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Review Article

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Maternal and Fetal Factors Affecting Cell-Free Fetal DNA (cffDNA) Fraction: A Systematic Review

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Abstract

Background: Cell-free fetal DNA (cffDNA) is a novel screening method for fetal aneuploidy that facilitated non-invasive prenatal testing (NIPT) through analysis of cffDNA in maternal plasma. However, despite increased sensitivity, it has a number of limitations that may complicate of its results interpretation. Therefore, elucidating factors affecting fetal fraction, as a critical limitation, guides its clinical application.

Methods: In this report, systematic search was carried out through PubMed, Web of Science, and Scopus databases until February 11, 2022 by using keywords consist of "noninvasive prenatal screening", "NIPT", "noninvasive prenatal", "cell free DNA" and "fetal fraction". The articles were screened for eligibility criteria before data extraction.

Results: A total of 39 eligible studies, most published between 2010 and 2020, were included. Based on the results of studies, a negative correlation between maternal age and BMI/body weight with fetal fraction was found. Furthermore, LDL, cholesterol, triglyceride level, metformin, heparin and enoxaparin therapy, hemoglobin-related hemoglobinopathies, and physical activity showed to have negative associations. Interestingly, it seems the ethnicity of patients from South and East Asia has a correlation with fetal fraction compared to Caucasians. Positive correlation was observed between gestational age, free β-hCG, PAPP-A, living in high altitude, and twin pregnancy.

Conclusion: Considering each factor, there was significant inconsistency and controversy regarding their impact on outcomes. Indeed, multiple factors can influence the accuracy of NIPS results, and it is worth noting that the impact of these factors may vary depending on the individual's ethnic background. Therefore, it is important to recognize that NIPS remains a screening test, and comprehensive pre- and post-NIPS counseling should be conducted as part of standard clinical practice.

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Introduction

Circulating cell-free DNA (cfDNA) has been proven to be useful for non-invasive prenatal screening/testing (NIPS, NIPT) of fetal aneuploidies by assessing cell free fetal DNA (cffDNA) in maternal plasma/serum (1-3). The detection rates of NIPT for most common fetal aneuploidies including trisomy 21, trisomy 18, and trisomy 13 are >99%, 98%, and 99%, respectively, with false positive rate (FPR) of 0.13% when all are combined (4). Despite the use of

NIPT for aneuploidy screening, cffDNA could be an important source for genetic and epigenetic screening of fetus, particularly for paternally inherited and de novo mutations. However, a great challenge remains in the detection of maternally inherited variants owing to the substantial background of maternal cfDNA.

The cffDNA fragment size (less than 200 base pairs) is smaller than that of maternal cell free DNA (5, 6) and it originates from apoptotic placental cells (trophoblasts) derived from the embryo (7). cffDNA concentration (fetal fraction) is about 10%–15% of total cfDNA between 10 and 20 weeks of pregnancy, which can be detected from the fourth week of gestation and can be quickly cleared from maternal blood following 2 hours after delivery (8-10). The use of cffDNA is still limited because cffDNA encompasses a minor proportion of total cfDNA in the plasma of pregnant women and commonly a minimum of 4% fetal fraction is required to provide a reliable test result (11-13). Different factors are reported to affect fetal fraction and there is high controversy in the literature for most factors. However, several factors' correlation with fetal fraction is supported by a more consistent literature, such as maternal weight or BMI and gestational age (14-16). In this study, a review of literature was performed to assess factors affecting fetal fraction of cfDNA and evaluate the individual influence of each factor.

Methods

Search strategy: A systematic search was carried out through MEDLINE/PubMed, Scopus, Cochrane library, and Web of Science (WoS) databases until February 11, 2022 by two reviewers (AS, OK) independently. The following keywords were used to retrieve relevant studies: ("noninvasive prenatal screening" [Title/Abstract] OR "NIPT"[Title/Abstract] OR "noninvasive prenatal" [Title/Abstract]) AND ("cell free DNA" [Title/Abstract] OR "fetal fraction" [Title/Abstract]). Furthermore, papers that were not identified by the above databases were included by evaluating the reference sections of relevant studies. The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) checklist was used for the selection process in our systematic review (Figure 1).

Study selection and data extraction: Randomized clinical trials, observational studies (cross-sectional, case–control, or cohort), and case series/reports were included. The inclusion criteria were the articles including pregnant women who underwent NIPT test and factors affecting fetal fraction of cell free DNA as one of the primary objectives of the papers. EndNote citation management software was used for the study selection process and to manage the obtained articles. The title and abstract of the studies were assessed based on the inclusion criteria after duplicate papers were rejected. Finally, a thorough screening of the full texts was done. The selection was carried out independently by two authors (AS, OK). Two researchers (AS, HS) independently extracted the following information including author, year, country, type of study, population, number of total patients, method of evaluating fetal fraction, and final outcome. A third reviewer resolved disagreements (OK).

Results

Study selection: Our preliminary search result yielded 2071 references. After duplicates were removed, a total of 1407 articles were screened, using titles and abstracts. The full-text of 57 studies were retrieved and after an evaluation based on our inclusion criteria, 39 studies were found eligible (Figure 1).

Study characteristics: A summary of the characteristics of the included studies evaluating the factors affecting fetal fraction is available in supplementary table 1. Most of the studies were published between 2010 and 2020 which investigated singleton pregnancies including pregnancies at 11-13 weeks of gestation. Of 38 studies about fetal fraction, 18 investigated maternal age, 27 investigated maternal weight/BMI, 23 investigated gestational age, PAPP-A, and free β-hCG, 9 studies fetal fraction, 8 studies fetal gender, 10 studies smoking, and 9 investigated racial origin. A total of 5 studies investigated the effect of twin/multiple pregnancy on Fetal Faction (FF). In 9 studies, other factors were cited (Figure 2).

Maternal age: Of all the studies included, 18 reported the relationship between maternal age and fetal fraction (8, 17-33). Among them, 6 studies found decreased fetal fraction with increasing age (24-27, 29, 30). Interestingly, a study by Scott et al. reported a significant negative correlation between maternal age and FF; however, in their multivariate analysis, the influence of maternal age was found to be nonsignificant (8).

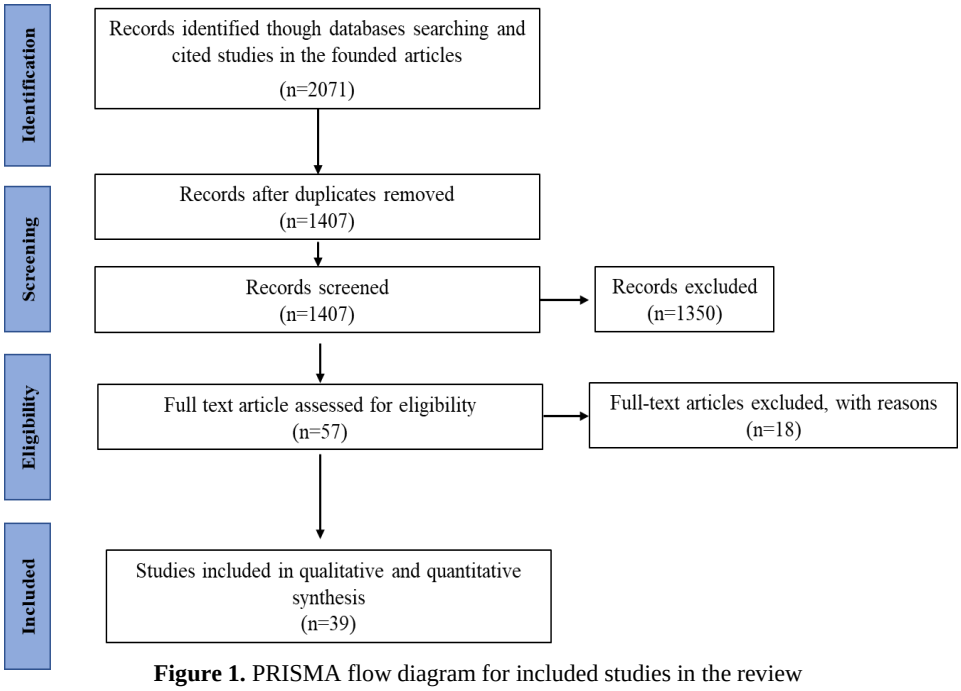

Figure 1. PRISMA flow diagram for included studies in the review

* The process of selecting and refining articles ultimately led to a final set of 39 included articles

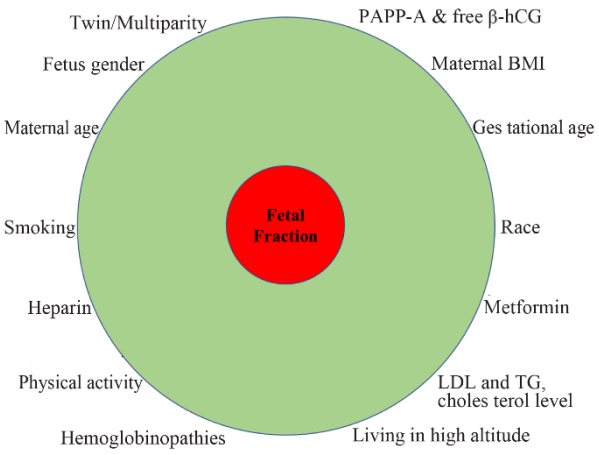

Figure 2. Reported factors affecting fetal fraction

* Negative correlation between maternal age and BMI/body weight with fetal fraction was detected. Likewise, LDL, cholesterol, triglyceride level, metformin, heparin and enoxaparin therapy, hemoglobin-related hemoglobinopathies, and physical activity showed negative associations. Positive correlation was seen between gestational age, free β-hCG, PAPP-A, living in high altitude, and twin pregnancy. There appears to be a potential race-dependent association with fetal fraction

In a large cohort study done by Hou et al. (25), samples from 13661 singleton pregnancies were analyzed in 5 groups (group 1: 18–24 years old, group 2: 25–29 years old, group 3: 30-34 years old, group 4: 35-39 years old, and group 5: ≥40 years old). Compared with the first group, the fetal fraction of all other groups was decreased significantly. Additionally, when compared to the second group, a significant decline was also seen in the 30-34, 35-39, or 40 year old cases. Similarly, the fetal fraction in the third group was higher than that in the 35–39 year-old cases or the group above forties. Another study by Sarno et al. (29) which was performed on 10698 women with singleton pregnancies undergoing NIPT investigated the rate of failure in each of these trisomies and provided a further option in collecting information from failed NIPTs. In their study, they have shown possible effects of multiple fetal and maternal factors, including maternal age. They found that in both univariate and multivariate analysis, the rate of test failure increased with increasing maternal age. Although some research demonstrated that maternal age had a substantial impact on fetal fraction, the majority of the studies that were included found a nonsignificant correlation (17-23, 28, 31-33).

Maternal weight/BMI: Of the total studies included, 27 reported the relationship between maternal weight/BMI and the fetal fraction. All studies found the relationship between decreased fetal fraction and increasing maternal weight/BMI (8, 18-44). Although all included studies proved the negative correlation between fetal fraction and

maternal weight and BMI, there is some inconsistency regarding the strength of such correlation. Rava et al. have found a weak but significant correlation between FF and maternal BMI (35). The idea was further supported by Suzumori et al. who found a nonsignificant correlation in the 10-20 weeks of gestation (34). In contrast, some studies reported a strong negative correlation between FF and BMI. In a cohort by Scott et al., they reported a significant decrease of FF from 12% to 7% in individuals with a BMI of <24 kg/m^2 and >30 kg/m^2, respectively (8). Additionally, they have discovered that there is little impact when sample collection is delayed due to the positive effects of increasing gestational age on FF.

Gestational age: Of the total studies included, 23 reported the relationship between gestational age and the fetal fraction (8, 17-33). Also, 18 studies showed increased fetal fraction with increasing gestational age (24-27, 29, 30). A weak correlation between gestational age and FF was observed by the majority of these studies. Moreover, 5 studies reported a nonsignificant difference regarding gestational age (19, 31, 34, 35, 44). During early weeks of gestation (typically between 10 and 20 weeks), there was no significant change in FF. However, a rapid increase in fetal fraction percentage was observed after 20 weeks of gestation (23, 36). Also, 2 studies predicted the increase of fetal fraction during early gestational age and after (36, 40). Wang et al. showed that between 10-21 weeks of gestation, an increase of 0.1% per week is predictable. Compared with the earlier weeks, the percentage of FF rose at a rate of 1% per week beginning at 21 weeks of gestation (36). This rate was observed to grow by 0.44% each week between 10 and 12.5 weeks of gestation and by 0.083% each week between 12.5 and 20 weeks, according to a study by Kinnings et al. (40).

The biochemical markers in the first trimester: PAPP-A and free β-hCG were used as markers in the first trimester. There was a significant correlation between PAPP-A, free β-hCG, and fetal fraction in 9 studies (8, 18, 19, 21, 28, 29, 30, 33, 37). Regarding free β-hCG, all included studies showed a significant rise in fetal fraction with increasing free β-hCG. Regarding PAPP-A, only a single study found a nonsignificant positive correlation between PAPP-A and FF (21). The association between FF and biochemical markers in the first trimester suggests a strong correlation between FF and placental size and function.

Fetal gender: Of the total studies included, 8 reported the relationship between fetal gender and the fetal fraction (20-23, 28, 33, 37, 40) and 3 studies reported that the fetal fraction in female fetuses is significantly higher than in male fetuses (20, 23, 28). However, 5 studies showed there was no significant association between fetal gender and FF (21, 22, 33, 37, 40).

Smoking: Of the total studies included, 10 studies reported the relationship between smoking and the fetal fraction (21, 22, 28, 29, 30, 31, 33, 37, 44, 45). Furthermore, 3 studies reported that the fetal fraction is significantly correlated with smoking status and FF decreases among individuals who are active smokers (28, 33, 37). Also, 7 studies showed there was no significant correlation between smoking status and FF (21, 22, 29, 30, 31, 44, 45). A study by Tarquini et al. specifically investigated the impact of maternal smoking on FF during the first trimester of pregnancy (45). Using the DYS14 sequence as a fetal marker and the quantitative real time PCR for DNA analysis, the fetal fraction of cell free DNA of a total of 177 non-smokers, 18 smokers, and 22 ex-smokers was assessed. The results of their study showed that there was no significant difference between people who were active or ex-smokers and the non-smoker group.

Ethnicity: Of the total studies included, 9 studies reported the relationship between racial origin and the fetal fraction (21, 27, 28, 29, 30, 31, 33, 37, 38). Among them, 6 studies found a significant negative correlation in individuals with South Asian ethnicity compared to Caucasians (21, 27, 29, 30, 37, 38). Four studies demonstrated a significant negative correlation in ethnicity of East Asians compared to Caucasians (21, 30, 37, 38). Four studies found a significant negative correlation in ethnicity of East Asians compared to Caucasians (29, 30, 33, 37). Two studies did not find any correlation between ethnicity and FF (28, 31).

Twin/multiple pregnancy: A total of five studies investigated the effect of twin/multiple pregnancy on FF (24, 29, 32, 41, 44). A study by Sarno et al. examined failure rate of NIPT between twin and singleton pregnancies (29). They found a higher risk of failure among twins and the median fetal fraction in this group was lower compared to singleton pregnancies (8% *vs.* 11%). Hedriana et al. reported that the average rate of FF among twin pregnancies is 32% higher than that of singletons, although this finding was not consistent when it

comes to comparing FF contribution between singleton and twin pregnancies (41).

Other factors: Several other factors affecting the rate of fetal fraction include anticoagulation therapy (43), lipid metabolism (42), gestational diabetes (46), medication intake (39), heparin treatment (47), hemoglobinopathies (48), abnormal miRNA expression (17), physical activity (49), and high altitude (50).

Decreased fetal fraction was observed with increased low-density lipoprotein (LDL), cholesterol and triglyceride (TG) levels (42), metformin (39), heparin and enoxaparin therapy (43, 47), hemoglobin-related significant hemoglobinopathies (48), and physical activity (49). A positive correlation was observed in individuals living in high altitude (50). No association was found between FF and gestational diabetes (46).

Discussion

In this study, factors affecting fetal fraction of cfDNA in plasma/serum of pregnant woman were evaluated. Based on the results of included studies, a negative correlation between maternal age and BMI/body weight with fetal fraction could be suggested. Although there was controversy on significance of this correlation among included studies, no study reported positive correlation. Decreased fetal fraction in patients with high BMI/body weight could be due to higher inflammation and necrotic adipose tissue in these patients (51).

Another factor that seems to have an interesting correlation with fetal fraction is gestational age. FF seems to increase with increasing gestational age and this increase is more significant after 20th week of gestation. PAPP-A and free β-hCG were reported to have a positive correlation with fetal fraction. Low levels of PAPP-A are found to be correlated with placental dysfunction (52-55); therefore, placental dysfunction could be the cause of negative correlation between PAPP-A and fetal fraction though further investigations are required to confirm the hypothesis. Interestingly, fetal fraction of pregnant women with female fetuses seems to be higher than pregnant women with male fetuses.

Tarquini et al. worked on the effect of smoking and they concluded that maternal smoking has no effect on fetal fraction during the first trimester (45), but there is controversy among other studies that reported smoking as one of their outcomes. In case of race and ethnicity, it seems the ethnicity of patients from South and East Asia has a negative correlation with fetal fraction compared to Caucasians.

Zhou et al. reported that the fetal fraction shared with each fetus in twin pregnancy has no significant difference with singleton fetus (32). Two studies reported that combined fetal fraction for monozygotic and dizygotic pregnancies is significantly higher than singleton pregnancies and dizygotic twins had a significantly higher fetal fraction compared to monozygotic twins (41, 44). Interestingly, Qiao et al.'s study showed that using smaller fragments of DNA for NIPT could improve fetal fraction in twin pregnancies (24).

Several studies reported that aneuploidies could affect fetal fraction. In a study by Zhou et al., it was found that pregnancies with trisomy 21 have higher fetal fraction (32). This finding is consistent with previously reported studies (33). However, a study by Lopes et al. showed nonsignificant reduction in fetal fraction in all trisomies (22). Increased fetal fraction in pregnancies with trisomy 21 could be due to higher levels of oxidative stress leading to more placental necrosis (56-59). This finding was reversed in pregnancies with trisomy 18, and most studies indicated a decreased fetal fraction in such pregnancies (30, 32, 34). On the other hand, two studies reported no significant association between trisomy 18 and fetal fraction (33, 60). Smaller placentas in trisomy 18 could be the cause of decreased fetal fraction in these patients (60). The study by Wataganara et al. reported a significant elevation in fetal fraction in patients with trisomy 13, but the outcome in other studies was not consisted with this one (30, 32, 34). Two main limitations among the included studies were inadequate adjustment of outcomes and the omission of fetal fraction as their primary focus of investigation.

Conclusion

Based on the reported studies, a negative correlation between maternal age and BMI/body weight, LDL, cholesterol, triglyceride level, metformin, heparin and enoxaparin therapy, hemoglobin-related hemoglobinopathies, and physical activity with fetal fraction could be suggested. It seems the ethnicity of patients from South and East Asia has a correlation with fetal fraction compared to Caucasians. Positive correlation was observed between gestational age, free β-hCG, PAPP-A, living in high altitude, and twin pregnancy. However, it is imperative to conduct studies that specifically focus on factors influencing fetal fraction,

particularly those that have been sources of controversy, using adjusted populations based on BMI and gestational age.

Conflict of Interest

The authors declare that they have no competing interests.

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Supplementary Table 1. Characteristics of included studies and quality assessment results

Author	Year	Country	Type	Population	Total patients (n)	Outcome	Ref
Talbot et al.	2020	Denmark	Retrospective cohort	Women with singleton ongoing pregnancy following either a fresh ET or a frozen ET in a modified natural cycle	292	Decreasing FF with increasing maternal BMI ($p<0.0001$) and with decreasing values of β -hCG (MoM) No association between FF and maternal age, gestational age, PAPP-A (MoM) or the thickness of the nuchal translucency	(19)
Scott et al.	2018	Australia	Retrospective cohort	All women with singleton pregnancies who underwent NIPT	5267	The association of high body mass index (BMI), early GA and decreased placental biomarkers (free β-hCG, PAPP-A, PlGF) with decreased FF Maternal age or nuchal translucency has no association with FF The correlation between trisomy 21 and increased FF	(8)
Schlütter et al.	2014	Denmark	Case series	Singleton pregnant women known to carry a male fetus when they attended the first trimester nuchal translucency scanning	9	The association between physical activity and decreased FF	(49)
Rolnik et al.	2018	Australia	Retrospective cohort	Women with singleton pregnancies who underwent cfDNA testing and reported fetal fraction	4317	The association between low FF and increased risk for pregnancy complication such as PE, risk of PE and FGR	(27)
Revello et al.	2016	UK	Cohort	Women with singleton pregnancies undergoing screening for fetal trisomies 21, 18, and 13 by cfDNA testing at 10–14 weeks of gestation	10698	The association between decreased FF and increasing body mass index and maternal age FF is lower in South Asian women than Caucasians FF increased with fetal crown–rump length and higher levels of free β-hCG and PAPP-A	(30)
Qiao et al.	2019	China	Cohort	Women pregnant with male fetuses	2903	The association of increased maternal BMI and decreasing FF The association of early GA and decreasing FF cfDNA sizes have negative correlation with FF	(24)
Putra et al.	2021	USA	Retrospective case-control	Women with a diagnosis of HSH using NIPS from a commercial laboratory	35	The relation between HBB-related significant hemoglobinopathies and low FF	(48)
Poon et al.	2013	USA	Cohort	Women with singleton pregnancies attending for their routine first hospital visit in their first-trimester	1949	The association between higher fetal and maternal cfDNA plasma concentration and higher levels of free β-hCG and PAPP-A Higher fetal and maternal cfDNA plasma concentration in Afro-Caribbeans and East-Asians than in Caucasians Lower fetal and maternal cfDNA plasma concentration in in smokers Fetal cfDNA level has an inverse relation with maternal weight and uterine artery pulsatility index Maternal cfDNA increased with maternal weight	(37)
Miltoft et al.	2020	Denmark	Cohort	Pregnant Danish-speaking women ≥18 years of age	321	FF decreased with maternal BM FF is lower in Asian women FF increased with β-hCG levels and gestational age FF is lower before 10 weeks of GA	(21)
Miltoft et al.	2018	Denmark	Cohort	Women aged ≥18 years with a singleton pregnancy, a cFTS risk for T21 of ≥1 in 1000 and no language barrier	6449	FF decreased with increasing maternal weight FF increased with the level of β-hCG and PAPP-A among female fetuses	(28)
Lopes et al.	2020	USA	Cohort	Pregnant women who underwent NIPT	2374	FF decreased in trisomy pregnancies and NIPS failure is because of high BMI/early gestational age FF is not significantly associated with maternal age, fetal gender, IUFD, IUGR, preeclampsia, hypertension, use of advanced reproductive technologies to achieve pregnancy, or smoking	(22)

Contd. Supplementary Table 1. Characteristics of included studies and quality assessment results

Author	Year	Country	Type	Population	Total patients (n)	Outcome	Ref
Lee et al.	2018	Australia	Retrospective cohort	Women with singleton pregnancies underwent cffDNA screening for trisomies 21, 18, 13 and sex chromosome aneuploidies (SCA) from 10 weeks of gestation	5625	The association between IVF conception, increased BMI, earlier gestational age and South and East Asian ethnicities and low FF	(38)
Kuhlmann-Capek et al.	2019	USA	Retrospective cohort	Women with singleton pregnancies undergoing cfDNA testing or genetic counseling	1051	The association of increase maternal BMI and low FF The association of early GA and low FF The a ssociation of exposure to metformin and low FF The association of two or more medications (heparin) and lower FF	(39)
Krishna et al.	2016	USA	Retrospective cohort	Singleton pregnancies with quantification of fetal fraction and pregnancy outcome information. All women undergoing NIPT	435	The association of ethnicity of African Americans and high body mass index with low FF Low FF caused more adverse perinatal outcomes in women with preterm birth (<37weeks) and pregnancy-associated hypertensive disorders is more in low FF Significant association between gestational diabetes or preterm premature rupture of membranes and low FF FF has no association with IUGR, induction of labor, mode of delivery, postpartum complications, or NICU admission Live birth decreases in low FF	(31)
Kinnings et al.	2015	USA	Cohort	Singleton pregnancies with a minimum gestational age of 10 weeks were included in this study	140377	FF increases with GA FF decreases with maternal BMI or blood volume The effect of aneuploidy status on FF varies with GA	(40)
Hou et al.	2019	China	Retrospective cohort	Women with singleton pregnancies who underwent NIPS	13661	FF increased with increase of GA FF decreased with increasing maternal BMI Maternal age has no relation with FF	(25)
Hopkins et al.	2020	USA	Retrospective cohort	All women who had cfDNA aneuploidy screening between 10 and 20 weeks of gestation	3206	Low FF is correlated with maternal obesity No relationship between FF and development of GDM up to 20 weeks	(46)
Hedriana et al.	2020	USA	Cohort	Twin and singleton pregnancy (MZ and DZ)	4615 twin and 121 446 singleton pregnancies	FF is higher in twins than singletons but the per fetus FF was lower. There are differences in the two FFs in dizygotic twin pregnancies	(41)
Gou et al.	2019	China	Retrospective cohort	Women with singleton pregnancies who underwent NIPT at 14 to 25 weeks of gestation	8129	FF has negative correlation with BMI, maternal age, nulliparity, and history of spontaneous preterm birth FF was positively correlated with GA FF has no correlation with assisted reproduction or hepatitis B surface antigen (HBsAg)	(26)
Dolatkhah et al.	2021	Iran	Cross-sectional study	Singleton pregnant women aged 20-47 years at 11+0 to 13+6 weeks of pregnancy	308	cffDNA has a negative correlation with maternal weight cffDNA has a positive correlation with PAPP-A and free β-hCG values cffDNA in male fetuses is higher than female fetuses cffDNA was not related with CRL and maternal age	(18)
Chen et al.	2019	China	Cohort	Pregnant women who opted for NIPT	362	FF has a positive correlation with GA in twin and triplet pregnancies FF has a same correlation like singleton pregnancy with fetal gender and chromosomal aneuploidy in twin and triplet pregnancies	(64)

Contd. Supplementary Table 1. Characteristics of included studies and quality assessment results

Author	Year	Country	Type	Population	Total patients (n)	Outcome	Ref
Cao et al.	2021	China	Retrospective cohort	Pregnant women at 12–26 weeks of gestation who underwent NIPT sequencing and serum lipid measurements	4514	FF decreased with increased LDL, cholesterol, and TG levels FF has a positive correlation with HDL-C level and the time interval between the two tests (in high TG levels)	(42)
Burns et al.	2017	USA	Retrospective cohort	Women with singleton pregnancies who had cfDNA screening at 10–25 weeks of gestation	2890	Enoxaparin therapy and obesity are related to low FF	(43)
Bevilacqua et al.	2015	Spain	Cohort	cfDNA testing was performed in twin pregnancies at 10–28 weeks of gestation	515	FF in twin pregnancies was lower than singleton pregnancies	(44)
Ashoor et al.	2013	UK	Cohort	Singleton pregnancies at 11–13 weeks of gestation and cf-DNA testing	1949	FF was associated with maternal weight, height, Afro-Caribbean origin, cigarette smoking, fetal CRL, trisomy 21 karyotype, log10 PAPP-A-MoM and log10 β-hCG-MoM Fetal fraction increased with fetal CRL FF was not associated with maternal age, method of conception, fetal gender, trisomy 18 karyotype or delta NT	(33)
Zhou et al.	2015	China	Case-control	Pregnant women that received non-invasive prenatal testing	23067	FF has a positive correlation with GA and increased rapidly after the 21 weeks of gestation FF has a negative association with maternal BMI FF was higher in trisomy 21 fetus and lower among trisomy 18 A 1.6-fold incensement of fetal fraction was observed in twin fetuses comparing to singleton pregnancy FF is lower in women with preexisting hypertension FF was not associated with preexisting diabetes, hyperthyroidism, or HBsAg carriers	(32)
Zhong et al.	2004	China	Case control	Pregnant women, Han Chinese at sea level, Lhasa Han Chinese, Han Chinese with preeclampsia, Tibetans with preeclampsia	19+21+27+15+11=83	FF increased by preeclampsia, fetal growth restriction, preterm labor, pregnancies with aneuploidy fetuses, and placental pathology FF increased in preeclampsia FF increased in high altitudes	(50)
Zhao et al.	2019	China	Cohort	Women with singleton pregnancies using non-invasive prenatal testing for aneuploidy via NIPT	2638	FF has a positive correlation with GA and negative correlation with BMI In female fetus, cfDNA is higher than male	(23)
Yan et al.	2000	Switzerland	Case-control	Pregnant women with an increased risk for an aneuploid fetus who were about to undergo an invasive prenatal diagnostic procedure	29+28	FF demonstrated a significant increase in trisomy 21, while its association with trisomy 18 did not reach statistical significance	(65)

Contd. Supplementary Table 1. Characteristics of included studies and quality assessment results

Author	Year	Country	Type	Population	Total patients (n)	Outcome	Ref
Wataganara et al.	2003	USA	Case-control	Five pregnant women confirmed to be carrying a singleton male fetus with trisomy 13 and serum samples from other 5 pregnant women confirmed a singleton male fetus with trisomy 18 Four or five serum samples from pregnant women carrying presumably normal euploid male fetuses, and 1 or 2 presumably normal euploid female fetuses as negative controls, matched for both gestational age and duration of specimen storage in the freezer	20	FF increased in trisomy 13 but FF decreased in trisomy 18	(60)
Wang et al.	2013	USA	Cohort	Women with singleton pregnancies at 10 weeks of gestation undergoing the harmony prenatal test	2234	FF increases with gestation FF decreases with increasing maternal weight	(36)
Tarquini et al.	2015	Italy	Case-control	344 plasma samples were taken from non-smoking pregnant women, 38 from active smoking pregnant women, and 33 from ex-smoking pregnant women, all of whom were in the first trimester of pregnancy	415	FF levels are not altered by smoking and previous smoking habits in pregnancy during first trimester	(45)
Alberry et al.	2009	UK/Egypt	Case-control	Women with singleton male pregnancies (n: 138) were recruited (over a period of 18 months in 2006-2007)	138	Free fetal DNA in normal pregnancies increased with gestational age Results were significantly higher in preeclampsia and fetal growth restriction groups than in normal pregnancy and were higher in severe preeclampsia than in milder disease	(66)
Suzumori et al.	2016	Japan	Prospective cohort	Pregnant women with high risk for fetal aneuploidy and singleton gestation were enrolled at 10 to 20 weeks of gestation The high-risk indications included maternal age of ⩾35 years at the time of delivery, abnormal fetal ultrasound, abnormal serum screening, personal history of a child with aneuploidy or a parent carrying a balanced Robertsonian translocation with an increased risk of trisomy 13 or 21	7740	FF decreased with increasing maternal weight Ff increased with gestation FF in triosomy 21 is higher than 13 and 18	(34)
Serapinas et al.	2020	Lithuanian	Cohort	Women with singleton pregnancy who underwent NIPT The exclusion criteria were multiple gestation and gestational age ≥21 weeks	862	FF increased with gestation FF decreased with maternal weight	(20)

Contd. Supplementary Table 1. Characteristics of included studies and quality assessment results

Author	Year	Country	Type	Population	Total patients (n)	Outcome	Ref
Sarno et al.	2016	UK	Cohort	438 twin and 10698 singleton pregnant women underwent screening for fetal trisomies by cfDNA testing at 10+0 to13+6 weeks of gestation	438+10698	FF is lower in twin than singleton pregnancies FF decreased with increasing maternal weight	(29)
Santoro et al.	2021	Italy	Case-control	Caucasian pregnant women who had undergone NIPT from 10 + 0 to 14 + 1 gestational weeks as a primary or secondary screening test for fetal chromosomal abnormalities	12	Aberrant maternal miRNA expression is associated with low FF	(17)
Nakamura et al.	2020	Japan	Retrospective cohort	Patients underwent NIPT singleton pregnancies	2651	Heparin use increased the short-sized cfDNA and affected the NIPT result	(47)
Guo et al.	2019	China	Retrospective cohort	Women with singleton pregnancies who underwent noninvasive prenatal testing at 14 to 25 weeks of gestation	8129	The fetal fraction of cell-free DNA was negatively correlated with body mass index, maternal age, nulliparity, and history of spontaneous preterm birth, positively correlated with gestational age, and not correlated with assisted reproduction or hepatitis B surface antigen (HBsAg)	(26)