



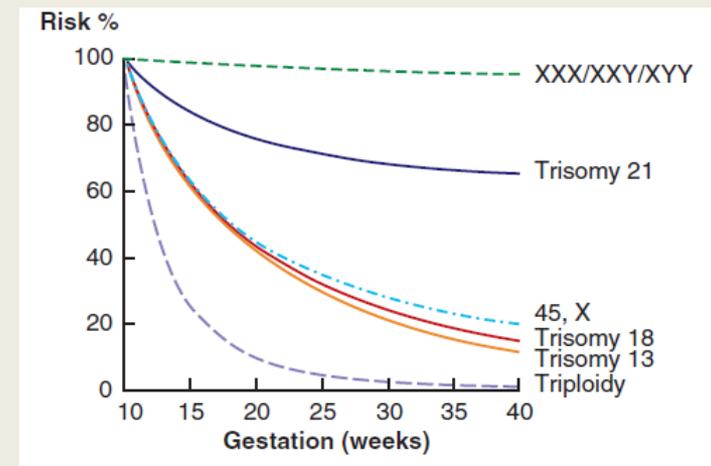
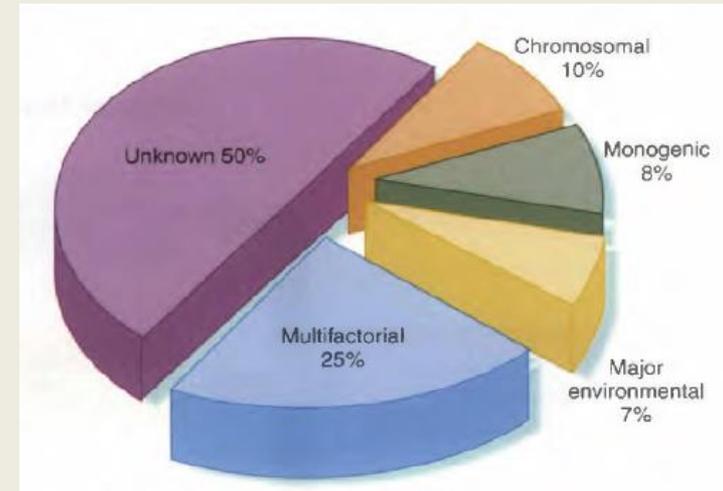
Non-Invasive Prenatal Testing Actual Place in Prenatal Diagnostic

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- Chromosomal abnormalities are:
 - present in 0,9% of the newborns
 - 10% -15% of conceptions are chromosomally abnormal.
 - the main cause of pregnancy loss.
- Almost 95% of abnormal chromosomal pregnancies are lost before term.
- The most severe anomalies lead to an early pregnancy loss, others allow the evolution of the pregnancies until the third trimester or even the birth of a viable newborn.
- They are an important cause of infantile mortality and morbidity
- **Aneuploidy** is the most common human chromosomal abnormality and is present in 3% - 4% of pregnancies.



- The most common aneuploidy detected at birth is **Trisomy 21**, with an incidence of 1/700 new-borns.
- Trisomy 21 is responsible for **95% of Down syndrome**
- **The risk of trisomy increase with maternal age**
- In trisomy 21 the fetal demise rate is:
 - 10% between 12-16 weeks
 - 20% between 17-40 weeks
 - **70% will arrive at term**
- *Because T21 is the most common type of **non-lethal** trisomy, it is the main point of prenatal screening and diagnostic protocols*

Table 2-4

Population Frequency of Specific Chromosomal Disorders

	Per 1000 Live Births*
Trisomy 21	1.5
Trisomy 18	0.12
Trisomy 13	0.07
47XXY (Klinefelter syndrome)	1.5
45X (Turner syndrome)	0.4
XYY syndrome	1.5
XXX syndrome	0.65

From Harpers PS: Practical Genetic Counseling, 6th Edition

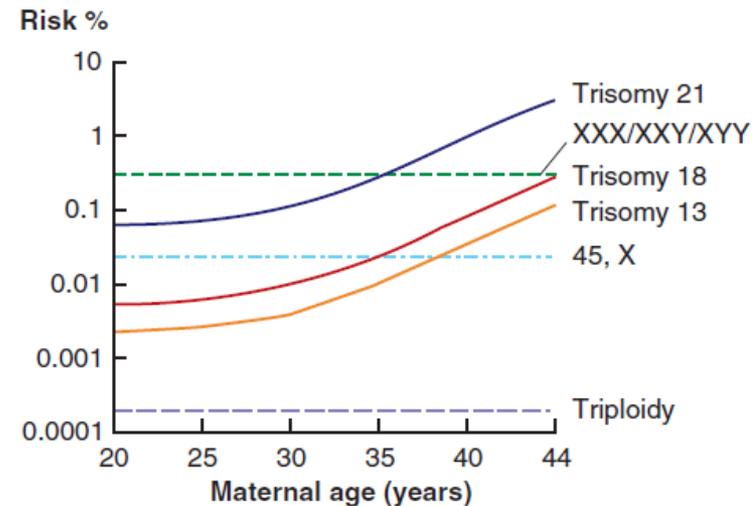


FIGURE 12-1 Maternal age-related risk for selected aneuploidies. (Redrawn from Nicolaides, 2004, with permission.)

Aneuploidy screening – History

- Advanced maternal age > 35 years - risk 1/375
- Second trimester screening by triple/quad test
- Genetic ultrasound (16-23 SA weeks) with anomaly detected: 75%
- First trimester screening (serum & ultrasound)
- Screening test for **all** pregnancies before 20 weeks.
- Combination between methods in order to improve detection rate

ACOG – 2007

- Irrespective of age, **all** women are **counseled** between the difference of **screening** and **diagnostic** tests

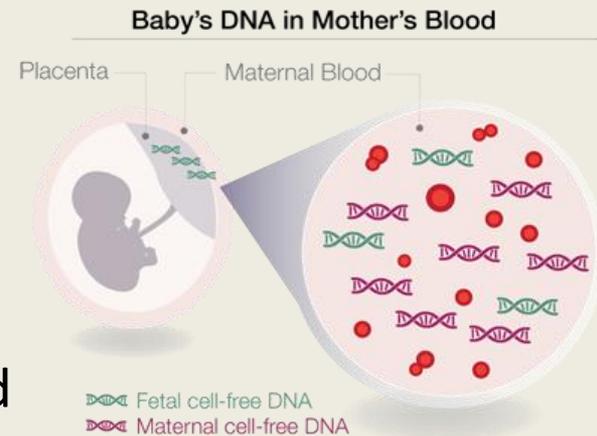


Aneuploidy screening – performance of classic techniques

First trimester	%
Maternal age	30-50
PAPP-A, hCG, MA	60-63
NT measurement and MA	74-80
Combined test (NT, PAPP-A, hCG, MA)	86-90
Combined test plus nasal bone, tricuspid flow, ductus venosus and facial angle	95
Second trimester	
Maternal age	30-50
2 nd trimester double test (AFP, hCG, MA)	60
Triple test (AFP, hCG, E3, MA)	68
Quadruple test (AFP, hCG, E3, Inhibin A, MA)	79
Ultrasound (16-23 weeks) with anomaly screening	75
Invasive diagnostic testing	
Chorionic villus sampling	close to 100
Amniocentesis	close to 100

Non Invasive Prenatal Testing -NIPT

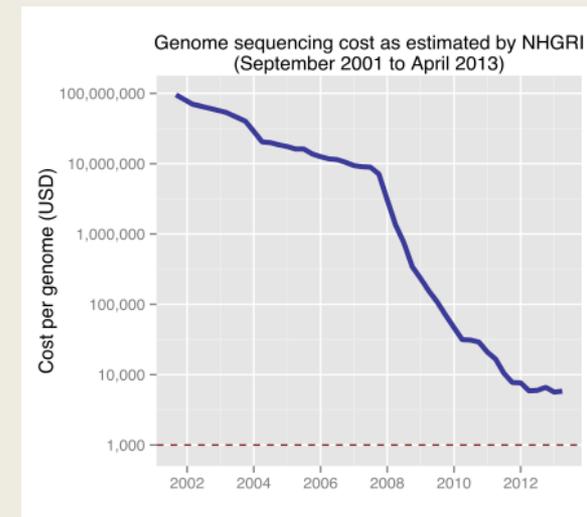
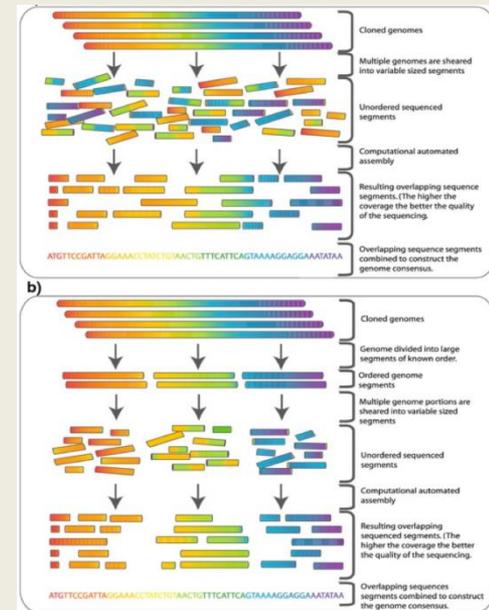
- In **1997 cell free fetal DNA** (*cff* DNA) was detected in maternal circulation from apoptotic placental cells.
- Actual techniques do not permit the complete separation of fetal DNA from maternal DNA in women plasma
- But, modern technique of DNA **sequencing** allow a very exact measurement of DNA fragments, and so, the **additional DNA fragments** that result from a trisomy or other fetal chromosomal anomaly detected in the maternal plasma can be very precisely identified.



Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.

Sequencing cff DNA techniques

- Shotgun Massively Parallel Sequencing (**s-MPS**) – next generation
 - DNA molecules from maternal plasma are randomly sequenced and the proportion of molecules of the chromosome of interest (cr. 21 i.e.) are compared with the proportion sequenced in other region of the genome.
 - The method can potentially detect all the genetic or chromosomal anomalies in the genome (potentially the sequencing of all the fetal genome from maternal plasma)
 - Require an important mathematical processing abilities,
 - Disadvantage: it sequence also zones of the genome that are not of diagnostic interest and price
- Targeted Massively Parallel Sequencing (**t-MPS**)
 - There are selected for sequencing only genomic regions from chromosomes with risk of trisomy and references regions.
 - The sequencing power is focused only on the interest regions.
- Single nucleotide polymorphism (**SNP**)-based approaches



Clinical utility of cff DNA

- Fetal **Rh group detection** in pregnancies of Rh negative woman.
- Determination of **fetal gender** in X-linked diseases.
- Prenatal diagnostic of **chromosomal, sub chromosomal** and **monogenic** anomalies
- The techniques applied have developed:
 - DNA – methylation
 - Analyze of fetal free RNA
 - Counting the rapport of alleles (fetal –maternal)
- The use of methods that allow single-molecule counting techniques using digital PCR
- Very fast evolution of **indications** also:
- **Towards prenatal fetal whole genome sequencing**

The indications for cffDNA

- TRISOMIES
 - Trisomy 21 (Down syndrome)
 - Trisomy 18 (Edwards syndrome)
 - Trisomy 13 (Patau syndrome)
 - Trisomy 16
 - Trisomy 22
- FETAL SEXUAL CHROMOSOMES
 - Fetal gender
 - 45,X (Turner syndrome)
 - 47 XXY (Klinefelter syndrome)
 - 47 XXX (Triple X syndrome)
 - 47 XYY
- MICRODELETIONS
 - 22q11.2 (DiGeorge syndrome)
 - 5p (Cri du chat syndrome)
 - 1p36 deletion syndrome
 - Syndrome Prader-Willi
 - Syndrome Angelman

Results of NIFT in aneuploidy screening

- Very good for T21 and T 18 when sequencing is successful with sensibility and specificity of 98,6- 100%
- Results for T13 have rapidly improved (Panorama, LIFECODEXX, Illumina >99%)
- **Today it is considered as a very good screening test. It is not a diagnostic test**

Table 2 Down syndrome and Edwards syndrome results in seven cell-free DNA studies carried out in high-risk pregnancies

Trial	Down syndrome		Edwards syndrome	
	DR (n (%))	FPR (n (%))	DR (n (%))	FPR (n (%))
Chiu <i>et al.</i> ^{65*}	86/86 (100)	3/146 (2.1)	—	—
Ehrich <i>et al.</i> ⁶⁶	39/39 (100)	1/410 (0.24)	—	—
Palomaki <i>et al.</i> ^{67,72}	209/212 (98.6)	3/1471 (0.20)	59/59 (100)	5/1688 (0.30)
Bianchi <i>et al.</i> ⁶⁸	89/90 (98.9)	0/410 (0.00)	35/38 (92.1)	0/463 (0.00)
Sparks <i>et al.</i> ⁷⁰	36/36 (100)	1/123 (0.81)	8/8 (100)	1/123 (0.81)
Ashoor <i>et al.</i> ⁶⁹	50/50 (100)	0/297 (0.00)	49/50 (98.0)	0/297 (0.00)
Norton <i>et al.</i> ⁷¹	81/81 (100)	1/2888 (0.03)	37/38 (97.4)	2/2888 (0.06)
Total	590/594 (99.3)	9/5745 (0.16)	188/193 (97.4)	8/5459 (0.15)

*2-plex and 8-plex data were reported but only the more favorable 2-plex results are included here. DR, detection rate; FPR, false-positive rate.

Ultrasound Obstet Gynecol 2013; 42: 15–33 Non-invasive prenatal testing for aneuploidy: current status and future prospects
P. BENN*, H. CUCKLE† and E. PERGAMENT‡

DNA Sequencing versus Standard Prenatal
Aneuploidy Screening

Diana W. Bianchi, M.D., R. Lamar Parker, M.D., Jeffrey Wentworth, M.D., Rajeevi Madankumar, M.D., Craig Saffer, M.D., Anita F. Das, Ph.D., Joseph A. Craig, M.D., Darya I. Chudova, Ph.D., Patricia L. Devers, M.S., C.G.C., Keith W. Jones, Ph.D.,

DNA SEQUENCING VS. STANDARD ANEUPLOIDY SCREENING

Table 3. Test Performance.*

Trisomy	No. of Cases	cfDNA Testing	Standard Screening
		% (95% CI)	
Trisomy 21	5		
Sensitivity		100 (47.8–100)	100 (29.2–100)
Specificity		99.7 (99.3–99.9)	96.4 (95.4–97.2)
Positive predictive value		45.5 (16.7–76.6)	4.2 (0.9–11.7)
Negative predictive value		100 (99.8–100)	100 (99.8–100)
Trisomy 18	2		
Sensitivity		100 (15.8–100)	100 (2.5–100)
Specificity		99.8 (99.6–100)	99.4 (99.0–99.7)
Positive predictive value		40.0 (5.3–85.3)	8.3 (0.2–38.5)
Negative predictive value		100 (99.8–100)	100 (99.8–100)

Conclusions

- Very efficient in high-risk group and also in low-risk group
- In a general obstetrical population (low-risk), prenatal testing with the use of cffDNA had significantly lower false positive rates and higher positive predictive values for Detection of trisomies 21 and 18 than standard screening.

Table 3: Clinical trials validated cffDNA analysis for detection of fetal aneuploidies

Studies	Trisomy 21		Trisomy 18		Trisomy 13	
	Sensitivity % (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity % (95% CI)	Sensitivity % (95% CI)	Specificity % (95% CI)
Palomaki <i>et al.</i> , 2011 ^[40]	98.6 (95.9-99.7)	99.8 (99.4-99.9)	-	-	-	-
Palomaki <i>et al.</i> , 2012 ^[41]	-	-	100 (93.9-100)	99.7 (99.3-99.9)	91.7 (61-99)	99.1 (98.5-99.5)
Bianchi <i>et al.</i> , 2012 ^[42]	100 (95.9-100)	100 (99.1-100)	97.2 (85.5-99.9)	100 (99.2-100)	78.6 (49.2-99.9)	100 (99.2-100)
Dan <i>et al.</i> ; 2012 ^[43]	100	99.96	100	99.96	-	-
Norton <i>et al.</i> ; 2012 ^[44]	100 (99.5-100)	99.97 (99.8-99.99)	97.4 (86.5-99.9)	99.93 (99.75-99.98)	-	-
Ashoor <i>et al.</i> ; 2012 ^[45]	100	100	98	100	-	-
Sparks <i>et al.</i> ; 2012 ^[46]	100	99.2	100	100	-	-
Ashoor <i>et al.</i> T 13; 2012 ^[47]	-	-	-	-	80 (49-94.3)	99.9 (99.7-100)
Nicolaidis <i>et al.</i> ; 2012 ^[26]	99	99.9	99	99.9	-	-

Advantages of NIPT

- False negative rate almost zero
- False positive rate much smaller than all the other screening tests but no zero!
- Technical possibilities of detecting also microdeletions, duplications, translocations and others chromosomal abnormalities.
- A negative NIPT exclude the necessity of an invasive test.
- Reduce with almost 89% the number of unnecessary amniocentesis and *CVS secondary* to a positive screening test
- Reduce the number of pregnancy loss due to an unnecessary invasive test
- Can be utilized like a contingency test, for patients with results in the alarm-zone.
- Can be utilized in patients with a positive screening test who refuse amniocentesis.

Universal screening with NIFT

100.000 pregnancies				
	Trisomy 21 N = 200		Normal pregnancies 99.800	
Screening method	Detection rate	Detected	False positive	
Maternal age	30%	60	5%	4990
Triple/quad test	70%	140	5%	4990
Combined test at 11-13 weeks	90%	180	5%	4990
cffDNA	99.5%	199	0.06%	80

At 1% pregnancy lost at invasive procedure, it means that 49 healthy pregnancies will be lost, and 4951 unnecessary invasive procedures will be performed.

Disadvantages of NIPT

- It is still expensive
- If it is directed only for the most frequent aneuploidies it will not detect others chromosomal anomalies (the cytogenetic FISH technique has the same risk)
- It will sometimes detect anomalies with an undetermined significance and unknown prognostic that necessitate a fetal (ultrasound) and maternal (cancer, karyotype) follow-up

Error factors influencing the NIPT

- Gestational age before 10 weeks (rapidly diminishing)
- **Fetal fraction of cffDNA**
- Maternal obesity
- Multiples pregnancies (twins):
 - Require an ultrasound examination prior to sampling to determine chorionicity (surrogate for zygosity).
 - Allow the determination of zygosity and the aneuploidy risk for each fetus in dizygotic twins. - 2 studies
- Maternal diseases: cancer, maternal chromosomal abnormalities, somatic mosaicism
- Confined placental mosaicism
- Maternal copy-number imbalance

Error factors influencing the NIPT

Table 1. True and False Positive Cases with Nonmosaic Karyotypes.

Chromosomal Abnormality	True Positive Result (N = 238)	False Positive Result (N = 56)
	<i>no./total no. (%)</i>	
Trisomy 13	14/26 (54)	12/26 (46)
Trisomy 18	40/52 (77)	12/52 (23)
Trisomy 21	161/177 (91)	16/177 (9)
Monosomy X	8/21 (38)	13/21 (62)
XXX or XXY	15/17 (88)	2/17 (12)
XYY	0/1	1/1 (100)

Fetal fraction

- Represent the percentage of cffDNA of fetal origin in maternal plasma
- An aneuploidy sample with a lower fetal fraction has a higher probability of resulting in a false negative result. (T. Musci 2013)
- Fetal fraction
 - 0-4% = too low to report. Require **resampling**
 - 4-8% = low fetal fraction that gives a decrease sensitivity with actual techniques. Require **additional work-up**
 - > 8% = fetal fraction adequate for best performance
- **Ask the providers of NIFT to mention the fetal fraction in their results !!!**

NIFT in multiple pregnancies

- Antenatal diagnostic in twins pregnancies is mandatory because the number of aneuploidies is increased and the risk of invasive diagnostic procedures for confirmation is also increased
- The **zigosity** is the main factor that influence the results
- In monozygotic monochorionic twins the 2 fetuses contribute with the same amount of cffDNA – the aneuploidy risk and the testing is the same like in single fetus pregnancy
- In dizygotic twins the contribution of each fetus to the DNA amount is **unequal**, sometimes at 1:2 ratio
 - In a dizygotic pregnancy, with a fetus with aneuploidy, the affected fetus may have a *fetal fraction lower than 4% and thus giving a false negative result*
- **in twins the fetal fraction is lower than in a single fetus pregnancy (7.4% in twins versus 10% in singleton)**

NIFT in multiple pregnancies

- In multiples it was proposed to use in the assessment the **lower fetal fraction**, and not the general fetal fraction. This approach will result in:
 - More cases without a result for a fetal fraction $<4\%$
 - Less cases with false - negative results
 - In cases with low FF $< 4\%$ an option is to rely on the results of combined test for the decision of an invasive testing
- In twins discordant for aneuploidy:
 - the use of **SNP** - single nucleotide polymorphism – allow the determination of zygosity and the distribution of fetal DNA for each fetus
 - The use of ultrasound evaluation may help in the identification of the fetus that needs an invasive procedure.
- **The results of NIFT in twins is feasible, with results almost as good as in single fetus pregnancy, but the rate of reporting results is lower (lower FF)**

Cell-Free DNA Analysis for Trisomy Risk Assessment in First-Trimester Twin Pregnancies

Maria del Mar Gil Maria Soledad Quezada Barbara Bregant Argyro Syngelaki
Kypros H. Nicolaides

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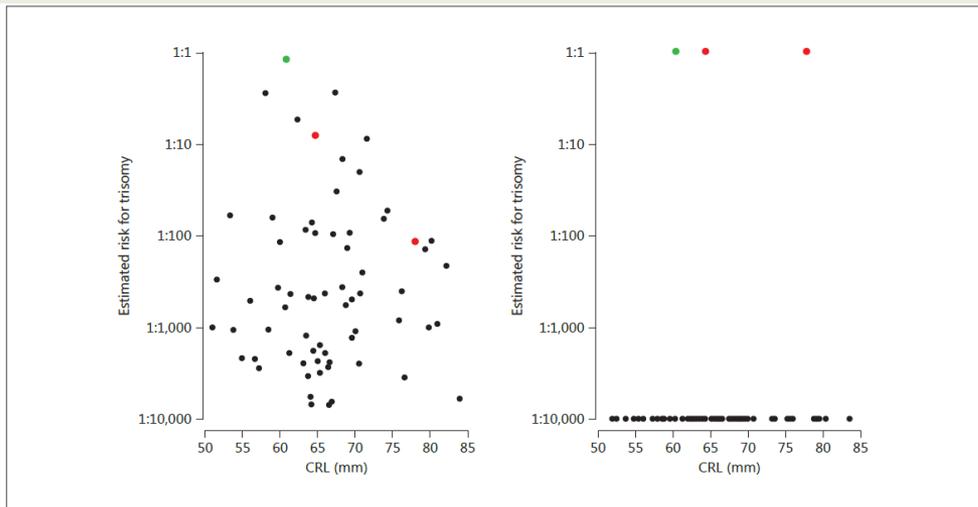


Fig. 1. Estimated risk for trisomies 21, 18 or 13 in the pregnancies with trisomy 21 (red circle), trisomy 18 (green circle) and assumed euploid (black circle) fetuses by the combined test (left) and maternal blood cfDNA test (right).

Other major advantages of cffDNA testing, compared to the combined test, are:

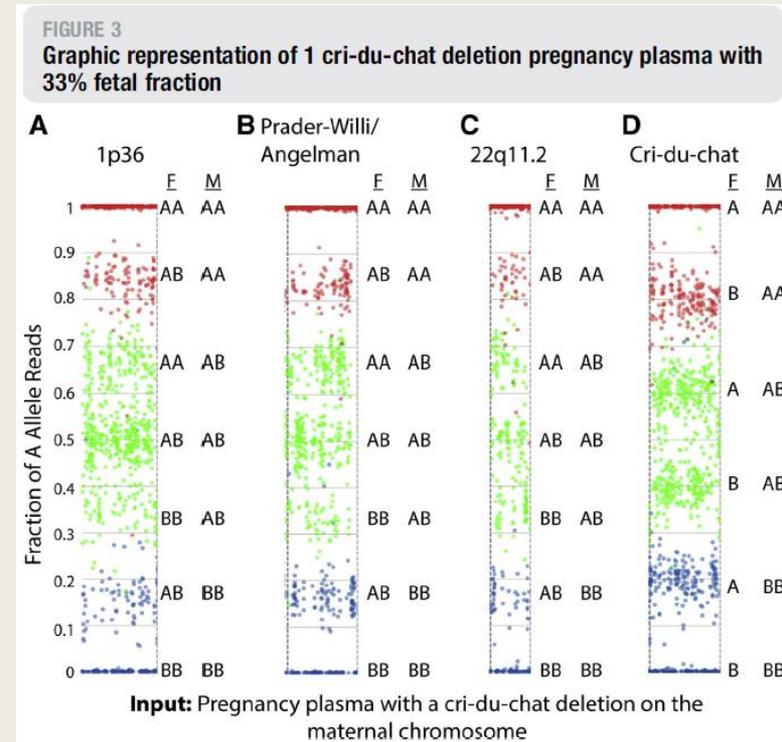
- reporting of results as a **very high** or **very low risk** which makes it easier for parents to decide in favor or against invasive testing
- a substantial reduction in the false positive rate.

NIFT in Microdeletions

- Represent subchromosomal abnormalities (microdeletions and duplication) that may result in **physical and/or intellectual impairments** that can be more severe than whole chromosome abnormalities.
- The incidence of subchromosomal copy number variations (CNVs) is **independent of the maternal age**
- Clinically significant microdeletions affect **1-1,7%** of all structurally normal pregnancies
- **In young women the risk of microdeletion is higher than the risk of T 21**
- In many cases the diagnostic is made during childhood, thus missing the benefit from an early therapeutic intervention
- In support of this, it is recommended that chromosome **microarray CGH** analysis be offered to **all women** who undergo invasive diagnostic testing.
- The possibility of using NIFT (targeted SNP-based approach) to detect the microdeletion syndromes with clinically severe phenotypes.

NIFT in Microdeletion

- 22q11.2 (Di George syndrome)
- 5p (Cri du chat syndrome)
- 1p36 deletion syndrome
- Prader-Willi Syndrome
- Angelman Syndrome



Expanding the scope of noninvasive prenatal testing:
detection of fetal microdeletion syndromes

Ronald J. Wapner, MD; Joshua E. Babiarez - Am J Obstet
Gynecol 2015;212:332.e1-9.

- The screening for microdeletion **is possible**.
- Because each microdeletion is rare, it is important that the false-positive rate for each is very low, while retaining
 - a high positive predictive value and
 - a very high negative predicted value

TABLE 4
Estimated positive predictive value and negative predictive value

Disorder	Incidence (1:n)	Frequency of deletion evaluated	Positive predictive value, ^a %	Negative predictive value, ^b %
22q11.2 del	2000	0.87	5.3	>99.99
Prader-Willi	10,000	0.28	4.6	>99.99
Angelman	12,000	0.28	3.8	>99.99
1p36 del	5000	0.60	17.0	>99.99
Cri-du-chat	20,000	0.65	5.3	>99.99

Expanding the scope of noninvasive prenatal testing: detection of fetal microdeletion syndromes Ronald J. Wapner, MD; Joshua E. Babiarez - Am J Obstet Gynecol 2015;212:332.

NIFT in Triploidy

- The affected embryos have an additional chromosomal haploid set of
 - maternal origin (digynic triploidy) or of
 - paternal origin (diandric triploidy)
- In the first trimester the frequency is 1/2.000
- In the 2nd and 3rd trimester the frequency is 1/250.000
- First trimester associated screening can collaterally identify 85% of cases with triploidy with a false positive rate of 5%
- cffDNA testing by targeted sequencing and allelic ratio analysis of single nucleotide polymorphisms covering ch. 21, 18, 13, X, and Y can
 - **detect diandric triploidy** (FF very high, may suggest also dizygotic twins)
 - **raise the suspicion of digynic triploidy.** (FF < 0.5%)

[Fetal Diagn Ther.](#) 2014;35(3):212-7. doi: 10.1159/000355655. **Prenatal detection of fetal triploidy from cell-free DNA testing in maternal blood.** [Nicolaidis KH¹](#), [Syngelaki A](#), [del Mar Gil M](#), [Quezada MS](#), [Zinevich Y](#)

Detection of monogenic diseases

- **The most frequent are de novo mutations**
- A monogenic disease can be diagnosed in a targeted manner in cases with familial history or with suggestive ultrasound markers. (short limbs for thanatophoric dysplasia or achondroplasia)
- It involves the detection of a paternally inherited fetal mutation that is not present in the mother's genome (Amicucci et al., 2000; Chiu et al., 2002).
- An approach called **relative mutation dosage** that could be used for the NIPT of virtually all monogenic diseases (Lun et al., 2008).
- *This approach allows one to deduce a fetal genotype based on the measurement of **subtle differences in concentrations of a mutant and a normal gene, as well as between two alleles of a SNP, using digital PCR.***

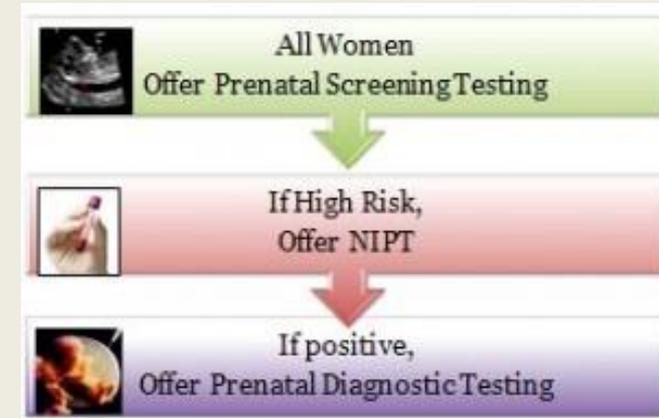
- This method has now been implemented successfully for the NIPT of **hemophilia** (Tsui et al., 2011) and **sickle cell anemia** (Barrett et al., 2012).
- It can potentially be applied to any genetic disease
 - **B-thalassemia**
 - **Cystic fibrosis**
 - **Tay Sachs**
 - **Muscular dystrophy**
 - **Achondroplasy**

Cystic fibrosis

- Mutations of the gene CFTR with autosomal recessive transmission
- Fetal risk of 25% if one of the parents is a carrier
- Is a treatable disease with a life expectancy of 40 years.
- With the diagnostic possible in the first trimester it is probable that the majority of these pregnancies will be interrupted.

Screening strategies for NIPT

- ACOG 2011 :
 - Screening in high-risk pregnancies (age > 35, aneuploidy at previous pregnancy or in family)
 - Contingent test with first trimester screening with moderate or high risk results
 - **Ultrasound anomalies**
- Canada 2013:
 - In high risk pregnancy like an alternative for direct invasive testing
 - If positive, amniocentesis before pregnancy termination .
- Can be used like a **primary screening procedure in all pregnancies**, also in the low-risk group. (NEJM feb 2014, 75% of aneuploidies are in the low-risk group)
- RCOG – 2014 – take in consideration all the possibilities for the NHS (National Health System), but **favors the primary screening with NIPT**

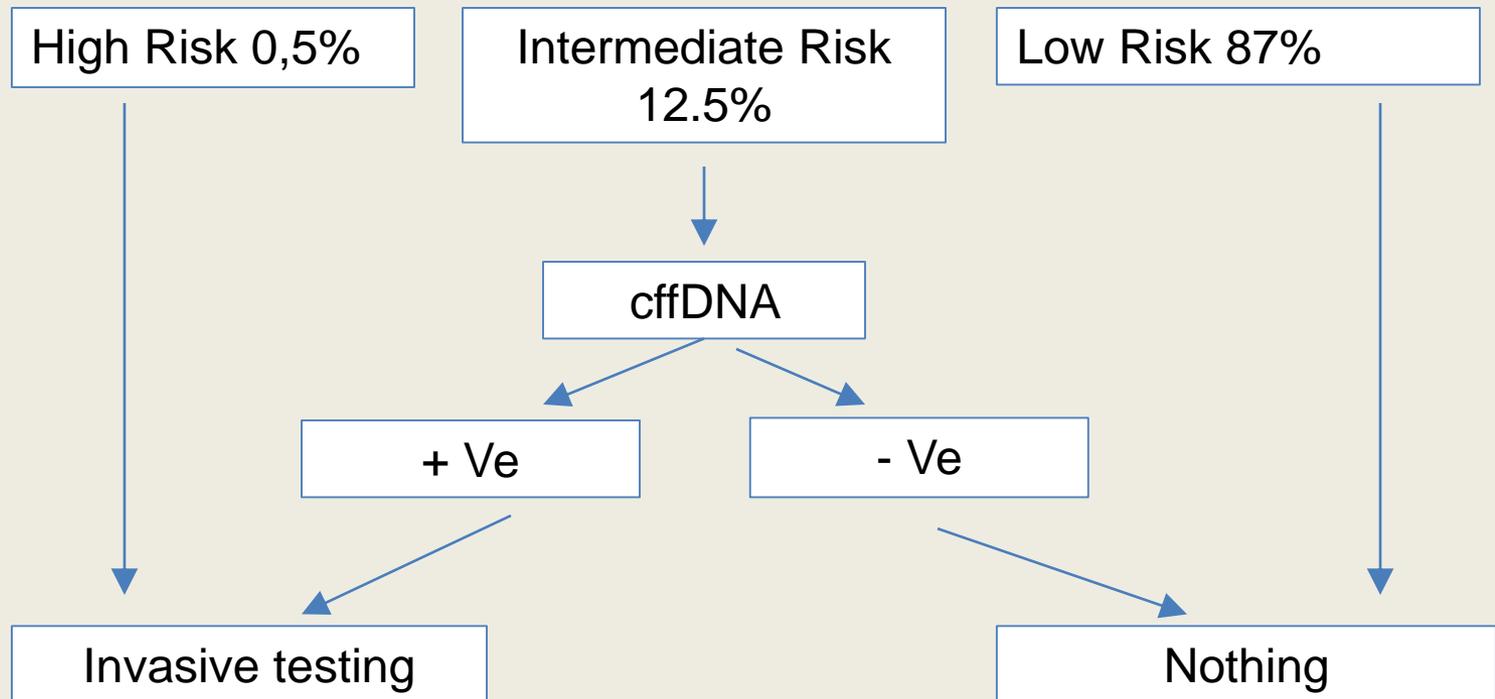


ACOG 2013

“Positive findings on noninvasive prenatal screening must be followed by invasive prenatal diagnostic testing before any irreversible decisions are made”

Contingent screening with cffDNA

Combined testing at 11-13 SA (serum & ultrasound)



Contingent screening with cffDNA

- Advantages:
 - Low cost for best results
- Disadvantage:
 - Long time to wait for the results

Relation of NIPT with ultrasound

- The ultrasound examination keep its place in the diagnostic of congenital malformations – early morphological scan at 12-14 weeks
- Because NIPT at this moment do not test for all the chromosomal anomalies and genetic rearrangements, if at an ultrasound examination an anomaly was detected, an invasive test will be proposed.
- If the NIPT detects an anomaly with a unknown signification, ultrasound examination is required.
- The early ultrasound scan at 11-14 weeks can detect others anomalies in which nuchal translucency is increase (some triploidies and sub chromosomal anomalies not yet tested by NIFT)
- In the second trimester NIFT can be proposed to patients with a significant malformations or with more soft-markers.
- NIPT will be indicated in a targeted mode for ultrasound detected abnormalities that are well-known to be associated with punctual genic mutations.

- NIPT do not detect all the significant chromosomal anomalies¹.
- The frequency of these anomalies can reach 23%
- These anomalies appears more frequently in cases with:
 - NT >3,5 mm
 - free β -HGC (<0.2 or \geq 5.0 (MoM))
 - PAPP-A <0.2MoM.
- These reasons justify the use of ultrasound at 11-14 weeks associated with NIFT



“Ultrasound will remain an important tool to detect malformations that indicate a possible trisomy, genetic anomalies that are not specifically tested for, and noninherited anomalies, as well as to provide information on phenotypes” – RCOG 2014

1 Ultrasound Obstet Gynecol 2014; 43: 265–271

Potential diagnostic consequences of applying non-invasive prenatal testing: population-based study from a country with existing first-trimester screening

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Conclusion

- The bioethical principle of nonmaleficence (the Hippocratic Oath: “**first, do no harm.**”) justifies revising the current standard of care.
- Offer **all** expectant mothers prenatal screening testing
 - Ultrasound and biochemical serum testing
- If the screening test indicates a higher likelihood of having a child with Down syndrome, or there exist other recognized factors to consider the mother “high risk,” then offer NIPT
- If the NIPT result is positive, then offer invasive diagnostic testing.

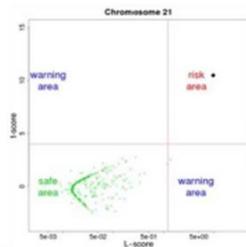
Conclusion

- It is important to address the ethical, legal and social issues surrounding the developments of NIFT
- These aspects need to be discussed by all the parties involved in prenatal care
- Materno-fetal specialists and obstetricians must know these tests, their indications, possibilities and limitations, in order to counsel the patients about the strategy that satisfied the best their interest in the choice of prenatal tests.

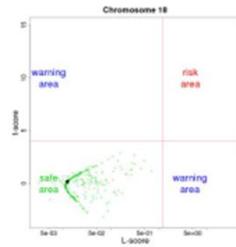
Thank you

Results:

Chromosome 21



Chromosome 18



Sample quality test: Passed

