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Retinopathy severity correlates with RANTES concentrations and CCR5-positive microvesicles in diabetes

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Abstract: Introduction: RANTES regulates leukocyte recruitment to areas affected by the inflammatory process. Microvesicles (MVs) belong to a subpopulation of extracellular vesicles and show proangiogenic potential by transferring bioactive molecules to target cells.
Objectives: The aim of this study was to determine the relationship between circulating proangiogenic factors (MVs and RANTES) and diabetes complications in patients with different severities of diabetic retinopathy (DR). CCR5 (CD195) receptors transported by annexin V-labeled MVs were also investigated.
Patients and Methods: Diabetic patients (n = 61), among whom 35 had confirmed DR classified according to guidelines, and controls (n = 25) were included. MVs were isolated by centrifugation and analyzed using flow cytometry, RANTES was assessed by ELISA.
Results: The study group differed from the control group with respect to BMI, age, heart rate and systolic blood pressure. Additionally, glucose and creatinine concentrations were significantly increased: 5.30 [5.09–5.62] vs. 9.38 [7.48–11.55] ($p<0.0001$) mmol/l and 74.59 [64–84] vs. 89.00 [77.11–105.44] μmol/l ($p = 0.0005$), respectively. RANTES concentrations were significantly increased in diabetic patients compared to those of controls (15.5 (9.7–18.1) vs. 8.9 (0.9–14.6) μg/ml ($p = 0.011$)), and RANTES

concentration significantly increased with respect to nonproliferative DR progression. Moreover, the number of CCR5-positive MVs was significantly increased in patients with heavy nonproliferative diabetic retinopathy (HNPDR) compared to those with soft nonproliferative DR (SNPDR): 1178 [836–2254] vs. 394 [275–799] counts/μl.

C o n c l u s i o n s: Correlation of RANTES concentrations with the stage of nonproliferative DR and the statistically significant dependence of CCR5-positive MVs with disease progression suggest that MVs and RANTES can be considered new biomarkers.

Key words: biomarkers, diabetic retinopathy, ectosomes, microangiopathy.

Introduction

RANTES (C-C motif ligand 5, Regulated-on-Activation-Normal-T-cell-Expressed-and-Secreted, also called CCL5) belongs to the family of C-C chemokines that are secreted by different kinds of cells, including T lymphocytes, macrophages, platelets, synovial fibroblasts, renal tubular epithelial cells and some types of cancer cells [1].

The production of RANTES is stimulated by the activation of HIF-1 (hypoxia inducible transcription factor 1). The main biological role of RANTES is the recruitment of leukocytes, mainly T lymphocytes, macrophages, eosinophils and basophils, to the area affected by inflammatory processes. RANTES works *via* CCR1, CCR3 and its main receptor CCR5 [1]. These receptors are G-protein coupled receptors, and after their stimulation, the cell polarizes and activates the PI3K/PKB-NFκB or Ras/MEK-ERK signal transduction pathway inside the cell; as a result, polymerization of G-actin to F-actin is observed, and activation of the actomyosin system leads to contraction and stimulates the cell to relocate [2, 3]. Interestingly, many studies have indicated that RANTES and CCR5 are also associated with type 2 diabetes mellitus (T2DM), glucose intolerance, obesity and atherosclerosis [4, 5]. On the other hand, RANTES is increasingly seen as a chemokine involved in angiogenesis, although its participation in this process has not yet been accurately described. The chemokine RANTES is described as both an antiangiogenic and a proangiogenic molecule [6]. The main mechanism of action of RANTES is associated with the recruitment of circulating proinflammatory cells that produce growth factors affecting the formation of blood vessels. RANTES also participates in the recruitment of endothelial progenitor cells from circulation and has the ability to stimulate endothelial cells to migrate and proliferate [7, 8]. In CCR5 knockout mice, permanent inhibition of corneal neoangiogenesis was observed [9].

In terms of pathologic and regenerative angiogenesis, extracellular vesicles (EVs) and their larger subpopulation, namely, microvesicles (MVs), also called ectosomes, should be considered as regulators in metabolic and vascular diseases [10]. In addition to MV procoagulant properties, which have already been well described,

MVs may show essential proangiogenic potential [11]. First, MVs have the ability to present tissue factor (TF) on their surface. Second, TF is the main initiator of the coagulation cascade and a strong stimulator of VEGF expression [12, 13]. Additionally, circulating MVs may contribute in transporting of cytokines and angiogenic factors in patient with diabetic retinopathy (DR) [14]. In an *in vitro* model, on the surface of endothelial and platelet origin MVs, the presence of matrix metalloproteinases (MMPs), which are strongly involved in angiogenesis, was confirmed [15]. In diabetic patients tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and TIMP2) were significantly elevated in MVs, which confirms the regulatory role of MVs in diabetic vascular complications [14]. Among the aforementioned proangiogenic factors that can be transferred *via* MV machinery, CCR5 receptors exposed on the MV surface were recognized [16]. Therefore, there is a strong possibility that CCR5 transferred *via* MVs contributes to the development of vascular complications in patients with diabetes. Moreover, MVs may transfer pro-and anti-angiogenic short noncoding RNA (microRNA), which was confirmed in number of clinical studies [17, 18].

The aim of this study was to determine the relationship between circulating proangiogenic factor levels (MVs and RANTES) and diabetic complications in patients with different severities of diabetic retinopathy (DR). To achieve this objective, in our study, we compared the concentration of RANTES in plasma, plasma fractions enriched in EVs and EV-depleted plasma. The presence of CCR5 receptors transported by annexin V-labeled MVs was also analyzed. The study was conducted with consideration of diabetes severity and the severity of diabetic complications, namely, DR.

Materials and Methods

The study group

The study included adults over 18 years of age. The study group consisted of patients diagnosed with type 1 diabetes mellitus (T1DM) and type 2 (T2DM) who were recruited at the outpatient clinic "Oko Laser" in Krakow and in the Interventional Cardiology Department at the Lesser Poland Cardio-Vascular Center AHoP in Chrzanów. The study included 61 patients with confirmed vascular complications, which were mainly retinopathy.

Patients were divided according to the Polish Diabetes Association (PTD) guidelines into the group of patients with controlled diabetes (CD), where the $HbA1_C$ levels were ≤7% and the group of patients with uncontrolled diabetes (UD) with $HbA1_C$ levels >7% [19]. Ophthalmic research was conducted in 35 out of 61 patients, and DR progression was classified into four groups according to PTD guidelines:

1. SNPDR, *soft nonproliferative DR*;

2. MNPDR, *moderate nonproliferative DR*;
3. HNPDR, *heavy nonproliferative DR*; and
4. PDR, *proliferative DR*.

Obesity and insulin resistance were not criteria for exclusion. Any patients or controls with acute coronary syndrome (ACS), acute ischemic stroke (IS) or critical limb ischemia were excluded from this study if they occurred within 6 months prior to study enrollment. Other criteria for exclusion were patients with a history of cancer, renal and liver failure, and past or present systemic inflammation as defined by high-sensitivity C-reactive protein (hs-CRP) levels above 10 mg/L. The clinical characteristics of the study subjects are listed in Table 1.

Table 1. Characteristics of the study group and control group.

Parameter	Study group (n = 61)	Control group (n = 25)	p
Epidemiological parameters			
sex: [female / male] [N]	24/37	14/11	0.158
age [years]	**63 (59–68)**	**50 (45–56)**	**<0.0001**
Type of diabetes: [T2DM / T1DM] [N]	10/51	—	—
duration of diabetes [years]	14 (9–45)	—	—
Clinical parameters			
ophthalmological examination [N/%]	35/57.4	25/100	—
confirmed retinopathy [N/(%)]	31/50.8	—	—
confirmed maculopathy [N/(%)]	23/37.7	—	—
BMI [kg/m^2]	**31.2 (26.5–36.2)**	**23.3 (22.1–26,8)**	**<0.0001**
heart rate [beats/min.]	**80 (74–90)**	**70 (60–70)**	**<0.0001**
systolic blood pressure [mmHg]	**130 (130–140)**	**120 (110–125)**	**<0.0001**
diastolic blood pressure [mmHg]	80 (70–80)	80 (80–85)	0.410
statin treatment [N/%]	26/42	—	—
aspirin treatment [N/%]	21/34.4	—	—
Laboratory parameters			
WBC [10^3/µl]	**7.42 ± 1.85**	**5.86 ±1.35**	**0.0002**
RBC [10^6/µl]	4.62 ± 0.46	4.84 ± 0.44	0.053
Hb [g/dl]	13.84 ± 1.41	14.20 ± 1.40	0.297
Hct [%]	40.88 ± 3.94	41.67 ±3.51	0.390
MCV [fl]	**88.66 ± 5.13**	**86.04 ± 4,55**	**0.029**
MCH [pg]	30.06 ±1.84	29.30 ± 1.71	0.081

Table 1. Cont.

Parameter	Study group (n = 61)	Control group (n = 25)	p
MCHC [g/dl]	33.80 (33.3–34.5)	34.2 (33.2–34.7)	0.567
PLT [10^3/µl]	227 (173–272)	228 (187–268)	0.651
NEUT [10^3/µl]	**4.15 (3.25–5.45)**	**3.2 (2.4–3.5)**	**0.003**
LYMPH [10^3/µl]	1.92 (1.65–2.24)	2.01 (1.7–2.10)	0.877
MONO [10^3/µl]	0.57 (0.44–0.76)	0.49 (0.41–0.65)	0.146
EO [10^3/µl]	0.19 (0.11–0.33)	0.14 (0.09–0.17)	0.087
BASO [10^3/µl]	0.00 (0.00–0.05)	0.00 (0.00–0.00)	0.351
Ret [‰]	11.80 (7.70–14.95)	11.00 (8.30–12.30)	0.546
HbA_{1C} [%]	7.70 (6.90–8.40)	NA	—
GLU [mmol/l]	**9.38 (7.48–11.55)**	**5.30 (5.09–5.62)**	**<0.0001**
hs CRP [mg/l]	1.39 (0.76–2.84)	0.86 (0.55–2.01)	0.327
CREA [µmol/l]	**89.00 (77.11–105.44)**	**74.59 (64.00–84.00)**	**0.0005**
eGFR (MDRD) [ml/min/1.73 m^2]	**74.67 (58.47–90.16)**	**92.20 (83.26–101.04)**	**0.0003**
TCHOL [mmol/l]	**4.74 ± 1.34**	**5.45 ± 1.06**	**0.022**
CHOL LDL [mmol/l]	**2.58 ± 1.14**	**3.51 ±0.98**	**0.0006**
CHOL HDL [mmol/l]	**1.10 (0.96–1.40)**	**1.32 (1.19–1.65)**	**0.011**
TG [mmol/l]	**1.83 (1.26–2.47)**	**1.03 (0.83–1.56)**	**0.0002**

The data in the table are shown as the median (lower — upper quartile) for variables with a distribution different from normal and average ± SD for variables with a normal distribution, in bold, the statistically significant differences between the groups were determined ($p < 0.05$). BMI — body mass index; WBC — the number of white blood cells; RBC — the number of red blood cells; Hb — hemoglobin concentration; Hct — hematocrit; MCV — mean volume of the red blood cell; MCH — mean mass of hemoglobin in the red blood cell; MCHC — mean hemoglobin concentration in the red blood cell; PLT — platelet count, NEUT — neutrophil count, LYMPH — lymphocyte count; MONO — monocytes count; EO — eosinophils count, BASO — basophils count; Ret — reticulocyte count; GLU — glucose; hsCRP — C-reactive protein; CREA — creatinine; eGFR — estimated glomerular filtration rate; TCHOL — total cholesterol; CHOL LDL — LDL fractional cholesterol; CHOL HDL — HDL fractional cholesterol; TG — triglycerides.

Ethics committee approval and patient consent

The study complied with the Declaration of Helsinki, and the study protocol was approved by the Jagiellonian University Bioethics Committee (permission no. KBET/206/B/2013, extended until December 31, 2017). Informed consent was obtained from all the study subjects.

Plasma preparation and determination of circulating MVs

Plasma, serum and whole blood were used for the tests. Double centrifuged citrate plasma samples (15 min at 2500 × *g*) were used to obtain the fraction of EVs and were stored frozen before analysis. For MV flow cytometry analysis, plasma samples were thawed in a water bath (at 37°C) to avoid cryoprecipitation. Subsequently, 350 µl of plasma was transferred to 1.5 ml Eppendorf tubes, which were spun once more for 90 min at 16000 × *g* [18]. The lower plasma fraction enriched with extracellular vesicles was obtained (EV — enriched fraction) and had a volume of 50 µl. The remaining plasma at a volume of 300 µl was also used for the assays as the S-fraction.

In the present study, large EVs (MVs) were determined using flow cytometry with a CytoFLEX analyzer (Beckman Coulter, Inc. USA), which allowed for identification of objects from approximately 100 nm. The CytoFLEX cytometer was equipped with three lasers (488 nm, 638 nm, and 405 nm) and 13 fluorescence channels, allowing for the identification of several antigens simultaneously. Circulating MVs were enumerated after annexin V labeling (Pacific Blue annexin V, Biolegend, San Diego, CA, USA), and specific monoclonal antibodies against CCR5 (PE/Cy7 anti-human CD 195 (CCR5) IgG2b, κ — clone J418F1, Biolegend, San Diego, CA, USA) were used. Cytometer calibration was performed with Gigamix beads (see Supplementary file).

Determination of biochemical parameters

Biochemical parameters, including triglyceride, LDL-C, and HDL-C levels, were determined using a MaxMat analyzer with ELITech Clinical Systems tests (Puteaux, France), with a limit of detection (LoD) of 0.06 mmol/L. hs-CRP was determined using the APTEC Ultra-Sensitive CRP test (APTEC Diagnostics NV, Belgium), with an LoD of 0.13 mg/L. $HbA1_C$ levels were measured by performing HPLC with a D-10 Hemoglobin Testing System (Bio-Rad Laboratories, Inc., Hercules, California, USA), certified by the National Glycohemoglobin Standardization Program (NGSP) organization. RANTES concentration was determined by ELISA (Human RANTES ELISA Kit, cat. no 201-12-0085, Sunred Biological Technology, China).

Statistical analysis

Statistical analyses were performed using the Statistica 12.0 package (StatSoft Polska, Kraków, Poland). The distribution of variables was examined using the

Kolmogorov–Smirnov normality test. Quantitative variables were characterized using descriptive statistics, i.e., mean and standard deviation (SD) for normally distributed data or median and interquartile interval (Q1–Q3) for nonnormally distributed data. Qualitative variables were compared using two-sided Chi-square and Fisher's exact tests. Student's *t*-test (normally distributed data) or Mann–Whitney *U* test (nonnormally distributed data) was used to determine significant differences between variables. Statistical significance was set at $p < 0.05$.

Results

The characteristics of the study group and control group are presented in Table 1. Significant differences between the control and study groups were observed in age, BMI and biochemical parameters, such as glucose creatinine (eGFR) and lipid profile. Interestingly, the study group (T2DM/T1DM) had good balanced total cholesterol and LDL fraction levels. Hematology parameters (RBC, WBC, NEUT, and MCV) also trended toward or were significantly different than those of control group.

RANTES concentrations and circulating MV numbers in patients and controls

RANTES concentration was determined in plasma and in two plasma fraction: the fraction that was enriched with EVs ($RANTES_{EV}$) and the fraction lacking EVs ($RANTES_{S}$). Circulating MVs identified by flow cytometry had a size of 100–900 nm, and they were divided into two groups: small MVs (MV^{AnnV+} <300 nm) and large MVs (MV^{AnnV+} ≥300 nm). MVs were also labeled for the presence of CCR5 using a cytometer. Data are shown in Appendix (Table 4).

RANTES concentrations and circulating MV numbers with respect to diabetes control

In the comparison, which included the level of diabetes control, significantly higher concentrations of total RANTES and RANTES in the S fraction were confirmed in the patient group compared to those of the control group. The results are shown in Table 2. *Post hoc* analysis confirmed significantly higher RANTES concentrations in the uncontrolled diabetes (UD) group in comparison to those of the control group:

— RANTES, $p = 0.028$;
— $RANTES_{S}$, $p = 0.049$; and
— $RANTES_{EV}$, $p = 0.048$.

Table 2. Comparison of the studied factors in groups with different levels of diabetes control and in the control group.

Parameter	UD (n = 45)	CD (n = 16)	Control group (n = 25)	p
RANTES [µg/ml]	**15.5 (10.9–18.0)**[a]	**15.5 (0.6–18.1)**	**8.9 (0.9–14.6)**	**0.034**
$RANTES_S$ [µg/ml]	**15.1 (10.5–18.9)**[a]	**15.9 (0.6–19.9)**	**8.4 (0.9–14.1)**	**0.049**
$RANTES_{EV}$ [µg/ml]	15.2 (11.0–19.0)[a]	14.2 (0.5–16.4)	6.7 (0.9–14.1)	0.052
MV^{AnnV+} 100–900nm [counts/µl]	820 (320–1715)	650 (254–1291)	805 (507–1581)	0.645
MV^{AnnV+} <300nm [counts/µl]	165 (41–456)[a]	113 (47–452)[a]	279 (148–714)	0.055
MV^{AnnV+} ≥300nm [counts/µl]	583 (286–1148)	444 (209–849)	647 (377–895)	0.521
$MV^{AnnV+/CCR5}$ [counts/µl]	66 (19–167)[a]	57 (36–432)	108 (49–293)	0.136

Bold indicates a statistically significant difference ($p < 0.05$); [a] statistically significant difference from the control group in *post hoc* tests. The values in the table are shown as the median (lower quartile — upper quartile); UD — a group of patients with uncontrolled diabetes; CD — a group of patients with controlled diabetes; MV^{AnnV+} — microvesicles labeled with annexin V; $MV^{AnnV+/CCR5}$ — microvesicles labeled with annexin V, which also exhibited the expression of CCR5.

The statistically significant difference in the analysis of the number of small MVs was confirmed in the UD group ($p = 0.029$) and in the CD group ($p = 0.049$) in comparison to that of the control group.

Post hoc analysis of the number $MV^{AnnV+/CCR5}$ showed a significantly reduced number of $MV^{AnnV+/CCR5}$ in patients from the UD group ($p = 0.048$).

RANTES concentrations and circulating MV numbers with respect to DR progression

The highest concentrations of RANTES were observed in patients with advanced nonproliferative DR. The *post hoc* analysis confirmed statistically significant differences in nonproliferative DR. Higher RANTES concentrations in all assessed fractions were found in the group with more advanced retinopathy than in the group the less advanced stage (HNPDR with regard to SNPDR):

— RANTES, $p = 0.022$;
— $RANTES_{EV}$, $p = 0.041$;
— $RANTES_S$, $p = 0.017$;
— MV^{AnnV+} 100–900 nm, $p = 0.001$;
— MV^{AnnV+} <300 nm, $p = 0.0007$; and
— MV^{AnnV+} ≥300 nm, $p = 0.001$.

In the case of $MV^{AnnV+/CCR5}$, no significant difference was observed, while the upward trend from the SNPDR to HNPDR stage was evident. The results are presented in Table 3 and Fig. 1.

Table 3. Comparison of the studied factors in groups with different degrees of severity of diabetic retinopathy.

Parameter	SNPDR (n = 7)	MNPDR (n = 5)	HNPDR (n = 13)	PDR (n = 6)
RANTES [μg/ml]	0.5 (0.4–10.3)	18.2 (0.6–18.4)	17.1 (12.2–20.9)[a]	1.9 (0.6–17.3)
$RANTES_S$ [μg/ml]	0.6 (0.4–13.3)	13.1 (0.7–15.1)	17.0 (12.3–19.1)[a]	2.7 (0.6–22.6)
$RANTES_{EV}$ [μg/ml]	0.6 (0.4–7.4)	16.7 (0.5–21.8)	15.7 (11.9–21.8)[a]	10.5 (0.6–13.5)
MV^{AnnV+} 100–900nm [counts/μl]	664 (320–1004)	1393 (571–1715)	1995[a] (1179–2728)	1427 (632–1661)
MV^{AnnV+} <300nm [counts/μl]	161 (42–203)	610 (206–622)	661[a] (292–831)	331 (202–740)
MV^{AnnV+} ≥300nm [counts/μl]	394 (275–799)	778 (383–1113)	1178[a] (836–2254)	978 (428–1149)
$MV^{AnnV+/CCR5}$ [counts/μl]	28 (11–185)	167 (88–364)	185 (106–439)	77 (28–178)

[a] statistically significant difference in RANTES concentration between HNPDR in regard to SNPDR groups. The values in the table are shown as the median (lower quartile — upper quartile); SNPDR — soft non-proliferative diabetic retinopathy; MNPDR — Moderate non-proliferative diabetic retinopathy; HNPDR — heavy non-proliferative diabetic retinopathy; PDR — proliferative diabetic retinopathy; MV^{AnnV+} — microvessicles labeled with annexin V; $MV^{AnnV+/CCR5}$ — microvesicles labeled with annexin V, which also exhibited the expression of CCR5.

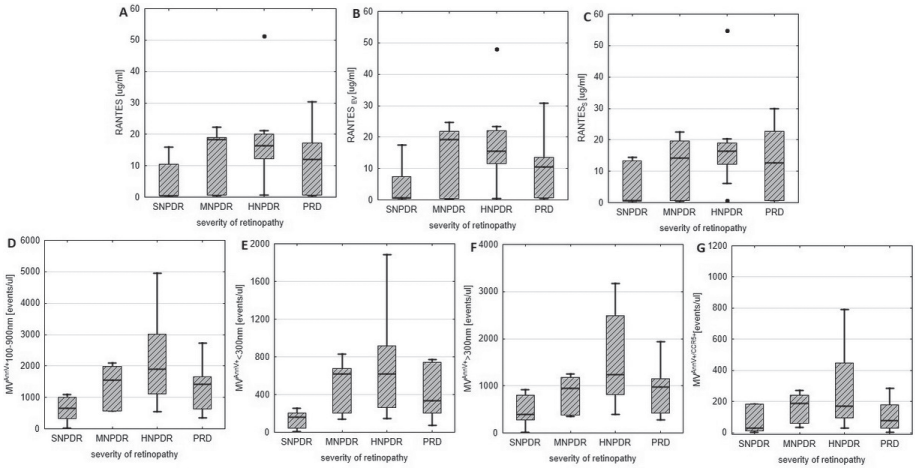

Fig. 1. Differences in concentrations of RANTES in subsequent fractions and numbers of $MV^{AnnV+/CCR5}$ depending on the severity of diabetic retinopathy. **A.** Total concentration of RANTES; **B.** EV fraction — $RANTES_{EV}$; **C.** supernatant fraction — $RANTES_S$; **D.** number of total EVs — MV^{AnnV+} 100–900 nm; **E.** number of small EVs — MV^{AnnV+}<300 nm; **F.** number of large EVs — MV^{AnnV+} ≥300 nm and **G.** number of CCR5-positive EVs — $MV^{AnnV+/CCR5}$ in patients with various intensities of nonproliferative retinopathy. Data are presented as median (horizontal dash), lower-upper quartile (frame), range of nonoccurring values (whiskers) and extreme values (•).

Significant relationships between the examined factors

In the control group, a significant relationship was observed between the $RANTES_{EV}$ concentration and the number of MV^{AnnV+} <300 nm (R = 0.553; p = 0.005). However, no statistically significant correlations were found in the study group. Upon further analysis, only patients with confirmed DR were included (n = 31). A statistically significant positive relationship was demonstrated between the concentration of $RANTES_{EV}$ and the number of MV^{AnnV+} 100–900 nm (R = 0.403; p = 0.024). A positive correlation was also observed between the concentration of $RANTES_{EV}$ and the number of small MV^{AnnV+} <300 nm (R = 0.397; p = 0.026) (Fig. 2A–B).

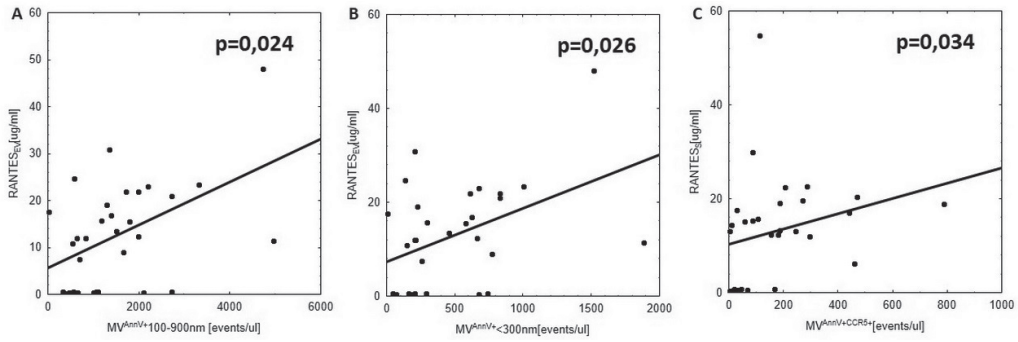

Fig. 2. Correlation between changes in the concentration of $RANTES_{EV}$ and the number of MV^{AnnV+}. **A.** MV^{AnnV+} 100–900 nm; **B.** MV^{AnnV+} <300 nm and **C.** $MV^{AnnV+/CCR5+}$.

With further analysis, the level of diabetes control was taken into account. In the group of patients with compensated diabetes, a negative correlation was demonstrated between the number of small MV^{AnnV+} and the total concentration of RANTES (R = –0.541; p = 0.030), as well as the in the fractions of $RANTES_S$ (R = –0.562; p = 0.023) and $RANTES_{EV}$ (R = –0.555; p = 0.025).

Next, the analysis took into account the number of receptors and antigens transferred by MV^{AnnV+}. In the group of patients with confirmed DR, a positive correlation was demonstrated between the number $MV^{AnnV+/CCR5+}$ and the concentration of $RANTES_S$ (R = 0.381; p = 0.034) (Figure 2C). Additionally, correlations between eGFR and changes in the concentration of RANTES in subsequent fractions were analyzed (Fig. 3A–C).

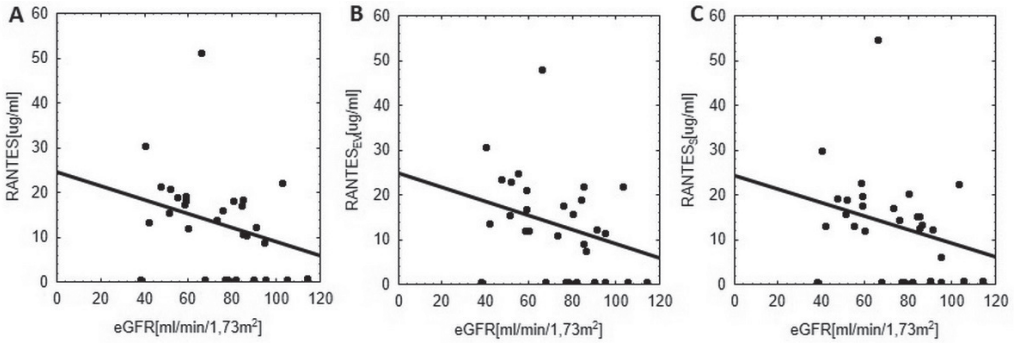

Fig. 3. Correlation between eGFR and changes in the concentration of RANTES in subsequent fractions. **A.** Total concentration of RANTES; **B.** EV fraction — $RANTES_{EV}$; and **C.** supernatant fraction — $RANTES_{S}$.

Discussion

Diabetic retinopathy treatment includes strict control of blood sugar and blood pressure, laser surgery (photocoagulation), vitrectomy surgery and medication injections. Currently, laser photocoagulation is usually very effective if it is performed in the early stage of disease and remains the gold standard for the treatment of DR. Pharmacological treatment is the method of choice to help prevent new blood vessel proliferation. Angiogenesis inhibitors are the most important group of drugs used; anti-VEGF preparations (bevacizumab and ranibizumab), although new, more effective drugs based on similar mechanisms are still being sought. The main objective of our study was to determine the relationship between circulating proangiogenic factor levels (MVs and RANTES) and diabetes complications in patients with different extents of DR.

In our study, we examined the concentration of the chemokine RANTES, the number of annexin V-labeled MVs and the number of CCR5 receptors transferred by MV^{AnnV+}, which can have a new impact on the diagnosis and treatment of diabetes complications, including DR. In particular, we assessed whether RANTES concentration and the MV number were related to the level of diabetes control and the severity of retinopathy.

We found that higher RANTES concentrations were observed in diabetic patients with respect to the control group. The RANTES concentration results obtained were similar to the research results of Dworack *et al.* and Maier *et al.* [20, 21]. First, they showed the correlations between changes in RANTES concentration and parameters determining the level of carbohydrate metabolism. However, the analysis of similar relationships conducted for the needs of the present study was not confirmed. There was no statistically significant correlation between RANTES concentration and

$HbA1_C$ levels in the present work, except glucose concentrations; $HbA1_C$ levels were the only parameter that reflected the level of carbohydrate metabolism in the group of patients. In studies conducted by Maier *et al.*, aside from RANTES in the serum, the concentration was also determined in vitreous fluid [21]. The concentration of this chemokine in the vitrectomy material proved to be below the detection limit. Due to the type of changes in the retina and the severity of retinopathy among patients in the present study, patients were not classified for vitrectomy. Thus, the analysis did not include the determination of RANTES concentration in vitreous fluid.

In addition to increased RANTES concentrations in the patient group and lack of effect of diabetic control on RANTES levels in the study, we showed that this chemokine may differentiate the degree of nonproliferative retinopathy between SNPDR (soft nonproliferative DR) and HNPDR (heavy nonproliferative DR) with statistical significance. A similar approach was made by Meleth *et al.* in their patient study, and a significant increase in RANTES was observed with the progression of nonproliferative retinopathy [22]. Higher RANTES concentrations have also been demonstrated in patients with nonproliferative retinopathy by Chen *et al.*; this group proposed the use of RANTES as a potential factor involved in the development of DR and determining the risk of DR [23]. Local and systemic inflammatory mediators were mostly investigated in the aqueous humor from an eye, and this kind of "liquid biopsy", has been proven as useful for personalized treatment of DR. Vujesevic *et al.* in their proteomic study showed significant increase of the RANTES levels in vitreous fluid obtained from patients with severe DR [24], In our study, we demonstrated that a significant increase in RANTES was correlated with DR severity and its drop was observed in proliferative DR Our finding is in concordance with observations made Chen *et al.* who showed the similar relationship in vitreous fluid from DR patients [25]. Additionally, we observed interesting was correlation between RANTES concentrations and the MV number, which suggest the role of MVs in a peripheral RANTES transfer in DR.

The chemokine RANTES is mainly produced by T lymphocytes, monocytes, and macrophages [1]. Its expression was also observed in human mesangium cells [26]. Monocytes found in the kidneys express CCR5 on their surface and are activated and stimulated to differentiate by RANTES [27]. Polymorphic variants of the RANTES/CCR5 genes that have been correlated with the occurrence of diabetic nephropathy have also been identified [28]. In the progression of diabetic renal disease, mesangial hyperplasia is one of the symptoms. In our study, we demonstrated that in the patient group with DR, there was a negative correlation between changes in RANTES concentration and the glomerular filtration rate (eGFR). This finding is very contributory for our knowledge about renal complications in diabetes, as increased RANTES expression by cells of mesangium may stimulate the progression of diabetic nephropathy.

We also found that the number MV^{AnnV+} in the size range of 100–900 nm and the number MV^{AnnV+} in the size range of 300–900 nm showed no significant differences between the patient group and the control group. The number of small MV^{AnnV+} was significantly lower in the study group compared to that of the control group (Table 4). Considering the level of diabetic alignment in the study group, we found this difference to be more significant in the UD group than in the control group. These results are different from those quoted in the reviews [29–31]. The measured number of MV^{AnnV+} in the control group (median — 805 counts/μl) was close to the number of MV^{AnnV+} in the control group in studies conducted by Sabatier *et al.* (average — 810 counts/μl) [32]. In contrast, the number of MV^{AnnV+} in patients with diabetes was approximately 50% higher than in our study. On the other hand, the results obtained by the same group of researchers showed that there is no statistically significant difference in the number of platelet MVs between the T2DM group and the control group. These findings are in agreement with our study. The number of MV^{AnnV+} in the size range of 100–900 nm and the number of MV^{AnnV+} in the size range of 300–900 nm showed no differences between the groups. We may speculate that this is because the dominant part of the surveyed population of MV^{AnnV+} was of platelet origin and because our study group consisted mostly of T2DM patients.

A detailed analysis of medical records showed that a significant part of the group of patients with diabetes was treated with statin therapy (declared by approximately 42% of patients), and over 1/3 of patients were treated with acetylsalicylic acid (ASA). This is another argument that can explain the lack of differences in the number of MV^{AnnV+} between the analyzed groups. Studies on simvastatin carried out by Nomura *et al.* provided arguments for this contradictory effect of statins on EV release (or internalization) that were observed as a reduced number of MVs in patients with T2DM [33]. Bulut *et al.* showed that platelet-derived MV levels decrease by more than 60% and endothelial-derived levels decreased by approximately 30% after 8 weeks of ASA therapy [34]. The use of statins and ASA in the group of patients with diabetes probably contributes to the balancing of all measured numbers, such as MV^{AnnV+} and large MV^{AnnV+} and the reduced number of small MV^{AnnV+} with respect to that of the control group.

By analyzing the patient group for the advancement of DR, the largest number of MV^{AnnV+} was demonstrated in the HNPDR group. The gradual increase in MV quantity was also strongly noticeable in MV^{AnnV+} along with the degree of advancement of nonproliferative DR. In the studies conducted by Ogata *et al.*, a correlation between the increase in platelet and monocyte MV numbers with the severity of DR was observed [35, 36]. In these studies, patients were divided into four groups: patients without retinopathy, patients with mild or moderate nonproliferative DR, patients with advanced nonproliferative DR, and patients with proliferative DR. In our study, the distribution of mild and moderate retinopathy to two separate groups

was used: mild nonproliferative DR and moderate nonproliferative DR. Despite the slight discrepancies in the applied division of the study group, a similar tendency was observed. The increased number MV^{AnnV+} in the HNPDR group may be used as a good prognostic biomarker of retinal blood vessel proliferation for DR treatment, and the most significant finding may be considered as evidence of MV involvement in the proliferation process.

The ability of MVs to transfer receptors for RANTES is still poorly documented and scarcely understood; hence, only a few studies have described this phenomenon. Studies on the CCR5 receptor were mainly carried out in the context of HIV infection. It turned out that mutation in the gene encoding this receptor in homozygotes results in resistance to HIV-1 infection [37]. It was in the context of HIV-1 infection that the ability of MVs to transfer this receptor was studied by Mack *et al.* [16]. The assays performed provide completely new information about the study group of patients with T1DM and T2DM. The evaluation of MV-mediated proangiogenic receptors in our study showed a reduced number of $MV^{AnnV+/CCR5+}$ in all patients relative to that of the control group. This phenomenon can be interpreted as an example of the antiangiogenic action of MVs by lowering the amount of the transferred proangiogenic receptor.

Our study brought new insight into the field of biomarkers of DR risk and progression, demonstrating that the correlation between changes in RANTES concentrations with the stage of nonproliferative DR and statistically significant dependence of concentration changes of $RANTES_{EV}$ with the number of MV^{AnnV+} 100–900 nm and the number of small MV^{AnnV+}. A similar relationship was confirmed in the studies by Nomura *et al.*, who showed significant dependence of changes in RANTES concentration with the number of circulating MVs of platelet, endothelial and monocytic origins [38]. It is not entirely clear whether this was due to the coexistence of two simultaneous phenomena or perhaps was due to the transfer of RANTES by MVs. The experiments carried out by the author did not confirm that EVs carry this chemokine. However, research conducted by Ohtsuka *et al.* showed that platelet-based MVs have the ability to release RANTES [39]. Lack of confirmation of the transfer of RANTES in the present study could have resulted from the chosen preparation method in which lysis of MV subjects was not performed or from the possibility of transferring RANTES by Ex, which were not analyzed in the present study. Explanation of the two phenomena concept is supported by other studies that showed that monocytes stimulated by cytokines, such as IL-8, increase the production of RANTES [40]. Therefore, in the future, we can try to explain the increased concentration of this chemokine as well as MV^{AnnV+} as the result of an inflammatory reaction associated with diabetes.

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Conception of the study (E.Ł.S.), study and methods design (A.T., B.K.-C., E.Ł.S., I.S.), Patients enrollment (A.Ż., I.S., M.K.), data collection and interpretation (B.M., M.K., I.S., A.Ż.), data analysis and interpretation (A.T., B.K.-C., B.M., E.Ł.S., P.H.), drafting of the article (A.T., E.Ł.S., P.H.), critical revision for important intellectual content, final approval of the version to be published (all authors).

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Appendix

Table 4. Comparison of the examined factors in the group of patients and in the control group.

Parameter	Study group (n = 61)	Control group (n = 25)	p
RANTES [μg/ml]	15.5 (9.7–18.1)	8.9 (0.9–14.6)	0.011
$RANTES_S$ [μg/ml]	15.1 (6.1–19.1)	8.4 (0.9–14.1)	0.014
$RANTES_{EV}$ [μg/ml]	14.9 (8.9–17.8)	6.7 (0.9–14.1)	0.028
MV^{AnnV+} 100–900 nm [counts/μl]	695 (265–1507)	805 (507–1581)	0.511
MV^{AnnV+} <300 nm [counts/μl]	161 (41–456)	279 (148–714)	0.016
MV^{AnnV+} ≥300 nm [counts/μl]	576 (229–1069)	647 (377–895)	0.493
$MV^{AnnV+/CCR5}$ [counts/ul]	62 (21–185)	108 (49–293)	0.049

The data in the table are shown as the median (lower — upper quartile) Bold indicates a statistically significant difference ($p < 0.05$). RANTES — it is the concentration of this chemokine in the dextrose; $RANTES_S$ — it is the concentration of this chemokine in plasma deprived of EV; $RANTES_{EV}$ — it is the concentration of this chemokine in plasma enriched with EV; MV^{AnnV+} — microvesicles labeled with annexin V; $MV^{AnnV+/CCR5}$ — microvesicles labeled with annexin V, which also exhibited the expression of CCR5.