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fast track

# Noninvasive Prenatal Methylomic Analysis by Genomewide Bisulfite Sequencing of Maternal Plasma DNA

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**BACKGROUND:** Epigenetic mechanisms play an important role in prenatal development, but fetal tissues are not readily accessible. Fetal DNA molecules are present in maternal plasma and can be analyzed noninvasively.

**METHODS:** We applied genomewide bisulfite sequencing via 2 approaches to analyze the methylation profile of maternal plasma DNA at single-nucleotide resolution. The first approach used maternal blood samples and polymorphic differences between the mother and fetus to analyze the fetal methylome across the genome. The second approach used the methylation profile of maternal blood cells and the fractional fetal DNA concentration in maternal plasma to deduce the placental methylomic profile from maternal plasma DNA-sequencing data.

**RESULTS:** Because of the noninvasive nature of these approaches, we were able to serially assess the methylation profiles of fetal, placental, and maternal plasma with maternal blood samples collected in the first and third trimesters and after delivery. Gestation-related changes were observed. The fetal methylation profile deduced from maternal plasma data resembled that of the placental methylome, both on a genomewide level and per CpG site. Imprinted genes and differentially methylated regions were identified from the maternal plasma data. We demonstrated one potential clinical application of maternal plasma bisulfite sequencing with the successful detection of fetal trisomy 21.

**CONCLUSIONS:** We successfully analyzed fetal and placental methylomes on a genomewide scale, noninvasively and serially. This development offers a powerful method for research, biomarker discovery, and clinical testing for pregnancy-related disorders.



Prenatal development involves a series of highly orchestrated genetic and epigenetic events. Abnormalities in the epigenetic control of developmental processes are implicated in infertility, spontaneous abortion, intrauterine growth abnormalities, and postnatal consequences *(1–3)*. DNA methylation is an important epigenetic mechanism and is best known in the context of the addition of a methyl group to the 5′ carbon of cytosine residues in CpG dinucleotides. Cytosine methylation adds a layer of control to DNA function and is known to be associated with repression of gene expression.

The human placenta exhibits a plethora of special characteristics involving DNA methylation *(4, 5)*. On a global level, placental tissues are hypomethylated compared with most somatic tissues *(5, 6)*. At the gene level, the methylation status of particular genomic loci is a specific signature of placental tissues *(6, 7)*, and this signature shows gestational age–dependent changes *(8)*. Imprinted genes, namely genes for which expression is dependent on the parental origin of alleles, serve key functions in the placenta *(9)*. The study of the DNA-methylation profile of placental tissues has provided insights into the pathophysiology of pregnancy-associated or developmentally related diseases, such as preeclampsia *(10)* and intrauterine growth restriction *(2)*.

A myriad of approaches have been used to investigate the placental methylome *(11, 12)*. Despite much effort, no practical means has become available to monitor the dynamic changes in the fetal or placental methylation profile on a genomewide scale throughout pregnancy and during disease processes. In this study, we aimed to develop a platform that would enable researchers to interrogate the fetal and placental methylation profile noninvasively, both serially and on a genomewide scale. We applied genomewide bisulfite sequencing *(13–17)* to the analysis of cell-free fetal DNA molecules found in the circulation of pregnant women *(18)*. We devised 2 approaches to interrogate

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the methylation status of fetal/placental DNA molecules in maternal plasma. The first approach exploits the polymorphic differences between the mother and the fetus to identify the fetal-specific DNA molecules. The second approach is not dependent on fetal polymorphisms. The placental methylome is assembled noninvasively by using the methylation profile of maternal blood cells and the fractional fetal DNA concentration in maternal plasma.

## Materials and Methods

### CASE RECRUITMENT AND SAMPLE PROCESSING

Pregnant women attending the antenatal clinic at the Department of Obstetrics and Gynaecology, Prince of Wales Hospital, Hong Kong, were recruited with written informed consent and with ethics committee approval. Chorionic villi were sampled upon a clinical indication for aneuploidy testing. Maternal peripheral blood was collected in EDTA-containing tubes before chorionic villus sampling.

For a single pregnancy, peripheral venous blood samples were collected in the first trimester (gestational age, 13 weeks and 4 days) before chorionic villus sampling, at 37 weeks and 5 days before elective cesarean section, and within 24 h after delivery. A portion of a chorionic villus sample (CVS)[4] was collected in the first trimester, and a portion of the placenta was collected immediately after delivery. The fetus was confirmed to be male and karyotypically normal. Samples were processed as previously reported *(19)* (see Supplemental Materials and Methods in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue11).

Maternal plasma was collected from another 12 pregnancies at gestational ages between 13 and 14 weeks when the women presented for conventional first-trimester screening for chromosomal aneuploidies. Five of the women were confirmed to be carrying trisomy 21 fetuses, and the remaining cases involved karyotypically normal fetuses.

### PREPARATION OF BISULFITE-TREATED DNA LIBRARIES AND SEQUENCING ANALYSIS

DNA libraries were prepared with the Paired-End DNA Sample Preparation Kit (Illumina) and the use of methylated adapters (Illumina). After 2 rounds of purification with Agencourt® AMPure XP magnetic beads (Beckman Coulter), we split the ligation products into 2 portions, one of which was subjected to 2 rounds of bisulfite modification with an EpiTect Bisulfite Kit (Qiagen). The adapter-ligated DNA molecules (either treated or untreated with sodium bisulfite) were enriched by 10 PCR cycles. Bisulfite-treated and untreated DNA libraries were sequenced for 75 bp in a paired-end format on HiSeq 2000 instruments (Illumina). Sequence reads were processed in a methylation data–analysis pipeline [Methy-Pipe *(20)*]. See the Supplemental Materials and Methods for additional details.

## Results

### SERIAL METHYLOMIC ANALYSIS OF A PREGNANCY

We performed whole-genome sequencing of the bisulfite-converted DNA libraries *(13)* prepared from the pregnancy with serially collected samples, which included blood cells of the first-trimester maternal blood sample, the CVS, the placental tissue collected at term, and the maternal plasma samples from the first trimester, third trimester, and postpartum. We also analyzed blood cell and plasma DNA samples obtained from 1 adult male and 1 adult nonpregnant female. We generated a total of $9.5 \times 10^9$ pairs of raw sequence reads. The sequencing coverage of each sample is shown in Table 1 and in Table 1 of the online Data Supplement. The sequence reads that were uniquely mappable to the human reference genome reached mean haploid genomic coverages of 51-fold, 34-fold, and 28-fold for the first-trimester, third-trimester, and postdelivery maternal plasma samples, respectively. The coverage of CpG sites in the genome ranged from 81% to 92% for the samples obtained from the pregnancy. The sequence reads that spanned CpG sites amounted to mean haploid coverages of 66-fold, 46-fold, and 38-fold for the first-trimester, third-trimester, and postdelivery maternal plasma samples, respectively. The mean bisulfite-conversion efficiency for all samples was 99.96% (range, 99.94%–99.98%).

### GENOMEWIDE METHYLATION PROFILES OF PLACENTAL TISSUES AND MATERNAL BLOOD CELLS

Between 71% and 72% of the sequenced CpG sites were methylated in the DNA extracted from blood cells obtained from the pregnant woman, the nonpregnant woman, and the adult male (Table 1). Consistent with reports about the hypomethylated nature of placental tissues, 55% and 59% of the CpG sites were methylated in the CVS and term placental tissue, respectively (Table 1). We also studied the placental methylome with an oligonucleotide array platform that covered about 480 000 CpG sites in the human genome (Illumina) *(17, 21)*. The data from the 2 platforms were highly concordant (see Fig. 1 in the online Data Supplement). Our sequencing data were also concordant with those reported by Chu et al. *(22)* (see Table 2 in the online

[4] Nonstandard abbreviations: CVS, chorionic villus sample; SNP, single-nucleotide polymorphism; DMR, differentially methylated region.

**Table 1. Summary of genomewide bisulfite-DNA sequencing results.**

| Sample | Total mappable reads, $\times 10^8$ | Whole genome depth, -fold[a] | CpG seq[b] depth, -fold | CpG methylation density, % | Genomic C seq depth, -fold | Genomic C methylation density, % | CHG seq depth, -fold | CHG methylation density, % | CHH seq depth, -fold | CHH methylation density, % |
|---|---|---|---|---|---|---|---|---|---|---|
| Maternal blood cells (1st trimester) | 4.90 | 22.48 | 26.13 | 71.72 | 16.21 | 5.64 | 21.74 | 0.08 | 13.65 | 0.08 |
| CVS | 3.72 | 16.67 | 23.39 | 55.35 | 15.18 | 4.30 | 19.71 | 0.17 | 12.78 | 0.19 |
| Term placenta | 5.82 | 18.68 | 25.53 | 59.13 | 17.67 | 4.36 | 22.04 | 0.24 | 14.80 | 0.29 |
| Maternal plasma (1st trimester) | 27.1 | 50.69 | 66.35 | 66.93 | 42.13 | 5.18 | 55.20 | 0.11 | 36.68 | 0.11 |
| Maternal plasma (3rd trimester) | 9.18 | 33.80 | 46.00 | 68.22 | 30.85 | 5.05 | 40.72 | 0.14 | 27.03 | 0.16 |
| Maternal plasma (postdelivery) | 9.62 | 28.08 | 37.72 | 73.16 | 24.91 | 5.45 | 32.90 | 0.12 | 21.77 | 0.12 |
| Adult male blood cells | 1.70 | 8.22 | 12.36 | 71.43 | 7.40 | 5.85 | 9.90 | 0.10 | 6.22 | 0.10 |
| Adult female blood cells | 1.76 | 8.52 | 12.07 | 71.93 | 7.44 | 5.71 | 9.81 | 0.10 | 6.32 | 0.10 |
| Adult male plasma | 0.804 | 3.65 | 5.38 | 71.15 | 3.39 | 5.54 | 4.59 | 0.10 | 2.91 | 0.10 |
| Adult female plasma | 0.533 | 2.31 | 3.59 | 72.46 | 2.17 | 5.87 | 2.91 | 0.11 | 1.86 | 0.10 |

[a] After removal of ambiguous and duplicated reads.
[b] seq, sequenced; H is an A, C, or T nucleotide in CHG or CHH.

Data Supplement). The rates of non-CpG methylation were <1% the maternal blood cells, the CVSs, and placental tissues (Table 1), consistent with results reported for nonpluripotent cells *(14, 15)*.

#### PLASMA METHYLOMES

The genomewide proportions of CpG sites methylated in DNA of plasma samples obtained from the male and nonpregnant female were almost the same as those in the corresponding blood cell DNA (see Table 1; see Fig. 2 in the online Data Supplement). We next evaluated the methylation density of each 100-kb bin in the human genome by determining the total number of unconverted cytosines at CpG sites as a proportion of all of the CpG sites covered by sequence reads mapped to that 100-kb region. The Pearson correlation coefficient ($r$) and the $r^2$ value were 0.963 and 0.927, respectively, for the plasma DNA sample and the corresponding blood cell DNA sample obtained from the nonpregnant female. The values for the corresponding samples from the male were 0.953 and 0.908 (see Fig. 3 in the online Data Supplement). These data are consistent with our previous findings that hematopoietic cells are the predominant source of DNA in human plasma *(23)*.

For DNA from maternal plasma, the overall proportions of methylated CpGs were 67% and 68% for the first- and third-trimester maternal plasma samples, respectively. Unlike the results obtained for the nonpregnant individuals, these proportions are lower than the proportion for the maternal blood cell sample but higher than for the CVS and the term placental tissue sample (Table 1). Of note is that DNA from the postdelivery sample of maternal plasma had a methylated-CpG percentage of 73%, which is similar to that of the blood cell data (Table 1). These trends were observed for CpGs distributed over all autosomes and chromosome X, and they spanned both the nonrepeat regions and multiple classes of repeat elements of the human genome (Fig. 1).

#### NONINVASIVE FETAL METHYLOMES

Fetal DNA molecules circulate in the maternal plasma against a background of a majority of maternal DNA *(24)*. We used single-nucleotide polymorphism (SNP) differences between the mother and the fetus to identify the fetal DNA molecules in maternal plasma. The aim was to identify SNP loci for which the mother was homozygous and the fetus was heterozygous. Genomic DNA from the maternal blood cells was genotyped. The mother was homozygous at 1 945 516 loci on the autosomes. Analysis of maternal plasma DNA-sequencing reads revealed the presence of a nonmaternal allele at 107 750 loci; these loci were considered informative. At each informative SNP, the allele not from the mother was termed a "fetal-specific allele," and the other allele was termed a "shared allele." From the allelic differences, we obtained fetal DNA percentages for the first-trimester, third-trimester, and postdelivery samples of maternal plasma of 14.4%, 33.9%, and 4.5%, respectively. The corresponding fetal DNA percentages calculated by using the numbers of chromosome Y reads were 14.2%, 34.9%, and 3.7%.

We assembled the fetal methylome from maternal plasma by using pairs of sequence reads that spanned ≥1 informative fetal SNP site and contained ≥1 CpG site. Reads that showed fetal-specific alleles were included in the assembly of the fetal methylome. Reads that showed the shared allele (i.e., the non–fetal-specific allele) were included in the assembly of the non–fetal-specific methylome, which predominantly consisted of maternally derived DNA molecules. The fetal-specific reads covered 218 010, 263 611, and 74 020 autosomal CpG sites for the first-trimester, third-trimester, and postdelivery maternal plasma samples, respectively. The shared reads had mean coverages of these CpG sites of 33.3-, 21.7-, and 26.3-fold, respectively. The fetal-specific reads had mean coverages of these CpG sites of 3.0-, 4.4-, and 1.8-fold for the first-trimester, third-trimester, and postdelivery maternal plasma samples, respectively. The coverage of these CpG sites by fetal-specific reads was proportional to the fetal DNA percentage in the sample. For the first-trimester maternal plasma sample, the genomewide percentage of methylated CpG sites among the fetal-specific reads was 47.0%, whereas the percentage for the shared reads was 68.1%. For the third-trimester maternal plasma sample, the percentage of methylated CpG sites for the fetal-specific reads was 53.3%, whereas that for the shared reads was 68.8%.

We determined the methylation density of each 1-Mb region in the genome. The fetal-specific and non–fetal-specific methylomes assembled from the maternal plasma sequence reads are presented in Fig. 2. In both the first- and third-trimester plasma samples, the fetal methylomes were more hypomethylated than the methylomes based on the shared reads. The overall methylation profile of the fetal methylomes more closely resembled that of the CVS or placental tissue samples. On the contrary, the DNA-methylation profile of the shared reads for plasma, which was predominantly maternal DNA, more closely resembled that of the maternal blood cells. We then systematically compared methylation densities per CpG site for the maternal plasma DNA reads and the maternal or fetal tissue DNA reads. Statistically significant correlations were observed (see Fig. 4 in the online Data Supplement).

#### FETUS-SPECIFIC DNA-METHYLATION SIGNATURES AT IMPRINTED LOCI

We sorted the list of imprinted loci reported by Woodfine et al. *(25)* for those that contained SNPs within the

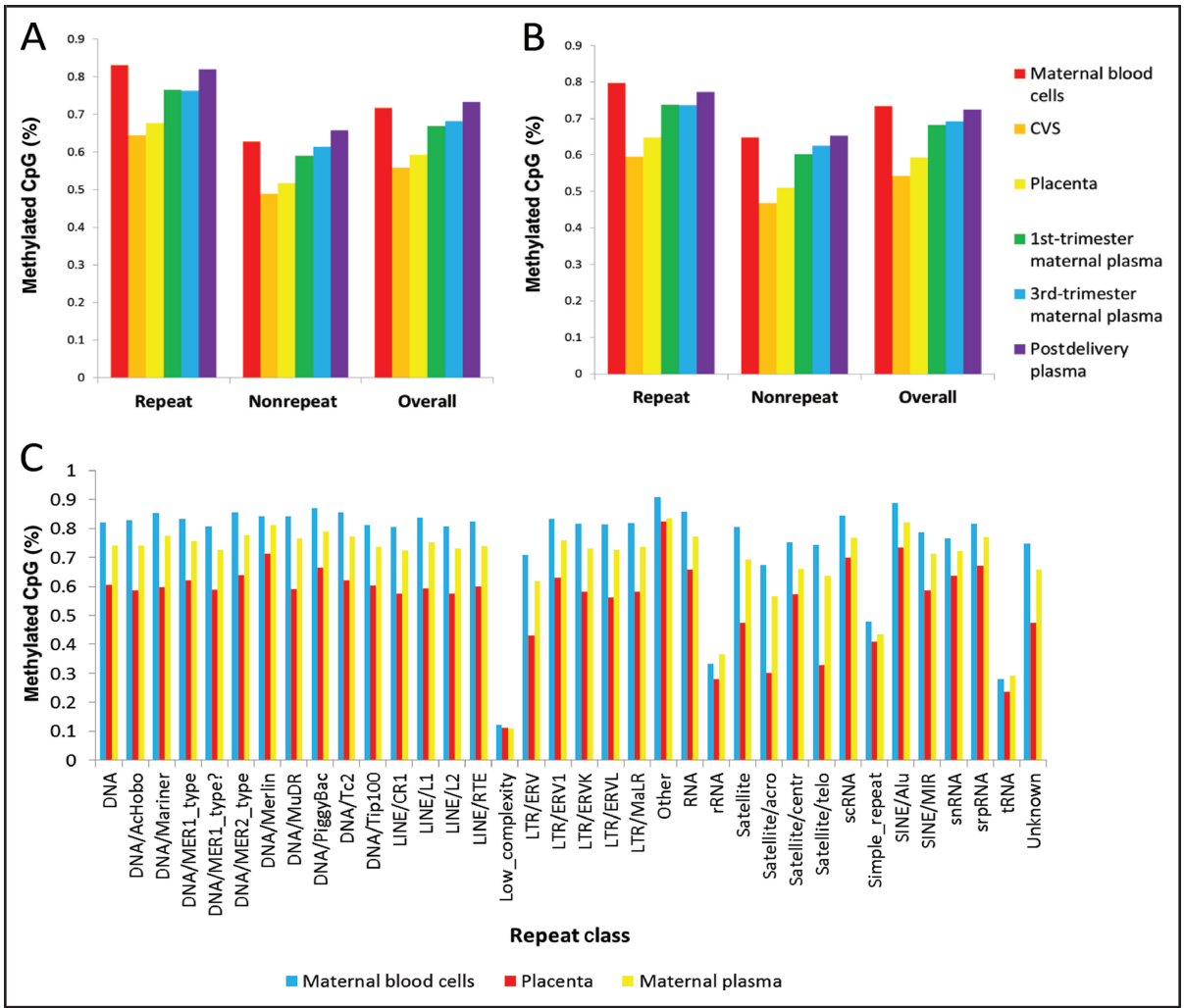


**Fig. 1. Bar charts of percentages of methylated CpG sites within the repeat and nonrepeat regions of the reference genome among samples collected from the pregnancy.**

"Overall" refers to data in both the repeat and nonrepeat regions of the genome (A), autosomes (B), and chromosome X (C). The various repeat classes are presented as defined by the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgTrackUi?g=rmsk). Data shown are from the first-trimester samples.

imprinting-control regions. Four loci fulfilled the criteria: *H19*[5] [H19, imprinted maternally expressed transcript (non-protein coding)], *KCNQ1OT1* [KCNQ1 opposite strand/antisense transcript 1 (non-protein coding)], *MEST* (mesoderm specific transcript), and *GNAS* (GNAS complex locus). By studying the SNP alleles and the methylation status of these loci in the sample of maternal blood cells, we can interpret the imprinting status of these loci from the maternal plasma data. The maternally inherited fetal reads, the paternally inherited fetal reads, and their respective methylation status could be deduced from the bisulfite DNA–sequencing data for maternal plasma (see Fig. 5 in the online Data Supplement).

### DIFFERENTIALLY METHYLATED LOCI IN PLACENTAL TISSUES AND MATERNAL BLOOD CELLS

The placenta is known for its tissue-specific methylation signatures *(7, 26, 27)*. Fetal-specific DNA-methylation markers have been developed for their detection in maternal plasma and for noninvasive prenatal diagnostic applications. These markers are

[5] Human genes: *H19*, H19, imprinted maternally expressed transcript (non-protein coding); *KCNQ1OT1*, KCNQ1 opposite strand/antisense transcript 1 (non-protein coding); *MEST*, mesoderm specific transcript; *GNAS*, GNAS complex locus.

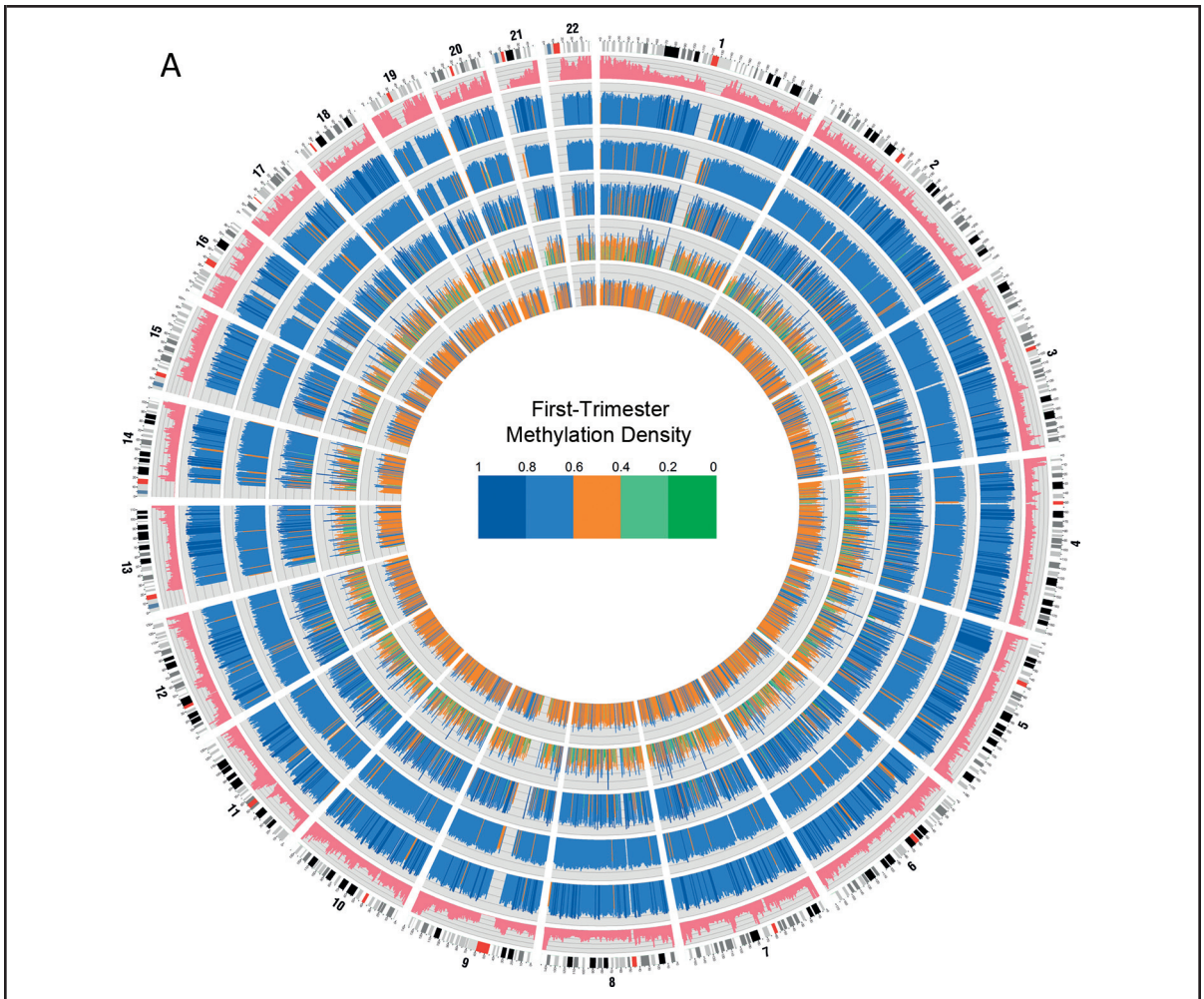


**Fig. 2. Circos plots of methylation density per 1-Mb bin.**

Chromosome ideograms (outermost ring) are oriented pter-qter in a clockwise direction (centromeres are shown in red). The second track inwards shows the number of CpG sites in the corresponding 1-Mb regions, up to 20 000 sites. The methylation densities of the corresponding 1-Mb regions are shown in the other tracks according to the color scheme presented in the center of each plot. A, Results for first-trimester samples. From inside to outside: CVS, fetal-specific reads in maternal plasma, shared reads in maternal plasma, combined fetal and nonfetal reads in maternal plasma, and maternal blood cells. B, Results for third-trimester samples. From inside to outside: term placental tissue, fetal-specific reads in maternal plasma, shared reads in maternal plasma, combined fetal and nonfetal reads in maternal plasma, postdelivery maternal plasma, and maternal blood cells (from the first-trimester blood sample).

*Continued on page 1589*

based on loci that are differentially methylated in placental tissues and maternal blood cells *(22, 28, 29)*. We mined our data on a genomewide basis for differentially methylated regions (DMRs). The key to success in developing fetal-specific DNA-methylation markers in maternal plasma is to have the methylation status of the maternal blood cells to be either as highly methylated or as unmethylated as possible *(28)*. We thus focused on loci in which the methylation densities of the DNA from the maternal blood cells were either ≤20% or ≥80%. DMRs were then identified among the subset of loci for which the methylation densities in the CVS or the term placental tissue sample were different by ≥20% from the methylation densities of the maternal blood cells. We identified 11 729 hypermethylated and 239 747 hypomethylated loci in the first trimester and identified 11 920 hypermethylated and 204 768 hypomethylated loci in the third trimester (see Table 3 in the online Data Supplement). We used 33 loci that had previously been reported *(26, 28–30)* to be differentially methylated in maternal

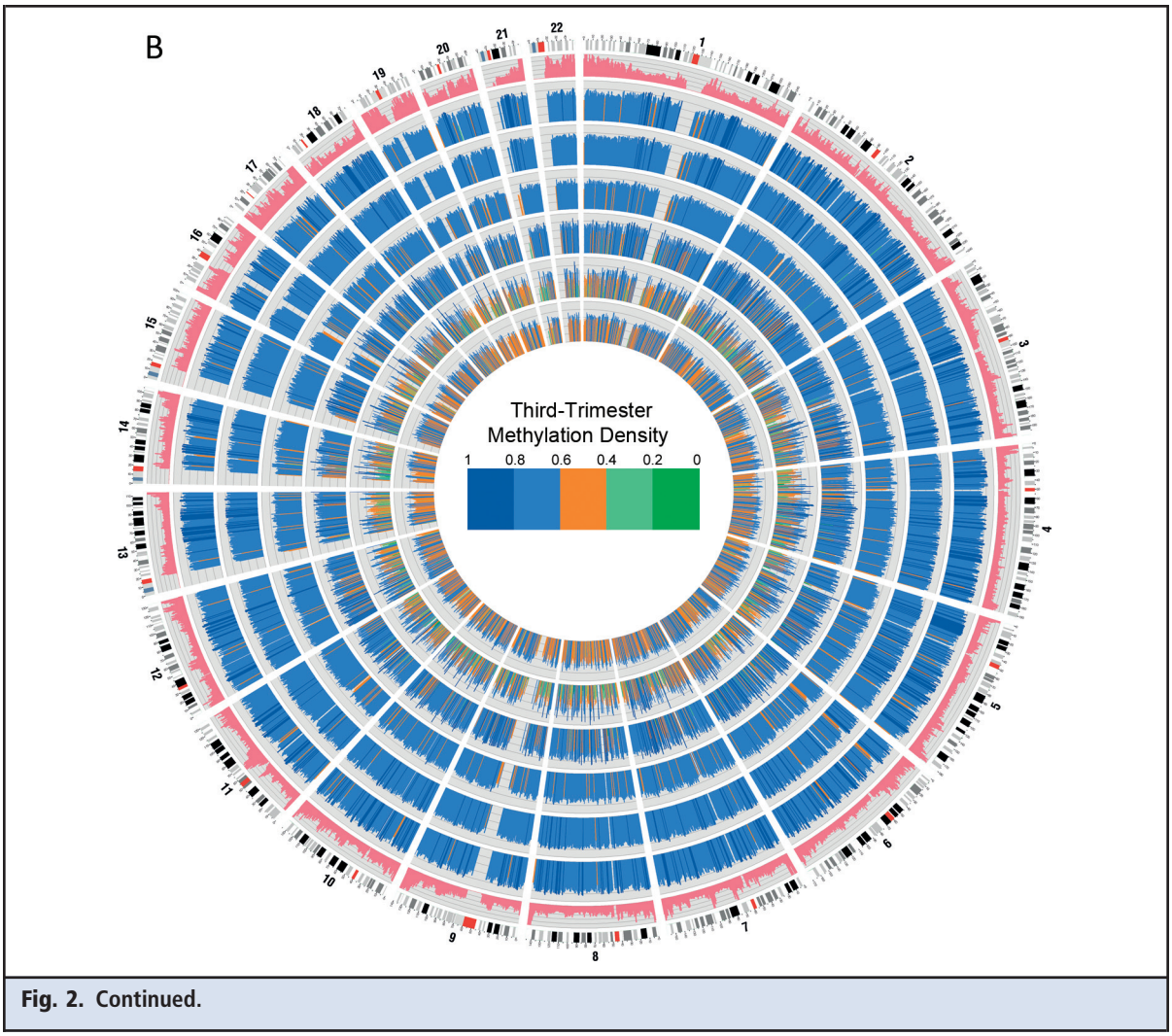


**Fig. 2. Continued.**

blood cells and first-trimester placental tissues to validate our list of first-trimester candidates. Our algorithm was able to identify 79% of the 33 loci as DMRs (see Table 4 in the online Data Supplement).

#### DIRECT AND NONINVASIVE IDENTIFICATION OF DIFFERENTIALLY METHYLATED REGIONS FROM THE MATERNAL PLASMA SEQUENCING DATA

The placenta is the predominant source of fetal DNA in maternal plasma *(31 )*. In this study, we showed that the methylation status of fetal-specific DNA in maternal plasma is correlated with the placental methylome. We therefore hypothesized that an algorithm could be developed to predict the methylation density of the placental DNA molecules from the maternal plasma DNA-sequencing data, which we termed the "predicted value." Again, we focused on loci that were either ≤20% or ≥80% methylated in the maternal blood cells. To deduce loci that were hypermethylated in placental tissues with respect to maternal blood cells, we sorted for loci that showed ≤20% methylation in maternal blood cells and ≥60% methylation according to the predicted value, with a ≥50% difference between the blood cell methylation density and the predicted value. To deduce loci that were hypomethylated in the placental tissues compared with maternal blood cells, we sorted for loci that showed ≥80% methylation in maternal blood cells and ≤40% methylation according to the predicted value, with a difference of at least 50% between the blood cell methylation density and the predicted value. Table 2 shows the numbers of loci deduced to be hypermethylated or hypomethylated in placental tissues. We used the bisulfite DNA–sequencing data for the placental tissues and maternal blood cells to verify the validity of the loci deduced from the maternal plasma DNA-sequencing data. The majority of the noninvasively deduced loci (Table 2; see Table 5 in the online Data Supplement) showed the expected methylation pattern in the

**Table 2. Numbers of hypermethylated or hypomethylated loci predicted from direct analysis of the maternal plasma bisulfite-sequencing data.**

| | First trimester | | Third trimester | |
|---|---|---|---|---|
| | Hypermethylated loci[a] | Hypomethylated loci[b] | Hypermethylated loci[a] | Hypomethylated loci[b] |
| Predicted loci, n | 3081 | 44 455 | 1746 | 14 930 |
| Loci with methylation densities >40% in the placental tissue, n[c] | 1678 | N/A[d] | 1525 | N/A[d] |
| Loci with methylation densities <60% in the placental tissue, n[c] | N/A[e] | 23 468 | N/A[e] | 13 475 |
| Loci overlapping with DMRs mined from the placental tissue[c] and maternal blood cells, n | 1457 | 21 812 | 1279 | 12 677 |

[a] Loci in which the placenta is ≥20% more hypermethylated than the maternal blood cells. The search for hypermethylated loci started from the list of loci showing methylation densities of <20% in maternal blood cells.
[b] Loci in which the placenta is ≥20% less methylated than the maternal blood cells. The search for hypomethylated loci started from the list of loci showing methylation densities of >80% in maternal blood cells.
[c] Bisulfite-sequencing data from the CVS and the term placental tissue were used to verify the first-trimester maternal plasma data and the third-trimester maternal plasma data, respectively.
[d] Not applicable to the search of hypomethylated loci.
[e] Not applicable to the search of hypermethylated loci.

tissues and overlapped with the DMRs mined from the tissue data and described in the earlier section.

#### GESTATIONAL VARIATION IN PLACENTAL AND FETAL METHYLOMES

The overall proportion of methylated CpGs was 55% in the CVS and 59% for the term placenta (Table 1). More hypomethylated DMRs could be identified from the CVS than from the term placenta, whereas the 2 tissues were similar with respect to the number of hypermethylated DMRs. Thus, it is evident that the CVS was more hypomethylated than the term placenta. This gestational trend was also apparent in the maternal plasma data. The proportion of methylated CpGs among the fetal-specific reads was 47.0% in the DNA from first-trimester maternal plasma but was 53.3% in DNA from third-trimester maternal plasma. DNA from first-trimester and third-trimester maternal plasma samples had similar numbers of validated hypermethylated loci (1457 and 1279 loci, respectively), but the first-trimester sample had substantially more hypomethylated loci (21 812 loci) than the third-trimester sample (12 677 loci; Table 2).

#### RELATIONSHIP BETWEEN METHYLATION STATUS AND THE SIZE OF DNA MOLECULES IN MATERNAL PLASMA

We used paired-end sequencing to determine the lengths of plasma DNA molecules *(23, 32)*. In a previous study, we showed that plasma DNA molecules had sizes that approximated those of mononucleosomes and that fetal DNA molecules were shorter than maternal ones *(32)*. In this study, we explored whether the methylation status of plasma DNA molecules bore any relationship to their sizes. For sequence reads that covered ≥1 CpG site, we determined the methylation densities for DNA molecules of the same size. We then plotted the relationship between the sizes of the DNA molecules and their methylation densities (Fig. 3). We considered a read containing a fetal-specific SNP allele to be a fetal DNA molecule and a read containing a maternal-specific SNP allele to be a maternal DNA molecule. In general, DNA molecules with high methylation densities were longer. This trend was present for both the fetal and maternal DNA molecules, for both the first and third trimesters. The overall sizes of the fetal DNA molecules were shorter than the maternal DNA molecules, as previously reported *(32, 33)*. The plasma DNA sample from the adult nonpregnant female also showed the same relationship between the size and the methylation state of DNA molecules (Fig. 3C). On the other hand, genomic DNA samples were fragmented by an ultrasonication step before massively parallel sequencing analysis and did not reveal the same trend.

#### DETECTION OF TRISOMY 21 BY METHYLATION DENSITY ASSESSMENT OF CHROMOSOME 21

As a demonstration of one potential clinical application of maternal plasma methylome analysis, we assessed the methylation density of chromosome 21 for 12 pregnancies. For each maternal plasma sample, we normalized the methylation density of plasma DNA molecules originating from chromosome 21 to the pooled methylation density of plasma DNA molecules

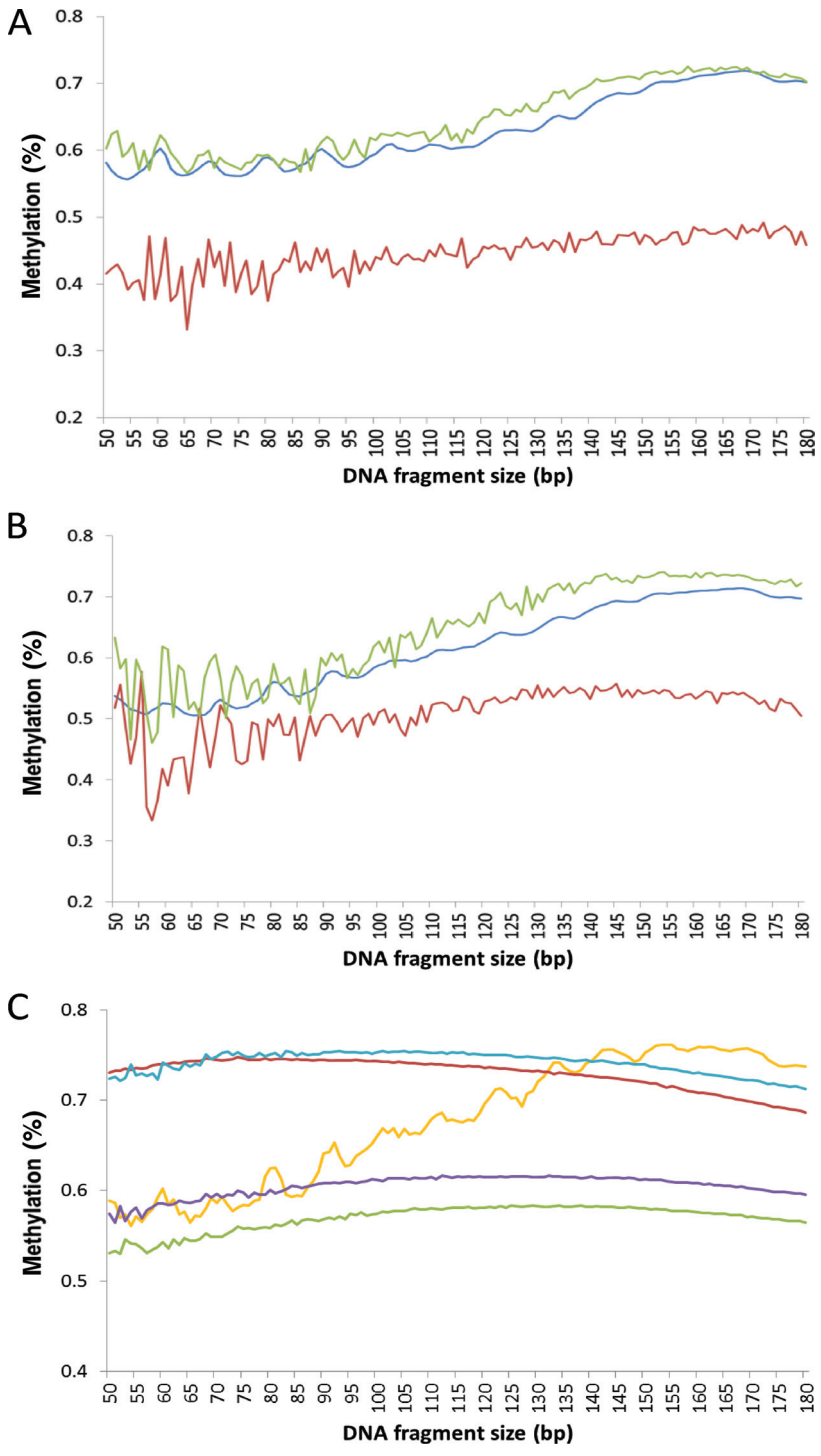


**Fig. 3. Plots of methylation densities and sizes of plasma DNA molecules.**

(A), First-trimester maternal plasma. (B), Third-trimester maternal plasma. Presented are data for all of the sequenced reads that cover ≥1 CpG site (blue curve), for reads that also contain a fetal-specific SNP allele (red curve), and for reads that also contain a maternal-specific SNP allele (green curve). (C), Indicated are adult female plasma (yellow), adult female blood cells (blue), maternal blood cells (red), term placenta (purple), and CVS (green).

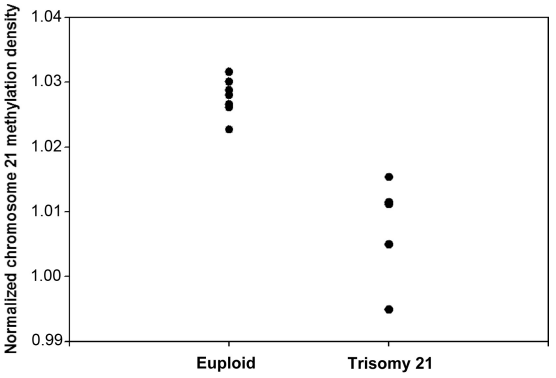


**Fig. 4. Plot of normalized chromosome 21 methylation densities of maternal plasma samples collected from euploid and trisomy 21 pregnancies.**

from all of the autosomes except chromosomes 13, 18, and 21. Owing to the presence of an additional dose of fetal chromosome 21 for the affected fetuses, the normalized chromosome 21 methylation densities were significantly lower for the trisomy 21 cases than for the euploid cases ($P = 0.003$, Mann–Whitney rank sum test; Fig. 4).

## Discussion

We used whole-genome bisulfite sequencing to analyze DNA methylomes from plasma DNA. We applied the approach to assess the methylation profile of a fetus and the placenta by sequencing DNA samples prepared from maternal plasma. We were able to perform noninvasive fetal and placental methylomic analyses during the pregnancy and monitor the changes serially as the pregnancy progressed. The comprehensiveness of the sequencing data allowed us to study the maternal plasma methylomes on a genomewide scale at single-nucleotide resolution.

With the genotype information of the mother, we were able to deduce the methylation profile of the fetal DNA from the maternal plasma data. We also developed an algorithm to noninvasively predict the methylation profile of the placenta without having to use genotype differences between the fetus and the mother. Thus, information conventionally obtained via the study of placental tissues (e.g., genomic-imprinting status, tissue-specific methylation status, and gestational variation) could be assessed directly from maternal plasma. Given the known association between altered DNA-methylation status and the many pregnancy-associated conditions *(1–3)*, the approach we have described could be used for pathophysiological studies and biomarker development. The platform also has the potential to be applied directly to prenatal assessment of fetal or pregnancy-associated diseases, as we showed for the detection of trisomy 21.

The high level of resemblance between the methylomes of blood cells and plasma for the male and nonpregnant female, as well as between the methylomes of maternal blood cells and the postdelivery maternal plasma sample, further affirm that hematopoietic cells are the main sources of DNA in human plasma *(23)*. Yet, the overall proportions of methylated CpGs in the first-trimester and third-trimester maternal plasma samples were reduced compared with the data from the maternal blood cells or the postdelivery maternal plasma sample. We suspected that the reduced methylation levels during pregnancy were due to the hypomethylated nature of the fetal DNA molecules present in maternal plasma. Indeed, by separately analyzing the fetal-specific and shared sequence reads, we demonstrated that the circulating fetal DNA molecules were much more hypomethylated than the background DNA molecules. A comparison of the methylation densities of corresponding loci in the fetal-specific maternal plasma reads and the placental tissue data for both the first and third trimesters revealed high levels of correlation. These data provide genome-level evidence that the placenta is the predominant source of fetal-derived DNA molecules in maternal plasma and represent a major step forward, compared with previous evidence based on selected loci *(31)*. The reversal of the methylation profile in the postdelivery maternal plasma sample to become more similar to that of the maternal blood cells suggests that the fetal DNA molecules were removed from the maternal circulation *(34)*. Calculation of the fetal DNA concentrations based on SNP markers of the fetus indeed showed that the concentration changed from 33.9% before delivery to just 4.5% in the postdelivery sample.

Interestingly, our data revealed a relationship between methylation status and the size of plasma DNA molecules. Plasma DNA molecules are known to exist in circulation in the form of short molecules, with the majority of molecules being about 160 bp *(23, 32)*. The characteristic size profiles of plasma DNA molecules suggest that many of these molecules are associated with mononucleosomes, possibly derived from enzymatic degradation during apoptosis. In this study, we showed that hypomethylated molecules were shorter than hypermethylated ones. The same trend was observed in both the fetal and maternal DNA molecules. Given that DNA methylation is known to influence nucleosomal packing *(35)*, our data suggest that the hypomethylated DNA molecules were less densely packed with histones and were therefore more susceptible to enzymatic degradation. On the other hand, the data presented in Fig. 3 also show that despite the fetal

DNA being much more hypomethylated than the maternal reads, the size profiles of the fetal and maternal DNA overlap. This observation suggests that the hypomethylated state of fetal DNA is not the only factor that accounts for its relative shortness compared with maternal DNA.

In summary, we have successfully assembled DNA methylomes via massively parallel bisulfite-sequencing analysis of plasma DNA. The present approach offers a noninvasive way to investigate or clinically monitor important pregnancy-associated conditions that is based on genome-scale methylomic analysis. We believe that the approach has an exciting future role to play in prenatal testing, monitoring, and research. We also foresee an application of this approach to other areas of medicine where plasma DNA analysis is of interest. For example, the methylomes of cancers could be determined from the plasma DNA of cancer patients *(36)*, or the methylomes of transplanted organs could be determined from the plasma DNA of organ transplant recipients *(23, 37)*.

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