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Renin-angiotensin system gene variants and risk of early- and late-onset preeclampsia: A single center case-control study

Lucia Maria Procopciuc[a], Georgiana Nemeti[b], Elena Buzdugan[c], Mihaela Iancu[d,*], Florin Stamatian[b], Gabriela Caracostea[b]

[a] Department of Medical Biochemistry, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
[b] Gynecological Clinic 1, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
[c] Medical Clinic V, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
[d] Department of Medical Informatics and Biostatistics, Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania

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ABSTRACT

Background: Changes in the renin-angiotensin-aldosterone system's (RAAS) activity due to different genetic variations could represent risk factors for the onset of preeclampsia.
Objective: To test and quantify the relationships of 8 RAAS gene polymorphisms (angiotensinogen (AGT)-M235T, AGT-T174M, angiotensin converting enzyme (ACE)-I/D, ACE8-A2350G, angiotensin II type 1 receptor (AGTR1)-A1166C, angiotensin II type 2 receptor (AGTR2)-C3123A, renin (REN)-G83A, aldosterone synthase (CYP11B2)-T344C) with susceptibility to early- (EOPE) and late-onset preeclampsia (LOPE).
Study design: We performed polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) analysis in 217 pregnant women, of whom 87 pregnant women with EOPE/LOPE and 130 normal pregnant women. The relationship between the studied RASS gene polymorphisms and EOPE/LOPE was tested by multiple logistic regressions.
Results: The multivariate logistic regression analysis showed that AGT-M235T (adjusted OR = 4.63), AGT-*T174M* (adjusted OR = 4.13), REN-*G83A* (adjusted OR = 3) and CYP11B2-*C344T* (adjusted OR = 3.13) gene polymorphisms remained independent risk factors for EOPE. Moreover, ACE-*I/D* (adjusted OR = 4.04), ACE-*A2350G* (adjusted OR = 3.5), AGTR1-*A1166C* (adjusted OR = 2.73), and REN-*G83A* (adjusted OR = 2.67) polymorphisms remained independent risk factors for LOPE. The frequency of overweight was significantly different ($p = 0.001$) in pregnant women with EOPE, LOPE and the control group (LOPE:16, 29.6% vs. EOPE:12, 36.4% vs. control group:16, 12.3%). Pregnant women with EOPE had babies with a significantly lower mean birth weight (2067.9 ± 887.9) in comparison to women with LOPE (mean ± SD: 2860.1 ± 771.1, $p < 0.001$) and women with normal pregnancies, respectively (mean ± SD: 3324.9 ± 484.9, $p < 0.001$).
Conclusion: We confirmed the influence of the renin-angiotensin-aldosterone system through these 8 genetic variations on the onset of preeclampsia.

1. Introduction

Preeclampsia, the most common of pregnancy complications, with high rates of morbidity and mortality, alongside with a high impact on both maternal and fetal health, is characterized by hypertension (higher than 140/90 mmHg) and proteinuria (more than 300 mg/24 hours) developed after 20 weeks of gestation [1,2]. Mothers with preeclampsia have a higher risk to develop cardiovascular, renal diseases or diabetes later in life. There is the same risk for babies whose mothers experienced preeclampsia [3].

Although the literature reports a series of risk factors for preeclampsia, including inflammation, endothelial dysfunction, oxidative stress, angiogenic imbalance, thrombophilia, or dyslipidemia, the pathogenesis of preeclampsia remains unknown [4,5].

It is well-known that the compensatory alterations in the rennin-angiotensin-aldosterone system (RAAS) contribute to the salt-water balance and appropriate placental perfusion during normal pregnancy and it is characterized by an increase in almost all the components of RAAS. On the other hand, preeclampsia is characterized by abnormal placentation with incomplete invasion of the cytotrophoblasts into the spiral arteries [6,7]. Hypertension during pregnancy occurs as a consequence of reduced placental perfusion, placental ischemia/hypoxia,

* Corresponding author.
E-mail address: miancu@umfcluj.ro (M. Iancu).

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endothelial dysfunction, which in turn determine vasoconstriction. In preeclampsia there is a disturbance of RAAS equilibrium both for circulation and the uteroplacental unit which leads to modification in both the levels and function of the components with decreased renin level, increased ACE activity, reduced AngII levels and increased responsiveness to ANG II. These observations suggest that the heritable allelic variations, especially the genetic polymorphisms in RAAS, could be part of the foundation for the genetics of preeclampsia and hence are related to the development of preeclampsia [8,9].

RAAS has an important role in blood pressure regulation and also in the regulation of electrolyte balance. The components of RAAS are renin (REN), angiotensinogen (AGT), angiotensin-converting enzyme (ACE), angiotensin I (ANGI), angiotensin II (ANGII), angiotensin 1–7 (ANG1-7), angiotensin II type 1 receptor (AGTR1), angiotensin II type 2 receptor (AGTR2), and aldosterone synthase (CYP11B2). Renin is an enzyme involved in the first step of RAAS and has a role in converting angiotensinogen into a decapeptide, angiotensin I. Angiotensin-converting enzyme, a component of the circulating RAAS, converts angiotensin I into angiotensin II, an octapeptide [10]. There are two angiotensin II receptors, with similar affinity for angiotensin II, angiotensin II type 1 and 2 receptors. Through its principal receptor, AGTR1, angiotensin II promotes its role as a vasoconstrictive substance, modulation of vascular tone, degradation of bradykinine, sodium reabsorption, aldosterone secretion [8,11].

Because hypertension is one of the characteristics of preeclampsia, spiral arteries, fetal tissues or the human placenta express angiotensinogen, ACE, ANGI, ANGII, REN, AGTR1, AGTR2, and RAAS has a role in blood pressure regulation, changes in the activity of the renin-angiotensin-aldosterone system due to different genetic variations, which could represent risk factors for preeclampsia [12,13].

Angiotensinogen (AGT) is expressed by spiral arteries, and *M235T* polymorphism located in exon 2 of the AGT gene (*T704C*, rs 699) is associated with higher AGT levels. Moreover, the T allele is associated with abnormal spiral artery remodeling, which is why this polymorphism could be considered a possible risk factor for preeclampsia development. The second common AGT polymorphism, *T174M*, located in exon 2 of the gene *(C521T,* rs 4762), could modify plasma AGT concentration and is in linkage disequilibrium with AGT-*M235T* polymorphism [14].

The insertion/deletion of a 287 bp Alu (I/D- rs 4646994) located in intron 16 of the ACE gene is associated with the modified ACE activity. The presence of the D allele means higher ACE activity, a higher rate of conversion of angiotensin I to angiotensin II, vasoconstriction, increased blood volume and increased blood pressure, which is why ACE plays an important role in cardiovascular pathology [15,16]. On the other hand, the presence of the I allele is associated with low ACE activity [6,9,21]. Higher ACE levels, due to the presence of the ACE-I/D polymorphism, determine higher uterine artery resistance, which is associated with the risk for preeclampsia [9,17]. *A2350G* polymorphism is located in exon 16 of the ACE2 gene (rs 4343), with a higher influence on plasma ACE levels, higher than that determined by ACE-I/D. It was associated with hypertension [18].

The angiotensin II/AGTR1/AGTR2 axis with a role in hypertension development also has an important role in the development of endothelial dysfunction in preeclampsia [8]. Two polymorphisms have been described in the AGTR1 and AGTR2 genes, namely *A1166C* located in the 3′untranslated region of the AGTR1 gene (rs 5186) and *C3123A* located in the 3′untranslated region of the AGTR2 gene (rs 11091046). The *A1166C* polymorphism can modify the response to ANGII, which means a higher influence on RAAS activity. Because AGTR2 induces apoptosis and probably throphoblast apoptosis too, it has been speculated that there is an association between AGTR2-*C3123A* polymorphism and preeclampsia [19]. Moreover, AGTR2-*C3123A* polymorphism influences the higher AGTR2 expression in the placenta.

Environmental and behavioral risk factors, such as smoking, oral contraceptive use, body mass index (BMI), etc. could also be involved in susceptibility to preeclampsia [20,21].

The objectives of this study were to test and quantify the relationships of 8 RAAS gene polymorphisms (AGT-*M235T,* AGT-*T174M,* ACE-*I/D,* ACE8- *A2350G,* AGTR1-*A1166C,* AGTR2-*C3123A,* REN-*G83A,* CYP11B2-*T344C*) with susceptibility to early- and late-onset preeclampsia.

2. Methods

2.1. Patients' selection

We performed a cross-sectional case-control study using a convenience sample with 217 pregnant women of which 87 pregnant women with preeclampsia and 130 normal pregnant women, selected in the Maternal-Fetal Medicine Department of Gynecology Clinic I, an academic tertiary hospital from Cluj-Napoca, Romania, in the period 2009–2010. PE was defined according to the International Society for the Study of Hypertension in Pregnancy (ISSHP) as blood pressure ≥ 140/90 mmHg on two occasions 4–6 h apart, after the 20th week of gestation in previously normotensive women, accompanied by one or more of the following new-onset conditions: proteinuria > 300 mg/24 h, other maternal organ dysfunction as renal insufficiency/failure (creatinine > 1.02 mg/dL), liver involvement (e.g. elevated transaminases twice the upper limit of normal), neurological complications (e.g. eclampsia), hematological complications (e.g. thrombocytopenia < 150,000/dL) or uteroplacental dysfunction with intrauterine growth restriction (IUGR) [22]. Preeclamptic women were grouped in early onset (EOPE) and late onset preeclampsia (LOPE). EOPE and LOPE were defined using 34 weeks of gestation as cut-off between the two groups. IUGR was defined as fetal abdominal circumference < 10th percentile and abnormal umbilical artery PI > 95th percentile, irrespective of the presence of absent or reversed end-diastolic flow. The gestational age was assessed according to the last menstrual period and first trimester ultrasound examination for all fetuses.

The exclusion criteria applied for both groups were: twin pregnancy, pregnancy obtained through assisted reproductive techniques, fetal malformations, previous maternal cardiopathy, PE superimposed on chronic hypertension, immune disorders, pregestational renal disease, pregestational diabetus mellitus, smoking habits and pregestational dyslipidemia.

Obstetrical ultrasonographic assessments were performed using a Voluson 730 Expert machine (GE Healthcare) with a 4–6 MHz curved array probe.

Blood pressure was assessed using a standard, calibrated, electronic sphygmomanometer Omron, with the woman in a sitting position with their legs not crossed. Systolic blood pressure (SBP) was considered to be high when over > 140 mmHg, while high diastolic blood pressure (DBP) was considered when over > 90 mmHg. After a resting period of 5 minute, 2 simultaneous measurements were taken into consideration for each arm and the average of the four values was registered onto the data sheets.

We collected the obstetric data (maternal age (years), gestational age on delivery (weeks), birth weight (grams), parity, BMI, SBP, DBP) from the obstetric data sheets. The selected behavioral risk factors included oral contraceptive (OC) use, smoking habits, and alcohol consumption.

The written informed consent was obtained from all the participants before their inclusion in the study, and the approval of the Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy was also given (study number 12/3.02.2009).

2.2. Methods

The genetic analysis of all pregnant women included in the study

was performed. In order to identify the 8 genetic RAAS variations, genomic DNA was extracted from blood drawn in vacutainers with ethylenediaminetetraacetic acid (EDTA). Polymerase chain reaction (PCR) or polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) analysis was carried out. For DNA isolation, a Zymo Research protocol (Zymo Research Corporation, Freiburg, Germany, (https://www.zymoresearch.de) was used. The 20 ng genomic DNA was amplified in 25 µl mixture containing 0.2 µM forward and reverse primers, 2.0 mM $MgCl_2$, 200 mM dNTPs (dATP, dGTP, dCTP, dTTP), 10 X buffer (750 mM Tris-HCl (pH 8.8 at 25 °C), 200 mM, $(NH_4)_2SO_4$ 0.1% (v/v) Tween 20) and 0.6U *Taq polymerase*.

After PCR amplification was performed in an iCycler (BioRad, California, USA, http://www.bio-rad.com), enzymatic digestion using specific endonucleases was carried out. We incubated the PCR fragments at 37 °C in 10 µl mixture using 5U restriction enzymes. The fragments obtained by restriction digest were visualized in UV light after agarose gel electrophoresis, stained with ethidium bromide solution. All PCR-RFLP reagents were from Fermentas (Thermo Fisher Scientific Inc. http://www.thermoscientific.com/fermentas), except for the primers, which were from Eurogentec (Kaneca Corporation, www.eurogentec.com). The PCR-RFLP conditions were specified in Table 1 [23,24].

The biochemical determination of high density lipoproteins (HDL-cholesterol), low density lipoproteins (LDL-cholesterol) and triglycerides (TG) levels was performed using Enzyme Linked Immunosorbent Assay (ELISA) methods (reagents from ADALTIS, Italy, http://www.adaltis.net/).

2.3. Statistical analysis

The statistical analysis was conducted with R-advanced software for statistical computing (version R 3.5.2).

Measures of centrality and dispersion as mean ± sample standard deviation (SD) or median with interquartile range (IQR) were used to describe the distributions of quantitative variables. The reporting of descriptive measures was based on the test results for the presence of normal distribution. The Student-t test for two independent samples and Mann-Whitney test were used to compare the distributions of quantitative anthropometric characteristics of PE and the control groups, while univariate analysis of variance (ANOVA) and Kruskal-Wallis test were used to compare the early-onset, late-onset preeclampsia and control groups. In the case of significant results, the analysis was followed by a post-hoc test.

The bivariate analysis consisting of a comparison of the studied groups regarding their qualitative clinical characteristics was performed using the absolute and relative frequency (% in relation to the size of each studied group). The distributions of the qualitative characteristics of the studied groups were compared using the asymptotic chi-square or Fisher's exact tests. The comparison of the nominal characteristics distributions in multiple groups (early-onset PE, late-onset PE and controls) was followed by Pairwise comparisons using Fisher's test with p-values adjusted by the Benjamini-Hochberg procedure in order to identify the source of differences between groups.

For all statistical tests, the chosen significance level was set at 0.05.

The relationship between the studied RASS gene polymorphisms and early- and late-onset PE was tested by multiple logistic regressions. The covariates included in the multivariable model were as follows: maternal age ($\geq$30 years/ $<$ 30 years), parity (multiparous vs. primiparous), smoking (yes/no), and overweight (BMI $\geq$ 25 kg/m2). Because of multiple comparisons, we used a Bonferroni correction. The cut-off value for the significance level was chosen by dividing 0.05 by the number of independent gene polymorphisms and it was set as $\alpha = 0.05/5 = 0.01$. Effect size was described by adjusted odds ratio with an associated 99% confidence interval.

The evaluation of Hardy-Weinberg equilibrium and linkage disequilibrium was performed using the "genetics" R package.

3. Results

3.1. Characteristics of the pregnant women in the study, regarding the presence of preeclampsia

The study included 217 pregnant women, 87 pregnant women with preeclampsia (PE), along with 130 control pregnant women. According to table 2, out of the women with preeclampsia, 33 (37.9%) developed preeclampsia before 34 weeks of gestation (early onset preeclampsia-EOPE) and 54 (62.1%) developed it after 34 weeks of gestation (late onset preeclampsia-LOPE).

No significant difference was found regarding the mean age of the control group and preeclampsia cases ($p = 0.721$), but there was a significant difference in the distribution of SBP and DBP values of the two groups ($p < 0.001$). There was a significant association between

Table 1
PCR-RFLP conditions for identification of RAAS polymorphisms.

Genetic variation	Gene (chr)	Primers	Annealing temperature (°C)	PCR fragment	Restriction enzyme	Restriction fragments
G83A (rs2368564)	*REN* (chr 1q32.1)	5′-GAGGTTCGAGTCGGCCCCCT-3′ 5′-TGCCCCAAACATGGCCACACAT-3′	66	250 bp	*MboI*	*G83*: 171,79 bp *A83*: 250 bp
M235T (T704C) (rs 699)	*AGT* (Chr 1q42-q43)	5′-CAGGGTGCTGTCCACACTGGACCCC-3′ 5′-CCGTTTGTGCAGGGCCTGGCTCTCT-3′	64	165 bp	*Tth111I*	*T235*:141,24 bp *M235*:165 bp
T174M (C521T) (rs 4762)	*AGT* (chr1q42-q43)	5′-GATGCGCACAAGGTCCTGTC-3′ 5′-CAGGGTGCTGTCCACACTGGACCCC-3′	57	303 bp	*NcoI*	*M174*:211,92 bp *T174*: 303 bp
I/D (rs1799752)	*ACE* (chr 17q23.3)	5′-CTGGAGACCACTCCCATCCTTTCT-3′ 5′-GATGTGGCCATCACATTCGTCAGC-3′	69	D allele: 290 bp I allele: 390 bp		
A2350G (rs 4343)	*ACE8* (chr 17q23.3)	5′-CTGACGAATGTGATGGCCGC-3′ 5′-TTGATGAGTTCCACGTCTTTCG-3′	59	122 bp	*BstUI*	*G2350*:100, 22 bp *A2350*: 122 bp
A1166C (rs 5186)	*AGTR1* (chr3q21-q25)	5′-ATAATGTAAGCTCATCCACC-3′ 5′-GAGATTGCATTTCTGTCAGT-3′	57	350 bp	*DdeI*	*C1166*: 211,139 bp *A1166*:350 bp
C3123A (rs 11091046)	*AGTR2* (chr xq22-q23)	5′-GGATTCAGATTCTCTTTGAA-3′ 5′-GCATAGGAGTATGATTTAATC-3′	52	312 bp	*AluI*	*A3123*:215,107 bp *C3123*:312 bp
T344C (rs 1799998)	*CYP11B2* (chr X)	5′-CAGGAGGAGACCCCATGTGAC-3′ 5′-CTCCACCCTGTTCAGCCCAAAT-3′	64	536 bp	*HaeIII*	*C344*:202,138,125,71 bp *T344*:278,138,125 bp

PCR-RFLP- polymerase chain reaction-restriction fragment length polymorphism; RAAS- renin angiotensin aldosteron system; REN-renin; AGT-angiotensinogen; ACE- angiotensin converting enzyme; AGTR1- angiotensin II receptor type 1; AGTR2-angiotensin II receptor type 2; CYP11B2- aldosterone synthase; chr- chromosome, bp-base pair.

Table 2
Characteristics of the studied groups.

Variables	Control group (N = 130)	PE group (N = 87)	p-value*	LOPE (N = 54)	EOPE (N = 33)	p-value#
Maternal age, years[a]	28.4 ± 4.7	28.7 ± 5.1	0.721	28.9 ± 5.1	28.3 ± 5.3	0.835
Age[b]						
< 30 years	88 (67.7)	62 (71.3)	0.577	39 (72.2)	23 (69.7)	0.817
≥30 years	42 (32.3)	25 (28.7)		15 (27.8)	10 (30.3)	
SBP, mmHg[c]	130[120;130]	160[150;170]	< 0.001	160[150;170]	160[160;170]	< 0.001
DBP, mmHg[c]	75[70;80]	100[100; 110]	< 0.001	100[100;110]	100[100; 110]	< 0.001
BMI, kg/m2[c]	21.8[19.8; 23.7]	22.5[20.1; 26.1]	0.026	21.4[19.9; 25.4]	23.7[20.7; 27.7]	0.039
BMI status						
< 25	114 (87.7)	59 (67.8)	< 0.001	38 (70.4)	21 (63.6)	0.001
≥ 25	16 (12.3)	28 (32.2)		16 (29.6)	12 (36.4)	
Parity[b]			0.017			
Primipara	79 (60.8)	66 (75.9)	0.027	39 (72.2)	27 (81.8)	0.05
Multipara	51 (39.2)	21 (24.1)		15 (27.8)	6 (18.2)	
Smoking habits[b]			0.073			
No	124 (95.4)	77 (88.5)	0.067	48 (88.9)	29 (87.9)	0.105
Yes	6 (4.6)	10 (11.5)		6 (11.1	4 (12.1)	
OC[b]			0.093			
No	118 (90.8)	74 (85.1)	0.278	47 (87.0)	27 (81.8)	0.330
Yes	12 (9.2)	13 (14.9)		7 (13.0)	6 (18.2)	
Gestational age at delivery, weeks	39[38; 40]	35[32; 38]	< 0.001	37.5[36; 39]	31[28; 33]	< 0.001
Type of delivery[c]						
spontaneous	108 (83.1)	18 (20.7)	< 0.001	10 (18.5)	8 (24.2)	< 0.001
cesarean section	22 (16.9)	67 (77.0)		43 (79.6)	24 (72.7)	
induced or EHP	0 (0)	2 (2.3)		1(1.9)	1 (3.0)	
Maternal complications[b]						
no	130 (100)	83 (96.5)	0.062	51 (96.2)	32 (97.0)	0.062
yes	0 (0)	3 (3.5)		2 (3.8)	1 (3.0)	
Birth weight, grams	3324.9 ± 484.9	2559.7 ± 899.6	< 0.001	2860.1 ± 771.1	2067.9 ± 887.9	< 0.001
Birth weight[b]						
< 1500	0 (0)	11 (12.6)	< 0.001	2 (3.7)	9 (27.3)	< 0.001
1500–2500	5 (3.8)	30 (34.5)		17 (31.5)	13 (39.4)	
> 2500	125 (96.2)	46 (52.9)		35(64.8)	11 (33.3)	
Gender[b]						
F	66 (50.8)	47 (54.0)	0.638	27 (50.0)	20 (60.6)	0.564
M	64 (49.2)	40 (46.0)		27 (50.0)	13 (39.4)	
NICU[b]						
no	130 (100)	69 (81.2)	< 0.001	49 (90.7)	20 (64.5)	< 0.001
yes	0 (0)	16 (18.8)		5 (9.3)	11 (35.5)	
Neonatal morbidity [b]						
no	130 (100)	76 (89.4)	< 0.001	52 (96.3)	24 (77.4)	< 0.001
yes	0 (0)	9 (10.6)		2 (3.7)	7 (22.6)	
Neonatal complications[b]						
no	130 (100)	71(81.6)	< 0.001	47 (87.0)	24(72.7)	< 0.001
yes	0 (0)	16 (18.4)		7 (13.0)	9 (27.3)	
Laboratory determinations						
TG, mg/dL	205.5[176; 240]	280[237.5; 298]	< 0.001	271[190; 298]	280[260; 298]	< 0.001
LDL-cholestrol, mg/dL	86.5[65.4; 108]	143[115.7; 175]	< 0.001	132[99.6; 170]	163[134; 175]	< 0.001
HDL-cholesterol, mg/dL	59.5[48.4; 77.3]	38.0[34.1; 45]	< 0.001	39.8[33.5; 48]	38[35; 43]	< 0.001

PE- preeclampsia; EOPE- early-onset preeclampsia; LOPE- late-onset preeclampsia; SBP- systolic blood pressure; DBP- diastolic blood pressure; BMI- body mass index; TG- triglycerides; LDL-cholesterol- low density lipoproteins; HDL-cholesterol- high density lipoproteins; NICU- neonatal intensive care unit; [a] data described by arithmetic mean ± standard deviation; [b] data described by number of cases and percentages; [c] data described by median[first quartile; third quartile]; p-values obtained from Student-t test, Mann-Whitney test, ANOVA, Kruskal-Wallis, chi-square or Fisher's exact tests; * p-values from comparison between control and PE groups; * p-values from comparison between control and PE group #p-values from comparison between control, early-onset and late-onset PE groups. BMI and smoking habits are values presented before pregnancy

PE and parity (p = 0.017) and overweight (p < 0.001), and a tendency towards statistical association between PE and smoking habits (p = 0.073) and oral contraceptive use (p = 0.093).

Pregnant women with PE had lower values for gestational age on delivery compared to normal pregnant women, (median [IQR]: 35 [32; 38] vs. 39 [38; 40] weeks, p < 0.001). Newborns of mothers with preeclampsia had a lower mean birth weight (mean ± SD: 2559.7 ± 899.6 vs. 3324.9 ± 484.9) compared to those of the control group (p < 0.001).

3.2. Characteristics of the pregnant women in the study, regarding the onset of preeclampsia

The frequency of overweight was significantly different (p = 0.001) in pregnant women with EOPE, LOPE and the control group (LOPE: 16, 29.6% vs. EOPE: 12, 36.4% vs. control group: 16, 12.3%).

Pregnant women with EOPE had babies with a significantly lower mean birth weight (2067.9 ± 887.9) compared to the women with LOPE (mean ± SD: 2860.1 ± 771.1) (Games-Howell test: p < 0.001) and those with normal pregnancies, respectively (mean ± SD: 3324.9 ± 484.9) (Games-Howell test: p < 0.001).

3.3. Distribution of RAAS genotypes in the studied groups

The distribution of heterozygous and homozygous genotypes for all the 8 genetic variations is presented in Table 3. In the PE group, the studied AGT-*M235T* (p = 0.659), ACE-*I/D* (p = 0.326), AGTR1-*A1166C* (p = 0.064), AGTR2-*C3123A* (p = 0.168) gene polymorphisms did not deviate from Hardy-Weinberg equilibrium, while AGT-*T174M* (p = 0.033), ACE8-*A2350* (p = 0.004), CYP11B2-*T344C* (p = 0.0007)

Table 3
Distribution of genotypes of the studied RAAS gene variants in the studied groups.

Gene variants/ Genotypes	Control group (N = 130), n (%)	LOPE (N = 54), n (%)	EOPE (N = 33), n (%)	p-value[#]
AGT-M235T				
MM	70 (53.8)	17 (31.5)	7 (21.2)	0.002
MT	45 (34.6)	23 (42.6)	18 (54.5)	
TT	15 (11.5)	14 (25.9)	8 (24.2)	
AGT-T174M				
TT	104 (80.0)	32 (59.3)	17 (51.5)	0.003
TM	19 (14.6)	16 (29.6)	11 (33.3)	
MM	7 (5.4)	6 (11.1)	5 (15.2)	
ACE-I/D				
II	49 (37.7)	7 (13.0)	4 (12.1)	0.002
ID	51 (39.2)	28 (51.9)	17 (51.5)	
DD	30 (23.1)	19 (35.2)	12 (36.4)	
ACE8-A2350G				
AA	72 (55.4)	17 (31.5)	7 (21.2)	< 0.001
AG	37 (28.5)	20 (37.0)	10 (30.3)	
GG	21 (16.2)	17 (31.5)	16 (48.5)	
AGTR1-A1166C				
AA	94 (72.3)	29 (53.7)	18 (54.5)	0.059
AC	28 (21.5)	19 (35.2)	10 (30.3)	
CC	8 (6.2)	6 (11.1)	5 (15.2)	
AGTR2-C3123A				
CC	92 (70.8)	32 (59.3)	17 (51.5)	0.210
CA	29 (22.3)	17 (31.5)	12 (36.4)	
AA	9 (6.9)	5 (9.3)	4 (12.1)	
REN-G83A				
GG	94 (72.3)	26 (48.1)	19 (57.6)	0.009
GA	30 (23.1)	23 (42.6)	9 (27.3)	
AA	6 (4.6)	5 (9.3)	5 (15.2)	
CYP11B2-T344C				
TT	82 (63.1)	21 (38.9)	17 (51.5)	0.004
TC	34 (26.2)	16 (29.6)	11 (33.3)	
CC	13 (10.0)	17 (31.5)	5 (15.2)	

LOPE- late onset preeclampsia; EOPE- early onset preeclampsia; AGT- angiotensinogen; ACE-angiotensin converting enzyne; AGTR1- angiotensin II type 1 receptor; AGTR2- angiotensin II type 2 receptor; REN-renine; CYP11B2- aldosteron synthase; N = number of cases (% calculated within group or subgroup); p-chi-square tests, df = 2, df = degrees of freedom; #p-values from comparison between control, early-onset and late-onset PE groups; significance level $\alpha = 0.05$.

presented deviations from Hardy-Weinberg equilibrium.Table 4.

Compared to the preeclampsia group, in the control group the studied REN-*G83A* (p = 0.103) and AGT-*M235*T (p = 0.087) did not deviate from Hardy-Weinberg equilibrium, while AGT-*T174M* (p = 0.00005), ACE-*I/D* (p = 0.0326), ACE8-*A2350G* (p = 0.0004), AGTR1-*A1166C* (p = 0.013), AGTR2-*C3123A* (p = 0.007), CYP11B2-*T344C* (p = 0.005) gene polymorphisms were not within Hardy-Weinberg equilibrium.

3.4. Association between the studied gene polymorphisms and PE onset

The results of the univariate regression analysis showed that the risk to develop EOPE was significantly increased in carriers of the AGT-*M235T* variant genotypes MT + TT (unadjusted OR = 4.86), AGT-*T174M* variant genotypes TM + MM (unadjusted OR = 4.27) and CYP11B2-*C344T* variant genotypes TC + CC (unadjusted OR = 3.21) gene polymorphisms. Also, the risk to develop LOPE was significantly increased in carriers of the variant genotypes for AGT-*M235T* (unadjusted OR = 2.46), AGT-*T174M* (unadjusted OR = 2.59), ACE-*I/D* (unadjusted OR = 4.23), ACE-*A2350G* (unadjusted OR = 3.72) and AGTR1-*A1166C* gene polymorphisms (unadjusted OR = 2.61).

The multivariate logistic regression analysis showed that AGT-*M235T* (adjusted OR = 4.63), AGT-*T174M* (adjusted OR = 4.13), REN-*G83A* (adjusted OR = 3) and CYP11B2-*C344T* (adjusted OR = 3.13) gene polymorphisms remained independent risk factors for EOPE, while ACE-*I/D* (adjusted OR = 4.04), ACE-*A2350G* (adjusted OR = 3.5), AGTR1-*A1166C* (adjusted OR = 2.73), and REN-*G83A* (adjusted OR = 2.67) polymorphisms remained independent risk factors for LOPE (Table 3).

4. Discussion

The components of the RAAS system are involved in placentation, as well as in the remodeling of spiral arteries. Because RAAS polymorphisms affect RAAS activity, they may be associated with placental abnormalities and thus, with preeclampsia. Moreover, the aforementioned RAAS polymorphisms, that have been studied, related to different diseases in which the endothelial dysfunction is involved [12].

In a previous study, we investigated the effect of the interaction between maternal/fetal genotypes for AGT-*M235T*, AGT-*T174M*, ACE-*I/D*, ACE-*A2350G*, AGTR1-*A1166C*, AGTR2-*C3123A*, REN-*G83A* in 36 mother/newborn pairs as a risk factor for preeclampsia [23].

To the best of our knowledge, this is the first study on a cohort of preeclamptic women from Transylvania in which we attempted to quantify the associations between eight genetic variations (AGT-*M235T*, AGT- *T174M*, ACE-*I/D*, ACE8-*A2350G*, AGTR1-*A1166C*, AGTR2-*A3123C*, REN- *G83A*, CYP11B2- *T344C*) and the onset of preeclampsia and also, to establish independent predictors for them. We selected these polymorphisms considering their relation with hypertension and because they targeted all the RAAS components. They have been extensively studied in relation to hypertension during pregnancy, but with conflicting results. Furthermore, we did not find studies in preeclampsia patients to simultaneously investigate these genetic variants as predictive factors for the onset of preeclampsia.

Afshariani et al, *Aunga et* and two meta-analyses conducted by *Lin et al* and *Shanshan et al*, but not the study conducted by *Agharwal et al* showed that the presence of TT genotype (*M235T* polymorphism) is associated with hypertension during pregnancy [25–29]. Recently, *Zitouni et al* confirmed the implication of *T174M* polymorphism in the pathogenesis of preeclampsia, while *Shahvaisizadeh* et al reported that this polymorphism influences the risk of early-onset preeclampsia [30,31]. The GOPEC Consortium found no association with preeclampsia for *M235T* or *T174M* polymorphism [32 32].

Even though some studies consider that there is a link between elevated ACE levels and the presence of the D allele, the results regarding the role of the D allele in preeclampsia are controversial [11,12,33]. *González-Garrido et al*, *Eman et al*, *Haram et al* and showed differences between preeclamptic women and normal pregnant women regarding ACE-I/D genotypes, and considered that women carrying the D allele have an increased risk for preeclampsia because they have higher uterine artery resistance [16,34,35]. Moreover, *Eman et al* and *Uma et al* suggested that women carrying the DD genotype had higher risk to develop early-onset preeclampsia, but not late onset preeclampsia [34,36]. Also, *Chen et al* performed a meta-analysis on preeclampsia and reported a higher risk for preeclampsia in the presence of the D allele [11]. *Zhang et al* found a significantly higher risk for preeclampsia in association with the ACE8- *A2350G* polymorphism [37].

Regarding the AGTR1-*A1166C* variant, the study performed by *Seremak-Mrozikiewicz et al* revealed a higher risk for preeclampsia in the presence of the heterozygous and homozygous genotypes, but the review conducted by *Zhao et al* did not confirm the results [38,39]. However, the study conducted by *Eman et al* showed a weak association of the AGTR1-*A1166C* variant and late onset preeclampsia [33]. AGTR2-*C3123A* was associated with the risk of preeclampsia according to the study carried out by *Zhou et al* [19].

Conflicting results were obtained regarding the association of the *C344T* polymorphism and the risk of preeclampsia. This genetic variation, located in the 5′-flanking region of the CYP11B2 gene (involved

Table 4
Associations between RAAS gene polymorphisms and preeclampsia onset.

Gene variants/ Dominant models	Control group (N = 130), n (%)	LOPE (N = 54), n (%)	EOPE (N = 33), n (%)	LOPE vs. control group				EOPE vs. control group			
				COR[a] (99%CI)	p-value*	AOR[b] (99%CI)	p-value*	COR[a] (99%CI)	p-value*	AOR[b] (99%CI)	p-value*
AGT-M235T											
MM	70 (53.8)	17 (31.5)	7 (21.2)	1 (Reference)	0.007	1 (Reference)	0.018	1 (Reference)	0.001	1 (Reference)	0.002
MT + TT	60 (46.2)	37 (68.5)	26 (78.8)	2.46 [1.04;5.85]		2.32[0.93;5.75]		4.86 [1.38;17.1]		4.63 [1.30;16.5]	
AGT-T174M											
TT	104 (80.0)	32 (59.3)	17 (51.5)	1 (Reference)	0.007	1 (Reference)	0.039	1 (Reference)	0.0005	1 (Reference)	0.001
TM + MM	26 (20.0)	22 (40.7)	16 (48.5)	2.59 [1.05;6.39]		2.16[0.83;5.66]		4.27 [1.44;12.6]		4.13 [1.36;12.6]	
ACE-I/D											
II	49 (37.7)	7 (13.0)	4 (12.1)	1 (Reference)	0.001	1 (Reference)	0.003	1 (Reference)	0.013	1 (Reference)	0.014
ID + DD	81 (62.3)	47 (87.0)	29 (87.9)	4.23 [1.35;13.2]		4.04[1.22;13.5]		4.08 [0.95;17.5]		4.16 [0.94;18.5]	
ACE8-A2350G											
AA	72 (55.4)	17 (31.5)	7 (21.2)	1 (Reference)	0.0002	1 (Reference)	0.0008	1 (Reference)	0.023	1 (Reference)	0.029
AG + GG	58 (44.6)	37 (68.5)	26 (78.8)	3.72 [1.49;9.3]		3.50 [1.34;9.2]		2.60 [0.88;7.74]		2.56 [0.85;7.77]	
AGTR1-A1166C											
AA	94 (72.3)	29 (53.7)	18 (54.5)	1 (Reference)	0.004	1 (Reference)	0.005	1 (Reference)	0.231	1 (Reference)	0.301
AC + CC	36 (27.7)	25 (46.3)	15 (45.5)	2.61 [1.11;6.13]		2.73 [1.08;6.88]		1.65 [0.56;4.84]		1.56 [0.51;4.76]	
AGTR2-C3123A											
CC	92 (70.8)	32 (59.3)	17 (51.5)	1 (Reference)	0.043	1 (Reference)	0.098	1 (Reference)	0.175	1 (Reference)	0.152
CA + AA	38 (29.2)	22 (40.7)	16 (48.5)	1.95 [0.83;4.58]		1.83 [0.71;4.69]		1.75 0.61;5.05]		1.88 [0.60;5.88]	
REN-G83A											
GG	94 (72.3)	26 (48.1)	19 (57.6)	1 (Reference)	0.014	1 (Reference)	0.006	1 (Reference)	0.0	1 (Reference)	0.009
GA + AA	36 (27.7)	28 (51.9)	14 (42.4)	2.26 [0.96;5.32]		2.67 [1.06;6.7]		2.79 [0.97;7.99]	12	3.00 [1.01; 8.93]	
CYP11B2-T344C											
TT	83 (63.8)	21 (38.9)	17 (51.5)	1 (Reference)	0.048	1 (Reference)	0.109	1 (Reference)	0.005	1 (Reference)	0.007
TC + CC	47 (36.2)	33 (61.1)	16 (48.5)	1.90 [0.82;4.37]		1.73[0.72;4.18]		3.21 [1.10;9.41]		3.13 [1.05;9.38]	

RAAS- renin angiotensin aldosteron system; EOPE- early-onset preeclampsia; LOPE- late-onset preeclampsia; AGT- angiotensinogen; ACE-angiotensin converting enzyne; AGTR1- angiotensin II type 1 receptor; AGTR2- angiotensin II type 2 receptor; REN-renine; CYP11B2- aldosteron synthase; [a] Crude odds-ratio; [b] Adjusted odds-ratio for known maternal predictors of PE such as maternal age (≥ 30 years/ < 30 years), parity (multiparous/primiparous), smoking (yes/no), overweight (BMI ≥ 25 kg/m^2), * p-values obtained from multinomial logistic regression; significance level $\alpha = 0.01$ because of multiple comparisons.

in aldosterone synthesis), was positively associated with preeclampsia in a study conducted by *Escher et al* [40]. The study performed by *Remirez-Salazar et al* showed a negative association between preeclampsia and *C344T* polymorphism [41].

In the present study, the distribution of gestational age on delivery and birth weight was significantly different between preeclamptic women and controls, with lower values for pregnant women with preeclampsia. The frequency of neonatal complications and morbidities was significantly higher in the newborns of mothers with preeclampsia.

Neonates of mothers with early-onset preeclampsia had a significantly lower mean birth weight compared to newborns of mothers with late-onset preeclampsia or controls.

Results of univariable statistical analysis revealed a significant association between EOPE and AGT-*M235T* (p = 0.001), AGT-*T174M* (p = 0.0005), CYP11B2-*C344T* (p = 0.005) gene variants. In addition, we found a significant association between LOPE and AGT-*M235T* (p = 0.007), AGT-*T174M* (p = 0.007), ACE-*I/D* (p = 0.001), ACE-*A2350G* (p = 0.0002), AGTR1-*A1166C* (p = 0.004), and CYP11B2-*C344T* (p = 0.048) gene variants.

According to the multivariable model, the associations between AGT-*M235T* (p = 0.002), AGT-*T174M* (p = 0.001), REN-*G83A* (p = 0.009) and CYP11B2-*C344T* (p = 0.007) with EOPE remained significant as did the associations between ACE-*I/D* (p = 0.003), ACE-*A2350G* (p = 0.0008), AGTR1-*A1166C* (p = 0.005) and REN-*G83A* (p = 0.006) and LOPE.

Our results revealed that both AGT polymorphisms- *M235T* and AGT-*T174M* are independent predictors for EOPE but not for LOPE in this Romanian sample. On the other hand, ACE-I/D, ACE-A235G variants are independent predictors for LOPE but not for EOPE in this Romanian sample. According to our results, AGTR2-*C3123A* polymorphism does not represent an independent predictor neither for early, nor for late onset preeclampsia. REN-G83A is an independent predictor for both EOPE and LOPE. Moreover, CYP11B2- *C344T* polymorphism represents an independent risk factor for EOPE. However, no significant association between this genetic variation and LOPE was found.

The risk for preeclampsia is increased in smokers (OR = 2.44) and in the case of oral contraceptive use (OR = 1.96), with a tendency to statistical significance.

With regard to other known risk factors for PE, such as BMI, *Gonzales-Garrido et al* reported, in one of their studies, an increased risk for preeclampsia considering BMI changes [16]. In the current study, the onset of preeclampsia was significantly associated with the presence of overweight, the post-hoc analysis (pairwise comparisons by Fisher's exact test) highlighting a significant association both between EOPE and overweight (corrected p-values: $p_{FDR} = 0.009$), and between LOPE and overweight (corrected p-values: $p_{FDR} = 0.003$).

Over the past few years, an ever increasing number of studies have attempted to bring additional data on predictive factors for preeclampsia, especially on predicting its onset. This is due to the fact that

preeclampsia is an obstetric disorder with a major impact on maternal and fetal health, both in the short and long term. The first strength of the current study lies on the fact that it is the first study, as far as our knowledge goes, based on testing the 8 genetic variations targeting RAAS in a multivariable model in the Romanian population in order to establish the independent predictive genetic factors for early and late onset of preeclampsia. The second strength of the present study consists in quantification of the effect size for the studied gene variants adjusted for known maternal predictors of PE such as maternal age (≥ 30 years/ < 30 years), parity (multipara/primipara), smoking (yes/no), and overweight (BMI ≥ 25 kg/m^2).

The limitations of this study, however, refer to the small number of women diagnosed with preeclampsia, and also to the fact that information regarding smoking habits and oral contraceptive use was obtained by directly interviewing the women included in the study. Furthermore, assessments were performed at the beginning of the pregnancy, and not during the pregnancy period.

5. Conclusions

We confirmed the association between the renin-angiotensin-aldosterone system genetic variations in blood pressure regulation and the onset of preeclampsia. *M235T* and AGT-*T174M* are independent predictors for EOPE but not for LOPE, while ACE-I/D, ACE-A235G variants are independent predictors for LOPE but not for EOPE in this Romanian sample. REN-G83A is an independent predictor for both EOPE and LOPE.

Because the goal in the management of preeclampsia is to identify factors for improving the prevention of preeclampsia, to differentiate between early- and late-onset preeclampsia, we consider that other hypotheses, as well as epigenetic modifications should be investigated in the future.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.08.006.

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