ROLE OF CLUSTERIN IN PREDICTING DEVELOPMENT OF EARLY- AND LATE-ONSET PREECLAMPSIA IN THE FIRST TRIMESTER OF PREGNANCY

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Pre-eclampsia (PE) occurs in 2–8% of pregnancies. It is one of the leading causes of maternal and perinatal morbidity and mortality. Today, there are no tests adopted by the practitioners that enable accurate prediction of early (weeks 20 through 34) or late (after week 34) onset of PE when the pregnancy is in its 11th to 14th week. This study aimed to evaluate the feasibility of using secretory clusterin quantification to predict early or late PE during the first trimester of pregnancy. The choice of this protein is determined, on the one hand, by the specificity of its expression for cytotrophoblast, syncytiotrophoblast, and extracellular trophoblast cells, and, on the other hand, by the proven negative effect of clusterin on the invasive properties of trophoblastic cells and gestational transformations of uterine vessels, which play a key role in the pathogenesis of PE. The study included 40 pregnant women aged 27–40 years who underwent a comprehensive screening examination in the first trimester of pregnancy. Western blotting revealed a significant increase in the level of secretory clusterin (40 kDa) in the extravesicular fraction of blood serum of pregnant women in the first trimester compared to physiological pregnancy: in early-onset PE, a twofold increase in the level of clusterin in the vesicular and extravesicular fractions of blood serum (p = 0.03 and p = 0.004, respectively), with late-onset PE — a threefold increase only in the extravesicular fraction of blood serum (p = 0.002). According to logistic regression models, the level of secretory clusterin in the extravesicular fraction of blood serum of pregnant women in the first trimester has prognostic significance in assessing the likelihood of developing early-onset PE (AUC = 0.97, Se = 1, Sp = 0.875, cutoff = 0.3877) and late-onset PE (AUC = 1, Se = 1, Sp = 1, cutoff = 0.5).

Keywords: peripheral blood serum, vesicles, placenta, clusterin, preeclampsia, Western blotting, miRNA, quantitative real-time PCR

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Compliance with ethical standards: the study was approved by the Ethics Committee of VI. Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology (Minutes #13 of December 10, 2020), conducted in accordance with the requirements of the Declaration of Helsinki of 1964, Federal Law “On the Fundamentals of Protecting the Health of Citizens in the Russian Federation” #323-FZ of November 21, 2011 All patients signed a voluntary informed consent form to participate in the study.

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РОЛЬ КЛАСТЕРИНА В ПРОГНОЗИРОВАНИИ РАЗВИТИЯ РАННЕЙ И ПОЗДНЕЙ ПРЕЭКЛАМПСИИ В ПЕРВОМ ТРИМЕСТРЕ БЕРЕМЕННОСТИ

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Преэклампсия (ПЭ) встречается в 2–8% беременностях, является одной из важнейших причин материнской и перинатальной заболеваемости и смертности. На сегодняшний день не используем в клинической практике тест-систем, позволяющих с высокой точностью прогнозировать на 11–14-й неделе беременности ранний дебют ПЭ (с 20-й по 34-ю неделю) или поздний дебют ПЭ (после 34-й недели). Целью исследования было оценить возможности использования количественного определения секреторной формы клеточного фактора в прогнозировании развития ранней и поздней ПЭ в первом триместре беременности. Выбор данного белка обусловлен специфичностью его экспрессии для клеток цитотрофобласта, синцитиотрофобласта и внеклеточного трофобласта, а также доказанным негативным влиянием клеток на инвазивные свойства трофобластических клеток и гестационные преобразования сосудов матки, играющих ключевую роль в патогенезе ПЭ. В исследование включены 40 беременных в возрасте от 27–40 лет, проходивших комплексное скрининговое обследование в первом триместре беременности. Методом Вестерн-блоттинга обнаружено значимое повышение уровня секреторного клеточного фактора (40 кДа) в сыворотке крови беременных в случае развития ПЭ при нарушении физиологической беременности: при ранней ПЭ — двукратное увеличение уровня клеточного фактора в везикулярной и вневезикулярной фракции сыворотки крови (p = 0.03 и p = 0.004 соответственно), при поздней ПЭ — трехкратное увеличение только во вневезикулярной фракции сыворотки крови (p = 0.002). Согласно моделям логистической регрессии уровень секреторного клеточного фактора в везикулярной фракции сыворотки крови беременных в первом триместре обладает диагностической значимостью, при оценке вероятности развития ранней ПЭ (AUC = 0.97, Se = 1, Sp = 0.875, cutoff = 0.3877) и поздней ПЭ (AUC = 1, Se = 1, Sp = 1, cutoff = 0.5).

Ключевые слова: сыворотка периферической крови, везикулы, плацента, клеточная и экскрессия, Вестерн-блоттинг, miРНК, количественная ПЦР в реальном времени

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Вклад авторов: А. В. Тимофеева — планирование исследования, проведение количественной ПЦР в реальном времени, проведение Вестерн-блоттинга, написание и редактирование рукописи; И. С. Федоров — пробоподготовка, проведение Вестерн-блоттинга, статистическая обработка данных; А. М. Тарасова — пробоподготовка и проведение Вестерн-блоттинга; К. А. Горина — клиническая характеристика пациенток; Ю. В. Сухова — формирование групп пациенток для исследования, В. А. Гусар — анализ полученных данных; Т. Ю. Иванец — скрининг в 1-м триместре беременности.


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Preeclampsia (PE) is a multisystem complicating disease that develops in 3 to 8% of all pregnant women [1] and causes 16–18% of all maternal deaths and 40% of fetal and neonatal deaths [2]. International Society for the Study of Hypertension in Pregnancy (ISSHP) defines PE as hypertension (blood pressure above 140/90 mm Hg) developing after 20 weeks of pregnancy, combined with proteinuria (at least 0.3 g/l per day) or signs of acute renal failure, liver dysfunction, neurological disorders, hemolysis or thrombocytopenia, or intrauterine growth retardation. PE may be early and late, depending on the time of onset of clinical symptoms (before or after the 34th week of pregnancy, respectively) [3] [https://cr.minzdrav.gov.ru/ schem/637_1]. Early-onset PE is characterized by the most severe course and accounts for 5–20% of all types of PE. For the fetus, the detrimental effect associated with PE comes from chronic hypoxia, intrauterine growth retardation (a highly frequent consequence); the subsequent complications are linked to prematurity and include respiratory distress syndrome, infectious and inflammatory diseases, intraventricular hemorrhages, cerebral palsy, cognitive retardation, autism, psychomotor, behavioral disorders and/or learning disabilities [4, 5].

Maternal and/or placental factors play a fundamental role in the pathogenesis of PE, which determines the time of onset of clinical manifestations of the condition and their severity. Placental factors include impaired proliferation and differentiation of trophoblast cells at the pre-implantation stage in case the embryonic program runs with errors, and at subsequent stages of implantation if there are inflammation-driven changes in the decidual layer that affect interactions between trophoblast and endometrial cells [6–8]. Impaired cell differentiation of the extravillous trophoblast leads to insufficient remodeling of the spiral uterine arteries: first, in the decidual one, then in the segments of the myometrium from the 16th to the 18th week of pregnancy weeks 25–33. High risk of developing PE, with condition manifestations at pregnancy weeks 34–37; 4) 11 women at high risk of developing PE whose pregnancy was normal and who gave birth to full-term babies; 2) 9 women at high risk of developing PE whose pregnancy was normal and who gave birth to full-term babies (according to the Astraia screening done in the first trimester) for prediction of development of placenta accreta, vesicular and extravesicular for prediction of development of placenta accreta, vesicular and extravesicular for prediction of development of placenta accreta, vesicular and extravesicular for prediction of development of placenta accreta, vesicular and extravesicular for prediction of development of placenta accreta, vesicular and extravesicular for prediction of development of placenta accreta, vesicular and extravesicular for prediction of development of placenta accreta, vesicular and extravesicular for prediction of development of placenta accreta, vesicular and 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The second cohort included 27 pregnant women aged 25–38 years who delivered by caesarean section. They formed four groups (Table 2): 1) 6 women with full-term normal pregnancy (37–39 weeks); 2) 7 women with placenta previa and premature rupture of membranes at 25–31 weeks of gestation; 3) 7 women with early-onset preeclampsia (pregnancy weeks 25–30); 4) 7 women with late-onset preeclampsia (weeks 36–38).

The exclusion criteria for both cohorts were as follows: pregnancy through assisted reproductive technology application, multiple pregnancy, aggravated somatic history of the woman, fetal/neonatal instability. The participants underwent the following examinations/tests: blood examination (clinical and biochemical), ultrasonography of pelvic organs and the fetus, Doppler imaging of feto-placental circulation, cardiotocography, blood pressure measurement, urine protein test, determination of the concentration of PLGF, sFlt-1, PAPP, β-hCG in serum blood.

Blood serum (800 µl) of each patient from the first cohort was centrifuged for 10 minutes at 300 g at 4 °C and the supernatant was re-centrifuged for 10 min at 3000 g. All data except for “edema of legs and feet” are given as means (minimum; maximum).

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Table 2. Clinical characteristics of the second cohort patients, normal and complicated pregnancy groups

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy</th>
<th>Complicated pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery</td>
<td>Planned caesarean section</td>
<td>Emergency caesarean section because of the risk of early pregnancy failure</td>
</tr>
<tr>
<td>Group of pregnant women (number of patients)</td>
<td>I (6), n &gt; 34</td>
<td>II (7), n &lt; 34</td>
</tr>
<tr>
<td>Preeclampsia manifestation time (weeks)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Delivery time (weeks)</td>
<td>38.0 (37.0; 39.0)*</td>
<td>29.0 (25.0; 32.0)*</td>
</tr>
<tr>
<td>Severe preeclampsia (number of people)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild preeclampsia (number of people)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Edema of legs and feet (number of people)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urine protein level (0.0–0.2 g/l)</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Blood pressure – systolic</td>
<td>112 (107; 119)*</td>
<td>116 (112; 120)*</td>
</tr>
<tr>
<td>diastolic</td>
<td>68 (65; 71)*</td>
<td>77 (74; 81)*</td>
</tr>
<tr>
<td>ALT (up to 31.0 U/l)</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>AST (up to 31.0 U/l)</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>Alkaline phosphatase (up to 239.0 U/l)</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>Platelets of peripheral blood (150–390 thou/mm²)</td>
<td>228 (166; 290)*</td>
<td>238 (183; 293)*</td>
</tr>
<tr>
<td>PLGF (250–1200 pg/ml)</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>sFLT-1 (950–2800 pg/ml)</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td>sFLT-1/PLGF</td>
<td>No data</td>
<td>No data</td>
</tr>
</tbody>
</table>

Note: * — the data are given as means (minimum; maximum) registered at admission to the hospital.

Follows: 15 min at 95 °C, subsequent 40 cycles (15 s at 94 °C, 30 s at the primer annealing temperature, and 30 s at 70 °C) in a StepOnePlusTM amplifier (Applied Biosystems; USA). The relative level of cDNA expression was estimated by the ΔCt method, where ΔCt = CtCi – CtCi, where (CtCi) is the value of the threshold amplification cycle for cDNA of miRNA analyzed in the sample; (CtCi) is the value of the threshold amplification cycle for cDNA of the reference cel-miR-39 RNA in the sample.

The remaining 500 µl of purified blood serum from the first cohort patients were used to isolate microvesicles using the miRCURY Exosome Kits (Qiagen; Germany), the process involving addition of 200 µl of a precipitating solution and 14-hour incubation at 4 °C, followed by centrifugation at 1500 g for 30 min at 20 °C. The supernatant was collected in a clean tube, diluted 100-fold with addition of Laemmli Sample Buffer (#1610737, BioRad; USA) and used for Western blotting. Two hundred and seventy µl of resuspension buffer were added to the precipitate containing the vesicles; the final 1000-fold dilution of the vesicles supplemented with Laemmli Sample Buffer (#1610737, BioRad; USA) with 5% (v/v) 2-mercaptoethanol (Am-O482-0.1, VWR Life Science AMRESCO; USA) and used for Western blotting.

Samples of the placental tissue collected from the second cohort patients no later than 10 minutes after delivery were tissue sections 5 mm thick that presented all payers of the placenta and the collected placental tissue samples were washed in 0.9% NaCl and instantly frozen in liquid nitrogen for subsequent storage at –80 °C. The tissue was ground to a powder in liquid nitrogen vapor; 10 mg of the tissue were lysed in RIPA Lysis Buffer System (sc-24948, Santa Cruz; USA). After incubation on ice for 30 min and centrifugation of the lysate at 10,000 g, we measured the concentration of soluble protein fraction with the help of the biuret method and a NanoDrop One spectrophotometer (ThermoScientific; USA). For subsequent Western blotting analysis, we took 40 µg of protein from each sample.

Western blotting was used to quantify the level of the secretory clusterin’s alpha subunit in peripheral blood serum (first cohort) and placenta (second cohort). Before fractionation in a 10% polyacrylamide gel in hydroxymethylaminomethaneetrine buffer (100 mM hydroxymethylaminomethane, 100 mM tricine, 0.1% sodium dodecyl sulfate), the samples were denatured at 70 °C for 1 min in Laemmli Sample Buffer (#1610737, BioRad; USA) containing 5% (v/v) 2-mercaptoethanol (Am-O482-0.1, VWR Life Science AMRESCO; USA). To determine molecular weight of the analyzed protein we introduced a PageRuler protein molecular weight marker, 10–250 kDa (#26619, Thermo Fisher Scientific; USA), into each membrane. When electrophoresis was over, the proteins were moved to a nitrocellulose membrane (0.45 µm, BioRad; USA), the transfer done semi-dry with 10 mM 3-cyclohexylamino-1-propanesulfonic acid (SW18880, Sigma-Aldrich; USA), pH 10.5, 10% ethanol. After membrane blocking in 5% skimmed milk (Blotting-Grade Blocker, #1706404, BioRad; USA), 0.1% Tween20 (#1706531, BioRad; USA), 50 mM Tris (T4661, Sigma; USA), pH 7.5, 150 mM NaCl (A1371, AppliChem Panreac ITW Companies; Germany) for 2 h, we incubated it for 1 h with primary antibodies to the clusterin’s alpha subunit at a dilution of 1 : 400 (B-5, sc-5289, Santa Cruz Biotechnology; USA), or to actin at a dilution of 1 : 400 (H-6, sc-376421, Santa Cruz Biotechnology; USA), in 5% skim milk, 0.1% Tween20, 50 mM Tris, pH 7, 5, 150 mM NaCl, washing the membrane three
times for 5 min in 0.05% Tween20, 50 mM Tris, pH 7.5, 150 mM NaCl, and then incubated for 1 hour with secondary polyclonal antibodies conjugated to horseradish peroxidase at a dilution of 1 : 2000 (HAF007, R&D Systems; USA) in 1% skim milk, 0.1% Tween20, 50 mM Tris, pH 7.5, 150 mM NaCl. After washing the membrane three times for 5 min in 0.05% Tween20, 50 mM Tris, pH 7.5, 150 mM NaCl, peroxidase activity was measured by adding Clarity MaxTM Western ECL Substrate (#1705062, BioRad; USA) and detecting chemiluminescence in the ChemiDoc MP gel documentation system (#12003154, BioRad; USA).

Statistical analysis of the data

Microsoft Excel and RStudio (Posit; USA) software were used for the purposes of statistical processing of the data. When the distribution did not obey the normal distribution law, we used the pairwise Mann–Whitney tests to do the statistical analysis. When the distribution of attributes differed from normal, they were described as a median (Me) and quartiles Q₁ and Q₃ in the Me (Q₁; Q₃) format. The significance threshold value was adopted at \( p = 0.05 \). To assess the possibility of classifying patients into groups based on the data obtained, we developed logistic regression models and verified their quality with the help of a ROC curve and sensitivity and specificity calculations.

RESULTS

Analysis of the content of secretory clusterin in the blood serum of patients of the first cohort

At the first stage of the study, we applied Western blotting with primary antibodies to the alpha subunit of the protein to retrospectively quantify secretory clusterin in the blood serum of the patients. At that time, on average, they were at the 12th week of pregnancy. Depending on the outcome of pregnancy, patients of the first cohort (Table 1) were divided into four groups (see “Patients and methods”). The miRCURY Exosome Kit (Qiagen; Germany), the action of which relies on precipitation in the presence of polyethylene glycol, allowed obtaining two fractions of blood serum: vesicular fraction, which included microvesicles, exosomes, apoptotic bodies, and a vesicle-free fraction (supernatant). Fig. 1 shows the results of the blood serum’s vesicular fraction analysis. Top part of the figure contains blots with chemiluminescent bands representing 40 kDa clusterin alpha subunit as registered in samples collected in the N (normal), Nhr (normal, high risk of PE) groups (according to the Astraia screening results), and ePE and lPE groups. In order to register the efficiency of protein transfer from gel to membrane and record differences in exposure during imaging in the gel-documenting system, we applied a reference sample (P) from the N group to one of the wells of each gel (same sample in all cases), thus enabling comparison of the chemiluminescence values in every sample. Compared to the N group, ePE group exhibited a significant \( p = 0.03 \) two-fold increase in the level of secretory clusterin in the vesicular fraction of blood serum of patients in the first trimester of pregnancy, as indicated in the diagram of Figure 1. As for the lPE group, it did not differ significantly from the N group in terms of the level of secretory clusterin in blood serum’s vesicular fraction.

Using the Spearman’s rank correlation coefficient, we revealed an inverse correlation between the level of secretory clusterin in the blood serum’s vesicular fraction and CRL \( (r = -0.31; p = 0.052) \), as well as a direct correlation between the level of this clusterin and the value of \( \beta \)-hCG in the blood serum \( (r = 0.28; p = 0.082) \) of women at the 12th week of pregnancy.

Fig. 2 presents the results of Western blotting aimed at establishing the level of secretory clusterin’s alpha subunit in the
vesicle-free fraction of blood serum. As the diagram of Fig. 2 shows, compared to the N group, ePE and IPE groups (p = 0.004 and p = 0.002, respectively) exhibited a significant increase of the level of secretory clusterin (40 kDa) in the blood serum’s extravesicular fraction (samples collected during the 1st trimester of pregnancy), this increase being 2.2-fold and 3-fold, respectively. Moreover, for IPE the level of clusterin in the extravesicular fraction was 1.5 times higher than for ePE (p < 0.001). As for the comparison of N and Nhr groups, we found no significant differences in the level of secretory clusterin in vesicular and extravesicular fractions of the blood serum (Fig. 1 and 2).

Using the Spearman’s rank correlation coefficient, we revealed an inverse correlation between the level of secretory clusterin in the vesicle-free fraction and β-hCG MoM (r = –0.3; p = 0.0627).

Quantification of miR-25-3p, miR-92a-3p, miR-320a and miR-17-5p in the blood serum of the first cohort patients.

According to data from miRWalk, miRanda, RNA22, and Targetscan databases, the potential regulators of the clusterin expression level are miR-320a, miR-930a-5p, miR-17-5p, miR-21-5p, miR-30c-5p, miR-1323, miR-25-3p, miR-138-5p, miR-34a-5p, miR-92a-3p. In a study investigating the relationship between the levels of clusterin and miRNAs regulating it in placenta accreta cases [24], we found significant inverse correlations between the content of secretory clusterin in the peripheral blood plasma of pregnant women, with the values being “−ΔCt” miR-25-3p, miR-92a-3p, miR-320a, miR-17-5p at the time of delivery. Due to the fact that trophoblastic cells in placenta accreta and preeclampsia cases have directly opposite invasive properties, it seemed interesting to us to trace the possible relationships between miRNA data and clusterin in the blood serum of patients from the first cohort at 11–14 weeks of pregnancy. The values of the relative content of miR-25-3p, miR-92a-3p, miR-320a, miR-17-5p in the serum of pregnant women were obtained using the method of quantitative real-time RT-PCR as “−ΔCt” values (see “Patients and methods”). Spearman’s rank correlation method revealed a statistically significant positive correlation between the content of secretory clusterin in the extravesicular fraction of blood serum of pregnant women and the miR-17-5p “−ΔCt” value (r = 0.34; p = 0.0356) of blood serum. It should be noted that, according to the miRTargetLink 2.0 database (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2/network/a7aae41-7676-4e3b-875c-43c926dedae5), clusterin is an experimentally proven target for miR-17-5p.

Spearman’s rank correlation method revealed statistically significant positive correlations between blood serum “−ΔCt” miR-16-5p and uterine artery pulsation index (UA (PI): r = 0.37, p = 0.021; UA (PI) MoM: r = 0.32, p = 0.046). In turn, inverse relationships were found between the uterine artery pulsation index and pregnancy-associated plasma protein A (UA (PI) and PAPP-A: r = –0.41; p = 0.01; UA (PI) MoM and PAPP-A MoM: r = –0.35, p = 0.0296).

Evaluation of the probability of development of early- and late-onset PE by the level of secretory clusterin in two fractions (vesicular and extravesicular) of the blood serum of women in the first trimester of pregnancy.

Based on the values of the content of secretory clusterin in the blood serum of women in the first cohort (Table 1), who underwent screening in the first trimester of pregnancy, we built logistic regression models to calculate the probability of development of early and late PE (Fig. 3). It was found that the best prognostic accuracy (with high specificity and sensitivity) is provided by the models built to
assess probability of occurrence of clinical manifestations of ePE and lPE after the 20th week of pregnancy, three assessment based on the level of secretory clusterin in the vesicle-free fraction of the blood serum of patients (but not in the vesicular fraction) by 11–14 weeks of pregnancy. The formulas for calculating the probability of development of early PE (formula 1) and late PE (formula 2) are given below:

\[
\begin{align*}
\text{Probability of development of ePE} & = \frac{1}{1 + e^{5.71 - 10.9x}} \\
\text{Probability of development of lPE} & = \frac{1}{1 + e^{267.11 - 152.58x}}
\end{align*}
\]

Analysis of the content of secretory clusterin in placental tissue collected from the second cohort patients at the time of delivery

The second cohort of patients was analyzed to identify secretory clusterin in placental tissue collected from women suffering ePE and lPE, the analysis including comparison with groups of the corresponding gestational age (N < 34 weeks, N > 34 weeks) without signs of PE (Table 2). The chemiluminescence data obtained for clusterin were correlated with the chemiluminescent signal from actin registered in the same sample. Comparing to the N group, we found a significant decrease in the level of secretory clusterin with a molecular weight of 40 kDa in the placenta from women that had PE, the decrease being 2.3-fold for ePE \( (p = 0.001) \) and 2.6-fold for lPE \( (p = 0.013) \), as shown on the diagram in Fig. 4.

**DISCUSSION**

In the present study, we decided to focus on quantification of the secretory clusterin in blood serum collected from women at the 11–14th weeks of pregnancy; the goal was to identify possible differences in the pathogenesis of ePE and lPE, which could form the basis of mathematical models enabling prediction of these complications in the first trimester before PE starts to clinically manifest itself.

We established that, compared to normal pregnancy, both ePE and lPE cause a significant increase in the level of secretory clusterin (40 kDa) in the extravesicular fraction of the blood serum of patients in the first trimester of pregnancy (two-fold and three-fold increase, respectively). Despite a more pronounced increase in the level of clusterin secretion in case of lPE (compared to ePE), the total amount of secretory clusterin circulating in the blood serum in ePE cases is much greater than that in lPE patients because of the vesicular fraction, where the level of clusterin is 2.7 times higher in ePE in compared to lPE. Moreover, since there were 10 times more vesicular fraction of blood serum than extravesicular fraction taken for Western blotting, it can be concluded that clusterin is more functionally important in the composition of extracellular vesicles circulating in the blood in ePE cases compared to lPE cases.

The data obtained in the present work on the increase of the level of clusterin in the peripheral blood of pregnant women with PE are consistent with the results of a study that used semi-quantitative nano LC/MS and found a statistically
significant increase in the level of clusterin in the blood serum of women at the 10–20th week of pregnancy, followed by the development of hypertensive disorders after the 20th gestational week [29]. However, in that work, pregnant women with ePE were not analyzed: it only included two groups of patients, with IPE and with hypertensive disorders without proteinuria. In other studies, analysis of blood plasma of pregnant women at the time of delivery revealed a statistically significant increase in the level of clusterin in the group of women with PE relative to the group of women with normal pregnancy [30, 31]; moreover, pregnant women with PE in combination with fetal growth retardation had a more significant increase in clusterin levels than pregnant women with PE with normal fetometric parameters [31]. The induction of clusterin synthesis during PE may be caused by the promoter region holding the gene encoding it in the binding sites for such factors as SP1, NF1, AP-1, HSF1, YB-1, p53, B-MYB, the level of which under conditions of oxidative stress, hypoxia and apoptosis rises sharply [32–35].

In turn, clusterin regulates the activity of the transcription factor NF-κB, which plays an important role in cell viability, their motility, proliferation, phenotypic transformation and inflammation [36]. Besides, the expression of clusterin, like any other protein, can be regulated at the post-transcriptional level by miRNA. In this work, quantification of potential regulators of clusterin expression (miR-25-3p, miR-92a-3p, miR-320a-3p and miR-17-5p) in the blood serum of women in the first trimester of pregnancy revealed a significant correlation between the secretory clusterin content in blood serum’s extravesicular fraction and the miR-17-5p “–ΔCt” value. One of the articles describes in detail the involvement of miR-25-3p, miR-92a-3p, miR-320a-3p, and miR-17-5p in the induction of the epithelial-mesenchymal transition [37]. It is possible that the participation of these miRNAs in the molecular transformations of extravillous trophoblast cells and subsequent remodeling of the uterine artery wall is reflected in the positive correlation we found between “–ΔCt” miR-16-5p blood serum of pregnant women and the uterine artery pulsation index (UA (PI): r = 0, 37, p = 0.021; UA (PI) MoM: r = 0.32, p = 0.046), the values of which were inversely correlated with the level of plasma pregnancy-associated protein A (UA (PI) and PAPP-A: r = 0.41, p = 0.01; UA (PI) MoM and PAPP-A MoM: r = 0.35, p = 0.0296).

Since there are three forms of clusterin in eukaryotic cells (nuclear, secretory, and cytosolic) [25], we deemed it interesting to analyze the possible differences between ePE and IPE cases in terms of the level of secretory clusterin (40 kDa) in placental tissue at the time of delivery as compared with placental samples from patients (similar pregnancy term) without signs of PE. We discovered a significant two-fold decrease of clusterin expression in the placental tissue of pregnant women with ePE and IPE. It is possible that the expression of secretory clusterin in the placenta of women with PE is reduced because of the excessive level of its secretion, which we observed in the participants of this study as early as in the first trimester of pregnancy (the participants that subsequently developed PE).

Another possible reason is the increased transition of secretory clusterin from the placenta into the maternal blood in case of PE, which is caused by the oxidative stress and hypoxic/ischemic processes in the placental tissue peculiar to this pregnancy complication. First, clusterin enters cytosol from the ER [38–40], then transitions to the maternal bloodstream as part of microvesicles and exosomes, or as part of apoptotic bodies in case of severe syncytiotrophoblast and cytotrophoblast ER stress [1]. It was found that oxidative stress and activation of ER stress markers, as well as the release of placental microvesicles into the bloodstream, are more pronounced in case of ePE than in the group of women with normal pregnancy

Fig. 4. Western blotting of clusterin in placental tissue at the time of delivery, ePE and IPE groups. The diagrams show the clusterin to actin content ratio in IPE cases [22, 41]. Moreover, the concentration of exosomes in the woman’s blood serum increases only when she suffers ePE but not IPE [42]. In our study, we established that the level of clusterin as part of the vesicles grows significantly in patients with ePE, while there no significant changes in clusterin content in the vesicular fraction of serum in patient with IPE. It was also proven that secretory clusterin in the blood serum of pregnant women can have a negative effect on proliferation, invasion, and survival of the trophoblast cells [27, 29], forming a positive feedback: “ER stress of syncytiotrophoblast cells — an increase in extratrophoblastic clusterin — aggravation of ER stress of syncytiotrophoblast cells and apoptotic/necrotic processes in them — replenishment of the extratrophoblastic clusterin fraction in the maternal circulation”.

Since for the two types of PE (early and late) statistically significant changes in the level of secretory clusterin were found in the extra-vesicular fraction of the blood serum of women in the first trimester of pregnancy compared with normal pregnancy, it is advisable to use this fraction to predict the development of PE at the stage of the first pregnancy screening, applying the logistic regression models developed in this study.

CONCLUSIONS

In the context of this study, we developed logistic regression models based on the level of secretory clusterin that allow predicting early and late PE long before the onset of clinical manifestations of any of them. However, before practical application of these models it is necessary to verify the obtained data on a larger sample. New pathogenetic mechanisms of the development of early- and late-onset PE were clarified based on the quantitative analysis of secretory clusterin in two fractions (vesicular and extravesicular) of the blood serum of women in the first trimester of pregnancy and in the placental tissue at the time of delivery (Fig. 5).
Early PE

- Impaired differentiation of trophoblast cells during early embryogenesis
- Violation of the 1st and 2nd wave of trophoblast invasion, impairment of remodeling of the spiral uterus arteries, hypoxia/ischemia of the placenta, ↑ sFLT/PLGF

Late PE

- Somatic diseases of the mother (CAG, diabetes mellitus, obesity, chronic kidney disease, liver disease)
- Dysfunction of the endothelium of placental vessels, violation of trophism of placental tissue

Stress of the endoplasmic reticulum (ER): imbalanced protein synthesis and folding

↑ Of concentration of chaperones in the ER, including clusterin

↑ Clusterin secretion via exocytosis:
  ↑ level of clusterin in the extra-vesicular fraction of blood serum (Fig. 2)

!! ePE and lPE may be predicted in the 1st trimester of pregnancy (Fig. 3)

References

5. Van Beek PE, Rijken M, Broeders L, Ter Horst HJ, Koopman-


Литература


