UNIVERSIDADE D
COIMBRA

Ana Luísa Teixeira Fontes

Insights into molecular cytogenetic routine diagnostics and characterization of repeated showing up constitutive chromosomal translocations

Dissertação no âmbito do Mestrado em Genética Clínica Laboratorial orientada pelo Professor Doutor Thomas Liehr e pela Professora Doutora Joana Barbosa de Melo e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Setembro de 2023

Faculdade de Medicina da Universidade de Coimbra

Insights into molecular cytogenetic routine diagnostics and characterization of repeated showing up constitutive chromosomal translocations

Ana Luísa Teixeira Fontes

Dissertação no âmbito do Mestrado em Genética Clínica Laboratorial orientada pelo Professor Doutor Thomas Liehr e pela Professora Doutora Joana Barbosa de Melo e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Setembro de 2023

UNIVERSIDADE D
COIMBRA

I would like to thank my supervisor, Professor Thomas Liehr, for enabling me to learn and improve my skills, for guiding me and for the permanent availability. Thank you for always answering my doubts, for your comprehension and for allowing me to have this opportunity.

To Niklas, Steffi and Luisa I would like to thank you all for the patience and help whenever I needed and for always being nice even when things went wrong.

For my team blue, Clem, Helo and Victor, for always putting a smile on my face even on the most difficult days. *Merci* for the trips, even the ones that never happened, and for being the best companions and support in Jena.

À minha família por me ter permitido esta aventura e por todos os esforços que me permitem estar onde estou hoje; agradecendo em especial à minha mãe.

Agradecer às professoras Isabel, Joana e Marta pela oportunidade dada e pela ajuda e apoio no meu percurso neste mestrado. Um obrigado também aos meus colegas, em especial à minha Rita, minha compincha de tudo, que me ouviu e ajudou e esteve lá sempre para mim nestes últimos dois anos.

Agradecer à doutora Marisa Marques por me ter incentivado a não desistir e ter-me ajudado a ultrapassar as minhas dificuldades e continuar, pois, tudo iria compor-se mesmo que devagar.

Aos meus amigos por estarem sempre lá para me ouvir, animar e incentivar quando mais precisava. Obrigada Mariana, Margarida e Carina e, em especial, obrigado à Ana pela companhia na Alemanha, ao Pedro por me ouvir sempre que precisei e à Castro por toda a força e suporte, acreditando mais em mim que eu própria.

Agradecer aos Bioqs pela família que foram durante todos estes anos de companheirismo e entreajuda, até durante o Covid, todo este percurso não teria sido a mesma coisa sem vocês. Um especial agradecimento aos meus meninos e melhor família que podia pedir. Ao Luís e à Ana por toda a força e companhia, ao Joel pela disponibilidade constante e companhia em todas as tardes de estudo, à Sofia por ouvir todos os meus desabafos e dar os melhores conselhos, ao Falcão por simplesmente estar lá, aos meus rijos, Margarida, Rita e Rafa, porque estes anos não teriam sido os mesmos sem vocês, e à Daniela, Grácio, Bea, Raquel, Silvério… e a tantos outros que não cabem aqui, mas que do fundo do meu coração vos agradeço.

Por fim, queria agradecer ao Chico, por toda a paciência e disponibilidade mesmo quando a mesma era escassa. Por estares sempre presente e me acompanhares durante o melhor e, sobretudo, o pior deste último ano, sem nunca teres desistido de mim. *Obrigada.*

ABSTRACT

Chromosomal aberrations are defined as changes in chromosomes, structural or numeric, that can result in genetic diseases. They are formed by chromosomal breakage or unequal crossing-over and can affect the content and shape of different chromosomes, altering the distribution of the genes within the genome. Translocations are part of structural aberrations and involve the exchange of segments between chromosomes, being classified as reciprocal or Robertsonian. Breakpoints are often not well characterized in repeatedly appearing translocations, being important their further characterization and narrow down. Thus, it is necessary to better characterize recurring translocations by molecular cytogenetics.

Banding cytogenetics allows the characterization of chromosomes based on alternating light and dark regions, a commonly used and very informative method, however, it has limitations that can be overcome by fluorescence *in situ* hybridization (FISH). This technique enables the simultaneous characterization of several chromosomal subregions smaller than a chromosomal band. The applied probes can be specific for individual chromosomes, regions of them or even genes, allowing the identification of various rearrangements. The specific FISH-signals can be detected using a fluorescence microscope. To characterize the breakpoints in selected translocations cases multicolor FISH was performed applying locus-specific and whole chromosome painting (WCP) probes.

Four translocations were analyzed in this work. The first translocation in analysis is the t(11;22)(q23.3;q11.21); here the goal was to verify the published breakpoints and to establish a probe set for its reliable characterization. Also, two new possible recurrent translocations are reported and their breakpoint regions were characterized, i.e. the translocations t(5;16)(q13.3;p13.3) and the t(Y;12)(q12;q12). Finally, a der(X)t(X;Y)(p22.32;p11.31) was studied; it could be characterized as carrying yet not reported breakpoints, adding one more variant to the already ~25 known ones.

Keywords: chromosomal aberrations; translocations; breakpoints; cytogenetics; fluorescence in situ hybridization (FISH)

Alterações cromossómicas definem-se como mudanças, estruturais ou numéricas, nos cromossomas que podem resultar em doenças genéticas. São formadas ou por quebras dos cromossomas, ou por um *crossing-over* desigual, e podem afetar o conteúdo e forma de diferentes cromossomas, levando a uma alteração da distribuição dos genes no genoma. As translocações são alterações estruturais que envolvem a troca de segmentos entre cromossomas, podendo ser classificadas como recíprocas ou Robertsonianas. Frequentemente, os pontos de quebra não se encontram bem caracterizados em translocações recorrentes, sendo importante a sua melhor caracterização e delimitação. Assim, recorrendo à citogenética molecular é necessário melhor caracterizar estas translocações recorrentes.

A citogenética convencional permite a caracterização dos cromossomas com base no seu padrão alternado entre bandas claras e escuras, um método habitualmente usado e muito informativo que, no entanto, apresenta limitações, as quais podem ser ultrapassadas recorrendo à técnica de FISH (*fluorescence in situ hybridization*). Esta técnica permite a caracterização simultânea de diversas regiões cromossómicas mais pequenas que uma banda cromossómica e as sondas utilizadas podem ser específicas para cada cromossoma, para as suas regiões ou até para genes, permitindo a identificação de vários rearranjos. Os sinais de FISH obtidos são depois detetados utilizando um microscópio de fluorescência. Para caracterizar os pontos de quebra nos casos selecionados de translocações, *multicolor* FISH foi realizado, aplicando-se sondas especificas de *locus* e de pintura cromossómica total (WCP).

Quatro translocações foram analisadas neste trabalho. A primeira translocação em análise foi a t(11;22)(q23.3;q11.21), onde se pretendia verificar os pontos de quebra já publicados e estabelecer um novo conjunto de sondas fiável para a sua caracterização. Igualmente, duas novas translocações possivelmente recorrentes são reportadas e os seus pontos de quebra foram caracterizados; as translocações t(5;16)(q13.3;p13.3) e a t(Y;12)(q12;q12). Por fim, um caso da translocação der(X)t(X;Y)(p22.32;p11.31) foi estudado e caracterizado, tendo-se observado a existência de pontos de quebra ainda não reportados, adicionando-se uma nova variante às já ~25 conhecidas.

Palavras-chave: alterações cromossómicas; translocações; pontos de quebra; citogenética; fluorescence in situ hybridization (FISH)

acro – Acrocentric

AT – Adenine-Thymidine

BAC – Bacterial artificial chromosome

Bio – Biotin

C-banding – Centromeric banding

DAPI – 4,6-diamidino-2-phenylindole.2HCl

DEAC – Diethylaminocoumarin-5-dUTP

der – Derivative

Dig – Digoxigenin

DS – Dextran sulphate

DSBs – Double-strand breaks

GTG-banding – Giemsa-Trypsin banding

FISH – Fluorescence *in situ* hybridization

LCRs – Low copy repeats

LSPs – Locus specific probes

Mb – Mega base (pair)

mFISH – Multicolour fluorescence *in situ* hybridization

PATRRs – Palindromic AT-rich repeats

PBS – Phosphate saline buffer

PCP – Partial chromosome painting

PCR – Polymerase chain reaction

Q-banding – Quinacrine fluorescence banding

rpm – Revolutions *per* minute

RT – Room temperature

SO – SpectrumOrange-dUTP

SRY – Sex-determining region Y

SSC – Saline sodium citrate

sSMC – Small supernumerary marker chromosome

TR – TexasRed

Tw20 – Tween 20

WCP – Whole chromosome painting

The work was based first on gaining some insights into routine diagnostics in the molecular cytogenetic host laboratory in Jena, and, after developing some autonomy, in working on a scientific question. Thus, the task described here is to do a detailed FISH-based study of a number of cases of translocation from the laboratory's case collection (http://cs-tl.de/DB/CA/BPs/0-Start.html) with seemingly same breakpoints for a further and more precise characterization of the breakpoints involved.

Such analysis is important hence translocations can either be private mutations, being unique in breakpoints, or be observed repeatedly in unrelated individuals of a population. In the latter case, the rearrangement can be due to a founder event or due to a specificity in the human genome which promotes formation of the identical translocation event. It is important to be aware of these three groups; to distinguish them it is at first necessary to be able to characterize potentially repeatedly occurring translocation events from spontaneous ones.

In this study a probe set to characterize the known, most-frequent non-Robertsonian translocation, t(11;22)(q23.3;q11.21) translocation was established and tested in nine patients. Also, one case, a male with karyotype 46,XX with known der(X)t(X;Y)(p22.32;p11.31), and two yet not reported translocations known events having been found in two and one case respectively, were included in this study: t(5;16)(q13.3;p13.3) and t(Y;12)(q12;q12).

The *Institute of Human Genetics* in Jena, Germany is a diagnostic and investigation centre whose focus is the better understanding of constitutional and acquired hereditary disorders (including cancer). The whole spectrum of genetic changes from chromosomal rearrangements, epigenetic and (molecular) genetic changes is studied. The services provided are realized in close cooperation with the *University Centre for Ambulant Medicine of Human Genetics* in Jena and all are certified, with regular participations in quality controls. The institute has a wide variety of groups that focus on specific fields, like molecular genetics, molecular haematology, cytogenetics, tumour cytogenetics and, the group where I developed my work, molecular cytogenetics, mainly applying the fluorescence *in situ* hybridization (FISH) technique. The molecular cytogenetics' group research focuses are, among other subjects, chromosomal rearrangements, small supernumerary marker chromosomes (sSMCs) and aberrations in acute lymphocytic leukaemia and acute myelocytic leukaemia.

My dissertation work focused on analysing the breakpoints of recurrent and potentially recurrent translocations, more specifically these four translocation events: t(11;22)(q23.3;q11.21), t(5,16)(q13.3;p13.3), t(Y;12)(q12;q12) and der(X)t(X;Y)(p22.32;p11.31). For that, FISH procedures were conducted, and microscopic analyses were performed.

1.1 Chromosomal Aberrations

When talking about genetic disorders, we can separate these in at least four different categories: single-gene defects, multifactorial conditions, epigenetic defects, and chromosomal aberrations/ abnormalities. The later can be described as alterations, structural or numerical, in single or multiple chromosomes, affecting both autosomes and sex chromosomes. They can be due to an error in cell division during mitosis or meiosis, that can occur in prenatal, postnatal, or during the preimplantation period. These affect the normal function of the organism and may also affect growth and development, since severe clinical consequences can arise from these abnormalities, such as spontaneous abortions, intellectual disabilities, neonatal death, malformations, and identifiable syndromes. When it is possible to identify these chromosomal abnormalities, prevention strategies, genetic counselling, and the appropriate treatment can be

established[1]. Chromosomal aberrations can be constitutional, which emerge during gametogenesis or early embryogenesis and so affect all cells, or acquired, which are normally related to tumours or leukaemia, since they develop after birth and only affect a specific group of cells[1,2]. Numerical abnormalities are more common than structural ones[1]; the latter result from breaks in chromosomes and unequal exchange between them. Deletions, duplications, inversions, ring chromosomes, isochromosomes and translocations, among others, are all structural aberrations[1,2]. These abnormalities can be inherited from a carrier parent, balanced or unbalanced, or can occur as *de novo* events, being formed in a single gamete or zygote[2].

1.1.1 TRANSLOCATIONS

The concept of chromosomal translocations has been part of the scientific knowledge for almost eight decades. A translocation involves the break of a specific zone in a chromosome and respective fusion with another chromosome, resulting in an exchange[2,3]. They can occur between two or more chromosomes and can be divided in reciprocal and Robertsonian. Reciprocal translocations are based on the exchange and fusion of the proximal portion of a chromosome with the distal of another, and the other way around[3]; whereas Robertsonian translocations involve only acrocentric chromosomes and a fusion of the long arms of two acrocentric chromosomes occurs due to a breakage at the centromere level, resulting on the loss of the short arms of the chromosomes involved and creating a new single chromosome, the result of the junction of the long arms[1,2]. Contrarily to terminal deletions, which occur when there is a break in a single chromosome, and inversions and ring chromosomes that occur due to two breaks in a single chromosome, translocations are due to two or more breaks in different chromosomes[2].

Double-strand breaks (DSBs) are single strand breaks in both strands of the DNA and problems in the DNA DSB repair system, an essential process in the stability of the genome, are the cause of translocations. When a DSB is formed, the two chromosomal fragments can move away from each other, emerging the possibility of connection to another chromosome spatially proximate. Cells have ways of dealing with these lesions, namely recurring to nonhomologous end joining and homology-dependent repair. Normally, both ends of a DSB are reconnected by these mechanisms, occurring only a small loss of genetic information; however, one or both ends of a DSB can end up incorrectly re-joined to another DSB, ending up in a translocation. Some factors

that can cause DSBs in cells are DNA replication errors and inappropriate action of cytidine deaminases, among others[3].

When balanced, translocations represent important mutations causative of Mendelian diseases and cancer, being important biological tools in the mapping of *loci* and cis-regulating regions of genes involved in diseases, while also being the cause of them[4].

1.1.1.1 Clinical Relevance of Translocations

Taking into consideration that about 0.4 to 0.9% of new-borns have chromosomal abnormalities, the clinical significance of these becomes evident[1]. Balanced translocations normally result in phenotypically normal individuals, which, however, are more prone to reproductive abnormalities, such as infertility (male carriers are associated with an increased risk of oligospermia or complete azoospermia), spontaneous abortions and abnormal offsprings[2,4]. These translocations can be transmitted from generation to generation, being sometimes only discovered in couples with reproductive problems or unbalanced offspring, since the meiotic process can be stopped or unbalanced gametes are produced by the carrier progenitor[4].

Unbalanced progeny is caused because of problems that can arise from the alteration of the meiotic process. In a translocation carrier, in pachytene a quadrivalent (reciprocal translocation) or a trivalent (Robertsonian translocation) is formed, resulting in normal, balanced, or unbalanced gametes during the segregation step[2,4]. There are four types of segregation that can occur in reciprocal translocations: alternate segregation, where all the gametes formed are balanced; adjacent I segregation, where normally compatible with life gametes are produced; adjacent II segregation, normally not compatible with life; and 3:1 segregation, where, when fertilization is carried out by a normal gamete, there is an expedient of chromosomes, which leads to inviable gametes. In a Robertsonian translocation six types of gametes are formed, one normal, one carrier and four that may lead to viable or non-viable zygotes, depending on if it leads to monosomies or trisomies, and on which acrocentric chromosomes are involved, when relating to trisomy[2].

The bulk of the reciprocal translocations are unique, making it difficult to assess the reproductive risk of unbalanced progeny in couples where one partner is a carrier of a translocation[4].

Translocations can also cause the reposition of proto-oncogenes resulting in dysregulation of the cell cycle which may result in tumours or leukaemia[2]. Translocations first gained significant

clinical relevance when it was found that a recurring chromosomal translocation was causative of leukaemia, best known as Philadelphia chromosome[3]. However, tumour genetics was not part of this work.

1.1.2 Breakpoints Characterization

There is evidence that the breakpoints involved in balanced rearrangements, from which translocations are part of, have a non-random distribution. The chromosomes in which breaks are more frequent are chromosome 9, 2 and 3, followed by 1, 4, 11, 10, being chromosomes X, 17, 19 to 22 and 13 the least involved in these breaks. This ascertains that there are mechanisms preferable for producing said chromosomic breaks at specific regions such as low copy repeats (LCRs) and fragile sites[5].

In reciprocal translocations there is no loss of chromatin in the exchange that occurs, however, depending on the breakpoints' location, the derivative chromosomes may have different morphologies[2]. Also, it is important to notice that in up to 6% of the balanced translocations the phenotype observed in abnormal[6], which can be caused by gene disruption, displace of regulatory regions from the genes they regulate, deletions, and position effects; however, the abnormal phenotype can also be due to a completely different reason like independent genetic alterations in one or more other genes[4,6,7]. Besides, translocations can be unique, disclosed to one single family, or more frequent in a population. In the latter case it is important to characterize seemingly independent translocation events in several families on the molecular level. Furthermore, translocations with identical breakpoints can be formed repeatedly (like the t(11;22)(q23.3;q11.21) being associated with Emanuel syndrome), or may have been formed once and spread as more or less harmless variants in a population (like the der(Y)t(Y;15)(q12;p11.2)) – founder effect[8–10]. This shows the importance of precise breakpoint mapping of a balanced chromosomal rearrangement, allowing the better understanding of the pathogenic causes of cases with clinical alterations, while also providing insights into the mechanisms underlying the formation of translocations[7].

Since breakpoints are often not well characterized in repeatedly appearing translocations, the attempt to characterize them in a few translocation cases is this work's major study objective.

1.1.3 The Development of cytogenetics: from karyotyping to FISH

It was 1956 when the correct number of chromosomes was characterized, marking the beginning of the modern era of human cytogenetics. This discovery was possible due to improvements on the methodology of the chromosomes analysis done by Tjio and Levan, such as colchicine-induced arrest at the metaphase stage of mitosis; the use of a hypotonic treatment to separate chromosomes; and fixation of chromosomes using a mixture of methanol and acetic acid before staining; these improvements allowed the accumulation and improved separation of chromosomes. Since that year multiple improvements and refinements to the chromosomic analysis techniques have been made, resulting in progress in medical genetics, namely better diagnosis not only at the phenotype level, but also at the DNA level[11,12].

Cytogenetics is an area of investigation important for the understanding of chromosomes characterization, allowing the identification of changes in the normal pattern of bands and its size variations, or changes in the chromosome itself[13]. Chromosomes identification by its own banding pattern was improved in 1971 with the introduction of staining techniques, allowing the recognition of new syndromes involving translocations, deletions, and duplications[12]. Quinacrine fluorescent staining (Q-banding), Giemsa-trypsin banding (GTG-banding) and centromeric banding (C-banding), are some of the banding techniques used for karyotyping and detection of constitutive chromosomal anomalies in clinical laboratories[14].

GTG-banding is the preferable and most used karyotyping technique for identification of various chromosomal alterations, such as translocations, inversions, and deletions/duplications[14]. However, classical genetics only enables the analysis of chromosome morphology in combination with observation of the black and white banding pattern and changes occurring within its normal pattern, any size variations occurring in the chromosome or in bands within it, and changes in the centromere index. Also, although the highly informative *in situ* view of the human genome, it has a low resolution[15]. These limitations can be overcome with the FISH method, which combines molecular and cytogenetic approaches providing an intermediate degree of resolution between DNA analysis and chromosomal investigations, while also giving information regarding the single-cell level[16].

1.2 Fluorescence *in situ* hybridization (FISH)

FISH is a molecular cytogenetic technique introduced around the 1980's [13] with advantages such as speed of analysis, sensitivity, stability, and higher precision and resolution, as well as being less laborious and time consuming in the analysis of chromosome aberrations, relatively to conventional cytogenetics techniques[14]. All developments due to this technique were only possible because of the advances in recombinant DNA technology[11], since it was feasible to create fluorescence labelled DNA probes which hybridize with specific chromosomic regions, allowing the better study of the structure and segregation of chromosomes by extending the contributions of conventional technologies. Since this technique allows the understanding of chromosomes' structure and behaviour during meiosis and mitosis, it enabled the analysis of some syndromes that were not possible to analyse by light microscope, revolutionizing the applications of cytogenetics in clinical and research[12]. For example, in mentally retarded patients or prenatal cases with specific sonography signs, the traditional cytogenetics methods show normal results, however, the clinical signs are evidence of some syndromes, such as microdeletion or microduplication syndromes. FISH is an essential method that can confirm or discard such suspicions, since the selection of specific FISH probes to analyse those syndromes is possible[15].

Due to improvements in the sensitivity, specificity, and resolution of the FISH technique, in combination with technologic advances in digital imaging, fluorescence microscopy and availability of the bioinformatic resources, the diversification of this technique in different protocols was prometed[16].

1.2.1 MULTICOLOUR-FISH (MFISH)

Multicolour-FISH (mFISH) is a technique that involves the use of at least three different ligands or fluorochromes for the specific DNA labelling, with the exclusion of counterstain, allowing the simultaneous visualization of multiple targets[13,15]. Due to the possibility of using different probes and combining labelling with different chromosomes, this technique makes it possible to detect all the 24 human chromosomes given that each specific combination results in a unique colour presentation for each chromosome[12], something that revolutionized the field of molecular cytogenetics[14]. In addition to these 24 chromosome probes, we can also have other probes added to the sets used, like single copy probes and chromosome-region-specific probes[13].

1.2.2 Classification of FISH Probes

A FISH probe set can be defined as a set that makes it possible to analyse and characterize several chromosomal subregions smaller than a chromosome arm, with the exclusion of the short arms of acrocentric chromosomes. This definition covers all the FISH methods, since all of them produce a specific chromosomic banding even if with different methodologies[13,15].

The FISH probes can be categorized as repetitive probes, painting probes and locus specific probes (LSPs)[12].

Repetitive probes can produce signals in both metaphase chromosomes and interphase nuclei, being the most common the α-satellite sequences, located within the centromeric region of all chromosomes. These probes can sometimes identify the same centromeric region, which is the case of the chromosomes 13 and 21, since the α-satellite composition in these chromosomes' centromeres is almost the same[12], a situation also verified in the heterochromatic region of all the acrocentric short arms[17]. Probes for telomeric sequences are also part of this group[17].

Painting probes consist of specific DNA sequences that can identify part of a chromosome or its entirety. These probes are useful in analysis of chromosomes in metaphases where they suffer complex rearrangements, helping analysing cancers and structurally abnormal chromosomes[12]. Whole chromosome paints (WCP) and partial chromosome paints (PCP) are included in these[15,17]. WCP are the most frequent probes used in diagnostics sets[15], given that WCP sets allow the identification of specific chromosomes and the better understanding of certain rearrangements[13], having increased the resolution of translocation's detention in samples[14].

LSPs can identify a single gene locus in different sizes ranging from 1,000 to 1,000,000 base pairs. These are used in the localization of genes or to identify deletions and duplications at the submicroscopic level[12], being able to be used both in metaphases and interphases[17]. These probes can be recreated by every laboratory albeit many are commercially available. They take advantage of bacterial artificial chromosome (BAC) probes, because of their easy tracking in genome browser and commercial offer[15].

BAC probes have defined sequences and size, and lead to very bright, intense, and easy to assess FISH results. In translocations, BACs enable the localization and characterization of breakpoints once having known sequences. If a breakpoint region is located within a BAC probe, we will see a split signal. Otherwise, they can simply flank a breakpoint[18].

1.2.2.1 Applications of mFISH Probe Sets

Various fields in pre- and post-natal studies like clinical genetics, tumour cytogenetics, neuroscience, reproductive medicine, evolutionary and chromosome biology, and comparative genomics are addressed using these probe sets[15,16]. The advances made in FISH technology derived from the development of different probes widened the use of this technique. For example, the development of LSPs improved the detection of tumours alterations, such as genes amplifications or losses; plus, these probes and chromosomes specific centromeric probes are also being used in routine of cytogenetic laboratories, helping detect congenital abnormalities such as trisomies[14]. The analysis and diagnostic of microdeletion syndromes, cryptic translocations, marker chromosomes and mosaicism were possible, due to the potential of combining WCP, PCP, BACs and centromeric probes in a single set[15,16].

1.2.2.2 Direct and Indirect Labelling

In 1988, polymerase chain reaction (PCR) was introduced and helped with mutation testing and gene mapping. This technique allows the amplification of DNA samples recurring to random DNA primers, having applications in the production and labelling of probes used in FISH, namely chromosome-specific ones[11].

The development of the field allowed the generation of a variety of direct and indirect DNA probe labelling methods with a wide spectrum of hybridization signal detection[14]. Direct probe labelling consists in the incorporation of fluorescent nucleotides, whereas indirect probe labelling is done with the incorporation of reporter molecules, like haptens, that can be detected by the incorporation of fluorescent-tagged antibodies or other affinity molecules[16,19]. Directly labelled probes lead to quicker results, since there is no detection step necessary[17].

1.2.3 FISH Procedure

This technique is possible to be done on metaphase chromosomes, fresh tissue sections, paraffinized tissue sections and interphase nuclei. Since the DNA analysed can vary from large to small sections, probes are synthesized according to that[14]. Normally, structural rearrangements like translocations are studied on metaphase chromosomes[15].

The most used human tissue in FISH protocols is peripheral blood due to its ease of obtaining and only requiring a short-term culture to get high quality metaphase spreads. Amniotic fluid, chorion tissue and skin fibroblasts are also very frequently used tissues, being the former two important in prenatal diagnostics[20].

In this technique DNA probes are hybridized to their complementary sequence, which have been previously fixed on slides[16]. The DNA probes used can be synthetized or grown in a vector, followed by a process of purification. The used probes and target sequences need to be denatured by resorting to heat and/or chemicals like formamide to break the double-strand connection. Following this, the hybridization with the homologous DNA occurs and some washes are realized to remove the excess of probe. The finished result can be observed with a fluorescent microscope and whether the target is present or not is asserted by the signal given[12].

1.2.3.1 Overcoming Technical Issues

The inability to discriminate between fluorochromes and misinterpretation of the results due to crossed hybridization of probes are some technical issues to be taken in consideration[12].

Efficiency and specificity can be influenced by various aspects during the hybridization process. It is essential that an adequate protease digestion with minimal tissue damage is carried out, and that the probes used are supplemented with COT1 DNA for crossed hybridization with DNA repetitive sequences to be supressed[21].

To reduce the number of false-positive and false-negative results the use of control probes is essential, as well as evaluating the hybridization adequacy of a probe to the DNA in analysis[22]. It is fundamental that the target in question is well understood before choosing any kind of probe, being it commercial or "in-house" probes, since it is the most critical component of ensuring specificity of hybridization. When producing "in-house" probes, which can be done by nick translation, random priming, or PCR, a rigorous quality control is fundamental[21].

In this study, WCP and locus specific BAC based probes were the preferable ones used in FISH analysis. This helped to characterize the specific breakpoints of the analysed translocations. The FISH protocol is explained in detail in ***Chapter 3: Materials and Methods***.

In this chapter a better understanding of each of the four translocations studied is done, denoting studies already performed, if that was the case. Also, the reason for the study of each individual translocation is explained and the answers of such will be found in the following chapters: ***Chapter 4: Results*** and ***Chapter 5: Discussion, Future Work and Conclusions***.

2.1 t(11;22)(q23.3;q11.21) translocation

The t(11;22)(q23;q11) translocation (Figure 1) is the most common recurrent non-Robertsonian translocation in humans[8,23], having been already reported in more than 160 unrelated families[24]. Just like what is normal in balanced carriers of constitutional translocations, patients are phenotypically normal, arising problems, which leads to their diagnosis, when infertility, spontaneous abortions, or unbalanced progeny appear[8,23].

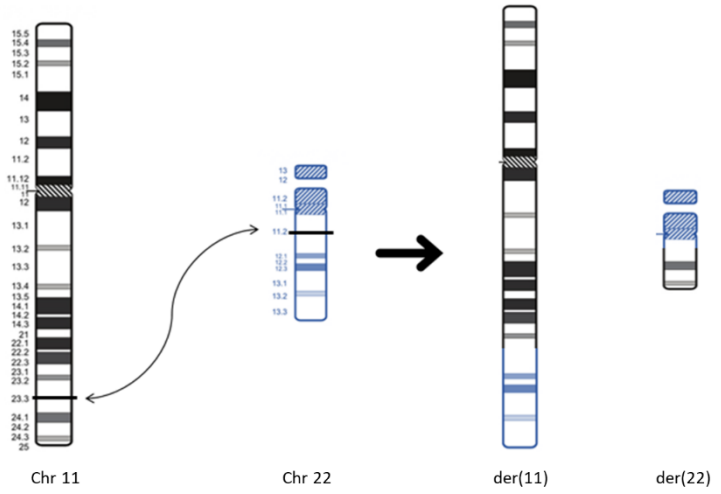

Figure 1. *Representative scheme of the t(11;22)(q23.3;q11.21) translocation.*

While offspring with karyotypes 46,XN,der(11)t(11;22)(q23.3;q11.21) or 46,XN,der(22)t(11;22)(q23.3;q11.21) are not viable, viable offspring of such a translocation t(11;22)(q23.3;q11.21) carrier can be characterized by a 47th chromosome to an extra der(22)t(11;22)(q23.3;q11.21), the result of a 3:1 segregation during meiosis (Figure 2). This is

best known as Emanuel syndrome, a disorder where the carriers have mental retardation, preauricular tag or sinus, ear anomalies and heart defects, among other symptoms. Normally, the appearance of an offspring with this syndrome is the reason of study of the parents, which leads in most cases to the identification of t(11;22) carriers[8].

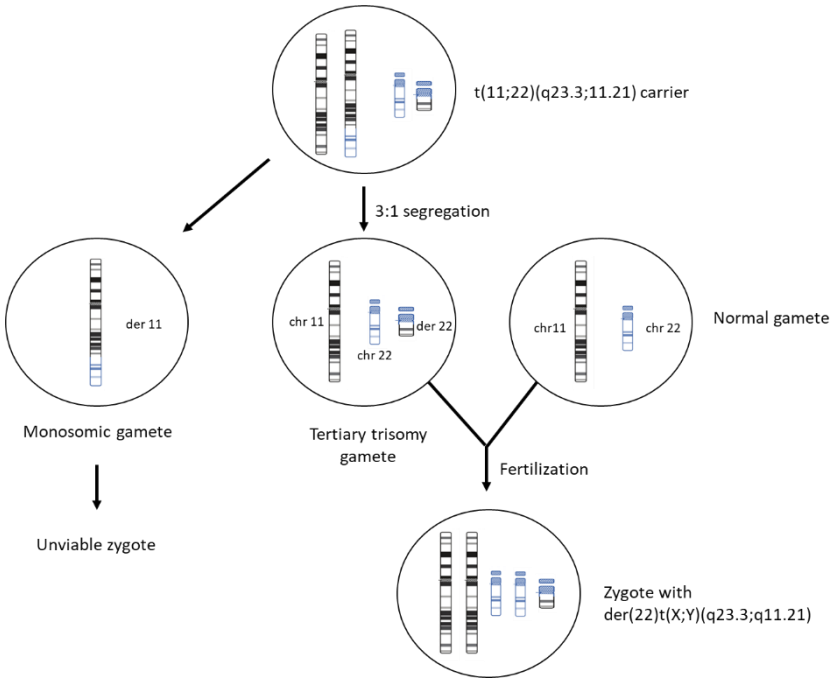

Figure 2. *Representation of the 3:1 segregation of a t(11;22)(q23.3;q11.21) carrier during meiosis and respective reproductive outcome.*

Due to the recurrent nature of this translocation, its breakpoint sites have been the subject of several studies. On both regions of the breakpoint, palindromic AT-rich repeats (PATRRs) were able to be found, being the formation of this translocation proposed to be based on such structures[8,23]. The 11q23 PATRR has approximately 450 base pairs, whereas the 22q11 PATRR has approximately 590, having both a high AT content[8]. The breakpoints of this translocation have been mapped and identified in Alu repeats on both chromosomes, being referred that an Alu-Alu recombination is in its basis[24].

The 22q11 region is known to have a high propensity to rearrangements, being involved in other aberrations causative of disorders, such as chronic myeloid leukaemia, caused by t(9;22)(q34;q11) translocations; the DiGeorge syndrome and velo-cardio-facial syndromes, caused by deletions in this region; and the cat-eye syndrome, caused by duplications in it[23]. This

region is designated as a hotspot, since numerous translocation breakpoints have been shown to occur in it, which is also the case of the t(17;22)(q11;q11) translocation whose breakpoint is located on the same interval as the one of the t(11;22) translocation[8].

In the literature various probe sets have been already described to characterize the breakpoint of this translocation[24,25]. The reason for such study lies in the fact that the host lab had no access to the published probes; an own probe set should be developed and probed in the available cases to characterize the known translocation.

2.2 der(X)t(X;Y)(p22.32;p11.31) translocation

This translocation is characterized by the unbalanced exchange of the distal segments of the short arms of both chromosomes (Figure 3). The carriers of the der(X)t(X;Y)(p22.32;p11.31) translocation are infertile 46,XX males, since the sex-determining region Y (SRY) is located on the distal region of the Yp chromosome[26]. It has been stated that the translocation involving these chromosomes may be due to an extension of the crossing over that occurs in male meiosis, resulting in an unequal Y/X interchange which results in the translocation of the *SRY* gene to the X chromosome[27].

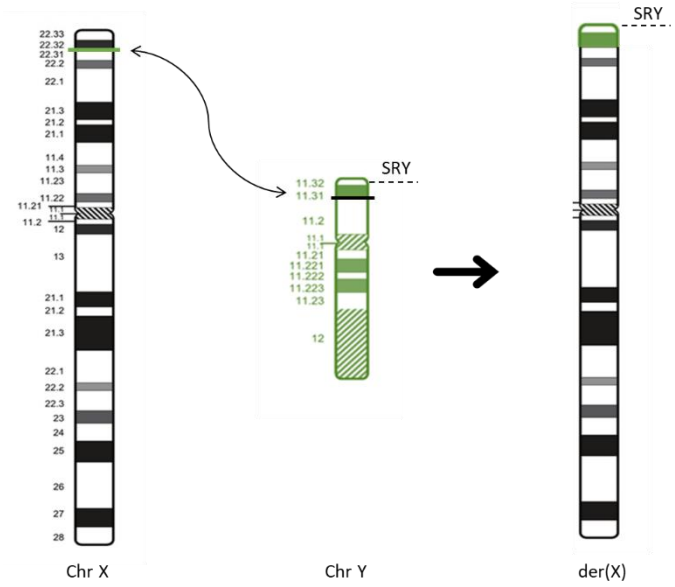

Figure 3. *Representative scheme of the der(X)t(X;Y)(p22.32;p11.31) translocation.*

This 46,XX testicular disorder of sex development, as it is called, is a rare disorder first described in 1964, occurring with a frequency of approximately 1 in 20,000[27]. Most of the cases reported are due to the translocation between chromosomes X and Y, having the others no such translocation reported or have a cryptic sex chromosome mosaicism involving Y cells in at least the Sertoli cells[26].

These patients suffer from azoospermia and may develop certain clinical features similar to the ones in Klinefelter syndrome, such as atrophic testes, abnormal penis size, abnormal hair distribution, and gynecomastia, since there is a disomy of the X chromosome and loss of the majority of the Y chromosome (including AZF region). Carriers are also smaller in size than normal XY males, which may be due to the absence of specific growth-related genes, located within the long arm of the Y-chromosome[26,27].

In Capron *et al.* (2022) different cases of 46,XX,SRY positive patients (with a translocation involving chromosomes X and Y) and all the different breakpoints occurring in a set of translocations appearing are described and characterized[27].

In this dissertation two samples regarding this translocation were analysed to see if the breakpoints observed where within the ones already presented in the literature. Also, the samples belonged to patients from different countries, which, since this is a translocation shown to have multiple breakpoints characterized, could mean the present of different breakpoints in both patients, something also in analysis to better characterize the recurrency of this translocation.

2.3 t(5;16)(q13.3;p13.3) translocation

The t(5;16)(q13.3;p13.3) translocation involving the short arm of chromosome 16, and long arm of chromosome 5, found in two of our patients (Figure 4), has not been described in the literature yet.

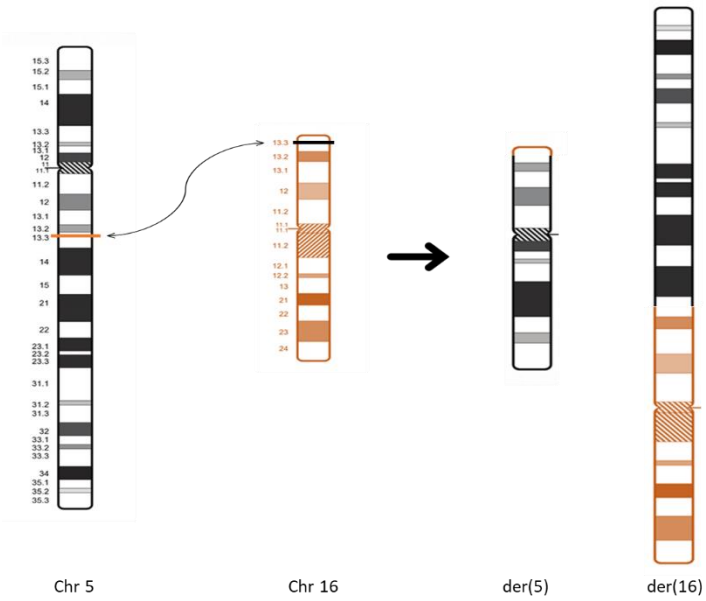

***Figure 4**. Representative scheme of the t(5;16)(q13.3;p13.3) translocation.*

In Mrasek *et al.* (2010) it was described that the 5q13 region is associated with fragile site FRA5K[28]. Regarding these chromosomes, it is also known that the 16p13.3 region is rich in LCRs and is a hotspot for recurrent microdeletions/duplications[29].

These cases were studied to check if they have the same breakpoint or different ones, to identify if we were dealing with two private mutations or a familiar one.

2.4 t(Y;12)(q12;q12) translocation

The long arm of the Y-chromosome is frequently affected by changes in its size and structure, called chromosomal heteromorphisms. A der(Y)t(Y;acro)(q12;p1?2) has been repeatedly seen in human cytogenetic findings, being the majority of the cases associated to the chromosome 15[9]. However, the reciprocal translocation of the Y-chromosome with an autosome is a rare event highly linked to male infertility[30], and translocations with chromosome 12 have not been reported in literature. This said, to better characterize this translocation (Figure 5), a study was conducted on the samples available from the lab collection.

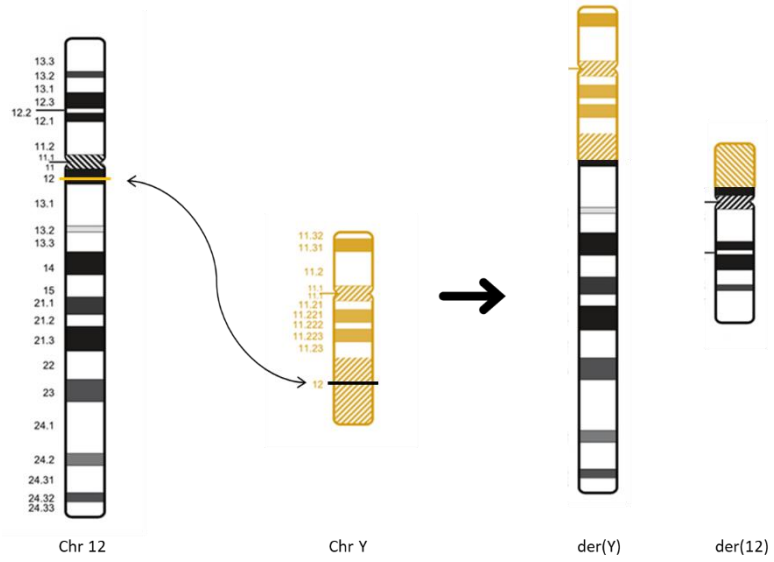

Figure 5. *Representative scheme of the t(Y;12)(q12;q12) translocation.*

Similar to the t(5;16) translocation, these cases were studied to allow the identification of private mutations or a familiar one.

As previously mentioned, various tissues can be used in the cytogenetic study of a sample, being the peripheral blood lymphocytes the most used thanks to its ease of collection and short-term culture (see ***Chapter 1: Introduction***). This said, all the FISH procedures done in this dissertation have resorted to peripheral blood suspensions collected throughout the years and stored in the laboratory. Also, there was a karyotyping protocol done from beginning, where it was possible to follow and carry out the process of the samples' preparation, which is based on the one described in Weise *et al*. (2017)[20]. The FISH protocol followed was based on the one described in Liehr *et al*. (2017)[19]. The cell pellets were provided for this work by the cytogenetic lab of the institute. FISH-probes were labelled in parts in frame of the present work.

3.1 Patients

A list of patients from the laboratory' case collection known to have the translocations desired to the study is indicated on Table 1. In this study, we were able to do a FISH analysis in all the patients where enough material for the study was available, and that would allow us to draw some feasible conclusions. In some patients known to have the translocations a better analysis was not possible to be done once the samples were too old or material was no longer available.

Table 1. *Translocation cases from the laboratory's collection and respective availability.*

Translocation in study	Case	Gender and Age	Material
45,XX,rob(13;14)(q10;q10)	A1*	FA	E
t(11;22)(q13.3;p13.3)	B1	FA	E
	B2		RO
	B3		E
	B4		RO
	B5		E
	B6		E
	B7		RO
	B8	MA	E

Translocation in study	Case	Gender and Age	Material
t(11;22)(q13.3;p13.3)	B9*	MP	L
	B10	FA	E
	B11	n.a.	E
	B12		E
der(X)t(X;Y)(p22.32;p11.31)	C1	MA	E
	C2		RO
	C3		L
t(5;16)(q13.3;p13.3)	D1	FA	E
	D2	MA	E
t(Y;12)(q12;q12)	E1	MA	E
	E2		RO

F: Female; M: Male; A: Adult; P: Prenatal; n.a.: not available; E: Enough; L: Little; RO: Run Out.

*Cases where the study performed was not due to infertility.

First and foremost, every patient arriving to the laboratory must have documentation related to itself. For each patient a formular is created, in which all the information needed about the patient and relatively to the analysis necessary is annotated, and a respective identification number is given. From here on, every sample from respective patient is identified by that number.

3.2 Preparation of the blood samples

After a blood sample was collected, 1 ml of it was well mixed with 9 ml of *LymphoGrow* (Cytogen GmbH) medium, a special medium for lymphocyte cells. Then, the mix was incubated for 72 h at 37 °C/ 5% CO_2. These steps were to be performed under sterile conditions. Having passed the 72 h, 100 µl of Colcemid (0.1 µg/ml; Biochrom GmbH) and 100 µl of ethidium bromide (Sigma Aldrich) working solution were added per culture bottle and incubated for 90 min. Afterwards, the cell culture was carefully shaken, for all the cells to come of the bottom of the culture flasks. The cell suspension obtained was transferred to Greiner tubes and centrifuged for 5 min at 1500 revolutions *per* minute (rpm) with a 12 cm radius rotor. The supernatant was aspirated with a vacuum pump until approximately 1 cm of the bottom (it was essential that the pellet remained

untouched). 10 ml of a hypotonic solution (0.075 M KCl (Merck)) preheated in advanced at 37 °C was added to the residue and resuspended well with vigorous shaking. Next, there was an incubation of 20 min at 37 °C and a fixation done by adding 0.5 ml of fixative (2-8 °C). The fixative was well shaken and centrifuged for 5 min at 1500 rpm with a 12 cm radius rotor. This fixative consists of a mix of methanol (EMSURE®) and glacial acetic acid (ROTH) in a 3:1 portion.

Next was a washing step that started with the removal of the supernatant with the vacuum pump and followed by the addition of 10 ml of fixative. This mix was shaken and centrifuged for 5 min at 1500 rpm on a 12 cm radius rotor. This washing procedure was repeated 1 to 3 times until the supernatant was clear, and the sediment obtained was white. The suspension was left overnight at -20 °C before we could prepare our slides.

3.2.1 Slides preparation and metaphase spreads

The slides preparation depended on what they were needed for: if it was for C-banding and karyotyping, then we would drop the cells on a slide and leave it at 60 °C overnight before its use on the following day; if it was for FISH analysis, then we would take the suspension from the freezer and drop our cells on a slide and after add a mix of fixative freshly prepared that would have been stored at -20 °C before its use. All the used slides would first need to be cleaned. This procedure consisted of first cleaning our slides with microscopic lens paper and after passing them 3 times in distilled water, leaving them in it on the freezer for 10 to 15 min or for 30 min on the fridge.

After cleaning, our slides were then ready to receive cells spreads. About 40-80 µl of sample (depending on the amount of pellet) would be dropped on the clean and humid slides from a considerable height. The same amount of fixative would be added, and our slides would be placed on a wet paper on top of a hot plate to evaporate the water until they were fully dried. We then would need to ascertain that we had a minimum of ten metaphases in our slides before moving on to the next stage of the FISH procedure. The slides would then stay at room temperature (RT) for about 24 h to naturally age[a] or placed on the oven for 2 h at 60 °C to fasten this process, enabling the start of the FISH protocol on the same day.

[a] The fresh slides are too fragile for immediate use and the DNA can be damaged in the following steps.

3.3 Karyotyping

A GTG-banding procedure was able to be performed. It consisted of passing the prepared slides for 7 seconds in trypsin (Sigma-Aldrich), followed by 3 min in Giemsa solution (Merck). The slides would then be ready for analysis under light microscope after drying.

3.4 FISH Protocol

3.4.1 Probe labelling

This part of the process was performed under a sterile hood. We started by making an initial mix with the reagents present in Table 2. The amount of each component was dependent on how many probes we were labelling. When preparing this mix, we would always make an extra amount in case anything went wrong. Everything would be well mixed before we took the amount necessary for each probe we were preparing. 2 µl of the respective DNA would then be added as well as the nucleotide. The quantity of nucleotide added was dependent on which one we were using:

- If labelling a Digoxigenin (Dig) or Biotin (Bio) probe, we would add 0.8 µl in the tube.
- If labelling a Spectrum-Orange-dUTP (SO) probe, we would add 2 µl in the tube.
- If labelling a Diethylaminocoumarin-5-dUTP (DEAC) probe, we would add 0.7 µl in the tube.
- If labelling a Texas Red (TR) probe, we would add 0.3 µl in the tube.

Table 2. *Reagents used in the initial mix needed for the labelling process.*

Reagent, stock concentration	For 1 sample*	2	3	4	5
H_2O	11.08	22.16	33.24	44.32	55.4
Label-PCR buffer, 10x NP40	2	4	6	8	10
DOP primer, 40 µM	1	2	3	4	5
Label-mix	2	4	6	8	10
$MgCl_2$, 25 mM	3	6	9	12	15
AmpliTaq®	0.12	0.24	0.36	0.48	06
Sum	**19.2**	**38.4**	**57.6**	**76.8**	**96**

* These amounts are for single sequence probes and repeated sequences probes, there is a need of duplication of these values for WCP probes.

After every component was added, our tubes would be introduced in the *Thermocycler* (MJ Research Inc) and the program "LABEL30" would be run. This would take about 3 h and after it our DNA would be amplified.

After taking our tubes from the *Thermocycler* we would follow the process with the precipitation step. In this we would add 10 µl of tRNA (Roche), 5 µl of 3M Natriumacetat pH 5.2 (Merck) and 100 µl of 100% ethanol (ROTH) to our tubes and let them stay at -20 °C for the night. The next day we would start by centrifuge our tubes for 20 min at 4 °C and 15,300 rpm. After finishing we would remove the supernatant, putting our tubes in the *Speedvac Concentrator* (Thermo Electron Corporation) to guarantee that there was not any liquid in our precipitate. We would finish this process by adding 80 µl of dextran sulphate (DS) (Sigma Aldrich) to our BAC solution (40 µl if we were dealing with WCPs), vortex them and let them in the *Eppendorf Thermomixer* (Eppendorf) at 37 °C until we would need them. After a minimum of 1 h our probes would be ready to be used.

Before we were able to analyse our chromosomes on the fluorescence microscope, we would need to prepare our slides the day before. The FISH protocol can be divided into 4 steps: Slides pretreatment, probes denaturation and pre-hybridization, slides denaturation and post-hybridization washes.

3.4.2 SLIDES PRE-TREATMENT

The first step was the washing of the slides in a continuously more concentrated alcohol series (70%, 95% and 100%) for 2 min each. When fully dried, we put our slides on a solution of acid pepsin preheated at 37 °C and incubated our covered slides for 3 min in *ThermoBrite* (Abbott Molecular Inc) at 37 °C covered by a coverslip (24 x 60). Next, we washed them in phosphate saline buffer (PBS) 1x for 5 min at RT. The post-fix stage, the next one, was done by incubating our slides for 10 min at RT with 100 µl of post-fix solution. The coverslip was then removed, and the slide was washed in PBS 1x for 5 min at RT. We finished this step with the repetition of the first step, the ethanol one, and let our slides out to dry completely.

3.4.3 PROBES DENATURATION AND PRE-HYBRIDIZATION

In this stage we had to prepare our probes to put them in the *Thermocycler* in the specific program, which varied depending on if we were preparing BACs and WCPs, CEPs or commercial probes. Before going to the *Thermocycler,* we needed to prepare our tubes with the probes, DS (if necessary) and the COT1 DNA. The wanted final volume would be 12 µl *per* slide. The amount of COT1 DNA and DS depended on the number of BACs used (Table 3). After the tubes' preparation and mix, then we could put them on the *Thermocycler* in the pre-hybridization program. The BACs' program consists of 5 min at 75 °C, 2 min at 4 °C and 30 min at 37 °C. The CEPs and commercial probes' program consist only of 10 min at 75 °C and do not require addition of DS and COT1 DNA, only addition of specific buffers that depend on which probe from which label were being used. When the program was done the BACs were ready to use.

Table 3. *Quantities of DS and COT1 DNA needed for the preparation of the tubes for denaturation and pre-hybridization.*

Number of BACs used (quantity)	DS (µl)	COT1 DNA (µl)
1 (3 µl)	9	5
2 (6 µl)	6	
3 (9 µl)	3	
+3 (12 µl or more)	-	10

3.4.4 SLIDES DENATURATION

We started by putting 100 µl of formamide 70% in our slides and let it stay covered while it denatures for 3 min on the electric plaque heated beforehand at 73 °C. Following, we would intend to give a heat shock to our slides, so we put them in 70% ethanol at -20 °C also for 3 min. The slides preparation was finished with the washing of them for 2 min in 95% ethanol and then 100% ethanol for the same amount of time. Once our slides were completely dried, we were able to add to them 12 µl of the probes mix prepared on the second stage of our protocol.

Then our slides would be covered with a coverslip, sealed with Fixogum™ (Marabu) and introduced on a wet chamber at 37 °C where they would stay overnight.

3.4.5 POST-HYBRIDIZATION WASHES

The following morning, everything would be carefully removed once our slides leave the wet chamber and the post-hybridization washes would start. The first step was the slides' introduction in 0.4 x SSC/Tw20 solution for 2 min. This solution would be pre-heated so it would be at 64 – 65 °C. Next, they would be introduced in 4 x SSC/Tw20 solution for 1 min at RT in a shaker. 100 µl of marvel were then added to the slides, which was covered afterwards and would go to the incubator (Heraeus Instruments) at 37 °C for 15 min, in a wet chamber. Once we took our slides out, we would wash them in 4 x SSC/Tw20 for 1-2 min at RT in a shaker and then add a solution of 100 µl of antibody previously prepared and our slides would again go to the incubator, in a wet chamber, at 37 °C for approximately 35 minutes.

The antibody preparation was dependent on the type of probe we were using in our analysis. The DEAC, TR or SO probes, were direct probes, so there was no need of adding any kind of antibody; in case of using the Dig or Bio probes, this step was crucial. There was necessary an extra carefulness when adding the antibody because we needed to analyse which colours were needed. In here was essential to not have different probes in the same coloration. The preparation of each solution for the slides consists of adding 100 µl of the marvel previous prepared and a quantity of antibody depending on the colour wanted (Table 4).

***Table 4**. Antibodies used in the coloration of the Dig and Bio probes.*

		Signal's colour wanted	Antibodies used
Probes	Dig	Green	1 µl FL
		Red	10 µl Rhod
	Bio	Green	1 µl FICT
		Yellow	5 µl Cy5

The last step consisted of taking the excess of antibody from our slides. First, we would put them in 4 x SSC/Tw20 at RT in a shaker for about 2-5 min, then we would wash them in a series of ethanol for 2 min each (like the first step of the FISH protocol). The slides would then be air dried in a dark chamber and a DAPI (4,6-diamidino-2-phenylindole.2HCl) dissolved solution would be added to them. The slides would be covered with a coverslip and would be ready to observe in the microscope.

3.4.6 MICROSCOPIC ANALYSIS

This was the last step of the process. In here we were able to see our chromosomes with various magnifications (20x, 40x, 100x). We needed to use an immersion oil (ZEISS Immersol™), to be able to see our metaphases with a 100x magnification and be able to select the best metaphases for analysis. We needed to select at least ten metaphases from each sample where our chromosomes would be well spread. This was the part where we could see if everything went well during the entire process because any mistakes would make it difficult to do a good analysis of our metaphases, like if our chromosomes where blurry due to an excess of DAPI[b], and make sure all our probes were working well. It was essential to specifically choose the program that would allow us to see all the colours used.

To interpret our results, we would need to carefully chose a metaphase and separate the chromosomes involved in the translocation that we wanted to analyse. Then we just needed to make sure that our probes were working well and interpret the results according to the signals obtained.

[b] To solve this, we need to pass the slides for 2 min in 100% ethanol, let them dry and add DAPI again in a smaller amount.

In this chapter we can observe the results of a karyotype study and of the FISH tests realized for the four translocations in study. A detailed analysis is done, for which the respective conclusions reached are possible to be checked on ***Chapter 5: Discussion, Future Work and Conclusions***. All the images used on this chapter are property of the *Institute of Human Genetics* in Jena.

4.1 Karyotype

A karyotype study was possible to be done and analysed on a known carrier of a Robertsonian translocation involving chromosomes 13 and 14 to better understand and gain experience of the basis of cytogenetics and to verify the advantages of the complementary study via FISH methods. The karyotype analysed is shown on Figure 6.

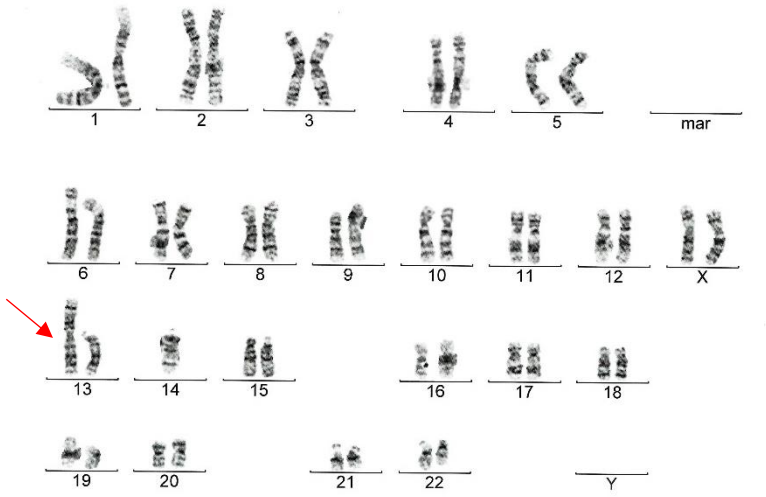

Figure 6. *Karyotype of a carrier of a 45,XX,rob(13;14)(q10;q10) translocation.*

An additional FISH test was conducted for a better clarification of the karyotype presented, resorting to WCPs of both chromosomes, centromeric probes of them, and a mid54 probe which covers for the short arms of the acrocentric arms. The FISH analysis confirmed the dicentricity of the derivative, with the break and fusion having occurred in the 13p11.2 and 14p11.2 regions, the usual in these cases. We can then indicate that the karyotype presented is:

45,XX,der(13;14)(q10;q10).ish der(13;14)(13qter->13p11.2::14p11.2->14qter), something that was not able to be postulated merely on the karyotype analysis.

4.2 t(11;22)(q23.3;q11.21) translocation

The probes used to analyse the cytogenetic location of the breakpoint of the translocation in study are indicated on Table 5 and the results obtained are presented in Figure 7. The set of probes was first tested on a normal patient, and, after noticing that the probes were working well, nine carriers of this translocation were tested. This way to establish the probes sets was also repeated in the other translocations cases.

***Table 5**. Probes used to detect the t(11;22)(q23.3;q11.21) translocation.*

Probes*	Cytogenetic Location	Base Pairs Involved (Mb)
RP11-356E17 (dig)	11q23.3	116,325,596-116,536,280
RP11-35P15 (bio)	11q23.3	117,517,615-117,689,361
RP11-379N11 (SO)	22q11.21	21,433,073-21,616,240
WCP 22 (DEAC)	Chromosome 22	-

* RP11 – BAC clones from the RPCI-11 BAC library.

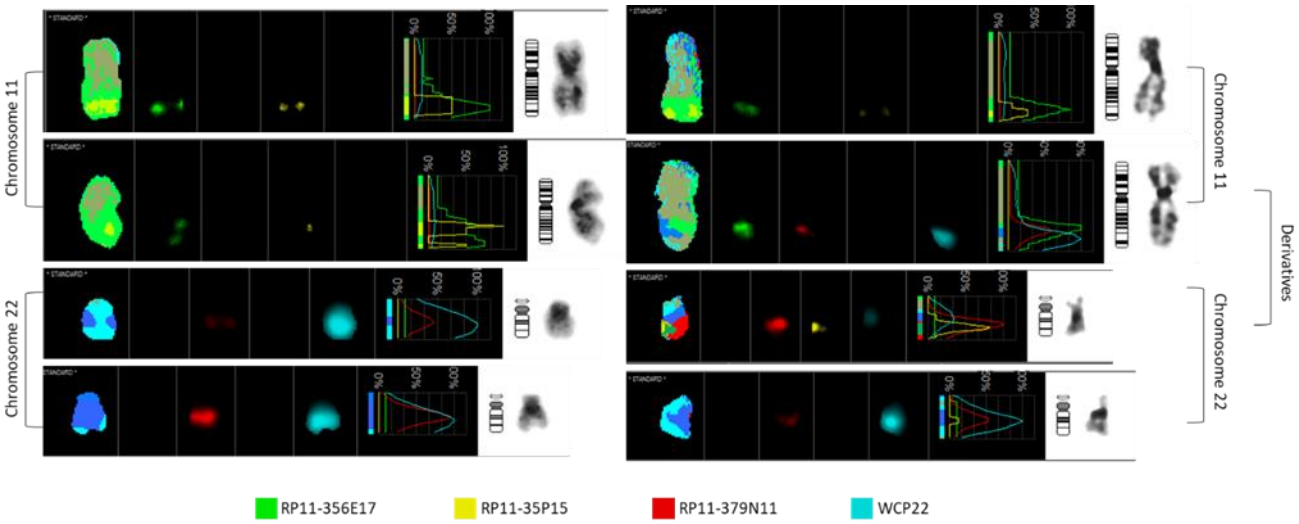

***Figure 7.** Results regarding the probes used in the study of the t(11;22)(q23.3;q11.21) translocation. Comparison between a normal person (left) and a carrier of the translocation (right).*

On the left of figure 7 we have a patient without the translocation in analysis, allowing a comparation point for the translocation carriers' results. On the right we have a carrier where we can easily see the split zone due to the different location of some of the used probes.

The SO signal regarding the 22q11.21 region of chromosome 22 (probe RP11-379N11), was found in both the chromosome 22 and the derivative of chromosome 11, indicating a signal split. This means that the breakpoint on the chromosome 22 is in the 21,433,073 Mb and 21,616,240 Mb region. Regarding chromosome 11, we spot the presence of the green signal and the yellow one on different derivatives, indicating a split zone between the RP11-356E17 and the RP11-35P15 probes. This means that the breakpoint on the chromosome 11 must be between 116,536,280 Mb and 117,517,615 Mb. This result was found in all our patients, except for patient B9, which was noticed to be a carrier of trisomy 22 (Figure 8). This is verified with the observation of four DEAC and SO signals (two normal chromosomes 22 and two derivatives). This patient has a 47,XX,+der(22)t(11;22)(q23.3;q11.21) karyotype.

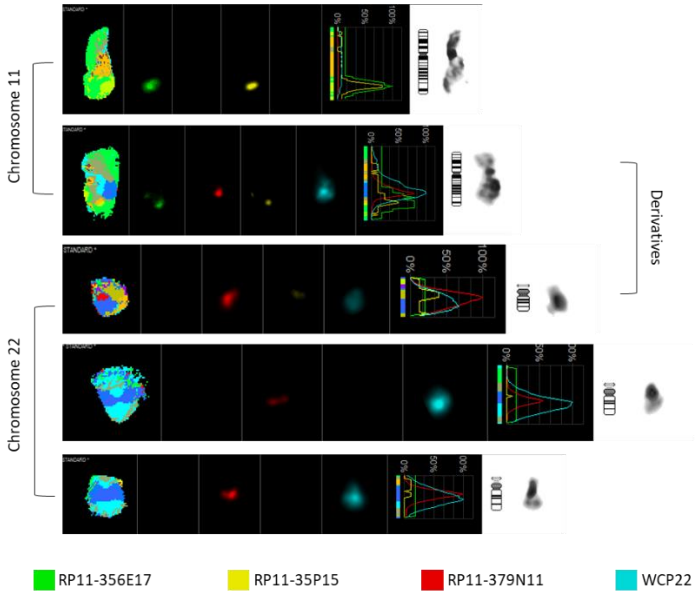

Figure 8. *Results regarding patient B9; the karyotype presented is 47,XX,+der(22)t(11;22)(q23.3;q11.21).*

4.3 der(X)t(X;Y)(p22.32;p11.31)

The probes used to analyse the cytogenetic location of the breakpoint of the translocation in study are indicated on Table 6 and the results obtained are presented in Figure 9. These sets were used in the analysis of samples from two patients, C1 and C3.

Table 6. *Probes used to detect the der(X)t(X;Y)(p22.32;p11.31) translocation.*

Probes	Cytogenetic Location	Base Pairs Involved (Mb)
RP11-1M18 (SO)	Xp22.31	6,564,614-6,724,938
RP11-126O22 (bio)	Xp22.31~22.2	9,358,753-9,500,420
RP11-299M10 (dig)	Xp22.2	11,499,342-11,685,070
RP11-122L9 (dig)	Yp11.2	4,857,081-5,017,603
RP11-35D7 (TR)	Yp11.2	6,051,700-6,206,231
RP11-115H13 (bio)	Yp11.2	6,692,454-6,859,727
WCP X (DEAC)	Chromosome X	-

On patient C1, the SO signal regarding the RP11-1M18 probe only appeared on the X chromosome and not in the derivative, however the yellow signal from the RP11-126O2 probe appeared in both the chromosome X and its derivative, indicating that the split zone is located between these probes (Figure 9, left). This means that the breakpoint on the chromosome X was between 6,724,938 Mb and 9,358,753 Mb. Regarding chromosome Y, the presence on the derivative of the RP11-35D7 probe, represented in TR, but not of the RP11-115H13 probe, represented in yellow (the signal that seems to be present is background), indicates that the split zone is located between these probes (Figure 9, right). This means that the breakpoint on the chromosome Y must be between 6,206,231 Mb and 6,692,454 Mb.

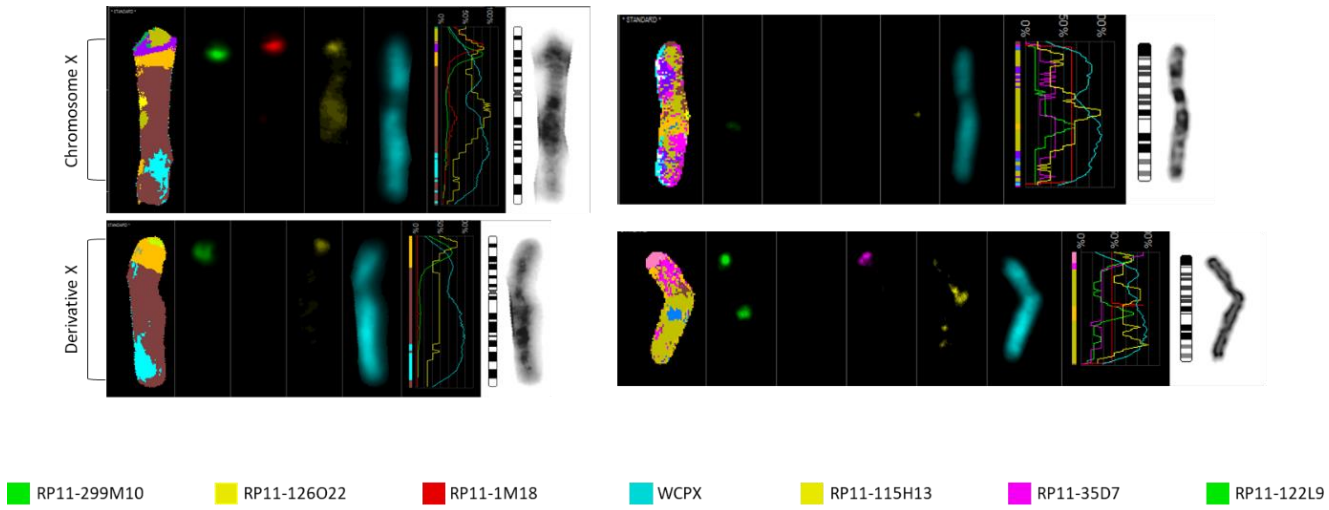

Figure 9*. Results regarding the probes used in the study of the t(X;Y)(p22.32.3;p11.31) translocation from patient C1.*

The same probe set was tested on patient C3, which came from a different country, however, the same split zone was not established. We were not able to further characterize the variant in this patient due to the lack of more material to do so.

4.4 t(5;16)(q13.3;p13.3)

The probes used to analyse the cytogenetic location of the breakpoint of the translocation in study are indicated on Table 7 and the results obtained are presented in Figures 10 and 11.

Table 7. *Probes used to detect the t(5;16)(q13.3;p13.3) translocation.*

Probes*	Cytogenetic Location	Base Pairs Involved (Mb)
CTD-2524J22 (TR)	16p13.3	588,659-789,507
CTC-357L21 (bio)	16p13.3	1,347,261-1,484,290
RP11-619A23 (SO)	16p13.3	3,720,076-3,914,571
RP11-115I6 (SO)	5q13.2	71,623,857-71,787,690
RP11-97L2 (dig)	5q13.3	73,780,538-73,931,815
CTD-2200O3 (DEAC)	5q13.3	75,565,882-75,697,435
CTC-431G16 (TR)	5q14.1	79,057,985-79,183,782

***** BAC clones from BAC/PAC Chori.

The first combination was tested on patient D1 and was in regard of finding the breakpoint on chromosome 16 (Figure 10, top). The TR signal appeared in both derivatives, meaning the split zone is in the region covered by the CTD-2524J22 probe. This means that the breakpoint on the chromosome 16 seems to be between 588,659 Mb and 789,507 Mb. This probe was then tested, together with WCPs of the chromosomes involved, in patient's D2 sample and the same breakpoint was identified, once it is possible to observe the TR signal in chromosome 16 and in both derivatives (Figure 10, bottom).

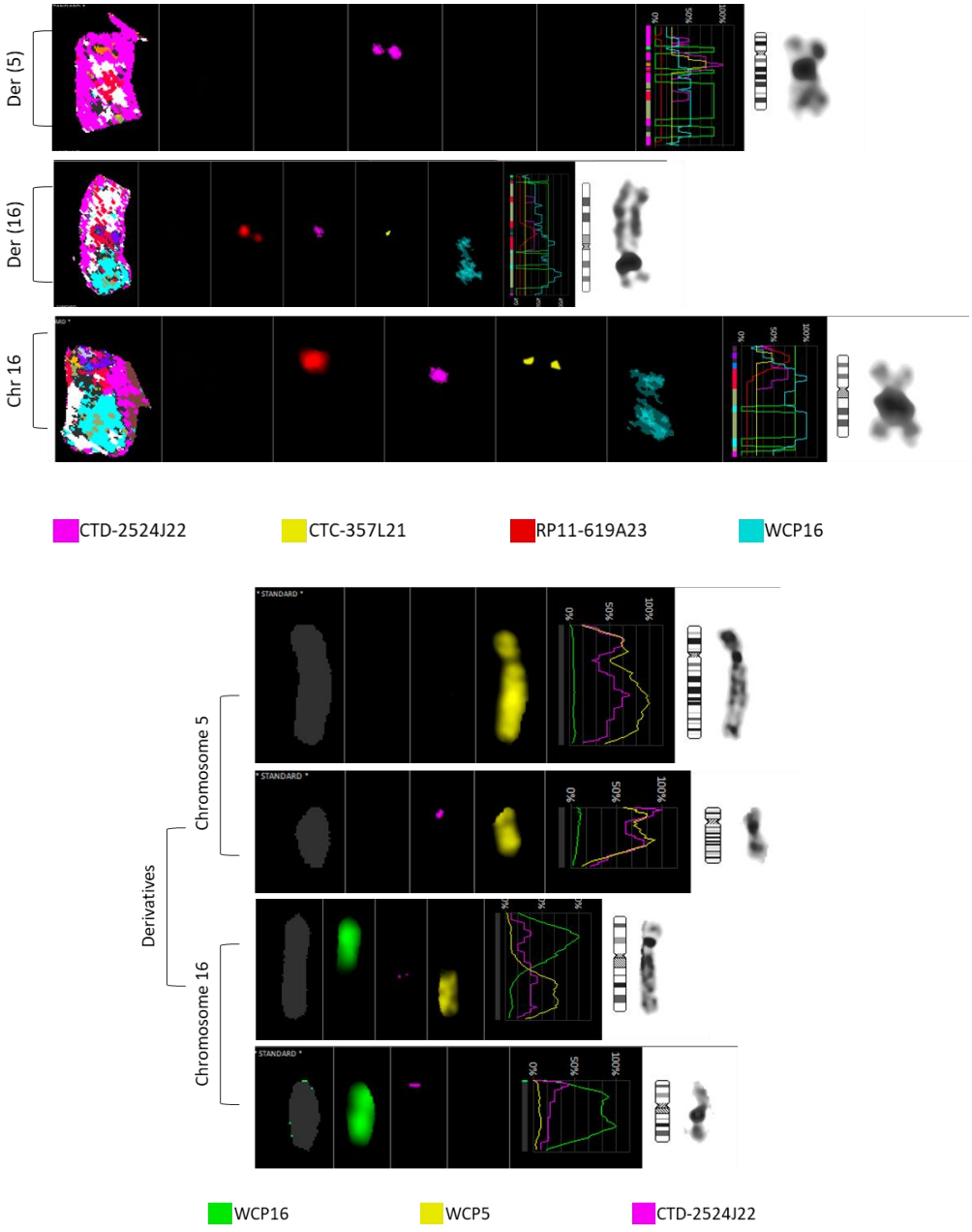

Figure 10. *Analysis of the breakpoint in the chromosome 16 of the t(5;16)(q13.3;p13.3) translocation in two patients recurring to the set probes indicated. First patient tested, D1, (top) narrowed down the probes used, and the probe suspected of having the breakpoint was tested with WCPs on patient D2 (bottom).*

Regarding the breakpoint of chromosome 5, the first set of probes was tested jointly with a WCP 5 probe on patient D1 (Figure 11, top). The presence of the green signal in the derivative of chromosome 5 and the presence of the TR signal in the derivative of chromosome 16, means that the split zone is located between the RP11-97L2 and the CTC-431G16 probes. This means that the breakpoint on the chromosome 5 is between 73,931,815 Mb and 79,057,985 Mb. The

same set probe switching RP11-115I6 for the WCP 16 probe, tested on patient D2, also allows to make the same deduction regarding the breakpoint location (Figure 11, bottom). The blue signal suffered from crossed hybridization, not contributing to the conclusions withdrawn, although in the patient D1 results it seems to be present in both derivatives, which is in accordance with the conclusions for the breakpoint.

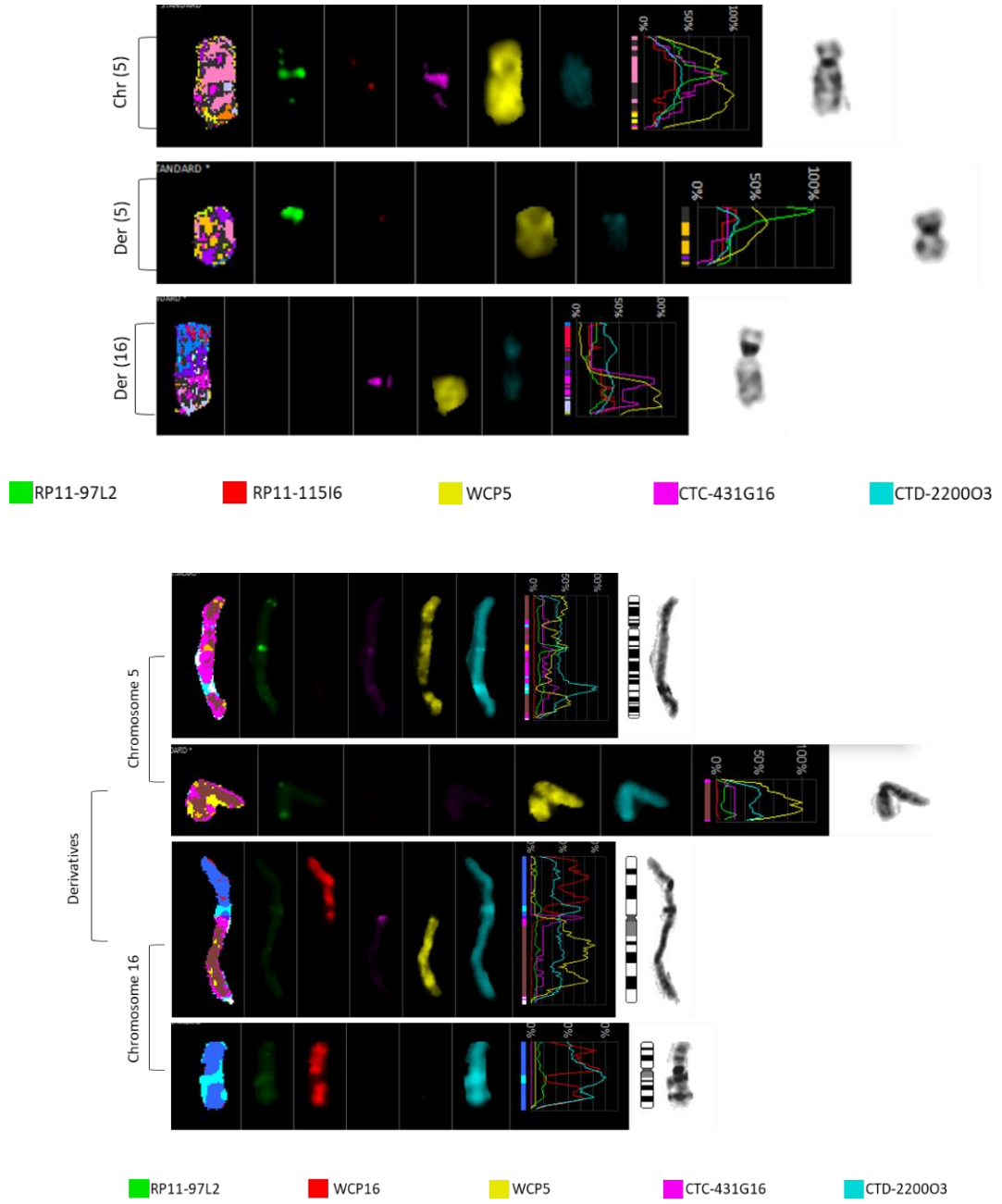

Figure 11. *Analysis of the breakpoint in the chromosome 5 of the t(5;16)(q13.3;p13.3) translocation in two patients recurring to the set probes indicated. The test on patient D1 (top) allowed the exclusion of one probe and the other probes together with WCPs were tested on patient D2 (bottom).*

4.5 t(Y;12)(q12;q12)

The probes used to analyse the cytogenetic location of the breakpoint of the translocation in study are indicated on Table 8 and the results obtained are presented in Figure 12.

Table 8. *Probes used to detect the t(Y;12)(q12;q12) translocation.*

Probes	Cytogenetic Location	Base Pairs Involved (Mb)
RP11-424J12 (dig)	Yq11.23	26,531,842-26,539,305
RP11-497C14 (bio)	Yq11.23	27,656,954-27,794,030
RP11-270H4 (SO)	Yq11.23	28,215,812-28,388,853
RP11-657P13 (dig)	12q11	36,281,502-36,416,018
RP11-115F18 (SO)	12q12	40,706,793-40,855,223
WCP 12 (DEAC)	Chromosome 12	-

The presence of the 3 probes tested from the bottom of the long arm of Y chromosome on the Y derivative, seems to indicate that the breakpoint is between the probe RP11-270H4, represented in SO, and the previously tested on diagnostics DYZ1 probe (Figure 12, left). This means that the breakpoint on the chromosome Y was between 28,388,853 Mb and 28,800,001 Mb. Regarding chromosome 12, the presence of both probes used on chromosome 12 and on the Y derivative, indicates that the split zone goes from the top of the probe RP11-657P13 (36,281,502 Mb) to the centromere (Figure 12, right).

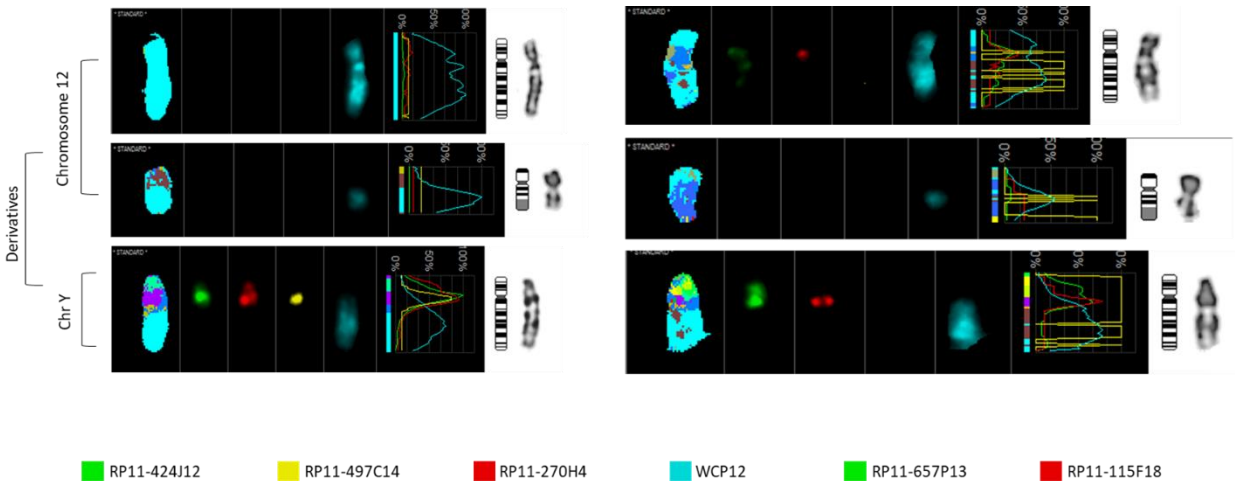

Figure 12. *Results regarding the probes used in the study of the t(Y;12)(q12;q12) translocation from patient E1.*

The karyotype analysis allows the confirmation of the translocation sought, however, more information regarding its breakpoint and a further clarification of the karyotype, and confirmation of the recurrency or not of the translocation was only possible resorting to FISH methodologies. The realization of a karyotype permitted a hands-on-hands insight on the FISH method necessity, having proved to be essential in the further characterization and clarification of translocations and respective breakpoints, the core of this dissertation.

In Hou (2003) it was established that the breakpoint of the t(11;22)(q23.3;q11.21) translocation on chromosome 11 was located between 116,585,061 Mb and 116,774,263 Mb and the one on chromosome 22 was between 21,502,000 Mb and 21,767,000 Mb[31], which is in accordance with the results obtained with the probe set tested by us. This said, we demonstrate that our results are in accordance with previous studies, reinforcing the non-familiar heritage of this recurrent translocation since patients from different backgrounds and countries present the same breakpoint. This, once more, shows that this translocation is due to a specificity on the human genome which promotes its formation, something already proven, as stated on the ***Chapter 2: Literature Review***. Here, we present a new probe set that can be used to identify and characterize this translocation with efficiency and ease.

Regarding our result of the der(X)t(X;Y)(p22.32;p11.31) translocation on patient C1, since the SRY is located within the 2,654,896-2,655,792 region as indicated in Capron *et al.*(2022)[27] and our analysis shows that the breakpoint of our patient is located proximal to that, we can assess that the SRY is present in the patient analysed. Also, in the same article it is indicated that most of the breakpoints involve the protein kinase X-linked gene, located between 3,522,384 Mb and 3,631,675 Mb, and the protein kinase Y-linked pseudogene, located between 7,142,013 Mb and 7,249,588 Mb. Also, none of the breakpoints reported are similar to the ones we have in our sample, so we can affirm that we present a new variant of the der(X)t(X;Y) translocation that was not yet characterized. The patient analysed suffered from infertility, which is in accordance with the established for the carriers of it. Also, since there was another patient from a different country studied under the same probe set, we could conclude that the breakpoint was not the same as the one from patient C1, denotating the already established non-recurrence of the breakpoints of this translocation. Since this is a translocation shown to present multiple

breakpoints regions, a deeper investigation regarding all the breakpoint regions already described in literature would be an interesting study to be performed to try to establish a connection and better comprehend the reason of such diverse propensity of breaks. Additionally, once this translocation is characterized by a multiplicity of breakpoints, the allocation of it to a group is complicated, since some breakpoints are found repeatedly, some of which seeming to be associated to specific regions in the chromosomes, while other only appear once, being due to private mutations, however, this is a complex rearrangements that occurs with some frequency and that has been the subject of several studies throughout the years and other ca be made to better comprehend this translocation.

The two patients analysed with the t(5;16)(q13.3;p13.3) translocation, were shown to have the same breakpoint. Because of the rarity of this translocation, its *de* novo occurrence in patients from unrelated families from the same region is extremely unlikely, arising the possibility of a familiar relation in both patients, similarly to the case described in Genest (1972)[10]. This makes us ascertain that this rearrangement, observed in two supposed unrelated individuals, belongs to the group of translocations characterized to have a founder event in its origin, meaning that a possible same ancestral passed the translocation through generations. This is a translocation never reported before, and, due to its rarity, an in-depth investigation of it would be interesting to be done, so the mechanisms behind its formation could be better comprehended. Also, a study of other family members from both patients, to better understand the origin of this translocation, would be an asset to be done.

The study of the t(Y;12)(q12;q12) translocation was started, and, due to the rarity of this translocation, the host lab only had samples from two patients from the last 20 years, with only one being able to undergo the study. This said, the analysis started is important and can be taken up again in the future as soon as another similar case shows up in routine GTG-banding analysis, and a better comprehension of the translocation can be done, since there is not much we can conclude from the results obtained from the single patient analysed. This said, with the single patient result we were able to narrow down the breakpoint, but we cannot assign this translocation to a specific group; however, we can suspect that it is probably due to a founder event or a private mutation, belonging to the former group if the other sample would have presented the same breakpoint, just like the previous case. The patient studied, and the one that was not possible to be study but was known to have this translocation, suffered from infertility problems, which is in accordance with the literature regarding Y/autosomes

translocations. Since there are some cases regarding chromosome Y/autosomes translocations, a propensity for such occurrence can be due to a specific mechanism in chromosome Y, which would be an interesting study, however, as already stated, t(Y;12) translocations have not been reported in the literature so this probably is not the case here. So, we can only say that in this work we describe a new translocation, which, as the other translocations involving chromosome Y and an autosome, is suspected to cause infertility problems in its carriers.

In conclusion, although there was not a broad number of samples and patients to analyse, we were able to report two new translocations and a new variant of an already established translocation, opening doors to more studies that can be continued in the future. This work is important because it can help the better diagnosis and understanding of the specifications of patients of certain translocations. In here we can perceive that there are rare translocations not yet reported and that more can exist since they normally do not cause phenotypical problems in its carriers, being frequently found during reproductive problems. This to say that various genetic problems are often not well characterized or found, and many may cause problems that are not associated to them, bolstering the importance of studies like the one presented here, that can help us assign our translocation to one group and access its reoccurring or spontaneous nature.

1. Queremel Milani DA, Tadi P. Genetics, Chromosome Abnormalities. [Updated 2023 Apr 24]. In: StatPearls [Internet]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK557691/ (visited on 11/07/23).
2. Moore CM, Best RG. (2001). Chromosomal Genetic Disease: Structural Aberrations. In: *eLS*. doi:10.1038/npg.els.0001452.
3. Baird DM, Hendrickson EA. (2018). Telomeres and chromosomal translocations: There's a ligase at the end of the translocation. In: *Adv Exp Med Biol*. Vol 1044:89-112. Springer New York LLC. doi:10.1007/978-981-13-0593-1_7.
4. Wilch ES, Morton CC. (2018). Historical and clinical perspectives on chromosomal translocations. In: *Adv Exp Med Biol*. Vol 1044:1-14. Springer New York LLC. doi:10.1007/978-981-13-0593-1_1.
5. Liehr T, Kosayakova N, Schröder J, Ziegler M, Kreskowski K, Pohle B, Bhatt S, Theuss L, Wilhelm K, Weise A, Mrasek K. (2011). Evidence for correlation of fragile sites and chromosomal breakpoints in carriers of constitutional balanced chromosomal rearrangements. In: *Balkan J Med Genet*. Dec;14(2):13-16. doi:10.2478/v10034-011-0042-z.
6. Simioni M, Artiguenave F, Meyer V, Sgardioli IC, Viguetti-Campos NL, Monlleó IL, Maciel-Guerra AT, Steiner CE, Gil-da-Silva-Lopes VL. (2017). Genomic Investigation of Balanced Chromosomal Rearrangements in Patients with Abnormal Phenotypes. In: *Mol Syndromol*. Jun 20;8(4):187-194. doi:10.1159/000477084.
7. Melaragno MI, Moysés-Oliveira M. (2017). Breakpoint Mapping of Balanced Chromosomal Rearrangements Using Array CGH of Microdissection-Derived FISH Probes. In: *Fluorescence In Situ Hybridization (FISH)*. Springer Protocols Handbooks. 587-596. doi:10.1007/978-3-662-52959-1_56.
8. Kurahashi H, Inagaki H, Ohye T, Kogo H, Tsutsumi M, Kato T, Tong M, Emanuel BS. (2010). The constitutional t(11;22): implications for a novel mechanism responsible for gross chromosomal rearrangements. In: *Clin Genet*. Oct;78:299-309. doi:10.1111/j.1399-0004.2010.01445.x.

9. Fuchs S, Lisfeld J, Kankel S, Person L, Liehr T. (2021). The acrocentric part of der(Y)t(Y;acro)(q12;p1?2) contains D15Z1 sequences in the majority of cases. In: *Hum Genome Var*. Jul 28;8(1). doi:10.1038/s41439-021-00163-9.
10. Genest P. An eleven-generation satellited Y chromosome. (1972) In: *Lancet*. May 13;299(7759):1073. doi:10.1016/S0140-6736(72)91258-5.
11. Ferguson-Smith MA. (2008) Cytogenetics and the evolution of medical genetics. In: *Genet Med*. Aug;10(8):553-559. doi:10.1097/GIM.0b013e3181804bb2.
12. Pergament E. (2000). New molecular techniques for chromosome analysis. In: *Bailliere's Best Pract Res Clin Obstet Gynaecol*. Aug;14(4):677-690. doi:10.1053/beog.1999.0104.
13. Liehr T, Starke H, Weise A, Lehrer H, Claussen U. (2004) Multicolor FISH probe sets and their applications. In: *Histol Histopathol*. Jan;19(1):229-237. doi:10.14670/HH-19.229.
14. Balajee AS, Hande MP. (2018). History and evolution of cytogenetic techniques: Current and future applications in basic and clinical research. In: *Mutat Res Genet Toxicol Environ Mutagen*. Dec;836:3-12. doi:10.1016/j.mrgentox.2018.08.008.
15. Liehr T, Weise A, Hamid AB, Fan X, Klein E, Aust N, Othman MA, Mrasek K, Kosyakova N. (2013). Multicolor FISH methods in current clinical diagnostics. In: *Expert Rev Mol Diagn*. Apr;13(3):251-255. doi:10.1586/erm.12.146.
16. Volpi EV., Bridger JM. (2008). FISH glossary: An overview of the fluorescence in situ hybridization technique. In: *Biotechniques*. Oct;45(4):385-409. doi:10.2144/000112811.
17. Liehr T. (2017). Classification of FISH Probes. In: *Fluorescence In Situ Hybridization (FISH)*. Springer Protocols Handbooks. 43-47. doi:10.1007/978-3-662-52959-1_4.
18. Liehr T. (2017). Homemade Locus-Specific FISH Probes: Bacterial Artificial Chromosomes. In: *Fluorescence In Situ Hybridization (FISH)*. Springer Protocols Handbooks. 101-106. doi:10.1007/978-3-662-52959-1_8.
19. Liehr T, Kreskowski K, Ziegler M, Piaszinski K, Rittscher K. (2017). The Standard FISH Procedure. In: *Fluorescence In Situ Hybridization (FISH)*. Springer Protocols Handbooks. 109-118. doi:10.1007/978-3-662-52959-1_9.
20. Weise A, Liehr T. (2017). Pre- and Postnatal Diagnostics and Research on Peripheral Blood, Bone Marrow, Chorion, Amniocytes, and Fibroblasts. In: *Fluorescence In Situ Hybridization (FISH)*. Springer Protocols Handbooks. 171-180. doi:10.1007/978-3-662-52959-1_17.
21. Bartlett JM. (2004). Fluorescence In Situ Hybridization Technical Overview. In: *Methods Mol Med*. 97;77-87. doi: 10.1385/1-59259-760-2:077.

22. Gu J, Smith JL, Dowling PK. (2017). Fluorescence in situ hybridization probe validation for clinical use. In: *Methods Mol Biol*. 1541:101-118. doi:10.1007/978-1-4939-6703-2_10.

23. Tapia-Páez I, Kost-Alimova M, Hu P, Roe BA, Blennow E, Fedorova L, Imreh S, Dumanski JP. (2001). The position of t(11;22)(q23;q11) constitutional translocation breakpoint is conserved among its carriers. In: *Hum Genet*. Aug;109(2):167-177. doi:10.1007/s004390100560.

24. Hill AS, Foot NJ, Chaplin TL, Young BD. (2000). The most frequent constitutional translocation in humans, the t(11;22)(q23;q11) is due to a highly specific alu-mediated recombination. In: *Hum Mol* Genet. Jun 12;9(10):1525-1532. doi: 10.1093/hmg/9.10.1525.

25. Weier HU, Munné S, Fung J. (2000). Patient-specific probes for preimplantation genetic diagnosis of structural and numerical aberrations in interphase cells. In: *J Assist Reprod Genet*. Apr;16(4):182-191. doi:10.1023/A:1020360706317.

26. Suzuki Y, Sasagawa I, Yazawa H, Tateno T, Nakada T, Saito H, Hiroi M. (2000). Localization of the sex-determining region-Y gene in XX males. In: *Archives of Andrology.* 44(2):133-136. doi: 10.1080/014850100262308.

27. Capron C, Januel L, Vieville G, Jaillard S, Kuentz P, Salaun G, Nadeau G, Clement P, Brechard MP, Herve B, Dupont JM, Gruchy N, Chambon P, Abdelhedi F, Dahlen E, Vago P, Harbuz R, Plotton I, Coutton C, Belaud-Rotureau MA, Schluth-Bolard C, Vialard F. (2022). Evidence for high breakpoint variability in 46, XX, SRY-positive testicular disorder and frequent ARSE deletion that may be associated with short stature. In: *Andrology*. Nov;10(8):1625-1631. doi:10.1111/andr.13279.

28. Mrasek K, Schoder C, Teichmann AC, Behr K, Franze B, Wilhelm K, Blaurock N, Claussen U, Liehr T, Weise A. (2010). Global screening and extended nomenclature for 230 aphidicolin-inducible fragile sites, including 61 yet unreported ones. In: *Int J Oncol*. Apr;36(4). doi:10.3892/ijo_00000572.

29. Tassano E, Alpigiani MG, Calcagno A, Salvati P, De Miglio L, Fiorio P, Cuoco C, Gimelli G. (2015). Clinical and molecular delineation of a 16p13.2p13.13 microduplication. In: *Eur J Med Genet*. 58(3):194-198. doi:10.1016/j.ejmg.2014.12.016.

30. Ghevaria H, Naja R, SenGupta S, Serhal P, Delhanty J. (2017). Meiotic outcome in two carriers of Y autosome reciprocal translocations: selective elimination of certain segregants. In: *Mol Cytogenet*. Feb 2;10(1):1-8. doi:10.1186/s13039-017-0303-y.

31. Hou JW. (2003). Supernumerary chromosome marker Der(22)t(11;22) resulting from a maternal balanced translocation. In: *Chang Gung Med J*. Jan;26(1):48-52. PMID: 12656309.

Table 9. *Resume of the preparation of the reagents needed for the FISH procedure with the respective mode of storage.*

Reagent required	Mode of preparation	Storage
Fixative	3:1 methanol (EMSURE®)/ glacial acetic acid (ROTH)	*
Ethanol 70%	350 ml 100% ethanol (ROTH) + 150 ml distilled water	RT
Ethanol 95%	475 ml 100% ethanol + 25 ml distilled water	RT
Acid Pepsin Solution	50 ml 0,2n HCl + 5ml pepsin stock solution (Sigma) + 950 ml distilled water	4 °C
Post-fix Solution	35 ml paraformaldehyde (Sigma) + 450 ml PBS + 50 ml 1mol $MgCl_2$ (Merck)	4 °C
1x PBS	47,75 g PBS (Biochrom) diluted in 5 l distilled water	RT
5 µl COT1 DNA	5 µl COT1 DNA stock (1 mg/ml; Roche Diagnostics) + 10 µl 100% ethanol (15 min *SpeedVac*)	- 20 °C
10 µl COT1 DNA	10 µl COT1 DNA stock (1 mg/ml) + 20 µl 100% ethanol (15 min *SpeedVac*)	- 20 °C
Hybridization Buffer	2g of DS dissolved in 10 ml 50% deionized formamide/ 2 x SSC/ 50 mM PBS for 3 h at 70 °C	- 20 °C
70% Formamide	700 ml formamide (Merck) + 100 ml 20 x SSC + 200 ml distilled water	4 °C
0,4 x SSC/Tw20	10 ml 20 x SSC (Invitrogen) + 490 ml distilled water + 1 ml Tween20 (Sigma) (pH 7-7,5)	TA
4 x SSC/Tw20	100 ml 20 x SSC + 400 ml distilled water + 250 µl Tween20 (pH 7-7,5)	TA
Marvel	0,1 g of dusted milk + 2 ml of 4 x SSC/Tween20 (centrifuged for 5 min at 2500 rpm)	*
DAPI Solution	10 µl 1 M DAPI (Merck) dissolved in 1 ml of VECTASHIELD® antifade (Vector Laboratories)	4 °C

* Always necessary to make fresh.