

ORIGINAL ARTICLE

Implementation of non-invasive prenatal testing by semiconductor sequencing in a genetic laboratory

Annelies Dheedene¹, Tom Sante¹, Matthias De Smet¹, Jean-François Vanbellinghen², Bernard Grisart³, Sarah Vergult¹, Sandra Janssens¹ and Björn Menten^{1*}

¹Center for Medical Genetics Ghent, Ghent University Hospital, Ghent University, Ghent, Belgium

²Plateforme de Biologie Moléculaire, Département des Laboratoires, Cliniques Universitaires Saint-Luc, Bruxelles, Belgium

³Centre de Génétique Humaine, Institut de Pathologie et Génétique, Charleroi, Belgium

*Correspondence to: Björn Menten. E-mail: bjorn.menten@ugent.be

ABSTRACT

Objectives To implement non-invasive prenatal testing (NIPT) for fetal aneuploidies with semiconductor sequencing in an academic cytogenomic laboratory and to evaluate the first 15-month experience on clinical samples.

Methods We validated a NIPT protocol for cell-free fetal DNA sequencing from maternal plasma for the detection of trisomy 13, 18 and 21 on a semiconductor sequencing instrument. Fetal DNA fraction calculation for all samples and several quality parameters were implemented in the workflow. One thousand eighty-one clinical NIPT samples were analysed, following the described protocol.

Results Non-invasive prenatal testing was successfully implemented and validated on 201 normal and 74 aneuploid samples. From 1081 clinical samples, 17 samples showed an abnormal result: 14 trisomy 21 samples, one trisomy 18 and one trisomy 16 were detected. Also a maternal copy number variation on chromosome 13 was observed, which could potentially lead to a false positive trisomy 13 result. One sex discordant result was reported, possibly attributable to a vanishing twin. Moreover, our combined fetal fraction calculation enabled a more reliable risk estimate for trisomy 13, 18 and 21.

Conclusions Non-invasive prenatal testing for trisomy 21, 18 and 13 has a very high specificity and sensitivity. Because of several biological phenomena, diagnostic invasive confirmation of abnormal results remains required. © 2016 The Authors. *Prenatal Diagnosis* published by John Wiley & Sons, Ltd.

Funding sources: Supported by a concerted research actions funding from BOF (Bijzonder Onderzoeksfonds) Ghent University, grant BOF15/GOA/011.

Conflicts of interest: None declared

INTRODUCTION

Non-invasive prenatal testing (NIPT) using cell-free fetal DNA is rapidly being adopted as a screening test for the detection of fetal aneuploidies of chromosomes 13, 18 and 21. Since the first proof of concept studies in 2008,^{1,2} many clinical studies have shown that NIPT can detect these chromosomal aneuploidies with high specificity and sensitivity.^{3–6} Those studies primarily focused on the implementation of NIPT for high-risk pregnancies. However, with NIPT outperforming the currently used screening tools for the detection of fetal aneuploidies, this test is very likely to replace standard prenatal trisomy 21 screening for all pregnancies in the near future.^{7–9} Recent studies have shown that with NIPT, also other autosomal aneuploidies (other than trisomy 13, 18 and 21) and even segmental chromosomal aberrations can be detected.^{10–13} Further validation and clinical studies in larger cohorts are however warranted to assess the added value, sensitivity and specificity of these results.¹⁴

Multiple technologies can be applied for performing NIPT. Shallow whole-genome sequencing can be used for complete genome profiling,^{1,2} while targeted NIPT makes use of enrichment strategies to specifically analyse loci of interest (chromosome 13, 18 and 21).^{15–18} For NIPT using whole-genome sequencing with counting statistics, usually a Z-score is calculated.^{1,2,19–22}

Most NIPT protocols based on whole genome sequencing make use of Illumina sequence-by-synthesis technologies. Recently however, it has been proven that also semiconductor sequencing is capable of reliably detecting fetal aneuploidies in maternal plasma.^{23–25}

In this study, we have applied semiconductor sequencing and further studied the impact of two critical parameters that should be taken into account when calculating both specificity and sensitivity for NIPT, that is, (1) the percentage of fetal DNA in the pool of maternal cell-free DNA (fetal fraction) and (2) the standard deviation on normal results, and hence, the amount of sequencing reads.

Gestational age and maternal weight are key characteristics that influence the fetal DNA fraction.^{26,27} Because a low fetal fraction leads to a dramatic decrease in sensitivity, fetal fraction assessment is of utmost importance.²⁸ The most convenient measurement of fetal fraction is based on the presence of the Y chromosome in the plasma sample (FF-Y). Evidently, this fetal fraction calculation is only possible for pregnancies with a male fetus. Other methods for fetal fraction measurements rely on single nucleotide polymorphism differences between maternal and fetal DNA. These calculations, however, mostly require additional testing, hence, rendering the overall test more costly. Interestingly, fetal cell-free DNA fragments are overall shorter than the corresponding maternal cell-free DNA fragments.^{29,30} This inherent information can be used for fetal DNA fraction measurements and eliminates the need of an additional test for fetal fraction measurement.³¹ Recently, Kim *et al.* developed a new strategy, called seqFF, for fetal fraction calculation.³² This method is based on the fact that the genomic origin or representation of cfDNA from fetal and maternal origin differs slightly. The method being described uses the sequence data that are investigated for the NIPT analysis itself, making an extra test for fetal fraction calculation redundant. More importantly, fetal fraction can be determined for pregnancies with male as well as female fetuses.

In addition to the issues raised in the preceding texts concerning sensitivity and specificity for NIPT, the use of cell-free fetal DNA in maternal blood also implies some further inherent issues that can give rise to discordant results with the genuine fetal karyotype. First, the fetal cfDNA in maternal plasma is derived from cytotrophoblasts,³³ and hence, (confined) placental mosaicism can lead to both false positive as well as false negative results. Also, maternal mosaicism for aneuploidies is a potential source for aberrant NIPT results.^{34,35} Secondly, yet another possible source for discordant results are maternal copy number variations (CNVs)^{36,37} or maternal malignancies.^{38–40} Although different data-analysis methods can be used to overcome part of these issues, these biological phenomena implicate that NIPT for chromosomal aneuploidies will always remain a screening test.

In order to further assess the validity of NIPT in relation to the issues raised in the preceding texts, we have validated NIPT on 201 normal and 74 aneuploid samples. Furthermore, we describe our findings in >1000 samples in a diagnostic setting.

MATERIALS AND METHODS

Validation and patient cohort

The validation cohort consists of 201 euploid and 74 aneuploid samples respectively that showed a normal aneuploid karyotype after invasive testing or a normal result after NIPT performed in a secondary laboratory. After explaining the study and signing an informed consent form, 10 mL of blood samples was collected in Cell-Free DNA BCT™ tubes (Streck) or EDTA tubes prior to the invasive procedure. Gestational age ranged from 11 to 32 weeks.

In total, 1081 patient samples have been tested in our centre during a period of 15 months. In Belgium, no reimbursement is

in place yet, and hence, no restriction on the indication for NIPT is applied. This way, high-risk as well as low-risk patients are included. Seventeen twin pregnancies were tested: one monochorionic/monoamniotic, seven monochorionic/diamniotic and nine dichorionic/diamniotic twins.

cfDNA isolation and sequencing

Maternal blood samples were centrifuged within 24 h of collection at 1600g for 10 min at 4 °C to separate the plasma from the blood cells. Plasma was transferred to microcentrifuge tubes and centrifuged at 16 000g for 10 min at 4 °C. Supernatant was transferred to a new microcentrifuge tube and stored at –80 or –20 °C until further processing. cfDNA was extracted from 3.5 mL of plasma using the QIAamp® Circulating Nucleic Acid Kit (Qiagen) following the manufacturer's instructions. DNA concentrations were measured using the Qubit® dsDNA HS Assay Kit (Invitrogen, Thermo Fisher), and 5 ng of cfDNA was used as input for library preparation.

Library construction was performed according to the Ion Plus Fragment Library Kit (Thermo Fisher) with small adjustments. Briefly, end-repair of the plasma cfDNA was performed with T4 DNA polymerase and T4 polynucleotide kinase. Ion Proton compatible adapters with barcodes (Ion Xpress Barcode Adapters, Thermo Fisher) were ligated to the cfDNA using DNA ligase. Libraries were amplified using Platinum® PCR SuperMix High Fidelity (Thermo Fisher) in a thermal cycler [5 min 95 °C, (15 s 95 °C, 15 s 58 °C, 1 min 70 °C) 9 cycles]. In between, libraries were purified using Agencourt AMPure XP beads (Beckman Coulter). After the amplification, double-size selection was performed using Agencourt AMPure XP beads for removal of residual adaptors and primers and potential contaminating genomic maternal DNA. Libraries were quantified with the Ion Library Quantitation Kit (Thermo Fisher). Template preparation and PI V2 or PI V3 chip loading were performed on Ion Chef™ (Thermo Fisher) using resp. 60 or 70 pM of equimolar pooled libraries. The samples were sequenced on an Ion Proton instrument using 400 flows.

Data analysis

Reads were demultiplexed and aligned to the NCBI GRCh37 human genome reference by the Ion Torrent Server (TMAP aligner). Subsequently, the reads were filtered on MAPQ >30, and both the Wisecondor algorithm²¹ as well as the QDNAseq algorithm¹⁹ were used for further downstream analysis. For the QDNAseq algorithm, the genome was divided in 100 kb bins, and read counts were calculated for each bin. After normalization for the total read count (genome wide average = 1), bin counts were log2 transformed, and the average bin count per chromosome was calculated. Finally, mean and standard deviation were calculated for all autosomes for 201 reference samples. With these values, a Z-score (standard score) for each chromosome in each sample could be calculated. QDNAseq values were imported and processed in Vivar.⁴¹ Aberrant results (for all chromosomes) were manually checked on a comprehensive chromosome profile by Wisecondor and Vivar to exclude large (maternal) segmental aberrations.

Quality parameters

Genome-wide standard deviation. For all samples, a standard deviation over all autosomal bins normalized by QDNAseq was calculated. The mean of this standard deviation over all reference samples was calculated at 0.117, with a standard deviation of 0.014. The samples with a standard deviation on their genome wide bincounts >0.160 ($=\text{mean} + 3 * \text{SD}$) were excluded for further analysis and needed resequencing.

Chromosomal standard deviation (Q-score). A second quality parameter is calculated as the standard deviation on the absolute value of all autosomal Z-scores, excluding chromosomes 13, 18 and 21. For the reference set, a mean Q-score of 0.675 was reached, and a cut-off of 1.36 ($=0.675 + 3 * \text{SD}$) was defined. The mean and standard deviation on all bin means for all chromosomes were calculated on all reference samples (refer to Table S1, Fig. S1).

Sequence reads. We aimed to obtain 10 million sequencing reads after quality filtering ($\text{MAPQ} > 30$). To achieve this, six samples were combined on one PI chip. The samples with <5 M reads needed resequencing.

Fetal fraction

Fetal fraction based on Y chromosome-specific reads (FF-Y). Fetal fraction for all pregnancies with a male fetus was calculated as described.² In brief, after stringent filtering ($\text{MAPQ} > 50$), the amount of Y-chromosome-specific reads is normalized for the total read count, and by a linear model based on both male and female reference samples, the fetal fraction was calculated.

Fetal fraction based on genomic representation. For all samples, the seqFF method was implemented as described by Kim *et al.*³² Both weighted rank selection criterion (WRSC) as well as elastic net (Enet) values were calculated. The mean of both was used as seqFF value.

Fragment length distribution. For each sample, a read length distribution is generated by measuring the read count for each length from 25 bp up to 240 bp. The read length distribution is subsequently corrected for total read count, and fetal fraction was predicted using the logistic regression model elasticnet trained on the reference set.³¹

Finally the average of Enet, WRSC and readlength predictions were used as combined fetal fraction (cFF) predictor and compared with FF-Y values.

ISO 15189 accreditation was obtained for the NIPT workflow.

RESULTS

Validation study

A reference set of 201 cfDNA samples from normal euploid pregnancies was tested for validating NIPT for trisomy 21, 18 and 13 (Table S1). On average, 13.6 million uniquely mapped reads were used for the analysis after quality filtering ($\text{MAPQ} > 30$) (minimum 5.2 million).

Using the mean and standard deviation per chromosome, a Z-score for each chromosome in each sample could be calculated. A Z-score higher than 5 was used as a cut-off for calling trisomy. A grey zone was defined for samples showing Z-scores between 3 and 5, and repeat analysis was performed before reporting the results. If the Z-score was again elevated (>3), this sample was called aberrant. Using the described protocol, all 201 reference samples showed a Z-score for chromosomes 13, 18 and 21 below 3, yielding a specificity of 100% in our validation cohort (Table S1).

From the means and standard deviations in the reference set, a theoretical calculation of the detection ratio for several fetal fractions could be made (Figure S2). Sensitivity of NIPT depends strongly on the fetal DNA fraction in a cfDNA sample. The higher the fetal fraction, the higher the sensitivity/detection rate of the test. With a fetal fraction of 4%, 2.68% of trisomy 21 samples will be missed based on the observed standard deviation on our reference, while with a fetal fraction of 5%, only 0.08% of trisomy 21 samples will be missed. Because most samples have a fetal fraction $>5\%$ (Figure S3), the sensitivity for most samples is very high. Based on these observations, we propose that a NIPT result with a fetal fraction below 4% should be reported with caution and considered for a second blood sampling.

For the calculation of the sensitivity of NIPT, 52 trisomy 21, 17 trisomy 18 and 5 trisomy 13 samples were analysed (Table S1). In total, 51 out of 52 trisomy 21 samples showed a Z-score >3 . One trisomy 21 sample had a Z-score of -1.05 (T21_3). This sample had a fetal fraction of only 2.7%, giving an explanation for the failed detection of trisomy 21. Two samples were from a dizygotic twin pregnancy, with a discordant fetal karyotype where one fetus had a trisomy 21, while the other one had a normal karyotype following invasive testing (T21_45 and T21_49). In previous studies, it has been shown that there is a high correlation between the fetal fraction and the Z-score calculation.^{42–44} T21_45 and T21_49 showed a Z-score of 12.21 and 9.50 respectively, while the fetal fraction was 22.52 and 16.19%. For a full trisomy 21 result, a higher Z-score would be expected based on the fetal fraction in the sample (Figure 1c). The discrepancy in Z-score/fetal fraction measurement hence suggests a deviation from a full trisomy 21 karyotype. The same holds true for T21_23, where a mosaic trisomy 21 was detected on invasive testing. The Z-score (5.30) in this positive sample is lower than what is expected based on the fetal fraction calculation (13.6%).

Sixteen out of 17 trisomy 18 samples showed a Z-score >3 for chromosome 18. Sample T18_16 had a Z-score of 1.07 with a fetal fraction of 9.5%; hence, one false negative was present in our training cohort (refer to supplemental info for additional details). Out of the five samples, five had a correct trisomy 13 call.

In total, we obtained a sensitivity of 98% (95% CI: 94–100%), 94% (95% CI: 88–100%) and 100% for trisomy 21, trisomy 18 and trisomy 13 detection on our validation set.

Fetal fraction calculation

Figure 2 shows length distributions from three samples with a different fetal fraction. The length plot clearly demonstrates a shift towards shorter read lengths in samples with a higher fetal fraction. Although there is a strong correlation between read length distribution and fetal fraction ($r=0.761$, Figure 3a),

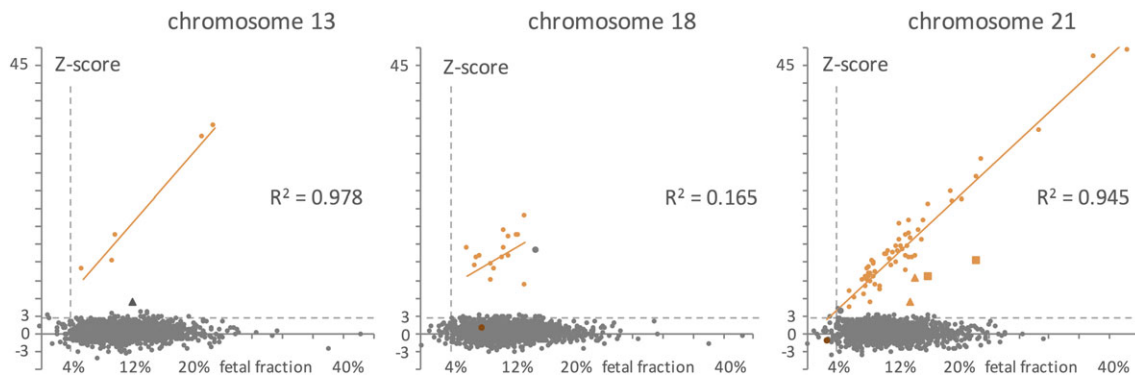


Figure 1 There is high correlation between the fetal fraction and the Z-score for aneuploid pregnancies. Z-scores for chromosome 13 (a), 18 (b) and 21 (c) in relation to the fetal fraction for all analysed samples (validation and patient cohort) are shown. Trisomic samples are indicated as orange dots; euploid samples are indicated as grey dots. There is a high correlation between the fetal fraction and Z-score for samples with a trisomy 13 ($r^2 = 0.978$) and 21 ($r^2 = 0.945$). Samples with deviation of the expected Z-score are indicated: maternal duplication on chromosome 13 (case_15, grey triangle), false negative results (T18_16 and T21_3, red dots), discordant trisomy 18 (case_14), twins with discordant karyotypes (T21_45 and T21_49, orange squares) and mosaic trisomy 21 samples (T21_23 and T21_51, orange triangles)

unfortunately, no clear cut-off could be set to point out the 4% fetal fraction boundary.

For validating the newly described seqFF method,³² a comparison of fetal fraction calculation based on FF-Y and seqFF (Enet and WRSC) for 609 pregnancies with male fetuses was performed. This comparison gives a correlation of $r = 0.909$ for the seqFF method (0.869 for Enet and 0.909 for WRSC) (Figure 3b and c). When we combine WRSC, Enet, as well as the read length prediction on fetal fraction, a Pearson correlation of 0.926 for this cFF was observed (Figure 4). Especially for low fetal fractions (<5%), combining these three methods performed better. For some samples, we observed a discordance between the FF-Y and cFF method. Dizygotic twins from different sex can explain the difference in fetal fraction calculation between FF-Y and cFF in four samples (ref_114 and T21_45 — Table S1; case_19 and case_20 — Table 1). In those samples, the fetal fraction based on the Y chromosome (FF-Y) is about half the fetal fraction based on

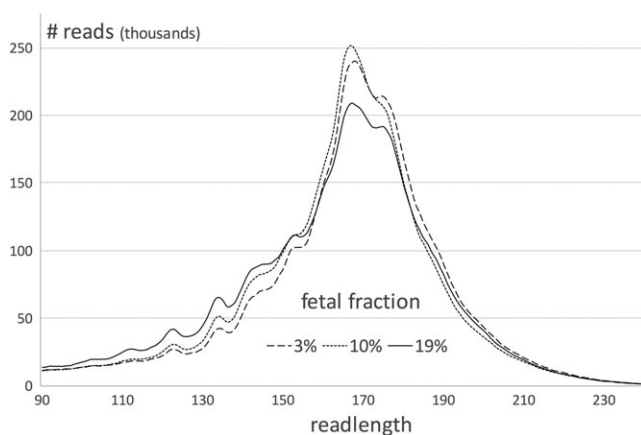


Figure 2 Fetal cfDNA fragments are shorter than maternal cfDNA. Read length distribution of three samples with a different fetal fraction (3, 10 and 19%). The sample with the highest fetal fraction (solid line, 19%) has more sequencing reads in shorter regions

the overall cFF method.

To get an overall estimation of the distribution of the fetal cfDNA fraction, the cFF method was implemented for all analysed samples (male and female fetuses) (Figure S2). Only 1.8% of all samples show a fetal fraction that is below 4%, hence, giving less reliable NIPT results. A blood redraw is indicated for these patients. It is well known that obese women have a higher risk for lower fetal fractions,²⁶ and indeed with a BMI above 30, the chance of having a fetal fraction below 4% raises to 8.7% ($p < 0.005$) in our cohort.

Clinical samples and special cases

Since the introduction of NIPT at the Center for Medical Genetics Ghent, 1081 clinical samples have been analysed. In total, 17 samples showed an abnormal result (Table 1).

In total, 14 trisomy 21 samples were detected by NIPT, 12 of which could be confirmed by invasive prenatal testing. For two samples, no invasive follow-up sample was received. For one patient, we simultaneously received a NIPT sample and CVS (case_12). NIPT showed a Z-score of 9.32 for chromosome 21 (cFF = 14.27%), while FISH on chorion villi revealed a mosaic karyotype with 40% of investigated cells showing three signals for chromosome 21. Abnormalities on ultrasound examination at 15 weeks of gestation were suggestive for a fetal trisomy 21, and subsequent amniocentesis showed a trisomy 21 in all cells analysed. Case_17 had a Z-score of 4.11 for chromosome 21 and a fetal fraction of 4.21%. Although this result was in the 'grey zone', an immediate follow-up amniocentesis was performed, accompanied with a novel blood redraw for NIPT analysis. Both tests showed a normal result (Z-score of -0.35 for chromosome 21 in blood redraw). No trisomy 21 could be detected on amniocytes. Five other samples showed a Z-score in the grey zone (z-scores ranged from 3.03 to 3.35). Upon repeat analysis, all had Z-scores below 3 and were reported as normal.

Case_14 had a Z-score of 13.78 for chromosome 18, with a FF of 12.30% (Figure 1b). Wiscandor analysis also showed an abnormal profile indicative for full trisomy 18. No

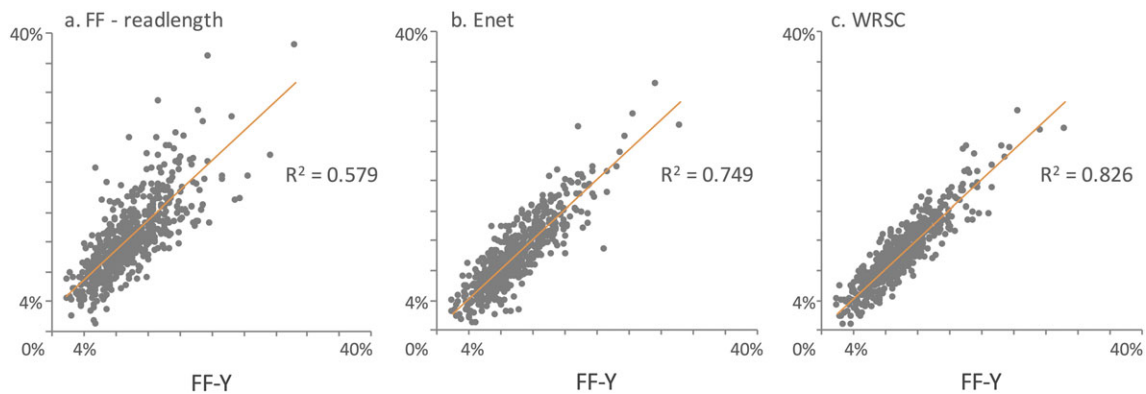


Figure 3 Both fragment length as well as the genomic representation of cfDNA are good indicators for fetal fraction. Correlation plot between fetal fraction as calculated on read length (a), elastic net (b) and weighted rank selection criterion (WRSC) (c) versus fetal fraction calculated based on the amount of Y-chromosome-specific reads (FF-Y) for 609 euploid male pregnancies. All three predictions give a good estimate of the fetal fraction with Pearson's r^2 ranging from 0.579 (readlength prediction) to 0.826 (WRSC)

abnormalities on ultrasound examination were observed at 11 weeks of gestation, and follow-up amniocentesis revealed a normal fetal karyotype, hence, showing a discordant NIPT result for trisomy 18. NIPT on a second blood sample again showed a positive result for trisomy 18 ($Z_{18} = 13.10$), and sample swap was excluded. The pregnancy is ongoing at time of writing, and placental tissue at birth is requested to evaluate a possible placental mosaicism for trisomy 18.

One sample showed a Z-score of 5.41 for chromosome 13, pointing to a trisomy 13 (case_15, Figure 1a). Wisecondor analysis, however, revealed that a maternal duplication of ~3 Mb in chromosome band 13q21.33 is responsible for the aberrant

Z-score in the cfDNA sample (Figure 5). We could not evaluate whether the fetus is also carrier of this duplication, as this result was not communicated to the patient, and no follow-up invasive testing was performed.

Case_16 showed a Z-score for chromosome 16 of 7.9. The sample was taken at 12 weeks (12w5d) of gestation for increased risk for trisomy 21, 18 and 13 (respectively $1/89$, $1 > 50$, $1 > 50$) after a first trimester combined test. No ultrasound abnormalities were apparent at that time point. Although detection of trisomy 16 is not included in the scope of our NIPT protocol, it was decided to report this incidental finding to the patient. Trisomy 16 is usually a mosaic condition, which is only viable in low-grade percentages. In prenatal setting, mosaic trisomy 16 has been described in confined placental mosaicism and can be associated with, for example, intra-uterine growth retardation.^{45–49} Extensive ultrasound follow-up of the pregnancy was advised after this NIPT result. After genetic counselling, the couple opted for invasive confirmation of the NIPT result by amniocentesis. Both FISH and arrayCGH analyses revealed a low-level mosaicism for trisomy 16 of 5–10% in uncultured amniotic cells. Because of complications related to the invasive procedure, the pregnancy was prematurely aborted at 19 weeks of gestation. FISH analysis on placental and fetal skin biopsy showed fetal mosaicism for trisomy 16 of 80 and 10% respectively, hence, confirming the NIPT results.

One NIPT result detected a male fetus based on the presence of Y chromosome material (case 18). However, at term, a healthy baby girl was born, raising questions about the correctness of the NIPT result at 11 weeks of gestation. Analysis was repeated, yielding the same results, and STR analysis excluded a sample swap. When comparing the fetal fraction calculated based on the Y chromosome (FF-Y) with the fetal fraction based on the cFF method, a large discrepancy in fetal fraction was noted (6.11% by FF-Y vs 13.51% by cFF). This deviation in fetal fraction measurements could be a result of the initial presence of a vanished (male) twin brother. Retrospectively, however, there was no indication for ultrasound anomalies nor evidence for an early twin gestation.

DISCUSSION

This article describes the implementation of non-invasive prenatal testing for chromosomal aneuploidies in an

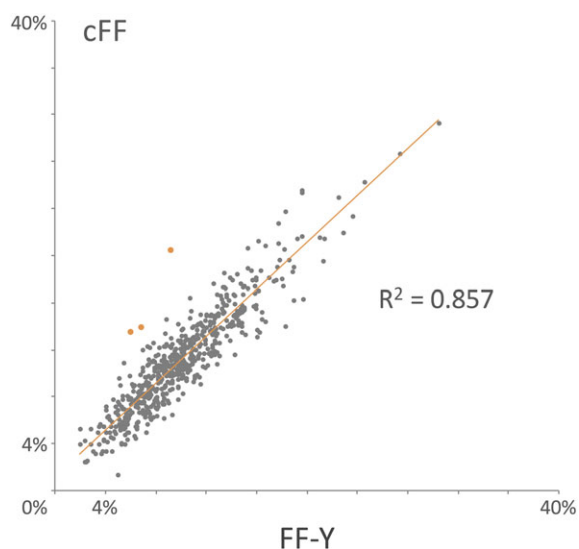


Figure 4 Combined fetal fraction prediction gives a very high correlation to fetal fraction as predicted by FF-Y. Correlation plot between combined fetal fraction prediction (combined fetal fraction, average between readlength prediction, elastic net and WRSC) versus fetal fraction calculated based on the amount of FF-Y for 609 euploid male pregnancies (grey dots). A very high correlation is noted between both predictions (Pearson's $r^2 = 0.857$). Orange dots represent three euploid, but dizygotic twin pregnancies with both male and female fetuses (ref_114, case_19 and case_20)

Table 1 Summary of aberrant and unusual results

Case	Indication	Result	Reported NIPT	FF-Y	cFF	Follow-up
Trisomy 21						
Case_1	Combined test 1/17 for T21	Z21 = 22.14	Trisomy 21	18.72%	19.44%	Confirmed fetal trisomy 21 by amniocentesis
Case_2	Combined test >1/50 for T21	Z21 = 10.26	Trisomy 21	Girl	9.10%	Confirmed fetal trisomy 21 by amniocentesis
Case_3	Combined test 1/268 for T21	Z21 = 12.78	Trisomy 21	Girl	13.20%	Confirmed fetal trisomy 21 by amniocentesis
Case_4	Not specified	Z21 = 13.79	Trisomy 21	10.28%	10.80%	No follow-up – termination of pregnancy
Case_5	Not specified	Z21 = 7.87	Trisomy 21	7.66%	9.77%	Confirmed fetal trisomy 21 by amniocentesis
Case_6	Maternal age	Z21 = 13.07	Trisomy 21	Girl	10.77%	Confirmed fetal trisomy 21 by amniocentesis
Case_7	Combined test 1/141 for T21	Z21 = 23.73	Trisomy 21	Girl	19.10%	Confirmed fetal trisomy 21 by amniocentesis
Case_8	Vanishing twin	Z21 = 12.6	Trisomy 21	Girl	14.03%	Confirmed fetal trisomy 21 by amniocentesis
Case_9	T21 in previous pregnancy	Z21 = 7.82	Trisomy 21	Girl	7.88%	Confirmed fetal trisomy 21 after spontaneous abortion
Case_10	Not specified	Z21 = 9.19	Trisomy 21	7.36%	7.90%	Confirmed fetal trisomy 21 by CVS
Case_11	NT = 6 mm	Z21 = 6.96	Trisomy 21	5.53%	5.87%	Confirmed fetal trisomy 21 by amniocentesis
Case_12	NT = 3.3 mm	Z21 = 9.32	Trisomy 21	Girl	14.27%	40% mosaic trisomy 21 on CVS, full trisomy 21 by amniocentesis
Case_13	Combined test 1/94 for T21	Z21 = 8.53	Trisomy 21	Girl	8.62%	No follow-up – termination of pregnancy – dysmorphic features on clinical examination
Trisomy 18						
Case_14	Not specified	Z18 = 13.78	Trisomy 18	Girl	15.18%	Normal female karyotype on amniocentesis, redraw NIPT Z18 = 13.01
Special results						
Case_15	Combined test 1/1590 for T21	Z13 = 5.41	Normal	Girl	12.30%	Maternal duplication on chromosome 13
Case_16	Combined test 1/89 for T21, >1/50 for T18, >1/50 for T13	Z16 = 7.9	Trisomy 16	7.53%	5.95%	True mosaicism trisomy 16
Case_17	Combined test 1/3494 for T21, >1/50 for T18, >1/50 for T13	Z21 = 4.11	Trisomy 21	5.17%	4.21%	Normal male karyotype on amniocentesis, redraw NIPT Z21 = -0.35
Case_18	Maternal age	Normal	Normal	6.11%	13.51%	NIPT reported male, healthy girl born (vanished twin?)
Case_19	Pregnancy after ART	Normal	Normal	6.95%	13.90%	Dizygotic twin (DC/DA)
Case_20	Pregnancy after ART, combined test 1/500 for T21	Normal	Normal	9.27%	20.39%	Dizygotic twin (DC/DA)

NT, nuchal translucency

DC/DA, dichorionic/diamniotic

academic cytogenomic laboratory. To this end, we applied an in-house developed protocol for shallow whole genome sequencing of cfDNA on a semiconductor instrument. NIPT for trisomy 21, 18 and 13 reached a specificity of 100% and a sensitivity of 98, 94 and 100% in our validation cohort. Several other studies describe a lower sensitivity for chromosome 13; hence, caution should be made given the limited number of positive validation samples.

Since its implementation, 1081 NIPT samples have been processed, and 17 showed an abnormal result. Most samples (14/17, 82%) showed a trisomy 21, but also other abnormalities have been observed. A total of 12 out of 14 (79%) positive trisomy 21 samples could be confirmed through subsequent invasive testing. For two samples, no follow-up information is available. One mosaic trisomy 21 case was detected and confirmed by CVS and amniocentesis. A case with discordant fetal sex shows that biological phenomena such as a vanishing

twin can possibly confound the NIPT results. One case showed a true mosaicism for trisomy 16, which was confirmed by amniocentesis and on fetal tissue. Trisomy 16 is the most common trisomy in spontaneous abortions. While full trisomy 16 has never been observed in live borns, mosaic trisomy 16 has been reported prenatally and postnatally. Mosaic trisomy 16 is commonly associated with intrauterine growth retardation, *mors in utero* or a high risk of abnormal outcome.^{45–49}

Four months after completion of the study, no false negative results for trisomy 21, 18 or 13 have been reported, yielding a sensitivity of 100%.

In this 1000 sample cohort already multiple aberrant, unusual or discordant findings were noted, warranting specialized follow-up and genetic counselling. We therefore advocate a close collaboration between NIPT providers, gynaecologists and genetic diagnostic laboratories to reduce maternal anxiety, enhance counselling and instigate

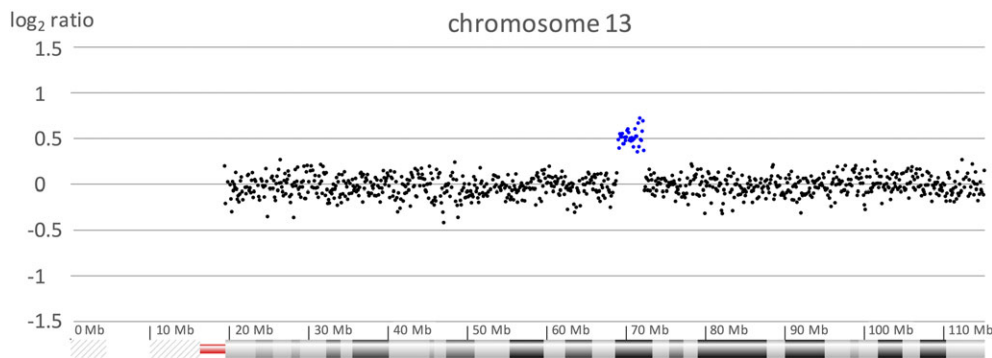


Figure 5 Maternal duplication on chromosome 13 leads to false positive trisomy 13 call. Comprehensive chromosome profile of chromosome 13 for case_15 showing a duplication (in blue) on chromosome band 13q21.33. The log₂ ratio of the duplication is higher than expected for a fetal cfDNA aberration (fetal fraction 12.30%), hence pointing to a duplication of maternal origin

confirmatory diagnostic testing. NIPT is a screening test based on cell-free fetal DNA measurements, and hence, false positive as well as false negative results are inherent to the technology. However, several methods can be implemented to reduce those false positive as well as false negative NIPT results, such as rigorous quality control, fetal fraction determination and exclusion of potential confounding maternal CNVs.

In our patient cohort, one sample had a maternal CNV on chromosome 13 that led to an increased Z-score for chromosome 13 (Z-score 13 = 5.41), giving a false positive call for trisomy 13. Generating a comprehensive chromosome profile for this sample clearly showed a maternal duplication on chromosome 13. Hence, a normal NIPT result for trisomy 13 was reported. Earlier studies also showed that maternal CNVs can be a confounding factor for reliable NIPT interpretation,^{36,37} advocating for a comprehensive chromosome profiling.

We calculated that a fetal fraction of minimum 4% is crucial to obtain a sensitivity warranted for reliable NIPT results (Figure S2). Although a higher sequencing depth can improve the sensitivity for these low fetal fractions, this does not scale well, and hence, a repeat blood sample was requested in our workflow. Moreover, a reliable calculation of the fetal fraction can help in the interpretation of Z-scores in the grey zone, as they might be indicative for mosaicism or twins with a discordant result. Because the fetal DNA fraction is low early in pregnancy and increases as the pregnancy advances, NIPT is currently performed from 10–11 weeks of gestation onwards.⁵⁰

It has been shown that obese mothers have on average lower fetal cfDNA fractions.^{26,27} Indeed, while in the entire cohort only 1.8% of samples showed a fetal fraction below 4%, this number increased to 8.7% for women with a BMI > 30. Measuring the fetal fraction in NIPT samples can give a better estimation on the reliability of NIPT results, and hence, less false negatives.

Only a limited number of laboratories currently make use of Ion Torrent sequencing technology for NIPT.^{13,23–25} We show that Ion Proton sequencing is appropriate for NIPT and can reliably detect fetal trisomy 13, 18 and 21. We and others recently showed that this technology is equally suitable for other shallow sequencing-based applications.^{51,52} The seqFF method for detecting the fetal fraction in maternal plasma, regardless the fetal gender, was successfully

applied to our Ion Proton NIPT data. This method showed a high correlation ($r=0.909$) with the fetal fraction calculation based on Y chromosome sequences (FF-Y). This shows that although the seqFF method was originally generated for Illumina technologies, it can be applied to Proton data and can be used for fetal DNA fraction measurements. Moreover, by including the read length distribution inherent to Ion Proton sequencing, we can improve the fetal fraction prediction even more ($r=0.926$).

In conclusion, we demonstrate that semiconductor sequencing is very well suited for the implementation of NIPT in a diagnostic genetic laboratory. Moreover, we have shown that whole chromosome profiling is advantageous to reduce the number of false positive and false negative results. The implementation of an easily established fetal fraction calculation for all pregnancies improves reliability of NIPT and is useful for the interpretation of Z-scores.

ACKNOWLEDGEMENTS

The authors would like to thank Shalina Baute, Lies Vantomme, Dimitri Broucke, Peter Degrave, Tine De Prêtre and Melek Yörük for their excellent technical support and Frank Speleman for critical proofreading the final manuscript.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Non-invasive prenatal testing (NIPT) for fetal aneuploidies has been applied worldwide and is mainly offered by commercial service providers.

WHAT DOES THIS STUDY ADD?

- This study demonstrates the performance of non-invasive prenatal testing in a genetic laboratory by semiconductor sequencing.
- Advantages of fetal fraction calculation and comprehensive chromosome profiling are discussed in this article and illustrated by several clinical examples.

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