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LC/MS/MS assessment of changes in placental angiotensin I metabolism in preeclampsia

Dominika Stettner-Kołodziejska[1], Beata Bujak-Giżycka[2], Anna Wiśniewska[3], Magdalena Łomnicka[3], Michał Kołodziejski[1], Marcin Wiecheć[1], Krzysztof Rytlewski[1], Hubert Huras[1], Rafał Olszanecki[3]

[1]Chair of Gynecology and Obstetrics, Jagiellonian University Medical College, Kraków, Poland
[2]Department of Clinical Pharmacology, Jagiellonian University Medical College, Kraków, Poland
[3]Chair of Pharmacology, Jagiellonian University Medical College, Kraków, Poland

Corresponding author: Beata Bujak-Giżycka, Ph.D.
Department of Clinical Pharmacology, Chair of Pharmacology, Jagiellonian University Medical College
ul. Grzegórzecka 16, 31-531 Kraków, Poland
Phone: +48 12 421 11 68; E-mail: beata.bujak-gizycka@uj.edu.pl

Abstract: B a c k g r o u n d: Preeclampsia (PE) is a condition characterized by high blood pressure and significant proteinuria in pregnant women. It affects about 7% pregnancies and can be cause of fetal and maternal morbidity and mortality. During pregnancy, a physiological overexpression of the Renin-Angiotensin System (RAS) components is observed, including increased plasma Ang II level. Dysregulation of RAS in placenta may contribute to preeclampsia and uterine growth retardation. The aim of the study was to evaluate the Ang I metabolism in human preeclamptic placentas and to compare to normal pregnancies condition.
M e t h o d: Fragments of placental tissues were collected right after ceasarian section from PE and physiological pregnancies. Tissues were incubated in Krebs buffer in the presence of Ang I. Evaluation of Ang I metabolites in incubating fluid was performed by LC/MS/MS method. mRNA expression of main RAS components was measured by RT-PCR.
R e s u l t s: Pattern of angiotensin metabolites did not differ between groups. The main products were Ang 1–7 and Ang II. Comparing to control group, more than 3-fold lower production of Ang II and Ang 1–7 in preeclampsia was observed. mRNA expressions of ACE and AT1 were significantly decreased in preeclamptic placentas, whereas higher expression of mRNA of ACE2 and MAS receptor were observed.
C o n c l u s i o n s: Production of Ang 1–7 by PE placentas was significantly lower than in control group. Significantly decreased mRNA expression of ACE and AT1 receptor and lower production of Ang II in placentas of PE patients suggest that placental Ang II/ACE/AT1r pathway could be less important than Ang 1–7/ACE-2/MASr pathway in development of preeclampsia, but this requires further investigations.

Keywords: angiotensinogen metabolism, placenta, preeclampsia, angiotensin II, RAS.

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Introduction

The renin-angiotensin-aldosterone system (RAAS) plays a crucial role in regulation of arterial blood pressure and the water and electrolyte balance.

In the plasma, angiotensin II (Ang II) is the predominant product of angiotensin I (Ang I) metabolism, while in tissues and organs, many different products are created, with angiotensin-(1–7) [Ang-(1–7)] and angiotensin II as main metabolites. The biological effects of the latter are widely described in literature [1–3]. It is well known that its increased production is responsible for a number of pathological changes in the cardiovascular system (remodeling of arterial walls and heart muscle, activation of thrombotic and inflammatory processes and oxidative stress) during the development of hypertension, atherosclerosis, and diabetes. It was demonstrated that Ang II via AT1 receptor stimulates the production of major vasoconstrictor — endothelin I and impairs the production and activity of vasodilatory NO and PGI_2 [4–6]. Importantly, Ang-(1–7) has been demonstrated to counteract the Ang II and exert vasodilatory, antiproliferative, anti-inflammatory, and anti-angiogenic actions [7, 8]. Ang-(1–7) is produced primarily in tissues on three major pathways: as a direct product of Ang I conversion under the influence of neutral endopeptidase (NEP), as a result of the decomposition of Ang-(1–9) (under the influence of ACE, NEP) or conversion of Ang II (under the influence of ACE2). Ang (1–7) was shown to act through specific Mas receptor, that differs to the AT1 and AT2 receptors for Ang II [7, 8].

During pregnancy, the activity of RAAS, both in blood and in many tissues, is typically increased [9–11]. The ACE is the only RAAS component, which concentration in the blood is decreased [12, 13]. It was demonstrated that Ang II participates in regulating uterine and placental vascular resistance and blood flow — its high concentrations decrease uterine-placental blood flow, while low concentrations contribute to its increase [14–16]. Abdul-Karim, Assalin *et al.* were probably the first to notice that pregnancy is characterized by decreased vascular sensitivity to the vasoconstrictive effect of Ang II [17]. Gant *et al.* explained this "Ang II resistance" by increased production of progesterone and prostacycline during pregnancy [18, 19]. However, according to recent reports, the "Ang II resistance" may result from the differences in structure of the AT1 receptor, which is monomeric in physiological pregnancy, whereas in non-pregnant patients — i.e. in a state of sensitivity to Ang II — it is heterodimeric. It has been shown, that the monomeric form of AT1 receptor exerts lower physiological activity, and may be easier inactivated by reactive oxygen species than dimeric receptor, what contributes to the control of RAAS activity during physiological pregnancy [20]. It may well be, that disturbances in local activity of RAAS, especially in terms of disbalance between Ang II and Ang-(1–7) may play an important role in conditions involving placental pathology, such as preeclampsia.

Severe preeclampsia remains one of the leading causes of maternal mortality (10–15%) and increases the risk of premature birth (15%), as the only ultimately effective causal treatment of preeclampsia or eclampsia is the delivery of the baby [21–24]. It is a systemic and multisystem disease which involves an increase in peripheral vascular resistance, increased platelet aggregation, and endothelial dysfunction [25].

In spite of continuous progress in medical research, the etiopathogenesis of pre-eclampsia still has not been fully understood. Factors listed as potentially related to its occurrence include the mother's age, first pregnancy, presence of inflammatory diseases in the mother, insufficient supply of nutrients (calcium, vitamins), insulin resistance, and genetic factors. Immunological disorders are a significant element of the disease and, in consequence thereof, oxidative stress, abnormal trophoblast invasion, and impaired maternal-fetal tolerance [26–29].

Literature still does not offer much information about the function (or dysfunction) of the local placental RAAS in preeclampsia. Compared to physiological pregnancy the deregulation of plasma RAAS is observed — the levels of renin, aldosterone, Ang I, and Ang II are lower, as well as the levels of Ang-(1–7) [30, 31]. In spite of overall lowering of the expression of RAAS components, in preeclampsia the increased sensitivity to Ang II has been demonstrated e.g. evidenced by increased vasospastic response to Ang II [32]. A number of studies have confirmed the presence of all RAAS components in the placenta [33–36]. Consequently, the participation of a local placental RAAS in the pathogenesis of pre-eclampsia, or intrauterine growth restriction has been argued, however the matter remains unclear, largely due to methodological difficulties requiring the use of many different approaches to measurements of angiotensins. We have shown that the use of mass spectrometry to assess the major networks of Ang I metabolism in various tissues ex vivo may provide valuable insight into the balance between local Ang II / Ang-(1–7) generation in tissues [37, 38]. In this study we evaluate the ability of the preeclamptic placental tissue to produce various metabolites of Ang I using a previously established ex-vivo model and an LC/MS/MS method and to compare to results obtained from normal pregnancies condition.

Material and Methods

Study design

A total of 28 women took part in the study, hospitalized at the Clinical Ward of the Obstetrics and Perinatology Clinical Department at the University Hospital of the Jagiellonian University Medical College in Kraków. 14 patients constituted the study group (with diagnosed pre-eclampsia); 14 patients were the control (comparative) group (physiological pregnancies, with normal course of pregnancy, carried to term, without any concomitant diseases or obstetric complications).

All pregnant patients participating in the study, were asked to give their informed consent to take part in the research, have samples of their placentas collected and tested, and for basic data to be collected. The study possesses resp. consent of the Bioethical Committee of the Jagiellonian University no. KBET/345/B/2012.

The **inclusion criteria** for the study group included: hypertension after the end of the 20th week of gestation >140/90 mmHg with concomitant proteinuria >300 mg/day or ≥3+ in a strip test (if the time of admission to hospital or the progress of the disease made daily urine collection impossible, with no concomitant diseases that could distort the results). The control group included healthy pregnant women with unifetal pregnancies carried to term, admitted to hospital in order to deliver the baby by a planned Cesarean section.

The **exclusion criteria** included multiple pregnancies, congenital diseases in the fetus, concomitant diseases other than gestational hypertension (including diabetes, cardiovascular diseases, immunological diseases), and lack of consent to participation in the study.

Patients who had a C-section performed to deliver their babies were qualified for analysis (both in the study group and in the control group). This was caused by the fact that this is the most common way of delivering babies in patients with severe pre-eclampsia or imminent eclampsia, due to the risk to the mother's and/or fetus' life.

In the control group, indications for C-section primarily included state after C-section in the previous pregnancy, breech position in the case of primiparas or maternal-fetal disproportion. It was decided that the same manner of delivery in both groups contributes to maximal possible homogeneity of comparisons.

Tissue collection

Fragments of placental tissue were collected right after its extraction during Caesarean section from women with pre-eclampsia and in physiological pregnancy.

Placenta pieces measuring approx. 1 × 1 cm in area and approx. 3 cm across the entire thickness of the placenta were collected from the peri-umbilical region using a sterile scalpel and surgical scissors (the region of the placenta was selected on the basis of preliminary studies [35]). The placental tissue was washed in a cold 0.9% NaCl solution, then placed in a sterile container filled with cold 0.9% NaCl, and transported to the laboratory (time between collection and delivery of the material did not exceed 60 minutes in any of the patients).

Delivered tissue fragments were immediately divided into three parts: for histopathological evaluation (protected in 10% buffered formalin, stored in a refrigerator), for RT-PCR tests (in RNA later, frozen at –80˚C), and for LC-MS/MS tests (placed in Krebs buffer, immediately tested).

Histopathology

Placenta samples taken from the peri-umbilical area in plastic containers in a 10% buffered formalin solution were delivered to the laboratory, where they were processed by a pathologist, placed in histological cassettes and again in buffered formalin for 24 hours in order to thoroughly fix the material. After this time, the sections were placed in a vacuum tissue processor where they were subjected to an automatic processing process. Next, the sections were embedded in paraffin blocks and then cut into sections with a thickness of 4 μm. The sections were stained with hematoxylin and eosin.

The sections placed on slides were deparaffinized in xylene, rehydrated through graded alcohols to water. Next, the slides were stained with Harris hematoxylin and eosine (15 min) according to standard protocol. The stained sections were examined under an Olympus microscope with a digital camera.

Ex vivo conversion of angiotensinogen fragments

Small fragments of placental tissue (in triplicates) were washed with cold, standard Krebs-Henseleit solution and cleaned of thrombi and tissue remnants. Then, tissue fragments were incubated in Krebs buffer (at 37°C) in the presence of Ang I (at final concentration of 1 uM). After 15 minutes of incubation, supernatants were collected and frozen in –80°C until further analysis. Placentas fragments were dried at 60°C and weighted to allow estimation of peptides production per mg of dry tissue.

Sample purification for LC/MS/MS analysis

After thawing, supernatants were purified and concentrated using Ultra-Micro Spin C-18 columns (Harvard Apparatus, USA). Acidified samples were applied on and centrifuged (2 min, 1000 × g). Then, columns were washed with 300 μl of 0.1% TFA and angiotensin peptides were eluted by centrifugation with 300 μl of 0.1% TFA in 40% acetonitrile. Samples eluates were lyophilized, and dry residues were reconstituted in 500 μl of 0.1% FA for further LC/MS/MS analysis. Samples for calibration curves of each examined peptide (mixture of standards in Krebs buffer) were prepared in the same mode as above.

LC/MS/MS analysis

Angiotensin peptides — angiotensins: I (Ang I), II (Ang II), III (Ang III), IV (Ang IV), 1–9 (Ang 1–9), 1–7 (Ang 1–7), and 1–5 (Ang 1–5), were purchased from Bachem (USA). All reagents and solvents used in the LC/MS/MS analysis — formic acid (FA), trifluoroacetic acid (TFA) (both purchased from Fluka) and acetonitrile (ACN; from JT Baker) were LC/MS grade.

Separation of angiotensin peptides was performed on a reversed-phase HPLC system (Dionex Ultimate 3000, Thermo Scientific USA), using an Acclaim® PepMap 100 C18 column (150 mm × 300 μm ID, 5 μm particle size, 100 Å) with a guard column C18 PepMap 100 (5 mm × 300 μm, 5 μm, 100 Å) (Thermo). The mobile phase solvents were: 0.1% FA in 2% ACN (phase A) and 0.1% FA in 80% ACN (phase B). Samples were injected onto chromatographic column (oven temperature 40°C) in a volume of 10 μl and separated at a flow rate of 2.5 μl/min with a 60 min. linear gradient from 10% to 60% B over 42 min.

Mass spectrometric detection was performed using an LCQ ion-trap mass spectrometer (Thermo Scientific, USA), with nanospray liquid junction ion source (source voltage 1.5 kV; capillary temperature 250°C, positive ion mode). For detection, selected reaction monitoring (SRM) mode was used (collision energy 35%; maximum injection time 100 msec, 2 μscans, isolation width 1.0 amu). Monitored ion pairs (parent ion — daughter ion) were as follows: 649–784 (Ang I), 1183–1045 (Ang 1–9), 524–784 (Ang II), 899–784 (Ang 1–7), 931–913 (Ang III), 775–513 (Ang IV) and 665–550 (Ang 1–5). The acquired data was analyzed by Xcalibur Software v.2.07.

Real-time RT-PCR

Total RNA was extracted from 30 mg pieces of human placentas using the RNeasy Fibrous Tissue Mini Kit (QIAGEN) with provided standard protocol. The concentration and purity of RNA was determined on an EPOCH microplate spectrophotometer (BioTek). A reverse transcription reaction was performed with 1 ug of RNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). All cDNA samples were diluted 10-fold prior to Real-Time PCR amplification.

Relative gene expressions analysis in human placentas was carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems). qPCR reaction was performed on 96-wells plate with TaqMan primers and probes (Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol. TaqMan Fast Advanced Master Mix and commercially available TaqMan Gene Expression Assays (Applied Biosystems) for human ACE (Hs00174179_m1, NM_00789.3), ACE2 (Hs01085333_m1, NM_021804.2), MME (Hs00153510_m1, NM000902.3), AGT (Hs01586213_m1, NM_000029.3), AGTR1 (Hs00258938_m1, NM_004835.4) and MAS1 (Hs00267157_s1, NM_002377.2), were used according to the manufacturer's instructions. The normalization was performed using the geometric mean of the two housekeeping genes, GAPDH (Hs99999905_m1, NM_002046.4) and HPRT1 (Hs01003267_m1, NM_000194.2). Endogenous control genes were selected on the basis of a pilot experiment. Relative expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) using DataAssist Software (Applied Biosystems).

Statistical analysis

Data analysis started with a statistical description of the study and control groups, verifying the lack of differences between the groups with the use of the chi-square test in the case of qualitative data and the Student's t-test for continuous data, with the adopted level of statistical significance of $p < 0.05$. This way, the age, BMI, origin, parity, previous miscarriages, pregnancy duration at the moment of delivery, and the baby's sex were compared. Then, the frequency of the occurrence of complications and the mean values of the patients' clinical parameters, differentiating both groups, were assessed. In the case of qualitative data, the chi-square test was used, whereas for quantitative data — the Student's t-test or, if the assumptions were not met, the non-parametric Mann–Whitney U test. The adopted statistical significance for all calculations was $p < 0.05$. It was agreed that the groups did not differ if Levene's test of homogeneity of variance and Student's t-test indicated $p > 0.05$. For qualitative variables, similar assumptions were met by the chi^2 test. Statistical analysis was performed using Statistica software, version 12.0.

The concentrations of angiotensin peptides were expressed as pg/mg of dry tissue. All the values placed at the figures and in the text are expressed as mean ± SD of *n* observations. Peptide levels were compared using a non-parametric Mann–Whitney test. The Student's t-test was used in the analysis of the RT-PCR data. A *p* value of less than 0.05 was considered statistically significant.

The correlation between the level of major angiotensins and systolic/diastolic blood pressure, ACE and AT1R expression was performed using the non-parametric Mann–Whitney test with the non-parametric Spearman's correlation coefficient. All the statistical analyses were performed using GraphPad Prism 5.0.

Results

Demographical data

The study and control groups were not significantly different in terms of age, BMI, place of residence or the sex of the baby, they did differ, however, in terms of the duration of pregnancy and parity. In all cases, the indication for an emergency C-section was imminent eclampsia. Among patients from the control group, multiparas were predominant (85%), with pregnancies carried to term, and the indications for delivery included obstetric reasons unrelated to any disease entities (primarily state after previous C-section — n = 8; lack of progress of labor — n = 4; pubic symphysis diastasis — n = 2). Due to the presence of pre-eclampsia in the study group, the differences between the groups were also observed in the clinical parameters and the condition of the baby after birth. No differences were observed in circulation in the

fetus, taking into consideration the Doppler parameters — pulsatility index of umbilical artery and middle cerebral artery (PI UA, PI MCA). The newborn's body mass and length were significantly larger in the control group, and so was the condition of the newborn evaluated using the Apgar score, which results from preterm delivery in the study group (Table 1).

Table 1. General characteristics of the study and control groups — quantitative and qualitative variables.

Compared factor	Preeclampsia (n = 14)	Control (n = 14)	p-value
Age [years] [mean +/– SD (95% CI)]	30.1 +/– 6.7 (26.2–34.0)	31.6 +/– 4.8 (28.8–34.3)	0.5032 (1)
BMI [mean +/– SD (95% CI)]	28.2 +/– 4.8 (25.4–31.0)	27.2 +/– 3.0 (25.5–28.9)	0.5291 (1)
Gestational age at delivery	33w4d; (30w4d–37w)	39w0d; (38w4d–39w)	**0.0001** (1)
Inhabitancy (city/village)	5/9 (35.7/64.3)	9/5 (64.3/35.7)	0.1306 (2)
Gravidity (primi-/multigravida)	11/3 (78.6/21.4)	2/12 (14.3/85.7)	**0.0006** (2)
Miscarriage in the past	6 (42.9%)	5 (35.7%)	0.6988 (2)
Indications for termination of pregnancy (urgent/elective)	14/0 (100/0)	3/11 (21.4/78.6)	**<0.0001** (2)
Gender (male/female)	5/9 (35.7/64.3)	7/7 (50/50)	0.4450 (2)
Systolic BP [mmHg] [mean +/– SD (95% CI]	164 +/–17 (153–174)	109 +/–11 (103–116)	**<0.0001** (1)
Diastolic BP [mmHg] [mean +/–SD (95% CI)]	103 +/–13 (96–111)	73 +/–10 (67–79)	**<0.0001** (1)
MAP [mmHg] [mean+/–SD (95% CI)]	123 +/–14 (116–131)	85 +/–9 (80–91)	**<0.0001** (1)
Proteinuria [g] [mean+/–SD (95% CI)]	4.98 +/–6.04 (1.49–8.47)	0	**0.0001** (3)
Birth weihght [g] [mean +/–SD (95% CI)]	1767 +/–726 (1348–2186)	3363 +/–511 (3068–3659)	**<0.0001** (1)
Infant length [cm] [mean +/–SD (95% CI)]	44 +/–6 (40–47)	53 +/–3 (51–55)	**<0.0001** (1)
APGAR score [>8/≤8 n (%)]	8/6 (57/43)	14/0 (100/0)	**0.0057** (2)

BP — blood pressure; MAP — mean arterial pressure; (1) student's T-test; (2) Chi-squared test; (3) Mann–Whitney U test

Histopathological assessment

In all the placentas of patients with pre-eclampsia, histopathological changes typical for the condition were found, with examples presented in Figures 1–4.

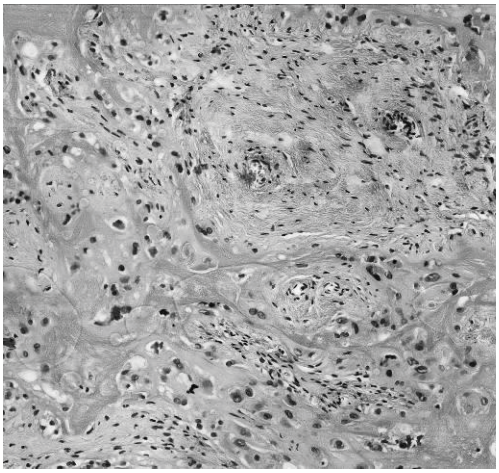

Fig. 1. Decidual spiral arteries — thickened wall and narrowed lumen — HE 100×.

Fig. 2. Consequences of vascular changes — placental infarction and blood clot inside vessel lumen HE 50×.

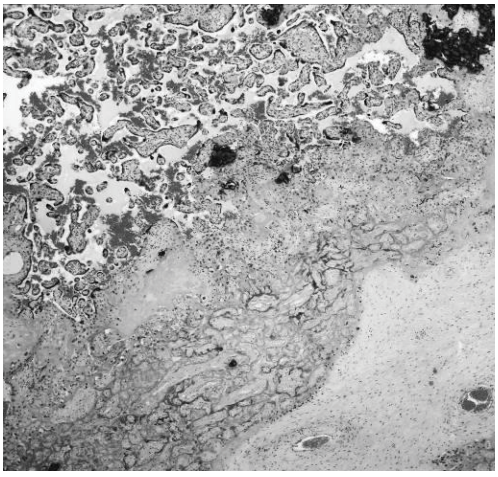

Fig. 3. Placental infarction and calcifications. HE 50×.

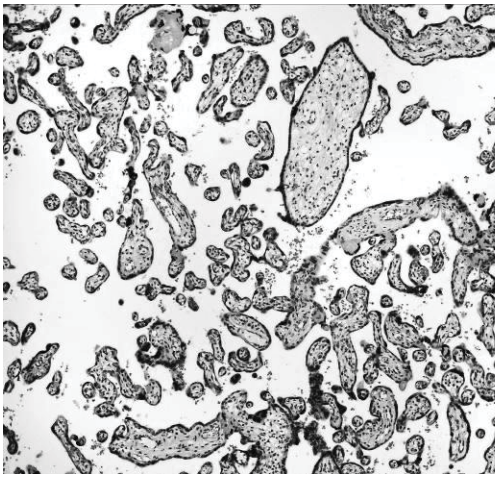

Fig. 4. Distal villous hypoplasia. HE 50×.

Assessment of the metabolism of exogenous Ang I in placental tissue ex vivo

The metabolism of exogenous Ang I in the placental tissues was very efficient, and the method used enabled precise estimation of the formation of individual peptides. All the expected metabolites were identified. Ex vivo, the main product of the breakdown of Ang I in the placenta was Ang-(1–7), the remaining metabolites (Ang II, Ang III, Ang IV, Ang-(1–9), and Ang-(1–5)) were formed at a much lower level. During initial measurements, the activity of a typical ACE inhibitor was examined. The inhibitor was administered to the tube where parts of the placenta were incubated. As expected, pre-incubation with perindoprilat caused a considerable decrease in the production of Ang II, both in the study group and in the control group, on average by about 90% (study group — 86.02%, control group — 90.05%). These results indirectly show the predominant role of ACE in the conversion of Ang I to Ang II in the placental tissue.

The metabolism of exogenous Ang I proceeded in a similar way in the study and control groups. Ang-(1–7) was the main product of the breakdown of Ang I in both groups, which shows a similar activity of enzymes responsible for the conversion of Ang I to Ang-(1–7) (Fig. 1).

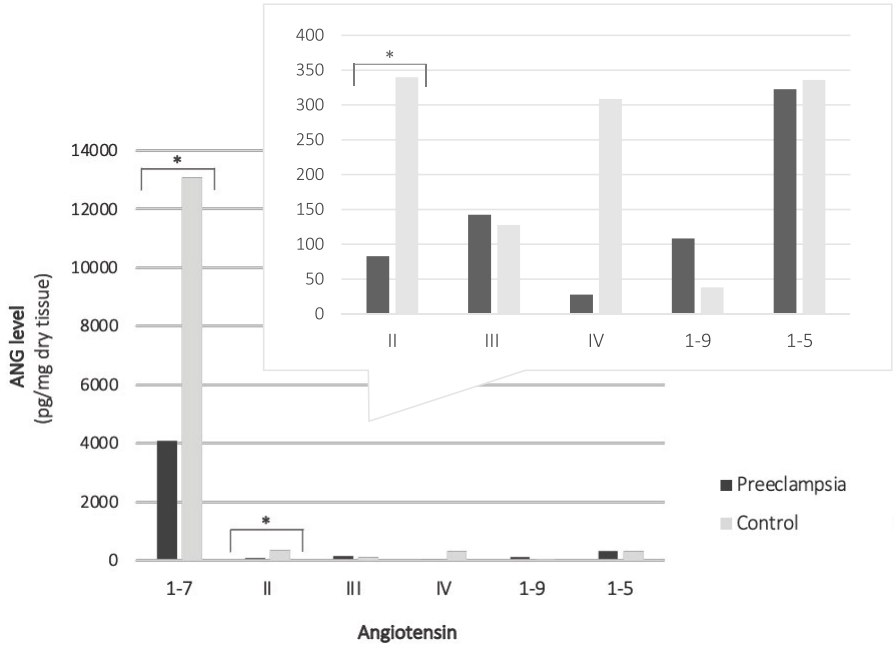

Fig. 1. Comparison of Ang-I metabolites production in human placenta (study and control group) — mean values and standard deviations (pg/mg dry tissue). The insert presents enlarged part of the chart showing the metabolites other than Ang-(1–7). P-value for individual angiotensins: Ang-(1–7)-0.0086; Ang II-0.0086; Ang III-0.2145; Ang IV-0.7272; Ang-(1–9)-0.1856; Ang-(1–5)-0.1932. * p <0.05

The formation of basic metabolites of Ang I, i.e. Ang II and Ang-(1–7), was considerably lower ($p < 0.01$) in the placentas of patients with pre-eclampsia compared to the control group (Fig. 1). The average ratio of Ang-(1–7) to Ang II concentrations, providing a general overview of the relative dominance of individual metabolite formation, was lower in the study group, however, the difference was not statistically significant (Table 2).

Table 2. Comparison between main products of Ang I in PE and control group.

	Preeclampsia	**Control**	**Mann–Whitney U test**
Ang II	96.23 (14.30–285.23)	405.44 (82.15–1207.85)	** **p <0.01**
Ang 1–7	3807.19 (97.23–14,358.57)	12926.28 (1342.84–37,752.32)	** **p <0.01**
Ang 1–7/Ang II	38.79 (2.23–128.17)	71.37 (2.29–287.76)	ns.

The levels of other Ang I metabolites were also lower in the study group than in the control group, yet they were not statistically significant either. The absence of statistical significance in spite of the clear differences between the groups — visible in the figure — in terms of average angiotensin levels, in particular in the case of Ang IV, resulted from large dispersion of measurement values in individual patients and from the small size of the groups (Fig. 1).

Expression of mRNA of main RAS components in the placenta

The results obtained from the measurement of gene mRNA expression for individual components of RAS in the placentas indicate lower mRNA expression for the AT1 receptor (statistically significant difference, to an expression of less than 50% in the control placenta); in the placental tissue of patients with pre-eclampsia, mRNA expression was also lower for ACE (to the level of 60% in the control placenta), yet without statistical significance. mRNA levels for the elements of an alternative conversion pathway of Ang I (ACE2, NEP, Mas receptor) demonstrated a slightly higher expression in patients with pre-eclampsia than in the control group, but the differences were not statistically significant (Fig. 2).

The correlation between the values of systolic and diastolic blood pressure and the levels of key metabolites of Ang I — Ang II and Ang-(1–7) — formed ex vivo, was also examined. Both in the control group and in the group of patients with pre-eclampsia, no correlation between the levels of Ang II, Ang-(1–7) or the values of the Ang-(1–7)/Ang II ratio or mRNA expression for ACE in the placenta and the values of systolic or diastolic blood pressure were found, therefore, the results are not presented herein.

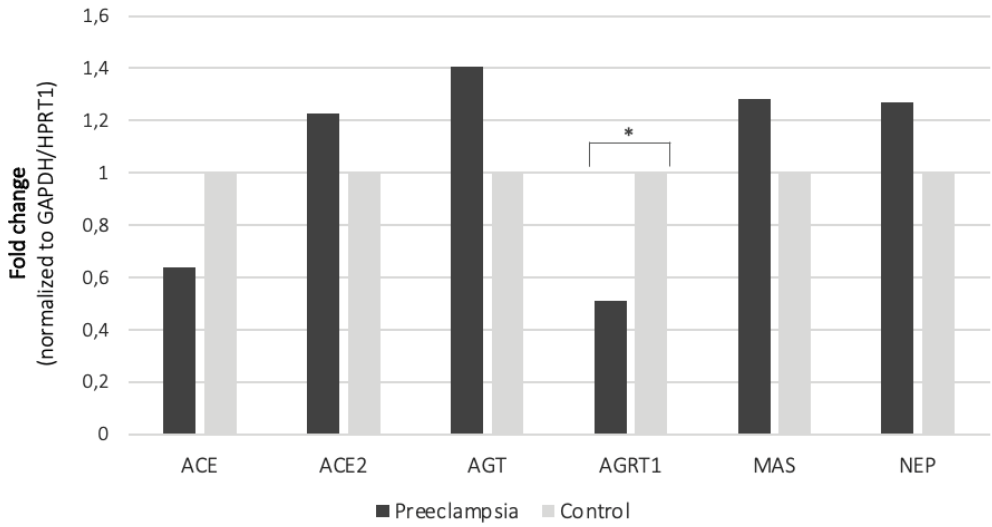

Fig. 2. Gene expression for individual components of RAA system in PE and control.

Discussion

In this study, we compared the Ang I metabolism in placental tissues from preeclamptic and physiological pregnancies, using previously established ex vivo model and LC/MS method [37, 38].

The study showed that in ex vivo conditions, placental tissues efficiently metabolize exogenous Ang I to an entire range of angiotensin peptides, which represents yet another confirmation of the activity of the placental RAAS and the argument that the angiotensins produced there may cause important biological effects. The overall lower levels of most peptides in the study group might indicate lower RAAS activity in the placenta in pre-eclampsia. Our results seem to be consistent with data found in literature which shows that compared to physiological pregnancies, where increased RAS activity occurs both in the blood and in many tissues, in patients with pre-eclampsia the plasma levels of individual RAS components (renin, aldosterone, Ang I, and Ang-(1–7)) are lower. It should be noted, however, that a systemic deficit does not necessarily have to go hand in hand with a deficit in the tissues. There is only few data found in literature about the formation and/or activity of different angiotensins in the placenta in pre-eclampsia. In a study in which the authors examined the level of Ang-(1–7) and ACE2 in the placenta, a similar expression of both these RAS components was found in the samples from placentas from physiological pregnancies and pre-eclampsia [39]. Kalenga *et al.* did not demonstrate qualitative or quantitative changes

in RAS activity (active renin, prorenin, ACE, and Ang II) in placentas from physiological pregnancies and pregnancies complicated by pre-eclampsia, either. Anton *et al.* demonstrated higher levels of Ang II in the placentas of women with preeclampsia [40]. Similarly, Gao *et al.* also described an increased level of Ang II and a similar level of Ang-(1–7) in the blood in the maternal side of the placenta in preeclampsia compared to physiological pregnancy [41]. While in the former cited article the authors concluded that the lack of differences in the tissues from a physiological pregnancy and preeclampsia does not exclude a cause and effect relationship between Ang-(1–7)/ACE2 deficiency in preeclampsia (which is indicated by other studies describing a lower level of circulating Ang-(1–7) in PE), the latter paper primarily raised the issue of decreased physiological response to a higher quantity of Ang II (dependent on the lowered expression of AT1R) as the main indicator of placental vessel dysfunction in preeclampsia. In the context of the article by Gao *et al.*, our results suggest deep, global impairment of RAS activity in patients with pre-eclampsia. A confirmation of this observation and explanation of the significance of such impairment in the pathogenesis of preeclampsia requires further research.

In this study, in both groups, Ang-(1–7) turned out to be the main metabolite of Ang I in the placenta. At the same time, using our ex vivo model, we confirmed that it is ACE, that is primarily responsible for the production of Ang II in the placenta, which expression tends to be lower in patients with preeclampsia. Compared to the control group, the levels of expression of ACE2 and NEP in the placenta in the study group did not change, and it may be speculated that it is the decrease in ACE expression [and subsequent lower activity of decomposition pathway of Ang I to Ang-(1–7)] with maintained NEP activity, that may be collectively responsible for the relative accumulation of Ang-(1–9) and decreased production of Ang-(1–7) in the group of patients with preeclampsia. It should be noted that in the study group, Ang-(1–7) was still generated by placental tissues in much larger quantities than Ang II. Our results seem to be inconsistent with the results of Anton *et al.*, who demonstrated almost twofold increase of the quantity of Ang II in placental villi in pre-eclampsia, with no significant differences (as compared to physiological pregnancies) in the levels of Ang I or Ang-(1–7) [42]. This discrepancy may be explained by differences in the methodology of measurements and the interpretation of results — in the cited studies, the authors measured the level of peptides in placental homogenates using the radioimmunoenzymatic method, and interpreted them as the actual *levels in the tissue*, whereas the results obtained in this study should be interpreted as an indicator of *the tissue's ability to convert Ang I to individual products*. Controversies regarding the profile of formation of individual Ang I products in the placenta in physiological pregnancy and in patients with placental abnormalities/dysfunction warrant the need for further research on this issue, with the use of various methods and experimental systems.

It should be noted that regardless of the method used, the assessment of local formation of angiotensins constitutes an important, yet not the only factor enabling the assessment of the physiological activity of RAAS in the placenta. In this context, the assessment of RAAS effector molecules — in particular angiotensin receptors — is also very important.

Our results indicate decrease of expression of AT1R mRNA in the placental tissue of patients with PE. It is contradictory to the most studies, which emphasize an increase in the level of AT1R. An increase in the expression of the AT1 receptor in pre-eclampsia, demonstrated with the use of immunohistochemical tests, as well as the RT-PCR and Western blot techniques, was observed by Thapa *et al.* and Leung *et al.* [43, 44]. An increase in AT1R expression was also demonstrated by Knock *et al.* using autoradiography in the villi of patients with PE [45]. In another, more extensive study, using immunofluorescence methods and RT-PCR, much higher AT1R expression was demonstrated in the placenta compared to the decidua, regardless of preeclampsia, yet still in the decidua of patients with pregnancies complicated with pre-eclampsia AT1 receptor expression was five times higher than in patients with physiological pregnancies (it should be noted, however, that such differences between groups were absent in placental expression) [46]. Anton *et al.* also demonstrated a threefold increase in the AT1 receptor mRNA regulation in placental villi of patients with pre-eclampsia, however, the receptor binding strength did not differ between the study and control groups. The surprising discrepancy between our results and data from literature clearly requires further studies, especially those precisely locating AT1R in the individual components of the placenta.

Noteworthy, the data available in literature concerning changes in the placental expression of ACE — an angiotensin converting enzyme connected with the "classic" conversion pathway of Ang I to Ang II — are strikingly inconclusive. In our study, in patients with PE, mRNA expression for ACE tended to be lower, while Herse and Kalenga found no differences in ACE, ACE2 and NEP levels between the placentas from physiological pregnancies and pre-eclamptic patients [32, 47]. Other authors, demonstrated higher activity of ACE in the umbilical cord venous endothelial cells than in arterial cells, and therefore suggested that the placental and umbilical cord venous endothelial cells may be the primary location of Ang I conversion to Ang II affecting the fetal-placental circulation [48–50]. Our results indicate no considerable changes in the expression of NEP, ACE2 and Mas in the placenta of patients with PE. There is very little data about the levels of these "non-classic" components of RAAS in the placenta. Anton *et al.* demonstrated that the level of Mas receptor mRNA is lower in the villi from the placentas of patients with pre-eclampsia than from physiological pregnancies (which could indicate impaired activity of Ang-(1–7)). Interestingly, they also showed that the level of AT2 receptor mRNA was exceptionally low (below detection level) [42]. All the discrepancies

described above clearly indicate high heterogeneity of RAAS in the placenta and the need for further comprehensive research on the functioning of this important system in the physiopathology of the placenta.

On account of the use of a new experimental system and analytical technique, our study should be considered as preliminary. A definite advantage of the study is the careful selection of patients — in order for them to constitute the most homogeneous group possible. Thus one can assume that all differences between the groups (parameters such as arterial pressure, proteinuria, parity, duration of pregnancy, newborn body mass and length) result from the natural course of the disease. In the study group, all of the babies were delivered by an emergency C-section, so both the gestational age and the body mass and length were lower. The lack of significant differences in the Doppler flows in the umbilical cord artery and the middle cerebral artery can be explained by the fact that they were performed as part of standard procedure on admission to hospital, by different physicians, not at the moment of deterioration of the patients' clinical condition and the decision to deliver the baby — which is unfortunately not always possible due to the clinical condition and the need for an emergency C-section. Moreover, the small size of the groups and no standardization of measurements (i.e. proper technique, equipment settings, trained ultrasound specialist) make it impossible to draw conclusions regarding these parameters. The article focuses on the assessment of the functioning of RAS in the placenta, not the usability of the biophysical techniques in the assessment of pre-eclampsia. In the case of quantitative assessment of the individual components of RAS, the differences at the consecutive stages of pregnancy described to date refer to trimesters, not the successive weeks [51]. Because in the case of all of the patients, both in the study group and the control group, babies were delivered in the third trimester, the several weeks' differences (33 weeks vs. 39 weeks on average) should not considerably distort the obtained results.

The results of our study, consistent with the stream of research on the functioning of RAS in the placenta, support the design of further studies, conducted on larger groups of patients and combining several assessment techniques (LC-MS, immunoenzymatic techniques, immunofluorescence, chemiluminescence, autoradiography).

In conclusion our study showed that the ex vivo production of Ang 1–7 by placentas from patients with preeclampsia was significantly lower than in control group. Significantly decreased mRNA expression of ACE and AT1 receptor and lower production of Ang II in placentas of PE patients suggest that placental Ang II/ACE/AT1r pathway could be less important than Ang 1–7/ACE-2/MASr pathway in development of preeclampsia, but this requires further investigations.

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Conflict of interest

None declared.

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