Detection of Paternally Inherited Fetal Point Mutations for β-Thalassemia Using Size-Fractionated Cell-Free DNA in Maternal Plasma

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Context Currently, fetal point mutations cannot be reliably analyzed from circulatory fetal DNA in maternal plasma, due to the predominance of maternal DNA sequences. However, analysis of circulatory fetal DNA sequences in maternal plasma have been shown to selectively enrich for fetal DNA molecules on the basis of a smaller molecular size than maternal DNA.

Objective To examine the prenatal analysis of 4 common β-thalassemia point mutations: IVSI-1, IVSI-6, IVSI-110, and codon 39.

Design, Setting, and Patients A total of 32 maternal blood samples were collected at 10 to 12 weeks of gestation (mean, 10.7 weeks) between February 15, 2003, and February 25, 2004, in Bari, Italy, from women with risk for β-thalassemia in their newborns immediately prior to chorionic villous sampling. Samples in which the father and mother did not carry the same mutation were examined. Circulatory DNA was size-fractionated by gel electrophoresis and polymerase chain reaction (PCR) amplified with a peptide-nucleic-acid clamp, which suppresses amplification of the normal maternal allele. Presence of the paternal mutant allele was detected by allele-specific real-time PCR.

Main Outcome Measure Detection of paternally inherited β-globin gene point mutations.

Results Presence or absence of the paternal mutant allele was correctly determined in 6 (86%) of 7 cases with the IVSI-1 mutation, 4 (100%) of 4 with the IVSI-6 mutation, 5 (100%) of 5 with the IVSI-110 mutation, and 13 (81%) of 16 with the codon 39 mutation. One false-positive test result was scored for the IVSI-1 mutation. Two cases with the codon 39 mutation were classified as uncertain and 1 case was excluded due to lack of a diagnostic test result at the time of analysis. These results yielded an overall sensitivity of 100% and specificity of 93.8%, with classified cases removed.

Conclusion Our recently described technique of the size-fractionation of circulatory DNA in maternal plasma may be potentially useful for the noninvasive prenatal determination of fetal point mutations.

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PATERNALLY INHERITED FETAL POINT MUTATIONS FOR \(\beta\)-THALASSEMIA

This approach, however, is unsuitable for the analysis of fetal loci that do not differ largely from the maternal alleles, due to the vast predominance of cell-free maternal DNA in the maternal samples. As such, the analysis of fetal point mutations has been restricted to single-case articles.\(^{10,11}\) It has recently been shown that circulatory fetal DNA sequences are generally smaller (<300 base pairs [bp]) than comparable circulatory maternal DNA species (>500 bp).\(^{12,13}\) By exploiting this observation, we have previously shown that this phenomenon can be used to selectively enrich for fetal DNA molecules, which permitted the detection of otherwise masked highly polymorphic fetal microsatellite markers.\(^{12}\) We examined whether this approach will permit the detection of fetal point mutations. The advantage of such a development is that it would permit the detection of paternal mutations, which could be used to determine which pregnancies are at risk for a compound heterozygous genetic disorder. We have focused on one of the most common monogenic disorders, \(\beta\)-thalassemia, and have examined 4 point mutations, which occur with high frequency in the Mediterranean population.\(^{14,15}\)

**METHODS**

**Sample Collection and Processing**

Following ethical approval from both participating institutions’ review boards and written informed consent from all participants, blood samples were obtained from 32 pregnant women with risk for \(\beta\)-thalassemia in their newborns between February 15, 2003, and February 25, 2004, in Bari, Italy. No one refused to participate and all women were self-declared white (southern Italian origin). Approximately 18-ml maternal blood samples were collected into two 9-ml EDTA blood collection tubes (Sarstedt, Sevelen, Switzerland) at 10 to 12 weeks of gestation (mean, 10.7 weeks; median, 11.2 weeks) before chorionic villous sampling. Initially, 21 samples were sent as whole blood by overnight commercial express courier service. Because of concern that this 24-hour delay before processing of the maternal plasma sample might be detrimental, the remaining 11 samples were processed directly on-site in Bari, Italy, and the plasma was shipped frozen to Basel, Switzerland.

All samples were sent coded and examined to Basel in a blinded manner. None of the samples examined have been used in any prior investigations. Plasma was prepared from the maternal blood samples by high-speed centrifugation as described previously and stored at –70°C before analysis.\(^{12}\) In addition, the frozen plasma samples shipped from Bari were again subjected to high-speed centrifugation (16000 g for 10 minutes) before analysis.\(^{16}\) We focused exclusively on samples in which the father was a carrier for 1 of the 4 following \(\beta\)-globin gene mutations (IVSI-1, IVSI-6, IVSI-110, and codon 39) and the mother had been genotyped to carry another \(\beta\)-globin gene mutation.

The chorionic villus sampling was obtained by transabdominal puncture with a 23-gauge needle under ultrasonic guidance. The samples were processed and analyzed at the diagnostic laboratory at the University of Bari, using an allele refractory mutation system and PCR procedure, followed by combined reverse dot blot analysis.\(^{7,8}\)

**Circulatory DNA Extraction and Size-Fractionation**

Circulatory DNA was extracted from 5- to 10-ml maternal plasma using commercial column technology (Roche High Pure Template DNA Purification Kit; Roche, Basel, Switzerland) in combination with a vacuum pump.\(^{12}\) After extraction, the DNA was separated by agarose gel (1%) electrophoresis (Invitrogen, Basel, Switzerland), and the gel fraction containing circulatory DNA with a size of approximately 300 bp was carefully excised. The DNA was extracted from this gel slice by using an extraction kit (QI-AEX II Gel Extraction Kit; Qiagen, Basel, Switzerland) and eluted into a final volume of 40-µl sterile 10-mM tris-hydrochloric acid, pH 8.0 (Roche).\(^{12}\) Strict anticontamination procedures were used throughout the procedure, including the analysis of on average 2 blank gel slices per samples examined, which were all negative.

**PCR Amplification Using a Peptide-Nucleic-Acid Clamp**

Peptide-nucleic-acids (PNAs) bind with very high affinity to specific DNA sequences (eg, to a wild-type or mutant allele), which may differ by as little as a single-base change.\(^{17}\) These molecules can be used when examining DNA samples that contain a mixture of wild-type and mutant alleles to suppress the specific amplification of either allele.\(^{17}\) In this manner, the mutant or wild-type allele can be selected specifically from a mixture of both alleles. We used a PNA sequence specific for the maternal normal allele to suppress amplification of the wild-type maternal allele, thereby enriching for the presence of paternally inherited mutant sequences. The PCR/PNA clamping reactions were performed in a total volume of 30 µl, consisting of 8-µl size-separated circulatory DNA, 1 x buffer with 3.5-mM magnesium, 0.2-mM dNTPs (nucleotides), 0.13-µM of each primer (all the primers used in this study were synthesized by Microsynth, Basel, Switzerland), and high performance liquid chromatography (HPLC) purified, and 0.6-U TaqGold DNA polymerase (Applied Biosystems, Rotkreuz, Switzerland), using the following PNA probe concentrations (Applied Biosystems): 0.67-µM for the IVSI-1 mutation, 0.5-µM for IVSI-6 mutation, 1-µM for IVSI-110 mutation, and 1-µM for codon 39 mutation. The detailed primer sequences and PCR/PNA clamping reactions are shown in Table 1. The clamping reaction was performed in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany).

**Allele-Specific Real-Time PCR**

Following the PCR/PNA clamping step, the presence of the mutant paternal allele was detected by a real-time allele-specific PCR reaction, which was performed on a sequence detector (Perkin Elmer Applied Biosystems 7000 Se-
Allele-Specific Real-Time PCR

The specificity of each of the allele-specific assays for the 4 β-globin gene mutations was optimized by evaluating a series of conditions concerning buffers composition (magnesium ions), temperature and length of PCR amplification cycles, as well as use of different oligonucleotide primers. These experiments were performed on artificial mixtures of mutant DNA diluted into wild-type DNA. The final conditions are listed in Table 1. For the real-time PCR analysis, the mixed solution (1X SYBR Green Master Mix, Applied Biosystems) was used to monitor the PCR reaction. The quantitative process used by real-time PCR makes use of a defined threshold value, which is determined by the cross-ing of a defined threshold by the accumulated PCR product, which is termed the threshold value or Ct. This value can be used for the accurate determination of the exact amount on specific input template DNA, by comparison with a standard curve. To measure the quantitative differences between 2 genetic loci (eg, wild-type and mutant), the difference between the respective Ct values (ΔCt) can be used. We used this ΔCt system to determine the ratio of wild-type to mutant, whereby the extent of the amplification of the normal wild-type allele (CtM) was subtracted from that of the mutant allele (Ctn).

By the use of this ΔCt(M:N) approach, we observed a clear discrimination of normal wild-type DNA samples from those samples heterozygous for the mutant allele, even with experimental conditions in which the mutant allele constituted less than 10% of the total DNA examined. This analysis also permitted us to assign arbitrary ΔCt(M:N) cut-off areas for the 4 allele-specific PCR assays; the normal allele yielding higher and the mutant allele yielding lower values (FIGURE).

Statistical Analysis
The χ2 test was used to evaluate whether a significant difference existed between the results obtained by the analysis of size-fractionated circulating DNA and by the analysis of total-circulatory DNA. The analysis was performed by using Stata version 8.0 (StataCorp LP, College Station, Tex).

RESULTS
The laboratory components of the study were performed from October 1, 2003, through May 30, 2004, in Basel, Switzerland. Four distinct point mutations of the β-globin gene—IVSI-1 (n=7), IVSI-6 (n=4), IVSI-110 (n=5), and codon 39 (n=16)—were examined. For each of these mutations, an allele-specific real-time PCR assay was developed. In the case of the codon 39 mutation, the development of an allele-specific assay was more complex due to the number of repetitive sequences in the vicinity of the mutation, which initially hindered the specificity of the PCR amplification. Because we were concerned that circulatory fetal DNA sequences may still be outnumbered by maternal DNA sequences, even after selective enrichment on the basis of size, an additional PCR step was used before the allele-specific PCR assay to ensure that the presence of mutant fetal alleles could be detected in a mixture of mutant and wild-type DNA.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers and PNA Probes</th>
<th>Conditions of PCR Clamping</th>
<th>Allele-Specific Real-Time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSI-1</td>
<td>F-primer: GTG AAG GAT GTG</td>
<td>Incubated at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 30 s, final extension at 72°C for 5 min</td>
<td>( \text{CT}_M )</td>
</tr>
<tr>
<td></td>
<td>R-primer: TCT CCT TAA ACC GGT</td>
<td>Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 30 s, final extension at 72°C for 5 min</td>
<td>( \text{CT}_N )</td>
</tr>
<tr>
<td></td>
<td>PNA primer: O-O-GAT ACC AAC CTG CCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVSI-6</td>
<td>F-primer: GTG AAG GAT GGT</td>
<td>Incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62°C for 30 s, final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-primer: TCT AAA CCT GTC TTA AAT TGA</td>
<td>Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 30 s, final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PNA primer: O-O-GAT ACC AAC CTG CCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVSI-110</td>
<td>F-primer: ACT CTT GGG TTG</td>
<td>Incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 62°C for 30 s, final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-primer: CAG CCT AAG GGG AAA ATA TGA</td>
<td>Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 30 s, final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PNA primer: O-O-TAG ACC AAT AGG C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Codon 39</td>
<td>F-primer: CTC TGG CTA TTG</td>
<td>Incubated at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 30 s, final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-primer: ATG CCC AAA GGA CTC AAA GAA CCC</td>
<td>Denatured at 95°C for 10 min, followed by 25 cycles of 95°C for 15 s, 70°C for 30 s, final extension at 72°C for 5 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PNA primer: O-O-ACC TGT GGG TCA A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: M, mutant allele; N, wild-type allele; OO, ethylene glycol linker; PCR, polymerase chain reaction; PNA, peptide-nucleic-acid.

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tand and wild-type alleles. In this additional PCR step, a PNA sequence was used that binds with high affinity to the wild-type maternal allele, thereby blocking its amplification during the PCR procedure. In this manner, an additional selective enrichment was performed for the paternally inherited mutant fetal allele. This step effectively suppressed the amplification of the wild-type allele by at least a factor of 1000-fold, whereas the mutant allele was amplified with normal efficacy.

Because a mixture of wild-type and mutant alleles was still present, a further safety measure was added to our analysis to ensure accurate determination of the presence of the paternal mutant allele. This was achieved by subtracting the extent of amplification of CTN from that of CTM in the subsequent real-time PCR assay. This approach permitted a clear discrimination between the 2 alleles, even with experimental conditions whereby the mutant allele was diluted in normal wild-type DNA of the order expected in maternal plasma samples (Figure, A). These assessments also permitted establishment of tentative cut-off ranges that could serve to distinguish between samples in which the mutant allele was present and those samples in which it was absent.

Confident that our assays were functional, we next tested a series of 32 clinical samples in a blinded manner, after which these results were compared with those obtained from the analysis of chrorionic villous material that had been performed by an independent routine diagnostic laboratory at the University of Bari.

These results (Table 2 and Table 3) and the codon 39 mutation (Figure, B) indicated the presence or absence of the mutant paternal allele in 6 (86%) of 7 cases for IVSI-1, 4 (100%) of 4 cases of IVSI-6, 5 (100%) of 5 cases for IVSI-110, and 13 (81%) of 16 cases for the codon 39 mutation. One false-positive test result was scored for our analysis of a IVSI-1 mutation. Because multiple negative controls and stringent anticontamination procedures were used throughout our analysis, it is unlikely that this is due to a contamination, but is most likely attributable to the concentration of input template DNA being too low to permit accurate analysis (44, 37, and 31 genome-equivalents, respectively). One of these cases, labeled as uncertain, had to be excluded because the diagnostic result from the chorionic villus sampling analysis was not available at the time of our analysis and preparation of our data set.

Our analysis of all 4 paternal mutant loci provide an overall sensitivity of 92.8% and specificity of 88.2% (Table 3). This was improved to a sen-

**Figure.** Allele Specific Real-Time PCR Analysis of the Codon 39 Mutation

- **A:** Mutant vs Wild-Type DNA
- **B:** Size-Fractionated DNA With PNA
- **C:** Total Plasma DNA With PNA
- **D:** Size-Fractionated DNA Without PNA

Real-time polymerase chain reaction (PCR) makes use of a defined threshold value (Ct). To measure the quantitative differences between wild-type (Ct,w) and mutant (Ct,m) alleles, the difference between the respective Ct values (ΔCt) can be used. A, Clear discrimination of the wild-type allele from the mutant allele on experimental serial dilutions of the mutant alleles into wild-type DNA. Following the ΔCtanalysis, wild-type alleles cluster above a ΔCt value of 6, while mutant alleles cluster at a value of less than 4. The arbitrarily assigned ΔCt cut-off values between 3.5 to 4.5 (shaded area) were used to distinguish between the presence of normal and mutant alleles. B, Analysis of size-fractionated circulatory DNA from maternal plasma samples in combination with the PCR/peptide-nucleic-acids (PNA) clamping step results in the correct appraisal of the presence (n=4) or absence (n=9) of the mutant allele. Three cases were classified as uncertain. C, Analysis of total cell-free DNA without size-fractionation results in the incorrect appraisal of 3 of 4 cases at the presence of the mutant allele even when using the PCR/PNA clamping step. D, Analysis of size-fractionated circulatory DNA without the use of the PCR/PNA clamping step results in the incorrect appraisal of the presence of the mutant allele in 2 cases.
sitivity of 100% and specificity of 93.8%, if the 2 cases classified as uncertain and the case without a diagnostic test result from the chorionic villus sampling analysis were excluded.

To verify the validity of our approach concerning the enrichment of circulatory fetal DNA sequences on the basis of their smaller size vs maternal DNA sequences, a parallel assessment of maternal plasma DNA samples that had not been subjected to size-fractionation was performed. This led to the incorrect evaluation of 6 (46%) of 13 cases that had inherited the paternal mutation for all 4 mutations examined, which is significantly different from our results obtained using size-fractionated DNA ($\chi^2$ test, $P = .02$). For the codon 39 mutation, these 3 cases are represented in the Figure, C. We also evaluated the value of the PCR/PNA clamping step to suppress amplification of the normal maternal allele on some of our samples ($n = 16$). This analysis indicated that in the absence of this suppression step incorrect results were recorded in 2 of 4 cases, even when using size-separated circulatory DNA (codon 39 mutation: Figure, D).

We initially only examined 21 maternal whole blood samples shipped directly from Bari to Basel by express courier. Nevertheless, it was quite apparent that the samples had been altered considerably during the 24-hour freight period, which has been suggested to hinder the analysis of fetal alleles in maternal plasma.\(^9\) Therefore, we also examined 11 plasma samples that had been directly prepared on site in Bari and shipped frozen to Basel for analysis. An examination of the total circulatory DNA in these 2 sets of samples indicated that considerable apoptosis of maternal cells had occurred in the samples shipped as whole blood (data not shown). Because this apoptotic process increases the amount of maternal DNA present in the gel fraction selected for the enrichment of fetal DNA species, it could thereby hinder the analysis of the mutant fetal allele. Our analysis of these samples indicated that the presence of this increased amount of maternal DNA did not hinder the detection of the mutant fetal allele with the conditions we had chosen.

**COMMENT**
Based on our results, which made use of a combination of size-separation and PCR/PNA clamping step, our strategy probably can be used for samples that have been shipped considerable distances (>$1600$ kilometers) by express courier service ($<24$ hours). The advantage of detecting these paternal mutations in compound heterozygous Mendelian disorders is that their absence can be used to exclude pregnancies at risk for these disorders, such as $\beta$-thalassemia major, thereby obviating the need for an invasive prenatal diagnostic procedure. Furthermore, a decrease of these risk-associated procedures can be achieved.\(^9\)

Our study indicates that fetal genetic traits involving point mutations can be detected from the analysis of circulatory fetal DNA in maternal plasma by selecting circulatory DNA sequences with a size of less than 300 bp. This step per-

<table>
<thead>
<tr>
<th>Case</th>
<th>Parent</th>
<th>Genotype</th>
<th>Circulating Fetal DNA</th>
<th>Chorionic Villus Sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mother</td>
<td>IVSI-1/N</td>
<td>IVSI-1</td>
<td>IVSI-1/N</td>
</tr>
<tr>
<td>2</td>
<td>Mother</td>
<td>IVSI-1/N</td>
<td>No IVSI-1 allele</td>
<td>N/N</td>
</tr>
<tr>
<td>3</td>
<td>Mother</td>
<td>IVSI-1/N</td>
<td>No IVSI-1 allele</td>
<td>N/N</td>
</tr>
<tr>
<td>4</td>
<td>Mother</td>
<td>IVSI-1/N</td>
<td>IVSI-1</td>
<td>IVSI-1/N</td>
</tr>
<tr>
<td>5</td>
<td>Mother</td>
<td>IVSI-1/N</td>
<td>False-positive (IVSI-1)</td>
<td>IVSI-745/N</td>
</tr>
<tr>
<td>6</td>
<td>Mother</td>
<td>IVSI-1/N</td>
<td>IVSI-1</td>
<td>IVSI-1/N</td>
</tr>
<tr>
<td>7</td>
<td>Mother</td>
<td>IVSI-1/N</td>
<td>IVSI-1</td>
<td>IVSI-1/codon 39</td>
</tr>
</tbody>
</table>

(continued)

**Table 2. Comparison Between the Analysis of Circulating Fetal DNA and Invasive Procedure (Chorionic Villus Sampling)**

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mits a selective enrichment of the fetal DNA species. Nevertheless, we have determined that despite this step, the reliable detection of fetal mutant alleles involving point mutations requires an additional safe-guard to ensure their optimal analysis by allele-specific PCR. In our case, this was achieved by using a PNA probe that binds with high affinity to the normal allele, thereby suppressing the amplification of maternal wild-type sequences. We showed that the combination of these 2 procedures permits the ready detection of paternally inherited fetal mutant alleles for 4 common β-thalassemia mutations.

Our study indicates that an analysis of 32 maternal blood samples, taken at 10 to 12 weeks of gestation, resulted in only 1 misdiagnosis, provided the 2 cases classified as uncertain, as well as the case with no clinical diagnostic result, are excluded. This yielded a sensitivity of 100% and a specificity of 93.8%. The goal of 100% sensitivity is far-reaching, as even the analysis of simple fetal genetic loci completely absent from the maternal genome, such as the RhD gene in pregnancies at risk for hemolytic disease of the newborn, cannot be achieved with 100% sensitivity on first trimester samples, with 2 of 9 RhD fetuses not being detected correctly.

The 1 false-positive test result observed for the detection of the IVSI-1 mutant allele was probably due to an insufficient amount of sample DNA (<50 genome-equivalents), and these conditions may lead to abnormal amplification.

Table 2. Comparison Between the Analysis of Circulating Fetal DNA and Invasive Procedure (Chorionic Villus Sampling) (cont)

<table>
<thead>
<tr>
<th>Case</th>
<th>Patient Sex</th>
<th>Genotype</th>
<th>Circulating Fetal DNA</th>
<th>Chorionic Villus Sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mother Codon 39/N</td>
<td>IVSI-6/N</td>
<td>Uncertain</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mother Codon 39/N</td>
<td>IVSI-6/N</td>
<td>No codon 39 allele</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Mother Codon 39/aa</td>
<td>IVSI-110/N</td>
<td>No codon 39 allele</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>No codon 39 allele</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>No codon 39 allele</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
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<tr>
<td>12</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
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<td>13</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
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<tr>
<td>14</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
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</tr>
<tr>
<td>15</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Mother Codon 39/N</td>
<td>IVSI-110/N</td>
<td>Codon 39</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: N, wild-type allele.

Table 3. Summary of the Results of the 4 Mutant Alleles by the Analysis of Circulating Fetal DNA

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. of Cases</th>
<th>Presence of Paternal Mutation</th>
<th>Absence of Paternal Mutation</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSI-1</td>
<td>7</td>
<td>4/4</td>
<td>2/3</td>
<td>100</td>
<td>66</td>
</tr>
<tr>
<td>IVSI-6</td>
<td>4</td>
<td>2/2</td>
<td>2/2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IVSI-110</td>
<td>5</td>
<td>3/3</td>
<td>2/2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Codon 30*</td>
<td>15</td>
<td>4/5†</td>
<td>9/10†</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>Total (including uncertain cases)</td>
<td>31</td>
<td>13/14</td>
<td>15/17</td>
<td>92.8</td>
<td>88.2</td>
</tr>
<tr>
<td>Total (excluding uncertain cases)</td>
<td>29</td>
<td>13/13</td>
<td>15/16</td>
<td>100</td>
<td>93.8</td>
</tr>
</tbody>
</table>

*One case excluded due to lack of a diagnostic test result at the time of analysis.
†Two cases classified as uncertain.
tion during the PNA-clamping process. Alerted that low-input DNA levels may be a problem, our subsequent analysis of 16 samples at risk for paternal inheritance for a codon 39 mutation contained 3 cases that were identified as uncertain because the quantity of the target DNA was low and on this basis reliable diagnostic results could not be determined. One of these cases had to be excluded from our analysis due to lack of a diagnostic result at the time of our data set preparation.

Our method also functioned in samples that had been shipped as whole blood, indicating that this approach probably would be suited for the analysis of clinical samples, which frequently have to be sent to specialized laboratories for analysis. In our opinion, a 20-mL maternal blood sample should be sufficient to permit an efficacious analysis.

In the context of detecting paternally inherited fetal point mutations, the use of mass spectrometry for the analysis of fetal point mutations from circulating plasma DNA has recently been reported. Although the determination of the presence of the paternal \( \beta \)-globin gene point mutation could be determined with high degrees of accuracy, these results were based on only 5 pertinent cases. An advantage of the mass spectrometry approach over our analysis is that it does not require any additional processing of the sample, such as size-fractionation. It is also much more amenable to high-throughput automated analysis. A disadvantage of this alternative development is that it requires sophisticated and expensive equipment not readily available to the majority of diagnostic or research laboratories.

Our approach, in contrast, is relatively simple and can be performed without the need for complex machinery, as it relies on technologies consistent with those currently used in many routine diagnostic and research laboratories. Moreover, we have estimated that the cost of the single analysis may be as low as US $8. This low-cost and use of simple equipment is especially suitable for the screening of at-risk pregnancies in developing countries. This method is also useful for detection of other fetal single-gene mutations, such as achondroplasia.

Size-fractionation may potentially provide an alternative approach for the noninvasive prenatal assessment of fetal single-gene disorders involving compound heterozygous mutations. The fetal genotype in those cases in which both partners are carriers for the same disease allele, frequently the case for cystic fibrosis, could be determined by an analysis of paternally and maternally inherited single-nucleotide polymorphisms associated with the mutant allele, as had recently been shown for a similar case at risk for \( \beta \)-thalassaemia. In the near future, the approach we have outlined may bring this desired goal in prenatal medicine closer to a clinical application.

Author Contributions: Dr Li had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Li, Di Naro, Vitucci, Holzgreve, Hahn. Acquisition of data: Li, Di Naro, Vitucci, Zimmermann. Analysis and interpretation of data: Li, Zimmermann, Hahn. Drafting of the manuscript: Li, Hahn. Critical revision of the manuscript for important intellectual content: Li, Di Naro, Vitucci, Holzgreve, Hahn. Statistical analysis: Li. Obtained funding: Holzgreve, Hahn. Administrative, technical, or material support: Di Naro, Vitucci, Zimmermann, Hahn. Study supervision: Hahn.

Financial Disclosures: Drs Li, Zimmerman, Holzgreve, and Hahn have filed for a patent covering the use of size-fractionated DNA for noninvasive prenatal diagnosis. This patent is owned by the University Hospital, Basel, Switzerland. Dr Di Naro and Vitucci reported no financial disclosures.

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REFERENCES


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of serving bowl size on consumption was statistically significant for men ($P = .02$) but not women ($P = .17$).

In the sensitivity analysis to estimate the potential impact of the 5 nonparticipants, the effect of bowl size remained significant ($P = .02$).

Comment. Small environmental factors can have a large influence on food consumption. At this party, large serving bowls led to a 56% greater intake (a mean of 142 more calories/person). The size of a serving bowl (or of a portion) may provide a consumption cue that implicitly suggests an appropriate amount to eat. Larger bowls, like larger packages or portions, may suggest that a proportionately larger amount is appropriate to consume. Although this study was not conducted in a medical setting, it is possible that if a physician giving diet-related advice recommends using smaller serving bowls, patients may serve themselves smaller portions.

Portion distortion has generally focused on how consumption cues lead people to overeat less healthy, energy-dense foods. An appropriate area for further research is whether these same cues, ie, larger serving bowls, can be used to encourage people to eat greater amounts of healthier foods such as fruits and vegetables.

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CORRECTIONS

Incorrect Data: In the Clinical Review entitled “A Simplified Approach to the Management of Non–ST-Segment Elevation Acute Coronary Syndromes” published in the January 19, 2005, issue of JAMA (2005;293:349-357), incorrect data were reported. In the “Anticoagulation” rows of the Table on Page 352, “creatinine clearance <60 mL/min” should have been reported as “<30 mL/min.” Also, in the center column on page 353, “creatinine clearance <60 mL/min [1.0 mL/s]” should have been reported as “<30 mL/min [0.5 mL/s].”

Incorrect Information: In the Medical News & Perspectives article “Michael E. DeBakey: Father of Modern Cardiovascular Surgery” published in the February 23, 2005, issue of JAMA (2005;293:913-918), President John F. Kennedy was erroneously described as one of the world leaders who were treated by DeBakey. DeBakey worked with Kennedy on medical legislation for Medicare.

Reference Error: In the Review entitled “Bariatric Surgery: A Systematic Review and Meta-analysis” published in the October 13, 2004, issue of JAMA (2004;292:1724-1737), there was a reference error. The Swedish Obese Subjects Intervention Study has not published any of its mortality data. On page 1736, column 2, first full paragraph, sentences 4 and 5 should be deleted. Sentence 6 should be “MacDonald et al reported that diabetic patients treated with an oral hypoglycemic had a 4.5% annual mortality rate for 9 years of follow-up compared with a 1% mortality rate in diabetic patients who underwent gastric bypass.”

Error in Table: In the Preliminary Contribution entitled “Detection of Paternally Inherited Fetal Point Mutations for β-Thalassemia Using Size-Fractionated Cell-Free DNA in Maternal Plasma” published in the February 16, 2005, issue of JAMA (2005;293:843-849), information (3 columns) was switched for mother and father. For example, in case 1 for paternal IVSI-1 mutation, “Codon 39/N” and “IVSI-1” and “IVSI-1/N” should be in the row with “Mother,” and “IVSI-1/N” should be in the row with “Father” in that order. The subsequent rows of genotype and results information should be switched for each case for the rest of the Table. Also, on page 848, the column heading “Patient Sex” should read “Parent.”