

NON-INVASIVE PRENATAL MOLECULAR DIAGNOSIS

Recent Advances

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IMPORTANCE OF PRENATAL SCREENING AND DIAGNOSIS

- **FETAL CHROMOSOMAL
ABNORMALITIES (ANEUPLOIDES)**
- **FETAL SINGLE-GENE GENETIC
DISORDERS**
- **FETAL MORE SUBLTE
CHROMOSOMAL
ABNORMALITIES**

INDICATIONS FOR PRENATAL SCREENING AND DIAGNOSIS

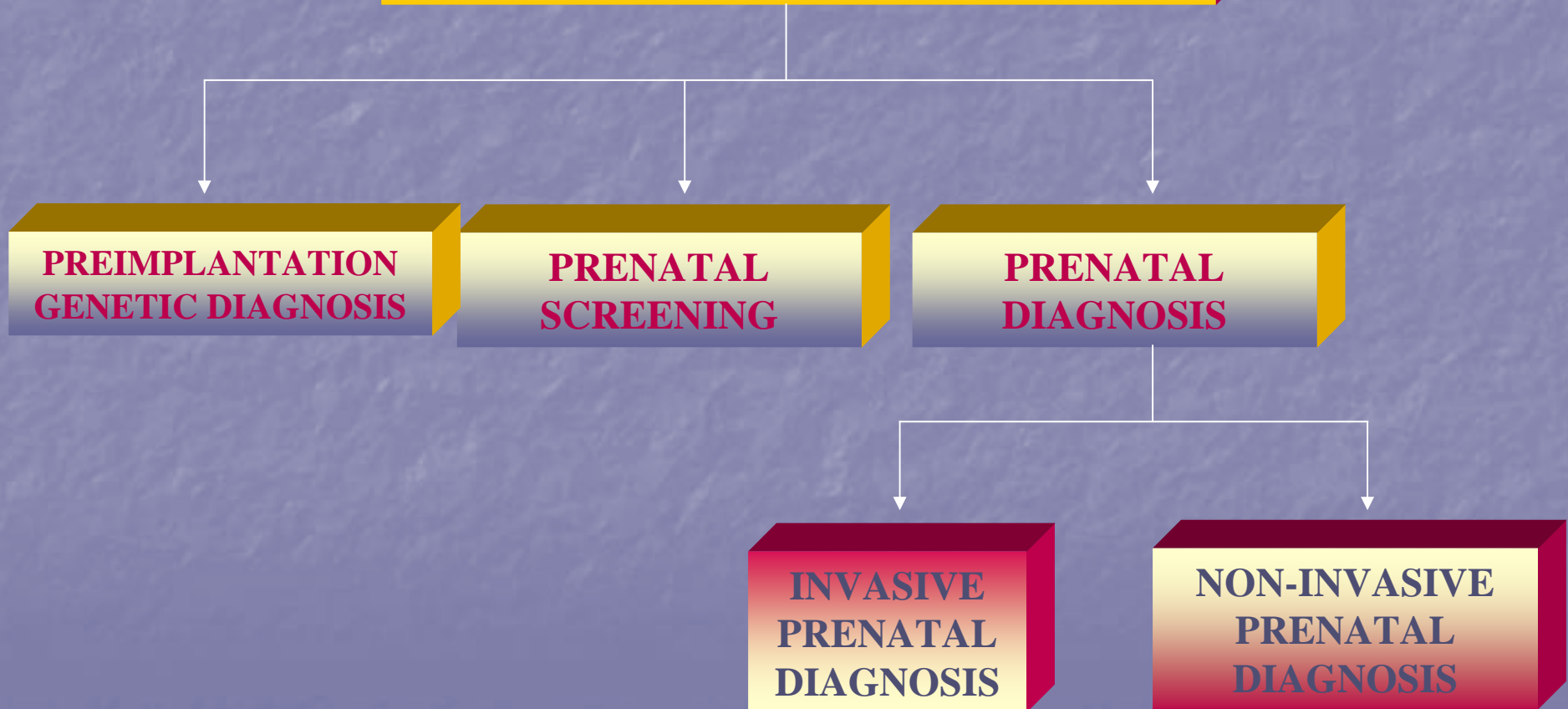
- **Advanced maternal age**
- **Abnormal fetal ultrasound findings**
 - nuchal thickening
 - cystic hydroma
 - heart abnormalities
- **Positive maternal serum screening**
- **Previous pregnancy or child with chromosomal abnormality**
- **Chromosome rearrangement in either member of the couple**
- **Increased risk for single gene disorder or X-linked disorder**
- **Increased risk of neural tube defect**
 - high AFP in maternal serum
 - previous child with neural tube defect
 - pregnancy exposure to valproic acid, carbamazepine, or other known teratogens
 - gestational or insulin-dependent diabetes mellitus

USEFULNESS OF PRENATAL SCREENING AND DIAGNOSIS

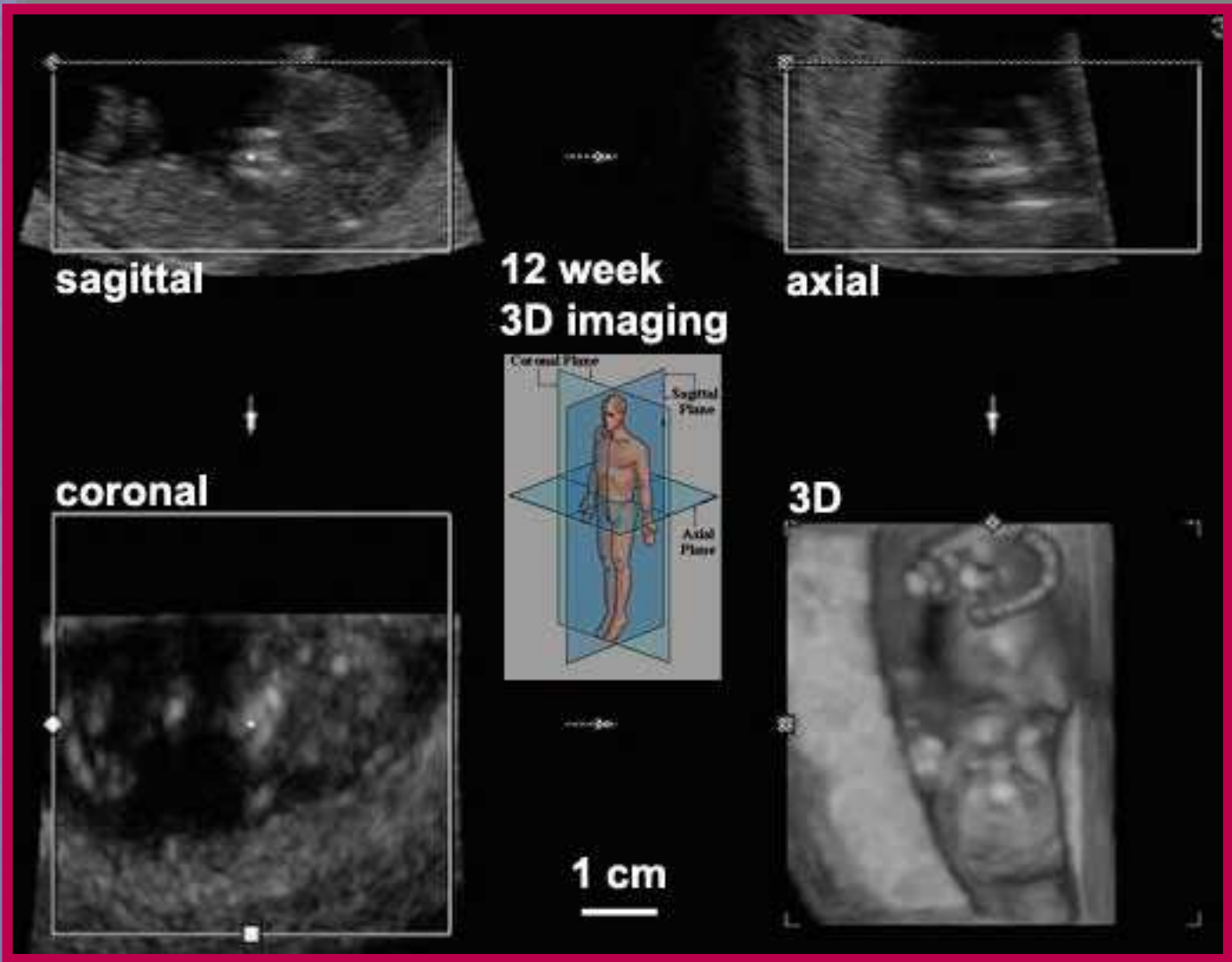
- **Intervention reducing the incidence of the condition**
- **Specific treatment that may improve the outcome of the condition**
- **Increase of the time that the family and medical providers have to prepare for managing the condition, and provide information for future family planning**

PRENATAL SCREENING AND DIAGNOSIS

PRENATAL TESTING



ULTRASOUND



BIOCHEMICAL TESTING

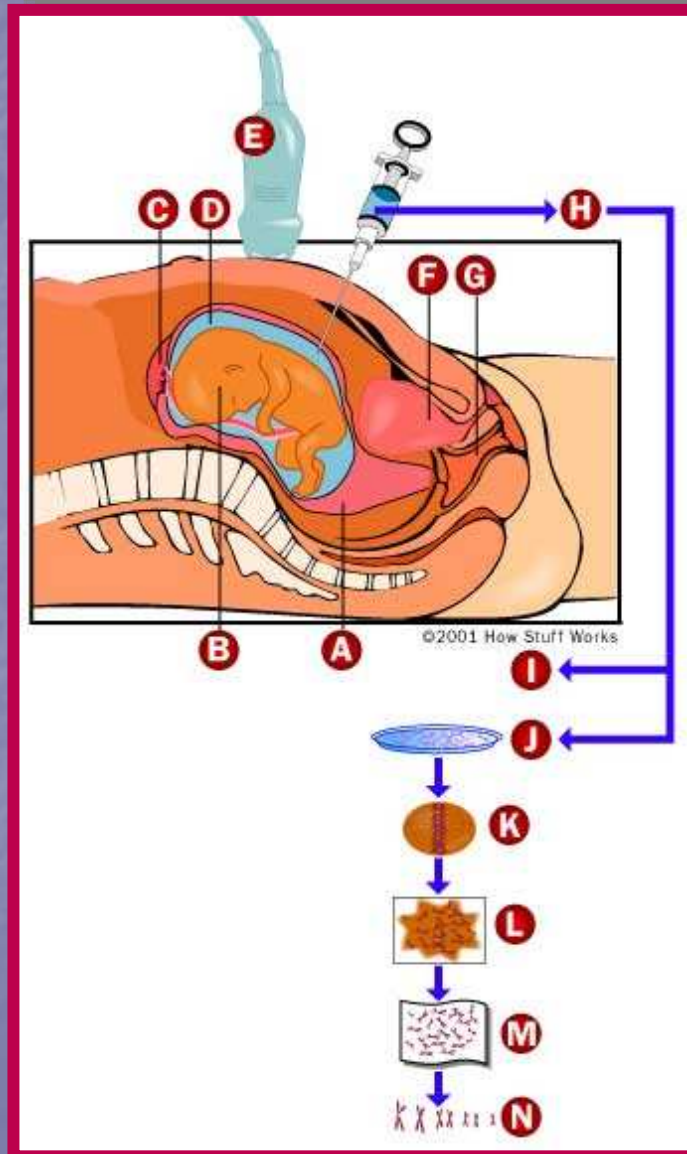
- **FIRST TRIMESTER PAPP-A TEST (PAPP-A, free β -hCG)**
- **SECOND TRIMESTER A-TEST (β -hCG, AFP, free Estriol, Inhibin-A)**

LIMITATIONS OF PRENATAL SCREENING

- SCREENING ONLY FOR DOWN SYNDROME
- NO DETECTION OF ANEUPLOIDES
- NO DETECTION OF SINGLE-GENE DISORDERS
- NO DETECTION OF MORE SUBTLE CHROMOSOMAL ABNORMALITIES
- NO DIAGNOSIS, RATHER ESTIMATION OF WOMAN'S ADJUSTED (or POSTERIOR) RISK OF VARIOUS GENETIC DISEASES

AMNIOCENTESIS

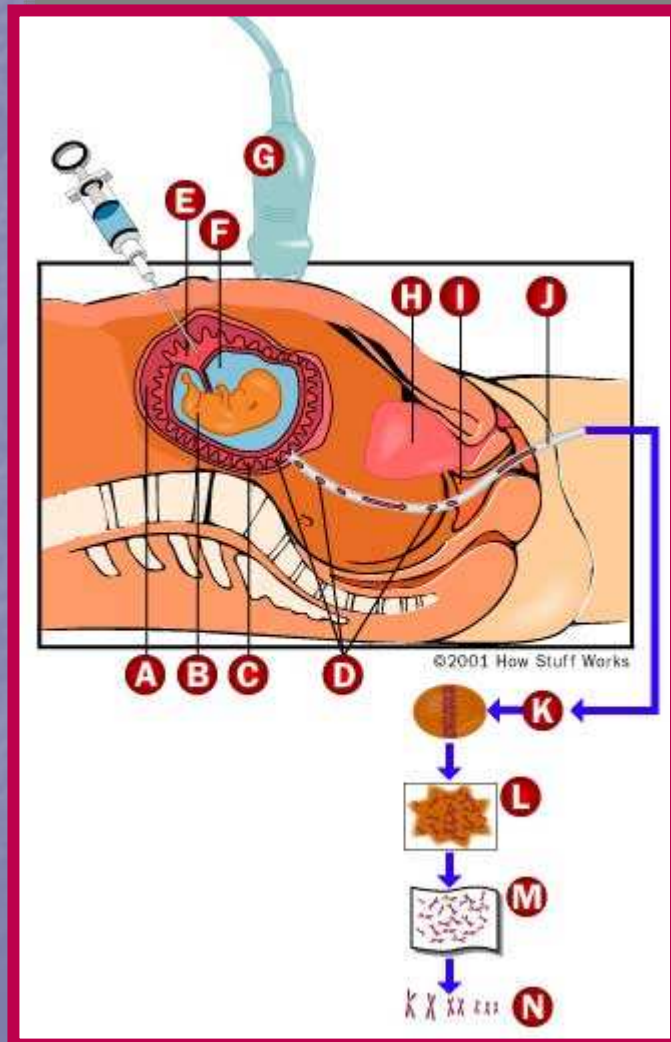
(AFTER 15th WEEK OF GESTATION)



- A** Uterus
- B** Fetus
- C** Placenta
- D** Amniotic sac
- E** Ultrasound probe
- F** Bladder
- G** Vagina
- H** Sample of amniotic fluid removed
- I** Analysis of chemicals in amniotic fluid
- J** Cells from amniotic fluid grown in culture
- K** 2-3 weeks of cell growth
- L** Treated cells are squashed on a microscope slide
- M** Chromosomes are photographed
- N** A karyotype is prepared

CHORIONIC VILLI SAMPLING

(11th – 14th WEEK OF GESTATION)



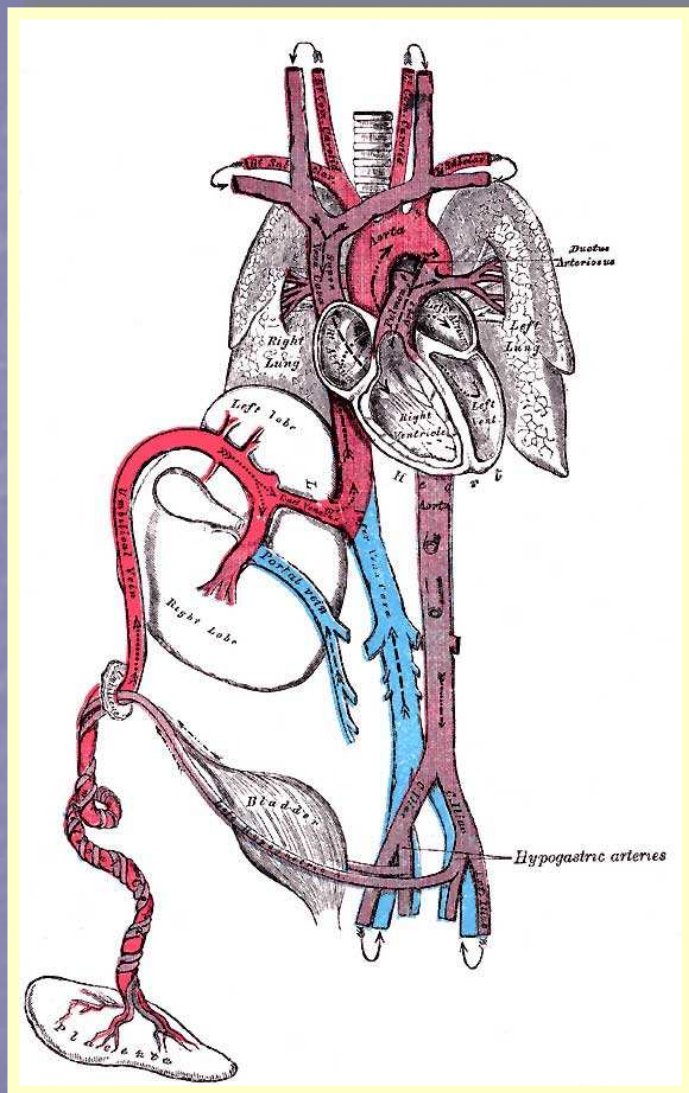
- A** Uterus
- B** Fetus
- C** Chorion
- D** Chorionic villi
- E** Placenta
- F** Amniotic fluid
- G** Ultrasound probe
- H** Bladder
- I** Vagina
- J** Sampling tube
- K** Next day
- L** Treated cells are squashed on a microscope slide
- M** Chromosomes are photographed
- N** A karyotype is prepared

LIMITATIONS OF PRENATAL INVASIVE DIAGNOSIS

- **SOME DEGREE OF RISK TO THE FETUS AND/OR MOTHER**
- **ABORTION OWING TO HEMORRAGE OR INFECTION OCCURS IN 0,2 – 0,4% OF PREGNANCIES IN WHICH AMNIOCENTECIS IS PERFORMED**
- **CVS CARRIES A POTENTIAL RISK OF FETAL LIMB MALFORMATION IN 0,01 – 0,03% OF CASES**
- **MOTHERS' DISTURBANCE**

NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

- **FETAL CELLS IN MATERNAL CIRCULATION**
- **FETAL NUCLEIC ACIDS (DNA, mRNA) IN MATERNAL CIRCULATION**



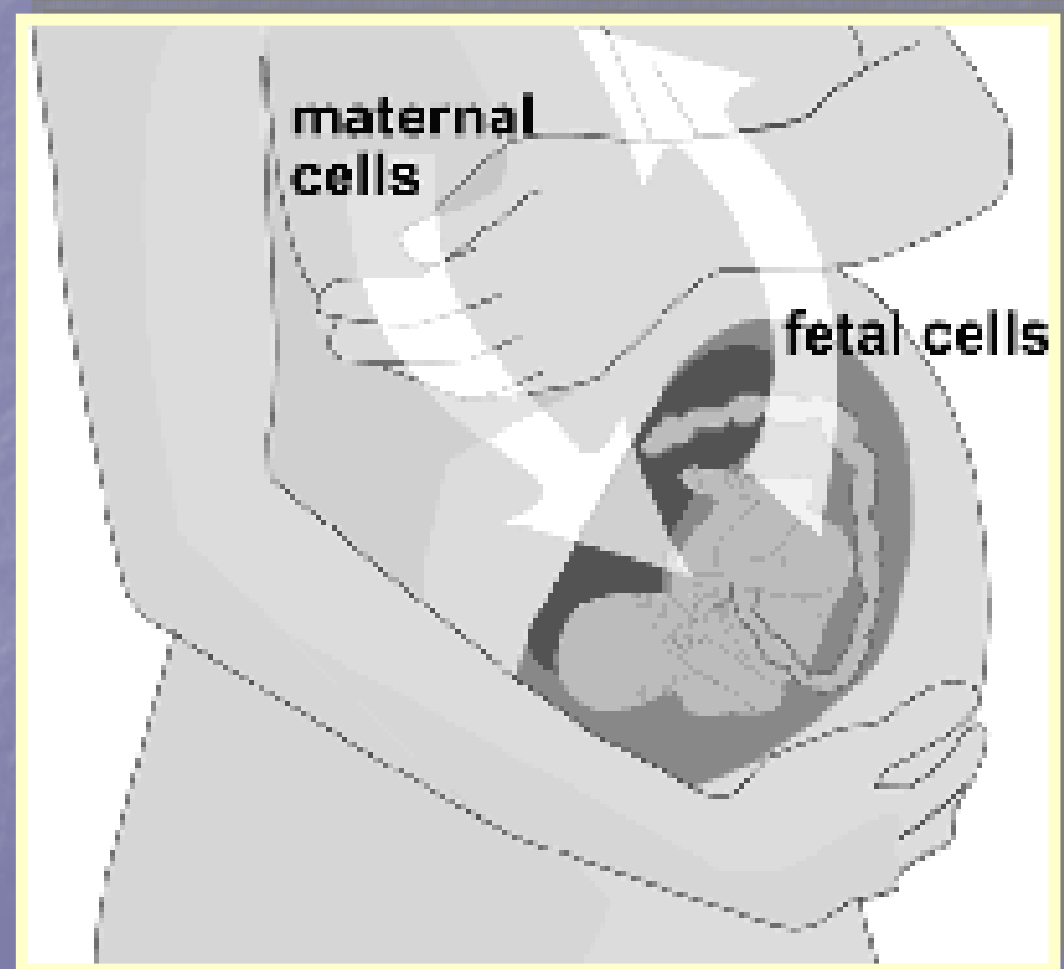
The historic diagram shows the general organisation of blood flow in the fetus including the connection with the placenta.

In General

Maternal Blood | -> umbilical vein -> liver -> anastomosis -> sinus venosus -> atria ventricles-> truncus arteriosus -> aortic sac -> aortic arches-> dorsal aorta-> pair of umbilical arteries | Maternal Blood

FETAL CELLS IN MATERNAL CIRCULATION

Fetomaternal cell trafficking results in the presence of fetal cells in the maternal circulation throughout pregnancy



TYPES OF FETAL CELLS IN MATERNAL CIRCULATION

- **TROPHOBLASTS**
- **LEUKOCYTES**
- **NUCLEATED RED BLOOD
CELLS (ERYTHROBLASTS)**

ADVANTAGES OF FETAL CELLS IN NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

- **PURE SOURCE OF THE ENTIRE FETAL GENOME, WITHOUT THE POSSIBLE INCLUSION OF ANY MATERNAL GENETIC MATERIAL**
- **POSSIBILITY FOR DIAGNOSIS OF ANY GENETIC DISEASE (INCLUDING ALL MENDELIAN DISORDERS) OR CHROMOSOMAL ABNORMALITY**

LIMITATIONS OF FETAL CELLS IN NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

- **SCARCITY OF INTACT FETAL CELLS IN THE MATERNAL CIRCULATION (around one cell per ml of maternal blood)**
- **LOW EFFICIENCY OF ENRICHMENT**
- **DIFFICULTIES WITH CHROMOSOMAL ANALYSIS ASSOCIATED WITH ABNORMALLY DENSE NUCLEI IN SOME CELLS**

ENRICHMENT METHODS OF FETAL CELLS FROM MATERNAL BLOOD

- **Fluorescence Activated Cells Sorting (FACS)**
- **Magnetic Activated Cell Sorting (MACS)**
- **Density gradient centrifugation**
- **Lectin-based methods**
- **Autodetection**

DNA ANALYSIS METHODS

- **Fluorescence In-Situ Hybridization (FISH)**

- **Polymerase Chain Reaction (PCR)**

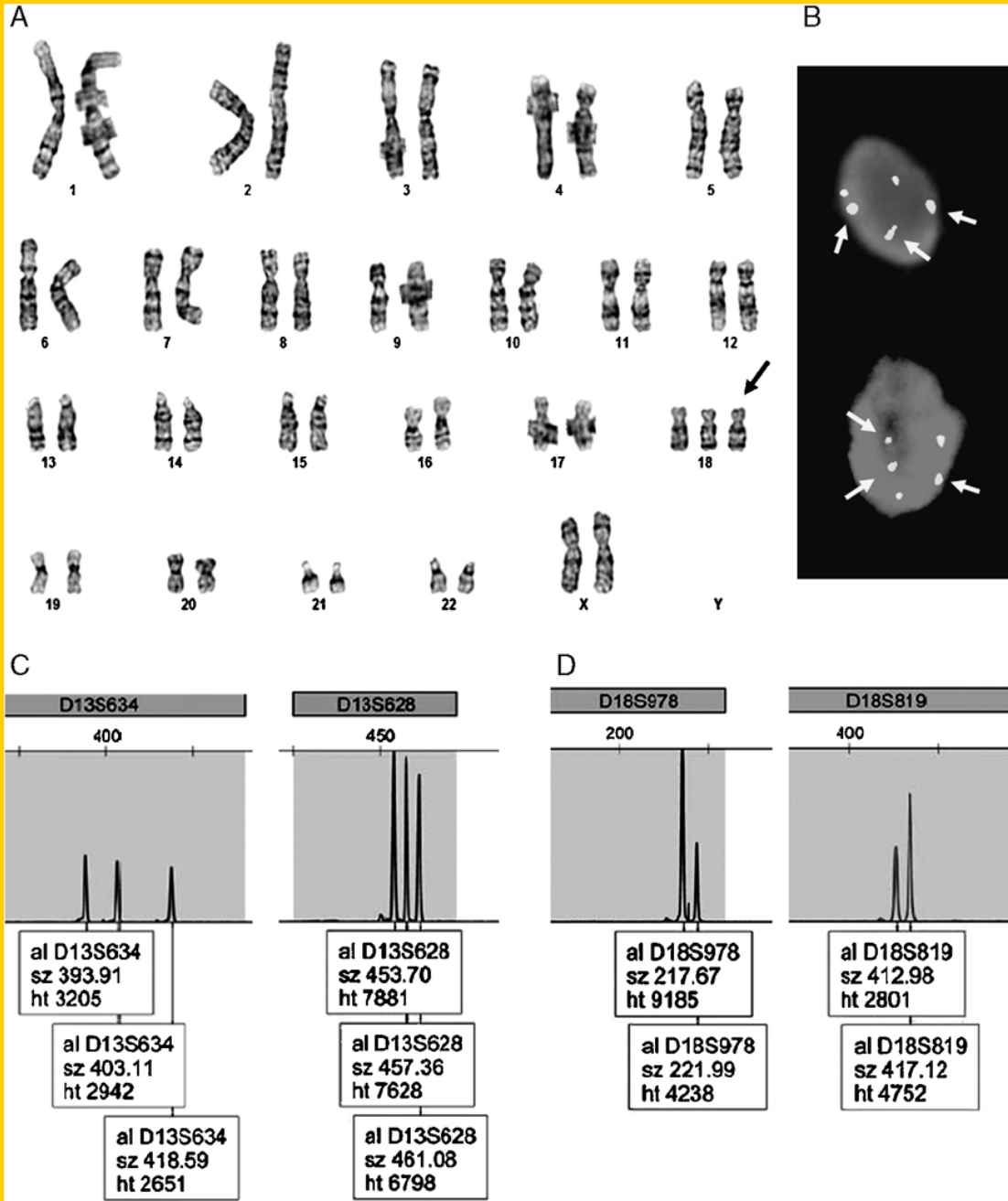
 - Real Time Quantitative PCR**

 - Nested PCR**

 - Pyrophosphorolysis-activated
polymerization PCR**

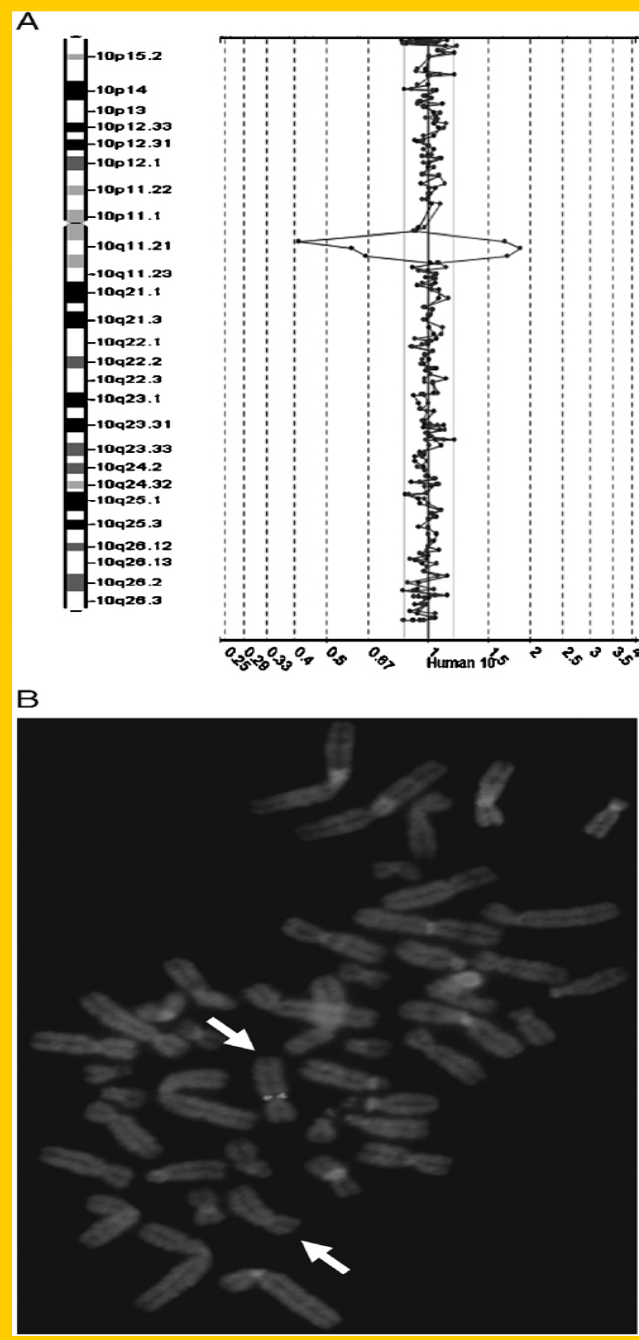
 - Digital PCR**

- **Mass Spectrometry**



Comparison of full chromosome analysis, rapid FISH, and QF-PCR for aneuploidy detection. A, Chromosomes from cultured amniocytes from a female fetus with trisomy 18. Arrow marks the additional chromosome 18. B, FISH analysis on uncultured amniocytes with probes to the centromeres of chromosomes X and 18 showed 3 copies of the chromosome 18 centromere (arrows) and 2 copies of the chromosome X centromere (unmarked signals) suggesting a female fetus with trisomy 18 (note: actual signals for these 2 sites are in different colors, which cannot be differentiated in this black and white reproduction). C, QF-PCR results for 2 STRs on chromosome 13 revealed 3 unique alleles for each STR, consistent with trisomy 13. Peaks are labeled with the STR locus, size of PCR product, and peak area, which is necessary for quantification of allele copy number. D, QF-PCR results for 2 STRs on chromosome 18 indicate 2 copies of 1 allele and 1 copy of a different allele for each STR, consistent with trisomy 18 owing to total of 3 alleles. Determination of the allele copy number was assessed by comparing the ratio of each peak area. Ratios between 0.8 and 1.4 indicated each peak represented a single allele, ratios of >1.8 or <0.65 were considered to be indicative of 1 peak representing two or more alleles.

Array Comparative Genomic Hybridization



aCGH for the detection of a subtle chromosome imbalance. A, Ratio plot of the intensity of the 2 fluorochromes at each target spot representing chromosome 10 from an aCGH assay on a patient referred for developmental delay. The reciprocal deviation seen for the target DNAs representing 10q11.22 to 10q11.23 indicates a deletion of this genomic segment in the patient. B, FISH using a probe that hybridizes to one of the targets in the 10q11.22 to 10q11.23 region is used to confirm the deletion in metaphase cells from the patient's sample. Arrows mark the normal 10 with the FISH signal visible just below the centromere (upper), and the deleted 10 that fails to hybridize with the FISH probe owing to the interstitial deletion (lower).

FETAL CELL-FREE NUCLEIC ACIDS IN NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

- **DNA-type**
- **mRNA-type**

BIOLOGY OF FETAL CELL-FREE DNA

- Product of **APOPTOSIS** or **NECROSIS** of placenta cells (trophoblasts) derived from the embryo, resulting in fragmentation and ejection of chromosomal DNA from the cell
- 3-6% of the total cell-free DNA in maternal circulation
- Predominantly short DNA fragments rather than whole chromosomes (80% are < 193 bp in length)
- Detection from the 4th week of gestation, though reliably from 7th week
- Concentration increases with gestational age – from equivalent of 16 fetal genomes per ml of maternal blood in the first trimester to 80 in the third trimester – with a sharp peak during the last 8 weeks of pregnancy
- Rapidly cleared, mainly by the renal system, from the maternal circulation with a half-life of 16 min and undetectable 2h after delivery

ADVANTAGES OF FETAL CELL-FREE NUCLEIC ACIDS IN NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

- **Significantly more present than fetal cells in maternal circulation by a factor almost 1000**
- **Direct fetal gender determination**
- **Detection of single-gene disorders**
- **Direct detection of the unique paternally inherited mutations in genetic diseases that are caused by more than one mutation, and in which the father and mother carry different mutations**
- **Tracing the inheritance of the mutant or normal chromosome by the fetus through the analysis of linked SNPs, for situations in which the father and the mother carry the same mutation**
- **Detection amenable to automation**

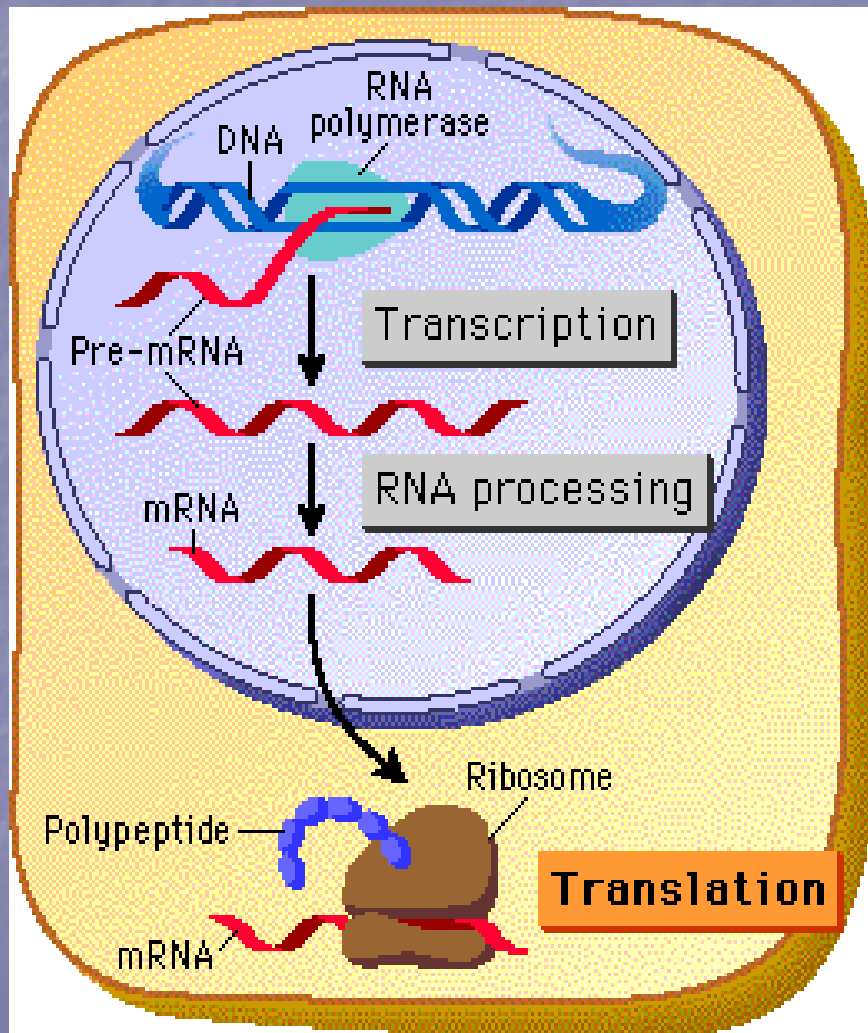
LIMITATIONS OF FETAL CELL-FREE NUCLEIC ACIDS IN NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

- **NO DETECTION OF ANEUPLOIDES**
- **RELATIVELY LOW CONCENTRATION IN MATERNAL BLOOD**
- **VARIATION BETWEEN INDIVIDUALS**
- **FETAL CELL-FREE NUCLEIC ACIDS ARE OUTNUMBERED 20 : 1 BY MATERNAL CELL-FREE NUCLEIC ACIDS**
- **THE FETUS INHERITS HALF ITS GENOME FROM THE MOTHER**

ENRICHMENT METHODS OF FETAL CELL-FREE DNA FROM MATERNAL CIRCULATION

- **Selective enrichment of fetal DNA based on the difference in the average physical length and maternal DNA fragments**
- **Suppression of maternal DNA by the addition of formaldehyde, a chemical that is thought to stabilize intact cells, thereby inhibiting further release of maternal DNA into the sample and increasing the relative proportion of fetal DNA**

Translation in a Eukaryotic Cell



In a eukaryotic cell, mRNA is synthesized in the nucleus and translated on ribosomes in the cytoplasm.

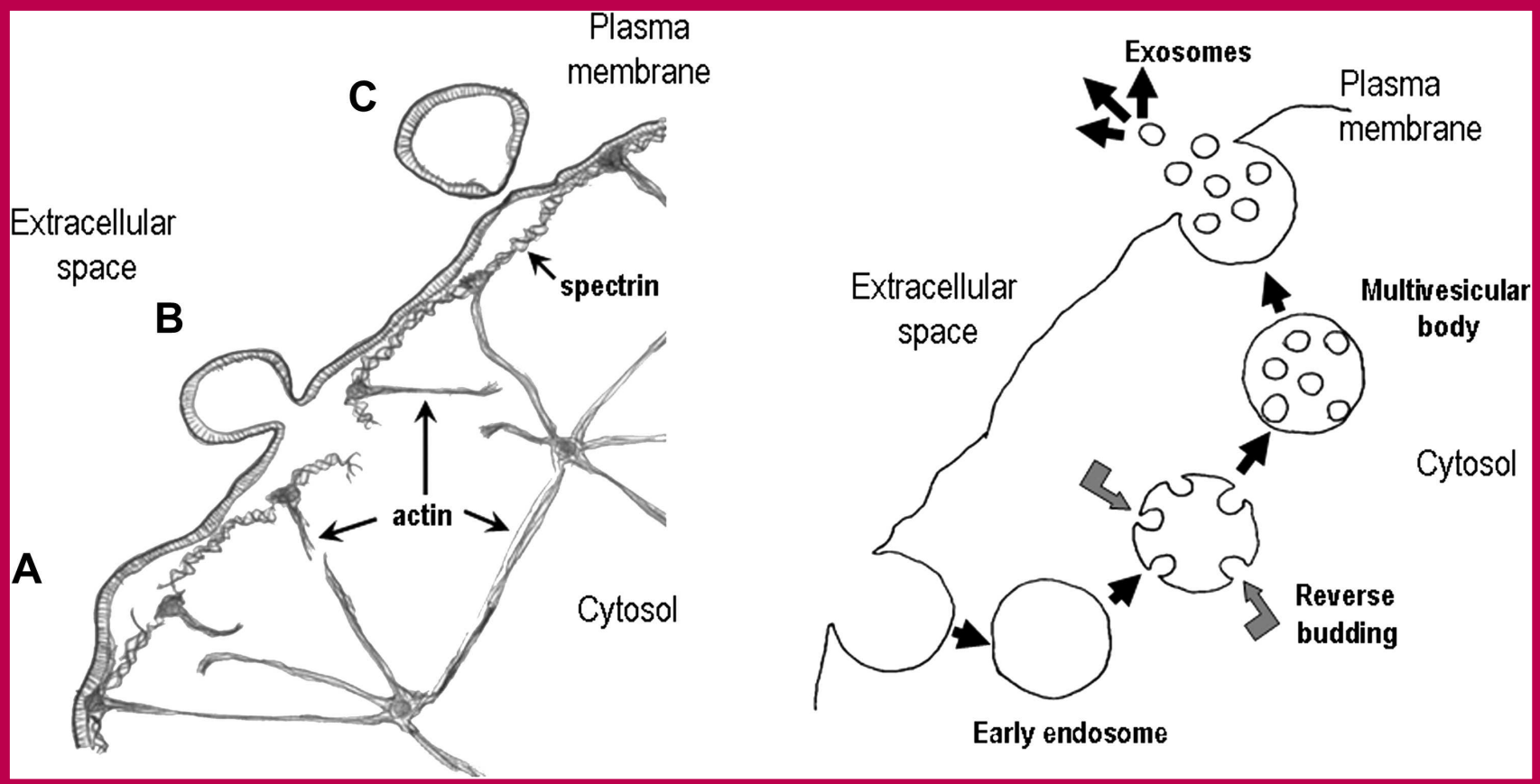
A protein-coding gene is transcribed into a pre-mRNA.

Pre-mRNA is processed into a mature mRNA.

mRNA exits the nucleus.

mRNA is translated on ribosomes to produce the polypeptide chain.

SYNCYTIOTROPHOBLAST MICROPARTICLES



ADVANTAGES OF FETAL CELL-FREE mRNA IN NON-INVASIVE PRENATAL DIAGNOSIS (NIPD)

- **POTENTIAL DISEASE
SPECIFICITY**
- **BROAD POPULATION COVERAGE,
IRRESPECTIVE OF FETAL
GENDER AND POLYMORPHISMS**

UNIVERSAL FETAL MARKERS

- **Single nucleotide polymorphisms (SNPs) or point mutations which differ between the maternal and paternal genomes but may not be linked directly to a specific disease**
- **Polymorphic segments of DNA that vary between the maternal and paternal genomes, such as short tandem repeats (STRs)**
- **Epigenetic modifications, specifically DNA methylation of certain genes, which differs between cells of the mother versus the growing fetus (SERPINB5)**
- **Detection of mRNA derived from genes that are uniquely active in the placenta or fetus (PLAC4)**
- **Detection of proteins derived from genes that are uniquely expressed in the placenta or fetus**

CLINICAL APPLICATIONS OF FETAL CELL-FREE NUCLEIC ACIDS IN NON-INVASIVE PRENATAL DIAGNOSIS

- **Sex determination** – by detecting cff-DNA sequences on the Y chromosome
- **Single-gene disorders** – by detecting a paternally inherited allele in cff-DNA
- **Pregnancy-related disorders** – by detecting either the presence of a working copy of the Rhesus gene or an elevation in the absolute concentration of cff-DNA
- **Aneuploidy** – by detecting an abnormal concentration of a particular chromosome, potentially using cff-RNA specific to the fetus and chromosome of interest

SEX DETERMINATION BY FETAL CELL-FREE DNA NON-INVASIVE TESTING

- **Male fetus at risk of a sex-linked disease, such as haemophilia or Duchene muscular dystrophy**
- **Ambiguous development of external genitalia**
- **Some endocrine disorders such as congenital adrenal hyperplasia**

SINGLE-GENE DISORDERS DIAGNOSIS BY FETAL CELL-FREE DNA PRENATAL NON-INVASIVE TESTING (I)

- **Diagnosis of dominant diseases that are paternally inherited (or occur *de novo* as a result of spontaneous mutations arising during oocyte or sperm formation**

Huntington's disease

Achondroplasia

Myotonic dystrophy

SINGLE-GENE DISORDERS DIAGNOSIS BY FETAL CELL-FREE DNA PRENATAL NON-INVASIVE TESTING (II)

■ Autosomal recessive diseases – fetal carrier status

Cystic fibrosis

Haemoglobinopathy

Congenital adrenal hyperplasia

DETECTION OF PREGNANCY-RELATED DISORDERS BY FETAL CELL-FREE DNA PRENATAL NON-INVASIVE TESTING

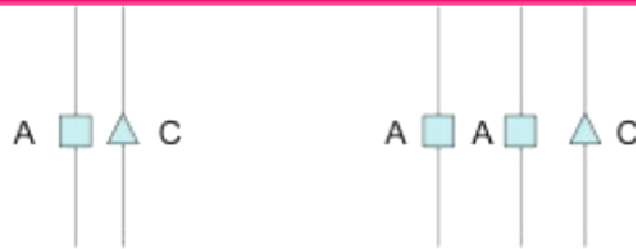
- **Fetal Rhesus blood group, by detection of a fetal Rhesus antigen gene**
- **Abnormal formation and functioning of the placenta, causing elevation of the cff-DNA concentration, which could be used as a diagnostic marker**

(preeclampsia, preterm labour, hyperemesis gravidarum, invasive placentation, IUGR, feto-maternal haemorrhage and polyhydramnios)

ANEUPLOIDY DETECTION BY FETAL CELL-FREE NUCLEIC ACIDS PRENATAL NON-INVASIVE DIAGNOSIS

- **Detection and quantification of chromosome specific markers which must necessarily be altered by aneuploidy**
- **Spontaneous detection of hundreds of paternally inherited SNPs on the chromosome of interest**
- **Exploitation of differences in the DNA methylation pattern between placenta and maternal cells, of a gene containing an informative SNP on the chromosome of interest (SERPINB5 in chromosome 18)**
- **Detection of uniquely placentally derived RNA, which contains an informative SNP, from the chromosome of interest (PLAC4 in chromosome 21)**

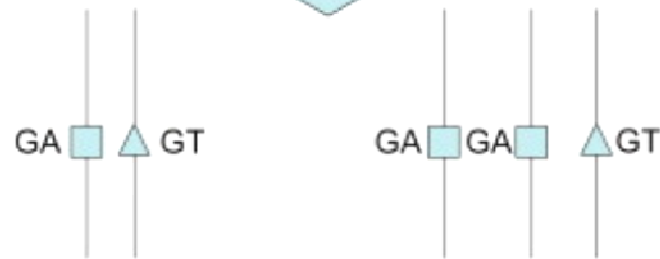
Heterozygous *maspin* locus
in placenta (unmethylated)



Release of unmethylated *maspin* cff-DNA into maternal plasma



Bisulfite-conversion (C→T)
of fetal *maspin* sequences



Methylation specific PCR and allele specific extension

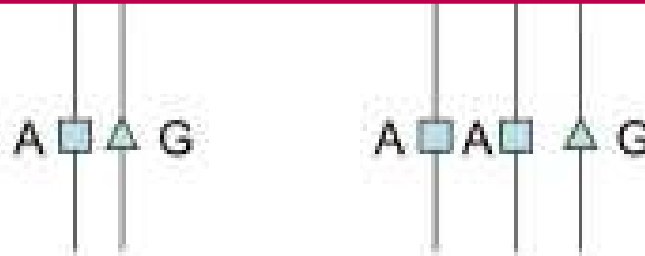


Quantitation of GA:GT
allelic ratios by MS

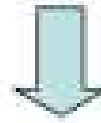


Schematic representation of the non-invasive detection of fetal trisomy 18 via the analysis of epigenetically modified *maspin* sequences. cff-RNA, placentally derived cell-free fetal messenger RNA; MS, mass spectroscopy; PCR, polymerase chain reaction.

Heterozygous
PLAC4 gene



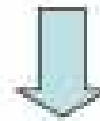
Placental transcription into mRNA



Heterozygous cff-RNA
in maternal plasma



RT-PCR and MS analysis



Quantitation of SNP
allelic ratios by MS



Schematic representation of the non-invasive detection of fetal Down syndrome via the analysis of placentally derived *PLAC4* cf-RNA. cff-RNA, placentally derived cell-free fetal messenger RNA; mRNA, messenger RNA; MS, mass spectroscopy; RT-PCR, real-time polymerase chain reaction; SNP, single nucleotide polymorphism.

SENSITIVITY :
90%
SPECIFICITY :
97%

FETAL FREE-CELL NUCLEIC ACID OF MATERNAL CIRCULATION IN CLINICAL PRACTICE

- **SEX DETERMINATION**
- **FETAL RHESUS BLOOD
GROUP**

On the cost issue, judging from the current platforms of circulating nucleic acid-based testing, this methodology should compare favourably with existing invasive methods of prenatal diagnosis, especially when the relative expensive clinicians' time involvement is factored in.

As like many other rapidly developing areas of research, the SOCIAL, ETHICAL and REGULATORY discussions tend to lag behind the technological progress, although this will hopefully be rectified as we enter the second decade of the field.

MAIN ACTIVE RESEARCH TARGETS

- **Research into fetal cell sorting techniques in the maternal blood**
- **Research into fetal cell-free nucleic acids in the maternal blood – new fetal- or placenta-specific molecular markers**



CLINICAL AND RESEARCH LAB FOR THERAPEUTIC INDIVIDUALIZATION (CRLTI)

ARETAEIO UNIVERSITY HOSPITAL

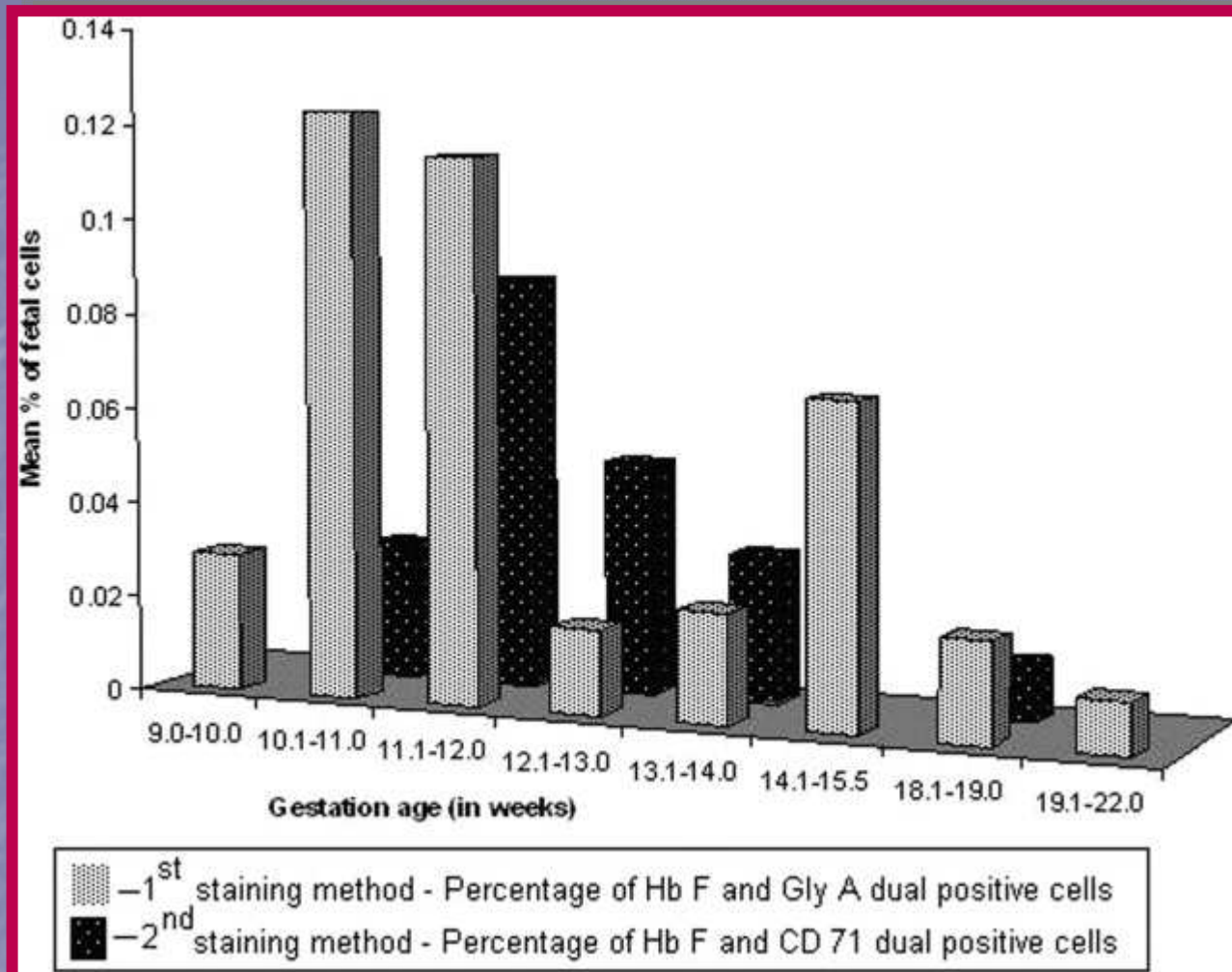


RELEVANT RESEARCH TARGETS IN CRLTI

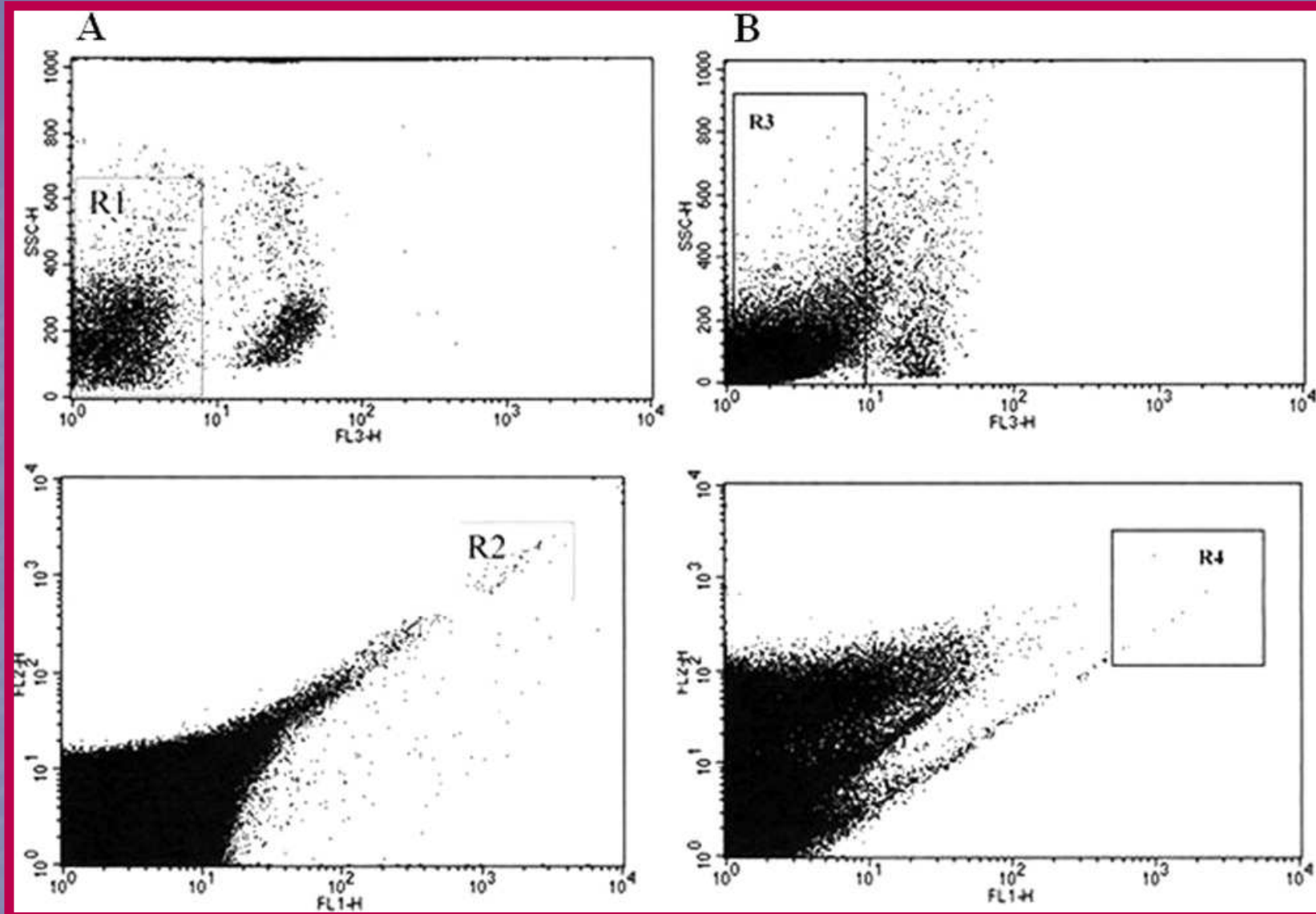
- **FACS sorting of fetal erythroblasts in maternal circulation appropriate to molecular analysis**
- **Detection and enumeration of fetal syncytiotrophoblast microparticles in maternal circulation**

PROTOCOL TECHNIQUES

- **Double discontinuous density gradient of Percoll for the separation of NRBCs from the maternal cell population**
- **Flow-cytometry NRBC detection using two sets of monoclonal antibodies**
 - 1st set : anti-CD45 (glycophorin A) and anti-fetal hemoglobin**
 - 2nd set : anti-CD45 (glycophorin A) and anti-fetal hemoglobin plus anti-CD71 (transferrin receptor)**



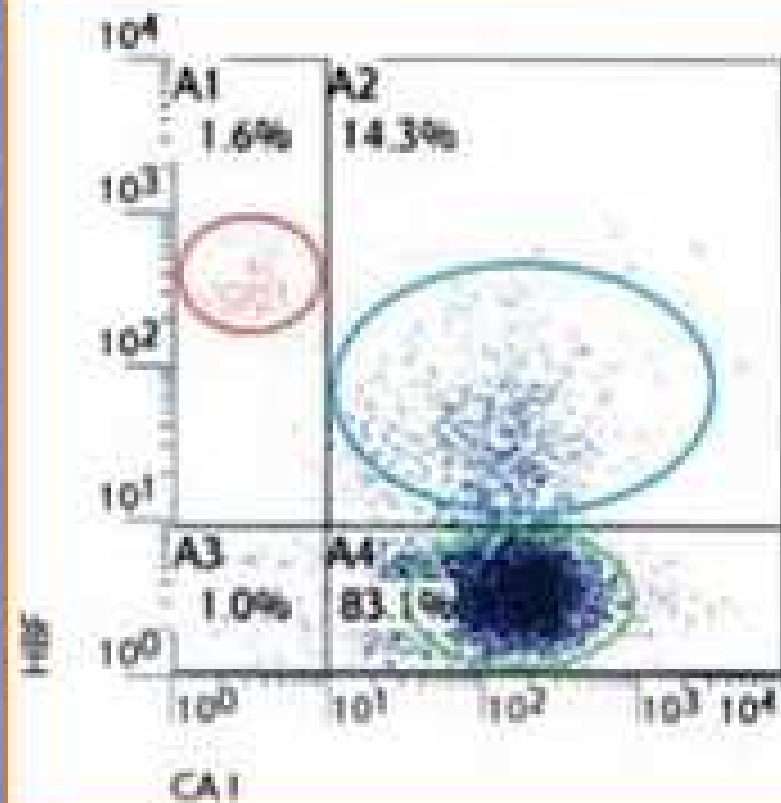
Comparison between the two combinations of antibodies over increasing gestational age. The graph shows a comparison of the two sets of antibodies over an increasing gestational age, which shows that we were able to obtain a comparatively better yield of fetal cells using CD45, anti-HbF, and Gly A beginning at 10 weeks.



Sorting strategy employed for the isolation of fetal NRBCs using the two sets of antibodies. **A:** In the first sorting panel, the first histogram displays both the CD45 positive and negative populations. In gate R1, we have selected for the CD45 negative population. The second histogram displays the cells in the gated R1 population. We selected for the anti-HbF-FITC positive cells on the X-axis and the Gly A-PE positive cells on the Y-axis. Thus the dual positive cells in Gate R2 were sorted. **B:** In the second sorting panel, Gate R3 represents the CD45 negative population and in the gate R4, we have selected for the dual positive anti-HbF-FITC on the X-axis and CD71-PE on the Y-axis.

FETO-MATERNAL TRANSFUSION DIAGNOSIS IN ROUTINE

- **Abdominal trauma**
- **Cases with possible RhD incompatibilities**
- **Preeclampsia**
- **Blood loss during pregnancy**
- **Intra-uterine transfusion**
- **Neonatal alloimmune thrombocytopenia
(anti-human platelet antigen HPA-1a)**
- **Hemolytic disease of the fetus and neonate
(fetal D-positive red cells)**



- = fetal RBCs HbF⁺⁺
- = maternal F-cells HbF⁺/CA⁺⁺
- = maternal RBCs HbF⁻/CA⁺⁺

Fetal Cell Count™ - unique concept using anti-HbF and anti-CA to allow the discrimination between fRBCs and maternal F cells

FLOW-CYTOMETRIC DETECTION OF SYNCYTIOTROPHOBLAST MICROPARTICLES

- **ANTI-HUMAN PLACENTAL
LACTOGEN**
- **ANTI-HUMAN CHORIONIC
GONADOTROPHIN**
- **ANTI-PLATELET GLYCOPROTEIN III_a**

CLINICAL AND RESEARCH LAB FOR THERAPEUTIC INDIVIDUALIZATION

- **Associate Professor Dr E. Kouskouni, Director of the Lab**
- Mr V. Tsamadias, B.Sc., Molecular Biologist – Genetist, Research Scientific Assistance
- Mrs S. Demeridou, Technologist, Technical Assistance
- Mrs E. Samara, Technician, Technical Assistance
- Mr E. Papakonstantinou, student in Molecular Biology, Routine Scientific Assistance
- Miss I. Zografou, student in Molecular Biology, Routine Scientific Assistance

Messages to take home

- **Non-invasive prenatal diagnosis of fetal aneuploides or genetic disorders has become realistic goal in routine prenatal care**
- **Fetal cell-free nucleic acids can be detected in maternal plasma after 7 weeks**
- **Fetal DNA within maternal plasma can be used for accurate fetal gender determination and fetal RhD blood typing in Rh- pregnant women**
- **It can be also applied to the identification of the paternally inherited diseases and sporadic genetic disorders**
- **Fetal DNA from maternal plasma cannot be used to diagnose maternally inherited diseases**
- **Recently fetal DNA was used to diagnose fetal aneuploides with a sensitivity of 90 % and price as cheap as any other relevant invasive procedure**
- **Analysis of NRBC in maternal blood retains some advantages**
- **Recent progress with regard to lectin separation, autoimage analysis, and FISH technology, makes the possibility of non-invasive prenatal diagnosis of aneuploidy more likely**
- **The development of techniques for non-invasive prenatal diagnosis using cell-free DNA and fetal cells in maternal blood will contribute greatly to the field of perinatal medicine and result in safer antenatal care**



THANK YOU FOR YOUR ATTENTION