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VALIDATION OF THE DIAGNOSTICS ALGORITHM TO MONITOR COAGULATION PARAMETERS IN PREGNANT WOMEN

D. S. KOROLOVA[1], A. O. PAVLENKO[1], A. ALTORJAY[2], S. I. ZHUK[3], I. V. US[3], Y. TSARYK[1], A. SURANYI[2], V. O. CHERNYSHENKO[1]✉

[1]Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;
[2]Albert Szent-Györgyi Medical School, University of Szeged, Hungary;
[3]P. L. Shupyk National Medical Academy of Postgraduate Education, Kyiv, Ukraine;
✉e-mail: bio.cherv@gmail.com

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Thrombotic events are among the most dangerous complications of pregnancy. Therefore, selection of appropriate tests and standardization of techniques used for accurate diagnostics of blood coagulation system state is of great importance. In this present study, we monitored several molecular markers of the dangers of intravascular thrombus formation and estimated the platelet function in pregnant women during gestation. We performed independent measurements using the same methodology for different cohorts of patients recruited in Kyiv (Ukraine) and in Szeged (Hungary). D-dimer and soluble fibrin were measured using ELISA. Protein C (PC) level was estimated using chromogenic substrate assay. Fibrinogen concentration was measured by spectrophotometry using thrombin-like enzyme. Platelet function was estimated by aggregometry. Statistical data analysis was performed using the Kruskal-Wallis test. Statistically significant increases of fibrinogen concentration from first to third gestational trimester was shown for both studied cohorts of patients (5-6 mg/ml at third trimester on average). Applied methods allowed us to detect the same tendencies of decreases in PC level as well as the appearance of moderate amounts of D-dimer (up to 300 ng/ml) and SF (up to 10-15 ug/ml). Platelet function was increased on the first trimester of pregnancy and decreased during following trimesters slightly. Results indicated the changes in the blood coagulation system of pregnant women during gestation with the same effectiveness independently of the selected cohorts, time and place of measurements. The application of the proposed diagnostics algorithm may allow estimating the risk of thrombotic complications during pregnancy.

K e y w o r d s: pregnancy, platelets, thrombosis, soluble fibrin, D-dimer, fibrinogen.

Thrombosis is among the most dangerous complications in pregnancy that can affect both mother and fetus' health. It is a major cause of maternal death in the developed world [1]. Early detection and effective prevention of deep vein thrombosis (DVT) and pulmonary embolism are important issues during gestation [2, 3]. Prognosis of the risk of intravascular thrombus formation during pregnancy is based mainly on clinical data, retrospective analysis, genetic markers, etc. Blood coagulation system parameters are often omitted in current algorithms [4].

Taking into account the necessity of blood coagulation parameters analysis, some diagnostic strategies include the measurement of D-dimer [5]. Authors are focusing on measuring D-dimer values for diagnosing the danger of intravascular blood clotting in the nonpregnant population. However, preg-

nancy leads to the physiological gradual increase in circulating D-dimer so this parameter may be helpful in the diagnosis of DVT only in early pregnancy [6]. This allowed some researchers to suggest that D-dimer has no practical diagnostic use in ruling out such pathologies as venous thromboembolism in the third trimester. D-dimer concentration is simply increasing during gestation as is the concentration of fibrinogen [7].

Most diagnostic algorithms do not include fibrinogen or D-dimer. It is much safer to prescribe heparins to the pregnant women during the third trimester without additional analysis [8]. It was even reported that thrombophilia evaluations should not be performed in pregnant women as far as women normally become prothrombotic in pregnancy, with increases in many procoagulant factors and a decrease in anticoagulant factors [9]. That is why anticoagulants are being prescribed independently of blood coagulation parameters during the late periods of gestation[10].

In the present study, we applied recent findings for diagnosing the danger of intravascular blood clotting to develop the algorithm for detecting a prethrombotic condition during pregnancy. We selected simultaneous D-dimer and soluble fibrin (SF) measurements, added estimation of anticoagulant protein C (PC) level as well as fibrinogen concentration and platelet function estimations using aggregometry [11-14].

We proposed easy-to-use, well-defined and informative methods as part of the diagnostic algorithm. To prove their usefulness, we studied the changes in measured parameters during gestation. Also, we approbated the algorithm independently in two different medical facilities, one in Ukraine (Kyiv Perinatal Center) and the other in Hungary (Department of Obstetrics and Gynecology, University of Szeged) using the same methodology for different cohorts of pregnant women.

Our aim was to prove selected methods and to confirm their applicability for the detection of the danger of intravascular thrombus formation during pregnancy.

Materials and Methods

Materials. PC activator and control donor blood plasma were from Siemens (Munich, Germany). Monoclonal antibodies to D-dimer or SF, thrombin-like enzyme from snake venom were obtained from Palladin Institute of Biochemistry of NAS of Ukraine (Kyiv, Ukraine); PC-specific chromogenic substrate S2236 (p-Glu-Pro-Arg-pNa) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Cohorts. Somatically healthy women 18–42 years old with spontaneous pregnancies that did not receive specific antithrombotic treatment were enrolled in the study at the Kyiv Perinatal Center (n = 70; 12.2021-03.2023) and Department of Obstetrics and Gynecology, University of Szeged (n = 132; 09.2022-12.2022).

Ethical statement. This study was approved by the Ethics Commission of the Kyiv Perinatal Center (protocol № 3, 05.05.2020) and the University of Szeged (32/2014 SZTE).

We examined 70 pregnant patients who sought consultation at an obstetrician's specialized hematology office, were observed in a specialized women's consultation or were undergoing inpatient treatment at the KNP "Perinatal Center of Kyiv." All patients were divided into groups according to their period of pregnancy: Group I – 15 pregnant women in the first trimester (up to 13 weeks of gestation), Group II – 22 pregnant women in the second trimester (13–26 weeks), Group III – 33 pregnant women in the third trimester of pregnancy (27–41 weeks).

One hundred thirty-two patients were recruited in the Department of Obstetrics and Gynecology, University of Szeged. All patients were divided into groups according to the period of pregnancy: Group I – 9 pregnant women in the first trimester (up to 13 weeks of gestation), Group II – 113 pregnant women in the second trimester (13–26 weeks), Group III – 10 pregnant women in the third trimester of pregnancy (27–41 weeks).

Inclusion criteria: age of patients 18–40 years, singleton pregnancy that occurred naturally, without severe pregnancy complications and severe extragenital pathology. Exclusion criteria: age less than 18 or more than 40 years, multiple pregnancy, pregnancy resulting from IVF, taking antiplatelet drugs during pregnancy, severe extragenital pathology, chromosomal pathology and fetal malformations.

All women provided oral and written informed consent for their inclusion in the study. Blood for testing was taken using vacuum systems in tubes with 3.8% sodium citrate.

Fibrinogen. Fibrinogen concentration in the blood plasma was determined by the modified spectrophotometric method. Blood plasma (0.2 ml) and phosphate-buffered saline (1.7 ml) were mixed in a glass tube. Coagulation was initiated by the addition of 0.1 ml thrombin-like enzyme from *Agkis-*

trodon halys halys snake venom (1 NIH/ml) to prevent fibrin cross-linking. Mixture was incubated for 30 min at 37 °C. The fibrin clot was removed and resolved in 5 ml of 1.5% acetic acid. The concentration of protein was measured using Spectrophotometer OPTIZEN POP (Daejeon, South Korea) at 280 nm (ε = 1.5) [15].

Soluble fibrin. SF was detected using sandwich ELISA with monoclonal antibodies produced at the Palladin Institute of Biochemistry of NAS of Ukraine. Fibrin-specific monoclonal antibody I-3C was used as capture antibody. Biotinilated monoclonal antibody II-4d that has epitope at the NH_2-terminal fragment of γ-chain of D-region of fibrinogen molecule was used as a tag antibody. Optical density was measured at 492 nm using Multiplate Reader RT-2100C (Rayto, China) [16].

D-dimer. D-dimer was detected using sandwich ELISA as described above for SF with modification. Biotinilated DD-specific monoclonal antibody III-3B that has epitope at the NH_2-terminal fragment of Bβ-chain of D-region of fibrin(ogen) was produced at the Palladin Institute of Biochemistry of NAS of Ukraine used as the capture antibody [17].

Protein C. Total PC level in blood plasma was determined using PC activator and specific chromogenic substrate S2236 (p-Glu-Pro-Arg-pNa) [18]. In a well of 96-well plate, 0.02 ml of studied blood plasma sample, 0.03 ml of S2236 solution (0.25 mM) and 0.03 ml of PC activator solution were admixed in the tris-buffered saline with 0.001 M $CaCl_2$ at final volume of 0.25 ml. The generation of colorful p-nitroaniline (pNa) was monitored at 405 nm using Thermo Multiskan (Thermo Fisher Scientific, USA). Results were presented as percentages from control values.

Platelet aggregation. Platelet aggregation was measured based on changes in the turbidity of human platelet-rich plasma (PRP). In a typical experiment, 250 µl of PRP was incubated with 25 µl of 0.025 M $CaCl_2$ and 25 µl of 12.5 µM adenosine diphosphate (ADP) at 37 °C. Aggregation was monitored for 10 min using the SOLAR analyzer of platelets aggregation AP2110 model (Minsk, Belarus) [19].

Statistics. Statistical data analysis was performed using the Kruskal-Wallis test (https://www.socscistatistics.com/tests/kruskal/default.aspx). All assays were replicated three times. Results are presented as boxplot diagrams with median, maximal and minimum values and interquartile range. Data were considered significant when $P < 0.05$.

Results

It is known fact that increasing fibrinogen concentration is observed during pregnancy. Our method of direct estimation of fibrinogen concentration was effective and showed reliable results in both studied cohorts (Fig. 1). Fibrinogen concentration elevation up to 5 mg/ml during the third trimester of pregnancy was statistically significant.

The excessive elevation of D-dimer concentration was detected for both cohorts (Fig. 2). Such increase was not dramatic and did not exceed 500 ng/ml but indicated the digestion of stabilized fibrin deposits by the fibrinolytic system. As it is shown on Fig. 3, concentration of SF, which indicates intravascular generation of active thrombin, was gradually

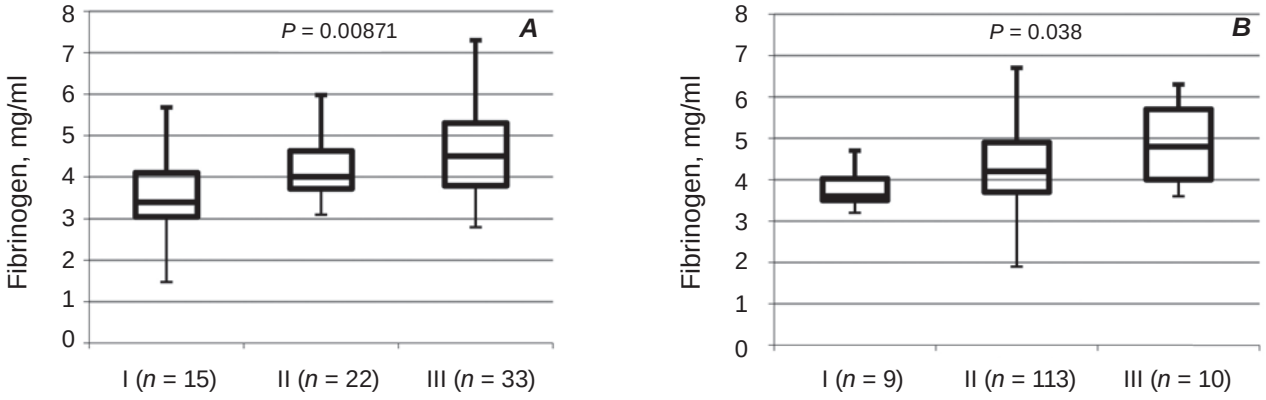

*Fig. 1. Changes in fibrinogen concentration in pregnant women during gestation. Data collected at the Kyiv Perinatal Center, Ukraine (**A**) or in Department of Obstetrics and Gynecology, University of Szeged, Hungary (**B**). Results analyzed by Kruskal-Wallis test. Results assumed as significant when $P < 0.05$. The normal range of fibrinogen concentration in blood plasma of healthy donors is 3.0 ± 0.5 mg/ml*

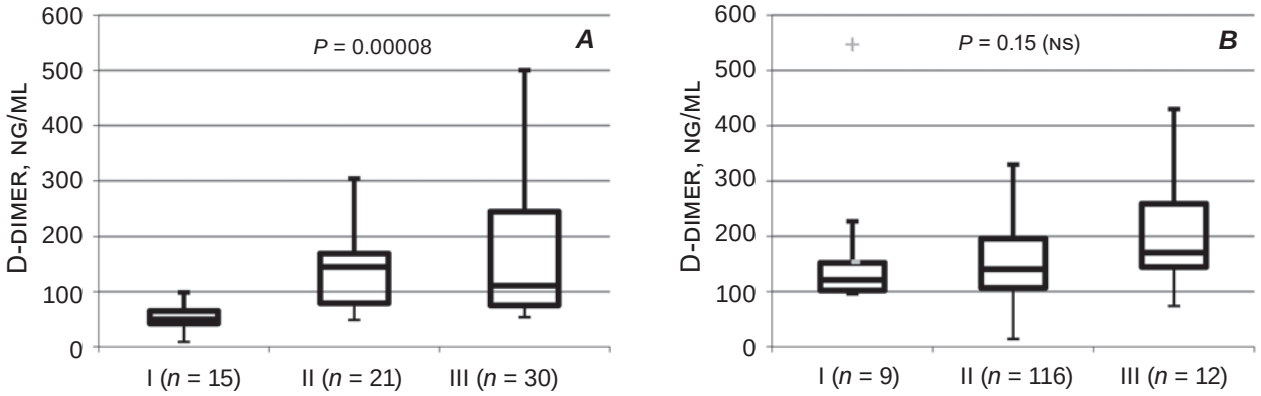

*Fig. 2. Changes in D-dimer concentration in pregnant women during gestation. Data collected at the Kyiv Perinatal Center, Ukraine (**A**) or in Department of Obstetrics and Gynecology, University of Szeged, Hungary (**B**). Results analyzed by Kruskal-Wallis test. Results assumed as significant when $P < 0.05$. The normal range of D-dimer concentration in blood plasma of healthy donors does not exceed 100 ng/ml*

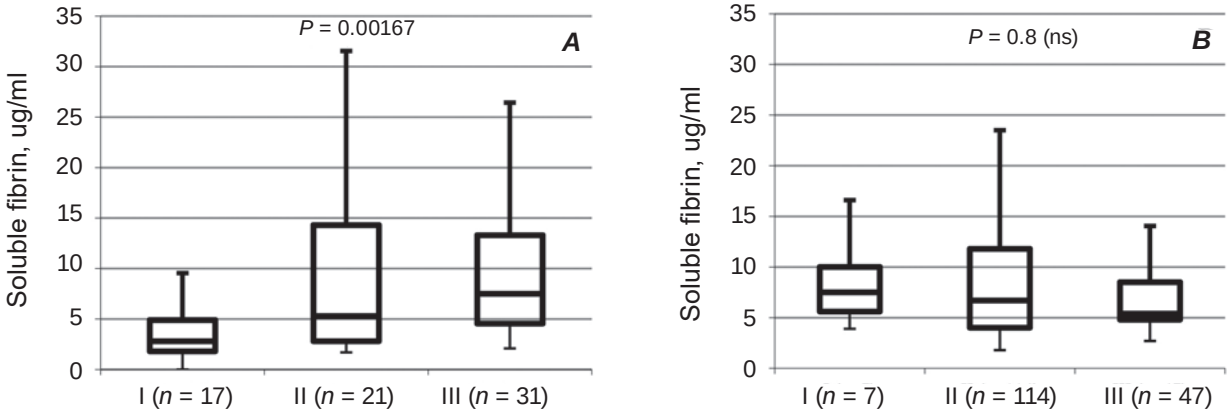

*Fig. 3. Changes in soluble fibrin concentration in pregnant women during gestation. Data collected at the Kyiv Perinatal Center, Ukraine (**A**) or in Department of Obstetrics and Gynecology, University of Szeged, Hungary (**B**). Results analyzed by Kruskal-Wallis test. Results assumed as significant when $P < 0.05$. The normal range of soluble fibrin concentration in blood plasma of healthy donors does not exceed 3 μg/ml*

increased during gestation in the first cohort (Kyiv) and was not changed in the second cohort (Szeged). However, some patients demonstrated elevated concentrations that must be precisely analyzed to prevent intravascular blood coagulation.

Both studied cohorts demonstrated the decrease of PC level from the first to the third trimesters (Fig. 4). It was correlated to SF accumulation. Notably some of the studied patients exhibited a drop in PC level down to 60%, which is an alarming sign. However, overall PC level remained within the normal range.

Analysis of platelet aggregation demonstrated a much more reliable change during gestation. Platelets were activated by ADP and speed and rate of platelet aggregation were monitored. It is important to emphasize that the same reagents and same preparation protocols were used for both studied cohorts.

Both rate of platelet aggregation (Fig. 5) and speed of platelet aggregation (Fig. 6) increased during the first trimester of pregnancy compared to the healthy nonpregnant population. However, both parameters decreased as the pregnancy continued in both studied cohorts. This tendency was obvious, but statistical significance was found only in the first cohort.

Thus, platelet ability to aggregate was higher during the first trimester and decreased to the end of pregnancy; although, it remained higher than usual. Interestingly, the drop in platelet aggregation (indicating suppressed blood clotting) was accompanied by acceleration in concentrations of fibrinogen, SF

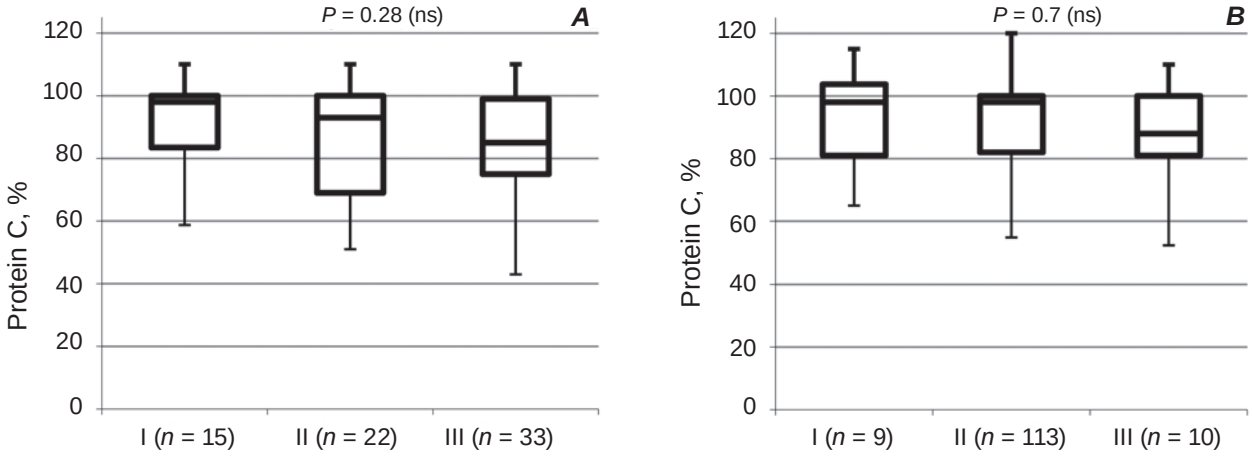

*Fig. 4. Changes in protein C level in pregnant women during gestation. Data collected at the Kyiv Perinatal Center, Ukraine (**A**) or in Department of Obstetrics and Gynecology, University of Szeged, Hungary (**B**). Results analyzed by Kruskal-Wallis test. Results assumed as significant when $P < 0.05$. The normal range of protein C level in blood plasma of healthy donors is 100 ± 15%*

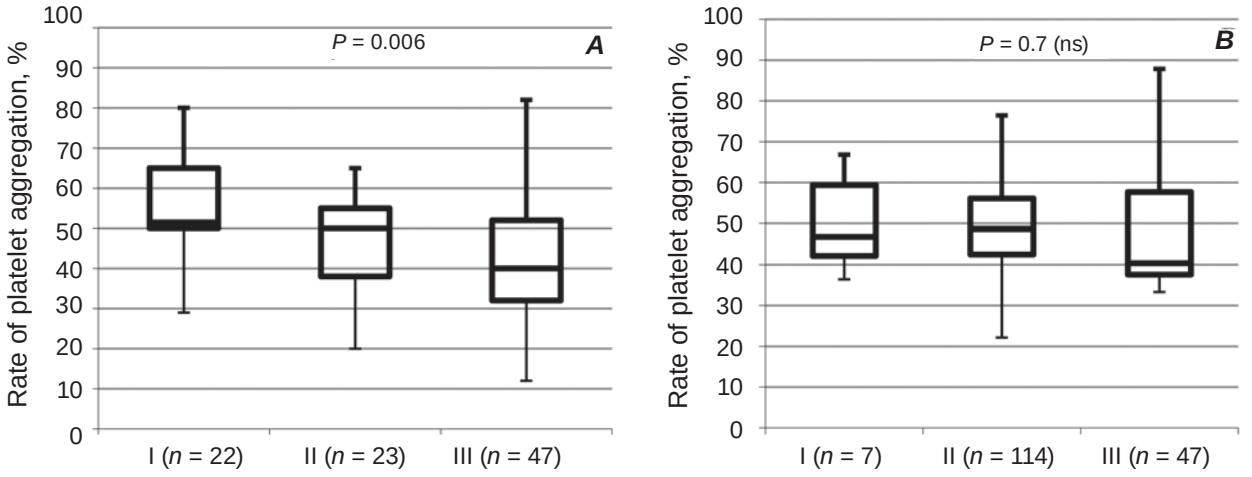

*Fig. 5. Changes in platelet aggregation rate in pregnant women during gestation. Data collected at the Kyiv Perinatal Center, Ukraine (**A**) or in Department of Obstetrics and Gynecology, University of Szeged, Hungary (**B**). Results analyzed by Kruskal-Wallis test. Results assumed as significant when $P < 0.05$. The normal range of the rate of platelet aggregation in blood plasma of healthy donors is 30 ± 10%*

and D-dimer that are known markers of elevated blood clotting.

Discussion

Complex analysis of the state of the blood coagulation system in blood plasma of pregnant women during gestation was performed independently in Kyiv (first cohort) and Szeged (second cohort). In performing measurements at different times and in different countries, we used exactly the same approaches that allowed us to analyze changes in studied parameters and to verify the usefulness of the proposed methods.

The concentration of fibrinogen was analyzed by modified spectrophotometric method. This approach allowed us to evacuate fibrinogen from plasma easily and to measure its concentration directly using spectrophotometer. Elevation of the fibrinogen level in both studied cohorts during gestation corresponded to recent knowledge about increasing fibrinogen concentration during pregnancy [20]. This also proves the adequacy of applied methods and measurements.

The monoclonal antibodies to D-dimer and to SF were used for the detection of these protein markers using sandwich-ELISA technique.

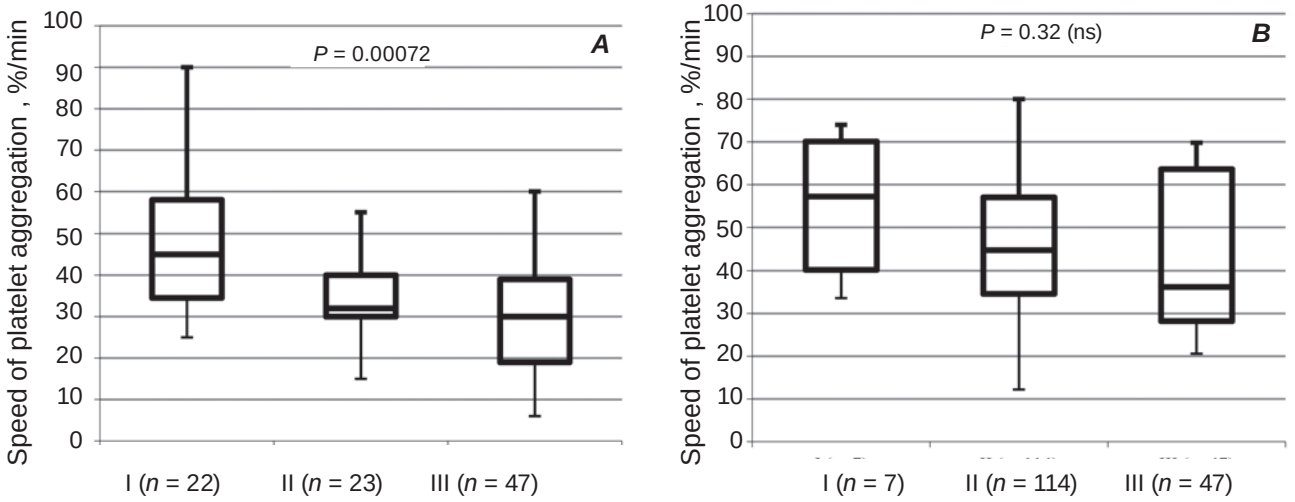

*Fig. 6. Changes in platelet aggregation speed in pregnant women during gestation. Data collected at the Kyiv Perinatal Center, Ukraine (**A**) or in Department of Obstetrics and Gynecology, University of Szeged, Hungary (**B**). Results analyzed by Kruskal-Wallis test. Results assumed as significant when $P < 0.05$. The normal range of the speed of platelet aggregation in blood plasma of healthy donors is 15 ± 5%/min*

Increasing of D-dimer concentration was shown previously for late trimesters of pregnancy [21]. However, current articles show an interconnection between D-dimer and SF. Actually SF, as a marker of the danger of intravascular blood coagulation, is not applied routinely in clinical diagnostics; however, some studies indicate the importance of such simultaneous analysis [22, 23]. SF appears at the time of clot formation and is an intermediate product in fibrin formation – the level of which does not depend on extravasal deposits of the fibrin. Unlike D-Dimer, which is considered a post-thrombotic factor, SF can be used as a prethrombotic marker.

We detected an increase of SF concentrations in both studied cohorts during gestation. This indicated an increased risk of intravascular thrombus formation that grows with pregnancy progression. Increase of SF concentration (accompanied by D-dimer concentration elevation) was statistically significant in the first cohort. This indicates the more intensive activation of the blood coagulation system. Unlike D-Dimer, which is considered a post-thrombotic factor, SF can be used as a prethrombotic marker.

At the same time, moderate generation of D-dimer can be a sign of effective clearance of fibrin deposits from the bloodstream in first cohort patients. From this point of view, the appearance of SF without accumulation of D-dimer can indicate the suppression of fibrinolysis and requires the inclusion of fibrinolytic system parameters into the diagnostic protocol for such patients.

Results of a decrease in the concentration of D-dimer in the third trimester, and a statistically significant increase in the levels of SF obtained in our study, may indicate a significant increase in prothrombotic potential with increasing gestational age due to the predominance of the prethrombotic marker – SF – under the conditions of suppression of the activity of wall fibrinolysis in order to minimize blood loss during childbirth.

As for PC, the main component of the anticoagulant system, we did not find statistically significant changes in its activity for any of the cohorts. Most scientists are focusing on hereditary deficiency of PC during pregnancy indicating the importance of this protein for normal hemostasis and fetus development [24]. That is why those pregnant who demonstrated the decreased level of PC must be assumed to be a risk group needing more detailed analysis.

In the present work, we performed a study of platelet aggregation in pregnant women during gestation. These anuclear cells are underestimated contributors to healthy development during pregnancy; however, are a source of proinflammatory mediators, which might interact with different trophoblast subtypes of the developing placenta [25]. Several studies indicate no statistically significant changes during gestation [26]. Others research agreed, but focused on the decrease in platelet count that is accompanied by notable change in the granule content [27].

Our findings demonstrated the overall hyperreactivity of platelets during all trimesters of pregnancy. However, the rate and speed of ADP-induced platelet aggregation were decreased from first to third trimesters in both studied cohorts. The nature of this phenomenon (that is also accompanied by higher risk of intravascular thrombus formation) must be studied more precisely, possibly with the use of other agonists in platelet aggregation.

Conclusions. In conclusion, we propose diagnostic tests that cover different subsystems of hemostasis: blood coagulation (fibrinogen, SF), blood coagulation and fibrinolysis (D-dimer), cellular branch of hemostasis (platelet aggregation) and anticoagulant system (PC). Independent analysis performed in different cohorts, from different facilities and even from different countries demonstrated the same trends in the change of studied parameters during gestation. First of all, the study confirmed the adequacy of selected tests for detection of the risk of intravascular thrombus formation that is elevating during the later trimesters of gestation. On the other hand, we were able to select some important peculiarities that must be studied more precisely, in particular, the interrelation of D-dimer and SF concentrations during pregnancy, the nature of platelet aggregation suppression during the third trimester of gestation and individual peculiarities of pregnant women with decreased PC level.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ВАЛІДАЦІЯ ДІАГНОСТИЧНОГО АЛГОРИТМУ ДЛЯ МОНІТОРИНГУ ПОКАЗНИКІВ КОАГУЛЯЦІЇ У ВАГІТНИХ

Д. С. Корольова[1], А. О. Павленко[1], А. Алторай[2], С. І. Жук[3], І. В. Ус[3], Ю. О. Царик[1], А. Шураньї[2], В. О. Чернишенко[1]✉

[1]Інститут біохімії ім. О. В. Палладіна НАН України, Київ;
[2]Медична школа імені Альберта Сент-Дьєрдья, Сегедський університет, Сегед, Угорщина;
[3]Національна медична академія післядипломної освіти імені П. Л. Шупика, Київ, Україна;
✉e-mail: bio.cherv@gmail.com

Тромбози є одними з найнебезпечніших ускладнень вагітності. Тому важливе значення має підбір відповідних тестів і стандартизація методик точної діагностики стану системи зсідання крові. У цій роботі ми досліджували декілька молекулярних маркерів загрози внутрішньосудинного тромбоутворення та оцінювали функцію тромбоцитів у вагітних протягом терміну гестації. Ми провели незалежні вимірювання за тією ж методологією для різних когорт пацієнтів, вибраних у Києві (Україна) та в Сегеді (Угорщина). D-димер і розчинний фібрин вимірювали за допомогою медоту ELISA. Рівень протеїну С оцінювали за допомогою аналізу хромогенного субстрату. Концентрацію фібриногену вимірювали спектрофотометричним методом із використанням тромбіноподібного ферменту. Функціональну активність тромбоцитів оцінювали за допомогою агрегатометрії. Статистичний аналіз даних проводили за допомогою тесту Краскела-Уолісса. Статистично достовірне підвищення концентрації фібриногену від першого до третього триместру вагітності було показано для обох досліджуваних когорт пацієнток (у середньому 5-6 мг/мл на третьому триместрі). Застосовані методи дозволили виявити ті ж тенденції зниження рівня протеїну С, а також появу помірних кількостей D-димеру (до 300 нг/мл) і розчинного фібрину (до 10-15 мкг/мл). Функціональна активність тромбоцитів була підвищена в першому триместрі вагітності

та незначно знижувалася протягом наступних триместрів. Результати показали зміни в системі зсідання крові вагітних під час гестації з однаковою ефективністю незалежно від обраних когорт, часу та місця вимірювань. Застосування на практиці запропонованого алгоритму діагностики може дозволити оцінити ризик тромботичних ускладнень під час вагітності

Ключові слова: вагітність, тромбоцити, тромбоз, розчинний фібрин, D-димер, фібриноген.

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