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CIRCULATING CELL-FREE DNA IS A BIOMARKER OF PREMATURE BIRTH AND COVID-19 AND PREDICTS PRENATAL CEREBRAL ISCHEMIA IN NEWBORNS

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Circulating cell-free DNA is a possible marker of not only apoptosis, but also COVID-19 in pregnant, which can also be a trigger factor for premature birth and predicts prenatal hypoxic-ischemic encephalopathy injury in newborns. DNA fragmentation in tissues and blood plasma was measured with the diphenylamine assay. The material for the study was the peripheral blood from pregnant women and newborns, cord blood, tissue of the placenta. A comparison of the level of cfDNA in the serum of healthy pregnant and pregnant women with premature birth suggests a high level of it in women with premature birth. Pregnant women with COVID-19 had significantly higher cfDNA values as compared to those in healthy pregnant women in cord blood, placenta and in newborns from women with COVID-19. The level of cfDNA increased with the severity of neonatal hypoxic-ischemic encephalopathy injury in newborns from women with premature birth.

Key words: cfDNA, preterm birth, COVID-19, prenatal hypoxic-ischemic encephalopathy.

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ЦИРКУЛЮЮЧА БЕЗКЛІТИННА ДНК Є БІОМАРКЕРОМ ПЕРЕДЧАСНИХ ПОЛОГІВ ТА COVID-19 І ПЕРЕДБАЧАЄ ПРЕНАТАЛЬНУ ЦЕРЕБРАЛЬНУ ІШЕМІЮ У НОВОНАРОДЖЕНИХ

Циркулююча безклітинна ДНК є можливим маркером не тільки апоптозу, але і COVID-19 у вагітних, що також може бути тригерним фактором для передчасних пологів і передбачає у новонароджених пренатальну гіпоксично-ішемічну енцефалопатію. Фрагментацію ДНК у тканинах та плазмі крові вимірювали за допомогою аналізу на дифеніламін. Матеріалом для дослідження стала периферична кров вагітних жінок та новонароджених, пуповинна кров, тканини плаценти. Порівняння рівня cfDNA у сироватці крові здорових вагітних та вагітних жінок з передчасними пологами свідчить про високий його рівень у жінок з передчасними пологами. Вагітні жінки з COVID-19 мали значно вищі значення cfDNA порівняно із здоровими вагітними жінками у пуповинній крові, плаценті та у новонароджених від жінок хворих на COVID-19. Рівень cfDNA збільшувався у новонароджених із пренатальною гіпоксично-ішемічною енцефалопатією від жінок з передчасними пологами.

Ключові слова: cfDNA, передчасні пологи, COVID-19, пренатальна гіпоксично-ішемічна енцефалопатія.

The study is a fragment of the research project "Development of the strategy of targeted neuroprotection of prenatal cerebral ischemia in newborn children", state registration No. 0118U001026.

About 15 million premature newborns are born annually in the world. One million of them die [12]. The most common cause of infant mortality is a perinatal brain injury, which is due to the degree of functional maturity of newborn infants. Preterm birth is rising globally. The frequency of preterm birth is geographically heterogeneous: from 5 % to 9 % in Europe, from 10.6 % in North America to 11.9 % in Africa [2]. However, amazing data comes from around the world. Namely, an unprecedented reduction of preterm birth was registered during the COVID-19 lockdown in Ireland [14], Canada, Denmark [9], but was not in Sweden, where there was no lockdown [11].

The pathogenesis of spontaneous preterm birth is largely unknown. Fetal cell-free DNA (cfDNA) is present in the mother's bloodstream and increases with the age of gestation and some complications of pregnancy (preeclampsia). Extracellular DNA may be a proinflammatory inducer due to DNA sensitivity of the Toll-like receptor 9 [3]. Toll-like receptor 4 (TLR4) activation by bacterial infection, in inflammatory diseases of the respiratory tract [15] or by sterile inflammatory insult is a primary trigger of spontaneous preterm birth [6].

Uncertainty prevails about whether vertical transmission of COVID-19 may occur in any phase of pregnancy. Placentas, amniotic fluid samples or newborns (directly after delivery) with positive RT-PCR results have not been described, which means that there is no virological evidence of intrauterine infection at the maternal-fetal interface [5].

Our hypothesis is based on cfDNA as a possible marker of not only apoptosis, inflammation, but also COVID-19 in pregnant, which can also be a trigger factor for premature birth.

The purpose of the study was to perform the circulating cell-free DNA analysis as a biomarker of premature birth and predicts neonatal hypoxic-ischemic encephalopathy injury in newborns from women with premature birth and COVID-19.

Materials and methods. The studies were carried out on the basis of city and regional hospitals in Lysychansk, Severodonetsk, Rubizhne, Kreminna and departments of the Belovodsk regional hospital of the Luhansk region in 2015–2021.

To test our hypothesis, we included in the study patients:

– 47 healthy non-pregnant female donors aged 18 to 42 years (control group);

– 120 pregnant women aged 17 to 46 years in terms of pregnancy from 8 to 41 weeks in the absence of signs of preeclampsia and placental insufficiency, whose pregnancy ended with the birth of live children at gestational age: 26–36 weeks – 25 pregnant women (19 %); 37 weeks – 15 pregnant women (11 %); 38 weeks – 14 pregnant women (11 %), 39 weeks – 37 pregnant women (29 %), 40 weeks and> – 28 pregnant women (22 %).

– 10 pregnant women with COVID-19 (8 %) – childbirth at 36-41 gestation;

– 14 women with a positive diagnosis of COVID-19 according to PCR analysis;

– 95 healthy newborns;

– 25 premature newborns, of them 15 newborns with prenatal cerebral ischemia (PCI);

– 10 newborns from women with COVID-19.

Preterm birth was defined as a delivery before 37 completed weeks of gestation and categorized as spontaneous or iatrogenic. Gestational age (GA) was based on first trimester crown-rump length. If the first Trimester scan was not performed, the estimated due date was calculated using the last menstrual period or a second- or third-trimester ultrasound.

The material for the study was the peripheral blood from the cubital vein of pregnant women, decidual tissue and villous chorion tissue of the mature placenta. In accordance with the provisions of the Helsinki Declaration of the World Medical Association of the last revision, prior to inclusion in the study, all pregnant women received informed consent for the use of biological material (Ethics Committee of the Lugansk State Medical University, Rubizhne, Ukraine, reference number 27/2015).

DNA fragmentation in tissues and blood plasma was measured with the diphenylamine assay as reported previously [13]. The material for the study was the peripheral blood from the cubital vein of pregnant women, cord blood, decidual tissue and villous chorion tissue of the mature placenta. 10 ml of blood was collected in vacuum tubes (BD Vacutainer, EDTA). The blood tubes were inverted 5–6 times to mix the blood with the anticoagulant and placed on ice. Then the blood was centrifuged at 2000 g at 4° C for 15 minutes to separate plasma on a refrigerated centrifuge K-24 (Germany). Serum was aliquoted and transferred to cryogenic tubes for storage at –40 °C prior to the study.

Placental villous tissue biopsies were collected after delivery. Placental tissues were washed five times in ice-cold phosphate-buffered saline (PBS) to clear the maternal blood and then homogenized using a homogenizer in 9 vol of a lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5 % Triton X-100) for 30 min at 4°C. Then the homogenate is filtered through a double layer of cheesecloth and 250 µl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer) is added.

A 1 ml sample (aliquot) is taken from the homogenate or a 1 ml sample from plasma eluate. The amount of fragmented DNA (f-DNA) was calculated in percentage as the ratio of the amount of extracted DNA (in the supernatant) to the total amount of DNA in the sample.

Data Processing. Statistical and graphical analyses were done using STATISTICA 7.0 StatSoft software and using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA) software. Data were summarized as mean (standard error) (Mean±SEM). MedCalc statistical software was used to perform ROC curve analysis and to determine the specificity, sensitivity, positive-predictive values and negative

predictive values for all the possible thresholds of the ROC curve. A p-value below 0.05 was considered statistically significant.

Results of the study and their discussion. We identified 130 women with a singleton gestation during our study period. Our primary outcome was the spontaneous birth of pregnant women with COVID-19. Secondary outcomes were overall preterm birth (PTB) before 37 weeks, spontaneous PTB between 26 and 36 weeks, between 37 and 38 weeks and between 39 and 40–41 weeks of gestation, and gestational age (GA) at birth.

The dynamics of cfDNA increases with the period of gestation, and in the postpartum period, it decreases sharply, but it does not reach the level of the control group of non-pregnant women (table 1).

□□bl□1

The level of cfDNA, % (M ± m)

cfDNA, %	Pregnant women with COVID-19[1], n=10	Gestational age at delivery				
		26–36 weeks of gestation[2] n=25	37 weeks of gestation n=15	38 weeks of gestation n=14	39 weeks of gestation n=37	40–41 weeks of gestation n=28
Serum	64.76±1.25	70.2±0.62	66.4±1.05	62.6±0.6	62.9±0.7	61.2±0.78
Kruskal-Wallis test		p^1=0.049	p^1=1.000	p^1=1.000	p^1=1.000	p^1=0.806
			p^2=0.249	p^2=0.00001	p^2=0.0000001	p^2=0.0000001
Mann-Whitney U Test		p^1=0.0008	p^1=0.47	p^1=0.159	p^1=0.217	p^1=0.0325
			p^2=0.0024	p^2=0.000001	p^2=0.0000001	p^2=0.0000001
Cord blood	88.9±0.98	82.2±0.91	79.1±1.12	75.03±1.4	74.4±0.8	74.0±1.1
Kruskal-Wallis test		p^1=0.8496	p^1=0.0662	p^1=0.000151	p^1=0.000001	p^1=0.0000001
			p^2=1.000	p^2=0.0225	p^2=0.000054	p^2=0.000019
Mann-Whitney U Test		p^1=0.0004	p^1=0.0001	p^1=0.000042	p^1=0.000002	p^1=0.000016
			p^2=0.04	p^2=0.0003	p^2=0.000001	p^2=0.0000001
Placenta	78.9±0.96	75.4±0.77	70.9±1.1	68.5±1.4	65.3±0.9	66.4±1.04
Kruskal-Wallis test		p^1=1.000	p^1=0.1249	p^1=0.0056	p^1=0.000001	p^1=0.000001
			p^2=0.739	p^2=0.028	p^2=0.0000001	p^2=0.000001
Mann-Whitney U Test		p^1=0.016	p^1=0.0003	p^1=0.000068	p^1=0.000003	p^1=0.000016
			p^2=0.0032	p^2=0.0002	p^2=0.000001	p^2=0.0000001
New-borns. 3-th day	54.6±2.98	36.5±1.3	27.9±0.8	28.1±0.9	28.7±0.6	29.2±0.7
ANOVA. LSD test		p^1=0.0000001	p^1=0.0000001	p^1=0.0000001	p^1=0.0000001	p^1=0.0000001
			p^2=0.0000001	p^2=0.000001	p^2=0.0000001	p^2=0.0000001
T-test Students		p^1=0.0000001	p^1=0.0000001	p^1=0.0000001	p^1=0.0000001	p^1=0.0000001
			p^2=0.00003	p^2=0.00007	p^2=0.0000001	p^2=0.000006

Note: p^1 – significant differences between group pregnant women with COVID-19 group and test other groups; p^2 – significant differences between 26–36 weeks of gestation group and test other groups of gestational age at delivery.

However. a comparison of the level of cfDNA in the serum of healthy pregnant and pregnant women with premature birth suggests a high level of it in women with premature birth. The difference in serum levels of cfDNA in the postpartum period in healthy women and women with premature birth was significant: 26–36 weeks of gestation – 70.2±0.62 %; 37 weeks of gestation – 66.4±1.05 % (p=0.249 – significant differences between the group of 26–36 weeks of gestation); 38 weeks of gestation – 62.6±0.6 % (p=0.00001 – significant differences between the group of 26–36 weeks of gestation); 39 weeks of gestation – 62.9±0.74 % (p=0.0000001 – significant differences between the group of 26–36 weeks of gestation); 40–41 weeks of gestation – 61.2±0.78 % (p=0.0000001 –significant differences between the group of 26–36 weeks of gestation). The level of cfDNA in umbilical cord blood, placenta and neonatal serum were also higher with preterm birth.

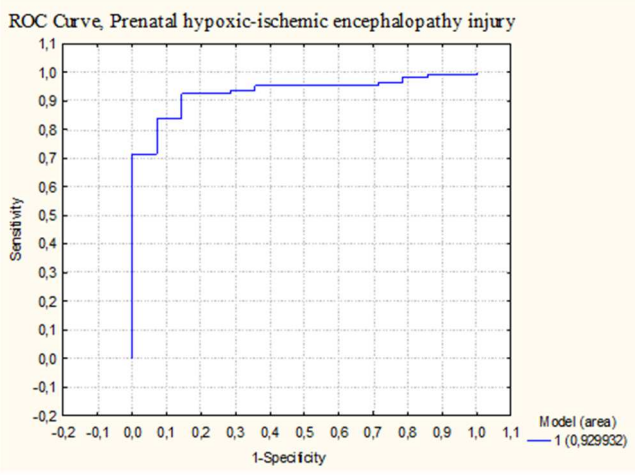
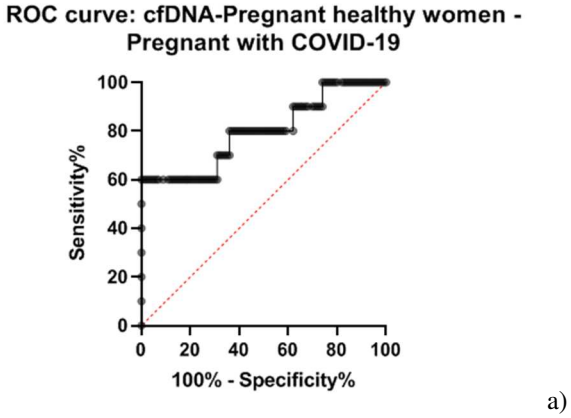


Fig.1. Receiver operationg characteristic (ROC) curves for the cfDNA in 26–36 weeks of gestation in women with premature birth.

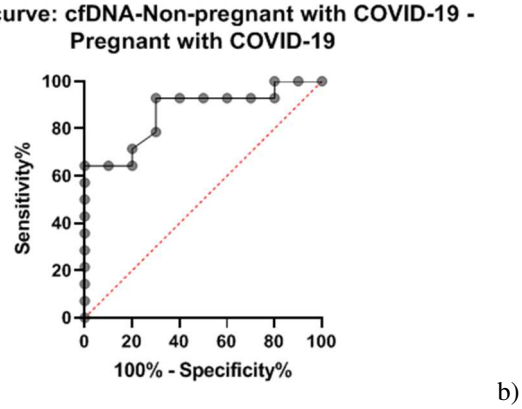
Note: Here and in the following figures: p<0.001 – calculated by univariate logistic regression analysis of the area under the curve (AUC) in group.

The ROC curves were analyzed by Neuranal Networks of Statistica 7.0 StatSoft software to evaluate the diagnostic accuracy of cfDNA for healthy pregnant women and women with premature birth in 26–36 weeks of gestation (fig.1).

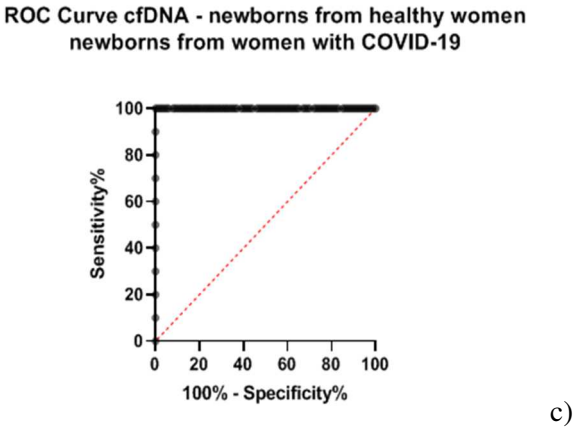
The area under the curve (AUC) could be mapped to compare different screening genes. In the ROC analysis. cfDNA showed the highest AUC value (0.931489) for the prediction of premature birth supporting potential role as diagnostic indicators.



a)



b)



c)

Fig. 2. ROC analysis: receiver operating characteristic (ROC) curves for cfDNA measured in a) healthy pregnant women and pregnant-COVID-19-women; b) non-pregnant COVID-19-women and pregnant-COVID-19-women; c) newborns from healthy women and newborns from women with COVID-19.

The non-pregnant COVID-19 patients exhibited high levels of cfDNA. However. the levels of cfDNA in pregnant women with COVID-19 were not as high (64.76±1.25, p=0.000839) as non-pregnant COVID-19 patients (74.88±1.58), which may be attributed to the initial low levels of them as exhibited in healthy non-pregnant women.

Pregnant women with COVID-19 had significantly higher cfDNA values as compared to those in healthy pregnant women in cord blood (88.9±0.98), placenta (78.9±0.96) and in newborns from women with COVID-19 (54.6±2.98).

ROC-analysis was used to evaluate the diagnostic performance of cfDNA profile in the differentiation between pregnant women and newborns from healthy women and women with COVID-19 (fig.2., table 2).

□□bl□2

The sensitivities, specificities and the cutoff value of cfDNA concentration in pregnant women and newborns from healthy women and women with COVID-19

Groups		Cut-off value	Area, AUC 95 % CI	Sensitivity	95 % CI	Specificity	95 % CI	p-value
healthy pregnant women	pregnant-COVID-19-women	< 87.13	0.7970 0.6243-0.9697	60.0	31.27-83.18 %	99.0	94.55-99.95 %	0.0020
non-pregnant COVID-19-women	pregnant-COVID-19-women	< 80.60	0.8679 0.7224-1.000	92.86	68.53-99.63 %	70.0	39.68-89.22 %	0.0026
newborns from healthy women	newborns from women with COVID-19	> 39.44	1.0000 1.000-1.000	100.0	72.25-100.0 %	100.0	96.3-100.0 %	< 0. 0001

Note: p-value was calculated by univariate logistic regression analysis of the area under the curve (AUC) in the group.

In the ROC analysis. cfDNA showed the highest AUC value for pregnant-COVID-19-women (AUC=0.7970) and in newborns from women with COVID-19 (AUC=1.0).

Next, we studied the level of the cfDNA in the □□rd bl□□d □□d □□rum □f healthy premature newborns and □□wb□r□□w□th hypoxic-ischemic encephalopathy injury (fig. 3).

The level of cfDNA was significantly higher in cord blood of premature newborns. Its level increased with the severity of neonatal hypoxic-ischemic encephalopathy injury in newborns from women with premature birth.

The ROC curves were analyzed by Neuranal Networks to evaluate the diagnostic accuracy of cfDNA for □□wb□r□□w□th hypoxic-ischemic encephalopathy injury (fig. 4).

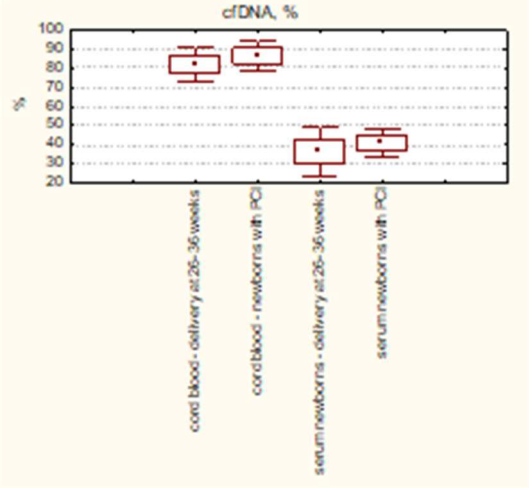


Fig. 3. The level of the cfDNA in the □□rd bl□□d □d □□rum □f healthy premature newborns (n=25) and □□wb□r□□ w□th hypoxic-ischemic encephalopathy injury (n=14).

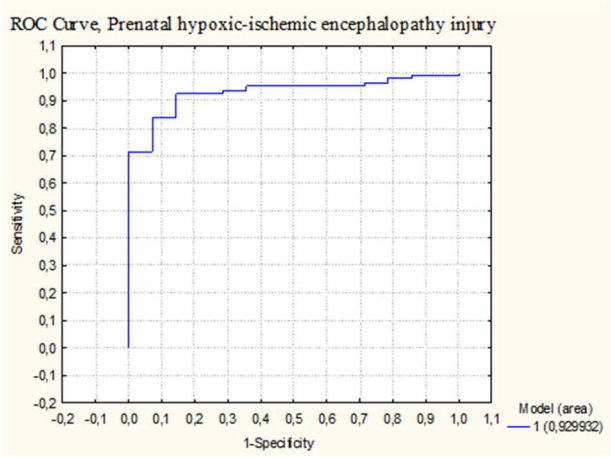


Fig. 4. Receiver operationg characteristic (ROC) curves for the cfDNA in cord blood in women with premature birth – the prediction of hypoxic-ischemic encephalopathy injury in premature newborns.

In the ROC analysis, cfDNA showed the highest AUC value for the prediction of hypoxic-ischemic encephalopathy injury in premature newborns. Moreover, the AUC was significantly higher in cord blood (AUC=0.929932) than in serum in pregnant women with preterm birth at 26–36 weeks of gestation (AUC=0.853617).

Interest in the use of cell-free nucleic acids (CFNAs) as clinical non-invasive biomarker panels for prediction and prevention of multiple diseases has greatly increased over the last decade. Indeed, circulating CFNAs are attributable to many physiological and pathological processes such as imbalanced stress conditions, physical activities, extensive apoptosis of different origin, systemic hypoxic-ischemic events and tumour progression [7].

The majority of fetal cell-free DNA in the maternal blood results from release from the syncytiotrophoblast as a result of cellular apoptosis and necrosis. Elevated levels of fetal cell-free DNA may be indicative of underlying placental dysfunction, which has been associated with preterm birth. Preliminary studies have demonstrated that fetal cell-free DNA is increased in pregnancies complicated by spontaneous preterm birth. Elevated fetal fraction levels at 14-20 weeks' gestation were significantly associated with an increased incidence of preterm birth [8]. There are limited data on the association between fetal cell-free DNA levels and fetal fraction and preterm birth in asymptomatic women in the third trimester. Our findings confirm that cfDNA in 26–36 weeks of gestation can significantly distinguish pregnant women with an increased risk of preterm birth among healthy pregnant women. ROC analysis was also used to evaluate the diagnostic performance of cfDNA in the differentiation between healthy pregnant and pregnant women with premature birth. Collectively, these findings suggest that an elevation in cell-free fetal DNA concentrations in the maternal circulation precedes the physiological process of labor at term and the pathological process of preterm labor.

COVID-19 infection in pregnancy leads to increased risk in pregnancy complications such as preterm birth. PPROM, and may possibly lead to maternal death in rare cases [1]. Although there is no evidence that pregnant women are more susceptible to SARS-CoV-2 infection, they may be at increased risk of developing severe illness when contracting SARS-CoV-2 infection [4]. Currently, there is limited evidence regarding the possibility of mother–fetal intrauterine vertical transmission in COVID-19. Most descriptions of SARS-CoV-2-infected pregnant women reported infections during the third trimester of pregnancy [4]. Our findings confirm that pregnant women with COVID-19 had significantly higher cfDNA values as compared to those in healthy pregnant women in cord blood, placenta and in newborns from women with COVID-19. In the ROC analysis, cfDNA showed the highest AUC value pregnant-COVID-19-women and in newborns from women with COVID-19.

Neonatal hypoxic-ischemic encephalopathy causes high mortality and long-term morbidity rates. The magnitude of the neuronal damage depends on the duration and severity of the initial insult combined with the deleterious effects of reperfusion and apoptosis [10]. Acquired our data revealed cfDNA concentration to predict cerebral ischemia in preterm infants.

Conclusions

1. An application of liquid biopsy is a promising approach to identify biomarker patterns specific for for tissue damage in physiological conditions (for example, childbirth) and in pathological conditions such as premature birth, COVID-19 and prenatal hypoxic-ischemic encephalopathy.

2. CfDNA – biochemical marker of fetal apoptosis in physiological pregnancy and pregnancy complicated by premature birth.

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