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Μοριακός καρυότυπος στην προγεννητική διάγνωση

ΠΑΟΛΑ ΕΥΑΓΓΕΛΙΔΟΥ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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Πρόλογος

Η εκπόνηση αυτής της διατριβής δεν θα ήταν δυνατή χωρίς την παρότρυνση, καθοδήγηση, επιμονή και υπομονή του Φίλιππου Πατσαλή τον οποίο και ευχαριστώ από τα βάθη της ψυχής μου. Τον ευχαριστώ ολόθερμα που στάθηκε πλάι μου σαν φίλος και μέντορας και που πίστεψε σε μένα όλα αυτά τα χρόνια της στενής μας συνεργασίας, πολλές φορές πιο πολύ κι από ότι πίστεψα εγώ στον ίδιο μου τον εαυτό. Δεν θα βρισκόμουν στο επίπεδο που είμαι σήμερα, σαν επιστήμονας, χωρίς τα εφόδια που μας παρείχε ο Φίλιππος, ατομικά και σαν ομάδα, θέτοντας τον πήχη ψηλά. Ευχαριστώ για τις αμέτρητες ώρες, μέρες, μήνες και τελικά χρόνια που αφιέρωσε σε μένα με αμείωτο ζήλο χωρίς να με εγκαταλείψει, ούτως ώστε να μπορέσω να ολοκληρώσω τη μελέτη αυτή. Του είμαι ευγνώμων που με δίδαξε ότι πάντα μπορείς να έχεις πάθος για αυτό που κάνεις και να στοχεύεις ψηλά όσο ακατόρθωτος και να φαίνεται ο τελικός στόχος.

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1) Introduction

1.1) Prenatal Diagnosis

Prenatal Diagnosis is the application of various techniques to determine whether the unborn fetus or embryo is affected with a genetic disorder or condition before birth. Such birth defects include Down syndrome, neural tube defect, chromosome abnormalities, thalassemia, sickle cell anaemia, Duchenne's muscular dystrophy and many others. The detection for each of these conditions depends on the method used for diagnosis. Prenatal Diagnosis can also be used to determine the sex of the unborn baby. Currently most of the diagnostic methods are applied using invasive procedures to obtain fetal material for the purposes of prenatal diagnosis.

1.2) Non-invasive Prenatal Screening

There are however several non-invasive screening tests that can be offered to all pregnant women and not just a subgroup. Non-invasive methods, called "screens", can only evaluate the risk of a condition and cannot determine 100% if the fetus has a condition. Findings from the non-invasive screening tests will determine whether or not there is a need for the pregnant woman to subsequently be offered an invasive prenatal procedure.

The non-invasive techniques include: a) procedures that allow fetal visualization and can be used to follow fetal growth and detect structural abnormalities like Ultrasound, Fetal echocardiography, MRI, Radiography, b) Listening to the fetal heartbeat, c) Screening for neural tube defects and d) Sequential screening [1,2], the process where to calculate the individual patient-specific risk for chromosomal defects one needs to take into account the background risk and multiply by a series of factors. These factors depend on the results of a series of screening tests carried out during the course of the pregnancy. Every time a test is carried out the background risk is multiplied by the test factor to calculate the new risk, which then becomes the

background risk for the next test. In the first trimester maternal serum screening can check levels of free β-hCG and PAPP-A in the prospective mother's serum, and combine these with the measurement of nuchal translucency (NT). Some institutions also look for the presence of a fetal nasal bone on the ultrasound.

New non-invasive tests are more extensively explored in discussion.

1.3) Indications for prenatal diagnosis

Prenatal Diagnosis can be invasive or non- invasive. As invasive prenatal procedures are associated with a risk for causing miscarriage (estimated to be around 1- 0.5%) it is necessary to evaluate the absolute need for testing. Therefore it is suggested that invasive procedures whether earlier in pregnancy (Chorionic Villus Biopsy) or later (amniocentesis, fetal blood) is reserved for pregnancies that are considered to be at high risk. The main indications for prenatal diagnosis are: a) advanced maternal age (above 35 years old). Maternal age alone is though a poor predictor, b) abnormal maternal serum biochemistry [for PAPP(A) and β- HCG] and/or abnormal ultrasound findings, c) fetal anomaly detected by ultrasonography, d) pregnancy history: previous abortus, stillbirth or livebirth with a chromosomal abnormality (mostly aneuploidy), e) transmissible chromosomal rearrangement (Pregnant woman or partner is a carrier of a chromosomal rearrangement), f) pregnant woman is a carrier of an X-Linked disorder (e.g. Fragile X), g) pregnant woman and partner carriers of a recessive genetic disorder like thalassemia, cystic fibrosis etc., h) exposure to viral infections, such as rubella or cytomegalovirus.

1.4) Benefits of prenatal Diagnosis

Invasive prenatal testing as mentioned above can be carried out earlier or later in pregnancy and it can offer the future couple the most suitable obstetric management by having: a) the choice to decide on the outcome of the pregnancy once the genetic or other result is available, b) help in determining whether to continue the pregnancy,

c) an estimate of the complications in the pregnancy, d) preparation of the couple for the birth of a child with an abnormality, and potentially offer education about the specific disorder and preparation for the special care that will be required of a handicapped child and e) a prognosis for future pregnancies for themselves and/or their immediate and extended families.

Therefore clinicians and patients should weigh the relative risks and benefits of invasive prenatal diagnosis performed later as compared to earlier in pregnancy.

1.5) Types and time periods of invasive procedures

- Chorionic Villus Sampling, (First trimester, 11-14 weeks gestational age)
- Amniocentesis, (Early; second trimester 15-27 weeks, Late; Third trimester 28-to term)
- Cordocentesis, FB (Fetal Blood, after 16 weeks of gestation)

For decades, Chorionic Villus Sampling (CVS- Figure 1.5.1.1) and Amniocentesis (AF- Figure 1.5.1.2) have been the two most common prenatal diagnostic procedures. Both are invasive procedures requiring the need of needle being passed through the cervix or through the abdominal wall into the uterus under ultrasound guidance. Depending on the procedure, a sample of chorionic villi surrounding the sac is obtained for CVS, or 10-20 mL of amniotic fluid from the amniotic cavity inside the uterus is collected for amniocentesis. AF contains cells from amnion, fetal skin, fetal lungs and urinary tract epithelium; CVS contains chorionic villi which are microscopic, finger-like projections that emerge from the chorionic membrane and eventually form the placenta. The cells that make up the chorionic villi are of fetal origin.

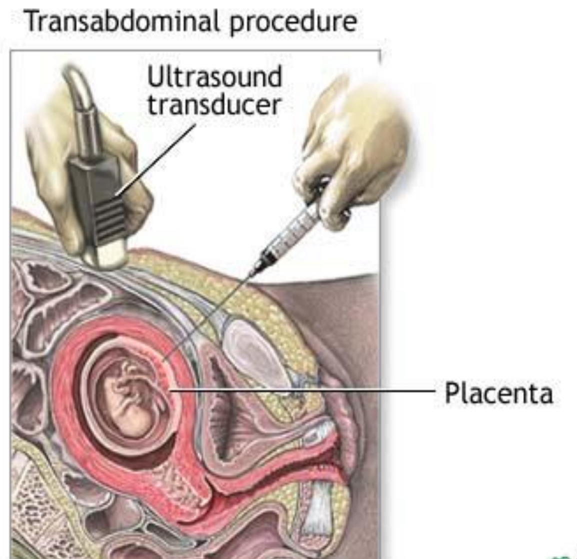


Figure 1.5.1.1: Trans-abdominal procedure for CVS. With ultrasound guidance the doctor locates the placenta and a long needle is inserted through the woman's abdominal wall, through the uterine wall and to the chorionic villi. The sample is obtained by applying suction from the syringe.

Both procedures are safe with an equivalent risk of 0.5% of procedure-induced pregnancy loss. Prospective comparative studies have demonstrated that with equally experienced operators, CVS and second trimester amniocentesis have similar procedure-induced miscarriage rates. When CVS procedures are performed after 10 weeks gestation, no increased risk of fetal anomalies has been demonstrated. On the contrary, when CVS is carried out prior to 10 weeks of gestation there may be an increased risk for limb reduction defects; when the amniocentesis is done prior to the 15 weeks it has an increased risk for talipes equinovarus. Laboratory analysis for both procedures is equally reliable. When carrying out chromosomal analysis for CVS, the karyotype, is identical to that of the fetus in over 98% of cases; in the remaining 1 to 2% confined placental mosaicism (CPM) occurs and therefore there is the need for a second invasive procedure to be performed to exclude confined placental mosaicism [3].

First-trimester CVS has the advantage over second-trimester amniocentesis, in that, it allows earlier prenatal diagnosis of various genetic and cytogenetic disorders in the fetus, thus giving the prospective couple the choice of earlier termination should they decide that to be the outcome of the pregnancy [4]. Test results for amniocentesis, whether done earlier or later in pregnancy, are usually available only after the 18th

week of gestation to the best of circumstances. Therefore CVS has developed to avoid the medical and psychological complications of later prenatal diagnosis by amniocentesis; CVS has rapidly become a primary tool for the diagnosis of fetal cytogenetic, molecular, and biochemical disorders. In addition, its development has led to an improved understanding of several biological processes, including confined placental mosaicism and uniparental disomy [5].

Fetal Blood sampling, also known as cordocentesis, is the third type of invasive prenatal diagnosis. It is performed after the 16 week of gestation and the sample is acquired in a similar way as CVS and AF. A needle is inserted into the umbilical cord under ultrasound guidance, and fetal blood is collected from the umbilical vein for chromosome analysis and/or other genetic diagnosis. An advantage of Fetal Blood is the rapid rate at which lymphocytes grow, allowing prompt genetic diagnosis. This technique is also useful for evaluating fetal metabolism and hematologic abnormalities.

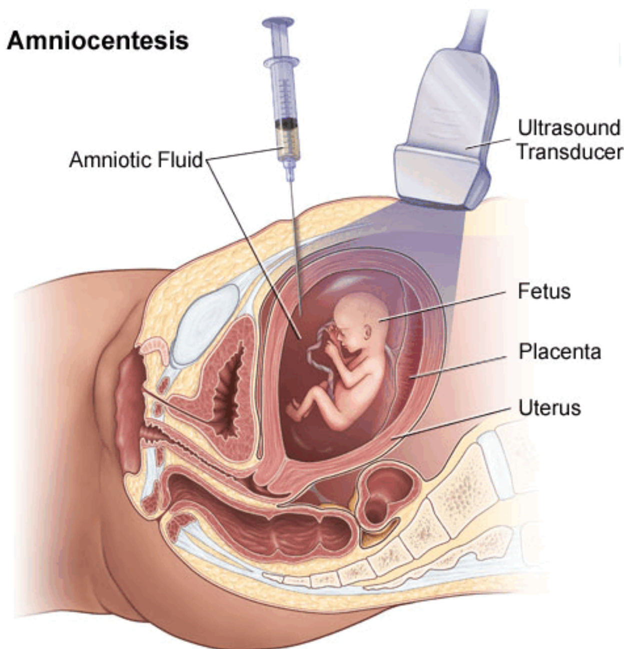


Figure 1.5.1.2: Trans-abdominal procedure of Amniotic Fluid sampling. A long needle is inserted through the woman's abdominal wall, through the uterine wall with ultrasound guidance, into the amniotic sac to withdraw a small sample of the amniotic fluid for examination. The amniotic fluid contains cells shed by the fetus.

1.5.1) Types of samples in invasive procedures

1.5.1.1) CVS

Once collected the villi are dissected under an inverted microscope from the maternal decidua. Following an enzymatic dissociation of the sample it is set up into cultures to eventually harvest metaphase cells (fibroblasts) for chromosome analysis to be carried out. This will determine karyotype of the fetus.

Alternatively, DNA can be extracted from the dissociated tissue for molecular analysis like Quantitative Fluorescent Polymerase Chain Reaction (QF PCR) or microarray Comparative Genomic Hybridization (aCGH) analyses. DNA analysis of CVS specimens is helpful for early diagnosis of hemoglobinopathies.

Confined placental mosaicism with CVS may result in diagnostic ambiguity, leading to the need for additional invasive diagnostic tests.

1.5.1.2) Amniotic Fluid

The Amniotic Fluid contains a heterogeneous population of cells and depending on the gestational age they are arising from the amnion, skin and the urogenital or respiratory tract. The numbers of fetal cells present in the AF sample increase with gestational age, but the viable cells are decreasing in numbers as the pregnancy progresses. Usually 10-20 ml of amniotic fluid is collected and presented to the laboratory for chromosomal, biochemical, and/or molecular analyses. The AF is set up into cultures to eventually harvest metaphase cells (fibroblasts) for chromosome analysis to be carried out. The same as with the CVS, DNA can be extracted from the sample for molecular analysis like QF PCR or aCGH analyses.

1.5.1.3) Fetal Blood

Fetal blood is the sample that is the most fetal in origin and it would be the most reliable material to use. Unfortunately though the availability of sampling expertise is

insufficient and the obstetric complications are relatively high so it is not always possible to have this sample available for analysis. One drawback of fetal sampling is the fact that it can be performed after the 16th week of gestation which falls well into the second trimester; results however can be out much earlier compared to the other two tissues which are usually used for prenatal diagnosis, CVS and AF. If however it is received by the laboratory part of the whole blood is cultured into culture medium to achieve growth and to eventually harvest metaphase cells (lymphocytes) for chromosome analysis to be carried out. DNA can be extracted from whole (fetal) blood for other molecular analyses.

1.5.1.4) Evacuated Products Of Conception (POC)

In the event of spontaneous miscarriage of a pregnancy, evacuated products of conception could be sent to the laboratory in order to determine if the miscarriage had occurred due to genetic abnormalities. When this type of sample is received by the laboratory for work up an effort is made to retrieve fetal material so that cultures can be set up for chromosomal or other molecular analyses. If this is a freshly first trimester miscarriage there are good chances of finding intact limbs from the fetus; these could be selected for culture as they will grow excellently. Most of the times, however, these samples do not contain any obvious fetal parts therefore the laboratory should try and collect anything that is fetal. Good substitutes in this case would be chorionic villi, embryonic sac or membranes. A major disadvantage of POC samples is the fact that together with the presumably fetal material, maternal tissue is also collected which can also grow in culture. This can interfere with the interpretation of the results. In addition, a number of these kinds of samples fail to grow *in vitro* or bacterial/fungal contamination occurs that makes it impossible to conclude the analysis. If something like this occurs DNA can be extracted from the same tissue that was used for setting up cultures, for analysis with alternative molecular methods.

The rationale behind all these different tissues to be used for chromosomal analysis is that following set up of the cultures a mitotic inhibitor (colchicine, colcemid) is added to the cultures to stop cell division at mitosis which allows an increased yield of mitotic cells for analysis. The cells are then centrifuged and media and the mitotic inhibitor are replaced with a hypotonic solution. This causes the red blood cells to lyse (for blood samples) and the white blood cells or fibroblasts to swell so that the chromosomes will spread when added to a slide. After the cells have been allowed to sit in hypotonic solution, Carnoy's fixative (3:1 methanol to acetic acid) is added. This kills the cells and hardens the nuclei of the remaining white blood cells or fibroblasts. The cells are generally fixed repeatedly to remove any debris or remaining red blood cells. The cell suspension is then dropped onto specimen slides. After aging the slides they are ready for banding and analysis. Analysis of banded chromosomes is carried out under a microscope and generally 20 cells are analyzed. This is done to rule out mosaicism with a confidence of 95%.

As the logic behind prenatal diagnosis lies with the principle that the constitutional karyotype of an individual is determined at conception, and that mitosis copies this genotype in all tissues derived thereafter, by karyotyping cells from different tissues the fetal karyotype can be determined [6]. In 99% of cases the fetal karyotype is reliably found. The remaining 1% of the cases is more problematic due to mosaicism. Mosaicism occurs when there is a mitotic error early in fetal life and depending on the stage of fetal development that this error takes place it will be apparent in the placenta, or the extra-embryonic tissues or even the embryo.

1.6) Methods

1.6.1) Cytogenetics-Classical chromosomal analysis

Cytogenetics includes routine analysis of G banded chromosomes, and/or other cytogenetic banding techniques (C- Banding, NOR, Q Banding), as well as molecular cytogenetics, such as Fluorescence In Situ Hybridization (FISH) and metaphase

Comparative Genomic Hybridization (mCGH). The aim of Classical Cytogenetics is to count and to structurally analyze the chromosomes for phenotype- genotype correlation, or for prenatal referrals to correlate sonographic markers or other indications to the genotype. Each of our 46 chromosomes has a characteristic structure with a distinctive banding pattern (dark and light bands) which is generated in the laboratory with the use of chemicals (trypsin, Giemsa or Leishman stain). These features are highly conserved in all humans thus making it easy to identify and distinguish from each other under the microscope. They define what is known as "normal Karyotype" and any deviation from this could cause a chromosome abnormality (Figure 1.6.1). Cytogenetics has been used since 1970 for prenatal diagnosis and it is still used as the primary detection method for prenatal samples. Chromosomal analysis is usually carried out at the 550 Band Level (G Banding) and aims to detect numerical or structural abnormalities in the unborn child. This method is capable of detecting rearrangements anywhere in the genome. The only limitation being its detection level and this falls between 5-10Mb. While some chromosome abnormalities are harmless variations, most are associated with clinical disorders. Half of all spontaneous abortions are due to chromosome abnormalities but the incidence in live births falls to less than 1 per cent. Loss or gain of whole chromosomes can cause severe disorders as they can affect the copy number of thousands of genes.

Few of the numerical abnormalities are compatible with development or even with life either because that chromosome has few genes (13, 18, 21, Y-chromosome) or because there is a natural mechanism to adjust gene dosage even in normal people (X-chromosome). The most common numerical abnormalities are listed in Table 1.6.1.

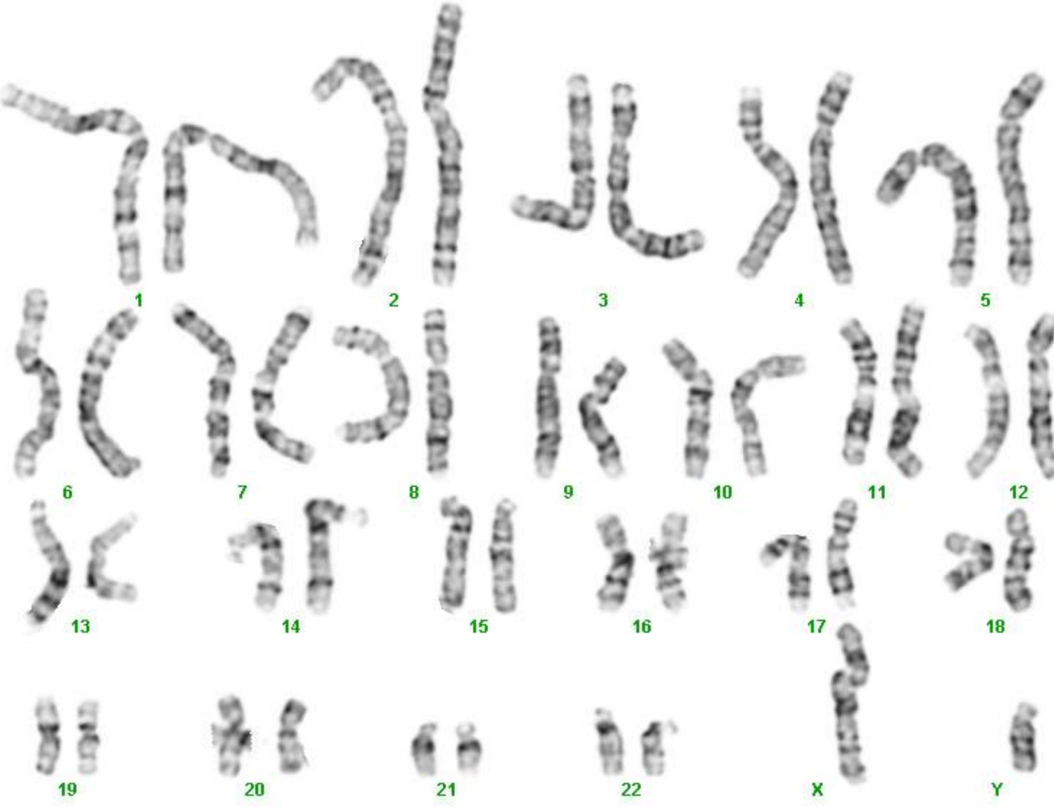


Figure 1.6.1: G- Banded male karyotype from an amniotic fluid sample showing 46 chromosomes.

Table 1.6.1: The major numerical abnormalities that survive to term. (Taken from: http://genome.wellcome.ac.uk)

Syndrome	Abnormality	Incidence per 10 000 births
Down	Trisomy 21	15
Edwards	Trisomy 18	3
Patau	Trisomy 13	2
Turner	Monosomy X	2 (female births)
Klinefelter	XXY	10 (male births)
XXX	XXX	10 (female births)
XYY	XYY	10 (male births)

Chromosomal analysis can successfully identify structural abnormalities either balanced or unbalanced, unless they are subtle. In balanced structural abnormalities there is rearrangement of genetic material, but overall there is no gain or loss (inversions, translocations). The major consequence of a balanced rearrangement is however the prevention of normal chromosome pairing at meiosis, which leads to

production of sperm and eggs with incomplete or partially duplicated chromosome, sets. Although most carriers of balanced translocations are phenotypically normal, an association of cytogenetically balanced translocations with phenotypic abnormalities has been reported [7]. The reason for this being: i) the disruption of a dosage-sensitive gene at the breakpoints or expression a recessive gene , ii) position effect with variable expression of genes near the translocation breakpoint iii) uniparental disomy (if the chromosome involved is subjected to imprinting) due to post-conceptional "correcting" loss of the homolog from the normal non-carrier parent iv) the rearrangement is not truly balanced at the DNA level or in familial cases there may be additional unbalanced subtle rearrangements which occurred during meiosis v) the rearrangement may host 'cryptic' complex chromosomal rearrangements (CCRs).

The clinical significance of prenatal and postnatal identification of cryptic CCRs is extremely important, as CCRs are associated with reproductive problems, multiple miscarriages, stillbirths or in patients with malformations, mental retardation, dysmorphic features or congenital anomalies [8]. As it is concluded in this study there is evidence that the unknown link between an apparently balanced rearrangement and the appearance of abnormal phenotype in the family may often explained due to the presence of cryptic CCRs [8]. The presence or absence of CCRs could only be identified with the use of other molecular methodologies (FISH, aCGH see sections 1.6.2 and 1.6.4)

Unbalanced abnormalities are very similar to numerical abnormalities, the only difference being the fact that there is partial and not complete gain or loss of a chromosome. This partial gain or loss could include large or smaller parts of chromosomes. But even if the copy number gain or loss is a tiny chromosome fragment it could have severe effects to the phenotype as it encompasses several genes. This is what happens in the subtelomeric as well as the microdeletions/microduplications syndrome regions. This is where Fluorescent *in situ*

hybridization (FISH) comes into use. There are some limitations of conventional cytogenetics and these include: a) it cannot reliably detect rearrangement of genomic segments smaller than 3-10Mb, and those located in G-negative band can be missed, b) the turnaround time for karyotyping is increased by the need for cells to be cultured 15-21 days before analysis, c) it is time consuming and requires highly skilled staff, d)it may not identify the origin of supernumerary marker chromosomes or ring chromosomes present in the analysis and will require the use of other methods to further investigate it and e) it cannot detect Uniparental Disomy (UPD)

1.6.2.) Molecular cytogenetics- Fluorescence In Situ Hybridization (FISH)

FISH is a molecular cytogenetics technique that uses fluorescently labeled DNA probes to hybridize to the specific locus of interest. It can be used as a diagnostic method for the microdeletions/microduplications syndromes and the subtelomeric regions (Figure 1.6.2) that are beyond the resolution of conventional cytogenetics or as a complimentary method to conventional cytogenetics. It can be used to confirm or further investigate a chromosomal abnormality found during chromosomal analysis. Furthermore, it can be used for rapid aneuploidy testing in interphase nuclei in Prenatal Diagnosis.

Subtelomeric FISH analysis made its appearance in 1996 after the National Institute of Health and the Institute of Molecular Medicine reported the isolation and characterization of the first generation set of subtelomeric clones [9]. After the second generation of subtelomeric probes was out in 1999 [10] subtelomeric FISH started to be extensively used. The introduction of Multiprobe technique (Cytocell Limited) in 1999[11] was a breakthrough for laboratories back then, as that was the only approach at the time to test patients with idiopathic Mental Retardation. This technique could simultaneously analyze the telomeres of patients. In a large study, by Knight et al., it was shown that rearrangements in subtelomeric regions, which are very gene rich, have been associated with mental retardation. In this study it was indicated by the

results that subtle chromosomal abnormalities, involving the subtelomeres, occurred in 7.4% of the moderately to severely affected individuals and 0.5% of the mildly affected [11]. In this study the authors concluded that: "once recognizable syndromes have been excluded, abnormalities that include the ends of chromosomes are the commonest cause of mental retardation in children with undiagnosed moderate to severe mental retardation".

FISH methodology however has its limitations and these are: a) it is locus specific and therefore one locus per test can be examined (except for the application of Multiprobe subtelomeric FISH where all subtelomeres of a patient could be examined at one single reaction), b) it is a targeted method; it requires clinical suspicion that a specific locus in the genome has a deletion in order to test for it, c) it mainly detects deletions and may fail to detect duplications even with interphase FISH and d) it cannot detect Uniparental Disomy (UPD).

Furthermore, in prenatal diagnosis the only microdeletion which has specific symptoms and recognizable ultrasound findings during fetal life (conotruncal cardiac defect) is the 22q11.2 causing DiGeorge/ Velocardiofascial syndrome (VCFS) [12]. There are a large number of microdeletion and microduplication syndromes known today. Most of these however remain undetected until after birth due to the fact that they have non- specific or no symptoms during fetal life [13]. A diagnostic test such as array Comparative Genomic Hybridization (aCGH, see section 1.6.4) could be performed prenatally and detect these conditions prior to birth. In this way the prospective parents would be offered the option to terminate an affected pregnancy or to prepare for the birth of an affected child or to make the necessary arrangements should a condition detected needs immediate attention after birth.

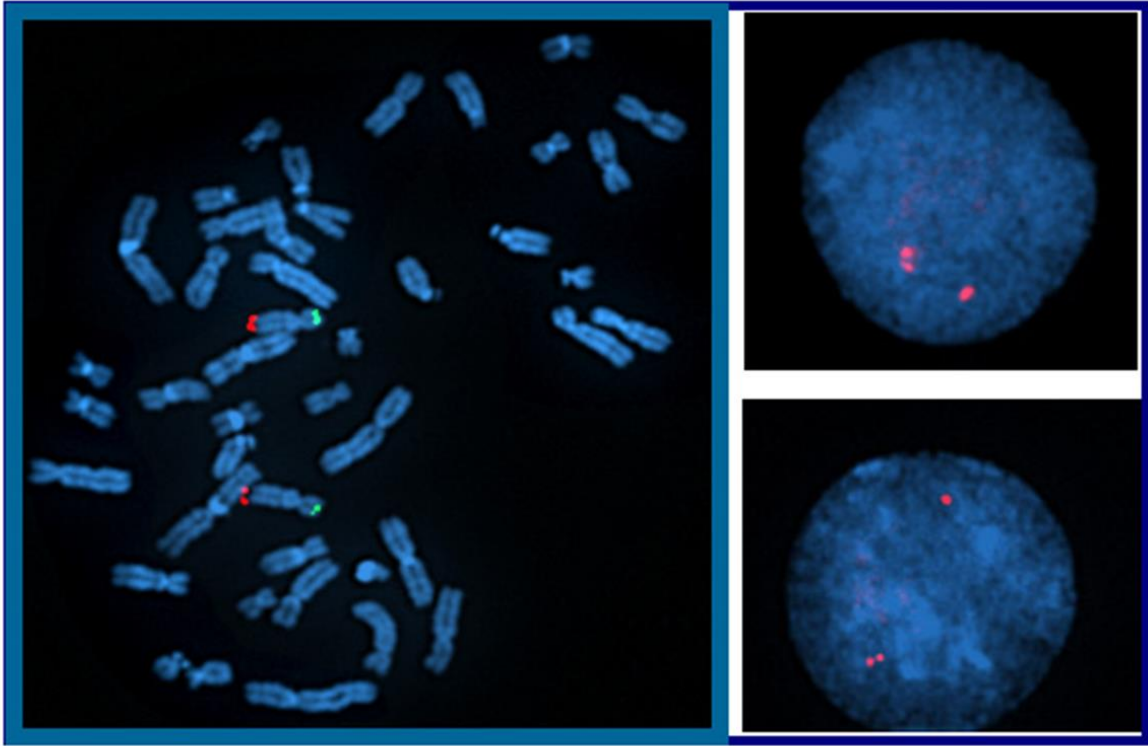


Figure 1.6.2: Subtelomeric FISH analysis on metaphase and interphase cells of a patient with a duplication of the long arm subtelomere for chromosome 10. Subtelomeric specific probes for the subtelomeres of chromosome 10 were used; labeled with FITC fluorophore (Green) is the short (p-arm) and labeled with TRITC fluorophore is the long (q- arm) of chromosome 10. The duplication in more clear on the interphase cells.

1.6.3) Metaphase CGH (mCGH)

Before array CGH was implemented into clinical practice metaphase Comparative genome hybridization mCGH was widely used since 1992 [14] especially in cancer cytogenetics. It was based on *in situ* hybridization of differentially labeled patient DNA and normal reference DNA to normal human chromosome spreads. Briefly, DNA is extracted from a control individual with a known, normal karyotype and a testing individual with an unknown karyotype. These two DNA samples are differentially labeled with two different fluorochromes and applied to metaphase spreads of a normal human. After hybridization the intensity ratio between the patient and normal fluorescence is measured and copy number variations in the patient DNA were detected. Metaphase CGH was widely used in cancer cytogenetics. This method has some limitations and these are: a) it can only detect unbalanced copy number changes and it cannot reveal any other abnormalities such as balanced

translocations or inversions, b) the resolution of mCGH is limited to that of the metaphase spreads and is therefore not higher than the one obtained from chromosomal analysis (3-10Mb) and c) there is a relative length of time of this assay which may be prohibitive for prenatal diagnosis [15].

An advancement of mCGH was High Resolution metaphase CGH (HR-mCGH) where it did not refer to a more stretched chromosome preparation, but rather to a further level of sophistication of the computer software that was used to analyze the images previously. With HR-mCGH small imbalances (3-5Mb) could now be identified [16]. In this study out of the 253 clinical cases examined 47 abnormalities were detected. Among 144 dysmorphic and mentally retarded subjects with normal karyotype, 15/144 (10%) had small deletions or duplications, of which 11/144 (7.6%) were interstitial. Among 25 dysmorphic individuals and mentally retarded individuals carrying apparently balanced translocations four had deletions at the translocation breakpoints and two had deletions elsewhere in the genome. In the same study 17 out of 19 complex rearrangements were clarified. These data shows the value of HR-mCGH at the time it was used.

Furthermore the authors add that the 7.6% detection rate for interstitial abnormalities are similar to the detection rate of subtelomeric FISH which was shown to be 7.5% in mentally retarded subjects previously studied by others. In conclusion, the present data suggest that chromosomal abnormalities may be detected in approximately 15% (7.5% + 7.6%) of mentally retarded and dysmorphic patients if both subtelomeric screening and HR-CGH are applied [16]. In the same study, HR-mCGH was also applied to de novo apparently balanced translocations detected prenatally. No abnormalities were detected in the six prenatal cases investigated in this survey. The authors estimate that when analyses of more such cases are performed, imbalances are bound to be found in some of them, but they will not appear as frequently as in the dysmorphic and mentally retarded patients with apparently

balanced karyotypes, since the majority of the prenatal cases are not expected to be associated with disease [16].

1.6.4) Microarray CGH or array CGH (aCGH)

In contrast to mCGH, array CGH permits a more detailed analysis with refined resolution even at the level of genes. The fundamental principle is the same as mCGH; it is a comparative genomic hybridization using array rather than a metaphase spread as a substrate (Figure 1.6.4). The microarray is comprised of thousands of spots of reference DNA sequences, applied in a precisely gridded manner on a slide. The resolution of the aCGH depends on how many spots of reference DNA exist on the slide. A slide with 3000 spots would have a resolution of 1 Mb across the entire genome. These spots could be Bacterial Artificial Chromosome (BAC) clones, Oligonucleotides, cDNA. Nowadays the resolution of aCGH has increased since there are microarrays that are spotted with up to two million oligonucleotides. Array CGH could detect gains and losses of very short genomic segments at multiple loci in a genome in a single assay, which gives it an advantage over conventional cytogenetics and FISH. Some of these gains and losses, Copy Number Variants (CNVs) found across the genome will be familial and they can be numerous and common. Some of these CNVs are of no clinical significance as they were seen in both phenotypically normal and abnormal individuals; others will have clinical significance and the remaining CNVs are of unclear significance. More cases with the same unclear significance CNVs need to be investigated in order to draw a conclusion whether they are benign or pathogenic. This is one problem in aCGH because in the analysis of one patient there may be a number of CNVs that will need further investigation before the final result could be reported. Further investigation would mean further testing of the patient and very often the parents with aCGH, FISH or other molecular methods. This in turn will mean longer turnaround times for the final result to reach the patient and higher

costs. In prenatal diagnosis longer turnaround times will mean longer periods of anxiety of the prospective parents. Array CGH has some limitations however and they include: a) it cannot reveal any balanced rearrangements such as balanced translocations or inversions, b) it cannot detect polyploidy, c) mosaicism below 20% cannot be detected and finally d) the method is unable to detect small or point-recessive mutations.

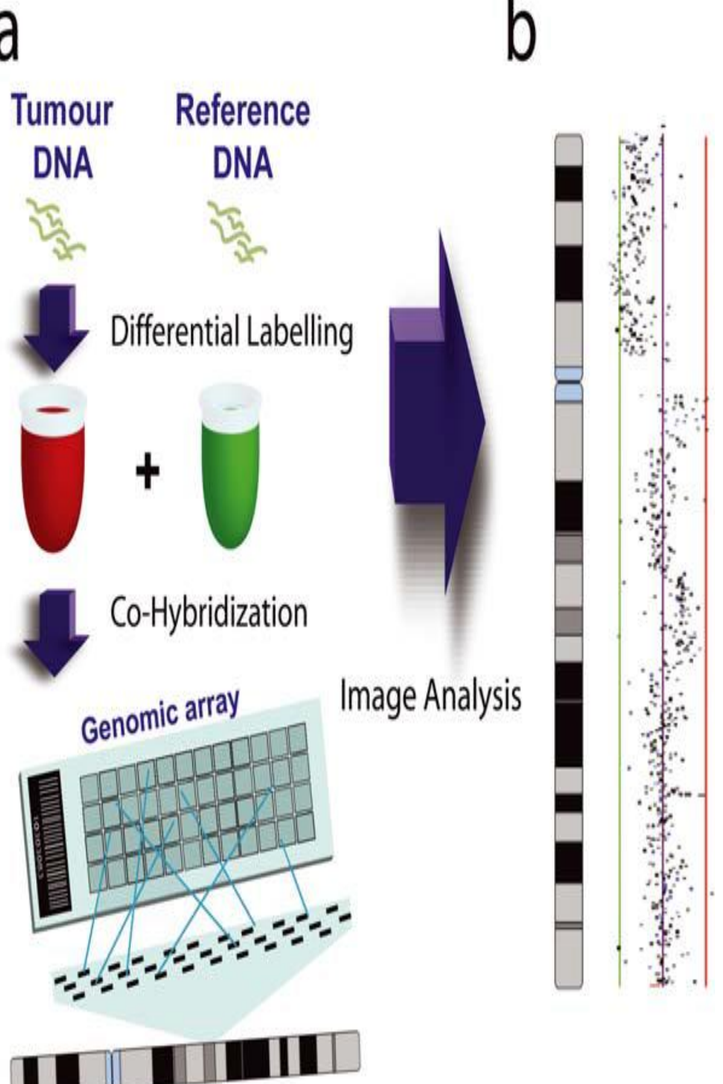


Figure 1.6.4: From [17] General principles of array comparative genomic hybridization. (a) Normal and patient, in this case DNA from a tumor of a patient, samples are isolated and used to create fluorescently labeled probes, commonly with cyanine 3 (Cy3; green) and cyanine 5 (Cy5;red) dyes. The probes are pooled and competitively co-hybridized to a glass slide spotted with a known array of mapped genomic clones. The arrays are analyzed with a microarray scanner, producing an image that is used to assess the log2 ratios of the Cy5 to Cy3 intensities for each clone. (b) A log2 ratio profile is assembled to determine relative copy number changes between the cancer and tumor samples. Each dot on the graph represents a clone. Values to the left of the '0' line indicate a loss of a genomic region, values to the right indicate a gain or amplification, and values at '0' indicate no change.

1.6.5) Quantitative Fluorescence Polymerase Chain Reaction (QF PCR)

QF PCR was first reported in the early 1990s. It is a rapid aneuploidy detection method for the common aneuploidies (chromosomes 13, 18, 21, X and Y) and is

usually carried out in conjunction with chromosomal analysis. In QF PCR highly polymorphic Short Tandem Repeats (STRs) for these chromosomes are amplified using fluorescence primers and PCR in a multiplex assay, followed by automated analysis of the fluorescence intensity of the alleles in a genetic analyzer [18]. These aneuploidies account for more than 80% of clinically significant chromosomal abnormalities diagnosed in the prenatal period [15]. The accuracy of QF PCR for these aneuploidies has been similar to that of interphase FISH. Advantages of QF PCR over FISH aneuploidy screening are that: 1) it is less expensive, 2) less labor intensive, 3) fast and 4) larger numbers of samples could be analyzed simultaneously by a single operator and has therefore replaced FISH in many laboratories. Another advantage of QF PCR is that it can identify the presence of maternal contamination in the processed samples. This could serve as a tool later on in the samples used for array CGH. This method has some limitations however and they include: a) mosaicism of less than 20-30% cannot be detected, b) it is designed to identify only the abnormalities that are specifically looked for, c) it cannot detect most structural abnormalities and d) there is a residual risk of chromosome aberration after QF PCR (or FISH) show a normal result; this risk was estimated to be 0.9% for all indications for invasive prenatal diagnosis and in 0.4% of all the invasive tests the chromosome aberration was of clinical significance [19].

1.6.6) Multiplex Ligation-dependent Probe Amplification (MLPA)

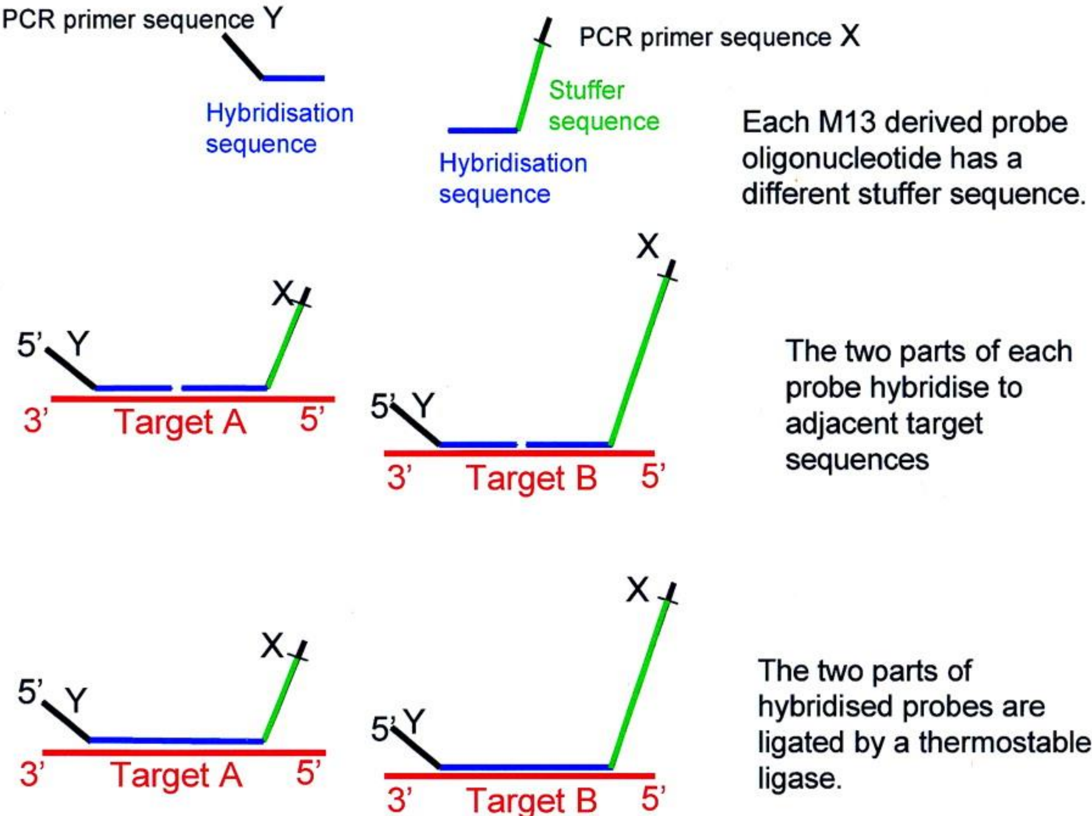
MLPA was first described in 2002 by Schouten et al. [20]. This method (Outlined in Figure 1.6.6) was initially designed to give relative quantification of 40 different DNA sequences in one reaction using only 20ng of human DNA. In MLPA, probes that are added to the samples are amplified and quantified. The amplification of the probes by PCR will depend on the presence of the target sequences in the sample examined. Each probe consists of two oligonucleotides, one synthetic and one M13 derived that hybridize to adjacent sites of the target sequence. The hybridized probe

oligonucleotides are ligated, permitting subsequent amplification. All ligated probes have identical end sequences, permitting simultaneous PCR amplification using only one primer pair. Each probe produces an amplification product of a unique size between 130 and 480 base pairs as one of the oligonucleotides contains a “stuffer” sequence of unique length for each probe, which by capillary electrophoresis allows separation and quantification of the single fragments to their length and fluorescence intensity. The relative quantity of each of the PCR products is proportional to the number of copies of the target sequence. Results are given as allele copy numbers as compared to a normal control. A ratio of 1 denotes normal allele pattern, a ratio of 0.5 shows an absence of an allele and a ratio of 1.5 is given when an allele is duplicated. Currently many MLPA kits for the detection of different conditions/disorders are commercially available by MRC Holland.

One advantage of MLPA over QF PCR is that it doesn’t have the problem with the non-informative polymorphic markers which often appears in QF PCR analysis. In addition MLPA can detect uniparental disomy. It has some limitations however and these are: a) it does not detect structural aberrations, b) it does not detect all types of triploidies (69,XXX) and c) maternal cell contamination will not be apparent from the analysis.

MLPA, Multiplex Ligation-dependent Probe Amplification

- Denatured genomic DNA is hybridized with a mixture of 40 probes.
- Each MLPA probe consists of two oligonucleotides, one synthetic and one M13-derived.



All probe ligation products are amplified by PCR using only one primer pair.

Amplification products are separated by electrophoresis. Relative amounts of probe amplification products reflect the relative copy number of target sequences.

Figure 1.6.6: Outline of MLPA reaction [20]

1.7) Abnormalities detected in prenatal diagnosis

1.7.1) Numerical Abnormalities

The most frequent abnormalities in prenatal diagnosis are numerical when instead of the normal two copies of chromosomes there are 3 copies (trisomy) or only one copy (monosomy). Four copies or five copies of chromosomes can exist at times but this mostly involves the sex chromosomes. Most numerical abnormalities are non-viable; viable autosomal monosomies are extremely rare and certain autosomal trisomies that can be viable are very frequently lost during pregnancy. Sex -chromosome aneuploidies are in general the ones that are mostly more tolerated. Approximately 30% of affected fetuses of gestational age between 12 weeks and term will miscarry. In addition the estimated rate of lethality between 16 weeks and term is 20% [1]. It is very rare for any other autosomal trisomy than 13, 18 and 21 (Also refer to Table 1.6.1), to survive through or even near to term with an exception of mosaic or partial trisomies, for chromosomes 8, 9 and 12. Table 1.7.1 shows the association of certain numerical abnormalities, encountered prenatally, with sonographic markers and maternal serum biochemistry.

1.7.1.1) Trisomy 21, Down Syndrome

Down syndrome (trisomy 21) is the most commonly recognized genetic cause of mental retardation and it appears in 1 out of 650 births. It is named after John Langdon Down, a British physician who described the syndrome in 1866. Trisomy 21, the presence of one extra chromosome 21, is present in 95% of persons with Down syndrome. Mosaicism, a mixture of normal diploid and trisomy 21 cells, occurs in 2%. The remaining 3% have a Robertsonian translocation in which all or part of an extra chromosome 21 is fused with another acrocentric chromosome. Most chromosome- 21 translocations are sporadic (75%), however, some are inherited from a parent who carries the translocation balanced by a chromosome

deletion (25%) [21]. Molecular genetic studies reveal that 95% of occurrences of trisomy 21 result from nondisjunction during meiotic division of the primary oocyte. Most trisomy 21 pregnancies prove to be nonviable. Only 25% of fetuses with trisomy 21 survive to term. The exact mechanism for this meiotic error remains unknown but it is related to maternal age. The risk of having a child with Down syndrome increases in a gradual, linear fashion until about age 30 and increases exponentially thereafter. The risk of having a child with Down syndrome is 1/1,300 for a 25-year-old woman; at age 35, the risk increases to 1/365. At age 45, the risk of a having a child with Down syndrome increases to 1/30. Characteristics of a Down syndrome child include : Flat facial profile (90% frequency), poor moro reflex (85%), hypotonia (80%, hyperflexibility of large joints (80%), loose skin on back of neck (80%), slanted palpebral fissures (80%), dysmorphic pelvis on radiographs (70%), small round ears (60%), hypoplasia of small finger, middle phalanx (60%), single palmar crease(45%).

Down syndrome persons usually have mild to moderate mental retardation (some can be severe), school-aged children often have difficulty with language/communication/ problem-solving skills, adults with Down syndrome have a high prevalence of early Alzheimer's disease, further impairing cognitive function, a number of congenital malformations and acquired diseases occur with increased frequency in persons with Down syndrome. Congenital heart disease and pneumonia are leading causes of mortality, especially in early childhood [22]. Prenatally the diagnosis for Trisomy 21 can be suspected at 11-14 weeks when increased Nuchal Translucency combined with maternal age, serum biochemistry and presence/absence of the nasal bone give a high risk for Down syndrome. If all these are combined together a detection rate of up to 95% can be achieved. [2]

Trisomy 21 can be diagnosed by conventional cytogenetics, QF PCR (98% of the cases), MLPA or aCGH methods.

Table 1.7.1: Association of chromosome abnormalities with sonographic markers and maternal serum biochemistry at the 11-14 week gestational age scan according to Cicero et al. 2003

Abnormality	βHCG (2MoM)	PAPP- A (0,5 MoM)	Sonographic markers
Trisomy 21	Increased	Decreased	Increased NT, absent nasal bone in60-70% of trisomies
Trisomy 18	Decreased	Decreased	Early onset IUGR, relative bradycardia, associated exomphalos in30% of the cases
Trisomy 13	Decreased	Decreased	Fetal tachycardia in 60% of cases, early onset IUGR, holoprocencephaly or exomphalos in 30% of cases
Turner syndrome	Normal	Lower	Fetal tachycardia in 50% of cases, early onset IUGR
Triploidy- diandric	Greatly increased	Mildly decreased	Early onset asymmetrical IUGR, relative bradycardia, holoprocencephaly, exomphalos or posterior fossa cyst in 40% of cases,, molar changes in the placenta in 30 % of cases
Triploidy- digynic	Markedly decreased	Markedly decreased	Early onset asymmetrical IUGR, relative bradycardia, holoprocencephaly, exomphalos or posterior fossa cyst in 40% of cases,, molar changes in the placenta in 30 % of cases

1.7.1.2) Trisomy 13, Patau Syndrome

Trisomy 13 or Patau syndrome is a rare genetic disorder in which a person has three copies of genetic material from chromosome 13, instead of the usual two copies. Rarely, the extra chromosome 13 could be attached to another chromosome (translocation). Trisomy 13 could also occur in a mosaic state. It was first observed by Thomas Bartholin in 1657, but the chromosomal nature of the disease was ascertained by Dr.Klaus Patau in 1960. The disease is named in his honor. Trisomy 13 occurs in about 1 out of every 10,000 new-borns. More than 80% of children with Patau syndrome die within the first year of life.

The symptoms that appear in Patau Syndrome include: clenched hands (with outer fingers on top of the inner fingers), close-set eyes -- eyes may actually fuse together into one, decreased muscle tone, polydactyly, hernias, hole/ split/ or cleft in the iris (coloboma), low-set ears, severe mental retardation, scalp defects (missing skin),

seizures, single palmar crease, skeletal (limb) abnormalities, small eyes, microcephaly, micrognathia, cryptorchidism.

Complications begin almost immediately after birth. They may include: breathing difficulty or lack of breathing (apnea), deafness, feeding problems, heart failure, seizures, and vision problems. Most infants with trisomy 13 have congenital heart disease.

Treatment could be offered but varies from child to child and depends on the specific symptoms.

Trisomy 13 can be diagnosed by conventional cytogenetics, QF PCR (98% of the cases), MLPA or aCGH methods.

1.7.1.3) Trisomy 18, Edwards Syndrome

Trisomy 18 is a genetic disorder in which a person has a third copy of material from chromosome 18, instead of the usual two copies, either full or partial and even in mosaic state. It is named after John H. Edwards, who first described the syndrome in 1960. It is the second most common autosomal trisomy, after Down syndrome that carries to term. Edwards syndrome occurs in around 1 in 6,000 live births and around 80% of those affected are female. The syndrome has a very low rate of survival, resulting from heart abnormalities, kidney malformations, and other internal organ disorders. Half of infants with this condition do not survive beyond the first week of life. Some children have survived to the teenage years, but with serious medical and developmental problems. The incidence increases as the mother's age increases.

The symptoms that appear in Edwards Syndrome are: clenched hands, crossed legs (preferred position), feet with a rounded bottom (rocker-bottom feet), low-set ears, mental deficiency, small head (microcephaly), small jaw (micrognathia), underdeveloped fingernails, undescended testicles, unusual shaped chest (pectus carinatum).

Treatment of children with Trisomy 18 is planned on a case-by-case basis depending on the patient's individual condition.

Trisomy 18 can be diagnosed by conventional cytogenetics, QF PCR (98% of the cases), MLPA or aCGH methods.

1.7.1.4) Sex Chromosome abnormalities

Sex chromosome abnormalities have an overall incidence of 1 in 250-300 at prenatal diagnosis [23]. The most common conditions are XXY, XXX, XYY and 45,X. Two of these conditions are further discussed below. Sex chromosome abnormalities can be diagnosed by conventional cytogenetics, QF PCR (98% of the cases), MLPA or aCGH methods.

1.7.1.4.1) Monosomy X, Turner Syndrome

Turner syndrome is caused by the absence of all or part of one copy of the X chromosome. The condition only occurs in females. Turner's syndrome is named after Henry Turner. Most commonly, the female patient has only one X chromosome. Others may have two X chromosomes, but one of them is incomplete. Both of these states can occur in a mosaic form. In 75% of the cases it is the paternal X chromosome that is absent and it is mostly a meiotic error. Turner syndrome occurs in 1 to 5000 births. Postnatally the diagnosis is prompted by the characteristic clinical findings: short stature, swelling, broad chest, low hairline, low- set ears, webbed neck, gonadal dysfunction, concurrent health concerns (congenital heart disease, hypothyroidism, diabetes, vision or hearing concerns, and many autoimmune diseases), cognitive deficits is often observed, with particular difficulties in visuo-spatial, mathematical and memory areas.

There is no treatment for Turner syndrome. Administration of growth hormone, either alone or with a low dose of androgen, will increase growth. In addition Estrogen

replacement therapy can be used to promote development of secondary sexual characteristics.

Prenatally the diagnosis is a bit of a more complex process which is driven by abnormal ultrasound findings or discovered in the results of a routine prenatal chromosomal study carried out for other reasons (i.e. advanced maternal age, abnormal maternal serum screening). In a study carried out by Papp et al.[24] it was shown that only 68.1% of the fetuses with Turner syndrome showed symptoms on sonography. Ultrasound findings, in the 16-23 weeks scan, that could indicate the presence of a Turner Syndrome fetus include nuchal cystic hygroma, hydrops, aortic arch hypoplasia, short femurs and renal anomalies[24,25]. Monosomy X has a very high in utero lethality with 75% abortion of 45,X cases detected following amniocentesis[26]. The lethal type according to Cicero et al., presents with high large nuchal cystic hygromata, generalized edema, mild pleural effusion and ascites, cardiac anomalies and horseshoe kidney[2].

1.7.1.4.2) Klinefelter Syndrome 47, XXY

Klinefelter syndrome is caused by the presence of an additional X chromosome in a male person. The syndrome was named after Dr Harry Klinefelter, who, in 1942, worked with Fuller Albright at Massachusetts General Hospital in Boston, Massachusetts and first described it in the same year. The syndrome exists in roughly between 1 to 500 and 1 to 1000 live male births. Many of these people may not show symptoms. The physical traits of the syndrome become more apparent after the onset of puberty, if at all. They include: androgen deficiency, small testes, gynecomastia, IQ is diminished by 10-15 points, learning difficulties at school are expected.

This genetic variation is irreversible; however, testosterone treatment is an option for some individuals who desire a more masculine appearance and identity. In addition if gynecomastia exists it can be treated surgically.

Klinefelter syndrome doesn't have any particular characteristics to attract the attention of the ultrasonographer when performing the regular scans in pregnancy. It is rather discovered by chance when performing prenatal diagnosis for other reasons (i.e. Advanced Maternal Age).

1.7.1.5) Presence of supernumerary marker chromosome or ring chromosome

The presence of an additional marker chromosome or ring chromosome could cause imbalance in a patient. A marker chromosome is a segment of genetic material, usually small- less than G chromosome size- that cannot be identified by standard cytogenetics and additional studies have to be carried out in order to determine its origin and its clinical significance. FISH and aCGH prove to be valuable tools for the investigation of such findings.

A ring chromosome could exist as additional material in the karyotype or it could replace a chromosome. When it exists as an additional material it could be either small or large or it could be present in a mosaic form with two cell lines; one with a small and one with a larger ring chromosome. Most of the times the origin of the ring chromosome could be speculated, but confirmation with other methods is necessary (FISH, aCGH).

1.7.1.6) Polyploidy

Cells with 69 or 92 chromosomes are referred to as triploid or tetraploid respectively. Triploidy is not consistent with life and it is not uncommon in early pregnancies (1%-3%), but about 99.9% are lost during the 10- to 20 week gestational age. Very rarely triploidy can occur in a mosaic state with normal cells. In triploidy there is a double chromosomal contribution to the conceptus from one parent (diandry or digyny if the

double contribution comes from the father or the mother respectively). Diandry is usually caused by the fertilization of an oocyte with two sperms; very rarely it is caused by the fertilization of a diploid sperm. Digyny is most commonly caused with the fertilization of a diploid egg by a haploid sperm.

Tetraploidy in a term pregnancy is very rare and the usual mechanism could be normal division of chromosomes but failure of the cytoplasmic cleavage at the first division of the zygote. The other possibility is the fertilization of an oocyte in which Meiosis I has failed, by two sperms. Tetraploidy could also be a cultural artefact.

Polyploidy can be diagnosed by conventional cytogenetics, QF PCR (98% of the cases), but not with aCGH.

1.7.2) Structural Abnormalities

Structural aberrations are the result of chromosomal breaks that occur during meiosis. They can affect one or two chromosomes and they can be balanced or unbalanced.

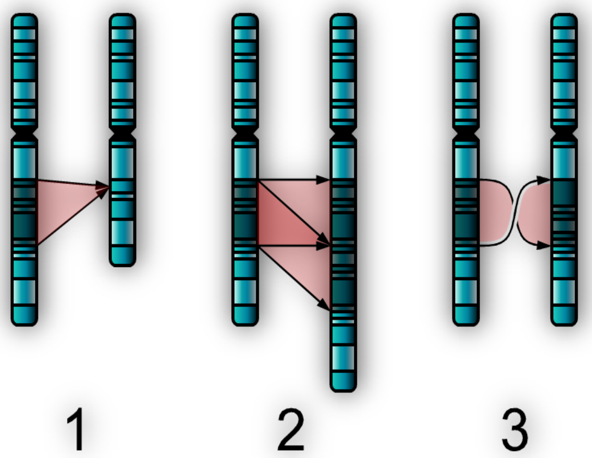


Figure 1.7.2.1: Structural rearrangements affecting a single chromosome; deletion (1), duplication (2) and inversion (3).

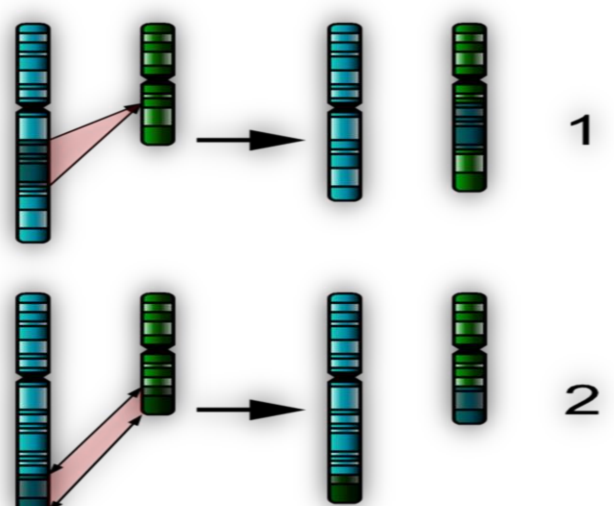


Figure 1.7.2.2: Structural Rearrangements affecting two chromosomes; insertion (1) and Translocation (2)

1.7.2.1) Balanced rearrangements

1.7.2.1.1) Translocations

Balanced reciprocal rearrangements are produced by the interchange of parts of two chromosomes without visible loss of chromosomes material (Figure 1.7.2.2, Number 2). When the translocation involves an acrocentric chromosome it is called Robertsonian. The great majority of apparently balanced translocations are usually not associated with abnormal phenotypes. In the normal population 1 in1000 people carry a balanced rearrangement. There is a risk of phenotypic abnormalities, however, in 6.1% of *de* novo apparently balanced translocation carriers [27]. In conventional cytogenetics a translocation may seem apparently balanced, but studies have shown that this is not always true. Even if a translocation is confirmed by FISH analysis using subtelomeric specific and whole chromosome paints, it may not always be truly balanced. Sismani et al. [28] demonstrated by aCGH that 3 out of 12 (25 %) postnatal balanced translocation cases, both familial and *de novo*, with abnormal phenotype, carried cryptic imbalances which could not be identified by classical cytogenetics and FISH analyses. In two of these cases the imbalance was near the translocation breakpoints and the third case had an aberration on another

chromosome unrelated to the translocation. In a prenatal study of 25 fetuses with normal or balanced karyotype and abnormal ultrasound findings we showed that the use of aCGH revealed copy number changes in 3 out 25 cases (12%,) two of which (8%) were considered clinically significant; one of these cases was an "apparently" balanced translocation case and the deletion was located at the translocation breakpoint [29].

These results highlight the need for using aCGH in diagnosis. It needs to be stressed out though that truly balanced translocations cannot be diagnosed with the use of aCGH.

1.7.2.1.2) Inversions

An inversion occurs when there are two breaks on a single chromosome and a 180 degree rotation of the section between the breaks (Figure 1.7.2.1 Number 3).The breaks could either take place on the same arm (Paracentric inversions) or on different arms (Pericentric Inversions). Inversions can be identified by conventional cytogenetics, confirmed by FISH most of the times. They cannot be detected by aCGH.

1.7.2.1.3) Insertions

An insertion occurs when there is loss of chromosomal material from one chromosome and inserted (inverted or in the same direction) at a different point of the same chromosome or on another chromosome (Figure 1.7.2.2, Number 1). This is an apparently balanced rearrangement and it can be detected by classical cytogenetics but not by aCGH. Array CGH would be proven useful in this type of aberration if the insertion was not truly balanced. Array CGH would be the only way to detect if there were any cryptic copy number changes.

1.7.2.2) Unbalanced rearrangements

1.7.2.2.1) Deletions

Deletions occur when there is loss of genetic material (Figure 1.7.2.1, Number 1). It could be terminal or interstitial and it could involve cytogenetically visible segments or segments that need the use of other techniques to be seen. Very small interstitial deletions are called microdeletions and some of those have been associated with particular disorders (DiGeorge/ Velocardiofacial syndrome, Smith Magenis, Miller-Dieker etc.). If a clinician suspects that a patient has one of these syndromes he/she could directly ask to rule out that syndrome by requesting FISH analysis with specific probes for that particular disorder; for example the 22q11.2 deletion syndrome. These deletions are at the limits of the optical resolution of extended chromosomes and might be missed by classical cytogenetics. Terminal deletions when suspected at classical cytogenetics analysis could be confirmed by subtelomeric FISH analysis. Otherwise small terminal deletions that escape chromosomal analysis will remain unidentified. Finally interstitial deletions that exist on other locations than the ones that exhibit the microdeletion syndromes, if they are really small (<3-5 Mb) they could again remain undetected by cytogenetic analysis. This is the main advantage of aCGH; a single assay that can detect all possible abnormalities present in a patient that cannot be identified by classical cytogenetics. Follow up is of course necessary with such findings as they need to be confirmed in the patient and the parents to determine whether the deletion is *de novo* or familial, so that the recurrence risk can be estimated; also to determine the clinical significance of the finding. Bateman et al. and Filges et al. report on cytogenetically visible interstitial deletions one *de novo* and one familial respectively of no phenotypic effect [30,31]. In the *de novo* case the patient had a 9.3Mb-10.7Mb deletion; she was of normal intelligence, had no dysmorphic features and had experienced 3 miscarriages. In the familial case there was a 14.5Mb deletion in three generations with no relevant phenotypic effect. Follow up is

important and it can be usually carried out with the application of aCGH in the parents and/or FISH analysis in the patient and the parents.

1.7.2.2.2) Duplications

Duplications occur when a section of genetic material is duplicated. Duplications can be seen cytogenetically and they can be identified as the duplicated material appears next to itself in the same or in an inverted orientation (Figure 1.7.2.1 Number 2). The exact location of duplications identified by array CGH cannot be known. The use of FISH would be needed in order to determine the physical location of the duplicated segment[32]. This is very important in diagnosis especially for *de novo* duplications in affected children where the duplication could mean the presence of an insertional translocation in one of the parents. The clinical significance of this finding lays with the determination of the recurrence risk in future pregnancies.

1.7.3) Uniparental Disomy (UPD)

Uniparental Disomy (UPD) is the presence of a chromosome pair derived from one parent in a disomic cell line [33]. Twenty years ago it was thought that UPD would be a rare event, but today there are more than 1,100 UPD clinical cases in the literature [34]. UPD is considered an important diagnostic [35] and prognostic factor for special syndromes [36,37]. According to Gardner and Sutherland [21] there are three types of UPD:

- For the entire chromosomal complement either maternal or paternal, leading to benign cystic ovary and complete hydatidiform mole respectively
- For a complete chromosome
- Segmental UPD

Furthermore, there are two subtypes of UPD:

- Heterodisomy (hUPD); meaning the inheritance of both chromosome from one parent
- Isodisomy (iUPD); meaning the inheritance of two copies of the same chromosome from one parent.

hUPD and iUPD can both cause a disease if they are affecting a gene underlying genomic imprinting or in iUPD it can cause a recessive disease in the offspring of a carrier parent . UPD has not been reported for all chromosomes. No maternal UPD for chromosomes 19 and Y and no paternal UPD for chromosomes 4, 17, 18 and 19, have been reported [33]. Examples of imprinting disorders regarded and registered in the database Online Mendelian Inheritance of Man [38] are:

- PatUPD(6) causing transient neonatal diabetes (OMIM #601410)
- MatUPD(7) causing Silver Russel syndrome (OMIM # 180860)
- PatUPD(11) causing Beckwith-Wiedemann Syndrome (OMIM #130650)
- MatUPD (14) causing Temple Syndrome (OMIM #605636, #176270)
- PatUPD (14) causing the paternal UPD (14) syndrome (OMIM #608149)
- MatUPD (15) causing Prader Willi syndrome (OMIM #176270)
- PatUPD (15) causing Angelman syndrome (OMIM #105830)

The frequency of UPD in new-borns is considered to be about 1 in 3,500 births- a rate of 0.029% [39]. In one third of the cases where UPD is identified it is in connection with a chromosomal abnormality. This stresses the need for chromosomal analysis to be carried out especially in the presence of UPD for one of the acrocentric chromosome 13, 14, 15, 21 and 22. It is known that the presence of a Robertsonian translocation contributes to the formation of UPD; over than 10% of the acrocentric chromosome derived UPDs summarized in the Liehr's database have a Robertsonian translocation [34].

Furthermore in 8% of published UPDs an abnormal balanced karyotype is reported having the presence of isochromosomes, inversions, balanced translocations and Robertsonian translocations. In 16% of reported UPD cases there is a connection to an unbalanced karyotype; the presence of a Small Supernumerary Marker chromosome for example. Finally 11% of all known UPD are of the segmental type. This type of UPD arises due to postzygotic somatic recombination between maternal and paternal homologues or with numerical and/or structural abnormalities (partial trisomies for example) [21].

UPD could be detected with MLPA and SNP arrays.

1.8) A new era in Cytogenetics with the use of array CGH

This revolutionary technology was first developed as a research tool for the investigation of genomic alterations in cancer. As previously mentioned aCGH compares DNA content from two differentially labeled genomes, a test/patient and a reference/control. After labeling, these two genomes are co-hybridized onto to a solid support, usually a glass microscope slide, on which cloned or synthesized DNA fragments are immobilized.

Arrays have been developed in a variety of designs over the years. They have been constructed to span the whole genome and they use various-sized targets from synthetically synthesized oligonucleotides to Bacterial Artificial Chromosome (BAC). Each BAC or oligonucleotide has a known position within the human genome. One of the first arrays used, was the 1 Mb BAC array with 1Mb backbone with additional BACs at regions known to be involved in the major human genetic disorders (Cytochip, BlueGnome 2006). Whole genome array also included the Tiling path BAC arrays consisting of 26,574 clones and covering 93.7% of euchromatic regions introduced by Fiegler et al. [40]. In addition to the whole genome arrays targeted arrays also exist for a specific region of the genome. It could be targeted to study a specific chromosome[41], or chromosome segment [42] or to detect and identify specific DNA

dosage abnormalities in individuals with suspected microdeletion syndromes [43] or subtelomeric rearrangements [44]. The resolution of the arrays is defined by 1) the size of the nucleic acid target on the array and 2) the density of the coverage over the genome. The smaller the nucleic acid sequence and the more contiguous the targets the higher the resolution of the array will be.

The arrays have been designed to provide redundancy with high sensitivity and specificity for the detection of clinically significant genomic imbalances.

In addition to BAC and oligonucleotide arrays Single Nucleotide Polymorphism (SNP) arrays are also available by several manufacturers.

The resolution of aCGH has the potential of being much higher than that of conventional cytogenetics because the resolution is determined by the size of the target arrayed on the solid platform and the coverage or density of those targets.

Array CGH has the ability, as mentioned previously, to investigate simultaneously thousands, or even more, loci on a single assay. This is an advantage of aCGH over classical cytogenetics and FISH. However, as Shaffer and Bejjani very well discuss, microarray analysis is not a stand-alone test in the diagnostic laboratory as other methodologies are needed to be carried out in order to be able to determine the chromosome rearrangement that occurred in the discovery of a copy number change[45]. This is extremely important, in the discovery of an aberration in a child, in order to be able to offer parents counseling for the condition of their child as well as recurrence risk.

In addition, aCGH analysis has revealed that many familial DNA gains or losses across the genome are abundant [46]. Some of these Copy Number Variants (CNVs) are of no clinical significance as they have been seen in both phenotypically normal and abnormal individuals. Others are believed to have clinical significance. And finally a number of CNVs cannot be classified as more cases with the same genomic

imbalances need to be identified and evaluated in order to categorize them as being causative or not. The discovery of CNVs in a diagnostic setting creates problems to the analyzers as they need to go through publicly available or in house databases in order to determine whether the CNVs found have any clinical significance.

The main limitations of aCGH are its inability to detect balanced rearrangements and low mosaicism. This is a drawback for is implementation in prenatal diagnosis.

1.9) Array CGH and prenatal Diagnosis

Array CGH is increasingly performed for the evaluation of individuals with birth defects, dysmorphic features and mental retardation. Genome-wide arrays are rapidly replacing conventional karyotyping in postnatal diagnostics and some studies suggest that it should be used as a First-Tier clinical diagnostic test for individuals with developmental delay [47]. Its introduction however in prenatal diagnosis is still limited but will definitely increase in the near future.

At the beginning of this study, 5 years ago, the work being done on prenatal diagnosis was extremely limited. Since then a number of groups has worked with the application of aCGH in prenatal diagnosis and have proven its usefulness, as well as its limitations, in using this technique in prenatal diagnosis. The question remains though as to whether it can be fully integrated in prenatal diagnosis, solely or in conjunction with other assays and replace conventional cytogenetics.

The main points that need to be thought about before implementing aCGH in prenatal diagnosis are:

- For which pregnancies aCGH should be carried out. Whether it will be for all pregnancies or for pregnancies with ultrasound abnormalities.
- Which array platform to use
- The need to set the appropriate calling criteria
- Which methods will be used to confirm aCGH findings

- Pre-test counselling

The last point is especially important in the prenatal setting. Pre-test counseling should be carried out to inform parents of the possibility of the fortuitous discovery of a CNV unrelated to the phenotype during array CGH analysis. It should be explained to the parents that there may be asymptomatic/pre-symptomatic results with aCGH analysis and they should be free to decide whether they wish to be informed of these findings or not [48].

1.10) Challenges

The major challenges faced in the routine diagnostic clinical setting after the implementation of the aCGH methodology involve primarily the interpretation of the results, confirmations, incidental findings, polymorphisms, incomplete penetrance and variable expression of certain copy number changes, intra-familial variability, mosaicism, availability of parental samples and genetic counseling.

Copy number variants (CNVs) are unexpectedly common in the human genome and many are without apparent clinical consequence [49]. Classifying a copy number change as pathogenic or benign is not always a straight forward answer. Careful assessment of the available databases of pathogenic and benign CNVs (e.g. DGV, DECIPHER, UCSC), analysis of parental samples to determine whether the CNV is a de novo or inherited is required. Phenotypic variability and incomplete penetrance of certain copy number changes inherited from apparently unaffected parents must always be considered when interpreting array data. Very often a CNV is of questionable clinical significance and categorization is not possible. This is one of the reasons that the use of aCGH in prenatal diagnosis is limited. Even though aCGH has distinct advantages over conventional cytogenetics, as mentioned previously, this technology cannot currently replace classic cytogenetics in prenatal diagnosis.

One challenge for the future will be to perform non-invasive aCGH on free fetal DNA isolated from maternal circulation. It was previously demonstrated that aCGH can be

performed on very small amounts of DNA with or without whole genome amplification[50], so it may enhance the potential for success of prenatal diagnosis from noninvasively sampled fetal DNA, either as cell-free DNA from maternal plasma or blood [51] or as fetal cells from the cervix [52].

1.11) Aim of the study

Initially the purpose of the study was the application of high-resolution microarrays in prenatal diagnosis for the detection of cryptic microduplications and microdeletions in fetuses with ultrasound abnormalities and normal karyotype. The study was further expanded to not only include fetuses with normal karyotypes and ultrasound findings, but fetuses with balanced or unbalanced rearrangements with or without ultrasound findings as well. This research aims to provide new scientific knowledge and benefits in fetal medicine and genetics. Some of the benefits that will arise from this research will be the ability to apply higher diagnostic resolution in prenatal diagnosis with, more accurate and detailed genetic counselling, and the association of abnormal ultrasound findings with genetic abnormalities and/or the description of new syndromes. Finally with this research we will aim to contribute to the introduction of molecular karyotype with the use of high-resolution microarrays in prenatal diagnosis.

2) Materials and Methods

2.1) Patients and Samples

A total of 202 patients and 64 parental samples were analyzed. Samples were however divided into three groups based on the different analysis procedures used.

- **Group 1**: prenatal samples which were received for prenatal diagnosis using G-banded karyotype and whole-genome array CGH methodology (See Table 3.1.1 in Chapter 3)
- **Group 2**: prenatal samples from terminated pregnancies with normal karyotype and ultrasound findings (See Table 3.1.2 in Chapter 3)
- **Group 3**: Products of Conception (POC) or Skin Biopsies (SB) from aborted fetuses/Intrauterine death/Stillbirth, samples which were initially received for chromosomal analysis. Tissue cultures for these POC samples failed to be established and therefore chromosomal analysis was not performed. They were analyzed using alternative methods. Several of these samples would have been referred for prenatal diagnosis if the miscarriage hadn't preceded. (See Table 3.1.3 in Chapter 3)

Groups 1 and 2 consisted of prenatal samples and their respective parental samples wherever available. Group 1 included 95 samples and 50 parental samples and Group 2 included 34 and 14 parental samples. The study also comprised of 73 POC samples which form Group 3.

2.2) Clinical Data

Ultrasound screening was carried out during the first trimester of pregnancies for all Group 1 and Group 2 CVS and AF samples received. The ultrasound findings for Groups 1 and 2 are detailed in section 3.2 in Chapter 3. Clinical follow up of newborns was done postnatally were possible. No fetopsies were performed on any

of the first and second and trimester fetuses. For Group 3 samples the reason for referral is listed in table 3.1.5 in chapter 3.

2.3) Conventional Cytogenetics and FISH analyses

Conventional G banding was carried out on fibroblast [Amniotic Fluid (AF), Chorionic Villi biopsies (CV), Skin biopsy (SB)] or fetal lymphocyte cultures from of all prenatal cases included in the study at the 550 band level, using standard cytogenetic methodologies [53]. Fluorescence In Situ Hybridization (FISH) was performed using commercially available probes according to the manufacturer's recommendation (VYSIS. Inc., Cytocell Co.).

2.4) DNA Isolation

DNA was extracted from CV/AF/POC (Chorionic Villi or Skin for POCs) samples, as well as from uncultured peripheral/fetal blood using the QIAGEN Mini and Midi kit, respectively following the manufacturer's recommendations (QIAGEN. Co.). Concentration and purity of the extracted DNA was measured with the NanoDrop spectrophotometer (NanoDrop Technologies, Inc.).

As the maximum absorbance for nucleic acids and proteins reaches 260 and 280nm respectively, the ratio of absorbances at these wavelengths is used to measure the purity in both nucleic acid and protein extractions. A ratio of ~1.8 is considered "pure" for DNA and a ratio of ~ 2.0 is accepted as pure for RNA. Absorbance at 230nm is accepted as being the result of other contamination. For this reason the ratio of A260/230 is being calculated. The 260/230 values for pure nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values, which will determine the purity of the DNA, are in the range of 1.8- 2.2; the values for 260/280 should be around 1.8. If these values fall outside the accepted ratios the DNA cannot be used for further testing.

2.5) Molecular Methods

2.5.1) Quantitative Fluorescent Polymerase Chain Reaction (QF PCR)

QF PCR was carried out [54] for all prenatal cases before chromosomal analysis was completed. For POC samples, for which tissue culture failed to grow and no chromosomal analysis would be possible QF PCR analysis was carried out following DNA extraction from the available sample. In QF PCR, 27 highly polymorphic short tandem repeats (STRs) on chromosomes 13, 18, 21, X and Y were amplified using fluorescent primers and PCR in an in-house multiplex assay (Tables 2.5.1a and 2.5.1b). STRs were divided into two mixes. In addition for sex determination a non-polymorphic marker was used which is part of the amylogenin gene (AMEL). This specific primer produces a 103bp fragment in females and two fragments of 103bp and 111bp in males. In addition TAF9L is used to rule out Turner Syndrome. This specific primer produces a 142bp fragment located at 3p24 and a 144bp fragment located at Xq13. Automated analysis of the fluorescence intensity of the alleles was followed with the aid of a genetic analyzer [15]. In order to further exclude the common aneuploidies (chromosome 15, 16, and 22) which are very often encountered in first and second trimester miscarriage the commercially available Devyser Extend M1 kit (Devyser AB.) was used only for the POC samples (Table 2.5.2). Maternal contamination was also excluded based on the absence of a second genotype in the QF PCR analysis.

Quantification was performed using peak heights (Figure 2.5.1). Every marker was distinguished by the size in base pairs, which were present on the electropherogram as peak heights. Normal loci have peak ratios 1:1 (1-1.4). However, for alleles which were separated by more than 24 bp a ratio up to 1.5 is acceptable. Trisomic loci have peak ratios 2:1 (<1.8) or 1:1:1 and non-informative loci have peak ratios 1.4-1.8 (Figure 2.5.1). Results were considered informative for a specific chromosome when at least two of the chromosomal markers from both mixes gave conclusive results.

TABLE 2.5.1a: Primers for QF PCR, their chromosome position and PCR product size in base pairs. First mix of probes out of two (Mix A)

MIX A	PCR product Size(bp)	Chromosome position	Sequence Forward	Sequence Reverse
D21S1435	163-196	21q21.3	PET-CCCTCTCAATTGTTTGTCTACC	ACAAAAGGAAAGCAAGAGATTTCA
DXYS218	266-294	PAR1	PET-TGTGTTTGGGTTTCCTCTGT	CGAAACTCCGTCTCAAAATA
D18S1002	340-370	18q11	PET-GTTTGATGGGAGGAAGCTATCTAT	GTGAAGTAGCGGAAGGCTGTAAT
D21S226	440-470	21q22.1	PET-GCAAATTTGTGGATGGGATTAACAG	AAGCTAAATGTCTGTAGTTATTCT
AMEL	103/111	Xp22.1/Yp11.2	NED-CCCTGGGCTCTGTAAAGAATAGTG	ATCAGAGCTTAAACTGGGAAGCTG
D13S258	180-296	13q21.33	NED-ACCTGCCAAATTTTACCAGG	GACAGAGAGAGGGAATAAACC
D18S386	330-400	18q22.1	NED-TGAGTCAGGAGAATCACTTGGAAC	CTCTTCCATGAAGTAGCTAAGCAG
MBP18	220-228	18q23	VIC-GGACCTCGTGAATTACAATC	ATTTACCTACCTGTTCATCC
XHPRT	263-299	Xq26.1	VIC-ATGCCACAGATAATACACATCCCC	CTCTCCAGAATAGTTAGATGTAGG
SRY	470	Yp11.3	VIC-GAATATTCCCGCTCTCCGGA	GCTGGTGCTCCATTCTTGAG
D18S391	140-180	18p11.31	6FAM-GGACTTACCACAGGCAATGTGACT	TAGACTTCACTATTCCCATCTGAG
D21S11	205-245	21q21.1	6FAM-TGTATTAGTCAATGTTCTCCAG	ATATGTGAGTCAATTCCCCAAG
D18S51	279-323	18q21.33	6FAM-CAAACCCGACTACCAGCAAC	GAGCCATGTTCATGCCACTG
D21S1412	384-418	21q22.2	6FAM-CGGAGGTTGCAGTGAGTTG	GGGAAGGCTATGGAGGAGA

TABLE 2.5.1b: Primers for QF PCR, their chromosome position and PCR product size in base pairs. Second mix of probes out of two (Mix B)

MIX B	PCR product Size(bp)	Chromosome position	Sequence Forward	Sequence Reverse
TAF9L	142/144	3p24/Xq13	PET-TGCCTAATGTTTTGTGATT	GACCCAAAACTACCTGTC
DXS8377	213-252	Xq28	PET-CACTTCATGGCTTACCACAG	GACCTTTGGAAAGCTAGTGT
D13S628	425-470	13q21.33	PET-TAACATTCATTGTCCCTTACAGAT	GCAAGGCTATCTAACGATAATTCA
AMEL	103/111	Xp22.1/Yp11.2	NED-CCCTGGGCTCTGTAAAGAATAGTG	ATCAGAGCTTAAACTGGGAAGCTG
X22	194-241	PAR2	NED-TCTGTTTAATGAGAGTTGGAAAGAAA	ATTGTTGCTACTTGAGACTTGGTG
D21S1414	328-443	21q21	NED-AAATTAGTGTCTGGCACCCAGTA	CAATTCCCCAAGTGAATTGCCTTC
DXS6803	106-125	Xq21.31	VIC-GAAATGTGCTTTGACAGGAA	CAAAAAGGGACATATGCTACTT
D21S1411	256-340	21q22.3	VIC-ATAGGTAGATACATAAATATGATGA	TATTAATGTGTGTCCTTCCAGGC
D13S634	385-440	13q21.33	VIC-GGCAGATTCAATAGGATAAATAGA	GTAACCCCTCAGGTTCTCAAGTCT
D18S535	455-500	18q12.3	VIC-CAGCAAACTTCATGTGACAAAAGC	CAATGGTAACCTACTATTTACGTC
D21S1437	110-140	21q21.1	6FAM-ATGTACATGTGTCTGGGAAGG	TTCTCTACATATTTACTGCCAACA
D13S631	190-210	13q32.1	6FAM-GGCAACAAGAGCAAAACTCT	TAGCCCTCACCATGATTGG
DXS981	230-260	Xq11.1	6FAM-CTCCTTGTGGCCTTCCTTAAATG	TTCTCTCCACTTTTCAGAGTCA
D13S305	430-465	13q13.3	6FAM-GCCTGTTTGAGGACCTGTCGTTA	TGGTTATAGAGCAGTTAAGGCA

Table 2.5.2: Primers for Devyser M1 extended, their chromosome position and PCR product size in base pairs

Devyser Mix	PCR product Size(bp)	Chromosome position
16G	122-154	16q22.3
15C	160-212	15q12
16C	258-310	16q24.1
22A	127-167	22q13.1
15A	189-237	15q15.1
16D	242-290	16p11.2
15B	232-362	15q26.2
22C	367-415	22q11.2
22E	115-175	22p13
22D	192-236	22q11.2
15D	240-312	15q12
16E	317-352	16q13
16F	365-401	16q11.2
22B	405-467	22q13.1

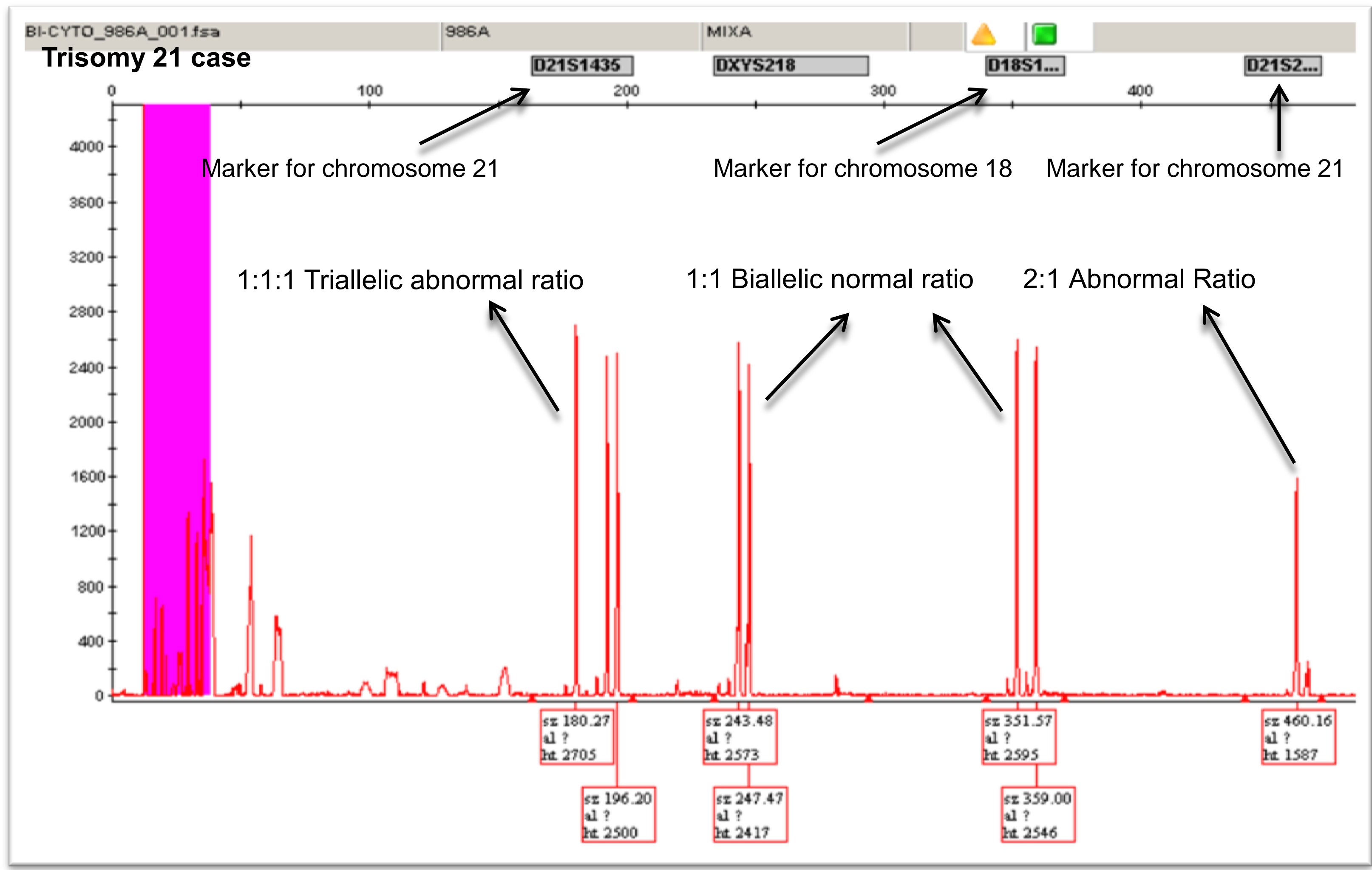


Figure 2.5.1: Electropherogram of QF PCR analysis carried out on a trisomy 21 case. Results from Mix A labeled with PET (Red). Polymorphic markers for chromosomes 18, 21, X and Y appear in this figure. The markers shown discriminate between the triallelic 1:1:1 ratio for marker D21S1435 and 2:1 ratio for marker D21S2226 both of which show trisomy for chromosome 21, whereas markers DXYS218 and D18S1002 show a normal biallelic 1:1 ratio.

2.5.2) Whole- Genome Array Comparative Genomic Hybridization (aCGH)

Following DNA extraction, the test and reference DNA of the same gender were co-hybridized to the array of choice, as previously described [40] (Figure 2.5.2).

Briefly, patient DNA was labeled by random priming using Bio Prime labelling kit (Invitrogen, Carlsbad, CA, USA) with Cyanine 3 and Cyanine 5 (Amersham Biosciences, UK) fluorescent dyes. DNA concentration varied from 150ng to 500ng depending on the method used. Pooled genomic DNA from peripheral blood leukocytes of phenotypically normal males or females from Promega (Promega, Madison, WI, USA) was used as reference for Groups 1 and 3. For all Group 2 samples, pooled DNA was not used as the reference DNA, but a normal male reference DNA was used instead, namely NA10851 (Coriell Cell Repositories, Coriell Intitute). DNA was then hybridized on the arrays using an automated slide processor (HS 4800, Tecan Inc., Mannedorf, Switzerland). Array images were then acquired using an Agilent laser scanner G2565B and image files were quantified using Agilent's Feature extraction software (V9.5.3.1) and analyzed with the BlueFuse for microarrays software package (BlueGnome, Ltd.UK). Group 2 samples were performed in duplicate with DNA labeling color reversal (dye swap) and their results were fused before the ratios were calculated by the Bluefuse software[40].

Many different array platforms were used in the study. For Groups 1 and 3 samples Cytochip BlueGnome arrays were used. BAC Cytochip array versions 1, 2 or 3 (with a content of 3574, 4212 and 5385 Clones respectively) and Oligonucleotide arrays with 105,000 or 180,000 oligos were applied. Whole-genome BAC arrays are commercially available and have a median resolution of 0.5-1Mb. For Group 2 samples, the Sanger Tiling Path arrays with 32,000 overlapping clones were used after extensive investigation of the different platforms available at the time (Refer to subsections 2.5.3 and 3.5.2.1 in Chapters 2 and 3 respectively under the title "Platform comparison"). Confirmations of the Copy Number Changes (CNCs)

revealed in array analysis of Group 2 samples were performed with 244,000 oligo arrays (Agilent Technologies Inc.). The 244K array is a slide which contains 244,000 immobilized oligonucleotides. These oligonucleotides represent region which cover the entire human genome, thus making it possible for finding unknown mutations (genetic imbalances) which may be involved in various genetic diseases.

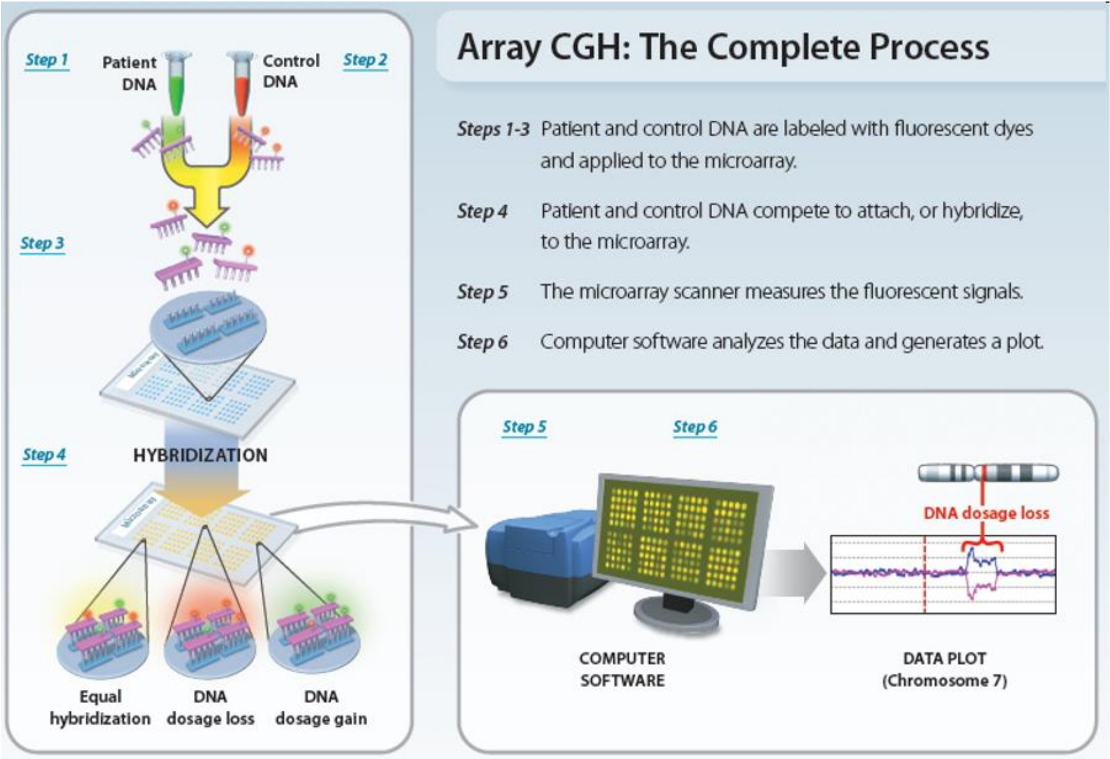


Figure 2.5.2: Diagrammatic representation of array CGH methodology (from 2008 Nature Education)

The calling thresholds set for BAC arrays were for at least two consecutive clones to fall outside the normal ranges ($\log_2[1/2]= -1$ for deletions and $\log_2[3/2]= 0.58$ for duplications) to discriminate deletions/duplication from normal copy number. For oligo arrays the minimum resolution was set at 200kb. For a Copy Number Change (CNC) to be considered as clinically significant/pathogenic the following criteria should apply:

(1) the aberration should be de novo, or inherited from an affected parent

(2) the region should contain genes and /or overlaps with a known syndrome or with a DECIPHER (DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources - http://decipher.sanger.ac.uk) entry

(3) the region should not be listed as polymorphic DGV (Database of Genomic Variants- http://projects.tcag.ca/variation)

(4) it was not previously found in the cohort of our patient dataset

If an aberration met criteria 2 and 3 but was found in a normal parent, and was not previously reported as recurrent syndromes with variable phenotype due to incomplete penetrance, it was classified as unclear significance.

All prospective parents were offered genetic counseling by the referring clinician and consented prior to the testing.

2.5.3) Platform comparison

The fact that there were a number of array platforms available created the need to search for the most suitable one for Group 2 samples of the study. Three different type of microarrays were tested:

1. DNA microarrays from “Vlaams Interuniversitair Instituut Voor Biotechnologie-VIB with 3341 specific probes
2. Wellcome Trust Sanger Institute Tiling Path Microarrays with 32000 probes
3. CytoChip Version 1.0, BlueGnome Ltd. With 3352 BAC probes

These microarrays were tested with DNA from amniotic fluid, CVS and Fetal Blood. The arrays were also checked with known abnormal cases to see the dynamic range of each array to call for abnormalities. Based on the results the Wellcome Trust Sanger Institute Tiling Path Microarrays were selected for Group 2 samples.

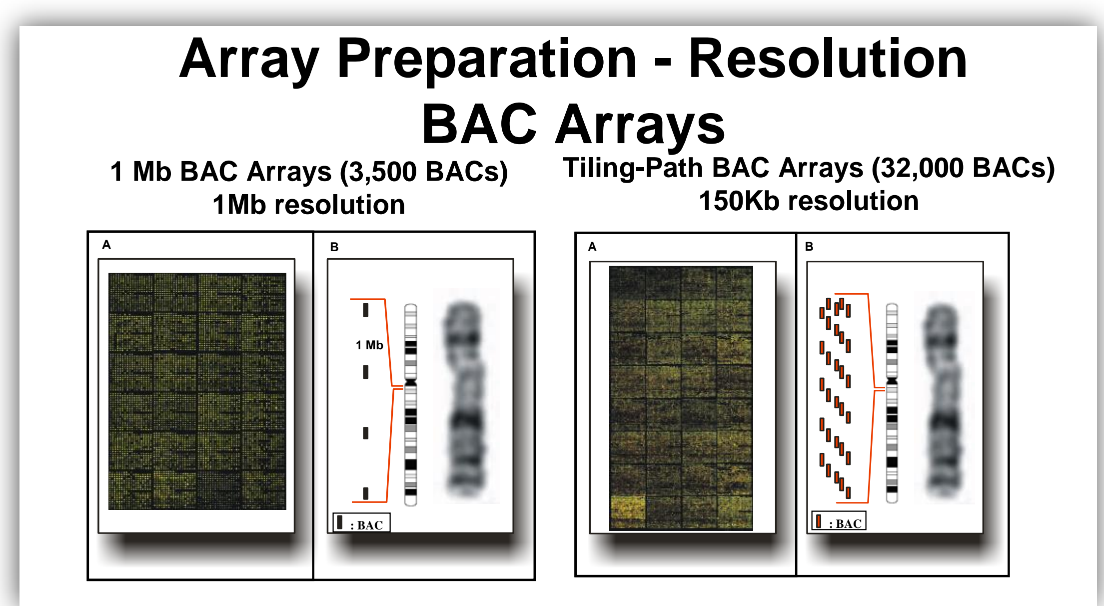


Figure 2.5.3: Schematic view of the difference in resolution between 1Mb array and the Wellcome Trust Tiling Path BAC microarray for chromosome X

2.5.4) Real- Time Polymerase Chain Reaction (PCR)

Real-Time PCR was used as a confirmatory test for several CNCs detected by aCGH analysis using previously described standard procedures [55]. Real-Time PCR primers were uniquely designed using Primer3 [56] and the selected sequences were then aligned against the human genome using the BLAT program to ensure their high degree of homology with each particular location [57]. Real-Time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and reactions were run on the BioRad Real- Time PCR system. Tables 2.5.41, 2.5.42 and 2.5.43 show the primers used for confirmation of array CGH results in Group 1 patient P24 and Group 2 patients P31 and P45 respectively.

Table 2.5.41: Sequence and parameters of the 1q31.2 test primer set. Three sets of primers were designed from within regions of unique sequence of 1q31.2.

Primer Name	Genomic Location of Amplicon	Primer Size	Primer Sequence- Forward	Primer Sequence- Reverse
RPG22-1	190244952-190245082	131bp	**Forward:** 60.0 TTCTGAGACATGCGTTTTGC	**Reverse:** 60.0 CAAACTGCATGGGTTTGTTG
RPG22-2	190195398-190195563	166bp	**Forward:** 60.0 AGCTCACCAAGGCAGAAAAA	**Reverse:** 60.0 GCCTGATAGCTGCTGTTTCC
RPG22-3	190268800-190268904	105bp	**Forward:** 60.0 TCTCTTTGTGGCCTTTGCTT	**Reverse:** 60.0 GACATCTGGCAGCAGCATAA

Table 2.5.42: Sequence and parameters of one primer set designed for each location that needed to be confirmed for patient Group 2 P31. Four separate locations were tested in total.

Primer Name	Genomic Location of Amplicon	Primer Size	Chromosome and Chromosomal Band	Primer Sequence- Forward	Primer Sequence- Reverse
28 A	46502818-46502949	125bp	Chr10q11.22	Forward: 60.0 C TTTGCTCACAGCATCTCACC	Reverse: 60.0 C CACCAGAGCTGGACAAGACA
28B	47081154-47081278	132bp	Chr10q11.22	Forward: 60.0 C AGAGTGGCTCCCTAGTGCAA	Reverse: 60.0 C CGCTAGTCTCAGGGCCTATG
29 A	69507085-69507218	134bp	Chr16q22.2	Forward: 60.0 C GTGGTGGGTAGTGCCCTAGA	Reverse: 60.0 C caagaccagcccaaagagag
29 B	69680586-69680692	107bp	Chr16q22.2	Forward: 60.0 C CATAGGTGGTTGCACACTGG	Reverse: 60.0 C GACTGAGGCCTGCCTTGTAG
30 A (Homology on chr4 Reverse primer)	70223473-70223569	97bp	Chr4q13.2	Forward: 60.0 C AGGGACACTTTTTCCCGTCT	Reverse: 59.9 C GGGTCAAAAAGTCCCAGTGA
31A	31931243-31931358	116bp	Chr17q12	Forward: 60.0 C GCCTGAAGAAACTGGCTTTG	Reverse: 60.0 C GAGAGGCTGTTTGTGGCTTC
31B	32922995-32923118	124bp	Chr17q12	Forward: 60.0 C GGAGATCGGGAAGAACATGA	Reverse: 60.0 C AACGAGTAGGCATGGGTTTG

Table 2.5.43: Sequence and parameters of one primer set designed for each location that needed to be confirmed for patient Group 2 P45. Three separate locations were tested in total.

Primer Name	Genomic Location of Amplicon	Primer Size	Chromosome and Chromosomal Band	Primer Sequence- Forward	Primer Sequence- Reverse
32 A	39373653-39373739	87bp	Chr8p11.23	Forward: 60.0 C GCCTGCAGTCAGAGAAAACC	Reverse: 60.0 C CCCCAGAATGGAAGAGATGA
32 B	39403600-39403746	147bp	Chr8p11.23	Forward: 60.0 C CACAGTTGCCAACAAAATGG	Reverse: 60.0 C CAGCCTCCTTTGCAGTTAGG
33 A	7809605-7809749	145bp	Chr10p14	Forward: 60.0 C TCCAGAATGGGATTTTCTGC	Reverse: 60.0 C TGGGGTCTAACAGTCCCAAG
33 B	7805216-7805335	120bp	Chr10p14	Forward: 60.0 C GAGCGGAATGTTCTGCTAGG	Reverse: 60.1 C GAGGGGTCTACAGGGAGGAG
34 A	80245014-80245096	83bp	Chr6q14.1	Forward: 60.1 C TGCATTTTATGGCACCTGAA	Reverse: 60.0 C AGGGGTGGGGATGAAAATAG
34B	80235181-80235292	112bp	Chr6q14.1	Forward: 59.9 C TTCCACCCTTTAAGCCAATG	Reverse: 60.0 C TGCTCTCCTCCCTTGAGTGT

2.5.5) Multiplex Ligation-dependent Probe Amplification (MLPA)

Another method that was used as a confirmatory test for several CNCs detected by aCGH analysis was MLPA; a multiplex PCR method detecting abnormal copy numbers of up to 50 different genomic DNA or RNA sequences, which is able to distinguish sequences differing in only one nucleotide [20]. Briefly, DNA is denatured and incubated overnight with a mixture of MLPA probes. MLPA probes consist of two separate oligonucleotides, each containing one of the PCR primer sequences. The two probe oligonucleotides hybridize to immediately adjacent target sequences and are then ligated during the ligation reaction. Only ligated probes will be exponentially amplified during the subsequent PCR reaction and therefore the number of probe ligation products will serve as the measure for the number of target sequences in the sample. The amplification products are separated using capillary electrophoresis. Probe oligonucleotides that are not ligated will only contain one primer sequence and will not be amplified exponentially and as a result they will not generate a signal. Comparing the peak pattern obtained to that of reference samples indicates which sequences show aberrant copy numbers. For the confirmation of the array findings, P033 probe mixture (MRC Holland) was used.

3) Results

3.1) Patients and Samples

3.1.1) Group 1

This group included 95 cases which were subcategorized in 6 categories according to the chromosomal analysis and/or results' by other methods and the ultrasound findings (Table 3.1.1):

- 59 had normal karyotypes and abnormal ultrasound findings **(A)**
- 7 had normal karyotype without ultrasound findings (aCGH was not initially requested but was carried out either because of: 1) maternal anxiety due to previous abnormal pregnancy, 2) a possible abnormality was revealed during analysis either by G Banding or QF PCR or MLPA) **(B)**
- 8 had an apparently balanced structural aberration with abnormal ultrasound findings **(C)**
- 11 had an apparently balanced structural aberration without abnormal ultrasound findings **(D)**
- 2 had an abnormal Karyotype with abnormal ultrasound findings **(E)**
- 8 had an abnormal Karyotype/ MLPA/QF-PCR without abnormal ultrasound findings **(F)**

In each subcategory "2" stands for the use of BAC array and "3" stands for the use of oligonucleotide array. For example if in the table a case is subcategorized as "A3" it means that it had a normal karyotype, abnormal ultrasound findings and was analyzed by oligonucleotide array.

A total of 53 Amniotic Fluid, 37 Chorionic villus, 2 skin biopsy and 3 fetal blood samples were included in this category.

Table 3.1.1: Overview of Group 1 prenatal cases

Case	Sample	Reason For Referral	Array Type	KARYOTYPE	Sub Cat.	GA
1	AF	*de novo* Balanced Rearrangement	1Mb BAC array	46,XY,t(5;16)(q33;q24)dn	D2	21,5
2	CVS	U/S Findings/ Myocardiopathy	1Mb BAC array	46,XY	A2	27
3	AF	*de novo* Balanced Rearrangement	1Mb BAC array	46,XX,t(2;12)(q31;q13)dn	D2	18,2
4	AF	Investigation of Abnormal Karyotype	1Mb BAC array	46,XY,+der(11)t(11;22)(q23;q11.2),-22	F2	18
5	CVS	U/S Findings/Increased NT	1Mb BAC array	46,XY	A2	12,3
6	AF	Ultrasound Finding/Nasal bone, Cardiac abnormalities	1Mb BAC array	46,XY	A2	22
7	AF	U/S Findings	1Mb BAC array	46,XX	A2	N.A.
8	CVS	U/S Findings/IUGR, Single Umbilical Artery, Pyelic Cyst	1Mb BAC array	46,XX	A2	13,4
9	CVS	Familial Balanced rearrangement	1Mb BAC array	46,XX,inv(3)(p11.2q11.2)pat	D2	N.A.
10	CVS	U/S Findings	1Mb BAC array	46,XX	A2	12,2
11	AF	U/S Findings	1Mb BAC array	46,XY	A2	N.A.
12	AF	U/S Findings/IUGR	1Mb BAC array	not available	A2	22,4
13	CVS	Maternal Anxiety	1Mb BAC array	46,XY	B2	12,4
14	AF	Maternal Anxiety	1Mb BAC array	46,XX	B2	N.A.
15	AF	U/S Findings	1Mb BAC array	46,XY	A2	N.A.
16	AF	U/S Findings	1Mb BAC array	46,XY	A2	N.A.
17	AF	U/S Findings/ Nasal Bone	1Mb BAC array	46,XY	A2	19,3
18	AF	U/S Findings/Increased NT	1Mb BAC array	46,XX	A2	14
19	AF	Investigation of Abnormal Karyotype	1Mb BAC array	46,XY,del(6)(q14,1q16,1)	F2	18
20	CVS	U/S Findings	1Mb BAC array	46,XY	A2	N.A.
21	AF	U/S Findings/Increased NT	1Mb BAC array	46,XY,t(17;21) (p11.2;q22.3)	C2	17

Case	Sample	Reason For Referral	Array Type	KARYOTYPE	Sub Cat.	GA
22	Skin	U/S Findings	1Mb BAC array	46,XY	A2	N.A.
23	AF	U/S Findings/Increased NT	1Mb BAC array	46,xy,20qh+ mat	A2	N.A.
24	CVS	*de novo* Balanced Rearrangement	1Mb BAC array	46,XY,t(1;2)(q25;q21)dn	D2	13,3
25	CVS	U/S Findings/ Familial Balanced rearrangement	1Mb BAC array	46,XY,inv(2)(p11.2q34)mat	C2	N.A.
26	AF	Investigation of Abnormal Karyotype	1Mb BAC array	47,XY,+mar	F2	21
27	AF	*de novo* Balanced Rearrangement	1Mb BAC array	46,XY,t(3;8) (p13;q24.22)de novo	D2	19,3
28	AF	U/S Findings/ Hydronephrosis, Aortic Arch	1Mb BAC array	46,XX,inv(20)q13.1q13.3)pat	C2	N.A.
29	CVS	Investigation of Abnormal Karyotype	1Mb BAC array	46,XY,der(4)t(4;7)(q21.1;q32.2),der(7)t(4;7)del (7)(q22.1q32.10dn	F2	13
30	CVS	Maternal Anxiety/ Previous child with aberration	1Mb BAC array	46,XX	B2	13
31	AF	U/S Findings/Facial Cleft	1Mb BAC array	46,XY	A2	22,5
32	AF	Maternal Anxiety/ Previous pregnancy with aberration	1Mb BAC array	46,XX,t(11;13)(p10;q10)dn	D2	16,5
33	AF	U/S Findings/ Talipes	1Mb BAC array	46,XY	A2	20,5
34	AF	U/S Findings/Short Limbs/ male fetus with female genitalia	1Mb BAC array	46,XY	A2	23,1
35	CVS	U/S Findings/ Nasal Bone	1Mb BAC array	46,XX,del(21)(q21q22.1)mat	E2	24,1
36	Skin	U/S Findings/ Brain	105K Oligo array	46,XX	A3	22,3
37	CVS	Maternal Anxiety	1Mb BAC array	46,XX	B2	13
38	FB	U/S Findings/Congenital Heart Disease	1Mb BAC array	46,XY,?17cenh+	A2	N.A.
39	CVS	Investigation of Possible abnormality	1Mb BAC array	46,XY,inv(17)(p11,2q21,31)mat	D2	17,1
40	AF	U/S Findings/Familial Balanced rearrangement	105K Oligo array	46,XY,t(3;16)(p25;p13.3)mat	C3	N.A.
41	AF	*De novo* Balanced Rearrangement	1Mb BAC array	46,XY,t(11;16)(q23.1;q23)dn	D2	21

Case	Sample	Reason For Referral	Array Type	KARYOTYPE	Sub Cat.	GA
42	CVS	Maternal Anxiety	1Mb BAC array	46,XX	B2	12,3
43	FB	U/S Findings/Increased NT	105K Oligo array	46,XY	A3	N.A.
44	CVS	*de novo* Balanced rearrangement	105K Oligo array	46,XY,t(4;11)(q28.2;q13)dn	D3	N.A.
45	AF	Investigation of Abnormal Karyotype	105K Oligo array	47,XY,+mar	F3	17
46	CVS	U/S Findings	105K Oligo array	46,XY	A3	N.A.
47	CVS	Investigation of Possible abnormality	105K Oligo array	46,XX	B3	N.A.
48	AF	Investigation of Abnormal Karyotype	105K Oligo array	47,XX,+mar [22]/46,XX[18]	F3	N.A.
49	AF	U/S Findings/Hydronephr osis of Kidney	1Mb BAC array	46,XY	A2	N.A.
50	CVS	U/S Findings	1Mb BAC array	46,XY	A2	11,6
51	CVS	U/S Findings/ IUGR	105K Oligo array	46,XX	A3	N.A.
52	CVS	Ultrasound Abnormalities/ Hypoplastic Nasal Bone	105K Oligo array	46,XX,del(7)(q34q35)	E3	13
53	CVS	U/S Findings	105K Oligo array	46,XX	A3	14,2
54	AF	U/S Findings/Enlarged Cisterna Magna	105K Oligo array	46,XY	A3	22,3
55	AF	U/S Findings/Increased NT	1Mb BAC array	46,XY	A2	21,4
56	AF	U/S Findings/NT Thickness	105K Oligo array	46,XY	A3	18
57	AF	U/S Findings/ Ventriculomegaly	1Mb BAC array	46,XY	A2	25
58	CVS	Maternal Anxiety	1Mb BAC array	46,XY	A2	12,1
59	AF	Familial Balanced rearrangement	105K Oligo array	46,Y,inv(X)(p11.3q27)mat	D3	N.A.
60	CVS	U/S Findings	105K Oligo array	46,XX,t(4;11)(q31.3;q22.1)dn	C3	N.A.

Case	Sample	Reason For Referral	Array Type	KARYOTYPE	Sub Cat.	GA
61	AF	U/S Findings/IUGR, Echogenic Bowel	105K Oligo array	46,XY	A3	22,1
62	AF	U/S Findings/ Talipes	105K Oligo array	46,XY	A3	22,3
63	AF	U/S Findings/ Echogenic Bowel, Short Limbs, Ventriculomegaly	1Mb BAC array	46,XY	A2	21,6
64	AF	U/S Findings/ Bilateral talipes equinovarus/de novo Balanced rearrangement	105K Oligo array	46,XY,t(5;13)(q10;q10)dn	C3	27
65	AF	U/S Findings	1Mb BAC array	46,XY	A2	N.A.
66	CVS	U/S Findings	1Mb BAC array	46,XY	A2	N.A.
67	AF	U/S Findings/Hypoplastic middle phalanx small finger, fetal anomaly scan	1Mb BAC array	46,XY	A2	N.A.
68	AF	de novo Balanced rearrangement	1Mb BAC array	46,XX,t(4;6)(p15.2;q25.1)dn	D2	N.A.
69	AF	U/S Findings/ Hydronephrosis, Choroid plexus cyst	105K Oligo array	46,XY	A3	N.A.
70	AF	U/S Findings/Short Limbs, Cystic adenomatoid malformation on right lung	105K Oligo array	46,XY	A3	22,2
71	AF	U/S Findings/Tetralogy of Fallot, Hydrops, Hydramnios, Non-Visible stomach	105K Oligo array	46,XY	A3	30,3
72	CVS	U/S Findings	105K Oligo array	46,XX	A3	12,1
73	CVS	U/S Findings/Increased NT	105K Oligo array	46,XX	A3	20,1
74	CVS	U/S Findings/Increased NT	105K Oligo array	46,XY	A3	N.A.
75	CVS	U/S Findings/Increased NT	105K Oligo array	46,XY	A3	13,3
76	CVS	U/S Findings	105K Oligo array	46,XX	A3	12
77	CVS	U/S Findings	105K Oligo	46,XY	A3	13,6

Case	Sample	Reason For Referral	Array Type	KARYOTYPE	Sub Cat.	GA
			array			
78	AF	U/S Findings/Micrognathia, Hypoplastic Fingers Phalanx	105K Oligo array	46,XY,t(1;20)(q35.3;q13.3)mat	C3	23,3
79	AF	U/S Findings	105K Oligo array	46,XY,t(1;15)(q25;q15)dn	C3	N.A.
80	CVS	Investigation of Abnormal Karyotype	105K Oligo array	47,XX,+mar/46,XX	F3	12,2
81	CVS	U/S Findings/Increased NT	180K Oligo array	46,XX	A3	12,4
82	CVS	Investigation of Possible abnormality	105K Oligo array	46,XY	B3	N.A.
83	AF	Investigation of Abnormal results with MLPA	105K Oligo array	46,XY	F3	17
84	AF	U/S Findings/Brain-Ventriculomegaly Inferior Vermis	105K Oligo array	46,XX	A3	22,4
85	AF	U/S Findings/Fetal Anomaly, Thorax Hydrothorax, Hydrops	180K Oligo array	46,XX	A3	N.A.
86	CVS	U/S Findings	105K Oligo array	46,XY	A3	13,2
87	AF	U/S Findings/Short Limbs, Choroid Plexus Cysts	180K Oligo array	46,XY	A3	22,5
88	CVS	U/S Findings/NT Thickness	180K Oligo array	46,XY	A3	12
89	FB	U/S Findings/ Polydactyly, Micrognathia	180K Oligo array	46,XY	A3	N.A.
90	AF	U/S Findings/Tetralogy of Fallot	180K Oligo array	46,XX	A3	N.A.
91	AF	U/S Findings/Ventriculomegaly	180K Oligo array	46,XY	A3	N.A.
92	AF	U/S Findings/Increased NT	180K Oligo array	46,XX	A3	17,2
93	AF	U/S Findings/ IUGR	180K Oligo array	46,XX	A3	33,3

Case	Sample	Reason For Referral	Array Type	KARYOTYPE	Sub Cat.	GA
94	AF	U/S Findings/Fetal Anomaly, Extremities Artrogryposis	180K Oligo array	46,XX	A3	25
95	CVS	U/S Findings/Increased NT	180K Oligo array	46,XY	A3	12,6

AF, Amniotic Fluid; CVS, Chorionic Villus Sample; FB, Fetal Blood; NT, Nuchal Translucency; IUGR, Intrauterine Growth Retardation; Inh., Inheritance Status; U/S Findings, Ultrasound Findings; GA, Gestational Age; SubCat, SubCategory; N.A; Not Available

3.1.2) Group 2

This group initially consisted of 74 samples out of which only 35 samples passed the DNA quality criteria and were selected for this study based on the DNA quality inclusion criteria (refer to DNA Isolation section). In this group, 34 fetuses (35 DNA samples in total as P44 and P45 are the same fetus but analysed from two different tissue types) from pregnancies which had a normal karyotype (46,XX or 46,XY) during chromosomal analysis but were terminated due to ultrasound findings, were included (Table 3.1.2).

Table 3.1.2: Overview of Group 1 prenatal cases

Case	DNA No.	Sample Type	Array Type	CLINICAL INFORMATION (U/S FINDINGS)
P1	30749	AF	Tiling Path Array	Increased NT, Subcutaneous Oedema
P4	30752	AF	Tiling Path Array	Increased NT
P6	30754	AF	Tiling Path Array	Increased NT
P7	30755	AF	Tiling Path Array	Rule Out cystic fibrosis, echogenic bowel on ultrasound
P9	30757	AF	Tiling Path Array	Polyhydramnios, Bilateral Hydrothorax, Oedema
P10	30758	AF	Tiling Path Array	Increased NT
P11	30759	AF	Tiling Path Array	Increased NT
P14	30762	AF	Tiling Path Array	Omphalocoele
P15	30763	AF	Tiling Path Array	Increased NT
P16	30764	AF	Tiling Path Array	Hydropericardium, Ovarian cyst
P17	30765	AF	Tiling Path Array	Cystic Hygroma
P21	30769	AF	Tiling Path Array	Cystic Hygroma
P23	30777	AF	Tiling Path Array	Pronounced bilateral club feet, Micrognathia

Case	DNA No.	Sample Type	Array Type	CLINICAL INFORMATION (U/S FINDINGS)
P24	30778	AF	Tiling Path Array	Meningocoele, Dandy- Walker malformation
P25	30779	AF	Tiling Path Array	Symmetrical IUGR, Hydrocephaly, Bilateral radius Aplasia
P26	30780	AF	Tiling Path Array	Phocomelia
P27	30781	AF	Tiling Path Array	Unbalanced AVSD (atrioventricular septal defect), DIRV (double inlet right ventricle), DORV (double outlet right ventricle)
P28	30782	AF	Tiling Path Array	Radius aplasia (?left side), Cardiopathy, SVA (sinus of Valsalva aneurysm)
P29	30783	AF	Tiling Path Array	AVSD (atrioventricular septal defect), SVA (sinus of Valsalva aneurysm), small stomach
P30	30784	AF	Tiling Path Array	Fetal akinesia , Distal arthrogryposis
P31	30785	AF	Tiling Path Array	Detailed U/S findings were not given
P32	30786	AF	Tiling Path Array	Unilateral Megalencephaly (hemimegalencephaly), Hydrocephaly
P33	30787	AF	Tiling Path Array	Unilateral Megalencephaly (hemi-megalencephaly), Right-sided
P34	30788	AF	Tiling Path Array	Polymalformations, Checkered limb, Neural tube defect
P35	30467	FB	Tiling Path Array	Enlarged liver, Facial Dysmorphisms, Extremities with limited movement, Polyhydramnion
P36	30233A	FB	Tiling Path Array	Cleft Lip
P37	30345	FB	Tiling Path Array	Absence of Corpus callosum
P38	31097	AF	Tiling Path Array	Fetal Abnormalities (Brain, Kidney)
P39	31099	AF	Tiling Path Array	Cleft Lip
P41	30647B	AF	Tiling Path Array	
P42	30977	AF	Tiling Path Array	Fetal Abnormalities (Brain)
P43	31248	AF	Tiling Path Array	Myocardiopathy
P44	27316	FB	Tiling Path Array	Absence of Corpus callosum
P45	28608	Skin	Tiling Path Array	Absence of Corpus callosum
P46	30446	AF	Tiling Path Array	Micropthalmia, Single umbilical artery, Polyhydramnion

AF, Amniotic Fluid; CVS, Chorionic Villus Sample; FB, Fetal Blood; NT, Nuchal Translucency; IUGR, Intrauterine Growth Retardation; N.A; Not Available;U/S Findings, Ultrasound Findings

Included in this group were:

- 30 Amniotic Fluid (AF)
- 4 Fetal Blood (FB)
- 1 Skin Biopsy samples (SB)

Blood samples and /or DNA were also received from both future parents were possible for subsequent testing.

3.1.3) Group 3

This group included 73 cases of POC and SB samples initially received for chromosomal analysis. Table 3.1.3 lists all the samples included in this group. Samples from all three trimesters were included (Table 3.1.31):

- 41 cases from 1st trimester (Gestational age 0-13 weeks)
- 25 were of 2nd trimester (Gestational age 14- 27 weeks)
- 7 were of 3rd trimester (Gestational age 28 -40 weeks)

These samples were initially set up and cultured for several days. In order for chromosomal analysis to be carried out actively dividing cells are required. For these 73 samples this failed to be accomplished and therefore chromosomal analysis could not be performed, either because of bacterial/fungal contamination in the culture or simply because the cells failed to adhere on the culture vessel despite repeated efforts to establish the culture. Therefore, Group 3 samples were analyzed using alternative methods.

Table 3.1.4: Overview of Group 3 patients

Case	Sample	Method of detection	Gestational Age	Reason For Referral
1	Skin	180K oligo array	37	IUD
2	Fetus & Placenta	180K oligo array	20,2	IUD
3	Skin	180K oligo array	24	Stillbirth
4	Fetus	1Mb BAC array	17,2	IUD
5	Products Of Conception	QF PCR	7,3	Missed Abortion, Recurrent Miscarriages
6	Fetus & Placenta	180K oligo array	12,4	Termination of Pregnancy, IVF pregnancy
7	Skin	1Mb BAC array	18	Missed Abortion
8	Products Of Conception	QF PCR	9	Missed Abortion
9	Fetus & Placenta	QF PCR	13	Missed Abortion, Unembryonic Gestation
10	Products Of Conception	QF PCR	8	Missed Abortion, IVF pregnancy
11	Fetus & Placenta	105K Oligo array	34	IUD

Case	Sample	Method of detection	Gestational Age	Reason For Referral
12	Fetus	1Mb BAC array	25	IUD
13	Products Of Conception	1Mb BAC array	8	Missed Abortion, Recurrent Miscarriages, Abnormal embryos after IVF
14	Products Of Conception	1Mb BAC array	6	Missed Abortion
15	Products Of Conception	1Mb BAC array	10	Missed Abortion
16	Products Of Conception	QF PCR	8	Missed Abortion
17	Skin & Muscle	1Mb BAC array	22,4	Termination of Pregnancy due to U/S findings (severe developmental Delay and ADT)
18	Products Of Conception	105K Oligo array	16	IUD
19	Products Of Conception	1Mb BAC array	7,2	Missed Abortion
20	Products Of Conception	QF PCR	9,5	Missed Abortion
21	Fetus & Placenta	1Mb BAC array	14	Missed Abortion
22	Products Of Conception	QF PCR	11	Missed Abortion
23	Skin	1Mb BAC array	17	Missed Abortion
24	Products Of Conception	QF PCR	14	Missed Abortion
25	Amniotic Fluid	105K Oligo array	22,3	Termination of Pregnancy due to Ultrasound abnormalities (Anophthalmia, Hydrocephaly, Hypoplastic Cerebellum)
26	Embryo and Placenta	QF PCR	15	Missed Abortion, Recurrent Miscarriages X4
27	Products Of Conception	QF PCR	10	Missed Abortion, No fetal heart detected
28	Products Of Conception	QF PCR	8	Missed Abortion, Recurrent Miscarriages X2
29	Products Of Conception	105K Oligo array	8	Missed Abortion
30	Products Of Conception	1Mb BAC array	8	Missed Abortion, Recurrent Miscarriages X2
31	Skin	1Mb BAC array	33	IUD
32	Embryo	QF PCR	15	Missed Abortion
33	Products Of Conception	1Mb BAC array	9,6	Missed Abortion, Recurrent Miscarriages X2
34	Products Of Conception	QF PCR	8	Missed Abortion
35	Skin	1Mb BAC array	39	Stillbirth (Uncomplicated pregnancy)
36	Products Of Conception	1Mb BAC array	9	Missed Abortion, IVF pregnancy

Case	Sample	Method of detection	Gestational Age	Reason For Referral
37	Skin	1Mb BAC array	39	IUD
38	Skin	1Mb BAC array	11	Missed Abortion
39	Products Of Conception	1Mb BAC array	8	Missed Abortion, Failed In Vitro Fertilization (IVF) X5
40	Fetus	1Mb BAC array	15,3	Missed Abortion
41	Fetus	1Mb BAC array	13,2	Missed Abortion, Cisterna Magna
42	Products Of Conception	1Mb BAC array	5,4	Missed Abortion
43	Skin	1Mb BAC array	16	IUD
44	Products Of Conception	QF PCR	8,1	Missed Abortion, Recurrent Miscarriages X2
45	Products Of Conception	1Mb BAC array	7	Missed Abortion, Recurrent Miscarriages
46	Products Of Conception	1Mb BAC array	5,4	Missed Abortion, Unembryonic Gestation
47	Products Of Conception	1Mb BAC array	13	Missed Abortion, Recurrent Miscarriages X2
48	Skin	1Mb BAC array	18	IUD
49	Skin, Placenta	1Mb BAC array	22	Termination of Pregnancy due to U/S Findings Increased NT
50	Products Of Conception	1Mb BAC array	9	Missed Abortion, Recurrent Miscarriages
51	Products Of Conception	1Mb BAC array	8	Missed Abortion
52	Products Of Conception	1Mb BAC array	12	Missed Abortion
53	Products Of Conception	1Mb BAC array	7	Missed Abortion
54	Skin	1Mb BAC array	19	Missed Abortion, Recurrent Miscarriages X2
55	Products Of Conception	1Mb BAC array	11	Unembryonic Gestation
56	Products Of Conception	1Mb BAC array	8	IUD
57	Products Of Conception	QF PCR	6,2	Missed Abortion, Previous Abortion was an abnormal male triploid karyotype
58	Products Of Conception	1Mb BAC array	8	Missed Abortion
59	Products Of Conception	QF PCR	8	Missed Abortion, Recurrent Miscarriages X2
60	Products Of Conception	1Mb BAC array	8	Missed Abortion, Empty Sac, Recurrent Miscarriages X3
61	Skin	1Mb BAC array	36	IUD
62	Products Of Conception	1Mb BAC array	9	Missed Abortion, Unembryonic Gestation

Case	Sample	Method of detection	Gestational Age	Reason For Referral
63	Products Of Conception	1Mb BAC array	8	Missed Abortion
64	Products Of Conception	1Mb BAC array	20	IUD
65	Products Of Conception	1Mb BAC array	16	Missed Abortion
66	Skin	1Mb BAC array	23,2	IUD
67	Skin	1Mb BAC array	12	Missed Abortion
68	Skin	1Mb BAC array	10	Missed Abortion
69	Products Of Conception	1Mb BAC array	20	Missed Abortion, Fetal anomalies: Large Ventricular septal defect , Dextrocardia, Increased NT
70	Products Of Conception	1Mb BAC array	14,5	Missed Abortion, History of Miscarriages x2
71	Skin	1Mb BAC array	20	Missed Abortion
72	Skin	1Mb BAC array	27	IUD, Short Femur
73	Skin, placenta	1Mb BAC array	33	IUD

NT, Nuchal Translucency; IUGR, Intrauterine Growth Retardation; Inh., Inheritance Status; U/S Findings, Ultrasound Findings; GA, Gestational Age

Table 3.1.31: Number of POC samples received per trimester and method analyzed

Trimester	QF PCR	BAC arrays	Oligo arrays	Total
1st trimester (1-13 weeks)	13	26	2	41
2nd trimester (14-27 weeks)	3	18	4	25
3rd Trimester (28- 40 weeks)	0	5	2	7
Total				73

3.2) Clinical Data

For most of Group 1 and Group 2 samples the reason for performing array CGH testing was based on the first trimester ultrasound screening findings. Detailed Ultrasound findings, were available, for each case, are listed in Tables 3.1.1 and 3.1.2. for each group respectively. The reason for referral for Group 3 samples is listed in Table 3.2 and it includes Missed abortion with or without Ultrasound findings, Termination of Pregnancy due to ultrasound findings, intrauterine death and stillbirth.

If recurrent miscarriage hadn't occurred for a number of Group 3 samples they would have probably been referred for prenatal diagnosis.

Table 3.2: Reason for Referral for Spontaneously Aborted Fetuses N=73

Reason for referral	1st Trimester 1-13 weeks	2nd trimester 14-27 weeks	3rd trimester 28-40 weeks
Missed Abortion/ No U/S Findings	40	11	
Missed Abortion with U/S findings		1	
TOP with U/S findings	1	3	
IUD		9	6
Stillbirth		1	1

U/S,Ultrasound;TOP,Termination of Pregnancy; IUD,Intrauterine death

3.3) DNA Isolation

Based on the quality criteria discussed in Chapter 2, section 2.3, 40 samples appeared not to meet the acceptable standards. Therefore phenol: chloroform extraction was carried out in an effort to clean up those DNA samples. (Protocol is shown in ANNEX).

3.4) Conventional Cytogenetics and FISH analyses

Results from chromosomal analyses carried out prior to array CGH testing can be found in table 3.1.1.

FISH was carried out in Cases 80 and 86 to confirm findings following aCGH analyses. In case 80, whole chromosome paint for chromosome 16 was used (Figure 3.4.1) and in case 86 subtelomeric specific probes for chromosome 9 and 17 were used (Figures 3.4.2 A and B). In both patients FISH analysis confirmed the aCGH results.

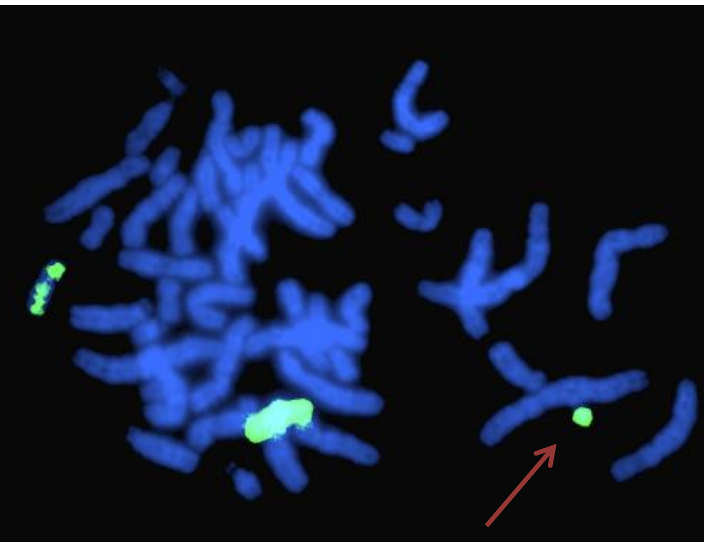


Figure 3.4.1: G- banding analysis determined the presence of a Marker chromosome in Chorionic Villus and amniotic fluid samples of this prenatal case (Case 80). Array CGH analysis showed a gain on chromosome 16 suggesting that the marker chromosome was of chromosome 16 origin. FISH analysis was performed with whole chromosome paint probe for chromosome 16, labeled with FITC fluorophore (Green), and confirmed the array results. The marker chromosome is depicted by the arrow in the figure. The picture also illustrates normal hybridization pattern of the two normal chromosomes 16.

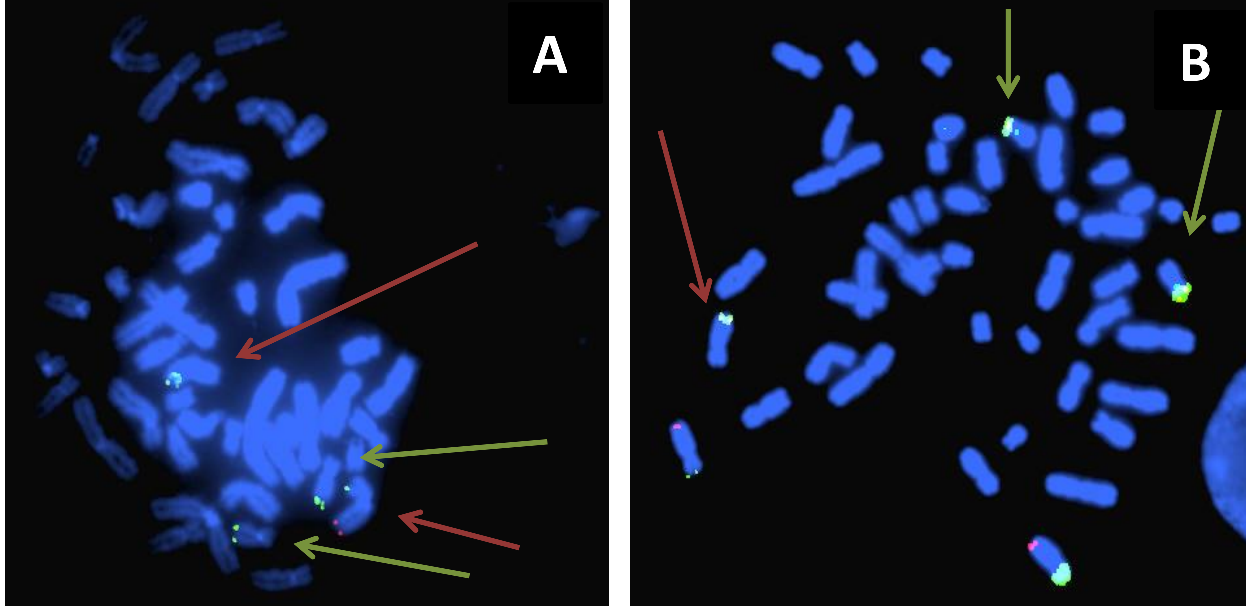


Figure 3.4.2: G- banding analysis determined a normal female karyotype in amniotic fluid sample of this prenatal case (Case 86). Array CGH analysis was also carried out because of Increased Nuchal translucency shown on ultrasound and identified a gain on the short arm (p- arm) of chromosome 17 and a deletion on the long arm (q- arm) of chromosome 9. FISH analysis was performed using two subtelomeric specific probes sets. One set included subtelomeric specific probes for the p (FITC-Green) and q (TRITC- Red) arms of chromosome 9 and the q (FITC+TRITC) arm of chromosome 17 (A); the second set , included subtelomeric specific probes for the p (FITC) and q (TRITC) arms of chromosome 8 and the p (FITC + TRITC) arm of chromosome 17 (B). In (A) the red arrows point at chromosome 9; the top arrow points at the abnormal one which has the q terminal deleted and the green arrows point at chromosome 17 which have normal hybridization on their q arms. In (B) the red arrow points at chromosome 9 which has chromosomal material from chromosome 17p on its long arm and the green arrows point at chromosome 17 which have normal hybridization on their p arms. FISH analysis confirmed the array CGH results and also determined that the copy number changes observed were the product of an unbalanced translocation.

3.5) Array CGH analysis and Confirmations

3.5.1) Group 1

A total of 95 cases were included in this group. Fifty of these samples were analyzed with 1Mb BAC arrays, 34 were analyzed with 105K oligo arrays and 11 were analyzed with 180K oligo arrays. Seventeen abnormal cases (17/95, 17.9%) were determined by array CGH analysis and the aberrations are listed in Table 3.5.1. Seven out of the sixteen (8/17, 47%) abnormal cases detected were from pregnancies which had ultrasound abnormalities but a normal karyotype, whereas 9 of the abnormalities were investigations of abnormalities detected by other methods (G Banding, MLPA, QF PCR) (9/17, 53%). If we exclude the nine abnormalities previously detected with other methods the detection rate of this method would actually be 9.3 % (8 out of 86 samples). Figure 3.5.11 shows the profile of an abnormal case (Case 86) as it was extracted from the Bluefuse software and figure 3.5.12 shows the aberrant regions in the publicly available databases.

3.5.1.1) Confirmation with Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA analysis was performed on Group 1 Case 17 for confirmation of array CGH results. G Banding analysis carried out on this sample showed normal karyotype 46,XY. BAC array CGH analyses carried out on fetal as well as parental DNA samples, revealed three CNCs of maternal origin. The CNCs included one deletion on the short arm of chromosome 17, on chromosomal band 17q11.2, of approximately 1.1Mb in size and two duplications on the short arm of chromosome 9 of approximately 0.4Mb and 0.3Mb on chromosomal bands 9p24.1 and 9p24.2 respectively. MPLA method using probe mixture P033 (MRC Holland) was used and confirmed the aberration on both the fetus and the mother.

3.5.1.2) Real- Time Polymerase Chain Reaction (PCR)

Real time PCR was used to confirm or exclude a CNC that was revealed by array CGH in one case. More specifically for Group 1 Case 24, G Banding analysis showed a *de novo* apparently balanced reciprocal translocation between the long arms of chromosomes 1 and 2, 46,XY,t(1;2)(q25;q21)dn, and array CGH analysis showed a possible deletion of one clone which was in the translocation breakpoint. Real Time PCR was performed on fetal and parental DNA samples to confirm the deletion and also determine whether it was familial or *de novo*. Real Time PCR confirmed the deletion and showed that it was *de novo* in origin. Figure 3.5.1.2 shows the schematic representation of the results.

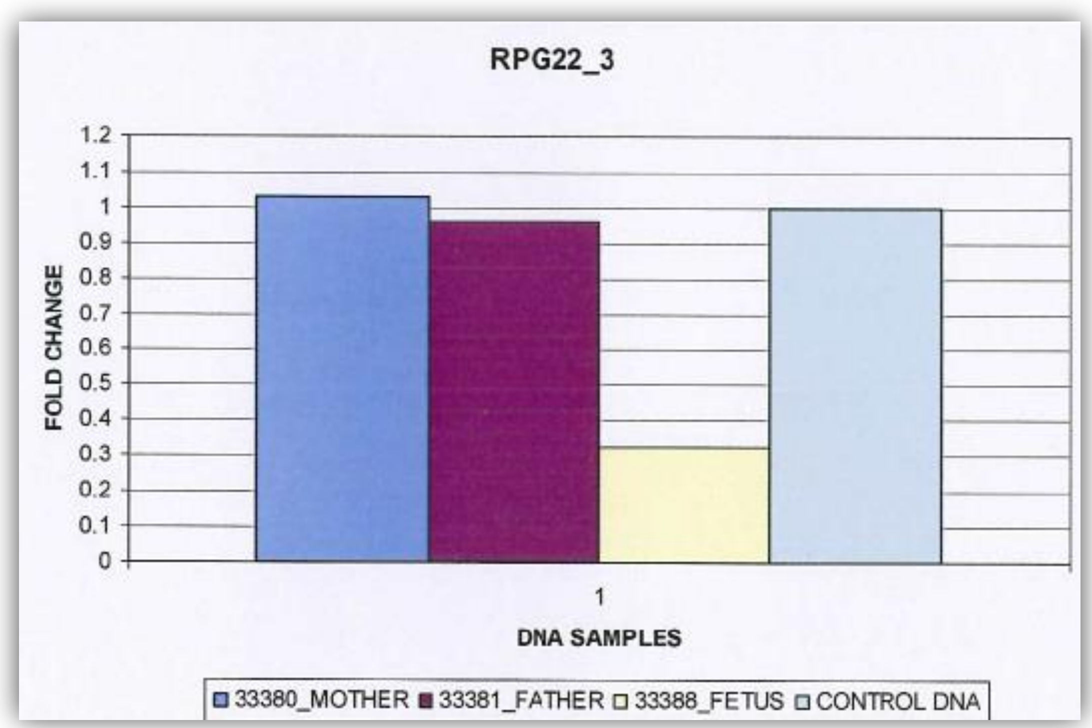


Figure 3.5.1.2: Figure represents the Real Time PCR experiment with one of the three primers (RPG22-3) used to confirm the deletion in the fetus, (Case 24). Fold copy number change of value equal to 1 (test sample /normal sample) indicate an equal ratio of the target and reference, which corresponds to no loss; a deleted region is expected to give a ratio value of 0.5±0.15 whereas a duplicated region is expected to give a value of 1.5 ±0. Fetus from case 24 is represented here by the yellow column showing a fold copy number change of 0.3 indicating a deletion, whilst the parents represented by the blue and maroon columns show fold copy number changes of 1± 0.35. Control DNA showing the same fold copy number change as in the parents is shown in light blue.

Table 3.1.2: Overview of Group 1 abnormal prenatal cases

Case	Sample	Reason For Referral	GA	Result	Status	Inh.	Array Type	KARYOTYPE	Sub Cat.
4	AF	Investigation of Abnormal Karyotype	18	arr cgh 11q23.3q25(RP11-4N9->RP11-469N6)x3,22q11.1q22.2(RP11-437O02->RP11-50L23)x1-Cytochip 2.0	Del, Dup	*De novo*	1Mb BAC array	46,XY,+der(11)t(11;22)(q23;q11.2),-22	**F2**
17	AF	U/S Findings/ Nasal Bone	19,3	arr cgh 9p24.2(P11-320E16->RP11-526D20)x3 mat,9p24.1(RP11-307L3->RP11-106A1R)x3,17p11.2(RP11-27J23->RP11-385D13)x1 mat	Del(1.1Mb), Dup(0.3Mb), Dup(0.4Mb)	Mat	1Mb BAC array	46,XY	**A2**
18	AF	U/S Findings/Increased NT	14	arr cgh 22q11.2(RP11-800B02->RP11-330P17)x3-Cytochip 2	Dup(0.7Mb)	N/A	1Mb BAC array	46,XX	**A2**
19	AF	Investigation of Abnormal Karyotype	18	arr cgh 6q14.1q16.1(RP11-379B8->RP11-21G12)x1 Cytochip 2	Del(10.5Mb)	*De novo*	1Mb BAC array	46,XY,del(6)(q?q?)	**F2**
24	CVS	*de novo* Balanced Rearrangement	13,3	arr cgh 1q31.2(RP11-440G22)x1-Cytochip 3.0	Del(0.2-1.35Mb)	*De novo*	1Mb BAC array	46,XY,t(1;2)(q25;q21)dn	**D2**
26	AF	Investigation of Abnormal Karyotype	21	arr cgh 21q11.2q21.1(RP1-126N20->RP11-28M9)X3-Cytochip 3.0	Dup(18.4Mb)	*De novo*	1Mb BAC array	47,XY,+mar	**F2**
29	CVS	Investigation of Abnormal Karyotype	13	arr cgh 7q22.1q32.1(RP11-44M6->RP11-21K15)x1 Cytochip 3	Del(26.2Mb)	*De novo*	1Mb BAC array	46,XY,der(4)t(4;7)(q21.1;q32.2),der(7)t(4;7)del(7)(q22.1q32.10)dn	**F2**
35	CVS	U/S Findings/ Nasal Bone	24,1	arr 21q21.1q22.11(19,389,989-33,824,529x1)mat	Del(14Mb)	Mat	1Mb BAC array	46,XX,del(21)(q?q?)mat	**E2**
45	AF	Investigation of Abnormal Karyotype	17	mos 47,XY,+mar. arr 21q11.2q21.1(13,539,832-15,716,987)x3~4,21q21.3(27,787,566-28,368,946)x3 dn	Dup(2.1Mb), Dup(0.5Mb)	*De novo*	105K Oligo array	47,XY,+mar	**F3**

Case	Sample	Reason For Referral	GA	Result	Status	Inh.	Array Type	KARYOTYPE	Sub Cat.
52	CVS	U/S Findings/ Hypoplastic Nasal Bone	13	arr 7q34q35(139,107,925-145,455,647x1)dn	Del(6.3Mb)	*De novo*	105K Oligo array	46,XX,del(7)(q?q?)	**E3**
56	AF	U/S Findings/ Increased NT	18	arr 5p14.3p14.2(22,344,207-24,523,053)x3 pat,15q25.2q25.3(81,011,096-83,478,823)x1 dn	Dup(2.2Mb), Del(2.4Mb)	Pat, *de novo*	105K Oligo array	46,XY	**A3**
80	CVS	Investigation of Abnormal Karyotype	12,2	47,XX,+mar/46,XX. arr 16p11.2p11.1(29,727,747-35,004,980)x2~3 dn	Dup(5.2Mb)	*De novo*	105K Oligo array	47,XX,+mar/46,XX	**F3**
83	AF	Investigation of Abnormal results with MLPA	17	arr 22q11.21(17,274,865-19,891,492)x3 mat	Dup(2.6Mb)	Mat	105K Oligo array	46,XY	**F3**
86	CVS	U/S Findings	13,2	arr 9q34.3(139,754,208-141,102,496)x1 mat,arr 17p13.3(48,569-2,002,395)x3 mat	Del(1.35Mb), Dup(1.95Mb)	Mat	105K Oligo array	46,XY	**A3**
88	CVS	U/S Findings/ Increased NT	12	arr 7q31.1(112,763,119-113,252,118)x3 mat	Dup(0.5Mb)	Mat	180K Oligo array	46,XY	**A3**
90	AF	U/S Findings/Tetralogy of Fallot		arr 9q34.3(139,754,208-141,102,496)X3,17p13.3(48,569-2,002,395)X1 mat	Dup(1.35Mb), Del(1.95)	Mat	180K Oligo array	46,XX	**A3**
94	AF	U/S Findings/Fetal Anomaly, Extremities Artrogryposis	25	arr 10p15.3(1,011,902-1,396,788)x3 pat,15q21.1(49,491,651-49,809,467)x1 mat	Dup(0,38Mb), Del(0.32Mb)	Pat, Mat	180K Oligo array	46,XX	**A3**

Amniotic Fluid, AF; Chorionic Villus Sample,CVS; Nuchal Translucency,NT; Inheritance Status, Inh.; Ultrasound Findings,U/S Findings; deletion, del; duplication,dup; maternal,mat; paternal,pat; Not Applicable, N/A; Subcategory, SubCat.

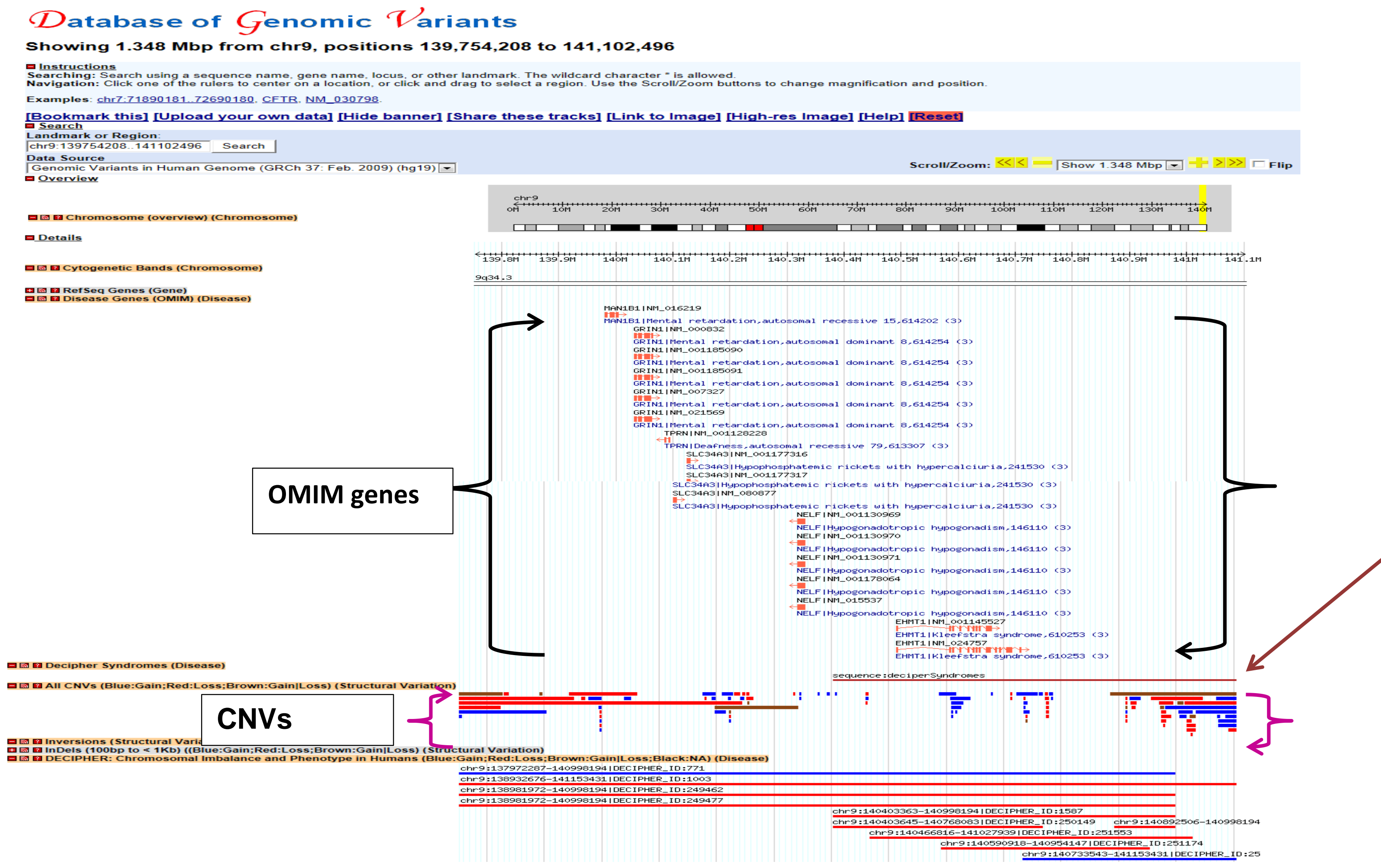


Figure 3.5.12: Representation of the chromosomal and genomic location region on chromosome 9 that has the copy number change in the Database of Genomic Variants (DGV). A loss of 1.35Mb in size which encompasses several OMIM genes (shown in brackets) and overlaps with a DECIPHER syndrome (the 9q microdeletion syndrome- shown by the red arrow). The area is not covered by a significant number of CNVs determining that it is not polymorphic.

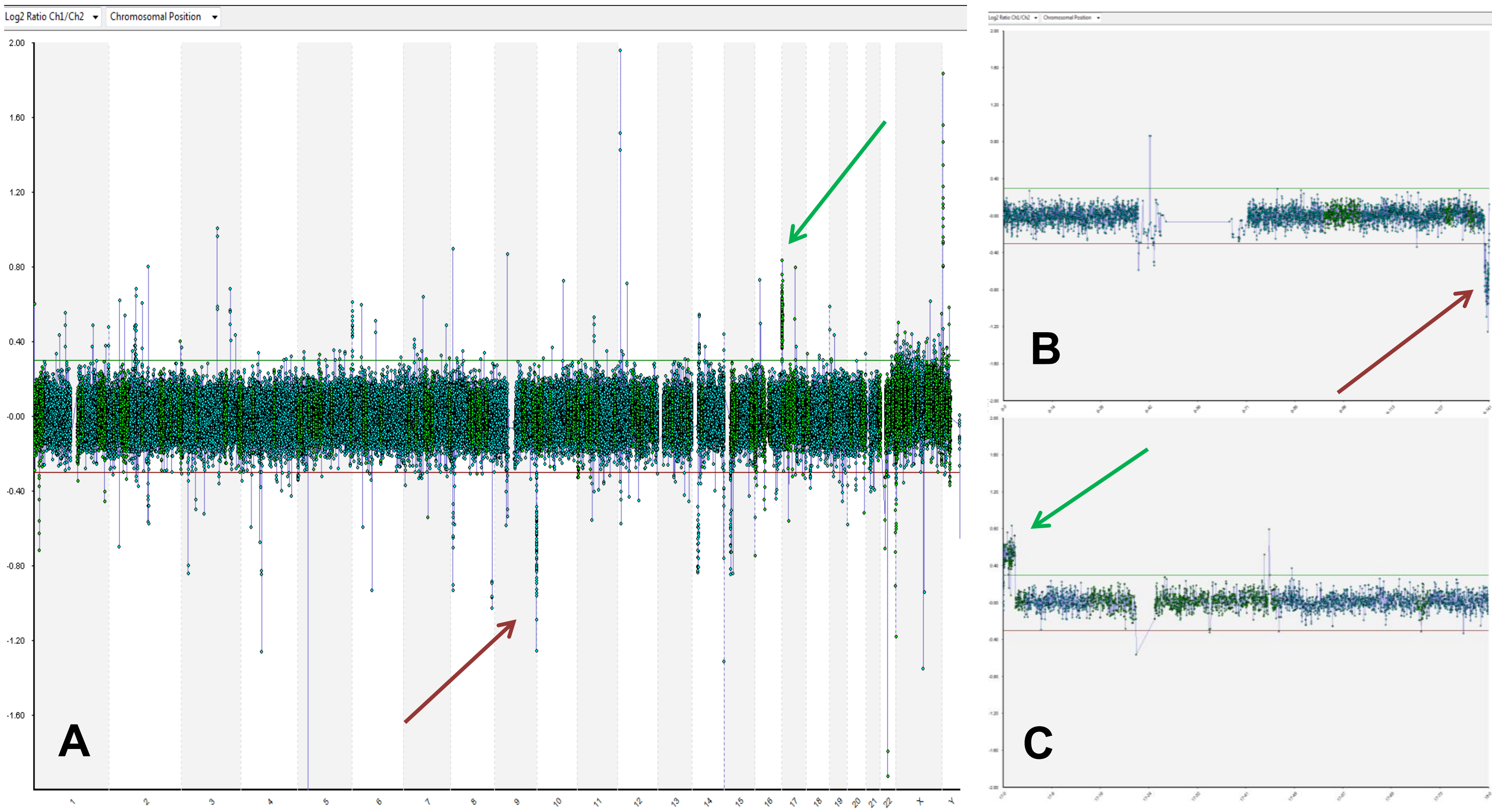


Figure 3.5.11: Profile of case Case 86 as it was extracted from the Bluefuse software (A). Patient 86 had normal karyotype on chromosomal analysis and array CGH analysis determined copy number loss on the long arm terminal of chromosome 9 (B) and copy number gain on the short arm of chromosome 17 (C). The red arrows show the loss on chromosome 9 and the green arrows show the gain on chromosome 17

3.5.2) Group 2

3.5.2.1) Platform comparison

The first test was made with DNA from a fetal blood sample of patient P36 with the CytoChip microarray Version 1.0 (BlueGnome Ltd. UK) and the result passed the quality criteria. The Cytochip BlueGnome array is a commercially available whole-genome BAC array with a median resolution of 0.5-1Mb and includes 3352 BAC. (Figure 3.5.2.1.1).

For array CGH, the test and reference DNA of the same gender were co-hybridized to the Cytochip (BlueGnome, Ltd., UK,) whole-genome BAC array, as previously described [40]. The reference DNAs were derived from pooled peripheral blood leukocytes of phenotypically normal males and females (Promega, Madison, WI, USA).

Next we tested the first (Lot # 2006) out of two Lots of microarrays which were obtained from “Vlaams Interuniversitair Instituut Voor Biotechnologie- (VIB), which were also of 0.5-1Mb median resolution and included 3341 BAC clones. Testing was carried out on two samples namely P35 (Figure 3.5.2.1.2) and P37 (Figure 3.5.2.1.3). Statistical data analysis with Bluefuse software determined that the quality of the results was poor and more noisy than the Cytochip microarrays.

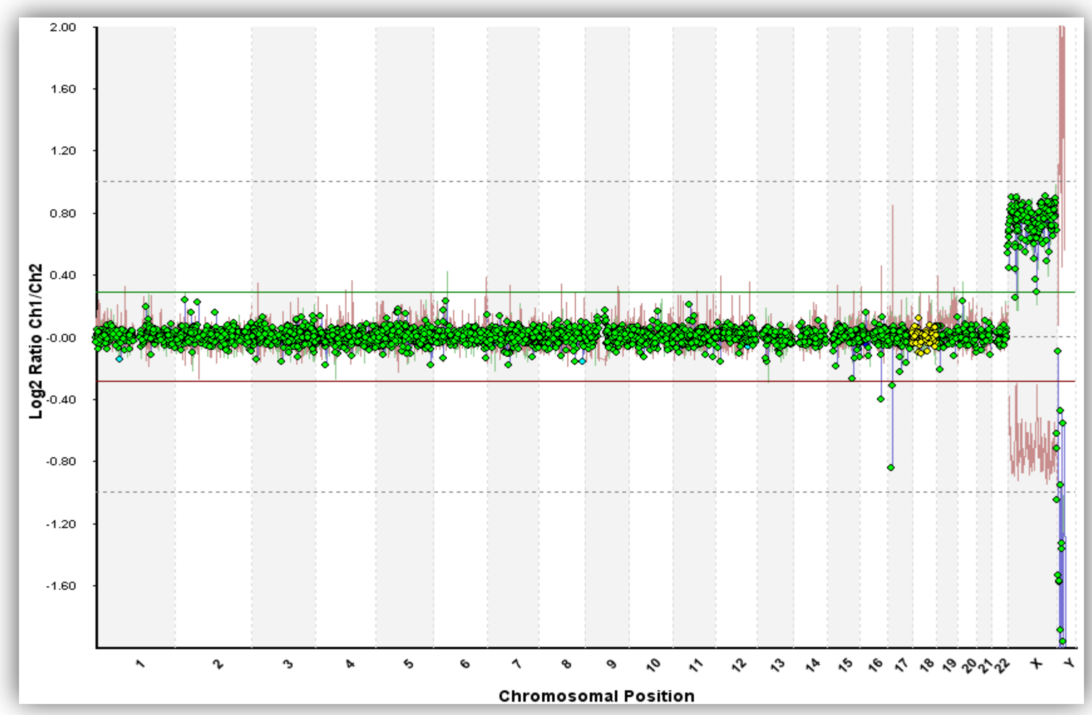


Figure 3.5.2.1.1: Array CGH analysis of patient P36 with version 1.0 Cytochip BlueGnome microarray.

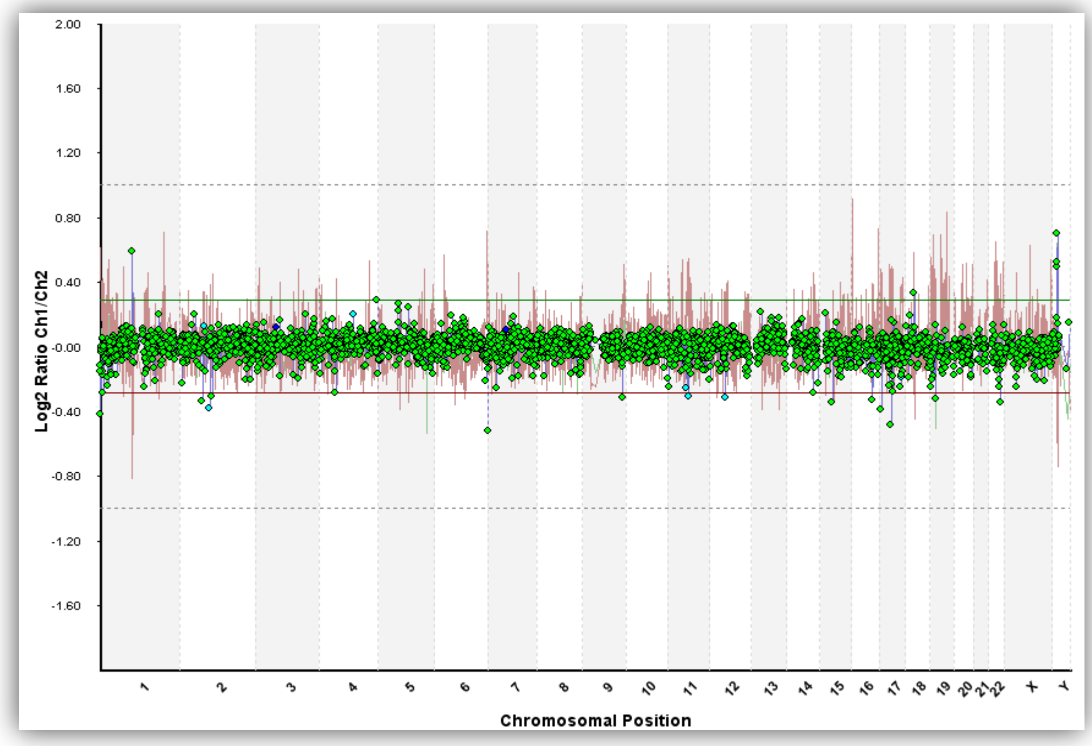


Figure 3.5.2.1.2: Array CGH analysis of patient P35 with the use of VIB microarray LOT # 2006 VIB microarray.

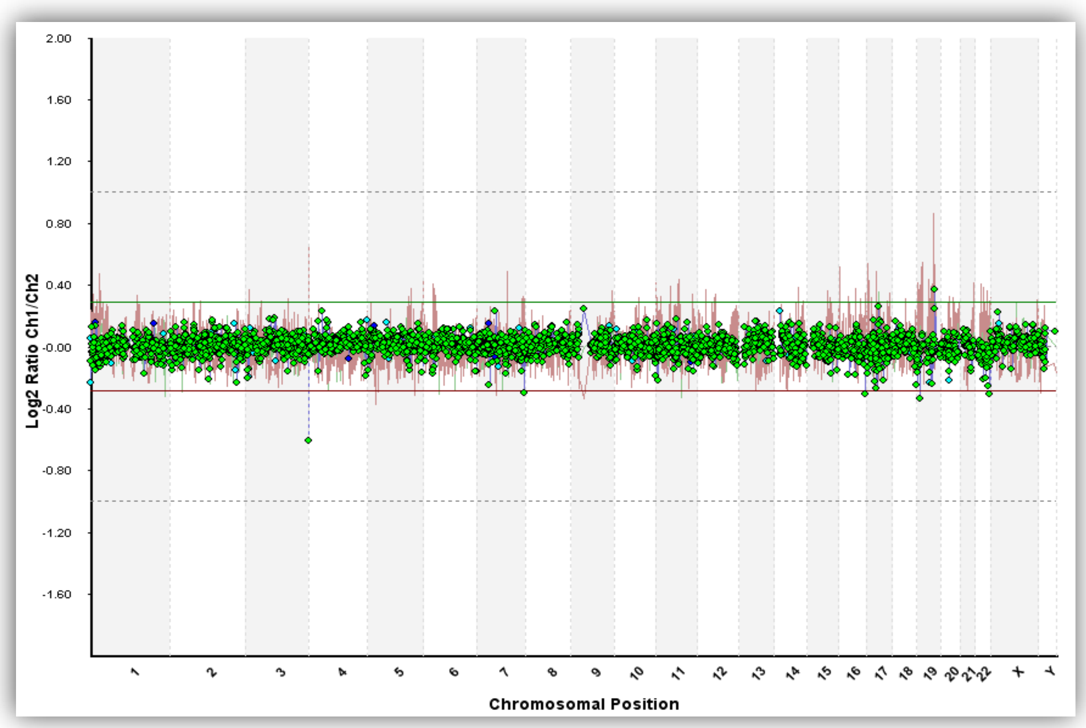


Figure 3.5.2.1.3: Array CGH analysis of patient P37 with the use of LOT # 2006 VIB microarray.

In order to determine whether the noisy results we received in the analysis using VIB arrays was due to poor DNA quality or poor array quality, we used the same LOT (LOT # 2006) microarrays for a patient (Control a) we had previously tested with an alternative microarray and gave good quality results. We chose a positive control patient with a known aberration to be able to test the dynamic range of this platform as well. In parallel, we analyzed DNA from patient P47 again using LOT# 2006 VIB microarrays (Figures 3.5.2.1.4 and 3.5.2.1.5).

The conclusion from the last test was that even though the microarray could sufficiently call the known abnormality in the control patient and with a good dynamic range, it did not give a clear profile with low noise for P47.

Next we tested another two samples (P46 and P61) with the VIB microarrays. For patient P46 we used LOT # 2007 microarrays and because the results we received

where not very good, for P61 we used the last microarray we had from LOT # 2006 (Figures 3.5.2.1.6 and 3.5.2.1.7).

As previously done in order to determine whether the poor results we received for P46 was due to the DNA or the array quality we performed the following:

1) we applied array CGH on P46 using Cytochip microarray (BlueGnome, Ltd, UK) (Figure 3.5.2.1.8)

2) we applied the VIB array LOT # 2007 on a patient (Control b) who was previously tested with another platform, gave good results and had a known aberration. (Figure 3.5.2.1.9).

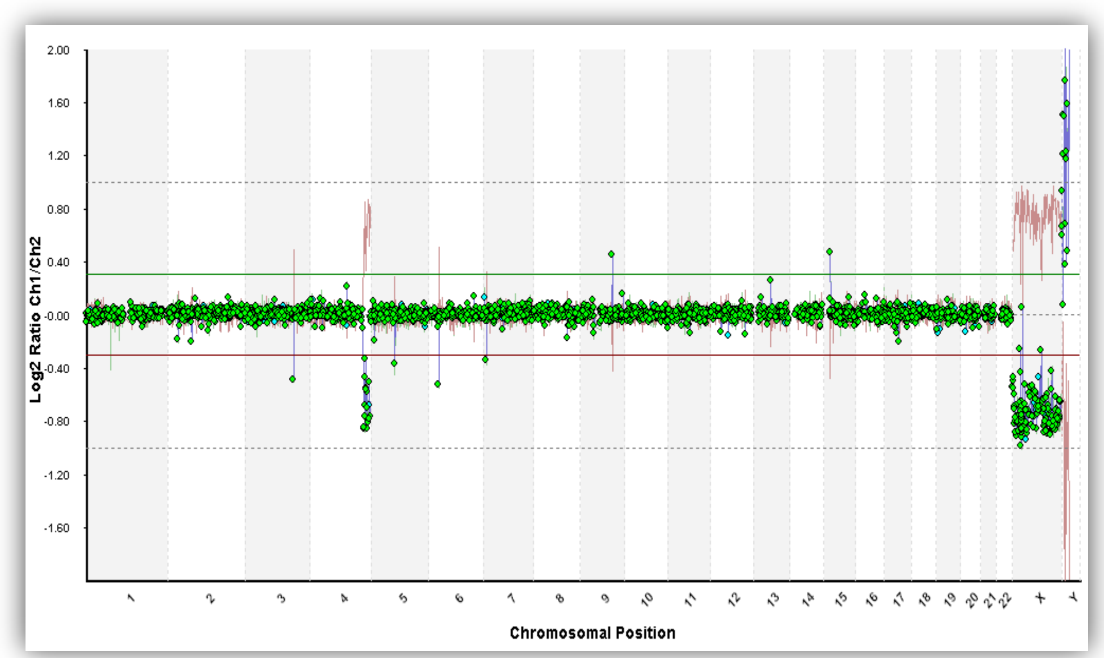


Figure 3.5.2.1.4: Array CGH analysis of the control patient (Control a) who carries a known CNC on chromosome 4. VIB microarray LOT 2006 was used.

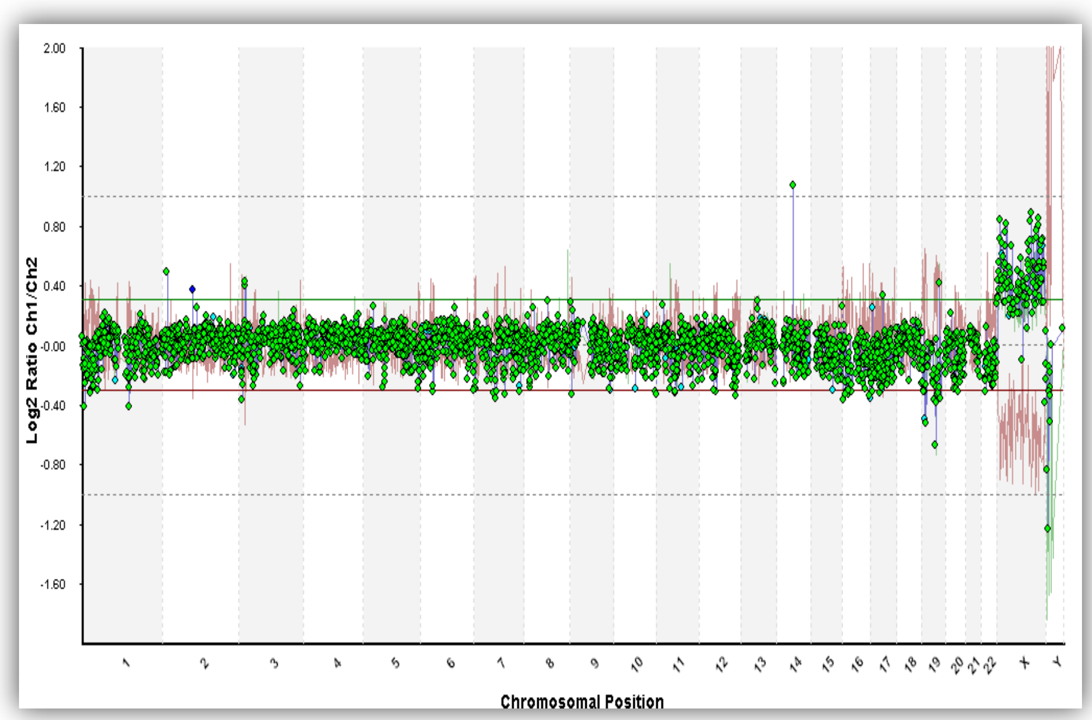


Figure 3.5.2.1.5: Array CGH analysis of patient P47 with the use of VIB microarray LOT # 2006. The profile for this patient appears to be very noisy.

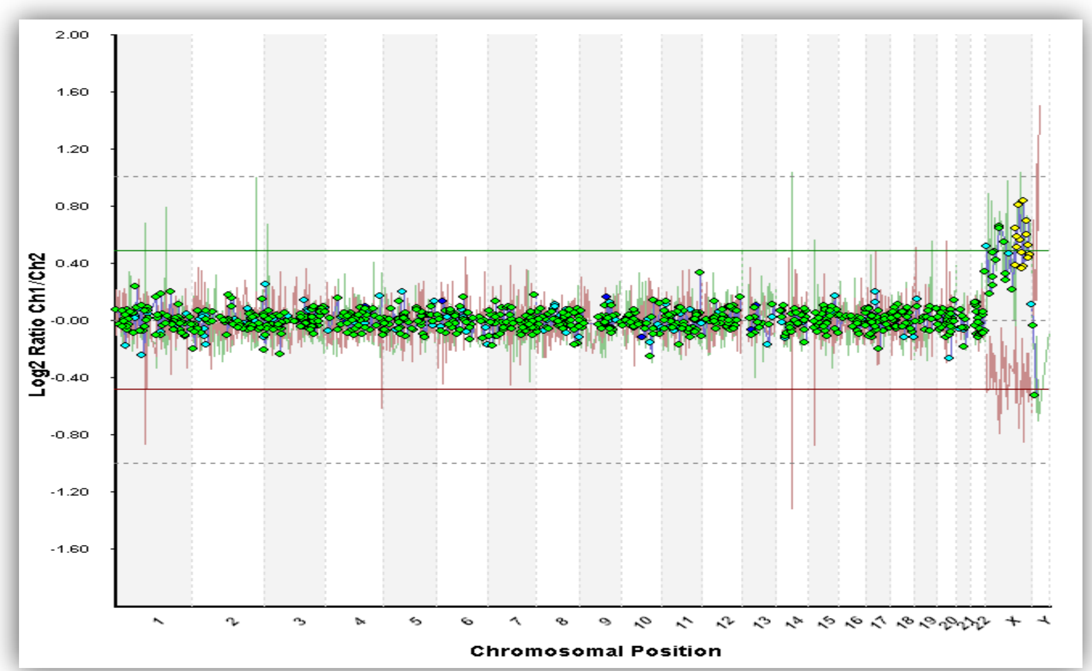


Figure 3.5.2.1.6: Array CGH analysis of patient P46 with the use of VIB microarray LOT # 2007.

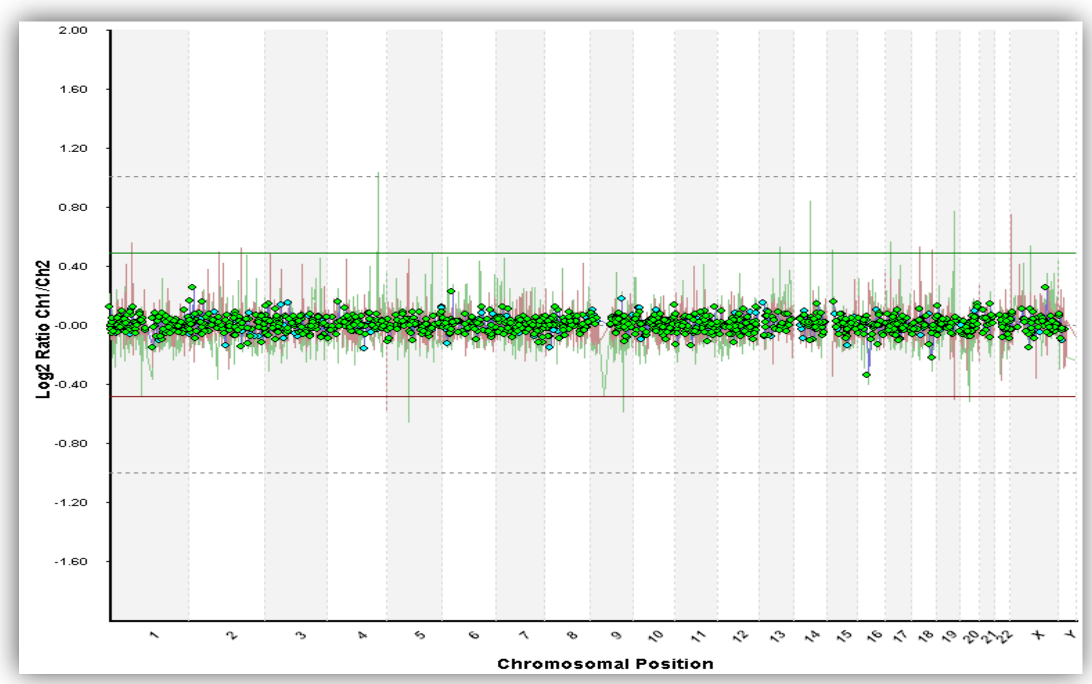


Figure 3.5.2.1.7: Array CGH analysis of patient P61 with the use of VIB microarray LOT # 2006.

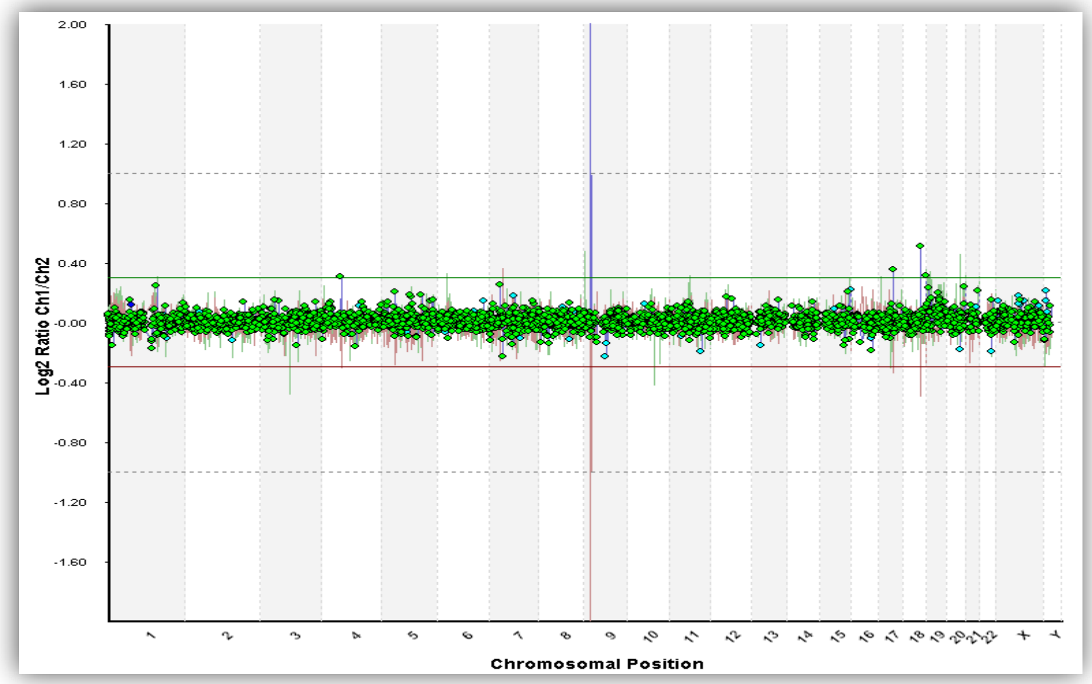


Figure 3.5.2.1.8: Array CGH analysis of patient P46 with the use of Cytochip microarray (BlueGnome).

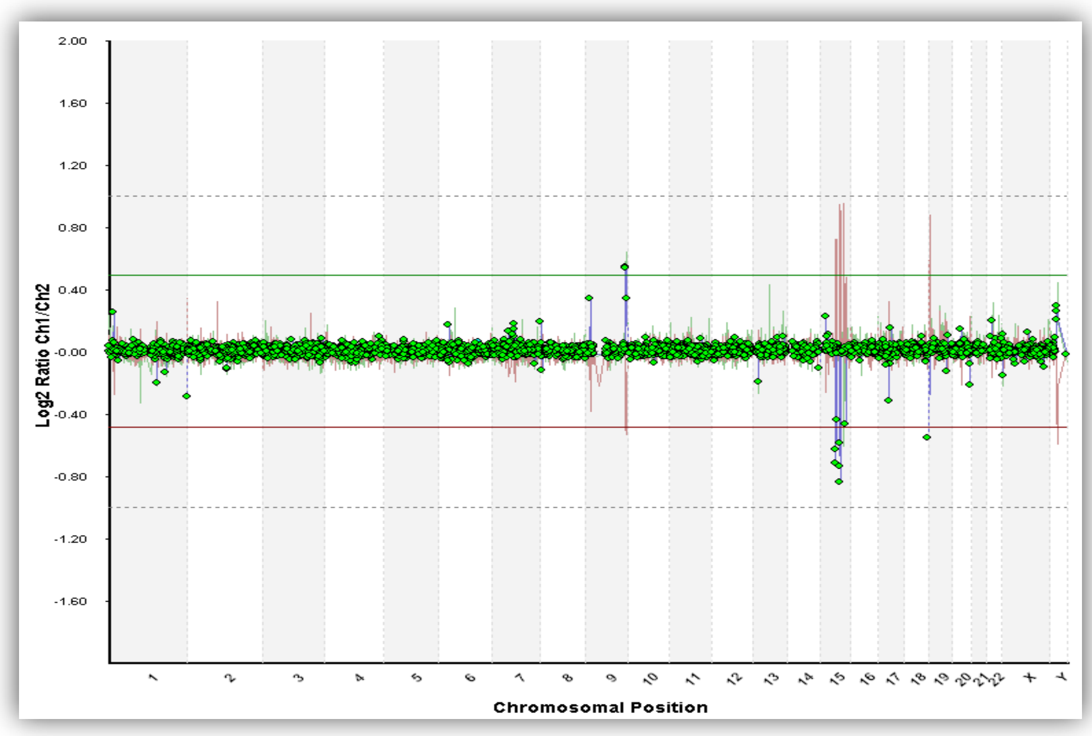


Figure 3.5.2.1.9: Array CGH analysis of the control patient (Control b) who carries a known CNC on chromosome 15. VIB microarray LOT # 2007 was used.

The conclusion from this last part of experimenting between platforms was that the DNA from P46 was not of poor quality after all since it gave a tight profile with the Cytochip BlueGnome array (Figure 3.5.2.1.8) in contrast to the more noisy profile with the VIB array (Figure 3.5.2.1.6). It also showed that the VIB array LOT # 2007 was not of inferior quality either since when it was used for Control Patient b the results were satisfactory.

Continuing with testing platforms three DNA samples (P1, P46, P47) were sent to the Wellcome Trust Sanger Institute (Hinxton, UK) for array CGH analysis using the CGH Wellcome Trust Tiling Path BAC microarrays. The difference between these microarrays from the ones used thus far is that they contain 32,000 clones instead of 3,552 included in the arrays tested up until now with an increased resolution of 60-200Kb as opposed to 0.501Mb.

Testing of VIB arrays LOT# 2007 was however continued on another two patients namely P48 (Figure 3.5.2.1.10) and P49 (Figure 3.5.2.1.11).

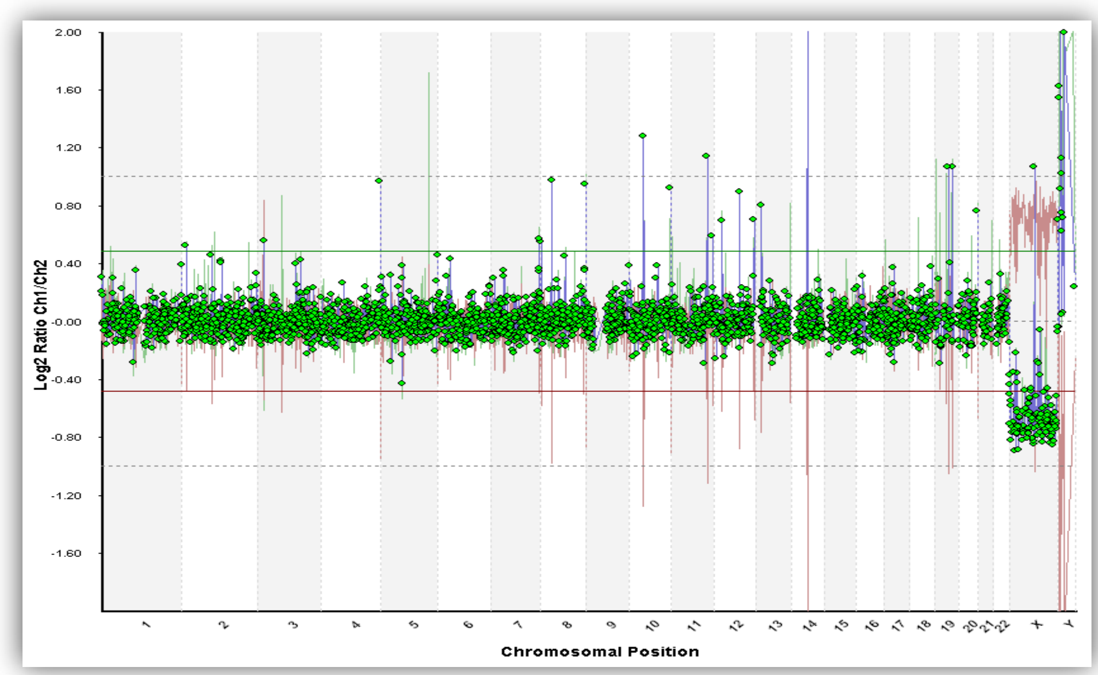


Figure 3.5.2.1.10: Array CGH analysis of patient P48 with the use of VIB microarray LOT # 2007 showing a noisy profile.

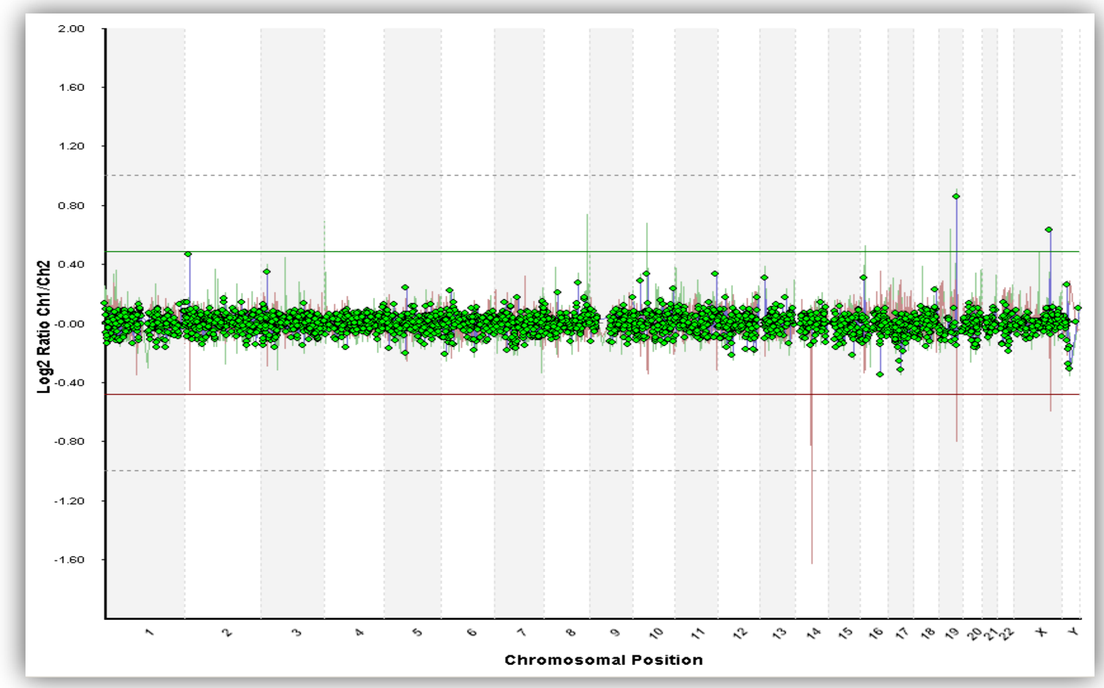


Figure 3.5.2.1.11: Array CGH analysis of patient P49 with the use of VIB microarray LOT 2007

Analysis of the array results from the last two patients did not give the expected results but we did not proceed with any further testing because we were expecting the analysis from the three patients we sent at the Wellcome Trust Sanger Institute.

We received electronically the results from these three patients (P1, P46, P47) but the Sanger Institute informed us that P47 (Figure 3.5.14) did not pass their quality control, therefore only the results from patients P1(Figure 3.5.12) and P46 (Figure 3.5.13) were informative; patient P47 was excluded from the study. Based on the good results from the other two patients (P1 and P46) we decided that this would be the platform to use for the rest of the samples in this Group (Group 2). Considering the fact now that the DNA from P1 did not pass the quality control we proceeded to the clean-up of this and another 30 samples from this group with phenol chloroform extraction.

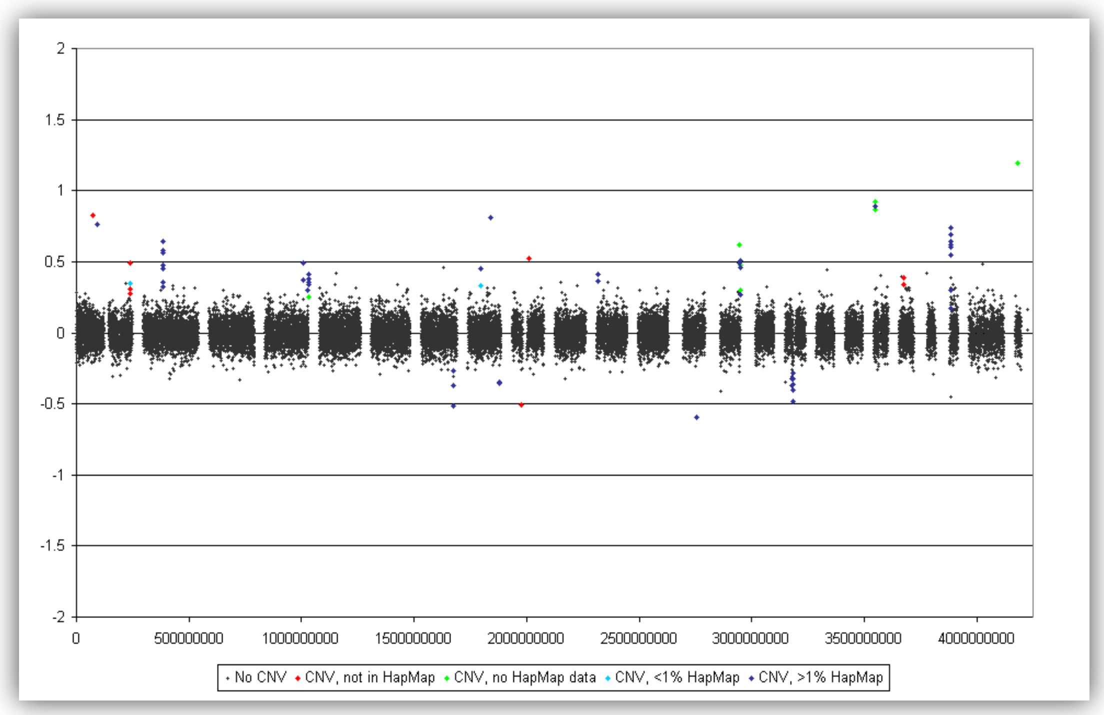


Figure 3.5.2.1.12: Array CGH analysis of patient P1 with the use of the Wellcome Trust Tiling Path BAC array. Analysis was done with the Institute's in house software.

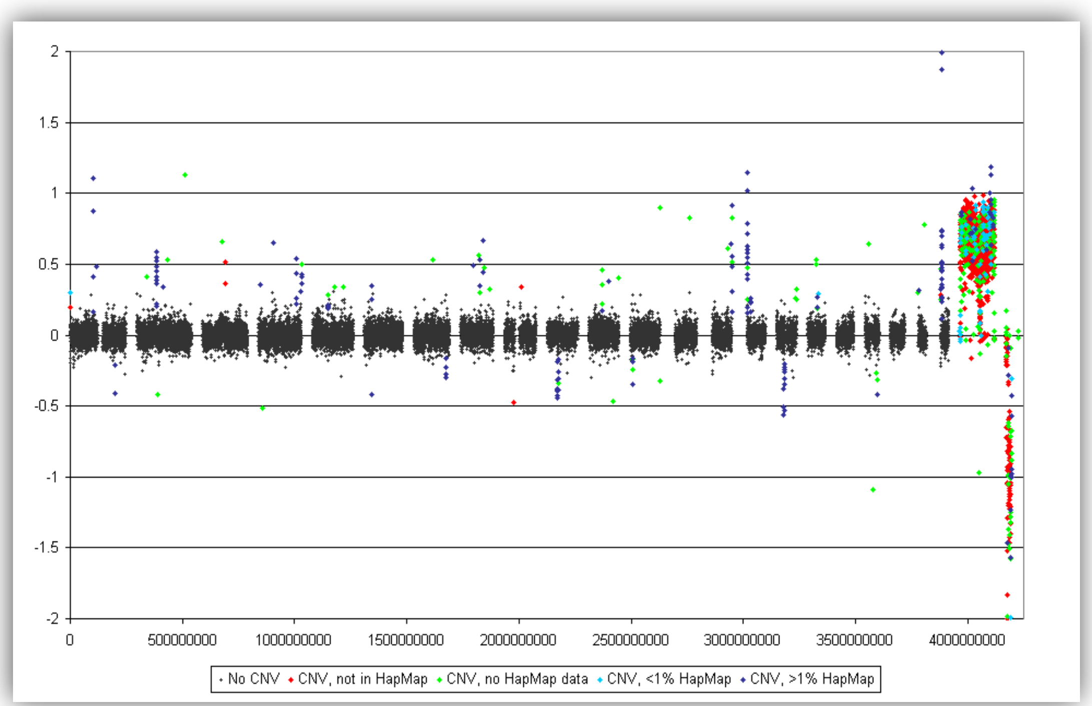


Figure 3.5.2.1.13: Array CGH analysis of patient P46 with the use of the Wellcome Trust Tiling Path BAC array. Analysis was done with the Institute's in house software.

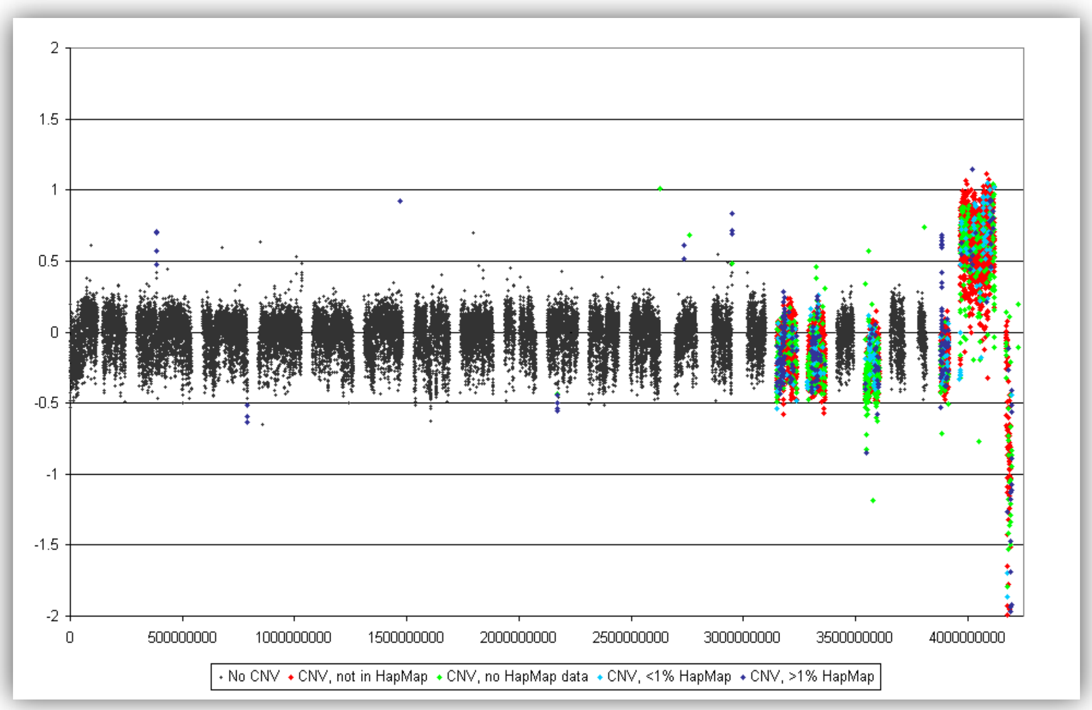


Figure 3.5.14: Array CGH analysis of patient P47 with the use of the Wellcome Trust Tiling Path BAC array. Analysis was done with the Institute's in house software.

As mentioned previously out of the 74 samples initially collected for this Group only 35 passed the quality control for the application of array CGH with the Wellcome Trust Tiling Path BAC microarrays. One such sample is P47 as shown in Figure 3.5.2.1.14, which gave a poor profile and was excluded from the study.

3.5.2.2) Array CGH analysis results

Array CGH analysis of the 34 patients in Group 2 revealed 627 Copy Number Changes (CNC). All detected CNC were compared to known aberrations listed in publically available databases, such as the DECIPHER (**D**atabas**E** of **C**hromosomal **I**mbalance and **P**henotype in **H**umans using **E**nsembl **R**esources http://decipher.sanger.ac.uk) and the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation/) using NCBI136/hg18 UCSC assembly. Based on the information obtained from these databases the CNCs that were found within normal copy number variant regions were excluded from any further investigation. These CNCs were considered of no clinical significance as they could also be found in normal controls and thus were most probably not associated with the clinical findings. Table 3.5.2.2 lists the common CNCs among all or some of the fetuses in this group.

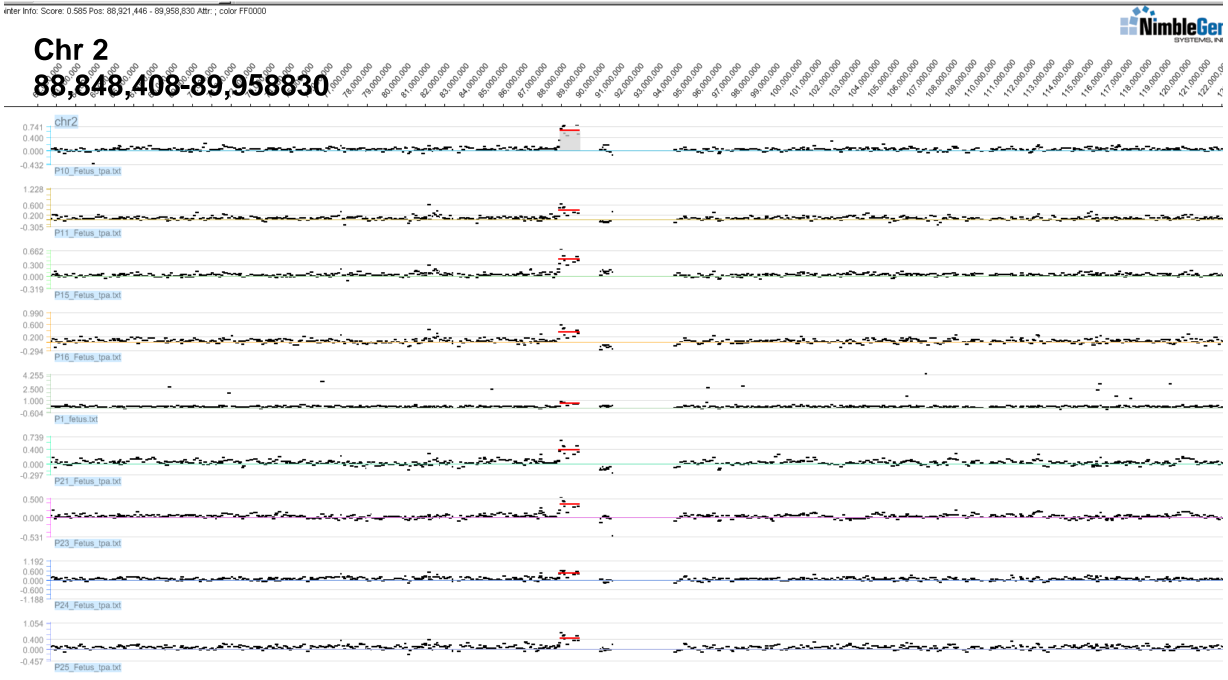
Table 3.5.2.2: Common CNCs found during analysis of the 34 fetuses with the Wellcome Trust Tiling Path microarray.

Fetus	Chromosome	Position(NCBI Build 36, Hg18)	CNV
P9,P6,P24,P25,P29,P41,P44	1	377-3,454,889	Dup
P1,P4,P6,P7,P9,P10,P11,P15,P16,P21,P23,P24, P25, P26,P27,P28,P30,P31,P32,P33, P34,P35,P36,P37,P38,P41,P43, P44,P45,P46	2	88,848,408-89,958830	Dup
P15,P26,P33,P35,P37,P39,P41	5	69,109,876-70,425,468	Dup
P6,P9,P24,P29	7	42,475-1,656,473	Dup
P16,P44,P45	7	143,370,074-143,828,603	Del
P15,P33,P34,P38,P43	7	143,370,074-143,828,603	Dup
P6,P9,P16,P29	7	61,087,267-62,014,044	Dup
P7,P9,P10,P15,P21,P23,P24,P25, P26,P27,P30,P31,P32,P34,P35, P37,P38,P39,P43,P44,P45,P46	10	46,232,533-47,972,148	Del
P6,P9,P11,P29	14	103,592,318-105,545,377	Dup
P4,P21,P24,P25,P27,P44	14	103,592,318-106,339,477	Dup

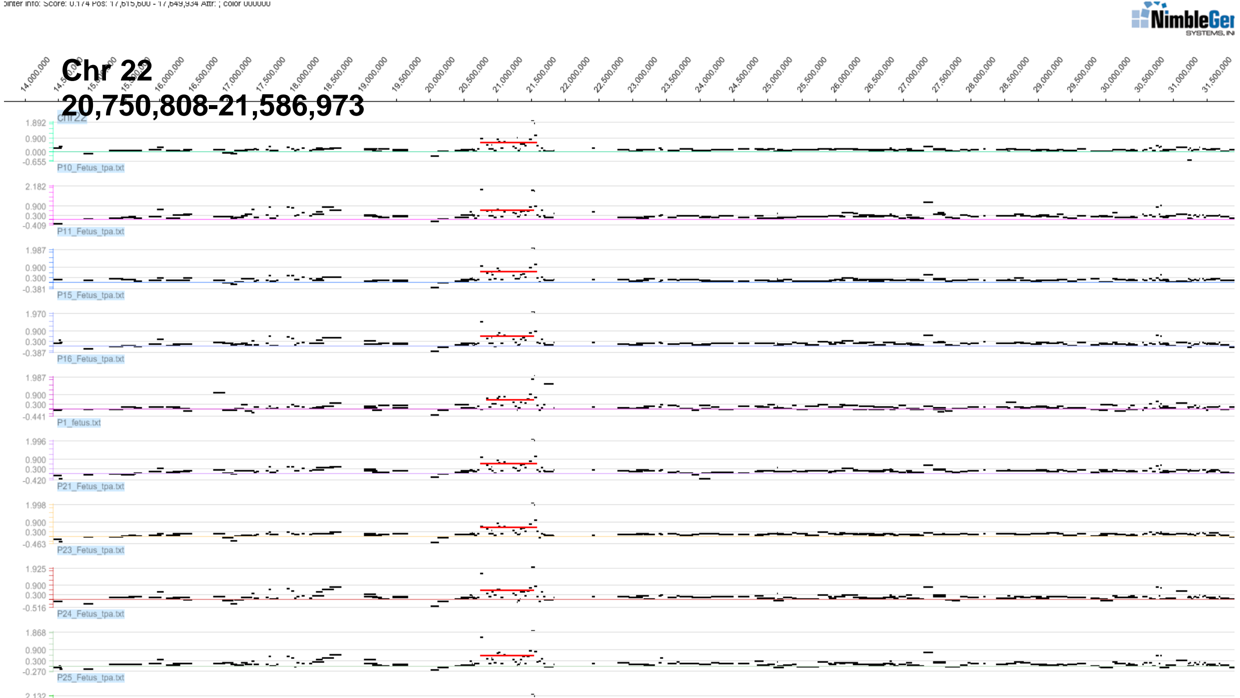
Fetus	Chromosome	Position(NCBI Build 36, Hg18)	CNV
P10,P16,P34	14	105,264,727-106,339,477	Dup
P7,P15,P23,P26,P28,P30,P31,P32,P35,P37,P43, P45,P46	14	105,264,727-105,954,605	Dup
P15,P30,P33,P41,P46	15	18,263,733-20,224,003	Dup
P26	15	18,263,733-20,224,003	Del
P16,P29,P34	15	18,263,733-21,365,850	Del
P23,P28,P45	17	41,439,734-42,110,774	Dup
P4,P6,P11,P15,P21,P24,P25,P28, P29,P30,P35,P44	19	74,843-2,018,523	Dup
P1,P4,P6,P7,P10,P11,P15, P16,P21,P23,P24,P25,P26,P27, P28,P30,P31,P32,P33,P34,P35, P36,P37,P38,P39,P41,P43,P44,P45,P46	22	20,750,808-21,586,973	Dup

Most of the 627 CNCs were considered benign. Figure 3.5.2.2 shows the two most common CNCs in this group:

- Copy number gain of approximately 1,1Mb on chromosome 2
- Copy number gain of approximately 836kb on chromosome 22.



A



B

Figure 3.5.2.2 : Most common CNCs found in the analysis of the 34 fetuses, with normal karyotype and ultrasound findings, analyzed with the Wellcome Trust Tiling Path BAC microarray, on chromosomes 2 (A) and 22 (B). Figure shows only part of the samples that showed the same polymorphism

3.5.2.3) Confirmations with 244,000 array

In 8 cases however it was determined from the analysis that confirmation of the findings was necessary (Figure 3.5.2.3.1). Confirmation was carried out with a higher resolution array of 244,000 oligonucleotides (Agilent Technologies) on these 8 fetuses and their parents (trio analysis- P4, P6, P11, P15, P27, P28, P31 and P45.). In addition to these patients', confirmation with 244K array was also carried out on another three samples for which we did not have parental DNA (P29) or we had DNA from one of the parents (P9, P17). The reason for carrying out these confirmations despite the fact that we did not have parental DNA or had DNA only from one parent, was so that we could confirm the findings with a second platform, other than the "Wellcome Trust Sanger Institute Tiling Path Array". Table 3.5.2.3 lists the abnormalities found on these patients and their confirmations.

More specifically for case P4 five CNCs were identified (Figure 3.5.2.3.2) three of which were inherited form both parents, one was only identified in the mother another one which was found on all of the 34 cases studied in this group.

Inherited from both parents:

- Copy number gain of approximately 350kb in size on chromosome 2
- Copy number gain of approximately 526kb in size on chromosome 14
- Copy number gain of approximately 73kb on chromosome 22

Inherited only from the mother:

- Copy number gain of approximately 77kb on chromosome 15

Found in all the cases in this group:

- Copy number gain of approximately 690kb on chromosome 22

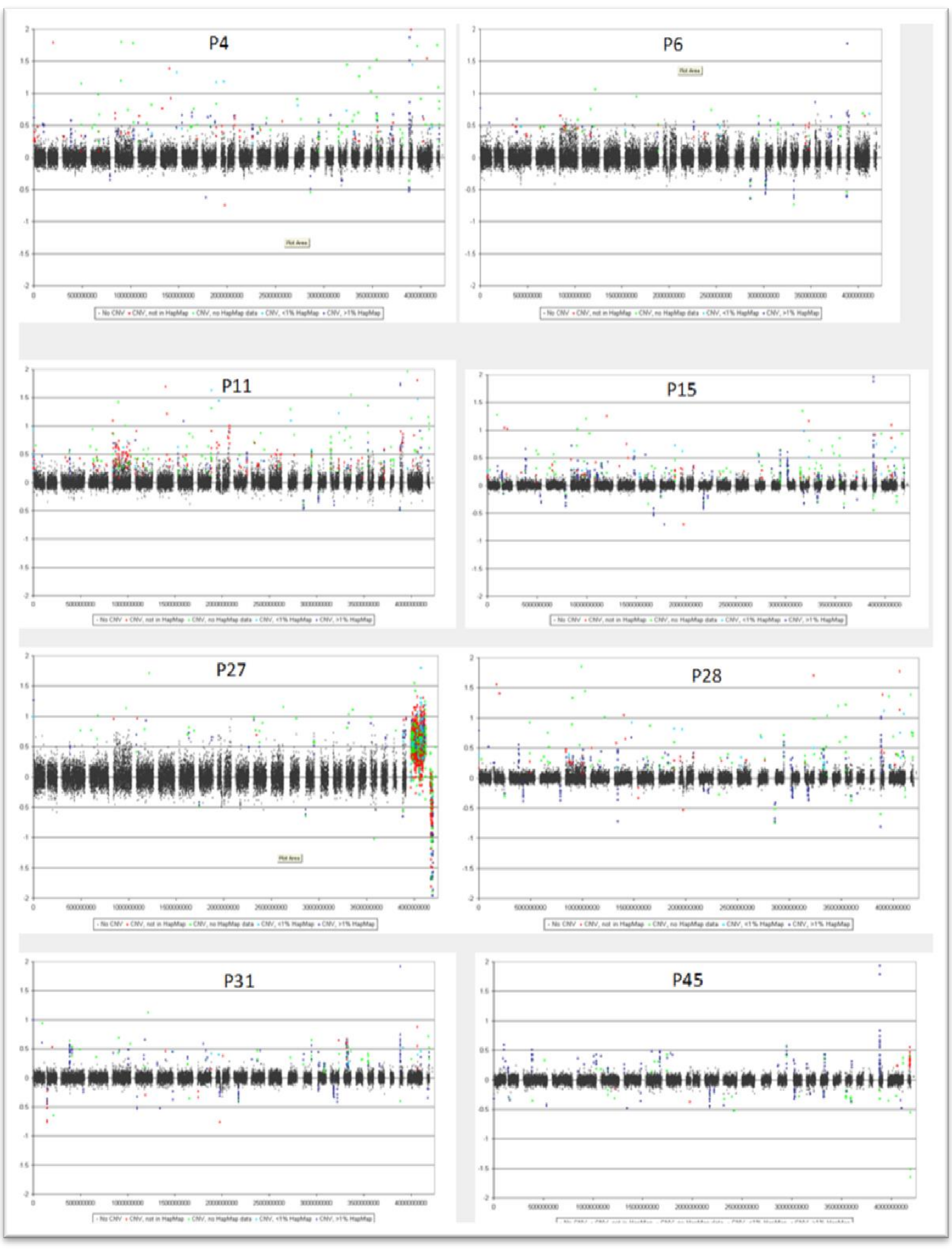
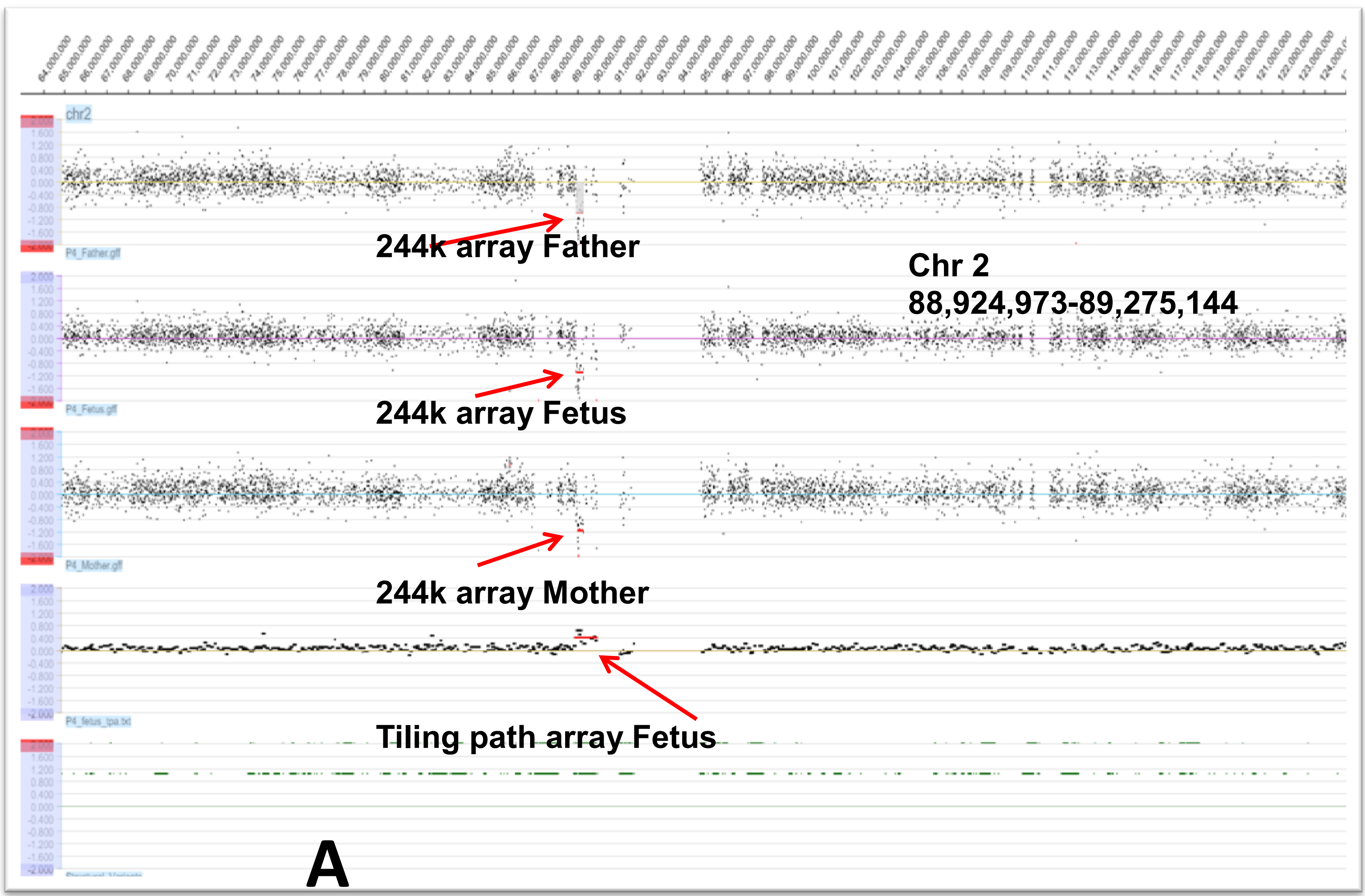
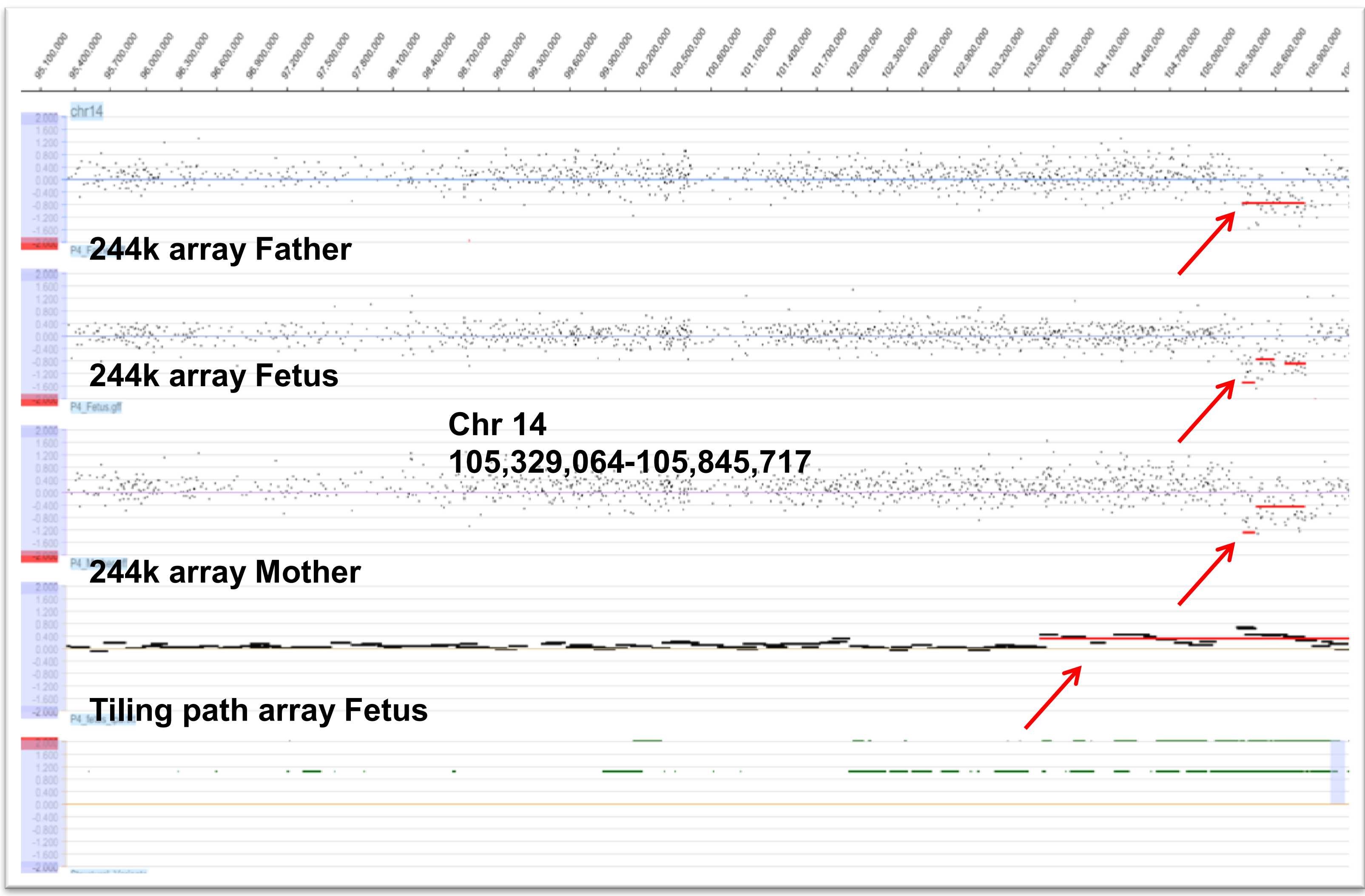
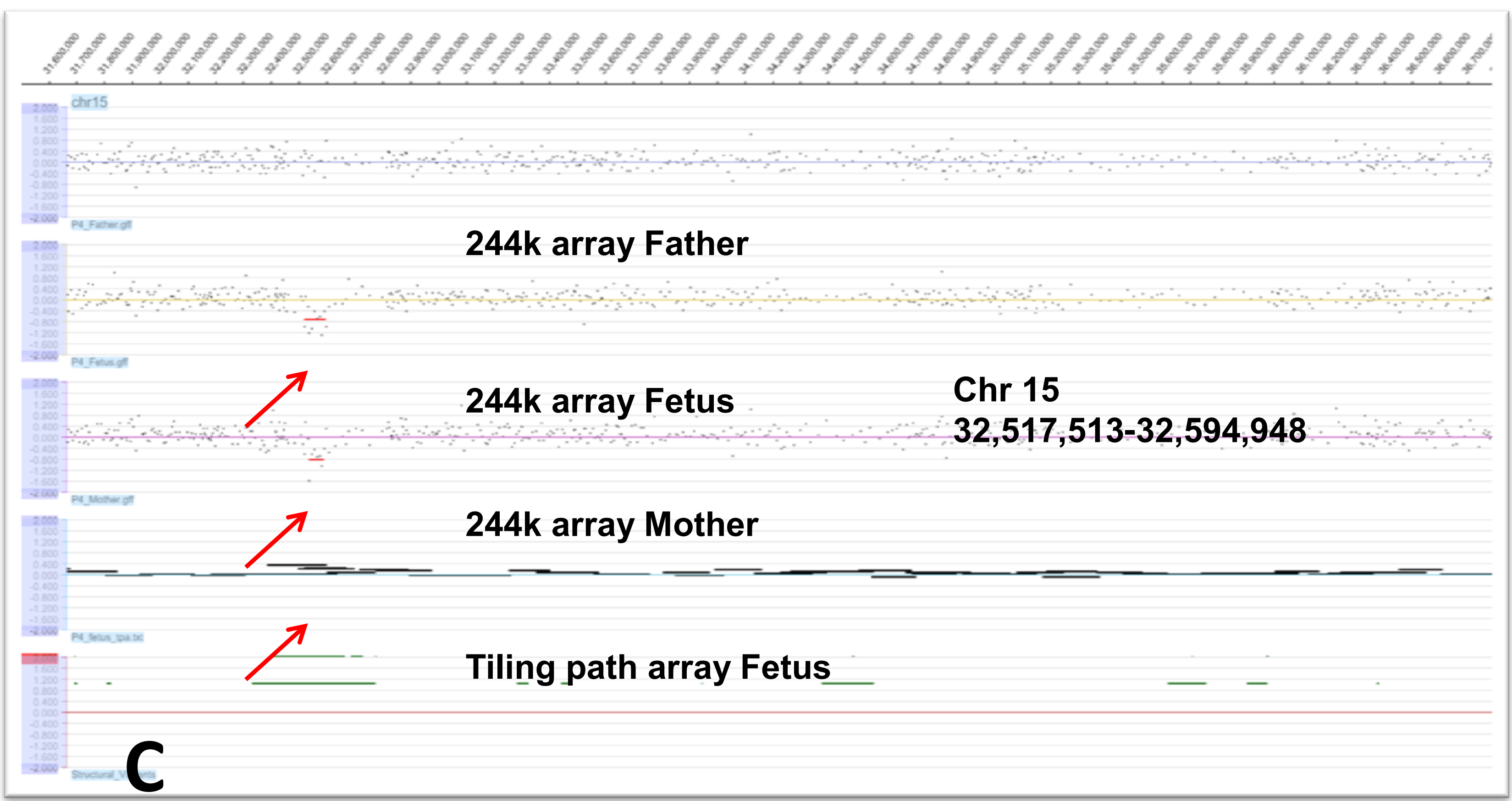


Figure 3.5.2.1: Array CGH analysis of patient P4, P6, P11, P15, P27, P28, P31 and P45 with the use of the Wellcome Trust Tiling Path BAC array. Analysis was done with the Institute's in house software


chr2
244k array Father
Chr 2
88,924,973-89,275,144
244k array Fetus
244k array Mother
Tiling path array Fetus
A



B


chr15
244k array Father
244k array Fetus
Chr 15
32,517,513-32,594,948
244k array Mother
Tiling path array Fetus
C

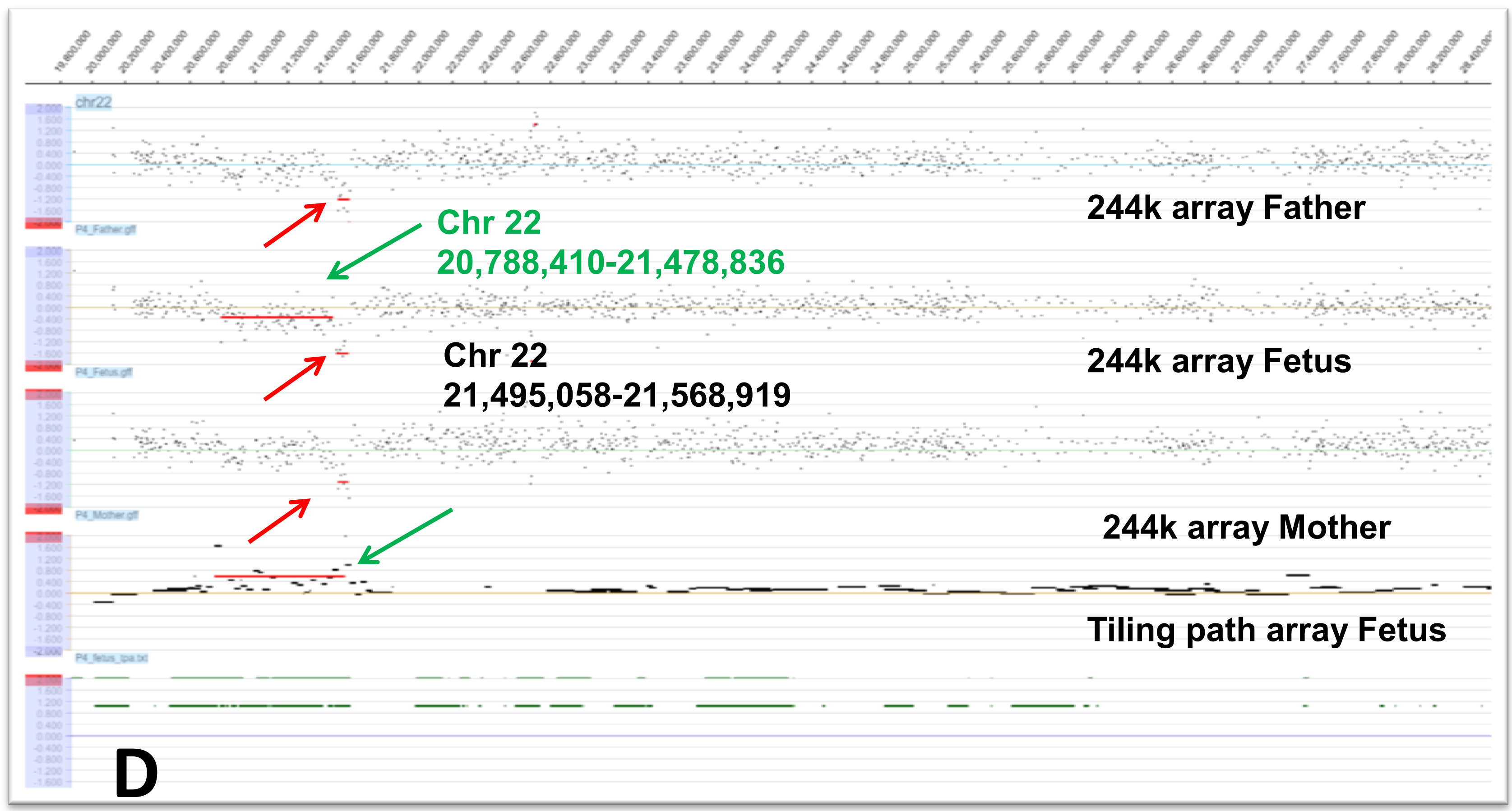


Figure 3.5.2.3.2: Array CGH analysis with 244K Agilent Technologies arrays in the fetus and the parents in parallel with the "Wellcome Trust Sanger Institute Tiling Path Array" in the fetus. Data analysis was done using SignalMap by Nimblegen. Shown here are CNCs in patient P4 which were inherited from the parents: chromosome 2 (A), chromosome 14 (B), chromosome 15 (C) and chromosome 22 (D). In images A, B and D the red arrows show inheritance from both parents whereas in image (C) inheritance was only from the mother. In addition in image (D) the green arrow shows the CNC which was also found in the rest of the patients in the study.

For case P6, 8 CNVs were identified, two of which were inherited from both parents, three were found only in the mother and three were found only in the father.

Inherited from both parents:

- Copy number gain of approximately 63kb in size on chromosome 22
- Copy number loss of approximately 18.5Mb in size on chromosome 22

Inherited from the mother:

- Copy number loss of approximately 35kb in size on chromosome 1
- Copy number gain of approximately 108kb in size on chromosome 4
- Copy number gain of approximately 75kb in size on chromosome 5

Inherited from the father:

- Copy number gain of approximately 956kb in size on chromosome 2
- Copy number gain of approximately 124kb in size on chromosome 14
- Copy number loss of approximately 83kb in size on chromosome 19

For case P11, 11 CNVs were identified eight of which were inherited in both parents, two were found only in the mother and one only in the father.

Inherited from both parents:

- Copy number gain of approximately 351kb in size on chromosome 2
- Copy number gain of approximately 459kb in size on chromosome 14
- Copy number gain of approximately 213kb in size on chromosome 15
- Copy number loss of approximately 219kb in size on chromosome 16
- Copy number gain of approximately 47kb in size on chromosome 17
- Copy number gain of approximately 795kb in size on chromosome 22
- Copy number gain of approximately 73kb in size on chromosome 22
- Copy number gain of approximately 72kb in size on chromosome 22
-

Inherited from the mother:

- Copy number loss of approximately 101kb in size on chromosome 2
- Copy number gain of approximately 34kb in size on chromosome 6

Inherited from the father:

- Copy number loss of approximately 70kb in size on chromosome 11

For case P15, 12 CNVs were identified six of which were inherited from both parents, five were also found in the mother and one was found in the father.

Inherited from both parents:

- Copy number gain of approximately 639kb in size on chromosome 1
- Copy number gain of approximately 350kb in size on chromosome 2
- Copy number gain of approximately 47kb in size on chromosome 6
- Copy number gain of approximately 689kb in size on chromosome 14
- Copy number gain of approximately 16kb in size on chromosome 15
- Copy number gain of approximately 123kb in size on chromosome 22

Inherited from the mother:

- Copy number gain of approximately 270kb in size on chromosome 3
- Copy number loss of approximately 19kb in size on chromosome 7
- Copy number gain of approximately 113kb in size on chromosome 12
- Copy number gain of approximately 174kb in size on chromosome 15
- Copy number gain of approximately 48kb in size on chromosome 17

Inherited from the father:

- Copy number loss of approximately 211kb on chromosome 2

For case P27, 8 CNVs were found of which six were inherited form both parents and two were also found in the mother.

Inherited from both parents:

- Copy number gain of approximately 351kb in size on chromosome 2
- Copy number gain of approximately 85kb in size on chromosome 5
- Copy number gain of approximately 136kb in size on chromosome 8
- Copy number loss of approximately 728kb in size on chromosome 10
- Copy number gain of approximately 99kb in size on chromosome 14
- Copy number gain of approximately 690kb in size on chromosome 22

Inherited from the mother:

- Copy number gain of approximately 459kb in size on chromosome 14
- Copy number gain of approximately 135kb in size on chromosome 7

For case P28, 6 CNVs were identified out of which four were inherited from both parents, one was found in the mother and one also found in the father.

Inherited from both parents:

- Copy number gain of approximately 1.1Mb in size on chromosome 2
- Copy number gain of approximately 80kb in size on chromosome 4
- Copy number gain of approximately 689kb in size on chromosome 14
- Copy number gain of approximately 839kb in size on chromosome 22

Inherited from the mother:

- Copy number gain of approximately 107kb in size on chromosome 5

Inherited from the father:

- Copy number gain of approximately 752kb in size on chromosome 14

For case P31, 12 CNVs were identified five of which were inherited from both parents, two were also found in the mother, and two were also found in the father. In addition one CNV identified in this case was also found in the entire 34 cases cohort of this group. Furthermore, two CNVs were identified which even though they are polymorphic in the normal population they were not found in either one of the parents and are therefore *de novo.*

Inherited from both parents:

- Copy number gain of approximately 359kb in size on chromosome 2
- Copy number gain of approximately 1.3Mb in size on chromosome 10
- Copy number gain of approximately 74kb in size on chromosome 12
- Copy number gain of approximately 196kb in size on chromosome 14
- Copy number gain of approximately 73kb in size on chromosome 22

Inherited from the mother:

- Copy number loss of approximately 70kb in size on chromosome 11
- Copy number gain of approximately 1.5Mb in size on chromosome 17

Inherited from the father:

- Copy number loss of approximately 2.2Mb in size on chromosome 1
- Copy number gain of approximately 118kb in size on chromosome 15

Found in all the cases in this group:

- Copy number gain of approximately 795kb in size on chromosome 22

De novo CNVs:

- Copy number gain of approximately 80kb in size on chromosome 4 (Figure 3.5.2.3.3A)
- Copy number loss of approximately 353kb in size on chromosome 16 (Figure 3.5.2.3.3B)

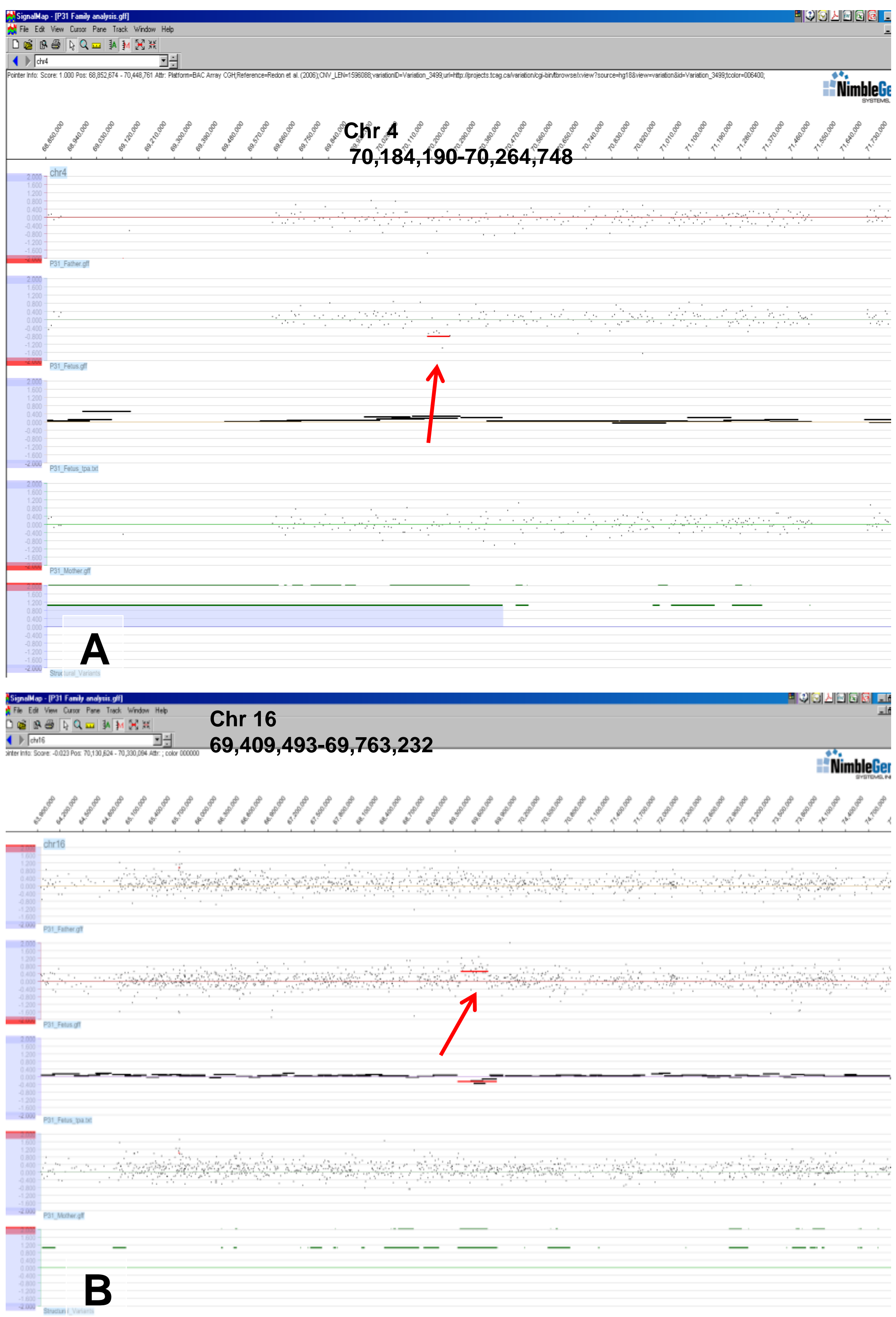


Figure 3.5.2.3.3: Array CGH analysis with 244K microarray by Agilent Technologies in the fetus (Patient P31) and the parents in parallel with the "Wellcome Trust Sanger Institute Tiling Path Array" only in the fetus. Data analysis was done using SignalMap by Nimblegen. Shown here are two *de novo* CVNs: one in chromosome 4 (A) and one in chromosome16 (B).

For case P45, 16 CNVs were identified three of which were inherited from both parents, two were also found in the mother and nine were also found in the father. Furthermore, two CNVs were identified which even though they are polymorphic in the normal population they were not found in either one of the parents and are therefore *de novo.*

Inherited from both parents:

- Copy number gain of approximately 689kb on chromosome 14,
- Copy number gain of approximately 224kb on chromosome 15
- Copy number gain of approximately 690kb on chromosome 22.

Inherited from the mother:

- Copy number gain of approximately 812kb in size on chromosome 1
- Copy number gain of approximately 97kb in size on chromosome 6

Inherited from the father:

- Copy number gain of approximately 242kb in size on chromosome 1
- Copy number gain of approximately 359Kb in size on chromosome 2
- Copy number gain of approximately 80kb in size on chromosome 4
- Copy number gain of approximately 97kb in size on chromosome 5
- Copy number gain of approximately 114kb in size on chromosome 5
- Copy number gain of approximately 72kb in size on chromosome 8
- Copy number gain of approximately 1.36Mb in size on chromosome 10
- Copy number gain of approximately 88kb in size on chromosome 16
- Copy number gain of approximately 551kb in size on chromosome 17

de novo CNVs:

- Copy number gain of approximately 148kb in size on chromosome 8 (Figure 3.5.2.3.4A)
- Copy number gain of approximately 39kb in size on chromosome 10 (Figure 3.5.2.3.4B)

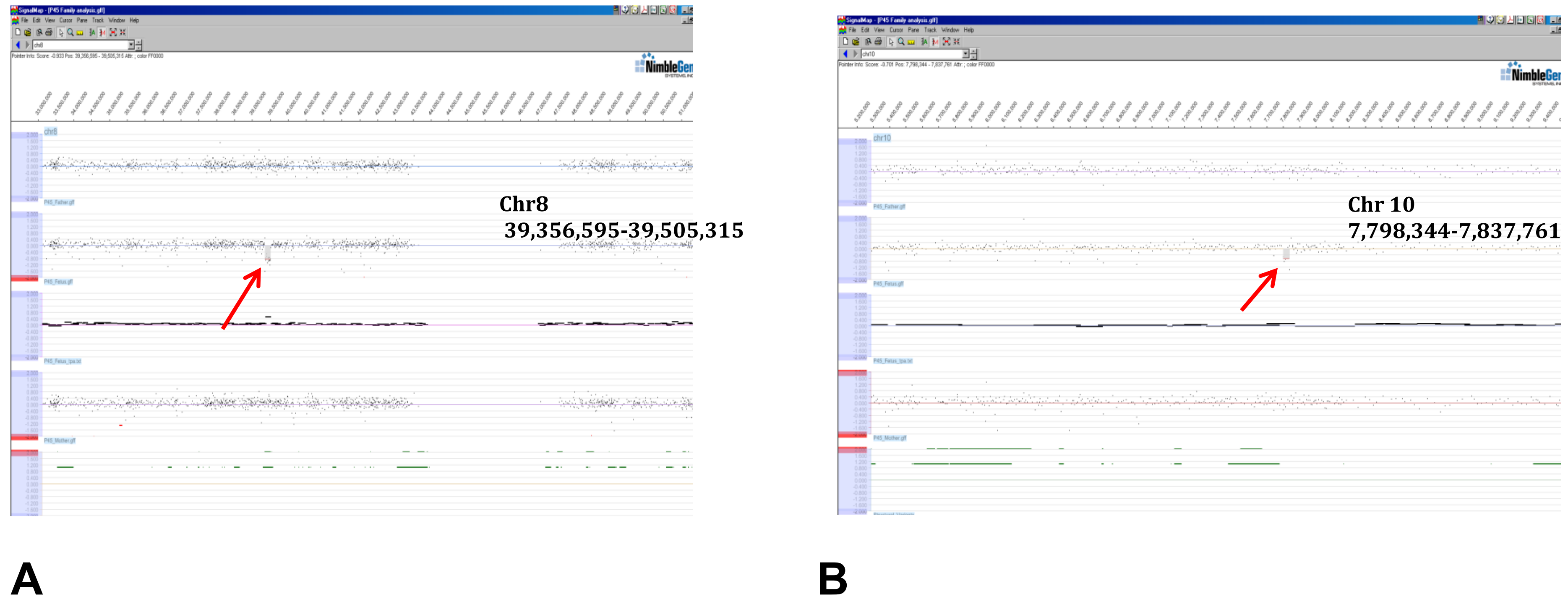


Figure 3.5.2.3.4: Array CGH analysis with 244K microarray by Agilent Technologies in the fetus (Patient P45) and the parents in parallel with the "Wellcome Trust Sanger Institute Tiling Path Array" only in the fetus. Data analysis was done using SignalMap by Nimblegen. Shown here are *de novo* CVNs: copy number gain in chromosomes 8 (A) and 10 (B).

Table 3.5.2.3: Copy number changes found after trio analysis with 244,000 Agilent arrays

Chromosome	Gain	Loss	Position	Size	Genome build	Inherited from	Tiling Path array	Real Time PCR Confirmations
P4								
2	√		88,924,973-89,275,144	350Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
14	√		105,329,064-105,845,717	526Kb	NCBI36/ hg18	Mother/Father	103,592,318-106,339,477 (2,7Mb)	Not Applicable
15	√		32,517,513-32,594,948	77Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
22	√		20,788,410-21,478,836	690Kb	NCBI36/ hg18	*de novo*	Same Abnormality Detected	Not Applicable
22	√		21,495,058-21,568,919	73Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
P6								
1		√	111,652,087-111,687,863	35kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
2	√		88,932,397-89,889,158	956Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
4	√		69,057,735-69,165,872	108Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
5	√		802,518-878,341	75Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
14	√		105,343,150-105,467,576	124Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
19		√	20,356,550-20,439,964	83Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable

Chromosome	Gain	Loss	Position	Size	Genome build	Inherited from	Tiling Path array	Real Time PCR Confirmations
22		√	20,750,808-21,546,701	18,5Mb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
22	√		21,495,058-21,558,869	63Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
P11								
2		√	214,539,670-214,641,428	101Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
2	√		88,932,397-89,284,297	351Kb	NCBI36/ hg18	Mother/Father	88,848,408-89,958,830 (1,1Mb)	Not Applicable
6	√		29,975,388-30,010,293	34Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
11		√	55,124,730-55,195,049	70Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
14	√		105,422,205-105,881,323	459Kb	NCBI36/ hg18	Mother/Father	103,592,318-105,545,377 (1,9Mb)	Not Applicable
15	√		32,454,294-32,667,567	213Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
16		√	28,732,295-28,952,277	219Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
17	√		41,577,520-41,624,530	47Kb	NCBI36/ hg18	Mother/Father	part of larger CNC in parents	Not Applicable
22	√		20,750,808-21,546,701	795Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
22	√		21,495,058-21,568,919	73Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable

Chromosome	Gain	Loss	Position	Size	Genome build	Inherited from	Tiling Path array	Real Time PCR Confirmations
22	√		22,677,959-22,750,254	72Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
P15								
1	√		147,307,637-147,946,964	639Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
2	√		88,924,973-89,275,144	350Kb	NCBI36/ hg18	Mother/Father	88,848,408-89,958,830 (1,1Mb)	Not Applicable
2		√	242,505,261-242,717,069	211Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
3	√		60,347,122-60,617,826	270Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
6	√		29,962,849-30,010,293	47Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
7		√	142,159,154-142,178,797	19Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
12	√		7,922,474-8,036,459	113Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
14	√		105,264,727-105,954,605	689Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
15	√		18,362,555-20,079,994	1,7Mb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
15	√		32,454,294-32,628,738	174Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
17		√	7,168,709-7,217,488	48Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable

Chromosome	Gain	Loss	Position	Size	Genome build	Inherited from	Tiling Path array	Real Time PCR Confirmations
22	√		21,452,488-21,575,888	123Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
P27								
2	√		88,932,397-89,284,297	351Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
4	√		70,159,690-70,264,748	105Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
5	√		763,494-848,803	85Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
8	√		39,368,509-39,505,315	136Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
10		√	46,404,919-47,133,339	128Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
14	√		105,314,254-105,413,767	99Kb	NCBI36/ hg18	Mother/Father	whole area duplicated 103,592,318-106,339,477 (2,7Mb)	Not Applicable
14	√		105,422,205-105,881,323	459Kb	NCBI36/ hg18	Mother/Father		Not Applicable
15	√		32,443,495-32,578,542	135Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
22	√		20,788,410-21,478,836	690Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
P28								
2	√		88,848,408-89,958,830	1,1Mb	NCBI36/ hg18	Partially Mother/Father	Same Abnormality Detected	Not Applicable
4	√		70,184,190-70,264,748	80Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable

Chromosome	Gain	Loss	Position	Size	Genome build	Inherited from	Tiling Path array	Real Time PCR Confirmations
5	√		771,310-878,341	107Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
14	√		18,732,531-19,485,397	752Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
14	√		105,264,727-105,954,605	689Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
22	√		20,750,808-21,586,973	836Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
P31								
1		√	144,967,596-147,203,336	2,2Mb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
2	√		88,924,973-89,284,297	359Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
2		√			NCBI36/ hg18		111,616,254-112,379,068 (762Kb)	Not Applicable
4	√		70,184,190-70,264,748	80Kb	NCBI36/ hg18	Deletion in parents	Same Abnormality Detected	Dup in parents del in fetus
10		√	46,396,163-47,735,531	1,3Mb	NCBI36/ hg18	Not Confirmed	Same Abnormality Detected	Deletion not confirmed
11		√	55,124,730-55,195,049	70Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
12	√		131,486,691-131,561,277	74Kb	NCBI36/ hg18	Deletion in parents	Same Abnormality Detected	Not Applicable
14	√		105,405,952-105,602,815	196Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
15	√		32,536,263-32,654,620	118Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable

Chromosome	Gain	Loss	Position	Size	Genome build	Inherited from	Tiling Path array	Real Time PCR Confirmations
16		√	69,409,493-69,763,232	353KB	NCBI36/ hg18	Not Confirmed	Same Abnormality Detected	Deletion not confirmed
17	√		31,799,968-33,322,352	1,5Mb	NCBI36/ hg18	Father	Same Abnormality Detected	Present in 3 copies in fetus and in two copies in father
22	√		21,495,058-21,568,919	73Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
22	√		20,750,808-21,546,701	795Kb	NCBI36/ hg18	*de novo*	Same Abnormality Detected	Not Applicable
P45								
1	√		12,822,189-13,065,179	242Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
1	√		147,134,175-147,946,964	812Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Not Applicable
2	√		88,924,973-89,284,297	359Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
4	√		70,184,190-70,264,748	80Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
5	√		763,494-878,341	114Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
5		√	104,204,320-104,302,184	97Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
6	√		80,199,703-80,297,179	97Kb	NCBI36/ hg18	Not Confirmed	Same Abnormality Detected	Dup not confirmed
8	√		39,356,595-39,505,315	148Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Confirmed and also found in mother
8	√		57,188,409-57,260,887	72Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable

Chromosome	Gain	Loss	Position	Size	Genome build	Inherited from	Tiling Path array	Real Time PCR Confirmations
10	√		7,798,344-7,837,761	39Kb	NCBI36/ hg18	Mother	Same Abnormality Detected	Confirmed and also found in mother
P45								
10		√	46,371,243-47,735,531	1,4Mb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
14	√		Smaller duplications		NCBI36/ hg18	Mother/Father	105,264,727-105,954,605 (689Kb)	Not Applicable
15	√				NCBI36/ hg18		Same Abnormality Detected	Not Applicable
16		√	32,443,495-32,667,567	224Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable
17	√		41,559,185-42,110,774	551Kb	NCBI36/ hg18	Father	Same Abnormality Detected	Not Applicable
22	√		20,788,410-21,478,836	690Kb	NCBI36/ hg18	Mother/Father	Same Abnormality Detected	Not Applicable

3.5.2.4) Confirmations with Real- Time Polymerase Chain Reaction (PCR)

Real time PCR was used to confirm or exclude a CNC that was revealed by array CGH in two cases. More specifically:

- Group 2 Case P31

G banding analysis showed normal karyotype and Tiling path array CGH analysis showed possible CNCs on chromosomes 4q13.2, 10q11.22, 16q22.2 and 17q12. Real Time PCR analysis on fetal and parental DNA samples was carried out and DID NOT confirm the CNCs on chromosomes 10 and 16. The CNC on chromosome 4 was shown to be deleted in the parents and of normal Copy Number in the fetus. The CNC on chromosome 17 appears to be in a polymorphic region and it is present in 3 copies in the fetus and in two copies in the father. Figure 3.5.2.4.1 shows the schematic representation of the results.

- Group 2 Case P45

G banding analysis showed normal karyotype and Tiling path array CGH analysis showed possible CNCs on chromosomes 6q14.1, 8p11.23, and 10p14. Real Time PCR analysis on fetal and parental DNA samples was carried out and DID NOT confirm the CNCs on chromosome 6. The CNC on chromosomes 8 and 10 were confirmed and it was revealed that they were of maternal inheritance. Figures 3.5.2.4.1 and 3.5.2.4.2 show the schematic representation of the results.

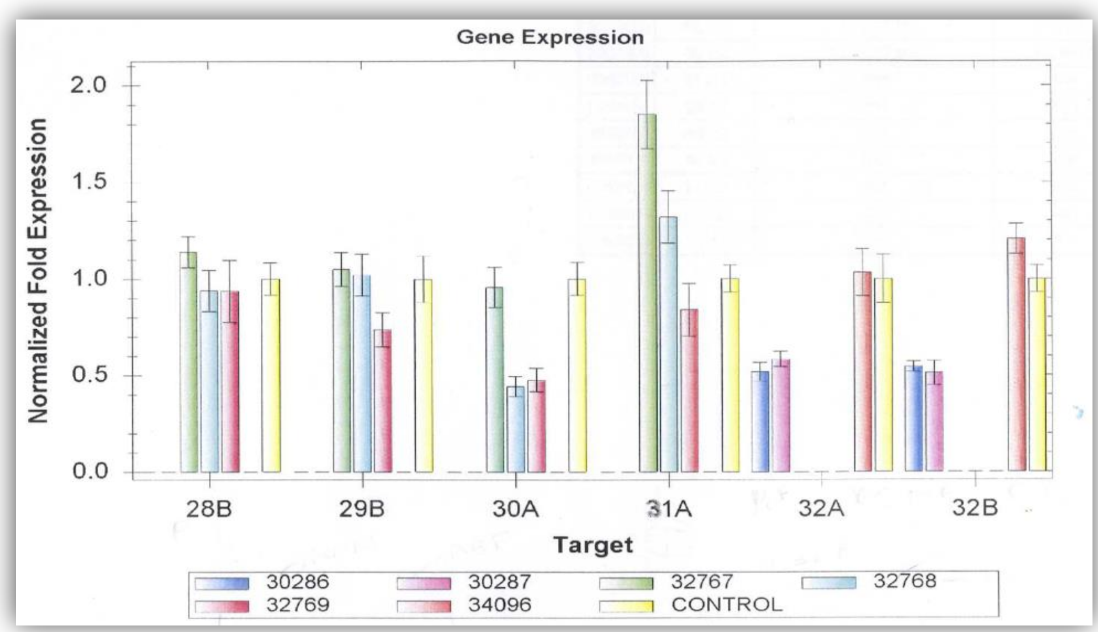


Figure 3.5.2.4.1: Figure represents the Real Time PCR experiment with six setsof primers used to confirm the copy number changes found in the 244K oligo array in fetuses P31 and P45. Fold copy number change of value equal to 1 (test sample /normal sample) indicate an equal ratio of the target and reference, which corresponds to no loss;a deleted region is expected to give a ratio value of 0.5±0.15 whereas a duplicated region is expected to give a value of 1.5 ±0.2. Primer sets 28, 29, 30 and 31 were used for Fetus from case P31. Primer sets 32A and 32B were used for fetus P45. Control DNA showing fold copy number change equal to 1 is shown in yellow.

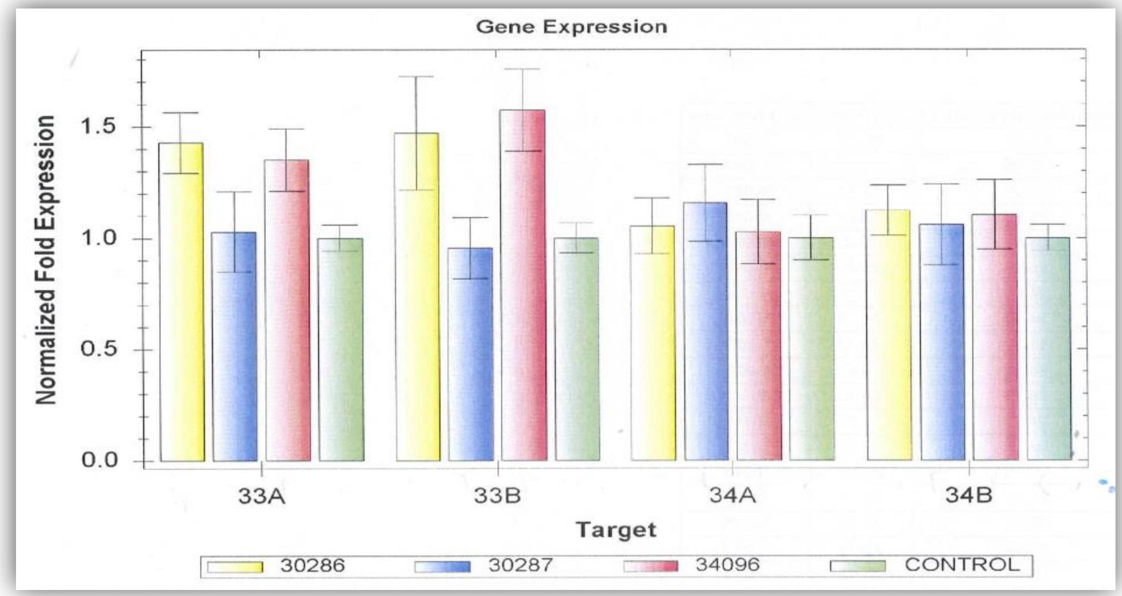


Figure 3.5.2.4.2: Figure represents the Real Time PCR experiment with four sets of primers used to confirm the copy number changes found in the 244K oligo array in fetus P45. Fold copy number change of value equal to 1 (test sample /normal sample) indicate an equal ratio of the target and reference, which corresponds to no loss; a deleted region is expected to give a ratio value of 0.5±0.15 whereas a duplicated region is expected to give a value of 1.5 ±0.2. Primer sets 33 and 34 were used for fetus from case P45. Control DNA showing fold copy number change equal to 1 is shown in green.

3.5.3) Group 3

Out of the 73 POC samples, sixteen cases were completed by QF PCR analysis only as the causative abnormality was detected and there was no need to proceed with any further investigation and 57 cases were further investigated using array CGH analysis. Out of the 16 abnormalities detected with QF PCR analysis 13 were first trimester and 3 were second trimester miscarriages; 4 triploidies, 8 autosomal trisomies and 4 sex chromosome abnormalities. Out of the 57 cases investigated with array CGH, 49 were analyzed using BAC arrays and 8 were analyzed using Oligo arrays. A total of 9 aneuploidies were detected by BAC arrays analysis, of which 7 were autosomal and 2 were sex chromosome aneuploidies. Eight of those aneuploidies were first trimester and one was second trimester miscarriage. In addition 1 benign familial CNC was detected by oligo array analysis. The abnormalities are listed in Table 3.5.31.

Figure 3.5.3 compares the abnormalities determined by QF PCR analysis versus the abnormalities found by array CGH analysis and Table 3.5.32 lists the abnormalities per trimester and method analyzed.

Table 3.5.31: Abnormalities revealed on POC samples

Case	Sample	Result	Status	Method of detection	GA	Reason For Referral/ Diagnosis/ Diagnostic Info
1	Products Of Conception	arr 20p13(566,613-758,298)x3 mat	Familial Benign CNC	105K Oligo array	16	Intrauterine Death (IUD), Karyotype 46,XX
2	Products Of Conception	arr 16p13.3q24.3(1-88,674,699)x3	Trisomy 16	1Mb BAC array	5,4	Missed Abortion
3	Products Of Conception	arr 15q11.2q26.3(1-100,171,678)x3	Trisomy 15	1Mb BAC array	8	Intrauterine Death
4	Products Of Conception	arr 16p13.3q24.3(1-88,447,848)x3	Trisomy 16	1Mb BAC array	8	Missed Abortion, Recurrent Miscarriages X2
5	Products Of Conception	arr 22q11.1q13.33(1-49,265,116)x3	Trisomy 22	1Mb BAC array	8	Missed Abortion, Empty Sac, Recurrent Miscarriages X3
6	Products Of Conception	arr 3p26.3q29(1-199,134,692)x3	Trisomy 3	1Mb BAC array	8	Missed Abortion, Failed In Vitro Fertilization (IVF) X5
7	Products Of Conception	arr(1-22)x2,(X)x1-Cytochip	Turner Syndrome	1Mb BAC array	8	Missed Abortion
8	Products Of Conception	arr 9p24.3q34.3(1-140,195,965)x3	Trisomy 9	1Mb BAC array	9	Missed Abortion, IVF pregnancy
9	Products Of Conception	arr 14q11.1q32.33(1-106,284,846)x3	Trisomy 14	1Mb BAC array	10	Missed Abortion
10	Skin, Placenta	arr(1-22)x2,(X)x3-Cytochip	Trisomy X	1Mb BAC array	22	Termination of Pregnancy due to Ultrasound abnormalities (NT)
11	POC	QF PCR analysis consistent with triploidy-male	Triploid	QF PCR	6,2	Missed Abortion, Previous Abortion was an abnormal male triploid karyotype
12	Products Of Conception	Consistent with trisomy 22	Trisomy 22	QF PCR	7,3	Missed Abortion, Recurrent Miscarriages
13	Products Of Conception	Consistent with trisomy 13	Trisomy 13	QF PCR	8	Missed Abortion, Recurrent Miscarriages X2
14	Products Of Conception	Consistent with trisomy 16	Trisomy 16	QF PCR	8	Missed Abortion
15	Products Of Conception	Consistent with trisomy 16	Trisomy 16	QF PCR	8	Missed Abortion, Recurrent Miscarriages X2
16	Products Of Conception	Consistent with Turner Syndrome	Turner Syndrome	QF PCR	8	Missed Abortion
17	Products Of Conception	Consistent with Turner Syndrome	Turner Syndrome	QF PCR	8	Missed Abortion, IVF pregnancy
18	Products Of Conception	Consistent with trisomy 16	Trisomy 16	QF PCR	8,1	Missed Abortion, Recurrent Miscarriages X2
19	Products Of Conception	Consistent with triploidy	Triploid	QF PCR	9	Missed Abortion

Case	Sample	Result	Status	Method of detection	GA	Reason For Referral/ Diagnosis/ Diagnostic Info
20	Products Of Conception	Consistent with Turner Syndrome	Turner Syndrome	QF PCR	9,5	Missed Abortion
21	Products Of Conception	Consistent with trisomy 21 (QF-PCR)	Trisomy 21	QF PCR	10	Missed Abortion, No fetal heart detected
22	Products Of Conception	Consistent with trisomy 18	Trisomy 18	QF PCR	11	Missed Abortion
23	Fetus & Placenta	Consistent with trisomy 21 (QF-PCR)	Trisomy 21	QF PCR	13	Missed Abortion, Unembryonic Gestation
24	Products Of Conception	Consistent with mosaic Klinefelter Syndrome in Products of Conception	Klinefelter Syndrome	QF PCR	14	Missed Abortion
25	Embryo and Placenta	Consistent with triploidy	Triploid	QF PCR	15	Missed Abortion, Recurrent Miscarriages X4
26	Embryo	QF PCR analysis consistent with triploidy-female	Triploid	QF PCR	15	Missed Abortion

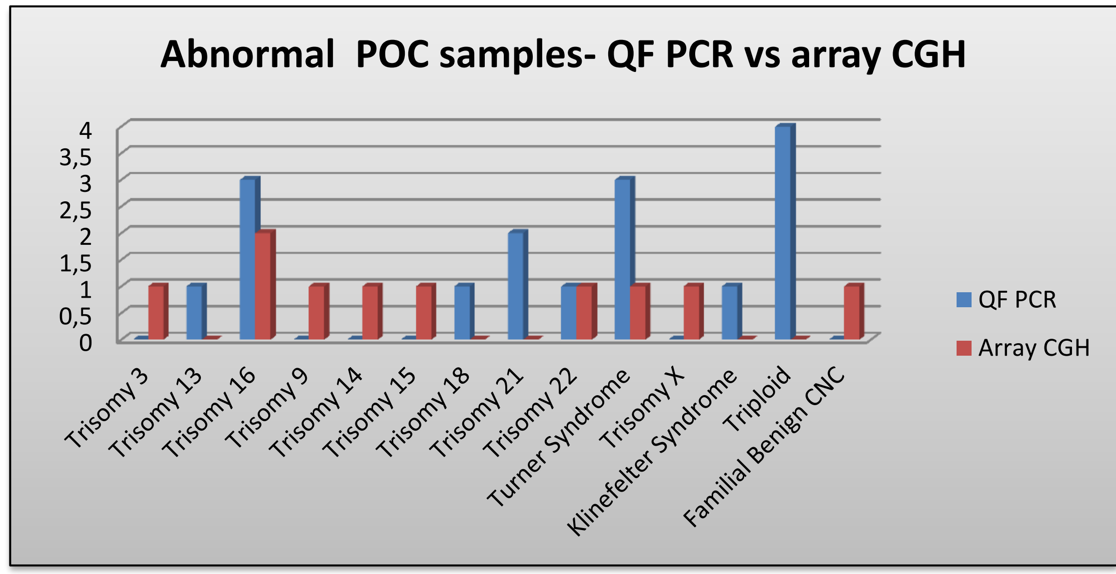


Figure 3.5.3: Comparison of abnormalities determined in POC samples with QF PCR vs array CGH analysis.

Table 3.5.32: Abnormalities detected in Group 3 samples per trimester and method analyzed

Trimester	QF PCR		BAC arrays		Oligo arrays		Total
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	
1st trimester (1-13 weeks)	0	13	18	8	2	0	41
2nd trimester (14-27 weeks)	0	3	17	1	3	1	25
3rd Trimester (28- 40 weeks)	0	0	5	0	2	0	7
Total							73

3.5.3.1) Quantitative Fluorescent Polymerase Chain Reaction (QF PCR)

QF PCR successfully identified the reason of the spontaneous abortion by revealing abnormal results as shown in figure 3.5.3.1, in 16 out of the 73 Group 3 cases and included:

- 4 triploidies,
- 8 autosomal trisomies
- 4 sex chromosome abnormalities

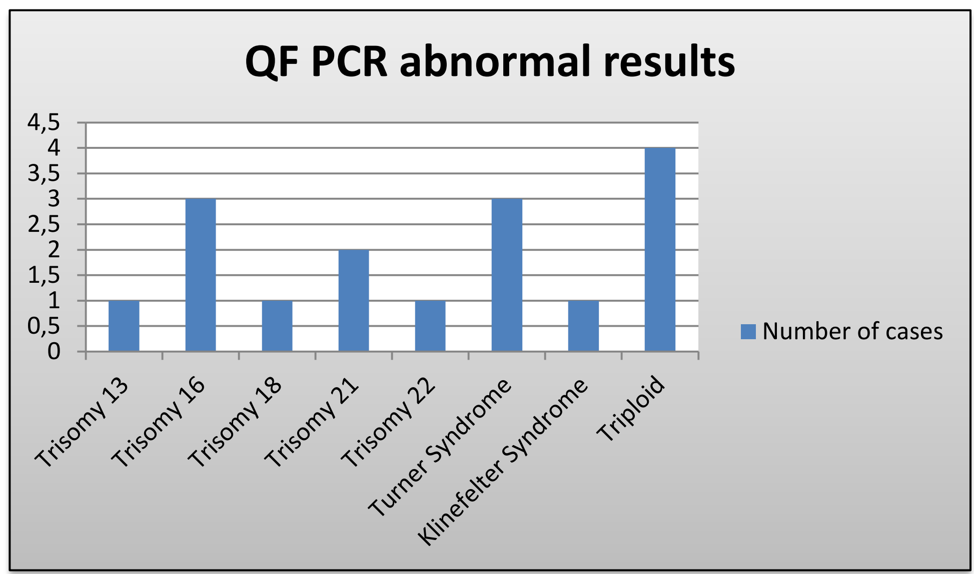


Figure 3.5.3.1.1: Schematic view showing the different abnormalities found in POC samples with QF PCR

FIGURES 3.5.3.1.2 and 3.5.3.1.3 in Annex show an electropherogram of a case with maternal contamination and an abnormal triploid case.

4) Discussion

4.1) Array CGH detection rate in Prenatal Diagnosis

Array CGH is a high throughput method which can be applied and detect copy number changes to a resolution of even as low as 1Kb. It has replaced chromosomal analysis in postnatal diagnosis, for certain referrals in many laboratories and is currently used as a First-Tier clinical diagnostic test for individuals with developmental delay [47]. In prenatal diagnosis chromosomal analysis still remains the First-Tier test. Many groups have demonstrated that by applying array CGH there was an additional detection of clinically significant genomic imbalances of 3.6% when the karyotype was normal, regardless of the indication of the referral for chromosomal analysis. This detection rate increased to 5.2% when the pregnancy had a structural malformation on ultrasound [58-65]. In these studies the overall detection of array CGH over chromosomal analysis was 12%. When benign CNVs were removed and considered as normal results the detection rate dropped to 3.6%[66]; this percentage included the pathogenic CNVs as well as the Variants of Unknown Significance (VOUS) with a potential of being pathogenic. The presence of VOUS was found in 1.1 % of cases [66]. As mentioned above the detection rate was increased for the cases where the referral included ultrasound abnormalities and a normal karyotype. In these studies the overall detection of array CGH was 11.2% and when benign variants were excluded and included in the normal results the detection rate dropped to 5.2% [59,61-65]. Furthermore, the presence of VOUS was in 1.9% of the studies. The ultrasound findings included cardiac abnormalities, increased nuchal translucencies, cystic hygromata or hydrops or central nervous system abnormalities. Most of these studies used Targeted BAC arrays [58-63,65] and some used both targeted and whole genome arrays [59,60,65]. The resolution for the arrays varied from 287 to 4685 BAC probes and 44,000 to 946,000 oligonucleotide probes.

Tyreman et al. conducted a retrospective analysis of 106 karyotypically normal referrals with ultrasound findings using the GeneChip 6.0 SNP array from Affymetrix. This platform provides uniquely high resolution coverage of the genome with over 1.8 million probes, using oligonucleotide targets that provide copy number information only and Single Nucleotide Polymorphisms (SNPs) oligonucleotide targets which provide genotyping as well as copy number information. In this study a total of 35 rare CNVs were identified, 10 (9%) of which were considered to be pathogenic, 12 were likely to be benign (11%) and 13 were VOUS (12%). The percentage of VOUS is slightly higher than the other studies because parental testing was not used in this study for their clarification. In addition in this study a case with a cryptic mosaic trisomy for chromosome 10 was identified as well as a case with Loss of Heterozygosity (LOH).The same platform can detect triploidy as well which is a major advantage; one of the limitations of aCGH is its inability to detect triploidies [64]. Table 4.1 shows the comparison between these studies.

Table 4.1: Comparison between various studies which used array CGH in Prenatal diagnosis

Study	Array Type	Karyotype/ Reason for Referral	Results	Clinical Significance of Results
Kleeman et al., 2009	Signature prenatal targeted BAC chip V, signature whole genome chip	Normal karyotype, sonographic anomalies	4/50 abnormal	2% clinically significant, 6% inherited or benign variant
Vialard et al., 2009	Targeted Genosensor BAC/PAC array	Normal karyotype, multiple congenital abnormalities	4/37 abnormal	10.8% clinically significant
Bi et al., 2008	BCM V6 oligonucleotide array	Normal karyotype, maternal age, sonographic anomalies, family history, miscarriages	3/15 abnormal	13% clinically significant, 7% inherited or benign variant
Shaffer et al., 2008	Prenatal targeted BAC array	149/151 normal karyotype, maternal age, sonographic anomalies, family history, parental anxiety	15/151 abnormal	1.3% clinically significant, 8% benign, 0.5% unclear significance

Study	Array Type	Karyotype/ Reason for Referral	Results	Clinical Significance of Results
Sahoo et al., 2006	BCM V4 targeted BAC array	93/98 normal karyotype, maternal age, sonographic anomalies, family history	5/98 abnormal of which one had additional abnormalities	5% clinically significant
Tyreman et al., 2009	GeneChip SNP whole genome oligonucleotide array	sonographic abnormalities	35/106 abnormal	9% likely pathogenic, 12% likely benign, 13% unclear significance
Coppinger et al., 2009	Signature V 4.0, prenatal targeted BAC array and whole genome array	Normal karyotype, maternal age, sonographic anomalies, family history, anxiety	**Whole genome**: 22/180 abnormal. **Targeted**: 7/62 abnormal	**Whole genome**: 2.7% clinically significant, 0.5% unclear significance, 8.8% benign variants. **Targeted**: 0.9% clinically significant, 0.5 unclear significance, 8% benign variants
Fiorentino et al., 2011	Cytochip Focus BAC array	maternal age, sonographic anomalies, family history, anxiety	Whole Genome	**3.3%** clinically significant, 13% benign variants.

In another study completed by Fiorentino et al. [67] pregnant women were referred for chromosomal and array CGH analyses. Both methods were carried out concurrently in order to compare results. A total of 1037 prenatal samples were studied and the reason for referral of these samples included advanced maternal age, ultrasound findings, parental anxiety and family history of a genetic condition or chromosome abnormality. Array CGH was carried out using whole-genome BAC array with a resolution of 1Mb across the genome and ~100kb resolution in 139 regions associated with constitutional disorders. From the analysis it was determined that 13% of the samples had likely benign and of no clinical significance CNVs. Furthermore, array CGH revealed clinically significant chromosome alterations in 3.3% of the samples. In 0.9% of the samples aCGH provided diagnosis of clinically significant chromosomal abnormality which was not detected by chromosomal analysis and would have otherwise gone undetected. Clinically significant results were also identified by conventional cytogenetics as well in 73.5% of the total

abnormalities also detected by aCGH (25/34) and in 2.4% of the total number of samples.

In the first group out of 95 patients studied, 17 abnormal cases (17/95, 17.9%) were determined by array CGH analysis. Eight out of the seventeen (8/17, 47%) abnormal cases detected were from pregnancies which had ultrasound abnormalities but a normal karyotype (n=7) or had a *de novo* balanced translocation (n=1), whereas nine of the abnormalities were investigations of abnormalities detected by other methods (G Banding, MLPA or QF PCR) (9/17, 53%). If we exclude the nine abnormalities previously detected by other methods the overall detection rate of this method would actually be 9.3% (8 out of 86 samples) which is comparable to the studies mentioned previously. Out of the 8 abnormal cases, the CNVs detected in six cases were likely to be pathogenic (7%) and 2 were benign as they were inherited from normal parents (2.3%). The detection of clinically significant CNVs was higher by 1-2 % from the studies previously discussed. So if we exclude the two cases with benign CNVs and classify them as normal cases the overall detection rate would drop to 7.1% from 9.3% (6 out of 84). This 7.1% of clinically significant CNVs would have remained undetected if chromosomal analysis alone was carried out in these prenatal cases, which supports the use of array in prenatal diagnosis in combination with chromosomal analysis. With the exception of the case with *de novo* balanced translocation which also carried a likely pathogenic CNV, the rest of the cases had a normal karyotype and ultrasound findings.

4.1.1) CNVs with variable expressivity

In addition to the clinically significant findings aCGH analysis revealed a case (Case 18) with a duplication of 0.7Mb in size, at the 22q11.2 microdeletion/microduplication syndrome region which was inherited form the mother. This was from a 14 week pregnancy referred for chromosomal and array CGH analyses due to an increased

nuchal translucency seen on ultrasound. Even though this finding was reported as likely to be pathogenic the exact risk for the fetus could not be estimated as this region is known to have variable expressivity and intrafamilial variability. This is one of the problems that arise with the use of aCGH in the prenatal setting; it is difficult to correlate such findings with the phenotype and this increases the anxiety for the future parents. Like the 22q11.2 microdeletion/microduplication syndrome there are other regions of variable expressivity and/or intrafamilial variation for instance the 16p11.2 [68], 16p13.11 [69], 7q11.23 [70].

4.1.2) Coincidental Findings

In another case, (Case 17) three CNVs were identified in total all of which were inherited from the mother; it was initially referred for chromosomal and array CGH analyses because of absence of the nasal bone on ultrasound screening. Array CGH analysis revealed two independent duplications on chromosome 9 (0.3Mb and 0.4Mb) which were of unclear clinical significance, but as they were inherited from the mother they were considered benign. In addition to these duplications the case revealed a deletion of 1.1Mb on chromosome 17 at 17p11.2. This region includes the PMP22 gene (Peripheral Myelin Protein 22) and is consistent with Hereditary Neuropathy with Liability to Pressure Pulsies (HNPP). This is a neuropathy with or without symptoms and while this finding was coincidental and unrelated to the reason for referral it was reported as causative. Findings such as these ones pose dilemmas to clinicians as it is hard to deal with. The fact that a CNV is also found in a parent will not always mean that it is benign. In addition to this, coincidental findings are a major issue that needs to be discussed in pre-test counseling extensively to discuss its implications and to give the patients the choice to choose whether they want to know or not. In this case the coincidental finding of the deletion of the PMP22 gene was not hard to report as it was later revealed to us that the mother had the

symptoms and at the end this proved to be a diagnosis for her condition rather than a problem in reporting the finding in the fetus. Not all cases of coincidental findings though will turn out to be such easy cases; there could be findings in late-onset diseases for which the patient would not have liked to know and would not otherwise know if testing wasn't done for other reasons. Coincidental findings could also occur in cancer genes like BRCA1 (hereditary breast cancer). Some carriers of mutations in BRCA1 will develop breast cancer and some will not. By disclosing such information to the prospective parents of a girl we are withholding the right of not – to- know of the patient. This kind of information would be of no use to a baby girl and it is not at their interest to be tested this early in life. So by reporting this finding we are hampering with the decisional autonomy of the prospective parents and to the child's future autonomy. Further to the implications such incidental finding may have to the unborn child it will extend to the mother as well. Once this information is released the mother, and consequently other family members, will start having thoughts about whether they want to be tested or not, whether they want to deal with this information or not and this will increase their anxiety. One could argue of course that such a finding could be regarded as prognosis and early preventive measures for the development of breast cancer could be taken. This could be correct for families that already know they are at high risk for breast cancer. However, it still remains the patients' decision to know or not. In addition this is an ethical issue that will be further discussed by the scientific community.

4.1.3) Pathogenic findings

4.1.3.1) Detection of *de novo* pathogenic findings

The third prenatal case (Case 24) in which a CNV was found was from a 13 week pregnancy referred for array CGH due to a *de novo* translocation identified during chromosomal analysis. The CNV was a deletion of 0.2Mb to 1.35Mb in size located at the translocation breakpoint and it was also *de novo* in origin, as confirmation of

the deletion was carried out in the parents as well by Real Time PCR. Due to fact that the deletion was *de novo* in origin, it was located at the translocation breakpoints and there was an entry with similar aberration in the DECIPHER this finding was reported as having an increased risk for phenotypic effect in the fetus. Three things need to be pointed out here:

- The use of BAC arrays which had a resolution of 1Mb did not permit us to estimate the exact size of the deletion which further creates problems in the interpretation. BAC arrays were used at the beginning of the study before oligonucleotide arrays were available. Once these became available they were used in prenatal testing
- It is important to test *de novo* balanced translocations by array CGH to lower the phenotypic risk of 6% [27] found in these cases as discussed by [28].
- Finally the reliable interpretation of CNV data is part of the aCGH analysis and reporting. This is a challenging task and one that requires expertise and knowledge which is present in various resources. In addition to the laboratory's own dataset several Internet resources are available to guide us through this complex task of interpreting the CNVs found. It is imperative that these databases are used to be able to discriminate between likely pathogenic, benign or variables of unclear significance. This will aid us further to correlate the genotype to the phenotype or the ultrasound findings.

Case 56 was a CVS sample from an 18 week pregnancy which was referred initially for chromosomal analysis only, due to increased Nuchal Translucency identified on ultrasound. QF PCR analysis was carried out as usual and revealed a normal diploid complement for chromosomes 13, 18, 21 and normal complement for chromosomes X and Y determining that the fetus was male. The sample was also treated as usually to establish cultures for chromosomal analysis, but even after several days there was

no growth in the culture. The physician was notified on the 14th day and it was suggested that we carried out aCGH analysis on the DNA that was extracted from the initial sample in order to avoid a second invasive procedure with the accompanied risk for miscarriage, to acquire a new sample for culturing. Array CGH analysis, using 105K oligonucleotide arrays, revealed a duplication on chromosome 5 of 2.1 Mb in size inherited from the healthy father and a *de* novo deletion on chromosome 15 of 2.4Mb in size. The duplication on chromosome 5 was classified as likely benign as it was inherited from the normal father, consequently stressing the necessity of confirming the presence/ absence of CNVs in the parents to further categorize them .The deletion on chromosome 15 was reported as likely pathogenic as it was relatively large in size, it was *de novo*, the deleted region contained many genes and was not listed as pathogenic in the publicly available databases. It was stressed in the report that detailed ultrasound was necessary. Furthermore, such single segmental imbalances even though they were determined by array CGH to be *de novo,* they could be the consequence of the unbalanced transmission of a derivative chromosome involved in an insertional balanced translocation (IT) in the parents [71]. Nowakowska et al. demonstrated that ITs underlie ~ 2.1% of apparently de *novo* interstitial CNVs. Such information may not be important to further evaluate the risk for the current fetus, but it is important for the accurate estimation of the recurrence risk to family members. Therefore chromosome visualization after microarray analysis is essential for delineating the rearrangement and assessing for further potential imbalance (in the immediate or even in the extended family). In the current case chromosomal analysis carried out in the parents did not detect an insertional translocation. The deletion was rather small in size for chromosomal analysis to detect (2.5Mb) therefore FISH analysis would be have been necessary to visualize exactly the nature of the imbalance. As this was a prenatal case and there

was not enough time to carry out customized FISH, a disclaimer was written on the report regarding this point.

Finally, in the current case aCGH analysis was also carried out with BAC arrays prior to the implementation of 105K oligo arrays. It was the time when with the availability of oligonucleotide arrays we were validating the platforms to switch to the higher resolution arrays. It is important to point out that the BAC array failed to detect the duplication on chromosome 5 most probably due to the lower resolution of the array and/or the particular calling criteria set at the time (in order to be called a copy number change had to be present in at least two consecutive clones).

The importance of carrying out confirmatory tests to the parents as well as the fetuses can also be seen in the other two prenatal cases; CNVs found in the fetuses were classified as benign as they were also present in healthy parents. Case 88 , a 12 week pregnancy, was referred for chromosomal and array CGH analyses because of Increased Nuchal Translucency. Array CGH analysis revealed a duplication of 0.5Mb in size on chromosome 7 which was classified to be benign as it was also present in the healthy mother. Case 94, a 25 week pregnancy was referred for chromosomal analysis due to Ultrasound Findings (Artrogryposis). Array CGH analysis revealed a duplication of 0.38Mb in size on chromosome 10 and a deletion of 0.32Mb in size on chromosome 15. Array CGH analyses carried out in the parents determined that the duplication was of paternal origin and the deletion was of maternal origin, determining that both CNVs were likely benign as each one was present in each one of the healthy parents.

It has to be pointed out that in the previous two cases array CGH analyses were carried out in the parents after extensive review of the publicly available databases (DGV, DECIPHER) as well as our own dataset. These databases did not show the CNVs found in these two cases to be common variants and that is why parental

aCGH was subsequently carried out and showed that those CNVs were specific to that family. About classifying a CNV please see subsection “CNV Classification”.

4.1.3.2) Detection of familial pathogenic findings

Finally, in the same group of patients, a 12 week pregnancy (Case 86) was referred for chromosomal analysis and aCGH due to increased Nuchal translucency (7.1mm). Chromosomal analysis was normal (46,XY), but array CGH revealed double segmental imbalance which is usually an indication for the presence of an unbalanced translocation. Array CGH carried out with 105K oligonucleotide array showed a terminal deletion on the long arm of chromosome 9 approximately 1.35Mb in size and a terminal duplication on the short arm of chromosome 17 approximately 1.95Mb in size. FISH analysis, using subtelomeric specific probes for chromosome 9 and 17, was then performed in order to visualize whether the findings occurred due to the presence of an unbalanced translocation. FISH analysis confirmed the array CGH results and determined the presence of an unbalanced translocation. Retrospective analysis of the fetus’s karyotype could not detect any of the abnormalities, as expected, since the imbalances (1.35Mb and 1.95Mb) were beyond the resolution of the karyotype. Chromosomal and FISH analyses carried out in the parents revealed the presence of a balanced translocation in the mother between the long arm terminus of chromosome 9 and the short arm terminus of chromosome 17. It is important to point out that the translocation was not visible in the karyotype of the mother. This is a cryptic translocation which under other circumstances would have been missed. The imbalances found are likely to be causative and related to the reason for referral as the deleted region on chromosome 9 overlaps with the 9q subtelomeric deletion region and includes many genes several of which are OMIM genes. In addition, the duplicated region on chromosome 17 contained many genes including two OMIM genes and partially overlapped with the Miller-Dieker syndrome region. The couple went through counseling for further explanation of the implications

of the findings for the current pregnancy, as well as, for future pregnancies; the couple elected to terminate the pregnancy. The importance of Genetic Counseling is further discussed in the specific subsection.

The usefulness of the additional information array CGH provided in the diagnosis in this case is obvious, without it would have remained undetected. Furthermore the information acquired from this case will be used from the family for the better management of their pregnancies in the future. After careful evaluation of this couple's reproductive and medical history, it was revealed that they had a previous pregnancy (Case 90) which was terminated due to multiple severe ultrasound findings (Tetralogy of Fallot, talipes and other). In addition the couple also had an affected child. Both the previous pregnancy and the child were previously karyotyped by our laboratory and the results were normal. As expected, retrospective G Banding analysis of both the child and the previous pregnancy did not detect the abnormalities, and the parents consented to perform array CGH on stored genetic material from their previous pregnancy and their affected child. Array CGH analysis revealed related findings to the current case and contributed to the diagnosis for their affected child. The importance of having the pedigree of a family being investigated is paramount as shown in this case. Had the parents informed the clinicians during the previous pregnancy that they already had an affected child the management of the first pregnancy might have been different. The first pregnancy was investigated by chromosomal analysis on Amniotic Fluid sample on the 16th week and revealed normal karyotype. It was terminated based on the ultrasound findings despite the fact that the karyotype was apparently normal. Had the parents known at the time that their born child had a chromosomal abnormality which was inherited from the mother, they would have opted for an earlier prenatal diagnosis on their first pregnancy perhaps by chorionic villus sampling. This would have lessened their anxiety.

4.1.4) Copy Number Variations (CNVs) - Polymorphisms

A different approach was used with the second group of samples as compared to the first group of samples. In an attempt to map copy number variations of the Cypriot population, a single Male reference DNA was used as our control DNA as opposed to pooled male/female control DNA used in the other array CGH analyses. The main idea was to build a database which would contain all this information to be used later on as a reference for analysis. The huge amount of CNVs picked up in the analysis of this group, most of which turned out to be common polymorphisms shared in the population, could not be directly compared to the other groups. There were only two CNVs which could be possibly pathogenic but more cases with the same ultrasound findings have to be studied in order to determine if these findings are related to that particular CNV.

4.2) Detection of aneuploidies/CNVs in POC/Intrauterine Death/stillbirth samples

The third group of patients consisted of 73 POC/Intrauterine death/stillbirths samples which were referred for chromosomal analysis in order to determine if the reason of the spontaneous miscarriage/intrauterine death/stillbirth was due to a chromosomal abnormality. Of all the recognized pregnancies, about 10-15% ends in clinical miscarriage or spontaneous abortion, usually towards the end of the first trimester. Out of these about 50% are shown to have a chromosomal abnormality, if they are all successfully cultured [72,73]. Fritz et al. suggest an even higher aneuploidy rate (72%) in specimens that failed to grow in vitro and were analyzed with metaphase CGH.

In our study, if alternative molecular methods were not applied the patients belonging in this group wouldn’t have received any results. The advantage of these assays is that they circumvent technical problems associated with tissue culturing. Some of the cases included in this group would have been referred for prenatal diagnosis if the spontaneous miscarriages hadn’t previously occurred.

The method used initially was QF PCR using extracted DNA from frozen tissue to exclude the most common aneuploidies seen in first trimester pregnancies which include aneuploidies for chromosomes 13, 15, 16, 18, 21, 22, X and Y as well as triploidies. Out of the 73 cases 41 were first trimester miscarriages. Sixteen cases (13 of which were first trimester) were diagnosed with the QF PCR analysis the results of which were consistent with the miscarriage. The abnormalities detected included four triploidies, three trisomies 16, three monosomies X, one 47,XXY, two trisomies 21, and one of each trisomies 13, 18 and 22. Once these abnormalities were ruled out, array CGH analysis was performed on the remaining of the sample to further exclude other aneuploidies or large copy number changes. In fifty one POC/intrauterine death/stillbirths samples QF PCR did not detect any abnormalities therefore we proceeded with the application of array CGH analysis. In an additional six cases QF PCR could not be carried out for technical reasons and array CGH only was applied for those cases. For the majority (49/57) of the samples BAC arrays were used and for the remaining oligonucleotide arrays (4/57 with 105K arrays and 4/57 with 180K arrays). Oligo arrays did not offer additional diagnostic information as all but one of the abnormalities found were aneuploidies. Oligo arrays detected a CNV of unclear significance which after parental analysis it was reclassified as familial benign CNV. BAC arrays detected nine aneuploidies: trisomies one of each for chromosomes 3, 9, 14, 15, 22 and X, two trisomies for chromosome 16, and two monosomies X. It is important to point out that six out of the nine abnormal cases identified here would have been identified by QF PCR if it was carried out.

The overall detection rate for abnormal cases in this group is 35% (26/73). However, if we exclude second and third trimester miscarriages/intrauterine death/stillbirth the detection rate is elevated to 51.2% for first trimester miscarriages. Trisomies accounted for the 57.7% (15/26) of all the first trimester cytogenetic abnormalities, 33% of those being trisomy 16. From the total of the abnormalities 15% (4/26) were

triploid cases and 15% (4/26) were 45,X . Furthermore the detection rate for abnormal cases in second trimester miscarriages was 16% (4/25) and 0% for third trimester POC/intrauterine death/stillbirths. Second trimester abnormalities included one trisomy X detected by 1Mb BAC array, one Klinefelter and two triploidies detected by QF PCR. Triploidies are usually cases with partial moles and they typically present as threatened, incomplete or missed abortion during the late first or early second trimester [21]. This is why in the cohort of our cases most triploidies appear in the first trimester group. Our results are similar to those reported in the literature [74].

Comparable results to ours are shown in a study of 26 first trimester fetuses that failed to grow in vitro analyzed with 1Mb BAC array [75]. In this study 15 out of 26 POC samples had abnormal profiles (57.7%) 13 of those being chromosomal aneuploidies (86.6%). The remaining two had a single clone deleted in one and a single clone duplicated in the other. Based on our calling criteria where, for BAC arrays, two consecutive clones have to deviate in order to be called a CNV, these two cases couldn't be considered abnormal unless further testing was carried out. The same study also noted the detection of autosomal monosomies a finding that is not normally detected in cultured Products of Conception. The most likely explanation for this being the fact that these specimens containing these chromosomal abnormalities do not do well when cultured and fail to produce analyzable metaphases for conventional cytogenetics. This could further explain the failure of some samples to grow in vitro.

We need to stress out that all the abnormalities detected within this group of samples were present in cases which had no ultrasound findings. The abnormalities shown here are very similar to those found in POCs that grow in culture; so it is obvious that for such cases, arrays with higher resolution do not offer additional diagnostic information therefore for the purposes of this analysis it seems unnecessary to use

higher resolution arrays. The higher costs and the possibilities of unsolicited findings during the analysis of these cases do not make high resolution arrays an appealing application. There are commercial arrays from some companies which are more suitable for these types of samples as they have lower resolution to serve the purpose of the analysis. For example the 15,000 oligonucleotide arrays manufactured by Oxford Gene Technologies, offer a good alternative of not such a high resolution as the 105,000 or 180,000 oligo arrays and as low as the 1Mb BAC arrays.

Studies where the application of aCGH was on fetuses with multiple malformations appear to have different results compared to the fetuses with no ultrasound findings. There are several examples in the literature showing that the detection rate in microdeletions/microduplications is higher in those samples that were presented with a number of serious ultrasound findings. In a study of 49 fetuses with multiple malformations and normal karyotype, targeted BAC array was used and a detection rate of 8% (4/49) causative imbalances was reported. [63]. Another group applied aCGH retrospectively in 50 fetuses with multiple malformations using a 44,000 oligonucleotide array and identified causative imbalances in 10% (5/50)[76]. Vialard et al. demonstrated a 10.8% detection rate by performing aCGH on 39 consecutive fetuses with multiple congenital abnormalities; 37 had normal karyotype and 2 had a *de novo* unbalanced karyotype. Targeted BAC array successfully characterized further the 2 abnormalities detected by cytogenetic analysis and detected another 4 abnormalities (4/37) [61].

Finally in a study where whole-genome aCGH was applied on fetuses presenting with at least one malformation detected on ultrasound, but for whom standard genetic analyses failed to provide a diagnosis showed clinically significant aberrations in 8.2% of tested fetuses. It also showed unclear clinical significant

results in 12.2% of the tested subjects [77]. Table 4.2 shows the comparison between these studies.

These data supports and suggest the implementation of aCGH, as its application offers additional diagnostic information in as much as 10% of cases were the fetuses have malformations and a normal karyotype. However it also presents us with the problem arising with variables of unclear significance.

Table 4.2: Comparison between studies which used array CGH in POC/Intrauterine Death or Still birth Samples

Study	Array Type	Karyotype/ Reason for carrying out array CGH	Results	Clinical Significance of Results
Benkhalifa et al., 2005	1 Mb BAC/PAC targeted array	Unknown/ Failure to grow in vitro	15/26 Abnormal	57.7% Causative
LeGaignec et al., 2005	BAC/PAC Targeted array	Unknown/ Multiple Malformations	5/49 Abnormal	8.2% Causative, 2% Unclear significance
Valduga et al., 2010	44,000 Oligonucleotide array	Unknown/ Multiple Malformations	5/50 Abnormal	10% Causative
D'Amours et al., 2012	Whole genome array	Normal Karyotype/ At Least one malformation	10/49 Abnormal	8,2% Causative, 12,2% Unclear Significance

The benefits these methods (QF PCR and array CGH) offer, in POC/intrauterine death/stillbirths samples are evident considering the fact that around 30% (73/250) of the total of these samples received by the laboratory over a year would have failed and no results would reach the patients. Moreover, the turnaround time for reporting POC/intrauterine death/stillbirths with these methods is dramatically decreased when it is compared to how long it would need if it were analyzed by G Banding. Finally a very small amount of DNA is required for both of the analyses to be carried out. The limitations of the methods lie with the fact that QF PCR is not a genome-wide analysis method and aCGH cannot detect balanced rearrangements and triploidies.

4.3) CNV Classification

By reviewing the publicly available databases a CNV can be classified as common or rare. Common CNVs usually represent normal genomic variation or benign CNVs that are mostly not involved in disease risk. In some occurrences a common CNV can represent a susceptibility locus. CNVs that are rare will more likely be penetrant for a disease, but some will be benign while other will still remain of unclear clinical significance [78]. It is important when comparing CNVs to compare gains with gains and losses with losses as the potential clinical consequences may differ significantly. The steps followed in interpreting CNVs are:

- Comparison with in-house and international datasets
- Comparison with in-house and international affected individual datasets
- Gene content and literature studies

One of the publicly available databases is the Database of Genomic Variants (DGV http://projects.tcag.ca/variation/) and it provides a useful catalogue of control data for studies aiming to correlate genomic variation with phenotypic data. Its difference from other databases is that it focuses solely on control samples. It is continuously updated with new data from published research studies. High quality studies only are included in this database; they undergo a series of reviews and only if they fulfil the inclusion criteria are then imported in DGV. Variants of greater than 50bp and smaller than 3Mb are included in DGV. For variants included in DGV a comparison is carried out with the regions associated with genomic disorders listed on DECIPHER to ensure that variants in control individuals do not coincide with known disease-causing variants [78]. Once it is determined that a CNV was not identified in a control set the next step is to determine whether it was previously found in a patient with similar phenotype. Databases that show genotype-phenotype correlation exist and are freely available to search from. Such databases are DECIPHER, ISCA, ECARUCCA. We mainly use the DECIPHER which is an interactive Web-based

database with tools that help us in the interpretation of subtle chromosomal abnormalities. DECIPHER retrieves information from a variety of resources which are relevant to the imbalance found in the patient. Known and predicted genes within an aberration are listed in the DECIPHER patient report, with consent a brief description of the phenotype is available, genes of clinical importance are highlighted and common copy- number changes in control populations are displayed [78].

Finally, once these databases have been consulted other resources will need to be searched in order to determine the function of a specific gene that was included in the aberrant region, a primary resource for connecting genes to disease related phenotypes in a general rather than case based manner is the Online Mendelian Inheritance in Men database (OMIM). It contains curated records of genetically inherited human disorders with references to causative genes or genetic loci.

Deciding to report or not to report a CNV as benign must be done with caution as they may sometimes contribute to pathogenicity if:

- There is a deletion on one allele and a mutated gene on the other allele [79]
- The same deletion is present in both alleles; Two benign heterozygote deletions generating a deleterious homozygous deletion [80]
- Each parent has a different benign (heterozygous) deletion in the same gene, which, when both are inherited there is a deleterious effect on the offspring (compound heterozygote)
- The region contains an imprinted gene with possible difference in pathogenicity [81]
- The CNV is on chromosome X and was inherited by a male offspring from his unaffected mother [82]
- The CNV is inherited from a mosaic carrier, who is not or only mildly affected[83]

- The CNV occurs in combination with another CNV and together these lead to a pathogenic defect [84]

In these cases any benign CNV will become pathogenic and it must be reported as such with a detailed explanation in the report.

4.4) New microdeletion syndromes that emerged from high resolution aCGH analysis

Over the years array CGH has contributed to the characterization of new microdeletion/ microduplication syndromes by screening large patient cohorts with intellectual disabilities. This is a benefit as diagnosis was provided to even more patients with intellectual disabilities. Even more of these novel syndromes may be identified in the future with the obvious value it will offer to the medical society. Array has facilitated a “reverse dysmorphology” approach in contrast to the earlier “phenotype first” approach. What this means is that the array results from a large cohort of patients are used to define a “critical “chromosome region that is deleted/duplicated in several patients. This is followed by comparison and study of the clinical features of the patients, to determine the essential phenotypic findings. Then if the clinical phenotype is distinctive enough searching for other patients who haven’t been screened, with similar features could identify others with the same abnormality [85]. Examples of such syndromes are the:

- 17q21.31 microdeletion and microduplication syndromes
- 15q13.3 deletion syndrome
- 16p11.2 deletion
- 16p11p12.1 deletion syndrome
- 2p15p16.1 deletion syndrome
- 15q24deletion syndrome
- 1q41q42 deletion syndrome

- 9q22.3 deletion syndrome

The choice of the array type to be used is critical in order to be able to uncover new microdeletion/microduplication syndromes. The use of targeted arrays for example could minimize the chance of novel syndromes to be identified as in order to reduce the probability to detect CNVs of unclear significance, they examine loci of known clinical significance. On the contrary the use of whole genome arrays offers more chances of previously undetected aberrations to be discovered, even though is not always a straight forward answer as there is a higher detection rate of CNVs with whole genome arrays.

Targeted arrays, though, with enriched probes to potential “hotspots” in the human genome associated with rearrangements, could lead to the identification of novel deletion/duplication syndromes. Such “hotspots’ are segmental duplications in certain chromosomes.

4.5) Can array CGH analysis fully replace karyotyping?

Arrays are being introduced in prenatal diagnosis in conjunction to chromosomal analysis but it cannot yet fully replace karyotyping for the following reasons: a) it cannot detect balanced rearrangements such as translocation, balanced insertions and inversions. This is especially important in Robertsonian translocations as carriers of such are at high risk for uniparental disomy [86], and the risks UPD implies as they were discussed previously. Even in the case were SNP arrays are used which can detect isodisomy [87] they cannot detect heterodisomy which is the most common form of UPD. In addition to Robertsonian translocation, balanced rearrangements especially *de novo* reciprocal translocations or insertions are important to be detected as they can sometimes lead to abnormal phenotypes for the reasons previously mentioned. Furthermore knowing the presence of a balanced rearrangement can provide the couple future risk assessments for an unbalanced

offspring and information useful for reproductive planning, b) it cannot detect low level mosaicism often seen in prenatal diagnosis. Mosaicism is detected in 1-2 % of CVS samples and in 0.2% of amniotic fluid samples [21]. Even though in about 84% of mosaic cases in CVS, the mosaicism is confined to the placenta [88] the remaining cases would have remained undetected if array CGH was the only method applied, c) it cannot always detect the presence of marker chromosomes even in the non-mosaic state. Marker chromosomes are encountered in about 0.1% of prenatal diagnoses [21] and very often in the mosaic form. Depending on which chromosome they were derived from, their size, their inheritance mode and whether are euchromatic or heterochromatic the phenotypic risk can be determined. In a study of 55 cases with marker chromosome it was demonstrated that out of the 26 non-mosaic markers only 14 were detected leaving 46% of array results normal. Even if this percentage reflects that the markers are mainly heterochromatic, the lack of detection does not completely exclude a possible phenotypic effect [89] and d) it cannot visualize the type of rearrangement in the event were deletion or duplication detected by array CGH is proven to be *de novo* after parental tesing.

4.6) Genetic Counseling

As genome-wide analysis is being introduced into prenatal diagnosis pre-test counseling is of paramount importance due to the nature of the test and the findings that may emerge from the analysis. Counselors should explain everything very clearly and offer information in a nondirective way, so that prospective parents can make their own decision having their future child's best interest in mind.

It is imperative that the following information is given by the prospective parents:

- Medical history of both parents
- Medical history of the pregnancy which should include any ultrasound findings
- Family pedigree of both parents up to three generations

Counselors should be aware of the state of mind parents-to–be are in, right after an ultrasound abnormality has been detected. Parents may not be able to absorb any information given to them at the time so it is good practice to have everything written down as well so that it is available for them to read later on. Following this, parental consent should be obtained. Prospective parents should be informed of the test, and its limitations should be further explained. They should know that the array technique cannot detect every single disease or well –known syndrome. In a study of 141 fetuses with ultrasound abnormalities and normal array results, there was a diagnosis in 15% of them when they were reviewed postnatally [90].

If, in the course of testing the fetus, whole-genome array analysis is needed to be carried out for the parents, they should be counseled appropriately including informed consent on what information they want to receive.

The parents should be aware of all the possible outcomes of the array testing which could either be normal or abnormal. It should be explained to them that if CNVs are detected they could:

- Explain the fetal ultrasound abnormalities
- Be *de novo* and of unknown clinical significance
- Be inherited and of unknown clinical significance
- Be an unsolicited finding unrelated to the ultrasound findings

Variables of unknown significance and incidental findings are the most challenging for counselors. This is why it is of prime importance to inform parents of such possible findings; an example is a late-onset inherited disease either *de novo* or inherited in the family. Its implications should be explained and a distinction should be made between treatable (hereditary cancer) and non-treatable (Huntington's disease) late-on-set diseases. There is no straight forward guideline on how this should be carried out, but for example in Europe the current tendency is to ask

parents whether they want to be informed about treatable late-onset diseases. Some laboratories even have a policy of not reporting unsolicited CNVs to non-treatable diseases [90]. There are many ethical questions arising from all these one of them being the extent to which pregnant women and their partners should be allowed to determine the range of possible outcomes that will or will not be reported back to them [91].

National guidelines in the use of array CGH in prenatal diagnosis remain to be established.

4.7) Future approaches in Prenatal Diagnosis

The introduction of Non-Invasive Prenatal Diagnosis (NIPD) will overcome the problem of who should be screened or not; whether it will be all pregnant women or those at high risk. In the near future it is possible that NIPD will replace all current biochemical screening tests or be the first-tier test after an indication of Down syndrome by a biochemical screening [92]. As array CGH was introduced in prenatal diagnosis, in the same way Next Generation Sequencing, a new emerging technology, could be applied in prenatal diagnosis. Either through NIPD for Down Syndrome [93,94] or genetic diseases like thalassemia or cystic fibrosis, or for whole exome or even whole genome sequencing[95].

NIPD for Down syndrome is rapidly evolving. Recent research shows that trisomy 21 can be reliably determined from the analysis of cell-free fetal DNA from maternal plasma[96,97]. Until today two methodologies have accomplished the development of NIPD methods for Down syndrome with positive results. They are the “next-generation sequencing” technologies and the “Methylation Dependent ImmunoPrecipitation (MeDIP) real time quantitative PCR” based approach [98].

The next-generation method can analyze the nucleotide sequences of millions to billions of DNA molecules in one run, being able to identify and count the frequency

distribution of DNA molecules in a sample. Based on the fact that maternal plasma DNA could be sequenced to identify the chromosomal origin of each DNA molecule, the proportion of molecules from a potentially aneuploid chromosome (for example 21) could be determined. Based on this Lo et al. demonstrated that the proportion chromosome 21 DNA molecules in plasma of pregnant women carrying a trisomy 21 fetus were elevated compared to that of euploid pregnancies [99]. This approach is highly accurate and very promising, as proven by two groups [99,100], for the direct detection of trisomy 21. The only drawback for this method is the fact that it is high cost and low throughput, only a small number of cases can be analyzed simultaneously and the results take several days to be available.

The MeDIP real time quantitative PCR methodology is built on the fact that there are differences in methylation between the mother and the fetus. Papageorgiou et al [93] developed a method which was based on the investigation of fetal specific methylation markers using the methylated DNA Immunoprecipitation methodology in combination with Real Time quantitative PCR. In the first trials of the method it provided 100% sensitivity and specificity. The MeDIP real time quantitative PCR methodology is a new, fast, and cost- effective NIPD for Down syndrome that can be offered as early as the 10th week of gestation. Once the larger scale validation study is completed (700-1000 samples) this method can be used in clinical practice [94].

The field of NIPD is evolving and it is possible that in the future it will be offered for aneuploidies for other chromosomes as well, or even small rearrangements. Further studies are needed, to establish whether it could, completely replace invasive prenatal diagnosis methods.

5) Concluding remarks

Karyotyping has been the golden standard method for prenatal diagnosis for decades, being able to sufficiently diagnose numerical and large structural abnormalities (<3-10Mb). With the introduction of array CGH analysis in postnatal analysis and its use as a first-tier test in cases of intellectual disabilities, it has been postulated that this method might someday actually replace conventional cytogenetics in prenatal diagnosis as well. Array CGH in a postnatal setting, has been demonstrated to be a high throughput, comprehensive and fast to detect copy number changes that can go undetected by light microscopy.

The current study has demonstrated that the usefulness of array CGH in prenatal diagnosis depends on the selection of the appropriate platform and reference DNA. More importantly, it has clearly shown through several examples presented in the thesis, that array CGH is a valuable tool in prenatal diagnosis, both in cases with fetal malformations and normal karyotype as well as in cases were an abnormality was detected with another method and further investigated with array CGH. Array CGH provided valuable information for phenotype-genotype correlation and provided more accurate information regarding the clinical significance and the risk in the current and future pregnancy of the respective patient. Another critical factor for accurate CNV classification is parental testing to determine between familial and *de novo* CNVs. Appropriate pre and post- test genetic counceling offer the prospective parents tools to decide on the management of their pregnancy. However, one of the problems posing dilemmas to genetics councelors and something that array CGH has to overcome is the fact that it can detect coincidental findings, variants of unknown significance as well as variants with variable expressivity.

Furthermore array CGH could be used in POC/intrauterine death/stillbirths samples were malformations exist in the fetuses, using the same platform as in prenatal cases, as it offers an increase in detection rate in this category of samples. Array

CGH can also be applied in samples were there are no ultrasound findings in the fetus, after they have been analyzed with QF PCR to exclude common aneuploidies. For this category of samples lower resolution arrays could be used.

Currently the ideal setting to advance prenatal diagnosis and increase its resolution would be to apply array CGH in high risk pregnancies in conjunction with chromosomal analysis with a microarray designed especially for prenatal diagnosis. As we have seen this increases the detection rate for likely pathogenic CNVs up to 5%. To avoid interpretation problems (previously discussed) these arrays should cover all known pathogenic CNVs and have a low –resolution backbone for the detection of relatively large CNVs thus keeping the detection of CNVs of unclear significance to the minimum. A shared database specifically dedicated to prenatal diagnosis coupled with the growing amount of data regarding CNVs and dosage sensitive genes could make it easier to interpret genomic arrays.

6)Summaries

6.1) Summary in English

Karyotyping has been the golden standard method for prenatal diagnosis for decades, for the diagnosis of numerical and large structural abnormalities (<3-10Mb). With the introduction of array Comparative Genomic Hybridization (CGH) analysis in postnatal analysis and its use as a first-tier test in cases of Intellectual disabilities, it has been postulated that this method might also become the first-tier test in prenatal diagnosis as well. Array CGH is a technology that has demonstrated that it is a high throughput, comprehensive and fast and has proven its ability to detect copy number changes that can go undetected by light microscopy.

The aim of this study was the application of high-resolution microarrays in prenatal diagnosis for the detection of cryptic microduplications and microdeletions in fetuses with ultrasound abnormalities and normal karyotype, and also to further investigate the abnormalities in fetuses with balanced or unbalanced rearrangements with or without ultrasound findings. This research aimed to provide new scientific knowledge and benefits in fetal medicine and genetics, and to prove that the application of higher diagnostic resolution in prenatal diagnosis is possible.

Array CGH was carried out to detect submicroscopic chromosomal imbalances, using commercially available Bacterial Artificial Chromosome (BAC) and oligonucleotide microarrays (Cytochip BlueGnome), as well as the Sanger Tiling Path BAC arrays. All Copy Number Changes (CNCs) revealed in array analysis were confirmed by a second method. A total of 202, out of which 129 were prenatal, and 64 parental samples were analyzed. Prenatal samples were tested either because: 1) they had normal karyotype and ultrasound abnormalities, 2) apparently balanced rearrangement and ultrasound abnormalities, 3) an apparently balanced structural

aberration without abnormal ultrasound findings, 4) an abnormal karyotype with abnormal ultrasound findings, 5) an abnormal karyotype/ MLPA/QF-PCR without abnormal ultrasound findings which required further investigation, 6) even though the karyotype was eventually normal and there were no ultrasound findings array CGH was carried out because of maternal anxiety due to an abnormal previous pregnancy or to rule out a possible abnormality which was revealed during chromosomal/ MLPA/ Quantitative Fluorescence Polymerase Chain Reaction (QF-PCR) analyses.

Fifty prenatal samples were analyzed with 1Mb BAC arrays, 34 with 105K and 11 with 180K oligonucleotide arrays and 34 samples were analyzed with Tiling path BAC array. Seventeen abnormal cases (17/95, 17.9%) were determined by array CGH analysis. Seven of the abnormal cases were from pregnancies which had ultrasound abnormalities and a normal karyotype, whereas 9 of the abnormalities were investigations of abnormalities detected by other methods (G Banding, MLPA, QF PCR) (9/17, 53%). By excluding the nine abnormalities previously detected with other methods the detection rate of this method is 8.1% (7 out of 86 samples).

The current prenatal cohort included 34 cases that had a normal karyotype and were terminated due to major ultrasound abnormalities. Array CGH analysis of theses 34 fetuses revealed 627 Copy Number Changes (CNC). After comparison to known aberrations listed in publically available databases, (DECIPHER (**D**atabas**E** of **C**hromosomal **I**mbalance and **P**henotype in **H**umans using **E**nsembl **R**esources, Database of Genomic Variants DGV) it was determined that these CNCs were found within normal copy number variant regions and were excluded from any further investigation. These CNCs were considered of no clinical significance as they could also be found in normal controls and thus were most probably not associated with the clinical findings.

Finally among the 202 samples there were 73 cases of Products of Conception/ intrauterine death /Stillbirth (POC/SB) samples which were initially received for chromosomal analysis. Due to failure of these samples to grow *in vitro* , additional tissue kept in storage was used for DNA extraction, so that they could be used in (QF- PCR) and array CGH analyses.

Out of the 73 POC/SB samples, 16 cases were completed by QF PCR analysis only, as the causative abnormality was detected and there was no need to proceed with any further investigation and 57 cases were further investigated using array CGH analysis. Out of the 16 abnormalities detected with QF PCR analysis 13 were first trimester and 3 were second trimester miscarriages; 4 triploidies, 8 autosomal trisomies and 4 sex chromosome abnormalities. Out of the 57 cases investigated with array CGH, 49 were analyzed using BAC arrays and 8 were analyzed using Oligo arrays. A total of 9 aneuploidies were detected by BAC arrays analysis, of which 7 were autosomal and 2 were sex chromosome aneuploidies. Eight of those aneuploidies were first trimester and one was second trimester miscarriage. In addition 1 benign familial CNC was detected by oligo array analysis.

Array CGH analysis has been proven to be a valuable tool for the determination of copy number changes in children with congenital abnormalities and it has replaced karyotyping in some laboratories. It has proven its value in Products of Conception/ Intrauterine death /Stillbirth as shown in this study by having the advantage to circumvent technical problems associated with tissue culturing thus leading to the failure of providing results by classical cytogenetics.

In prenatal diagnosis chromosomal analysis is still the primary choice of testing; it has to overcome some of its limitations, such as the detection of CNCs of unclear significance or coincidental findings, before it can fully replace chromosomal analysis in prenatal diagnosis. As it was shown, by our results and those of others, the

detection rate in clinically significant copy number changes is increased by 8% with the application of array CGH in cases were the karyotype is normal and there are sonographic malformations in the fetus it can be used more extensively in prenatal diagnosis as well. Furthermore in 9 cases it has provided additional information, valuable for the clinical interpretation of the findings detected by other methodologies.

6.2) Περίληψη στα Ελληνικά

Η χρωμοσωμική εξέταση είναι η μέθοδος που χρησιμοποιείται στην προγεννητική διάγνωση για δεκαετίες, για τη διάγνωση αριθμητικών και μεγάλων δομικών ανωμαλιών (< 3-10 Mb). Με την εισαγωγή της μεθοδολογίας του συγκριτικού γενωμικός υβριδισμού με μικροστυστοιχίες (array CGH) στην μεταγεννητική ανάλυση και τη χρήση τους ως την πρώτη επιλογή εξέτασης σε περιπτώσεις ατόμων με διανοητική αναπηρία, έχει διατυπωθεί η άποψη ότι η μέθοδος αυτή μπορεί κάποια μέρα να αποτελέσει τη πρώτη μέθοδο επιλογής και στην προγεννητική διάγνωση. Η array CGH έχει αποδειχθεί να είναι μια τεχνολογία υψηλής απόδοσης, περιεκτική και γρήγορη. Έχει επίσης την ικανότητα να ανιχνεύει αλλαγές σε γενετικό υλικό που να είναι μη ανιχνεύσιμες στο μικροσκόπιο.

Σκοπός της μελέτης αυτής ήταν η εφαρμογή υψηλής ευκρίνειας μικροσυστοιχιών στην προγεννητική διάγνωση για τον εντοπισμό κρυπτικών μικροδιπλασιασμών και μικροελλειμάτων σε έμβρυα με φυσιολογικό καρυότυπο αλλά με σοβαρά υπερηχογραφικά ευρήματα (συγγενείς ανωμαλίες, δυσμορφίες) και επίσης να διερευνήσει περαιτέρω σε έμβρυα με ήδη ανιχνευμένες, με άλλες μεθόδους, ανωμαλίες με ισορροπημένες ή μη ισορροπημένες ανακατατάξεις με ή χωρίς υπερηχογραφικά ευρήματα. Αυτή η έρευνα είχε ως στόχο να προσφέρει νέες επιστημονικές γνώσεις και οφέλη στην εμβρυϊκή ιατρική και στη γενετική, και να αποδείξει ότι είναι δυνατή η εφαρμογή της array CGH στην προγεννητική διάγνωση.

Array CGH πραγματοποιήθηκε για την ανίχνευση μικροσκοπικών χρωμοσωμικών ατυπιών, χρησιμοποιώντας εμπορικά διαθέσιμες μικροσυστοιχίες με BAC κλώνους και μικροσυστοιχίες ολιγονουκλεοτιδίων (Cytochip, BlueGnome, UK), καθώς και με μικροσυστοιχίες με BAC κλώνους με μερική αλληλοεπικάλυψη του Ινστιτούτου Sanger. Οποιεσδήποτε αλλαγές ανιχνεύθηκαν στον αριθμό αντιγράφων του DNA επιβεβαιώθηκαν και από δεύτερη μέθοδο. Αναλύθηκαν συνολικά 202 περιστατικά, εκ

των οποίων τα 129 ήταν προγεννητικές, και 64 δείγματα από γονείς. Οι προγεννητικές παραπομπές έγιναν είτε διότι: 1) είχαν φυσιολογικό καρυότυπο ενώ ταυτόχρονα είχαν υπερηχογραφικά ευρήματα, 2) ισορροπημένη ανακατάταξη στον καρυότυπο και υπερηχογραφικά ευρήματα, 3) ισορροπημένη ανακατάταξη στον καρυότυπο χωρίς όμως υπερηχογραφικά ευρήματα, 4) ανωμαλία στον καρυότυπο και υπερηχογραφικά ευρήματα, 5) ανίχνευση ανωμαλίας στον καρυότυπο / MLPA/QF-PCR χωρίς όμως υπερηχογραφικά ευρήματα τα οποία χρειάζονταν περαιτέρω διερεύνηση, 6) κάποια περιστατικά που παρόλο ότι είχαν φυσιολογικό καρυότυπο και καθόλου υπερηχογραφικά ευρήματα, διενεργήθηκε array CGH είτε λόγω ανησυχίας των γονιών λόγω ύπαρξης ανωμαλίας σε προηγούμενη κύηση ή για να αποκλείσει μια πιθανή ανωμαλία που αποκαλύφθηκε κατά τη ανάλυση με καρυότυπο / MLPA / QF-PCR.

Πενήντα προγεννητικές αναλύθηκαν με BAC arrays ευκρίνειας μίας Μεγαβάσης, 34 με μικροστυστοιχίες 105,000 ολιγονουκλεοτιδίων και 11 με μικροστυστοιχίες 180,000 ολιγονουκλεοτιδίων, και 34 δείγματα αναλύθηκαν με Tiling Path BAC array. Ανιχνεύθηκαν 17 ανώμαλα περιστατικά με την array CGH ποσοστό που ανέρχεται στο 17.9% (17/95). Επτά από τα ανώμαλα περιστατικά προέρχονταν από εγκυμοσύνες με φυσιολογικό καρυότυπο και ευρήματα στους υπέρηχους, ενώ 9 (9/17, 53%) από τις ανωμαλίες που ανιχνεύθηκαν αποτελούσαν διερεύνηση περιστατικών με ανωμαλίες που διαγνώστηκαν με άλλες μεθόδους (χρωμοσωμική εξέταση, MLPA, QF- PCR). Αποκλείοντας τα εννέα αυτά περιστατικά από τους υπολογισμούς μας το ποσοστό ανίχνευσης της μεθόδου αυτής είναι 8.1% (7/86).

Το σύνολο των περιστατικών της μελέτης αυτής περιλάμβανε και 34 περιστατικά με φυσιολογικό καρυότυπο στα οποία έγινε τερματισμός εγκυμοσύνης λόγω σοβαρών υπερηχογραφικών ευρημάτων. Η ανάλυση κατά array CGH των 34 αυτών εμβρύων φανέρωσε 627 αλλαγές στον αριθμό αντιγράφων του DNA (CNC). Κατόπιν συγκρίσεως των αλλαγών αυτών στη γενετική σύσταση αυτών των εμβρύων με

γνωστές ανωμαλίες που απαριθμούνται σε δημόσια διαθέσιμες βάσεις δεδομένων, DECIPHER (**D**atabas**E** of **C**hromosomal **I**mbalance and **P**henotype in **H**umans using **E**nsembl **R**esources), DGV (Database of Genomic Variants) καθορίστηκε ότι οι πλείστες περιέχονται στη λίστα πολυμορφισμών και εξαιρέθηκαν από περαιτέρω διερεύνηση. Αυτές οι αλλαγές δεν θεωρήθηκαν κλινικής σημασίας εφόσον βρέθηκαν και σε φυσιολογικούς μάρτυρες, κι επομένως το πιο πιθανόν να μην σχετίζονται με τις υπερηχογραφικές ανωμαλίες των εμβρύων αυτών.

Τέλος ανάμεσα στα 202 δείγματα υπήρχαν και 73 περιστατικά από αυτόματες αποβολές/ ενδομήτριος θάνατος /Θνησιγένεια (POC/SB) τα οποία είχαν αρχικά παραπεμφθεί για χρωμοσωμική εξέταση. Λόγω όμως του γεγονότος ότι τα περιστατικά αυτά δεν αναπτυχθήκαν στις καλλιέργειες επιπρόσθετο υλικό που παρακρατήθηκε από αυτά χρησιμοποιήθηκε για απομόνωση DNA, ούτως ώστε να χρησιμοποιηθεί για ανάλυση με άλλες μεθόδους (QF- PCR και array CGH).

Από τα 73 POC/SB αυτά δείγματα, 16 περιστατικά ολοκληρώθηκαν μόνο με τη μέθοδο της Ποσοτικής Φθορίζουσας Αλυσιδωτής αντίδρασης της Πολυμεράσης (QF-PCR), καθώς με αυτή ανιχνεύθηκε επιτυχώς ο λόγος της αποβολής και δεν υπήρχε λόγος να προχωρήσουμε σε περαιτέρω διερεύνηση. Στα υπόλοιπα 57 περιστατικά εφαρμόστηκε η array CGH.

Από τις 16 ανωμαλίες που ανιχνεύθηκαν με την μέθοδο QF-PCR, οι 13 ήταν αποβολές πρώτου τριμήνου και οι τρεις ήταν αποβολές δευτέρου τριμήνου. Σ’ αυτές συμπεριλαμβάνονταν 4 τριπλοειδίες, 8 αυτοσωμικές τρισωμίες και 4 ανωμαλίες των φυλετικών χρωμοσωμάτων. Από τα 57 περιστατικά των οποίων η ανάλυση διεκπεραιώθηκε με array CGH, τα 49 αναλύθηκαν με μικροσυστοιχίες BAC και τα 8 αναλύθηκαν με μικροσυστοιχίες ολιγονουκλεοτιδίων. Συνολικά ανιχνεύτηκαν 9 ανευπλοειδίες με μικροσυστοιχίες BAC, από τις οποίες οι 7 ήταν αυτοσωμικές και οι 2 ήταν ανευπλοειδίες των φυλετικών χρωμοσωμάτων. Οκτώ από τις ανευπλοειδίες

αυτές ήταν πρώτου τριμήνου και μία ήταν δευτέρου. Επιπρόσθετα με εφαρμογή μικροσυστοιχίας ολιγονουκλεοτιδίων ανιχνεύθηκε και μια καλοήθης CNC κληρονομημένη από τον ένα γονιό.

Η εφαρμογή της μεθόδου array CGH έχει αποδειχθεί ένα πολύτιμο εργαλείο για τον προσδιορισμό των αλλαγών στον αριθμό αντιγράφων σε παιδιά με συγγενείς ανωμαλίες και έχει αντικαταστήσει τη χρωμοσωμική ανάλυση σε αρκετά εργαστήρια. Έχει αποδειχθεί επίσης η αξία της στη χρήση της και σε αποβολές/ Ενδομήτριου θανάτου /Θνησιμότητας, όπως φαίνεται στην παρούσα μελέτη έχοντας το πλεονέκτημα να παρακάμψει τις τεχνικές δυσκολίες που συναντούμε συχνά και σχετίζονται με την αδυναμία ανάπτυξης ινοβλάστων σε καλλιέργεια, και ακολούθως στη αποτυχία διάγνωσης του περιστατικού με την κλασσική κυτταρογενετική.

Στη προγεννητική διάγνωση η χρωμοσωμική ανάλυση εξακολουθεί να αποτελεί την πρώτη επιλογή εξέτασης. Η μέθοδος αυτή πρέπει να ξεπεράσει κάποιους από τους περιορισμούς της πριν να μπορέσει να αντικαταστήσει ίσως τη χρωμοσωμική εξέταση. Έχει αποδειχθεί όμως τόσο από εμάς όσο και από αποτελέσματα άλλων, ότι η συχνότητα ανίχνευσης των αλλαγών στον αριθμό αντιγράφων με κλινική σημασία αυξάνεται κατά 8%, με την εφαρμογή της array CGH σε περιπτώσεις με φυσιολογικό καρυότυπο και υπερηχογραφικά ευρήματα στο έμβρυο, οπότε η μέθοδος αυτή ίσως να μπορεί να χρησιμοποιηθεί ευρύτερα και στην προγεννητική διάγνωση. Επιπρόσθετα σε 9 περιπτώσεις η μέθοδος αυτή παρείχε πληροφορίες πολύτιμες για την ερμηνεία των ευρημάτων που εντοπίστηκαν από άλλες μεθόδους.

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Annex

PROTOCOLS

Phenol: Chloroform Extraction for DNA clean up

1) Label one set of Eppendorf and one set of screw cap tubes with DNA number
2) Add H_2O to the DNA sample and make volume up to 400µl
3) Add 400µl of 1 part phenol and 1 part chloroform and mix
4) Shake gently until a “bubbly” solution forms
5) Centrifuge at 5000rpm for 20 minutes
6) Transfer supernatant into a new tube
7) Add 1/10volume Sodium Acetate 3M
8) Add 2 volumes ice cold 96% Ethanol and shake gently
9) Place at -20ºC for at least 20 minutes

   STOP POINT: you can leave DNA samples at -20ºC for longer period of time if needed
10) Spin at 10000rpm for 15-20 minutes at 4 ºC
11) Remove supernatant and add ~200µl 70% Ethanol at Room Temperature (RT)
12) Spin at 10000rpm for 5 minutes at RT (if necessary repeat steps 11,12 to remove any salts)

    NOTE: If pellet is visible and dislodged be careful not to remove it
13) Dry the pellet at RT
14) Resuspend pellets in H_2O (15-20 µl)
15) Measure DNA concentration on NanoDrop Spectrophotometer

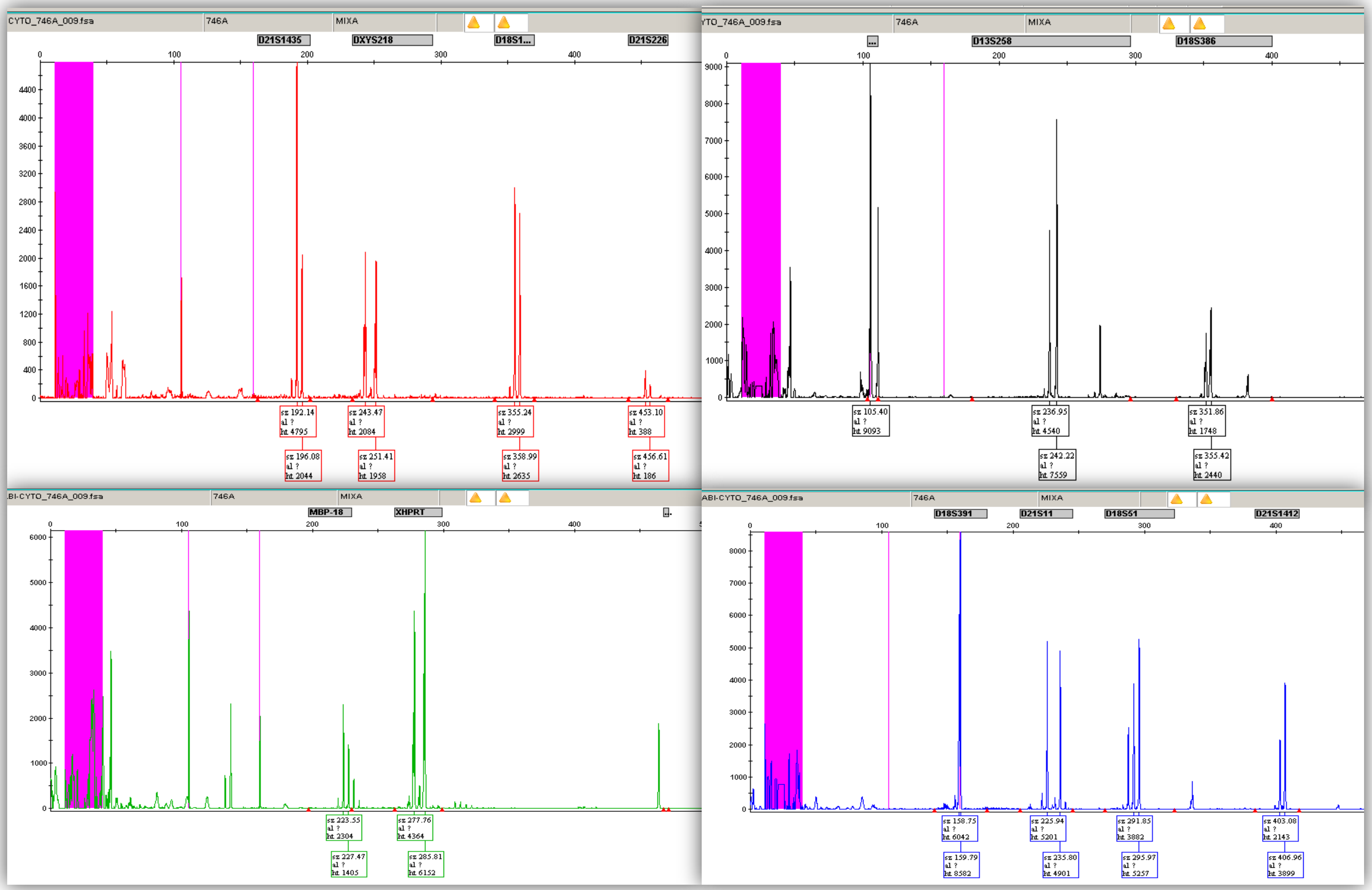

Figure 3.5.3.1.2: Electropherogram of QF PCR analysis carried out on a case with maternal contamination. Polymorphic markers for chromosomes 18, 21, X and Y appear in this figure representing Mix A . Labeled with PET (Red) are markers D21S1435 (ratio 2:1), DXYS218 (ratio 1:1), D18S1002(ratio 1:1), D21S2226 (ratio 2:1). Labeled with NED (Black) are markers for D13S258 (ratio1.8), D18S386 (Ratio 1.8) and non- polymorphic AMEL (ratio 1.8). The presence of two extra peaks are indicative of a second genotype. Labeled with VIC (Green) are polymorphic markers for MBP-18 (ratio1.7 and the presence of a third peak), XHPRT (ratio 1:2) and the presence of SRY. Labeled with FAM (blue) the polymorphic markers for D18S391, DS21S11(1:1), D18S51(ratio 1.4 and presence of an extra peak) and D21S1412 (1.7). The presence of a second genotype and of non-informative markers determine the presence of maternal contamination in the sample.

Triploid

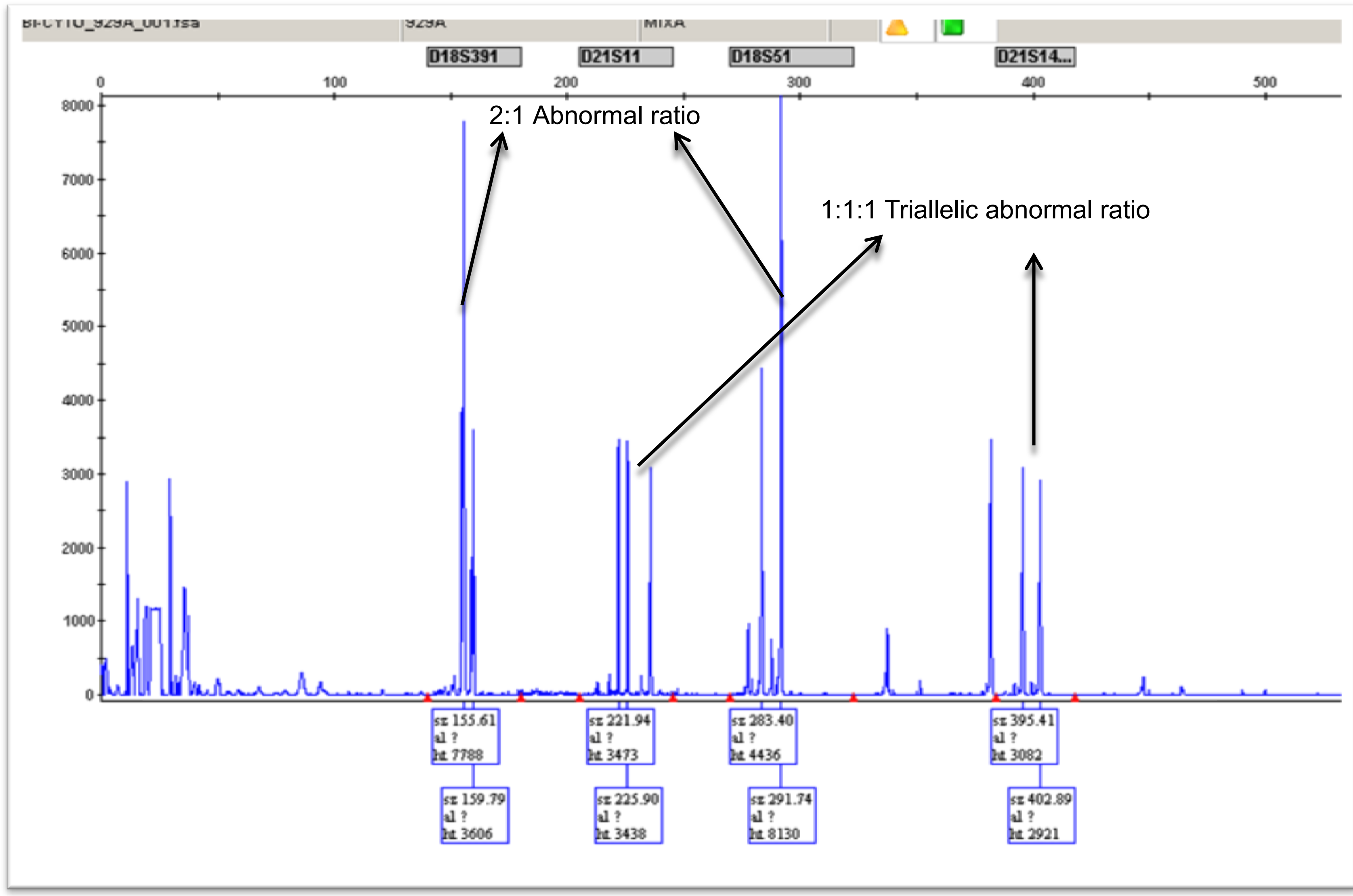

Figure 3.5.3.1.3: Electropherogram of QF PCR analysis carried out on a triploid case. Results from Mix A labeled with FAM (Blue). Two polymorphic markers used for each of chromosomes 18 and 21 are appear in this figure. The markers shown in the figure discriminate between the triallelic 1:1:1 ratio for markers D21S11 and D21S1412 and 2:1 ratio for markers D18S391 and D18S51 all of which show the presence of three copies of chromosomes 18 and 21.