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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 hipoehogenog plaka (HoP) i fenotip hiperehogenog 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

Correspondence to: Ana Kolaković, University of Belgrade, Vinča, Institute of Nuclear Sciences – National Institute of the Republic of Serbia, Department of Health and Environmental Research, Laboratory for Radiobiology and Molecular Genetics, Mike Petrovića Alasa 12–14, 11 351 Belgrade, Serbia. E-mail: anakolakovic@vin.bg.ac.rs

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 uznapredovaloj 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 rupture [1]. The characterization of carotid plaque (CP) morphology by non-invasive ultrasound (US) may provide insight into the composition of the plaque and its structure [2]. Dominantly echolucent, hypoechogenic plaques (HoPs) of the carotid artery (CA), which are lipid-rich with increased macrophage density [3], 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 calcification [4]. 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 stability [6]. 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 locally [7]. 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 system [10].

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 lesions [13] or prevents early atherosclerotic lesion formation [14] and increases plaque stability [15].

ACE is expressed in multiple tissues [16], various cells in blood vessels, and human atheroma [17], whereas its homolog, ACE2, is expressed in endothelium, macrophage foam cells, and vascular SMC [18]. The development of As is delayed by ACE2 activation [18], and adverse vascular remodeling is prevented [19].

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 mice [20]. TMEM27 regulates arterial BP [20] and is expressed in the vascular endothelium and kidney [21].

Previously, the variation in the expression of some RAS family members, e.g., the MasR1, in human CP tissue was related to different PPs [22]. Moreover, treatment with the oral formulation of Ang(1-7) enhances a more stable phenotype in atherosclerotic CP [22], 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 As [18]. 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 atheromas [17] but not related to CA phenotype [22]. The TMEM27 was detected previously in carotid tissue in our preliminary study [24].

Sex and gender play an important role in cardiovascular research when addressing disease prevalence, risk factors, diagnostic evaluation, and overall health outcomes [25]. 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 CAs [32]. 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 described [34].

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 out [28]. 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 detail [28] 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 blot [35]. 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 $2^{-\Delta CT}$ [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 ($\times 10^9$/L)	7.83 ± 1.50	8.97 ± 2.69	0.61*
Red blood cells ($\times 10^{12}$/L)	4.49 ± 0.35	4.78 ± 0.27	0.07*
Platelets ($\times 10^9$/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. females (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

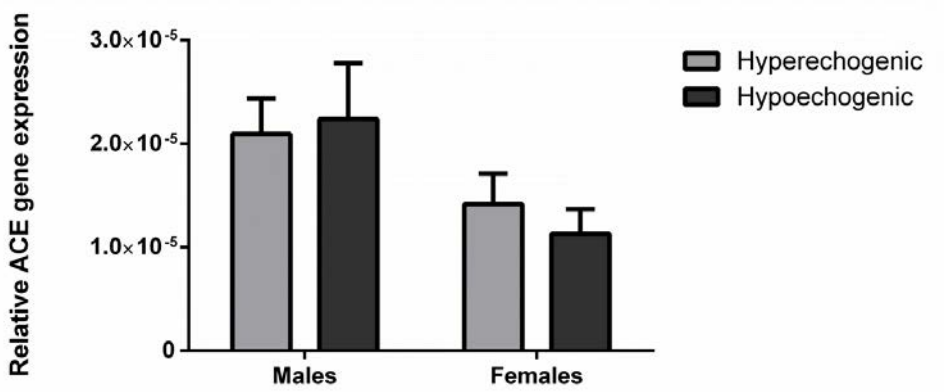

Fig. 1 – Relative angiotensin-converting enzyme *(ACE)* gene expression in carotid plaque (CP) tissue. **There was no significant difference in *ACE* gene expression between plaque phenotypes: males – HerP phenotypes (n = 32) vs. HoP phenotypes (n = 14), $p = 0.99$; females – HerP phenotypes (n = 17) 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.**

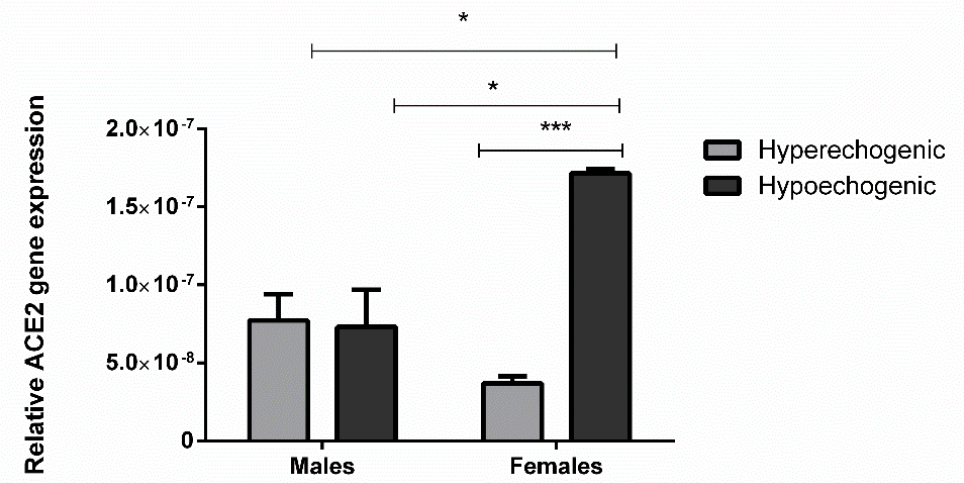

Fig. 2 – Relative angiotensin-converting enzyme *(ACE)2* gene expression in carotid plaque tissue. ***** significant difference between females with HoP phenotypes (n = 3) vs. females with HerP phenotypes (n = 9), $p < 0.001$; *significant difference between females with HoP phenotypes (n = 3) vs. males 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 expression 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).

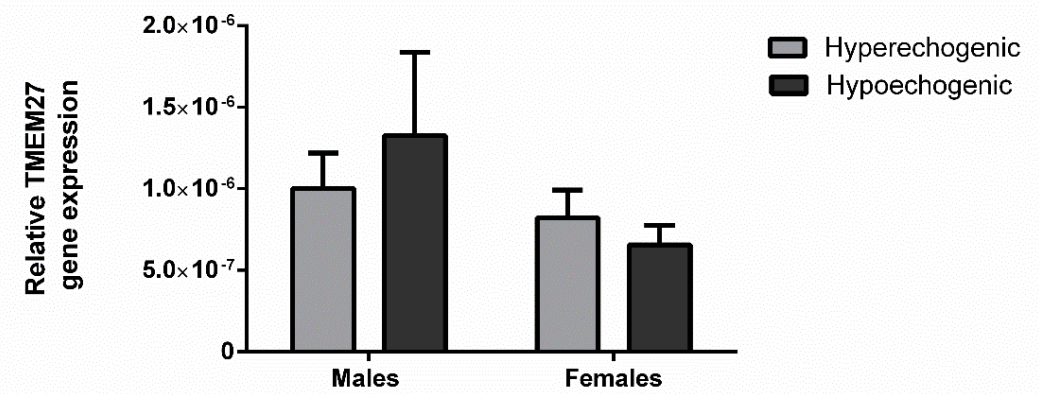

Fig. 3 – Relative transmembrane protein 27 (*TMEM27)* gene expression in carotid plaque tissue. There was no significant difference in *TMEM27* gene expression between plaque phenotypes: males – HerP phenotypes (n = 32) vs. HoP phenotypes (n = 14), $p = 0.86$; females – HerP phenotypes (n = 17) vs. HoP 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 ± 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 ($p < 0.0001$). Significantly higher ACE protein level was found in males with HerP phenotype of CP in comparison to males with HoP phenotypes ($p = 0.0007$, Tuckey *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 ($p = 0.86$).

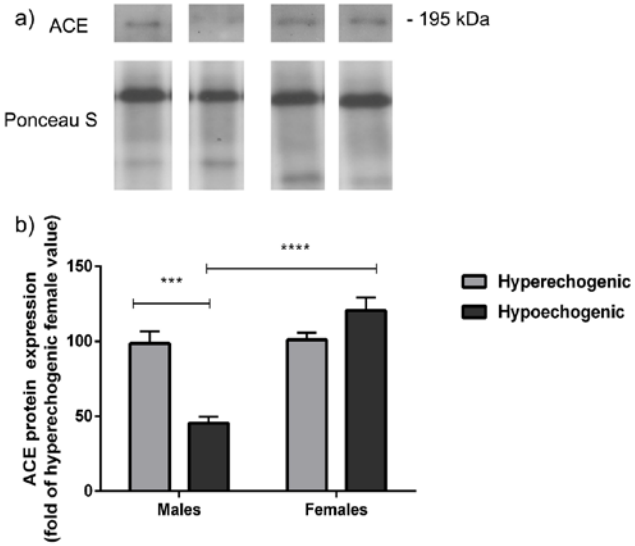

Fig. 4 – Western blot of angiotensin-converting enzyme *(*ACE*)* protein expression in carotid plaque tissue: a) representative image of Western blot: band No. 1 – HerP males; band No. 2 – HoP males; band No. 3 – Herp females; band No. 4 – HoP females; b) graphic display of Western blot results. Ponceau 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 ± standard error of the mean. For abbreviations, see Table 1.**

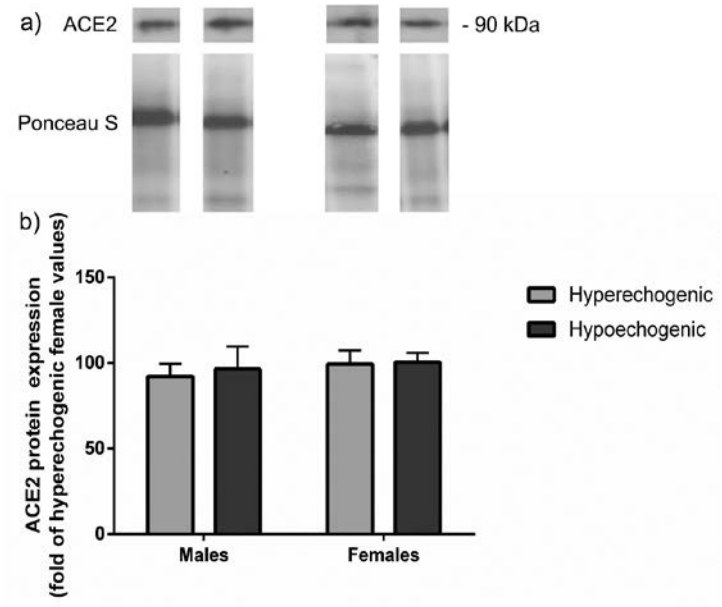

Fig. 5 – Western blot of angiotensin-converting enzyme *(*ACE*)*2 protein expression in carotid plaque tissue: a) representative image of Western blot: band No. 1 – HerP males; band No. 2 – HoP males; band No. 3 – Herp females; band No. 4 – HoP females; b) graphic display of Western blot results.
Ponceau 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 ± 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 As [42]. 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 data [22] showed no difference in ACE mRNA intraplaque 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 events [46]. Sex is associated with the presence of atherosclerotic plaque hemorrhage, and it reshapes the relation between plaque hemorrhage and cardiovascular outcome [46], potentially biassing 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 females [47], 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 mice [48], 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 abundances [52]. 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 transcripts [53].

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 CP [54]. Histological analyses of plaques have revealed differences in cell species, inflammation, and neovascularization status between males and females [47]. 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 activity [56]. 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 axis [57]. 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 lesion [18]. However, in a previous study, ACE2 mRNA expression was very weak in human CP [22]. ACE2 protein was expressed in macrophages, SMC, and in the endothelial cells of *vasa vasorum* from atherosclerotic CP [18]. 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 stages [15]. Even the local pattern of shear stress forces in blood vessels affects the expression of ACE2 in atherosclerotic plaques [59]. It was shown that ACE2 activation improves endothelial function *in vivo* [60], attenuates the formation of thrombi, and decreases platelet adhesion to injured vessels [61]. The intraplaque levels of ACE2 mRNA in our study were significantly different among the groups regarding sex and PPs, as shown in our preliminary study [24] and herein. However, 44% of plaques in our study had detectable mRNA. Previously, in some studies, ACE2 expression was not detectable [17], while others detected a very weak level of ACE2 mRNA in human CP, only in a few specimens [22]. 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 As [18]. Furthermore, data so far point to the possibility that ACE2 functions as a tissue-specific negative feedback regulator of the activated RAS [18]. Due to the capacity of ACE2 to exert cardioprotective effects shown in animal models [62], 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 superoxide [20]. This result suggested that activation of TMEM27 might be beneficial in the regulation of BP by affecting nitric oxide bioavailability and vascular damage [63]. 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.

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