Cell-free fetal DNA at 11-13 weeks of gestation is not altered in complicated pregnancies

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Abstract. Non-invasive maternal cell-free fetal DNA (cffDNA) is a promising biomarker for screening common genetic syndromes. Alterations in the expression levels of cffDNA in the maternal circulation have been demonstrated in abnormal pregnancies. However, the results are conflicting. The present study aimed to investigate whether cffDNA levels are associated with pregnancy complications. The study group comprised pregnant women who presented with pregnancy complications, such as preterm birth, gestational hypertension, intrauterine growth retardation, gestational diabetes, polyhydramnios, oligohydramnios, vaginal bleeding and placental abruption. The control group comprised women who had a normal pregnancy course. Blood samples were obtained from 500 pregnant women between 11-13 weeks of gestation. cffDNA was amplified, sequenced and analyzed using the next-generation aneuploidy test of a Panorama-Natera kit. Nuchal translucency (NT) thickness as well as pregnancy associated plasma protein-A (PAPP-A) and β-human chorionic gonadotropin (β-hCG) levels were also assessed. Statistical analysis was performed in 494 out of the 500 samples collected with SPSS v.26 using non-parametric methods. The parameters were normalized by the multiples of median (MoM) method. The expression levels of PAPP-A, β-hCG, and the NT mean MoM values were significantly different between the study and control groups (P=0.005, P<0.001 and P=0.007, respectively). However, the expression levels of cffDNA and the mean MoM values were not significantly different between these two groups (P=0.687). The findings of the present study support the conclusion that cffDNA expression is not altered in a series of pregnancy complications. The prognostic value of cffDNA in predicting adverse pregnancy outcomes requires further investigation.

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Introduction

Prenatal testing is an integral part of daily obstetric practice in most developed countries (1). The current methods include non-invasive prenatal screening, recommended in all pregnancies and prenatal diagnosis, which can include invasive analysis of fetal material with minimal risk of miscarriage (2). Based on this evidence, novel approaches and non-invasive methods of obtaining fetal material are systematically sought, which can result in the identification of predictive or diagnostic indicators for the detection or prediction of chromosomal abnormalities, genetic diseases, or pathological conditions of pregnancy that lead to adverse events of the fetus/newborn or the pregnant mother (3). In recent years, the application of non-invasive prenatal testing (NIPT) is based on the isolation of cell-free fetal DNA (cffDNA) from maternal blood samples (4). Potential sources of cffDNA include the fetal nucleated red blood cells that undergo apoptosis in maternal circulation, but the most likely source of origin is the placenta (4,5). cffDNA is used as a diagnostic or predictive biomarker and its application has attracted considerable research interest (5-10).

Several studies that examined the application of NIPT indicated that the number of embryonic cells and the cffDNA concentration changed not only with gestational age, but also in the presence of various pregnancy complications, such as preterm birth, idiopathic hydramnios, placenta previa, intrauterine growth retardation (IUGR), vaginal bleeding, threatened miscarriage and recurrent pregnancy loss (11-19). Moreover, a well-documented association has

been observed between altered concentration of cffDNA and preeclampsia (8,20,21) haemolysis, elevated liver enzyme activity levels and low platelet count syndrome (HELLP), or eclampsia (22,23). Previous studies that examined the application of cffDNA in pregnancy complications are controversial with regard to the successful use of this marker in predicting adverse pregnancy outcomes (24).

The present prospective study aimed to investigate the association between the levels of cffDNA in the serum of pregnant women and the occurrence of pregnancy complications. In addition, the potential application of the predictive value of cffDNA was explored. Pregnant women were monitored at the Department of Obstetrics and Gynecology University Hospital of Heraklion, Crete, particularly at the Human Reproduction Unit, the Embryo Medicine Unit and the Outpatient unit of the Obstetrics Clinic.

Patients and methods

Peripheral blood samples were obtained at 11-13 weeks of pregnancy from all the participants at the Department of Obstetrics and Gynecology, University Hospital of Heraklion, Crete, Greece. During the first trimester, a routine screening test was used to assess aneuploidy by the use of biochemical markers [β-human chorionic gonadotropin (β-hCG) and pregnancy associated plasma protein-A (PAPP-A)] and sonographic markers, such as nuchal translucency (NT) thickness, nasal bone presence, ductus venosus blood flow and examination for tricuspid regurgitation.

The study group comprised pregnant women aged 19 to 41 years old with an age range of 22 years who presented with pregnancy complications, such as preterm birth, idiopathic hydramnios, placenta previa, IUGR, vaginal bleeding, threatened miscarriage, preeclampsia/eclampsia and HELLP syndrome eclampsia. The control group comprised women who did not present with any of the aforementioned complications and had a normal pregnancy course and an optimal perinatal outcome. Recruitment and sample collection started on November, 2014 and was completed October, 2016.

The samples were collected in tubes with EDTA and stored at -80˚C. In addition, data were collected regarding the somatometric and demographic characteristics of pregnant women, the individual and medical history, as well as the previous obstetric history. In pregnancies, the following parameters were recorded: i) The parameters of fetal development as well as other elements of the course of pregnancy, including the presentation of any complications (aforementioned) and the perinatal outcome; ii) the parameters of childbirth, such as birth weight at birth, gestation week at delivery, mode of birth and condition of the newborn and iii) the clinical, sonographic and laboratory parameters used to assess the pregnancy course and those that are indicative or diagnostic for the pathological function of the placenta (Doppler of uterine and fetal vessels), the cardiotocograph, the biophysical activities of the fetus, the amount of amniotic fluid and specific biochemical indicators.

Sequencing and analysis of the samples was performed by Natera, Inc. Cell-free DNA was amplified, sequenced, and analyzed using a custom Natera collection kit. The test contains an SNP algorithm to determine fetal fraction and ploidy status. Fetal fraction was measured using an SNP-based cfDNA prenatal test (Panorama™; Natera, Inc.) as previously described (25-27). In brief, 13,926 SNPs in maternal plasma cfDNA were amplified and sequenced. Custom QC metrics based on the sequencing data were used and 50-bp single-end reads were used. A maximum likelihood method which differentiates maternal from fetal alleles was used to determine the presence or absence of fetal aneuploidy, and simultaneously return a fetal fraction measurement. NextSeq™ 500/550 High Output Kit v2.5 (75 cycles; cat. no. 20024911; Illumina, Inc.) was used as sequencing kit. A custom data pipeline and algorithms were used to analyze the data (PanoramaTM; Natera, Inc.) (25-27)

In the present study, a total of 2x10 ml (a single draw) of blood was used from each subject for analysis. An average of $1.22x10^7$ reads were mapped for each sample when sequenced at normal depth-of-read. Samples not generating sufficient information were resequenced at a higher depth of read (average, $2.45x10^7$ mapped reads per sample). The fetal fraction was reported as a percentage of the identified fetal (placental) DNA to the total cell-free DNA present in maternal blood.

The research protocol was approved (approval no. 882, 20/29-10-1014) by the Ethics and Bioethics Scientific Committee of University Hospital of Heraklion-PAGNI (Heraklion, Greece).

Statistical analysis. Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) v.26 (IBM Corp.) using non-parametric methods. The parameters were normalized by the multiples of median (MoM) method (28,29), which is the standard for reporting serum maternity test results (5). Shapiro-Wilk normality test was performed and the data indicated that the demographic parameters did not follow a normal distribution. Non-parametric tests, such as χ^2, Mann-Whitney U and Kruskal-Wallis were used. The correlations between the demographic factors and the measurements of the parameters examined were investigated using Spearman correlation analysis. The correlation coefficient was considered to be weak (<0.4), moderate (0.5-0.7) and strong (>0.7). Statistical significance was defined as a value of $P<0.01$.

Results

Statistical analysis was performed in 494 out of the 500 samples collected from pregnant women. The demographics for all available data are presented in Table I. A total of 248 out of 494 (50.2%) neonates were male with an average weight of 3,308 g, while 242 were females (49%) with an average weight of 3,135 g. The average difference in weight was significantly different, with male infants being heavier by 173 g than females ($P<0.001$).

A total of 461 newborns were delivered naturally and 31 by cesarean section, while induction of delivery was performed in 28 neonates (Table I). Only 8 infants were placed in the intensive care unit (ICU) (1.6%), whereas conception was performed via *in vitro* fertilization (IVF) for 21 cases (4.3%). The fetal development parameters (biometrics) and the data on the course of pregnancy (amniotic fluid volume and fetal biophysical activities) were monitored. A total of 459 out of 494 infants (92.9%) did not present with a pathological

Table I. Demographics for available data of enrolled pregnancies.

Parameters		N	N%	χ^2
Delivery type	Normal	461	93.3	P<0.001
	C-section	31	6.3	
Delivery week	33	2	0.4	P<0.001
	36	114	23.1	
	37	124	25.1	
	38	122	24.7	
	39	113	22.9	
	40	16	3.2	
Induced	Yes	28	5.7	P<0.001
	No	424	85.8	
Sex	Male	248	50.2	P=0.786
	Female	242	49.0	
ICU	No	486	98.4	P<0.001
	Yes	8	1.6	
Conception	Automatic conception	471	95.3	P<0.001
	IVF	21	4.3	

ICU, intensive care unit; IVF, *in vitro* fertilization.

condition (Table II). The demographic information of the relevant pathological pregnancies is listed in Table II.

The pathological complications were recorded in 14 out of 494 (2.8%) of the fetuses, where preeclampsia was noted in their corresponding mothers. In addition, a total of 4 pregnant women suffered from pre-hypertension and placental abruption/bleeding, whereas oligohydramnios was present in 5 and polyhydramnios in 2 pregnant women. Gestational diabetes was present in 4 women, of whom 3 were treated with diet restrictions and 1 with insulin. The histogram of weight distribution was prepared according to the pathological conditions present (Fig. 1A). No significant differences were noted following comparison of the sex with the pathological or demographical parameters.

'MoM' is a measure of the deviation of an individual result from the median and is commonly used to report the results of medical screening tests, particularly where the results of individual tests are highly variable. The mean MoM values of PAPP-A, β-hCG and cervical transparency measurement were used to perform comparisons between samples derived from pregnancies with pathological features in the fetus and those derived from normal pregnancies. The MoM distributions for PAPP-A and β-hCG, NT and cffDNA between the two groups of pregnancies are revealed in Fig. 1B. The expression levels of PAPP-A, β-hCG and the NT mean MoM values were significantly different between these two groups (P=0.005, P<0.001 and P=0.007, respectively). However, the expression levels of cffDNA and the mean MoM values were not significantly different between these two groups (P=0.687).

By comparing all pathological conditions individually, the data indicated that β-hCG levels and NT mean MoM values were significantly different despite the limited number of samples used. In contrast to these findings, the levels of cffDNA and the mean MoM values did not demonstrate a significant difference in IUGR and gestational diabetes cases, as well as in preeclampsia and preterm delivery cases, although their values were somewhat different. The MoM values for all the biochemical markers investigated according to each pathological condition are revealed in Table III.

Discussion

cffDNA can serve as a pathological marker or be used to provide genetic material for personalized medicine (30). The utilization of cffDNA, which is present in the blood circulation of pregnant women (5), has modernized prenatal care for genetic disorders and aneuploidies (31,32); cffDNA has also been used for >20 years for fetal blood group prediction (9,33). It is considered that non-invasive prenatal diagnosis using fetal DNA in maternal blood may play an increasingly important role in the future practice of prenatal testing. However, it is important to address the ethical, legal and social issues regarding this application. The advantage of non-invasive testing compared with invasive testing is to avoid harming the fetus. However, this method offers limited precision, compared with that of specific diagnostic tests, such as chorionic villus sampling or amniocentesis (10,34). In addition, the false-positives and false-negatives in NIPT, possibly related to the placental origin of fetal DNA, remain an important issue to be addressed (35). Moreover, this method of testing does not offer additional genetic information (10,34).

The main objective of the present study was to investigate the predictive value of the increased levels of cffDNA in the maternal circulation during the first trimester of pregnancy with the subsequent onset of a number of serious pathological conditions of pregnancy.

The findings indicated a lower C-section rate of pregnant women, which was in stark contrast with the results reported by Antoniou *et al* and the cited WHO report published in 2020 (36,37). This further illustrated the requirement for a more formal information source pertinent to WHO, which can provide official data for C-sections in an effort to limit unwarranted use. The mean MoM values of PAPP-A, β-hCG and NT, reported in the present study, were affected in samples derived from cases that presented with pathological conditions, which is in agreement with previous studies (38-44). By contrast, the mean MoM values of cffDNA were somewhat different between the IUGR and the gestational diabetes groups, as well as between the preeclampsia and the preterm delivery groups (14,45-48); however, these differences did not reach statistical significance in the present study. cffDNA levels are increased throughout the course of normal pregnancy as well as in certain pathological conditions (6,49) making it hard to measure incremental differences. Cell-free DNA is present in healthy individuals. The 'pathogenic' value cut-offs cannot be easily distinguished from the corresponding normal value cut-offs. Specific values of 6 (low range) and 650 (high range) ng/ml have been measured in healthy men, indicating the potential weaknesses of the quantification methods used (50-52).

A review article (12) that summarized previous findings on preterm birth and other adverse pregnancy outcomes was inconclusive as to the role of cffDNA in preterm births; this report described the technical and standardization issues

Table II. Pathological pregnancy conditions and demographics.

		Pathological conditions																			
		Normal		Preeclampsia		Gestational hypertension		Bleeding-placental abruption		IUGR		Gestational diabetes with with diet		Gestational diabetes with insulin		Preterm delivery		Oligohydramnios		Polyhydramnios	
Parameters		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Delivery	Normal	437	95.2	11	78.6	2	50	3	75	0	0	3	100	0	0	1	100	2	40	2	100
	C-section	21	4.6	2	14.3	2	50	1	25	1	100	0	0	1	100	0	0	3	60	0	0
Delivery	33	0	0	0	0	0	0	0	0	1	100	0	0	0	0	1	100	0	0	0	0
week	36	112	24.4	2	14.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	37	122	26.6	2	14.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	38	112	24.4	2	14.3	2	50	1	25	0	0	1	33.3	0	0	0	0	2	40	2	100
	39	98	21.4	8	57.1	2	50	0	0	0	0	1	33.3	1	100	0	0	3	60	0	0
	40	12	2.6	0	0	0	0	3	75	0	0	1	33.3	0	0	0	0	0	0	0	0
Induced	Pre-term	19	4.1	3	21.4	1	25	3	75	0	0	1	33.3	0	0	0	0	1	20	0	0
	Normal	408	88.9	9	64.3	1	25	0	0	0	0	2	66.7	0	0	1	100	1	20	2	100
Sex	Male	232	50.5	8	57.1	2	50	1	25	1	100	1	33.3	0	0	1	100	2	40	0	0
	Female	223	48.6	6	42.9	2	50	3	75	0	0	2	66.7	1	100	0	0	3	60	2	100
ICU	No	458	99.8	11	78.6	4	100	3	75	0	0	3	100	1	100	0	0	4	80	2	100
	Yes	1	0.2	3	21.4	0	0	1	25	1	100	0	0	0	0	1	100	1	20	0	0
Conception	Natural conception	443	96.5	12	85.7	4	100	3	75	0	0	3	100	0	0	1	100	3	60	2	100
	IVF	15	3.3	2	14.3	0	0	1	25	1	100	0	0	0	0	0	0	2	40	0	0

IUGR, intrauterine growth retardation; ICU, intensive care unit; IVF, *in vitro* fertilization.

Table III. MoM values for the cffDNA, the biochemical markers (PAPP-A and β-hCG) and NT, investigated according to each pathological condition.

		Pathological conditions										
MoM		Normal	Preeclampsia	Gestational hypertension	Bleeding-placental abruption	IUGR	Gestational diabetes with diet	Gestational diabetes with insulin	Preterm delivery	Oligohydramnios	Polyhydramnios	Kruskal Wallis test
cffDNA	N	459	14	4	4	1	3	1	1	5	2	0.293
	Mean	1.01	0.88	1.08	0.94	0.40	1.20	1.79	0.60	1.00	1.47	
	SD	0.42	0.42	0.34	0.37	0	0.77	0	0	0.44	0.20	
PAPP-A	N	459	14	4	4	1	3	1	1	5	2	0.06
	Mean	1.07	1.14	1.24	1.14	0.87	1.44	1.37	0.89	1.10	1.24	
	SD	0.23	0.23	0.22	0.21	0	0.18	0	0	0.16	0.10	
β-hCG	N	459	14	4	4	1	3	1	1	5	2	0.032
	Mean	1.03	1.04	1.15	1.15	1.08	1.23	1.24	1.11	1.18	1.41	
	SD	0.20	0.15	0.17	0.13	0	0.16	0	0	0.10	0.36	
NT	N	459	14	4	4	1	3	1	1	5	2	0.015
	Mean	1.01	1.07	1.61	1.30	0.69	1.17	1.88	1.06	1.30	1.06	
	SD	0.26	0.34	0.31	0.56	0	0.61	0	0	0.37	0.35	

MoM, multiples of median; IUGR, intrauterine growth retardation; cffDNA, cell-free fetal DNA; PAPP-A, pregnancy associated plasma protein-a; β-hCG, β-human chorionic gonadotropin; NT, nuchal translucency.

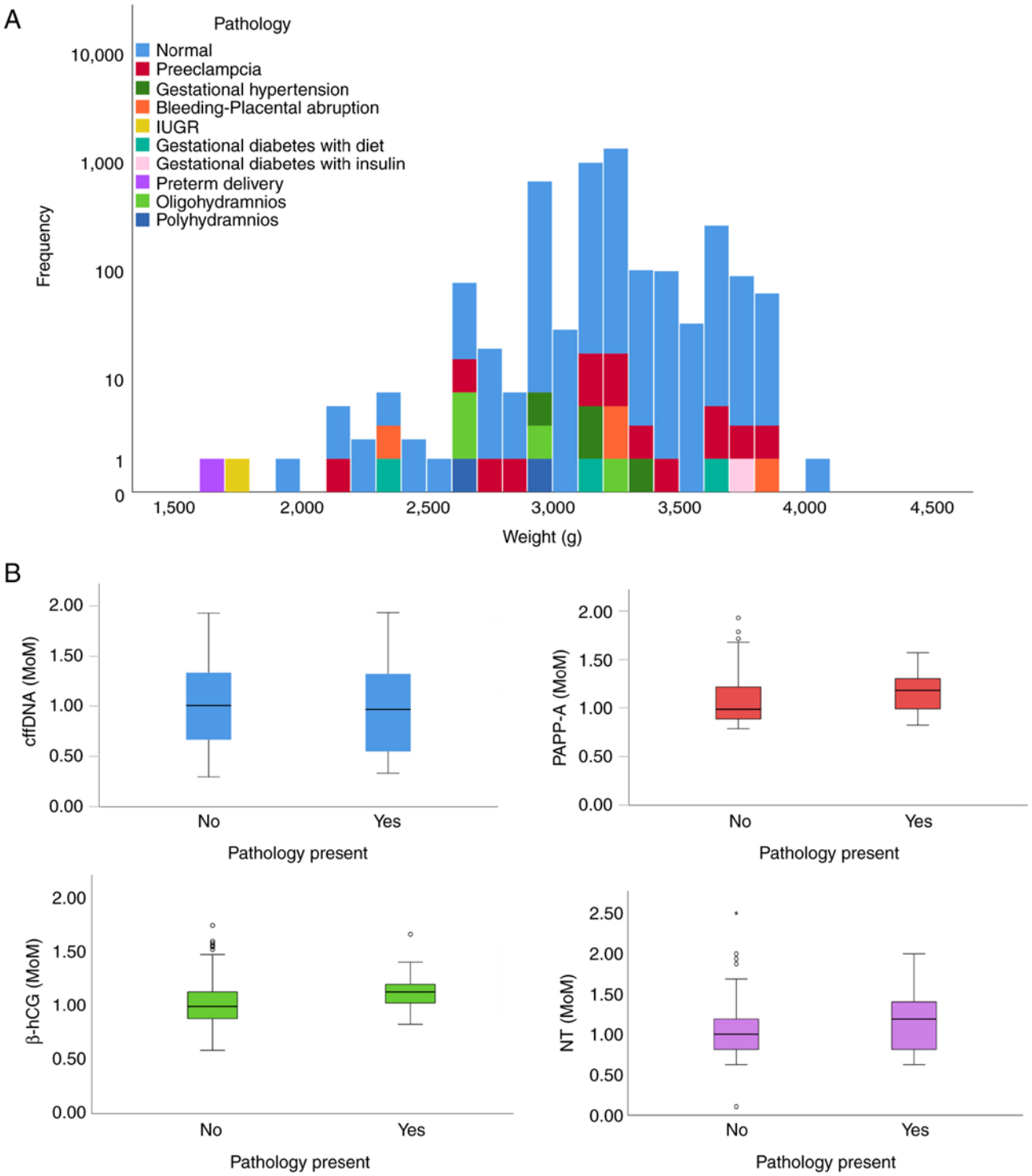

Figure 1. (A) Log scale histogram of weight distribution according to each pathological condition. (B) Bar charts of cffDNA, PAPP-A, β-hCG and NT MoM distribution according to the presence of each pathological feature. *, Outlier value. cffDNA, cell-free fetal DNA; PAPP-A, pregnancy associated plasma protein-A; β-hCG, β-human chorionic gonadotropin; NT, nuchal translucency; MoM, multiples of median; IUGR, intrauterine growth retardation.

between the pertinent studies that examined the role of cffDNA in preterm birth and highlighted the requirement to establish a normal range cut-off related to cffDNA levels. In addition, two different systematic reviews have been published on the use of cffDNA and the prediction of preeclampsia (53,54). These studies concluded that cffDNA is indeed a marker that can be used from the beginning of the second trimester and onwards; after this period, its predictive value is reduced. The studies also proposed the limitation of the heterogeneity of the published data regarding cffDNA levels and the challenges encountered during the interpretation of the findings. A large study (55) of 1,949 singleton pregnancies concluded that cffDNA concentration levels were variable and that maternal weight was affecting cffDNA MoM values; however, it was not significantly altered in pregnancies with pathological findings, such as preeclampsia.

In the present study, cffDNA was unaffected by weight or any of the related factors. Thurik *et al* performed a nested first-trimester case-control study investigating preeclampsia, hypertension, gestational diabetes and preterm birth (48). This study converted first-trimester cffDNA values to MoM values, failing to present predictive values for preeclampsia. Based on this evidence, it is unknown whether cffDNA is actually a valid marker for the identification of pathologies during pregnancies (24). Notably, for preeclampsia, it may not provide added value to the existing screening methods (8,56); this conclusion has also been claimed for preterm births (57). The latest review by Merriel *et al* (24) is sceptical regarding the use of cffDNA as a pathological marker since conflicting results are presented in the reviewed studies. The authors of this study also highlighted the lack of common guidelines, biochemical tests and units, and the requirement for a normal concentration range and specific time period of sample collection as factors that pose serious challenges in the interpretation of the results. Merriel *et al* (24) could not identify a role for the use of cffDNA in clinical NIPT testing for high-risk pregnancies. The present study reports similar conclusions. A previous review (20) provided evidence and evaluated the total cell-free DNA as a more appropriate alternative index, especially for preeclampsia. Total cell-free DNA comprises placental cffDNA and maternal cell-free DNA from maternal leukocytes.

The evidence for the use of cffDNA in predicting adverse pregnancy outcomes is controversial. However, this research is vital for developing a better understanding of disease processes. Currently cffDNA testing does not have any clinical application for the prediction of pregnancy complications and additional development is required for possible use in clinical practice. Large-scale studies that will investigate the possible alterations in cffDNA, in the process of the pregnancies that exhibit severe complications, are required. Despite the small number of cases, the present study revealed no alterations in the first trimester; however, it would be interesting to address whether the cffDNA is altered at the second or the third trimester of complicated pregnancies, and, whether it may have any clinical usefulness as a diagnostic or a predictive marker. The current use of NIPT in prenatal diagnosis can be potentially added to more novel technologies of personalized medicine, such as next-generation sequencing and chromosomal microarray analysis (58) if they can be applied in a non-invasive manner.

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Availability of data and materials

The datasets analyzed and/or generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZK, SS, EM and KR conceived and designed the study. ZK, EP, EM, AP and SS acquired, analyzed and interpreted the data. ZK, SS and IP confirmed the authenticity of the raw data. ZK, EP, IP and SS drafted the manuscript. All authors critically reviewed the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study protocol conformed to the globally accepted regulations on clinical studies involving human data and approval was conferred by the Ethics and Bioethics Scientific Committee of the University Hospital of Heraklion-PAGNI (Crete, Greece) (approval no. 822, 20/29-10-2014; Heraklion, Greece). Written informed consent was obtained from all of the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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