Cytogenomic Internship and Prenatal Diagnosis Study using aCGH for Genotype–Phenotype Correlation in 772 Fetuses

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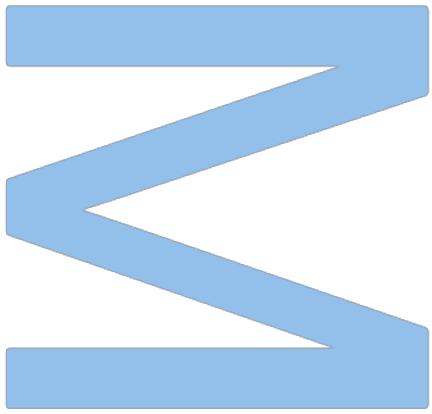
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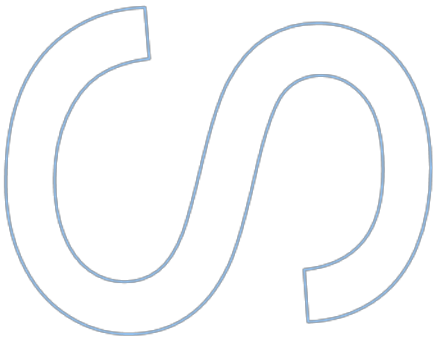
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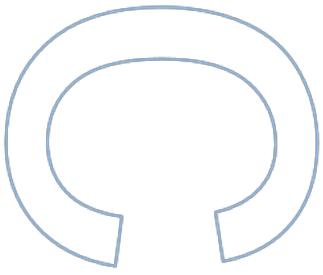

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Resumo

A citogenética humana consiste no estudo da morfologia e função dos cromossomas. Com os avanços tecnológicos observados nos últimos anos, ocorreu uma evolução dos aparelhos de microscopia e técnicas de cultura e bandeamento, levando a uma explosão de conhecimento nesta área. Isto levou à criação de uma nova área: a citogenética clínica, uma perspetiva genómica aplicada à medicina, que visa identificar anomalias cromossómicas e correlacioná-las com fenótipos e patologias.

Inúmeras patologias estão associadas a distúrbios genéticos, como deficiência intelectual, infertilidade e malformações congénitas.

A identificação de rearranjos cromossómicos pode ser realizada recorrendo a diferentes técnicas, em contexto pré e pós-natal, sendo que cada uma tem vantagens e desvantagens inerentes. As técnicas de citogenética convencional estudam cromossomas em divisão celular e permitem uma análise global de todo o genoma, detetando a maioria das anormalidades cromossómicas, com resolução de 5-10Mb. As técnicas citogenómicas fundem princípios citogenéticos com moleculares, focando-se no nível microscópico/submicroscópico e a resoluções moleculares.

Neste estágio, foram abordadas várias técnicas: técnicas de citogenética convencional (cultura celular, manipulação, espalhamento e bandeamento) para obter o cariótipo e técnicas de citogenética molecular como Hibridização Fluorescente *in situ* (FISH), Hibridização Genómica Comparativa em Array (aCGH) e Marcação de *nicks* por dUTP e Deoxinucleotidil Terminal Transferase (TUNEL).

Foram descritos sete casos clínicos, envolvendo protocolos citogenéticos e/ou citogenómicos, com o intuito de elucidar toda a dinâmica por trás do processo de diagnóstico e como as técnicas se complementam de forma a atingir um melhor resultado de diagnóstico e uma correta relação genótipo-fenótipo.

Além disso, foi realizado um estudo na área de pré-natal utilizando a técnica aCGH para uma correlação genótipo-fenótipo numa coorte de 772 fetos. O aCGH está na linha da frente do diagnóstico genético pré-natal. As indicações para realizar estudos genéticos são variáveis e é importante correlacionar os achados clínicos com as alterações genéticas. O objetivo é avaliar a prevalência de variações do número de cópias (CNVs) anormais detetadas por aCGH em amostras de pré-natal, correlacionar CNVs patogénicas com achados clínicos, analisar a prevalência das variantes de

significado incerto (VUS) e salientar a importância clínica do aCGH no diagnóstico pré-natal.

Os nossos resultados demonstram uma taxa de deteção de 8,3% (6.4-10.5%, intervalo de confiança de 95% (CI)) de CNVs patogénicas. Dentro deste grupo, a indicação mais frequente foi malformações estruturais (57%), maioritariamente envolvendo o sistema nervoso central, esquelético e cardíaco. O número de resultados patogénicos detetados em casos com malformações fetais múltiplas foi estatisticamente superior que em casos com malformações num sistema isolado ($p<0.001$). A segunda indicação mais frequente foi aumento da translucência da nuca (5-6.4mm). No entanto, a taxa de CNVs patogénicas não apresentou diferenças estatisticamente significativas entre os grupos de malformações estruturais e não-estruturais ($p>0,001$), elucidando a relevância do estudo genético por aCGH em todos os casos. Foram identificados 217 fetos com CNVs classificadas como VUS, envolvendo principalmente os cromossomos X, 1 e 16. Os nossos resultados demonstram um aumento de 4,9% (4,2-5,6%, CI 95%) no rendimento de diagnóstico usando aCGH em comparação com o uso do cariótipo convencional isoladamente, confirmando que o aCGH pode melhorar o rigor do diagnóstico pré-natal.

Este trabalho fornece uma análise genótipo-fenótipo completa, que pode ser clinicamente útil para a classificação de variantes e para a compreensão de fenótipos, de forma a que os resultados de diagnóstico sejam cada vez mais corretos e claros.

Palavras-chave: Citogenética convencional, citogenómica, diagnóstico, alterações cromossómicas, pré-natal, aCGH, malformações estruturais, variação do número de cópias, VUS.

Abstract

Human cytogenetics is the study of the morphology and function of chromosome. With the technological development observed in the last years, microscopy devices, culture and banding techniques were improved, which gave rise to an explosion of knowledge in this area. This led to a new area: clinical cytogenetics, a genome-wide perspective applied to medicine, which aims to identify chromosomal abnormalities and correlate them with phenotypes and pathologies. Numerous pathologies are associated with genetic disorders, such as intellectual disability, infertility, and congenital malformations.

The identification of chromosomal rearrangements can be carried out by different techniques, in prenatal and postnatal areas, each of which has inherent advantages and disadvantages. Conventional cytogenetic techniques study chromosomes in cell division and allows a global screen of the entire genome by detecting the majority of chromosomal abnormalities, with a resolution of 5-10Mb. Cytogenomics techniques, which merge cytogenetic and molecular principles, are focused on the microscopic/submicroscopic level and at molecular resolutions.

In this internship, several techniques were addressed: conventional cytogenetic techniques (cell culture, manipulation, spreading and banding) to obtain the karyotype, and the molecular cytogenetic techniques Fluorescence *In Situ* Hybridization, Array Comparative Genomic Hybridization (aCGH) and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

Seven clinical cases were described, involving the different cytogenetics and/or cytogenomics protocols, aiming to elucidate all the dynamics behind the diagnostic process and how the techniques could complement each other to reach a better report and a correct genotype-phenotype relationship.

Furthermore, it was also conducted a study in the prenatal area using the aCGH technique for genotype-phenotype correlation in a cohort of 772 fetuses. aCGH is at the forefront of prenatal genetic diagnosis. The indications for performing genetic studies are variable and it is important to correlate the clinical findings with the genetic alteration. The aim was to evaluate the prevalence of abnormal copy number variations (CNVs) detected by aCGH, in prenatal samples, correlate pathological CNVs with clinical findings, analyse the prevalence of variants of uncertain significance (VUS) and highlight the clinical importance of aCGH in prenatal diagnosis.

Our results demonstrated 8.3% (6.4-10.5%, 95% confidence interval (CI)) detection rate of pathogenic CNVs. Within this group, the main indication was structural malformations (57%) mainly involving the central nervous system, skeleton and cardiac systems. Pathogenic results in cases with multiple malformations was statistically higher than in cases with isolated anatomical system malformations ($p<0.001$). The second most frequent indication was increased nuchal translucency (5-6.4mm). However, the rate of pathogenic CNVs did not show significant differences between structural and non-structural malformations ($p>0.001$), highlighting the relevance of genetic study by aCGH in all cases. A total of 217 fetuses with CNVs classified as VUS were identified, mainly involving chromosomes X, 1 and 16. Our findings demonstrate 4.9% (4.2-5.6%, 95% CI) increased in the diagnostic yield using aCGH compared to the use of conventional karyotype alone, confirming that the aCGH can improve the accuracy of prenatal diagnosis.

Our survey provides a full genotype-phenotype analysis that can be clinically useful for the classification of variants and for the understanding of phenotypes so that the diagnostic result is increasingly reliable and clear.

Keywords: Conventional cytogenetics, cytogenomics, diagnosis, chromosomal abnormalities, prenatal, aCGH, structural malformations, copy number variations, VUS.

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Abbreviation List

FISH	Fluorescence *in situ* Hybridization
MLPA	Multiplex Ligation-Dependent Probe Amplification
aCGH	Array Comparative Genomic Hybridization
NGS	Next Generation Sequencing
DNA	Deoxyribonucleic Acid
NOR	Nucleolus Organizer Region
G1	Gap 1
S	Synthesis
G2	Gap 2
GL/FMUP	Genetic Laboratory, included in the Pathology Department of the Faculty of Medicine of University of Porto
CNVs	Copy Number Variations
ISCN	International System of Chromosome Nomenclature
ACOG	The American Congress of Obstetricians and Gynecologists
CVS	Chorionic Villus Sampling
PUBS	Percutaneous Umbilical Blood Sampling
PCR	Polymerase Chain Reaction
GenQA	Genomics Quality Assessment
FBS	Fetal Bovine Serum
HEPA	High-Efficiency Particulate Air
t-Flasks	Tissue Culture Flasks
MTX	Methotrexate
QF-PCR	Quantitative Fluorescent Polymerase Chain Reaction
PBS	Phosphate-Buffered Saline
EDTA	Ethylenediaminetetraacetic Acid
GTL staining	G-Bands using Trypsin and Leishman Stain
C-banding	Centromeric Heterochromatin Staining
DAPI	4’,6-Diamidino-2-Phenylindole

DA	Distamycin A
DA-DAPI staining	Distamycin-DAPI staining
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
ISH	*In Situ* Hybridization
COBRA	Combined Binary Ratio Labeling FISH
SKY	Spectral Karyotyping FISH
M-FISH	Multicolor FISH
TDT	Terminal Deoxynucleotidyl Transferase
dUTPS	Triphosphated Deoxyuridine Nucleotides
FITC	Fluorescein Isocyanate
ASRM	American Society For Reproductive Medicine
CMA	Chromosomal Microarray Analysis
SNPs	Single Nucleotide Polymorphism
VUS	Variants Of Uncertain Significance
SMFM	Society For Maternal Fetal Medicine
P	Pathogenic
LP	Likely-Pathogenic
B	Benign
LP	Likely-Benign
DGV	Database Of Genomic Variants
OMIM	Online Mendelian Inheritance In Man
SRY	Sex-Determining Region of Y Chromosome
CHRNA7	Cholinergic Receptor Nicotinic Alpha 7 Subunit

I. Introduction

1 Historical perspective of genetics and clinical cytogenetics

Clinical genetics focuses on the study of human biological variation and its relationship to health and disease. It encompasses mechanisms of inheritance, cytogenetics, molecular and biochemical genetics.

Its discovery was not a punctual work of a single scientist, but a gradual and lengthy process resulting from the research of countless scientists and the correlation of several discoveries spaced in time until reaching the knowledge we have today.

We cannot discuss genetics without mentioning the work of Gregor Mendel and Charles Darwin, two contemporary scientists of the 19th century considered the pioneers of genetics. Darwin studied the mechanisms of heredity, presenting the first developmental theory of heredity - Pangenesis. Mendel studied the evolutionary phenomenon by proposing that cells contained some factor that passed from generation to generation, transmitting information. Later, these revolutionary ideologies would become the inspiration and basis of future theories, however, at that time, the lack of scientific means to deepen these ideologies made their works go unnoticed in the scientific community[1,2].

By the middle of the 19th century, it was already consensual that the cells derived from other cells and that the hereditary information was contained in the nucleus, nevertheless the nature of the hereditary material was still uncertain. In 1882, Walther Flemming, a German anatomist, published the first illustrations of human chromosomes, referring to the stainable portion of the nucleus as chromatin, deduced the movement of chromosomes during cell division and applied the term "mitosis" for the first time (Figure 1). This discovery marks the beginning of cytogenetics, a field of genetics focused on the study of cell structures, mainly chromosomal analysis, and its relationship with heredity[3].

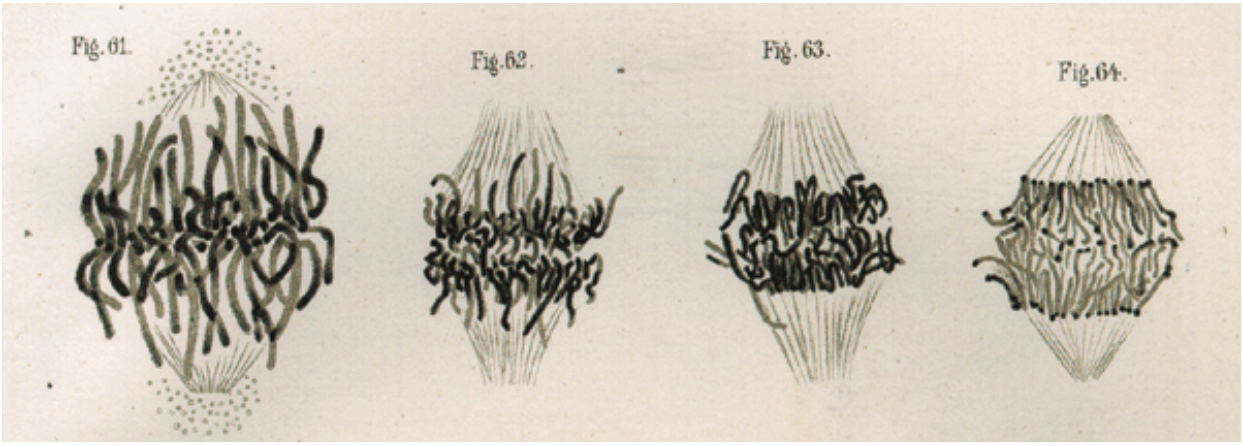


Figure 1 - The first illustrations of mitosis and chromosomes by Walter Flemming, which mark the beginning of cytogenetics[4].

The word chromosome was used for the first time in 1888 by Heinrich Waldeyer referring to what Gregor Mendel named “Kopplungsgruppen“ (“linked up groups” in German)[5]. Subsequently, the chromosome-theory of inheritance was proposed by Walter Sutton and Theodor Boveri in 1902/1903, being also observed by Sutton similarities between chromosomal behavior and the Mendelian segregation of genes[6,7].

The following years were characterized by technological advancements, namely in the manipulation methods and improvement of optical lenses that allowed the perpetuation of cytogenetic studies[3].

The beginning of the 20th century was marked by the attempt to determine the correct number of the human sex chromosomes per cell despite the staining procedures were still very rudimentary. Von Winiwarter, in 1912, reported that men and women have 47 and 48 chromosomes, respectively [8]. In 1921, Painter also reported that the number of human chromosomes was 48[9].

Although technical improvements continued, the visualization and distinction of individual chromosomes was not absolutely clear, which led to the estimate of 48 chromosomes not only being taken as a fact for decades, but also other studies "confirmed" this same result [10]. One example is the study by Hsu, in 1952, that due to an error in the procedure, cell cultures were washed with a hypotonic solution instead of an isotonic solution. This caused the entry of water into the cells, promoting the swelling and the separation of the chromosomes, facilitating their visualization and even so, 48 chromosomes were reported[11]. However, due to this accidental discovery, four years later, Tjio and Levan combined this technique with the application of colchicine which

resulted in the spread of chromosomes with sufficient quality to perform the first clear count, concluding that the human diploid chromosome number was 46[12].

With the knowledge of the number of chromosomes in a normal diploid cell, it did not take long for the discovery of the first chromosomal syndromes, thus giving rise to a new area: clinical cytogenetics, a genome-wide perspective applied to medicine [13].

In 1959, Lejeune, Gautier and Turpin reported for the first time a chromosomal disorder [14], caused by an extra chromosome 21, called trisomy 21 and further Down syndrome. Subsequently, other aneuploidies were reported in the following years such as Turner's syndrome [15], Edward's syndrome [16] and Patau's syndrome [17].

Until this time, the differentiation of chromosomes was taking into account the size and appearance/general structure, which did not allow the clear identification of all chromosomes. The first chromosome banding technique was reported in 1968 by Torbjörn Caspersson. Through his work on plant cells, the Swedish biologist realized that each chromosome, when labeled with fluorescent quinacrine compounds, did not fluoresce uniformly along the entire arms, but rather that each pair of chromosomes presented a unique pattern that made it possible to clearly distinguish each chromosomal pair [18]. Caspersson carried out the same study on human chromosomes and reported the characteristic “banding” pattern of each pair of chromosomes [19].

The technical difficulties of the protocol led to the discovery of other banding techniques. Drets and Shaw described a method of producing similar chromosomal banding patterns, dependent on the heterochromatin present along each chromosome, using an alkali and saline pretreatment followed by staining with Giemsa [20]. This method has undergone several modifications over the years but remains the golden standard of classical cytogenetic analysis.

With the widespread of the staining techniques, several new syndromes were reported, followed by a large an investment in the discovery of new techniques that could overcome some of the disadvantages of classical cytogenetics. Despite conventional cytogenetics offering a low-cost view of the complete genome at the single cell level that allows the detection of numerical and structural alterations, this technique is quite time-consuming because it involves cell culture, also requires highly qualified technicians, and has a resolution of only 5-10Mb[21,22].

Molecular cytogenetic techniques were then introduced, which had greater resolution and more specificity. The fluorescence in situ hybridization (FISH) was the first

technique to be developed, allowing the detection of smaller alterations not observable on the karyotype, using probes that mark the region of interest.

Subsequently, other new techniques began to emerge that allowed the evaluation of copy number variations, such as Multiplex Ligation-dependent Probe Amplification (MLPA), Array Comparative Genomic Hybridization (aCGH) and Next Generation Sequencing (NGS), a more detailed technique that allow the sequencing of millions of small DNA fragments and enable the detection of variants at the DNA sequence level[23,24]. These techniques were initially applied in the postnatal context, but the application in prenatal diagnosis also began to emerge after realizing that amniotic fluid could be a source of fetal DNA and be cultured[25].

All these discoveries and technological advances over the years resulted in an explosion of knowledge in this area: the development of microscopes allowed images to become witnesses, reliable and easily reproducible protocols led to their application throughout the world and international research projects such as the Human Genome Project were initiated[26,27]. All this culminated in an increased accuracy and resolution rate leading to a better diagnostic yield and, as such, leading to the progress of medical genetics.

Both conventional cytogenetics and molecular cytogenetics (also called cytogenomics) can be applied in the clinical field for studies in the context of pre and postnatal diagnosis. Each technique has advantages and disadvantages, that will be addressed later, so different approaches may be used in order to complement the results. The genetic study should be seen as a dynamic process and it is the responsibility of the medical team, cytogeneticists and technicians to analyse each case individually to understand which techniques are most appropriate.

2 DNA, Chromosomes and Cell cycle

The nucleus of cells contains deoxyribonucleic acid (DNA), the molecule that carries all the genetic information of an organism. DNA is not found in its naked form in the cell, but in association with various proteins, namely histones, in a complex called chromatin. Each set of histone octomer forms a disk structure around 150 base pairs of DNA, called a nucleosome. These nucleosome chains are packed into a secondary helical chromatin structure, which will be highly compacted to form units: the chromosomes[28].

Human beings have 46 chromosomes per diploid cell, arranged in 23 pairs: 22 pairs of chromosomes called autosomes and the remaining pair called sex chromosomes, which can be X or Y. The set of autosomes is the same in women and men, however the complement of sex chromosomes in women is XX and in men is XY. Under normal conditions, offspring inherits one chromosome from each pair from each parent.

Chromosomes, formed by two sister chromatids, structurally have a short arm, designated p, and a long arm, designated q. Functionally, they can be divided into, centromeres, telomeres, and nucleolus organizer region (NOR), in the case of acrocentric chromosomes (Figure 2). The centromere is a constriction where there is union of the two sister chromatids, visible in the metaphase chromosomes. Telomeres are the terminal region of chromosomes and perform structural, protective functions and ensure complete DNA replication. The NOR regions, found on the satellite stalks of acrocentric chromosomes, participate in nucleolus formation and are responsible for rRNA production [3]. The positions of the different regions of each chromosome are not random, there is order. The entire organization of the chromosome is fundamental for the regulation of gene expression[29].

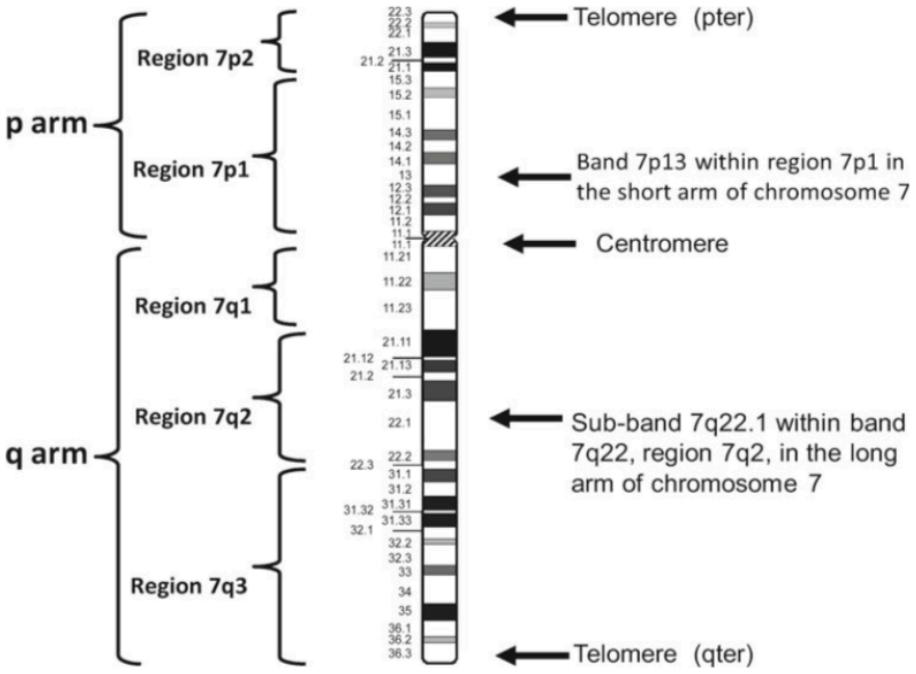


Figure 2 - Morphological and structural features of a human chromosome, in this example chromosome 7[3].

2.1 Cell Cycle

For the development of cytogenetics, it was fundamental to understand the cell cycle and the behavior of chromosomes throughout the different phases. The cell cycle consists of an important orderly set of events that involves cell growth and cell division in order to produce new cells. This ensures the production of sex cells and the growth, development, repair and replacement of body cells throughout life.

There are two types of cell division: mitosis and meiosis. Mitosis consists of the division of somatic cells, resulting in two cells genetically identical to the cell that gave rise to them (diploid or 46 chromosomes). Meiosis consists of the division of germ cells, giving rise to gametes, with one chromosome of each pair (haploid or 23 chromosomes)[28].

The cell cycle lasts approximately 17-18 hours and could be divided into 2 main phases: interphase and M phase[3].

Interphase is the longest phase of the cell cycle, characterized by intense metabolic activity, and subdivided into three stages: Gap 1 (G1), Synthesis (S) and Gap 2 (G2). The G1 phase is normally the longest, and it is during this stage that important components for the entire cell cycle are synthesized, from membranes, organelles, ribosomes, proteins, etc. that cause the cells to increase their size. If conditions are favorable for cell growth and division, cells enter the next stage, the S phase, where complementary and semi-conservative DNA replication takes place. That is, cells have twice the amount of normal human DNA (4n). Then, the G2 phase occurs, where the cell prepares to enter the division phase through the synthesis of RNA and proteins[30].

2.1.1 Mitosis

After interphase is completed, cells enter M phase, which has a period of nuclear division (mitosis) that includes prophase, metaphase, anaphase, telophase, and a period of cytoplasmic division, cytokinesis.

During prophase, the previously duplicated chromosomes condense, the spindle begins to form, and the nucleus membrane and nucleolus disappear. In metaphase, the chromosomes present their maximum level of condensation, being easily observable under the microscope, which is why cytogenetic studies are carried out at this stage. The chromosomes adopt a position in the equatorial plane and attach to the spindle

microtubules at the centromere. In anaphase, sister chromatids divide and migrate to opposite poles of the spindle as chromosomes. Upon reaching the spindle pole, telophase begins, with the formation of the nuclear membrane of the two new cells.

Finally, the physical division of the two cells takes place by the process called cytokinesis[3,30].

2.1.2 Meiosis

As mentioned earlier, in germ cells there is a different type of cell division called meiosis. In meiosis there are two cycles: meiosis I, where there is a reduction division of the genetic material and meiosis II, where there is an equational division of the genetic material. This process results in haploid cells (23 chromosomes), and the diploid complement is restored upon fertilization.

Meiosis I is divided into four distinct phases: prophase I, metaphase I, anaphase I and telophase I. In prophase I, chromosome condensation begins and homologous chromosomes pair (each pair of homologous chromosomes has four chromatids). In this stage, crossing over processes occur, which leads to recombination of the genetic material. Later, in metaphase I, the nuclear membrane disappears and the meiotic spindle is formed with the chromosomes positioned along the equatorial plane. In anaphase I, the centromeres of each bivalent are separated, and each component of the pair migrates to one of the poles of the cell. With the arrival of each set of haploid chromosomes at the pole of the cell, telophase I begins and the nucleolus reorganizes.

After this first cycle of meiosis, cytokinesis occurs obtaining two daughter cells with 23 chromosomes, each containing two chromatids[3,30].

Subsequently, meiosis II proceeds similarly to mitosis with prophase II, metaphase II, anaphase II, telophase II, with an equational division, in males [29], resulting in four daughter cells, each containing 23 chromosomes of only one chromatid, which after differentiation produces four sperm cells. In females, is produced only one oocyte (with 23 chromosomes), and two polar bodies.

3 Laboratory characterization

The Genetic Laboratory, included in the Pathology Department of the Faculty of Medicine of University of Porto (GL/FMUP), focuses its activity on teaching, community

support and research. Directed by Professor Filipa Carvalho, the Genetic laboratory is divided into two major areas: Cytogenetics, coordinated by Professor Sofia Dória, and Molecular Genetics, coordinated by Professor Filipa Carvalho and Doctor Susana Fernandes. It provides services to the community directly through the cytogenetics and molecular genetics laboratories and indirectly through the collaboration established with the Centro Hospitalar Universitário de São João and other Hospital Centers.

Some of the molecular genetic tests performed at the GL/FMUP are not performed by any other public national laboratory. The implementation of molecular diagnostic techniques for diseases in which there is no diagnostic offer in Portugal is one of the strategies of the laboratory. In addition, GL/FMUP has actively participated in several research projects in different national and international areas.

It is integrated in a quality management system, certified by the ISO 15189 standard. The laboratories covered by this standard are internationally recognized and a reference for safety and quality in the clinical field.

In addition, the GL/FMUP is currently under the accreditation process by the Instituto Português de Acreditação. Accreditation consists of the demonstration and recognition, through an assessment carried out by an Accreditation Corporation, of the technical and management competence of an entity to carry out specific conformity assessment activities.

4 Objectives

The internship, included in the scope of the Master's degree in Biochemistry, aims to integrate a diagnostic laboratory, with a multidisciplinary team, acquiring skills and knowledge of clinical cytogenetics and cytogenomics.

By integrating a diagnostic laboratory, it is intended to understand the different stages of a diagnostic process, following the cases from entry, the procedures, analysis, discussion of results and final report. Be able to be autonomous in different laboratory technologies, understanding the advantages and limitations of each technique, and have a critical thinking to understand which technique is best suited to be used, according to the clinical indication. It is intended to interpret the results obtained, prepare the reports and relate learned concepts, applying the correct nomenclature. Moreover, enter the routine of a diagnostic laboratory, from laboratory standards, ensure quality, regulation and work ethic.

At the same time, it was proposed a research work in order to develop specific skills in research and scientific writing. Therefore, it was conduct a retrospective study of 772 prenatal cases with clinical indication for genetic study by aCGH technique, of Centro Hospitalar Universitário de São João and Centro Hospitalar de Vila Nova de Gaia e Espinho, from March 2013 to June 2022. The following objectives were established:

- Re-evaluate the classification attributed to the alterations found in the cases;
- Identify the main clinical indications for prenatal diagnosis and compare with the literature, in studies with the similar and larger sample size;
- Evaluate the prevalence of abnormal copy number variations (CNVs) in prenatal samples
- Correlate pathological CNVs with clinical findings;
- Evaluate the prevalence of VUS and which chromosomes are most involved;
- Report variants rarely discussed in the literature;
- Compare the diagnostic yield of aCGH with conventional cytogenetic;
- Highlight the clinical importance of aCGH in prenatal diagnosis.

II. Clinical Cytogenetics

1 Chromosomal abnormalities

Although all cellular processes, including cell division, are highly regulated with multiple checkpoints throughout the various stages, errors do occur and can result in chromosomal abnormalities. In addition, external factors such as the consumption of certain drugs can also lead to errors. Clinical cytogenetics aims to identify these alterations and correlate them with the observed phenotype, identifying syndromes and recurrent regions in order to make a diagnosis as clear as possible. Chromosomal abnormalities are the main type of genetic diseases, with an incidence of 1 in 554 births and can result in the most diverse clinical manifestations, from congenital malformations, intellectual disability, miscarriage, and infertility[29].

Chromosomal alterations can be numerical or structural and can involve autosomes and/or sex chromosomes. The severity of the phenotype will depend on several factors: for numerical alterations will depends on whether it is a gain or loss and which chromosome is involved, larger chromosomes with more genetic information result in more severe phenotypes or are incompatible with life, ending in a miscarriage; in the case of structural alterations, it also depends on whether it is a gain or loss of genetic material, the location of the breakpoints, the chromosome itself and the genetic constitution of the region involved[31].

1.1 Numerical Chromosomal Abnormalities

Numerical anomalies can be classified into two groups: poliploidies (alteration of the entire chromosomal complement (n)) and aneuploidies (alteration of the number of individual chromosomes). The causes for the occurrence of these anomalies are variable[32].

The origins of poliploidies can be due to errors in fertilization, for example an egg fertilized by two sperm, errors in mitosis, namely the occurrence of an endoreplication (two replications before mitosis) or induction by drugs, such as colchicine which blocks

metaphase or cytochalasin B which inhibits cytokinesis culminating in binucleated (tetraploid) cells[29].

The etiology of aneuploidies is meiotic or mitotic nondisjunction. Meiotic nondisjunction in either meiosis I or meiosis II results in the formation of nullisomic and disomic gametes (Figure 3). Mitotic non-disjunction leads to the formation of monosomic and trisomic cells, which may occur in mosaic, where the phenotypic expression will depend on the frequency of the altered cells: when the non-disjunction is later, the greater the prevalence of altered cells, the greater the phenotypic expression of the syndrome (Figure 3). A low frequency of recombination is one of the main predisposing factors for non-disjunction, however it is also known that advanced maternal age is also related to the occurrence of aneuploidies. The most common aneuploidies are trisomy 21, trisomy 18 and trisomy 13[3].

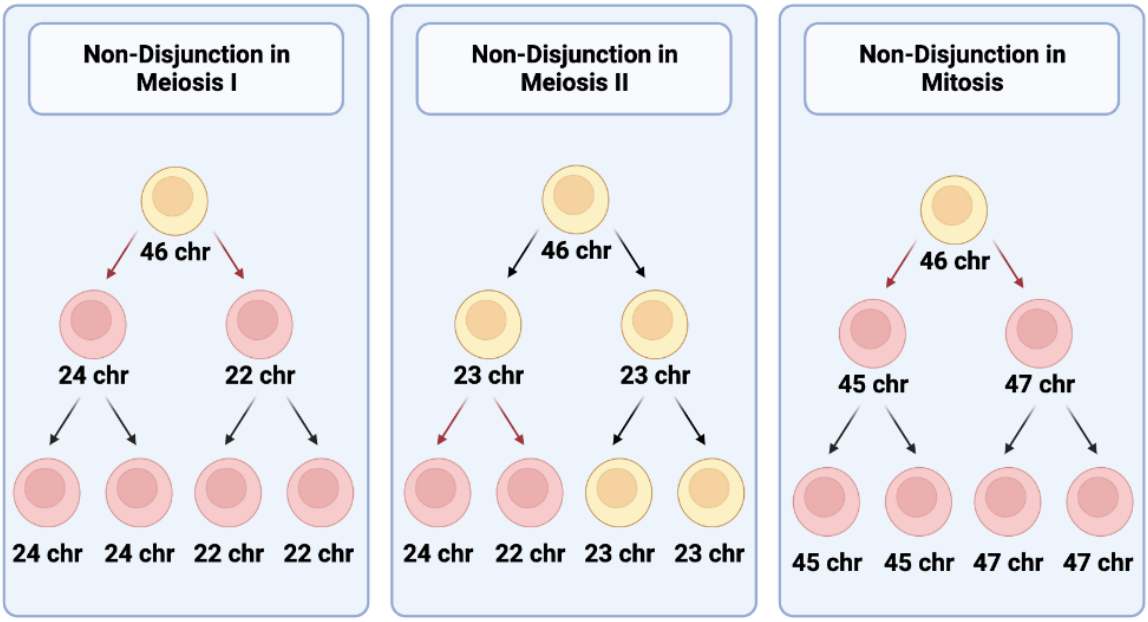


Figure 3 - Numerical chromosomal abnormalities resulting from non-disjunction in meiosis I, meiosis II and mitosis. Chr: Chromosome.

1.2 Structural Chromosomal Abnormalities

Structural alterations involve only a certain region of the chromosome, but since the chromosomes are able to rearrange themselves in countless different combinations, there are infinite possibilities for structural alterations.

These alterations can be divided into two groups: balanced structural alterations (where there is no increase or loss of DNA with active genes, culminating in a normal phenotype) or unbalanced (with increase/loss of DNA in active genes, associated with abnormal phenotypes). Although carriers of balanced abnormalities do not usually have an abnormal phenotype, the alterations can be passed on to offspring and result in an unbalanced rearrangement. Balanced structural alterations can be inversions, insertions, and translocations, unbalanced ones can be duplications, deletions, ring chromosomes, isochromosomes, and marker chromosomes (Figure 4). Like numerical abnormalities, these alterations can happen in every cells of an individual or in mosaic[29,33].

These alterations can occur due to errors in meiosis, such as unequal crossing over between chromosomes that are not fully paired (deletions and duplications) or due to unbalanced meiotic segregation from pairing chromosomes with balanced inversions or translocations (partial trisomies and monosomies). It may also be due to chromosomal breaks in one or more chromosomes with consequent loss or rearrangement of segments due to failure of the DNA repair machinery. These breaks can be caused by environmental factors or cellular aging, and the incidence of structural abnormalities in gametes is influenced by paternal age[3].

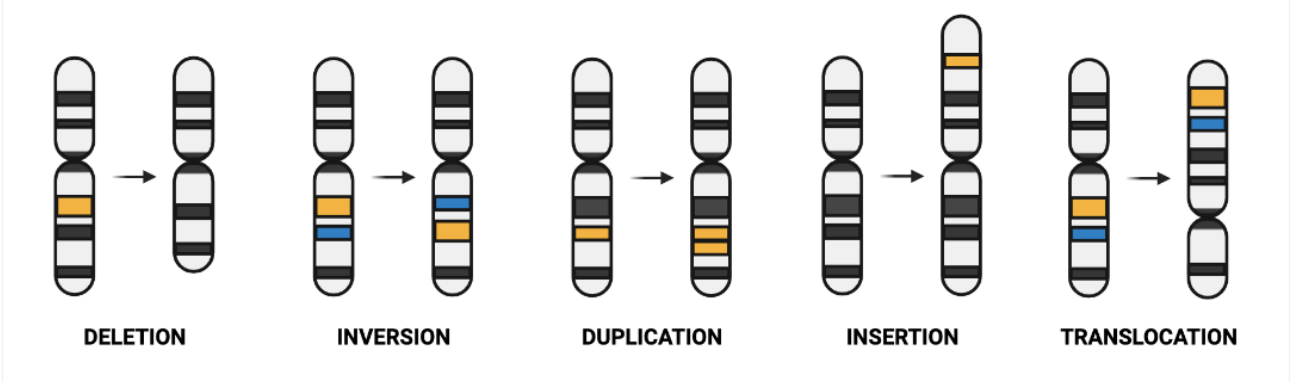


Figure 4 - Examples of structural alterations, namely deletion, inversion, duplication, insertion and intrachromosomal translocation.

2 Human Chromosome Nomenclature

With the advances on cytogenetics, emerged the necessity to develop a common language that would allow cytogeneticists all over the world to describe and debate their results, create databases and encourage international partnerships.

In 1971, the Paris Conference took place, where the International System of Chromosome Nomenclature (ISCN), created a nomenclature based on G-band patterns for karyotype assembly. In this nomenclature the 46 human chromosomes would be numbered from 1 to 22 in the case of autosomes, by decreasing size (except for chromosome 21, which is smaller than 22), and the sex chromosomes by X and Y, in that order. Each alteration has its own name and there are several rules for writing normal and pathogenic karyotypes[32].

In order to maintain the purpose of this nomenclature, it is periodically updated by an elected committee. The standardization of the nomenclature was of extreme importance for reporting results in diagnostic laboratories[34].

The ISCN is currently an abbreviation for the International System for Human Cytogenomic Nomenclature, and the most recent version that should be used is the ISCN 2020, presenting rules not only for conventional cytogenetics but also for several molecular cytogenetic techniques[35].

3 Prenatal Cytogenetics

The aim of prenatal testing is to enlighten parents and pregnant women about the risk for fetus malformations or genetic disorders and provide information on possible consequences, choices available and how to manage the risk. Prenatal testing may be performed as a screening test or as diagnosis. Taking into account the prenatal context, which may be a stressful period for parents and family, both screening and diagnostic tests should be as less invasive as possible, but efficient, economical, reliable and should be available in early stages of pregnancy to allow a proper counseling of the couple, manage all the information along with the emotional stress and be able to safely carry out any interventions [36,37]. Although the terms “screening” and “diagnosis” are commonly used as synonyms, these terms, despite being correlated, have different purposes.

The goal of prenatal screening is to access fetuses at high risk for congenital anomalies or chromosomal alterations, subsequently offering prenatal diagnosis for pregnancies identified in these conditions or provide reassurance in pregnancies that are not at high risk. There are several types of screening tests, usually noninvasive, including serum screening, carrier screening, and ultrasound, all of which are available for all ongoing pregnancies, depending on the time of pregnancy[38].

Prenatal diagnosis term applies to testing fetuses already known to be at high risk for birth defects, to determine if the fetus is affected or not with the malformation in question and should be as definitive as possible. Clinical indications for prenatal diagnosis are described in international guidelines and include[3,29]:

- Previous child with *de novo* chromosomal abnormalities;
- Presence of chromosomal abnormalities in one of the parents;
- Family history of a genetic disorder;
- Risk for a neural tube defect;
- Increased risk as determined by screening tests, e.g. fetal ultrasound malformations, positive maternal serum screening;
- Advanced maternal age (≥35 years); (this indication is not consensual namely when is considered as the only indication; may also be used other cut-offs, (≥38 years or ≥40 years)
- Maternal Request. The American Congress of Obstetricians and Gynecologists (ACOG) released "ACOG Practice Bulletin No. 226," that establish that this test can be performed and recommended in all pregnancies, if the patients wish to undergo these studies, however regulations differ throughout Europe and depend on each individual professional guidelines [39,40]

Prenatal diagnosis allow couples to understand and learn about the care of the infant condition and prepare for the birth of a newborn with special needs. Additionally, may allow in utero intervention that may improve survival and neonatal outcomes and for some couples may mean choosing to terminate the pregnancy. Altogether, allow couples, who know they are at high risk for having a child with birth defects, the opportunity to clarify their uncertainties and the possibility of also having healthy children, which was not possible before prenatal diagnosis and would make the parents choose to forego having children.[29]

However, there are disadvantages inherent to these tests, such as increased stress, anxiety and the fact that in some cases the result may be inconclusive. Additionally, genetic prenatal diagnosis often requires an invasive procedure such as chorionic villus sampling (CVS), amniocentesis or percutaneous umbilical blood sampling (PUBS) to acquire fetal cells for analysis, which has been associated with a slightly increase in the rate of fetal loss.

3.1 Chorionic villus sampling (CVS)

CVS is a technique performed to biopsy tissue from the villi of the chorion. It can be carried via transcervical or transabdominal, depending on placental site and according to the operator's experience (Figure 5A). Chorionic villi are derived from the trophoblast, the outer layer of the blastocyst, and are a great source of fetal tissue for biopsy[3,37].

CVS is usually performed between 11th to 12th weeks of pregnancy and the additional risk of fetal loss has been reported to vary between 0.2% and 2%[41].

Since it is performed in the first trimester of pregnancy, it has the advantage of obtaining results at an early stage of pregnancy, giving the couple more time for genetic counseling, reflection and, if desired, allowing a safer termination of pregnancy. However, it is estimated that about 1 to 2% of CVS results show mosaicism confined to the placenta, which does not reflect true mosaicism. This means that the alteration found in the trophoblast biopsy may not reflect the chromosomal constitution of the fetus. Furthermore, cell culture failure is more common with CVS and is more susceptible to maternal contamination[38,42].

3.2 Amniocentesis

Amniocentesis is another invasive technique, where an ultrasound guided needle, displaced from any fetal part, placenta or large vessels, is introduced into the amniotic sac and removes a sample of amniotic fluid (Figure 5B). Besides the study of genetic disorders, it also evaluates intra-amniotic or fetal infection through cell culture or polymerase chain reaction (PCR) and assess the presence of neural tube defects by measuring amniotic fluid alpha-fetoprotein and acetylcholinesterase[38,43].

This technique should be preferentially performed between 15 and 17 weeks of pregnancy and procedure-specific risk of miscarriage is 0.11% (95% CI, 0.04 to 0.26%)[41].

The number of viable cells present in the amniotic fluid is inversely proportional to the time of gestation and for this reason, performing an amniocentesis at a later stage of pregnancy may increase the difficulty in obtaining viable cells for culture, on the other hand, it should not be performed in an earlier stage of pregnancy due to increased risk

for amniotic fluid leakage, higher culture failure rates, less amniotic fluid and more difficult access [3,37].

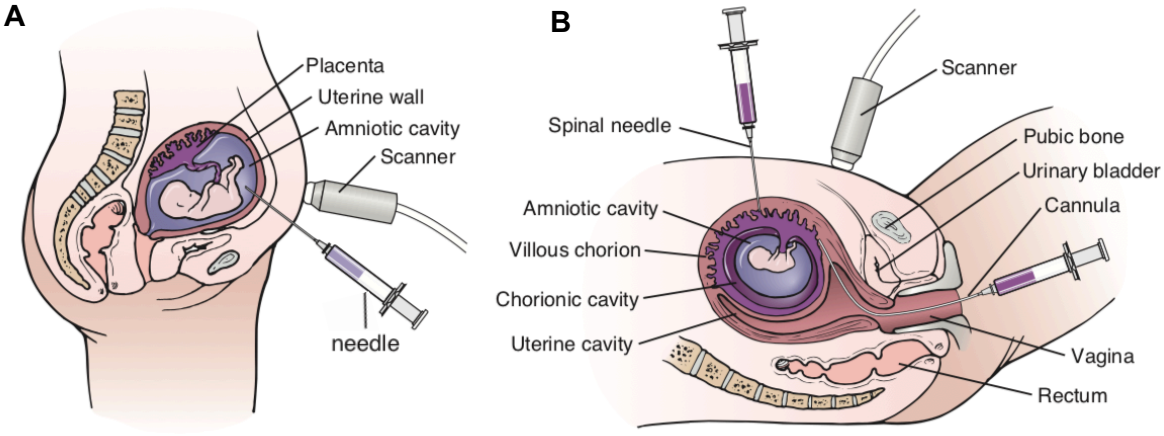


Figure 5 – (A) Amniocentesis procedure and respective instrumentation. (B) Chorionic villus sampling (CVS) procedure and respective instrumentation. Adapted from Robert Nussbaum et al.[29].

3.3 Percutaneous Umbilical Blood Sampling (PUBS)

PUBS also known as Fetal Blood Sampling or Cordocentesis, involves inserting a thin needle guided by ultrasound through the walls of the abdomen and uterus into the umbilical cord at the site where the cord enters the placenta, and obtaining a blood sample from the fetus (Figure 6). This technique is used less frequently in prenatal diagnosis, must be performed at or after 18–20 weeks and carries a risk of fetal death higher than CVS or amniocentesis, estimated to be around 1–2% when performed by an experienced professional[37,44].

Since it is the only technique that provides direct access to fetal circulation, it is mainly used in suspected fetal anemia and also in cases of congenital infections, metabolic disorders, fetal growth restriction and hematologic disorders. It is also widely used in cases where it was not possible to obtain significant clinical information through amniocentesis, CVS or ultrasound and, as such, an additional study is necessary to clarify the previous results, with the additional advantage of allowing a short-term lymphocyte culture to be established for chromosomal analysis[45,46].

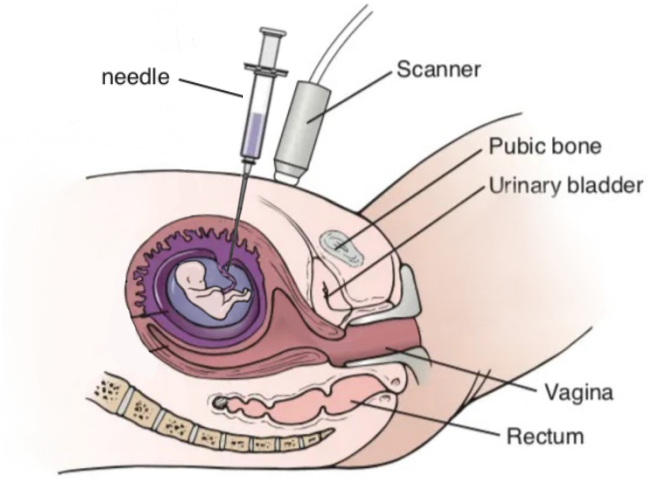


Figure 6 - Percutaneous Umbilical Blood Sampling, procedure and respective instrumentation. Adapted from Robert Nussbaum et al [29].

With the acquisition of cells from the fetus by the invasive techniques mentioned, cells can be analysed through different techniques to obtain the result. They can be analysed using conventional cytogenetics, the karyotype that requires cell culture in order to obtain a reasonable number of cells to proceed with the chromosomal analysis. They can also be analysed using cytogenetics/cytogenomics techniques such as aCGH that use DNA extracted from the biological sample and not require cell culture or FISH that can be performed in uncultured cells, which significantly reduces the response time. Other molecular techniques such as MLPA, QF-PCR or NGS are also available.

Each technique has advantages and disadvantages and these will be discussed later. The decision of the most appropriate technique depends on each case and should take into account the indication for carrying out the genetic study.

4 Postnatal Cytogenetics

The goal of postnatal genetic diagnosis is to detect chromosomal abnormalities underlying a genetic disorder, reduced fertility, congenital malformations, intellectual disability, the likelihood of having a child with an inherited genetic disorder, among others. As previously mentioned, genetic tests can be performed using different technologies and the laboratory must select the most suitable one as well as the type of sample, given the referral reason. The biological samples most frequently used in

postnatal diagnosis are peripheral blood and semen, and the main clinical indications described in international guidelines for performing postnatal genetic studies are:[47,48]

- Abnormal clinical phenotype and dysmorphisms;
- Intellectual disability;
- Multiple congenital anomalies;
- Age-inappropriate structure or growth;
- Primary or secondary amenorrhea or ovarian insufficiency;
- Ambiguous genitalia;
- Idiopathic infertility;
- Sperm abnormalities (azoospermia or severe oligospermia);
- Three or more miscarriages;
- Familial history of chromosome rearrangements/genetic disorders;

Depending on the indication, there are more adequate techniques, namely in cases where phenotypes are typically associated with microdeletion/microduplication syndromes (such as minor facial dysmorphia or intellectual disability), molecular cytogenetic techniques (aCGH or FISH) are more suitable since they have higher resolution than the karyotype. In cases of amenorrhea or ambiguous genitalia, the analysis of the constitutional karyotype is recommended, since these cases may be related to large rearrangements/deletions/duplications on X chromosome and mosaicism[48,49].

Despite the internationally established guidelines for both prenatal and postnatal diagnosis, local policies might differ throughout countries, and the decision depends on the clinician and the patient's will.

5 Quality Assurance and Practices Standards

Quality assurance includes all activities and methodologies impregnated in a laboratory that ensure the high quality of a service to its users. Since a clinical laboratory plays an important role in the health system, this issue must be well addressed to minimize the occurrence of errors without compromising the dynamics and efficiency of the process. There are several steps from obtaining the sample for

cytogenetic/cytogenomic study to the preparation of the final report, and many variables can expose samples/data to different conditions that must be managed aiming to reach a proper diagnosis[47,50]. As such, the GL/FMUP, in addition to external quality control by Genomics Quality Assessment (GenQA) and other national/international guidelines, established internal regulations for the pre-analytical, analytical and post-analytical phases in order to make the whole process more harmonious in the laboratory itself but also to facilitate communication between the various organizations and elements involved.

5.1 Sample Preparation

The GL/FMUP established protocols and requirements for cytogenetic and molecular cytogenetic studies in order to standardize and consequently minimize potential errors and delays, thus individuals who are not intimately familiar with the procedure can easily perform it. The protocols specify the ideal specimen volume, suitable transport containers, appropriate transport time and temperature to optimize sample growth, labeling form, and laboratory contacts.

Peripheral blood samples from newborn, children, and adults, for conventional cytogenetic study should be collected in sterilized tubes containing sodium heparin and should be discarded if outdated. Heparin is a natural anticoagulant that interferes with the conversion of prothrombin to thrombin, therefore its use is recommended in blood sample collections and is identified by being a tube with a green cap. Peripheral blood samples for molecular genetic studies should be collected in sterilized tubes containing EDTA K2, as it preserves cellular integrity and is identified with a purple cap. The volume collected should between 1mL and 4mL for molecular studies. Lymphocyte cultures are normally performed within 24 hours of collection, but no later than 72 hours. In the hold time between harvest and culture, samples are kept at 4ºC. Culture medium can be added to small blood amounts samples due to the tendency to dry up.

In amniotic fluid samples, 15mL should be collected in a sterile falcon tube but the first few milliliters of an amniotic tap are the most likely to be contaminated with maternal cells and should not be submitted to the cytogenetics laboratory.

For chorionic villus samples and tissue samples, these should be placed in sterile falcon tubes with culture medium provided by the laboratory, with approximately 10 chorionic villi and fragments larger than 0.5 cm^3. In the case of the chorionic villus

sample, maternal blood should be sent together to screen for maternal contamination. Cell culture is performed immediately upon receipt of the samples in the laboratory.

Transport of all samples must be ensured at room temperature and temperature extremes must be avoided.

If these requirements are not met a repeat sample should be requested, but because obtaining a new sample is not always practical or possible, in such cases the laboratory must attempt to retrieve the original sample.

After the samples enter the laboratory, which must be accompanied by the requisition with all the necessary clinical data, a unique identifier to each sample is assign, distinguishing it from other samples and from previous studies of the patient, then all sample registration and labeling are done. The label contains this identification code, the initials of the patient's name, requisition number, date of birth, date and other relevant information, if necessary.

Then, the samples go to the laboratory where the analytical procedures begin.

5.2 Procedures/Analysis Standards and Storage

The protocols used in each technique are duly documented and accessible by any technician/intern in the laboratory and are annually re-evaluated. All material used in the processing of one sample have the same label, thus ensuring security and confidentiality of the process. Every equipment is cleaned and calibrated regularly, and the date and technician who performed it are recorded. All the results of any methodology are analysed by more than one technician and both must be in agreement, in case of doubts they are discussed in a meeting.

After the analysis of each process, the analysed material is stored under specific conditions and for a certain period. With respect to conventional cytogenetics, remaining peripheral blood that was not cultured is stored for 1 month at 4ºC, amniotic fluid/chorionic villus/tissue cultures are kept under culture until the case is done, the analysed slides are stored for 12 months wrapped and at room temperature, and the remaining fixed cell suspension that was not used in the spreading is stored at -20ºC for 5 years or indefinitely, if the result was normal or abnormal, respectively.

Slides for observation under a fluorescence microscope are stored in light-protected boxes at -20ºC and the extracted DNA used for aCGH is stored indefinitely at 4ºC.

5.3 Reporting Standards

It is the responsibility of the cytogeneticist to clearly and unambiguously describe the cytogenetic findings and provide an explanation of the clinical significance of the results through the final report. Reports should be succinct and clear so that non-experts in the field such as clinicians, healthcare workers and even the patient can easily understand.

Every report should include the general information above:

- Clinical indication and reason for referral;
- Date of referral and date of report;
- Patient identification (using at least 2 different types of identification);
- Laboratory identification;
- Laboratory unique sample identification;
- Requesting clinician;
- Cytogeneticist(s) responsible for analysis and reporting;
- Biological sample type;
- Total number of cells scored and analysed (if applicable);
- Limitations of the methodology
- Report result

In case of abnormal result also:

- Result written according to updated ISCN nomenclature;
- Clear written interpretation;
- State of the art;
- Recommendations for genetic counselling;
- Relevant additional comments;
- Request for follow up of family members, if appropriate;

In addition to this information, depending on the techniques performed, other specific information must be included in the report, having been specified in the corresponding following chapters.

One aspect that the laboratory must consider to guarantee the quality of the procedures is the response time. That is, the time from the sample entering the laboratory until the final report is completed, and this must be as short as possible. In

Table 1, the period recommended and applied in the GL/FMUP for each type of analysis is indicated.

Table 1 - Response times depending on the type of biological sample available for analysis. Adapted from Ros Hastings et al.[50]. FISH: Fluorescence in situ hybridization. TUNEL: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling.

Analysis	Response Time
Amniotic fluid and long term CVS cultures	17 days
Short term CVS cultures (directs)	7 days
Lymphocytes or solid tissue cultures	28 days
Prenatal samples for aCGH	17 days
Postnatal samples for aCGH (proband)	60 days
Postnatal samples for aCGH (proband + parents)	90 days
Samples from parents after proband aCGH result	60 days
FISH	30 days
TUNEL	30 days

The results, captured images of the cases, as well as other relevant information, are recorded on the digital platform of GL/FMUP, called Klims, indefinitely. This is important because some genetic tests results may have implications for other family members or have late-life effects, so it should be possible to access the original results years later.

III. Material and Methods

1 Conventional Cytogenetics

Conventional cytogenetics consists in the study of chromosomes in cell division, through optical microscopy. It is possible a global screen of the entire genome by detecting the majority of chromosomal abnormalities, whether balanced or unbalanced, with a resolution of 5-10Mb, in various types of samples, such as peripheral blood, amniotic fluid, chorionic villi, fibroblasts and abortion products [3,21].

The technical name is Chromosome analysis, or Karyotyping, and consists of culturing samples under specific conditions in the presence of reagents that stimulate cell division and arrest as many cells as possible in metaphase. After banding techniques, it is possible to directly access all 23 pairs of chromosomes and analyse if there is any alteration, since normal chromosomes have specific banding patterns.

Due to the broad character of the analysis, this technique can be applied in a wide range of cases. Examples of the clinical indications for Karyotype are family history of chromosomal rearrangements, suspected deletion/duplication syndromes, infertility of unknown etiology, developmental delay, dysmorphism, premature menopause, and, in prenatal cases, abnormal fetal ultrasound and positive maternal serum screening result indicative of an increased risk to a fetus with chromosomal abnormalities[39].

The conventional cytogenetic study procedure, in general, consists of the following steps: cell culture, harvesting and spreading, chromosomal banding and chromosomal analysis.

1.1 Cell culture

Cells must be grown and divided in culture aiming to obtain a desirable cell concentration for perform chromosomal analysis [32]. Cell culture is a laboratory technique that aims to grow cells under appropriate physiological conditions, mimicking the natural extracellular and intracellular microenvironment [51].

This microenvironment is possible through the use of an incubator, which allows to establish ideal conditions of temperature, humidity, oxygen and carbon dioxide. Also,

through cell culture basal medium and its supplements, providing the cells with all the necessary nutrients such as vitamins, carbohydrates, amino acids, hormones, minerals, growth factors and components that control physicochemical properties such as pH and cellular osmotic pressure[51].

There is a huge variety of commercially available culture mediums, and the choice is dependent on the type of samples for analysis. Most of which are incomplete, that is, they do not contain all the nutrients necessary for cell growth and therefore must be supplemented. Some of the most used additives are L-Glutamine, essential for proper cell growth and fetal bovine serum (FBS) which provides growth factors and hormones that act as a carrier of lipids, enzymes, micronutrients, which must be in an amount between 10-30 %. The addition of antibiotics is also important, such as gentamicin which inhibits bacterial growth. Mitotic stimulators should also be used, especially in lymphocyte cultures since they do not spontaneously undergo cell division. The most used is phytohemagglutinin, a mitotic agent capable of binding to the cell membrane, altering its permeability and leading to an increase in lymphocyte cell division[3].

Another important factor to consider in cell culture is ensuring an aseptic work environment. Microbiological infections are one of the main problems for the quality and maintenance of *in vitro* cultures. Smaller levels of contamination can lead to difficult to interpret or untrue results, ultimately a higher level of contamination can lead to cell death. As such, GL/FMUP has adopted several procedures to minimize these issues, decreasing cell loss, resources, and time[51].

The vertical laminar flow chamber contains a high-efficiency, bacterial-retentive filter called high-efficiency particulate air (HEPA), so the purified air circulates on the work surface in parallel lines at uniform velocity, filtering almost all microorganisms from the air and protecting not only the cultures but also the operator himself. In addition, laboratory staff wear disposable gloves and lab coats for all procedures, use only sterile material and disinfect the work surface with 70% ethanol, as well as all equipment before use, such as incubator, centrifuge, and inverted microscope[52,53].

It is important that all technicians are aware of signs of contamination so that they can take appropriate measures, that is removing the contaminated culture, disinfecting all surfaces that came into contact and discovering the source of contamination.

Generally, bacterial contamination will cause the medium to look cloudy. Furthermore, the products synthesized by the metabolic pathways of bacteria can

change the pH of the medium, and since the media have phenol red as a pH indicator, in case of contamination, the natural reddish color of the medium becomes yellowish[3].

The choice of culture method depends on the type of sample and the clinical indication of the case. Blood samples are made up of single, free-floating cells. Sterile centrifuge tubes or tissue culture flasks (T-flasks) can be used for such suspension cultures. Cells from amniotic fluid and chorionic villi must addere to a surface in order to grow. These samples could be grown in T-flasks or *in situ*[3]. The first change of culture medium is performed after 5-6 days and it is not discarded as it may contain some cells in suspension with proliferative capacity. As such, a subculture is carried out, placing into a new flask to start a new culture, functioning as a reserve of the primary culture.

Each sample must be grown at least in two different flasks/cell chambers to obtain independent cultures, by different operators. In this way, the quality of the diagnosis is safeguarded in case of failure of any of these parameters in one of the cultures and suspected artifacts can be clarified.

1.1.1 High resolution lymphocyte culture (Flask Method)

In each culture flask, 9ml of complete medium, namely RPMI 1640 medium with gentamicin and FBS are added together with 200 µL of phytohaemagglutinin (Figure 7). Subsequently, 1 mL of peripheral blood is added in all samples except in cases of children under 1 year, that only 0.5ml is added (Figure 8). The culture flasks are incubated for 48h or 72h in incubators at 37ºC.

After this time, the cultures are synchronized with the addition of 40µL of methotrexate (MTX), which is a folic acid antagonist that prevents nucleic acid synthesis by blocking cells in S phase[32,54]. The flasks are homogenized and incubated again at 37ºC for 16h30.

Afterwards, the flasks are homogenized, their contents transferred to culture tubes and centrifuged at 1600 rpm for 6min at room temperature. With the aid of the vacuum pump, the supernatant is discarded. The pellet is resuspended and 6 ml of complete medium and 120µL of thymidine are added, which leads to the breakdown of pyrimidine bases, unblocking the action of methotrexate and leading the cells to advance in the cell cycle[3].

After a 4h45m incubation at 37°C, the cell cycle is blocked in the pro-metaphase by adding 120µl of Colcemid and incubated for 15min at 37°C, followed by the harvesting

phase. Colcemid is a synthetic analogue of colchicine, which blocks the formation of the mitotic spindle preventing cells from entering anaphase [32].

This methodology leads to high resolution bands.

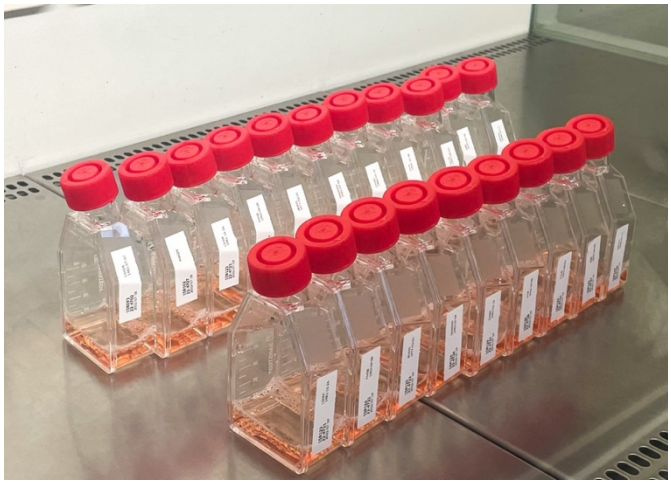


Figure 7 - Preparation of flasks with RPMI 1640 culture medium, gentamicin, FBS and phytohaemagglutinin.

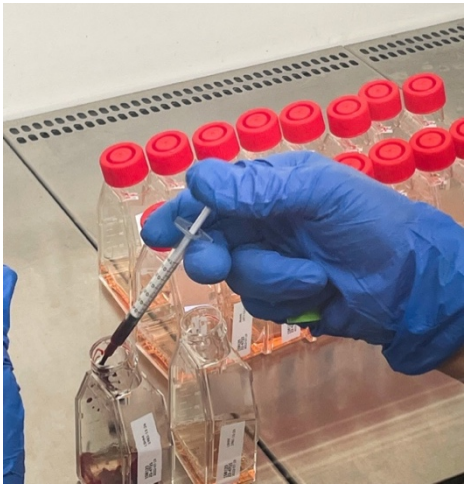


Figure 8 - Addition of the peripheral blood sample to the complete culture medium to later establish the culture.

1.1.2 Amniocytes culture (Flask Method)

Before performing the cell culture, the color of the amniotic fluid is evaluated, which should be yellowish/transparent. If a redder tone is observed, indicating traces of maternal blood, screening for maternal contamination should be performed using the Quantitative fluorescent PCR (QF-PCR) technique.

In the laminar flow chamber, 2.5mL of amniotic fluid contained in the syringe is transferred into a conical tube, properly identified, and the amniotic fluid is centrifuged at 1100rpm for 10min.

The supernatant is discarded almost entirely with a pasteur pitette and, duly homogenized, 2 ml of complete medium is added. Then, 1ml is transferred to each culture flask, where were previously added 4ml of complete medium - Amniomax Medium with Amniomax Supplement. The flasks are incubated for 5 days in a CO_2 incubator (5%) at 37°C.

After 5 days, the growth of the cultures is daily checked under the inverted microscope and the medium is changed every 48 hours, until harvesting. The first removed medium is transferred to another t-flask and will also be cultured (Figure 9).

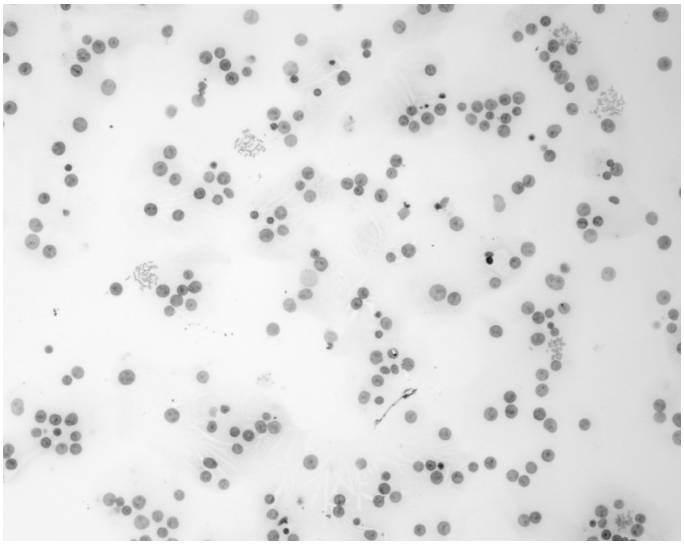


Figure 9 – Amniocyte culture by flaks method, visualized under a phase-contrast microscope.

1.1.3 Amniocytes culture (*In situ* Method)

In the laminar flow chamber, 2.5mL of amniotic fluid contained in the syringe is transferred into a conical tube, properly identified, and the amniotic fluid is centrifuged at 1100rpm for 10min.

Additionally, 2ml of complete medium, (Amniomax Medium with Amniomax Supplement), is added to each culture slide, that are small chambers where the cells will grow directly in the slide.

Still in the laminar flow chamber, the supernatant is discarded, and 1ml of complete medium is added. After homogenizing the content, 0.5 ml are transferred to each culture slide, already with 2ml of medium, and incubated in a CO_2 incubator (5%) at 37°C.

On the 5th day of culture, the medium is replaced: all content is removed (and subcultured into a new culture slide) and 2.5ml of new culture medium is added.

Cell density is daily monitored under a microscope and the culture medium is replaced every 48h until the visualization of the cells colonies with metaphases, then are harvested (Figure 10).



Figure 10 - Two amniocyte colonies cultured by in situ method, visualized under a phase-contrast microscope.

1.1.4 Chorionic villi culture (Flask Method)

Firstly, the chorionic villi are prepared by placing them on a sterile plate and washed with sterile phosphate-buffered saline (PBS) using a Pasteur pipette. With a magnifying glass the clots are removed, and the villi are separated. Washes are done with sterile PBS to try to remove all the blood present (Figure 11).

Subsequently, the tissue is cut into very small fragments and an enzymatic digestion is carried out. First, 2ml 0.5% Trypsin/Ethylenediaminetetraacetic acid (EDTA) (10x) is added for 20min at 37°C and then the fragments are transferred to a conical tube and vortexed. After, PBS is added to wash the fragments, the tubes are vortexed and centrifuged at 1100rpm for 10min. The supernatant is removed and 1ml Collagenase type I is added for 30min, at 37°C. While trypsin breaks the bonds, collagenase allows the cells to disaggregate.

Then, 2ml of complete culture medium is added. After centrifuge, the majority of the supernatant is discard; the tube is homogenized and about 30µL of these is removed to an eppendorf to be used for the maternal contamination test performed by QF-PCR. Then, 0.2 to 0,6 ml of culture medium is added in the tube depending on the number of *in situ* chambers that will be cultivated. The cells are then divided and transferred to *in situ* chambers (0,1ml for each *in situ* chambers). Previously was added 4.8 ml of complete culture in each flask.

The flasks are incubated in a CO_2 incubator (5%), at 37°C. On the 5th day of culture, the medium is replaced, removing all existing content (that will be subcultured) and 5ml of new culture medium is added. The cell density continues to be monitored under the microscope and the next procedure is decided. If greater cell growth is required, the medium is replaced every 48h.

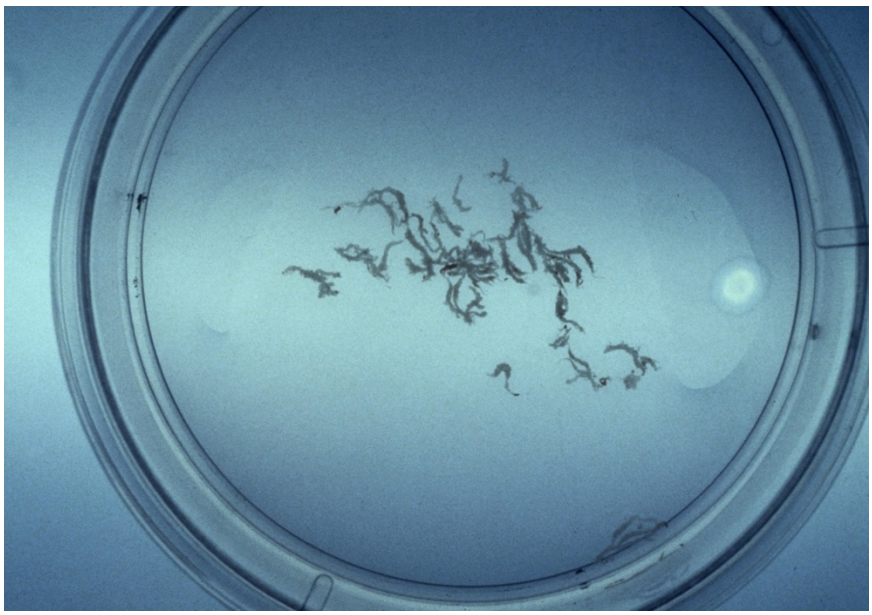


Figure 11 - Chorionic villi after separation from maternal material[55].

1.1.5 Chorionic villi culture (*In situ* Method)

The chorionic villi are prepared in the same way as in flask cultures.

Subsequently, 0.1 mL of the cell pellet is added to each properly labeled culture slide chamber already containing 2.4 mL of culture medium.

The culture slides are incubated in a CO_2 incubator (5%) at 37°C and cell growth is monitored under a microscope from the 2nd day of culture. On the 3rd or 5th day of culture, the medium is replaced, removing all the existing content (that will be subcultured) and 2.5 ml of culture medium is added.

Cell density is continuously evaluated to decide on the next procedure: if greater cell growth is required, the medium is replaced every 48h.

1.2 Harvesting and Spreading

1.2.1 Lymphocytes, Amniocytes and Chorionic villi (Flask method)

In the case of flask cultures, the harvesting procedure for the cultures addressed is slightly different, despite the spreading being identical

After addition of colcemid, the contents of the tube are homogenized and centrifuged for 6min at 1600 rpm, at room temperature, discarding the supernatant with the vacuum pump. It is important to strictly comply the time of colcemid exposure since it is a fundamental step for the good quality of the metaphases. A reduced time leads to chromosomes being too long with several overlaps, however, a longer time leads to a higher mitotic index but with much more condensed chromosomes which could compromise the identification of structural chromosomes abnormalities[3].

A hypotonic solution is added gradually, previously heated to 37°C, for 10min at room temperature. The hypotonic solution has less salt concentration than the cell cytoplasm, allowing water to move into the cell by osmosis. This causes the cells to swell, what is necessary for proper chromosome spread on the slide[56].

Then the sample fixation process begins using 3 solutions with methanol and acetic acid in different proportions, all made on the same day of use. After centrifugation, the supernatant is discarded under the same conditions and the Ibrahimov solution (92 mL of distilled water, 3mL of methanol and 5mL of acetic acid) is added to ¾ of the culture tube, slowly and stirring. After another centrifugation, the supernatant is discarded and methanol up to ¾ of the tube is added. A final centrifugation is performed, the supernatant is discarded, and the pellet is resuspended in 5 ml of cold fixative solution, drop by drop. The cold fixative solution is three parts absolute methanol to one-part glacial acetic acid and is used to block the action of the hypotonic solution and retain the cells swollen, thus concluding the fixation of the biological material. The tubes are stored at 4ºC until spread.

In the case of amniocytes and chorionic villi culture, for harvesting, 120µl of colcemid is added at each culture and incubated at 37°C for 2h30min. Posteriorly, the supernatant is discarded and 4.6ml of a previously heated Trypsin/EDTA/PBS solution are added and placed in the incubator at 37°C for 10min.

The trypsin, a proteolytic enzyme, allows the resuspension of membrane-anchored cells, so that they are free in the culture medium, allowing their manipulation.

After this step, must be confirmed under the microscope if the cells are detached from the base of the culture flask and are suspended in the solution. In such case, 6.6 ml of previously heated distilled water are added, and then transferred to a tube properly identified. Finally, after a centrifugation at 1600 rpm for 6min at room temperature, removal of the supernatant and resuspension of the pellet, cold fixative is added slowly,

stirring constantly (drop-by-drop fixation) until about ¾ of the volume of the tube, thus fixing the biological material.

This step should be repeated about 2 to 3 times. Tubes are stored at –20°C until spread.

The next phase is spreading and consists of placing the cell suspension on slides that will be later stained and visualized under a microscope.

After homogenizing the contents of the tubes with fixed cells from suspension cultures, with a Pasteur pipette, these are dropped onto glass slides slightly inclined from a height of approximately 30cm, inside the flow chamber (Figure 12). The conditions established in the flow chamber should be temperature at 23ºC and a humidity of 42%. For each culture in each case, two slides are spread, thus obtaining 4 slides per case.



Figure 12 - Spreading the suspension of fixed cells on a slide.

The turgid and fragile cells hit the slide, and the fixative's surface tension pressure the cells, causing them to become compressed between the slide and the meniscus of the fixative. As the fixative evaporates, the cells stretch until they become flat, taking up more area on the slide. The longer it takes for evaporation to occur, more spread the cells and chromosomes become. The slides are placed vertically and allowed to dry inside the flow chamber. After they are dry, they are aged at 90ºC for 1h05 to enhance chromosome banding.

1.2.2 Amniocyte and Chorionic villi (*In situ* Method)

When the cultures are for harvesting, 30µl of chromosome resolution additive are add for 1h30min, which has the function of improving the resolution of the chromosomes. Subsequently, 30µl of colcemid are added for 2h30. At the end of this time, all the medium is removed and 2ml of preheated sodium citrate (which function as a hypotonic solution) are gradually added.

After 30min, the previous solution is discarded and another 2ml of sodium citrate is added again. Following 10min, 1ml of cold fixative is slowly added over sodium citrate. Following 5min, the entire content was aspirated, again adding 1ml of cold fixative and let it act for 1min.

This step is repeated 2-3 times. Finally, inside the flow chamber, the ideal conditions for *in situ* cultures are established, namely temperature of 22ºC and humidity of 48.5%. When these conditions are stabilized, the fixative is removed and the slide is separated from the box (Figure 13), allowing to dry horizontally inside the flow chamber.

After drying, the slides are aged at 90ºC for 1h05.

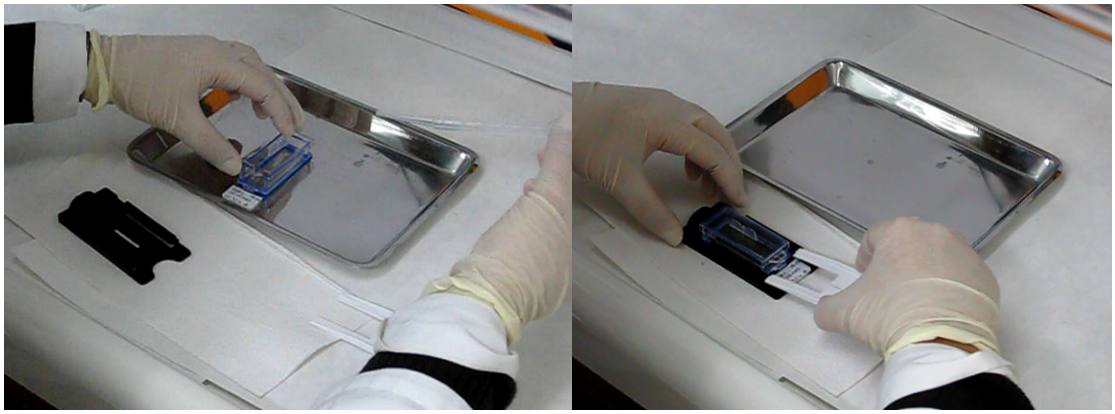


Figure 13 – Harvesting and spreading in cultures by the *in situ* method.

1.3 Chromosomal Banding

Banding techniques are fundamental for chromosomal analysis as they allow to identify all the chromosomes and possible rearrangements when present. The goal is to produce slides with many metaphases, with well-dispersed and non-overlapping chromosomes[57].

Chromosomes analysed in a metaphase, after banding techniques, have specific and individualized banding patterns. That is, each pair of chromosomes presents the same pattern of bands that is unique and restricted to that pair, facilitating the distinction of each chromosome. Furthermore, as the banding patterns of normal chromosomes have already been described, these landmarks allow the identification of chromosome breaks, alterations, and locate specific genes[58].

During this internship, G-bands using trypsin and Leishman stain (GTL staining) was addressed, which is a whole-chromosome banding method routinely applied in the

laboratory. Three other techniques of selective chromosome banding that highlight particular regions of chromosomes were also addressed, namely the NOR Staining, Centromeric Heterochromatin Staining (C-banding) and Distamycin-DAPI Staining (DA-DAPI staining) techniques, which are used for specific situations.

1.3.1 G-bands, using Trypsin and Leishman stain (GTL staining)

G bands are generated through a pretreatment with the trypsin enzyme and stained with Leishman stain. The result of this staining gives rise to positive bands, of darker tone, enriched in regions of adenine and thymine that are poor in genes, and negative bands, of light tone, rich in cytosine and guanine, with many genes [32].

The procedure is carried out automatically by the Mirastainer device, having programmed the time for which the slides must be in each of the solutions, transferred them sequentially (Figure 14).

First, all the solutions are made and placed in the respective tank, then the slides are placed on the arm of the device with the identification of the case at the top.

In the first program, the slides are submerged in hydrogen peroxide for 3min30sec, which leads to the aging of the chromosomal material, important to obtain sharper/clear bands. Then, the slides are dehydrated in methanol for 10sec with agitation. The slides are removed from the arm and placed horizontally on the bench on filter paper to air dry. When they are dry, the blades are put back on the arm.

In this second program the slides will be submerged in trypsin for 4sec. The chromosomes are pretreated with trypsin as its proteolytic action leads to a partial digestion of the chromosomes, allowing the incorporation of the staining solution. It is important that this step is optimized as insufficient trypsinization leads to slides with unbanded chromosomes, but excessive time leads to faded chromosomes with staining around them[58].

After, slides are washed in a phosphate buffered saline called Sorensen for 12sec, followed by Leishman stain solution for 10min. Subsequently, emerged on another phosphate buffer named Gurr for 3sec. Both buffer washes are intended to remove salts, proteins, and other contaminants. At the end, the slides are washed in running water for 3sec and dried at 45ºC for 2 min (Figure 15).

Slides are grouped by case, wrapped in paper, and stored until analysis.



Figure 14 - Slide staining by the GTL method using the Mirastainer device.

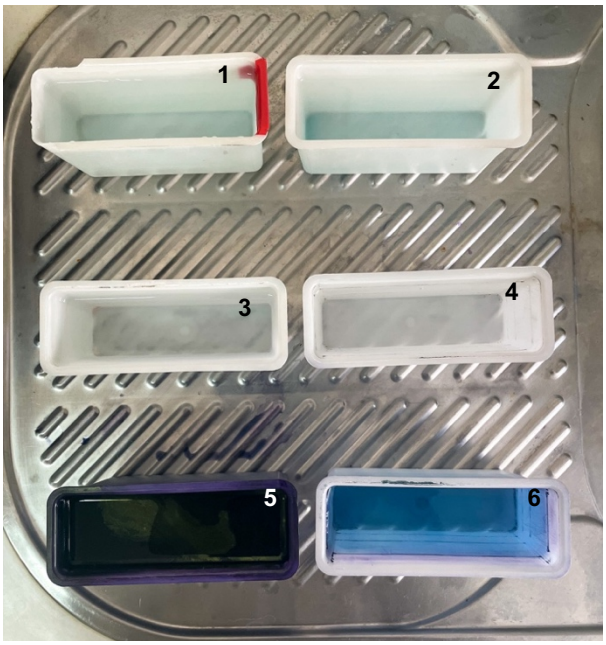


Figure 15 - Solutions used for staining slides by the GTL method, placed in the Mirastainer device. 1: Hydrogen Peroxide. 2: Methanol. 3: Trypsin. 4: Sorensen. 5: Leishman Stain. 6: GURR.

1.3.2 Centromeric Heterochromatin Staining (C-banding)

C-banding allows centromeric labeling of each chromosome and additionally other heterochromatin-containing regions, such as the regions adjacent to the centromeres at 1q, 9q, and 16q, as well as the distal region of Yq, regions which contain highly repetitive and rapidly renaturing DNA. This is possible through a mild alkali pretreatment followed by Leishman staining. As a result of this banding, gray chromosomes with dark markings in the mentioned areas are observed [32,58].

This type of banding, despite marking regions that apparently have no clinical relevance, is useful to determine the presence of dicentric/pseudodicentric chromosomes and also to check if there are heterochromatic regions on marker chromosomes[3].

The slides first undergo an acid purification step in order to depolymerize the DNA[59] being submerged in a hydrochloric acid solution for 30min.

After these, the slides are washed in distilled water and submerged in the barium hydroxide solution, for 10min, leading to DNA denaturation.

The slides are then washed thoroughly with distilled water and placed in a 2xSSC solution at 60°C for 1h. This treatment with hot salt leads to removal of chromosomal material in all regions except those where DNA has renatured, the regions that will be stained by the Leishman.

At the end of this time, it is washed again with distilled water and allowed to air dry. Finally, the slides are stained in a Leishman stain solution for 10 min, washed and when they are dry, they are ready to be analysed under the microscope (Figure 16).



Figure 16 – Metaphase from lymphocyte culture, stained by the C-banding, visualized under an optical microscope.

1.3.3 Nucleolar-Organizer-Region Staining (NOR staining)

The NOR-banding technique stains NOR regions, located on the satellite stalks of acrocentric chromosomes (13, 14, 15, 21, and 22) that contain genes for ribosomal RNA. Two types of staining are used in this technique, silver nitrate (a protein stain) and a Leishman stain [32] (Figure 17).

NOR staining is useful for studying some chromosome polymorphisms and identifying satellite stalks in acrocentric chromosomes[58].

Four drops of the silver nitrate solution and 2 drops of the gelatin solution are spilled on a slide. The gelatin is a colloidal developer, and the silver nitrate has affinity

for the NOR regions, staining them. The two solutions are mixed by gently sliding a coverslip over the surface of the liquid, leaving the coverslip over the slide.

Then, the slide is placed on the hybright at 70°C, until the solution turns golden brown (approximately 2min). Afterwards, the coverslip is removed by washing with distilled water.

The slide is submerged in a Leishman stain solution for 10min and washed with running distilled water to remove excess dye and dried at room temperature.

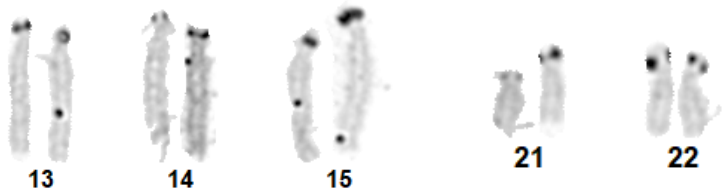


Figure 17 - NOR staining on chromosomes 13, 14, 15, 21, and 22, visualized under an optical microscope.

1.3.4 Distamycin A-DAPI Staining (DA-DAPI staining)

DA/DAPI staining is another selective banding technique, which involves the use of 4',6-diamidino-2-phenylindole (DAPI) which is fluorescent, and distamycin A (DA) a non-fluorescent antibiotic. Both reagents bind to double-stranded A-T-rich regions of DNA, resulting in a fluorescent signal in the heterochromatin regions of chromosomes 1, 9, 15, 16 and the heterochromatic portion of Yq chromosome (Figure 18). Unlike the other visualized banding techniques, this technique requires the use of a fluorescence microscope [3,60].

In a 24x22mm coverslip, 20µl of the DA solution, stored at -20°C, is placed. The slide is overlaid on the coverslip, with the material side facing down.

The slide is incubated for 30min at room temperature and then washed with distilled water, allowing the coverslip to fall off by itself.

Subsequently, the slide is washed in a 2xSSC solution and on a 24x50mm coverslip, a drop of DAPI is placed and the slide (still wet) is overlaid on the coverslip, as in the previous step. The slide is stored at 4ºC, in the dark, until analysed.

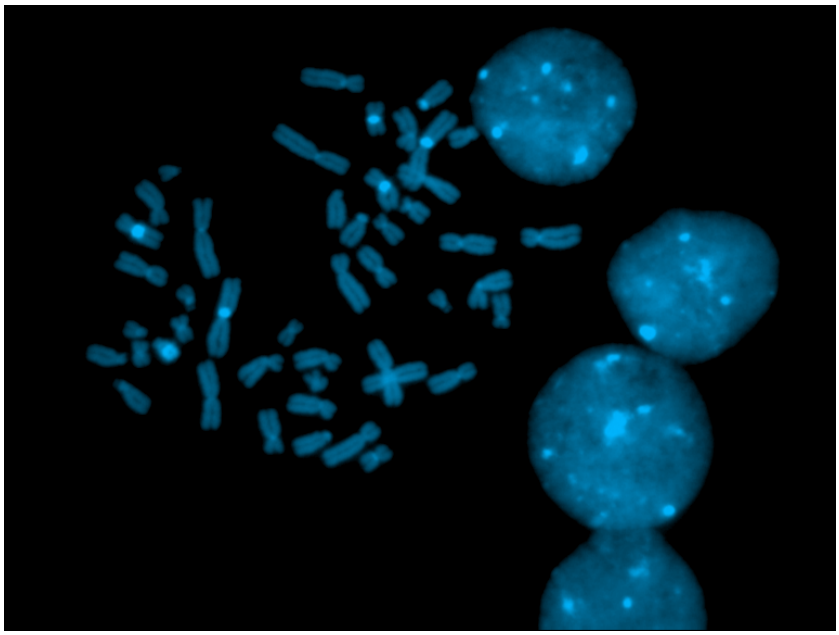


Figure 18 - Metaphase with DAPI staining, visualized under a fluorescence microscope.

1.4 Chromosomal Analysis

After GTL staining, chromosomes are analysed under an optical microscope and the karyotype organized. In lymphocyte cultures, high resolution with 550-850 bands bands are obtained, in the remaining cultures, about 400-550 bands are usually achieved[32].

First, with a 10x objective, the slide is analysed in a continuous and methodical way, region by region, looking for metaphases with the ideal characteristics for the analysis, namely chromosomes with a good spread and size, with few overlaps. When a metaphase in this condition is found, with the 100x immersion objective, the number of chromosomes are counted and recorded on the report sheet, including the reference of the sex chromosomes (XX or XY). This step is performed in both cultures several times. Depending on the clinical indication the total number of metaphases scored by case is different. In cases with a history of infertility, repeated miscarriages, suspected mosaicism, or pathology associated with the sex chromosomes (such as Klinefelter's or Turner's Syndromes) are scored 30 metaphases (25 scored metaphases plus 5 structurally analysed metaphases). In the remaining cases, more than 20 metaphases are scored (15 scored metaphases plus 5 structurally analysed metaphases). If two metaphases with the same anomaly or suspected low-grade mosaicism are identified,

the number of scored metaphases can be increased, taking into account the following criterion (Table 2):

Table 2 - Metaphases scored and respective probability of identifying mosaicism.

Metaphases scored	Probability of identifying mosaicism
14	>20%
29	>10%
58	>5%

At least 5 metaphases are captured and completely analysed, recording the microscopic coordinates. Using the *Cytovision* program, the karyotype is organized. When the study is completed, a second cytogeneticist observes the karyotyped cells to confirm the cytogenetic result.

Subsequently, the report is prepared and should mention:

- Number of cells scored;
- The microscopic coordinates of the cells captured in image;
- Result described according to cytogenomic nomenclature (ISCN 2020);
- Correlation of other techniques performed to complement the study, if applicable

Despite the advantages of conventional cytogenetics (Figure 19), it also has several limitations. Since it is a technique that requires cell culture, it is quite time consuming, taking about two weeks to get results. Furthermore, this technique requires highly qualified technicians and the result is operator dependent. Cases with few metaphases available, more frequent in prenatal cases, may lead to suboptimal results. Submicroscopic alterations are not detected and these represent one of the major causes of intellectual disability and congenital malformations. Furthermore, abnormalities caused by molecular genetic mutations or environmental factors are also not detected[21].

In order to overcome these limitations, molecular cytogenetics techniques were used to complement or, in some cases, to replace Karyotype.

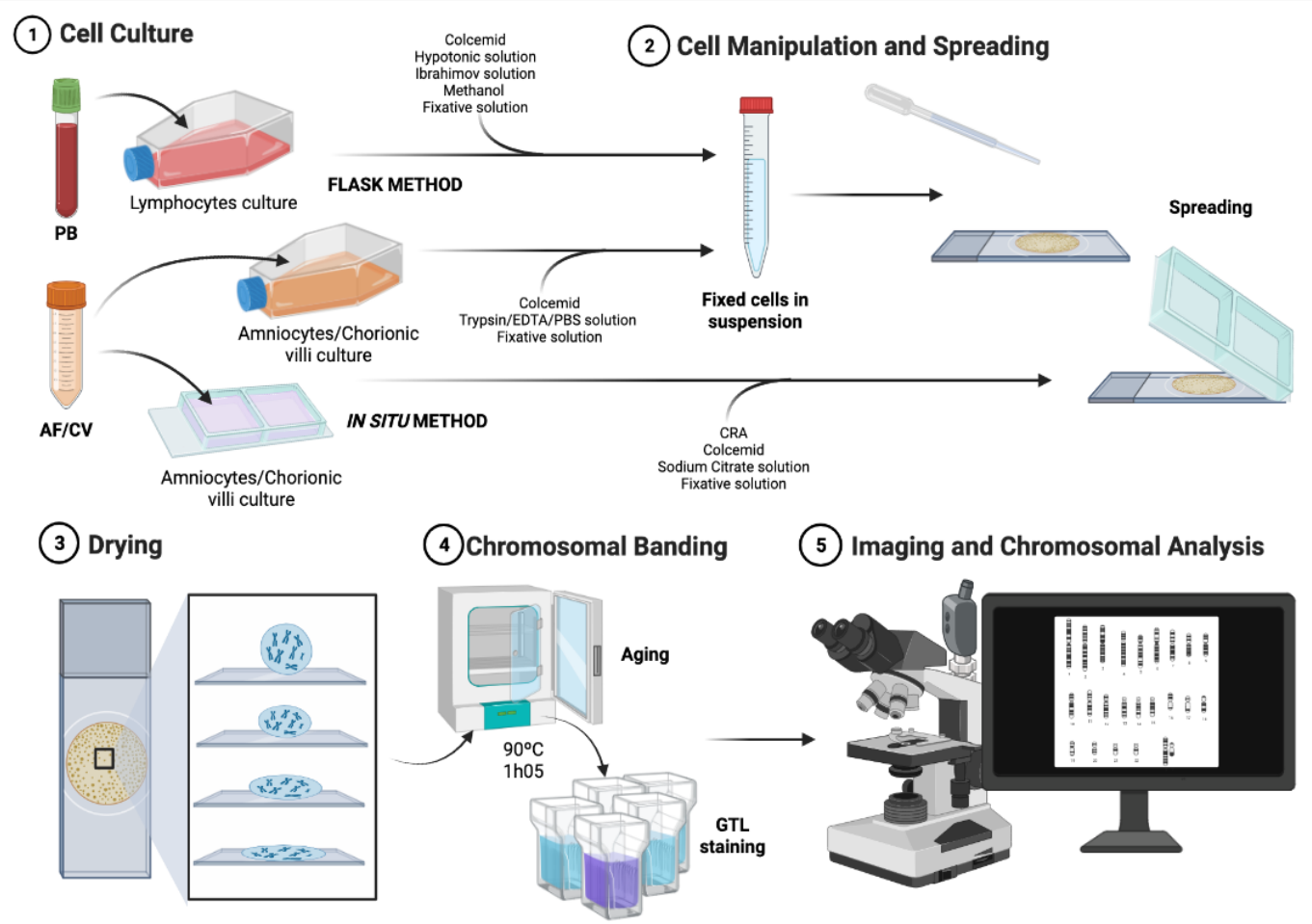


Figure 19 - Representative scheme of all the steps of conventional cytogenetics: cell culture of lymphocytes, amniocytes and chorionic villi by the flask or in situ method; cell manipulation and spreading; drying; chromosomal banding by the GTL method; imaging.

2 Molecular Cytogenetics

Molecular cytogenetics (or cytogenomics) combines both molecular and cytogenetic techniques and has greatly expanded the scope and accuracy of the clinical genetic analysis.

In this internship, Fluorescence *In Situ* Hybridization, Terminal deoxynucleotidyl transferase dUTP nick end labeling and Array Comparative Genomic Hybridization were addressed.

2.1 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization is a versatile technique that allows the visualization of chromosomal segments at the single-cell level. By using fluorochrome-coupled nucleotides incorporated with DNA fragments, which act as a probe, it targets complementary sequences resulting in colored signals that can be detected using a fluorescence microscope, thus allowing to assess the presence or absence of specific chromosomal segments in cells [61].

In situ hybridization (ISH) techniques have been used since 1960, using radioactive RNA/DNA probes labeled with radioactive isotopes, with the hybridization sites detected by autoradiography. In 1980, with the discovery of fluorescent labels, these quickly replaced radioactive labels because of their greater security, stability, and ease detection, therefore FISH technique began to be commonly used for diagnosis[62,63].

After FISH, new and more specific techniques emerged, such as Combined Binary Ratio Labeling FISH (COBRA), Spectral Karyotyping FISH (SKY) and multicolor FISH (M-FISH)[64,65].

The probes used for FISH determine the resolution of the cytogenomic study. Currently, there is a multitude of commercial probes available, which can be used both for diagnosis and research, with distinct excitation and emission characteristics and different target DNA. FISH probes can specifically target repetitive DNA sequences, individual DNA sequences or whole chromosomes. Repetitive sequence probes are mainly used for centromeric or pericentromeric regions. Unique sequence probes are useful for detecting genomic rearrangements, as well as gains and losses of specific genes. Probes that hybridize across the length of an entire chromosome, are also known as chromosome paints (Figure 20), can be used to confirm/characterize chromosomal rearrangements identified by conventional cytogenetics and for the study of aneuploidies, centromeric probes are used [63,66].

Most FISH probes are derived from bacterial artificial chromosomes and include genomic inserts between 100 and 300Kb [67].

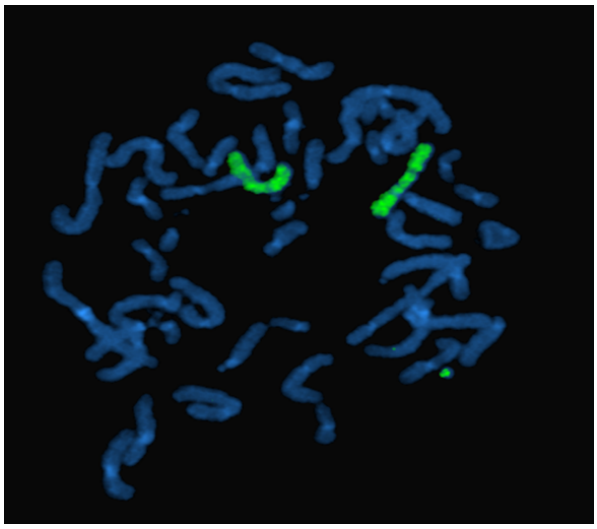


Figure 20 - FISH technique, whole chromosome painting of chromosome 7.

The FISH technique can be applied to different types of cells or tissues and, although there are slight differences in the protocols, the basis of the methodology is the same. First, it is necessary to select the appropriate probe taking into account the study target and also select a control probe, that should not target a region close to the one under study, in order to ensure the accuracy of the FISH result. Subsequently, since DNA can be reversibly denatured and renatured, in this methodology the target DNA and the probe are denatured using high temperature incubation in a formamide/salt solution, obtaining single-stranded DNA molecules and single-stranded probes. Then, both are incubated together under conditions that promote renaturation and due to the complementarity, would result in the hybridization of the probe to the target DNA strand. Following hybridization, the cells are subjected to washing procedures to remove unspecified bound sequences, and the preparations are examined under a fluorescent microscope [21,63,67].

FISH technology has now become a wide-spread tool for gene mapping and chromosomal abnormalities characterization, with ease and increase precision. Because the target DNA remains intact, unlike in other molecular genetic studies, information regarding the positions of probes in the chromosome bands is directly obtain. Additionally, one of the major advantages of FISH studies is that can be performed on

interphase cells (uncultured preparations) what allows for rapid screening, from fresh or aged samples[61,67].

Despite the high specificity and sensitivity of the technique, it is limited to the genomic region covered by the probe, which implies selecting previously the probes that will be used. Furthermore, it analyses just a few alterations at the same time and single-gene point mutations. Small gains/losses smaller than 30kb will not be detected, depending on the probe design[21].

2.1.1 FISH on lymphocytes, amniocytes and chorionic villi

In the GL/FMUP, FISH studies are mainly performed in cells from lymphocytes, chorionic villi and amniocytes.

The main clinical indications are: suspicion of a microdeletion/duplication syndrome, mosaicisms detected on the karyotype to confirm cell lines percentages, and cases with inherited cryptic abnormalities not detected on the Karyotype. It is also widely used in the follow up of the karyotype to confirm which chromosomal segments and breakpoints are involved in the rearrangement, to characterize supernumerary marker chromosome, and in cases where the karyotype has low quality[50].

Regarding the preparation of samples for hybridization by FISH, a few drops of the sample are spread on 1 to 2 slides and air-dried, to promote aging. A region with a high number of metaphases is marked with a diamond pen, on the opposite side of the slide with the biological material. Subsequently, the sample is dehydrated by passing through PBS, distilled water, 70% ethanol, 96% ethanol, 100% ethanol and air-dried. Whenever large amounts of cytoplasm are observed, a pepsin digestion should be performed to remove cytoplasm excess and make the metaphases accessible to the entrance of the probe.

On a coverslip, a mix of probe, buffer and water is overlaid on the slide in the previously delimited region and set on the thermal plate at 75ºC for 3min to promote denaturation. Subsequently, the slide is placed at 37°C, on a humid chamber well insulated from light, in the incubator for 3 hours or overnight, to promote hybridization. Then, post-hybridization washes are carried out to remove excess unbound probe. First, the slides are placed in a solution of 2xSCC with formamide, which is an ionizing solvent [68], then in 2xSCC and finally in 4xSSC with Tween, which is a stable non-ionic surfactant with low toxicity, that acts as a detergent[69].

Slides are placed back in a solution of PBS, distilled water, 70% ethanol, 96% ethanol and 100% ethanol, so that all the unpaired probes are removed. Finally, DAPI is applied to the slides and placed in -20ºC for a few minutes. Then, using fluorescence microscopy, the sample is analysed and studied (Figure 21).

The analysis of a FISH consists in evaluating the percentage of cells with the positive or negative probe signals. The number of cells scored and the values from which an alteration is considered relevant depends on the clinical indication. For suspected mosaicisms, at least 200 are scored, but can be extended up to 500. To identify microdeletions/duplications, only a few metaphases are scored.

When preparing the final report, it should be mentioned:

- Information about the probes used (i.e., type, target locus, manufacturer);
- Number of cells scored;
- Percentage of abnormal cells;
- Result described according to cytogenomics nomenclature (ISCN 2020)
- Correlation with karyotype or aCGH result, if available;
- Mosaic findings should be related to the referral indication, where appropriate;
- Make clear the limitations inherent to the technique;
- Recommendations for appropriate clinical follow up;

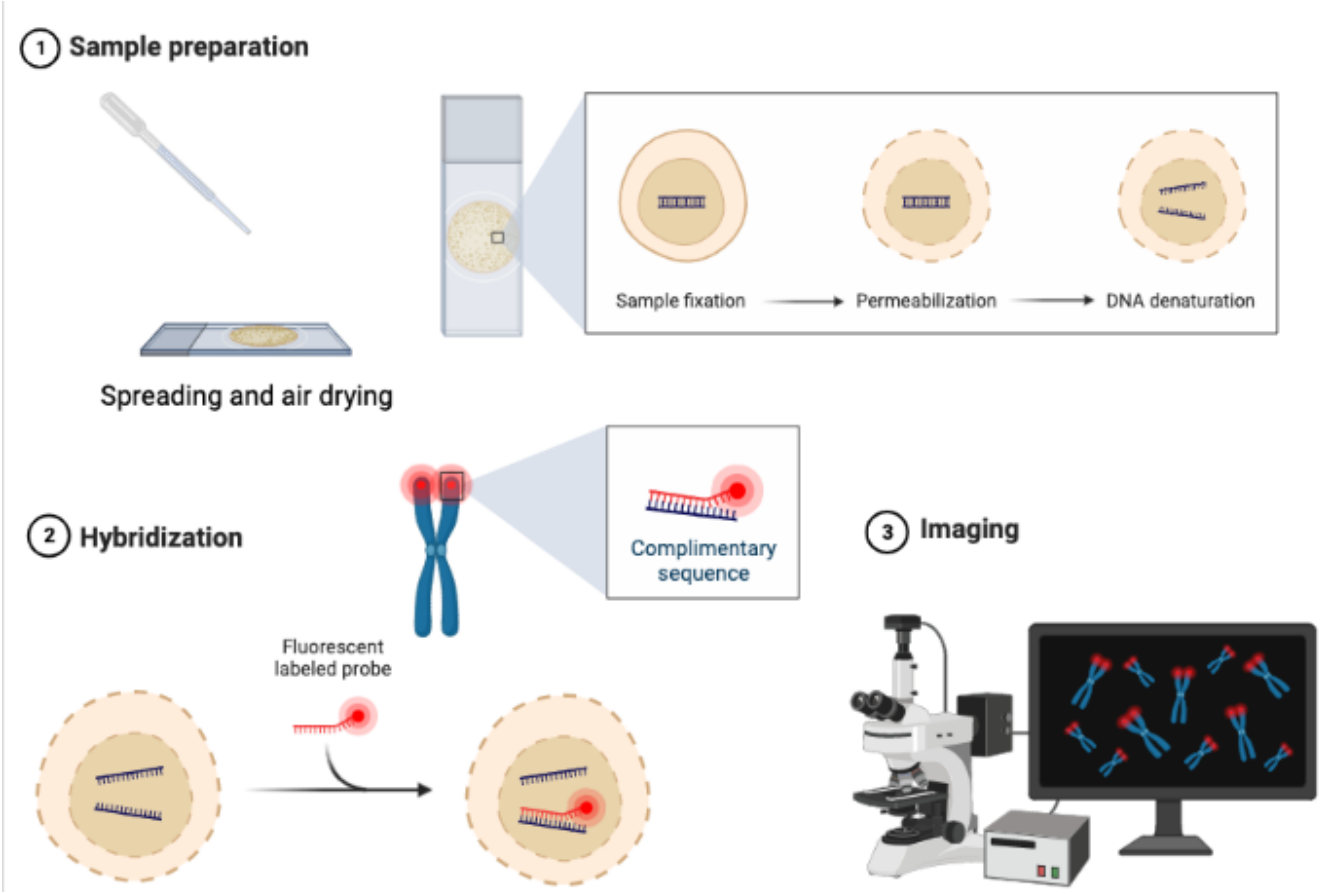


Figure 21 - Representative scheme of all the steps of FISH: sample preparation, hybridization and imaging.

2.1.2 FISH on sperm cells

FISH has also been widely used in cases of male infertility. Infertility is defined as the inability to conceive despite attempting for one year, affecting about 12% of the reproductive age population. About 50% of these cases could be attributed to male factors, however, the mechanisms that lead to infertility are not yet fully understood and can often be multifactorial. Some of the well-documented male infertility causes are sperm abnormalities (motility, volume, vitality, concentration, and morphology), stress and about 50% due to chromosomal abnormalities [70,71].

The first study conducted to investigate the chromosomal constitution of spermatozoa from infertile individuals using FISH was published in 1995, and since then many other studies have been carried out. It is consensual in the literature that the

population of infertile men has a significantly higher percentage of sperm aneuploidies, compared to fertile men[72].

FISH is the most simple, precise, and trustable test to determine the frequency of autosomes and sex chromosomes aneuploidies in sperm nuclei of men with clinical indication of infertility, couples with recurrent pregnancy loss and failure in assisted reproductive technologies [70,73]. This technique provides results with high statistical power since thousands of sperm are analysed in a single assay, but it turns out to be time-consuming, highly laborious and all the clinical consequences of an abnormal FISH sperm result are still not fully understood or consensual in the literature [72].

In this assay, ejaculated, epididymal, or testicular sperm is studied to define the frequency of chromosomal aneuploidies on chromosomes X, Y, 13, 18 and 21. Ideally, the individual's complete set of chromosomes should be analysed, however, due to the costs of the assay, only the chromosomes most involved in life-compatible/viable aneuploidies with more clinical relevance are studied, namely the ones mentioned above[71].

To apply FISH on sperm cells, the sample must first be prepared through washing and then fixed on slides. The ejaculated sperm sample should be prepared within 30min after collection. After centrifuged, the seminal fluid is removed. Two further washes are done with PBS and fixative is slowly added. Following another centrifugation, the sample fixed in suspension is stored at -20°C.

To perform the technique, the previously prepared sample is spread on 2 slides. Slides are washed in 2xSCC and dehydrated in 70% ethanol, 96% ethanol, 100% ethanol and then submerged in a DTT solution at 37°C, for decondensation of the sperm head allowing probes to access the DNA of the spermatozoon[70].

After washed again in 2xSCC and dehydrated in 70% ethanol, 96% ethanol, 100% ethanol, slides are air-dried. The slides are then denatured in a formamide solution at 75°C for 5min for DNA denaturation and dehydrated again. Two probe mixes are made and applied on different slides: in one is applied a mix with probes for chromosomes X, Y and 18 and in the other a mix with probes for chromosomes 13 and 21 (Figure 22). These are hybridized overnight at 37ºC in a humid chamber.

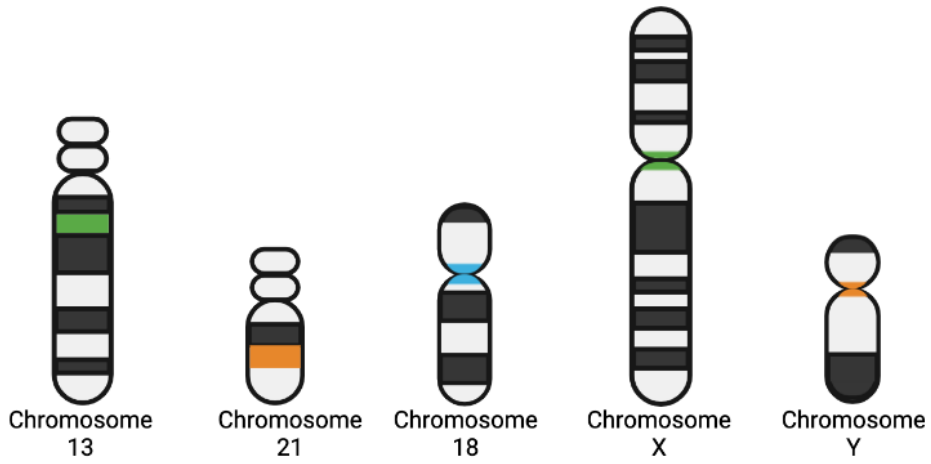


Figure 22 - Chromosomal region marked by probes used in sperm FISH. LSI 13 (13q14), LSI 21 (D21S259, D21S341, D21S342; 21q22.13-q22.2), CEP 18 (D18Z1) and CEP X(DXZ1), CEPY (DYZ3).

On the next day, slides are washed in two solutions with Triton X, one at 73ºC for 2min, the other at room temperature for 1min. After drying, DAPI II is added and visualized under a fluorescence microscope (Figure 23 and Figure 24).

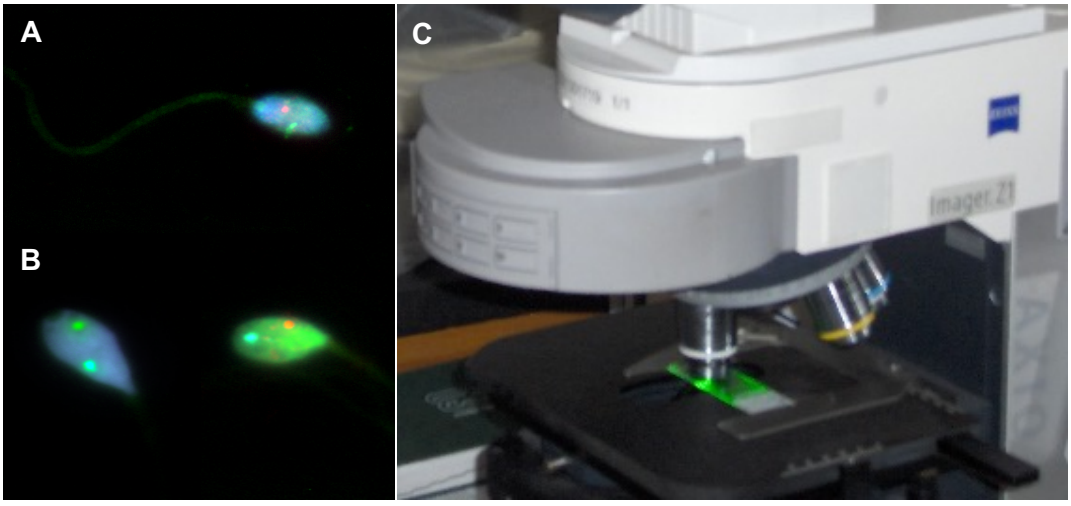


Figure 23 - Spermatozoa visualized under a fluorescence microscope after the FISH technique. (A) Sperm labeled with probes for chromosome 13 and 21. (B) Sperm labeled with probes for chromosomes 18, X and Y. On the left we have a sperm with fluorescence for chromosomes 18 and X, on the right with fluorescence for chromosomes 18 and Y. (C) Fluorescence microscope, used in FISH analysis.

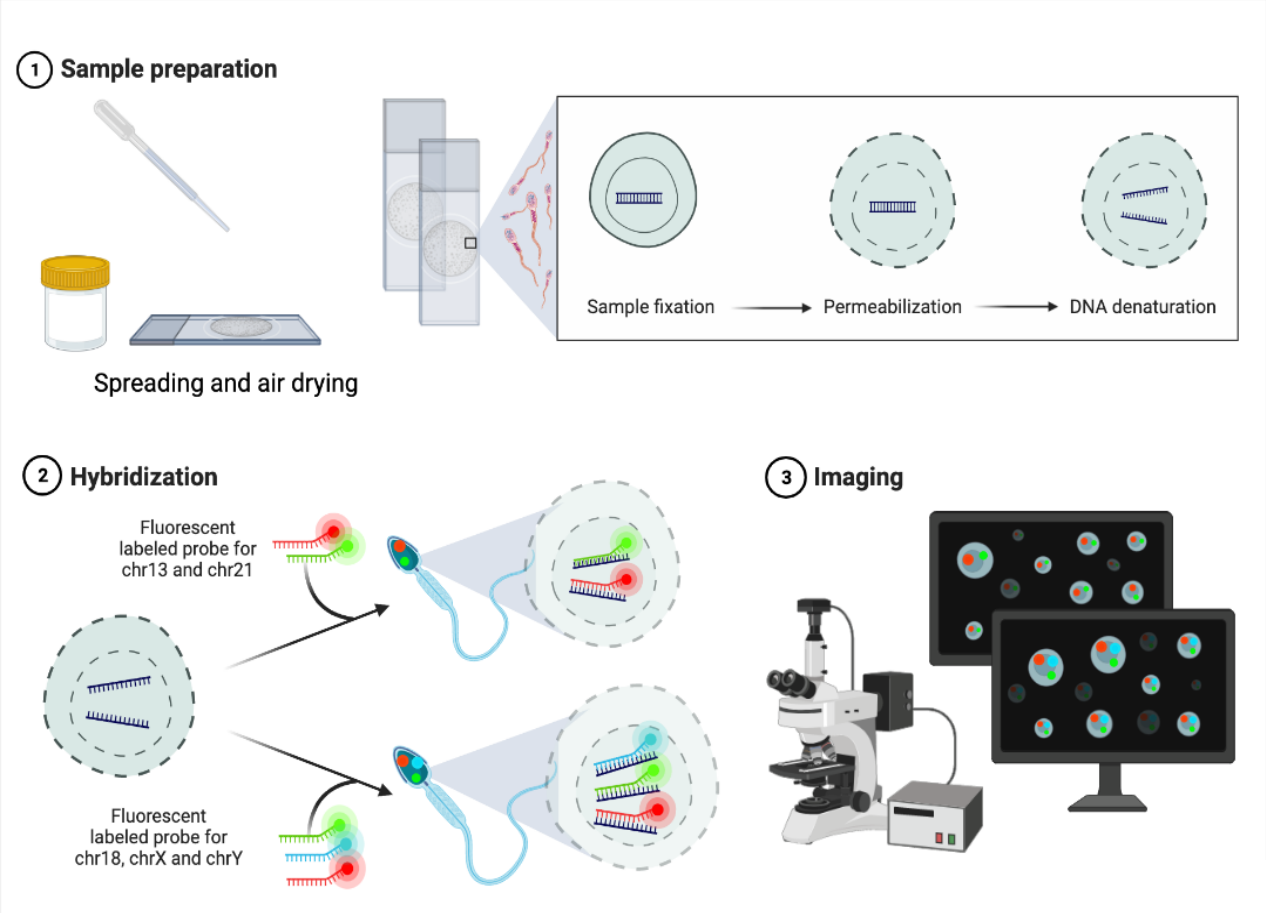


Figure 24 - Representative scheme of all the steps of FISH in sperm cells: sample preparation, hybridization and imaging.

At least 1000 spermatozoa are counted on each slide, but sperm with bad-defined or overlapping heads are not scored. According to literature, the reference values of disomy that are expected in a normal sperm sample technique are described in Table 3 and all this information must be specified in the final report.

Table 3 - Reference values of disomy expected in a normal sperm sample.

Rates of disomy	Reference value
Chromosome 13	<0.29%
Chromosome 18	<0.40%
Chromosome 21	<0.49%
Chromosome XX	<0.71%
Chromosome XY	<0.43%
Chromosome YY	<0.61%

2.2 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Terminal deoxynucleotidyl transferase dUTP nick end labeling or TUNEL is a simple, sensitive, and precise technique for directly assessing DNA fragmentation in spermatozoa, originally described in 1992 [74].

Although DNA fragmentation is a natural phenomenon of sperm and the level of DNA damage may vary from one sperm to another, an increase in these levels is correlated with fertility problems. This parameter is not evaluated in the routine sperm analysis, where motility, volume, vitality, concentration and morphology are studied, nonetheless spermatozoa can show normal values in all these parameters and still have DNA damage. As such, there is a need to carry out this study particularly in cases with idiopathic infertility [75,76].

Several mechanisms can lead to sperm DNA fragmentation, namely: (1) apoptosis during the process of spermatogenesis, also called abortive apoptosis, where abnormal sperm that should be eliminated becomes highly fragmented but manage to escape apoptosis and will appear in the ejaculated sperm; (2) oxidative stress, when sperm production of free radicals exceeds antioxidant activity, especially during transport from the seminiferous tubules to the epididymis; (3) due to intrinsic factors (e.g. varicocele, diabetes) and extrinsic factors (e.g. lifestyle, radiation treatments)[76,77].

Regardless of the mechanism, DNA fragmentation in sperm is irreversible since sperm cannot repair damaged DNA [78].

The principle behind the TUNEL technique is based on the identification of blunt ends of the strand DNA breaks by the terminal deoxynucleotidyl transferase (TDT) enzyme. This template-independent polymerase catalyzes the addition of triphosphated deoxyuridine nucleotides (dUTPS) fluorescently labeled with fluorescein isocyanate (FITC), to the 3'-hydroxyl termini of DNA fragments. This produces a green fluorescence signal proportional to the number of strand breaks, which can be visualized using either flow cytometry or fluorescence microscopy[75,79,80].

Samples are first prepared by adding PBS and centrifuged, at least 2 times, to remove seminal fluid, and the sperm concentration is adjusted with PBS. Ejaculated sperm sample should be prepared within 30min of collection. Subsequently, a smear is made on 5 slides, and the procedure will be applied on one slide.

For sample fixation, the slide is subjected to paraformaldehyde with PBS for 1 hour and permeabilized in a solution of sodium citrate and triton-x for 2min at 4ºC. After two washes in PBS, the TUNEL mix is added to a coverslip and overlaid on the slide and incubated at 37ºC for 1 hour in a light-protected box. After being washed again in PBS, DAPI is added, and observation is carried out under a fluorescence microscope (Figure 25).

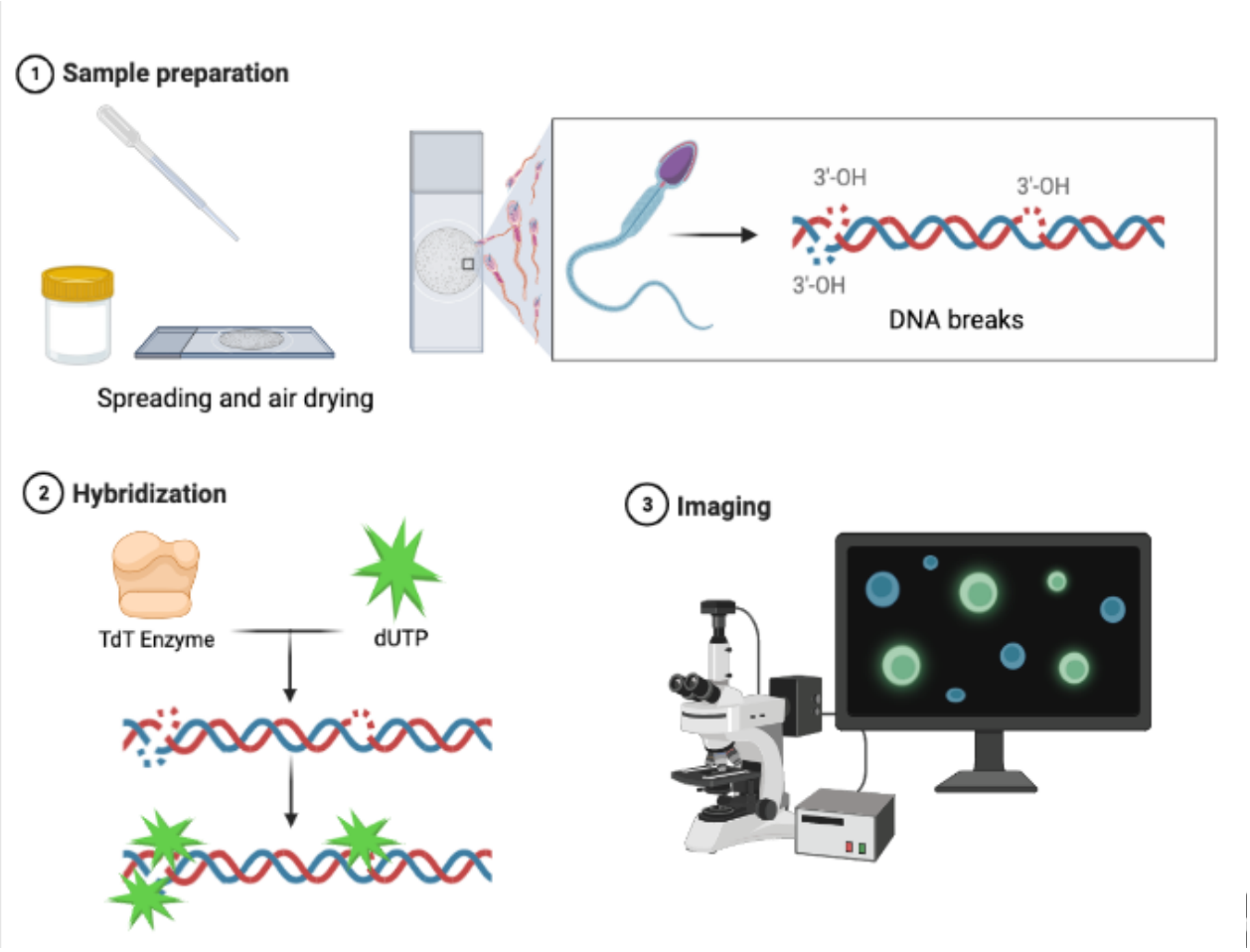


Figure 25 - Representative scheme of all the steps of TUNEL in sperm cells: sample preparation, hybridization and imaging.

For the analysis, at least 1000 sperm are scored, a sperm with a positive TUNEL result (that is, with fragmentation) will appear with green fluorescence, a sperm with a negative TUNEL result (without fragmentation) will appear with no fluorescence (Figure

26). This TUNEL procedure is performed twice for each patient, on different days, and the average of the percentages obtained is calculated to obtain the result.

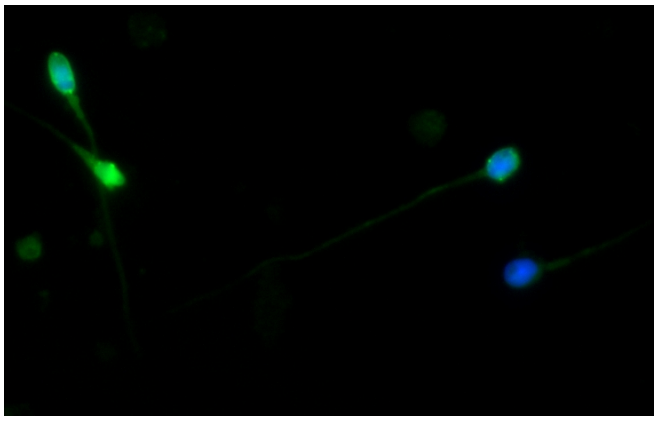


Figure 26 - Result of the TUNEL technique, sperm cells observed under a fluorescence microscope. On the left are two cells labeled with green fluorescence, that is, with fragmentation. On the right are two cells without fluorescence, that is, without fragmentation.

According to the Practice Committee of the American Society for Reproductive Medicine (ASRM), a value higher than 36% of fragmented spermatozoa is considered an abnormal result [81].

In the final report, the number of analysed spermatozoa, the percentage of fragmentation and reference value must be specified.

Unlike other techniques, the TUNEL assay has the advantage of concurrently detecting single and double-strand breaks. However, it is a non-qualitative technique revealing the number of cells with damaged DNA, but not the degree of damage. Additionally, another disadvantage is that TUNEL staining will also detect non-apoptotic cells, including necrotic degenerating cells, mechanically damaged cells and cells undergoing DNA-repairing or gene transcription [80,82].

2.3 Array comparative genomic hybridization (aCGH)

Chromosomal microarray analysis (CMA) is a relatively recent technique, introduced in 1990 that has revolutionized diagnostics in cytogenetics by providing genome-wide coverage for chromosomal abnormalities with high resolution in a short period of time [83]. CMA can be performed either by aCGH or combined with the

identification of single nucleotide polymorphism (SNPs) array. In GL/FMUP, the technique used is the aCGH.

This technique allows detection of submicroscopic rearrangements in the entire genetic complement of an individual, also known as copy number variations (CNVs), which represents the variation in the number of copies of an individual's DNA segment when compared to other different individuals' genomes [84]. Submicroscopic imbalances are also referred to as microdeletions and microduplications, especially when they involve genomic regions that may be associated with clinical features. The estimated incidence of syndromes associated with microdeletions/duplications is 1:1000[85,86]. However, CNVs do not necessarily have to be associated with an adverse phenotype, in fact, CNVs are present in about 12% of all human beings genome, the majority benign variants[3].

Karyotype, as previously referred, only detects rearrangements above 5-10Mb, therefore, aCGH. represents the great added value in the identification of CNVs below this size. Besides, the data obtained informs not only about the precise chromosomal segment involved in rearrangements but also the genes involved, allowing correlation with international databases. The turnaround time may be shorter since it does not require cell culture and can be performed on DNA extracted from peripheral blood, uncultured chorionic villi, amniotic fluid, and fetal tissue[3,84].

Nonetheless, aCGH does not detect balanced rearrangements (such as inversions or reciprocal translocations) which can have a high impact on reproductive success. Additionally, cannot detect low-level mosaicism, point mutations, unbalanced rearrangements in genomic regions not covered by the probes and polyploidy. This potential inability to detect polyploidy (e.g. triploidy) is not a major clinical concern, as a large proportion of these pregnancies are life-incompatible and will result in miscarriages or have major sonographic abnormalities[87,88].

There are several types of array platforms available commercially, with different characteristics in terms of resolution, genome coverage and recommended use for diagnosis. The resolution of the array depends on the distance between the probes and whether are distributed mostly in gene-rich regions or evenly throughout the genome. In our laboratory, cases were analysed by aCGH, using Agilent SurePrint G3 Human Genome 4x180K or 8x60k microarrays platforms (Agilent Technologies, Santa Clara, CA), with microarrays containing approximately 180,000 probes and a 13kb average or 60,000 probes and 41Kb of probe spacing, respectively. The 4x180K configuration (with

≅60Kb resolution) is preferentially applied in the postnatal context and the 8x60k format (with ≅200Kb resolution) in prenatal cases. The array resolution depends on the genome-wide median probe spacing, considering that at least 3 contiguous probes should indicate the presence of a deletion or a duplication[89–91].

The resolution of the aCGH must be carefully considered since with the increasing resolution, also increases submicroscopic changes not commonly observed in the population and with few associated studies, so its pathogenicity can neither be confirmed nor ruled out. These submicroscopic changes are called variants of uncertain significance (VUS) [92]. This turns out to be a disadvantage of the technique and the main adversity encountered in the interpretation of an aCGH result, namely in the prenatal context. However, with more experience using aCGH, the number of CNVs interpreted as VUS will decrease, also the size and genes involved in the variation help to determine its pathogenicity. Additionally, through parental testing it can be determined whether the CNVs was inherited from one of the parents or if it occurred *de novo*. It is not a rule, but if the CNVs is inherited from a healthy parent it is less likely to be pathogenic[85].

In the context of the clinical application of the technique, as it is already well established in the literature the association between microdeletion/microduplication syndromes with intellectual disability, developmental delay, congenital anomalies and autism spectrum disorder, the American Academy of Pediatrics has recommended CMA as a first-tier test in these situations[86,93,94].

In 2013, the ACOG alongside the Society for Maternal Fetal Medicine (SMFM) recommended that CMA should be the gold standard test for cytogenetic analysis of all pregnancies where ultrasound abnormalities were found. Furthermore, this test can be performed and recommended in all pregnancies, even in the absence of ultrasound abnormalities and regardless of the mother's age, if the patients wish to undergo these studies[95].

Regarding the procedure of the technique, it is used DNA extracted from the patient sample (which can be peripheral blood, LA, tissues, etc.) and DNA from a female or male control sample, matching de sex of the patient. Both DNAs are labeled with a fluorescent probe, the control labeled with a green probe (Cyanine 3) and the test with a red probe (Cyanine 5). Subsequently, the samples are mixed and co-hybridized on an array slide that contains thousands of immobilized complementary probes representative of the entire human genome, and there being competition between the sample DNA and the

control DNA for the binding sites. Hybridization takes place in the presence of Cot-I to minimize nonspecific hybridization.

After this process, the slide is read by a scanner and the fluorescence ratio of the hybridization signals of the test and the control samples is determined at different positions along the genome using the *CytoGenomics* software (different versions; Agilent Technologies, Santa Clara, CA).

Unbalanced chromosomal regions will show deviant log ratio of the intensities of the signals: a value of 0 (log_2) indicates a balance of the number of copies and that therefore no changes in this region, a value of 0,58 (log_2) or -1 (log_2) indicates a duplication or deletion, respectively[96,97].

This is because the unaltered chromosomal regions show an equal contribution from the green and red probes, resulting in an orange/yellowish color. However, if a chromosomal region is deleted in our sample, that region will fluoresce predominantly green. Likewise, if a chromosomal region is amplified in the sample, the corresponding chromosomal region will appear redder (Figure 27).

After identifying the CNVs presents, they are classified according to the American College of Medical Genetics – ACMG standards and guidelines, as pathogenic (P), likely-pathogenic (LP), benign (B), likely-benign (LB) or VUS [98]. For that end, several databases are used, namely, UCSC Genome Browser, Database of Genomic Variants (DGV), Online Mendelian Inheritance in Man (OMIM), ClinGen, PubMed and inhouse laboratory's database.

Subsequently, the report is prepared, which must contain:

- Complete cytogenetic location (chromosome number, band, coordinates and CNVs size with genome build [e.g. GRCh37 (hg19)] using cytogenomic nomenclature (ISCN 2020)
- Report if a gain or loss of genetic material was found;
- Classification and interpretation of the CNVs found, including evidence and references;
- Genes involved;
- Make clear the limitations inherent to the technique;
- Recommendations for appropriate clinical follow up;

Sometimes the interpretation of the clinical significance of CNVs can be complex, especially if it is a VUS or if there is no relationship between the phenotype and the CNVs

found. In these cases, the clinical geneticist might discuss with the requesting clinician additional clinical findings that may add important information to clarify the pathogenicity[99,100].

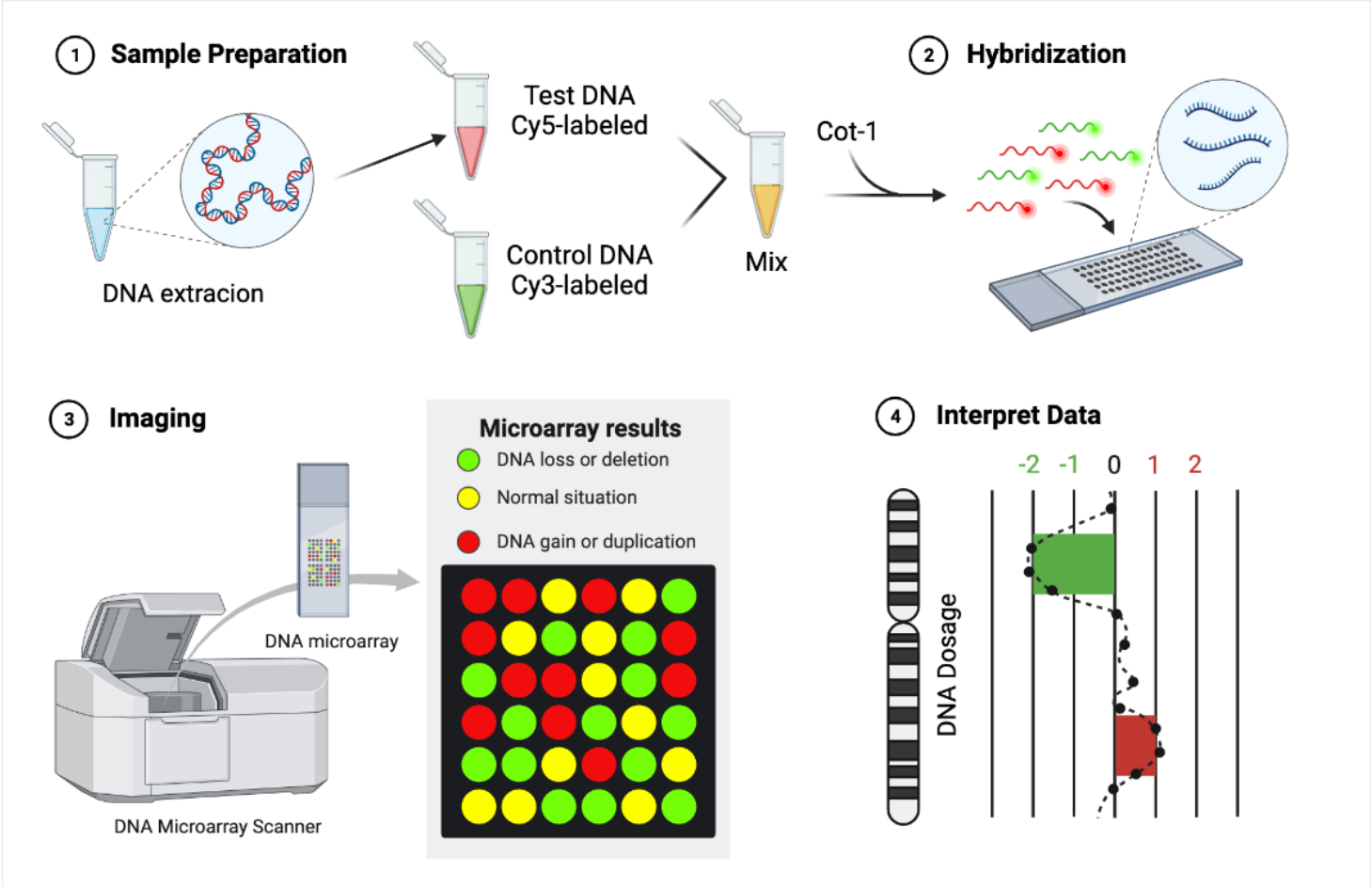


Figure 27 - Representative scheme of all the steps of Array CGH: sample preparation with labeling, hybridization, imaging and interpret data.

IV. Case Studys

During my cytogenomics internship, I had opportunity to monitor several cases of the GL/FMUP, since their entry, the procedures, analysis, discussion of results and final report. Among these, 7 will be addressed, involving the different cytogenetics or cytogenomics protocols. It will be included prenatal and postnatal cases, in order to elucidate all the dynamics behind the diagnostic process and how the techniques could complement each other to reach to a better report.

Table 4 - Summary of the 7 cases analysed below, with indication of the area, clinical indication and technique(s) performed.

Case Study	Context	Clinical Indication	Technique(s) performed
I	Postnatal	Couple with primary infertility	Karyotype FISH DA/DAPI
II	Postnatal	Couple with primary infertility	Karyotype FISH in sperm cells TUNEL
III	Prenatal	Fetus with increased nuchal translucency and other skeletal malformations	Karyotype
IV	Postnatal	10-month-old female child with sleep disturbances, microcephaly, axial hypotonia and other minor dysmorphia	aCGH Karyotype FISH
V	Prenatal	Fetus with congenital anomaly in the central nervous system, namely bilateral ventriculomegaly	aCGH
VI	Postnatal	1-year-old child with hypotonia and dysmorphia.	aCGH Karyotype
VII	Prenatal	Prenatal diagnosis due to obstetrical history	aCGH

1 Case Study I

Couple with primary infertility

In this case, a karyotype was performed in a couple with primary infertility (female with 33 years and male with 36 years). Primary infertility is a term used to describe couples that have never been able to conceive. This condition is relatively common in humans, affecting about 15% of couples trying to conceive, and may be associated with chromosomal abnormalities [101].

To obtain the couple's karyotype, lymphocyte cultures from peripheral blood samples were done, followed by a GTL staining as a staining method for the analysis of chromosomal bands.

In both cases, 30 metaphases from two different cell lines were scored: the female had a normal female karyotype (46,XX) (Figure 28). On the other hand, the male karyotype revealed the presence of a marker chromosome and apparently the absence of the Y chromosome (46,X,+mar) (Figure 29, indicated with the arrow).



Figure 28- Karyotype of the case I female - 46,XX. Normal karyotype for a female individual.

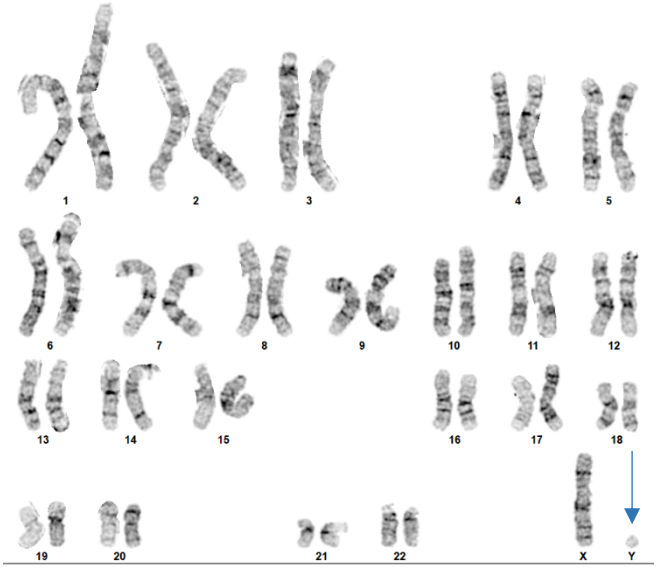


Figure 29 - Karyotype of the case I male - 46,X,+mar. This represents an abnormal karyotype.

As such, it was suspected that this marker chromosome could part of Y chromosome. To analyse this possibility, a FISH study was performed with a probe for the Sex-determining Region of Y chromosome (SRY) gene, which is found only on the

Y chromosome and is responsible for the development of male genitals [102]. As control, a centromeric probe (DXZ1), for the X chromosome, was used.

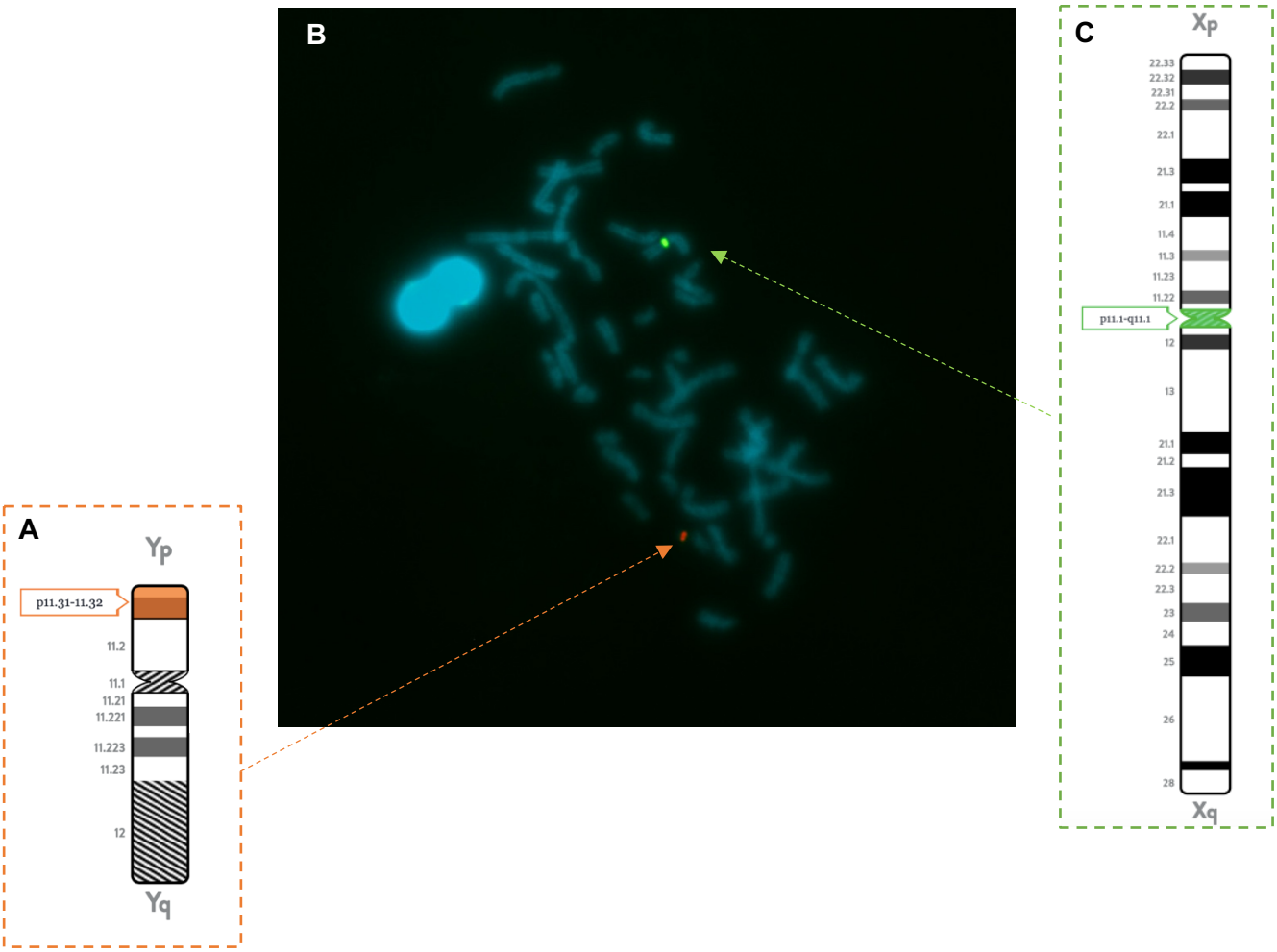


Figure 30 - (A) Vysis LSI SRY SpectrumOrange Probe with cytogenomic localization Yp11.3 (Orange signal)[119]. (B) Metaphase of the case I male, after performing the FISH technique, visualized under a fluorescence microscope. The orange/red marking demonstrates that the marker chromosome originates from the Y chromosome. (C) Vysis CEP X (DXZ1) SpectrumGreen Probe with cytogenomic localization Xp11.1-q11.1 (green signal)[120].

Figure 30, shows the hybridization signals of the control probe and the SRY probe, which confirms that the marker was originated from the Y chromosome. These data combined with the chromosome morphology observed in the karyotype suggested that, in fact, it was a Y ring chromosome.

This type of structure results when a chromosome breaks in two sites and the ends fuse together to form a circular “ring” structure. Due to the instability of ring

chromosomes during cell division, they frequently arise spontaneously and are rarely inherited. There are several possible mechanisms for the formation of this ring chromosome, namely fusion of two breakpoints in one or two euchromatic regions forming a "sticky end", with consequent loss of acentric fragments in the short arm and/or long arm or fusion of two telomeres without loss of chromosomal segments.[33]

For better understanding of this structure, a DA/DAPI stain was carried out and no fluorescence was observed on the Y ring chromosome (Figure 31). This means that this structure lost, at least, the heterochromatic region, and point out that the formation mechanism of the ring chromosome should be probably the first mechanism mentioned, that includes losses of genetic material, which could be the cause for infertility [103].

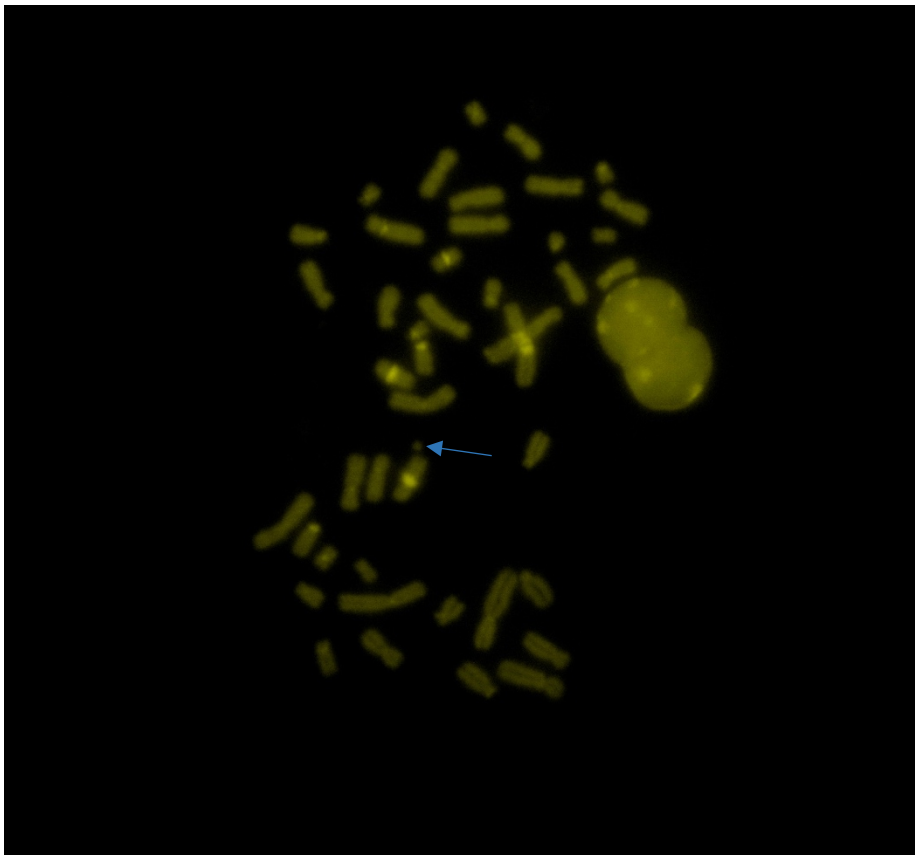


Figure 31 - Metaphase of a the case I male, after DA/DAPI staining, visualized under a fluorescence microscope.

2 Case study II

Couple with primary infertility

Karyotype was performed in a couple with primary infertility (female with 31 years and male with 44 years) through lymphocyte cultures from peripheral blood samples, followed by GTL staining. Chromosomal evaluation of 30 metaphases from two cell lines from both revealed normal karyotypes (Figure 32 and Figure 33). Therefore, a FISH on sperm cells and TUNEL studies in the male were requested.

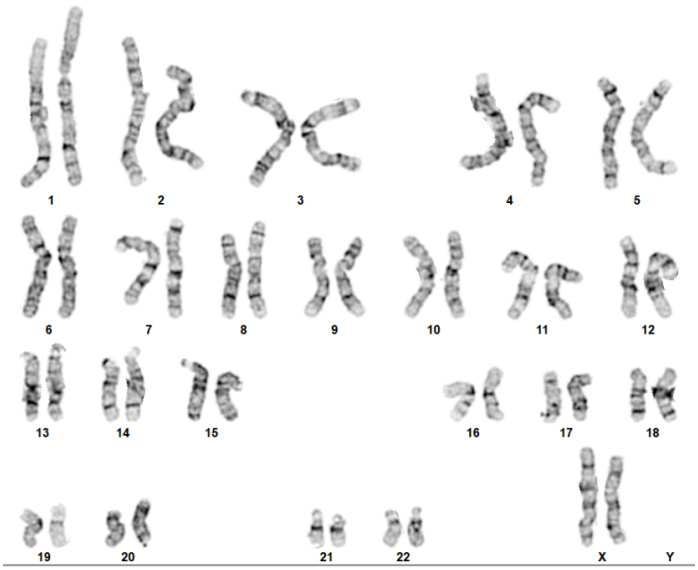


Figure 32 – Karyotype of the case II female - 46,XX. Normal karyotype for a female individual.

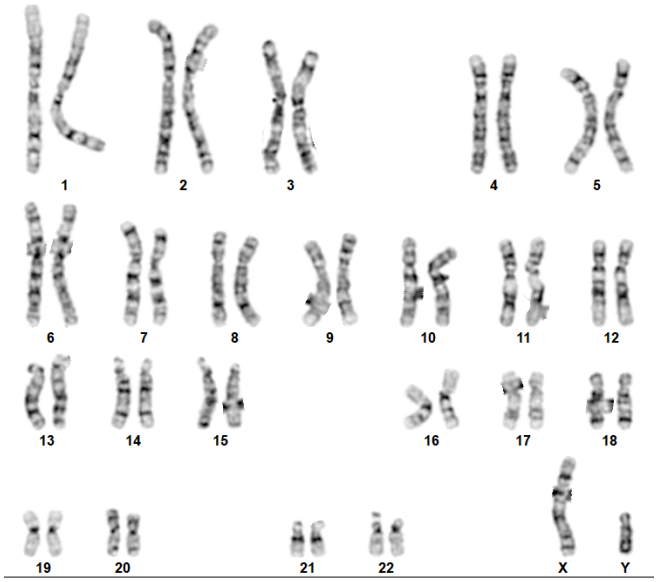


Figure 33 - Karyotype of the case II male - 46,XY. Normal karyotype for a male individual.

The FISH technique was performed with the previously described probes (CEP X(DXZ1), CEPY (DYZ3), LSI 13 (13q14), CEP 18 (D18Z1), and LSI 21 (21q22.13-q22.2)). The analysis of 1003 spermatozoa (probes 13 and 21) and 1004 spermatozoa (probes 18, X, and Y), revealed that 99.7% of the sample had a normal constitution for the analysed chromosomes (Figure 34 and Figure 35). The rates of disomy observed were:

- disomy 13 = 0% (rv: <0.29%)
- disomy 18 = 0% (rv: <0.40%)
- disomy 21 = 0.1% (rv: <0.49%)
- disomy XX = 0% (rv: <0.71%)

- disomy XY = 0.1% (rv: <0.43%)
- dyssomia YY = 0% (rv: <0.61%) (rv: reference value)

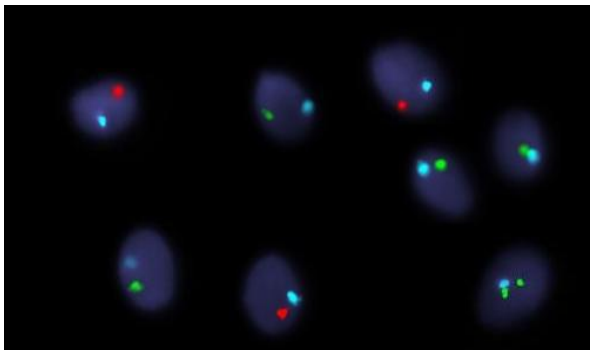


Figure 34 - Sperm cells of the case II male visualized under a fluorescence microscope after the FISH technique. Sperm labeled with probes for chromosomes 18 (blue signal), X (green signal) and Y (orange signal).

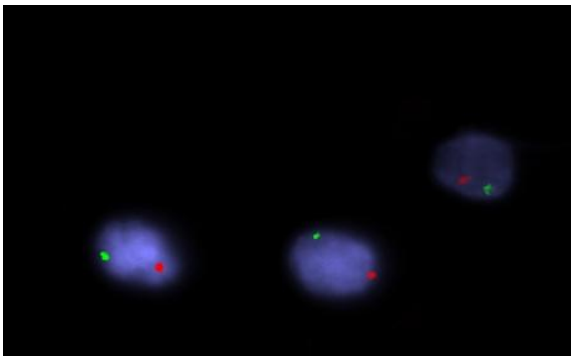


Figure 35 – Sperm cells of the case II male visualized under a fluorescence microscope after the FISH technique. Sperm labeled with probes for chromosome 13 (green signal) and 21 (orange signal).

Subsequently, the TUNEL technique was performed, where 2033 spermatozoa were scored and 1256 spermatozoa with fragmented DNA were observed, thus obtaining a percentage of sperm with DNA fragmentation of 61.8% (Figure 36). This value is above the limit considered normal by the ASRM Practice Committee (<36%), which leads us to conclude that this could be related to the infertility.

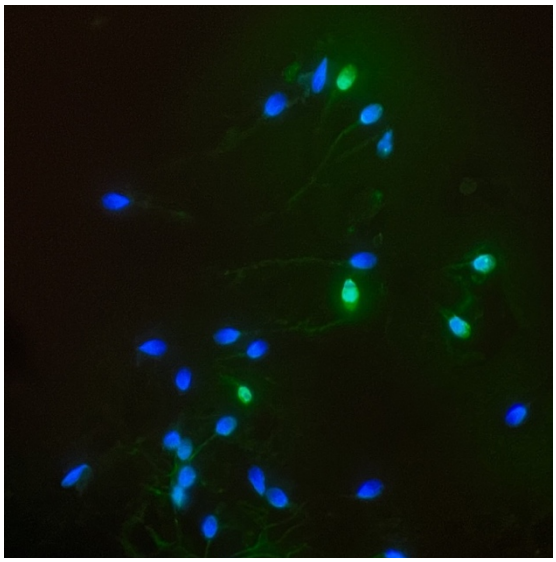


Figure 36 - Sperm cells of the case II male visualized under a fluorescence microscope after performing the TUNEL technique.

3 Case Study III

Fetus with increased nuchal translucency and other skeletal malformations

Structural malformations were detected in a fetus at 23 weeks, namely increased nuchal translucency (3.7mm), hypoplasia of the middle phalanx of the 5th toe, with the toes in flexion.

These malformations may be associated with a chromosomal disorder, namely Down's syndrome (trisomy 21), so a karyotype was requested.

Karyotype was performed through amniocyte culture and GTL staining.

Chromosomal evaluation of 20 metaphases from two cell lines and *in situ* cultures revealed a male karyotype with the an extra chromosome 21 (47,XY,+21), compatible with trisomy 21/Down Syndrome (Figure 37, indicated with the arrow).

The great majority of aneuploidies arise *de novo* and the risk of recurrence is approximately 1%, however, prenatal diagnosis should be offered in future pregnancies due to the advanced maternal age (the mother is 40 years old), which is a main factor for the predisposition of chromosome non-disjunction [104].

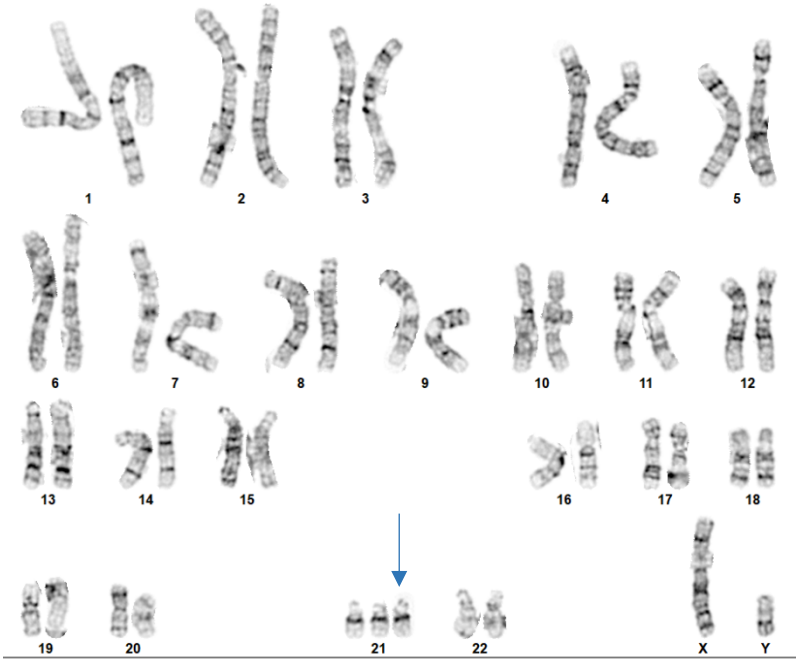


Figure 37 - Karyotype of the case III male fetus showing a trisomy 21 - 47,XX,+21.

4 Case Study IV

10-month-old female child with sleep disturbances, microcephaly, axial hypotonia and other minor dysmorphia

An aCGH was request in a 10-month-old female child with sleep disturbances, microcephaly, axial hypotonia and other minor dysmorphia. DNA was extracted from peripheral blood of the patient and the 4x180k configuration array was used to search for clinical relevant CNVs, with an average resolution of 60Kb.

The aCGH analysis revealed a heterozygous deletion of approximately 5.3Mb, on the short arm of chromosome 5 involving the p15.33-p15.32 regions within genomic positions 26142-5296560, and in addition, a heterozygous duplication, on the same chromosome, of approximately 1Mb, involving the p15.32-p15.31 regions, with genomic positions at 5343166-6475347 (Figure 38).

The described deletion is in the region of the already known Cri-du-Chat Syndrome (OMIM: 123450). Cri-du-Chat Syndrome or Cat Cry Syndrome is a chromosomal disorder resulting from a total or partial deletion in the short arm of chromosome 5, with an estimated incidence of 1:50,000 live births [105]. The phenotype of this syndrome is variable, with the main clinical manifestations being the characteristic high-pitched, cat-like cry that gave rise to the syndrome's name, intellectual disability, hypotonia, congenital heart disease, microcephaly, epicanthus, wide nasal bridge and other facial dysmorphisms[106].

In this case, the patient had the typical phenotype of the syndrome, but it appears to be milder. It is described in the literature that the degree of the other clinical manifestations is typically proportional to the size of the deletion, and genomic studies suggest that haploinsufficiency for specific regions within 5p14-p15 may largely contribute to the severity of intellectual disability [29]. At the same time, a 1Mb duplication was also detected in a region almost contiguous to the deletion. Taking into account that the deletion does not involves the 5p14-p15 region, and the simultaneously presence of a duplication in the region characteristic of the syndrome that might counterbalanced the loss-of-function in some genes, may justify a less severe phenotype in the patient.

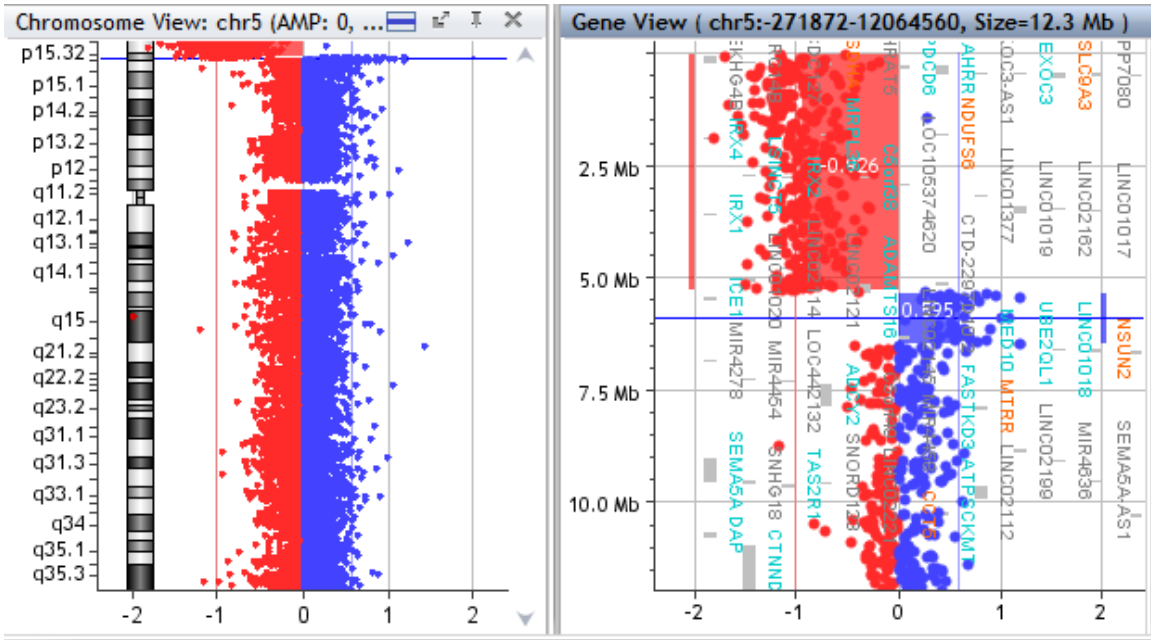


Figure 38 - aCGH result of the case IV female child, for chromosome 5 – arr[GRCh37]5p15.33-p15.32(26142-5296560)x1, 5p15.32-p15.31(5343166-6475347)x3. A log ratio of , -0.926 is indicative of a deletion, 0.595 is indicative of a duplication.

Parent's follow up was recommended by karyotype once the size of the deletion allows to be detected with this technique.

The study on the mother revealed a female karyotype with the same deletion on the short arm of chromosome 5, that was found in the daughter (46,XX,del(5p15.33-p15.32) (Figure 39).

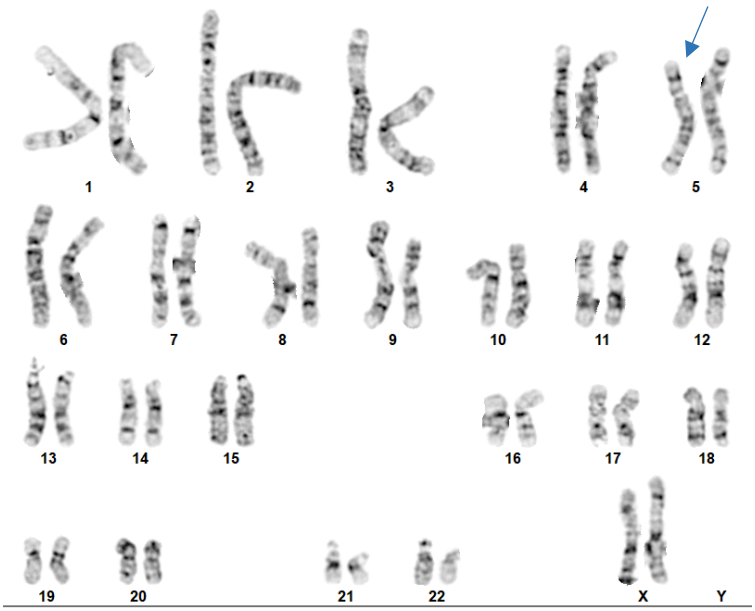


Figure 39 - Karyotype of the case IV female progenitor showing a deletion on 5p chromosome - 46,XX,del(5p15.33-p15.32). This represents an abnormal karyotype and indicates that the abnormal chromosome 5, also present in the daughter (case 4), was inherited from the mother.

Because the duplication size is above 5Mb, could not be identified on the karyotype, so the FISH study was performed with a subtelomeric probe for chromosome 5 (LPT5/G probe, cytocell). The FISH analysis showed 3 hybridization signals, thus demonstrating that both the deletion and the duplication found in the daughter were inherited from the mother (Figure 40).

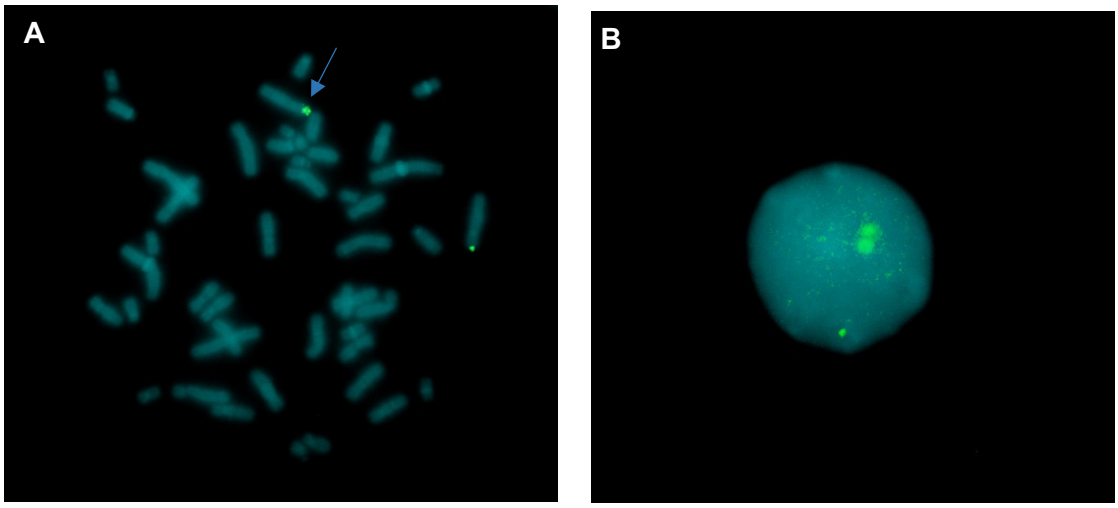


Figure 40 – (A) Metaphase of the case IV female progenitor after performing the FISH technique with a subtelomeric probe for chromosome 5 (LPT5/G probe, cytocell) in the mother´s cells of the proband. In this image there are two hybridization signals, but in the chromosome indicated by the arrow, the signal size is larger suggesting that may correspond to two overlapped signals. (B) Nucleus in interphase of the case IV female progenitor visualized under a fluorescence microscope after performing the FISH technique with the LPT5/G probe. In this cell it is evident that there are 3 hybridization signals, confirming the presence of the duplication in the mother as in the proband

Out of the expected, the father's karyotype also revealed an chromosomal abnormality, an inversion (balanced rearrangements) on the long arm of the chromosome 1 [46,XY,inv(1)(q23.3q32.1)], which was not inherited by the daughter (Figure 41).

Therefore, both parents had chromosomal alterations with a 50% probability of being inherited by the offspring. In this case the child received the abnormal chromosome 5 from the mother and the normal chromosome from the father. After consultation the clinical geneticist confirmed that the mother also had a mild phenotype characteristic of the syndrome.

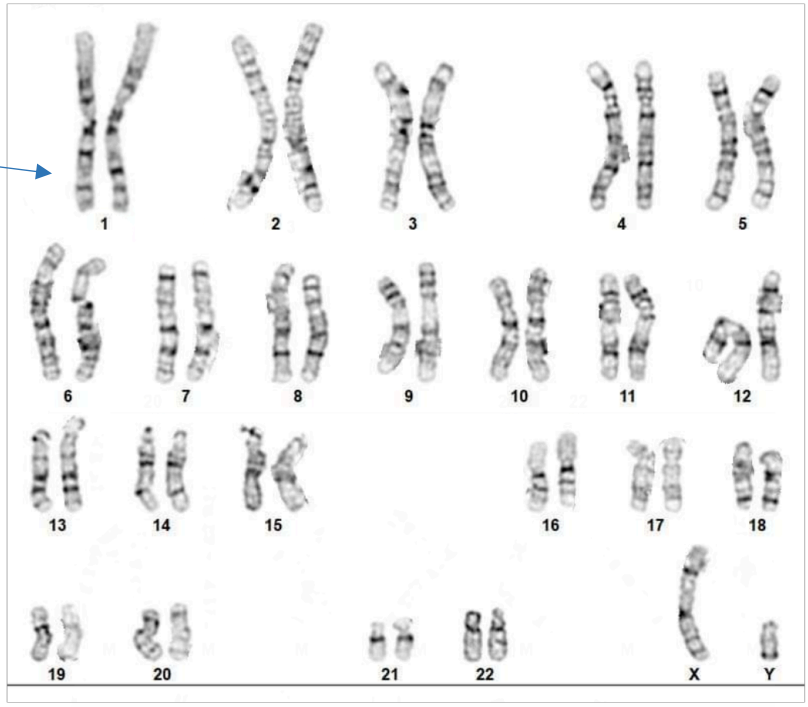


Figure 41 - Karyogram of the case IV male progenitor – (46,XY,inv(1)(q23.3q32.1). This represents an abnormal karyotype and this alteration was not found in the daughter.

5 Case Study V

Fetus with congenital anomaly in the central nervous system, namely bilateral ventriculomegaly

An aCGH was performed in a fetus with a bilateral ventriculomegaly detected in the ultrasound at 20 weeks. This malformation is an enlargement of the cerebral ventricular diameter (≥10 mm) and is often associated with chromosomal disorders [107].

The aCGH technique was performed in DNA extracted from amniotic fluid using 8x60k configuration, with an average resolution of 200Kb.

The aCGH analysis revealed the presence of two duplications of approximately 1.9Mb and 679Kb involving chromosomes 15 and 16, respectively.

The duplication on chromosome 15 involves the q13.2q13.3 region, between the genomic positions 31014508-32914140, which includes the genes *FAN1, TRPM1, MIR211, KLF13, OTUD7A, CHRNA7, ARHGAP11A, LOC100288637, HERC2P10, MTMR10, LOC283710, GOLGA8K, ULK4P3, ULK4P1, ULK4P2, GOLGA8O, WHAMMP1, LOC100996255* and *GOLGA8N*. This CNV has already been recognized as a recurrent syndrome (15q13.3 microduplication syndrome), involving the regions BP4-BP5 on chromosome 15 and including the genes MTMR15, MTMR10, TRPM1, KLF13, OTUD7A and CHRNA7 (Figure 42).

There is a huge spectrum of clinical manifestations described in the literature associated with this disorder, from normal individuals to neurodevelopmental problems, linguistic delay, mood disorders, epilepsy, intellectual disability seizures, autism spectrum disorder, attention deficit hyperactivity disorder and heart problems [108,109].

The duplication genomic interval observed in our patient overlaps several other reported cases, all of them encompassing the cholinergic receptor nicotinic alpha 7 subunit (CHRNA7) gene, which encodes for the neuronal alpha7 nicotinic acetylcholine receptor. This gene is considered the main candidate for the neuropsychiatric phenotype[110].

Additionally, a duplication was also detected in 16q22.1, between nucleotide positions 68494255-69173835 and encompassing the genes ZFP90, CDH3, CDH1, HAS3, CHTF8, MIR7641-2, TANGO6, UTP4, classified as likely-pathogenic (Figure 43).

Several cases involving this region have been described, associated with hypoplasia, heart problems, dysmorphism, hypotonia, among others [111,112].

Brisset et al. reported a phenotypic map of partial 16q trisomy, where the 16q22 region was associated only with club feet[113]. Moreover, recently a case report of a microdeletion in 15q13.3 and a duplication in 16q22.1 was described in the literature, where the authors argue that the presence of a second alteration can exert an additive or epistatic effect, altering the risk associated with the presence of the allele that determines the phenotype[114].

To assess whether it is an inherited or *de novo* alteration, the study of the progenitors was performed using aCGH on peripheral blood using 4x180k Agilent format.

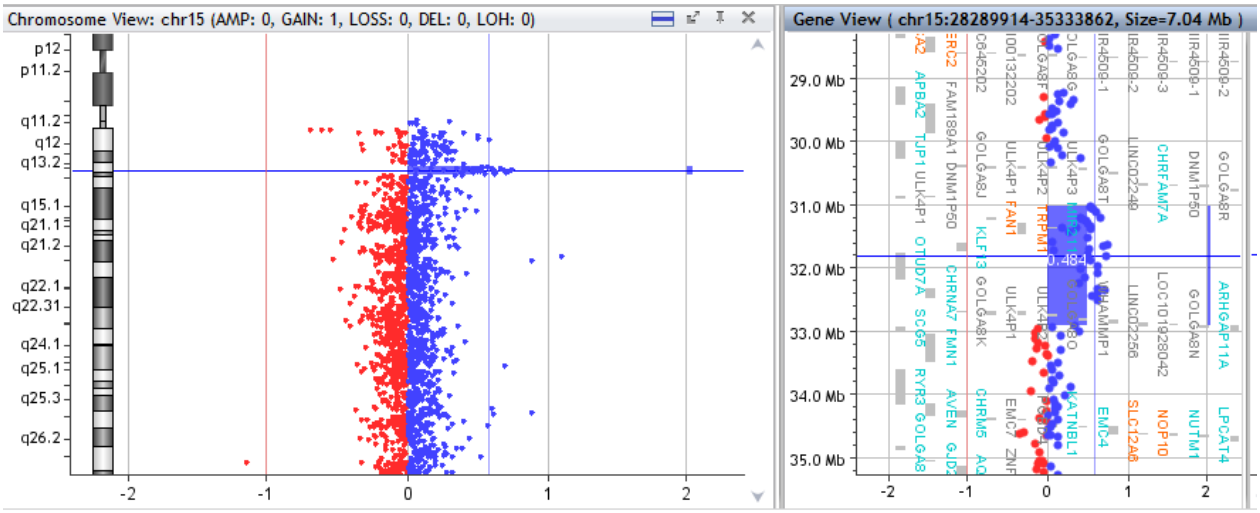


Figure 42 - aCGH result if the case V fetus, for chromosome 15 – (arr[GRCh37]15q13.2q13.3(31014508-32914140)x3). A log ratio of 0.484 is indicative of a duplication.

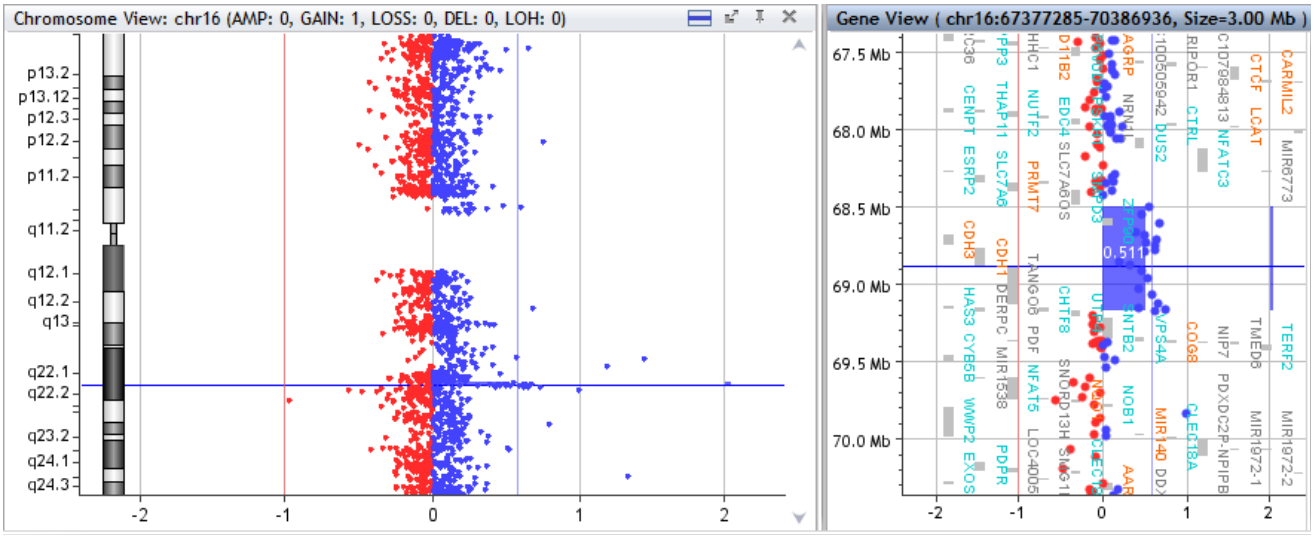


Figure 43 - aCGH result if the case V fetus for chromosome 16 – arr[GRCh37]16q22.1(68494255-69173835)x3. A log ratio of 0.511 is indicative of a duplication.

The study of the parents revealed that the duplication of chromosome 15, the main responsible for the phenotype, was inherited from the father and the duplication of chromosome 16 was inherited from the mother, both parents being apparently healthy (Figure 44). This information supports the additive effect, that the presence of the two pathogenic/probably pathogenic CNVs are associated with a high risk of a more severe phenotype. The parents were referred to a genetic counseling consultation, where they chose to perform pregnancy termination. Prenatal diagnosis should be offered in future pregnancies.

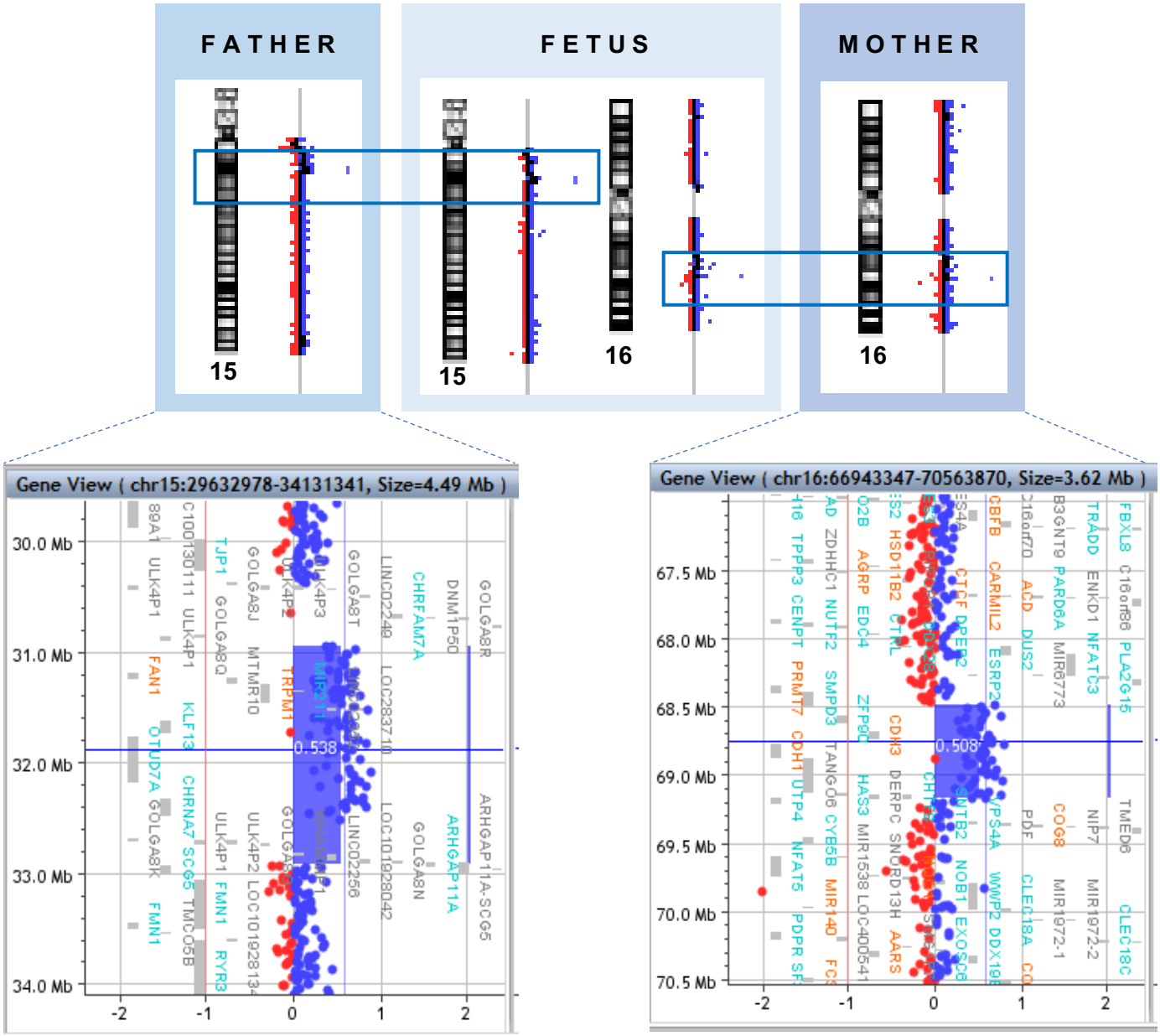


Figure 44 - On the left, aCGH result of the case V male progenitor, for chromosome 15 – arr[GRCh37]15q13.2q13.3(31014508-32914140)x3. A log ratio of 0.538 is indicative of a duplication. On the right, aCGH result of the case V female progenitor for chromosome 16 – arr[GRCh37]16q22.1(68494255-69173835)x3. A log ratio of 0.508 is indicative of a duplication.

6 Case Study VI

1-year-old child with hypotonia and dysmorphia

An aCGH was requested in a 1-year-old female child with hypotonia and dysmorphia.

The aCGH technique was performed on DNA from peripheral blood (4x180k configuration), revealed a deletion of approximately 58Mb on the short arm of chromosome X, within genomic positions at 61091-58051765, and at same time a duplication of approximately 93Mb in the long arm also in the X chromosome, between the genomic positions 61931689-155190083 (Figure 45).

This result is compatible with the presence of 1 copy of the short arm and 3 copies of the long arm of the X chromosome, indicative of an X isochromosome. An isochromosome is a chromosome that has lost one of its arms, but the other is in duplicate in a mirror-image fashion.

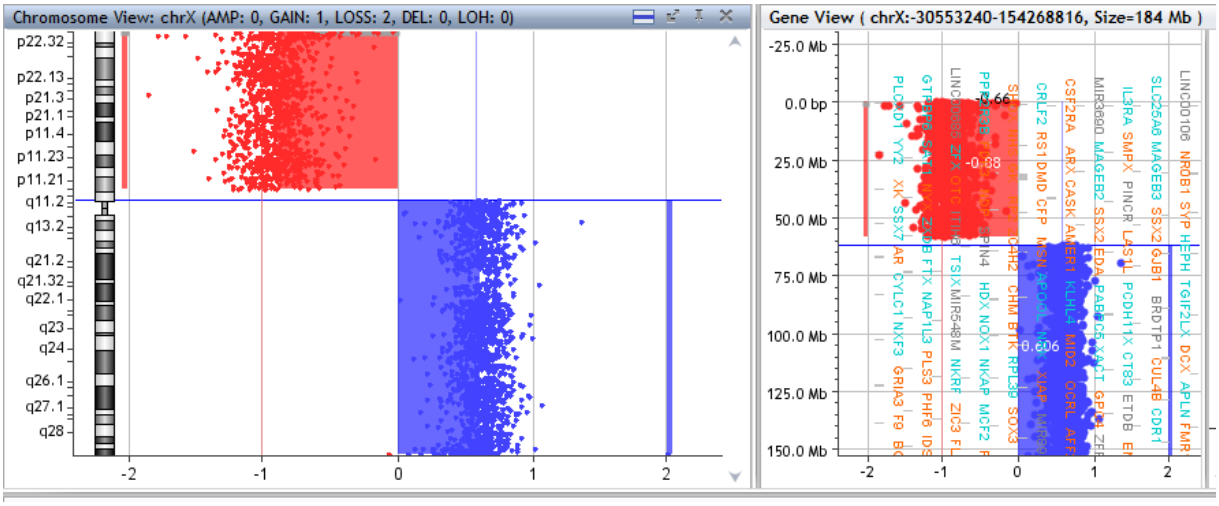


Figure 45 - aCGH result of the case VI female child for chromosome X – arr[GRCh37]Xp22.33p11.21(61091-58051765)x1, Xq11.1q27.3(61931689-155190083)x3.

In order to validate this alteration and confirm the chromosome structure, a karyotype study was performed. Chromosomal evaluation of 30 metaphases from two cell lines revealed the presence of an isochromosome X (Figure 46). The nomenclature was described as following: 46,X,i(X)(q10).

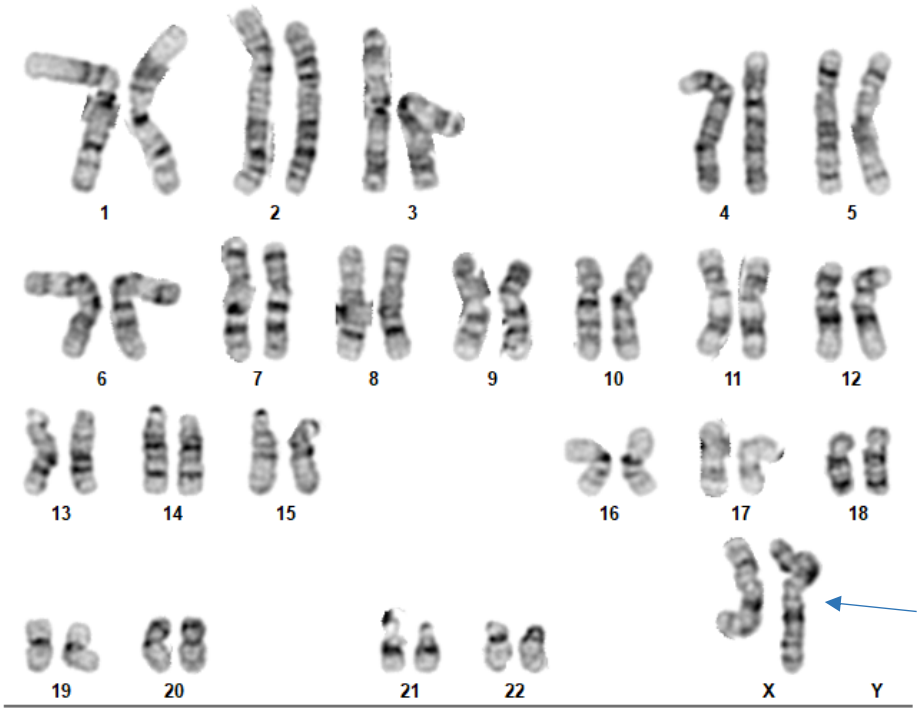


Figure 46 – Karyotype of the case VI female child, showing the abnormal chromosome X in case 5 (46,X,i(X)(q10)). This represents an abnormal karyotype with an X isochromosome.

The most common form of isochromosomes is, in fact, the ones involving the X chromosome. Most patients with this unbalanced rearrangement have this chromosome inactivated. Because of this preferential inactivation of the abnormal X, X chromosome abnormalities have less impact on phenotype than other unbalanced abnormalities of similar size (or even smaller) in autosomes, which explains the less severe phenotype observed[29].

This result and its implications should be discussed in a genetic counseling consultation and prenatal diagnosis may be offered in future pregnancies.

7 Case Study VII

Prenatal diagnosis due to obstetrical history

In this case, chorionic villi biopsy was performed at 15 weeks of gestation due to a previous peripartum death with hydrops, in spite of the fetus did not appear to have any malformation. The genetic study was performed using the aCGH technique. Previously, the DNA used was screened for maternal contamination by QF-PCR technique.

aCGH analysis revealed a triplication on one of the chromosome 18, with a size of approximately 1114Kb, on the short arm involving the p11.31p11.23 region, and between genomic positions 6942021-8055875 (Figure 47). This interval encompass the genes LAMA1, PTPRM, LOC101927188, LRRC30 and was classified as Likely pathogenic. The *LAMA1* gene (encoding Laminin alpha-1) belongs to the family of Laminins, which are heterotrimeric glycoproteins present in the extracellular matrix and essential for cell membrane formation and embryonic development[115].

Homozygous mutations or deletions of the *LAMA1* gene have been associated with cerebellar dysplasia, delayed motor development, pathological myopia and Poretti-Boltshauser syndrome [116,117].

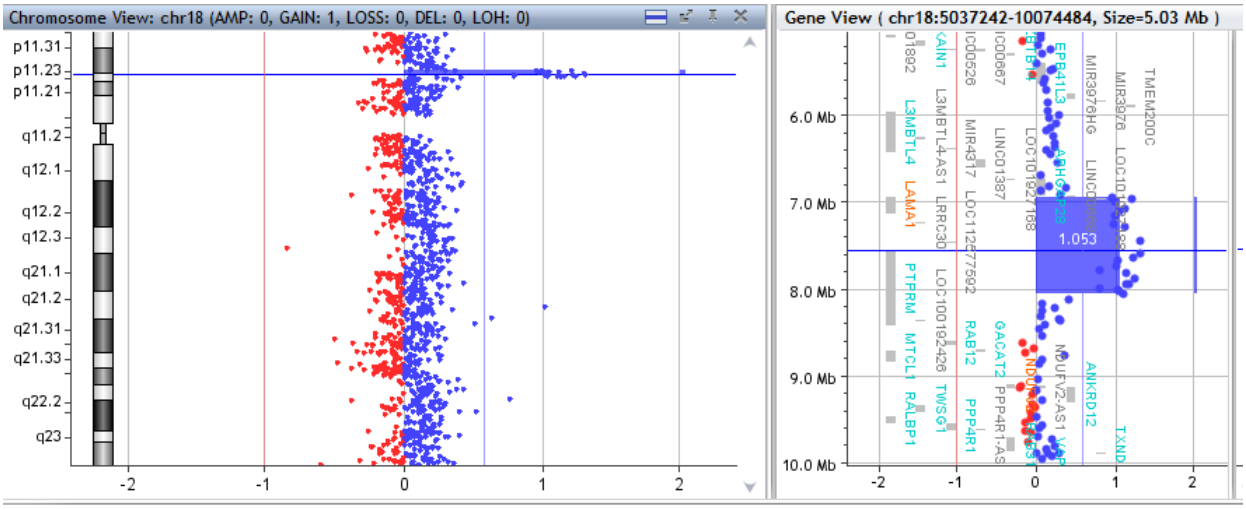


Figure 47 – aCGH result of case VII fetus for chromosome 18 – arr[GRCh37] 18p11.31p11.23(6942021_8055875)x4. A log ratio of 1.053 is indicative of the presence of 4 copies (triplication in the abnormal chromosome).

The identified duplication may interrupt the *LAMA1* gene (partial duplication) and the break point is in exon 63 (reverse strand) so the possibility of the gene functioning in haploinsufficiency should also be considered. A CNV partially overlapping ours were described in the literature, involving the *LAMA1* gene and the child presented

psychomotor delay, hypoplasia of the cerebellum vermis, chorioretinal coloboma, short stature with GH deficiency and deafness[118].

The study of the progenitors was carried out by aCGH, revealing that the father was a carrier of the same triplication found in the fetus (Figure 48 and Figure 49).

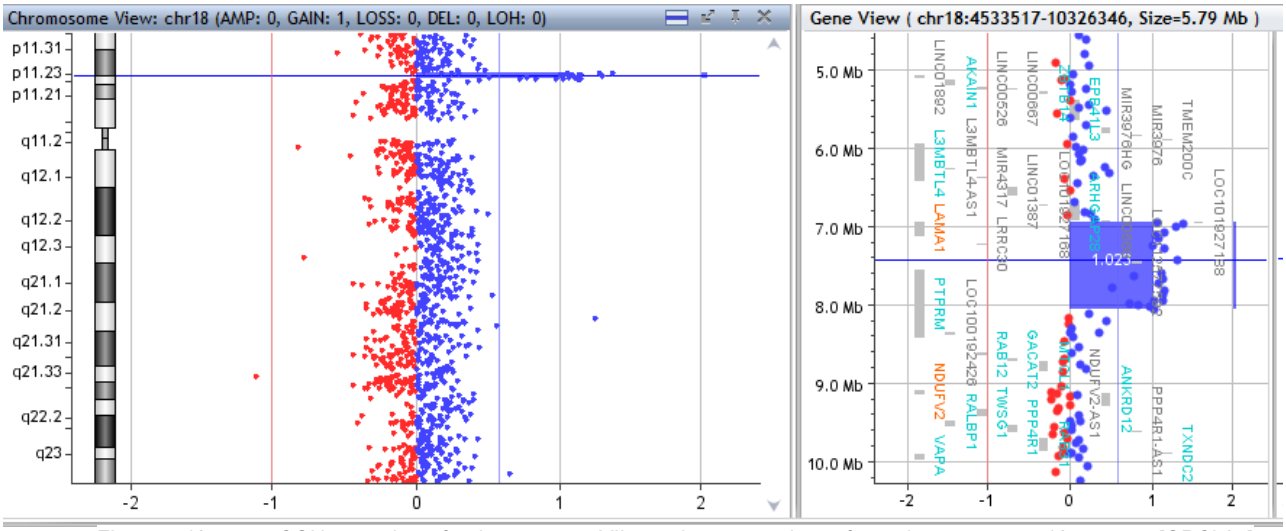


Figure 48 - aCGH result of the case VII male progenitor for chromosome 18 – arr[GRCh37] 18p11.31p11.23(6942021_8055875)x4). A log ratio of 1.023 is indicative of the presence of 4 copies (triplication in the abnormal chromosome).

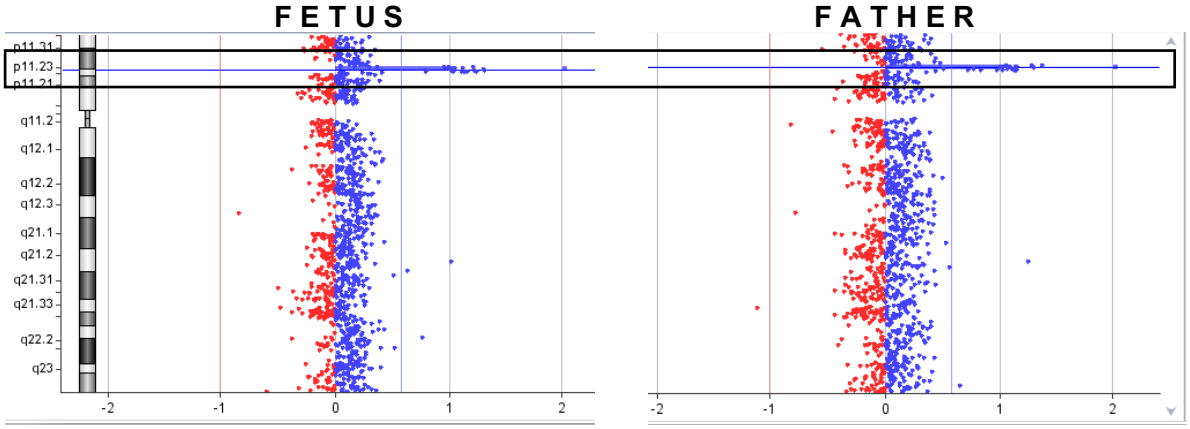


Figure 49 - Comparation of aCGH result for chromosome 18 in the fetus and male progenitor.

The parents were referred for a genetic counseling consultation and decided to proceed with the pregnancy but resulted in a full-term delivery of a stillborn with hydrops fetalis. Prenatal diagnosis should be offered in future pregnancies.

No association was found between hydrops and relevant genes on 18p11. This case was included and will be reported in the Paper – Prenatal diagnosis study using aCGH for genotype-phenotype correlation in 772 fetuses.

V. Paper – Prenatal diagnosis study using aCGH for genotype-phenotype correlation in 772 fetuses

In parallel with the cytogenomic internship, a research project called "Prenatal diagnosis study using aCGH for genotype-phenotype correlation in a cohort of 772 fetuses" was conduct.

Microarray-based comparative genomic hybridization is at the forefront of prenatal genetic testing and can detect large and submicroscopic alterations, that can have clinically significance for fetuses. Through the retrospective analysis of a large sample size of two tertiary hospitals, the prevalence and report of abnormal CNVs, the correlation between pathological CNVs with clinical findings and the prevalence of VUS were evaluated. The reporting of these findings is clinically useful for the classification of new or rare variants making the diagnosis clearer and improving genetic counseling. These data also allow to highlight the importance of aCGH in prenatal diagnosis (Figure 50).

This work was submitted to the Annals of Diagnostic Pathology, a peer-reviewed scientific journal which focuses on morphologic studies using standard diagnostic techniques and clinicopathological correlations, in the various fields of medicine. The article is currently published.

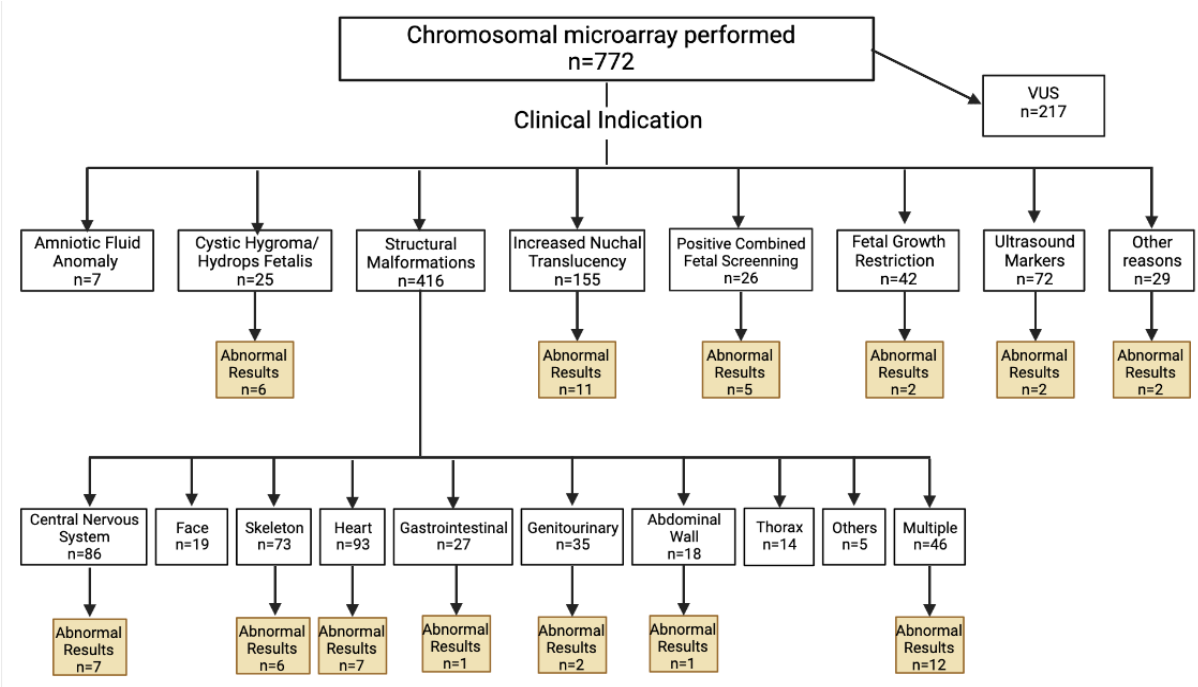


Figure 50 – Workflow of chromosomal microarray results and clinical indications.

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Original Contribution

Prenatal diagnosis study using array comparative genomic hybridization for genotype-phenotype correlation in 772 fetuses

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VUS

ABSTRACT

Objective: The aim was to evaluate the main indications for prenatal diagnosis, the prevalence of abnormal copy number variations (CNVs), correlate them with clinical findings, analyze the prevalence of VUS, report the rare variants found and additionally highlight the clinical importance of microarray-based comparative genomic hybridization (aCGH) in prenatal diagnosis.
Study design: We retrospectively analyzed a cohort of 772 fetuses with indication for genetic study in two tertiary hospitals, in a 9-years-period, using aCGH.
Results: Our results demonstrated 8.3 % (6.4–10.5 %, 95 % CI) detection rate of pathogenic CNVs. Within this group, the main indication was structural malformations (57 %) mainly involving central nervous system, skeletal and cardiac systems. Pathogenic results in cases with multiple malformations were higher than in cases with isolated anatomical system malformations showing statistical significant differences ($p < 0.001$). The second indication where we found more pathogenic CNVs was increased nuchal translucency (5–6.4 mm). In fact, the rate of pathogenic CNVs did not show significant differences between structural and non-structural malformations ($p > 0.001$), highlighting the relevance of genetic study by aCGH also in cases with no structural malformations.
A total of 217 fetuses with CNVs classified as VUS were identified, mainly involving chromosomes X, 1 and 16.
Conclusion: Our findings demonstrate 4.9 % (4.2–5.6 %, 95 % CI) increased in the diagnostic yield using aCGH compared to the use of conventional karyotype alone, confirming that the aCGH can improve the accuracy of prenatal diagnosis. Our survey provides a full genotype-phenotype analysis that can be clinically useful for the classification of variants in the context of prenatal setting, helping to provide a better reproductive genetic counselling.

1. Introduction

In the prenatal period, screening and diagnostic tests are performed regularly to check for abnormalities of the fetus. These should be as less invasive as possible but efficient, economical, reliable and should be available in early stages of pregnancy to allow proper counselling of the couple, manage all the emotional stress and be able to safely carry out any interventions [1,2].

When fetal abnormalities are detected in the screening tests, it is important to carry out a genetic diagnostic study to try to understand if it's just an isolated finding or if can be associated with a genetic disorder [2-4].

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CORRECTED PROOF

Note: Low-resolution images were used to create this PDF. The original images will be used in the final composition.

In the past, prenatal genetic diagnosis has relied mostly on conventional G-banded karyotyping [5], which analyses all the chromosomal complement and detect numerical and structural aberrations larger than 5-10 Mb. However, this methodology involves cell culture, requires more time for reporting [6,7] and do not detect submicroscopic rearrangements (microdeletions and microduplications), that represent a major cause for congenital anomalies and neurodevelopmental delay [8,9].

Comparative genomic hybridization (aCGH) provides genome-wide coverage for detection of chromosomal unbalanced rearrangements and aneuploidies in a short period of time, with much higher resolution than karyotype [7,10,11]. It allows detection of submicroscopic changes also known as copy number variations (CNVs), which represents the variation in the number of copies of an individual's DNA segment when compared to a reference genome [12-14].

In 2013, the American College of Obstetricians and Gynecologists (ACOG) alongside Society for Maternal Fetal Medicine (SMFM) recommended that aCGH should supplement and, in some cases, replace karyotype. According to this, aCGH should be the gold standard test for cytogenetic analysis when ultrasound structural abnormalities are detected [15].

Currently, one great challenge in the interpretation of an aCGH result is the presence of variants of uncertain significance (VUS) once there is not enough knowledge in the literature if these CNVs could be or not causative of disease, and so may be either benign or pathogenic [16].

This study retrospectively evaluates the prevalence of abnormal CNVs detected by aCGH, in 772 prenatal samples in two tertiary hospitals, during a 9-years period. Furthermore, we evaluate the main clinical indications for aCGH test, correlate pathogenic/likely-pathogenic CNVs with clinical findings, analyze the prevalence of VUS as well as highlighting the clinical aCGH relevance in prenatal diagnosis.

2. Material and methods

2.1. Clinical data

We retrospectively analyzed 772 cases with prenatal indication for aCGH (Fig. 1). Cases were selected from the Centro Hospitalar Universitário de São João and Centro Hospitalar de Vila Nova de Gaia/Espinho from March 2013 to June 2022.

Cases were divided according to the indication for the invasive procedure: structural malformations (n = 416, 54 %), amniotic fluids anomaly (AFA) (n = 7, 1 %), cystic hygroma/hydrops (CH/HF) (n = 25, 3 %), increased nuchal translucency (NT) (n = 155, 20 %), positive combined screening (PCS) (n = 26, 3 %), fetal growth restriction (n = 42, 6 %), ultrasound markers (n = 72, 9 %) and other reasons (n = 29, 4 %).

The structural malformations were subdivided considering the anatomical system where the malformations were found: Central Nervous System (CNS), Face, Skeleton, Heart, Gastrointestinal, Genitourinary, Abdominal Wall, Thorax, Others (when the structural malformations didn't fit into the groups previously mentioned) and Multiple (when the fetal malformations involved more than one anatomical system, specifying which ones were affected). Detailed information about all groups and subgroups can be found in Supplemental file 1.

Table 1 describes the number of cases for each group and subgroup including all clinical information, as well as the number of pathogenic CNVs identified. Abnormal results were described in more detail in Table 2. It should be noted that all the clinical information accessed to perform the invasive test may not be a formal indication for carrying out the genetic study, representing different referencing criteria of the clinician.

2.2. aCGH platform

Cases were analyzed by aCGH, using Agilent SurePrint G3 Human Genome 4x180K or 8x60k microarrays platforms (Agilent Technologies, Santa Clara, CA). This offers genome-wide coverage, with microarrays containing approximately 180,000 probes and a 13 kb average or 60,000 and 41 kb of probe spacing, respectively. The results obtained were analyzed using the CytoGenomics software (different versions; Agilent Technologies, Santa Clara, CA). Details of the array designs and software are available from Agilent Technologies (https://www.agilent.com).

The interpretation of the results was made after literature review and using international databases OMIM, DECIPHER, and CLINGEN. CNVs were classified according to the American College of Medical Genetics Standards guidelines as pathogenic (P), likely- pathogenic (LP),

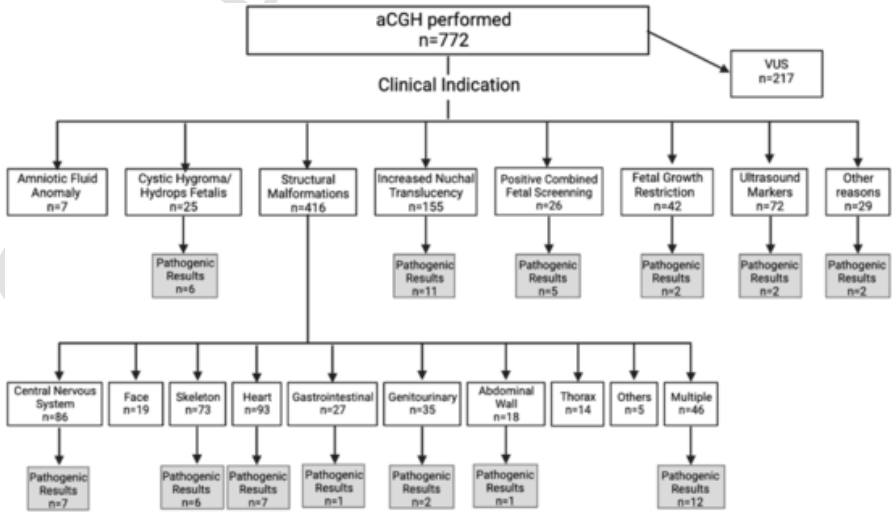


Fig. 1. Workflow of the 772 fetuses analyzed by aCGH, with the respective clinical indications and pathogenic cases found in each group;

Table 1
Ultrasound abnormalities found in the 772 fetuses and respective number of cases with pathogenic CNVs. Some patients presented more than one malformation/abnormality.

	Total	Increased NT	CH/HF	AFA	PCS	Pathogenic
Structural malformations	**416**	**14**	**1**	**4**	**6**	**36**
Central nervous system	**86**	**1**			**2**	**7**
Absent septum pellucidum	2					
Acrania	9					
Agenesis/hypoplasia of corpus callosum	10					
Brain hemorrhage	1					
Choroid plexus cyst	2				1	1
Diastematomyelia	1					
Encephalocele	3					
Encephalocele, agenesis of corpus callosum, ventriculomegaly	1					
Holoprosencephaly	1					
Hypoplastic cerebellum	2					
Macrocephaly	2					1
Microcephaly	5					
Posterior fossa malformation	8	1				2
Ventriculomegaly/hydrocephalus	39				1	3
Face	**19**					
Cleft lip and palate	8					
Cleft lip only	9					
Cleft palate only	2					
Skeleton	**73**	**1**	**1**	**1**	**1**	**6**
Hands and limb malformation	14			1		
Shortened long bones	21					3
Skeletal dysplasia	5	1				
Talipes	26		1		1	1
Vertebral defect	7					2
Heart	**93**	**2**		**1**		**7**
Arch abnormalities	6					2
AVSD	14	1				2
Cardiomegaly	1					
Cardiopathy	48					2
Coarctation of the aorta	3					
Dextrocardia	1					
Dilatation of the right heart chambers	1			1		
HLHS	5					
Isomerism	2					
Situs inversus	1					
Tetralogy of Fallot	7	1				1
Transposition of great arteries	4					
Gastrointestinal	**27**			**2**		**1**
Bowel obstruction	1					
Dilation of intestinal loops	2					
Duodenal atresia	2					
Esophageal atresia	5			2		1
Gallbladder not visualized	2					
Intestinal hyperechogenicity	10					
Intestinal hyperechogenicity and dilation of intestinal loops	5					
Genitourinary	**35**	**1**			**1**	**2**
Bladder exstrophy	2					
Horseshoe kidney	1					
Hydronephrosis	6				1	1
Hydronephrosis and megabladder	1					
hypospadias	4					
kidney malformation	5					
Megabladder	6	1				
Micropenis	1					
Pelvic kidney/agenesis unilateral	3					
Unilateral multicystic kidney	5					**1**
Ureterocele	1					
Abdominal wall	**18**	**4**			**1**	**1**
Abdominal wall herniation	1					
Body stalk anomaly	1	1				
Omphalocele	16	3			1	1
Thorax	**14**					
Bronchogenic cyst	1					
Chaos syndrome	1					
Cystic adenomatoid malformation	3					
Diaphragmatic hernia	8					
Lung abduction	1					
Others	**5**					
Cervical cystic nodules	1					
Cervical lymphagioma	1					
Intra-abdominal calcifications	1					
Sacrococcygeal teratoma	2					
Multiple	**46**	**5**			**1**	**12**
CNS, facial	1					
CNS, genituriary	2					
CNS, heart	6	1				2
CNS, skeleton	5					1
CNS, thorax	1	1				
Geniturinary, abdominal wall	1					
Geniturinary, gastrointestinal	3					1
Geniturinary, heart	5					
Geniturinary, skeleton	3					
Heart, face	1	1				1
Heart, gastrointestinal	2					
Heart, skeleton	5	1			1	2
Heart, thorax	1					
Skeleton, gastrointestinal	1					
Thorax, gastrointestinal	1					1
CNS, face, skeleton	1					1
CNS, geniturinary, heart	1					1
CNS, geniturinary, skeleton	1					
CNS, heart, thorax	1					
Geniturinary, gastrointestinal, skeleton	1					
Geniturinary, heart, skeleton	1	1				1
Geniturinary, skeleton, thorax	1					1
Heart, abdominal, skeleton	1					
Amniotic fluids anomaly (AFA)	**7**			–		
Cystic hygroma/hydrops fetalis (CH/HF)	**25**	**4**	–			**6**
increased nuchal translucency (NT)	**155**	–	**1**		**2**	**11**
Positive combined screening (PCS)	**26**				–	**5**
Fetal growth restriction	**42**					**2**
Ultrasound markers	**72**				**2**	**2**
Absent/hypoplastic nasal bone	7					
Agenesis of ductus venosus	4					
ARSA	15					
Echogenic intracardiac focus	3					
Increased nuchal fold	42				2	2
Single umbilical artery	1					
Other reasons	**29**			**1**		**2**
Discrepancy in fetal weights (twin pregnancy)	2					
Family history	17					2
Macrosomy	1			1		
Maternal anxiety	9					
Total	**772**					**64**

Abbreviations: AVSD - atrioventricular septal defect, HLHS - hypoplastic left heart syndrome, ARSA - aberrant right subclavian artery, CNS - central nervous system, NT - increased nuchal translucency, CH/HF - cystic hygroma/hydrops, AFA - amniotic fluids anomaly, PCS - positive combined screening. numbers a bold represents the total of cases for each group of ultrasound malformations/abormalities

CORRECTED PROOF

Table 2
Pathogenic or likely pathogenic copy number variations found in fetuses with indication for genetic study by aCGH.

Patient	BM	aCGH result	Dimension (kb)	Classification	Interpretation	Clinical Findings	Inheritance	Outcome
1	VC	arr[GRCh37] 1p31.1p22.1(77587977_92568170)x1	14,980	P	1p31 microdeletion syndrome	Isolated heart and increased NT	*De novo*	TOP
2	LA	arr[GRCh37] 1p36.33(759762_1946178)x1, 15q25.2qter(82404371_102465355)x3	1180 + 20,060	P	1p36 loss and 15q25.2qter gain	Isolated CNS and PCS	*De novo*	TOP
3	LA	arr[GRCh37] 1q21.1(145291711_145833054)x1	540	P	TAR syndrome	Multiple: heart and skeleton	Mat	TOP
4	VC	arr[GRCh37] 2p24.3(14726071_16185337)x1	1460	P	2p24.3 loss	Isolated: CNS	Pat	Live born
5	VC	arr[GRCh37] 2pter-p16.3(42444_47938187)x3, 12p13.33(230421_1601369)x1	47,890 + 1370	P	2pterp16.3 gain and 12p13.33 loss	Multiple: Skeleton and heart and increased NT and *CF*/HF	*De novo*	TOP
6	VC	arr[GRCh37] 5q35.2-q35.3(175437847_177539953)x1, 11p15.3-p15.2 (10822054_14362283)x3, 11p14.3p14.1 (24563723_28910541)x3	2100 + 3540 + 4340	P (chr5)	Sotos syndrome	Increased NT	*De novo*	Infant death at 3 months
7	LA	arr[GRCh37] 6q26qter(165203779_170890108)x3, 10q26.1qter(121032524_135404523)x1	5680 + 14,370	P	10q26.1qter loss and 6q26qter gain	Increased NT e soft markers	Mat	TOP
8	LA	arr[GRCh37] 7q11.23(72726578_74139390)x1	1410	P	7q11. 23 duplication syndrome	Isolated: Skeleton	N/A	TOP
9	VC	arr[GRCh37] 8p11.21q21.2(42552632-86393161)x3 [0.5]	43,840	P	8p11.21q21.2 duplication	Increased NT	*De novo*	TOP
10	VC	arr[GRCh37] 8pter-p23.1(221611_8930202)x1, 12pter-p12.1(289648_24861284)x3	8700 + 24,570	P	8pter-p23.1 loss and 12pter-p12.1 gain	Multiple: CNS and heart, increased NT	Pat	TOP
11	VC	arr[GRCh37] 9q34.2q34.3(136407014_141018984)x1	4610	P	Kleefstra syndrome 1	Increased NT and CH/HF	*De novo*	TOP
12	LA	arr(13)x3	115,170	P	Patau syndrome	Increased NT	*De novo*	Miscarriage
13	LA	arr[GRCh37] 16p11.2(29042059_30197341)x3	1160	P	16p11.2 duplication syndrome	PNA e soft markers	Mat	Live born
14	LA	arr[GRCh37] 16p11.2(29673954_30198600)x3	520	P	16p11.2 duplication syndrome	Multiple thorax and gastrointestinal	Pat	Neonatal death
15	LA	arr[GRCh37] 16p12.2(21951379_22407931)x1	460	P	16p12.2 microdeletion	B	Mat	TOP
16	LA	arr[GRCh37] 16p13.11 (15110668_16292235)x3	1180	P	16p13.11 microduplication syndrome	Isolated: Heart	Mat	Live born
17	VC	arr[GRCh37] 16p13.11(15492317_16276115)x3	783	P	16p13.11 microduplication syndrome	Increased NT and CH/HF	Mat	Live born
18	LA	arr[GRCh37] 16p13.11p12.3(15492317_18112776)x3	2620	P	16p13.11 microduplication syndrome	Fetal growth restriction	Mat	Live born
19	LA	arr[GRCh37] 17q12(34817422_36168104)x1	1350	P	17q12 deletion syndrome	Isolated genitourinary	*De novo*	No information
20	VC	arr(18)x3	78,077	P	Edwards Syndrome	Isolated: Skeleton	*De novo*	TOP
21	LA	arr(18)x3	78,077	P	Edwards syndrome	Multiple: skeleton, CNS and face	*De novo*	TOP
22	LA	arr[GRCh37] 18p11.32p11.21(118760_14951330)x1, 18q12.1q12.3(26341636_40131891)x3, 18q12.3q21.2(42248207_54436252)x3, 18q22.3q23(71343626_77992312)x3	14,830 + 13, 790 + 12,180	P	partial trisomy 18	CH/HF	*De novo*	Fetal death
23	LA	arr[GRCh37] 18q22.1q23(61838447_77982126)x1	16,140	P	18q22.1q23 loss	Isolated: Skeleton	*De novo*	TOP
24	VC	arr(20)x3	63,030	P	Trisomy 20	Increased NT and soft markers	*De novo*	TOP
25	LA	arr[GRCh37] 5q13.2(68849594_70807140)x1, 6q26(162727410_163036272)x3, 20p12.1q11.2(13254618_26075841)x3	1900 + 300 + 12, 820	P (chr20)	20p12.1q11.2 gain	PCS	*De novo* for supernumerary chromosome 20	TOP
26	LA	arr(21)x3	48,130	P	Down syndrome	Multiple: CNS and skeleton	*De novo*	TOP
27	VC	arr[GRCh37] 22q11.2(18641409_20311763)x3	1670	P	22q11.2 microduplication syndrome	Increased NT	*De novo*	TOP
28	LA	arr[GRCh37] 22q11.21(18641409_21440514)x3	2800	P	22q11.2 microduplication syndrome	PCS	Mat	Live born

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CORRECTED PROOF

Table 2 (*continued*)

Patient	BM	aCGH result	Dimension (kb)	Classification	Interpretation	Clinical Findings	Inheritance	Outcome
29	VC	arr[GRCh37] 22q11.21(18635797_21440514)x3	2800	P	22q11.2 microduplication syndrome	Increased NT	Mat	Live born
30	LA	arr[GRCh37] 22q11.21(18919942_21440514)x1	2520	P	22q11 deletion syndrome	Isolated: Heart	*De novo*	Ongoing pregnancy
31	LA	arr[GRCh37] 22q11.21(18919942_21926320)x3	3010	P	22q11.2 microduplication syndrome	Increased NT	Mat	Live born
32	LA	arr[GRCh37] 22q11.21(19084543_20311763)x1	1230	P	22q11 deletion syndrome	Isolated heart and CH/HF	*De novo*	Miscarriage
33	LA	arr[GRCh37] 22q11.22q11.23 (22998284_24988455)x3	1990	P	22q11.2 microduplication syndrome	Fetal growth restriction	Mat	Live born
34	LA	arr[GRCh37] 22q12.1(28924876_29130415)x1	210	P	22q12.1 gain	Isolated: Skeleton	Mat	No information
35	VC	arr[GRCh37] 22q13.2q13.33 (42029866_50255802)x1	8230	P	Phelan-McDermid syndrome (without the gene SHANK3)	Isolated: genitourinary and PCS	*De novo*	TOP
36	LA	arr[GRCh37] 22q13.2q13.33 (42556272_51178264)x1	8620	P	Phelan-McDermid syndrome (without the gene SHANK3)	Isolated CNS	*De novo*	Live born
37	VC	arr(X)x3	155,270	P	Trisomy X	Multiple genitourinary, heart and skeleton and increased NT	*De novo*	TOP
38	LA	arr(X)x3	155,270	P	Trisomy X	Isolated: skeleton and PCS	*De novo*	Live born
39	LA	arr(X)x2,(Y)x1	155,020	P	Klinefelter syndrome	Isolated gastrointestinal	*De novo*	Live born
40	LA	arr(X)x2,(Y)x1	155,270	P	Klinefelter syndrome	PCS	*De novo*	Live born
41	VC	arr(X)x1	155,270	P	Turner syndrome	Increased NT	*De novo*	Miscarriage
42	VC	arr(X)x1	155,270	P	Turner syndrome	CH/FH and increased NT	*De novo*	Miscarriage
43	VC	45,X/46,X,+mar. arr[GRCh37] Xp11.3q21.1(42892771_78873820)x2	35,980	P	Xp11.3q21.1 gain	CH/HF	*De novo*	TOP
44	LA	arr[GRCh37] Xp11.21q21.1(56239496_76052355)x2	19,810	P	Xp11.21q21.1 gain	PCS	*De novo*	Live born
45	VC	arr[GRCh37] Xp11.3(44777230_44970749)x0	190	P	Xp11.3 loss	Increased NT	Mat	Miscarriage at 16 weeks
46	LA	arr[GRCh37] Xp22.31(6552712_8097511)x0	1540	P	Xq22.3 loss	Isolated: Heart	N/A	Live born
47	LA	arr[GRCh37] Xp22.31(6552712_8097511)x1	1540	P	Xq22.3 loss	Multiple: Heart and CNS	Mat	TOP
48	LA	arr[GRCh37] Xp22.31(7218800_7744191)x0~1	530	P	Xq22.3 loss	Isolated abdominal wall and increased NT	Not maternal but without father information	Miscarriage at 15 weeks
49	LA	arr[GRCh37] Xp22.33 or Yp11.32 (505608_1000682 or 455680_950682)x1	495	P	Xq22.3 loss	Isolated: Skeleton	N/A	Live born
50	VC	arr[GRCh37] Xp22.33p11.23(61091_49706442)x1	49,650	P	Xq22.3 loss	Increased NT, CH/HFe soft markers	N/A	miscarriage
51	LA	arr[GRCh37] Xq28(152684501_153822717)x2	1140	P	Xq28 duplication syndrome	Isolated: Heart	Mat	TOP
52	LA	arr[GRCh37] Xq28(154120738_154560375)x1	440	P	Xq28 loss	Isolated CNS	Mat	Ongoing pregnancy
53	LA	arr[GRCh37] 15q13.2q13.3(31014508_32914140)x3, 16q22.1(68494255_69173835)x3	1890 + 680	P(chr15) LP (chr16)	15q13.3 microduplication syndrome	Isolated: CNS	Mat (chr16) Pat (chr15)	TOP
54	LA	arr[GRCh37] Xq28(152101362_155232907)x3, 10p15.3(299304_470231)x3	3130 + 170	P (chrX) LP (chr10)	Xq28 duplication syndrome	Multiple: CNS, genitourinary and heart	N/A	Live born
55	LA	arr[GRCh37] 4q34.1q34.3(175090451_178044012)x1	2950	LP	4q34.1q34.3 loss	PNA	Pat	Live born
56	LA	arr[GRCh37] 5p15.2(11689576_11843365)x1	150	LP	Partial overlap with cri-du-chat syndrome	Multiple: genitourinary and gastrointestinal	N/A	Live born
57	LA	arr[GRCh37] 6q22.31(118757440_119149967)x3	390	LP	6q22.31 gain	Multiple: genitourinary, skeleton and thorax	Not maternal but without father information	TOP
58	LA	arr[GRCh37] 14q23.1(58872942_59109596)x3	240	LP	14q23.1 gain	Isolated: Heart	*De novo*	Live born

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CORRECTED PROOF

Table 2 (*continued*)

Patient	BM	aCGH result	Dimension (kb)	Classification	Interpretation	Clinical Findings	Inheritance	Outcome
59	LA	arr[GRCh37] 15q11.2(22509195_23179948)x3	670	LP	15q11 duplication syndrome	Isolated: CNS	Pat	Live born
60	VC	arr[GRCh37] 17p13.1(7306518_7460864)x1	150	LP	17p13.1 loss	PCS	Pat	Live born
61	VC	arr[GRCh37] 18p11.31p11.23 (6942021_8055875)x4	1110	LP	18p11.31p11.23 gain	B	Pat	Term delivery of a stillborn
62	LA	arr[GRCh37] 20q13.33(62494619_62704695)x1	210	LP	20q13.33 loss	Isolated: CNS	N/A	TOP
63	VC	arr[GRCh37] 22q11.21(20754422_21440514)x1	690	LP	partial overlap with 22q11.2 microdeletion syndrome	Multiple: Heart and face and increased NT	*De novo*	TOP
64	VC	arr[GRCh37] Xp11.22(50339590_51307620)x2	970	LP	Xp11.22 gain	Increased NT e soft markers	Mat	Live born

Abbreviations: BM - biological material, mat - inherited from mother, pat - inherited from father, TOP - termination of pregnancy, N/A - not available.

benign (B), likely-benign (LB) or VUS [17]. The genetic study of the parents was carried out, whenever possible, following a pathogenic or likely-pathogenic.

2.3. Statistical analysis

The statistical analysis in this study was performed using IBM SPSS Statistics 27.0. Cases with multiple structural malformations and pathogenic CNVs were compared with the ones with isolated structural using Fisher Exact Test. The pathogenic CNVs from fetuses with structural malformations and non-structural abnormalities were compared using χ^2 test. A value of $p < 0.001$ was considered statistically significant.

2.4. Ethics declaration

This study was performed in accordance with the Centro Hospitalar Universitário de São João (CHUSJ) Ethics Committee with formal approval (authorization request CE-34-2022). All procedures and experiments in this study follow the guidelines and regulations.

3. Results

The maternal age of the participants ranged between 16 and 47 years at time of testing, with an average age of 32.4 ± 5,7 years, wherein 36.4 % (n = 281) of mothers were advanced maternal age (≥35 years). The samples included 73 % (n = 563) amniotic fluid and 27 % (n = 209) chorionic villi.

In 708 cases (91.7 %), only benign (common polymorphisms) and/or likely benign CNVs were detected, therefore a normal result was reported and, because it is not considered clinically relevant, were not communicated to the parents. In the remaining 64 cases (8.3 %), pathogenic or likely pathogenic CNVs were detected so an abnormal result was reported and the parents were referred for genetic counselling. Among the cases with pathogenic results, 57 % were due to structural malformations detected on ultrasound (36/64), 17 % increased NT (11/64), 9 % CH/HF (6/64), 8 % PCS (5/64), 3 % FGR (2/64), 3 % CM (2/64) and 3 % due to other reasons (2/64).

Regarding cases with structural malformations, the detection rates in each subgroup were 8 % in CNS (7/86), 8 % in skeleton (6/73), 8 % in heart (7/93), 4 % in gastrointestinal (1/27), 6 % in genitourinary (2/35), 6 % in abdominal wall (1/18), 26 % in fetuses with multiple malformations (12/46) and were not found in the thorax (0/14), face (0/19) and others (0/5). In the other cases with non-structural abnormalities, detection rates were 24 % in the CH/HF group (6/25), 7 % in TN (11/155), 19 % in PCS (5/26), 5 % in FGR (2/42), 3 % CM (2/72) and 7 % in other reasons (2/29).

Of the 64 alterations detected by aCGH, 38 (53 %) were submicroscopic (<5 MB), this means that more than half would not be detected by conventional karyotype.

3.1. Pathogenic and likely pathogenic CNVs

From the 64 cases with abnormal result, the majority were associated with known genetic disorders or already reported in the literature as being pathogenic (n = 54). All this information is described in Table 2.

Regarding likely pathogenic CNVs (n = 10), once these are associated with a more complex prenatal counselling, a more detail description beyond the information included on Table 2 is thereafter provided.

In Patient 55, 4q34.1q34.3 loss was detected, and this interval contains the *VEGFC* gene that encodes an enzyme that promotes angiogenesis and endothelial cell growth, and therefore may affect the permeability of blood vessels. Dysfunction of this gene appears associated with lymphedema [18]. In our case, an increased nuchal fold was detected. The 19-month-old child appears to have an age-appropriate development.

Patient 56 showed a small CNV partial overlapping the Cri-du-Chat syndrome region, encompassing the *CTNND2* gene, which has been implicated in the intellectual disability associated with this syndrome [19]. The ultrasound malformations identified included left renal agenesis and small loop dilatation which may be associated with the syndrome. Currently the child has several skeletal malformations compatible with Cri-du-Chat syndrome phenotype.

A 6q22.31 gain was detected in case 57. This alteration encompasses the *PLN* gene, which performs important functions in terms of cardiac contractility and relaxation and, as such, mutations of the *PLN* gene (Phospholamba) in homozygosity or heterozygosity/deletions have been associated with hypertrophic cardiomyopathy manifestation in adulthood (with the possibility of incomplete penetrance) [20]. Our case present right diaphragmatic hernia, hydronephrosis, clubfoot, thin limbs and parents chose to terminate the pregnancy. Autopsy revealed arachnodactyly, bilateral non-communicating diaphragm defect, with severe pulmonary hypoplasia.

Patient 58 showed a 14q23.1 gain, according to OMIM, this duplication could be associated with the syndrome Townes-Brocks syndrome type 2, since the duplication could interrupt the function of the *DACT1* gene. This syndrome is characterized by the triad of imperforate anus, dysplastic ears often associated with sensorineural and/or conductive hearing impairment, and thumb malformations. Kidneys and heart malformations could also be present [21]. In our case, currently with 10 months, only aortic arch malformation was detected.

A 15q11 duplication syndrome was found in case 59 presenting a fetus with Chiari malformation type 2 and a posterior fossa malformation. Literature is not consensual in the classification of this CNV, and moreover it is also associated with low penetrance. Chiari malformation type 2 and a posterior fossa malformation are not previously reported associated with this duplication, being the developmental delay and behavioral problems the most common phenotype that cannot be evaluated in a prenatal context [22]. The parents decided to proceed with the pregnancy and an occipital encephalomeningocele was visual-

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ized in a late ultrasound. The newborn was submitted to surgical correction and referred to a development consultation.

In case 60, with 17p13.1 loss, no ultrasound alterations were found, the indication for performing the invasive test was a PCS. According to the literature, the *POLR2A* gene, included in genomic interval, may be associated with neurodevelopmental disorder with hypotonia, variable intellectual disability and behavioral anomalies [23]. The parents chose to maintain the pregnancy. The newborn at 5-month-old appears to be healthy.

In case 61, an 18p11.31p11.23 gain (triplication on one of the chromosome 18) was detected, encompassing the *LAMA1* gene which is important for cell membrane formation and embryonic development. A CNV partially overlapping ours were described in the literature, with a child showing psychomotor delay, hypoplasia of the cerebellar vermis, chorioretinal coloboma, short stature with GH deficiency [24]. The aCGH study was performed due to a previous peripartum death with hydrops. The CNV was inherited from the healthy father and pregnancy resulted in a full-term delivery of a stillborn with hydrops fetalis.

Case 62 showed 20q13.33 loss, this CNV encompasses the gene *DNAJC5* which, according to the literature, is associated with neuronal ceroid lipofuscinosis, clinically characterized by motor and cognitive progressive deterioration, eyesight problems and premature death [25]. In our case, posterior fossa malformation was present, which could be related to the reported pathology. Termination of pregnancy (TOP) was performed and the autopsy revealed hindbrain developmental anomalies indicating a Dandy-Walker Malformation.

The CNV found in case 63 partial overlaps with 22q11.2 microdeletion syndrome, but was much smaller, not including the *TBX1* gene. In DECIPHER (https://www.deciphergenomics.org), several similar CNVs similar were described and, in general, patients presented neurodevelopmental disorders and several congenital malformations. In our case a cystic formation in the nose and mouth (6x5mm), tricuspid regurgitation and increased TN was detected. After TOP, autopsy confirmed these malformations and additionally micrognathia, low-implanted external auricular pavilions and camptodactyly of the fingers, bilaterally.

Patient 64, presents an Xp11.22 gain, which overlaps the region of the Xp22.22 microduplication syndrome [Xp11.22p11.23 recurrent region (includes *SHROOM4*) Clingen], however it is smaller (970 kb) and only includes *SHROOM4*, that is in fact one of the main genes that is considered responsible for the phenotype. The recurrent duplication comprises about 4,5 Mb including several OMIM genes additional to *SHROOM4*. The CliGen triplosensitivity (TS) score is 3 (sufficient evidence for triplosensivity). Although, it is important to stress that our genomic interval is smaller and is also present in the healthy mother.

Mutations in *SHROOM4* gene were also associated with Stocco dos Santos intellectual disability syndrome [26] and currently the ClinGen ID/Autism Gene Curation Expert is Panel disputing this gene-disease relationship (data from ClinGen). Since the expected phenotype is associated with neurodevelopment disability, it is not possible to correlate with prenatal findings. The indication for aCGH test in our case increased NT and venous ductus with inverted A-wave. The parents chose to keep the pregnancy and at 4-month-old the newborn appears to be healthy.

3.2. VUS detected in the cohort

We found 217 cases with VUS (28.1 % 217/772), 13 of which associated with a pathogenic CNV and the remaining 204 in cases with a normal aCGH result, Supplemental file 2. Since some cases had more than one CNV with VUS classification, the total number of VUS CNVs was 296. We found VUS CNVs for all chromosomes, with the X chromosome being the most frequent (n = 44), followed by chromosome 1 (n = 27), chromosome 16 (n = 26) and the least involved ones were chromosomes 18 (n = 2), 12 (n = 3), and 21 (n = 3).

The most frequent CNV involved was Xp22.33 (n = 15 cases) followed by the 16p11.2 (n = 12) and 1q25.2 region (n = 10). No correlation was observed between clinical findings and these VUS.

3.3. Inheritance and pregnancy outcomes

The study of the parents was carried by aCGH and/or Karyotype whenever was possible in the presence of pathogenic or likely pathogenic CNVs. In 9 cases complete information was not available, either because the parents chose not to undergo genetic testing or because there was only one of them available. In 30 cases the CNVs were *de novo*, in one case it was inherited by both parents (two different CNVs), and in 17 and 7 cases the inheritance was from the mother and father, respectively. Of these cases, 23 chose to continue the pregnancy, two are still ongoing, 26 decided to perform TOP, 8 resulted in spontaneous abortions, one resulted in a stillbirth, one child died after three months, one resulted in neonatal death and two abandoned the consultation.

3.4. Increased nuchal translucency and its association with other indications

The most common indication for carrying out the genetic study was increased NT (n = 173), being isolated in 155 cases and in 18 cases in association with another abnormality. Among these cases, 152 had a normal result (87.9 %) and 21 had a pathogenic or likely pathogenic CNV (12.1 %). In Table 3, the cases are distributed by different NT cut-off points (mm) in the following intervals: 3 to 3.4, 3.5 to 4.9, 5 to 6.4 and ≥6.5. In some cases, it was not possible to obtain information about the value of increased NT. The cut-off point that contains the highest percentage of pathogenic cases is between 5 and 6.4, with 31.2 %, followed by the interval ≥6,5, 3–3.4 and 3.5–4.9, with 26.7 %, 10.3 %, 7.4 %, respectively.

4. Discussion

Using aCGH technique, our findings indicate 8.3 % (6.4–10.5 %, 95 % CI) detection rate of pathogenic or likely pathogenic CNVs in fetuses with indication for invasive test. These findings are consistent with literature, with diagnostic rates of 6.5 %, 7.8 % and 9.6 %, being the differences reported related with the size of cohort and the inclusion criteria [16,27,28].

In our study, pathogenic results were mainly associated with structural malformations (57 %). Within this group, those with multiple structural malformations showed the highest yield of abnormal CNVs (25 %).

The detection rate for fetuses with multiple structural malformations was statistically higher than those with structural malformations in an isolated anatomical system ($p < 0.001$) and this result seems to be consensual in the literature [29,30]. This highlights the importance of carrying out a genetic study in cases with multiple structural malformations, especially when they involve cardiac, CNS and skeleton systems.

Table 3
Distribution of cases (normal or pathogenic) according to the nuchal translucency size.

	TN size (mm)				No information	Total
	3.0–3.4	3.5–4.9	5.0–6.4	≥6.5		
Normal result	52 (89.7 %)	63 (92.6 %)	11 (68.8)	11 (73.3 %)	15	152
Pathogenic result	6 (10.3 %)	5 (7.4 %)	5 (31.2 %)	4 (26.7 %)	1	21
Total	58	68	16	15	16	173

Abbreviations: NT - nuchal translucency.

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The isolated systems with the highest yields of abnormal CNVs are similar to those previously reported, with the CNS, skeletal and cardiac systems being the most associated with pathogenic/likely pathogenic CNVs. In Society of Obstetricians and Gynaecologists of Canada (SOGC) Genetics Committee and the Canadian College of Medical Geneticists (CCMG) guidelines and other studies, gastrointestinal malformations seem to have similar percentages to these groups, but in our study this association is much lower (4 %) [30-32]. This can be explained by the smaller number of cases with gastrointestinal malformations in our cohort or due to the genetic variability of the Portuguese population. Specifically, the individual structural malformations most associated with pathogenic CNVs were shortened long bones, ventriculomegaly/hydrocephalus, vertebral defect, posterior fossa malformation, arch abnormalities AVSD and unspecified cardiopathy.

Regarding cases with pathogenic CNVs, the most frequent alterations were Xq22.3 loss (n = 5), 22q11.2 microduplication (n = 5) and 16p13.11 microduplication (n = 3) syndromes. The associated phenotypes of the pathogenic CNVs are well established in the literature, and correlation between our clinical findings and the aCGH result was observed. In cases where both pathogenic and likely-pathogenic CNVs were detected, the genotype-phenotype correlations were more difficult to establish and therefore the interpretation was mainly focused on the pathogenic CNV.

In respect of cases with only likely-pathogenic CNVs, the correlation with the ultrasound findings is more complex, since the relationship between CNV and phenotype is not yet well established, making genetic counselling more difficult. Thus, there is a need to report these rarer alterations in order to find similar cases and, in the future, establish better genotype-phenotype correlations, making the prenatal counselling easier.

Increased NT, above 3 mm, is a risk indicator for chromosomal aneuploidies [31,33] and was the most common clinical indication to perform genetic study and the most frequently associated with pathogenic CNVs. Of the 173 cases, 12 % showed a pathogenic/likely pathogenic CNV. Our findings showed a higher association with abnormal CNVs in the range of values between 5.0 and 6.4 mm (31.2 %) and ≥6.5 mm (26.7 %), following the tendency of the higher risk being associated with higher nuchal translucency thickness [34]. Our results are also in agreement with the EACH study, which concluded that the rate of pathogenic CNVs was higher in fetuses with a NT >5 mm than in those in the 3.5–4.9 mm range [35].

Besides increased NT group, other abnormalities like CH/HF (24 %), PCS (19 %), FGR (4.8 %), and CM (2.8 %), also present a significant association to abnormal CNV.

In our study, considering these last 5 groups and AFA group as non-structural abnormalities, the detection rate of abnormal CNVs in fetuses with structural malformations detected on ultrasound (8.7 %, 36/416) was not statistically different (p > 0.001) from fetuses with non-structural abnormalities (6.4 %, 26/356). This underlines the importance of performing genetic studies not only in cases with structural malformations. However, more studies with larger cohorts to evaluate the prevalence of abnormal CNVs in cases with ultrasound markers or minor findings need to be done.

As mentioned before, one of the main adversities in the interpretation of an aCGH result is the classification of VUS. With the application of high resolution aCGH technique or other molecular techniques as whole exome sequencing in prenatal diagnosis, the number of VUS detected will increase, so it is important to report these CNVs in order to achieve common VUS that could be classified as benign or likely benign CNVS and reassure parents.

In our study VUS (28.1 % 217/772) were found in 217 cases, 13 of which associated with a pathogenic CNV. We found a total of 296 VUS CNVs covering all chromosomes, with the X chromosome being the most frequent (n = 44), followed by chromosome 1 (n = 27), chromosome 16 (n = 26) and the least involved ones were chromosomes 18 (n = 2), 12 (n = 3), and 21 (n = 3).

The most frequent CNV involved was Xp22.33 (n = 15 cases) followed by the 16p11.2 (n = 12) and 1q25.2 region (n = 10). No correlation was observed between clinical findings and these VUS.

Using the aCGH technique, our findings demonstrate 4.9 % (4.2–5.6 %, 95 % CI) increased in the diagnostic yield comparing to conventional karyotype, once only 26/64 cases would be detected in the karyotype (40 %). However, this value would be even lower since some cases present more than one submicroscopic alteration.

Our results are slightly superior to those reported in the literature with larger cohorts, such as the EACH study that identified an increase in diagnostic yield of 3.5 % and in Hillman et al. systematic review that reported a 3.6 % increase [32,35]. These slight changes in the results can be explained by the differences in the array resolution and due to the reporting practices of each individual laboratory.

5. Conclusion

In this study we retrospectively analyzed 772 cases with indication for genetic study and demonstrate that aCGH technique offers a large increase in diagnostic yield compared to the conventional karyotype alone.

Cases with structural malformations were the most frequent indication for genetic study. As expected, pathogenic CNVs were found to be associated more frequently to multiple malformations than those only affecting one anatomical system. Although, taking into account that we did not find statistically significant differences between the number of pathogenic CNVs in structural and non-structural malformations cases, we suggest that aCGH test should be performed for both indications. In this work we also addressed all the VUS CNVs found in our cohort. Reporting VUS could allow to better recognize in the future which these variants are benign or pathogenic, allowing a better genotype-phenotype correlation and improving genetic prenatal counselling.

In conclusion, our survey provides a full genotype-phenotype analysis that can be clinically useful for the classification of variants in the context of prenatal setting, helping to provide a better reproductive genetic counselling.

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CRediT authorship contribution statement

Beatriz C. Costa contributed to the analysis, planning, interpretation of the data and wrote the manuscript. Ana Grangeia contributed to the analysis, interpretation of the ultrasonographic malformations, provided access to the clinical information at CHUSJ and made critical review of the manuscript. Diane Vaz contributed to the interpretation of the data. Joana Galvão and Mónica Melo provided access to the clinical information at CHVNG and made critical review of the clinical data. Teresa Carraca contributed to the interpretation of fetuses' malformations and made critical review of the clinical data. Carla Ramalho contributed to the interpretation of the clinical findings and critical review of the manuscript. Sofia Dória conceived the idea, supervised the work, analysis of aCGH database and made the critical review of the manuscript. All authors contributed to editing of the final manuscript. Carla Ramalho and Sofia Dória approve the final revisions.

Declaration of competing interest

The authors have no conflict of interests to declare.

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VI. Concluding Remarks and Perspectives

During my curricular internship at GL/FMUP I had the opportunity to integrate the routine of a clinical cytogenetics laboratory.

I participated in all stages of a diagnosis, from the pre-analytical, analytical and post-analytical phases. I acquired skills in work planning, management of reagents, materials and equipment preparation in order to optimize the analytical phase. I independently performed conventional cytogenetic and molecular techniques, knowing the advantages and disadvantages of each technique, learning when and which to use, taking into account the clinical indication.

The work carried out included the preparation, establishment of flask and *in situ* cultures of peripheral blood, amniotic fluid and chorionic villi, harvesting and spreading. I performed the GTL staining, NOR staining, C-banding and DAPI staining in order to identify chromosomal alterations. Analysed these abnormalities under a microscope, assembled the karyotypes and write reports using the nomenclature described by the ISCN and according to laboratory standards. Performed the FISH technique on cultured and uncultured lymphocytes, amniocytes and chorionic villi and on sperm cells. In addition, performed the aCGH and TUNEL technique.

Together with the practice of all these techniques, this internship allowed me to distinguish the differences between pre and postnatal diagnosis and the importance of genetic counselling. Furthermore, allowed me to consolidate and increase my knowledge in the cytogenetic field, stimulate critical thinking, autonomy and acquire a rhythm of work in a clinical environment where responsibility is a crucial factor.

As such, I conclude that the internship objectives were achieved. The work developed allowed me to foster my interest in genetics and diagnosis and prepare myself for a professional career in the clinical cytogenetic area and to work in a hospital environment.

The goals proposed in the research "Prenatal diagnosis study using aCGH for genotype-phenotype correlation in a cohort of 772 fetuses" were also achieved.

Abnormal results were found in 64 cases, indicating a 8.3% (6.4-10.5%, 95% CI) detection rate of pathogenic or likely-pathogenic CNVs in fetuses with indication for invasive test. Among these, 57% were due to structural malformations (36/64), followed by 17% increased NT (11/64), 9% CH/HF (6/64), 8% PCS (5/64), 3% FGR (2/64), 3% CM (2/64) and 3% due to other reasons (2/64).

Within the group of structural malformations, the groups with the highest percentage of abnormal cases were the central nervous system, skeleton and heart, which is in agreement with the literature. Pathogenic results in cases with multiple malformations was statistically higher than in cases with isolated anatomical system malformations ($p<0.001$).

Within the cases with non-structural abnormalities, the groups with the highest percentage of abnormal cases were NT, CH/HF, and PCS. Because the NT group was the most common indication and the second more frequent indication for the diagnostic study, it was divided into different cut-off points (mm), and the range of values that contains the highest percentage of pathogenic cases is between 5-6.4, with 31.2% of the cases. The detection rate of abnormal CNVs in fetuses with structural malformations (8.7%, 36/416) was not statistically different ($p>0.001$) from fetuses with non-structural abnormalities (6.4%, 26/356). This finding underlines the importance of performing genetic studies in all cases.

Among all the abnormal CNVs found about 53% (38/64) were submicroscopic meaning that more than half would not be detected by conventional karyotype, demonstrating a 4.9% (4.2-5.6%, 95% CI) increased in the diagnostic yield comparing to karyotype alone, what highlights the importance of the role of aCGH in prenatal diagnosis.

The pathogenic alterations found are mostly known, the most frequent alterations were AMME complex (n=5), 22q11.2 microduplication (n=5) and 16p13.11 microduplication (n=3) syndromes. However, 10 cases with rare alterations were reported, correlating with phenotype and clinical follow up when possible, namely: a 4q34.1q34.3 loss, a CNV partial overlapping the Cri-du-Chat syndrome region, a 6q22.31 gain, a 14q23.1 gain, a 15q11 microduplication syndrome, a 17p13.1 loss, a 18p11.31p11.23 gain, 20q13.33 loss, a CNV partial overlapping the 22q11.2 microdeletion syndrome and a Xp11.22 gain.

The interpretations of VUS is one of the main adversities in a aCGH result. A total of 217 fetuses with CNVs classified as VUS were identified in our cohort and reported, the majority in cases reported as normal, with the most involved chromosomes being X, 1 and 16.

The strengths of our study include the detailed assessment of all indications for carrying out the invasive study, the detailed description of cases with abnormal CNVs along with proper interpretation. However, the cases were collected from two hospitals

in the North of Portugal, which may limit generalizability. In the future, this study could be performed in partnership with more hospitals, in order to increase the sample size.

Since the identification of VUS is extremely important for the detection of new pathologies, in the future it is important to assess whether the most prevalent VUS are associated with phenotypes and explore this field. In addition, some follow ups were not possible because the cases were still recent, so a reassessment of the reported cases should be carried. It would also be enriching to carry out this study with other high-resolution techniques, e.g. NGS, and compare diagnostic yields.

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VIII. Appendix

1 Paper Supplemental file 1: Detailed clinical data of each group and subgroup of structural malformations and abnormalities found in fetuses.

The structural malformations were subdivided considering the anatomical system where the malformations were found: Central Nervous System (CNS), Face, Skeleton, Heart, Gastrointestinal tract, Genitourinary, Abdominal Wall, Thorax, Others and Multiple.

The CNS abnormalities found were absent septum pellucidum, acrania, agenesis or hypoplasia of corpus callosum, brain hemorrhage, choroid plexus cyst, diastematomyelia, encephalocele, holoprosencephaly, hypoplastic cerebellum, macrocephaly, microcephaly, posterior fossa malformation (including Dandy-Walker malformation and Chiari malformation type 2), ventriculomegaly or hydrocephalus and simultaneously encephalocele, agenesis of corpus callosum and ventriculomegaly. Respectively to facial abnormalities, cases with cleft lip, cleft palate and both simultaneously were identified.

The skeleton abnormalities found were hand and limb malformation (which includes syndactyly, clinodactyly, permanently clenched hands, bifid thumb and other malformations of the hands, foot and limb), Shortened long bones, skeletal dysplasia, Talipes (unilateral or bilateral clubfoot and clubhands) and vertebral defects.

Corresponding to heart abnormalities, it was found arch abnormalities (including aortic arch hypoplasia and inverted aortic arch), atrioventricular septal defect (AVSD), cardiomegaly, cardiopathy not specified, coarctation of the aorta, dextrocardia, dilatation of the right heart chamber, hypoplastic left heart syndrome (HLHS), isomerism, inversus situation, tetralogy of Fallot and transposition of great arteries.

The gastrointestinal tract abnormalities found were bowel obstruction, dilation of intestinal loops, duodenal atresia, esophageal atresia, galbladder not visualized, intestinal hyperechogenicity and simultaneously intestinal hyperechogenicity and dilation of intestinal loops.

Genitourinary abnormalities included exstrophy bladder, horseshoe kidney, megabladder, hydronephrosis, both hydronephrosis and megabladder, hypospadias,

kidney malformation, micropenis, pelvic kidney/unilateral agenesis, unilateral multicystic kidney, ureterocele. Successively, the alterations in the abdominal wall found were abdominal wall herniation, body stalk anomaly and omphalocele.

The thorax abnormalities were bronchogenic cyst, chaos syndrome, cystic adenomatoid malformation, diaphragmatic hernia and lung abduction. Other structural malformations were also found that did not fit into the groups mentioned above, which were called "others", namely cervical cystic nodules, cervical lymphagioma, intra-abdominal calcifications and sacrococcygeal teratoma.

When the fetal malformations involved more than one anatomical system it was called "multiple", specifying which ones were affected.

Regarding abnormalities that are not considered structural malformations but that are also warning signs and indicative for performing an invasive test, we have amniotic fluids anomaly (AFA), which includes hydramnios and oligohydramnios, cystic hygroma/hydrops fetalis (CH/HF) which also includes pleural effusion and fetal ascites, nuchal translucency (when this value is equal or greater than 3mm), positive combined fetal screening, fetal growth restriction, ultrasound markers (containing absent/hypoplastic nasal bone, agenesis of ductus venosus, aberrant right subclavian artery (arsa), echogenic intracardiac focus, increased nuchal fold or neck swelling and single umbilical artery.

Lastly, some cases presented other reasons that didn’t fit into the established groups, specifically discrepancy in fetal weights (in a twin pregnancy), family history of genetic disorders or bad obstetrical history, macrosomy and maternal anxiety.

2 Paper Supplemental file 2: Genomic region of the variants of uncertain significance (VUS) detected in each patient of the cohort.

Patient	aCGH result	VUS geomic region (1)	VUS geomic region (2)	VUS geomic region (3)	VUS geomic region (4)	VUS geomic region (5)
1	Normal	22q11.23				
2	Normal	5q34	11p15.3			
3	Normal	19q13.42				
4	Normal	22q11.23				
5	Normal	Xq22.2				
6	Normal	3q13.32				
7	Normal	10q11.22	20q12-13.11			
8	Normal	1p32.2				
9	Normal	2q37.3	3q28			
10	Normal	16p11.2/p11.1				
11	Normal	11q14.3				
12	Normal	9q33.1				
13	Normal	Xp22.31				
14	Normal	1p31.3/p31.2	4q13.2	4q31.21	9p23	9q33.1
15	Normal	Xp22.2				
16	Normal	16q22.2				
17	Normal	1q25.2	2p12			
18	Normal	1q43				
19	Normal	10q26.2	11p11.12			
20	Normal	1q25.2				
21	Normal	15q13.2				
22	Normal	9p23	9q33.1			
23	Normal	8p11.21				
24	Normal	Yq11.222				
25	Normal	4p15.31				
26	Normal	1q25.2	14q32.2			
27	Normal	1p31.3-p31.2				
28	Normal	Xq21.2-q21.31				
29	Normal	6q21.32				
30	Normal	1q25.2	3q13.31	14q32.2		
31	Normal	17q24.2				
32	Normal	Xp22.31				
33	Normal	20p13				
34	Normal	1q25.2	6q22.31			
35	Normal	5p13.3	17p13.1			
36	Normal	1q21.1				
37	Normal	16p11.2				

Patient	aCGH result	VUS geomic region (1)	VUS geomic region (2)	VUS geomic region (3)	VUS geomic region (4)	VUS geomic region (5)
38	Normal	5q15	14q21.1			
39	Normal	8q21.3	Xp22.33			
40	Normal	10q24.2				
41	Normal	16p11.2				
42	Normal	16p11.2				
43	Normal	10q11.22	19q12-q13.11	Xp22.33		
44	Normal	16p11.2				
45	Normal	15q25.2q25.3				
46	Normal	1q25.3	5q23.1	9q33.1		
47	Normal	20q13.13				
48	Normal	Xp22.33				
49	Normal	Yq11.21				
50	Normal	3q25.32q25.33				
51	Normal	Xq28				
52	Normal	14q12				
53	Normal	4p16.3	4q13.3	Xp22.33/Yp11.32	Xp22.33/Yp11.32	
54	Normal	22q13.31				
55	Normal	7q36.2	8q11.23	8q11.23	Xp22.33	
56	Normal	Xp22.33/Yp11.32				
57	Normal	1p31.3/p31.2				
58	Normal	3q22.1	21q11.2			
59	Normal	1q25.2				
60	Normal	9q33.1				
61	Normal	19q12q13.11				
62	Normal	13q12.1	19q12-q13.11			
63	Normal	1q25.2				
64	Normal	19q12q13.11				
65	Normal	19q12q13.11	Xq21.33	Xq27.1		
66	Normal	6p21.32				
67	Normal	3q13.31	19q12-q13.11			
68	Normal	11p14.1	13q12.12	16q13		
69	Normal	Xq28/Yq12				
70	Normal	11p14.1	14q23.2	16q13	Xq28	
71	Normal	14q31.1				
72	Normal	4p16.1	5q31.1	7p21.1	9q33.2	
73	Normal	4q31.23	6p21.31	14q31.1	16q13	
74	Normal	4q26				
75	Normal	9p23				
76	Normal	9q33.1				
77	Normal	1q25.1-q25.2				
78	Normal	6q26				
79	Normal	3p22.1	3q25.1			
80	Normal	14q23.2				

Patient	aCGH result	VUS geomic region (1)	VUS geomic region (2)	VUS geomic region (3)	VUS geomic region (4)	VUS geomic region (5)
81	Normal	4p16.3				
82	Normal	14q31.1				
83	Normal	Xp22.33				
84	Normal	3q11.2				
85	Normal	16q13				
86	Normal	7q21.13				
87	Normal	2q32.2				
88	Normal	Xp22.33	Xq28			
89	Normal	10q26.13				
90	Normal	14q31.1				
91	Normal	2q31.1	6q16.1			
92	Normal	2q24.1q24.2				
93	Normal	9q33.2				
94	Normal	Xq13.1	Xq27.1q27.2			
95	Normal	5q11				
96	Normal	3q25.1				
97	Normal	7q21.11				
98	Normal	2p22.3				
99	Normal	15q11.2				
100	Normal	6q26	16q13			
101	Normal	4q22.2				
102	Normal	3q25.1	4q31.23	11p14.1	16q13	
103	Normal	22q12.2				
104	Normal	12q24.31				
105	Normal	12p12.3	14q31.1			
106	Normal	17p13.3				
107	Normal	15q26.3				
108	Normal	Xp22.31				
109	Normal	2q32.2	Yq11.223			
110	Normal	1p22.1				
111	Normal	10q21.3				
112	Normal	1q25.1				
113	Normal	16p11.2				
114	Normal	13q21.2q21.31				
115	Normal	Xp22.33/Yp11.32				
116	Normal	Xp21.1				
117	Normal	2p11.2	2p11.2-p11.1	4p16.1	16p11.2-p11.1	21p11.2-p11.1
118	Normal	11q14.1				
119	Normal	4q31.3				
120	Normal	Xp22.33				
121	Normal	8q21.3				
122	Normal	Xp22.31				
123	Normal	10q21.3				

Patient	aCGH result	VUS geomic region (1)	VUS geomic region (2)	VUS geomic region (3)	VUS geomic region (4)	VUS geomic region (5)
124	Normal	16p11.2				
125	Normal	Yq11.21	Yq11.221			
126	Normal	3p26.1				
127	Normal	Yq11.21				
128	Normal	16q11.2				
129	Normal	Xp22.33				
130	Normal	11q22.3				
131	Normal	Xp22.11				
132	Normal	21q22.2				
133	Normal	16q11.2				
134	Normal	8p22	10p11.21			
135	Normal	6q22.31				
136	Normal	1p36.11	Yq11.221			
137	Normal	14q21.3				
138	Normal	13q33.2q33.3				
139	Normal	17q24.2				
140	Normal	15q21.2				
141	Normal	6q22.1				
142	Normal	1p31.3	2p22.3	17q24.1		
143	Normal	7q22.1				
144	Normal	Yq11q22.1				
145	Normal	4q21.3				
146	Normal	8p23.1	19p13.12	19q13.42		
147	Normal	9q32				
148	Normal	10q26.3				
149	Normal	Yq11.22				
150	Normal	16q23.1				
151	Normal	Xp22.2				
152	Normal	10q24.2	Xq21.33			
153	Normal	14q12				
154	Normal	20p13	Yq11.21	Yq11.221		
155	Normal	2q33.1				
156	Normal	20p12.1p11.23				
157	Normal	16p11.2				
158	Normal	Xp21.1				
159	Normal	Xp21.1				
160	Normal	8p21.3				
161	Normal	5q22.1				
162	Normal	1p21.3				
163	Normal	Xq27.3				
164	Normal	6q26				
165	Normal	1q31.1				
166	Normal	3q29				

Patient	aCGH result	VUS geomic region (1)	VUS geomic region (2)	VUS geomic region (3)	VUS geomic region (4)	VUS geomic region (5)
167	Normal	20p12.1				
168	Normal	18p11.21				
169	Normal	18p11.21				
170	Normal	19q13.33				
171	Normal	13q12.2				
172	Normal	6q26				
173	Normal	8q11.21				
174	Normal	Xp22.33/Yp11.32				
175	Normal	10q21.3				
176	Normal	1q31.3	6p22.3			
177	Normal	10q21.1				
178	Normal	Xq22.2				
179	Normal	2q34	6p21.31			
180	Normal	16q21				
181	Normal	6q26				
182	Normal	11q14.1				
183	Normal	8q22.1				
184	Normal	2q37.3				
185	Normal	1p36.32				
186	Normal	Xp21.1				
187	Normal	17q12				
188	Normal	10p15.3				
189	Normal	11p11.12				
190	Normal	10q21.3q22.3				
191	Normal	Xq28				
192	Normal	16q23.3	Xp11.22			
193	Normal	13q13.3				
194	Normal	16q23.1				
195	Normal	9q34.11				
196	Normal	2q37.3	Xq22.2			
197	Normal	7q36.1				
198	Normal	8q11.21				
199	Normal	16p11.2				
200	Normal	Xp22.33				
201	Normal	11p11.12				
202	Normal	16p11.2	Xp22.13 -p22.12			
203	Normal	17p13.2				
204	Normal	2q32.2q32.3				
205	P (Patient 3 in table 2)	1q25.2				
206	P (Patient 6 in table 2)	1q25.2	9q33.1			

Patient	aCGH result	VUS geomic region (1)	VUS geomic region (2)	VUS geomic region (3)	VUS geomic region (4)	VUS geomic region (5)
207	P (Patient 13 in table 2)	4q13.3	4q21.1	14q24.3		
208	P (Patient 16 in table 2)	12q21.31-q21.32	Xq22.3			
209	P (Patient 21 in table 2)	15q21.1				
210	P (Patient 25 in table 2)	9q31.3	17q24.2			
211	P (Patient 31 in table 2)	9p22.2				
212	P (Patient 32 in table 2)	21p11.2				
213	P (Patient 38 in table 2)	14q31.1	16q13			
214	P (Patient 40 in table 2)	14q24.2	16p11.2/p11.1			
215	P (Patient 41 in table 2)	1q25.3				
216	P (Patient 49 in table 2)	Xp22.33				
217	P (Patient 57 in table 2)	1p31.3/p31.2				