



The expression of renin-angiotensin system components in human carotid plaque

Ekspresija komponenti renin-angiotenzin sistema u humanom karotidnom plaku

Ana Kolaković*, Maja Bundalo*, Tamara Djurić*, Igor Končar^{†‡},
Aleksandra Stanković*, Maja Živković*

University of Belgrade, *Institute of Nuclear Sciences “Vinča”, Department of Health and Environmental Research, Laboratory for Radiobiology and Molecular Genetics,
[†]Faculty of Medicine, Belgrade, Serbia; [‡]University Clinical Center of Serbia, Clinic for Vascular and Endovascular Surgery, Belgrade, Serbia

Abstract

Background/Aim. The renin-angiotensin system (RAS) is linked to the development of atherosclerosis (As), including its initiation and progression. Besides the well-known angiotensin-converting enzyme (ACE), two newer RAS family members are related to vascular remodeling – ACE2 as a homolog of ACE and collectrin [transmembrane protein 27 (TMEM27)] as a homolog of ACE2. Up to now, a limited number of studies have examined the expression of these RAS components in advanced carotid plaque (CP) tissue based on the sex of the patients and plaque phenotypes (PPs). There are two ultrasonographically defined PPs – the hypoechogenic plaque (HoP) and the hyperechogenic plaque (HerP) phenotype. The aim of the study was to investigate whether there was a correlation between the expression of RAS components in the CP and the sex and PPs of patients. **Methods.** We examined 74 patients with advanced CP who underwent carotid endarterectomy. The intraplaque expression of RAS components was determined with the real-time polymerase chain reaction, using the TaqMan[®] gene expression assays and Western blot. A two-way ANOVA followed by a *post-hoc* Tukey test was performed for the statistical analysis of results. **Results.** No interaction was recorded between

the sex of the patients and PPs in influencing the relative expression of ACE and TMEM27 messenger RNA (mRNA) ($p > 0.05$). In 56.06% of plaque samples, no expression of ACE2 mRNA was detected. Among the plaques where ACE2 mRNA expression was detected, its expression level was higher in females with the HoP phenotype compared to females with the HerP phenotype ($p < 0.001$). In patients with the HoP phenotype, females had higher expression of ACE2 mRNA than males ($p < 0.05$). In the male study group, ACE protein levels were significantly lower in the HoP phenotype compared to the HerP phenotype ($p < 0.001$). Females with the HoP phenotype had significantly higher ACE protein levels than males with the HoP phenotype ($p < 0.0001$). **Conclusion.** Our results revealed alterations in the expression levels of ACE and ACE2, at the mRNA and protein levels, in advanced carotid As. These alterations are impacted by sex and PP and may indicate a switch from the balanced RAS/ACE/ACE2 axis in the healthy blood vessel to the unbalanced axis in vascular remodeling due to As.

Key words:

carotid artery diseases; gene expression; plaque, atherosclerotic; proteins; renin angiotensin system; rna, messenger.

Apstrakt

Uvod/Cilj. Renin-angiotenzin sistem (RAS) povezan je sa razvojem ateroskleroze (As), uključujući njen nastanak i progresiju. Pored dobro poznatog angiotenzin-konvertujućeg enzima (*angiotensin-converting enzyme* – ACE), dva nova člana RAS familije povezana su sa remodelovanjem zidova krvnih sudova – ACE2 kao homolog ACE i kolektrin [*transmembrane protein 27* (TMEM27)] kao homolog ACE2. Do sada je mali broj studija ispitivao ekspresiju komponenti RAS sistema u tkivu uznapredovalog karotidnog plaka (KP) u odnosu na pol

bolesnika i fenotip plaka (FP). Postoje dva tipa KP definisana primenom ultrazvuka – fenotip hipoechogenog plaka (HoP) i fenotip hiperechogenog plaka (HerP). Cilj rada bio je da se ispita da li postoji korelacija između ekspresije komponenti RAS u KP i pola i FP bolesnika. **Metode.** Ispitano je 74 bolesnika sa uznapredovalim KP koji su bili podvrgnuti operativnoj proceduri karotidne endarterektomije. Ekspresija komponenti RAS sistema u tkivu plaka utvrđena je lančanom reakcijom polimeraze u realnom vremenu (*real-time polymerase chain reaction*) primenom eseja TaqMan[®] tehnologije i metode *Western blot*-a. Dvosmerna analiza varijanse i Tukey *post-hoc* test korišćeni

su za statističku obradu rezultata. **Rezultati.** Nije utvrđena interakcija FP i pola bolesnika u uticaju na relativnu ekspresiju informacione RNK (iRNK) za ACE i TMEM27 ($p > 0,05$). U 56,06% uzoraka plaka nije detektovana ekspresija iRNK za ACE2. U plakovima u kojima je detektovana ekspresija iRNK za ACE2, njen nivo bio je viši kod žena sa HoP u poređenju sa ženama sa HerP ($p < 0,001$). U grupi bolesnika sa fenotipom HoP, žene su imale viši nivo iRNK za ACE2 nego muškarci ($p < 0,05$). U grupi muškaraca, nivoi ekspresije ACE proteina bili su značajno niži u fenotipu HoP u poređenju sa HerP ($p < 0,001$). Žene sa fenotipom HoP imale su značajno viši nivo ACE

proteina u poređenju sa muškarcima sa HoP ($p < 0,0001$). **Zaključak.** Naši rezultati pokazali su da postoje promene nivoa ekspresije ACE i ACE2, na nivou proteina i iRNK u uznapređovanoj karotidnoj As. Te promene zavise od pola i FP i mogu ukazivati na to da balans ose RAS/ACE/ACE2 koji postoji u zdravom krvnom sudu postaje poremećen tokom remodelovanja zida krvnog suda usled As.

Ključne reči:

aa.carotis, bolesti; geni, ekspresija; aterosklerotički plak; proteini; renin-angiotenzin sistem; rnk, informaciona.

Introduction

Atherosclerosis (As) is a chronic inflammatory disease of the arterial wall characterized by the development of atherosclerotic lesions (plaques) that can interrupt the blood flow in vessels but also be vulnerable to rupture¹. The characterization of carotid plaque (CP) morphology by non-invasive ultrasound (US) may provide insight into the composition of the plaque and its structure². Dominantly echolucent, hypoechogenic plaques (HoPs) of the carotid artery (CA), which are lipid-rich with increased macrophage density³, may confer a higher risk for clinical complications (ischemic events) compared to dominantly echogenic plaques (hyperechogenic plaques – HerP) of the CA which are rich in fibrous tissue and sometimes calcification⁴. Recently, it has been shown that plaques stratified by their echogenicity, as determined by the US, have molecular signatures attributed to iron homeostasis, calcification, the balance of cell survival, and lipid transdifferentiation of the smooth muscle cell (SMC)⁵.

The renin-angiotensin system (RAS) has a significant role in the regulation of numerous physiological functions, including blood pressure (BP), electrolyte balance, inflammation, oxidative stress, fibrosis, and cell proliferation. Therefore, RAS is integrally associated with the progression of As, remodeling of blood vessel walls, and plaque stability⁶. The RAS expression in various tissues highlights the importance of this system in tissues in which angiotensin (Ang) II, as the key active peptide, could be generated locally⁷. The discovery of Ang-converting enzyme (ACE) 2, a homolog of ACE^{8, 9}, demonstrated that the RAS functions through two distinct routes with opposing effects: the traditional ACE/Ang II/Ang III type1 receptor (AT1R) pathway and the novel ACE2/Ang(1-7)/mitochondrial assembly (Mas) receptor 1 (MasR1) axis pathway. ACE2 is known as an endogenous antagonistic regulator of the RAS, which inhibits detrimental Ang II signaling. It reduces BP by hydrolyzing Ang II into the vasodilator Ang(1-7) and has anti-inflammatory effects on the cardiovascular system¹⁰.

Deletion of ACE2 in As-prone mice and apoE-deficient (ApoEKO) mice promotes upregulation of pro-inflammatory mediators of As: monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)- α , interleukin (IL)-6, vascular cell

adhesion molecule (VCAM)-1, matrix metalloproteinase (MMP)-9 and MMP-2 in aorta/artery tissues^{11, 12}. On the other hand, overexpression of ACE2 in other animal models of As either decreases the level of Ang II, ACE, and AT1R protein in atherosclerotic lesions¹³ or prevents early atherosclerotic lesion formation¹⁴ and increases plaque stability¹⁵.

ACE is expressed in multiple tissues¹⁶, various cells in blood vessels, and human atheroma¹⁷, whereas its homolog, ACE2, is expressed in endothelium, macrophage foam cells, and vascular SMC¹⁸. The development of As is delayed by ACE2 activation¹⁸, and adverse vascular remodeling is prevented¹⁹.

Collectrin [also known as transmembrane protein 27 (TMEM27)], a homolog of ACE2, is another new molecule that belongs to the RAS family that has a role in endothelial dysfunction, as shown in collectrin mice²⁰. TMEM27 regulates arterial BP²⁰ and is expressed in the vascular endothelium and kidney²¹.

Previously, the variation in the expression of some RAS family members, e.g., the MasR1, in human CP tissue was related to different PPs²². Moreover, treatment with the oral formulation of Ang(1-7) enhances a more stable phenotype in atherosclerotic CP²², which is important as it is known that ACE2 is the main enzyme in Ang(1-7) synthesis. ACE2 messenger RNA (mRNA) is expressed in both early and advanced human carotid atherosclerotic lesions in another study, suggesting no significant differences according to the progression of As¹⁸. However, the ACE2 expression was not detected in some studies^{17, 22}, and thus, it requires further research. ACE expression was localized in atherosclerotic CA^{23, 24}, and other atheromas¹⁷ but not related to CA phenotype²². The TMEM27 was detected previously in carotid tissue in our preliminary study²⁴.

Sex and gender play an important role in cardiovascular research when addressing disease prevalence, risk factors, diagnostic evaluation, and overall health outcomes²⁵. Recently, sex differences in atherosclerotic PPs have been described (lipid-rich plaques in males, fibrous plaques in females)²⁶. RAS activity is also modulated by androgens and estrogens and reveals sex-specific cardiovascular pathologies^{27, 28}. Sex differences in ACE activation have been suggested^{28, 29}. Likewise, sex differences in ACE and ACE2 modulation of Ang(1-7) levels in males and females have been suggested^{30, 31}. This implies that men and women should be studied separately by sex in cardiovascular re-

search, especially in research on ACE and ACE2 expression in CP.

To our knowledge, there are no studies that investigated the expression of RAS components in advanced CP tissue depending on sex. Therefore, the goal of this study was to compare the expression of RAS family members (ACE, ACE2, and TMEM27) in advanced CP tissue regarding sex and different PPs (HerP/HoP).

Methods

Carotid atherosclerotic tissue specimens and ultrasound evaluation of the carotid artery

In the current investigation, we tested CP tissue specimens ($n = 200$) consecutively collected from patients who underwent carotid endarterectomy (CEA). The US evaluation of the CAs included the bifurcations as well as the common CA and internal CA. The North American Symptomatic Carotid Endarterectomy Trial method was used to measure the degree of stenosis, which was greater than 70%, and define the presence of plaque in the CAs³². According to Gray-Weale et al.³³, we have classified advanced CP tissue as HerP (dominantly echogenic) and HoP (dominantly echolucent) phenotypes. Patients' tissue samples were collected two weeks after the US examination. Tissue samples were immediately frozen in liquid nitrogen after being obtained from the patient and were kept at -80°C . In this study, 74 tissues from patients not receiving ACE inhibitors or Ang receptor blockers antihypertensive treatment underwent further processing for RNA and protein extraction.

The Ethics Committee of the University Clinical Center of Serbia evaluated and approved the research protocol (No. 136/8, from July 21, 2016). Participants who took part in the study gave their informed consent.

Measurement of biochemical parameters

After being admitted for the planned CEA, biochemical analyses were performed via the hospital's routine laboratory protocol for all patients, as previously described³⁴.

Reverse transcription and quantitative real-time PCR

As previously reported, RNA was extracted, its amount and structural integrity were assessed, and reverse transcription was carried out²⁸. After RNA quality assessment, only specimens with high RNA quality ($n = 66$) were converted to complementary DNA (cDNA).

For detection and quantification of ACE, ACE2, and TMEM27, TaqMan® Gene Expression assays were utilized, as follows: Hs00174179_m1, Hs00222343_m, and Hs00252907_m1, respectively (Applied Biosystems, Foster City, CA). The relative mRNA levels of ACE, ACE2, and TMEM27 were normalized to 18s rRNA (Hs99999901 s1), which was used as an endogenous control. All reactions were performed on a Real-time 7500 system according to recommended protocols (ABI, Foster City, CA).

Extraction of proteins and Western blot

We employed the techniques previously described in detail²⁸ to prepare tissue lysates from carotid tissue specimens ($n = 20$), determine the protein concentration, and prepare samples for Western blot. Primary antibodies (sc-20791 and sc-20998, Santa Cruz Biotechnology, USA, dilution 1:200) were incubated with polyvinylidene difluoride membranes to detect the presence of ACE and ACE2 proteins, respectively. Membranes were then washed and incubated with an appropriate secondary anti-rabbit antibody (dilution 1:10,000). The membranes were stained with Ponceau S (Sigma-Aldrich P3504) as a loading control for the Western blot. This reversible Ponceau S staining has been proven as the adequate methodology for assessing equal gel loading in Western blot³⁵. Using ImageJ software (NIH, Bethesda, USA), signals produced by enhanced chemiluminescence reagents on films and signals obtained on membranes were scanned, and protein levels were quantified afterward.

Statistical analysis

Statistica Version 8.0 software (Stat Soft Inc., Tulsa, Oklahoma) and GraphPadPrism Version 6.0 software (GraphPad Software Inc., San Diego, CA), were used for statistical analysis. Unless otherwise noted, the results for continuous variables were reported as means with standard error of the mean. For all continuous variables, the Kolmogorov-Smirnov test was used to check for a normal distribution. Unpaired Student's t -test was used to compare mean values for continuous variables with normal distribution, while the Mann-Whitney U test was used for variables with skewed mean value distribution. The Chi-square test was applied for categorical variables. To ascertain whether the sex and PPs, as well as their interactions, alter the expression of RAS components, we performed a two-way ANOVA for multiple comparisons followed by the Tuckey *post-hoc* test. Differences with a two-tailed alpha-probability p -value of 0.05 or less were considered statistically significant. Using $2^{-\Delta\text{CT}}$ ³⁶, the relative gene expression levels of the ACE, ACE2, and TMEM27 genes were calculated and normalized to 18s rRNA.

Results

Patients with carotid atherosclerosis

Table 1 shows the demographic, biochemical, and clinical parameters with regard to the classification of US-defined PPs (HerP vs. HoP). Biochemical parameters in the blood that were measured, including inflammatory and hemostasis mediators (IL-6, C-reactive protein, plasminogen, fibrinogen, plasminogen activator inhibitor-1, factor VII, antithrombin III, von Willebrand factor, and protein C), did not differ between the two different kinds of PPs. Age, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, total cholesterol levels, lipoprotein(a), white blood cell counts, red blood cell counts, and platelet counts were similar between the two groups with different PPs.

Table 1

Demographic, biochemical, and clinical parameters of patients regarding ultrasonographically defined plaque phenotypes

Parameters	Plaque phenotype		p-value
	HerP (n = 49)	HoP (n = 17)	
Age (years)	63.93 ± 8.84	67.82 ± 7.38	0.11•
Sex			
male	32 (65.31)	14 (82.35)	0.22#
female	17 (34.69)	3 (17.65)	
Total cholesterol (mmol/L)	5.63 ± 1.09	5.79 ± 1.54	0.76•
LDLC (mmol/L)	3.47 ± 0.86	3.62 ± 1.13	0.71•
HDLC (mmol/L)	1.26 ± 0.27	1.17 ± 0.29	0.43•
Triglycerides (mmol/L)	1.97 ± 0.99	2.18 ± 0.87	0.91*
Lipoprotein(a) (mg/dL)	35.16 ± 44.63	31.26 ± 47.55	0.88*
Hypertension	43 (87.76)	17 (100.00)	0.17#
Treatment with statins	10 (20.41)	4 (23.53)	0.88#
Antiplatelet therapy	49 (100)	17 (100)	0.99 #
Smoking	43 (87.76)	16 (94.12)	0.58#
Cerebrovascular insult	9 (18.37)	0 (0.00)	0.05#
Transient ischemic attack	7 (14.29)	4 (23.53)	0.40#
Coronary comorbidity	18 (36.73)	9 (52.94)	0.27#
Symptomatic [□]	19 (38.78)	8 (47.06)	0.59#
Diabetes mellitus	10 (20.41)	5 (29.41)	0.47 #
PAOD	13 (26.53)	4 (23.53)	0.77 #
Glucose (mmol/L)	7.31 ± 3.20	6.43 ± 2.45	0.51*
ApoA-I (g/L)	1.81 ± 0.34	1.82 ± 0.38	0.99•
ApoB (g/L)	1.14 ± 0.18	1.27 ± 0.34	0.82*
Factor VII (g/L)	115.42 ± 34.56	102.38 ± 24.62	0.37•
Fibrinogen (g/L)	5.64 ± 1.83	5.84 ± 1.97	0.79•
vWf (IU/dL)	171.50 ± 46.82	150.63 ± 35.73	0.30•
Plazminogen (g/L)	138.88 ± 21.78	136.44 ± 19.72	0.78•
D-dimer (mg/L)	0.19 ± 0.20	0.22 ± 0.17	0.73*
PAI-1 (U/mL)	4.26 ± 1.01	4.22 ± 1.38	0.94•
Antithrombin III (g/L)	106.88 ± 13.16	103.89 ± 16.88	0.62•
Protein C (g/L)	126.35 ± 34.58	118.78 ± 34.38	0.60•
C-reactive protein (mg/L)	5.48 ± 4.55	4.53 ± 3.49	0.93*
Interleukin-6 (pg/mL)	6.07 ± 6.71	3.96 ± 3.43	0.53*
White blood cells (×10 ⁹ /L)	7.83 ± 1.50	8.97 ± 2.69	0.61*
Red blood cells (×10 ¹² /L)	4.49 ± 0.35	4.78 ± 0.27	0.07*
Platelets (×10 ⁹ /L)	285.41 ± 78.37	250.11 ± 62.18	0.25•
TAS (mmol/L)	1.60 ± 0.13	1.62 ± 0.13	0.72•
Superoxide dismutase (IU/L)	1,397.78 ± 161.79	1,430.11 ± 150.30	0.64•
Glutathione peroxidase (IU/L)	46.58 ± 16.27	64.93 ± 27.17	0.05•
Glutathione reductase (IU/L)	57.61 ± 10.93	58.06 ± 10.21	0.92•

HerP – hyperechogenic plaque; HoP – hypoechogenic plaque; LDLC – low-density lipoprotein cholesterol; HDLC – high-density lipoprotein cholesterol; PAOD – peripheral arterial occlusive disease; ApoA-I – apolipoprotein A-I; ApoB – apolipoprotein B; vWf – von Willebrand factor; PAI-1 – plasminogen activator inhibitor-1; TAS – total antioxidative status.

□ Patients who previously had symptoms of ipsilateral stroke or transient ischemic attack of the carotid artery territory.

• Student's t-test; # Pearson Chi-square test; *Mann-Whitney U test.

Results are presented as mean ± standard deviation or numbers (percentages).

We also analyzed mean values of the parameters of oxidative stress in plasma and did not find any difference in these parameters between HerP and HoP (Table 1).

ACE, ACE2, and TMEM27 gene expression in carotid plaque tissues

We examined the *ACE* gene expression in human CP tissue in relation to PPs [HerP phenotypes (n = 49) vs. HoP phenotypes (n = 17)] and sex [males (n = 46) vs. fe-

males (n = 20)]. The *ACE* mRNA level in CP tissue was not significantly different between different US-defined PPs (Figure 1). We also analyzed the gene expression of *ACE* in CP tissue with regard to sex and found no difference. Two-way ANOVA did not reveal a significant effect of sex and PP interaction on *ACE* mRNA expression (*p* = 0.727).

We detected the *ACE2* gene expression in 29 CP samples out of the 66 (43.94%) CP samples. Considering that in 56.06% of samples *ACE2* mRNA was not detected, we checked if the distribution of sex and PPs was different

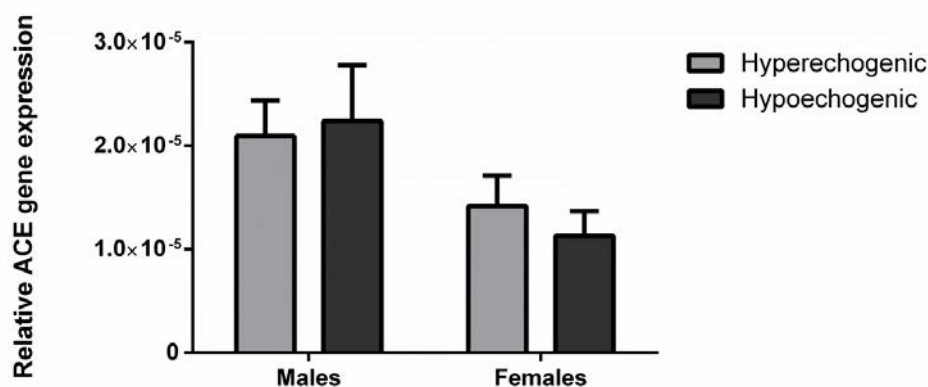


Fig. 1 – Relative angiotensin-converting enzyme (ACE) gene expression in carotid plaque (CP) tissue.

There was no significant difference in ACE gene expression between plaque phenotypes: males – HerP phenotypes (n = 32) vs. HoP phenotypes (n = 14), $p = 0.99$; females – HerP phenotypes (n = 17) vs. HoP phenotypes (n = 3), $p = 0.99$. The cDNA from CP tissue samples was used as a template in RT-qPCR for relative quantification of intraplaque mRNA expression of ACE. For each sample, the expression level of ACE mRNA was normalized to the housekeeping gene for 18S rRNA. The statistical significance was assessed by two-way ANOVA, followed by a *post-hoc* Tukey test. The results are expressed as $2^{-\Delta CT}$ values. Values are presented as mean ± standard error of the mean. For other abbreviations, see Table 1.

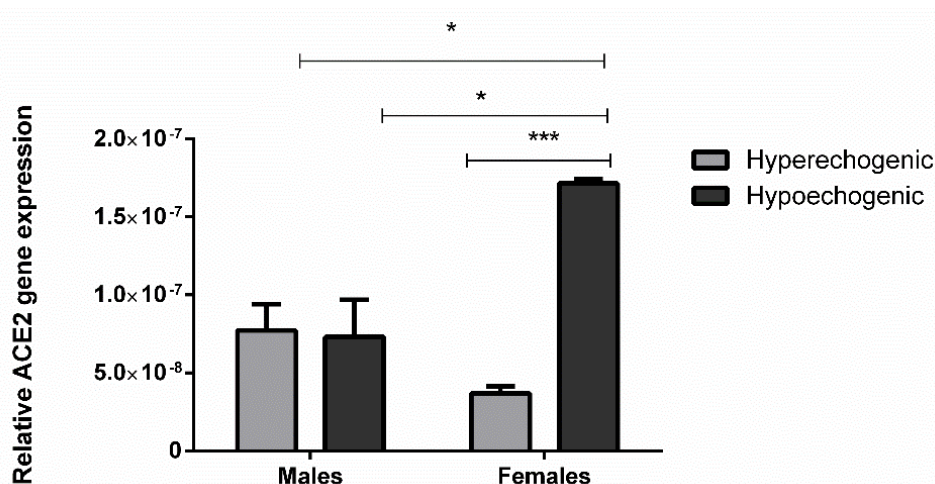


Fig. 2 – Relative angiotensin-converting enzyme (ACE)2 gene expression in carotid plaque tissue.

*** significant difference between females with HoP phenotypes (n = 3) vs. females with HerP phenotypes (n = 9), $p < 0.001$; *significant difference between females with HoP phenotypes (n = 3) vs. males with HoP phenotypes (n = 11) and vs. males with HerP phenotypes (n = 6), $p < 0.05$. The cDNA from CP tissue samples was used as a template in RT-qPCR for relative quantification of intraplaque mRNA expression of ACE2. For each sample, the expression level of ACE2 mRNA was normalized to the housekeeping gene for 18S rRNA. The statistical significance was assessed by two-way ANOVA, followed by a *post-hoc* Tukey test. The results are expressed as $2^{-\Delta CT}$ values. Values are presented as mean ± standard error of the mean. For other abbreviations, see Table 1.

between groups with and without detected expression. We did not find any significant differences in distributions (Chi-square test, $p = 0.63$). Hence, we compared the ACE2 gene expression in HerP phenotypes (n = 20) vs. HoP phenotypes (n = 9) in males (n = 17) and females (n = 12). Significant correlations between sex and PPs and gene expression were found using two-way ANOVA ($p = 0.001$). Females with HoP CP had significantly higher levels of ACE2 gene expression than females with HerP, according to a *post-hoc* analysis (Tuckey *post-hoc* test) ($p = 0.0007$) (Figure 2). Furthermore, we detected significantly higher ACE2 gene ex-

pression in the HoP phenotype of females in comparison to males with the HoP phenotype ($p = 0.02$). There was no statistically significant difference between HerP and HoP CP tissue in the male patient group ($p = 0.99$).

The TMEM27 mRNA expression was not significantly different either with regard to sex or the US-defined PPs (Figure 3). Sex and PPs did not significantly interact, according to two-way ANOVA ($p = 0.61$). We analyzed the TMEM27 gene expression in a total of 65 human CP tissue samples with regard to the PPs – HerP phenotypes (n = 49) vs. HoP phenotypes (n = 16), and sex – males (n = 45) vs. females (n = 20).

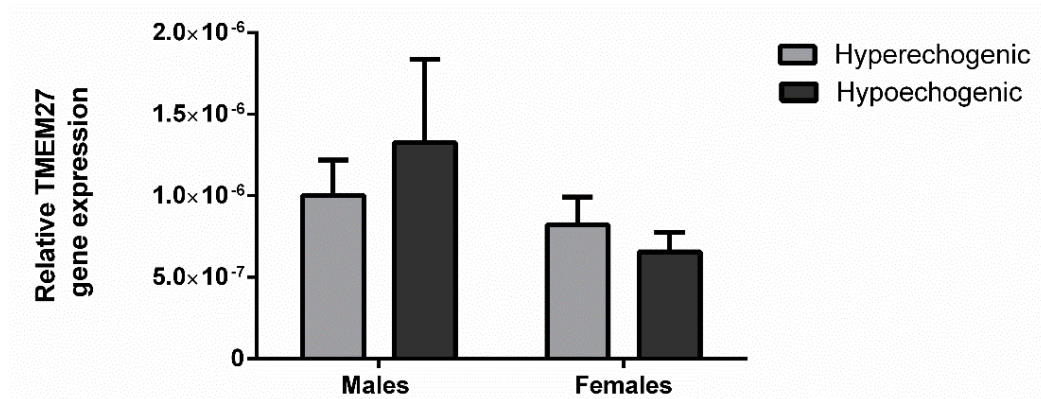


Fig. 3 – Relative transmembrane protein 27 (TMEM27) gene expression in carotid plaque tissue. There was no significant difference in TMEM27 gene expression between plaque phenotypes: males – HerP phenotypes (n = 32) vs. HoP phenotypes (n = 14), $p = 0.86$; females – HerP phenotypes (n = 17) vs. HoP phenotypes (n = 3), $p = 0.99$. The cDNA from CP tissue samples was used as a template in RT-qPCR for relative quantification of intraplaque mRNA expression of TMEM27. For each sample, the expression level of TMEM27 mRNA was normalized to the housekeeping gene for 18S rRNA. The statistical significance was assessed by two-way ANOVA, followed by a *post-hoc* Tukey test. The results are expressed as $2^{-\Delta CT}$ values. Values are presented as mean $\pm$ standard error of the mean. For other abbreviations, see Table 1.

ACE and ACE2 protein expression detected by Western blot

To analyze whether ACE and ACE2 protein levels were altered regarding PPs (HerP vs. HoP) and sex (males vs. females), we performed a Western blot analysis. Two-way ANOVA revealed a significant correlation between sex and PPs and the ACE protein expression ($p < 0.0001$). Significant-

ly higher ACE protein level was found in males with HerP phenotype of CP in comparison to males with HoP phenotypes ($p = 0.0007$, Tukey *post-hoc* test). Furthermore, we detected higher ACE protein levels in females with HoP phenotypes than in males with the same PP ($p < 0.0001$) (Figure 4).

Two-way ANOVA of ACE2 protein levels showed that the effects of sex and PPs were not significant (Figure 5) and also that their interactions were not significant ($p = 0.86$).

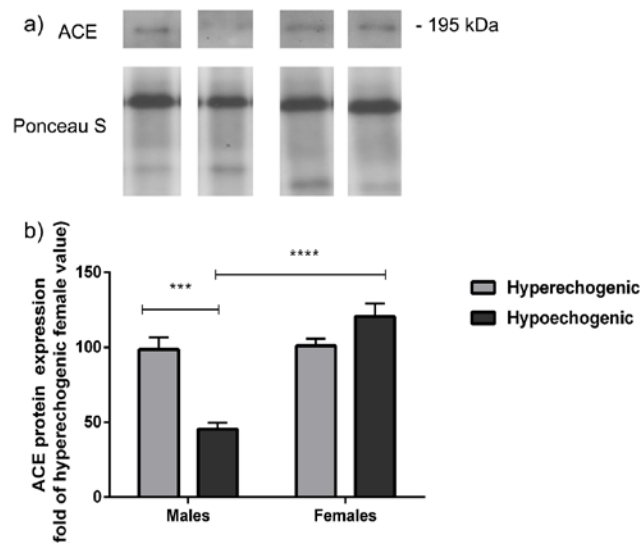


Fig. 4 – Western blot of angiotensin-converting enzyme (ACE) protein expression in carotid plaque tissue: a) representative image of Western blot: band No. 1 – HerP males; band No. 2 – HoP males; band No. 3 – HerP females; band No. 4 – HoP females; b) graphic display of Western blot results.

Ponceau S staining was used as a loading control. *** significant difference between males with HoP phenotypes (n = 4) vs. males with HerP phenotypes (n = 4), $p < 0.001$; **** significant difference between males with HoP phenotypes vs. females with HoP phenotypes (n = 6), $p < 0.0001$. The statistical significance was assessed by two-way ANOVA, followed by a *post-hoc* Tukey test. In all tests, the differences with two-tailed alpha-probability and $*p < 0.05$ were considered statistically significant. Results are presented as mean percent change $\pm$ standard error of the mean.

For abbreviations, see Table 1.

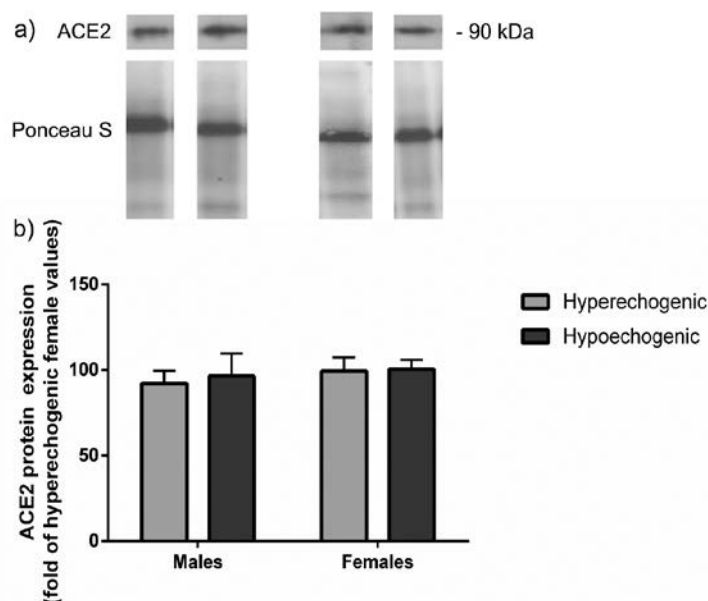


Fig. 5 – Western blot of angiotensin-converting enzyme (ACE)2 protein expression in carotid plaque tissue: a) representative image of Western blot: band No. 1 – HerP males; band No. 2 – HoP males; band No. 3 – HerP females; band No. 4 – HoP females; b) graphic display of Western blot results.

Ponceau S staining was used as a loading control. There is no significant difference in ACE2 protein expression between plaque phenotypes in males or females, $p > 0.05$. The statistical significance was assessed by two-way ANOVA, followed by a post-hoc Tukey test. Results are presented as mean percent change $\pm$ standard error of the mean.

For abbreviations, see Table 1.

Discussion

Ang II plays a role in As development, including the onset, progression, and destabilization of atherosclerotic lesions^{37–39}. The discovery of the new ACE2/Ang(1-7)/MasR1 axis has challenged the role of the traditional ACE/Ang II/AT1R axis in some complications of chronic cardiovascular diseases^{40, 41}. Moreover, RAS works via two opposing arms, resulting in either proatherogenic or atheroprotective effects. These effects may be influenced by the types of Ang peptides available and their levels, which are produced by two enzymes, ACE and ACE2, as well as the locally expressed receptors. A recent study established how sex affects circulating levels of the ACE2/Ang(1-7)/MasR1 axis in coronary As⁴². Since recent findings emphasize the significance of sex as an important variable in cardiovascular disease and genomics^{43–45}, our goal was to elucidate whether tissue intraplaque expressions of RAS family enzyme components, ACE, ACE2, and TMEM27, differ depending on PPs and sex. In our study group, we revealed a significant effect of sex and PPs on the expression levels of intraplaque ACE proteins. Lower protein levels of ACE were found in HoP compared to HerP in the male study group. Looking at the HoP phenotype only, the expression of the ACE gene was significantly lower in males than in females. ACE mRNA levels generally showed a trend of higher expression in males than in females, although not significant compared to females. Previous data²² showed no difference in ACE mRNA intra-

plaque levels in asymptomatic vs. symptomatic patients. There could be several reasons for such discrepancies in results. In the current study, we do have a higher number of males than females, although the ratio of HerP vs. HoP is not different regarding sex. Yet, the number of females is limited, which is common in consecutive sample collections and represents one of the major study limitations. In addition, in males, As starts approximately ten years earlier than in females, and females in our study are of similar age as males, leading to a possibility of the presence of systemic prominent As. Likewise, there is a higher proportion of females who are symptomatic in comparison to males and with a greater percentage of coronary comorbidity in females (although not significantly) in our study group. It could contribute to a burden on the intraplaque expression of ACE in females with HoP in this study. However, it should be noted that plaques in males are more often associated with plaque hemorrhage and clinical events⁴⁶. Sex is associated with the presence of atherosclerotic plaque hemorrhage, and it reshapes the relation between plaque hemorrhage and cardiovascular outcome⁴⁶, potentially biasing the study group in terms of surviving prior events.

It has been known for a while that PPs could be different among the sexes. It was shown that males develop unstable PPs, rich in inflammatory intraplaque content, more often than females⁴⁷, which is in agreement with the higher number of males in the HoP group. It is still an intriguing topic leading to novel research on the transcriptomic level of sex differences in atherosclerotic PPs (lipid-rich plaques in men,

fibrous plaques in women)²⁶. In addition, the recent study shows that an increase in macrophage ACE in atherosclerotic mice reduces the As in comparison to wild-type mice⁴⁸, suggesting a new ACE effect, which they claim to be independent of Ang II. Since the mRNA and protein expression in our study are not in alignment, it is noteworthy that the correlation between expression levels of protein and mRNA in mammal tissue is not always high^{49–51}. Many processes occur after mRNA forming: *post*-transcriptional, translational, and protein degradation regulation, so steady-state transcript abundances only partially predict protein abundances⁵². Indeed, a previous deep proteome and transcriptome analysis of 29 healthy human tissues revealed a strong difference between mRNA and protein quantities and that protein expression was often more stable across tissues than that of transcripts⁵³.

Bearing in mind the complexity of As as an inflammatory process, differences in expression might exist in intraplaque inflammatory cells infiltrate, state of activation, and interaction of different cell populations invading CP⁵⁴. Histological analyses of plaques have revealed differences in cell species, inflammation, and neovascularization status between males and females⁴⁷. Furthermore, using data on DNA methylation status in cells, it was discovered that female plaques contain more potential SMC-like cells, whereas male plaques contain more immune-like cells (macrophages, mast cells, T cells, B cells)⁵⁵.

Although ACE is expressed in a variety of cell types, it has been demonstrated that ACE produced by SMC directly causes As in both male and female mice, independently of BP and circulating ACE activity⁵⁶. According to those recent findings, females in our study may have more SMC than expected in the same type of plaques compared to males, as well as possible different methylation status in cells that could reflect the observed sex differences in the ACE protein level.

There is still missing knowledge about the role and activation of the ACE2/Ang(1-7)/MasR1 axis in carotid atheroma instability. Numerous harmful phenotypes associated with cardiovascular disease may be inhibited by the activation of this RAS counter-regulatory axis⁵⁷. It was proposed that the beneficial effect of ACE2 in thrombosis could be due to a concomitant increase in the production of Ang(1-7) and degradation of Ang II^{40, 58}. Both ACE2 mRNA and protein expression were present in all layers of the vessel wall. In addition, ACE2 protein was present in human veins, non-diseased mammary arteries, and atherosclerotic arteries. In human carotid atherosclerotic lesions, ACE2 mRNA expression was present in an early and advanced lesion¹⁸. However, in a previous study, ACE2 mRNA expression was very weak in human CP²². ACE2 protein was expressed in macrophages, SMC, and in the endothelial cells of *vasa vasorum* from atherosclerotic CP¹⁸. Overexpression of ACE2 in the rabbit model of As was shown to reduce the size of atherosclerotic lesions, stabilize already existing lesions, and inhibit lesion growth at early stages of the disease but not at advanced stages¹⁵. Even the local pattern of shear stress forces in blood vessels affects the

expression of ACE2 in atherosclerotic plaques⁵⁹. It was shown that ACE2 activation improves endothelial function *in vivo*⁶⁰, attenuates the formation of thrombi, and decreases platelet adhesion to injured vessels⁶¹. The intraplaque levels of ACE2 mRNA in our study were significantly different among the groups regarding sex and PPs, as shown in our preliminary study²⁴ and herein. However, 44% of plaques in our study had detectable mRNA. Previously, in some studies, ACE2 expression was not detectable¹⁷, while others detected a very weak level of ACE2 mRNA in human CP, only in a few specimens²². It is not clear why ACE2 is not detectable in a certain percentage of plaques. In our study group, we found significantly higher intraplaque ACE2 mRNA levels in females with HoP compared to females with HerP and in comparison to males. This result must be taken with caution since the group of females with HoP is rather small. Previously it was found that ACE2 protein levels did not differ with regard to PPs and were similar in all stages of As¹⁸. Furthermore, data so far point to the possibility that ACE2 functions as a tissue-specific negative feedback regulator of the activated RAS¹⁸. Due to the capacity of ACE2 to exert cardioprotective effects shown in animal models⁶², its role requires further studies in humans.

Regarding levels of TMEM27 mRNA, we did not detect any significant differences concerning sex and plaque type. To our knowledge, we were the first to detect TMEM27 mRNA levels in intraplaque tissue of CAs. A previous study showed that TMEM27 was expressed in the primary endothelial cells of the lungs and regulated the balance of nitric oxide and superoxide²⁰. This result suggested that activation of TMEM27 might be beneficial in the regulation of BP by affecting nitric oxide bioavailability and vascular damage⁶³. Still, more functional research is needed to determine the involvement of TMEM27 in As.

Our study has several limitations. First of all, since this was a single-center study with a small sample size of patients, additional research, including a bigger sample size, is required. Second, the asymptomatic patient group in our study was more frequently diagnosed during a routine CA US. Some of the individuals in the study also had concomitant conditions, such as coronary artery disease, diabetes mellitus, and/or hypertension. At the moment, our findings related to the expression of components of the RAS gene family were detected in the overall intraplaque tissue, so the cellular source of these components is not known. We did not evaluate tissue and circulating levels of ACE and ACE2 simultaneously, thus failing to test the hypothesis that the increase in tissue ACE will be reflected in increased circulating activity. Likewise, we did not measure protein levels of TMEM27.

Conclusion

In summary, our study may provide additional information regarding the relationship between the intraplaque expression of components of the RAS family based on sex and PP in advanced carotid As. The main novelties of this study

are sex specific differences in the expression of RAS components in different CP phenotypes and the detection of TMEM27 mRNA levels in the CP. Further studies are needed to resolve and elucidate sex-specific differences and mechanisms involved in complex RAS components interplay in As.

Acknowledgement

The research was funded by the Serbian Ministry of Education, Science and Technological Development (grant No 451-03-66/2024-03/200017).

R E F E R E N C E S

- Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature* 2011; 473(7347): 317–25.
- Kawasaki M, Takatsu H, Noda T, Ito Y, Kunishima A, Arai M, et al. Noninvasive quantitative tissue characterization and two-dimensional color-coded map of human atherosclerotic lesions using ultrasound integrated backscatter: comparison between histology and integrated backscatter images. *J Am Coll Cardiol* 2001; 38(2): 486–92.
- Grønholdt ML, Nordestgaard BG, Bentzon J, Wiebe BM, Zhou J, Falk E, et al. Macrophages are associated with lipid-rich carotid artery plaques, echolucency on B-mode imaging, and elevated plasma lipid levels. *J Vasc Surg* 2002; 35(1): 137–45.
- Nordestgaard BG, Grønholdt ML, Sillesen H. Echolucent rupture-prone plaques. *Curr Opin Lipidol* 2003; 14(5): 505–12.
- Rykażewska U, Zhao Q, Saliba-Gustafsson P, Lennquist M, Kronqvist M, Bergman O, et al. Plaque Evaluation by Ultrasound and Transcriptomics Reveals BCLAF1 as a Regulator of Smooth Muscle Cell Lipid Transdifferentiation in Atherosclerosis. *Arterioscler Thromb Vasc Biol* 2022; 42(5): 659–76.
- Kolaković A, Živković M, Stanković A. Involvement of the Renin-Angiotensin System in Atherosclerosis. In: *Tolekova AN*, editor. *Renin-Angiotensin System – Past, Present and Future*. London: IntechOpen; 2017. p. 268.
- Van Kats JP, Danser AH, van Meegen JR, Sassen LM, Verdouw PD, Schalekamp MA. Angiotensin production by the heart: a quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation* 1998; 98(1): 73–81.
- Donoghue M, Hsieh F, Baronas E, Godbont K, Gosselin M, Stagliano N, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 2000; 87(5): E1–9.
- Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* 2000; 275(43): 33238–43.
- Tikellis C, Thomas MC. Angiotensin-Converting Enzyme 2 (ACE2) Is a Key Modulator of the Renin Angiotensin System in Health and Disease. *Int J Pept* 2012; 2012: 256294.
- Thomas MC, Pickering RJ, Tsorotes D, Koitka A, Sheehy K, Bernardi S, et al. Genetic Ace2 deficiency accentuates vascular inflammation and atherosclerosis in the ApoE knockout mouse. *Circ Res* 2010; 107(7): 888–97.
- Sabara M, Ikutomi M, Morita T, Minami Y, Nakajima T, Hirata Y, et al. Deletion of angiotensin-converting enzyme 2 promotes the development of atherosclerosis and arterial neointima formation. *Cardiovasc Res* 2014; 101(2): 236–46.
- Zhang C, Zhao YX, Zhang YH, Zhu L, Deng BP, Zhou ZL, et al. Angiotensin-converting enzyme 2 attenuates atherosclerotic lesions by targeting vascular cells. *Proc Natl Acad Sci U S A* 2010; 107(36): 15886–91.
- Zhang YH, Zhang YH, Dong XF, Hao QQ, Zhou XM, Yu QT, et al. ACE2 and Ang-(1-7) protect endothelial cell function and prevent early atherosclerosis by inhibiting inflammatory response. *Inflamm Res* 2015; 64(3–4): 253–60.
- Dong B, Zhang C, Feng JB, Zhao YX, Li SY, Yang YP, et al. Overexpression of ACE2 enhances plaque stability in a rabbit model of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2008; 28(7): 1270–6.
- Nehme A, Cerutti C, Dhaouadi N, Gustin MP, Courand PY, Zibara K, et al. Atlas of tissue renin-angiotensin-aldosterone system in human: A transcriptomic meta-analysis. *Sci Rep* 2015; 5: 10035.
- Nehme A, Cerutti C, Zibara K. Transcriptomic Analysis Reveals Novel Transcription Factors Associated With Renin-Angiotensin-Aldosterone System in Human Atheroma. *Hypertension* 2016; 68(6): 1375–84.
- Sluimer JC, Gasc JM, Hamming I, van Goor H, Michaud A, van den Akker LH, et al. Angiotensin-converting enzyme 2 (ACE2) expression and activity in human carotid atherosclerotic lesions. *J Pathol* 2008; 215(3): 273–9.
- Patel VB, Zhong JC, Fan D, Basu R, Morton JS, Parajuli N, et al. Angiotensin-converting enzyme 2 is a critical determinant of angiotensin II-induced loss of vascular smooth muscle cells and adverse vascular remodeling. *Hypertension* 2014; 64(1): 157–64.
- Cechova S, Zeng Q, Billand M, Mutchler S, Rudy CK, Straub AC, et al. Loss of collectrin, an angiotensin-converting enzyme 2 homolog, uncouples endothelial nitric oxide synthase and causes hypertension and vascular dysfunction. *Circulation* 2013; 128(16): 1770–80.
- Chu PL, Gigliotti JC, Cechova S, Bodonyi-Kovacs G, Wang YT, Chen L, et al. Collectrin (Tmem27) deficiency in proximal tubules causes hypertension in mice and a TMEM27 variant associates with blood pressure in males in a Latino cohort. *Am J Physiol Renal Physiol* 2023; 324(1): F30–F42.
- Fraga-Silva RA, Saverini SQ, Montecucco F, Nencioni A, Caffa I, Soncini D, et al. Treatment with Angiotensin-(1-7) reduces inflammation in carotid atherosclerotic plaques. *Thromb Haemost* 2014; 111(4): 736–47.
- Fukuhara M, Geary RL, Diz DI, Gallagher PE, Wilson JA, Glazier SS, et al. Angiotensin-converting enzyme expression in human carotid artery atherosclerosis. *Hypertension* 2000; 35(1 Pt 2): 353–9.
- Kolaković A, Živković M, Djurić T, Končar I, Stanković A. The expression of renin-angiotensin-system components (ACE, ACE2 and collectrin (TMEM27)) in the human carotid plaques depending on gender and plaque phenotype. *Atherosclerosis* 2018; 275: e135.
- Mensab GA, Fuster V. Sex and Gender Differences in Cardiovascular Health. *J Am Coll Cardiol* 2022; 79(14): 1385–7.
- Sakkers TR, Mokry M, Civelek M, Erdmann J, Pasterkamp G, Diez Benavente E, et al. Sex differences in the genetic and molecular mechanisms of coronary artery disease. *Atherosclerosis* 2023; 384: 117279.
- McGuire BB, Watson RW, Pérez-Barriocanal F, Fitzpatrick JM, Docherty NG. Gender differences in the renin-angiotensin and nitric oxide systems: relevance in the normal and diseased kidney. *Kidney Press Res* 2007; 30(2): 67–80.
- Bundalo MM, Živković MD, Romić S, Tepavčević SN, Koricanac GB, Djurić TM, et al. Fructose-rich diet induces gender-specific changes in expression of the renin-angiotensin system in rat heart and upregulates the ACE/AT1R axis in the male rat aorta. *J Renin Angiotensin Aldosterone Syst* 2016; 17(2): 1470320316642915.

29. Zapater P, Novalbos J, Gallego-Sandín S, Hernández FT, Abad-Santos F. Gender differences in angiotensin-converting enzyme (ACE) activity and inhibition by enalaprilat in healthy volunteers. *J Cardiovasc Pharmacol* 2004; 43(5): 737–44.
30. Liu J, Ji H, Zheng W, Wu X, Zhu JJ, Arnold AP, et al. Sex differences in renal angiotensin converting enzyme 2 (ACE2) activity are 17 β -oestradiol-dependent and sex chromosome-independent. *Biol Sex Differ* 2010; 1(1): 6.
31. Bhatia K, Zimmerman MA, Sullivan JC. Sex differences in angiotensin-converting enzyme modulation of Ang (1-7) levels in normotensive WKY rats. *Am J Hypertens* 2013; 26(5): 591–8.
32. North American Symptomatic Carotid Endarterectomy Trial Collaborators; Barnett HJM, Taylor DW, Haynes RB, Sackett DL, Peerless SJ, et al. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. *N Engl J Med* 1991; 325(7): 445–53.
33. Gray-Weale AC, Graham JC, Burnett JR, Byrne K, Lusby RJ. Carotid artery atheroma: comparison of preoperative B-mode ultrasound appearance with carotid endarterectomy specimen pathology. *J Cardiovasc Surg (Torino)* 1988; 29(6): 676–81.
34. Djurić T, Stanković A, Končar I, Radak D, Davidović L, Alavantić D, et al. Association of MMP-8 promoter gene polymorphisms with carotid atherosclerosis: preliminary study. *Atherosclerosis* 2011; 219(2): 673–8.
35. Romero-Calvo I, Ocón B, Martínez-Moya P, Suárez MD, Zarzuelo A, Martínez-Augustín O, et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem* 2010; 401(2): 318–20.
36. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3(6): 1101–8.
37. Weiss D, Kools JJ, Taylor WR. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation* 2001; 103(3): 448–54.
38. Mazzeoli L, Duchosal MA, Korber M, Bouzourene K, Aubert JF, Hao H, et al. Endogenous angiotensin II induces atherosclerotic plaque vulnerability and elicits a Th1 response in ApoE^{-/-} mice. *Hypertension* 2004; 44(3): 277–82.
39. Da Cunha V, Martin-McNulty B, Vincelette J, Choy DF, Li WW, Schroeder M, et al. Angiotensin II induces histomorphologic features of unstable plaque in a murine model of accelerated atherosclerosis. *J Vasc Surg* 2006; 44(2): 364–71.
40. Fraga-Silva RA, Da Silva DG, Montecucco F, Mach F, Stergiopoulos N, da Silva RF, et al. The angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas receptor axis: a potential target for treating thrombotic diseases. *Thromb Haemost* 2012; 108(6): 1089–96.
41. Paz Ocaranza M, Riquelme JA, García L, Jalil JE, Chiong M, Santos RAS, et al. Counter-regulatory renin-angiotensin system in cardiovascular disease. *Nat Rev Cardiol* 2020; 17(2): 116–29.
42. Zhou X, Zhang P, Liang T, Chen Y, Liu D, Yu H. Relationship between circulating levels of angiotensin-converting enzyme 2-angiotensin-(1-7)-MAS axis and coronary heart disease. *Heart Vessels* 2020; 35(2): 153–61.
43. Hartman RJG, Owsiany K, Ma L, Koplev S, Hao K, Slenders L, et al. Sex-Stratified Gene Regulatory Networks Reveal Female Key Driver Genes of Atherosclerosis Involved in Smooth Muscle Cell Phenotype Switching. *Circulation* 2021; 143(7): 713–26.
44. Hartman RJG, Huisman SE, den Ruijter HM. Sex differences in cardiovascular epigenetics-a systematic review. *Biol Sex Differ* 2018; 9(1): 19.
45. Gasbarrino K, Di Iorio D, Daskalopoulou SS. Importance of sex and gender in ischaemic stroke and carotid atherosclerotic disease. *Eur Heart J* 2022; 43(6): 460–73.
46. Vrijenboek JE, Den Ruijter HM, De Borst GJ, de Kleijn DP, De Vries JP, Bots ML, et al. Sex is associated with the presence of atherosclerotic plaque hemorrhage and modifies the relation between plaque hemorrhage and cardiovascular outcome. *Stroke* 2013; 44(12): 3318–23.
47. Wendorff C, Wendorff H, Pelisek J, Tsantilas P, Zimmermann A, Zernecke A, et al. Carotid Plaque Morphology Is Significantly Associated With Sex, Age, and History of Neurological Symptoms. *Stroke* 2015; 46(11): 3213–9.
48. Cao D, Khan Z, Li X, Saito S, Bernstein EA, Victor AR, et al. Macrophage angiotensin-converting enzyme reduces atherosclerosis by increasing peroxisome proliferator-activated receptor α and fundamentally changing lipid metabolism. *Cardiovasc Res* 2023; 119(9): 1825–41.
49. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* 2012; 13(4): 227–32.
50. Kosti I, Jain N, Aran D, Butte AJ, Sirota M. Cross-tissue Analysis of Gene and Protein Expression in Normal and Cancer Tissues. *Sci Rep* 2016; 6: 24799.
51. Kousounadis A, Langdon SP, Um IH, Harrison DJ, Smith VA. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Sci Rep* 2015; 5: 10775.
52. De Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels. *Mol Biosyst* 2009; 5(12): 1512–26.
53. Wang D, Eraslan B, Wieland T, Hallström B, Hopf T, Zolg DP, et al. A deep proteome and transcriptome abundance atlas of 29 healthy human tissues. *Mol Syst Biol* 2019; 15(2): e8503.
54. Grufman H, Schiopu A, Edsfield A, Björkbacka H, Nitulescu M, Nilsson M, et al. Evidence for altered inflammatory and repair responses in symptomatic carotid plaques from elderly patients. *Atherosclerosis* 2014; 237(1): 177–82.
55. Hartman RJG, Siemeling MA, Huijtema S, Dekkers KF, Slenders L, Boltjes A, et al. Sex-dependent gene regulation of human atherosclerotic plaques by DNA methylation and transcriptome integration points to smooth muscle cell involvement in women. *Atherosclerosis* 2021; 331: e217.
56. Chen X, Howatt DA, Balakrishnan A, Moorleghen JJ, Wu C, Cassis LA, et al. Angiotensin-Converting Enzyme in Smooth Muscle Cells Promotes Atherosclerosis-Brief Report. *Arterioscler Thromb Vasc Biol* 2016; 36(6): 1085–9.
57. Clarke C, Flores-Muñoz M, McKinney CA, Milligan G, Nicklin SA. Regulation of cardiovascular remodeling by the counter-regulatory axis of the renin-angiotensin system. *Future Cardiol* 2013; 9(1): 23–38.
58. Wysocki J, Ye M, Rodríguez E, González-Pacheco FR, Barrios C, Evora K, et al. Targeting the degradation of angiotensin II with recombinant angiotensin-converting enzyme 2: prevention of angiotensin II-dependent hypertension. *Hypertension* 2010; 55(1): 90–8.
59. Fraga-Silva RA, Montecucco F, Costa-Fraga FP, Nencioni A, Caffa I, Bragina ME, et al. Diminazene enhances stability of atherosclerotic plaques in ApoE-deficient mice. *Vascul Pharmacol* 2015; 74: 103–13.
60. Fraga-Silva RA, Costa-Fraga FP, Murça TM, Moraes PL, Martins Lima A, Lautner RQ, et al. Angiotensin-converting enzyme 2 activation improves endothelial function. *Hypertension* 2013; 61(6): 1233–8.
61. Fraga-Silva RA, Sorg BS, Wankbede M, Dedend C, Jun JY, Baker MB, et al. ACE2 activation promotes antithrombotic activity. *Mol Med* 2010; 16(5–6): 210–5.

62. Bošković M, Živković M, Koricanac G, Tepavčević S, Zec M, Debeljak-Martić J, et al. Walnut supplementation after fructose-rich diet is associated with a beneficial fatty acid ratio and increased ACE2 expression in the rat heart. *Front Physiol* 2022; 13: 942459.
63. Yang G, Chu PL, Rump LC, Le TH, Stegbauer J. ACE2 and the Homolog Collectrin in the Modulation of Nitric Oxide and Oxidative Stress in Blood Pressure Homeostasis and

Vascular Injury. *Antioxid Redox Signal* 2017; 26(12): 645–59.

Received on October 28, 2022
Revised on November 20, 2023
Revised on January 12, 2024
Accepted on January 21, 2024
Online First March 2024