Clinical Chemistry 62:6
848–855 (2016)

Cell-Free DNA Analysis of Targeted Genomic Regions in Maternal Plasma for Non-Invasive Prenatal Testing of Trisomy 21, Trisomy 18, Trisomy 13, and Fetal Sex

George Koumbaris,[1] Elena Kypri,[1] Kyriakos Tsangaras,[1] Achilleas Achilleos,[1] Petros Mina,[1] Maria Neofytou,[2] Voula Velissariou,[1] Georgia Christopoulou,[1] Ioannis Kallikas,[3] Alicia González-Liñán,[4] Egle Benusiene,[5] Anna Latos-Bielenska,[6] Pietryga Marek,[7] Alfredo Santana,[8] Nikoletta Nagy,[9] Márta Széll,[9] Piotr Laudanski,[10] Elisavet A. Papageorgiou,[1] Marios Ioannides,[1] and Philippos C. Patsalis[1,2*]

BACKGROUND: There is great need for the development of highly accurate cost effective technologies that could facilitate the widespread adoption of noninvasive prenatal testing (NIPT).

METHODS: We developed an assay based on the targeted analysis of cell-free DNA for the detection of fetal aneuploidies of chromosomes 21, 18, and 13. This method enabled the capture and analysis of selected genomic regions of interest. An advanced fetal fraction estimation and aneuploidy determination algorithm was also developed. This assay allowed for accurate counting and assessment of chromosomal regions of interest. The analytical performance of the assay was evaluated in a blind study of 631 samples derived from pregnancies of at least 10 weeks of gestation that had also undergone invasive testing.

RESULTS: Our blind study exhibited 100% diagnostic sensitivity and specificity and correctly classified 52/52 (95% CI, 93.2%–100%) cases of trisomy 21, 16/16 (95% CI, 79.4%–100%) cases of trisomy 18, 5/5 (95% CI, 47.8%–100%) cases of trisomy 13, and 538/538 (95% CI, 99.3%–100%) normal cases. The test also correctly identified fetal sex in all cases (95% CI, 99.4%–100%). One sample failed prespecified assay quality control criteria, and 19 samples were nonreportable because of low fetal fraction.

CONCLUSIONS: The extent to which free fetal DNA testing can be applied as a universal screening tool for trisomy 21, 18, and 13 depends mainly on assay accuracy and cost. Cell-free DNA analysis of targeted genomic regions in maternal plasma enables accurate and cost-effective noninvasive fetal aneuploidy detection, which is critical for widespread adoption of NIPT.

The discovery of free fetal DNA (ffDNA)[11] in maternal circulation *(1)* marked the beginning of the noninvasive prenatal testing (NIPT) era, and allowed the development of the first noninvasive prenatal tests. ffDNA has been successfully used for the determination of fetal sex and fetal rhesus D status in maternal plasma *(2, 3)*. These methods have become routine in a number of clinical laboratories worldwide. However, direct analysis of the limited amount of ffDNA in the presence of an excess of maternal DNA presents a great challenge for NIPT.

The percentage of ffDNA in maternal circulation was originally estimated to be 3%–6% of the total cell free DNA *(4)*. However, recent studies suggest that fetal fraction can be as high as 10%–20% *(5)*. The presence of such high amounts of maternal DNA in maternal circulation in relation to the limited amount of fetal DNA poses a major challenge for the quantification of fetal DNA and the detection of fetal aneuploidies.

Over the last decade a large number of different methods have been applied to allow the discrimination or enrichment of ffDNA from circulating maternal DNA *(6)*. The DNA-based approaches include sequencing and epigenetics based assays, which focus on the investigation of the methylation status of fetal DNA either using sodium bisulfite DNA treatment *(7)*, methylation-sensitive restriction enzymes, or antibodies specific to the

[1] NIPD Genetics, Ltd, Nicosia, Cyprus; [2] The Cyprus Institute of Neurology and Genetics; [3] AAK Ultrasound and Fetal Medicine Center, Nicosia, Cyprus; [4] Andros Day Surgery Clinic, Reproductive Medicine Unit. Palermo, Italy; [5] Department of Human and Medical Genetics, Faculty of Medicine, Vilnius University, Vilnius, Lithuania; [6] Center for Medical Genetics Genesis, Poznan, Poland; [7] Gynecology and Obstetrics, Poznan University of Medical Sciences, Poland; [8] Clinical Genetics, Childhood Hospital Materno-Infantil, Las Palmas GC, Canary Islands, Spain; [9] Department of Medical Genetics, University of Szeged, Szeged, Hungary; [10] Department of Perinatology and Obstetrics Medical University of Bialystok, Poland.

* Address correspondence to this author at: NIPD Genetics, Ltd, Nicosia, Neas Engomis 31 Nicosia, NA, Cyprus 2409. Fax +357-22-266-899; e-mail p.patsalis@nipd.com.

Received November 26, 2015; accepted March 21, 2016.
Previously published online at DOI: 10.1373/clinchem.2015.252502

[11] Nonstandard abbreviations: ffDNA, free fetal DNA; NIPT, noninvasive prenatal testing; NGS, next generation sequencing; T21, trisomy 21; T18, trisomy 18; T13, trisomy 13; TACS, target capture sequences; BAM, binary; tririsk, trisomy risk; CV, chorionic villus; FN, false negative; FP, false positive; TFM, true fetal mosaicism; CPM, confined placental mosaicism.

5-methylcytosine residues of CpG dinucleotides across the genome *(8–10)*. Alternative approaches have targeted fetal-specific mRNA *(11)* or have focused on the investigation of fetal-specific proteins *(12)*.

The use of next generation sequencing (NGS) technologies in NIPT has revolutionized the field. In 2008, 2 independent groups demonstrated that NIPT of trisomy 21 (T21) could be achieved using massively parallel shotgun sequencing *(13, 14)*, ushering in a new era of NIPT and opening new possibilities for the use of these technologies in clinical practice. On the basis of these findings, biotechnology companies and independent groups initiated clinical studies and developed new NIPT tests *(15–21)*.

More recently, targeted NGS approaches, in which only specific sequences of interest are used, have been developed. A single nucleotide polymorphism–based NGS approach involving multiplex targeted amplification and analysis of single nucleotide polymorphisms and a quantitative NGS approach that uses ligated probes that are then amplified and sequenced have been described *(20, 21)*. Targeted approaches have the potential to increase throughput and reduce cost because they require substantially less sequencing than whole genome sequencing approaches.

Nevertheless, the development of even more accurate, cost-effective NIPT methods is greatly needed. In particular, approaches that can target specific sequences of interest, thereby reducing the amount of sequencing needed compared to whole genome–based approaches, can be extremely advantageous. Here we present a highly accurate and cost-effective method for the detection of fetal trisomies 21, 18, and 13, which overcomes many of the limitations of the current NIPT technologies.

Materials and Methods

SAMPLE COLLECTION

Plasma samples were obtained anonymously from pregnant women of at least 18 years of age from the 10th week of gestation. Only singleton pregnancies were analyzed. Protocols used for sample collection were approved by the National Bioethics Committees and informed consent was obtained from all participants. Referring centers were provided with all relevant information about eligibility criteria, benefits, and limitations of participating in this study *(22)*. The aneuploid cases enrolled in this study consisted of T21, trisomy 18 (T18), and trisomy 13 (T13) pregnancies, which were confirmed via invasive testing.

SAMPLE COLLECTION AND PREPARATION

A mean of 8 mL of peripheral blood was collected from each subject into EDTA-containing tubes. A mean of 4 mL of plasma was isolated via a double centrifugation protocol of 1600 × *g* for 10 min, followed by 16 000 × *g* for 10 min. Plasma samples were given a unique identifier and were stored at −80 °C until subsequent analysis. ffDNA was extracted from 4 mL plasma using the Qiasymphony DSP Virus/Pathogen Midi Kit (Qiagen).

Table 1. Blind validation study results.

Karyotype	No. of samples	Correct call
Normal	538	538 (100%, 99.3–100)
Trisomy 21	52	52 (100%, 93.2–100)
Trisomy 18	16	16 (100%, 79.4–100)
Trisomy 13	5	5 (100%, 47.8–100)
Sex determination	611	611 (100%, 99.4–100)

SEQUENCING LIBRARY PREPARATION

Extracted DNA was processed using standard library preparation methods with minor modifications *(23)*. Negative-control libraries were also prepared. In summary, 5′ and 3′ overhangs were filled-in using T_4 polymerase (NEB) and 5′ phosphates were attached using T_4 polynucleotide kinase (NEB). Reaction products were purified using the MinElute kit (Qiagen). Subsequently, sequencing adaptors were ligated to both ends of the DNA using T_4 DNA ligase (NEB), followed by purification using the MinElute kit (Qiagen). Nicks were removed in a fill-in reaction using Bst polymerase (NEB) with subsequent incubation at 65 °C for 25 min and then 12 °C for 20 min. Library amplification was performed using Fusion polymerase (Agilent Technologies) and all samples were assigned a unique barcode. Sequencing library products were purified using the MinElute purification kit (Qiagen).

DESIGN AND PREPARATION OF TARGET CAPTURE SEQUENCES

Custom target capture sequences (TACS) of approximately 250 bp were designed to capture selected loci on chromosomes 21, 18, 13, and Y (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue6). The genomic target-loci were selected on the basis of GC content, distance from repetitive elements, and absence of surrounding complex genomic architecture. The TACS were prepared by polymerase chain reaction using MyTaq polymerase (Bioline) and primers designed to amplify the target-loci in normal DNA. Amplicons were verified by agarose gel electrophoresis and were purified using standard PCR clean up kits such as the Qiaquick PCR purification kit (Qiagen) or the NucleoSpin 96 PCR clean-up kit (Macherey

Nagel). TACS concentration was measured using the Nanodrop spectrophotometer (Thermo Scientific).

TACS were pooled equimolarly, and were blunt ended using the Quick Blunting kit (NEB). Following purification using the MinElute kit (Qiagen), they were biotinylated using the Quick Ligation Kit (NEB) and were purified using the MinElute kit (Qiagen). The TACS (1500 ng) were then immobilized on streptavidin-coated magnetic beads (Invitrogen) as previously described *(24)*.

HYBRIDIZATION

Amplified libraries were mixed with hybridization buffer (Agilent), blocking agent (Agilent), blocking oligonucleotides *(25)*, Cot-1 DNA (Invitrogen), and salmon sperm DNA (Invitrogen). Sequencing library hybridization mixtures were then denatured at 95 °C for 3 min and were incubated at 37 °C for 20 min before being added to the biotinylated TACS. The samples were then incubated for 12–48 h at 66 °C and were washed as previously described *(24)*. Captured sequences were eluted by heating. Eluted sequences were amplified using outer-bound adaptor primers. Enriched amplified products were pooled equimolarly and were sequenced on a MiSeq, NextSeq 500, or Hiseq 2500 sequencing platform (Illumina).

DATA ANALYSIS

Alignment to the human reference genome. Paired-end read fragments of each sample were processed using the Cutadapt software *(26)* to remove adaptor sequences and poor-quality reads. The remaining sequences were aligned to the human reference genome build hg19 (UCSC Genome Bioinformatics) using the Burrows-Wheeler alignment algorithm *(27)*. The Picard tools software suite [Broad Institute (2015) *Picard*] was used to remove duplicate read entries and convert aligned reads to a binary (BAM) file containing uniquely aligned read entries. Per base read-depth information was retrieved from this final BAM file using the SAMtools software suite. Single nucleotide polymorphism information across the targeted sequences was obtained using the bcftools suite of functions and the vcfutils.pl script, which accompany the SAMtools software suite *(27)*.

Classification of fetal aneuploidy. The sequencing procedure introduces read-depth discrepancies across many regions of interest. This bias is in part dependent on the GC-content of each sequenced region *(28)*. GC-bias alleviation was achieved by estimating each region's GC content and subsequently grouping the read-depth of similar GC-content regions together to create matching groups. Matching groups from the test chromosome were compared to the corresponding matching groups on the reference chromosomes using 3 statistical tests: a paired *t*-test, a bivariate nonparametric bootstrap, and a stratified permutation test. The score obtained from each method was used to calculate a weighted sum. To account for run-to-run bias *(29)*, each weighted sum was normalized by subtracting the run-specific median and then dividing by a multiple of the empirical standard deviation of euploid samples. The run-specific median was calculated from the weighted sums of all samples in a sequencing run. The theoretical variance of the random variable denoted by the weighted sum of the 3 methods was estimated from a training set of 100 euploid samples. This normalized score was used to estimate the trisomy risk (tririsk) of each sample. Scores above a specific threshold were classified as high-risk for trisomy.

Estimation of fetal fraction. A finite (binomial) mixture model based on Bayesian inference *(30)* was developed and used to compute the posterior distribution of fetal DNA fraction using allelic counts at heterozygous loci in maternal plasma. Three possible informative combinations of maternal/fetal genotypes were used within the model to identify fetal DNA fraction values that were strongly supported by the observed data. The posterior distribution of fetal fraction was calculated using a Metropolis-Hastings algorithm *(31)*. The lower bound of the 95% credible interval of this posterior probability distribution was subsequently inferred.

Results

A total of 631 plasma samples were analyzed in this blind study, including 52 T21, 16 T18, and 5 T13 pregnancies from women who had undergone invasive procedures (Fig. 1). One sample did not pass the sequencing library quality control criteria and was excluded from the analysis. Another 19 samples (14 normal, 3 T18, 2 T13) exhibited an insufficient ffDNA fraction of $<4\%$ and were excluded from the analysis. Fig. 2 summarizes the demographic characteristics of all 631 samples. The median maternal age was 36 years, the median maternal weight was 63 kg, and the median gestational age was 16 weeks.

Tririsk scores for T21, T18, and T13 were assigned to the 611 samples that passed all quality control criteria (Fig. 3). Samples with a tririsk score exceeding a threshold of 1 were classified by the classification analysis algorithm as trisomic. *A posteriori* analysis of the validation data set suggested that this threshold could be as low as 0.91 (see online Supplemental Fig. 1). T21 was detected in 52/52 cases (95% CI, 93.2% to 100%) (Fig. 3A). T18 was detected in 16/16 cases (95% CI, 79.4% to 100%) (Fig. 3B) and T13 was detected in 5/5 cases (95% CI, 47.8% to 100%) (Fig. 3C). These results are summarized in Table 1.

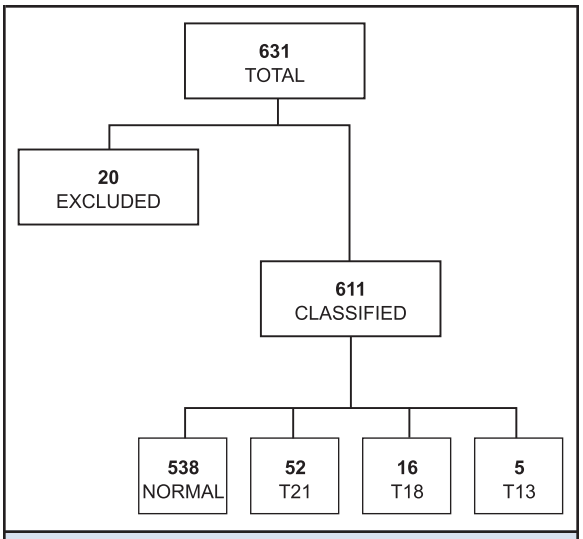

Fig. 1. Flow diagram displaying sample information.
Nineteen samples were excluded from the analysis because of low fetal fraction, and 1 sample was excluded because of technical reasons. The remaining cohort of 611 samples consisted of 538 normal samples, 52 T21 samples, 16 T18 samples, and 5 T13 samples.

The fetal fraction distribution of all cases can be seen in online Supplemental Fig. 2. The mean fetal fraction of all samples was 10.9% with an SD of 4.1%. As shown in Fig. 4, there was no association between fetal fraction and tririsk scores in normal samples (Pearson correlation test p-values >0.4 for all 3 aneuploidy tests), although there was a clear association between these variables in trisomic samples. Specifically, a Pearson correlation test evaluating the association between tririsk scores and fetal fraction in T21, T18, and T13 samples resulted in p-values of 0.0014, 0.0002, and 0.0164 respectively.

Discussion

This study used a targeted assay that employed target capture sequences and a novel analytical algorithm to detect fetal trisomies 21, 18, and 13. In a blind validation study, which included 631 pregnant women of at least 10 weeks of gestation, the assay results exhibited 100% diagnostic sensitivity and specificity and correctly classified 52/52 cases of T21, 16/16 cases of T18, and 5/5 cases of T13, in all samples that passed quality control criteria ($n = 611$). The test also correctly identified fetal sex in all cases.

In this study we focused our analysis on chromosomes 21, 18, and 13, and determined that an optimized set of approximately 1500 loci was sufficient to enable highly accurate fetal aneuploidy detection. We also tested alternative sets consisting of fewer TACS and/or TACS of variable GC content. These experiments allowed us to determine that the most important technical factor affecting the performance of the assay was the number of TACS on different chromosomes that exhibited similar GC-content characteristics, thus allowing for more robust GC-bias correction. We observed that this was more pronounced on chromosome 18, where 1 T18 sample was classified as normal when sets of TACS that were not optimally matched for GC-content were used. These results indicated that the assay was sensitive to TACS GC-content differences, and enabled us to construct an optimal set of TACS on chromosomes 21, 18, and 13 that resulted in the correct classification of all normal and trisomic cases (Fig. 3).

Our assay employs a robust analysis algorithm that minimizes random and systemic variation between sequencing runs and is sensitive enough to distinguish between euploid and aneuploid samples. There is a clear separation between the risk scores of trisomic and disomic samples (Fig. 3), allowing a binary classification scheme.

The targeted test described here constitutes an integrated assay that incorporates simultaneous determination of fetal fraction and accurate detection of fetal aneuploidies. The algorithm uses a Bayesian approach to estimate fetal DNA fraction. As such, additional information can be easily incorporated into the model. In addition, instead of inferring a point estimate of fetal DNA fraction, the algorithm calculates the posterior distribution of the fetal DNA fraction in each sample. It subsequently uses the lower bound of the corresponding 95% credible interval to determine whether a sample has adequate fetal fraction. This conservative approach of estimating fetal fraction ensures that the lowest possible fetal fraction of each sample is considered for classification purposes, thus minimizing the possibility of incorrect calls that could potentially arise from low proportions of fetal DNA. This novel fetal fraction estimation algorithm was also independently and thoroughly validated using Y-chromosome loci in male samples. The fetal fraction estimation algorithm was also tested using nonpregnant samples. The algorithm correctly identified the absence of fetal DNA in these samples.

This study identified 3 T18 and 2 T13 samples that had low fetal fraction. This further illustrates the need for accurate fetal fraction estimation in NIPT to avoid false negative (FN) results *(32)*. The targeted assay described here is inherently characterized by high depth of sequencing, which allows highly accurate fetal fraction quantification and aneuploidy detection. In the clinical setting it is of paramount importance that low fetal fraction

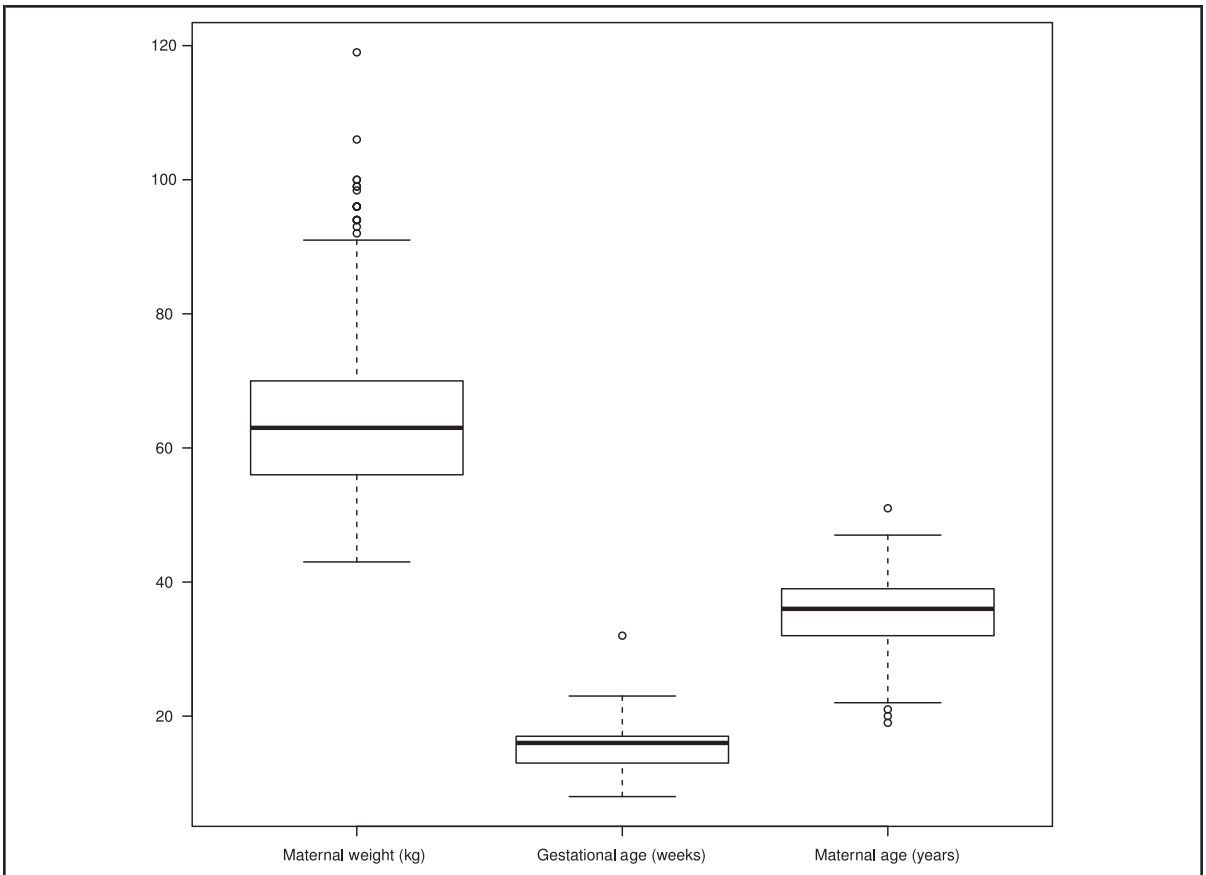

Fig. 2. Tukey boxplots illustrating demographic characteristics of the 631 samples.

The bottom and top of each box represent the interquartile range (IQR) of the described characteristic and the band inside the box represents the median value. The "whiskers" of each box illustrate the range of data found within 1.5×IQR. Values greater than 1.5×IQR are illustrated as empty circles.

samples are identified so that a redraw sample is requested for reanalysis.

The current study evaluated samples from multiple centers in the form of a simple streamlined assay that can be easily implemented in a clinical setting. Future work will focus mainly on first trimester samples and low-risk pregnancies, because NIPT tends to gradually migrate from second to first trimester screening, and from high to intermediate and low-risk pregnancies. Our data suggests that this assay will exhibit the same exceptional accuracy in both low and high risk pregnancies.

The targeted noninvasive prenatal assay described here has several advantages compared to whole genome sequencing methods. Whole genome sequencing requires a very large number of reads and only allows the simultaneous analysis of very few samples. The inherently limited throughput of whole genome methods imposes a significant financial and logistical burden. In contrast, the targeted method described here uses only specific genomic regions and significantly reduces the number of required reads. This results in a dramatic increase in efficiency and a significant reduction in overall costs. At the same time, the enrichment of only specific genomic regions allows for optimal GC-bias correction and enables high enrichment levels, which result in very accurate aneuploidy detection. The targeted nature of the assay also ensures extremely high accuracy by enabling robust fetal fraction estimation and by avoiding copy number variants or other complex genomic architectural elements which can cause false positive (FP) or FN results *(33, 34)*.

Although NIPT has major advantages compared to conventional screening approaches, a number of challenges remain. It has been noted that feto-placental mosaicism can result in discordant findings between NIPT and fetal karyotyping *(35)*. Chromosomal mosaicism in chorionic villus samples is detected in 1%–2% of cases,

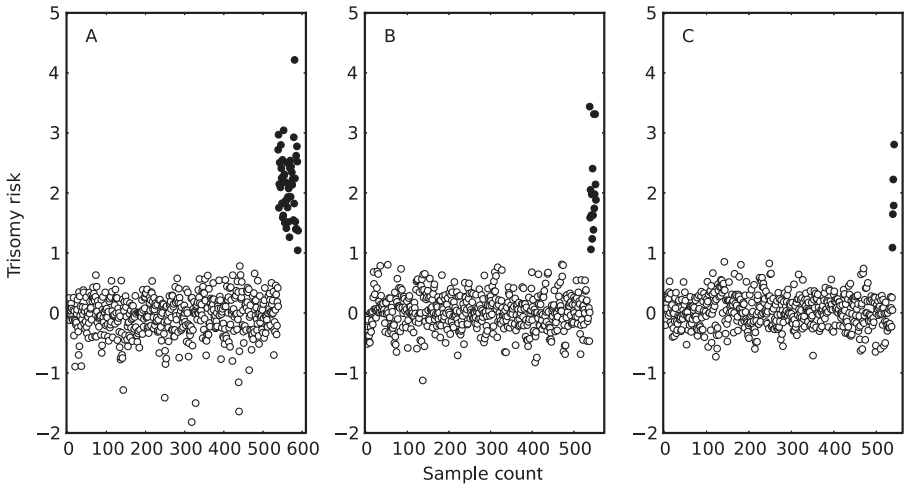

Fig. 3. Tririsk scores of the 611 classified samples.

T21 cases (A) T18 cases (B) and T13 cases (C). Normal samples are shown as empty circles, trisomic samples as black circles.

and can involve different numerical and structural chromosomal abnormalities and feto-placental lineages *(36)*. True fetal mosaicism (TFM) is confirmed in only 13% of these cases, whereas in 87% the chromosomal abnormality is confined to the placenta [confined placental mosaicism (CPM)] *(37)*. It is known that ffDNA circulating in maternal plasma originates from apoptosis of the cells of the outer layers of the placenta, i.e., the cytotrophoblast and syncytiotrophoblast cells *(38)*. Cases of mosaicism, in which the chromosomal constitution of the cytotrophoblast is different from that of the fetus, are potential sources of FP and FN results. CPM type I and III with an abnormal cytotrophoblast and normal amniocytes can cause FP results, whereas TFM type V with a normal cytotrophoblast and abnormal amniocytes can cause FN results *(36)*. The largest monocentric study

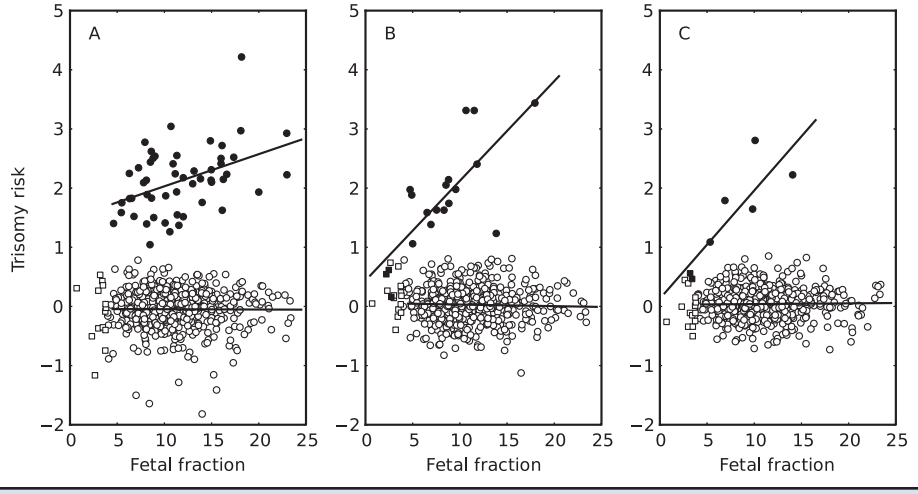

Fig. 4. Association of fetal fraction with tririsk scores in normal and trisomic samples.

T21 samples (A), T18 samples (B), T13 samples (C). Normal samples of sufficient fetal fraction for analysis are shown as empty circles and normal samples of insufficient fetal fraction for analysis (<4%) are shown as empty squares. Trisomic samples of sufficient fetal fraction for analysis are shown as black circles and trisomic samples of insufficient fetal fraction for analysis (<4%) are shown as black squares. Line of best fit illustrates the lack of association between tririsk score and fetal fraction in normal samples and conversely the presence of association between tririsk score and fetal fraction in trisomic samples.

examining chromosomal mosaicism in relation to NIPT results includes a retrospective audit of 52673 chorionic villus samples in which cytogenetic analysis of the cytotrophoblast (direct) and villus mesenchyme (culture) was performed, followed by confirmatory amniocentesis in chorionic villi mosaic cases *(36)*. According to this study the combined FP rate for T13, T18, and T21, would be 1 in 3006 cases, and the FN rate would be 1 in 107. Because both T13 and T18 pregnancies are highly likely to have abnormalities detectable by ultrasound investigation and will spontaneously abort between 12 weeks and term *(39)*, the main concern remains for FP and FN T21 results. Taking into consideration the incidence of T21 in the general population *(40)* and the incidence of TFM type V *(36)*, the number of FN T21 cases is estimated to be approximately 1 in 100 000 NIPTs. Also, assuming that at least 70% CPM is needed to produce a FP T21 result *(36)*, the FP T21 rate would be approximately 1 in 13 000. Although these figures are very low, it is important to be aware of the genetic physiology of the placenta and the limitations it imposes on NIPT when contemplating its integration into safe clinical prenatal care.

A major objective in the field of prenatal testing is the reduction of the number of unnecessary invasive procedures. ffDNA testing can significantly reduce procedure-related losses while maintaining high detection rates. It provides clinicians and prospective parents with a powerful tool to help them make informed decisions regarding the need for an invasive procedure, without posing any risk to the pregnancy. The clinical impact of ffDNA testing has been significant as indicated by its quick adoption in prenatal care. The extent to which ffDNA testing can be applied as a universal screening tool for T21, 18, and 13 depends mainly on assay accuracy, low number of nonreportable tests, and cost. In this study we presented the development and validation of a novel, cost-effective and exceptionally accurate method for NIPT of aneuploidies of chromosomes 21, 18, and 13 and fetal sex.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: G. Koumbaris, NIPD Genetics; E. Kypri, NIPD Genetics, Ltd, Nicosia, Cyprus; K. Tsangaras, NIPD Genetics; A. Achilleos, NIPD Genetics; P. Mina, NIPD Genetics; 1. A. Latos-Bielenska, Department of Medical Genetics, Poznan University of Medical and Centers of Medical Genetics Genesis; E.A. Papageorgiou, NIPD Genetics Ltd; M. Ioannides, NIPD Genetics Ltd; P.C. Patsalis, NIPD Genetics, Nicosia, Cyprus.
Consultant or Advisory Role: None declared.
Stock Ownership: G. Koumbaris, NIPD Genetics Stock option; E. Kypri, NIPD Genetics, Ltd, Nicosia, Cyprus; K. Tsangaras, NIPD Genetics; A. Achilleos, NIPD Genetics; P. Mina, NIPD Genetics; E.A. Papageorgiou, NIPD Genetics Ltd; P.C. Patsalis, NIPD Genetics, Nicosia, Cyprus.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Patents: G. Koumbaris, Patent number: Provisional Application No. 62/165,593/; E. Kypri, Patent number 62/165,593; K. Tsangaras, Patent number: Provisional Application No. 62/165,593/; P. Mina, Patent number: Provisional Application No. 62/165,593; E.A. Papageorgiou, Patent number: 62/165,593; P.C. Patsalis, Patent number: 62/165,593.
Other Remuneration: E.A. Papageorgiou, NIPD Genetics Ltd.

Role of Sponsor: No sponsor was declared.

Acknowledgments: We thank Mitera Hospital, Athens, Greece, for recruiting participants for this study during the period 2009–2013.We also thank Jacqueline Donoghue-Nadalis, cytogeneticist, for her valuable comments and suggestions.

References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–7.
2. Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. N Engl J Med 1998;339:1734–8.
3. Bianchi DW, Avent ND, Costa JM, van der Schoot CE. Noninvasive prenatal diagnosis of fetal Rhesus D: ready for Prime (r) Time. Obstet Gynecol 2005;106: 841–4.
4. Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet 1998;62:768–75.
5. Lun FM, Chiu RW, Chan KC, Leung TY, Lau TK, Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008;54:1664–72.
6. Chan KC, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, et al. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem 2004;50:88–92.
7. Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, Lo YM. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 2008; 54:482–90.
8. Papageorgiou EA, Fiegler H, Rakyan V, Beck S, Hulten M, Lamnissou K, et al. Sites of differential DNA methylation between placenta and peripheral blood: molecular markers for noninvasive prenatal diagnosis of aneuploidies. Am J Pathol 2009;174:1609–18.
9. Papageorgiou EA, Karagrigoriou A, Tsaliki E, Velissariou V, Carter NP, Patsalis PC. Fetal specific DNA methylation ratio permits non-invasive prenatal diagnosis of trisomy 21. Nat Med 2011;17:510–3.
10. Papageorgiou EA, Koumbaris G, Kypri E, Hadjidaniel M, Patsalis PC. The epigenome view: an effort towards non-invasive prenatal diagnosis. Genes (Basel) 2014; 5:310–29.
11. Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS, et al. mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 2003;100: 4748–53.
12. Avent ND, Plummer ZE, Madgett TE, Maddocks DG, Soothill PW. Post-genomics studies and their application to non-invasive prenatal diagnosis. Semin Fetal Neonatal Med 2008;13:91–8.
13. Chiu RW, Chan KC, Gao Y, Lau VY, Zheng W, Leung TY, et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci U S A 2008;105:20458–63.
14. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl

Acad Sci U S A 2008;105:16266–71.
15. Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet Med 2011;13: 913–20.
16. Ehrich M, Deciu C, Zwiefelhofer T, Tynan JA, Cagasan L, Tim R, et al. Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: a study in a clinical setting. Am J Obstet Gynecol 2011;204: 205 e1–11.
17. Chen EZ, Chiu RWK, Sun H, Akolekar R, Chan KCA, Leung TY, et al. Noninvasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma DNA sequencing. PLoS One 2011;6:e21791.
18. Sehnert AJ, Rhees B, Comstock D, de Feo E, Heilek G, Burke J, Rava RP. Optimal detection of fetal chromosomal abnormalities by massively parallel DNA sequencing of cell-free fetal DNA from maternal blood. Clin Chem 2011;57:1042–9.
19. Zimmermann B, Hill M, Gemelos G, Demko Z, Banjevic M, Baner J, et al. Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. Prenat Diagn 2012;32:1233–41.
20. Nicolaides KH, Syngelaki A, Gil M, Atanasova V, Markova D. Validation of targeted sequencing of single-nucleotide polymorphisms for non-invasive prenatal detection of aneuploidy of chromosomes 13, 18, 21, X, and Y. Prenat Diagn 2013;1–5.
21. Sparks AB, Wang ET, Struble CA, Barrett W, Stokowski R, McBride C, et al. Selective analysis of cell-free DNA in maternal blood for evaluation of fetal trisomy. Prenat Diagn 2012;32:3–9.
22. Matthijs G, Souche E, Alders M, Corveleyn A, Eck S, Feenstra I, et al. Guidelines for diagnostic next-generation sequencing. Eur J Hum Genet 2016;24: 2–5.
23. Meyer M, Kircher M. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harb Protoc 2010;2010: pdb.prot5448.
24. Tsangaras K, Siracusa MC, Nikolaidis N, Ishida Y, Cui P, Vielgrader H, et al. Hybridization capture reveals evolution and conservation across the entire Koala retrovirus genome. PLoS One 2014;9:e95633.
25. Maricic T, Whitten M, Paabo S. Multiplexed DNA sequence capture of mitochondrial genomes using PCR products. PLoS One 2010;5:e14004.
26. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 2011;17:10–2.
27. Durbin RM, Abecasis GR, Altshuler DL, Auton A, Brooks LD, Gibbs RA, et al. A map of human genome variation from population-scale sequencing. Nature 2010;467: 1061–73.
28. Chen YC, Liu T, Yu CH, Chiang TY, Hwang CC. Effects of GC bias in next-generation-sequencing data on de novo genome assembly. PLoS One 2013;8:e62856.
29. Aird D, Ross MG, Chen WS, Danielsson M, Fennell T, Russ C, Jaffe DB. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biol 2011;2:R18.
30. McLachlan G, Peel D. Finite mixture models. New York: John Wiley & Sons; 2004.
31. Chistophe A. An introduction to MCMC for machine learning. Machine Learning 2003;50:5–43.
32. Benn P, Cuckle H. Theoretical performance of non-invasive prenatal testing for chromosome imbalances using counting of cell-free DNA fragments in maternal plasma. Prenat Diagn, 2014;34:778–83.
33. Snyder MW, Simmons LE, Kitzman JO, Coe BP, Henson JM, Daza RM, et al. Copy-number variation and false positive prenatal aneuploidy screening results. N Engl J Med 2015;372:1639–45.
34. Phillips ST, Freeman K, Geppert J, Agbebiyi A, Uthman OA, Madan J, et al. Accuracy of non-invasive prenatal testing using cell-free DNA for detection of Down, Edwards and Patau syndromes: a systematic review and meta-analysis. BMJ Open, 6:e010002, 2016.
35. Bianchi DW, Wilkins-Haug L. Integration of noninvasive DNA testing for aneuploidy into prenatal care: what has happened since the rubber met the road? Clin. Chem 2014;60:78–87.
36. Grati FR, Malvestiti F, Ferreira JC, Bajaj K, Gaetani E, Agrati C, et al. Fetoplacental mosaicism: potential implications for false-positive and false-negative noninvasive prenatalscreening results. Genet In Med 2014;16: 620–4.
37. Grati FR, Bajaj K, Malvestiti F, Agrati C, Grimi B, Malvestiti B, et al. The type of feto-placental aneuploidy detected by cfDNA testing may influence the choice of confirmatory diagnostic procedure, Prenat Diagn 2015;35:1–11.
38. Faas BH, de Ligt J, Janssen I, Eggink AJ, Wijnberger LD, van Vugt JM, et al. Non-invasive prenatal diagnosis of fetal aneuploidies using massively parallel sequencing-by-ligation and evidence that cell-free fetal DNA in the maternal plasma originates from cytotrophoblastic cells. Expert Opin Biol Ther 12 Suppl 1:S19–26, 2012.
39. Nicolaides KH. Screening for fetal aneuploidies at 11 to 13 weeks, Prenat Diagn 2011;31:7–15.
40. EUROCAT Website Database: http://www.eurocat-network.eu (Accessed March 2016).