UNIVERSIDADE D
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Insights into molecular cytogenetic routine diagnostics

Characterization of repeatedly showing up constitutive chromosomal inversions

Dissertação no âmbito do Mestrado em Genética Clínica Laboratorial, orientada pelo Professor Doutor Thomas Liehr, coorientada pela Professora Doutora Isabel Maria Marques Carreira e entregue à Faculdade de Medicina da Universidade de Coimbra

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INSIGHTS INTO MOLECULAR CYTOGENETIC ROUTINE DIAGNOSTICS

CHARACTERIZATION OF REPEATEDLY SHOWING UP CONSTITUTIVE CHROMOSOMAL INVERSIONS

Dissertation submitted to the Faculty of Medicine of the University of Coimbra for the Master's Degree in Clinical Laboratory Genetics

Orientated by Professor Doctor Thomas Liehr

Coorientated by Professor Doctor Isabel Maria Marques Carreira

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Resumo

Inversões pericêntricas e paracêntricas referem-se a anomalias cromossómicas que resultam de quebras em ambos os braços ou num único braço de um cromossoma. Estas anomalias cromossómicas não estão, normalmente, relacionadas com alterações fenotípicas, sendo, normalmente, associadas com problemas de infertilidade. Embora a maioria das inversões sejam *de novo* e únicas, inversões com pontos de quebra repetidos já foram relatadas.

Ao longo desta dissertação de mestrado, foram estudados os pontos de quebra de cinco inversões, quatro paracêntricas e uma pericêntrica, em vinte pacientes que sofriam de problemas de fertilidade, para deteminar se estes são recorrentes ou se ocorrem em locais diferentes do cromossoma. Os pacientes tinham já sido diagnosticados por citogenética convencional. Neste trabalho, a técnica *Fluorescence in situ Hybridization* multicolor (m-FISH) foi aplicada usando várias sondas locus específico, centroméricas e/ou pintura em preparações citogenéticas derivadas de sangue periférico, isto é, cromossomas metafásicos.

A interpretação dos resultados obtidos permitiu identificar pontos de quebra recorrentes em todas as inversões estudadas, em pelo menos um dos pontos de quebra envolvidos. No entanto, também foram encontrado pontos de quebra com localização única. Este trabalho corrobora a hipótese de que alterações cromossómicas equilibradas aparentemente únicas podem vir a ser identificadas em indivíduos não relacionados, representado, desta forma, variantes cromossómicas raras que se encontram na população em geral.

Palavras-chave: Citogenética clínica; Inversões paracêntricas; Inversões pericêntricas; Pontos de quebra recorrentes; *Fluorescence in situ hybridization*

Abstract

Pericentric and paracentric inversions refer to chromosomal aberrations that are the result of breakpoints found in both arms or in the same arm of a chromosome. It is often the case that such aberrations are not associated with phenotypic alterations being nonetheless possibly related with fertility problems. Although the majority of inversions are *de novo* and unique, there have been reports of recurrent breakpoints, as well.

Throughout the course of this master's dissertation, the breakpoints of five inversions, four paracentric and one pericentric, within twenty patients who were suffering from fertility problems were studied to determine whether the inversions are recurrent or occur at different locations of the corresponding chromosomes. Inversion carriers have been identified by routine banding cytogenetics. Here multicolour fluorescence in situ hybridization (m-FISH) technique was applied using various locus-specific, centromeric and/or painting probes on peripheral blood derived cytogenetic preparations, this is metaphase chromosomes.

The interpretation of the results obtained allowed the identification of recurrent breakpoints within all the studied inversions, in at least one of the breakpoints involved. However, breakpoints with unique location were also found. This work corroborates with the hypothesis that seemingly unique unrecognized balanced aberration may be identify in unrelated individuals, representing rare chromosomal variants potentially spreading in populations.

Key words: Clinical cytogenetics; Paracentric inversions; Pericentric inversions; Recurrent breakpoints; Fluorescence in situ hybridization

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List of abbreviations

% – Percentage

µl - Microlitre

µM – Micromolar

acc. – according

BAC – Bacterial artificial chromosome

bio – Biotin

ºC – Celsius degree

CCD – Charged coupled device

CDK – Cyclin dependent kinases

CEP – Centromeric probes

CGH – Comparative genomic hybridization

chr – Chromosome

cm – Centrimeter

CNV – Copy number variant

Cy5 – Cyanine5

DAPI – 4,6-diamidino-2-phenylindol.2HCl 1

dATP – Deoxyadenosine triphosphate

dCTP – Deoxycytidine triphosphate

ddH_2O - Double-distilled water

dGTP – Deoxyguanosine triphosphate

dig – Digoxigenin

DNA – Deoxyribonucleic acid

DOP – Degenerate oligonucleotide primer

DSB – Double-strand breakage

dTTP – Deoxythymidine triphosphate

dUTP – Deoxyuridine triphosphate

FISH – Fluorescence in situ hybridization

FITC – Fluorescein isothiocyanate

Fl – Anti-digoxigenin fluorescein

FoSTeS – Fork Stalling and Template Switching

g - Gram

G1 – Gap 1

G2 – Gap 2

G, GTG – Giemsa, G-bands by trypsin using giemsa

HCl – Hydrochloric acid

IBD – Identical by descent

i.e. – That is

inv – Inversion

kb – Kilobase

KCl – Potassium Chloride

LCRs – Low Copy Repeats

L.Pu – Label buffer

M – Molar

Mb – Megabase

m-FISH – Multicolour fluorescence in situ hybridization

$MgCl_2$ – Magnesium chloride

ml – Millilitre

MLPA – Multiplex ligation-dependent probe amplification

mm – Millimetre

mM - Milimolar

M – Mitosis

mol - Mole

MW – Molecular weight

NAHR – Non-Allelic Homologous Recombination

NGS – Next generation sequencing

NHEJ – Non-Homologous Recombination

no – Number

NP – Nonionic polyoxyethylene

PBS – Phosphate buffered saline

pcp – Partial chromosome paint

PCR – Polymerase chain reaction

PRINS – Primed in situ hybridization

Rhod – Anti-digoxigenin rhodamine

RNA – Ribonucleic acid

Rpm – Revolutions per minute

S – Synthesis

SA – Strepavidin

SG – Spectrum Green

SO – Spectrum orange~

SSC – Saline-sodium citrate

SR – Spectrum red

TR – Texas red

TRITC – Tetramethylrhodamine

t-RNA – Tranfer RNA

Tw - Tween

UCSC – University of California Santa Cruz

UV – Ultravioleta

wcp – Whole chromosome paint

w/v – Weight per volume

YAC – Yeast artificial chromosome

1. Introduction

1.1. The cell and the human genome

The cell is the fundamental unit of life and allows the transmission of genetic material in all organisms. All cells in the human body are divided into cytoplasm and nucleus, the latter contains a copy of large amounts of deoxyribonucleic acid (DNA), organized into forty-six chromosomes, constituting the human genome [1–3]. The human karyotype is arranged in twenty-two autosomal pairs of homologous chromatids (diploid) and two sex chromosomes that alter according to gender (XX for females and XY for males) (Figure 1). Each of them contains information written in the same chemical code of four nucleotides, which allows the production of proteins, ensuring the maintenance of all the processes of embryogenesis, development, growth, metabolism, and reproduction [1,4].

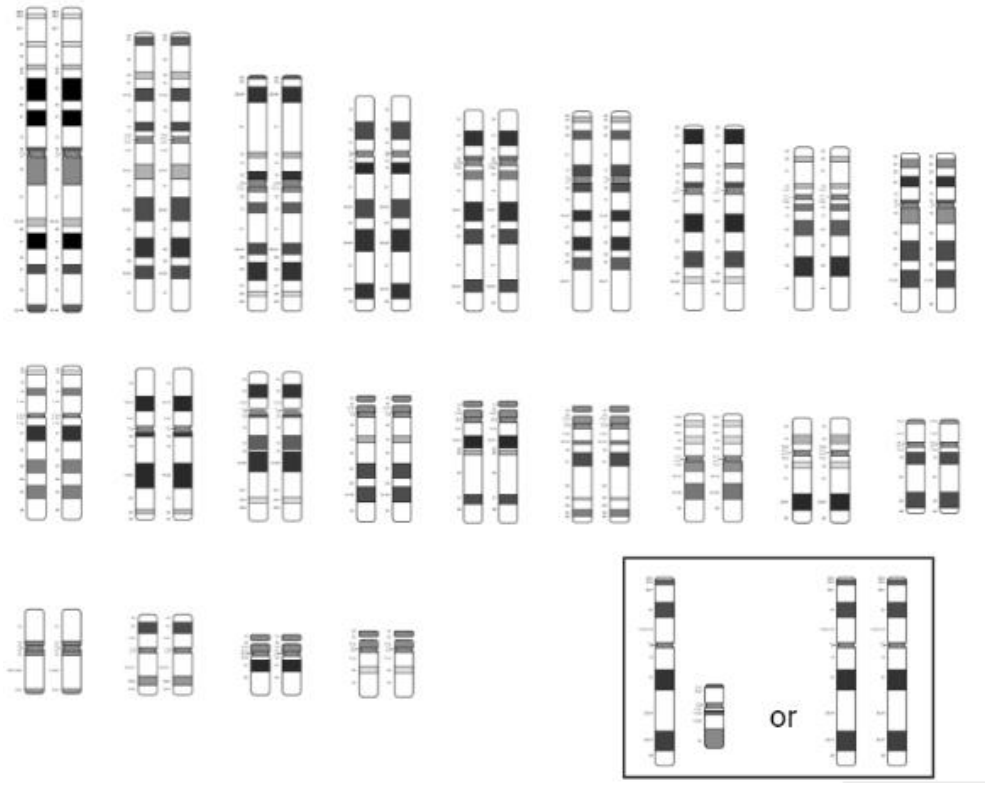

Figure 1. Idiogram of the banding pattern of normal human chromosomes. [Created in BioRender.com]

During the cell cycle and in the body's different cells, DNA is found in several levels of chromatin organization, where the double helix is folded and packed due to bonds with histone and nonhistone proteins. Histones are a family of proteins that organize the double helix into nucleosomes and these into looped domains that coil more or less and separate the chromatin into structurally and functionally distinguished compartments: heterochromatin and

euchromatin[1,5,6]. These two constitutions of DNA represent one mechanism for the regulation of the expression of the ≈ 21,500 genes in the human genome [7]. Heterochromatin is a condensed territory of the chromosome with a small number of genes and little or no transcription of these to proteins. In contrast, a higher proportion of genes are highly transcribed in euchromatin, as transcription machinery can reach the DNA since the chromatin is less condensed [1,5]. This way, the cells can determine which genes are expressed through the structural and chemical organization of the chromosomes and several different signalling mechanisms, to differentiate and provide the body with cells having distinct functions [1,4,5].

1.2. The cell cycle

The cell cycle includes all processes required to form two daughter cells from one initial cell. Although mitosis is critical for growth and differentiation, it is the period where the cell spends the shortest time during the cell cycle. Most of the time a cell is in interphase, which is divided into G1 (Gap1), S phase (synthesis phase), and G2 (Gap 2). During G1 and G2 the cell is metabolically active to provide all the proteins and ribonucleic acid needed not only for the synthesis of DNA but also for both daughter cells to be able to survive. During G1 phase, there is only one chromatid of each chromosome in the nucleus and by the end of G1 phase, chromosomes are thin and extended, so that the replication machine can reach them. Cells are only able to transmit the genetic material to the offspring due to its ability to duplicate every DNA molecule into identical copies, called sister chromatids. G2 is a relatively short step, in which the genetic content is duplicated, and the chromosomes begin to condensate. The sister chromatids result from DNA replication that occurs in the S phase of the cell cycle. Both chromatids are joined by cohesins, i.e. multisubunit protein complexes holding together the two identical DNA double helices along their lengths, and by the centromere, formed by a class of repetitive DNA and specific proteins, being responsible to attach chromatids to each other and, together with kinetochores, to attach the chromosomes to microtubles during mitosis. After replication, the chromatids are ready to be separated during M phase (mitosis phase), so the cell can divide into two genetically identical daughter cells [3,6,8].

The M phase includes a continuous process of nuclear division divided into distinct stages (mitosis), and cytokinesis, where cell division occurs. The five stages of mitosis are prophase, pro-metaphase, metaphase, anaphase, and telophase. Prophase initiates mitosis with the condensation of chromosomes and separation of the centrosomes to opposite poles of the cell, to form the mitotic spindle. In pro-metaphase the nuclear membrane breaks, allowing the

chromosomes to attach to the mitotic spindle by their kinetochores. During metaphase, the chromosomes are at the maximum stage of contraction and are arranged along the middle of the cell, due to the equal forces exerted at the connection point by the microtubules from the centromeres on both sides. The chromatids separate and the single double helixes migrate toward opposite directions in anaphase. In telophase, the chromatids are completely separated, and two nuclear membranes are reconstructed around the two groups of daughter chromosomes, which start to uncoil and become decondensed again to end mitosis. The cytoplasm is separated during cytokinesis, a process in which filaments in the plasma membrane contract and divide the cytoplasm [4,6,8,9].

Meiosis is the process that takes place in the germ cells of the ovaries and testes. It produces gametes with twenty-three chromosomes, which are combined during fertilization to restore the diploid number and form a zygote. During meiosis, genetic material replicates in interphase similarly to mitosis, followed by two cell divisions (meiosis I and meiosis II), each with prophase, metaphase, and anaphase. Meiosis enables reproduction of diploid organisms because it allows children to receive half of their chromosome-set from each parent. It plays a crucial role in genetic diversity because of recombination that occurs during meiosis I [4,8,9].

Meiosis I is a reductive division in which the number of chromosomes is divided by half. The first division occurs in four stages: prophase I, metaphase I, anaphase I, and telophase I. In this process, one sister chromatid comes from the mother and the other from the father, and the homologous are paired locus by locus. The pairs of homologous are called bivalents, or tetrad because is possible to see four chromatids. Multiple identical or very similar segments of DNA are exchanged between the maternal and paternal chromatids of each bivalent in a process called crossing-over. The bivalents are also separated by the spindle, resulting in two sets of 23 recombinant sister chromatids with random combinations of paternal and maternal material [4,6,8,10]. Meiosis II is similar to an ordinary mitotic division, although the number of chromosomes is only twenty-three [6].

1.3. Chromosomal aberrations

Although the cell cycle is highly protected and controlled with checkpoints, determining if the genome and the cell are in a condition to progress to the next phase, the karyotype of a cell is always at risk of being altered by the formation of chromosomal aberrations, which can have disastrous consequences. These aberrations might originate during normal cellular processes

such as DNA replication, DNA repair, and cell division. However, their occurrence might be influenced by age and exposure to environmental factors, such as radiation and chemicals, either natural or manufactured [1,3,11]. During DNA replication, sometimes wrong nucleotides are incorporated into the sequence. This means that the daughter cells are going to receive different genetic information. One of them is going to carry a mutant karyotype and pass it on to its offspring. Although the enzyme responsible for the assembly of new nucleotides on the complementary strand, called DNA polymerase, is faithful in the duplication of the double helix, this process needs to be remarkably accurate. Therefore, there are DNA repair enzymes that replace the wrong nucleotides, avoiding errors in DNA replication. Nucleotides can also be damaged due to chemical factors and even if DNA repair enzymes identify the damage, it might not be well corrected[8,12,13]. On the other hand, some disturbances arise from events during meiosis, such as homologs that occasionally fail to separate properly resulting in malsegregation or nondisjunction, or errors during cross-over, like mispairing of non-homologous sequences and repair mechanisms for double-strand breakage (DSB) that might prioritize the reconnection of the strands over the accuracy of such reconnection [2,8,11,12].

Chromosomal abnormalities resulting from errors that might appear during the cell cycle have the potential to be transmitted to the next generation of cells. The moment when such errors occur is critical to determine whether the abnormality is present in all cells of the body or not. A constitutional abnormality involves all the body since it occurs in very early stages of development as a result of problems in fertilization, in the first mitotic division of the embryo, or during meiosis for the formation of the sperm or the egg involved. If the abnormality is present only in particular tissues or cells, a somatic abnormality that was acquired later in the formation of the organism is present. The existence of cell lines with different chromosomal content that are derived from the same zygote is called mosaicism [9,11,14].

Within the many rearrangements, it is possible to find in a given population unrelated individuals with similarities in breakpoints, sizes, and genomic content. These recurrent rearrangements happen due to regions of genome instability with a predisposition to rearrange, caused by specific architectural features. However, there are also non-recurrent abnormalities that are unique and do not share genomic features with non-related individuals. Disorders in the chromosome, whether constitutional or acquired, can be categorized as numerical or structural. [9,11,14]. These abnormalities have been increasingly identified as the reason of infertility, although fertility problems are mainly related to idiopathic factors [15].

1.3.1. Numerical chromosomal abnormalities

Numerical chromosomal abnormalities are related to an incorrect amount of genetic material and involve either euploidy or aneuploidy. In case of a wrong fertilization process, euploid embryos may be formed, with more or fewer chromosome complements than is normally observed in somatic cells. It is expected to observe forty-six chromosomes after fertilization, but occasionally 69 or 92 chromosomes are observed, which means that one or two complete extra chromosome sets, respectively, are present. These events are called triploidy or tetraploidy. They are explained by the fecundation of the egg with two sperm or by the involvement of diploid gametes in fertilization, due to failure in one of the meiotic divisions. Both cases are – if not present in low mosaic - incompatible with life. Triploids are observed in 1-3% of human pregnancies and although the infants can survive to term, they are not able to live for more than a few days after birth [3,8,9,16].

Aneuploidy only involves one or more individual chromosomes that are present in an extra or missing copy. It is the most frequent type of human chromosome disorder, occurring in approximately 5% of pregnancies [8]. The absence of one member of a chromosome pair is called monosomy, and is rarely compatible with life, except for monosomy of the X chromosome. Trisomy is described as the presence of an extra chromosome, and it might not be lethal if the chromosomes involved are 13, 18, or 21. One of the main causes of aneuploidy is the failure of disjunction during one of the meiotic divisions or the delay of a chromosome or a chromatid during anaphase that fails to enter the nuclei of the daughter cells, in a mechanism called anaphase lag [4,8,9].

1.3.2. Structural chromosomal abnormalities

Structural chromosomal abnormalities are the result of chromosome breakage followed by incorrect reconstruction that can result in gain, loss, or reallocation of the chromosomal segments involved. Chromosomal rearrangements occur spontaneously and although they exist in many forms, they are still altogether less common than aneuploidies [6,16]. There are several mechanisms by which these rearrangements can be formed, including DNA recombination, repair, and replication processes, which are initiated after a double-strand break (DSB). Theoretically, breakpoints can occur anywhere in the human genome, but there is evidence that there are areas with higher susceptibility [6,14] Non-Allelic Homologous Recombination (NAHR)

is the cause of the most recurrent rearrangements (Figure 2). This event occurs within Low Copy Repeats (LCRs), which are DNA blocks that range in size from 1 to 500 kb, share more than 97% of sequence identity, and are present throughout the genome. NAHR is a repair event that occurs after a DSB during meiosis or mitosis. This is due to an incorrect alignment due to LCR sequence identity. The resulting abnormality is dictated by the orientation of the LCR segments, their size, and if the NAHR takes place intrachromosomal, interchromatin, or interchromosomal [6,14].

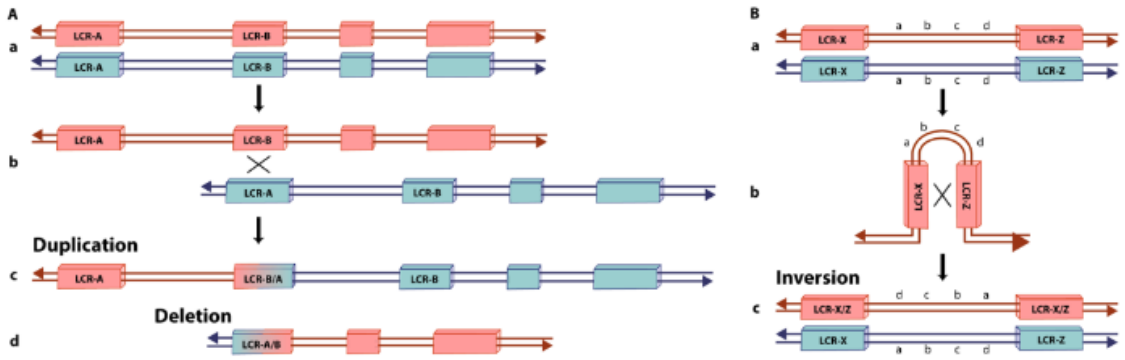

Figure 2. ***Non-Allelic Homologous Recombination (NAHR) mechanism.*** *(A) The mechanism can lead to the formation of duplication and deletion. Instead of normal pairing and aligning (a), Low Copy Repeats (LCRs) are misaligned due to their high level of sequence identity (b) and can generate (c) duplication or (d) deletion. (B) NAHR as the genesis event of inversions. Instead of normal pairing and aligning (a), Low Copy Repeats (LCRs) in the same DNA strand with similar sequences but in opposit orientations align with each other (b), leading to unequal crossing over that can generate an inversion (c). [Created by BioRender.com].* *(Adapted from* 14.)

Non-Homologous End-Joining (NHEJ) is the repair mechanism that brings two-ended DSBs with no homology at the breakpoints together (Figure 3). This can happen without editing of the ends or involving the addition or loss of nucleotides. Non-recurrent abnormalities through NHEJ are associated with repetitive sequences. NHEJ is the primary formation mechanism for balanced chromosomal alterations [14,17].

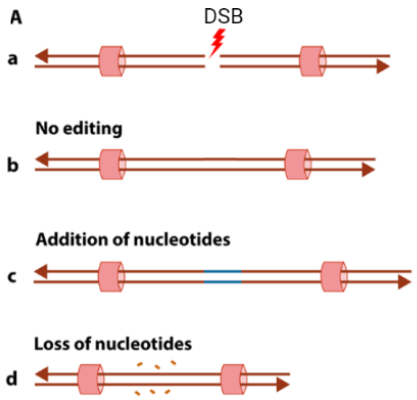

Figure 3. ***Non-Homologous End-Joining (NHEJ) mechanism.*** *After a Double Strand Break (DSB) (a), the broken ends are molecularly bridged and rejoined. This can happen without nucleotide edition (b), or with editing the broken ends with addition (c) or loss (d) of nucleotides. [Created by BioRedner.com]* *(Adapted from* 14)

Another common cause of chromosomal structural rearrangements is replication-based mechanisms. This happens when the replication machinery encounters an obstacle or error when DNA strands are separated to be used as templates for complementary strands synthesis. They are the genesis of complex rearrangements involving single-strand errors. Fork Stalling and Template Switching (FoSTeS) mechanism is an example (Figure 4). In this event, the replication fork stops due to an obstacle at one position. The replication machinery sometimes switches templates to a nearby homologous or non-homologous region and restarts DNA synthesis. This can lead to the invasion of other replication forks that may be downstream or upstream on the same strand or on a different chromosome [14,17].

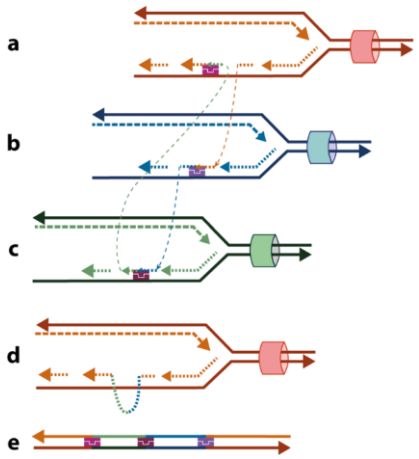

Figure 4. ***Fork Stalling and Template Switching (FoSTeS) mechanism.*** *A replication fork that stalls (a) disengages the lagging strand from its original template and, due to microhomology (purple), invades another replication fork (dashed line) and restarts DNA synthesis (b). Invading other replication forks is possible when the lagging strand disengages again (c). It is eventually possible for the strand to return to its original template and start synthesis again (a, d). As a result of this microhomology, segments from different parts of the genome are brought together to create the final product (e). [Created by BioRender.com].* (*Adapted from* 14).

Rearrangements involving the structure of chromosomes are divided into balanced and unbalanced. Unbalanced structural rearrangements have additional or missing information and are the genesis of the phenotype of clinically affected individuals. On the other hand, in balanced structural rearrangements, the chromosomes are still present with complete genetic information. The carriers are mostly phenotypically normal and normally are not even detected, although the alteration might lead to progeny with unbalanced karyotypes [6,18]. There is a kind of structural abnormalities that are difficult to categorize as balanced or unbalanced. In this case, all the genetic material is present however, a gene was disrupted, resulting in a rearrangement that is not truly balanced. The results may vary between the absence of protein, the production of nonfunctional protein, or the fusion of chromosomal segments that leads to

the production of new proteins with a different function [6,18]. Whether unbalanced or balanced, structural abnormalities can either be intrachromosomal or interchromosomal, if they involve only one chromosome or more than one, respectively [19].

1.3.2.1. Unbalanced chromosomal rearrangements

Unbalanced chromosomal rearrangements include deletions, duplications, extra marker-, ring-, iso-, or dicentric chromosomes. In most carriers of unbalanced rearrangements, the phenotype is abnormal because the normal amount and organization of genes are disturbed [8].

In deletions and duplications, the loss or gain of segments of a chromosome is involved, resulting in partial monosomy or partial trisomy. The size of deletions may vary from a single base pair up to more than 20 Mb [6,19]. They can happen at the terminal ends of the chromosome, involving a single breakage, or interstitially, in which the two breakpoints re-join. The duplicated segments might be adjacent to each other or be in distinct parts of the same chromosome, and they might be oriented in the same or opposite directions. Extra marker chromosomes are described as very small unidentified chromosomes and add genetic material to the genome that may result in foetal abnormality, depending on the origin of the marker. A ring chromosome is formed by the fusion of two ends of the same chromosome. This may or not involve loss of chromosomal regions. It is normally found in mosaicism due to the difficulty of going through mitosis. In iso-chromosomes, one of the arms is missing and the other is duplicated, leading to monosomy of one arm and trisomy of the other, or tetrasomy if the iso-chromosome is there in addition. The centromere can either be normal or be duplicated. Dicentric chromosomes are the result of the fusion of segments of two different chromosomes, or chromatids, where both have an active centromere and lose the acentric fragments [4,6,8,16,19].

1.3.2.2. Balanced chromosomal rearrangements

Balanced chromosomal rearrangements are listed as insertions, translocations, and inversions. An insertion occurs when a fragment is removed from one chromosome and inserted into another one in the usual orientation or inverted. This involves three breaks, two on the donor chromosome and one on the recipient. The segment can also be misplaced within the same chromosome, resulting in intrachromosomal insertions. Translocations happen when there are two breaks in nonhomologous chromosomes, and the genetic material in between is exchanged. Translocations can be reciprocal or Robertsonian. In reciprocal translocations, as the name suggests, there is a reciprocal exchange of chromosomal segments between nonhomologous

chromosomes with the remaining chromosome number. Robertsonian translocations involve acrocentric chromosomes, such as 13, 14, 15, 21, and 22. They occur when the long arms of two of these chromosomes join near the centromere region and the short arms are lost. The chromosome number is altered to forty-five, but the karyotype remains balanced. This is because all the short arms of acrocentric chromosomes have the same information to produce ribosomal RNA. Although carriers of translocations are usually clinically normal, the behaviour of these chromosomes during segregation in meiosis is deregulated due to the incapacity to pair normally. This can result in aborts, infertility, or children with an abnormal phenotype. Inversions involve only two intrachromosomal breakpoints and a 180 degrees rotation of the segment between them. They are divided into pericentric inversions and paracentric inversions. The first one affects both arms of the chromosome, and the second one is confined to one of the arms [8,17,19].

1.3.2.2.1. Inversions

Inversion carriers are typically phenotypically normal, like most people with structural rearrangements. The breakpoints usually do not disturb genes and the opposite orientation of DNA does not seem to influence its function. Both pericentric and paracentric inversions have been reported on all twenty-three chromosomes. Most of these disorders are associated with reproductive problems, with no direct phenotypic effects. Most breakpoints found in inversions are unique, however, recurrent breakpoints have been identified. They can either be the result of independent events, which means that they were formed several times due to the existence of susceptible areas [20], or identical by descent (IBD), that were inherited from a single common ancestor [21]. Even when the carriers seem to be apparently unrelated individuals, it is possible to conclude if they have a single or small number of common ancestors through the study of polymorphic markers [17,22,23].

1.3.2.2.1.1. Pericentric inversions

A frequency of 0.12% to 0.7% has been estimated for pericentric inversions in humans. They involve breakpoints in the short (p) and long (q) arms with perturbation of the centromere and the arm proportion. The breakage can occur closer or farther from the centromere. The production of unbalanced gametes is determined by the size of the inverted segment and by the meiotic behaviour of the affected chromosome, like the number of chiasmata. During the crossing-overs in meiosis I, homologous chromosomes pair through their homologous locus

and exchange genetic information. When a bivalent consists of a normal chromosome and a chromosome with an inversion, a reverse loop is formed because it is not possible for a linear pairing to happen. The inverted segment is reverted to pair with its homologous region of the normal chromosome and the distal non-inverted segments pair normally [6,17].

The number of crossovers determines whether the recombinant chromosomes are balanced or unbalanced (Figure 5). When an even number of crossovers occur involving the same sister chromatids, they cancel each other, and the resultant chromosomes are normal and contain all the genetic information. On the other hand, in the case of an odd number of crossovers within the reverse segment, the products are one normal chromatid, one balanced chromatid with the inversion and the other two have complementary duplications and deletions of the distal segments on both short and long arms[6].

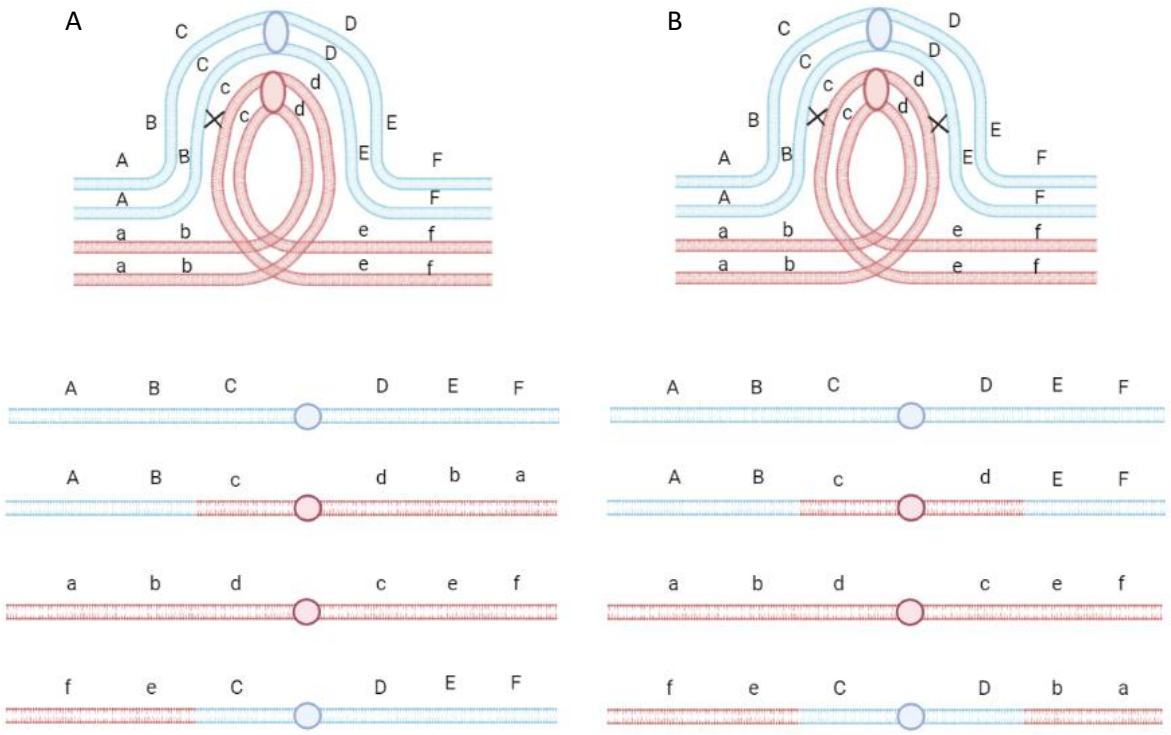

Figure 5. ***Representation of the meiotic products as a result of heterozygotic crossing-over of a pericentric inversion.*** *The results depend on how many chiasmata happen. If only one or an odd number (a), there are four possible chromatids that can be formed. The products are a normal chromatid, a chromatid with inversion, and two chromatids with deletion and duplication. If there are two or an even number of chiasmata involving the same sister chromatids (b), they cancel each other and there is the formation of balanced chromosomes, two without the inversion, and two with the inversion. [Created by BioRender.com]*

The size of the inverted segment determines if the recombinants are viable. If the inverted material involved in the reserve loop is of small dimensions, the distal segments are large, and because of this, the amount of duplicated or deleted DNA is also large. The risk of miscarriage,

in this case, is high. Contrarily, the larger the segments the bigger possibility of taking a pregnancy to term and of postnatal survival of children with unbalanced karyotypes. There is also the possibility that the chiasmata occur outside the inverted segment and the final chromosomes are balanced [8].

Other models represent what happens during meiosis that involves varied factors. One of those being the size of the inverted material. If the inversion involves only a very small part of the chromosome, there is the possibility that the unaffected regions of the chromatids pair linearly. In this case, synapsis does not happen in the inverted material located and chiasmata just happen within the normal part of the DNA. This leads to the formation of balanced chromosomes. However, if the inverted segment is of very large proportions, the synapsis will only occur within the breakpoints and the distal segments do not pair. Crossing-over only happens within paired regions, leading to the production of chromosomes in an equivalent way that happens in the basic reverse loop [6].

1.3.2.2.1.2. Paracentric inversions

Paracentric inversions occur at an estimated frequency of 0.1-0.5%. Both breakpoints are positioned in one of the arms of the affected chromosome without the involvement of the centromere. Production of unbalanced and balanced chromosomes after meiosis is determined by the number of crossovers, and whether the crossing-over occurs in the inverted segment. The classical model for pairing homologous is the reverse loop, but in this case, the centromeres are in the distal part [6].

The formation of a reverse loop can result in many outcomes. Two things can happen that do not represent a risk for the formation of unbalanced chromosomes. The first one is if the chiasmata are formed within the distal segment. The second is if the crossovers happen in even numbers within the inverted segment and involving the same chromatids because they are going to cancel each other. On the other hand, an odd number of crossovers within the inversion loop results in the production of a dicentric chromatid, an acentric fragment, and two balanced chromatids, one normal and one with inversion (Figure 6A). There is still the possibility of producing four unbalanced chromatids if there is the formation of two chiasmata in the reverse loop that involve different pairs of chromatids (Figure 6B). In this case, the results are two acentric fragments and two dicentric chromatids [6,24].

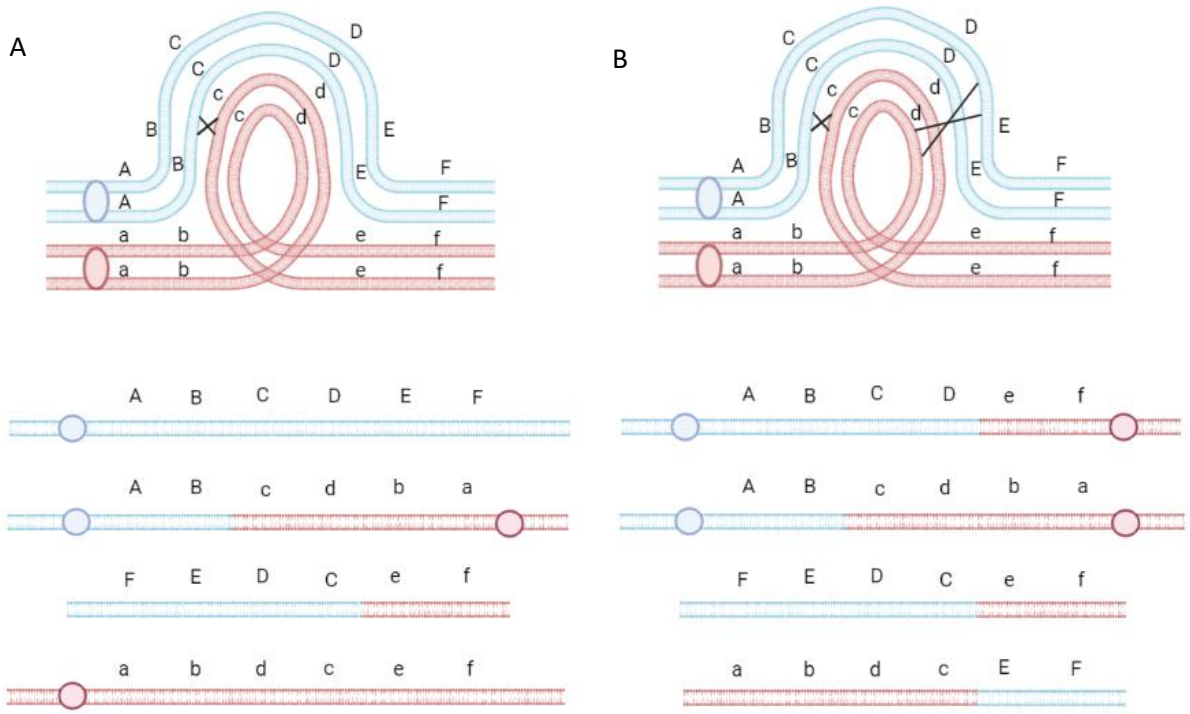

Figure 6. ***Representations of the meiotic products as a result of heterozygotic crossing-over of a paracentric inversion****. The results depend on how many chiasmata happen. If only on or an odd number (a) there are four possible recombinants: one normal chromatid, one balanced with inversion, and one dicentric and another acentric chromosome. If there are two or an even number of chiasmata involving different sister chromatids (b) two acentric fragments and two dicentric chromatids are formed. [Created by BioRender.com]*

Acentric fragments are parts of the chromosome that lack centromeres. They do not have the ability to attach to simple fibres. Which ends up with their loss during cell division when the chromosomes are separated into different poles of the cell. Dicentric chromosomes are composed of two centromeres and are able to join themselves to the spindle. However, they also represent a problem during cell division. The centromeres can be pulled in opposite directions, which can result in broken or stretched chromosomes. This can result in a bridge between the two daughter cells, resulting in an unequal distribution of genetic information. Due to the mitotic instability of both dicentric and acentric recombinant chromosomes, they have been reported in the offspring of paracentric inversion carriers in very few cases [6,14,25].

It has been reported that most of the children who carry a recombinant coming from a paternal paracentric inversion have a monocentric chromosome with duplications and deletions of the inverted fragment. The explanations for this phenomenon include breakage of dicentric recombinants, unequal crossing-over, and a U-loop type of exchange event. This atypical recombination is caused by the breakage of the double strand of the sister chromatids and the incorrect repair of the broken ends in a symmetric U form. Dicentric chromosomes are formed,

which break during cell division and result in two monocentric chromosomes with reciprocal deletions and duplications [14,26,27].

1.3.2.2.1.3. Consequences to inversion carriers and their offspring

Inversion carriers are normally not affected phenotypically, but there are inversions associated with health complications. Some of these pathogenic consequences are immunodeficiency, mental retardation, schizophrenia, bipolar disorder, and deafness [28]. One of the reasons for abnormal phenotypes is the disruption of genes at the time of the breakage. This can result in changes in the organism's normal function [28,29]. It is also possible that the inversion separates the gene from its expression regulation elements, leading to gene expression differences [28,30].

The formation of unbalanced recombinants might be the origin of reproductive problems and represents a risk for the offspring to carry an unbalanced chromosome associated with abnormal phenotypic characteristics [31,32]. In most cases, acentric and dicentric chromosomes resulting from paracentric inversions are not mitotically stable. Their production results in infertility problems and early pregnancy loss [32,33]. In the case of pericentric inversions, unbalanced gametes might fertilize. The embryos have partial monosomy or trisomy that, depending on the affected chromosome and the location and size of the inverted segment, and its proportion to the size of the chromosome, might result in congenital defects [33]. This way, carriers of paracentric inversions have a better chance of normal progeny because the recombination products are non-viable [25].

1.4. Genetic approaches to determined chromosomal / genetic changes

1.4.1. Cytogenetics

Cytogenetics is the field of genetics that studies the structure, function, and behaviour of chromosomes, including their relationship to human health and disease. It involves the examination of chromosomal aberrations and the identification of genetic mutations. The understanding of chromosome arrangement, organization, and pathogenic changes is critical to clarify the genetic basis of cancer, infertility, and other diseases. This field is mainly divided into two: banding cytogenetics and molecular cytogenetics. While conventional cytogenetics, such as G-banding karyotyping, is used for the detection of imbalances of large dimensions, molecular cytogenetics provides higher resolution. Comparative genomic hybridization (CGH) can find submicroscopic alteration through analysis of copy numbers in the genome. However,

most techniques are used together with fluorescence in situ hybridization (FISH), to gain insight into the position and orientations of the alterations [14,34].

Banding cytogenetics involves the analysis of chromosomes under a microscope. The cultured cells are treated to stop mitosis during metaphase and are then stained to be examined through a microscope. In metaphase, the level of condensation is the highest, allowing the visualization and distinction of chromosomes. The distinction is based on their size, centromere positions, and band pattern. These staining techniques are able to identify large-scale chromosomal abnormalities, such as missing or extra chromosomes, deletions, duplications, inversions, and translocations [34,35].

The most widely used technique for chromosome banding is Giemsa banding (G-banding or GTG-banding). The staining is done with a trypsin treatment followed by a Giemsa dye, which results in light or dark bands depending on the level of gene density and chromatin structure of the regions. Light bands correspond to gene-rich regions and the darker the band the fewer genes are present in that region [35]. As the first banding technique, Q-banding uses quinacrine fluorochromes to stain the chromosomes, producing similar patterns to those of G-banding but having the problem of quenching the fluorescence [36]. Other banding techniques are R-, C-, and NOR-banding. Reverse banding (R-banding) produces an opposite banding pattern than G-banding due to an additional step of heating before Giemsa staining. C-banding stain selectively heterochromatic centromeric regions of chromosomes. The staining of nucleolar organizer regions (NOR-banding), which are regions involved in the production of ribosomal RNA, is done with silver nitrate [36,37].

Analysing chromosomes using conventional cytogenetics is a cost-effective way to obtain valuable information for research and clinical settings in a cost-efficient way. It makes it possible to study all the chromosomes simultaneously [34]. However, there are some limitations associated that must be considered. Its low resolution of 5-10 megabase pairs (Mb) causes small genomic imbalances to be missed, as well as the ones that involve no change in the banding pattern. Because of this, it is limited to the detection of ploidy and big structural abnormalities. It cannot detect point mutations or other types of molecular alterations, such as submicroscopic changes. These techniques typically require fresh tissue and cell culture, which is time-consuming and might lead to nonviable cells [35,38]. To overcome such limitations and obtain a more comprehensive analysis of the genetic material of cells, conventional cytogenetics should be used in conjunction with more sophisticated techniques, such as molecular cytogenetics [38].

Molecular cytogenetics combines conventional cytogenetic and molecular techniques to study genetic alterations. This approach requires the use of DNA sequences or chromosome specific fluorescent-labelled probes that bind to certain parts of DNA sequences, taking advantage of the fact that complementary nucleotide sequences hybridize with each other. This allows researchers to visualize specific regions of the chromosomes to detect small-scale chromosomal aberrations and unique genetic mutations or alterations that are important biomarkers for constitutional syndromes and acquired diseases [34,39]. Examples of molecular cytogenetics techniques are fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), and PRINS (primed in situ hybridization) – the latter two are not further treated as they were not used in this work.

In situ hybridization began to be used in the late 1960s, resorting to the use of radioactive isotopes, and was later adapted to visualize chromosomes using fluorescent-labelled probes, which allowed the widespread use of FISH [35,37,40]. In this technique, DNA fragments are labelled with fluorochromes (probes) and hybridized with a complementary sequence of the single stranded DNA in study. To perform FISH, a target DNA and a probe DNA are required. The target DNA can be obtained from native cells, tissue sections, interphase or metaphase nuclei, and pure DNA and was to be fixed to a slide. It needs to be intact, non-degraded, and of high molecular weight. The most common sources of target DNA in humans are peripheral blood lymphocytes, bone marrow cells, skin fibroblasts, buccal mucosa, hair root cells, urine derived cells, amniotic fluid, chorion biopsy derived cells, sperm, oocytes, and tumour cells and tissues. The main reaction is based on consecutive denaturation and renaturation of both DNAs (Figure 7). The hybridization is followed by washing steps. The visualization of fluorescence probes and the chromosomes is done under a fluorescent microscope [34,35,41].

Locus specific probes are fragments of human genomic DNA generated from molecular clones of bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), plasmids, and cosmids. They contain the specific DNA sequence to be targeted, with a minimum size of 12 kb. The target sequence can represent a functional gene, or a particular chromosome region or locus. These probes are normally used for the diagnosis of small rearrangements. Repetitive sequence probes consist of monomers repeated hundreds to thousands of times targeting centromeres (centromeric or α-satellite), telomeres, or other repetitive regions. They are easily visualized as strong signals. Centromeric probes (CEP) allow chromosome differentiation because the centromere repetitive sequences are specific to each chromosome. This excludes

chromosomes 13/21 and 14/22 that share centromere homology. Whole and partial (at least 1 or 2 euchromatic subbands) chromosome paints (wcps and pcps) are generated by microdissection or flow sorting, allowing the staining of the entire chromosome, parts of it, and fragments of unknown origin [34,38,42]. It is possible to use simultaneously three or more probes with different ligands or fluorochromes that are complementary to different regions of the karyotype, in a technique called multicolour FISH (m-FISH) [43].

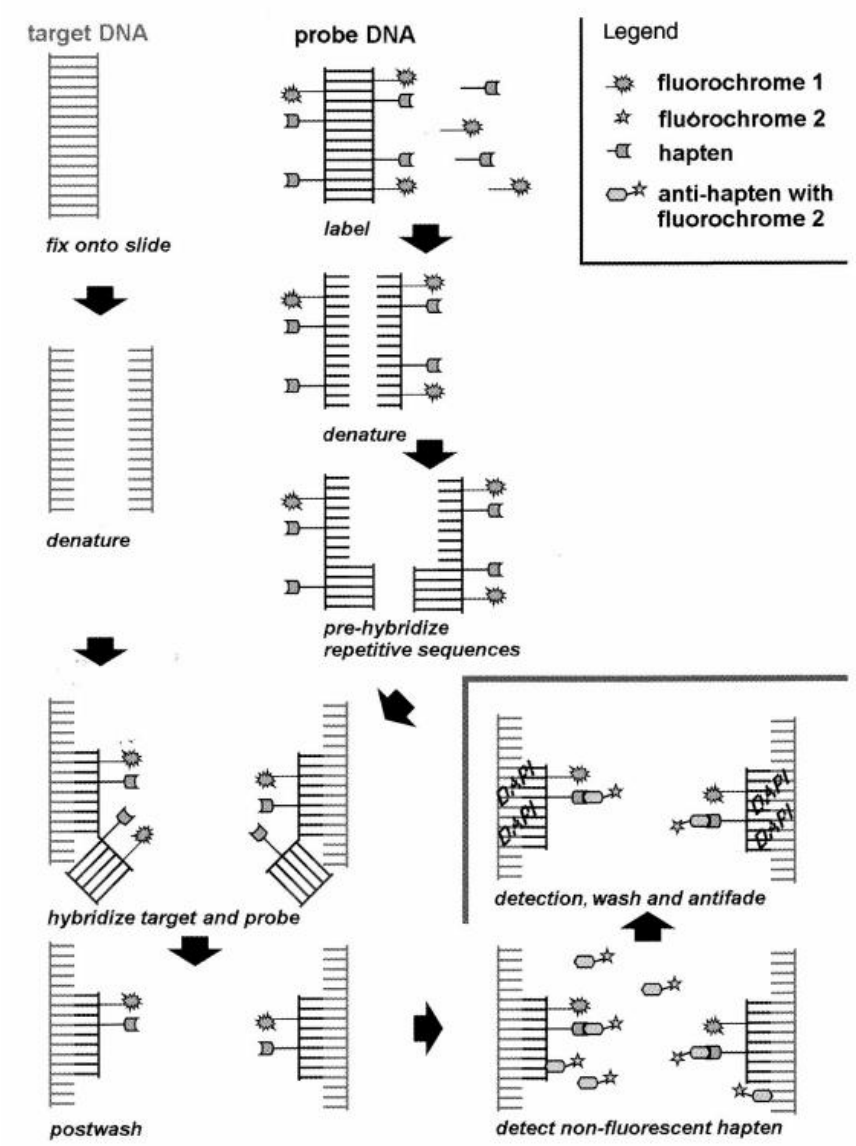

Figure 7. ***Flow diagram of fluorescence in situ hybridization (FISH).*** *DNA target (light gray) is fixed on the slide surface and denatured. Meanwhile, a fluorochrome and/or nonfluorescent hapten must be used to label the probe DNA (black), before denatured and pre-hybridize with unlabelled repetitive DNA (dark gray). Hybridization is then performed by combining probe DNA with target DNA. During post washing, unbound single-stranded DNA and nonspecifically bound DNA are removed. In the case of nonfluorecent hapten, it must be detected by a fluorescence coupled anti-hapten. FISH is completed after detection, washing, and application of an antifade solution containing DAPI (4,6-diamidino-2-phenylindol.2HCl 1). The slide is ready for inspection under microscope.* (*Adapted from* 44)

The probes used can either be labelled directly or indirectly. Directly labelled probes have the fluorochrome incorporated, whereas indirectly labelled probes require an additional step of antibody reaction. The fluorochromes incorporated in the directly labelled probes can emit green (fluorescein isothiocyanate – FITC, and Spectrum Green - SG), red (tetramethylrhodamine – TRITC, Texas Red – TR, and Spectrum Red - SR), orange (Spectrum Orange - SO), and blue (Streptavidin - SA) [34,41]. Indirect probes are usually labelled with the haptens digoxigenin (dig) or biotin (bio). The most common antibodies used for digoxigenin Rhod (anti-digoxigenin rhodamine) and Fl (anti-digoxigenin fluorescein), and for biotin are avidin-Cy5 (sulfo-cyanine5) or avidin-FITC [34,41,45]. There are a lot of commercially available probes, but it is also possible to prepare homemade locus-specific FISH probes using BACs [46].

FISH is a fast technique with high sensitivity and specificity, reliability, and reproducibility, used for the detection of both numerical and structural abnormalities. The major advantages are the ability to use nondividing cells (in interphase) from recent or aged samples, the fact that the results can be available within 24-48 hours, and the high resolution. The main limitations associated are the cost of execution and the fact that the results will only include the limited genomic regions targeted with the probes [34,37,39].

1.4.2. Molecular genetics

Molecular genetics is focused on the study of genetic material at a molecular level to perform evaluations of heredity, genetic variation, and mutation through sequencing of chromosomes and analysing gene expression. Many gene sequencing techniques require a first step of polymerase chain reaction (PCR), which amplifies a specific region of human DNA to make multiple copies of it [35]. Multiplex ligation-dependent probe amplification (MLPA) is a method based on PCR used to detect CNVs, which play critical roles in the development of genetic diseases. MLPA is highly sensitive and detects CNVs with high accuracy, using a low amount of DNA, without needing cell culture. However, it is not capable of detecting ploidy, balanced rearrangements, and low-level mosaicism [34,47]. Other techniques are widely used for DNA sequencing, such as Sanger sequencing and next-generation sequencing (NGS). Sanger is commonly used for targeted sequencing of specific regions of the genome, being able to detect small disease-causing variants [34,35]. NGS techniques can sequence millions of DNA fragments simultaneously, and technological platforms can be used to detect alteration in DNA samples by comparing them to a reference[34,35]. These techniques are not further treated, as they were not used in this work.

1.5. Objectives

This work aimed to characterize the breakpoints of constitutive chromosomal inversions that seemed to appear repetitively in middle-European population, as they were observed during 25 years of routine diagnostics in the host laboratory more than 2 times.

Five different inversions were studied in a total of 20 unrelated carriers with origin from Germany. Inversion carriers were initially identified by GTG banding of metaphase chromosomes after being referred for karyotype analysis, mainly for fertility problems; the breakpoints have been further studied in routine diagnostics by whole and/or partial chromosome paints.

Here further molecular cytogenetic characterization of the inversion breakpoints was done, applying selected locus-specific probes form a collection of >7000 BAC probes of the host institute.

Therefore, the scientific objective of this work can be summarized in the following question:

Do the five different inversions studied in 20 unrelated carriers share identical breakpoints?

2. Material and Methods

2.1. Material

2.1.1. Studied cases

The analysed cases were referred for cytogenetic analysis because of a history of infertility and/or prenatal problems. Inversions in chromosomes 9, 11, 12, 14, and Y were analysed, as specified in Table 1.

Table 1. Initial suggested breakpoints of the twenty evaluated cases.

Patient no	Initial breakpoints
1a	inv(9)(q32q34.3)
1b	inv(9)(q32q34.3)
1c	inv(9)(q32q34.3)
2a	inv(11)(q21q23.3)
2b	inv(11)(q21q23.3)
2c	inv(11)(q21q23.3)
2d	inv(11)(q21q23.3)
3a	inv(12)(q13.1q24.1)
3b	inv(12)(q13.1q24.1)
3c	inv(12)(q13.3~14.1q24.11~24.12)
3d	inv(12)(q13.3~14.1q24.11~24.12)
4a	inv(14)(q22q32.1)
4b	inv(14)(q22.3q24.3)
4c	inv(14)(q22.3q24.3)
5a	inv(Y)(p11.2q11.222)
5b	inv(Y)(p11.2q11.222)
5c	inv(Y)(p11.2q11.222)
5d	inv(Y)(p11.2q11.222)
5e	inv(Y)(p11.2q11.222)
5f	inv(Y)(p11.2q11.222)

2.1.2. Reagents

Chromosome studies were performed by FISH using different probe sets in the metaphases of the 20 patients. To perform this technique, it was required to be equipped with all the essentials for a molecular cytogenetic laboratory. These essentials included reagents and solutions, probe

sets, and image acquisition systems and software. The following more specific items were needed.

- Cell culture medium: Lymphogrow Medium (Cytogen)
- Colcemid
- Ethidium bromide
- Hypotonic solution: 0.075 M KCl
- Fixative: 3 volumes of methanol added to 1 volume of acid acetic and stored at -20 ºC after mixed
- Double-distilled water (ddH_2O) = Aqua ad iniectabilia
- Applied Biosystems^{TM} AmpliTaq DNA polymerase.
- Biotin-16-dUTP (bio)
- Digoxigenin-11-dUTP (dig)
- Spectrum-Orange-dUTP (So)
- Spectrum-Green-dUTP (Sg)
- Spectrum-Red-dUTP (Sr)
- Texas Red-dUTP (Tr)
- Label-mix: 50 µl of dTTP 10 M and 100 µl of dATP, dCTP, and dGTP 10 mM were added to 150 µl of ddH_2O.
- DOP (degenerate oligonucleotide primer) 4 µM: 6MW primer 5'-CCG ACT CGA GNN NNN NAT GTG G-3' [48]
- Label Buffer, 10 x NP40: 0,5 µl of 0,05% NP40 were added to 1 ml of 10 x PCR II - Buffer
- $MgCl_2$ 25 mM
- Tube with Cot10 DNA: 10 µl of Cot1-DNA human (1mg/ml) were added to 20 µl of 100% Ethanol and the tubes were dried in a SpeedVac Vacuum Concentrator
- Ribonucleic acid transfer - t-RNA
- 3M Sodium acetate: 24.6 g sodium acetate was added to 100 ml ddH_2O; the pH was adjusted to 5.2 with acetic acid
- Antibodies (Cy5-Streptavidin (Cy5), Anti-Digoxigenin Fluorescein (Fl), Fluorescein Avidin DCS (FITC), Anti-Digoxigenin Rhodamine (Rhod))
- Dextran sulfate (DS): 2 g of Dextran sulfate powder were added to 2 ml of 20 s SSC and 2 ml of 0.5 mol Sodium Phosphate buffer at pH 7.
- Hybridization buffers
- Ethanol 100%, 90%, and 70% at room temperature

- Ethanol 100% at -20 ºC
- PBS 1x (phosphate buffered saline)
- Pepsin solution: 50 ml 0.2 M HCL was added to 950 ml of Aqua Deion and heated at 37 ºC, then 5 ml of pepsin stock solution 2 % (w/v) were added.
- Postfix solution: 500 ml of 2% paraformaldehyde were mixed with 450 ml of 1 x PBS and 50 ml 1 M $MgCl_2$
- DAPI solution: 1.5 µl of DAPI stock solution were dissolved in 1 ml Vectashield antifade.
- 70 % Formamide solution: 700 ml of Formamide and 100 ml f 20xSSC were added to 200 ml of Aqua Deion
- Rubber cement: Fixogum™
- 0.4xSSC/Tw solution: 10 ml of 20xSSC (saline sodium citrate) and 1 ml of Tween 20 with a pH of 7 to 7.5 were added to 490 ml of Aqua Deion
- 0.2xSSC solution: 50 ml of 20xSSC were added to 450 ml of Aqua Deion
- 4xSSC/Tw solution: 100 ml of 20 x SSC and 250 µl of Tween 20 with a pH of 7 to 7.5 were added to 400 ml of Aqua Deion
- Marvel solution: 2 ml of 4xSSC/Tw solution were added to 0.1g of Marvel powder (non-fat dry milk) and the mixture was centrifuged at 2500 rpm for 5 minutes at room temperature and the supernatant was used.
- DAPI: antifade solution

2.1.3. FISH probes

Multicolour FISH was performed with three, four, and five fluorochromes and one counterstain [49]. For image acquisition, it was necessary to use a microscope equipped with six filter sets, connected to a computer through a charge-coupled device (CCD) camera. Acquisition and evaluation of these images are only possible with specific software. In this work, ISIS software (MetaSystems, Altlussheim, Germany) was used.

For the evaluation of the breakpoints through FISH studies, combinations of specific probes were used (Table 2). In the cases reported as inv(9)(q32q34.3) the following combinations were used: 1) RP11-570D4 in 9q32 (chr9: 115,825,791-116,002,725) with RP11-78H18 in 9q32~9q33.1 (chr9:117,567,550-117,726,266) and wcp9, and 2) RP11-5N16 in 9q34.13 (chr9:134,994,187-135,087,298) with RP11-153P4 in 9q34.2 (chr9:136,541,741-136,720,872) and wcp9. In the cases reported as inv(11)(q21q23.3) the following combinations were used:

1) RP11-16K5 in 11q21 (chr11:95,929,949-96,073,030) with RP11-25P2 in 11q21 (chr11:96,650,441-96,797,156) and CEP11; 2) of RP11-356E17 in 11q23.3 (chr11:116,325,596-116,536,280) with CTD-3245B9 in 11q23.3 (chr11:118,643,767-118,890,685) and CEP11, and 3) CTD-3245B9 in 11q23.3 (chr11:118,643,767-118,890,685) with RP11-46D5 in 11q23.3 (chr11:119,222,049-119,392,148) and CEP11. For the cases reported as inv(12)(q13.1q24.1) and inv(12)(q13.3~14.1q24.11~24.12) the following combinations were used: 1) RP11-410D16 in 12q14.1 (chr12:61,926,855-62,084,090) with RP11-209I21 at 12q14.1 (chr12:64,569,968-64,719,641) and wcp12, and 2) RP1-305I20 in 12q24.11 (chr12:110,679,236-110,799,763) with RP11-90D13 in 12q24.12 (chr12:112,504,265-112,680,932) and wcp12. In the cases reported as inv(14)(q22q32.1) and inv(14)(q22.3q24.3) the following combination were used: 1) RP11-550M19 in region 14q23.1 (chr14:58,200,882-58,319,921) with RP11-701B16 in region 14q23.1 (chr14:59,969,361-60,140,574) and CEP14, 2) RP11-676P5 in region 14q23.3 (chr14:64,559,678-64,698,723) with RP11-701L2 in region 14q23.2~23.3 (chr14:64,698,730-64,882,102) and CEP14, and 3) RP11-285P21 in region 14q24.3 (chr14:78,388,349-78,591,383) with RP11-242P2 in region 12q31.1 (chr14:80,030,106-80,193,689) with CEP14. For the cases reported as inv(Y)(p11.2q11.222) the following combination were used: 1) RP11-35D7 in Yq11.2 (chrY:6,051,700-6,206,231) with RP11-506A3 in Yp11.2 (chrY:6,563,521-6,763,534) and CEPY, and 2) RP11-209I11 in Yq11.223 (chrY:23,449,035-23,619,769) with RP11-5C5 in Yq11.223 (chrY:24,820,670-24,977,625) and CEPY.

Locus-specific bacterial artificial chromosome (BAC) probes have been originally obtained from BACPAC Chori as bacteria stocks; DNA was extracted and labelled in the laboratory and was available for this work as DNA (Table 2). The wcp probes are homemade microdissection derived. The CEP probes used were obtained commercially from Abbott/Vysis – Mannheim, Germany, (CEP9, CEP11, CEPY), Cytocell – OGT, Oxford, UK (CEP12, CEPY), and Zytovision – Bremerhaven, Germany (CEP14/22).

Table 2. ***FISH homemade probe sets for detection of inversions in chromosomes 9, 11, 12, 14, and Y*** *with chromosome position of breakpoints. * There were found two breakpoints in the 11q23.3 cytogenetic band, thus two different combinations were used. Probe CTD-3245B9 is included in one of the breakpoints but not in the other. ** Probe RP11-35D7 is supposed to be on top of RP11-507A3 but in the images obtained with the use of test suspension is possible to see that they appear upside down. This visualization is confirmed by the results in patients.*

Chr	Cytogenetic band location	FISH probe	Inclusion in the inverted segment	Cytogenomic position acc. to GRCh37/hg19
9	9q32	RP11-9H12	No	115,825,791-116,002,725
9	9q32~9q33.1	RP11-78H18	Yes	117,567,550-117,726,266

9	9q34.13	RP11-5N16	Yes	134,994,187-135,087,298
9	9q34.2	RP11-153P4	No	136,541,741-136,720,872
11	11q21	RP11-16K5	No	95,929,949-96,073,030
11	11q21	RP11-25P2	Yes	96,650,441-96,797,156
11	11q23.3	RP11-356E17	Yes	116,325,596-116,536,280
11	11q23.3	CTD-3245B9	No/Yes *	118,643,767-118,890,685
11	11q23.3	RP11-46D5	No	119,222,049-119,392,148
12	12q14.1	RP11-410B16	No	61,926,855-62,084,090
12	12q14.1	RP11-209I21	Yes	64,569,968-64,719,641
12	12q24.11	RP1-305I20	Yes	110,679,236-110,799,763
12	12q24.13	RP11-90D13	No	112,504,265-112,680,932
14	14q23.1	RP11-550M19	No	58,200,882-58,319,921
14	14q23.1	RP11-701B16	Yes	59,969,361-60,140,574
14	14q23.2	RP11-676P5	No	64,559,678-64,698,723
14	14q23.2~23.3	RP11-701L2	Yes	64,698,730-64,882,102
14	14q24.3	RP11-285P21	No	78,388,349-78,591,383
14	14q31.1	RP11-242P2	Yes	80,030,106-80,193,689
14	14q24.1	RP11-179B8	Yes	69,155,047-69,336,025
14	14q24.1	RP11-486O13	Yes	70,543,612-70,729,879
14	14q24.3	RP11-368K8	Yes	76,140,845-76,326,753
14	14q24.3	RP11-463C8	Yes	77,583,547-77,763,874
Y	Yp11.2	RP11-35D7**	Yes	6,051,700-6,206,231
Y	Yp11.2	RP11-507A3**	No	6,563,521-6,763,534
Y	Yq11.223	RP11-209I11	Yes	23,449,035-23,619,769
Y	Yq11.223	RP11-5C5	No	24,820,670-24,977,625

2.2 Methods

2.2.1. Probe labelling

The work was done under a sterile and DNA free hood. Thus, UV light was on for 15 minutes before the beginning of the procedure. A MIX solution was prepared with the required quantities of each component (Table 3A). 19.2 µl of the MIX solution were added to each tube of 0.5 ml, together with fluorochromes. The quantity added is dependent on which fluorochrome is needed (Table 3B). With pipets reserved for DNA solutions, 2 µl of each unlabelled probe were added to the tubes. After a short centrifugation and vortex, the tubes were placed in a thermocycler that followed a labelling program (Table 4). A precipitation step

was performed by adding 10 µl of tRNA, 5 µl of 3 M sodium acetate with a pH of 5.2, and 100 µl of 100% ethanol. The solution was left to precipitate at 20 ºC overnight (16 hours).

Table 3. A) ***Reagents and required quantities for the MIX solution used in the labelling process of BACs.*** *B)* ***Required quantity of the distinct fluorochromes for the labelling process of BACs.***

A

Reagent	Quantity for one sample
H_2O	11,06 µl
L.Pu., 10x NP40	2 µl
DOP primer, 40 µM	1 µl
Label-mix	2 µl
$MgCl_2$ 25 mM	3 µl
Ampli Taq	0.12 µl

B

Fluorochrome	Quantity per sample
SO	2 µl
SG	2 µl
SR	2 µl
TR	0.3 µl
Bio	0.8 µl
Dig	0.8 µl

Table 4. ***Temperature and time of each step of the labelling program in the thermocycler****.*

	Time	Temperature
	3 minutes	94 ºC
31 x	1 minute	91 ºC
	1 minute	56 ºC
	2 minutes	70 ºC
	0.1ºC/second till	74 ºC
	5 minutes	72ºC
	∞	4 ºC

The solution was centrifuged at 15,300 rpm for 20 minutes at 4ºC to separate the DNA from unwanted reagents. The supernatant was discarded, and the pellet was dried in a vacuum centrifuge for 10-15 minutes. 80 µl of DS were added to the pellet, which was dissolved with the help of a shaker at 37 ºC. The probes were stored at -20 ºC until used.

2.2.3. Cell and slide preparation

Metaphases were obtained from peripheral blood, according to the protocol used in the laboratory, which is here described, as it was also performed for control slides in this work; cytogenetically worked up patient material was provided for this work. 1 ml of heparinized blood was added to 9 ml of cell culture medium, and the suspension was mixed carefully under sterile conditions. The mixture was incubated for 72 hours at 37 ºC. For each patient, two cell cultures were prepared. Ninety minutes before harvesting the cells, 0.05 ml of colcemid and

0.05 ml of ethidium bromide working solution were added to each culture bottle, mixed, and incubated at 37 ºC. The cell cultures were carefully shaken in the flasks for the cells to gather in the bottom of the bottle to be transferred into 15 ml tubes (Greiner tubes). The solutions were centrifuged at room temperature for 5 minutes at 1500 rpm on a 12 cm radius rotor. The supernatants were discarded by aspirating them carefully with a vacuum pump, up to the bend in the tubes to avoid loss of material. The pellets were resuspended in 5 ml of preheated hypotonic solution at 37 ºC and incubated at 37 ºC for 20 minutes. Afterward, 0.5 ml of fixative at 2 ºC – 8 ºC were added slowly and mixed carefully. The centrifugation was repeated, and the supernatants were aspirated as much as possible. 5 ml of fixative at 2 ºC – 8 ºC were used to resuspend the pellets. The washing steps of centrifugation and resuspension of the pellets were repeated 1 to 3 times until the supernatants were clear and the sediments were white. Cell suspensions were left overnight at -20 ºC and centrifuged with the same previous settings. Finally, the supernatants were aspirated, and the sediments were resuspended in 1 ml of fixative and stored at -20 ºC until the suspension is used. Clean slides were washed three times with distilled water and placed in Coplin jars with water at -20ºC for 10 minutes. For this study, samples from 2010 to 2022 were used. For suspensions from before 2019, the cells were centrifuged at 1500 rpm for 5 minutes at 21 ºC and the supernatants were replaced with fixative. The amount of fixative varies according to the size of the pellet. The pellets were resuspended and 60 – 80 µl of the cell suspensions were dropped on the ice-cold humid slides from a significant distance. The slides were held at a 45 ºC angle and 60 – 80 µl of fixative were spread downward. The drying process was accelerated on a hotplate at 50 ºC. The slides were visualized under a phase-contrast microscope to ensure enough metaphases in each slide to do a proper evaluation. Aging of slides was realized by leaving them at room temperature for 24 hours or incubating them at 65 ºC for 2 hours. After the aging process, the slides are ready to go through pre-treatment steps for FISH.

2.2.4. Slide pre-treatment and FISH-procedure

Slide pre-treatment for FISH procedures leads to better results, facilitating the evaluation and interpretation of the results, with brighter signals and less background. The time of pepsin treatment was adapted to optimize the balance between chromosome preservation and chromosome digestion. Chromosomes need to be digested for the probes to be able to reach the chromosomes. However, if they are too digested it might not be possible to interpret the results. The post-fixation solution was used to stabilize the chromosome morphology and harden the

chromosomes before the washing and dehydration steps. Formamide is an efficient reagent in the denaturation of target DNA [49]. The steps involving post-fix solution and formamide were realized inside a hood and the solutions were managed with care, due to their carcinogenic properties.

Pre-treatment started with a series of ethanol dehydration of slides in Coplin jars with 70%, 95%, and 100% ethanol at room temperature for 3 minutes each. After air-drying, 300 µl of preheated pepsin (37ºC) was added to each slide to suffer pepsin digestion. A 24 mm x 60 mm coverslip was put on the slides that were incubated for 2 minutes at 37ºC. The coverslips were removed, and the slides were washed in a Coplin jar with 1 x PBS for 5 minutes at room temperature. The slides were passed through a dehydration series of 70%, 95%, and 100% ethanol in Coplin jars for 3 minutes each and allowed to air-dry. The slides were submitted to 100 µl of post-fixation solution and incubated for 10 minutes at room temperature with 24 mm x 60 mm coverslips. After removing the coverslip, slides were immersed in 1xPBS and washed for 5 minutes at room temperature. Dehydration series of 70%, 95%, and 100% ethanol were performed in Coplin jars for 3 minutes at room temperature and the slides were air-dried. 100 µl of formamide was added to the slides for a denaturation step for 3 minutes at 73ºC. This was followed by a thermal shock realized by the immersion of the slide in a Coplin jar with 70% ethanol at -20ºC for 3 minutes. One last step of dehydration of the slides was performed with a series of 95% and 100% ethanol in Coplin jars at room temperature for 2 minutes each. The probe solutions were added to the air-dried denatured slides and covered with 24 mm x 24 mm coverslips on the region of interest. The solution was sealed with rubber cement and the slides were incubated in a humid chamber at 37ºC overnight (16 – 60 hours).

For the preparation of the probe solution, it was mandatory to consider the kind of probes involved. Depending on whether probes were labelled directly or indirectly, the solutions are prepared differently. All the combinations used in this study included indirectly labelled probes, which require a pre-hybridization treatment. 3 µl of each probe were added to a tube with Cot10 DNA and submitted to a denaturation and prehybridization program in a thermocycler. The program goes through one step of 5 minutes at 75ºC, followed by 2 minutes at 4 ºC, and finishes with 30 minutes at 37 ºC. Cot DNA is used to prevent cross-hybridization between the probes. When WCP probes are being used, 3 µl of it is added to the probe solution to go through the prehybridization program. The commercialized CEP probes are diluted in a hybridization buffer from the supplier and are heated to 37ºC by a variable time determined by the supplier.

Post-hybridization wash steps are required to remove probes that bonded to non-specific sites and that did not bond at all. It was necessary to optimize the stringency wash conditions of the probes used. Stringency is directly related to the temperature and inversely related to the salt concentration of the wash buffers [41]. Marvel solution is used to block cross-hybridization and eliminates non-specific ligation sites [50]. The addition of the antibodies solution allows the visualization of indirect labelling probes, together with the direct labelled ones.

The rubber cement and the coverslips were removed with forceps and the slides were post-washed for 2 minutes at 0,4 x SSC/Tw solution in Coplin jars at a water bath of 70 ºC. The next post-washing step was performed for 1 minute in Coplin jars with 2 x SSC solution at room temperature on a shaker. The slides were incubated with 100 µl of marvel solution for 15 minutes at 37ºC in a humid chamber, with 24 mm x 60 mm coverslips. The coverslips were removed, and the slides were washed with 4 x SSC/TW solution in Coplin jars at room temperature for 1 minute in a shaker. An antibody solution of 100 µl of marvel and the required quantity of the needed antibodies (Table 5) was prepared and added to slides, which were covered with 24 mm x 60 mm coverslips and incubated in a humid chamber at 37 ºC for 35 - 45 minutes. A last dehydration step was performed in a series of 70%, 95%, and 100% ethanol for 3 minutes each at room temperature. The slides were air-dried in the dark, counterstained with two drops of DAPI solution, and covered with 24 x 60 mm coverslips. The products of this procedure were slides with stained metaphase and visible probes, which were observed under a fluorescence microscope. At least 10 metaphases of each case and hybridization were evaluated, to assure that the visualized alteration is present in all cells.

Table 5. ***Quantity required of each antibody for the preparation of the antibodies solution****.*

Antibody labelled with	Quantity
Fl	1 µl
Cy5	5 µl
FITC	10 µl
Rhod	1 µl

3. Results

Breakpoints of five inversions were analysed with multicolour-FISH and with different probe combinations. It was possible to determine four pairs of breakpoints that are repeated in two or more cases (Table 6).

Table 6. ***Breakpoints found for each patient****, with cytogenetic band location and chromosome position of the* ***FISH probes used to characterize such breakpoints*** *and indication of which patients share the same breakpoints.*

Initial breakpoints	Cytogenetic band location	FISH probe	Cytogenomic position acc. to GRCh37/hg19	Patient	Same breakpoints found
inv(9)(q32q34.3)	9q32	RP11-9H12	115,825,791-116,002,725	1a	Yes
	9q32~9q33.1	RP11-78H18	117,567,550-117,726,266	1b	Yes
				1c	No
	9q34.13	RP11-5N16	134,994,187-135,087,298	1a	Yes
	9q34.2	RP11-153P4	136,541,741-136,720,872	1b	Yes
				1c	No
inv(11)(q21q23.3)	11q21	RP11-16K5	95,929,949-96,073,030	2a	No
	11q21	RP11-25P2	96,650,441-96,797,156	2b	Yes
				2c	Yes
				2d	Yes
	11q23.3	RP11-356E17	116,325,596-116,536,280	2a	Yes
	11q23.3	CTD-3245B9	118,643,767-118,890,685	2b	Yes
	11q23.3	CTD-3245B9	118,643,767-118,890,685	2c	Yes
	11q23.3	RP11-46D5	119,222,049-119,392,148	2d	Yes
inv(12)(q13.1q24.1)	12q14.1	RP11-410B16	61,926,855-62,084,090	3a	Yes
	12q14.2	RP11-209I21	64,569,968-64,719,641	3b	Yes
				3c	Yes
				3d	Yes
inv(12)(q13.3~14.1 q24.11~24.12)	12q24.11	RP1-305I20	110,679,236-110,799,763	3a	Yes
	12q24.13	RP11-90D13	112,504,265-112,680,932	3b	Yes
				3c	Yes
				3d	Yes
inv(14)(q22q32.1) inv(14)(q22.3q24.3)	14q23.1	RP11-550M19	58,200,882-58,319,921	4a	Yes
	14q23.1	RP11-701B16	59,969,361-60,140,574	4b	Yes
	14q23.2~23.3	RP11-701L2	64,698,730-64,882,102	4c	No
	14q24.3	RP11-285P21	78,388,349-78,591,383	4a	No
	14q31.1	RP11-242P2	80,030,106-80,193,689		
	-	-	-	4b	No
	-	-	-	4c	No
inv(Y)(p11.2q11.222)	Yp11.2	RP11-35D7	6,051,700-6,206,231	5a	No
	Yp11.2	RP11-507A3	6,563,521-6,763,534	5b	Yes
				5c	Yes
				5d	No
				5e	Yes
				5f	Yes
	Yq11.223	RP11-209I11	23,449,035-23,619,769	5a	No
	Yq11.223	RP11-5C5	24,820,670-24,977,625	5b	Yes
				5c	Yes
				5d	No
				5e	Yes
				5f	Yes

3.1. Results of cases 1a, 1b and 1c

Cases 1a, 1b, and 1c were referred to as inv(9)(q32q34.3) and various multicolour-FISH with different probe combinations were performed, in order to characterize the breakpoints (Figure 12). Patients 1a and 1b presented the same breakpoints on both ends, despite being from two apparently unrelated families. It was determined that these two cases are actually carriers of inv(9)(q32q34.13~34.2) (Table 7).

*Table 7. **Initial and final breakpoints of patients 1a, 1b, and 1c**, with the chromosome positions.*

Patient	Initial breakpoints	Final breakpoints	**Cytogenomic position acc. to GRCh37/hg19**
1a	inv(9)(q32q34.3)	inv(9)(q31~q33.1q34.13~34.2)	116,002,725-117,567,550 and 135,087,298-136,541,741
1b	inv(9)(q32q34.3)	inv(9)(q31~q33.1q34.13~34.2)	116,002,725-117,567,550 and 135,087,298-136,541,741
1c	inv(9)(q32q34.3)	-	-

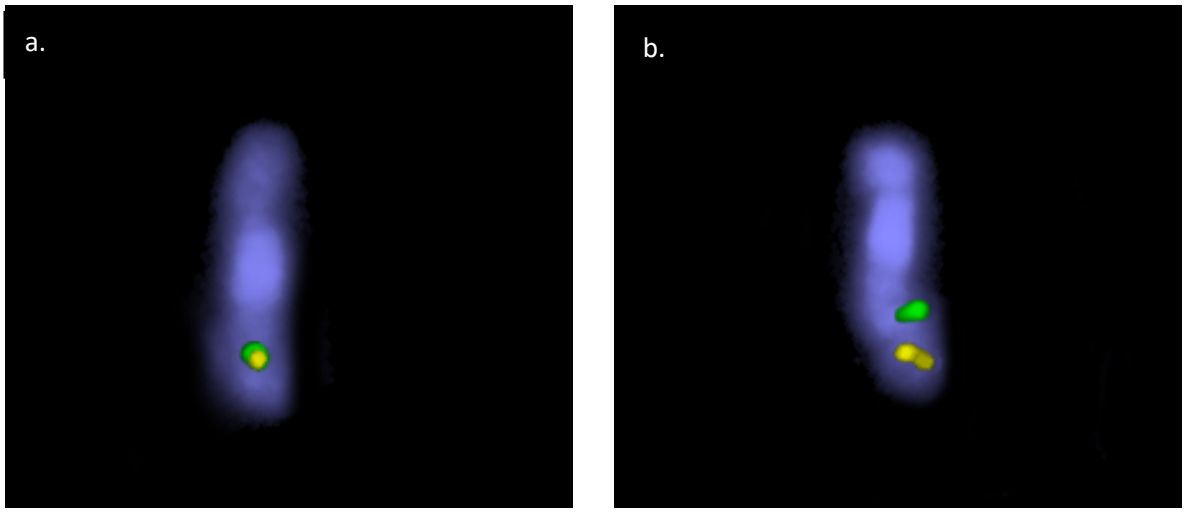

*Figure 8. **FISH results for the normal chromosome 9** (a.) **and the chromosome with an inversion** (b.) **of patient 1b.** Probes of the 9q32 breakpoint, RP11-9H12 and RP11-78H18, are green and yellow, respectively.*

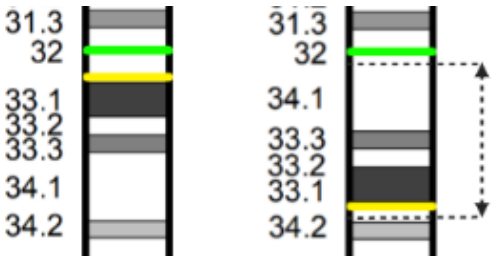

*Figure 9. **Graphic representation of the position of probes RP11-9H12** (green) **and RP11-78H18** (yellow) **in a normal chromosome** (left) **and in a chromosome with the inversion** (right). [Created with BioRender.com]*

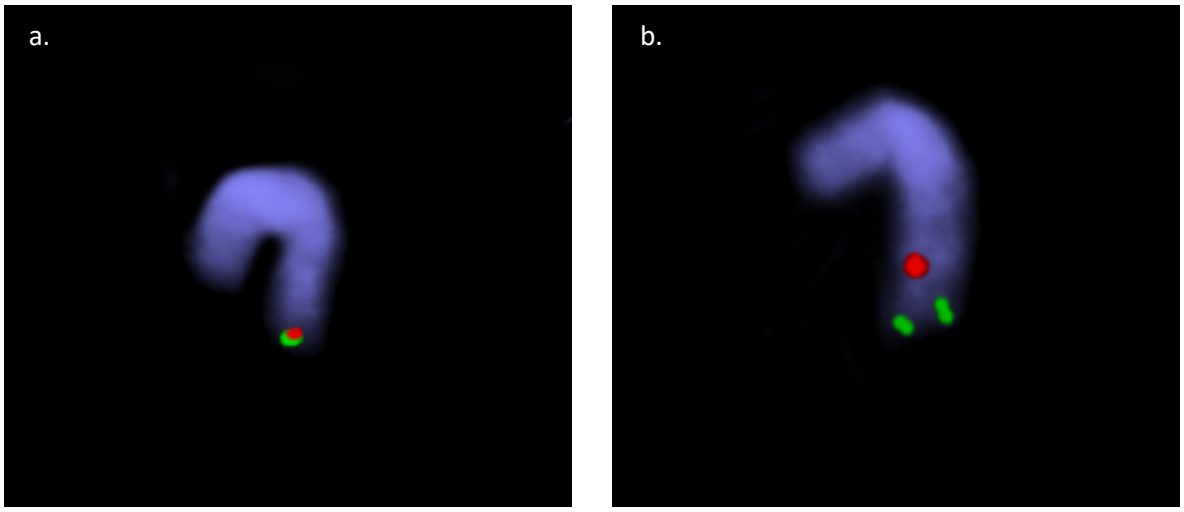

Figure 10. ***FISH results for the normal chromosome 9*** *(a.)* ***and the chromosome with the inversion*** *(b.)* ***of patient 1b.*** *Probes of the 9q33.13~34.2 breakpoint, RP11-5N16 and RP11-153P4, are red and green, respectively.*

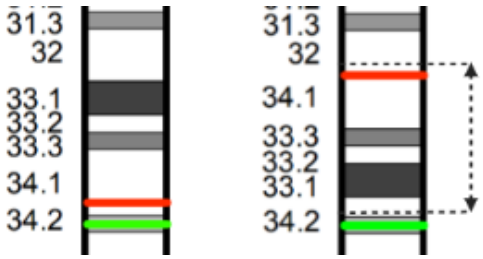

Figure 11. ***Graphic representation of the position of probes RP11-5N16*** *(red)* ***and RP11-153P4*** *(green)* ***in a normal chromosome*** *(left)* ***and in a chromosome with the inversion*** *(right). [Created with BioRender.com].*

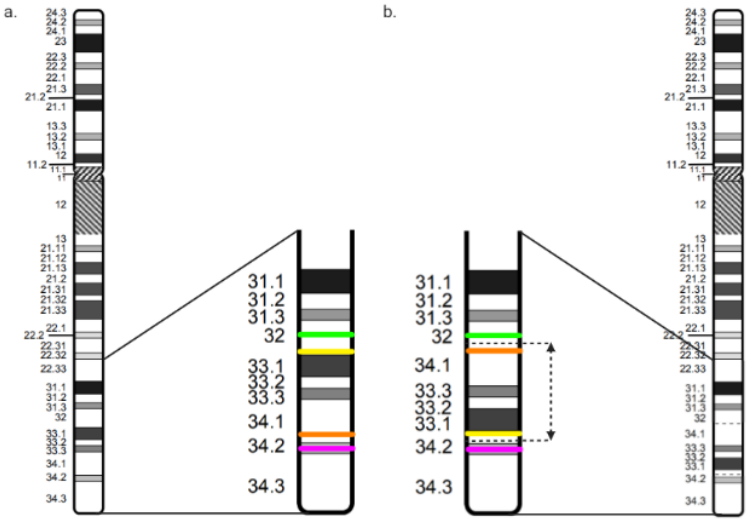

*Figure 12****. Graphic representation of the normal position of the probes*** *(a)* ***and the position observed in the FISH results for the inverted chromosome 9 of patients 1a and 1b*** *(b.) with the representation of both normal and inverted chromosome 9. Probes of the 9q32 breakpoint, RP11-9H12 and RP11-78H18, are represented as green and yellow, respectively. Probes of the 9q34.13~34.2 breakpoint, RP11-5N16 and RP11-153P4, are represented as orange and magenta, respectively.*

According to the results, the 9q32 breakpoint is between probes RP11-9H12 and RP11-78H18, which means that the breakpoint is the chromosome position 116,002,725-117,567,550 8

(Figures 8 and 9). On 9q34.13~34.2 the breakpoint is between probes RP11-5N16 and RP11-153P4, being located in the chromosome position 135,087,298-136,541,741 (Figure 10 and 11). It was not possible to determine the breakpoints for patient 1c due to the lack of metaphases in good condition, associated with the age of the sample.

3.2. Results of cases 2a, 2b, 2c, and 2d

Cases 2a, 2b, 2c, and 2d were referred to as inv(11)(q21q23.3) and various multicolor-FISH with different probe combinations were performed, in order to characterize the breakpoints. (Figure 17)

Table 8. ***Initial and final breakpoints of patients 2a, 2b, 2c, and 2d****, with chromosome positions.*

Patient	Initial breakpoints	Final breakpoints	Cytogenomic position acc. to GRCh37/hg19
2a	inv(11)(q21q23.3)	inv(11)(?q23.3)	Unknown and 116,536,280-118,643,767
2b	inv(11)(q21q23.3)	inv(11)(q21q23.3)	96,073,030-96,650,441 and 116,536,280-118,643,76
2c	inv(11)(q21q23.3)	inv(11)(q21q23.3)	96,073,030-96,650,441 and 118,890,685-119,222,049
2d	inv(11)(q21q23.3)	inv(11)(q21q23.3)	96,073,030-96,650,441 and 118,890,685-119,222,049

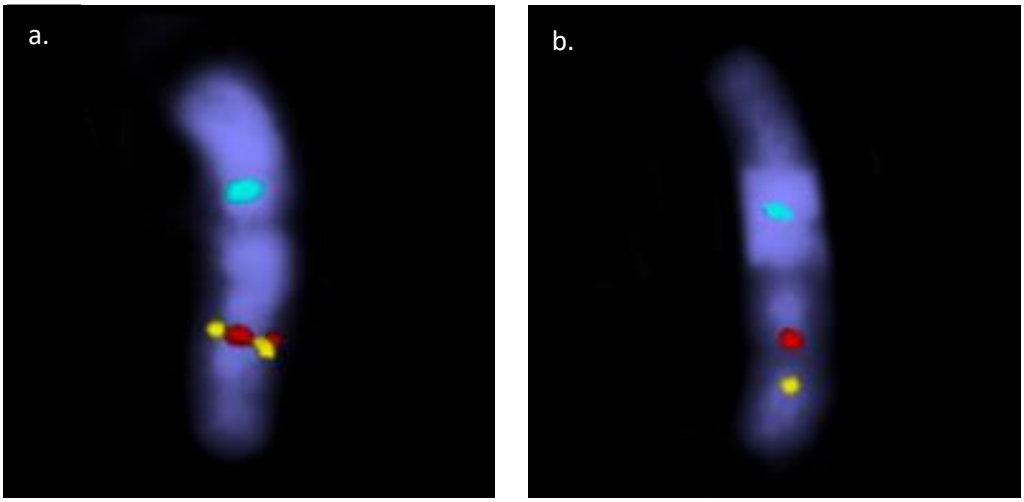

Figure 13. ***FISH results for the normal*** *(a.)* ***and the inverted*** *(b.)* ***chromosomes 11 of patient 2d,*** *Probes of the 11q21 breakpoint, RP11-16K5 and RP11-25P2, are red and yellow, respectively, and CEP11 is blue.*

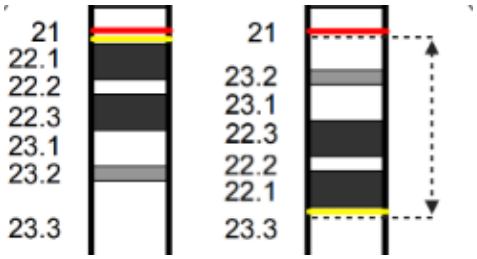

Figure 14. ***Graphic representation of the position of probes RP11-16K5*** *(red)* ***and RP11-25P2*** *(yellow)* ***in a normal chromosome*** *(left)* ***and in a chromosome with the inversion*** *(right). [Created with BioRender.com].*

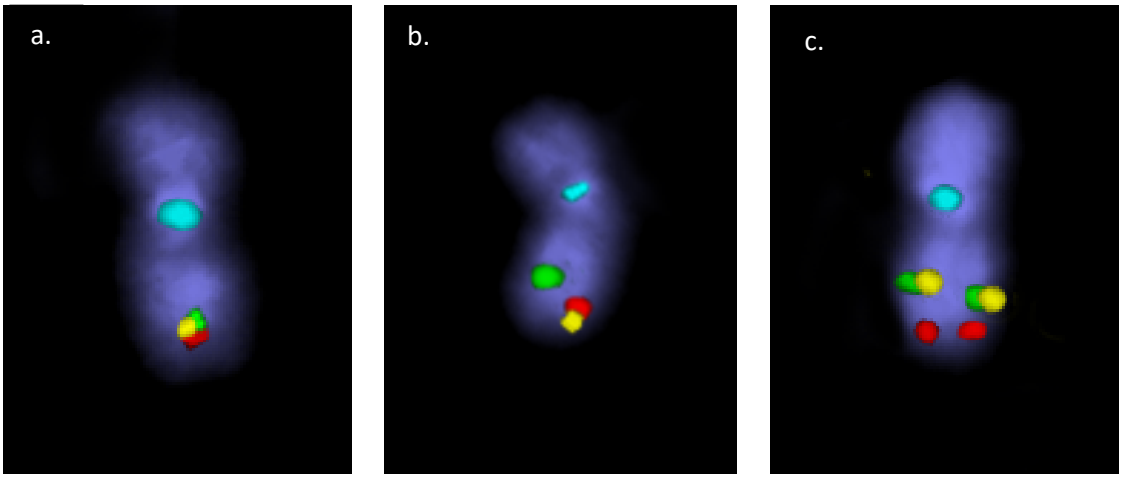

Figure 15. ***FISH results for the normal chromosome 11 of patient 2b*** *(a.)* ***and inverted chromosomes 11 of patient 2b*** *(b.)* ***and patient 2d*** *(c.).* *Probes of the 11q23.3 breakpoint, RP11-356E17, CTD-3245B9, and RP11-46D5, are green, yellow, and red, respectively, and CEP11 is blue.*

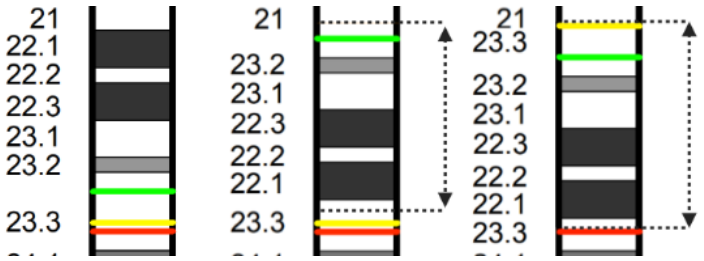

Figure 16. ***Graphic representation of the position of probes RP11-356E17*** *(green),* ***CTD-3245B9*** *(yellow)* ***and RP11-46D5*** *(red)* ***in a normal chromosome*** *(left) and in chromosomes with the inversion (middle and right). [Created with BioRender.com].*

Patients 2b, 2c, and 2b present the same breakpoint for 11q21, located between probes RP11-16K5 and RP11-25P2 namely, in the chromosome position 96,073,030-96,650,441 (Figure 13 and 14). Patient 2a presented a different 11q21 breakpoint, located above the determined position. No further studies were realized to characterize the exact location. According to the results, two positions were found for the 11q23.3 breakpoint. Cases 2a and 2b represent a breakpoint between probes RP11-356E17 and CTD-3245B9, being in the chromosome position 116,536,280-118,643,767 (Figure 15 and 16). Whereas cases 2c and 2d have the breakpoint between probes CTD-3245B9 and RP11-46D5, that is, in the chromosome position 118,890,685-119,222,049 (Figure 15 and 16). This way, patients 2b, 2c, and 2d are all carriers of inv(11)(q21q23.3), but 2b present a different breakpoint in q23.3 than 2c and 2d (Table 8).

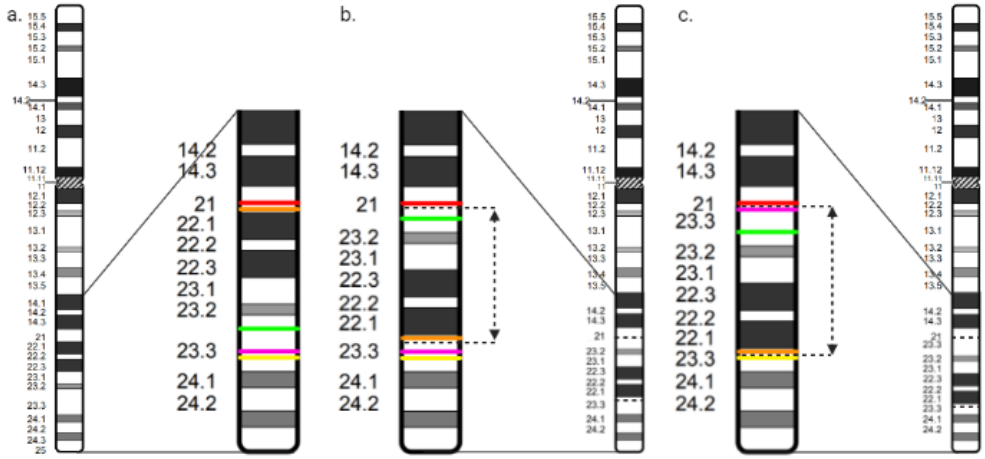

Figure 17. ***Graphic representation of the normal position of the probes*** *(a)* ***and the position observed in the FISH results for the inverted chromosomes of patient 2b*** *(b.)* ***and for patients 2c and 2d*** *(c.). Probes of the 11q21 breakpoint, RP11-16K5 and RP11-25P2, are represented by red and orange, respectively. Probes of the 11q23.3 breakpoint, RP11-356E17, CTD-3245B9 and RP11-46D5, are represented by green, magenta, and yellow, respectively.*

3.3. Results of cases 3a, 3b, 3c, and 3d

Cases 3a and 3b were referred to as inv(12)(q21q23.3) and cases 3c and 3d as inv(12)(q13.3~14.1q24.11~24.12). Various multicolour-FISH with different probe combinations were performed, in order to characterize the breakpoints (Figure 22). All patients presented the same breakpoints on both ends, despite being from two apparently unrelated families. It was determined that these four cases are actually carriers of inv(12)(q14.1~14.2q23.11~24.13) (Table 9).

Table 9. ***Initial and final breakpoints of patients 3a, 3b, 3c, and 3d****, with chromosome positions.*

Patient	Initial breakpoints	Final breakpoints	Cytogenomic position acc. to GRCh37/hg19
3a	inv(12)(q13.1q24.1)	inv(12)(q14.1~14.2q24.11~24.13)	62,084,090-64,569,968 and 110,799,763-112,504,265
3b	inv(12)(q13.1q24.1)	inv(12)(q14.1~14.2q24.11~24.13)	62,084,090-64,569,968 and 110,799,763-112,504,265
3c	inv(12)(q13.3~14.1q24.11~24.12)	inv(12)(q14.1~14.2q24.11~24.13)	62,084,090-64,569,968 and 110,799,763-112,504,265
3d	inv(12)(q13.3~14.1q24.11~24.12)	inv(12)(q14.1~14.2q24.11~24.13)	62,084,090-64,569,968 and 110,799,763-112,504,265

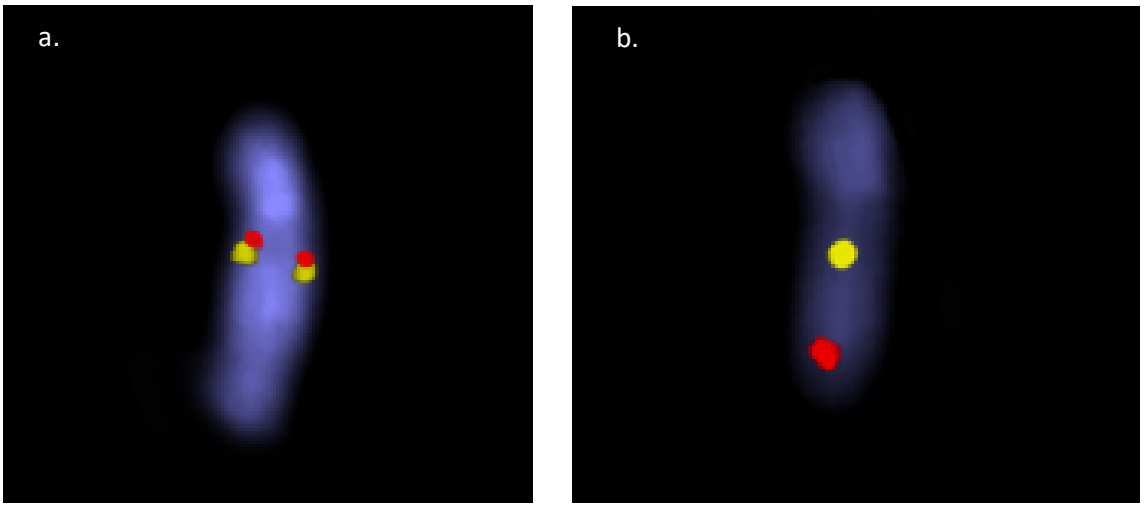

Figure 18. ***FISH results for the normal chromsome 12*** *(a.)* ***and the chromosome with the inversion*** *(b.)* ***of patient 3a.*** *Probes of the 12q14.1~14.2 breakpoint, RP11-410B16 and RP11-209I21, are yellow and red, respectively.*

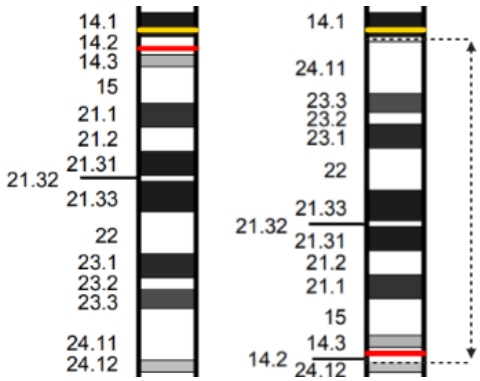

Figure 19. ***Graphic representation of the position of probes RP11-410B16*** *(yellow)* ***and RP11-209I21*** *(red)* ***in a normal chromosome*** *(left)* ***and in a chromosome with the inversion*** *(right). [Created with BioRender.com].*

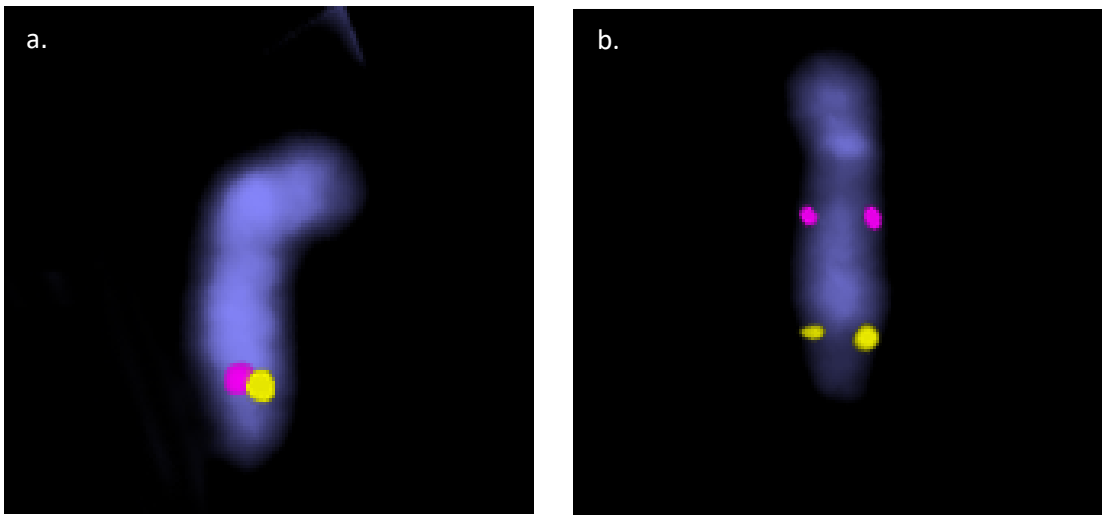

Figure 20. ***FISH results for normal chromosome 12*** *(a.)* ***and the chromosome with the inversion*** *(b.)* ***of patient 3a.*** *Probes of the 12q24.11~24.13 breakpoint, RP11-305I20 and RP11-90D13, are magenta and yellow, respectively.*

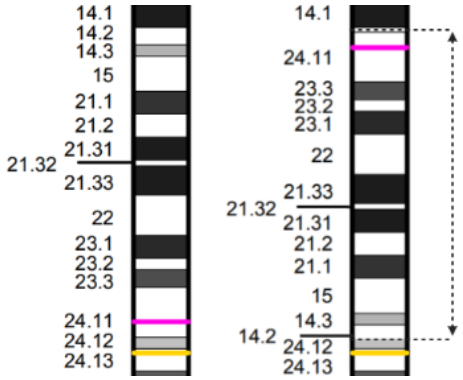

Figure 21. ***Graphic representation of the position of probes RP11-305I20*** *(magenta)* ***and RP11-90D13*** *(yellow)* ***in a normal chromosome*** *(left)* ***and in a chromosome with the inversion*** *(right). [Created with BioRender.com].*

According to the results, the 12q14.1~14.2 breakpoint is between probes RP11-410B16 and RP11-209I21, which means that the breakpoint is the chromosome position 62,084,090-64,569,968 (Figures 18 and 19). On 12q24.11~24.13 the breakpoint is between probes RP11-305I20 and RP11-90D13, being located in the chromosome position 110,799,763-112,504,265 (Figures 20 and 21).

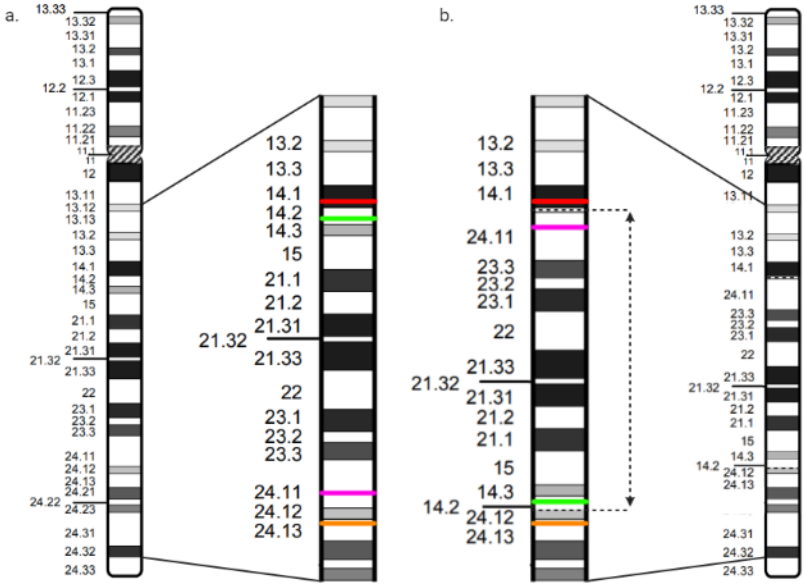

Figure 22. ***Graphic representation of the normal position of the probes*** *(a)* ***and the position observed in the FISH results for the inverted chromosomes of patients 3a, 3b, 3c, and 3d*** *(b.). Probes of the 12q14.1~14.2 breakpoint, RP11-410B16 and RP11-209I21, are represented as red and green, respectively. Probes of the 12q24.11~24.13 breakpoint, RP11-305I20 and RP11-90D13, are represented as magenta and orange, respectively.*

3.4. Results of cases 4a, 4b, and 4c

Case 4a was referred to as inv(14)(q22q32.1) and cases 4b and 4c as inv(14)(q22.3q24.3). Various multicolour-FISH with different probe combinations were performed, in order to

characterize the breakpoints (Figures 28). All patients presented different breakpoints. Expected the breakpoints in 14q23.1 on patients 4a and 4b, which are the same (Table 10).

Table 10. ***Initial and final breakpoints of patients 4a, ab, and 4c****, with chromosome positions.*

Patient	Initial breakpoints	Final breakpoints	Cytogenomic position acc. to GRCh37/hg19
4a	inv(14)(q23.1q32.1)	inv(14)(q23.1q24.2~31.1)	58,319,921-59,969,361 and 78,591,383-80,030,106
4b	inv(14)(q23.1q24.3)	inv(14)(q23.1q?)	58,319,921-59,969,361 and ?
4c	inv(14)(q23.2q24.3)	inv(14)(q23,2q?)	64,698,730-64,882,102 and ?

According to the results, patients 4a and 4b present the same breakpoint in region 14q23.1, which was determined by using the probes RP11-550M19 and RP11-701B16 and visualizing them in different chromosome bands. This makes the breakpoint to be in the chromosome region 58,319,921-59,969,361 (Figures 23, 24, and 25).

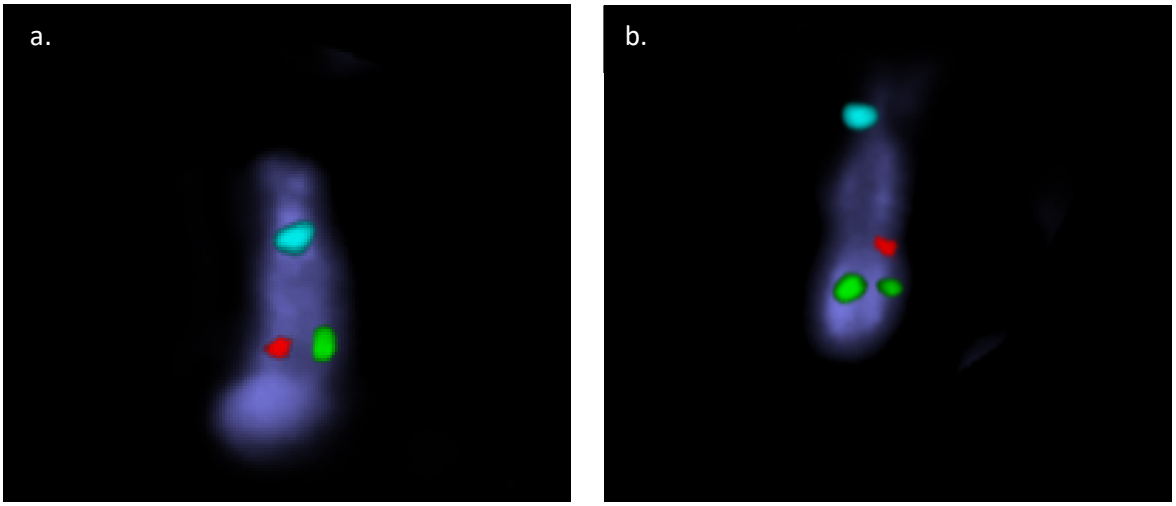

Figure 23. ***FISH results of normal chromosomes 14*** *(a.)* ***and the chromosome with the inversion of patients 4a*** *(b.). Probes of breakpoint 14q23.1, RP11-550M19 and RP11-701B16, are red and green, respectively. CEP14 is blue in both images*

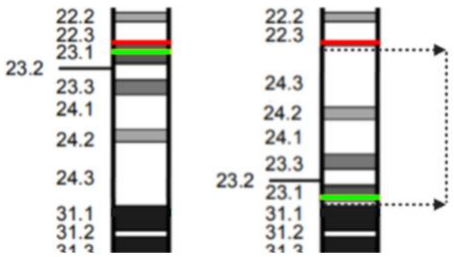

Figure 24. ***Graphic representation of the position of probes RP11-550M19*** *(red)* ***and RP11-701B16*** *(green)* ***in a normal chromosome*** *(left)* ***and in a chromosome with the inversion*** *(right). [Created with BioRender.com].*

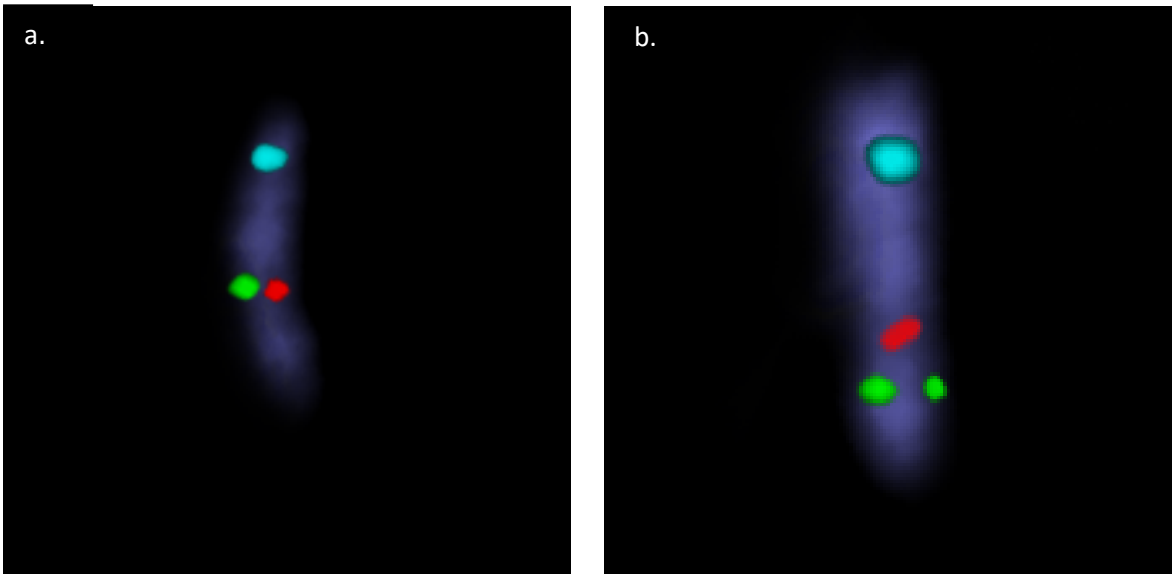

Figure 25. ***FISH results of normal chromosomes 14*** *(a.)* ***and the chromosome with the inversion of patients 4b*** *(b.). Probes of breakpoint 14q23.1, RP11-550M19 and RP11-701B16, are red and green, respectively. CEP14 is blue in both images*

Results for patient 4c show probe RP11-701L2 split and probe RP11-676P5 in region 14q23.2. These results allowed to conclude that the breakpoint is located within the chromosomal region of RP11-701L2, which corresponds to 64,698,730-64,882,102 (Figure 26).

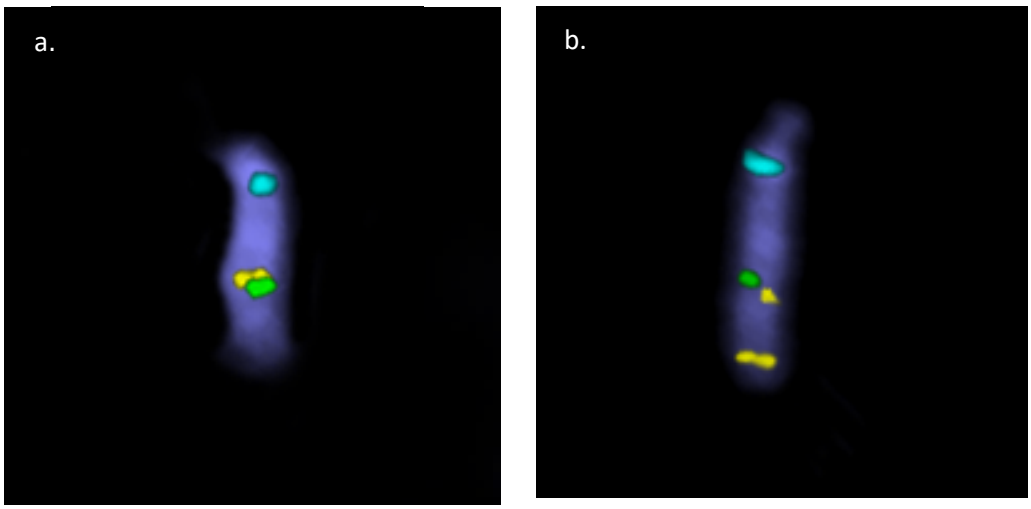

Figure 26. ***FISH results for normal*** *(a.)* ***and inverted*** *(b.)* ***chromosomes of patient 4c****. Probes of the 14q23.2 breakpoint, RP11-701L2 and RP11-676P5, are yellow and green, respectively CEP14 is blue in both images*

For the bottom breakpoints, different probes combinations were used to characterize them in each patient. On patient 4a, the 14q24.1~31.1 breakpoint was characterized with the use of probes RP11-285P21 and RP11-242P2, which means its chromosomal position is 78,591,383-80,030,106 (Figure 27).

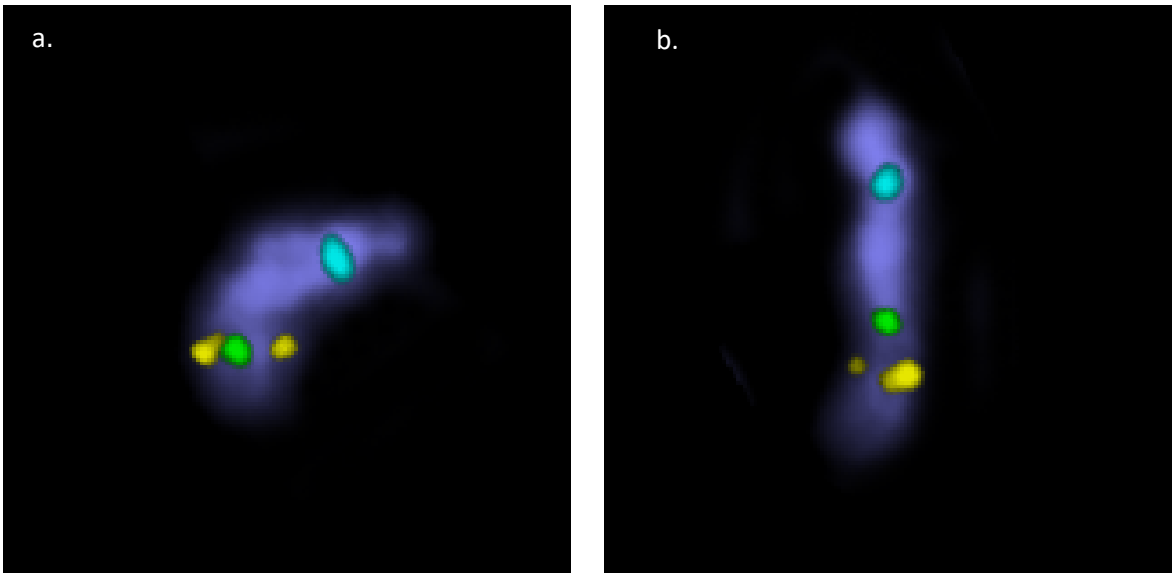

Figure 27. ***FISH results for normal*** *(a.)* ***and inverted*** *(b.)* ***chromosomes of patient 4c.*** *Probes of the 14q23.2 breakpoint, RP11-285P21 and RP11-242P2, are green and yellow respectively. CEP14 is blue in both images.*

For patients 4b and 4c, the breakpoint was not totally identified. Results show that patient 4b has a breakpoint below probe RP11-486O13 and patient 4c has a breakpoint below probe RP11-463C8. This is possible to visualize because all the probes in the used combinations are inverted, which means that they are included in the inversion segment. These results allowed to determine that patient 4a has an inv(14)(q23.1q24.2~31.1). It was not possible to identify the exact breakpoints for patients 4b and 4c.

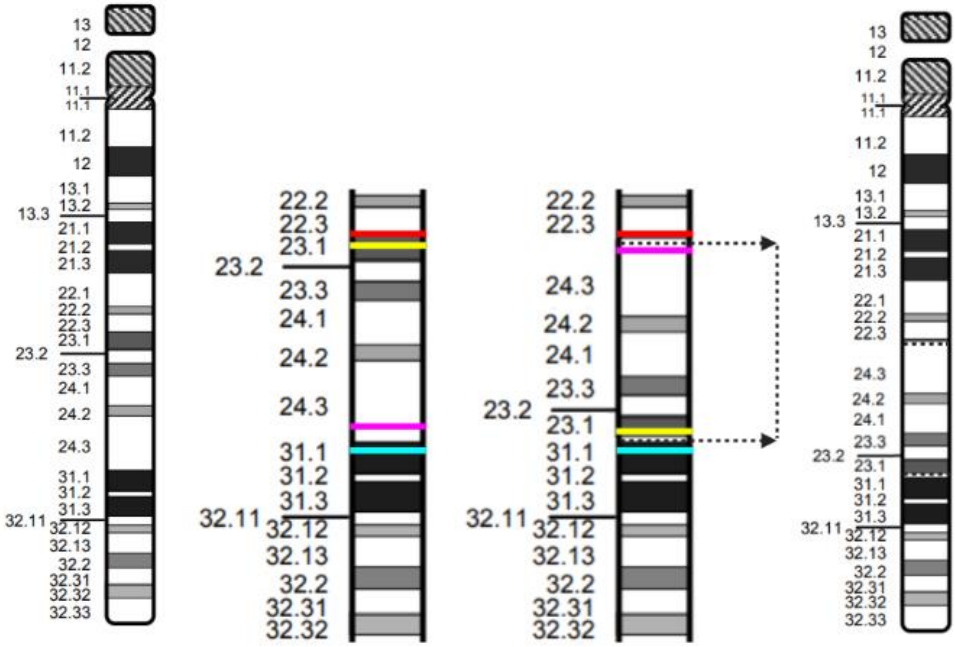

Figure 28. ***Graphic representation of the normal position of the probes*** *(a)* ***and the position observed in the FISH results for the inverted chromosome 14 of patient 4a****. Probes involved in the 14q23.1 breakpoint, RP11-550M19 and RP11-701B16, are red and yellow, respectively. Probes of the 14q24.1~31.1, RP11-285P21 and RP11-242P2, are magenta and blue, respectively.*

3.5. Results of cases 5a, 5b, 5c, 5d, 5e, and 5f

Cases 5a, 5b, 5c, 5d, 5e, and 5f were referred as inv(Y)(p11q11.222). Various multicolor-FISH with different probe combinations were performed, in order to characterize the breakpoints (Figure 33). All patients presented the same breakpoints on both ends, despite being from two apparently unrelated families. Except for patients 5a and 5d in which it was not possible to determine the breakpoints due to the lack of metaphases in good condition, associated with the age of the sample. It was determined that the other four cases are actually carriers of inv(Y)(p11.2q11.223) (Table 11).

Table 11. ***Initial and final breakpoints of patients 5a, 5b, 5c, 5d, 5e and 5f****, with chromosome positions.*

Patient	Initial breakpoints	Final breakpoints	Cytogenomic position acc. to GRCh37/hg19
5a	inv(Y)(p11.2q11.222)	-	-
5b	inv(Y)(p11.2q11.222)	inv(Y)(p11.2q11.223)	Unknow and 23,619,769-24,820,670
5c	inv(Y)(p11.2q11.222)	inv(Y)(p11.2q11.223)	Unknow and 23,619,769-24,820,670-24
5d	inv(Y)(p11.2q11.222)	-	-
5e	inv(Y)(p11.2q11.222)	inv(Y)(p11.2q11.223)	Unknow and 23,619,769-24,820,670-24
5f	inv(Y)(p11.2q11.222)	inv(Y)(p11.2q11.223)	Unknow and 23,619,769-24,820,670-24

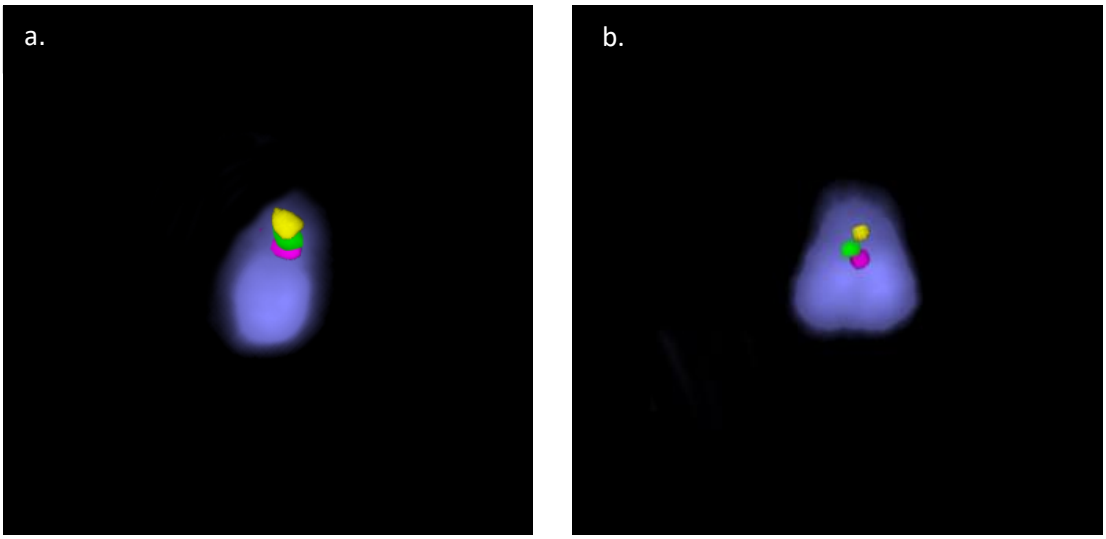

Figure 29. ***FISH results for normal chromosome Y of test suspension*** *(a.)* ***and for the inverted chromosome Y of patient 5b*** *(b.). On the left, probes of the Yp11.2 breakpoint, RP11-35D7 and RP11-507A3, are green and yellow, respectively, and CEPY probe is magenta (a.). On the right, probes of the Yp11.2 breakpoint, RP11-35D7 and RP11-507A3, are magenta and yellow, respectively, and CEPY probe green (b.).*

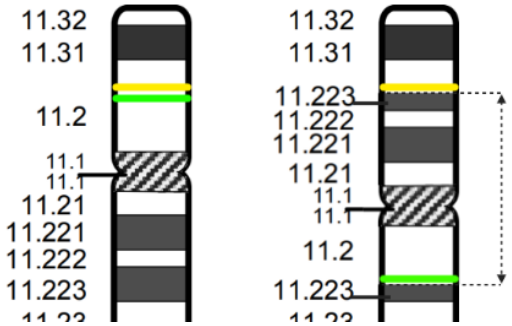

Figure 30. ***Graphic representation of the position of probes 35D7*** *(yellow)* ***and RP11-507A3*** *(green)* ***in a normal chromosome*** *(left)* ***and in a chromosome with the inversion*** *(right). [Created with BioRender.com].*

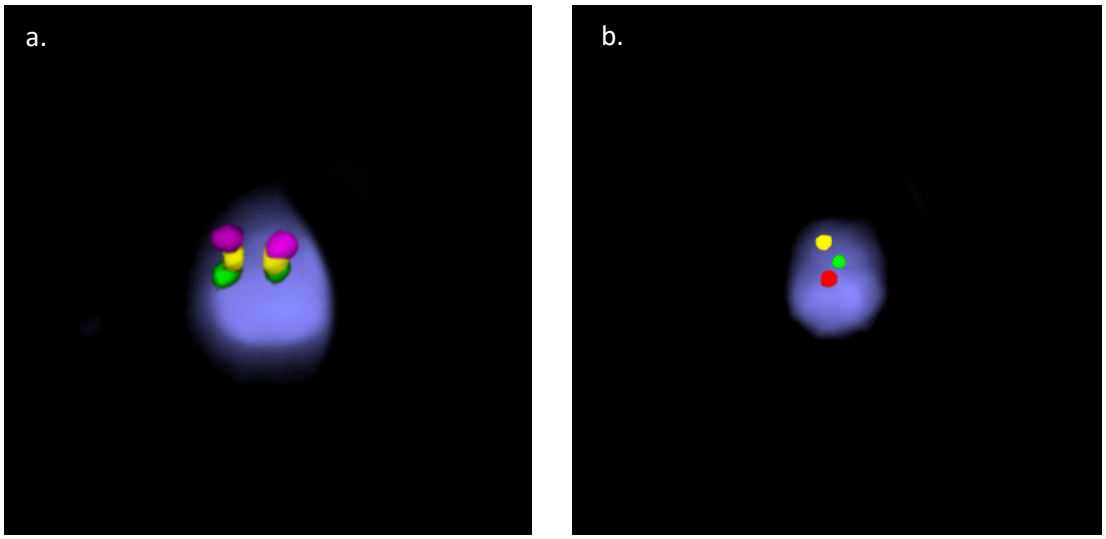

Figure 31. ***FISH results for normal chromosome Y of test suspension*** *(a.)* ***and for the inverted chromosome Y of patient 5b*** *(b.).* ***On the left,*** *probes of the Yq223 breakpoint, RP11-209I11 and RP11-5C5, are yellow and green, respectively, and CEPY probe is magenta (a.). On the right, probes of the Yq223 breakpoint, RP11-209I11 and RP11-5C5 are yellow and red, respectively, and CEPY probe is green (b.).*

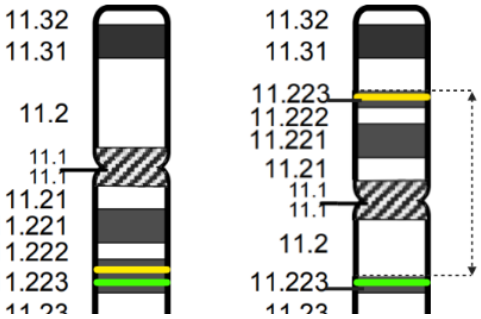

Figure 32. ***Graphic representation of the position of probes 209I11*** *(yellow)* ***and RP11-5C5*** *(green)* ***in a normal chromosome*** *(left)* ***and in a chromosome with the inversion*** *(right). [Created with BioRender.com].*

According to the results, the Yp11.2 breakpoint is between probes RP11-35D7 and RP11-507A3 (Figures 29 and 30). These two probes have an unknown chromosome position since their signals appear in reverse order. RP11-35D7 should be on 6,051,700-6,206,231 and RP11-507A3 should be on 6,563,521-6,763,534, but the signal of the second one is shown on top of the first one, in normal samples (Figure 29). On Yq11.223 the breakpoint is between probes RP11-209I11 and RP11-5C5, being located in the chromosome position 23,619,769-24,820,670-24 (Figures 31 and 32).

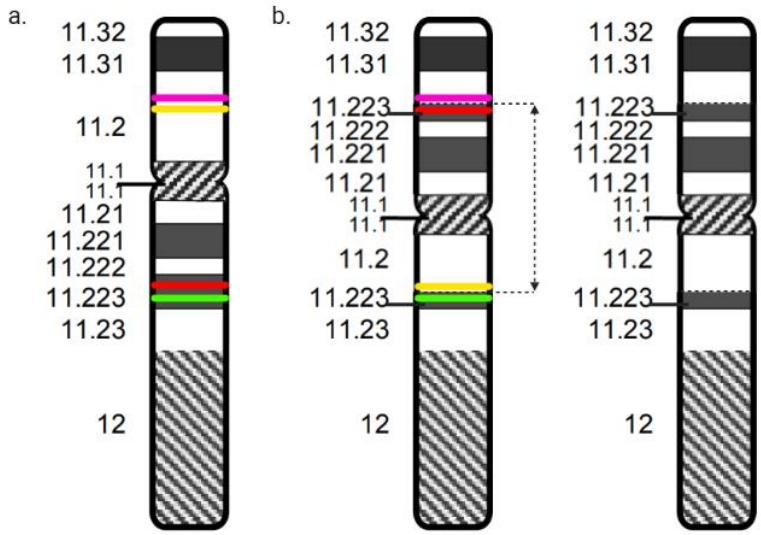

Figure 33. ***Graphic representation of the normal position of the probes*** *(a)* ***and the position observed in the FISH results for the inverted chromosome Y of patients 5b, 5c, 5e, and 5f*** *(b.). Probes of the Yp11.2 breakpoint, RP11-35D7 and RP11-507A3, are represented as yellow and magenta, respectively (a.). Probes of the Yq223 breakpoint, RP11-209I11 and RP11-5C5, are represented as red and green, respectively (b.)*

4. Discussion

Pericentric and paracentric inversions might have an impact on the progeny of carriers, and to study them is important to determine the estimated risk of having chromosomally abnormal offspring, related to delays and issues that may occur during meiosis [51]. The majority of inversions are formed by unique breakpoints, but recurrent inversions that share the same breakpoints have already been reported [52]. It is important to have the knowledge of repetitive inversions because genetic laboratories might be confronted with them more often and the study of their behaviour influences genetic counselling for affected individuals and couples [53].

All the 20 patients studied here were known inversion carriers that were referred to routine diagnosis due to infertility, and characterized by GTG banding. Conventional karyotyping has limitations related to resolution, not being able to detect rearrangements with smaller sizes than 5 Mb. For this reason, FISH technique was used to check whether the inversions were involving of the same breakpoints. FISH was at a resolution of 100kb - 1Mb [20].

Two patients that are carriers of a paracentric in chromosome 9 were studied and it was observed that both share the same breakpoints in 9q32 and 9q34.13~34.2. Three patients with a paracentric inversion in chromosome 11 share the same breakpoint in 11q21. Two of them share the same breakpoint on 11q23, while the other two have the breakpoint in 11q23 more distally. Four patients with a paracentric inversion in chromosome 12 were referred to as having different breakpoints, but in the course of this study, it was observed that all of them share breakpoints in 12q14.1~14.2 and 12q23.11~24.13. Two cases with a paracentric inversion on chromosome 14 were found to share the same breakpoint in 14q23.1. Four pericentric inversions in chromosome Y of apparently unrelated individuals were characterised as having the same breakpoints in Yp11.2 and Yq11.223.

The presence of repeated breakpoints in apparently unrelated individuals opens the possibility that they are truly recurrent breakpoints. However, it is necessary to realize follow up studies to confirm that they are genuinely recurrent or if the inversions have been transmitted IBD. The possible common ancestors may be closer or further down the generations. To discard IBD it would be necessary to measure the degree of allele sharing between the patients [20].

In case the repeated breakpoints are not a result of IBD, there is the possibility that they are events mediated by repetitive sequence elements, like LCRs. For this, the length of the location of the breakpoint would have to be narrowed down with appropriate techniques, so the genetic

environment of each inversion could be explored, using the human reference genome (GRCh37/hg19) at the UCSC genome browser [21]. The study of the origin of breakpoint is important to understand the way affected chromosome interact and evolved from one generation to another.

Unique breakpoints were also observed within these patients. The 11q23.3 breakpoint on patient 2b was not identified in other cytogenetically comparable patients. The same was the case for patient 4a, that presents a different breakpoint on the region 14q24.2~31 than the others. Although breakpoints were not determined exactly in all patients, it was possible to conclude that there are also such being localized in different positions. The breakpoints that were not exactly identified on the patients with inversions on chromosomes 11 and 14 should continue to be studied, using combinations of probes that are located above the probes that were used in this study. It is still a possibility that patients 4b and 4c present the same location for the more distal breakpoint.

Probes RP11-35D7 and RP11-507A3 should be further investigated to determine their exact position, in order to establish the breakpoint involved in this pericentric inversion of chromosome Y.

4.1. Recurrent breakpoint 9q32 and 9q34.13~34.2

Breakpoints 9q32 and 9q34.13~34.2 were identified in all the carriers that were possible to analyse in this work. These regions have been associated with duplications, translocations, and diseases [54–57]. This inversion is a paracentric one that occurs in the distal part of the q arm of chromosome 9. There is a possibility for the formation of inversion loops during meiosis that can result in acentric, dicentric, inverted and normal chromosomes. Because acentric and dicentric chromosomes are not mitotic stable, only the gametes with inverted and normal chromosomes will be able to fertilize and result in offspring. For this reason, the patients may relate to fertility problems.

4.2. Recurrent breakpoint 11q21 and 11q23

Breakpoint 11q21 was identified in all four analysed patients. Two different breakpoints were found in the region 11q23, that are localized 246,918 to 2,569,233 bp apart from each other. The inv(11)(q21q23) have been reported before at least in the Netherlands, in Germany, and in the United States of America [53,58–60]. Still, the breakpoints have not been determined in more detail yet. In general, this inversion is harmless, despite being related with spontaneous and

repeated abortions. This is a paracentric inversion that is not associated with abnormal offspring, although it is possible to transmit the inversion.

4.3. Recurrent breakpoints 12q14.1~14.2 and 12q23.11~24.13

Breakpoints 12q14.1~14.2 and 12q23.11~24.13 were identified in the four carriers that were studied. Subbands 12q14.1-q14.2 have been related with colorectal cancer and 12q14 have been related with benign lipoma [61,62]. There is a cyclin-dependent kinase (CDK) that was identified in the 12q14.1-14.2 region [62]. The inv(12)(q14.1~14.2q23.11~24.13) is a paracentric inversion that can be involved in the formation of inverted loop during meiosis. This can lead to the formation of various recombinants, but the only viable ones are a normal chromosome and a chromosome with the same inversion. Therefore, it is probably not associated with abnormal offspring.

4.4. Recurrent breakpoint 14q23.1

Breakpoint 14q23.1 was found is two of the three analysed carriers of a paracentric inversion in chromosome 14. This region has been associated with multiple disease such as anomalies in the branchial arches [63], colorectal cancer [64], and multiple congenital anomalies-intellectual disability [65]. As for the inversion itself, it is a paracentric inversion that is normally not associated with abnormal offspring, due to the mitotic instability of the acentric and dicentric recombinants that can be formed during crossing-over in meiosis in the event of inverted loop. This way, offspring can only receive a normal or an inverted chromosome with the same breakpoints. The fact that acentric and dicentric recombinants are also formed, explains the infertility problem of the carriers.

4.5. Recurrent Yp11.2 and Yq11.223

Breakpoints in Yp11.2 and Yq11.223 were identified in all the four carriers possible to analyse. The inv(Y)(p11.2q11.223) have been reported before [66], as well as a similar inv(Y)(p11.2q11.222), that is related with retardation features [67]. Gain of region Yp11.2 have been reported as a rare variant of Klinefelter syndrome [68], and this region is also in related with deletions in the Chinese Han population [69]. This is a pericentric inversion, in which the centromere is involved, and the proportion of the arms is changed. The recombinants resulting from crossing-over within a reverse loop in meiosis can include duplication and deletion of the distal parts of the inverted chromosome. In this case, one of the recombinants would have a duplication of region Ypter-Yp11.2 and a deletion of Yq11.223-Yqter, the other one would

have the complementary deletion and duplication and the other two would be one normal chromosome and one with the same inversion. To the best of my knowledge the breakpoints were not yet characterized in more detail in any other study.

Overall, the question to be worked on formulated in 1.5 of this work:
Do the five different inversions studied in 20 unrelated carriers share identical breakpoints?
can be answered with – yes, they do.

Still, we encountered some limitations like the lack of metaphases in good conditions in older samples that made it impossible to characterize their breakpoints. Other than that, further studies should be conducted to narrow the position of breakpoints for better characterization and understanding of the results.

5. Conclusions

The present work represents the first time that a study has been conducted to examine whether or not there is a possibility that the five studied constitutional inversions found among middle-European / German populations are repetitive. It is not unlikely that more balanced aberrations, which have yet to be recognized, will be detected in unrelated individuals with fertility problems. This has been demonstrated recently in a study of a population from southeast Europe with the inversion inv(9)(p23q22.3) [21].

By analysing the 20 present cases using FISH technique, this work has shown that the breakpoints studied were not all recurrent, however, at least one of the breakpoints in all of the inversions was recurrent. Similar breakpoints were found on both sides of the inversions on chromosomes 9 and 11 as well as chromosome Y. The breakpoints for chromosome 11 and Y have also been reported in infertile individuals previously [53,66].

In the end this means that it is still possible to carefully analyse seemingly unique inversions in infertile in more detail in case they show up more than one time. The contribution of such rearrangements to the genetic variety of human population is not yet fully accessed.

6. Final considerations

The worked carried out to fulfil the requirements necessary to obtain the Master's degree in Clinical Laboratory Genetics took place at the Institut für Humangenetik, Universitätsklinikum Jena, Germany. During the period I was in the institute, I had the opportunity to learn, follow and participate in the daily routine of a molecular cytogenetics laboratory.

As part of the work, I had the opportunity to independently perform the FISH molecular cytogenetics technique with BACs, CEPs and WCP probes, in samples of peripheral blood. I also performed microscopic analysis with image capture of metaphases with the help of ISIS software. I accompanied the process of preparation of culture and manipulations of peripheral blood cells and bone marrow cells. I also had the opportunity to follow the staining and chromosomal banding techniques (GTG- and C-banding), the molecular cytogenetic techniques Fiber-FISH and FISH in tissue, as well as in fish cells, and the molecular genetics techniques Real-Time PCR and NGS.

In addition to the technical skills acquired in molecular cytogenetics, additional skills acquired such as work planning, reagent preparation, and autoclaving were also consolidated.

Having had the opportunity of working with the entire team at the Institut für Humangenetik, Universitätsklinikum Jena, in a work environment characterized by rigor and responsibility was an honour and a privilege.

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