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MicroRNA-126 and micro-/macrovascular complications of type 1 diabetes in the EURODIAB Prospective Complications Study

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MicroRNA-126 and Micro/Macrovascular Complications of Type 1 Diabetes in the EURODIAB Prospective Complications Study --Manuscript Draft--

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| Abstract: | AIMS. Increasing evidence suggest a potential role of circulating miRNAs as clinical biomarkers and loss of miRNA-126 has been proposed as a predictor of type 2 diabetes onset. However, a systematic analysis of circulating miRNAs in type 1 diabetic patients with micro/macrovascular complications has not yet been performed. METHODS. A cross-sectional nested case-control study from the EURODIAB Prospective Complications Study of 455 type 1 diabetic patients was performed. Case subjects (n=312) were defined as those with one or more complications. A differential miRNA expression profiling was performed in pooled serum samples from cases and controls. Furthermore, miR-126 levels were quantified by qPCR in all individual samples and associations with diabetic complications investigated. RESULTS. 25 miRNAs differed in pooled samples from cases and controls. miR-126 levels were in case than in control subjects, even after adjustment for age and sex. In logistic regression analyses, miR-126 was negatively associated with all complication examined separately. This was likely dependent of diabetes as associations were no longer significant 25% risk reduction, independent of age, sex, A1C and diabetes duration, was still observed for proliferative retinopathy (OR=0.75, 95% CI 0.59-0.95). CONCLUSIONS: In this large cohort of type 1 diabetic subjects, we found that miR-126 | | | |

| | levels are associated with vascular complications of diabetes, particularly with proliferative retinopathy. |
|------------------------|---|
| Response to Reviewers: | Reviewer #1: The Authors conducted a systematic analysis of circulating miRNAs in type 1 diabetic patients with and without micro/macrovascular complications. Differential miRNA profiling showed that expression of 25 miRNAs differed in pooled serum samples from cases and control subjects. In particular, miR-126 levels were significantly lower in case than in control subjects, even after adjustment for age and sex. In logistic regression analyses, miR-126 was negatively associated with all complications as well as with each micro/macrovascular complication examined separately. However, associations were no longer significant after adjustment for both hyperglycemia and diabetes duration, except for proliferative retinopathy, thus suggesting that loss of miR-126 might be a signature of type 1 diabetes, as previously shown for type 2 diabetes. |
| | The paper is interesting, though it has some limitations: |
| | 1. The cross sectional design, which did not allow to assess temporal relationship, though the large EURODIAB database should contain the information on the time of onset of each complications and this could be better analysed using individual samples. |
| | We agree with the Reviewer that a prospective study would have given more information on the temporal relationship between changes in microRNA levels and development of complications. However, we could not perform a prospective study on the cohort of type 1 diabetic patients from the EURODIAB study because serum samples collected at the baseline examination were not stored for subsequent analyses; therefore, miRNA measurements could only be performed in serum samples collected at the follow-up visit. Moreover, time of onset of complications was not recorded in the EURODIAB PCS as patients were re-assessed 7 years after the baseline visit rather than annually. To acknowledge this limitation, pointed by the Reviewer, a sentence has been included in the discussion (page 10 lines 2-4). |
| | 2. As loss of miR-126 seems to be related to the presence of diabetes rather than of its complications, the appropriate control group should be composed of age- and sex-maged non-diabetic individuals. Results of our logistic regression analyses showed that the association between the risk of most complications and miR-126 loss was dependent of long exposure to hyperglycemia (diabetes duration, HbA1c). This finding is in line with the results of the Brunick study in type 2 diabetic patients and with several other reports in vivo and in vitro suggesting that hyperglycemia lowers circulating miR-126 levels. Therefore, we agree with the Reviewer that loss of miR-126 is likely related to hyperglycemia, independently of its underlying cause (i.e. type 1, type 2, IGT). However, the study question was not if miR-126 is link to hyperglycemia in type 1 diabetes, but if miR-126 is associated with diabetic complications beyond the effect of hyperglycemia and may serve as additional biomarker, which was not the case except for retinopathy. Therefore, we did not include a control group of non-diabetic patients as this would have not help clarifying the role of miR-126 in diabetic complications. In addition, the nested case-control study was performed almost twenty years ago and non-diabetic control subjects recruited now could not be compared to patients, who were recruited long time ago |

Click MicroRNA-126 and Micro/Macrovascular Complications of Type 1 Diabetes in the EURODIAB **Prospective Complications Study.**

Running title: miRNAs and type 1 diabetes complications

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ABSTRACT

AIMS. Increasing evidence suggest a potential role of circulating miRNAs as clinical biomarkers and loss of

1miRNA-126 has been proposed as a predictor of type 2 diabetes onset. However, a systematic analysis of $\frac{3}{4}$ circulating miRNAs in type 1 diabetic patients with micro/macrovascular complications has not yet been eperformed.

⁸METHODS. A cross-sectional nested case-control study from the EURODIAB Prospective Complications $^{10}_{11}$ Study of 455 type 1 diabetic patients was performed. Case subjects (n=312) were defined as those with one or ¹3more complications of diabetes; control subjects (n=143) were those with no evidence of any complication. A ¹⁵₁₆differential miRNA expression profiling was performed in pooled serum samples from cases and controls. 18Furthermore, miR-126 levels were quantified by qPCR in all individual samples and associations with diabetic ²⁰₂₁complications investigated.

23RESULTS. 25 miRNAs differed in pooled samples from cases and controls. miR-126 levels were significantly ²⁵lower in case than in control subjects, even after adjustment for age and sex. In logistic regression analyses, ²/₂₈miR-126 was negatively associated with all complications (OR=0.85, 95% CI 0.75-0.96) as well as with each ³⁰micro/macro-vascular complication examined separately. This was likely dependent of diabetes as associations $_{33}^{32}$ were no longer significant after adjustment for both hyperglycemia and diabetes duration. However, a 35significant 25% risk reduction, independent of age, sex, A1C and diabetes duration, was still observed for $^{37}_{38}$ proliferative retinopathy (OR=0.75, 95% CI 0.59-0.95).

40CONCLUSIONS: In this large cohort of type 1 diabetic subjects, we found that miR-126 levels are associated $^{42}_{43}$ with vascular complications of diabetes, particularly with proliferative retinopathy.

- ⁴⁷Keywords: type 1 diabetes, microRNAs, retinopathy, complications.

INTRODUCTION

Micro- and macrovascular complications are a major cause of morbidity and mortality in type 1 diabetic 1 2patients and there is an increasing quest to find novel biomarkers to identify and treat individuals at high risk.

- ⁴₅ MicroRNAs (miRNAs) are small, non-protein-encoding RNAs that post-transcriptionally regulate gene ⁶₇expression via suppression of target mRNAs [1]. MiRNAs are critically involved in many biological processes ⁹and accumulating evidence points to an important role of miRNAs in the pathogenesis of both diabetes and ¹¹adiabetes-related complications [2-4]. MiRNAs are also present in the circulation in a remarkable stable form as ¹³¹⁴packaged in microvesicles that protect them from endogenous RNase activity. Circulating miRNAs can display ¹⁶₁₇unique expression profiles in pathological conditions, suggesting that distinctive miRNA signatures may be ¹⁸₁₉exploited as innovative diagnostic/prognostic tools [5-8].
- 21 Despite the growing interest in miRNAs, there is relatively little knowledge on circulating miRNAs in 22 23 24both diabetes and diabetic chronic complications. A distinct circulating miRNA profile has been shown in 25 $^{26}_{27}$ children with newly diagnosed type 1 diabetes [9] and reduced miR-126 levels were a significant predictor of 28 29type 2 diabetes in a systematic analysis of circulating miRNAs from the population-based Bruneck study [10]. 30 $^{31}_{32}$ Moreover, both miR-27b and miR-320a have been proposed as biomarkers of diabetic retinopathy in type 1 33 34 diabetes patients [11], while levels of specific TGF-β1-regulated miRNAs (let-7c-5p, miR-29a-3p, let-7b-5p, 35 $^{36}_{37}$ miR-21-5p) appear to predict progression to end-stage renal disease (ESRD) in proteinuric type 1 diabetes 38 39 patients with normal renal function [12]. However, a comprehensive analysis of the relationship between serum 40 $^{41}_{42}$ miRNAs and micro/macrovascular diabetic complications is still lacking.
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$^{53}_{54}$ MATERIALS AND METHODS

- 55 56**Patient Sample**
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The EURODIAB PCS (1997-1999) is a follow-up of the EURODIAB IDDM Complications Study (1989-1991), which was designed to explore risk factors for diabetes complications in 3,250 randomly selected people 1with type 1 diabetes, aged 15-60 years, attending 31 diabetes centers in 16 European countries [13,14].

³A cross-sectional nested case-control study was designed at the 1997-1999 follow-up examination [15-19]. The ⁵gresponse rate at follow-up examination was 57.8% (20). Case subjects were selected to have the greatest ⁷acomplication burden possible to provide sufficient numbers for subgroup analyses. Thus, case subjects were all ¹⁰1those with cardiovascular diseases or retinopathy, or albuminuria at follow-up. Control subjects were selected ¹²1sto be completely free of complications. This design allowed us to compare individuals with single or multiple ¹⁵16complications with individuals free of complications, according to the study question, as efficiently as possible. ¹⁷18Applying these criteria, this yielded 312 cases and 143 control subjects with full data on complications and ¹⁹21samples available for analyses.

22 23Patient evaluation for the presence of cardiovascular risk factors [hypertension, body mass index (BMI), waist-24 ²⁵to-hip ratio (WHR), smoking, cholesterol, triglycerides, A1c] is described elsewhere (15,19). Retinopathy was 27 28graded according to the EURODIAB protocol [20]. Albumin excretion rate (AER), assessed on two 24-h urine 29 ³⁰collections by immunoturbidimetric method, was categorized as normoalbuminuria ($<20 \mu g/min$), 31 32 ³/₃₃microalbuminuria (20-200 µg/min), and macroalbuminuria (≥200 µg/min). Estimated glomerular filtration rate 34 ³⁵(eGFR) was determined using the four-component abbreviated equation from the Modification of Diet in Renal 36 $^{37}_{38}$ Disease study [21]. Subjects with an eGFR <60 ml/min/1.73m² were defined as having chronic kidney disease 39 40(CKD). Distal symmetrical polyneuropathy (DSP) was diagnosed based on (i) presence of one or more 41 $^{42}_{43}$ neuropathic symptoms, (ii) absence of two or more ankle or knee reflexes, and (iii) abnormal vibration 44 45perception threshold, measured by centrally calibrated biothesiometers (Biomedical, Newbury, OH) on the right 46 ⁴⁷big toe and on the right medial malleolus. Cardiovascular disease (CVD) was defined as physician-diagnosed 49 ¹₅₀myocardial infarction, angina, coronary artery bypass graft, or stroke and/or ischemic changes on centrally 51 ⁵²Minnesota-coded electrocardiogram. Soluble vascular cell adhesion molecule (sVCAM-1) levels were 53 ${}^{54}_{55}$ measured by a commercially available ELISA (R&D Systems, Oxon, UK) [15].

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57Differential miRNA expression profiling in pooled serum samples

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⁵⁹Pooled serum samples from cases and controls were used to assess expression of 377 individual miRNAs. Total ⁶¹ ⁶²RNA was extracted using the Trizol reagent (Invitrogen, Milan, Italy) and RNA quality assessed by capillary

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electrophoresis on an Agilent-2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). MiRNAs were reverse transcribed using the Megaplex Primer Pool A, Human Pool A v2.1 (Applied Biosystems). RT reaction lproducts were pre-amplified using the Megaplex PreAmp Primers (Primers A v2.1) and the TaqMan® PreAmp ² Master Mix. MiRNA expression profiling was performed by Human TaqMan miRNA Array A on an 7900HT ⁵ 6Fast Real-Time PCR System. Raw Ct values were calculated using the SDS software and standardized to both ⁸U6 snRNA and the spike-in C.elegans-miR-39. MiRNAs were excluded if both samples had Ct values \geq ¹⁰ 1³S/undetermined. Relative expression was calculated using the comparative Ct method (2^{- $\Delta\Delta$ Ct}). MiRNAs were ¹² ¹³considered differentially expressed if they exhibited greater than twofold expression differences.

¹⁵₁₆miR-126 Expression in individual samples

¹⁸Expression of miR-126 was quantitated in individual serum samples from all 455 patients. Diluted pre-²⁰ ²⁰ ²¹amplification products were combined with Taqman miRNA Assay and Taqman Universal PCR Master Mix ²² ²³No AmpErase UNG, then a qPCR was performed using a specific Taqman microRNA Assay (002228) on an ²⁴ ²⁵Applied Biosystems 7900HT thermocycler. All samples were run in triplicates and standardized to both U6 ²⁶ ²⁷₂₈snRNA and the spike-in C.elegans-miR-39 using the SDS2.2 software.

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³⁰Statistical analyses. ³¹

³²₃₃Variables distributed normally are presented as means (standard deviation, SD), while variables with skewed ³⁴ ³⁵distribution were analyzed after log transformation (miR-126, triglycerides, AER, creatinine, sVCAM-1,) and ³⁷₃₆presented as geometric means and interquartile range. Logistic regression analyses were used to estimate the ³⁹ ⁴⁰codd ratios (ORs) of miR-126 for any complication (AER \geq 20 µg/min, retinopathy, neuropathy, CVD), ⁴¹ ⁴²independently of confounders and known risk factors. The likelihood ratio test was used to compare nested ⁴³ ⁴⁴ ⁴⁵models examining the role of age, sex, diabetes duration, A1C, and sVCAM-1. In light of the hypothesis of a ⁴⁶ ⁴⁷different role of miR-126 in the pathogenesis of different micro/macrovascular complications, logistic ⁴⁸ ⁴⁹₅₀regression models were also fitted separately for each complication/subgroup.

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52**RESULTS** 53

⁵⁴₅₅Characteristics of case and control subjects

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⁵⁷The study population (n = 455) had a mean age of 39.5 years, average diabetes duration of 21.5 years, and an $^{58}_{60}$ equal proportion of men and women. Case subjects with vascular complications had a more adverse risk factor

⁶¹ ⁶²profile than control subjects (Table 1). Of the 312 case subjects, nephropathy was present in 179 (41.3% micro-

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and 58.7% macroalbuminuria, retinopathy in 249 (background 47.8% and proliferative 52.2%), DSP in 176 (56.4%). Most people, however, had more than one complication; indeed, 161 (51.6%) individuals had both $1AER \ge 20 \mu g/min$ and retinopathy, 113 (36.2%) had both $AER \ge 20 \mu g/min$ and DSP, and 108 (34.6%) had $AER \ge 20 \mu g/min$, DSP, and retinopathy. CVD was present in 126 subjects (40.4%), all of whom also had at least one $\frac{5}{6}$ microvascular complication who had CVD only.

⁸Differential miRNA expression profiling

¹⁰₁₁Differential miRNA profiling showed that expression of 25 miRNAs differed in pooled serum samples from ¹² ¹³cases and control subjects. As detailed in the supplementary online Table, 10 miRNAs (miR-139-5p, miR-133a, ¹⁴ ¹⁵miR-106a, miR-16, miR-222, miR-17, miR-140-3p, miR-574-3p, miR-486-3p, miR-885-5p) were up-regulated ¹⁷ ¹⁸and 15 down-regulated (miR-155, miR-92a, miR-126, miR-483-5p, miR-29a, miR-320, miR-145, miR-146a, ¹⁹

²⁰₂₁miR-191, miR-342, miR-223, miR-24, miR-150, miR-486-5p, miR-484) in cases as compared to controls.

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23miR-126 expression

²⁵Out of the 25 differentially expressed miRNAs, miR-126 was chosen for further analysis by qRT-PCR in all ²⁷₂₈recruited subjects because miR-126 has been involved in pathophysiological processes of relevance to diabetic ³⁰complications, such as angiogenesis, vascular repair, and inflammation [3].

³²₃₃miR-126 was measurable in all 455 samples with a right-skewed distribution of values. Consistent with the ³⁴ ³⁵profiling results, qRT-PCR analysis showed that miR-126 levels were significantly lower in cases than in ³⁶ ³⁷₃₈control subjects (Table 1) and results were unchanged after adjustment for age and sex (p=0.045). In cases with ³⁹ ⁴⁰micro-macroalbuminuria (0.78 vs 1.28, p=0.03) or retinopathy (0.77 vs 1.29, p=0.02) or DSP (p=0.016) age-⁴¹ ⁴²and sex-adjusted miR-126 levels were also lower than in controls.

44 45**Logistic regression analyses**

⁴⁷Logistic regression analyses were performed to assess whether lower levels of miR-126 conferred an increased ⁴⁹ $_{50}^{00}$ OR of having any complication, independently of main risk factors and confounders. In the unadjusted model ⁵¹ $_{52}^{10}$ (Model 1), miR-126 levels were negatively associated with all complications (OR=0.85, 95% CI 0.75-0.96) as ⁵⁴ $_{55}^{54}$ well as with CVD, nephropathy, DSP and retinopathy examined separately. After inclusion of age and sex into ⁵⁶ $_{57}$ the model (Model 2), the association was only marginally significant for all complications, but retained ⁶⁰ $_{60}^{59}$ statistical significance for nephropathy and retinopathy. This was likely dependent of hyperglycemia and ⁶¹ $_{62}$ diabetes duration as associations were no longer significant after further adjustment for these variables (Model

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3). Of interest, a 21% and 25% risk reduction independent of age, sex, A1C and diabetes duration was still observed for macroalbuminuria (OR=0.79, 95% CI 0.63-1.00) and proliferative retinopathy (OR=0.75, 95% CI 10.59-0.95), respectively (Model 3). After inclusion into the model of sVCAM-1, an established miR-126 target 3 [22], OR for proliferative retinopathy only remained marginally significant (OR=0.79, 95% CI 0.61-1.02) 6(Model 4). A minority of case subjects (n: 29) were under treatment with aspirin and their exclusion did not 7 ⁸modified results [macroalbuminuria: OR 0.79 (0.62-1.00) (Model 3) and OR 0.87 (0.67-1.12) (Model 4); 10 ¹₁₁proliferative retinopathy: OR 0.75 (0.58-0.95) (Model 3) and OR: 0.79 (0.61-1.02) (Model 4)]. 12 13DISCUSSION 14 $^{15}_{16}$ Our study is the most extensive study to date on circulating miRNAs in serum samples from type 1 diabetic 18patients and our results expand upon a growing body of literature that highlights the role of miRNAs in diabetic 19 $^{20}_{21}$ complications. 22 23Using an unbiased approach, we assessed the differential expression of 377 miRNAs in pooled serum samples 24 ²⁵from type 1 diabetic patients with and without vascular complications. Among them, 25 miRNAs were 27 $\frac{1}{28}$ differentially expressed in subjects with vascular complications; however, these results may underestimate real 29 ³⁰differences as pooling of samples may mask opposing changing in individual samples. 31 $^{32}_{33}$ In our profiling, levels of both miR-29a and miR-155 were lower in cases compared to controls. Of interest, a 34 ³⁵previous study has shown that miR-29a is diminished in proteinuric compared to normoalbuminuric type 1 36 ³⁷₃₈diabetic patients and a predictor of rapid progression towards ESRF [12]. Moreover, a lower content of miR-39 40155, which is known to suppress angiotensin II receptor activity [23,24], was previously found in urinary 41 $^{42}_{43}$ exosomes from type 1 diabetic patients with microalbuminuria as compared to normoalbuminuric patients [25]. 44 45At variance with a recent study on biomarkers of both incidence and progression diabetic retinopathy in type 1 46 ⁴⁷diabetic patients, our profiling did not reveal differences in miR-27 expression and levels of pro-angiogenetic 49 $\frac{1}{50}$ miR-320 were even lower in cases as compared to controls [11]. However, profiling data on pooled serum from 51 52patients with multiple complications of diabetes cannot be compared to data obtained from individual 53 ${}^{54}_{55}$ measurements performed on a well-selected cohort of patients with retinopathy, particularly in respect to 56 57miRNAs affecting angiogenesis that is known to undergo opposing deregulation in different vascular beds of 58 ⁵⁹₆₀diabetic complications.

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Several other differentially expressed miRNAs were previously linked to vascular diseases in either type 2 diabetic patients or non-diabetic subjects. We found that circulating levels of endothelial cell-enriched (miR-1126 and miR-92a), inflammation-associated (miR-155), and smooth muscle-enriched (miR-145) miRNAs were 2 ³lower in type 1 diabetic patients with complications. Consistently, previous studies have shown that these 5 miRNAs were significantly reduced in patients with stable of coronary artery diseases (CAD) compared to 7 ⁸healthy controls [26]. In our study, miR-16 was enriched in pooled samples from case subjects and a rise in 10 ¹₁₁serum miR-16 levels has been previously reported in type 2 diabetic patients with chronic complications [27]. 12 ¹Furthermore, in a prospective study, baseline miR-16 levels correlated with amputation and restenosis at 14 ¹⁵₁₆follow-up in type 2 diabetic patients who underwent re-vascularization [28]. Finally, miR-133a levels, which 17 18are enhanced in non-diabetic patients with CHD and positively correlated with the severity of the coronary 19 $^{20}_{21}$ artery stenosis [29], were also greater in pooled serum from case subjects.

²²₂₃Surprisingly, miR-885-5p, the miRNA displaying the most pronounced upregulation in cases, has not been ²⁴²⁵previously studied in the context of either diabetes or vascular diseases; however, mir-885-5p has potent anti-²⁷₂₈proliferative, pro-apoptotic, and pro-senescence effects; therefore, its enrichment might be of relevance and ²⁹ ³⁰deserves further investigation [30].

³²₃₃Among differential expressed miRNAs, we have chosen miR-126 for further assessment by measurement in all ³⁴ ³⁵individual serum samples from the EURODIAB nested case-control study. Consistent with the profiling results, ³⁶ ³⁷mean miR-126 levels were significantly lower in case than in control subjects and negatively associated with all ³⁹ ⁴ccomplications in logistic regression analysis.

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 $^{42}_{43}$ miR-126 is highly enriched in endothelial cells and plays a pivotal role in maintaining endothelial homeostasis 44 45and vascular integrity [31]. Furthermore, miR-126 controls endothelial inflammation, at least in part by 46 ⁴⁷lowering VCAM-1 expression [22,32]. As endothelial cell activation and inflammation are common features of 48 49 $\frac{1}{50}$ micro-macrovascular diseases in diabetes, this may provide a plausible link between miR-126 loss and diabetes 51 ⁵²complications. In line with this hypothesis, microvesicles released from endothelial cells cultured under 53 ⁵⁴₅₅standard conditions promote vascular repair via transfer of miR-126; by contrast, microvesicles from 56 57endothelial cells exposed in vitro to high glucose to mimic hyperglycemia show reduced miR-126 content and 58 ⁵⁹₆₀ impaired regenerative potential [33]. Whether miR-126 is a marker of diabetic complications or a mediator of 61

6 2vascular injury in diabetes remains, however, to be established.

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A previous pioneer work has identified a plasma miRNA signature for type 2 diabetes that includes loss of endothelial miR-126 [10] and results have been later confirmed by an independent laboratory [34]. Our results lexpand these findings and suggest that a measurable reduction of circulating miR-126 may be a marker not only ³of pre-diabetes, but also of diabetes-induced endothelial dysfunction. In logistic regression analyses, the inverse ⁵cassociations between miR-126 and vascular complications remained only marginally significant after ⁸adjustment for A1C and diabetes duration, suggesting a major role of hyperglycemia in mediating the ¹⁰1¹relationship between miR-126 and both micro and macrovascular complications of diabetes. Although this ¹²3¹finding may be important on the pathogenic standpoint, it reduces miR-126 relevance as potential new clinical ¹⁴biomarker for early diagnosis of complications.

18In logistic regression analysis, the OR of proliferative retinopathy was 25% lower for each unit increment of 19 $^{20}_{21}$ miR-126 levels. This was statistically significant and independent of age, sex, A1C, and diabetes duration. 22 23Although results from subgroup analyses have to be taken with caution, this finding is of potential relevance as 24 ²⁵miR-126 controls expression/signalling of angiogenetic factors, such as VEGF and SDF-1 [35-38], and 26 27 28 downregulation of miR-126 has been proven in the retina from diabetic rats [37] and causally linked to 29 ³⁰angiogenesis in animal models of cancer [35,36]. The association between miR-126 and proliferative 31 32 $^{32}_{33}$ retinopathy was partially dependent of sVCAM-1. In this regard it is noteworthy that overexpression of SDF-1, 34 ³⁵a miR-126 target, has been shown to contribute to proliferative retinopathy development, at least in part, 36 $^{37}_{38}$ through VCAM-1 upregulation on retinal endothelial cells, resulting in enhanced recruitment of endothelial cell 39 40precursors from hematopoietic stem cells [39].

⁴²Anti-platelet treatment may represent a potential confounding factor in the assessment of circulating levels of ⁴⁴ ⁴⁵platelet-derived miRNAs and platelets are another important source of circulating miR-126 besides endothelial ⁴⁶ ⁴⁷cells [40]. Therefore, we cannot exclude the possibility that anti-platelet therapy administered to type 1 diabetic ⁴⁹ ⁵⁰patients with vascular complications may explain their lower circulating miR-126 levels. However, in the ⁵¹ ⁵²EURODIAB PCS only a minority of cases were under anti-platelet treatment and exclusion of these subjects ⁵³ ⁵⁴from the analyses did not modify results, making less likely this possibility.

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⁵⁷There are certain limitations to our study. Firstly, this is a cross-sectional study and this restricts our ability to ⁵⁸ ⁵⁹assess temporal relationships between miR-126 and vascular complications and to identify causal biological ⁶¹ ⁶²mechanisms underlying this association. However, no data on miR-126 in large groups of type 1 diabetes

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patients exist; therefore this study may serve as a reasonable starting point to explore the role of this miRNA in type 1 diabetes. Although the Eurodiab PCS is a prospective study, serum samples collected at the baseline lexamination were not stored for subsequent analyses; therefore, miRNA measurements could only be 2 ³performed in serum samples collected at the follow-up visit. Secondly, the number of controls was lower than the overall number of cases, thus reducing the power of analyses; comparisons between controls and cases with 7 ⁸single complications allowed a more favorable case/control ratio, but multiple comparisons within the same 10 incase-control study base might have caused significant results due to chance. Thirdly, although serum samples 12 ¹³were adequately stored, the possibility of protein degradation cannot be excluded, though miRNAs are stable in 14 $^{15}_{16}$ biological fluids. Unlike previous studies, a key strength here is the ability to account for confounding by other 17 18risk factors and complications, and the large sample size provides sufficient power for these analyses. In 19 $^{20}_{21}$ addition, our patients were from a representative sample of people with type 1 diabetes across Europe, and our 22 23 results, therefore, are likely to be generalisable. This is the first study profiling miRNAs in a large group of type 24 ²⁵1 diabetic patients and our results provide evidence that miR-126 levels are associated with vascular 26 27 28 complications of diabetes and in particular with proliferative retinopathy. Further studies are required to 29 ³⁰determine causal relationships and elucidate underlying mechanisms. 31

³²₃₃ACKNOWLEDGMENTS

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³⁷₃₈**CONFLICT OF INTEREST:** None

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40ETHICAL APPROVAL: All procedures followed were in accordance with the ethical standards of the ⁴ institutional and/or national research committee and with the 1964 Helsinki declaration and its later ⁴²amendments or comparable ethical standards.

44 45INFORMED CONSENT: Informed consent was obtained from all individual participants included in the 46study.

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⁴⁸₄₉**REFERENCES**

- $\frac{1}{50}$ 1. He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5: 522-531. 51
- ⁵²². Filios SR, Shalev A (2015) β-Cell microRNAs: small but powerful. Diabetes 64:3631-3644.
- ⁵³3. Beltrami C, Angelini TG, Emanueli C (2014) Noncoding RNAs in diabetes vascular complications. J Mol 54 Cell Cardiol 89:42-50. 55
- 5*6*4. Shantikumar S, Caporali A, Emanueli C (2012) Role of microRNAs in diabetes and its cardiovascular complications. Cardiovasc Res 93:583-593. 57
- ⁵⁸5. Mitchell PS, Parkin RK, Kroh EM, et al (2008) Circulating miRNAs as stable blood-based markers for 59 cancer detection. Proc Natl Acad Sci U S A 105:10513-10518.
- 60 ₆₁6. Tanaka M, Oikawa K, Takanashi M, et al (2009) Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. PLoS One 4:e5532. 62
- 63 64

- 7. Laterza OF, Lim L, Garrett-Engele PW, et al (2009) Plasma miRNAs as sensitive and specific biomarkers of tissue injury. Clin Chem 55:1977-1983.
- 8. McManus DD, Ambros V (2011) Circulating miRNAs in cardiovascular disease. Circulation 124:1908-1910.
- 19. Nielsen LB, Wang C, Sorensen K, et al (2012) Circulating levels of microRNA from children with newly
- ² diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell
- ³ function and glycaemic control during disease progression. Exp Diabetes Res 896362
- ⁴₅10. Zampetaki A, Kiechl S, Drozdov I, et al (2010) Plasma microRNA profiling reveals loss of endothelial 6 miR-126 and other microRNAs in type 2 diabetes. Circ Res 107:810-817
- 711. Zampetaki A, Willeit P, Burr S, et al (2016) Angiogenic microRNAs linked to incidence and progression of
 diabetic retinopathy in type 1 diabetes. Diabetes 65:216-227.
- ⁹ 12. Pezzolesi MG, Satake E, McDonnell KP, et al (2015) Circulating TGF- β 1-regulated miRNAs and the risk 11 of rapid progression to ESRD in type 1 diabetes. Diabetes 64:3285-3293.
- ¹¹1213. The EURODIAB IDDM Complications Study Group: (1994) Microvascular and acute complications in IDDM patients: the EURODIAB IDDM Complications Study. Diabetologia 3:278-285.
- ¹⁴14. Chaturvedi N, Sjoelie AK, Porta M, et al (2001) EURODIAB Prospective Complications Study.: Markers of insulin resistance are strong risk factors for retinopathy incidence in type 1 diabetes. Diabetes Care 24: 284-289.
- 1815. Schram MT, Chaturvedi N, Schalkwijk CG, et al (2005) EURODIAB Prospective Complications Study
- ¹⁹ Group.: Markers of inflammation are cross-sectionally associated with microvascular complications and cardiovascular disease in type 1 diabetes: the EURODIAB Prospective Complications Study. Diabetologia 48:370-378.
- 48:370-378.
 2316. Gruden G, Bruno G, Chaturvedi N, et al (2009) EURODIAB Prospective Complications Study Group.:
 ANTI-HSP60 and ANTI-HSP70 antibody levels and micro/ macrovascular complications in type 1
 diabetes: the EURODIAB Study. J Intern Med 266:527-536.
- ²⁶2717. Gruden G, Bruno G, Chaturvedi N, et al (2008) EURODIAB Prospective Complications Study Group.:
 ²⁸Serum heat shock protein 27 and diabetes complications in the EURODIAB prospective complications
 ²⁹study: a novel circulating marker for diabetic neuropathy. Diabetes 57:1966-1970.
- ³⁰18. Burt D, Bruno G, Chaturvedi N, et al (2009) Anti-heat shock protein 27 antibody levels and diabetes ³¹ complications in the EURODIAB study. Diabetes Care 32: 1269-1271.
- ³² Complications in the ECRODIAB study. Diabetes Cate 32, 1209-1271.
 ³² Study. Study. Diabetes Cate 32, 1209-1271.
 ³² Chaturvedi N, Schalkwijk CG, Abrahamian H, et al (2002) EURODIAB Prospective Complications Study.
 ³⁴ Group.: Circulating and urinary transforming growth factor beta1, Amadori albumin, and complications of type 1 diabetes: the EURODIAB prospective complications study. Diabetes Cate 25:2320-2327.
- ³⁶20. Giunti S, Bruno G, Lillaz E, et al (2007) EURODIAB IDDM Complications Study Group. Incidence and
- ³⁷ risk factors of prolonged QTc interval in type 1 diabetes: the EURODIAB Prospective Complications
 ³⁹ Study. Diabetes Care 30:2057-2063.
- 4021. Levey AS, Bosch JP, Lewis JB, et al (1999) 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 130:461-470.
- ⁴³₄₄22. Harris TA, Yamakuchi M, Ferlito M, et al (2008) MicroRNA-126 regulates endothelial expression of 45 vascular cell adhesion molecule 1. Proc Natl Acad Sci USA 105:1516-1521.
- 4623. Martin MM, Lee EJ, Buckenberger JA, et al (2006) MicroRNA-155 regulates human angiotensin II type 1
 ⁴⁷ receptor expression in fibroblasts. J Biol Chem 281:18277-18284.
- ⁴⁸/₄₉24. Martin MM, Buckenberger JA, Jiang J, et al (2007) The human angiotensin II type 1 receptor +1166 A/C polymorphism attenuates microrna-155 binding. J Biol Chem 282:24262-24269.
- 5125. Barutta F, Tricarico M, Corbelli A, et al (2013) Urinary exosomal microRNAs in incipient diabetic nephropathy. PLoS One 8:e73798.
- ⁵³26. Fichtlscherer S, De Rosa S, Fox H, et al (2010) Circulating microRNAs in patients with coronary artery disease. Circ Res 107:677-684.
- 5₅₆27. Wang C, Wan S, Yang T, et al (2016) Increased serum microRNAs are closely associated with the presence of microvascular complications in type 2 diabetes mellitus. Sci Rep 6:20032.
- ⁵⁸28. Spinetti G, Fortunato O, Caporali A, et al (2013) MicroRNA-15a and microRNA-16 impair human circulating proangiogenic cell functions and are increased in the proangiogenic cells and serum of patients with critical limb ischemia. Circ Res 112:335-346.
- 62
- 63

- 29. Wang F, Long G, Zhao C, et al (2013) Plasma microRNA-133a is a new marker for both acute myocardial infarction and underlying coronary artery stenosis. J Transl Med 11:222.
- 30. Afanasyeva EA, Mestdagh P, Kumps C, et al (2011) MicroRNA miR-885-5p targets CDK2 and MCM5, activates p53 and inhibits proliferation and survival. Cell Death Differ 18:974-984.
- 131. van Solingen C, Bijkerk R, de Boer HC, et al (2015) The role of microRNA-126 in vascular homeostasis.
 ² Curr Vasc Pharmacol 13:341-351.
- ³32. Asgeirsdóttir SA, van Solingen C, Kurniati NF, et al (2012) MicroRNA-126 contributes to renal $\frac{4}{5}$ microvascular heterogeneity of VCAM-1 protein expression in acute inflammation. Am J Physiol Renal
- ⁶ Physiol 302:F1630-1639.
- 733. Jansen F, Yang X, Hoelscher M, et al (2013) Endothelial microparticle-mediated transfer of MicroRNA-126
 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial
 microparticles Circulation 128:2026-2038.
- ¹⁰₁₁34. Zhang T, Lv C, Li L, et al (2013) Plasma miR-126 is a potential biomarker for early prediction of type 2 12 diabetes mellitus in susceptible individuals. Biomed Res Int 2013:761617.
- ¹³³⁵. Zhang Y, Wang X, Xu B, et al (2013) Epigenetic silencing of miR-126 contributes to tumor invasion and angiogenesis in colorectal cancer. Oncol Rep 30:1976-1984.
- ¹⁵₁₆36. Chen H, Li L, Wang S, et al (2014) Reduced miR-126 expression facilitates angiogenesis of gastric cancer through its regulation on VEGF-A. Oncotarget 5:11873-11885.
- 1837. Ye P, Liu J, He F, et al (2013) Hypoxia-induced deregulation of miR-126 and its regulative effect on VEGF and MMP-9 expression. Int J Med Sci 11:17-23.
- ²⁰₂₁38. van Solingen C, de Boer HC, Bijkerk R, et al (2011) MicroRNA-126 modulates endothelial SDF-1
- expression and mobilization of Sca-1(+)/Lin(-) progenitor cells in ischaemia. Cardiovasc Res 92:449-455.
- ²339. Butler JM, Guthrie SM, Koc M, et al (2005) SDF-1 is both necessary and sufficient to promote proliferative
 retinopathy. J Clin Invest 115:86-93.
- ²⁵40. McManus DD, Freedman JE (2015) MicroRNAs in platelet function and cardiovascular disease. Nat Rev
 Cardiol 12:711-717.

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|---|-------------------------------------|------------------------------------|----------|
| 15 16 | Case subjects | Control subjects | Р |
| 17 10 N | 312 | 143 | |
| $^{18}_{19}$ Age (vears) | 41.5 ± 10.1 | 35.4 ± 7.3 | < 0.0001 |
| 20 Diabetes duration (years) | 24.5 ± 9.4 | 15.1 ± 6.7 | < 0.0001 |
| 21 Males (%) | 52.5% | 48.2% | 0.45 |
| 22 BMI (Kg/m ²) | 25.0 + 3.5 | 23.7 + 2.6 | < 0.0001 |
| ²³ WHR | 0.89 ± 0.13 | 0.87 ± 0.16 | 0.35 |
| 24 (%) | 89 ± 16 | 77 + 12 | <0.001 |
| 26 Systolic blood pressure (mmHg) | 126 7+ 21 6 | 1143 ± 129 | <0.0001 |
| ²⁷ Disstolic blood pressure (mmHg) | 75.8 ± 11.3 | 73.2 ± 10.3 | 0.04 |
| ²⁸ Hypertension (%) | 53 5% | 10.5% | <0.04 |
| 29 Total cholesterol (mmol/l) | 5.570 5.45 + 1.21 | 4.88 ± 1.08 | <0.0001 |
| 30 I otal choicsterol (mmol/l) | 3.45 ± 1.21 3.60 + 1.11 | 4.00 ± 1.00 3.04 ± 0.95 | <0.0001 |
| 32 HDL cholesterol (mmol/l) | 3.00 ± 1.11 1 50 ± 0.44 | 1.66 ± 0.42 | <0.0001 |
| ³³ Triglycoridog (mmol/l) | 1.39 ± 0.44 1 18 (0 81 1 58) | 1.00 ± 0.42 | <0.10 |
| ³⁴ miD 126 | 1.10(0.01-1.30) | 1.20(0.64, 2.11) | <0.0001 |
| 35 mik-120 | 0.83 (0.33-2.48) | 1.50(0.04-5.11) | <0.011 |
| $_{36}$ Data are expressed as mean \pm 5D, percentage 0 | i geometric mean (interquarti | le range) for log-transformed d | ata. |
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¹²Table 1. Characteristics of the 455 subjects with type 1 diabetes subjects recruited in the cross-sectional ¹³nested case-control study of the EURODIAB PCS.

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| ¹⁴ Table 2 Odds ratios for | · diabetes complication | ons hy miR-126 l | evels in the nest | ed case-control s |
| ¹⁵ ₁₆ the EURODIAB PCS st | udy | | | |
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| 18 | MODEL 1 | MODEL 2 | MODEL 3 | MODEL 4 |
| 19 | | | | |
| 20 | OR (95% CI) | OR (95% CI) | OR (95% CI) | OR (95% CI) |
| ²¹ All complications | 0.85 (0.75-0.96) | 0.89 (0.77-1.03) | 0.91 (0.77-1.07) | 0.93 (0.79-1.10) |
| ²² ₂₃ CVD (<i>n</i> =126) | 0.82 (0.70-0.96) | 0.87 (0.72-1.06) | 0.89 (0.72-1.10) | 0.91 (0.73-1.13) |
| 24 Nephropathy ($n=179$) | 0.83(0.72-0.95) | 0.84(0.72-0.99) | 0.88 (0.73-1.06) | 0 95 (0 78-1 17) |

study within

| 18 | | MODEL 1 | MODEL 2 | MODEL 3 | MODEL 4 |
|----------|--|------------------|------------------|------------------|-------------------|
| 19 | | | | | |
| 20 | | OR (95% CI) | OR (95% CI) | OR (95% CI) | OR (95% CI) |
| 21 | All complications | 0.85 (0.75-0.96) | 0.89 (0.77-1.03) | 0.91 (0.77-1.07) | 0.93 (0.79-1.10) |
| 22 23 | CVD (<i>n</i> =126) | 0.82 (0.70-0.96) | 0.87 (0.72-1.06) | 0.89 (0.72-1.10) | 0.91 (0.73-1.13) |
| 24 | Nephropathy (n=179) | 0.83 (0.72-0.95) | 0.84 (0.72-0.99) | 0.88 (0.73-1.06) | 0.95 (0.78-1.17) |
| 25 | Microalbuminuria (<i>n</i> =74) | 0.88 (0.74-1.05) | 0.89 (0.73-1.08) | 0.96 (0.77-1.20) | 0.99 (0.78-1.25) |
| 26 | Macroalbuminuria (n=105) | 0.77 (0.65-0.91) | 0.78 (0.63-0.97) | 0.79 (0.63-1.00) | 0.86 (0.67-1.10) |
| 27 | DSP (<i>n</i> =176) | 0.84 (0.73-0.97) | 0.89 (0.75-1.06) | 0.89 (0.74-1-08) | 0.92 (0.75-1.11) |
| 28 | | 0.00 (0.70.0.04) | 0.04 (0.72.0.00) | 0.06 (0.72.1.04) | 0.00 (0.72, 1.07) |
| 29 | Retinopathy (n=249) | 0.82 (0.72-0.94) | 0.84 (0.72-0.99) | 0.86 (0.73-1.04) | 0.88 (0.73-1.07) |
| 30 | Background Retinopathy (<i>n</i> =119) | 0.85 (0.73-1.00) | 0.89 (0.73-1.07) | 0.91 (0.74-1.11) | 0.91 (0.74-1.12) |
| 31 | Proliferative Retinopathy (<i>n</i> =130) | 0.78 (0.67-0.91) | 0.75 (0.61-0.92) | 0.75 (0.59-0.95) | 0.79 (0.61-1.02) |

33Model 1: unadjusted

³⁴Model 2: adjusted for age, sex

³⁵₃₆Model 3: adjusted for age, sex, diabetes duration, A1C, ₃₇Model 4: adjusted for age, sex, diabetes duration, A1C, sVCAM-1