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ORIGINAL ARTICLE

MicroRNA-423 may regulate diabetic vasculopathy

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Abstract
To test the hypothesis that microRNAs may play a role in diabetic retinopathy, we measured the levels of different markers [microRNAs, vascular endothelial growth factor (VEGF), nitric oxide (NO), and total antioxidant capacity (TAO)] in patients with type 2 diabetes mellitus (T2DM) and microvascular complications. Sixty-nine patients were recruited: 22 healthy subjects, ten T2DM patients without retinopathy, 22 with nonproliferative diabetic retinopathy, and 15 with proliferative diabetic retinopathy (PDR). Serum levels of NO, VEGF, TAO and 16 candidate microRNAs were measured. Additionally, the mRNA levels of endothelial nitric oxide synthase (eNOS), induced NOS (iNOS), C reactive protein (CRP), VEGF, tumor necrosis factor α (TNFα), PON2, p22, and SOD2 were measured in human vascular endothelial cells cultured in the presence of pooled sera from the subject groups. Plasma miR-423 levels showed a significant ~ twofold decrease in patients with PDR compared to controls. P lasma NO levels were significantly higher in retinopathy, VEGF levels were significantly lower, and TAO was significantly decreased. eNOS mRNA levels were lower in the cells of T2DM patients without retinopathy, but higher in PDR. PON2, p22, and SOD2 mRNA levels were all significantly lower in PDR. CRP, TNFα, iNOS, and VEGF mRNA levels showed no significant association with disease status. Lowered miR-423 levels in diabetic patients showed a correlation with VEGF and an inverse correlation between NO and eNOS expression. Our findings suggest a cross talk between miR-423 and VEGF signaling, affecting eNOS function. miR-423 may be involved in the regulation of diabetic vascular retinal proliferation.

Keywords miR-423 · NO · VEGF · NO-dependent pathways

Introduction

microRNAs (miRNAs) are short noncoding RNAs functioning as negative regulators of gene expression [1]. Although miRNAs are primarily intracellular molecules, they are detectable in body fluids and specifically in the blood [2], where they are contained in small membranous vesicles (exosomes, microparticles), within HDL, or linked to proteins [3–6]. It is now evident that extracellular miRNAs have physiological roles that involve immune and other intercellular communication mechanisms [7].

This study aimed to explore the possible involvement of extracellular miRNAs in microvascular complications of type 2 diabetes mellitus (T2DM), We recruited patients with T2DM at different stages of diabetic retinopathy (DR), which is a standard way of estimating severity of diabetic microvascular complications, as well as healthy controls. To identify the circulating miRNAs that could be associated with DR, we measured the serum levels of 16 candidate miRNAs identified in our and others' prior studies (miR-423, miR-486-3p, miR-320a-3p, miR-320b, miR-200b-3p, miR-221-3p, miR-146a-5p, miR-183-5p, miR-122-5p, miR-126-5p, miR-30d, miR-93-5p, miR-21, miR-27b-3p, let-7f-5p, and miR-16-2-3p. We also measured the serum levels

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of vascular endothelial growth factor (VEGF), nitric oxide (NO), and total antioxidant capacity (TAO) in our cohort.

To identify mechanistic pathways possibly affected by the altered serum miRNA levels, we measured by qRT-PCR the mRNA levels of endothelial nitric oxide synthase (eNOS), induced NOS (iNOS), C reactive protein (CRP), tumor necrosis factor α (TNFα), paraoxonase 2 (PON2), p22, and SOD2 in cultured human vascular endothelial cells (HUVEC) exposed to pooled sera from patients with different stages of retinopathy.

Our hypothesis was that one or more of the miRNAs may serve as a biomarker of disease status and severity and furthermore may have a regulatory role in NO-mediated pathways, responsible for the deregulated microvasculature proliferation and the oxidative stress that together with pro-inflammatory cytokines are responsible for aggravating endothelial dysfunction and accelerated atherosclerosis.

Methods

Cohort description

The study was approved by the Research Ethics Committee of the Baruch Padeh Medical Center, Israel, and participants gave fully informed oral and written consent. Sixty-nine patients were recruited from the ophthalmology outpatient clinic in the Baruch Padeh Medical Center. Every patient was examined by an ophthalmologist to determine retinopathy status. Based on the clinical classification, we had a group of 22 healthy subjects (ten women, 39 ± 10 years old), ten patients with T2DM without retinopathy (four women, mean age 64 ± 10 years old), 22 patients with nonproliferative diabetic retinopathy (NDPR) (ten women, mean age 66 ± 7 years old), and 15 patients with proliferative diabetic retinopathy (PDR) (seven women, mean age 65 ± 9 years old). The investigator who performed the laboratory experiments was blinded to the patients' clinical data. Venous blood samples were drawn from an antecubital vein into ethylenediaminetetraacetic acid-containing tubes. All blood samples were centrifuged at 3000×*g* for 5 min at 25 °C. Patients' sera were aliquoted for the measurement of nitric oxide (NO), vascular endothelial growth factor (VEGF), and I-CAM levels, for RNA isolation and for treatment of cultured cells as outlined below.

NO, VEGF, I-CAM, and total antioxidant capacity levels in sera were determined using nitric oxide assay kit cat. 780051-192 (Cayman, Ann Arbor, Michigan, USA), Human VEGF Immunoassay (Colorimetric) cat. DVE00 (R&D Systems, Minneapolis, Minnesota, USA), Human I-CAM-1/CD54 Allele-specific Immunoassay (Colorimetric) cat. DCD540 (R&D Systems Minneapolis, Minnesota, USA), and Total Antioxidant Capacity Assay Kit cat. Ab65329 (Abcam, Cambridge, UK), respectively, according to the manufacturer's instructions. The assays employed a sandwich ELIZA technique.

Analysis of miRNA levels in sera

Previously, we have systematically compared several methods of RNA extraction from serum/plasma [8]. Total RNA isolation used the Qiagen miRNeasy Serum/Plasma kit (Cat. 217184), with an elution volume of 14 µl nuclease-free water. Samples were lysed in QIAzol lysis reagent (Qiagen Cat. 79306). After elution, samples were stored at − 80 °C. Reverse transcription and quantitative PCR for candidate miRNAs were performed using TaqMan Advanced miRNA assays (Applied Biosystems), with a scaled-down version of the manufacturer's protocol. Briefly, the polyadenylation of miRNAs was performed in a final volume of 3 µl, using 2 µl of RNA; adapter ligation was performed in a final volume of 9 µl; reverse transcription was performed in a final volume of 18 µl; the miR-Amp reaction was performed in a final volume of 30 µl; and qPCR was performed in a final volume of 5 µl, in duplicates, on an Applied Biosystems ABI-7900HT Sequence Detection System (Thermo Fisher, Warrington, UK) equipped with a 384-well block. Results were analyzed using the comparative Ct approach with subsequent global normalization in SDS 2.3 (Thermo Fisher) and Microsoft Excel software.

Choice of tested miRNAs

Candidate miRNAs were gathered from 17 published studies of miRNAs showing altered levels in the plasma/serum of subjects with diabetes [9–25]. We focused on 16 candidate miRNAs: Let-7f-5p [9, 10], miR-16-2-3p [11], miR-27b-3p [12], miR-28-3p [11, 13, 14], miR-30d [15, 16], miR-93-5p [17], miR-122-5p [16, 18, 19], miR-126-5p [15, 20], miR-146a-5p [15, 21], miR-183-5p [22], miR-200b-3p [23], miR-221-3p [24, 25], miR-320a-3p [12, 17], miR-320b [17], miR-423 [11, 17], and miR-486-3p [11].

Cell culture

The cell line HUVEC (C-2519A) was obtained from Lonza (Basel, Switzerland) and grown in EGM-2 Bullekit medium cat. CC-4176 (Lonza, Basel, Switzerland). Subsequently, cells were incubated in 5% CO_2 at 37 °C.

HUVEC were exposed to serum pools from the subject groups as described above (10% in medium) overnight in vitro. Subsequently, cells were lysed for RNA isolation as outlined below.

Quantification of gene expression in cultured cells

RNA was extracted from cell pellets with TRI Reagent cat. T9424 (Sigma-Aldrich Israel Ltd., Rehovot, Israel). Each cDNA sample was produced from 1 µg total RNA, with VERSO reverse transcription kit cat. AB1453B (Thermo Fisher Scientific, Waltham, Massachusetts, USA) on an Applied Biosystems ABI-2720 thermal cycler (Thermo Scientific) using the following thermal profile: 70 °C for 5 min, 42 °C for 1 h, 52 °C for 30 min, and 95 °C for 2 min.

The cDNA of the mRNA transcripts was amplified using a Rotor-GeneTM 6000 qPCR cycler (Corbett Research, Sydney, Australia), using the PerfeCta SYBR Green Fast-Mix cat. 95072-012-4 (Quantabio, Beverly, Massachusetts, USA), and the following thermal profile: 95 °C for 3 min, followed by 55 cycles of 95 °C for 3 s, 60 °C for 30 s, followed by 72 °C for 10 min. Specific primers used are listed in Table 1. All primers were tested for efficiency (by serial dilutions) and specificity (by melting peak analysis). Results were analyzed using the comparative Ct approach with subsequent normalization to β-actin.

Statistics

Student's *t* test and Mann–Whitney test were used to assess statistical significance of differences between groups.

Table 1 qPCR primers used

Gene	Primer sequence (5′–3′)
β-Actin	Forward: GCCCTGGACTTCGAGCAAGA Reverse: TGCCAGGGTACATGGTGGTG
TNFα	Forward: CCCATGTTGTAGCAAACCCTC Reverse: ATGAGGTACAGGCCCTCTGA
CRP	Forward: CAGACAGACATGTCGAGGAAG Reverse: GTCGAGGACAGTTCCGTGTA
eNOS	Forward: CCCCGGAGAATGGAGAGAGCTTTG Reverse: GCCGAGCCCGAACACACAGA
iNOS	Forward: CGCATGACCTTGGTGTTTGG Reverse: CATAGACCTTGGGCTTGCCA
p22phox	Forward: CAGTGCGCGCCTAGCAGTGT Reverse: AACACGCCCGCCACAATGGA
PON2	Forward: CGACTTAAAGCCTCCAGAGAA Reverse: GGGAATTTTAGACCCACACTAAA
SOD2	Forward: GCACTAGCAGCATGTTGAGC Reverse: GCGTTGATGTGAGGTTCCAG
VEGF	Forward: CCCCAAACCAGTAACAATC Reverse: CACAGGCACATTTTCCAG

Results

Decreased serum miR-423 levels in PDR

To check whether serum miRNA levels show changes in association with DR status, we measured the levels of 16 candidate miRNAs (let-7f-5p, miR-16-2-3p, miR-27b-3p, miR-28-3p, miR-30d, miR-93-5p, miR-122-5p, miR-126-5p, miR-146a-5p, miR-183-5p, miR-200b-3p, miR-221-3p, miR-320a-3p, miR-320b, miR-423, and miR-486-3p) by qRT-PCR. Of all miRNAs tested, miR-423 levels showed an overall negative trend in correlation to diabetic retinopathy progression, with significantly lower levels in the PDR group ($p = 0.03$) compared to healthy controls (Fig. 1a). All other miRNAs were not significantly changed in the different stages of diabetic retinopathy.

Nitric oxide (NO) level in serum was measured using the nitrate/nitrite assay, where nitrate was converted into nitrite by nitrate reductase and was detected by a fluorescent product. NO levels were similar in healthy controls and in T2DM nonretinopathy patients, but were significantly higher in patients with NPDR ($p = 0.03$) and further increased in patients with PDR ($p = 0.005$) (Fig. 1b).

Vascular endothelial growth factor (VEGF) levels in serum were significantly higher in patients with T2DM without retinopathy ($p = 0.01$) but lower in patients with NPDR and significantly lower in patients with PDR ($p = 0.008$) (Fig. 1c).

Total antioxidant capacity (TAO) level in serum was significantly lower in patients with T2DM no-retinopathy ($p = 0.0007$) and in patients with NPDR ($p = 0.01$) and was further inhibited in patients with PDR ($p = 0.0006$, all compared to healthy volunteers) (Fig. 1d).

To study the effects of secreted factors on gene expression in endothelial cells in the context of microvascular complications of diabetes, cultured HUVEC were exposed to serum pools from the different subject groups (10% in medium) overnight in vitro, and mRNA levels were measured by qRT-PCR. Endothelial nitric oxide synthase (eNOS) mRNA levels were significantly lower in patients with T2DM compared to healthy volunteers ($p = 0.01$) (Fig. 2a). No significant change was observed in patients with NPDR, but eNOS mRNA levels were significantly higher in patients with PDR ($p = 0.02$) (Fig. 2a).

Induced nitric oxide synthase (iNOS) mRNA levels did not differ significantly between the groups (Fig. 2b).

C reactive protein mRNA levels were not significantly increased in diabetic patients but were reduced in patients with NPDR ($p = 0.05$) and with PDR (nonsignificant) (Fig. 2c).

Vascular endothelial growth factor (VEGF) mRNA levels showed a trend of up-regulation in all diabetic groups

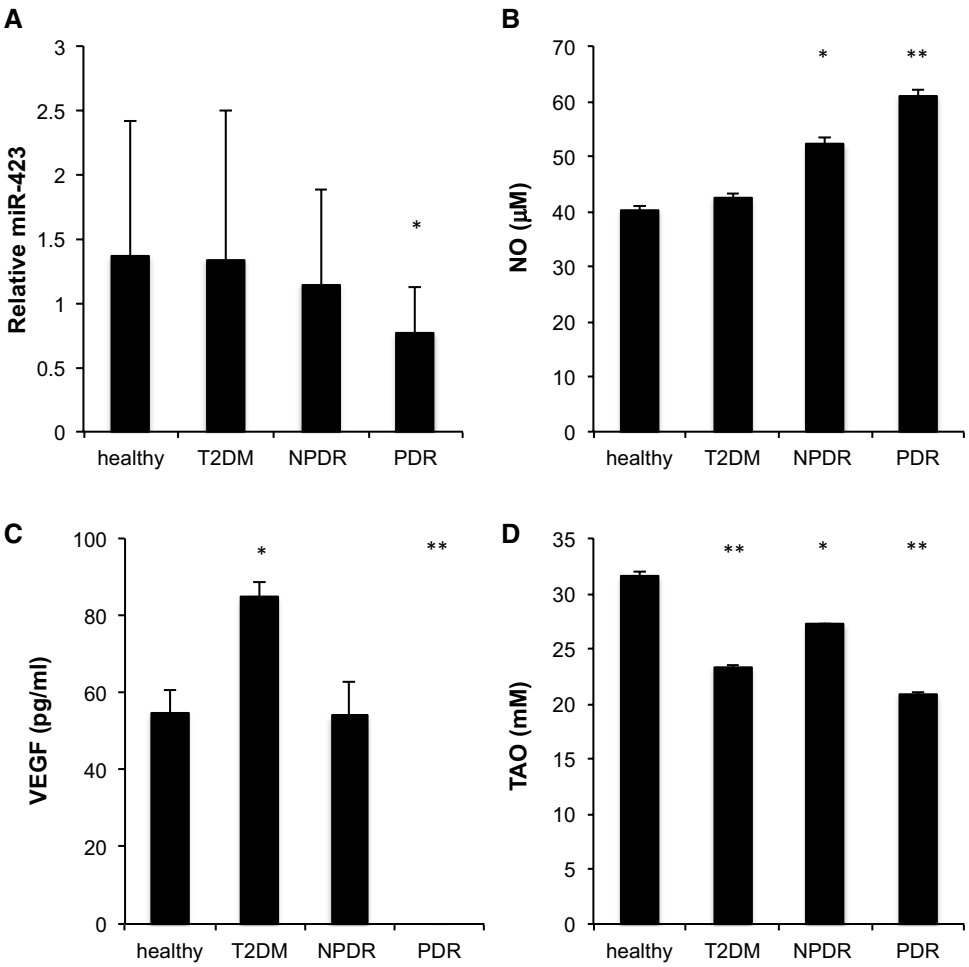

Fig. 1 **a** miR-423, **b** nitric oxide (NO), **c** vascular endothelial growth factor (VEGF) and **d** total antioxidant capacity (TAO) measurements in sera of groups as indicated: healthy ($N=22$), type 2 diabetes (T2DM, $N=10$), nonproliferative diabetic retinopathy (NPDR, $N=22$), and proliferative diabetic retinopathy (PDR, $N=15$). Bars, SD. * $p<0.05$; ** $p<0.01$ (Mann–Whitney test)

compared to healthy controls; however, only in the NPDR group, the difference was significant ($p=0.03$) (Fig. 2d).

Tumor necrosis factor α (TNFα) mRNA levels were higher in patients with T2DM compared with healthy controls ($p=0.05$), but did not differ significantly in the other groups (Fig. 2e).

Serum paraoxonase/arylesterase 2 (PON2) mRNA levels were significantly higher in the T2DM nonretinopathy group ($p=0.02$) but lower in the NPDR and PDR groups ($p=0.02$, 0.03, respectively) compared with healthy controls (Fig. 2f).

p22 mRNA levels were significantly lower in patients with PDR compared with healthy controls ($p=0.03$) (Fig. 2g).

SOD2 mRNA levels were also significantly lower in patients with PDR compared with healthy controls ($p=0.03$) (Fig. 2h).

Discussion

We found that serum levels of miR-423 were negatively associated with retinopathy progression, reaching a significant change in patients with PDR, concomitantly with a significant decrease in VEGF levels and significantly elevated NO levels. Exposure of HUVEC to sera from patients with PDR led to an increase in eNOS expression, in agreement with the higher serum NO levels. No change was observed in iNOS expression, so the increased NO levels were not due to induced NO production. In general, we did not observe increased inflammatory activity in patients with diabetic retinopathy; both CRP and TNFα expressions were lower in patients with PDR. However, it is important to mention that the sample size was small, and in order to consolidate our findings, a larger study should be done.

Prior studies have shown that miR-423 was decreased in patients with T2DM [23] and in morbidly obese subjects [24] which suggests a possible role in the pathway leading from obesity to diabetes mellitus.

Hypoxia is a potent trigger of VEGF production and secretion. It has been reported that microRNAs regulate VEGF signaling [26]; however, the mechanism(s) are still unclear. Hypoxia induces the accumulation of p53 [27, 28], which could regulate the expression of miRNAs that affects the production and secretion of VEGF. Also, the hypoxia-inducible factor 1 α (HIF-1α) down-regulates

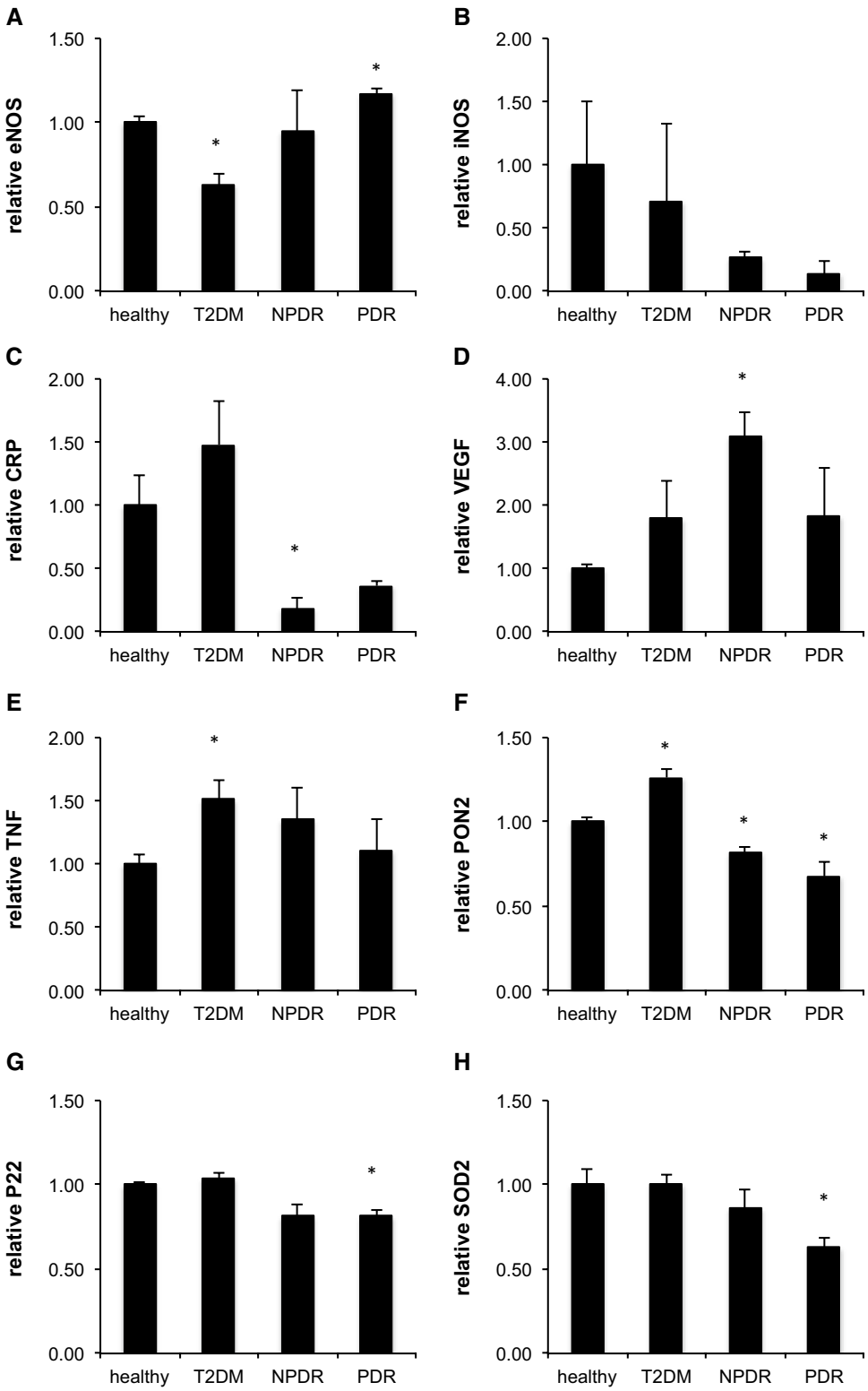

Fig. 2 qRT-PCR quantification of gene expression in HUVEC cultured in the presence of pooled sera from groups as indicated: healthy ($N=22$), type 2 diabetes (T2DM, $N=10$), nonproliferative diabetic retinopathy (NPDR, $N=22$), and proliferative diabetic retinopathy (PDR, $N=15$). **a** eNOS—endothelial nitric oxide synthase; **b** iNOS—induced nitric oxide synthase; **c** CRP—C reactive protein; **d** VEGF—vascular endothelial growth factor; **e** TNFα—tumor necrosis factor α; **f** PON2—paraoxonase 2; **g** P22—P22phox; **h** SOD2—superoxide dismutase 2. Results were normalized to β-actin. Bars, SE ($n=3$). * $p<0.05$ (Mann–Whitney test)

c-Myc-activated genes [29, 30] and may also down-regulate different microRNAs. Additionally, some microRNAs are transcriptionally regulated by NFκB which shows that it can directly activate miR-146 expression. In turn, miR-146 overexpression inhibited interleukin-1-β-induced NFκB activation in endothelial cells in the retina, a negative feedback mechanism [31]. A similar mechanism could have been activated in our patients with advanced diabetic retinal vasculopathy.

We have previously shown that diabetic patients without retinopathy had higher CRP, vascular cell adhesion molecule-1 (VCAM-1), and VEGF levels. However, when patients' retinopathy reached the proliferative stage (PDR), all markers of inflammation and angiogenesis were inhibited [32].in agreement with our current study.

miR-423 expression was dramatically increased in patients with dilated cardiomyopathy, and it suppressed proliferation and induced apoptosis of cardiomyocytes of patients with dilated cardiomyopathy [33]. On the other hand, miR-423 levels in microvesicles were enhanced by ischemia post-conditioning in cardiac fibroblasts, which was found to exert cardio-protective effects during the acute phase of ischemic reperfusion injury [34].

NO is synthesized as a by-product of the conversion of L-arginine to L-citrulline by endothelial nitric oxide synthases (eNOS) and acts as a pleiotropic intracellular messenger affecting physiological and pathological conditions [35]. Most of the NO in plasma is produced by eNOS, but there are stressful conditions like inflammation, where inducible NO is formed [36]. Low levels of NO are beneficial and help to keep homeostasis of vascular tonus, help to keep the blood in a normal equilibrium in aspects of coagulation and inflammation, and have anti-proliferative properties. However, high NO levels may cause uneventful effects through production of reactive oxygen species [37, 38]. Excessive reactive oxygen species (ROS) react with NO radicals and form the peroxynitrite anion (a toxic oxidant causing damage to biological molecules) [39–41]. In diabetic patients, hyperglycemia enhances the production of glycation end products, polyol, protein kinase C, and hexosamine pathways that may lead to oxidative stress [42, 43]. NO is oxidized in vivo, producing stable NO products nitrate and nitrite (NOx). There is a causal relationship between NO and plasma NOx; NOx level in plasma reflects NO bioavailability [38, 43]. Studies have demonstrated increased NO levels in diabetic patients [43–47], but other studies reported a decrease in NO level in diabetics [48–50]. A systemic review and meta-analysis of 30 published papers from different populations showed that serum or plasma NOx levels are higher—both in T1DM and T2DM—compared with nondiabetic controls [51]. Previous studies have shown that hyperglycemia enhanced NO production [52, 53] and/or decreased its bioavailability, leading to increased superoxide by-products [54]. Increased NO levels activated PKR-like endoplasmic reticulum kinase (a key signaling molecule in endoplasmic reticulum stress, involved in inflammation and apoptosis) that leads to apoptosis [55]. Additionally, high NO levels are cytotoxic for pancreatic beta cells, inhibit insulin secretion, and induce lipid peroxidation and apoptosis [56, 57].

We observed elevated NO levels apparently produced and secreted due to increased eNOS (but not iNOS) activity in patients with PDR.

Patients with diabetes mellitus have an impaired ability to build coronary collaterals and to regenerate damaged blood vessels, mainly due to a reduction in endothelial progenitor cells (EPCs) count and an impaired function of these cells [58]. Hyperglycemia has been demonstrated to reduce EPCs count and impair the function of EPCs (migration and proliferation) through an inhibitory effect on the phosphatidylinositol-3 kinase (PI 3 K)/protein kinase B (PKB)/Akt/endothelial nitric oxide synthase (eNOS)/nitric oxide signaling pathway [59–61]. Possible mechanisms that connect hyperglycemia and stem cells may include a reduction in NO bioavailability [62] and accelerated senescence through activation of p38 mitogen-activated protein kinase [63], oxidative stress [64], and through a direct inhibition of eNOS and excessive production of superoxide anions [65].

Our group has demonstrated that diabetic patients have an impaired ability to grow colonies of EPCs, but patients with PDR had almost no ability to produce EPCs in culture, which means that patients in this stage of microvascular disease have no regenerative capability [66]. We have also shown that patients with PDR have low levels of inflammatory proteins and low levels of VEGF [32].

VEGF induces NO production and secretion. Exposure of endothelial cells of rat aortic rings to VEGF increased significantly eNOS mRNA and eNOS protein levels, associated with an increase in cGMP in the cells [67]. This effect could be inhibited by tyrosine kinase inhibitors. The signaling pathway activated by VEGF (leading to an increase in eNOS) is led by at least two receptors—VEGFR1 and VEGFR2 [68]. Adding VEGF to cultured bovine aortic endothelial cells enhanced tyrosine phosphorylation of several proteins including VEGFR1, VEGFR2, phospholipase C, phosphatidylinositol 3-kinase, Ras GTPase activating protein and the oncogenic adaptor protein NcK, all leading to a proliferative response [69]. It seems that VEGF needs NO in order to induce angiogenesis in a cGMP-dependent pathway. Inhibition of eNOS (by L-NNA) blocked VEGF-induced NO release and angiogenesis [70]. VEGF and NO have bidirectional effects. NO also can enhance VEGF synthesis, and inhibition of NO decreased VEGF synthesis [71].

In our study, inhibition of TAO paralleled retinopathic severity, reaching the lowest levels in PDR. All three antioxidants measured (PON2, p22, and SOD2) showed lower expression in patients with PDR, which agrees with the reported inhibition of antioxidant activity in diabetic patients with severe retinopathy [72, 73].

Our findings suggest a link between miR-423, VEGF, and NO (through eNOS) in the molecular pathology of diabetic retinopathy. miR-423 may serve as a biomarker and a regulator of vascular growth/proliferation or inhibition, depending on the pathological process and/or the organ involved.

Limitations of the study

Our study was limited by the relatively small sample size. Future studies will need to recruit larger and more diverse populations. Further functional studies are required to test the mechanism of action of miR-423 in the context of diabetic complications.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215–33.
2. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. 2010;101:2087–92.
3. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to rewired networks. Cardiovasc Res. 2012;93:555–62.
4. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol. 2011;13:423–33.
5. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res. 2011;39:7223–33.
6. Ortega FJ, Mercader JM, Moreno-Navarrete JM, Rovira O, Guerra E, Esteve E, et al. Profiling of circulating microRNAs reveals common microRNAs linked to type 2 diabetes mellitus that change with insulin sensitization. Diabetes Care. 2014;37(5):1375–83.
7. Thomou T, Mori MA, Dreyfuss JM, Konishi M, Sakaguchi M, Wolfrum C, et al. Adipose-derived circulating miRNAs regulate gene expression in other tissues. Nature. 2017;542(7642):450–5.
8. Meerson A, Ploug T. Assessment of six commercial plasma small RNA isolation kits using qRT-PCR and electrophoretic separation: higher recovery of microRNA following ultracentrifugation. Biol Methods Protoc. 2016;1:bpw003.
9. Pezzolesi MG, Satake E, McDonnell KP, Major M, Smiles AM, Krolewski AS. Circulating TGF-β1-regulated miRNAs and the risk of rapid progression to ESRD in type 1 diabetes. Diabetes. 2015;64:3285–93.
10. Jones A, Danielson KM, Benton MC, Ziegler O, Shah R, Stubbs RS, Das S, Macartney-Coxson D. miRNA signatures of insulin resistance in obesity. Obesity (Silver Spring). 2017;25:1734–44.
11. Meerson A, Najjar A, Saad E, Sbeit W, Barhoum M, Assy N. Sex differences in plasma microRNA biomarkers of early and complicated diabetes mellitus in Israeli Arab and Jewish Patients. Non-Coding RNA. 2019;5:32.
12. Zampetaki A, Willeit P, Burr S, Yin X, Langley SR, Kiechl S, Klein R, Rossing P, Chaturvedi N, Mayr M. Angiogenic microRNAs linked to incidence and progression of diabetic retinopathy in type 1 diabetes. Diabetes. 2016;65:216–27.
13. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, Mayr A, Weger S, Oberhollenzer F, Bonora E, et al. Plasma microRNA profiling reveals loss of endothelial MiR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107:810–7.
14. Jimenez-Lucena R, Rangel-Zúñiga OA, Alcalá-Díaz JF, Lopez-Moreno J, Roncero-Ramos I, Molina-Abril H, Yubero-Serrano E, Caballero-Villarraso J, Delgado-Lista J, Castaño JP, et al. Circulating miRNAs as predictive biomarkers of type 2 diabetes mellitus development in coronary heart disease patients from the CORDIOPREV study. Mol Ther Nucl Acids. 2018;12:146–57.
15. Seyhan AA, Nunez Lopez YO, Xie H, Yi F, Mathews C, Pasarica M, Pratley RE. Pancreas-enriched miRNAs are altered in the circulation of subjects with diabetes: a pilot cross-sectional study. Sci Rep. 2016;6:31479.
16. de Candia P, Spinetti G, Specchia C, Sangalli E, La Sala L, Uccellatore A, Lupini S, Genovese S, Matarese G, Ceriello A. A unique plasma microRNA profile defines type 2 diabetes progression. PLoS ONE. 2017;12:e0188980.
17. Hirota K, Keino H, Inoue M, Ishida H, Hirakata A. Comparisons of microRNA expression profiles in vitreous humor between eyes with macular hole and eyes with proliferative diabetic retinopathy. Graefes Arch Clin Exp Ophthalmol. 2015;253:335–42.
18. Blum A, Yehuda H, Geron N, Meerson A. Elevated levels of miR-122 in serum may contribute to improved endothelial function and lower oncologic risk following bariatric surgery. Isr Med Assoc J. 2017;19:620–4.
19. Pastukh N, Meerson A, Kalish D, Jabaly H, Blum A. Serum miR-122 levels correlate with diabetic retinopathy. Clin Exp Med. 2019;2:255–60.
20. Wang X, Sundquist J, Zöller B, Memon AA, Palmér K, Sundquist K, Bennet L. Determination of 14 circulating microRNAs in Swedes and Iraqis with and without diabetes mellitus type 2. PLoS ONE. 2014;9:e86792.
21. Kong L, Zhu J, Han W, Jiang X, Xu M, Zhao Y, Dong Q, Pang Z, Guan Q, Gao L, et al. Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. Acta Diabetol. 2011;48:61–9.
22. Flowers E, Kanaya AM, Fukuoka Y, Allen IE, Cooper B, Aouizerat BE. Preliminary evidence supports circulating microRNAs as prognostic biomarkers for type 2 diabetes. Obes Sci Pract. 2017;3:446–52.
23. McArthur K, Feng B, Wu Y, Chen S, Chakrabarti S. MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. Diabetes. 2011;60:1314–23.
24. Carreras-Badosa G, Bonmatí A, Ortega F-J, Mercader J-M, Guindo-Martínez M, Torrents D, Prats-Puig A, Martinez-Calcerrada J-M, Platero-Gutierrez E, De Zegher F, et al. Altered circulating miRNA expression profile in pregestational and gestational obesity. J Clin Endocrinol Metab. 2015;100:E1446–56.
25. Farr RJ, Januszewski AS, Joglekar MV, Liang H, McAulley AK, Hewitt AW, Thomas HE, Loudovaris T, Kay TWH, Jenkins A, et al. A comparative analysis of high-throughput platforms for validation of a circulating microRNA signature in diabetic retinopathy. Sci Rep. 2015;5:10375.
26. Hua Z, Lv Q, Ye W, Chung-Kwun A, Cai G, Gu D, et al. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PLoS ONE. 2006;1(1):e116. https://doi.org/10.1371/journal.pone.0000116.
27. Graeber TG, Peterson JF, Tsai M, Monika K, Fornace AJ, et al. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol. 1994;14:6264–77.
28. Koumenis C, Alarcon R, Hammond E, Sutphin P, Hoffman W, et al. Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. Mol Cell Biol. 2001;21:1297–310.

29. Giaccia AJ, Simon MC, Johnson R. The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes Dev. 2004;18:2183–94.
30. Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, et al. HIF-1 alpha induces cell cycle arrest by functionally counteracting Myc. EMBO J. 2004;23:1949–56.
31. Kovacs B, Lumayag S, Cowan C, Xu S. microRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. Investig Ophthalmol Vis Sci. 2011;52:4402–9.
32. Blum A, Socea D, Ben-Shushan RS, Keinan-Boker L, Naftali M, Segol G, Tamir S. A decrease in VEGF and inflammatory markers is associated with diabetic proliferative retinopathy. Eur Cytokine Netw. 2012;23(4):158–62.
33. Li Q, Yu Q, Na R, Liu B. MiR-423-5p inhibits human cardiomyoblast proliferation and induces cell apoptosis by targeting Gab 1. Int J Clin Exp Pathol. 2016;9(9):8953–62.
34. Luo H, Li X, Li T, Zhao L, He J, Zha L, Qi Q, Yu Z. Exosomes/microvesicles microRNA-423-3p derived from cardiac fibroblasts mediates the cardioprotective effects of ischemic postconditioning. Cardiovasc Res. 2018. https://doi.org/10.1093/cvr/cvy231.
35. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem. 1994;63:175–95.
36. Caimi G, Hopps E, Montana M, Noto D, Canino B, Lo Presti R, et al. Evaluation of nitric oxide metabolites in a group of subjects with metabolic syndrome. Diabetes Metab Syndr. 2012;6(3):132–5.
37. Zou MH, Cohen R, Ullrich V. Peroxynitrite and vascular endothelial dysfunction in diabetes mellitus. Endothelium. 2004;11(2):89–97.
38. Pacher P, Beckman JS, Liauder L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87(1):315–424.
39. Beckman JP, Crow JP. Pathological implications of nitric oxide, superoxide and peroxynitrite formation. Biochem Soc Trans. 1993;21(2):330–4.
40. Honing ML, Morrison PJ, Banga JD, Stroes ES, Rabelink TJ. Nitric oxide availability in diabetes mellitus. Diabetes Metab Rev. 1998;14(3):241–9.
41. Forstermann U. Nitric oxide and oxidative stress in vascular disease. Pflugers Arch. 2010;459(6):923–39.
42. Brownlee M. The pathology of diabetic complications: a unifying mechanism. Diabetes. 2005;54(6):1615–25.
43. Pitocco D, Zaccardi F, Di Stasio E, Romitelli SA, Santini SA, Zuooi C, et al. Oxidative stress, nitric oxide, and diabetes. Rev Diabet Stud. 2010;7(1):15–25.
44. Adela R, Nethi SK, Bagul PK, Barui AK, Mattapally S, Kuncha M, et al. Hyperglycemia enhances nitric oxide production in diabetes: a study from South Indian patients. PLoS ONE. 2015;10(4):e0125270.
45. Aydin A, Orhan H, Sayal A, Ozata M, Sahin G, Ismer A. Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: effects of glycemic control. Clin Biochem. 2001;34(1):65–70.
46. Hoeldtke RD, Bryner KD, McNeill DR, Warehime SS, Van Dyke K, Hobbs G. Oxidative stress and insulin requirements in patients with recent-onset type 1 diabetes. J Clin Endocrinol Metab. 2003;88(4):1624–8.
47. Mylona-Karayanni C, Gourgiotis D, Bossios A, Kamper EF. Oxidative stress and adhesion molecules in children with type 1 diabetes mellitus: a possible link. Pediatr Diabetes. 2006;7(1):51–9.
48. Pitocco D, Zaccardi F, Di Stasio E, Romitelli F, Martini F, Scaglione GL, et al. Role of asymmetric dimethyl L arginine (ADMA) and nitrite/nitrate (NOx) in the pathogenesis of oxidative stress in female subjects with uncomplicated type 1 diabetes mellitus. Diabetes Res Clin Pract. 2009;86(3):173–6.
49. Daimon M, Sugiyama K, Saitoh H, Yamaguchi A, Hirata A, Ohnuma H. Increase in serum ceruloplasmin levels is correlated with a decrease of serum nitric oxide levels in type 2 diabetes. Diabetes Care. 2000;23(4):559–60.
50. Krause J, Rodrigues-Krause J, O'Hagan C, De Vito G, Boreham C, Susta D, et al. Differential nitric oxide levels in the blood and skeletal muscle of type 2 diabetic subjects may be consequence of adiposity: a preliminary study. Metab, Clin Exp. 2012;61(11):1528–37.
51. Shiekh GA, Ayub T, Khan SN, Dar R, Andrabi KI. Reduced nitrate level in individuals with hypertension and diabetes. J Cardiovasc Dis Res. 2011;2(3):172–6.
52. Yang P, Cao Y, Li H. Hyperglycemia induces inducible nitric oxide synthase gene expression and consequent nitrosative stress via c-Jun N-terminal kinase activation. Am J Obstet Gynecol. 2010;203(2):185 e5-11.
53. Zhang X, Fu Y, Xu X, Li M, Du L, Han Y, et al. PERK pathway are involved in NO-induced apoptosis in endothelial cells cocultured with RPE under high glucose conditions. Nitric Oxide. 2014;40:10–6.
54. Cai S, Khoo J, Channon KM. Augmented BH4 by gene transfer restores nitric oxide synthase function in hyperglycemic human endothelial cells. Cardiovasc Res. 2005;65(4):823–31.
55. Smukler SR, Tang L, Wheeler MB, Salapatek AM. Exogenous nitric oxide and endogenous glucose-stimulated beta-cell nitric oxide augment insulin release. Diabetes. 2002;51(12):3450–60.
56. Kurohane Kaneko Y, Ishikawa T. Dual role of nitric oxide in pancreatic beta cells. J Pharmacol Sci. 2013;123(4):295–300.
57. Eizirik DL, Flodstrom M, Karlsen AE, Welsh N. The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. Diabetologia. 1996;39(8):875–90.
58. Cha HN, Kim YM, Kim JY, Kim YD, Song IH, Min KN, et al. Lack of inducible nitric oxide synthase does not prevent aging-associated insulin resistance. Exp Geront. 2010;45(9):711–8.
59. Lambiase PD, Edwards RJ, Anthopoulos P, Rahman S, Meng YG, Bucknall CA, et al. Circulating humoral factors and endothelial progenitor cells in patients with differing coronary collateral support. Circulation. 2004;109:2986–92.
60. Chen YH, Lin SJ, Lin FY, Wu TC, Tsao CR, Huang PH, et al. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide related but not oxidative stress-mediated mechanisms. Diabetes. 2007;56:1559–68.
61. Krankel N, Adams V, Linke A, Gielen S, Erbs S, Lenk K, et al. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells. Arterioscler Thromb Vasc Biol. 2005;25:698–703.
62. Callaghan MJ, Ceradini DJ, Gurtner GC. Hyperglycemia-induced reactive oxygen species and impaired endothelial progenitor cell function. Antioxid Redox Signal. 2005;7:1476–82.
63. Ma FX, Zhou B, Chen Z, Ren Q, Lu SH, Sawamura T, et al. Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. J Lipid Res. 2006;47:1227–37.
64. Kuki S, Imanishi T, Kobayashi K, Matsuo Y, Obana M, Akasaka T. Hyperglycemia accelerated endothelial progenitor cell senescence via the activation of p38 mitogen-activated protein kinase. Circ J. 2006;70:1076–81.
65. Thum T, Fraccarollo D, Schultheiss M, Froese S, Galuppo P, Widder JD, et al. Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes. Diabetes. 2007;56:666–74.
66. Blum A, Socea D, Pastukh N, Jabaly H. Colony forming unites-endothelial progenitor cells: a surrogate marker for diabetic retinopathy and high cardiovascular mortality rate. Int J Pharma Res Rev. 2016;5(5):57–62.
67. Bouloumie A, Schini-Kerth VB, Busse R. Vascular endothelial growth factor up-regulates nitric oxide synthase expression in endothelial cells. Cardiovasc Res. 1999;41:773–80.

68. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem. 1994;269:26988–95.
69. Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. J Biol Chem. 1995;270:6729–33.
70. Cudmore M, Ahmed S, Al-Ani B, Hewett P, Ahmed S, Ahmed A. VEGF-E activates endothelial nitric oxide synthase to induce angiogenesis via cGMP and PKG-independent pathways. Biochem Biophys Commun. 2006;345(4):1275–82.
71. Dulak J, Jozkowicz A, Dembinska-Kiec A, Guevera I, Zdzienicka A, Zmudzinska-Grochot D, Florek I, Wojtowicz A, Szuba A, Cooke JP. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2000;20(3):659–66.
72. Bajaj S, Khan A. Antioxidants and diabetes. Indian J Endocrinol Metab. 2012;16(2):S267–71.
73. Rehman K, Akash MSH. Mechanism of generation of oxidative stress and pathophysiology of type 2 diabetes mellitus: how are they interlinked? JCell Biochem. 2017;118(11):3577–85.