EMERGING PREDICTION OF PREECLAMPSIA BASED ON THE EXPRESSION OF EXOSOMAL SUMO PROTEINS

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The cellular response to various types of stress underlying placental vascular dysfunction is under the sumoylation control. Consequently, SUMO homeostasis is closely related to the maintenance of angiogenic balance, the disruption of which is a feature of preeclampsia (PE). The goal of the research is to search for exosomal markers of such a disorder. The expression and prognostic potential of exosomal SUMO 1–4, UBC9 and hnRNPA2/B1 were evalueted in 39 pregnant women (cohort I) in the first trimester using Western blotting technology. The expression of these proteins in the placenta (cohort II, 27 pregnant women) at the time of delivery was also assessed. The expression of their conjugated forms was significantly changed in pregnant women with early-onset (SUMO 1, $p = 0.03$; SUMO 2/3/4, $p = 0.03$) and late-onset PE (SUMO 1, $p = 0.03$; SUMO 2/3/4, $p = 0.04$; UBC9 and hnRNPA2/B1, $p < 0.0001$, respectively). This change may be due to the functional specificity of SUMO isoforms in the context of their subcellular targets upon exposure to stressful stimuli. Significant changes in the expression of these proteins were also found in the placenta. Significant correlations were established between the expression of exosomal SUMO 2/3/4 ($r = –0.59$; $p = 0.01$) and UBC9 ($r = –0.88$; $p = 0.0001$) with PlGF in early-onset PE. In late-onset PE, hnRNPA2/B1 ($r = –0.48$; $p = 0.03$) and UBC9 ($r = –0.48$; $p = 0.03$) was correlated with β-hCG, and SUMO 2/3/4 with PAPP-A ($r = –0.60$; $p = 0.006$) in the blood serum of pregnant women. The analyzed proteins also significantly correlated with uterine artery pulsation index (SUMO 1 ($r = 0.59$; $p = 0.01$), SUMO 2/3/4 ($r = 0.54$; $p = 0.02$), hnRNPA2/B1 ($r = 0.75$; $p = 0.0001$)) and mean arterial pressure (UBC9 ($r = 0.53$; $p = 0.03$)). Based on the data the logistic models have been created to predict the risk of developing early-onset (UBC9 (AUC = 0.88; Se-0.72; Sp-1)) and late-onset PE (SUMO 1 (AUC = 0.79; Se-0.8; Sp-0.77)) at 11–14 weeks of pregnancy.

Keywords: exosomes, sumoylation, SUMO, prediction, placental dysfunction, preeclampsia

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НОВЫЕ ВОЗМОЖНОСТИ ПРОГНОЗИРОВАНИЯ ПРЕЭКЛАМПСИИ НА ОСНОВЕ ЭКСПРЕССИИ ЭКЗОСОМНЫХ БЕЛКОВ SUMO

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Клеточная реакция на стресс, лежащий в основе сосудистой дисфункции плаценты, находится под контролем сумоилирования. Следовательно, SUMO-гомеостаз тесно связан с поддержанием ангиогенного баланса, нарушение которого характерно для преэклампсии (ПЭ). Цель работы — поиск экзосомных маркеров подобного нарушения. Оценивали экспрессию и прогностический потенциал экзосомных SUMO 1–4, UBC9 и hnRNPA2/B1 у 39 беременных (когорта I) в первом триместре с помощью вестерн-блоттинга. В когорте II (27 беременных) оценивали экспрессию данных белков в плаценте на момент родов. Экспрессия экзосомных конъюгированных форм значимо изменялась у беременных с ранней (SUMO 1, $p = 0,03$; SUMO 2/3/4, $p = 0,03$) и поздней ПЭ (SUMO 1, $p = 0,03$; SUMO 2/3/4, $p = 0,04$; UBC9 и hnRNPA2/B1, $p < 0,0001$ соответственно), что может быть обусловлено функциональной специфичностью изоформ SUMO в контексте их субклеточных мишеней при воздействии стрессовых стимулов. В плаценте также обнаружены значимые изменения экспрессии конъюгированных форм данных белков. При ранней ПЭ установлены значимые корреляционные связи экспрессии экзосомных SUMO 2/3/4 ($r = –0,59$; $p = 0,01$) и UBC9 ($r = –0,88$; $p = 0,0001$) с уровнем PlGF, а при поздней ПЭ — hnRNPA2/B1 ($r = –0,48$; $p = 0,03$), UBC9 ($r = –0,48$; $p = 0,03$) с β-ХГЧ, и SUMO 2/3/4 ($r = –0,60$; $p = 0,006$) с концентрацией PAPP-A в сыворотке крови беременных. Анализируемые белки достоверно коррелировали с пульсационным индексом маточной артерии (SUMO 1 ($r = 0,59$; $p = 0,01$), SUMO 2/3/4 ($r = 0,54$; $p = 0,02$), hnRNPA2/B1 ($r = 0,75$; $p = 0,0001$)) и средним артериальным давлением (UBC9 ($r = 0,53$; $p = 0,03$)). На основе полученных данных созданы логистические модели прогнозирования риска развития ранней (UBC9 (AUC = 0,88; Se-0,72; Sp-1)) и поздней ПЭ (SUMO 1 (AUC = 0,79; Se-0,8; Sp-0,77)) на сроке 11–14 недель беременности.

Ключевые слова: экзосомы, сумоилирование, SUMO, прогнозирование, плацентарная дисфункция, преэклампсия

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The role of placental dysfunction proven by numerous studies in the basis of adverse pregnancy outcomes, including preeclampsia (PE) and fetal growth restriction (FGR), is undeniable. The molecular component of these syndromes is represented by key processes combined with stress — hypoxic, oxidative/nitrate, mitochondrial and endoplasmic reticulum [1–4], resulting from abnormal endovascular invasion of spiral arteries by trophoblasts. As a result, various pro-inflammatory factors that disturb the angiogenic balance are released into the mother's bloodstream [5]. The resulting therefrom pathological changes lead to a higher risk of metabolic, cardiovascular and nephrological diseases in women and their fetuses in the long term [6, 7].

In general, PE is defined as a multisystem disease, with new onset hypertension with (or without) significant proteinuria after 20 weeks of gestation [8, 9]. Over the past three decades, this definition has been expanded. Nowadays the definition include timing of symptom onset in early or late pregnancy, with delivery before or after 34 weeks, and different phenotypes due to adverse effects on the fetus (PE with or without IGR) [8]. Of note is the recent discussion that argues that the maternal cardiovascular system is the etiological cause of PE [5,10]. However, the linking mechanism between placental dysfunction and maternal cardiovascular maladaptation is angiogenic-antiangiogenic imbalance [10]. During physiological pregnancy, the levels of angiogenic placental growth factor (PLGF) and anti-angiogenic factor sFlt-1 (soluble fms-like tyrosine kinase 1) are balanced, but under conditions of hypoxia and oxidative stress, there is an increase in the secretion of sFlt-1 by cytotorophoblasts, leading to impaired angiogenesis. There are propose various pathways modulating PLGF expression [11]. One of them is mediated by the transcription factor GCM-1, which plays a critical role in maintaining the balance between proliferation and differentiation of syncytiotrophoblast in the first trimester of pregnancy [12] and its upstream target DREAM [13]. It is important to emphasize that the modulation of the activity of the latter occurs at the post-translational level, including through sumoylation [14]. Interestingly, this modification is also a key player in controlling cellular responses to heat shock, inflammation, and various types of stress (oxidative, hypoxic, mitochondrial) underlying placental dysfunction [15–18].

Sumoylation is a dynamic reversible process carried out by SUMO (Small Ubiquitin-like MOdifier) proteins, which have four isoforms. Their conjugation (the formation of an isopeptide bond between SUMO and the target protein) is performed by the UBC9 enzyme [19]. Sumoylation has attracted increasing attention in the context of regulating the expression of molecules mediating placental function and angiogenesis. However, data on the study of sumoylation in placental diseases are essentially limited. In particular, Baczyk et al. revealed a significant increase of SUMO 1 and SUMO 2/3 in the placenta in severe early-onset PE [20]. And the WGCNA evolutionary approach has identified gene co-expression modules that play a critical role in the pathogenesis of PE, including SUMO 1 and the heterogeneous nuclear protein (hnRNP), as candidates genes for positive selection [21].

The coordinated interface between the fetoplacental and maternal systems is a complex multidimensional array of tissues, resident and circulating factors, covering the developing fetus, placenta, decidua, and the mother's dynamic cardiovascular system [1]. It is carried out through extracellular vesicles (exosomes, microvesicles, apoptotic vesicles), which are secreted by various types of cells, carry a certain cargo (proteins, lipids, mRNA transcripts), and also have the ability to modulate the function of target cells and have therapeutic potential [22, 23]. In particular, exosomes are selectively packaged with signaling molecules such as microRNAs (miRNAs). Their secretion by syncytiotrophoblast increases with placental dysfunction [24–26]. Most notably, the selectivity and loading of miRNAs into exosomes is mediated by sumoylation of the heterogeneous nuclear protein hnRNPA2/B1 [27].

Progress in understanding the molecular processes consociating placental dysfunction and the maternal cardiovascular system suggests that disturbances in the angiogenic-antiangiogenic balance may be a target for the search for exosomal prognostic markers. Our previous studies demonstrated a regulatory mechanism along the miR-652-3p/ SUMO 2/3/4/ UBC9/ GCM-1/ PlGF axis in the placenta of pregnant women with early-onset PE [28] and changes in SUMO 1–4 and UBC9 expression in exosomes of pregnant women with early-onset PE at the time of delivery [29]. Further to these studies, we focused our attention on evaluating the expression of exosomal SUMO 1–4, UBC9, and hnRNPA2/B1 proteins as predictors of placental dysfunction at early gestational periods (11–14 weeks) before the manifestation of clinical signs of this pathology. Beyond that, to the best of the authors' knowledge, such studies have not previously been conducted.

METHODS

Study Design and Patient Cohort

This study included pregnant women who were under observation at the “National Medical Research Center for Obstetrics, Gynecology, and Perinatology named after Academician V. I. Kulakov” of the Ministry of Healthcare of the Russian Federation. The total sample of patients of reproductive age consisted of 66 pregnant women, divided into 2 cohorts (Fig. 1). Inclusion criteria are singleton pregnancy, patients aged from 25 to 40 years, delivered vaginally and by cesarean section. The study of both cohorts did not include pregnant women with multiple pregnancies, resulting from assisted reproductive technologies, positive somatic history and genetic pathologies in the mother and fetus. Cohort I included 39 pregnant women and was divided into groups: pregnant women who subsequently developed early-onset (11 pregnant women) and late-onset (10 pregnant women) PE; pregnant women with a high risk of developing PE according to combined prenatal screening in the first trimester and a favorable pregnancy outcome (9 pregnant women); pregnant women with a physiological course of pregnancy (9 pregnant women). The expression of SUMO, UBC9 and hnRNPA2/B1 proteins was assessed in exosomes of pregnant women at 11–14 weeks of gestation. Cohort II included 27 pregnant women with early-onset (7 pregnant women), late-onset PE (7 pregnant women) and a control group of the corresponding period (7 and 6 pregnant women, respectively) to assess protein expression in placental samples. The detailed clinical characteristics of pregnant women included in study are presented in Table 1–2.

Exosome Purification from the Serum Blood Samples of Pregnant Women

Whole blood samples were collected from pregnant women at 11–14 weeks of pregnancy (cohort I). Previously, the samples were centrifuged for 20 minutes, +4 °C at 300 g. The upper phase was then carefully transferred into a clean conical bottom tube and centrifuged again for 10 min at +4 °C at

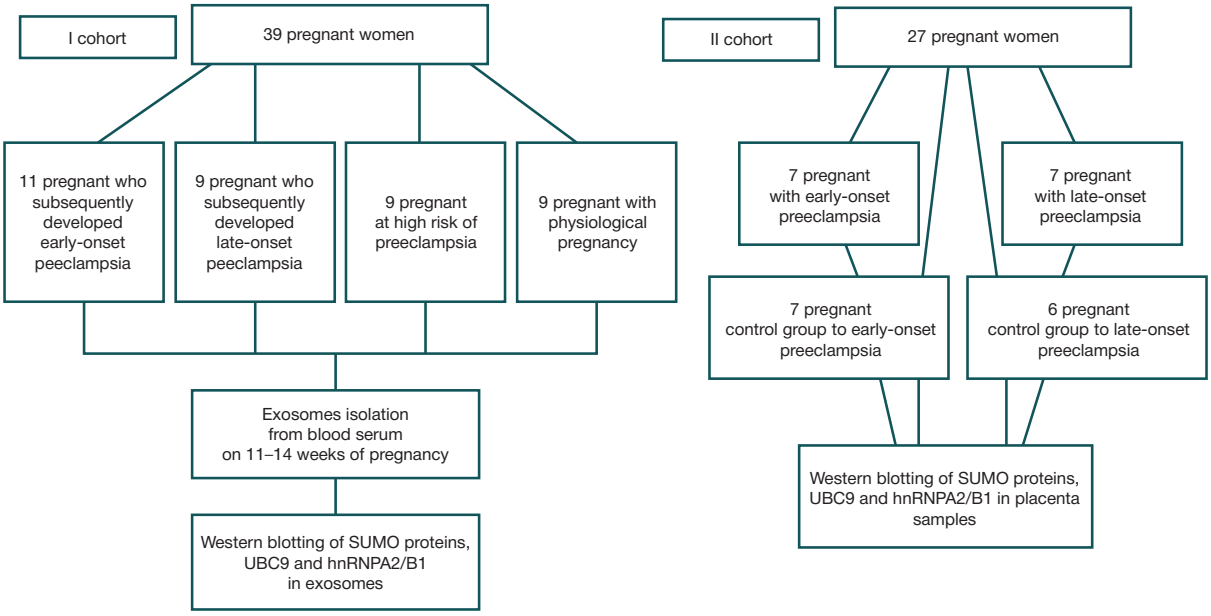

Fig. 1. Flowchart of the study population

16,000 g to remove cell debris. From the prepared samples, 600 µl was used for purification of exosomes using miRCURY Exosome Serum/Plasma Kit (cat. no. 76603; Germany, Qiagen) according to the manufacturer's instructions, and subsequent Western blotting.

Western Blotting of exosomal and tissue proteins

A placental tissue (a cross-section through the maternal and fetal part of the placenta no more than 5 mm in thickness, obtained immediately after delivery) from pregnant women (cohort II) was used for protein extraction. Powdered tissue samples preliminarily ground in liquid nitrogen were homogenized in a RIPA Lysis Buffer System (sc-24948; Santa Cruz Biotechnology, Inc.; Dallas, Texas, USA). Separation of proteins (20 µg per gel lane) was performed in Tris/Tricine/SDS Buffer (12.5%). Exosome and tissue protein transfer to nitrocellulose membrane (0.45 µm, cat. no: 1620115; Bio-Rad, USA) was performed using Trans-Blot SD™ (cat. #. 170-3957; Bio-Rad, USA) in 10 mM CAPS + 10%C_2H_5OH, pH = 11. The membranes were blocked with 5% NFDM/TBST for 2 h. Incubation with primary antibodies: SUMO 1 (1:500; sc-5308; Santa Cruz Biotechnology, USA), SUMO 2/3/4 (1:500; sc-393144; Santa Cruz Biotechnology, USA), UBC9 (1:500; sc-271057; Santa Cruz Biotechnology, USA), hnRNPA2/B1 (1:500; sc-374053; Santa Cruz Biotechnology, USA), and Actin (1:100; sc-376421; Santa Cruz Biotechnology, USA) were performed for 2h (RT). Secondary HRP-conjugated antibodies (goat anti-mouse IgG-HRP: sc-2031; Santa Cruz Biotechnology, USA) were incubated for 1 h (RT) in 1% NFDM/TBST. A SuperSignal West Femto Maximum Sensitivity Substrate Kit (cat. no: 34096, Thermo Scientific™, USA) was used as a detection reagent. Densitometric analysis was performed using Bio-Rad ImageLab 6.0 software. The expression of tissue proteins SUMO 1–4, UBC9 and hnRNP A2/B1 was normalized to that of actin, and the level of SUMO 1–4, UBC9 and hnRNP A2/B1 in exosomes was normalized to the concentration of total protein.

Evaluation of biochemical parameters of prenatal screening in blood serum of pregnant women

The concentration of placental factors (sFlt-1, PLGF) and hormones (PAPP-A, b-HCG) in the blood serum of pregnant women at 11–14 weeks of gestation was determined by immunochemical analyzer "Cobas e411" (Roche Diagnostics GmbH; Germany) using commercial kits of this manufacturer.

Statistical Analysis

The statistical significance of the difference between the clinical parameters and the proteins expression in study groups was assessed by the Wilcox–Mann–Whitney test using scripts written in the R language (https://www.R-project.org/). The Spearman nonparametric rank correlation method was used to evaluate the relationship between the protein expression and clinical parameters of pregnant women. Logistic regression models for proteins expression were created to test their predictive ability. The efficiency of the created models was evaluated by ROC analysis.

RESULTS

SUMO 1–4, UBC9 and hnRNPA2/B1 Expression in Exosomes at 11–14 weeks of pregnancy

The expression of SUMO 1–4, UBC9, and hnRNPA2/B1 in exosomes isolated from the serum blood of pregnant women with physiological pregnancy, which subsequently manifested early-onset and late-onset PE, as well as pregnant women with a high risk of developing PE according to combined prenatal screening at 11–14 weeks of gestation, was assessed by Western blotting. There were several conjugated forms of these proteins differing in molecular weights detected, while no free forms were found in exosomes (Fig. 2 A–D).

Comparative analysis revealed that in the exosomes of pregnant women with early-onset PE, the expression of the conjugated form SUMO 1 (~50–52 kDa) was significantly reduced (p = 0.03), and SUMO 2/3/4 (~55–58 kDa) was increased relative to physiological pregnancy (p = 0.03). Expression of approximately molecular weight conjugated UBC9 (~54–63 kDa) and hnRNPA2/B1 (~51–55 kDa) was also increased, but without statistically significant differences (p = 0.07; p = 0.3, respectively). Presumably, these forms are a SUMO 1 + UBC9 conjugate, as the molecular weight of their free forms is ~12 kDa and ~18 kDa, respectively. And if

Table 1. The clinical characteristics of pregnant women (cohort I)

	11–14 weeks of pregnancy						
	Pregnant who subsequently developed EPE (*n* = 11)	Pregnant who subsequently developed LPE (*n* = 10)	Pregnant at high risk of PE (*n* = 9)	Pregnant with PP (*n* = 9)	*p*-value EPE vs.PP	*p*-value LPE vs.PP	*p*-value high risk vs.PP
Gestational age at the time of screening, weeks	12.1 (11.8; 12.25)	12.15 (12.1; 12.47)	12.1 (11.6; 12.3)	12.35 (12.03; 12.9)	0.1	0.3	0.3
History of PE, *n* (%)	0	0	0	0	–	–	–
Mean arterial pressure, MoM	1.06 (1; 1.11)	0.98 (0.94; 1.1)	0.96 (0.95; 0.98)	1 (0.96; 1.04)	0.08	1	0.07
PlGF (17.6–70.0, pg/ml; 12 week of gestation)	11.7 (6.3; 18.0)	15.8 (15.6; 23.08)	18.8 (18.8; 18.8)	18.05 (16.01; 26.5)	0.001	0.2	0.8
β-hCG (0.5–2.0, MoM)	0.92 (0.65; 1.07)	0.72 (0.52; 0.8)	0.74 (0.58; 1.22)	1.38 (1.18; 1.8)	<0.001	<0.001	0.07
PAPP-A (0.5–2.0, MoM)	0.9 (0.72; 1.1)	0.65 (0.57; 1.17)	1.07 (0.9; 1.32)	0.87 (0.74; 1.28)	0.7	0.3	0.5
Ultrasonography:							
CRL (43–84 mm)	57.7 (56.5; 60.2)	58.6 (57.7; 62.5)	57.6 (56; 61.2)	64.4 (55.7; 66.0)	0.11	0.3	0.4
NTS (1.6–1.7 mm)	1.6 (1.38; 1.73)	1.5 (1.42; 1.85)	1.5 (1.29; 1.9)	1.25 (1.2; 1.6)	0.2	0.1	0.6
PI UtA (0.76–1.1, MoM)	1.14 (1; 1.21)	0.99 (0.76; 1.3)	1.1 (1.02; 1.19)	1.02 (0.91; 1.13)	0.3	0.7	0.3
PI DV	0.98 (0.98; 0.98)	0.97 (0.97; 0.97)	0.95 (0.95; 0.95)	1.06 (0.99; 1.14)	0.02	0.02	0.03
	Clinical characteristics at the time of delivery						
Gestational age at the time of delivery, weeks	32.1 (30.7; 33.35)	37.2 (36.47; 38.27)	38.3 (37.6; 39.2)	38.8 (38.35; 39.35)	<0.001	0.01	0.4
Systolic blood pressure (110–130 mmHg)	150 (145; 160)	140 (130; 150)	140 (115; 147)	115 (110; 131.2)	<0.001	0.006	0.08
Diastolic blood pressure (65–80 mmHg)	100 (90; 100)	90 (90; 95)	90 (75; 90)	77.5 (71.2; 85.2)	<0.001	0.003	0.1
Proteinuria (0–0.2 g/L)	1.98 (1.08; 2.5)	0.27 (0.13; 0.95)	0.09 (0; 0.1)	0 (0; 0.1)	<0.001	0.01	0.5
Peripheral edema, *n* (%)	5 (45.4)	4 (40.0)	1 (11.1)	3 (33.3)	–	–	–
Ratio of placental dysfunction markers (sFLT-1/PlGF; 1.5–7)	316.6 (116.7; 433.9)	120.1 (78.8; 156.9)	133.7 (117.9; 173.4)	54.4 (54.4; 54.4)	0.01	<0.001	<0.001
Platelet level (150–400 × 10^9 c/L)	220 (146; 233)	244 (142; 262.5)	212 (186; 234)	247.5 (235.7; 270.2)	0.03	0.07	0.07
ALT level (0–40 u/L)	30.9 (21.7; 68.05)	20.6 (15.4; 22.3)	19.2 (18.1; 26.1)	31.8 (18.2; 31.8)	0.09	1	0.7
AST level (0–40 u/L)	27.3 (21.7; 43.15)	25.1 (20.05; 32.6)	17.4 (16.3; 34.5)	19.7 (19.7; 20.6)	0.08	0.3	0.8
Ultrasonography PI UtA (average value)	1.24 (1.05; 1.38)	0.9 (0.74; 0.99)	0.68 (0.55; 0.85)	0.57 (0.52; 0.6)	<0.001	0.001	0.1
Birth weight, grams	1350 (1195; 1572)	2721 (2409.25; 2992.5)	3040 (2750; 3266)	3320 (3257; 3612.5)	<0.001	0.01	0.05
Apgar 1 score	7 (7; 7)	8 (8; 8)	8 (8; 8)	8 (8; 8)	<0.001	1	0.2
Apgar 5 score	8 (7; 8)	9 (9; 9)	9 (9; 9)	9 (9; 9)	<0.001	0.3	0.8
Newborns outcomes:							
IP, *n* (%)	7 (63.6)	0	0	0	–	–	–
IVH, *n* (%)	5 (45.4)	0	0	0	–	–	–
RDS, *n* (%)	2 (18.1)	0	0	0	–	–	–

Note: EPE is early-onset preeclampsia; LPE is late-onset preeclampsia; PP is physiological pregnancy; PlGF is a placental growth factor; sFLT-1 is a soluble Fms-like tyrosine kinase 1; PAPP-A is a pregnancy-associated protein A; b-HCG is a human chorionic gonadotropin, subunit b; CRL is a crown-to-rump length; NTS is a nuchal translucency scan; PI UtA is a Pulsatility Index of the uterine artery; PI DV is a Pulsatility Index of ductus venosus; IP is an intrauterine pneumonia; IVH is an intraventricular hemorrhage; RDS is a respiratory distress syndrome; The median (Me) and quartiles Q_1, Q_3 in the format Me (Q_1–Q_3) were used in the case of non-normal distribution.

hnRNPA2/B1 (free form ~36/38 kDa) is attached as a SUMO 1 target, their total molecular weight may be ~50–56 kDa.

There is also a significant decrease in the expression of the conjugated SUMO 1 (~50–52 kDa; $p = 0.03$) in late-onset PE. While the expression of the conjugated SUMO 2/3/4 (~55–58 kDa; $p = 0.04$), UBC9 (~54–63 kDa; $p < 0.0001$) and hnRNPA2/B1 (~51–55 kDa; $p < 0.0001$) significantly increased relative to normal pregnancy (Fig. 3 A–D).

Interestingly, the analysis of the studied proteins expression in the group of pregnant women with a high risk of developing PE also revealed a significant decrease in the conjugated SUMO 1 expression (~50–52 kDa; $p = 0.007$) and an increase in the conjugated UBC9 (~54–63 kDa), hnRNPA2 /B1 (~51–55 kDa) relative to physiological pregnancy ($p = 0.01$ and $p = 0.001$, respectively).

SUMO 1–4, UBC9 and hnRNPA2/B1 Expression in Placenta Tissue in early-onset and late-onset PE

Taking into account significant changes in the conjugated SUMO 1–4, UBC9 and hnRNPA2/B1 expression in exosomes of pregnant women secreted by syncytiotrophoblasts at 11–14 weeks of gestation, their expression in the placenta in pregnant women with early-onset and late-onset PE was evaluated. Western blotting identified conjugated fragments of these proteins, differing in molecular weights, corresponding to those found in exosomes.

Comparative analysis revealed a significant decrease in the conjugated SUMO 1 expression (~50–55 kDa, $p = 0.04$; $p = 0.04$, respectively), SUMO 2/3/4 (~55–59 kDa; $p = 0.008$, $p = 0.05$, respectively) and hnRNPA2/B1 (~54 kDa; $p = 0.01$,

Table 2. The clinical characteristics of pregnant women (cohort II)

	Pregnant with EPE (n = 7)	Control group (n = 7)	p	Pregnant with LPE (n = 7)	Control group (n = 6)	p
Gestational age at the time of delivery, weeks	29 (27; 30)	30 (26.7; 30.5)	0.5	37 (36; 37.5)	38 (38; 38.75)	0.03
Manifestation PE, weeks	25 (23.5; 25)	absent	–	36 (36; 36)	absent	–
Systolic blood pressure (110–130 mmHg)	150 (145; 170)	115 (109.2; 117.5)	0.002	140 (140; 147.5)	110 (110; 113.7)	0.03
Diastolic blood pressure (65–80 mmHg)	100 (92.5; 106.5)	70 (67.5; 72.9)	0.002	100 (90; 100)	70 (70; 70)	0.005
Proteinuria (0–0.2 g/L)	2.08 (0.82; 3.72)	absent	–	1.07 (0.39; 1.81)	absent	–
Peripheral edema, n (%)	1 (14.2)	absent	–	5 (71.4)	absent	–
Ratio of placental dysfunction markers (sFLT-1/PLGF; 1.5–7)	413 (315.65; 546.43)	NA	–	219.79 (80.9; 289.9)	NA	–
Platelet level (150–400 × 10^9 c/L)	129 (102.5; 185)	246 (190.5; 269)	0.01	233 (219; 254.5)	233.5 (192; 287.7)	1
ALT level (0–40 u/L)	64.3 (22.15; 91.22)	NA	–	22.6 (20; 28.65)	NA	–
AST level (0–40 u/L)	37.4 (24; 48.37)	NA	–	28.3 (22.6; 32.95)	NA	–
Birth weight, grams	826.17 (590; 1068.5)	VLBW	–	2725 (2495; 2774.5)	3325 (2892.5; 3401.2)	0.1

Note: EPE is early-onset preeclampsia; LPE is late-onset preeclampsia; NA here means "not analyzed"; VLBW is very low birth weight; The median (Me) and quartiles Q_1, Q_3 in the format Me (Q_1–Q_3) were used in the case of non-normal distribution.

p = 0.009, respectively) in the placenta in early-onset and late-onset PE. Their conjugated forms approximately correspond to the molecular weights of fragments detected in exosomes. Meanwhile, the expression of conjugated UBC9 (~53–55 kDa; p = 0.04) is significantly reduced only in late-onset PE relative to the comparison group of the corresponding gestational age (Fig. 4 A–D).

Correlation of exosomal SUMO 1–4, UBC9 and hnRNPA2/B1 expression with clinical assessments of pregnant women screening. Predictive ability

Considering the significant change in the expression of the studied proteins in exosomes, we assessed the relationship of these changes with the indices of combined prenatal screening of the first trimester in pregnant women with manifestations of early and late forms of PE using the Spearman nonparametric rank correlation method (Table 3).

The exosomal SUMO 1 levels (r = 0.59; p = 0.01), SUMO 2/3/4 (r = 0.54; p = 0.02), and hnRNPA2/B1 (r = 0.75; p = 0.0001) were significantly correlated with the pulsation index of the uterine artery of pregnant women with early-onset PE. Whereas the UBC9 level is correlated with the mean arterial pressure (r = 0.53; p = 0.03). Moreover, a high invert correlation of SUMO 2/3/4 (r = –0.59; p = 0.01) and UBC9 (r = –0.88; p = 0.0001) with the concentration of PlGF was observed. Interestingly, a correlation was established with the biochemical parameters of combined screening in pregnant women with late-onset PE: the concentration of β-hCG with the exosomal UBC9 (r = –0.48; p = 0.03) and hnRNPA2/B1 (r = –0.48; p = 0.03), as well as PAPP-A concentrations with a SUMO 2/3/4 level (r = –0.60; p = 0.006).

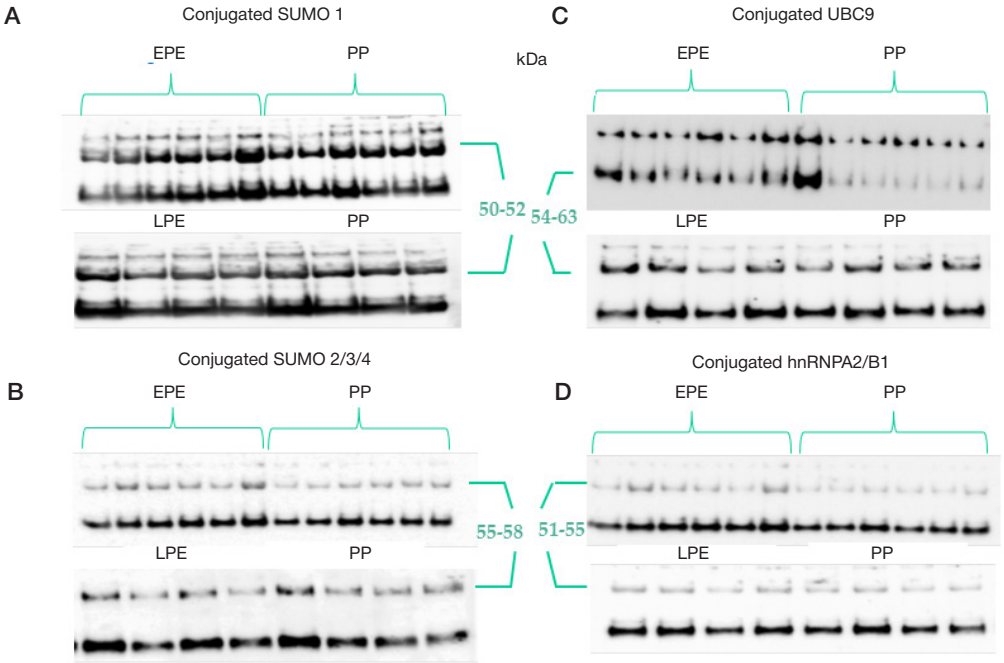

Fig. 2. Western blot of membrane with conjugated exosomal forms of SUMO 1 **(A)**, SUMO 2/3/4 **(B)**, UBC9 **(C)** and hnRNPA2/B1 **(D)** in pregnant women with subsequently developed early-onset PE (EPE), late-onset PE (LPE), and physiological pregnancy (PP)

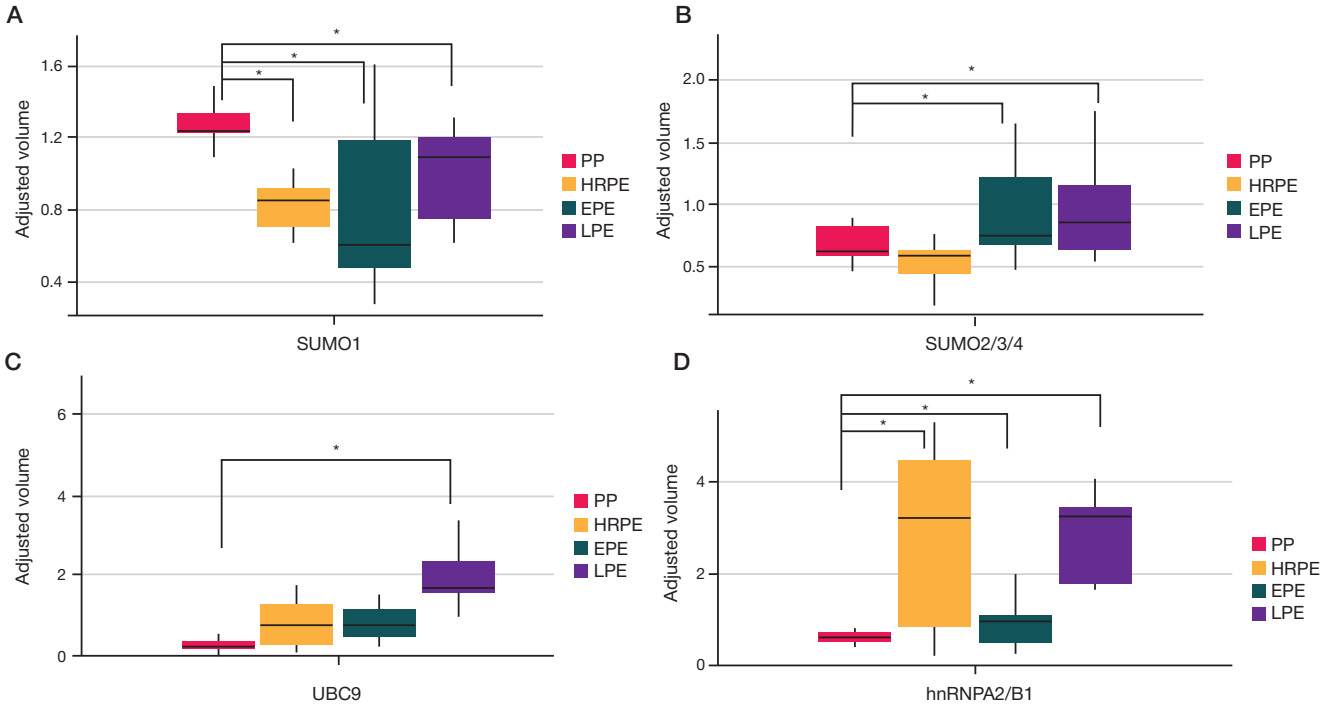

Fig. 3. Comparative analysis of SUMO 1 **(A)**, SUMO 2/3/4 **(B)**, UBC9 **(C)** and hnRNPA2/B1 **(D)** expression in exosomes in pregnant women with physiological pregnancy (PP), who subsequently developed early-onset PE (EPE), late-onset PE (LPE), and high risk of PE (HRPE). Total densitometry of proteins was quantified and normalized to loading control sample. Data presented in the format Me (Q_1; Q_3); *: significance level $p \leq 0.05$ when compared with PP

The logistic regression models were created with the view to assess the possibility of using exosomal SUMO 1–4, UBC9 and hnRNPA2/B1 as potential predictors of the development of PE in early pregnancy (Fig. 5 A–D, Table 4).

ROC curves for logistic models are represented by various combinations, among which significant ones were chosen (the formulas for them are given below the Table 4). Importantly, the selected models also make it possible to differentiate pregnant women at high risk of developing PE according to the combined prenatal screening of the first trimester from pregnant women with PE and pregnant women with a normal pregnancy.

DISCUSSION

The progress of a successful pregnancy is based on the mechanism of communication between the fetoplacental and maternal compartments, which is carried out through the release of bioactive molecules and extracellular vesicles

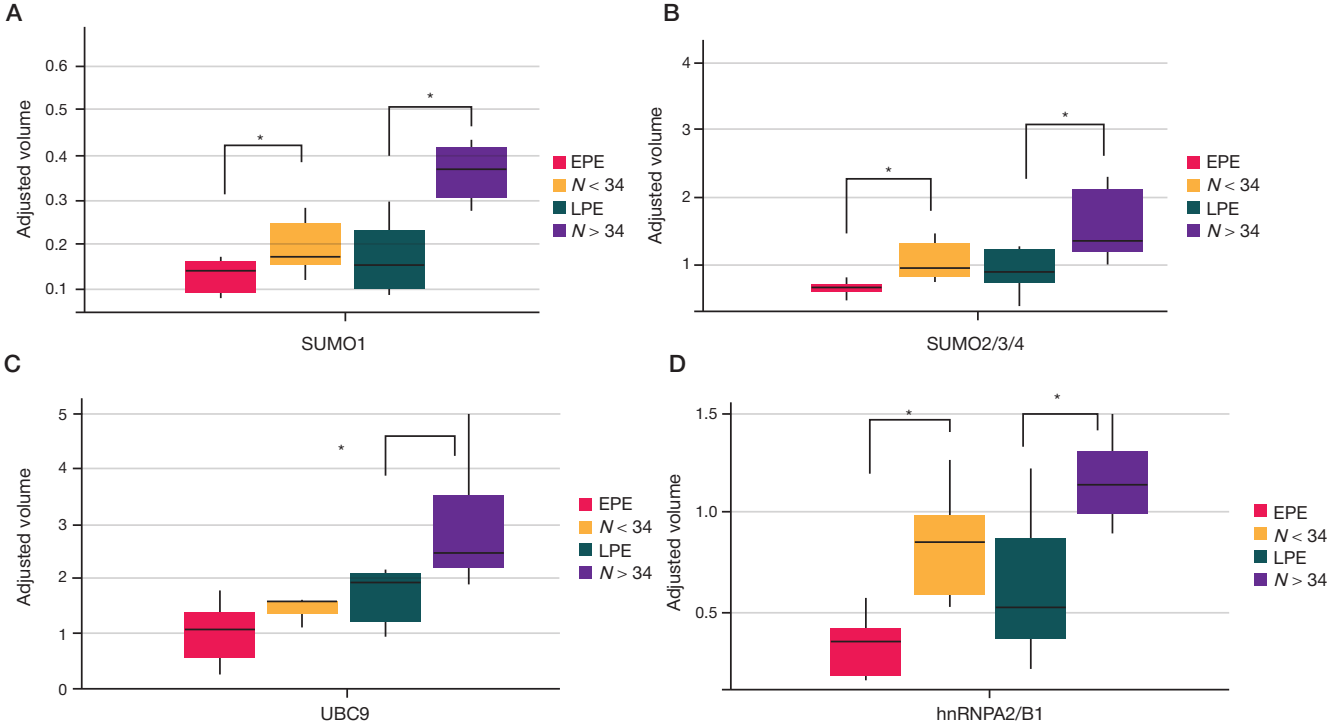

Fig. 4. Comparative analysis of SUMO 1 **(A)**, SUMO 2/3/4 **(B)**, UBC9 **(C)** and hnRNPA2/B1 **(D)** expression in placental tissue in pregnant women with early-onset (EPE) and late-onset (LPE) PE, and age-matched controls ($N < 34$, $N > 34$). Total densitometry of proteins was quantified and normalized to loading control actin. Data presented in the format Me (Q_1; Q_3); *: significance level $p \leq 0.05$ when compared with age-matched controls

Table 3. The results of a correlation of protein expression in exosomes with indices of combined prenatal screening of pregnant women with early-onset and late-onset PE

Parameter	SUMO 1		SUMO 2/3/4		UBC9		hnRNPA2/B1	
	*r**	*p***	*r**	*p***	*r**	*p***	*r**	*p***
	Early-onset PE							
Mean arterial pressure, MoM	ns	ns	ns	ns	0.53	0.03	ns	ns
PI UtA, MoM	0.59	0.01	0.54	0.02	ns	ns	0.75	0.0001
PlGF	ns	ns	–0.59	0.01	–0.88	0.0001	ns	ns
	Late-onset PE							
β-hCG, MoM	ns	ns	ns	ns	–0.48	0.03	–0.63	0.004
PAPP-A, MoM	ns	ns	–0.60	0.006	ns	ns	ns	ns

Note: * *r* is a Spearman rank correlation coefficient; ** *p* is the statistical significance of correlation; Ns is not statistically significant.

reflecting the pathophysiological state of donor cells and can modulate the functions of target cells [30]. It is known that placental exosomes mediate the adaptation of maternal vessels to pregnancy, and their plasma concentration increases with the progression of pregnancy and correlates with blood flow in the uterine arteries [31]. Such functional uniqueness allows them to be considered as dynamic biomarkers capable of real-time monitoring of placental dysfunction. [32].

An interesting aspect that attracts attention is exosomal content, which determines the bioactivity of exosomes, and its loading is controlled by one of the posttranslational modifications, sumoylation [27]. Reversible conjugation of the small SUMO peptide with target proteins is critical for cell function and various cellular processes, including transcription, DNA repair, cell cycle regulation, chromatin remodeling, nucleocytoplasmic transport, and apoptosis [33]. The expression of SUMO 2 and SUMO 3 isoforms, which are 97% identical to each other, and SUMO 1 by 46%, was found in all eukaryotic cells [19, 34]. Recent discoveries have demonstrated that another isoform, SUMO 4, is expressed in the placenta [35]. At the same time, disorders of sumoylation homeostasis are associated with various pathological conditions [36, 37]. Despite the available data on the involvement of sumoylation in placental dysfunction [13, 20], there are no data of the exosomal SUMO proteins expression in this pathology.

In the context of the above, we evaluated the SUMO proteins expression in exosomes of pregnant women at 11–14 weeks of gestation (cohort I). It should be noted that we have previously revealed an increase in conjugated SUMO 1–4 and UBC9 expression in exosomes of pregnant women with early-onset PE at the time of delivery [29] and free forms of SUMO 1–4, UBC9 in the placenta in early-onset PE [28]. In the present study, the conjugated SUMO 1 expression in exosomes of pregnant women with early-onset PE at 11–14 weeks of gestation was significantly reduced, while the SUMO 2/3/4 expression was increased relative to normal pregnancy. A similar picture was observed with regard to direction of the conjugated SUMO 1 and SUMO 2/3/4 expression in exosomes in pregnant women with late-onset PE. As previously shown by Baczyk D. et al., the expression pattern of SUMO proteins may be due to their unique spatiotemporal distribution in trophoblast layers during pregnancy, as well as their activity in response to oxidative stress and inflammation. In particular, free SUMO 1 and SUMO 4 are predominantly expressed in cytotrophoblasts

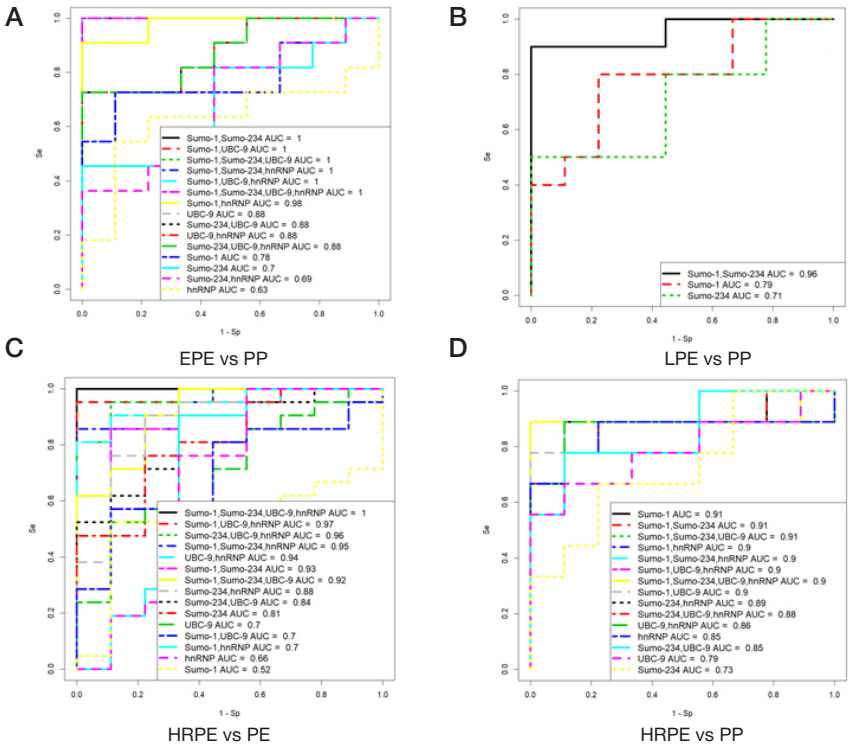

Fig. 5. ROC curves for a logistic model for the risk assessment of PE at early gestation by SUMO 1–4, UBC9 and hnRNPA2/B1 expression in exosomes **(A–D)**. EPE is early-onset PE; LPE is late-onset PE; HRPE is high risk of PE; PP is physiological pregnancy

Table 4. Predictive values with parameters of logistic models for SUMO 1–4, UBC9 and hnRNPA2/B1

	AUC	Sensitivity	Specificity	Cutoff	*p*-value	Parameters	Formulas
EPE vs. PP							
UBC9	0.88	0.72	1	0.72	0.03	i — 2.65 UBC9 — 6.73	$\frac{1}{1+e^{2.65-6.73x_1}}$
LPE vs. PP							
SUMO 1	0.79	0.8	0.77	0.43	0.05	i — 7.4 SUMO1 — 6.29	$\frac{1}{1+e^{-7.4+6.29x_1}}$
HRPE vs. PE							
UBC9; hnRNPA2/B1	0.94	0.80	1	0.79	0.02/0.01	i — 0.18 UBC9 — 6.23 hnRNPA2/B1 — 2.19	$\frac{1}{1+e^{-0.18-6.23x_1+2.19x_2}}$
HRPE vs. PP							
SUMO 1	0.91	0.88	1	0.58	0.02	i — 9.78 SUMO1 — 9.073	$\frac{1}{1+e^{-9.78+9.073x_1}}$

Note: EPE is early-onset PE; LPE is late-onset PE; HRPE is high risk of PE; PP is physiological pregnancy; AUC is area under curve.

in the first and second trimesters of pregnancy, with a shift to the syncytiotrophoblast in the third trimester. In particular, free SUMO 1 and SUMO 4 are predominantly expressed in cytotrophoblasts in the first and second trimesters of pregnancy, with a shift to the syncytiotrophoblast in the third trimester. SUMO 2/3 stable expressed throughout the trophoblast layer during pregnancy. However, the activity and redistribution of SUMO 1 and SUMO 4 into syncytium from the cytotrophoblast is observed in response to hypoxic/oxidative stress, and the SUMO 2/3 expression increases during inflammatory stress [38]. It is noteworthy that the results of our study, in general, are consistent with the above data of Baczyk D. et al., demonstrating multidirectional activation of the expression of exosomal conjugated SUMO 1 and SUMO 2/3/4 in response to cellular stress in placental dysfunction. Nevertheless, the all SUMO isoforms expression and respectively their subsequent secretion should increase in cells under the influence of various stress stimuli. However, SUMO 1 expression in exosomes was reduced in both early-onset and late-onset PE in our study. Previously, Saitoh H. et al. found a decrease in pool of the SUMO 2/3 free in cells and the accumulation of its high molecular weight conjugates in response to cellular stress, in contrast to SUMO 1 [39]. Taking into account these data, we hypothesized that hypoxic and oxidative stress that occurs with early placental dysfunction activates the SUMO 2/3 free to form a large number of conjugates with target proteins and induce stress-sensitive signaling cascades through exosomes. Whereas, SUMO 1 is able to selectively conjugate to targets in response to stress. This is evidenced by a decrease in the expression of its conjugated form in exosomes. Interestingly, we did not find free forms of these proteins in exosomes. And the molecular weight of the conjugated forms, presumably, conforms to the total weight of their free form with UBC9. Moreover, the conjugated UBC9 expression was also significantly increased only in late-onset PE relative to normal pregnancy. It should be noted that UBC9 is the only enzyme that conjugates SUMO 1–4 with target proteins, in contrast to the ubiquitination system [19]. Therefore, its expression level is critical for sumoylation. Based on the previously demonstrated spatiotemporal distribution of SUMO 1–4 in trophoblast cells (38), we suggested a certain specificity of UBC9 conjugation with SUMO 1 and SUMO 2/3 in the context of their subcellular targets. Even more so, evidence of its different immunoreactivity with SUMO proteins has already been obtained on model objects depending on the cell population [40].

Sumoylation is known to modulate the transcriptional activity and localization of many nuclear [41] and cytoplasmic proteins (42), thereby regulating a wide range of biological processes. And certain interest is the recent discovery of the sumoylation of the heterogeneous nuclear ribonucleoprotein (hnRNPs) family members, which provides a universal mechanism for regulating their RNA-binding activity and subsequent selective sorting of transcripts into exosomes [27, 43]. Sumoylation of hnRNPA2/B1 by SUMO 1 is a necessary condition for its binding to specific miRNA exomotifs and their subsequent loading into exosomes. At the same time, inhibition of sumoylation can impair protein binding to miRNA. In our previous study, exomotifs of a number of microRNAs sensitive to hypoxia were identified, and their expression correlated with the of SUMO 2/3/4 level in the placenta of pregnant women with early-onset PE [29]. Based on this relationship, we assessed the hnRNPA2/B1 expression. It was significantly increased in exosomes of pregnant women with late-onset PE at 11–14 weeks, similarly to UBC9. Since hnRNPA2/B1 is a SUMO substrate [44], it is likely that its expression in exosomes may also be regulated by SUMO 2/3/4, if the level of SUMO 1 is reduced. Moreover, providing the specificity of UBC9 conjugation, it may be suppose that the hnRNPA2/B1 expression is activated by conjugation with UBC9 in late-onset PE. And this is necessary for loading microRNAs into exosomes that regulate targets associated with vascular dysfunction. It is worth noting no less interesting results on protein expression in pregnant women with a high risk of developing PE according to the combined prenatal screening of the first trimester. Despite the presence of markers of placental dysfunction, the outcome of their pregnancies was favorable. However, the direction of the conjugated SUMO 1, UBC9 and hnRNPA2/B1 expression coincided with that in early-onset and late-onset PE, the exclusion of SUMO 2/3/4, the level of which did not change.

Considering the revealed changes in the expression of conjugated proteins in the exosomes of pregnant women with PE at 11–14 weeks of gestation, it seemed interesting to evaluate their expression in the placenta of pregnant women with early-onset and late-onset PE (cohort II). In the placenta, we found a decrease in the conjugated SUMO 1–4 and hnRNPA2/B1 expression in both early-onset and late-onset PE. And the level of UBC9 significantly decreases only in late-onset PE. The results were unexpected, just as intriguing. As noted earlier, the process of sumoylation, being the most important regulator of the cellular response to stress, includes an increase in the level of high-molecular-weight SUMO conjugates, as well as free forms. Moreover, a previous study found an increase in SUMO free in early-onset PE [28]. Such multidirectional

expression of free and conjugated forms of the studied proteins in the placenta may be explained by the fact that free forms are expressed exclusively in response to stressful stimuli, performing an adaptive function. While, the formation of SUMO conjugates is necessary to trigger regulatory cascades. Thereby, the change in their expression depends on what is needed at a given time — sumoylation or desumoylation of the target protein. Our assumption has been confirmed in a number of studies. In particular, Bhattacharjee J. et al. demonstrated that the level of SUMO free increases in early pregnancy (9–10 weeks), and this coincides with physiological placental hypoxia. However, overexpression of the SUMO 2/3 can inhibit the activity of HIF 1A (hypoxia inducible factor) at 10–12 weeks of gestation. In order to preserve the stability of latter, the SENP 3 desumoylation protein reduces the expression of SUMO 2/3 [45]. Other authors provide evidence for the role of the SENP 1 desumoylation protein in endothelial cells as a positive regulator of hypoxia-induced VEGF expression and angiogenesis [46, 47]. With regard to placental dysfunction, changes in GCM-1 expression induced by hypoxia and regulated by the p45 NF-E2 transcription factor [48] are associated, among other things, with desumoylation [49,50]. It is important to note that factors that increase global sumoylation do not necessarily lead to changes in sumoylation of all SUMO substrates. Sumoylation of individual proteins occurs in a substrate-specific manner and often without global changes [51].

The discovery of placental factors has contributed to a surge in research on their use as biomarkers for diagnosing and predicting the risk of developing PE and FGR since 2003 [52]. However, the most reliable results were obtained when predicting the early-onset PE. Researchers attribute this to the fact that a change in their level is a reflection of syncytiotrophoblast stress, that is, placental dysfunction in general, and not a biomarker of PE [53]. In addition, the provided diagnostically significant level of pro- and anti-angiogenic factors circulating in the mother's blood can be achieved at the onset of a pathological condition, and not at the initial stage of impaired endovascular invasion of spiral arteries by trophoblast [54]. In this regard, it is necessary to look for specific markers. Interestingly, a decrease in angiogenic PLGF and an increase in anti-angiogenic sFlt-1 during the third trimester correlate with changes in the redistribution of SUMO 1 and SUMO 4 from the cytotrophoblast to the syncytium. The given type of placental cell is the only which directly contacts with the maternal circulation [38]. As previously reported, we found a correlation between changes in miR-423-3p, miR-652-3p expression, the level of SUMO 2/3/4, UBC9 in the placenta and a decrease in the concentration of PLGF in the blood of pregnant women with early-onset PE at the time of delivery [28]. Based on the results, a search was made for correlations with indices of combined prenatal screening of pregnant women in the first trimester. An increased level of exosomal SUMO 2/3/4 and UBC9 correlated inversely with the PLGF in the blood serum of pregnant women in the early gestation, that is in good agreement with our previous data. Moreover, the relationship confirms the regulation of placental factor through sumoylation and indicates the specificity of SUMO 2/3/4 and UBC9 in relation to PLGF. Interestingly, this relationship was revealed only for the early-onset PE. It is noteworthy that, beyond the association with the placental factor, significant correlations were found the expression of all conjugated SUMO isoforms and hnRNPA2/B1 with the uterine artery pulsation index. And UBC9 correlated with mean arterial pressure. The highlight was an increased level of SUMO 2/3/4 was inversely correlated with PAPP-A, while UBC9 and hnRNPA2/B1 were inversely correlated with the concentration of β-hCG in the late-onset PE. At the same time, no correlation was found between SUMO 1 and any of the indices. Low concentrations of β-hCG and PAPP-A in the first trimester of pregnancy are associated with the risk of developing PE and FGR [55–57].

Given the correlation results, as well as the lack of data on the predictive potential of SUMO modifications, we created logistic regression models with ROC-curves to assess the possibility of using the studied exosomal proteins as potential predictors of the risk of developing PE. Significant coefficients were determined for UBC9 (AUC = 0.88; Se-0.72; Sp-1) in early-onset PE prediction model, and for SUMO 1 (AUC = 0.79; Se-0.8; Sp-0.77) in late-onset PE. In addition, it is possible to differentiate pregnant women with a high risk of developing PE from pregnant women with PE and with physiological pregnancy based on the assessment of UBC9, hnRNPA2/B1 expression (AUC = 0.94; Se-0.80; Sp-1) and SUMO 1 (AUC = 0.91; Se-0.88; Sp-1), respectively. We payed attention to SUMO 2/3/4 did not reach a significant level in any of the models. Nevertheless, the results suggest a differential specificity of UBC9 and SUMO 1 in the pathogenesis of PE subtypes, as well as a different functional role of SUMO proteins in placental dysfunction in general, that is important for a preventive therapeutic strategy. Undoubtedly, the use of predictive models will require subsequent validation in a large cohort of pregnant women with relevant clinical outcomes, including isolated PE and FGR.

CONCLUSIONS

The primary value of this research is that it opens up several avenues for predicting conditions associated with placental dysfunction based on the study of the exosomal contents sumoylation pattern. This is the first data on the exosomal expression of conjugated SUMO 1–4, as well as UBC9 and hnRNPA2/B1 that differentially change in early gestation in pregnant women with PE. The possibility of predicting this pathology may be due to the functional specificity of SUMO isoforms, as well as the conjugation/deconjugation mechanism that coordinates the signaling pathways. It is worth noting that our study has limitations due to the small cohorts. We brought this to our attention because the validation of predictive models requires an expansion of the pregnant women cohort. Moreover, we did not evaluate desumoylating protein expression in exosomes. But this is rather seen as a prospect for future research, as well as the study of the SUMO substrates involved in the regulation of placental dysfunction.

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