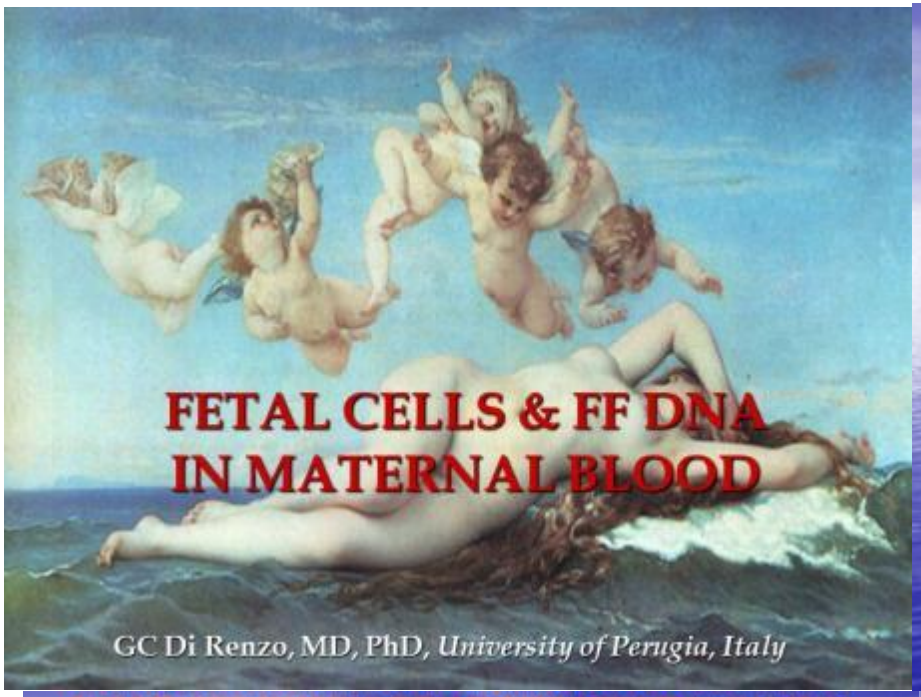


CURRENT PRENATAL DIAGNOSIS TOOLS

INVASIVE

Villocentesis

Amniocentesis

Cordocentesis

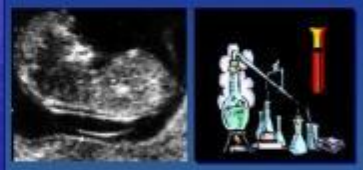
Offered to women at risk for:

- maternal age
- positive screening test
- chromosomal abnormalities
- previous affected child

NON INVASIVE

Ultrasound screening

Biochemical screening



Low sensitivity and specificity (< 100%)

2

NEED TO DEVELOP NEW NON INVASIVE PRENATAL DIAGNOSTIC TESTS

- SIMPLE
- EASY
- LEAST AGGRESSIVE
- LEAST ANXIOUS
- MORE SENSITIVE
- MORE SPECIFIC

NEW APPROACHES

FETAL CELLS IN MATERNAL BLOOD

FREE FETAL DNA IN MATERNAL BLOOD

FREE FETAL RNA IN MATERNAL BLOOD

3

GENERAL APPROACH TO FETAL CELL ISOLATION FROM MATERNAL BLOOD

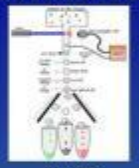
SAMPLING → ENRICHMENT → ANALYSIS

venipuncture

- Density gradient centrifugation (Oosterwijk JC et al., 1996; Samura O et al., 2000)
- Density gradient centrifugation after treating the sample with RosetteStep antibody (Bischoff FZ et al., 2003)
- Density gradient centrifugation of cells with density previously modified by non-physiological conditions (Sifar G et al., 2005)

- Micromanipolation of individual cells (Takabayashi H et al., 1995; Vona G et al., 2002)

FACS
(Fluorescence activated cell sorting)



MACS
(Magnetic activated cell sorting)

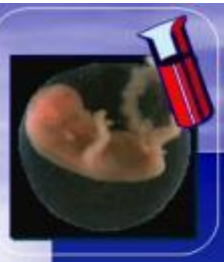
FISH

PCR

4

KEY BIOLOGICAL QUESTIONS

- Which is the ideal fetal cell type for a non invasive prenatal diagnosis?
- Which is the frequence of fetal cells in maternal blood?
- Which are suited laboratory approaches to enrich and to purify fetal cells in maternal blood?
- Are fetal cells always present in maternal blood during gestation?
- Are the fetal cells, isolated from maternal blood, sufficient for genetic diagnosis?
- Which is the best timing to retrieve fetal cells from maternal blood?



FETAL CELL TYPES IN MATERNAL BLOOD DURING GESTATION

Studies on fetal blood obtained by cordocentesis have been able to strengthen the knowledge of the composition and development of fetal blood component throughout pregnancy

- LYMPHOCYTES
- ERYTHROBLASTS
- TROPHOBLASTS
- HEMATOPOIETIC STEM PROGENITOR CELLS
- MESENCHYMAL STEM CELLS

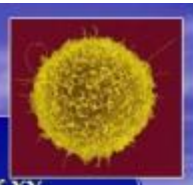
NUMBER OF FETAL CELLS

- 1 fetal cell/ 10^5 – 10^8 maternal cells
  (Price JO et al., 1991; Hamada H et al., 1993; Langlois S et al., 1993)
- 1 fetal cell/ ml of maternal blood
  (Bianchi D et al., 1997)
- 2 –6 fetal cells/ml maternal blood
  (Krabchi et al., 2001)
- 0 – 2 fetal progenitor cells/ ml maternal blood
  (Guetta et al., 2003)
- Numerous studies demonstrated that in women carrying fetus with trisomy 21 or 13 and in pregnancies complicated by preeclampsia, the mean number of fetal cells increase in respect to normal pregnancies.
  (Holzgreve W et al., 2007)

TIME OF APPEARENCE OF FETAL CELLS IN MATERNAL CIRCULATION

- After 40 days of gestation
- From 4th week of gestation
  (Peault B et al., 2003; Lo YMD et al., 1996)
- 11 - 16 weeks of gestation
  (Ideal time for isolating fetal cells from maternal blood)

LYMPHOCYTES



- Presence in maternal blood: discovered by Walknowka et al. with the detection of XY cariotype in women carrying a male fetus (Lancet, 1969)
- Identification: HLA monoclonal antibody
- Frequency: small number in early gestation

$$\frac{\text{Fetal lymphocytes}}{\text{Maternal lymphocytes}} = 1/800\text{-}1/60{,}000$$

- Purity: 0.01%-3.7%
- Disadvantages:
  - necessity of performing HLA typing of both parents prior to fetal enrichment
  - possible persistance of fetal lymphocytes in the maternal blood (0.01%-0.1% one year after birth)

TROPHOBLASTS

- Presence in maternal blood: discovered by Douglas et al. by using cytological methods (Am J Obstey Gynecol, 1959).
- Identification: H315 (Johnson et al., 1981), FD066Q and FD0338P (Mueller et al., 1990), HLA-G (vanWijk et al., 2001) for trophoblast; GB 17, 21, 25 for syncytiotrophoblast and cytotrophoblast (Bruch et al., 1991).
- Frequency of fetal trophoblasts expressing HLA-G: 1 fetal trophoblast/1 ml of maternal blood
- Advantages: unique morphology and intimate contact with maternal blood.
- Disadvantages:
  - adsorption of trophoblast antigens onto the cell surface of maternal leucocytes;
  - multinucleate cells (2-80 nuclei);
  - possible discrepancy between fetal and placental kariotype.

- In progress: *in vitro* expansion of trophoblast cells. Preliminary results showed a fivefold increase in the yield (Guetta et al., 2005).

ERYTHROBLASTS

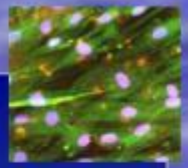
- Presence in maternal blood: the first research groups to focus on this cells were those of Bianchi (Proc Natl Acad Sci USA,1990) and of Simpson (Hum Reprod, 1991) and Holzgreve (Am J Reprod Immunol, 1993)
- Identification: CD71, CD36, GPA, HbF by direct selection; CD45, CD14 by indirect selection
- Frequency: the most abundant cells of fetal blood in first trimester
- NIFTY-I (Prenat. Diagn. 2002; 22: 609-615):
  - Study design:
    - 5 years of prospective study with 2744 enrolled pregnant women;
    - to evaluate the real possibility of using fetal erythroblasts in non invasive prenatal diagnosis of chromosome abnormalities
  - Results and conclusions:
    - erytroblasts selection by MACS is better than by FACS;
    - correct fetal gender in 41.4% of patients and 11.1% false positive results
    - correct diagnosis of aneuploidies in 74.4% of patients and 0.6%-4.0% false positive results

ERYTHROBLASTS

- Advantages:
  - mononucleate cells virtually absent in the adult circulation
  - limited life-span (~90 days)
  - morphologically well distinguished
- Disadvantages:
  - insufficient number of cells after enrichment
  - erythroblasts from maternal blood are principally of maternal origin (Slunga-Talberg, 1995)
  - erythroblasts could be impervious to FISH analysis because of their dense compact nucleus with apoptotic character (Hahn S et al., 2005)

- In progress: research of new antibodies for their selection by MACS (Alvarez et al., 2005)

MESENCHYMAL STEM CELLS (MSCs)



- Presence in maternal blood: MSCs circulate in 1st and early 2nd trimester fetal blood (Campagnoli et al., 2001)
- Identification: MSCs have been isolated from maternal blood at 7th week of gestation
- Frequency: MSCs represent 0,4% of fetal nucleated cells in fetal blood

- Work in progress: MSCs have been only recently considered as target cells for non invasive prenatal diagnosis and so the feasibility of their use should be evaluated moreover (Fisk et al., 2005)

13

HEMATOPOIETIC STEM PROGENITOR CELLS (HSPCs)

- Presence in maternal blood: HSPCs are present in maternal circulation from 4th weeks of gestation whereas their concentration decrease after 20 weeks.
- Identification: CD34, CD133 monoclonal antibodies;
- In vitro culture expansion has been studied and proposed by Lo et al. (Lancet, 1994), Little et al. (Blood 1997) and Di Renzo et al. (Journal of Hematotherapy & Stem Cell Research 2000).
- Frequency: fetal/maternal cell ratio is 1 per 4.75×10^6 - 1.6×10^7 cells.
- Advantages:
  - Clonogenicity;
  - Increased clonogenicity in fetal blood during early 2nd trimester;
  - Versatility to culture and to proliferate extensively in vitro.

- Work in progress:
  - persistence in maternal blood after pregnancy?
  - new fetal HSPCs markers

14

A NEW METHODOLOGY OF FETAL STEM CELL ISOLATION, PURIFICATION, AND EXPANSION: PRELIMINARY RESULTS FOR NON INVASIVE PRENATAL DIAGNOSIS

F. Tilesi, G. Coata, G.C. Di Renzo et al.

Journal of Hematotherapy & Stem Cell Research 2000; 9: 583-590

15

RESULTS

An enrichment of

33 times of BFU-E/CFU-E and

16 times of CFU-GM colonies after

miniMACS CD34+ HSPCs purification

was obtained

16

RESULTS

Results of FISH analysis with X and Y, 21 chromosome fluorescent probes in cultured cells

Slide Identific.	Fetal karyotype	Number examined cells by FISH	Number cells with XY signals	Number cells with Y signals	Number cells with trisomy 21 signals	Fetal/maternal cell ratio
*27	46, XY	669	5	-	-	1/133
*19	46, XY	1433	6	-	-	1/238
†3	46, XY	570	-	11	-	1/52
†4	46, XY	1050	-	4	-	1/262
°18	47, XX+21	659	-	-	19	1/34
°15	47, XY, +21	100	-	-	8	1/12

* cases studied by X- and Y chromosome fluorescent probe

† cases studied by Y chromosome fluorescent probe

° cases studied by chromosome 21 fluorescent probe

PRENATAL DIAGNOSIS OF GENETIC ABNORMALITIES USING FETAL CD34+ STEM CELLS IN MATERNAL CIRCULATION AND EVIDENCE THEY DO NOT AFFECT DIAGNOSIS IN LATER PREGNANCIES

G. Coata, G.C. Di Renzo et al.

Stem Cells 2001; 19: 534-542.

RESULTS

- We found an higher number of fetal cells after the culture of CD34+ HSPCs.
- We also observed an expansion of fetal cells in all 7 samples cultured before and after CD34+ HSPCs enrichment.

IMPROVEMENTS IN SLIDE PREPARATION AND MICROSCOPY SCORING OF NUCLEI FROM CULTURED CD34+ HSPCs FOR FISH ANALYSIS IN NON INVASIVE PRENATAL DIAGNOSIS

Data 2007-2008

FISH OPTIMIZATION

OBJECTIVE

Optimization of FISH technique for high detection efficiency of rare fetal CD34+ HSPCs:

- to improve
  - quality of nuclei to retain as near a native nuclei structure as possible during FISH slide preparation
  - quality of nuclei scoring
- to avoid false positive and false negative detection of trisomic nuclei
- to compare manual and automated motorized microscopy scoring

PATIENTS

- 20 pregnant women at 14 -16 week of gestation
-10 non-pregnant women and male adult patients

METHODS

We compared:

- classic methodology: FISH on dropped nuclei
- new methodology: FISH on cytospinned nuclei → manual scoring / automated scoring

CEP XY and LSI 21 specific probes were used

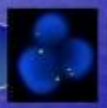
21

FISH OPTIMIZATION

Classic methodology

Cells lysed by using hypotonic solution and nuclei manually dropped after fixing by Carnoy solution

FISH

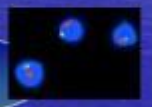


Manual scoring

New methodology

Cells spread on slides by cytospin, fixed by Carnoy solution

FISH



Manual scoring

Automated scoring

22

FISH OPTIMIZATION
RESULTS (1)

Classic methodology → IMPROVEMENTS in slide preparation → New methodology

- ↑ percentage of target area without overlapping nuclei
- ↑ number of nuclei with good morphologic characteristics (unbroken nuclei and enlarged nuclei with intact boundaries)
- ↓ false positive results as background fluorescence and contaminants: clearly recognizable
- ↓ time consuming (half time gain)

Manual microscopy scoring → COMPARING → Automated motorized microscopy scoring

Mean of 3000 nuclei scored for each slide in both analysis

- The number of detected fetal cells was correlated
- Scoring Time (h) → Manual: 1.5 for CEP XY and 3-4 for LSI 21; Automated: 2.5 for CEP XY and 4-5 for LSI 21
- Reproducibility of scoring area

23

FISH OPTIMIZATION
RESULTS (2)

1) Comparison between two methodologies for enumeration of XY chromosomes.

Classic methodology	New methodology
86,96% of cases (male and female fetuses) were correctly classified	We correctly classified all the samples tested

2) Comparison between two methodologies for enumeration of chromosome 21

Classic methodology	New methodology
100% of trisomic fetuses were correctly identified	100% of trisomic fetuses were correctly identified
False positive findings: hardly recognizable	False positive findings: clearly recognizable

24

FISH OPTIMIZATION

CONCLUSIONS

- We improved the overall efficiency of FISH results in terms of:

1) adequate nuclei density and homogeny on slides with a decreased number of overlapping nuclei

2) good maintenance of nuclei morphology

3) ability to differentiate between close and split signals with decreasing false and negative findings

4) reproducibility of target area

- Manual vs. automated motorized microscopy scoring:

1) the scoring time using automated microscopy system slight increases (only 1 hour) compared to manual microscopy

2) Advantages of automated detection of rare cells:
   a. More reproducible
   b. Less human time spent (45 min vs. 3-4 h)
   c. Possibility of scanning slides 24/24 (day and night)

MOTORIZED MICROSCOPE WITH AUTOMATED ACQUISITION SYSTEM

Microscope BX-61 Olympus with software BX-UCB Olympus

Objective changer

Motorized table with 4 sides of reading

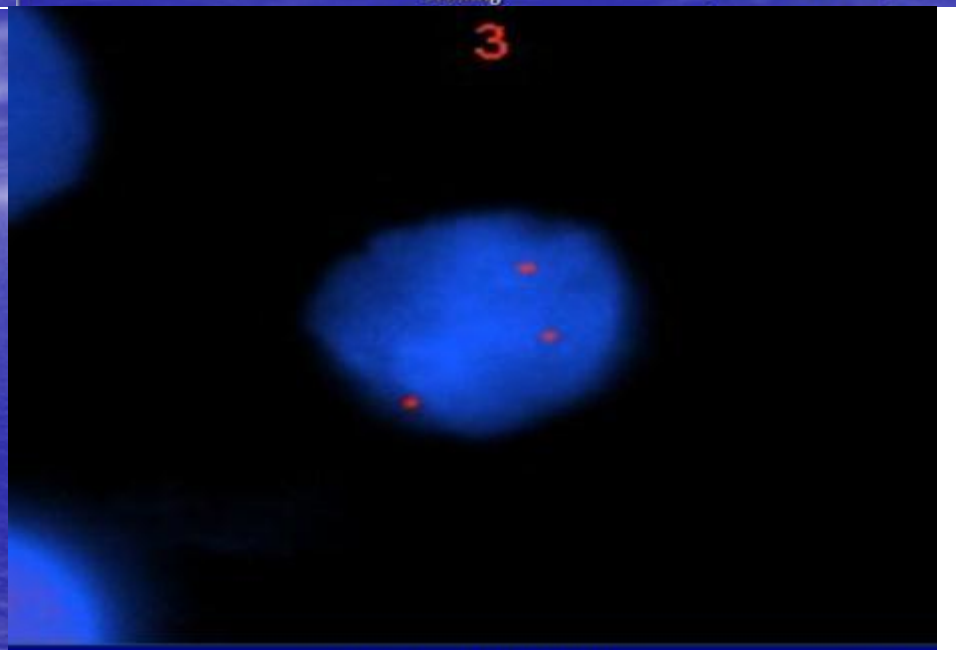
Fluorescence lamp (100Watt) at hight pression of mercury

FISH PERFORMED BY USING LSI 21 PROBE FOR THE NON INVASIVE DIAGNOSIS OF FETAL TRISOMY 21

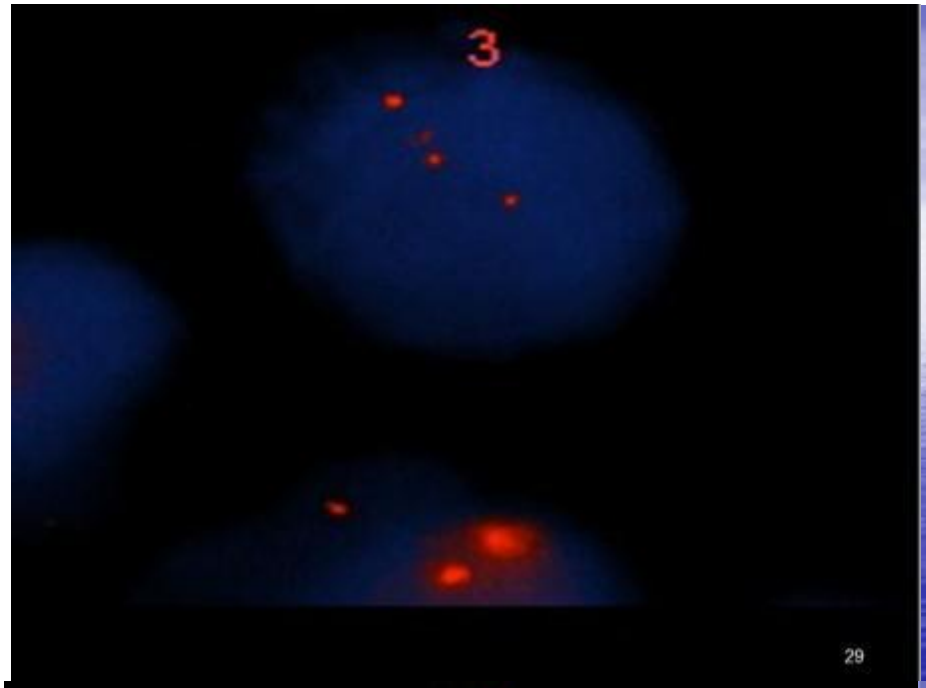
A and B: Frames obtained by using the automated mycroscope

A: Two disomic nuclei for the chromosome 21

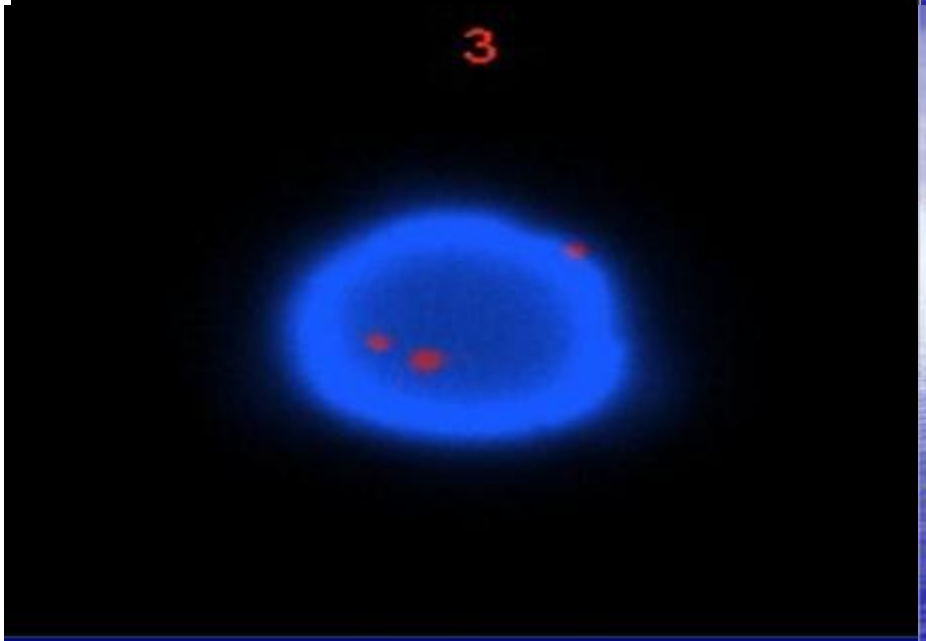
B: Fetal trisomic nucleus for the chromosome 21



SAFE TEST: TRISOMY 21



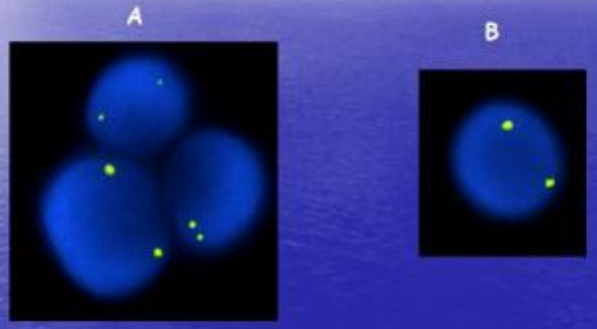
29



SAFE TEST: TRISOMY 13

32

FISH PERFORMED BY USING LSI 13 PROBE FOR THE NON INVASIVE DIAGNOSIS OF FETAL TRISOMY 13

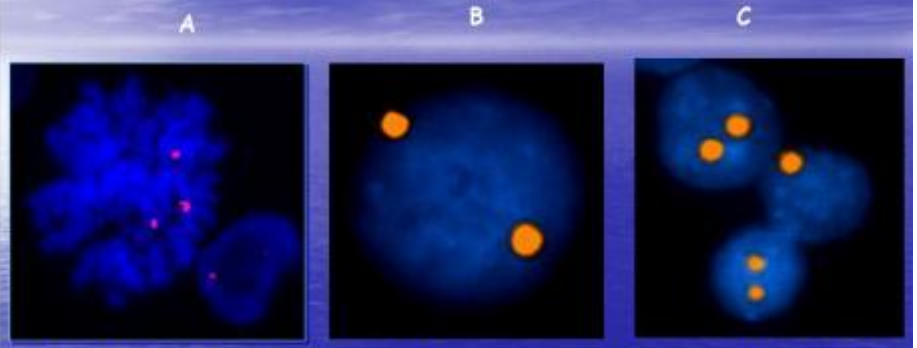


A and B: Frames obtained by using the automated mycroscope

A and B: Each nucleus showed is disomic for the chromosome 13

31

FISH PERFORMED BY USING CEP 18 PROBE FOR THE NON INVASIVE DIAGNOSIS OF FETAL TRISOMY 18

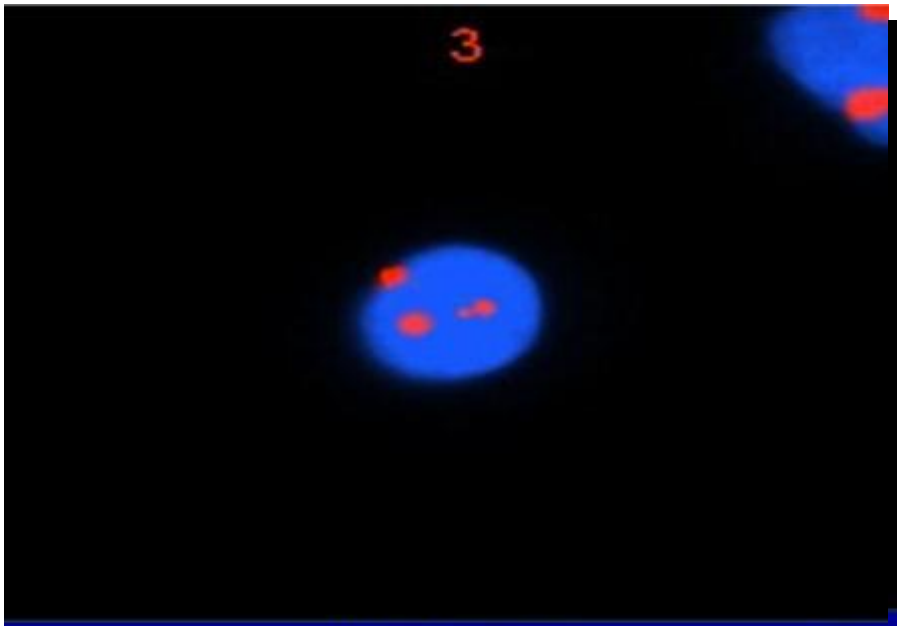


A: Fetal metaphase shows three 18 orange spots and a maternal nuclei with two 18 orange spots

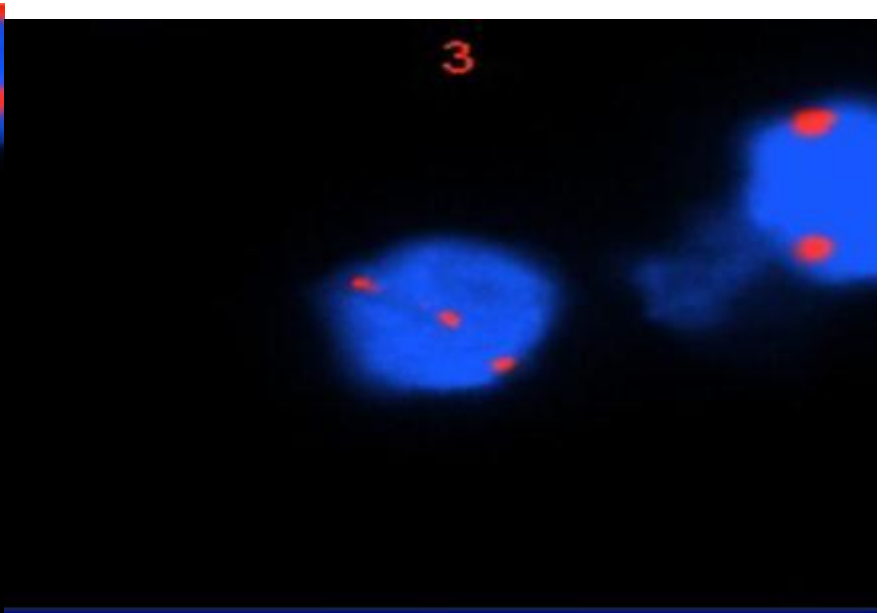
B and C: Frames obtained by using the automated mycroscope

B: Disomic nucleus for the chromosome 18

C: Two disomic nuclei and one monosomic nucleus for the chromosom[illegible]18

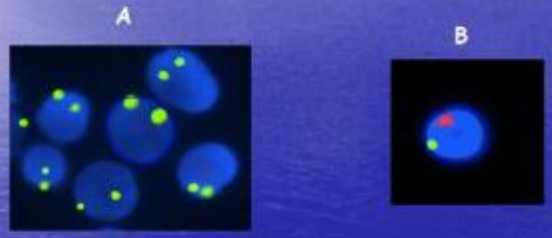


SAFE TEST: TRISOMY 18 34



SAFE TEST: TRISOMY 18 35

FISH PERFORMED BY USING CEP XY PROBE FOR THE NON INVASIVE DIAGNOSIS OF FETAL GENDER



A and B: Frames obtained by using the automated mycroscope

A: XX nuclei

B: Fetal XY nucleus

36

RESULTS SAFE TEST 2006-2009

- 1562 tests: checked by CVS, amniocentesis, birth genetic map
- 15 trisomy 21
- 5 trisomy 18
- 1 trisomy 13
- 1 Klinefelter

- Detection rate 100%
  Sensitivity 100% Specificity 94%

- Chr 21 sens 100% spec 89%
- Chr 18 sens 100% spec 92%
- Chr 13 sens 100% spec ND
- Chr X & Y sens 100% spec 100%

37

"FETAL CD34+ HSPCs PERSISTENCE" AND "FETAL MICROCHIMERISM" IN MATERNAL BLOOD AFTER DELIVERY

G Coata, G C Di Renzo et al.
AJOG 2008

38

FETAL CD34+ CELL PERSISTENCE ? FETAL MICROCHIMERISM?

- Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum.

  Bianchi DW, DeMaria MA et al. Proc Natl Acad Sci USA 1996; 93: 705-708

  "Decades after delivery, male fetal CD34+ and CD34+CD38+ cells have been identified in 75% of women with sons"

- Prenatal diagnosis of genetic abnormalities using fetal CD34+ stem cells in maternal circulation and evidence they do not affect diagnosis in later pregnancies.

  Coata G, Di Renzo GC et al. Stem Cells 2001; 19: 534-542

  "Using a well-described PCR amplification protocol, we identified Y positivity in samples from 3/13 mothers who gave birth male offspring and 1/12 women who had never been pregnant"

- Male microchimerism in women without sons: Quantitative assessment and correlation with pregnancy history.

  Yan Z, Nelson JL et al. Am J Med 2005; 118 (8): 899-906

  "Male microchimerism was not infrequent in women without sons"

39

FETAL CD34+ CELL PERSISTENCE ? FETAL MICROCHIMERISM?

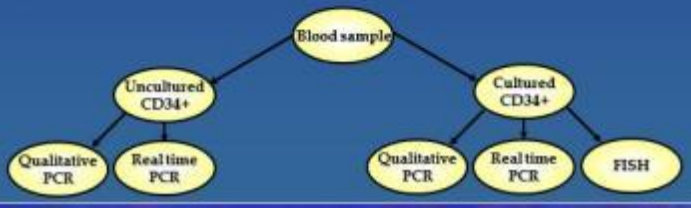
PATIENTS

-10 non pregnant women with at least one male son
- 10 never pregnant women*
- 5 male adult patient (positive control)

* We used a strictly criteria of enrollment: well-documented reproductive history (no spontaneous fetal loss, no elective termination, no extra-uterine pregnancy) and no blood transfusion, no autoimmune disease, no twin brother or sister

MATERIALS MEDHODS

- CD34+ cells: before and after culture in semi-solid medium (MethoCult GF+ H4435, Stem Cell Technologies)
- DNA extraction with QIAamp DNA mini kit (Qiagen)
- qualitative PCR by using primer specific for SRY single copy gene (SRY-109F - SRY-245R)
- real time PCR by using the same primer of qualitative PCR and SRY specific TaqMan probe
- FISH analysis by using centromeric XY probes (Vysis)

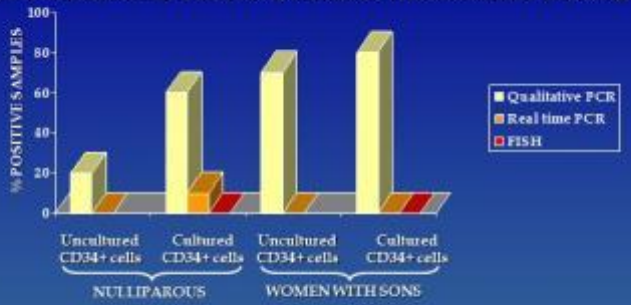


40

FETAL CD34+ CELLS PERSISTENCE ? FETAL MICROCHIMERISM?

RESULTS

COMPARISON AMONG THE RESULTS OBTAINED BY THE 3 DIFFERENT TECHNIQUES



- By using qualitative PCR, we obtained an elevated number of positive samples from women with sons and women who have never been pregnant; while we obtained only 1 positive sample in the group of nulliparous by real time PCR and anyone by FISH
- There is no agreement between qualitative PCR and real time quantitative PCR findings; while there is a good agreement between real time quantitative PCR and FISH findings for both the studied groups

41

FETAL CD34+ CELLS PERSISTENCE ? FETAL MICROCHIMERISM?

CONCLUSIONS

- By using qualitative PCR, we found a considerable number of SRY positive samples in both the studied groups; however, the specificity of these results must be evaluated
- Real time quantitative PCR and FISH techniques do not detect male microchimerism in both the studied groups
- Therefore, even if fetal CD 34+ cells could persist in maternal blood, their number are evidently very low and does not influence the quantitative PCR and FISH results for diagnosis of fetal trisomies in subsequent pregnancies

NEW POSSIBILITIES FROM FREE FETAL DNA

New possibilities in the field of prenatal non invasive diagnosis

- *Schmorl, 1893: presence of fetal cells in maternal blood*
- *Lo et al., 1997: presence of free fetal DNA (ffDNA o cfDNA) in maternal blood*
- *Lo et al., 1998: ffDNA concentrations correspond to 3,4% of total maternal plasma during first trimester of pregnancy, but to 6,2% in the late third trimester*
- *Lo et al., 1998, Bianchi et al., 1998; Watanagara et al., 2005: ffDNA amount increases by advancing in gestational age*
- *Lo et al., 1998: ffDNA levels are almost 1,000-fold higher than the DNA present in intact fetal cells circulating in maternal plasma*
- *Lo et al., 1999: The circulating cfDNA mean half-life is of about 72 hours after delivery*

ffDNA applications in prenatal non invasive diagnosis

- *Huge diagnostic potentiality of ffDNA*
- *Monogenic disorders: About 9000 known monogenic disorders; frequency of 3,6 affected newborns per 1000 living newborns*
- *X-linked diseases: Incidence of 1/2000; only for a small number of these diseases are available specific genetic tests*
- *Non invasive prenatal diagnosis of fetal RhD status: pregnant woman RhD- at risk for developing isoimmunization against RhD+ fetus*