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Association of serum MicroRNA-145-5p levels with microvascular complications of type 1 Diabetes: The EURODIAB prospective complications study

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ABSTRACT

Aims: To investigate whether serum miR-145-5p levels were associated with micro-macrovascular chronic complications in patients with type 1 diabetes (DM1).
Methods: A nested case-control study from the EURODIAB Prospective Complications Study was performed. Cases ($n = 289$) had one or more complications of diabetes, whereas controls ($n = 153$) did not have any complication. We measured miR-145-5p levels by qPCR and investigated the association with diabetes complications.
Results: Mean miR-145-5p levels were significantly lower in cases with microangiopathy [2.12 (0.86–4.94)] compared to controls [3.15 (1.21–7.36), $P < 0.05$] even after adjustment for age, gender, and diabetes duration. In logistic regression analysis, miR-145-5p levels in the lowest tertile were associated with an over three-fold increased odds ratio (OR) of albuminuria [3.22 (1.17–8.81)], independently of both demographic and diabetes-related factors. In addition, mir145-5p levels in the lowest tertile were independently and inversely associated with arterial hypertension [1.96 (1.08–3.56)] and hypertension was the mediator of the relationship between miR-145-5p and albuminuria.
Conclusions: In this large cohort of DM1 patients, we found an inverse association between miR-145-5p and albuminuria that was mediated by systemic hypertension.

1. Introduction

Diabetes mellitus is associated with long-term vascular complications that affect both quality of life and mortality rate [1]. Both hyperglycemia and hypertension are major determinants in the development of diabetic complications. Moreover, formation of advanced glycation end products (AGEs) and altered production of cytokines, including the pro-sclerotic cytokine transforming growth factor-β1 (TGF-β1) and the proangiogenic cytokine vascular endothelial growth factor (VEGF), are believed to play an important role in mediating the deleterious effects of hyperglycemia [2]. Currently available clinical and biohumoral markers of diabetic complications are insufficient for the early diagnosis and the prognostic stratification of patients at high risk. Therefore, there is the need to identify new diagnostic/prognostic biomarkers.

MicroRNAs (miRNAs) are evolutionarily conserved small sequences of non-coding RNAs that control gene expression at the post-transcriptional level by targeting the 3′ untranslated region of mRNAs [3]. MiRNAs regulate most biological processes and have been involved in the pathogenesis of chronic diabetic complications [4,5]. Although miRNAs function cell-intrinsically to regulate gene expression,

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extracellular miRNAs are also present in body fluids. Circulating miRNAs are protected from degradation as they are either enclosed in extracellular vesicles (EV) [6,7] or bound to lipoprotein complexes [8]. Serum profiles of miRNAs may change in pathological conditions in a disease-specific manner and this together with the remarkable stability of miRNAs in biofluids make miRNAs an attractive new class of potential biomarkers [9,10].

Among miRNAs, miR-145-5p is of particular interest in the context of vascular complications. MiR-145-5p is highly expressed by vascular muscle cells (VSMC) and to a lesser extent by endothelial cells (EC), monocyte-macrophages, fibroblasts, and both glomerular and tubular epithelial cells [11–19]. MiR-145-5p is induced by high glucose, TGF-β1 [20], and shear stress [15,16], and has several vasoprotective effects. In particular, miR-145-5p promotes both differentiation and contractility of VSMC with a shift from a proliferative/synthetic (atherogenic) to a quiescent/contractile (non-atherogenic) phenotype [12,13,21], limits vascular fibrosis by downregulating the TGF-β1 receptor of type 2 [22], and reduces inflammation by downregulating the junctional adhesion molecule-A on EC [15,16] and by lowering macrophage polarization towards a M1 pro-inflammatory phenotype [17]. In microvascular retinal EC, miR-145-5p represses VEGF and attenuates high glucose-induced oxidative stress [23,24]. In renal cells, miR-145-5p reduces high glucose-induced podocyte apoptosis [25], inhibits both cell proliferation and release of inflammatory cytokines by mesangial cells [26], and suppresses tubular-mesenchymal transition [27]. Consistently, studies in both experimental animals and humans have shown changes in miR-145-5p expression in various diseased vascular beds. MiR-145-5p is downregulated in the atherosclerotic plaques of experimental animals. However, there is an increase in miR-145-5p expression in advanced human atherosclerotic plaques as well as in the atherosclerotic plaques of hypertensive patients [28], suggesting that both severe vascular injury and hypertension may upregulate miR-145-5p.

There is relatively little information on serum miR-145-5p levels in patients with vascular diseases. Circulating miR-145-5p levels were elevated in patients with unstable angina [29,30] and myocardial infarction, but reduced in patients with coronary artery disease (CAD) [31–34] and inversely associated with CAD severity [35]. However, there is no information on circulating miR-145-5p in vascular diseases in the context of diabetes.

We previously reported that miR-145-5p was one of the 25 differentially expressed miRNAs in a profiling analysis performed in pooled serum samples from patients with type 1 diabetes (T1DM) patients with and without chronic complications [36]. Herein, we explored the potential independent associations of miR-145-5p with micro/macrovascular complications of T1DM by measuring miR-145-5p in individual serum samples from T1DM patients of the EURODIAB PCS nested case-control study.

2. Methods

2.1. Patient sample

The EURODIAB IDDM Complications Study (1989–1991) was performed to identify risk factors for vascular diabetes complications in 3,250 patients with T1DM [37]. Participants were 15–60 years old and they were randomly selected in 31 European diabetes centres.

Approximately 6 to 8 years after baseline examinations, participants were recalled for follow-up assessment (1997–1999, EURODIAB Prospective Complication Study) and 1,880 subjects (57.8%) returned for examination (median follow-up 7.3) [38].

A nested case-control study was designed at the follow-up examination [39]. Cases (n = 356) were subjects with CVD, diabetic retinopathy, or albuminuria, while controls (n = 185) were completely free of complications [40,41]. The design allowed to efficiently compare individuals with one or more complications with individuals free of complications.

Of these 541 individuals, clinical data and serum samples for miR-145-5p measurement were available for 460 subjects (300 cases and 160 controls) (Fig. 1). Eighteen samples were excluded because both miR-145-5p and the endogenous control U6 were undetectable; therefore, the present analyses were performed on 289 cases and 153 controls. Case and control subjects were unmatched, so that the impact of key variables could still be assessed, and any adjustments were taken care of at the analysis stage. The EURODIAB study was approved by the Ethical Committee and the procedures were in accordance with the Helsinki Declaration.

2.2. Definitions and measurements

Hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg and/or use of antihypertensive agents [42]. Retinopathy was diagnosed and graded based on the EURODIAB protocol [43]. The presence of diabetic nephropathy was assessed by measuring albumin excretion rate (AER) in 24-hour urine collections and classified in normoalbuminuria (<20 μg/min), microalbuminuria (20–200 μg/min), and macroalbuminuria (≥200 μg/min). Glomerular filtration rate (eGFR) was estimated using the Modification of Diet in Renal Disease study equation [44]. CVD was defined as myocardial infarction, coronary artery bypass graft, angina or stroke and/or ischemic ECG-changes that were centrally classified based on the Minnesota coding system. ELISA kits were used to measure both Amadori albumin and serum TGF-β1 levels (R&D Systems. Oxon, UK), as previously described [45].

2.3. RNA isolation

Serum samples (200 μl) were added to 750 μl of TRIZOL®LS reagent (Thermo Fisher, Milan, Italy). Mixtures were left for 15 min at room temperature (RT), then Cel-miR-39 (3 μl spike-in) and chloroform (200 μl) were added. After mixing, incubation (RT for 5 min), and centrifugation, the upper aqueous phase was collected and incubated with isopropanol (500 μl) for 10 min at RT. After centrifugation, pellets were washed with ethanol (75%), air-dried, and then re-suspended in RNAse-free H_2O (25 μl). Quality of RNA was evaluated by capillary electrophoresis on an Agilent-2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

2.4. Reverse transcription and pre-amplification

TaqMan MicroRNA Reverse Transcription Kit was used for reverse transcription (RT). Briefly, a fixed volume of RNA (3 μl) was reverse transcribed on a Veriti thermocycler (Thermo Fisher, Milan, Italy) as follows: 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. RT products were pre-amplified using the Megaplex PreAmp Primers (Thermo Fisher, Milan, Italy). Both RT and PreAmp products were stored at − 20 °C.

2.5. Taqman qPCR assay

Expression of miR-145-5p, U6 snRNA, and Cel-miR-39 was assessed using specific Taqman miRNA Assays. Diluted pre-amplification products were combined with Taqman miRNA Assay and Taqman Universal PCR Master Mix No AmpErase UNG to a final volume of 10 μl and amplified on an Applied Biosystems 7900HT thermocycler (95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min). The SDS software was used to calculate raw Ct values. Results were normalised to both U6 snRNA and Cel-miR-39. If Ct of both miR-145-5p and U6 were ≥ 35 cycles or undetermined, samples were excluded from the analyses. Samples with Cel-miR-39 ≥ 35 cycles/undetermined were rerun. Relative expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

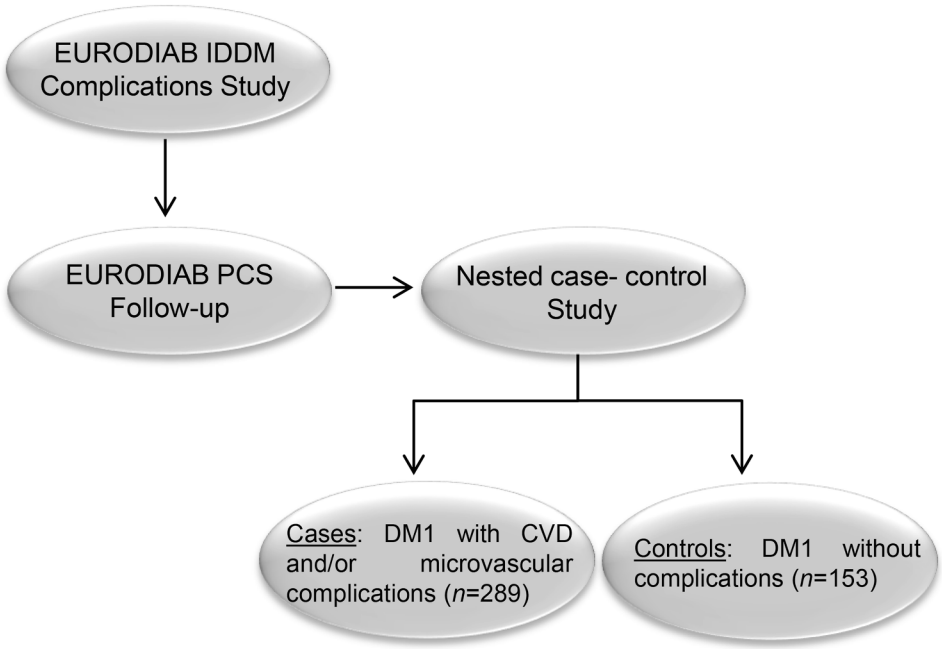

Fig. 1. Design of the study.

2.6. Statistical analyses

Results were expressed as mean (standard deviation, SD). Non-normally distributed variables (miR-145-5p, TGF-β1) were expressed as geometric means (25–75 percentiles) and log-transformed prior to analyses. Linear correlations of miR-145-5p values with clinical variables were assessed using Pearson's correlation coefficients.

Logistic regression analyses were performed to estimate the odd ratios (ORs) of miR-145-5p for all and individual diabetes complications (nephropathy, retinopathy, CVD), independently of confounders and known risk factors. The likelihood ratio test was used to compare nested models examining the role of age, sex, diabetes duration, HbA1c, Amadori albumin, TGF-β1, and hypertension. Analyses were hypothesis oriented and variables were retained in the model if they added significantly to the likelihood of models or to the estimated coefficients of predictors. MiR-145-5p levels were categorized by tertile distribution in controls. Logistic regression analysis was also performed to estimate the ORs of miR-145-5p for hypertension, independently of age, sex, total cholesterol, HbA1c, diabetes duration, smoking. Analyses were performed on the SPSS software (Version 27).

3. Results

3.1. Characteristics of patients

Participants (n = 442) had a mean age of 39.5 years and a similar percentage of men (51.1%) and women (48.9%). Average diabetes duration was 21.7 years. Risk factor profile was more adverse in cases than in controls (Table 1). Arterial hypertension was present in 56.4% of cases and 13.1% of controls. Among cases, 115 subjects had CVD (40%). Nephropathy was present in 178 (41.6% microalbuminuria and 58.4% macroalbuminuria) and retinopathy in 253 (background 47.4% and proliferative 52.6%); however, most cases had both microvascular complications (56.7%).

3.2. Serum miR-145-5p levels

Individual Ct values of miR-145-5p, U6, Cel-miR-39C are reported in the Suppl. Table 1. In the overall population, distribution of miR-145-5p values was left-skewed and serum miR-145-5p levels were significantly (P = 0.002) lower in hypertensive [1.83 (0.75–3.99)] than in normotensive [2.96 (1.13–6.75)] subjects, even after adjustment for age, sex, and diabetes duration (P = 0.002) (Fig. 2A). Values of miR-145-5p were directly correlated with HDL-cholesterol ($r = 0.17$, $p < 0.001$) and inversely correlated with BMI ($r = -0.11$, $p < 0.05$), SBP ($r = -0.15$, $p < 0.001$), A1c ($r = -0.12$, $p < 0.05$), and serum creatinine ($r = -0.13$, $p < 0.01$). Table 2 shows the correlation matrix between miR-145-5p levels and other continuous variables.

Comparisons between patients with and without complications showed that serum miR-145-5p levels were significantly (P = 0.014) lower in cases than in controls [2.12 (0.86–4.94) vs. 3.15 (1.21–7.36)] (Fig. 2B). The difference was still statistically significant after adjustment for age, gender, and duration of diabetes (P = 0.010). Subgroup

Table 1
Characteristics of the 442 subjects with T1DM recruited in the cross-sectional nested case-control study of the EURODIAB PCS.

	Case subjects	Control subjects	P
N	289	153	
Age (years)	41.6 ± 10.8	35.6 ± 7.57	<0.001
Diabetes duration (years)	25.1 ± 9.2	15.3 ± 7.13	<0.001
Males (%)	52.9%	47.7%	0.43
BMI (Kg/m^2)	24.9 ± 3.5	23.7 ± 2.63	<0.001
WHR	0.9 ± 1.2	0.9 ± 0.16	0.136
HbA1c (mmol/mol)	76 ± 0.8	61 ± 1.2	<0.001
HbA1c (%)	9.1 ± 1.6	7.7 ± 1.2	<0.001
SBP (mmHg)	127.5 ± 21.8	114.8 ± 13.5	<0.001
DBP (mmHg)	76.0 ± 11.5	73.4 ± 10.7	<0.05
Hypertension (%)	56.4%	13.1%	<0.001
Total cholesterol (mmol/l)	5.5 ± 1.2	4.9 ± 1.1	<0.001
LDL-cholesterol (mmol/l)	3.3 ± 1.1	2.8 ± 1.0	<0.001
HDL-cholesterol (mmol/l)	1.6 ± 0.4	1.7 ± 0.4	<0.05
Triglycerides (mmol/l)	1.2 (0.84–1.58)	0.8 (0.66–1.06)	<0.001
Amadori albumin (U/ml)	47.0 ± 13.5	42.5 ± 12.6	<0.01
eGFR (ml/min/1.73 m^2)	90.2 ± 25.2	106.0 ± 14.0	<0.01
Serum TGF-β1 (ng/ml)	6.3 (3.70–9.07)	5.6 (3.40–8.75)	<0.01

Data are expressed as mean ± SD, percentage or geometric mean (25°-75° percentile) for log-transformed data. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure, eGFR, estimated glomerular filtration rate.

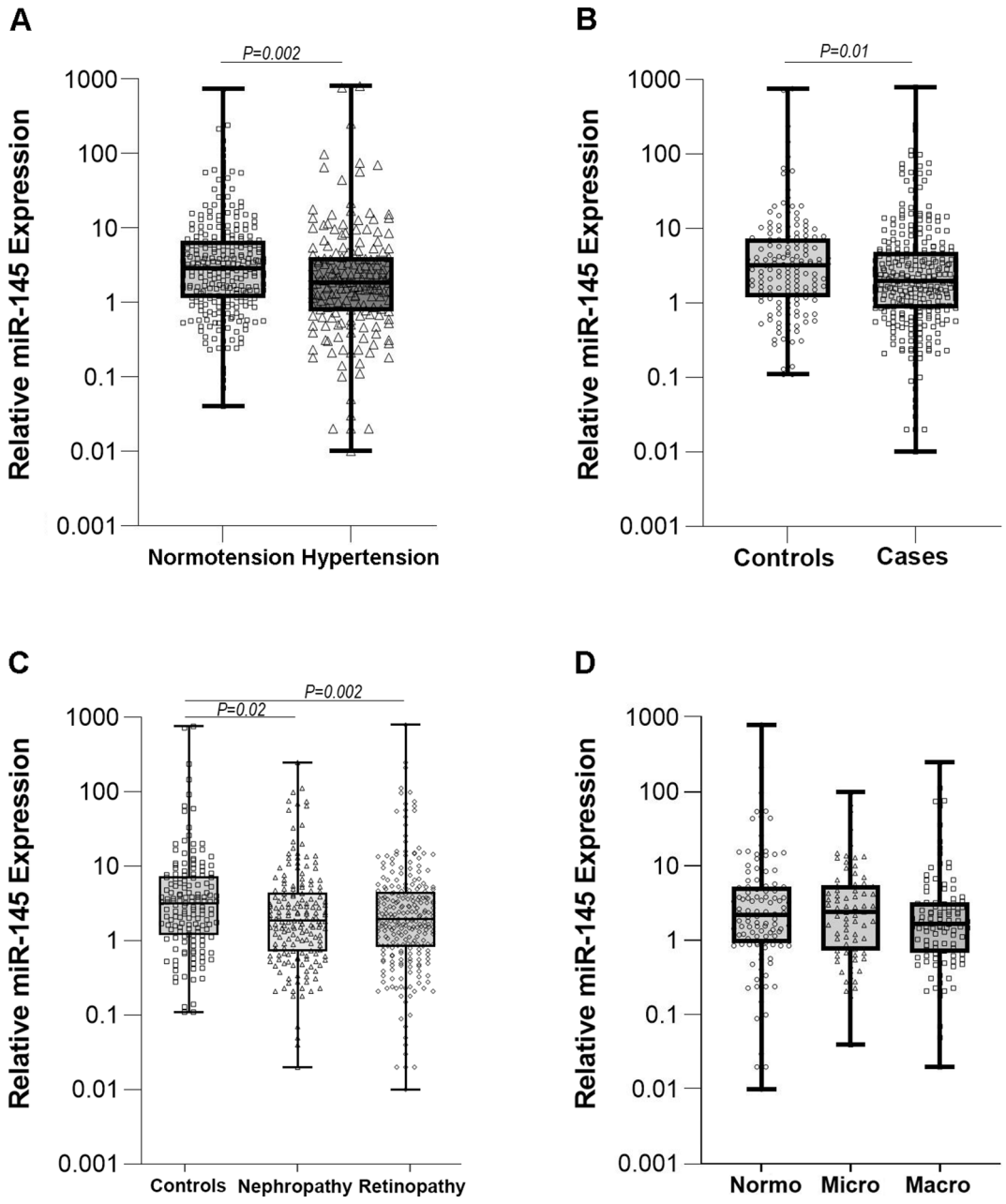

Fig. 2. Serum miR-145 expression. **(A)** miR-145-5p levels in T1DM patients with (n = 183 or without hypertension (n = 259) (P = 0.002). **(B)** miR-145-5p levels in T1DM patients with (cases; n = 289) and without (controls; n = 153) micro-macrovascular complications (*p < 0.01 cases vs. controls). **(C)** miR-145-5p levels in T1DM controls, T1DM cases with nephropathy (n = 178; P = 0.02) and T1DM cases with retinopathy (n = 253; P = 0.002). **(D)** miR-145-5p levels in T1DM patients with normo, micro or macroalbuminuria.

analyses by individual complications showed that miR-145-5p values were significantly lower in cases with nephropathy [1.99 (0.72–4.41) P = 0.007)] and retinopathy [1.98 (0.83–4.61) P = 0.005] compared to controls (Fig. 2C,D). Differences remained significant (nephropathy P = 0.02; retinopathy P = 0.002) after adjustment for age, sex, and diabetes duration. On the contrary, no difference in miR-145-5p levels was observed between cases with CVD [2.23 (0.93–5.39) P = 0.078] and controls.

3.3. Logistic regression analyses

Logistic regression analyses were carried out to assess the association between miR-145-5p levels and T1DM complications, independently of main risk factors and confounders. As shown in Table 3, miR-145-5p levels in the lowest tertile were associated with an increased OR of all complications [2.02 (1.12–3.64)], nephropathy [2.16 (1.11–4.21)], and retinopathy [2.35 (1.24–4.45)] independently of age, sex, and diabetes duration (Model 1). There was no association between miR-145 and CVD and exclusion of subjects treated with renin-Angiotensin system inhibitors (6 controls and 106 cases) did not modify the results adjusted for age, sex and diabetes duration [I°tertile OR 1.46 (0.66–3.24), II°tertile 0.92 (0.40–2.12), III° tertile 1.00].

After further adjustment for HbA1c, Amadori albumin, and TGF-β1 (Model 2), the association with all complications and retinopathy was no longer significant. However, there was still a 3.22-fold increased risk of macroalbuminuria [3.22 (1.17–8.81)]. The independent and inverse association between miR-145-5p and macro-albuminuria was mediated by systemic hypertension as it was abolished by the inclusion of

Table 2
Pearson correlation coefficient of clinical variables.

		Log miR-145	Age	BMI	DM Duration	SBP	DBP	HbA1c	T-Chol	HDL-Chol	LDL-Chol	sCr	Log TGF-β1
Log miR-145	r	1	−0.044	−0.005	−0.114	−0.145	−0.051	−0.121	−0.025	0.172	−0.066	−0.128	−0.025
	P	0.360	0.017	0.002	0.920	0.281	0.011	0.598	0.000	0.000	0.078	0.007	0.593
Age	r	−0.044	1	0.666	0.192	0.405	−0.012	0.014	0.278	0.085	0.216	0.067	−0.009
	P	0.360		0.000	0.000	0.000	0.800	0.764	0.000	0.073	0.000	0.162	0.849
BMI	r	**−0.114**	0.192	0.102	1	0.247	0.200	0.068	0.191	0.109	0.266	0.019	−0.049
	P	**0.017**	0.000	0.033		0.000	0.000	0.156	0.000	0.022	0.000	0.687	0.310
DM Duration	r	−0.005	0.666	1	0.102	0.391	−0.029	0.107	0.254	−0.204	0.186	0.104	0.027
	P	0.920	0.000		0.033	0.000	0.542	0.026	0.000	0.000	0.002	0.029	0.570
SBP	r	**−0.145**	0.405	0.391	0.247	1	0.578	0.074	0.288	−0.038	0.314	0.350	0.077
	P	**0.002**	0.000	0.000	0.000		0.000	0.125	0.000	0.424	0.000	0.000	0.107
DBP	r	−0.051	−0.012	−0.029	0.200	0.578	1	0.005	0.208	−0.033	0.259	0.256	0.071
	P	0.281	0.800	0.542	0.000	0.000		0.921	0.000	0.488	0.000	0.000	0.138
HbA1c	r	**−0.121**	0.014	0.107	0.068	0.074	0.005	1	0.136	−0.107	0.127	−0.012	0.171
	P	**0.011**	0.764	0.026	0.156	0.125	0.921		0.005	0.025	0.041	0.809	0.000
T-Chol	r	−0.025	0.278	0.254	0.191	0.288	0.208	0.136	1	0.165	0.945	0.172	0.089
	P	0.598	0.000	0.000	0.000	0.000	0.000	0.005		0.000	0.000	0.000	0.062
HDL-Chol	r	**0.172**	0.085	0.109	−0.204	−0.038	−0.033	−0.107	0.165	1	−0.112	−0.123	0.093
	P	**0.000**	0.073	0.022	0.000	0.424	0.488	0.025	0.000		0.068	0.010	0.052
LDL-Chol	r	−0.066	0.216	0.186	0.266	0.314	0.259	0.127	0.945	−0.112	1	0.296	0.055
	P	0.282	0.000	0.002	0.000	0.000	0.000	0.041	0.000	0.068		0.000	0.368
sCr	r	**−0.128**	0.067	0.104	0.019	0.350	0.256	−0.012	0.172	−0.123	0.296	1	0.064
	P	**0.007**	0.162	0.029	0.687	0.000	0.000	0.809	0.000	0.010	0.000		0.182
logTGF-β1	r	−0.025	−0.009	0.027	−0.049	0.077	0.071	0.171	0.089	0.093	0.055	0.064	1
	P	0.593	0.849	0.570	0.310	0.107	0.138	0.000	0.062	0.052	0.368	0.182	

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; sCr, serum creatinine.

Table 3
Odds ratio values for diabetes complications by miRNA-145-5p values in the nested case-control study of the EURODIAB PCS study.

	MODEL 1 OR (95% IC)	MODEL 2 OR (95% IC)	MODEL 3 OR (95% IC)
All complications			
logMir-145 ≤ 1.671	**2.02 (1.12–3.64)**	1.70 (0.89–3.24)	1.47 (0.75–2.86)
1.671–5.065	1.28 (0.71–2.33)	1.10 (0.57–2.12)	0.97 (0.76–2.86)
≥ 5.065	1.00	1.00	1.00
CVD			
≤ 1.671	1.76 (0.85–3.65)	1.49 (0.67–3.29)	1.45 (0.65–3.26)
1.671–5.065	1.12 (0.53–2.39)	1.08 (0.48–2.43)	1.11 (0.49–2.53)
≥ 5.065	1.00	1.00	1.00
Nephropathy			
≤ 1.671	**2.16 (1.11–4.21)**	1.75 (0.81–3.79)	1.44 (0.63–3.30)
1.671–5.065	1.32 (0.67–2.62)	0.95 (0.43–2.09)	0.77 (0.34–1.78)
≥ 5.065	1.00	1.00	1.00
Microalbuminuria			
≤ 1.671	1.77 (0.81–3.87)	1.31 (0.52–3.31)	1.32 (0.50–3.47)
1.671–5.065	1.08 (0.48–2.43)	0.84 (0.33–2.15)	0.85 (0.32–2.24)
≥ 5.065	1.00	1.00	1.00
Macroalbuminuria			
≤ 1.671	**3.64 (1.51–8.80)**	**3.22 (1.17–8.81)**	2.23 (0.75–6.65)
1.671–5.065	1.74 (0.71–4.26)	1.23 (0.44–3.42)	0.93 (0.31–2.79)
≥ 5.065	1.00	1.00	1.00
Retinopathy			
≤ 1.671	**2.35 (1.24–4.45)**	2.00 (0.99–4.06)	1.82 (0.87–3.81)
1.671–5.065	1.61 (0.84–3.09)	1.39 (0.68–2.85)	1.32 (0.62–2.78)
≥ 5.065	1.00	1.00	1.00

Model 1: adjusted for age, sex, diabetes duration.
Model 2: Model 1 + HbA1c, Amadori Albumin, log-serum TGF-β levels.
Model 3: Model 2 + hypertension.
Likelihood ratio chi-square test and Wald test were used.

hypertension into the model (Model 3) [2.23 (0.75–6.65)].

Logistic regression analysis performed to assess the relationship between hypertension and miR-145-5p levels showed that miR-145-5p levels were independently and inversely associated with hypertension. As shown in Table 4, lower miR-145-5p levels were associated with a more than 1.8-fold increase OR of hypertension independently of age, sex, and diabetes duration. After adjustment for HbA1c, total cholesterol and smoking, the strength of the association was even greater [1.96 (1.08–3.56)].

Table 4
Odds ratios for arterial hypertension by miR-145-5p values 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)
Hypertension			
≤ 1.671	**1.91 (1.09–3.37)**	**1.82 (1.02–3.27)**	**1.96 (1.08–3.56)**
1.671–5.065	1.76 (0.97–3.20)	1.76 (0.95–3.27)	1.77 (0.94–3.32)
≥ 5.065	1.00	1.00	1.00

Model 1: adjusted for age, sex, diabetes duration, HbA1c.
Model 2: Model 1 + BMI, total cholesterol.
Model 3: Model 2 + smoking.

4. Discussion

The present study investigated the potential independent association between serum miR-145-5p levels and chronic complications of T1DM in the nested case-control study of the EURODIAB PCS, which is one of the largest European studies on diabetic complications.

Levels of miR-145-5p in the lower tertile were associated with an increased OR for all complications that was predominantly driven by a significantly enhanced risk of both retinopathy and nephropathy. Similarly, we have previously reported that both miR-126 and miR-146-5p were associated with retinopathy and CVD in T1DM patients of the EURODIAB nested case-control study [36,46].

Hyperglycemia and advanced glycation end products play a key role in the pathogenesis of microvascular diabetic complications by inducing oxidative stress, inflammation, fibrosis both directly and indirectly through expression of deleterious cytokines, such as TGF-b1 and VEGF. In vitro studies in target cells of microvascular complications have shown that miR-145-5p is induced by both high glucose and TGF-b1 [20] and that miR-145-5p can limit the deleterious effects of high glucose by suppressing TGF-b1 signalling [22], inflammation [23,26], oxidative stress [23,24], apoptosis [25], and VEGF expression [24]. Consistent with the hypothesis that miR-145-5p may have a vaso-protective effect in the context of diabetes, we found an inverse association between serum miR-145 levels and microvascular complications that was mediated by HbA1c, Amadori Albumin, and TGF-β1. Lower circulating levels of miR-145-5p may identify the subgroup of patients

with an insufficient compensatory miR-145-5p response to hyperglycemia and thus more prone to vascular complications.

After adjustment for diabetes-related factors, miR-145-5p levels in the lowest tertile were still inversely associated with an over three-fold increased OR of overt nephropathy. We previously reported an increase in miR-145-5p content in the urinary exosomes from T1DM patients with persistent microalbuminuria and normal renal function [18]. The reason why miR-145-5p is increased in the urinary exosomes of microalbuminuric T1DM patients and reduced in sera from macroalbuminuric T1DM subjects is unknown; however, a different cellular origin of miR-145-5p is a possible explanation. Serum miR-145-5p likely derives from EC, monocytes, and VSMC, while urinary exosomal miR-145-5p may derive from renal cells. Accordingly, exposure of mesangial cells to high glucose enhances miR-145-5p expression and induces the release of exosomes enriched in miR-145-5p [18]. Moreover, our previous study was performed in T1DM patients with microalbuminuria and we cannot exclude that urine exosomal miR-145-5p content drops when overt nephropathy develops.

Given the key role of arterial hypertension in the pathogenesis of diabetic nephropathy, we explored the potential role of hypertension in the association between albuminuria and miR-145-5p. We found an inverse association between miR-145-5p and arterial hypertension that was independent of age, sex, diabetes duration, total cholesterol, HbA1c, and smoking. Moreover, the association between miR-145-5p and macroalbuminuria was no longer significant after inclusion of arterial hypertension into the model. This is the first evidence of an independent relationship between arterial hypertension and serum miR-145-5p in a large cohort of T1DM patients, though a previous study showed that miR-145-5p content is reduced in blood mononuclear cells from a small sample of non-diabetic hypertensive subjects [47].

The relationship between miR-145-5p and systemic hypertension is complex and given the cross-sectional design of our study, we cannot establish if reduced serum miR-145-5p levels were either a cause or a consequence of systemic hypertension. Both in vitro and in vivo studies have shown that miR-145-5p plays a crucial role in regulating both vascular tone and contractility and that mice knockout for miR-145-5p have reduced vascular tone and blood pressure levels [11,48]. On the other hand, exposure of VSMC to either stretching or angiotensin II to mimic hypertension in vivo lowers miR-145-5p expression [49,50]. However, whether circulating miR-145-5p levels in humans mirror miR-145-5p expression in VSMC is unknown.

In our study miR-145-5p levels were similar in CVD cases and controls and there was no association between miR-145-5p and CVD in logistic regression analysis. Therefore, our results do not support the hypothesis that miR-145-5p is a potential biomarker of CVD in patients with T1DM [31–34]. MiR-145-5p can increase the activity of the renin-angiotensin system by reducing both angiotensin-converting enzyme (ACE) expression and ACE2 shedding [51]. However, exclusion of patients treated with RAS inhibitors did not modify the results, suggesting that treatment with RAS inhibitors was not a confounder. The lack of changes in circulating levels of miR-145-5p in T1DM patients with CVD may be the net result of opposite effects on miR-145-5p expression in conditions of diabetes-induced chronic vascular stress. In keeping with this hypothesis, in vitro studies have shown that shear stress, high glucose, TGF-β1, and inflammatory cytokines induce miR-145-5p expression in EC, VSMC, monocytes [15,16,20]. while vascular injury [12] and mechanical stretch [50] downregulates miR-145-5p in VSMC.

Previous studies reported a reduction in circulating miR-145-5p levels in non-diabetic subjects with CAD [31–35], possibly reflecting a deficiency of protective miR-145-5p expression in the vascular bed. On the other hand, there are reports of enhanced circulating miR-145-5p levels in unstable angina and myocardial infarction [29,30]. Likely, in these acute conditions miR-145 is released into the circulation by apoptotic/necrotic cells and is a marker of vessel/heart injury. In addition, miR-145 reduces myocardial infarction size in experimental animals by accelerating cardiomyocyte autophagy [52], suggesting that miR-145 overexpression may represent a mechanism of repair.

In conclusion the study shows an inverse association between serum miR-145-5p levels and microvascular diabetes complications that is independent of demographic confounders, such as age, sex and diabetes duration. The association with retinopathy is mediated by hyperglycemia and its direct consequences. On the contrary, the association with albuminuria is independent of diabetes-related factors and due to the strong inverse association between miR-145-5p and hypertension that is a well-known determinant of diabetic nephropathy.

Our study has several limitations. Although the EURODIAB study had a prospective design, samples were not collected at baseline; therefore, miR-145-5p levels could only be measured at follow-up. The cross-sectional design of the study makes it impossible to establish causal and temporal relationships. The power of the analyses was reduced by the lower number of controls compared to cases. The possibility of miR-145-5p degradation during storage cannot be excluded, though miRNAs are very stable in biofluids. Data on statin use were not collected in the EURODIAB study. Finally, cases and controls were not matched for clinical variables and cases had a more adverse risk factor profile than controls; however, adjustments for age, diabetes duration, and HbA1c were made at the analysis stage. A key strength of our work is the large sample size and the possibility to correct for the confounding effect of other risk factors and complications; however, a significant and independent association was only found in subgroup analyses including a smaller sample of patients. Moreover, multiple comparisons within the same case-control study base might have caused significant results due to chance.

There is increasing interest in miRNAs as potential biomarkers of diabetes complications; however, most of the studies were performed on very small numbers of patients [53]. Moreover, the majority of the studies on diabetic nephropathy and miRNAs in T1DM were carried out on urinary samples and there is little information on serum levels of miRNAs [18,53]. Pezzolesi et al. found that let-7c-5p and miR-29a-3p were associated with an over 50% reduction of the risk of rapid progression to ESRD, while let-7b-5p andmiR-21-5p were associated with a 2.5-fold increase in ESRD risk [54]; however, this study was performed in patients with advanced nephropathy. Our study was performed on serum samples from a relatively large and representative group of T1DM patients and investigated the relevance of a yet unexplored miRNA. Our findings show an inverse and independent relationship between miR-145-5p and overt diabetic nephropathy that was mediated by arterial hypertension. In the view of potential future clinical application, it would be of interest to establish in prospective studies if miR-145 has a predictive value and its addition to currently available clinical markers and scores improves identification of subgroups of patients at high risk. Finally, the evidence of a strong and independent association between hypertension and serum miR-145-5p deserves further investigation in subjects with and without diabetes to clarify if it may be exploited for clinical purposes.

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CRediT authorship contribution statement

Federica Barutta: Conceptualization, Methodology, Formal analysis, Writing – original draft, Supervision. **Stefania Bellini:** Conceptualization, Methodology, Writing – original draft. **Simonetta Guarrera:** Investigation. **Giuseppe Matullo:** Writing – review & editing. **Casper Schalkwijk:** Data curation. **Coen D. Stehouwer:** Data curation. **Nish**

Chaturvedi: Data curation. **Sabita S. Soedamah-Muthu:** . **Marilena Durazzo:** Writing – review & editing. **Gabriella Gruden:** Conceptualization, Methodology, Writing – original draft, Supervision, Funding acquisition.

Declaration of Competing Interest

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.

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