

Non-Invasive Prenatal Diagnosis of Down Syndrome

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Philippos C. Patsalis
Chief Executive Medical Director



**THE CYPRUS INSTITUTE OF
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Prenatal Diagnosis

- Prenatal diagnosis is testing for diseases or conditions in the fetus before it is born.

Indications for Diagnostic Tests

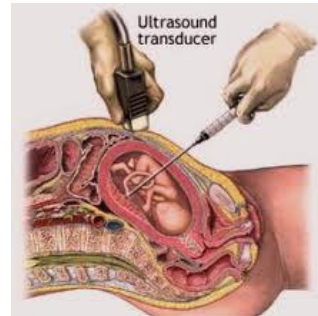
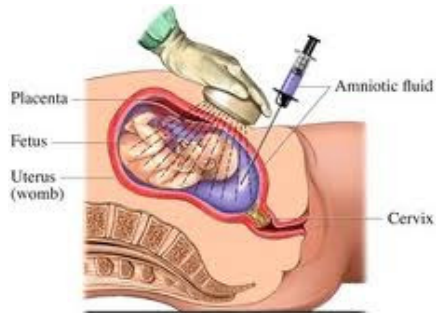
- Advanced maternal age (>35 y), the most common indication
- Previous offspring with chromosomal anomalies or birth defects
- Parental balanced translocation, inversion, or both
- Suggestive fetal U/S findings
- Positive maternal screening test findings
- Mendelian genetic trait in the parents
- Mother having a disease or being exposed to drugs, medications, or infections known to be associated with congenital abnormalities

- **Prenatal testing**

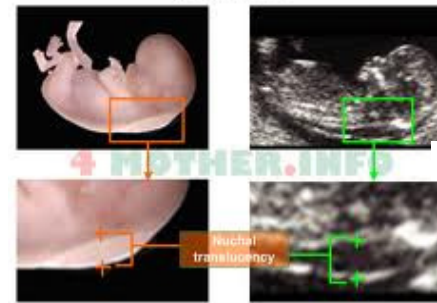
- screening (non inherited disorders)
- diagnostic (inherited disorders)

Prenatal Diagnosis

• Invasive



• Non-Invasive



- Advantages
 - Accurate ~100%
- Disadvantages
 - 1% risk for spontaneous abortion
 - Can not be applied to all pregnancies
 - Can not have effective prevention

- Advantages
 - No risk for spontaneous abortion
 - Can be applied to all pregnancies
- Disadvantages
 - Not that accurate ~80-90%
 - Can not have effective prevention

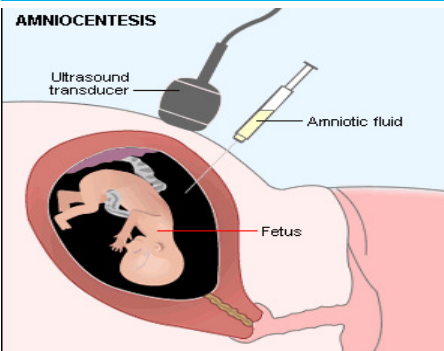
Prenatal Diagnosis of Down Syndrome

- Down Syndrome is one of the most common syndromes and the most common cause of Intellectual Impairment (1/700 births)



NIPD tests will be based on blood collection from pregnant women and investigation of the fetal DNA present in maternal circulation

• Invasive



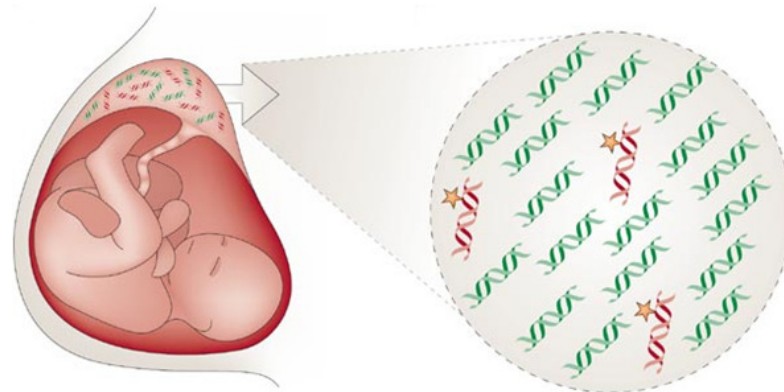
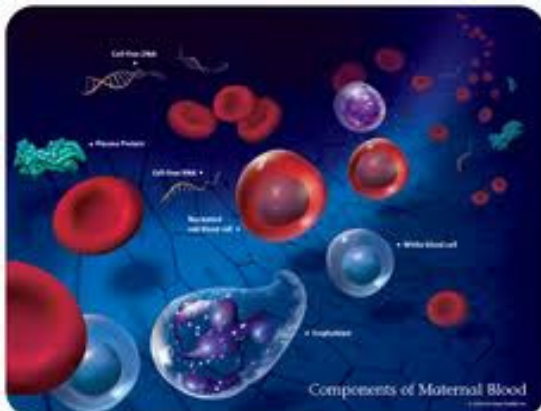
• Non-Invasive



- Urgent need for the development of Non Invasive Prenatal Diagnosis
 - Do not put the fetus at risk for spontaneous abortion
 - Can be offered to all pregnancies
 - Provide a more effective prevention

Towards the development of non-invasive prenatal diagnostic

- Identification of plasma nucleic acids in healthy and sick individuals. Mandel and Metais (C R Seances Soc Biol Fil., 142(3-4):241-243, 1948)
- Isolation of fetal cells from maternal blood. Very limited amount, present in high maternal background. Bianchi DW et al. (Am. J. Hum. Genet., 61:822-829, 1997)
- Identification of small amounts free fetal DNA in maternal plasma. Limitations: Very limited amount (3-6%), and ffDNA is 50% identical with the maternal DNA background. Lo YM et al. (Lancet, 359:485-487, 1997)



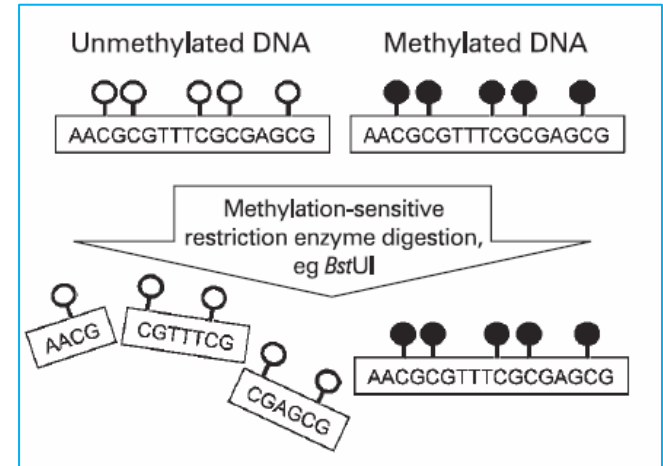
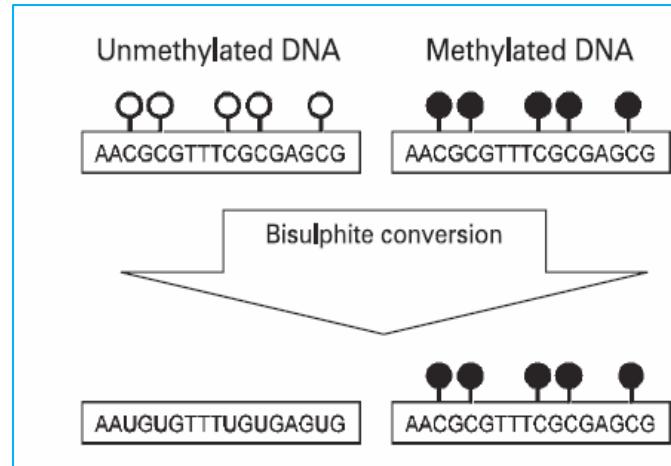
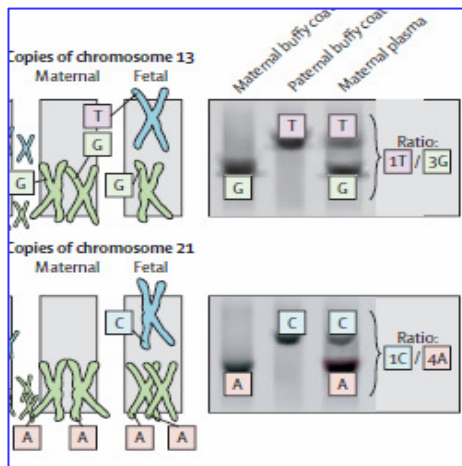
Identification of fetal-specific markers

- Identification of fetal DNA in maternal plasma and serum. Furthermore, they identified Y chromosome sequences in pregnancies bearing a male fetus demonstrating NIPD for X-linked disorders. Avent ND and Citty. Prenatal Diagnosis, 26:598-603, 2006),
 - Demonstration of non-invasive fetal RhD genotyping using maternal plasma. Lo YM et al. The New Engl. J. of Med. 339:1734-1738, 1998.
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- RNAs (ZFY and HLA-G mRNAs) in maternal plasma. Poon LLM et al. (Lo YM), Clinical Chemistry, 46:1832-1834, 2000.
 - RNA marker on chr. 21, the PLAC4. Lo YM et al. (Nat. Med., 13(2):218-223, 2007).
 - Epigenetic marker IGF2-H19 locus and a SNP in this region . Poon LLM et al, (Lo YM), Clinical Chemistry, 48(1): 35-41, 2002.
 - Epigenetic marker SERPINB5 gene on chr. 18 and a SNP in this region. Chim SSC et al. (Lo YM), PNAS, 102(41): 14753-14758, 2005
 - 3 DMRs on chr. 21. Old RW et al. (Hulten MA), Reprod. Biomed, 15(2):227-235, 2007.
 - 22 DMRs on chr.21. Chim SSC et al. (Lo YM), Clinical Chemistry, 54(3):500-511, 2008.

**Non-Invasive Prenatal Diagnostic
approaches for chromosomal
aneuploidies**

NIPD for Trisomy 21

- Using informative SNPs. Dhalla R et al. (Varney J), Lancet, 369:474-481, 2007.; Deng YH et al. (Chen HY), Clin. Chem. Lab. Med., doi: 10.1515/CCLM.2011.099, 2011.
- Using sodium bisulfite conversion in combination with restriction enzymes and SNPs. Tong YK et al. (Lo YM), Clinical Chemistry, 56(1):90-98, 2010.
- Using methylation sensitive-restriction endonucleases to in combination with real-time qPCR or Digital PCR. Tong YK et al. (Lo YM), Plos one, 5(12): e15244, 2011.
- SNP in mRNA marker, using primer extension and mass spectrometry and digital PCR. Lo YM et al., Nat. Med. 13(2):218-223, 2007); Tsui et al. (Lo YM), Clinical Chemistry, 56(1):73-81, 2010



NIPD using Digital PCR

Digital RNA-SNP approach:

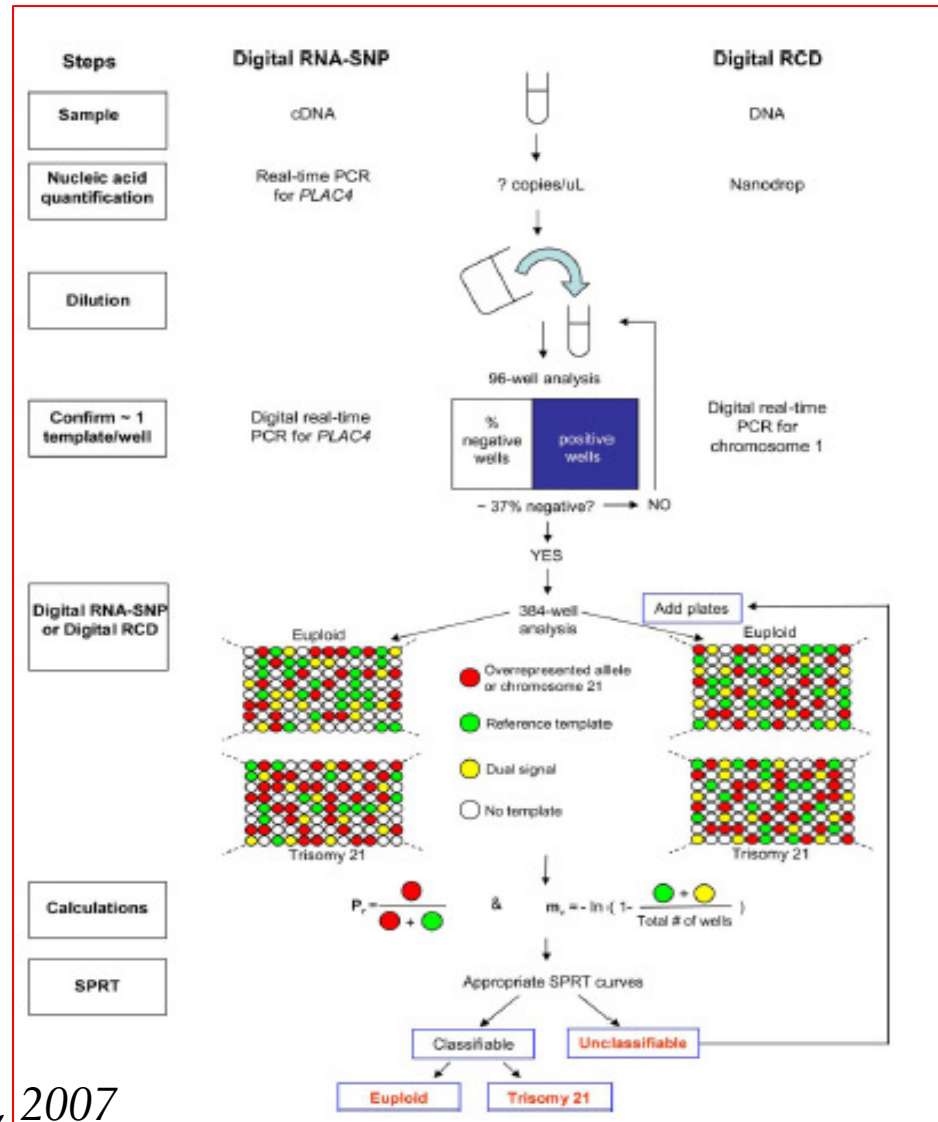
- A heterozygous fetal allele in *PLAC4* RNA in the presence of maternal homozygous allele is necessary for the analysis to take place

Digital RCD approach:

- A non-polymorphic site on chr21 is used and compared with a reference locus on chromosome 1

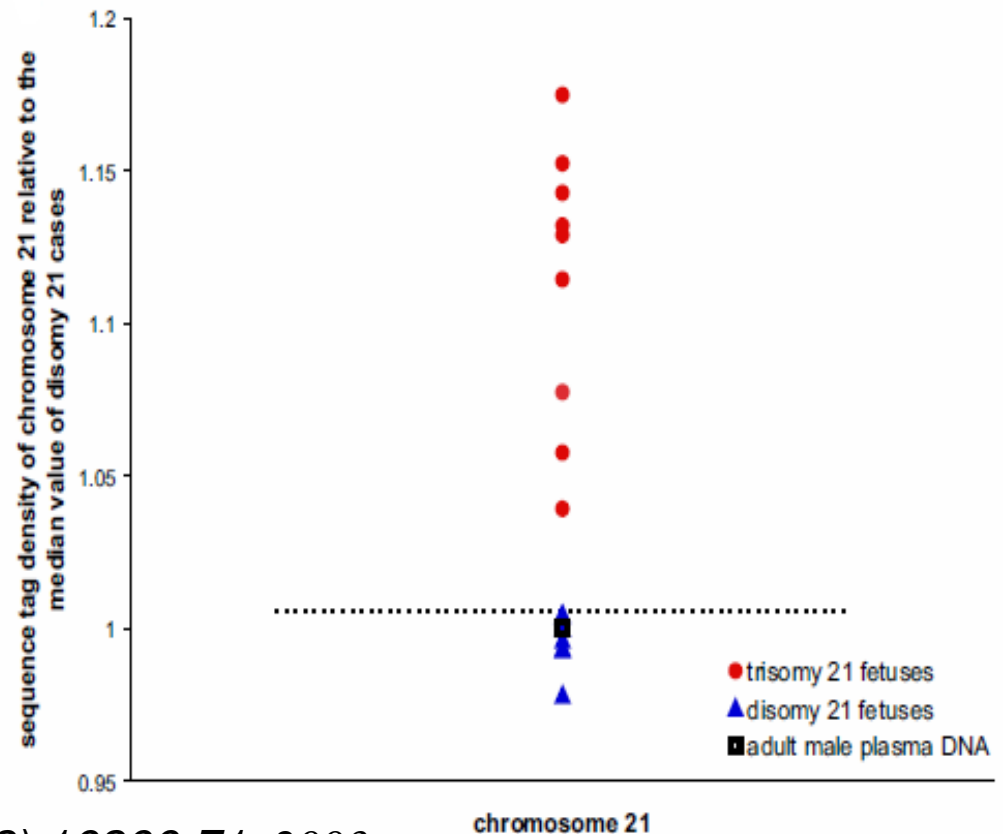
Results: In total 9 euploid and 4 T21 cases were tested with 100% correct classification

Lo YM et al. PNAS, 104(32):13116-13121, 2007



NIPD using Next-Generation Sequencing

- Approach: Direct sequencing of cell-free DNA with high-throughput shotgun sequencing from plasma of pregnant women, obtaining, on average, 5 million sequence tags per patient sample.
- Analysis: The ratio of each chromosome's sequence tag density over the median tag density of all autosomes was calculated. These values obtained were used for comparison among different samples
- Results: In total 9 Trisomy 21, 2 Trisomy 18, 1 Trisomy 13, 6 normal cases were tested with 100% correct classification



NIPD using Next-Generation Sequencing

- AIM: Application of massively parallel genomic sequencing to quantify maternal plasma DNA sequences for NIPD of Trisomy 21.
- Analysis: For a Trisomy 21 case, a high z-score for % chr21 was expected when compared with the mean and standard deviation of % chr21 of euploid cases.
- Samples: 28 plasma samples of which 14 derive from trisomy 21 fetuses (study of 2008).
 - Results: All correctly identified.
- Samples: 753 plasma samples of which 86 derive from Trisomy 21 cases (study of 2011).
 - Results: 8-plex protocol :79.1% and 98.9% sensitivity and specificity respectively. 2-plex protocol: 100% and 97.9% sensitivity and specificity respectively.

Chiu RWK et al. (Lo YM), PNAS, 105(51):20458-20463, 2008

Chiu RWK et al. (Lo YM), BMJ, doi:10.1136/bmj.c7401, 2011)

Limitations

Limitations towards NIPD methods

- Accuracy: Aim ~100% sensitivity and ~100% specificity
- Limited number of fetal markers
- Even more limited number of informative SNPs or methylation sensitive sites on these regions
- Using sodium bisulfite conversion: (a) Complete bisulfide conversion is rarely achieved, (b) Degradation of DNA always obtained after sodium bisulfide treatment
- Digital PCR, next-generation sequencing are highly complex and expensive methodologies

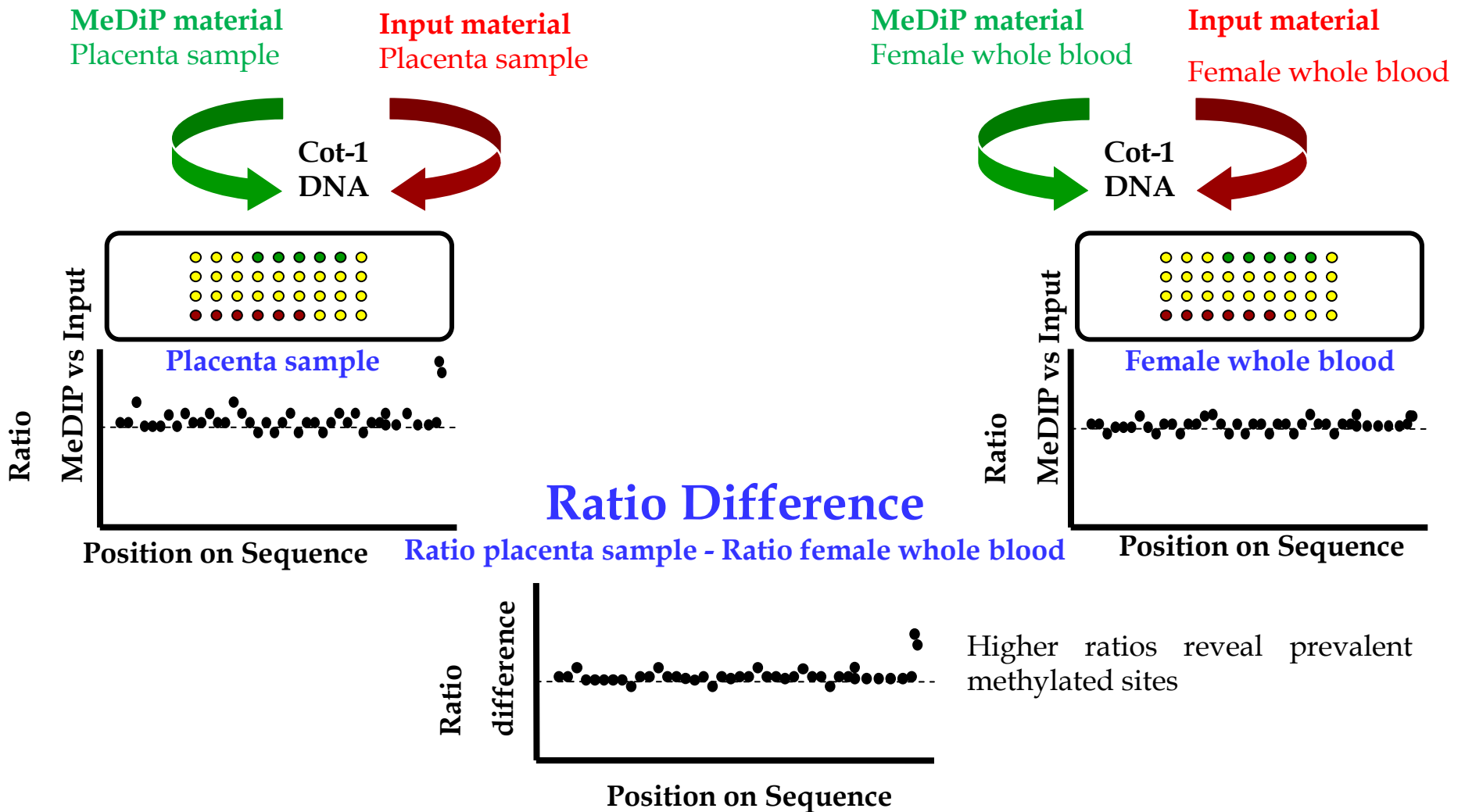
To overcome the above limitations we aimed a new NIPD method using Methylated DNA Immunoprecipitation (MeDiP) and RT qPCR

Papageorgiou EA et al. ((Patsalis PC), Am J. Pathol., 174(5):1609-1618, 2009).

Papageorgiou EA et al. ((Patsalis PC), Nat. Med., doi:10.1038/nm.2312, 2011).

Develop new fetal markers DMRs

Methylated DNA Immunoprecipitation (MeDiP)
with high resolution oligo array



Methylated DNA Immunoprecipitation (MeDiP) with high resolution oligo array

- Oligonucleotide microarray platform specific for chromosomes 13, 18, 21, X and Y.
- Each probe on chromosome is 50-60bp long and the median probe spacing of
 - 225bp for chr13, 170bp for chr18, 70bp for chr21,
 - 340bp for chrX 20bp for chrY
- Methylation DNA immunoprecipitation was followed by ligation-mediated PCR (LM-PCR)
- The differentially methylated regions were identified using the SW-ARRAY algorithm previously used for CNV calling as described by *Price, T.S. et al., Nucleic Acid Research 16:3555-3464, 2005*

Identification of DMRs on chromosome 21 between the fetus and the mother

An example the oligo array results for a region located on chromosome 21



Methylated DNA Immunoprecipitation (MeDiP) with high resolution oligo array

Differentially Methylated Regions in Placental Compared with Whole Blood DNA Samples Across Chromosomes 13, 18, 21, X, and Y

CHR*	Trimester	No. of hyper [†]	No. of hypo [‡]	Total no.	% Of hyper [§]	% Of hypo [¶]
13	First	1310	1336	2646	49.5	50.5
13	Third	1311	1318	2629	49.9	50.1
18	First	1967	1888	3855	51.0	48.9
18	Third	1957	1944	3901	50.2	49.8
21	First	1063	1015	2078	51.1	48.8
21	Third	1042	1040	2082	50.0	49.9
X	First	1992	1951	3943	50.5	49.5
X	Third	1995	1989	3984	50.1	49.9
Y	First	1192	1120	2312	51.6	48.4
Y	Third	1986	1990	3976	49.9	50.0

*Chromosome.

[†]Number of hypermethylated regions.

[‡]Number of hypomethylated regions.

[§]Percentage of hypermethylated regions.

[¶]Percentage of hypomethylated regions.

- Some DMR are intragenic
- Some DMRs are intergenic
- Some DMRs are within the promoter regions

Methylation status of DMRs on chromosome 21.

CHR.	TRIMESTER	N ^o OF ENR.	N ^o OF DEPL.	TOTAL N ^o .	% OF ENR.	% OF DEPL.
21	FIRST	1063	1015	2078	51.15	48.84
21	THIRD	1042	1040	2082	50.05	49.95

Differentially methylated regions located within genes across chromosome 21

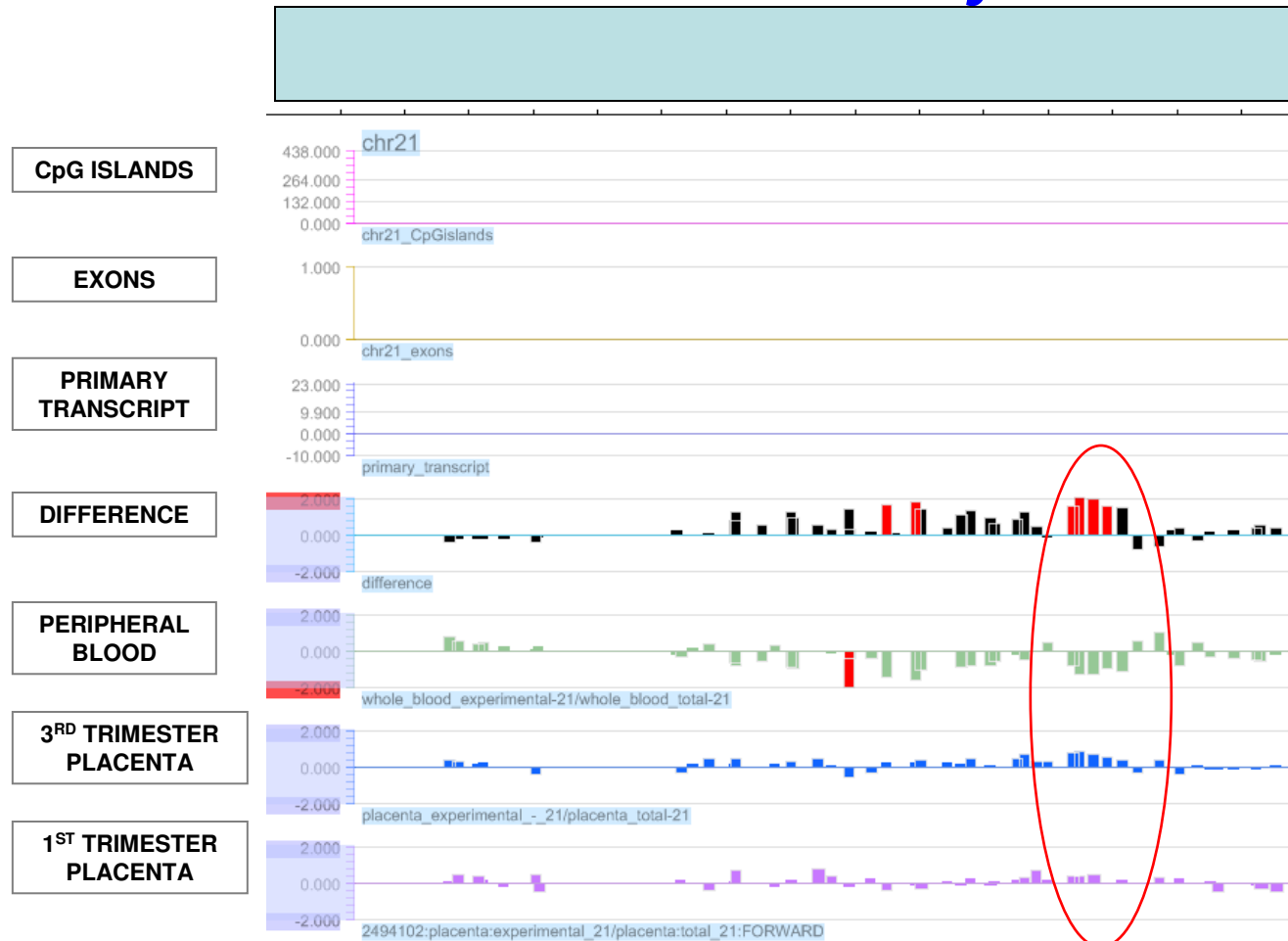
CHR.	TRIMESTER	N ^o OF ENR.	N ^o OF DEPL.	TOTAL N ^o .	% OF TOTAL	% OF ENR.	% OF DEPL.
21	FIRST	362	545	907	43.65	39.91	60.09
21	THIRD	338	486	824	39.58	41.02	58.98

- About the same number of hyper Vs hypo DMRs
- About 40% within genes, 60% out of genes
- The same conclusions in the other chromosomes

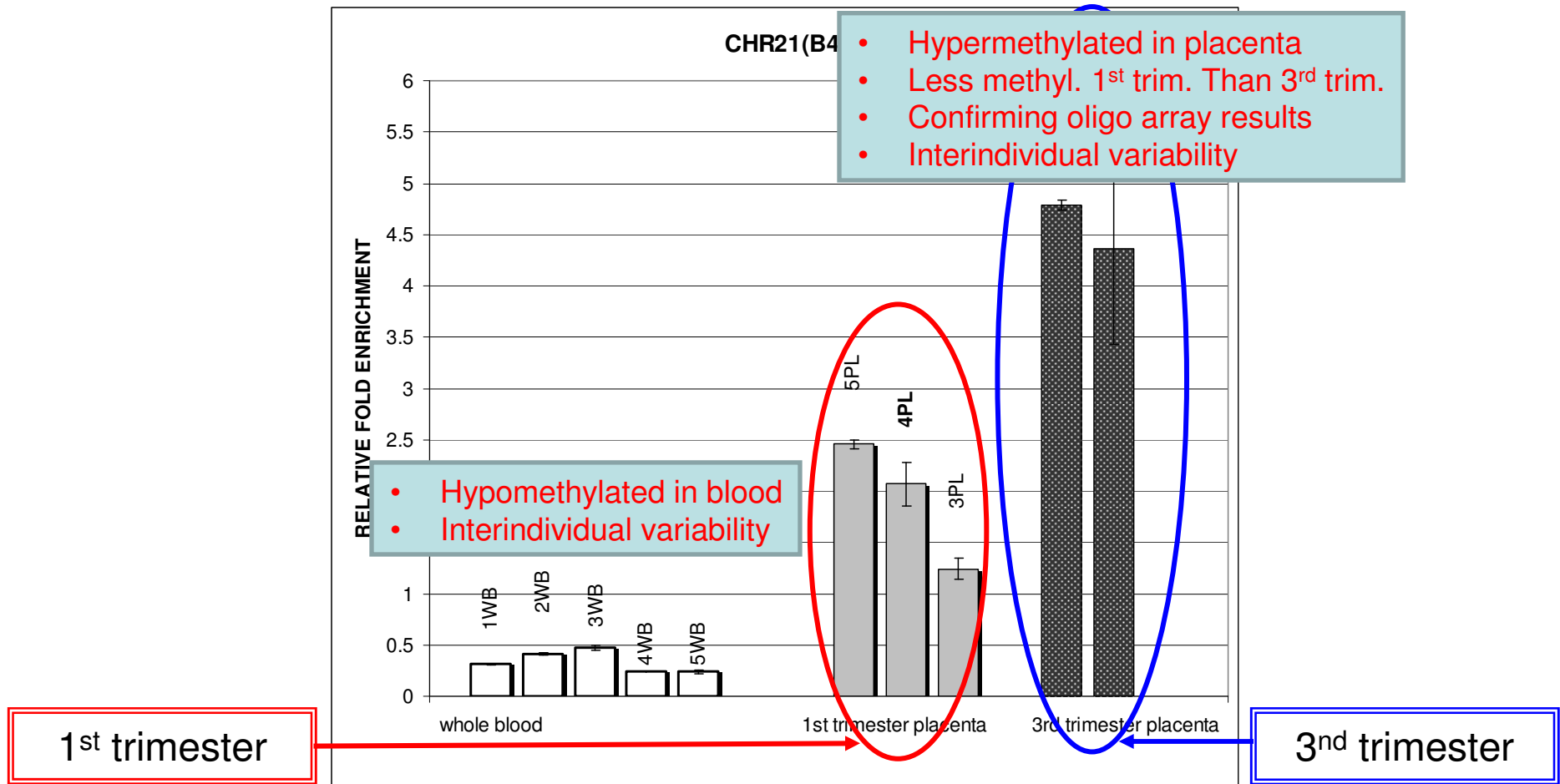
Selection of DMRs to develop a NIPD for trisomy 21

- **Selection criteria:**
 1. The regions should be hypermethylated in placenta and hypomethylated in peripheral blood.
 2. The methylation status is the same in 1st and 3rd trimester placentas.
 3. The methylation level is above a threshold value given by the microarray analysis.

Selection of DMRs to develop a NIPD for trisomy 21



Evaluating the methylation status of the selected DMR in different individuals



Immunoprecipitated samples:

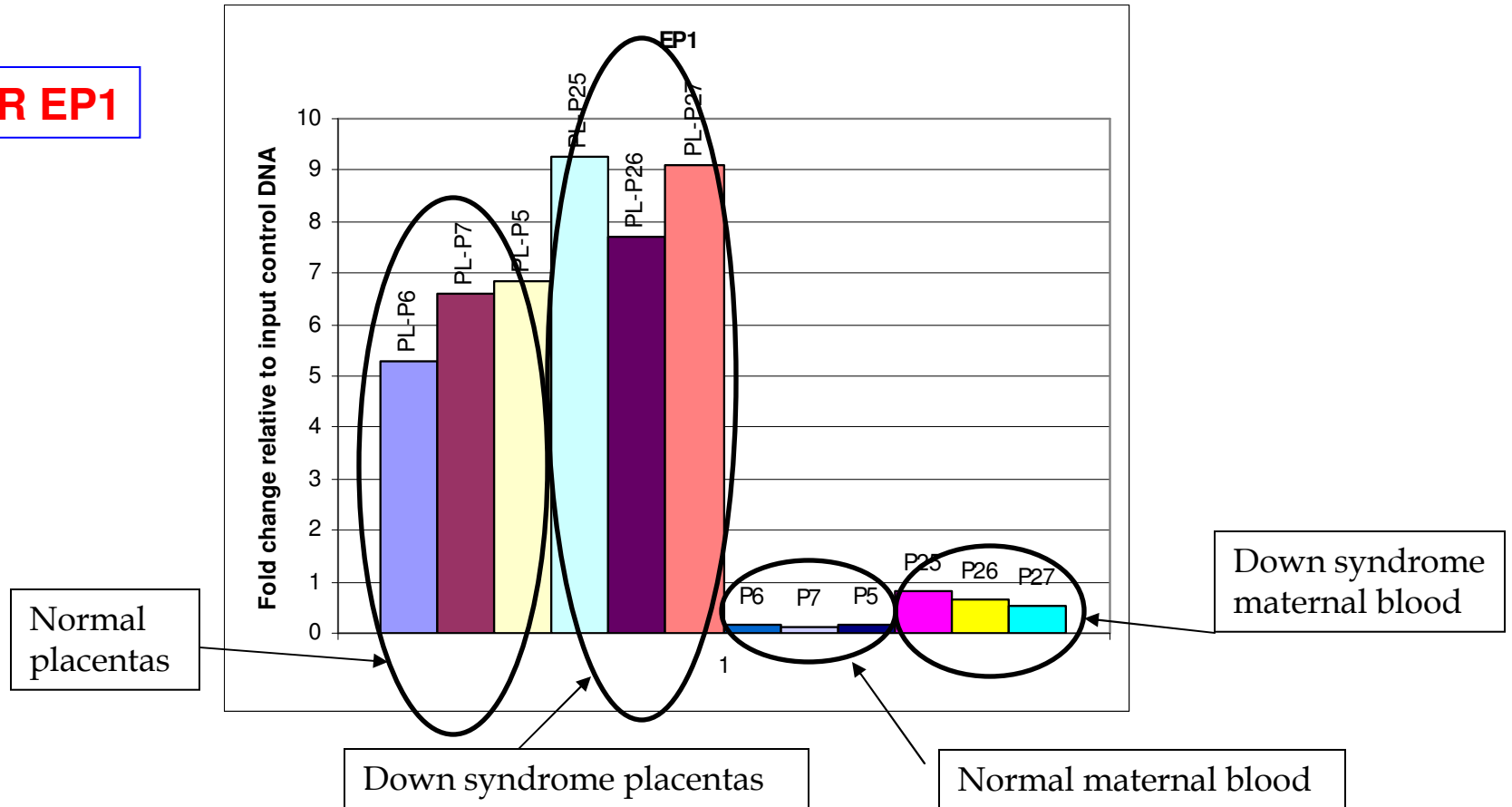
Whole blood non-pregnant,

1st trimester placenta,

3rd trimester placenta

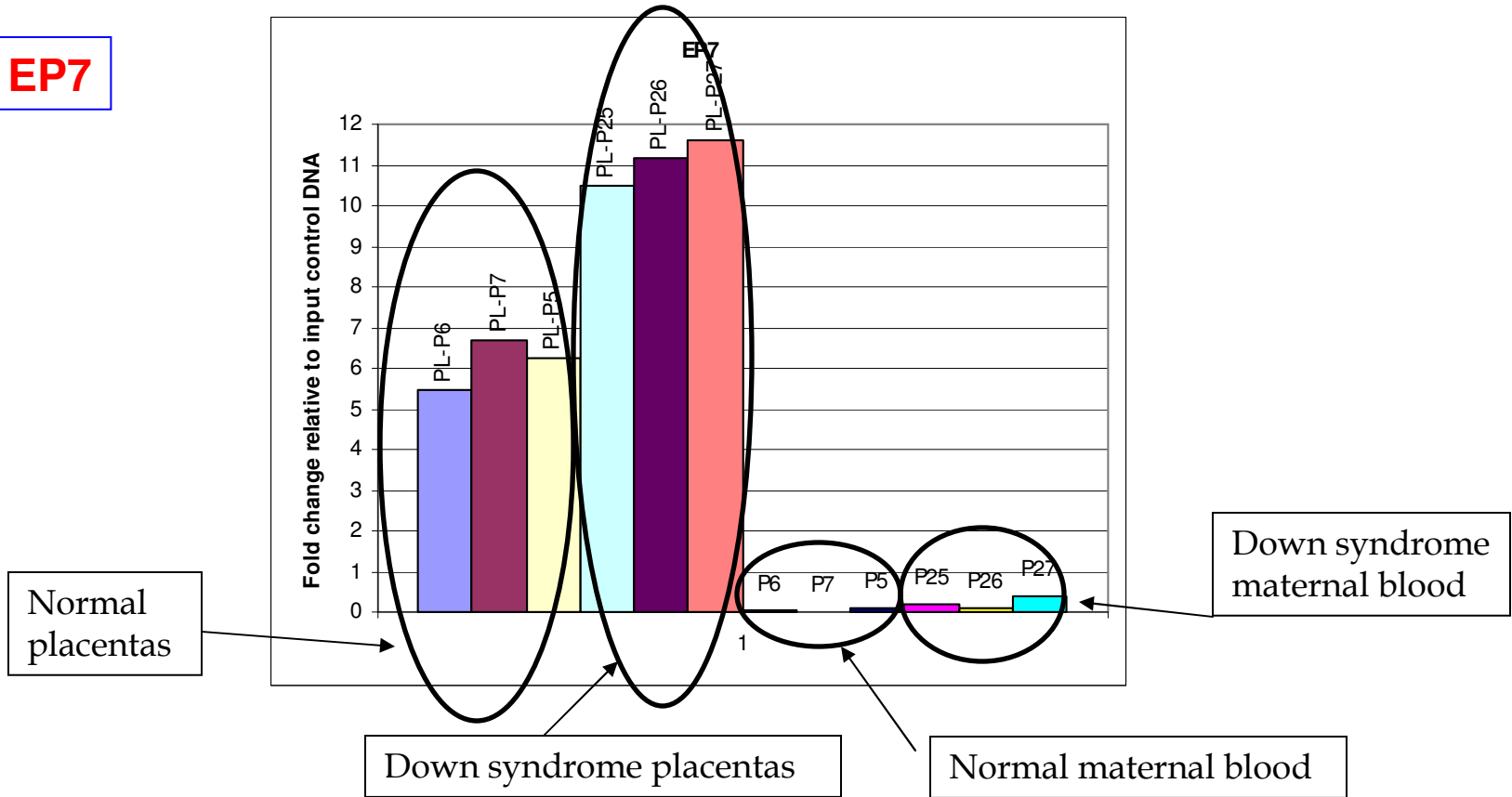
Identification of fetal DNA in maternal peripheral blood and discrimination of normal from Down syndrome cases

DMR EP1

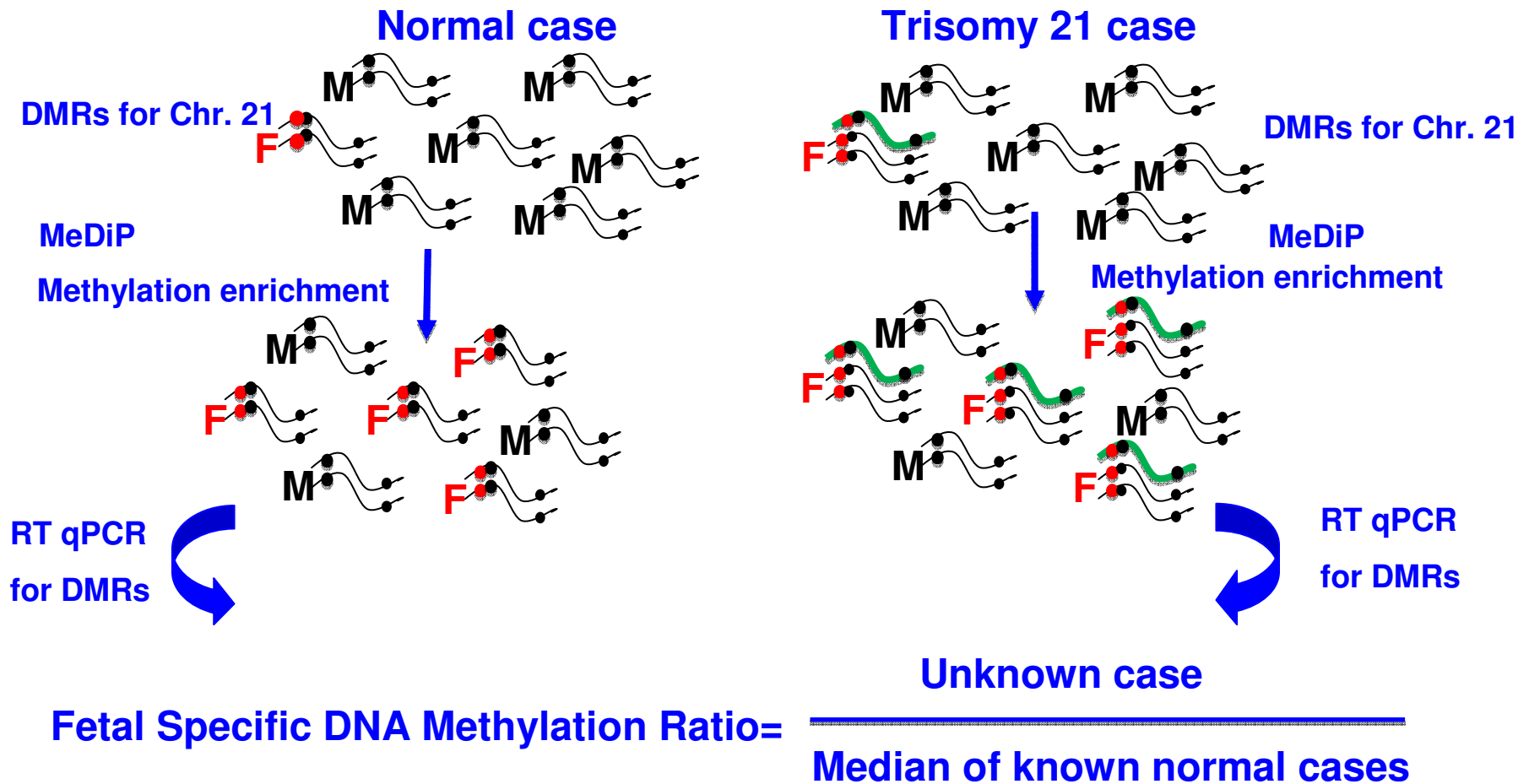


Identification of fetal DNA in maternal peripheral blood and discrimination of normal from Down syndrome cases

DMR EP7



Fetal-specific DNA methylation ratio permits NIPD of Trisomy 21

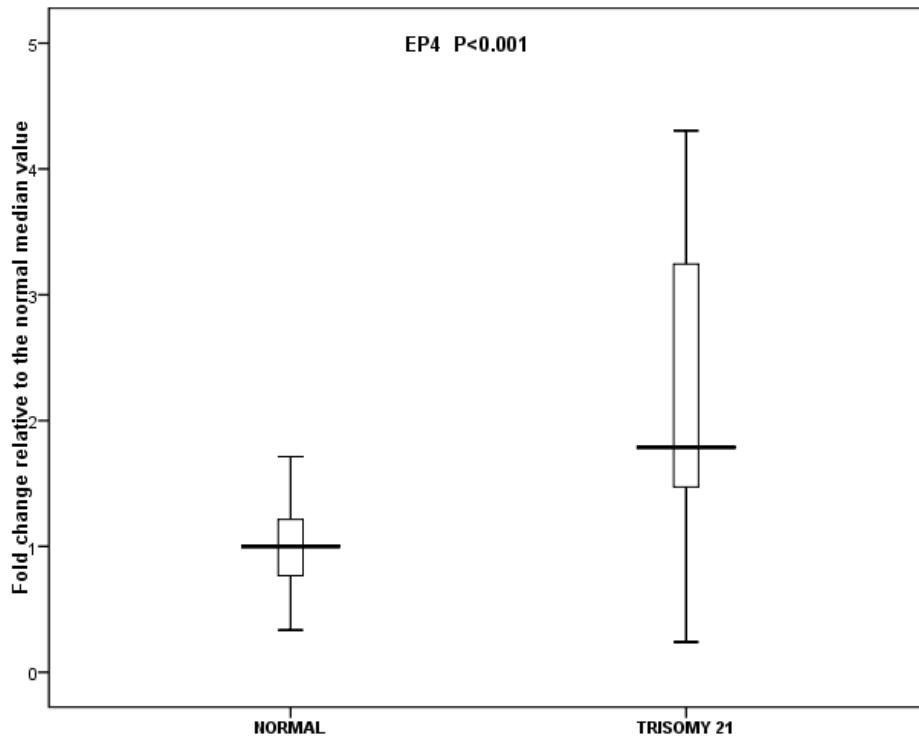


Fetal-specific DNA methylation ratio permits NIPD of Trisomy 21

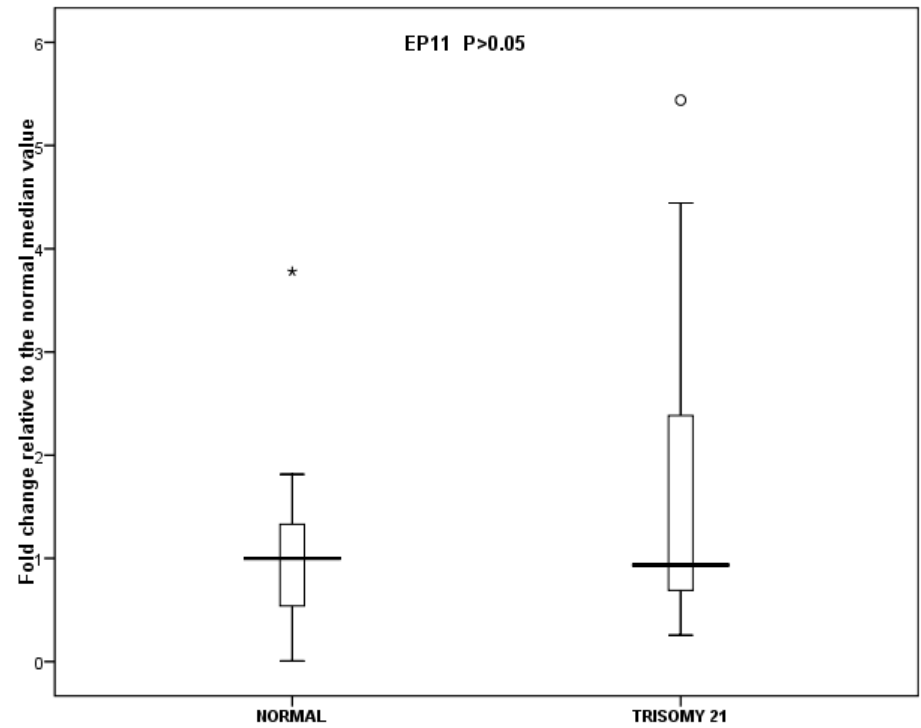
- We used 40 maternal peripheral blood DNAs including
 - samples from 20 women bearing a normal fetus
 - samples from 20 women bearing a Down fetus.
- AIM:
 - Confirm the preliminary results indicating differences of the methylation levels between normal and Down syndrome pregnancies.
 - Investigate the behavior of different regions.
 - Develop a novel non-invasive prenatal diagnostic test for Down Syndrome.

Fetal-specific DNA methylation ratio permits NIPD of Trisomy 21

Statistically significant region

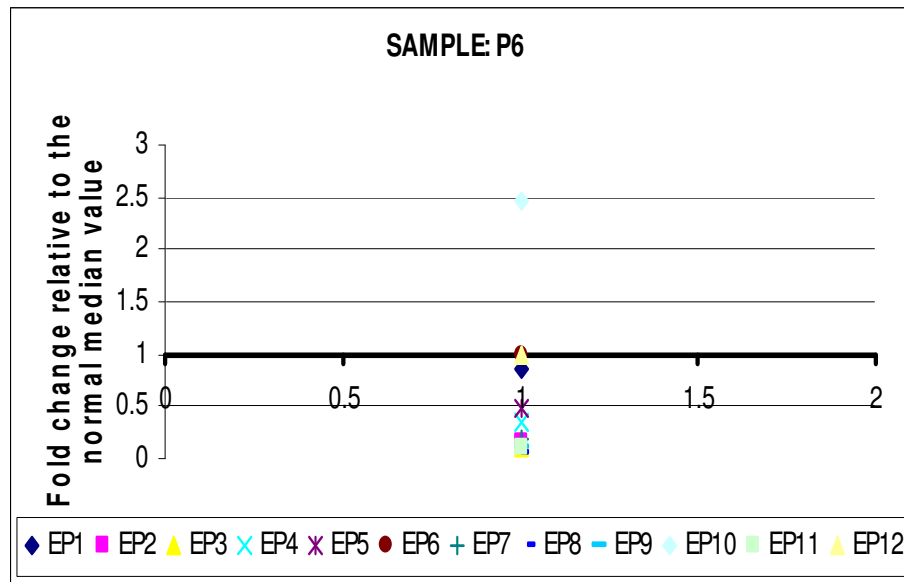


Non-Statistically significant region

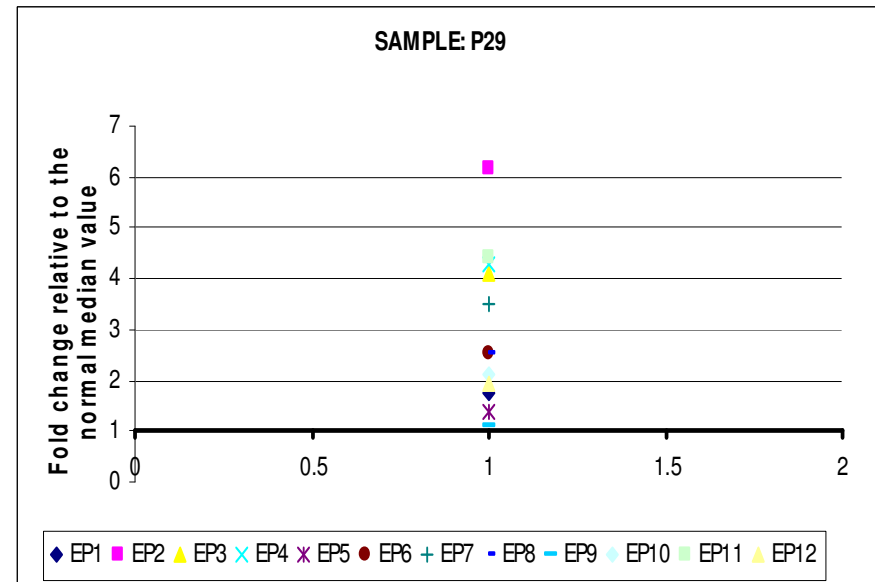


Fetal-specific DNA methylation ratio permits NIPD of Trisomy 21

Normal case



Down syndrome case



Fetal-specific DNA methylation ratio permits NIPD of Trisomy 21

- We designed a diagnostic equation by using the statistical software SPSS v16.0.
- The statistical analysis showed that the combination of the results obtained from eight out of the twelve regions tested lead to the correct diagnosis of all the normal and Down syndrome pregnancies tested.

$$D = - 6.331 + 0.959 X_{EP4} + 1.188 X_{EP5} + 0.424 X_{EP6} + 0.621 X_{EP7} + 0.028X_{EP8} \\ + 0.387 X_{EP10} - 0.683 X_{EP11} + 0.897 X_{EP12}$$

- where X_{EPi} = ratio value Sample; EPi

Fetal-specific DNA methylation ratio permits NIPD of Trisomy 21

- We have completed validation and blind study application of the new NIPD method in 80 blood samples from pregnant women (46 samples with normal pregnancy and 34 with trisomy 21 pregnancy).
- All pregnancies were correctly classified providing 100% sensitivity and 100% specificity.

Papageorgiou EA et al. ((Patsalis PC), Nat. Med., doi:10.1038,2011).

Fetal-specific DNA methylation ratio permits NIPD of Trisomy 21

Prediction values obtained from normal and trisomy 21 cases when using the diagnostic equation

Sample	Status	Prediction value
P41	Normal	-2.34
P42	Normal	-1.16
P43	Normal	-3.54
P44	Normal	-2.79
P45	Normal	-3.19
P71	Trisomy 21	2.41
P72	Trisomy 21	7.27
P73	Trisomy 21	1.62
P74	Trisomy 21	6.26
P75	Trisomy 21	24.16

Normal values: ≤ 0

Abnormal values: > 0

Future directions

- Perform a larger-scale clinical study which is essential in order to enable the introduction of the new test into clinical practice.
- Provide an accurate, fast, simple and cost effective test that can be safely used in the clinical practice
- Further development of the new test so it can be utilized for the non-invasive prenatal diagnosis of other syndromes such as trisomy 13, 18 and aneuploidies associated with chromosomes X and Y.
- Development of non-invasive prenatal diagnosis for other diseases and syndromes