



University of Siena

Department of Biotechnology

Ph.D in Medical Genetics

***Technical improvements in
prenatal diagnosis***

Dr. Rondinella Dalila

Tutor: Prof. Francesca Mari

Supervisor: Prof. Alessandra Renieri

Doctoral School in Oncology and Genetics

Academic year 2010-2011

XXIII cycle

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1. Introduction

1. INTRODUCTION

1.1 Overview of Human Cytogenetics

Cytogenetics is the study of the structure, function, and evolution of chromosomes, the vehicles of inheritance that reside in the cell nucleus. Cytogenetics deals with chromosome behavior during the division of the somatic and early germline cells. Human cytogenetics is particularly concerned with how these processes may go wrong and how structural changes arise, because changes in the number or structure of chromosomes are major causes of intellectual disability, multiple malformations, cancer, infertility, and spontaneous abortions.

Human cytogenetics had its beginning in the nineteenth century, aided by the development of the compound microscope, fixatives for preserving cell structure, and chemical dyes that preferentially stain nuclei and chromosomes. The first study of human chromosomes, by Fleming in 1889, provided limited information.¹ Few studies on human chromosomes were published before 1952. That of Painter in 1923 was responsible for the notion that the human chromosome number is 48, a mistake that went uncorrected for the next 33 years.² It may simply have been the result of the inadequate methods available to the early investigators, who had to examine serial section of testes because the badly overlapping chromosomes were not even in one focal plane. Accurate studies of human chromosomes became possible only after several technical developments. Improved cell culture methods provided a ready source of individual dividing cells that could be squashed on a slide. Blakeslee and Eigsti showed in 1936 that colchicine destroys the mitotic spindle and blocks cells in metaphase, facilitating their accumulation and study.¹ Hsu discovered in 1952 that treatment of cells with hypotonic salt solution before fixation gave a marked improvement in chromosome

spreading.³ Taking advantage of these new methods, Tjio and Levan (1956) established that the correct human diploid chromosome number is 46 (Fig.1), based on their study of cultured embryonic lung cells from several individuals.⁴

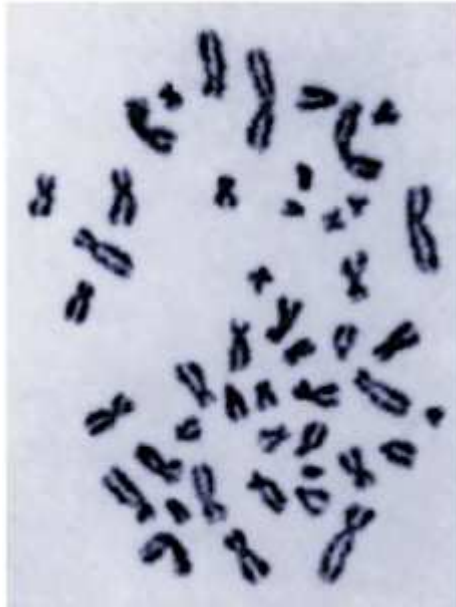


Fig. 1 A human metaphase plate, from the original Tjio and Levan paper, showing Human metaphase chromosomes.⁴

Methods continued to improve. Air-drying cell suspensions directly on microscope slides gave better spreading and flattened the entire metaphase spread into a thin focal plane. An important innovation in cell culture technique came with the discovery in 1960 by Moorhead and his associates that peripheral blood lymphocytes can be induced to divide after a few days in culture in the presence of phytohemagglutinin, a bean extract.⁵ Because blood samples are so readily available, chromosome studies could be carried out quickly and easily on virtually anyone. Such cultures are still one of the most widely used sources of human chromosomes. An important additional source is amniotic fluid. In 1966, Steele and Breg reported that cells cultured from amniotic fluid could be used to determine the chromosome

constitutions of the fetus.⁶ This is the technique that is still most widely used for prenatal chromosome studies, although a lot of studies are also carried out on cells cultured from biopsies of chorionic villi taken from the placenta during the first trimester of pregnancy.

The new techniques were soon applied to individuals who were mentally retarded or had multiple malformations. Lejeune et al. (1959) found that Down syndrome in several subjects was caused by the presence of three copies (trisomy), instead of the normal two, of number 21.⁷ The same year, Jacobs and Strong found a male with Klinefelter syndrome who had an XXY complement, while Ford and his collaborators reported females with Turner syndrome who had a single X (monosomy X) or were X/XX mosaics, with both X and XX cells.^{8,9} They also reported the first case of double aneuploidy: an extra sex chromosome and an extra chromosome 21 (XXY-trisomy 21) in a man with 48 chromosomes who had both Klinefelter and Down syndrome.⁸ These observations indicated that sex determination in humans depends upon the presence or absence of a Y chromosome and not on the ratio of X chromosomes to autosome sets, as it does in *Drosophila*.

The presence of multiple malformations in 21 trisomic patients led to the search for trisomy of other autosomes among these patients. Trisomy 13 and trisomy 18 were discovered in 1960 by groups headed by Patau and Edwards, respectively.^{10,11} No additional trisomies were found in liveborns, so attention turned to a search for chromosome abnormalities in spontaneously aborted embryos or fetuses, based on the assumption that trisomy for these autosomes might act as embryonic lethals. Only structural aberrations that produced large changes in the length or arm ratio of chromosome could be detected with the methods available before 1970. These included Robertsonian translocations, which involve the long arms of two acrocentric

chromosomes, such as numbers 14 and 21. Their discovery by Penrose, Fraccaro, and others was the result of studies of exceptional cases of Down syndrome in which the mother was young or there was an affected relative.^{12,13} A number of deletions were also detected. Nowell and Hungerford noted in 1960 the consistent presence of a deleted G group chromosome (Philadelphia) in chronic myelogenous leukemia cells.¹⁴ Later, using banded chromosomes, Rowley showed that the aberration was really a specific translocation.¹⁵

The first example of a deletion of a D group chromosome was found in 1963 in a patient with retinoblastoma by Penrose's group, who pointed out that if the deletion was responsible for the disease, a gene for retinoblastoma must be in the deleted segment.¹⁶ Deletions were shown to cause some characteristic and previously unrecognized clinical syndromes: Lejeune's cri du chat (cat cry) syndrome by a deletion of the short arm of chromosome 5 and the Wolf-Hirschorn syndrome by a telomeric deletion of the short arm of chromosome 4.¹

Until 1970, chromosome identification, and particularly the identification of structural changes, was severely limited. Normal chromosomes could be sorted into seven groups on the basis of length and arm ratio, but only a few chromosomes can be individually recognized. The introduction of chromosome banding techniques revolutionized human cytogenetics. In 1970, Caspersson et al. discovered that quinacrine mustard produces consistent fluorescent banding patterns along each human chromosome that are so distinctive that every chromosome can be individually identified.¹⁷ This discovery was followed by a flood of additional banding techniques, whose use greatly simplified chromosome studies and made possible the identification of an enormous range of chromosome abnormalities, especially structural aberrations,

such as translocations, inversions, deletions, and duplications, that were previously undetectable.

However, even high resolution karyotypes are unreliable for detecting many known microdeletion syndromes, which range from 3–5 Mb in size, and cannot detect smaller aberrations.¹⁸

A challenge in prenatal diagnosis is the finding of supernumerary marker chromosomes. The term *supernumerary marker chromosome* has been used to refer to any unidentifiable small chromosome present in addition to the normal chromosome complement. Classification of such marker chromosomes is important for phenotype/karyotype correlation, which is mandatory for proper counseling.¹⁹ The frequency of marker chromosomes identified at birth is 0.14-0.72 in 1.000 births, whereas their frequency in prenatal diagnostic studies is slightly elevated to 0.65-1.5 in 1.000.^{20,21} The elevated frequency seen in prenatal studies is most likely associated with the advanced maternal age seen in the prenatal population.²⁰ Before the use of molecular cytogenetic techniques for the identification of marker chromosomes, risks used in counseling were largely based on the gross morphologic characteristic of the markers. A satellite *de novo* marker appeared to carry a better prognosis than a nonsatellited *de novo* marker chromosome (10.9 versus 14.7 percent).¹⁹

Today using molecular cytogenetic techniques in combination with banding studies, the origin of all marker chromosome are potentially identifiable.²² Markers can be derived from any chromosome, although supernumerary chromosome derived from chromosomes 15, 22, and isochromosomes as i(12p), i(18p), i(9p) are most common. They appear to be more *de novo* than inherited.¹⁹

However, little is known about the mechanism of formation of these markers or their phenotypic consequences in many cases.²⁰

Occasional presence of mosaicism represent another great concern in prenatal diagnosis. The finding of karyotypically abnormal cells in otherwise normal cells chromosome preparations may reflect “true mosaicism”, which is defined as the presence of multiple fetal cell lines. Alternatively, the abnormal cells could indicate “pseudomosaicism”, i.e. abnormality arising in culture, or confined placental mosaicism (CPM), i.e. abnormalities derived from extrafetal tissue and not representative of the fetus.¹⁹ CPM is usually detected in 1-2 percent of pregnancies studied by chorionic villus sample (CVS) and is confirmed in the fetus in 5-25% of these cases.²³

1.2 Molecular cytogenetic techniques

In the 1990s the introduction of molecular cytogenetic techniques into the clinical laboratory setting represented a major advance in the ability to detect known syndromes and identify chromosomal rearrangements of unknown origin. Fluorescence in situ hybridization (FISH), which is the annealing of fluorescently labelled locus-specific probes to their complimentary sequences in the genome, allowed the detection of specific microdeletion syndromes (Fig. 2).²⁴ There are currently a number of commercially available FISH probes for the most common disorders, and this method is still predominantly used when the clinical phenotype is suggestive of a particular disorder. In fact FISH is a targeted method which requires prior knowledge of the region of interest and can be used to screen only one or several genomic regions at a time. Several other FISH-based methods, including spectral karyotyping (SKY), multicolour FISH (m-FISH),²⁴ and comparative genomic hybridization (CGH) have proven extremely useful in the identification of unknown chromosomal material. SKY and m-FISH rely mainly on the principal of differentially labelling each chromosome

using a unique combination of fluorochromes and are especially beneficial for identifying the origin and content of supernumerary marker chromosomes (SMCs) and complex chromosome rearrangements (CCRs) that involve more than two chromosomes.

CGH was originally introduced for the cytogenetic analysis of solid tumors, which can be difficult to culture, and involves the differential labeling of DNA from a test sample and a reference sample (Fig. 3).²⁴ The fluorescently labelled reactions are combined and hybridized to metaphase spreads from chromosomally normal individuals. Gains and losses of the genome in the test sample relative to the control sample are represented as ratios that are quantified from digital image analysis. This method allows the investigation of the whole genome and is very useful for determining the origin of unknown genetic material, such as SMCs and other unbalanced rearrangements.²⁴ However, CGH does not detect balanced rearrangements, and the resolution is on the order of 5–10 Mb, and consequently many genomic disorders cannot be detected.²⁵

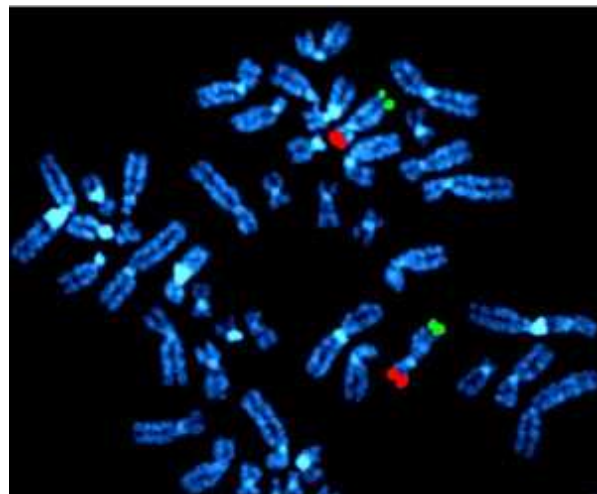


Fig. 2 Fluorescence in situ hybridization (FISH) of metaphase human chromosomes. The probes are to the 5p telomere (red) and to the *HAPLN1* gene at 5q14 (green).

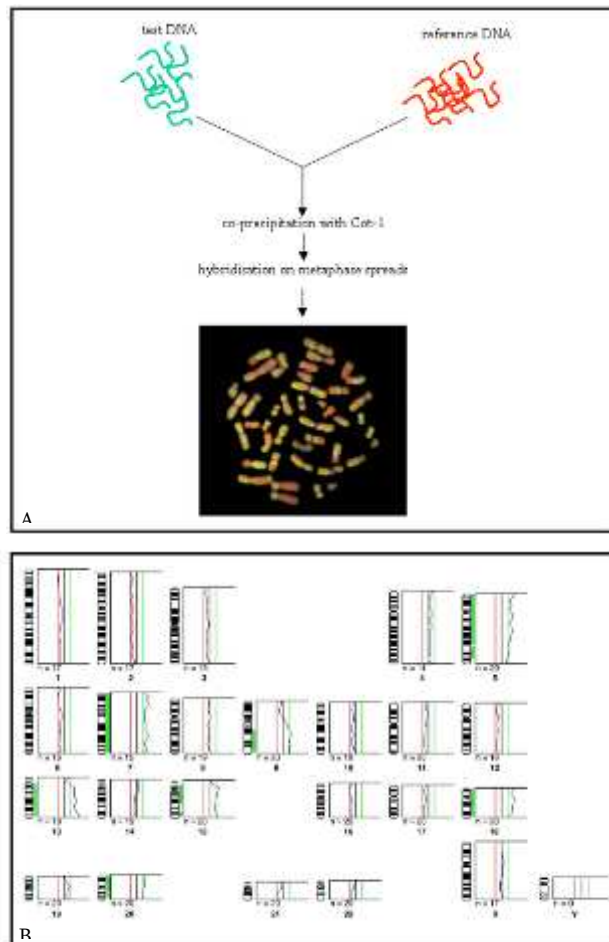


Fig.3 Comparative Genomic Hybridisation experiment. A) Test and reference DNAs are labelled in SpectrumGreen and SpectrumRed respectively and simultaneously hybridised to a normal metaphase spread. B) Color ratio peak profile of the CGH experiment. The central black graph bar represents a ratio of 1. Areas to the right and to the left represent gains or losses in DNA copy number. The green line indicates the threshold value (1.25) for copy number gains and the red line indicates the threshold value for copy number losses (0.75). *n* represents the number of chromosomes analysed (adapted from Florida G. et al, 2005)

The need to screen the whole genome at a resolution that surpassed the existing technologies led to the implementation of microarray based CGH. The principle is very similar to that employed for traditional CGH, where two differentially labelled specimens are cohybridized in the presence of Cot 1 DNA; however, instead of metaphase spreads, the hybridization targets are DNA substrates immobilized on a glass slide.^{24,26,27} Subsequently, the arrays are scanned and the resultant data are analyzed by

software that computes the log₂ ratios for a variety of copy number differences between a patient and reference sample (Fig. 4).

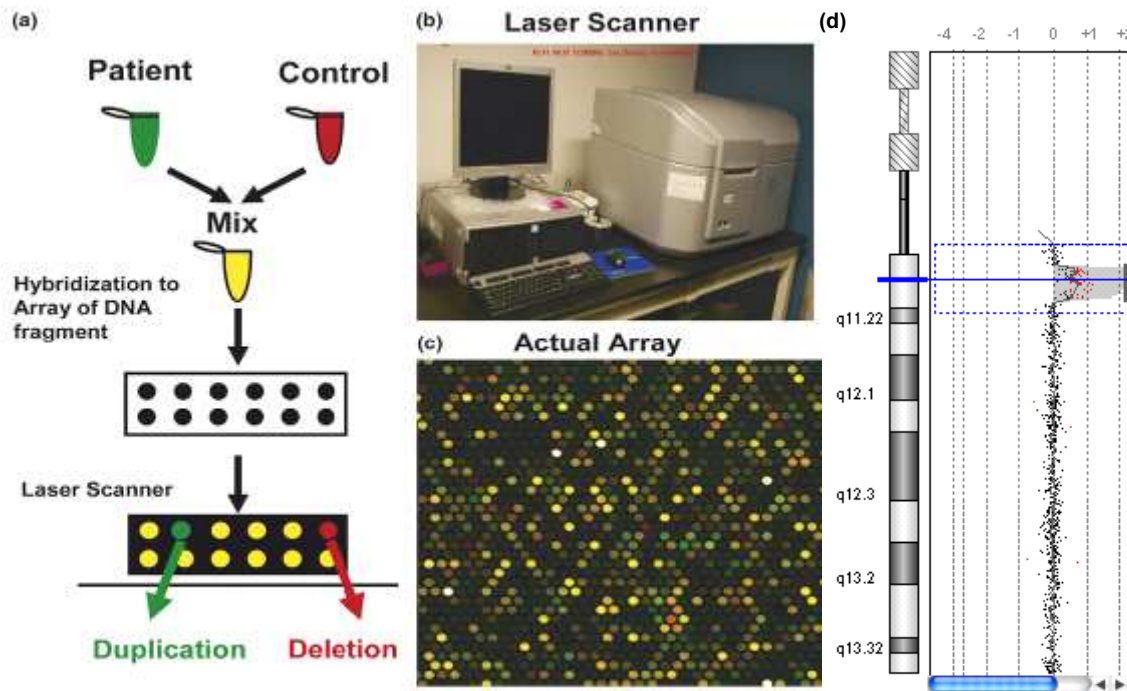


Fig. 4 Schematic representation of an array-CGH experiment. a) Test and reference DNA are differentially labelled, co-precipitated and hybridised to an array. b) and c) After wash procedures, the slides are analysed through a scanner and fluorescence intensities of each probe are determined. d) After imaging processing and data normalization, the log₂ ratios of the probes are plotted as a function of chromosomal position. Probes with a value of zero represent equal fluorescence intensity ratio between sample and reference. Each dot represents a single probe spotted on the array. In this representation, copy number loss shift the ratio to the left and copy number gains shift the ratio to the right.

Array-CGH is an entirely molecular technique with a cytogenetic application and represents a hybrid method that requires the expertise of both specialties. The current limitations of the technology include the inability to detect balanced chromosome rearrangements and the equivocal nature of copy number alterations of unknown significance that may be identified. Nevertheless, it is being used routinely in the clinical setting with a normal chromosome result in cases of intellectual disability

and/or multiple congenital anomalies (ID/MCA); as a result the diagnostic yield in this patient group has increased considerably.²⁵

1.3 Application of array-CGH in prenatal diagnosis

Conventional chromosome analysis has been the gold standard method in prenatal genetic diagnosis for over 30 years, since the development of banding techniques in 1970. However, it lacks the resolution necessary to detect more subtle chromosome abnormalities (those less than 5-6 Mb).²⁸ A significant proportion of fetal developmental defects, including various anomalies associated with growth retardation and abnormalities of amniotic fluid, are caused by smaller chromosomal rearrangements, below the current resolution of detection.^{29,30} In particular, the technique of array-CGH, also called "molecular karyotype", is able to detect microscopic and submicroscopic chromosomal imbalances on a genome-wide scale. This includes all of the common aneuploidies as well as microdeletions and microduplications smaller than 100 kb in size.³¹

Array-CGH overcomes many of the limitations of both conventional chromosome analysis and FISH analysis and has become widely used in the clinical setting for both postnatal and, more recently, prenatal diagnosis.^(29,31-36) Array-CGH has a number of advantages compared to conventional chromosome analysis, such as the use of direct fetal samples, which eliminates the need for cell culture and decreases the overall turn around time. In addition, array-CGH analysis is more sensitive and less labor intensive than chromosome analysis, and has the capacity to be automated.^{32,35,37} As many diagnostic testing methods, array-CGH has several limitations: mosaicism below 10-20%³⁸ and chromosomal aberrations with no gain or no loss of genetic material relative

to the rest of genome, such as completely balanced translocations, inversions or polyploidy, will not be detected. However the biggest advantage of array-CGH is the ability to detect genomic copy number changes below the resolution of standard chromosome analysis.^{34,39} It is also a high-throughput method, detecting hundreds or thousands of discrete loci in a single simultaneous assay.⁴⁰

Recently array-CGH has been validate for the clinical diagnosis of copy number abnormalities in prenatal samples.^{32,35,36} There are at least two situations for which the augmentation of conventional karyotype with genome-wide arrays has become the gold standard for accurate prenatal diagnosis and proper genetic counseling. The first one is in the setting of a fetal sonographic abnormality with a normal karyotype. General practice suggests that sonographic anomalies, such as increased nuchal translucency, association of two or more ecographic markers of aneuploidy, amniotic fluid volume alteration and/or intrauterine growth retardation associated with major structural abnormalities may benefit from this technology. The second circumstance for which the genome-wide array is now considered essential is the finding of chromosome rearrangements for which the clinical significance remains undefined, such as in case of apparently balanced *de novo* chromosome rearrangements, supernumerary marker chromosomes of unknown origin and structural abnormalities not obviously classifiable as clinically irrelevant polymorphisms.⁴¹

Given that oligonucleotide arrays are becoming more widely used for prenatal diagnosis, it is anticipated that the detection rate will increase as more testing is performed on these higher resolution platforms. One drawback to increased array resolution is the increased likelihood of finding copy number variations (CNVs) of uncertain clinical significance, which is a serious concern in prenatal diagnosis. In order to minimize uncertainty in prenatal cases, guidelines are generally set forth at each

laboratory as to the minimum size of CNV to be reported and the genomic regions considered clinically significant.^{36,42}

Because of this uncertainty of interpretation of the CNVs is difficult to predict if array-CGH will eventually replace karyotyping and FISH for rapid prenatal detection of chromosomal imbalances. For now, it is clear that these tests are necessary for confirming and clarifying abnormal array-CGH results, especially when parental studies are inconclusive.^{35,36,43}

In Italy the application of array-CGH in prenatal diagnosis is not regulated due to lack of official guidelines from scientific societies who are working on this important issue. At the moment prenatal analysis by array-CGH is offered on research basis in some selected cases, while in private centers the situation is less regulated.

1.4 Quantitative Fluorescence Polymerase Chain Reaction

As previously described, in the last years alternative methods to conventional cytogenetic analysis have been developed to reduce the reporting time, the work load and to allow the introduction of automatic methods. Three rapid tests are presently used to detect the most common aneuploidies (trisomies 13, 18, 21, and sex chromosomes aneuploidies): FISH on cells in interphase, quantitative fluorescent polymerase chain reaction (QF-PCR), and multiplex ligation-dependent probe amplification (MLPA). The reporting time of these techniques is reduced to 2-3 days, allowing decisions on pregnancy management to be made earlier.⁴⁴ In addition to these techniques, in the last years array-CGH, which has higher resolution and provides information about the copy number of the whole genome, has also been suggested as a potential screening method. Overall, QF-PCR is presently considered the preferred technique for its cost/benefit

ratio. However, it is unable to detect clinically significant structural chromosome abnormalities or aneuploidies on chromosomes different from those included in the assay.

Several studies of prenatal cases performed by both QF-PCR and conventional cytogenetic analysis, allowed to assess the advantages and limitations of applying the molecular technique to fetal samples collected by amniocentesis and chorionic biopsy. The overall results confirm that QF-PCR is a rapid, simple and accurate diagnostic test and provides correct diagnoses for the majority of prenatal cases.⁴⁵ In the cases of normal pregnancies, which were correctly diagnosed by QF-PCR, parents could be informed about the outcome of the test within 24-48 hours from sample collection. QF-PCR could detect aneuploidies for chromosomes 21, 18, 13, X and Y. Of course, as the tests were not set up to investigate all chromosomal disorders, aneuploidies affecting other chromosome were missed. In several cases, the great efficiency and reliability of QF-PCR allowed early termination of affected fetuses without further waiting for the completion of cytogenetic analysis. The main limitation of QF-PCR is that only major chromosome disorders are detected using the restricted number of selected markers, missing rare trisomies and duplications or deletions. Moreover, in agreement with previous studies⁴⁶, low levels of mosaicism (less than 20%) may not be detected.

The first approach of QF-PCR as stand alone test comes from UK experience.⁴⁷ From May 2007, consistent with National Screening Committee (NSC) guidelines on fetal chromosome analysis, the South East England Genetics Clinical Network offers rapid aneuploidy exclusion using QF-PCR alone for women found to be at increased risk of Down's syndrome following a nationally recognized screening test and it offers karyotype, after performing QF-PCR, for the following referral categories:

- Ultrasound detection of any major structural abnormality including nuchal translucency (NT)>3mm before 14 weeks gestation or a nuchal fold measuring 6mm or greater between 14 and 20 weeks gestation.
- Ultrasound detection of two or more minor markers of aneuploidy.
- History of chromosome abnormality indicative of increased risk for future pregnancies. Chromosome abnormality may be present in the woman or her partner or in a previous pregnancy (excluding non-viable aneuploidy). If there is a family history, karyotyping of the woman or her partner should be undertaken first in order to establish whether prenatal diagnosis is indicated.
- Non-routine cases not fulfilling the above criteria after discussion and agreement between the referring clinician and a senior staff member from Cytogenetic Unit.

In some Italian centers QF-PCR is now offered to women who undergo invasive prenatal diagnosis by amniocentesis or villocentesis as a choice alternative to karyotype. QF-PCR in addition to the subsequent full chromosome analysis of cultured cells is performed only in some selected cases following the same criteria adopted by UK.

2. Rationale, aims and outline

2. RATIONALE, AIMS and OUTLINE

During the last years improvements in prenatal diagnosis have been made and at the moment QF-PCR is considered the favorite technique for its low cost, high degree of automation and speed of results (24-48 hours). The short time for completing the analysis decreases the anxiety of parents and in case of a positive result allows earlier termination of pregnancy, without waiting for the completion of conventional cytogenetic analysis. This rapid test can be carried out even in samples contaminated with maternal cells obtaining successful results in the most cases, unlike karyotype. QF-PCR has the limitation that, using a restricted number of selected markers for chromosomes 21, 18, 13, X and Y, can detect only major chromosome disorders, missing clinically significant structural chromosome abnormalities or aneuploidies on chromosomes different from those included in the assay.

Conversely, the array-CGH technique is able to detect microscopic and submicroscopic chromosomal imbalances on a genome-wide scale, including all the common aneuploidies as well as microdeletions and microduplications as small as than 100 kb in size, and thus 100 folds more than the current resolution of karyotype.

The aim of my thesis was to evaluate the appropriateness of the employment in the prenatal diagnostic setting of three different available genetic techniques: conventional karyotype, QF-CPR and array-CGH. In particular, the thesis aimed to estimate if the QF-PCR rapid test could be used as single diagnostic test replacing conventional cytogenetic analysis and to verify the positive and negative sides of array-CGH employment in prenatal diagnosis.

In the last three years, a total of 1474 prenatal samples were collected. On 478 samples both QF-PCR and karyotype were performed. According to specific clinical

and genetic criteria, a selected group belonging to this latter cohort, was analyzed by array-CGH. The inclusion criteria were: presence of specific ultrasound abnormalities and/or presence of specific fetal chromosomal abnormalities.

3. Materials and methods

3. MATERIALS AND METHODS

3.1 Samples collection

In this study were included 1474 prenatal samples: 959 amniotic fluids and 515 chorionic villus samples. Samples were collected in the Medical Genetics Unit of the Biotechnology Department (University of Siena) from May 2008 to July 2011. All women underwent genetic counseling and were given detailed information on the advantages and limitations of QF-PCR assay, karyotype and array-CGH; written informed consent was obtained in all cases. In the absence of specific indications, women could choose between QF-PCR or karyotype, whereas in the presence of specific indications such as ultrasound abnormalities both techniques were performed. Regarding CVS, direct method was replaced with QF-PCR.

Array-CGH was proposed in the frame of a national multicenter prospective study to women in the presence of: de novo apparently balanced chromosomal rearrangements, small supernumerary marker chromosomes, nuchal translucency >4 mm, an association of more soft markers ultrasound (choroid plexus cysts, intestinal hyperechogenicity, kidney pielectasia, single umbilical artery, cardiac echogenic foci), abnormal amniotic fluid volume and/or fetal growth retardation in association with minor ultrasound markers, congenital heart defects, diaphragmatic hernia, abnormalities of the central nervous system (excluding anencephaly). In all these conditions, array-CGH represented an integration to karyotype, not an alternative.

In all cases tested by array-CGH blood drawing of both parents was obtained. Array-CGH results were always discussed with the couple during a follow up genetic counseling in order to provide clarification of possible outcome of the investigation and implications for the unborn child.

3.2 Karyotype analysis

Samples for conventional cytogenetic analysis (amniocytes and chorionic villi) were cultured and processed with different procedures.

Amniocytes

Amniotic fluid was centrifuged at 1000 rpm for 10 minutes, and the pellet was cultured in two flasks with Chang Medium (Irvine Scientific, Santa Ana, CA). The cultures were grown with 5% CO₂ in a 37° incubator. After evaluation of culture growth, approximately 10 days later, the flask was treated with 100 µl of Colcemid (Irvine Scientific, Santa Ana, CA) and incubated at 37°C for 1 hour and 30 minutes. Then the flask was treated with 2 ml of Trypsin-EDTA 1X (Irvine Scientific, Santa Ana, CA) for 4 minutes at 37°C. When the cells were detached, 2 ml of Chang Medium were used to inactivate Trypsin activity. Cells were transferred in a tube and centrifuged at 1500 rpm for 8 minutes. After discarding the supernatant, 8 ml of hypotonic solution (0.56 g KCl in 100 ml of water) were added to the pellet that is incubated at 37°C for 8 minutes. Cell breakage was facilitated by pipetting with a Pasteur. A fixative composed by acetic acid and methyl acid in a proportion of 1:3 was used to wash the pellet for 4 times. Finally the pellet was shot on a slide at controlled temperature and humidity. The slide was dried at 37°C overnight. The day after the slide was treated with Trypsin solution (Sigma-Aldrich, St. Louis, Missouri, USA) for approximately 7 seconds and stained with Giemsa (Merck, Darmstadt, Germany) for approximately 7 minutes.

Chorionic Villus Samples

Chorionic villus sample, previously washed in physiological solution, was observed at the inverted microscope in order to discard the deciduous tissue. The villus were transferred in a tube, treated with 2.5 ml of Pronase 1 mg/ml (Roche Diagnostics, Mannheim, Germany) for 5 minutes at room temperature and 2.5 ml of Collagenase 1 mg/ml (Sigma-Aldrich, St.Louis, Missouri, USA) for 20 minutes at 37°C in order to disrupt the villus. The tube was centrifuged and the pellet spread in 3-5 amnioidishes (EuroClone, Pero, Italy) with 2 ml of Chang Medium. The cultures were grown with 5% CO₂ in a 37° incubator. After evaluation of culture growth, approximately 10 days later, some selected amnioidishes of each sample were treated with 80 µl of Colcemid and incubated at 37°C for 2 hours. In a room at controlled temperature and humidity, Chang Medium was removed, the amnioidishes were treated with 2.5 ml of hypotonic solution (0.5 g Trisodio Citrato + 0.15 g NaCl in 100 ml of water) for 15 minutes. A fixative composed by acetic acid and ethanol in a proportion of 1:3 was used to wash the amnioidishes for 3 times. Finally the dish was removed from the amnioidish and dried first in a Optichrome (EuroClone, Pero, Italy) at controlled temperature and humidity for 30 minutes and then at 37°C overnight. The day after the dish was mounted on a slide and subsequently treated with Trypsin for approximately 12 seconds and stained with Giemsa for approximately 12 minutes.

Slides obtained from both amniotic fluids and chorionic villus samples were observed with Axioskop 40 system (Zeiss, Gottingen, Germany) and the software Ikaros was used for karyotype analysis.

For each sample, 25 metaphases were analyzed and the result of karyotype analysis obtained within 21 days from the amniocentesis or villocentesis.

A standard system of chromosome nomenclature for banded chromosome and chromosome abnormalities was developed through a series of conferences and publications; a standing committee now publishes comprehensive booklets incorporating the accepted nomenclature and modifications necessitated by new developments in the field. The most recent is ISCN (2009), an international system for human cytogenetics nomenclature. Telomeres, centromeres, and a number of prominent bands are used as landmarks. A section of a chromosome between two landmarks is called a *region*, and these regions are numbered 1,2,3, and so on, in both p and q directions, starting from the centromere. The bands within the regions are numbered according to the same rule. Thus, the first band in the second region of the short arm of chromosome 1 is 1p21. The increasing use of high-resolution banding has led to an extension of this system. To indicate a subband a dot is used, followed by the number of the subband (they are numbered sequentially from the centromere). About designation of chromosome abnormalities, an extra or a missing chromosome is denoted with a plus or a minus sign respectively before the number of the chromosome.¹

3.3 QF-PCR analysis

QF-PCR consists of amplification of specific STRs for chromosomes 21, 18, 13, X, and Y using fluorescent primers. Primers producing amplicons of similar sizes are labelled with different fluorochromes in order to be amplified in the same multiplex QF-PCR reaction.

Genomic DNA was extracted from 1,5 ml amniotic fluid or two independent small villi fragments using a Chelex based procedure (Instagene Matrix, Bio-Rad

Laboratories, CA) as previously described.⁴⁸ This DNA extraction procedure allows similar DNA concentrations to be obtained on different samples, so that QF-PCR can be carried out in the same conditions.

Aneuploidy screenings were performed using the commercially available Aneufast™ QF-PCR kit (CE-IVD) with selected markers distributed in two multiplex assays (S1 and S2) in order to reduce the risk of sample mishandling. These two assays allow to amplify simultaneously four STRs on chromosomes 21, 18 and 13, two pseudoautosomal STRs, one X linked, and also two non-polymorphic sequences, Amelogenin (AMXY) and SRY, for identification of sex. Briefly, 2.4 µl DNA for each sample were added in two different wells, respectively with 5µl of S1 and 5µl of S2. PCR conditions were as follows: 95°C for 15 minutes, followed by 25 cycles consisting of melting at 95°C for 40 seconds, annealing at 60°C for 1.30 minutes, and then extension at 72°C for 40 seconds, ended by a final extension at 60°C for 30 minutes. After amplification, samples were prepared for electrophoresis:

- 1- In a 1.5 ml tube the necessary amount of size standard for all the samples to be analysed was prepared by combining 40 µl of Hi-Di Formamide and 0.3 µl of GeneScan-500 LIZ;
- 2- 20 µl of this mix were used to inject 1.5 µl of each Aneufast™ S1 and S2 product collected in the same well of a plate;
- 3- The sample plate with Formamide and Size Standard was denatured for 2 minutes at 95°C;
- 4- Samples were loaded on the Genetic Analyser.

The resulting PCR products can be visualized and quantified as peak areas of the respective repeat lengths by capillary electrophoresis on an ABI 3130 automated DNA

sequencer using Gene-Mapper 4.0 Software (Applied Biosystems, Foster City, CA) as previously described.⁴⁹ In normal heterozygous subjects, the QF-PCR product of each STR should show two peaks with similar fluorescent activities and thus a ratio of peak areas close to 1:1 (ranging from 0.8 to 1.4:1). A trisomy is suspected when the ratio is above or below this range (peak area ratios ≤ 0.6 and ≥ 1.8) (trisomic diallelic); otherwise there are three alleles of equal peak area with a ratio of 1:1:1 (trisomic triallelic) (Fig. 5). The presence of trisomic triallelic or diallelic patterns for at least two different STRs on the same chromosome is considered as evidence of trisomy. Trisomic patterns observed for all chromosome-specific STRs are indicative of triploidy.

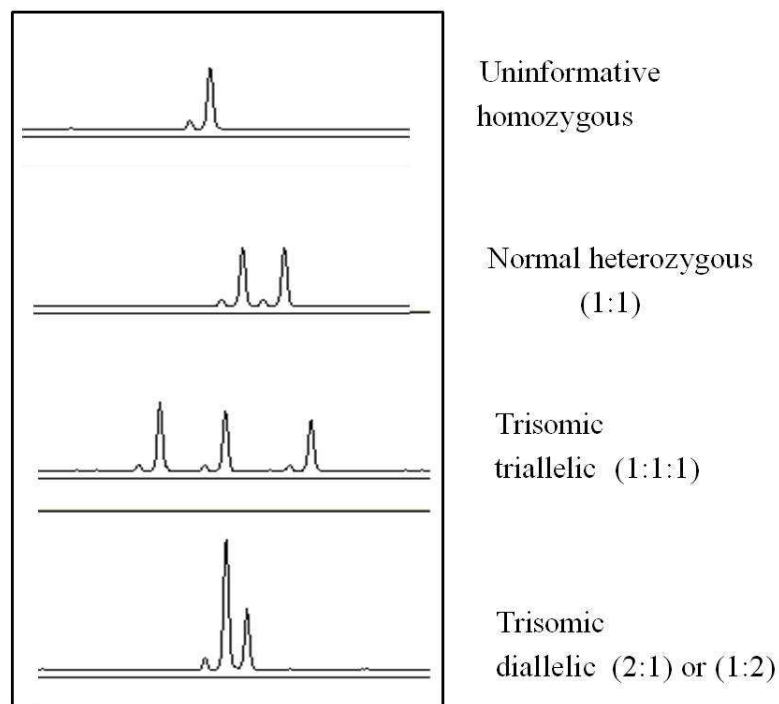


Fig. 5 Allele plots generated by Gene Mapper 4.0

Samples with less than two informative markers on each chromosome were retested using chromosome-specific multiplex PCR assays including up to five STRs on chromosomes 21 and 18, seven STRs on the X, and four markers on chromosome 13. These sets of additional markers were also used to confirm sample identity in all aneuploid cases by testing a second aliquot obtained from the original sample. All prenatal samples were processed and reported within 24-48 hours. Recent improvements of the AneufastTM QF-PCR kit with the addition of new markers were very useful. In particular the MXY assay has been improved with the inclusion of primers to amplify chromosome-specific sequences of the paralogous gene TAF9L. This gene has a high degree of sequence identity between chromosome 3 and chromosome X; primers on this gene amplify a 3 b.p. deletion generating a chromosome X specific product of 110 b.p. and a chromosome 3 specific product of 107 b.p. Therefore accurate X chromosome dosage can be assessed by TAF9L marker allowing to perform diagnosis of X monosomy.

3.4 Array-CGH analysis

3.4.1 Sample preparation

Genomic DNA was extracted from 4-5 ml amniotic fluid or four independent small villi fragments using a QIAamp DNA Mini Kit according to the manufacturer protocol (Qiagen, www.qiagen.com). In the event that insufficient DNA is obtained, array CGH using whole genome amplification (WGA) was performed. Genomic DNA of normal controls was obtained from Promega. The OD260/280 method on a photometer was used to determine the appropriate DNA concentration. Ten micrograms

of genomic DNA both from the patient and from sex-matched controls (Promega) were sonicated to produce a homogeneous smear DNA extending from approximately 600 bp to 2 kb. Test and reference DNA samples were subsequently purified using dedicated columns (DNA Clean and Concentrator, Zymo Research, Orange, CA) and the appropriate DNA concentrations were determined by a DyNA QuantTM200 Fluorometer (GE Healthcare, www.gehealthcare.com).

3.4.2 Human oligonucleotides array

Array based CGH analysis was performed using commercially available oligonucleotide microarrays containing about 43,000 60-mer probes with an estimated average resolution of about 75-100 kb (Human Genome CGH Microarray 44B Kit, Agilent Technologies). Physical positions of the probes correspond to the UCSC genome browser - NCBI build 36, March 2006. (<http://genome.ucsc.edu>).

DNA labeling was carried out according to the Agilent protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis 2.0v) using the Bioprime DNA labelling system (Invitrogen). Briefly, genomic DNA (2 µg) was mixed with 20 µl of 2.5X Random primer solution (Invitrogen) and MilliQ water to a total volume of 41 µl. The mix was denatured at 95°C for 7 min and then incubated in ice/water for 5 minutes. Each sample was added with 5 µl of 10X dUTP nucleotide mix (1.2 mM dATP, dGTP, dCTP, 0.6 mM dTTP in 10 mM Tris pH 8 and 1 mM EDTA), 2.5 µl of Cy5-dUTP (test sample) or 2.5 µl of Cy3-dUTP (reference sample) and with 1.5 µl of Exo-Klenow (40 U/µl, Invitrogen).

Labeled samples were subsequently purified using CyScribe GFX Purification kit (Amersham Biosciences) according to manufacturer protocol. Test and reference DNA

were pooled and mixed with 50 µg of Human Cot I DNA (Invitrogen), 50 µl of Blocking buffer (Agilent Technologies) and 250 µl of Hybridization buffer (Agilent Technologies). Before hybridization to the array the mix was denatured at 95°C for 7 minutes and then pre-associated at 37°C for 30 minutes. Probes were applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 40 hours at 65°C in a rotating oven (20 rpm). The array was subsequently disassembled and washed according to the manufacturer protocol with wash buffers supplied by Agilent. The slides were dried and scanned using an Agilent G2565BA DNA microarray scanner. Image analysis was performed using the CGH Analytics software v. 3.4.40 with default settings.

The software automatically determines the fluorescence intensities of the spots for both fluorochromes performing background extraction and data normalization, and compiles the data into a spreadsheet that links the fluorescent signal of every probe on the array to the oligonucleotide name, its position on the array and its position in the genome. The linear order of the oligonucleotides is reconstituted in the ratio plots consistent with an ideogram. The ratio plot is arbitrarily assigned such that gains and losses in DNA copy number at a particular locus can be observed as a deviation of the ratio plot from a modal value of 1.0.

4. Results

4. RESULTS

From May 2008 to July 2011, a total of 1474 prenatal samples were collected from three different prenatal diagnostic centers (Siena - Azienda Ospedaliera Universitaria Senese; Nottola - Ospedali Riuniti Valdichiana Senese; Campostaggia - Ospedale Valdelsa). Prenatal samples included 959 amniotic fluids (AF) and 515 chorionic villus samples (CVS) obtained between 15-18 and 11-13 weeks of gestation, respectively. The indication of referral was in most cases advanced maternal age (84%) followed by positive screening test (6%), ultrasound abnormalities (4%), positive family history for chromosomal abnormalities and parental rearrangements (4%) and other (2%) (Fig. 6).

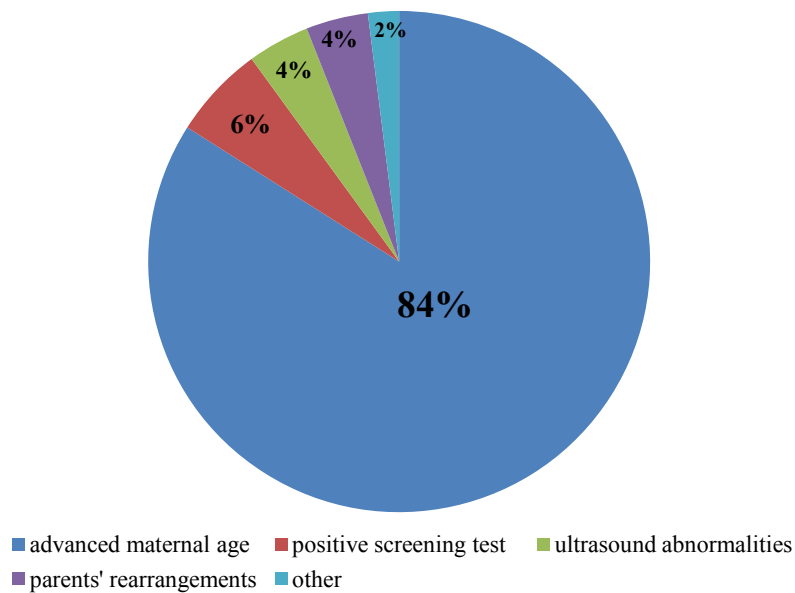


Fig. 6 Graphical representation of clinical indications to invasive procedure in prenatal samples.

4.1 QF-PCR and karyotype analyses

Among the 1474 prenatal samples, 412 samples (289 AF, 123 CVS) were analyzed only by QF-PCR while 584 AF only by karyotype. The remaining 478 samples (86 AF, 392 CVS) were analyzed by both QF-PCR and karyotype.

In total, 40 positive samples were identified (21 AF, 19 CVS): 16 cases of trisomy 21, 2 cases of trisomy 21 with Robertsonian translocation, a case of trisomy 21 associated with a translocation involving chromosome 8, 6 cases of trisomy 18, a case of partial trisomy 18, a case of trisomy 13, 3 cases of monosomy X, a case of triploidy, a case of triple X, a trisomy 21 mosaicism, a case of monosomy X mosaicism, a case of marker mosaicism, a case of constitutional chromosomal marker, 3 cases of Robertsonian translocation, and a case of complex rearrangement involving chromosome 1 and 5. (Fig. 7-8, Tab. 1).

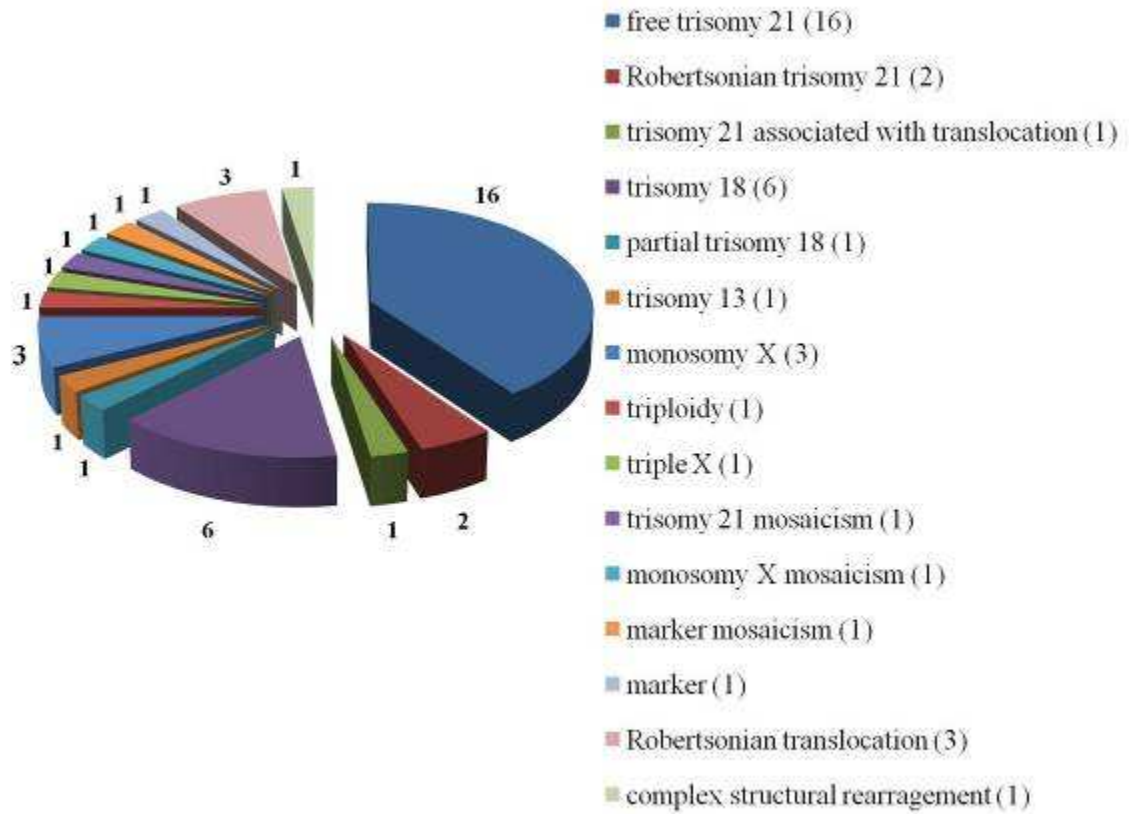


Fig. 7 Graphical representation of positive cases. Number of cases is shown in brackets.

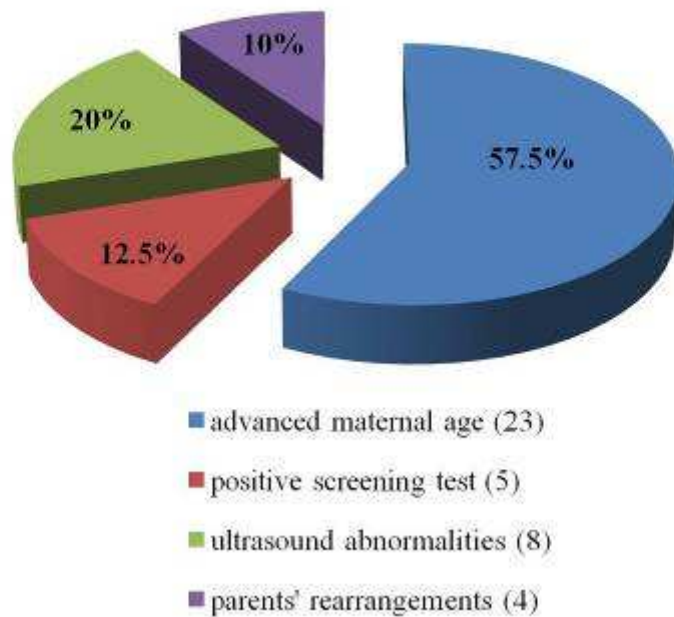


Fig. 8 Graphical representation of indications to invasive test for positive cases. Number of cases is shown in brackets.

Tab. 1 Overview of positive cases identified either by QF-PCR or karyotype.

CASES	INDICATION TO INVASIVE TEST	QF-PCR	KARYOTYPE {n° of cases}
7	Advanced maternal age	Trisomy 21 (3F) (4M)	47,XX,+21 {3} 47,XY,+21 {4}
1	Positive screening test	Trisomy 21 (M)	47,XY,+21
1	Positive screening test, NT=5.2mm at 12 weeks	Trisomy 21 (F)	47,XX,+21
4	Advanced maternal age	Trisomy 21 (3F) (1M)	-
2	Advanced maternal age	-	47,XX,+21 47,XY,+21
1	Positive screening test	-	47,XX,+21
1	Positive screening test, oligoidramnios	Trisomy 21(F)	46,XX,rob(21)(q10;q10)
1	Advanced maternal age	-	46,XX,rob(21)(q10;q10)
2	Positive screening test	Trisomy 18 (M)	47,XY,+18
1	Advanced maternal age, NT=3.92mm at 12 weeks	Trisomy 18 (F)	47,XX,+18
1	NT=6.7mm at 12 weeks, advanced maternal age	Trisomy 18 (F)	47,XX,+18
2	Advanced maternal age	Trisomy 18 (1F) (1M)	47,XX,+18 47,XY,+18
1	Advanced maternal age	Trisomy 13 (F)	47,XX,+13
1	NT=4.99mm at 11 weeks	Turner	45,X
1	Advanced maternal age, NT=3.5mm at 11 weeks	Turner	45,X
1	Advanced maternal age	Turner	45,X
1	Advanced maternal age	Turner mosaicism	failed
1	Advanced maternal age	Triple X	47,XXX
1	IUGR, oligoidramnios	Triploidy	69,XXY
2	Parental balanced translocation	Normal (F)	45,XX,rob(13;14)(q10;q10)
1	Parental balanced translocation	Normal (M)	45,XY,rob(14;21)
1	Advanced maternal age	Normal (M)	46,XY[75]/47,XY,+21[5]
1	Advanced maternal age, positive screening test	Trisomy 21 (F)	47,XX,+21,t(8p;8q)
1	Parental balanced translocation	Partial trisomy 18 (M)	46,XY,der(11)t(11;18)(q25;q22)mat
1	Advanced maternal age, NT=2.8mm at 12 weeks	Normal (F)	47,XX,+mar
1	Advanced maternal age, ultrasound abnormalities identified at 19 weeks	Normal (F)	46,XX,add(5)(p15.3)
1	Advanced maternal age	Normal (M)	46,XY,[16]/47,XY,+mar[4]

NT = nuchal translucency

M = male

F = female

IUGR = intrauterine growth retardation

Considering the 478 samples where both QF-PCR and karyotype analyses were performed, QF-PCR was able to detect 29/29 constitutional aneuploidies of chromosomes 21, 18, 13, X or Y without false negative results (100% sensitivity and specificity for the analyzed chromosomes) (Tab. 1).

Conversely, QF-PCR, according to its technical limitation, was clearly unable to detect chromosomal rearrangement not including the tested chromosomes and low-grade mosaicism (Tab. 1).

A case of partial chromosomal imbalance.

An interesting case is that of a woman with a balanced translocation (11;18)(q25;q22) who performed villocentesis followed by QF-PCR and karyotype. The rapid test revealed 2 markers of chromosome 18 with a trisomic pattern, and 3 markers with a normal profile (Fig. 9). The trisomic markers were located in 18q22.1 and 18q22.2, while normal markers were located in 18pter-18p11.22, 18q12.2 and 18q21.32-18q21.33 suggesting that it was a partial trisomy involving the translocated region of the mother. After the QF-PCR result, karyotype analysis was performed. It identified a derivative chromosome 11 (Fig. 10). A further analysis by array-CGH revealed a partial terminal duplication of the long arm of chromosome 18 (see for details Results 4.2 - Array-CGH analysis).

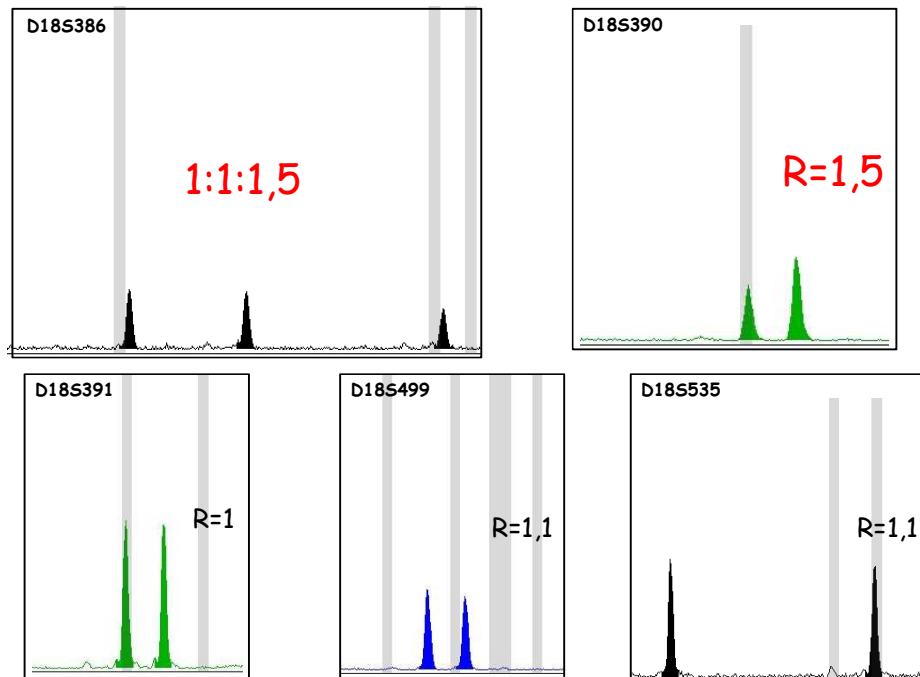


Fig. 9 STR patterns of a case diagnosed as partial trisomy 18 by QF-PCR.

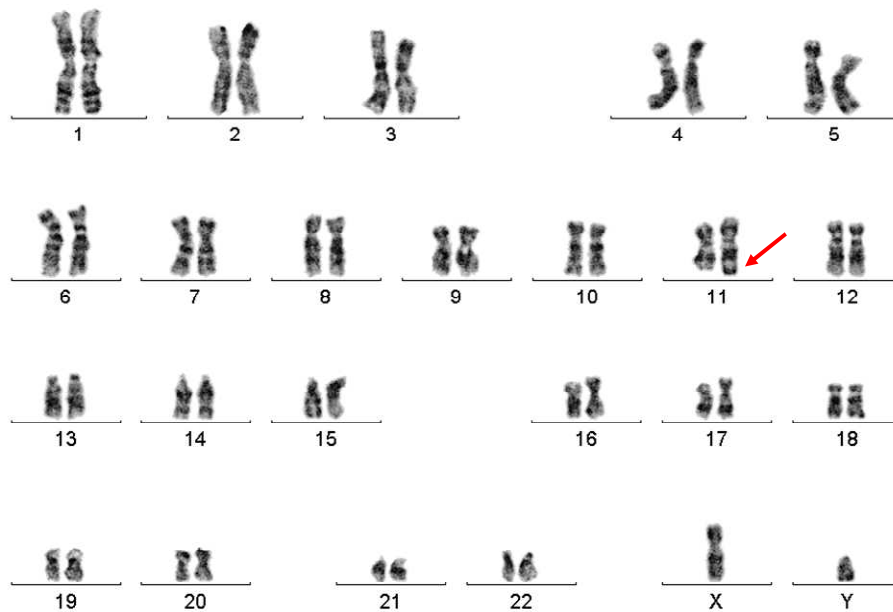


Fig. 10 Identification of a derivative chromosome 11 by karyotype analysis (see arrow).

Cases with ascertained contamination.

In three samples (2 CVS and 1 AF) QF-PCR revealed a contamination pattern which resulted in STR profiles with extra allele peaks for all chromosome markers that are not compatible with normal, trisomic or triploidy cases (Fig. 11). In all contaminated cases a blood sample of mother was requested in order to confirm or exclude the maternal origin of extra alleles. In all three prenatal samples it was ascertained the maternal contamination.

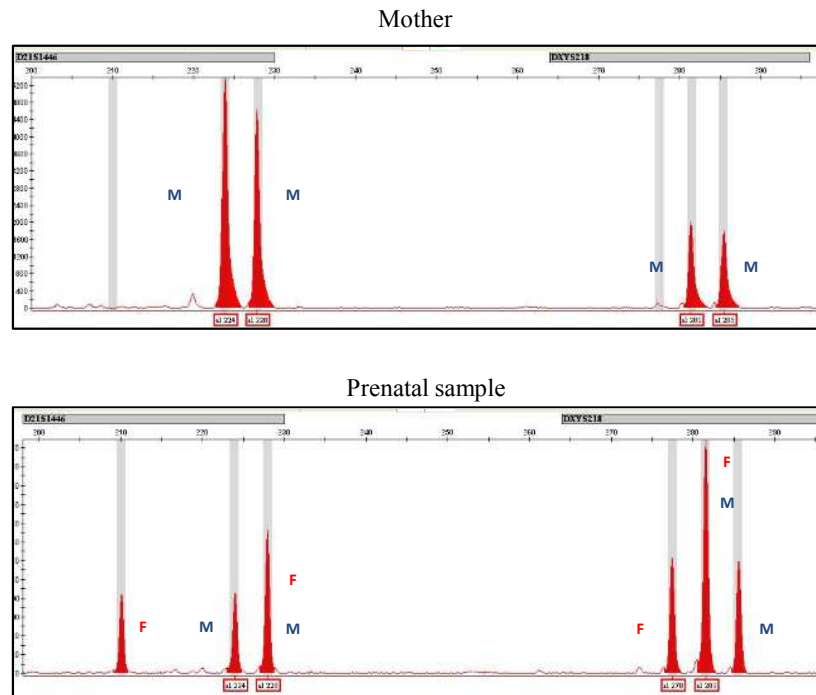


Fig. 11 Comparison of STR profiles between mother and prenatal sample in order to establish the origin of extra alleles (M = mother, F = fetus).

Cases of mosaicism.

In a case of AF, karyotype analysis revealed a marker in mosaic state {46,XY[16]/47,XY,+mar[4]} calculated at about 20%. Evidently, this case was not revealed by QF-PCR. It was a true mosaicism since multiple abnormal cell lines were observed at least in two colonies originating from two independent cultures.

By karyotype analysis we have found a CVS with trisomy 21 mosaicism {46,XY[75]/47,XY,+21[5]} calculated at about 6%. QF-PCR was not able to detect it because lower than 20%. Subsequently amniocentesis was performed and resulted normal both by QF-PCR and karyotype, suggesting that it was a case of confined placental mosaicism (CPM).

In one CVS, QF-PCR analysis detected an altered profile of sex chromosomes interpreted as monosomy X mosaicism (Fig. 12). Karyotype analysis of this sample failed due to poor cell growth. Although the QF-PCR kit is not specifically designed to detect mosaicism, such condition should be suspected in particular when unusual sex chromosome patterns are observed. The proportion between the two cell lines may be directly deduced by the ratio between the peaks of sex chromosomes that should fall outside the limits of normal ranges. In this case, in order to assess mosaicism degree, the average of peaks ratios for all markers of X chromosome was determined in $R=0.23$ and thus mosaicism grade was interpreted as 77%. The additional marker TAF9L which is present in the new kit of Aneufast (see Materials and methods section) was very useful to confirm these data. Peaks ratio of TAF9L was calculated as $R=1.65$ giving a mosaicism value of 82,5% (Fig. 13). Therefore, the grade of mosaicism was definitely determined at about 80%. In addition, QF-PCR analysis results indicated a possible mitotic origin of the aneuploidy and thus a probable case of CPM since all informative abnormal markers showed a trisomic diallelic pattern. In order to ascertain this

mechanism, amniocentesis was performed and both QF-PCR and karyotype analyses resulted normal, thus confirming that mosaicism for monosomy X identified at CVS derived from extra-fetal tissues.

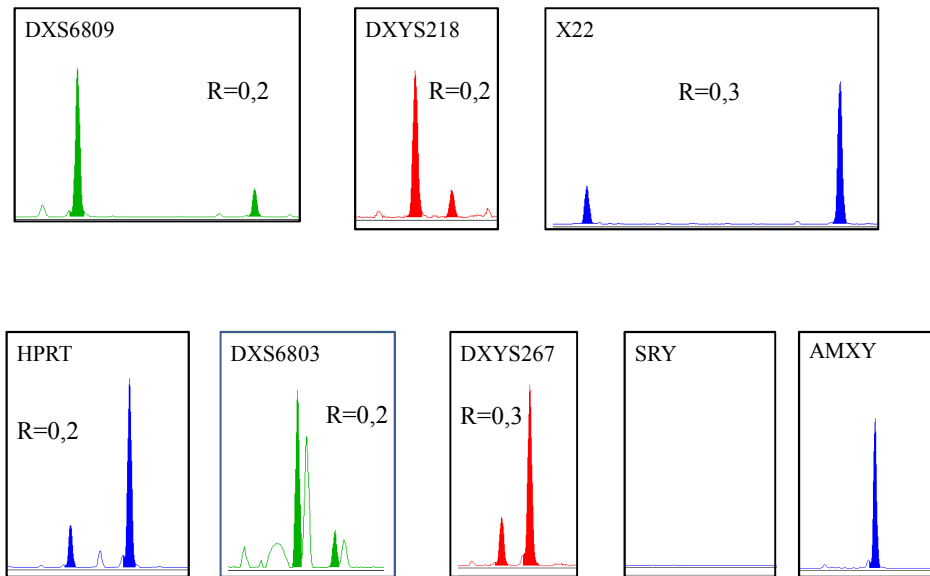


Fig. 12 Sample with altered STR profiles characterized by skewed ratios between alleles of sex chromosomes with an average of $R=0,23$ indicating a monosomy X mosaicism (about 80%). The absence of Y-specific products of AMXY and SRY is also shown.

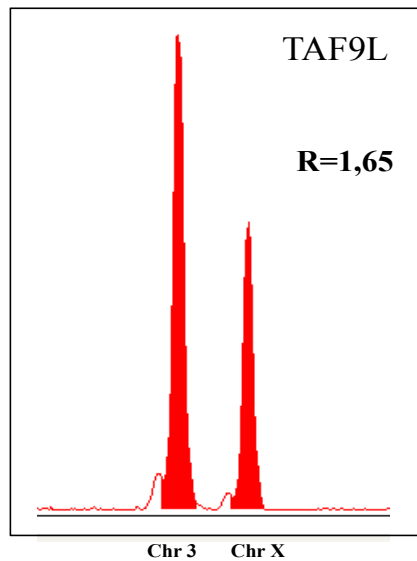


Fig. 13 Detection of X chromosome aneuploidies by QF-PCR analysis of TAF9L.

- X monosomy is determined by the double dose of chromosome 3-specific product compared to the X (ratio 2:1). In this case, single alleles should be detected for all STR markers on the sex chromosomes in absence of Y-specific sequences of the AMXY and SRY.
 - Two peaks of equal fluorescent intensity (ratio 1:1) indicate the presence of two X chromosomes (normal female or Klinefelter Syndrome).
 - Skewed ratio (0,7:1) in favor of the X specific product indicates the presence of three X chromosomes. In this case, STR markers on the sex chromosomes should result in trisomic pattern.
- This picture shows peaks area ratio of TAF9L (R=1.65) in a case of monosomy X mosaicism at 80%.

Sex chromosomes aneuploidies.

In addition to the case already described, other 5 cases of sex aneuploidies were found: 3 cases of monosomy X (45,X) plus a case with triple X (47,XXX) identified at CVS and a triploidy (69,XXY) identified in AF (Tab. 1). One of the monosomy X cases and the triple X had advanced maternal age as indication to perform villocentesis. However, the main indication to invasive test for all cases of Turner syndrome was an increased nuchal translucency. The sample diagnosed as triploidy had IUGR and oligoidramnios. In all these cases of sex aneuploidies both QF-PCR and karyotype were concordant (Fig. 14-16, Tab. 1).

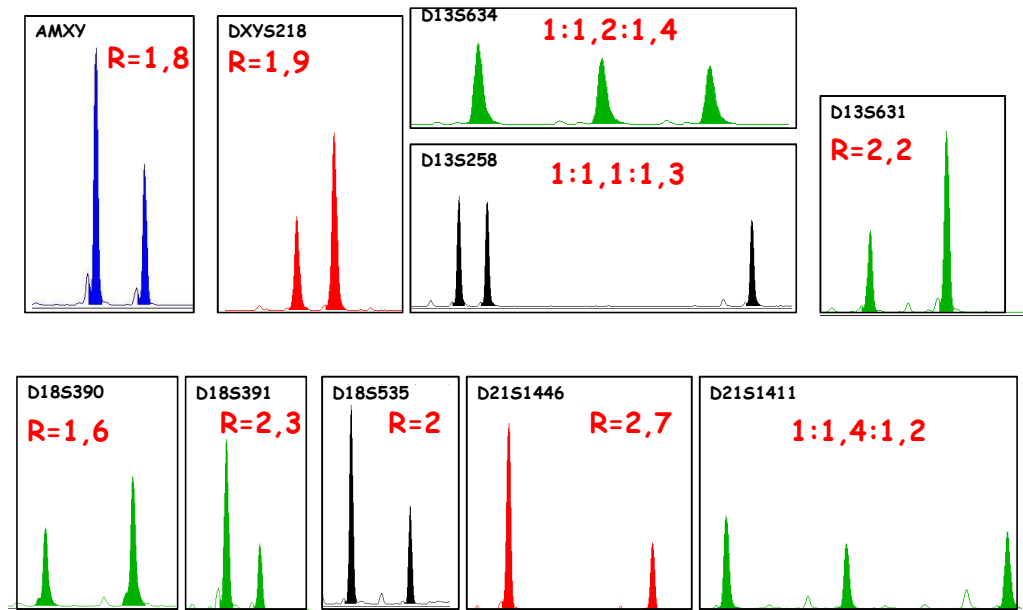


Fig. 14 QF-PCR profiles of a case of triploidy. Markers for all chromosomes were altered with peaks area ratios about 2:1 or 1:1:1. These STR profiles indicate the presence of an extra copy of each chromosome defined as triploidy.

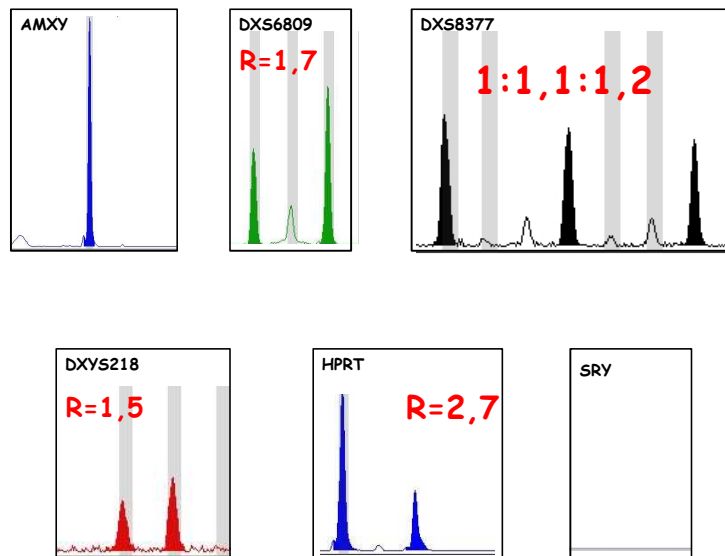


Fig. 15 A case of triple X identified by QF-PCR. Skewed peaks area ratios for sexual markers indicating an extra copy of chromosome X. The absence of SRY peak and the AMXY with only the peak of X chromosome exclude the presence of chromosome Y. STR profiles of other chromosomes were normal.

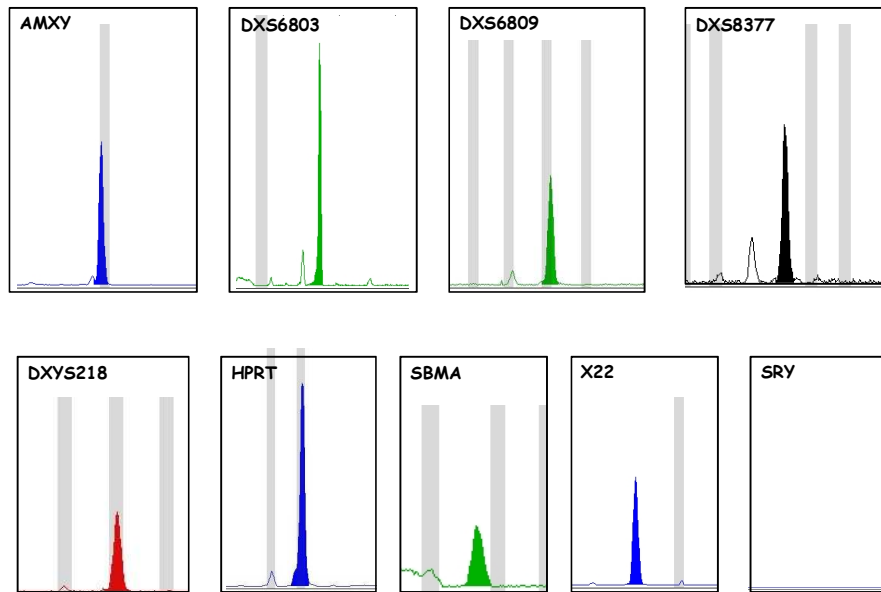


Fig. 16 A case of monosomy X identified by QF-PCR. The amplification of a single peak for all markers of sexual chromosomes and the absence of SRY peak indicate the presence of a single X chromosome corresponding to Turner syndrome.

4.2 *Array-CGH*

Array-CGH was performed in the following cases: 1- characterization of fetal chromosomal rearrangements; 2- characterization of fetal chromosomal markers; 3- increased nuchal translucency (>4 mm) with normal fetal karyotype.

Array-CGH analysis was performed in a total of 6 samples including 3 cases of chromosomal rearrangements, 2 cases of chromosomal markers, and one case with increased NT (>4mm) and normal QF-PCR and karyotype analyses (Tab. 2).

Tab. 2 Cases selected for array-CGH analysis.

CASES	INDICATION TO INVASIVE TEST	QF-PCR	KARYOTYPE	ARRAY-CGH
1	Advanced maternal age, positive screening test	Trisomy 21 (F)	47,XX,+21,t(8p;8q)	arr(1-20,22,X)x2,(21)x3
1	Advanced maternal age, NT=2.8mm at 12 weeks	Normal (F)	47,XX,+mar	Normal (F)
1	Advanced maternal age	Normal (M)	46,XY[16]/47,XY,+mar[4]	Normal (M)
1	Parental balanced translocation	Partial trisomy 18 (M)	46,XY,der(11)t(11,18)(q25;q22)mat	arr18q22.1q22.3(59,581,756-75,074,982)x3
1	Advanced maternal age, ultrasound abnormalities identified at 19 weeks	Normal (F)	46,XX,add(5)(p15.3)	arr1q41q44(221,213,018-247,179,232)X3 arr5p15.33(148,243-4,200,304)X1
1	NT=4.8mm at 12 weeks	Normal (F)	46,XX	Normal (F)

NT = nuchal translucency
M = male
F = female

In a case of trisomy 21 identified by QF-PCR in which karyotype revealed an associated translocation {47,XX,+21,t(8p;8q)}, array-CGH analysis confirmed the result of QF-PCR indicating that the translocation involving chromosome 8 was balanced (Fig. 17,18).

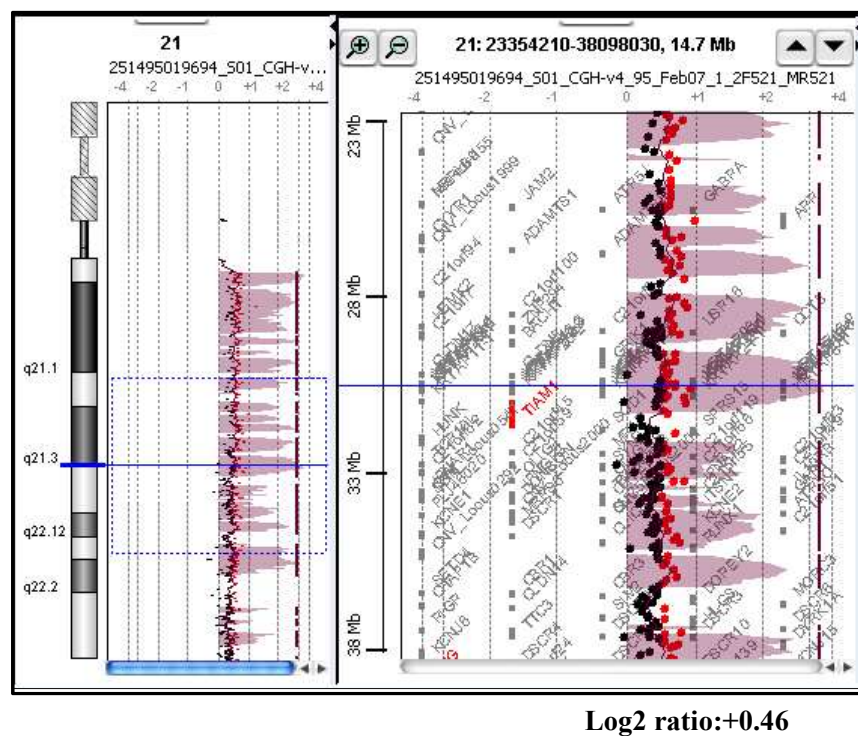


Fig. 17 Array-CGH ratio profiles of chromosome 21, indicating trisomy 21. On the left, the chromosome 21 ideogram. On the right, the log₂ ratio of the chromosome 21 probes plotted as a function of chromosomal position. Copy-number gain shifts the ratio to the right.

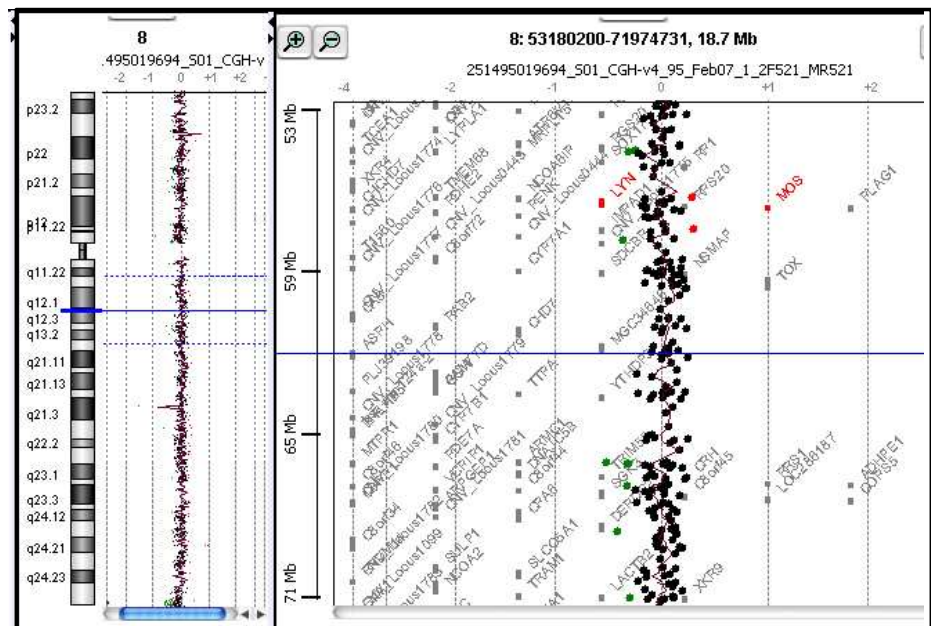


Fig. 18 Array-CGH profile of chromosome 8. On the left, the chromosome 8 ideogram. On the right, the log₂ ratio of the chromosome 8 probes plotted as a function of chromosomal position. There are no CNVs.

In order to characterize a *de novo* marker mosaicism, revealed by karyotype, array-CGH was performed and resulted normal. The discrepancy was probably due to the fact that mosaicism level was below 20% and therefore array-CGH could not detect it. Parents' karyotype showed that marker was not inherited. A clinical assessment after birth was performed (1 year of age) and it revealed normal growth and neurocognitive development. At child examination no particular cranio-facial and physical features were appreciated. The karyotype from peripheral blood of the child confirmed the presence of mosaicism for the supernumerary marker chromosome, detected in the prenatal period, however at a lower grade of mosaicism (2.5%).

In a case with NT of 4.8 mm and normal result of both QF-PCR and karyotype, array-CGH analysis was performed and it excluded that the ultrasound anomaly was due to a genomic imbalance.

In another sample with increased NT (NT=2.8mm) karyotype analysis revealed a supernumerary marker chromosome (47,XX,+mar) (Fig. 19), while QF-PCR result was negative. The presence of a marker induced us to perform array-CGH analysis that resulted normal. Karyotype analysis of parents demonstrated that chromosome marker was inherited from the mother.

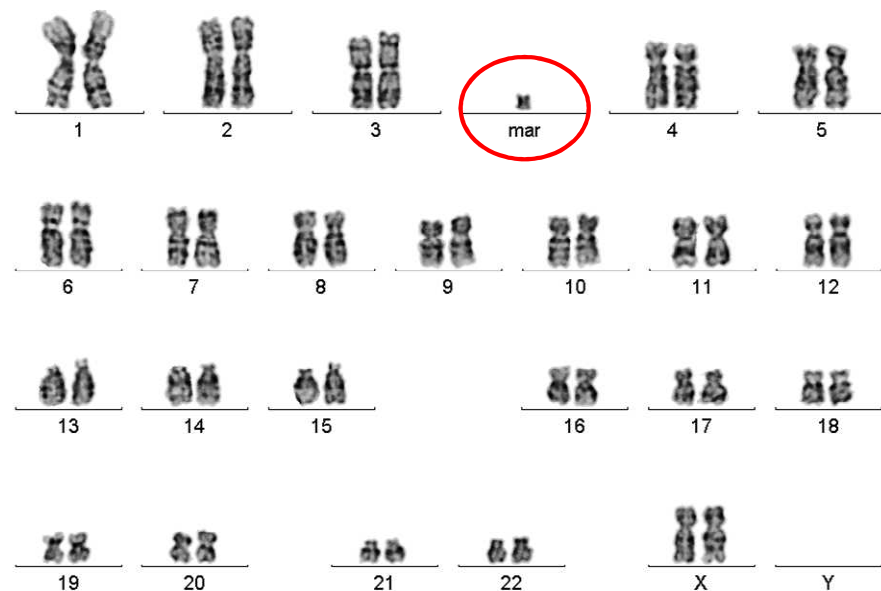


Fig. 19 Karyotype analysis of a CVS that shows the presence of a supernumerary chromosome marker (see circle).

Array-CGH was also employed in the case of a partial trisomy 18 identified by QF-PCR and a derivative chromosome 11 identified by karyotype in order to define exactly the genomic rearrangement. The array-CGH identified a 15,5 Mb duplication of

18q22.1q22.3 {arr 18q22.1q22.3(59,581,756-75,074,982)x3}. The poor quality of the experiment did not allow the analysis of chromosome 11 (Fig. 20).

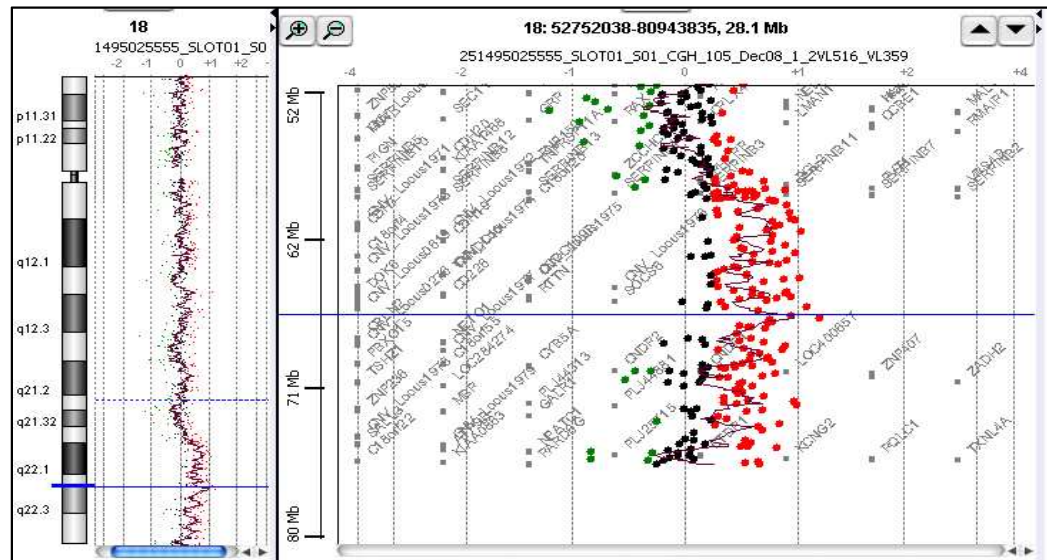


Fig. 20 Array-CGH ratio profile of a CNV on 18q22.1. On the left, the chromosome 18 ideogram. On the right, the log₂ ratio of the chromosome 18 probes plotted as a function of chromosomal position. Copy-number gain shifts the ratio to the right.

4.3 A prenatal case of trisomy 1q41-qter and monosomy

5p15-pter.

A 36 year-old woman was referred to prenatal genetic counseling for advanced maternal age. Family history was negative for congenital malformations or other genetic diseases. Both parents were healthy and consanguinity was excluded.

Chorionic villus sampling was performed at 12⁺⁶ weeks of gestation and ultrasound examination found no fetal malformations. Thirty metaphases of chorionic villus cells in four separate cultures were analyzed. In all, the fetal karyotype was 46,XX,add(5)(p15.3), showing an aberrant chromosome 5 with additional material at its

short arm (Fig.21a). Karyotype performed on lymphocytes of both parents was normal, suggesting a *de novo* origin of the rearrangement identified in the fetus.

Array-CGH analysis showed that the additional material on chromosome 5p originated from chromosome 1q and defined the extension and the breakpoints. The telomeric duplication size on chromosome 1 was about 26.0 Mb [arr1q41q44(221,213,018-247,179,232)X3] with proximal normal breakpoint mapped in 1q41 and located in 221.18 Mb (according to UCSC Genome Browser, <http://genome.ucsc.edu>, on Human May 2006 Assembly). The telomeric deletion size on chromosome 5p was about 4 Mb [arr5p15.33(148,243-4,200,304)X1] with the proximal normal breakpoint mapped in 5p15.33 and located in 4.70 Mb (Fig. 21b,c).

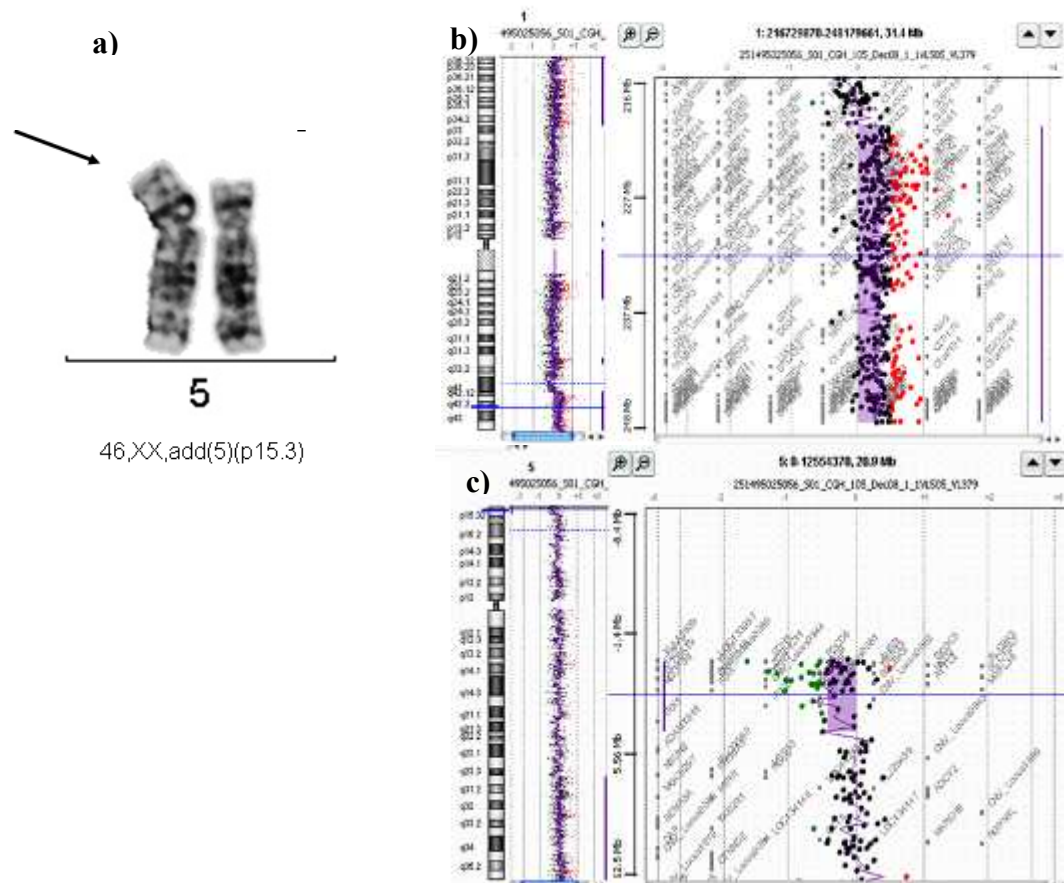


Fig. 21 a) Partial fetal karyotype showing an aberrant chromosome 5 with additional material at its short arm (see arrow). **b)** Array-CGH profile of chromosome 1 from DNA of chorionic villus cells showing the rearrangement of the long arm. In red, the duplication (average log₂ ratio:+0.6). **c)** Array-CGH profile of chromosome 5 from DNA of chorionic villus cells showing the rearrangement of the short arm of chromosome 5. In green, the deleted region (average log₂ ratio:-0.96).

A subsequent ultrasound examination performed at 19 gestational weeks to better examine fetal structures showed both anatomical and functional anomalies. Continuous movements and abnormal hands positioning together with contraction of 3th and 4th fingers of both hands were present (Fig. 22a). Moreover, a duodenal stenosis was shown at detailed investigation of gastrointestinal system. A “double bubbles” image, evident throughout exam duration, suggested the possibility of an organic impairment of the duodenum lumen (Fig. 22b). After counseling, the parents opted for termination of the pregnancy.

Autopsy findings revealed a female fetus with fairly peculiar facial features, including long face, elongated and flattened philtrum, wide and flat nasal bridge, and abnormal ears (posteriorly rotated and poorly formed). No other relevant external anomalies were found even if fingers and toes were slender and elongated. At brain examination, corpus callosum agenesis and a cerebellar hypoplasia with a reduced number and dimension of gyra was observed. No other macroscopic alterations of the internal organs, including heart gastrointestinal and urinary tracts were found. At histological examination, the morphology of the female gonads was relatively normal, with a reduced number of primordial follicles and numerous oogones.

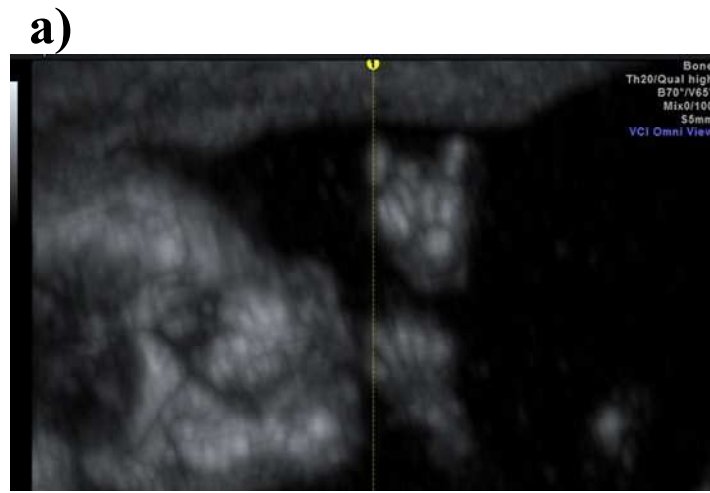


Fig. 22 a) Image showing contraction of the 3th and 4th finger of the left hand. **b)** Ultrasound pictures show the double bubble by 2-3D transverse scan.

5. Discussion

5. DISCUSSION

The analysis of a large cohort of prenatal samples by QF-PCR, karyotype and, in some selected cases, by array-CGH, allowed us to assess the advantages and limitations of applying these three different techniques in prenatal diagnosis.

QF-PCR was able to correctly identified 100% of constitutional trisomies of chromosomes 21, 18, 13, and aneuploidies involving chromosomes X and Y (100% specificity). QF-PCR due to its limitations, did not identify 7 cases (7/478; 1.5%) for the presence of structural rearrangements or mosaicism below 20%. All these 7 cases were detected by karyotype. However, it is important to underline that all these rearrangements, except one, involved other chromosomes respect to those analyzed by QF-PCR, and that the majority of these cases (5/7) revealed not to be pathogenic: i.e. 3 cases of balanced Robertsonian translocation, a mosaic trisomy 21 confined to placenta and an inherited constitutional chromosomal marker. Only the complex structural rearrangement between chromosomes 1 and 5 had a clear clinical impact. In fact, fetal ultrasound anomalies were ascertained at 19 gestation weeks, however, these findings would have prompt to perform further genetic investigations.

QF-PCR was also able to detect a partial rearrangement involving chromosome 18. In this case QF-PCR profiles showed two altered contiguous markers on chromosome 18q22-ter, while the other markers throughout the chromosome were normal suggesting a partial trisomy 18. This interpretation of results was also compatible with the notion that the mother had a balanced reciprocal translocation $t(11;18)(q25;q22)$, involving chromosome 18.

As expected, QF-PCR could not distinguish free trisomies 21 from those present in the contest of Robertsonian translocations. Given this limitation, after the

identification at QF-PCR of a trisomy involving the acrocentric chromosomes 13 or 21, fetal karyotype and/or parents' karyotype were always performed in order to ascertain a correct recurrence risk for the couple.

QF-PCR analysis was also useful to establish fetal zygosity in all cases of multiple pregnancies with uncertain chorionicity in order to ascertain the correct diagnosis if the same sex of twins is identified.

Another of the advantages of QF-PCR is that it could be carried out even in samples contaminated with maternal cells if maternal DNA is also tested. This allows to distinguish fetal from maternal STR profiles. Contamination with bacteria did not affect the QF-PCR results, while it often interferes with cell culture enabling conventional cytogenetic analysis. In total conventional cytogenetics analysis was not performed in 16 prenatal cases because of cell culture failure due to poor cell growth, contamination by bacterial or fungal cells or maternal cell overgrowth. In 15 of these cases QF-PCR tests were successful and gave normal results; thus, a second invasive procedure could be avoided. Only in one case of CVS, QF-PCR showed the presence of high level maternal cell contamination and no result could be obtained other than fetal sex. Amniocentesis was then performed.

Overall, our results confirm that QF-PCR is an accurate diagnostic test, providing correct diagnoses for the majority of prenatal cases (471/478, 98.5%) with the advantage to obtain results more rapidly than karyotype.

A particular attention should be paid to cases of mosaicism that are generally the most difficult to detect and interpret, especially in prenatal diagnostic setting, when in a restricted period of time "true mosaicism" has to be distinguished from "pseudomosaicism", or CPM. In our study a case of low-level trisomy 21 mosaicism (undetectable by QF-PCR) and a case of high-level monosomy X mosaicism (detectable

by QF-PCR) were identified. Further analyses on amniotic fluids demonstrated that they were CPM, therefore clinically insignificant since there is no statistical evidence of the adverse effect of CPM for X chromosome monosomy or trisomy 21 on fetal outcome.²⁰

Another interpretation issue is about supernumerary marker chromosomes. In our study 2 cases of supernumerary chromosome were detected: one in a mosaic state, one constitutional. As already pointed out both markers were not identified by QF-PCR. In the first case the marker was identified in about 20% of analyzed metaphases. The couple was informed that the low grade of mosaicism together with normal ultrasound fetal scans represented a favorable prognostic factor, even if the phenotype could not be specifically defined. As already discussed the clinical assessment of the child performed at 1 year of age did not reveal any abnormality confirming what was hypothesized during prenatal time. Regarding the case of the constitutional chromosomal marker, parents' analysis revealed that the marker was inherited from the mother, and in other maternal relatives suggesting that it was not pathogenic in line with C- and Q- banding techniques results that determined the heterochromatin nature of marker chromosome.

Overall it can be stated that QF-PCR is able to detect the majority of chromosomal clinically relevant abnormalities present in prenatal diagnosis and that the undetected anomalies when pathogenic are usually associated with fetal ultrasound abnormalities that would have prompted to perform additional tests such as karyotype and array-CGH. Thus, our findings suggest the possibility to use QF-PCR as the only approach in prenatal diagnosis for certain referral categories, replacing conventional cytogenetic analysis with this rapid molecular assay.

Selected cases of our cohort were also studied by array-CGH in order to better characterize chromosomal markers and chromosome structural aberrations.

In particular two cases with chromosomal markers were analyzed by this technique. In the case of the constitutional inherited chromosomal marker, array-CGH helped to definitely estimate its clinical significance. In fact, array-CGH did not detect any genomic imbalances and let us consider it as non pathogenic. Conversely, array-CGH did not add useful information in the case of marker mosaicism. The absence of CNVs could not exclude that the marker was constituted by euchromatin since array-CGH is unable to detect mosaic at a low-grade.

Array-CGH was also employed to study a fetus with normal QF-PCR and karyotype results and increased NT and it established the absence of genomic imbalances. This result induced us to reassure the parents even if a skeletal dysplasia or a heart defect could not be excluded and a third level ultrasound examination was suggested to the couple.

Array-CGH technique was useful to better characterize three structural chromosomal abnormalities: an apparently balanced translocation 8p;8q associated with trisomy 21, a case with partial trisomy 18, identified by QF-PCR, and a complex rearrangement between chromosomes 1 and 5. In the first case the analysis excluded the presence of CNVs in chromosome 8, demonstrating that the translocation was balanced. In the second case, array-CGH allowed us to exactly define the exact genomic region involved in the rearrangement and consequently to better define the prognosis of fetus. Finally, array-CGH successfully characterized a case of derivative chromosome 5 identified by karyotype. The array-CGH analysis was able to rapidly detect that the rearrangement was complex and included a trisomy 1q41-qter and a monosomy 5p15-pter.

Partial 5p deletions ranging from 5 to 40 Mb result in Cri-du chat syndrome (CDCS). The main clinical characteristics of CDCS are low birth weight, microcephaly,

neonatal hypotonia, speech symptoms, round face with peculiar facial features including downward slanting palpebral fissures, hypertelorism, broad nasal bridge, epicanthal folds, micrognathia, low-set ears, abnormal dermatoglyphics and a typical cat-like cry.⁵⁰ Genotype-phenotype correlation studies revealed that most clinical variations of CDCS are related to the size and location of the deletion. Two critical regions in CDCS have been defined: one in 5p15.3, associated with the cat-like cry and speech symptoms, and the other in 5p15.2 associated with dysmorphisms and intellectual disability.⁵¹ Partial trisomy 1q syndrome (PT1qS) is a rare chromosomal abnormality, which has been described either isolated or in most cases accompanied by other chromosome aberrations such as monosomy. To date, two major PT1qS with regard to the breakpoint localization have been described, 1q32-qter and 1q42-qter, with the former described as “proximal partial trisomy 1q” and the latter as “distal partial trisomy 1q”.⁵² The clinical phenotype of partial trisomy 1q syndrome varies widely, due to the different breakpoints on chromosome 1 and the extent of the monosomic segment of the involved autosomes. Duplication of 1q42-qter are usually associated with low birth weight, macrocephaly with large fontanelles, prominent forehead, broad nasal bridge, facial nevi, low-set ears, downward slanting palpebral fissures, hands and feet abnormalities and psychomotor retardation. Partial trisomy 1q32 syndrome is usually characterized by more severe phenotypes, including urogenital anomalies and severe cardiac defects.⁵³

The case reported in this thesis represents the first *de novo* unbalanced translocation involving chromosomes 1 and 5 identified during prenatal period. So far, only two cases with associated distal 5p deletion and partial trisomy 1q have been reported in postnatal time.^{50,52} In 2001 Emberger *et al* identified a patient with a partial trisomy 1q combined with CDCS caused by a *de novo* unbalanced translocation 46,XX,der(5)(5qter-5p13.1::1q41-1qter).⁵² In 2010 Flores-Ramirez *et al.* reported a case

of a complex rearrangement involving chromosome 1 and 5, characterized by FISH and derived from a balanced maternal translocation $t(1;5)(q32;p13)$.⁵⁰ These rearrangements were very similar but larger than ours. Interestingly, the phenotype of these two cases partially overlaps with that of our case. Common manifestations included wide nasal bridge, dysmorphic ears, and foot and hand anomalies. In addition the case described by Flores-Ramirez showed heart disease and urogenital abnormalities. The absence of urogenital abnormalities and severe heart anomalies in our case is in accordance with the fact that our deletion is distal as these features seem to be characteristic of the “proximal PT1qS”.⁵⁴ Interestingly, although macrocephaly is suggestive of distal trisomy 1q, our case presents microcephaly typical finding in CDCS, suggesting that some phenotypic expressions were obviously the result of a combination of both partial aneuploidies. This fact demonstrates the complexity of genotype-phenotype correlation and the uncertainty of prognosis in this rare complex chromosomal aberrations.⁵²

The important data that emerge from the description of this case is the significant impact of array-CGH technique which allowed us to better characterize the anomalies detectable by standard karyotype. In fact, detailed breakpoint characterization of partial trisomy and partial monosomy syndromes will allow a more precise genotype-phenotype correlation on dependence of gene involvement.

Our results together with literature data suggest that genome-wide array analysis may act as a substitute for conventional cytogenetics in prenatal diagnosis, although the latter remains irreplaceable to complete diagnosis and proper genetic counseling to the parents in selected cases.

Application of array-CGH requires great care for the possibility to detect CNVs of uncertain clinical significance that is a great concern in prenatal diagnosis. About two

thirds of genomic imbalances detected by prenatal array-CGH have been interpreted as probably benign and of no clinical significance.³⁶

Common protocols for the application and interpretation of genomic arrays in prenatal diagnosis would be desirable and hopefully be capable of decreasing the risk of unexpected findings. It seems likely that in the future, the availability of shared databases specifically dedicated to prenatal diagnosis coupled with the growing amount of data regarding CNVs could make it easier to interpret genomic arrays. Bui et al. (2011) suggest a possible workflow for prenatal molecular karyotype, completely eliminating conventional cytogenetics as follows: fetal sampling and parental blood collection → DNA extraction and quality controls → *SRY* PCR for fetal sex determination → Microsatellite analysis to exclude maternal contamination → Genome-wide array analysis and quality controls on results → Evaluation of detected CNVs → Possible evaluation of parental DNAs → Reporting (available in 7 days if parental DNAs have to be analyzed).²⁵

Given the present *limited* scientific knowledge on CNV variability of the human genome, targeted platforms containing probes covering both regions whose deletion/duplication are clearly associated with fully penetrant disease, and regions traditionally considered at risk (such as subtelomeric and pericentromeric regions) may significantly reduce the risk of uncertain findings. However, such platforms require continuous updating for pathogenetic deletions/duplications and obviously could miss important genomic imbalances that could be associated with a disease in the future.

Overall, the superior diagnostic power of array-CGH far outweighs these concerns and this technique will be surely applied in prenatal settings more and more extensively. In this early period all such issues have to be addressed during pre- and post-test counseling and certainly their impact will further diminish as the technology continues

to develop and experience with its prenatal diagnostic use grows.⁵⁵ In the near future we hope to greatly reduce the load of conventional cytogenetic analyses in the prenatal diagnosis of chromosome disorders using QF-PCR and array-CGH, accompanied by careful monitoring of pregnancies by non-invasive screening test.

6. Future perspectives

6. FUTURE PERSPECTIVES

In the last 2-3 years, the robustness of molecular karyotype technologies, such as array-CGH, has become obvious to the medical and laboratory community involved in prenatal diagnostic testing. Evidences regarding the increased diagnostic yield of this technique with respect to conventional karyotype makes its use tempting in a routine prenatal setting, although the debate on possible pitfalls of this approach is still ongoing essentially concerning the possible detection of copy-number variations (CNVs) of uncertain or unknown clinical significance.

On the basis of the recent debates about the role of traditional cytogenetics and whether array-CGH can be considered as a replacement for this routine testing and in light of results obtained with our study that go mainly against conventional karyotyping in favor of array-CGH, in collaboration with 3 colleagues I decided to establish a spin-off that will have the aim to provide pre- and post-natal diagnostic analysis through the use of innovative techniques, such as QF-PCR and array-CGH. Spin-off services will be also directed to pregnant women aged <35 years and without any pathologic indication who do not have the possibility to choose invasive prenatal diagnosis under the National Health Service. Potential users of the services provided by the spin-off will be mainly public or private gynecology centers or single users interested in expanding the number and quality of available tests.

7. References

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8. AKNOWLEDGEMENTS

I would like to thank prof. Alessandra Renieri for giving me this opportunity and believing in me. This doctorate school taught me a lot and enriched me with ways of approaching a life time.

A thank to all my colleagues. I learned and shared a lot from them. In particular I would like to thank Veronica and Chiara for their help, but most of all for their friendship.

A special thank to my family that always encouraged me and supported.