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ORIGINAL ARTICLE

WILEY PRENATAL DIAGNOSIS

Creating basis for introducing non-invasive prenatal testing in the Estonian public health setting

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Abstract

Objective: The study aimed to validate a whole-genome sequencing-based NIPT laboratory method and our recently developed NIPTmer aneuploidy detection software with the potential to integrate the pipeline into prenatal clinical care in Estonia.

Method: In total, 424 maternal blood samples were included. Analysis pipeline involved cell-free DNA extraction, library preparation and massively parallel sequencing on Illumina platform. Aneuploidies were determined with NIPTmer software, which is based on counting pre-defined per-chromosome sets of unique k-mers from sequencing raw data. SeqFF was implemented to estimate cell-free fetal DNA (cffDNA) fraction.

Results: NIPTmer identified correctly all samples of non-mosaic trisomy 21 (T21, 15/15), T18 (9/9), T13 (4/4) and monosomy X (4/4) cases, with the 100% sensitivity. However, one mosaic T18 remained undetected. Six false-positive (FP) results were observed (FP rate of 1.5%, 6/398), including three for T18 (specificity 99.3%) and three for T13 (specificity 99.3%). The level of cffDNA of <4% was estimated in eight

samples, including one sample with T13 and T18. Despite low cffDNA level, these two samples were determined as aneuploid.

Conclusion: We believe that the developed NIPT method can successfully be used as a universal primary screening test in combination with ultrasound scan for the first trimester fetal examination.

1 | INTRODUCTION

Aneuploidies are a major cause of perinatal morbidity and mortality. In many countries, including Estonia, prenatal testing for fetal aneuploidies relies on the first trimester combined test (FCT), including the analysis of maternal serum biomarkers, pregnancy-associated plasma protein-A (PAPP-A) and free β-human chorionic gonadotropin (fβ-hCG), and fetal nuchal translucency (NT) ultrasound, after which women who are deemed to be at high risk are offered an invasive confirmatory test. The main issue associated with FCT is relatively high false-positive (FP) rate, about 5%, due to which ~90% of the women eligible for the following invasive testing carry a healthy baby.[1] At the same time, invasive methods – amniocentesis and chorionic villus sampling (CVS) result in a small but concerning risk for miscarriage, even if the procedure is performed by an experienced specialist.[2] The number of unnecessary invasive testing can be reduced significantly if more precise primary screening methods are properly utilised.

Since 2011, non-invasive prenatal testing (NIPT) is routinely used for the detection of common fetal trisomies, such as trisomy 21 (T21), 18 (T18) and 13 (T13), demonstrating sensitivity and specificity up to >99%.[3,4] Several NIPT technologies have been developed for that; including massively parallel sequencing (MPS), chromosome-selective or targeted sequencing and SNP (single nucleotide polymorphism) based approaches. In MPS-NIPT, plasma DNA random or "shotgun" whole-genome sequencing is used, and a z-score of >3 standard deviations away from the expected read count of a specific chromosome is considered as high-risk indicator for fetal trisomy of a test sample.[5] On the contrary, in targeted sequencing, the plasma DNA fragments from chromosomes 13, 18 and 21 are amplified, and the enriched fragments are sequenced instead of the entire genome, thus revealing only these three trisomies. Similarly, in SNP-based NIPT, counting of the fetal alleles and calculating the allele ratio is used to predict the fetal trisomy for chromosomes 13, 18 or 21.[6]

Presently NIPT testing is growing constantly worldwide as it is recognized as a highly precise screening test that is not more hazardous for a pregnant woman than a simple blood draw procedure. Given that NIPT offers safe and accurate selection of high-risk pregnancies and enables to avoid a large number of unnecessary invasive procedures, we aimed to validate a whole-genome MPS-based NIPT laboratory method and our recently developed NIPT analysis software (NIPTmer)[7] for detecting T13, T18 and T21, and sex chromosome aberrations, with the potential to integrate NIPT into the prenatal clinical care in Estonia.

What is already known about this topic?

- The developed NIPT approaches are different in terms of the genetic and bioinformatic technologies used, computational resources required, data analysis speed, and finally even in robustness and reliability.

What does this study add?

- Novel in-house developed NIPTmer program counts predefined per-chromosome sets of unique k-mers from sequencing data.
- NIPTmer uses less computer resources and is faster than available NIPT-tools.
- NIPTmer was validated for detection of fetal aneuploidies, demonstrating its readiness for use in prenatal testing.

2 | METHODS

2.1 | Samples and study design

The study was approved by the Research Ethics Committee of the University of Tartu (#246/T-21) and written informed consent was obtained from all participants. A total of 408 samples from pregnant women were collected at Tartu University Hospital (Tartu, Estonia) and at East-Tallinn Central Hospital (Tallinn, Estonia) during 2015 to 2018. Singleton pregnancy with the gestational age of ≥10 weeks was the mandatory requirement for enrolment.

The cohort included cases with both high (referred as high-risk pregnancies; n=259) and low (referred as general population; n=149) risk of aneuploidy. The high-risk factors included increased FCT-based trisomy risk of >1/300 for any common fetal trisomies (T21, T18 and/or T13, n=208), family history of genetic diseases (including fetal trisomies, n=25), ultrasound abnormalities (n=15), and in rare cases also elevated maternal age of ≥36 years or other reason (n=11). All women from the high-risk group underwent invasive prenatal diagnostic procedure (amniocentesis or CVS followed by karyotyping, fluorescence *in situ* hybridisation (FISH) or chromosomal microarray analysis). The type of the invasive procedure (amniocentesis or CVS) is shown in Table S1, CVS was performed only using the long-term culture method.

Pregnant women from general population had low risk results at FCT and no detectable fetal abnormalities on ultrasound examination. In this group of women, the general health status of a baby was determined at birth by a neonatologist.

The mean age of participants in high-risk group was 35 years (median 36, range 17-47, and 25th and 75th percentiles 30 and 39, respectively) and 29 years in general population (median 30, range 16-41, and 25th and 75th percentiles 26 and 33, respectively). The mean gestational age at NIPT in high-risk group was 15 weeks (median 15, range 11-21, and 25th and 75th percentiles 13 and 17, respectively) and 25 weeks in general population (median 25, range 10-40, and 25th and 75th percentiles 20 and 33, respectively). General characteristics of the study cohorts are summarized in Table S1.

Samples from general population were analysed by Illumina technology using massively parallel sequencing to generate preliminary reference dataset. This was initially used to analyse the data obtained from the high-risk group samples. The reference dataset was updated constantly by including euploid samples from high-risk group, which makes further analysis more precise.

In addition, 16 cell-free DNA samples from the Center for Human Genetics, UZ Leuven, Belgium enriched for common trisomy cases (T21 n=2, T18 n=3 and T13 n=3) were blindly analysed using the same method. The results were compared with KU Leuven laboratory results.

Laboratory personnel performing NIPT were blinded to the results of karyotyping obtained from the invasive procedures in high risk pregnancies. As this was a validation study, no results were returned to participants.

2.2 | Sample processing and sequencing

Samples (n=424) were processed according to previously published guidelines from KU Leuven, with minor modifications.[8] Briefly, peripheral blood samples were collected in cell-free DNA BCT tubes (Streck, USA) and plasma was separated within 72h by standard dual centrifugation. Cell-free DNA was extracted from 4-5 ml plasma using QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany). Whole-genome libraries were prepared using TruSeq ChIP Library Preparation kit (Illumina Inc., USA) as described in Bayindir et al., with 12 cycles for the final PCR enrichment step.[8] Following quantification, equal amounts of 24 libraries were pooled and the quality of the pool was assessed on Agilent 2200 TapeStation (Agilent Technologies, USA). Next-generation sequencing was performed on the NextSeq 500 instrument (Illumina Inc.) with an average coverage of 0.32× (minimum 0.08 and maximum 0.42) and producing 85 bp single-end reads.

2.3 | Fetal DNA fraction (FF) calculation

Sequencing read data was mapped with Bowtie 2.3.4.1[9] against GRCh37 pre-built Bowtie index file with pre-set option of 'very-sensitive' and the maximum fragment length of 500 for valid paired-end alignments.[10] Mapped data was filtered by mapping quality of 30, after which SeqFF[10] with minor modifications (to optimise the file handling in SeqFF calculation workflow) was used to calculate cell-free fetal DNA (cffDNA) fraction (FF) for all samples.

2.4 | Detection of fetal trisomies

The plasma DNA sequencing data was analysed with our novel in-house developed NIPTmer software package for fetal aneuploidy detection, which is based on counting pre-defined per-chromosome sets of unique k-mers (sequences of nucleotides) from raw sequencing data and applying linear regression model on the counts, thus avoiding time-consuming read-mapping step.[7] Although whole-genome sequencing was performed, only the risks for T13, T18 and T21, and sex chromosome abnormalities were blindly analysed using NIPTmer software and compared to the results of the invasive prenatal testing, while other autosomal aberrations were only retrospectively analysed, if detected by invasive procedure in high-risk pregnancies.

NIPTmer calls aneuploidies of the chromosome of interest based on the z-score (difference between expected and observed values, normalized to the standard deviation of expected values) of the detected number of specific k-mers compared to the expected number predicted by linear model. The model makes prediction based on the k-mer counts of other autosomes and GC content of the sample and is trained on the dataset of samples with known karyotype. NIPTmer is significantly faster than whole-genome mapping-based NIPT tools, uses less computer resources and does not require previous experience with mapping of next-generation sequencing reads. The time to analyse a single sequenced sample with NIPTmer is less than 5 minutes on 32-core server and 20 minutes on two-core laptop. In comparison, mapping sequenced reads to reference genome with Bowtie2 or BWA takes more than hour, being the time-limiting step in mapping-based tools. Moreover, NIPTmer allows on-the-fly updating of both control and aneuploidy datasets so the prediction accuracy gets the better the more analyses are performed. The program is also more robust as the non-unique and polymorphic genome regions are discarded before the actual analysis, in k-mer list generation step, and thus do not introduce additional variability in test results.

3 | RESULTS

A total of 424 samples were analysed at the gestational weeks of 16-41, while 323 (76.2%) samples were analysed during the usual prenatal screening window of 10 to 20 completed weeks of gestation. FF estimates by SeqFF were between 3-49% (with average of 12%), and average FF was lower in the high-risk group compared to the general population (10% (range 3-29%) vs 17% (range 5-49%), respectively), which may be explained by a wider gestational age range of the latter. The average cffDNA for all 323 tests within the usual prenatal screening window was 10% (range 3-31%).

The results of NIPT and invasive fetal testing are summarized in Table 1. Z-score threshold for common trisomies (T21, T18 and T13) was set at 3.0, which allowed correct identification of all non-mosaic T21 (15/15), T18 (9/9) and T13 (4/4) cases (sensitivity of the test

TABLE 1 NIPT results for common aneuploidies (T21, T18 and T13), 45,X karyotype and follow-up data of the study population (n=424).

	Estonian cohort		Belgium cohort			
	High-risk group	General population	Samples enriched for aneuploid cases	Total	FP	FN
Number of samples	259	149	16	424		
T21						
NIPT	13	0	2	15	0	0
Invasive prenatal diagnosis	13	-	2	15		
Neonatal follow-up	-	0	-			
T18						
NIPT	8	1	3	12	3	1[§]
Invasive prenatal diagnosis	7	-	3	10		
Neonatal follow-up	-	0	-			
T13						
NIPT	1	3	3	7	3	0
Invasive prenatal diagnosis	1	-	3	4		
Neonatal follow-up	-	0	-			
45,X						
NIPT	4	0	0	4	0	0
Invasive prenatal diagnosis	4	-	0	4		
Neonatal follow-up	-	0	-			

FP - false-positive results; FN - false-negative results.

[§]Mosaic T18 case with 20% of T18 cells.

for all common trisomies 100%; the 95% confidence intervals 78.2-100% for T21, 66.4-100% for T18 and 39.8-100% for T13). However, one mosaic T18 remained undetected (z-score of 2.43). FISH on chorionic villi revealed the presence of T18 in ~20% of the cells, while karyotyping using the GTG (G-bands by trypsin using Giemsa) banding technique of the same sample was not able to establish the percentage of mosaicism because of the small number of the cells analysed. Six FP results were observed (FP rate of 1.5%, 6/398), including three for T18 (specificity 99.3%; the 95% confidence interval 97.8-100%) and three for T13 (specificity 99.3%; the 95% confidence interval 97.8-100%), while no FPs for T21 were observed (specificity 100%; the 95% confidence interval 99.1-100%). Two FP NIPT results for T18 in the high-risk group were refuted by the results of the invasive procedure, showing the normal fetal karyotype. Four FPs were detected among general population pregnancies (Table S1), with the babies confirmed healthy at birth and after follow-up of the newborns by the neonatologist. When only the NIPT tests within the usual prenatal screening window of 10 to 20 completed weeks gestation were considered, the sensitivities for all common trisomies and the specificity for T21 remained 100%, the specificity for T13 increased to 100% (as FP T13 tests were recruited beyond the screening window) and the specificity for T18 decreased slightly to 99.1%. This means that the positive predictive value (PPV) for the NIPT tests for 10-20 weeks of gestation was 100% for T13 and T21, and 75% (9/12 with three FP T18 tests) for T18. In addition, CVS with long-term culture method detected one mosaic trisomy 8 (karyotype 47,XY,+8(11)46,XY(29)), but there was no evidence of it with NIPT, if sequencing data was analysed retrospectively.

Fetal sex was determined correctly for all samples (Table S1). Five cases with sex chromosome aneuploidies (SCA) from high-risk group were identified by invasive testing, of which four monosomy X (45, X; Turner syndrome; both sensitivity and specificity of 100%) cases were determined by NIPT (z-scores for X chromosome between -8.1 to -16.4), however one 47,XXX karyotype remained undetected (z-score -3.3) as seen in Table S1.

For eight samples the test failed (1.9%, 8/424), as the proportion of cffDNA in maternal plasma remained <4% (2.83-3.99%). Slightly higher test failure rate of 2.5% (8/323) was observed for the NIPT tests done for pregnant women from gestational weeks of 10 to 20, representing the timeframe for NIPT usual clinical use. Six out of these eight samples were from the high-risk pregnancies and two were from Belgium cohort. Despite low cffDNA fraction in two aneuploid samples from Belgium, we were able to correctly identify T13 and T18 cases. All the rest six cases with cffDNA fraction <4% from the high-risk Estonian pregnancies were carrying fetuses with normal karyotype.

4 | DISCUSSION

The aim of the current study was to adjust previously described experimental protocol from KU Leuven[8] and our in-house developed NIPTmer analysis pipeline for detection of common fetal aneuploidies[7]

in order to create a basis for introducing NIPT in the Estonian public health setting. To date, our validated NIPT is already implemented in Estonian healthcare system under NIPTIFY™ name.

We analysed 424 samples including high- and low-risk pregnancies and correctly identified all non-mosaic samples aneuploid for chromosomes 21 (15/15), 18 (9/9) and 13 (4/4). In the current study z-score cut-off of 3 was applied for T21, T18 and T13. The relative coverage (or z-score) distribution among euploid and aneuploid pregnancies depends on both technological (cell-free DNA extraction method, sequencing library preparation, sequencing technology, and equipment used) and biological (gestational age and body mass index) factors. Thus, the actual cut-offs for calling the elevated risk of a trisomy should be defined in every NIPT laboratory separately.[7] These cut-offs determine the predictive power and the proportions of FP and false-negative (FN) NIPT results. Setting cut-off values stringently minimizes the number of FP results and eventually enables to reduce significantly the number of invasive prenatal tests, which is one of the issues addressed by NIPT. However, one should keep in mind that this would introduce additional FN cases that must be avoided. With the currently established threshold value of 3, six FP results were introduced, with the FP rate of 1.5%. Therefore, the further improvement of the current approach is possible as the FP rate of <0.1% is considered as the golden standard for NIPT studies, at least for Down syndrome.[11] However, the FP rate for T21 in the current study was 0%, as all FP tests were reported for T13 and T18. All the NIPT FP results resulted in the birth of healthy children, as confirmed by invasive prenatal testing or follow-up of the newborns by a neonatologist. The patients in our study did not receive any feedback on NIPT results. However, in a clinical service, confirmatory invasive testing should be offered to all pregnant women with high-risk testing results as NIPT represents a screening not a diagnostic test.[12]

In addition to the common trisomies in our patients' cohort, a case of mosaic trisomy 8 (47,XY,+8(11)/46,XY(29)) was detected by CVS but not retrospectively by NIPT. Inability to pick up this abnormality can be explained by <30% mosaicism and relatively low cffDNA level (6%). The detection of chromosomal abnormalities other than T21, T18 and T13 (sex chromosome aneuploidies, other autosomal trisomies, triploidy and microdeletions) has been reported and is included in some commercial NIPT platforms with variable detection rates.[13] However, this is not recommended by the American College of Obstetricians and Gynecologists (ACOG) and debate over clinical utility continues.[14] As current MPS-NIPT platforms are not robust enough to reliably detect aneuploidies and clinically relevant microdeletions of all autosomal chromosomes with high accuracy, the potentially less expensive targeted NIPT methods might be more cost-effective, and could become a useful mass screening tool, as we have recently demonstrated.[15]

With regard to SCAs, four cases of monosomy X were correctly determined with current NIPT pipeline, however, a case of 47,XXX, a syndrome with clinically mild phenotypic changes, was not detected. As in three out of four pregnancies with Turner syndrome fetuses the enlarged NT was observed, invasive fetal studies would likely have been offered to these women. Moreover, screening for SCAs is controversial due to the varying severity of these conditions, and the fact that NIPT is less accurate in prediction of SCA compared to common trisomies. Some studies find that FP rate is especially high for Turner syndrome,[16,17] which might be partially explained by maternal mosaicism for monosomy X that can be a part of normal aging.[18] Despite possible frequent false positive results, however, the health problems associated with Turner syndrome are the reasons why parents need this information to make decisions about their pregnancies. For these reasons, the present clinical study included NIPT screening for the high-risk of Turner syndrome.

Another question concerns the possible target groups for NIPT, which is also relevant for Estonia. It has been shown that there is no significant difference in NIPT screening performance between high-risk, routine and mixed populations.[19] Therefore, the question arises what is the better way to implement NIPT into clinical practice and who are the main target groups for this testing. In general, three models have been proposed for incorporation of NIPT into current screening programs.[20] The first one assumes that NIPT is used as a second-tier screening test where women first undergo conventional FCT and those with elevated risk are subsequently offered NIPT or invasive testing. In this case, NIPT would be offered to ~5% of women, thus reducing costs and FP screening results,[20] but the detection rate of the fetal trisomies would still be limited by the detection rate of the FCT. According to the second model, women deemed to be at high risk (e.g. ≥1:100) after conventional FCT would be offered to choose between NIPT, invasive testing or no follow-up, and those with the intermediate risk (e.g. 1:101-2,500) would be offered NIPT or no further testing. This model is likely to offer better balance between added cost and improved detection rate.[21] However, the use of NIPT as a secondary screening test mainly lowers the FP rate, while does not diminish the FN rate of the initial FCT. The third model would be to offer NIPT to all women as a primary screening test combined with ultrasound. This would result in a higher detection rate compared with the FCT. Recently, this last option has been chosen by countries such as the Netherlands and Belgium where NIPT is offered to all pregnant women since 2017. As this approach is likely to provide the best detection rate, it is also our preferred option for NIPT implementation in Estonia.

Various national studies evaluating NIPT performance have shown that NIPT significantly reduces the extent of invasive testing by between 60%[22] and 75%.[23] Although, there is still controversy on the magnitude of the fetal loss rate caused by invasive testing, the reduction of invasive procedures is certainly perceived as better care by both health care providers and pregnant women.[22] In addition, offering NIPT to high-risk pregnant women before invasive prenatal testing helped to drop the number of women refusing further testing,[24] leading to increased detection of fetal chromosomal aberrations.[25]

In our study, 15 women from high-risk group of patients demonstrating fetal abnormalities in the ultrasound examination with or without increased combined risk for common trisomies were enrolled (Table S1). In four of these pregnancies chromosomal aberrations were found using NIPT (two cases of T18, a single case of T13 and a monosomy

X), which were concordant with the findings from the invasive testing. However, it is very important to emphasize that pregnancies with the identified fetal malformations are certainly not the patients for whom the NIPT should be offered, as the invasive testing is the primary choice for them. In the present study, NIPT analysis was conducted in parallel to the invasive prenatal testing in order to get the valuable feedback about the accuracy of the developed NIPTmer method.

The reference group (general population, low risk for fetal trisomies) for NIPTmer analysis in our study consisted of patients with wide range of gestational ages (10-40 weeks). No limitations on gestational age were applied to the reference group in order to validate NIPTmer and SeqFF programs in samples with low as well as with high FF levels. The high-risk group, however, comprised of patients with gestational age suitable for NIPT analysis prior to invasive testing. The gestational age is one of the most important variables that affects the size of FF, which in turn can lead to testing failure, and possible FN results, if the cffDNA percentage is low in early stages of pregnancy. The test failure rate of 1.9% was observed in the present study for all NIPT tests, as the proportion of cffDNA in maternal plasma remained <4% in 8 samples out of 424, which is a widely accepted minimum that allows reliable detection of common trisomies.[5,12,14] In these cases, the recommendation is to repeat the testing until the cut-off of 4% of cffDNA is achieved.

We also demonstrated that by limiting the analysis with the samples representing the usual prenatal screening window of 10-20 completed weeks of gestation, that had lower FF, did not influence the accuracy of testing in terms of sensitivity and specificity for the analysed chromosomal conditions. However, we saw slightly increased test failure rate of 2.5% for the NIPT tests from 10-20 weeks of gestation, when compared to 1.9% if all tests were included, indicating that the lower fraction of cffDNA at earlier stages of pregnancy may increase failure rates.

In conclusion, we validated next-generation sequencing-based NIPT method and original NIPTmer software in Estonian population setting. Our NIPT methodology proved to perform efficiently in detecting common fetal aneuploidies T21, T18, T13 and monosomy X with 100% sensitivity, and 99.3-100% specificity. We believe that the developed NIPT method can successfully be used as a universal primary screening test in combination with ultrasound scan for the first trimester fetal examination.

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CONFLICTS OF INTEREST

Here described NIPT method is implemented to Estonian healthcare system under NIPTIFY™ name that belongs to Competence Centre on Health Technologies. The authors do not have commercial interests in connection with NIPTIFY testing.

DATA AVAILABILITY STATEMENT

All the data is provided in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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