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RESEARCH ARTICLE

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Non-Invasive prenatal testing with rolling circle amplification: Real-world clinical experience in a non-molecular laboratory

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Abstract

Background: Non-invasive prenatal testing (NIPT) using cell-free DNA (cfDNA) circulating in maternal blood provides a sensitive and specific screening technique for common fetal aneuploidies, but the high cost and workflow complexity of conventional methodologies limit its widespread implementation. A unique rolling circle amplification methodology reduces cost and complexity, providing a promising alternative for increased global accessibility as a first-tier test.

Methods: In this clinical study, 8160 pregnant women were screened on the Vanadis system for trisomies 13, 18, and 21, and positive results were compared to clinical outcomes where available.

Results: The Vanadis system yielded a 0.07% no-call rate, a 98% overall sensitivity, and a specificity of over 99% based on available outcomes.

Conclusion: The Vanadis system provided a sensitive, specific, and cost-effective cfDNA assay for trisomies 13, 18, and 21, with good performance characteristics and low no-call rate, and it eliminated the need for either next-generation sequencing or polymerase chain reaction amplification.

KEYWORDS
aneuploidies, cell-free fetal DNA, cfDNA, next-generation sequencing, NIPS, NIPT, non-invasive prenatal testing, prenatal screening, rolling circle amplification, trisomies

1 | INTRODUCTION

Non-invasive prenatal testing (NIPT) has revolutionized prenatal testing, impacting expectant women worldwide since its clinical introduction in 2011.[1] This sensitive and specific screening technique for common aneuploidies detects and analyzes cell-free DNA (cfDNA).[1,2] Originating from the placenta, cfDNA representing the pregnancy can be found circulating in maternal blood starting in early pregnancy up until hours after delivery, making it a prime target for prenatal testing.[1,2] NIPT allows for direct screening from maternal plasma for fetal chromosomal abnormalities as early as 10 weeks' gestation.[1,3]

Technological advances in analyzing cfDNA by next-generation sequencing (NGS) have provided a commonly used platform capable of the precision needed to detect the small changes in copy number required for fetal aneuploidy analysis with NIPT.[1] Targeted aneuploidies include trisomy 21 (T21, Down syndrome), trisomy 18 (T18, Edwards syndrome), and trisomy 13 (T13, Patau syndrome), among others.[1,2,4,5] CfDNA-based NIPT boasts a very high sensitivity for detecting Down syndrome, Edwards syndrome, and Patau syndrome

combined with a very low false-positive rate.[4] The highly accurate results of NIPT reduce the number of invasive testing procedures that could result in a miscarriage.[1,4] However, many NIPT technologies do exhibit higher rates of non-reportable results, or no-calls, than conventional aneuploidy screening.[6–9]

While NIPT technologies have vastly improved sensitivity and specificity for trisomies 13, 18, and 21 compared to that of conventional screening tools such as biochemical maternal blood tests and/or ultrasound, NIPT is still not widely offered within many laboratories globally due to the cost and workflow complexity.[10,11] Due to this limited accessibility, cfDNA-based NIPT has been used in some settings as a follow-up procedure for high-risk pregnancies detected with traditional first-trimester screening, limiting the advantage of the higher sensitivity.[10,11] Removal of the cost and complexity roadblocks faced by many laboratories may allow for more widespread implementation of NIPT, along with the performance benefits it presents.

Recently, a newer NIPT technology has been introduced and validated, which provides a much-needed reduction in cost and workflow complexity by eliminating the need for NGS and/or polymerase chain reaction (PCR) amplification, along with the associated infrastructure.[10,11] The Vanadis® system (PerkinElmer, Waltham, MA, USA) screens chromosomes by converting chromosome-unique fragments into digitally quantifiable objects through rolling circle replication.[10–12] With this methodology, target fragments from a chromosome of interest are directly captured using specific probes before being converted into circular DNA. This DNA is then amplified via rolling circle replication to form a single rolling circle product (RCP), which is fluorescently labeled for counting by the imaging system.[10] (Figure 1).

The goal of our study at Women's Health Connecticut (WHCT) was to determine the performance metrics and clinical utility of this newly developed rolling circle replication method.

2 | METHODS

2.1 | Study population and sample collection

Between March 16, 2021 and March 31, 2022, physicians within the Women's Health Connecticut (WHCT) practice, a 90-location practice with 250 physicians were offered general population screening of pregnant patients using the Vanadis system, to screen for the detection of trisomies 13, 18 and 21. Eight thousand one hundred and sixty samples were collected, and the participants included 5870 women under the age of 35. Inclusion criteria included women whose fetuses had a gestational age ≥10 weeks. Thirty-two patients were excluded from the study due to the gestational age of the fetus. Two 10 mL tubes of whole blood were collected in physicians' offices or collection sites into Cell-free DNA BCT tubes (Streck, Omaha, NE, USA) and transported to WHCT for NIPT testing with the Vanadis system. Following a double-centrifugation step, as per Vanadis system protocols, plasma was extracted from both tubes and frozen at −80°C until ready for further processing. If available, the second tube collected from each patient was used as a backup for any test failures and repeats during the study.

2.2 | Test method using Vanadis system: Clinical analysis

2.2.1 | Extraction and amplification

All samples were tested and analyzed using the Vanadis system according to the manufacturer's manuals and kit inserts. An overview of the methodology can be seen in Figure 1. Briefly, this consisted of extracting cfDNA from plasma using a bead-based protocol on the Vanadis Extract® Instrument. In the Vanadis Core® Instrument,

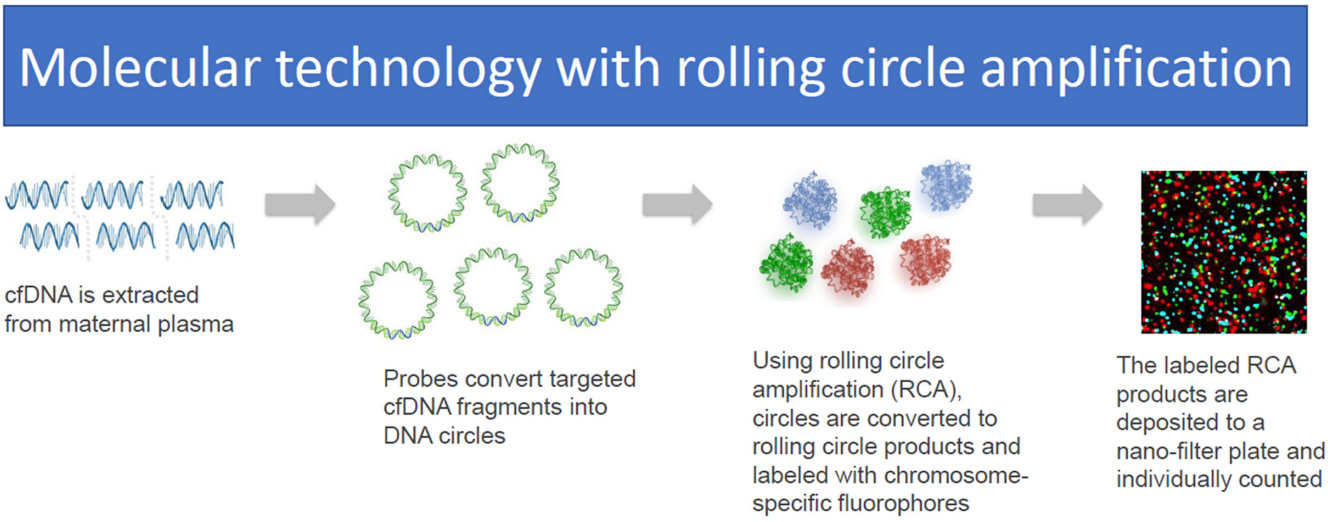

FIGURE 1 Molecular Technology with Rolling Circle Amplification. Flowchart of procedure used for NIPT analysis with the Vanadis system. Abbreviations: cfDNA, cell-free DNA; RCA, rolling circle amplification.

locus-specific oligonucleotide probes and DNA ligase were used to convert targeted fragments into circular DNA. The circular DNA was then amplified by DNA polymerase to form rolling circle products and fluorescently labeled according to the chromosome of origin in preparation for imaging.[10]

cfDNA from 8160 samples was processed in the Vanadis system and repeat runs on the second tube were conducted only for samples with z-scores within an intermediate range for trisomy 18 or that failed the first run, as detailed in the next section.

2.2.2 | Image analysis and sample classification

Samples were imaged and counted within the Vanadis View® Instrument.[13] The Vanadis system software was used for quality assessment prior to analysis of the normalized chromosomal ratio and z-score calculation using Lifecycle™ software version 7.0 Rev 3 RUO (PerkinElmer Inc.). Based upon these z-scores, a high- or low-risk designation was made for each sample using z-score cutoffs of 3.5 for trisomy 21, and 3.15 for trisomy 13. For trisomy 18, there was a reflex protocol that was dependent on the z-score. Samples with z-scores <2.70 on the first run were called low-risk. Samples ≥5.00 were called high-risk. For z-scores between 2.70 and 4.99, the sample was repeated if a second tube was available, starting with cfDNA extraction. Then for the second run, samples with a z-score <2.00 were reported low risk, and samples ≥2.00 were reported as high-risk. For samples which did not have a second tube, the trisomy 18 call was based on the original tube using a 3.15 z-score cutoff. All z-score cutoffs were taken from the Vanadis system's IVD (In Vitro Diagnostic) kit inserts.

2.2.3 | Fetal sex determination calculations

Fetal sex determination was calculated by examining the ratio of Y-chromosome RCPs relative to autosomal RCPs using an adaptive binary classifier.[10,11]

2.3 | Clinical outcome evaluation

Clinical outcomes were available for some patients, and these outcomes were included in the study for comparison to the Vanadis system results. The clinical outcomes were determined using one or more of the following methods: birth examinations, chorionic villus sampling, ultrasound, and/or amniocentesis. Due to the close relationship between our lab and our clinicians, discrepant results between clinical outcomes and Vanadis system results were reported to the laboratory when detected, so although we were unable to collect outcomes on all screen negatives, we were made aware immediately when a false-negative arose, thus all of these patients were likely accounted for in our calculations.

3 | RESULTS

Of the 8160 samples tested with the Vanadis system at WHCT, 38 were excluded from analysis (32 for gestational age and six due to no-call results). (Figure 2) Of the 32 samples excluded for gestational age, two samples were excluded because gestational age was not provided and 30 samples because gestational age was outside the range for inclusion in the study.

There was a first-pass no-call rate of 0.98% (80 samples). However, 92.5% of these first-pass no-calls resolved with a second run of the patient's sample, for a final no-call rate of 0.07% (six samples) from a single draw. Reasons for first-pass testing no-calls are provided in Table S1. Traditionally, "no-call" results are provided on samples where the laboratory cannot provide a result (often due to technical reasons—so-called "test failures") or choose not to provide results (often as the laboratory is not confident enough in the given sample's results). With the Vanadis system, the high number of quality metrics that each sample must pass, along with the exclusion of a fetal fraction cutoff, ensures that the only causes for the system not generating a result are technical or biological. Due to this, we will often use the terms "test failures" and "no-call results" interchangeably.

After exclusions, 8122 samples were processed on the Vanadis system and provided screening results for trisomies 13, 18, and 21. Of the 8122, 5867 patients were under 35 and 2255 patients were 35 or older. The patients' mean age was 31 years, ranging from 14 to 50 years. The mean gestation age was 12 weeks, 1 day (range 10 weeks, 0 days–40 weeks, 0 days). All samples were run at least once on the Vanadis system. Repeat runs were conducted for samples with z-scores within an intermediate range for trisomy 18 (2.70–4.99) or that failed the first run. Ninety-two samples screened positive and 8030 screened negative. (Figure 2) (Table 1).

Of the 92 screen-positive samples, 75 (81.5%) had an available clinical outcome, and 17 (eight T21, six T18, and three T13) did not have an available clinical outcome (Table 1). The 17 samples that had unavailable outcomes were only used in screen-positive rate calculations, but not in the other performance metrics. For the 75 samples that had screened positive with the Vanadis system and that had available clinical outcomes, 43 were clinically affected and 32 were clinically unaffected. (Table 1).

During the course of the study, we were notified of three trisomy 21 false-negative (FN) results. One of the FN results (Case #1) had a z-score of −0.63 and a post-natal blood chromosome analysis identified as the standard 47,X*,+21 Down syndrome karyotype. The second (Case #2) was T21 FN with a z-score of 3.04 and was reported to be confirmed via karyotype on the newborn. The final (Case #3) FN had a z-score of 0.82 and was identified to have a 21q;21q translocation, which in the literature has been shown to be overrepresented in FN cfDNA screening results involving Down syndrome.[14]

For Case #1 and 2, there was additional frozen plasma available for retesting. A comprehensive examination of the instruments and factors which could have caused these FN results provided no

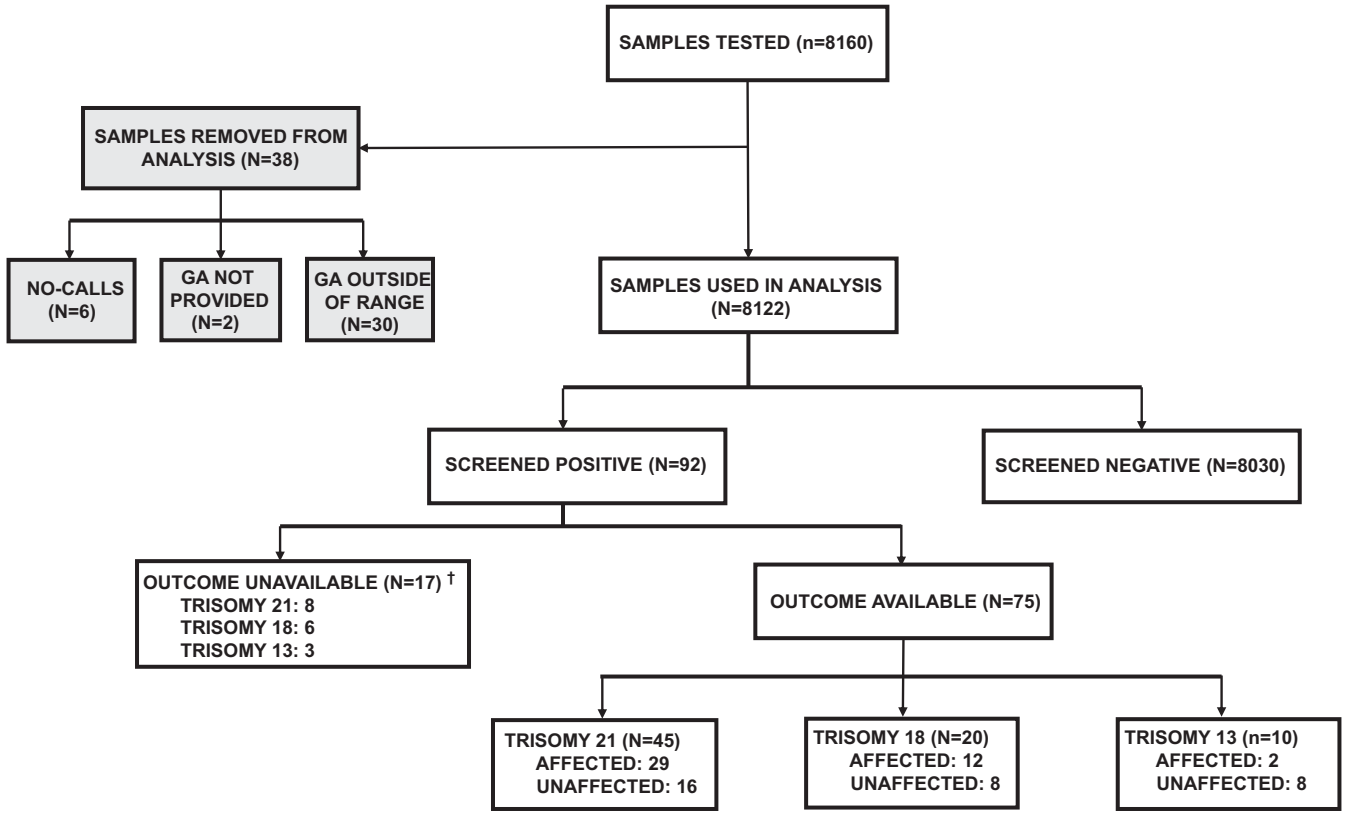

FIGURE 2 Flowchart for Clinical Study. Flowchart of sample processing for clinical study, with sample inclusions/exclusions and outcomes. Abbreviations: GA, Gestational Age. †Only used in screen-positive rate calculations.

insight, and so these samples were sent to an external commercial NIPT laboratory that performs NGS in order to evaluate concordance of results against another platform. The NIPT assay run on the external NGS platform also resulted in a negative (missed) result for Case #1. Case #2 was positive, however, the external commercial NIPT laboratory was unable to provide z-scores for this sample as per their protocol. As a result, we do not know whether this would have been considered a borderline result in their assay or a more strongly positive result.

For more comprehensive evaluation, we sent Case #1 and #2 in a cohort of 164 other random clinical samples to the same external commercial NIPT laboratory and found complete concordance with the additional samples in regard to affected status (excluding Case # 1 and #2) and fetal sex determination. Of note, the commercial NIPT laboratory had three no-call results in this cohort (1.8% no-call rate), while the Vanadis system did not have any (0% no-call rate).

Based on the assessment of the FN cases, it is presumed that Case #1 and #3 both had an underlying biological basis for the screen-negative results by NIPT. For Case #1, given the significantly low z-score and concordance of a negative result on a different NIPT platform, the pregnancy is suspected to have mosaicism, particularly true fetal mosaicism type V.[15] As mentioned, Case #3 was noted to have a 21q;21q translocation, which has been reported to occur in other NIPT trisomy 21 FN cases.[14] Previous studies on this phenomenon state that there is likely a postzygotic event that leads to the formation of this rearrangement in which there is placental mosaicism that is undetectable by NIPT.[14] Parental karyotypes were also performed for Case #3 and neither parent is a carrier of this 21q;21q rearrangement; hence this is suspected to be a de novo event. For Case #2, in which the z-score was 3.04, the call had been close to the cutoff used by our laboratory for a positive trisomy 21 (≥3.5) result and thus, it is likely that this may have been a case of mosaicism or low fetal fraction. As other laboratories often use a z-score cutoff of 3.0 for trisomy 21, this case may have been called differently if it were run at a laboratory that used this common cutoff.[16] As these cases were identified postnatally, placental tissue was not assessed or saved following delivery. Therefore, we are unable to determine with certainty if there was in fact placental mosaicism involved in any of these cases.

In light of this information, the test performance for chromosome 21 was calculated with the presumption that two of the three FN cases (Cases #1 and #3) would have been missed by virtually all NIPT platforms due to the suspected normal or mosaic placental make-ups of these pregnancies. However, an argument can be made that these samples could be considered as true additional FNs. To account for that possibility, if we compared the performance of the system with these FN excluded; with these FN included; and with how the system has performed based on a summary of the eight previously published Vanadis-based studies,[10–13,17–20] it would show a consistent 99.8% specificity and sensitivities of 96.7%, 90.6%, and 99.5% respectively.

The performance of the Vanadis system was assessed by comparing results to that of the available clinical outcomes. Based on

these comparisons, and a screen-positive rate of 1.13%, we obtained an overall specificity of 7995 of 8027, a sensitivity of 43 of 44 and a final no-call rate of six of 8160.

We retested 10 positive T18 samples which had borderline z-scores that fell within an intermediate range (2.70–4.99). (Table 2) Retesting resulted in the Vanadis system providing five screen-positive results and five screen-negative results. Follow-up of these five screen-negative samples revealed that all results were consistent with the clinical phenotype. Of the five screen-positive results, three had clinical phenotypes or diagnostic test results that were concordant with the results. Thus, by using the retesting protocol, we were able to identify two extra true positives while only increasing the false positives by one (as compared to a single pass run).

Sex determination was assessed in 7767 samples. We received notification that 13 (three true males and 10 females) of the 7767 were discrepant; hence, 99.8% provided a concordant result.

TABLE 1 Vanadis system test results.

	Affected	Unaffected	Pending
T13			
Screen positive	2	8	3
Screen negative	0	8109	
T18			
Screen positive	12	8	6
Screen negative	0	8096	
T21[a]			
Screen positive	29	16	8
Screen negative	1	8066	
Overall[a]			
Screen positive	43	32	17
Screen negative	1	8027	

Note: Total samples: 8122.

[a]Two classified Trisomy 21 false-negative samples removed—please see explanation within text.

4 | DISCUSSION

To achieve global widespread adoption of improved first-line screening for common aneuploidies, an accomplishment which would benefit both patients and providers, accurate and cost-effective prenatal tests using cfDNA are needed.[10,11] While NGS-based NIPT testing has been suggested to be this first-line screen, some drawbacks have prevented its widespread implementation, including the cost and complexity of NGS analysis, which requires complex bioinformatic analysis, high run costs, and extensive hands-on time.[12] With the lower degree of complexity and substantially automated workflow associated with the Vanadis system, this methodology provided a solution for our clinicians and patients. Our laboratory experienced a reduced start-up time and decreased costs, by eliminating the need for building and developing dedicated pre- and post-PCR rooms and complicated bioinformatics servers. As a non-molecular laboratory, the removal of the complexities involved with PCR and NGS, on a system that produced accurate results and exhibited clinical utility, made the operation of this system simpler and more streamlined for our staff. Furthermore, the highly automated workflow, reduced IT/bioinformatics dependence, and non-molecular focus eliminated the need to hire additional staff and allowed the current staff to perform additional duties. Based on our current numbers and processes, we calculated that our current staff would be able to run at least 14,000 samples a year without needing to hire any additional staff or working overtime.

The Vanadis system performance met or exceeded the performance of other types of prenatal testing methodologies as reported in other studies, with a greater sensitivity in detecting trisomies as compared with Integrated Screening (first- and second-trimester biochemical measurements, combined with a first-trimester nuchal translucency ultrasound). (Table 3)[12,13,21] In our clinical study, performance of the Vanadis system was comparable to the reported performance of a variety of other next-generation sequencing-based NIPT methodologies for screening for trisomies 18 and 13,[22–25] as well as other NIPT methodologies that we have used in the past for screening aneuploidies.

TABLE 2 Retested borderline T18 samples by Vanadis

ID	Tube A18	Tube B18	Vanadis final	Clinical outcome	Method of clinical determination
2106300973B	4.34	3.30	T18	FP	Birth phenotype
2105190920B	2.99	2.08	T18	TP—VUS	Amniocentesis
2105251253B	3.80	3.11	T18	TP	Ultrasound anomalies
2109171247B	2.73	1.06	Normal	TN	Normal ultrasound
2109211144B	2.98	−0.36	Normal	TN	Normal ultrasound
2109141510B	2.99	4.82	T18	TP	FISH
2112130124B	2.80	2.41	T18	FP	Birth phenotype
2111130552B	2.72	1.78	Normal	TN	Normal ultrasound
2111231172B	2.81	0.00	Normal	TN	Normal ultrasound
2202221155B	2.78	0.57	Normal	TN	Normal ultrasound

Abbreviations: FISH, Fluorescence In Situ Hybridization; FP, false positives; TP, true positives, TN, true negatives; VUS, Variant of uncertain significance.

TABLE 3 Vanadis versus Integrated Screening Performance for Trisomy 21.

Performance metric	Vanadis[a]	Integrated screening[b]
Sensitivity	99%	96%
False-positive rate	0.19%	5.00%
No-call rate	0.09%	0%
Number needing counseling	49	474
Number pursuing additional testing	38	375

[a]Performance metrics as reported in literature and including our data.[10–13,17–20]

[b]Performance metrics as reported in literature.[21,39–41]

Regarding trisomy 21, the three postnatally reported FN cases did result in a lower sensitivity point estimate than normally observed for trisomy 21 by NIPT screening. However, upon further investigation, we concluded that two of the three FN cases were most probably due to a biological limitation that would not likely have been identified by any NIPT platform. Thus, if excluding those cases, the performance of the Vanadis system achieved in our laboratory for trisomy 21 screening was closer to that of other NIPT platforms as well as to that of other laboratories using Vanadis for trisomy 21 screening. As concluded in a study by Palomaki, et al., "when comparing these [detection] rates to published rates from clinical cfDNA tests, the relevant test failure rates must be considered" as tests with high failure rates "are likely to mask false-negative findings [within their no-call results] and are not included in their reported detection rates."[18] This is also supported by a meta-analysis which stated that studies that included test failures "decreased sensitivity estimates by 1.7% for Down syndrome".[26] Given that the Vanadis system had an exceptionally low no-call rate in our study (and thus is including many samples that other tests would have excluded), this would likely affect our final detection rates as compared to other systems.

One of the distinguishing features of the Vanadis system in our clinical study was the observed low no-call rate of 0.07% with 92.5% of first-pass no-call results resolved with a second run. No-call rates typically seen in other NIPT assays have ranged between 1.9% and 6.3%.[6–9] This 96%–99% reduction in the number of no-call results as compared to other systems benefited our clinicians and their patients. Mitigating the occurrence of test failures is critical, especially with the potential global adoption of NIPT as a first-line screening for general population pregnant women.[27] For patients with no-call results from cfDNA screening, it is recommended that they receive additional genetic counseling, as well as comprehensive diagnostic testing and ultrasound, as the risk of aneuploidies is higher.[16] Fewer no-call results mean fewer invasive procedures, which translates to fewer miscarriages of likely healthy fetuses. Associated reductions in healthcare costs and clinical burden (counseling and follow-up appointments) also provide benefits to both patient and provider.[16,27] In addition, 50% of patients are left without definitive NIPT results when offered repeat testing due to a no-call result, as there is poor patient compliance and hesitancy in submitting samples for repeat testing.[10] Finally, the uncertainty around test failure, along with the possible increased risk for an affected baby, is known to increase anxiety in expectant mothers.[27]

Using other methodologies, samples are most often dropped from analysis due to low fetal fraction; in fact, 46%–80% of canceled samples fall in this category, as observed across multiple studies.[28–31] Providing a no-call result can significantly impact an NIPT assay's performance,[32] making it a significant consideration in the evaluation and comparison of NIPT testing methodologies.[16] Since the Vanadis system does not measure fetal fraction due to the high variability found in the methodologies for the measurement of fetal fraction,[33] the number of no-call results was reduced in our study.

If a population of 9000 women, which is approximately our yearly screening rate, were screened for Down syndrome through Vanadis (99% detection rate, 0.19% false-positive rate and 0.09% no-call rate based on all the published studies and our current data for the no-call rate)[10–13,17–20] and Integrated Screening (96% detection rate,[21] 5.0% false-positive rate[34] and 0% no-call rate), using a Down syndrome prevalence of 1/340,[35] then 474 women would be offered genetic counseling following Integrated Screening, whereas only 49 would be offered genetic counseling after Vanadis screening. As 85% of women pursue diagnostic testing after a screen-positive result from NIPT testing,[36,37] 24% of women pursue diagnostic testing with no-call results[38] and 79% of women who screen positive with Integrated Screening pursue additional testing,[39] this would translate to 375 women pursuing a diagnostic test following Integrated Screening versus 38 women following Vanadis screening. This equates to a 90% reduction in both required clinical follow-up and additional testing. Vanadis screening would thus impact patients by lowering unnecessary emotional burden, invasive diagnostic testing (with possible associated miscarriages), and would significantly lower medical costs. In our clinical experience, this lower no-call rate has been noticed by both our participating clinicians and maternal-fetal medicine consultants and demonstrates the clinical utility of the Vanadis system.

As mentioned, other NIPT technologies typically have a 1.9%–6.3% no-call rate before redraw of patient blood samples, as compared to the no-call rate before redraw of 0.07% (with only six no-calls) with the Vanadis system in our clinical study.[6–9] Therefore, if a different NIPT technology was used on our cohort, it would have equated to an additional 154–512 failed samples, which is significant when considering that all these women received results promptly with our system, eliminating the need for further counseling and diagnostic testing, as recommended by medical societies.[6]

The clinical study confirmed that the performance of the Vanadis system was enhanced by adding an additional run to screen borderline samples. By using the rerun protocol for T18 testing of samples with borderline z-scores, two extra true positives were detected at the expense of only one false positive. This is an important consideration for future clinical work, as repeat runs should be considered for borderline samples.

A limitation of our study was that clinical outcomes of completed pregnancies relied on reporting from newborn evaluation, which is contained in the chart of the newborn, not the birth parent. Because

all users of the Women's Health Connecticut laboratory are Women's Health Connecticut providers, they are in constant communication with the laboratory and have attempted to discover any unanticipated outcomes through contact with pediatricians, and postpartum interaction with the birth parent. There is an exceptionally close collaboration between the laboratory and our clinical parties, including the sharing of a common electronic health record database. Due to these facts, we are confident that we have identified all the false-negative results for our population. Regardless, we were not able to confirm all screen-negative results.

In conclusion, the Vanadis system provided a low-cost, high-throughput NIPT assay with excellent performance characteristics and a superior no-call rate compared to a commercial NGS-based NIPT system. The improved patient care and reduced costs that are possible with the Vanadis system provide ample evidence of its clinical utility which supports our continued use of this system in our organization, WHCT, and warrants wider use as a screening methodology for aneuploidies.

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy concerns.

PATIENT CONSENT STATEMENT

Enrolled patients for the clinical studies signed consent forms prior to non-invasive prenatal testing (NIPT) testing.

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SUPPORTING INFORMATION

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

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