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Original Research

The current fetal cell-free DNA in the blood of pregnancies without complications and with gestational and diabetes 2 type mellitus

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Abstract

Background and Aim: Non-invasive prenatal testing (NIPT) has another name for non-invasive prenatal screening (NIPS) – this area of prenatal diagnosis is rapidly developing and using the latest technologies. New generation sequencing for detection of fetal extracellular DNA in maternal plasma or other methods for evaluating extracellular DNA is the basis of NIPT and is used in 80% of cases to detect the main aneuploidies – trisomy 13, 18, 21. This study was aimed at comparing two simplified methods for isolating fetal DNA from peripheral blood, and then comparing the results in pregnant women without complications and pregnancies with one type of complication. **Results:** We tested peripheral blood samples from 64 normal and 29 abnormal pregnancies fetuses. The gestational age had a mean of 21 weeks. Maternal age has a median of 29 years. From 10 mL of maternal blood, we isolated a mean of $10.3 \pm 1.15 \times 10^6$ mononuclear cells. The median number of isolated fetal nucleated red blood cells, corrected for 10 mL blood, was 31.2×10^4 cells in group II 72.4×10^4 – group I after magnetic-activated sorting and 11.7×10^4 cells in group abnormal pregnancy, 29.5×10^4 – normal after hemoglobin enrichment. There is a significant statistical difference between the numbers of total NRBC isolated by the two techniques and research groups ($p < 0.001$). **Conclusion:** Using a modified single-cell – based droplet digital PCR (sc-ddPCR) NIPT, researchers conducted a proof-of-concept study that successfully assessed the genetic information of extremely rare fetal cells in patients with uncomplicated pregnancy and women with gestational diabetes mellitus, as well as in pregnant women with type 2 diabetes complicated by hypertension.

Keywords: cell-free fetal DNA, gestational diabetes, magnetic-activated cell sorting, non-invasive prenatal testing.

Introduction

Non-invasive prenatal testing (NIPT) has another name for non-invasive prenatal screening (NIPS) – this area of prenatal diagnosis is rapidly developing and using the latest technologies. New generation sequencing for detection of fetal extracellular DNA in maternal plasma or other methods for evaluating extracellular DNA is the basis of NIPT and is used in 80% of cases to detect the main aneuploidies – trisomy 13, 18, 21 [1, 2, 3,4].

There is evidence that the improvement of this technology will soon increase the reliability of detecting micro-deletions/duplications throughout the genome [8], which have been identified as a common cause of a number of human diseases.

Many non-invasive prenatal screening tests today analyze fetal extracellular DNA that is mixed in with large amounts of cell-free DNA in the mother's blood. However, it has long been known that a very small number of fetal or placental cells, estimated at fewer than 10/mL, circulates in pregnant women in the peripheral blood. The currently used NIPS, based on extracellular cfDNA, detect only the fetal fraction, which is about 5–20% of the total DNA, this affects the reliability and specificity of the study. Many authors indicate that the reliability is significantly influenced by such factors: history of malignant neoplasms, chromosomal mosaicism of a pregnant woman, body mass index (BMI), copy number variants (CNV), organ transplantation. When conducting NIPT, the above factors can affect the reliability, which will lead to false-positive or false-negative results [1–4] When analyzing the literature data, it can be concluded that NIPT is a screening test that requires diagnostic confirmation (by such methods as FISH). However, the determination of methods for isolating extracellular cfDNA for subsequent analysis remains the most actuality.

Although much of our work has utilized a maternal-white-blood-cell-depletion method to enrich for target-cell candidates, we have also collaborated on a positive enrichment strategy using magnetic-activated cell sorting (MACS) [6, 7, 9]. In this research, we have returned to positive selection with MACS.

This study was aimed at comparing two simplified methods for isolating fetal DNA from peripheral blood, and then comparing the results in pregnant women with and without complications.

Materials and Methods

Patients

This study included women who had previously signed an informed consent: with a singleton pregnancy; undergoing planned prenatal diagnostics and antenatal care. After obtaining informed consent, approved by the Ethics Committee of the Ukrainian Association of Biobanks, 10 mL of peripheral venous blood (which did not exceed the volume of standard studies) was taken into K2 EDTA vacuum units and processed for 1–4 hours. All women underwent an ultrasound examination, the results of which were diagnosed with the normal development of the fetus (absence of structural or chromosomal abnormalities).

Scientists at the Ukraine Association of Biobank and Department of Genetics, Obstetrics, Gynecology and Fetal Medicine Kharkiv Medical Academy of Postgraduate Education (Kharkiv, Ukraine) recruited 64 pregnant women without any obstetrical complications (group I) and abnormalities malformations (group II) as determined by fetal ultrasonography. Group II included 29 women in total: 18 of them had gestational diabetes mellitus and 11 cases with hypertension during pregnancy + type 2 diabetes (Table 1).

Isolation of nucleated cells from the peripheral blood of pregnant women

At the first stage, the samples that arrived at the laboratory for 15 minutes were mixed on a roller, followed by a 1:1 dilution of Dulbecco's phosphate buffered saline (PBS) without Ca and Mg (Biochrom AG), layered on a double density gradient of 1.119 g/mL and 10 mL 1.077 g/mL Percoll (Fluka). At the second stage, the samples

Table 1: The type of pregnant women with pregnancy complications.

The type of pregnancy complications	Group research	The number of cases (n)
Pregnancy-induced hypertension+diabetes 2 type	Group II	11
Gestational diabetes mellitus	Group II	18
Normal	Group I	64

were centrifuged at 500 *g* for 30 minutes at a temperature of –20°C.

The next step was to select the cell ring at the border of the two density gradients. For this, it was taken with a Pasteur pipette and washed twice with Dulbecco's PBS solution at a 1:1 dilution, followed by centrifugation at 500 *g* for 10 minutes. Cellular localization was performed using a density marker – green beads 1.102 g/mL and orange 1.035 g/mL, Sigma.

In the third step, the supernatant was removed and the cells were resuspended in 3 mL buffer at a ratio of 1:10 of the MACS BSA stock solution with autoMACS™ wash solution (buffer B). Cells sorted by magnetic activation sorting (MACS) were resuspended, processed, and stained cells were observed using a fluorescence microscope system (EVOS life, Thermo Scientific, USA).

Separation and generation sequencer of nucleated cells from the maternal blood

While this technology uses a sequencing-by-synthesis method and emulsion PCR (emPCR) similar to other platforms, it differs in that it doesn't use fluorescence or chemiluminescence. Instead, it measures the H^+ ions released during base incorporation (Ion Torrent's, Ion OneTouch 2 System, USA). The Ion OneTouch™ ES employs proven magnetic bead technology to isolate template-positive Ion Sphere™ particles that can be loaded directly onto the ion semiconductor chip—delivering automated, highly reproducible enrichment with every run.

Statistical analysis

The data were expressed as mean ± standard error of the mean. Statistical differences among the prospective groups and their counterparts were analyzed using one-way analysis of variance (ANOVA) as part of an SPSS software package (v.16.0 for Windows, 2007; SPSS, Inc., Chicago, IL) by a post hoc test followed by Dunnett's for several comparison tests to compare treated groups against respective controls. Significant differences were indicated by p values < 0.05.

Results and Discussion

Glucose intolerance first diagnosed during pregnancy is gestational diabetes mellitus (GDM) [10]. A diagnosed GDM has a long-term adverse effect on the health of a pregnant woman, increases the risk of adverse outcomes in childbirth, and also predisposes the child to coronary heart disease, obesity, metabolic syndrome, and type 2 diabetes at a later age [10–12].

From the point of view of a number of researchers, fetal nuclear red blood cells (NRBC) are a reliable candidate for analysis, since they are well morphologically differentiated and because they contain a representation of the entire fetal genome and, although they have a limited lifespan, they have specific markers and allow analysis with magnetically activated cell sorting [13–15].

We analyzed and tested peripheral blood samples from 64 pregnant women without pathology and 29 samples from women with abnormal pregnancies. The mean gestational age of all women was 21 weeks (range 12–30). The average age of the pregnant woman was 29 years (range 20–42). As a result of isolation of nucleated cells from 10 mL of peripheral blood of pregnant women, on average, $10.3 \pm 1.15 \times 10^6$ mononuclear cells were sequenced (Figure 1) (mean ± SE).

We did not find the variations of isolated total NRBC with gestational age like others, using a magnetic-activated cell sorting.

The median number of isolated fetal nucleated red blood cells, corrected for 10 mL blood, was 31.2×10^4 cells in group II 72.4×10^4 – group I after magnetic-activated sorting and 11.7×10^4 cells in group abnormal pregnancy, 29.5×10^4 – normal after hemoglobin enrichment. There is a significant statistical difference between the numbers of total NRBC isolated by the two techniques and research groups ($p < 0.001$) (Figure 2).

The standard protocol for dPCR is designed for the analysis of nanograms of genomic DNA; however, the amount of cfDNA

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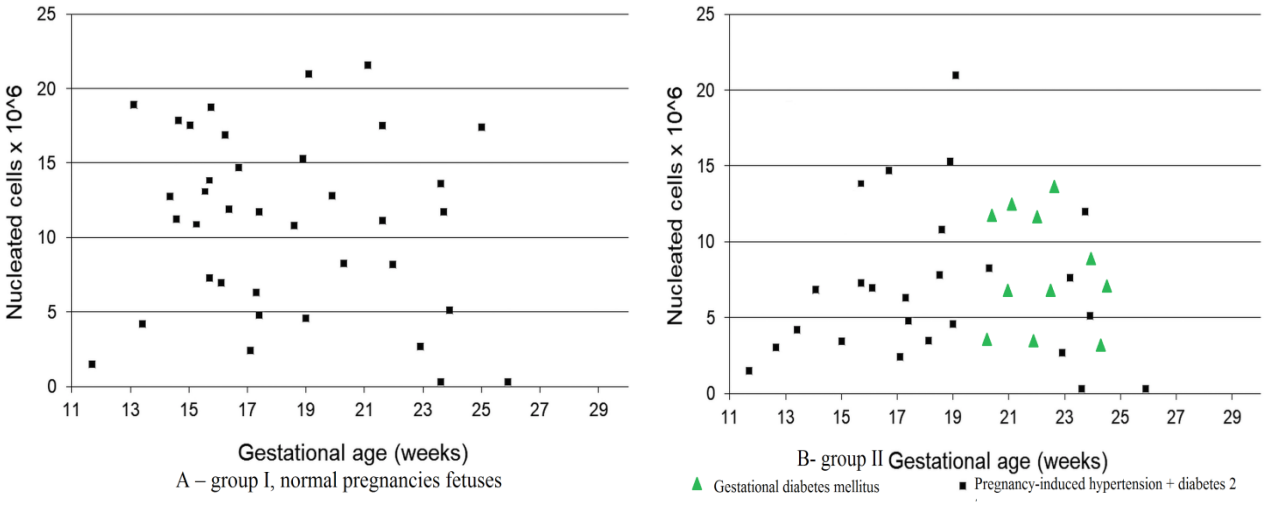

Figure 1: The level of NRBC isolated by centrifugation in the density gradient depending on gestational age (A – group I, B – group II).

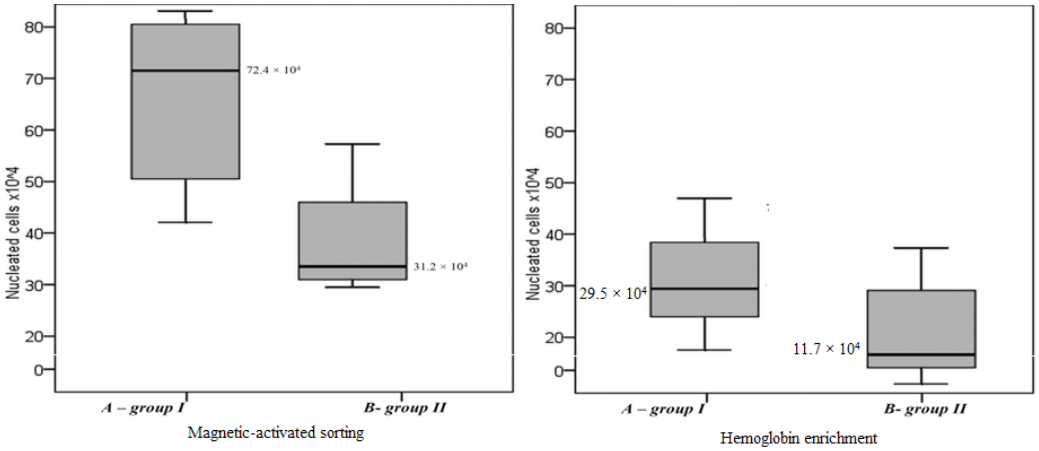

Figure 2: The median number of isolated NRBC, corrected for 10 mL blood in group of abnormal and normal pregnancy.

in plasma samples is much lower. The optimal number of pre-amplified target molecules for dPCR at normal and abnormal pregnancy. The cfDNA was extracted from the plasma mixtures and conducted to pre-amplification PCR. After purification, dilution series from 10^7 to 10^3 molecules per chip of the pre-amplification products were analyzed by dPCR. The analysis of 10^6, 10^5, and 10^4 molecules revealed similar results of normal and abnormal pregnancy. In women with hypertension and type 2 diabetes arising during pregnancy, a low level of target/total cells was established and corresponded to 10^7 molecules, a similar trend was observed for the level of antibodies/total cells, which also corresponded to 10^7 molecules. Lower numbers of molecules (12.7×10^4 and 19.7×10^3) in pregnancy-induced hypertension + diabetes 2 type lead to overestimation of the target/total ratio. The results obtained from 106 molecules were the most robust; thus, 10^6 pre-amplified molecules were used in all pregnancy group dPCR (Table 2).

We found an average of five cells in 10 mL blood for paramagnetic selection, and three cells in 10 mL blood for anti-CD71 and anti-CD14 selection. We evaluated CD14– CD71+ cell quantity and the efficiency of group patient's abnormal and normal pregnancy. A 24-hour incubation in control medium was finally selected as differentiation protocol. Cells became adherent and the expression of recognized macrophage markers, CD71 analyzed by immunofluorescence staining to confirm the monocyte-to-macrophage differentiation in group normal pregnancy, also clearly increased. The expression of CD14, which decreases with macrophage differentiation [19], was also studied

and confirmed the differentiation. Cells were then fixed and immunolabeled for CD14 and CD71 using specific antibodies (green). Nuclei were detected with To-pro3 (blue) (Figure 3).

Table 2: Results from digital PCR analysis in dilution series of pre-amplified cell-free DNA from normal and abnormal pregnancy.

Group	Preamplifier molecules	Target/ total, %
Pregnancy-induced hypertension + diabetes 2 type	10^7	5.05 ± 1.33
	10^6	4.27 ± 0.72
	10^5	7.8 ± 0.86
	10^4	12.74 ± 1.15
	10^3	19.7 ± 0.96
Gestational diabetes mellitus	10^7	5.75 ± 1.4
	10^6	5.1 ± 0.85
	10^5	8.6 ± 1.3
	10^4	14.55 ± 1.55
	10^3	23.1 ± 1.25
Normal	10^7	6.13 ± 1.1
	10^6	5.3 ± 0.92
	10^5	9.4 ± 1.4
	10^4	16.2 ± 1.4
	10^3	29.4 ± 1.34

Mean ± SD values from biological triplicates.

The stationary capture of fetal nucleated erythroblasts in the magnetic column through a single separation cycle allowed the elimination of non-specific cells with a 92% efficiency. However, these values are lower than those obtained by Huang et al.

Dragos Nemescu et al. in a similar experiment (median separation efficiency 98.6%, percentiles 20 and 80, 98.1% and 99%, respectively) [16–18]. However, other groups, using a similar method, have obtained significantly more CD71 positive cells (mean of 34–149 × 10^4 cells/10 mL blood), with a lower depletion rate (94–97%) [14, 15]. Variations in the initial cell number and the different measurement methods (automatic vs. manual) can explain these differences.

Conclusion

Using a modified single-cell–based droplet digital PCR (sc-ddPCR) NIPT, researchers conducted a proof-of-concept study that successfully assessed the genetic information of extremely rare fetal cells in patients with uncomplicated pregnancy and women with GDM, as well as in pregnant women with type 2 diabetes complicated by hypertension.

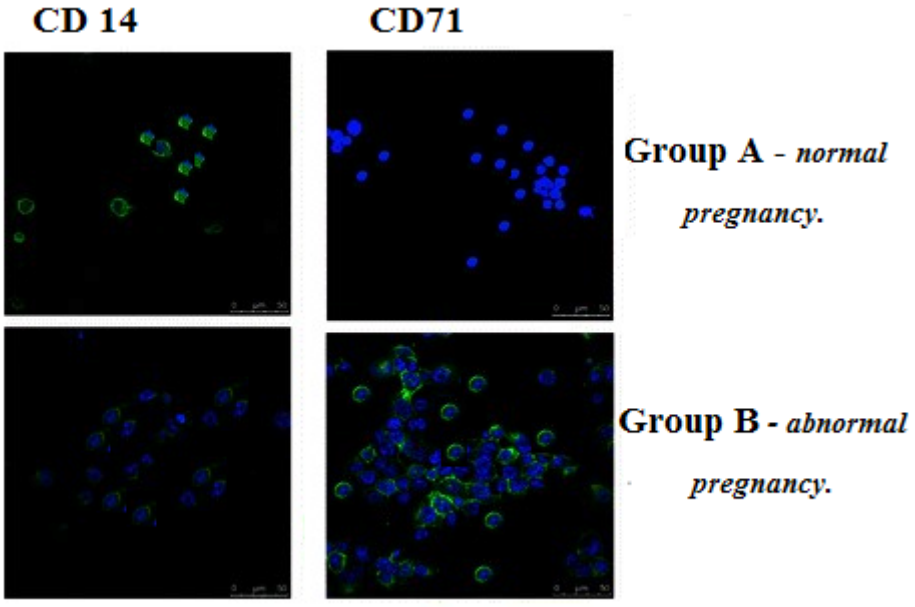

Figure 3: The CD14–, CD71+ cell quantity and the efficiency of group patient's abnormal and normal pregnancy. The fixed and immune-labeled for CD14 and CD71 using specific antibodies (green).

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This modified magnetic-activated cell sorting system makes it possible to directly assess single-cell DNA information from live cells without cell-fixation, cell-staining, and whole genome amplification steps.

The results obtained will allow identifying candidates for participation in early interventional studies and criteria for close monitoring of pregnant women with an increased risk of gestational diabetes mellitus and hypertension associated with pregnancy associated with type 2 diabetes.

Conflict of Interest

The authors declare no conflict of interest.

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