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Clinical Research Article

Serum MicroRNA-191-5p Levels in Vascular Complications of Type 1 Diabetes: The EURODIAB Prospective Complications Study

Stefania Bellini,[1] Simonetta Guarrera,[2,3] Giuseppe Matullo,[1,4] Casper Schalkwijk,[5] Coen D. Stehouwer,[5] Nish Chaturvedi,[6] Sabita S. Soedamah-Muthu,[7,8] Federica Barutta,[1,*] and Gabriella Gruden[1,*]

[1]Department of Medical Sciences, University of Turin, 10126 Turin, Italy
[2]Italian Institute for Genomic Medicine, IIGM, 10060 Candiolo, Italy
[3]Candiolo Cancer Institute, FPO-IRCCS, 10060 Candiolo, Italy
[4]Medical Genetics Unit, AOU Città della Salute e della Scienza, 10126 Turin, Italy
[5]Department of Internal Medicine and Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, 6221 Maastricht, the Netherlands
[6]Institute of Cardiovascular Science, University College London, London WC1E 6BT, UK
[7]Center of Research on Psychology in Somatic Diseases (CORPS), Department of Medical and Clinical Psychology, Tilburg University, 5048 Tilburg, the Netherland
[8]Institute for Food, Nutrition and Health, University of Reading Reading RG6 6UR, UK

Correspondence: Federica Barutta, PhD, Department of Medical Sciences, University of Turin, Corso Dogliotti 14, 10126 Turin, Italy. Email: federica.barutta@unito.it.
*Co-last authors.

Abstract

Context: MicroRNA-191-5p regulates key cellular processes involved in the pathogenesis of diabetic complications such as angiogenesis, extracellular matrix deposition, and inflammation. However, no data on circulating microRNA-191-5p in the chronic complications of diabetes are available.

Objective: To assess whether serum levels of microRNA-191-5p were associated with micro- and macrovascular disease in a large cohort of subjects with type 1 diabetes mellitus (DM1) from the EURODIAB Prospective Complication Study.

Design and Setting: Levels of microRNA-191-5p were measured by quantitative PCR in 420 patients with DM1 recruited as part of the cross-sectional analysis of the EURODIAB Prospective Complication Study. Cases (n = 277) were subjects with nephropathy and/or retinopathy and/or cardiovascular disease (CVD). Controls (n = 143) were patients without complications. Logistic regression analysis was performed to evaluate the potential independent association of microRNA-191-5p levels with chronic complications of diabetes.

Results: Levels of microRNA-191-5p were significantly reduced ($P < .001$) in cases compared with controls even after adjustment for age, sex, and diabetes duration. Logistic regression analysis revealed that microRNA-191-5p was negatively associated with a 58% reduced odds ratio (OR) of chronic diabetes complications, specifically CVD, micro-macroalbuminuria, and retinopathy (OR, 0.42; 95% CI, 0.23-0.77), independent of age, sex, physical activity, educational levels, diabetes duration, glycated hemoglobin, total insulin dose, hypertension, smoking, total cholesterol, albumin excretion rate, estimated glomerular filtration rate, serum vascular cell adhesion molecule-1, and tumor necrosis factor-α. Analyses performed separately for each complication demonstrated a significant independent association with albuminuria (OR, 0.36; 95% CI, (0.18-0.75) and CVD (OR, 0.34; 95% CI, 0.16-0.70).

Conclusions: In DM1 subjects, microRNA-191-5p is inversely associated with vascular chronic complications of diabetes.

Key Words: microRNAs, type 1 diabetes, diabetic complications, cardiovascular diseases, albuminuria, diabetic nephropathy

Abbreviations: AER, albumin excretion rate; AMI, acute myocardial infarction; CV, cardiovascular; CVD, cardiovascular disease; DM1, type 1 diabetes mellitus; DM2, type 2 diabetes mellitus; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; OR, odds ratio; PCS, Prospective Complication Study; sVCAM1, soluble vascular cell adhesion molecule-1.

Individuals with type 1 diabetes mellitus (DM1) have a greater risk of having micro-macrovascular complications and identification of novel biomarkers for early diagnosis, risk stratification, and progression prediction is clinically relevant.

MicroRNAs (miRs) are a class of noncoding RNAs containing 18 to 24 nucleotides (1). They regulate a vast array of biological processes and participate in the pathogenesis of chronic complications of diabetes (2, 3). Although miRs exert their function intracellularly, they are also found in body fluids. Moreover, miRs are very stable in the circulation (4-6), where they often display a disease-specific expression profile. Therefore, circulating miRs have been proposed as non-invasive clinical biomarkers in several pathophysiological conditions, including diabetes complications (7-9).

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MicroRNA-191-5p is predominantly expressed by platelets and endothelial cells (10, 11). MicroRNA-191-5p can modulate a vast array of cellular processes, including proliferation, differentiation, migration, and apoptosis, by targeting both cell cycle-associated genes and transcription factors (12, 13). Moreover, it can restrain inflammation by inhibiting the CCAAT enhancer binding protein β/NLR Family Pyrin Domain Containing 3 pathway (14) and is considered a marker of platelet activation/function (15).

Data on circulating miR-191-5p levels in individuals with micro-macrovascular diseases are scarce. However, serum levels of miR-191-5p were reduced in subjects with acute myocardial infarction (AMI) (16, 17). Lower miR-191-5p values were prospectively associated with an enhanced risk of reinfarction (18). Conversely, elevated circulating miR-191-5p levels were found in an animal model of ischemic stroke (19) and in children with nephrotic syndrome (20).

In the context of diabetes, Zampetaki et al found reduced serum miR-191-5p levels in those with type 2 diabetes (DM2) compared with controls (21); this was confirmed by a subsequent case-control study (22). Moreover, miR-191-5p appears to delay wound healing in those with DM2 with peripheral vascular disease by inhibiting both migration and angiogenesis (11). Data in patients with DM1 are lacking; however, we previously demonstrated that miR-191-5p was 1 of the 25 miRs deregulated in a profiling study performed on pooled samples obtained from serum of those with DM1 with and without vascular complications (23). In this study, we assessed the potential associations of microRNA-191-5p with chronic complications of DM1 by assessing serum miR-191-5p levels in patients with DM1 from the EURODIAB Prospective Complication Study (PCS).

Materials and Methods

EURODIAB Study

The EURODIAB Insulin-dependent diabetes mellitus Complications Study (1989-1991) was carried out to determine risk factors for micro-macrovascular diabetes complications in subjects with DM1 (n = 3250) (24). Participants were aged between 15 and 60 years and were enrolled from 31 centers in 16 European countries. DM1 was clinically defined as a diagnosis made before the age of 36 years, with a continuous need for insulin therapy within 1 year of diagnosis.

Six to 8 years after baseline examinations, 1880 participants were reexamined (1997-1999) in the follow-up study (EURODIAB Prospective Complication Study PCS). Data on complications were available on 1296 patients (25). Among those who were not reexamined, a total of 437 individuals had been recruited from centers that were not involved in the follow-up process. Additionally, 101 individuals died, 465 participants only provided morbidity data, and 367 individuals could not be traced or were otherwise unavailable.

Nested Case-control Study: Patient Selection

At the follow-up examination of the EURODIAB PCS, a nested case-control study was designed (26). Cases were selected to have the greatest complication burden as possible to provide sufficient numbers for subgroup analyses (27, 28). Controls were selected to be completely free of complications. Thus, cases were all those with cardiovascular disease (CVD) or proliferative retinopathy or macroalbuminuria at follow-up and all those with microalbuminuria and some degree of retinopathy (n = 356). Control subjects were individuals who had no evidence of CVD, retinopathy, or neuropathy and were normo-albuminuric at follow-up (n = 185). Controls and cases were unmatched, so that the impact of key variables could still be assessed, and any adjustments were performed at the analysis stage.

Of these 541 individuals, full clinical data and serum samples for miR-191-5p measurement were available for 447 subjects (293 cases and 154 controls) (Fig. 1). Twenty-seven samples were excluded because both miR-191-5p and the endogenous control U6 were undetectable or because of poor RNA quality; therefore, the analyses were performed on 277 cases and 143 controls.

All data and samples, including the presence of micro-macrovascular diabetes-related complications, were exclusively collected at the follow-up stage. Serum samples were immediately aliquoted, frozen, and stored in a dedicated −80 °C freezer. Only new aliquots, which had not undergone any freeze-thaw cycles, were used. The study was approved by the Ethical Committee, the procedures were in accordance with the Helsinki Declaration, and informed consent was obtained from all subjects involved in the study.

Definitions and Measurements

Hypertension was defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg and/or treatment with blood pressure-lowering drugs (29). Retinopathy was assessed according to the EURODIAB protocol (30). Twenty-four-hour urine collection was performed to measure albumin excretion rate (AER) and AER was categorized as normo-albuminuria (<20 μg/min), micro-albuminuria (20-200 μg/min), and macro-albuminuria (>200 μg/min) (31). The Modification of Diet in Renal Disease Study equation was used to calculate the estimated glomerular filtration rate (eGFR) (32). CVD was defined as coronary artery bypass graft, AMI, angina, ischemic/hemorrhagic stroke, and/or ischemic electrocardiogram changes. Information on the total daily insulin corrected for body weight (IU/kg/day), educational levels (age at completion), and physical activity was collected through a questionnaire. Physical activity was assessed based on sports participation, walking distance, regular bicycling, and expressed as a dichotomous variable. Specifically, physically inactive participants

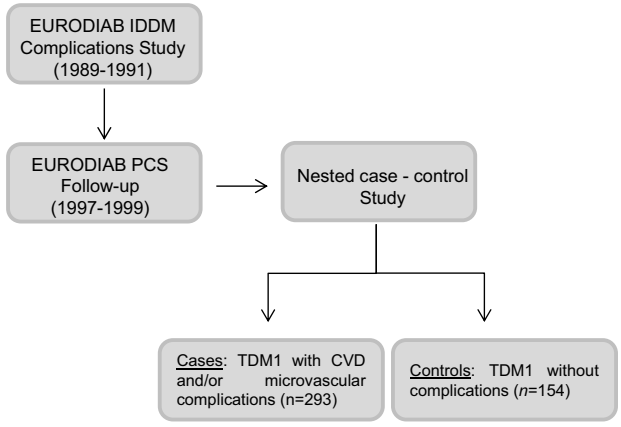

Figure 1. Design of the study.

were those who reported walking less than 1.5 km on an average weekday, no regular bicycling, and no participation in sports. Participants who reported walking 1.5 km or more on an average weekday, cycling regularly, or playing in any sport were considered to be the physically active group (33).

Soluble vascular cell adhesion molecule-1 (sVCAM1) and TNF-α were measured by ELISA (Cat# DTA00D, RRID: AB_2941365; Cat# DVC00, RRID:AB_2941366, R&D Systems), as previously reported (34).

Total RNA Extraction and miR Expression Analysis

RNA was extracted from 200 µL of serum samples using TRIzol LS reagent (Thermo Fisher, Milan, Italy). The spike-in Cel-microRNA-39 (3 µL) was added immediately before RNA extraction. The RNA quality was evaluated by automated electrophoresis (Agilent Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA, USA) and an RNA integrity number ≥7.0 was considered acceptable. Expression of miR-191-5p, Cel-miR-39, and U6 snRNA was carried out following the manufacturer's guidelines (Thermo Fisher). In brief, reverse transcriptase reactions containing RNA (3 µL), reverse transcriptase, deoxynucleotide triphosphates, RNAse inhibitor, specific stem-loop primers, and 1X buffer were incubated in a Veriti Thermocycler (Thermo Fisher) at 16 °C for 30 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes and then held at 4 °C. The reverse transcriptase products were preamplified using the Megaplex PreAmp Primers (Thermo Fisher) by heating the samples at 95 °C for 10 minutes, followed by 12 cycles at 95 °C for 15 seconds and 60 °C for 4 minutes. Quantitative PCR for miR expression levels was performed by combining the preamplification products with TaqMan Universal PCR Master Mix (No AmpErase UNG) and TaqMan miRNA Assay on an Applied Biosystems 7900HT thermocycler (95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 second and 60°C for 1 minute). The following TaqMan miRNA Assay were used: microRNA-191-5p (002299), U6 snRNA (001973), and Cel-microRNA-39 (000200). Both Cel-microRNA-39 and U6 snRNA were used to normalize the results. Samples with Ct of both U6 snRNA and miR-191-5p ≥35 cycles/undetermined were not included in the analyses. If Ct of Cel-miR-39 were ≥35 cycles/undetermined, samples were rerun. The relative expression was calculated by using the comparative Ct method (2-ΔΔCt).

Data Presentation and Statistical Analysis

Data were presented as mean ± SD. Normality was tested using both the Shapiro-Wilk and the Kolmogorov-Smirnov tests. Additionally, a Q-Q plot was generated to visually evaluate normality. Nonnormally distributed variables (miR-191-5p, triglycerides, AER, TNF-α, sVCAM-1) were expressed as median (25th-75th percentiles) and they were log-transformed before analyses. Pearson's correlation coefficient analysis was used to explore the relationship between miR-191-5p values and clinical variables. Logistic regression analysis was performed to estimate the odd ratios (ORs) of miR-191-5p for any complication (micro-macroalbuminuria, retinopathy, CVD), independently of known risk factors and confounders. The likelihood ratio test was used to compare nested models examining the role of age, sex, physical activity, education level, smoking, diabetes duration, hypertension, total insulin dose, glycated hemoglobin (HbA1c), AER, eGFR, total cholesterol, TNF-α, and sVCAM1.

Secondary analyses

Given the hypothesis of a distinct role of miR-191-5p in various micro/macrovascular complications, logistic regression models were separately applied to each complication and to patients with micro- and macro-albuminuria. Furthermore, to address the potential confounding influence of aspirin, which is known to decrease miR-191-5p levels, logistic regression analysis was also rerun after the exclusion of subjects under therapy with aspirin.

All analyses were performed using SPSS 28.0 software. $P < .05$ was considered significant.

Results

Characteristics of Patients

Participants (n = 420) had a mean age of 39.5 (± 10.1) years, an average diabetes duration of 21.6 (± 9.6) years, and a similar proportion of women (48.9%) and men (51.1%). Table 1 shows the risk factor profile in both cases and controls. Among cases, 112 patients had CVD (40.4%). Diabetic nephropathy was present in 170 (micro-albuminuria [40.6%] and macro-albuminuria [59.4%]) and diabetic retinopathy in 243 subjects (background [47.7%] and proliferative [52.3%]). However, most cases (56.7%) had both nephropathy and retinopathy.

Serum miR-191-5p Levels

Individual Ct values of miR-191-5p, U6 snRNA, and Cel-microRNA-39 are reported in Table 2. The distribution of miR-191-5p values was left-skewed and miR-191-5p was significantly ($P < .001$) reduced in cases (4.42 [1.91-10.59]) compared with controls (7.12 [2.88-19.14]) even after adjustment for sex, age, and diabetes duration ($P < .001$) (Fig. 2A). Subgroup analyses by each complication revealed that circulating miR-191-5p levels were significantly reduced in subjects with nephropathy (3.92 [6.84-10.42]; $P < .001$), retinopathy (4.62 [7.71-10.82], $P < .001$), or CVD (4.43 [6.70-10.86], $P < .001$) compared with controls (7.12 [2.88-19.14]) (Fig. 2B). Adjustment for age, sex, and diabetes duration did not modify the results. The majority of the patients (91.4%) had normal eGFR values (≥60 mL/min) and mean eGFR levels were similar among patients with CVD, nephropathy, and retinopathy (CVD, 87.51 ± 25.8; nephropathy, 87.88 ± 38.22; retinopathy, 89.44 ± 26.29).

Linear Correlations

Values of miR-191-5p correlated directly with eGFR ($r = 0.17$, $P = .000$) and inversely with age ($r = -0.13$, $P = .004$), diabetes duration ($r = -0.14$, $P = .003$), AER ($r = -0.14$, $P = .001$), TNF-α ($r = -0.14$, $P = .006$), and sVCAM1 ($r = -0.10$, $P = .043$). There was no significant correlation between miR-191-5p and body mass index ($r = -0.04$, $P = .44$), HbA1c ($r = 0.02$, $P = .64$), systolic blood pressure ($r = -0.04$, $P = .43$), diastolic blood pressure ($r = -0.03$, $P = .44$), triglycerides (−0.08, $P = .07$), and total cholesterol (−0.08, $P = .11$).

Logistic Regression Analyses

Logistic regression analyses were carried out to evaluate whether lower miR-191-5p values conferred an enhanced OR of having complications independently of main confounders and risk factors. In model 1 adjusted for age, sex, HbA1c,

Table 1. Characteristics of the 420 subjects with DM1 recruited in the nested case-control study of the EURODIAB PCS

	Case subjects	Control subjects	*P*
N	277	143	
Age, y	41.5 ± 10.7	35.6 ± 7.10	<.001
Diabetes duration, y	25.0 ± 9.1	15.3 ± 7.12	<.001
Males, %	52.9	47.7	.43
BMI, kg/m^2	24.8 ± 3.5	23.7 ± 2.60	<.001
HbA1c, mmol/mol	76 ± 0.8	61 ± 1.2	<.001
SBP, mm Hg	127.3 ± 21.5	114.8 ± 13.5	<.001
DBP, mm Hg	75.9 ± 11.3	73.5 ± 10.7	<.05
Hypertension, %	55.9	13.4	<.001
Total cholesterol, mmol/L	5.5 ± 1.2	4.9 ± 1.1	<.001
LDL-cholesterol, mmol/L	3.3 ± 1.1	2.8 ± 1.0	<.001
HDL-cholesterol, mmol/L	1.6 ± 0.4	1.7 ± 0.4	.157
Triglycerides, mmol/L	1.14 (0.81-1.59)	0.82 (0.63-1.10)	<.001
eGFR, mL/min/1.73 m^2	90.3 ± 25.3	106.0 ± 14.0	<.01
Serum sVCAM1, ng/mL	408.0 (315-409)	362.0 (341-504)	<.001
Serum TNF-α, pg/mL	3.18 (2.39-4.32)	2.30 (1.74-2.87)	<.001

Data are expressed as mean ± SD, percentage or median (25%-75% percentile) for log-transformed data.
Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCS, Prospective Complication Study; SBP, systolic blood pressure; sVCAM-1, soluble vascular cell adhesion molecule-1.

and diabetes duration, miR-191-5p values were negatively associated with all complications (OR, 0.44 [95% CI, 0.27-0.72]) as well as with CVD, albuminuria, and retinopathy examined separately (Table 3). After inclusion of AER and eGFR into the model (model 2), the association was still significant for all complications (OR, 0.49 [95% CI, 0.28-0.86]), CVD (OR, 0.45 [95% CI, 0.24-0.86]), and albuminuria (OR, 0.46 [95% CI, 0.25-0.84]), but it was no longer significant for retinopathy. The inverse association between miR-191-5p levels and all complications, CVD, and albuminuria was strengthened by the adjustment for cardiovascular (CV) risk factors (hypertension, smoking, total cholesterol) (model 3), markers of inflammation-driven vascular injury (TNF-α, sVCAM-1) (model 4), and other demographic and clinical parameters (physical activity, educational level, and total insulin dose) (model 5). In the fully adjusted model (model 5), an increase of 1 unit in miR-191-5p levels was associated with a 58% decrease in the OR for all complications, a 66% decrease in the OR for CVD, and a 64% decrease in the OR for albuminuria. Subanalyses performed separately in patients with micro- and macroalbuminuria revealed that miR-191-5p levels were inversely and independently associated with both degree of albuminuria (micro OR, 0.41 [0.18-0.91]; macro OR, 0.25 [0.08-0.75]). Finally, results of logistic regression analyses were not modified by the exclusion of the 19 subjects under treatment with aspirin (Table 4).

Discussion

The present study assessed the potential association between serum moR-191-5p levels and chronic DM1 complications in the EURODIAB PCS. Results demonstrated an independent and inverse relationship between serum miR-191-5p levels and the risk of chronic complications of diabetes.

Levels of miR-191-5p were significantly reduced in cases compared with controls even after adjustment for age, sex, and diabetes duration. Furthermore, in logistic regression analysis miR-191-5p levels were inversely associated with the risk of chronic diabetes complications, specifically CVD, micro-macroalbuminuria and retinopathy, independently of age, sex, physical activity, educational level, smoking, diabetes duration, total insulin dose, HbA1c, hypertension, total cholesterol, albuminuria, and eGFR. This suggests that measurement of circulating miR-191-5p levels may have an additional value over traditional risk factor assessment in identifying subjects at risk of complications.

The inclusion of TNF-α and sVCAM-1 in the logistic regression model did not affect the strength of the association. Therefore, the inverse association between miR-191-5p and vascular complications was not mediated by inflammation. In line with this, a positive rather than a negative correlation between serum miR-191-5p and inflammatory cytokines has been previously reported (11).

Platelets release miR-191-5p-enriched extracellular vesicles and treatment with anti-platelet agents reduces circulating miR-191-5p levels (35). Therefore, we cannot exclude the possibility that antiplatelet therapy administered to patients with DM1 and micro/macrovascular complications may explain their lower circulating miR-191-5p levels. However, only a minority of cases was treated with antiplatelet agents; exclusion of these patients did not modify the results.

Analyses carried out separately for each complication demonstrated an inverse and independent relationship of miR-191-5p with both CVD and albuminuria. The association between miR-191-5p and diabetic retinopathy was likely mediated by albuminuria because most patients with albuminuria also had retinopathy and the association was no longer significant after adjustment for eGFR and albuminuria. A role of renal function in mediating the relationship is unlikely as most patients had normal renal function and eGFR levels were similar in patients with retinopathy and nephropathy.

In the fully adjusted model, the OR of albuminuria was 64% lower for each unit increment of log-miR-191-5p levels. Furthermore, a significant and independent association

Table 2. Mean Ct values for miR-191-5p, U6 snRNA, and Cel-miR-39 in both controls and cases

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
1	19.14	14.68	25.61
2	16.95	19.58	21.01
3	15.84	16.18	22.78
4	19.55	13.07	23.71
5	19.66	16.19	27.24
6	19.31	17.84	22.63
7	20.09	22.08	24.74
8	18.80	12.87	24.06
9	18.39	14.70	21.56
10	16.93	13.78	21.58
11	17.17	13.73	21.89
12	20.67	14.89	27.88
13	20.39	20.84	24.62
14	16.78	16.19	25.79
15	20.03	19.63	25.42
16	17.14	11.89	25.73
17	20.54	15.23	24.39
18	20.29	13.95	27.07
19	18.48	14.98	21.88
20	19.05	16.26	25.82
21	20.50	16.19	25.29
22	16.30	13.10	20.53
23	18.78	15.48	20.99
24	19.90	13.14	24.83
25	19.70	18.57	24.02
26	19.71	14.97	24.16
27	20.38	13.57	21.61
28	18.01	13.70	23.28
29	20.17	13.19	22.65
30	18.04	13.27	23.67
31	18.78	17.69	23.04
32	19.90	16.19	22.95
33	17.36	13.42	21.45
34	21.34	20.99	23.21
35	19.94	13.93	21.55
36	19.74	17.05	28.60
37	19.20	14.38	23.01
38	17.98	15.20	23.63
39	18.90	15.22	21.30
40	20.13	19.38	25.24
41	16.36	13.90	23.46
42	19.53	15.31	25.77
43	22.55	19.63	27.21
44	21.17	16.65	23.99
45	20.52	13.38	22.67
46	21.16	15.74	24.96
47	19.46	15.75	21.98
48	20.56	15.20	27.08
49	16.82	13.53	25.50
50	24.26	12.34	22.31
51	18.45	13.74	22.92
52	20.84	18.37	29.55
53	20.65	15.06	23.76
54	19.29	15.06	29.81
55	23.63	15.20	27.16
56	18.18	17.44	25.81
57	19.84	12.92	26.09
58	19.03	16.56	23.25
59	19.16	11.94	21.63
60	19.93	20.26	23.81
61	19.12	15.29	27.05
62	20.48	15.74	22.99
63	18.16	12.83	25.03
64	18.16	17.05	22.67
65	18.51	15.25	23.98
66	20.11	14.31	23.24
67	21.36	23.96	26.17
68	19.29	16.29	21.76
69	17.69	15.10	22.37
70	19.93	13.12	27.14
71	18.57	13.53	24.14
72	22.26	18.30	23.09
73	18.21	17.17	26.88
74	22.70	27.41	20.30
75	21.15	15.06	23.87
76	18.70	13.11	23.86
77	17.95	13.17	20.30
78	19.93	12.75	24.37
79	20.69	17.18	25.22
80	21.40	16.49	26.34
81	19.94	14.69	23.76
82	19.67	22.22	19.92
83	20.82	22.39	23.41
84	19.21	13.99	28.39
85	22.07	18.78	26.64
86	20.40	19.69	19.75
87	21.97	17.80	23.71
88	20.16	17.59	24.86
89	20.89	19.76	24.48
90	22.38	15.01	23.56
91	19.99	13.94	21.67
92	21.30	24.60	25.14
93	20.99	16.61	22.63
94	17.91	16.09	25.09
95	21.27	17.37	23.55
96	17.18	15.77	24.72
97	21.08	16.32	25.08
98	18.10	14.09	20.49
99	21.57	20.10	25.93
10	22.51	14.91	23.94
101	20.82	15.62	24.94

(*continued*)

(*continued*)

Table 2. Continued

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
102	21.03	17.99	23.54
103	18.41	16.12	25.71
104	20.59	24.44	20.52
105	19.92	18.21	24.60
106	27.97	16.68	32.15
107	18.58	17.19	22.52
108	18.25	13.47	23.24
109	20.71	20.66	23.99
110	18.91	14.78	20.46
111	21.44	16.70	26.34
112	17.47	15.82	21.42
113	21.13	12.87	25.42
114	18.78	17.66	25.81
115	20.22	15.09	23.35
116	19.05	19.55	21.44
117	18.31	16.53	23.99
118	17.19	13.42	22.42
119	18.43	12.10	23.01
120	18.29	16.91	25.19
121	17.33	15.56	24.06
122	17.21	13.63	23.74
123	17.10	18.56	23.07
124	32.33	25.76	31.55
125	18.65	15.78	28.27
126	18.63	18.95	26.61
127	17.03	14.60	21.70
128	19.93	17.57	22.50
129	17.62	14.58	22.75
130	20.57	21.47	25.16
131	21.53	15.94	24.98
132	17.42	13.88	24.40
133	20.17	15.52	23.41
134	17.25	13.05	22.30
135	19.99	15.00	23.89
136	19.04	15.16	23.86
137	20.12	23.28	20.67
138	28.24	27.18	31.15
139	18.34	15.60	28.49
140	17.53	14.31	22.66
141	17.07	14.92	21.38
142	16.75	14.44	23.93
143	18.77	13.98	20.84
144	18.22	23.06	22.31
145	18.77	14.94	21.86
146	21.08	13.80	24.69
147	18.95	13.78	22.33
148	17.85	13.32	20.20
149	17.99	17.58	25.01
150	22.21	18.84	26.95
151	17.49	12.67	22.29
152	18.54	19.42	22.10

(*continued*)

Table 2. Continued

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
153	19.59	15.61	25.44
154	19.08	12.87	20.77
155	18.52	15.30	24.82
156	21.09	18.74	23.09
157	12.85	19.17	14.74
158	19.45	21.45	19.83
159	19.06	11.57	25.70
160	19.26	13.68	27.38
161	20.51	14.49	21.43
162	21.54	15.32	28.11
163	17.63	11.64	26.62
164	21.88	14.31	28.51
165	20.46	16.49	24.05
166	19.24	17.00	24.44
167	20.71	14.85	27.44
168	19.17	11.99	24.03
169	28.43	22.48	26.35
170	21.71	16.74	26.47
171	20.67	16.31	25.85
172	18.49	13.46	23.01
173	25.44	13.17	24.02
174	20.13	15.29	24.12
175	20.39	15.20	24.32
176	18.04	13.04	22.81
177	21.31	20.18	24.98
178	23.01	13.36	23.33
179	18.25	15.52	23.06
180	20.43	17.13	25.22
181	20.26	15.48	24.32
182	19.11	14.02	22.26
183	20.61	16.10	24.94
184	23.80	16.57	24.71
185	20.45	20.40	24.74
186	22.51	15.76	25.83
187	20.31	17.48	23.62
188	18.95	12.76	24.51
189	19.77	14.24	25.74
190	17.56	20.79	22.66
191	21.64	17.18	28.27
192	21.44	20.16	28.96
193	19.40	19.34	24.08
194	17.62	18.07	23.23
195	20.12	15.98	23.95
196	20.49	16.00	22.98
197	18.70	17.83	22.76
198	19.27	13.97	22.94
199	18.49	19.22	23.56
200	19.18	15.19	24.16
201	18.62	12.85	23.32
202	17.53	17.34	27.29
203	17.80	14.15	25.97

(*continued*)

Table 2. Continued

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
204	18.08	13.26	21.52
205	23.29	18.63	22.09
206	18.92	16.36	24.88
207	19.90	10.92	25.52
208	24.53	21.78	21.70
209	18.87	15.12	22.89
210	24.86	16.76	27.76
211	18.38	12.68	22.06
212	19.77	19.35	24.07
213	20.42	18.89	22.35
214	19.42	14.72	28.13
215	20.11	17.97	23.21
216	18.07	14.42	22.34
217	17.25	12.74	21.76
218	19.24	19.39	21.77
219	17.08	13.63	22.65
220	19.01	17.90	25.28
221	32.33	15.10	23.13
222	19.52	20.43	22.37
223	32.33	26.81	23.36
224	16.97	15.03	22.93
225	21.89	16.95	22.48
226	16.91	13.70	25.71
227	21.85	16.61	25.06
228	18.74	15.11	21.39
229	17.82	17.73	24.63
230	17.30	14.90	22.94
231	17.71	16.26	22.60
232	19.51	21.08	23.13
233	16.10	12.56	24.93
234	17.05	16.68	22.70
235	18.23	15.18	26.59
236	17.91	13.26	24.91
237	17.23	12.71	23.93
238	19.18	14.56	24.59
239	20.17	16.68	23.79
240	20.68	15.36	25.63
241	18.47	13.48	22.99
242	18.37	15.36	25.68
243	16.75	14.00	21.68
244	19.90	14.82	23.18
245	26.02	21.13	26.81
246	18.46	13.33	23.47
247	17.81	18.10	22.75
248	17.74	16.50	22.66
249	26.58	21.12	24.78
250	19.28	13.69	23.43
251	20.09	17.88	22.73
252	19.57	16.03	26.30
253	19.26	15.50	25.30
254	22.06	17.12	26.58

(*continued*)

Table 2. Continued

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
255	20.83	14.10	28.59
256	18.31	13.03	28.43
257	18.15	14.78	24.48
258	17.77	14.70	26.80
259	23.79	16.31	24.74
260	18.77	14.78	26.86
261	21.19	16.58	28.44
262	16.32	17.48	22.58
263	18.70	20.27	29.67
264	18.98	13.84	22.42
265	21.59	15.95	25.52
266	19.76	15.27	26.57
267	20.56	16.33	23.26
268	18.94	16.52	22.02
269	21.92	19.67	26.13
270	24.02	13.81	24.61
271	16.42	14.93	23.05
272	17.45	12.64	20.24
273	18.86	13.85	20.56
274	16.89	13.88	19.10
275	17.33	18.77	23.76
276	19.26	13.01	23.39
277	15.71	11.71	17.64
278	18.68	13.56	25.12
279	18.34	14.83	22.77
280	17.77	19.94	25.13
281	21.42	14.38	24.60
282	16.50	12.70	23.40
283	15.07	13.84	21.42
284	16.25	12.85	22.67
285	19.72	11.42	24.27
286	19.58	12.56	26.07
287	18.21	12.53	24.82
288	24.44	12.20	24.76
289	19.95	17.54	26.07
290	16.51	12.06	23.76
291	18.47	19.55	24.45
292	18.39	15.50	28.68
293	21.40	15.69	24.18
294	17.50	12.16	21.43
295	19.67	17.63	30.43
296	23.10	14.41	23.27
297	16.29	19.04	21.23
298	18.17	15.67	26.62
299	18.31	17.84	23.24
300	29.66	16.82	28.09
301	18.16	12.34	21.74
302	21.00	16.33	21.16
303	16.34	17.71	21.96
304	19.92	14.37	30.56
305	18.70	14.51	25.24

(*continued*)

Table 2. Continued

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
306	17.62	16.11	22.43
307	15.98	16.98	22.01
308	14.54	18.42	17.12
309	18.90	19.25	25.73
310	18.57	14.20	24.04
311	21.39	15.84	27.08
312	16.72	13.84	22.70
313	19.53	15.26	22.05
314	19.47	13.08	21.69
315	18.32	13.78	24.59
316	16.68	12.67	16.11
317	14.39	14.97	21.60
318	23.07	21.58	24.88
319	27.64	23.45	24.77
320	15.49	13.26	17.12
321	21.51	19.17	24.49
322	20.90	19.10	22.31
323	19.25	18.78	23.94
324	18.26	17.17	20.87
325	18.07	12.19	18.43
326	20.42	14.54	26.25
327	20.60	15.63	24.12
328	20.84	15.94	29.05
329	18.68	16.46	20.77
330	20.58	21.19	28.24
331	18.97	11.79	24.97
332	21.36	14.40	27.15
333	18.21	12.93	24.62
334	18.45	13.54	22.34
335	18.07	13.50	24.16
336	20.23	12.64	24.01
337	17.54	14.57	21.65
338	18.91	15.34	24.54
339	21.75	22.59	25.57
340	15.72	13.74	23.93
341	18.17	16.73	23.86
342	24.86	13.08	21.77
343	23.39	18.53	23.43
344	17.22	13.45	23.52
345	19.28	17.71	22.17
346	18.63	13.23	23.86
347	20.22	17.87	22.59
348	18.51	17.24	20.59
349	17.95	20.35	20.98
350	21.97	19.15	26.45
351	18.38	13.95	24.06
352	19.47	15.14	27.66
353	19.73	13.98	25.25
354	17.93	13.10	24.55
355	18.33	14.33	24.79
356	21.82	18.82	24.51

(*continued*)

Table 2. Continued

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
357	17.80	16.45	22.90
358	18.31	13.19	25.71
359	17.65	19.31	22.50
360	17.24	15.86	23.96
361	18.20	11.89	20.74
362	18.55	15.57	25.27
363	20.27	16.22	23.93
364	19.03	13.41	20.09
365	18.39	19.93	21.35
366	17.48	13.54	23.21
367	17.18	13.12	24.76
368	19.85	19.58	27.68
369	17.50	12.28	21.49
370	30.20	28.48	25.51
371	19.03	13.14	27.80
372	19.56	15.88	24.57
373	18.56	15.23	23.65
374	18.70	12.59	21.36
375	18.17	19.49	26.28
376	17.32	14.63	26.56
377	17.25	12.81	23.28
378	18.47	17.99	22.99
379	17.44	12.51	23.79
380	16.30	17.08	24.19
381	17.20	14.17	22.19
382	17.34	14.87	28.36
383	18.94	14.12	28.02
384	20.79	16.57	24.20
385	16.16	14.81	23.72
386	18.08	15.19	23.80
387	27.83	15.72	23.15
388	21.21	23.18	23.34
389	17.95	12.96	22.67
390	17.38	15.22	23.59
391	18.21	13.84	22.72
392	17.81	18.07	24.16
393	17.89	14.95	24.31
394	17.41	18.65	22.02
395	21.03	20.42	24.77
396	22.08	19.54	25.78
397	20.47	20.93	25.90
398	20.15	16.06	27.80
399	17.70	14.61	24.45
400	17.87	17.25	25.54
401	17.74	14.96	22.18
402	20.49	15.17	24.02
403	19.47	16.70	26.35
404	16.89	12.88	19.40
405	15.74	17.01	22.93
406	20.73	15.90	28.43
407	19.07	16.87	26.91

(*continued*)

Table 2. Continued

ID	microRNA-191	Cel-microRNA-39	U6 snRNA
408	18.15	14.89	21.03
409	24.15	25.08	24.53
410	15.27	11.74	19.79
411	18.47	16.10	23.76
412	16.90	19.32	21.79
413	17.05	14.58	21.61
414	17.76	13.80	22.07
415	17.85	16.51	25.15
416	19.49	13.75	21.87
417	20.28	18.58	27.25
418	20.34	22.47	25.13
419	22.18	13.53	24.65
420	18.24	13.47	22.00

between albuminuria and miR-191-5p persisted even when micro- and macroalbuminuria were analyzed separately. The underlying mechanism is unknown; however, treatment with miR-191-5p reduced renal histological damage, inflammatory cytokine production, and apoptosis in a model of acute kidney damage via oxidative stress responsive kinase 1 inhibition (36). This suggests that lower miR-191-5p levels may favor the activation of deleterious pro-apoptotic/pro-inflammatory pathways involved in the development of albuminuria. At variance with our findings, previous studies reported increased miR-191-5p levels in both renal tissue and sera from children with nephrotic syndrome (20, 37) and greater miR-191-5p content in plasma exosomes from subjects with nondiabetic hypertension and albuminuria (38). However, the different clinical context and/or biological samples do not allow direct comparisons.

We also found a direct and significant correlation between miR-191-5p and eGFR. In keeping with this, miR-191-5p was an independent determinant of eGFR in a previous study performed in nondiabetic individuals with hypertension (39). However, in our study, the association of miR-191-5p with albuminuria was independent of eGFR. The number of patients with eGFR below 60 mL/min/1.73 m^2 was too small to assess whether low miR-191-5p conferred an increased OR of stage 3 chronic kidney disease.

Logistic regression analysis also showed an inverse relationship between miR-191-5p and the risk of CVD that was independent of both demographic and diabetes-related confounders. Of interest, the strength of the association increased after inclusion of smoking, total cholesterol, and arterial hypertension into the model. The mechanism is unclear; however, these CV risk factors may increase both CVD and miR-191-5p, either directly or through platelet activation, and hence partially mask the inverse association between miR-191-5p and CVD. In line with this hypothesis, cigarette smoke extracts have been shown to increase miR-19-5p levels in vitro (40). Moreover, miR-191-5p targets the proprotein convertase subtilisin/kexin type 9 enzyme, suggesting an interplay between miR-191-5p and cholesterol metabolism (41).

Previous data on the role of miR-191-5p in CVD are limited, and most available studies were carried out in the acute setting. An miRNA expression profile identified miR-191-5p as an miRNA downregulated in subjects with AMI; this finding was validated in subsequent small case-control studies (16, 17). Recently, a prospective nested case-control study performed on patients with ST-elevation myocardial infarction, who underwent primary percutaneous coronary intervention, showed that miR-191-5p levels were reduced in patients who experienced major adverse cardiovascular events during the 2-year follow up compared with individuals who remained free of CV events (18). Moreover, in logistic regression analysis miR-191-5p was inversely associated with AMI recurrence. These data combined with our results raise the possibility that miR-191-5p levels may represent a novel prognostic biomarker in subjects at high CV risk.

The mechanism of the inverse relationship between miR-191-5p and CVD is unknown. However, miR-191-5p has protective and antiapoptotic effects on the endothelium. Moreover, miR-191-5p enhanced cell viability and reduced apoptosis in cardiomyocytes exposed to hypoxia through modulation of TNF receptor associated factor 3 signalling

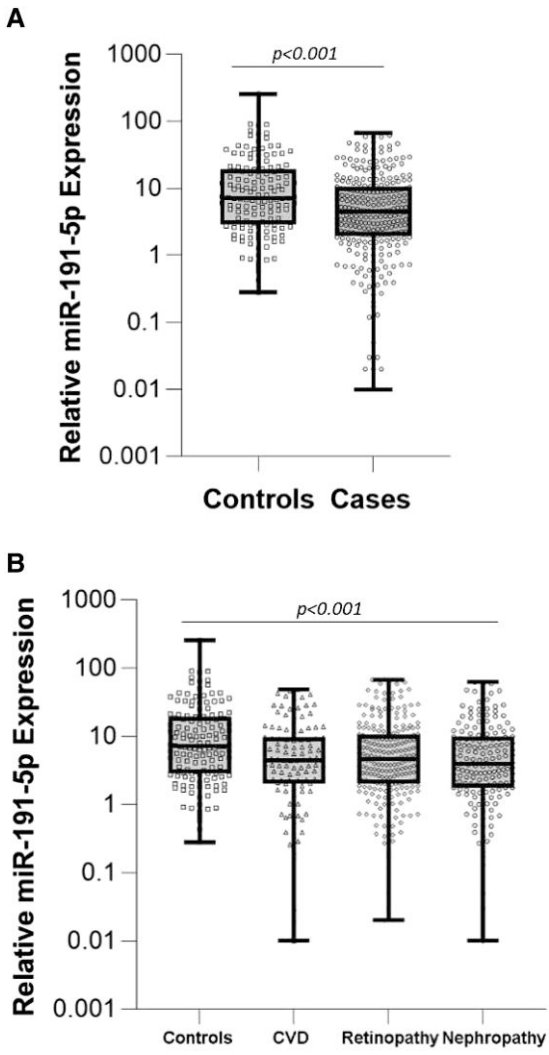

Figure 2. Serum miR-191-5p levels in patients with DM1 from the EURODIAB nested case-control study. (A) Cases (n = 277) with vascular complication vs. controls (n = 143) without any complication ($P < .001$). (B) Controls vs. patients with cardiovascular diseases (CVD; n = 112), diabetic retinopathy (n = 243), and diabetic nephropathy (n = 170) ($P < .001$ controls vs. others).

Table 3. Odds ratios (ORs) for diabetes complications by microRNA-191-5p levels in the nested case-control study of the EURODIAB PCS

	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)	Model 5 OR (95% CI)
All complications	0.44 (0.27-0.72)	0.49 (0.28-0.86)	0.46 (0.25-0.83)	0.44 (0.24-0.81)	0.42 (0.23-0.77)
CVD	0.46 (0.26-0.83)	0.45 (0.24-0.86)	0.36 (0.18-0.72)	0.33 (0.16-0.69)	0.34 (0.16-0.70)
Albuminuria[a]	0.38 (0.21-0.69)	0.46 (0.25-0.84)	0.44 (0.23-0.85)	0.39 (0.20-0.80)	0.36 (0.18-0.75)
Micro	—	—	—	—	0.41 (0.18-0.91)
Macro	—	—	—	—	0.25 (0.08-0.75)
Retinopathy	0.51 (0.29-0.89)	0.58 (0.31-1.10)	0.53 (0.26-1.06)	0.52 (0.25-1.08)	0.49 (0.23-1.02)

Model 1: adjusted for age, sex, glycated hemoglobin, diabetes duration.
Model 2: model 1 + log-AER, eGFR.
Model 3: model 2 + hypertension, total cholesterol, smoking.
Model 4: model 3 + log-TNF-α, log-VCAM-1.
Model 5: model 4 + physical activity, educational level, total insulin dose.
Abbreviations: AER, albumin excretion rate; CVD, cardiovascular diseases; eGFR, estimated glomerular filtration rate; PCS, Prospective Complications Study; sVCAM-1, soluble vascular cell adhesion molecule- 1.
[a]Not adjusted for log-AER.

Table 4. Odds ratios (ORs) for diabetes complications by microRNA-191-5p levels in the nested case-control study of the EURODIAB Prospective Complications Study

	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)	Model 5 OR (95% CI)
All complications	0.45 (0.27-0.74)	0.52 (0.29-0.91)	0.48 (0.26-0.87)	0.47 (0.25-0.86)	0.45 (0.24-0.83)
CVD	0.50 (0.27-0.90)	0.48 (0.25-0.92)	0.38 (0.19-0.77)	0.36 (0.17-0.75)	0.36 (0.17- 0.76)
Albuminuria[a]	0.39 (0.22-0.71)	0.47 (0.26-0.86)	0.45 (0.23-0.86)	0.40 (0.20-0.81)	0.38 (0.18-0.77)
Retinopathy	0.53 (0.30-0.92)	0.61 (0.32-1.18)	0.56 (0.27-1.14)	0.57 (0.27-1.18)	0.53 (0.25-1.11)

Model 1: adjusted for age, sex, glycated hemoglobin, diabetes duration.
Model 2: model 1 + log-AER, eGFR.
Model 3: model 2 + hypertension, total cholesterol, smoking.
Model 4: model 3 + log-TNF-α, log-VCAM-1.
Model 5: model 4 + physical activity, educational levels, total insulin dose.
Abbreviations: AER, albumin excretion rate; CVD, cardiovascular diseases; eGFR, estimated glomerular filtration rate; sVCAM-1, soluble vascular cell adhesion molecule-1.
[a]Not adjusted for log-AER.

(42). Finally, miR-191-5p targets the transcriptional regulator early growth response protein 1, which is induced by injury of vascular smooth muscle cells and activates pathways involved in the development of vascular disease. Importantly, a mimic of miR-191-5p was shown to suppress intimal thickening in vivo after carotid injury via Erg-1 suppression (43).

Our study had several strengths: the elevated sample size and the ability to correct the results for potential confounding effects, risk factors, and other complications. In addition, the patients came from a representative sample of European patients with DM1, making the results generalizable. There are also several limitations in this study. Although the design of the EURODIAB study was prospective, samples collection at baseline was lacking; therefore, miR-191-5p levels could only be measured at follow-up. It is not possible to prove temporal and causal relationships because of the cross-sectional design of the study. We cannot exclude the possibility of miR-191-5p degradation during storage period; however, miRNAs are extraordinarily stable in biofluids. Finally, cases and controls were unmatched for clinical variables and cases showed a risk factor profile worse than controls; however, adjustments were performed at the analysis stage.

In conclusion, the potential role of microRNAs as circulating biomarkers of diabetes complications has garnered significant interest among researchers and clinicians alike. Our study contributes to this growing body of knowledge by demonstrating a significant inverse and independent association between serum miR-191-5p levels and DM1 vascular complications and in particular with CVD and albuminuria. Our findings suggest that miR-191-5p could serve as a potential biomarker for assessing the risk of vascular complications in individuals with DM1. However, it is essential to acknowledge that further studies are necessary to validate and extend these initial findings. In particular, replication of the study results in other cohorts would enhance the robustness of the observed associations. Additionally, exploring the temporal relationship between miR-191-5p levels and the development of DM1 vascular complications would provide crucial insights into its potential as a predictive biomarker.

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Author Contributions

F.B., G.G., and S.B. undertook conceptualization and writing-original draft preparation. F.B. and G.G. provided methodology, supervision, and funding acquisition. S.B. undertook formal analysis. S.G. undertook investigation. C.S., C.D.S., N.C., and S.S.S. did data curation. G.M. did writing-review and editing. All authors reviewed the manuscript.

Disclosures

Authors affirm that the work submitted for publication is original and has not been published other than as an abstract or preprint in any language or format and has not been submitted elsewhere for print or electronic publication consideration. The authors also affirm that each person listed as authors participated in the work in accordance with ICMJE authorship guidelines and is prepared to take public responsibility for it. All authors consent to the investigation of any improprieties that may be alleged regarding the work. Each author further releases and holds harmless the Endocrine Society from any claim or liability that may arise therefrom. The authors have nothing to disclose.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Some datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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