

## Original Article

# Role of cell-free fetal DNA in the maternal plasma in the prenatal diagnosis of chromosomal abnormalities

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**Abstract:** Objective: This study aimed to investigate the role of cell-free fetal DNA (cff-DNA) in the maternal plasma in the prenatal diagnosis of chromosomal abnormalities. Methods: The results of cell-free DNA detection of maternal plasma were retrospectively reviewed in 1440 pregnant women, and the pregnancy outcomes were followed up. Results: (1) Of 1440 pregnant women, 20 had a high risk for trisomy 21, and invasive prenatal examination showed the karyotype of 47, XN, +21; 22 had a high risk for trisomy 18, invasive prenatal examination in 19 women showed the karyotype of 47, XN, +18, but 3 women did not receive karyotyping. (2) When the cut-off value was 1/750, both the sensitivity and detection rates were 100% in the screening of trisomy. (3) Fetal chromosomal aneuploidy was significantly associated with NT thickening, AFP MOM,  $\beta$ -HCG MOM ( $\geq 3.5$ ) and apparent ultrasound structural abnormalities ( $P < 0.05$ ). Conclusion: The cff-DNA detection is a fast, safe and high-throughput method for the prenatal examination of fetal chromosomal abnormalities. It is expected to replace the invasive prenatal diagnostic techniques and bring good prospects for the non-invasive prenatal diagnosis.

**Keywords:** Cell-free fetal DNA, down syndrome, non-invasive prenatal diagnosis

## Introduction

Trisomy 21 (T21) and trisomy 18 (T18) are the most common fetal chromosomal abnormalities in clinical practice, account for 0.2%-0.3% of full-term pregnancies and have been major birth defects that should be controlled and prevented currently [1-3]. In recent years, increasing studies reveal that detection of cell-free fetal DNA (cff DNA) in the maternal plasma has high sensitivity and specificity in the diagnosis of T21 and T18, and may serve as an effective supplement to the current pre-natal screening and prenatal diagnosis [3-5]. In this study, the results from cff-DNA detection in 1440 pregnant women between January 2014 and April 2015 were retrospectively revised. Herein, we reported our findings.

## Materials and methods

### General information

A total of 1440 pregnant women who received cff-DNA detection in the Second People's

Hospital of Huai'an between January 2014 and April 2015 were divided into 4 groups: (1) Advanced age group: the pregnant women was no younger than 35 years ( $n=312$ ); (2) abnormalities in serum screening: women were younger than 35 years and serum screening showed the risk ratio of T21 or T18 was  $\geq 1/1000$ , or AFP,  $\mu E3$  and  $\beta$ -HCG MOM  $< 0.5 / > 2.5$  was present ( $n=940$ ). (3) Abnormalities from ultrasound examination: women were younger than 35 years, serum screening showed low risk, but ultrasound examination showed NT thickening or abnormalities were present in the systemic screening of the fetus ( $n=34$ ); (4) other factors group: the mother and/or father had chromosomal abnormality, the mother had history of abnormal pregnancy/delivery or exposure to toxic substance in pregnancy, or had a family history of imbecile, or had no serum screening or ultrasound screening ( $n=154$ ). All the women had single fetus, and were aged 21-44 years. There were 1070 primiparas and 370 multiparas. Informed consent was obtained before pre-natal examination.

### Methods

**Measurement of nuchal translucency (NT):** NT was measured at the gestational age of 11-14 weeks (fetal crown-rump length: 45-84 mm). Criteria for screening: the middle sagittal image was obtained and amplified. The maximum thickness of the subcutaneous translucency was measured between the skin and the soft tissue overlying the cervical spine. The  $\geq 3$  mm was a criterion for abnormal NT (NT thickening).

**Serum screening in the middle trimester:** Serum screening was performed at gestational age of 15~19<sup>+6</sup> weeks. The time-resolved fluorescence immunoassay was employed for the detection of AFP,  $\mu$ E3 and  $\beta$ -HCG. Finland prenatal screening management system was used to calculate the risk values of T21, T18 and neural tube defects. The cut-off value was 1/270 for high risk for T21, and 1/270~1/1000 for critical risk for T21. The cut-off value was 1/350 for high risk for T18 and 1/350-/1000 for critical risk for T18. The normal range of AFP,  $\mu$ E3 and  $\beta$ -HCG MOM was 0.5-2.5.

**Ultrasound screening in the middle trimester:** Ultrasound screening was performed in gestational age of 18-27 weeks. The biparietal diameter (BPD), head circumference (HC), lateral ventricle (LV), posterior cranial fossa (PCF), cerebellar diameter (CD), lip line (LL), abdominal circumference (AC), humeral length (HL), femoral length (FL), placenta (PL), amniotic fluid (AF), umbilical artery (UA), ductus venosus (DV) and middle cerebral artery (MCA) were measured, and at least 20 images were captured and stored: cerebral thalamus section, cerebellum section, nasolabial line plane, orbital section, longitudinal axis of the spine section, Four-chamber view of the heart, three-vessel section of the heart, left ventricular outflow tract section, ductus arteriosus section, abdominal magenblase section, bilateral renal section, long bone section, cord blood flow map, bladder and double umbilical artery graph, ductus venosus flow map and Doppler color flow map of the heart.

**Detection of cff-DNA in the peripheral blood of pregnant women:** Peripheral blood (3-5 ml) was collected from each pregnant woman and anti-coagulated. The blood was stored at -4°C and the plasma was separated and frozen. The plasma was subjected to gene screening in the

BGI Shenzhen Biotech Co., Ltd. When the detection of plasma cff-DNA of pregnant women showed chromosomal abnormalities, amniocentesis or fetal cord blood puncture was performed for confirmed diagnosis. If negative results were present in the cff-DNA detection, the women were followed up until delivery.

**Collection of general information:** The age, number of pregnancy, number of delivery, NT, risk values of T21, T18 and NTD, AFP,  $\mu$ E3 and  $\beta$ -HCG MOM, results from systemic screening of the fetus, cff-DNA detection, and karyotyping, pregnancy outcome, method of conception and history of unsuccessful pregnancy were recorded.

**Statistical analysis:** Statistical analysis was performed with SPSS version 17.0. Quantitative data are expressed as mean  $\pm$  standard deviation, and compared with one way analysis of variance if homogeneity of variance was present (F test). Rates were compared with Chi square test. Categorical data were subjected to multivariate logistic regression. A value of  $P < 0.05$  was considered statistically significant.

### Results

#### *cff-DNA detection and karyotyping*

cff-DNA detection was performed in 1440 pregnant women, and positive results were found in 42 women accounting for 2.92% (42/1440). High risk for T21 was noted in 20 women, and then invasive prenatal examination was performed and showed the karyotype was 47, XN, +21 in all these women (1.39; 20/1440). High risk for T18 was found in 22 women accounting for 1.53% (22/1440), and invasive prenatal examination was performed in 19 patients and showed the karyotype was 47, XN, +18 in these women and 3 women did not receive karyotyping. Tetralogy of Fallot was found in a woman in the middle trimester and MRI showed ventricular septal defect, aortic saddle, ascending aortic dilatation and small main pulmonary artery. Induction of labour was performed in one woman due to termination of pregnancy at gestational age of 17 weeks, and ultrasound examination showed the NT was 6.5 mm and the fetal head was lemon-like. Another woman already had 3 healthy children and thus refused karyotyping, and induced labour was performed directly. In 1398 women receiving cff-DNA detection, negative results were observed, and

## Role of cell-free fetal DNA in diagnosing chromosomal abnormalities

**Table 1.** Age, number of pregnancy and number of delivery among T21 women, T18 women and healthy pregnant women

| Group         | n    | Age (yr)   | Pregnancy (n) | Delivery (n) |
|---------------|------|------------|---------------|--------------|
| T21 women     | 20   | 31.57±5.56 | 1.71±0.76     | 0.43±0.14    |
| T18 women     | 22   | 31.50±7.49 | 2.40±1.90     | 0.50±0.07    |
| Healthy women | 1398 | 30.09±4.64 | 2.04±1.29     | 0.46±0.09    |
| <i>F</i>      |      | 0.780      | 0.609         | 1.281        |
| <i>P</i>      |      | 0.459      | 0.544         | 0.278        |

**Table 2.** NT thickness and apparent structural abnormalities in ultrasound examination among T21 women, T18 women and healthy pregnant women

| Group         | n    | NT thickening |       | Apparent structural abnormalities |       |
|---------------|------|---------------|-------|-----------------------------------|-------|
|               |      | n             | %     | n                                 | %     |
| T21 women     | 20   | 2             | 10.00 | 0                                 | 0     |
| T18 women     | 22   | 10            | 45.45 | 18                                | 81.82 |
| Healthy women | 1398 | 10            | 0.72  | 16                                | 1.14  |
| <i>P</i>      |      |               | 0.368 |                                   | 0.368 |

**Table 3.** AFP,  $\beta$ -HCG and  $\mu$ E3 MOM among T21 women, T18 women and healthy pregnant women

| Group           | n    | AFP MOM   | $\beta$ -HCG MOM | $\mu$ E3 MOM |
|-----------------|------|-----------|------------------|--------------|
| T21             | 20   | 0.27±0.07 | 4.08±2.09        | 0.45±0.07    |
| T18             | 22   | 0.55±0.07 | 1.96±1.05        | 0.45±0.07    |
| Healthy control | 1398 | 0.98±0.46 | 2.65±1.67        | 1.00±0.78    |
| <i>F</i>        |      | 3.276     | 4.847            | 0.972        |
| <i>P</i>        |      | 0.039     | 0.008            | 0.379        |

neonates with abnormal chromosomes were not found after delivery.

One way analysis of variance of general characteristics among T21 women, T18 women and healthy pregnant women: There were no significant differences in the age, number of pregnancy and number of delivery among T21 women, T18 women and healthy pregnant women ( $P>0.05$ ) (**Table 1**).

NT thickness and apparent structural abnormalities in ultrasound examination among T21 women, T18 women and healthy pregnant women were shown in **Table 2**.

AFP,  $\mu$ E3 and  $\beta$ -HCG MOM among T21 women, T18 women and healthy pregnant women

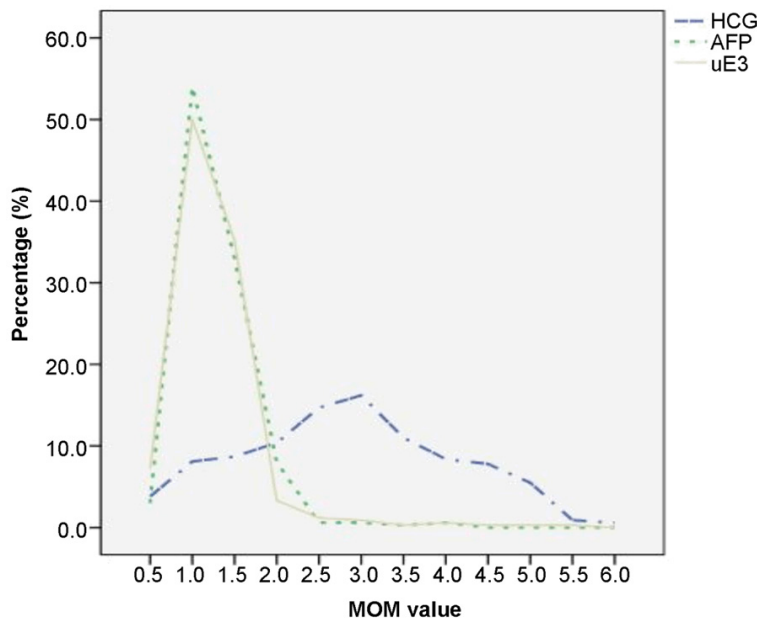
The AFP MOM was compared with Levene test among three groups and homogeneity of vari-

ance was present ( $F=1.184$ ,  $P=0.307>0.10$ ). Intergroup comparison showed significant difference in the AFP MOM among three groups ( $F=3.276$ ,  $P=0.039<0.05$ ). Further LSD-t test showed the AFP MOM in T21 group was significantly lower than in healthy control group ( $P=0.029<0.05$ ), but no significant difference was observed in the AFP MOM between T21 group and T18 group ( $P=0.543>0.05$ ) as well as between healthy control group and T18 group ( $P=0.183>0.05$ ) (**Table 3**). (1) The  $\beta$ -HCG MOM was compared with Levene test and homogeneity of variance was present ( $F=1.446$ ,  $P=0.237>0.10$ ). Intergroup comparison showed significant difference in  $\beta$ -HCG MOM among three groups ( $F=4.847$ ,  $P=0.008$ ). Further LSD-t test showed the  $\beta$ -HCG MOM in T21 group was significantly higher than in healthy control group ( $P=0.005<0.05$ ) and T18 group ( $P=0.004<0.05$ ), but no marked difference was observed between healthy control group and T18 group ( $P>0.05$ ) (**Table 3**). (3)  $\mu$ E3 MOM was compared with Levene test, and homogeneity of variance was present ( $F=0.317$ ,  $P=0.728>0.10$ ). Intergroup comparison showed the  $\mu$ E3 MOM was comparable among three groups ( $F=0.972$ ,  $P=0.379>0.05$ ) (**Table 3**).

*Serum screening of AFP,  $\beta$ -HCG and  $\mu$ E3 MOM in the middle trimester of pregnant women (Figure 1)*

Sensitivity and specificity of different cut-off value in T21: the risk for T21 was divided into high risk + critical risk and low risk, and the results from cff-DNA detection are shown in **Table 4**. For 10 cases of T21, the sensitivity of the cut-off value at 1/750 was 100% and the detection rate was 100% in high risk + critical risk cases. In healthy pregnant women, 138/699 was used as a cut-off value indicating low risk for T21 and had the specificity of 19.74% (**Table 4**).

Logistic regression analysis of factors related to the chromosome aneuploidy in the fetus: Logistic regression analysis showed the chromosome aneuploidy in the fetus was related to the NT thickening, abnormal AFP MOM,  $\beta$ -HCG



**Figure 1.** AFP,  $\beta$ -HCG and  $\mu$ E3 MOM detected in the middle trimester of pregnant women.

**Table 4.** Sensitivity and specificity of different cut-off values in the diagnosis of T21

| Cut-off value | T21          |                    |
|---------------|--------------|--------------------|
|               | Sensitivity  | Specificity        |
| 1/270         | 40% (8/20)   | 74.39% (1040/1398) |
| 1/500         | 60% (12/20)  | 39.34% (550/1398)  |
| 1/750         | 100% (20/20) | 19.74% (276/1398)  |
| 1/1000        | 100% (20/20) | 4.01% (56/1398)    |

MOM  $\geq 3.5$ , and ultrasound apparent structural abnormalities, but not to the advanced age of the pregnant women, abnormal integrated risk value, history of unsuccessful pregnancy or delivery and method of conception (**Table 5**).

## Discussion

Clinical value of detection of cff-DNA in the plasma of pregnant women: There are about 5 million neonates with birth defects each year worldwide and about 1.2 million new cases of birth defects every year in China. The genetic abnormalities are the cause of birth defects in 70% of neonatal patients, and chromosomal abnormalities and gene mutation are the major causes of these genetic diseases. Thus, prenatal screening and prenatal diagnosis are clinically important for the prevention of birth defects.

In 1997, Lo et al reported the presence of cff-DNA in the peripheral blood of the mother, which initiates the application of detection of maternal peripheral blood in the diagnosis of fetal chromosomal diseases and genetic diseases [6].

In 2012, Norton et al [7] applied MPGS in the detection of cff-DNA in the maternal blood and found it had the sensitivity of 100% and specificity of 99.97% in the diagnosis of T21 and the sensitivity of 97.4% and specificity of 99.93% in the diagnosis of T18, suggesting that MPGS is an efficient test in the detection of cff-DNA in the maternal blood, but not a diagnostic test. Thus, the pregnant women should be informed before sample collection that positive results do not imply the abnormal chromosomes of the fetus, and negative results not definitely indicate the normal chromosomes [8].

Invasive diagnostic examination can be used to detect the whole chromosomal karyotype or be applied on the basis of fluorescence in situ hybridization. Thus, it may provide a large amount of additional information, besides generally abnormal triploid. Of note, it is invasive and might cause complications such as intra-uterine infection, bleeding, miscarriage and stillbirth. That is the reason why some pregnant women refuse the invasive prenatal examination. Currently, methods used for prenatal screening can not only be used in the aneuploidy screening, but, together with other biochemical and biophysical markers, be helpful for the early effective screening of pregnancy complications such as pre-eclampsia and preterm birth [9]. Detection with MPGS requires a small amount of samples, has high throughput and high detection rate and is non-invasive. However, the traditional detection requires complicated algorithm, several blood examinations and ultrasound examination. Thus, this technique may serve as a supplement to the current techniques, and cff-DNA detection may further

**Table 5.** Logistic regression analysis of factors related to the chromosome aneuploidy in the fetus

|                                                     | Regression coefficient (95% CI) | P       |
|-----------------------------------------------------|---------------------------------|---------|
| Advanced age ( $\geq 35$ years)                     | 2.186 (0.939~5.086)             | 0.070   |
| NT thickening ( $\geq 3$ mm)                        | 70.213 (10.849~454.397)         | <0.0001 |
| Abnormal AFP MOM ( $<0.5$ or $>2.5$ )               | 6.481 (1.164~36.097)            | 0.033   |
| Abnormal $\beta$ -HCG MOM                           |                                 |         |
| $2.5 < \text{MOM} < 3.5$ or $\text{MOM} < 0.5$      | 0.437 (0.083~2.317)             | 0.331   |
| $\text{MOM} \geq 3.5$                               | 0.135 (0.038~0.482)             | 0.002   |
| Abnormal $\mu\text{E3}$ MOM ( $<0.5$ or $>2.5$ )    | 0                               |         |
| Abnormal comprehensive risk value ( $\leq 1/1000$ ) | 0.686 (0.209~2.247)             | 0.533   |
| Ultrasound apparent structure abnormalities         | 10.047 (2.748~36.735)           | <0.0001 |
| Unsuccessful pregnancy or delivery                  | 0                               |         |
| Method of conception                                |                                 |         |
| Natural Pregnancy                                   | 0                               |         |
| Ovulation stimulation                               | 0.489 (0.081~2.963)             | 0.436   |
| IVF                                                 | 0.039 (-0.238~0.314)            | 0.793   |

screen high risk pregnant women who require other diagnostic techniques. In this study, screening was performed in 1440 pregnant women, and 20 were T21 positive. Then, amniocentesis was performed, and karyotyping showed T21, indicating the diagnostic accuracy of 100%. In addition, T18 was found in 22 women of whom karyotyping was done in 19 women and all were diagnosed with T18, and direct induced labour was performed in 3 women who did not receive karyotyping. Of 1398 women with negative results in cff-DNA detection, trisomy was not found in the fetuses born before the publication of this paper.

The accuracy of cff-DNA detection in screening aneuploidy is dependent on the detection accuracy and plasma cff-DNA concentration, but not the incidence of a disease in general population. The accuracy of non-invasive prenatal screening of aneuploidy in high risk population is also applicable in general population [10]. However, more studies are required to confirm the range of chromosomal abnormalities that can be identified by cff-DNA detection.

#### *Detection of cff-DNA in different groups*

When the pregnant women are relatively old, they are exposed to more radiations and harmful substances, and thus the chance of mutations in the genetic materials increases. Generally, the chromosomes will not separate during the cell division, resulting in the disease. Thus, the advanced age of the mother is one of

important factors affecting the chromosome number. However, the abnormality in the fetal chromosome number occurs in about 60-85% of pregnant women younger than 35 years. Thus, the age of pregnant women may not be used as a criterion in the screening of Down syndrome. Our results also showed the age of pregnant women was similar in T21 women, T18 women and healthy control group, and fetal chromosomal aneuploidy had no relationship with the advanced age of pregnant women.

MOM is a measure of how far an individual test result deviates from the median. In this study, MOM refers to the times of the serum marker in the blood of a specific pregnant woman as compared to that of the health pregnant women (median). In the middle trimester of pregnancy, AFP,  $\beta$ -HCG and  $\mu\text{E3}$  are the most common parameters used for the prenatal screening of Down syndrome in which the risk for fetal Down syndrome is evaluated in a specific pregnancy. However, the MoM of each parameter should be calculated, aiming to avoid the influences of races, age of pregnant women, body weight and detection methods. Then, multivariate normal model is used to estimate the likelihood ratio and the risk for fetal Down syndrome. As shown in **Figure 1**, the AFP,  $\mu\text{E3}$  and  $\beta$ -HCG showed positive skewness distribution, steep convergence was found in AFP and  $\mu\text{E3}$  MOM curves, and flat and decentralization were found in  $\beta$ -HCG MOM curve, which were consistent with previously reported [11]. In our study, the serum AFP MOM in T21 group was signifi-



cantly lower than in healthy control group ( $P<0.05$ ), and serum  $\beta$ -HCG MOM in T21 group was markedly higher than in T18 group and healthy control group ( $P<0.05$ ). Logistic regression analysis showed the fetal chromosome aneuploidy was closely associated with AFP MOM and  $\beta$ -HCG MOM ( $\geq 3.5$ ). Thus, pregnant women with abnormal MOM (especially the  $\beta$ -HCG MOM  $\geq 3.5$ ) require genetic consultation. That is, not only high risk pregnant women, but low risk pregnant women identified on the basis of MOM require genetic consultation, aiming to screen abnormal fetus.

In the available studies, the cut-off value of critical risk has never been determined in Down syndrome. In China, the cut-off value is determined as the range between cut-off value of high risk population and cut-off value of healthy population. In our study, the cut-off value of critical risk was determined as 1/1000. In T21 group, the sensitivity was 100% (95% CI, 95.5~100%) and detection rate was 100% for high risk and critical risk if the cut-off value was 1/750. In healthy pregnant women, the cut-off value of low risk for T21 was 276/1398 which had the specificity of 19.74% (95% CI, 99.8~99.99%). Thus, the cut-off value of critical risk was determined as 1/750 with which the proportion of pregnant women requiring cff-DNA detection reduces without compromising the detection rate and sensitivity. The cut-off value of critical risk at 1/750 was more clinically important for the clinical screening as compared to 1/1000. However, the number of patients with confirmed chromosomal abnormalities was small, and thus there was the possibility of Selection bias. More future studies with large sample size are required to confirm our findings.

A large number of studies have shown that NT thickness is positively associated with the incidence of infant deformity [12]. If the screening on the basis of NT has become mature and been promoted, it may definitely improve the diagnosis. Thus, medical interventions against birth defects may be administered in the first trimester and the pregnancy is discontinued as early as possible, which may reduce the physical and mental burden of the mother and maximize the advantageous and harmless principle of medical ethics. In the middle trimester, ultrasound examination may show some signs such

as endocardial cushion defect, multiple malformations and dandy-walker, which may be indicative of T18 and should be paid attention to. In the present study, of 42 cases of chromosome aneuploidy, NT thickening was found in 12 cases, and ultrasound apparent structural abnormalities were noted in 18. Logistic regression analysis showed fetal chromosome aneuploidy was closely related to the NT thickening and ultrasound structural abnormalities. In our study, cff-DNA detection in 16 pregnant women showed negative, but ultrasound examination indicated significantly structural abnormalities in the fetus, and abnormalities were not found in further karyotyping. Thus, when ultrasound examination shows NT thickening or structural abnormalities (such as omphalocele, former holoprosencephaly, huge bladder and diaphragmatic hernia), invasive diagnosis is required although cff-DNA detection displays negative results [13]. At gestational age of 20 weeks, invasive diagnosis is performed depending on the results from cff-DNA detection and ultrasound examination. The ultrasound examination may serve as a complementary technique to cff-DNA detection to increase the detection rate of deformities.

In our studies, chromosomal abnormalities were not observed in other factors group, which may be ascribed to (1) the small number of pregnant women receiving prenatal screening; (2) the low incidence of Down syndrome; (3) short follow up.

### *Issues and limitations*

Not all the pregnant women who received cff-DNA detection in our hospital were followed up due to the territorial management of pregnant women. In addition, 3 women positive for T18 in cff-DNA detection refused amniocentesis and cord blood puncture, and direct induced labour was performed. Karyotyping was not performed for the confirmed diagnosis in all the pregnant women with negative results in cff-DNA detection, and identification of euploid neonates was based on the absence of phenotype of aneuploid.

### **Conclusions**

Detection of cff-DNA in the peripheral blood of pregnant women is an efficient technique for the prenatal screening. It can be performed

rapidly, is safe and not influenced by the serum markers, has very low false positive rate and false negative rate, is not dependent on the age of pregnant women and can be used in a wide range of gestational age [14]. Thus, it may serve as a supplement to the current techniques for prenatal screening and has been widely accepted by pregnant women. Fetal chromosome aneuploidy is closely related to NT thickening, AFP MOM,  $\beta$ -HCG MOM ( $\geq 3.5$ ) and ultrasound apparent ultrastructural abnormalities ( $P < 0.05$ ). cff-DNA detection together with other techniques may reduce the incidence of birth defects and improve the birth quality.

Currently, cff-DNA detection is application in only a few diseases and has a high cost. In addition, the new technique has the concerns on ethics and law, and there is still controversy on the role of this new technique. However, detection of cff-DNA in the peripheral blood of pregnant women opens a door for the non-invasive prenatal diagnosis. We believe that these problems will be resolved with the development of science and technology and the improvement of living standard [15]. Further studies are required to confirm the accuracy of cff-DNA detection in low risk population and expand the range of chromosomal abnormalities for the cff-DNA detection. Whether cff-DNA detection may replace Amniocentesis as a gold standard in clinical practice is required to be validated in future multicentered studies with large sample size.

## Disclosure of conflict of interest

None.

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## Role of cell-free fetal DNA in diagnosing chromosomal abnormalities

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