

# Cell-Free Fetal DNA Testing

Guideline Number: MMG089.R  
 Effective Date: January 1, 2021

[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) for members with a singleton pregnancy in any one of the following circumstances:

- Maternal age or oocyte age of 35 years or older at delivery; or
- Fetal ultrasound findings indicating an increased risk of aneuploidy; or
- History of a prior pregnancy with a trisomy; or
- Positive first- or second-trimester screening test results for aneuploidy; or
- Parental balanced Robertsonian translocation with an increased risk of fetal trisomy 13 or trisomy 21; or
- Screening after pre-test counseling from a board-certified genetic counselor or from the prenatal care physician or healthcare professional using [Shared Decision-Making \(SDM\)](#)

Due to insufficient evidence of efficacy, DNA-based noninvasive prenatal tests are unproven and not medically necessary for all other 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

#### Cell-Free Fetal DNA Testing

Medical office notes documenting the following, when applicable:

- 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
- Counseling provided by genetic counselor or prenatal provider on the risks and benefits of testing using Shared Decision Making

## 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).

**Shared Decision-Making (SDM):** SDM is a process by which physicians and individuals work together to choose the treatment option that best reflects the clinical evidence and the individual's values and preferences (Armstrong and Metlay, 2020).

**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
0060U	Twin zygosity, genomic targeted sequence analysis of chromosome 2, using circulating cell-free fetal DNA in maternal blood
0168U	Fetal aneuploidy (trisomy 21, 18, and 13) DNA sequence analysis of selected regions using maternal plasma without fetal fraction cutoff, algorithm reported as a risk score for each trisomy
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

*CPT® is a registered trademark of the American Medical Association*

Diagnosis Code	Description
G91.2	(Idiopathic) normal pressure hydrocephalus
O09.00	Supervision of pregnancy with history of infertility, unspecified trimester
O09.01	Supervision of pregnancy with history of infertility, first trimester
O09.02	Supervision of pregnancy with history of infertility, second trimester
O09.03	Supervision of pregnancy with history of infertility, third trimester
O09.10	Supervision of pregnancy with history of ectopic, unspecified trimester
O09.11	Supervision of pregnancy with history of ectopic, first trimester
O09.12	Supervision of pregnancy with history of ectopic, second trimester
O09.13	Supervision of pregnancy with history of ectopic, third trimester
O09.211	Supervision of pregnancy with history of pre-term labor, first trimester
O09.212	Supervision of pregnancy with history of pre-term labor, second trimester

Diagnosis Code	Description
O09.213	Supervision of pregnancy with history of pre-term labor, third trimester
O09.219	Supervision of pregnancy with history of pre-term labor, unspecified trimester
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
O09.30	Supervision of pregnancy with insufficient antenatal care, unspecified trimester
O09.31	Supervision of pregnancy with insufficient antenatal care, first trimester
O09.32	Supervision of pregnancy with insufficient antenatal care, second trimester
O09.33	Supervision of pregnancy with insufficient antenatal care, third trimester
O09.40	Supervision of pregnancy with grand multiparity, unspecified trimester
O09.41	Supervision of pregnancy with grand multiparity, first trimester
O09.42	Supervision of pregnancy with grand multiparity, second trimester
O09.43	Supervision of pregnancy with grand multiparity, third 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
O09.611	Supervision of young primigravida, first trimester
O09.612	Supervision of young primigravida, second trimester
O09.613	Supervision of young primigravida, third trimester
O09.619	Supervision of young primigravida, unspecified trimester
O09.621	Supervision of young multigravida, first trimester
O09.622	Supervision of young multigravida, second trimester
O09.623	Supervision of young multigravida, third trimester
O09.629	Supervision of young multigravida, unspecified trimester
O09.70	Supervision of high risk pregnancy due to social problems, unspecified trimester
O09.71	Supervision of high risk pregnancy due to social problems, first trimester
O09.72	Supervision of high risk pregnancy due to social problems, second trimester
O09.73	Supervision of high risk pregnancy due to social problems, third trimester
O09.811	Supervision of pregnancy resulting from assisted reproductive technology, first trimester
O09.812	Supervision of pregnancy resulting from assisted reproductive technology, second trimester
O09.813	Supervision of pregnancy resulting from assisted reproductive technology, third trimester
O09.819	Supervision of pregnancy resulting from assisted reproductive technology, unspecified trimester
O09.821	Supervision of pregnancy with history of in utero procedure during previous pregnancy, first trimester
O09.822	Supervision of pregnancy with history of in utero procedure during previous pregnancy, second trimester
O09.823	Supervision of pregnancy with history of in utero procedure during previous pregnancy, third trimester

Diagnosis Code	Description
O09.829	Supervision of pregnancy with history of in utero procedure during previous pregnancy, unspecified trimester
O09.891	Supervision of other high risk pregnancies, first trimester
O09.892	Supervision of other high risk pregnancies, second trimester
O09.893	Supervision of other high risk pregnancies, third trimester
O09.899	Supervision of other high risk pregnancies, unspecified trimester
O09.90	Supervision of high risk pregnancy, unspecified, unspecified trimester
O09.91	Supervision of high risk pregnancy, unspecified, first trimester
O09.92	Supervision of high risk pregnancy, unspecified, second trimester
O09.93	Supervision of high risk pregnancy, unspecified, third trimester
O09.A0	Supervision of pregnancy with history of molar pregnancy, unspecified trimester
O09.A1	Supervision of pregnancy with history of molar pregnancy, first trimester
O09.A2	Supervision of pregnancy with history of molar pregnancy, second trimester
O09.A3	Supervision of pregnancy with history of molar pregnancy, third trimester
O26.20	Pregnancy care for patient with recurrent pregnancy loss, unspecified trimester
O26.21	Pregnancy care for patient with recurrent pregnancy loss, first trimester
O26.22	Pregnancy care for patient with recurrent pregnancy loss, second trimester
O26.23	Pregnancy care for patient with recurrent pregnancy loss, third trimester
O26.841	Uterine size-date discrepancy, first trimester
O26.842	Uterine size-date discrepancy, second trimester
O26.843	Uterine size-date discrepancy, third trimester
O26.849	Uterine size-date discrepancy, unspecified trimester
O26.851	Spotting complicating pregnancy, first trimester
O26.852	Spotting complicating pregnancy, second trimester
O26.853	Spotting complicating pregnancy, third trimester
O26.859	Spotting complicating pregnancy, unspecified trimester
O26.891	Other specified pregnancy related conditions, first trimester
O26.892	Other specified pregnancy related conditions, second trimester
O26.893	Other specified pregnancy related conditions, third trimester
O26.899	Other specified pregnancy related conditions, unspecified trimester
O26.90	Pregnancy related conditions, unspecified, unspecified trimester
O26.91	Pregnancy related conditions, unspecified, first trimester
O26.92	Pregnancy related conditions, unspecified, second trimester
O26.93	Pregnancy related conditions, unspecified, third trimester
O28.0	Abnormal hematological finding on antenatal screening of mother
O28.1	Abnormal biochemical finding on antenatal screening of mother
O28.2	Abnormal cytological finding on antenatal screening of mother
O28.3	Abnormal ultrasonic finding on antenatal screening of mother
O28.4	Abnormal radiological finding on antenatal screening of mother
O28.5	Abnormal chromosomal and genetic finding on antenatal screening of mother
O28.8	Other abnormal findings on antenatal screening of mother
O28.9	Unspecified abnormal findings on antenatal screening of mother

Diagnosis Code	Description
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
O35.2XX0	Maternal care for (suspected) hereditary disease in fetus, not applicable or unspecified
O99.210	Obesity complicating pregnancy, unspecified trimester
O99.211	Obesity complicating pregnancy, first trimester
O99.212	Obesity complicating pregnancy, second trimester
O99.213	Obesity complicating pregnancy, third trimester
O99.280	Endocrine, nutritional and metabolic diseases complicating pregnancy, unspecified trimester
O99.281	Endocrine, nutritional and metabolic diseases complicating pregnancy, first trimester
O99.282	Endocrine, nutritional and metabolic diseases complicating pregnancy, second trimester
O99.283	Endocrine, nutritional and metabolic diseases complicating pregnancy, third trimester
O99.284	Endocrine, nutritional and metabolic diseases complicating childbirth
O99.285	Endocrine, nutritional and metabolic diseases complicating the puerperium
O99.310	Alcohol use complicating pregnancy, unspecified trimester
O99.311	Alcohol use complicating pregnancy, first trimester
O99.312	Alcohol use complicating pregnancy, second trimester
O99.313	Alcohol use complicating pregnancy, third trimester
O99.320	Drug use complicating pregnancy, unspecified trimester
O99.321	Drug use complicating pregnancy, first trimester
O99.322	Drug use complicating pregnancy, second trimester
O99.323	Drug use complicating pregnancy, third trimester
O99.330	Smoking (tobacco) complicating pregnancy, unspecified trimester
O99.331	Smoking (tobacco) complicating pregnancy, first trimester
O99.332	Smoking (tobacco) complicating pregnancy, second trimester
O99.333	Smoking (tobacco) complicating pregnancy, third trimester
O99.340	Other mental disorders complicating pregnancy, unspecified trimester
O99.341	Other mental disorders complicating pregnancy, first trimester
O99.342	Other mental disorders complicating pregnancy, second trimester
O99.343	Other mental disorders complicating pregnancy, third trimester
O99.810	Abnormal glucose complicating pregnancy
O99.814	Abnormal glucose complicating childbirth
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)

Diagnosis Code	Description
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
Z34.00	Encounter for supervision of normal pregnancy, unspecified trimester
Z34.01	Encounter for supervision of normal pregnancy, first trimester
Z34.02	Encounter for supervision of normal pregnancy, second trimester
Z34.03	Encounter for supervision of normal pregnancy, third trimester
Z34.80	Encounter for supervision of other normal pregnancy, unspecified trimester
Z34.81	Encounter for supervision of other normal pregnancy, first trimester
Z34.82	Encounter for supervision of other normal pregnancy, second trimester
Z34.83	Encounter for supervision of other normal pregnancy, third trimester
Z34.90	Encounter for supervision of normal pregnancy, unspecified, unspecified trimester
Z34.91	Encounter for supervision of normal pregnancy, unspecified, first trimester
Z34.92	Encounter for supervision of normal pregnancy, unspecified, second trimester
Z34.93	Encounter for supervision of normal pregnancy, unspecified, third trimester
Z36.0	Encounter for antenatal screening for chromosomal anomalies
Z36.1	Encounter for antenatal screening for raised alphafetoprotein level
Z36.2	Encounter for other antenatal screening follow-up
Z36.3	Encounter for antenatal screening for malformations
Z36.4	Encounter for antenatal screening for fetal growth retardation
Z36.5	Encounter for antenatal screening for isoimmunization
Z36.81	Encounter for antenatal screening for hydrops fetalis
Z36.82	Encounter for antenatal screening for nuchal translucency
Z36.83	Encounter for fetal screening for congenital cardiac abnormalities
Z36.84	Encounter for antenatal screening for fetal lung maturity
Z36.85	Encounter for antenatal screening for Streptococcus B
Z36.86	Encounter for antenatal screening for cervical length

Diagnosis Code	Description
Z36.87	Encounter for antenatal screening for uncertain dates
Z36.88	Encounter for antenatal screening for fetal macrosomia
Z36.89	Encounter for other specified antenatal screening
Z36.8A	Encounter for antenatal screening for other genetic defects
Z3A.09	9 weeks gestation of pregnancy
Z3A.10	10 weeks gestation of pregnancy
Z3A.11	11 weeks gestation of pregnancy
Z3A.12	12 weeks gestation of pregnancy
Z3A.13	13 weeks gestation of pregnancy
Z3A.14	14 weeks gestation of pregnancy
Z3A.15	15 weeks gestation of pregnancy
Z3A.16	16 weeks gestation of pregnancy
Z3A.17	17 weeks gestation of pregnancy
Z3A.18	18 weeks gestation of pregnancy
Z3A.19	19 weeks gestation of pregnancy
Z3A.20	20 weeks gestation of pregnancy
Z3A.21	21 weeks gestation of pregnancy
Z3A.22	22 weeks gestation of pregnancy
Z3A.23	23 weeks gestation of pregnancy
Z3A.24	24 weeks gestation of pregnancy
Z3A.25	25 weeks gestation of pregnancy
Z3A.26	26 weeks gestation of pregnancy
Z3A.27	27 weeks gestation of pregnancy
Z3A.28	28 weeks gestation of pregnancy
Z3A.29	29 weeks gestation of pregnancy
Z3A.30	30 weeks gestation of pregnancy
Z3A.31	31 weeks gestation of pregnancy
Z3A.32	32 weeks gestation of pregnancy
Z3A.33	33 weeks gestation of pregnancy
Z3A.34	34 weeks gestation of pregnancy
Z3A.35	35 weeks gestation of pregnancy
Z3A.36	36 weeks gestation of pregnancy
Z3A.37	37 weeks gestation of pregnancy
Z3A.38	38 weeks gestation of pregnancy
Z3A.39	39 weeks gestation of pregnancy
Z3A.40	40 weeks gestation of pregnancy
Z3A.41	41 weeks gestation of pregnancy
Z3A.42	42 weeks gestation of pregnancy
Z3A.49	Greater than 42 weeks gestation of pregnancy



## 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. Only conventional prenatal diagnosis (i.e., chorionic villus sampling (CVS) or amniocentesis) can definitively diagnose fetal trisomies. (American College of Obstetricians and Gynecologists [ACOG], 2020).

During pregnancy, there are cfDNA fragments from both the mother and fetus in maternal circulation. The tests detect the fetal component 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 (ACOG, 2020).

## Clinical Evidence

### Aneuploidy for Trisomies 21, 18, and 13

Hong et al. (2020) reported the results of several NIPT tests from a single center and confirmed their accuracy and reliability. NIPT data was retrospectively collected from 1,591 women from CHA Gangnam Medical Center from March 2014-November 2018. Karyotype was confirmed based on chorionic villus sampling, amniocentesis or postnatal cord/peripheral blood. Of the 1,591 women, 1,544 (97.0%) of cases were reported to have negative NIPT results and 40 (2.5%) had positive NIPT results. Overall, for chromosome abnormalities, the sensitivity and specificity were reported as 96.29% and 99.93%, respectively. The PPV and NPV values were 72.22% and 99.93%, respectively. There was a decrease in the mean number of amniocentesis performed from 31.5 per month to 21.7 per month after utilization of NIPT as a screening tool. The authors concluded from their study that NIPT is an accurate and specific screening test for trisomy 21 due to its high PPV and can reduce potential procedural-related risks. Additional studies for confirmatory testing to predict cases of trisomy 13, 18, and sex chromosome aneuploidy were suggested.

Van der Meij et al. (2019) published a report on the nationwide implementation of NIPS as a first-tier screening test to women who are at average risk for fetal aneuploidy. Of 73,239 NIPS results, 343 were screen positive for common aneuploidy with confirmation of abnormal results in 289 women. This false positive rate of 0.07% is significantly lower than the 5.4% false positive rate of first trimester screening (FTS) reported by Norton et al. 2015 from the same cohort.

Table 1: Fetal aneuploidy screening methods vary in PPV by maternal age. References that can be utilized to learn about the statistics for each method in more detail.

Trisomy	PPV and Test	Maternal Age					Reference
		20	25	30	35	40	
Trisomy 21	PPV, NIPT	38%-80%	51%	61%	73-95%	91-99%	Perinatal Quality Foundation; ACOG, 2020
	PPV, FTS	3.04%	3.12%	3.5%	3.99%	5.76%	Spencer, 2001
Trisomy 18	PPV, NIPT	14%	15%	21%	39%	69%	Perinatal Quality Foundation
	PPV, FTS	0.58%-15%					UK NSC, 2013
Trisomy 13	PPV, NIPT	6%	7%	10%	21%	50%	Perinatal Quality Foundation
	PPV, FTS	0.94%-2.03%					UK NSC, 2013

Note: Perinatal Quality Foundation Calculator, available at <https://www.perinatalquality.org/Vendors/NSGC/NIPT/>

The Washington State Health Care Authority commissioned a health technology assessment (HTA) on cell-free fetal DNA from the Center for Evidence-based Policy Oregon Health & Science University, and a final report was issued in December 2019 (Washington State, 2019). The report reviewed available peer reviewed literature and concluded that for all age groups, cell-free fetal DNA testing for aneuploidy has a lower false positive rate than conventional screening and results in a lower rate of invasive testing than conventional aneuploidy screening.

The Province of Ontario, Canada commissioned a HTA of NIPT in 2019 (Ontario, 2019). The HTA committee reviewed available evidence in peer-reviewed literature and concluded that NIPT “NIPS is an effective and safe prenatal screening method for trisomies 21, 18, and 13 in the average-risk or general population.” They noted that NIPT was more accurate with an improved sensitivity and specificity than traditional forms of aneuploidy screening.

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 mosaics, 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.

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.

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.

Quezada et al. (2015) studied 2905 women with singleton pregnancies between 10-11 weeks gestation who were undergoing routine FTS. The average maternal age was 37, and 67% of the participants were over age 35. Results were available on a total of 2851 cases. Fifty four cases did not get results because of lost sample (n=1), low fetal fraction (n=38), and test failure (n=15). In the cohort that did not have a NIPS result were 49 euploid pregnancies, 2 Trisomy 21, and 3 miscarriages that did not have chromosome results available. FTS and NIPS identified all cases of common aneuploidy. The primary distinction between the two tests was the false positive rate; FTS had a false positive rate of 4.4% and NIPS was 0.25%.

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  $\geq 35$  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.

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%).

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.

### ***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.

### ***QNatal***

The performance of cell-free DNA screening (QNatal advanced) for trisomies 21, 18, 13 and sex chromosome aneuploidies (SCA) was conducted on a population of both average and high risk pregnancies using massive parallel sequencing and a GC content correction algorithm. Pregnancy outcome was obtained by genetic counselors and /or Quest diagnostics client services. The mean age at delivery was 35.2 +/- 5.8 years; 69% were advanced maternal age. Twins and higher order multiples represented 2.0% and 0.02% of specimens, respectively. 69,794 specimens were subjected to cell-free screening. 87% were high risk pregnancies; 13% were classified as average risk. 1,359 (1.9%) had a positive result. Of the confirmed cases, PPV for trisomies 21,18, 13, SCA and microdeletions was 98.1%, 88.2%, 59.3%, 69.0% and 75.0%. The overall PPV was 87.2%, sensitivity was 97.9%, and specificity was 99.9%. The authors attribute the higher PPVs obtained in this study to possible technological differences between QNatal advanced and alternate laboratory assays. The authors also add that the high PPV for confirmed SCA outcomes (69.0%) with an incidence of 0.36% for both average and high risk patients may have been due to prospective analysis of possible maternal SCA that was performed to avoid false-positive results (Guy et al., 2019).

### ***verifi***

In a multicenter comparative study, 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).

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.

### ***Harmony Prenatal Test***

Jones et al. (2018) examined the performance of cfDNA analysis of the X and Y chromosomes using microarray quantitation for determination of SCA probability in singleton pregnancy and fetal sex in twin and singleton pregnancies. Banked maternal plasma from 791 singleton and 51 twin pregnancies was collected as part of an ongoing multicenter clinical study (NCT02201862 and NCT01451671) from King's College London, UK; karyotypes were obtained for each sample. Y-chromosome specific DANSR assay was used to determine fetal sex in singleton and twin pregnancies. Results were reported based on the presence or absence of the Y chromosome fragments. For sex chromosome aneuploidy (SCA) evaluation, 742 samples were qualified. Fifteen cases of SCA were correctly classified (100% sensitivity; 95% CI, 79.6-100%). For the 727 disomic pregnancies, 725 were correctly identified as low-risk for sex chromosome aneuploidy (99.7% specificity; 95%CI, 99.0-99.9%). 748/752 singleton and 39/39 twin pregnancies had results obtained for fetal sex determination. Fetal sex was concordant with karyotypic sex in 786/787 cases (99.9% concordance). All twin cfDNA results accurately reflected either the presence of two females (n=18) or at least one male fetus (n=21). The authors concluded that targeted cfDNA testing was performed with high accuracy for fetal sex assessment in both singletons and twins, and correctly identified all SCAs with high specificity. However, the observed positive predictive value in this group may not be applicable to routine prenatal screening populations.

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%.

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).

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).

### ***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 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 nanofilter-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  $\beta$ -chorionic gonadotropin (hCG) were determined. Thirteen trisomy 21 pregnancies were correctly identified, and no false positives were found.

## Sex Chromosome Aneuploidies

There is a lack of studies that demonstrate clinical utility of using NIPT for determination of sex chromosome aneuploidy testing. Additional studies with large numbers of pregnancies with abnormal cfDNA sex chromosome aneuploidy screening need to be undertaken prior to acceptance of this screening.

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.

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 aneuploidy 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

Using cfDNA for determination of aneuploidies other than trisomies 13, 18, and 21 is in the early stages of development. Evidence at this time is limited and has shown a high number of false positive results.

Xue et al. (2019) conducted a retrospective evaluation of prospectively gathered NIPT information for the detection of rare fetal chromosome aneuploidy (RCA). Analysis was performed for all 24 chromosome aneuploidies among 57,204 pregnancies in Suzhou China. A total of 586 positive cases were identified following NIPT; 92 were positive for RCA. Forty-three cases underwent prenatal diagnosis and only 1 case of trisomy 22 was confirmed. Chromosome 7 aneuploidy accounted for 25/92 positive RCA NIPT results which is consistent with previous reports of trisomy 7 being the most frequently detected chromosome abnormality. Follow-up revealed that several false-positive RCA cases were caused by confined placental mosaicism, maternal aneuploidy and maternal cancer. The authors conclude that NIPT accuracy for RCAs remains limited.

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 FTS markers; pregnancy-associated plasma protein-A (PAPP-A), free  $\beta$  human chorionic gonadotrophin, and placental growth factor (PIGF). 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 PIGF 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 segmental 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

The evidence is insufficient to support the use of cfDNA for the screening of microdeletions and copy number variants. Further validation studies are needed to determine the sensitivity and specificity of this screening test for microdeletion syndromes.

Cui et al. (2019) evaluated the clinical utility of non-invasive prenatal testing (NIPT) for the detection of copy number variants (CNVs) by reporting on 161 pregnancies with ultrasound findings and negative NIPT results for chromosomal aneuploidy. Fetal CNVs were diagnosed by CNV sequencing; fetal and parental karyotypes were obtained by G-banding. NIPT revealed 11 CNVs  $\geq 1$ Mb in 9 samples, including two CNVs in each one of two separate samples. CNV sequencing on amniotic fluid was performed for 137 samples and 24 samples of fetal tissue. Fetal karyotypes were obtained for 78 cases and 7 cases were diagnosed as abnormal. The sensitivity and specificity of NIPT for detecting CNV  $>1$ Mb were 83.33% and 99.34%, respectively. The PPV and NPV were 90.91% and 98.68%, respectively. The sensitivity and specificity for CNVs 1Mb-5Mb was higher than for those  $\geq 5$ Mb. The authors claimed that NIPT can be performed for pregnancies with structural fetal anomalies for CNV detection, however due to the residual chromosomal aneuploidy risks for pregnancies with soft ultrasound markers, women with structural ultrasound anomalies should be offered invasive procedures for diagnosing CNVs. This study is difficult to generalize to the average screening population, as only pregnancies with ultrasound anomalies and negative NIPT results were selected for analysis. Future studies are needed for NIPT and CNV detection.

From March 2016-May 2017, Hu et al. (2019) studied 8,141 single pregnancies with a genome wide SSP- NIPT in order to establish positive predictive value for sub-chromosomal microdeletions and microduplications at 3 Mb resolution (4.89 million reads). Maternal age ranged from 15-46 with 13.79% over age 35 and 40.88% between ages 25-29. 13.79% presented for NIPT because of advanced maternal age; 26 cases had abnormal ultrasound findings. Other indications included: poor fertility and abnormal serum screening. Fifty-one (0.63%) were positive for chromosome microdeletions or microduplications, however 13 (36.11%) were true positives. Twenty-three (63.89%) were false-positives, and 15 were unverified cases. Of the 13 true positives, 9 were de novo mutations and 4 were inherited mutations. Seven cases were identified as disease-causing and 6 were of unknown clinical significance. The authors state that microdeletion NIPT has a low PPV and has not demonstrated an acceptable low false-positive rate to be considered practical for prenatal screening. Diagnostic testing is recommended.

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.

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.

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 karyotype or fluorescent in situ hybridization (FISH). After adjusting the algorithms and using a deeper read depth, 29 of 31 subchromosomal 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

There is insufficient evidence to support the use of repeat NIPT screening due to low fetal fraction results. The limited studies available have not provided clinical utility to support this testing.

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).

Benn et al. (2019) reviewed 159,574 Natera SNP-based NIPT samples between January 1, 2016 and October 1, 2016 to identify cases with a “no call” result and that underwent subsequent redraw. The dataset included 2,959 cases with a no-call result due to low fetal fraction (FF). Risks for trisomy 13, 18, and triploidy were evaluated using a FF-based algorithm. For each sample, a

FF z-score (number of standard deviations that the FF departed from the mean after adjustment for patient weight/gestational age) and a fetal fraction-based risk (FFBR) score were calculated. Using a risk cut-off of 1/100, the FFBR algorithm was introduced into clinical practice and provided an increased risk for the relevant condition as opposed to reporting an uninformative NIPT result; genetic counseling, comprehensive ultrasound and prenatal diagnosis were then recommended. Women receiving an FFBR risk <1/100 do not demonstrate an increased risk and can be offered the option of repeat testing. Risk-unchanged women were subsequently analyzed with a different regression model to determine the likelihood of an informative redraw. Of 2,644 samples with an uninformative result and redraw, 1,147 (43.4%) were high risk for trisomy 13,18 or triploidy. 1,497 (56.6%) were risk unchanged and of these, 975 (65.1%) had an informative redraw (80% of the original no-calls had informative results). Initial FF, maternal weight and time between blood samples were directly related to the likelihood of a successful redraw ( $p < .001$ ). Using the revised FFBR algorithm which uses FF as a biomarker after adjustment for maternal weight and gestational age for women with unchanged results, the number of women with an uninformative NIPT result was reduced to 0.7%. This study had several limitations including the dataset as a whole. The analysis is based on high-risk women with suspected chromosome abnormalities and the actual pregnancy outcomes are unknown for the cases in this study. The model has not been validated in a prospective study group. The authors concluded that this new algorithm and predictive model may be informative for redraw information for women with low FF and uninformative NIPT. Additional studies with outcome analysis are needed.

White et al. (2019) retrospectively studied factors that influence obtaining results on repeat NIPT following insufficient fetal fraction from initial specimen. A total of 2,906 samples were submitted for repeat testing by Harmony NIPT assay. Maternal age, weight, gestational age, time of sample, method of conception and number of fetuses were ascertained. A fetal fraction with a minimum of 4% was required. A result was obtained in 53% of subsequent specimens. The likelihood of obtaining a result was associated with the interval time between draws (per day, OR 1.040, 95% CI 1.031-1.051) and maternal weight (per kg, OR 0.988, 95% CI 0.985-0.991). An association was not made based on maternal age, gestational age at testing, IVF status or twin/singleton status. For every day the redraw interval increases, it is expected to see a 4% increase in the likelihood of obtaining a result. However, there is a 1.2% decrease in the likelihood of obtaining results for every kg increase in the maternal weight. A total of 246 pregnancies were redrawn more than once with 51.0% reporting results with the second submission. The authors acknowledged that the study findings should be applied after a normal ultrasound has been confirmed. No information was available about karyotype/fetal outcomes. Furthermore, the authors concluded that the decision to redraw should take into consideration ultrasound findings, other screening results, maternal factors, gestational age and parental preferences for follow-up.

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 FTS (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 mosaicism 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, totaling 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

There is a lack of evidence to support the use of cfDNA testing for determination of fetal RhD genotyping. Limited studies have been performed and the study populations are lacking diversity.

A prospective cohort, systematic review and meta-analysis was performed by Yang et al. (2019) to assess the diagnostic accuracy of high-throughput NIPT for fetal RhD status in RhD-negative women not known to be sensitized to the RhD antigen. Databases scanned for this meta-analysis included MEDLINE, EMBASE and Science Citation Index and were searched through February 2016. Included for review were 3,921 identified studies. The study population included RhD negative pregnant women known to not be sensitized to the RhD antigen and the index test was high-throughput cell-free DNA on maternal plasma.



Serological cord blood testing at birth was considered the reference standard and eligible studies were required to report diagnostic accuracy data including true positive, false positive, true negative and false negative absolute numbers. Diagnostic accuracy of NIPT varied by gestational age with data suggesting that NIPT was consistently accurate any time after the first trimester. The false negative rate (those incorrectly classified as RhD negative) was 0.34% (95% CI 0.15-0.76) and the false positive rate (incorrectly classified as RhD positive) was 3.86% (95% CI 2.54-5.82). Because this study is a meta-analysis, the authors described the risk of bias in the original articles and several of the included studies were deemed to be high-risk for bias due to the selected populations and the reference standards. The authors concluded that the use of NIPT for fetal RhD screening in all RhD-negative women is possible. Results would significantly reduce the need for unnecessary prenatal anti-D prophylaxis, while marginally increasing the risk of sensitization due to false negative results.

Manfroi et al. (2018) performed fetal RhD genotyping with polymerase chain reaction using cell-free DNA from maternal plasma to determine the diagnostic accuracy of non-invasive fetal genotyping at different gestational ages. A commercial multiple-exon assay was used to determine the accuracy of fetal RhD genotyping. Samples from RhD negative women (n=367) with RhD positive partners or partners with unknown RhD phenotype were collected between 24-28 weeks gestation; due to lack of available first trimester samples the analysis was restricted to 24-28 weeks during which fetal genotyping is usually performed for prenatal RhIg administration. Neonatal results were provided for 284 pregnancies. The reported sensitivity and specificity were 100% and 97.5%, respectively. Diagnostic accuracy was 96.1% including 9/284 inconclusive results. The low number of early gestational age samples is a weakness of the study and the authors attribute a false negative result to this. The authors concluded that cell-free DNA for RhD genotyping is an accurate and reliable tool for fetal immunoprophylaxis.

Saramago et al. (2018) conducted a health technology assessment of the use of cell-free DNA 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 sensitized 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 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

The use of cfDNA testing to evaluate single gene disorders is in the early stages of development. The evidence available at this time is limited and contains small sample sizes. Further studies with a larger number of patients is needed to determine the clinical utility of this approach.

Young et al. (2020) performed a retrospective analysis for non-invasive prenatal diagnosis (NIPD) single-gene testing for pregnancies at risk for cystic fibrosis (CF), spinal muscular atrophy (SMA) and X-linked Duchenne/Becker muscular dystrophies (DMD/BMD) by utilizing the relative haplotype dosage (RHDO) method. RHDO uses a capture-based targeted enhancement, followed by massive parallel sequencing and analysis by relative haplotype dosage. The requirements for NIPD using RHDO include known family history of disorder, confirmed molecular diagnosis, and necessary reference samples when possible. RHDO allows both paternal and maternal inheritance to be determined by measuring allelic imbalance between two haplotypes in cfDNA with phasing conducted through SNP sequencing; multiple single gene disorders can be performed during the same sequencing run and the same assay can be used for all families at risk for a particular condition which eliminates preliminary work-up prior to pregnancy. RHDO for DMD/BMD was performed in at-risk pregnancies following confirmation of a male fetus by cfDNA and requires maternal haplotype with a male reference sample (previous affected, previous unaffected, other male relative affected, or unaffected maternal grandfather) providing the mutated or normal haplotype. Due to the 12% chance for DMD/BMD gene recombination, RHDO was performed 5'-3' and 3'-5'. For CF/SMA, maternal and paternal haplotypes in addition to a reference child (i.e. previously affected child, an unaffected non-carrier child) or a carrier child if parents had different mutations were used for phasing. From September 2016 to October 2019, 152 at-risk pregnancies were referred to the

West Midlands Regional Genetics Laboratory. Follow-up genetic testing was performed for 70 of the 146 pregnancies to date for which a diagnostic result was issued. In all cases, follow-up testing confirmed the RHDO result and no discrepancies were reported demonstrating 100% concordance. For an additional 39 cases no postnatal discrepancies have been reported to date. The authors concluded that NIPD by RHDO can be performed clinically for both autosomal recessive and X-linked disorders with a high sensitivity and specificity. However, this study is difficult to generalize to a non-selected population as the families selected needed to have a known family history of the disorder. In addition, this study had a lack of follow up data for many cases. In conclusion, the authors emphasized the importance of prenatal counseling for patients undergoing NIPD for single gene disorders and having access to NIPT for routine aneuploidy screening at 10 weeks of pregnancy. Further testing is needed to validate this method for clinical use.

Zhang et al. (2019) developed and reported their clinical validation experience with a novel method for non-invasive prenatal sequencing for a panel of causative genes for frequently occurring monogenic, dominant disorders. Maternal cfDNA was barcoded and enriched and analyzed by next generation sequencing (NGS) for target regions of 30 genes. Low level fetal variants were then determined by a statistical analysis adjusted for NGS read count and fetal fraction. Likely pathogenic and pathogenic variants were confirmed by a secondary amplicon-based cfDNA test. Clinical testing was performed on 422 pregnancies, with or without ultrasound findings. Of these 422 cases, 390 had negative testing and 32 had positive results. Follow-up testing on cases was limited and only included 233 of the 422 original cases. The researchers stated that this study revealed 20 true-positives, 127 true-negatives, zero false-positives and zero false-negatives. A significant limitation of this study was the lack of follow up data for many cases therefore the clinical sensitivity and specificity is limited to only cases with outcomes. The authors concluded that by using this novel NIPT NGS method, a large number of dominant, monogenic disorders can be identified however additional validation studies are needed.

Camunes-Soler et al. (2018) developed a method of noninvasive prenatal diagnosis of inherited single-gene disorders using droplet digital PCR from cfDNA. cfDNA and fetal fraction were determined using TaqMan assays which target highly variable SNPs. Next, a ratio of healthy and diseased alleles in maternal plasma was quantified using the Taqman assay to target parental mutations. The study involved enrolling pregnant patients who are carriers of mutations causing autosomal-recessive or X-linked disorders for both single mutations and compound heterozygous mutations. Nine pregnancies at risk for different single-gene disorders including: hemophilia, ornithine transcarbamylase deficiency (OTC), cystic fibrosis, B-thalassemia, mevalonate kinase deficiency, acetylcholine receptor deficiency and DFNB1 nonsyndromic hearing loss were testing in the study. For each specimen, the fetal fraction and total cfDNA was measured using ddPCR. Primers were designed to amplify the mutation regions and TaqMan probes labeled with different fluorophores against healthy and mutated alleles. Accurate NIPT, according to the authors, relies on comparing the ratio of mutated and healthy alleles in maternal blood with the ratios expected for a healthy or affected fetus. Two affected and 7 unaffected pregnancies were confirmed by follow-up neonatal testing.

Xiong et al. (2018) conducted a feasibility study on patients of South East Asian descent to determine if targeted sequencing and relative mutation dosage 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 dosage 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  $\beta$ -thalassaemia carriers at a risk for having a child with  $\beta$ -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 amplification, 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 to the 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.

## Twin Gestation

The evidence is insufficient to support the use of NIPT in twin gestations due to lack of prospective studies and the inability to determine which twin would be affected. As testing is advanced it may be possible to use NIPT in the future.

Chibuk et al. (2020) presented data from cfDNA screening in twins over a 4.5 year period at a large clinical laboratory. Cytogenetic and SNP microarray diagnostic results from CVS, amniocentesis or products of conception samples were submitted to LabCorp and Integrated Genetics Laboratories between September 2013 to June 2018. The retrospective study attempted to evaluate the performance of genome-wide cfDNA screening for common aneuploidies in twin pregnancies with matched karyotype and/or microarray results from diagnostic specimens for 422 twin pregnancies. 371 reportable cfDNA samples yielded a sensitivity of 98.7% and a specificity of 93.2% for trisomies 21,18 and 13 collectively (95% CI). 100% sensitivity was seen for trisomies 18 and 13. 78.7%, 84.6%, and 66.7% were the positive predictive values for trisomy 21, 18 and 13, respectively (95% CI). The negative predictive value for patients receiving a negative result for all three aneuploidies was 99.6% (95 CI, 97.7,100). The group concluded that cfDNA screening in their cohort of twin pregnancies demonstrated superior performance with higher sensitivity and positive predictive values for trisomy 21 compared to traditional serum biochemical screening, adding that cfDNA screening may also be a reliable screening tool for trisomies 18 and 13. While this study was able to demonstrate significant values for sensitivity and specificity, there were several limitations in regards to the samples that were used. The sample set was from patients that opted for additional diagnostic testing, which created a bias towards cases that may be positive for aneuploidy. In addition, this sample cohort contained cases that were non-reportable from cfDNA screening. This sample set was also only a subset of patients from a larger population, so the results may not be generalizable. Another limitation that the authors note is that the analysis involved filtering of cfDNA samples for inclusion and that samples were selected based on the number of fetuses listed on the test requisition. The authors state that this may not accurately reflect the gestational status of the ongoing pregnancy. Studies that determine test performance in larger screening groups are needed.

The Dyr et al. (2019) retrospective study reported on Sequenom's clinical laboratory experience of more than 30,000 multifetal samples (twins, triplets and higher order) using cell-free DNA. This was the largest to date cfDNA multigestation study and the only known study with experience in triplets and higher-order gestation. Sequencing data was analyzed from 2011-2107 using the MaterniT21 platform to identify autosomal trisomies and other subchromosomal events. All samples were tested for trisomy 21, 18, 13. Select, opt-in samples (58%, n=16,951) were evaluated for trisomies 16 and 22 and seven common microdeletions including 22q-, 5p-,15q-, 1p36-, 11q-, 8q- and 4p-. Fetal fraction was adjusted in proportion to fetal number. Feedback was provided in 50 cases; 6 positive results for microdeletions and 7 positive results for trisomies 16 were included; 6.7 (86%) reported missed abortion or co-twin demise. 4/6 positive microdeletions had diagnostic testing and there were 3 true positives; two who did not have diagnostic testing had suggestive clinical findings. Non-reportable rate was 5.95% and the positivity rate for trisomy 21, 18 and 13 in multifetal samples was 1.50%, 0.47%, and 0.21%, respectively. It was concluded by the authors that cfDNA for multifetal gestation offers accurate screening for fetal aneuploidy that meets and exceeds performance of original clinical validation studies. However, in this retrospective study, the absence of outcome data is a significant limitation.

By combining their results with those identified in a systematic literature review, Gil et al. (2019) reported on the clinical performance of cfDNA for trisomies 21, 18 and 13 in twin pregnancies. Their prospective study population included self-referred women and women selected for cfDNA testing following routine first-trimester combined screening from the Fetal Medicine Center in London. MEDLINE, EMBASE, CENTRAL, ClinicalTrials.gov and World Health Organization International Clinical Trials Registry Platform was utilized to identify peer-reviewed publications on clinical validation of cfDNA for trisomies 21,18 and 13 in twin pregnancies. Using the authors data set of 997 twin pregnancies and those from the literature search including seven relevant studies, a meta-analysis was performed. cfDNA correctly identified 16/17 (94.1%) of trisomy 21; 9/10

(90%) of trisomy 18; 1/2 (50%) of trisomy 13 and 962/968 (99.4%) of those without any trisomies. When combining the author data set with those reported in the literature search, there were 56 trisomy 21 and 3,718 non-trisomy 21 twin pregnancies. The pooled detection rate (DR) and false-positive rate (FPR) was 98.2% (95% CI, 83.2-99.8%) and 0.05% (95% CI, 0.01-0.26%), respectively. For the combined 18 cases of trisomy 18 and 3,143 non-trisomy 18 pregnancies, the pooled DR and FPR were 88.9% (95% CI, 64.8-97.2%) and 0.03% (95% CI, 0.00-0.33%), respectively. There were only three cases of trisomy 13, 2 (66.7%) were detected by cfDNA with an AFP of 0.19%. The authors presented that cfDNA accuracy for trisomy 21 in twin pregnancies is similar to reports in singleton pregnancies and is superior to first-trimester combined testing or second-trimester biochemical screening. The majority of the studies had a high risk of bias because the cfDNA testing was not carried out or did not provide results in all cases or there was incomplete follow up. The authors stated that these studies used were classified as having a high risk of concerns regarding applicability to the general population. The testing was not carried out in the general population and some patients had additional aneuploidy screening tests.

## Twin Zygosity

The evidence is insufficient to support the use of NIPT in twin zygosity due to the lack of prospective studies that establish clinical utility. As testing is advanced it may be possible to use NIPT in the future.

In the first cohort study of twins in which NIPT-based zygosity was correlated with provider-assigned chorionicity, Jelsema et al. (2019) compared ultrasound assignment of chorionicity with single nucleotide polymorphism (SNP) based NIPT to determine zygosity (monozygotic MZ vs. dizygotic DZ) using a Natera proprietary algorithm. Between October 2017 and May 2018, 4,885 twin samples received SNP-based NIPT zygosity determination and established likelihood of aneuploidy for the pregnancy. Chorionicity was determined for 3,949/4,885 (80.8%) of patients. Monochorionicity (MC) was determined in 553 (11.3%); dichorionicity (DC) in 2,330 (47.7%); “Don’t know” for 1,066 (21.8%) and “Not Recorded” for 936 (19.2%). SNP based NIPT established that 1,450/4,885 (29.7%) were MZ twins and 2,435/4,885 (70.3%) were DZ. Of the “Don’t Know” or unrecorded cases (41%), 30.7% were determined to be MZ. Of the twins identified by their provider as MC, 3.4% were found to be DZ and of the DC twins identified by their provider, 12.9% were determined to be MZ. It was reported by the authors that as approximately half of the unspecified/unreported chorionicity samples were determined to be MZ, early and accurate assignment is essential for risk assessment for adverse pregnancy outcome. For the 3.4% of MC determined to be DZ, significant cost reduction for twin-twin transfusion syndrome (TTTS) monitoring and maternal anxiety reduction was achieved. With ultrasound findings, accurate NIPT determination allows for better risk counseling for the possibility of one vs. both fetuses having a genetic disorder. SNP-based NIPT can also ensure that experienced providers evaluate MZ twins as early in pregnancy as possible for chorionicity. Prospective studies were recommended by the authors to establish the clinical utility of SNP-based NIPT in twins with assignment of both zygosity and aneuploidy risk.

Norwitz et al. (2019) performed a prospective blinded study of 126 twin pregnancies to examine the validity of SNP based NIPT for zygosity. Ninety-five cases (30MZ; 65DZ) were used for zygosity determination. A combined fetal fraction for MZ was used and two different fetal fraction estimates were used for DZ. Two samples did not report results for zygosity due to low FF (1/30 MZ (3.3%) and 1/65DZ (1.5%). 29/30 MZ (96.7%) and 64/65 DZ (98.5%) were identified correctly giving a MZ sensitivity (DZ specificity) of 100% (29/29; 95% CI, 88.1%-100%) and an MZ specificity (DZ sensitivity) of 100% (64/64; 95%CI, 94.4%-100%). The overall zygosity test performance was 100% (93/93; 95% CI, 96.1%-100%).

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.

## 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.
- Offer aneuploidy screening other than NIPT for patients with a history of bone marrow or organ transplantation from a male donor or donor of uncertain biological sex.

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 referencing screening for chromosome abnormalities (ACOG, 2020).

Level A recommendations regarding cfDNA include:

- Prenatal genetic screening (serum screening with or without nuchal translucency (NT) ultrasound or cell-free DNA screening) and diagnostic testing should be offered to all pregnant women regardless of maternal age or risk for chromosome abnormality.
- If screening is accepted, patients should only have one screening performed and not multiple screening tests performed simultaneously.
- Cell-free DNA is the most sensitive and specific screening test for the common fetal aneuploidies. Nevertheless, it has the potential for false-positive and false-negative results. Furthermore, cell-free DNA testing is not equivalent to diagnostic testing.
- Patients with positive screening should have genetic counseling, comprehensive ultrasound and be offered diagnostic testing.
- Patients whose cfDNA are not reportable are at increased risk for chromosomal aneuploidy and should be offered genetic counseling, comprehensive ultrasound and diagnostic testing.

Level B recommendations regarding:

- The use of cfDNA as follow-up for patients with a screen positive serum-analyte test results is an option for patients who want to avoid diagnostic testing.
- In situations of isolated, soft ultrasound markers and no prior screening has been performed cfDNA, quad screen or diagnostic testing should be offered.

- cfDNA screening can be performed for twin pregnancies. Overall performance of screening for trisomy 21 by cfDNA in twin pregnancies is encouraging, but the total number of reported affected cases is small. Given the small number of affected cases it is difficult to determine an accurate detection rate for trisomy 18 and 13.

Level C recommendations regarding cfDNA include:

- In multifetal gestations, if a fetal demise, vanishing twin, or anomaly is identified in one fetus, there is a significant risk of an inaccurate test result if serum-based aneuploidy screening or cell-free DNA is used.
- Patients with unusual or multiple aneuploidies detected by cell-free DNA should be referred for genetic counseling.

An ACOG practice advisory recognizes the emerging technology and availability of cell-free DNA screening for single-gene disorders but emphasized that there is insufficient evidence to demonstrate accuracy and positive and negative predictive values for general population use (ACOG, 2019; reaffirmed 2020). For this reason, ACOG does not recommend single gene cfDNA screening in pregnancy.

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; reaffirmed 2019).

### ***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).

### ***International Society for Prenatal Diagnosis (ISPD)***

ISPD guidance on the use of cfDNA for aneuploidy screening noted that cfDNA should be a primary screening test offered to all pregnant women. The guidance also noted that it was appropriate as a secondary test for women found to be at high risk due to an abnormal serum screening result or ultrasound finding. cfDNA is significantly better than conventional screening with a much high odds of being affected given a positive result (OAPR). The authors concluded that while the performance of the test has been initially established in high risk women, there was now sufficient evidence to support the use of testing in women of average risk (Benn 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 joint committee opinion with ACOG (SMFM, 2020), SMFM recommends the following points of consideration regarding cfDNA testing for aneuploidy:

- cfDNA screening appears to be the most accurate test for trisomy 21
- cfDNA screening does not screen for all chromosome conditions and will not identify risk for the range of disorders potentially identified with traditional screening
- cfDNA screening has false positive and false negative results which is particularly true for lower risk women
- Diagnostic confirmation with CVS or amniocentesis is recommended for women with abnormal cfDNA results. Irreversible decisions for pregnancy termination should not be undertaken based on cfDNA results
- A negative cfDNA result does not definitively rule out trisomy 21 or other chromosome abnormalities
- Women with failed cfDNA have an increased risk for aneuploidy, need genetic counseling and should be offered diagnostic testing
- SMFM recommends payers provide adequate reimbursement for genetic counseling services
- All genetic screening is elective. Aneuploidy screening, diagnostic testing or no testing is a personal decision and any of these is a reasonable option.

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, 2015b).

## **U.S. Food and Drug Administration (FDA)**

This section is to be used for informational purposes only. FDA approval alone is not a basis for coverage.

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: <https://www.fda.gov/medical-devices/ivd-regulatory-assistance/clinical-laboratory-improvement-amendments-clia>. (Accessed January 30, 2020)

### **Additional Product Information**

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

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# Guideline History/Revision Information

Date	Summary of Changes
01/01/2021	<p><b>Template Update</b></p> <ul style="list-style-type: none"> <li>Reformatted policy; transferred content to new template</li> </ul> <p><b>Coverage Rationale</b></p> <ul style="list-style-type: none"> <li>Replaced language indicating:           <ul style="list-style-type: none"> <li>“DNA-based noninvasive prenatal tests of fetal aneuploidy are proven and medically necessary as screening tools for trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome) or trisomy 13 (Patau syndrome) in any one of the [listed] circumstances” with “DNA-based noninvasive prenatal tests of fetal aneuploidy are proven and medically necessary as screening tools for trisomy 21 (Down syndrome), trisomy 18 (Edwards syndrome) or trisomy 13 (Patau syndrome) <i>for members with a singleton pregnancy</i> in any one of the [listed] circumstances”</li> <li>“DNA-based noninvasive prenatal tests are unproven and not medically necessary for all other <i>fetal</i> conditions [not listed as proven and medically necessary]” with “DNA-based noninvasive prenatal tests are unproven and not medically necessary for all other conditions [not listed as proven and medically necessary]”</li> </ul> </li> <li>Added coverage criterion requiring “screening after pre-test counseling from a board-certified genetic counselor or from the prenatal care physician or healthcare professional using Shared Decision-Making (SDM)”</li> <li>Removed language indicating DNA-based noninvasive prenatal tests of fetal aneuploidy in conjunction with biochemical marker testing of maternal blood for maternal conditions (e.g., PAPP-A testing for pre-eclampsia) is unproven and not medically necessary due to insufficient evidence of efficacy</li> </ul> <p><b>Applicable Codes</b></p> <ul style="list-style-type: none"> <li>Added ICD-10 diagnosis codes O09.00, O09.01, O09.02, O09.03, O09.10, O09.11, O09.12, O09.13, O09.211, O09.212, O09.213, O09.219, O09.30, O09.31, O09.32, O09.33, O09.40, O09.41, O09.42, O09.43, O09.611, O09.612, O09.613, O09.619, O09.621, O09.622, O09.623, O09.629, O09.70, O09.71, O09.72, O09.73, O09.811, O09.812, O09.813, O09.819, O09.821, O09.822, O09.823, O09.829, O09.891, O09.892, O09.893, O09.899, O09.90, O09.91, O09.92, O09.93, O09.A0, O09.A1, O09.A2, O09.A3, O26.20, O26.21, O26.22, O26.23, O26.841, O26.842, O26.843, O26.849, O26.851, O26.852, O26.853, O26.859, O26.891, O26.892, O26.893, O26.899, O26.90, O26.91, O26.92, O26.93, O28.0, O28.1, O28.2, O28.4, O28.8, O99.210, O99.211, O99.212, O99.213, O99.280, O99.281, O99.282, O99.283, O99.284, O99.285, O99.310, O99.311, O99.312, O99.313, O99.320, O99.321, O99.322, O99.323, O99.330, O99.331, O99.332, O99.333, O99.340, O99.341, O99.342, O99.343, O99.810, O99.814, Z34.00, Z34.01, Z34.02, Z34.03, Z34.80, Z34.81, Z34.82, Z34.83, Z34.90, Z34.91, Z34.92, Z34.93, Z36.0, Z36.1, Z36.2, Z36.3, Z36.4, Z36.5, Z36.81, Z36.82, Z36.83, Z36.84, Z36.85, Z36.86, Z36.87, Z36.88, Z36.89, Z36.8A, Z3A.09, Z3A.10, Z3A.11, Z3A.12, Z3A.13, Z3A.14, Z3A.15, Z3A.16, Z3A.17, Z3A.18, Z3A.19, Z3A.20, Z3A.21, Z3A.22, Z3A.23, Z3A.24, Z3A.25, Z3A.26, Z3A.27, Z3A.28, Z3A.29, Z3A.30, Z3A.31, Z3A.32, Z3A.33, Z3A.34, Z3A.35, Z3A.36, Z3A.37, Z3A.38, Z3A.39, Z3A.40, Z3A.41, Z3A.42, and Z3A.49</li> </ul> <p><b>Documentation Requirements</b></p> <ul style="list-style-type: none"> <li>Updated list of applicable documentation requirements to include “counseling provided by genetic counselor or prenatal provider on the risks and benefits of testing using Shared Decision-Making”</li> </ul> <p><b>Definitions</b></p> <ul style="list-style-type: none"> <li>Added definition of “Shared Decision-Making (SDM)”</li> </ul> <p><b>Supporting Information</b></p> <ul style="list-style-type: none"> <li>Updated <i>Description of Services</i>, <i>Clinical Evidence</i>, and <i>References</i> sections to reflect the most current information</li> <li>Archived previous policy version MMG089.Q</li> </ul>

## 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 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.