Cell-free DNA screening for fetal aneuploidy using the rolling circle method: A step towards non invasive prenatal testing simplification

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Abstract

Objective: To assess the efficacy of cell-free (cf)DNA screening for aneuploidy using the automated system based on rolling circle replication.

Methods: A prospective study among women referred for invasive prenatal diagnosis between July 2018 and December 2019. The plasma fraction was extracted within 5 days from blood collection, stored at −20°C and cfDNA measured between January and December 2019.

Results: A total of 805 women were recruited; 778 with singleton pregnancies and 27 twins. There were 48 Down syndrome, 25 Edwards syndrome and 3 Patau syndrome cases. Overall, the no-call rate was 2.6% (95% confidence interval 1.6%–3.9%) which reduced from 4.7% to 1.1% after relocation of the system (p < 0.002) to ensure a constant ambient temperature below 25°C. In singletons the Down syndrome detection rate (DR) was 100% (93%–100%) and false-positive rate (FPR) 0.14% (0.00%–0.79%). The Edwards syndrome DR was 96% (80%–100%) and FPR 0.78% (0.29%–1.7%). One false-positive had a confined placental trisomy 18 and the remaining five a z-score requiring sample repetition; all the false-positives occurred before system relocation (p < 0.005). Patau syndrome DR and FPR were 67% (9.4%–99%) and 0.26% (0.03%–0.95%).

Conclusion: The cfDNA rolling circle method yields similar results to other methods provided that room temperature is adequately controlled.

Key points

What is already known about this topic?
• Cell-free DNA has considerably better screening performance than conventional tests
• Performance of the rolling circle method, in a single study, was similar to the three widely used methods

What does this study add?
• Confirmation of the performance of the rolling circle method
• Demonstration that it can be readily incorporated into a conventional screening laboratory
INTRODUCTION

Currently, in Italy screening for Down and Edwards syndromes is by either the conventional “Combined” test, based on first trimester maternal serum pregnancy associated plasma protein (PAPP)-A, free-β human chorionic gonadotrophin (hCG) and ultrasound nuchal translucency (NT), or the “Integrated” test which incorporates first trimester PAPP-A and NT with second trimester serum markers – in the Piedmont region they are α-fetoprotein (AFP), unconjugated estriol (uE3) and hCG.

A single maternal plasma marker of Down, Edwards and Patau syndromes, cell-free (cf)DNA, has considerably better screening performance than all conventional multi-marker screening tests. This has prompted us to consider an improvement of the screening protocol in Piedmont. Three cfDNA policies can be considered: “Primary” offering cfDNA to women identified as high risk because of a screen-positive conventional test or another indication; and “Contingent” offering it to those with screen-positive/borderline screen-negative results, or borderline screen-positive/screen-negative results, and very high risk pregnancies directly referred to invasive prenatal diagnosis. Several studies have shown that at current cfDNA costs, Primary screening is expensive. Secondary screening is cost-neutral or better and Contingent screening is affordable (see review). Moreover, the Contingent approach would have an additional economic and practical advantage if cfDNA testing can be carried out in the same biochemical laboratory as the conventional screening.

Three laboratory methods of cfDNA assay are currently in widespread use: whole-genome massively parallel sequencing (MPS) to sequence and count large numbers of unique cfDNA fragments and assign them to the chromosome of origin; a similar MPS approach but targeting specific chromosome regions of interest for enrichment and amplification; and analyzing single nucleotide polymorphisms thus determining the relative quantitative contributions of maternal and fetal DNA in the plasma. These methods require specialized equipment and staff as facilities for polymerase chain reaction (PCR) and massive sequencing not generally available in a biochemical screening laboratory.

Recently, a new method has been developed which incorporates target regions into circular DNA that can undergo rolling circle replication, fluorophore labeling of the products, and detection following nanoparticle filtration. From the only published clinical study this seems to have a screening performance similar to that of the other methods with the advantage of being highly automated without requiring next generation sequencing expertise.

This study was carried out in the Regional referral center for prenatal screening of Piedmont. The aim was to investigate the efficacy of the new automated cfDNA assay in a cohort of high risk women attending prenatal invasive diagnosis for different indications thus comparing the results of the non-invasive test with the fetal karyotype. Most women had already had Combined or Integrated tests and the rest were tested retrospectively so as to estimate cfDNA efficacy in women who would be selected in a Contingent screening policy based on borderline screen-positive/screen-negative results.

METHODS

Participants

This was a prospective study carried out among women referred for invasive prenatal diagnosis to the Ultrasound and Prenatal Diagnosis Unit at St Anna Hospital between July 2018 and December 2019. All women aged 18–55 referred after 10 weeks’ gestation were recruited to the study. Exclusion criteria were: severe hypotension episodes or other conditions that may complicate the blood drawing or other pregnancy related conditions; maternal aneuploidy; cancer; and invasive prenatal diagnosis earlier in pregnancy.

Blood samples

For those agreeing to participate in the study a 10 ml blood sample was collected in a blood collection tube (Streck®) and sent to the Prenatal Screening laboratory for processing, storage and analysis. Women referred for chorionic villus sampling (CVS) without having had conventional screening were included in the study provided they agreed to have a retrospective Combined test in which case a further 2 ml of blood was collected for serum markers testing. Such women, as well as those who had already had a Combined test, were also asked to have an Integrated test. In such cases a further 2 ml of blood was collected for serum marker testing and a second blood sample was taken three weeks later. Women referred for amniocentesis without having had a conventional screening test were not included in the study. The results of these screening tests were not used clinically.

Cytogenetics

Genetic analysis of samples obtained at amniocentesis or CVS was performed in the Medical Genetics Unit. Diagnosis was mostly based on karyotyping whereas a smaller number had array comparative genomic hybridization or trisomies assay by quantitative fluorescent PCR (QF-PCR). In a very small number of pregnancies the fetal material was insufficient for karyotyping and the normal diploid status was assessed on the basis of clinical outcome.

cfDNA test

Plasma was extracted using a double centrifugation protocol of 1300 g for 30 min followed by 2400 g for 20 min. The plasma fraction was transferred to an intermediate container following the first
centrifugation step and to a Sarstedt tube after the second centrifugation. The plasma fraction was extracted within 5 days of storage at room temperature following the blood draw and then stored at −20°C until processing.

Testing was carried by the rolling circle method between January and December 2019 using the automated Vanadis® system (PerkinElmer) in 20 analytical runs. The system extracts and selects thousands of unique DNA fragments from the target chromosomes and converts them into large spherical DNA objects as described previously. These objects were counted by a simple digital readout. After automated quality assessment, the results were classified as high risk or low risk according to the z-score. The z-score cut-offs were 3.50 for chromosome 21 and 3.15 for chromosomes 18 and 13. Before the Vanadis system was installed in the laboratory, cfDNA was extracted from plasma using the Microlab STARlet workstation (Hamilton) (71.4% of samples) while testing was completed with the Vanadis system. Samples that failed quality assessment were reported as no-calls. The cfDNA results were not used clinically.

Laboratory technicians, in training biologists and health managers had a one-week training course on site. Three technicians were involved in the study and worked in pairs, dedicating part of their time over three days for each of the 20 analytical runs.

Screening performance of cfDNA was assessed in terms of the no-call rate and, in the accepted samples, the detection rate (DR) and false-positive rate (FPR) for each syndrome. The DR was the proportion of affected pregnancies with high risk results for the corresponding trisomy. The FPR was the proportion of pregnancies without that syndrome, including those with other aneuploidies, with high risk results for the corresponding trisomy.

During the course of the study it was noticed that the quality of analytic runs was systematically decreased possibly affecting results, although not all runs failed the quality control criteria. This was investigated and high room temperature (>25°C) during the summer months exceeded the requirements set for the instruments. During the investigation, a part of the Vanadis Extract® instrument (EXU) was replaced and additional air conditioning was installed in July 2019. Because the problem persisted, the Vanadis system was relocated to a different laboratory space in September 2019. Screening performance was assessed separately in the runs before and after relocation of the system to the new laboratory.

### 2.5 Conventional screening

Maternal serum PAPP-A and free-β hCG (Delfia Xpress, PerkinElmer), AFP, uE₂ and hCG (AutoDELFI, PerkinElmer) assays were performed in the Prenatal screening laboratory. The levels of these markers, together with the NT measurement, information on maternal age, gestational age, maternal weight, smoking status, assisted reproduction and insulin-dependent diabetes were used to compute the risk of Down and Edwards syndrome using the Alpha Software (Logical Medical Systems). The same laboratory and software systems were used to calculate risks in women whose additional blood samples were retrospectively tested; for women who already had an Integrated test, the blood sample was taken from storage for free-β hCG testing in order to complete a Combined test. Results were classified as borderline if the risk of Down syndrome was in the range 1 in 100-1 in 2500 at term or the risk of Edwards syndrome was 1 in 100-1 in 1000.

### 3 RESULTS

A total of 805 women were recruited to the study and the characteristics of their pregnancies are summarized in Table 1. There were 778 singletons and 27 twins including five “vanishing” twins, that spontaneously reduced to singletons before recruitment; of the non-vanishing twins 9 were mono-chorionic, 10 dichorionic and three with unknown chorionicity. The series included 48 pregnancies affected by Down syndrome, one of which was a twin that had reduced to a singleton, 25 Edwards syndrome and 3 Patau syndrome. Among pregnancies without any of the three common trisomies, 5.5% (40/729) had other chromosomal or genomic variants (28 pathogenic, including placental mosaicisms, 6 benign and 6 of uncertain significance); 5% (35/703) in singletons and 19% (5/26) in twins. Of those enrolled in the study, four women refused the planned invasive prenatal diagnosis, and in two cases the fetal cell culture failed. With respect to these six cases, five women had an unaffected pregnancy on the basis of clinical examination of the infant whereas one fetus had ultrasound sign of Patau syndrome (holoprosencephaly) and the pregnancy was terminated.

There were 438 women who had not had conventional screening before enrollment and except for 9, all singletons, including 2 with Down syndrome, they were screened as part of the study. Of the 796 screened before or subsequently, a Combined test was carried out in 782 and an Integrated test in 515, with both tests performed in 501 cases.

Table 2 shows for singletons the no-call rate according to diagnosis. The rates are shown for all women, separately for the period before and after the Vanadis system was relocated, and separately for those with and without borderline conventional screening results (i.e., risk between 1 in 100 and 1 in 2500 for Down syndrome or 1 in 1000 Edwards syndrome). The overall no-call rate was 2.6% (20/778; 95% confidence interval [CI] 1.6%–3.9%). There was no statistically significant difference in this rate according to diagnosis or the screening result. But the relocation of the Vanadis system was associated with a statistically significant reduction in no-calls from 4.7% to 1.1% (15/322 compared with 5/456; p < 0.002, Chi-squared test).

Table 3 shows the DR and FPR in the 758 singleton pregnancies after excluding the no-calls. As in Table 2 the results are shown for all women and broken down into subgroups. All Down syndrome pregnancies were detected by cfDNA with only one false-positive result which had a chromosome 21 z-score of 3.62, just above the cut-off, while in the true-positive singleton pregnancies the z-score was 7.35–37.7 (median 16.59). The DR for Edwards
syndrome was lower (96%) due to one false-negative cfDNA result but the overall false-positive was considerably higher. All six false-positives occurred before the instrument was moved (p < 0.005). One of them was indeed a true chromosome 18 trisomy confined to the placenta (47,XX,+18 in 20 metaphases) while the karyotype on amniotic fluid cells was normal without any evidence of fetal mosaicism (46,XX in 32 metaphases and two signals for chromosome 18 centromere in 115 interphase nuclei by FISH assay). The other five false-positive results had a chromosome 18 z-score of 3.43–4.25 compared with 4.25–19.78 in the true-positive singleton pregnancies (median 9.11). Of the three Patau syndrome cases one was a cfDNA false-negative and there were two false-positives. None of the affected pregnancies had a false-positive result for a different syndrome. There were no statistically significant differences in the FPRs between those with and without borderline conventional screening results.

The cfDNA tests on the 27 twins did not result in any no-calls, the Down syndrome case (a vanishing twin) had a true-positive result and all the unaffected pregnancies had true-negative results.

### TABLE 1 Patient and pregnancy characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>Median (IQR) 36 (33–39)</td>
</tr>
<tr>
<td>Gestational age at CVS (days)</td>
<td>Median (IQR) 85 (82–89)</td>
</tr>
<tr>
<td>Gestational age at amniocentesis (days)</td>
<td>Median (IQR) 117 (113–126)</td>
</tr>
<tr>
<td>Maternal weight (kg)</td>
<td>Median (IQR) 62 (55–70)</td>
</tr>
<tr>
<td>Origin</td>
<td>Italy (632 (78.5%)) Other European (88 (10.9%)) Africa (38 (4.7%)) Elsewhere or unknown (47 (5.8%))</td>
</tr>
<tr>
<td>Fetuses</td>
<td>Singleton (778 (96.6%)) Twin (27 (3.4%)) Of which vanishing twin 5 (0.6%)</td>
</tr>
<tr>
<td>Main reason for referral</td>
<td>NT, positive biochemical screening and/or NIPT done elsewhere 360 (44.7%) Advanced maternal age onlyb 236 (29.3%) Recurrence risk for chromosomal, genomic or genetic diseases 122 (15.2%) Ultrasound fetal abnormalities 66 (8.2%) Infection and other 21 (2.6%)</td>
</tr>
<tr>
<td>Invasive prenatal diagnosis</td>
<td>CVS (475 (59.0%)) Amniocentesis (295 (36.6%)) Both (31 (3.9%)) Neither 4 (0.5%)</td>
</tr>
<tr>
<td>Genetic diagnosis</td>
<td>Karyotype (743 (92.3%)) Array CGH or QF-PCRa 39 (4.8%) Both 17 (2.1%) Not done or failure 6 (0.7%)</td>
</tr>
<tr>
<td>Screening testc</td>
<td>Combined test only (281 (34.9%)) Integrated test only 14 (1.7%) Both 501 (62.2%) Not done/incomplete 9 (1.1%)</td>
</tr>
</tbody>
</table>

Abbreviations: CGH, comparative genomic hybridization; CVS, chorionic villus sampling; IQR, inter-quartile range; NIPT, non invasive prenatal testing; NT, nuchal translucency; QF-PCR, quantitative fluorescent polymerase chain reaction.

a Only one QF-PCR was performed.

b Age ≥35 is currently an indication, regardless of the screening result if done, although this policy is about to change.

c Before the study or subsequently.
This study shows a considerable screening performance for the new highly automated method of cfDNA testing. A high degree of automation, obviating the need for expertise in next generation sequencing, makes the method suitable for biochemical screening laboratories.

The singleton Down syndrome DR of 100% (95% CI 93%–100%) and FPR of 0.14% (95% CI 0.00%–0.79%) are similar to that found in the only other published study of testing clinical samples using the Vanadis method with rates of 100% (112/112, 95% CI 97%–100%) and 0.00% (0/1037, CI 0.00%–0.36%). In a meta-analysis of 47 published studies using other cfDNA methods the weighted pooled estimate of DR was 99.7% (95% CI: 99.1%–99.9%) and FPR 0.04%.
The meta-analysis included both prospective studies on samples drawn in conventional screening programs and retrospective studies in women with samples mostly drawn before invasive prenatal diagnosis. Prospective studies are affected by incomplete follow-up and 'viability' bias due to the inclusion of detected non-viable cases. Including just the 22 retrospective studies the overall, pooled but not weighted, DR was 99.3% (95% CI 98.6–99.7%) and FPR 0.11% (95% CI 0.06%–0.18%).

The singleton Edwards syndrome DR was 96% (95% CI 80%–100%) and FPR 0.78% (95% CI 0.29%–1.7%). By comparison, in the other Vanadis clinical study the rates were 89% (32/36, 95% CI 74%–97%) and 0.48% (5/1037, CI 0.16%–1.1%) respectively; and in the meta-analysis of retrospective studies 97% (95% CI 94%–98%) and FPR 0.09% (95% CI 0.04%–0.16%) respectively. Whilst the CIs for the DRs using the new method overlap with those for the other methods, the FPR in the current study is relatively high and the lower confidence limit exceeds the upper limit for the other methods. However, one false-positive result occurred in a case of chromosome 18 trisomy confined to the placenta while fetal cells from amniotic fluid had normal karyotype thus representing discordance between CVS and amniocentesis results. The remaining five false-positive results had chromosome 18 z-scores within a borderline zone where the Vanadis kit instructions recommend repeating the test on a second sample. The current study did not allow test repetition because each woman provided only one sample. Moreover, with outcome based on amniotic fluid karyotype – three out of five false-positives – placental mosaicism cannot be excluded, nor maternal chromosomal abnormalities or residual cfDNA derived from a vanishing twin.

The singleton Patau syndrome DR and FPR were 67% (95% CI 9.4%–99%) and 0.26% (95% CI 0.03%–0.9%); the clinical Vanadis study 100% (10/10, 95% CI 69%–100%) and 0.10% (1/1037, CI 0.00%–0.54%)4; and the meta-analysis of retrospective studies 90% (95% CI 83%–95%) and FPR 0.18% (95% CI 0.11%–0.28%). The poor Patau syndrome DR was evaluated on a very limited number of cases; in a Contingent cfDNA approach such pregnancies might be referred for an invasive procedure because of their high risk conventional screening test results.

Overall, among singletons there were 20 no-calls, a rate of 2.6% (95% CI 1.6%–3.9%). In the meta-analysis 26 studies reported a singleton no-call rate, after excluding failures due to blood collection and transportation to the laboratory. The median no-call rate was 2.3% and for studies giving the reason for no-calls, in about half the samples the reason was a fetal fraction (FF), the proportion of cfDNA derived from the fetus, below 4%. This cut-off is widely used because the cfDNA test is expected to be less accurate at such levels.

However, unlike other cfDNA methods, Vanadis does not measure FF. This is because of reduced assay variability due to not having a DNA amplification step which allows test accuracy at FF below 2%. Moreover, the measurement of FF itself has been shown to introduce considerable variability. In the other prospective study of the Vanadis method the no-call rate was only 0.42% (5/1200, 95% CI 0.14%–0.97%). The much higher no-call rate in the current study is attributable to the decreased quality of assay runs in the early part of the study, possibly due to high temperature in the laboratory space. After the system had been moved to a more suitable space the no-call rate was 1.1% (95% CI 0.36%–2.5%) and consistent with the other study. The difference in no-call rate before and after the move cannot be attributed to factors such as gestational age and maternal weight, which are known to influence FF. In fact, first trimester samples analyzed in the two time periods belonged to women with a median gestational age of 86 and 85 days, respectively (p = 0.13, Wilcoxon Rank Sum Test) and the median maternal weight was 63.0 and 61.5 kg (p = 0.55, Wilcoxon Rank Sum Test).

The DRs reported in the meta-analyses and in the two studies using the Vanadis method exclude no-calls but in practice the effective DR will depend on how no-calls are treated. Referring all of them for invasive prenatal diagnosis, as some medical bodies currently suggest, will increase the effective DR but considerably increase the FPR, taking no further action will reduce the effective DR and FPR, whilst testing a repeat blood sample will resolve about two-thirds of no-calls due to low FF. The resolution of no-calls using the Vanadis method has been assessed in 8 out of 545 samples (1.5%) tested in Finland; in the repeated samples only one was a no-call. It is unclear how many of the studies in the meta-analysis reported no-call rates after repeat testing but neither of the Vanadis studies had repeat testing as only one sample was available.

As with conventional multi-marker screening, the performance of cfDNA testing in twin pregnancies is expected to be less than in singletons due to the underrepresentation of the supernumerary chromosome in twins discordant for a trisomy. Although in theory detection in twins should be lower than in singletons, it can be offset to some extent by a higher FF in twins, estimated to be one-third in one study. In fact, in a meta-analysis of 11 published studies of discordant twins the DRs were similar to singles: Down syndrome 97% (57/59, 95% CI 88%–100%), Edwards syndrome 89% (8/9, 95% CI 0.52%–100%), and Patau syndrome 100% (2/2, 95% CI 16%–100%). The FPR was 0.05% (1/1952, 95% CI 0.00%–0.58%). FF may differ between the two fetuses and a policy has been adopted whereby both FFs are estimated and the smaller is used to decide on a no-call. This will lead to a higher no-call rate in twins than singletons; 9.4% and 2.9% in one study. The current study had too few twins to make any comparisons and the previous clinical study of the Vanadis method excluded twins.

From a practical perspective a known vanishing twin might be a contra-indication for cfDNA testing but the five in the current study were included as twins. There were no false-positives or false-negatives and so if they had been classified as singletons the no-call, DR and FPR would not have been materially altered.

The screening performance of cfDNA using any of the four methods is substantially higher than the Combined or Integrated tests. For example, at 11 weeks’ gestation the Combined test model predicted Down syndrome DR – for a 5% FPR using parameters based on meta-analysis and a standardized maternal age distribution – is 87%. For the Integrated test the predicted DR increases to 94% although this would be lower without second trimester inhibin-A, as in Piedmont.
When considering a Contingent cfDNA screening policy it is relevant to assess the test performance in those with borderline conventional screening results (i.e., risk 1 in 100-1 in 2500 for Down syndrome or 1 in 1000 Edwards syndrome) compared with the remainder. This study found a lower overall no-call rate in the Combined test borderline group of 1.8% (7/383) compared to the screen-negatives where it was 2.9% (11/373) although this difference did not reach statistical significance ($p = 0.31$, Chi-squared test). There was no material difference in no-call rate according to the Integrated test results. There were too few affected pregnancies with borderline results to compare DRs but there were sufficient unaffected or other pregnancies to compare FPRs. Given that a pregnancy with a syndrome could be a false-positive for another syndrome, the overall rate in the Combined test borderline group was 1.1% (4/376) compared with 1.4% (5/362) in screen-negatives which was not statistically significant ($p = 0.69$). For the Integrated test borderline group, the corresponding rates were 1.4% and 1.9% ($p = 0.62$).

The Contingent cfDNA policy examined here is based on women with borderline Down or Edwards syndrome risks being offered a cfDNA test and those with risks above the cut-offs being referred directly for invasive prenatal diagnosis. Since the study was based on women referred for invasive testing, many of whom had a positive conventional screening test as the indication, it cannot be used to directly estimate the DR and FPR of the Contingent cfDNA policy. However, published model predicted estimates are available in the literature and local details such as the maternal age distribution, type of conventional screening test and specific cut-off risks defining the borderline are unlikely to make a substantial difference.

The rolling circle method, in common with other cfDNA tests, can also accurately detect fetal gender. The current study is focused on using the method in screening for aneuploidy and gender determination was not investigated.

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CONFLICT OF INTEREST
Prof. Cuckle is a paid consultant of Perkin Elmer Life Science which supplies the Vanadis system. All authors received no external funding for this work.

ETHICS STATEMENT
The study protocol was approved by the regional ethics committee, decision numbers 0010199 and 0009379.

DATA AVAILABILITY STATEMENT
Access to the data is by direct contact with the authors.

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REFERENCES