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

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Preeclampsia (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 blood serum of pregnant women in the case of PE 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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РОЛЬ КЛАСТЕРИНА В ПРОГНОЗИРОВАНИИ РАЗВИТИЯ РАННЕЙ И ПОЗДНЕЙ ПРЕЭКЛАМПСИИ В ПЕРВОМ ТРИМЕСТРЕ БЕРЕМЕННОСТИ

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Преэклампсия (ПЭ) встречается в 2–8% беременностей, является одной из важнейших причин материнской и перинатальной заболеваемости и смертности. На сегодняшний день нет используемых 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 кластерина 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 кластерина на инвазивные свойства трофобластных 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 кластерина (40 кДа) в сыворотке крови беременных в случае развития ПЭ относительно физиологической беременности: при ранней ПЭ — двукратное увеличение уровня кластерина 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 кластерина во вневезикулярной фракции сыворотки крови беременных в первом триместре обладает прогностической 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, кластерин, преэклампсия, Вестерн-блоттинг, мкРНК, количественная ПЦР в реальном времени

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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/schema/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 segment before the 10th week of pregnancy in the form of reduced arterial obstruction by endovascular trophoblast cells, which translates into damage to the placental villi by reactive oxygen and nitrogen species [9], and then in the segments of the myometrium from the 16th to the 18th week of pregnancy [10]. The result of abnormal restructuring of the uterine arteries is increased resistance of the uterine arteries, mechanical damage to the placental villi due to increased pressure of blood entering the intervillous space [11–14], and, as a result, hypoxic/ischemic changes in the placental tissue due to the impaired uteroplacental blood flow [1, 15]. Placenta suffering ischemia releases various biological factors that damage vascular endothelium and trigger acute multiple organ failure in the mother. PE is linked to such changes in the level of placental factors circulating in the blood as decreased concentrations of pregnancy-associated plasma protein A (PAPP-A) and placental growth factor (PlGF), as well as to the increase of formation of soluble fms-like tyrosine kinase-1, level of vascular endothelial growth factor A (VEGF-A), inhibin A, activin A, procoagulant P-selectin, pro-inflammatory interleukin 2 and tumor necrosis factor alpha, etc. [1, 16–18]. Maternal pathogenetic factors include genetic predisposition, immunological factors, chronic diseases in the mother (metabolic syndrome, diabetes mellitus, chronic arterial hypertension), which can contribute to the regulation of the placentation process, as well as aggravate the maternal susceptibility to the factors secreted by ischemic placental tissue, which accelerates the onset of clinical symptoms in the mother [19].

Looking for reasons behind temporal differences in manifestation of the clinical symptoms of PE, researchers have compared the profiles of DNA methylome of trophoblast cells, placental transcriptome and maternal proteome peculiar to early and late PE cases [20]. Other studies revealed that in women with PE, blood serum secretome may trigger stress of trophoblast's endoplasmic reticulum (ER), i.e., functional overload of the protein secretion apparatus resulting from faults in the folding of protein molecules [21]. Moreover, the degree of activation of the misfolded proteins utilization system differs in early and late PE cases [22, 23].

Thus, a thorough analysis of changes in the placental secretome will allow understanding the differences in the pathogenesis of early- and late-onset PE. In our previous study [24], we found a decrease in the level of the secretory form of clusterin in blood plasma of women with placenta accreta, a pregnancy complication characterized by excessive invasion of trophoblast cells and an increased level of angiogenic factors, i.e., processes radically opposite to those observed in PE. Clusterin is an intra- and extracellular chaperone. It plays an important role in stress-induced protein homeostasis (proteostasis); its activity depends on the degree of glycosylation in the ER [25, 26]. Clusterin is expressed in many tissues of a human body, including cells of cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast [27]. Clusterin is known to inhibit the epithelial-mesenchymal transition during phenotypic transformation of trophoblast cells, which lowers their migration and invasion by suppressing the expression of matrix metalloproteinase 9 and vimentin and increasing the expression of E-cadherin [27]. Under physiological conditions, clusterin is mainly secreted into the extracellular space after post-translational modification in the ER and Golgi apparatus, then it binds with misfolded proteins into aggregates that are internalized by receptor-mediated endocytosis and then sent to autophagosomes for degradation. When ER is under stress (e.g., as a consequence of oxidative stress), clusterin is released therefrom into cytosol to create aggregates with misfolded proteins, which are transported directly to proteasomes for degradation [28]. The pronounced expression of ER stress markers leads to activation of signaling pathways involved in inflammation and apoptosis, which support the accumulation of misfolded protein molecules and exacerbate the pathological process.

Since clusterin participates in the processes induced by stress of the ER, which manifests vividly in PE, and because of the specifics of clusterin expression in trophoblast cells and it having a secretory form, we designed and conducted this study, which aimed to evaluate the significance of secretory clusterin found in different fractions of blood serum of women (vesicular and extravesicular) for prediction of development of early or late PE during the first trimester of pregnancy.

METHODS

All patients that participated in this study applied to the V. I. Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology for pregnancy follow-up.

The first cohort of patients included 40 pregnant women aged 27–40 years who underwent a set of examinations as part of the first trimester screening routine. They formed four groups (Table 1): 1) 10 women at low risk of developing PE (according to the Astraia screening done in the first trimester) 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; 3) 10 women at high risk of developing PE, with condition manifestations at pregnancy weeks 34–37; 4) 11 women at high risk of developing PE, with condition manifestations at pregnancy weeks 25–33.

Table 1. Clinical characteristics of the first cohort patients, all groups, screened in the first trimester of pregnancy

	Normal, N (n = 10)	Normal, at high risk of developing PE, Nhr (n = 9)	Late-onset preeclampsia, lPE (n = 10)	Early-onset preeclampsia, ePE (n = 11)
	First pregnancy trimester screening			
Gestational age	12.5 (12.0; 13.4)	12.1 (11.2; 13.1)	12.2 (11.6; 12.5)	12.0 (11.2; 12.4)
Crown rump length, CRL (43.0–84.0 mm)	62.5 (54.0; 74.7)	59.1 (50.0; 69.0)	59.6 (55.1; 64.0)	57.4 (50.0; 62.0)
Nuchal translucency thickness, NT (1.6–1.7 mm)	1.4 (1.1; 2.2)	1.5 (1.0; 2.0)	1.6 (1.3; 2.0)	1.7 (1.1; 2.9)
Uterine artery pulsatility index, UA (PI), 0.9–2.6 (5th and 95th percentiles)	1.6 (0.4; 2.2)	1.8 (1.2; 2.5)	1.7 (0.7; 2.4)	2.1 (1.3; 3.5)
UA (PI) MoM	0.9 (0.3; 1.3)	1.1 (0.8; 1.4)	1.0 (0.4; 1.5)	1.1 (0.3; 2.1)
b-hCG (50.0–55.0 IU/ml)	68.7 (52.3; 89.8)	47.1 (23.1; 114.6)	36.4 (27.8; 53.6)	43.4 (15.6; 94.3)
b-hCG (0.5–2.0 MoM)	1.5 (1.1; 2.3)	1.1 (0.4; 2.5)	0.8 (0.5; 1.6)	0.9 (0.3; 1.7)
PAPP-A (0.7–6.0 IU/L)	3.1 (1.6; 6.9)	2.4 (1.1; 4.2)	2.7 (0.6; 5.0)	2.4 (0.8; 6.2)
PAPP-A (0.5–2.0 MoM)	1.2 (0.5; 3.2)	1.2 (0.4; 2.4)	0.9 (0.4; 2.7)	1.1 (0.5; 2.9)
	Delivery			
Gestational age	38.6 (36.0; 40.6)	37.7 (31.0; 40.2)	37.3 (35.4; 38.5)	31.9 (28.2; 35.6)
Alanine-aminotransferase, ALT (up to 31.0 U/l)	31.8 (8.8; 95.0)	24.1 (11.8; 46.1)	34.2 (12.4; 165.1)	78.2 (11.8; 352.2)
Aspartate aminotransferase, AST (up to 31.0 U/l)	19.7 (11.1; 25.5)	24.3 (13.0; 40.5)	48.9 (10.9; 262.3)	68.7 (13.6; 282.4)
Alkaline phosphatase (up to 239.0 U/l)	182.3 (130.8; 292.6)	130.2 (94.2; 183.0)	208.8 (154.3; 319.6)	119.8 (87.1;169.2)
Lactate dehydrogenase, LDH (130.0–220.0 U/l)	345.6 (271.0; 408.2)	362.2 (296.8; 422.2)	435.8 (36.4; 743.1)	598.7 (351.4;1680.0)
BP systolic (20 to 40 years: 120–127 mm Hg)	118 (90; 140)	135 (105; 170)	140 (127; 160)	152 (140; 170)
BP diastolic (75–80 mm Hg)	77 (60; 90)	86 (70; 110)	91 (80; 105)	98 (90; 110)
Protein level in urine (0.0–0.2, g/l)	0.1 (0.1; 0.1)	0.1 (0.0; 0.1)	0.4 (0.2; 0.9)	2.1 (0.2; 3.4)
Peripheral blood leukocytes (4.0–9.0 thou/mm^3)	9.5 (5.2; 15.6)	8.8 (7.8; 10.5)	10.8 (7.7; 24.4)	11.5 (3.3; 23.2)
Platelets of peripheral blood (150–390 thou/mm^3)	261.8 (201.0; 390.0)	209.9 (146.0; 287.0)	210.1 (93.0; 300.0)	203.5 (82.0; 359.0)
PLGF (250–1200 pg/ml)	115.3 (94.4; 143.8)	74.9 (43.2; 113.4)	83.6 (34.2; 152.0)	56.8 (22.2; 109.7)
sFLT-1 (950–2800 pg/ml)	6271.0 (5168.0; 7763.0)	11895.6 (5190.0; 19418.0)	9651.7 (4027.0; 14131.0)	10722.8 (5216.0; 19738.0)
sFLT-1/ PLGF	54.4 (53.9; 54.8)	173.4 (107.3; 430.1)	129.4 (66.8; 233.6)	285.4 (48.8; 636.2)
Edema of legs and feet (number of patients)	3	1	4	5
Full-term fetus weight, 3200–3500 g	3396.5 (2880.0; 3952.0)	2764.4 (780.0; 3550.0)	2744.9 (2132.0; 3518.0)	1424.2 (900.0; 2582.0)
Placenta weight at full-term pregnancy, 390–415 g	463.1 (303.0; 650.0)	324.4 (106.0; 449.0)	370.3 (257.0; 465.0)	230.2 (119.0; 371.0)
Mean uterine artery PI (39th week, 5th and 95th percentiles: 0.47–0.91)	0.6 (0.5; 0.7)	0.8 (0.5; 1.7)	0.9 (0.6; 1.1)	1.2 (1.0; 1.5)
Umbilical artery PI (39th week, 5th and 95th percentiles: 0.76–1.03)	0.8 (0.6; 1.4)	1.1 (0.7; 2.3)	0.9 (0.7; 1.0)	1.4 (0.8; 1.8)
Middle cerebral artery PI (39th week, 5th and 95th percentiles: 0.93–1.73)	1.4 (1.2; 1.7)	1.4 (1.2; 1.6)	1.3 (0.6; 1.7)	1.6 (1.1; 2.4)
Cerebro-placental ratio, > 1	1.8 (1.1; 2.5)	1.4 (0.6; 2.0)	1.5 (1.1; 2.3)	1.3 (0.8; 1.9)

Note: all data except for "edema of legs and feet" are given as means (minimum; maximum).

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 without clinical manifestations of preeclampsia; 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 aneuploidy. 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 at 4 °C to remove impurities from blood cells. Purified serum (200 out of 700 μl) was used for RNA isolation with the help of the miRNeasy Serum/Plasma kit (Qiagen; Germany) with preliminary addition of 5.6×10^8 copies of synthetic cel-miR-39 RNA (Qiagen; Germany) after serum incubation with phenol mixture Qiazol to control the efficiency of RNA extraction and cDNA synthesis as recommended by the manufacturer. Seven μl of the RNA eluate were used for reverse transcription done with the miScript II RT Kit (Qiagen; Germany) as recommended by the manufacturer. The synthesized cDNA (2 μl) served as a template for real-time PCR analysis that employed a sense primer specific to the studied miRNA (miR-320a-3p, MIMAT0000510, 5'-aaaagctgggttgagagggcga, annealing temperature with template 59.5°C; miR-17-5p, MIMAT0000070, 5'-caaagtgcttacagtgcaggtag, 55°C; miR-25-3p, MIMAT0000081, 5'-cattgcacttgtctcggtctga, 56°C; miR-92a-3p, MIMAT0000092, tattgcacttgt, °Cgg06tcc), cel-miR-39 (miScript Primer Assay, Ce_miR-39_1, 55°C; Qiagen; Germany), and miScript SYBR Green PCR Kit (Qiagen; Germany) containing miScript Universal Primer (antisense) and PCR mix SYBR Green PCR MasterMix. The PCR reaction conditions were as

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

	Normal pregnancy	Complicated pregnancy		
Delivery	Planned caesarean section	Emergency caesarean section because of the risk of early pregnancy failure	Caesarean section because of early preeclampsia	Planned caesarean section because of late preeclampsia
Group of pregnant women (number of patients)	I (6). $n > 34$	II (7). $n < 34$	III (7). ePE	IV (7). lPE
Preeclampsia manifestation time (weeks)	No	No	24.5 (22.0; 28.0)*	36.1 (36.0; 37.0)*
Delivery time (weeks)	38.0 (37.0; 39.0)*	29.0 (25.0; 32.0)*	28.2 (25.0; 30.0)*	36.9 (36.0; 38.0)*
Severe preeclampsia (number of people)	0	0	7	1
Mild preeclampsia (number of people)	0	0	0	6
Edema of legs and feet (number of people)	0	0	1	5
Urine protein level (0.0–0.2 g/l)	Normal	Normal	2.3 (0.2; 4.6)*	1.4 (0.1; 4.1)*
Blood pressure – systolic – diastolic	112 (107; 119)* 68 (65; 71)*	116 (112; 120)* 77 (74; 81)*	155 (125; 180)* 100 (80; 120)*	144 (120; 175)* 93 (70; 100)*
ALT (up to 31.0 U/l)	No data	No data	74 (11; 215)*	23 (12; 32)*
AST (up to 31.0 U/l)	No data	No data	55 (11; 194)*	29 (16; 48)*
Alkaline phosphatase (up to 239.0 U/l)	No data	No data	110 (54; 179)*	165 (79; 252)*
Platelets of peripheral blood (150–390 thou/mm^3)	228 (166; 290)*	238 (183; 293)*	145 (68; 243)*	238 (181; 308)*
PLGF (250–1200 pg/ml)	No data	No data	30 (14; 47)*	101 (54; 216)*
sFLT-1 (950–2800 pg/ml)	No data	No data	11957 (5615; 23226)*	14657 (7489; 24990)*
sFLT-1/ PLGF	No data	No data	444 (126; 847)*	193 (42; 348)*

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 = (Ct)si – (Ct)ri, where (Ct)si is the value of the threshold amplification cycle for cDNA of miRNA analyzed in the sample; (Ct)ri 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) with 5% (v/v) 2-mercaptoethanol (Am-O482-0.1, VWR Life Science AMRESCO; 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) was 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 included both the fetal and maternal parts thereof from the chorionic plate to the decidua. 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 hydroxymethylaminomethanetricine buffer (100 mM hydroxymethylaminomethane, 100 mM tricine, 0.1% sodium dodecyl sulfate), the samples were denatured at 70 °C for 10 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 PAAG well. 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 (SW18805, 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

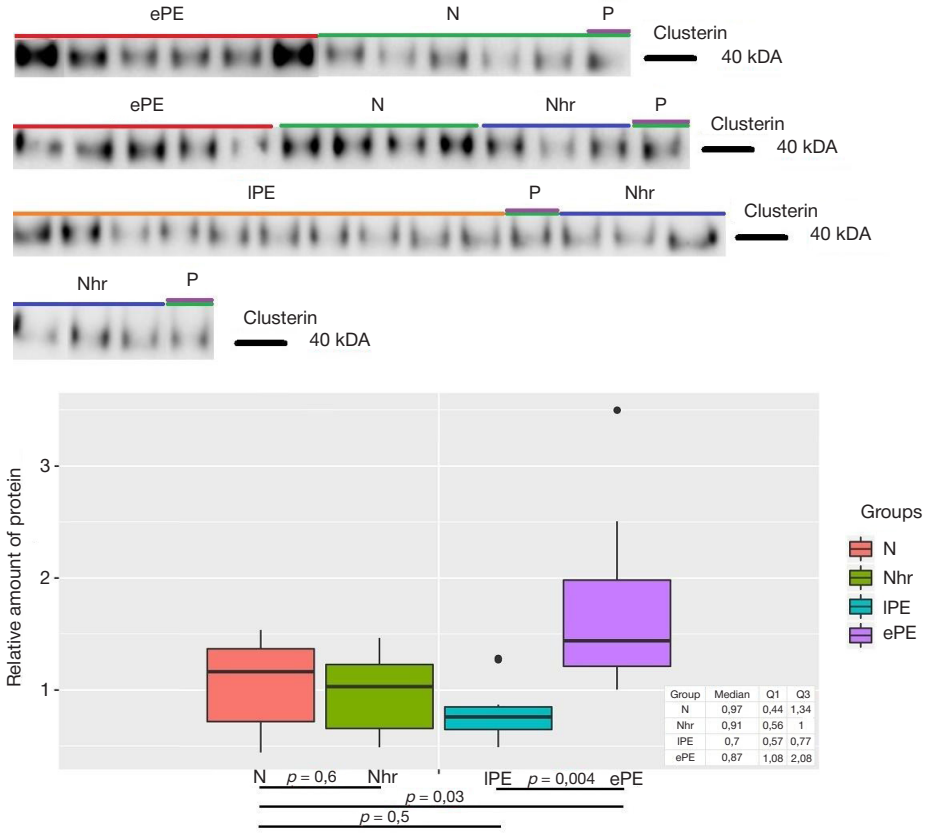

Fig. 1. Western blotting of clusterin in the vesicular fraction of blood serum of patients from the first cohort, 12th week of pregnancy

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 Q_1 and Q_3 in the Me (Q_1; Q_3) format. The significance threshold value was adopted at $p = 0.05$. The differences were considered significant 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 IPE 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 IPE 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 β-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

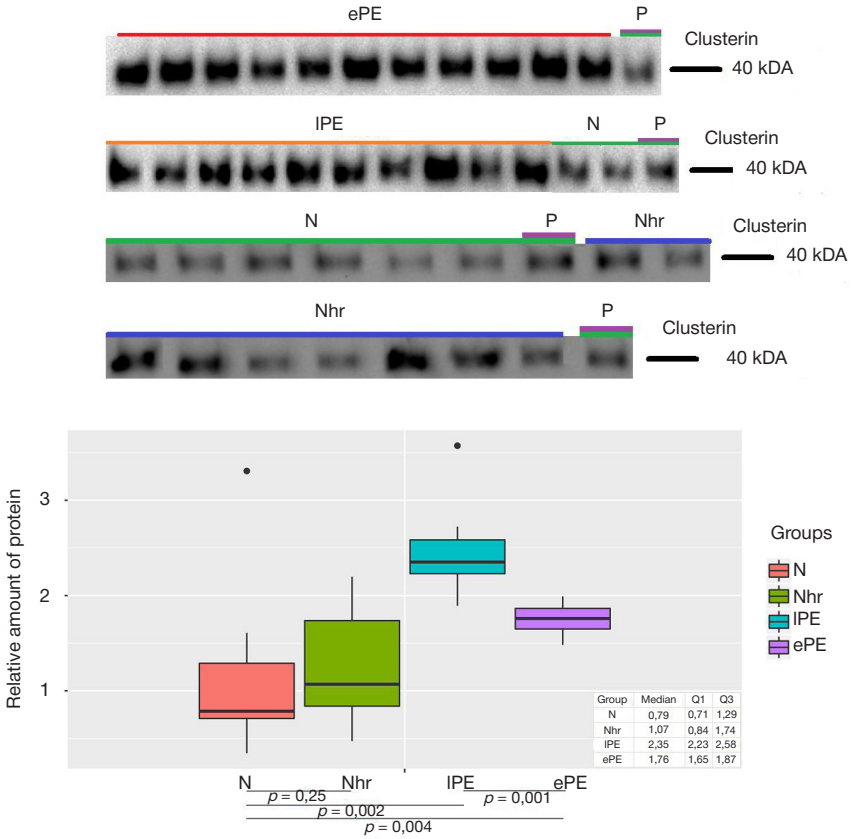

Fig. 2. Western blotting of clusterin in the vesicle-free fraction of blood serum of patients from the first cohort, 12th week of pregnancy

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 blood serum's 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-30a-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/a7aa6e41-7676-4e3b-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

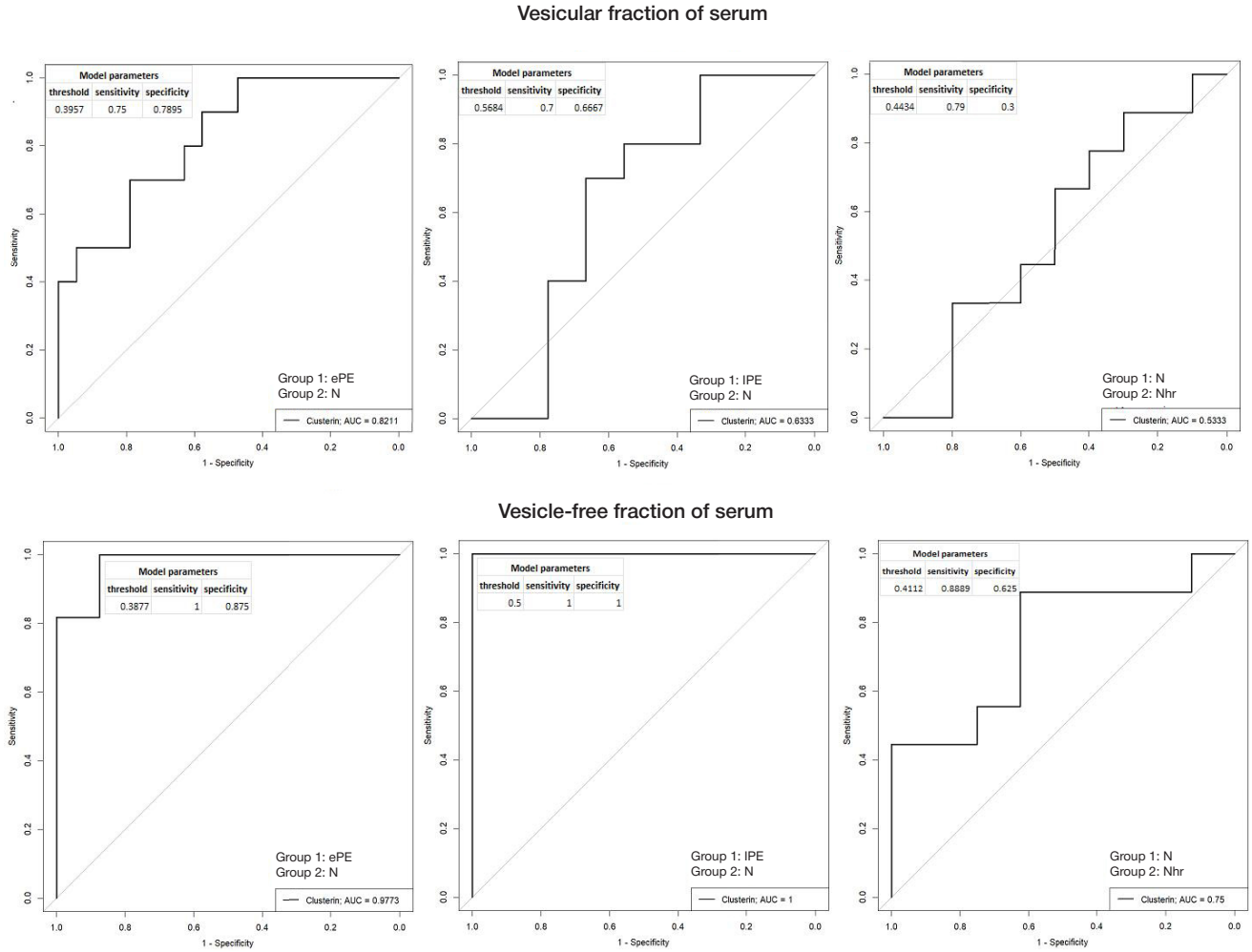

Fig. 3. Logistic regression models predicting development of ePE and IPE by the level of secretory clusterin in the vesicular and vesicle-free fractions of serum collected from the patients at 12th week of pregnancy

assess probability of occurrence of clinical manifestations of ePE and IPE 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:

$$\frac{1}{1 + e^{15.71-10.9x}}, \quad (1)$$

$$\frac{1}{1 + e^{267.11-152.58x}}. \quad (2)$$

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 IPE, 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 IPE (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 IPE, 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 IPE 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 IPE (compared to ePE), the total amount of secretory clusterin circulating in the blood serum in ePE cases is much greater than that in IPE patients because of the vesicular fraction, where the level of clusterin is 2.7 times higher in ePE in compared to IPE. 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 IPE 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 phenotypic transformation 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

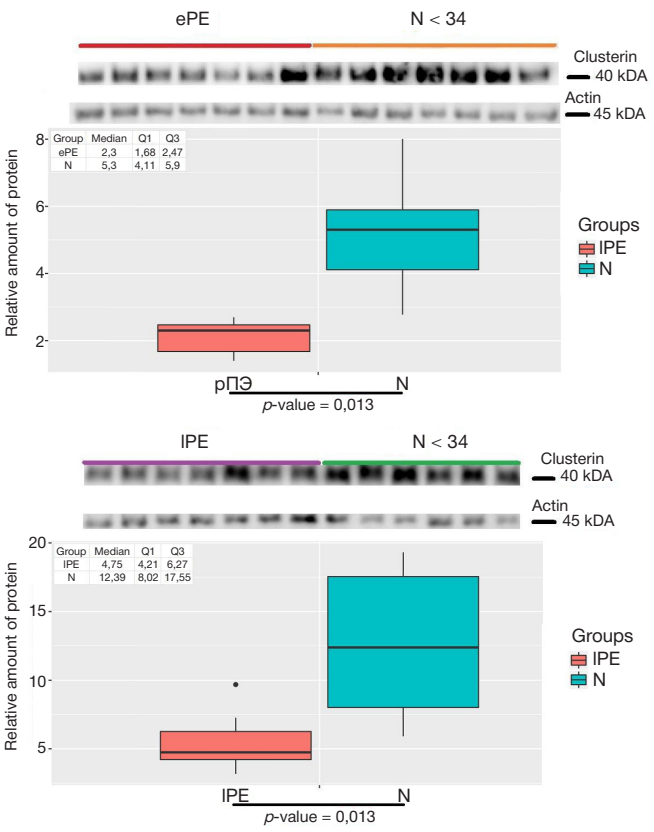

Fig. 4. Western blotting of clusterin in placental tissue at the time of delivery, ePE and IPE groups. The diagrams show the clusterin to actin content ratio

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).

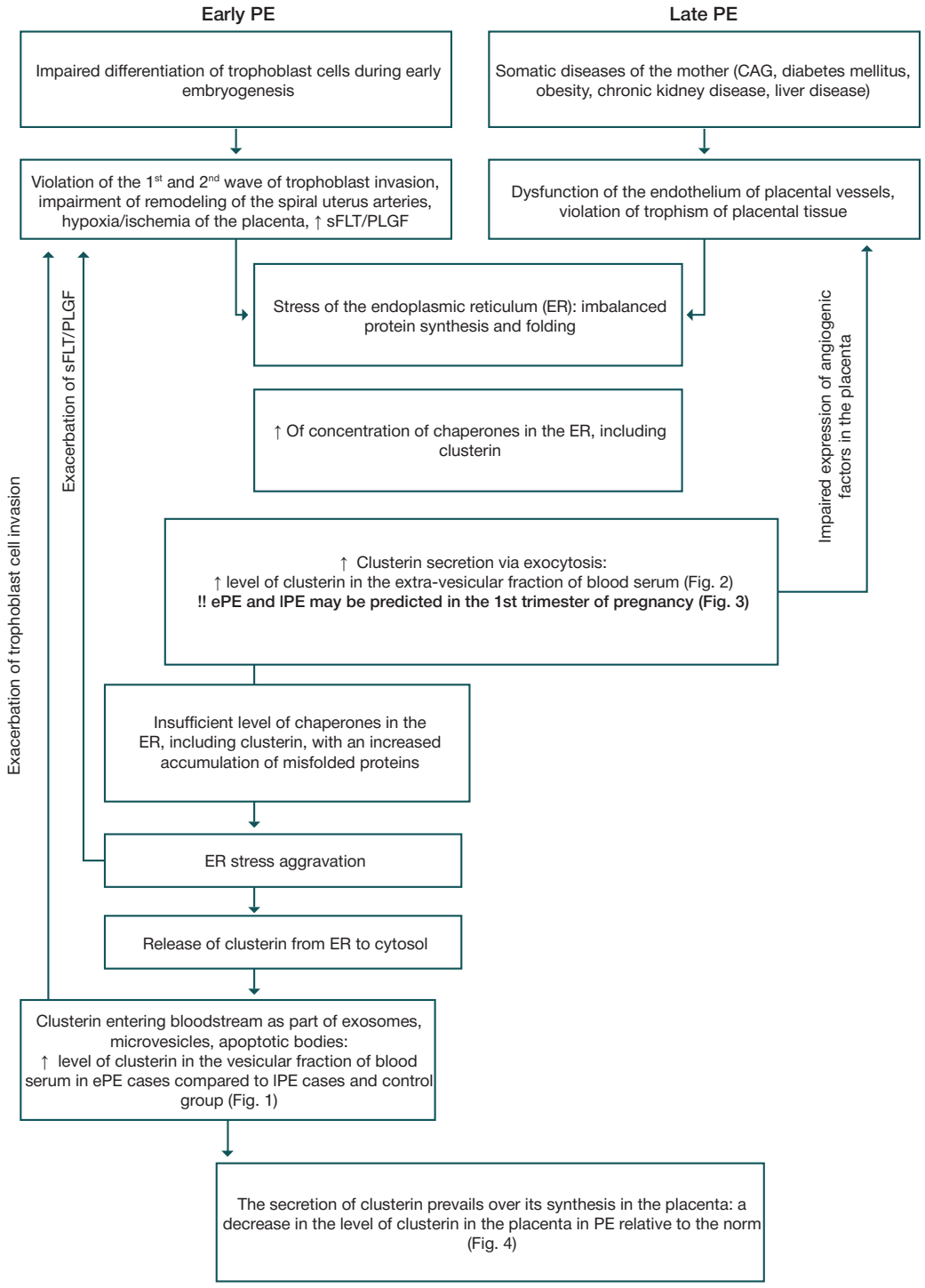

Fig. 5. Schematic representation of the role of secretory clusterin in the pathogenesis of early- and late-onset PE

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