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Article

Assessment of Placental Chromosomal Mosaicism during Prenatal Cell-Free DNA Screening Refines Positive Predictive Values for Fetal Trisomy

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BACKGROUND: Confined placental mosaicism can cause false-positive prenatal cell-free DNA (cfDNA) screening results, thereby reducing the positive predictive value (PPV) of the test. We sought to investigate how PPVs for the common fetal trisomies can be refined based on the presence or absence of chromosomal mosaicism in cfDNA sequencing data.

METHODS: The study cohort included singleton pregnancies tested between March 2019 and December 2021. Outcome data were requested for high-risk results. Mosaic ratio (MR) generated by VeriSeq NIPT Solution v2 was used to classify high-risk cfDNA results as mosaic trisomy ($MR < 0.7$) or non-mosaic trisomy ($MR \geq 0.7$) and the PPVs calculated.

RESULTS: The cohort consisted of 821 high-risk results from 76 329 tests (1.08%). Prior to applying MR, PPVs for T21, T18 and T13 were 93.3% [95% CI 90.2–95.5], 81% [95% CI 73.1–87.0], and 55.3% [95% CI 44.7–65.4], respectively. After applying MR, PPVs for non-mosaic trisomy results were significantly higher ($P < 0.001$) than the PPVs for mosaic trisomy results; T21: 99.3% and 50%, T18: 97.6% and 22.7%, T13: 93.9% and 0%, respectively.

CONCLUSIONS: Mosaic ratio can be used to calculate more specific PPVs for the common trisomies. There is currently limited guidance on the application of VeriSeq v2 MR. Our approach provides a framework for laboratories to consider using MRs to refine PPV estimates for the common trisomies. High-risk cfDNA screening results are distressing for tested individuals. A refined PPV incorporating the presence or absence of mosaicism provides patients with more accurate information on the likely outcome of the diagnostic testing result, helping guide genetic counseling, choice of prenatal procedure, and overall pregnancy management.

Introduction

Prenatal cell-free DNA (cfDNA) screening, or non-invasive prenatal testing (NIPT), assesses placental cfDNA in maternal blood to determine the chance of fetal aneuploidy. Prenatal cfDNA screening, performed commonly for trisomy (T) 21, T18, and T13, is reported with high sensitivity (T21: 99%, T18 and T13: 98%) and specificity (99.9%) and very low false positive (FP) and false negative (FN) rates (1).

The positive predictive value (PPV) is the chance a screen-positive result will be confirmed after diagnostic testing and is an important metric for counseling of high-risk screening results (2, 3). It is primarily determined by the FP rate of the test and the prevalence of the condition being screened. The PPV for T21 (91.78%) is higher than the PPVs for T18 (65.77%) and T13 (37.23%) (4). This reflects the higher prevalence of T21 and higher rates of confined placental mosaicism (CPM) causing FP results for T13 and T18 (5, 6). Other biological causes of FP cfDNA screening results include co-twin demise, maternal chromosome mosaicism, and, rarely, maternal malignancies (7). Technical and statistical causes also contribute to discordant cfDNA screening results.

The placental cfDNA in maternal circulation is derived from apoptosis of cytotrophoblast cells, an outer cell layer of the chorionic villi (8). These cells do not always provide a true representation of the fetal karyotype due to CPM, which affects up to 1% to 2% of pregnancies (9–11). CPM occurs when a chromosome anomaly exists in the placenta but not the fetus. Only diagnostic testing can confirm if a trisomy is present in the fetus, although identifying chromosome mosaicism during cfDNA screening can modify the chance of the trisomy being confirmed (12).

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Pertile et al. (13) analyzed possible trisomy mosaicism in cfDNA by comparing the trisomic fraction with the fetal fraction in a cohort of pregnancies with suspected rare autosomal trisomies. When this ratio was high (for example, 1.0 or 100% trisomy), pregnancies had an increased risk for fetoplacental complications associated with a high proportion of trisomic cells in the placenta, most commonly from a trisomic conception. The authors proposed that estimation of placental mosaicism might assist in identifying pregnancies at higher risk of adverse outcomes (13). Since then, several custom bioinformatic approaches to predict cytotrophoblast mosaicism in cfDNA sequencing data have been described (12, 14–16). Most recently described as the "mosaicism ratio," Rafalko et al. (12) reported that samples with higher mosaic ratios (MR) involving the common trisomies were more likely to reflect true positive (TP) results (MR $\geq$ 0.7), and samples with the lowest MR were more likely to reflect FP results (MR 0.2–0.49).

The VeriSeq™ NIPT solution v2 (Illumina, Inc.; VeriSeq v2) is a commercially available, whole-genome sequencing cfDNA screening assay released in 2019 and now registered for use in multiple countries (16). The VeriSeq v2 test analysis output includes a MR, calculated for screen-positive whole chromosome and partial chromosome anomalies. Presently, there is no guidance on how this metric could be used for result interpretation (16).

In 2019, Victorian Clinical Genetics Services (VCGS) introduced the VeriSeq v2 assay for prenatal genome-wide cfDNA screening (16). We sought to determine how users of this assay can employ the MR to aid interpretation of T21, T18, and T13 cfDNA screening results and how mosaicism impacts the corresponding PPVs.

Materials and Methods

ETHICS STATEMENT

Genetic testing was performed in accordance with the approved ethical guidelines of the Melbourne Children's Campus, Royal Children's Hospital, Melbourne, Australia. As the intention of the study was to monitor, evaluate, and improve health services only, according to the Australian Health Records Act, this is a quality activity and does not require organizational Human Research Ethics Committee review. Pregnant individuals having NIPT through VCGS were informed about the test by their healthcare practitioner (obstetrician, midwife, general practitioner, or genetic health professional) and provided consent prior to testing. This included consent to collect pregnancy outcome data for the purposes of quality assurance and test evaluation.

STUDY COHORT

The study cohort consisted of consecutively screened singleton pregnancies of at least 10 weeks gestational age tested between March 2019 and December 2021 at VCGS, Melbourne, Australia. Outcome data were sought for all screen-positive T21, T18, or T13 cases.

SAMPLE PROCESSING

Maternal blood was collected into a cell-free DNA BCT® tube (Streck). Samples were tested using the VeriSeq v2 (Illumina) screening test, as previously described (16). The assay was run in genome-wide mode.

BIOINFORMATICS METRICS OF THE VERISEQ V2 ASSAY

VeriSeq v2 classified samples as "anomaly detected" or "no anomaly detected." A log likelihood ratio (LLR) was computed for each region tested, using the t-statistic and the estimated fetal fraction (FF). The t-statistic is a signal-to-noise metric of the difference in coverage between the region and the rest of the genome, compared to variation in the sample. Two FF estimates are available to users: (*a*) an NIPT report that provides FF as a rounded integer percentage (e.g., 8%), and (*b*) a supplementary report that provides FF as an unrounded decimal number (e.g., 0.0835). The LLR is the probability of a sample being affected given the observed coverage (t-statistic) and FF vs the probability of a sample being unaffected given the observed coverage. Every autosomal chromosome has a specific, predetermined LLR threshold for trisomy. The MR is a metric calculated for screen-positive whole chromosome and partial chromosome anomalies. The MR is calculated from the ratio of the FF inferred from the coverage of the region to the FF of the sample. This ratio can give an estimation of placental cytotrophoblast trisomy (and monosomy) mosaicism. Ratios can fall outside the expected range between 0 and 1 due to variability in the estimate of the FF used in the calculation (17).

SAMPLE EXCLUSION

cfDNA screen-positive data were manually reviewed and curated. For a subset of results that were suspected false-positive cases, testing was either repeated or the sample was reported as test failed and a new sample requested (Supplemental Methods). Cases with multiple aneuploidies or known co-twin demise were excluded from analysis.

CYTOGENETIC AND CLINICAL OUTCOME STUDIES

Confirmatory diagnostic testing options were offered to all individuals who received high-risk T21, T18, and T13 results. Samples for prenatal diagnosis were obtained via chorionic villus sampling (CVS) or amniocentesis. Postnatal samples for study included products of

conception (POC), fetal tissue, placental tissues, newborn blood, and saliva. For some cases with false-positive cfDNA results, placental biopsies were taken following birth to test for CPM. Diagnostic testing was performed using chromosomal microarray (Infinium GSA-24 v2.0 and v3.0, Illumina Inc. when tested at VCGS), conventional karyotyping, fluorescent in situ hybridization, and/or quantitative fluorescent PCR.

Pregnancy outcome data were obtained through diagnostic testing records at VCGS or from the patient's referring practitioner. Cases were classified as TP or FP based on diagnostic testing results.

MOSAICISM PREDICTION AND DESCRIPTIVE STATISTICS

As the FF decreases, variance in the t-statistic could be attributed to either technical noise or true deviation due to cytotrophoblast trisomy mosaicism. To determine the FF at which mosaicism cannot be differentiated from technical noise, the linear regression ±2 SD of the t-statistic to the unrounded decimal number FF (VeriSeq v2 supplementary report) was calculated for TP and true negative (TN) screening results. The FF where the TP boundary intersected the TN boundary was considered the FF below which mosaicism cannot be accurately calculated. For these calculations, TP included only those cases where the screen-positive chromosome was cytogenetically confirmed with non-mosaic trisomy, and TN was determined from the same cases where the screen-negative chromosomes were cytogenetically confirmed with disomy.

Based on prior research and our own empirical evidence estimating mosaicism for rare autosomal and common trisomies and correlating this with known cytogenetic outcomes, a MR cutoff of ≥0.7 was used to classify a screen-positive result as "non-mosaic" trisomy (12, 13, 18). When the MR was <0.7, we classified the result as "mosaic" trisomy. For context, a T21 result with an MR of 0.5 indicates 50% trisomy mosaicism in cytotrophoblast and was classified as a mosaic T21 finding.

PPV were calculated [PPV = TP/(TP + FP)] for each screen-positive trisomy prior to applying the MR. The fetal fraction and the MR were then used to divide the screen-positive cohort into 3 subgroups, and the PPV was again calculated for(*a*) non-mosaic trisomies, (*b*) mosaic trisomies, and (*c*) trisomic cases where the FF was below the intersect for determining mosaicism. CIs are 2-sided 95% CIs based on the Wilson score method. For PPV calculations, TP cases included any case confirmed by diagnostic testing with non-mosaic trisomy or true fetal mosaicism for trisomy.

Descriptive statistics were used to describe maternal age and gestational age. Statistical significance of the difference in proportions of TPs was determined using the Fisher exact test, while Student t-tests were employed for comparing groups. A P value less than 0.05 was considered significant. Statistical analyses were performed using the R statistical package (software version 4.1.2; R Foundation for Statistical Computing).

Results

PREGNANCY CHARACTERISTICS AND OUTCOMES

A total of 76 329 women with cfDNA screening were included in the study cohort. The median gestational age was 10 weeks and 6 days, with 94.8% of tests performed before 14 weeks gestation. The median maternal age was 33.6 years at testing. Most tests (86.9%) were performed as a primary screening test. Samples associated with fetal ultrasound anomalies were not commonly referred for screening (0.8%).

There were 821/76 329 (1.08%) singleton pregnancies reported as high risk for one of the common trisomies; T21 525/821 (63.9%), T18 173/821 (21.1%), and T13 123/821 (15.0%). For screen-positive results, the median maternal age was 37.2 years, and the median gestational age was 10 weeks and 6 days. There was a significant difference in median maternal age ($P = 2.2 \times 10^{-16}$) at the time of testing between the screen-positive and total cohorts. Of 821 high-risk cases, diagnostic cytogenetic testing results were available for 565 (68.8%), pregnancy outcome information without cytogenetic testing was available for 164 (20.0%), and outcomes were unavailable for 92 (11.2%) (Fig. 1).

During the study, there were an additional 63/76 329 (0.08%) trisomies called by VeriSeq v2 (32 T21, 18 T18, and 13 T13) that were suspected false-positive results due to borderline log likelihood ratio and/or t-statistic relative to the fetal fraction, sometimes seen in suspected poor-quality samples (e.g., collection issue, transport delay). These samples were retested (Supplemental Methods). After retesting, 53/63 (84.1%) cases were reported as low risk for these trisomies, and 3/63 (4.8%) cases were reported as high risk (1x T21, 2x T18) and included in the screen-positive cohort. No result was generated for 7/63 (11.1%) retested cases (Supplemental Results). Outcomes are recorded in Supplemental Table 1.

FF AND MOSAICISM PREDICTION

Chromosomes 21, 18, and 13 showed different levels of technical noise in the t-statistic when true cases of trisomic (TP) and disomic (TN) chromosomes were compared. For chromosome 21 (329 TP and 205 TN), the FF intersect between TP and TN cases was 5.24% (Fig. 2, left panel), for chromosome 18 (98 TP and 437 TN) it was 4.01% (Fig. 2, middle panel), and for chromosome 13 (46 TP and 474 TN) it was 3.69% (Fig. 2, right panel). For subsequent sample analyses, the intersects applied where MR could not be determined on trisomic cases

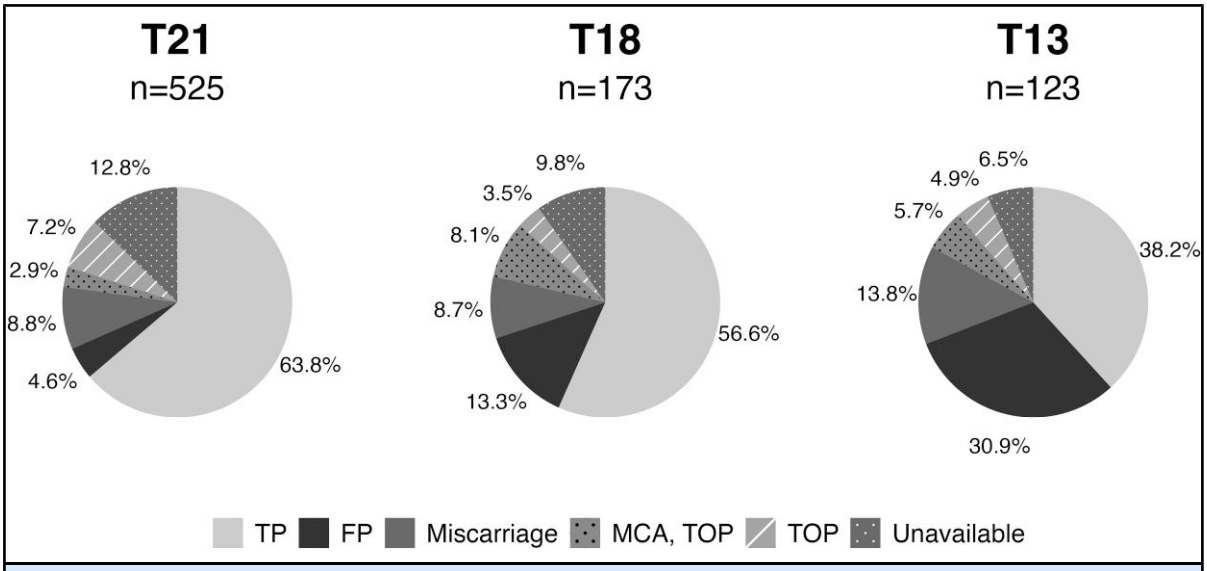

Fig. 1. Outcomes of high-risk T21/T18/T13 results. For the aneuploidy detected, T21 (left), T18 (middle), and T13 (right), each slice represents the percentage of each pregnancy outcome. Abbreviations: MCA, miscarriage (no cytogenetic testing); TOP, termination of pregnancy with major congenital anomalies (no cytogenetic testing); TOP, termination of pregnancy (no cytogenetic testing); Unavailable, diagnostic testing declined or pregnancy outcome information unavailable.

were <5.0% FF for T21 and <4.0% FF for T18 and T13. Mosaic ratios were only used to classify screen-positive trisomic cases with FFs above these values using the unrounded decimal number. For example, a screen-positive T21 result with an unrounded decimal number FF of 0.0493 (rounded FF 5%) could not be classified using MR, as the unrounded decimal number FF is below the 5.0% T21 intersect. A screen-positive T21 result with an unrounded decimal number FF above this intersect (e.g., 0.0544) could be classified using MR as non-mosaic trisomy (MR ≥0.7) or as mosaic trisomy (MR <0.7). The unrounded decimal number FF and the MR value are available in the VeriSeq v2 Supplementary report (example in Supplemental Table 2).

MOSAICISM RATIO

When the FF was below the intersect, TP and FP cases had wider distributions of MR ($P = 0.01$) when compared with those having a FF above the intersect ($P = 2.2 \times 10^{-16}$) (Fig. 3). When the FF was above the intersect, the median MR for TP results was 1.07, suggesting a non-mosaic trisomy in the cfDNA, whereas the median MR for FP results was 0.38, suggesting a mosaic trisomy in the cfDNA.

MOSAICISM DETECTION USING MOSAIC RATIO

When MR was used to classify screen-positive trisomic results (Fig. 4), T13 had the highest proportion of cases classified as mosaic trisomy (35.5%), while T21 had the lowest (5.3%). Cases could not be accurately classified as mosaic or non-mosaic trisomy when the FF was below the intersects. This affected 22% of T13 results and approximately 13% and 14% of T18 and T21 results, respectively (Fig. 4).

DIAGNOSTIC CONFIRMATION OF PLACENTAL TRISOMY MOSAICISM

Chorionic villi (CV) samples were available for testing for 21/95 mosaic cfDNA screening results (10 T21, 7 T18, 4 T13). Of these 21 cases, 10 were confirmed with mosaic trisomies in the CV by cytogenetic testing (5 T21, 1 T18, 4 T13), and 2 were confirmed as non-mosaic T21 (Supplemental Table 3). For the remaining 9/21 cases (3 T21, 6 T18), analysis of CV showed no evidence of the trisomy. A further five mosaic T21 screening results had only amniotic fluid studied, which demonstrated mosaicism for T21, suggesting these results were also mosaic in the CV (Supplemental Table 3).

PPV CALCULATIONS USING MOSAIC RATIO

Prior to applying MR, the PPV for T21 was 93.3% [95% CI: 90.2, 95.5], for T18 it was 81% [95% CI: 73.1, 87.0], and for T13 it was 55.3% [95% CI: 44.7, 65.4] (Table 1). After applying MR, non-mosaic trisomy screening results (MR ≥0.7) were significantly more

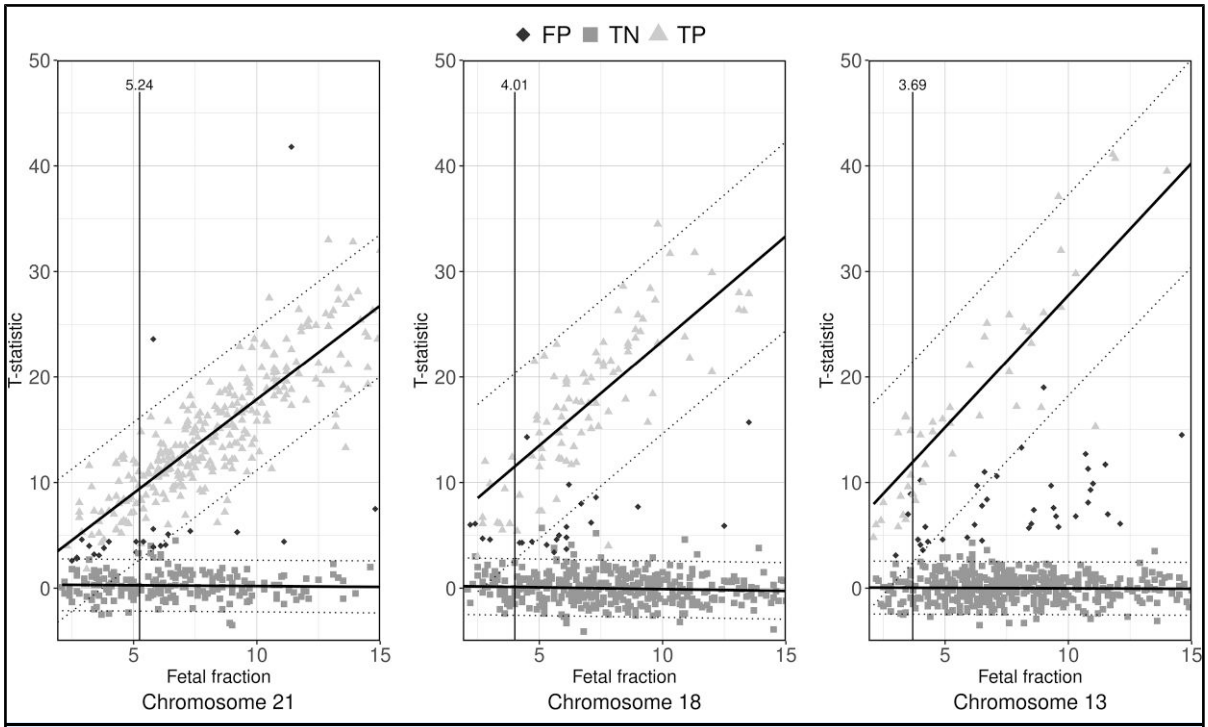

Fig. 2. T-statistic in relation to the fetal fraction for chromosomes 21 (left), 18 (middle), and 13 (right). The x-axis shows fetal fraction; the y-axis shows t-statistic. TP cases (triangles) were those with cytogenetically confirmed non-mosaic trisomy. TN (squares) were those with cytogenetically confirmed disomy. FP cases (diamonds) were screen-positive cases where non-mosaic trisomy could not be confirmed. Solid lines mark the linear regression of TP (diagonal line) and TN (horizontal line). The dashed lines represent ±2 SD. Solid vertical lines represent the fetal fraction where the TP −2 SD (lower boundary) and the TN +2 SD (upper boundary) intersect.

likely to be confirmed in the fetus than mosaic trisomy screening results (MR <0.7) (all *P* values <0.001). The PPV for all trisomies exceeded 90% when classified as non-mosaic, with the largest improvement seen for T13 (from 55.3% to 93.9%). In contrast, T13 screening results classified as mosaic were never confirmed (Table 1).

When mosaic trisomy cfDNA results were confirmed in the fetus (13 T21, 5 T18), the fetus was more likely to have a non-mosaic trisomy (8/13 T21, 5/5 T18) than true fetal mosaicism (TFM).

If the 60 screen-positive trisomies that were retested were included as FP results, the PPV for the non-mosaic trisomies would remain unchanged, while the PPV for the mosaic and unclassified groups would be reduced further, as these FP results were restricted to mosaic calls or fetal fractions that were below the intersect for calling mosaicism (Supplemental Results, Supplemental Table 4). Therefore, Supplemental Table 4 represents the VeriSeq v2 PPV performance in a general risk cohort when users adhere strictly to the algorithm's output and no screen-positive results are retested.

TRUE FETAL MOSAICISM

TFM was determined in 7 cases (6/7 T21, 1/7 T13) following diagnostic cytogenetic testing. Of true fetal mosaics for T21, 5/6 were classified mosaic on cfDNA screening, and 1/6 was classified non-mosaic. The one T13 true fetal mosaic was classified as non-mosaic on cfDNA screening.

DISTRIBUTION OF TP AND FP RESULTS USING MR

Figure 5 shows the MR and FF of screen-positive cases classified by cytogenetic outcome. Of note, most TP results were classified as non-mosaic trisomies (MR ≥ 0.7), and most FP results were classified as mosaic (MR <0.7). True fetal mosaicism was seen more commonly in cfDNA screening results classified as mosaic (TP TFM). Placental trisomy mosaicism suggested in cfDNA (MR <0.7) was cytogenetically confirmed in several cases where chorionic villi was tested (FP CPM).

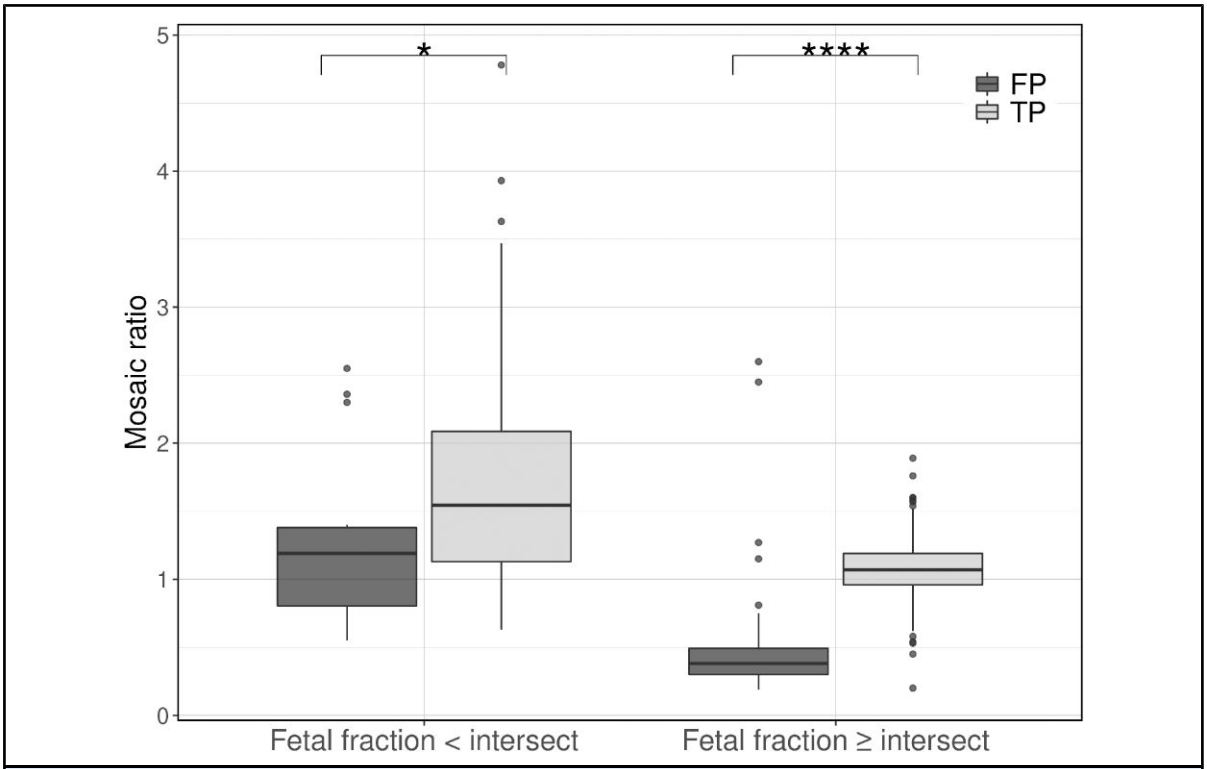

Fig. 3. MR distribution plots for all cases called as trisomy 21, trisomy 18, or trisomy 13 by VeriSeq v2, with cytogenetic outcome available. The *x*-axis shows cases with a FF <intersect (left) and ≥intersect (right). The solid line within each box marks median MR; lower and upper limits mark the first and third quartiles. Outliers are marked with dots. Statistical significance between FP (dark grey) and TP (light grey) groups is indicated by stars (* for $P < 0.05$, ** for $P < 0.0001$). Five samples had MR <0 or >6 for one or more test chromosomes and are excluded (FF ranged from 1.2% to 2.4%).**

PREVALENCE AND PPV

PPV depends on disease prevalence. The PPV for trisomy is expected to be lower at younger maternal ages and increase with advancing maternal age as the prevalence of trisomy increases. The difference in the proportion of TP non-mosaic trisomy cfDNA screening results was compared between women <35 and ≥35 years. The PPV for women <35 years was 96.7% (118/122) and ≥35 years was 99.3% (277/279). This difference in TP proportions approached significance ($P = 0.07$). In contrast, there was no difference in the proportion of TP mosaic cfDNA screening results, with 17.0% (8/47) TP in women <35 years compared with 30.3% (10/33) in women ≥35 years ($P = 0.18$). This is consistent with most CPM cases occurring from post zygotic gain of the trisomic chromosome, where the parental origin of the trisomic chromosome is random (19).

Discussion

CPM is detected in 1% to 2% of CVS cases, and the interpretation is well established from the CVS literature; amniocentesis is usually required to resolve the fetal karyotype (9–11, 20). CPM has also emerged as a well-documented cause of FP NIPT results, as the analysis uses fragmented cfDNA derived from apoptotic cytotrophoblast cells of the CV (21–23). An advantage of CVS over amniocentesis is the opportunity for an earlier diagnosis, but concern exists about the possible need for amniocentesis if placental mosaicism is found (24, 25). Our data demonstrates that using MR can help mitigate this concern by predicting the presence or absence of trisomy mosaicism in the CV. This finding can then be used to help guide genetic counseling, choice of prenatal procedure, and overall pregnancy management (12, 14, 26).

In our cohort, screening results classified as non-mosaic trisomy were significantly more likely to be confirmed in the fetus than results classified as mosaic. Our data support employing an MR of ≥0.7 to classify non-mosaic T21/T18/T13 results when using the VeriSeq v2 platform. This value has been reported previously by Rafalko et al. (12) to differentiate between non-mosaic and mosaic results when using another NIPT platform. When we compare the non-mosaic trisomy results, our study showed similarly high PPVs to Rafalko et al. (12) (T21: 99.3% vs 98.4%, T18: 97.6% vs 96.3%, T13: 93.9% vs 93.9%). However, mosaic trisomies showed differences. For example, we had 0/32 mosaic T13 cases confirmed (PPV 0.0%) compared with 21/65 (PPV 32.3%). We also calculated lower PPVs for mosaic T21 and T18, in keeping with these mosaic trisomies being associated with CPM. These differences might be explained by platform specific calculation of FF and MR, a more general risk population screened in our study cohort, a dynamic estimate of low FF samples (VeriSeq v2) instead of a fixed threshold of 4%, and our determination of the FF below which mosaicism cannot be accurately calculated.

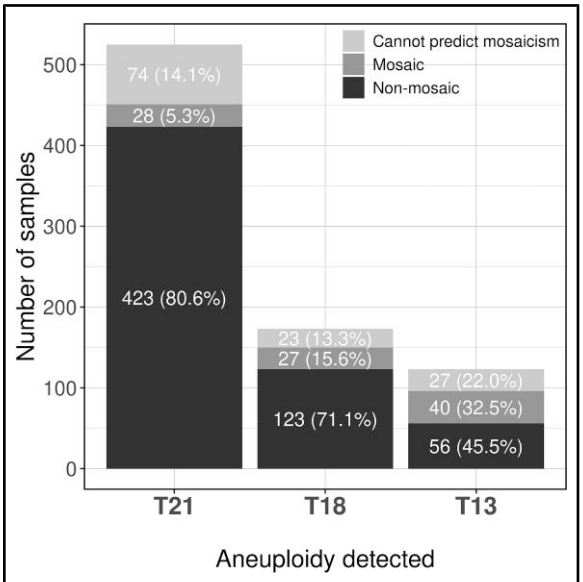

Fig. 4. Frequency of mosaic ratio classifications and trisomy detected in the overall screen-positive cohort (n = 821). The *x*-axis shows the trisomy detected and the *y*-axis the frequency. Cases with fetal fraction below the intersect are dark grey, mosaic trisomy results are grey, and non-mosaic trisomies are light grey.

Cytogenetic testing of CV does not always confirm a trisomy predicted by cfDNA screening, possibly due to a localized or uneven distribution of abnormal cells in the placenta (25). Nonetheless, we were able to confirm trisomy mosaicism in the CV of many suspected mosaic cases. An undiagnosed twin or co-twin demise pregnancy is another possibility to consider when trisomy mosaicism is suspected. If one twin is trisomic, a decreased MR occurs due to the placental contributions from both the normal and aneuploid twin (27, 28). A statistical rather than biological FP result might also explain a lack of cytogenetic confirmation in the CV of some cases.

Posttest genetic counseling is recommended for patients receiving screen-positive NIPT results (29–31). Such results are associated with distress about being on an unexpected path and worry about whether

Table 1. Observed positive predictive values for the common trisomies.[a]

	Total cohort (uncategorized)	cfDNA screening result		
		Non-mosaic trisomy	Mosaic trisomy	FF < Intersect
Condition	PPV (TP/TP + FP; 95% CI)	PPV (TP/TP + FP; 95% CI)	PPV (TP/TP + FP; 95% CI)	PPV (TP/TP + FP; 95% CI)
Trisomy 21 ($P = 3.3 \times 10^{-14}$)	93.3 (335/359; 90.2–95.5)	99.3 (282/284; 97.5–99.8)	50.0 (13/26; 32.1–67.9)	81.6 (40/49; 68.6–90.0)
Trisomy 18 ($P = 2.1 \times 10^{-13}$)	81.0 (98/121; 73.1–87.0)	97.6 (82/84; 91.7–99.3)	22.7 (5/22; 10.1–43.4)	73.3 (11/15; 48.0–89.1)
Trisomy 13 ($P = 1.6 \times 10^{-16}$)	55.3 (47/85; 44.7–65.4)	93.9 (31/33; 80.4–98.3)	0.0 (0/32; 0–10.7)	80.0 (16/20; 58.4–91.9)

[a]Two-sided 95% CI based on the Wilson score method. *P* value (for the Fisher exact test of independence).

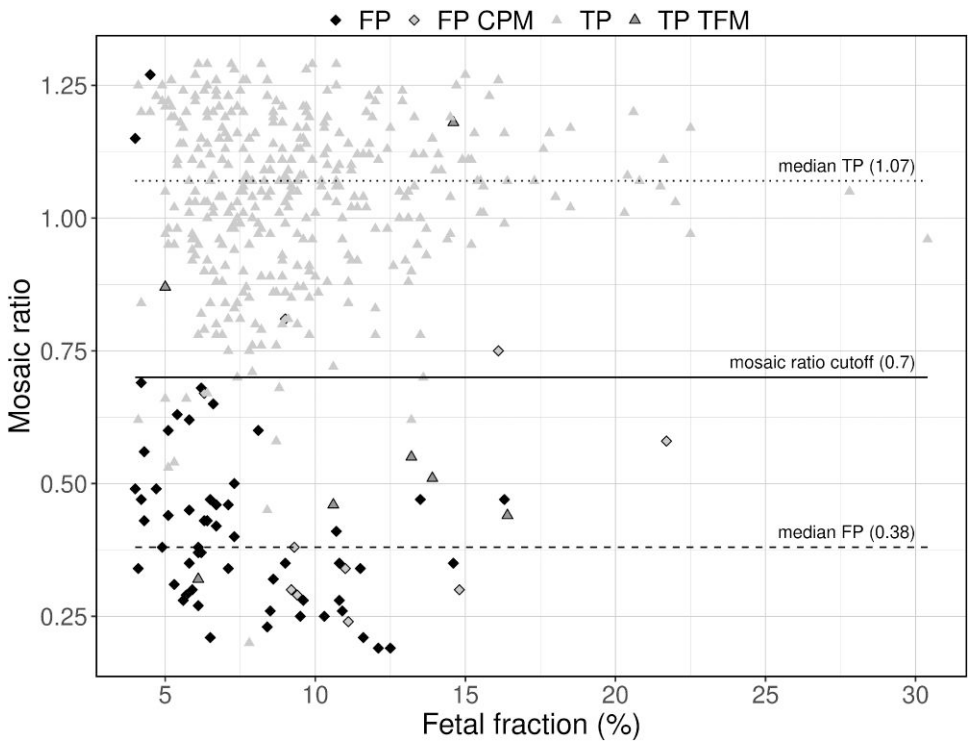

Fig. 5. Mosaic ratio and fetal fraction distribution for TP and FP results with FF ≥intersect. The *x*-axis shows fetal fraction, and the *y*-axis shows MR. The solid line marks the MR cutoff. The dotted line represents the median MR of TP results. The dashed line represents the median MR of FP results. Cases with MR >1.3 not displayed (TP = 40, FP = 1, FP CPM = 1).

the result will be confirmed on diagnostic testing. The counseling should include PPV information and available options for prenatal diagnosis (30). When offering guidance on choice of prenatal procedure, healthcare professionals need to consider gestational age, rates of CPM, ultrasound findings, and maternal factors (32). In many cases, CVS will provide an early definitive diagnostic result, allowing the option for surgical termination of pregnancy, although some patients may elect amniocentesis because of concern about the chance of CPM. Having a refined PPV that incorporates the presence or absence of mosaicism provides patients with more accurate information on the likely outcome of the diagnostic testing result, enabling the pregnant patient and their partner time to prepare and adjust.

The presence or absence of ultrasound abnormalities is another important consideration to help guide the choice of prenatal procedure (5, 33, 34). Scott et al. (33) assessed how the use of late first-trimester ultrasound findings impacted the PPV following a screen-positive NIPT result. The PPVs for all trisomies were high when abnormal ultrasound findings were present (T21: 99%, T18: 97%, and T13: 93%). However, many TP cases of T21 and T18 had no abnormal ultrasound findings, and a normal ultrasound was not predictive of a normal karyotype. Although PPVs for cases with normal ultrasound were reduced, more than half of these cases were confirmed with trisomy. In comparison, the PPVs for predicted non-mosaic trisomy in our cfDNA screening cohort (T21: 99.3%, T18: 97.6%, T13: 93.9%) are essentially identical to those reported by Scott et al. (33) when fetal anomalies were identified by expert sonographers. For a non-mosaic trisomy on cfDNA screening, the chance of a FP result due to placental mosaicism was low. Thus, for patients who want an earlier diagnostic result, CVS can be performed with higher confidence following a non-mosaic T21/T18/T13 screening result, even when fetal ultrasound is normal.

The use of cfDNA screening to predict placental mosaicism has been described by several groups and is not dependent on a specific NIPT platform (13–15). Using VeriSeq v2 to classify trisomies in our cohort, T13 had the highest rate of mosaic cfDNA findings (32.5%), followed by T18 (15.6%) and T21 (5.3%). These results compare with 2 previous studies, which also reported the highest rates of mosaicism in cfDNA for T13 (13.3%–49%), followed by T18 (12.8%–26%) and T21 (3.2%–5%) (12, 14). This is also reflected in the historical CVS data, with T13 showing the highest rate of mosaicism in cytotrophoblast and T21 the lowest (5).

The PPVs for non-mosaic trisomies exceeded 90% in our general risk cohort. However, FP results were still recorded, emphasizing the importance of prenatal diagnosis to confirm screening results if termination of pregnancy is being considered (29, 30). There were relatively few cases of TFM reported, with 7/413 (1.7%) trisomic cases representing TFM. Mosaic T21 cfDNA screening results were associated with the highest rate of TFM, with 5/13 (38.5%) TP results confirmed. The clinical presentation of TFM is more variable than it is for non-mosaic trisomy (35). When a mosaic T21 cfDNA screening result is communicated, the prospect of a full T21 or mosaic T21 finding in the fetus should be considered, together with the possibility of a normal karyotype outcome.

There are several limitations to our study. This is a retrospective cohort, and outcome data are not available for all cases. The median maternal age of 33.6 years at screening is higher than the median maternal age at delivery in Victoria, Australia, in 2020 and 2021 (32.2 and 32.4 years) (36). It is also higher than the median maternal age reported in some other nonselected NIPT cohorts (e.g., Dutch and Belgian national studies had a median age of 32 years at screening and a mean age of 30.7 years at delivery, respectively) (37, 38). The increased median maternal age in our cohort is expected to lead to an increased prevalence of the common trisomies and higher PPVs.

When the fetal fraction is <5.0% for T21 and <4.0% for T18 and T13, mosaicism cannot be reliably predicted. Consequently, we could not predict mosaicism for 14.1% of T21, 13.3% of T18, and 22% of T13 results. Our cohort was screened early in pregnancy, and our results are only applicable to pregnancies screened primarily at 10 to 12 weeks of gestational age. However, the same principles for MR apply at later gestations, when fetal fractions are, on average, higher (39). In a population where screening is performed later in pregnancy, higher FFs should mean fewer uncategorized cases. Our dataset had additional quality control applied to the VeriSeq v2 output that minimized some FP results. We have accounted for this in Supplemental Table 4, which calculates the PPVs based on the original VeriSeq v2 output.

The VeriSeq v2 assay is being used in many countries, with 68 NIPT laboratories enrolled in a 2023 European external quality scheme (EMQN/GenQA) using this platform (40). However, there is currently limited guidance for VeriSeq v2 users on the application of MR (16). Our approach provides a framework for laboratories to consider mosaic ratios to refine PPV estimates for the common trisomies to help guide genetic counseling, choice of prenatal procedure, and overall pregnancy management. MR can also be used to classify other screen-positive cfDNA results, including some sex chromosome aneuploidies, rare autosomal aneuploidies, and segmental chromosome anomalies >7 Mb. We encourage laboratories that incorporate MR to report on their experience.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: cfDNA, cell-free DNA; NIPT, noninvasive prenatal testing; T, trisomy; FP, false positive; FN, false negative; PPV, positive predictive value; CPM, confined placental mosaicism; MR, mosaic ratio; TP, true positive; VeriSeq v2, VeriSeq™ NIPT solution v2; VCGS, Victorian Clinical Genetics Services; LLR, log likelihood ratio; FF, fetal fraction; CVS, chorionic villus samples; POC, products of conception; TN, true negative; CV, chorionic villi; TFM, true fetal mosaicism.

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