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Lekić, Andrica; Brekalo, Zdrinko; Kvesić, Ante; Kovačević, Miljenko; Baričev Novaković, Zdenka; Šutić, Ivana; Bulog, Aleksandar; Šutić, Ingrid; Pavišić, Valentino; Mrakovčić-Šutić, Ines

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Crosstalk Between Enzyme Matrix Metalloproteinases 2 and 9 and Regulatory T Cell Immunity in the Global Burden of Atherosclerosis

A. Lekic*, Z. Brekalo†, A. Kvesic†, M. Kovacevic‡, Z. Baricev-Novakovic§, I. Sutic§, A. Bulog¶, I. Sutic**, V. Pavisic†† & I. Mrakovcic-Sutic††

*Department of Basic Medical Sciences, Faculty of Health Studies, University of Rijeka, Rijeka, Croatia; †Department of Surgery, University Hospital Mostar, Mostar, Bosnia and Herzegovina; ‡Department of Cardiovascular Surgery, Medical Faculty, University of Rijeka, Rijeka, Croatia; §Department of Family Medicine, Medical Faculty, University of Rijeka, Rijeka, Croatia; ¶Department of Public Health, Medical Faculty, University of Rijeka, Rijeka, Croatia; **Medical Faculty, University of Rijeka, Rijeka, Croatia; and ††Department of Physiology and Immunology, Medical Faculty, University of Rijeka, Rijeka, Croatia

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Correspondence to: I. Mrakovcic-Sutic, Department of Physiology and Immunology, Medical Faculty, University of Rijeka, Brace Branchetta 20, Rijeka, Croatia.
E-mail: ines.mrakovcic.sutic@medri.uniri.hr

Andrica Lekic and Zdrinko Brekalo contributed equally to this work.

Abstract

Changes in immune and inflammatory responses may play a crucial role in the development and progression of atherosclerosis, as an autoimmune, chronic and progressive inflammatory disease. Immunological activity and vascular inflammation during atherosclerosis can be modulated by autoimmune responses against self-antigens, according to changeable risk factors (cholesterol, oxidized low-density lipoprotein (ox-LDL) in the vascular wall, fatty acids, etc.), and accompanied by accumulation of leucocytes and proinflammatory cytokines, which stimulate the transcription of matrix metalloproteinases (MMPs), whose concentration are increased in foam cell-rich regions. Regulatory T cells (Tregs) represent a unique subpopulation of T cells specialized in the regulation of immune response and in the suppression of proatherogenic T cells. The aim of our study was to examine the interactions between the concentration of enzyme matrix metalloproteinases 2 and 9 (MMP-2 and 9) in urine and the percentage of Tregs in peripheral blood of two groups of patients: with carotid artery stenosis (CAS), undergoing surgery and with mild atherosclerosis (A) from general practice. The method of enzyme immunoassay (ELISA) was used to determine enzyme MMP expression, and Tregs was examined by flow cytometric analysis. Our data have showed a large increase in the enzyme MMP-2 and 9 in the urine of CAS and A patients in comparison with healthy controls and indicated this method as an easy marker for the monitoring of the development of atherosclerosis. Simultaneously, the diminished number of Tregs in the same patients pointed the importance of these regulatory mechanisms in the etiopathogenesis of atherosclerosis and possible Tregs-mediated therapy.

Introduction

Cardiovascular diseases (atherosclerosis, coronary artery disease, peripheral vascular disease, stroke) are leading causes of morbidity and mortality in all developed and developing countries and are subject to examination by numerous groups of researchers of different orientations, which have the same goal: a better understanding of the disease, and risk factors that can lead to disease, early recognition and treatment in order to better forecasts, reducing complications and consequently better public health. Atherosclerosis represents an inflammatory, chronic metabolic disorder of the vessel wall, accompanied with unregular innate and adaptive immune responses, which may cause the progression of the disease. It seems that the beginning is recruitment and activation of monocytes and macrophages caused by abnormal innate immunity, which consequently leads to the accumulation of modified lipids in the arterial intima, formation of vascular plaques, following with inflammation, fibrosis and cell death [1, 2]. Accumulation of oxidized low-density lipoprotein (oxLDL), which together with heat-shock proteins acts as autoantigens presented by antigen-presenting cells (APC), promotes the inflammation and leads to secretion of interleukins, chemokines and proteases. Inflammatory process is stimulated by innate signals and migration of T cells and monocytes in the arterial wall which is crucial for atherogenesis [3]. Ox-LDL *in vitro* can contribute to Treg/Th17 balance in the periphery inducing the apoptosis of Tregs and the proliferation of Th 17 [4]. Furthermore, it seems that Tregs may become apoptotic through Fas/FasL pathway, leading to their alteration and reduction in the

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number of Tregs, what happened in acute coronary syndrome [5]. Th1 pathway accompanied with interferon-γ (IFN-γ) production is involved in immunopathogenesis of atherosclerosis, while Th2-driven response depends of the stage of the illness and of the lesion's site [6]. Immune response probably occurs also in atherosclerotic lesions via adaptive T cells after antigen presenting by antigen-presenting cells (macrophages or dendritic cells) to naive T cells. Influential determinants of plaque growth may be recognized by regulatory type of cytokines such as IL-10 and TGF-β, which are in the same time possible markers for the expression of Tregs lineage. CD4+/CD25+/FoxP3+ regulatory T lymphocytes (Tregs) are a special lineage of T cells, which play a crucial role in the prevention of autoimmunity and maintaining tolerance of allogenic transplants. There is accumulating evidence that Tregs are an important part of normal immune system and responsible for balancing the immunological self-tolerance and homeostasis. Their anomaly may cause an aberrant or excessive immune responses, including autoimmune and immunopathological diseases. Recent findings suggest the role of Tregs in suppression of various innate and acquired immune responses in different types of autoimmune disorders and their experimental models, such as arthritis [7, 8], colitis [9, 10], diabetes [11], lupus [12], gastritis [13, 14] and thyroiditis [15, 16], as well as their crucial involvement in tumour immunity [17–21], environmental diseases [22], as well as in atherosclerosis [23–27]. The matrix metalloproteinases (MMPs) play a key role in angiogenesis together with migration and/ or invasion of endothelial cells in surrounding stroma and tissues. MMPs are involved in degrading of extracellular matrix (ECM), which consequently lead to facilitate invading of endothelial cells [28] and in the same time stimulate the releasing of extracellular matrix-sequestered proangiogenic factors (ECM-sequestered proangiogenic factors), integrins, adhesion receptors and different growth factors and receptors [29–32].

The aim of our study was to examine the interactions between the concentration of enzyme matrix metalloproteinases 2 and 9 (MMP-2 and 9) in urine and the number of Tregs in peripheral blood of patients with mild atherosclerosis (A patients) and with carotid arteries stenosis (CAS patients)) who were undergoing the surgical procedure and comparison their results with these in healthy blood donors. We hypothesized that circulating levels of MMPs were abnormal in patients who had atherosclerotic changes and these levels were compared with those in matched controls.

Material and methods

Patients' database. Patients were selected from a stratified sample of the population of adult patients of both sexes who are controlled or treated in ambulance of Family Medicine of Primorsko-Goranska County with diagnosed atherosclerotic changes in medium- and large-sized arteries and accompanied with high level of cholesterol and triglycerides, as well as from a stratified population sample of adult patients of both sexes with carotid artery stenosis undergoing surgical procedure at the Department of Toracovascular Surgery in Clinical Hospital Centre Rijeka. Voluntary blood donors (without hypercholesterolaemia and triglyceridemia) were used as control group. The patients who underwent the surgical procedure were divided into two groups (patients with symptomatic carotid artery disease and asymptomatic patients in whom surgery is necessary). Forty patients with atherosclerosis of carotid artery disease and 40 patients with mild atherosclerosis (diagnosed by ultrasound) as well as 20 healthy volunteers comprised the study. All data from patients and healthy volunteers as well as their blood samples and urine were acquired in accordance with the published International Health Guidelines outlined in the declaration of Helsinki 'Ethical principled for medical research involving human subjects'. The study protocol was approved by Ethics Committee of the Faculty of Medicine, University of Rijeka. All patients and healthy volunteers subscribed the written informed consent. All patients with acute or chronic inflammatory disease, patients who were previously treated with immunosuppressive or radiation therapy, or those with some other immunological diseases or those who underwent some other surgical proceeding were excluded from the study.

Quantitative determination of human MMP-2 and MMP-9. The method of enzyme immunoassay (ELISA) was used to determine enzyme expression of matrix metalloproteinases 2 and 9 (MMP 2 and 9) in urine. In our experiment, we used The Ray Bio Human MMP-2 and 9 Enzyme-Linked Immunosorbent Assay Kit [33, 34]. All urine samples were prepared following the manufacture instructions. The kit was used as a highly selective *in vitro* ELISA kit for quantitative measurement of proactive and active matrix metalloproteinases 2 and 9 (MMP-2 and 9) enzyme concentrations in human urine. Using the special computer program (TECAN, Magellan, USA) used as an informational support for reading absorbance and concentrating readings on TECAN readers, the mean values of absorbance of proactive and active forms of the matrix metalloproteinase 2 and 9 enzymes in our samples were read by logarithmic programming. All standards, samples and blinds worked in duplicates. We used the Magellan program to determine the mean absorbance value for each standard, sample and blind test.

Isolation of peripheral blood mononuclear cells (PBMC). Heparinized ten millilitres of peripheral blood was acquired in Vacutainer (Becton Dickinson, Franklin Lakes, NY, USA), overlaid onto density gradient Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged for 20 min at 800′*g*. After the centrifugation, the cells from the interface were collected and washed twice in Roswell

Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Auckland, NZ, USA) and resuspended at a final concentration of $1'10^6$ peripheral blood lymphocytes per sample in fluorescent-activated cell sorting (FACS) buffer. The viability of PBL was >95% assessed with propidium iodide 0.5 mg/ml/10^6 cells (Sigma–Aldrich Chemi), and a flow cytometer analysis was performed on FACSCalibur (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

Immunofluorescent staining and flow cytometry technique. Immunophenotypic profiles of peripheral blood regulatory T lymphocyte subsets were analysed using human regulatory T cell staining kit (eBioscience), which contains CD4 (FITC), CD25 (APC) and Foxp3 (PE). Samples were prepared according to the manufacturer's recommendations. All blood samples had adequate isotypic controls. PBL were gated on the basis of forward and side scatter. A minimum of 10^4 cells was analysed usinf flow cytometer FACSCalibur (Becton Dickinson). Thresholds for positive staining were set at <2% using the negative control, and percentages of positive cells were obtained by subtracting the value of the control.

Statistical analysis. For statistical analysis, we used a personal computer and software package Statistica 12 (Stat.Soft, Inc., Tulsa, OK, USA). The numerical evaluation of the collected data (concentration of MMP2 and MMP9) was carried out, using descriptive statistics to determine the mean values and a measure of deviation (variability), and the absolute or relative frequencies to express categorical data. Normality of distribution was tested using the Kolmogorov–Smirnov test. A parametric test was used if the data distribution is normal, and if the distribution does not follow the normality of nonparametric tests. Of the nonparametric tests, we used the Mann–Whitney test for two independent groups of samples or the Kruskal–Wallis test for multiple samples. Sample size was determined the required strength test and the level of statistical significance. In our research, we use the normal force test in biomedicine and 80% significance level of the usual 5% ($P < 0.05$). In a regression analysis of the data was included more than one independent variable, and we do multivariate regression analysis. In this way, we predicted one criterion with the help of several independent predictors and evaluated which predictor has the highest weight. For a graphical representation of the data, we used MS Excel. To examine the picture view of correlation, we made a point diagram (scatter plot) and the regression line was drawn in Statistics 12.

Results

Demographic data

In Table 1, we can see the distribution of patients by age and sex. Men suffer from atherosclerosis more often than women.

Concomitant diseases

We can notice that the majority of patients had cardiomyopathies (56%). Diabetes mellitus had 30% of patients, while peripheral diseases had 5% and chronical obstructive pulmonary disease had 3%. In our investigated group, 6% were smokers (Fig. 1).

Distribution of the concentration of enzymes MMP-2 and 9

Figure 2 shows a statistically significant augmentation of the distribution of the concentration of enzyme MMP-2 in urine of atherosclerotic patients (with hypercholesterolaemia and triglyceridemia who were treated only in the ambulance of Family Medicine in comparison with healthy controls). Moreover, patients from Department of Cardiovascular Surgery, who were the candidates for surgical procedure due to the large stenosis of the of carotid arteries, have also statistically significant increase in the MMP-2 enzyme concentration, compared to atherosclerotic patients from the ambulance of general medicine and in comparison with voluntary blood donors. The difference was analysed using the Kruskal–Wallis ANOVA test, $P < 0.001$. Individual differences among patients with atherosclerosis and stenosis ($P = 0.009$), patients with atherosclerosis and the control group ($P < 0.001$) and patients with stenosis and the control group ($P < 0.001$) were also determined. There was a statistically significant difference in the MMP-9 enzyme concentration in patients with atherosclerosis, stenosis and the control group (Fig. 3). The difference was also analysed using the Kruskal–Wallis ANOVA test, $P < 0.001$. Individual differences among patients with atherosclerosis and stenosis are also statistically significant

Table 1 Distribution of patients by age and sex.

Age	Male	Female
<50	0	0
51–60	10	0
61–70	24	3
71–80	31	21
81–90	3	8
>90	0	0
Percentage	68	32

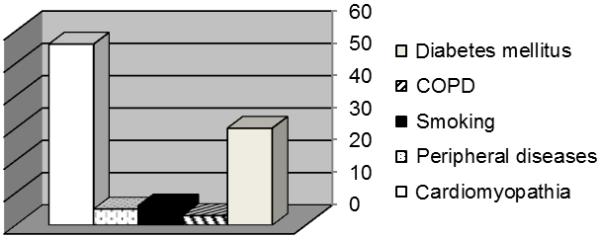

Figure 1 The percentage of concomitant diseases in patients with atherosclerotic changes.

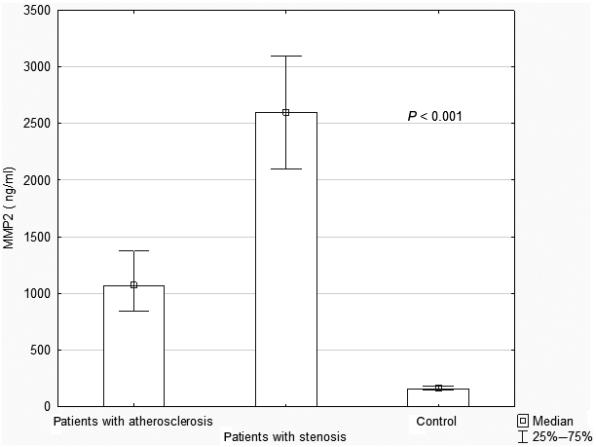

Figure 2 Distribution of the concentration of enzyme MMP-2 in urine (ng/ml) of atherosclerotic patients, patients with carotid artery stenosis and voluntary blood donors. The difference was analysed by using the Kruskal–Wallis ANOVA test, $P < 0.001$. Individual differences among patients with atherosclerosis and stenosis ($P = 0.009$), patients with atherosclerosis and the control group ($P < 0.001$), and patients with stenosis and the control group ($P < 0.001$) were also determined.

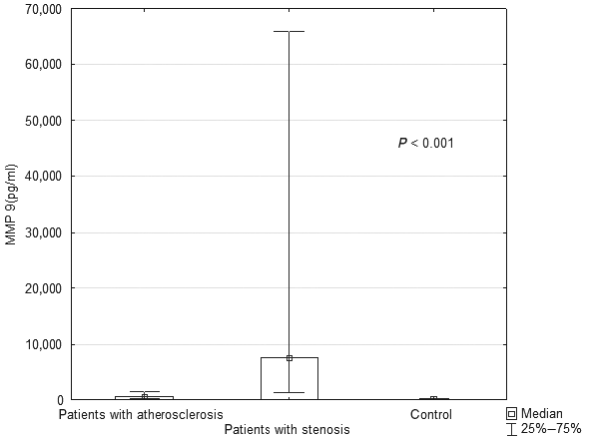

Figure 3 Distribution of the concentration of enzyme MMP-9 in urine (pg/ml) of atherosclerotic patients, patients with carotid artery stenosis and voluntary blood donors. The difference was analysed by using the Kruskal–Wallis ANOVA test, $P < 0.001$. Individual differences among patients with atherosclerosis and stenosis ($P = 0.007$), patients with atherosclerosis and the control group ($P < 0.001$), and patients with stenosis and the control group ($P < 0.001$) were also determined.

($P = 0.007$), as well as the differences between patients with atherosclerosis and the control group ($P < 0.001$) and patients with stenosis and the control group ($P < 0.001$).

The percentage of regulatory T cells (Tregs)

We observed that patients with atherosclerotic changes have statistically significant decreased ($P < 0.001$) the percentage of regulatory T lymphocytes with characteristic phenotype CD4+CD25+Foxp3+ (Fig. 4) in peripheral blood, compared to healthy blood volunteers, which point to the autoimmune etiopathogenesis of atherosclerosis. There was a statistically significant difference in the percentage of regulatory T cells in patients with atherosclerosis, stenosis and the control group. Patients with stenosis have statistically lower percentage of Tregs compared with patients with atherosclerosis ($P = 0.004$), while patients with atherosclerosis have statistically diminished values of Tregs in comparison with the control group ($P < 0.001$), as well as patients with stenosis and the control group ($P < 0.001$). To examine the possible interactions between regulatory immune process and the level of enzymes MMP-2 and 9 in atherosclerotic process, we made correlations (Figs. 5 and 6). There are downregulations of the enzymes level of MMP-2 and Tregs (Fig. 5; $P < 0.004$ and Spearman′s correlation coefficient is negative $r = -0.59$), as well as MMP-9 and Tregs (Fig. 6; $P < 0.005$ with Spearman′s correlation negative coefficient and $r = -0.48$.).

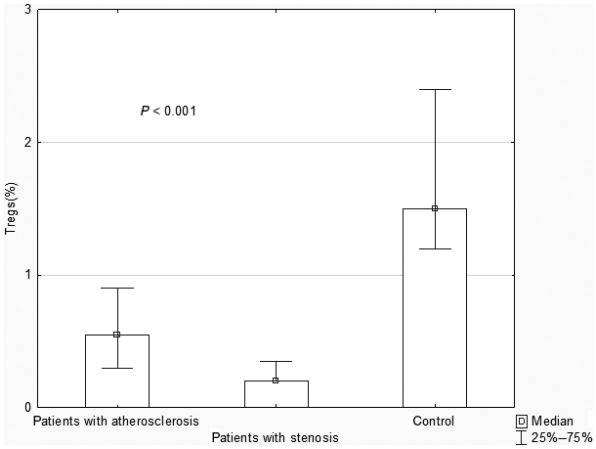

Figure 4 The percentage of regulatory T cells (Tregs: CD4+CD25+ FoxP3+) in peripheral blood mononuclear cells (PBMC) of patients with atherosclerosis and with carotid artery stenosis in comparison with healthy volunteers (level of statistical significance: $P < 0.001$). Individual differences among patients with atherosclerosis and stenosis ($P = 0.004$), patients with atherosclerosis and the control group ($P < 0.001$), and patients with stenosis and the control group ($P < 0.001$) were also determined.

The characteristics of atherosclerotic plague

We have noticed on Table 2 that average values of MMP-9 in urine increased in patients with higher level of carotid artery stenosis and with presents of symptoms. Group of patients with level of carotid artery stenosis between 79 and 99% have the most intensive augmentation of MMP-9 values (average values 3679 pg/ml), while the group of patients with stenosis <60% has the concentration of MMP-9 approximately 859.7 pg/ml. Similar results are connecting to MMP-2, but in this enzyme, this differences were not so strong. In the group of patients with the highest concentration of MMP-9 are also present more

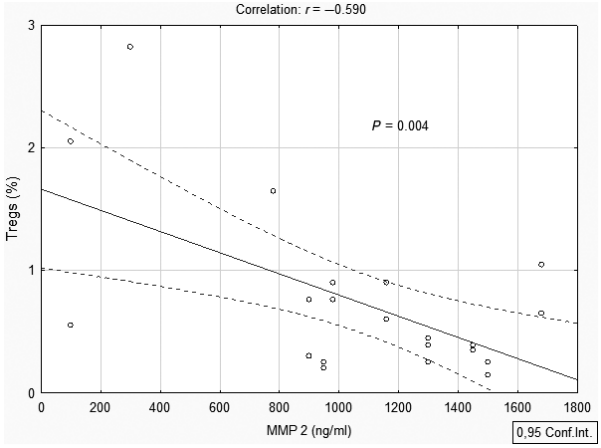

Figure 5 Correlation between the percentage of regulatory T cells (Tregs) in peripheral blood mononuclear cells (PBMC) and the concentration of the enzyme MMP-2 in urine of patients with atherosclerosis. Level of statistical significance $P = 0.004$. Spearman′s correlation coefficient is negative $r = -0.59$.

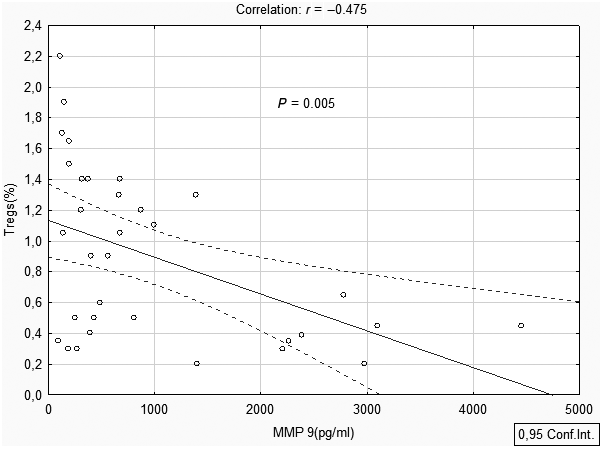

Figure 6 Correlation between the percentage of regulatory T cells (Tregs) in peripheral blood mononuclear cells (PBMC) and the concentration of the enzyme MMP-9 in urine of patients with atherosclerosis. Level of statistical significance $P = 0.005$. Spearman′s correlation coefficient is negative and is $r = -0.48$.

often the complications of atherosclerotic process, like TIA (transitory ischaemic attaché) and stroke (Table 3). Complications (TIA and stroke) of atherosclerotic changes in carotid arteries were enhanced in group with stenosis of 79–99%. Symptoms were absent in group of patients with stenosis <60%, compared to the group with stenosis of 79–99%, where 86% had symptoms.

Discussion

MMPs are involved in the creation of atheroma plaques and remodelling of extracellular matrix and cellular infiltration or migration. Dysregulation of extracellular matrix metabolism contribute to inappropriate vascular remodelling and consequently to the developing of atherosclerosis. Although many studies investigated MMPs family and their role in pathogenesis of atherosclerosis in last decade, there are still many controversies about their action: MMPs participate in all stages of plaque progression, consequently leading to plaque rupture. Matrix metalloproteinases (MMPs) play a key role in the physiology of connective tissue development, in morphogenesis and in wound healing, and their unregulated activity has been implicated in numerous disease processes including arthritis, tumour cell metastasis and atherosclerosis [35, 36]. There are complex network of signals which initiate angiogenesis. Proangiogenic cytokines are accompanied with growth factors (transforming growth factor—TGF β, tumour necrosis factor-α—TNF α, vascular endothelial growth factors—VEGFs, fibroblast growth factors—FGFs, angiopoietins, platelet-derived growth factors—PDGFs, epidermal growth factor (EGF), interleukins, etc.) from inflammatory cells or tumour cells. Some of them may induce angiogenesis by proliferation via binding to their receptors on endothelial cells, or indirectly stimulating local stromal or inflammatory cells [37]. In this study, we have found significant augmentation of enzymes MMP 2 and 9 in urine of patients with atherosclerosis, as well as in patients with CAS. Moreover, these values are higher in patients with CAS than in patients with mild atherosclerosis, which is treated medicamentously in the ambulance of Family Medicine. Unstable atherosclerotic plaque appears to have less vascular smooth muscle (VSM) cells and augmentation of macrophage-derived foam cells. Enhanced activity of MMP contributes to reducing the strength of the fibrous cup and to plaque rupture. On the other hand, some members of MMPs family contribute migration and proliferation of VSM cells, making plaque stable with promoting atherosclerotic plaque cap growth. Recent studies have different findings, but it seems that MMP-3 and 9 have protective roles, inhibiting plaque growth; MMP-7 has no effect on stability of plaque, but can reduce VSM cells in plaque, while MMP-12 is involved in destabilization and rupture of plaque [38]. Although these are controversial data, our findings point out that increased levels of MMP 2 and 9 are accompanied with higher stenosis (Table 2) and more pronounced symptoms of homogeneous and heterogeneous plaques. Simultaneously, with augmentation of MMP 2 and 9 and higher level of stenosis, here are more frequent complications (TIA and stroke) in comparison with patients that have diminished level of MMP 2 and 9 and less level of stenosis. Moreover, MMP concentration in blood was found increased in patients with acute myocardial infarction and unstable angina, as well as after coronary angioplasty, contributing to formation of restenosis lesions. Our data have first shown the increased MMP-2 and 9 concentrations in urine in patients with atherosclerosis and with carotid artery stenosis, which is easy and economic method, underline the crucial role of MMP-2 and 9 in pathogenesis of

Table 2 Average values of MMP-9 in urine, level of carotid artery stenosis and incidence of symptoms (percentage display).

			Homogeneous plaque (%)		Heterogeneous plaque (%)	
Average values of MMP-9 in urine (pg/ml)	Patients (%)	Level of stenosis, %	Symptomatic plaque	Asymptomatic plaque	Symptomatic plaque	Asymptomatic plaque
859.7	4.8	<60	0	0	6.8	0
1987.6	42.8	60–79	12.8	6.8	11.8	0
3679.5	52.4	79–99	17.3	11.9	27.8	4.8
Total	100		30.1	18.7	46.4	4.8

Table 3 Average values of MMP-9 in urine, level of carotid artery stenosis and the most common symptoms (percentage display).

Average values of MMP-9 in urine (pg/ml)	Level of stenosis (%)	TIA (%)	Stroke (%)	Asymptomatic patients (%)
859.7	<60	0	0	0
1987.6	60–79	21	10	3
3679.5	79–99	24	10	14

atherosclerosis. These findings may contribute to better understanding and monitoring of this enzyme, simultaneously opening the possible therapeutic way to prevention the rupture of atherosclerotic plaque and restenosis.

The relationship between the stenosis degree of atherosclerotic alteration of the carotid artery wall and the ultrasound examination of the plaque structure found in symptomatic patients on the one hand, and the expression of the symptoms on the other hand was also the subject of many studies [39]. There was a statistically significant correlation between plaque heterogeneity and incidence of symptoms compared to stenosis and symptoms. Carotid artery plaques were characterized by ultrasound on homogeneous and heterogeneous, and in each of this group we could classified the patients with symptoms or without them. With the augmentation of stenosis, comparatively we noticed the increasing percentage of patients with plaques, accompanied with statistically significant augmentation of MMP-9 levels in urine (Table 2). Furthermore, in the group of patients with the highest stenosis of carotid arteries (79-99%), there are also the most intensive symptoms (TIA and stroke) and this group has the biggest level of MMP-9 in urine (Table 3). It is concluded that plaque materials could be more credible signs of a certain risk of stroke than the stenosis.

However, during tissue injury, there are increased endogenous danger signals, as well as pathogenic infection, and there may be inducers of lesion inflammation, contributing the generating of endogenous anti-angiogenic compounds. There are many investigations whose purpose is to give the evidence for the potential involvement of Tregs in atherosclerosis, to prove their action on plaque destabilization. There are some data that have shown that patients with developed atherosclerosis have reduced plasma concentration of TGF-β and IL-10, which are the products of Tregs [40, 41]. Our results indicate that decreased percentage of Tregs was accompanied with more difficult stage of atherosclerosis. Significant diminished per cent of Tregs are found in blood of patients with CAS compared with patient with mild atherosclerosis ($P = 0.004$), as well as with healthy controls ($P < 0.001$). Also, the statistical decreased concentration is found in patients with mild atherosclerosis versus healthy controls ($P < 0.001$) (Fig. 4). Del Porto F *et al.* [42] have found that the concentrations of Tregs were significantly increased in patients with symptomatic CAS compared with those with asymptomatic stenosis, while Tregs/Th17 ratio was significantly reduced in asymptomatic CAS versus symptomatic CAS. Quite contrary, Liu Zd *et al.* [43] reported that Treg cells, as well as Treg-related cytokines (IL-10 and TGF-β1), and Foxp3 mRNA were diminished in patients with CAS accompanied with unstable plaques in comparison with those patients with stable plaques. Treg cells may have a protective role against the formation of atherosclerotic plaques. Our data have shown statistically significant inverse correlation between the percentage of regulatory T cells (Tregs) with characteristic phenotype CD4+CD25+Foxp3+ in peripheral blood mononuclear cells (PBMC) and the concentration of the enzymes MMP-2 and 9 in urine of patients with atherosclerosis, pointed the importance of these regulatory mechanisms in the etiopathogenesis of atherosclerosis.

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Conflict of interest

The authors declare that they have no conflict of interest. All the authors participated in the investigations and do not have any financial or personal relationships with other

people or organizations that could inappropriately influence this work in order to potential conflicts of interest including employment, stock ownership, consultancies, honoraria or other.

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