FETAL ANEUPLOIDY TESTING USING CELL-FREE FETAL NUCLEIC ACIDS IN MATERNAL BLOOD

Guideline Number: MMG089.L **Effective Date**: May 1, 2019

Instructions for Use

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Related Medical Management Guideline

- Chromosome Microarray Testing (Non-Oncology Conditions)

COVERAGE RATIONALE

DNA-based noninvasive prenatal tests of fetal aneuploidy are proven and medically necessary as screening tools for trisomy 21 (Down syndrome), trisomy 18 (Edwards's syndrome) or trisomy 13 (Patau syndrome) in ANY ONE of the following circumstances:

- Maternal age of 35 years or older at delivery
- Fetal ultrasound findings indicating an increased risk of aneuploidy
- History of a prior pregnancy with a trisomy
- Positive first- or second-trimester screening test results for aneuploidy
- Parental balanced Robertsonian translocation with an increased risk of fetal trisomy 13 or trisomy 21

Due to insufficient evidence of efficacy, DNA-based noninvasive prenatal tests are unproven and not medically necessary for all other fetal conditions including, but not limited to, the following:

- Multiple gestation pregnancies
- Twin zygosity
- Repeat testing due to low fetal fraction
- Screening for the following:
  - Aneuploidy other than trisomies 21, 18, or 13
  - Microdeletions
  - Single gene disorders
  - Fetal RhD status

Genetic Counseling

Genetic counseling is strongly recommended prior to fetal screening or prenatal diagnosis in order to inform persons being tested about the advantages and limitations of the test as applied to a unique person.

DOCUMENTATION REQUIREMENTS

Benefit coverage for health services is determined by the member specific benefit plan document and applicable laws that may require coverage for a specific service. The documentation requirements outlined below are used to assess whether the member meets the clinical criteria for coverage but do not guarantee coverage of the service requested.

Required Clinical Information

Fetal Aneuploidy Testing Using Cell-Free Fetal Nucleic Acids in Maternal Blood

Medical notes documenting **all** of the following:

- Maternal age
- History of prior pregnancy with a trisomy, if applicable
- History of parental balanced Robertsonian translocation
- Abnormal first- or second-trimester screening test result

DEFINITIONS

Aneuploidy: A normal human cell has 23 pairs of chromosomes. The gain or loss of chromosomes is called Aneuploidy (Genetics Home Reference, 2018d).

Cell Free Fetal DNA (cffDNA or cfDNA): Small fragments of fetal DNA that cross the placenta and enter the maternal blood. Fragments can be measured using different DNA testing techniques in the first trimester (Allyse and Wick, 2018).

Comparative Genomic Hybridization (CGH): CGH is a technology that can be used for the detection of genomic copy number variations (CNVs). Tests can use a variety of probes or Single Nucleotide Polymorphisms (SNPs) to provide copy number and gene differentiating information. All platforms share in common that individual and reference DNA are labelled with dyes or fluorescing probes and hybridized on the array. A scanner then measures differences in intensity between the probes, and the data is expressed as having greater or less intensity than the reference DNA (South et al., 2013).

Massively Parallel Sequencing (MPS): Also referred to as Next Generation Sequencing (NGS), as well as Massively Parallel Shotgun Sequencing (MPSS), this technology allows for the simultaneous sequencing of multiple genes at the same time on a solid surface like a glass slide or bead (Alekseyev et al., 2018).

Mosaicism: An error in cell division may cause an individual to have two or more different populations of cells that have different chromosomes. One example is mosaic Turner syndrome, where some cells are 46,XX and others are 45,X due to the loss of a chromosome (Genetics Home Reference, 2018d).

Next Generation Sequencing (NGS): New sequencing techniques that can quickly analyze multiple sections of DNA at the same time. Older forms of sequencing could only analyze one section of DNA at once (Alekseyev et al., 2018).

Non-Invasive Prenatal Testing/Screening (NIPT/NIPS): A common term used to describe different types of analysis of cell-free fetal DNA (cffDNA) (Allyse and Wick, 2018).

Single Nucleotide Polymorphisms (SNPs): Small variations in DNA between individuals occur about every 300 nucleotides. These small differences, SNPs, usually have no impact on health or development but help identify specific chromosomal locations in the DNA (Genetics Home Reference, 2018d).

Trisomy 13 (Patau Syndrome): A chromosomal condition with an extra chromosome 13. It is associated with multiple congenital anomalies and significant developmental delay. Most infants die in the first month after birth, with only 5-10% surviving past the first year. The risk of having a child with trisomy 13 increases with a mother's age (Genetics Home Reference, October 2018c).

Trisomy 18 (Edwards Syndrome): A chromosomal condition with an extra chromosome 18. It is associated with multiple congenital anomalies and developmental delay. Most infants die in the first year of life, with only 5-10% surviving past the first year. The risk of having a child with trisomy 18 increases with a mother's age (Genetics Home Reference, October 2018b).

Trisomy 21 (Down Syndrome): A chromosomal condition with an extra chromosome 21. It is associated with intellectual disability, a characteristic facial appearance and poor muscle tone (hypotonia) in infancy. The degree of intellectual disability varies, but it is usually mild to moderate. Individuals with Down syndrome may be born with a variety of birth defects, including heart defects and digestive abnormalities. The risk of having a child with trisomy 21 increases with a mother's age (Genetics Home Reference, 2018a).

Whole Genome Sequencing (WGS): WGS determines the sequence of the entire DNA in a person, or a tissue type, such as a tumor, which includes the protein making (coding) as well as non-coding DNA elements (Genetics Home Reference, 2018e).

APPLICABLE CODES

The following list(s) of procedure and/or diagnosis codes is provided for reference purposes only and may not be all inclusive. Listing of a code in this guideline does not imply that the service described by the code is a covered or non-covered health service. Benefit coverage for health services is determined by the member specific benefit plan document and applicable laws that may require coverage for a specific service. The inclusion of a code does not imply any right to reimbursement or guarantee claim payment. Other Policies and Guidelines may apply.

CPT Code	Description
0009M	Fetal aneuploidy (trisomy 21, and 18) DNA sequence analysis of selected regions using maternal plasma, algorithm reported as a risk score for each trisomy
0060U	Twin zygosity, genomic targeted sequence analysis of chromosome 2, using circulating cell-free fetal DNA in maternal blood
81420	Fetal chromosomal aneuploidy (e.g., trisomy 21, monosomy X) genomic sequence analysis panel, circulating cell-free fetal DNA in maternal blood, must include analysis of chromosomes 13, 18, and 21
81422	Fetal chromosomal microdeletion(s) genomic sequence analysis (e.g., DiGeorge syndrome, Cri-du-chat syndrome), circulating cell-free fetal DNA in maternal blood
81479	Unlisted molecular pathology procedure
81507	Fetal aneuploidy (trisomy 21, 18, and 13) DNA sequence analysis of selected regions using maternal plasma, algorithm reported as a risk score for each trisomy

ICD-10 Diagnosis Code	Description
G91.2	(Idiopathic) normal pressure hydrocephalus
O09.291	Supervision of pregnancy with other poor reproductive or obstetric history, first trimester
O09.292	Supervision of pregnancy with other poor reproductive or obstetric history, second trimester
O09.293	Supervision of pregnancy with other poor reproductive or obstetric history, third trimester
O09.299	Supervision of pregnancy with other poor reproductive or obstetric history, unspecified trimester
O09.511	Supervision of elderly primigravida, first trimester
O09.512	Supervision of elderly primigravida, second trimester
O09.513	Supervision of elderly primigravida, third trimester
O09.519	Supervision of elderly primigravida, unspecified trimester
O09.521	Supervision of elderly multigravida, first trimester
O09.522	Supervision of elderly multigravida, second trimester
O09.523	Supervision of elderly multigravida, third trimester
O09.529	Supervision of elderly multigravida, unspecified trimester
O28.3	Abnormal ultrasonic finding on antenatal screening of mother
O28.5	Abnormal chromosomal and genetic finding on antenatal screening of mother
O28.9	Unspecified abnormal findings on antenatal screening of mother
O35.0XX0	Maternal care for (suspected) central nervous system malformation in fetus, not applicable or unspecified
O35.1XX0	Maternal care for (suspected) chromosomal abnormality in fetus, not applicable or unspecified
O35.1XX1	Maternal care for (suspected) chromosomal abnormality in fetus, fetus 1
O35.1XX2	Maternal care for (suspected) chromosomal abnormality in fetus, fetus 2
O35.1XX3	Maternal care for (suspected) chromosomal abnormality in fetus, fetus 3
O35.1XX4	Maternal care for (suspected) chromosomal abnormality in fetus, fetus 4
O35.1XX5	Maternal care for (suspected) chromosomal abnormality in fetus, fetus 5
O35.1XX9	Maternal care for (suspected) chromosomal abnormality in fetus, other fetus

ICD-10 Diagnosis Code	Description
O35.2XX0	Maternal care for (suspected) hereditary disease in fetus, not applicable or unspecified
Q90.0	Trisomy 21, nonmosaicism (meiotic nondisjunction)
Q90.1	Trisomy 21, mosaicism (mitotic nondisjunction)
Q90.2	Trisomy 21, translocation
Q90.9	Down syndrome, unspecified
Q91.0	Trisomy 18, nonmosaicism (meiotic nondisjunction)
Q91.1	Trisomy 18, mosaicism (mitotic nondisjunction)
Q91.2	Trisomy 18, translocation
Q91.3	Trisomy 18, unspecified
Q91.4	Trisomy 13, nonmosaicism (meiotic nondisjunction)
Q91.5	Trisomy 13, mosaicism (mitotic nondisjunction)
Q91.6	Trisomy 13, translocation
Q91.7	Trisomy 13, unspecified
Q92.0	Whole chromosome trisomy, nonmosaicism (meiotic nondisjunction)
Q92.1	Whole chromosome trisomy, mosaicism (mitotic nondisjunction)
Q92.2	Partial trisomy
Q92.5	Duplications with other complex rearrangements
Q92.61	Marker chromosomes in normal individual
Q92.62	Marker chromosomes in abnormal individual
Q92.7	Triploidy and polyploidy
Q92.8	Other specified trisomies and partial trisomies of autosomes
Q92.9	Trisomy and partial trisomy of autosomes, unspecified
Q95.0	Balanced translocation and insertion in normal individual
Q95.1	Chromosome inversion in normal individual
Q95.2	Balanced autosomal rearrangement in abnormal individual
Q95.3	Balanced sex/autosomal rearrangement in abnormal individual
Q95.5	Individual with autosomal fragile site
Q95.8	Other balanced rearrangements and structural markers
Q95.9	Balanced rearrangement and structural marker, unspecified

DESCRIPTION OF SERVICES

Routine screening tests for trisomies 21, 18, and 13 include first-trimester screening (which involves an ultrasound and a blood test), maternal serum screening (a blood test) and a high-resolution ultrasound evaluation in the second trimester. These tests may identify women with an increased risk of having a child with trisomy 21, 18, or 13, but they cannot diagnose, confirm or exclude the possibility of a chromosomal disorder. Conventional prenatal diagnosis (i.e., chorionic villus sampling (CVS) or amniocentesis) can definitively diagnose fetal trisomies, although these invasive procedures are associated with a risk of miscarriage (Hayes, 2018).

Tests that detect fetal trisomies, without the need for CVS or amniocentesis, analyze cell-free DNA (cfDNA) fragments in maternal blood. During pregnancy, there are cfDNA fragments from both the mother and fetus in maternal circulation. The tests detect an increased amount of chromosomal material in maternal blood and can be offered as early as 9 weeks of pregnancy. Available tests use different methodologies and algorithms for data analysis. Depending on the test, the methodology may involve massively parallel sequencing (MPS), targeted sequencing of specific chromosomal segments or directed sequence analysis of single nucleotide polymorphisms (SNPs) (Hayes, 2018). All tests were validated in high-risk couples. It is unknown whether they are as accurate for low- to average risk couples.

In addition to screening for common fetal aneuploidies, cfDNA panels are also being used to screen for microdeletion syndromes which are caused by a chromosomal deletion that is too small to be detected by conventional cytogenetic testing (Hayes, 2018).

CLINICAL EVIDENCE

Aneuploidy for Trisomies 21, 18, and 13

Studies evaluating the analytic validity of NIPT analysis showed that analytic sensitivity depended on the conditions being tested. For this assay, the test sensitivity was 944% to 100% for T21, 87.5%-100% for T18, and 40%% to 100% for T13, with a test specificity > 99%. In five studies evaluating the NIPT assay in multiple-gestation pregnancies, the overall data was missing critical outcome information, resulting in insufficient information to draw conclusions about the sensitivity and specificity of testing in twins. (Hayes, 2018).

Chen et al. (2018) studied the ability for NIPT to detect chromosome aneuploidy in a cohort of 4194 women undergoing cytogenetic prenatal diagnosis at the Changzhou Maternity and Child Health Care Hospital between January 2009 and October 2017. Patients were over 35 years old and between 17-23 weeks gestation. Cell-free fetal DNA was analyzed using MPSS. Routine karyotyping identified 233 fetuses with chromosome abnormalities, for an overall incidence of 5.6%. There were 132 cases of Trisomy 21, of which 4 were caused by a Robertsonian translocation, 14 were mosaic, and 21 cases had an abnormal chromosome structure. There were 37 Trisomy 18, 4 Trisomy 13, 5 monosomy X, 32 other sex chromosome abnormalities, and 2 other autosomal aneuploidies. In comparison, NIPT identified 204 of the cases with abnormal chromosomes. The cases missed included 1 Trisomy 21, 1 Trisomy 18, 2 other autosome aneuploidy, 6 chromosome aneuploidy mosiacs, all 21 structurally abnormal chromosomes resulting in Trisomy 21. The authors conclude that using NIPT alone will miss 12.4% of fetal chromosome abnormalities if it completely replaces prenatal diagnosis for women of advanced maternal age, but they noted that in this study, the anomalies missed are not associated with advanced maternal age.

Five clinical laboratories in the Netherlands, Australia, Denmark, and Brazil collaborated to review their outcome records for true positive trisomy 21 and false negative cases (Huijsdens-van Amsterdam et al., 2018). The labs represented all major NIPT technologies of massively parallel shotgun sequencing (MPSS), targeted MPS (tMPS), targeted microarray (tMA), and targeted single nucleotide polymorphism (tSNP). Overall the labs documented 646 confirmed cases of trisomy 21 and 9 false negative cases, resulting in a false negative rate of 1.4%. There were four cases missed of classic trisomy 21, and five cases with a *de novo* 21q:21q rearrangement. Two of the five were confirmed to be isochromosomes. In one case, placental material was available and placental mosaicism was confirmed. The authors noted that a low trisomic fraction relative to the fetal fraction suggests placental mosaicism. The average fetal fraction in 21q:21q cases was 10%. When combined with the literature on false negative trisomy 21 results, 21q:21q rearrangements represented 28% of all false negatives.

Mackie et al. (2017) conducted a meta-analysis of cohort studies reporting on cell-free fetal DNA NIPT results in singleton pregnancies. They reviewed peer reviewed publications identified through Medline, Embase, CINAHL, and the Cochrane Library, with publication dates from 1997 to April 2015. Inclusion criteria were singleton pregnancies of any gestation with results confirmed by karyotype or phenotype, cohort studies, and had more than five participants. Case studies, pre-implantation testing, or other fetal cell testing studies were excluded. A total of 117 studies met criteria for 18 conditions. The sensitivities and specificities for each condition were determined by bi-variate meta-analysis. For trisomy 21, there were 148,344 tests identified with a respective sensitivity and specificity of 99% and 99%. For trisomy 18, there were 146,940 tests identified with a respective sensitivity and specificity of 97% and 99%. Monosomy X had 6,712 tests identified with a respective sensitivity and specificity of 93% and 99%. RhD had 10,290 tests identified with a respective sensitivity and specificity of 99% and 98%. Trisomy 13 was evaluated by univariate analysis, and there were 134,691 tests identified with a respective sensitivity and specificity of 90% and 100%. The authors concluded that NIPT could be considered diagnostic for determining fetal sex and RhD status. However, they determined that NIPT for chromosome 21, 13, and 18 aneuploidies is a screening test because of the lower disease prevalence, role of confined placental mosaicism, and lower sensitivity and specificity.
Hayes evaluated the clinical utility of cfDNA screening in populations at low risk for aneuploidy. The overall quality of evidence was low for fetal trisomy 21, 18, and 13 in women with low-risk singleton pregnancies and very low in women with multiple gestation pregnancies. Study limitations included varied and small patient populations with few chromosomally abnormal pregnancies detected, incomplete reporting of results and exclusion of women with cfDNA screening failures. Studies directly comparing clinical outcomes of cfDNA screening with those of routine screening strategies for low-risk or general obstetric patients in a real-world setting are needed (Hayes, 2017a).

In a multicenter, observational study, Palomaki et al. (2017) assessed the clinical utility of cfDNA-based screening for aneuploidies offered through primary obstetrical care providers to a general pregnancy population. Among 2,681 tests reported, 16 women (0.6%) were screen-positive for trisomy 21, 18, or 13. Twelve were confirmed (PPV, 75%) and four were false-positives (0.15%). Of 150 test failures (5.6%), 79% had a negative serum or subsequent cfDNA test. There were no reported cases of aneuploidy among cfDNA test failures.

Iwarsson et al. (2017) conducted a systematic review and meta-analysis to assess the performance of cfDNA testing for the detection of trisomy 21, 18, and 13 in a general pregnant population and update the data on high-risk pregnancies. In a general pregnant population, the authors noted moderate evidence of a pooled sensitivity of 0.993

and specificity of 0.999 for the analysis of trisomy 21. Pooled sensitivity and specificity for trisomies 13 and 18 were not calculated in this population due to the low number of studies. In a high-risk pregnant population, the authors noted moderate evidence of pooled sensitivities for trisomies 21 and 18 of 0.998 and 0.977, respectively, and low evidence of a pooled sensitivity for trisomy 13 of 0.975. The pooled specificity for all three trisomies was 0.999. The authors concluded that cfDNA performs well as a screen for trisomy 21 in a general pregnant population; however, more data is needed for trisomies 18 and 13.

Norton et al. (2016) compared the performance of sequential and cfDNA screening for detection of fetal chromosomal abnormalities in a general prenatal cohort. Of 452,901 women who underwent sequential screening; 2,575 (0.57%) had a fetal chromosomal abnormality. The DR of sequential screening for all aneuploidies in the cohort was greater than cfDNA. The authors concluded that cfDNA should not be adopted as a primary screening tool without further evaluation of the implications for detection of all chromosomal abnormalities and how to best evaluate no results cases.

Taylor-Phillips et al. (2016) conducted a systematic review and meta-analysis to measure the accuracy of NIPT for Down, Edwards, and Patau syndromes using cfDNA. Pooled sensitivity was 99.3% for Down, 97.4% for Edwards, and 97.4% for Patau syndrome. The pooled specificity was 99.9% for all three trisomies. Sensitivity was lower in twin than singleton pregnancies, reduced by 9% for Down, 28% for Edwards, and 22% for Patau syndrome. Pooled sensitivity was also lower in the first trimester of pregnancy in studies of the general obstetric population. NIPT using cfDNA has very high sensitivity and specificity for Down syndrome, with slightly lower sensitivity for Edwards and Patau syndrome. NIPT should not be used as a final diagnosis for positive cases.

Zhang et al. (2015) reported the clinical performance of MPS-based NIPT in detecting trisomies 21, 18, and 13 and to compare its performance in low-risk and high-risk pregnancies. NIPT was performed and results obtained in 146,958 samples, for which outcome data were available in 112,669 (76.7%). Repeat blood sampling was required in 3213 cases and 145 had test failure. Aneuploidy was confirmed in 720/781 cases positive for trisomy 21, 167/218 cases positive for trisomy 18, and 22/67 cases positive for trisomy 13. Nine false negatives were identified, including six cases of trisomy 21 and three of trisomy 18. The overall sensitivity of NIPT was 99.17%, 98.24%, and 100% for trisomies 21, 18, and 13, respectively, and specificity was 99.95%, 99.95%, and 99.96% for trisomies 21, 18, and 13, respectively. In the low-risk population, the PPV was 81.36% for trisomy 21. The reduced PPV in the low-risk group as a consequence of lower disease prevalence, reaffirmed that NIPT should not be used as a diagnostic test and that confirmation by invasive testing is still necessary. The authors reported no significant difference in test performance between 72,382 high-risk and 40,287 low-risk pregnancies (sensitivity, 99.21% vs. 98.97% ; specificity, 99.95% vs. 99.95%). The major factors contributing to false-positive and false-negative results were maternal copy number variant and fetal/placental mosaicism. An author noted limitation was the incomplete follow-up of NIPT results, which could introduce bias into the performance evaluation.

Mersy et al. (2013) conducted a systematic review of seven studies that examined noninvasive prenatal testing (NIPT) of fetal trisomy 21 using MPS. The authors reported sensitivities between 98.58% and 100% and specificities between 97.95% and 100%. MPS with or without pre-selection of chromosomes exhibits a negative predictive value (NPV) (100%); however, PPVs were lower, even in high-risk pregnancies (19.7-100%).

Dar et al. (2014) reported clinical experience of a SNP-based NIPT in high- and low-risk women. Samples were received from 31,030 patients, 30,705 met study criteria and 28,739 passed quality-control metrics and received a report detailing aneuploidy risk. Samples that passed quality control were analyzed for trisomy 21, trisomy 18, trisomy 13, and monosomy X. Results were reported as high or low risk for fetal aneuploidy for each chromosome. Follow-up on outcome was sought for a subset of high-risk cases. False-negative results were reported voluntarily by providers. PPV was calculated from cases with an available prenatal or postnatal karyotype or clinical evaluation at birth. In all, 507 patients received a high-risk result for any of the 4 tested conditions (324 trisomy 21, 82 trisomy 18, 41 trisomy 13, 61 monosomy X; including one double aneuploidy case). Within the 17,885 cases included in follow-up analysis, 356 were high risk, and outcome information revealed 184 (51.7%) true positives, 38 (10.7%) false positives, 19 (5.3%) with ultrasound findings suggestive of aneuploidy, 36 (10.1%) spontaneous abortions without karyotype confirmation, 22 (6.2%) terminations without karyotype confirmation, and 57 (16.0%) lost to follow-up. This yielded an 82.9% PPV for all aneuploidies, and a 90.9% PPV for trisomy 21. The overall PPV for women aged ≥35 years was similar to the PPV for women aged <35 years. Two patients were reported as false negatives. The authors reported that the results suggest the clinical performance of this SNP-based NIPT in a mixed high- and low-risk population is consistent with performance in validation studies. However, the authors also noted that the main limitation of the study is the incomplete follow-up data, particularly on low-risk patients. This lack of follow-up precludes precise calculation of sensitivity and specificity and determination of NPV.

In a prospective, multicenter, blinded study, Norton et al. (2015) evaluated cfDNA testing in the assessment of risk for trisomy 21 in a large, routine prenatal screening population. Pregnant women presenting for aneuploidy screening at 10 to 14 weeks of gestation were assigned to undergo both standard screening (with measurement of nuchal

translucency and biochemical analytes) and cfDNA testing. The study also evaluated the performance of cfDNA testing and standard screening in the assessment of risk for trisomies 18 and 13. Determination of the birth outcome was based on diagnostic genetic testing or newborn examination. Of 18,955 women who were enrolled, results from 15,841 were available for the primary analysis. The mean maternal age was 30.7 years, and the mean gestational age at testing was 12.5 weeks. Trisomy 21 was detected in 38 of 38 women (100%) in the cfDNA-testing group, as compared with 30 of 38 women (78.9%) in the standard screening group. False positive rates were 0.06% in the cfDNA group and 5.4% in the standard-screening group. The PPV for cfDNA testing was 80.9%, as compared with 3.4% for standard screening. In a secondary analysis of 11,994 women with low-risk pregnancies based on a maternal age <35, cfDNA testing identified 19 of 19 women with trisomy 21, with six false positive results. Among the 14,957 women for whom standard screening showed a risk of less than 1 in 270, cfDNA testing identified eight of eight women with trisomy 21, with eight false positive results. The PPV for cfDNA testing was 76.0% for women under the age of 35 years and 50.0% for those with a negative result on standard screening. Of the ten cases of trisomy 18 in the primary analysis population, cfDNA testing identified nine and standard screening identified eight. cfDNA testing had a false positive rate of 0.01% and a PPV of 90.0%, as compared to a false positive rate of 0.31% and a PPV of 14.0% with standard screening. Among the 11,185 women who underwent both cfDNA testing and standard screening for trisomy 13, there were two confirmed cases. Of these, cfDNA testing identified two and standard screening identified one. cfDNA testing had a false positive rate of 0.02% compared to 0.25% for standard screening.

Verweij et al. (2012) conducted a systematic review on the diagnostic accuracy of noninvasive prenatal diagnosis (NIPD) of fetal trisomies using cfDNA or RNA in maternal blood to detect fetal trisomy 21. With a total of 681 pregnancies included, overall sensitivity was 125/125 (100%) and specificity 552/556 (99.3%). NIPD of fetal trisomy 21, using fetal nucleic acids in maternal plasma, appears to have a high diagnostic accuracy.

Published evidence regarding the clinical utility of noninvasive prenatal testing is limited. Prospective data is needed in which test results are acted upon clinically, showing that results lead to a change in patient management and/or outcomes (Simpson, 2012). For example, data must demonstrate that physicians have sufficient confidence in both positive and negative test results to refrain from performing more invasive testing, e.g., amniocentesis, for the purpose of confirming the previously obtained test results.

Limited data is available regarding improvement in patient outcomes if cfDNA is offered as a screening test to all pregnant women. While some study results suggest making cfDNA-based testing available to all pregnant women, regardless of risk, these studies fail to address the fact that a test's positive predictive value (PPV) is highly dependent on the patient's pretest trisomy risk. cfDNA-based testing's very low PPV in patients at low risk for trisomy must be considered in any discuss ion of expanding current guidelines to offer cfDNA testing as a first-line screen in this population.

MaterniT21

In a prospective multicenter observational study of pregnant women at high risk for fetal aneuploidy, Porreco et al. (2014) evaluated the clinical performance of MPA of cfDNA in maternal blood. A total of 3430 patients were included in the analysis. There were 137 fetuses with trisomy 21, 39 with trisomy 18, and 16 with trisomy 13. There were no false-negative results for trisomy 21, three for trisomy 18, and two for trisomy 13. All false-positive results (3) were for trisomy 21. The PPVs for trisomies 18 and 13 were 100% and 97.9% for trisomy 21. The authors concluded that noninvasive prenatal analysis of cfDNA from maternal blood is an accurate advanced screening test with extremely high sensitivity and specificity for trisomy 21 (>99%) but less sensitivity for trisomies 18 and 13. Despite high sensitivity, there was modest positive predictive value for the small number of common sex chromosome aneuploidies because of their very low prevalence rate.

Canick et al. (2012) conducted a study on prenatal testing for Down syndrome (trisomy 21), trisomy 18 and trisomy 13 by massively parallel shotgun sequencing (MPSS) of circulating cfDNA in pregnant women with multiple gestations. Among a cohort of 4664 high-risk pregnancies, maternal plasma samples were tested from 25 twin pregnancies (17 euploid, five discordant and two concordant for Down syndrome; one discordant for trisomy 13) and two euploid triplet pregnancies. Seven twin pregnancies with Down syndrome, one with trisomy 13, and all 17 twin euploid pregnancies were correctly classified [detection rate 100%, false positive rate 0%], as were the two triplet euploid pregnancies. Although study size is limited, the authors concluded that this data provides evidence that MPSS testing can be reliably used as a secondary screening test for Down syndrome in women with high-risk twin gestations. Further studies, with larger patient populations, are needed to confirm these results.

In the largest and most comprehensive study to date, Palomaki et al. (2011) evaluated the analytic validity of a noninvasive prenatal screening test for Down syndrome that measures circulating cfDNA in maternal plasma. Test results were compared to those obtained after chorionic villus sampling or amniocentesis. A total of 4664 pregnant women, each considered at high risk for having a child with trisomy 21 (based on maternal age, screening test results and/or ultrasound results), were recruited from 27 prenatal diagnostic centers and included in this blinded, nested case-control study. Of the 4664 cases, 279 (6%) were excluded from the analysis for various reasons. Of the

remaining cases, 218 (5%) fetuses were diagnosed with trisomy 21 based on the results of invasive testing. The first 212 cases were selected for analysis. Fetal karyotyping was compared with an internally validated, laboratory-developed test based on next-generation sequencing. Down syndrome detection rate was 98.6% (209/212), the false-positive rate was 0.20% (3/1471) and the testing failed in 13 pregnancies (0.8%). The authors concluded that, when applied to high-risk pregnancies, measuring maternal plasma DNA detects nearly all cases of Down syndrome at a very low false-positive rate. This method can substantially reduce the need for invasive diagnostic procedures and attendant procedure-related fetal losses.

Using the same cohort of patients, Palomaki et al. (2012) reported additional data indicating that maternal plasma cfDNA sequencing also has the capability to detect other aneuploidies, such as trisomy 18 and trisomy 13. Sixty-two pregnancies with trisomy 18 and 12 with trisomy 13 were included in the analysis. Among the 99.1% of samples interpreted, observed trisomy 18 and 13 detection rates were 100% (59/59) and 91.7% (11/12) with false-positive rates of 0.28% and 0.97%, respectively.

Chiu et al. (2011) validated the clinical efficacy and practical feasibility of massively parallel maternal plasma DNA sequencing to screen for fetal trisomy 21 among high risk pregnancies clinically indicated for amniocentesis or chorionic villus sampling. The diagnostic accuracy was validated against full karyotyping, using prospectively collected or archived maternal plasma samples. Results were available from 753 pregnancies with the 8-plex sequencing protocol and from 314 pregnancies with the 2-plex protocol. The performance of the 2-plex protocol was superior to that of the 8-plex protocol. With the 2-plex protocol, trisomy 21 fetuses were detected at 100% sensitivity and 97.9% specificity, which resulted in a positive predictive value (PPV) of 96.6% and NPV of 100%. The 8-plex protocol detected 79.1% of the trisomy 21 fetuses and 98.9% specificity, giving a positive predictive value of 91.9% and negative predictive value of 96.9%. The authors concluded that multiplexed maternal plasma DNA sequencing analysis could be used to rule out fetal trisomy 21 among high risk pregnancies.

Ehrich et al. (2011) evaluated a MPSS assay for noninvasive trisomy 21 detection using circulating cfDNA. A total of 480 plasma samples from high-risk pregnant women were analyzed. Thirteen samples were removed due to insufficient quantity or quality. Eighteen samples failed prespecified assay quality control parameters. In all, 449 samples remained: 39 trisomy 21 samples were correctly classified; one sample was misclassified as trisomy 21. The overall classification showed 100% sensitivity and 99.7% specificity. The authors reported that extending the scope of previous reports, these results warrant clinical validation in a larger multicenter study.

verifi

The MELISSA (MatErnal Blood IS Source to Accurately Diagnose Fetal Aneuploidy) trial was conducted as a prospective, multicenter observational study to determine the diagnostic accuracy of MPS (verifi™) to detect fetal aneuploidy from maternal plasma. Blood samples were collected from 2,882 women undergoing invasive prenatal diagnostic procedures. Within an analysis cohort, the following were classified correctly: 89 of 89 trisomy 21 cases (sensitivity 100%, 95% CI), 35 of 36 trisomy 18 cases (sensitivity 97.2%, 95% CI) and 11 of 14 trisomy 13 cases (sensitivity 78.6%, 95% CI). The authors reported high sensitivity and specificity for the detection of trisomies 21, 18, 13 in women with singleton pregnancies at an increased risk for aneuploidy. Further studies are needed to build confidence in the diagnostic performance of the test in low-risk populations and in multiple gestations. ClinicalTrials.gov NCT01122524 (Bianchi et al., 2012)

Sehnert et al. (2011) evaluated an optimized algorithm for use with massively parallel DNA sequencing of cfDNA to detect fetal chromosomal abnormalities. Existing algorithms focus on the detection of fetal trisomy 21 (T21); however, these same algorithms have difficulty detecting trisomy 18 (T18). Blood samples were collected from 1014 patients prior to undergoing an invasive prenatal procedure. The DNA extracted from 119 samples underwent massively parallel DNA sequencing. Fifty-three sequenced samples came from women with an abnormal fetal karyotype. To minimize the intra- and interrun sequencing variation, investigators developed an optimized algorithm by using normalized chromosome values (NCVs) from the sequencing data on a training set of 71 samples with 26 abnormal karyotypes. The classification process was then evaluated on an independent test set of 48 samples with 27 abnormal karyotypes. Sequencing of the independent test set led to 100% correct classification of T21 (13 of 13) and T18 (8 of 8) samples. Other chromosomal abnormalities were also identified. The authors concluded that MPS is capable of detecting multiple fetal chromosomal abnormalities from maternal plasma when an optimized algorithm is used.

In a multicenter comparative study funded by Illumina, Bianchi et al. (2014) evaluated the performance of noninvasive prenatal testing using MPS of cfDNA in maternal blood for detecting fetal aneuploidy in low-risk women. The primary series included 1914 women with an eligible sample, a singleton fetus without aneuploidy, results from cfDNA testing and a risk classification based on standard screening. For trisomies 21 and 18, the false positive rates with cfDNA testing were significantly lower than those with standard screening (0.3% versus. 3.6% for trisomy 21; and 0.2% versus. 0.6% for trisomy 18). The use of cfDNA testing detected all cases of aneuploidy (5 for trisomy 21, 2 for trisomy 18 and 1 for trisomy 13; NPV, 100%. The PPVs for cfDNA testing versus standard screening were 45.5%

versus 4.2% for trisomy 21 and 40.0% versus 8.3% for trisomy 18. Further studies are needed to determine the clinical validity of sequence-based fetal aneuploidy testing in this patient population.

Another study, which involved 6123 patients with blood samples submitted for testing with the Verifi Prenatal Test, 155 (2.6%) fetuses were identified as having trisomy 21, 66 (1.1%) with trisomy 18, 19 (0.3%) with T13, and 40 (0.7%) with Turner syndrome (45,X). Unclassifiable results were obtained in up to 1% of cases for each of the chromosomes analyzed. Overall, of 280 fetuses with aneuploidy detected by NIPT, 94 (33.6%) were confirmed or the pregnancies resulted in miscarriage, and 14 (0.2%) yielded discordant (likely false-positive) results. Among cases with euploid NIPT results, there were five (0.08%) known false-negative cases (2 trisomy 21, 2 trisomy 18, and 1 45,X). Follow-up information was not available for the remaining cases at the time of publication (Futch et al., 2013).

Harmony Prenatal Test

Gil et al. (2014) performed a meta-analysis of cfDNA in maternal blood in screening for aneuploidies. Weighted pooled detection rates (DR) and false-positive rates (FPR) in singleton pregnancies were 99.0% and 0.08%, respectively, for trisomy 21; 96.8% and 0.15% for trisomy 18; 92.1% and 0.20% for trisomy 13; 88.6% and 0.12% for monosomy X and 93.8% and 0.12% for sex chromosome aneuploidies other than monosomy X. For twin pregnancies, the DR was 94.4% and the FPR was 0% for trisomy 21. The authors concluded that screening for aneuploidies analyzing cfDNA in maternal blood provides effective screening for trisomies. The same group of authors updated the meta-analysis with data from 37 studies published since the initial review. The studies reported cfDNA results in relation to fetal karyotype from invasive testing or clinical outcome. Weighted pooled DR and FPR in singleton pregnancies were 99.2% and 0.09%, respectively, for trisomy 21; 96.3% and 0.613% for trisomy 18; 91.0% and 0.13% for trisomy 13; 90.3% and 0.23% for monosomy X and 93.0% and 0.14% for sex chromosome aneuploidies other than monosomy X. For twin pregnancies, the DR for trisomy 21 was 93.7% and the FPR was 0.23% (Gil et al., 2015). In a 2017 update, weighted pooled DR and FPR in singleton pregnancies were 99.7% and 0.04%, respectively, for trisomy 21; 97.9% and 0.04% for trisomy 18; 99.0% and 0.04% for trisomy 13; 95.8% and 0.14% for monosomy X and 100% and 0.004% for sex chromosome aneuploidies other than monosomy X. For twin pregnancies, the DR for trisomy 21 was 100% and the FPR was 0.0%. This meta-analysis also highlighted low-risk and mixed-risk populations. The overall incidence of aneuploidy was higher than anticipated for the low or mixed risk population. When studies were broken out by technology, the sample sizes for the different types of tests was individually low. This suggests that more data is needed to determine the positive predictive value for the low risk or mixed risk populations (Gil et al., 2017).

Ashoor et al. (2013) assessed the performance of the Harmony Prenatal Test for the detection of trisomy 13. A two-phase, blinded, case-control study was performed to optimize and validate the trisomy 13 algorithm. In the first phase, trisomy 13 risk scores were given for 11 cases of trisomy 13 and 145 euploid cases at 11-13 weeks' gestation. The test identified seven (63.6%) cases of trisomy 13 with no false positives. The trisomy 13 algorithm was subsequently modified and the trisomy 13 risk score was > 99% in all 11 cases of trisomy 13 and < 0.01% in all 145 euploid cases. In the second phase, the new algorithm was used to generate trisomy 13 risk scores for 10 cases of trisomy 13 and 1939 euploid cases. The trisomy 13 risk scores were > 99% in eight (80.0%) cases of trisomy 13. In the 1939 euploid cases the risk score for trisomy 13 was < 0.01% in 1937 (99.9%), 0.79% in one and > 99% in one. Therefore, at the predefined risk cut-off of 1% for classifying a sample as high or low risk, the false-positive rate (FPR) was 0.05%.

Nicolaides et al. (2012) conducted a cohort study of 2049 pregnant women undergoing routine screening for aneuploidies at 11-13 weeks' gestation. Plasma cfDNA analysis using chromosome-selective sequencing was used. Laboratory testing on a single plasma sample was carried out blindly and results were provided as risk score (%) for trisomies 21 and 18. Trisomy risk scores were given for 95.1% (1949 of 2049) of cases including all eight with trisomy 21 and two of the three with trisomy 18. The trisomy risk score was >99% in the eight cases of trisomy 21 and 2 of trisomy 18 and <1% in 99.9% (1937 of 1939) of euploid cases. The authors concluded that noninvasive prenatal testing (NIPT) using chromosome-selective sequencing in a routinely screened population identified trisomies 21 and 18 with a false-positive rate of 0.1%. However, the authors cautioned that because the sensitivity and specificity of NIPT is not 100%, the test should not be considered a diagnostic replacement for invasive testing in high-risk pregnancies.

Norton et al. (2012) conducted a multicenter cohort study evaluating the performance of a noninvasive prenatal test for fetal trisomy 21 (T21) and trisomy 18 (T18) using cfDNA from maternal plasma. Chromosome-selective sequencing was performed with reporting of an aneuploidy risk (high risk or low risk) for each subject. Of the 81 T21 cases, all were classified as high risk for T21 and there was one false-positive result among the 2888 normal cases, for a sensitivity of 100% and a false-positive rate of 0.03%. Of the 38 T18 cases, 37 were classified as high risk and there were two false-positive results among the 2888 normal cases, for a sensitivity of 97.4% and a false-positive rate of 0.07%. The authors concluded that chromosome-selective sequencing of cfDNA and application of an individualized risk algorithm is effective in the detection of fetal T21 and T18.

Sparks and colleagues (2012a) described the initial development of the Harmony Prenatal Test. Blood samples were obtained from 298 women with singleton pregnancies at 10 weeks or later. Invasive prenatal diagnosis identified 39

fetuses with trisomy 21 and seven fetuses with trisomy 18. Samples were analyzed using a novel, highly multiplexed assay, referred to as digital analysis of selected regions (DANSR™). All (100%) aneuploid samples were correctly identified.

Sparks et al. (2012b) evaluated a novel biochemical assay and algorithm for the prenatal evaluation of risk for fetal trisomy 21 (T21) and trisomy 18 (T18) using cfDNA obtained from maternal blood. In a blinded analysis of 167 pregnant women, digital analysis of selected regions (DANSR), in combination with a novel algorithm, fetal-fraction optimized risk of trisomy evaluation (FORTE), correctly identified all 36 cases of T21 and eight cases of T18. The investigators assayed cfDNA from a training set and a blinded validation set of pregnant women, comprising 250 disomy, 72 T21 and 16 T18 pregnancies. In the training set, 163/171 subjects passed quality control criteria. FORTE produced an individualized trisomy risk score for each subject, and correctly discriminated all T21 and T18 cases from disomic cases. All 167 subjects in the blinded validation set passed quality control and FORTE performance matched that observed in the training set correctly discriminating 36/36 T21 cases and 8/8 T18 cases from 123/123 disomic cases. The authors concluded that while DANSR and FORTE enable accurate noninvasive fetal aneuploidy detection in a high-risk population, larger studies that include low- and average-risk pregnancies are needed.

In a nested case-control study, Ashoor et al. (2012) assessed the prenatal detection rate of trisomy 21 and 18 by chromosome-selective sequencing of maternal plasma cfDNA. cfDNA was examined in stored plasma that was obtained at 11-13 weeks before chorionic villous sampling from 300 euploid pregnancies, 50 pregnancies with trisomy 21 and 50 pregnancies with trisomy 18. The authors reported that the sensitivity for detecting trisomy 21 was 100% (50/50 cases); the sensitivity for trisomy 18 was 98% (49/50 cases) and the specificity was 100% (297/297 cases).

In the first study of 284 obstetrical patients who were evaluated by both the Harmony Prenatal Test and traditional first-trimester screening, all patients received NIPT results indicating a low likelihood (< 0.01%) of fetal trisomies 21, 18, or 13. A single patient, who had a first-trimester screening result of one in five for trisomy 21, elected to have invasive prenatal diagnosis, which revealed a normal fetal karyotype. The clinical outcomes for the remaining pregnancies were not known at the time of publication, although the NIPT results were consistent with the risk estimations provided by first-trimester screening (Fairbrother et al., 2013).

In a second study involving the Harmony Prenatal Test, 1005 pregnant women were tested with both NIPT and traditional first-trimester screening. The detection rates for trisomies 21 and 18 were 100% and 80%, respectively. In addition, assuming the pregnancies that had not yet delivered did not have an undiagnosed aneuploidy (missed by both NIPT and first-trimester screening), the false-positive rate was 0.1% for NIPT and 3.4% for first-trimester screening (Gil et al., 2013).

Panorama Prenatal Test

Pergament et al. (2014) evaluated the analytic validity of a SNP-based NIPT in a high- and low-risk population. A total of 1064 maternal blood samples from seven weeks of gestation and beyond were included. Of these, 1,051 were within specifications and 518 (49.3%) were low risk. Nine hundred sixty-six samples (91.9%) successfully generated a cfDNA result. Among these, sensitivity was 100% for trisomy 21 (58/58) and trisomy 13 (12/12) and 96.0% for trisomy 18 (24/25). Specificity was 100% for trisomies 21 and 13 and 99.9% for trisomy 18. However, 16% (20/125) of samples did not return a result. Sensitivity and specificity did not differ in low-risk and high-risk cohorts. Level of evidence: II.

Nicolaides et al. (2013) validated the Panorama Prenatal Test (Natera Inc.) in a population of 242 women with singleton pregnancies undergoing chorionic villus sampling (CVS) at 11 to 13 weeks of gestation. Women were referred because first-trimester screening indicated an increased risk for trisomy 21, 18, or 13. Maternal blood samples were sent to the Natera laboratory, where testing involved the targeted sequencing of 19,488 SNPs. Sequencing data were analyzed using the NATUS algorithm. Results were provided for 229 (94.6%) of the 242 cases. Thirty-two cases were correctly identified as aneuploid, including trisomy 21 [n = 25; sensitivity = 100%, specificity = 100%], trisomy 18 (n = 3), trisomy 13 (n = 1), Turner syndrome (n = 2) and triploidy (n = 1), with no false positive or false negative results.

Zimmermann et al. (2012) evaluated a noninvasive prenatal test using targeted sequencing of SNPs (Panorama Prenatal Test). Blood samples were obtained from 166 pregnant women at nine weeks of gestation or later. It was not specified whether these pregnancies were high- or average-risk. Of the 166 fetuses, 11 had trisomy 21, three had trisomy 18, and two had trisomy 13. Twenty-one samples did not pass the DNA quality test and were excluded from the analysis. All aneuploidies were correctly identified for a sensitivity and specificity of 100%.

Vanadis NIPT

Dahl et al. (2018) reports on a new approach to NIPT testing that uses novel molecular probe technology to label target chromosomes, then use a nanofilter to enrich single molecules for imaging and counting. This allows for identification of aneuploidy without the need for DNA amplification, microarrays or sequencing. The authors developed

probes that were designed to capture and label rolling circle replication products (RCPs) from 3500 loci on chromosome 21, as well as an optically transparent nanofilter 96-well plate to capture the RCPs. Four enzymatic processes result in labelled RCPs. First, the target chromosomes are broken up into cfDNA targets, and then the fragments are mixed with, and hybridized to, a complementary probe set that allows for chromosome specific labelling. The probes are designed so that when perfect hybridization occurs, a circle is formed. Next, exonucleases are used to remove all remaining linear DNA and unused probes. In the final step, primers are added and DNA circles are combined with polymerases so the circles are copied to a clonal concatenated RCP. Each original cfDNA target fragment generates one clonally amplified RCP that collapses in solution into a sub-micron sized DNA bundle. The RCPs are labelled with fluorescent nucleotides, and then added to the nanfilter-96 well plate. The labelled RCPs are imaged through the bottom of the plate using the Vanadis plate scanner, which quantifies the labelled RCPs. The authors used data from a known dataset of 10, 698 pregnancies from gestational weeks 10–14 to determine the precision of the assay to identify the different fetal fraction levels to eliminate to achieve the maximal detection rate and low false positive rate. The specificity of probe capture was determined using cell lines and was >99%, and reference samples with pre-determined amounts of trisomy DNA were used to identify the fetal fraction measurable. Fetal fraction at 4% or higher were clearly identified. Proof of concept on clinical samples was performed on a blinded set of 17 trisomy 21 samples in a cohort of 165 normal samples. Using an age adjusted risk cutoff of 1% risk, all affected and normal samples correctly identified. A prospective, high risk singleton pregnancy sample cohort of 104 women was also tested. All patients were examined by ultrasound to record the gestational age by measurement of the crown-rump length, to diagnose major fetal abnormalities and to measure NT thickness. Maternal serum levels of pregnancy associated plasma protein (PAPP)-A and free β-chorionic gonadotropin (hCG) were determined. Thirteen trisomy 21 pregnancies were correctly identified, and no false positives were found.

Sex Chromosome Aneuploidies

Sex chromosome aneuploidies (SCA) have a prevalence of about 1 in 460 in pregnancies undergoing amniocentesis, making them as common as autosomal aneuploidies in the average risk population. NIPT has limitations in identifying SCA because of placental mosaicism, fetal mosaicism, and maternal X chromosome variations. Ramadaney et al. (2018) retrospectively examined a cohort of 136 women with singleton pregnancies who underwent NIPT and received positive results for a SCA. The testing occurred between January 2013 to September 2017. All women had post-test genetic counseling in which the PPV of the test, its limitations, and clinical history of the associated SCA was reviewed. All patients were offered invasive prenatal diagnosis, and the benefits, risks and limitations were reviewed, and all patients had an ultrasound at the time of genetic counseling or when diagnostic testing was performed. The median patient age was 32. Seventy-three (54%) patients had NIPT positive for 45,X. Two pregnancies were fetal demises at the time of consultation, and confirmational testing was not pursued on the products of conception. Twenty five (35%) had ultrasound findings consistent with 45,X. Two patients were lost to follow up, four decided to have prenatal diagnosis, and one of these had normal karyotype results. The remaining 19 patients with ultrasound findings declined testing. One terminated her pregnancy, eight later had a fetal demise. Ten patients are thought to have had live births, but six were lost to follow up. Four had post-natal confirmational testing that confirmed X abnormalities, including one baby with a 7;X translocation. Amongst the 46 pregnancies positive for 45,X and no ultrasound abnormalities, 24 chose prenatal diagnosis. Twenty-one had normal results, and the three abnormalities were mosaic. The other 62 patients had positive NIPT results for 47, XXY (n=27), 47,XXX (n = 22), 47,XYY (n = 13), and one other (48,XXYY). Fifteen women chose prenatal diagnosis, and 11 had confirmed abnormal karyotypes. In the cohort that declined invasive testing, four had ultrasound abnormalities, including one fetus with clubfoot and ambiguous genitalia. Post-natal testing identified this baby had 48, XXYY. Post-natal testing was recorded in another 13 patients. Nine of the 14 post-natal tests were consistent with NIPT results. Overall, the majority of patients declined prenatal diagnosis (65%) even when ultrasound abnormalities were present. In the 64 women with outcome information available, the NIPT result was confirmed in 30 (47%). The authors concluded that comprehensive pre- and post-test counseling is recommended, and that practice guidelines need to address provider responsibilities for post-natal testing when a positive NIPT for SCA is identified.

Hayes evaluated the clinical utility of cfDNA screening for fetal sex chromosome aneuploidy in women with singleton or multiple gestation pregnancies. The overall quality of evidence was very low. Current clinical utility studies demonstrate a wide variability in the impact of cfDNA sex chromosome aneuploidy screening in decision-making outcomes. Study limitations include varied and small patient populations with few women with abnormal cfDNA screening results for individual sex chromosome aneuploidies, conflicting results or information that limited available data, confusing results presentation and limited patient follow-up. Additional studies with large numbers of women with abnormal cfDNA sex chromosome aneuploidy screening are needed. No studies were identified evaluating cfDNA screening for sex chromosome aneuploidy in multiple gestation pregnancies (Hayes, 2017b).

Bianchi et al. (2015) reviewed the experience of a commercial laboratory testing fetal sex chromosomes in 18,161 samples undergoing NIPT analysis. The average maternal age was 35, and the average gestational age was 14 weeks. Genome-wide massively parallel sequencing of cell-free DNA isolated from maternal plasma was performed and sex chromosome results were classified into six categories: XX, XY, monosomy X, XXX, XXY, and XYY based on the normalized chromosome values obtained for both X and Y. Results positive for sex anueploidy were phoned to the

ordering providers office, and outcome data was requested by phone, and subsequently by fax. Fetal sex was reported as XY (9,236) or XX (8,721) in 17,957 cases without sex aneuploidy. Thirty two cases were reported to be discordant. In four of these cases, a biological explanation was found; two cases had documented co-twin fetal demise, one mother had a kidney transplant from a male donor, and one fetus had ambiguous genitalia. There were 204 cases of sex chromosome aneuploidy (SCA) reported. These included monosomy X (n=148) followed by XXX (n=38), XXY (n=12), and XYY (n=6). Outcome information was available in 61 cases. In 44 cases of monosomy X with outcome data, 35 were false positives. In 13 cases of XXX with outcome data, 12 were false positive. In two cases of XXY with outcome data, no false positives were reported. In two cases with outcome data for XYY, one fetus was concordant but the other had a genotype of XXY. Because outcome data was not available for all cases, the true false positive rate for SCA cannot be established but ranges from 0.26% and 1.05%. The authors conclude that NIPT results for fetal sex and SCA can be confounded by maternal variables. Maternal history, ultrasound data, fetal and maternal chromosome analysis are important variable to include in the resolution of NIPT sex chromosome results.

Other Aneuploidies

Wan et al. (2018) studied the clinical utility of expanded NIPT screening in 15,362 pregnancies to identify autosomal aneuploidies other than trisomies 13, 18, and 21. The study is a retrospective review of a cohort of NIPT cases identified at a single prenatal care center, Guangzhou Women and Children's Medical Centre in China, from the beginning of the service in February 2015 to January 2018. Pregnancies were > than 12 weeks gestation. NIPT was performed using single-end sequencing by whole genome sequencing of plasma DNA. Routine karyotyping and microarray analysis were provided to patients who chose invasive testing. Overall, 59 aneuploidies other than Trisomies 13, 18 or 21 were identified, with a screen positive rate of 0.38%. The average age was 33, and the average gestational age was 15 weeks. The results included Trisomy 7 (n=18), Trisomy 22 (n=6), Trisomy 8 and 16 (n=5). There were four monosomies for chromosome 14, and two for chromosome 16. Thirty-six women had invasive prenatal diagnosis, and the remaining had follow up confirmation at a later time. The majority of cases were found to be false positives, with only three cases of 59 having confirmed aneuploidy in later testing. One Trisomy 7 was found to be a fetal mosaic, and after extensive counseling, the parents chose to terminate the pregnancy. One case involving trisomy 2 was verified with microarray analysis demonstrating chromosome 2 uniparental disomy, and loss of heterozygosity of 7q11.23 was identified as the cause of one NIPT Trisomy 7 finding. Both pregnancies had normal outcomes. Two pregnancies associated with a chromosome 16 aneuploidy result by NIPT ended with fetal demise. The authors hypothesized that the discordant NIPT results could be from confined placental mosaicism. They concluded that autosomal aneuploidies other than Trisomies 13, 18, and 21 are often false positives and associated with good pregnancy outcomes, and invasive testing should be used with caution.

Scott et al. (2018) reported on the results of a prospective NIPT study using the Illumina sequencing platform to assess aneuploidy for all chromosomes. From March 2015 to August 2017 samples were collected from participating prenatal clinics on 23,388 enrolled women with singleton pregnancies who were at least 10 weeks pregnant. All women had pre-test counseling, an ultrasound and then blood was collected. Patients were excluded if ultrasound revealed any fetal abnormality, gestation was under 10 weeks, or there was insufficient sample. One third of patients also had extra blood collected to concurrently measure the first trimester screening markers; pregnancy-associated plasma protein-A (PAPP-A), free β human chorionic gonadotrophin, and placental growth factor (PlGF). The average maternal age was 35, and 93% of samples were collected in the first trimester. Genome-wide MPS of cfDNA was performed using Illumina Next-Seq 500/550 sequencers according to the Illumina TruSeq Nano 16 sample protocol. Normal chromosome values (NCV) were calculated for 13, 18, 21, X and Y per the usual NIPT protocol, and in addition, NCV was determined for the remaining 22 autosomes. NIPT results identified 186 pregnancies at risk for trisomy 21, 53 at risk for trisomy 18, and 38 for trisomy 13. There were 114 pregnancies at risk for a SCA. However, the focus of this study was the outcomes for the remaining autosome results. Twenty-eight pregnancies were identified as high risk for a rare autosomal trisomy (RAT). The most common result was trisomy 7 (n = 6), followed by trisomy 16 (n = 4) and trisomy 22 (n = 3). Six pregnancies resulted in miscarriage. Two were found to have true fetal mosaicism. Nine fetuses had intrauterine growth retardation (IUGR), and six had ultrasound anomalies. In all cases of IUGR where first trimester marker results were also available, the PAPP-A levels were below the second percentile, and the PlGF levels were also reduced. Five of eighteen live births were premature. Four pregnancies were terminated. Four fetuses had structural anomalies despite a normal chromosome microarray on amniocentesis, causing the authors to hypothesize that the RAT had an effect on organ system development yet was not detectable through the amniocentesis. Overall, only nine of the 28 cases (32%) were full term, live born, and phenotypically normal. Because outcomes were only collected on the 28 RAT cases, the authors were not able to determine sensitivity or specificity.

GENOMA Laboratory in Rome reported on their experience of offering genome-wide NIPT in a general population of pregnant women in Fiorentino, et al. (2018). Their methodology relies on massively parallel sequencing (MPS) of cffDNA, followed by bioinformatic analysis for the common trisomies, and then a subsequent bioinformatic analysis for rare trisomies and segemental genomic imbalances. Samples were excluded if the fetal fraction was <2%, or the assay failed. From December 2015 through May 2016, testing was offered to an unselected consecutive series of pregnant women seeking NIPT for common trisomies. Only singleton pregnancies qualified, and the gestational age was at least 10 weeks. Indication for testing included advanced maternal age, positive maternal serum screening,

prior pregnancy with a trisomy, patients <age 35 who wanted screening, or fetal anomaly found on ultrasound. All patients were followed to determine outcome. Those with positive NIPT results had confirmational testing by CGH or karyotype. Normal results were confirmed by newborn physical or genetic testing. Overall, there were 12,114 reportable results. One hundred and sixty-six were positive for a common trisomy, including sex chromosome aneuploidies, and 151 were confirmed as true positives. The cases classified as false positive included one trisomy 21, one trisomy 18, one trisomy 13, ten monosomy X, one XXX, and one XXY. The authors concluded that the sensitivity was 100% and the specificity was 99% for the common aneuploidies. For rare trisomies and genomic imbalances, there were 30 pregnancies with a positive result. Rare trisomies were reported for 17 samples and confirmed in ten, three of which were low level mosaicisms and seven resulted in spontaneous miscarriage. One pregnancy was positive for trisomy 15, and invasive testing revealed a diploid 15 with uniparental disomy of chromosome 15 because of a rare trisomy rescue. Thirteen pregnancies had subchromosomal imbalances that was confirmed in eight cases. In two cases, the fetus was found to have an unbalanced chromosome translocation, inherited from a parent that was unaware they had a balanced translocation. The authors concluded that genome wide screening detected 12 viable pregnancies with clinically relevant abnormalities that would have been missed using standard NIPT screening.

Van Opstal et al. (2018) reported on the presence of rare trisomies and other abnormalities found by the Trial by Dutch laboratories for Evaluation of Noninvasive Prenatal Testing (TRIDENT) study. The TRIDENT study was a trial where NIPT was offered as an alternative to pregnant women considering invasive prenatal diagnosis between April 2014 and April 2015. NIPT testing was performed using whole-genome shallow massively parallel shotgun sequencing. Of 3306 enrolled cases, 753 were analyzed only for chromosomes 21, 13 and 18. All others, 2553, were analyzed for all chromosomes and for segmental subchromosomal abnormalities. Results were reported in 2527 cases. In 78, a common trisomy was found, and follow up information was reported elsewhere. Forty-one cases of another type of chromosome abnormality were identified. One case of reported trisomy 8 was terminated at a private clinic before any follow up was available. In the remaining 40 cases, ten were confirmed to be true positives. These included two cases of trisomy 9, which were confirmed to be mosaic in the fetus. Both resulted in live births with multiple congenital anomalies. One case of a dual trisomy 15 and trisomy 22 was reported, and fetal tissue confirmed a mosaic trisomy 15. The pregnancy resulted in a live birth with no identifiable anomalies. One trisomy 22 was identified, confirmed as a mosaic trisomy 22. The pregnancy had multiple anomalies and was terminated. Six of the 10 cases were genomic imbalances that included dup 2p, del 6q, del80/dup8q, del 9p, del 12q, and del 18p. All were confirmed through amniocentesis. In 22 of the 40 positive cases, placental testing confirmed that confined placental mosaicism was the likely cause of the NIPT results, and in this group there were ten infants with some impact, ranging from small for gestational age to multiple congenital anomalies. The authors conclude that genome wide screening for NIPT results in identification of chromosomal aberrations other than trisomy 21, 18 or 13 in about one-third of screen positive results, and this information is important for pregnancy management.

Rare trisomies were the focus of the analysis of Pertile, et al. (2017). Two cohorts of patients were studied. The first was 72,932 subjects derived from 84,945 continuous NIPT tests performed between October 2013 and September 2014 in the Illumina Northern California Clinical Services laboratory. The average maternal age was 35, and the average gestational age was 12 weeks. Cohort 2 was independently derived from 16,885 continuous NIPT tests performed between April 2015 and August 2016 in the Australian Victorian Clinical Genetics Services laboratory. The average maternal age was 34, and the average gestational age was 10 weeks. Both labs used a similar, but not identical, whole genome sequencing methodology and a similar quality control parameter, normalized chromosome denominator quality. Cohort 1 had 328 abnormal results related to rare trisomies dysploidy, or copy number variants. Cohort 2 had 71 abnormal results, and rare trisomies represented 60 of these cases. Outcome data was available in 52 patients and included miscarriage, true fetal mosaicism, and confirmed or suspected uniparental disomy. Seventeen cases had amniocentesis and seven resulted in normal karyotypes. The clinical utility of a whole-chromosome analysis is still not entirely clear, but this study strongly suggests that a full analysis of all chromosomes may have clinical relevance, particularly with respect to TFM and pathogenic UPD, and in monitoring for an increased risk of IUGR in ongoing pregnancies. The authors concluded that the presence of a rare trisomy, particularly at a proportion similar to the fetal fraction, was associated with serious pregnancy complications, and patients should be given the option to learn this information. However, further research is needed to determine the full clinical utility of reporting autosomal aneuploidy for any chromosome.

Microdeletions and Copy Number Variants

DiGeorge syndrome, also known as velocardiofacial syndrome or 22q11 deletion syndrome, is one of the most common microdeletion syndromes with an incidence of 1 in 3,000-6,000 births. Affected individuals have a wide array of clinical manifestations, including congenital heart defects, immune dysfunction, hypocalcemia, mild-to-severe learning disabilities, and an increased risk of mental health disorders. Ravi et al. (2018) reported on the clinical validity of using a SNP based NIPT assay to detect fetal 22q11.2 deletions during pregnancy. Women from six prenatal centers were enrolled in the study, and were undergoing invasive prenatal diagnosis for a variety of reasons. At the time of blood draw, information about gestational age, maternal age and weight, and time between the invasive procedure and blood draw were collected. Samples from patients that were <9 weeks gestation, had a fetal demise, had atypical 22q distal deletions on invasive testing, or equivocal invasive test results were excluded. Patients with

inconclusive or no call NIPT results were excluded and no redraws were requested. The study was internally blinded, but ultimately included ten patients with confirmed fetal 22q11.2 deletions and 390 with unaffected pregnancies. The mean age was 28, and the gestational age averaged 21 weeks for affected pregnancies and 12.8 weeks for unaffected pregnancies. Samples were tested at Natera using a massively multiplexed PCR (mmPCR) amplification targeting SNPs covering chromosomes 13, 18, 21, 22, X, and Y. The target set contained 13,926 distinct genetic loci, including 1,351 SNPs spanning a 2.91 Mb section of the 22q11.2 region that constitutes ~87% of all deletions detected in individuals with the 22q11.2 deletion syndrome. Risk status for the 22q11.2 deletion was assigned as high or low risk, or risk unchanged/no call. High-risk calls with maternally deleted haplotypes were sequenced at a higher depth of read to confirm high-risk status. For cases with a fetal fraction of 2.8–6.5%, the sample was evaluated only for the presence or absence of the paternally-inherited haplotype. Of the ten affected pregnancies, nine were identified as test positive, or high risk. Of the 390 unaffected samples, one false positive was found. Overall the study found the sensitivity to be 90%, the specificity to be 99.7%, and based on a prevalence of 22q11.2 deletions of 1 in 1,442 in pregnancy, the estimated positive predictive value (PPV) is 19.6%.

Schwartz et al. (2018) reported on a cytogenetic laboratory's experience with the follow up of NIPT results positive for microdeletions. Three hundred and forty-nine patients were positive by NIPT for microdeletions 1p, 4p, 5p, 15q, or 22q and had an invasive diagnostic test with chromosome microarray analysis as a follow up. Thirty two (9.2%) patients had a microdeletion confirmed. Of those, 39% of the cases had additional abnormal microarray findings. Unrelated abnormal microarray findings were detected in 11.8% of the patients tested. This cohort had stretches of homozygosity in the microdeletion region which could be an explanation for false positive NIPT results. In this study the NIPT positive predictive value of the NIPT microdeletion test was 9.2%.

Petersen et al. (2017) similarly reported on a commercial cytogenetic laboratory's experience with the follow up of NIPT results positive for aneuploidies and microdeletions. In this cohort of 712 patients, 492 were referred due to being high risk for trisomies 21, 13, and 18. One hundred and thirty-eight were positive for SCA, 52 microdeletions, and 4 were positive for large genomic deletions/duplications. Confirmatory diagnostic testing was performed either prenatally through CVS or amniocentesis, or postnatally on infant blood. Tests included fluorescence in situ hybridization (FISH), karyotype, and chromosomal microarray analysis (CMA). Trisomy 13 was confirmed in 34 of 76 screen positives, and trisomy 18 was confirmed in 82 of 106 screen positives. There were 268 screen positives for trisomy 21, of which 228 were confirmed. The positive predictive values for trisomy 13, 18, and 21 were consistent with previous reports at 45%, 76%, and 84%. Monosomy X was confirmed in 24 of 89 cases, 17 of 20 XXY cases were confirmed, and 4 of 4 XYY cases were confirmed. Detection of sex chromosomal aneuploidies had positive predictive values of 26% for monosomy X, 50% for 47,XXX, and 86% for 47,XXY. Rare autosomal trisomies were screen positive in 12 cases, and confirmed only in 1. For microdeletion syndromes, there were ten screen positive for Prader Willi syndrome, and no confirmed cases. DiGeorge syndrome had 28 positives and six confirmations, del1p36 had seven screen positive cases and one confirmation, and there were seven screen positives for Cri du Chat syndrome, and no confirmed cases. The positive predictive values ranged from 0% for detection of Cri-du-Chat syndrome and Prader-Willi/ Angelman syndrome to 14% for 1p36 deletion syndrome and 21% for 22q11.2 deletion syndrome. Four cases were screen positive for large deletions or duplications, and two cases were confirmed. The authors concluded that the positive predictive values were aligned with the literature for common aneuploidies, but note that continued monitoring of its performance after introduction into clinical practice is necessary to fully establish its clinical utility. The need to provide ongoing education to clinicians and patients on the limitations of NIPT testing was highlighted.

Martin et al. (2018) reported on the experience of a commercial laboratory using SNP based technology to identify five microdeletion syndromes. The study period was February 2014 to February 2015, and for each maternal blood sample, the maternal age, maternal weight, gestational age, reason for testing, and informed consent were collected. Two cohorts were used; the first was 42,326 samples which had screening for all five microdeletions, and another cohort of 21,948 samples that only had 22q11 deletion analysis. The SNP was designed to cover 672 SNPs within a 2.91 Mb section of the 22q11.2 region that is deleted in approximately 87% of individuals diagnosed with 22q11.2 deletion syndrome, and 1152 SNPs in each of the following: a 10 Mb region deleted in approximately 60% of patients diagnosed with 1p36 deletion syndrome, a 20 Mb region deleted in approximately 65% of patients diagnosed with cri-du-chat syndrome, and a 5.85 Mb region deleted in approximately 28% of patients diagnosed with Prader-Willi/Angelman syndromes. Results were calculated to be high risk, low risk, or risk unchanged based on the disease prevalence, SNP data, and whether one or two alleles were successfully analyzed. Follow up was requested from ordering providers via phone or email, and if outreach during the pregnancy was not successful, at least two more attempts were made after the estimated delivery date. Of the 80,449 samples received for microdeletion testing, 5511 samples did not meet criteria for inclusion. For the 22q11 deletion syndrome, there were 283 high risk cases, 2808 risk unchanged results, and 71,841 low risk results. Follow up was available for 153 high risk cases, and 24 (8.5%) were confirmed true positive and 129 (45.6%) were false positive. The remaining 4 microdeletion syndromes collectively had 215 high risk cases. Seven were true positive, 117 were false positive, and 91 had no follow up available. A revised risk calculation protocol for determining high risk was developed and applied to 268 of the original 283 high risk cases, and 92 of the original false positive cases were reclassified to low risk, and one true positive was

also reclassified to low risk. The positive predictive value of the original test was 15.7% for 22q11.2 deletion syndrome, and 5.2% for the other four disorders combined. With the revised protocol, these values increased to 44.2% for 22q11.2 and 31.7% for the others.

Lo et al. (2016) developed a segmentational algorithm in their NIPT bioinformatics calling pipeline to identify subchromosomal abnormalities. Maternal blood samples were collected from women undergoing invasive procedures for clinical indications in 40 maternity clinics around the UK as part of the RAPID (Rapid Accurate Prenatal Noninvasive Diagnosis) project, and a subset of samples with known outcomes were selected for use in a proof of concept study. This included 31 test samples with known unbalanced chromosome rearrangements and 534 samples with known normal chromosomes by karotype or fluorescent in situ hybridization (FISH). After adjusting the algorithms and using a deeper read depth, 29 of 31 subchromosal abnormalities were correctly identified. In the 534 normal samples, there were 2 false positive results. The authors noted that when using the standard read depth utilized in NIPT testing, only rearrangements >6Mb could be found, and few <6Mb unless maternally inherited. They concluded that because standard NIPT can only detect the larger chromosomal rearrangements and requires knowledge of fetal fraction, it is not yet ready for routine clinical implementation.

Gross et al. (2016) evaluated the ability of a SNP based NIPT test to detect 22q11 deletion syndrome in a commercial lab. A retrospective analysis was performed for 21,948 consecutive samples for fetal aneuploidy and microdeletion screening received over a 6-month period from February to August 2014. Demographic information received included indication for testing, gestational age, maternal date of birth, maternal weight, and whether the mother was a known microdeletion carrier. A paternal sample was requested but not required, and 5,912 (26.9%) cases included a paternal sample. Prior to analysis of 22q11.2, the standard panel testing for aneuploidy at chromosomes 13, 18, 21, X and Y was conducted; samples that failed quality control at this step were not evaluated for 22q11.2. This region was analyzed by 672 SNPs targeting the 2.91-megabase (Mb) loci associated with the 22q11.2 deletion syndrome. Fetal results were predicted based on the pattern of SNPs, fetal fraction and paternal results when available. Ninety-five cases were reported as high risk. Of these, 84 had some outcome data available. Invasive testing was performed in 48 cases, and 11 had post-natal testing, and testing was declined by the remaining patients. Of those with follow up diagnostic testing, 11 were true positives, and 50 were false positives. Seventy seven high risk patients had ultrasound data available, and 26 had anomalies observable on ultrasound, of which nine were true positives. There were three pregnancy terminations related to screening results of 22q11.2 deletion, two of which were confirmed as true positive. The authors conclude that the availability of genetic counseling and other resources to manage high risk 22q11.2 cases is an important aspect of this screening test.

While individually rare, subchromosomal abnormalities occur in 1.6% of pregnancies. Helgeson et al. (2015) reported on the development of an algorithm to be applied to cell-free fetal DNA testing to support identifying 5pdel, 22q11del, 15qdel, 1p36del, 4pdel, 11qdel, and 8qdel in routine testing. Low coverage whole genome massively parallel sequencing was used to analyze cell-free fetal DNA, and used a statistical method to search for consistently under-represented regions followed by a decision tree to differentiate whole-chromosome events from regional deletions. A cohort of 175,393 high risk pregnancies was used to test the algorithm. Samples were collected from October 2013 to October 2014. Fifty five cases were screen positive for subchromosomal events. Outcome data was available for 53 cases. Chromosome microarray or FISH confirmed the findings in 41 (77%) cases. Nine cases did not have confirmational testing, but had clinical features on ultrasound consistent with the deletion. Three cases were false positives. The false negative rate and sensitivity were not conclusively determined.

Wapner et al. (2015) estimated the performance of a SNP-based NIPT for five microdeletion syndromes. After validation of the SNP-targeted assay, a cohort of 469 test samples was evaluated. Detection rates were 97.8% for a 22q11.2 deletion (45/46) and 100% for Prader-Willi (15/15), Angelman (21/21), 1p36 deletion (1/1), and cri-du-chat syndromes (24/24). False-positive rates were 0.76% for 22q11.2 deletion syndrome (3/397) and 0.24% for cri-du-chat syndrome (1/419). No false positives occurred for Prader-Willi (0/428), Angelman (0/442), or 1p36 deletion syndromes (0/422). Further validation studies are needed to determine the sensitivity and specificity of this screening test for microdeletion syndromes.

Zhao et al. (2015) reported on the development of a novel approach to identifying microdeletions in NIPT. The team used shallow whole genome sequencing followed by a rigorous statistical approach, applying a circular binary segmentation (CBS) method, to detect consistently increased or decreased regions in the normalized data. Additionally the log odds ratio was calculated to determine the likelihood of a microdeletion event being true at the measured fetal fraction. The methodology was tested on 183 samples with known outcomes derived from amniocentesis, of which NIPT results were obtained on 178 samples. Of these, 16 were known to have microdeletions and 162 were normal. The average fetal fraction was 9.2%. The test identified 13 of the 16 affected samples. Of the three false negatives, one was anticipated to be a trisomy 8. Further analysis of this case found that the sample originated from a low level mosaicism case and the sample was exempted from further testing. The second false negative did identify an abnormality that was reported to be a maternal, rather than fetal event. Both the mother and fetus had the same 4q34 deletion. The third false negative is likely due to a low fetal fraction of 4.8%. In the 162

samples predicted to be normal, five positive results were found. Only the amniocentesis results were available for concordance, no other fetal tissue or information, so to further assess these five potential false positives, deeper sequencing was performed on the samples. One sample was reclassified as normal, but the other four had the same positive result. The authors concluded that their algorithm correctly identified 17 of 18 cases with microdeletions and 156 of 157 unaffected cases, and the limitations of the approach include fetal fraction, microdeletion size, and the variability in the even region.

Fetal Fraction

The proportion of fetal cell-free DNA is called the Fetal-Fraction (FF), and is an important aspect of NIPT testing. If FF is too low, an NIPT result cannot be accurately determined. FF can be impacted by maternal weight, gestational age, and fetal aneuploidy. The lower limit of fetal cell-free DNA is 4%. Repeating an NIPT due to low fetal fraction is controversial, as before 20 weeks of gestation, the fetal fraction increases < 0.1% per week. (Gregg et al., 2016).

McKanna et al. (2019) evaluated the role of low FF in identifying pregnancies at increased risk for trisomies 13 and 18, as well as triploidy. The authors used data from >165,000 singleton pregnancies to construct an algorithm (FFBR) using maternal weight, gestational age, and FF distributions from normal and affected pregnancies. The algorithm was validated on a blinded set of SNP-based NIPT results from 1148 cases that had no-call results, as well as outcome data available. The average age of the cohort was 34, the average weight was 208 pounds, the average gestational age was 12.3 weeks, and the average FF was 3.1%. The primary reason for referral were advanced maternal age (55%), routine screening for average risk women (33%), abnormal maternal serum screen (4.2%), abnormal ultrasound (3.7%), or a positive family history (3.2%). FFBR scores were calculated for the cohort, and 49% (564/1148) had a high score associated with a $\geq$ risk of trisomies 13, 18, or triploidy. The rate of scores differed between referral groups as follows: advanced maternal age, 63.7%; maternal serum screen positive, 41.7%; abnormal ultrasound, 39.5%; family history, 40.5%; and the average risk group, 28.3%. Based on the characteristics of the high FFBR score cohort, the expected rate of trisomy 13, trisomy 18 and triploidy was expected to be 0.7%, however the observed rate in this group was significantly higher at 5.7%. In the low FFBR score cohort, the incidence was not significantly different at 0.5% than was expected (0.2%). Unexplained pregnancy loss was also higher in the high FFBR cohort at 14.7%, compared to what was expected of 10.4%. In this cohort, the positive predictive value of the high FFBR score was 5.7% with a sensitivity of 91.4% for trisomy 13, trisomy 18 and triploidy.

To determine if a redraw is useful when no result is obtained on an initial NIPT test, Benn et al. (2018) performed a retrospective review of samples referred to Natera laboratories between January 2016 and October 2016 that were considered to be 'no result.' The lab uses a SNP based approach to NIPT analysis, and classifies tests as 'no result' when the fetal fraction is less than 2.8%, or the fetal fraction was less than 7% and SNP patterns could not be interpreted with a high degree of confidence. Of the 242,607 samples received in this time period, there were 8,605 cases that did not receive a result. Of these, 3,355 redraw samples were received. Cases that had no result because of inadequate sampling, because the test was cancelled, or for certain findings, such as large regions of homozygosity, were excluded from review. Cases that met the criteria for a redraw due to low fetal fraction or low confidence in the SNP pattern were analyzed and included 2,959 samples. A result was obtained in 1,861 (62.9%). Fetal fraction was generally higher at the time of redraw, which was, on average, 14 days after the first sample. When the initial fetal fraction was 1.5-2%, the informative redraw rate was 27.8%. If it was >4%, the informative redraw rate was 86.5%. The authors also looked at maternal weight as a factor in a successful redraw, and noted that the informative redraw rate for women <180 pounds was 73%, and for women >240 pounds was 47.7%. Regardless of maternal weight, the initial fetal fraction percent was the most informative for determining redraw success. The authors highlight American College of Obstetricians and Gynecologists states that women whose initial test results are not reported should receive further genetic counseling and be offered comprehensive ultrasound evaluation and diagnostic testing because of an increased risk of aneuploidy, but does not preclude repeat NIPT as an option. In contrast, the American College of Medical Genetics and Genomics states that a repeat blood draw is not appropriate and diagnostic testing should be offered. Benn et al. (2018) conclude that their data provides the option of using gestation age, maternal weight, and initial fetal fraction to further inform women about the possibility of an informative redraw when considering repeating NIPT, doing maternal serum screening, or having an invasive test.

Placental mosaicism is the primary cause of false positive results in NIPT analysis. Brison et al. (2018) reported on their efforts to develop a method to detect placental chromosomal mosaicism using cell-free fetal DNA by combining FF with genome wide aneuploidy detection. A consecutive non-selected series of 19,735 pregnant women who were at least 10 weeks gestation, were tested for common fetal aneuploidies by NIPT. The categories of pregnant women included average risk (61.7%), advanced maternal age (over 36 years of age) (24.2%), increased risk for fetal aneuploidy as indicated by first trimester screening (12.2%), familial history of congenital or hereditary diseases (1.8%), or NIPT following pre-implantation genetic diagnosis and in vitro fertilization (0.1%). A retrospective analysis of the cohort for placental moscaicism was conducted by collecting genome wide read counts that were normalized, GC corrected and aggregated per chromosome to produce a chromosomal read count (CRC). Calls were determined for all chromosomes for trisomy, monosomy, borderline aneuploidies, fetal sex and maternal copy number variants. Samples with undetermined fetal sex or poor quality were excluded. FF was estimated using the SeqFFA formula.

Placental mosaicism was predicted using an algorithm incorporating the mean CRC, standard deviation CRC, with the FF. Respectively 3.2% (5/154), 12.8% (5/39), and 13.3% (2/15) of trisomies 21, 18, and 13 were predicted and confirmed to be mosaic. The incidence of rare autosomal trisomies was ~0.3% (58/19,735), 45 of which were predicted to be mosaic. Twin pregnancies with discordant fetal genotypes were predicted and confirmed.

Hudecova et al. (2014) investigated if differences in fetal DNA fractions existed between different pregnancy risk groups, as FF is an important factor in determining the reliability of the NIPT analysis. Seven hundred and twenty six pregnant women were consecutively recruited from the Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, between November 2011 and January 2013. The inclusion criteria were male singleton pregnancies with recorded data for risk group stratification. The high risk (HR) group was defined as those pregnancies with a risk of trisomy 21 was greater than 1 in 250, the intermediate risk (IR) group had a with risk between 1 in 250 to 1 in 1000, and the low risk group (LR) had a risk of trisomy 21 lower than 1 in 1000. In total 337 cases met criteria. Statistical analysis found that a minimum of 127 cases were needed for analysis, so the authors selected 138 LR cases and all HR and IR for study, totalling 195 pregnancies. Fetal DNA fractions were compared between the three fetal aneuploidy risk groups (HR, IR, LR) and assessed for correlation with the FTS test parameters; gestational age, crown-rump length (CRL), free b-subunit of human chorionic gonadotropin (free bhCG), nuchal translucency (NT), pregnancy-associated plasma protein A (PAPP-A) as well as maternal weight. The authors found no statistically significant difference in fetal DNA fractions across the high, intermediate and low risk groups. FF showed a strong negative correlation with maternal weight, and weak but significant correlations with gestational age, crown-rump length, multiple of medians of free b-subunit of human chorionic gonadotropin and pregnancy-associated plasma protein A. The researchers conclude that the aneuploidy screening by NIPT is likely to offer similar analytical reliability without respect to the a priori fetal aneuploidy risk because of similar fetal DNA fractions in maternal plasma between high, intermediate and low risk pregnancies in the general population.

RhD Genotyping

Saramago et al. (2018) conducted a health technology assessment of the use of cell-free cffDNA to determine fetal RhD status. The authors searched MEDLINE and other databases, from inception to February 2016, for studies of high-throughput NIPT free-cell fetal deoxyribonucleic acid (DNA) tests of maternal plasma to determine fetal RhD status in RhD-negative pregnant women who were not known to be sensitised to the RhD antigen. Inclusion criteria for all reviews included pregnant women who were RhD negative and not known to be sensitized to the RhD antigen. For examining diagnostic accuracy, inclusion criteria was prospective cohort studies reporting absolute numbers, and for clinical effectiveness, studies that used high throughput NIPT in which anti-D prophylaxis was given and clinical outcomes were reported. The inclusion criteria for implementation outcomes was any publication that reported issues related to the implementation of, or practical advice, relating to NIPT. Eight studies were included in the diagnostic accuracy review, seven studies were included in the clinical effectiveness review and 12 studies were included in the review of implementation. The meta-analysis found that women in the studies were at at least 11 weeks gestation or later, and mostly Caucasian with singleton pregnancies. The false negative rate (at risk of sensitization) was 0.34%, and the false positive rate (receiving unnecessary anti-D prophylaxis) was 3.86%. Clinical outcome data was limited to confirm the true sensitization rate, but there was no evidence of potential adverse effects. The authors concluded that there was limited data on clinical effectiveness of NIPT for fetal RhD status, and more studies were needed for non-Caucasians and multiple gestations.

Single Gene Disorders

Xiong et al. (2018) conducted a feasibility study on patients of South East Asian descent to determine if targeted sequencing and relative mutation doseage can be used to correctly identify maternal beta-thalassemia mutations in fetal cell-free DNA. Samples were collected from 49 couples at risk to have a child with beta-thalassemia, and genomic DNA was evaluated from the parents, cell-free fetal DNA and either amniocentesis or CVS. Common *HBB* mutations were targeted using nested PCR. Relative mutation doseage was used to determine if the fetus had the wild type allele or the maternal carrier allele. Forty-eight of the samples were able to be classified using cell-free fetal DNA (98%). The correct result was obtained in 44 of the 48 cases (91.7%), and there was one false positive and three false negatives. The overall sensitivity was 87.5% and the specificity was 95.8% for the inheritance of the maternal allele.

Byrou et al. (2018) presented their approach to developing a sensitive and reproducible assay for identifying paternally inherited beta-thalassemia genes in cell-free fetal DNA. Seventeen couples that were β-thalassaemia carriers at a risk for having a child with β-globin gene disorder participated, and were counseled on the 11th week of pregnancy about the study. Consent was obtained. Cell-free DNA was extracted from maternal plasma, and COLD PCR was initially used for amplication, which preferentially amplifies minor alleles that are present in mixtures with excess major alleles. A panel of 49 SNPs located on the beta-globin cluster on chromosome 11 and exhibiting high degree of heterozygosity (>6%) in the Cypriot population was used for *HBB* mutation detection. COLD PCR did not provide satisfactory results, so the authors modified the protocol to utilize temperature gradient (TG) COLD PCR. Using TG COLD PCR allowed for the correct identification of the paternal *HBB* alleles in the cell-free fetal DNA in all cases, which

was concordant with CVS analysis. The authors concluded that using TG COLD PCR can be a cost effective approach for all labs and these results are an encouraging step forward in developing a NIPT assay for beta-thalassemia.

Duchenne (DMD) and Becker (BMD) muscular dystrophy are X-linked disorders caused by mutations in the dystrophin gene, and DMD is found in 1 in 3500 male births. Most cases are caused by large deletions of the dystrophin gene (60-65%), small mutations (25-30%) and the rest by partial deletions (5-10%). Parks et al. (2016) reported on developing a test using cell-free fetal DNA to provide a screening tool for pregnant women. They developed an assay using massively parallel sequencing on an Illumina MiSeq by targeted capture enrichment of single nucleotide polymorphisms (SNPs) across the dystrophin gene, which then underwent relative haplotype dosage analysis. They recruited seven healthy pregnancies and two pregnant DMD carriers th NIPSIGEN study ('NIPSIGEN: clinical translation of NIPD for SGDs'; REC approval number: 13/NW/0580). Tissue from the healthy donor CVS sample and blood from the DMD carriers was used to make a reference haplotype. When the fetal fraction was >4%, the results from the NIPT test matched the fetal sample in all cases, including a dystrophin gene recombination event in one patient. The authors conclude that this initial validation study demonstrates that screening for DMD through NIPT can be accurate and reliable.

Studies to date show promised that screening for fetal single gene disorders holds promise, but more studies are needed to determine clinical validity and utility.

Twin Zygosity

Qu et al. (2013) studied the ability of MPSS to assess twin zygosity using cffDNA. Eight women with twin pregnancies were consented for the study. The sets included four dichorionic, diamniotic twins, three monochorionic diamniotic twins, and one monochorionic monoamniotic. Cord blood was collected separately from each twin at the time of delivery. Microarray genotyping was used to assess the cffDNA, and regions of DNA heterozygosity were compared within each sample. Little regional variation was expected for identical twins, while greater variation was expected for fraternal twins. This method was able to accurately identify the monozygotic and dizygotic twin pregnancies, and in dizygotic twins could determine the contribution of each twin to the fetal fraction.

Leung et al. (2013) utilized MPSS of eight twin pregnancies, six of which had euploid twins, and two of which were known to have one aneuploid twin, and eleven known euploid singleton pregnancies. Their goal was to assess the ability of cffDNA to provide an individualized assessment of trisomies in twins, as well as zygosity and fetal fraction. Here we report on the zygosity results. Zygosity was determined using an algorithm to analyze the ratio of fetal-specific allele to major allele for specific loci. For identical twins it is expected that the fetal fraction calculated would be the same across all loci, but for fraternal twins, the fetal fraction would vary across various loci. Using this information, the algorithm correctly identified four twin pregnancies as identical, and the other two twin pregnancies as fraternal. The authors concluded that knowing the zygosity not only contributes to better aneuploidy prediction in twin gestations for NIPT testing, but also has additional clinical utility since monozygotic pregnancies have a higher complication rate, and knowing this information early could help the clinician better monitor the pregnancy. In addition, the authors identified the need for more studies before this could be used clinically.

More studies are needed on the analytical validity and clinical utility of using cffDNA to determine twin zygosity.

Professional Societies

American College of Medical Genetics and Genomics (ACMG)

An ACMG position statement addresses the advantages and disadvantages of noninvasive prenatal screening for fetal aneuploidy and offers recommendations for pre- and post-test genetic counseling. The statement includes the following recommendations:

- Provide up-to-date, balanced and accurate information early in gestation to optimize patient decision making, independent of the screening approach used.
- Laboratories should work with public health officials, policymakers and private payers to make NIPT, including the pre- and post-test education and counseling, accessible to all pregnant women.
- Allow patients to select diagnostic or screening approaches for the detection of fetal aneuploidy and/or genomic changes that are consistent with their personal goals and preferences.
- Inform all pregnant women that diagnostic testing (CVS or amniocentesis) is an option for the detection of chromosome abnormalities and clinically significant copy-number variants.
- Inform all pregnant women that NIPT is the most sensitive screening option for traditionally screened aneuploidies (i.e., Patau, Edwards, and Down syndromes).
- Offer diagnostic testing for a no-call result due to low fetal fraction. Repeat testing is not appropriate.
- Offer aneuploidy screening other than NIPT in women with extreme obesity.
- Labs should always report fetal fraction results and report the reason for a no-call result.
- Do not offer NIPT for autosomal aneuploidies other than chromosomes 13, 18, and 21.
- Do not offer NIPT to screen for genome wide copy number variants.

ACMG emphasizes that all genetic screening has residual risk and there are pros and cons to any screening approach (Gregg et al, 2016).

American College of Obstetricians and Gynecologists (ACOG)/Society for Maternal-Fetal Medicine (SMFM)

ACOG and SMFM jointly published a practice bulletin on screening for fetal aneuploidy. The document states that no one screening test is superior to other screening tests in all test characteristics. Screening for aneuploidy should be an informed patient choice with shared decision making. The guidelines state that because cfDNA is a screening test with the potential for false-positive and false-negative test results, such testing should not be used as a substitute for diagnostic testing. Most published experience is based on studies conducted on high-risk populations. The sensitivity and specificity in the general obstetric population are similar to the levels published for the high-risk population. However, cfDNA screening cannot have the same accuracy in low-risk pregnancies because the positive predictive value is affected by the prevalence of the disorder in the population. The positive predictive value is lower in the general obstetric population because of the lower prevalence of aneuploidy in this population. All women with a positive cfDNA test result should have a diagnostic procedure before any irreversible action is taken. cfDNA screening tests for microdeletions have not been validated clinically and are not recommended at this time (ACOG, 2016).

In Committee Opinion 181, ACOG notes that while there is improved accuracy of noninvasive fetal RhD genotyping, comparisons with current routine prophylaxis of anti-D immunoglobulin at 28 weeks of gestation have not shown a consistent benefit, and noninvasive assessment of fetal RhD status is not recommended for routine use at this time (ACOG, 2017).

American Society of Human Genetics (ASHG)/European Society of Human Genetics (ESHG)

In a joint statement, ASHG and ESHG present different scenarios for NIPT-based screening for common autosomal aneuploidies. The statement suggests that trade-offs involved in these scenarios should be assessed in light of the aim of screening, the balance of benefits and burdens for pregnant women and their partners. The statement includes the following recommendations:

- NIPT offers improved accuracy when testing for common autosomal aneuploidies compared with existing tests such as combined first-trimester screening. However, a positive NIPT result should not be regarded as a final diagnosis: false positives occur for a variety of reasons. Women should be advised to have a positive result confirmed through diagnostic testing if they are considering a possible termination of pregnancy.
- Expanding NIPT-based prenatal screening to also report on sex chromosomal abnormalities and microdeletions is not recommended.

When the test is assessed in terms of its predictive value, the low prevalence of the relevant conditions in the target population must be taken into consideration. For instance, the PPV in a general risk population can result in a higher rate of false alarms because of the lower prevalence of aneuploidies in this population (Dondorp et al., 2015).

National Society of Genetic Counselors (NSGC)

An NSGC practice guideline (Wilson et al., 2013) provides a summary of screening and diagnostic testing options for chromosome aneuploidy. A decision tree and comparison tables are included to help providers select the test which best suits their patient's needs. The guideline also states that referral to a genetic counselor or other qualified provider may be appropriate if a patient could benefit from additional discussion prior to making a decision regarding screening and diagnostic testing options. NIPT is only recommended for patients from high-risk populations, including advanced maternal age, positive screening test, abnormal ultrasound suggestive of aneuploidy or prior pregnancy with chromosome aneuploidy. It is recommended that a positive NIPT be followed by confirmatory diagnostic testing prior to making pregnancy decisions.

In a separate position statement, the NSGC supports NIPT screening as an option for pregnant patients. Because cfDNA screening cannot definitively diagnose or rule out genetic conditions, qualified providers should communicate the benefits and limitations of cfDNA screening to patients prior to testing. Many factors influence cfDNA screening performance, therefore it may not be the most appropriate option for every pregnancy. Prior to undergoing cfDNA screening, patients should have the opportunity to meet with qualified prenatal care providers who can facilitate an individualized discussion of patients' values and needs, including the option to decline all screening or proceed directly to diagnostic testing. Clinicians with expertise in prenatal screening, such as genetic counselors, should provide post-test genetic counseling to patients with increased-risk screening results. Diagnostic testing should be offered to patients with increased-risk results to facilitate informed decision making. Released October 2016 (NSGC website).

Society for Maternal-Fetal Medicine (SMFM)

In a statement clarifying recommendations regarding cfDNA testing, SMFM asserted that it does not recommend that cfDNA aneuploidy screening be offered to all pregnant women. Limited data on the effectiveness and clinical utility for improving patient outcomes preclude a recommendation that cfDNA be actively offered to all pregnant women.

However, SMFM believes that the option should be available to women who request additional testing beyond what is currently recommended by professional societies (SMFM, 2015).

U.S. FOOD AND DRUG ADMINISTRATION (FDA)

Laboratories that perform DNA-based prenatal tests for trisomy 21, 18 and 13 are regulated by the FDA under the Clinical Laboratory Improvement Amendments. See the following web site for more information: http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/IVDRegulatoryAssistance/ucm124105.htm. (Accessed October 17, 2018)

Additional Product Information

- Harmony™ Prenatal Test (Ariosa® Diagnostics/Roche)
- MaterniT21® PLUS (LabCorp®/Sequenom Laboratories)
- Panorama™ Prenatal Test (Natera™ Inc.)
- QNatal® Advanced (Quest Diagnostics™)
- Vanadis™ NIPT Test (Vanadis Diagnostics)
- verifi® Prenatal Test ((Illumina®, Inc.)
- VisibiliT™ (LabCorp®/Sequenom Laboratories)

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GUIDELINE HISTORY/REVISION INFORMATION

Date	Action/Description
07/01/2019	**Template Update** • Added *Documentation Requirements* section
05/01/2019	• Updated coverage rationale; added language to clarify the listed services are unproven and not medically necessary *due to insufficient evidence of efficacy* • Updated supporting information to reflect the most current FDA information and references • Archived previous policy version MMG089.K

INSTRUCTIONS FOR USE

This Medical Management Guideline provides assistance in interpreting UnitedHealthcare standard benefit plans. When deciding coverage, the member specific benefit plan document must be referenced as the terms of the member specific benefit plan may differ from the standard benefit plan. In the event of a conflict, the member specific benefit plan document governs. Before using this guideline, please check the member specific benefit plan document and any applicable federal or state mandates. UnitedHealthcare reserves the right to modify its Policies and Guidelines as necessary. This Medical Management Guideline is provided for informational purposes. It does not constitute medical advice.

UnitedHealthcare may also use tools developed by third parties, such as the MCG™ Care Guidelines, to assist us in administering health benefits. UnitedHealthcare West Medical Management Guidelines are intended to be used in connection with the independent professional medical judgment of a qualified health care provider and do not constitute the practice of medicine or medical advice.

Member benefit coverage and limitations may vary based on the member's benefit plan Health Plan coverage provided by or through UnitedHealthcare of California, UnitedHealthcare Benefits Plan of California, UnitedHealthcare of Oklahoma, Inc., UnitedHealthcare of Oregon, Inc., UnitedHealthcare Benefits of Texas, Inc., or UnitedHealthcare of Washington, Inc.