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High-Resolution Profiling of Fetal DNA Clearance from Maternal Plasma by Massively Parallel Sequencing

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BACKGROUND: With the advent of massively parallel sequencing (MPS), DNA analysis can now be performed in a genomewide manner. Recent studies have demonstrated the high precision of MPS for quantifying fetal DNA in maternal plasma. In addition, paired-end sequencing can be used to determine the size of each sequenced DNA fragment. We applied MPS in a high-resolution investigation of the clearance profile of circulating fetal DNA.

METHODS: Using paired-end MPS, we analyzed serial samples of maternal plasma collected from 13 women after cesarean delivery. We also studied the transrenal excretion of circulating fetal DNA in 3 of these individuals by analyzing serial urine samples collected after delivery.

RESULTS: The clearance of circulating fetal DNA occurred in 2 phases, with different kinetics. The initial rapid phase had a mean half-life of approximately 1 h, whereas the subsequent slow phase had a mean half-life of approximately 13 h. The final disappearance of circulating fetal DNA occurred at about 1 to 2 days postpartum. Although transrenal excretion was involved in the clearance of circulating fetal DNA, it was not the major route. Furthermore, we observed significant changes in the size profiles of circulating maternal DNA after delivery, but we did not observe such changes in circulating fetal DNA.

CONCLUSIONS: MPS of maternal plasma and urinary DNA permits high-resolution study of the clearance profile of circulating fetal DNA.

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The discovery of cell-free fetal DNA in maternal plasma has offered new opportunities for noninvasive prenatal diagnosis *(1)*. The use of cell-free fetal DNA for fetal rhesus D genotyping *(2–4)* and fetal aneuploidy detection *(5–8)* has been introduced into clinical service. Despite the rapid developments in clinical applications, much about the biology of circulating fetal DNA remains to be studied.

In 1999, Lo et al. used real-time PCR targeting the *SRY* (sex determining region Y) gene to analyze serial postdelivery samples of plasma collected from women carrying male fetuses and found that fetal DNA was cleared from plasma with a mean half-life of approximately 16 min *(9)*. Smid et al. reported that 47 of 105 women had very low fetal DNA concentrations detectable within 2 days after delivery *(10)*. Today, rapid clearance of fetal DNA from maternal plasma is a consensus in the field *(10–12)*.

Previous publications speculated that nucleases in the plasma, liver *(9)*, and kidneys *(13–15)* might be involved in the clearance of fetal DNA. In particular, there has been controversy regarding transrenal excretion of circulating fetal DNA *(16–18)*. Very recently, Tsui et al. showed that fetal DNA could be detected in maternal urine with massively parallel sequencing (MPS)[4] but that it became undetectable by 24 h after delivery *(19)*.

Certain pathologic conditions associated with damage to and dysfunction of the liver and kidneys, such as preeclampsia, have been associated with impaired clearance of fetal DNA *(20)*. Prolonged postpartum persistence of fetal DNA has been reported in a pregnant woman with acute fatty liver disease *(21)*. These studies shed light on the possible mechanisms for the removal of fetal DNA from maternal plasma. Investigations into the clearance of plasma DNA have improved our understanding of the biology of plasma DNA and the pathology of a number of conditions. Furthermore, the study of plasma DNA clearance has potential clinical utility. For example, researchers have studied the clearance kinetics of tumor-derived Ep-

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[4] Nonstandard abbreviations: MPS, massively parallel sequencing; SNP, single-nucleotide polymorphism; LOD, limit of detection.

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stein–Barr virus DNA in the plasma of nasopharyngeal carcinoma patients after surgical resection *(22)* and radiotherapy *(23)*. For radiotherapy in particular, the clearance of Epstein–Barr virus DNA from the plasma is a strong prognosticator *(24)*.

With MPS, we can precisely quantify fetal DNA in maternal plasma *(25)*. Through paired-end sequencing, size profiles for fetal and maternal DNA can be obtained *(26)*. We therefore adopted paired-end MPS in this high-resolution study of the clearance profile of circulating fetal DNA after delivery. We hypothesized that if plasma nucleases play a major role in the clearance of fetal DNA, the fetal DNA remaining in maternal plasma after delivery would become progressively shorter. On the other hand, if renal clearance represents a major clearance route, we would expect fetal DNA molecules smaller than the glomerular filtration barrier to disappear first *(27–29)*. We also explored the contribution of transrenal excretion on the clearance of circulating fetal DNA.

Materials and Methods

Details of sample processing, DNA extraction, single-nucleotide polymorphisms (SNPs) genotyping, and real-time PCR are provided in the Supplemental Methods (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue8).

STUDY PARTICIPANTS AND SAMPLE COLLECTION

The study was approved by the institutional ethics committee. We recruited 13 women with uncomplicated singleton pregnancies that were delivered by elective cesarean section at the Prince of Wales Hospital, Hong Kong. Informed written consent was obtained from all patients. The indications for cesarean section were obstetrics related, such as previous cesarean section(s), breech presentation, and oligohydraminos, rather than due to maternal diseases. Women with clinically important concurrent diseases were excluded from the study. Ten milliliters of peripheral blood was collected from each participant into EDTA-containing tubes upon her admission to the labor ward. Each individual consented to one of the following postdelivery protocols, in which 5 mL of blood was collected into EDTA-containing tubes: protocol 1 (cases 1–5: 3 min, 10 min, 15 min, 30 min, 1 h, 2 h, and 24 h postpartum), protocol 2 (cases 6–8: 40 min, 70 min, 2 h, 6 h, 12 h, and 18 h postpartum), and protocol 3 (cases 9–13: 24 h, 48 h, 72 h, and, for 1 case, 96 h postpartum).

To reduce the degree of discomfort for each participant, we used this study design to keep both the number of blood samplings and the blood volume collected to a minimum. For the participants who consented to protocol 2, we also collected timed urine samples from the catheter bag and recorded the total volume of urine produced within each time interval. For all cases, we collected cord blood samples and recorded the birth weight of each baby.

DNA SEQUENCING

We prepared sequencing libraries of plasma and urinary DNA with the Paired-End Sequencing Sample Preparation Kit (Illumina) *(30)*. For library preparation, we used 10–20 ng of extracted plasma DNA and 10–100 ng of extracted urine DNA. The adaptor-ligated plasma DNA was enriched with a 10-cycle PCR, and the adaptor-ligated urinary DNA was enriched with a 15-cycle PCR. Each library was paired-end sequenced in 1 lane of a flow cell on a HiSeq 2000 sequencer (Illumina) (50 bp × 2).

SEQUENCE ALIGNMENT

We trimmed any adaptor sequences from the sequencing reads *(19)* before alignment to the nonrepeat masked human reference genome (NCBI Build 36.1/hg18) (http://genome.ucsc.edu). The Short Oligonucleotide Alignment Program 2 (SOAP2) (http://soap.genomics.org.cn/) was used. For SNP calling, we allowed up to 2 nucleotide mismatches for each member of the paired-end reads. We included only paired-end reads with both ends aligned to the same chromosome in the correct orientation. The size of each fragment was determined from the start and end coordinates of the mapped paired-end reads, and we included only fragments from 20 bp to 600 bp for subsequent analysis. We filtered all but 1 duplicated read with identical start and end coordinates.

DETECTION OF FETAL DNA IN MATERNAL PLASMA AND URINE

We calculated the fractional fetal DNA concentration (fetal percentage) of the maternal plasma and urine samples by first identifying from the genotyping data all SNP loci that were homozygous (i.e., AA) in the mother and heterozygous (i.e., AB) in the fetus. We then used the sequencing data to determine the number of fetal-specific allele (B allele) counts and the number of shared allele (A allele) counts. We calculated the fetal DNA percentage according to the following equation *(26)*:

$$\text{Fetal DNA percentage} = \frac{\text{Fetal-specific allele count} \times 2}{\text{Fetal-specific allele count} + \text{Shared allele count}} \times 100\%.$$

We considered fetal DNA to be present in the maternal plasma or urine if the fetal DNA percentage was above the limit of detection (LOD). We evaluated the perfor-

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mance of genotyping with the BeadChip array (Illumina) by comparing the genotype calls made with the array and with MPS. Genotyping results from the 2 platforms showed 99.1% concordance (data not shown). Therefore, the LOD for measuring fetal DNA would be mainly affected by sequencing and alignment errors.

To determine the LOD for measuring fetal DNA with MPS, we analyzed SNPs in which the mother and the fetus were both homozygous for the same allele (i.e., AA). We used the sequencing data to determine the number of true allele (A) counts and the number of unexpected allele (non-A) counts. We then calculated the sequencing error with the following equation:

$$\text{Sequencing error (\%)} = \frac{\text{Unexpected allele count}}{\text{True allele count} + \text{Unexpected allele count}} \times 100\%.$$

We determined the mean and SD of the sequencing error for all samples and calculated the LOD for fetal DNA with the following equation:

$$\text{LOD} = [\text{Mean sequencing error} + (3 \times \text{SD}_{\text{sequencing error}})] \times 2.$$

Because most of the urinary DNA molecules were <100 bp, the number of nucleotides that could be used for alignment was reduced, which would affect the alignment accuracy for urinary DNA. Hence, different LODs were used for plasma and urinary fetal DNA.

CALCULATION OF THE ABSOLUTE FETAL DNA CONCENTRATION

We determined the absolute fetal DNA concentration according to the following equation:

$$\text{Absolute fetal DNA concentration} = \text{Plasma DNA concentration} \times \text{Fetal percentage},$$

with DNA concentration expressed in genome equivalents per microliter.

The plasma DNA concentrations for cases 1–5 and cases 6–8 were determined with the real-time assays for the β-globin- and leptin-encoding genes, respectively.

Results

SEQUENCING PERFORMANCE

On average, 165×10^6 and 150×10^6 raw paired-end reads were obtained for sequencing of each maternal plasma sample and each maternal urine sample, respectively, with 1 lane of a flow cell on a HiSeq 2000 sequencer (see Tables 1 and 2 in the online Data Supplement). We uniquely mapped 80.6% and 73.0% of the raw paired-end reads to the reference genome for the plasma and urine samples, respectively (see Tables 1 and 2 in the online Data Supplement). Duplicated reads accounted for 5.9% and 4.2% of the aligned reads for plasma and urine samples, respectively (see Tables 1 and 2 in the online Data Supplement).

DETECTION OF FETAL DNA IN POSTDELIVERY MATERNAL PLASMA

On average, we identified 198 236 informative SNPs from the genotyping data in each case for the detection of fetal DNA (see Table 3 in the online Data Supplement). We calculated the fractional fetal DNA concentrations for the maternal plasma samples with these informative SNPs, as previously described (see Table 3 in the online Data Supplement and Materials and Methods).

In total, we analyzed 82 maternal plasma samples from 13 women. We obtained a mean (SD) sequencing error of 0.41% (0.04%) (see Table 4 in the online Data Supplement). Thus, the LOD for measuring plasma fetal DNA was 1.06%. Fetal DNA was considered present in the maternal plasma sample if the fetal DNA percentage was >1.06%.

Even with the clearance of fetal DNA postpartum, we were able to detect fetal DNA with MPS in all samples collected at 2 h after delivery (Table 1). Six of the 10 plasma samples collected at 24 h after delivery had detectable fetal DNA (Table 1). One of the 5 patients who had plasma samples collected daily after delivery had a detectable fetal DNA concentration up to 2 days postpartum (Table 1). Therefore, circulating fetal DNA in normal pregnancies disappeared by 1 to 2 days postpartum.

CLEARANCE KINETICS OF CIRCULATING FETAL DNA

Because we observed fluctuations in the total concentration of plasma DNA after delivery (see Fig. 1 in the online Data Supplement), we investigated the clearance kinetics of circulating fetal DNA by determining the absolute fetal DNA concentration of each maternal plasma sample from the plasma DNA concentrations measured by quantitative PCR and the fetal percentages measured by MPS. The clearance profiles for the absolute fetal DNA concentrations were similar for cases 1–8 (Fig. 1). In addition, in cases 1–5 we observed an increase in the absolute concentration of fetal DNA immediately after delivery, i.e., at 3 min (Fig. 1A). This finding is consistent with observations in previous studies *(9, 20)* that were suggested to be due to delivery-associated traumatic procedures.

Plotting the natural logarithm of the absolute fetal DNA concentration against time demonstrated that cases 6–8 had a 2-phase clearance pattern—a rapid initial phase followed by a slow phase (Fig. 2). For cases 1–8, we also determined the slopes of the plots and

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Table 1. Fractional fetal DNA concentrations in predelivery and serial postdelivery maternal plasma.

Sample collection time	Fractional fetal DNA concentration, %[a]												
	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11	Case 12	Case 13
Before delivery	26.4	17.7	52.4	26.5	18.3	31.1	29.9	24.6	25.1	35.6	42.2	15.3	24.6
After delivery													
3 min	26.3	17.2	50.6	24.8	21.6	—	—	—	—	—	—	—	—
10 min	22.4	14.0	43.5	19.2	17.4	—	—	—	—	—	—	—	—
15 min	19.1	11.9	37.6	16.3	14.1	—	—	—	—	—	—	—	—
30 min	15.6	7.4	26.9	10.3	8.9	—	—	—	—	—	—	—	—
40 min	—	—	—	—	—	17.5	8.9	12.0	—	—	—	—	—
60 min	9.0	4.4	16.1	5.5	4.0	—	—	—	—	—	—	—	—
70 min	—	—	—	—	—	12.8	4.9	7.0	—	—	—	—	—
2 h	6.6	3.5	7.5	4.0	2.1	7.1	2.4	4.2	—	—	—	—	—
6 h	—	—	—	—	—	2.0	2.6	1.6	—	—	—	—	—
12 h	—	—	—	—	—	1.6	2.6	1.3	—	—	—	—	—
18 h	—	—	—	—	—	1.3	2.2	<LOD	—	—	—	—	—
24 h	3.0	2.1	3.1	<LOD[b]	<LOD	—	—	—	<LOD	1.3	1.3	1.3	<LOD
48 h	—	—	—	—	—	—	—	—	<LOD	<LOD	<LOD	1.2	<LOD
72 h	—	—	—	—	—	—	—	—	<LOD	<LOD	<LOD	<LOD	<LOD
96 h	—	—	—	—	—	—	—	—	—	<LOD	—	—	—

[a] Fetal DNA percentage $= \frac{\text{Fetal-specific article count} \times 2}{\text{Fetal-specific allele count} + \text{Shared allele count}} \times 100\%$.

[b] Samples with a fetal percentage below the LOD are marked as <LOD.

calculated the half-lives with the following equation (see Table 5 in the online Data Supplement):

$$\text{Half-life} = -\frac{\ln(2)}{\text{Slope}}.$$

The mean half-lives of the rapid phase (0–2 h after delivery) and the slow phase (6–18 h after delivery) were approximately 1 h and 13 h, respectively (see Table 5 in the online Data Supplement).

CORRELATION OF BIRTH WEIGHT WITH ABSOLUTE AND FRACTIONAL FETAL DNA CONCENTRATIONS

We explored whether the birth weight of the baby was correlated with the absolute and fractional fetal DNA concentrations in the predelivery samples of maternal plasma. No significant Spearman correlations were found [absolute fetal DNA concentration, $r = -0.226$ ($P = 0.459$); fractional fetal DNA concentration, $r = -0.359$ ($P = 0.229$)]. See Fig. 2 in the online Data Supplement.

TRANSRENAL CLEARANCE OF CIRCULATING FETAL DNA

To study the contribution of transrenal excretion on the clearance of circulating fetal DNA, we collected serial samples of urine and plasma from 3 study participants. We analyzed a total of 21 urine samples from the 3 women. We calculated the fractional fetal DNA concentrations in the urine samples by using the informative SNPs identified from the genotyping data (see Table 6 in the online Data Supplement). We obtained a mean (SD) sequencing error of 0.40% (0.16%) (see Table 4 in the online Data Supplement). Thus, the LOD for measuring urinary fetal DNA was 1.76%.

For each of the studied time intervals, we measured the absolute fetal DNA concentrations in the samples of maternal plasma collected at the beginning (T_1) and at the end (T_2) of the time period (see Tables 7 and 8 in the online Data Supplement). Assuming a plasma volume of 3850 mL *(31)*, we estimated the amount of fetal DNA present in the maternal circulation at T_1 and T_2, and we calculated the amount of fetal DNA cleared from the maternal circulation during this period by subtracting the amount of fetal DNA at T_2 from that at T_1 (see Tables 7 and 8 in the online Data Supplement).

To determine the amount of fetal DNA excreted in the urine during each time interval, we measured the total volume of urine produced and determined the concentration of leptin-encoding urinary DNA with real-time PCR (see Table 9 in the online Data Supple-

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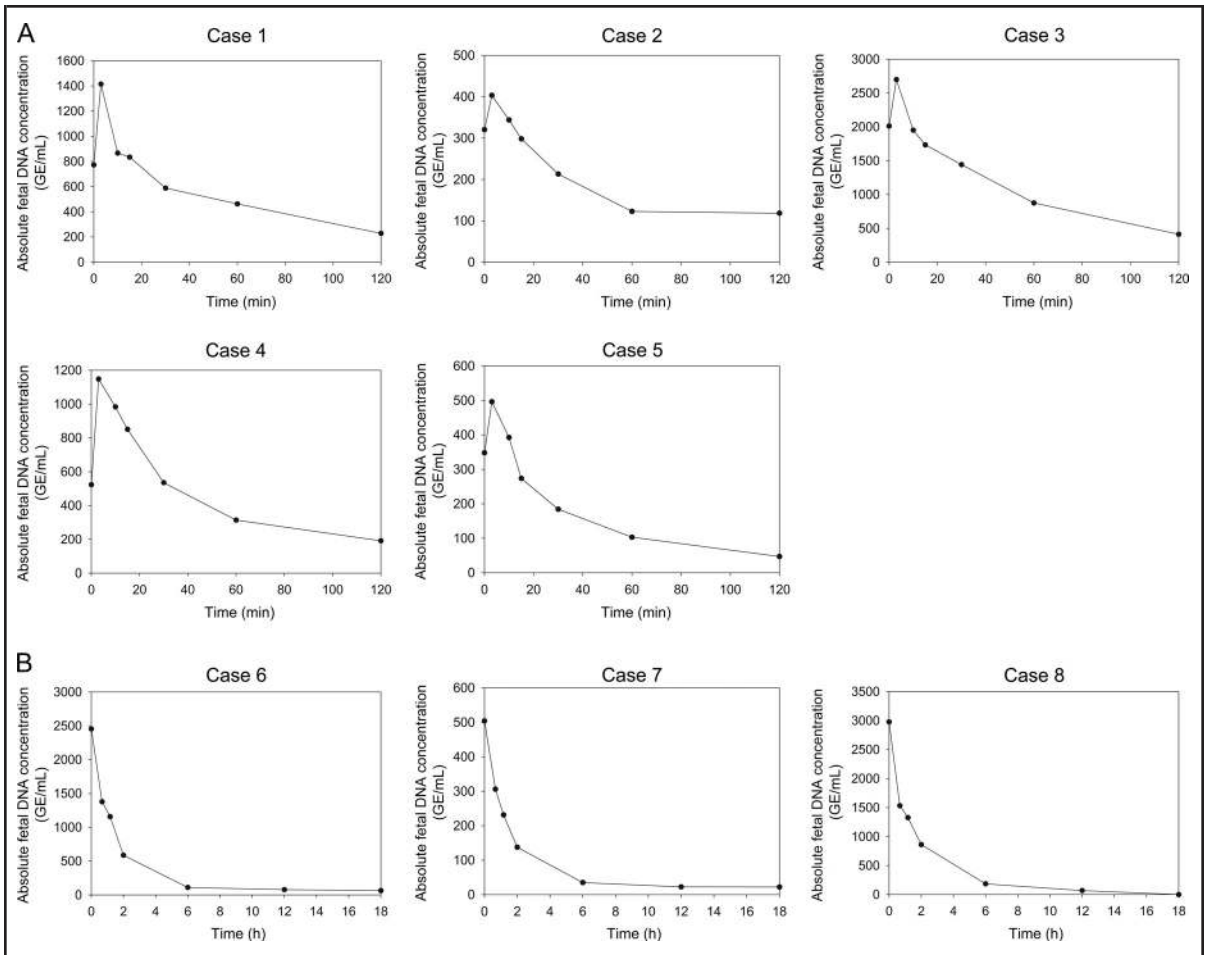

Fig. 1. Dynamics of fetal DNA clearance.

(A), Cases 1–5 (within 2 h after delivery). (B), Cases 6–8 (within 18 h after delivery). Time is plotted on the *x* axis, and the absolute fetal DNA concentration in genome equivalents (GE) per milliliter is plotted on the *y* axis. Value at time 0 indicates that for the predelivery sample.

ment). The median sizes for plasma DNA and urinary DNA were 165–175 bp and 39–178 bp, respectively (see Table 10 in the online Data Supplement). Unlike plasma DNA molecules, >99% of which had sizes equal to or greater than the amplicon size of the leptin DNA assay (data not shown), a substantial proportion of urinary DNA molecules were shorter than 62 bp (see Table 9 in the online Data Supplement). Therefore, we quantitatively corrected for the total amount of urinary DNA on the basis of the proportion of urinary DNA with sizes ≥62 bp (see Table 9 in the online Data Supplement). After the correction, we used the fetal DNA percentage determined by MPS to calculate the amount of urinary fetal DNA produced in each time interval (see Table 9 in the online Data Supplement).

With knowledge of the amount of fetal DNA cleared from the maternal circulation and the amount of fetal DNA produced in the urine during each time interval, we estimated the contribution of transrenal excretion to the clearance of circulating fetal DNA with the following equation:

$$\text{Percentage cleared transrenally} = \frac{\text{Amount of fetal DNA produced in urine}}{\text{Amount of fetal DNA cleared from plasma}} \times 100\%.$$

Hence, 0.2%–19.0% of plasma fetal DNA was cleared through the transrenal route during the studied time intervals (Table 2).

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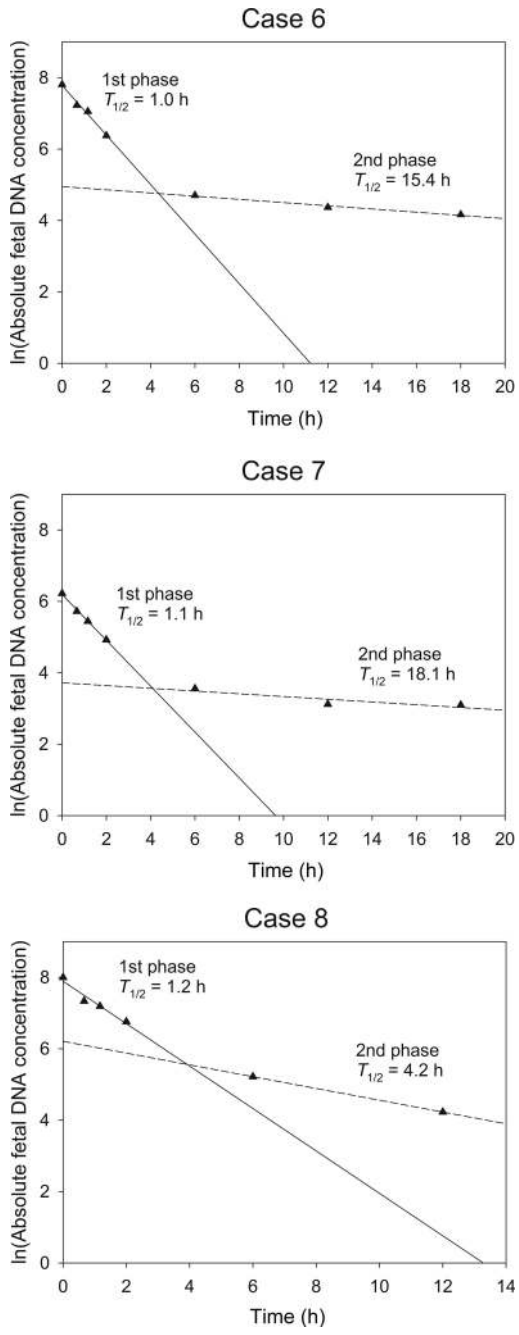

Fig. 2. Plots of the natural logarithm of the absolute fetal DNA concentration against time for cases 6–8.

Time after delivery is plotted on the *x* axis, and the natural logarithm of the absolute fetal DNA concentration is plotted on the *y* axis. Linear regression lines for the rapid phase and the slow phase are represented by solid and dashed lines, respectively. The corresponding half-lives ($T_{1/2}$) for the 2 phases are shown.

Table 2. Contribution of transrenal excretion to clearance of circulating fetal DNA.

Time after delivery	Fetal DNA amount, GE[a] Cleared from plasma[b]	Produced in urine[c]	Fetal DNA cleared transrenally, %[d]
Case 6			
0–40 min	4 142 353	204 768	4.9
40–70 min	849 514	54 185	6.4
70 to 2 h	2 195 937	96 340	4.4
2–6 h	1 834 485	348 566	19.0
Case 7			
0–40 min	762 591	1729	0.2
40–70 min	287 688	517	0.2
70 min to 2 h	360 598	1265	0.4
Case 8			
0–40 min	5 562 770	428 073	7.7
70 min to 2 h	1 791 338	6845	0.4

[a] GE, genome equivalents.
[b] Amount of fetal DNA cleared from maternal circulation during the studied time interval (see Tables 7 and 8 in the online Data Supplement for the calculation).
[c] Amount of fetal DNA in the urine produced during the studied time interval (see Table 9 in the online Data Supplement for the calculation).
[d] The percentage of circulating fetal DNA cleared through the transrenal route during the studied time interval is calculated as follows:

$$\text{Percentage cleared transrenally} = \frac{\text{Amount of fetal DNA produced in urine}}{\text{Amount of fetal DNA cleared from plasma}} \times 100\%.$$

CHANGES IN THE SIZE PROFILES OF PLASMA DNA AFTER DELIVERY

We constructed size profiles for fetal DNA and maternal DNA by using paired-end reads containing fetal-specific and maternal-specific alleles, respectively. We used SNP loci that were heterozygous (AB) in the mother but homozygous (AA) in the fetus to identify maternal DNA fragments. In general, the size distributions of both fetal DNA (Fig. 3; see Fig. 3 in the online Data Supplement) and maternal DNA (Fig. 3; see Fig. 4 in the online Data Supplement) in all the predelivery and postdelivery plasma samples resembled those of a first-trimester case reported previously *(26)*. All showed a predominant peak at 166 bp and a 10-bp periodicity below 150 bp.

Next, we calculated the ratio of the short DNA fragments to the long DNA fragments with the following equation:

$$\text{Ratio of short to long DNA fragments} = \frac{\text{CF (size} \leq 150\text{ bp)}}{\text{CF (size 163} - \text{159 bp)}},$$

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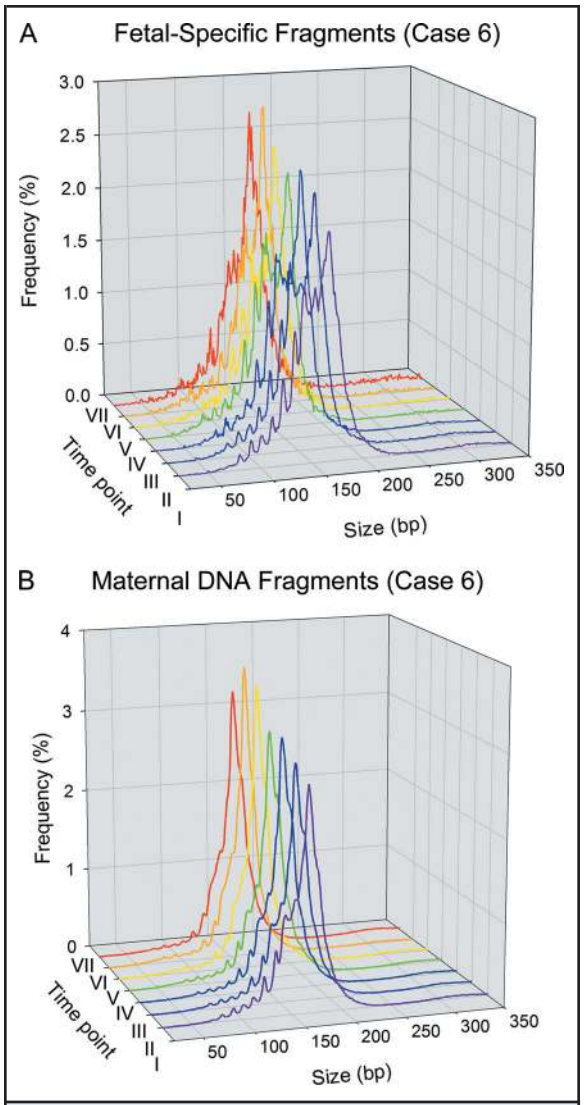

Fig. 3. Three-dimensional plots for the changes in the size distributions of fetal DNA (A) and maternal DNA (B) after delivery for case 6.

Time point I represents the predelivery sample. Time points II–VII represent postdelivery samples collected at 40 min, 70 min, 2 h, 6 h, 12 h, and 18 h after delivery. Size profiles for different time points are represented by lines of different colors.

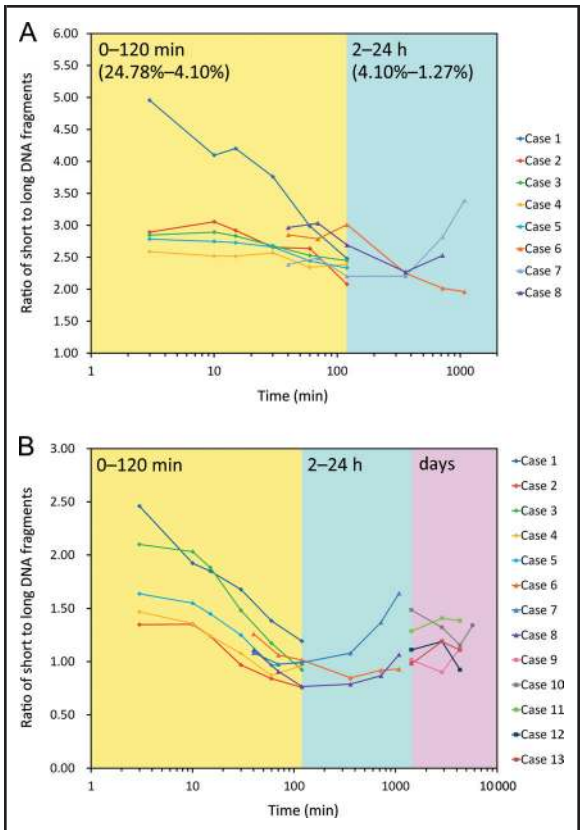

Fig. 4. Changes in the ratio of short to long plasma DNA fragments after delivery for fetal-specific DNA (from 3 min up to 18 h postpartum) (A) and maternal-specific DNA (from 3 min up to 4 days postpartum) (B).

Time after delivery is plotted on the *x* axis on a logarithmic scale. Each case is represented by a line and symbol of unique color, and cases from the same time series are represented by the same symbol (i.e., ◆, ▲, or ■). The median fractional fetal DNA concentrations of the first and last time points of each time period are indicated in parentheses.

where CF (size ≤150 bp) is the cumulative frequency of fragments with sizes ≤150 bp and CF (size 163–169 bp) is the cumulative frequency of fragments with sizes of 163–169 bp. With the exception of 2 cases, no substantial change was apparent in the ratio of short to long fetal DNA fragments after delivery (Fig. 4A). For maternal DNA, the ratio of short to long DNA fragments seemed to decrease in the first 2 h after delivery (Fig. 4B).

We compared the ratio of short to long DNA fragments at 3 min and 120 min postpartum for both fetal DNA and maternal DNA for cases 1–5 (see Tables 11 and 12 in the online Data Supplement). For fetal DNA, we found no significant changes between the 2 time points in the ratio of short to long fragments ($P = 0.104$, paired *t*-test). Maternal DNA had significantly more long DNA fragments at 120 min postpartum ($P = 0.006$, paired *t*-test).

In summary, we observed a significant downward trend for maternal DNA in the ratio of short DNA fragments to long DNA fragments within 2 h after delivery. In other words, the size profile of maternal DNA

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shifted to longer DNA fragments after delivery. We observed no such changes in circulating fetal DNA.

Discussion

This work represents the first attempt to use MPS of maternal plasma and urine for studying the clearance profile of circulating fetal DNA. In contrast to previous studies that used locus-specific quantitative PCR *(9–12)*, in this study we used paired-end MPS to explore this phenomenon with higher analytical sensitivity and precision and in a genomewide fashion. This approach also allowed us to concurrently study the size profile in plasma.

In the present study, 6 of the 10 samples of maternal plasma collected at 24 h after delivery had detectable fetal DNA. Of the 5 cases with daily collection of plasma samples after delivery, only one had a detectable fetal DNA concentration by 2 days postpartum. Smid et al. previously reported that 47 (45%) of 105 women had very low concentrations of detectable fetal DNA within 2 days after delivery *(10)*. Taking the results together, we postulate that the final disappearance of circulating fetal DNA in normal pregnancies is typically around 1–2 days postpartum.

Seven of the 8 women in our previous study had undetectable fetal DNA by 2 h postpartum *(9)*. In the present study using MPS, we detected fetal DNA in all samples collected at 2 h after delivery. Furthermore, the half-life of fetal DNA clearance in the previous study was 16 min *(9)*, whereas the present study revealed a half-life of 1 h for the rapid phase. The apparent discrepancies between the current results and our previous data can be explained from a number of angles. First, the 1999 study used only a single fetal target gene (i.e., the *SRY* gene) for quantitative PCR analysis *(9)*. In contrast, the current study used genomewide MPS in analyzing a mean of 198 000 fetal targets (i.e., informative SNPs) across the entire genome. Second, detection of fetal DNA with quantitative PCR requires the sizes of fetal DNA molecules to be at least equal to the amplicon size for the assay, which was 102 bp in the original study *(9)*. In comparison, using MPS allowed us to detect fetal DNA fragments with sizes as small as 22 bp. Third, the previous study *(9)* used 200 μL of maternal plasma for DNA extraction, whereas we extracted DNA from 1.2–5.6 mL of maternal plasma in the present study. Therefore, the discrepancies between the 2 reports can be explained by the increased sensitivity of MPS to detect fetal DNA and the larger volume of plasma used for analysis.

Lo et al. analyzed serial samples of maternal plasma collected at multiple time points within 2 h postpartum *(9)*. The present study extended the serial blood sampling to 18 h postpartum in 3 cases. Results from these 3 cases suggest that circulating fetal DNA is cleared in 2 phases. The clearance half-lives associated with the 2 phases are 1 h and 13 h. Given that the clearance half-life of fetal DNA in the first 2 h after delivery was 1 h and assuming the maternal contribution was constant, we expect that $<0.1\%$ $(1/2^{10})$ of fetal DNA present at the time of delivery would remain in the maternal plasma by 10 h after delivery. Hence, our finding of the presence of detectable concentrations of fetal DNA in 6 of the 10 samples of maternal plasma collected at 24 h after delivery provides further evidence for a biphasic clearance of circulating fetal DNA. This biphasic clearance pattern of circulating fetal DNA may have implications for the underlying mechanisms of DNA removal. For example, we speculate that relatively high concentrations of cell-free fetal DNA and fetal nucleated cells are present in the maternal blood toward the end of pregnancy. The first phase of removal could represent the rapid removal of cell-free fetal DNA from maternal plasma. The second phase may be related to the destruction of fetal nucleated cells by the maternal immune and/or reticuloendothelial systems, causing liberation of cell-free fetal DNA into the maternal plasma. Future studies that explore this biphasic clearance pattern would be worthwhile.

Through analyzing maternal plasma and urine samples collected serially after delivery, we have found that 0.2%–19.0% of the fetal DNA in plasma is cleared through the transrenal route. Previous studies of clearance with animals produced results consistent with these findings. Chused et al. intravenously injected 2 μg of radioactively labeled human cell line DNA into mice *(32)*. These investigators detected 10% of the injected radioactivity in the collected urine. Our data are thus consistent with those of Chused et al. and suggest that transrenal excretion is a minor route for the clearance of circulating fetal DNA. Furthermore, the proportion of plasma fetal DNA cleared through the transrenal route seems to be quite variable.

In this study, we monitored serial changes in the size distributions of both fetal and maternal DNA in maternal plasma after delivery. Interestingly, we found that no substantial changes in the size distributions of fetal DNA occurred after delivery. Our size data suggest that both plasma nucleases and the kidneys might not be the major mechanisms for the clearance of fetal DNA. This supposition implies that other mechanisms, in particular organ systems that do not show a size preference for the removal of fetal DNA, might be more important for fetal DNA clearance.

The significant downward trend in the ratio of short to long DNA fragments observed in this study implies an increase in the proportion of long maternal DNA fragments within 2 h after delivery. We believe

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that plasma DNA is released after cell death. Longer DNA fragments may represent the newly released DNA fragments from apoptotic and/or necrotic maternal cells, e.g., cells from the placentation site of the uterus.

Preeclampsia is reportedly associated with increased fetal DNA concentrations in maternal plasma *(33)*. The increased fetal DNA concentrations associated with this condition have been suggested to be due to both increased release from apoptotic cells *(34)* and impaired clearance *(20)*. Therefore, the use sequencing-based methods in future studies of the clearance of fetal DNA in preeclamptic pregnancies would also be worthwhile.

In summary, we have demonstrated that MPS is a powerful tool for studying the clearance kinetics of circulating fetal DNA. This work has implications for various physiological and pathologic conditions in which nonhost DNA can be found, e.g., pregnancy, cancer *(35, 36)*, and transplantation *(30, 37)*.

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