GENETIC STUDIES IN FETAL SAMPLES

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INTRODUCTION

The **genome** is the genetic information that an organism inherits from its parents and that characterizes an individual. The genome is organized into units called chromosomes. The human genome contains 23 pairs of chromosomes. A **chromosome** is made up of a double strand of DNA associated with proteins and contains genetic information divided into units of information called genes. A **gene** is the smallest fragment of a DNA molecule with a complete information for a given character. The human genome is made up of 3,000 million base pairs (Mb) and contains about 23,000 genes. They are arranged linearly along the chromosomes. Genetic abnormalities can be divided into 3 large groups: chromosomal abnormalities, submicroscopic abnormalities and monogenic abnormalities.

1.- **Chromosomal abnormalities** are defined as an alteration in the number or structure of the chromosomes detectable on karyotype. Chromosomal abnormalities are usually not hereditary diseases and therefore have a low risk of recurrence. We can distinguish between:

- **Numerical chromosomal abnormalities**. The most frequent chromosomal abnormality compatible with life is trisomy of chromosome 21 (Down syndrome), followed in frequency by trisomy 18 (Edwards syndrome) and trisomy13 (Patau syndrome). Of complete monosomies, only one is known. that is compatible with life, monosomy of the X chromosome (45,X) or Turner syndrome. During the prenatal period, polyploidies are also frequent, characterized by the presence of an extra complete chromosomal complement, mainly triploidy.
- **Structural chromosomal abnormalities**. They originate from an abnormal reconstruction after one or several chromosomal breaks in a single or more than one chromosome. The structural rearrangements are classified as unbalanced or balanced depending on whether genetic material is lost or gained or, on the contrary, there are no dose alterations. About 4% of cases of Down syndrome are caused by the presence of a Robertsonian translocation in one of the parents.

2.- **Microdeletion and microduplication syndromes**, also called submicroscopic anomalies because they are not seen on the karyotype, or copy number variations (CNV), are genetic abnormalities that involve genomic fragments between 10 kb and 10 Mb. These clinical syndromes are caused by a deletion or duplication involving various genes, and the most frequent are DiGeorge syndrome (del22q11.2), Williams syndrome (del7q11.23) or Prader-Willi syndrome (del15q11-13) and a growing number of syndromes of microdeletion and microduplication that are currently being described.

3.-**Monogenic diseases:** diseases caused by pathogenic genetic variants (mutations) or changes in the structure of a single gene that results in an altered protein or its absence. Monogenic diseases are divided according to their inheritance pattern into:

- Autosomal dominant. A prenatal diagnosis will be made when a parent is affected, since there is a 50% risk of transmission to the fetus. Prenatal diagnosis is also offered after the appearance of a "de novo" case in a previous child since, although the risk of recurrence is less than 5%, there could be germinal mosaicism in one of the parents. Some frequent diseases with autosomal dominant inheritance in our population are: Noonan syndrome, neurofibromatosis, tuberous sclerosis, Huntington's disease, myotonic dystrophy (Steinert), Marfan syndrome and achondroplasia

- Autosomal recessive. They require that both parents be carriers (healthy) or affected by the illness. In the most frequent case of healthy carriers, there are no cases in previous generations. Consanguineous couples are at increased risk of recessive diseases, as well as some ethnic groups where inbreeding is frequent. The current technological evolution allows a couple to undergo genetic screening to detect carrier status that analyzes up to 600 genes, and can determine thus the risk of having a child with a recessive genetic disease, due to the carrier state of their parents. In a consanguineous couple, carrier status should be ruled out in order to the most frequent recessive diseases in its population. Some of the most frequent recessive inheritance in our population are cystic fibrosis, congenital adrenal hyperplasia, beta-thalassemia (detectable on a blood count), spinal muscular atrophy, and Gaucher's disease.

- X Linked disorders. These are the so-called sex-linked diseases caused by a gene located on the X chromosome. Usually a healthy mother, but a carrier, transmits the X chromosome with the alteration to 50% of their offspring and 50% of males will be affected by the disease and 50% of women will be healthy carriers. Anyway, the women may also be affected in dominant sex-linked inheritance, although usually to a lesser degree, because of a phenomenon of preferential inactivation of the altered X chromosome. Some of the most frequent diseases with X-linked inheritance in our population are the syndrome of Fragile X, Duchenne muscular dystrophy, and hemophilia A and B.

Prenatal genetic tests are tests aimed at analyzing the fetal genome in order to identify the genetic cause of a certain disease. In case of family history, it will be necessary to have identified the mutation causing the disease in the family before doing the prenatal study. There exists various prenatal genetic diagnosis techniques and each test provides different specific information. Thus, in each case a specific test should be chosen, based on the diagnostic suspicion, on the individual risk and the preference and acceptance of the mother.

1.- QF-PCR (Quantitative Fluorescent Polymerase Chain Reaction)

QF-PCR quantifies the number of chromosomes 13, 18, 21, X and Y by studying various sequences of polymorphic DNA called short tandem repeats (STR) or microsatellite markers, located in each of the 5 chromosomes studied. The goal of the QF-PCR is the rapid diagnosis of the **most common aneuploidies**, which are those that involve these 5 chromosomes (trisomy 13, trisomy 18, trisomy 21 and sex aneuploidies). It is also able to detect triploidies and some tetraploidies.

QF-PCR allows detection of **maternal cell contamination** in a fetal sample. It is advisable rule out maternal contamination from a maternal sample, either from mouthwash or blood in situations where there is an increased risk of maternal contamination, such as in chorionic villi karyotypes from miscarriages, transplacental amniocentesis, or blood fluids.

QF-PCR is capable of detecting whether the nondisjunction causing a trisomy is of meiotic **origin** and therefore both present in the zygote (presence of three alleles in a 1:1:1 ratio for at least one of the markers studied) or probably mitotic and, therefore, post-zygotic (presence of 2 different alleles in relation 2:1 for all markers studied). The study with QF-PCR of the parents allows knowing the **parental origin** of the triploidies, digynic or diandric (associated with a partial mole), as well as of the chromosome in excess in a trisomy or, failing that, in monosomy X. In case of complete uniparental disomy (androgenetic) you can diagnose a complete mole. In the case of a multiple gestation, the QF-PCR is capable of detecting the zygosity of the twins, since the STR amplification pattern of all monozygotic twin markers will be identical.

The main **advantages** of this technique are:

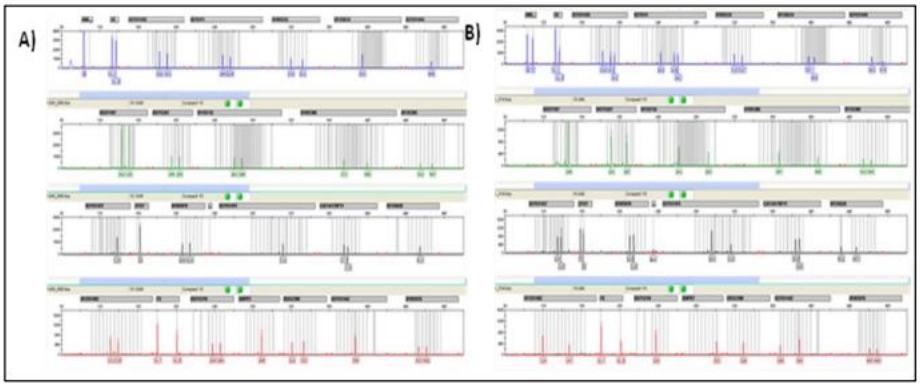
- its speed (results are available in 24-48 hours).
- the small sample volume required.
- no cell culture requirement.
- automated processing.
- obtaining a result in more than 99% of the samples.
- a relatively simple interpretation.
- a good cost-benefit ratio.

Among its **limitations** are:

- a study restricted to 5 chromosomes.
- no detection of monosomy except for Turner syndrome (since there are two markers that allow us to calculate the total number of sex chromosomes).
- non-detection of mosaics when they are low-grade (<20%) or the sample is contaminated maternal.
- autosomal trisomies cannot be classified as free trisomy or translocation trisomy.

QF-PCR has proven to be a very robust and careful technique and, although it does not require confirmation by another technique, it is not usually used in isolation. After a normal test result QF-PCR is advisable to perform either a CMA or a conventional karyotype, since there is a residual risk of

0.05% of an unfavorable phenotype after normal QF-PCR in low-risk cases aneuploidy. In our center it is the only chromosomal analysis that we carry out in cases of monochorionic twins, when they are pregnancies at low risk of aneuploidy. You can also accompany molecular studies, PCR for fetal infections or in the threat of preterm labor.

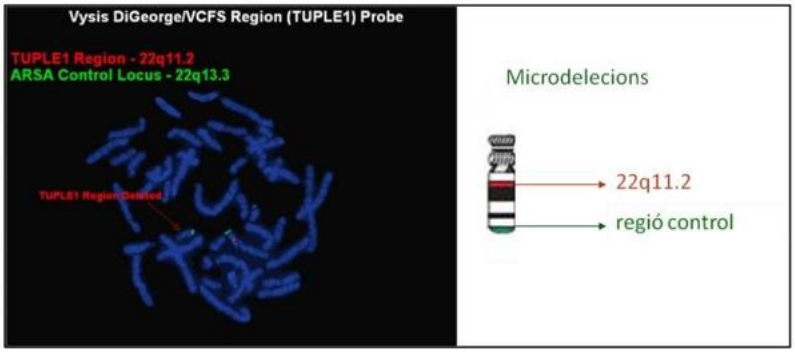


QF-PCR: A) Female fetus with a normal complement for chromosomes 13, 18, 21 and sex; B) Male fetus with trisomy 21.

2.-FISH (Fluorescent in situ hybridization)

FISH probe analysis can be performed on interphase nuclei, if there are enough cells, or on metaphase chromosomes after cell culture. The FISH study has been used in prenatal diagnosis with 3 main purposes:

1. Chromosomal characterization: useful when discovering chromosomal rearrangements or marker chromosomes (of unknown origin). Currently replaced by the CMA.
2. Study of microdeletions: it has been used for specific alterations, such as the syndrome of DiGeorge (del22q11.2) or Williams syndrome (del7q11.23). Each microdeletion requires a specific probe. It is currently being replaced by the CMA, except for the parental studies.
3. Rapid diagnosis of typical aneuploidies with the use of a probe for each of the following chromosomes 13,18, 21, X and Y on uncultured interphase nuclei. It has been replaced by QF-PCR, since the latter technique has some advantages (detection of contamination maternal, zygosity in multiples, origin of the parental alleles).



FISH: Study of the 22q11.2 microdeletion.

3.- KARYOTYPE

The **karyotype or cytogenetic study** consists of the analysis of the number and structure of all the chromosomes in metaphase, by studying the specific band pattern for each chromosome. It shows all the chromosomes in a single test and is therefore considered a complete genome analysis ("genomewide"), similar to CMA and unlike QF-PCR, FISH or MLPA (Multiplex Ligations Probe Amplification), the karyotype allows the identification of all numerical chromosomal abnormalities (including autosomal and sex aneuploidies), and structural anomalies with a segment chromosomal involved greater than 5-10 Mb (including balanced translocations, translocations unbalanced, deletions, duplications, inversions and insertions).

Sample processing and karyotyping are largely manual, and their interpretation requires expert staff. The minimum number of bands for a prenatal karyotype can vary between 250-400, depending on the tissue of origin and the indication for the analysis. Usually, the study chromosome is based on 20 metaphases. When the number of analyzable metaphases is lower, the result will be suboptimal and should be noted in the report. The diagnostic accuracy of the karyotype is greater than 99% for aneuploidy and structural chromosomal abnormalities with imbalances greater than 5-10Mb. It has a good cost-benefit ratio.

There are 3 **methods of obtaining metaphases** from fetal samples:

- **Short-term culture or semi-direct method in chorionic villi:** From the spontaneously replicating trophoblast cells, a karyotype can be obtained in 3-7 days. Studies the main cell type that originates cell-free fetal DNA circulating in maternal plasma studied in non-invasive aneuploidy tests. Culture failure is exceptional if you have a minimal number of villi. It does not present a risk of maternal contamination, since the maternal cells do not replicate spontaneously, but have a 2% risk of detecting abnormalities confined to the placenta, specifically the trophoblast. It has a risk of 1/3000 of false negatives. In many centers short-term culture has been replaced by QF-PCR.

- **Long-term culture (in chorionic villi or amniotic fluid):** It consists of a cell culture of 10-15 days duration necessary to obtain enough metaphases of mesenchymal cells from chorionic villi or desquamation cells present in the amniotic fluid. The chromosomes that are obtained usually have more band quality than those of short-term culture. Culture failure is more frequent when the sample comes from a fetal demise, but it is rare in chorionic villi or amniotic fluid samples in viable fetuses. There is a risk of maternal contamination if maternal cells are inadvertently cultured. Compared to short-term, the long-term culture has fewer false negatives and has a lower chance of detecting an abnormality confined to the placenta, so always after a short-term culture in chorionic villi, a long-term culture it is required.

- **Fetal blood culture:** A culture similar to that of postnatal peripheral blood is performed, consisting of stimulation of the lymphocytes.

It may be the case that a cytogenetic study fails to detect **mosaicism** if the various cell lines are not present in the sample obtained for analysis, or if it is a low-grade mosaicism frequency. Thus, a mosaic of 20% is detected in 99% of cases, if it is possible to study a minimum of 20 metaphases. In case of suspicion or discovery of mosaicism, it will be advisable to study a minimum of 50 metaphases. In case of diaphragmatic hernia, 50-100 metaphases/nuclei will be studied in the karyotype/FISH in amniotic fluid, since tetrasomy 12p causing Pallister-Killian syndrome that may be associated with diaphragmatic hernia is usually mosaic.

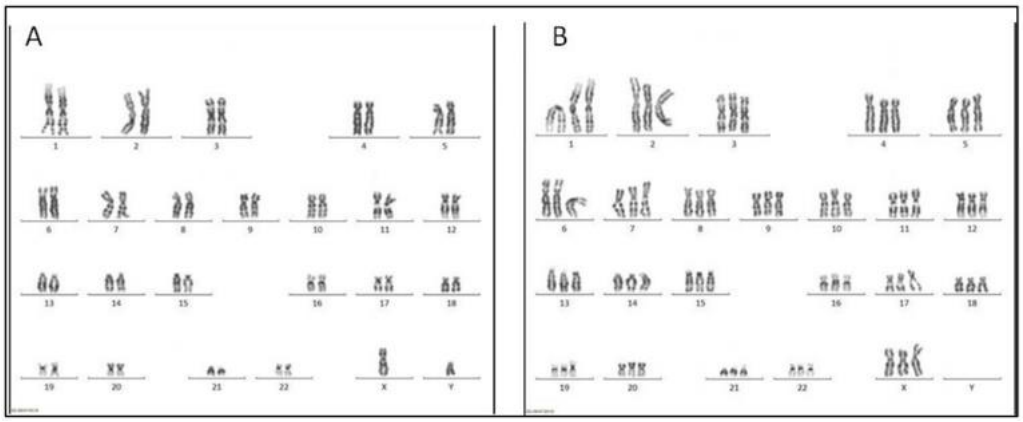
Feto-placental discrepancies: Prenatal cytogenetic studies on chorionic villi have evidenced the existence of feto-placental discrepancies in 1-2% of cases. So, to confirm some abnormal karyotypes, detected in villi samples, abnormalities detected in cells of origin strictly fetal as those of the amniotic fluid. Results are considered reliable in the following homogeneous (not mosaic) anomalies:

- trisomy 21
- trisomies of the sex chromosomes (47, XXX, 47, XXY, 47, XYY)
- triploidy
- familial structural anomalies.

On the contrary, it is necessary to check in amniotic fluid, in the absence of fetal ultrasound signs related to the anomaly:

- the rest of homogeneous anomalies (trisomies 13 and 18 and monosomy X)
- any mosaic abnormality with a normal cell line.

These recommendations apply whether the anomaly is found only in the short-term culture (suspected mosaic confined to the placenta, CPM type 1), only in the long-term culture (suspected CPM type 2), or in both cultures (suspected CPM type 3). And also if it is found in the QF-PCR, replacing the short-term culture.



Karyotypes: A) Male fetus with a normal karyotype. B) Female fetus with triploidy 69, XXX

Currently, the karyotype is being replaced by the chromosomal microarray analysis (CMA) in all invasive procedures, exception of the cases of diagnosis by QF-PCR of a trisomy 21, 13 or a monosomy X. It is preferable to cancel the CMA and perform a karyotype because it is able to show whether the trisomy is a free trisomy or a translocation trisomy, and whether monosomy X is pure line or mosaic.

4.-CHROMOSOMAL MICROARRAY ANALYSIS (CMA)

The **CMA** also called microarray, molecular karyotype or simply array, is a method of genetic analysis of the entire genome, with a resolution of 10 to 1000 times greater than that of the conventional karyotype, since it detects anomalies from 10Kb - 1Mb, depending on the chosen resolution. It allows to identify both aneuploidies and submicroscopic changes that would not be detected by a conventional karyotype.

There are two main microarray technologies:

· Comparative genomic hybridization microarray (array-CGH). It is the most used prenatally. It consists of the competitive hybridization of two differently labeled DNAs, a reference DNA and fetal DNA, on a solid support with DNA probes arranged according to their position in the genome and, therefore, allows detection of gain or loss of genetic material.

· SNP-array or "*single nucleotide polymorphisms array":* compares the intensity of DNA hybridization fetal with a previously determined control signal.

The **DNA probes** currently used in array-CGH are oligonucleotides. These probes are selected based on 2 criteria, since they are combined:

· probes distributed throughout the genome, with a uniform spacing ("backbone coverage")
· probes with a higher density in the regions causing known disorders ("hot spots")

Any **fetal sample** with sufficient DNA is valid for the CMA, such as chorionic villi, amniotic fluid, blood, or other fetal tissue or fluid. The extraction of 15-20 cc of amniotic fluid is recommended (30 cc in case of polyhydramnios) or 20-40 mg of chorionic villi. It is convenient to set always a backup culture, to be used in case more DNA needs to be extracted (more often in amniotic fluid than in chorionic villi), or to perform subsequent diagnostic techniques.

The **turn-around time** of a CMA performed directly on the sample is 7-10 days, but 2 additional weeks for culture may be required if not enough DNA has been obtained.

The losses and gains of genetic material detected by the CMA are called **copy number variations (CNV).** In any case, the CMA does not detect alterations in the DNA sequence, neither point mutations nor balanced rearrangements. According to their clinical relevance, CNVs are classified into:

· Benign CNVs: present in the general population, also called polymorphisms

· Pathogenic or likely pathogenic CNV: associated with abnormal phenotypes. can present incomplete penetrance (not all individuals carrying this CNV have the disease) or variable expressivity (the disease has a variable expression).

· Variant of uncertain significance (VUS): when there is not enough evidence in the literature or in the databases of their presence in the general healthy population, nor its association with abnormal phenotypes.

Similar to the karyotype, the CMA may not detect some cases of **mosaicism**, if it is not present in the analyzed cell sample or when one of the two lines is present in less than 20% cells.

In prenatal diagnosis, the CMA has more detection capacity as compared to the conventional karyotype in all indications of invasive testing. In case of fetal anomaly, it allows to detect an additional 6-8% ("incremental yield") of abnormalities above the karyotype. This rate varies by depending on the type of malformation and can be up to 17% in the case of congenital heart disease. In the cases in which the CMA has been performed without a clear medical indication or solely due to maternal advanced age, there is about a 1% incremental yield, that can be explained because microdeletion and microduplication syndromes are, together, a very relevant cause of pathology detectable antenatally.

A **previous analysis by QF-PCR** to the CMA allows:

· To rule out common aneuploidies and triploidies. It is important to take into account that array-CGH does not detect all triploidies (69,XXX).

· To determine the fetal sex.

· To rule out maternal cell contamination in the samples.

The result of the QF-PCR will indicate how the study should be continued:

· Abnormal QF-PCR: no need to be continued with the CMA. A karyotype can be used for genetic counselling of future pregnancies (mainly in trisomies 21 and 13 and monosomy X).

· If QF-PCR detects maternal contamination: several washings of the backup culture will be performed until no maternal contamination is detected, and then DNA will be extracted again for the CMA.

· Normal QF-PCR: continued with the CMA.

Although it would be ideal to obtain **parental samples** (blood, buccal swab or saliva) at the same time as the fetal sample, in order to ascertain the "de novo" or parental inherited origin of any CNV found, this is not a common practice. When a pathogenic CNV of variable expressivity or

low penetrance is found, parental samples should be studied “a posteriori” to determine if it is inherited or “de novo” appearance.

Informed consent: During the pre-test genetic counseling, the center's policy in relation to report the different types CNVs should be discussed. In our center, neither benign nor likely-benign CNVs or VUS in normal ultrasound are reported. In case of a VUS found in a malformed fetus, a committee formed “ad hoc” by geneticists and Fetal Medicine specialists will decide whether parental samples should be obtained after inform the couple. Pathogenic and likely pathogenic CNVs should be reported. In our center we give the option to the couple to be informed about the incidental findings that would include the following specific types of pathogenic CNV responsible for:

- Late-onset diseases, taking into account that one of the parents - may be carriers of the same alteration and present the disease before their child.
- Low penetrance diseases (only few individuals with this genetic change have the disease).
- Secondary findings which are defined as those actively searched for variants in 59 genes defined as "actionable" in the prevention of cancer and cardiovascular diseases are not currently reported in our center.

Array-CGH results: A) male hybridization profile without pathogenic variants: arr(X,Y)x1,(1-22)x2 and B) female hybridization profile with a 22q11.21 deletion: arr[CRGh37] 22q11. 21 (18894835_21505417)x1

Indications of the CMA: In our center, the CMA is the primary test in genetic invasive procedures and has already replaced the conventional karyotype. In any other center where karyotypes are still performed, the CMA should be the test of choice in case of:

1. Fetal structural abnormality on ultrasound: identification of a major malformation or more than one minor, as well as markers or findings suggestive of birth defects.
2. Fetal growth restriction (FGR): defined as an estimated fetal weight / biometrics < 3rd percentile
    a. isolated presentation, before 24 weeks.
    b. associated with any marker (including femur length < -3 SD and polyhydramnios, but not oligohydramnios) before 28 weeks.
    c. associated with malformations at any gestational age.
3. Increased nuchal translucency (>99th percentile)
4. Previous child with a cryptic deletion or duplication 'de novo' (not detectable by karyotype and therefore detected by CMA/FISH). There is a possibility of germinal mosaicism in one of the parents. Targeted FISH or MLPA could also be used, but the genomewide coverage of CMA recommends its use in case of invasive testing.
5. Family history of chromosomal rearrangement of risk for an ongoing pregnancy:
    a.Balanced reciprocal parental translocation or pericentric inversion.
    b. Familial cryptic deletion or duplication with significant transmission risk, penetrance, and clinical relevance.
    c. Marker chromosome mosaicism present in one parent that can be inherited by the fetus and potentially pathogenic character
6. Findings in the fetal karyotype:
    a. Reciprocal translocation.
    b. Apparently balanced 'de novo' inversion.
    c. Marker chromosome (especially ring-type and non-satellite marker). In these cases, a CMA with the highest possible resolution is recommended.
7. Second trimester pregnancy loss or intrauterine fetal demise: The fact of not requiring a cell culture allows it to be applied to non-viable cells, which would not grow in a culture, so in case of fetal demise this will be the technique of choice.

5.- MOLECULAR PRENATAL DIAGNOSIS OF MONOGENIC DISEASES

In pregnancies at high risk of monogenic diseases, either due to family history or due to sonographic findings, a prenatal diagnosis of the genetic disease "in utero" can be made, ideally when the gene is identified and the causative pathogenic variant is also known. There are several **molecular diagnostic techniques** that are applied according to the clinical suspicion and the type of causative alteration and it will be the laboratory specialist who must decide which is the most appropriate

technique to carry out the study. The indications of molecular studies in ultrasound findings are the following:

1. Specific genetic tests in suspicion of a specific disease:

a) Achondroplasia/hypochondroplasia: in case of short femur: < -3 SD or < -2.5 SD and femur/foot ratio <0.85. A femoral angle > 130º is the best predictor of achondroplasia.

b) Cystic fibrosis: in case of hyperechogenic bowel and/or multiple intra-abdominal calcifications. The current in house panel of 50 mutations has a coverage of 83% of the cases of cystic fibrosis in our population. The ethnicity should be specified, because the panels can be tailored to ethnicity. If one parent is a carrier of a *CFTR* gene mutation, the entire gene of the partner should be sequenced and gene CNVs studied to achieve a 99% detection.

c) Steinert myotonic dystrophy: in case of severe polyhydramnios (ILA>30).

d) Smith-Lemli-Opitz: in the finding of hypospadias + normal Doppler (< 28 weeks) associated with microcephaly (<-3 SD) (≥28 weeks). It is easier to perform the biochemical study of sterols than gene sequencing.

2. Known mutation study by classical Sanger sequencing in the following situations:

a. Segregation studies in the parents of a pathogenic or likely pathogenic variant found by Next-generation Sequencing (NGS) in a fetus using a multigene panel or the clinical exome.
b. Segregation studies of a mutation(s) in a new pregnancy of a family(ies) that can be inherited by the fetus, in either a dominant inherited disease, or recessive where the 2 parents are usually the carriers, or sex-linked where the mother is the carrier. In dominant "de novo" diseases, the risk of recurrence is 1%, explained by the presence of a germinal mosaicism in one of the parents.
c. Confirmation of a new variant found by NGS in a fetus (using a multigene panel or a clinical exome) in case of insufficient coverage (< 30 *reads)* or in *frameshift* mutations.

3. Multigene panel: It is a technique that simultaneously determines the sequence of various genes by NGS. Usually, the whole exome is sequenced but interpretation is directed to the genes of interest. It has the advantage of minimizing incidental results. It is used when a specific disease or abnormality can be caused by pathogenic variants in a large number of different known genes. In our environment we use the following panels, usually after having a normal result at CMA and without requiring blood from both parents:

a) Hydrops - RASopathies panel in:

- hydrops fetalis after a normal QF-PCR and in parallel to the CMA.
- or nuchal translucency ≥ 5 mm after normal QF-PCR and parallel to the CMA.

- or persistent nuchal fold ≥ 5 mm (16-17 weeks) or ≥ 6 mm (18-22 weeks) after an increased nuchal translucency > 99th percentile at 11-13 weeks.
- or nuchal translucency > 99th percentile and pulmonary stenosis or cardiomyopathy.

b) CAKUT panel *(congenital anomalies of the kidney and urinary tract):* in bilateral hyperechoic or dysplastic/polycystic kidneys.

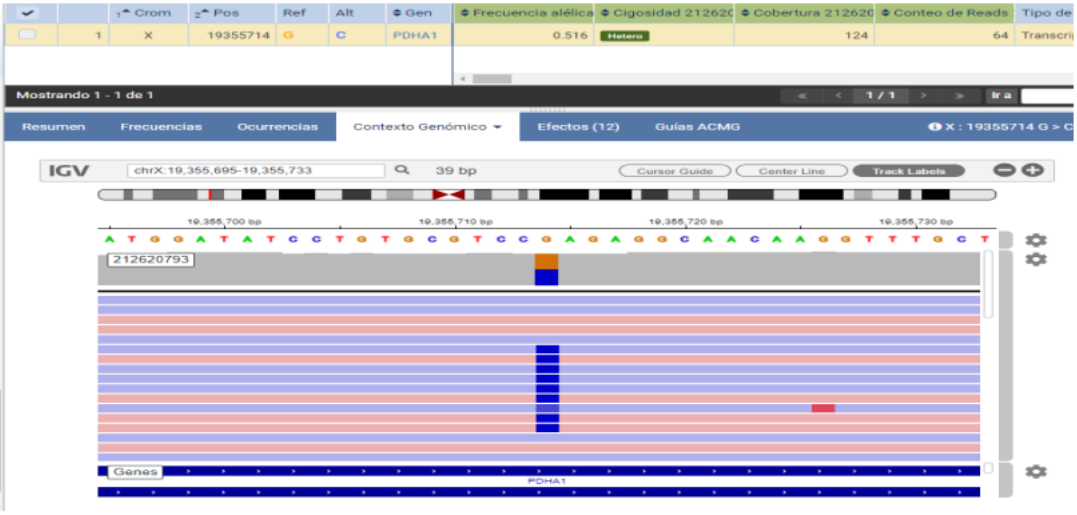
c) Osteogenesis imperfecta panel: in bent or fractured bones.

d) Craniosynostosis panel: in ultrasound suspicion of craniosynostosis

e) Tuberous sclerosis panel: in cardiac rhabdomyomas.

f) Fetal phenotype-driven panel (HPO codes): used by some centers before expanding it to clinical exome.

When a pathogenic or likely pathogenic variant is found, it should be segregated in the parents and Sanger sequencing of the gene involved performed in the parents and the fetus to check if it has been inherited or if it is "de novo".



Detection of the c.788G>C variant; p.Arg263Pro in the PDHA1 gene (NM_000284.4) in heterozygosis as displayed in the "Integrative Genome Viewer (IGV)" tool.

4. Clinical exome sequencing (CES): It is a technique that uses NGS to determine the sequence of all coding exons of all genes in the genome, but restricts the interpretation to the 4,000 OMIM genes associated with a clinical phenotype. The exome accounts for only 1-2% of the genome and contains about 85% of the variants known to cause disease. It is used when a specific disease or condition can be caused by pathogenic variants in many unknown genes. In our center, CES is performed, after obtaining a normal CMA, in case of:

a. Pregnancy with a recurrent fetal malformation
b. Fetus with malformations of 2 different systems (multisystem)
c. FGR with malformations of 2 systems (except hypospadias) or biometrics (FL or CC) < -3 SD without signs of placental insufficiency.

d. Skeletal dysplasia. In case of lethal skeletal dysplasia, it is preferrable to wait for the results of post-mortem studies
e. Complex abnormalities of the central nervous system.
f. Cardia defect or structural abnormality with hypoplastic/absent olfactory grooves (suspect CHARGE).

For exome sequencing a blood drawn and DNA extraction is required from both parents. In ongoing pregnancies when the turn-around time matters, exome sequencing will be performed using the "trio" approach, that is, sequencing the exomes of the fetus, mother and father simultaneously. Otherwise, the fetus alone will be sequenced and the parenteral DNA will be used to segregate the candidate variants. All results are discussed by a Clinical Review Committee where the pathogenicity of the variants is reviewed, and the overlap between the candidate variants and the phenotype of the fetus and other studied members of the family. In negative cases, the Committee will indicate which genes related to the observed fetal phenotype whose coverage should be reviewed. These committees should ideally include a molecular geneticist, one/two clinical geneticists (prenatal and pediatric), a perinatal pathologist and a genetic counseler.

Advice post termination of pregnancy (TOP): In case of TOP in a malformed fetus without a previous genetic diagnosis (with normal QF-PCR, karyotype or CMA) and without saved DNA, the obtention of a fetal sample (amniotic fluid, fetal tissue, or blood) prior to TOP is desirable to allow the performance of future molecular studies driven by the post-mortem findings, and a radiography in case of skeletal dysplasia.

6.- EPIGENETICS: STUDIES OF UNIPARENTAL DISOMY AND METHYLATION

Epigenetics studies the changes in gene expression produced by a mechanism other than DNA sequence changes. In the human genome there are more than 100 "imprinted" genes, with a monoallelic expression (preferential or exclusive) of a single copy of the gene depending on its parental origin. Thus, there are diseases that appear due to the silencing of a correct sequence of DNA, usually by cytosine methylation, or by having the 2 copies of an imprinted gene that comes from the same parent (uniparental disomy, UPD).

1. **Methylation studies** on imprinted genes relevant to fetal development and differentiation are performed using the MS-MLPA technique *(Methylation-Specific Multiplex ligation-dependent probe amplification).* For suspicion of Silver-Russell or Beckwith-Wiedemann we will carry out studies of methylation in case of:
    a. FGR with relative macrocephaly HC/AC>90th percentile or long bone asymmetry >15%: 11p15 methylation MLPA studies for suspicion of Silver-Russell syndrome, since a 30-50% of cases are caused by hypomethylation of the paternal IC1 imprinting centre.

b. Isolated omphalocele or associated with organomegaly, asymmetry or macroglossia: 11p15 methylation MLPA, for suspicion of Beckwith Wiedemann syndrome since it may already be caused by hypomethylation of the maternal IC2 imprinting center (40-50%) or a hypermethylation of the maternal IC1 (5-10%), or by both changes appearing in a paternal UPD (20%), or finally for changes in the 11p15.5 region.
c. Omphalocele with reduced thorax or polyhydramnios: 14q31-32 MS-MLPA for suspicion of Kagami-Ogata syndrome.

2. **Uniparental disomy** (UPD) occurs when the 2 homologous chromosomes of the same pair come from the same parent. It can be classified in **isodisomy** if both chromosomes are identical and heterodisomy if they correspond to 2 alleles of a parent. The incidence of uniparental disomy transmission of any chromosome in live newborns has been estimated at 1/3500.

   The clinical relevance of **uniparental disomy** depends mainly on whether the chromosome involved is one of the 6 chromosomes that present regions subject to imprinting (chromosomes 6,7,11,14,15 and 20). Therefore, the heterodisomy study with DNA samples from both parents will be indicated when any of the 6 imprinted chromosomes are involved in:

   a. Trisomies confined to the placenta, pure or in mosaic. In trisomy 15 confined to the placenta there is Prader-Willi /Angelman risk.
   b. Robertsonian translocation involving chromosomes 14 or 15.
   c. Marker chromosome derived from imprinted chromosomes
   d. Suspected Silver-Russell syndrome: maternal UPD7 in amniotic fluid (10% of cases) in case of normal methylation studies.

Microsatellite study of chromosome 15 showing a maternal uniparental disomy causing Prader-Willi syndrome.

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