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ORIGINAL PAPER

THE ROLE AND RELATIONSHIP OF ENDOTHELIN-1 AND MATRIX METALLOPROTEINASES-2 AND –9 IN THE DESTABILIZATION OF ATHEROMATOUS PLAQUE

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Abstract

Introduction. Matrix metalloproteinases (MMP-2 and MMP-9) have a crucial role in the pathogenesis of atherosclerosis, because they are responsible for the degradation of laminae elastica in the arteries and the formation of aneurysms. The MMP-2 is also liable for cleavage of a big endothelin-1 (ET-1), resulting in the release of the vasoactive ET-1 peptide responsible for vasoconstriction and smooth muscle proliferation, activating the process of athero- and thrombogenesis.

The objective of the study was to determine the distribution of ET-1, MMP-2, and MMP-9 in the vascular wall of abdominal aorta in dead patients, with and without atherosclerotic changes by immunohistochemical staining.

Material and methods. The 24 segments of human abdominal aortas collected at autopsy within five hours of death were used: group 1 (G1) included 20 atherosclerotic segments and group 2 (G2) included 4 non-atherosclerotic segments. G1 was further divided into two sub-groups: G1-A and G1-B. Group G1-A included plaques complicated with calcifications, erosions, hemorrhages, and group G1-B uncomplicated atheromatous plaques.

Résumé

Le rôle et la relation de l'endothéline-1 et des métalloprotéinases matricielles-2 et –9 dans la destructuration de la plaque athéromateuse chez les patients décédés de diverses maladies

Introduction."Les métalloprotéinases matricielles (MMP-2 et MMP-9) ont un rôle crucial dans la pathogenèse de l'athérosclérose car elles sont responsables de la dégradation des laminae elastica dans les artères et de la formation d'anévrismes. La MMP-2 est également responsable du clivage d'une grande endothéline-1 (ET-1), entraînant la libération du peptide vasoactif ET-1 responsable de la vasoconstriction, de la prolifération et de la migration des muscles lisses, activant le processus d'athéro- et la thrombogénèse.

L'objectif de notre étude vise à déterminer la distribution de ET-1, MMP-2 et MMP-9 dans la paroi vasculaire de l'aorte abdominale chez les patients décédés avec et sans modifications athérosclérotiques par coloration immunohistochimique.

Materiel et méthodes. Les 24 segments des aortes abdominales humaines prélevés à l'autopsie dans les 5 heures suivant le décès ont été utilisés : (G1) comprend 20 segments athérosclérotiques et (G2) contient

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Results. The results showed the local overrepresentation of ET-1, MMP-2, and MMP-9 in the atherosclerotic segments. The intensity of staining in G1 was significantly higher in comparison to G2. The increased staining of ET-1, MMP-2, and MMP-9 in macrophages, smooth muscle cells, and endothelial cells correlates with atherosclerosis severity.
Conclusions. Our study showed the active participation of ET-1, MMP-2, and MMP-9 in the pathogenesis of atherosclerosis by triggering a vicious circle responsible for the atherosclerotic plaque's destabilization.

Keywords: atherosclerosis, ET-1, MMP-2, MMP-9, immunohistochemistry.

Abbreviations
ECM: extracellular matrix
MMPs: matrix metalloproteinases
ET-1: endothelin-1
VSMCs: vascular smooth muscle cells
IL: interleukin

Résultats. Nos résultats ont montré la surreprésentation locale d'ET-1, MMP-2 et MMP-9 dans les segments athérosclérotiques. L'intensité de la coloration dans G1 était significativement plus élevée par rapport à G2. La coloration accrue d'ET-1, MMP-2 et MMP-9 dans les macrophages, les cellules musculaires lisses et les cellules endothéliales est en corrélation avec la gravité de l'athérosclérose.
Conclusions. Notre étude a montré la participation active des ET-1, MMP-2 et MMP-9 dans la pathogenèse de l'athérosclérose en déclenchant un cercle vicieux responsable de la déstabilisation de la plaque d'athérosclérose.

Mots-clés: athérosclérose, ET-1, MMP-2, MMP-9, immunohistochimie.

Introduction

Atherosclerosis is a systemic disease associated with lipid metabolic disorders affecting mainly large and medium arteries. It is characterized by the deposition of lipids in the intima of the arteries which leads to the formation of atherosclerotic plaques.Atherosclerosis is the most common cause of death in developing countries, despite various modern methods of treatment. Atherosclerosis is a complex process involving the interplay of genetic and environmental factors and the involvement of multiple cell types. An essential role in the development of atherogenesis is attributable to dyslipidemia, endothelial dysfunction, oxidative stress and inflammation[1]. The development of atherosclerosis includes several phases: the lesion initiation, progression and complication[2]. During the formation of atherosclerotic plaques, circulating monocytes enter the wall of the vascular endothelium and are transformed into macrophages, which by ingestion of modified lipoproteins turn into foam cells, signs of atherosclerotic lesions.

The development of atherosclerosis occurs with the destruction of the extracellular matrix (ECM), deposition of various components, formation of fatty streaks and fibrous plaques. There are three main types of cells involved in the pathogenesis of atherosclerosis: macrophages, smooth muscle cells, and endothelial cells[3]. A reduction in the ECM can result in the erosion and rupture of fibrous plaques, leading to various ischaemic heart disease manifestations. The balance between the cells' function and enzyme activity to degrade the ECM and endogenous inhibitors is an essential factor in determining atherosclerosis progression and plaque stability[4]. Matrix metalloproteinases-2 and 9 (MMP-2 and MMP-9) represent the group of gelatinases that have a similar effect, resulting in collagen type IV degradation. An increased activity of MMP-2 and MMP-9 is associated with the degradation of laminae elastica in the arteries and the formation of aneurysms. Changes in expression of MMP-2 and MMP-9 in the vascular wall, in various pathological processes, have prognostic significance for the course and outcome of disease[5]. Scientific interest in the role and influence of MMPs on the vascular wall and the search for effective drugs to treat their activity remains high[6,7].

Another factor actively involved in the pathogenesis of atherosclerosis is endothelin-1 (ET-1). ET-1 is a vasoactive peptide released by the endothelial and vascular smooth muscle cells (VSMCs). ET-1 leads to vasoconstriction, smooth muscle proliferation, and migration, activating the process of athero- and thrombogenesis. The specific endopeptidase pre-pro-ET-1 is converted to Big-ET-1[8,9]. Hypoxia, haemodynamic vascular stress, angiotensin II, catecholamines, thrombin, thromboxane A2, oxidized lipoproteins, etc., lead to endothelial dysfunction and inflammation of the vascular wall, and are the leading causes of ET-1 secretion[10]. In addition to the widely known vasoconstrictor effect, ET-1 causes vascular fibrosis and stimulates reactive oxygen species production. There are authors claiming that ET-1 induces pro-inflammatory mechanisms by increasing

the expression of tumour necrosis factor-α, interleukin-1 (IL-1), IL-6, and other cytokines[11], which are involved in the destruction of the ECM and together with monocytes are a source for the overproduction of the MMPs[12]. MMP-2 is liable for the cleavage of a big ET-1, resulting in the release of the vasoactive ET-1 peptide[13]. There is a vicious circle for the overproduction of ET-1, MMP-2, and MMP-9, responsible for destabilization of atheromatous plaque.

The objective of the study was to determine the distribution of ET-1, MMP-2, and MMP-9 in the wall of abdominal aorta, in patients with and without atherosclerotic changes, by immunohistochemical staining.

Materials and methods

Tissue specimens

Twenty-four segments of human abdominal aortas from patients (aged 18 to 90 years) with various diseases were collected within 5 hours of death in the Department of Pathology of the University Hospital Pleven, Bulgaria. All resected specimens were fixed in 10% phosphate buffered formalin (pH 7.4) for 24 h. The tissue was dehydrated in graded series of alcohol and embedded in a paraffin block. The samples were divided in two groups in accordance to the American Heart Association[14]: G1 included 20 atherosclerotic segments and G2 included 4 non-atherosclerotic segments. G1 was further divided into two sub-groups: G1-A and G1-B. Group G1-A included complicated plaques with calcifications, erosions, haemorrhages, and group G1-B contained uncomplicated atheromatous plaques. The study was approved by the Research Ethics Committee of Medical University-Pleven. The control carotid arteries have only diffuse thickening of the intima, without inflammatory cellular infiltrate, as assessed by light microscopic examination of haematoxylin-eosin-stained areas.

Immunohistochemistry of ET-1, MMP-2, and MMP-9

The segment of the aortic wall embedded in paraffin was cut and mounted on slides. Three micron-thick sections were deparaffinized, rehydrated in a series of alcohols, and stained with polyclonal antibodies for ET-1(N-8-R, sc-21625-R), MMP-2 (8B4, sc-13595), and MMP-9 (C-20, sc-6840) from Santa Cruz Biotechnology, following the manufacturer's instructions. The visualization was achieved with Dako-EnVision Detection Kit (Dako, USA) and counterstained with Mayer's haematoxylin. The general background was blocked by normal serum from the antibody's host (Jackson ImmunoResearch, UK), and the endogenous enzymes were inactivated with hydrogen peroxide. Immunohistochemical (IHC) analysis of ET-1, MMP-2, and MMP-9 was evaluated semiquantitatively by visual scoring the intensity of the staining. The evaluation was performed by the pathologist involved in a project. We used the following scale for the intensity of the estimated: 0 – no colouring; 1 – weak; 2 – moderate; 3 – strongly[15]. IHC analysis is considered positive if dark brown nuclear staining for ET-1 and MMP-9 and dark purple nuclear staining for MMP-2 are present in the sections. Each sample was subjected to routine histological testings by applying haematoxylin & eosin (H&E) and Van Gieson (VG).

Statistical analysis

For IHC study we used nonparametric test – Mann Whitney, by comparing the median values, with SPSS 20 statistical software. Data were presented as mean ± standard error of the mean (SEM), with $p < 0.05$ as the limit for statistical significance.

Results

Sample characteristics

The control group included four fragments of the abdominal aorta of people who died of non-cardiovascular disease (3 men and one women), aged 18 to 44 years (average age 32 years). Of the 20 atherosclerotic samples, 11 were obtained from males and 9 from females. Their age ranged from 38 to 90 years (median age, 70 years), Figure 1.

Plaques from 24 autopsied patients were analysed. The baseline demographic features are listed in Table 1. The age and risk factors did not differ significantly between the groups.

Histological results

According to histological criteria, atherosclerotic lesions are considered advanced if the accumulation of lipids, cells, and matrix components, including minerals, leads to structural disorganization and deformation of the arterial wall. Lesions considered advanced do not always narrow the arterial lumen and therefore may not always be visible on angiography, but such lesions may be clinically significant, as complications may develop suddenly. Clinical symptoms and mortality from atherosclerosis are often the result of disruptions on the lesions surface, haematoma or haemorrhage and thrombotic deposits. In this case, these violations can be classified as complicated lesions. Complicating features may be due to differences in risk factors, tissue reactions and changes in the activity of some enzymes, for example metalloproteinases[14]. Fig. 2 (A, B, C, D) illustrates vessels without atherosclerosis (G2) and different microscopic degrees of abdominal aortic atherosclerotic damage (G1).

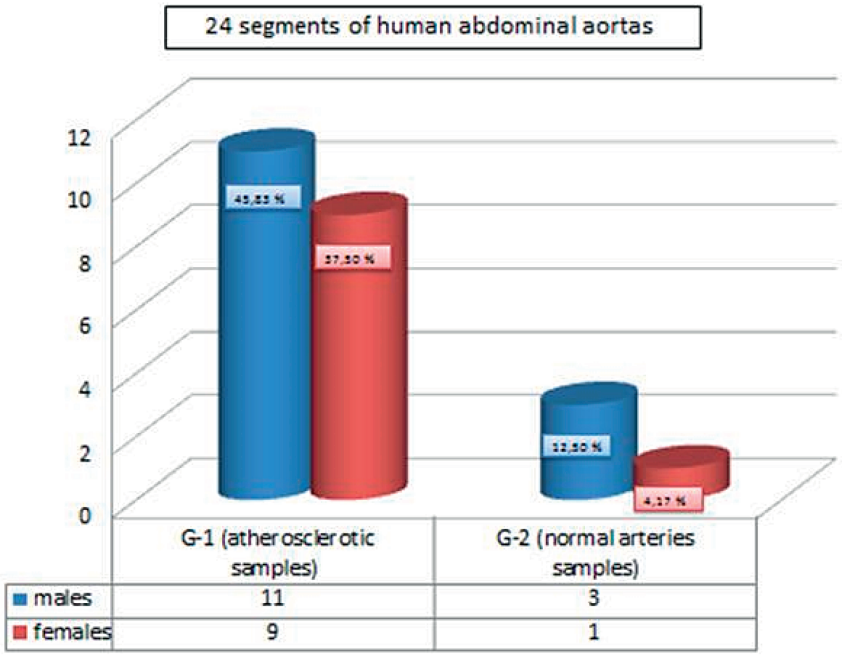


Fig. 1. Distribution of patients in groups G1 and G2, by sex and atherosclerotic changes.

Table 1. Baseline characteristics of the subjects. Data are n (%) values except where stated otherwise

Characteristic	*N (%)*
Age (years; mean±SD)	67.9±2.7
Sex (*n*, male/female)	14/10
Cause of death	
• *Gastrointestinal causes: ileus, pancreatitis, ulcer, cholecystitis, cirrhosis*	8 (33.33)
• *Myocardial infarction*	6 (25.00)
• *Hypothermia*	2 (8.33)
• *Pulmonary pathology: chronic obstructive pulmonary disease, pneumonia*	6 (25.00)
• *Trauma*	2 (8.33)
Diabetes	3 (12.50)
Hyperlipidaemia	12 (50.00)
Smoking	9 (37.50)
Macroscopic:	
• *Plaque ulceration*	7 (29.17)
Microscopic:	
• *Rupture*	2 (8.33)
• *Thromus*	5 (20.83)
• *Cap thinning*	7 (29.17)
• *Large lipid core*	8 (33.33)
• *Calcification*	13 (54.17)

Immunohistochemical results

Staining of ET-1, MMP-2 and MMP-9 in atherosclerotic altered vessels G1A and G1B was immunohistochemically examined and compared to control group G2.

Immunohistochemical staining of the ET-1

Endothelial cells, macrophages and VSMCs demonstrate the staining for ET-1 (Fig. 3). Their immunoreactivity was greater in atherosclerotic altered vessels in group G1-A compared to the control group G2 ($p<0.05$), and in group G1-B compared to the control group G2 ($p<0.05$). There was also a significant difference in ET-1 expression between GI-A and GI-B, with the staining intensity being significantly higher in G1A than in G1B ($p<0.05$). Table 2 shows the quantitative assessment of ET-1 expression in the three groups: G1-A, G1-B and G2. Figure 3 shows IHC staining of ET-1 in GI-A, GI-B, and GII groups.

A variable immune response to ET-1 (moderate to strong) has been shown in VSMCs. IHC analysis is considered positive in the presence of dark nuclear brown staining (arrow).

Immunohistochemical staining of the MMP-2

Endothelial cells, macrophages and VSMCs demonstrate the staining for MMP-2 (Fig. 4). Their immunoreactivity was greater in atherosclerotic altered vessels in group G1-A compared to the control group G2 ($p<0.05$) and in group G1-B compared to the control group G2. There was also a significant difference in MMP-2 expression between GI-A and GI-B, with the staining intensity being significantly higher in G1A than in G1B ($p<0.05$). Figure 4 shows IHC staining of MMP-2 in GI-A, GI-B, and GII groups.

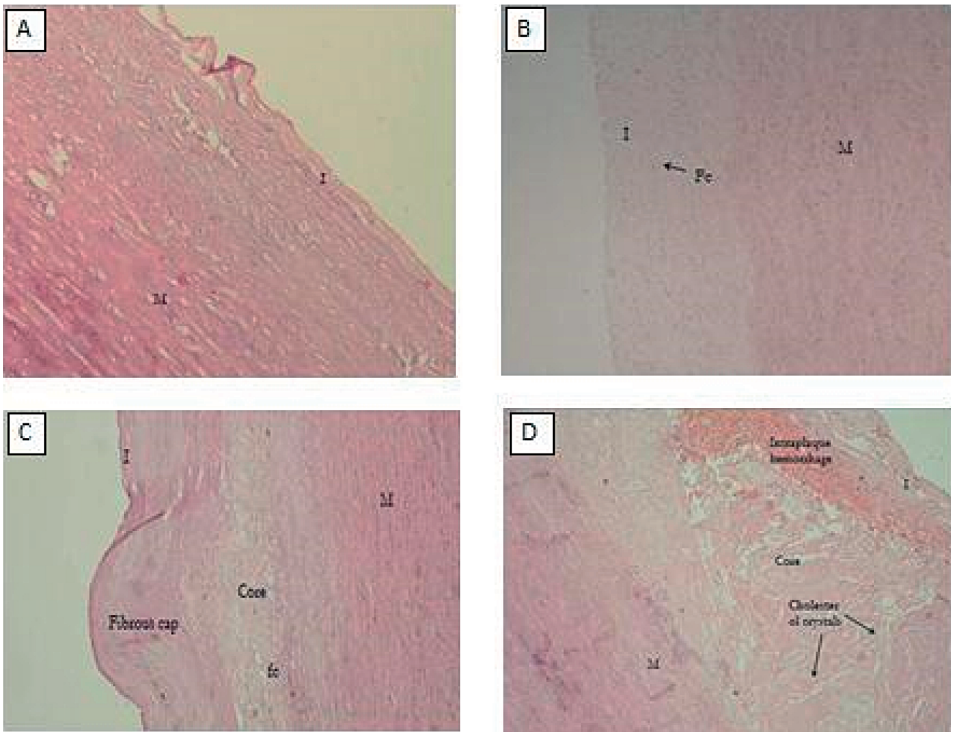


Fig. 2 A. Segment of the abdominal aorta without atherosclerosis. I-intima; M-media. HE x 200. **B.** Segment of the abdominal aorta. Mild atherosclerosis with thickened intima, lipid spot and presence of foam cells (fc). I-indicates intima; M- media. HE x 100. **C.** Atherosclerosis on the abdominal aorta with advanced lesions. Extracellular lipid forms a confluent core in the musculoelastic layer. Foam cells (fc) overlie core. Uncomplicated atheromatous plaques. I-indicates intima; M-media. HE x 400. **D.** Complicated plaque on the abdominal aortic with calcification. The lipid core also contains cholesterol crystals (arrows). Macrophage foam cells (fc) overlie core. I- indicates intima; M, media. HE x 400.

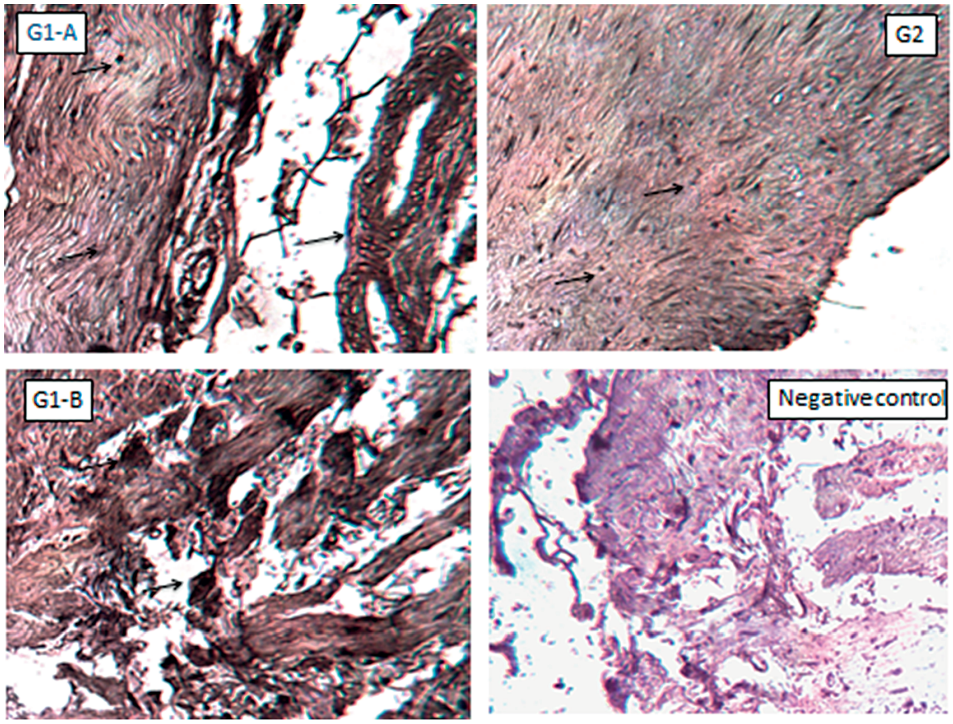


Fig. 3. IHC nuclear staining of ET-1 in preparations of human abdominal aortas.
G1-A: the group with complicated atherosclerosis: strong staining of ET-1 (3) in the VSMCs of the intimal and medial layers. Magnification x 400. **G1-B**: group with noncomplicated atherosclerosis: moderate staining of ET-1 (2) in the VSMCs of intimal and medial layers. Magnification x 400. **G2**: control group: weak staining of ET-1 (1) in the VSMCs of the medial layers aortic rings. Magnification x 400.

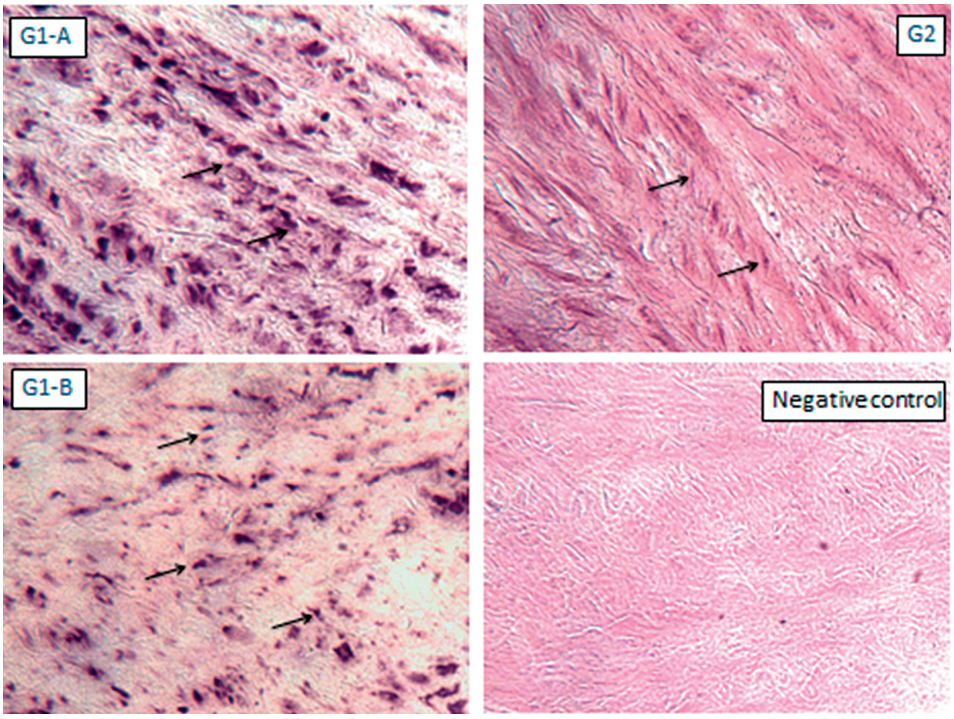


Fig. 4. IHC nuclear staining of MMP-2 in preparations of human abdominal aortas. A variable immune response to MMP-2 (moderate to strong) has been shown in VSMCs. IHCl analysis is considered positive in the presence of dark purple nuclear staining (arrow). **G1-A:** group with complicated atherosclerosis: strong staining of MMP-2 (3) was observed in the medial VSMCs. Magnification x 400. **G1-B:** group with noncomplicated atherosclerosis: moderate staining of MMP-2 (2) was observed in the medial VSMCs. Magnification x 400. **G2:** control group: weak staining of MMP-2 (1) in medial VSMCs. Magnification x 400.

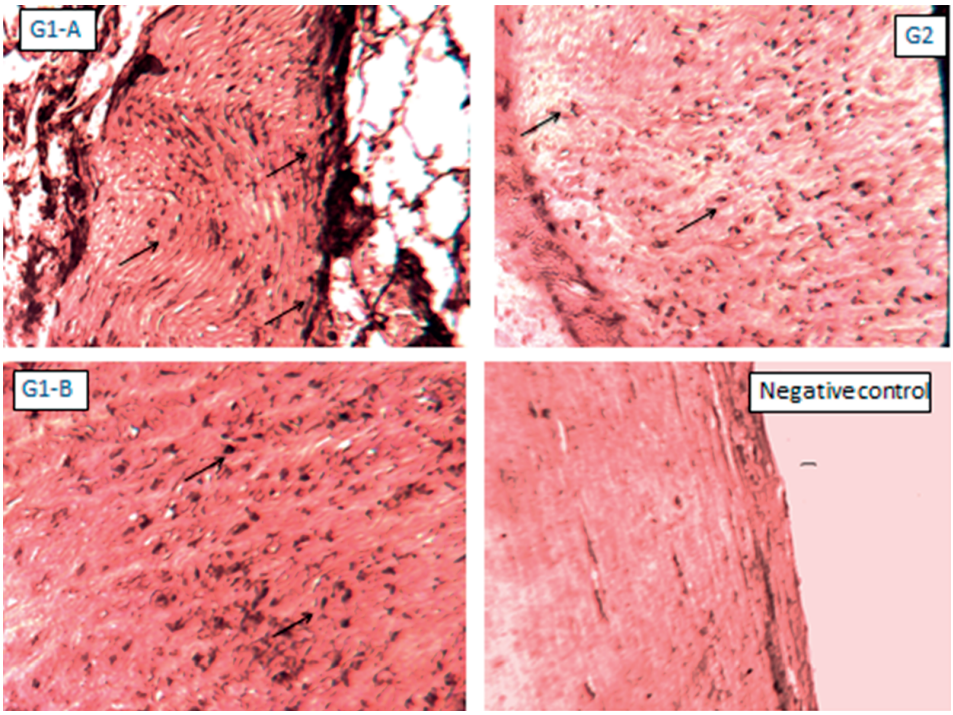


Fig. 5. IHC nuclear staining of MMP-9 in preparations of human abdominal aortas. A variable immune response to MMP-9 (moderate to strong) has been shown in VSMCs. IHC analysis is considered positive in the presence of dark nuclear brown staining (arrow). **G1-A:** group with complicated atherosclerosis: strong staining of MMPs-9 (3) in VSMCs in medial layers. Magnification x 400. **G1-B:** group with noncomplicated atherosclerosis: moderate staining of MMPs-9 (2) in VSMCs in medial layers. Magnification x 400. **G2:** control group: weak staining of MMP-9 (1) in medial VSMCs. Magnification x 400.

Immunohistochemical staining of the MMP-9

IHC staining of MMP-9 provided information about the localization and cellular origin of the protease. The immunostaining revealed that macrophages and VSMCs demonstrate the staining for MMP-9 (Fig. 5). Their immunoreactivity was greater in atherosclerotic altered vessels around inflammatory foci in group G1-A compared to the control group G2 ($p<0.05$) and in group G1-B compared to the control group G2 ($p<0.05$). There was also a significant difference in MMP-9 expression between GI-A and GI-B, with the staining intensity being significantly higher in G1A than G1B ($p<0.01$). Figure 5 shows IHC staining of MMP-9 in GI-A, GI-B, and GII groups.

Discussion

Inflammation plays a key role in atherogenesis, mediating all stages of the disease, from onset to progression and complications. Elevated markers of inflammation predict outcomes in patients with acute coronary syndromes. The presence of chronic inflammation, evidenced by high levels of C-reactive protein, determines the risk of atherosclerotic complications. In addition, some treatments that reduce coronary risk also reduce inflammation. Inflammation in atherosclerosis has practical clinical applications for risk prevention and targeted therapy. In a global perspective, atherosclerosis can be considered a form of chronic inflammation resulting from the interaction between modified lipoproteins, monocyte macrophages, T cells and normal cellular elements of the arterial wall. Inflammatory process eventually leads to the development of complex lesions or plaques entering the arterial lumen. Plaque rupture and thrombosis lead to acute complications such as myocardial infarction and stroke[16].

The assessment of inflammatory processes in the development of atherosclerosis by examining specific markers involved in plaque destabilization remains important. ET-1, MMP-2 and MMP-9 are abundantly present in human arteries and in different stages of atherosclerotic plaque evolution[17,18]. In healthy individuals, ET-1 is weak expressed by endothelial cells[19], MMP-2 and MMP-9 are weak expressed mainly by VSMCs[20]. In coronary vessels with atherosclerotic alterations ET-1 and MMPs-2, 9 are present in the endothelium, subendothelium and intima of the vessel and are expressed by a variety of cells, but mostly by VSMCs (which actively proliferate and migrate into subendothelial space) and macrophages[21]. ET-1 and MMPs-2, 9 are markers of chronic inflammation and are present in very early stages of plaque development[17,18]. Our study showed that ET-1 and MMPs –2, 9 staining are increased in atherosclerotically damaged vessels compared to normal vessels. The increased staining intensity of ET-1 in macrophages, VSMCs and endothelium cells correlates with MMPs – 2, 9 staining and the severity of atherosclerosis. Studies showed that ET-1, apart from vasoconstrictor activity, has a chemotactic effect on monocytes, neutrophils and it participates in platelet activation[22]. ET-1 is also involved in the overproduction of MMPs – 2, 9, being generated by the same inflammatory cells[20,23]. This explains and demonstrates the active participation of ET-1 and MMPs –2, 9 in the pathogenesis of atherosclerosis.

Conclusions

In conclusion, the increased immunostaining of ET-1, MMPs-2 and –9 in atheromatous plaques of abdominal aorta confirms that they are leading factors for destabilization of atheromatous plaque. A vicious circle is formed from their generation through interaction with proinflammatory cytokines.

Author Contributions:

Conceptualization, A.V.G.; methodology, T.M.B.; software, A.V.G.; validation, T.M.B.; formal analysis, A.A.D. and A.V.G.; investigation, T.M.B.; resources, T.M.B., A.A.D. and A.V.G.; data curation, A.A.D.; writing original draft preparation, A.V.G.; writing review and editing, T.M.B., and A.A.D.; visualization, A.V.G.; supervision, A.A.D.; project administration, A.V.G. All the authors have read and agreed with the final version of the article.

Compliance with Ethics Requirements:

"The authors declare no conflict of interest regarding this article"

"The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law."

This study was approved by the Scientific Ethics Committee of MU-Pleven, Bulgaria (approval № 223).

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