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ORIGINAL ARTICLE

WILEY PRENATAL DIAGNOSIS

Clinical validation of a novel automated cell-free DNA screening assay for trisomies 21, 13, and 18 in maternal plasma

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Abstract

Objective: To evaluate clinical performance of a new automated cell-free (cf)DNA assay in maternal plasma screening for trisomies 21, 18, and 13, and to determine fetal sex.

Method: Maternal plasma samples from 1200 singleton pregnancies were analyzed with a new non–sequencing cfDNA method, which is based on imaging and counting specific chromosome targets. Reference outcomes were determined by either cytogenetic testing, of amniotic fluid or chorionic villi, or clinical examination of neonates.

Results: The samples examined included 158 fetal aneuploidies. Sensitivity was 100% (112/112) for trisomy 21, 89% (32/36) for trisomy 18, and 100% (10/10) for trisomy 13. The respective specificities were 100%, 99.5%, and 99.9%. There were five first pass failures (0.4%), all in unaffected pregnancies. Sex classification was performed on 979 of the samples and 99.6% (975/979) provided a concordant result.

Conclusion: The new automated cfDNA assay has high sensitivity and specificity for trisomies 21, 18, and 13 and accurate classification of fetal sex, while maintaining a low failure rate. The study demonstrated that cfDNA testing can be simplified and automated to reduce cost and thereby enabling wider population-based screening.

Olle Ericsson, Tarja Ahola, Filip Karlsson, and Fredrik Persson prepared the first version of the manuscript. All authors have contributed, read, and approved the final version of the manuscript. Fredrik Dahl and Olle Ericsson jointly supervised this work.

1 | INTRODUCTION

Prenatal screening for trisomies 21, 18, and 13 is currently performed using multiple maternal serum and ultrasound markers. During the last decade, clinical implementation studies of maternal plasma cell-free (cf)DNA testing has demonstrated a much higher sensitivity and specificity for these trisomies.[1] However, where cfDNA screening has been adopted in public health programs, it is mostly as a second-tier test.[2,3] Health economic analyses have identified the necessity of more cost-effective cfDNA tests to enable a wide adoption of first-line screening.[4-10]

Attempts to reduce costs have been made by using target enrichment approaches with sequencing or microarray readout instead of whole genome sequencing. Targeted tests require less sequencing for comparable trisomy 21 sensitivity and specificity but suffer from higher no-call rates of approximately 3% to 5%. The majority of these no-calls are due to low fraction of fetal-derived cfDNA in the maternal plasma (fetal fraction). Targeted tests are also based on high complexity genetic analysis platforms that require a relatively advanced laboratory setup.[11,12] Other aneuploidy tests measuring cfDNA using digital PCR and qPCR have been demonstrated; however, clinical validation studies demonstrating comparable performance for trisomy 21 detection is lacking. In addition, these PCR-based tests do not easily scale up to include detection of trisomies 18 and 13, which are included in contemporary screening programs. We believe the high-cost structure and complexity of sequencing and microarray-based cfDNA testing is the main reason why cfDNA testing has not yet been widely adopted as a first-tier aneuploidy test.

To enable high-performance cost-effective aneuploidy screening, we have developed an automated Vanadis noninvasive prenatal testing (NIPT) assay targeting specific chromosomes based on digital molecular quantification in a 96-well microplate format. The assay enables automated high precision cfDNA analysis from primary blood tubes. Previously, the Vanadis NIPT assay has been demonstrated to enable high precision measurement of aneuploidies and clinical classification of euploid and aneuploid trisomy 21 pregnancies[13] and is here extended to trisomies 13 and 18. Targeted NIPT tests using PCR typically remove samples with low fetal fraction since these cannot be classified accurately, resulting in a high assay failure rate. Using direct quantification of targeted chromosomal fragment labelled by rolling circle replication, the Vanadis NIPT has been demonstrated to correctly classify reference samples well below 4% fetal fraction.[13]

The current study was carried out to demonstrate the performance of the Vanadis NIPT assay in the detection of trisomies (T) 21, 18, and 13 as well as the accuracy of fetal sex determination.

What's already known about this topic?

- Maternal plasma cell-free (cf)DNA analysis with next-generation sequencing has a high sensitivity and specificity for fetal trisomy 21 and other common autosomal trisomies.
- A new amplification-free, nonsequencing, and targeted cfDNA assay has been developed.
- Proof-of-principle analysis found the new assay has promising results in screening for trisomy 21.

What does this study add?

- The new assay has high sensitivity and specificity for trisomies 21, 18, and 13 in singleton pregnancies.
- It can accurately determine fetal sex.
- It is suitable for use in biochemical screening laboratories since it is highly automated and does not require specialized personnel.

2 | METHODS

2.1 | Study population

A total of 1200 samples were analyzed, from either prospectively or retrospectively collected high-risk cohorts. All study sites had the required ethical approvals and informed consent. Subjects were at least 18 years of age with a pregnancy of at least 10-week gestation and classified as high-risk for chromosomal aneuploidies by first trimester screening, maternal age, abnormal ultrasound findings, or prior trisomic pregnancy.

Reference outcomes were determined by amniocentesis or chorionic villi sampling (CVS), followed by cytogenetic testing (karyotyping, FISH or QF-PCR) or clinical examination of neonates. Study exclusion criteria were confirmed placental mosaicism, multiple gestation, partial trisomy or translocation, confirmed vanishing twin, maternal malignancy, unknown or insufficient reference outcome information, and other chromosomal abnormalities.

Four hundred thirty-three prospective study samples were collected at King's College Hospital, London, UK (n = 219) and Sahlgrenska Hospital, Gothenburg, Sweden (n = 214). Blood samples (10 mL) were taken prior to CVS because the first-trimester combined test identified these women as being at high risk of trisomies. This cohort included 36 cases of T21, 19 of T18, four of T13, and 374 unaffected samples.

A second cohort of stored plasma samples (n = 757) had been collected in association with routine clinical testing at Cerba laboratory, France, from women referred for cfDNA testing based on physician's assessment or invasive diagnostic testing because the routine NIPT result was positive for one of the major trisomies. The population included 76 cases of T21, 10 of T18, three of T13, and 668 unaffected cases. These samples were analyzed with Vanadis NIPT on site by the study site personnel. Upon shipping and installation of a Vanadis NIPT system to this site, a training and familiarization period of 3 weeks was conducted before the main study phase was initiated by the study site personnel.

In addition, 10 plasma samples collected from women with confirmed aneuploidies following diagnosis of trisomy 18 (n = 7) and trisomy 13 (n = 3) were analyzed. Samples were collected at Karolinska University Hospital, Huddinge, Sweden, from women who had invasive testing; blood draw was performed at 7 days or more after the invasive testing procedure.

In the analyzed cohorts, there were in total 112 cases of T21, 36 of T18, and 10 of T13.

2.2 | Sample collection

Samples (10 mL) were collected in Cell-Free DNA BCT tubes (Streck, Omaha, NE) and processed to plasma using a double centrifugation protocol of either 1300 *g* for 30 minutes followed by 2400 *g* for 20 minutes or 1600 *g* for 15 minutes followed by 2500 *g* for 10 minutes. The plasma fraction was transferred to an intermediate container following the first centrifugation step and transferred to a storage tube after the second centrifugation step. The plasma fraction was extracted within 5 days of receipt and stored at −80°C until processing at the Vanadis Diagnostics Laboratory. Processing and analysis of plasma samples using Vanadis NIPT was performed blinded to the reference outcomes.

2.3 | Test method

The Vanadis NIPT assay relies on a series of enzymatic steps that specifically generate labelled rolling circle replication products (RCPs) from chromosomal cfDNA targets, as previously described.[13] Automated extraction of cfDNA from plasma was performed using the Vanadis Extract platform, followed by continued processing on the Vanadis Core platform to generate labelled RCPs, which were imaged and counted using the Vanadis View instrument.

Samples in the present study were processed in batches of 60 to 82 samples per run on the Vanadis Core instrument, which resulted in hands-on times of approximately 45 to 60 minutes. The total runtime was 40 to 50 hours per run. Since no manual pipetting or intervention is required, once processing has started on each instrument, the workflow allows for processing up to 20 000 samples per year by a single laboratory technician.

2.4 | Image analysis

Images were converted to chromosome specific RCP counts using a prototype version of the Vanadis View image processing platform. The image processing works by first estimating and correcting for spectral crosstalk as well as masking of large bright regions caused by, eg, dust particles. After these steps, the signal from the fluorescent RCPs is enhanced using a custom wavelet filter and the RCPs are distinguished from the background using an adaptive thresholding algorithm. Low quality and deviating images were automatically removed. The output from the Vanadis View instrument consists of four chromosome specific RCP counts, corresponding to chromosomes 21, 18, 13, and Y, and several quality metrics for each sample such as signal intensity and focus quality.

2.5 | Sample classification

Automated data analysis and quality assessment is performed, and chromosomal ratio calculations are calculated for all approved samples as previously described.[13] The approved samples were classified into low or high risk with a *z*-score approach based on each normalized chromosomal ratio and the sample-specific standard deviation. The cutoffs used were a *z* score of 3.5 for chromosome 21 and 3.15 for chromosomes 18 and 13. The samples that failed the quality assessment were rejected and classified as no-calls.

The fetal sex was classified from the number of detected RCPs from chromosome Y relative to the number of RCPs from the measured autosomal chromosomes using an adaptive binary classifier.

2.6 | Technical evaluation of trisomy 21 performance

The point estimate for sensitivity was estimated using the confirmed positive cases in the clinical study results. The trisomy 21 sensitivity was also modelled by fitting a distribution to the observed data using the following assumptions: (a) Since the *z* score of trisomy 21 samples is linearly correlated with the fetal fraction of the sample, they are assumed to be described by the same type of distribution.[13] (b) The fetal fraction distribution is well described by a model such that the square root of fetal fraction is normally distributed.[14] False positive rate is modeled under the assumption that the measured chromosomal ratio of the euploid samples follow a normal distribution.

2.7 | Study IRB numbers

Sample collection in France: AC-2015-2418
Sample collection in UK: 03-04-070
Sample collection in Sweden: Stockholm 2011/156-31/2, Gothenburg EPN 647-15

3 | RESULTS

3.1 | Clinical population demographics

The median maternal age of the study population of 1200 samples was 38 years (interquartile range [IQR]: 34-41), the median gestational age was 14 weeks (IQR: 13-15), and the median maternal weight was 64 kg (IQR: 57-73). Demographics differed slightly between the three cohorts. Median gestational age was 14, 14, and 13 weeks; maternal age was 41, 38, and 36 years; and maternal weight was 69, 62, and 67 kg for the prospective, retrospective, and postdiagnosis cohorts, respectively.

3.2 | Vanadis NIPT test performance

The performance of the Vanadis NIPT test is shown in Table 1. For T21 the sensitivity was 100% (95% CI, 96.8%-100%) and specificity was 100% (95% CI, 99.6%-100%); the respective values for T18 were 89% (95% CI, 73.9%-96.9%) and 99.5% (95% CI, 98.9%-99.8%) and for T13 were 100% (95% CI, 69.2%-100%) and 99.9% (95% CI, 99.5%-100%).

Five unaffected samples failed to pass the automated sample quality assessment criteria resulting in a first-pass assay failure rate of 0.4%. In the current study, only one sample was analyzed from each patient, ie, no reruns or redraws were performed. Four of the assay failures were due to high number of counts, most likely due to high cfDNA concentration of the sample. One assay failure was due to abnormal signal intensity distribution among the counted objects for that sample.

Sex classification was performed in 979 of the samples by analysis of chromosome Y; 99.6% (975/979) provided a concordant result and four provided discrepant results (three males and one female).

3.3 | Modelling trisomy 21 sensitivity

By fitting the observed data to a model where the square root of the fetal fraction is normally distributed, the *z*-score cutoff of 3.5 results in a sensitivity of 99.8% for trisomy 21 (Figure 1). The false positive rate at 3.5 standard deviations from the average normal ratio is estimated to be 0.003% for T21 under the assumption that normal samples follow a normal distribution. Technical evaluation of T18 and T13 performance was not performed due to the low number of affected cases in the study population.

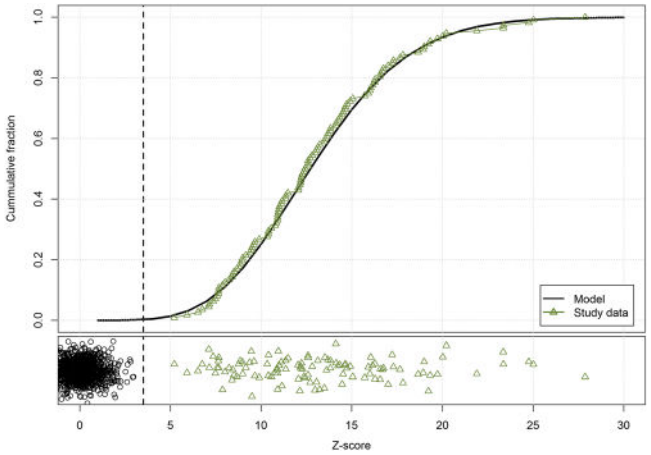

FIGURE 1 Top: Cumulative distribution of trisomy 21 *z* scores as modeled based on published fetal fraction distribution (black line) and from the 112 trisomy 21 cases analyzed in this study (green line). The study trisomy 21 data points are well described by the theoretical model. The model predicts that 99.8% of trisomy 21 cases will have *z*-score values above the cutoff used for trisomy 21 classification (dashed black line). Bottom: Individual *z* scores of unaffected (circles) and trisomy 21 cases (triangles) analyzed in this study [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

The findings of this clinical evaluation of the Vanadis NIPT system demonstrate that the sensitivity of the test was 100%, 89%, and 100% for T21, T18, and T13, respectively, and the respective specificities were 100%, 99.5%, and 99.9%, while maintain a first pass assay failure rate of only 0.4%. In addition, the sex classification provided a correct classification in 99.6% of all tested pregnancies.

The results from the present study indicate lower sensitivity for T18 compared with T21 and T13. These results are consistent with a NIPT meta-analysis also reporting lower T18 sensitivity as compared with T21 and T13.[1] It should also be noted that biological factors such as confined placental mosaicism can contribute to nonconcordant results from cfDNA analysis.

In this study, only a single sample per patient was analyzed. A rerun strategy based on leftover samples of the first pass assay failures is likely to resolve any technical failures since they are not related to sample characteristics. The majority of failures in this study were due to a high number of counted objects, most likely due to high plasma cfDNA levels; thus, they can be diluted and reanalyzed.

Unpublished data suggests that 90% of the first pass failures can be resolved by a rerun of leftover samples, including samples that has gone through a plasma dilution step prior to reanalysis. This high resolve rate is made possible by not failing low fetal fraction samples, which are likely to fail again in a reanalysis.

The fetal fraction of samples collected post diagnosis could in theory be increased by fetomaternal transfusion of cfDNA caused by the invasive testing procedure. However, the *z*-score result of T18 (n = 7) and T13 (n = 3) samples collected post confirmatory diagnosis was on average 1.5 *z* scores lower compared with the average of the other T18 (n = 29) and T13 (n = 7) cases included in this study, indicating no increase in fetal fraction due to the invasive procedure. This is in line with data published by Samuel et al who reported a downward

TABLE 1 Vanadis NIPT results for trisomy 21, trisomy 18, and trisomy 13 screening versus reference outcome

Condition	Trisomy (n)	Nontrisomy (n)	Sensitivity (95% CI)	Specificity (95% CI)
Trisomy 21	112	1037	100% (96.8%-100%)	100% (99.6%-100%)
Trisomy 18	36	1037	89% (73.9%-96.9%)	99.5% (98.9%-99.8%)
Trisomy 13	10	1037	100% (69.2%-100%)	99.9% (99.5%-100%)

Abbreviation: NIPT, noninvasive prenatal testing.

trend in cell-free fetal DNA in maternal plasma at 24 hours and 7 days following CVS.[15]

Modelling of assay performance based on the analyzed cases for T21 show a strong separation of the affected and unaffected population and indicates a sensitivity well above 99% (99.8%). This is achieved by having a high precision measurement, and the assay does not identify and discard samples with low fetal fraction in order to achieve this performance.

The Vanadis technology is developed to eliminate complexity and facilitate high levels of automation to minimize hands-on time while still retaining the high measurement precision achieved by counting individual molecules without prior amplification.[13] As part of the study, the Vanadis system was installed at an external laboratory and operated by laboratory technicians without previous experience of genetic testing. This demonstrates that the Vanadis NIPT platform can be easily adopted with minimal operator training and hands-on sample processing time to provide a cost-effective automated screening solution for trisomies 21, 18, and 13.

The majority of screening programs only provide testing to a subset of women identified by prior screening as being at high risk of aneuploidies. Providing cfDNA testing as a second-tier test reduces the false positive rate but does not increase the detection rate since these pregnancies are missed in the first-tier screening test. The Vanadis system enables a reduction in cost and workflow complexity without compromising on performance; consequently, the test can be made available to a wider population of pregnant women.

CONFLICT OF INTEREST

Authors with affiliation to Vanadis Diagnostics were at some point during the project employed by PerkinElmer or Vanadis Diagnostics that holds the commercial rights to the technology presented herein.

B.J. has performed clinical diagnostic trials for Ariosa, Natera, and Vanadis Diagnostics with reimbursement for costs per patient. No personal reimbursements.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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