David Geffen School of Medicine

Center for Fetal Medicine & Women's Ultrasound

NIPS: The Science, Limitations and Utility

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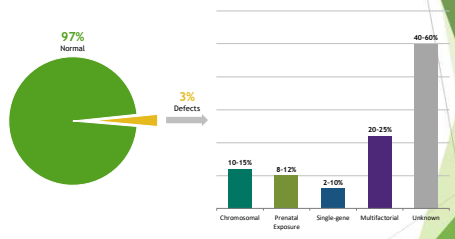
1

Disclosure

- GE Medical Systems
  - Research Support
  - Speakers' Bureau
- Illumina
  - Research Support
  - Clinical Advisor
- Natera
  - Speakers' Bureau

2

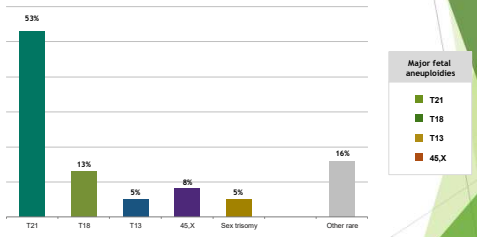
Birth Defects
Rates and causes in live births



Adapted from Stevenson, RE and Hall, J. *Human Malformations and Related Anomalies*, 2nd ed. Oxford University Press, Oxford, 2006

3

Prenatal Prevalence
Of reported chromosomal abnormalities



Data adapted from Wellesley, D, et al., Rare chromosome abnormalities, prevalence and prenatal diagnosis rates from population-based congenital anomaly registers in Europe. *Eur J of Hum Gen* 11 January 2012.

4

Conventional Prenatal Screening Options
Detection rates for Trisomy 21

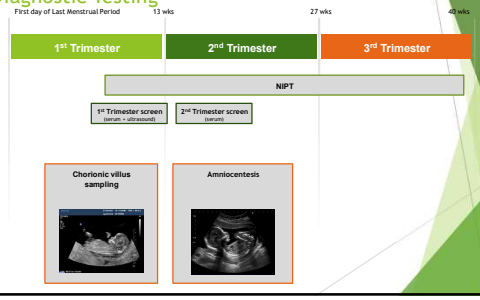
		Detection rate (%)
1st Trimester:	NT Ultrasound	64-70
1st Trimester:	1st Trimester blood screen › NT Ultrasound	82-87
2nd Trimester:	Triple screen	69
2nd Trimester:	Quadruple screen	81
Integrated screen:	1st Trimester blood screen › NT Ultrasound › 2nd Trimester blood screen	94-96
Serum integrated:	1st Trimester blood screen › 2nd Trimester blood screen	85-88
Sequential screen:	1st Trimester blood screen › NT Ultrasound › 2nd Trimester blood screen	95
	False positive rate:	~5%

LDP1

American College of Obstetricians and Gynecologists. Screening for fetal aneuploidy. Practice Bulletin No. 163. *Obstet Gynecol.* 2016; 127(5):e123-37.

5

Current Paradigm of Prenatal Screening and Diagnostic Testing



6

Slide 5

LDP1 Lawrence D. Platt, 3/11/2019

Factors Affecting Analyte Levels in Traditional Screening

Gestational age[1]

- First trimester:
  - Human chorionic gonadotropin (hCG) levels fall rapidly; Pregnancy-associated plasma protein A (PAPP-A) increases rapidly
- Second trimester:
  - Alpha-feto protein (AFP) and unconjugated estriol increase; hCG declines; Inhibin-A remains stable
- Errors in estimation can lead to false positive or false negative results

Maternal Weight[2,3]

- Inversely correlated to analyte levels due to the larger blood volume of heavier women

Racial/Ethnic background[4]

- African American women tend to have higher AFP, PAPP-A, and hCG levels and lower Inhibin-A levels than Caucasian women

Diabetes Mellitus[5]

- Women with insulin dependent diabetes mellitus have lower levels of AFP and unconjugated estriol compared to pregnant women without diabetes

1. American College of Medical Genetics. *Standards and Guidelines for Clinical Genetics Laboratories*. Second Edition, 1999
2. Lambert-Messerlian G, Palomaki GE, and Canick JA. Adjustment of serum markers in first trimester screening. *J Med Screen*. 2009;16(2):102-3.
3. Wald NJ, Kennard A, Hackshaw A, and McGuire A. Antenatal screening for Down's syndrome. *J Med Screen*. 1997;4(4):181-246. Review. Erratum in: *J Med Screen* 1998;5(2):110. *J Med Screen* 1998;5(3):164.
4. Spencer K, Heath V, El-Sheikhah A, Ong CY, and Nicolaides KH. Ethnicity and the need for correction of biochemical and ultrasound markers of chromosomal anomalies in the first trimester: a study of Oriental, Asian and Afro-Caribbean populations. *Prenat Diagn*. 2000;20(6):465-9.
5. Baumgarten A and Robinson J. Prospective study of an inverse relationship between maternal glycosylated hemoglobin and serum alpha-fetoprotein concentrations in pregnant women with diabetes. *Am J Obstet Gynecol*. 1988;159(1):27-31.

7

NIPT / NIPS

The Science

8

Introduction: NIPT/NIPS

- Quantification of the "fetal" cell-free circulating DNA in the maternal plasma (a.k.a." cfDNA testing" or "non-invasive prenatal testing/screening," NIPT/NIPS)
  - Aimed at detecting pregnancies at an increased risk for trisomies 21, 18, and 13
- cfDNA fragments derived from both the mother and conceptus, specifically from the apoptosis of the cytotrophoblast
- Maternal cfDNA contribution exceeds the placental contribution (a.k.a. "fetal fraction")
  - At 10 weeks of gestation, the average ratio of maternal-to-fetal contribution is approximately 9:1

9

Noninvasive Prenatal Testing (NIPT)

Objectives of NIPT

- Reduce exposure of fetus to risk
- Reduce false positives
- Testing that can easily be offered to pregnant women
- Enable a high detection rate

10

10

Objectives of NIPS

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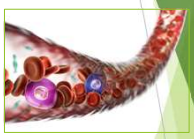
11

Cell-Free DNA (cfDNA) in Maternal Blood

An ideal analyte for aneuploidy testing

Maternal blood contains both maternal and fetal cfDNA

- 2-20% of total cfDNA in maternal blood is placental (cytotrophoblastic)[1,2]
- Released into bloodstream through apoptosis (cell death)



Detected after 7+ weeks gestation and undetectable within hours postpartum[1]

1. Barrett, A, Zimmerman BG, Wang D, Holloway A, Chitty L. Implementing prenatal diagnosis based on cell-free fetal DNA: Accurate identification of factors affecting fetal DNA yield. *PLoS One*. 2011;6(10):e25202.
2. Nigam A, Saxena P, Prakash A, Acharya A. Detection of fetal nucleic acid in maternal plasma: A novel noninvasive prenatal diagnostic technique. *J Internat Med Sci Acad*.2012; 25(3): 119-120.

12

LIMITATIONS and the implication #1

- **Limitation to current approaches of cfDNA:**
  - *It cannot efficiently physically separate these two components (maternal and fetal) for two components of analysis*
- **Implication #1:** Assessment of trisomy presents a quantitative challenge of detecting the additional genetic information contributed by the third chromosome against the background of maternal DNA
  - **Cause:** Sophisticated algorithms must be used when investigating fetal trisomies. Algorithms that can detect minute increments of circulating chromosomal fragments.

13

LIMITATIONS and the implication #2

- **Implication #2:** Chromosome imbalance affecting either the maternal or placental compartment may also be detected by cfDNA testing, thereby limiting the ability todiscriminate between maternal and placental / fetal abnormalities
  - "Needle in the haystack" phenomena impacts the interpretation and management of po / high-risk cfDNA testing results

14

cfDna testing strategies

- Massively Parallel Shotgun Sequencing (MPSS)
- Targeted Next Generation Sequencing (t-NGS)
- Digital Analysis of Selected Regions (DANSR)
- Single Nucleotide Polymorphisms (SNP)-based

MPSS | t-NGS | DANSR | SNP

15

cfDNA Testing Strategies

- **Initially, all four methodologies were based in t-NGS. DANSR technology shifted from NGS to microarray platform**
- **Analysis of the chromosome is performed by two different approaches.**
- **For t-NGS, DANSR, and SNP-based methods, the analysis is limited to a portion of the total circulated cfDNA fragments**
  - **Based on the initial capture / amplification stage**
  - **Using specific molecular probes that will only target circulating fragments**
- **For MPSS, the targeting of the region of interest is performed in the subsequent bioinformatic phase**
  - **Only after sequencing, alignment, and counting of all circulating fragments**

16

Massively Parallel Shotgun Sequencing (MPSS)

- **Whole-genome technology based on NGS of ~25M maternal and fetal cfDNA fragments belonging to all chromosomes**
- **Main stages of the process:**
  - **Library preparation**
  - **Sequencing of the cfDNA fragments**
  - **Sequence alignment**
  - **Fragment counting**
  - **Statistical analysis**
  - **Reporting**

17

Massively Parallel Shotgun Sequencing (MPSS)

- Sample analysis is performed in a multiplex fashion … parallel sequencing in one NGS reaction
- All individual cfDNA fragments are:
  - Molecularly barcoded for recognition and assignment to the specific patient in the subsequent analytic stages
  - Samples are "normalized" to obtain the same concentration for each DNA sample and mixed in a unique sequencing reaction (cell flow)
  - Normalization is necessary to prevent a disproportionate number of sequence reads in a flow cell n occur thereby reducing the number of reads available to reliably determine the trisomy risk
- The number of cfDNA fragments that can be sequenced for each sample (sequencing depth) depends on the number of samples and the ability to normalize DNA concentration across all samples
  - A 4-fold variation in the median reads/sample might be observed for a 12-plex reaction
  - The higher the sample input, the lower the sequencing depth and consequently, the poorer the
  - analytic performance
- **MPSS-based available tests may not offer the same analytical conditions (sensi/speci)**
- **and could vary from one lab to another**

18

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19

Massively Parallel Shotgun Sequencing (MPSS)

Continued

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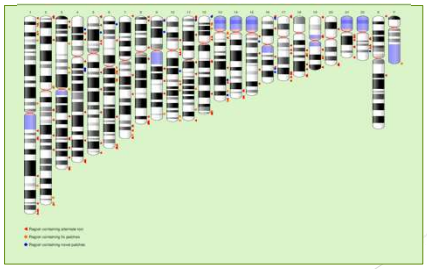
20

Massively Parallel Shotgun Sequencing (MPSS)

- After sequencing the first nucleotides of each circulating fragment (~25-36 base pairs, bp):
  - the chromosome of origin of each individual read is determined by mapping the obtained sequence against a Human Reference Genome
  - ~50% of these reads are mapped uniquely to the human genome covering 4% of the entire genome; other reads remain unmapped
  - After alignment, sequences belonging to each chromosome are counted and the data analyzed to provide the fetal trisomy risk score
    - Compared to euploid and trisomic reference samples and usually presented as z-scores
    - Z-score refers to the number of standard deviations from the mean of the reference euploid data set

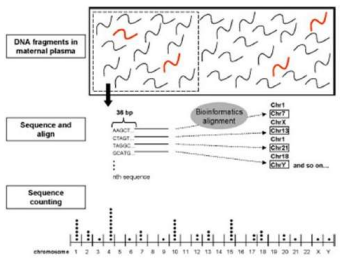
21

Human Genome Reference



22

NIPT Aneuploidy Testing with Massively Parallel Genomic Sequencing
Massively Parallel Shotgun Sequencing (MPSS)



23

Targeted NGS (t-NGS)

- **t-NGS is a variation of MPSS that analyzes only chromosomes of interest and a few selected reference chromosomes for comparison**
- **Special complementary probes are used to select the fragments of interest**
  - **Similar to MPSS, the fragments are sequenced and the resulting sequencing reads are aligned to the Human Genome Reference**
  - **Chromosomes are counted and compared to determine fetal risk**
  - **Selective sequencing of cfDNA fragments containing SNP loci allows for the estimation of the fetal fraction percentage by SNP genotyping**

24

Directed Analysis of Selected Regions (DANSR)

- Two different sets of DANSR probes are used to capture cfDNA fragments from chromosomes of interest
  - Each probe provides different types of information (trisomy risk score and fetal fragment estimation)
  - Risk score is determined by capturing and counting a selected number of fragments belonging to chromosomes 21, 18, and 13 and reference chromosomes (1-12)
  - Fetal fragment is determined by genotyping a selected number of cfDNA that belong to the reference chromosomes (1-12) and mapping SNP loci

25

Directed Analysis of Selected Regions (DANSR)

- Microarray platform with a single-plex approach is used for analysis
  - Each sample is individually analyzed in each subarray, allowing standardization of analytical considerations
  - Output are analyzed with the Fetal Fraction Optimized Risk of Trisomy evaluation (FORTE) algorithm
    - FORTE incorporates different risk factors , e.g., maternal and gestational age, to provide a fetal trisomy risk

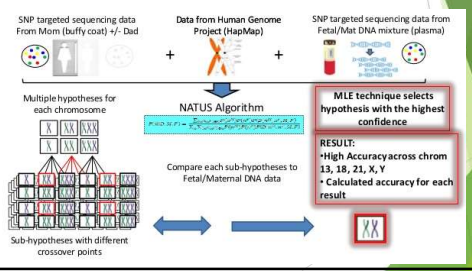
26

Single Nucleotide Polymorphisms (SNP)-based

- Targets specific chromosomes and differs from other technologies
  - Does not require reference chromosomes for comparison
- Based on targeted amplification and sequencing of cfDNA fragments belonging to chromosomes of interest
  - **Test has undergone several revisions since first introduced to improve performance, i.e., fetal fragment percentage cutoff decreased from 4% to 2.8%**
- SNP performs both a quantitative and a qualitative analysis of the obtained SNP profiles
  - Qualitative analysis allows formation of the fetal trisomy risk using NATUS algorithm

27

NATUS – Next-generation Aneuploidy Testing Using SNPs



28

Fetal Fraction Estimation

- Fetal fraction estimation using MPSS are based on:
  - Sex chromosome ratio or Trisomy fraction
    - **FF is measured in male, monosomy X, or trisomic pregnancies using the count data of X and Y chromosomes or chromosomes of interest, allowing the FF assessment of a subgroup (male and abnormal) pregnancies**
  - Differences between maternal and fetal cfDNA characteristics (methylation)
  - Distribution of maternal and placental cfDNA fragment size
  - Indirect sequencing variables
  - DNA digestion of fetal and maternal cfDNA related to different nucleosome positioning, and
  - Preferred ending sites for maternal and cfDNA
- Remaining methods are based on differences in metabolic and enzymatic cell processes affecting maternal and trophoblastic chromatin remodeling

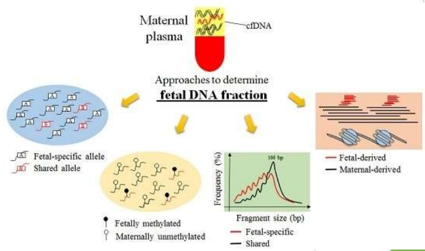
29

Fetal Fraction Estimation

- Methods for FF estimate based on fragment size indirectly deduce this information from
  - The overall plasma DNA size distribution for each maternal plasma DNA
  - Regression equation obtained from a training set of tested pregnancies: without a specific molecular assay
  - Principle behind this methodology is that there is a difference of average size of fetal and maternal fragments. These methods were found to be less reliable than methods
- FF estimation by SNP genotyping is applied by DANSR and SNP-based cfDNA tests and by t-NGS methods based on the selection of cfDNA fragments napping SNP loci
  - Based on the quantification of SNP variants circulating in maternal plasma differing from those of the mother
  - SNP genotyping of a pure female sample DNA detects an SNP profile, including two homozygous genotypes (AA and BB) together with the heterozygous (AB)

30

Fetal Fraction



31

Test Methodology can Minimize the Effects on Fetal Fraction Variation

- Assay Quality
  - Lowers the limit of detection (LOD)
    - Higher LOD is more likely to be affected by FF
  - Based on sequencing methodology and analysis method
- Fetal Fraction
  - Lower fetal fraction demands a lower LOD

It is important to know the Limits of Detection for the various cfDNA platorms

Assay Quality

Limit of Detection

Fetal Fraction

32

Fetal Fraction Estimation – Future Needs

- It is questionable whether all these methods are comparable
- **Need for comparative studies between labs comparing FF estimation methods**
- **Need to generate international standardization for FF estimation, ensuring comparability and consistency between labs**

33

Fetal Fraction
In cfDNA testing

Fetal fraction = amount of fetal cfDNA in total cfDNA

% fetal fraction (FF) affects ability of NIPT to detect fetal aneuploidy[1]

Threshold for fetal fraction depends on coefficient of variation obtained for an individual chromosome[1]

A robust test algorithm is less likely to be affected by fetal fraction[1]

1. Rava R, Srinivasan A, Sehnert AJ, Bianchi DW. Circulating Fetal Cell-Free DNA Fractions Differ in Autosomal Aneuploidies and Monosomy X. Clin Chem 2014; 60(1):243-50. doi: 10.1373/clinchem.2013.207951.

34

Factors Affecting Fetal Fraction

Gestational age[1-2]
- Slight increase from 10-21 weeks gestation
- Significant increase after 21 weeks gestation

Maternal BMI[1-3]
- Higher BMI is correlated with lower FF
- Women weighing more than 200 pounds will have ~10% lower FF
- Significant increase reported in maternal weight in samples with low FF versus samples that were successfully reported

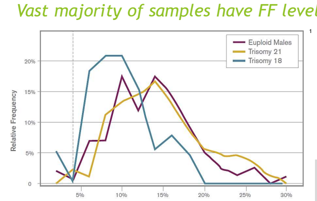
Small placenta[3]
- Fetal cell-free DNA is from placental cells undergoing apoptosis
- Number of apoptotic cells is proportional to placental mass

Aneuploidy[1,3-8]
- Fetal fraction is increased in pregnancies with T21
- Fetal fraction is decreased in pregnancies with T18, T13, and MX

1. Kinnings SL, Geis JA, Almasri E, et al. Factors affecting levels of circulating cell-free fetal DNA in maternal plasma and their implications for noninvasive prenatal testing. *Prenat Diagn*. 2015; 35(8):816-822.
2. Wang E, Batey A, Struble C, Musci T, Song K, andn Oliphant A. Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. *Prenat Diagn*. 2013; 33:662-666.
3. Ashoor G, Syngelaki A, Poon LC, Rezende JC, and Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11-13 weeks' gestation: relation to maternal and fetal characteristics. *Ultrasound Obstet Gynecol*. 2013;41:26-32.
4. Norton ME, Jacobsson B, Swamy GK, et al. Cell-free DNA analysis for noninvasive examination of trisomy. *New Engl J Med*. 2015; 372(17):1589-1597.
5. Rava RP, Srinivasan A, Sehnert AJ, Bianchi DW. Circulating fetal cell-free DNA fractions differ in autosomal aneuploidies and monosomy X. *Clin Chem*. 2014; 60(1):243-250.
6. Dar P, Curnow KJ, Gross SJ, et al. Clinical experience and follow-up with large scale single-nucleotide polymorphism-based noninvasive prenatal aneuploidy testing. *Am J Obstet Gynecol*. 2014; 211(5):527.e1-527.e17
7. Pergament E, Cuckle H, Zimmermann B, et al. Single-nucleotide polymorphism-based noninvasive prenatal screening in a high-risk and low-risk cohort. *Obstet Gynecol*. 2014; 124(2 Pt 1):210-218.
8. American College of Obstetricians and Gynecologists. Screening for fetal aneuploidy. Practice Bulletin No. 163. *Obstet Gynecol*. 2016; 127(5):e123-137.

35

Fetal Fraction in NIPT
Vast majority of samples have FF levels well above 4%



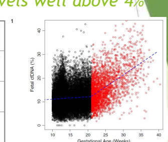


Figure 2
Relationship between percentage of fetal cfDNA and gestational age. Fetal cfDNA percentage for 22,384 pregnancies is plotted with respect to gestational age (weeks). At 10 weeks 0 days to 10 weeks 6 days gestation, the median fetal cfDNA percentage was 10.2%. Between 10 and 21 weeks gestation (black open circles), fetal cfDNA increased at 0.10% per week (p < 0.0001; blue dash line within black open circles) and 2% of the pregnancies during this period were below 4% fetal cfDNA. Starting at 21 weeks gestation (red open circles), the fetal cfDNA percentage increased at a rate of 1% per week (p <0.0001; blue dash line within red open circles), a tenfold increase in the amount of fetal cfDNA percentage per week compared with the weeks between 10 and 21 weeks gestation.

1. Rava RP, Srinivasan A, Sehnert AJ, Bianchi DW. Circulating Fetal Cell-Free DNA Fractions Differ in Autosomal Aneuploidies and Monosomy X. *Clin Chem*. 2014; 60(1):243-250.
2. Wang E, Batey A, Struble C, Musci T, Song K, Oliphant A. Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. *Prenat Diagn*. 2013; 33:662-666.

36

Why do Labs Measure Fetal Fraction?

Assays with lower quality use FF to eliminate difficult samples

- Eliminating samples with low fetal fraction artificially increases sensitivity and specificity

Even so, there is no improvement in performance statistics reported for labs that measure FF versus those that do not[1]

- Particularly no difference in Negative Predictive Value (NPV) or Observed Frequency of False Negative cases

1. Taneja PA, Snyder ML, de Feo E, et al. Noninvasive prenatal testing in the general obstetric population: clinical performance and counseling considerations in over 85,000 cases. Prenat Diagn. 2016; 36(3):237-243.
2. Mccullough RM, Almasri EA, Guan X, et al. Non-invasive prenatal chromosomal aneuploidy testing - clinical experience: 100,000 clinical samples. PLoS One. 2014; 9(10):e109172.
3. Norton ME, Jacobsson B, Swamy GK, et al. Cell-free DNA analysis for noninvasive examination of trisomy. New Engl J Med. 2015; 372(17):1589-97.
4. Dar P, Curnow KJ, Gross SJ, et al. Clinical experience and follow-up with large scale single-nucleotide polymorphism-based noninvasive prenatal aneuploidy testing. Am J Obstet Gynecol. 2014; 211(5):527.e1-527.e17.

37

A comparison of the technical characteristics of the NIPS technologies

	Fetal Ploidy Risk Determination			FF Determination		Technical Aspects				Test Examples
Technology	Principle	Statistics	Triploids	Principle	DNA Contamination Detection	Sample Analysis	Counting Depth	Max. Sample Throughput (Smples/Run)	Add Further Clinically Validated Targets?	
MPSS	CR	z-score/NCV	ND	Multiple approaches other than SNP-g	No, as SNPs are not tested	Multiplex by NGS	Variable	48	Possible by unlocking bioinformatic analysis of the fragment counts belonging from other chromosomes	HiSeq / NextSeq (Illumina) or Ion Proton (Thermo Fisher Scientific) NPSS platform-based technologies which are offerd under multiple local brands (e.g., VeriSeq, Verifi, G-test, Neobona, MaternIT, Tranquility, iONA InformaSeq, Prelude, Safe Test, etc.)
Targeted NGS	CR	z-score/NCV	ND	SNP-g or fragment size	Yes, if SNPs are tested	Multiplex by NGS	Variable	192	Possibly by adding new complementary molecular probes in the assay	Clarigo, Multiplexes, Agilent, Veracity (NIPD Genetics)
DANSR	CR	FORTE (incorporating FF%) - OR	ND	SNG-g	Yes	Singleplex by microarray	Standardized	384	Possible by adding new complementary DANSR probes in the assay	Harmoy Prenatal Test (Roche)
SNP-counting	SNP-g	NATUS (incorprating FF%) - Bayesian statistics with MLE	Detectable	SNP-g	Yes	Multiplex by NGA	Variable	48	Possible if enough informative SNPs are present in the target chromosome region	Panorama (Natera)

CR, Counting Ratio; SNP-g, SNP genotyping; ND, Not Detectable; MLE, Maximum Likelihood Estimation

38

Analytic Validity, Clinical Validity, and Clinical Utility

- As with any new technology, it is critical to determine the performance characteristics of the new test albeit in the "real" world and more importantly, if the test truly impacts care
- ACCE Model Project, developed by the CDC, is the model used to evaluate new genetic tests - still used around the world

39

Analytic Validity – Sensitivity

- **Sensitivity** (true positive rate or detection rate, DR) refers to the ability of a test to identify a genetic change when it is present
- To assess the sensitivity of cfDNA, the lab will use stored blood samples from mothers with a fetal aneuploidy to determine how often the test identifies the samples correctly
- The benchmark set by standard screening approaches were sensitivities of 50% to 95% for T21, depending on the screening strategy
- Since 2011, cfDNA testing was superior, with DR from 98.6% to 100% for T21
- cfDNA testing had the add'l benefit of higher sensitivities for other aneuploidies; some studies reporting sensitivity as high as 100% for T18, T13 and even monosomy X studies

40

Analytic Validity – Specificity

- **Specificity** (true negative rate; closely related to the false positive rate, FPR) refers to the ability of a test to report a negative screen result when in fact there is no aneuploidy which the FPR refers to the % of non-aneuploid samples that were initially reported as aneuploid
- Early cfDNA studies across all platforms demonstrated not only superior sensitivity, but also superior specificity for T21, 97% to 100%
- cfDNA testing also have very high specificities for other aneuploidies (93% to 100% for T18; 84% to 100% for T13; and 99% to 100% for monosomy X
- Compared to 95% specificity for standard screening, cfDNA testing was a substantial improvement

41

Clinical Validity

- Clinical validity speaks to the question … **how well will the best perform in a clinical setting once there is evidence that it works in the lab?**
- Common misconception that lab results with excellent sensitivities/specificities will translate in the at-large population. Not so as lab results are derived from known, usually stored samples

42

Clinical Validity

- There was the mistaken impression that cfDNA results were diagnostic due to the very high reported cfDNA testing sensitivities
  - Subsequent to a few high-profile FP cases, it was clear that more studies were needed to accurately assess PPV and NPV, especially in the average risk population
  - Prospective, multicenter blinded study (18,995 women), PPV of cfDNA testing for T21 was 80.9% - substantially better than standard screening methods with a PPV of 3.4%
  - Study clearly demonstrated that 100% sensitivity does not translate to 100% PPV, which is more closely correlated with specificity rather than sensitivity
  - For other aneuploidies, PPVs of 31.1% to 50.5% for T13 and monosomy X in cytogenetically-confirmed cases. Placental mosaicism may have influenced the lower PPV.

Confirmatory testing is strongly recommended in the presence of a positive report.

43

Large Clinical Trials

Study	Method	Trisomy 21				Trisomy 18				Trisomy 13			
		DR (%)	FPR (%)	NR* (%)	Unclass** (%)	DR (%)	FPR (%)	NR* (%)	Unclass* (%)	DR (%)	FPR (%)	NR* (%)	Unclass** (%)
1. Chiu et al (2011)	Shotgun	86/86 (100)	3/146 (2.1)	11/764 (1.4)									
2. Ehrich et al (2011)	Shotgun	39/39 (100)	1/410 (0.2)	18/467 (3.9)									
3. Palomaki et al (2011, 2012)	Shotgun	209/212 (98.6)	3/1471 (0.2)	13/1686 (0.8)		59/59 (100)	5/1688 (0.3)	17/1988 (0.9)		11/12 (91.7)	16/1688 (0.9)	17/1988 (0.9)	
4. Bianchi et al (2012)	Shotgun	89/89 (100)	0/404 (0)	16/532 (3.0)	7/503 (1.4)	35/36 (97.2)	0/461 (0)	16/532 (3.0)	5/502 (1.0)	11/14 (78.6)	0/488 (0)	16/532 (3.0)	2/502 (0.4)
5. Sparks et al (2012)	Targeted	36/36 (100)	1/123 (0.8)	8/338 (2.4)*		8/8 (100)	1/123 (0.8)	8/338 (2.4)*					
6. Ashoor et al (2012)	Targeted	50/50 (100)	0/297 (0)	3/400 (0.8)		49/50 (98.0)	0/297 (0)	3/400 (0.8)					
7. Norton et al (2012)	Targeted	81/81 (100)	3/2888 (0.1)	148/3228 (4.6)		37/38 (97.4)	3/2888 (0.1)	148/3228 (4.6)					
TOTAL		**590/593 (99.5)**	**11/5739 (0.2)**	**217/7415 (2.9)**	**7/503 (1.4)**	**188/191 (98.4)**	**9/5457 (0.2)**	**192/6486 (3.0)**	**5/502 (1.0)**	**22/26 (84.6)**	**16/2,176 (0.7)**	**33/2520 (1.3)**	**2/502 (0.4)**

Table 2. Large clinical trials of Non-invasive Prenatal Testing by Massively Parallel Sequencing (NIPT-MPS) for fetal trisomy 21, 18 and 13.

44

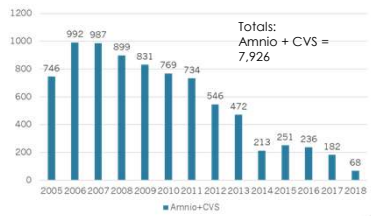
Evidence for NIPT Performance

	DR (%)	95% CI	FPR (%)	95% CI
Trisomy 21	99.2	98.5-99.6	0.09	0.05-0.14
Trisomy 18	96.3	94.3-97.9	0.13	0.07-0.20
Trisomy 13	91.0	85.0-95.6	0.13	0.05-0.26
Monosomy X	90.3	85.7-94.2	0.23	0.14-0.34
Other sex aneuploidies	93.0	85.8-97.8	0.14	0.06-0.24
Twins T21	93.7	83.6-99.2	0.23	0.00-0.92

Gil et al (2015). *Ultrasound Obstet Gynecol*, 45: 249-266

45

Decline in Invasive Procedures Since cfDNA Screening Introduced January, 2005 – May, 2018



46

Clinical Utility – Sex Chromosome Aneuploidies

Gil MM, et al. Ultrasound Obstet Gynecol 2017; 50 (3): 302-314

- What about clinical? Does the test impact care and outcomes?
- cfDNA testing has prompted major practice shifts related to invasive testing, significantly reducing the number of amniocentesis and chorionic villus sampling
  - This dramatic reduction in procedures is used as a measure of clinical utility because it has significantly impacted care. This has raised the question … ***"will there be an adequate number of CVS cases to train the next generation of maternal-fetal medicine specialists as well as cytogeneticists for karyotyping"***
- There is no established clinical utility for T13 and T18 as most will be picked up on ultrasound and testing is never ordered alone for these
- In a meta-analysis for Sex Chromosome Aneuploidies (SCA) beyond monosomy X, the DR was 100% with a FPR of 0.004%. Limitation of this analysis is the small sample size (combined total of 17 cases of SCA other than monosomy).
  - ACOG does not recommend SCA cfDNA screening as a front-line test, but can be made available to patients upon request

47

Clinical accuracy of abnormal cfDNA results for sex chromosomes

Scibetta EW, Platt, LD, et al. Prenat Diagn 2017; 37 (13): 1291-1297

- Retrospective cohort study of abnormal cfDNA results for sex chromosomes
- Results were deemed "abnormal" if they were
  - Positive, Inconclusive, or Discordant with U/S
- Primary outcome was concordance with karyotype or postnatal eval
- 31/50 (62%) were positive for SCA; 13/50 (26%) were inconclusive; 6/50 (12%) were sex discordant on U/S
- 19/31 (61%) were reported as 45,X; 12/31 (39%) were SC trisomy;
- Abnormal karyotypes confirmed in 8/23 (35%) of SC aneuploidy; 1/5 (20%) were inconclusive results
- Abnormal SC cfDNA results were associated with IVF (p=.001) and twins (p<.001)
- Sex discordance between cfDNA and U/S was associated with twins (p<.001)
- **Conclusion: Abnormal SC cfDNA results were associated with IVF and twins. Our results indicate cfDNA for sex prediction in twin of limited utility. PPV and sensi for SC determination were lower than previously reportsl,**

48

Clinical Utility : Twin Pregnancy

Fosler L, Platt LD. Ultrasound Obstet Gynecol 2017; 49: 470-477

- Objective of this study was to evaluate the use of NIPT in twin pregnancy
- Two sets of maternal blood samples from twin pregnancies were analyzed
  - 115 stored samples with known outcome (study A)
  - 487 prospectively collected samples which outcomes were requested from providers (study B)
  - NIPT used to screen for T13, T18, T21 and X and Y in all cases
  - Results compared with outcomes when known
- Study A: 115 samples classified correctly by NIPT
  - 3 cases T21 (1 fetus affected), 1 MC T18 (both fetuses affected) and 111 euploid
- Study B: NIPT results reported for 479/487 samples (98.4%)
  - Aneuploidy detected or suspected in 9 cases (1.9%); 7 cases T21 detected, 1 case T21 suspected and 1 case with T21 detected and T18 suspected
  - Aneuploidy outcome information available for 171 (35.7%) cases in Study B
  - 6/9 cases were confirmed to be a true positive in at least one twin-based on karyotype or birth outcome; 2 were suspected to be concordant-based on U/S findings
    - The 1 known discordant results was for the aneuploidy suspected case

▶No FN were reported

▶ **Conclusion: NIPT performed well in the detection of T21 in twin pregnancy, with a combined FP frequency for T13, T18, and T21 of 0% in Study A, and 0.2% in Study B**

49

Clinical Utility : Twin Pregnancy

Fosler L, Platt LD. Ultrasound Obstet Gynecol 2017; 49: 470-477

Table 2 Results of non-invasive prenatal testing (NIPT) in 115 twin pregnancies with known clinical outcome included in Clinical Study A

	Clinical outcome		NIPT result	
Sample size (n)	Twin A	Twin B	Aneuploidy	Chromosome Y
24	46,XX	46,XX	Not detected	Absent
45	46,XX	46,XY	Not detected	Present
42	46,XY	46,XY	Not detected	Present
2	47,XY + 21	46,XY	T21 detected	Present
1	Mosaic 47,XY + 21 [7]/46,XY [11]	46,XX	T21 detected	Present
1	47,XY + 18	47,XY + 18	T18 detected	Present
				DR: 91/91 (100%); Spec: 24/24 (100%)

50

Clinical Utility : Twin Pregnancy

Fosler L, Platt LD. Ultrasound Obstet Gynecol 2017; 49: 470-477

Table 3 Clinical outcome in nine twin pregnancies with non-invasive prenatal testing (NIPT) result of aneuploidy detected or suspected included in Clinical Study B

	NIPT result		Clinical outcome			
Sample	Aneuploidy	Chromosome Y	Twin A	Twin B	Source	Details
1	T21 detected	Present	XY + 21	XY	CVS	
2	T21 detected	Present	XY + 21	XY	CVS	
3	T21 detected	Present	XY + 21	XX	Amniocentesis	Triplet to dizygotic twins
4	T21 detected	Present	XY + 21	XX	Amniocentesis	
5	T21 detected	Present	XX + 21	XY	Visual exam at birth	
6	T21 detected	NA*	XY + 21	XX	Cord blood analysis	
7	T21 suspected	Present	XY	XX	Amniocentesis	1.27 Mb microduplication of 20q11 in one fetus
8	T21 detected	Absent	Abnormal findings†	Normal findings	Ultrasound exam	
9	T21 detected, T18 suspected	Present	Demise	NA		

51

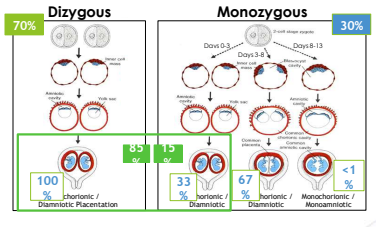
Clinical Utility : Twin Pregnancy

Liao H, et al. Prenat Diag 2017; 37 (9): 874-882
Gil, MM, et al. Ultrasound Obstet Gynecol 2017; 50 (3): 302-314

- In 2017 Liao et al performed a meta-analysis in twins and reported a pool sensitivity of 99% and a specificity of 100% for T21. However, the difference of DR between monozygotic and dichorionic twin pregnancies was not available for analysis as the number of monozygotic twins was limited.

Gil et al also determined strong performance characteristics with a DR for twins was 100% sensitivity, 100% specificity

52

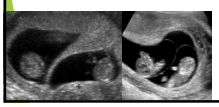


53

Accuracy of Sonographic Chorionicity Classification in Twin Gestations Blumenfeld e

MC = 90	**73**	**17 (DC)**		**81.1%**	**19% Missed Surveillance**
DC = 455	437	18 (MC)		96.0%	Increased Cost
Total = 545	510	25	11.4 vs 13.0 p=.03	93.6%	

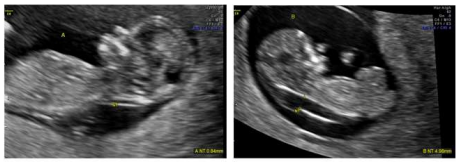
Natera Zygosity Validation Data
MZ 29/29
DZ 64/64



J Ultrasound Med 2014; 33:2187-2192

54

Clinical Utility : Twin Zygosity SNP



55

Clinical Utility - Microdeletions

- Few studies have described the success of cfDNA testing and the detection of deletions in chromosome 22 that can lead to the common 22q11.2 deletion syndrome
  - Syndrome can have major consequences if not detected early
  - Validation studies have been in high-risk populations; currently no studies in an average risk population
  - **Ultrasound may be able to detect ~80% of fetal 22q11.2DS because of the association with cardiac anomalies. The added benefit of cfDNA screening may be significantly reduced if ultrasound can detect associated anomalies.**
- **Further research is required before clinical utility in the average risk population and clinical utility data will be available to justify routine screening for all pregnant women**

56

Clinical Utility – Other Microdeletions

- A few papers have been published looking at cfDNA for testing non-22q deletions
- While tests are available, professional societies (ACOG, SMFM, ACMG) caution that cfDNA testing for microdeletion syndromes is not currently recommended.

Martin K, et al Clin Genet 2017; 93 (2): 293-300	Used SNP method, PPV 31.7% Overall FPV, 4 other major deletion syndromes, 0.07%
Hedgeson, J. et al. Prenat Diagn 2015; 35 (10); 999-1004	Used MPSS, 55 sub-chromosomal deletions Overall PPV 60% to 100% when follow-up available, with FPV 0.0017%
Minimal data on general risk population, no prospective data that speaks to there being tangible benefits with respect to outcome	

57

Association of CNV with specific U/S-detected anomalies -

Donnelly JC, Platt LD, et al. Obstet Gynecol 2014; 124 (1): 83-90

- Fetuses with structural anomalies were compared with those without detected abnormalities for the frequency of other-than-common copy number variants
- 1,082 fetuses with anomalies detected on U/S exam, 752 had a normal karyotype. CNV were present in 61 (8.1%) of these euploid fetuses
- Fetuses with >1 anomaly had a 13.0% frequency of benign CNV, significantly higher (P<.001) than the frequency (3.%) in fetuses without anomalies (n=1,922)
- Specific organ systems in which isolated anomalies were nominally significantly associated with benign CNV were the renal (P=.036) and cardiac system (P=.012), but did not meet significant after the adjustment
- Chromosomal microarray offers additional information over karyotype, the degree of which depends on the organ system involved

58

cfDNA screening in clinical practice: abnormal autosomal aneuploidy and microdeletion results

Valderramos SG, Platt LD et al. Am J Obstet Gynecol 2016; 215 (5): 626.e1-626.e10

- Investigate factors associated with the accuracy of abnormal autosomal cfDNA results
- 121 patients had abnormal cfDNA results for T21, T18, T13 and/or microdeletions
- 105 had abnormal cfDNA for T21, T18, T13
- 92 (87.6%) were positive and 13 (12.4%) were non-reportable
- Results of 92 positive cfDNA:
  - T21 (48, 52.2%)
  - T18 (22, 23.9%)
  - T133 (17, 18.5%)
  - Triploidy (2, 2.2%)
  - Positive for >1 parameter (3, 3.3%)
- **No association between FP rates and testing platform, but there was a difference between the 4 labs (P=.018)**
- **In all 26 patients had positive (n=9) or non-reportable (n=17) microdeletion results. 7/9 screen positive for microdeletions underwent confirmatory testing - all were FP**

59

cfDNA screening in clinical practice: abnormal autosomal aneuploidy and microdeletion results

Valderramos SG, Platt LD et al. Am J Obstet Gynecol 2016; 215 (5): 626.e1-626.e10

- Overall, PPV of cfDNA was 73.5% (61/83, 95% CI, 63-82%) for all trisomies
  - T21 83.0% (39/47, 95% CI, 69-92%)
  - T18 65.0% (13/20, 95% CI, 41-84%)
  - T13 43.7% (7/16, 95% CI, 21-70%)
- Abnormal cfDNA results were associated with positive serum screening
  - T21 (17/48, 70.8%)
  - T18 (7/22, 77.8%)
  - T13 (3/17, 37.5%)
  - Non-reportable (2/13, 16/7%)
  - P=.004
- Abnormal 1T U/S
  - T21 (25/24, 55.6%)
  - T18 (13/20, 65%)
  - T13 (6/14, 42/9%)
  - Non-reportable 1/13, 7/7%)
  - P=.003
- No association between FP rates and testing platform, but there was a difference between the 4 labs (P=.018)
- In all 26 patients had positive (n=9) or non-reportable (n=17) microdeletion results. 7/9 screen positive for microdeletions underwent confirmatory testing - all were FP

60

cfDNA screening in clinical practice: abnormal autosomal aneuploidy and microdeletion results

Valderramos SG, Platt LD et al. Am J Obstet Gynecol 2016; 215 (5): 626.e1-626.e10

- Conclusion:
  - PPV of 73.5% for cfDNA for autosomal aneuploidy is lower than reported
  - PPV for microdeletion testing was 0%
  - Diagnostic testing is needed to confirm abnormal cfDNA results for aneuploidy and microdeletions

61

Clinical experience of lab follow-up with NIPT using cfDNA and positive microdeletion results in 349 cases

Schwartz S, Plat LD, et al. Prenat Diagn 2018; 38 (3): 210-218

- Patient that were screened positive by NIPT for a microdeletion involving 1p, 4p 5p, 15q, or 22q - had invasive diagnostic studies by CVS or amnio
- Overall PPV for 349 patients was 9.2%
- 39.3% of the cases had additional abnormal microarray findings when microdeletion was confirmed
- Unrelated abnormal microarray findings were detected in 11.8% of patient in whom the screen positive microdeletion was not confirmed
- Stretches of homozygosity in the microdeletion were frequently associated with a FP cfDNA microdeletion result
- Conclusions::Confirmatory diagnostic microarray studies are imperative due to the high percentage of FP and the frequent additional abnormalities not delineated by cfDNA analysis.

62

Clinical Utility – Genome-Wide Imbalances

Ehrich M, et al. Genet Med 2017; 19 (12): 1332-1337

- Using MPSS that interrogate the genome beyond the typical trisomies and SCA,
  - Positive calls on 554 patients out of 10,000
  - 164 cases that would have been missed using only the typical cfDNA testing panel
  - Patients with known fetal anomalies, should be offered invasive testing using microarray analysis
    - If anomalies are already present, the delivery team and neonatal unit will be sufficiently prepared regardless if a screening test is available (limiting potential clinical utility)

63

DOI: 10.1002/pd.4583 PRENATAL DIAGNOSIS

ORIGINAL ARTICLE

Non-invasive prenatal diagnosis of achondroplasia and thanatophoric dysplasia: next-generation sequencing allows for a safer, more accurate, and comprehensive approach

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Clinical Chemistry 58:10
000—000 (2012)
Molecular Diagnostics and Genetics

Noninvasive Prenatal Diagnosis of Monogenic Diseases by Targeted Massively Parallel Sequencing of Maternal Plasma: Application to β Thalassemia

Kwan-Wood G. Lam,[1,2] Peiyong Jiang,[1,2] Gary J.W. Liao,[1,2] K.C. Allen Chan,[1,2] Tak Y. Leung,[3] Rossa W.K. Chiu,[1,2] and Y.M. Dennis Lo[1,2]*

64

Beyond screening for chromosomal abnormalities; advances in non-invasive diagnosis of single gene disorders and fetal exome sequencing

Hayward J, Chitty LS. Semin Fetal Neonatal Med 2018; 23 (2): 94-101

- The next general sequencing (NGS) offers new promises for safer prenatal genetic diagnosis
- These innovative approaches will
  - Improve screening for fetal aneuploidy
  - Allow definitive non-invasive prenatal diagnosis of single gene disorders at an early gestational state without the need for invasive testing
  - Improve our ability to detect monogenic disorders as the etiology of fetal abnormalities
- The introduction of whole genome, exome and targeted NGS produces unprecedented volumes of data requiring complex analysis and interpretation

65

Beyond screening for chromosomal abnormalities; advances in non-invasive diagnosis of single gene disorders and fetal exome sequencing

Hayward J, Chitty LS. Semin Fetal Neonatal Med 2018; 23 (2): 94-101

- The achievement of this goal requires the most progressive technological tools for rapid high-throughput generation at an affordable cost
  - Transforming modern healthcare and personalized medicine
- As larger proportions of patients with genetic disease are identified , appropriate genetic counseling to families and potential parents.
- Novel treatment targets will continue to be explored
  - Ethical considerations will likely be introduced as well, particularly if genome editing techniques are included in these targeted treatments

66

NIPT sequencing for multiple Mendelian monogenic disorders using cfDNA

Zhang J, et al. Nature 2019 [epub ahead of publication]

- Current NIPT screening is unable to detect many monogenic disorders associated with de novo mutations, despite their high incidence
- Zhang et al has been developing and validating a new approach for NIPT sequencing for a panel of causative genes for frequent dominant monogenic diseases (DMD)
- Pathogenic or likely pathogenic variants were confirmed by a secondary amplicon-based test on cfDNA
- 422 pregnancies w/w-out abnormal U/S findings or family history. F/U studies on bases with available outcomes resulted:
  - 20 confirmed true-positive
  - 127 true-negative
  - 0 false positive and 0 false negatives
- **Initial clinical study demonstrated that this NIPT can provide valuable molecular information for the detection of a wide spectrum of DMD, complementing current aneuploidy screening or carrier recessive disorder**

67

The questions being debated:

- Is cfDNA screening the best choice for *primary* screening for all patients?
- Is cfDNA the best choice for all types of pregnancies?
- Is cfDNA screening the best option for low risk patients?
- Is cfDNA a good choice for risks *other than* standard aneuploidies?

68

All patients deserve equal access

- ACOG indicates that testing should no longer be stratified by maternal age
- We've spent the past decade trying to abolish "advanced maternal age" and the age 35 cutoff
- It is not *ethical* to withhold this test
- But….There is a difference between *withholding* something for an individual patient, and recommending it for all patients as policy

69

Limitations of cfDNA Testing – Its Biological Reasons

- Although cfDNA testing accuracy is high, it is not a diagnostic test
- Small chance of discordance (FN/FP) when compared with the real fetal chromosomal constitution
- The reasons for the discordance may be due to technical issues or biological reasons (either fetal or maternal origin)
- Most relevant and frequent reason: insufficient/absent FF and fetoplacental mosaicism
  - Presence of fetoplacental mosaicisms is a major source for discordant cfDNA
  - If a confined placental mosaicism (Type I or III) is present, an FP cfDNA result may be detected.
  - Conversely, a true fetal mosaicism type V may result in a FN cfDNA result. The estimated FNR is independent of the level of mosaicism; 1/135 for T21; 164 for T18' and 1/136 for T13

70

Limitations of cfDNA Testing – Its Biological Reasons

- Insufficient fetal fragments can also cause discordant reults
  - Obese women tend to have more maternal circulating DNA causing a decrease in fetal fragment (diluting effect)
  - Gestation age can impact fetal fragments
    - Levels increase by 0.1% per week up to 21 weeks of gestation
    - Beyond 21 weeks, FF increases by 1% per week
- Fetal fragment in maternal plasma also increases with serum PAPP-A and β-hCG
  - Higher in Afro-Caribbean and East-Asian origins compared to Caucasian or non-Hispanic origin
  - Decreases in IVF and twin pregnancies
- Presence of T13 and T18, but not T21, can cause a decrease of FF due to decreased placental mass.

71

Limitations of cfDNA Testing – No Results

Gil MM, et al. Ultrasound Obstet Gynecol 2017; 50 (3): 302-314
Grati FR, et al. Ultrasound Obstet Gynecol 2017; 50 (1): 134-137

- cfDNA tests can fail. There are different reasons for the absence of a result:
  - Specimen handling issues, including administrative ones
  - Insufficient (<4%) fetal fragment
  - Technical reasons, e.g. quality metrics of the extracted DNA, such as reduced amplification
  - Biological reasons
- No result rate attributable to low fetal fragment ranges from 0.1% to 6.1%
- No result rate for reasons related to low quality assay ranges from 0.2% to 2.8%
- The reason for a "no-call" results is relevant because it influences the overall test performance
- "Theoretical modeling predicts that choosing a cfDNA-based platform with effective FF metrics and the lowest no result rates reduces the number of pregnancies in which the test has lower performance due to insufficient FF while increasing PPV in the reported population." [Grati]

72

Summary of the Definitions of Analysis of Performances, Clinical Performances, and Clinical Utility and the Study Designs Requested to Validate Them

	Performance Measure	Relevant Questions	Study Design
Analytic Validity - *Does this test work in the laboratory setting?*	Sensitivity (true positive rate)	How often is the test positive when an mutation is present	Usually retrospective case control design: Assess proportion of positive results among affected (often stored) samples that is correctly called
	Specificity (true negative rate)	How often is the test negative when a mutation is present?	

Modified from ACCE Model

73

Summary of the Definitions of Analysis of Performances, Clinical Performances, and Clinical Utility and the Study Designs Requested to Validate Them

	Performance Measure	Relevant Questions	Study Design
Clinical Validity- *Has this test been adequately validated on the populations to which it may be offered?*	Prevalence	What percentage of the population in question is affected with the specific disorder? The NPV and PPV are both dependent on prevalence.	Prospective blinded studies where patients receive both the new test and the previous "gold standard" test
	What is the positive predictive value (PPV)?	What is the chance that if the test is positive, the patient does have the disorder? PPV = number of true positives/(number of true positive + number of false positives)	
	What is the negative predictive value (NPV)?	What is the chance that if the test is negative the patient does not have the disorder? NPV = number of true negatives/(number of the true negatives + false negatives)	

Modified from ACCE Model

74

Summary of the Definitions of Analysis of Performances, Clinical Performances, and Clinical Utility and the Study Designs Requested to Validate Them

		Relevant Questions	Study Design
Clinical Utility- *What is the impact of the test on patient care?*	Are there effective interventions available in the case of a positive screen result?		Prospective, blinded, adequately powered randomized controlled trials to determine if there is any impact on clinical outcome (negative or positive)
	If applicable, are diagnostic tests available?		
	Is there general access to the intervention?		
	What are the financial costs and economic benefits associated with screening?		
	Are adequate educational materials in place?		
Modified from ACCE Model	What are the ethical, legal and social implications of this test?		

75

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- Prenatal diagnosis of fetal chromosomal anomalies has been a two-tier approach
  - 1st Tier – assess for risk osf T21, T18 or T13
  - 2nd Tier – apply a diagnostic technique to the screen positive cases
  - Superior DR for this broad range of fetal chromosomal anomalies relies on a FPR of 5% using standard "pre-cfDNA" screening approaches
- This reality has led to two very different thought patterns regarding prenatal diagnosis and the issue of FPR
  - Assumes goal of prenatal screening/diagnosis is to detect maternal age associated anomalies, T21, T18, and T13
  - Assumes goal of prenatal screening is to detect as many affected fetuses as possible
- Reasoning for the superiority of standard screening was based on routine karyotype as the ultimate test for prenatal diagnosis

76

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- **Dilemma:** ***Would the addition of cfDNA to standard screening be enough to compensate for the loss in the DR of other anomalies caused by a decrease in as FPR?***
- Norton et al partially answered the question
  - Study states that 16.9% of the karyotype anomalies diagnosed in women whose screening had been reported as "high risk" and have undergone fetal karyotyping would not have been detectable by cfDNA testing.
- Norton et al in a different study then reported a more accurate comparison between the DR of "sequential screening" and cfDNA test for all chromosomal anomalies diagnosed pre-/postnatally
  - DR for sequential screening was 81.6$ vs. 70% or 72% for cfDNA testing
- Two different results reported for cfDNA questions whether FF was reported and the related number of inconclusive cases

Norton ME, et al. Obstet Gynecol 2014; 124 (5):979-986
Norton ME, et al. Am J Obstet Gynecol 2015; 212 (1): S2

77

Rare chromosomal abnormalities: Can they be identified using convention 1T combined screening methods?

Kane DT, D'Alton ME, Malone F. Abstract #887 SMFM 2019 Am J Obstet Gynecol 2019; 220 (1): S576

- 36,120 patients underwent combined 1T screening. 129 were found to have 1 of the following chromosomal abnormalities: T21, T18, T13 or Turner
- 42 were found to have "other" rare chromosomal abnormalities e.g., triploidy, structural chromosomal abnormalities, SCA or unusual chromosomal abnormalities (47XX+16; incidence of 1.1/1,000 for RCA
- 2/42 (5%) had an NT measurement of ≥3 mm
- DR of combined 1T screening was 33% (14/42) – using a risk cut-off of ≥1:300
- 67% of cases of rarer fetal chromosomal abnormalities had a "normal" combined 1T screening risk (<1:300) and 95% had a "normal" NT (<3mm)

78

Rare chromosomal abnormalities: Can they be identified using convention 1T combined screening methods?

Kane DT, D'Alton ME, Malone F. Abstract #887 SMFM 2019 Am J Obstet Gynecol 2019; 220 (1): S576

- Conclusion:
  - Traditional 1T screening methods - unable to identify the vast majority of RCA
  - 1T NT measurement - very poor stand-alone screening test for rare chromosomal abnormalities (sensi 5%)
- Suggested: potential DR of RCA justifies the continued use of traditional NT/serum screening
- as the primary screening offered to pregnant women
- ***Our data suggest that a paradigm shift to NIPT as primary screening methodology should result in overall higher DR of aneuploidy with a significant reduction of FPR despite the lack of value of traditional NT/serum screening or RCA***

79

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- Summary: There are two competing views regarding prenatal testing of fetal genomic defects
  - One values the diagnosis of common aneuploidies. **cfDNA testing is the most efficient and accurate screening test and should be the screening test of choice t use in everyone.**
  - Second view values the diagnosis of as many genomic anomalies as possible as per the most advanced diagnostic techniques.
- Remains unclear … which approach has the highest DR for genomic abnormalities?
  - cfDNA based or serum/ultrasound based screening?

80

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- As previously discussed, there are two main reasons for failure:
  - Technical-related
  - Low FF
- Measuring FF is a best practice as it aids in explaining the reason for test failure and no result
- Current consensus among cfDNA testing providers … samples with <4% FF result should not be reported due to the increased FNR
- If result is reported as failed or no result for low quality metrics and >4% FF, a redraw may return a result.
- In obese patients, resampling for low quality metrics in combination with FF levels >4% to <10% on the first sample is less likely to be successful. The limitation of this type of testing should be raised during pre-test counseling
- Obese patients should be warned that their failure rate is likely to be higher, up to 24.3%

Yared E, et al. Am J Obstet Gynecol 2016; 215 ; 370.e1-6

81

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- When selecting the lab where to send your patients' samples, it is important that the physicians favor labs that
  - Report FF
  - Do not report results on samples with low FF
  - Have the lowest failed results rate for technical issues

Yared E, et al. Am J Obstet Gynecol 2016; 215 ; 370.e1-6

82

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- We've discussed the problem with fetal findings, but what about "maternal incidental findings"?
- Unintended consequence of cfDNA testing is the identification of maternal conditions, e.g., non-diagnosed cancer
- Prospective study reported a risk of maternal occult malignancy of 19% fo complex abnormalities detected by cfDNA testing
  - However, there is not enough data to reliably determine the DR, FPR, and the PPV for occult cancer in women undergoing cfDNA testing for fetal aneuploidy screening
- More frequently reported are the cases where the mother carries a mosaic cell line of 45,X or 46,XXX
  - An important source of FP for fetal SCAs in addition to confined placental mosaicism
  - Frequency of fetal SCAs among abnormal cfDNA testing results - around 40%

Wang E, et al. Prenatal Diagn 2013; 33:662-666

83

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- Absolute frequency of SCAs has been found to be between 0.4% to 0.6% of patient undergoing cfDNA testing for prenatal screening
- Wang et al found 8.7% (16/187) of patients diagnosed with SCAs by cfDNA as carriers of a maternal mosaic; 6 abnormal chromosome X gain and 10 had an abnormal chromosome X loss
- Frequency of maternal mosaicism for the X chromosome loss is likely to increase with maternal age. Mosaic 45,X was identified in 0.07% of girls <16 yrs and in 7.3% of women older than 65

Wang L, et al. Taiwan J Obstet Gynecol 2015; 54 (5): 527-531
Lau TK, et al. Ultrasound Obstet Gynecol 2014; 43 (3): 254-264
Zhang B, et al. J Int Med Res 2017; 45 (2): 621-630
Russell LM, et al. Cytogenet Geome Res 2007; 116 (3): 181-185
Reiss RE, et al. Prenat Diagn 2017; 37 (5): 515-520

84

Practical Problems Related to the Limitations of cfDNA Testing and How to Manage Them

- Another less common source of FP for fetal SCA … the presence of chromosome X maternal copy number variant (CNV)
- Wang et al identified this type of maternal genomic defect in 2/25 patients with
  - High-risk results for cfDNA testing for SCAs
  - Slight deviations of chromosomes X and Y from normal
  - Thus, an abnormal cfDNA test related to the number of X chromosomes should probably be investigated in the mother before the fetus. However, a finding in the mother does not exclude the same abnormality in the fetus

Wang S, et al Clin Chim Acta 2015; 444: 113-116

85

NIPT: need for informed enthusiasm

Han CS, Platt LD 2014; 211 (6): 577-580

- Since the inception of NIPT, the landscape of prenatal diagnosis has changed dramatically for genetic abnormalities
- NIPT is attractive to both patients and practitioners – earlier and improved DR at 10 weeks' gestation surpasses the 85=90% DR of traditional 1T screening with NT and serum analytes from 11-14 wks gestation
- Both pre-test and post-test genetic counseling is essential to ensure patients understand the limitations and benefits of NIPT before the test is ordered, as well as the results including FN, FP and no result of these euploid fetuses.

86

Conclusion

- Since introduction into clinical practice in 2010, the technical approach of cfDNA for fetal aneuploidy has evolved
- Our understanding of its potential applications and clinical implications has challenged existing prenatal diagnosis and screening paradigms
- Although a technically challenging test, cfDNA testing is easier to scale up than ultrasound-based screening
  - Easier to standardize, potentially decreases the error associated with the variation in the provider quality
- The number of labs providing cfDNA has increased
  - Increases the availability, competition, and consequent affordability expands the likelihood of error
- Reports from recent preliminary voluntary quality assessment survey to cfDNA testing reporting or MPSS-based technologies only. As such, uncovered suboptimal practices from some labs has been revealed.
- This corroborates the need for caution and care when selecting a lab and ensuring appropriate pre-/posttest counseling is provided

87

88

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