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Cytogenetics and Genomics analysis in prenatal and postnatal diagnosis

relatório de estágio

Dissertação no âmbito do Mestrado em Genética Clínica Laboratorial, orientada pela Professora Doutora Sofia Dória e Professora Doutora Maria Joana Melo e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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List of Abbreviations

aCGH- array Comparative Genomic Hybridization

ASRM- American Society for Reproductive Medicine

BAC- Bacterial Artificial Chromosome

cDNA- complementary DNA

CMA- Chromosomal Microarray Analysis

CNV- Copy Number Variant

DA - Distamycin A

DAPI- 4',6-Diamidino-2-Phenylindole, Dihydrochloride

dNTPs- Deoxynucleotide triphosphates

dTTPs- 2'-Deoxythymidine 5'-Triphosphate

dUTPs- 2´-Deoxyuridine 5´-Triphosphate

DTT- Dithiothreithol

DGV- Database of Genomic Variants

EDTA- EthyleneDiamineTetraAcetic Acid

FBS- Fetal Bovine Serum

FISH- Fluorescence In Situ Hybridization

ISCN- International System for Human Cytogenomic Nomenclature

Kb- Kilo base

Mb- Mega bases

MLPA- Multiplex Ligation-dependent Probe Amplification

MTX- Methotrexate

NOR- Nucleolar Organizer Regions

OGM- Optical Genome Mapping

OMIM- Online Mendelian Inheritance in Man

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PHA- Phytohemagglutinin

PL- Pregnancy Loss

QF-PCR- Quantitative Fluorescent Polymerase Chain Reaction

RPL- Recurrent Pregnancy Loss

RT-qPCR- Real Time-quantitative PCR

SDF- Semen DNA Fragmentation

SNP- Single Nucleotide Polymorphism

SNP-array- Single Nucleotide Polymorphism array

SSC- Saline-Sodium Citrate

STR- Short Tandem Repeat

Tdt- Terminal Deoxynucleotidyl Transferase

TUNEL- Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling assay

UNG- Uracil N-Glycosylase

VUS- Variants of Uncertain Significance

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Resumo

Este relatório integra vários trabalhos realizados no Serviço de Genética da Faculdade de Medicina da Universidade do Porto. São abordadas as diferentes metodologias realizadas de citogenética convencional e molecular em contexto de diagnóstico pré e pós-natal. Adicionalmente, realizaram-se dois estudos retrospetivos, uma avaliação de uma série de produtos de abortamento e um estudo de reavaliação da classificação de VUS (Variants of Uncertain Significance; variantes de significado incerto).

O projeto de análise de uma base de dados de produtos de abortamento teve como objetivo organizar a base de dados, dando uma perspetiva da forma como o diagnóstico foi feito durante os últimos 19 anos e comparar os resultados com a literatura. A etiologia da perda de gravidez esporádica ou recorrente é multifuncional e as causas genéticas podem ser estudadas com base nas técnicas de citogenética convencional e genética molecular. Uma boa seleção da técnica para fazer este estudo é essencial, já que as técnicas apresentam limitações e podem ser complementares. Foi demonstrado que, tal como na literatura há alguns problemas associados às técnicas executadas como *standard* aquando do processamento de uma amostra de produto de abortamento. O estudo apresentou um nível de não resposta de 29%. Para melhorar este índice de diagnósticos dados por número de amostra que chega ao laboratório é necessário aumentar a qualidade da amostra, por exemplo encurtando o tempo entre a recolha e o processamento, e é necessário enfatizar que o envio de sangue materno é crucial para garantir a exclusão da contaminação materna.

O segundo projeto teve como principal objetivo conduzir uma reanálise às VUS que tinham sido classificadas pela instituição ao longo de 10 anos. As VUS foram selecionadas a partir de base de dados com 3244 VUS, identificadas em 3166 pacientes, com o critério de estar presente em 2 ou mais destes. 440 VUS foram selecionadas, de onde 69 foram reclassificadas como benignas/ provavelmente benignas e uma foi reclassificada como provavelmente patogénica, segundo plataformas como OMIM e DGV. Este estudo realçou a importância da atualização constante das bases de dados, já que novas informações são descobertas frequentemente, e uniformização de decisões dentro da própria instituição para melhorar a precisão do diagnóstico.

Palavras-chave: Array-CGH; Cariótipo; Citogenética; Cromossomas; Produtos de abortamento.

Abstract

This report encompasses several projects carried out at the Genetics Service of the Faculty of Medicine, University of Porto. It addresses the various methodologies of conventional and molecular cytogenetics in prenatal and postnatal diagnosis. In addition, two retrospective studies were carried out, an evaluation of a series of products of abortion and a study to re-evaluate the classification of VUS (Variants of Uncertain Significance).

The project involved the analysis of a products of conception database aimed to organise the data, providing an overview of the diagnostic approach over the past 19 years and comparing the results with existing literature. The etiology of sporadic or recurrent pregnancy loss is multifunctional, and genetic causes can be investigated through conventional cytogenetics and molecular genetics techniques. The selection of the appropriate technique for this study is critical, as these methods have limitations and can be complementary. Similar to existing literature, the study revealed specific issues associated with standard techniques used to process products of conception samples. The study demonstrated a non-response rate of 29%. It is imperative to improve sample quality by reducing the time between collection and processing to enhance the diagnostic rate for the received samples. Furthermore, emphasising maternal blood submission is essential for excluding maternal contamination.

The second project's primary objective was to reanalyse VUS, which the institution had been classifying for over 10 years. The VUS were selected from a database containing 3244 instances, identified in 3166 patients, with the criterion of being present in 2 or more cases. Out of the 440 selected VUS, 69 were reclassified as benign/probably benign, and 1 was reclassified as probably pathogenic based on platforms such as OMIM and DGV. This study underscored the significance of continuous database updates, as new information is frequently discovered, and the importance of uniform decision-making within the institution to enhance diagnostic accuracy.

Keywords: Array-CGH; Cytogenetics; Chromosomes; Karyotype; Products of conception.

I – Introduction

1. History of clinical cytogenetics

In 1882, Flemming made the first draft of what a chromosome would be, a concept that would only be used for the first time in 1888 by Waldeyer (Flemming, 1882; Waldeyer, 1888). The analysis of chromosomes only became possible when, in 1952, Hsu accidentally discovered that by treating cells with a hypotonic product, the metaphase chromosomes spread, allowing individual analysis under the microscope (Hsu & Pomerat, 1953). Four years later, Tjio and Levan established the number of human chromosomes as 46, which contradicted what was thought to be 48 (Tjio & Levan, 1956).

Clinical cytogenetics emerged in 1959 when the extra copy of chromosome 21 was given as the cause of Down Syndrome and Jacobs & Strong gave the extra copy of X as the cause of Klinefelter Syndrome (Jacobs & Strong, 1959; Lejeune et al., 1959).

In the 1970s, with the discovery of banding techniques, initially by using fluorescent stains and later by using trypsin-treated slides stained with Giemsa, it became possible to visualise variations outside the common patterns. Cytogenetics stopped being used only to see numerical abnormalities and started detecting deletions, duplications and structural rearrangements that could be associated with specific syndromes (Dave & Sanger, 2007; Martin & Warburton, 2015).

Cytogenetic analysis studies the structure and number of chromosomes present in human and other eukaryotic cells (Montazerinezhad et al., 2020).It is, currently, subdivided into conventional cytogenetics and molecular cytogenetics. Conventional cytogenetic techniques require dividing cells division for chromosomes to be individually distinguished under the microscope light, as the chromosomes are best studied during metaphase when they are at their most contracted state (Gersen & Keagle, 2013).

Over the years, cytogenetic approaches to the study of chromosomes and their abnormalities have improved. Other techniques were developed, namely Fluorescence in situ hybridisation (FISH) and comparative genomic hybridisation (CGH), which combined cytogenetics with DNA-based methods, giving rise to molecular cytogenetics. These techniques were developed to detect some abnormalities that would not be seen by a conventional cytogenetics technique either because they were too small, as changes smaller than 4 Mb cannot be detected, or because they were not detectable by changes in the banding pattern alone (Chowdhury et al., 2020; Martin & Warburton, 2015).

In the molecular field of genetics, the structure and function of genes are studied at a molecular level. This information can determine patterns of descent and is it used to understand the mutations that cause certain diseases (Chowdhury et al., 2020).

In 1941 it surged the first idea that a specific gene would be responsible for a biochemical reaction. With Avery et al. experiments and the deoxyribonucleic acid (DNA) structure being proposed in 1953 by Watson and Crick, the idea that proteins were responsible for transmitting characteristics from generations was changed by the idea that the genetic information is stored in the DNA (Beadle & Tatum, 1941; Watson & Crick, 1953). This revolutionised the field, and it was the beginning of many discoveries in the 1950s and 1960s (Gayon, 2016). In 1993 a Nobel Prize was shared between Mullis and Smith for the discovery of the PCR technique (Durmaz et al., 2015). This technique made it easier to detect genomic mutations, using methods like restriction fragment length polymorphism and sequencing based methods. However, these methods were not able to detect every mutation, so it was important to develop new techniques. When the sequence of the gene in interest was unknown, it was challenging to interpret the results. Therefore, determining the DNA sequence was necessary for genetic diagnostics. This necessity was overcome with the introduction of Sanger sequencing in 1977, and the first draft of the human genome was published in 2001 (Lander et al., 2001; Sanger et al., 1977). The final product of the Human Genome Project was published in 2003, but it still didn't reveal the complete sequence, although leading to a massive improvement in sequencing technology. The automation of the Sanger method improved DNA sequencing efficiency, but it still needed to be more cost-effective and efficient. A new technology that we now call NGS was created to erase these disadvantages (Durmaz et al., 2015).

2. DNA, Chromosomes and Cell cycle

Genetic information is stored in cells as molecules called DNA, as mentioned. DNA is made up of twisted backbones made of deoxyribose-phosphate, connected by hydrogen bonds formed between nucleotide bases. There are two main types of nucleotide bases: purines, including adenine (A) and guanine (G), and pyrimidines, including cytosine (C) and thymine (T). Purines pair with pyrimidines to form base pairs, such as A-T and G-C (Korf & Sathienkijkanchai, 2009).

DNA in the cell nucleus is organized into chromosomes, which are compacted structures formed by wrapping DNA around proteins called histones. Humans have 46 chromosomes arranged in 23 pairs, including autosomes and sex chromosomes (X and Y). Females have two X chromosomes as their sex chromosome complement, while males have one X and one Y.

A chromosome consists of sister chromatids connected at the centromere. The chromosome also has distinct regions known as the short (p) and long (q) arms, separated by the centromere, as shown in Figure 1. The centromere, telomere, and nucleolar organizing regions are functional parts of the chromosome with important roles. The centromere is particularly essential for the chromosome's survival during cell division, while the telomere marks the physical end of the chromosome (Gersen & Keagle, 2013).

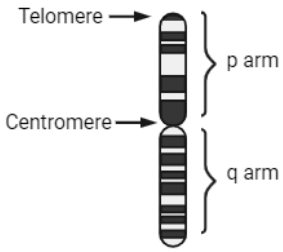

***Figure 1: R**epresentation of a chromosome 3 ilustrating the telomere, centromere, p and q arms.*

To comprehend cytogenetics, it is essential to grasp the concept of cell division. In order to study chromosomes through conventional cytogenetic methods, it is necessary to have cells that are actively dividing. Additionally, various abnormalities in cytogenetics arise from errors occurring during the process of cell division.

There are two types of cell division: mitosis and meiosis. Mitosis is the division of somatic cells, while meiosis is the division of germ cells. Mitosis results in two genetically identical daughter cells, each with 46 chromosomes (diploid cells). On the other hand, meiosis produces reproductive cells called gametes, with each cell containing 23 chromosomes, one from each pair (haploid cells).

The cell cycle consists of four main stages: gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M). G1 is the longest stage, lasting approximately 9 hours, where cells are active, and protein synthesis takes place. If a cell does not undergo further division, it enters a permanent arrested phase called gap zero (G0). The synthesis phase lasts about 5 hours and involves DNA replication, resulting in identical sister chromatids. G2, lasting around 3 hours, is a preparation phase for cell division. Finally, mitosis, lasting 1-2 hours, is the stage where cells reproduce and generate genetically identical daughter cells. This makes the cell cycle last around 17-18 hours (Gersen & Keagle, 2013; Korf & Sathienkijkanchai, 2009).

Mitosis consists of four phases: prophase, metaphase, anaphase, and telophase, as seen in Figure 2. Interphase is the time between two mitotic events. Each cell division

begins with DNA replication in the S phase, resulting in two sister chromatids for each chromosome. Prophase involves chromosome condensation, disappearance of the nucleolus and nuclear membrane, and the initiation of mitotic spindle formation. During metaphase, chromosomes align in the equatorial plane, but homologous chromosomes do not pair. Maximum chromosome condensation occurs in this phase. Anaphase is when chromosomes split at the centromere, and chromatids move to opposite poles. Telophase is marked by the formation of nuclear membranes and is usually followed by the cytokinesis or the division of the cytoplasm (Gersen & Keagle, 2013).

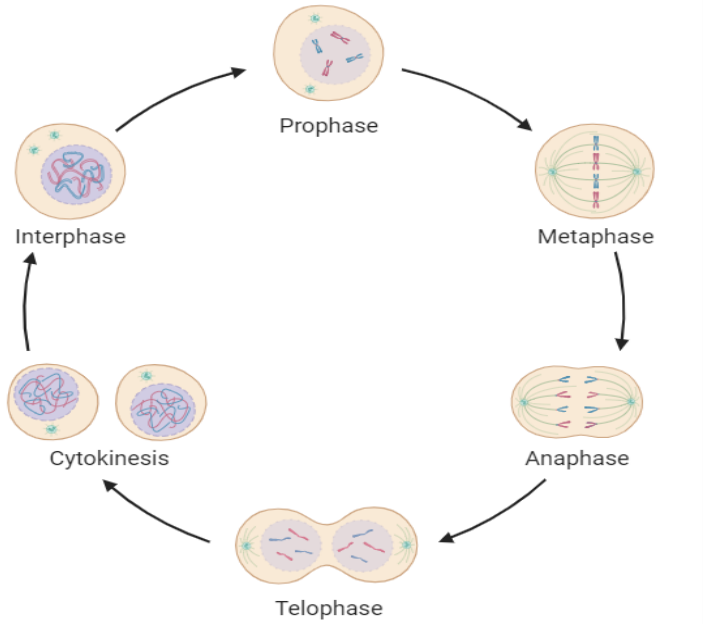

***Figure 2**: Schematic representation of Mitosis. Done in Biorender **(Gersen & Keagle, 2013)**.*

Meiosis is a process that involves a single DNA replication and two rounds of chromosome segregation. It consists of two stages: meiosis I and meiosis II. Just like mitosis, meiosis follows the phases of prophase, metaphase, anaphase, and telophase. However, in the prophase of meiosis I, homologous chromosomes pair up and undergo crossing over (genetic recombination). At the end of meiosis I, the number of chromosomes is halved. Consequently, the daughter cells formed after meiosis I are haploid, with 23 chromosomes each. These are the key differences between meiosis and mitosis (Gersen & Keagle, 2013).

3. Chromosomal abnormalities

Chromosome disorders fall into two main categories: numerical abnormalities (aneuploidy and polyploidy) and structural abnormalities (structural rearrangement).

Aneuploidy occurs when there is a deviation from the normal number of chromosomes, resulting in the loss or gain of one or more individual chromosomes from the diploid set, such as monosomy and trisomy. This usually results from a nondisjunction in the meiosis. Polyploidy is when the cell has more than two copies of the haploid genome. For instance, tetraploidy refers to a cell with 96 chromosomes (Korf & Sathienkijkanchai, 2009).

In structural chromosome abnormalities, the continuity of a chromosome is disrupted by breaks, followed by reconstruction in an abnormal combination. There are several forms of structural abnormalities, namely deletions, duplications, isochromosomes, inversions, ring chromosomes, and translocations, as can be seen in Figure 3.

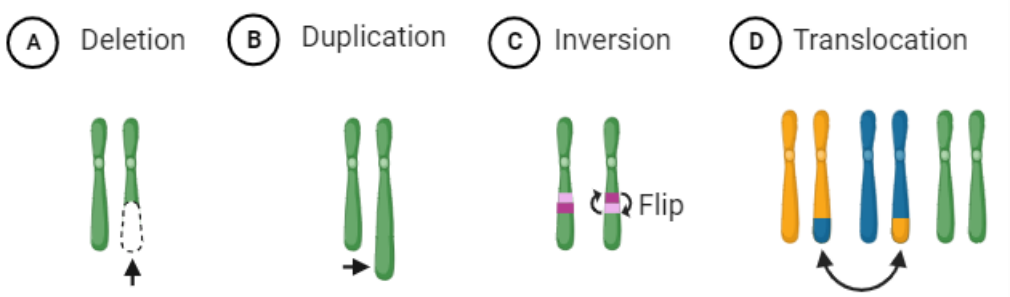

Figure 3: *Schematic representation of some of the structural anomalies. A- Deletion; B- Duplication; C- Inversion; D- Translocation. Done in Biorender.*

In deletion, a chromosome segment is lost due to a single break, resulting in the loss of the distal fragment. Alternatively, two breaks can occur, causing the loss of an interstitial segment. Duplication, on the other hand, involves the addition of a chromosomal segment. An isochromosome is a chromosome that has one arm duplicated invertedly while losing the other arm. In terms of inversions, they happen when a chromosomal fragment flips by 180 degrees as a result of breaks at two separate locations on one chromosome, followed by the reunion of the inverted region. Translocations entail the exchange of material between chromosomes, and they can be balanced or unbalanced depending on whether material is lost or gained. Balanced translocations and inversions typically do not cause any phenotype, but after crossing over in meiosis, they can result in unbalanced chromosomes, resulting in abnormal offspring (Korf & Sathienkijkanchai, 2009).

4. Context and Objectives

This report was carried out to obtain a Master's degree in Clinical Laboratory Genetics, and reports work done during a year of internship in the Genetics Service, Pathology Department of the Faculty of Medicine of the University of Porto (FMUP).

The Genetics Service has a protocol collaboration with Centro Hospitalar Universitário de São João and provides services to the community through its cytogenetics and molecular genetics Laboratory.

The work developed through the year focused on cytogenetic and genomic analysis of prenatal and postnatal cases. The main objective was to present the various diagnostic strategies performed in the Laboratory. It was intended to identify, analyse and select different cytogenetic technologies, understand the theoretical concepts, perform the techniques and know how to interpret the respective results. Additionally, it was also proposed acquiring skills in issuing reports, ethical principles, and naming rules according to the most recent International System for Human Cytogenomic Nomenclature (ISCN), as well as knowing the standards of good laboratory practice, namely all the rules necessary for the accreditation of a genetic test by the iso 15189 norm.

In this report, I will reflect on the work done over the year, focusing on two projects. The first project, titled "Products of Conception," emerged as a retrospective study, utilising a database of 1405 cases of pregnancy loss. The main objective was to organise the database, have an insight into how the diagnosis was made over the 19 years, and compare the Laboratory's results with those reported in the literature. Three clinical cases were included in this chapter to serve as examples of how we should proceed and what genetic studies should be conducted in the face of a specific clinical indication. The second project, "Assessing Variants of Uncertain Significance: A Retrospective Analysis of aCGH Cases," aimed to reanalyse VUS classified over 10 years, emphasizing the importance of continuous updates and reclassifications. This was submitted and accepted at the “14th European Cytogenomics Conference”.

II- Products of Conception

1. Introduction

A miscarriage is defined by the "Sociedade Portuguesa de Obstetrícia e Medicina Materno-fetal" as the spontaneous premature loss of a fetus before the 24th week of gestation (Monteiro et al., 2022). It is a prevalent complication in pregnancy, with approximately 10-15% of all clinically recognised pregnancies resulting in pregnancy loss. Most miscarriages occur during the first trimester of gestation (Gajjar et al., 2023; Reddy et al., 2012). Stillbirth refers to losing a fetus in the third trimester when the pregnancy is near term. This incidence is approximately 13.5% (Bender Atik et al., 2022)

Recurrent pregnancy loss (RPL) is a less common condition characterised by the loss of two or more pregnancies and it affects around 1–2% of couples (Bender Atik et al., 2022). Etiological investigations into RPL can be initiated after the second pregnancy loss, regardless of whether the losses were consecutive (Monteiro et al., 2022). It is essential to differentiate between primary and secondary RPL, with the latter defined by the occurrence of a previous viable pregnancy that progressed beyond 24 weeks (Bender Atik et al., 2022).

The etiology of both sporadic and recurrent pregnancy losses is multifactorial, as depicted in Figure 4, and can be classified into known and idiopathic causes, where no identifiable cause is found (Arias-Sosa et al., 2018). Known etiological factors may encompass environmental, anatomical, endocrine, immunological, thrombophilic, or genetic factors.

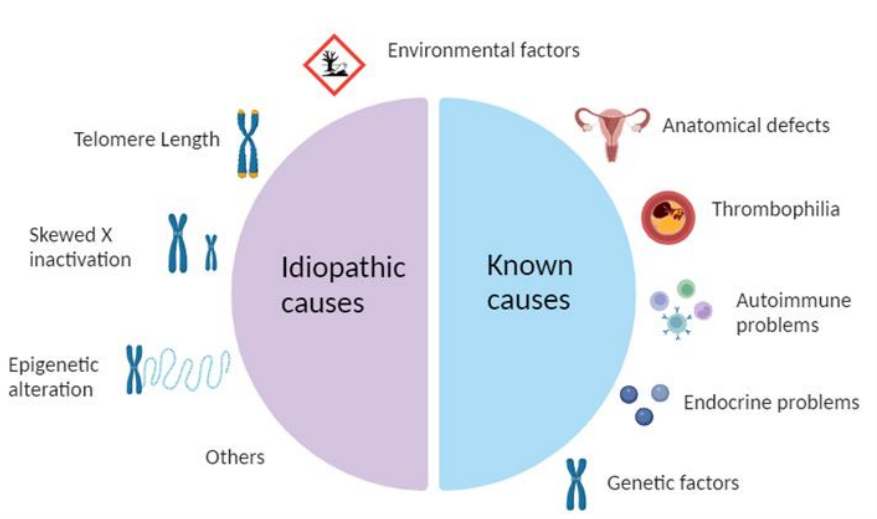

Figure 4: *Schematic illustration of the known and idiopathic causes of sporadic and recurrent pregnancy losses. Done in Biorender.*

Environmental factors associated with pregnancy losses include alcohol and tobacco consumption, exposure to toxic substances, and elevated stress levels. Maternal weight is a topic of debate, as low weight appears to be linked to sporadic pregnancy losses, while obesity is a risk factor for RPL (Monteiro et al., 2022; Teles et al., 2017). Anatomical anomalies in the mother, such as congenital uterine abnormalities, are also considered risk factors, with a prevalence of approximately 13-25% in women with RPL. Among endocrine factors, conditions like hypothyroidism, hyperthyroidism, and diabetes mellitus are known to be associated with infertility (Monteiro et al., 2022).

Chromosomal abnormalities have been reported in about 50% of pregnancy losses, with approximately 80-90% of these cases involving numerical chromosomal abnormalities. Trisomy 16 is the most observed chromosomal abnormality in products of conception, followed by other autosomal aneuploidies, monosomy X, and triploidies (Gajjar et al., 2023; Monteiro et al., 2022; Reddy et al., 2012; Teles et al., 2017; Warren & Silver, 2008). In the context of RPL, around 2-5% of couples have balanced chromosomal anomalies, with reciprocal translocations being the most common, followed by Robertsonian translocations (Monteiro et al., 2022; Warren & Silver, 2008). In addition to these anomalies, genetic factors include confined placental mosaicism and monogenic diseases (Warren & Silver, 2008).

Until recently, RPL was considered exclusively a female problem, but nowadays, male factors have been reported in cases of RPL. Although there is a high association between a high rate of DNA fragmentation and aneuploidies present in the spermatozoa and this condition, more studies need to be conducted to clarify this association (Arias-Sosa et al., 2018; Monteiro et al., 2022). High levels of DNA fragmentation were associated with male infertility and pregnancy loss, even with normal mobility, quantity, and morphology of spermatozoa. An increased rate of sperm aneuploidy of chromosomes 13, 18, 21, X, and Y has also been reported in association with RPL compared to control cases (Carrell et al., 2003). Adding to the male factors mentioned above, the presence of microdeletions in the azoospermia factor regions (AZFa, AZFb, and AZFc) on the Y chromosome are also known causes of infertility (Arias-Sosa et al., 2018).

Focusing on the idiopathic causes, many potential causes have been discussed, and recent studies have found associations with the disease, although there is no consensus.

Beyond the main causes mentioned before some studies also associate other factors to RPL, although not so consensual. One example is the telomere length. A few studies have reported that shortened length of the telomeres is associated with RPL, as this

could be related to a faster ageing rate. The telomeres are mainly composed of highly repetitive and no-coding DNA, crucial in maintaining chromosome integrity (Arias-Sosa et al., 2018).

Skewed X inactivation, as an epigenetic factor, has also been proposed as a possible cause of RPL.

Epigenetics is the study of mechanisms that regulate gene expression without involving changes in the DNA sequence but are heritable through cell division. Operationally, epigenetics relies on a diverse cellular machinery that controls genome function. It plays a crucial role in various processes, including development, physiological homeostasis, and adaptation to external stresses, whether physical, chemical or of biochemical/biological origins. The epigenetic marks that determine whether genes are expressed or repressed can be passed down through cell divisions, such as during mitosis, and in a limited number of cases, through the meiosis process.

X inactivation occurs normally in a random way in each cell, with both copies inactivated proportionately. However, when one of the X's is preferentially inactivated, this creates a skewed ratio of cells with a specific inactivated X chromosome. When this occurs, the normal X is preferentially inactivated, and the abnormal gene is preferentially expressed, which may result in an abnormal phenotype (Warren & Silver, 2008). Other epigenetic mechanisms associated with pregnancy loss have also been studied, including methylation abnormalities and genomic imprinting (Vasconcelos et al., 2019). Crucial changes in the epigenetic markers, which control gene expression, during pregnancy, are necessary for normal tissue remodelling and pregnancy maintenance. Modifications in this machinery are important in the biological regulation of RPL. One of these epigenetic phenomena of interest is DNA methylation, a process for silencing specific genes. Differences in methylation patterns in loci associated with RPL and imprinted genes in placenta samples were also reported by other groups (Arias-Sosa et al., 2018; Hanna et al., 2013).

Identifying the pregnancy loss cause is crucial to provide vital information for couples' genetic counselling. This helps to understand the risks of recurrence in future pregnancies and to make informed decisions about continuing to try a natural pregnancy (Monteiro et al., 2022).

Through the years, the techniques used to study the genetic causes of miscarriages have changed and evolved. Nowadays, the first line tests are the Quantitative Fluorescent Polymerase Chain Reaction (QF-PCR), used to detect the most common aneuploidies and maternal contamination and aCGH and/or cell culture.

Karyotype may be unsuccessful in half of the cases due to the presence of maternal tissue or to culture failure, as cells from the product tissue will sometimes not grow if it takes a long time to initiate the cell culture (Warren & Silver, 2008). In Portugal, array-CGH is the recommended technique because of the limitations of the cell culture technique, although the transition is still being made (Monteiro et al., 2022). In the FMUP Laboratory, a cell culture is always performed even when a karyotype is not required to allow the cells reservation. When an abnormal result is detected in the QF-PCR or array, the karyotype could be performed namely in cases with trisomies identified involving acrocentric chromosomes or suspicion of derivative or marker chromosomes in the aCGH profile. The parents may also be studied, (using a peripheral blood culture) when the anomaly detected in the prenatal study justifies, as some unbalanced anomalies can be inherited from the parents, which can be also a RPL cause. In these cases, a genetic counselling consultation is recommended to evaluate the risk of recurrence. Genetic counselling can help decide whether to keep trying a new pregnancy, give up or opt for a gamete donation, pre-implantation genetic testing, or prenatal diagnosis (Monteiro et al., 2022).

After two pregnancy losses with no cause identified, couples should be referred to genetic/reproduction consultations that will advise and conduct tests to try to identify the cause.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP- Nick end Labelling) technique is performed to evaluate DNA fragmentation, FISH in spermatozoa allows to identify aneuploidies, and Multiplex PCR detects Y microdeletions; these are some strategies that maybe used in cases with infertility. Alongside the different genetic diagnosis performed by routine, the FMUP Laboratory is also interested in studying epigenetic causes of idiopathic PL in a research context. So, RT-qPCR among other relevant techniques have also been done to evaluate the association between epigenetic factors and RPL. This issue is matter of a PhD project.

2. Methodology

During my internship I had the opportunity to participate in all the activities of the laboratory including performing by myself or monitoring the techniques described in the following chapter. So, after a three-month period of training I could conducted, under surveillance, about 430 lymphocyte cell cultures, harvesting and G-banding. The FMUP Laboratory is accredited by the ISO 15890 norm, therefore, the techniques cannot be carried out by other technicians not indicated in the role manual. Additionally, I performed other more sporadic cytogenetic techniques whenever was necessary, namely C-banding, NOR and DA/DAPI staining, on about 10 occasions.

TUNEL and FISH assays on sperm nuclei techniques were observed several times and in about 8 cases I carried out the protocol accompanied by the technician responsible for the procedure. Regarding the gene expression on pregnancy losses I had training in performing RT-PCR and then I executed all the protocol by myself on 7 cases.

2.1. Conventional Cytogenetics

Conventional cytogenetics is still considered for some clinical indications a standard test for diagnosis genetic pathologies (Dave & Sanger, 2007; Kang, 2018). It is exceptionally reliable for identifying aneuploidy and other major chromosomal rearrangements, like deletions, duplications, balanced or unbalanced translocations, insertions, and inversions (Chowdhury et al., 2020).

Karyotype is a visual exercise where the chromosomes are examined while lined up and organised by size, centromere location, and band pattern.

This technique is an excellent cost-benefit example, as it can detect any aneuploidy and many structural anomalies. However, it has a low-resolution limit (not detecting anomalies under 4Mb), and as it needs cultured cells, its response time for tissues, amniotic fluid or chorionic villi can alter between 1 to 3 weeks, being longer than molecular techniques.

2.1.1 Cell Culture

Some specimens contain natural proliferating cells, but others don´t have enough spontaneously proliferating cells, needing to be cultured in the laboratory. The critical requirement is that the cells must be alive when initiating the technique. How the specimen is collected and handled influences the cells' growth and the metaphases' quality. All the specimens must be labelled with the patient's information, and the laboratory must reject improperly labelled specimens (Gersen & Keagle, 2013).

All cells are grown and maintained in an aqueous growth medium, and some are formulated for specific cell types that require particular conditions, as AmnioMax and BIO-AMF-1 are used in this protocol for amniocyte culture (Gersen & Keagle, 2013). RPMI 1640 is a growth medium appropriate for a wide range of cell types and, in this case, is used for peripheral blood culture.

The culture media needs to maintain a proper pH to achieve optimum cell growth; therefore, various additives are included, such as salts, glucose, and a buffering system. Phenol red is used as a pH indicator, so if the medium turns yellow, it is too acidic, and if it turns pink/purple, it is too basic (Gersen & Keagle, 2013).

Most of the culture media are incomplete and contain only some necessary nutrients. They are supplemented with additives that will intensify the growth of the desired cells while preventing the growth of microorganisms. To promote cell growth, L-Glutamine, a serum, and a mitogen are added. L-Glutamine is an amino acid stored frozen and added just before use and as will be mentioned forward, Phytohemagglutinin (PHA) is the mitotic stimulant used to stimulate the division of T-lymphocytes (Gersen & Keagle, 2013). As a method to inhibit the growth of microorganisms, gentamicin is used. It is a bacterial inhibitor but does not dismiss the need for a good sterile technique, which is the best defence against contamination (Gersen & Keagle, 2013).

In this case, the mediums used for the amniocyte culture are complete mediums, where there is no need for supplements. While the medium used for peripheral blood culture is the RPMI 1640 + L-Glutamine + 25mM Hepes, which is incomplete and must be supplemented with Fetal Bovine Serum (FBS), gentamicine and PHA.

Once cultures are initiated, they grow under specific temperature, humidity, and pH conditions. Cells grow at 37ºC, so it is essential to have incubators that maintain this temperature. Cultures are held in either "open" or "closed" systems, where open systems allow the free exchange of gases between the atmosphere inside the flask and the environment of the incubator. An open system is used for tissue culture, keeping the CO2 level at 5% to maintain the ideal pH. The humidity is held at 97% to prevent cell death from dried-out cultures. A disadvantage of this system is that it is more susceptible to microbial contamination. For the lymphocyte culture, a "closed" system is needed. In this system, humidity is self-maintaining, and CO2 incubators are not required (Gersen & Keagle, 2013).

2.1.1.1 Tissue Culture, maintenance and cell harvest

The tissue from the product of conception arrives at the laboratory in an adequately labelled RPMI + Antibiotic-Antimycotic 50 ml conical tube.

From this moment, all steps are carried out in a Telstar Bio II Advance Plus Class II Microbiological Safety Cabinet. The tissue is placed in a sterile plate and washed with sterile PBS using a sterile Pasteur pipette. When the tissue is from the placenta, it should be washed several times with PBS to remove all the blood. The magnifying glass is used to isolate the skin, as seen in Figure 5, or the villi, Figure 6, from the tissue, removing any blood clots and maternal tissue.

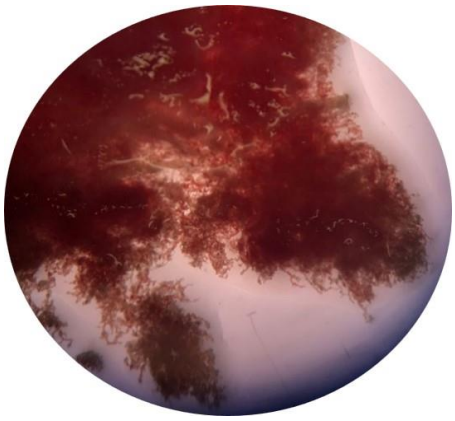

Figure 5: *Example of villis isolated from placental tissue observed under the magnifying glass.*

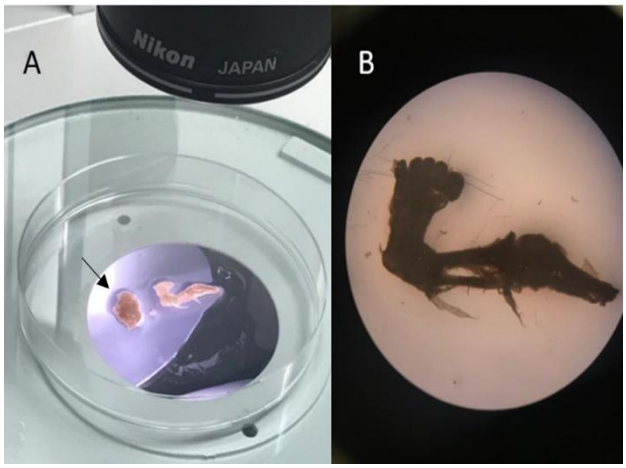

Figure 6: *Example of skin isolation from a tissue of a product of conception. Caption: A- The black arrow points to the tissue removed from the leg, it will be used to start the cell culture and to DNA extraction. B- The rest of the product of conception after.*

The solid tissue is usually too large to be cultured directly and needs to be disaggregated. To obtain single cells, the tissue is cut into small fragments using scalpels, and 1 ml of collagenase is added to achieve cell dispersion by enzymatic digestion of the sample (Gersen & Keagle, 2013). The plate is kept in the laboratory incubator at 37ºC for 1 hour.

The cell suspension is transferred to a 15 ml conical tube, and after the addition of 2 ml of growth medium, it is centrifuged at 1100 rpm for 10 minutes. The supernatant is removed with a Pasteur pipette, and the pellet is divided equally into two tissue culture flasks (T-flask) with 5 ml growth medium each. Each culture has its growth medium. This is because, as mentioned above, there is a risk of the culture being contaminated and infected. Media with different characteristics are used to increase safety and reduce the likelihood of contamination of both cultures.

To allow cell growth, T-flasks are incubated for 3-5 days. During this time, the cell density must be monitored using an inverted microscope, and the medium must be changed. In this case, the cells grow adhered to the surface of the culture flask. The culture is removed the next day when the cell density is ideal. Otherwise, the incubation time is increased, replacing the medium every two days. The medium must be changed (a process called “feeding the culture”) because an exhausted medium becomes acidic and does not have the ideal conditions to allow cell growth.

In this method, the culture period might be two weeks or more, in some cases (Gersen & Keagle, 2013).

After adding 120 µl of Colcemid®, the cell culture is incubated for 2 hours and 30 minutes at 37ºC and will present an aspect as seen in Figure 7. The Colcemid® prevents the separation of the sister chromatids in anaphase, as it binds to the protein tubulin, obstructing the formation of the spindle fibers or destroying those already present (Gersen & Keagle, 2013). The variations in Colcemid® incubation time can alter the results. Insufficient time will result in fewer metaphase spreads and longer, overlapping chromosomes. In contrast, longer incubation times will result in more cells in metaphase but in shorter and thicker chromosomes because chromosomes condense as metaphase progresses, becoming difficult to analyse (Howe et al., 2014).

To remove the cells from the surface of the culture flask, the supernatant is removed, and a Trypsin/PBS/EDTA solution is added, previously heated at 37º, before incubating for 10 minutes. The trypsin treatment releases the individual cells into the fluid and permits their harvest.

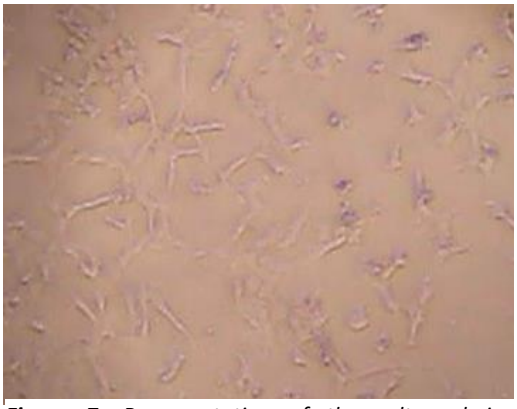

Figure 7: *Representation of the culture being observed under the inverted microscope after the Colcemid® addition.*

After verifying that the cells are suspended in the culture using the microscope, 6.6 ml of distilled water (at 37ºC) is added, and the solution is transferred to a 15 ml conical tube. The distilled water, acting like a hypotonic solution, will swell the cells to yield proper chromosome spreading without lysing it. The centrifugation for 6 minutes at 1600 rpm will allow the separation of the cells.

After removing the supernatant using a vacuum pump, a fresh Carnoy's fixative solution must be added while mixing the pellet. The steps where the vacuum pump is used are executed in a Labopur Filtration Hood. Carnoy's fixative solution of 3:1 absolute methanol - glacial acetic acid is used to stop the hypotonic solution's action and fix the cells in the swollen state while lysing any red blood cells still present in the sample.

As the fixative absorbs water from the atmosphere, affecting the chromosome quality, it must be prepared fresh before use (Gersen & Keagle, 2013; Howe et al., 2014).

The sample is now prepared for the next step, the slide preparation, and must be stored at 4ºC.

2.1.1.2 Lymphocytes Culture, maintenance and cell harvest

As shown in Figure 8, the lymphocyte culture procedure contains seven steps leading to chromosome observation and analysis obtained from peripheral blood. The protocol followed in the FMUP laboratory has some changes from many laboratories to improve the quality of the results and optimise the process.

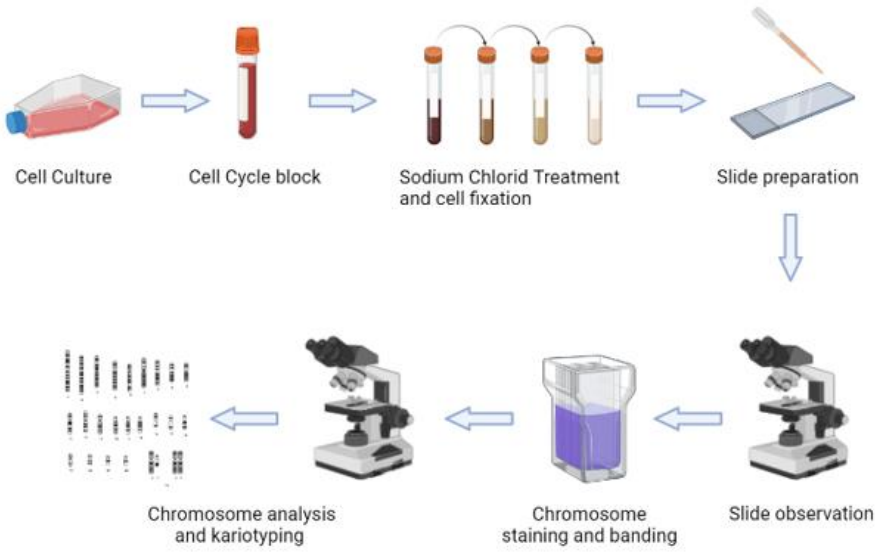

Figure 8: *Workflow to obtain chromosomes for cytogenetic analysis from peripheral blood done in BioRender.*

The peripheral blood sample is collected in the laboratory in a sterile syringe containing preservative-free sodium heparin. The culture should be started within 48 hours of collection for better results. Samples must be kept at room temperature or above 4ºC until they can be processed.

Every step of this procedure where biological substances are handled is carried out in Class II (Type A2) Biological Safety Cabinet - HERAsafe HS12. It ensures high product and operator protection from contamination.

To initiate the procedure for each T-flask, 9 ml of RPMI 1640 + L-Glutamine + 25mM Hepes medium, 200 µL of PHA, and 1 ml of blood is added before homogenising the content and incubating cultures for 48 or 72 hours at 37ºC. If the patient is less than one year old, only 0.5 ml of blood is used.

Usually, the procedure proceeds with the cell cycle block, but in FMUP laboratory, a cell synchronisation technique is used to achieve high-resolution banding. The cells are synchronised earlier in the cell cycle with the methotrexate (MTX)-thymidine synchronisation technique, preventing the chromosome from condensation (Gersen & Keagle, 2013). MTX blocks cells in the S phase of the cell cycle, while the thymidine releases the cells from the block to proceed through to the early stages of mitosis. This technique has proven successful in increasing the number of mitotic cells and obtaining longer chromosomes (Morris & Fitzgerald, 1985).

After adding 40 µl of MTX, flasks are returned to the incubator for 16 hours and 30 minutes. The contents are mixed and transferred to the conical tubes before the centrifugation for 6 minutes at 1600 rpm. The supernatant is removed using the vacuum pump, and the medium is renovated by adding 6 ml of complete medium and 120µl of thymidine for 4 hours and 45 minutes. This protocol executes all the steps where the vacuum pump is used in a Labopur Filtration Hood. Afterwards, 120 µl of Colcemid® is added, and the tubes are incubated for 15 minutes. As mentioned, the time variations in Colcemid® incubation can alter the results (Howe et al., 2014). Exposure time varies by specimen type; in this case, 15 minutes was the ideal balance between quantity and quality.

The procedure continues to the “Cell Harvest” part. At this stage, four solutions are added to the sample at separate times. Between each step, the tube is centrifuged for 6 minutes at 1600 rpm, and the supernatant is removed using the vacuum pump. The first solution to be added is the hypotonic solution, left at room temperature for 10 minutes. This hypotonic solution has a lower salt concentration and will allow water to enter the cells by osmosis, causing chromosomes to spread. If the exposure time is too long, the cells can burst, and if it is too short, cells will not swell sufficiently, resulting in poor spreading of the chromosomes (Gersen & Keagle, 2013).

Other procedures continue with the fixative solution, but in this case, the following solution to be applied is Ibraimov's. This solution consists of 3% methanol and 5% acetic acid in 92% distilled water. In 1983, Ibraimov (1983) showed that this application allows the destruction and elimination of erythrocytes, which increases the quality of lymphocyte fixation as the fixation process is directed towards the lymphocytes. In addition, the residue formed after the removal of the solution is loose and easily resuspended, facilitating the rest of the process.

Adding methanol, the third step, helps clean the cells that the Ibraimov solution destroyed before the fixation (Ibraimov, 1983). Finally, the last solution is Carnoy’s Fixative Solution. This solution must be fresh, and the addition must be done while

mixing the pellet to remove all the proteins before preparing the slides. If not, a yellow cap will form on the top of the pellet, complicating the rest of the procedures (Gersen & Keagle, 2013; Howe et al., 2014). The samples must be stored at 4ºC until the slide preparation. On that day, they must be washed two more times with a fixative solution, and to achieve optimal results, cell suspension concentration must be adjusted.

2.1.2 Preparing slides

Fixed cells are dropped onto glass slides, previously labelled, to allow chromosome staining and analysis. For better results, it is essential to ensure the slide is at a 45º angle and that the drop happens from at least 5 cm high to allow chromosomes to spread (Gersen & Keagle, 2013; Howe et al., 2014). Humidity and temperature affect how quickly the cell suspension dries on the slide, affecting chromosome spreading. Therefore, this step is carried out in a CDS-5 Thermotron Cytogenetic Drying Chamber, which provides the perfect conditions of humidity (42%) and temperature (23º).

After preparing the slides, they are examined with a phase contrast microscope to verify the metaphase quality and number. The ideal concentration has enough metaphases, but the slide cannot be too packed. The chromosomes must be well spread with minimal overlapping but with individual metaphases identifiable. For 1 hour and 5 minutes, the slides are incubated in a 90ºC chamber for the ageing process, which enhances chromosome banding.

2.1.3 Chromosome staining and Banding

Before the 1970s, the chromosomes were stained using a stain with an affinity for the chromatin, not having visible bands when observed under microscope light. Chromosomes were classified according to the length, centromere position, and proportion of the short and long arms, which could not be individually identified. Numerical aberrations could be identified but difficult to characterise, although structural aberrations were even more difficult to find and characterise (Gersen & Keagle, 2013).

Many banding and staining techniques are now available, and they can be divided into two groups: those that produce differential bands along the chromosome and those that stain a specific region of the chromosome. The first group allows the creation of a unique pattern for each chromosome, making it possible to identify a specific chromosome in metaphase and characterise if there is a structural abnormality. The second one is used in special circumstances to stain specifically certain regions of the

chromosomes namely centromeric, heterocromatic or NOR regions. (Gersen & Keagle, 2013).

Some of the staining and banding techniques are summarised in Table 1. Techniques carried out in the laboratory during the year are described in detail further on.

The first banding technique discovered was the Q-Banding technique using quinacrine in 1971. Shortly after, the G-Banding was introduced using Giemsa Stain after a pre-treatment with trypsin, also known as GTG banding (G bands produced with trypsin and Giemsa). Wright stain and Leishman stain can also be used in place of Giemsa. They are equally effective but produce slightly different contrasting properties (Gersen & Keagle, 2013; Moore & Best, 2001).

Table 1: *Summary of Staining and Banding Techniques.*

Type	*Stain*	*Area Stained*	*Application*
G-Banding	Giemsa/Leishman/ Wrigt	Not region specific; forms light (C-G rich) and dark bands (A-T rich).	Widely used to identify and characterize human chromosomes. Used in routine.
Q-Banding	Quinacrine dihydrochloride	Not region specific; brightly fluorescing A-T rich regions.	Not routine as a fluorescence microscope is needed. Useful to confirm the presence of Y material.
R-Banding	Various Techniques	Not region specific; C-G-rich stain darkly or fluoresce brightly and A-T-rich stain lightly or fluoresce dull	Standard technique in France
C-Banding	Giemsa	Constitutive heterochromatin	To detect the presence of dicentric chromosomes, study marker chromosomes
T-Banding	Giemsa	Telomers	Identify terminal regions of chromosomes
NOR banding	Giemsa	Nucleolar organizer regions	Identify marker chromosomes and polymorphisms in acrocentric chromosomes
DAPI/ DA staining	DAPI, with distamycin A	DAPI/DA fluoresces certain A-T-rich areas of constitutive heterochromatin	Identify variations in the polymorphic regions of chromosomes 1, 9, 16 and distal Yq, rearrangements of chromosome 15, and to study marker chromosomes with satellites

The G-Banding technique can be divided into two steps, and could be executed in MIRASTAINER II, performing an automatic slide staining; the conditions are shown in Table 2. In the first step (Figure 9), the slides are first immersed in a Perhydrol solution and then in methanol for a slide cleaning.

Figure 9: *Representation of the 1st step of the G-Banding technique. Caption: 1- Perhidrol; 2- Methanol.*

After being dry, they start step 2, emerging in 6 different tanks following the order: Trypsin solution, Sorensen's Buffer, Leishman stain, Gurr's Buffer, H20, and hot air, represented in Figure 10.

Table 2: *Time and Step conditions of MIRASTAINER II program for automatic G-Banding.*

Program 5	
Reagent	Timing
Perhydrol	3 min 30 sec
Methanol	10 sec
Program 3	
Trypsin	4 sec
Sorensen	12 sec
Leishman	10 min
Gurr	3s
Water	3sec
Hot air (45ºC)	2 min

Trypsin is an enzyme that denatures heterochromatic regions of chromosome with little to no transcriptional activity (A-T rich regions). These regions will be visualized as dark bands while euchromatic regions (C-G-rich regions) will stain lightly, as they are less condensed (Howe et al., 2014; Huang & Chen, 2017).

The right trypsin exposure timing is crucial. With a longer exposure, chromosomes will appear diffused and swollen, while short incubation time will result in chromosomes with indistinguishable bands and low contrast (Howe et al., 2014).

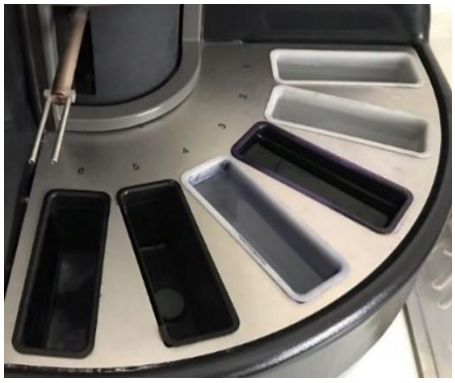

Figure 10: *Representation of the 2nd step of G-Banding. Caption: 1- Trypsin solution; 2- Sorensen; 3- Leishman stain; 4- Gurr; 5-H2O; 6- hot air.*

The Gurr Buffer is used to dilute the Leishman stain and the Sorensen Buffer to produce the trypsin solution. The Buffers are essential to keep the pH at a nearly constant value.

After the slides are dry, they are analysed under a microscope light, as shown in Figure 11.

Figure 11: *Example of a metaphase analysed under a microscope light in 10x resolution.*

C-banding or CBG banding (C-bands by barium hydroxide and Giemsa) selectively stains the constitutive heterochromatin darkly, and the rest of the chromatin remains pale. Noncoding constitutive heterochromatin is highly repetitive α-satellite DNA

sequences that replicate later in the cell cycle and have special stability characteristics under extreme heat and chemical exposure (Gersen & Keagle, 2013; Moore & Best, 2001). The bands are produced by treating chromatin with acidic (hydrochloric acid) and basic solutions (barium hydroxide - BaOH). BaOH will depurate and denature the DNA selectively, and the fragments are washed away by incubation in a warm salt solution (2x SSC - Saline-Sodium Citrate) (Gersen & Keagle, 2013; Moore & Best, 2001). The slides are first dipped in a hydrochloric acid /H2O solution for 30 minutes. After being washed with distilled water, they are in contact with BaOH for 10 minutes. The slides are washed with distilled water and then are in contact with 2x SSC at 60ºC for 1 hour. After getting dry, the slides are stained with Leishman and, after drying are ready to be observed under microscope light. The stained areas will be around the centromeres, the pericentromeric regions of chromosomes 1, 9, 16, and the distal Y long arm. Thus, the technique is not routine in the laboratory; it is mostly used to study the heterocromatic region of Y chromosome, dicentrics and pseudodicentric chromosomes, marker chromosomes, and to detect pericentric inversions of the chromosomes 1, 9, and 16 (Gersen & Keagle, 2013; Huang & Chen, 2017; Moore & Best, 2001).

The NOR staining, or silver staining, selectively stains the nucleolar organizer regions (NORs) located on the satellites of the acrocentric chromosomes. These segments contain genes for ribosomal RNA and can be stained by silver nitrate. This happens because the NORs contain acidic non-histone proteins that can bind silver ions, which are the only regions stained when using silver nitrate (Gersen & Keagle, 2013; Trerè, 2000). To the slide, 2 drops of gelatine and 4 drops of silver nitrate solution are dropped and homogenised using a coverslip over the liquid. The slide is then placed in the HYBriteTM at 70ºC until the slide gets a brown/gold colour (2 minutes). The coverslip is removed with distilled water, and the slide is stained for 10 minutes with the Leishman Stain. After being washed with water, they are dried and can be observed under a microscope light. The silver stains the activity, not the presence of rRNA genes, so per cell, not all NORs will be stained, simultaneously. More metaphases need to be observed to be certain.

The DA/DAPI staining combines DAPI (4,6-Diamino-2-Phenole-Indole), a fluorescent dye, with distamycin A, an antibiotic. These two form stable bonds to A-T rich heterochromatic regions that contain double-stranded, highly repetitive satellite regions of DNA (Gersen & Keagle, 2013; Lin et al., 1988). With a diamond pen, a circle is made in the slide to restrict the area that will be analysed. 20µL of DA solution is placed in a 24x22mm coverslip, and the slide will be placed above to be in contact with the solution. The slide is incubated for 30 minutes, protected from the light, and washed with distilled water and 2xSSC. In a 24x50mm coverslip, DAPI is dropped and

put in contact with the slide. It is stored at 4ºC in the dark until observed under fluorescent microscope light.

This staining technique is used to identify variations in polymorphic regions of chromosomes 1, 9, 16, and the distal Yq to study the rearrangements of the short arm of chromosome 15 and marker chromosomes with satellites (Gersen & Keagle, 2013).

2.1.4 Microscope Analysis

After any staining technique, the slides are analysed under microscope light, except for the DA/DAPI staining, which needs a fluorescent microscope light.

First, in a 10x magnification, individualized metaphases are searched thought the slide in a methodical way. An ideal metaphase must have well-spread, not overlapped chromosomes and a good size. When a good metaphase is found, it is analysed in a 1000x resolution (higher magnification).

When a metaphase with the ideal qualities is found, the microscope coordinates are noted, and with a 100x immersion objective, the chromosomes are counted, and the sex chromosomes are identified. Both cultures are analysed.

To effectuate the karyotype and the respective analysis, the program Cytovision is used. The karyotype is organized by arranging the chromosomes by size, location of centromeres and analysing the banding pattern. It starts with the longest chromosome, ending with the shortest, and all chromosomes have the short arm oriented towards the top.

Between twenty and thirty metaphases, depending on the clinical indication provided, must be analysed, and counted. In cases of infertility, RPL, suspected mosaicism and pathologies associated with the sex chromosomes, in total thirty metaphases are scored (twenty-five metaphases where chromosomes are counted plus five structurally analysed metaphases). In the other cases twenty metaphases are scored, from which five are analysed.

The report is then prepared, including the number of metaphases analysed, the microscope coordinates and the result according to cytogenomic nomenclature (ISCN 2020).

2.2. Molecular Cytogenetics

Over the years, cytogenetic approaches to studying chromosomes and their abnormalities have improved. Other techniques were developed, namely Fluorescence

in situ hybridisation (FISH) and comparative genomic hybridisation (CGH), which combined cytogenetics with DNA-based methods, giving rise to molecular cytogenetics. These techniques were developed to detect some abnormalities that a conventional cytogenetics technique would not detect.

2.2.1 FISH in sperm nuclei

As it was mentioned before, the FISH method was employed in the late 1980s to detect chromosomal abnormalities that could not be detected using standard Cytogenetic techniques, as FISH can detect up to 10 Kb (Martin & Warburton, 2015; Montazerinezhad et al., 2020).

FISH is based on the ability of a single-stranded DNA to hybridize with a complementary sequence to form a double-stranded DNA. In this technique, the DNA probes, a short sequence of single-stranded DNA, are tagged with a fluorescent dye. They are applied to the cell preparation and will attach to the complementary sequence in the specimen if the sequence is present. If there is a hybridization, the site will be visualized under a fluorescence microscope (Chowdhury et al., 2020).

One advantage of this technique is that it is not necessary to carry out a cell culture, making it possible to analyse interphase cells (Martin & Warburton, 2015).

There are a lot of FISH DNA probes available with different targets. The beta-satellite probes detect beta-satellite regions on the acrocentric chromosomes, while the alpha-satellite probes are specific to centromeric regions. There are classic satellite DNA probes that detect pericentric regions of all chromosomes, telomeric/subtelomeric probes specific for some regions, and unique gene sequences, used to identify specific regions on a chromosome. Probes that paint the whole chromosome are also available, useful for identifying the origin of an unknown chromosomal segment (Dave & Sanger, 2007).

In this context, the FISH technique is executed in decondensed sperm nuclei, included in the protocol to study infertile males. This technique aims to study the most common aneuploidies that could be present in spermatozoa and be the cause of numerous miscarriages.

The Aneuvysion Assay Kit (13/21; X/Y/18), a multicolour probe panel, is used for the FISH study in spermatozoa. In this kit, the probes used for the X/Y/18 study are CEP probes which consists in a mixture of probes specific for the D18Z1, DXZ1, and DYZ3 regions that detect alpha satellite sequences in the centromere regions of the three chromosomes. In contrast, the LSI 13/21 mix probe contains unique DNA sequences

that hybridize in the 13q14 region of chromosome 13 and on D21S259, D21S341, and D21S342 loci within the 21q22.13 to 21q22.2 region (Abbott Laboratories, 2014).

FISH is very useful in detecting chromosomal aneuploidies, including loss or gain of a whole chromosome but also for chromosomal regions. Although, one limitation of this technique is the fact that it is directed for a target. An abnormality can only be detected if there is a suspicion of the region to be studied as the probes are targeted (Chowdhury et al., 2020; Dave & Sanger, 2007). When using specific DNA probes and different combinations of probes, it is possible to evaluate aneuploidy frequencies for any chromosome and to study thousands of spermatozoa in a short time (Egozcue et al., 1997). The combination of probes is possible as long as these are marked with different fluorescent dyes (a maximum of three).

To produce slides with sperm spread, the sperm sample is centrifuged for 20 minutes at 1500 rpm to remove the seminal liquid. It is washed with PBS, incubated for 10 min at 37º, and centrifuged for 10 min to remove the supernatant. The sample is washed again with PBS and, after being centrifuged, is treated 2 times with fixative. A drop of semen is spread over 5 slides (for a possible need to repeat the procedure). The slides are observed under microscope light to confirm the concentration and a circle is draw with a diamond pen in a selected region. The slides are stored. For each procedure two slides are used; in one, the chromosomes X, Y, and 18 are studied, and the other is used to study chromosomes 13 and 21.

The sperm head has a highly compacted chromatin; thus, a decondensing treatment is needed for the DNA to be hybridized. In the laboratory, dithiothreitol (DTT) is a reducing agent, breaking the disulfide bridges in the sperm chromatin. It is a sensible treatment; if the timing is too short, the accessibility will be reduced, and if it is too long, a single probe may produce more than one signal by hybridizing to different chromatin domains (Egozcue et al., 1997).

To execute the decondensing treatment, the slides are washed with 2xSSC for 3 minutes, two times, and washed with 70%, 96%, and 100% ethanol for 2 minutes each, in this order. DTT solution is then applied to the slides, which are incubated at 37º; the slides with the 13/21 probes are set for 3 minutes, while those used to study the X/Y/18 chromosomes are incubated for 30 seconds to 1 minute. This timing is variable according to ambient conditions, and the times that the DTT has been used, the more often it is used, the longer it needs to act. As mentioned before, the slides are washed with 2xSSC and a progressive percentage of ethanol.

After getting dry, the slides are treated with 70% Formamide for 5 minutes at 73ºC. Formamide lowers the melting temperature of DNA, lowering its stability and

denaturing the molecule (Blake & Delcourt, 1996). After that, the slides are washed with 70%, 96%, and 100% ethanol for 1 minute each. Over an 18x18 coverslip, 5 µl of probes from the Aneuvysion Assay Kit are applied in the selected region. A parafilm is placed around the coverslip for the slide to hybridize overnight at 37ºC.

The next day, the coverslip is removed, and the slide is washed with 0.4%xSSC/0.3%NP-40 for 2 minutes at 73ºC before being washed with 2%xSSC/0.1%NP-40 for 1 minute. The slide is left to dry, and 10µl of DAPI II is placed in a 24x50mm coverslip that is applied over the slide. It is stored at -20ºC. The slides are observed under fluorescence microscope, and 1000 spermatozoa are counted. As it was mentioned before, there are two moments of cell counting. The percentage of each probe is noted, and the average is calculated to draw a conclusion.
Reference values of disomy that are expected in a normal sperm sample technique are described in Table 3 and 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%

2.2.2 TUNEL assay

After being created to measure DNA damage in somatic cells, the TUNEL assay is a dependable and sensitive technique used to identify DNA fragmentation in sperm cells. It makes use of TdT, or terminal deoxynucleotidyl transferase. To enable the detection of damaged or fragmented DNA by fluorescence microscopy, this polymerase catalyses the addition of fluoresceinated-2'-Deoxyuridine 5'-Triphosphate (dUTP) at the 3'-OH end of the DNA fragments. The test consists of fixation and permeabilization of sperm in a slide, marking DNA fragments, and staining and evaluating the sperm sample (Hamilton & Assumpção, 2019; Sharma et al., 2021).

Recurrent pregnancy loss is one of the many scenarios where Semen DNA Fragmentation (SDF) Testing is recommended as the chances to conceive, naturally or after an IVF and ICSI, are significantly reduced in men with fragmented DNA.

The DNA can be damaged due to several intrinsic factors like a defective maturation of the spermatozoa or the apoptotic process, and extrinsic. Some of the extrinsic factors are the presence of a male reproductive system pathology, environmental factors (smoking, using drugs), and the presence of a systemic disease (cancer or diabetes, for example) (Hamilton & Assumpção, 2019; Sharma et al., 2021).

There are several assays to evaluate Sperm DNA Fragmentation (SDF), subdivided into Direct and Indirect assays. In the Indirect assays, only the susceptibility of the DNA to acid denaturation or the double-single strand breaks is analysed. In contrast, the direct assays evaluate the integrity of the DNA (comet assay) or the DNA fragmentation (TUNEL) (Sharma et al., 2021).

TUNEL is described as the most common test used for evaluating SDF in spermatozoa as well as several end-point conditions in both natural and assisted reproduction and as the most predictive assay for miscarriage rate in a meta-analysis (Baskaran et al., 2019; Robinson et al., 2012).

For a proper analysis, the concentration of the spermatozoa needs to be adjusted to 2.5 × 106, so the first step is the sample preparation. The sample is collected in the Laboratory or maybe transported by the patient under required conditions. The sample is centrifuged at 1500 rpm for 10 minutes, and after the supernatant is removed, and then washed with PBS. This is done two times, but the second time, the PBS is added to adjust the concentration. A smear is done in 5 slides for each patient; after they are dry, they are stored. The procedure is done two times for each patient on separate days to count the spermatozoa and calculate the percentage of DNA fragmentation based on a media. The rest of the slides are used in case of any problem and to repeat the procedure if needed.

Before starting the procedure, the slides need to be fixated, covering the zone of the sample with 4% Paraformaldehyde/PBS (previously stored at 4ºC) for 1 hour at room temperature. The slides are washed in PBS, permeabilized at 4º C on a solution of 0.1% Sodium citrate /0.1%Triton-X (100 ml distilled water + 0.1g sodium citrate, 0.1 ml Triton-X) and then rewashed two times with PBS for 5 minutes. For preserving lipid components, paraformaldehyde is the best fixative agent (Jalali et al., 2023).

The extra amount of PBS is removed with a paper sheet around the sample (making a square).

For each slide, 48µL of TUNEL mixture is added to the sample zone, and the slide is covered with a parafilm coverslip. The slide is incubated for 1 hour in a dark chamber at 37ºC. The TUNEL mixture is done from 5µL of TdT enzyme + 45 µL of label solution

(fluorescein-dUTP) for each slide used. These reagents belong to the In Situ Cell Death Detection Kit, Fluorescein, and are stored at -20ºC.

The slides are washed four times for 2 minutes in PBS, let to dry, and DAPI is added before adding a coverslip. The slides are stored at -4ºC until observation.

The slides are observed under a fluorescence microscope, and 1000 cells are counted at two different time points. The percentage of cells exhibiting fragmentation is calculated for each moment, and then the average is determined. According to the American Society for Reproductive Medicine (ASRM) Practice Committee, the reference value for DNA fragmentation is less than 36%. The total number of cells counted, the percentage of fragmentation and the reference value must be specified in the report.

2.2.3 Array Comparative Genomic Hybridization (aCGH)

Array comparative genomic hybridisation (aCGH) is a type of Chromosomal Microarray Analysis (CMA). It is used to diagnose genomic duplications or deletions and was developed to detect genomic imbalances throughout the entire genome with a simple assay. It allows a high-resolution screening of the whole genome with a focus on the loss and gain of specific genetic regions (Dave & Sanger, 2007). It entered the clinical diagnostic procedure in 2004, initially containing bacterial artificial chromosome (BAC) clones corresponding to regions with clinical relevance. With technological evolution, BAC was substituted with oligonucleotide sequences, or SNP probes, that allowed the targeted region to be expanded to interrogate the whole genome (Martin & Warburton, 2015).

When adding more probes to the technique, the diagnostic yield increased, and the detection of tiny imbalances was allowed. The term copy number variant (CNV) was created to describe a variant resulting from the deletion or duplication of a genomic material with a size >1Kb (Martin & Warburton, 2015).

Unlike the first CGH technique developed (based on metaphase chromosomes), the aCGH uses thousands of short sequences of DNA/probes as targets printed on a chip. DNA of the test sample and the control sample are differently labelled with fluorescent dyes, mixed, and hybridized onto the glass slide. The DNA fragments hybridize with the complementary sequence in the array and are then scanned in a microarray scanner. It measures the fluorescence intensity of the two labelled dyes, and the software calculates the ration to identify the CNV in the genome (Chowdhury et al., 2020; C. Zhang et al., 2017). It reflects the copy number ratio of that DNA sequence in the sample compared to the control (C. Zhang et al., 2017).

This technique has a few resolutions available according to the number of probes included and consequently the space between them. For prenatal cases including pregnancy loss cases, the resolution used was 8x60K, meaning that approximately 60,000 oligos cover the whole genome, and eight samples can be used in only one chip.

Although aCGH technology has a much higher resolution than conventional chromosome studies, detecting smaller deletions/duplications all in one test, it cannot detect balanced abnormalities. Another disadvantage, namely in the prenatal context, is the detection of CNVs that still doesn't have a clinical significance described (VUS). For this reason, in prenatal cases the resolution chosen for the technique was lower than in postnatal cases (4x180k), to reduce the possibility of finding VUS.

To execute this technique, we need DNA already extracted and quantified from the samples. The DNA samples are then prepared in Eppendorf because the concentration must be adjusted with nuclease-free water according to the earlier quantification. For 8x60k arrays, 0.5 µg of gDNA is used in 13 µL total. As previously mentioned, paired controls of the same gender as the case are used. The control concentration is 250 ng/µl. After the samples are prepared with the right concentration, they are marked with fluorescence. A random primer (2.5 µl) is used in both samples and control, and they are put at 98ºC for 10 minutes before being refrigerated on ice for 5 minutes or at -20ºC for 2 minutes. Two Master Mixes are produced, one to be used for the samples and another for the controls. Table 4 shows the quantity used to make the Master Mix. The enzyme should be kept at -20ºC and only be added when the samples are ready.

Table 4: *Master Mix preparation conditions for the fluorescence marking step of the aCGH technique.*

Reagents	**Per reaction (µl)**	**8x60k x 8 (µl)**
5x Reaction Buffer	5	42.50
10x dNTPs	2.5	21.25
Cyanine 3-dUTP or Cyanine 5-dUTP	1.5	12.75
Exo (-) klenow	0.5	4.25
Total Volume	19/9.5	80.75

To the sample, 9.5 µl of the respective Master Mix is added, mixing the two with a short spin. For the cyanines to correctly mark the DNA, the samples are incubated for 2 hours at 37ºC, following 10 minutes at 65ºC, and left at 4ºC for at least 5 minutes.

After having marked DNA, the samples must be cleaned two times with Tris-EDTA buffer using purification columns with collective tubes. After being centrifuged, the final volume should be 20 µl for the marking process to be evaluated using Nanodrop. Absorbance is determined at A260nm (DNA), A550nm (cyanine 3), e A650nm (cyanine 5) to determine the quality of the marked DNA.

If the quantity and quality are not adequate, the samples can't be used.

The samples (and respective controls) are mixed in 0.5 ml eppendorfs, 8 µl each. The hybridization master mix should be prepared, as shown in Table 5.

Table 5: *Hybridization Mix Preparation conditions for the hybridization step of the aCGH technique.*

Reagents	Per reaction (µl)
Cot-1 DNA (1,0 mg/ml)	2
10x aCGH Blocking agent	4,5
2x HI-RPM Hybridization buffer	22,5
Final Volume	29

To each sample, 29 µl of the Mix is added, while mixing with an up and down movement. For the mix to work, the samples are put at 98ºC for 3 minutes and immediately changed to 37ºC for 30 minutes or more.

The gasket slide is prepared and placed in the hybridization chamber. A volume of 40 µl is dispensed from each sample into the respective well. The array is placed over the slide and the security is guaranteed. It is important to ensure that the well is filled with the sample, that there is no contamination between samples, and that all the samples hybridize to the array. The gasket is placed in the hybridization oven for 24 hours at 67ºC and 20rpm.

Before being scanned the array must be washed with Oligo aCGH Wash Buffer 1 and 2. The array is then placed in the slide holder of the scanner, and the fluorescence ratio of the hybridization signals is determined in different positions along the genome.

The results are analysed using the *Cytogenomics* software. Unbalanced chromosomal regions will exhibit a deviant log ratio of the intensity of the signals. A value of 0 (log2) shows a balance in the number of copies, while a value of 0,58 (log2) or -1 (log2) suggests a duplication or deletion, respectively. This occurs because the unmodified chromosomal regions have an orange/yellowish colour due to an equal contribution from the green and red probes. However, if a chromosomal region in our sample is deleted, the area will primarily fluoresce green. The appropriate chromosomal region will appear redder if a chromosomal region is amplified in the sample.

After identifying the CNVs, they must be classified according to American College of Medical Genetics, as pathogenic (P), likely-pathogenic (LP), benign (B), likely-benign (LB) or VUS (Kowalczyk et al., 2022). For this purpose, there are several online databases available, such as Database of Genomic Variants (DGV), ClinGen, Online Mendelian Inheritance in Man (OMIM), DECIPHER, Franklin, among others. The laboratory also has an internal database, where the CNVs are reported.

Because knowledge is always improving, it is important to constantly go to these databases to update some of the results and our internal database. When a VUS is detected, these databases should be visited more frequently.

When compiling a report, it is crucial to ensure that all necessary information is included. This includes providing the complete cytogenetic location using the cytogenomic nomenclature (ISCN 2020), as well as details about the CNV found, its classification, and interpretation, with supporting evidence and references. It is also important to mention the genes involved and any limitations associated with the technique used.

2.3. Molecular Genetics

2.3.1 DNA Extraction

The first DNA isolation was performed in 1869 by Friedrich Miescher when hoping to determine the cells' chemical composition (Tan & Yiap, 2009). Nowadays, DNA isolation/extraction can be done by several techniques, according to the biological sample, but irrespective of the protocol used, the DNA extracted must have high quality. For this quality to be assured, the cells must be disrupted, the proteins denatured, and the nucleases inactivated. It is important to ensure no contamination (Mullegama et al., 2019; Tan & Yiap, 2009).

This chapter will describe two methods of DNA extraction used in the laboratory.

After any DNA extraction protocol, the quality of the products is tested using the NanoDrop 2000c spectrophotometer. This spectrophotometer uses UV spectrophotometric measurements to determine the DNA concentration and purity (Mullegama et al., 2019). When using A260/A280 absorbance, the presence of proteins in the sample is evaluated; it is expected to have values between 1.8 and 2 to assure the gDNA high-quality. To evaluate the presence of phenol and other contaminants, the A260/A230 absorbance is used, and if the values are above 1, the purity of the DNA is proved.

2.3.1.1 Automated DNA extraction from peripheral blood with Prepito

The DNA extraction method depends on the sample used; in this case, the FMUP Laboratory uses an automatic method to extract the nucleic acids from the peripheral blood. It is performed in the Chemagic Prepito Instrument, using the Prepito DNA Blood250 Kit (PerkinElmer, 2019).

This protocol uses magnetic separation technology, as shown in Figure 12. The nucleic acid molecules are captured using M-PVA magnetic beads, which are highly specific binding agents. These beads are attracted to metal rods momentarily magnetised by an electromagnet. The rods transport the beads through the process solutions, and as the beads do not carry any liquid, the potential sample cross-contamination is avoided. The rotation of the rods is switched on after the electromagnet is deactivated, resuspending the particles during sample resuspension, and washing steps (chemagen Technologie GmbH, 2023b).

Figure 12: *Magnet beads function representation of the Prepito technique. Done In Biorender* (chemagen Technologie GmbH, 2023a).

In the wells, 260 µL of blood from each sample is pipetted, where there was, previously, 10 µL of protease.

2.3.1.2. Manual DNA extraction from tissues with Zymo Kit

By using different buffers and collection tubes with respective columns, DNA extraction is possible from solid tissues with a Quick-DNA™ Miniprep Plus Kit. This technology allows concentrated gDNA above 50 kb to be eluted into 35µl DNA Elution Buffer or water (Zymo Research, 2023).

Products of conception samples need to be digested before extracting the nucleic acids. This digestion occurs physically by cutting the sample into small fragments using a scalpel, as mentioned, and chemically by adding proteinase K and a solid tissue buffer. For a total of 200 µl, 95 µl of PBS and Solid Tissue Buffer and 10 µl of proteinase K are added to the sample. The proteinase will lyse the tissue. The sample is vortexed for 15 seconds and then incubated at 55ºC for 1-3 hours or overnight, not damaging the extraction quality. Before proceeding, the tissue must be solubilised, and the sample must be mixed. Then, 400 µl of Genomic Binding Buffer is added to the sample and vortexed for 20 seconds before continuing to the DNA purification. The final 900 µl of the samples are transferred to a Zymo-Spin Column, with collection tubes previously labelled, where the tube is centrifuged for 1 minute at 13500 rpm. When finished, the supernatant is rejected, and the collection tube is replaced. As seen in Figure 13, the first steps are repeated by adding DNA Pre-Wash Buffer and gDNA Wash Buffer two times, in this order. Between steps, the supernatant is always rejected, and before the elution step, the collection tube is also replaced by a new one. The Elution Buffer will allow the DNA to be precipitated and collected in the collection tube, being the column rejected in the end.

The DNA is in the collection tube in the final step. It is ready to be used in any procedure after having the quality and quantity checked using the Nanodrop 2000c spectrophotometer.

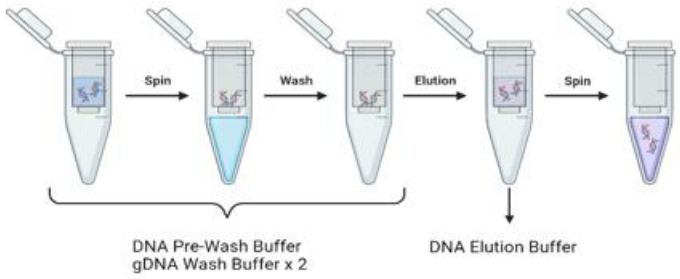

Figure 13: *Representation of the purification step of Zymo technique. Done in Biorender.*

2.3.2 Quantitative Fluorescent Polymerase Chain Reaction

Quantitative Fluorescent Polymerase Chain Reaction (QF-PCR) has emerged as rapid testing of uncultured cells to detect aneuploidies. This diagnosis approach surged in 1993 but was only reported with clinical application in 2001 (Langlois et al., 2011; Mann & Ogilvie, 2012). Compared to FISH, the previous technique implemented to shorten the response time of the diagnosis of aneuploidies, QF-PCR allows the

detection in more samples, is less labour-intensive, although having higher performance, and is less expensive (Mann & Ogilvie, 2012).

This technique is PCR based and consists in amplifying chromosome-specific sequences. These sequences are polymorphic repetitive regions (STRs) that differ in length between individuals. These products are labelled with fluorescence and then separated by capillary electrophoresis. The chromosomes studied are 13, 18, 21, X, and Y, and the copy number of each allele is determined by the ratio of peak area detected for each marker (Langlois et al., 2011; Mann & Ogilvie, 2012; Nicolini et al., 2004). To increase certainty for each chromosome, several STRs are studied; five specific for chromosome 13, five specific for chromosome 18, six specific for chromosome 21, three for chromosome X and, additionally, two non-polymorphic sequences: amelogenin (AMXY) and SRY (Y chromosome), which allows the identification of the gender of the sample.

Besides detecting aneuploidies and triploidies, this technique can detect mosaicism, although not in low percentages, and the presence of other cells in the sample, in contrast to the MLPA technique. So, QF-PCR is used to identify maternal cell contamination, which is essential to minimise misdiagnosis in products of conception. When comparing the tissue profile with the mother's sample, obtained from a peripheral blood sample, the lengths of the STR can be compared. If the peaks are coincident, maternal cell contamination can be confirmed (Mann & Ogilvie, 2012).

To initiate the technique, a PCR is performed to amplify the target STRs before going through capillary electrophoresis. The DNA extracted from the samples must be diluted at 4 ng/µl for 2.5 µl to be used. Before performing the PCR, 10 µL of QST*Rplusv2 reaction mix is added to each DNA to be tested, and the conditions of the reaction are shown in Table 6.

Table 6: *PCR conditions of temperature and time to QF-PCR technique.*

Temperature	Time	
95 ºC	15 min.	
95 ºC	30 seg.	26X
59 ºC	90 seg.	
72 ºC	90 seg.	
72 ºC	30 min.	
15 ºC	∞	

After the PCR is completed, the STRs are amplified, and they are to be quantitatively analysed using capillary electrophoresis. The mix used in this step is a compound of a digested product, HiDi formamide that denatures the samples and the molecular weight marker Liz 600. In each well of the 96-well optical plate, 13.5 µl of the mix is placed and added to 1 µl of the DNA from the PCR. The plate is covered and placed to denature at 94ºC for 2 to 3 minutes and then placed on ice for 1 minute. There should be no dry wells, so a solution, for example HiDi Formamide should be added in each empty well.

For the run, the plate is placed in the 3500 Genetic Analyzer, previously instructed, with the correct definitions.

The written report must contain pertinent information regarding the employed genetic markers, identified alterations, and an analysis of the outcomes. Furthermore, if the presence of maternal contamination is detected, the report should take note of its implications. In instances where a maternal sample is not provided and the results indicate 46,XX, it is imperative that the report acknowledges the possibility of a maternal contamination. This observation should be included to ensure the accuracy and validity of the results.

2.3.3 Y Microdeletions

The second most common genetic cause of male infertility is the Yq11.2 microdeletions, making this molecular diagnosis a standard clinical investigation in the study of severe male infertility. Most of these microdeletions occur in the AZF (Azoospermia Factor genes) regions, AZFa, AZFb, AZFbc, and AZFc (Witherspoon et al., 2021). This study is performed in case of abnormal spermogram indicative of secretory azoospermia or severe oligozoospermia.

The method used to detect these microdeletions is the PCR multiplex followed by capillary electrophoresis.

The primers used in this technique can be specific for genes or amplify anonymous MSY (male-specific region in the Y chromosome) regions. Using this technique, it is possible to implement an internal control. This gene exists in male and female DNA, distinguishing a negative result from a technique failure. As a positive control, the DNA from a male with normal spermatogenesis must be used (Witherspoon et al., 2021). The search for STRs in the AZF zones co-occurs with the SRY and other control regions, being this control of the DNA of a fertile man, DNA from a woman, and negative control. The primers are divided into four reactions to analyse the 22 STRS on Y chromosome.

To execute the multiplex PCR, the master mix and primers are mixed with water and then transferred to an Eppendorf with 3 µl of DNA for each reaction. The PCR condition is demonstrated in Table 7. After PCR, the product is analysed using Qiaxel with the AL420 method.

Table 7: *Multiplex PCR conditions of temperature and time for the Y microdeletion detection.*

Time	Temperature	Number of Cycles	Step
2 minutes	95ºC	1	Denaturation
3 seconds	95ºC (denaturation)	35	Amplification
60 seconds	57ºC, 60 ºC and 62 ºC (annealing)		
90 seconds	72 ºC (new DNA chain synthesis)		
30 minutes	60ºC	1	Final extension

2.3.4 Real Time -quantitative PCR (RT-qPCR)

When the objective is to quantify gene expression and not simply detect the presence of a gene, the Real Time PCR (qPCR- real time PCR or RT-qPCR) technique combined with reverse transcription should be used. This technique allows in real-time monitoring of the amplification reaction of a specific target. For this, we need to extract RNA and convert it into cDNA before the PCR reaction, which will tell us the degree of expression of a particular gene under a given set of conditions (Covey, 2021; Maddocks & Jenkins, 2017).

Starting with the RNA extraction this step is executed with the TRIzol® reagent. It allows simultaneous extraction of RNA, DNA, and proteins. It is a monophasic solution of phenol and guanidine isothiocyanate that presents a highly efficient capacity to inhibit the activity of RNases and, thus, to preserve the integrity of the RNA during the homogenisation of the tissue (Fisher Scientific, 2023).

Tubes are prepared with zirconium oxide beads and 1 ml of TRIzol® reagent, while the respective tissues are thawed in ice. Next, the thawed tissues are transferred to the prepared tubes and subjected to the action of the Minilys homogeniser. After total tissue lysis, the solution is transferred to a new 1.5 ml tube and a 5-minute incubation at room temperature is carried out, ensuring complete dissociation of the nucleoprotein complexes. Subsequently, 200 µl of chloroform is added to the dissociated tissue, being incubated for 3 minutes at room temperature.

A 12000xg centrifugation is performed for 15 minutes at 4°C, allowing the separation of the sample into three phases, a colourless upper aqueous phase containing the RNA, a white-coloured interface containing the DNA and a pink-coloured organic lower phase containing the protein fraction, as seen in Figure 14. The content of the aqueous phase is then used, and the RNA isolation phase begins with the transfer of this content to a 1.5 ml RNase-free tube and the addition of 500 µl of isopropanol. After inverting the tubes to mix the solution, they are incubated for 10 minutes on ice, followed by a 12 000xg centrifugation for 10 minutes at 4°C. A white cloud of precipitated RNA is observed, as observed in Figure 14, and the addition of 75% (v/v) ethanol allows the removal of salts that are still adhered to the RNA precipitate. A second centrifugation at 7500 x g for 5 minutes at 4°C is performed, and, again, the supernatant is discarded while the RNA pellet is left to dry at room temperature. Finally, the RNA is resuspended in RNase-free water and subjected to a 10 min incubation at 60°C. The concentration and purity of the extracted RNA are determined from the NanoDrop 2000c spectrophotometer.

Before the extracted RNA being used for the gene expression study, treating it with Dnase I endonuclease is essential. This treatment allows the digestion and, consequently, the removal of genomic DNA that might still exist in the RNA samples. In this procedure, the samples are treated with 10x Buffer with MgCL2, DNase I and DEPC-treated water. After being incubated for 30 minutes, EDTA is added to the samples, which, together with a second incubation of 10 minutes at 65°C, stops the enzymatic action of DNase I.

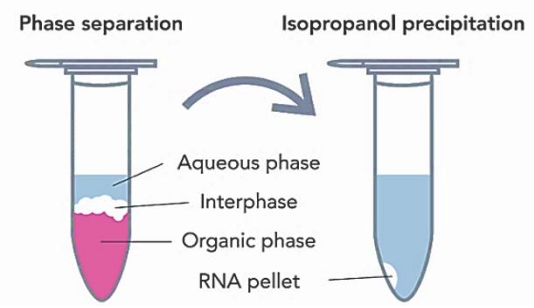

Figure 14: *Representation of the Separation phase after TRIZol addition and isopropanol treatment in the RNA extraction step for the qRT-PCR technique. Adapted from (ZYMO Research, n.d.)*

For the gene expression study, as mentioned before, it is necessary to perform reverse transcription previously, that is, the synthesis of cDNA from RNA. For this reaction to the DNase I-treated RNA resulting from the previous step, 5 µl of Braun sterile bi-distilled H2O and 4 µl of qScriptTM cDNA SuperMix are added. This SuperMix contains

a buffer, dNTPs, MgCl2, primers, RNase inhibitor protein, qScript reverse transcriptase and stabilisers. qScript is an RNase H(+) derivative of MMLV reverse transcriptase, which is a multifunctional enzyme with RNA- and DNA-dependent DNA polymerase activity and is responsible for the reverse transcription of single-stranded RNA into double-stranded DNA (Quanta BioSciences, 2007).

The contents are mixed, centrifuged, and subjected to incubation of different temperature cycles in a thermocycler. Thus, the samples are initially subjected to 25°C for 5 minutes, followed by 30 minutes at 42°C and finally 5 minutes at 85°C. At the end of this step, all cDNA samples can be stored at -20°C until being used.

The quantification of a target gene is made possible by detecting and quantifying the fluorescence produced during amplification cycles (CTs). Different fluorescent dyes can be used in this technique; an example is the SYBR Green dye, which mainly shows high affinity with the DNA double-strand, binding to it whenever present in the reaction. In this way, it is expected that the intensity of the fluorescent signal increases in proportion of molecules of PCR products. This signal generated during the exponential phase of the reaction allows information about the initial quantity of the amplification target to be obtained. If a specific target sequence is abundant in the sample under study, amplification will be observed in the first cycles of the reaction (Bio-Rad Laboratories, 2006).

The SYBR-based approach for RT-qPCR is commonly used once it is relatively inexpensive, as no probe design is required, only two primers are used, and it is adaptable to different targets. However, as the measurement of the signal generated in SYBR depends on the presence of double-stranded DNA, regardless of sequence, special care needs to be taken with the specificity of the technique and the interaction of the primers. Primers lacking specificity can bind not specifically (so-called off-targeting), generating false positive signals. Furthermore, primer-dimer interactions can generate false signals and increase the lower limit of assay detection (Covey, 2021).

Before performing RT-qPCR for each sample, a standard curve needs to be constructed to calculate the efficiency and specificity of the primers. The respective standard curves are obtained from a RT-qPCR reaction in which different cDNA dilutions are analysed in duplicate (1:2, 1:10, 1:50, 1:250, 1:1250). When the values between the duplicates are discordant, they are excluded from the analysis of the standard curves. The pattern of these curves allows different dilution points to be obtained and some important parameters to be determined, such as correlation coefficients, slope values and the efficiency of the PCR reaction.

As for the determination of the standard curves, the expression levels of all genes are evaluated using the StepOnePlusTM Real-Time PCR System. For all reactions, samples are run in duplicate to minimise within-plate variations. In addition, for each plate filled, a negative control (without any sample present) is also included, essential to detect any contamination during the procedure. Also, as prevention of possible contaminations, PowerUp SYBR Green Master Mix is used, which has the particularity of containing Uracil N-Glycosylase (UNG) and dUTPs, instead of 2'-deoxythymidine 5'-triphosphate (dTTPs). This characteristic prevents contamination with PCR products from previous reactions since, in an initial phase, the UNG degrades all the sequences present with dUTPs. At the same time, our sample is intact since its sequence contains dTTPs and not dUTPs. With the temperature change, this enzyme becomes inactive, the target sequence is amplified, and the new amplified products start incorporating dUTPs.

In each reaction, 5 µl of 2x PowerUp SYBR Green Master Mix, 0.25 µl of each primer, 2 µL of diluted cDNA and 2.5 µL of Braun Sterile Bidistilled H2O are added, giving a final volume of 10 µl per well. The reaction conditions are shown in Table 8.

***Table 8**: PCR conditions of temperature and time, with PowerUp SYBR Green Master Mix, for the RT-qPCR technique.*

Time	Temperature	Number of Cycles	Step
2 minutes	50ºC	1	Degradation of products from previous reactions
2 minutes	95ºC	1	
3 seconds	95ºC	40	Exponential amplification
30 seconds	60ºC		
15 seconds	95ºC	1	Melting Curve
1 minute	60ºC	1	
15 seconds	95ºC	1	

3. Results

3.1 Database with Genetic Results of Pregnancy Loss Cases

A database of 1405 cases of pregnancy losses was analysed. The database consisted of products of conception cases that had undergone genetic and anatomopathological analysis, between 2003 and the last month of 2022.

Both RPL and sporadic pregnancy loss cases were included. The database provided information on the karyotype, aCGH, FISH, and QF-PCR results, the patient's clinical

history, the trimester of pregnancy, and some clinical indications about the pregnancy loss, such as suspected causes.

Over the years, 123 FISH and 53 aCGH techniques were performed.

From the 1405 cases, as shown in Figure 15, 56% of the samples were female, and 36% were from the masculine sex. The sex was determined using QF-PCR, karyotype, or FISH results. In 8% of the cases, the sex couldn't be determined because the tissue was of insufficient quality.

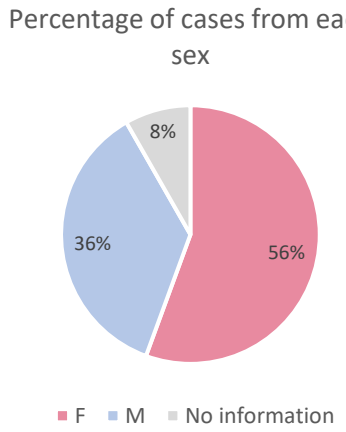

Figure 15: *Graphic representing the percentage of cases from each sex.*

Recurrent pregnancy losses were found in 37% of the cases.

We can observe in Figure 16 that most of the cases were material from the first trimester, followed by the second and then the third trimester. The first trimester was defined up to 12 weeks and the second trimester between 13 and 24 weeks. This information was previously obtained from the obstetrics records, and then confirmed after anatomopathologic study.

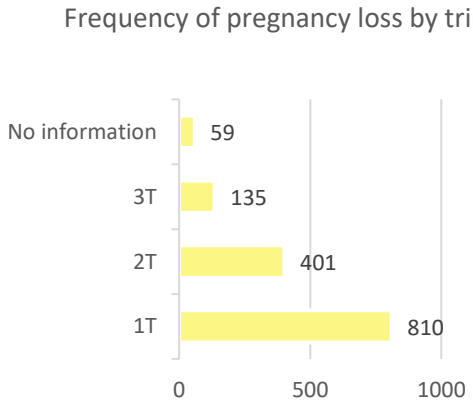

Figure 16: *Graphic of the frequency of pregnancy losses divided by trimester.*

In Figure 17 we can observed the percentage of cases with a normal/abnormal result or without a result, including all the studies performed. It shows that almost 50% of the cases had a normal result. It should be noted that being a retrospective database, not all techniques were performed in 2013, having been made available over the years. Additionally, the studies carried out also depend on what is requested by the hospital.

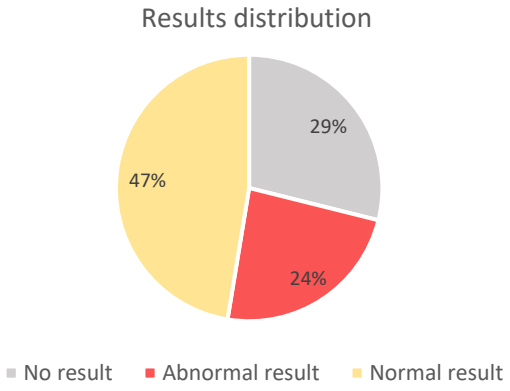

Figure 17: *Circle Graphic illustrating the distribution of the results.*

From the 1405 cases included, QF-PCR, to detect maternal contamination, was performed in 208 cases, from which 97 cases had no contamination detected. Maternal contamination was detected in the remaining 111 cases. These cases were included in the "no result" group unless an abnormal result was detected using other technique.

This "no result" group (29%) also includes cases where the cell culture did not grow due to the poor quality of the tissue received, cases where the cell culture did not provide metaphases to be analysed. In these cases, none of the techniques provided a reliable result. A cell culture was done in 1327 samples, 336 of them weren't successful (25%).

Focusing on the 333 cases with abnormal result, the majority were chromosomal numeric abnormalities (83% - 276 cases), 31 cases had structural anomalies detected (9%), and 8% were cases where more than one cell line were observed (mosaicism), Figure 18. The Robertsonian translocation between chromosomes 13 and 14 was the most common structural abnormality, while tetraploidy was the most observed mosaic abnormality.

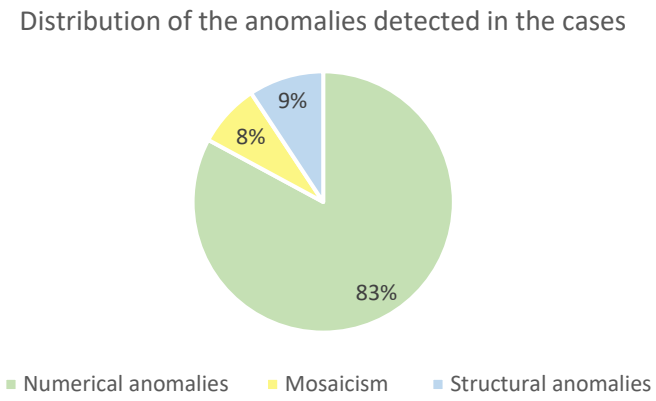

Figure 18: *Circle Graphic illustrating the distribution of the anomalies detected in the cases.*

Figure 19 shows the distribution of the numerical abnormalities by type of chromosome involved and including mosaic cases, with a total of 290 cases. The abnormalities present with a frequency less than or equal to two cases were added to the column "Others".

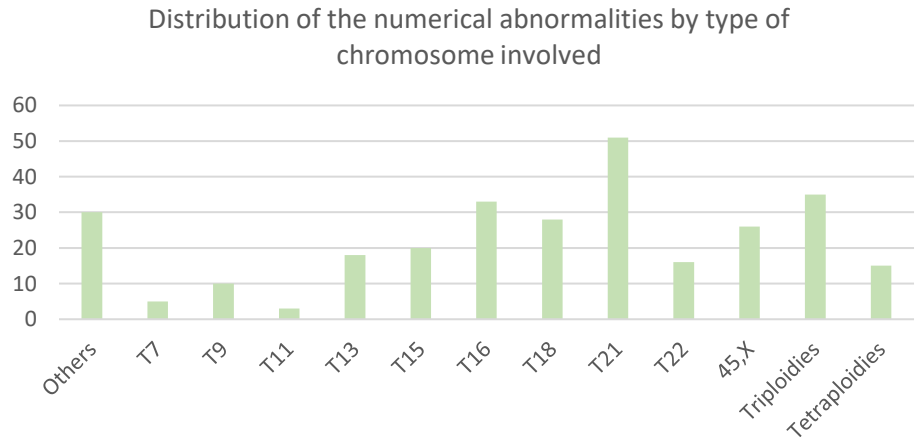

Figure 19: *Graphic illustrating the distribution of the numerical abnormalities by type of chromosome involved.*

The most frequent numerical anomaly was trisomy 21, followed by triploidy, trisomy 16 and 18, and then monosomy X.

Mosaic cases included more frequently tetraploidies and trisomy 16.

3.2 Clinical cases

3.2.1 Clinical Case 1

Samples (fetal tissue and placenta) from a 17-week loss of pregnancy arrived at the laboratory for genetic study, as the mother had experienced recurrent pregnancy losses. A QF-PCR was requested as first-line method, followed by an aCGH, in the tissue, if the result came back normal. Peripheral blood from the mother was also studied to rule out maternal contamination in the placental sample.

As previously mentioned, the copy number of each allele is determined by the ratio of peak area detected for each marker. A normal diploid sample contains two chromosomes, one from each parent. Two alleles of a marker for a specific chromosome are detected by the presence of two peaks with a ratio of 1:1 when the marker is heterozygous, and one peak when the marker is homozygous (having two alleles with the same length and the same number of repeats on each chromosome).

The observation of an extra STR allele in a three-peak pattern with a 1:1:1 ratio or a two-peak pattern with a 2:1 or 1:2 ratio is indicative of the presence of an additional sequence, which could represent an additional chromosome, as seen in the case of trisomies. When this happens in different STR markers could represent a triploidy, or a maternal contamination.

A comparison of the allelic profile of the fetal or placenta sample with the maternal sample is performed to exclude maternal contamination, by having two separate QF-PCR executed, one with the DNA from the placenta and one from the mother's DNA. The common alleles between the maternal sample and the product of the conception sample are identified for each STR. If they are identical (having the same genotype in both the product of conception and the mother), they are not counted to exclude maternal contamination. In the end, it is determined how many out of 19 STRs allow the exclusion of maternal contamination in the sample.

In Figure 20, we have part of the results obtained from the 2 QF-PCR tests, and the peaks shared between the mother and the placenta are marked in red (A and C - QF-PCR from the product of conception, and B and D - QF-PCR from the mother). As can be seen, only in STR D21S1446 is observed two equal peaks; in all the others, the tissue had one peak similar to that of the mother and the other from the father. In total, 3 out of 19 STRs are the same, confirming the exclusion of maternal contamination in the placenta sample.

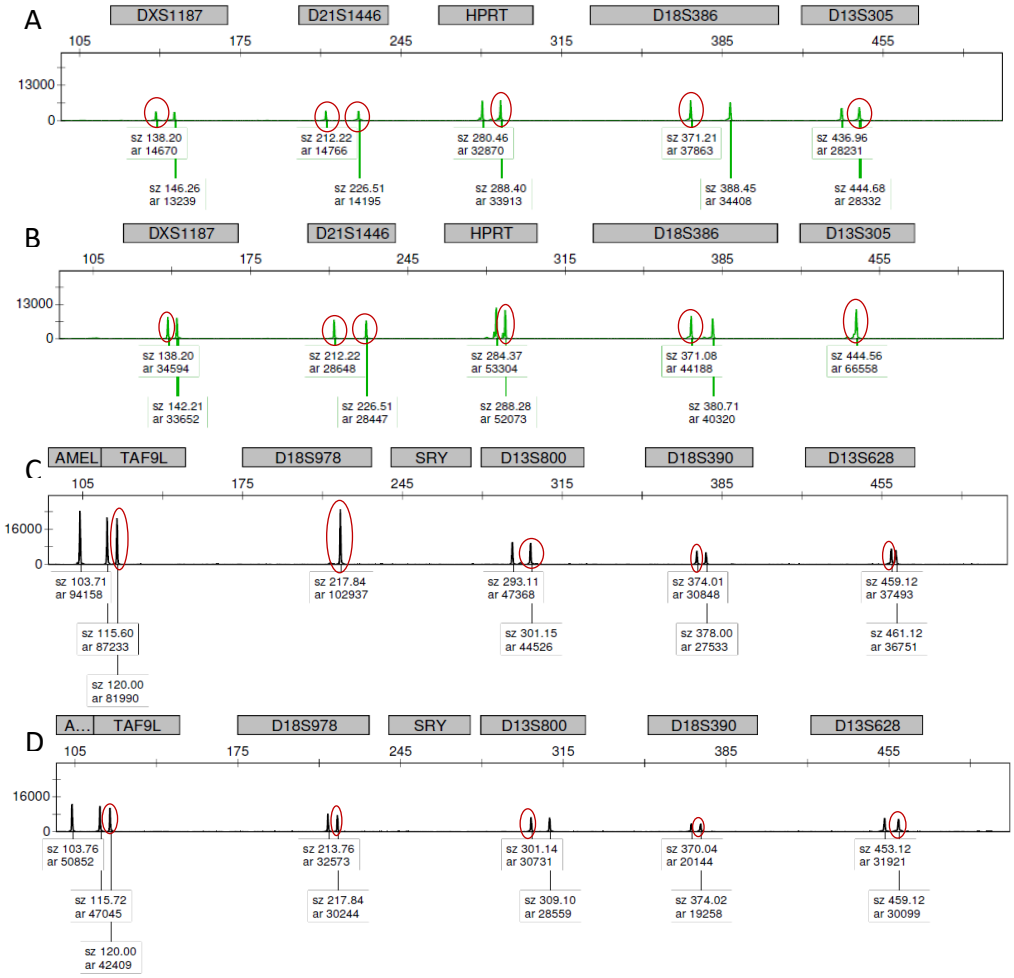

Figure 20: *Comparing the QF-PCR results from the peripheral blood of the mother and the case 1 placental sample. A and C- Part of the result of the QF-PCR of the placental sample. B and D- Part of the result of the QF-PCR of the peripheral blood.*

As can be observed in Figures 20A and C, all the STRs have two peaks, thus two alleles. The rest of the STRs from the results had two alleles identified, too; therefore, the sample didn't have any trisomy 13, 18 or 21. Analysing the STRs for sex chromosomes, all the ones from chromosome X had two alleles, and as can be seen in Figure 20C, the SRY STR didn't amplify; thus, the sample is an XX sample.

From this technique, we could conclude that the sample didn't have maternal contamination, that it is a female fetus, without aneuploidies of the studied chromosomes.

The aCGH with 8x60k configuration was then performed in the DNA extracted from the tissue. The results are presented in Figures 21, 22, and Table 9. In Figure 21, it is observed a Genome View, giving a general perspective of all the amplification and

deletions detected. There are several CNVs identified that are considered normal variant (polymorphisms), which are not reported.

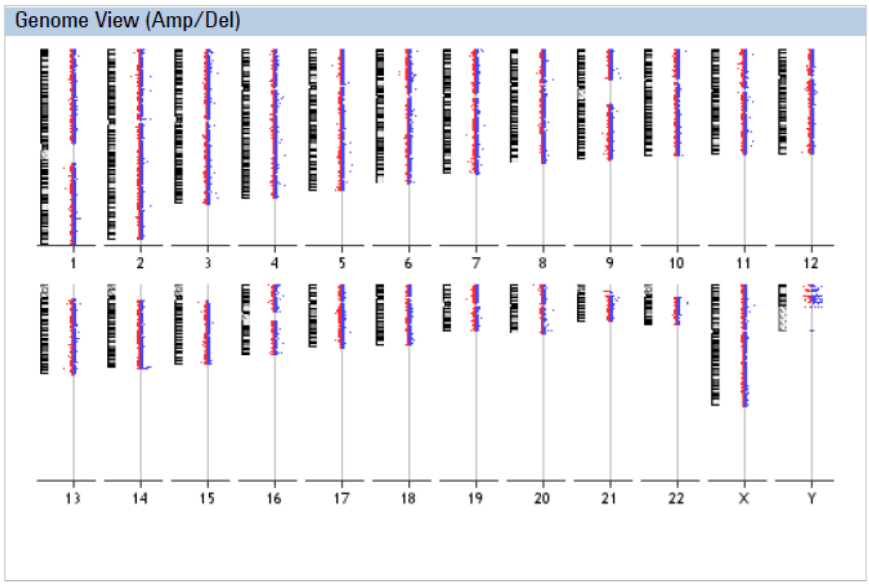

Figure 21: *aCGH results from the case 1 fetal tissue sample. Genome view from all chromosomes using a female reference.*

The aCGH analysis revealed a heterozygous duplication of approximately 525Kb on the short arm of the chromosome 16, involving the p11 region within genomic positions 29673954_30198600 as shown in Figure 22 and Table 9. There are 42 genes included in the interval, and a list with some of them could be also observed in Table 9. For analysis, several databases were used, namely: ClinGen (https://www.clinicalgenome.org), DECIPHER (http://decipher.sanger.ac.uk), OMIM (http://omim.org/), Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/), and internal database.

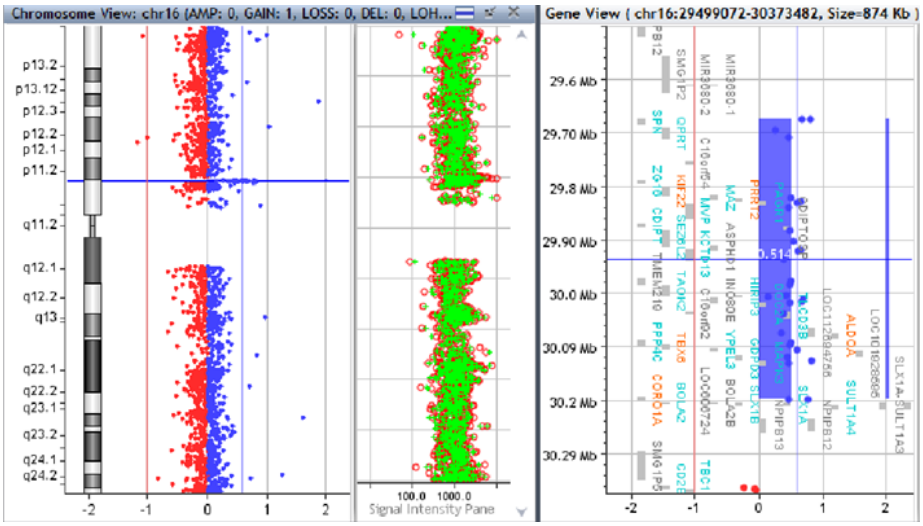

Figure 22*: aCGH result from the case 1 fetal tissue sample. Chromosome and gene view from chromosome 16.*

Resorting to the platforms mentioned above, the CNV arr[GRCH37] 16p11.2(29673954_30198600)x3 was classified as pathogenic; therefore, it was reported, as shown in Table 9.

According to ClinGen this region includes genes that are dosage sensitive, such as *ALDOA*, *CORO1A* and *PRRT2.* The clinical features already reported associated with 16p11.2 duplication (OMIM: 614671) may include developmental delays (speech, language, and motor delays), autism spectrum disorder, intellectual disability, seizures, microcephaly, decreased body mass index, behavioural problems, psychiatric disorders, congenital abnormalities, and others. There were cases where incomplete penetrance has been observed (D'Angelo et al., 2016; Steinman et al., 2016; Weiss et al., 2008). This is a phenomenon where the same genotype can either cause the expected clinical phenotype or be absent.

The presence of congenital abnormalities in a fetus could be the cause of the abortion (Bender Atik et al., 2022).

Table 9: *aCGH result of the case1 fetal tissue sample. Table detailing the CNV reported.*

Chr	Min Start-Stop(bp) Max Start-Stop (bp)	Min Cytoband Max Cytoband	Min Size (Kb) Max Size (Kb)	#Probes	Gain	Genes included	Classification
16	29673954-30198600 29133736-30363911	p11.2 p11.2	524.647 1,230.176	30	0.514	ALDOA, C16orf54, CORO1A, PRRT2, SPN, TBX6, ZG16…	Pathogenic

3.2.2 Clinical Case 2

A placenta and tissue sample, from a pregnancy loss, arrived for cytogenetic analysis with a suspicion of trisomy 21. The first technique to be performed was QF-PCR, along with cell culture, to execute a karyotype.

Analysing the QF-PCR result, as shown in Figure 23, and focusing on the first row (DXS6803), only one peak appears. This indicates that the sample has only one X chromosome. When determining the sex of the sample, it is essential to analyse the AMEL and SRY STR markers. Combining this information with the peak that appeared on the SRY and the two peaks identified in the AMEL confirms that the sample is from a male. The AMEL marker amplifies non-polymorphic sequences on the X (104 bp) and Y (110 bp) chromosomes.

Now, turning our attention to chromosome 21 and analysing the STRs (D21S1435, D21S11, D21S1437, and D21S1446), circled in red, we can observe that three of them show three peaks, representing the presence of three copies of chromosome 21 (trisomy 21). The only STR on chromosome 21 that does not show three peaks is D21S1437, which displays two peaks, with one of them being higher. This higher peak indicates the presence of two homozygous alleles. Only two alleles can be observed by analysing the other STRs from the remaining chromosomes.

In summary, this sample is from a male fetus with trisomy 21 (Down syndrome). Chromosomes 13 and 18 are normal.

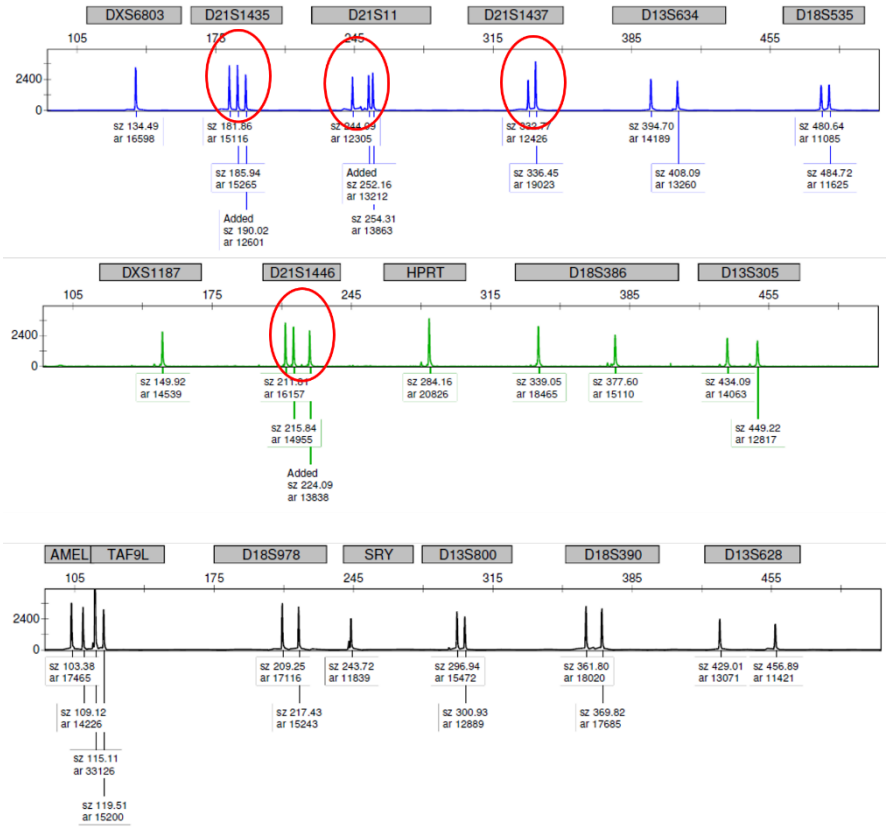

Figure 23: *QF-PCR results from the fetal tissue sample. Circled in red are the SRTs from chromosome 21.*

The karyotype from the fetal tissue is illustrated in Figure 24 showing a Robertsonian translocation between the two chromosomes 21 (circled in red). Although the existence of a Robertsonian translocation makes it appear that the case has the normal number of chromosomes (46), there is additional information for three chromosomes 21, confirming the trisomy detected in the QF-PCR. As mentioned in the last technique, a Y chromosome is present. The karyotype of the product of conception is 46,XY,+21,der(21;21)(q10;q10). This indicates that it is a male with 46 chromosomes,

presenting a Robertsonian translocation between two homologous chromosomes 21, with the breakpoints from both chromosomes located on q10.

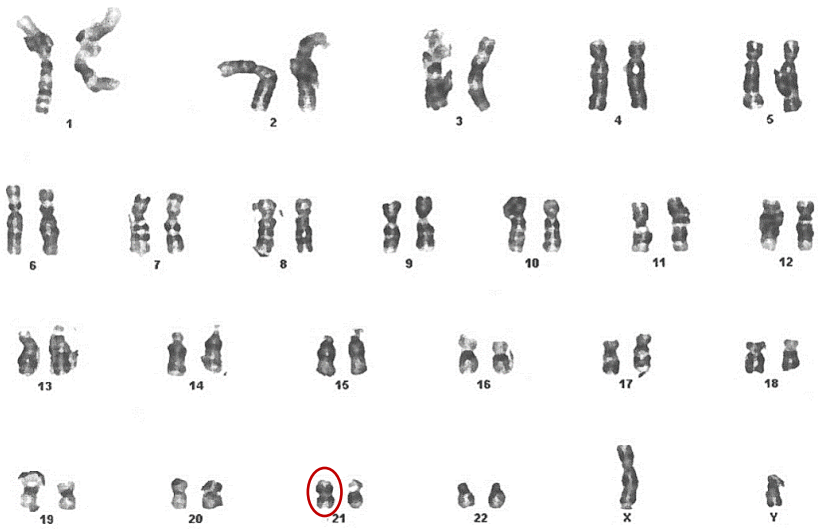

Figure 24: *Karyogram from the tissue sample – case 2. (46, XY,+21,der(21;21)(q10;q10)). This presents an abnormal karyotype.*

When a Robertsonian translocation is detected, it is important to study the parents because the risk of recurrence can be high, and reproductive risks can be discussed in a genetic counselling consultation. In this case, only the mother could be studied, and a cell culture was done to execute a karyotype.

The karyotype of the mother, as shown in Figure 25, appears to be normal 46, XX. Therefore, the translocation was not inherited from the female progenitor. The father was not available to perform the study.

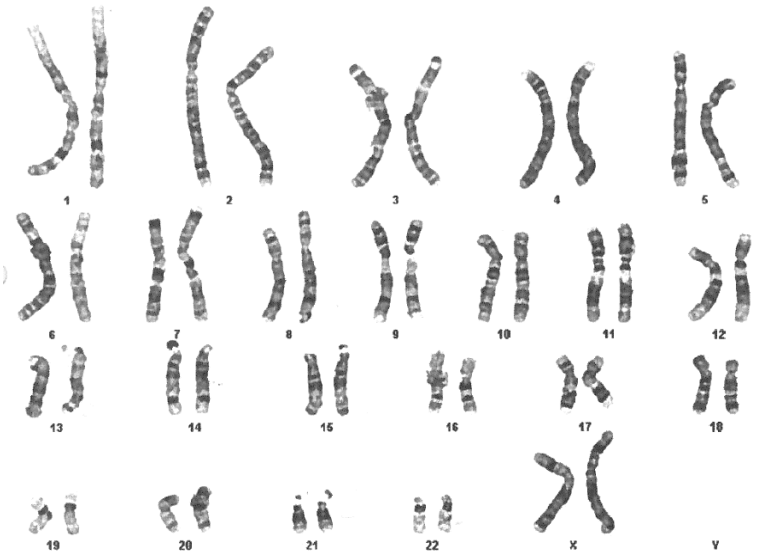

Figure 25: *Karyogram of the mother- case 2. (46,XX). This represents a normal karyotype.*

The risk of recurrence could not be established as the father's karyotype was not performing, not knowing if the translocation was inherited or de novo. In any case, the female parent could know that her individual risk of having a child with trisomy 21 overlaps that of the general population according to her age (excluding the possibility of the existence of gonadal mosaicism).

3.2.3 Clinical Case 3

A couple that experienced RPL, underwent genetic testing to study a possible male factor. A semen sample was sent to the FMUP and the TUNEL and FISH techniques were requested on spermatozoa.

FISH technique was performed in the sperm sample, using the Aneuvysion kit mentioned earlier. Two thousand and seven cells were counted in two separate moments, and the percentage of aneuploidies for each chromosome analysed was calculated.

Figure 26 represents an example of what could be visualized with the FISH technique. In Figure 26A, the probe that hybridized to the specific region of the chromosome 13 can be visualized with a green colour. In contrast, the one hybridised to the region of chromosome 21 is represented with an orange colour. Passing on to the next slide hybridised with the CEP probes, Figure 26B, chromosome 18 is visualised with a blue colour, the X is marked with a green probe, and the chromosome Y with an orange labelled probe.

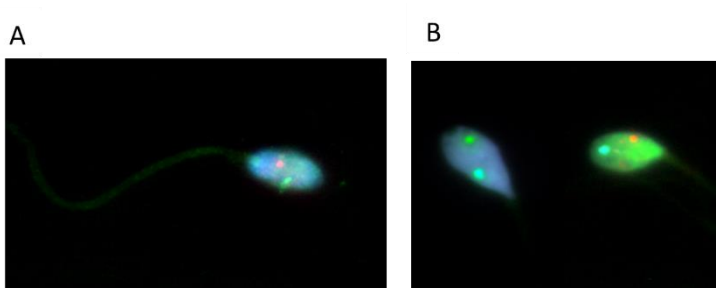

Figure 26: *Fish in spermatozoa results. A- Results using proves 13(green) and 21(red). B- Results using probes for X (green), Y(red) and 18(blue).*

The rates of disomy observed are shown in Table 10.

Table 10: *Rates of disomy observed in spermatozoa after FISH technique.*

Chromosome	Rates of disomy observed (rv: reference value)
Chromosome 13	0.01% (<0.29%)
Chromosome 18	0.00% (<0.40%)
Chromosome 21	0.10% (<0.49%)
Chromosome XX	0.01% (<0.71%)
Chromosome XY	0.01% (<0.43%)
Chromosome YY	0.00% (<0.61%)

This result indicates a low frequency of numerical chromosomal abnormalities involving chromosomes 13, 18, and 21, X and Y in the spermatozoa of the male partner.

After the TUNEL technique was also performed, 2009 spermatozoa were counted, in two separate moments, as shown in Table 11 and the percentage of cell's exhibiting fragmentation was calculated.

When DNA fragmentation is observed, the spermatozoa can be seen with green fluorescence, as represented in Figure 27.

Table 11: *Results of cell counting after TUNEL technique.*

	With fragmentation	Total cells counted
Moment 1	375	1006
Moment 2	388	1003

From the 2009 cells, 763 showed fragmentation signals, being the average percentage of fragmentation approximately 38%. This value is above the limit considered normal by the ASRM Practice Comitte (<36%), leading us to conclude that DNA fragmentation could be related to the infertility.

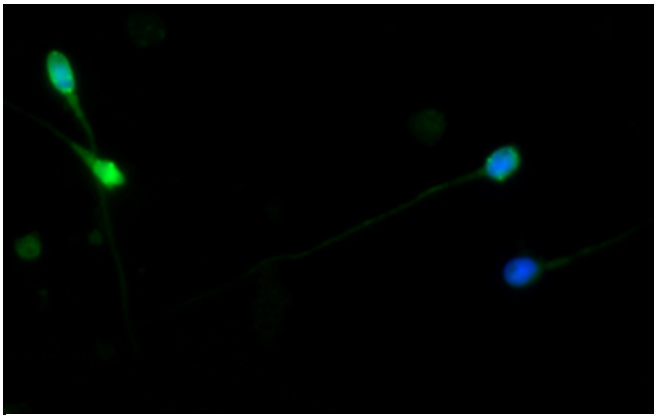

Figure 27: *Example of sperm cells visualized under fluorescence microscope after the TUNEL technique.*

3.3 Preliminary study of idiopathic cases

This project aimed to evaluate the gene expression of various genes involved in epigenetic mechanisms to determine if dysregulation of gene expression, leading to overexpression or under-expression, could be associated to spontaneous abortion.

We analysed the expression of 6 epigenetic modifiers, including IGF2, CDKN1C, KCNQ1, PHLDA2, MEST, and PEG10, in 31 placentas during the second trimester of gestation. This included 18 controls and 13 idiopathic cases. Additionally, we analysed 31 placentas from the third trimester, comprising 12 controls, 10 idiopathic cases, and 9 term placentas. The term “placentas” refers to placentas obtained from pregnancies that have reached full term (after 37 weeks) and have been through a successful birth. For both groups, the controls were selected from cases of abortion without identifiable genetic causes, such as infections, umbilical cord pathologies, or uterine anatomical abnormalities.

These genes were selected because some previous studies have shown the involvement of PEG10 and MEST in embryonic lethality, plus according to the literature, a decrease in MEST protein expression was observed in human placentas of missed abortions (Ono et al., 2006; Peng et al., 2016). Furthermore, the group had already shown some correlation between the alteration of expression of imprinted genes, namely CDKN1C, IGF2, KCNQ1 and PHLDA2, in placental samples of spontaneous abortions (Cordeiro et al., 2014; Dória et al., 2010).

After collecting the gene expression values for both control and idiopathic cases, the data was processed using the $2^{-\Delta\Delta Ct}$ formula. The data was then statistically analysed using the Mann-Whitney U test to determine if there was a correlation between the gene expression of each specific gene and abortion.

In Figure 28, differences in gene expression among the different studied groups for the second trimester can be observed.

As can be seen, in the second trimester, only the MEST gene showed a significant difference in expression values. The gene exhibited a tendency to be under-expressed in idiopathic cases.

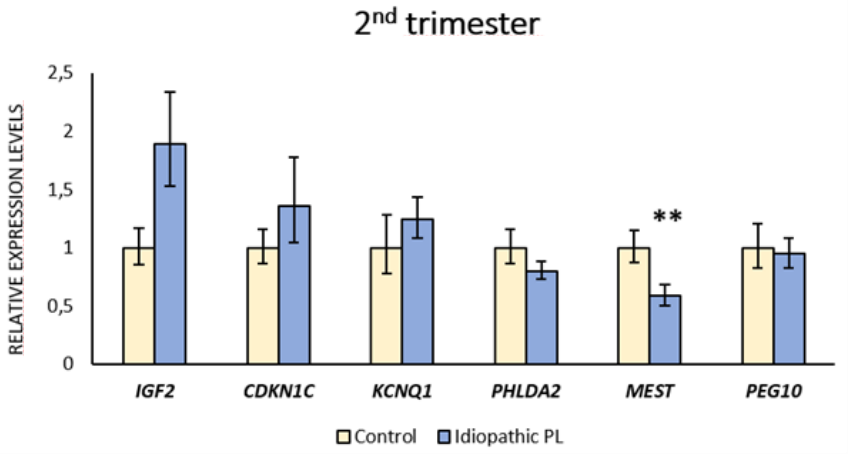

Figure 28: *Graphic presenting the differences in gene among the groups from the second trimester. Bars represent 2ΔΔCt ± SEM (standard error of the mean); * represents p < 0.05, ** represents p < 0.01, and *** represents p < 0.001.*

For the third trimester, the differences in gene expression between the three groups studied are shown in Figure 29.

In the third trimester, with a significant difference, the IGF2, KCNQ1, and PEG10 genes increased expression compared to term cases. As for the MEST and CDKN1C, genes showed a substantial under-expression in idiopathic cases compared to term cases. None of the genes demonstrated significant differences when comparing the idiopathic cases to the controls.

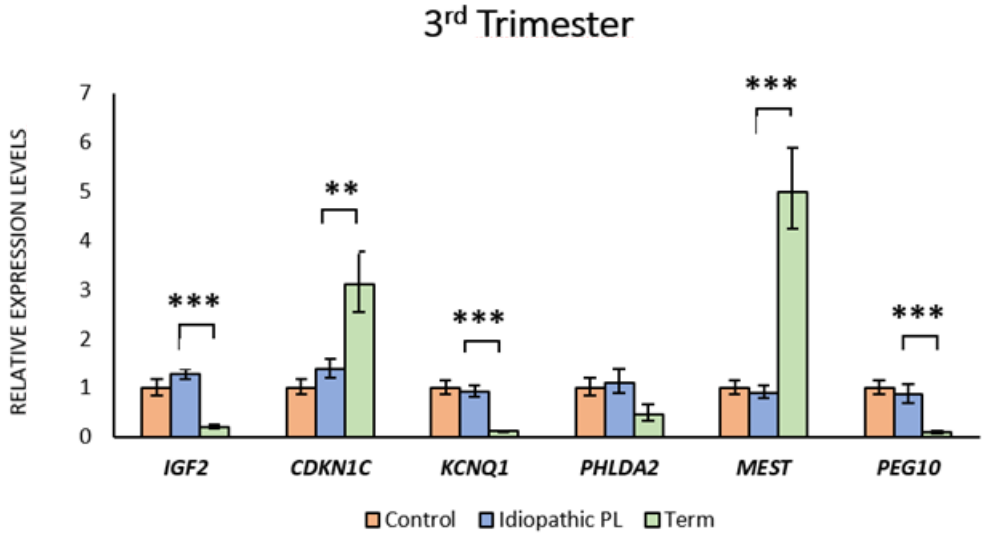

Figure 29: *Graphic presenting the differences in gene among the groups from the third trimester. Bars represent 2ΔΔCt ± SEM (standard error of the mean); * represents p < 0.05, ** represents p < 0.01, and *** represents p < 0.001.*

4. Discussion

Firstly, our results showed that out of the 1405 cases analysed, only 53 underwent aCGH testing. Although it is important to note that this technique was not available as a standard technique in the early years of the database. aCGH is now implemented as a routine technique in the study of pregnancy losses.

We observed 20% more female products of conception than males. However, it's essential to consider that the maternal contamination exclusion technique was not consistently performed, so some of the female cases might refer to maternal material. We also observed that most of the products of conception submitted to our service were from the first trimester which can be explained by the fact that spontaneous abortions are more frequent in the 1st trimester. It is also important to stress that the guidelines for investigating pregnancy losses have changed over the years, namely which techniques should be required. Nowadays, pregnancy loss case is sent for cytogenetic investigation if it is a case of RPL or a stillbirth (Monteiro et al., 2022).

Additionally, we noticed that in 29% of cases that yielded no result, poor cell culture quality was a common issue, highlighting one of the significant challenges encountered while presenting results. The number of unsuccessful cell cultures (25%) was lower than the expected 50%, as reported by Warren and Silver (2008), but it remains higher than desired. To improve this rate, efforts should focus on shortening the time between tissue harvest and culture initiation and ensuring better quality tissue is provided. Additionally, it's crucial to note that there might be cases resulting from maternal material contamination in the "normal" category (47%).

The number of numerical anomalies detected in our cases was significantly higher than the structural anomalies and mosaicism, which is consistent with what many authors have reported, including a previous study from the group (Dória et al., 2009; Monteiro et al., 2022; Warren & Silver, 2008).

Regarding the most prevalent trisomy in products of conception, there is a consensus among several authors that trisomy 16 is the most common, followed by other autosomal aneuploidies such as monosomy X and triploidies (Gajjar et al., 2023; Monteiro et al., 2022; Reddy et al., 2012; Teles et al., 2017; Warren & Silver, 2008). However, in our case, the most frequently observed aneuploidy was trisomy 21, followed by triploidies, trisomy 16 and 18, and then monosomy X. This discrepancy can be attributed to the fact that in many cases, only the QF-PCR technique was executed, which only detects anomalies in chromosomes 13, 18, and 21. As a result, many cases

were reported as normal when other anomalies could be involved, leading to a different pattern of observed trisomies in our study.

The chosen techniques to evaluate the causes of each abortion are crucial, as each technique has advantages and disadvantages.

Focusing on the first clinical case, the uses of both QF-PCR and aCGH technique was crucial. Firstly, it allowed the exclusion of maternal contamination, ensuring accurate results. Secondly, it enabled to identify a possible cause behind the loss of pregnancy. If only QF-PCR had been used, the 874Kb pathogenic duplication would not be reported, and a possible cause of the pregnancy loss would not be noticed. If a karyotype had been performed instead of aCGH, the duplication would have gone unnoticed, as karyotyping has a detection limit of 4 to 5Mb. Consequently, it is vital to determine which technique to perform firstly based on the clinical indication and then proceed with subsequent testing to enhance the reliability of the diagnosis.

In the second clinical case, it is essential to stress that performing a karyotype remains crucial. Relying solely on the QF-PCR technique would only allow us to identify a chromosome 21 trisomy without knowing its specific form. Trisomy 21 could be present either in a free form, resulting in a karyotype of 47,XY,+21 or 47,XX,+21, with a clearly identifiable extra chromosome, or as a result of a translocation (Antonarakis et al., 2020). There is a significant difference in the risk of recurrence between free trisomy 21 and a Robertsonian translocation. In cases of free trisomy, the likely causes are errors in maternal meiosis I or meiosis II, or eventually during mitosis after the formation of the zygote. The risk of recurrence in this scenario is about 1% in women under 30 years. For women over 30, the recurrence risk is due to advanced age risk (Gersen & Keagle, 2013). However, when a fetus is a carrier of a translocation, it becomes necessary to study both parents. If the translocation is inherited the risk of recurrence is near 100%, and a genetic counselling consultation is recommended. In the clinical case described, a Robertsonian translocation between both 21 homologous chromosomes was present in the fetus and if the same translocation was confirmed in one of the parents, the recurrence risk would be very high. In theory, all chromosome segregations within a carrier parent of a homologous Robertsonian translocation would produce monosomic or trisomic conceptions (Gersen & Keagle, 2013).
Due to these critical distinctions, it remains essential to continue performing karyotypes, as it is the only available technique that can differentiate between these types of cases.

On clinical case number 3, the fragmentation percentage is above the limit considered normal thus being considered a possible cause of the infertility. In this case of high

sperm DNA fragmentation, specialized techniques like Intracytoplasmic Sperm Injection (ICSI) may be employed. With ICSI, a single sperm is directly injected into an egg to facilitate fertilization. Some studies showed that sperm chosen with hyaluronan-selected sperm would decrease pregnancy loss rates compared with ICSI with sperm selected using standard methods (Bender Atik et al., 2022). Another approach suggested as an effective way to overcome unexplained ICSI failures is ICSI using testicular spermatozoa. In this case the spermatozoa are more protected from external factors that may cause SDF (Esteves et al., 2021).
The study of Microdeletions in Y chromosome should not be forgotten, once it could be another factor contributing to infertility.

Passing on to the preliminary study presented, it is important to emphasize that the selection of appropriate controls is crucial. In the second trimester the aim was to compare the expression values of the idiopathic cases to the cases of abortion without genetic causes.

On the third trimester, the research group established two types of controls to optimize the results. One of them was term placentas from healthy births to study the differences in gene expression between healthy pregnancies and spontaneous abortions. This would serve as the control for the expected result. The other one was chosen to include abortion cases with non-genetic cause as controls, as well. This decision was made because this cases with non-genetic causes went through the abortion process, whereas term placentas did not, which could be a variable. In addition, term placentas are of a gestational age of over 37 weeks, whereas the idiopathic cases studied and the cases with non-genetic causes cover gestational ages starting from 24 weeks. By comparing the idiopathic cases to both control groups, we can establish a better correlation with the gestational ages with the control cases with non-genetic causes, due to a better gestational age proximity.

This comprehensive approach ensures a more robust analysis and interpretation of the gene expression data.

5. Conclusion

Pregnancy loss is one of the most common complications during pregnancy. Currently, spontaneous abortion continues to present a significant medical challenge, making it crucial to study the underlying causes of human PL.

The analysis of the pregnancy losses database was very important to understand how different techniques have different advantages and disadvantages, and the choice of

the one to perform must be made according to the clinical indication provided ensuring optimal cost-effectiveness.

Each method has its strengths and limitations, and selecting the most appropriate one is essential. Currently, ensuring that the material from the product of conception arrives at the laboratory with high quality is of utmost importance to reduce the percentage of cases that remain unanswered due to poor sample quality. This is essential for accurate and reliable genetic analysis, as it can directly impact the success of various diagnostic techniques, such as karyotyping and aCGH. Healthcare professionals need to prioritize proper sample collection, handling, and transportation, involving following established guidelines and best practices for sample preservation and storage to prevent degradation or contamination. In addition, emphasizing the need for maternal blood samples is essential for conducting proper exclusion of maternal contamination. Maternal contamination can introduce false results, especially in cases where fetal genetic material is present in low quantities. By obtaining maternal blood samples in addition to the product of conception, laboratories can accurately distinguish between fetal and maternal genetic material, ensuring that the material analysed is from the fetus.

Research on this topic still holds hope, as efforts continue to decode more causes of recurrent pregnancy loss. This ongoing investigation has the potential to shed light on future treatments and offer alternatives for achieving a healthy pregnancy.

Regarding future prospects in this field, several techniques have been implemented to enhance the diagnostic process.

Optical Genome Mapping (OGM), a recent technique, has evolved into a highly promising approach for identifying extensive structural variants within human genomes. It employs linearized strands of high molecular weight DNA, which are significantly longer than the DNA segments studied in contemporary second- and third-generation sequencing techniques, thereby achieving average read lengths exceeding 200 Kb (Dremsek et al., 2021).

The methodology relies on the specific fluorescent labelling of high molecular weight DNA, that is loaded into the Saphyr instrument, wherein electrophoresis linearizes the DNA molecules. These molecules are then subjected to high-resolution imaging through a fluorescence microscope. The extracted DNA, identified through captured images, are individually recognized, aligned, and reconstructed from scratch to generate the distinctive labelled genomic pattern of the sample (Q. Zhang et al., 2023).

When comparing this new technique to karyotyping and aCGH, which are currently being used as first-tier tests in several clinical indications for genetic studies, and acknowledging the limitations of both, OGM has the capability to detect both balanced and unbalanced alterations with a resolution of up to 30Kb. This grants OGM distinct advantages over both techniques, as it can identify a broader range of alterations with higher precision. However, it's important to note that this method has a limitation in detecting triploidies and higher-order polyploidies. This limitation is of significant importance in this field, considering that these alterations are present in approximately 17% of miscarriage cases, as demonstrated in this project. Other variants that appear to be undetectable by OGM are Robertsonian translocations and other whole-arm translocations that involve the centromere (Dremsek et al., 2021). As another vantage we have the capability of the OGM to detect low-level mosaics of >5% (Dremsek et al., 2021).

To perform the technique, high molecular weight DNA is extracted from 1-1.5 million viable cells, according to Bionano protocols. This is a major drawback, especially for the implementation of OGM in prenatal diagnosis, as the cell culture process to achieve that many cells is time-consuming (Q. Zhang et al., 2023).

At present, the average expense for OGM per sample is approximately $500. This makes the technology a bit more expensive than karyotyping, but it is more affordable than FISH panels or CMA. Moreover, OGM is significantly less expensive than Whole Genome Sequencing and Whole Exome Sequencing (Levy et al., 2023).

The single nucleotide polymorphism array (SNP-array) is a CMA type distinct from aCGH. While aCGH compares the DNA sample under test to a standard reference DNA sample, revealing relative DNA quantities across different genome regions, SNP-array employs a different approach. It can identify triploidy and loss of heterozygosity (LOH) by binding the test DNA sample to the array platform and analysing the signal intensity of SNP probes. This is a capability that aCGH lacks, as it cannot detect triploidy. In practical terms, SNP-array can simultaneously detect aneuploidy, polyploidy, subtle chromosomal imbalances, uniparental disomy, and mosaic chromosomal abnormalities. This comprehensive detection ability significantly improves the identification of fetal chromosomal abnormalities in miscarriages. Moreover, compared to Next-Generation Sequencing, the SNP-array method offers a more straightforward analysis and is more cost-effective (Xiang et al., 2020; You et al., 2018).

OGM is still undergoing testing and implementation, whereas the SNP-array technique is a well-established method that will soon be integrated into the laboratory's practices.

III- Assessing variants of uncertain significance a retrospective analysis of aCGH cases

1. Introduction

aCGH has established itself as the gold standard test for investigating neurodevelopmental disorders and congenital abnormalities. The detection of CNVs through aCGH analysis allows classifying these variants into different categories, including pathogenic, likely pathogenic, likely benign, benign, and VUS (Kowalczyk et al., 2022). One of the major ethical issues often raised is how to deal with these VUS, as in many cases, these variants are so rare that little information is available. Further studies are necessary to ascertain whether a VUS is associated with a specific disease.

The reclassification of CNVs from a VUS to a clinically significant variant or benign status is paramount for genetic counselling. To achieve this, laboratories must consistently monitor the updating of CNV databases and stay abreast of the latest literature, ensuring the provision of the most accurate and up-to-date diagnoses. In this context, our primary aim was to conduct a comprehensive reanalysis of VUS classified within the institution over ten years.

2. Methods

Our database included 3166 patients studied from 2012 to 2022, and 440 VUS were selected to be reclassified, considering that the same CNV were present at least in two different patients. The aCGH was performed using the Agilent 4x180K platform. The main clinical information from all the patients was also collected. The platforms used to update the clinical significance were mainly DGV, OMIM, NCBI, ClinGen, and DECIPHER.

3. Results

Out of 3166, 1938 patients (61.2%) presented 3244 VUS. The main clinical indications detected were autism spectrum (25.6%), followed by psychomotor development disorders (18.6%), as shown in Figure 30.

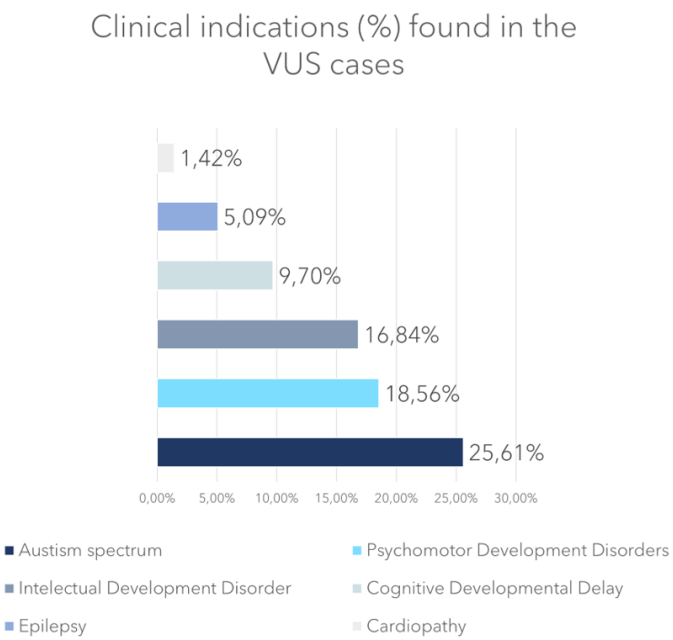

Figure 30: *Clinical indications found in the VUS cases.*

Analysing Figure 31, the chromosome with the highest frequency of VUS was chromosome X (12.6%), with 203 and 205 VUS overlapping the short and long arm, respectively, followed by chromosome 7 with a frequency of 7.4% overlapping mainly the long arm. Chromosome 21 registered the lowest number of VUS with a frequency of 1.8%.

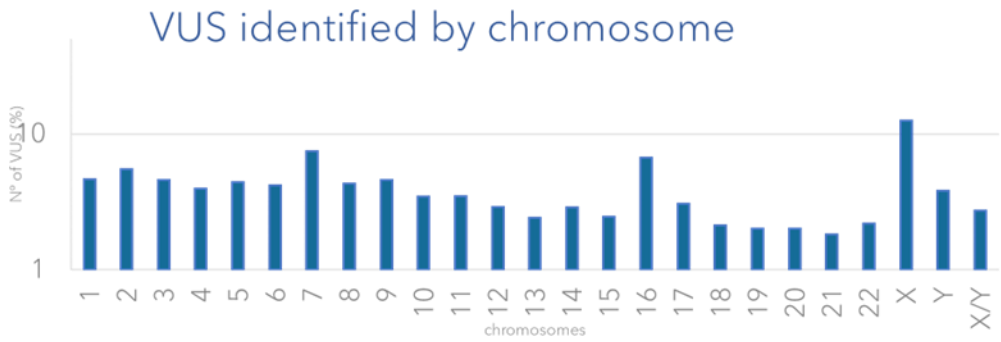

Figure 31: *Number of VUS (%) identified by chromosome.*

From the 440 VUS selected, 69 were reclassified as benign/likely benign variants, and one was reclassified as likely pathogenic.

Of these 69 VUS, 15.9% were from the X/Y chromosome, while chromosomes 18 and 21 had no CNVs reclassified, as shown in Figure 32. The second chromosome with the highest percentage of VUS reclassified was chromosome 16, with 11,6%.

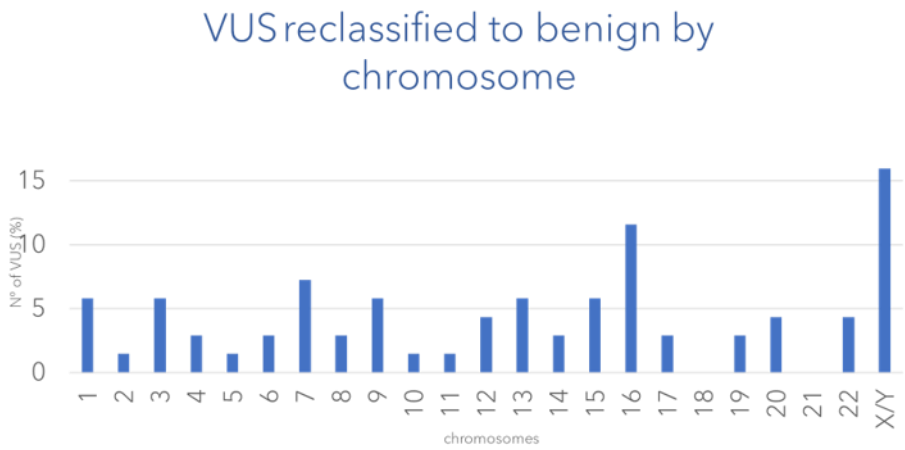

Figure 32: *Number (%) of VUS reclassified to benign by chromosome.*

The CNV reclassified to likely pathogenic was: arr[GRCH37] 7q11.22(69293697_69433076)x1, overlapping AUTS2 gene (Score HI:3 ClinGen).

4. Conclusion

The reclassification of VUS to pathogenic variants observed in this study highlights a critical issue in genetics. Despite ongoing efforts to update variant databases, there remains a significant inter-laboratory discrepancy in the classification of genetic variants. Moreover, even within a single laboratory, variant classification may change over time, reflecting the evolving understanding of genetic variations and their clinical implications.

Regular and systematic updates of variant databases are essential to establish a more robust and standardised classification of VUS. By doing so, we can better define the clinical significance of these variants, providing more accurate and reliable information to clinicians and patients. Particularly in prenatal diagnosis, where precise and definitive genetic information is of utmost importance, reporting these classification changes is crucial.

The reclassification of VUS to pathogenic variants has significant implications for genetic counselling. It allows for more informed and targeted discussions with patients

and their families, providing them with a clearer understanding of the potential risks and implications associated with specific genetic variations. By reducing the uncertainty and anxiety often associated with identifying VUS, we can empower individuals to make more informed decisions about their healthcare and family planning.

Moreover, sharing and disseminating the updated classification of VUS can contribute to advancing genetic knowledge on a broader scale. Collaborative efforts between laboratories and researchers can lead to a more comprehensive and standardised understanding of genetic variants and their clinical significance.

In conclusion, the reclassification of VUS to pathogenic variants underscores the importance of continuously updating variant databases and collaborative efforts in genetics. By striving for greater consistency in variant classification and reporting, we can improve the accuracy and impact of genetic counselling, particularly in the context of prenatal diagnosis, ultimately leading to better patient care and outcomes.

IV- Conclusion / Final Considerations

My internship at the Genetics Laboratory of FMUP allowed me to engage with various techniques in the field over nine months. During this time, I developed the ability to select the most suitable technique for each situation, effectively identifying and differentiating between them. I acquired hands-on laboratory knowledge, learning how to execute each technique, thereby applying the theoretical concepts I had learned during my master's studies.

Throughout the internship, I understood the significance of upholding ethical principles, good laboratory practices, and other key factors for laboratory accreditation. Ensuring quality at every step of the laboratory process was vital to producing accurate and swift results.

In summary, the internship had a profoundly positive impact on my personal and professional growth. I significantly enhanced my teamwork, responsibility, and professionalism. As for my professional development, the opportunity to engage with various techniques and gain experience in executing and analysing them has prepared me to enter the workforce in this field. The goals set at the beginning of the year were successfully achieved.

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VI – Annexes

Annex 1 – Reagents

Acetic acid glacial, NORMAPUR® 2,5L, 20104.323 – VWR/Prolabo

AmnioMAX™ C-100 Basal Medium, Gibco® 450 ml, 17001-074 – Alfagene/Life Technologies

AmnioMAX™ C-100 Suplement, Gibco® 75 ml, 12556-023 - Alfagene/Life Technologies

Aneuvysion Assay Kit (13/21; X/Y/18) / Vysis, 35-161075 (50 assays) – Abbot/Werfen Portugal

Antibiotic-antimycotic Gibco® 100X (PSA), 15240-062 – Alfagene/Life Technologies

Barium Hydroxide (BaOH), Merck®, 500 g, 1.01737.0500 – VWR/MERCK

Bio-AMF-1 Medium, 450 ml, BI01-190-1A – Bioportugal/Biological Industries

Bio-AMF-1 Suplement, 50 ml, BI01-192-1E – Bioportugal/Biological Industries

Braun sterile bi-distilled Water, 10 ml, 3653840, B/Braun

Colcemid® 10 µg/ml, Gibco® 10 ml, 15212-012 – Alfagene/Life Technologies

Collagenase Type 1, Gibco® 1 g, 17100-017 – Alfagene/Life Technologies

DAPI II, 125 ng/ml, 32-804931 (2x500 µl) – Abbot/Werfen Portugal

DTT, 2 g, 10 197 777 001 – Roche

EDTA (EthyleneDiamineTetraAcetic Acid), Sigma® 250 g, E-5134

FBS (Fetal bovine serum), Gibco®, 500 ml, 10270-106 – Alfagene/Life Technologies

Gelatine, Difco®, 0143-02

Gene Scan™- 600 LIZ™, 200 µL, 4408293, Applied Biosystems

Gentamicine 10mg/ml, Gibco® 100 ml, 15710-049 – Alfagene/Life Technologies

Gurr's Buffer Tablets pH 6.8+/-0.2, BDH Gurr® 100tablets, 363112P – VWR /BDH Prolabo

HiDi™ Formamide, 25 ml, 4311320, Applied Biosystems

Hydrochloric acid fuming 37%, Merck® 1 L, 1.00317.1000 – VWR/MERCK

Hydrogen peroxide 35% H_2O_2 130V, 1 L, Q0017 – Sociedade Portuense de Drogas, SA

In Situ Cell Death Detection Kit, Fluorescein, 50 tests, 11684795910 – Roche

Kit Elucigene QST*Rplusv2, 50 tests, AN0PLB2, Elucigene Diagnostics

Leishman Stain Solution, Sigma® 25 g, L6254 – Bioportugal/ Sigma

L-glutamine, 200 nM, Gibco®, 100ml, 25030-024 – Alfagene/Life Technologies

Methanol, NORMAPUR® 2,5 L, 20847.320 – VWR/Prolabo

Methotrexate 25 mg/ml, Tevaguard® 2 ml – HSJ Pharmacy

Netropsin 2 mg, N9653 – SIGMA

PBS (Phosphate Buffered Saline) 0,01M, pH7.4, Sigma®, P3813-10PAK – Sigma

Phytohemagglutinin-M (PHA-M), Gibco® 10 ml, 10576-015 – Alfagene/Life Technologies

Potassium Chloride (KCL), Emsure® 500 g, 1.04936.0500 – VWR/MERCK

Potassium dihydrogen phosphate (KH_2PO_4), Emsure® 1Kg, 1.04873.1000 – VWR/MERCK

RPMI 1640 + L-Glutamine + 25mM Hepes, 500 ml, L0495-500 – LabClinics/Biowest

Silver Nitrate ($AgNO_3$), EMSURE® 25 g, 1.01512.0025 – VRW/MERCK

Sodium Phosphate Dibasic Dihydrate ($Na_2HPO_4.2H_2O$)

SSC UltraPure™, 20X, Invitrogen™, 15557-036 – Alfagene/Life Technologies

Sodium Chloride, Merck® 1 Kg, 1.06404.1000 – VWR/MERCK

SureTag DNA Labelling Kit Ref.5190-3399

TritonTM-X-100, T8787 100 ml- Sigma

Thymidine, Sigma® 1 g, T-1895/T-9250 – Sigma

Trypsin, 1:250, Gibco® 100 g, 27250-018 – Alfagene/Life Technologies

Trypsin-EDTA (0.5%), 10X, Gibco® 100 ml, 15400-054 – Alfagene/Life Technologies

Annex 2 – Solutions

Complete Medium for lymphocytes culture: 100 ml of RPMI® 1640 + 0.5 ml of Gentamicine + 20 ml of FBS.

Transportation medium for tissue culture: 100ml de RPMI® + 1 ml PSA.

Medium for tissue culture 1: 44 ml of BIO-AMF-1 + 5 ml of BIO-AMF-1 supplement+ 0.5 ml PSA + 0.5 ml L-glutamine.

Medium for tissue culture 2: 45 ml of Amniomax medium + 7.5 ml of Amniomax supplement.

Hypotonic Solution: 2.25 gr of potassium chloride + 500 ml of distilled water. (Valid for 1 week)

Methotrexate (MTX) solution: 10 ml of RPMI® 1640 + 10 µl of Methotrexate ® (25 mg/ml). Aliquote (1,5ml) e store at -20ºC. (Valid for 15 days)

Thymidine Solution: 2 mg of Thymidine + 8 ml of distilled water. Aliquote (2 ml) e store at -20ºC. (Valid for 15 days)

EDTA solution (5%): Dissolve 0.5 g of EDTA in 10 ml of distilled water.

Solução de Tripsina/EDTA/PBS (destacamento para extensão): 20 mg of Trypsin 1:1 + 50 ml PBS + 0.3 ml EDTA (prepare daily).

Collagenase Solution: 40 mg of Collagenase + 15 ml of RPMI + Gentamicin and 5ml of FBS. (valid for 15 days)

Perhydrol Solution (300ml): 75 ml hydrogen peroxide at 35%, 130vol + 225 ml distilled water. (H_2O_2 must be kept at 4ºC. Prepare daily and use at room temperature)

Sorensen's Buffer: 9.47 g of Sodium Phosphate Dibasic Dihydrate + 9.073 of Potassium dihydrogen phosphate + 1 L of distilled water. (Must be kept at 4ºC. Prepare daily and use at room temperature. Valid for 2 weeks)

Gurr's Buffer: 1 Gurr's Buffer Tablet + 1 L of distilled water. (Must be kept at 4ºC. Prepare daily and use at room temperature. Valid for 2 weeks)

Trypsin Solution for G-Banding: 360 mg of trypsin + 300 ml of Sorensen's Buffer, stir for 10 minutes with a magnet at position 7. (Prepare daily)

Leishman Stain (standard solution): 0.75 g of Leishman Stain + 100 ml of Methanol, stir for 10 minutes with a magnet. Add 400 ml of Methanol and stir for 24 hours. Let rest for 2 days. (Leishman stain must be prepared in a 1000 ml Glass Balloon. Avoid contact with light during all procedure, having the glass balloon raped with aluminium foil)

Leishman Stain (usage solution): Filter 75 ml of the Leishman stain with 2 filter papers into a 100 ml beaker. Wah the beaker with 225 ml of Gurr's buffer, adding to the stain. Homogenise with a Pasteur's pipette. (Prepare daily)

Barium Hydroxide (BaOH) solution: 4 g of barium hydroxide + 100 ml of distilled water. Heat for best dilution (+/- 2 min in the microwave) homogenize and filter. Transfer to staining jar and leave at room temperature.

Hydrochloric Acid Solution (1N): 8.7 ml of Hydrochloric Acid + 91.3 ml of distilled water. Transfer to a container and protect from light.

Silver nitrate solution (50%): 0.5 g of Silver Nitrate + 1 ml of distilled water. Store away from light at 2-5^{o}C. (Valid for 6 months)

Distamycin (DA) solution: 10 mg of DA + 10 ml of McIlvaine buffer. Aliquot into plastic tubes and store at -20^{o}C.

20 x SSC solution: 175.3 g 3M of sodium chloride + 88.3 g 0.3M of sodium citrate + 1 L of distilled water. Transfer to staining jar.

2xSSC solution: 10 ml of 20xSSC + 90 ml of distilled water.

DTT solution: 0.604 g of TRIS + 1 ml of Triton-X-100 + 0.0772 g of DTT + 100 ml of distilled water. Prepare in a dark beaker. Stir in the dark. Measure pH and adjust to 7.4 with 37% hydrochloric acid (±0.2ml)

Formamide (70ml): 49 ml of Formamide + 14 ml of distilled water + 7 ml of 20xSSC.

Solution of 0.4xSSC + 0.3%NP-40 (250ml): 5 ml of 20xSSC (pH=5.3) + 220 ml of distilled water + 750 µl of NP-40. Adjust final volume to 250ml; Measure pH and adjust to 7-7.5 with sodium hydroxide; Filter and store at room temperature.

Solution of 2xSSC + 0.1%NP-40 (250ml): 25 ml of 20xSSC (pH=5.3) + 250 µl of NP-40 + 220 ml of distilled water. Adjust final volume to 250 ml. Measure pH and adjust to 7-7.5 with sodium hydroxide; Filter and store at room temperature.

Solution of 70% Formamide/2xSSC: 49 ml Formamide + 14 ml distilled water + 7 ml 20xSSC

Ethanol 70%: 70 ml of absolute ethanol + 30 ml of distilled water.

Ethanol 85%: 85 ml of absolute ethanol + 15 ml of distilled water.

Ethanol 90%: 90 ml of absolute ethanol + 10 ml of distilled water.

Ethanol 96%: 96 ml of absolute ethanol + 4 ml of distilled water.