNA-Map website (http://mirnamap.mbc.nctu.edu.tw/index.php) [32]. Only polymorphisms that had minor allele frequencies higher than 1% were considered. Moreover, *miRNA-146a* and *miR-155* rs767649 polymorphisms were also selected based on available literature: rs2910164 was reported as being associated with other autoimmune diseases [26] and diabetic chronic complications [33, 34], while rs767649 was associated with lung cancer [35].

Since *HLA* class II (DR and DQ) haplotypes may influence the association of non-HLA polymorphisms with T1DM [3], we also analyzed the frequencies of high-risk *HLA* haplotypes in case and control groups. For this, three polymorphisms of the *HLA* class II loci (rs3104413, rs2854275 and rs9273363) were genotyped in all samples using Custom TaqMan Genotyping Assay 20x, as described above. A study showed that these polymorphisms can predict *HLA-DR/DQ* haplotypes relevant to T1DM with an accuracy >99% [3]. Thus, using the method proposed by

Nguyen et al. [3], we determined the frequencies of the following *HLA-DR/DQ* haplotypes: high-risk haplotypes (DR4/DQ8, DR3/DR4-DQ8 or DR3/DR3), intermediate-risk haplotypes (DR3/DRx), and low-risk haplotypes (DRx/DRx or DR4/DQ7).

Although real-time PCR is not the gold standard for genotyping polymorphisms; nowadays, it is the most commonly technique used in epidemiological studies involving a large number of subjects and frequencies of known polymorphisms. Of note, 10% of the amplification reactions were performed twice, with a calculated error rate based on PCR duplicates of <0.5%. Moreover, genotyping success was more than 95% in our samples.

Statistical analysis

Allele frequencies were determined by gene counting, and departures from the Hardy–Weinberg equilibrium (HWE) were verified using Chi-square tests. Allele and genotype frequencies were compared between groups of subjects using Chi-square tests. Clinical and laboratory characteristics were compared between groups by using unpaired Student's *t* test or Chi-square, as appropriate. Variables with normal distribution are presented as mean ± SD. Variables with skewed distribution were log-transformed before analyses and are presented as median (minimum–maximum values). The magnitude of association of different genotypes with T1DM was estimated using odds ratio (OR) with 95% CI in logistic regression analysis, adjusting for age, ethnicity, and high-risk *HLA* haplotypes.

Power calculations (PEPI program, v. 4.0) showed that this study has a power of approximately 80% at a significance level of 0.05 to detect an OR of 0.6 or higher (for the presence of the minor alleles of each polymorphism). All analyses were performed with the SPSS 18.0 software (SPSS, Chicago, IL). *P* values <0.05 were considered significant.

Results

Sample description

The main clinical and laboratory characteristics of T1DM patients and nondiabetic subjects included in the study are described in Table 1. Mean age and mean body mass index (BMI) were higher in nondiabetic subjects than in T1DM patients ($P = 0.001$). As already mentioned, the percentage of black subjects was higher in the control group compared with cases ($P = 0.0001$). As expected, mean HbA1c, prevalence of arterial hypertension and frequencies of high-risk *HLA* haplotypes were higher in cases than control subjects ($P < 0.00001$). Mean age at T1DM diagnosis was 16 (1–29.9) years, 31.8% of cases had diabetic kidney disease (DKD), and 44.5% had diabetic retinopathy.

Genotype and allele distributions

Genotype distributions of *miR-146a* rs2910164 (C/G), *miR-155* rs767649 (T/A), and *miR-375* rs6715345 (C/G) polymorphisms were in agreement with those predicted by HWE in nondiabetic subjects ($P \geq 0.05$). Frequencies of miRNA minor alleles in white and black subjects were: 28.9 versus 32.1% for the *miR-146a* rs2910164C allele ($P = 0.27$), 6.5 versus 7.0% for *miR-155* rs767649A allele ($P = 0.72$), and 3.2 versus 3.4% for *miR-375* rs6715345C allele ($P = 0.99$).

Frequencies of *miR-146a* rs2910164, *miR-155* rs767649, and *miR-375* rs6715345 polymorphisms in T1DM patients and nondiabetic subjects are depicted in Table 2. *MiR-146a*

Table 1 Characteristics of T1DM patients and nondiabetic subjects included in this case–control study

Characteristic	T1DM patients ($n = 490$)	Nondiabetic subjects ($n = 469$)	*P* value
Age (years)	34.3 ± 12.1	40.3 ± 9.7	0.0010
Gender (% male)	50.3	55.8	0.06
Ethnicity (% white)	99.0	92.6	0.0001
HbA1c (%, mmol/mol)	8.7 ± 2.1 (71.6 ± 7.5)	5.3 ± 0.2 (34.4 ± 0.75)	0.0001
BMI (kg/m^2)	23.8 ± 3.4	26.8 ± 4.8	0.001
Hypertension (%)	30.6	5.2	0.0001
Smoking (%)	10.8	7.1	0.15
High-risk *HLA* haplotype (%)*	57.1	17.0	0.0001
Age at T1DM diagnosis (years)	16 (1–30)	–	–
Diabetic kidney disease (% yes)	31.8	–	–
Diabetic retinopathy (% yes)	44.5	–	–

P value was computed using Chi-square tests or Student's *t* test, as appropriated

HbA1c glycated hemoglobin, *BMI* body mass index

* High-risk *HLA* haplotypes: DR4/DQ8 or DR3/DR4-DQ8 or DR3/DR3

Table 2 Genotype and allele frequencies of miRNA polymorphisms in patients with type 1 diabetes mellitus (T1DM) and nondiabetic subjects

Polymorphism	T1DM patients	Nondiabetic subjects	Unadjusted OR (95% CI)/*P*	Adjusted OR (95% CI)/*P**
miR-146a rs2910164	$n = 431$	$n = 405$		
Genotype				
G/G	257 (59.6)	192 (47.4)	1	1
G/C	142 (33.0)	161 (39.8)	0.460 (0.285–0.742)/0.001	0.505 (0.220–1.156)/0.11
C/C	32 (7.4)	52 (12.8)	0.659 (0.492–0.883)/0.005	0.570 (0.353–0.919)/0.02
Dominant model				
G/G	257 (59.6)	192 (47.4)	1	1
C/C + C/G	174 (40.4)	213 (52.6)	0.499 (0.352–0.707)/0.0001	0.557 (0.355–0.874)/0.01
Recessive model				
C/G + G/G	399 (92.6)	353 (87.2)	1	1
C/C	32 (7.4)	52 (12.8)	0.544 (0.343–0.865)/0.01	0.637 (0.285–1.423)/0.27
Additive model				
G/G	257 (59.6)	192 (47.4)	1	1
C/C	32 (7.4)	52 (12.8)	0.460 (0.285–0.742)/0.001	0.505 (0.220–1.148)/0.10
miR-155 rs767649	$n = 490$	$n = 391$		
Genotype				
T/T	446 (91.0)	334 (85.4)	1	1
T/A	44 (9.0)	57 (14.6)	0.578 (0.381–0.878)/0.01	0.508 (0.265–0.973)/0.04
A/A	0	0	–	–
miR-375 rs6715345	$n = 429$	$n = 469$		
Genotype				
G/G	399 (93.1)	440 (93.8)	1	1
G/C	29 (6.7)	28 (5.9)	0.907 (0.057–14.546)/0.95	0.847 (0.363–1.975)/0.70
C/C	1 (0.2)	1 (0.3)	–	–
Dominant model				
G/G	399 (93.1)	440 (93.8)	1	1
C/C + G/C	30 (6.9)	29 (6.2)	0.877 (0.517–1.486)/0.63	0.923 (0.406–2.097)/0.85

Data are shown as *n* (%) or proportion

Considering the low frequency of minor alleles of *miR-155* rs767649 and *miR-375* rs6715345 polymorphisms only the dominant inheritance model was tested

* Adjusted by age, ethnicity, and high-risk *HLA* haplotypes

rs2910164C allele frequency was 23.9% in T1DM patients and 32.7% in controls ($P = 0.003$). Genotype frequencies of the rs2910164 polymorphism were different between T1DM and control groups ($P = 0.001$). Moreover, the C allele was associated with protection for T1DM under dominant, recessive, and additive inheritance models ($P = 0.0001$, $P = 0.01$, and $P = 0.001$, respectively). However, after adjustment for the presence of high-risk *HLA* haplotypes, age, and ethnicity, the *miR-146a* rs2910164 polymorphism remained associated with T1DM only for the dominant model (OR = 0.557, 95% CI 0.355–0.874; $P = 0.01$) (Table 2).

MiR-155 rs767649A allele frequency was 4.5% in T1DM patients compared with 7.3% in controls ($P = 0.006$). The frequency of the T/A genotype was also higher in T1DM cases than control subjects ($P = 0.01$), and this genotype remained associated with protection for T1DM after adjustment for high-risk *HLA* haplotypes, age, and ethnicity (OR = 0.508, 95% CI 0.265–0.973; $P = 0.04$) (Table 2). No subject carried the rare A/A genotype; thus, no genetic inheritance model was tested.

Interestingly, *miR-155* rs767649 and *miR-146a* rs2910164 polymorphisms seem to interact in the susceptibility for T1DM since the prevalence of T1DM was lower as more minor alleles of the two polymorphisms were present in the subjects (*P*-trend = 0.001, Table 3). Thus, the presence of two minor alleles of rs767649 and rs2910164 polymorphisms was 9.8% in T1DM patients compared with 15.1% in nondiabetic subjects (OR = 0.541, 95% CI 0.338–0.865; $P = 0.01$), but the presence of three minor alleles of the two polymorphisms was only 0.5% in diabetic patients compared with 2.5% in controls (OR = 0.181, 95% CI 0.038–0.866; $P = 0.03$). No subject carried four minor alleles of both polymorphisms.

Table 3 Interaction analysis between *miR-146a* rs2910164 and *miR-155* rs767649 polymorphisms

	T1DM patients ($n = 388$)	Nondiabetic subjects ($n = 338$)	Unadjusted OR (95% CI)/ *P*	*P*-trend
No minor alleles [TT-GG]	204 (52.6)†	148 (43.8)†	1	0.001
1 minor alleles [AT-GG or TT-CG]	144 (37.1)	131 (38.7)	0.797 (0.581–1.095)/0.16	
2 minor alleles [AA-GG, AT-CG or TT-CC]	38 (9.8)†	51 (15.1)†	0.541 (0.338–0.865)/0.01	
3 minor alleles [AA-CG or AT-CC]	2 (0.5)†	8 (2.4)†	0.181 (0.038–0.866)/0.03	

No subject carried 4 minor alleles of the two analyzed polymorphisms

† $P < 0.05$ in the standardized residual analysis

miR-375 rs6715345C allele frequency was 3.6% in T1DM patients and 3.3% in the nondiabetic group ($P = 0.59$). Genotype frequencies of the rs6715345 polymorphism were also similar between groups ($P = 0.89$), and the polymorphism was not associated with T1DM when considering the dominant model of inheritance ($P = 0.85$), adjusting for co-variables. Due to the low frequency of the C allele, no other inheritance model was tested (Table 2).

In an exploratory analysis, clinical, and laboratories characteristics were compared between T1DM patients carrying the minor alleles of the three analyzed miRNAs polymorphisms (data not shown).

Age, gender, age at T1DM diagnosis, systolic and diastolic blood pressure, BMI, lipid profile, and HbA1c were similar between subjects carrying the minor alleles of the *miR-146a* rs2910164, *miR-155* rs767649, and *miR-375* rs6715345 polymorphisms, analyzed individually, and subjects carrying ancestral homozygous genotypes ($P > 0.05$). However, T1DM patients carrying the *miR-375* rs6715345C allele (dominant model) had lower fasting plasma glucose levels compared to patients with the G/G genotype (127.0 ± 65.4 *vs*. 180.4 ± 107.6; $P = 0.001$). In nondiabetic subjects, age, gender, HbA1c, BMI, and prevalence of hypertension were similar between different genotypes of the analyzed polymorphisms (dominant model, all $P > 0.05$).

Discussion

MiRNAs are key regulators of gene expression and, like other genes, their coding sequences are subject to genetic variation. Polymorphisms in miRNA genes can have marked effects on miRNA functionality at all levels, including miRNA transcription, maturation and target specificity and, consequently, they may contribute to T1DM pathogenesis [24, 36]. Nevertheless, studies that evaluated the association between miRNAs and T1DM focused on gene expression profiles. Thus, for the first time, we analyzed the associations of *miR-146a* rs2910164, *miR-155* rs767649, and *miR-375* rs6715345 polymorphisms with T1DM. Although frequencies of the *miR-375* rs6715345 polymorphism were not significantly different between T1DM patients and nondiabetic subjects, *miR-146a* rs2910164 and *miR-155* rs767649 polymorphisms were associated with protection for T1DM, adjusting for high-risk *HLA* haplotypes, age, and ethnicity.

Growing evidence indicates that miRNA-146a has a role in innate immunity by negatively regulating the inflammatory response after recognition of bacterial components by TLRs on monocytes and macrophages [17, 37–44]. Proinflammatory cytokines such as TNF and IL-1β also activate *miR-146a* expression [10, 37, 45]. Then, upon activation by cytokines or lipopolysaccharide (LPS), in a NF-κB-dependent manner, miR-146a targets *TRAF-6* and *IRAK-1* expressions. These genes encode two key adapter molecules downstream of TLRs and cytokine receptors, indicating a role of miR-146a in controlling signaling from these receptors through a negative feedback loop involving downregulation of TRAF-6 and IRAK-1, which prevents overstimulation of the TLR pathway rather than totally abrogate it [13, 15, 17]. Because both TRAF-6 and IRAK-1 act in the same cascade, the cumulative effect of a decrease in their expressions would probably have a considerable effect on TLR signaling [15]. Moreover, miR-146a suppresses LPS-induced IκBα phosphorylation, decreasing NF-κB activation in both cardiomyocytes and macrophages [41].

MiR-146a also seems to participate in the adaptive immune system, being highly expressed in Tregs [13]. Lu et al. [46] found that this miRNA regulates Treg-mediated suppression of IFNγ-dependent Th1 responses and associated autoimmunity by directly targeting STAT-1, contributing to a severe failure of immune tolerance. MiR-146a is also induced in human primary T lymphocytes upon T-cell receptor (TCR) stimulation. In particular, *miR-146a* overexpression impairs both activator protein 1 (AP-1) activity and IL-2 production induced by TCR engagement, further suggesting a role of this miRNA in the modulation of adaptive immunity [47]. Accordingly, *miR-146a* was reported as being downregulated in serum and

peripheral blood mononuclear cells (PBMCs) from newly diagnosed T1DM patients compared with healthy control subjects [9]. Interestingly, decreased *miR-146a* expression was significantly associated with high serum glutamic acid decarboxylase antibody titers [9].

In contrast, Roggli et al. [10] reported that miR-146a was increased in pancreatic islets from non-obese diabetes (NOD) mice during development of pre-diabetic insulitis. Furthermore, exposure of MIN-6 cells and human beta-cells to IL-1β and TNF induced *miR-146* expression, which increased beta-cell apoptosis through an unknown mechanism. Blocking miR-146a in MIN-6 cells using an antisense molecule protected these cells from cytokine-induced apoptosis and also prevented the reduction in glucose-induced insulin secretion observed after IL-1β exposure [10].

The rs2910164 (G/C) polymorphism is located within the seed sequence of the *pre-miR-146a* (Fig. 1a), and the minor C allele is predicted to cause a mispairing within the hairpin stem, possible altering the interaction of miR-146a with its targets [24, 48]. Jazdzewski et al. [48] reported that the C allele affected the processing efficacy of *pre-miR-146a* into the mature form, causing an almost twofold decrease in the mature miR-146a in human bone osteosarcoma epithelial cells. This reduction in miR-146a led to less efficient inhibition of its target genes such as *TRAF-6* and *IRAK-1*. Similar results were found in a 293T cell line derived from human embryonic kidneys [49]; however, another study performed in a MCF-7 breast cancer cell line showed that the C allele resulted in elevated expression of mature miR-146a [50]. Several studies have reported the association of the *miR-146a* rs2910164 polymorphism with different cancers [48–50], autoimmune diseases [25–27], diabetic polyneuropathy and cardiovascular autonomic neuropathy in T2DM patients [34], and DKD in T1DM patients [33], corroborating a functional role of this polymorphism. Nevertheless, additional studies are needed to clarify the functional effect of the rs2910164 polymorphism in immune cells as well as pancreatic beta-cells.

MiR-155 is processed from an exon of a noncoding RNA transcribed from the *B-cell integration cluster* (*BIC*) located on chromosome 21 [15, 16]. *BIC/miR-155* is highly expressed in activated T and B cells as well as dendritic cells and macrophages [16] and is induced by inflammatory mediators including IFN-β, TNF, IFN-γ, and different bacterial- and viral-derived TLR ligands [14, 51–53]. Because miR-155 induction involves both NF-κB and the c-Jun N-terminal kinase (JNK), it fits the profile of a typical immune-response molecule [14, 15]. During normal conditions, activated NF-κB upregulates *miR-155* that, upon processing and maturation, downregulates IκB kinases (IKKs) to reduce the activation of NF-κB [51]. MiR-155

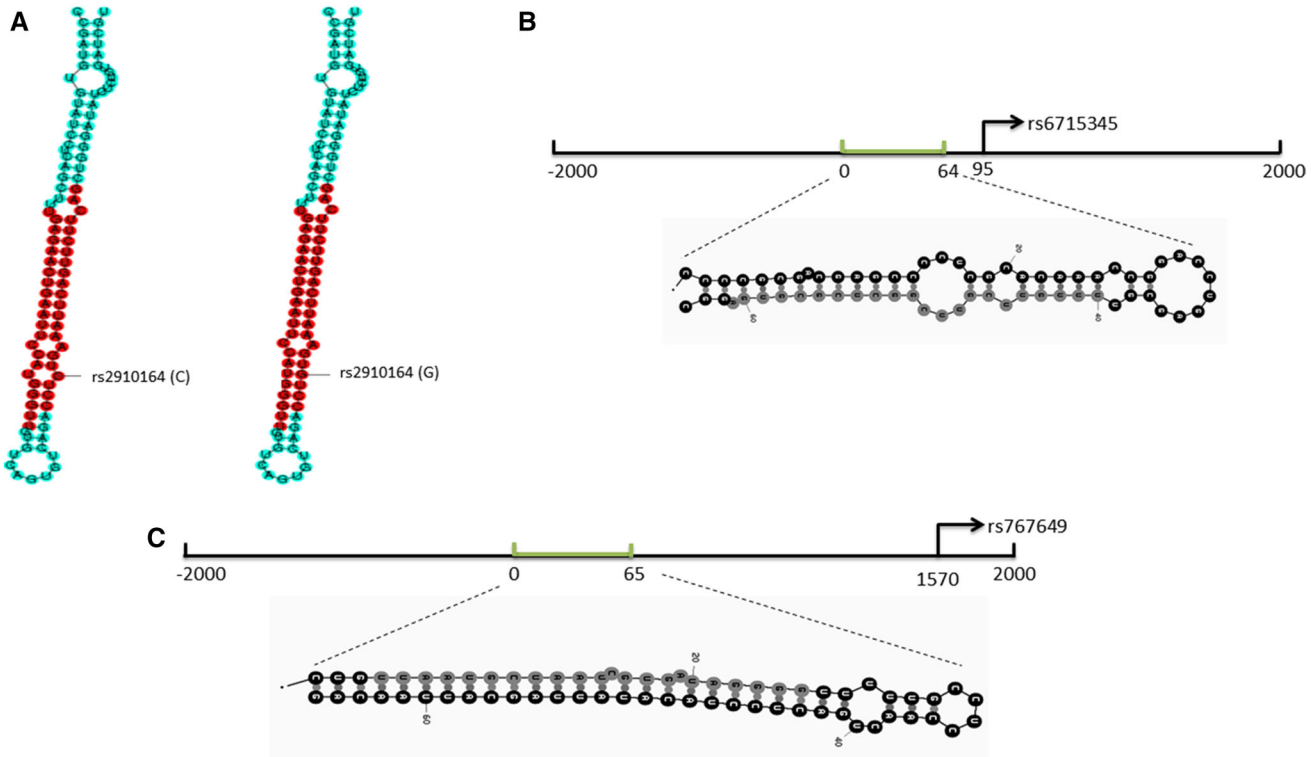

Fig. 1 Localization of the three analyzed polymorphisms in their corresponding pre-miRNA and flanking regions. **a** *miR-146a* rs2910164; **b** *miR-375* rs6715345; **c** *miR*-155 rs767649. Figures were performed based on miRNAMap and miRNASNP 2.0 websites

also positively regulates host antiviral immune response by promoting type I IFN signaling through targeting SOCS1, a negative regulator of type I IFN pathway [53]. This miRNA has been involved in numerous biological processes, including myeloid cell differentiation, inflammation, and innate and adaptive immunity [16, 38, 40, 52, 54–56]. Consequently, miR-155 dysregulation has been associated with different types of cancer, cardiovascular diseases, viral infections, RA, DKD, and other diabetic chronic complications [11, 16, 23, 51]. Moreover, this miRNA was downregulated in serum from newly diagnosed T1DM patients [18].

The rs767649 (T/A) polymorphism is located in the upstream flanking region of the *pre-miR-155* gene in a potential enhancer element (Fig. 1b), and it was predicted to disrupt the binding of NF-κB and other regulatory factors to the miR-155 [35, 57]. Xie et al. [35] reported that the rs767649T/T genotype was associated with risk to nonsmall cell lung cancer. Moreover, functional experiments showed that the T allele increased the transcriptional activity of *miR-155* in a lung cancer cell line (A549) and in turn promoted increased cell proliferation, migration, and invasion via the oxidative stress-related pathway [35]. The T/T genotype was also associated with increased risk and poor survival of hepatocellular carcinoma, and the T allele contributed to higher *miR-155* expression in both hepatocellular carcinoma and adjacent nontumor tissues [58]. To date, no study has evaluated the association between the *miR-155* rs767649 polymorphism and T1DM, T2DM, or diabetic chronic complications.

In the present study, *miR-155* rs767649 and *miR-146a* rs2910164 polymorphisms seem to interact in the susceptibility for T1DM as the prevalence of T1DM decreased as more minor alleles of the two polymorphisms were present in the subjects. Considering that rs767649A and rs2910164C alleles seem to be associated with decreased expression of the respective miRNAs, we therefore hypothesized that the reduced levels of mature miR-146a and miR-155 might have impaired ability to dampen NF-κB-mediated inflammation in beta-cells, predisposing to T1DM. Further studies are necessary to confirm this hypothesis in beta-cells.

miR-375 is the most abundant miRNA detected in islets and is important for development and maintenance of normal alpha- and beta-cell mass in mice [19, 59]. Erener et al. [60] showed that massive beta-cell loss elicited by administration of streptozotocin in C57BL/6 mice caused a dramatic increase in circulating levels of miR-375. Plasma levels of miR-375 were considerably increased in NOD mice 2 weeks before the onset of T1DM, suggesting that this miRNA can be used as a marker of beta-cell death [60]. Marchand et al. [21] reported that *miR-375* expression was lower in serum from newly diagnosed T1DM children (before insulin treatment) than in serum from nondiabetic pediatric controls. This miRNA was also downregulated in isolated human islets exposed to high glucose concentrations [21].

The rs6715345 polymorphism is located in the near upstream flanking region of the *pre-miR-375* (Fig. 1c), and it was predicted to be a potentially functional variant [61]. Here, we could not find any association between the rs6715345 polymorphism and T1DM. Nevertheless, T1DM patients carrying the *miR-375* rs6715345C allele had lower fasting plasma glucose levels compared to patients with the G/G genotype. This association is biologically plausible considering the recognized role of miR-375 in insulin secretion and beta-cell mass [59, 62]. In particular, miR-375 plays a negative regulatory role in glucose-induced insulin secretion since this miRNA targets *myotrophin* mRNA, which codifies a protein involved in insulin granule function [63]. Moreover, glucose seems to influence the cAMP-PKA pathway to repress *miR-375* expression, whereas lower *miR-375* levels promote insulin secretion to reduce blood glucose levels (reviewed in [19]). Accordingly, *miR-375* knockout mice are hyperglycemic and glucose intolerant despite normal insulin secretion [18]. Thus, we hypothesized that the *miR-375* rs6715345C allele might be associated with decreased *miRNA-375* expression, leading to increased insulin secretion by beta-cells. Further studies are needed to evaluate the role of the rs6715345 polymorphism in beta-cell function.

In conclusion, the present study indicates that *miR-146a* rs2910164 and *miR-155* rs767649 polymorphisms are associated with protection for T1DM in Brazilian subjects. These associations are biological plausible considering the involvement of miR-146a and miR-155 in immunity and inflammation, which are key players in T1DM pathogenesis. Further studies are required to confirm these associations in other populations.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical disclosure All procedures performed in studies involving human participants were in accordance with the ethical standards of the Hospital de Clínicas de Porto Alegre research committee (Number of Approval 14-0516) and with the 1964 Helsinki declaration and its amendments or comparable ethical standards.

Informed consent All subjects gave assent and written informed consent prior to participation.

References

1. American Diabetes Association (2015) Classification and diagnosis of diabetes. Diabetes Care 38(Suppl):S8–S16. doi:10.2337/dc15-S005
2. van Belle TL, Coppieters KT, von Herrath MG (2011) Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev 91(1):79–118. doi:10.1152/physrev.00003.2010
3. Nguyen C, Varney MD, Harrison LC, Morahan G (2013) Definition of high-risk type 1 diabetes HLA-DR and HLA-DQ types using only three single nucleotide polymorphisms. Diabetes 62(6):2135–2140. doi:10.2337/db12-1398
4. Pociot F, Lernmark A (2016) Genetic risk factors for type 1 diabetes. Lancet 387(10035):2331–2339. doi:10.1016/S0140-6736(16)30582-7
5. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136(2):215–233. doi:10.1016/j.cell.2009.01.002
6. Guay C, Regazzi R (2013) Circulating microRNAs as novel biomarkers for diabetes mellitus. Nat Rev Endocrinol 9(9):513–521. doi:10.1038/nrendo.2013.86
7. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19(1):92–105. doi:10.1101/gr.082701.108
8. Butz H, Kinga N, Racz K, Patocs A (2016) Circulating miRNAs as biomarkers for endocrine disorders. J Endocrinol Invest 39(1):1–10. doi:10.1007/s40618-015-0316-5
9. Yang M, Ye L, Wang B et al (2015) Decreased miR-146 expression in peripheral blood mononuclear cells is correlated with ongoing islet autoimmunity in type 1 diabetes patients. J Diabetes 7(2):158–165. doi:10.1111/1753-0407.12163
10. Roggli E, Britan A, Gattesco S et al (2010) Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. Diabetes 59(4):978–986. doi:10.2337/db09-0881
11. Churov AV, Oleinik EK, Knip M (2015) MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. Autoimmun Rev 14(11):1029–1037. doi:10.1016/j.autrev.2015.07.005
12. Xu WD, Lu MM, Pan HF, Ye DQ (2012) Association of MicroRNA-146a with autoimmune diseases. Inflammation 35(4):1525–1529. doi:10.1007/s10753-012-9467-0
13. Taganov KD, Boldin MP, Chang KJ, Baltimore D (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci USA 103(33):12481–12486. doi:10.1073/pnas.0605298103
14. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D (2007) MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci USA 104(5):1604–1609. doi:10.1073/pnas.0610731104
15. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD (2008) MicroRNAs: new regulators of immune cell development and function. Nat Immunol 9(8):839–845. doi:10.1038/ni.f.209
16. Faraoni I, Antonetti FR, Cardone J, Bonmassar E (2009) miR-155 gene: a typical multifunctional microRNA. Biochim Biophys Acta 1792(6):497–505. doi:10.1016/j.bbadis.2009.02.013
17. Rusca N, Monticelli S (2011) MiR-146a in Immunity and Disease. Mol Biol Int 2011:437301. doi:10.4061/2011/437301
18. Guay C, Roggli E, Nesca V, Jacovetti C, Regazzi R (2011) Diabetes mellitus, a microRNA-related disease? J Lab Clin Med 157(4):253–264. doi:10.1016/j.trsl.2011.01.009
19. Li X (2014) MiR-375, a microRNA related to diabetes. Gene 533(1):1–4. doi:10.1016/j.gene.2013.09.105
20. Erener S, Mojibian M, Fox JK, Denroche HC, Kieffer TJ (2013) Circulating miR-375 as a biomarker of β-cell death and diabetes in mice. Endocrinology 154(2):603–608. doi:10.1210/en.2012-1744
21. Marchand L, Jalabert A, Meugnier E et al (2016) miRNA-375 a sensor of glucotoxicity is altered in the serum of children with newly diagnosed type 1 diabetes. J Diabetes Res 2016:1869082. doi:10.1155/2016/1869082
22. Nielsen LB, Wang C, Sorensen K et al (2012) Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. Exp Diabetes Res 2012:896362. doi:10.1155/2012/896362
23. Khamaneh AM, Alipour MR, Sheikhzadeh Hesari F, Ghadiri Soufi F (2015) A signature of microRNA-155 in the pathogenesis of diabetic complications. J Physiol Biochem 71(2):301–309. doi:10.1007/s13105-015-0413-0
24. Cammaerts S, Strazisar M, De Rijk P, Del Favero J (2015) Genetic variants in microRNA genes: impact on microRNA expression, function, and disease. Front Genet 6:186. doi:10.3389/fgene.2015.00186
25. Li C, Fu W, Zhang Y et al (2015) Meta-analysis of microRNA-146a rs2910164 G > C polymorphism association with autoimmune diseases susceptibility, an update based on 24 studies. PLoS ONE 10(4):e0121918. doi:10.1371/journal.pone.0121918
26. Park R, Lee WJ, Ji JD (2016) Association between the three functional miR-146a single-nucleotide polymorphisms, rs2910164, rs57095329, and rs2431697, and autoimmune disease susceptibility: a meta-analysis. Autoimmunity. doi:10.3109/08916934.2016.1171854
27. Jimenez-Morales S, Gamboa-Becerra R, Baca V et al (2012) MiR-146a polymorphism is associated with asthma but not with systemic lupus erythematosus and juvenile rheumatoid arthritis in Mexican patients. Tissue Antigens 80(4):317–321. doi:10.1111/j.1399-0039.2012.01929.x
28. von Elm E, Altman DG, Egger M et al (2008) The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. J Clin Epidemiol 61(4):344–349. doi:10.1016/j.jclinepi.2007.11.008
29. Little J, Higgins JP, Ioannidis JP et al (2009) Strengthening the reporting of genetic association studies (STREGA)—an extension of the STROBE statement. Genet Epidemiol 33(7):581–598. doi:10.1002/gepi.20410
30. Assmann TS, Brondani Lde A, Bauer AC, Canani LH, Crispim D (2014) Polymorphisms in the TLR3 gene are associated with risk for type 1 diabetes mellitus. Eur J Endocrinol 170(4):519–527. doi:10.1530/EJE-13-0963
31. Gong J, Tong Y, Zhang HM et al (2012) Genome-wide identification of SNPs in microRNA genes and the SNP effects on microRNA target binding and biogenesis. Hum Mutat 33(1):254–263. doi:10.1002/humu.21641
32. Hsu SD, Chu CH, Tsou AP et al (2008) miRNAMap 2.0: genomic maps of microRNAs in metazoan genomes. Nucleic Acids Res 36(Database issue):D165–169. doi:10.1093/nar/gkm1012
33. Kaidonis G, Gillies MC, Abhary S et al (2016) A single-nucleotide polymorphism in the MicroRNA-146a gene is associated with diabetic nephropathy and sight-threatening diabetic retinopathy in Caucasian patients. Acta Diabetol. doi:10.1007/s00592-016-0850-4
34. Ciccacci C, Morganti R, Di Fusco D et al (2014) Common polymorphisms in MIR146a, MIR128a and MIR27a genes

contribute to neuropathy susceptibility in type 2 diabetes. Acta Diabetol 51(4):663–671. doi:10.1007/s00592-014-0582-2
35. Xie K, Ma H, Liang C et al (2015) A functional variant in miR-155 regulation region contributes to lung cancer risk and survival. Oncotarget 6(40):42781–42792. doi:10.18632/oncotarget.5840
36. Bulik-Sullivan B, Selitsky S, Sethupathy P (2013) Prioritization of genetic variants in the microRNA regulome as functional candidates in genome-wide association studies. Hum Mutat 34(8):1049–1056. doi:10.1002/humu.22337
37. Nahid MA, Pauley KM, Satoh M, Chan EK (2009) miR-146a is critical for endotoxin-induced tolerance: implication in innate immunity. J Biol Chem 284(50):34590–34599. doi:10.1074/jbc.M109.056317
38. Nahid MA, Satoh M, Chan EK (2011) MicroRNA in TLR signaling and endotoxin tolerance. Cell Mol Immunol 8(5):388–403. doi:10.1038/cmi.2011.26
39. Nahid MA, Satoh M, Chan EK (2011) Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. Open J Immunol 186(3):1723–1734. doi:10.4049/jimmunol.1002311
40. Doxaki C, Kampranis SC, Eliopoulos AG, Spilianakis C, Tsatsanis C (2015) Coordinated Regulation of miR-155 and miR-146a Genes during Induction of Endotoxin Tolerance in Macrophages. J Immunol 195(12):5750–5761. doi:10.4049/jimmunol.1500615
41. Gao M, Wang X, Zhang X et al (2015) Attenuation of cardiac dysfunction in polymicrobial sepsis by microRNA-146a is mediated via targeting of IRAK1 and TRAF6 expression. J Immunol 195(2):672–682. doi:10.4049/jimmunol.1403155
42. Saba R, Sorensen DL, Booth SA (2014) MicroRNA-146a: a dominant, negative regulator of the innate immune response. Front Immunol 5:578. doi:10.3389/fimmu.2014.00578
43. Meisgen F, Xu Landen N, Wang A et al (2014) MiR-146a negatively regulates TLR2-induced inflammatory responses in keratinocytes. J Invest Dermatol 134(7):1931–1940. doi:10.1038/jid.2014.89
44. Al-Quraishy S, Dkhil MA, Delic D, Abdel-Baki AA, Wunderlich F (2012) Organ-specific testosterone-insensitive response of miRNA expression of C57BL/6 mice to Plasmodium chabaudi malaria. Parasitol Res 111(3):1093–1101. doi:10.1007/s00436-012-2937-3
45. Nakasa T, Miyaki S, Okubo A et al (2008) Expression of microRNA-146 in rheumatoid arthritis synovial tissue. Semin Arthritis Rheum 58(5):1284–1292. doi:10.1002/art.23429
46. Lu LF, Boldin MP, Chaudhry A et al (2010) Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. Cell 142(6):914–929. doi:10.1016/j.cell.2010.08.012
47. Curtale G, Citarella F, Carissimi C et al (2010) An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes. Blood 115(2):265–273. doi:10.1182/blood-2009-06-225987
48. Jazdzewski K, Murray EL, Franssila K, Jarzab B, Schoenberg DR, de la Chapelle A (2008) Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. Proc Natl Acad Sci USA 105(20):7269–7274. doi:10.1073/pnas.0802682105
49. Xu T, Zhu Y, Wei QK et al (2008) A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma. Carcinogenesis 29(11):2126–2131. doi:10.1093/carcin/bgn195
50. Shen J, Ambrosone CB, DiCioccio RA, Odunsi K, Lele SB, Zhao H (2008) A functional polymorphism in the miR-146a gene and age of familial breast/ovarian cancer diagnosis. Carcinogenesis 29(10):1963–1966. doi:10.1093/carcin/bgn172
51. Ma X, Becker Buscaglia LE, Barker JR, Li Y (2011) MicroRNAs in NF-kappaB signaling. J Mol Cell Biol 3(3):159–166. doi:10.1093/jmcb/mjr007
52. Sun Y, Cai J, Ma F, Lu P, Huang H, Zhou J (2012) miR-155 mediates suppressive effect of progesterone on TLR3, TLR4-triggered immune response. Immunol Lett 146(1–2):25–30. doi:10.1016/j.imlet.2012.04.007
53. Wang P, Hou J, Lin L et al (2010) Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. J Immunol 185(10):6226–6233. doi:10.4049/jimmunol.1000491
54. Pareek S, Roy S, Kumari B, Jain P, Banerjee A, Vrati S (2014) MiR-155 induction in microglial cells suppresses Japanese encephalitis virus replication and negatively modulates innate immune responses. J Neuroinflammation 11:97. doi:10.1186/1742-2094-11-97
55. Curtis AM, Fagundes CT, Yang G et al (2015) Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1. Proc Natl Acad Sci USA 112(23):7231–7236. doi:10.1073/pnas.1501327112
56. Schulte LN, Westermann AJ, Vogel J (2013) Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing. Nucleic Acids Res 41(1):542–553. doi:10.1093/nar/gks1030
57. Karczewski KJ, Tatonetti NP, Landt SG et al (2011) Cooperative transcription factor associations discovered using regulatory variation. Proc Natl Acad Sci USA 108(32):13353–13358. doi:10.1073/pnas.1103105108
58. Ji J, Xu M, Tu J et al (2016) MiR-155 and its functional variant rs767649 contribute to the susceptibility and survival of Hepatocellular carcinoma. Oncotarget. doi:10.18632/oncotarget.11206
59. Poy MN, Hausser J, Trajkovski M et al (2009) miR-375 maintains normal pancreatic alpha- and beta-cell mass. Proc Natl Acad Sci USA 106(14):5813–5818. doi:10.1073/pnas.0810550106
60. Erener S, Mojibian M, Fox JK, Denroche HC, Kieffer TJ (2013) Circulating miR-375 as a biomarker of beta-cell death and diabetes in mice. Endocrinology 154(2):603–608. doi:10.1210/en.2012-1744
61. Zhu J, Yang L, You W et al (2015) Genetic variation in miR-100 rs1834306 is associated with decreased risk for esophageal squamous cell carcinoma in Kazakh patients in northwest China. Int J Clin Exp Pathol 8(6):7332–7340
62. El Ouaamari A, Baroukh N, Martens GA, Lebrun P, Pipeleers D, van Obberghen E (2008) miR-375 targets 3′-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. Diabetes 57(10):2708–2717. doi:10.2337/db07-1614
63. Poy MN, Eliasson L, Krutzfeldt J et al (2004) A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432(7014):226–230. doi:10.1038/nature03076

Capítulo 3

“MicroRNAs e a Doença renal do diabetes”

ARTIGO 4

MicroRNAs and diabetic kidney disease: systematic review and bioinformatic analysis

MicroRNAs and diabetic kidney disease: systematic review and bioinformatic analysis

Taís S. Assmann[1,2], Mariana Recamonde-Mendoza[3,4], Bianca M. de Souza[1,2], Andrea C. Bauer[1,2], Daisy Crispim[1,2*].

1- Endocrine Division, Hospital de Clínicas de Porto Alegre. Porto Alegre, Rio Grande do Sul, Brazil.

2- Postgraduation Program in Medical Sciences: Endocrinology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

3- Institute of Informatics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

4- Experimental Research Center, Hospital de Clínicas de Porto Alegre. Porto Alegre, Rio Grande do Sul, Brazil

* Correspondence to: Dr. Daisy Crispim. Rua Ramiro Barcelos 2350; prédio 12; 4° andar. Zip Code 90035-003. Porto Alegre, Rio Grande do Sul, Brazil. E-mail: dcmoreira@hcpa.edu.br

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ABSTRACT

Introduction: MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression. Emerging evidence has suggested a role for miRNAs in the development of diabetic kidney disease (DKD), indicating that miRNAs may represent potential biomarkers of this chronic diabetic complication. However, results are still inconclusive, with few miRNAs being consistently dysregulated among studies. Therefore, we performed a systematic review of the literature on the subject, followed by bioinformatic analysis, to point out which miRNAs are dysregulated in DKD patients and in which pathways they participate.

Methods: PubMed and EMBASE were searched to identify all studies that compared miRNA expressions between patients with DKD (cases) and diabetic patients without this complication or healthy subjects (control groups). MiRNA expressions were analyzed in kidney biopsies, urine/urinary exosomes or total blood/plasma/serum. MiRNAs consistently dysregulated in DKD patients were submitted to bioinformatic analysis to retrieve their putative target genes and identify potentially affected pathways under their regulation.

Results: Twenty-seven studies were included in the systematic review. Among 151 dysregulated miRNAs reported in these studies, 14 (9.2%) were reported in at least 3 studies. Of them, 6 miRNAs were consistently dysregulated in patients with different stages of DKD compared to control subjects: miR-21-5p, miR-29a-3p, miR-126-3p, miR-192-5p, miR-214-3p, and miR-342-3p. Bioinformatic analysis indicated that these 6 miRNAs are involved in pathways related to DKD pathogenesis, such as apoptosis, fibrosis, and extracellular matrix accumulation.

Conclusion: Six miRNAs seem to be dysregulated in patients with different stages of DKD, constituting potential biomarkers of this disease.

Keywords: Systematic review; microRNA; diabetic kidney disease; bioinformatic analyses.

INTRODUCTION

Diabetic kidney disease (DKD), also known as diabetic nephropathy (DN), is a common microvascular complication that affects approximately 40% of patients with type 1 and type 2 diabetes mellitus (DM) (1, 2). This complication is the leading cause of end-stage renal disease (ESRD) in patients starting renal replacement therapy and is associated with increased cardiovascular mortality (1). Usually, DKD is clinically characterized by albuminuria and a gradual reduction in the glomerular filtration rate (GFR) (3, 4). The progressive decline in renal function during DKD is a result of pathophysiological alterations in the kidneys, such as glomerular hypertrophy, mesangial expansion and tubulointerstitial fibrosis due to the accumulation of extracellular matrix (ECM) proteins, basement membrane thickening, and podocyte dysfunction (4, 5).

At the cellular level, chronic hyperglycemia leads to increased generation of advanced glycation end-products, reactive oxygen species, activation of protein kinase C and renin-angiotensin system, and production of transforming growth factor-β1 (TGF-β1), which is a master cytokine/growth factor in fibrosis and inflammation (4, 6). These alterations have been associated with activation of key transcription factors, such as Smads and NF-κB, leading to aberrant expression of pro-inflammatory, pro-fibrotic, cell-cycle, and ECM genes (5).

Although a tight glycemic control is able to reduce the development and delay the progression of DKD (7, 8), current therapies are still not totally effective in preventing progression to ESRD. It has not yet been clearly established by which mechanisms some diabetic patients will progress to loss of renal function and require dialysis while others will preserve normal renal function regardless of the glycemic

control (9). Therefore, additional mediators and mechanisms leading to DKD need to be identified for more effective diagnosis and treatment of this complication.

In this context, emerging evidence suggests a role for epigenetic factors, such as microRNAs (miRNAs), in the development of DKD (5, 9, 10). MicroRNAs (miRNAs) are short (≅ 19 – 25 nt) noncoding RNAs that regulate gene expression, primarily by translation repression or mRNA degradation (11, 12). More than 2,000 human miRNAs have been identified so far, and at least 60% of all protein-coding genes are regulated by them (10, 11). Consequently, miRNAs may affect the development or progression of many diseases (13-15).

Regarding kidney diseases, miRNAs can be induced in renal cells *in vivo* and *in vitro* under hyperglycemic conditions, promoting accumulation of ECM proteins related to fibrosis and glomerular dysfunction (10). TGF-β1 is able to regulate expressions of miR-21, miR-29, miR-192, miR-200, miR-214, miR-216a/217, miR-382, and miR-491 families (10, 16-19). In accordance with the role of miRNAs in renal fibrosis, a number of circulating or urinary miRNAs have been reported as being dysregulated in specific stages of DKD (20-28), suggesting that miRNA signatures may be exploited as diagnostic/prognostic biomarkers of DKD, as well as may provide important information about that mechanisms involved in this complication (29).

Despite the large number of studies that have reported associations between miRNA expressions and DKD, the majority of the findings failed to be reproduced in other populations. Therefore, to further clarify the repertoire of miRNAs associated with DKD, we performed a systematic review of the literature on the subject. Moreover, we also carried out bioinformatic analyses to investigate the regulatory and functional roles of these miRNAs in biological pathways.

METHODS

Search strategy and eligibility of studies

This systematic review was designed and described in accordance with current guidelines (30, 31), and its protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO) (http://www.crd.york.ac.uk/PROSPERO), with the number of CRD42017070885. PubMed and EMBASE repositories were searched to find all studies that investigated miRNA expressions in DKD samples. The following medical subject headings (MeSH) were used: ("diabetes mellitus" OR "diabetic kidney disease" OR "diabetes complications") AND ("microRNA" OR "RNA, small untranslated"). The search was limited to English, Spanish or Portuguese language papers and was finished on August, 2017. Reference lists from all articles were also manually checked in order to identify other relevant citations. To guarantee that important studies were not missed, searches in Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) and Array Express (www.ebi.ac.uk/arrayexpress) databases were also performed.

All original articles that evaluated miRNA expressions in patients with DKD (cases) and patients without this complication (controls) were included in this systematic review. Studies that did not have an appropriate control group or studies performed in cell lines or animal models were excluded. Two researchers (T.S.A and B.M.S) independently reviewed titles and abstracts of all articles retrieved to evaluate whether they were eligible for inclusion.

Data extraction and quality assessment of each study

Results were independently collected by two investigators (B.M.S. and T.S.A.) using a standardized abstraction form (30), and consensus was pursued in all extracted items. When consensus could not be achieved, differences in data extraction were resolved by a third reviewer (D.C.). Information extracted from each individual study were as follow: 1) characteristics of studies and samples; 2) information regarding miRNA expression, which included method used for quantification, tissue analyzed, and number of miRNAs investigated; and 3) miRNA expressions in each study group. Prior to analysis, all miRNA names were standardized based on miRBase v21 information.

Two investigators (T.S.A and B.M.S) evaluated the quality of each eligible study using The Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) (32). This tool contains 4 key domains (patient selection, index test, reference standard, and flow/timing) supported by 7 questions to help a decision on risk of bias, rating risk of bias, and about the applicability of studies. Each question can be answered with "yes", "no", or "unclear". A score of 1 is then given for each "yes" (low risk/high concern), a score of 0.5 for each "unclear", and a score of 0 for each "no" (high risk/low concern). Scores range from 0 to 7, with studies being classified as having "good quality" (scores 6-7), "fair" (scores 4-5), or "poor" quality (scores < 3).

Additionally, we evaluated if the articles followed the Minimum Information about a Microarray Experiment (MIAME) guideline v2.0 (33) or the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE)

guideline (34). Only articles in accordance with these guidelines were included in this review.

Evaluation of microRNA putative target genes and functional enrichment analysis

The list of miRNAs identified as consistently dysregulated in DKD patients in this systematic review was then submitted to bioinformatic analyses to search for their putative target genes and identify potential biological pathways under their regulation (**Supplementary Figure 1**). For the analysis of target genes, we combined information from experimentally validated miRNA-target gene interactions with results from target prediction algorithms in order to retrieve a comprehensive set of targets while controlling for false positive rates. Specifically, we queried the databases miRTarbase release 6.1 (35) and starBase v2.0 (36) of experimentally validated data regarding miRNA-target interactions, restricting the search for interactions classified as functional in miRTarBase, and interactions predicted by 2 or more software with at least one supporting experiment in starBase. Additional validated targets were also obtained from miRecords (data as of April 2013, last update) (37), and the union of all interactions retrieved from these resources was adopted as the set of validated miRNA-target gene interactions in our study.

For *in silico* analysis with target prediction algorithms, we used the web-based tools TargetScan v7.1 (38), Diana MicroT-CDS (39), and miRanda-mirSVR (August 2010 Release) (40, 41), which are well consolidated tools for this purpose. The complete list of miRNA-target gene interactions was downloaded from these websites and filtered using an in-house script in the R environment to collect only interactions associated with the miRNAs of interest in our study. To control for false positive

rates, the following additional filtering criteria were adopted: 1) for TargetScan v7.1, we considered interactions involving conserved miRNA sites and with Context++ scores < -0.1; 2); for Diana MicroT-CDS, we kept interactions with prediction scores > 0.7; and 3) for miRanda-mirSVR, we selected interactions involving conserved miRNAs with scores < -0.1. Finally, only miRNA-target gene interactions predicted by at least 2 *in silico* analysis tools were retained as the set of computationally predicted miRNA- target interactions. The combination of unique validated and predicted miRNA-target interactions was used for further analyses. MiRNAs and genes identifiers were mapped to miRBase v21 and Human Gene Nomenclature Committee (42, 43) standards, respectively.

Following the computational analysis of miRNA targets, we assessed the functional enrichment of target genes using pathways annotation from the KEGG Pathway Database (44, 45) and the clusterProfiler package in R/Bioconductor environment (46). This investigation was performed in two ways: 1) for targets of each individual miRNA; and 2) for targets of miRNAs grouped by their expression profile (up- or downregulation). Statistical significance for KEGG pathways enrichment was estimated with a hypergeometric test and adjusted to account for multiple hypotheses using the False Discovery Rate (FDR) procedure implemented in the qvalue R package (47). Pathways with a q-value < 0.05 were considered strongly enriched for the genes targeted by the selected miRNAs.

To clarify functional similarities between miRNAs of interest, we used the Jaccard Similarity Coefficient (JC), which provides a measure of similarity between two sets of elements. The JC is computed as the size of the intersection between the two sets divided by the size of their union, and provides a value in the range from 0 to 1 that reflects the degree of shared members between the sets, where the higher the

value the larger is their overlap (and consequently the similarity). We adopted this criterion to evaluate the similarity of miRNAs in a pairwise manner, both in terms of their putative target genes and enriched pathways.

RESULTS

Literature search, characteristics of the eligible studies, and quality assessment

The flow diagram showing the strategy used to select studies for inclusion in this systematic review is shown in **Figure 1**. After applying the search criteria, a total of 2849 publications were retrieved from databases; however, following full text analysis, only 27 articles fulfilled the eligibility criteria and were included in this review (20, 21, 23-28, 48-66). The main characteristics of these articles are shown in **Table 1**. Chien *et al*. (51) analyzed 2 samples that differed in the manner DKD was classified [according to urinary albumin to creatinine rate (UACR) or estimated GFR (eGFR) values]; thus, their results are shown separately in **Table 1**. Sample sizes in the included studies ranged from 9 to 690. The number of miRNAs analyzed ranged from 1 to 1066, with the number of miRNAs differentially expressed between groups varying from 1 to 29 (**Table 1**).

Most of the studies (60.7%) comprised patients with type 2 DM (T2DM), 17.9% of the articles included patients with type 1 DM (T1DM), and 21.4% of them did not report DM type. Moreover, studies applied different diagnostic methods for DKD classification: 53.6% of them used UACR and/or urinary albumin excretion (UAE), 32.1% performed kidney biopsies (some of them in combination with eGFR and UACR measurements), and 14.3% of them used only eGFR. Regarding UACR and UAE, patients were classified as normoalbuminuric, microalbuminuric or

macroalbuminuric; however, not all studies compared the three groups of patients. The Modification of Diet in Renal Disease (MDRD) equation was mainly used to calculate eGFR (67), and patients were usually categorized as having low eGFR (< 60 mL/min/1.73m^2) or high/normal eGFR (≥ 60 mL/min/1.73m^2) (**Table 1**).

Regarding the tissues analyzed, 40.7% of the studies evaluated circulating miRNA expressions in blood samples (total blood, serum or plasma), 40.7% in urine samples [whole urine, exosomes or extracellular vesicles (EVs)] and 18.6% in kidney biopsies. The results reported by Baker *et al*. (26) are shown separately since they analyzed miRNAs in two types of renal tissue (**Table 1**).

Quality of each study included in this review was assessed using QUADAS-2 scores. Overall, 40.7% of the studies were considered as having "good quality" (scores 6 to 7; **Supplementary Table 1**). The remaining studies scored between 4.5 and 5.5, being considered as of "fair quality". No study scored less than 4.5.

Differentially expressed miRNAs in DKD

Out of 151 differentially expressed miRNAs reported in the 27 articles that analyzed DKD patients and controls, 32 miRNAs were reported in at least two studies (**Supplementary Table 2**), and 14 of them were reported in three or more studies, being chosen for further evaluation (**Table 2**). Among these 14 miRNAs, 5 miRNAs (miR-21-5p, miR-29a-3p, miR-126-3p, miR-214-3p, and miR-342-3p) seem to be consistently upregulated in DKD cases compared to controls (T1DM controls or healthy subjects), while miR-192-5p seems to be downregulated in cases (**Table 2**). Eight miRNAs were reported as being downregulated in cases from one study, while upregulated in cases from another study. These contradictory results may be due to

differences in tissue types, methods used for DKD classification, or type of DM that was analyzed (**Table 2**).

Putative target genes of differentially expressed miRNAs

Bioinformatic analyses were performed to retrieve putative target genes for the 6 miRNAs (hsa-miR-21-5p, hsa-miR-29a-3p, hsa-miR-126-3p, hsa-miR-192-5p, hsa-miR-214-3p, and hsa-miR-342-3p) consistently dysregulated in DKD patients. Data from 6 distinct resources, including databases with experimentally validated miRNA-target interactions and computational target prediction tools, were systematically combined (**Supplementary Figure 1**). A total of 5374 interactions were retrieved for the miRNAs of interest. Among them, 3519 are from sources of experimentally validated data, while 1855 are from computational prediction tools (**Table 3; Supplementary Table 3**). An average of 895.7 ± 437.7 targets were found for the 6 miRNAs, with the largest number of targets retrieved for the miR-29a-3p (1272) and the smallest number for the miR-126-3p (55).

Next, we compared the list of putative targets among the 6 miRNAs by computing the JC values in a pairwise fashion (**Supplementary Figure 2A**). The highest JC values were obtained for miR-29a-3p in comparison to miR-21-5p (JC = 0.079) and miR-214-3p (JC = 0.068). Nonetheless, as the JC values suggest, these overlaps in the number of targets are very low, indicating that few targets are shared among the miRNAs of interest. Among the 4375 unique genes collectively targeted by the 6 miRNAs (i.e., size of the union of all targets identified), we found that the expression of the anti-apoptotic gene *BLC2* is potentially regulated by all miRNAs excepting miR-214-3p, and that 15 targets are modulated by 4 out of 6 miRNAs.

Pathways retrieved by functional enrichment analysis

To further extract insights regarding the functional consequences of dysregulation of the 6 selected miRNAs, we performed functional enrichment analysis of the putative target genes using pathway maps from the KEGG Pathway Database. This analysis was carried out for each miRNA evaluated individually and also for target genes grouped by miRNAs with similar expression profile. Out of the 319 pathways annotated in KEGG Database (assessed in September 2017), a total of 126 pathways were significantly overrepresented (q-values < 0.05) in the putative target lists analyzed, and 82 KEGG terms were enriched for more than one miRNA. No significant KEGG term was found for hsa-miR-342-3p considering the applied threshold.

As expected, results from pathway enrichment analysis based on predicted/validated targets of miR-21-5p, miR-29a-3p, miR-126-3p, miR-192-5p, and miR-214-3p revealed a number of already known DKD-associated pathways, such as AGE-RAGE signaling pathway in diabetic complications, longevity regulating pathway, TGF-β1, VEGFA, NF-κB, PI3K-Akt, and FoxO signaling pathways (**Supplementary Table 4**). Moreover, some pathways are not known to be involved in DKD pathogenesis, suggesting new potential pathways to be studied (**Supplementary Table 4**).

We also compared the similarity among the 6 miRNAs in terms of their enriched pathways evaluating the pairwise JC, and observed that despite the low overlap among their targets (**Supplementary Figure 2A**), a substantial overlap in the list of modulated pathways are found for some pairs of miRNAs, especially among miR-21-5p, miR-29a-3p and miR-126-3p (**Supplementary Figure 2B**). This may

indicate that these miRNAs act in shared pathways through distinct mechanisms and targets, thus having complementary roles in their modulation.

MiRNAs involved in the TGF-β1 pathway

TGF-β1, the most abundant isoform of TGF-β, is a major player in DKD pathogenesis, mainly because of its potent pro-fibrotic actions (4, 10, 16). The pro-fibrotic effects of TGF-β1 are primarily accomplished by Smad-dependent pathways; although, Smad-independent pathways, related to EGFR, MAPK, p53, and PI3K/AKT, might also be involved (68).

The TGF-β1/Smad signaling pathway regulates expression of several genes, including genes involved in miRNA biogenesis (**Figure 3**). Moreover, TGF-β1 positively or negatively regulates expressions of a number of miRNAs, including miR-21, miR-29, miR-192, and miR-214 families (10, 16-18), which is in accordance with our data, although only miR-21-5p reached a q-value < 0.5. In addition, several members of the TGF-β1 pathway are targeted by miRNAs, suggesting an autoregulatory feedback loop between TGF-β1 and some miRNAs (69).

Among the miRNAs that participate in the TGF-β1/Smad pathway (**Figure 3**), miR-21-5p, miR-29a-3p, miR-192-5p and miR-214-3p were found as being dysregulated in patients with DKD in our systematic review (**Table 2**). As shown in **Figure 4**, a positive feedback loop between TGF-β1 and miR-21-5p, through the miR-21 target Smad7, demonstrates a possible mechanism that amplifies TFG-β1 signaling during renal fibrosis (60). In addition, TGF-β1 activates PI3K/Akt signaling pathway by downregulating PTEN through miR-21-5p, miR-29a-3p and miR-214-3p actions, which leads to hypertrophy and fibrosis (60, 70, 71).

TGF-β1-induced downregulation of miR-192-5p in human proximal tubular cells (PTCs) increases the expression of E-cadherin repressors Zeb1 and Zeb2, decreasing the levels of E-cadherin, thus increasing collagen production and ECM accumulation (72) (**Figure 4**). Of note, contradictory results have been shown regarding TGF-β1 and miR-192-5p interactions, since TGF-β1 was also reported to increase miR-192-5p expression in mouse mesenchymal cells (MCs) through acetylation of the transcription factor Ets-1 (a negative regulator of miR-192) and histone H3 by Akt-induced p300 activation (22), which would accelerate the signaling downstream of TGF-β1.

Similarly to miR-192-5p, miR-29a-3p might have both anti-fibrotic and pro-fibrotic functions (**Figure 4**). Increased levels of miR-29a-3p indirectly inhibit TGF-β1 and production of many types of collagen, leading to decreased ECM accumulation and fibrosis (73). On the other hand, miR-29a-3p targets PTEN, increasing PI3K/Akt signaling pathway (71), which could potentially increase kidney hypertrophy, although this pathway was not studied in kidney cells.

DISCUSSION

Laboratory markers of DKD are still not accurate enough to predict which diabetic patients are at risk for developing kidney complications prior to its clinical stablished dysfunction (74). Thus, the discovery of new biomarkers capable of predicting DKD development is highly expected, since they may identify patients at higher risk in whom an early treatment may help delay or prevent the onset of this complication, as well as its progression to ESRD (74).

In this context, recent studies have demonstrated that miRNAs are key mediators in the pathogenesis of DKD, suggesting that circulating miRNAs can be used as biomarkers to assess DKD development and progression (6, 10, 16, 74). Circulating and urinary miRNAs are ideal noninvasive biomarkers because they are stable in biofluids and exosomes, and can be detected using stablished techniques for quantification, such as quantitative PCR (qPCR) (10, 15). However, miRNA profile studies in humans have shown contradictory results, with few miRNAs being consistently dysregulated among studies [reviewed in (6, 10, 16, 74)]. Therefore, as part of the ongoing effort to identify a profile of miRNAs that could be used as biomarkers of DKD, we performed a systematic review of all studies that evaluated miRNA expressions in tissues from patients with DKD (cases) and diabetic patients without DKD or healthy subjects (control groups). Six miRNAs (miR-21-5p, miR-29a-3p, miR-126-3p, miR-192-5p, miR-214-3p, and miR-342-3p) were consistently dysregulated in total blood/plasma/serum, urine/urinary exosomes or kidney biopsies of DKD patients.

MiR-21-5p is one of the most abundant miRNAs in human tissues (6), and it has been consistently reported as being upregulated in serum/plasma and kidney samples of both T1DM and T2DM patients with different stages of DKD (**Table 2**). This miRNA has been negatively correlated with eGFR and serum creatinine levels (sCR) and positively correlated with tubulointerstitial injury, glomerulosclerosis and proteinuria levels (26, 56, 60). Moreover, miR-21-5p was upregulated in DKD progressors [patients with a fast increase in sCR levels (51) or who progressed to ESRD (20)] compared to non-progressors, indicating the potential of this miRNA in monitoring DKD progression. Increased miR-21-5p expression was also observed in

renal transplanted patients with fibrotic kidney disease and in patients with IgA nephropathy (75).

The role of miR-21-5p in renal fibrosis is well stablished, although the mechanisms involved have not been completely clarified (6, 16, 74, 76). MiR-21-5p expression increases in kidney cells cultured with TGF-β1 or high glucose and downregulates PTEN, thereby activating PI3K/Akt (**Figure 4**) and mTOR, leading to MC hypertrophy and tubulointerstitial fibrosis (16, 60, 74, 77). Zhong *et al*. (76) reported that targeting Smad7 may be another mechanism by which miR-21-5p regulates renal injury because miR-21-5p knockdown in db/db mice restored Smad7 levels and inhibited the activation of TGF-β1 and NF-κB signaling pathways, protecting against renal fibrosis. Accordingly, other studies have shown that miR-21-5p inhibitors are effective in ameliorating DKD and fibrosis in mice, suggesting that this miRNA is a potential therapeutic target against the DKD progression (10, 56, 78-80).

The miR-29 family consists of 3 members (miR-29a, miR-29b and miR-29c) that share the same seed sequence and target almost the same set of genes (16, 81). MiR-29a-3p seems to be upregulated in serum/plasma, urine or kidney of both T1DM and T2DM patients with DKD (**Table 2**). Interestingly, Pezzolesi *et al*. (20) reported that 1-standard deviation increase in miR-29a-3p expression in plasma was associated with protection against fast progression to ESRD (OR= 0.39, 95% CI 0.20-0.76) in T1DM patients, suggesting that the upregulation of this miRNA in DKD patients may be an attempt of the organism to ameliorate renal dysfunction.

In line with this hypothesis, most experimental studies have indicated that miR-29a attenuates renal fibrosis, mainly by targeting different types of collagen via a Smad3-dependent mechanism, thus leading to decreased ECM accumulation (73, 74,

81-83). TGF-β1 and high glucose are known to downregulate miR-29a in tubular epithelial cells (TECs), MCs, and podocytes (73, 82, 84, 85), which might predispose to renal dysfunction. Accordingly, Lin *et al*. (84) reported that diabetic mice overexpressing miR-29a had better nephrin levels, podocyte viability and renal function and less glomerular fibrosis compared to wild-type mice. This renal protection was explained by a role of miR-29a in decreasing histone deacetylase activity, which restored nephrin levels (84). Interestingly, a recent study demonstrated that Linagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor used as an antidiabetic drug, can also confer renal protection and decrease fibrosis in a mouse model of DKD by inducing miR-29a, which targets DPP-4 (86).

MiR-192-5p is highly expressed in normal kidneys compared to the other organs (16), and it seems to be downregulated in urinary EVs, serum or urine of DKD patients (**Table 2**). Krupa *et al*. (72) observed a decreased miR-192-5p expression in kidney biopsies of patients with advanced DKD, which negatively correlated with tubule interstitial fibrosis. The decreased miR-192-5p expression in DKD patients might be due to the TGF-β1-induced downregulation of this miRNA, leading to increased expressions of E-cadherin repressors Zeb1 and Zeb2, which may increase collagen production and ECM accumulation, thus worsening renal fibrosis (72, 87) (**Figure 4**). Accordingly, miR-192-5p has also being negatively correlated with UACR, TGF-β1 and proteinuria levels (59, 64, 88).

On the other hand, studies in rodent models of DKD have indicated that TGF-β1 also induce miR-192-5p expression through promoter Smad-binding elements and epigenetic regulation via Ets-1 (10, 22). Consequently, TGF-β1-induced miR-192-5p upregulation in TECs and MCs has been linked to renal damage in streptozotocin (T1DM) and db/db (T2DM) models of DKD (10, 17, 22, 89-91). Interestingly, miR-

192-5p upregulates other miRNAs in MCs, including miR-200b/c, creating an amplifying circuit that further augments ECM accumulation in kidney (10). In agreement with a pro-fibrotic role of miR-192-5p in murine models of DKD, both miR-192 knockout mice and streptozotocin-induced diabetic mice treated with anti-miR-192 showed less severe phenotypes of DKD compared to wild-type mice (91, 92). The differences in miR-192-5p expressions between human and rodent could be partially explained based on the aligned sequences of this miRNA between species. The hsa-miR-192-5p and mmu-miR-192-5p share 93% of similarity in their seed sequences, while hsa-miR-192-5p and rno-miR-192-5p share only 91% of similarity (sequences retrieved from miRBase and aligned using MUSCLE tool). These differences in miR-192-5p seed sequences could affect expressions of different genes between species, thus having different effects on DKD pathogenesis.

Most of the studies included in this systematic review showed that miR-126-3p is upregulated in plasma, urine or kidney of DKD patients (**Table 2**), as well as in non-diabetic patients with ESRD (93). Importantly, a tight glycemic control was able to decrease miR-126-3p expression in urine of DKD patients (57). MiR-126-3p is highly expressed in endothelial cells and plays a key role in maintaining endothelial homeostasis and angiogenesis (94, 95). It has pro-angiogenic actions by repressing SPRED1 and PIK3R2, two negative regulators of VEGFA (95, 96). Moreover, miR-126-3p regulates renal vascular inflammation by lowering VCAM-1 expression (97, 98). Therefore, miR-126-3p downregulation may predispose to DKD due to its role in endothelial dysfunction, which is a common feature of diabetic complications (99).

MiR-214-3p has been shown to be upregulated in urine or kidney of DKD patients (**Table 2**) and also in murine models of renal injury (70, 100, 101); however, little is known about the mechanisms of action of this miRNA. TGF-β1 seems to

activate miR-214-3p that, in turn, targets PTEN, a repressor of the PI3K/Akt signaling pathway, leading to renal hypertrophy and fibrosis (70, 102). In mice, genetic deletion of miR-214-3p attenuated interstitial fibrosis induced by unilateral ureteral obstruction (UUO), while treatment of wild-type mice with an anti-miR-214 before UUO had anti-fibrotic effects (100), suggesting that miR-214-3p promotes renal fibrosis.

MiR-342-3p has been described as being upregulated in urine/urinary exosomes or kidney biopsies of DKD patients (**Table 2**), being positively correlated with UACR and sCR levels and negatively correlated with eGFR (21); however, no study has explained the molecular basis behind this association. MiR-342-3p dysregulation has also been reported in several diseases, including different cancers (103-105), obesity (106, 107), T1DM and T2DM (15), and acute kidney rejection (108). Our in silico analysis did not identify any significant pathway associated with this miRNA.

In conclusion, this systematic review and bioinformatic analysis suggest that 6 miRNAs (miR-21-5p, miR-29a-3p, miR-126-3p, miR-192-5p, miR-214-3p, and miR-342-3p) are consistently dysregulated in patients with DKD, constituting potential biomarkers of this chronic diabetic complication. These miRNAs and their targets participate in pathways of known relevance for DKD pathogenesis, such as TGF-β1, VEGFA, NF-κB, PI3K-Akt, and FoxO signaling pathways, reinforcing their pathogenic relevance. Further studies are necessary to confirm the involvement of these 6 miRNAs in different stages of DKD and to translate these findings to clinical practice.

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REFERENCES

1. Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T. Diabetic nephropathy: diagnosis, prevention, and treatment. Diabetes Care. 2005;28(1):164-76.
2. Macisaac RJ, Ekinci EI, Jerums G. Markers of and risk factors for the development and progression of diabetic kidney disease. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2014;63(2 Suppl 2):S39-62.
3. Ritz E, Zeng XX, Rychlik I. Clinical manifestation and natural history of diabetic nephropathy. Contributions to nephrology. 2011;170:19-27.
4. Kanwar YS, Sun L, Xie P, Liu FY, Chen S. A glimpse of various pathogenetic mechanisms of diabetic nephropathy. Annu Rev Pathol. 2011;6:395-423.
5. Reddy MA, Tak Park J, Natarajan R. Epigenetic modifications in the pathogenesis of diabetic nephropathy. Semin Nephrol. 2013;33(4):341-53.
6. Simpson K, Wonnacott A, Fraser DJ, Bowen T. MicroRNAs in Diabetic Nephropathy: From Biomarkers to Therapy. Current diabetes reports. 2016;16(3):35.
7. de Boer IH, Rue TC, Cleary PA, Lachin JM, Molitch ME, Steffes MW, et al. Long-term renal outcomes of patients with type 1 diabetes mellitus and microalbuminuria: an analysis of the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications cohort. Archives of internal medicine. 2011;171(5):412-20.
8. Nathan DM, Bayless M, Cleary P, Genuth S, Gubitosi-Klug R, Lachin JM, et al. Diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: advances and contributions. Diabetes. 2013;62(12):3976-86.
9. Thomas MC. Epigenetic Mechanisms in Diabetic Kidney Disease. Current diabetes reports. 2016;16(3):31.
10. Kato M, Natarajan R. MicroRNAs in diabetic nephropathy: functions, biomarkers, and therapeutic targets. Ann N Y Acad Sci. 2015;1353:72-88.
11. Esteller M. Non-coding RNAs in human disease. Nature reviews Genetics. 2011;12(12):861-74.
12. Butz H, Kinga N, Racz K, Patocs A. Circulating miRNAs as biomarkers for endocrine disorders. J Endocrinol Invest. 2016;39(1):1-10.
13. Lin S, Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer. 2015;15(6):321-33.
14. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. Journal of autoimmunity. 2009;32(3-4):189-94.
15. Assmann TS, Recamonde-Mendoza M, de Souza BM, Crispim D. MicroRNA expression profiles and type 1 diabetes mellitus: systematic review and bioinformatics analysis. Endocrine connections. 2017.
16. Chung AC, Yu X, Lan HY. MicroRNA and nephropathy: emerging concepts. Int J Nephrol Renovasc Dis. 2013;6:169-79.
17. Kato M, Arce L, Wang M, Putta S, Lanting L, Natarajan R. A microRNA circuit mediates transforming growth factor-beta1 autoregulation in renal glomerular mesangial cells. Kidney international. 2011;80(4):358-68.

18. Kato M, Putta S, Wang M, Yuan H, Lanting L, Nair I, et al. TGF-beta activates Akt kinase through a microRNA-dependent amplifying circuit targeting PTEN. Nature cell biology. 2009;11(7):881-9.
19. Park JT, Kato M, Yuan H, Castro N, Lanting L, Wang M, et al. FOG2 protein down-regulation by transforming growth factor-beta1-induced microRNA-200b/c leads to Akt kinase activation and glomerular mesangial hypertrophy related to diabetic nephropathy. The Journal of biological chemistry. 2013;288(31):22469-80.
20. Pezzolesi MG, Satake E, McDonnell KP, Major M, Smiles AM, Krolewski AS. Circulating TGF-beta1-Regulated miRNAs and the Risk of Rapid Progression to ESRD in Type 1 Diabetes. Diabetes. 2015;64(9):3285-93.
21. Eissa S, Matboli M, Bekhet MM. Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis. Biomed Pharmacother. 2016;83:92-9.
22. Kato M, Dang V, Wang M, Park JT, Deshpande S, Kadam S, et al. TGF-beta induces acetylation of chromatin and of Ets-1 to alleviate repression of miR-192 in diabetic nephropathy. Sci Signal. 2013;6(278):ra43.
23. Barutta F, Tricarico M, Corbelli A, Annaratone L, Pinach S, Grimaldi S, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. PloS one. 2013;8(11):e73798.
24. Argyropoulos C, Wang K, McClarty S, Huang D, Bernardo J, Ellis D, et al. Urinary microRNA profiling in the nephropathy of type 1 diabetes. PloS one. 2013;8(1):e54662.
25. Argyropoulos C, Wang K, Bernardo J, Ellis D, Orchard T, Galas D, et al. Urinary MicroRNA Profiling Predicts the Development of Microalbuminuria in Patients with Type 1 Diabetes. Journal of clinical medicine. 2015;4(7):1498-517.
26. Baker MA, Davis SJ, Liu P, Pan X, Williams AM, Iczkowski KA, et al. Tissue-Specific MicroRNA Expression Patterns in Four Types of Kidney Disease. Journal of the American Society of Nephrology : JASN. 2017.
27. Cardenas-Gonzalez M, Srivastava A, Pavkovic M, Bijol V, Rennke HG, Stillman IE, et al. Identification, Confirmation, and Replication of Novel Urinary MicroRNA Biomarkers in Lupus Nephritis and Diabetic Nephropathy. Clin Chem. 2017.
28. Jia Y, Guan M, Zheng Z, Zhang Q, Tang C, Xu W, et al. miRNAs in Urine Extracellular Vesicles as Predictors of Early-Stage Diabetic Nephropathy. J Diabetes Res. 2016;2016:7932765.
29. Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. Nat Rev Endocrinol. 2013;9(9):513-21.
30. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Journal of clinical epidemiology. 2009;62(10):1006-12.
31. Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. JAMA. 2000;283(15):2008-12.
32. Whiting PF, Rutjes AW, Westwood ME, Mallett S, Deeks JJ, Reitsma JB, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med. 2011;155(8):529-36.
33. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet. 2001;29(4):365-71.

34. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-22.
35. Chou CH, Chang NW, Shrestha S, Hsu SD, Lin YL, Lee WH, et al. miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. Nucleic Acids Res. 2016;44(D1):D239-47.
36. Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res. 2014;42(Database issue):D92-7.
37. Xiao F, Zuo Z, Cai G, Kang S, Gao X, Li T. miRecords: an integrated resource for microRNA-target interactions. Nucleic Acids Res. 2009;37(Database issue):D105-10.
38. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife. 2015;4.
39. Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 2013;41(Web Server issue):W169-73.
40. Betel D, Koppal A, Agius P, Sander C, Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol. 2010;11(8):R90.
41. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res. 2008;36(Database issue):D149-53.
42. Wain HM, Bruford EA, Lovering RC, Lush MJ, Wright MW, Povey S. Guidelines for human gene nomenclature. Genomics. 2002;79(4):464-70.
43. Gray KA, Yates B, Seal RL, Wright MW, Bruford EA. Genenames.org: the HGNC resources in 2015. Nucleic Acids Res. 2015;43(Database issue):D1079-85.
44. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 2016;44(D1):D457-62.
45. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27-30.
46. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16(5):284-7.
47. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing. 2013.
48. Al-Kafaji G, Al-Mahroos G, Al-Muhtaresh HA, Skrypnyk C, Sabry MA, Ramadan AR. Decreased expression of circulating microRNA-126 in patients with type 2 diabetic nephropathy: A potential blood-based biomarker. Exp Ther Med. 2016;12(2):815-22.
49. Bai X, Geng J, Zhou Z, Tian J, Li X. MicroRNA-130b improves renal tubulointerstitial fibrosis via repression of Snail-induced epithelial-mesenchymal transition in diabetic nephropathy. Scientific reports. 2016;6:20475.
50. Bijkerk R, Duijs JM, Khairoun M, Ter Horst CJ, van der Pol P, Mallat MJ, et al. Circulating microRNAs associate with diabetic nephropathy and systemic microvascular damage and normalize after simultaneous pancreas-kidney transplantation. Am J Transplant. 2015;15(4):1081-90.

51. Chien HY, Chen CY, Chiu YH, Lin YC, Li WC. Differential microRNA Profiles Predict Diabetic Nephropathy Progression in Taiwan. International journal of medical sciences. 2016;13(6):457-65.
52. Delic D, Eisele C, Schmid R, Baum P, Wiech F, Gerl M, et al. Urinary Exosomal miRNA Signature in Type II Diabetic Nephropathy Patients. PloS one. 2016;11(3):e0150154.
53. Eissa S, Matboli M, Aboushahba R, Bekhet MM, Soliman Y. Urinary exosomal microRNA panel unravels novel biomarkers for diagnosis of type 2 diabetic kidney disease. J Diabetes Complications. 2016;30(8):1585-92.
54. Fiorentino L, Cavalera M, Mavilio M, Conserva F, Menghini R, Gesualdo L, et al. Regulation of TIMP3 in diabetic nephropathy: a role for microRNAs. Acta Diabetol. 2013;50(6):965-9.
55. Huang Y, Liu Y, Li L, Su B, Yang L, Fan W, et al. Involvement of inflammation-related miR-155 and miR-146a in diabetic nephropathy: implications for glomerular endothelial injury. BMC Nephrol. 2014;15:142.
56. Kolling M, Kaucsar T, Schauerte C, Hubner A, Dettling A, Park JK, et al. Therapeutic miR-21 Silencing Ameliorates Diabetic Kidney Disease in Mice. Molecular therapy : the journal of the American Society of Gene Therapy. 2017;25(1):165-80.
57. Liu Y, Gao G, Yang C, Zhou K, Shen B, Liang H, et al. Stability of miR-126 in Urine and Its Potential as a Biomarker for Renal Endothelial Injury with Diabetic Nephropathy. International journal of endocrinology. 2014;2014:393109.
58. Lv C, Zhou YH, Wu C, Shao Y, Lu CL, Wang QY. The changes in miR-130b levels in human serum and the correlation with the severity of diabetic nephropathy. Diabetes Metab Res Rev. 2015;31(7):717-24.
59. Ma X, Lu C, Lv C, Wu C, Wang Q. The Expression of miR-192 and Its Significance in Diabetic Nephropathy Patients with Different Urine Albumin Creatinine Ratio. J Diabetes Res. 2016;2016:6789402.
60. McClelland AD, Herman-Edelstein M, Komers R, Jha JC, Winbanks CE, Hagiwara S, et al. miR-21 promotes renal fibrosis in diabetic nephropathy by targeting PTEN and SMAD7. Clin Sci (Lond). 2015;129(12):1237-49.
61. Peng H, Zhong M, Zhao W, Wang C, Zhang J, Liu X, et al. Urinary miR-29 correlates with albuminuria and carotid intima-media thickness in type 2 diabetes patients. PloS one. 2013;8(12):e82607.
62. Peng R, Liu H, Peng H, Zhou J, Zha H, Chen X, et al. Promoter hypermethylation of let-7a-3 is relevant to its down-expression in diabetic nephropathy by targeting UHRF1. Gene. 2015;570(1):57-63.
63. Shao Y, Ren H, Lv C, Ma X, Wu C, Wang Q. Changes of serum Mir-217 and the correlation with the severity in type 2 diabetes patients with different stages of diabetic kidney disease. Endocrine. 2017;55(1):130-8.
64. Wang G, Kwan BC, Lai FM, Chow KM, Li PK, Szeto CC. Urinary sediment miRNA levels in adult nephrotic syndrome. Clin Chim Acta. 2013;418:5-11.
65. Zhou J, Peng R, Li T, Luo X, Peng H, Zha H, et al. A potentially functional polymorphism in the regulatory region of let-7a-2 is associated with an increased risk for diabetic nephropathy. Gene. 2013;527(2):456-61.
66. Conserva F, Pontrelli P, Accetturo M, Cordisco G, Fiorentino L, Federici M, et al. MIRNA EXPRESSION IN HUMAN DIABETIC NEPHROPATHY. Nephrology Dialysis Transplantation. 2014;3(Supplement 3):168-77.

67. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150(9):604-12.
68. Trionfini P, Benigni A, Remuzzi G. MicroRNAs in kidney physiology and disease. Nature reviews Nephrology. 2015;11(1):23-33.
69. Butz H, Racz K, Hunyady L, Patocs A. Crosstalk between TGF-beta signaling and the microRNA machinery. Trends in pharmacological sciences. 2012;33(7):382-93.
70. Denby L, Ramdas V, McBride MW, Wang J, Robinson H, McClure J, et al. miR-21 and miR-214 are consistently modulated during renal injury in rodent models. The American journal of pathology. 2011;179(2):661-72.
71. Tumaneng K, Schlegelmilch K, Russell RC, Yimlamai D, Basnet H, Mahadevan N, et al. YAP mediates crosstalk between the Hippo and PI(3)K-TOR pathways by suppressing PTEN via miR-29. Nature cell biology. 2012;14(12):1322-9.
72. Krupa A, Jenkins R, Luo DD, Lewis A, Phillips A, Fraser D. Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy. Journal of the American Society of Nephrology : JASN. 2010;21(3):438-47.
73. Wang B, Komers R, Carew R, Winbanks CE, Xu B, Herman-Edelstein M, et al. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. Journal of the American Society of Nephrology : JASN. 2012;23(2):252-65.
74. DiStefano JK, Taila M, Alvarez ML. Emerging roles for miRNAs in the development, diagnosis, and treatment of diabetic nephropathy. Current diabetes reports. 2013;13(4):582-91.
75. Wang G, Kwan BC, Lai FM, Chow KM, Li PK, Szeto CC. Urinary miR-21, miR-29, and miR-93: novel biomarkers of fibrosis. American journal of nephrology. 2012;36(5):412-8.
76. Zhong X, Chung AC, Chen HY, Dong Y, Meng XM, Li R, et al. miR-21 is a key therapeutic target for renal injury in a mouse model of type 2 diabetes. Diabetologia. 2013;56(3):663-74.
77. Dey N, Das F, Mariappan MM, Mandal CC, Ghosh-Choudhury N, Kasinath BS, et al. MicroRNA-21 orchestrates high glucose-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes. The Journal of biological chemistry. 2011;286(29):25586-603.
78. Wang J, Gao Y, Ma M, Li M, Zou D, Yang J, et al. Effect of miR-21 on renal fibrosis by regulating MMP-9 and TIMP1 in kk-ay diabetic nephropathy mice. Cell Biochem Biophys. 2013;67(2):537-46.
79. Wang JY, Gao YB, Zhang N, Zou DW, Xu LP, Zhu ZY, et al. Tongxinluo ameliorates renal structure and function by regulating miR-21-induced epithelial-to-mesenchymal transition in diabetic nephropathy. American journal of physiology Renal physiology. 2014;306(5):F486-95.
80. Chau BN, Xin C, Hartner J, Ren S, Castano AP, Linn G, et al. MicroRNA-21 promotes fibrosis of the kidney by silencing metabolic pathways. Science translational medicine. 2012;4(121):121ra18.
81. He Y, Huang C, Lin X, Li J. MicroRNA-29 family, a crucial therapeutic target for fibrosis diseases. Biochimie. 2013;95(7):1355-9.
82. Qin W, Chung AC, Huang XR, Meng XM, Hui DS, Yu CM, et al. TGF-beta/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol. 2011;22(8):1462-74.

83. Liu Y, Taylor NE, Lu L, Usa K, Cowley AW, Jr., Ferreri NR, et al. Renal medullary microRNAs in Dahl salt-sensitive rats: miR-29b regulates several collagens and related genes. Hypertension. 2010;55(4):974-82.
84. Lin CL, Lee PH, Hsu YC, Lei CC, Ko JY, Chuang PC, et al. MicroRNA-29a promotion of nephrin acetylation ameliorates hyperglycemia-induced podocyte dysfunction. Journal of the American Society of Nephrology : JASN. 2014;25(8):1698-709.
85. Du B, Ma LM, Huang MB, Zhou H, Huang HL, Shao P, et al. High glucose down-regulates miR-29a to increase collagen IV production in HK-2 cells. FEBS letters. 2010;584(4):811-6.
86. Kanasaki K, Shi S, Kanasaki M, He J, Nagai T, Nakamura Y, et al. Linagliptin-mediated DPP-4 inhibition ameliorates kidney fibrosis in streptozotocin-induced diabetic mice by inhibiting endothelial-to-mesenchymal transition in a therapeutic regimen. Diabetes. 2014;63(6):2120-31.
87. Wang B, Herman-Edelstein M, Koh P, Burns W, Jandeleit-Dahm K, Watson A, et al. E-cadherin expression is regulated by miR-192/215 by a mechanism that is independent of the profibrotic effects of transforming growth factor-beta. Diabetes. 2010;59(7):1794-802.
88. Szeto CC, Ching-Ha KB, Ka-Bik L, Mac-Moune LF, Cheung-Lung CP, Gang W, et al. Micro-RNA expression in the urinary sediment of patients with chronic kidney diseases. Dis Markers. 2012;33(3):137-44.
89. Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, et al. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(9):3432-7.
90. Chung AC, Huang XR, Meng X, Lan HY. miR-192 mediates TGF-beta/Smad3-driven renal fibrosis. Journal of the American Society of Nephrology : JASN. 2010;21(8):1317-25.
91. Putta S, Lanting L, Sun G, Lawson G, Kato M, Natarajan R. Inhibiting microRNA-192 ameliorates renal fibrosis in diabetic nephropathy. Journal of the American Society of Nephrology : JASN. 2012;23(3):458-69.
92. Deshpande SD, Putta S, Wang M, Lai JY, Bitzer M, Nelson RG, et al. Transforming growth factor-beta-induced cross talk between p53 and a microRNA in the pathogenesis of diabetic nephropathy. Diabetes. 2013;62(9):3151-62.
93. Wang H, Peng W, Shen X, Huang Y, Ouyang X, Dai Y. Circulating levels of inflammation-associated miR-155 and endothelial-enriched miR-126 in patients with end-stage renal disease. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas. 2012;45(12):1308-14.
94. van Solingen C, Bijkerk R, de Boer HC, Rabelink TJ, van Zonneveld AJ. The Role of microRNA-126 in Vascular Homeostasis. Curr Vasc Pharmacol. 2015;13(3):341-51.
95. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Developmental cell. 2008;15(2):261-71.
96. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, et al. miR-126 regulates angiogenic signaling and vascular integrity. Developmental cell. 2008;15(2):272-84.
97. Asgeirsdottir SA, van Solingen C, Kurniati NF, Zwiers PJ, Heeringa P, van Meurs M, et al. MicroRNA-126 contributes to renal microvascular heterogeneity of

VCAM-1 protein expression in acute inflammation. American journal of physiology Renal physiology. 2012;302(12):F1630-9.
98. Harris TA, Yamakuchi M, Ferlito M, Mendell JT, Lowenstein CJ. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(5):1516-21.
99. Cheng H, Harris RC. Renal endothelial dysfunction in diabetic nephropathy. Cardiovascular & hematological disorders drug targets. 2014;14(1):22-33.
100. Denby L, Ramdas V, Lu R, Conway BR, Grant JS, Dickinson B, et al. MicroRNA-214 antagonism protects against renal fibrosis. Journal of the American Society of Nephrology : JASN. 2014;25(1):65-80.
101. Godwin JG, Ge X, Stephan K, Jurisch A, Tullius SG, Iacomini J. Identification of a microRNA signature of renal ischemia reperfusion injury. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(32):14339-44.
102. Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J, et al. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer research. 2008;68(2):425-33.
103. Fayyad-Kazan H, Bitar N, Najar M, Lewalle P, Fayyad-Kazan M, Badran R, et al. Circulating miR-150 and miR-342 in plasma are novel potential biomarkers for acute myeloid leukemia. Journal of translational medicine. 2013;11:31.
104. Van der Auwera I, Limame R, van Dam P, Vermeulen PB, Dirix LY, Van Laere SJ. Integrated miRNA and mRNA expression profiling of the inflammatory breast cancer subtype. Br J Cancer. 2010;103(4):532-41.
105. Ronchetti D, Lionetti M, Mosca L, Agnelli L, Andronache A, Fabris S, et al. An integrative genomic approach reveals coordinated expression of intronic miR-335, miR-342, and miR-561 with deregulated host genes in multiple myeloma. BMC Med Genomics. 2008;1:37.
106. Oger F, Gheeraert C, Mogilenko D, Benomar Y, Molendi-Coste O, Bouchaert E, et al. Cell-specific dysregulation of microRNA expression in obese white adipose tissue. The Journal of clinical endocrinology and metabolism. 2014;99(8):2821-33.
107. Wang L, Xu L, Xu M, Liu G, Xing J, Sun C, et al. Obesity-Associated MiR-342-3p Promotes Adipogenesis of Mesenchymal Stem Cells by Suppressing CtBP2 and Releasing C/EBPalpha from CtBP2 Binding. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2015;35(6):2285-98.
108. Anglicheau D, Sharma VK, Ding R, Hummel A, Snopkowski C, Dadhania D, et al. MicroRNA expression profiles predictive of human renal allograft status. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(13):5330-5.

Table 1. Characteristics of the studies included in the systematic review.

First author, Year [ref]	Study design	DKD classification	Tissue	Method	N° of dysregulated miRNAs in DKD		Observations
					Up	Down	
Al-Kafaji, 2016 (48)	T2DM patients: 50 DKD (micro + macro) and 52 N; 50 healthy subjects	UAE / UACR	Whole blood	qPCR	0	1	miR-126 was correlated with albuminuria and eGFR (-)
Argyropoulos, 2013 (24)	T1DM patients: DKD (micro + macro), and 10 N	UAE / sCR	Urine	Microarray	21	8	≠ miRNA profiles across stages of DKD
Argyropoulos, 2015 (25)	T1DM patients (17 micro and 10 N)	UAE	Urine	qPCR	9	9	Association of miRNAs with development of DKD
Bai, 2016 (49)	27 T2DM patients with DKD (proteinuria); 20 healthy subjects	Proteinuria / hematuria / drop in renal function	Plasma	qPCR	0	1	miR-130b was correlated with sCR, proteinuria and tubulointerstitial fibrosis (-)
Baker, 2017[a] (26)	19 patients with DKD; 14 healthy subjects	Biopsy	Glomeruli	Sequencing / qPCR	8	10	miR-21-5p was correlated with eGFR (-)
Baker, 2017[b] (26)	19 patients with DKD; 14 healthy subjects	Biopsy	Proximal tubules	Sequencing / qPCR	2	11	miR-21-5p, miR-155-5p and miR-182-5p were correlated with sCR (-); miR-21-5p was correlated with eGFR (-)
Barutta, 2013 (23)	T1DM patients: 12 DKD (micro) and 12 N; 10 healthy subjects	UAE / UACR	Urinary exosomes	qPCR	2	2	Association of miRNAs with DKD

Bijkerk, 2015 (50)	T1DM patients: 21 DKD (waiting list for SPK), 15 without DKD, and 37 after SPK; 19 healthy subjects	eGFR	Plasma	Megaplex arrays	11	2	miRNAs were dysregulated in DKD patients, but normalized after SPK
Cardenas-Gonzalez, 2017 (27)	58 T1DM or T2DM with DKD; 119 healthy subjects	eGFR / UACR / biopsy	Urine	Microarray	12	3	miR-2861, miR-1915-3p, and miR-4532 were correlated with eGFR (+), sCR and proteinuria (-)
Chien, 2016[a] (51)	T2DM patients: 21 overt DKD (proteinuria), 17 micro, and 12 N	UACR	Serum	qPCR	3	0	miR-21-5p and miR-29a/b/c were increased in DKD progressors (sCR changes) *vs*. nonprogressors
Chien, 2016[b] (51)	T2DM patients: 18 low eGFR (< 60ml/min) and 32 high eGFR (≥ 60ml/min)	eGFR	Serum	qPCR	1	0	miR-21-5p was upregulated in the low eGFR group
Conserva, 2014*	8 patients with DKD (proteinuria / low eGFR); 4 healthy subjects	Proteinuria / eGFR / biopsy	Kidney	Microarray / qPCR	18	0	These miRNAs target *VEGFA*, *SMAD3*, *BCL2*, and *SULF1*
Delic, 2016 (52)	T2DM patients: 8 DKD (eGFR <60ml/min), and 8 without DKD; 8 healthy subjects	eGFR / UACR	Urinary exosomes	microarray / qPCR	14	2	miR-320c was correlated with eGFR (-) and UACR (+)
Eissa, 2016[a] (53)	T2DM patients: 90 DKD (macro + micro) and 46 N; 44 healthy subjects	UACR	Urinary exosomes / urine pellet	Microarray / qPCR	6	0	miR-15b, miR-34a and miR-636 were correlated with sCR and UACR (+)
Eissa, 2016[b] (21)	T2DM patients: 110 DKD (macro + micro) and 56 N; 54 healthy subjects	UACR	Urinary exosomes	qPCR	3	0	miR-133b, miR-30a and miR-342 were correlated with UACR (+), sCR (+) and eGFR (-)

Fiorentino, 2013 (54)	8 patients with DKD; 4 healthy subjects	Proteinuria / eGFR / biopsy	Kidney	qPCR	1	0	miR-21 was increased in DKD patients
Huang, 2014 (55)	6 T2DM patients with DKD; 3 healthy subjects	Biopsy	Kidney	Microarray / qPCR	2	0	MiR-155 expression was correlated with sCR (+)
Kolling, 2017 (56)	26 DKD patients (low eGFR / proteinuria); 20 healthy subjects	Biopsy	Serum / urine / kidney	qPCR	1	0	miR-21 was correlated with chronic tubulointerstitial injury and proteinuria (+)
Jia, 2016 (28)	T2DM patients: 50 DKD (micro + macro), and 30 N; 10 healthy subjects	UACR	Urinary EV	qPCR	3 (micro)	3 (macro)	≅ miR-192, miR-194 and miR-215 expressions across DKD stages
Liu, 2014 (57)	T2DM patients: 92 DKD (macro) and 86 N; 85 healthy subjects	Overt DR /UAE	Urine	qPCR	1	0	↓ miR-126 after glycemic control treatment in DKD patients
Lv, 2015 (58)	T2DM patients: 190 DKD (micro + macro) and 137 N; 130 healthy subjects	UACR	Serum	qPCR	0	1	miR-130b was correlated with UAE, sCR, and HbA1c (-)
Ma, 2016 (59)	T2DM patients: 307 DKD (micro + macro) and 157 N; 127 healthy subjects	UACR	Serum	qPCR	0	1	miR-192 was correlated with UACR and *TGF-β1* (-)
McClelland, 2015 (60)	35 T2DM with DKD; 8 healthy subjects	Biopsy	Kidney	qPCR	1	0	miR-21 was correlated with eGFR (-), and proteinuria and glomerulosclerosis (+)
Peng, 2013 (61)	T2DM patients: 42 DKD (micro + macro) and 41 N	UACR	Urine	qPCR	1	0	miR-29a was correlated with UAE (+)

Peng, 2015 (62)	T2DM patients: 20 DKD (micro) and 20 N; 20 healthy subjects	UAE	Plasma	qPCR	0	1	↑ methylation in the let-7a-3 promoter in the DKD group *vs*. the other groups
Pezzolesi, 2015 (20)	T1DM patients: DKD with proteinuria (38 fast-progressors to ESRD and 38 nonprogressors) and 40 N patients	UACR and eGFR	Plasma	Multiplex qPCR	3	2	let-7c-5p and miR-29a-3p protected against fast-progression to ESRD; let-7b-5p and miR-21-5p were associated with risk of ESRD
Shao, 2016 (63)	T2DM patients: 309 DKD (micro + macro; eGFR > 60ml/min) and 186 N; 195 healthy subjects	UACR	Serum	qPCR	1	0	mir-217 was correlated with UACR and sCR (+), and was ≠ across DKD stages
Wang, 2013 (64)	20 patients with DGS and 10 healthy subjects	Biopsy and eGFR	Urine	qPCR	0	3	miR-192 was correlated with proteinuria (-), and miR-638, miR-29 and miR-150 with eGFR (+)
Zhou, 2013 (65)	T2DM patients: 108 DKD (micro), and 108 N; 62 healthy subjects	UAE	Whole blood	Microarray / qPCR	17	5	↓ let-7a in DKD patients, but N patients had higher levels than healthy subjects

ACR: albumin/creatinine ratio; AER: albumin excretion ratio; DKD: diabetic kidney disease; DR: diabetic retinopathy; eGFR: estimated glomerular filtration rate; ESRD: end-stage renal disease; EV: extracellular vesicles; micro: microalbuminuric; macro: macroalbuminuric; N: normoalbuminuric; q-PCR: quantitative polymerase chain reaction; sCR: serum creatinine; SPK: simultaneous pancreas-kidney transplantation; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; UACR: urinary albumin/creatinine ratio; UAE: urinary albumin excretion. *Abstract from Congress.

Table 2. MiRNAs differentially expressed in DKD patients and analyzed in at least three studies.

miRNA	First author, year	Tissue	Change of expression	Comparison groups	DM type
miR-21-5p	Baker *et al.*, 2017[a]	Glomeruli	Up	DKD *vs.* healthy subjects	DM
	Chien *et al.*, 2016[a]	Serum	Up	Overt DKD (proteinuric) > micro and N	T2DM
	Chien *et al.*, 2016[b]	Serum	Up	eGFR <60ml/min *vs.* eGFR >60ml/min	T2DM
	Fiorentino *et al.*, 2013	Kidney	Up	DKD (proteinuric / low eGFR) *vs.* healthy controls	DM
	Kolling *et al.*, 2017	Serum	Up	DKD (proteinuric / low eGFR) *vs.* healthy controls	DM
	McClelland *et al.*, 2015	Kidney	Up	Fast-progressors to dialysis *vs.* slow-progressors; late DKD *vs.* healthy controls	T2DM
	Pezzolesi *et al.*, 2015	Plasma	Up	Fast-progressors to ESRD *vs.* nonprogressors	T1DM
miR-29a-3p	Baker *et al.*, 2017[a]	Glomeruli	Up	DKD *vs.* healthy subjects	DM
	Chien *et al.*, 2016[a]	Serum	Up	Overt DKD (proteinuric) *vs.* N	T2DM
	Conserva *et al.*, 2014	Kidney	Up	DKD (proteinuric / low eGFR) *vs.* healthy controls	DM
	Peng *et al.*, 2013	Urine	Up	DKD (micro + macro) *vs.* N	T2DM
	Pezzolesi *et al.*, 2015	Plasma	Down	Fast-progressors to ESRD *vs.* nonprogressors	T1DM
miR-30a-5p	Argyropoulos *et al.*, 2015	Urine	Down	DKD (micro) *vs.* N	T1DM
	Baker *et al.*, 2017[a]	Glomeruli	Down	DKD *vs.* healthy subjects	DM
	Baker *et al.*, 2017[b]	Proximal tubules	Down	DKD *vs.* healthy subjects	DM
	Cardenas-Gonzalez *et al.*, 2017	Urine	Up	DKD *vs.* healthy controls	DM
	Eissa *et al.*, 2016[a]	Urine / urinary exosomes	Up	DKD (micro + macro) *vs.* N and healthy controls	T2DM
	Eissa *et al.*, 2016[b]	Urinary exosomes	Up	DKD (micro + macro) *vs.* N and healthy controls	T2DM

miR-30c-5p	Zhou *et al.*, 2013	Whole Blood	Up	DKD (micro) *vs.* N and healthy subjects	T2DM
	Cardenas-Gonzalez *et al.*, 2017	Urine	Up	DKD *vs.* healthy controls	DM
	Baker *et al.*, 2017[a]	Glomeruli	Down	DKD *vs.* healthy subjects	DM
	Baker *et al.*, 2017[b]	Proximal tubules	Down	DKD *vs.* healthy subjects	DM
miR-126-3p	Al-Kafaji *et al.*, 2016	Whole blood	Down	DKD (micro + macro) < N < healthy controls	T2DM
	Argyropoulos *et al.*, 2015	Urine	Up	DKD (micro) *vs.* N	T1DM
	Bijkerk *et al.*, 2015	Plasma	Up	DKD (waiting list for SPK) *vs.* healthy controls	T1DM
	Conserva *et al.*, 2014	Kidney	Up	DKD (proteinuric / low eGFR) *vs.* healthy controls	DM
	Liu *et al.*, 2014	Urine	Up	DKD (macro) *vs.* N and healthy controls	T2DM
miR-130b-3p	Bai *et al.*, 2016	Plasma	Down	DKD (proteinuric) *vs.* healthy controls	T2DM
	Bijkerk *et al.*, 2015	Plasma	Up	DKD (waiting list for SPK) *vs.* healthy controls	T1DM
	Lv *et al.*, 2015	Serum	Down	DKD (micro + macro) vs. N and healthy controls	T2DM
miR-148a-3p	Baker *et al.*, 2017 [a]	Glomeruli	Down	DKD *vs.* healthy subjects	DM
	Baker *et al.*, 2017 [b]	Proximal tubules	Down	DKD *vs.* healthy subjects	DM
	Conserva *et al.*, 2014	Kidney	Up	DKD (proteinuric / low eGFR) *vs.* healthy controls	DM
miR-155-5p	Barutta *et al.*, 2013	Urinary exosomes	Down	DKD (micro) *vs.* N and healthy controls	T1DM
	Baker et al., 2017 [b]	Proximal tubules	Up	DKD *vs.* healthy subjects	DM
	Huang *et al.*, 2014	Kidney	Up	DKD *vs.* healthy controls	T2DM
miR-192-5p	Jia *et al.*, 2016	Urinary EV	Up	DKD (micro) *vs.* N and healthy controls	T2DM
	Jia *et al.*, 2016	Urinary EV	Down	DKD (macro) *vs.* DKD (micro)	T2DM
	Ma *et al.*, 2016	Serum	Down	DKD (micro + macro) *vs.* N and healthy controls	T2DM
	Wang *et al.*, 2013	Urine	Down	DGS *vs.* healthy controls	DM
miR-214-3p	Argyropoulos *et al.*, 2013	Urine	Up	DKD (micro) *vs.* N	T1DM

	Baker et al., 2017 [a]	Glomeruli	Up	DKD *vs.* healthy subjects	DM
	Conserva *et al.*, 2014	Kidney	Up	DKD (proteinuric / low eGFR) *vs.* healthy controls	DM
miR-342-3p	Conserva *et al.*, 2014	Kidney	Up	DKD (proteinuric / low eGFR) *vs.* healthy controls	DM
	Eissa *et al.*, 2016[a]	Urine / urinary exosomes	Up	DKD (micro + macro) *vs.* N and healthy controls	T2DM
	Eissa *et al.*, 2016[b]	Urinary exosomes	Up	DKD (micro + macro) *vs.* N and healthy controls	T2DM
miR-486-3p	Argyropoulos *et al.*, 2013	Urine	Up	DKD (macro) *vs*. N	T1DM
	Baker *et al.*, 2017 [a]	Glomeruli	Down	DKD *vs.* healthy subjects	DM
	Baker *et al.*, 2017 [b]	Proximal tubules	Down	DKD *vs.* healthy subjects	DM
miR-486-5p	Zhou *et al.*, 2013	Blood	Up	DKD (micro) *vs.* N and healthy subjects	T2DM
	Baker *et al.*, 2017 [a]	Glomeruli	Down	DKD *vs.* healthy subjects	DM
	Baker *et al.*, 2017 [b]	Proximal tubules	Down	DKD *vs.* healthy subjects	DM
miR-638	Argyropoulos *et al.*, 2013	Urine	Up	DKD (micro) *vs*. N	T1DM
	Delic *et al.*, 2016	Urine	Up	DKD (eGFR <60ml/min) *vs.* diabetic and healthy controls	T2DM
	Wang *et al.*, 2013	Urine	Down	DGS *vs*. healthy controls	DM

DGS: diabetic glomerulosclerosis; eGFR: estimated glomerular filtration rate; ESRD: end-stage renal disease; EV: extracellular vesicles; Macro: macroalbuminuric; Micro: microalbuminuric; N: normoalbuminuric; SPK: simultaneous pancreas-kidney transplantation.

Table 3. Number of putative miRNA-target gene interactions for each consistently dysregulated miRNA in DKD samples.

microRNA ID	Total number of miRNA-target gene interactions	Interactions with experimental evidences*	Interactions obtained using computational prediction**
hsa-miR-126-3p	55	51	4
hsa-miR-192-5p	1144	1024	120
hsa-miR-21-5p	923	725	198
hsa-miR-214-3p	1111	476	635
hsa-miR-29a-3p	1272	810	462
hsa-miR-342-3p	869	433	436

* miRTarbase, starBase and miRecords databases were used to retrieve the experimentally validated data regarding miRNA-target interactions.

** TargetScan, DianaMicroT-CDS and miRanda-mirSVR databases were used for *in silico* analysis with target prediction algorithms.

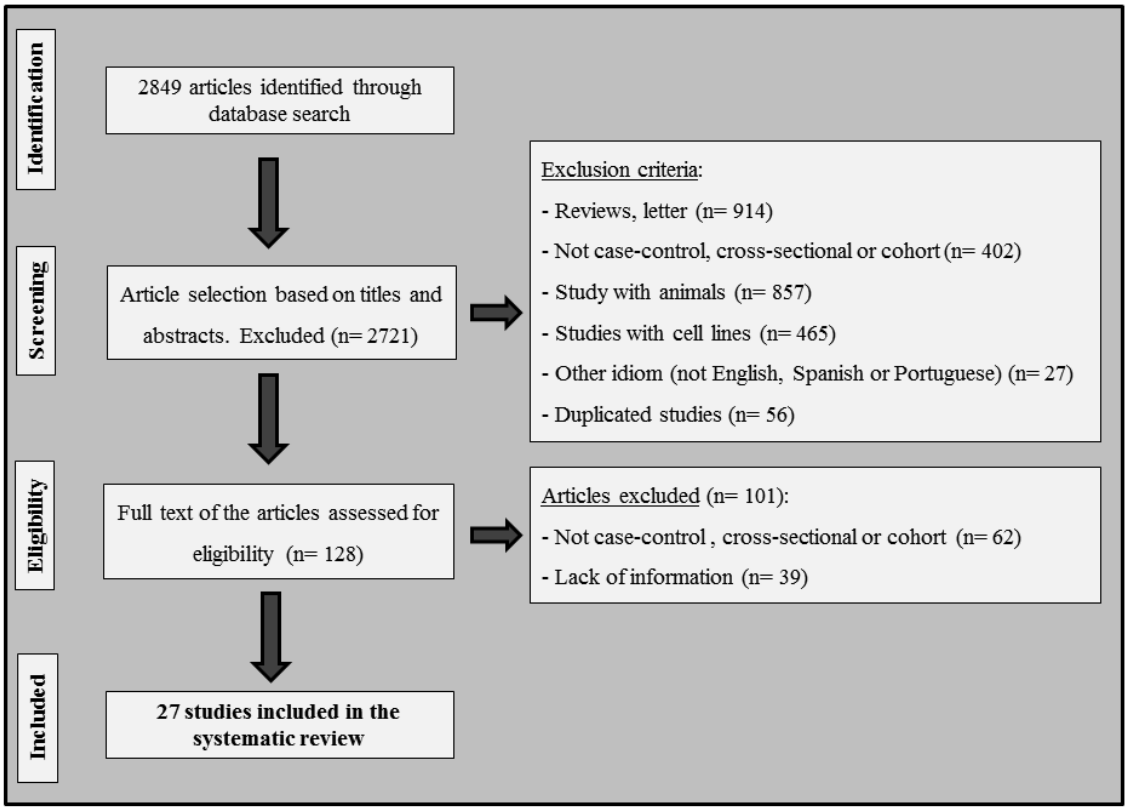
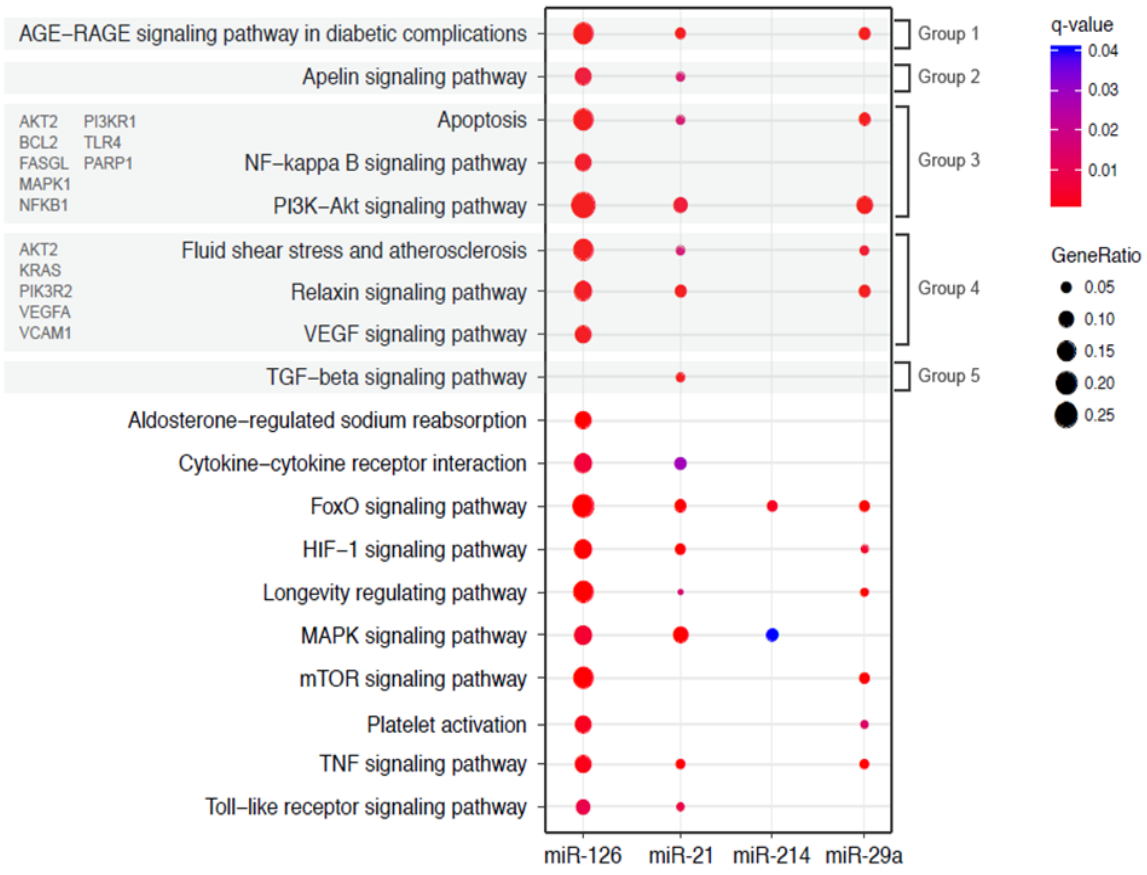


Figure 1. Flowchart illustrating the search strategy used to identify association studies between microRNAs and diabetic kidney disease.



<u>Figure 2</u>. Significant KEGG pathways regulated by the 6 consistently dysregulated miRNAs in patients with diabetic kidney disease. KEGG pathway enrichments are displayed in a scatter diagram, where each point represents the enrichment level; the color corresponds to the q-value, and the size corresponds to the number of genes enriched for each given pathway (GeneRatio). Pathways with a q-value ≤ 0.05 were considered as significantly enriched. The right y-axis represents the KEGG pathways, and the x-axis shows the 4 miRNAs that participated in each selected pathways. MiR-192-5p and miR-342-3p were not significantly enriched in the showed pathways. Pathways were clustered according to their function roles (Group 1 - 5). For group 3 and 4 more than one pathway was clustered, and the shared genes among these pathways are shown.

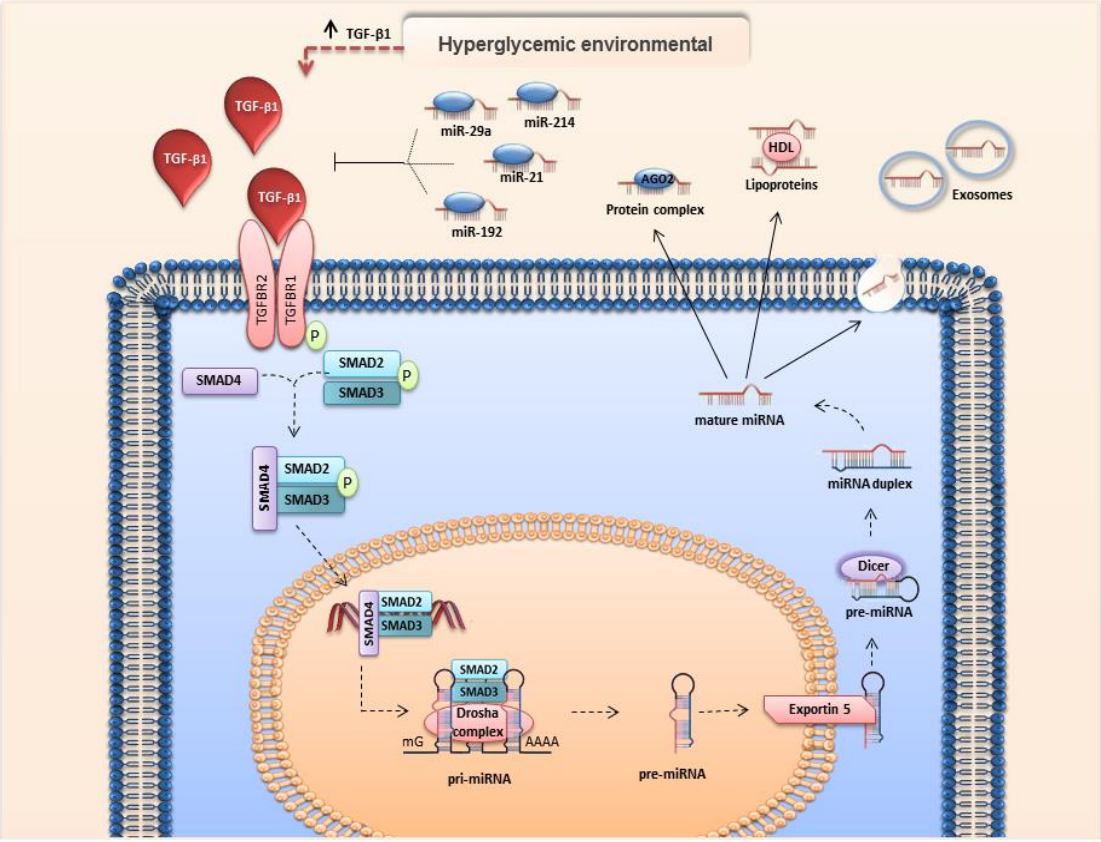


Figure 3. Interactions between TFG-β1/Smad signaling pathway and biogenesis, release and function of miRNAs. TGF-β1/Smad signaling can modulate miRNA expression by regulating the processing of pri-miRNA into pre-miRNA in the nucleus. The Drosha complex cleavages the pri-miRNA into pre-miRNA. Pre-miRNAs are then transported to the cytoplasm, through a process involving Exportin-5, and further cleaved by Dicer to yield 21–23 nts miRNA:miRNA duplexes. One strand of the miRNA:miRNA duplex can associate to the RISC complex and guide translational repression of target mRNAs or be released by the cells in association with RNA-binding proteins, such as Argonaute-2, or lipoproteins, like HDL. Besides that, miRNAs can be loaded in microvesicles formed by plasma membrane or in exosomes that are released in the extracellular space. In the Smad-dependent pathway, TGFBR1 activates Smad2 and Smad3 by phosphorylation, which in turn bind to Smad4 forming a complex that translocates to the nucleus and regulates gene

transcription, including genes related to miRNA biogenesis. In the same way as TGF-β1/Smad signaling can modulate miRNA expressions, some miRNAs, such as miR-21, miR-192, miR-214 and miR-29 families, can modulate TGF-β1 levels. *Abbreviations*: miRNA, microRNA; pre-miRNA, miRNA precursor; P, phosphorylation; pri-miRNA, primary miRNA transcript; RISC, RNA-induced silencing complex; TGF-β1, transforming growth factor-β1; TGFBR1, TGF-β receptor type 1; TGFBR2, TGF-β receptor type 2.

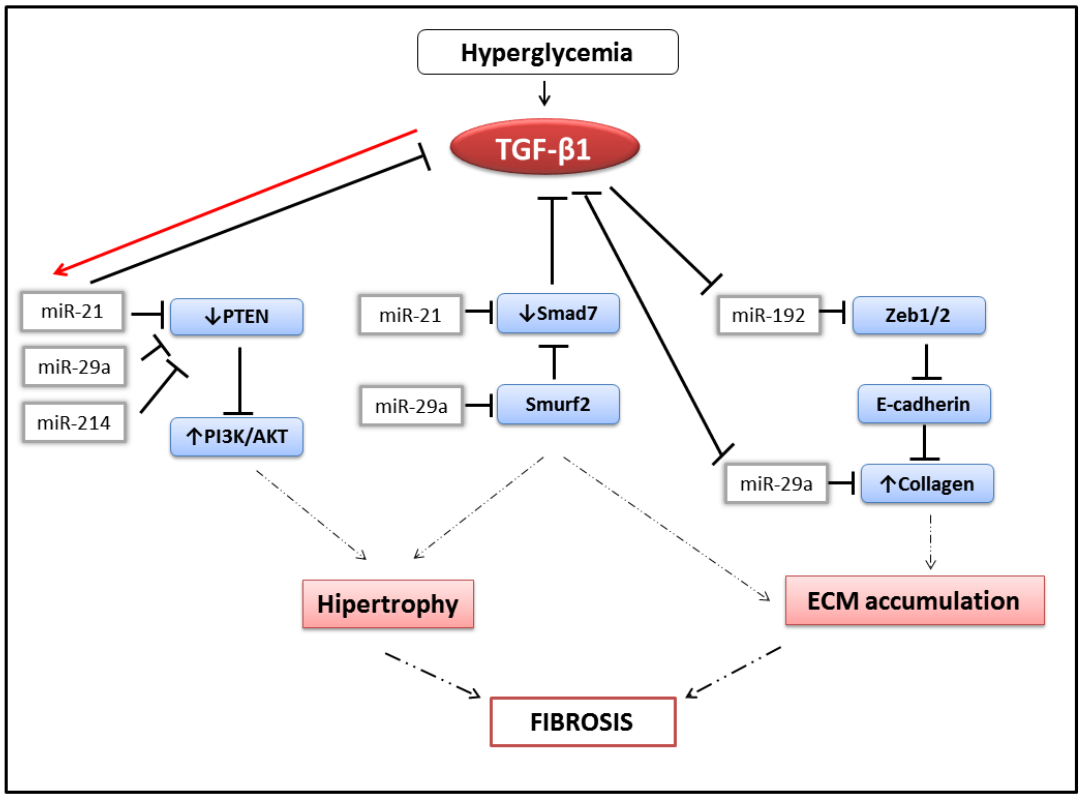


Figure 4. MiRNA-regulatory networks in response to TGF-β1. TFG-β1 induces the expression of several miRNAs and some of them also control TGF-β1 levels. TGF-β1 promotes fibrosis by regulating miR-21, miR-29a and miR-192 expressions during renal injury. MiR-192 and miR-21 play a pathological role in kidney fibrosis through a feed-forward loop that amplifies TGF-β1 signaling and promotes fibrosis. In contrast, miR-29a plays a protective role in renal fibrosis by inhibiting the deposition of extracellular matrix. This miRNA also has a pro-fibrotic role by inhibiting PTEN, consequently increasing PI3K/Akt signaling pathway. Some miRNAs shown in this figure have been directly connected to DKD through specific targets, while the specific details of others are yet to be clearly established.

SUPPLEMENTARY MATERIAL LIST

Supplementary Figure 1. Bioinformatic analysis flowchart.

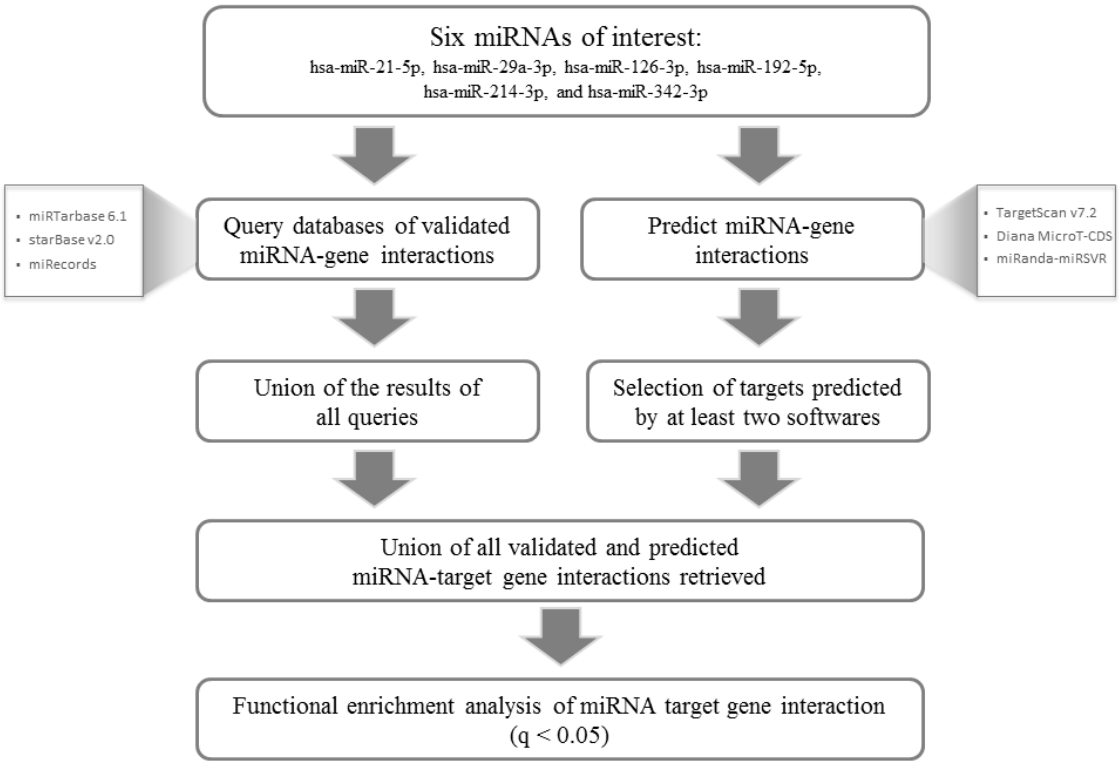
Supplementary Figure 2. Jaccard similarity coefficient. **A)** number of mRNA targets; **B)** number of pathways. The solid lines connecting molecules represent miRNA-mRNA interactions. Node size represents number of targets for each miRNA; edge width denotes overlap between miRNAs measured by the Jaccard similarity coefcient (JC); and nodes are colored based on the differential expression of the miRNAs: green represents the miRNA which is downregulated in patients with DKD and nodes in pink represent the miRNAs which are upregulated in patients with DKD.

Supplementary Table 1. QUADAS-2 quality results for the included studies.

Supplementary Table 2. MiRNAs analyzed in all included studies.

Supplementary Table 3. MiRNA-target gene interactions obtained for the 6 miRNAs consistently dysregulated in DKD patients. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 4. Results of pathway analyses for each consistently dysregulated miRNAs found in the systematic review. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).



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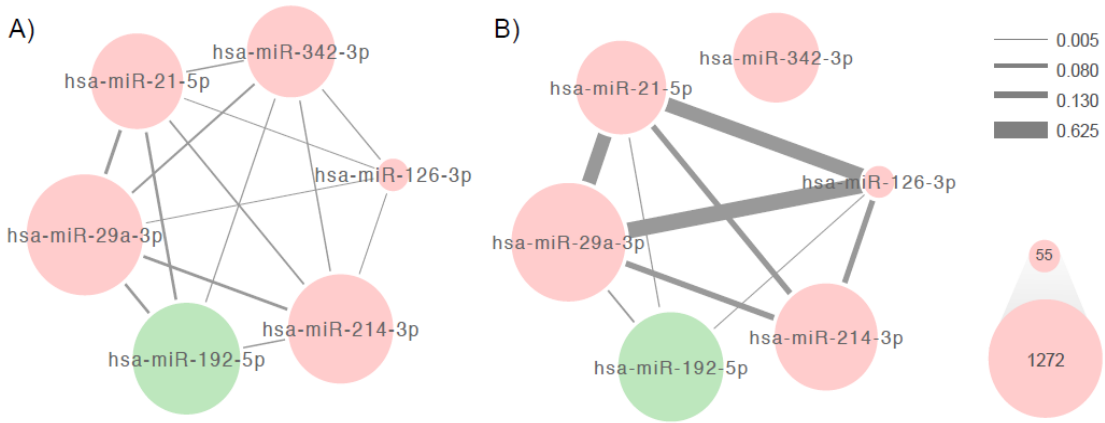

Supplementary Figure 2. Jaccard similarity coefficient. **A)** number of mRNA targets; **B)** number of pathways. The solid lines connecting molecules represent miRNA-mRNA interactions. Node size represents number of targets for each miRNA; edge width denotes overlap between miRNAs measured by the Jaccard similarity coefcient (JC); and nodes are colored based on the differential expression of the miRNAs: green represents the miRNA which is downregulated in patients with DKD and nodes in pink represent the miRNAs which are upregulated in patients with DKD.

Supplementary table 1. QUADAS-2 quality results.

Study	Risk of Bias				Applicability concerns			Total score
	Patient Selection	Index Test	Reference Standard	Flow and timing	Patient Selection	Index Test	Reference Standard	
Al-Kafaji *et al*., 2016	☺	☺	☺	☹	☺	☺	?	5.5
Argyropoulos *et al*, 2013	☺	☺	☺	☺	☺	☺	?	6.5
Argyropoulos *et al*, 2015	☺	☺	☺	☺	☺	☺	?	6.5
Bai *et al*, 2016	☺	☺	☺	☺	☺	☺	☺	7.0
Baker *et al*, 2017	☺	☺	☺	☹	☺	☺	☺	6.0
Barutta *et al*, 2013	☺	☺	☺	☹	☺	☺	?	5.5
Bijkerk *et al*, 2015	☺	☺	☺	☹	☺	☺	?	5.5
Cardenas-Gonzalez *et al*, 2017	☺	☺	☺	☺	☺	☺	?	6.5
Chien et al., 2016	☺	☺	☺	☹	☺	☺	?	5.5
Conserva *et al*, 2014	☺	☺	☺	☹	?	?	?	4.5
Delic *et al*, 2016	☺	☺	☺	☹	☺	☺	?	5.5
Eissa *et al*, 2016[a]	☺	☺	☺	☺	☺	☺	?	6.5
Eissa *et al*, 2016[b]	☺	☺	☺	☺	☺	☺	?	6.5

Fiorentino *et al*, 2013	☺	☺	☺	☹	☺	☺	☺	6.0
Huang *et al*, 2014	?	☺	☺	☹	☺	☺	☺	5.5
Kolling *et al*, 2017	☺	☺	☺	☺	☺	☺	☺	7.0
Jia *et al*, 2016	☺	☺	☺	☹	☺	☺	?	5.5
Liu *et al*, 2014	☺	☺	☺	☹	☺	☺	?	5.5
Lv *et al*, 2015	?	☺	☺	☹	☺	☺	☺	5.5
Ma *et al*, 2016	☺	☺	☺	☹	☺	☺	☺	6.0
McClelland *et al*, 2015	?	☺	☺	☹	☺	☺	☺	5.5
Peng *et al*, 2013	☺	☺	☺	☹	☺	☺	?	5.5
Peng *et al*, 2015	☺	☺	☺	☹	☺	☺	?	5.5
Pezzolesi *et al*, 2015	☺	☺	☺	☺	☺	☺	?	6.5
Shao *et al*, 2016	☺	☺	☺	☹	☺	☺	?	5.5
Wang *et al*, 2013	?	☺	☺	☹	?	☺	☺	5.0
Zhou *et al*., 2013	☺	☺	☺	☹	☺	☺	?	5.5

☺: low risk (yes; scores 1); ☹: high risk (no; scores 0); ?: unclear (scores 0.5). Scores range from 0 to 7, with studies being classified as having “good quality” (scores 6-7), “fair” (scores 4-5), or “poor quality (scores <3).

Supplementary Table 2. miRNAs analyzed in all studies included in the systematic review.

miRNA identity	First author, year	Tissue	Change of expression
miR-320a	Zhou, 2013	Blood	Up
let-7a-5p	Zhou, 2013	Blood	Down
let-7a-2	Peng, 2015	Plasma	Down
let-7a-3	Peng, 2015	Plasma	Down
let-7a-5p	Argyropoulos, 2015	Urine	Down
let-7b-3p	Argyropoulos, 2015	Urine	Up
let-7b-5p	Pezzolezi, 2015	Plasma	Up
	Baker, 2017	Proximal tubules	Down
let-7c-5p	Pezzolezi, 2015	Plasma	Down
let-7d-5p	Zhou, 2013	Blood	Down
let-7f-5p	Zhou, 2013	Blood	Down
miR-10a-5p	Cardenas-Gonzalez, 2017	Urine	Up
miR-100-5p	Cardenas-Gonzalez, 2017	Urine	Up
miR-101-3p	Conserva,	Kidney biopsy	Up
miR-106b-5p	Conserva, 2014	Kidney biopsy	Up
miR-1180-3p	Baker, 2017	Glomeruli	Down
	Cardenas-Gonzalez, 2017	Urine	Up
miR-1224-3p	Argyropoulos, 2013	Urine	Up
miR-122-5p	Argyropoulos, 2015	Urine	Up
miR-1227-5p	Delic, 2016	Urine	Up
miR-1234-5p	Delic, 2016	Urine	Up
miR-1247-5p	Argyropoulos, 2015	Urine	Down
miR-125b-5p	Baker, 2017	Proximal tubules	Up
miR-126-3p	Bijkert, 2015	Plasma	Up
	Bijkert, 2015	Plasma	Down
	Liu, 2014	Urine	Up
	Conserva, 2014	Kidney biopsy	Up
	Al-Kafaji , 2016	Blood	Down
	Argyropoulos, 2015	Urine	Up
miR-130a-3p	Barutta, 2013	Urinary exosome	Up
miR-130b-3p	Bijkert, 2015	Plasma	Up
	Lv, 2015	Serum	Down
	Bai, 2016	Plasma	Down
miR-132-3p	Bijkert, 2015	Plasma	Up
miR-133b	Eissa [a], 2016	Urine/Urinary exosomes	Up
	Eissa [b], 2016	Urinary exosome	Up
miR-140-5p	Conserva, 2014	Kidney biopsy	Up
miR-141-3p	Conserva, 2014	Kidney biopsy	Up
	Argyropoulos, 2013	Urine	Up
miR-145-5p	Barutta, 2013	Urinary exossome	Up
miR-146a-5p	Huang, 2014	Kidney biopsy	Up
	Conserva, 2014	Kidney biopsy	Up

miR-148a-3p	Conserva, 2014	Kidney biopsy	Up
	Baker, 2017	Glomeruli	Down
	Baker, 2017	Proximal tubules	Down
miR-150-5p	Conserva, 2014	Kidney biopsy	Up
	Baker, 2017	Glomeruli	Up
miR-152	Bijkert, 2015	Plasma	Up
miR-155-5p	Barutta, 2013	Urinary exossome	Down
	Baker, 2017	Proximal tubules	Up
	Huang, 2014	Kidney biopsy	Up
miR-15a-5p	Conserva, 2014	Kidney biopsy	Up
miR-15b-5p	Eissa [a], 2016	Urine/Urinary exosomes	Up
miR-16-5p	Conserva, 2014	Kidney biopsy	Up
	Baker, 2017	Proximal tubules	Down
miR-17-5p	Argyropoulos, 2013	Urine	Up
miR-17-5p	Argyropoulos, 2015	Urine	Down
miR-181a-5p	Bijkert, 2015	Plasma	Up
miR-181b-5p	Baker, 2017	Proximal tubules	Down
miR-185-5p	Conserva, 2014	Kidney biopsy	Up
miR-186-5p	Conserva, 2014	Kidney biopsy	Up
miR-188-3p	Argyropoulos, 2013	Urine	Down
miR-191-5p	Zhou, 2013	Blood	Up
miR-1912	Argyropoulos, 2013	Urine	Up
miR-1913	Argyropoulos, 2013	Urine	Up
miR-1915-3p	Zhou, 2013	Blood	Up
	Cardenas-Gonzalez, 2017	Urine	Down
miR-1915-5p	Delic, 2016	Urine	Up
miR-192-5p	Wang, 2013	Urine	Down
	Jia, 2016	Urinary exosome	Up
	Jia, 2016	Urinary exosome	Down
	Ma, 2016	Serum	Down
miR-195-5p	Jia, 2016	Urinary exosome	Up
	Jia, 2016	Urinary exosome	Down
miR-196a-5p	Baker, 2017	Proximal tubules	Down
miR-198	Wang, 2013	Urine	Down
miR-199a-3p	Argyropoulos, 2015	Urine	Up
miR-19a-3p	Conserva, 2014	Kidney biopsy	Up
miR-21-5p	Fiorentino, 2013	Kidney biopsy	Up
	McClelland [a], 2015	Kidney biopsy	Up
	Pezzolezi, 2015	Plasma	Up
	Chien, 2016	Serum	Up
	Baker, 2017	Glomeruli	Up
	Kolling, 2017	Plasma	Up
miR-214-3p	Conserva, 2014	Kidney biopsy	Up
	Baker, 2017	Glomeruli	Up
	Argyropoulos, 2013	Urine	Up

miR-214-5p	Argyropoulos, 2013	Urine	Up
miR-215	Jia, 2016	Urinary exosome	Up
	Jia, 2016	Urinary exosome	Down
miR-217	Shao, 2016	Serum	Up
miR-221-3p	Argyropoulos, 2013	Urine	Down
miR-222-3p	Argyropoulos, 2013	Urine	Up
miR-223-3p	Bijkert, 2015	Plasma	Down
miR-23a-3p	Baker, 2017	Glomeruli	Up
miR-25-3p	Bijkert, 2015	Plasma	Up
miR-26a-5p	Zhou, 2013	Blood	Down
miR-27a-3p	Bijkert, 2015	Plasma	Up
miR-2861	Delic, 2016	Urine	Up
	Cardenas-Gonzalez, 2017	Urine	Down
miR-29a-3p	Chien, 2016	Serum	Up
	Conserva, 2014	Kidney biopsy	Up
	Peng, 2013	Urine	Up
	Baker, 2017	Glomeruli	Up
	Pezzolezi, 2015	Plasma	Down
miR-29b-3p	Wang, 2013	Urine	Down
miR-29b-1-5p	Argyropoulos, 2013	Urine	Up
miR-29c-3p	Pezzolezi, 2015	Plasma	Down
miR-301a-3p	Conserva, 2014	Kidney biopsy	Up
miR-302a-3p	Argyropoulos, 2015	Urine	Up
miR-30a-3p	Baker, 2017	Glomeruli	Down
miR-30a-5p	Eissa [a], 2016	Urine/Urinary exosomes	Up
	Eissa [b], 2016	Urinary exosome	Up
	Baker, 2017	Proximal tubules	Down
	Baker, 2017	Glomeruli	Down
	Cardenas-Gonzalez, 2017	Urine	Up
	Argyropoulos, 2015	Urine	Down
miR-30c-5p	Zhou, 2013	Blood	Up
	Cardenas-Gonzalez, 2017	Urine	Up
	Baker, 2017	Glomeruli	Down
	Baker, 2017	Proximal tubules	Down
miR-30d-5p	Delic, 2016	Urine	Down
	Cardenas-Gonzalez, 2017	Urine	Up
miR-30e-5p	Delic, 2016	Urine	Down
	Cardenas-Gonzalez, 2017	Urine	Up
miR-3184-3p	Baker, 2017	Glomeruli	Down
miR-320	Bijkert, 2015	Plasma	Up
miR-320b	Zhou, 2013	Blood	Up
miR-320c	Delic, 2016	Urine	Up
	Zhou, 2013	Blood	Up
miR-320d	Zhou, 2013	Blood	Up
miR-320e	Zhou, 2013	Blood	Up

miR-323b-5p	Argyropoulos, 2013	Urine	Down
miR-326	Bijkert, 2015	Plasma	Up
miR-335-3p	Argyropoulos, 2013	Urine	Up
miR-340	Bijkert, 2015	Plasma	Up
miR-342-3p	Conserva, 2014	Kidney biopsy	Up
	Eissa [a], 2016	Urine/Urinary exosomes	Up
	Eissa [b], 2016	Urinary exosome	Up
miR-34a	Eissa [a], 2016	Urine	Up
miR-363	Zhou, 2013	Blood	Down
miR-3665	Zhou, 2013	Blood	Up
miR-371b-3p	Delic, 2016	Urine	Up
miR-373-3p	Argyropoulos, 2013	Urine	Up
miR-375	Wang, 2013	Urine	Down
miR-377	Conserva, 2014	Kidney biopsy	Up
miR-3940-5p	Zhou, 2013	Blood	Up
miR-3960	Zhou, 2013	Blood	Up
miR-420	Argyropoulos, 2013	Urine	Up
miR-423-5p	Baker, 2017	Glomeruli	Down
miR-424-5p	Barutta, 2013	Urinary exossome	Down
	Argyropoulos, 2013	Urine	Up
miR-4270	Delic, 2016	Urine	Up
miR-429	Conserva, 2014	Kidney biopsy	Up
	Argyropoulos, 2013	Urine	Up
miR-4286	Baker, 2017	Glomeruli	Up
miR-4301	Baker, 2017	Glomeruli	Down
	Cardenas-Gonzalez, 2017	Urine	Up
miR-433	Argyropoulos, 2013	Urine	Up
miR-4429	Zhou, 2013	Blood	Up
miR-4454	Zhou, 2013	Blood	Up
miR-4466	Zhou, 2013	Blood	Up
miR-4488	Zhou, 2013	Blood	Up
miR-451a	Baker, 2017	Proximal tubules	Down
miR-453	Argyropoulos, 2013	Urine	Down
miR-4532	Cardenas-Gonzalez, 2017	Urine	Down
miR-4536-3p	Cardenas-Gonzalez, 2017	Urine	Up
miR-4707-5p	Zhou, 2013	Blood	Up
miR-4739	Delic, 2016	Urine	Up
miR-4778-5p	Delic, 2016	Urine	Up
miR-486-3p	Argyropoulos, 2013	Urine	Up
	Baker, 2017	Glomeruli	Down
	Baker, 2017	Proximal tubules	Down
miR-486-5p	Zhou, 2013	Blood	Up
	Baker, 2017	Glomeruli	Down
	Baker, 2017	Proximal tubules	Down

miR-495	Argyropoulos, 2015	Urine	Down
miR-520h	Argyropoulos, 2013	Urine	Down
miR-524-5p	Argyropoulos, 2013	Urine	Down
miR-548c-3p	Argyropoulos, 2015	Urine	Down
miR-548o-3p	Argyropoulos, 2015	Urine	Down
miR-552	Argyropoulos, 2013	Urine	Up
miR-5585-3p	Baker, 2017	Glomeruli	Up
miR-589-5p	Argyropoulos, 2013	Urine	Down
	Baker, 2017	Glomeruli	Up
miR-572	Delic, 2016	Urine	Up
miR-574-3p	Bijkert, 2015	Plasma	Down
miR-6068	Delic, 2016	Urine	Up
miR-6126	Delic, 2016	Urine	Up
miR-6133	Delic, 2016	Urine	Up
miR-616-5p	Argyropoulos, 2015	Urine	Up
miR-619	Argyropoulos, 2013	Urine	Up
miR-628-5p	Argyropoulos et al, 2013	Urine	Up
miR-636	Eissa [a], 2016	Urine/Urinary exosomes	Up
miR-638	Argyropoulos, 2013	Urine	Up
	Delic, 2016	Urine	Up
	Wang, 2013	Urine	Down
miR-640	Argyropoulos, 2015	Urine	Up
miR-645	Argyropoulos, 2015	Urine	Up
miR-660	Bijkert, 2015	Plasma	Up
miR-665	Argyropoulos, 2015	Urine	Down
miR-6747	Cardenas-Gonzalez, 2017	Urine	Up
miR-6839	Cardenas-Gonzalez, 2017	Urine	Up
miR-6842-3p	Cardenas-Gonzalez, 2017	Urine	Up
miR-765	Argyropoulos, 2013	Urine	Up
miR-767-3p	Argyropoulos, 2015	Urine	Down
miR-770-5p	Argyropoulos, 2015	Urine	Up
miR-98-5p	Baker, 2017	Proximal tubules	Down
miR-92a-3p	Argyropoulos, 2013	Urine	Up
miR-92b-3p	Argyropoulos, 2013	Urine	Up

ARTIGO 5

Circulating microRNA expression profile associated with diabetic kidney disease: case-control and *in silico* analysis

Circulating miRNAs in diabetic kidney disease: case-control study and *in silico* analyses

Taís S. Assmann[1,2], Mariana Recamonde-Mendoza[3,4], Aline R. Costa[1], Márcia Puñales[5], Balduíno Tschiedel[5], Luís H. Canani[1,2], Andrea C. Bauer[1,2] and Daisy Crispim*[1,2].

1- Endocrine Division, Hospital de Clínicas de Porto Alegre. Porto Alegre, Rio Grande do Sul, Brazil.

2- Postgraduation Program in Endocrinology, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

3- Institute of Informatics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

4- Experimental Research Center, Hospital de Clínicas de Porto Alegre. Porto Alegre, Rio Grande do Sul, Brazil.

5- Instituto da Criança com Diabetes, Hospital Nossa Senhora da Conceição, Porto Alegre, Rio Grande do Sul, Brazil.

* Correspondence to: Dr. Daisy Crispim. Ramiro Barcelos Street 2350; building 12; 4° floor. Zip Code 90035-003. Porto Alegre, Rio Grande do Sul, Brazil. E-mail: dcmoreira@hcpa.edu.br

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ABSTRACT

Background: Diabetic kidney disease (DKD) is the major cause of end-stage renal disease, contributing to increased morbidity and mortality among diabetic patients. Recent studies have shown that microRNAs (miRNAs) play a key role in DKD pathogenesis; however, the identification of a specific miRNA profile involved in this diabetic complication remains to be achieved.

Aims: To investigate a miRNA expression profile in patients with type 1 diabetes mellitus (T1DM) with DKD (cases) or without this complication (controls), and to perform bioinformatic analysis to evaluate in which pathways these miRNAs are involved.

Methods: Expressions of 48 miRNAs were investigated in plasma of 58 T1DM patients (23 controls, 18 with moderate DKD, and 17 with severe DKD) and 10 healthy subjects using Stem-loop RT-PreAmp Real-time PCR and TaqMan Low Density Array cards (Thermo Fisher Scientific). Five dysregulated miRNAs were then selected for validation in an independent sample of 10 T1DM controls, 19 DKD patients and 10 healthy subjects, using RT-qPCR. Bioinformatic analyses were done to explore the putative target genes and biological pathways regulated by these miRNAs.

Results: Nine miRNAs were differentially expressed between DKD cases and T1DM controls. After validation, miR-21-3p and miR-378-3p were confirmed to be upregulated in patients with severe DKD; while miR-16-5p and miR-29a-3p were downregulated in this group of patients compared to T1DM controls. Our bioinformatic analyses indicate that these 4 miRNAs regulate genes from PI3K/Akt, longevity, fluid shear stress and atherosclerosis, AGE-RAGE, TGF-β1, and relaxin signaling pathways.

Conclusions: Our study indicates that 4 miRNAs are differently expressed in severe DKD patients, providing information about the biological pathways in which they are involved.

Keywords: microRNAs, diabetic kidney disease, bioinformatics, target prediction

INTRODUCTION

Diabetic kidney disease (DKD) is a common microvascular complication that occurs in approximately 40% of patients with diabetes mellitus (DM), and it is caused by dysfunction of various cell types of the kidneys, ultimately leading to end-stage renal disease (ESRD) (1-3). This complication is clinically characterized by albuminuria and a progressive decline in the glomerular filtration rate (GFR) (1, 4). Key pathological alterations of DKD include glomerular hypertrophy, mesangial expansion and tubulointerstitial fibrosis, caused by accumulation of extracellular matrix (ECM) proteins, thickening of basement membrane, and podocyte apoptosis and foot process effacement (5).

Usually, DKD is the cumulative result of inadequate metabolic control over many years of DM; hence, patients with chronic hyperglycemia have a greater risk for developing this complication than those with a tight glycemic control (6, 7). However, it has not yet been completely understood why some patients with intensive treatment and dedicated compliance still develop DKD, while others do not develop it despite poor glycemic control (6). In addition, the predictive power of the laboratorial tests currently used for DKD diagnosis is apparently not sufficient for reliable identification of which patients are at high risk of developing DKD or progress to ESRD (3, 8). Therefore, new biomarkers and pathological mechanisms need to be identified for more effective diagnosis, prognosis and treatment of DKD patients.

MicroRNAs (miRNAs) are a class of short noncoding RNAs that regulate expression of more than 60% of protein-coding genes by pairing to the 3′ untranslated regions (3′ UTR) of their target mRNAs, thus inducing their degradation or translational repression (5, 9, 10). Recent evidence has shown that miRNAs can be transported between cells as well as circulate as stable molecules in body fluids; constituting a new

cell-to-cell communication mode (11, 12). Moreover, miRNA expression profiles in body fluids usually reflect a tissue specific injury (13), being associated with the development of various diseases (11, 14-16). Therefore, circulating miRNAs are ideal noninvasive biomarkers of health status and disease progression because they are stable in body fluids, and can be detected using stablished techniques for quantification, such as quantitative PCR (RT-qPCR) (11, 17, 18).

The association between miRNAs and kidney dysfunction was initially suggested by experimental studies showing that podocyte-specific knockout of Dicer, an enzyme required for the production of mature miRNAs, caused proteinuria, podocyte foot effacement and apoptosis, glomerulosclerosis and tubulointerstitial fibrosis (19-21). Hyperglycemia is known to induce expression of several miRNAs in renal cells both *in vivo* and *in vitro*, which promotes the accumulation of ECM proteins related to fibrosis and glomerular dysfunction (22). Accordingly, over the past few years a number of circulating or urinary miRNAs have been reported as being dysregulated in specific stages of DKD (22-27); however, results are still inconclusive.

Therefore, taking into account that miRNAs might be biomarkers of DKD, the aim of this study was to investigate a circulating miRNA expression profile in plasma of T1DM patients with different stages of DKD. Additionally, we carried out bioinformatic analyses to investigate the putative targets and biological pathways under regulation of the miRNAs potentially associated to DKD.

METHODS

Study population and phenotype measurements

This case-control study was designed in accordance with STROBE guidelines (28), and included two independent samples of T1DM patients with DKD (cases) and without this complication (T1DM controls), called "screening sample" and "validation sample". The screening sample comprised 23 T1DM controls and 35 patients with DKD (18 with moderate DKD and 17 with severe DKD). The validation sample included 10 T1DM controls and 19 DKD cases (10 with moderate DKD and 9 with severe DKD). Additionally, a group of 10 healthy subjects was included in both screening and validation samples.

All T1DM patients included in the study were from the outpatient clinic at Hospital de Clínicas de Porto Alegre or Instituto da Criança com Diabetes at Grupo Hospitalar Conceição (Rio Grande do Sul, Brazil), and were recruited between August 2014 and September 2016. DKD was defined following the Kidney Disease Improving Global Outcomes (KDIGO) guidelines (29). T1DM patients were then divided into 3 groups according to their renal function: 1) patients without DKD and ≥10 years of T1DM diagnosis [urinary excretion of albumin (UEA) < 30mg/g and estimated GFR (eGFR) ≥ 60 ml/min/1.73 m²; **T1DM control**]; 2) patients with moderate DKD (UEA 30-300mg/g and/or eGFR 30-59 ml/min/1.73 m²); and 3) patients with severe DKD (UEA > 300 mg/g and/or eGFR 1-29 ml/min/1.73 m²). Therefore, the **case group** was constituted by patients having moderate or severe DKD. The inclusion criteria was age 18–40 years, and T1DM diagnosis was based on the American Diabetes Association criteria (30). Exclusion criteria for cases were any febrile illness during the last 3 months, chronic inflammatory or rheumatic diseases, hepatitis, HIV-positivity,

glucocorticoid treatment, liver or cardiac failure, kidney transplantation, hereditary dyslipidemia, and inborn or acquired errors of metabolism excepting DM.

In addition, nondiabetic groups in the screening and validation samples consisted of age- and gender-matched healthy blood donors recruited between August 2014 and September 2016. Exclusion criteria for these groups were: presence of alcoholism, obesity, nondiabetic chronic kidney disease (CKD), hypertension, smoking habits, family history of DM, occurrence of infections or inflammatory diseases, and glycated hemoglobin (HbA1c) $\geq$ 5.7%.

A standard questionnaire was used to collect information on age, age at diagnosis, T1DM duration, and drug treatment. The ethnic group was defined based on self-classification, and only white subjects were included in the study. All patients underwent physical and laboratory evaluations, as previous described (31). Serum creatinine was measured by the Jaffé reaction and UAE by immunoturbidimetry (Sera-Pak immuno microalbuminuria, Bayer, Tarrytown, NY, USA; mean intra and interassay coefficients of variance of 4.5% and 11% respectively) (32). Patients interrupted the use of angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists for at least one week before having their UAE measured. Estimated GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (33).

The study protocol was approved by Ethic Committees in Research from Hospital de Clínicas de Porto Alegre and Grupo Hospitalar Conceição/Instituto da Criança com Diabetes, and all patients gave their informed consent in writing.

RNA extraction

Peripheral blood samples of all subjects were collected at least 8 h after the last meal, always during the morning. After collection, blood samples were checked for

hemolysis; then, non-hemolyzed samples were centrifuged at 3500 rpm for 15 min at 4°C, and plasma aliquots were stored at -80°C until relative quantification of miRNA expressions.

Total RNA was extracted from 450 µl of plasma using the MiRVana PARIS miRNA Isolation Kit (Ambion, Thermo Fisher Scientific, DE, USA), following the manufacturer's instructions. Purity and concentration of RNA samples were evaluated using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Only samples that had acceptable purity ratios (A260/A280 = 1.9 - 2.1) were used for the analyses (34). RNA integrity was checked on agarose gels containing GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

Quantification of miRNA expressions using Custom TaqMan Array miRNA cards

Relative expression of 48 miRNAs was analyzed in plasma of all subjects from the screening sample using Custom TaqMan Array MicroRNA cards (Thermo Fisher Scientific), which contain probes for 45 target miRNAs and for 3 reference genes [*RNU44*, *RNU48* and *small nuclear RNA U6* (*U6snRNA*)], as described in the **Supplementary Table 1**. Selection of these miRNAs was performed by searching in the miRWalk 2.0 database (35) those miRNAs possible associated with DKD and also based on the available literature (36, 37).

Quantification of miRNA expressions were done in 3 separate reactions. First, total RNA (10 ng) was reverse-transcribed into specific miRNAs using the TaqMan miRNA Reverse-Transcription (RT) kit and Multiplex RT Pool Set (Thermo Fisher Scientific), which contains stem-loop RT primers that bind to their complementary miRNAs and initiate the RT reaction. Second, specific cDNAs for the 48 miRNAs of interest were pre-amplified with the TaqMan PreAmp Master Mix 2x kit and Custom

PreAmp Primer Pool (Thermo Fisher Scientific), according to the manufacturer's recommendations. The PreAmp product was then diluted with 175 µl of 0.1x TE buffer (pH 8.0).

Third, RT-qPCR reactions were done in a Vii™ 7 Fast Real-Time PCR System Thermal Cycler (Thermo Fisher Scientific), using the TaqMan Low Density Array (TLDA) block. Reactions were performed using 1.13 µl of the diluted PreAmp product and 56.25 µl of the TaqMan Universal PCR Master Mix II no UNG 2x (Thermo Fisher Scientific), in a total volume of 112.5 µl pipetted into the 384 micro-wells of the array plates by centrifugation. The thermal-cycling conditions were: 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Relative expressions were calculated using the $2^{-\Delta\Delta CT}$ method (38). For all analysis, only the *U6snRNA* was used as the reference gene because it showed the lowest variation among samples. Data are shown as n fold changes in relation to a calibrator sample.

Validation of Custom TaqMan Array miRNA results using individual RT-qPCR

Five miRNAs were chosen for confirmation of their expressions in the validation sample using individual RT-qPCR, accordingly to the MIQE guidelines (34). The criteria for choosing these miRNAs were: 1) those miRNAs with the highest differences in n fold changes between patients with DKD and T1DM controls from the screening sample; and/or 2) number of miRNA targets involved in pathways related to DKD pathogenesis, which was assessed by bioinformatic analysis.

RT-qPCR experiments were performed in two separate reactions: first, total RNA was reverse-transcribed into cDNA; then, cDNA was amplified by RT-qPCR. RT of 2 ng of RNA into cDNA was done using TaqMan miRNA RT kits (Thermo Fisher Scientific) specific for each of the 5 miRNA analyzed. RT-qPCR reactions were

performed in a Vii™ 7 Fast Real-Time PCR System Thermal Cycler, using 0.5 μl TaqMan miRNA Assays 20x (Thermo Fisher Scientific) for target miRNAs or *U6snRNA*, 5 μl TaqMan Universal PCR Master Mix II no UNG 2x, and 1 μl of cDNA (10 ng/μl), in a final volume of 10 μl. Each sample was evaluated in triplicate and a negative control was included in all experiments. Cycling conditions were an initial cycle of 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 90 s. Quantification of the 5 miRNAs were performed using the $2^{-\Delta\Delta CT}$ method and the *U6snRNA* gene as the reference gene (34), and are shown as n fold changes in relation to the calibrator sample.

In silico identification and analysis of putative miRNA target genes

Those dysregulated miRNAs chosen for validation by RT-qPCR were then submitted to bioinformatic analyses to investigate their putative target genes and to find possible biological pathways under their regulation (**Supplementary Figure 1**). For the analysis of putative target genes, we used both validated data and computational prediction tools. First, we collected experimentally validated miRNA-target interactions reported in humans from 3 distinct public resources: miRTarBase release 6.1 (39), starBase v2.0 (40), and Tarbase v7.0 (41). Data was pre-processed using in-house scripts in the R environment to retrieve only those interactions involving miRNAs of interest, and the following filtering criteria were adopted: 1) for mirTarBase, only interactions classified as functional were kept; 2) for starBase, we restricted for interactions with "low" stringency and predicted by at least 3 software; and 3) for TarBase, we considered only interactions classified as "positive", which was related to a direct association between miRNAs and their target genes. In addition, for TarBase data derived from HITS-CLIP and PAR-CLIP high-throughput experiments (classified as limited evidence), we only

considered interactions supported by 3 or more experiments. The union of all interactions retrieved from the 3 queried sources was considered as the set of validated miRNA-target gene interactions in our study.

Second, to complement the list of experimentally validated miRNA-target interactions, we used the following web-based *in silico* target prediction tools: TargetScan v7.1, where interactions involving conserved miRNA sites and with Context++ scores < -0.2 were selected (42); Diana MicroT-CDS, selecting interactions with scores > 0.8 (43); and Microcosm v5.0, retrieving interactions with P values < 0.1 (44). All interactions predicted by at least 2 computational tools were then included in the final list of predicted miRNA-target interactions. Lists of unique validated and predicted miRNA-target gene interactions were combined for use in the subsequent functional enrichment analyses. Of note, prior to the next analysis, miRNAs and genes identifiers were mapped to miRBase v21 (44) and Human Gene Nomenclature Committee (45, 46).

We investigated the functional enrichment of the retrieved target genes using pathways annotation from KEGG Pathway Database (47, 48) and statistical methods implemented in the package clusterProfiler in R/Bioconductor environment (49). This investigation was performed for targets of each individual miRNA of interest or for targets of these miRNAs grouped according to their expression profile (up- or downregulation). Significance for KEGG pathways enrichment was estimated using a hypergeometric test and adjusted to account for multiple hypothesis testing using the False Discovery Rate (FDR) procedure implemented in the q-value R package (50). Pathways with a q-value < 0.05 were considered strongly enriched for the inputted list of target genes; nonetheless, as this analysis is sensitive to the number of targets

retrieved for a given miRNA, pathways with P values < 0.1 and biological plausibility were also taken into consideration in the interpretation of miRNA functional roles.

Functional similarities between miRNAs of interest were analyzed using the Jaccard similarity coefficient (JC), which provides a measure of similarity of two sets of elements. The JC is computed as the size of the intersection between the two sets divided by the size of their union, and provides a value (0 to 1) that reflects the degree of shared members between the sets, where the higher the value the larger is their overlap.

Statistical analysis

Normal distribution was checked using Kolmogorov-Smirnov and Shapiro-Wilk tests. Variables with normal distribution are shown as mean ± standard deviation (SD). Variables with skewed distribution were log-transformed before analyses and are presented as median (25^{th}-75^{th} percentiles). Categorical data are shown as percentage. Clinical and laboratory characteristics as well as miRNA expressions were compared among groups of subjects using One-way ANOVA or χ^2 tests, as appropriate.

Correlations between quantitative variables were analyzed using Pearson's correlation tests. To investigate the discriminatory power of the dysregulated miRNAs to differentiate patients with or without DKDs, receiver-operating characteristic (ROC) curves were created and the respective areas under the curves (AUCs) were calculated. All analyses were performed using the SPSS statistical package (v.20.0) for Windows (SPSS Inc, Chicago, IL), and P values < 0.05 were considered statistically significant.

RESULTS

Clinical and laboratory characteristics of the subjects included in the study

Descriptive characteristics of T1DM controls, cases with moderate or severe DKD and healthy subjects included in this study are summarized in **Table 1**. Considering that distributions of these characteristics were similar between subjects from the screening and validation samples (data not shown), results are shown in Table 1 for both samples analyzed together. DKD cases, T1DM controls and healthy subjects from both samples did not differ significantly regarding age, gender, and mean body mass index (BMI). HbA1c levels were higher in patients with DKD compared to the other groups, but T1DM controls had intermediate levels between DKD cases and healthy subjects. Patients with severe DKD showed higher prevalence of hypertension and diabetic retinopathy when compared to the other diabetic groups. As expected, creatinine levels were increased and eGFR was decreased in patients with severe DKD compared to the other diabetic groups. UAE progressively increased from T1DM controls to moderate and severe DKD (**Table 1**).

Expression profile of 45 miRNAs in subjects from the screening sample

Expressions of 45 circulating miRNAs were evaluated in plasma of T1DM controls and DKD cases from the screening sample. Of these 45 miRNAs, 40 (88.9%) had detectable expression in plasma in >10% of the samples, allowing more reliable statistical analyses (**Figure 1** and **Table 2**). Nine of these 40 miRNAs were differentially expressed between T1DM controls and DKD cases (**Table 2**). Of note, miR-16-5p, miR-29-3p and miR-204-5p were significantly downregulated in patients with moderate and severe DKD compared to T1DM controls, while miR-503-5p was upregulated in moderate and

severe DKD. Interestingly, miR-21-3p and miR-378a-5p were upregulated only in patients with severe DKD compared to the other groups, while miR-141-3p, miR-192-5p and miR-215-5p were downregulated in patients with severe DKD (**Table 2**).

Expressions of the 9 dysregulated miRNAs in DKD patients were also compared between T1DM controls and healthy subjects to investigate if these miRNAs were also associated with T1DM *per se* (**Table 3**). As a result, we found that miR-16-5p and hsa-miR-29a-3p were significantly upregulated in healthy subjects compared to T1DM controls (P = 0.0001 and P = 0.039, respectively); thus, expressions of these 2 miRNAs progressively decreased from healthy controls to T1DM controls and DKD patients. Expressions of the other 7 dysregulated miRNAs were not significantly different between T1DM controls and healthy subjects (**Table 3**).

Validation of the five selected miRNAs by RT-qPCR

Out of the 9 miRNAs differently expressed among the analyzed groups in the screening sample, we choose 5 miRNAs to validate by RT-qPCR in the validation sample (miR-16-5p, miR-21-3p, miR-29a-3p, miR-378a-5p, and miR-503-5p) after applying the selection criteria described in the Methods section.

As depicted in **Figure 2A** and **2C**, miR-16-5p and miR-29a-3p were downregulated in patients with severe DKD compared to both moderate DKD and T1DM control groups, although the comparison of miR-29-3p expression between severe DKD and moderate DKD groups did not reach formal statistical significance (P = 0.090). MiR-21-3p and miR-378a-5p were increased in severe DKD patients compared to patients with moderate DKD and T1DM controls (**Figure 2 B and D**); however. In contrast with the array data, miR-503-5p expression was not significantly different among the 3 analyzed groups (**Figure 2E**).

For the subsequent analyses, both screening and validation samples were analyzed together in order to increase the statistical power. First, we calculated sensitivity and specificity of the 4 differently expressed miRNAs for detecting DKD, using ROC curve analyses (**Supplementary Table 2**). The highest AUC (0.804) was found for miR-29a-3p, with a cutoff of 0.143, resulting in 72.4% sensitivity and a low specificity of 13% for detecting DKD cases. MiR-16-5p, miR-21-3p and miR-378a-5p showed AUCs lower than 0.80 for detecting DKD cases. Regarding severe DKD, miR-21-3p showed the highest AUC (0.887) with a cutoff of 0.141, and 80.0% sensitivity and 87.0% specificity (compared to the moderate DKD group). Moreover, for miR-16-5p, miR-29a-3p and miR-378-5p we found that AUCs were 0.706, 0.680 and 0.875; with cutoffs of 0.004, 0.156 and 0.430, resulting in sensitivity of 76.9%, 76.9% and 90.0%, and specificity of 17.9%, 23.1% and 89.3; respectively (compared to the moderate DKD group; **Supplementary Table 2**).

Next, we evaluated correlations among the 4 dysregulated miRNA (miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p) validated in this study and DKD-related measurements (eGFR, creatinine and UAE levels), as well as HbA1c levels. MiR-21-3p and miR-378a-5p expressions were negatively correlated with eGFR ($r = -0.633$, $P = 0.004$; and $r = -0.455$, $P = 0.044$; respectively), while miR-378a-5p expression was positively correlated with UAE levels ($r = 0.338$, $P = 0.049$) in DKD cases. MiR-21-3p was also positively correlated with serum creatinine levels ($r = 0.616$, $P = 0.004$). None of the 4 validated miRNAs correlated with HbA1c levels ($P > 0.05$).

Target prediction for the dysregulated miRNAs analyzed in the validation sample

Target prediction of the 5 miRNAs chosen for validation by RT-qPCR (hsa-miR-16-5p, hsa-miR-29a-3p, hsa-miR-21-3p, hsa-miR-378a-5p, and hsa-miR-503-5p) were

performed using distinct resources, including experimentally validated data and computational target prediction tools, as described in the Methods Section. In general, 1670 genes were identified as putative targets of these 5 miRNAs (**Supplementary Table 3**), although only 200 genes were found to be modulated by 2 or more miRNAs (**Supplementary Figure 2**).

Individually, 983 target genes were found for hsa-miR-16-5p, 56 for hsa-miR-21-3p, 689 for hsa-miR-29a-3p, 34 for hsa-miR-378a-5p, and 126 for hsa-miR-503-5p (**Supplementary Table 3; Supplementary Figure 2**). Additionally, we obtained a total of 1291 experimentally validated interactions and 597 predicted interactions for the 5 miRNAs analyzed. The 3 upregulated miRNAs (hsa-miR-21-3p, hsa-miR-378a-5p, and hsa-miR-503-5p) target a total of 215 unique genes, while the downregulated miRNAs (hsa-miR-16-5p and hsa-miR-29a-3p) target a total of 1572 unique genes (**Supplementary Table 3).** The complete list of targets for each of the 5 analyzed miRNAs of interest is shown in the **Supplementary Table 4**.

We next compared the list of putative target genes shared among the 5 miRNAs analyzed in the validation sample by computing the JC in a pairwise fashion (**Figure 3A**), as described in the Methods Section. The highest JC was found for the comparison between hsa-miR-16-5p and hsa-miR-503-5p (0.095), whereas the smallest non-zero JC value was detected between hsa-miR-16-5p and hsa-miR-378a-5p (0.002), indicating that these 5 miRNAs target few genes in common.

Functional enrichment analysis for the 5 miRNAs analyzed in the validation sample

To explore the biological pathways possible affected by the 5 miRNAs chosen for validation by RT-qPCR (hsa-miR-16-5p, hsa-miR-21-3p, hsa-miR-29a-3p, hsa-miR-378a-5p, and hsa-miR-503-5p), we carried out functional enrichment analysis of their

target genes using pathways maps from the KEGG Database. A total of 136 pathways were enriched for these miRNAs considering P values < 0.1, among which 91 pathways have corrected q-values < 0.05 (**Supplementary Table 5**). We also analyzed functional enrichment of the 5 miRNAs of interest grouped by their expression profile (up- or downregulation), and observed that many of the enriched pathways consist of genes well known to be related to DKD pathogenesis, such as PI3K/Akt, p53, AMPK, longevity regulating, fluid shear stress and atherosclerosis, endocytosis, protein processing in endoplasmic reticulum, AGE-RAGE, TGF-β1, and relaxin signaling pathways (**Figure 4**).

We next compared the similarity among the 5 miRNAs of interest in terms of their enriched pathways using the pairwise JC (**Figure 3B**). Despite the low overlap among target genes of these miRNAs, as shown in **Figure 3A**, a substantial overlap in the list of regulated pathways are found for some pairs of miRNAs, especially among hsa-miR-16-5p, hsa-miR-29a-3p, and hsa-miR-503-5p (**Figure 3B**). This may indicate that these miRNAs act in shared pathways through distinct mechanisms and targets, thus having complementary roles in their modulation.

Noteworthy, in the **Supplementary Table 6** we show the list of target genes and significant pathways for the 4 miRNAs (hsa-miR-141-3p, hsa-miR-192-5p, hsa-miR-204-5p, and hsa-miR-215-5p) that were dysregulated in DKD patients from the screening sample, but were not chosen for validation by RT-qPCR**.**

DISCUSSION

Significant efforts have been made to identify biomarkers that could clinically detect early stages of DKD and the progressive decline in kidney function (22, 26). Circulating

and urinary miRNAs are not affected by glomerular filtration and dialysis since they circulate in body-fluids complexed to proteins, such as Argonaute-2, or packaged into microvesicles, exosomes, or apoptotic bodies (51, 52); potentially constituting ideal biomarkers of DKD. Therefore, considering that miRNAs might be biomarkers of DKD, we evaluated the expression profile of 45 circulating miRNAs in T1DM patients with this complication and in T1DM controls. In the screening sample, we found 9 miRNAs dysregulated in plasma of patients with different stages of DKD (miR-16-5p, miR-21-3p, miR-29a-3p, miR-141-3p, miR-192-5p, miR-204-5p, miR-215-5p, miR-378a-5p, and miR-503-5p). Five of these miRNAs were then chosen for validation in an independent sample. Among them, miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p maintained the expression trend observed in the screening sample, but miR-503-5p was not confirmed to be associated with DKD.

MiR-16-5p and miR-29a-3p were downregulated in patients with severe DKD compared to the other groups of patients. In line with our results, Baker *et al*. (53) reported that this miRNA was downregulated in proximal tubules of patients with DKD compared to healthy subjects. MiR-16-5p expression was also downregulated in serum of patients with nephrotic syndrome (54), and in plasma of critically ill patients with dialysis-dependent acute kidney injury compared to healthy subjects (55). In contrast, Neal *et al.* (56) showed that this miRNA was upregulated in plasma of nondiabetic patients with ESRD receiving dialysis compared to CKD patients with moderate renal impairment or normal renal function. Interestingly, miR-16-5p is regulated by vasopressin in kidney inner medullary collecting duct cells from rats (57). The vasopressin system has emerged as a factor strongly associated with kidney health in the general population as well as in patients with different stages of CKD (58). Moreover, miR-16-5p targets *VEGFA* (59), a well-known vascular growth factor that is important

for maintaining the integrity of the glomerular filtration barrier as well as a survival factor for the podocyte (60).

Experimental studies indicates that miR-29 family members (including miR-29a-3p) have potent anti-fibrotic activities, mainly by targeting different types of collagen via TGF-β1-Smad3-dependent mechanisms, which leads to decreased ECM accumulation and, consequently, renal fibrosis (61-64). MiR-29a-3p is downregulated by TGF-β1 in a variety of cell lines, including human and rat proximal tubule epithelial cells, mouse mesangial cells, and human podocytes (63-65), which might predispose to DKD. Accordingly, miR-29a knockdown in diabetic mice promoted histone deacetylase activity, decreasing nephrin expression and, consequently, causing podocyte apoptosis, proteinuria and subsequent renal dysfunction (65). Hence, diabetic mice overexpressing miR-29a showed improved renal function and podocyte viability and decreased glomerular fibrosis compared with the wild-type mice (65). In agreement with the present study, Pezzolesi *et al.* (25) found that miR-29a-3p was downregulated in plasma of fast-progressors to ESRD compared to nonprogressors, while 1-standard deviation increase in miR-29a-3p expression was protective against fast progression to ESRD in T1DM patients (25). However, other studies have shown that this miRNA is upregulated in serum, urine or kidney of type 2 diabetic patients (53, 66, 67). These contradictory results may be explained by differences in tissue types, methods used for DKD classification, or type of DM that was analyzed.

The present study also indicates that miR-21-3p and miR-378a-5p are upregulated in plasma of patients with DKD. MiR-21-3p was also reported to be increased in post-transplant renal biopsies of patients with acute kidney injury compared to matched-allografts without any pathology, twelve days after engraftment (68). However, the molecular mechanisms behind the association of this miRNA with kidney

injury are still unclear. Bang *et al*. (69) showed that miR-21-3p is produced by cardiac fibroblasts and acts as a potent paracrine-acting miRNA that induces cardiomyocyte hypertrophy. MiR-21-3p might mediate cardiac hypertrophic by targeting histone deacetylase 8 (*HDAC8*), which modulates the Akt/Gsk3b pathway (70). Therefore, there is a possibility that this miRNA might also lead to hypertrophy of renal cells. Accordingly, miR-21-3p is also induced by TGF-β1 in primary parenchymal lung fibroblasts, affecting their function (71). In addition, Jiao *et al*. (72) reported that miR-21-3p may be an oncogene for colorectal cancer by promoting cellular mobility through epithelial-to-mesenchymal transition (EMT). Interestingly, these authors showed that miR-21-3p targets *E-cadherin* and *Smad7* in a colorectal cancer cell line, leading to EMT promotion (72). It is known that E-cadherin decreases collagen production and, consequently, ECM accumulation and fibrosis in renal cells (5); thereby, there is a possibility that miR-21-5p may be triggering renal fibrosis by targeting *E-cadherin*. By targeting *Smad7*, a TGF-β1 negative regulator (5), miR-21-3p may also amplify the TFG-β1 signaling cascade during renal fibrosis. Additional studies are needed to confirm if miR-21-3p targets *E-cadherin* and *Smad7* in renal cells.

MiR-378a-5p was originally reported to be expressed in a number of cancer cell lines (73), and has been shown to directly affect *VEGFA* expression by competing with miR-125a for the same seed-region in *VEGFA* mRNA, causing upregulation of this vascular growth factor (74). Xing *et al*. (75) demonstrated that mesenchymal stromal cells overexpression rno-miR-378a-5p had increased expression of *VEGFA*, *PDGF-β* and *TGF-β1*, which have a recognized role in kidney dysfunction. This miRNA also seems to be involved in apoptosis in colorectal cancer cells (76). Further studies are necessary to clarify by which mechanisms miR-378-5p predispose to DKD.

Although not chosen for validation, miR-141-3p, miR-192-5p, miR-204-5p and miR-215-5p seem to be downregulated in DKD patients from the screening sample, and deserve to be further investigated in great depth. Among these miRNAs, miR-192-5p is highly expressed in normal kidneys compared to other organs, and has been consistently reported as being downregulated in urine or serum of patients with DKD (22, 26, 77, 78). In addition, this miRNA has been negatively correlated with urinary albumin to creatinine ratio, TGF-β1 and proteinuria levels (77-79). MiR192-5p downregulation in renal cells seems to lead to decreased *E-cadherin* expression, thus increasing collagen production and ECM accumulation, worsening renal fibrosis (80).

This study has a few limitations. First, although the limited sample size of the screening sample, we tried to validate the results of 5 miRNAs by RT-qPCR in an independent sample of DKD cases and controls. Of them, 4 miRNAs had their results confirmed in the validation sample, suggesting that the association with DKD is robust. Second, approximately 50% of the severe DKD group is constituted by patients in hemodialysis. Hence, we cannot exclude the possibility that the upregulation of some miRNAs in plasma of severe DKD patients may be due to a markedly decreased GFR in these patients, concentrating these miRNAs in plasma. Even though we did not evaluated miRNA expression in urine to answer this question, it seems that miRNAs complexed to exosomes are not influenced by GFR and hemodialysis (51, 52).

In conclusion, the present study identified a set of nine miRNAs dysregulated in T1DM patients with different stages of DKD. Four of them had their results validated in a different sample, confirming that miR-21-3p and miR-378a-5p are upregulated in patients with severe DKD; while, miR-16-5p and miR-29a-3p are downregulated in this group of patients. Additionally, these miRNAs regulate genes from several important pathways involved in mechanisms related to DKD development, such as podocyte

injury, proteinuria, mesangial proliferation, extracellular matrix accumulation, and endothelial cell injury. Experimental validation of the putative targets and perturbed pathways under their regulation, as well as further investigation of these miRNAs in different populations, are needed to confirm their utility as biomarkers.

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Declaration of interest

All authors declare no conflict of interest.

Author Contributions

TSA designed the study, acquired and analyzed the data, and drafted the manuscript. MRM performed the bioinformatics analysis and reviewed the manuscript. ACR acquired and analyzed the data. ACB, MP, BT and LHC interpreted the data and reviewed the manuscript. DC supervised the study, analyzed the data and drafted the manuscript. All authors approved the final version.

REFERENCES

1. Kanwar YS, Sun L, Xie P, Liu FY, Chen S. A glimpse of various pathogenetic mechanisms of diabetic nephropathy. Annu Rev Pathol. 2011;6:395-423.
2. Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T. Diabetic nephropathy: diagnosis, prevention, and treatment. Diabetes Care. 2005;28(1):164-76.
3. Macisaac RJ, Ekinci EI, Jerums G. Markers of and risk factors for the development and progression of diabetic kidney disease. American journal of kidney diseases : the official journal of the National Kidney Foundation. 2014;63(2 Suppl 2):S39-62.
4. Ritz E, Zeng XX, Rychlik I. Clinical manifestation and natural history of diabetic nephropathy. Contributions to nephrology. 2011;170:19-27.
5. Reddy MA, Tak Park J, Natarajan R. Epigenetic modifications in the pathogenesis of diabetic nephropathy. Semin Nephrol. 2013;33(4):341-53.
6. Thomas MC. Epigenetic Mechanisms in Diabetic Kidney Disease. Current diabetes reports. 2016;16(3):31.
7. Nadkarni GN, Yacoub R, Coca SG. Update on glycemic control for the treatment of diabetic kidney disease. Current diabetes reports. 2015;15(7):42.
8. Glassock RJ. Debate: CON position. Should microalbuminuria ever be considered as a renal endpoint in any clinical trial? American journal of nephrology. 2010;31(5):462-5; discussion 6-7.
9. Esteller M. Non-coding RNAs in human disease. Nature reviews Genetics. 2011;12(12):861-74.
10. Butz H, Kinga N, Racz K, Patocs A. Circulating miRNAs as biomarkers for endocrine disorders. J Endocrinol Invest. 2016;39(1):1-10.
11. Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. Nat Rev Endocrinol. 2013;9(9):513-21.
12. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clinical chemistry. 2010;56(11):1733-41.
13. Seyhan AA, Nunez Lopez YO, Xie H, Yi F, Mathews C, Pasarica M, et al. Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study. Sci Rep. 2016;6:31479.
14. Feng Y, Yu X. Cardinal roles of miRNA in cardiac development and disease. Sci China Life Sci. 2011;54(12):1113-20.
15. Lin S, Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer. 2015;15(6):321-33.
16. Assmann TS, Recamonde-Mendoza M, de Souza BM, Crispim D. MicroRNA expression profiles and type 1 diabetes mellitus: systematic review and bioinformatics analysis. Endocrine connections. 2017.
17. Fabbri M. miRNAs as molecular biomarkers of cancer. Expert Rev Mol Diagn. 2010;10(4):435-44.
18. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(11):4402-7.
19. Harvey SJ, Jarad G, Cunningham J, Goldberg S, Schermer B, Harfe BD, et al. Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. Journal of the American Society of Nephrology : JASN. 2008;19(11):2150-8.

20. Ho J, Ng KH, Rosen S, Dostal A, Gregory RI, Kreidberg JA. Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. Journal of the American Society of Nephrology : JASN. 2008;19(11):2069-75.
21. Shi S, Yu L, Chiu C, Sun Y, Chen J, Khitrov G, et al. Podocyte-selective deletion of dicer induces proteinuria and glomerulosclerosis. Journal of the American Society of Nephrology : JASN. 2008;19(11):2159-69.
22. Kato M, Natarajan R. MicroRNAs in diabetic nephropathy: functions, biomarkers, and therapeutic targets. Ann N Y Acad Sci. 2015;1353:72-88.
23. Huang Y, Liu Y, Li L, Su B, Yang L, Fan W, et al. Involvement of inflammation-related miR-155 and miR-146a in diabetic nephropathy: implications for glomerular endothelial injury. BMC Nephrol. 2014;15:142.
24. Argyropoulos C, Wang K, McClarty S, Huang D, Bernardo J, Ellis D, et al. Urinary microRNA profiling in the nephropathy of type 1 diabetes. PloS one. 2013;8(1):e54662.
25. Pezzolesi MG, Satake E, McDonnell KP, Major M, Smiles AM, Krolewski AS. Circulating TGF-beta1-Regulated miRNAs and the Risk of Rapid Progression to ESRD in Type 1 Diabetes. Diabetes. 2015;64(9):3285-93.
26. Chung AC, Yu X, Lan HY. MicroRNA and nephropathy: emerging concepts. Int J Nephrol Renovasc Dis. 2013;6:169-79.
27. Simpson K, Wonnacott A, Fraser DJ, Bowen T. MicroRNAs in Diabetic Nephropathy: From Biomarkers to Therapy. Current diabetes reports. 2016;16(3):35.
28. von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP, et al. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. J Clin Epidemiol. 2008;61(4):344-9.
29. Andrassy KM. Comments on 'KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease'. Kidney international. 2013;84(3):622-3.
30. American Diabetes A. (2) Classification and diagnosis of diabetes. Diabetes Care. 2015;38 Suppl:S8-S16.
31. Assmann TS, Brondani Lde A, Bauer AC, Canani LH, Crispim D. Polymorphisms in the TLR3 gene are associated with risk for type 1 diabetes mellitus. Eur J Endocrinol. 2014;170(4):519-27.
32. Zelmanovitz T, Gross JL, Oliveira JR, Paggi A, Tatsch M, Azevedo MJ. The receiver operating characteristics curve in the evaluation of a random urine specimen as a screening test for diabetic nephropathy. Diabetes care. 1997;20(4):516-9.
33. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150(9):604-12.
34. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-22.
35. Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. Nat Methods. 2015;12(8):697.
36. Barutta F, Tricarico M, Corbelli A, Annaratone L, Pinach S, Grimaldi S, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. PloS one. 2013;8(11):e73798.
37. DiStefano JK, Taila M, Alvarez ML. Emerging roles for miRNAs in the development, diagnosis, and treatment of diabetic nephropathy. Current diabetes reports. 2013;13(4):582-91.

38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
39. Chou CH, Chang NW, Shrestha S, Hsu SD, Lin YL, Lee WH, et al. miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. Nucleic Acids Res. 2016;44(D1):D239-47.
40. Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res. 2014;42(Database issue):D92-7.
41. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. Nucleic acids research. 2015;43(Database issue):D153-9.
42. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife. 2015;4.
43. Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 2013;41(Web Server issue):W169-73.
44. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic acids research. 2008;36(Database issue):D154-8.
45. Wain HM, Bruford EA, Lovering RC, Lush MJ, Wright MW, Povey S. Guidelines for human gene nomenclature. Genomics. 2002;79(4):464-70.
46. Gray KA, Yates B, Seal RL, Wright MW, Bruford EA. Genenames.org: the HGNC resources in 2015. Nucleic Acids Res. 2015;43(Database issue):D1079-85.
47. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 2016;44(D1):D457-62.
48. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27-30.
49. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16(5):284-7.
50. Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing. 2013.
51. Martino F, Lorenzen J, Schmidt J, Schmidt M, Broll M, Gorzig Y, et al. Circulating microRNAs are not eliminated by hemodialysis. PloS one. 2012;7(6):e38269.
52. Lorenzen JM, Thum T. Circulating and urinary microRNAs in kidney disease. Clinical journal of the American Society of Nephrology : CJASN. 2012;7(9):1528-33.
53. Baker MA, Davis SJ, Liu P, Pan X, Williams AM, Iczkowski KA, et al. Tissue-Specific MicroRNA Expression Patterns in Four Types of Kidney Disease. Journal of the American Society of Nephrology : JASN. 2017.
54. Zapata-Benavides P, Arellano-Rodriguez M, Bollain YGJJ, Franco-Molina MA, Rangel-Ochoa GA, Avalos-Diaz E, et al. Cytoplasmic Localization of WT1 and Decrease of miRNA-16-1 in Nephrotic Syndrome. BioMed research international. 2017;2017:9531074.
55. Lorenzen JM, Kielstein JT, Hafer C, Gupta SK, Kumpers P, Faulhaber-Walter R, et al. Circulating miR-210 predicts survival in critically ill patients with acute kidney injury. Clinical journal of the American Society of Nephrology : CJASN. 2011;6(7):1540-6.

56. Neal CS, Michael MZ, Pimlott LK, Yong TY, Li JY, Gleadle JM. Circulating microRNA expression is reduced in chronic kidney disease. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2011;26(11):3794-802.
57. Kim JE, Jung HJ, Lee YJ, Kwon TH. Vasopressin-regulated miRNAs and AQP2-targeting miRNAs in kidney collecting duct cells. American journal of physiology Renal physiology. 2015;308(7):F749-64.
58. Clark WF, Devuyst O, Roussel R. The vasopressin system: new insights for patients with kidney diseases: Epidemiological evidence and therapeutic perspectives. Journal of internal medicine. 2017;282(4):310-21.
59. Qu Y, Liu H, Lv X, Liu Y, Wang X, Zhang M, et al. MicroRNA-16-5p overexpression suppresses proliferation and invasion as well as triggers apoptosis by targeting VEGFA expression in breast carcinoma. Oncotarget. 2017;8(42):72400-10.
60. Brosius FC, Coward RJ. Podocytes, signaling pathways, and vascular factors in diabetic kidney disease. Advances in chronic kidney disease. 2014;21(3):304-10.
61. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. Physiological genomics. 2012;44(4):237-44.
62. He Y, Huang C, Lin X, Li J. MicroRNA-29 family, a crucial therapeutic target for fibrosis diseases. Biochimie. 2013;95(7):1355-9.
63. Qin W, Chung AC, Huang XR, Meng XM, Hui DS, Yu CM, et al. TGF-beta/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol. 2011;22(8):1462-74.
64. Wang B, Komers R, Carew R, Winbanks CE, Xu B, Herman-Edelstein M, et al. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. Journal of the American Society of Nephrology : JASN. 2012;23(2):252-65.
65. Lin CL, Lee PH, Hsu YC, Lei CC, Ko JY, Chuang PC, et al. MicroRNA-29a promotion of nephrin acetylation ameliorates hyperglycemia-induced podocyte dysfunction. Journal of the American Society of Nephrology : JASN. 2014;25(8):1698-709.
66. Chien HY, Chen CY, Chiu YH, Lin YC, Li WC. Differential microRNA Profiles Predict Diabetic Nephropathy Progression in Taiwan. International journal of medical sciences. 2016;13(6):457-65.
67. Peng H, Zhong M, Zhao W, Wang C, Zhang J, Liu X, et al. Urinary miR-29 correlates with albuminuria and carotid intima-media thickness in type 2 diabetes patients. PloS one. 2013;8(12):e82607.
68. Wilflingseder J, Sunzenauer J, Toronyi E, Heinzel A, Kainz A, Mayer B, et al. Molecular pathogenesis of post-transplant acute kidney injury: assessment of whole-genome mRNA and miRNA profiles. PloS one. 2014;9(8):e104164.
69. Bang C, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, et al. Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. The Journal of clinical investigation. 2014;124(5):2136-46.
70. Yan M, Chen C, Gong W, Yin Z, Zhou L, Chaugai S, et al. miR-21-3p regulates cardiac hypertrophic response by targeting histone deacetylase-8. Cardiovascular research. 2015;105(3):340-52.
71. Ong J, Timens W, Rajendran V, Algra A, Spira A, Lenburg ME, et al. Identification of transforming growth factor-beta-regulated microRNAs and the microRNA-targetomes in primary lung fibroblasts. PloS one. 2017;12(9):e0183815.

72. Jiao W, Leng X, Zhou Q, Wu Y, Sun L, Tan Y, et al. Different miR-21-3p isoforms and their different features in colorectal cancer. International journal of cancer. 2017;141(10):2103-11.
73. Jiang J, Lee EJ, Gusev Y, Schmittgen TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. Nucleic acids research. 2005;33(17):5394-403.
74. Hua Z, Lv Q, Ye W, Wong CK, Cai G, Gu D, et al. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PloS one. 2006;1:e116.
75. Xing Y, Hou J, Guo T, Zheng S, Zhou C, Huang H, et al. microRNA-378 promotes mesenchymal stem cell survival and vascularization under hypoxic-ischemic conditions in vitro. Stem cell research & therapy. 2014;5(6):130.
76. Wang Z, Ma B, Ji X, Deng Y, Zhang T, Zhang X, et al. MicroRNA-378-5p suppresses cell proliferation and induces apoptosis in colorectal cancer cells by targeting BRAF. Cancer cell international. 2015;15:40.
77. Ma X, Lu C, Lv C, Wu C, Wang Q. The Expression of miR-192 and Its Significance in Diabetic Nephropathy Patients with Different Urine Albumin Creatinine Ratio. Journal of diabetes research. 2016;2016:6789402.
78. Wang G, Kwan BC, Lai FM, Chow KM, Li PK, Szeto CC. Urinary sediment miRNA levels in adult nephrotic syndrome. Clinica chimica acta; international journal of clinical chemistry. 2013;418:5-11.
79. Szeto CC, Ching-Ha KB, Ka-Bik L, Mac-Moune LF, Cheung-Lung CP, Gang W, et al. Micro-RNA expression in the urinary sediment of patients with chronic kidney diseases. Dis Markers. 2012;33(3):137-44.
80. Wang B, Herman-Edelstein M, Koh P, Burns W, Jandeleit-Dahm K, Watson A, et al. E-cadherin expression is regulated by miR-192/215 by a mechanism that is independent of the profibrotic effects of transforming growth factor-beta. Diabetes. 2010;59(7):1794-802.

Table 1. Clinical and laboratory characteristics of all subjects included in the study considering the screening and validation samples together.

Characteristic	**T1DM controls (n = 33)**	**Moderate DKD (n = 28)**	**Severe DKD (n = 26)**	**Healthy subjects (n = 20)**	**P value***
Age (years)	25.2 ± 5.5	22.3 ± 5.0	26.8 ± 6.1	24.6 ± 5.9	0.247
Gender (% male)	55.6	58.8	51.8	54.9	0.814
BMI (kg/m²)	23.4 ± 2.9	23.6 ± 3.6	22.8 ± 3.7	25.7 ± 3.8	0.174
HbA1c (%)	8.4 ± 1.1[a]	10.1 ± 2.1[b]	9.3 ± 1.8[b]	5.3 ± 0.3[c]	0.0001
Hypertension (% yes)	11.1[a]	11.8[a]	71.4[b]	0.0	0.0001
Age at T1DM diagnosis (years)	9.0 (5.0 – 12.0)	6.0 (3.0 – 10.0)	6.0 (6.0 – 9.0)	-	0.608
Duration of T1DM (years)	15.0 (13.0 – 19.0)[a]	13.5 (10.0 – 21.0)[a]	19.0 (12.0 – 26.0)[b]	-	0.003
Insulin dose (unit/kg·day)	0.8 ± 0.2	0.9 ± 0.3	0.7 ± 0.3	-	0.442
Triglycerides (mg/dl)	74.0 (50.0 – 107.0)	110.5 (63.5 – 148.2)	114.0 (63.0 – 215.0)	-	0.068
Total-CT (mg/dl)	177.4 ± 33.4	193.6 ± 79.0	186.4 ± 42.9	-	0.727
HDL-CT (mg/dl)	49.1 ± 11.3	58.5 ± 14.9	62.1 ± 27.5	-	0.173
Creatinine (μg/dl)	0.8 (0.6 – 0.9)[a]	0.9 (0.8 – 1.2)[a]	5.1 (1.7 – 8.1)[b]	-	0.0001
eGFR (mL/min per 1.73m^2)	120.0 (109.0 – 127.0)[a]	120.0 (89.0 – 128.0)[a]	13.5 (6.0 – 61.5)[b]	-	0.0001
UAE (mg/g)	5.5 (3.3 – 9.1)[a]	77.0 (46.0 – 201.1)[b]	565.6 (3057.9)[c]	-	0.0001

Urea (mg/g)	36.0 (32.0 – 37.0)	42.0 (38.0 – 46.0)	105 (67.5 – 138.5)	-	0.124
Diabetic retinopathy (%)	7.7[a]	21.4[a]	78.6[b]	-	0.0001

Variables are shown as mean ± SD, median (25^{th}-75^{th} percentiles) %, as appropriate. * P values were computed using χ^2 or ANOVA, followed by post-hoc multiple comparison tests (residual analysis or Tukey's tests, respectively), as appropriate. Analyses with significant differences are indicated as follows: means, medians or % indicated by different letters differed from the other groups at $P < 0.05$. CT: cholesterol; BMI: body mass index; eGFR: estimated glomerular filtration rate; HbA1c: glycated hemoglobin.

Table 2. MiRNA expressions in the screening sample obtained using Custom TaqMan Array MicroRNA cards.

miRNA	T1DM patients without DKD (n = 23)	T1DM patients with moderate DKD (n = 18)	T1DM patients with severe DKD (n = 17)	P*
hsa-let-7b-5p	0.248 (0.065 – 1.356)	0.166 (0.002 – 0.387)	0.072 (0.010 – 0.186)	0.360
hsa-let-7c-5p	0.349 (0.037 – 0.941)	0.215 (0.045 – 0.521)	0.104 (0.003 – 0.653)	0.150
hsa-miR-10a-5p	0.153 (0.038 – 0.591)	0.079 (0.003 – 0.553)	0.009 (0.001 – 0.136)	0.145
hsa-miR-15a-5p	0.069 (0.006 – 0.157)	0.070 (0.001 – 0.138)	0.086 (0.009 – 0.200)	0.424
hsa-miR-16-5p	0.219 (0.039 – 1.000)[a]	0.054 (0.0008 – 0.391)[b]	0.010 (0.003 – 0.119)[b]	**0.014**
hsa-miR-17-5p	0.071 (0.009 – 0.374)	0.050 (0.001 – 0.463)	0.012 (0.001 – 0.123)	0.453
hsa-miR-20b-5p	0.089 (0.014 – 0.315)	0.041 (0.0005 – 0.302)	0.019 (0.003 – 0.089)	0.376
hsa-miR-21-3p	0.043 (0.0003 – 0.145)[a]	0.011 (0.0001 – 0.076)[a]	1.026 (0.339 – 1.587)[b]	**0.003**
hsa-miR-21-5p	0.017 (0.004 – 0.070)	0.058 (0.017 – 0.156)	0.091 (0.026 – 0.298)	0.110
hsa-miR-25-3p	0.116 (0.045 – 0.485)	0.116 (0.027 – 0.258)	0.023 (0.003 – 0.125)	0.067
hsa-miR-29a-3p	1.112 (0.397 – 1.757)[a]	0.371 (0.119 – 0.480)[b]	0.291 (0.126 – 0.614)[b]	**0.001**
hsa-miR-29b-3p	0.199 (0.103 – 0.332)	0.057 (0.031 – 0.265)	0.031 (0.014 – 0.120)	0.161
hsa-miR-29c-3p	0.076 (0.008 – 0.162)	0.060 (0.003 – 0.194)	0.007 (0.0005 – 0.045)	0.123
hsa-miR-92a-3p	0.148 (0.022 – 0.401)	0.084 (0.016 – 0.255)	0.016 (0.005 – 0.142)	0.156

has-miR-93-5p	0.156 (0.013 – 0.407)	0.065 (0.0008 – 0.515)	0.018 (0.007 – 0.207)	0.550
hsa-miR-126-3p	0.110 (0.018 – 1.562)	0.094 (0.011 – 0.689)	0.040 (0.009 – 0.417)	0.471
hsa-miR-132-3p	0.005 (0.0001 – 0.03)	0.006 (0.003 – 0.013)	0.0005 (0.0001 – 0.004)	0.065
hsa-miR-133a-3p	0.189 (0.026 – 0.918)	0.082 (0.008 – 1.234)	0.129 (0.014 – 0.426)	0.522
hsa-miR-141-3p	0.203 (0.075 – 0.955)[a]	0.040 (0.006 – 0.626)[a]	0.0001 (0.0001 – 0.112)[b]	**0.001**
hsa-miR-146a-5p	0.099 (0.010 – 0.197)	0.095 (0.008 – 0.281)	0.023 (0.008 – 0.239)	0.919
hsa-miR-155-5p	0.134 (0.027 – 0.355)	0.129 (0.027 – 0.443)	0.015 (0.007 – 0.356)	0.156
hsa-miR-182-5p	0.446 (0.101 – 1.210)	0.461 (0.155 – 2.861)	0.406 (0.020 – 2.939)	0.899
hsa-miR-183-5p	0.124 (0.075 – 0.980)	0.141 (0.045 – 0.502)	0.272 (0.001 - NO)	0.586
hsa-miR-192-5p	0.060 (0.033 – 0.418)[a]	0.051 (0.002 – 0.329)[a,b]	0.010 (0.0001 – 0.112)[b]	**0.010**
hsa-miR-200a-3p	0.173 (0.005 – 0.805)	0.047 (0.0001 – 0.193)	0.056 (0.004 – 0.084)	0.475
hsa-miR-200b-3p	0.034 (0.006 – 0.108)	0.011 (0.001 – 0.029)	0.005 (0.001 – 0.049)	0.426
hsa-miR-200c-3p	0.126 (0.009 – 0.361)	0.099 (0.004 – 0.586)	0.070 (0.019 – 0.208)	0.878
hsa-miR-204-5p	0.090 (0.017 – 0.224)[a]	0.009 (0.0001 – 0.032)[b]	0.004 (0.0001 – 0.028)[b]	**0.001**
hsa-miR-205-5p	0.327 (0.019 – 11.925)	0.0001 (0.0001 – 2.112)	NA	0.168
hsa-miR-210-3p	0.212 (0.015 – 0.602)	0.149 (0.021 – 0.302)	0.033 (0.007 – 0.468)	0.508
hsa-miR-211-5p	0.048 (0.008 – 1.317)	0.059 (0.002 – 1.455)	0.047 (0.010 – 0.223)	0.907
hsa-miR-215-5p	0.499 (0.143 – 1.964)[a]	0.340 (0.097 – 1.144)[a]	0.022 (0.0001 – 0.385)[b]	**0.021**

hsa-miR-216a-5p	0.062 (0.006 – 0.450)	0.003 (0.0001 – 0.075)	0.004 (0.0001 – 0.015)	0.179
hsa-miR-320a	0.469 (0.101 – 1.049)	0.452 (0.042 – 1.595)	0.171 (0.044 – 2.358)	0.715
hsa-miR-338-3p	0.835 (0.210 – 5.019)	0.565 (0.132 – 4.689)	0.501 (0.058 – 1.483)	0.361
hsa-miR-377-3p	14.480 (0.007 - NO)	0.150 (0.001 - NO)	NA	0.575
hsa-miR-378a-5p	0.052 (0.008 – 0.157)[a]	0.087 (0.014 – 0.316)[a]	2.812 (0.795 – 4.475)[b]	**0.008**
hsa-miR-486-5p	0.211 (0.044 – 1.245)	0.119 (0.007 – 0.764)	0.025 (0.007 – 0.543)	0.336
hsa-miR-503-5p	0.112 (0.0001 – 0.354)[a]	1.574 (0.977 – 2.549)[b]	5.676 (4.952 – 6.504)[b]	**0.006**
hsa-miR-638	5.442 (0.582 – 15.909)	1.005 (0.324 – 4.044)	4.646 (0.470 – 26.433)	0.269

Data are shown as median (25^{th} – 75^{th} percentiles) of n-fold values. *P values were obtained from One-way ANOVA followed by Tukey post-hoc tests using the log-transformed variable. Values with significant differences are indicated by different letters ($P < 0.05$), while similar values are indicated by the same letters. NO = non-observed (for these groups, it was not observed any subject with expression higher than the 50^{th} percentile). NA = non-available (no expression values were observed for this group).

Table 3. MiRNA expressions in T1DM controls and healthy subjects from the screening sample.

miRNA	**T1DM controls (n = 23)**	**Healthy subjects (n = 10)**	**P***
hsa-miR-16-5p	0.219 (0.039 – 1.000)	1.098 (0.644 – 1.583)	**0.0001**
hsa-miR-21-3p	0.043 (0.0003 – 0.145)	0.079 (0.009 – 0.741)	0.949
hsa-miR-29a-3p	1.112 (0.397 – 1.757)	1.450 (1.167 – 1.935)	**0.039**
hsa-miR-141-3p	0.203 (0.075 – 0.955)	2.295 (0.097 - NO)	0.256
hsa-miR-192-5p	0.060 (0.033 – 0.418)	0.211 (0.074 – 0.588)	0.096
hsa-miR-204-5p	0.090 (0.017 – 0.224)	0.037 (0.002 – 0.185)	0.794
hsa-miR-215-5p	0.499 (0.143 – 1.964)	0.415 (0.225 – 7.873)	0.161
hsa-miR-378a-5p	0.052 (0.008 – 0.157)	2.091 (0.274 – 6.580)	0.245
hsa-miR-503-5p	0.112 (0.0001 – 0.354)	NA	-

Data are shown as median (25^{th} – 75^{th} percentiles) of n-fold values. *P values were obtained by Student t-tests, using the log-transformed variable. NO = non-observed (for these groups, it was not observed any subject with expression higher than the 50^{th} percentile). NA = non-available (no expression values were observed for this group).

FIGURES

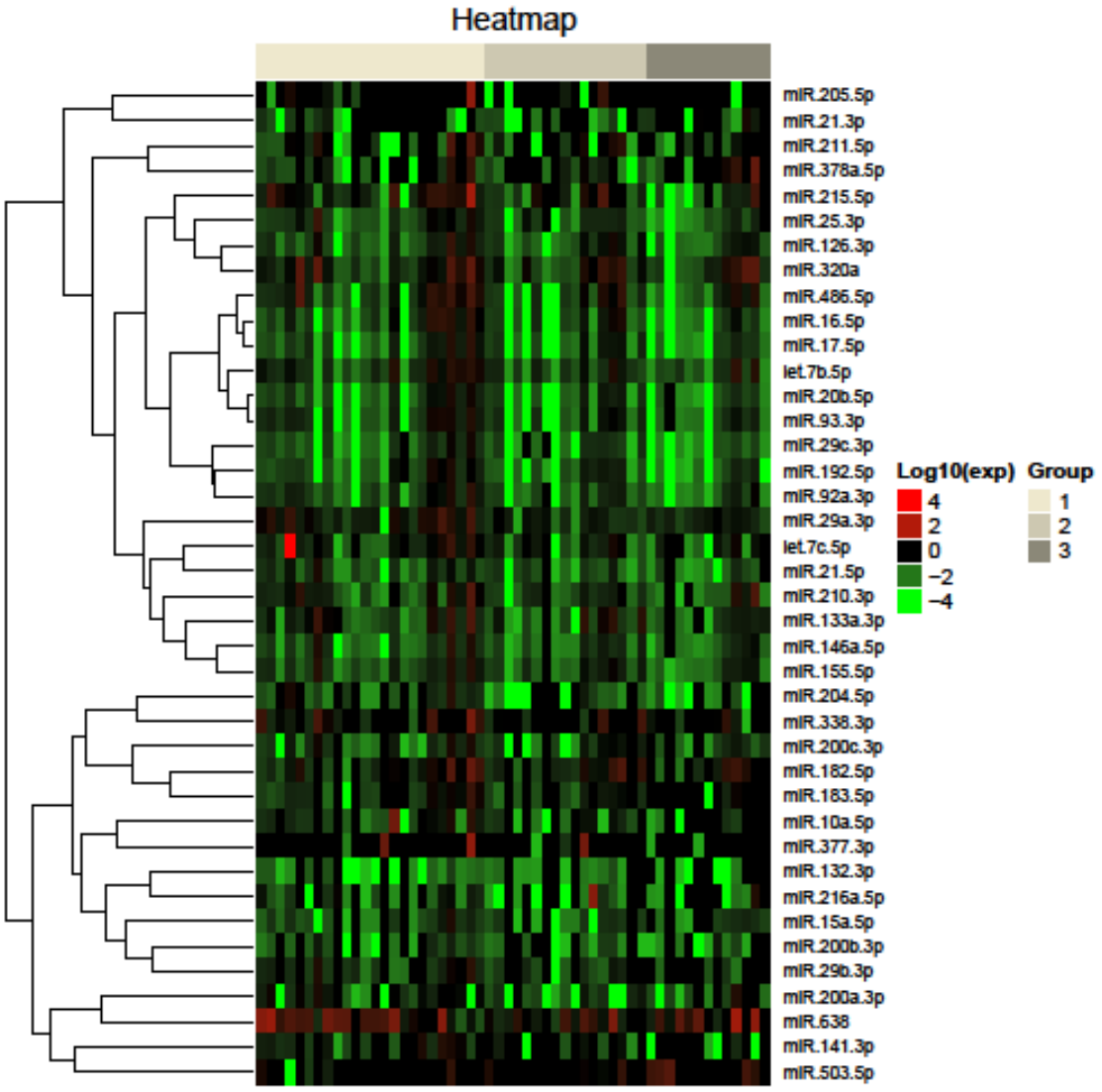
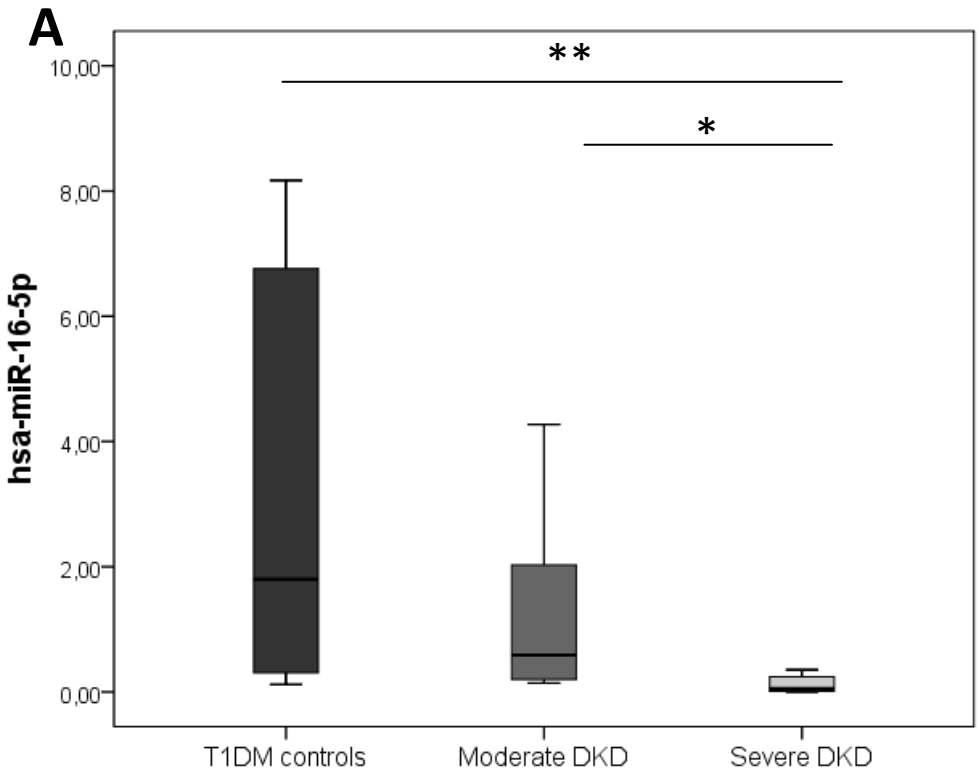


Figure 1. Distinct miRNA expression patterns in T1DM controls and in patients with moderate DKD or severe DKD. Heatmap of the 40 miRNAs expressed in subjects from the screening sample, derived from the array analysis. Each column represents an individual sample and each row represents an individual miRNA. Expression levels of miRNAs are shown in red (for upregulated miRNAs) and green (for downregulated miRNAs), with brighter shades indicating higher fold differences ($\log_{10}$ n-fold change values) in relation to the calibrator sample. Absence of difference

in expression levels is represented in black. Hierarchical clustering was performed using Pearson uncentered distance metric with complete linkage.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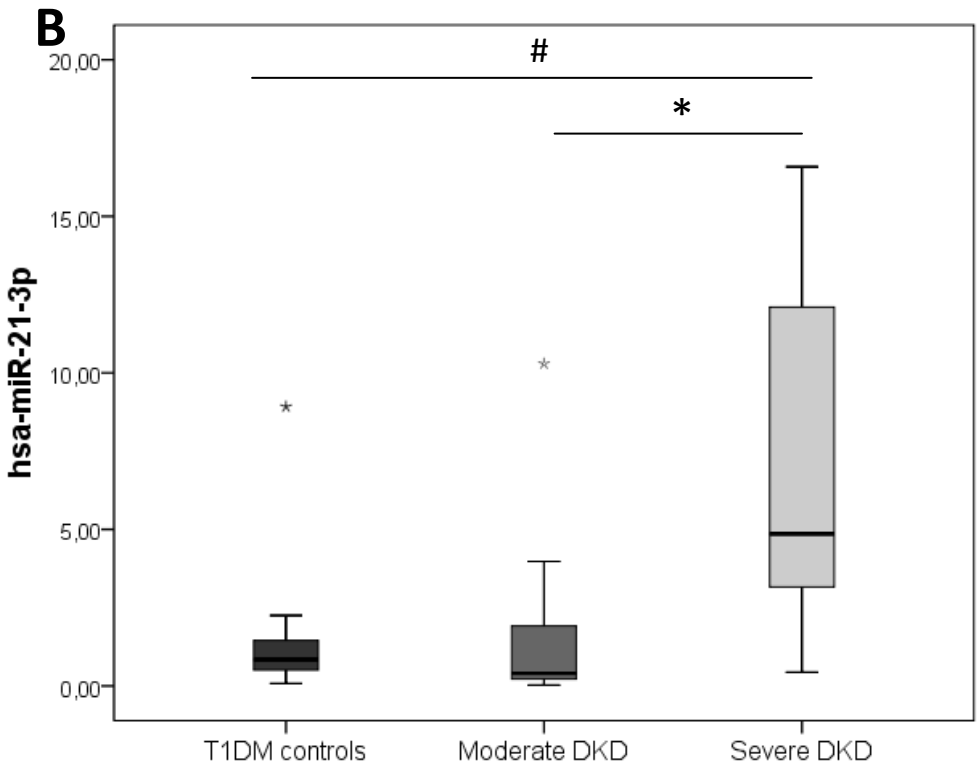
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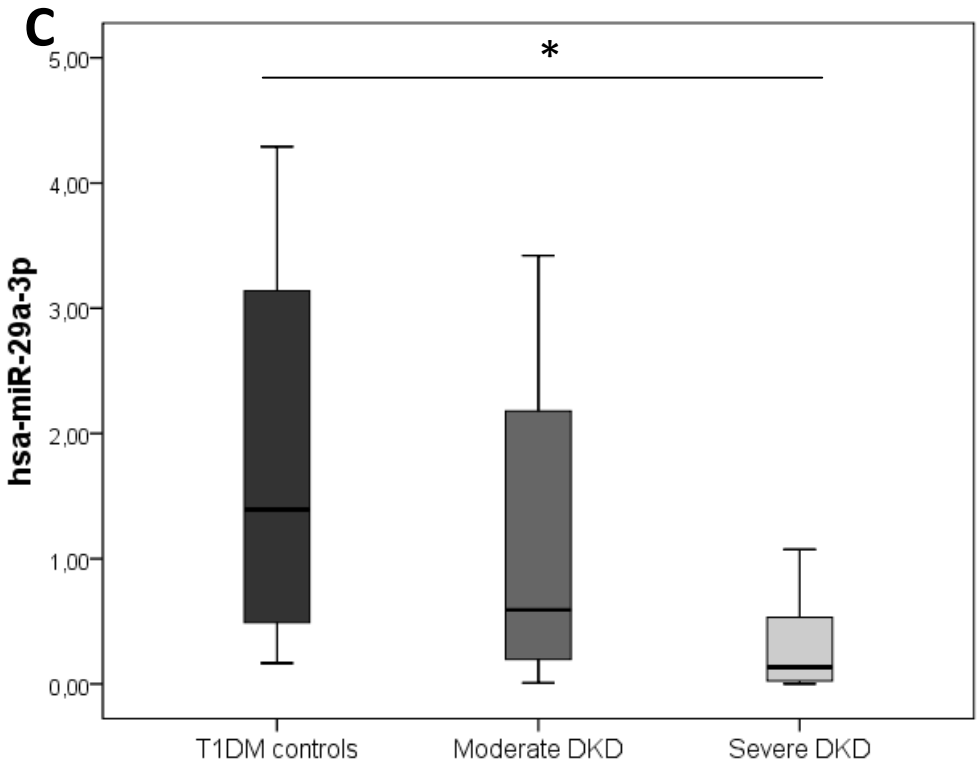
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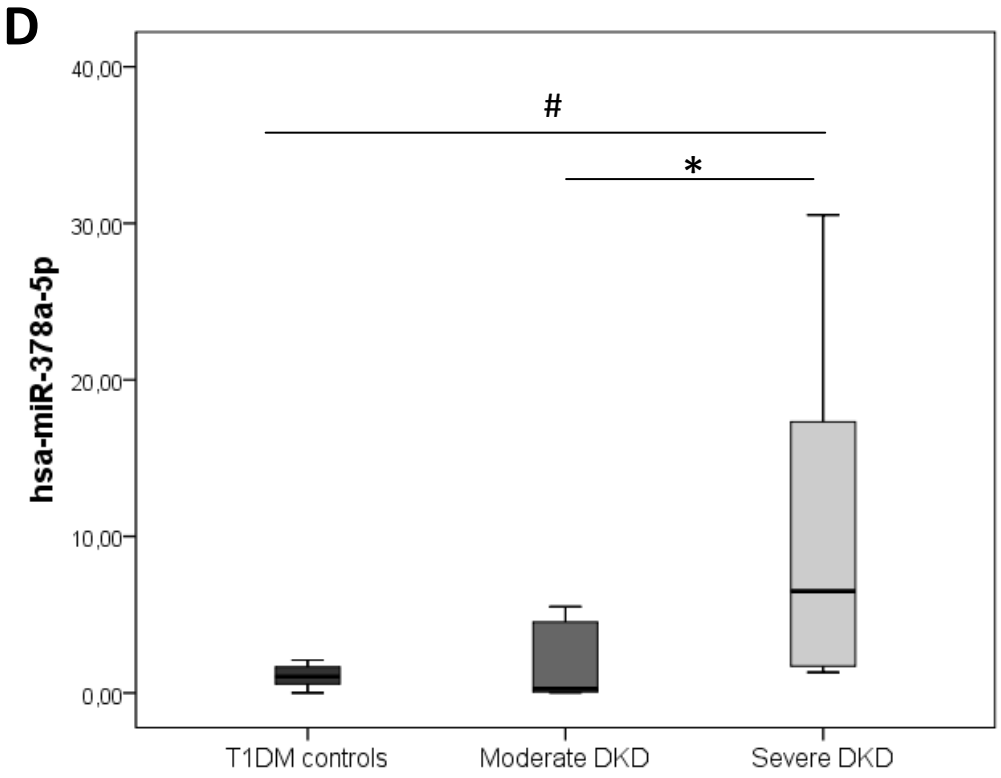
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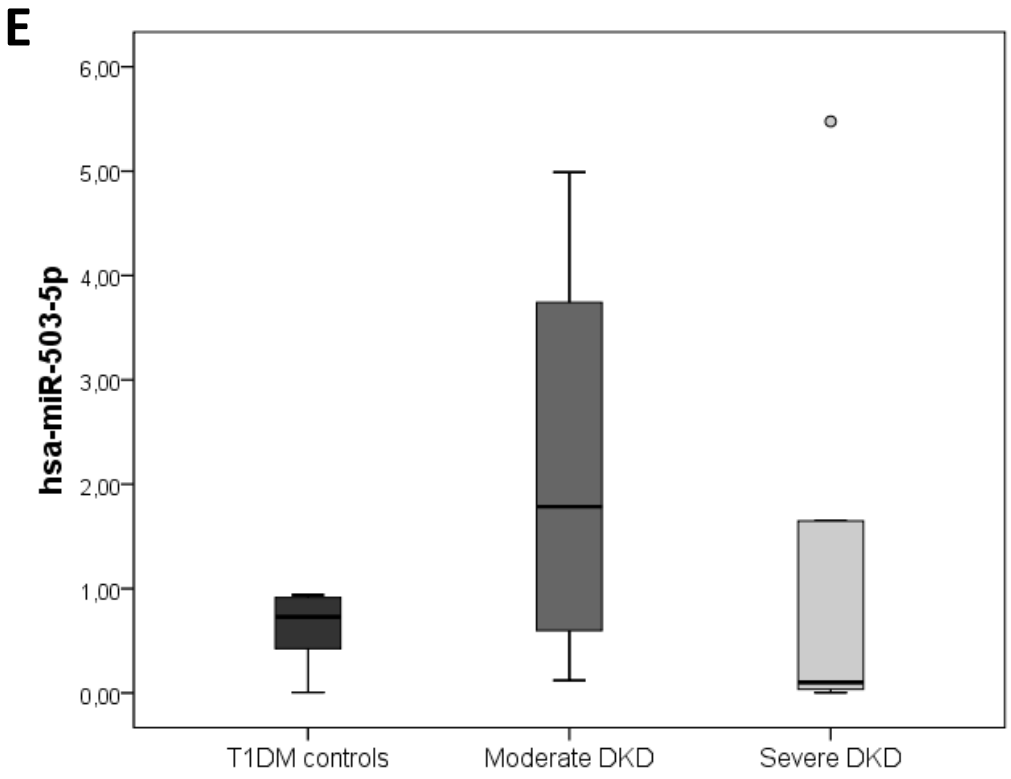


Figure 2. Dysregulated miRNAs in plasma from T1DM controls and patients with moderate or severe DKD from the validation sample. Relative expressions of (**A**) miR-16-5p, (**B**) miR-21-3p, (**C**) miR-29a-3p, (**D**) miR-378a-5p, and (**E**) miR-503-5p were evaluated using RT-qPCR. Results are expressed as n-fold changes in relation to calibrator sample ($\Delta\Delta C_t$ method), using the *U6 snRNA* as the reference gene, and are shown as median (25^{th} – 45^{th} percentiles). P-values were obtained using one-way ANOVA tests with Tukey's post-hoc tests. * $P < 0.050$, ** $P < 0.010$ and # $P < 0.100$.

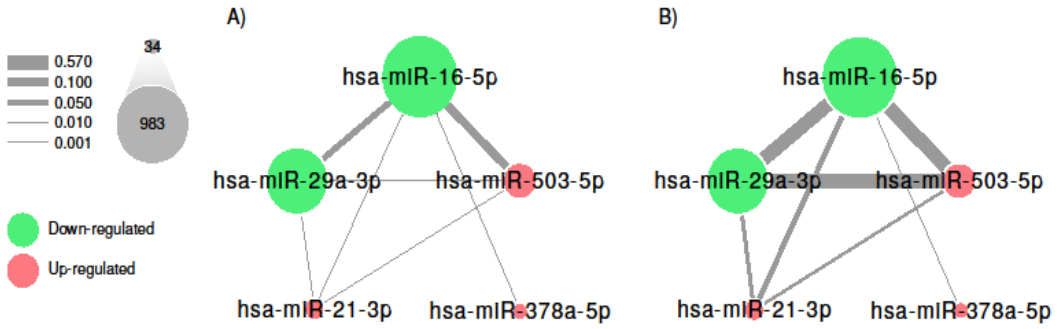
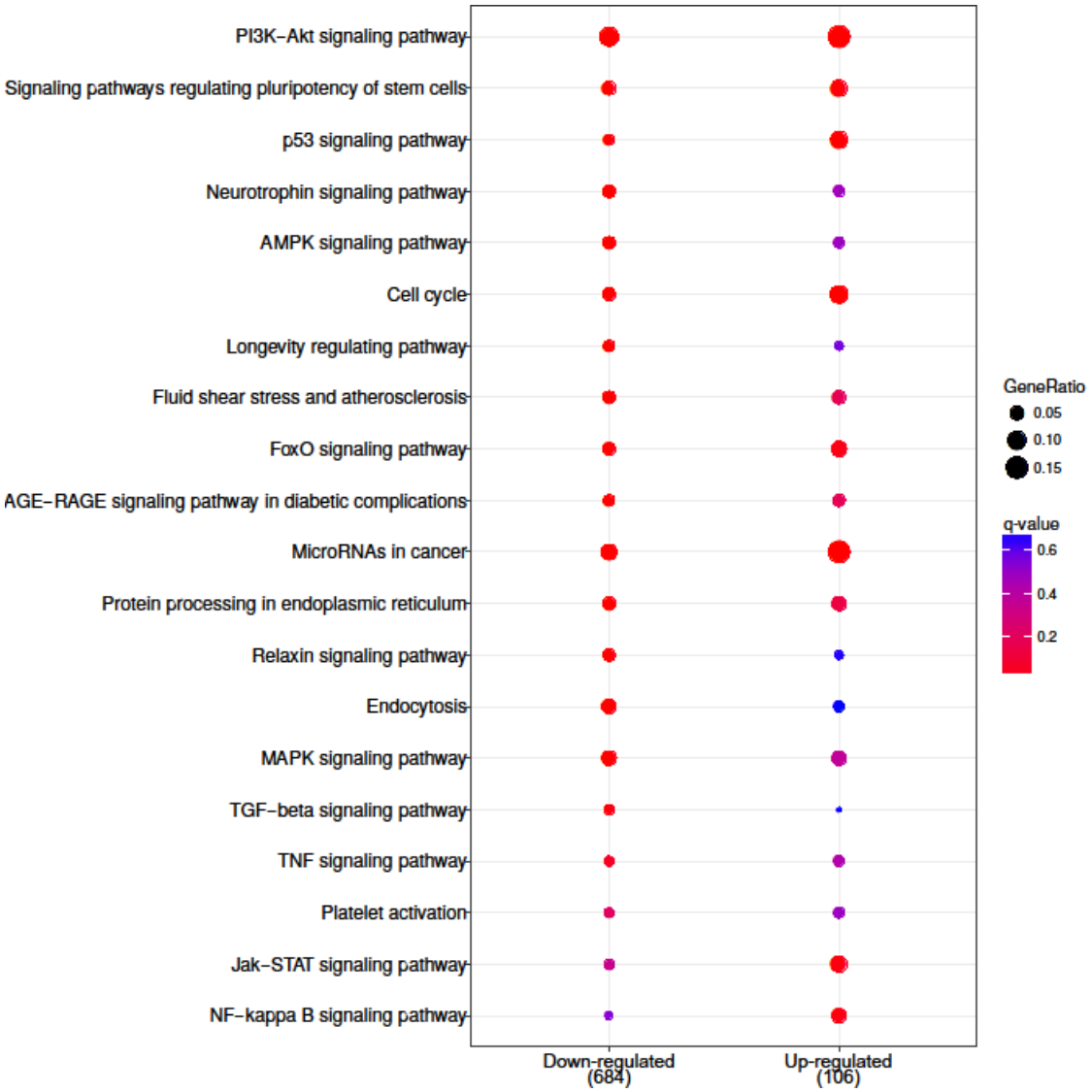


Figure 3. Jaccard coefficients (JC) computed in terms of (A) number of target genes and (B) number of pathways for miR-16-5p, miR-21-3p, miR-29a-3p, miR-378a-5p, and miR-503-5p. The solid lines connecting molecules represent the existence of shared targets or pathways (JC > 0). Node size represents number of targets or pathways for each miRNA; edge width is proportional to the overlap between miRNAs targets or pathways measured by the JC; and nodes are colored based on the differential expression of the miRNA in DKD cases: green represents those miRNAs downregulated in DKD patients; while pink represents miRNAs which are upregulated.



<u>Figure 4</u>. KEGG pathways potentially regulated by miR-16-5p, miR-21-3p, miR-29a-3p, miR-378a-5p, and miR-503-5p, according to their expression profile. Downregulated miRNAs in DKD patients: miR-16-5p and miR-29a-3p. Upregulated miRNA in DKD patients: miR-21-3p, miR-378a-5p, and miR-503-5p. The size of the circles denote the ratio of target genes involved in a given pathway (GeneRatio) and its color represents the pathway's q-value. Q-values: P values corrected for multiple tests using the Benjamini-Hochberg procedure, as described in the Methods Section.

SUPPLEMENTARY MATERIAL LIST

Supplementary Figure 1. Bioinformatics approach used to investigate the targets and pathways of the miRNAs of interest.

Supplementary Figure 2. Venn diagram demonstrating target genes shared among the 5 miRNAs chosen for validation by RT-qPCR.

Supplementary Table 1. Assay reference numbers for the 48 analyzed miRNAs included in the TaqMan Low Density Array cards**.**

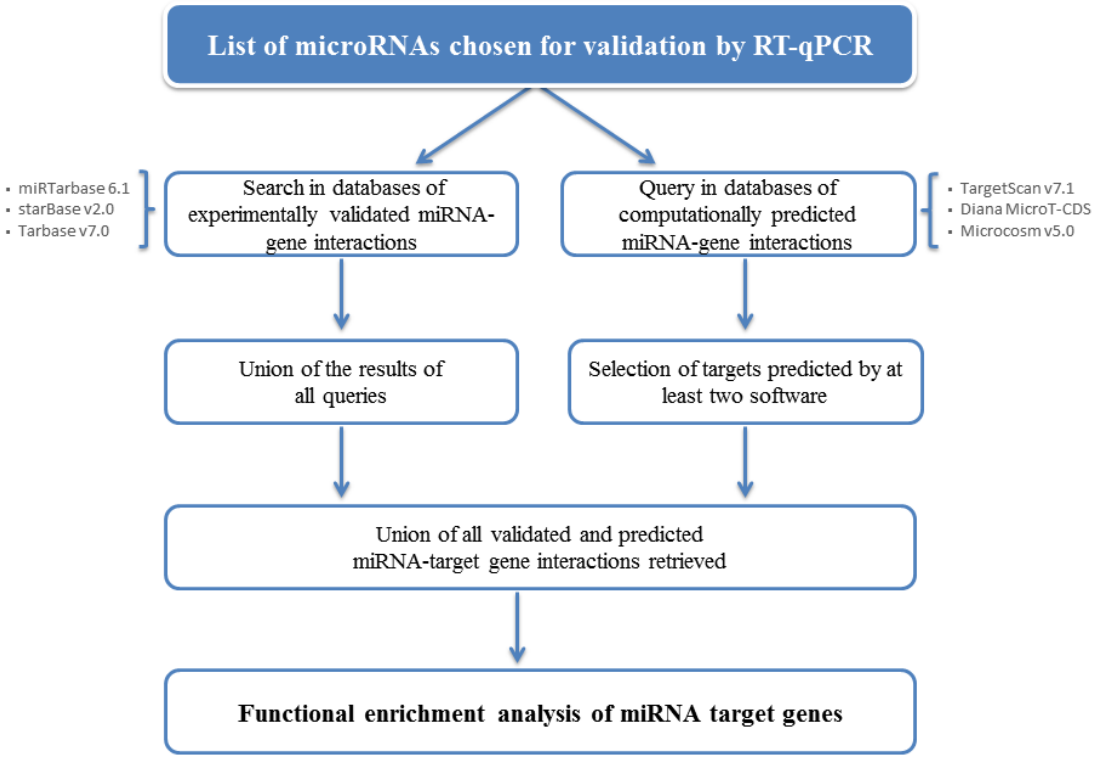
Supplementary Table 2. Receiver operating curves for miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p expressions in plasma of T1DM controls and DKD cases.

Supplementary Table 3. Total number of miRNA-target interactions for each of the 5 miRNA analyzed in the validation sample, both when considered individually and grouped by their expression profile (up- or downregulation in DKD cases).

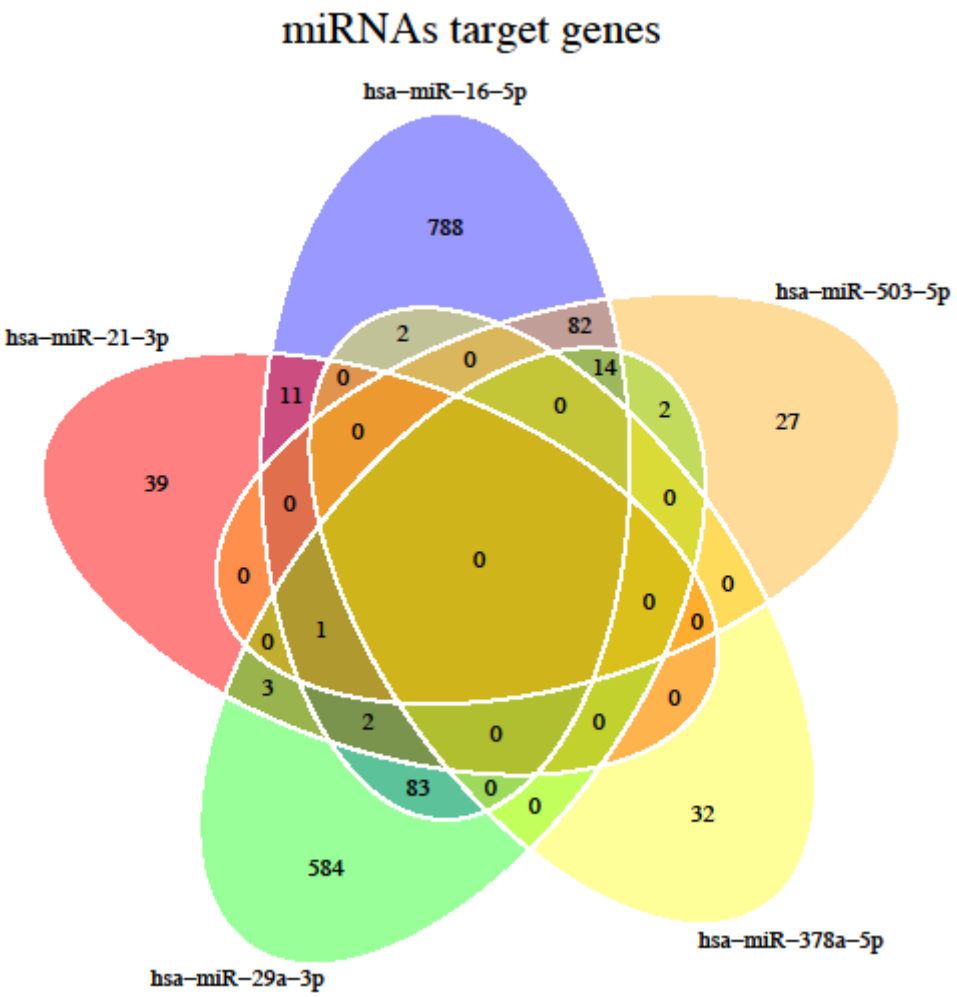
Supplementary Table 4. MiRNA-target gene interactions for the 5 miRNAs chosen for validation by RT-qPCR (miR-21-3p, miR-29a-3p, miR-378a-5p, and miR-503-5p, according to their expression). (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 5. Significant KEGG pathways obtained for the 5 miRNAs chosen for validation by RT-qPCR (miR-21-3p, miR-29a-3p, miR-378a-5p, and miR-503-5p, according to their expression). All terms with P values < 0.1 are shown. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).

Supplementary Table 6. Results of functional enrichment analysis for the 4 possibly dysregulated miRNAs without validation analysis: miR-141-3p, miR-192-5p, miR-204-5p, hsa-miR-215-5p. All terms with P values < 0.1 are shown. (*Tabela em formato excel encaminhada em arquivo digital anexo a tese*).



Supplementary Figure 1. Bioinformatics approach used to investigate the targets and pathways of the miRNAs of interest.



Supplementary Figure 2. Venn diagram demonstrating target genes shared among the 5 miRNAs chosen for validation by RT-qPCR.

Supplementary Table 1. Assay reference numbers of the 48 analyzed miRNAs included in the TaqMan Low Density Array cards**.**

microRNA	Assay ID*
hsa-let-7b-5p	002619
hsa-let-7c-5p	000379
hsa-miR-10a-5p	000387
hsa-miR-124-5p	002197
hsa-miR-126-3p	002228
hsa-miR-132-3p	000457
hsa-miR-133a-3p	002246
hsa-miR-141-3p	000463
hsa-miR-144-3p	002676
hsa-miR-146a-5p	000468
hsa-miR-155-5p	002623
hsa-miR-15a-5p	000389
hsa-miR-16-5p	000391
hsa-miR-17-5p	002308
hsa-miR-182-5p	002334
hsa-miR-183-5p	002269
hsa-miR-192-5p	000491
hsa-miR-200a-3p	000502
hsa-miR-200b-3p	002251
hsa-miR-200c-3p	002300
hsa-miR-204-5p	000508
hsa-miR-205-5p	000509
hsa-miR-20b-5p	001014
hsa-miR-21-3p	002438
hsa-miR-21-5p	000397
hsa-miR-210-3p	000512
hsa-miR-211-5p	000514
hsa-miR-215-5p	000518
hsa-miR-216a-5p	002220
hsa-miR-25-3p	000403

hsa-miR-29a-3p	002112
hsa-miR-29b-3p	000413
hsa-miR-29c-3p	000587
hsa-miR-320a	002277
hsa-miR-338-3p	002252
hsa-miR-377-3p	000566
hsa-miR-378a-5p	000567
hsa-miR-486-5p	001278
hsa-miR-503-5p	001048
hsa-miR-638	001582
hsa-miR-92a-3p	000431
hsa-miR-93-5p	001090
hsa-miR-96-3p	002140
RNU44	001094
RNU48	001006
U6 snRNA	001973
ath-miR-159a	000338

* Assay numbers for the miRNA catalogue of Thermo Fisher Scientific (DE, USA) from where the assays were acquired. ID = miRNA identity in miRBase v21.0. hsa = homo sapiens.

Supplementary Table 2. Receiver operating curves for miR-16-5p, miR-21-3p, miR-29a-3p, and miR-378a-5p expressions in plasma of T1DM controls and DKD cases.

Comparison	miRNAs	AUC (95% CI)	P value	Cut off	Sensitivity	Specificity
DKD cases *vs.* T1DM controls	Hsa-miR-16-5p	0.732 (0.595 – 0.868)	0.004	0.012	0.586	0.174
	Hsa-miR-21-3p	0.643 (0.454 – 0.832)	0.178	0.008	0.762	0.417
	Hsa-miR-29a-3p	0.804 (0.677 – 0.930)	0.0001	0.143	0.724	0.130
	Hsa-miR-378a-5p	0.692 (0.521 – 0.863)	0.046	0.067	0.727	0.562
Severe DKD *vs.* moderate DKD	Hsa-miR-16-5p	0.706 (0.554 – 0.858)	0.027	0.004	0.769	0.179
	Hsa-miR-21-3p	0.887 (0.678 – 0.999)	0.0001	0.141	0.800	0.870
	Hsa-miR-29a-3p	0.680 (0.536 – 0.852)	0.053	0.156	0.769	0.231
	Hsa-miR-378a-5p	0.875 (0.715 – 0.999)	0.001	0.43	0.900	0.893

AUC = area under the curve; CI = confidence interval. DKD = diabetic kidney disease; T1DM = type 1 diabetes mellitus.

Supplementary Table 3. Number of miRNA-target interactions for each miRNA analyzed in the validation sample, both when they were considered individually or grouped by their expression profile.

MiRNA/Group	Interactions from experimental evidences	Interactions from computational prediction	Total number of miRNA-target interactions
hsa-miR-16-5p	730	253	983
hsa-miR-21-3p	45	11	56
hsa-miR-29a-3p	416	273	689
hsa-miR-378a-5p	5	29	34
hsa-miR-503-5p	95	31	126
Upregulated miRNAs*	145	71	216
Downregulated miRNAs*	1146	526	1672

* Upregulated miRNAs: hsa-miR-21-3p, hsa-miR-378a-5p, and hsa-miR-503-5p.

* Downregulated miRNAs: hsa-miR-29a-3p and hsa-miR-16-5p.

CONCLUSÕES GERAIS

Os miRNAs tem um papel importante na regulação de genes que participam de diversas vias associadas ao DM1 e suas complicações crônicas, com a DRD. De fato, nossa revisão sistemática demonstrou que 11 miRNAs circulantes são diferencialmente expressos entre pacientes com DM1 e indivíduos não-diabéticos. Entre esses, os miR21-5p, miR-100, miR-148a, miR-181a, miR-210-5p e miR-375 estavam mais expressos, enquanto que os miR-146a-5p, miR-150, miR-342 e miR-1275 estavam menos expressos em pacientes com DM1 comparados aos controles. Esses miRNAs participam de vias previamente associadas com a patologia do DM1, como vias de apoptose, secreção da insulina e sistema imune. Essa revisão também evidenciou as limitações dos estudos que investigaram a expressão de miRNAs em pacientes com DM1, incluindo a falta de padronização da nomenclatura oficial dos miRNAs, não descrevendo qual fita do miRNA foi utilizada (-3p ou -5p), e o uso de diferentes metodologias para quantificação da expressão dos miRNAs, o que faz com que os resultados não possam ser diretamente comparáveis por meta-análise.

No estudo caso-controle, cinco miRNAs foram confirmados como diferencialmente expressos em pacientes com DM1 de diagnóstico recente comparados com pacientes com >5 anos de diagnóstico e indivíduos não-diabéticos. MiR-103a-3p, miR-155, miR-200a-3p, miR-210-5p estavam mais expressos, enquanto que miR-146a5p estava menos expresso em pacientes com DM1 de diagnóstico recente. A análise de bioinformática confirmou que esses miRNAs participam de vias previamente associadas como DM1, como vias de apoptose, insulina e sistema imunológico.

Em relação ao estudo que investigou polimorfismos em genes codificantes de miRNAs, encontramos que a presença dos alelos mais raros dos polimorfismos rs2910164 no gene miR-

146a e rs767649 no gene miR-155 foi associada com proteção ao DM1. Por outro lado o polimorfismo rs6715345 no gene miR-375 não foi associado com o DM1 na nossa população.

No que se refere à expressão de miRNAs em pacientes com DRD, a nossa segunda revisão sistemática evidenciou que seis miRNAs são diferencialmente expressos em pacientes com DRD comparados aos controles. Entre esses miRNAs, miR-21-5p, miR-29a-3p, miR-126, miR-214 e miR-342 são mais expressos, enquanto que o miR-192 foi descrito como menos expresso em pacientes com DRD comparado aos controles. Nossa análise de bioinformática demonstrou que esses seis miRNAs participam de vias previamente associadas com DRD, como PI3K/Akt, longevidade, TGF-β1 e relaxina. Além disso, essa revisão evidenciou as limitações dos estudos que analisaram a expressão de miRNAs em pacientes com DRD, como por exemplo, as diferentes formas de classificação dessa complicação, visto que alguns estudos usaram apenas a excreção urinária de albumina, outros apenas a taxa de filtração glomerular estimada e alguns poucos consideraram modificações histológicas em biópsia renal para confirmação da DRD. Da mesma forma que os estudos incluídos na revisão sistemática de miRNAs e DM1, também se observou a falta de padronização da nomenclatura oficial dos miRNAs e o uso de diversas metologias para quantificação da expressão dos miRNAs.

No estudo caso-controle, quatro miRNAs foram confirmados como diferencialmente expressos em pacientes como DM1 e DRD comparados com pacientes sem essa complicação (controles DM1). MiR-21-3p e miR-378a-5p estavam mais expressos em pacientes com DRD comparado aos controles DM1, enquanto que os miR-16-5p e miR-29a-3p estavam menos expressos em pacientes com DRD. A análise de bioinformática confirmou que esses miRNAs participam de vias previamente associadas com a patogênese da DRD, como TGF-□1, VEGFA, NF-κB, PI3K-Akt e FoxO.

OUTRA PRODUÇÃO BIBLIOGRÁFICA NO PERÍODO DO DOUTORADO

Além dos artigos que fazem parte da presente tese, ao longo do período do doutorado foram desenvolvidos os seguintes manuscritos:

1. Duarte GCK; **Assmann TS**; Dieter C; Souza BM; Crispim D. *GLIS3* rs7020673 and rs10758593 polymorphisms interact in the susceptibility for type 1 diabetes mellitus. **Acta diabetologica**, v. 54, p. 813-821, 2017.

2. Lemos NE; Dieter C; Dorfman LE; **Assmann TS**; Duarte GCK; Canani LH; Bauer AC; Crispim D. The rs2292239 polymorphism in ERBB3 gene is associated with risk for type 1 diabetes mellitus in a Brazilian population. **Gene**, 2017 (in press).

3. **Assmann TS**; Brondani LA; Bouças AP; Rheinheimer J; Souza BM; Canani LH; Bauer AC; Crispim D. Nitric oxide levels in patients with diabetes mellitus: A systematic review and meta-analysis. **Nitric Oxide** (Print), v. 61, p. 1-9, 2016.

4. Rheinheimer J; Bauer AC; Silveiro SP; Estivalet AAF; Bouças AP; Rosa AR; Souza BM; Oliveira FS; Cruz LA; Brondani LA; Azevedo MJ; Lemos NE; Carlessi R; **Assmann TS**; Gross JL; Leitão C; Crispim D. Human pancreatic islet transplantation: an update and description of the establishment of a pancreatic islet isolation laboratory. **Archives of Endocrinology and Metabolism**, v. 59, p. 161-170, 2015.

5. Sortica DA; Buffon MP; Souza BM; Nicoletto B; Santer A; **Assmann TS**; Crispim D; Canani LH. Association between the ENPP1 K121Q Polymorphism and Risk of Diabetic Kidney Disease: A Systematic Review and Meta-Analysis. **Plos One**, v. 10, p. e0118416, 2015.

6. Brondani LA; Boelter G; **Assmann TS**; Leitão C; Canani LH; Crispim D. Irisin-encoding gene (FNDC5) variant is associated with changes in blood pressure and lipid profile in type 2 diabetic women but not in men. **Metabolism, Clinical and Experimental** (Print), v. 64, p. 952-957, 2015.

7. Brondani LA; Souza BM; **Assmann TS**; Bouças AP; Bauer AC; Canani LA; Crispim D. Association of the UCP polymorphisms with susceptibility to obesity: case-control study and meta-analysis. **Molecular Biology Reports**, 2014 (in press).

8. **Assmann TS**; Lemos NE; Brondani LA; Carlessi R; Maldonado-Bernal C; Cruz M; Canani LH; Crispim D. Association between Asp299Gly and Thr399Ile Polymorphisms in Toll-Like Receptor 4 Gene and Type 2 Diabetes Mellitus: Case-Control Study and Meta-Analysis. **Journal of Diabetes & Metabolism**, v. 05, p. 1-10, 2014.

9. **Assmann TS**; Duarte GCK; Rheinheimer J; Cruz LA; Canani LH; Crispim D. The *TCF7L2* rs7903146 (C/T) polymorphism is associated with risk to type 2 diabetes mellitus

in Southern-Brazil. **Arquivos Brasileiros de Endocrinologia e Metabologia** (Impresso), v. 10, p. 1-0, 2014.

10. Brondani LA; **Assmann TS**; Souza BM; Bouças AP; Canani LH; Crispim D. Meta-analysis reveals the association of common variants in the uncoupling protein (*UCP*) 1-3 genes with body mass index variability. **Plos One**, v. 9, p. e96411, 2014.