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Original Article

Sequencing of short cfDNA fragments in NIPT improves fetal fraction with higher maternal BMI and early gestational age

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Abstract: Low fetal DNA fraction (< 4%) samples obtained during noninvasive prenatal testing (NIPT) are responsible for 0.5%-3% of "no calls". Maternal characteristics such as body mass index (BMI) and gestational age (GA) are the main factors that influence fetal fraction. Here, we improved fetal fraction by performing semiconductor sequencing of shorter fragments (107-145 bp) of cfDNA (traditional NIPT fragment is 160 bp). Multivariable linear regression modeling was used to evaluate the association between fetal fraction and maternal characteristics including BMI and GA. Among the 1495 shorter cfDNA sequencing samples, BMI and GA were negatively and positively correlated with fetal fraction, respectively. Compared with underweight pregnant women, sequencing of shorter fragments decreased the mean fetal fraction differences between BMI groups, especially in the obese women (~15%). We also showed that the average fetal fraction was 22.2% and 96.3% fetal fraction more than 10% in the obese women with the average GA of 17 weeks. Size selection slightly decreased the mean fetal fraction differences between different GA groups, and sequencing shorter cfDNA can yield a fetal fraction at an earlier GA. Collectively, our results support the strategy of sequencing shorter to improve fetal fraction in subjects with a high maternal BMI and earlier GA.

Keywords: Noninvasive prenatal testing, fetal fraction, size selection, obesity, gestational age

Introduction

Noninvasive prenatal testing (NIPT) is increasingly popular and is being utilized for aneuploidy screenings across the maternal age spectrum beginning at gestational age (GA) of 9-10 weeks and for subjects who are not significantly obese [1]. It is estimated that between 4 and 6 million pregnant women are now receiving NIPT each year worldwide, and the number is expected surpass 15 million within a decade [2, 3]. Because cell-free DNA (cfDNA) is a mixture of genomic DNA fragments of maternal and fetal (placental) origin [4, 5], NIPT utility is directly related to the fetal fraction [6]. Recent studies have reported that the average fetal fraction is 10% [7]. However, 0.5% to 3% of women and their clinicians will be frustrated by a cfDNA report without a result or a "no calls" due to a low fetal fraction (< 4%) [8-10]. Numerous maternal and fetal characteristics have been associated with reductions in fetal fraction including early GA, maternal obesity, and multiple pregnancies [8, 11, 12]. Maternal weight is inversely related to the fetal fraction [8]. Previous data suggested that a fetal fraction below 4% increased with maternal weight from < 1% at 60 kg to > 50% at 160 kg [13]. The clinical application of NIPT is therefore limited by the low fetal fraction (< 4%) of cfDNA in obese women. The rate of increase in fetal fraction is not constant across GA. From 10-12.5 weeks, 12.5-20 weeks, and > 20 weeks, the fetal fraction increases at rates of 0.44%, 0.083%, and

0.821% per week, respectively [6]. Waiting for a later GA and repeating sample collection is not a reliable approach to overcoming the low fetal fraction in subjects with higher body mass indexes (BMIs) and earlier GAs.

Early work, using quantitative polymerase chain reaction (qPCR) with different amplicon sizes targeting the leptin gene, revealed that maternal-derived DNA fragments are generally longer than fetal-derived ones [14]. A subsequent study demonstrated that circulatory fetal DNA can be enriched by size selection of fragment sizes < 0.3 kb by agarose gel electrophoresis and real-time PCR [15]. Recent studies reported that the most significant difference between fetal and maternal DNA in maternal plasma is the reduction in the 166-bp peak relative to the 143-bp peak [16, 17]. A possible mechanism for this shortening of circulating fetal DNA is that fetal-specific preferred end sites were mostly located at the border or within the nucleosome core. However, the maternal-specific end sites were mostly in the linker region, which was consistent with the size profile of DNA wrapping the nucleosome core and nucleosome spacing pattern in the genome [16, 17]. Based on this hypothesis, our group developed a new NIPT method that sequences shorter cfDNA fragments (107-145 bp) to improve the fetal fraction [18]. The objective of this study was to examine the impact of maternal characteristics, such as GA and BMI, on fetal fraction in shorter cfDNA fragment sequencing.

Materials and methods

Protocol for sequencing shorter cfDNA fragments for NIPT

After receiving approval by the reproductive medicine ethics committee of Suzhou municipal hospital (approval no. K901001), we retrospectively analyzed plasma samples from 1495 women pregnant with male fetuses by sequencing shorter cfDNA fragments (107-145 bp) for NIPT. All of samples had fetal karyotypes or clinical follow-up results. cfDNA was extracted from 600 μl plasma, a library was constructed by PCR, and recycled shorter fragments form PCR were produced by E-Gel® EX Gels (Invitrogen, Carlsbad, CA, USA). cfDNA and library concentrations were measured with the Qubit™ dsDNA HS Kit. Then, the recycled cfDNA were sequencing by Ion Proton system. Fetal DNA concentration was evaluated by calculating the proportion of reads from chromosome Y.

Statistical analysis

The distribution of fetal fraction was assessed for an approximately normal distribution with normal Q-Q plots. We used different linear regression models to examine the associations of fetal fraction and GA and BMI. We generated a categorical variable for BMI (< 18.5 kg/m^2 vs. 18.5-24.9 kg/m^2, 25-30 kg/m^2, ≥ 30 kg/m^2) and GA (21-26 weeks vs. 18-20 weeks, 15-17 weeks, 12-14 weeks). Relative to the referent category, we computed estimates and 95% confidence intervals (CIs) for the mean differences in fetal fraction for each category of BMI and GA. We used five different models. Model 1 is a univariate linear regression of the relationship between BMI or GA and fetal fraction. Model 2 was adjusted for GA (continuous numerical variables) and multiple gestations (categorical variables) or BMI (continuous numerical variables) and multiple gestations (categorical variables). Model 3 added mean size of cfDNA (continuous numerical variables) on the basis of model 2. Model 4 added maternal age (continuous numerical variables), maternal plasma cfDNA concentration (continuous numerical variables), library concentration (continuous numerical variables), and uniquely mapped reads (continuous numerical variables) on the basis of model 2. Model 5 added the mean size of cfDNA (continuous numerical variables) on the basis of model 4. We selected these confounders based on their associations with the outcomes of interest or a change in effect estimate > 10% [19]. All analyses were performed using SPSS version 24.0 (IBM Corp, Armonk, NY, USA). All *P* values were 2-sided, and P < 0.05 was considered statistically significant.

Results

Sample characteristics

The characteristics of the study population are presented in **Table 1**. The mean maternal age, BMI, GA, mean uniquely mapped reads, size of cfDNA, and fetal DNA concentration were 31.0 (range 18 to 47 years), 22.5 (range 15.4 to 44.2 kg/m^2, **Figure 1A**), 17.3 (range 12 to 26 week, **Figure 1B**), 2.4 Mb (range 1.0 to 9.0 Mb), 130 bp (range 107 to 145 bp) and 30.7% (range 5.8% to 93.6%), respectively. The number of uniquely mapped reads needed for short-

Table 1. Maternal and fetal characteristics of the study population (n = 1495)

Characteristic	Male fetus
Sample size	1495
Maternal age (years)	31.0 (18.0-47.0)
Maternal weight (kg)	58.3 (40-109)
Maternal height (cm)	160.8 (145-179)
Body mass index (kg/m^2)	22.5 (15.4-44.2)
Gestational age (week)	17.3 (12-26)
Number of pregnancy	2.5 (1-9)
Uniquely mapped reads (Mb)	2.4 (1.0-9.0)
Median size of cell-free DNA (bp)	130 (107-145)
Fetal DNA concentration (%)	30.7 (5.8-93.6)
Maternal plasma cell-free DNA concentration (ng/μl)	0.17 (0.04-0.71)
Library concentration (ng/μl)	8.3 (0.05-21.7)
Singleton pregnancies	1327
Monochorionic twins	7
Dizygotic twins	161

sequencing NIPT is significantly lower than that of traditional NIPT. In singleton and twin pregnancies, the mean fetal fractions were 31.0% (range 5.8% to 93.6%) and 28.3% (range 8.6% to 68.1%), which was higher than a previous study on fetal fraction in twin pregnancy research [12].

Fetal fraction decreases with BMI in sequencing of short cfDNA NIPT

BMI was negatively correlated with fetal fraction in sequencing of short cfDNA NIPT (**Figure 2A**). This result confirmed that fetal fraction was decreased in the high BMI group. As shown in **Figure 2B**, among pregnant women with the same BMI range, fetal fraction decreased with longer cfDNA fragment size. In the same cfDNA fragment size range, fetal fraction decreased with higher maternal BMI. The mean fetal fractions for subjects with maternal BMI < 18.5 kg/m^2, 18.5-24.9 kg/m^2, 25-30 kg/m^2, and ≥ 30 kg/m^2 were 33.7% (range 12.1% to 57.5%), 31.4% (range 6.4% to 93.6%), 27.5% (range 5.8% to 54.3%), and 22.2% (range 8.6% to 40.1%) (**Figure 2C**). Among the obese subjects, 96.3% of fetal fractions were more than 10%.

Sequencing of shorter cfDNA decreases fetal fraction differences among BMI groups

The multivariable-adjusted (model 4) mean fetal fraction differences across the categories of maternal BMI were -1.87% (95% CI: -3.75% to 0%) for a BMI of 18.5-24.9 kg/m^2, -5.03% (95% CI: -7.20% to -2.86%) for 25-29.9 kg/m^2, and -9.29% (95% CI: -13.37% to -5.20%) for BMI > 30 kg/m^2 compared with BMI < 18.5 kg/m^2 (P_{trend} < 0.0001). However, mean fetal fraction differences were -1.67% (95% CI: -3.41% to 0.07%) for 18.5-24.9 kg/m^2, -4.38% (95% CI: -6.39% to -2.37%) for 25-29.9 kg/m^2, and -7.78% (95% CI: -11.56% to -3.99%) for BMI > 30 kg/m^2 compared with BMI < 18.5 kg/m^2 (P_{trend} < 0.0001) after adjusting for confounding factors plus the average size of cfDNA (model 5, **Figure 3**), suggesting that sequencing shorter cfDNA significantly decreases mean fetal fraction differences between different BMI groups, especially in obese subjects (by ~15%).

Sequencing of shorter cfDNA reduces the screen failure rate for obese women

We sequenced shorter cfDNA of the first sample of obese women who had failed normal NIPT due to insufficient fetal fraction. The mean fetal fraction was 3.4% at first sample, increased to 4.73% at repeat sample collection (mean time interval 11 days), and reached 15.48% during NIPT with shorter cfDNA (mean size: 125 bp, **Figure 2D**). In one-third of samples, fetal fraction was still below the lower limit (4%) after repeat sample collection at later GA. The average increased time was 1.39 and 2.35 weeks at repeat sample collection and sequencing of shorter cDNA (**Figure 2E**). Fetal fraction was significantly increased by sequencing shorter cDNA, suggesting that this, rather than waiting for a later GA, is a reasonable strategy to reduce the probability of screening failure in obese subjects.

Relationship between GA and fetal fraction in sequencing short cfDNA

GA was positively correlated with fetal fraction (**Figure 4A**). The mean fetal fractions for of 12-14, 15-17, 18-20, and 21-26 weeks' gestation were 28.0% (range 11.8% to 58.0%), 29.6%

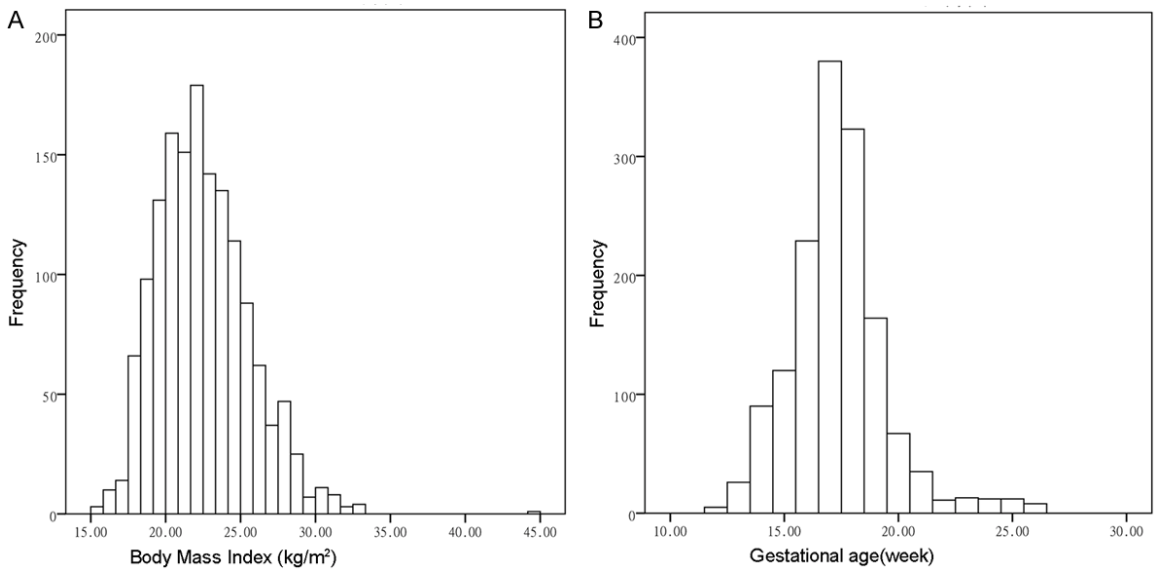

Figure 1. Distributions of body mass index (A) and gestational age across (B) all samples in this study.

(range 6.4% to 93.6%), 31.5% (range 5.8% to 71.4%) and 37.6% (range 14.0% to 79.3%) (**Figure 4C**). As shown in **Figure 4B**, pregnant women in the same GA range had lower fetal fractions with longer cfDNA fragment size. In the same cfDNA fragment size range, fetal fraction increased with older GA.

The mean fetal fraction differences were -6.07% (95% CI: -8.38% to -3.77%) for 18-20 weeks, -7.93% (95% CI: -10.20% to -5.67%) for 15-17 weeks, and -9.53% (95% CI: -12.36% to -6.70%) for 12-14 weeks compared with a GA of 21-26 weeks (P_{trend} < 0.0001) (**Figure 5**). Additional adjustments changed these effects only slightly, suggesting that the confounding factors of this study were not related to the mean fetal fraction differences between different GA groups. Notably, sequencing shorter cfDNA can yield a higher fetal fraction at earlier GAs (mean fetal fraction: 28%).

Discussion

We observed that higher BMI and earlier GA were associated with lower fetal fraction. Compared with normal NIPT (typically ~10% [7]), sequencing shorter cfDNA NIPT can yield higher fetal fractions in patients with higher BMI and earlier GA. Sequencing shorter cfDNA NIPT significantly decreases mean fetal fraction differences between different BMI groups, especially in the obese women (~15%). Sequencing shorter cfDNA is a more reliable method for improving fetal fraction than waiting for an older GA. However, the mean fetal fraction differences between different GA groups were not explained by confounding factors of this study.

The inverse association between maternal BMI and fetal fraction could be attributed to a diluting effect. It more likely that this is due to increased production of total cfDNA because decreased clearance would also likely lead to an increase in fetal cfDNA [20]. Another possible explanation for this association is that accelerated turnover of adipose cells and increased white blood cell count in obese women leads to greater release of cfDNA of maternal origin into the circulation [6, 20, 21]. Livergood, et al. reported increased odds of a screening failure among overweight and obese women compared to normal weight women with a significant upward trend from 2 to > 8-fold as BMI increases from overweight to class III obesity. The authors also reported that significant differences in NIPT failure rates between obese and normal weight women disappear at 21 weeks, when reproductive choices are more limited [8]. A recent report showed that among the 94 cases who were retested with a second blood sampling, 61% had either positive or negative results; our result (2/3) is comparable to this rate in obese pregnant women [22]. High-resolution plasma DNA size profiling revealed

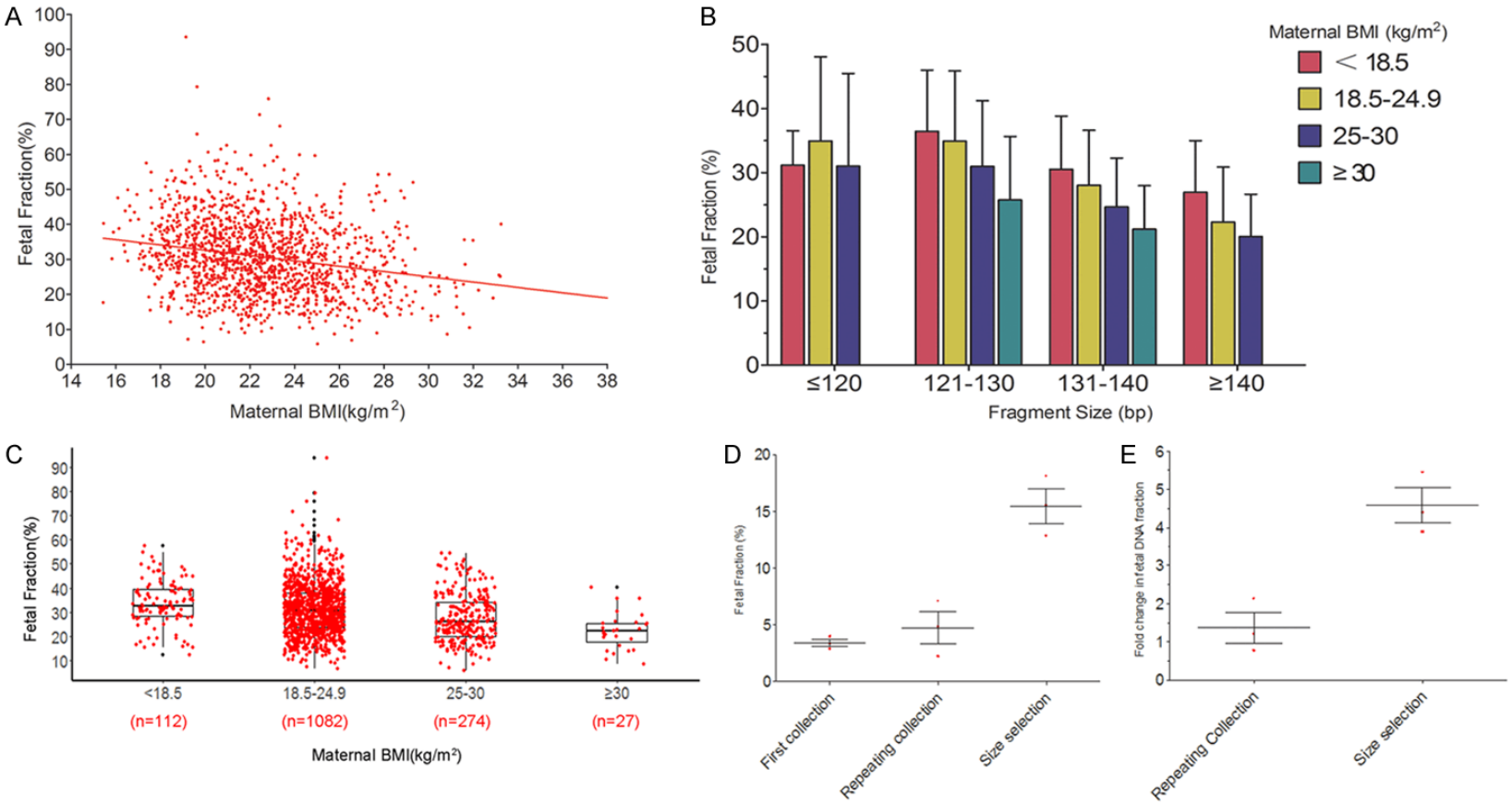

Figure 2. Relationship between fetal fraction and maternal BMI. A. BMI was negatively correlated with fetal fraction in sequencing of shorter cfDNA for NIPT. B. In pregnant women with the same body mass index range, fetal fraction decreased with longer cfDNA fragment size. In the same cfDNA fragment size range, fetal fraction decreased with maternal BMI. C. The mean fetal fractions for maternal BMI < 18.5, 18.5-24.9, 25-30, and ≥ 30 kg/m^2 were 33.7%, 31.4%, 27.5%, and 22.2%, respectively. The fetal fraction in obese pregnant women decreased significantly; however, the fetal fraction was > 10% in 96.3% of pregnant women by sequencing of short cfDNA NIPT. D. The mean fetal fraction was 3.4% at first sampling, 4.73% at repeat sample collection (mean interval 11 days), and 15.48% when sequencing of shorter cDNA NIPT (mean size of cell free DNA: 125 bp). E. The average increased times were 1.39 and 2.35 at repeat sample collection and shorter cfDNA sequencing, respectively.

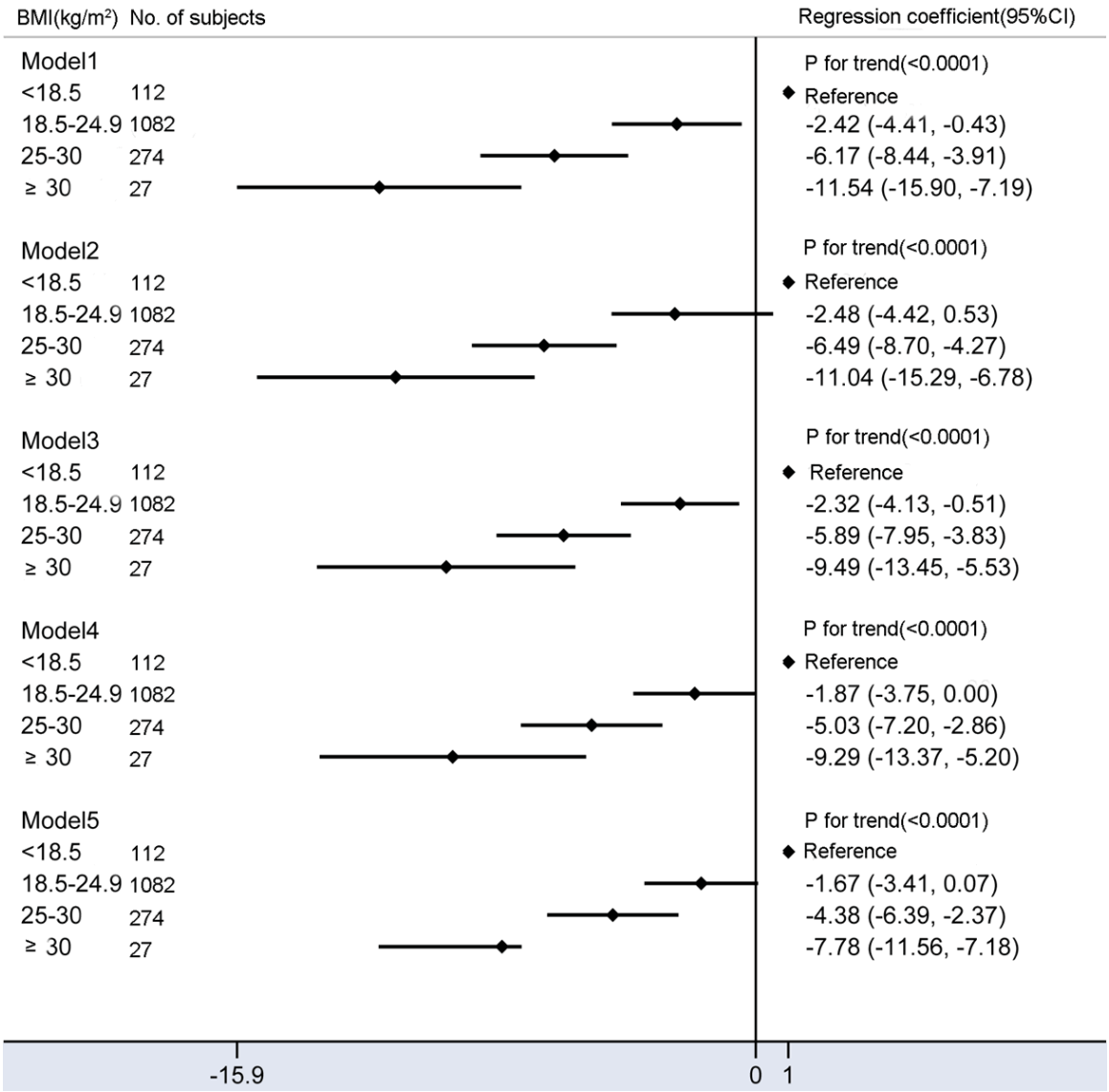

Figure 3. Mean differences in fetal fraction according to maternal BMI (kg/m^2). Model 1: Crude model. Model 2 were adjusted for GA and multiple gestations. Model 3 added average size of cell-free DNA on the basis of model 2. Model 4 added maternal age, maternal plasma cell-free DNA concentration, library concentration, and uniquely mapped reads on the basis of model 2. Model 5 added average size of cell-free DNA on the basis of model 4. Compared to models 2 and 4, models 3 and 5 included additional adjustments for confounding factors of the average size of cell-free DNA and significantly reduced fetal fraction differences between obese and underweight pregnant women.

the most striking difference between fetal and maternal cfDNA fragments is the relative reduction of the 166-bp peak and elevations of smaller peaks at sizes ≤ 143 bp for fetal cfDNA, suggesting that maternal-derived DNA is longer [16, 17, 23-25]. Hence, sequencing shorter cfDNA fragments (107-145 bp) may be a reasonable strategy to reduce the probability of low fetal fraction in obese subjects. Indeed, we found that the average fetal fraction was 22.2%, and 96.3% of fetal fractions were > 10% in obese women with an average GA of 17 weeks. Sequencing shorter cfDNA also significantly decreased mean fetal fraction differences between obese and normal weight subjects.

The positive association between maternal GA and fetal fraction in sequencing of short cfDNA NIPT is compatible with a published traditional NIPT study [13]. However, the mean fetal fraction differences between GA groups were not

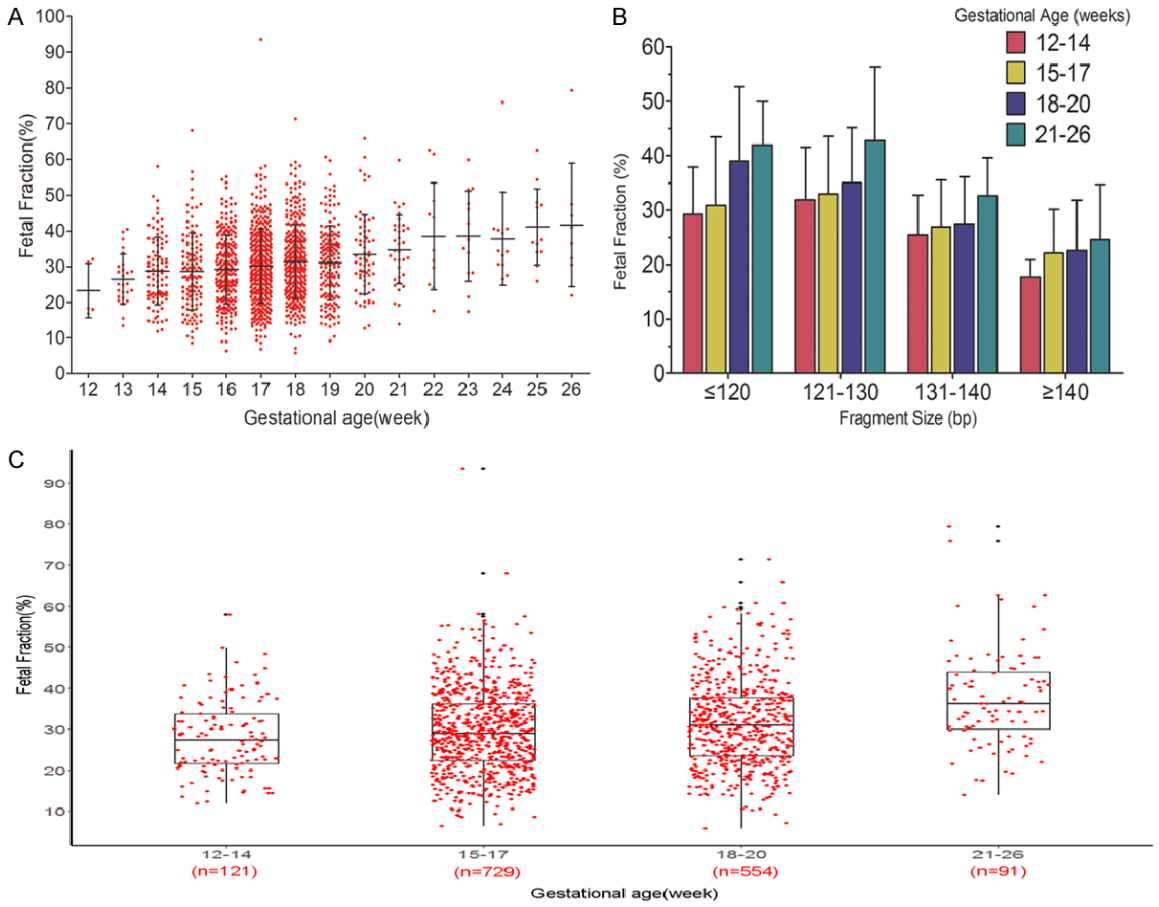

Figure 4. Relationship between fetal fraction and gestational age (week). A. Between 12 and 20 weeks' gestational age, mean fetal fraction increased slightly. Stating at 20 weeks, there was a greater weekly increase for fetal percent. B. In pregnant women with the same gestational age range, fetal fraction decreased with longer cfDNA fragment size. In the same cfDNA fragment size range, fetal fraction increased with gestational age. C. The mean fetal fractions of 12-14, 15-17, 18-20, and 21-26 weeks' gestation were 28.0%, 29.6%, 31.5%, and 37.6%. Compared with normal NIPT (typically ~10%), sequencing short cfDNA NIPT significantly increased fetal fraction at 12-14 weeks.

explained by confounding factors of this study. cfDNA in the peripheral blood sample of a pregnant woman derives from three tissues: placenta, maternal, and fetus [26]. Apoptotic placenta cells (trophoblasts) are a major source of fetal fragments in maternal plasma [27], and the number of apoptotic cells would be proportional to the placental mass. Hence, GA-related differences may be due to placental mass rather than maternal cell apoptosis or confounding factors of this study. However, we also noticed that the mean fetal fraction of slightly early GA (12-14 weeks) was much higher than that in normal NIPT (28.0% vs. 10%).

In summary, this is the first study to explore the relationship between maternal GA or BMI and fetal fraction adjusted for a considerable number of potential confounders in sequencing of shorter cfDNA NIPT. Sequencing shorter cfDNA fragments offers a solution to low fetal fraction due to maternal with higher BMIs and earlier GAs. However, the insufficient sample sizes of size-selection NIPT for obese subjects and earlier gestational ages means that further studies are required to validate these findings.

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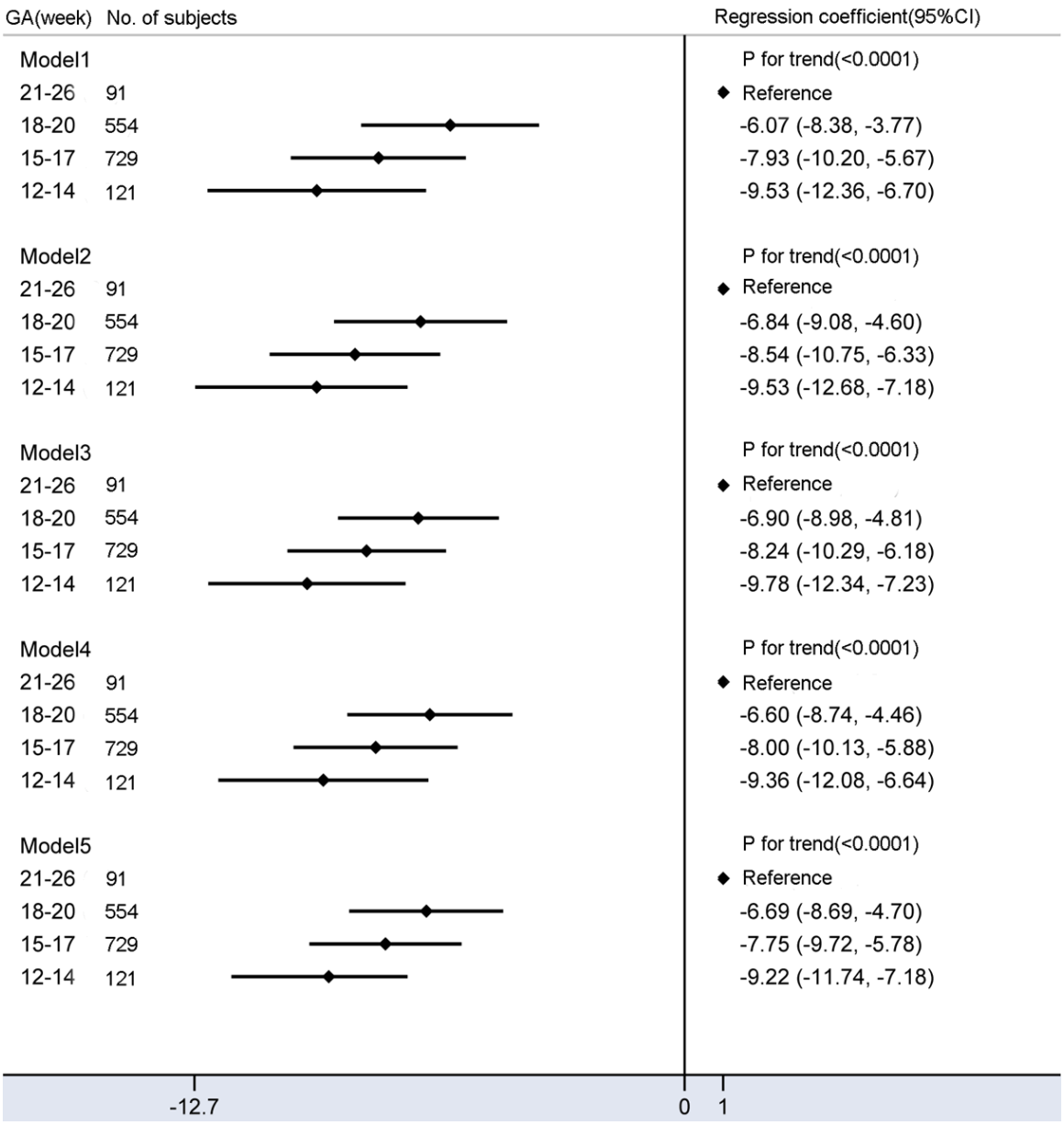

Figure 5. Mean differences in fetal fraction according to gestational age (week) category. Model 1 is a univariate linear regression of relationship between GA and fetal fraction. Model 2 was adjusted for BMI and multiple gestations. Model 3 added average size of cell-free DNA on the basis of model 2. Model 4 added maternal age, maternal plasma cell-free DNA concentration, library concentration, and uniquely mapped reads on the basis of model 2. Model 5 added average size of cell-free DNA on the basis of model 4. Compared with model 1, additional adjustment for confounding factors slightly reduced fetal fraction differences among different groups.

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Disclosure of conflict of interest

None.

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