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R. BADACZ[1], J. PODOLEC[1], T. PRZEWLOCKI[1,2], M. SIEDLINSKI[3], E. JOZEFCZUK[3], H. OLEKSY[1], J. BARAN[1], P. PIENIAZEK[1,2], K. ZMUDKA[1], A. KABLAK-ZIEMBICKA[1]

THE ROLE OF CHEMOKINE CCL5/RANTES AND METALLOPROTEINASE-9 AS INFLAMMATORY MODULATORS IN SYMPTOMATIC INTERNAL CAROTID ARTERY STENOSIS

[1]Department of Interventional Cardiology, Institute of Cardiology, Jagiellonian University Medical College and the John Paul II Hospital, Cracow, Poland; [2]Department of Cardiac and Vascular Diseases, Institute of Cardiology, Jagiellonian University Medical College and the John Paul II Hospital, Cracow, Poland; [3]Department of Internal and Agricultural Medicine, Faculty of Medicine, Jagiellonian University Medical College, Cracow, Poland

Up to 80% of all ischemic strokes (IS) attributed to internal carotid athero-occlusive artery stenosis (ICAS) are related to a thromboembolic mechanism. One athero-occlusive ischemic event increases the risk for ischemia in another vascular territory, resulting from inflammation within the atherosclerotic plaque induced by cytokines. Thus, ultrasonographic characteristics of vulnerable plaques in ICAS, including plaque echolucency and ulceration might correspond to cytokine activity. The present study aimed to investigate the associations between serum cytokines and atherosclerotic plaque characteristics and the 3-year risk of a major adverse coronary and carotid ischemic event (MACCE) in symptomatic patients treated for ICAS. Plaque characteristics on ultrasonography, serum levels of C-C motif chemokine ligand 5 (CCL5)/regulated on activation, normal T-cell expressed and secreted (RANTES), metalloproteinase-9 (MMP-9), interleukin-6 (IL-6), transforming growth factor beta (TGF-β), C-X-C motif chemokine ligand 16 (CXCL16), FAS ligand (FASL) and high sensivity C-reactive protein (hs-CRP) were analyzed in 103 symptomatic patients with ICAS prior to carotid revascularization. The incidence of MACCE: cardiovascular death (CVD), myocardial infarction (MI) and recurrent ischemic stroke (IS) were recorded prospectively for up to 5 years (median 37; IQR 21 – 40 months). Echolucent plaques, in comparison to echogenic plaques, displayed lower median levels of RANTES (P = 0.042) but higher median levels of IL-6 (P = 0.039). There was no relationship between plaque characteristics and median levels of MMP-9, TGF β, CXCL16, FASL, or hs-CRP (P = NS). During follow-up, MACCE occurred in 15 (14.6%) patients. Univariate Cox proportional hazard analysis indicated median RANTES levels < 45.5ng/mL (hazard ratio (HR) = 3.95; 95%CI = 1.10 – 14.2; P = 0.035), MMP-9 > 0.6 µg/mL (HR 4.5; 95%CI = 1.4 – 13.9; P = 0.009), renal impairment (HR 3.48; 95%CI = 1.29 – 9.34; P = 0.013) as potential MACCE risk factors. On multivariate Cox proportional hazard analysis, MMP-9 > 0.6 µg/mL and RANTES < 45.5 ng/ml were associated with a 4.72-fold (95%CI = 1.3 – 17.0; P = 0.017) and a 3.8-fold risk increase (95%CI = 1.07 – 13.89; P = 0.038) of MACCE. Kaplan-Meier analysis showed significant differences in MACCE-free survival rates depending on RANTES and MMP-9 median levels. We conclude that serum RANTES, IL-6, and MMP-9 were associated with plaque vulnerability and predicted adverse MACCE in patients treated for ICAS.

Key words: *ischemic stroke, internal carotid artery stenosis, C-C motif chemokine ligand 5/regulated on activation, normal T-cell expressed and secreted, metalloproteinase-9, cytokines, biomarkers, carotid plaque morphology*

INTRODUCTION

Annual worldwide mortality from ischemic stroke (IS) is estimated at 6.5 million, accounting for approximately 12% of total deaths worldwide and is a major cause of disability in developed countries (1, 2). Approximately 20% of cerebral ischemic events (CIE) are attributed to internal carotid artery stenosis (ICAS). The mechanism of cerebral ischemia involves either cerebral artery embolization, with debris released from a vulnerable carotid plaque and thrombus, or diminished cerebral blood flow causing hypoperfusion of brain structures (3-6).

A key feature of embolic CIE is an unstable plaque with active inflammation, liable to undergo rupture and thrombosis (7). The inflammatory process is both local and systemic, and the serum increase of inflammatory cytokines might help to identify vulnerable patients with unstable plaque formation (7-9).

Currently, a major point of interest is the assessment of serum interleukins (pathway from interleukin (IL)-1β to IL-6) or serum metalloproteinases (MMP-9) in the process of covering fatty streak formation in the presence of leukocytes.

CCL-5/RANTES (regulated on activation, normal T-cell expressed and secreted) recruits leukocytes into sites of

inflammation, which plays a key role in lymphocyte and macrophage mobilization and chemotaxis to the formed plaque. It participates in the process of inflammatory cell migration to the intima, further cell recruitment, and stimulates release of mediators. Many studies have shown significant correlations between RANTES levels and atherosclerotic plaque progression, cardiac injury, and markers of heart failure, even among patients with acute coronary syndromes complicated by sudden cardiac arrest (10).

As the inflammation leading to atherosclerosis is a generalized process, one athero-occlusive ischemic event increases the risk of ischemia in another vascular territory due to release of variable cytokines (11-13). Ultrasonographic characteristics of the vulnerable plaque in patients with ICAS, including plaque echolucency and ulceration, may correspond to the severity of the inflammatory process, and together with serum cytokine levels, can predict future cardiovascular outcome in patients with athero-occlusive disease.

The aim of the present study was to investigate the association between serum cytokines and atherosclerotic plaque characteristics and the long-term risk for major adverse coronary and carotid ischemic events (MACCE) in patients treated for ICAS following CIE.

MATERIAL AND METHODS

Patients

All patients gave written informed consent for participation in the study in accordance with requirements of the institutional local Ethics Committee. The study was performed in accordance with the requirements of the Declaration of Helsinki.

The prospective study comprised 103 patients (64.1% men, mean age 68.4 ± 9.4 years) with recently symptomatic ICAS (mean degree of stenosis 83.9 ± 14.8%) with IS (n = 80 subjects) or transient ischemic attack (TIA, n = 23) in median time of 24 days (IQR 14; 42) referred for carotid artery revascularization. The median NIHSS score assessed on admission was 2 (IQR 1 – 4). Exclusion criteria included occlusion of ICA, atrial fibrillation, or other known potential causes of CIE (coagulation disorders *etc*.), coronary instability, class III or IV congestive heart failure, chronic or acute inflammatory status, active cancer, or evidence of recent intracranial bleeding.

The data of index CIE: IS or TIA were obtained from the relevant stroke unit and sourced from available medical documentation, including brain imaging, either with computed tomography or magnetic resonance imaging. Neurological assessment was performed in all subjects prior to intervention to confirm the indications for carotid revascularization. Blood samples were collected on admission for further analysis. On admission, demographic data, presenting symptoms, as well as cardiovascular risk factors (age, gender, hypertension, hypercholesterolemia, diabetes mellitus, or active smoking) were obtained from each patient. Other information obtained included history of coronary artery disease (CAD), myocardial infarction (MI), history of coronary revascularization - both percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG), as well as interventions within the peripheral arteries.

Sample collection and blood analysis

All blood samples were collected on admission, prior to any intervention, immediately after written informed consent was obtained from the patients, and prepared for further analysis. Samples were allowed to coagulate for 30 min, centrifuged, and sera were frozen at –80°C until further biomarker analysis.

Concentrations of MMP-9, CCL5/RANTES, and IL-6 in serum samples were quantified using kits no. DMP900, DRN00B, and D6050 (R&D Systems, USA) respectively, according to the manufacturer's instructions. Final absorbance readings at 450 nm and 540 nm were performed using a Synergy H4 Microplate Reader (BioTek, USA). CXCL16, FASL, and transforming growth factor beta (TGF-β) serum levels were determined by ELISA (Human CXCL16 Immunoassay, Human FASL Immunoassay, Human TGF-β Immunoassay; R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Carotid ultrasonography

The severity of ICAS was assessed by high-resolution B-Mode, color and pulse Doppler ultrasonography (DUS) of carotid arteries with an ultrasound machine (Toshiba Aplio TUS-A300; Saronno, Italy) equipped with a linear-array 7.5 MHz transducer. Patients were examined in a supine position with the head tilted backwards.

In compliance with the joint recommendations for reporting degree of carotid ultrasound stenosis, the grade of stenosis in carotid arteries was assessed *via* measuring the increase in peak systolic velocity (PSV) > 2.3 m/sand a PSV ratio of > 4 (14). The degree of ICAS was also assessed according to NASCET criteria by measuring the vessel diameter at the point of maximal stenosis compared with the plaque-free vessel diameter distally from the lesion.

Carotid plaque morphology was classified as echolucent (*e.g*. plaque on DUS detectable on color-Doppler imaging only) or echogenic (visible in B-mode imaging) on DUS. Furthermore, plaque surface was described as smooth or ulcerated.

Follow-up

The incidence of MACCE, including cardiovascular death (CVD), myocardial infarction (MI), and recurrent CIE were recorded prospectively during the median follow-up period of 37 IQR (interquartile range) 21 – 40 months.

MI was diagnosed according to current guidelines of the European Society of Cardiology. Diagnosis of CIE was confirmed by available medical history from the Neurology Unit. CVD was defined as a fatal CIE, fatal MI, or other cardiovascular death (*i.e*. any sudden or unexpected death unless proven as non-cardiovascular on autopsy).

Statistical analysis

Continuous variables are presented as mean ± SD or median and interquartile range (IQR) for variables with no normal distribution. Categorical variables are expressed as frequencies and percentages. Means of analyzed parameters across groups were tested with the analysis of variance (ANOVA) test and frequencies were compared by the Chi-squared testfor independence. The normal distribution of studied variables was determined by the Shapiro-Wilk test. Differences between mean values were verified using the Mann-Whitney U test and Student's T-test for independent variables as appropriate. The median values of cytokines calculated from the whole study group, were used as cut-offs to evaluate risk of future MACCE. Potential independent prognostic markers of cardiovascular events during the follow-up period were established from the clinical, procedural, laboratory and angiographic variables with univariate Cox proportional hazard analysis (*Table 3*), and in case of a trend toward difference ($P < 0.1$), they were entered into a multivariate Cox proportional hazard model. Results of the multivariate Cox analysis are expressed as hazard ratio (HR)

and 95% confidence interval (95% CI). Survival analysis was performed with the use of Kaplan-Meier curves followed by the log-rank test to verify statistical significance. Due to a lack of normal distribution of variables (Shapiro-Wilk test), the non-parametric test was used for statistical analysis.

Statistical analysis was performed using Statistica 13.0 software. Statistical significance was assumed at $P < 0.05$.

RESULTS

The study comprised 103 (n = 66, 64% male) patients with symptomatic ICAS referred for carotid revascularization. Characteristics of study participants, as well as cytokine levels are presented in *Table 1*.

Table 1. Baseline characteristics of study participants.

	Total group n = 103	**MACCE group n = 15**	**NON-MACCE group n = 88**	**P value MACCE** versus **non-MACCE**
Demographic data				
Age (years),median (IQR)	70 (62 – 76)	73 (365 – 80)	68 (61 – 76)	0.297
Male, n (%)	66 (64.1%)	12 (80.0%)	54 (61.4%)	0.112
Smoking, n (%)	54 (52.4%)	10 (66.7%)	44 (50.0%)	0.461
Coronary artery disease, n (%)	54 (52.4%)	8 (53.3%)	46 (52.3%)	0.947
Previous MI, n (%)	16 (15.5%)	3 (20.0%)	13 (14.7%)	0.966
Previous CABG, n (%)	4 (3.9%)	2 (13.3%)	2 (2.3%)	0.287
Previous PCI, n (%)	16 (15.5%)	1 (6.7%)	15 (17.1%)	0.381
Peripheral arterial disease, n (%)	24 (23.3%)	6 (40.0%)	18 (20.5%)	0.044
Hypertension, n (%)	99 (96.1%)	15 (100%)	84 (95.5%)	0.430
Diabetes, n (%)	32 (31.1%)	6 (40.0%)	26 (29.6%)	0.948
Dyslipidemia, n (%)	89 (86.4%)	12 (80.0%)	77 (87.5%)	0.246
Renal dysfunction (eGFR < 60ml/min), n (%)	17 (16.5%)	5 (33.3%)	12 (13.6%)	0.027
Angiographic and imaging data				
ICA stenosis grade (%), median (IQR)	80 (75 – 84)	79 (63 – 85)	80 (75 – 85)	0.373
NIHSS score, median (IQR)	2 (1 – 4)	1.5 (0 – 3)	2 (0 – 4)	0.273
Laboratory tests results				
Fibrinogen (g/L), median (IQR)	3.53 (3.17 – 4.22)	3.58 (3.11 – 4.45)	3.53 (3.09 – 4.22)	0.764
hs-CRP (g/L), median (IQR)	2.74 (1.47 – 6.29)	2.22 (1.00 – 4.54)	2.74 (1.47 – 6.29)	0.659
LDL-cholesterol (mmol/L), median (IQR)	2.60 (2.03 – 3.01)	2.65 (2.03 – 3.24)	2.59 (2.03 – 2.95)	0.700
Creatinine (μmol/L), median (IQR)	82 (71 – 102)	97 (85 – 109)	79 (70 – 98)	0.041
CCL5/RANTES (ng/ml), median (IQR)	45.5 (28.9 – 68.9)	25.8 (15.8 – 62.7)	47.4 (31.9 – 69.5)	0.018
MMP-9 (ug/ml), median (IQR)	0.61 (0.37 – 0.91)	0.66 (0.49 – 1.03)	0.59 (0.37 – 0.87)	0.242
IL-6 (pg/ml), median (IQR)	4.73 (3.09 – 7.34)	4.51 (2.62 – 6.32)	4.78 (3.24 – 7.33)	0.433
TGF-β (ng/ml), median (IQR)	48.3 (42.0 – 64.9)	46.9 (31.1 – 59.2)	50.4 (43.1 – 65.0)	0.345
CXCL16 (ng/ml), median (IQR)	3.69 (3.29 – 4.31)	3.47 (3.13 – 3.81)	3.71 (3.29 – 4.32)	0.139
FASL (ng/ml), median (IQR)	55.7 (47.5 – 64.7)	59.0 (46.1 – 65.4)	47.3 (55.8 – 65.1)	0.839

Abbreviations: CABG, coronary artery bypass grafting; CCL5, C-C motif chemokine ligand 5; CXCL16, C-X-C motif chemokine ligand 16; eGFR, estimated glomerular filtration rate; FASL, FAS ligand; hs-CRP, high sensivity C-reactive protein; ICA, carotid athero-occlusive artery; IL-6, interleukin 6; LDL, low density lipoprotein; MACCE, major adverse coronary and carotid ischemic event; MI, myocardial infarction; MMP-9, metalloproteinase 9; NIHSS, National Institutes of Health Stroke Scale; PCI, percutaneous coronary intervention; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β, transforming growth factor beta.

The majority of patients presented with vulnerable plaque morphology on admission (*Table 2*). Ulcerated plaques, as compared to smooth plaques, were characterized by higher median levels of IL-6 (5.09; IQR 3.24 – 9.9 versus 3.76; IQR 2.83 – 5.81; P = 0.039); while echolucent plaques, as compared to echogenic plaques, had lower levels of serum

Table 2. Plaque morphology and cytokine levels.

	Echolucent plaque n = 71 (68.9%)	**Echogenic plaque** n = 32 (31.1%)	**P value**	**Ulcerated plaque** n = 36 (34.9%)	**Smooth plaque** n = 67 (65.1%)	**P value**
RANTES, median (IQR)	35.7 (25.5 – 52.9)	48.9 (32.5 – 76.6)	0.042	43.4 (25.5 – 62.7)	47.5 (29.4 – 68.3)	0.365
MMP-9, median (IQR)	0.62 (0.41 – 0.96)	0.49 (0.35 – 0.77)	0.258	0.56 (0.36 – 0.76)	0.61 (0.40 – 0.97)	0.442
IL-6, median (IQR)	4.92 (3.44 – 8.06)	4.06 (2.62 – 5.66)	0.039	5.09 (3.24 – 9.9)	3.76 (2.83 – 5.81)	0.040
CXCL-16, median (IQR)	3.62 (3.29 – 4.31)	3.74 (3.44 – 4.08)	0.395	3.81 (3.39 – 4.08)	3.61 (3.17 – 4.31)	0.978
TGF-β, median (IQR)	49.1 (42.1 – 58.7)	48.2 (41.9 – 65.0)	0.167	45.4 (37.7 – 57.2)	55.1 (43.6 – 68.7)	0.931
FASL, median (IQR)	55.9 (45.4 – 64.7)	55.6 (49.0 – 65.1)	0.187	63.1 (47.6 – 68.7)	54.8 (47.3 – 59.7)	0.976
hs-CRP, median (IQR)	2.77 (1.53 – 6.45)	2.55 (1.16 – 6.15)	0.256	2.56 (1.77 – 7.09)	2.83 (1.00 – 6.00)	0.730

Abbreviations: CXCL16, C-X-C motif chemokine ligand 16; FASL, FAS ligand; hs-CRP, high sensivity C-reactive protein; IL-6, interleukin 6; MMP-9, metalloproteinase 9; RANTES, regulated on activation normal T-cell expressed and secreted; TGF-β, transforming growth factor beta.

Table 3. Univariate Cox analysis for major adverse coronary and carotid ischemic event (MACCE) during follow-up.

Co-factor	**Hazard Ratio**	**95% Confidence Interval**	**P value**
Age	1.04	0.98 – 1.10	0.159
Male	3.36	0.75 – 15.0	0.112
Smoking	1.12	0.38 – 3.26	0.831
Coronary artery disease	1.83	0.29 – 2.42	0.745
Previous MI	0.59	0.16 – 2.13	0.424
Previous CABG	0.49	0.11 – 2.14	0.342
Previous PCI	1.14	0.27 – 5.02	0.866
Peripheral arterial disease	2.77	1.04 – 7.38	0.042
Hypertension	0.00	0.00 – 1.00	0.991
Diabetes	1.05	0.36 – 3.03	0.925
Dyslipidemia	1.55	044 – 5.46	0.492
Renal dysfunction (eGFR < 60ml/min)	3.48	1.29 – 9.34	0.013
Fibrinogen	1.04	0.68 – 1.70	0.761
hs-CRP	1.01	0.95 – 1.07	0.744
LDL-cholesterol	1.16	0.74 – 1.81	0.516
Creatinine	1.01	0.99 – 1.02	0.097
CCL5/RANTES	3.95	1.10 – 14.2	0.035
MMP-9	4.50	1.40 – 13.9	0.009
IL-6	0.96	0.95 – 1.09	0.604
TGF-β	0.98	0.94 – 1.01	0.206
CXCL16	0.53	0.19 – 1.49	0.231
FASL	1.01	0.96 – 1.04	0.686

Abbreviations: CABG, coronary artery bypass grafting; CCL5, C-C motif chemokine ligand 5; CXCL16, C-X-C motif chemokine ligand 16; eGFR, estimated glomerular filtration rate; FASL, FAS ligand; hs-CRP, high sensivity C-reactive protein; IL-6, interleukin 6; LDL, low-density lipoprotein; MI, myocardial infarction; MMP-9, metalloproteinase 9; PCI, percutaneous coronary intervention; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β, transforming growth factor beta.

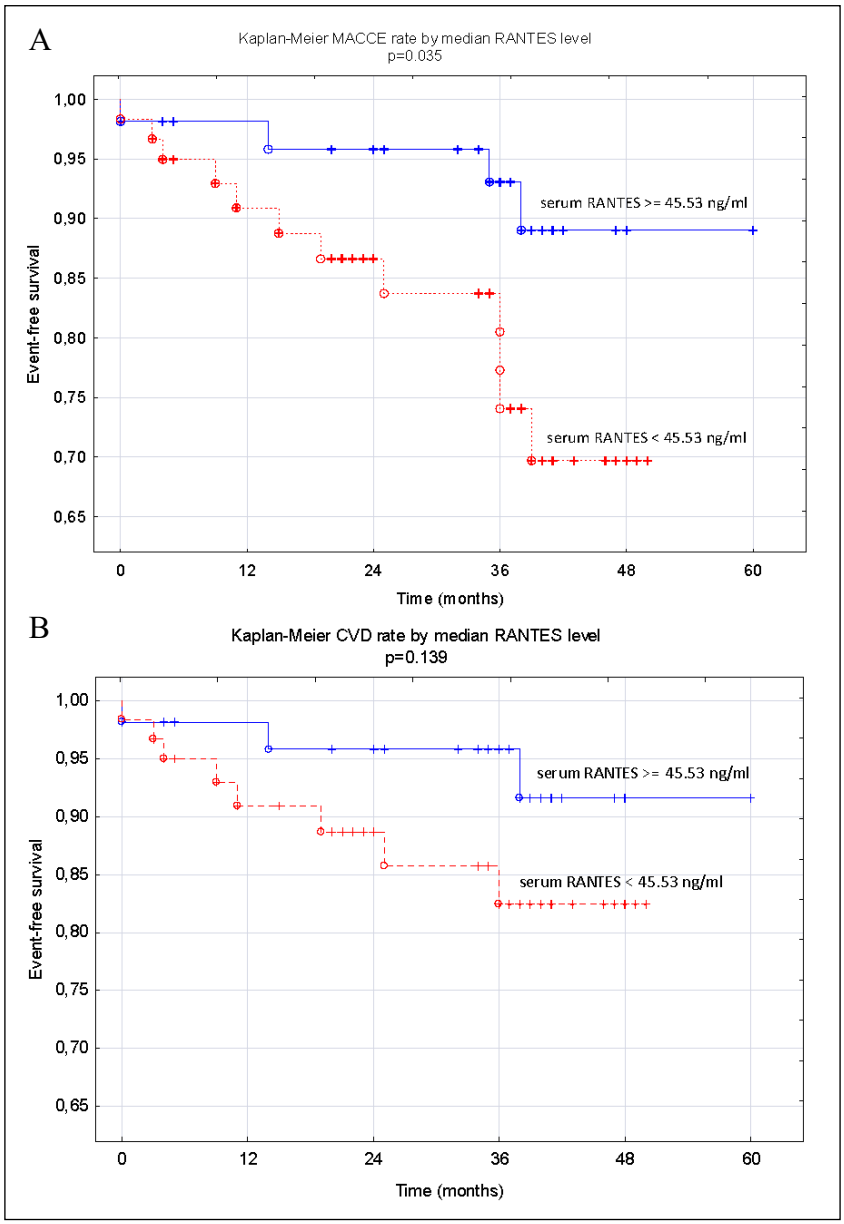

Fig. 1 A,B. Kaplan-Meier event-free survival curves stratified according to median levels of regulated on activation, normal T-cell expressed and secreted (RANTES) for (*A*): major adverse coronary and carotid ischemic event (MACCE), (*B*): cardiovascular death (CVD).

CCL5/RANTES (35.7; IQR 25.5 – 52.9 versus 48.9; IQR 32.5 – 76.6; P = 0.042) and higher levels of IL-6 (4.92; IQR 3.44 – 8.06 versus 4.06; IQR 2.62 – 5.66; P = 0.04). There were no statistically significant differences with respect to plaque characteristics and hs-CRP, FASL and TGF-β levels (P = NS) (*Table 2*).

We observed statistically significant correlations between CCL5/RANTES or IL-6 and degree of carotid stenosis (R = –0.20; P = 0.042 and R = –0.23; P = 0.019, respectively).

During a median follow-up of 37 (IQR 21 – 40) months, 22 MACCE occurred in 15 (14.6%) patients, including 10 (7.7%) CVD, 6 (5.8%) MI, and 6 (5.8%) CIE.

Lower median CCL5/RANTES levels (25.8; IQR 15.8 – 62.7 versus 47.4; IQR 31.9 – 69.5 ng/mL; P = 0.018) were observed in the MACCE group when compared with the non-MACCE group (88 patients). Similarly, lower CCL5/RANTES levels were observed in patients who died from CVD (25.8; IQR 16.9 – 45.8 versus 46.32; IQR 31.0 – 69.5 ng/mL; P = 0.034) and suffered from MI (23.4; IQR 12.1 – 26.6 versus 47.0; IQR 31.8 – 69.5 ng/mL; P = 0.001) when compared with non-CVD and non-MI groups, respectively.

Univariate Cox proportional hazard analysis indicated median CCL5/RANTES < 45.5 ng/mL (HR 3.95; 95%CI = 1.10 – 14.2; P = 0.035), MMP-9 > 0.6 µg/mL (HR 4.5; 95%CI = 1.4 – 13.9; P = 0.009), peripheral arterial disease (HR 2.77; 95%CI 1.04 – 7.38; P = 0.042) and renal impairment with estimated glomerular filtration rate (eGFR) < 60 ml/min (HR 3.48; 95%CI = 1.29 – 9.34; P = 0.013) as potential risk factors of MACCE (*Table 3*).

On multivariate Cox proportional hazard analysis, MMP-9 level > 0.6 µg/mL was associated with a 4.72-fold increase in risk of MACCE (95%CI = 1.3 – 17.0; P = 0.017), while CCL5/RANTES level < 45.5 ng/ml was associated with a 3.8-fold increase in risk of MACCE (95%CI = 1.07 – 13.89; P = 0.038).

The Kaplan-Meier curves showed significant differences in event-free survival rates depending on CCL5/RANTES and MMP-9 median levels (*Figs. 1* and *2*).

The 1-, 2-, and 3-year Kaplan-Meier MACCE-free survival curves were 98.1%, 98.1%, and 93.1% for patients with median

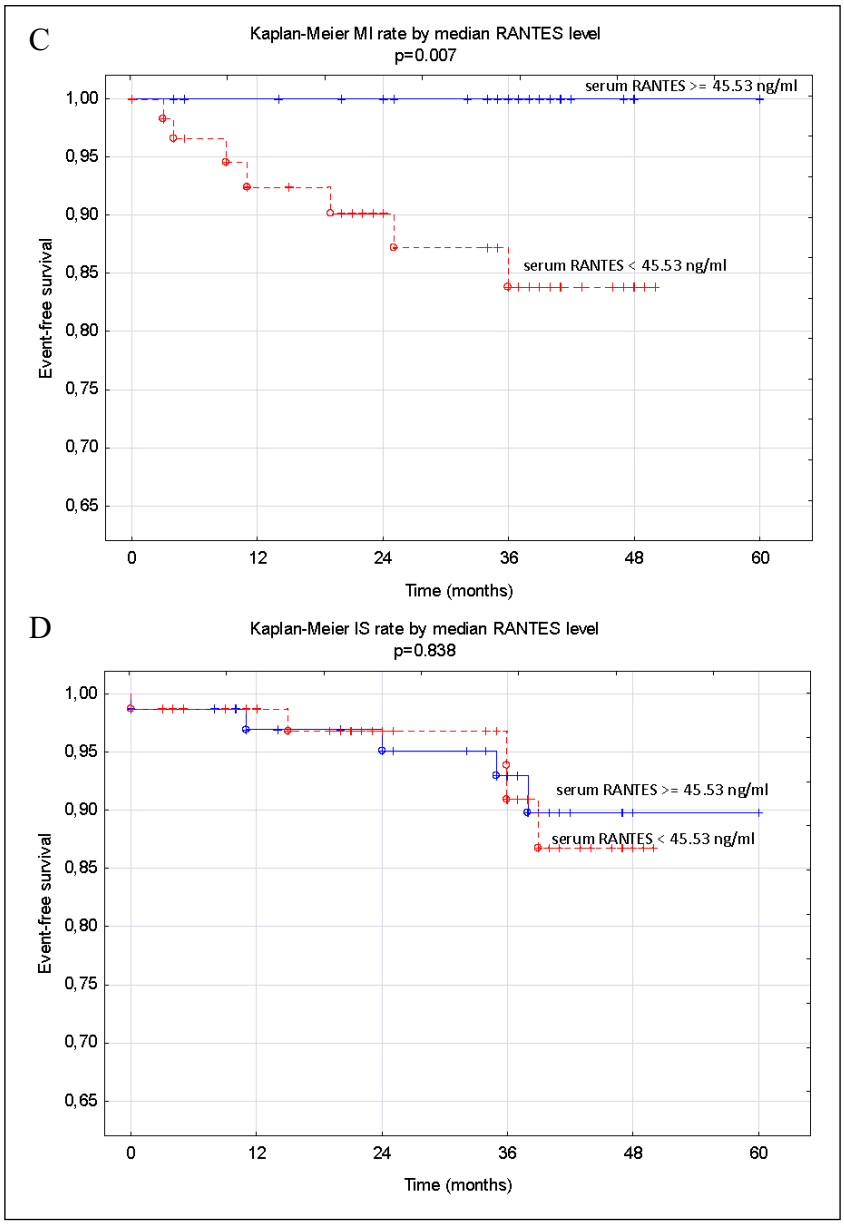

Fig. 1 C,D. Kaplan-Meier event-free survival curves stratified according to median levels of regulated on activation, normal T-cell expressed and secreted (RANTES) for (*C*): myocardial infarction (MI) and (*D*): ischemic stroke (IS).

CCL5/RANTES > 45.5 ng/ml, while the Kaplan-Meier MACCE-free survival curves were 90.8%, 86.6%, and 74.1% for CCL5/RANTES < 45.5 ng/ml (P = 0.035) (*Fig. 1A*).

MMP-9 levels > 0.6 µg/mL were associated with significantly lower MACCE-free survival: 90.5%, 86%, 79.9% at 1-, 2-, and 3-year follow-up, as compared to patients with MMP-9 levels < 0.6 µg/mL (98.1%, 95.9%, and 90.6%, respectively, P = 0.015) (*Fig. 2A*).

In addition, higher median MMP-9 levels were associated with increased IS incidence (P = 0.029), with no statistically significant association with CVD and MI (P = 0.086 and P = 0.193, respectively) (*Fig. 2B-2D*). Meanwhile, lower RANTES levels were associated with increased MI incidence (P = 0.007) (*Fig. 1C*).

DISCUSSION

In experimental studies using mice, IS due to surgical suture of the middle cerebral artery induced massive and rapid activation of the peripheral immune system. This led to significantly enhanced levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-6, monocyte chemoattractant protein 1 (MCP-1), IL-2, RANTES, and chemokine receptors: macrophage inflammatory protein 2 (MIP-2) and C-C chemokine receptors (CCR1, CCR2, CCR7, CCR8) (15). The activation of cytokines and chemokines attracts mononuclear cells and granulocytes, which cause further damage to the ischemic and surrounding areas of brain tissue (15). Immune response is a critical factor in determining the progress of neurodegeneration after stroke (16).

There is evidence supporting the role of inflammatory cytokines in the pathogenesis of athero-occlusive disease, including ICAS and its complications such as IS or TIA (17).

Our present study showed higher serum levels of IL-6 in patients with a soft - echolucent, ulcerated carotid plaque phenotype and positive correlations between IL-6 and RANTES with ICA stenosis. Moreover, significantly lower serum RANTES levels were found in patients with echolucent (vulnerable) plaques. Echolucent plaques are believed to contain

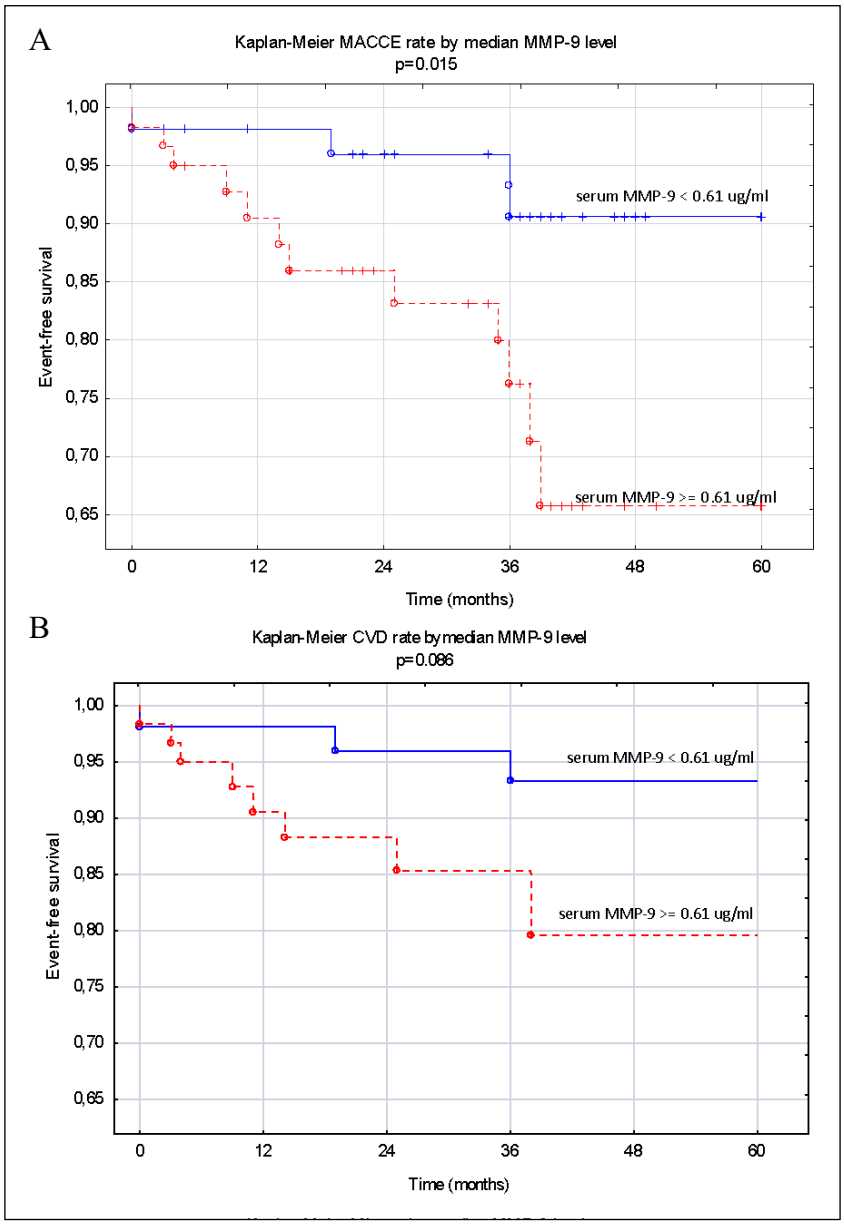

Fig. 2 A,B. Kaplan-Meier event-free survival curves stratified according to median levels of metalloproteinase-9 (MMP-9) for (*A*): major adverse coronary and carotid ischemic event (MACCE), (*B*): cardiovascular death (CVD).

inflammatory cells such as macrophages and leukocytes, specifically, T-cells (18).

In the Tromso study, IL-6 (OR 1.44, 95%CI 1.12 – 1.85 per SD increase in IL-6 level) was independently associated with rapid plaque progression (19), which is in line with results in our study. Kyriakidis *et al*. studied expression levels of cytokines in excised plaques obtained during carotid endarterectomy from 16 symptomatic and 8 asymptomatic patients (20). They found that symptomatic patients had type II and type III plaques (predominantly echolucent), which was related to higher levels of IL-6, MMP-1, and MMP-9, when compared to uniformly echogenic plaques (type IV) in asymptomatic patients, suggesting a link between inflammation and potential risk of plaque rupture (20).

Similarly, circulating biomarkers of extracellular matrix remodeling: MMP-2, MMP-9, as well as tissue inhibitor of metalloproteinases (TIMP)-1 were related with carotid stenosis, as well as aneurysm rupture through higher proteolytic activity within aneurysmal thrombus (21, 22).

In another study evaluating the levels of multiple cytokines within carotid plaques, Shalhoub *et al*. found higher levels of TNF-α, IL-1, IL-6, GM-CSF, CCL5/RANTES, CCL20, CXCL9, MMP-3, and MMP-9 in patients with symptomatic ICAS (23). In a study by Eilenberg *et al*., patients with vulnerable plaque characteristics on DUS and histology presented with higher MMP-9/NGAL levels (24). In another study, the multi-marker approach for predicting the course of symptomatic ICAS, with subsequent incidence of CIE, was proposed by Musialek *et al*., however, no single cytokine was identified as a prognostic marker of symptomatic ICAS, apart from HDL cholesterol level (25).

Controversially, data concerning CCL5/RANTES concentration are inconsistent. Li *et al*. demonstrated that macrophages were the major CCL5-expressing cells in plaques, which were significantly reduced in Batf3$^{-/-}$Apoe$^{-/-}$ mice (26). Furthermore, CD8α^{+} dendritic cells aggravated atherosclerosis, likely by inducing a Th1 cell response, which promoted CCL5 expression in macrophages and increased infiltration of leukocytes and lesion inflammation (26).

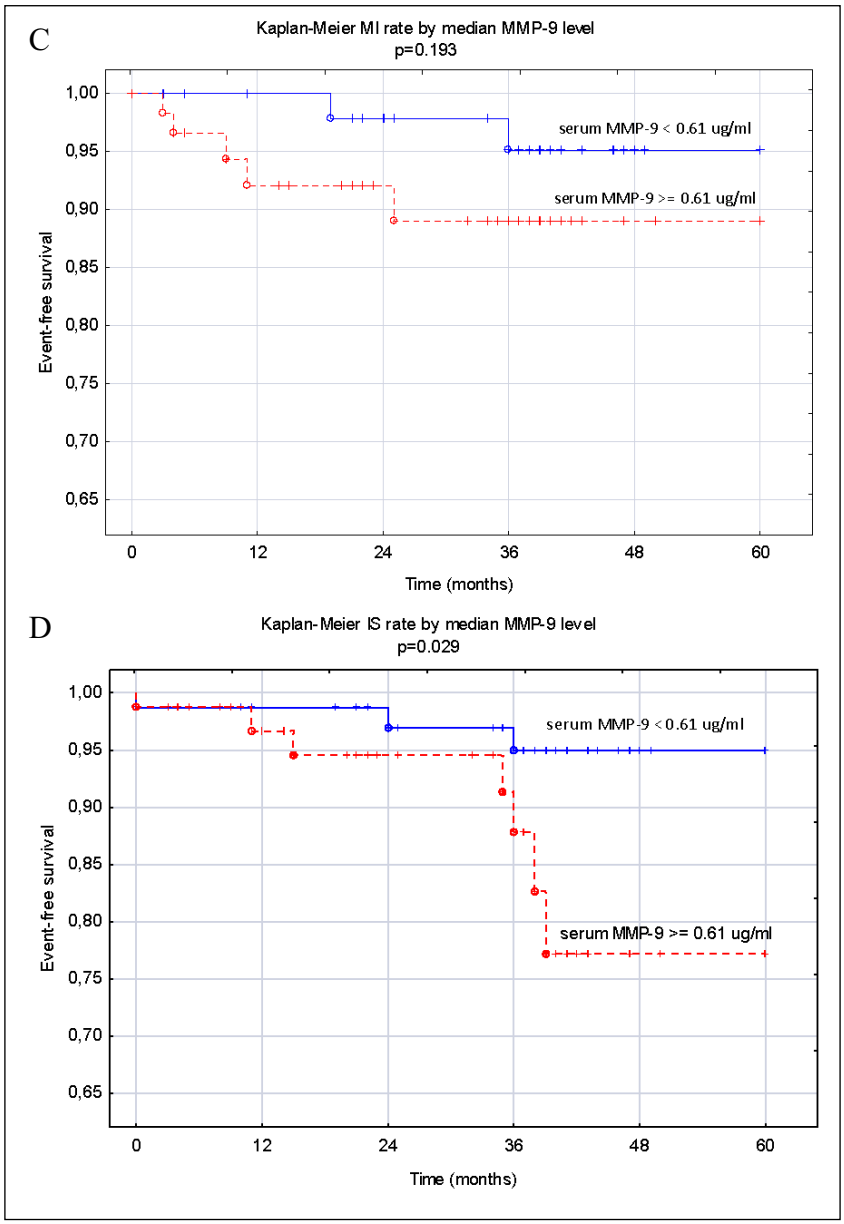

Fig. 2 C,D. Kaplan-Meier event-free survival curves stratified according to median levels of metalloproteinases-9 (MMP-9) for (C): myocardial infarction (MI) and (D): ischemic stroke (IS).

Experimental studies in mice showed that RANTES is overexpressed in atherosclerotic lesions and that genetic modification of mouse samples by deleting genes responsible for coding the CCL5/RANTES receptor CCR5 reduces progression of atherosclerosis and the risk of MI (27, 28). CCL5/RANTES was increased in patients with MI as compared to stable CAD patients and even higher levels were noted in patients with subsequent heart failure (29).

In contrast, there is evidence that serum level of CCL5/RANTES is decreased in patients with multi-vessel CAD (30). Controversially, in a study by Herder *et al*., high plaque RANTES levels were associated with an unstable plaque phenotype, while serum RANTES levels were neither associated with CAD nor incident MI (31). A negative correlation between serum chemokine RANTES level and left ventricular mass in patients with aortic stenosis, as measured by MRI, was found in patients with advanced hypertrophy and fibrotic remodeling of the left ventricle (32).

Studies concerning the role of RANTES in patients with ICAS are sparse. The recently published ARIC Carotid MRI study demonstrated that higher RANTES levels were associated with higher lipid-core volume, which can lead to formation ofhigh-risk plaques (33). Controversially, the same study showed a positive association between RANTES and fibrous cap thickness, which characterize stable plaques, significantly decreasing the reliability of CCL5/RANTES assessment in carotid plaque morphology (33).

In our present study, together with analysis of carotid plaque morphology and cytokine levels, we focused on the potential role of chemokines in predicting future MACCE, including risk of CVD, MI, or recurrent IS.

Although cytokines are not a part of routine laboratory diagnostics in patients with ICAS, their assessment could play an important role in future MACCE prediction and prognosis evaluation (8, 12, 19, 23). Although intervention in symptomatic ICAS results in decreased risk of recurrent IS resulting from index lesion, we observed that serum levels of RANTES and MMP-9 were associated with future adverse ischemic events in other arterial territories during the follow-up period.

In the present study, RANTES < 45.5 ng/mL increased the risk of MACCE by 3.8-fold while MMP-9 > 0.6 µg/mL

increased the risk of MACCE by 4.7-fold. Cavusoglu *et al*. showed that lower RANTES levels had an independent predictive value of CVD and MI in CAD patients undergoing coronary angiography, with similar results in diabetic patients (34). One possible explanation of this phenomenon may be due to chemokine migration to the interior of the arterial wall and to the plaque itself, decreasing its levels in peripheral blood. Similar results were presented in a paper by Versteylen *et al*., in which only CCL5/RANTES provided independent predictive value for obstructive CAD (HR 1.27 (1.02 – 1.59), P = 0.04) in patients undergoing coronary CT for suspected CAD in primary care patients (35). A review by Ammirati *et al*., covering the multi-level issue of inflammatory markers in patients with ICAS, claimed that cytokines such as IL-6 and TNF-α were shown to reliably predict carotid plaque characteristics, while novel markers such as members of the MMP family (including MMP-9) appear to be implicated in its destabilization (36). The results are similar in our study, in which we observed higher IL-6 levels in patients with echolucent plaque types, while MMP-9 was an independent predictor of future MACCE.

The possible prognostic value of cytokines, especially MMPs, was addressed in a study by Zhong *et al*, who found an association between higher levels of MMP-9 and increased risk of major disability and death in patients with acute stroke (37). Although the trial included patients with acute CIE, regardless of its etiology, as compared to symptomatic ICAS patients in our present study, it clearly demonstrated MMP-9 to be a potential predictor of adverse outcome (37).

There are many both bench and bed-site studies that address the problem of plaque instability and future MACCE. Many of them pointed out independent risk markers such as IL-1β, IL-6, MMP-9, hs-CRP, TGF-β, CXCL16, microRNAs, and many others (39-44). Their clinical relevance might be reflected by the introduction of the therapeutic approaches: *e.g*. studies on herbal decoction Ojeoksan that is natural inhibitor of TNF-α, MMP-2 and MMP-9 expression, downregulating reactive oxygen species/nuclear factor-κB signaling (45). Therefore, Ojeoksan may inhibit atherosclerosis development and progression (45).

We focused on RANTES as the potentially early-stage marker in atherosclerotic process, hence potential target for pharmacological intervention in the future. However, a main limitation of RANTES, leading to discrepant results, is the fact that commercially available assays measure total RANTES protein, although variant forms including truncated proteins and proteins with different oxidation, glycation and glycosylation patterns exist. Furthermore, CCL5/RANTES has variant genotype polymorphisms, although their significance was not confirmed so far (31). Our data demonstrate that levels of RANTES and IL-6 may identify patients with vulnerable plaque, while RANTES and MMP-9 levels might be used as predictors of patient cardiovascular risk. Of note, our study is small and preliminary such that further investigation is needed.

Study limitations

There are several aspects to be considered as study limitations. Firstly, the laboratory analysis of analyzed cytokines was performed only once, after the enrollment, prior to carotid revascularization, as procedure itself could influence further cytokines levels. Secondly, the plaque morphology was assessed only on Doppler ultrasound examination with no histological confirmation, as no plaque excision was possible in patients treated with carotid artery stenting. Lastly, this is preliminary pilot data, that requires further investigations.

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Author's address: Dr. Jakub Podolec, Department of Interventional Cardiology, Institute of Cardiology, Jagiellonian University, School of Medicine, The John Paul II Hospital, 80 Pradnicka Street, 31-202 Cracow, Poland.
E-mail: jjpodolec@gmail.com