Performance of a targeted cell-free DNA prenatal test for 22q11.2 deletions in a large clinical cohort

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Running Head: Performance of a cfDNA test for fetal 22q11.2 deletions

Key words: 22q11.2 deletion, cell-free DNA, NIPT, microdeletion, prenatal diagnosis

What are the novel findings of this work?

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This is a large-scale prospective clinical evaluation of the sensitivity and specificity of a targeted cell-free DNA test for fetal 22q11.2 deletions. In 735 pregnancies, including 46 with a 22q11.2 deletion, the cell-free DNA test accurately identified 69.6% of affected cases as “high probability” with no false positive results.

**What are the clinical implications of this work?**

As the most common microdeletion, 22q11.2 is associated with significant morbidities and mortality, but the widely variable clinical features make it notoriously difficult to identify. Routine implementation of this test could accelerate detection and guide pregnancy management without increasing the likelihood of false positive results.
Abstract

Objectives: 22q11.2 microdeletions are more common than trisomies 18 and 13 combined, but no routine approach to prenatal screening has been established. This study evaluates the clinical sensitivity and specificity of a targeted cfDNA test to screen for fetal 22q11.2 deletions in a large cohort through blinded analysis of prospectively enrolled pregnancies and collected clinical samples.

Study design: In order to analyze a meaningful number of cases with a 22q11.2 deletion, samples were obtained through a prospective, multicenter enrollment of pregnancies with fetal cardiac abnormalities and collection of clinical samples from a research sample bank. Fetal genetic status as evaluated by microarray analysis, karyotype with FISH, or comparable technology was available for every case enrolled. Samples were processed as previously described for the Harmony prenatal test with the addition of DANSR (Digital Analysis of Selected Regions) assays targeting the 3.0Mb region of 22q11.2. Operators were blinded to genetic truth. Sensitivity and specificity of the cfDNA test for 22q11.2 deletions were calculated based on concordance between the cfDNA result and fetal genotype.

Results: The final study group consisted of 735 clinical samples: 358 prospectively enrolled pregnancies and 377 collected clinical samples. Of 46 maternal plasma samples from pregnancies with a 22q11.2 deletion ranging in size from 1.25 to 3.25 Mb, 32 had a cfDNA result indicating a high probability of deletion (sensitivity 69.6%; 95% CI: 55.2-80.9%). All 689 maternal plasmas without 22q11.2 deletions were correctly classified to have no evidence of a 22q11.2 deletion by cfDNA analysis, a specificity of 100% (95% CI: 99.5-100).
Conclusions: This is a large-scale prospective clinical evaluation of the sensitivity and specificity of a targeted cfDNA test for fetal 22q11.2 deletions. Results demonstrate that this targeted cfDNA test can detect common and smaller, nested 22q11.2 deletions with a low (0-0.5%) false positive rate. Although the PPV observed in this study population was 100%, the expected PPV in the general pregnancy population can be calculated as 12.2% using 99.5% specificity and 41.1% at 99.9% specificity. Use of this cfDNA test to screen for 22q11.2 deletions could enhance identification of pregnancies at-risk for 22q11.2 deletion syndrome without significantly increasing the likelihood of maternal anxiety and unnecessary invasive procedures related to false positive results.
**INTRODUCTION:**

Deletions in the 22q11.2 chromosomal region are the most common of all microdeletions. They range in size and can lead to a wide spectrum of clinical features known as 22q11.2 deletion syndrome (22q11.2DS). 1,2 85% of 22q11.2DS is caused by a 3 Mb (2.54 Mb) deletion (“common”), and about 15% is due to smaller, nested deletions within the same region. 3 With a prevalence of about 1 in 1000 pregnancies, 22q11.2 deletions are the second most common genetic cause of congenital heart disease and developmental delay after Down syndrome. 4–6

Prenatal screening for 22q11.2 deletions has potentially high clinical value because it can influence pregnancy management. 22q11.2DS is associated with morbidities and premature mortality, but the extreme variability in clinical presentation can delay diagnosis for years after features are observed.7,8 The corresponding missed opportunities for early interventions, anticipatory care and access to services can increase the likelihood of premature mortality as early as the neonatal period. 9,10 Identification of a pregnancy at risk for a 22q11.2 deletion could direct care toward detailed ultrasound evaluations and diagnostic testing in effort to promote better outcomes. 1,11,12

The ability of cell-free DNA (cfDNA) analysis to detect fetal 22q11.2 deletions as early as the first trimester of pregnancy has been demonstrated in analytical validation studies and small cohorts.13–17 However, professional societies have been reluctant to recommend screening because the clinical performance of cfDNA analysis for 22q11.2 deletions requires further investigation.18–20 To date, no prospectively collected, large clinical validation study of prenatal cfDNA analysis for 22q11.2 deletions has been published.
The objective of this study was to define the clinical performance of a targeted cfDNA test to screen for fetal 22q11.2 deletions through blinded analysis of prospectively enrolled pregnancies with cardiac abnormalities and collected clinical samples. Sensitivity and specificity were determined by comparison of cfDNA results to fetal status as determined by the current gold standard genetic diagnostic testing on CVS, amniocentesis, or peripheral blood specimens.
METHODS:

The 1/1000 prenatal prevalence of 22q11.2 deletions means that a prohibitively large number of pregnancies would be needed to use a general clinical population to accurately evaluate cfDNA test performance. An alternative approach involving clinical samples with an increased likelihood of a 22q11.2 deletion was taken in this study to enable evaluation of performance in a meaningful sample size since sensitivity and specificity are characteristics of the test independent of prevalence.

The final study population consisted of two arms: prospectively enrolled pregnancies with fetal cardiac abnormalities, and clinical samples collected from a research biobank.

Prospectively enrolled pregnancies

Between June 2015 and 2019, 13 centers in Australia, Belgium, Germany, Italy, United States, and Taiwan enrolled pregnancies with fetal cardiac abnormalities as part of the Non-Invasive Chromosomal Evaluation of 22q11.2 (22Q) study (NCT02541058). These pregnancies would receive genetic diagnostic testing for 22q11.2 during the prenatal or neonatal period as standard of care. Inclusion criteria required the presence of a single gestation of at least 10 weeks and that women be at least 18 years of age at the time of enrollment. The cut-off date for enrollment was July 26, 2019.

Maternal blood samples were collected in Roche cell-free DNA Collection Tubes and sent to the Ariosa Diagnostics Inc. Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory for the Harmony® prenatal test.
Enrollment centers collected pregnancy characteristics such as maternal age, gestational age at time of blood collection, ultrasound results, *in vitro* fertilization (IVF) and egg donor status. The respective enrollment centers coordinated genetic diagnostic testing for every pregnancy per local standards through chromosomal microarray analysis (CMA), karyotype and fluorescent in situ hybridization (FISH) targeted to the 22q11.2 region and/or comparable technology (e.g., quantitative fluorescence polymerase chain reaction, QF-PCR; BACs-on-BEADS, BoBs) of chorionic villi, amniocytes, cord blood or buccal swab.

All enrollees provided written informed consent under clinical study protocol AD-202. The protocol was conducted per International Council for Harmonisation and Good Clinical Practice guidelines and was reviewed and approved by an ethics committee and/or the institutional review board at each enrollment center.

_Collected clinical samples_

Plasma samples from women carrying pregnancies with confirmed status for the presence or absence of a fetal 22q11.2 deletion were received from a sample bank created as part of the RAPID Non-Invasive Prenatal Testing (NIPT) evaluation study (RP-PG-0707-10107) with national research ethics approval (13/LO/0082).

Maternal age and gestational age at time of blood collection were provided. All participants gave written consent. Blood samples were collected prospectively in either Streck or EDTA tubes, double spun, and plasma stored at -80 degrees C.
Sample processing

Samples were processed for the Harmony prenatal test with the addition of 22q11.2 DANSR (Digital Analysis of Selected Regions) assays and analyzed in a single custom microarray. For each sample, a probability score incorporating the fetal fraction was generated by the fetal fraction optimized FORTE algorithm for 22q11.2 deletion. Samples with probability scores of 1% or greater for a 22q11.2 deletion were classified as “high probability”, which can be fetal or maternal, or both. Otherwise the sample was classified as “no evidence of a deletion observed.” Operators were blinded to ‘genetic truth’ defined as the 22q11.2 copy number status assessed by diagnostic testing. Results of cfDNA analysis were not communicated to study participants, since diagnostic test results were already available.

Data Analysis

Sensitivity and specificity of the cfDNA test for 22q11.2 deletions were calculated based on concordance between the cfDNA result and genetic truth. Pregnancies with chromosome abnormalities other than 22q11.2DS, such as a trisomy, were classified as deletion-negative. All confidence intervals were determined using the Wilson method.
RESULTS:

Characteristics of study populations

Characteristics of both the prospectively enrolled study participants and the collected clinical samples are shown in Table 1. 370 maternal plasmas were collected from the prospective pregnancy enrollment. 4 cases did not meet inclusion criteria and 8 samples did not yield a cfDNA result. The final prospectively enrolled study group consisted of 358 cases, of which 34 had a fetal 22q11.2 deletion; 8 of these deletions were smaller than 2.5Mb. 78 cases had other findings including whole chromosome aneuploidies and subchromosomal imbalances. The majority of pregnancies had chromosomal microarray analysis (88%) and amniocentesis was the most common method of diagnostic testing (83%). 8 pregnancies were conceived by in vitro fertilization. The clinical collection consisted of 377 samples, of which 217 have been reported previously.14 12 of the collected clinical samples were from pregnancies with a fetal 22q11.2 deletion, including one of 1.4 Mb.

Screening performance

Combining the prospectively enrolled pregnancies with the collected clinical samples yielded a total of 735 maternal plasma samples that were eligible for analysis.

24 of 34 prospectively enrolled pregnancies with fetal 22q11.2 deletions and 8 of 12 collected clinical samples with fetal 22q11.2 deletions were determined by cfDNA analysis to have a high probability of deletion. Therefore, in total, 32 of 46 samples were correctly identified as high probability for a 22q11.2 deletion by cfDNA analysis; a sensitivity of 69.6% (95% CI: 55.2-80.9). 6 of those that were not detected were smaller deletions of less than 2.5Mb. The small number of
cases with smaller deletions limits an analysis of sensitivity as a function of deletion size. There was no significant difference in sensitivity between the prospectively enrolled pregnancies (70.6%) and collected clinical samples (66.7%).

The 689 maternal plasmas without 22q11.2 deletions included 324 prospectively enrolled pregnancies and 365 collected clinical samples. There were no false positive results; all were correctly classified to have no evidence of a 22q11.2 deletion by cfDNA analysis, a specificity of 100% (95% CI: 99.5-100).

The combined performance is presented in Table 2.
DISCUSSION

Principal findings

735 maternal plasmas were evaluated by cfDNA analysis for the probability of a common or smaller, nested fetal 22q11.2 deletion. cfDNA results were compared to fetal status based on diagnostic testing to establish a sensitivity of 69.6% (95% CI: 55.2-80.9) and specificity of 100% (95% CI: 99.5-100). Notably, no false positive results were observed in this large-scale prospective clinical evaluation of cfDNA test sensitivity and specificity for fetal 22q11.2 deletions.

Interpretation of results

The availability of cfDNA screening for 22q11.2 deletions was initially supported by studies demonstrating the technical ability to detect deletions in laboratory-generated plasma mixtures.13–15 To date, most clinical studies have either evaluated very small numbers of affected pregnancies or larger data sets without complete outcome information.13–17,21–27

Ravi et al16 described the performance of a targeted single nucleotide polymorphism (SNP)-based cfDNA test in a retrospective cohort study of 400 clinical samples with confirmed genetic status for 22q11.2. One false positive was identified, correlating to a specificity of 99.74%. Only 10 maternal plasma samples from pregnancies with a 22q11.2 deletion were analyzed, all of which had the larger common deletion. Sensitivity was estimated to be 78.3% after adjusting for the exclusion of nested 22q11.2 deletions but with wide confidence intervals (95% CI 50-89.8) due to small sample size. Liang et al26 reported a >99.9% specificity and 86.7% sensitivity for 22q11.2 using a “genome wide” next generation sequencing assay in a study of >90,000 women. Clinical follow-up was obtained for 13 positive results; however, the lack of genetic outcome data on
screen negative cases precludes an accurate sensitivity calculation. The lower than expected frequency of 22q11.2 deletions found in the study population (less than 1/7,000 versus an expected 1/1000) suggests that many cases were not detected and the clinical sensitivity overestimated.

For the targeted cfDNA test used in the current study, Schmid et al\textsuperscript{14} previously reported an analytical sensitivity of 75.4\% (95\% CI: 67.1–82.2\%). Specificity was determined to be at least 99.5\% (95\% CI: 99.0–99.7\%) based on a clinical group of 1,604 presumed unaffected samples. A recent prospective study by Kagan et al\textsuperscript{28} used the test in 1,127 pregnancies. Three false positive results were identified, corresponding to a specificity of 99.7\%. The study was not intended to calculate sensitivity.

Collection of genetic outcome for every case in the current study enabled evaluation of both clinical sensitivity and clinical specificity. The 100\% specificity and 70\% sensitivity observed are consistent with previous studies of this targeted cfDNA test for 22q11.2 deletions\textsuperscript{14,28} and were established in a true clinical population including both common and smaller 22q11.2 deletions.

Fetal 22q11.2 deletions can manifest with a variety of severe to subtle features, only some of which are identifiable by second trimester ultrasound.\textsuperscript{11,12} Even in the absence of physical malformations, 22q11.2 deletions are associated with an increased risk for morbidities and mortality as early as the neonatal period.\textsuperscript{7,8} The widely variable clinical expression of 22q11.2 deletions, clinicians’ general lack of familiarity with the condition and lack of established pre- or post-natal screening protocol contribute to diagnostic delays - even when clinical signs are present.\textsuperscript{12,29} The introduction of cfDNA analysis for prenatal 22q11.2 deletion screening in the
first trimester could promote timely diagnosis, inform pregnancy management, and enable early interventions targeted to improve outcomes.

Our results demonstrate the ability of a targeted cfDNA test to provide prenatal screening for common and nested 22q11.2 deletions with a very low false positive rate (95% CI: 0-0.5%). While the established sensitivity, specificity and corresponding false positive rate are features of the test independent of population characteristics, the positive and negative predictive values (PPV and NPV) are influenced by the prevalence of 22q11.2 deletions in the population studied. In this population, where the incidence of 22q11.2 deletions was high (6.2%), the PPV was 100% and the NPV was 98%. In the general pregnancy population, where prevalence of 22q11.2 deletions is estimated to be 1/1000, the expected PPV is calculated to be 12.2% using 99.5% specificity and 41.1% at 99.9% specificity, while the expected NPV is calculated to be >99.9%.

Pregnancies with cardiac abnormalities are at increased risk for a variety of genomic imbalances beyond 22q11.2DS that cfDNA testing does not detect. Although such high-risk pregnancies were used in this study to establish a meaningful sample size, definitive diagnostic testing with chromosomal microarray analysis is the recommended approach in these cases. For patients who decline prenatal diagnosis after the identification of a fetal cardiac abnormality, cfDNA analysis could be helpful in that a high probability result would make a diagnosis of 22q11.2 deletion likely. However a low-probability result would be less useful.
Strengths and limitations

The strengths of this study include the prospective collection of a large number of pregnancies with fetal 22q11.2 deletion, inclusion of common and smaller 22q11.2 deletions, and the availability of a genetic study for every pregnancy assessed. The multi-center prospective pregnancy enrollment enabled a determination of test performance for both common and nested 22q11.2 deletions in a true clinical population. In total, 46 pregnancies with 22q deletions were collected and studied, representing a 4-fold increase over the next largest assessment of cfDNA sensitivity for 22q11.2 deletions.16

This study was limited to a single targeted cfDNA test and does not necessarily represent the performance of other targeted or non-targeted cfDNA methodologies.
Conclusion

Prenatal screening for 22q11.2 deletions has become clinically available with limited studies in clinical populations. This study has now established the sensitivity and specificity of a targeted cfDNA test for fetal 22q11.2 deletions in a large clinical population, which includes both the common and smaller nested deletions causing 22q11.2DS. Use in the first trimester to screen for 22q11.2 deletions in the general pregnancy population could be considered in effort to support early detection without significantly increasing the likelihood of false positive result. Adoption into clinical care could be further supported by studies evaluating PPV and NPV in a general obstetric population.
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## Table 1
Characteristics of the study population

| Fetal status       | MA, year Mean ±SD (Range) | GA, week Mean ±SD (Range) | FF, % Mean ±SD (Range) | MA, year Mean ±SD (Range) | GA, week Mean ±SD (Range) | FF, % Mean ±SD (Range) |
|--------------------|---------------------------|---------------------------|------------------------|---------------------------|---------------------------|------------------------|
| 22q11.2 del        | 33 (22-43) ± 5 ± 6.1 (13.9-36) | 24.3 ± 6.1 (13.9-36) | 16.8 ± 8.6 (6.3-36.7) | 29 (18-37) ± 5 ± 6.6 (10-34.1) | 20.9 ± 6.6 (10-34.1) | 14.8 ± 6.4 (7.5-26.1) |
| No 22q11.2 del     | 31 (16-47) ± 6 ± 6.3 (11.4-41) | 24.7 ± 6.3 (11.4-41) | 17.2 ± 7.5 (5.1-41.8) | 32 (19-47) ± 5 ± 5.7 (10.3-37.3) | 17.3 ± 5.7 (10.3-37.3) | 14.4 ± 5.8 (5.2-43) |

Abbreviations: MA, Maternal age. GA, Gestational age. FF, Fetal fraction. Del., deletion.
Table 2
Screening performance

|                         | Prospective pregnancy enrollment | Collected clinical samples* | Combined  |
|-------------------------|----------------------------------|-----------------------------|-----------|
| Total Samples, N        | 358                              | 377                         | 735       |
| Fetal 22q11.2 del, n/N  | 24/34                            | 8/12                        | 32/46     |
| No fetal 22q11.2 del, n/N | 324/324                          | 365/365                     | 689/689   |
| Sensitivity, % (95% CI) | 70.6 (53.8-83.2)                  | 66.7 (39-86.2)              | 69.6 (55.2-80.9) |
| Specificity, % (95% CI) | 100 (98.8-100)                    | 100 (99.0-100)              | 100 (99.5-100) |

Abbreviation: Del., deletion.

* Data from 217 collected clinical samples previously reported in Schmid et al. 14