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V.E. FRANKEVICH¹, E.N. NIKOLAEV^{2,3,4}, N.E. KAN¹, G.T. SUKHIKH¹**DIFFERENTIAL DIAGNOSIS OF HYPERTENSIVE DISORDERS IN PREGNANCY
BASED ON URINE PEPTIDOME PROFILE ANALYSIS**¹National Medical Research Center of Obstetrics, Gynecology and Perinatology named after Academician V.I. Kulakov, Ministry of Health of Russia, Moscow, Russia²Moscow Institute of Physics and Technology, Moscow, Russia³Emanuel Institute for Biochemical Physics, Russian Academy of Sciences, Moscow, Russia⁴Skolkovo Institute of Science and Technology, Skolkovo Innovation Center, Moscow, Russia**Objective.** To identify peptide panel allowing to discern different hypertensive disorders in pregnancy.**Materials and methods.** A case-control study enrolled 64 women that were classified into four groups: preeclampsia (PE), chronic hypertension (CAH), PE superimposed on CAH and control. Urine samples obtained from each patient were analyzed with chromatography coupled with mass-spectrometry followed by bioinformatics analysis.**Results.** For all four groups common typical 36 peptides were identified, which are mainly fragments of collagen (COL1A1; COL3A1, etc.), and one peptide uromodulin (UMOD). For patients with hypertensive disorders (PE, PE superimposed on CAH, CAH) characteristic 34 peptide fragments of collagen (COL1A1; COL3A1, etc.) and alpha-fibrinogen (FGA) were identified. For the group of patients with preeclampsia (PE, PE superimposed on CAH) characteristic panel of 16 peptides were identified: 13 of which are fragments of the protein alpha-1-antitrypsin (SERPIN1), 1 peptide is a fragment of alpha-1 chain collagen 1 (COL1A1), 1 peptide is a part of alpha-2-HS-glycoprotein (AHSG), 1 peptide is a fragment of apolipoprotein A-I (APOA1). Semiquantitative analysis of the data of the four groups (PE, PE superimposed on CAH, CAH, the control group), non-parametric tests Kruskal-Wallis and Mann-Whitney test showed the presence of 112 peptides, differentiating at least one pair of groups. Separately, a study was conducted on the peptide profile of urine from a patient with gestational arterial hypertension (GAH) from the time of diagnosis GAH (32-33 weeks) until delivery (36-37 weeks) in the dynamics. Correlation was established between increased levels of peptides protein SERPIN1(A1AT) and the emergence and increasing severity of PE.**Conclusion.** In the comparative analysis the panel of 12 peptides which can reliably differentiate hypertensive disorders in pregnant women was formed. Fragments of alpha-1-antitrypsin confirmed their importance as markers of preeclampsia suggested by the authors in earlier papers. The demonstrated dynamics of changes in the peptide profile in clinical manifestation of PE in the patient with GAH shows real possibilities of the use of peptide analysis in clinical practice in order to timely diagnose and predict PE. Further studies are needed to implement the results in clinical practice.**Key words:** peptidomics, preeclampsia, hypertensive disorders, mass-spectrometry, biomarkers, predictors, bioinformatics.**Authors' contributions.** Muminova K.T., Kononikhin A.S., Khodzhaeva Z.S., Shmakov R.G., Sergeeva V.A., Starodubtseva N.L., Bugrova A.E., Indeykina M.I., Zakharova N.V., Frankevich V.E., Nikolaev E.N., Kan N.E., Sukhikh G.T.: developing of research design, obtaining data for analysis, reviewing publications on the topic of the article, statistical analysis of the obtained data, article writing.**Conflict of interest.** Authors declare lack of the possible conflicts of interests.**Financing.** This work was supported by grants from the Russian Foundation for Basic Research No. 17-08-01537 A, No. 16-54-21011 ShNF.

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As preeclampsia (PE) accounts for high rate of maternal and perinatal morbidity and mortality and has short- and long-term consequences, its prediction and early (preclinical) diagnosis is of utmost importance [1–5]. Thus, differential diagnosis of hypertensive disorders in pregnancy is of current interest as these pathologies affect not only outcomes for the mother and fetus but their future quality of life as well. According to the international

recommendations proteinuria is no longer a criterion of PE as one cannot exclude preexisting urinary tract pathology [2–6]. Meanwhile there are data that proteinuria can precede PE [7]. Non-significant proteinuria often accompanies hypertension and proteome/peptidome analysis is a promising tool for accurate PE detection.

Current classification distinguishes chronic arterial hypertension (CAH), gestational arterial hypertension

(GAH), de novo emerged after 20 weeks of gestation, preeclampsia (PE), and PE superimposed on CAH [1-5].

Proteome fingerprints of 59 urine samples characteristic of severe PE requiring emergent delivery were revealed in the exploratory phase of one of the first studies with 284 participants [8]. In the challenge phase algorithm was tested on 225 women with high and low risk of PE. Then in translational phase identification and validation of biomarkers were performed by tandem mass-spectrometry in urine, blood serum and placenta. In the end, 5 SERPINA-1 and albumin peptides were proven to be PE biomarkers.

Later on serum peptide analysis was conducted in 62 pregnant women (31 with PE, 31 – control) [9]. Authors showed 19 marker-peptides, 13 of which belonged to fibrinogen, 1 to alpha-1-antitrypsin, 1 to apolipoprotein L1, 1 to H4 inter-alpha-trypsin inhibitor heavy chain, 2 to kininogen-1, and 1 to thymosin beta-4.

A case-control study [10] allowed to identify 50 peptides derived from collagen alpha-chain, fibrinogen alpha-chain, uromodulin, retinol-binding protein and some other protein precursors. Authors collected samples from women at 12, 16, 20, and 28 weeks of pregnancy. They showed a peptide panel at 28 weeks predictive for PE as well as its dynamics at 12 and 16 weeks. However, other researchers note that the same peptides can be identified in the urine of patients with chronic kidney disease [11] and heart failure [12, 13], sometimes as a consequence of CAH. In such disorders, as PE, endothelial dysfunction and systemic inflammatory response take place [14]. Given these facts, there is a need for more precise differential diagnosis of PE and other hypertensive disorders, CAH in particular.

The aim of our study was to identify peptide panel capable to discern hypertensive disorders in pregnant women: PE, PE superimposed on CAH and CAH.

Materials and methods

Urine samples were collected in National Research Medical Center of Obstetrics, Gynecology and Perinatology named V.I. Kulakov after the patients' informed consent. Severity of PE was defined according to Federal recommendations approved by Ministry of Healthcare of Russia [1]. Early-onset PE was defined as PE manifestation before 34 weeks. Normotensive healthy women with proteinuria at a level lower than 100 mcg/ml were enrolled into control group.

Urine samples were chosen as the object of study due to the fact that urine collection is known to be non-invasive and have high specificity of urine peptidome in different pathologies, especially associated with kidney function impairment [8–19]. Therefore, this approach may lead to development of promising minimally invasive diagnostic method.

The study included 64 patients: 5 of whom had late-onset severe PE, 11 patients had late-onset mild PE, 9 patients with early-onset severe PE, 3 patients with early-onset mild PE, 8 patients with PE superimposed on CAH, 8 patients with CAH, 20 patients – control.

Within 20 min after collection urine samples were centrifuged for 10 min at 2000 g, 4 °C. The supernatant was then stored at –80 °C. Peptides were extracted by

size-exclusion chromatography and then were analyzed by HPLC-MS/MS according to previously described protocols [16–18]. Briefly, 1.5 ml of urine was diluted with the denaturing buffer (4 M urea, 20 mM ammonium hydroxide, 0.2% sodium dodecyl sulfate) and underwent ultrafiltration and gel-filtration for separating large proteins, low-weight contaminants and changing the buffer. HPLC-MS/MS analysis was performed on a nano-HPLC Agilent 1100 system (Agilent Technologies, Santa Clara, CA, USA) using homemade capillary column (id 75 µm × length 12 cm, Reprosil-Pur Basic C18, 3 µm, 100 Å; Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) in combination with a 7-Tesla LTQ-FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanospray ion source (in-house system). Gradient chromatography was implemented by changing the relative concentration of solvent B (80% acetonitrile/0.1% formic acid) in flow of solvent A (0.1% formic acid). Main elution time was 15-45 min: linear gradient from 3% to 50% of solvent B, elution of the most hydrophobic peptides was 45-50 min: linear gradient from 50% to 90% of solvent B. Urinary peptides identification was achieved by the UniProtKB database (UniProt release 2015_10), database search using MaxQuant (version 1.5.3.30). Following parameters were used: non-specific enzyme, mass accuracy for the precursor ion was 20ppm, mass accuracy for MS/MS fragments was 0.50 Da; possible variable modifications were oxidation of methionine, lysine and proline residues, up to 5 variable modifications per peptide, minimal peptide length was 5 amino acids, maximal peptide weight was 10 kDa, FDR≤0.01, minimal score for unmodified peptides was 20, for modified - 40. Semiquantitative results per each identified protein were obtained by label-free method with alignment of chromatograms and normalization to the total intensity. Method of projection to latent structures was used for visualizing the obtained data. Statistical differences in urine protein constituents between groups studied were detected using the Kruskal-Wallis test. For chosen peptides, pairwise analysis was performed using Mann-Whitney test with Bonferroni correction.

Results and discussion

Mean age in PE, CAH, PE superimposed on CAH, and in control group was 32.02 ± 2.09 , 35.8 ± 1.77 , 34.07 ± 2.44 , and 31.07 ± 2.40 years, respectively. Mean BMI was 28.06 ± 5.5 kg/m², 31.6 ± 3.8 kg/m², 29.5 ± 2.8 , and 28.26 ± 4.5 kg/m², respectively. In the intergroup analysis, the incidence of hypertension in family history in PE, CAH and PE superimposed on CAH did not differ statistically between the groups: 13 (46.4%), 4 (50%) and 4 (50%), but was significantly higher compared to control: 4 (20%) ($p < 0.01$). Thus, family history of hypertension is a risk factor for hypertensive disorders in pregnancy. In group with PE, women were significantly more likely to have adverse pregnancy outcomes: antenatal fetal death ($n=2$; 7.1%), early neonatal death ($n=1$; 3.6%), history of preeclampsia ($n=5$; 17.9%) compared to women with PE superimposed on CAH (0, 0, and $n=1$; 12.5%, respectively), and to control group where these complications were not observed ($p < 0.05$).

Maternal characteristics are displayed in Table 1.

The duration of prolonged pregnancy from the moment of PE manifestation was 13.9 ± 8.2 days, respectively ($p < 0.01$). The rate of mandated preterm delivery due to fetal condition deterioration was significantly higher and accounted for 46.4% ($n=13$) in PE group. Aggravation of PE, ineffectiveness of antihypertensive therapy and neurological manifestations resulted in delivery in 53.6% ($n=15$).

To identify endogenous urine peptides obtained from 64 patients an analysis of 256 basic chromatograms with mass spectra (4 repeated analyses of each sample) was performed. Due to the presence of possible peptides modifications (oxidation of methionine, Proline, lysine), as well as non-specificity of N- and C-terminal groups, the search with full database of human proteins requires significant computational resources. To facilitate the search procedure, we created a lesser database consisting of 145 proteins, which allowed us to use the computing power of the laboratory to conduct the search, identification and semi-quantitative

analysis of the data obtained by HPLC-MS/MS. The design of the experiment is shown in Figure 1.

The PE group was analysed without classifying the patients according to time of the onset and severity of symptoms due to the small number of patients. Thus, the study comprised four groups: PE, PE superimposed on CAH, CAH, healthy pregnant women.

When performing projection to latent structures (PLS is one of the statistical methods of linear regression), a distribution of the first two components for all urine peptides in patients of all groups was made. The PLS model of two groups (control and CAH) is shown in Fig. 2: area 2 stands for control group (C), area 1 refers to CAH. Figure 3 shows the PLS-model of other clustered groups: area 2 reflects PE and PE superimposed on CAH (PE and PE+CAH, respectively), area 1 reflects CAH and controls (CAH and C, respectively). The statistical model is characterized by parameters $R^2 = 0.87$ and $Q^2 = 0.65$. The latter indicates a good predictive ability in cases of PE or PE superimposed on CAH. Although

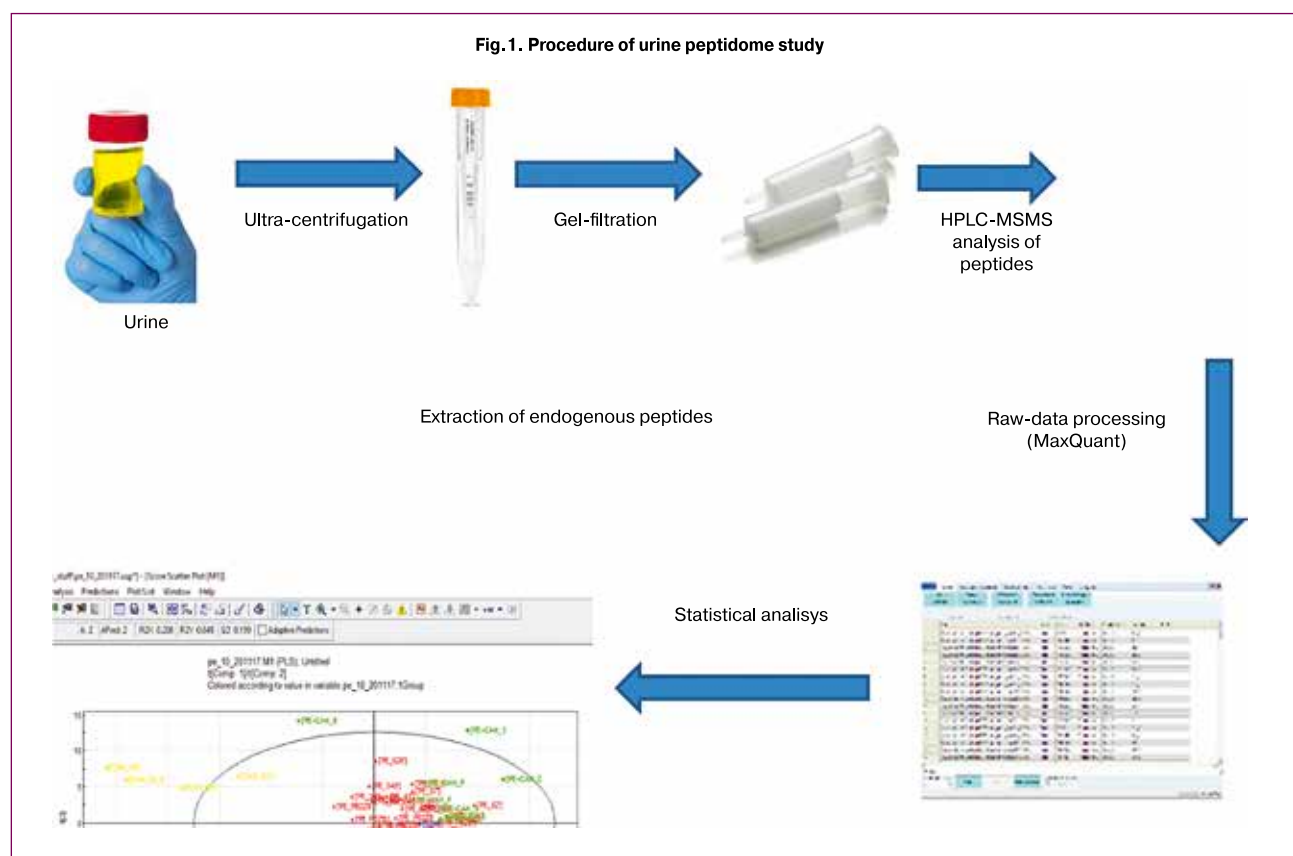


Table 1. Clinical and anamnestic features of the patients

	PE (n=28) 1	CAH (n=8) 2	PE superimposed on CAH (n=8) 3	Control (n=20) 4
Mean age, years	32.02 ± 2.09	35.8 ± 1.77	34.07 ± 2.44	31.07 ± 2.40
BMI, kg/m ²	28.06 ± 5.5	31.6 ± 3.8	29.5 ± 2.8	28.26 ± 4.5
Family history of hypertension	13(46.4%)	4(50.0%)	4(50.0%)	4(20.0%)*
Stillbirth	2(7.1%)	1(12.5%)	0	0
Early neonatal death	1 (3.6%)	0	0	0
History of PE	5(17.9%)	0	1 (12.5%)	0

* $p < 0.01$ comparing to other groups.

there are several outliers in each group, two clusters are well pointed out, between which a separation line can be drawn with a high degree of reliability.

Semiquantitative analysis of the four groups (PE, PE superimposed on CAH, CAH, the control group) by non-parametric tests of Kruskal-Wallis and Mann-Whitney revealed the presence of 112 peptides, distinguishing at least one pair of groups (Table 2).

More detailed distribution of these peptides is shown in the Venn diagram (Fig.4). The intersection of all four groups includes 36 peptides: 22 of which are derived from alpha-1 chain of collagen 1, 9 peptides are from the alpha-1 chain of collagen 3, 2 peptides are from the alpha-2 chain of collagen 1, 1 peptide is from the alpha-1 chain of collagen 1/18, and 1 peptide is from the uromodulin. The second largest intersection corresponding to groups with hypertensive disorders contains 34 peptides: 12 peptides are from the alpha-1 collagen chain 1, 10 peptides are from the alpha-fibrinogen, 8 peptides are from the alpha-1 collagen chain 3, and 4 peptides are from other collagen types. The presence of fibrinogen peptides may indicate arterial hypertension.

Fig.2. PLS-analysis graphics based on semiquantitative peptidomics data of two groups: CAH – area 1, control (C) – area 2

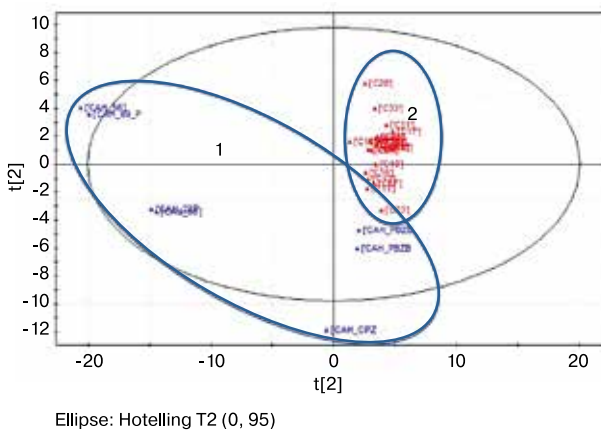
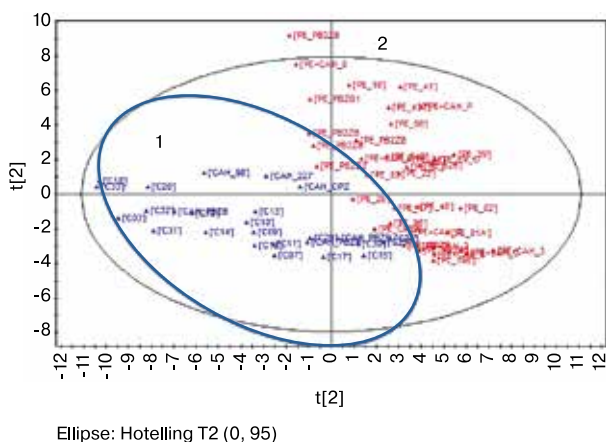


Fig. 3. PLS-analysis graphics based on semi-quantitative peptidomics data of four groups: control (C) and CAH – area 1, PE and PE superimposed on CAH – area 2



In this study, we do not find statistically significant peptides belonging exclusively to the PE group. However, what is more important, a group of 16 peptides specific to both PE and PE superimposed on CAH was discovered. Of these, 1 peptide is a fragment of the alpha-1 chain of collagen 1, 1 peptide is a fragment of alpha-2-HS - glycoprotein, 1 peptide is a fragment of apolipoprotein A - I, and the remaining 13 peptides are parts of alpha-1-antitrypsin. Fibrinogen fragments in urine indicate mainly an inflammation and cardiovascular diseases, meanwhile peptides of protein alpha-1-antitrypsin, in the native form inhibiting one of the most common proteases-trypsin, indicate changes in the metabolic pathways of protein degradation.

A complete sequence of alpha-1-antitrypsin is presented in Figure 5, where we outlined the identified marker peptides. These peptides are also presented in Table 1. It is noteworthy that only 4 peptides are located in the central region of the protein, and all the rest cover the C-terminal zone. Connections between almost every pair of amino acids have been broken in the area starting with Proline-393 and ending with glutamic acid-400. The exceptions are valine-395 and phenylalanine-396, and these amino acids are not included in any of the noted peptides. The mechanism for such a specific and intense protease activity has not been identified yet, however there is a theory that explains absence of fragments with 394-FVFLM-398 sequence among the detected peptides. Molecular dynamics methods [20] showed that this pentapeptide has an aggregation ability considerably surpassing the one of the 16-KLVFF-20 beta-amyloid fragment, which requires a slightly different approach in biochemical analysis compared to the basic protocol according to which this study was conducted. The role of amyloids in the pathogenesis of preeclampsia is ambiguous, however this theory still exists [20].

While comparing the received data of our study, in particular a group of 16 peptides specific to both PE and PE superimposed on CAH, with the results of other authors [17, 18], we revealed the presence of 12

Fig.4. Venn diagram for 112 peptides identified by Kruskal-Wallis test ($p < 0,05$) based on semiquantitative peptidomics data of four groups: control, CAH, PE, and PE superimposed on CAH

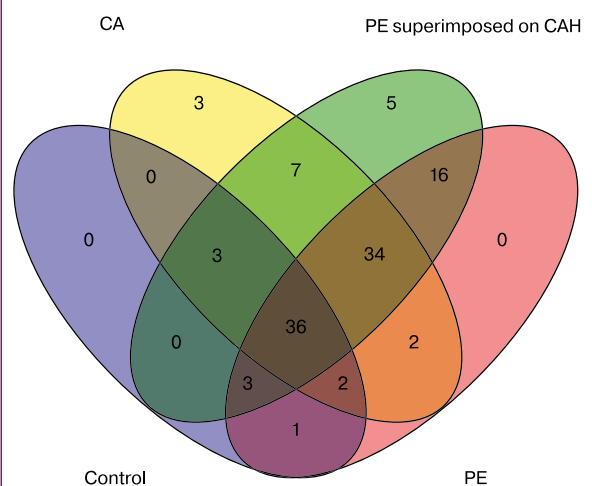


Fig.5. Amino acid sequence of SERPINA1 protein. Underlining displays the rate of a fragment appearance in marker peptides: one, two, three, four, five times

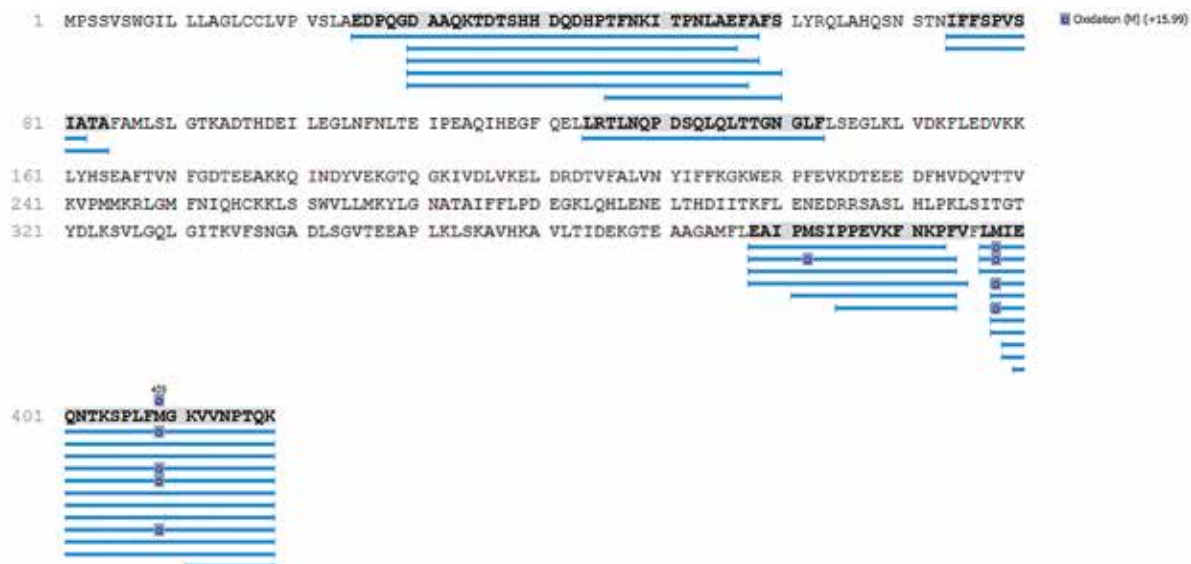


Fig.6. Dynamics of urine peptide profile changes in a patient with GAH starting from admission (32-33 weeks) till delivery when developing severe PE (36 weeks)

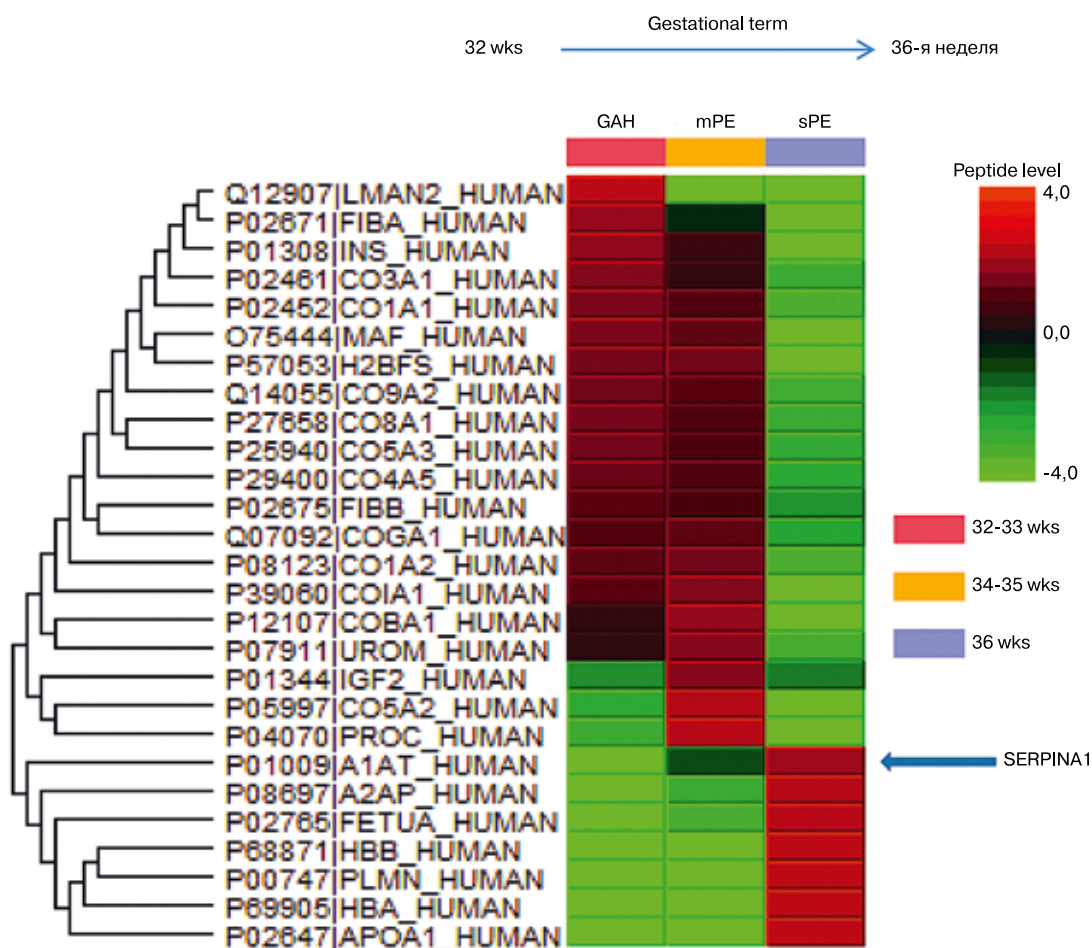


Table 2. Urine peptide panel allowing for precise diagnosis of certain hypertensive disorders in pregnancy (CAH, PE, and PE superimposed on CAH)

Peptide	Protein code	Gene	Protein	Mann-Whitney criterion, <i>p</i>					
				PE superimposed on CAH vs. control	PE superimposed on CAH vs. CAH	PE superimposed on CAH vs. PE	PE vs. control	PE vs. CAH	CAH vs. control
387EAIPIPIPEVKFNKPF404	P01009	SERPINA1	α_1 -antitrypsin	0.001	0.032	0.030	0.085	0.280	-
400EQNTKSPLFMGKVNPTQK418	P01009	SERPINA1	α_1 -antitrypsin	0.001	0.032	0.006	0.239	0.472	-
642GLPGTGGPPGKPG657	P02461	COL3A1	Collagen α_1 (III) chain	0.011	0.751	0.423	0.012	0.221	0.002
642GLPGTGGPPGKPGEP659	P02461	COL3A1	Collagen α_1 (III) chain	0.004	1.000	0.142	0.005	0.175	0.002
399IEQNT KSPL407	P01009	SERPINA1	α_1 -antitrypsin	0.005	0.076	0.029	0.239	0.472	-
397LMIEQNTKSPLFMGKVNPTQK418	P01009	SERPINA1	α_1 -antitrypsin	0.001	0.032	0.017	0.142	0.361	-
124LRLNQDLSQLLTTGNGL143	P01009	SERPINA1	α_1 -antitrypsin	0.005	0.076	0.010	0.422	0.640	-
397MIEQNTKSPL407	P01009	SERPINA1	α_1 -antitrypsin	0.001	0.032	0.030	0.085	0.280	-
591SVIDQSRVNLNLPITR606	P07911	UMOD	Uromodulin	0.157	0.110	0.320	0.326	0.005	0.001
1177VGPVPPGPPGPPGPPSA1193	P02452	COL1A1	Collagen α_1 (I) chain	0.003	0.031	0.090	0.000	0.098	0.134
1177VGPVPPGPPGPPGPPSA1194	P02452	COL1A1	Collagen	0.004	0.031	0.202	0.001	0.143	0.232
1177VGPVPPGPPGPPGPPSAG1195	P02452	COL1A1	α_1 (I) chain	0.009	0.041	0.154	0.002	0.189	0.162

common peptides. These peptides are related to alpha-1-antitrypsin and alpha-1(I and III) collagen chains (Table 2) and have confirmed their role as potential PE markers.

Moreover, we examined the dynamics of urine peptide profile in a patient with GAH transformed into severe PE from the onset of hypertension (32-33 weeks) to delivery (36-37 weeks). As a result, the possibility of utilization of the proposed panel of peptide markers for timely diagnosis and prediction of PE was shown (Fig. 6).

Conclusion

The results of the study suggest that endogenous urine peptides of pregnant women are specific to various hypertensive pathologies, in particular, to preeclampsia. Analysis of urine samples of 64 patients provided a panel of 112 peptides that allowed to distinguish healthy patients from the group of CAH, PE or PE superimposed on CAH. Peptides-fragments of alpha-1-antitrypsin and alpha-1 (I and III) collagen chains proved their importance as PE markers, previously proposed by other authors. A group of 16 peptides, peculiar for both PE and PE superimposed on CAH, confirms the presence of specific peptide signatures for PE. The demonstrated dynamics of changes in the peptide profile in clinical manifestations of PE in the patient with GAH transforming into severe PE shows the real possibilities of the use of peptide analysis of urine in clinical practice for the timely diagnosis and prediction of PE. Further studies will be needed to obtain detailed data to predict and diagnose PE early and more precisely. .

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