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Extracellular vesicles participate in the transport of cytokines and angiogenic factors in diabetic patients with ocular complications

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Abstract: Introduction: Extracellular vesicles (EVs), including circulating microvesicles (MVs) or microparticles (MPs) and exosomes, derived from cells or platelets are present in the peripheral blood and are important elements involved in the activation of the coagulation system, transport of macromolecules and intercellular communication. In patients with vascular complications (including diabetes), the number of EVs is significantly increased during the acute phase of the disease. However, less is known about EVs release in the chronic state of diabetes.
Objectives: To analyse the profile of inflammatory cytokines and angiogenic factors in EVs in diabetic patients with ocular and vascular complications.
Patients and methods: The study included patients with diabetes and varying degrees of ocular complications including retinopathy (n = 48) and the control group (n = 13). EV-enriched and EV-depleted fractions were obtained from platelet-poor plasma by means of the centrifugation method (16 000 g, for 90 min). In screening, the profile of cytokines with pro-angiogenic effects was preliminary assessed using the protein microarray technology for controlled diabetic patients — CD, uncontrolled diabetic patients — UD and for the control group. In all patients, concentrations of cytokines: RANTES (Regulated on Activation, Normal T-cell Expressed and secreted) and Ang-2 (angiopoietin-2) were assayed using the ELISA method. Common blood and biochemical tests were performed.

Results: In patients with diabetes, analysis of supernatant revealed significantly increased concentrations of basic fibroblast growth factor (bFGF) and soluble receptor for vascular endothelial growth factor 2 (V-EGFR2) when compared to the control group: 49 (10.5–122) vs. 24 (2–72.5) SD (p = 0.03) and 260 (195.5–351) vs. 360 (256–461.5) SD (p = 0.01). In UD patients, concentrations of RANTES, angiostatin, tumor necrosis factor-α (TNF), and tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and TIMP2) were relatively higher in the EV-enriched fraction when compared to the EV-depleted fraction. *Post hoc* analysis revealed significant differences between UC patients and the control group in RANTES (16.73 (14.41–18.93) vs. 14.62 (12.37–15.28) mg/ml; p = 0.0235) and Ang-2 (2.76 (2.23–4.64) ng/ml vs. 1.74 (1.54–1.93); p = 0.0316) concentrations. These analyses did not reveal any significant differences in RANTES and Ang-2 concentrations between CD patients and the control group.
Conclusions: The profiles of cytokines and angiogenic factors in EVs are significantly increased in patients with diabetes. Also, the formation of specific cytokines related to EVs is strongly influenced by disease duration and successful treatment. EVs seem to be the conveyors of upregulated cytokines and angiogenic agents in diabetic patients.

Key words: Extracellular vesicles (EVs), diabetes, retinopathy, cytokines, RANTES, Angiopoietin-2.

Introduction

Diabetic complications are currently one of the more serious social problems in Poland. Mortality and varying degrees of disability, which are a consequence of diabetic complications, are not only a major health issue but also can generate a number of social and economic problems. Currently, it is estimated that approximately 3 million Poles suffer from diabetes. Diabetes causes a high risk of vascular complications, of which up to 41% include eye damage [1]. Consequently, prevention and early diagnosis may play a very important role to achieve an improved therapeutic effect by determining new ways of effective treatment. The macula is a very small area at the centre of the retina, responsible for pinpoint vision. The main cause of blindness in the diabetic population is maculopathy, usually preceded by macular edema. This is the one of very primary symptoms of diabetic retinopathy developed when blood vessels in the retina are leaking fluids and cause hypoxia without perfusion of the retina [2]. It is suggested that hyperglycemia and hypoxia may interact *via* a common metabolic imbalance to initiate and exacerbate some complications and increase free NADH/NAD_c, which appears a candidate entangling metabolite causing complications of diabetes [3]. However, the mechanism of how local retinal hypoxia is associated with hyperglycemic conditions in diabetes is still poorly understood [4]. A wide range of phenomena that contribute to diabetic retinopathy includes the inflammatory response and increased permeability of the blood-retina barrier [5]. They can involve multiple factors that finally produce local changes in microcirculation and stimulate angiogenesis — the formation of new blood vessels [4, 5].

Currently, increased attention is focused on the development of new directions for retinopathy treatment based on the fundamentals of the anti-inflammatory and

anti-angiogenic approach [6]. Current research is also focused on extracellular vesicles (EVs), which are thought to have an increasingly important role in the progression of diabetic complications [7, 8]. EVs are considered to be not only a biomarker but also a therapeutic target in diabetic retinopathy [9].

In our study, we conducted experiments to find a meaningful link between the inflammatory state, angiogenesis and circulating EVs. Our working hypothesis assumed that in patients with some vascular diabetic complications (retinopathy), circulating EVs can participate in the transport of inflammatory cytokines and selected angiogenic factors [10]. To test such a formulated hypothesis we planned to perform a screening experiment to select specific angiogenic and inflammatory cytokines in the EV-enriched fraction of plasma obtained from patients with controlled diabetes mellitus (CD), uncontrolled diabetes mellitus (UD), and from the control group. Then, we quantitatively analyzed the expression of the cytokine regulated on activation, normal T-cell expressed and secreted (RANTES) and the angiogenic factor, the role of which is also related to endothelial dysfunction — angiopoietin-2 (Ang-2) in an extended group of patients with vascular complications with respect to control subjects.

Materials and methods

Study group

Twelve patients aged 40 to 64 years were enrolled into the screening study. The participants were classified into 3 groups: CD — controlled diabetes type 2 (n = 4); UD — uncontrolled diabetes type 2 (n = 4), and the control group which included healthy subjects (n = 4). The inclusion criteria were described in our previous paper [11]. The study groups were allocated according to the criterion of glycated hemoglobin (HbA1c) levels. According to the Polish Diabetes Association guidelines for 2014, an HbA1c level of 7% is a general criterion of carbohydrate metabolism compensation. Patients in whom glycated hemoglobin levels exceed 7% are considered as they have poorly controlled glucose management and they were assigned to the UD group [12]. All patients underwent medical examination and a structured interview to investigate any family history of vascular and cancer diseases, smoking and use of medications. In the screening group, 1 patient only had 1 microangiopathy disorder — retinopathy; 4 patients had 2 disorders: retinopathy and nephropathy; 2 patients had 2 types of vascular complications: microangiopathy and macroangiopathy, 1 patient had a disorder in the form of macroangiopathy only. The characteristics of the study and control groups used for the screening is presented in Table 1.

For the quantitative study, an extended group of 61 patients was analyzed including diabetic patients (n = 48) and controls (n = 13). Their demographic and clinical characteristics is presented in Table 3. In that group, 11 patients had only 1 microangiopathy disorder: retinopathy; 9 patients had 2 disorders: retinopathy and nephropathy; 2 patients

Table 1. Characteristics of the screening study group in comparison with the control group.

	Diabetes Mellitus n = 8	Control n = 4	P
Age (years)	61.5 (59.5–65.5)	56 (53–58.5)	0.104
BMI (kg/m^2)	26.87 (25.36–31.59)	24.03 (21.99–29.93)	0.445
Sex (men/women)	5/3	2/2	0.766
TC (mmol/l)	5.00 (4.07–7.26)	6.53 (5.88–6.99)	0.552
LDL-C (mmol/l)	2.88 (2.23–4.02)	4.51 (3.48–5.15)	0.203
HDL-C (mmol/l)	1.21 (1.02–1.45)	1.27 (1.06–1.67)	0.734
TG (mmol/l)	1.62 (0.93–3.25)	1.77 (1.22–2.11)	0.932
Glucose (mmol/l)	**12.65 (7.65–17.5)**	**5.70 (5.00–6.25)**	**0.034**
HbA1C (%)	8.25 (6.6–10.75)	NA	–
Creatinine (μmol/l)	**89.91 (77.1–117.1)**	**69.5 (64.5–75)**	**0.043**
hsCRP (mg/l)	1.62 (0.93–2.89)	2.17 (1.94–2.84)	0.445

Values are given as median (Q1–Q3). Abbreviations: BMI, body mass index; hsCRP, high sensitivity C-reactive protein; HbA1C, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides. Bold means statistically significant differences between the groups.

had 2 types of disorders: microangiopathy and macroangiopathy disorders, and 16 patients had a disorder only in the form of macroangiopathy.

Patients were recruited for the study from the Private Ophthalmology Practice OKO-LASER Outpatient Clinic (Krakow, Poland) and Department of Interventional Cardiology, American Heart of Poland SA (Chrzanów, Poland). The study was approved by the Ethics Committee of the Jagiellonian University Medical College (No. KBET/260/B/2013).

Sample collection

All blood samples were drawn at the same time of the day (between 08:00 and 10:00 am) by venipuncture of the antecubital vein with a > 21-gauge needle following application of a light tourniquet. The first 2–3 ml of blood was not included for EV analysis. The

blood collected into tri-sodium citrate tubes was centrifuged twice at 2500×g for 15 min to obtain platelet-poor plasma (PPP). For hematology and HbA1C, EDTA was used as an anticoagulant. For biochemistry and biomarkers, the blood was collected in serum separator tubes (SST). The plasma and serum samples were aliquoted and frozen at −80°C for further analyses.

For the analysis of EVs (including MVs or MPs and exosomes), 300 mL of PPP was thawed in water bath at 37°C to avoid cryoprecipitation, mixed and centrifuged at 16 000×g for 90 minutes at 4°C to obtain the EV-enriched fraction in the lower part of the supernatant (first 50 μl above the pellet). The rest of the supernatant was considered the EV-depleted fraction. The pellet containing apoptotic bodies and residual platelets was removed (Fig. 1).

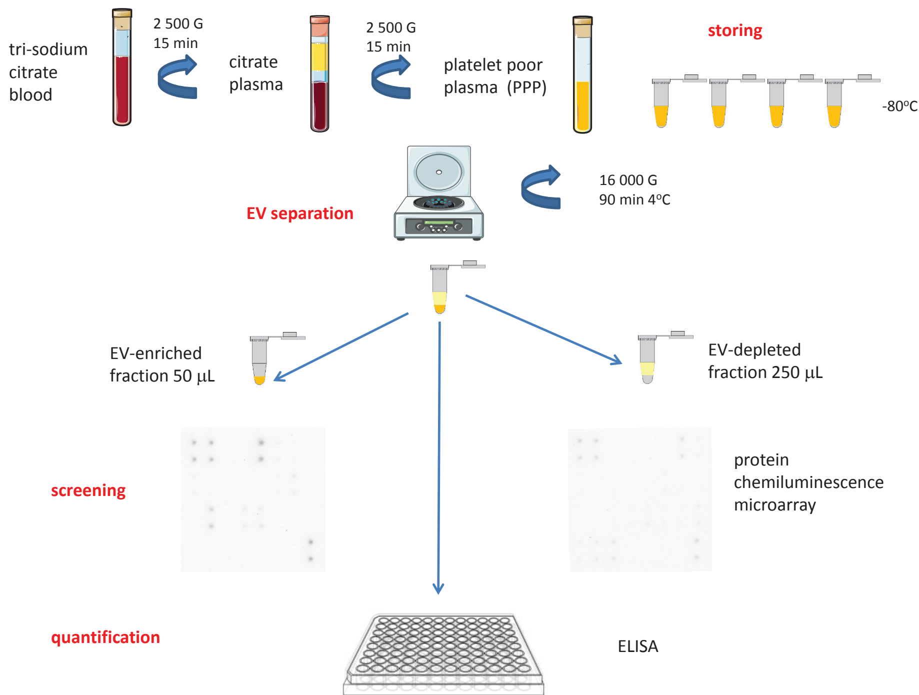

Fig. 1. Schematic representation of centrifugation protocol and analysis workflow. The figure was produced using Servier Medical Art.

Laboratory analyses

Standard blood tests including biochemistry (e.g. triglycerides, LDL-C, HDL-C) were performed on the MaxMat analyzer using ELITech Clinical Systems tests (Puteaux, France). Detection limit of this method was 0.06 mmol/l. For high-sensitivity C-reactive

protein, the APTEC Ultra Sensitive CRP test was used (APTEC Diagnostics nv, Belgium). Detection limit of this method was 0.13 mg/l. HbA1C concentration was measured on D-10 analyzer (D-10 Hemoglobin Testing System, Bio-Rad Laboratories, Inc, California, USA). This method is certified by the NGSP.

As a screening tool to compare expression levels of variety of proteins (cytokines, growth factors, proteases, soluble receptors, and other proteins), the Membrane-Based Human Angiogenesis Antibody Arrays by RayBiotech, Inc (cat. No AAH-ANG-1000-8, C-Series) were used. Chemiluminescence was detected by means of the EC3 BioSpectrum Imaging System (UVP, Jena, Germany) equipped with the Vision Works LS software. Semi-quantitative analysis was performed using Image J software with the Protein Array Analysis plugin by Gilles Carpentier [13]. Data are expressed as signal density [SD] units.

Quantitative analyses of selected factors were performed using commercially available immunoenzymatic assays (ELISA). RANTES (cytokine Regulated on Activation, Normal T-cell Expressed and Secreted) levels were determined by means of Human RANTES ELISA Kit (cat No 201-12-0085; SUN RED Biological Technology, Shanghai, China). Angiopoietin-2 (Ang-2) levels were determined by means of the Qantikine ELISA Human Angiopoietin-2 kit (cat. No DANG20; R&D Systems, Minneapolis, USA).

Statistical analysis of biochemical and epidemiological parameters

All analyses were carried out with Statistica version 10.0 (StatSoft Inc. USA). Array results are presented as median with interquartile range (Q1–Q3). To compare diabetic patients with the control, ANOVA analysis and the Kruskal-Wallis test were performed. For all results, the Shapiro-Wilk test was performed for testing the sample normal distribution. Continuous variables were presented as mean ±SD or median with interquartile range (Q1–Q3). Variables were compared by the unpaired *t*-test or the Mann-Whitney *U* (continuous) test or by the Fisher's exact test or chi-square test (categorical). A Duncan's *post-hoc* multiple range test was performed to analyze the non-interactive effect of diabetes duration and biomarker concentrations. P-values below 0.05 were considered significant.

Results

Screening microarray tests included semi-quantitative analyses of cytokines and angiogenic factors levels, both in the EV-enriched fraction (EVs) and in supernatant. At first, differences between patients with diabetes mellitus and the control group were analyzed (Table 1). Diabetic patients having vascular complications were characterized by a significant increase in Ang-2 (71 (27–127) vs. 33.5 (9.5–63) SD; p = 0.044), basic fibroblast growth factor — b-FGF (58 (18.5–134.5) vs. 19 (0–64.5) SD; p = 0.035), thrombopoietin — THPO (55 (27–128) vs.12.5 (2–49.5 SD); p = 0.008), receptor of urokinase-type plasminogen activator receptor — u-PAR (208 (133.5–237) vs. 121 (82–150.5 SD);

p = 0.034), vascular endothelial growth factor — VEGF (58.5 (14–110) vs. 15.5 (0–57) SD; p = 0.036) levels in supernatant and EV fraction (Fig. 2 A and C).

In the EV fraction, diabetic patients had lower vascular endothelial growth factor type 3 receptor (VEGFR-3) levels in comparison to control subjects (352.5 (261–417.5) vs. 349 (298.5–498) SD; p = 0.004) (Fig. 2 B and D). In plasma depleted of EVs, higher b-FGF levels were observed when compared to controls (49 (10.5–122) vs. 24 (2–72.5) S; p = 0.03). However, VEGFR2 levels were lower in diabetes patients than in control subjects (260 (195.5–351) vs. 360 (256–461.5) SD; p = 0.01).

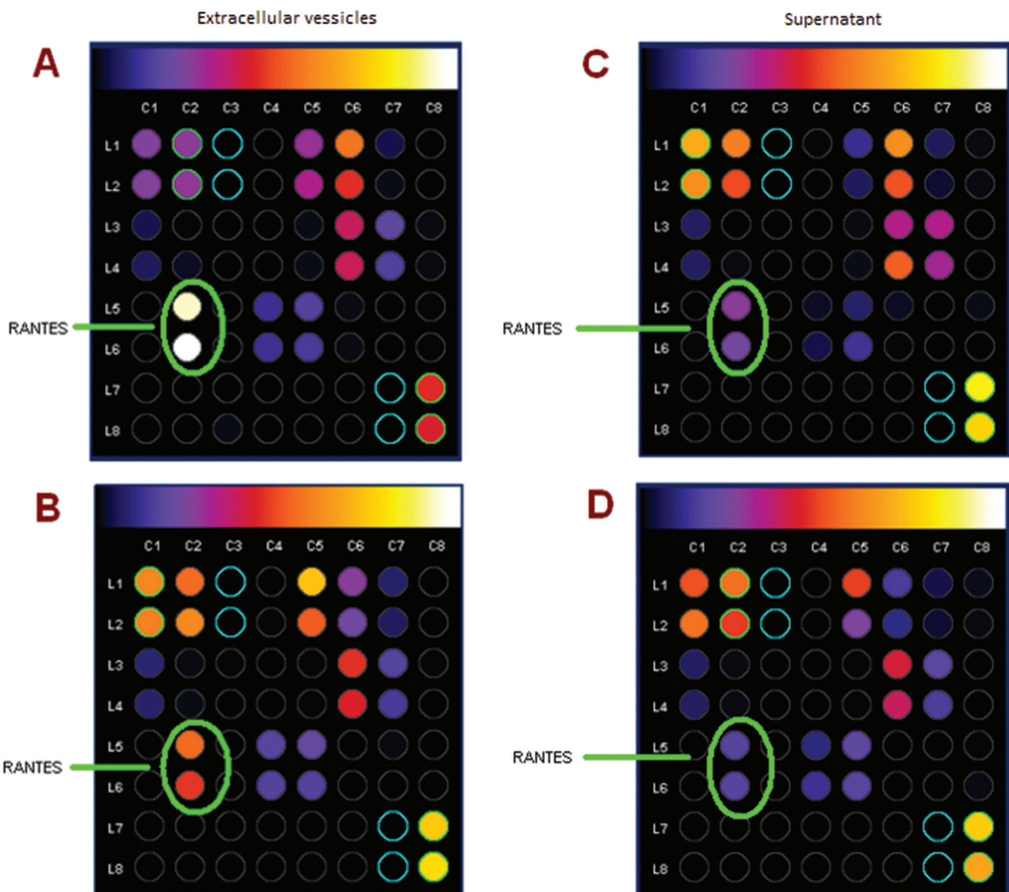

Fig. 2. Visual representation of protein microarray images.

The signal intensity for each pair of spots is proportional to the concentration of the antigen in the sample. Expression pattern of proteins in the EV-enriched fraction (A,B) and supernatant from patient plasma (C,D). Representative microarrays for UD patient (A,C) and CD patient (B,D) are presented. Chemiluminescence was detected by CCD camera and analyzed using Image J software with Protein Array Analysis plugin by Gilles Carpentier. Position C5/L1, C5/L2 represents angiostatin, C6/L1, C6/L2 — EGF, C2/L5, C2/L6 — RANTES, C4/L5, C4/L6 — TIMP-1, C5/L5, C5/L6 — TIMP-2, which are overexpressed in MPs.

Secondly, significant differences between the groups determined according to the HbA1c criteria were considered. The biochemical characteristics of those groups is presented in Table 2. In the control group and the group of patients with controlled diabetes mellitus (CD), no significant differences were observed in the levels of cytokines and angiogenic factors measured in the EV fraction and supernatant. However, in the uncontrolled diabetes (UD) group, significantly higher levels of RANTES (1101.5 (426–1760) vs. 270 (203.5–321) SD; $p = 0.007$) (Fig. 3 A), angiostatin (381 (287–561) vs. 243 (183.5–345.5) SD; $p = 0.05$) (Fig. 3 B), tumor necrosis factor alpha — TNFα (212 (143.5–256.5) vs. 143.5 (96.5–180.5) SD; $p = 0.046$), growth-related oncogene alpha — GROα (126.5 (95.5–240.5) vs. 87.5 (64–104.5) SD; $p = 0.031$) and metallopeptidase inhibitors type 1 — TIMP1 (242.5 (231.5–504) vs. 192.5 (89–200) SD; $p = 0.013$) and type 2 — TIMP2 (279 (221–718.5) vs. 206 (159–228) SD; $p = 0.031$) (Figs. 3 C and D) were observed.

Table 2. Characteristics of controlled diabetes type 2 (CD) and uncontrolled diabetes type 2 (UD) patients in comparison with the control group, in the screening study.

	CD n = 4	UD n = 4	C n = 4	P
Age (years)	61.5 (61–65)	60.5 (49–66.5)	56 (53–58.5)	0.223
BMI (kg/m^2)	26.87 (25.1–29.46)	28.82 (25.36–32.92)	24.03 (21.99–29.93)	0.368
Sex (men/women)	2/2	3/1	2/2	0.710
TC (mmol/l)	4.07* (3.78–4.5)	7.26* (6.21–7.46)	6.53 (5.88–6.99)	**0.049**
LDL-C (mmol/l)	2.28 (1.72–2.88)	4.02 (2.94–5.07)	4.51 (3.48–5.15)	**0.049**
HDL-C (mmol/l)	1.14 (0.85–1.33)	1.33 (1.10–1.59)	1.27 (1.06–1.67)	1.00
TG (mmol/l)	1.01 (0.76–2.59)	2.29 (1.53–5.03)	1.77 (1.22–2.11)	0.368
Glucose (mmol/l)	10.10 (7.65–12.65)	17.5 (9.65–21.45)	5.70 (5.00–6.25)	**0.049**
HbA1C (%)	6.60* (6.25–6.90)	10.75* (9.85–12.25)	NA	**0.018**
Creatinine (µmol/l)	75.49 (71.65–97.1)	107.66 (89.91–126.58)	69.5 (64.5–75)	**0.018**
hsCRP (mg/l)	1.48 (0.93–19.44)	2.04 (1.02–2.89)	2.17 (1.94–2.84)	0.368

Values are given as median (Q1–Q3). Abbreviations: BMI, body mass index; hsCRP, high sensitivity C-reactive protein; HbA1C, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides. Bold means statistically significant differences between the three groups.
*means statistically significant between the subgroups CD and UD ($p < 0.05$).

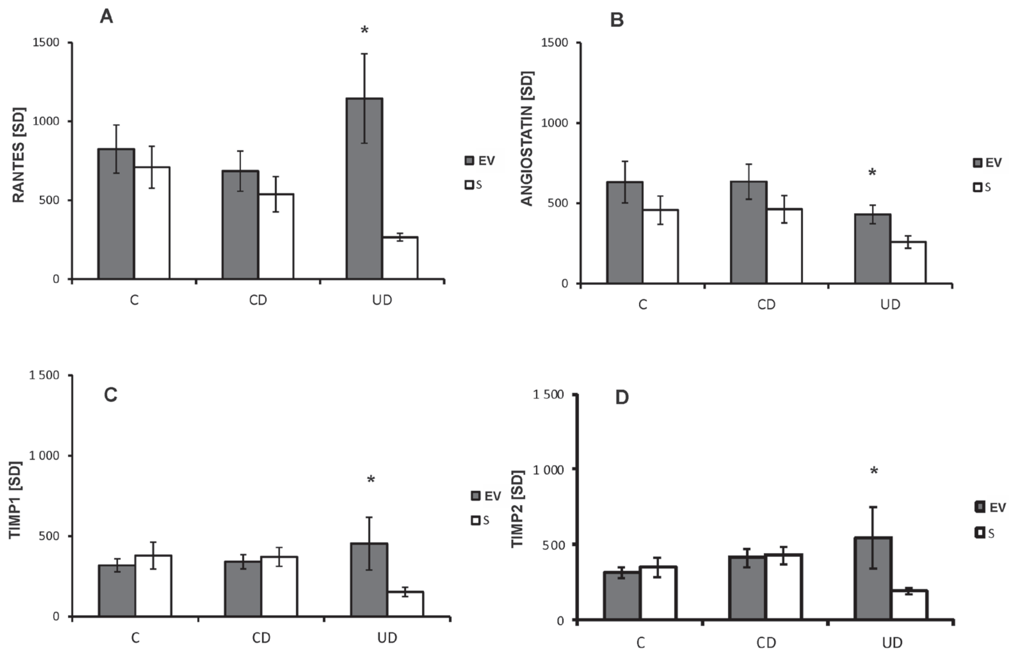

Fig. 3. Semi-quantitative analysis of selected angiogenic factors in the EV-enriched fraction (EV) and supernatant (S) in plasma from patients with controlled diabetes type 2 (CD) and uncontrolled diabetes type 2 (UD) in comparison with the control group.
Levels of angiogenic factor shown as Mean ± SEM of signal density [SD] for: RANTES (A), Angiostatin (B), TIMP1(C), TIMP2 (D).

Thirdly, quantitative analysis of two selected pro- and anti-angiogenic factors was thoroughly performed by means of the ELISA method: angiopoietin-2 (Ang-2) and RANTES. Ang-2 is considered to be a vessel stabilizing factor, while RANTES can act as a specific anti-angiogenic cytokine. Those studies were completed with the extended group of patients (n = 61); the epidemiological and clinical characteristics of these groups is presented in Table 3 and 4.

The analyses from the extended study confirmed results obtained from the microarrays assay. Increased levels of Ang-2 were observed in patients with diabetes in comparison with the control group. Significant differences were also observed between the groups determined according to the HbA1C criteria.

Post hoc analysis revealed a difference between the UC group and the control one in RANTES levels (p = 0.0235) and Ang-2 concentrations (p = 0.0316). These analyses did not reveal any significance in RANTES and Ang-2 in CD patients and controls.

Table 3. Characteristics of the extended study group in comparison with the control group.

	Diabetes Mellitus n = 48	Control n = 13	p
Age (years)	63.02 ± 9.92	52.58 ± 6.96	**0.001**
BMI (kg/m^2)	31.18 ± 5.21	26.14 ± 5.29	**0.007**
Sex (men/women)	29/19	5/8	0.164
TC (mmol/l)	4.69 ± 1.29	5.53 ± 1.00	**0.033**
LDL-C (mmol/l)	2.64 ± 1.14	3.53 ± 1.14	**0.014**
HDL-C (mmol/l)	1.11 (0.94–1.33)	1.45 (1.31–1.65)	**0.002**
TG (mmol/l)	1.67 (1.13–2.42)	0.88 (0.79–1.75)	**0.011**
Glucose (mmol/l)	10.15 (7.40–12.00)	5.28 (4.90–5.70)	**0.000005**
HbA1C (%)	10.04 (7.35–8.45)	NA	–
Creatinine (μmol/l)	90.54 (80.54–108.56)	71.0 (60–80)	**0.0003**
hsCRP (mg/l)	1.51 (0.81–2.98)	0.85 (0.71–2.14)	0.139
RANTES (μg/ml)	16.77 (14.75–18.7)	14.62 (12.37–15.28)	0.076
Ang-2 (ng/ml)	2.72 (1.90–4.50)	1.74 (1.54–1.93)	**0.008**

Values are given as average ± SD for parametric test or median (Q1–Q3) for nonparametric test. Abbreviations: BMI, body mass index; hsCRP, high sensitivity C-reactive protein; HbA1C, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; RANTES, regulated on activation, normal T-cell expressed and secreted; Ang-2,angiopoietin-2; Bold means statistically significant differences between the groups.

Table 4. Characteristics of extended subpopulations of controlled diabetes type 2 (CD) and uncontrolled diabetes type 2 (UD) patients in comparison with the control group.

	CD n = 11	UD n = 37	Control n = 13	p
Age (years)	60.91 ± 7.09*	63.65 ± 10.62*	52.58 ± 6.96*	**0.003**
BMI (kg/m^2)	31.36 ± 5.62*	31.12 ± 5.15*	26.14 ± 5.29*	**0.029**
Sex (men/women)	7/4	22/15	5/8	0.357
TC (mmol/l)	4.13 ± 0.48*	4.86 ± 1.41	5.53 ± 1.00*	**0.024**
LDL-C (mmol/l)	2.22 ± 0.59*	2.77 ± 1.24	3.53 ± 1.14*	**0.018**
HDL-C (mmol/l)	1.16 (0.96–1.34)	1.09* (0.93–1.29)	1.45* (1.31–1.65)	**0.021**

TG (mmol/l)	1.23 (0.83–1.83)	2.02* (1.24–2.48)	0.88* (0.79–1.75)	**0.011**
Glucose (mmol/l)	8.2* (6.50–12.00)	10.4* (7.90–12.00)	5.28* (4.90–5.70)	**0.0000**
HbA1C (%)	6.70 (6.30–6.90)	8.00 (7.60–8.60)	NA	–
Creatinine (μmol/l)	88.29 (74–100.9)	90.99* (81.08–109.91)	71.0* (60–80)	**0.0007**
hsCRP (mg/l)	1.65 (0.95–5.52)	1.46 (0.81–2.8)	0.85 (0.71–2.14)	0.319
RANTES (μg/ml)	16.89 (15.18–18.20)	16.73* (14.41–18.93)	14.62* (12.37–15.28)	**0.023**
Ang-2 (ng/ml)	2.11 (1.58–3.91)	2.76* (2.23–4.64)	1.74* (1.54–1.93)	**0.036**

Values are given as average ± SD for parametric test or median (Q1–Q3) for nonparametric test. Abbreviations: BMI, body mass index; hsCRP, high sensitivity C-reactive protein; HbA1C, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; RANTES, regulated on activation, normal T-cell expressed and secreted; Ang-2, angiopoietin-2 fraction.
Bold means statistically significant differences between the three groups.
*means statistically significant between the subgroups in post hoc analysis ($p < 0.05$).

Discussion

Our results obtained in the microarray assay in diabetic patients showed increased levels of common angiogenic factors like: VEGF, b-FGF, Ang-2. There are a number of data concerning the involvement of VEGF and its receptors and bFGF and the angiopoietin family in angiogenesis [5, 6, 14]. Additionally, some evidence of dominant involvement of these cytokines in the development of blood vessels are often cited and the role of VEGF and its receptors was emphasized [4–6]. However, a special role in this process should be assigned to Ang-2, which correlates better than Ang-1 with the extent of angiogenesis in patients with complicated diabetes [14].

Wohlfart and co-workers (2014) suggested that among typical angiogenesis factors, bFGF plays a pivotal role in diabetic retinopathy; it became overexpressed in the retinal tissue in obese Zucker diabetic rats [15]. In our screening study, we observed that bFGF was significantly increased in patients with vascular complications; nonetheless it was not confirmed that bFGF is transferred via EVs, mostly MVs. The level of bFGF in EVs was lower than in plasma depleted of EVs (supernatant). These data are consistent with results published by Chen and co-workers showing that bFGF is not abundant in MVs derived from human mesenchymal stem cells (MSCs) when compared to the parent cells [16]. However, in the EV-enriched plasma fraction from diabetic patients, VEGFR3 levels were decreased when compared to the control group. These data suggest

that circulating EVs in diabetic patients have a lower pro-angiogenic potential than it was previously hypothesized [9, 10].

Among those highly angiogenic factors, in the microarray assay we observed increased levels of u-PAR and thromboplastin. Remarkably, in hypoxic conditions, MVs derived from MSCs were augmented with u-PAR when compared to normoxic conditions [16]. Despite the fact that u-PAR is not a growth factor, its role in angiogenesis is undoubted. It is known that u-PAR enhances uPA function by localizing plasmin formation at the cell membrane. Thus, the increased expression of u-PAR in the diabetic retina may be involved in the breakdown of cell junctions and permeability barrier function [17]. In stable coronary artery disease (CAD) patients, thrombopoietin was inversely associated with platelet turnover parameters, and diabetes significantly increased its levels [18]. However, there are no data suggesting the involvement of MVs in thrombopoietin transport.

In our study, we assumed that diabetes mellitus has an influence on the release of angiogenic factors and inflammatory cytokines, and appropriate control of glucose levels may change the pattern of their transportation via EVs. We observed that in patients with higher levels of HbA1c (above 7%), increased abundance of anti-angiogenic factors was observed in EVs: angiostatin, RANTES, TIMP1 and TIMP2. The increased concentration of these factors in the EV-enriched fraction in plasma obtained from UD patients may suggest that EVs may participate in anti-angiogenic factors transfer as well. To the best of our knowledge, this is the first study showing the relationship between TIMP1/2, RANTES and angiostatin, and EV transport. In dogs with hyperglycemia, higher concentrations of angiostatin in myocardial interstitial fluid (MIF) correlated with impaired collateral perfusion and blocking of angiostatin with specific antibody restored endothelial cell tube formation evoked by MIF [19].

RANTES is a chemokine which is strongly related to retinopathy and angiopathy. Higher levels of RANTES were observed in patients with non-proliferative retinopathy [20]. It acts through two separate receptors: CCR1 and CCR5 [21, 22]. The antiangiogenic character of RANTES is achieved by activation of CCR1. The anti-angiogenic effect is observed at the beginning of the process [21]. On the other hand, genetic ablation of CCR5 results in the inhibition of neovascularization [22]. Consecutively, TIMP1 and TIMP2 have specific ability to inhibit endothelial cell morphogenesis. Thus, we may assume that TIMP1/2 can prevent neoangiogenesis in diabetic patients [23]. Moreover, TIMP2 inhibits bFGF-induced proliferation of microvascular endothelial cells [24].

In our study, we observed that circulating EVs participate in the transfer of both pro-angiogenic factors and anti-angiogenic cytokines. We confirmed that the profile of angiogenic factors reveals the relevance of blood glucose management in patients with diabetes mellitus. In diabetic patients, levels of pro-angiogenic factors are higher than in control ones; nevertheless in patients with uncontrolled diabetes levels of anti-angiogenic factors are significantly increased. We may assume that in specific conditions, EVs accomplish anti-angiogenic activity, which can produce additional vascular complications

in patients with poorly controlled glucose levels. Our observations are consistent with Yang and co-workers' study (2008). They reported that lymphocyte-derived MVs inhibit angiogenesis by stimulating oxidative stress and negatively regulating VEGF-induced pathways [25]. Moreover, endothelial-derived MVs negatively affect angiogenesis *in vitro* by inducing oxidative stress [26].

Interestingly, we observed that both RANTES and Ang-2 are upregulated in UD patients. In our study, we assumed that the HbA1C cut-off for poorly controlled diabetes is 7%. The 7% value is recommended as a target value by most of the recommendations and is used to discriminate patients with poorly controlled diabetes [12]. We may accept that even tiny variations in HbA1c levels may reflect EV cargo and influence their embarking with angiogenic and inflammatory factors. Taking into consideration our observations, we may assume that the EV profile can be used as a marker in prognosis and risk evaluation in patients with vascular complications [14, 27].

Additionally, our study suggests that even in unregulated and highly complicated diabetes mellitus, some mechanisms are activated which minimize complications. This may be a compelling alternative that we should consider when looking for new ways of treating and trying to improve the therapeutic effects in diabetic retinopathy.

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Conflict of interest

Authors declare no conflict of interest.

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