UNIVERSIDADE D
COIMBRA

Cristiana Alexandra Duarte Ferreira

GENETIC STUDIES IN ONCOLOGY: A CLINICAL LABORATORY APPROACH

Internship Report

Internship report for the conclusion of MSc in Clinical Laboratory Genetics, oriented by Professor Dra Isabel Carreira, co-oriented by Professor Dra Cecília Correia and presented to Faculty of Medicine of University of Coimbra.

June of 2022

This internship was in partnership with the Instituto Português de Oncologia do Porto Francisco Gentil- IPO-Porto, in the Service of Genetics.

Acknowledgments

Throughout the writing of this internship, I have received a great deal of support and assistance.
I would first like to thank my supervisor, Professor Dra. Isabel Carreira, whose expertise was invaluable in helping me obtaining this internship. Your insightful feedback pushed me to sharpen my thinking and brought my work to a higher level.
I would also like to thank my co-supervisor, Professor Dra. Cecília Correia, for her valuable guidance throughout my internship. You provided me with the tools that I needed to successfully complete my internship and with the writing of this thesis.
I would like to acknowledge all of the technicians from the Genetics Service of IPO-Porto for their wonderful support in everything I needed and for passing all of your knowledge in the easiest way for me to understand and be able to follow everything.
In addition, I would like to thank my parents for their wise counsel and sympathetic ear. For always listening to my complaints and having the patient to hear me train my presentation for weeks. You are always there for me. Finally, I could not have completed this dissertation without the support of my friends, Fabiana Ornelas, Pedro Veiga, Ana Bárbara and Pedro Marinho who provided stimulating discussions as well as happy distractions to rest my mind outside of my internship.

Abstract

The first steps in Portugal in the fight against cancer were due to Professor Francisco Soares Branco Gentil, in 1907, founding the Instituto Português de Oncologia. This institution has a mission, which includes providing healthcare focused on the patient. Also, it has a multidisciplinary approach to all cancer types offering a high standard treatment.

The Genetics Service is in the Department of Laboratory Diagnosis of IPO-Porto and has the main aim to provide genetic diagnosis in oncology. It is intended that every patient can have the genetic information relevant to the diagnosis, prognosis and/or response to therapy, and also, to identify families and individuals at risk for developing cancer and help them in the monitoring and prophylaxis.

The laboratory is divided in two functional areas: Cytogenetics, including conventional and molecular; and Molecular Genetics, which includes, germinative/predictive genetics and hemato-oncology/sarcomas.

The study of cancer genetics has a long history, beginning in the 1890's when a german pathologist, David von Hansemann, discovered that malignant cells from carcinoma biopsies had abnormal mitosis. Most cancers have origin in a single somatic cell and with the accumulations of genetic and epigenetic changes, the cell population can evade the normal regulation of the cell cycle.

Hematology is the study of the physiology of blood and the diseases associated with it and oncology is the study of all types of cancer. Hemato-oncology is the study of blood cancers.

Over 85% of human cancers are solid tumors. Solid tumors have a wide range of neoplasms, that are identified for their location, morphology and genetic characteristics. The effectiveness of treatment in this type of neoplasms depends on a variety of factors, such as, adequate delivery and treatment.

I came in contact with several techniques during my internship, such as, cell culture, cytogenetics, both conventional and molecular. And several molecular genetic techniques, such as, PCR, MLPA, fragment analysis, Sanger sequencing and NGS.

I was able to accompany various professional of the Genetics Service during their routine work in the laboratory. I present several clinical cases that represent my stay in IPO-Porto. I was at IPO-Porto from September 2021 to May 2022, acquiring laboratory experience in all areas of the Genetics Service.

Key-words: oncology; genetic methodologies; molecular genetics; cytogenetics; molecular cytogenetics

Index

Table's Index

Figure's Index

Abbreviation's List

AML - Acute myeloid leukemia

B-ALL - B-lymphoblastic leukemias

BL - Burkitt lymphoma

cDNA - Complementary DNA

CHKS - Caspe Healthcare Knowledge Systems

CLL/SLL - Chronic lymphocytic leukemia/ small lymphocytic lymphoma

CML - Chronic myeloid leukemia

ddNTP - Dideoxynucleotides triphosphate

DMR - Deep molecular response

EBV - Epstein-Barr virus

ET - Essential thrombocythemia

FAP - Familial adenomatous polyposis

FFPE - Formalin Fixed Paraffin Embedded

FISH - Fluorescence *in situ* Hybridization

HBOC - Hereditary Breast and Ovarian Cancer

HBV and HCV - Hepatitis B and C virus

HGBL - High-grade B-cell lymphoma

HPVs - High-risk papillomaviruses

HQS - Health Quality Service

HTLV-1 - Human T cell lymphotropic virus1

IL-2 - Interleukin-2

IPO-Porto - Instituto Português de Oncologia do Porto

ISCN - International System for Human Cytogenetic Nomenclature

ISO - International Organization for Standardization

ITD - Internal tandem duplications

KSHV - Kaposi's sarcoma herpesvirus

MDS - Myelodysplastic syndromes

MLPA - Multiplex ligation dependent probe amplification

MMax - MarrowMax

MMR - Major molecular response

MMR - Mismatch Repair

MSI - Microsatellite instability

MTX - Methotrexate

NCCN -National Comprehensive Cancer Network

NGS - Next Generation Sequencing

NORs - Nucleolar Organizing Regions

NSCLC - Non-Small Cell Lung Cancer

OC - Ovarian cancer

OECI - Organization of European Cancer Institutes

P.CCC - Porto Comprehensive Cancer Center

PCR - Polymerase chain reaction

Ph chromosome - Philadelphia chromosome

PHA - Phytohemagglutinin

PMF - Primary myelofibrosis

RT-qPCR - Quantitative Real Time PCR

SNS - *Sistema Nacional de Saúde*

TKI - Tyrosine kinase inhibitors

TMZ - Temozolomide

WHO -World Health Organization

WT - Wild type

1. Introduction

1.1. Laboratory layout

The first steps in Portugal in the fight against cancer were due to Professor Francisco Soares Branco Gentil, in 1907. In 1950, the construction site for the Instituto Português de Oncologia do Porto (IPO-Porto) was purchased. In 1974, exactly on the 17th of April the IPO-Porto initiates its functions. In the year of 2000, the building of the laboratories was functional for initiating activities. In 2003, the Center of Investigation was created. The accreditation HQS (Health Quality Service) was obtained in 2004 and the certification in 2008. Since then, reaccreditation and certification are regularly obtained. IPO Porto is also a member of the Organization of European Cancer Institutes (OECI) and, since 2011, is accredited by OECI as a Comprehensive Cancer Center (Porto Comprehensive Cancer Center, P.CCC) in collaboration with Instituto de Investigação e Inovação em Saúde (i3S).

In 2014, was the celebration of the 40th anniversary of IPO-Porto, and in 2015 it was awarded a gold medal for distinctive services in healthcare. Right now, this institution has over 47 years of history (Institucional - IPO-PORTO, 2021).

This institution has a mission, which includes providing healthcare, in useful time, focused on the patient, but not diminishing the prevention, investigation, education and training in Oncology to assure high quality, efficiency and humanism. The IPO-Porto vision is that with minimum treatment times and maximum healing ratios, the community shall view the oncological patient as a chronic patient, without stigmas and with quality of life.

This institution has a multidisciplinary approach to all cancer types offering a high standard treatment.

The values of IPO-Porto are also an important part and they compass five major areas:

Quality - assure high quality and clinical safety services for everyone; provide healthcare with high quality service, safe, effective, and focused on the patient.

Integrity - treating all patients with dignity and respect, promoting equity, diversity and offering high healthcare standards. All decisions should be made with honesty and responsibility in the best interest of the community it serves.

People - people are the core of all the services in *Sistema Nacional de Saúde* (SNS): everyone involved with the institution, whether it is a patient, a worker or a manager of the institution;

Excellence - everyone should trust the services provided. The institution should implement continuous improvements in the results of treatments and care it promotes, with the best available scientific evidence implementing effective services (including clinical results, use of financial resources, or others).

Community - besides being a hospital care provider, the institution is also a consumer and creator of waste, and has to work in partnership with the community, volunteers and other organizations to impact in a positive way people's life and minimize the environmental impact.

The Genetics Service is in the Department of Laboratory Diagnosis of IPO-Porto and has the main aim to provide genetic diagnosis in oncology, but also maintaining the scientific activity in cancer genetics and the ability to implement new techniques in diagnosing patients. It is also involved in academic education and training of health professionals. The Genetics Service aims to be a national and international reference in oncology diagnosis, and to contribute to the scientific and technological advances in this field. It is intended that every patient can have the genetic information relevant to the diagnosis,

prognosis and/or response to therapy, and also, to identify families and individuals at risk for developing cancer and help them in the monitoring and prophylaxis.
The service is located in the laboratory building of IPO-Porto, on the 6th floor. This floor is only dedicated to oncology genetics. The main focus is the genetic diagnosis in oncology but has also a scientific investigation component involved in various projects at national level. The director of the service is Professor Doctor Manuel Teixeira.
The service is divided in two areas, the genetic diagnosis through identification of genetic alterations in neoplastic cells, and studies of hereditary cancer predisposition.
The laboratory is divided in two functional areas:
- Cytogenetics, including conventional cytogenetics and molecular cytogenetics.
The karyotype is often important in the differential diagnosis and gives prognostic information to possibly classify the patients in differential prognostic groups. As for FISH, fluorescence in situ hybridization, searches for specific alterations that have a clinical relevance.
- Molecular Genetics, which includes, germinative/predictive genetics and hemato-oncology/sarcomas. In the first, the hereditary predisposition for cancer is determined, and besides the index case, the relatives are also tested for the genetic alteration. In the later, a search for genetic alterations in neoplastic cells is done, with emphasis in the alterations that give prognostic information and/or therapeutic significance (Serviço de Genética - IPO-PORTO, 2021).
The laboratories are strategically placed to avoid contaminations between samples (Figure 1). There are three sections, in which, the middle is used for storage, a wash room, the bathrooms/lockers and the reception/elevators/stairs. On corridor A, in one of the extremities is the office of Professor Doctor Manuel Teixeira. Right next door is the laboratory of the automatic sequencers, after is the main molecular laboratory (processing of samples and nucleic acid extraction), and subsequently is the electrophoresis laboratory. Alongside is the brightfield microscopy room and the adjacent dark room for fluorescence microscopy (FISH analysis), following the main cytogenetic laboratory of sample processing. The last two laboratories are the most distant, due to contaminations, the cell culture laboratory and the PCR preparation laboratory, respectively. On corridor B, first is the library and next door is the break room. This is followed by the real time PCR laboratory, alongside is the Next Generation Sequencing (NGS) laboratory and the adjacent room is the office for the professionals specialized in germinative/predictive genetics. Next comes the thermocyclers room, side-by-side with the office of the medical geneticists. The last two rooms are offices dedicated to colo-rectal cancer and hemato-oncology, respectively.

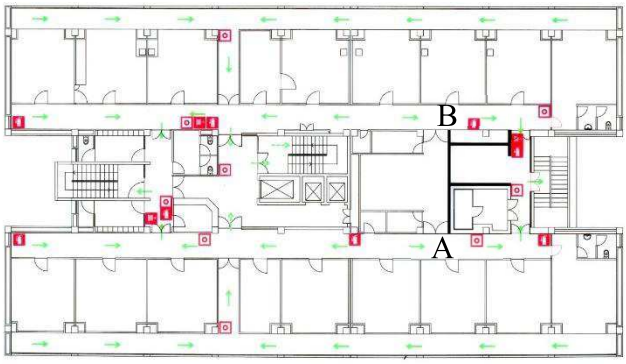


Figure 1. Plant of Emergency of Floor 6, Building E.

1.2. Quality and safety in laboratory

The International Organization for Standardization, ISO, based the standards that the United States of America military used for the manufacture and production of equipment and established standards for industrial manufacturing. The ISO 9001 (Quality Management System - Requirements) refers to the standards of quality in manufacturing and service industries, and can be applied to a variety of organizations, including hospitals and laboratories.

The IPO-Porto has implemented a quality management system, according to the ISO9001, provided by the Health Quality Service standard (HQS), currently designated Caspe Healthcare Knowledge Systems (CHKS) since 2004 and the certification since 2008. Since then, reaccreditation and recertification are regularly obtained.

Following a strategy of continuous improvement in the health care provided, in 2011 the institution obtained the Accreditation as a Comprehensive Cancer Center by the OECI (Organization of European Cancer Institutes).

To evaluate the quality in each laboratory it is needed to establish standards so it can be comparable between laboratories. ISO 15189 (Medical Laboratories – Requirements for Quality and Competence) is specific to laboratories and guaranties the quality and competence of medical laboratories. The ISO 15189 standard is divided in two major parts, the first refers to management requirements (generic requirements related to quality management systems) and the second refers to technical requirements (specific requirements related to activities carried out by clinical laboratories: the staff, facilities, procedures, reports and quality) (ISO, International Organization for Standardization).

In order to ensure the best quality in the laboratory it is needed to evaluate several factors. Among them, the laboratory environment; quality control procedures; communications; record keeping; trained technicians; and good quality reagents and equipment. For a laboratory to be recognized it should go through audits by a credible organization.

The space and facilities of the laboratory should be enough to ensure the quality of work and safety of the workers. A medical laboratory must be designed for biosafety level 2 or higher. Aerosols are the main contamination source in medical laboratories, and this can happen in very long distances. Laboratory biosafety and biosecurity activities are fundamental to protect the laboratory workforce and the wider community against unintentional exposures or releases of pathogenic biological agents (WHO, World Health Organization).

As a laboratory technician it is important to be aware of safety rules and processes and understand the safety and biosafety management issues when working with toxic chemicals, biological samples and physical hazards. The waste must be properly disposed, using a code of colors for each type of waste (such as: radioactive, halogenated liquids, non-halogenated liquids, sharp objects). Everything in the laboratory must be labeled.

It is very important that the patients and the samples don't have common pathways, and the sample should have a moving-forward path within the laboratory. The laboratory must have proper ventilation, the walls and floors must be easy to wash and disinfect, as well as work benches. A manual with procedures for safety and biosafety in the laboratory, organizing safety training and exercises must be done and all workers must be aware of these safety measures. The laboratory must have restricted access (WHO, World Health Organization).

Equipment and reagents management is essential to quality management. This helps to maintain a high-level laboratory performance, reduces variation in test results and lengthens instrument life and prevents interruption in breakdowns and failures. Reagents must always be available to the regular laboratory work, but overstocking or lack of

reagents should be avoided. All instruments and reagents must be registered in a document including various informations: manufacturer, calibrations, date of acquisition, dates of maintenance, validation dates. When a new equipment is acquired it is necessary to evaluate the performance, usually analyzing previous samples with known results (ISO, International Organization for Standardization).

Laboratory work is a complex process. There are a variety of procedures that need to be properly executed, even the procedures before the sample arrives to the laboratory need to be controlled (Lao et al., 2017).

There are three phases in a laboratory process: pre-analytical; analytical and post-analytical. To have a high-quality standard, a method to detect any errors made in each phase is required.

The pre-analytical phase involves everything before the test *per se*, namely, sample collection, transportation, sample identification and registration at arrival to the laboratory. A problem in one of these steps impairs all the steps following and can lead to delays in test's results, unnecessary redraws, incorrect diagnosis or treatment. Each sample must have essential information, such as: patient name, teste requested, date of sample collection, source of the sample, clinical data, name of the care provider, identification number. The pre-analytical phase is the most prone to errors. The most frequent problems arising from mistakes in tube filling, inappropriate specimen containers, and requesting procedures (Plebani et al, 2015). For the adoption of the ISO 15189 it is crucial to monitor and manage these errors.

The analytical phase involves the actual performance of the laboratory test and can also be prone to errors. Errors occur much less frequently in the analytic phase of laboratory testing than in either the pre-analytic or post-analytic phases. Some errors are related to degradation of reagents, human error, failure to comply with work instructions according to the manufacturer, equipment failure or calibration error.

The post-analytical phase is the final phase of the total testing process and involves evaluation of laboratory test results and release of test results. Inappropriate use of laboratory test results, critical result reporting, and transmission of correct results are areas of potential error in the post-analytical phase.

The results and the quality management of the laboratory relies on the accuracy and precision of the testing and reporting of the laboratory. If a laboratory gives inaccurate results some of the consequences may result in unnecessary treatment, treatment complications, failure to provide the proper treatment, delay in correct diagnosis and additional and unnecessary diagnostic testing (Plebani, 2006).

The current International Standard (ISO 15189) is an effective tool for improving quality, decreasing the risk of errors and increasing patient safety. The ISO standards are very rigorous and obtaining a certification from this organization requires a lot of planning and implementation of a quality system.

A laboratory audit ensures that the laboratory has quality systems in place, follows good laboratory practices, and generates data of integrity and quality. During an audit, the whole process from pre-analytical to post-analytical phase, the technicians and layout of the laboratory is evaluated.

The Genetic's Service is implementing the ISO 15189 for the quality and safety management of the laboratory.

1.3. Cancer

The study of cancer genetics has a long history, beginning in the 1890's when a german pathologist, David von Hansemann, discovered that malignant cells from carcinoma biopsies had abnormal mitosis. Theodor Boveri, based in this pathologist's information and other studies, came up with the theory that the neoplasm had origin in an acquired genetic change. At that time, this could not be proved due to the difficulties in observing the mammalian chromosomes. Meanwhile, scientific progress in the visualization of plant and insect's chromosomes with the squash and smear technique was achieved and this technique was experimented in the mammalian tissues. Tissue culturing was also expanding and improving, with the discovery that colchicine arrested the cell cycle and hypotonic solute helped with the spread of metaphases. This improvements in the various techniques culminated in the correct description of the human chromosome number, and some major human chromosomal syndromes in 1956. Also, the discovery of the mitogenic effect of phytohemagglutinin (PHA) on lymphocytes and a method for short-term cultures of peripheral blood had a major impact on the studies of cancer genetics (Heim and Mitelman, 2015).

Most cancers have origin in a single somatic cell and with the accumulations of genetics and epigenetic changes, the cell population can evade the normal regulation of the cell cycle (Ponder, 2001; Jorde, Carey and Bamshad, 2015).

In 1976, Peter Nowell, published a work where he explained his theory on the clonal evolution of tumor cell populations, known as the Monoclonal Origin of Cancer. This theory states that cancer has origin in one single cell, which acquires an alteration that gives the cell survival advantage and starts to proliferate at a higher rate than normal cells. This advantage in comparison with other cells permits the clonal expansion (Nowell, 1976).

The major six hallmarks of cancer, published by Hanahan and Weinberg, in 2000, are: evade signals to stop proliferation and to differentiate; continuous proliferation; evasion of apoptosis; capability of invasion and angiogenesis. A decade later the same authors published a second review with two more hallmarks, the reprogramming energy and evading immune response (Ponder, 2001; Hanahan, 2022).

Cancer is a complex disease, and several factors are involved in the development of cancer. Although there may be an underlying genetic predisposition to cancer, various risk factors have a major contribution (Lewandowska et al., 2019).

Risk factors include tobacco, radiation, diet and the production of free radicals. Some individuals are more susceptible to these external factors that lead to cancer due to particular genetic variations within the population. For example, individuals with fair complexion are more prone to skin cancer, and people with polymorphisms in the interleukin-1 gene are more prone to gastric cancer and hypochlorhydria induced by *Helicobacter pylori*.

These variations may explain the different cancer susceptibilities in the population, and the study of gene-environment interaction may lead to prevention strategies for those at risk.

Most cancers develop due to multiple factors thus having a multifactorial etiology (Turnbull and Hodson, 2005).

There are two major classes of genes involved in carcinogenesis, tumor suppressor genes and oncogenes.

Tumor suppressor genes are a group of genes involved in cell proliferation, differentiation, apoptosis and repair of DNA errors. When these genes are mutated and/or inactivated, the protective measures of the cell are altered, and the cell begins to accumulate more alterations and can escape apoptosis. There are three types of genes in

this group: gatekeeper genes: limit cell growth by regulating basic cell functions and controlling cell cycling, proliferation, differentiation, and apoptosis; caretaker genes: correct errors in and repair DNA; landscaper genes: regulate the cellular microenvironment.
Oncogenes are a group of genes that derive from the proto-oncogenes. Proto-oncogenes encode growth factors, membrane receptors and transcription factors.
Alterations in either of these classes of genes give rise to uncontrolled cell replication and proliferation leading to cancer.
The cancer predisposition syndromes are, usually, correlated to tumor suppressor genes. At cellular level, there is only one allele altered and a second hit is necessary for the development of cancer. Therefore, in inherited syndromes the carriers of the anomaly are more prone to the development of cancer. These individuals already have one allele altered that is inherited, therefore, there is only the need to occur one more alteration in the other allele for the development of cancer. Normally, the second hit in these individuals is due to environmental factors.
On the other hand, individuals with no inherited alteration need two events for the development of cancer. This is the Knudson two-hit hypothesis (Turnbull and Hodson, 2005; Kentsis, 2020).
Approximately 12% of cancers worldwide have origin in an oncovirus infection. This oncovirus infection it is not sufficient for cancer development, but persistent infections play a role in this process, as well as the immune system, that can either have a protective or deleterious role. Some of the most common oncovirus include hepatitis B and C virus (HBV and HCV), Epstein-Barr virus (EBV), high-risk papillomaviruses (HPVs), human T cell lymphotropic virus1 (HTLV-1), and Kaposi's sarcoma herpesvirus (KSHV) (Mesri, Feitelson and Munger, 2014; Kentsis, 2020).
Cancer classification is very important in medicine, these diseases must be described, defined and named, in order to allow a correct patient diagnosis that can lead to the best treatment of the case. A classification should not be static because in medicine everything is evolving, and the classification should be changed along with the new information that becomes available (WHO, 2017).

1.3.1. Epidemiology

In 2020, Portugal had a total population of 10196707 people, and the number of new cases of cancer was 60467, meaning that 0.593% of the total population had some type of cancer. In 2020, there were 30168 deaths due to cancer, representing 0.296% of the total population. And the 5-year prevalence was 169550 cases.
In 2020, Portugal's most common cancer was the colorectal cancer with 17.4% (10501 cases), followed by breast cancer with 11.6% (7041 cases). The third most common cancer was prostate cancer with 11.2% (6759 cases), the fourth and fifth most common are lung cancer 9% (5415 cases) and stomach cancer 4.9% (2950 cases), respectively.
The most common cancers in males are prostate cancer with 20% and colorectal cancer with 19%, followed by lung (11.6%), bladder (5.6%) and stomach (5.2%) cancers, respectively.
In females, the most common, by a great difference, is breast cancer with 26.4%, while the second most common with 15.3% is colorectal cancer, followed by lung (5.6%), thyroid (5.2%) and corpus uteri (4.6%).
Regarding new cancer cases in 2020, breast cancer and prostate cancer are on the top two places in Portugal, with 11.6% and 11.2%, respectively, followed by lung cancer with 9%.

In Portugal lung cancer had the highest mortality rate with 15.9% of the cases, followed by colorectal and stomach cancers, with 9.9% and 7.7%, respectively (GLOBOCAN 2020: New Global Cancer Data | UICC, 2022).
In Figure 2, it is represented the statistics of Portugal's cancer cases from GLOBOCAN.

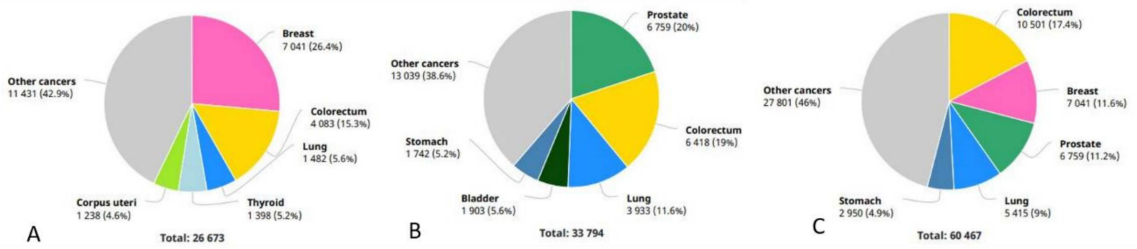


Figure 2. Statistics from GLOBOCAN Portugal 2020. A-Cancer cases in females. B-Cancer cases in males. C- Total cancer cases in Portugal. Source: GLOBOCAN 2020: New Global Cancer Data | UICC, 2022

1.4. Hemato-oncology

Hematology is the study of the physiology of blood and the diseases associated with it and oncology is the study of all types of cancer. Hemato-oncology is the study of blood cancers.

1.4.1. Hematopoietic neoplasms classification

The World Health Organization has the hematopoietic neoplasms classified according to certain characteristics and it is regularly updated to always be up-to-date with the new information that is gathered through investigation of these neoplasms (WHO, 2017). There are several entities that are characterized by the presence of specific genetic alterations, some of which are discussed below.

1.4.1.1. Myeloid neoplasms

Myeloproliferative Neoplasms

Myeloproliferative neoplasms
Chronic myeloid leukaemia, *BCR-ABL1*–positive
Chronic neutrophilic leukaemia
Polycythaemia vera
Primary myelofibrosis
Essential thrombocythaemia
Chronic eosinophilic leukaemia, NOS
Myeloproliferative neoplasm, unclassifiable

Figure 3. WHO classification of Myeloproliferative neoplasms. Source: WHO, 2017

Chronic myeloid leukemia, CML

CML is a clonal disease that is a myeloproliferative neoplasm (Figure 3) characterized by neoplastic overproduction of granulocytes (Heim and Mitelman, 2015). This disease accounts for approximately 15-20% of adult leukemias.
CML can be divided into three phases: the chronic phase, the accelerated phase and then the blast phase. Most cases of CML, in the developed world, are diagnosed at the chronic phase, and if left untreated it will eventually progress to the next phase, the accelerated and after the blast phase (National Comprehensive Cancer Network, 2022).

The first example of a consistent chromosome abnormality in a neoplasia was published in *Science* in 1960, by Nowell and Hungerford, and was named after the city where was presented: The Philadelphia chromosome.
This marker chromosome is the characteristic feature of this leukemia, and usually arises from a reciprocal translocation between chromosomes 9 and 22, giving origin to the fusion gene *BCR-ABL1* (Heim and Mitelman, 2015). However other non-CML leukemias can have this genetic alteration.
At the time of a CML diagnosis, a cytogenetic evaluation should be done for confirmation of the diagnosis and evaluation of other cytogenetic abnormalities. Molecular evaluation to detect the specific transcript of the patient and also a quantification of the mRNA transcripts is also mandatory. In the follow-up, quantitative RT-PCR should be done to evaluate the molecular response to therapy (National Comprehensive Cancer Network, 2022).
The main treatment for this leukemia is based in ABL1 tyrosine kinase inhibitors (TKI), that are very efficient and relatively well tolerated within the patients. This therapy dramatically improved the outcome for CML patients, and if addressed appropriately, it can lead to an optimal molecular response in the majority of CML patients and a life expectancy that approaches that of the general population.
The response to TKI is monitored, trough RT-qPCR. The patients can have an early molecular response, when the BCR-ABL1 transcripts are inferior to 10% in the first 3-6 months of therapy; major molecular response, when BCR-ABL1 transcripts are less than 0.1%, and a deep molecular response when the transcripts are less than 0.01%. If the patient achieves a stable deep molecular response, along with some other factors, namely the treatment duration, discontinuation of the therapy can be considered (Heim and Mitelman, 2015).
In some cases, point mutations in the *BCR-ABL1* kinase domain can occur and patients have a consequent resistance to TKI's, according to Table 1. This is called acquired resistance to treatment. When patients develop this type of resistance it is necessary to do a mutation test to understand which is responsible for the resistance and give options of new treatment to the patient. Therefore there are several lines of TKIs already in the market (Cerveira et al., 2021).

Table 1. Resistant mutations to each TKI therapy. Adapted from: Patel, O'Hare and Deininger, 2017

Therapy	Resistant mutations
Imatinib	L248R, E255V, T315I/V
Bosutinib	T315I, V299L, F317L, L248R
Dasatinib	T315I/A, F317L/V/I/C, T317I/C, V299L
Nilotinib	T315I, Y253H, E255K/V, F359V/C/I, E359C/I
Ponatinib	E255V

Myelodysplastic syndromes, MDS

Figure 4. WHO classification of Myelodysplastic syndromes. Source: WHO, 2017

Myelodysplastic syndromes, in Figure 4, are a very heterogenous group of disorders that are characterized by the dysplastic maturation of hematopoietic cells in conjunction with cytopenias and can even progress to AML. This group of disorders is more predominant in men and the incidence increases with age. Some of these disorders are chronic myelomonocytic leukemia, atypical chronic myeloid leukemia, juvenile myelomonocytic leukemia and MDS with sideroblasts and thrombocytosis. About 10-15% of MDS are related to previous therapy, in other words it is developed after chemotherapy and/or radiotherapy, but the majority of cases arise *de novo*, although the normal risk factors, such as, tobacco, solvents, radiation also play a role.

The cytogenetic evaluation is very important to confirm the diagnosis and to help to determine the prognosis, the survival and risk for progression. Therapy related MDS has a percentage of 95% of patients in which are detected chromosomal abnormalities, and primary MDS only 40-70% of patients have chromosome abnormalities (Heim and Mitelman, 2015; WHO, 2017).

In Table 2, are the most commonly alterations associated with MDS.

Table 2. Chromosomic/Genetic aberrations associated with MDS and their prognostic value. Adapted from: National Comprehensive Cancer Network, 2022

Aberration	**Prognostic value**
del(13q)	Low risk
T12	Low risk
SF3B1	Intermediate risk
NOTCH1	Intermediate risk
del(11q)	High risk
del(17p)	High risk
IGH	High risk
Complex Karyotype	High risk

Acute myeloid leukemia, AML

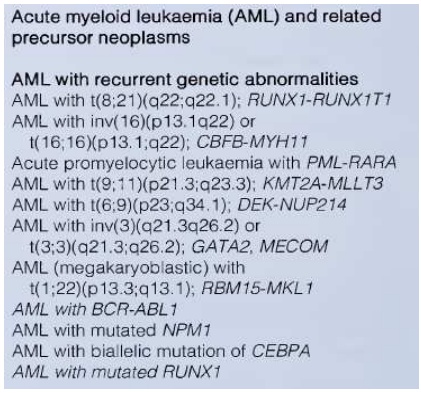


Figure 5. WHO classification of Acute Myeloid Leukemia. Source: WHO, 2017

Acute myeloid leukemia (Figure 5) is characterized by an accumulation of immature myeloid blasts in the bone marrow, peripheral blood or other tissue. This leukemia is predominant in adults, with men being slightly more affected than women. Studies shown that children have more abnormal karyotypes than adults in this pathology (WHO, 2017; National Comprehensive Cancer Network, 2022).

AML is a very complex disease, both morphologically and genetically. Nowadays, several gene mutations are known to be associated with AML, presented in Table 3. Three main groups of genes that when altered are involved in the pathogenesis of AML: transcription factors, when the genes involved are *CEBPA* and *NPM1*; signal transduction, when it is *FLT3*, *KRAS* and *NRAS*; and epigenetic changes, in *TET2*, *IDH1*, *IDH2* and *DNMT3A*.

WHO classification includes AML with recurrent genetic alterations.

The most common are AML with t(8;21)(q22;q22.1), that results in a *RUNX1-RUNX1T1* fusion gene; AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22), which results in the fusion gene *CBFB-MYH11*. Another subtype is acute promyelocytic leukemia with *PML-RARA*, characterized by abnormal promyelocytes predominating in the bone marrow. Another subtype is AML with mutated *NPM1*, this subtype is characterized by the anemia and thrombocytopenia. The *NPM1* gene is one of the most common alterations in AML, and the exon 12 is usually the most involved (Heim and Mitelman, 2015; WHO, 2017).

Table 3. Chromosomic/Genetic aberrations associated with AML and their prognostic value. Adapted from: National Comprehensive Cancer Network, 2022

Aberration	Prognostic value
t(8;21)(q22;q22.1): *RUNX1-RUNX1T1*	Low risk
inv(16)(p13.1q22)/ t(16;16)(p13.1;q22): *CBFB-MYH11*	Low risk
t(15;17)(q24.1;q21,2): *PML-RARA*	Low risk
t(9;11)(p21.3;q23.3): *MLLT3-KMT2A*	Intermediate risk
t(9;22)(q34.1;q11.2): *BCR-ABL1*	High risk
Mutated *TP53*	High risk
Mutated *RUNX1*	High risk
Complex Karyotypes	High risk
Monosomic Karyotypes	High risk

1.4.1.2. Lymphoid neoplasms

Precursor lymphoid neoplasms

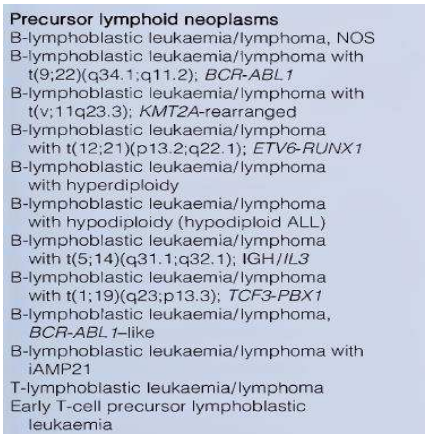


Figure 6. WHO classification of Precursor lymphoid neoplasms. Source: WHO, 2017

B-lymphoblastic leukemias, B-ALL

B-ALL is a precursor lymphoid neoplasm (Figure 6) characterized by small to medium sized blasts with scarce cytoplasm and that involves bone marrow and blood. It is more predominant in pediatric cases, occurring approximately in 75% of children with 6 years-old or less.

There are subtypes with recurrent genetic aberrations, such as rearranged *KMT2A*. This gene has a wide range of fusion partners and is more commonly rearranged in infants under 1 year-old. Another subtype is the t(12;21)(p13.2;q22.1), resulting in the fusion gene *ETV6-RUNX1*.This rearrangement is more predominant in children (Heim and Mitelman, 2015; WHO, 2017).

Table 2 shows the most common genetic alterations in ALL. There are other genes involved that are not proven of prognostic values, yet.

The majority of children have a karyotype with hyperdiploidy or t(12;21)(p13;q22) which are associated with a good prognosis (WHO, 2017).

Table 4. Genetic aberrations associated with ALL and prognostic value. National Comprehensive Cancer Network, 2022

Aberration	Prognostic value
Hyperdiploidy (>50 chr)	Low risk
Hypodiploidy (<40 chr)	High risk
t(9;22)(q34;q11): Ph chr	High risk
t(12;21)(p13;q22): *ETV6-RUNX1*	Low risk
t(8;14), t(2;8), t(8;22): x-*MYC*	High risk
***BCR-ABL1*-like**	High risk
Ikaros: *IKZF1*	High risk
iamp21: *RUNX1*	High risk

Mature B-Cell neoplasms

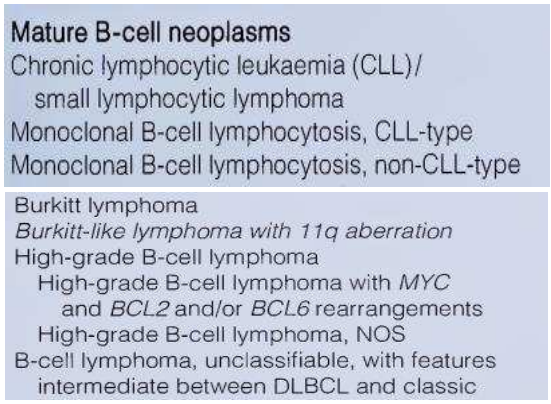


Figure 7. WHO classification of Mature B-Cell neoplasms (partial). Source: WHO, 2017

Chronic lymphocytic leukemia/ small lymphocytic lymphoma (CLL/SLL)

CLL/SLL is a Mature B-cell neoplasm (Figure 7) composed of monomorphic small mature B cells that co-express CDS and CD23. CLL is the most common leukemia of adults in western countries and most cases are diagnosed on the basis of routine blood analysis in asymptomatic subjects.

IGHV genes are mutated in 50- 70% of cases. The most common alterations are deletions in 13q14.3 and trisomy 12 or partial trisomy 12q13; less commonly, there is deletion in 11q22-23 (*ATM* and *BIRC3*), in 17p13 (*TP53*), or 6q21.

Burkitt lymphoma

Burkitt lymphoma is a Mature B-cell neoplasm that is often present in extranodal sites or as an acute leukemia, has monomorphic medium-sized B-cells and a rearranged *MYC* gene with an *IG locus*. A combination of techniques is required to correctly diagnose this type of lymphoma.

Sporadic BL is seen throughout the world, mainly in children and young adults. Endemic BL is present in equatorial Africa and in Papua New Guinea and is more predominant in children with 4-7 years (WHO, 2017).

High-grade B-cell lymphoma (HGBL)

HGBL is a group of aggressive B lymphomas that for biological and clinical reasons should not be classified as diffuse large B-cell lymphoma (DLBCL), or as Burkitt lymphoma (BL).

A sub-group of them is High-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements. This is an aggressive mature B-cell lymphoma that harbors a MYC rearrangement and a rearrangement in *BCL2* and/or in *BCL6*. These lymphomas are often called double-hit lymphomas, or triple-hit lymphomas if there are both *BCL2* and *BCL6* rearrangements in addition to the *MYC* rearrangement. This neoplasm is more common in elderly patients, above 60-70 years old (WHO, 2017).

1.4.2. Molecular genetics of hematopoietic neoplasms

1.4.2.1. Fusion genes

Fusion genes are constituted by joining parts of two different genes. This fusion can occur due to translocations and deletions. Fusion proteins produced by this change may lead to the development of some types of cancer, due to the deregulation of one of the genes involved. Fusion genes also showed their value by serving as a diagnostic tool to monitor treatment progress by measuring the disappearance of the fusion (Parker and Zhang, 2013).

***BCR-ABL1* fusion gene**

The *ABL1* gene is a tyrosine kinase that is essential for cell growth and differentiation, and when it is translocated, it has a constitutive activation that allows the cells to be continuously proliferating (Avery 2009).

There are several different types of transcripts, depending on where the breakpoints of the two genes, *BCR* and *ABL1*, occur. The most common transcripts, in CML, are b2a2 (e13a2) and b3a2 (e14a2) (p210) but rarer transcripts, for example, (e1a2) (p190) or e19a2 (p230), can also be found in a lower percentage of cases. (Branford and Hughes, 2006; De Braekeleer et al., 2012).

The molecular response of the patients to therapy, TKIs, tyrosine kinase inhibitors, should be measured through a real-time qPCR. Major molecular response, MMR, is defined as a 3-log reduction from the standardized baseline. With prolonged TKI therapy, deeper levels of response can be achieved, with some patients reaching levels where no disease can be detected. Patients achieve MMR when the *BCR-ABL1* transcript level is ≤0.1%. There are different levels of deep molecular response, DMR, MR3, MR4 and MR4.5, depending on the level of transcripts detected. The patients should be monitored always, even after they achieve DMR (Hochhaus et al., 2020; (Cerveira et al., 2021).

***PML-RARA* fusion gene**

PML-RARA fusion gene results from the t(15;17)(q22;q21) translocation, this fusion gene encodes a fusion protein that has a function of transcriptional repressor, not giving access of chromatin for transcription. The *PML* gene encodes a putative novel transcription factor and the *RARA* gene encodes a retinoic acid receptor-α.

The *RARA* gene has always the same breakpoint, unlike the PML that has three different breakpoints, one in intron 6, exon 6 and intron 3, giving the protein three isoforms depending in which breaking site of the *PML* gene it occurs (Gabert et al., 2003; van Dongen et al., 1999).

This fusion gene is very typical of promyelocytic leukemia, and the different isoforms of the fusion gene although associated with different phenotypes do not change the outcome. This type of leukemia accounts for about 10% of all AMLs. The *PML-RARA* fusion gene is present in about 90% of all promyelocytic leukemias, but it is one of the better prognoses within the AML's patients (De Braekeleer et al., 2014; van Dongen et al., 1999; Wang et al., 2017).

***CBFB-MYH11* fusion gene**

CBFB-MYH11 is a fusion gene that results from the inversion of the chromosome 16 or the translocation between the two chromosomes 16. The *CBFB* gene encodes an heterodimeric transcription factor, the core binding factor b subunit, and the *MYH11* encodes the smooth muscle myosin heavy chain protein. The action of the *CBFB-MYH11* is dominant in relation to the wild type *CBFB.* This type of alteration is usually associated with good prognosis (Castilla et al., 2004; van Dongen et al., 1999).

This fusion gene and the *RUNX1-RUNX1T1* both have subunits of the core binding factor; therefore, the treatment is very similar and they are included in the same category (Wang et al., 2017).

RUNX1-RUNX1T1 fusion gene

This fusion gene results from the t(8;21)(q22;q22) translocation and involves the genes *RUNX1* and *RUNX1T1*. The *RUNX1* encodes a transcription factor and has two breakpoint sites one in exon 5 and the second in exon 6, while the *RUNX1T1* encodes a transcriptional corepressor, CBFA2T1 protein, and has, also, two breakpoint sites, one in exon 1 and the other in exon 2.
When the fusion happens the transcription factor encoded by the *RUNX1* gene is no longer functional and there is inhibition of transcription. This type of alteration is of good prognosis for the patients (Wang et al., 2017).

Gene rearrangements can lead to fusion genes, but not only. The genes explained above are rearranged to create only fusion genes, whereas the genes discussed below can have fusion partners but it is not exclusively fusions.

MLL gene rearrangements

MLL gene is located on the long arm of the chromosome 11, region 2, band 3, and is involved in translocations, internal gene duplications, deletions or inversions of the 11q and insertions. Several fusion partners have been identified, being the most common in AML the *MLLT3*, *MLLT10* and *SEPT6* genes as for ALL the *AFF1*, *MLLT1* and *MLLT10* are the most common.
MLL gene is very frequent in infant leukemia, present in approximately 70%, and in adult leukemia accounts for about 10% of cases. Patients with *MLL* rearrangements have a bad prognosis and are treated with protocols for high-risk patients (Cerveira et al., 2012).

PAX5 gene rearrangements

PAX5 gene encodes a transcription factor, the B-cell lineage-specific activator protein, in the hematopoietic system this gene is only expressed in the B-cells.
PAX5 has a major role of enabling the expression of genes that are inappropriate of B-cells and activating the genes that need to be activated in B-cells, therefore if this gene is lost the cells won't have the specific lineage genes activated and the development stops. When the gene is fused with another, through a translocation, the binding part of the protein is still available, and it binds to the DNA but it's not clear if the chimera genes regulate the transcription the same way as a wild type PAX5 protein does or if it has an aberrant function.
There have been identified multiple genes that are fusion partners with *PAX5*, like *ETV6*, *ELN*, *FOXP1*, *XNF521* genes and others. These fusions give the patients a common ALL phenotype, and are considered of good prognosis (Nebral et al., 2009).

ETV6 gene rearrangements

The *ETV6* gene encodes a ubiquitously nuclear protein that belongs to a large family of transcriptional factors, the ETS family. The *ETV6* gene has 8 exons, and has two start codons, one at exon 1 and the second is located upstream of codon 3. It is located on the short arm of chromosome 12, region 1, band 3. These rearrangements are very common in human leukemias and myelodysplastic syndromes.
The *ETV6* protein is essential for normal embryonic development and hematopoietic regulation. The rearrangements involved in these gene are often structural chromosomal

abnormalities, and there are a various number of fusion partners identified. There are several classes of genes that can rearrange with *ETV6* and change leukemogenesis, fusion with tyrosine kinases genes can be treated with TKIs, transcription factors and others.
Other fusion partners are *FLT3*, the *ABL1*, the *JAK2*, *RUNX1* and *PAX5* genes. Different parts of the *ETV6* gene may be involved in the fusions. Also, depending in the genes involved different mechanisms can be altered like with the activation of the kinase activity, in transcription factors the function can be altered, like a transcriptional activator can become a repressor and vice versa, and another mechanism is the loss of function of the two genes that form the fusion gene (De Braekeleer et al., 2012).

***IGH locus* rearrangements**

The *IGH locus* is responsible for the production of antibodies, so it is a gene that normally is constantly activated and needs to recombine with different other genes for the production of the antibodies. This recombination can be abnormal and instead of the normal genes it happens with oncogenes and gives them an inadequate expression, resulting in leukemogenesis. Some recurrent fusion partners of *IGH* are *MYC*, *BCL* family and *CCND1* genes (Avery, 2009).

1.4.2.2. Major genes alterations

Hematopoietic neoplasms are a very heterogenous group of diseases and have a variety of important alterations, either for diagnosis and prognosis. Some individuals with hematopoietic neoplasms have a normal karyotype but it is advantageous to identify the alteration. Recently, with the development of methodologies, new genetic mutations associated with leukemia have been identified (Daver et al., 2019).
Below are discussed the major genes alterations in a variety of hematopoietic neoplasms that are evaluated in IPO-Porto.

***FLT3* gene**

FLT3 is a receptor tyrosine kinase, which plays a role in survival, proliferation and differentiation of lymphoid and myeloid lineages (Small, 2006). Alterations in these gene are secondary alterations and it is also, a therapeutic target along with chemotherapy. Alterations of the *FLT3* gene are common in AML whether ITD, internal tandem duplications, or point mutations in the tyrosine kinase domain. Both types of alterations lead to the constitutive activation of the receptor (Kutny et al., 2012). ITD alteration occurs in the juxtamembrane domain and/or TKD1 domain of the receptor, it is a driver mutation that confers an unfavorable prognosis and has a bad impact on the management of the patients (Daver, Schlenk, Russell and Levis, 2019). A large portion of the studies done that involve this gene, demonstrate that only the ITD has a bad outcome, but others report that the point mutation have worse outcomes in adults. In conjunction, the alterations in the *FLT3* gene are the most common in AML, and more prevalent in acute promyelocytic leukemia (Kutny et al., 2012). A group with especially worse outcomes is the group of patients that have ITD alterations and lost the WT, wild type, allele of *FLT3*. The ratio between the ITD allele and the WT allele is very important due to, patients that have a low ratio have better survival rates, to the point where it is similar to patients that don't have alterations in the *FLT3* gene (Small, 2006). It was observed that patients without *FLT3* alterations at diagnosis, can have these alterations when relapsing, and due to this it is necessary to test the patients at several points in time for these alterations.

Although, some studies found that these associations are weak and that other factors, such as, karyotype and alterations in the *NPM1* gene may influence the outcome and the prognostic value (Daver et al., 2019).

***JAK2* gene**

JAK2 is part of the Janus family of kinases. It is located in chromosome 9p24, and it has multiple fusion partners identified (Morgan and Gilliland, 2008). The *JAK*s are an essential part for normal hematopoiesis, and the *JAK-STAT* pathway is crucial for the signaling of other cytokines (Smith and Fan, 2008). The deletion of the JH2 domain negatively regulates *JAK2* activity, and the interaction between JH1 and JH2 is responsible for the inhibition of the JH1 domain that can never leave the activate conformation, the translocations of *JAK2* are responsible for the permanent activation of the same.

The *JAK2* V617F mutation is a point mutation at codon 617 of exon 14, where there is a substitution of a valine for a phenylalanine, it is a somatic and acquired mutation. This mutation is responsible for the constitutive phosphorylation of *JAK2* and erythropoietin hypersensivity. The gene dosage of *JAK2* is also a factor for the various diseases that *JAK2* is involved, where homozygous *JAK2* V617F have a worst outcome and people who are heterozygous and homozygous for WT for *JAK2* have a better outcome. The *JAK2* V617F point mutation has not been observed in simultaneous with other tyrosine kinase mutations (Morgan and Gilliland, 2008; Smith and Fan, 2008).

Several mutation tests can be used like DNA sequencing, which can detect new variants, allele-specific PCR, that can differentiate between the WT allele and the mutated allele, RT-qPCR, this type of PCR can also distinguish between the WT and mutated allele though it has a lower sensibility (Smith and Fan, 2008).

***MPL* gene**

Mutations in the *MPL* gene, also known as *TpoR*, human thrombopoietin receptor, are only found in patients with essential thrombocythemia, ET, and primary myelofibrosis, PMF (Levy et al., 2020). This gene is responsible for the maintenance of the hematopoietic stem cell, the development of the megakaryocytes and platelet production. The *MPL* gene has a hotspot for mutations in the exon 10 that encodes the transmembrane domain. It is reported that nearly 6 to 14% of patients with *JAK2* V617F have mutations in the transmembrane domain of the *MPL* gene, which cause gain of function and therefore lead to the permanent activation of the *JAK2* (Bridgford et al., 2020).

***NPM1* gene**

The nucleophosmin gene, *NPM1*, encodes a ubiquitous multifunctional shuttling protein 1 that has a more predominant location in the nucleolus. This gene is the most common mutated in AML. When it is mutated, the protein has a presence in the cytoplasm.

The mutations in these gene are always heterozygous and the majority in the exon 12, that probably are originated by replication errors. The most common type of mutations is the insertion of 4bp that cause a frameshift and alters the C-terminal of the protein. Due to, the alteration of the conformation of the C-terminal the protein cannot ancor itself to the membrane of the nucleolus (Falini et al., 2020; Martelli et al., 2021).

The rarer mutations that occur outside of exon 12 and fusions with other genes are also responsible for the presence of nucleophosmin in the cytoplasm. Mutations in these gene are always in hetorozigoty, because the loss of the two alleles is lethal and results in embryonic death. Patients with mutations in the *NPM1* gene have, normally, normal karyotype due to the fact that both normal and mutated alleles can regulate centrosome

duplication. *NPM1* mutations are driving mutations for the preleukemic clonal hematopoiesis, and there is evidence that if it possible to eliminate the mutated clones it is a possible cure for AML.
These types of mutations are rare in children, about 6.5%, have a higher predominance in the middle age and it lowers its frequency again after 70 years of age. *NPM1* mutations are considered as favorable prognosis for the patients, but if it appears simultaneously with *FLT3* mutations it becomes of poor prognosis (Falini et al., 2021).

1.5. Solid neoplasms

Over 85% of human cancers are solid tumors. Solid tumors have a wide range of neoplasms, that are identified for their location, morphology and genetic characteristics. The effectiveness of treatment in this type of neoplasms depends on a variety of factors, such as, adequate delivery and treatment (Jain et al. 2011).

1.5.1. Therapy-related genetic alterations

The majority of recently approved molecularly targeted cancer drugs are specific for oncoproteins encoded by somatically mutated genes. There are genes strongly associated with certain types of cancer that when altered are responsible for a better response or resistance to a certain therapy (Hahn et al. 2021).

Non-Small Cell Lung Cancer

Lung cancer is the leading cause of cancer death worldwide in men. In Portugal, it is the fourth most incident cancer in 2020 and the first in terms of mortality (GLOBOCAN 2020: New Global Cancer Data | UICC, 2022). Late diagnosis is a major obstacle to outcomes in this type of cancer.
Approximately 85-95% of lung cancer cases are related to smoking. The Non-Small Cell Lung Cancer (NSCLC), accounts for more than 80% of all lung cancers, is classified in adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell carcinoma and morphological undifferentiated.
In lung cancer, there are several targetable activating alterations in cancer genes, such as *EGFR, ALK, ROS1, MET, BRAF, RET* and *KRAS* (NCCN 2021).
The *EGFR* gene is a receptor tyrosine kinase that is found normally in the surface of epithelial cells. In this gene, the exon 19 deletions and the p.L858R point mutation are associated to better response to TKI therapy. Insertions in exon 19 are less common and are also associated to response to TKI therapy. Variants in exon 20 are very heterogenous and one particularly (T790M) is associated with resistance to target therapy with first- and second-generation EGFR-TKI. Everyday new variants are being identified as a result of the advancements on NGS technologies (Castellanos, Feld and Horn, 2017).
The *ALK* gene is a receptor of tyrosine kinase frequently involved in translocations that lead to gene fusions in lung cancer. The most common fusion partner is *EML4* (microtubule-associated protein-like 4). These fusions are associated with responsiveness to ALK inhibitors (Alexander, Kim and Cheng, 2020).
ROS1 is a receptor tyrosine kinase that undergoes genetic rearrangements in a variety of human cancers including NSCLC. This gene has several fusion partners, the most common being *CD74*, *SLC34A2*, *CCDC6* and *FIG* genes. These alterations are associated with response to ROS1 inhibitors (NCCN 2021).
BRAF is a serine/threonine kinase that interacts in the MAP/ERK signaling pathway. The V600E variant is a biomarker for this type of cancer, giving a poor prognosis.

The *KRAS* gene provides instructions for making a protein called K-Ras that is part of a signaling pathway known as the RAS/MAPK pathway. Activating mutations in *KRAS* are found in 25 to 30% of NSCLCs, representing the most prevalent genomic driver event in NSCLC. One of the most frequent KRAS mutations in patients with NSCLC is KRAS G12C, which is predictive of response to targeted treatment.
MET is a receptor tyrosine kinase. There are several alterations in this gene, including the skipping of exon 14, which is associated with response to MET TKIs. The exon 14 of the *MET* gene encodes the intracellular juxtamembrane (JX) domain. The alteration disrupts intronic splice sites that flank exon 14, including the splice acceptor site of intron 13 and the splice donor site of intron 14, or mutation within the exon 14 coding sequence itself, and all result in exon 14 skipping in the transcript (NCCN 2021).

Colon cancer

In Portugal, colon cancer is the fourth cancer with the highest rate of new cases in 2020 and the second in mortality rate (GLOBOCAN 2020: New Global Cancer Data UICC, 2022). However, the incidence and mortality of this type of cancer has been decreased, probably due to the improvements in the diagnosis and treatment.
Most colorectal cancers are sporadic cases, but it is estimated that about 20% occur in family clusters, and of these only a small amount is due to hereditary syndromes, being Lynch syndrome and familial adenomatous polyposis (FAP) the most frequent ones.
Biomarkers are important to identify patients that are good candidates for targeted therapy. Currently, the mutation status of *KRAS/NRAS* and BRAF and MSI/MMR status are important in defining therapy.
KRAS/NRAS are involved in the MAPK pathway and activating variants in these genes are strong indicators of insensibility to EGFR inhibitors.
BRAF V600E is present in approximately in 5 to 9% of colon cancers, this specific variant is responsible for a poor prognosis regardless the treatment given to the patient. Even though, they may benefit from EGFR inhibitors as first-line therapy (NCCN 2021).

Breast Cancer

Breast cancer is the cancer with the highest rate of new cases and the second in mortality rate, in Portugal, in 2020. In Portuguese women, it is the fifth in mortality (GLOBOCAN 2020: New Global Cancer Data | UICC, 2022).
All patients with breast cancer should be assigned a clinical stage, that helps the stratification for treatment. In 2000, four different molecular subtypes of breast cancer were identified, through gene expression profiling. Estrogen receptors, progesterone receptors, as well as *HER2* status should be tested on the primary tumor and/or metastases for everyone newly diagnosed with invasive breast cancer or a breast cancer recurrence, which are used to guide treatment. These biomarkers are tested with IHC (immunohistochemistry), in case the *HER2* result is equivocal it can be sent to the genetic laboratory for a FISH assay of this gene.
Some of the biomarkers used in decision of targeted therapies are mutation status of the *BRCA1/2* and *PIK3CA* genes (NCCN 2021).
The *BRCA1/2* is assessed in all patients with recurrent or metastatic (germinative alterations) breast cancer to identify the patients that would benefit from PARP inhibitors. Variants in these genes can be germline or somatic. These genes are DNA double strand repair genes and variants predispose patients to cancer due to the lack of ability to complete the recombination repair in the cells with damaged DNA strands (Yoshida, 2020).

The *PIK3CA* activating mutations are assessed in patients with hormone receptor positive/ *HER2* negative breast cancer. These types of variants are predictive of response to the alpha-selective PI3 kinase inhibitor alpelisib in combination with fulvestrant (Litton, Burstein and Turner, 2019).
HER2 positive breast cancer have several monoclonal antibodies that can be used in the treatment. These antibodies have binding sites in different sites of HER2 protein (Barzaman et al., 2020).

On the other hand, there are somatic alterations that are inherited and are, also, responsible for the response or resistance to therapy and are not related to the type of cancer present in the patient (Mansouri et al., 2019).

***MGMT* gene**

O6-methylguanine-DNA methyltransferase, *MGMT*, is a gene located on chromosome 10 and encodes a repair protein that reverses the alkylation process. The *MGMT* promoter has CpG islands, that when methylated there is a silencing of the gene.
Temozolomide, TMZ, is a chemotherapy widely used in the treatment of glioblastomas, GBM. This chemotherapy induces cytotoxic cell death in tumor cells by alkylating DNA at multiple sites. The repair of this alkylation events on the O6 group of guanines, that is the most toxic for a cell, is dependent on the *MGMT*. The methylation of the *MGMT* promoter silences the gene and there is not enough DNA repair and, therefore, greater response to TMZ (Mansouri et al., 2019).
There are several techniques used for determination of *MGMT* methylation. In IPO-Porto it is used the MS-MLPA assay.
MGMT methylation has been widely studied as a biomarker in predicting the prognosis of patients with GBM and their response to therapy. *MGMT* methylation is associated with a better overall survival in GBM in patients treated with temozolomide, TMZ. But this effect is not the same for all populations. In the Asian population the methylation of *MGMT* does not hold the same significance as it does in the Caucasian population. Also, age seems to have an impact on the prognosis (Rao, Quddusi and Shamim, 2018).

1.5.2. Hereditary syndromes

Approximately 5–10% of cancers are hereditary cancer predisposition syndromes. Identifying patients who have an inherited cancer predisposition syndrome has significant benefit to both the patient and at-risk relatives. For the index patient, the diagnosis of a hereditary cancer syndrome has implications for their surveillance strategy for multiple component tumors in terms of age of initiation and intervals between surveillance exams, and may lead to the consideration of prophylactic surgery (Syngal et al., 2015).

Lynch Syndrome

Lynch syndrome is known as one of the most common forms of inherited cancer predisposition. It is an autosomal dominant condition and is associated with increased risk to colon and endometrial cancers, but not exclusively (Pastorino et al., 2020).
This syndrome is caused by germline disruption in the mismatch repair genes, *MLH1, MSH2, MSH6,* or *PMS2*, and rarer in the *EPCAM* gene. Two major approaches to the diagnosis of Lynch syndrome are molecular screening of colorectal and endometrial tumor specimens for evidence of defective MMR function (MMR-D) or high-level MSI (MSI-H) to identify patients with cancer who should undergo germline testing for

pathogenic MMR gene variants; or direct germline testing performed on patients with personal and/or family histories of cancer are suspicious for Lynch syndrome (Yurgelun and Hampel, 2018).

Hereditary breast and ovarian cancer

Families with a history of multiple breast or ovarian cancers approximately account for 15% of all patients with breast cancer. Most cases of Hereditary Breast and Ovarian Cancer (HBOC) syndrome are caused by certain mutations in *BRCA1* or *BRCA2*. Individuals with HBOC syndrome may also have an increased risk of developing other types of cancer, including melanoma, pancreatic and prostate cancers (Yoshida, 2020).
The most commonly known ovarian cancer (OC) susceptibility genes, which include (Song et al., 2014).
Genetic alterations are also observed at a high frequency in groups that are or were geographically or culturally isolated, in which one or more of the ancestors harbored the altered gene. This is often called the founder effect or founder variant. Founder mutations of *BRCA1/2* have been widely reported in different regions and ethnic groups. However, genetic testing for *BRCA1/2* should include uniform sequence analysis along with deletion/duplication analysis. *BRCA1/2* is the most frequent cause of high penetrance among HBOCs and affects all ethnic groups and races (Yoshida, 2020).
Under the assumption that other genes contribute risk to these cancers, research focused on identifying additional major risk genes (Alenezi et al., 2020). Nearly all known HBOC susceptibility genes encode tumor suppressors that participate in genome stability pathways (Yoshida, 2020). They have been predominantly associated with an increased risk of breast cancer (*CDH1, TP53, PALB2, PTEN, STK11, ATM, CHEK2*), or ovarian cancer (*BRIP1, RAD51C, RAD51D, MLH1, MSH2*) (Marmolejo et al., 2021).
The *BRCA1* gene encodes a multi-domain protein that functions in a number of cellular pathways to maintain genomic stability, including cell cycle checkpoint activation as well as transcriptional regulation and apoptosis. *BRCA1* and *BRCA2* are also important for DNA repair, specifically in homologous recombination of double-strand DNA breaks. *BRCA1* and *BRCA2* mutations confer a very high life-time risk of breast cancer (Wendt and Margolin, 2019).
One of the most intensively studied tumor suppressors genes is tumor protein 53 (*TP53*) due to the loss of wild-type *TP53* activity is frequently detected in several tumor types. The protein regulates the cell cycle, interacts in DNA repair, apoptosis, cellular senescence and metabolism. The majority of alterations in this gene are missense variants in the DNA-binding domain. In addition, missense variants have been associated with earlier onset, compared to truncating variants, due to a dominant negative effect that interferes with the function of wild-type p53 (Wendt and Margolin, 2019).
Variants in *BARD1* could encode an aberrant protein affecting the interaction with *BRCA1* and predispose to BC and/or OC, especially in hereditary cancer syndrome families not accounted for by *BRCA1* or *BRCA2* (Alenezi et al., 2020).

1.5.3. Pharmacogenetics of cancer chemotherapy-induced toxicity

Pharmacogenetics refers to the genetic variation that is inherited and is responsible for drug response. This is important in defining the course of treatment and/or dosage of treatment given to patients. The alterations in loci responsible for drug response can predict the different responses of patients to each drug and its dosage and prevent serious complications, such as, toxicity (Lunenburg et al., 2019).

DPYD gene

The gene that encodes this enzyme is the *DPYD* gene, dihydropyrimidine dehydrogenase gene, that has 23 exons and located on chromosome 1. This gene is well characterized in the Caucasian population, but in the non-Caucasian population there isn't that much information. There are hundreds of variants identified in these gene, but only a few are well characterized. These ones are the ones that are known for being pathogenic in the Caucasian population, meaning that cause a lower production of the functional enzyme, c.1905+1G>A (DPYD *2A, IVS14+1G>A, rs3918290), c.2846A>T (p.D949V, rs67376798), c.1236G>A/HapB3, (rs56038477/ rs75017182), and c.1679T>G (DPYD*13, p.I560S, rs55886062) (White et al., 2021).

Fluoropyrimidine (5-FU) is a chemotherapy used in several types of cancers, for example, colon, breast and head and neck cancers. Some patients are at risk of developing toxicity to this chemotherapy, due to the deficiency in a crucial enzyme for the degradation of pyrimidines. Toxicity from this chemotherapy can lead to hospitalization, several complications and ultimately death. This is why the genotyping of the *DPYD* gene is very important, to identify patients which will develop toxicity and adjust the dosage of 5-FU before patients develop toxicity (Maillard et al., 2021) .

There are a number of methods that can be used to detect these alterations in the *DPYP* gene, including genotyping (Vogel et al., 2020).

Even though, it would be advantageous for patients to avoid serious complications due to this treatment, it wasn't possible to implement a method that meet the standards of an ideal test. It should have high sensitivity and specificity and be rapid, cheap and non-invasive (Ciccolini, Milano and Guchelaar, 2021).

UGT1A1 gene

UGT1A1, uridine disphosphate glucuronosyl transferase 1, gene belongs to a family of at least 12 UGT encoded by the UGT1 locus on chromosome 2. The difference between the isoforms in these gene is the number of exon 1 that the transcript has, each isoform has different specificity to different substrates. The *UGT1A1* is responsible for the glucuronidation of bilirubin and is integrated in the metabolism of irinotecan (Etienne-Grimaldi et al., 2015).

The genetic alterations that result in Gilbert's syndrome are dependent on the population. By molecular analysis, it has been shown that in the Caucasian population Gilbert's syndrome is most caused by a polymorphism in the *UGT1A1* gene. Depending on the number of TA insertions in the TATA box of the promotor region, the wildtype genotype is named (6/6), the heterozygous (6/7) and the homozygous genotype (7/7) or UGT1A1*28. Therefore, patients with the wild-type genotype (6/6) are homozygous with six repeats of the TA insertion. Patients with the (7/7) genotype are homozygous with seven TA repeats, whereas the heterozygous genotype (6/7) consists of one allele with six TA repeats and of one with seven TA repeats. These alterations result in lower quantities of enzyme being produced. The genotype UGT1A1*28 (7/7) is more predominant in Caucasians, whereas UGT1A1 (6/6) is more predominant in Asians (Schulz et al., 2009).

Most of the patients with Gilbert's syndrome are asymptomatic but can have episodes of mild intermittent jaundice due to the accumulation of bilirubin. Individuals with this syndrome have a severe toxicity when being treated with irinotecan-based chemotherapy. Irinotecan-based chemotherapy is used in the treatment of several cancers such as colorectal, lung, gastric and gynecologic cancers. The evidence suggests that an adjustment of dosage according to the genotype of the patient is the most advantageous course to these patients (Takano and Sugiyama, 2017). SN38 is the active form of

irinotecan and is metabolized in the biliary rout after glucuronidation by uridine diphosphate–glucuronosyltransferase 1A1 (*UGT1A1*). When the *UGT1A1* gene is altered there is a decrease in the *UGT1A1* enzyme and the deficient degradation of SN38, leading to toxicity in these patients (Etienne-Grimaldi et al., 2015).

1.5.4. Prosigna Breast Cancer Prognostic Gene Signature Assay

The breast cancer molecular subtypes differ in genomic complexity, in key genetic alterations and also in clinical prognosis. The molecular differences appear to be associated with both the clinical outcome and the response to chemotherapy. The St Gallen International Expert Panel recommends an intrinsic subtype-based approach to support adjuvant systemic therapies (endocrine, chemotherapy and anti-HER2 therapy) in breast cancer. Several gene signatures are currently being used to predict outcomes in breast cancer. PAM50 gene signature is a multigene expression assay to quantify mRNA expression of 50 genes. PAM50 test classifies tumor samples into one of the four intrinsic subtypes (luminal A, luminal B, HER2-enriched and basal-like) and provides an accurate estimate of the risk of distant recurrence in hormone receptor positive breast cancer when analyzed with the Prosigna® algorithm (Wallden et al., 2015; Pu et al., 2019). The Prosigna assay relies in certain parameters, tumor size and the metastasized nodes and in conjunction with the expression profile it defines the risk of recurrence (Baskota et al., 2020).

The PAM50 assay is carried out in the NanoString equipment, with mRNA from FFPE tissues that is extracted with a commercial kit. The downside of this assay is that it is extremely expensive (Wallden et al., 2015). But it can compensate the cost of the assay, by giving the women the right course of treatment. The main goal was to spare the women, that didn't need chemotherapy, of that treatment and with that save money. But after the assay was tested it was proved that the contrary was also happening, this means women that weren't indicated for chemotherapy, with the result of the PAM50 were receiving a new prognosis and were indicated for chemotherapy, and this was the opposite of the goal of the assay (Jensen et al., 2018).

The IPO-Porto has very restricted criteria for patients to perform this assay, because in some cases it is not necessary because it is very clear that the patient has a high-risk tumor and it will need chemotherapy and the assay would not change the subtype.

2. Objectives

Obtain theoretical and practical knowledge of the various methodologies in the laboratory.
Get experience with the technologies used in the laboratory.
Obtain theoretical and practical experience for better adaptation to the labor market.
Obtain experience in clinical laboratory diagnosis.
Obtain experience in oncology.
Get experience in conventional and molecular cytogenetic techniques.
Get experience in molecular genetic techniques.
Obtain experience in the genetic study of neoplastic diseases.

3. Methods and materials

During my internship in IPO-Porto I learned and worked in several areas of laboratorial diagnosis, such as, hemato-oncology, focusing in conventional and molecular cytogenetics, and general molecular genetics, focusing on several techniques, such as qualitative and quantitative PCR, Sanger Sequencing and NGS.
In the beginning, I acquired theorical knowledge and observed practical laboratorial work in conventional cytogenetics. Then, I was able to perform cell cultures, cytogenetic processing of the samples and other laboratory work, including banding techniques. Using the CytoVision software I learned karyotype assembly. In molecular cytogenetics, I learned to perform the routine FISH protocols applied to hemato-oncology samples, as well as protocols specific for FFPE tissues samples from solid tumors. I was able to analyze a few cases in the fluorescence microscope.
In laboratorial molecular work, I acquired a significant experience in nucleic acids extraction, including, apart from manual extraction, automatic extraction with QIAsymphony SP/SA, commercial column kits of DNA/RNA extraction, RNA extraction with Trizol, DNA/RNA extraction from FFPE tumor tissues and extraction of ctDNA for liquid biopsies.
In hemato-oncology molecular genetics, first I observed and then performed qualitative and quantitative PCR, executing analysis for *JAK2*, *FLT3*, *BCR-ABL* genes. Also, performed MLPA assays, Fragment analysis and Sanger Sequencing.
In molecular genetics, I learned several techniques and was able to execute assays for Idylla™, cobas® and Prosigna. In NGS, I had a more observational stand, as it is a very sensitive technique, and we were working with patient samples that need the result as soon as possible.
I was able to analyze with supervision the results from all the techniques I learn about.

3.1.Strategies used for hemato-oncology

There are various strategies used in the laboratory and this is a dynamic tool that depends on several variables: the neoplasm, the patient clinical information and the impact on the response to therapy.
Samples arrive at the Genetics Service reception and are separated according to the laboratory test requested (cytogenetics and/or molecular genetics).
For conventional cytogenetics, fresh samples are usually bone marrows or, rarely, peripheral bloods. For molecular cytogenetics, apart from the same sample as for karyotype, FFPE tissues also arrives. Rarely, lymph nodes or fresh tumors are received for culture and karyotyping and/or FISH analysis.
For molecular genetics, samples usually are bone marrow and/or peripheral blood for extraction of nucleic acids (DNA and/or RNA).
For karyotyping, short-term cultures of 24h are performed in all samples, except for those that have a referral for CLL or are a CLL follow-up, that need a 72h culture. Samples for FISH usually are uncultured, it is used cultured samples only if karyotype is also requested.
Cell cultures for follow-up karyotypes are routinely done, but analysis is only performed if molecular follow-up is uninformative.
All FFPE tissues are for FISH and/or molecular studies.
For molecular genetics, samples can be for extraction of DNA if there is a referral or if it is a follow-up of CLL and MDS. Or extraction of RNA, in cases of referral and follow-up of AML, ALL and CML.

In CML cases, approximately 10 to 20mL of peripheral blood, for RNA extraction with Trizol, is needed. At diagnosis, a qualitative RT-PCR is done initially, for the identification of the transcript present in the patient, and then, a RT-qPCR is performed for the quantification of the transcript. In follow-ups, RT-qPCR for the continuing monitoring of the disease need to be performed. If the patient gains acquired resistance to the inhibitors, it is necessary to identify the mutation responsible in the kinase domain of the fusion gene *BCR-ABL1*, and this can be done with semi-nested PCR, Sanger sequencing or NGS.
In CLL cases, extraction of DNA is performed from peripheral blood in order to allow the identification of possible *TP53* gene mutations, using Sanger sequencing.
In MDS samples, extraction of DNA is performed from bone marrow. An ASO-PCR, for the identification of point mutation V617F in *JAK2*, is performed. If the result is negative, Sanger sequencing is used to identify alterations in exon 12 *JAK2*, exon 9 of *CALR* gene and exon 10 of *MPL* gene.
In samples of patients with ALL, DNA and RNA extraction is performed from blood marrow. The RNA is used for the identification of fusion genes through qualitative RT-PCR and after an RT-qPCR for quantification of the transcripts is done. The DNA is used for identification of deletions of *IKZF1*, *ETV6* and other genes.
In AML, extraction of DNA and RNA from bone marrow is performed. The RNA is for the identification and quantification of fusion genes, and the DNA is used for detection of mutations in the *FLT3* and *NPM1* genes.
In follow-ups of these last two (ALL and AML), the transcripts identified in the diagnosis are quantified, until it is not possible to detect the transcripts in the bone marrow. After that, a qualitative PCR for the transcripts identified in the diagnosis is sufficient.

3.2. Techniques used in hemato-oncology

3.2.1. Cytogenetics

Cell culture

Cell culture should be done in the day of the reception of the sample and the culture choice depends on the referral reason and the request from the physician. Short term cultures are more efficient in this type of samples, normally, 24 hours culture. The exception is CLL, that needs more time (72h) to proliferate, and it is recommended the addition of the cytokine interleukin-2 and the synthetic oligonucleotide DSP-30 (that mimics immunostimulatory properties of bacterial DNA and acts as a mitotic stimulant in B-cell malignancies) (Rack et al., 2019).

Karyotyping

Walther Flemming, in the 1890s, used the dye aniline to assess the behavior of chromosomes during mitosis, and called chromatin to the structures that he observed. In 1888, Waldeyer-Hartz used the term chromosomes. Thenceforth, the techniques were always advancing to try to arrest the cells cultured in vitro at mitosis and then try to visualize the chromosomes. With these advances, Tjio and Levan (1956) were able to correctly describe the exact number of human chromosomes as 46 (Balajee and Hande, 2018).
Hauchka (1953), Levan (1956) and Makino (1956) helped to elucidate the evolution of the cancer cells and the possible role of the karyotypic changes in neoplasms. These early studies were done in metastatic tumors, where the karyotype alterations were very significant and numerous, so it was very difficult to draw conclusions for the role of these aberrations in early stages.

The first substantial success was when Nowell and Hungerford (1960) described the Philadelphia chromosome (Ph), in the bone marrow of patients with chronic myeloid leukemia (CML). But for a while, this was the only significant discovery in human cancer cytogenetics, due to the superabundance of karyotypic aberrations in other neoplasms and it was thought that these alterations occur during tumor progression (Heim and Mitelman, 2015).

Levan (1966) and van Steenis (1966), from the analysis of the data existing in chromosomal aberrations in neoplasms, mostly in gastric, mammary, uterine and ovarian carcinomas, suggested that there was a pattern of aberrations associated. Afterwards, these patterns were demonstrated in hematologic disorders and solid tumors.

In the 1970's, with the introduction of the banding techniques and its ability to distinguish each chromosome, various alterations were described in tumors.

Initially, the identification of the Ph chromosome as a deleted 22 chromosome, the identification of the trisomy 8 in acute myeloid leukemia, monosomy 22 in meningioma and del(20q) in polycythemia. Shortly after, the balanced translocations were beginning to be reported, for example, a t(8;21)(q22;q22) in bone marrow cells of patients with acute myeloid leukemia, and the Ph chromosome as the result of a t(9;22)(q34;q11) and not a simple deletion of the chromosome 22 (Heim and Mitelman, 2015).

The banding techniques discovered in 1970's, allow the identification of the chromosomes by their pattern of different light and dark bands (Balajee and Hande, 2018; Kashork, Theisen and Shaffer, 2010). First, Capersson et al. (1970) demonstrated that using quinacrine mustard the chromosomes had visible fluorescent bands. Independently, Arrighi and Hsu (1971) and Yunis et al. (1971) developed the heat or alkali denaturation and Giemsa staining. (Finaz and Grouchy, 1972).

In G-banding, G-positive bands (G-dark bands) are AT-rich, late replicating and gene poor while G-negative bands (G-light bands) are CG-rich, early replicating and gene rich. Some laboratories prefer to use the R-banding that is the reverse of G-banding, because it stains darker the telomeric regions.

Other banding techniques were developed, such as those that stain specific chromosomes structures: constitutive heterochromatin (C-bands), telomeric regions (T-bands) and Nucleolar Organizing Regions (NORs) (ISCN, 2020).

Cytogenetic methods rely in microscopic analysis of single cells and depend on several factors to be properly executed and give reliable results. It is important to have a correct sampling collection, sufficient quantity and the sample should be representative of the neoplasia. This last requirement is important, as it is unknown which cells are analyzed, except when in combination with flow cytometry, selecting the cells of interest. Also, in cultured samples an overgrowth of normal cells instead of the neoplastic cells can occur or it may exist a selection of certain clones or even new aberrations, not present *in vivo*, may emerge. This is why in the banding techniques it is preferable to have direct or short-term cultures (Heim and Mitelman, 2015).

It was necessary to establish an internationally accepted nomenclature for the diverse techniques and its patterns, for a better understanding in the scientific community, and in 1978 the first International System for Human Cytogenetic Nomenclature (ISCN 1978) was published. Since then, the nomenclature has been regularly updated enclosing the discoveries made (ISCN 2020).

More than 1200 neoplasms with abnormalities were reported during the first ten years of the advent of the banding techniques, and more than 60 recurrent chromosomal alterations identified. Between 1980 and 1989, more than 200 solid tumors were reported and nearly 200 structural aberrations identified. The identification of specific alterations was able to create associations between cytogenetics and clinic (Heim and Mitelman, 2015).

In the late 1970s, the International Workshops on Chromosomes in Leukemia provided an information-sharing area where cytogeneticists, clinicians and pathologists could share their data and could find associations between cytogenetic alterations and clinical prognostic and diagnostic. With this work, it was also possible to understand cytogenetic data was able to categorize the patients in subgroups according to specific cytogenetic alterations.
Scientists concluded that karyotypic alterations in cancer are not evenly distributed through the genome. Different chromosomes, regions and bands have a higher propensity to be involved in rearrangements, and it is now common knowledge that specific abnormalities are associated with different neoplasms (Heim and Mitelman, 2015).
Complex karyotypes can be difficult to be interpreted due to the presence of several alterations, such as, unbalanced translocations, markers or ring chromosomes or staining regions difficult to analyze (Arceci, 2012).

Fluorescence *in situ* hybridization (FISH)

This technique allows the visualization of the nuclei acid inside the cell and can detect possible alterations while maintaining the cell morphology (Waters, Barlow and Gould, 1998). FISH was developed in the early 1980's and the applications only increased since 1990's (Hu et al., 2014; Ratan et al., 2017).
Conventional cytogenetics is a very robust technique that allows the visualization of the entire set of chromosomes, but to a certain degree of resolution. Microdeletions, microduplications, some derivative chromosomes and translocations, which are below the microscopic resolution will be missed. Another disadvantage is that the classic cytogenetics needs metaphases and for that, cells in proliferation are required.
FISH not only allows the identification of poor morphology chromosomes but is also applicable in interphase nucleus and paraffin-embedded tissues (Waters, Barlow and Gould, 1998; Ratan et al., 2017). However, the analysis of interphase nuclei can be difficult, due to for example, overlapping signals. One single signal can represent a monosomy for that chromosome or just a random overlapping of the same color signals (Rautenstrauß and Liehr, 2012).
With the advance of technology, the labelling of FISH probes has become much more stable and efficient, giving the technique certain advantages (Waters, Barlow and Gould, 1998; Ratan et al., 2017). With the higher sensitivity of FISH, it is possible to detect gene alterations that can be targeted with a certain therapy, such as the amplification of the human epidermal growth factor receptor 2, *HER2*, in breast cancer or the anaplastic lymphoma kinase, *ALK*, in lung adenocarcinoma (Hu et al., 2014; Ratan et al., 2017).
There are some disadvantages, like in any other technique, such as the requirement for specialized professional staff, it is time consuming and there are also some variations inter-laboratory and even intra-laboratory (Yoon, Do and Cho, 2013).
FISH is based on the concept of base pair complementarity, hence the sequence of interest needs to be known. The main phases of FISH are: denaturation, hybridization, detection and analysis. The first step is to prepare probes that are complementary to the targeted sequence, and label it with fluorophores (Ratan et al., 2017). Nowadays most probes are commercialized and bought from specific suppliers. After, the probe and targeted DNA need to be denatured, so the probe can hybridize with the DNA. To minimize the background fluorescence, washes for elimination of excess of probe should be done and a nuclear counterstain should be applied (Hu et al., 2014; Ratan et al., 2017).
Molecular cytogenetics can clarify some molecular mechanisms that are altered in neoplasms. It is also very useful in diagnostic and prognostic and can also provide information that allow the use of targeted therapies personalized to each patient. Imatinib

is a good example, it is a targeted therapy for a fusion gene in CML. Another example is the crizotinib for the *EML4-ALK* fusion gene (Heim and Mitelman, 2015).

3.2.2. Molecular genetics

PCR

PCR, polymerase chain reaction, is an amplification technique that uses primers to flank the segment of DNA and a DNA polymerase for amplification of the target DNA. The PCR techniques are extensively used in diagnostic techniques (Bahakeem and Qadah, 2020). This technique was discovered by Mullis in 1983, from which he was awarded a Noble Prize in 1993.

The principal of the PCR technique is that with high temperatures it is possible to denature the double strand of the DNA and then with less high temperatures the primers can hybridize with the target DNA and after with a temperature stable DNA polymerase it is possible to amplify the segment of DNA between the two primers. This process is repeated several times having an exponential number of copies being produced.

This technique has three steps: denaturation, that uses high temperatures to separate the double-stranded DNA; annealing, that is when the primers hybridize with the target DNA; and amplification, that is the phase that happens the synthetization of the new DNA strand (Singh et al., 2014).

The PCR techniques have several advantages like the amounts of DNA needed in the reaction that is reduced, there is no need of live cells and it detects a variety of abnormalities, not only at the DNA level but at the chromosomal level too. The downside is that the technique is very targeted and only looks at a specific target and not the whole picture (Bahakeem and Qadah, 2020).

There are several types of PCR reactions: qualitative PCR, Reverse transcription PCR, Real time PCR, Allele specific PCR, Nested PCR and various others.

The most used in diagnostics are qualitative, reverse transcription and real time PCRs.

Qualitative PCR has no modification to the first technique discovered, it occurs the amplification and after the PCR products are submitted to an electrophoresis, with the molecular weight of the bands it is possible to distinguish the different segments of DNA. It can be used to understand which of the variants is present in the patient (Singh et al., 2014).

Reverse transcription PCR uses RNA to create a complimentary DNA, it's called cDNA. In this reaction it is used a different enzyme, the reverse transcriptase, that was first discovered in the Rous Sarcoma Virus, this enzyme is able to do transcriptase and reverse transcriptase that is essential for this virus. With time, it was discovered several other reverse transcriptases that have other advantages, like resistance to higher temperatures. This type of PCR reaction can be done in one step or two steps, one step uses all the reagents in one single reaction, this means that the reverse transcription and the amplification of the cDNA are done at the same time. On the other hand, two-step is when the reverse transcription is done first and then in another different reaction is done the amplification of the cDNA. It is used when the product that needs to be analyzed is in RNA form, and as it is known RNA is unstable so it is performed this PCR for more stability in the reactions that are performed after. For example, in fusion genes the RNA is smaller in size and this is advantageous (Singh et al., 2014).

Real time PCR detects fluorescence in real time and makes a standard curve to measure the quantity of DNA present in the sample. This type of PCR eliminates the post-amplification step, there is no need for an extra technique like in the types before, also it has a higher sensitivity, precise measurement and analysis (Singh et al., 2014).

This type of PCR cannot be carried out in a normal thermocycler, it needs to have a system to excite the fluorochromes and a system to capture the fluorescence and, also, a system for analyzing the data (Singh et al., 2014). It is largely used in the monitoring of minimal residual disease (Mocellin et al., 2003).

MLPA

Multiplex ligation dependent probe amplification, MLPA, is a PCR based technique, where the probe is the one that is amplified instead of the target DNA. The probe has a quencher sequence that has different sizes and that allows to identify which of the sequence is being amplified. It is used only one PCR primer, thus, enabling the amplification of up to 60 MLPA probes in one single reaction, and using the same protocol for all MLPA applications.

MLPA has a high reproducibility rate so it is possible to use a standard DNA, that doesn't have alterations in the regions of the probes, and compare the signal of this DNA to the signal of the patient's DNA, and it is possible to tell if it has a deletion or duplication and if it is in homozigosity or hetorozigosity. It is also included various standard probes to be used as a control to capacitate the comparison between different samples. In cancer, only genes that have a constant expression should be used in these types of standards.

Using various probes that are located in the same gene or chromosomic region gives a more accurate result and increases the sensibility of detection alterations in that region. It is possible to use a specific enzyme that is very sensitive to alterations in the ligation site, and thus it is able to detect point mutations in those sites. Therefore, a MLPA specific to point mutations can be performed to mutations that are relevant to the diagnosis, prognosis and treatment in cancer. For example, V600E is an activating mutation in the BRAF gene that is commonly found in melanomas, tumors of the colon, thyroid and lung. This mutation accounts for about 80% of all mutations in the BRAF gene, and thus MLPA can be useful in these situations, as for genes that have a higher rate of mutations like TP53 it is not very recommended, and sequencing might be more appropriate. Though, the specific point mutations probes are very helpful, if the probes are to compete for the binding sequence, to detect mutations that are very close in proximity it becomes a problem because they can bind to each other, and the results are not reliable. If it is necessary to use such probes, it is more reliable to do so in different reactions.

MLPA has been shown to have a higher resolution in detecting partial gene deletions in ALL than FISH or even SNP-arrays, even though it is important to always have in mind the percentage of tumor cells in the sample given, before the result is given.

This technique has some downfalls, like the reference DNA, it is very sensitive to variations of salt, the concentration of DNA and contaminations with iron or heparin influence the reaction. Therefore, it is recommended to use the same extraction method for all samples and use the denaturation controls and Q-fragments controls (MLPA, MRC-Holland).

MLPA presents advantages in terms of analyzing tumor DNA, for example, in a simultaneous it can detect different types of genetic aberrations, copy number variations, methylation alterations and point mutations can also be detected, otherwise it would be needed to perform several techniques to evaluate all of these parameters. In addition, as it is only one technique being used it saves time and reagents, and also, the amount of sample needed is less than if it was two or three different analyses. As MLPA needs extracted DNA a variety of samples can be used, like blood, bone marrow, fresh-frozen tumor DNA samples and highly fragmented FFPE tumor DNA samples. It does not need intact cells like karyotype or FISH. It is also a technique that is easily upgradable only by adding new probes or replacing them (Hömig-Hölzel and Savola, 2012).

Fragment Analysis

Fragment analysis is a rapid, sensitive and specific method for detection of variants that alter the length of the DNA fragment. This technique is able to detect the allele ration and the difference in length of the ITD in the *FLT3* gene, this is important for the prognosis of the patient because the allele ratio is one of the parameters used in the decision of the treatment and the prognosis of the patient.

The ITD in the *FLT3* gene and its allelic ration are difficult to detect through next generation sequence, so the traditional method, fragment analysis is still preferable in comparison (He et al., 2019). Fragment analysis is based on a PCR reaction with primers marked with fluorochromes that flank the mutation, the amplicons detected that are bigger than the wild type amplicons and are marked with the two fluorochromes are considered positive. The disadvantage of this technique is that the wild type is amplified at a greater rate than the mutated allele during PCR, affecting the accuracy of the allele ratio. After the first step, the PCR reaction, the PCR product is submitted to a capillary electrophoresis for the detection of the sizes of the PCR products (Kim et al., 2015).

Sanger Sequencing

The advent of Sanger Sequencing was in 1975, when Fred Sanger introduced his method of sequencing denominated “plus and minus”. After this, the sequencing methods only moved forward with the improvement of the Sanger sequencing and even more advanced methods of sequencing. In 1977, Sanger published an improvement of his first sequencing method (Hutchison, 2007).

The principle of the Sanger sequencing remains the same, by halting the elongation with a labeled, and thereby identifiable, dideoxynucleotides triphosphate (ddNTP), the length of the fragment can be utilized to identity of the terminating base. The ddNTPs lack the 3’ hydroxyl group that is needed for the extension of DNA chains, due to that, the amplification of that amplicon is stopped and forms a shorter fragment than the one being amplified.

In its current form, fluorescently labeled ddNTPs are mixed with regular, non-labeled, non-terminating nucleotides in a cycle sequencing reaction, elongation stops at all positions in the amplicon. After which, it is done a capillary electrophoresis for separating sequences by length and identification of the terminating base. Initially at a high cost, refinements and automation have improved cost effectiveness significantly (Pettersson et al., 2009).

3.3. Techniques used in solid neoplasms

3.3.1. Cytogenetics – FISH

***HER2* gene**

HER2, human epidermal growth factor 2, is a proto-oncogene that codes for a cell receptor and is involved in cell division and survival (Chrzanowska, Kowalewski and Lewandowska, 2020). *HER2* amplification is associated with a bad prognosis, a higher percentage of recurrence and lower response to therapies, as well as a larger tumor size. This gene is a biomarker for targeted therapy with a monoclonal antibody that has a high clinical effectiveness (Wesoła and Jeleń, 2015; Agersborg et al., 2018).

The *HER2* gene is located on the long arm of chromosome 17, and gene amplification and protein overexpression are not always correlated.

FISH is a very sensitive and specific tool to evaluate *HER2* gene amplification in breast cancer (Wesoła and Jeleń, 2015) particularly in immunohistochemical equivocal cases, where FISH is mandatory (Agersborg et al., 2018).
The IPO-Porto uses FISH when the result of the immunohistochemistry gives an equivocal result.

MDM2 gene

The *MDM2*, murine double minute 2, gene is located in chromosome 12, encoding an E3 ubiquitin ligase that has the ability of suppressing the expression of the *PT53* gene, and in consequence inhibits apoptosis and increases cell survival. The status of the *MDM2* gene can provide, not only a diagnosis, but also a prognosis for the patient (Mardekian et al., 2015; Suster et al., 2019).
FISH is very useful in cases where morphological and immunohistochemistry are very similar, and it is not possible to distinguish different lipomatous tumors. The amplification of the *MDM2* gene is present in atypical lipomatous tumors and well differentiated liposarcomas and FISH is a technique that is very helpful in the differential diagnosis (Asif et al., 2018; Sarwar et al., 2021).

3.3.2. Molecular genetics

Idylla™

Idylla™, Biocartis, is a fully automated system that makes molecular testing easy and fast. It eliminates the time of deparaffinization and extraction, doing it all in the cartridge. It helps in giving results in a shorter time for therapies to start as soon as possible, this time is crucial for patients that start targeted therapy. It is a PCR based technique and has high sensitivity, it is highly standardized and has a design for contamination control. And has a sample versatility that can be used in solid and liquid biopsies.
Idylla™, Biocartis, has different mutation tests, *EGFR* mutation test, *BRAF* mutation test, *KRAS* mutation test, *NRAS-BRAF* mutation test, gene fusion test and MSI test.
In the IPO-Porto it is used the *BRAF*, *KRAS* and *NRAS-BRAF* mutation tests, in biopsies from patients with lung cancer, for a shorter time of turnaround. This way the patients can start the therapy as soon as possible and have a better chance of responding to therapy.
The *BRAF* mutation test detects the alterations V600E/E2/D and V600K/R/M mutations in codon 600 of the BRAF gene.
The *KRAS* mutation test detects 21 alterations in codons 12, 13, 59, 61, 117 and 146 of the *KRAS* gene.
The *NRAS-BRAF* mutation test detection of 18 mutations in codons 12, 13, 59, 61, 117, 146 of the *NRAS* gene and 5 mutations in codon 600 of the *BRAF* gene (© 2022 Biocartis).

cobas® *EGFR/PIK3CA* test

The cobas® *EGFR* test is a real time PCR that detects 42 mutations in exons 18, 19, 20 and 21 of the epidermal growth factor receptor (*EGFR*) gene, and includes the T790M, in non-small cell lung cancer (NSCLC) patients. It has a fast turnaround time of results and it is possible to use both FFPE tissue as plasma specimens. Provides consistent, objective and reproducible results validated by clinical studies. The test is indicated as a companion diagnostic to aid in selecting NSCLC patients for treatment with *EGFR* tyrosine kinase inhibitors.
The cobas® *PIK3CA* test is used to identify patients with advanced or metastatic breast cancer whose tumors harbor phosphoinositide 3-kinase, catalytic, alpha subunit

(*PIK3CA*) mutations. It identifies 17 mutations in exons 2, 5, 8, 10, and 21 in the gene *PIK3CA* in DNA isolation from FFPE tissue.

NGS

Next generation sequencing, NGS, is a high-throughput, massively parallel sequencing technology that allows the rapid and precise sequencing of multiple genes, whole exomes and whole genomes (Heikamp and Pui, 2018; Leisch et al., 2019).

NGS has been widely implemented in cancer diagnosis and thus it has also been a helpful tool in better understanding cancer genomic variations, including point mutations, insertions and deletions, translocations, microsatellite instability, tumor mutation burden and copy number variations (Wakai et al., 2018).

Even though, NGS has evolved greatly, it is still acceptable that the variations found with this technique are confirmed with other techniques, such as, Sanger sequencing, MLPA and others (Beck, Mullikin and Biesecker, 2016). Most false-positive variants are caused by NGS technical errors due to regional and site-specific chemical and physical factors during library preparation and sequencing. One solution for this problem is to filter out sequencing artifacts by attachment of a unique molecular identifier (UMI) to each DNA molecule during library preparation, followed by bioinformatic identification of each replica from the same fragment through the UMIs. If one error only appears in the replicas from the same fragment, all have the same UMI, it can be discarded and considered an error (Willey et al., 2021).

As the number of gene-based therapies and the evidence supporting their increased efficacy grows, there is a demand for techniques that enable the identification of accurate biomarkers. Biomarkers range from variants in individual genes (BRAF V600E, EGFR) as well as others that take into account the broader mutational landscape of a tumor (Zhao et al., 2020). Identifying genetic biomarkers in tumor tissue can help in deciding the treatment needed and personalize each treatment according to the genetic profile of the tumor. In some patients it is not possible to perform a biopsy, therefore, there is no tumor tissue available for testing. NGS of plasma ctDNA can be useful in these cases, to detect the genetic biomarkers, without being invasive for the patient. Various studies have proven that plasma NGS has high sensitivity and a high concordance with tissue results, particularly in patients with non-small-cell lung cancer (Christopoulos et al., 2021; Schouten et al., 2021).

There are implemented, in IPO-Porto, the NGS panels TruSight™ Hereditary Cancer Panel, TruSight™ Oncology 500 (TSO500) and AVENIO ctDNA and Tumor Tissue Expanded Kit for FFPE tissues and liquid biopsies. It is used several databases and softwares to help with the annotation, identification and description of the variants, such as, OncoKB, ClinVar, NCBI and PierianDx.

TruSight™ Hereditary Cancer Panel is a next-generation sequencing assay that includes 113 genes related to cancer predisposition, 125 SNPs.

TruSight Oncology 500 is a next-generation sequencing (NGS) assay that enables in-house comprehensive genomic profiling of tumor samples. It supports identification of all relevant DNA and RNA variants implicated in various solid tumor types. In addition, it accurately measures key current immuno-oncology biomarkers: microsatellite instability (MSI) and tumor mutational burden (TMB) (© 2022 Illumina).

The AVENIO ctDNA and Tumor Tissue Expanded Kit is a NGS assay with a 77 gene panel containing genes in U.S. National Comprehensive Cancer Network (NCCN) Guidelines and emerging cancer biomarkers. The Expanded Kit is a pan-cancer assay that's specially optimized for lung cancer and colorectal cancer (© 2022 Roche Molecular Systems, Inc.).

4. Results

4.1. Hemato-oncology

There are 9 clinical cases in this chapter of hemato-oncology. All the cases were chosen to have an example of every scenario and technique that I accompanied during my stay in this area.

Clinical case 1

A bone marrow sample from a male individual arrived at the laboratory. Initially the patient's records were consulted in order to obtain the clinical information. This case was a follow-up after aloBMT (allogenic bone marrow transplant) due to a diagnosis of AML. Two cultures of 24h were set-up: one with MMax (MarrowMax, Gibco) without synchronization and one with RPMI (RPMI-1640, Gibco) synchronized with MTX (Methotrexate). After 24 hours, the cultures were processed, according to standard protocols. From each culture, two slides were made. G banding was performed after an enzymatic treatment and Leishman stain. The slides were inserted in the Automated CytoVision Leica Biosystem for photographic capture and then analyzed.
The bone marrow donor was a female, so it was expected that only female cells would be present.

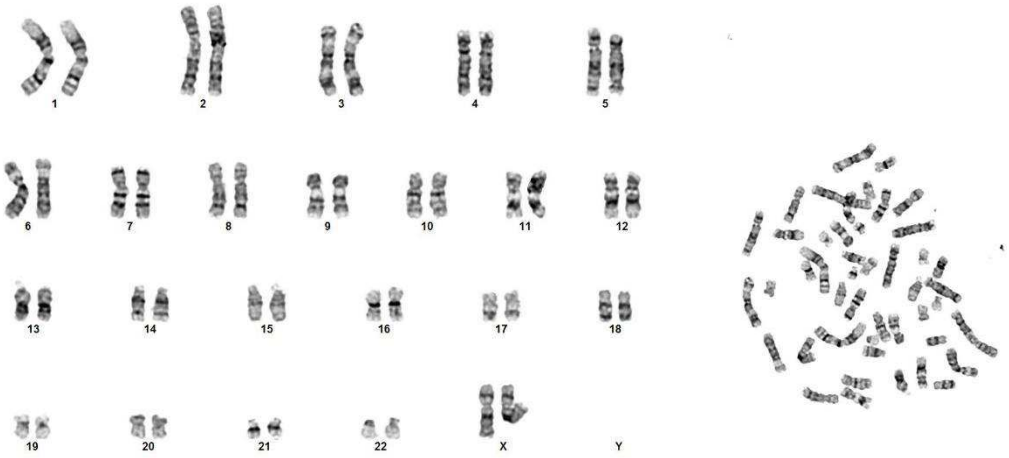


Figure 8. Metaphase and Karyogram of aloBMT, with female donor.

Result: 46,XX[20].
Interpretation: All twenty metaphases analyzed were female, reflecting the normal karyotype of the Bone Marrow donor. In Figure 8, it is possible to observe the karyogram of one of the cells present in this sample. The report mentioned that it was not detect any alterations with this technique.

Clinical case 2

A bone marrow sample from a male individual arrived at the laboratory. Initially the patient's records were consulted in order to obtain the clinical information. This case was a referral of CLL. One culture of 72h was set-up with DSP-30 and IL-2. After 72 hours, the cultures were processed, according to standard protocols. Two slides were made. G banding was performed after an enzymatic treatment and Leishman stain. The slides were inserted in the Automated CytoVision Leica Biosystem for photographic capture and then analyzed.

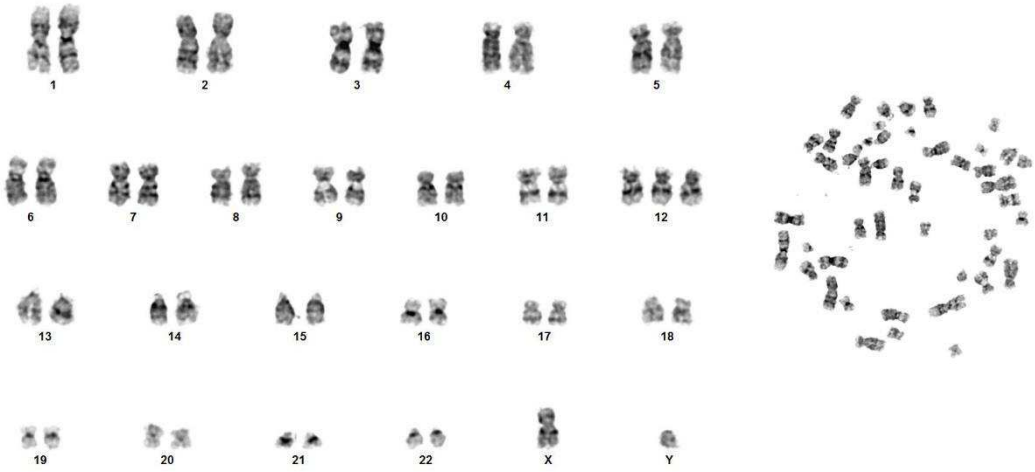


Figure 9. Metaphase and Karyogram, with trisomy of chromosome 12.

Result: 47,XY,+12[19]/46,XY[1].
Interpretation: Nineteen out of twenty cells analyzed presented trisomy 12 and the remaining cell had a normal karyotype. In Figure 9, it is possible to observe the karyogram of one of the cells present in this sample. Trisomy 12 is one of the most common cytogenetic alterations in CLL.

As requested, FISH for *IGH*, 11/*ATM*, Chr12, 13qter/*DLEU1*, 17/*TP53* (Kreatech, Leica Biosystems) was performed. This is the panel usually done in CLL suspicions.
The *IGH* probe is a Dual Color Break-Apart, which marks the 5' of the gene in green and the 3' in red (Figure 10). If the gene is intact two fusion signals will be seen. If it is rearranged, one red, one green and a fusion signal will be observed. After the assay, it was possible to evaluate the integrity of the *IGH* gene, concluding that there was no rearrangement in this gene (Figure 11).
Result: nuc ish(3'IGH,5'IGH)x2(3'IGH con 5'IGHx2)[100]

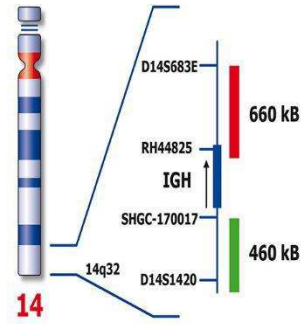


Figure 10. IVD IGH Break probe. (Source: Kreatech, Leica Biosystems).

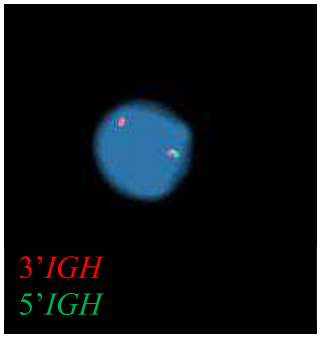


Figure 11. Cell with two fusion signals for IGH.

The probe for 11/ATM consists of two different probes, one that marks the *ATM* gene in red, and the other that labels the centromeric region of the chromosome 11 in green (Figure 12).
After the analysis, it was assessed the integrity of the *ATM* gene, and the conclusion was that there were no abnormalities detected in the *ATM* gene (Figure 13).
Result: nuc ish (D11Z1, ATM)x2[100]

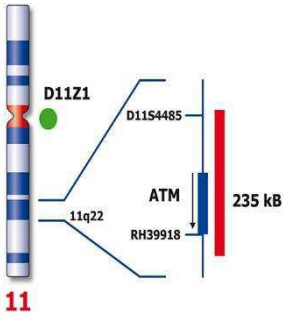


Figure 12. IVD-ATM-SE11 probe. (Source: Kreatech, Leica Biosystems)

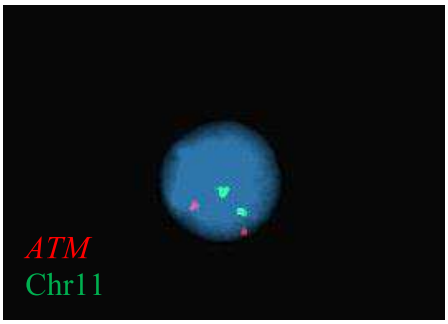


Figure 13. Cell with two signals for each probe.

The assay to evaluate the presence of numerical abnormalities of chromosome 12 uses two different probes, one that marks the centromeric region of the chromosome 6 in red (as a control), and the centromeric region of the chromosome 12 in green.
After the analysis, it was possible to say that there were, in fact, three chromosomes 12, confirming the result given with the karyotype (Figure 14).
Result: nuc ish(D6Z1x2,D12Z3x3)[60/100]

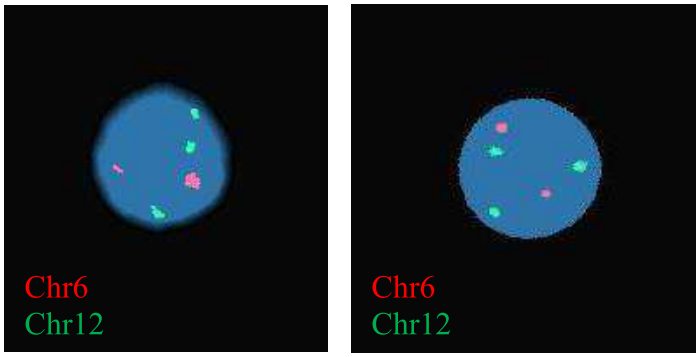


Figure 14. Cells with two signals, in red, for chromosome 6 and three signals, in green, for chromosome 12.

The probe for 13qter/*DLEU1* consists in two different probes, one that marks the *DLEU1* gene in red, and the terminal region of the long arm of the chromosome 13, 13qter, in green (Figure 15).
After the analysis, it was possible to say that no abnormalities were detected in the 13qter region (Figure 16).
Result: nuc ish (DLEU1, 13qter)x2[100]

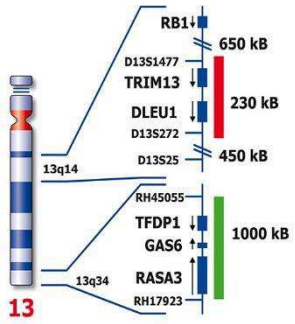


Figure 15. IVD-DLEU1-13qter probe. (Source: Kreatech, Leica Biosystems)

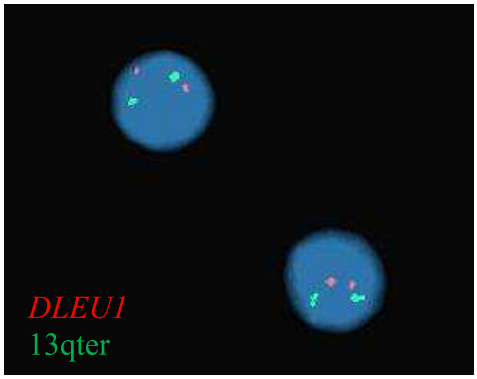


Figure 16. Cells with two signals, in red, for the DLEU1 gene and two signals, in green, for 13qter.

The probe for 17/TP53 consists in two different probes, one that marks the *TP53* gene in red, and the centromeric region of the chromosome 17 in green (Figure 17).
After the analysis, it was possible to say that there was a deletion of one copy of the *TP53* gene, in 40% of the nuclei observed (Figure 18).
Result: nuc ish(D17Z1x2,TP53x1)[40/100]

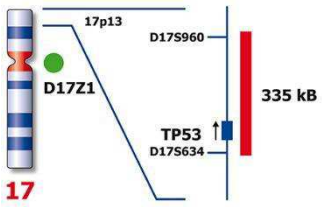


Figure 17. IVD-TP53-17 probe. (Source: Kreatech, Leica Biosystems)

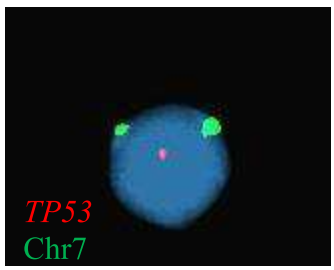


Figure 18. Cell with two signals, in green, for chromosome 17 and one signal, in red, for TP53 gene.

With the use of several probes in different critical genomic sites it was possible to reach a more accurate diagnosis and complete the patient's genomic data for diagnosis and prognosis.
Interpretation: The cytogenetics report mentioned the presence of Trisomy 12. And the FISH report mentioned Trisomy 12 and loss of one of the copies of the *TP53* gene.

Clinical case 3

One sample of bone marrow from a male individual arrived at the laboratory. Initially the patient's records were consulted to obtain the clinical information. This case was a referral of mantle lymphoma. One 72h culture with DSP-30 and IL-2 was set-up. After 72 hours, the cultures were processed according to standard protocols. Two slides were made and G banding was performed after an enzymatic treatment and Leishman staining. The case was inserted in the Automated CytoVision Leica Biosystem and then analyzed.

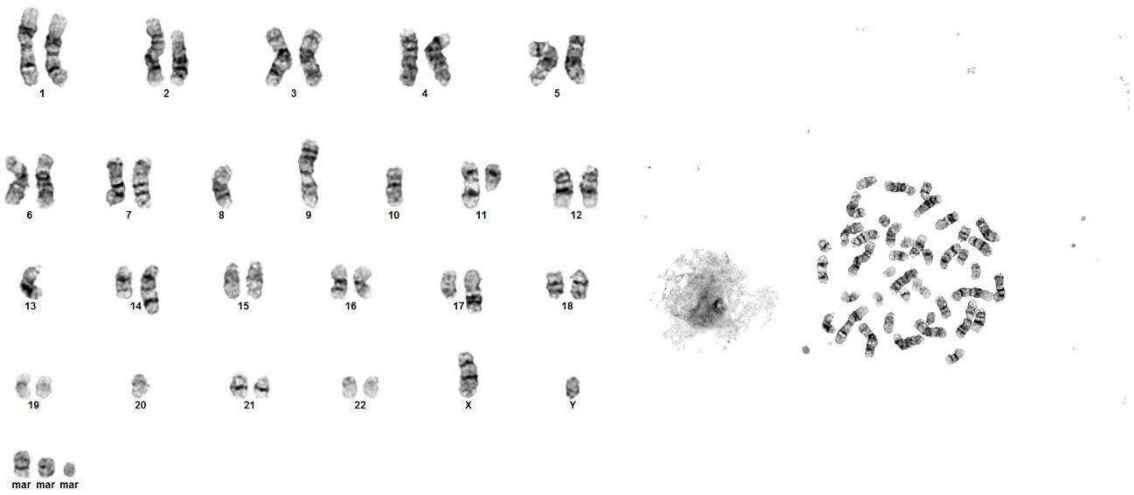


Figure 19. Metaphase and Complex Karyogram of a referral of mantle lymphoma.

Result: 44-47,XY,add(2)(p11.2),-8,-9,der(9)t(8;9)(q13;p12),-10,t(11;14)(q13;q32), del(13)(q12;q31),-16,add(17)(q25),+mar1,+mar2,+mar3[cp15]/46,XY[5]
Interpretation: Fifteen out of twenty cells analyzed presented various aberrations, showing karyotypic heterogeneity. The cells share several cytogenetic abnormalities but subclones cannot be distinguished. In Figure 19, it is possible to observe the karyogram of one of the cells present in this sample. Some of the aberrations in this karyotype are monosomy 8, monosomy 9, monosomy 16 and the typical mantle lymphoma translocation t(11;14)(q13;q32). The remaining five cells presented a normal male karyotype.
This translocation affects the *CCND1* and *IGH* genes, which is compatible with the diagnosis of mantle lymphoma.

Clinical case 4

One sample of bone marrow from a male individual arrived at the laboratory. Initially the patient's records were consulted in order to obtain the clinical information. This case was a CLL follow-up. One 72h culture with DSP-30 and IL-2 was set-up. After 72 hours, the cultures were processed according to standard protocols. Two slides were made and G banding was performed after an enzymatic treatment and Leishman staining. The case was inserted in the Automated CytoVision Leica Biosystem and then analyzed.

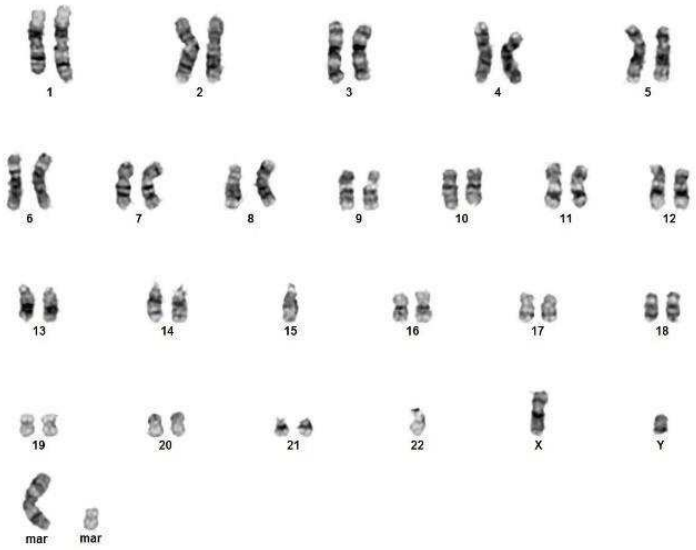


Figure 20. Complex Karyogram of a LLC follow-up.

Result: 46,XY,add(8)(p21),del(9)(p13),del(10)(q24q26),-15,-22,+mar1,+mar2[cp20]
Interpretation: The twenty cells analyzed presented various aberrations, showing karyotypic heterogeneity. The cells share several cytogenetic abnormalities but subclones cannot be distinguished. In Figure 20, it is possible to observe the karyogram of one of the cells present in this sample. Some of the aberrations in this karyotype are monosomy 15, monosomy 22 and a 9p13 deletion. This result is similar to the one at the moment of diagnosis, confirming the presence of the neoplasia.

Clinical case 5

One sample of peripheric blood arrived at the laboratory, with referral of pediatric ALL. RNA was extracted and quantified. After, it was done a qualitative PCR to identify if there were any translocations from the ALL-pediatric panel in the sample. The ALL-pediatric panel uses primers to identify the translocations t(1/19)(q23;p13) (*TCF3-PBX1*), t(4/11)(q21;q23) (*KMT2A/AFF1*), t(12/21)(p13;q22) (*ETV6-RUNX1*) and t(9/22)(q34;q11) (*BCR-ABL1*).
Results: In Figure 21, it is possible to observe the picture of the electrophoresis run of this sample.

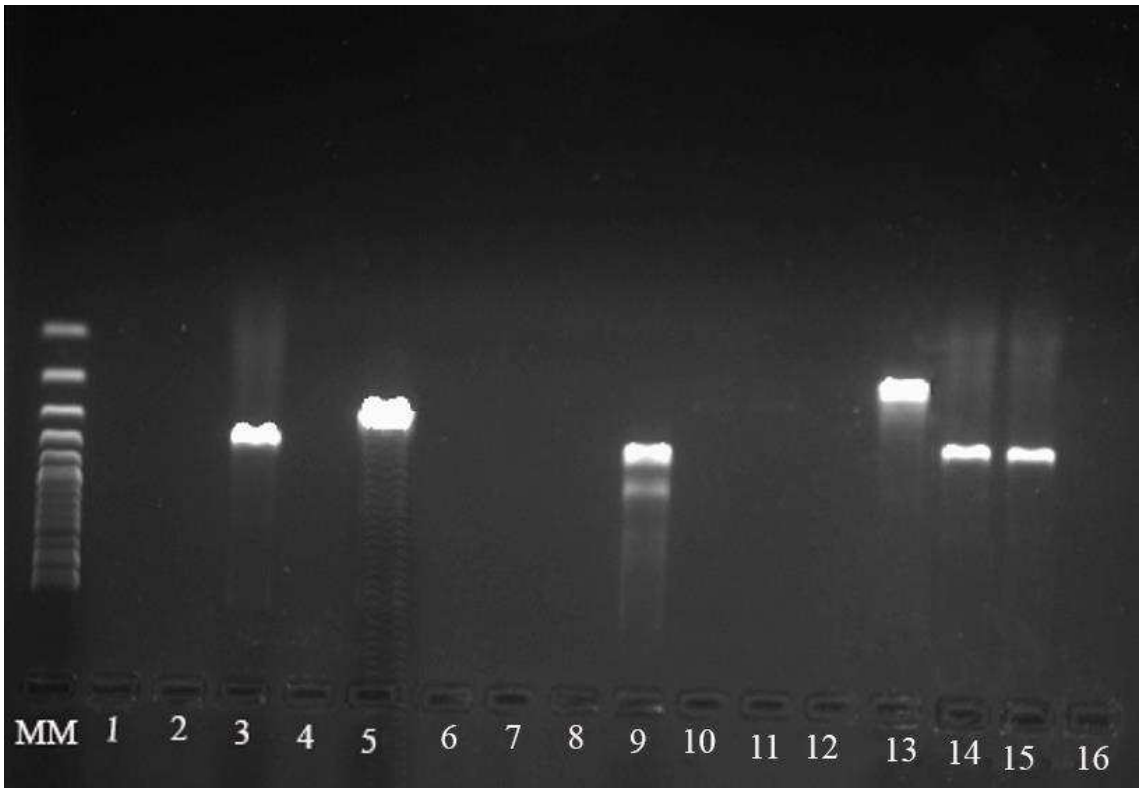


Figure 21. Electrophoresis of a qualitative PCR, from case 5. MM- molecular marker; 1-Sample 1; 2-Replica 1; 3- housekeeping gene β2; 4- NTC; 5- Control positive for 1/19; 6- Sample 1; 7- Replica 1; 8-NTC; 9- Control positive for 4/11; 10- Sample 1; 11- Replica 1; 12- NTC; 13- Control positive for 12/21; 14- Sample 1; 15- Replica 1; 16- NTC for 9/22.

Interpretation: The transcripts derived from the translocation t(1/19)(q23;p13) (*TCF3-PBX1*), t(4/11)(q21;q23) (*KMT2A/AFF1*), t(12/21)(p13;q22) (*ETV6-RUNX1*) and t(9/22)(q34;q11) (*BCR-ABL1*) were not detected. The report was given referring that no alterations were identified with this technique in the patient.

Clinical case 6

One sample of peripheric blood arrived at the laboratory, with referral of ALL. DNA was extracted from the sample, with QIAsymphony SP/AS equipment and quantified. It was performed a MLPA reaction, with a panel of genes usually screened for ALL cases.
Results: In Figure 22, it is possible to observe the graphics of the MLPA reaction. And, in Figure 23, it is possible to observe that all probes and controls are within the limit of 0.8-1.2, meaning that the result of this reaction is negative.

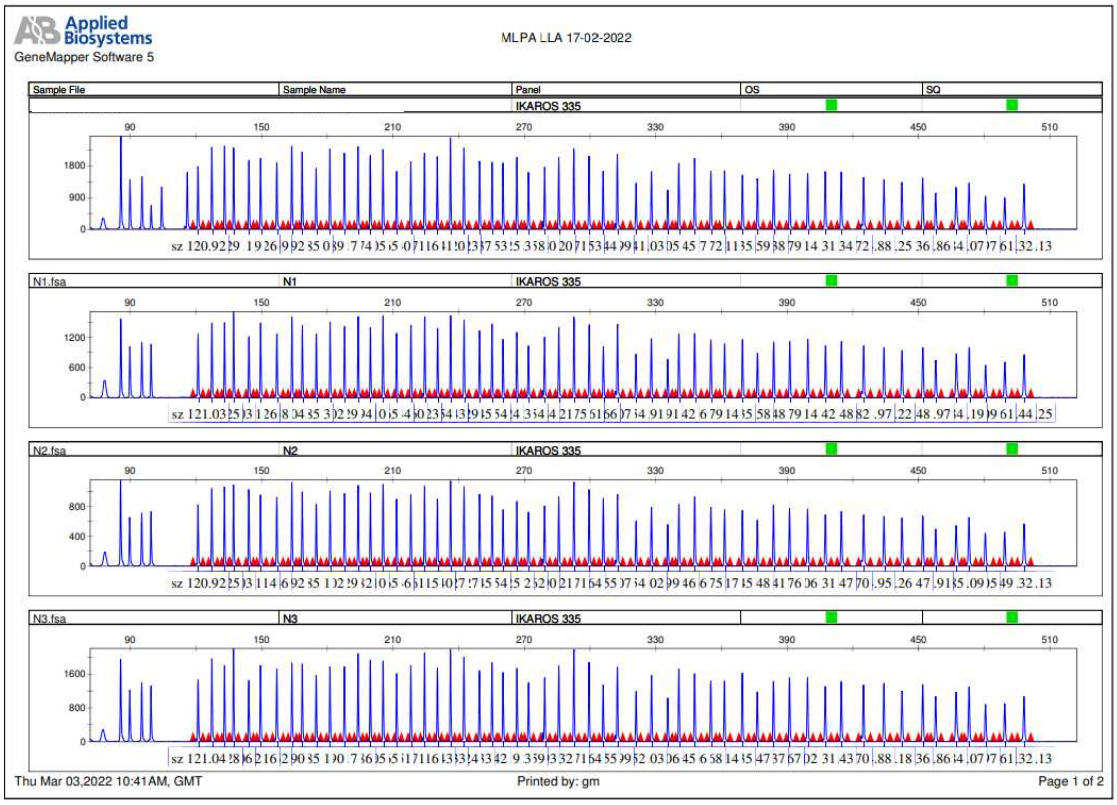


Figure 22. Graphical displays of a MLPA reaction, from case 6.

All probes were within the limits of 0.8-1.2 meaning that this case is negative.

Interpretation: The report was given referring that no deletions were found in the gene panel performed.

Clinical case 7

One sample of peripheric blood arrived at the laboratory, with referral of myeloproliferative syndrome. DNA was extracted from the sample, with QIAsymphony SP/AS equipment and quantified. This case was for Sanger sequencing of the *MPL* gene. Before preparing the sample for sequencing, an electrophoresis is run in order to confirm that genetic material was correctly amplified.
Results:

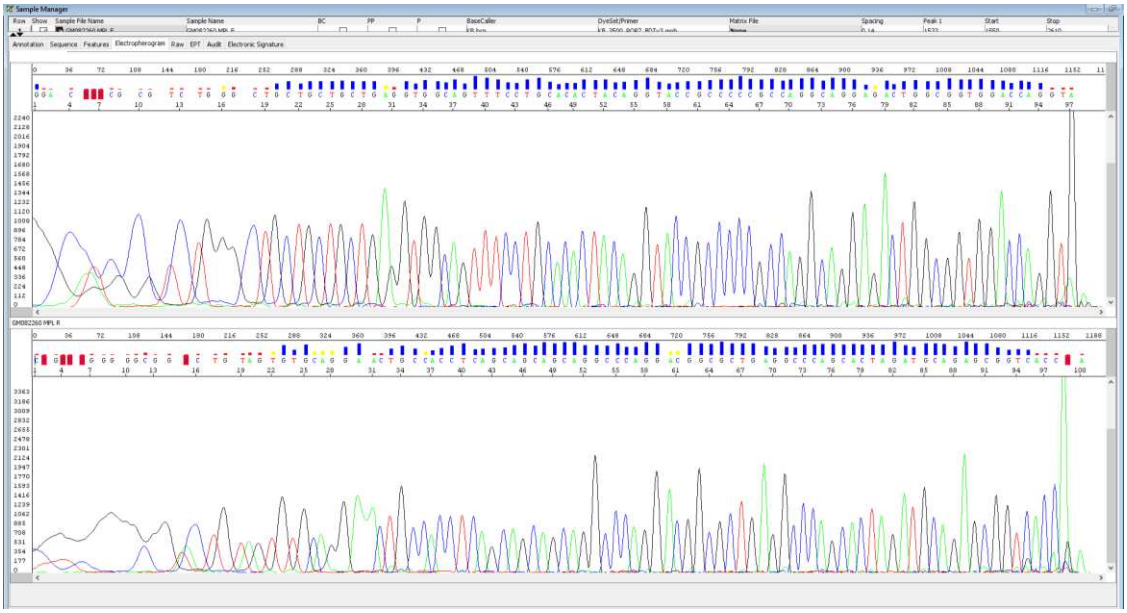


Figure 23. Sanger Sequencing of the MPL gene.

Interpretation: In Figure 23, it is possible to analyze the electropherogram of the sequencing and it is, also, possible to verify that no alterations were detected. The report was given according to this result.

Clinical case 8

A case of referral of AML arrived at the laboratory and the DNA extracted was used for detection of alterations in the *FLT3* gene, through fragment analysis. The samples are analyzed in duplicate, in this case both duplicates are consistent with each other.
Results: In the Figure 24, it is possible to identify a second pique in the two replicas of the sample. And it is confirmed, in Figure 25, where the sizes of the fragments are presented. The second pique is approximately 407bp, which means that the allele has extra duplications in relation to the WT allele, while the WT allele is in approximately 327bp.

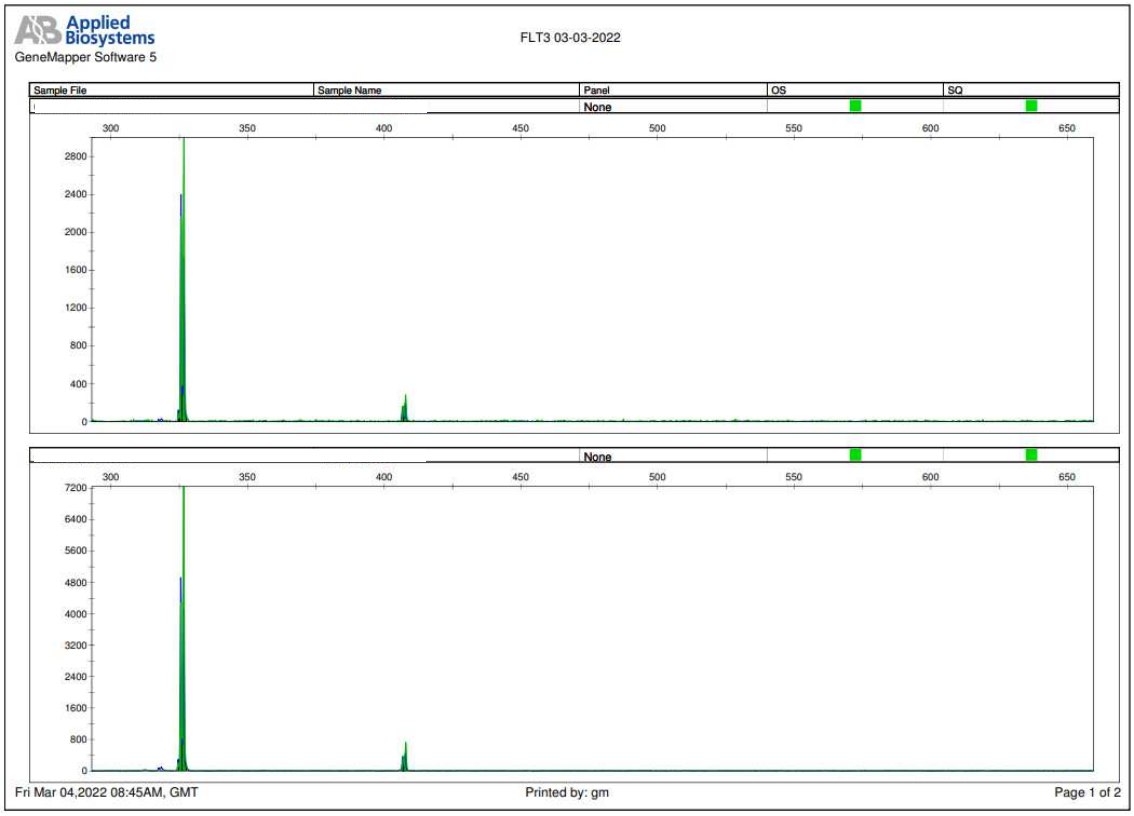


Figure 24. Graphical displays of the fragment analysis of FLT3 ITD.

Applied Biosystems
GeneMapper Software 5
FLT3 03-03-2022

		Dye/Sample Peak	Sample File Name	Marker	Size	Height	Area	Data Point
1		B,9	ITD REP.fsa		325.63	2403	11271	3823
2		B,10	ITD REP.fsa		326.64	1738	8727	3832
3		B,11	ITD REP.fsa		406.68	164	883	4541
4		B,12	ITD REP.fsa		407.7	194	1078	4550
5		G,10	ITD REP.fsa		325.63	2167	10305	3823
6		G,11	ITD REP.fsa		326.64	3003	15795	3832
7		G,12	ITD REP.fsa		406.79	169	898	4542
8		G,13	ITD REP.fsa		407.82	285	1723	4551
9		B,23	ITD.fsa		325.54	4930	23270	3830
10		B,24	ITD.fsa		326.56	4133	20437	3839
11		B,25	ITD.fsa		406.68	370	1962	4545
12		B,26	ITD.fsa		407.71	456	2412	4554
13		G,17	ITD.fsa		325.65	4295	20005	3831
14		G,18	ITD.fsa		326.56	7245	40451	3839
15		G,19	ITD.fsa		406.91	381	1886	4547
16		G,20	ITD.fsa		407.82	732	4396	4555

Figure 25. Size of the fragments from the Figure above.

Interpretation: This case was reported as having a *FLT3* ITD alteration.

Clinical case 9

A case with referral AML arrived at the laboratory and the DNA extracted was used for detection of alterations in the *FLT3* gene, through fragment analysis.
Results: It is possible to identify, in Figure 26, that the sample has a second pique in the one hundred, that is due to an enzymatic restriction that recognizes the site of the point mutation. And it is confirmed, in Figure 27, where the size of the fragments are presented. The second pique is approximately 127bp, which indicates that there was no cleavage done by the enzyme, while the wild type allele is in approximately 79bp.

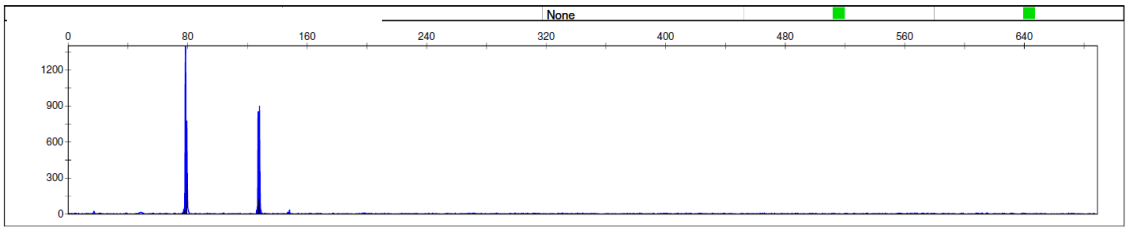


Figure 26. Graphical displays of the fragment analysis of FLT3 point mutation.

Applied Biosystems
GeneMapper Software 5

FLT3 17-02-2022

		Dye/Sample Peak	Sample File Name	Marker	Size	Height	Area	Data Point
1		B,1	DD.fsa		78.52	2522	12259	1620
2		B,2	DD_.fsa		78.52	1401	7714	1635
3		B,3	DD_.fsa		127.2	856	3463	2038
4		B,4	DD_.fsa		128.13	902	4373	2046
5		B,1	DD.fsa		78.53	3189	16552	1669

Figure 27. Size of the fragments from the Figure above.

Interpretation: This case was reported as having a *FLT3* point mutation.

4.2. Solid tumors

There are 6 clinical cases in this chapter of solid tumors. All the cases were chosen to have an example of scenarios and techniques that I accompanied during my stay in this area.

Clinical case 1

A formalin-fixed paraffin-embedded slide arrived with a suspect of a well-differentiated liposarcoma. Amplification of *MDM2* gene was requested. The probe for MDM2/12 marks the *MDM2* gene in red, and the centromeric region of the chromosome 12 in green (Figure 28).
Result: nuc ish(D12Z3x2,MDM2 amp)[100]
Interpretation: In Figure 29, it is possible to observe a capture of the slide, which demonstrates the amplification of the gene. *MDM2* amplification was reported.

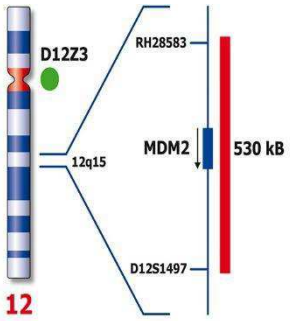


Figure 28. IVD-MDM2-12 probe. (Source: Kreatech, Leica Biosystems)

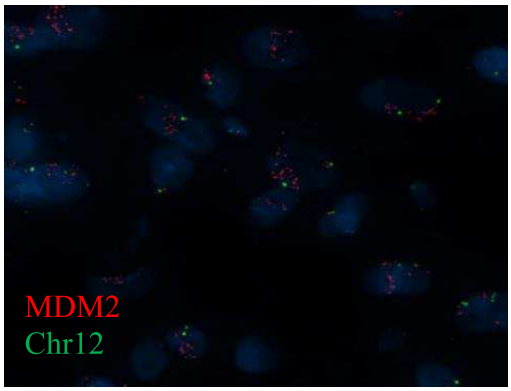


Figure 29. Cells with two signals for chromosome 12 and several signals for MDM2 gene.

Clinical case 2

Amplification of *ERBB2* gene in a formalin-fixed paraffin-embedded slide of a breast cancer case was requested. The probe for ERBB2/17 marks the *ERBB2* gene in red, and the centromeric region of the chromosome 17 in green (Figure 30).
Result: nuc ish (D17Z1x2,ERBB2 amp)[100].
Interpretation: In Figure 31, it is possible to observe a capture of the slide, which identifies the amplification of the gene. *ERBB2* amplification was reported.

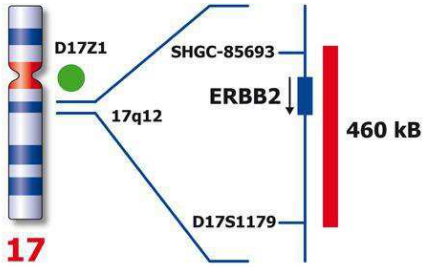


Figure 30. IVD-ERBB2-17 probe. (Source: Kreatech, Leica Biosystems)

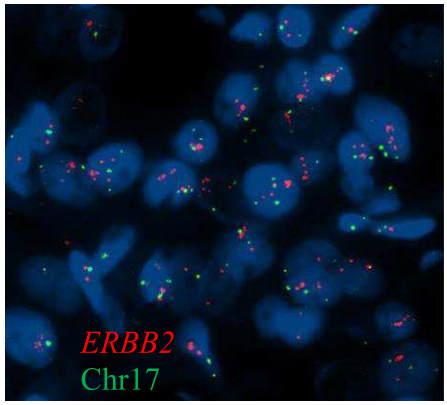


Figure 31. Cells with two signals for chromosome 17 and several signals for ERRB2

Clinical case 3

A formalin-fixed paraffin-embedded slide arrived at the laboratory with a request for PAM50. The woman had 54 years-old with a diagnosis of invasive ductal carcinoma grade 2, it was HER2 negative, estrogen receptors at 90% and progesterone receptors at 70%. It was done a tumorectomy plus analysis of the sentinel node, tumor had 2.4cm and Ki67<15 and the analysis of the nodes revealed a metastasized node (N1), giving the classification of Luminal A-like. With this classification the patient would only be treated with hormonotherapy.

From enriched tumor areas (>10% tumor cells), 1 to 6 sections of 10 µm were obtained for RNA extraction and purification. It was conducted using the High Pure FFPET RNA Isolation Kit (Roche Life Science) according to NanoString Technologies guidelines. Optical density of total RNA was measured at 260 and 280 nm to determine yield and purity using a low-volume spectrophotometer (Nanodrop, Thermo Scientific). RNA sample passed quality control if the measured concentration was ≥ 12.5 ng/µL and the A260/280 ratio was 1.7-2.5. Gene expression profiling was performed on a nCounter Analysis System using the PAM50 test according to the manufacturer's instructions. Data were interpreted from the report generated by Nanostring Technologies.

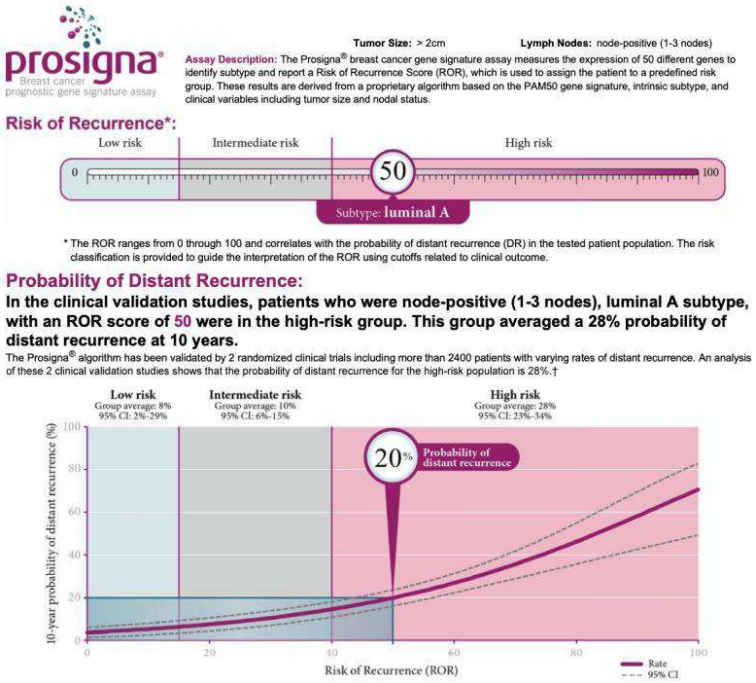


Figure 32. Report from the Prosigna-PAM50 assay

Interpretation: The assay gave a classification of luminal A of high risk and a probability of 20% of recurrence in the next 10 years if the patient doesn't do any type of therapy (Figure 32). With this result the patient has criteria for receiving hormonotherapy and chemotherapy. In this case, the subtype classification didn't change but the therapy to be offered to the patient has changed.

Clinical case 4

A formalin-fixed paraffin-embedded slide arrived at the laboratory with a request for PAM50. The woman had 54 years-old with a diagnosis of invasive ductal carcinoma grade 1, it was HER2 negative, estrogen receptors at 100% and progesterone receptors at 20%. It was done a tumorectomy plus analysis of the sentinel node, tumor had 2.1cm and Ki67=20 and the analysis of the ganglion revealed a metastasized node (N1), giving the classification of Luminal B-like. With this classification the patient would be treated with hormonotherapy and chemotherapy.
From enriched tumor areas (>10% tumor cells), 1 to 6 sections of 10 µm were obtained for RNA extraction and purification. It was conducted using the High Pure FFPET RNA Isolation Kit (Roche Life Science) according to NanoString Technologies guidelines. Optical density of total RNA was measured at 260 and 280 nm to determine yield and purity using a low-volume spectrophotometer (Nanodrop, Thermo Scientific). RNA sample passed quality control if the measured concentration was ≥ 12.5 ng/µL and the A260/280 ratio was 1.7-2.5. Gene expression profiling was performed on a nCounter Analysis System using the PAM50 test according to the manufacturer's instructions. Data were interpreted from the report generated by Nanostring Technologies.

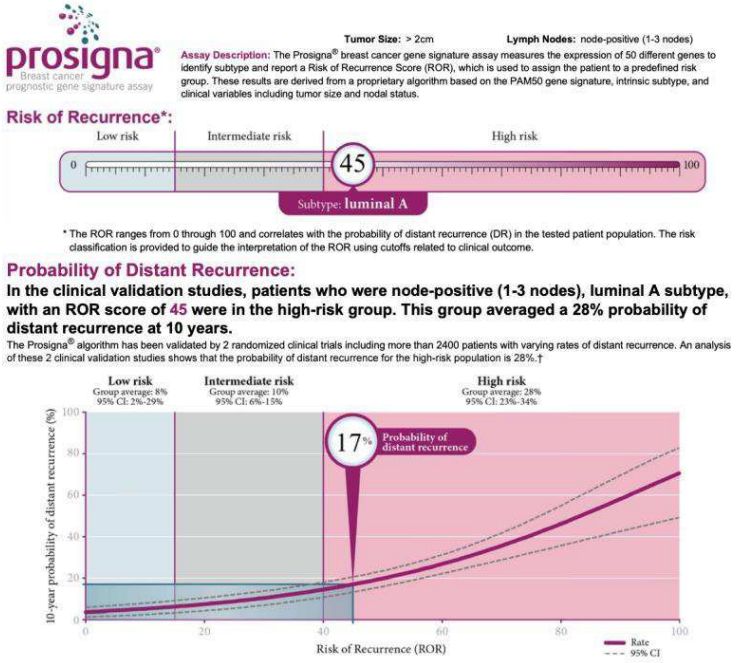


Figure 33. Report from the Prosigna-PAM50 assay

Interpretation: The assay gave a classification of luminal A of high risk and a probability of 17% of recurrence in the next 10 years if the patient doesn't do any type of therapy (Figure 33). With this result the patient has criteria for receiving hormonotherapy and chemotherapy. In this case, the subtype classification changed but the adjuvant treatment is maintained.

Clinical case 5

A 36-year-old man with colorectal cancer (Lynch syndrome) was referred to genetic testing. The patient has family history of colon cancer. In addition, the patient had loss of expression of *MSH2* and *MSH6* genes in the immunohistochemistry analysis. The doctor's request was for *MSH2* and *MSH6* gene analysis. DNA was extracted from peripheral blood with QIAsymphony SP/AS equipment and quantified.
Result: The analysis was done by NGS (TruSight colon panel) and confirmed that he had a pathogenic variant in the *MSH2* gene, c.2152C>T. In Figure 34, it is possible to observe the filtered variants and highlighted in red is the pathogenic variant. In Figure 35, it is possible to observe the IGV program, which shows the alteration in the sequencing and the coverage of the location of the variant.

##Variants of Sample

#Chr:ChrPos	Gene	Exon	Transcript	HGVSCoding	HGVSProtein	Coverage	VAF	FP	TP	SP	FG	TG	SG	Rs	1KG	GnExomes	GnomAD	ClinVar	ClinVar_CIP
2:47703652	MSH2	13	NM_000251.2	c.2152C>T	p.Gln718Ter	909	0,45	0,0006	1	1581	0,0010	4	3893	rs587779139	N/A	N/A	N/A	P	N/A
7:6013013	PMS2	15	NM_000535.7	c.*17G>C		210	0,12	0,0025	4	1581	0,0010	4	3893	rs556089649	0,0002	0,0000	0,0005	B	N/A
3:37067101	MLH1	intron	NM_000249.3	c.1039-27T>A		72	0,18	0,0487	77	1581	0,0216	84	3893	rs9862158	0,2081	0,0026	0,0073	B	N/A
2:48030838	MSH6	intron	NM_000179.3	c.3438+14A>T		303	0,51	0,0677	107	1581	0,0275	107	3893	rs2020911	0,4010	0,4118	0,3212	B	N/A
3:37083740	MLH1	intron	NM_000249.4	c.1668-19A>G		361	0,38	0,0708	112	1581	0,0288	112	3893	rs9876116	0,3123	0,3708	0,3995	B	N/A
7:6038722	PMS2	intron	NM_000535.7	c.705+17A>G		472	0,52	0,0816	129	1581	0,0331	129	3893	rs62456182	0,3257	0,3682	0,3403	B	N/A
2:47641560	MSH2	intron	NM_000251.2	c.942+24_942+29delAAAAAA		149	0,22	0,0980	155	1581	0,1360	529	3893	rs747112683	N/A	N/A	N/A	CIP	B(2),LB(1),VUS(1)
7:6781309	PMS2CL	intron				402	0,13	0,1080	171	1581	0,0878	342	3893	rs1240546128	N/A	N/A	0,0001	N/A	N/A
7:6045627	PMS2	2	NM_000535.5	c.59G>A	p.Arg20Gln	703	0,44	0,1180	186	1581	0,0902	351	3893	rs10254120	0,0757	0,0716	0,0804	B	N/A
7:6022629	PMS2	intron	NM_000535.5	c.2007-7C>T		272	0,31	0,1250	198	1581	0,0956	372	3893	rs55954143	0,1280	0,0758	0,1225	B	N/A
7:6786731	PMS2CL	5				797	0,29	0,1250	197	1581	0,0989	385	3893	rs111706670	0,0723	N/A	0,0927	N/A	N/A
7:6781312	PMS2CL	intron				410	0,15	0,2540	401	1581	0,1990	774	3893	rs1349514814	N/A	N/A	0,0000	N/A	N/A
7:6022626	PMS2	intron	NM_000535.5	c.2007-4G>A		296	0,20	0,2820	446	1581	0,2140	834	3893	rs1805326	0,1627	0,1608	0,1283	B	N/A
2:48018081	MSH6	2	NM_000179.2	c.276A>G	p.Pro92=	518	0,38	0,3450	546	1581	0,2670	1040	3893	rs1800932	0,0867	0,1347	0,1331	B	N/A
2:48010558	MSH6	1	NM_000179.2	c.186C>A	p.Arg62=	756	0,47	0,3470	548	1581	0,2600	1011	3893	rs1042820	0,0691	0,1444	0,1158	B	N/A
2:48010488	MSH6	1	NM_000179.2	c.116G>A	p.Gly39Glu	747	0,48	0,3570	565	1581	0,2700	1051	3893	rs1042821	0,2009	0,1820	0,1893	B	N/A
2:48023115	MSH6	3	NM_000179.2	c.540T>C	p.Asp180=	434	0,46	0,4900	775	1581	0,3770	1469	3893	rs1800935	0,1352	0,2130	0,2303	B	N/A
3:37053568	MLH1	8	NM_000249.3	c.655A>G	p.Ile219Val	533	0,44	0,5060	800	1581	0,4050	1577	3893	rs1799977	0,1296	0,2328	0,2301	B	N/A
7:6790900	PMS2CL	6				625	0,46	0,5480	867	1581	0,4190	1630	3893	rs200453156	N/A	N/A	0,3315	N/A	N/A
7:6013153	PMS2	15	NM_000535.5	c.2466T>C	p.Leu822=	574	0,45	0,5490	868	1581	0,4190	1630	3893	rs10000	0,0627	0,1104	0,1127	B	N/A
7:6026988	PMS2	11	NM_000535.5	c.1408C>T	p.Pro470Ser	915	0,50	0,6740	1066	1581	0,5160	2010	3893	rs1805321	0,3582	0,3878	0,3716	B	N/A
7:6037058	PMS2	intron	NM_000535.5	c.706-5_706-4delTT		339	0,42	0,6750	1067	1581	0,5350	2083	3893	rs776641246	0,0917	0,1021	0,0141	B/LB	N/A
2:48033891	MSH6	intron	NM_000179.2	c.4002-11_4002-10delTT		368	0,27	0,7910	1251	1581	0,6250	2432	3893	rs1491083972	N/A	N/A	0,0062	CIP	B(1),LB(1),VUS(1)
2:48033891	MSH6	intron	NM_000179.2	c.4002-10delT		368	0,46	0,8940	1413	1581	0,7070	2752	3893	rs1316556030	N/A	N/A	0,2911	LB	N/A
2:48032741	MSH6	intron	NM_000179.2	c.3557-4dupT		478	0,55	0,9080	1435	1581	0,7160	2787	3893	rs267608104	N/A	0,2966	0,1545	B	N/A
2:47635524	MSH2	intron	NM_000251.2	c.212-4dupT		605	0,14	0,9550	1510	1581	0,7460	2903	3893	rs757515274	N/A	0,2036	0,0085	B	N/A
7:6037058	PMS2	intron	NM_000535.5	c.706-4delT		337	0,29	0,9670	1529	1581	0,7260	2827	3893	rs60794673	0,4531	0,4308	0,4478	B/LB	N/A
7:6036980	PMS2	7	NM_000535.5	c.780C>G	p.Ser260=	414	0,99	0,9680	1531	1581	0,7430	2893	3893	rs1805319	0,8313	0,8018	0,8206	B	N/A
7:6026775	PMS2	11	NM_000535.5	c.1621A>G	p.Lys541Glu	591	1,00	0,9800	1550	1581	0,7530	2932	3893	rs2228006	0,8832	0,8408	0,8724	B	N/A
2:47635524	MSH2	intron	NM_000251.2	c.212-4delT		605	0,18	0,9940	1571	1581	0,7660	2983	3893	rs1485021746	N/A	0,1488	0,0075	B/LB	N/A

Figure 34. Filtered variants of the NGS data. Highlighted in red is the pathogenic variant in MSH2 gene.

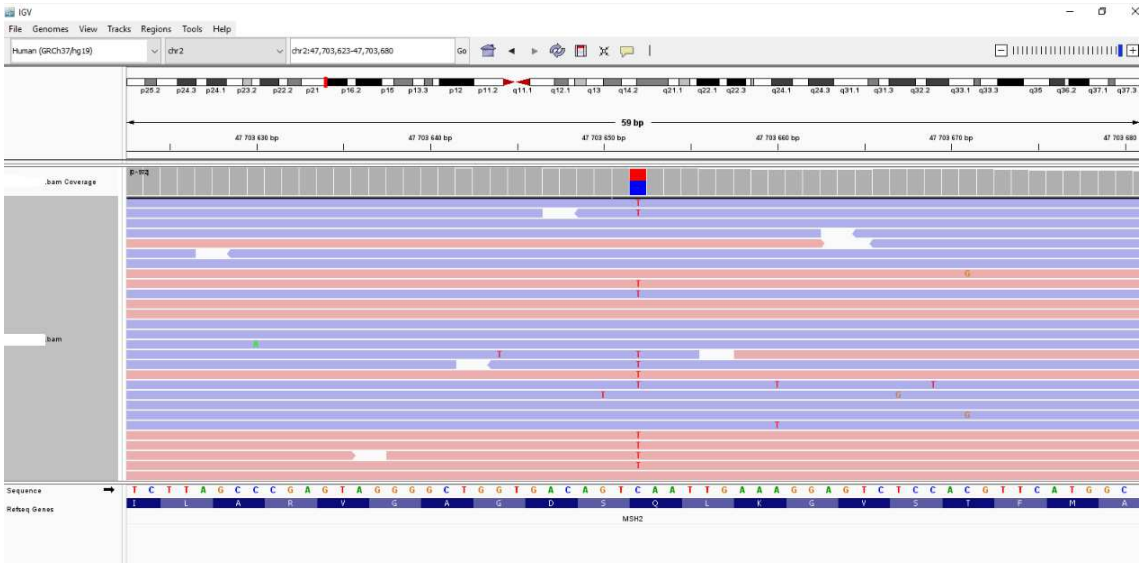


Figure 35. IGV view of the pathogenic variant in the MSH2 gene.

The pathogenic variant was confirmed with Sanger sequencing (Figure 36).
Interpretation: The report was given as positive for *MSH2* gene, c.2152C>T.

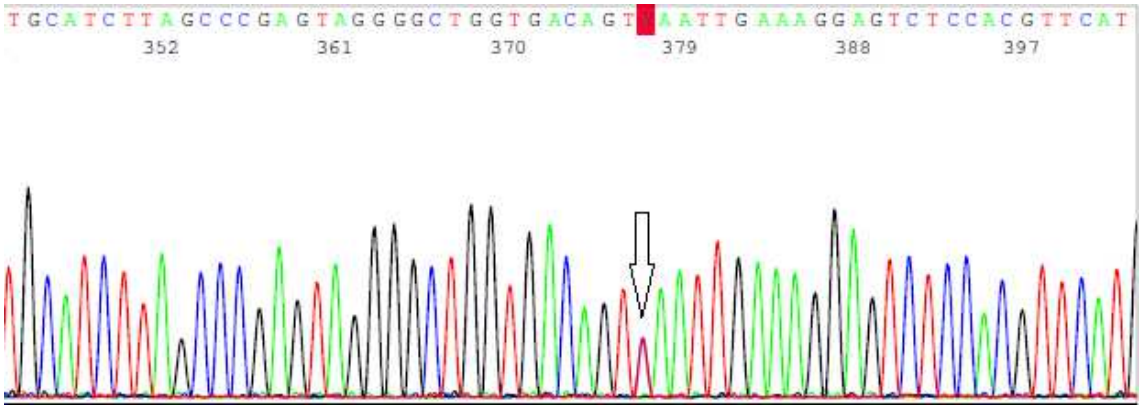


Figure 36. Sanger sequencing of the pathogenic variant in the MSH2 gene.

Clinical case 6

A 40-year-old woman with breast cancer was referred to genetic testing. The patient has no family history of relevance but had the first diagnosis at 38-years. Perhaps, the lack of family history of breast cancer is due to the family being mainly man. The doctor's request was for breast cancer gene panel.
Result: The analysis was done by NGS (TruSight breast panel) and confirmed that he had a pathogenic variant in the *BRCA2* gene, c.156_157insALU. It is possible to analyze, in Figure 3, the variant in the NGS data.

Sample				Dispersão Média	0,0012		
Control(s)							

Comparison Type:Multiple Controls - Average	100

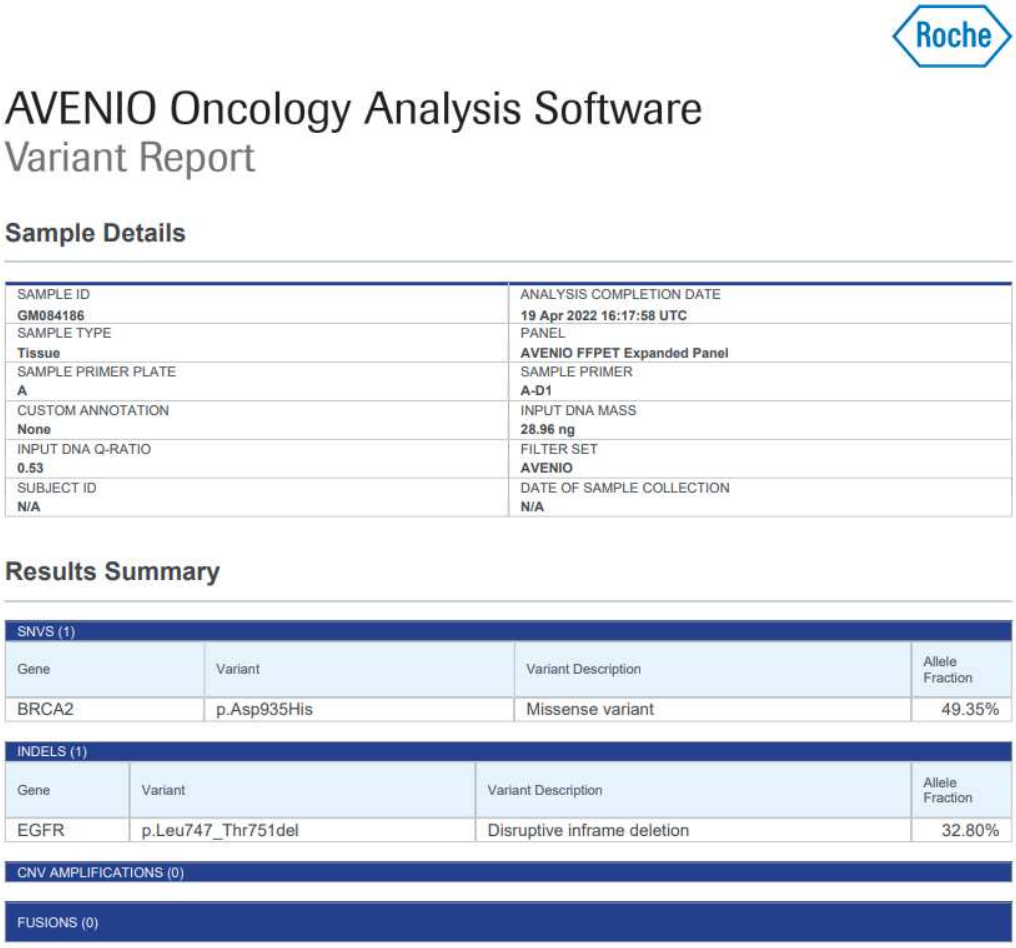
Gene	Exon	Ratio	Deletion Score	Normal Score	Duplication Score	HMM Calls	Normalized Read Counts(Sample;Control)
BRCA2	1	0,4943	0	80	0	Normal	1253,000;1282,000
BRCA2	2	0,4929	0	80	0	Normal	901,000;927,000
BRCA2	3	0,43	0	42,12	0	Normal	998,000;1323,000
BRCA2	Alu1	0,3841	28,5	0,01	0	Deletion	295,000;473,000
BRCA2	Alu2	0,3789	14,76	0,15	0	Deletion	158,000;259,000
BRCA2	4	0,4896	0	80	0	Normal	825,000;860,000
BRCA2	5	0,5045	0	67,48	0	Normal	849,000;834,000
BRCA2	6	0,5055	0	72,25	0	Normal	876,000;857,000
BRCA2	7	0,4981	0	80	0	Normal	1032,000;1040,000
BRCA2	8	0,499	0	37,23	0	Normal	490,000;492,000
BRCA2	9	0,4964	0	80	0	Normal	825,000;837,000
BRCA2	10	0,4969	0	80	0	Normal	5351,000;5417,000
BRCA2	10.1	0,4825	0	80	0	Normal	1754,000;1881,000
BRCA2	10.2	0,5044	0	80	0	Normal	3594,000;3531,000
BRCA2	11	0,4971	0	80	0	Normal	21375,000;21623,000
BRCA2	11.1	0,485	0	80	0	Normal	1821,000;1934,000
BRCA2	11.2	0,4941	0	80	0	Normal	2020,000;2068,000
BRCA2	11.3	0,504	0	80	0	Normal	2284,000;2248,000
BRCA2	11.4	0,5033	0	80	0	Normal	2375,000;2344,000
BRCA2	11.5	0,4967	0	80	0	Normal	1890,000;1915,000
BRCA2	11.6	0,4951	0	80	0	Normal	2063,000;2104,000
BRCA2	11.7	0,5061	0	80	0	Normal	2105,000;2054,000
BRCA2	11.8	0,4977	0	80	0	Normal	2287,000;2308,000
BRCA2	11.9	0,489	0	80	0	Normal	2246,000;2347,000
BRCA2	11.1	0,4989	0	80	0	Normal	2238,000;2248,000
BRCA2	12	0,4823	0	69,24	0	Normal	614,000;659,000
BRCA2	13	0,5187	0	33,69	0	Normal	680,000;631,000
BRCA2	14	0,5031	0	80	0	Normal	2543,000;2512,000
BRCA2	15	0,4891	0	80	0	Normal	1437,000;1501,000
BRCA2	16	0,4965	0	80	0	Normal	1278,000;1296,000
BRCA2	17	0,5	0	80	0	Normal	1327,000;1327,000
BRCA2	18	0,503	0	80	0	Normal	2155,000;2129,000
BRCA2	19	0,4936	0	80	0	Normal	1305,000;1339,000
BRCA2	20	0,5071	0	80	0	Normal	1254,000;1219,000
BRCA2	21	0,5051	0	80	0	Normal	1190,000;1166,000
BRCA2	22	0,4851	0	80	0	Normal	1517,000;1610,000
BRCA2	23	0,5096	0	69,24	0	Normal	1467,000;1412,000
BRCA2	24	0,5013	0	80	0	Normal	1186,000;1180,000
BRCA2	25	0,4895	0	80	0	Normal	1615,000;1684,000
BRCA2	26	0,507	0	80	0	Normal	1439,000;1399,000
BRCA2	27	0,5039	0	80	0	Normal	3412,000;3359,000
BRCA2	3UTR	0,5028	0	80	0	Normal	3619,000;3578,000

Figure 37. CNV analysis. Highlighted in red is the Alu insertion.

Interpretation: The report stated that it was found a pathogenic variant in the *BRCA2* gene, c.156_157insALU.

Clinical case 7

A 40-year-old woman with lung cancer was referred to genetic testing. The analysis was done by the AVENIO FFPE Expanded panel for lung cancer. DNA was extracted from FFPE tissue with macrodissection of the tumor area and extracted with a silica column kit and quantified.



AVENIO Oncology Analysis Software
Variant Report

Sample Details

SAMPLE ID **GM084186**	ANALYSIS COMPLETION DATE **19 Apr 2022 16:17:58 UTC**
SAMPLE TYPE **Tissue**	PANEL **AVENIO FFPET Expanded Panel**
SAMPLE PRIMER PLATE **A**	SAMPLE PRIMER **A-D1**
CUSTOM ANNOTATION **None**	INPUT DNA MASS **28.96 ng**
INPUT DNA Q-RATIO **0.53**	FILTER SET **AVENIO**
SUBJECT ID **N/A**	DATE OF SAMPLE COLLECTION **N/A**

Results Summary

SNVS (1)

Gene	Variant	Variant Description	Allele Fraction
BRCA2	p.Asp935His	Missense variant	49.35%

INDELS (1)

Gene	Variant	Variant Description	Allele Fraction
EGFR	p.Leu747_Thr751del	Disruptive inframe deletion	32.80%

CNV AMPLIFICATIONS (0)

FUSIONS (0)

Figure 38. Summary of the variant report of the AVENIO FFPE Expanded panel.

Results: In Figure 38, it is possible to observe that variant in the *BRCA2* gene was identified. Although, it was not reported because it was described as a benign variant in the literature. And it was identified a pathogenic variant in the *EGFR* gene (Figure 39).

EGFR INDEL DESCRIPTION			
GENE	EGFR		
GENOMIC POSITION	chr7:55174774	VARIANT DEPTH / UNIQUE DEPTH	581/2463
ALLELE FRACTION	32.80%		
TRANSCRIPT	ENST00000275493.6		
CODING CHANGE	c.2240_2254delTAAGAGAAGCAACAT	AMINO ACID CHANGE	p.Leu747_Thr751del
VARIANT DESCRIPTION	Disruptive inframe deletion	EXON NUMBER OVER TOTAL EXON	19/28

Database Annotations			
Population Databases			
EXAC OVERALL FREQUENCY	Not listed	1000 GENOMES FREQUENCY	Not listed
HIGHEST SUBPOPULATION FREQUENCY IN EXAC	Not listed	HIGHEST SUBPOPULATION FREQUENCY IN 1000 GENOMES	Not listed
DBSNP ID	rs121913442	HIGHEST FREQUENCY IN DBSNP	Not listed
COSMIC			
ID	COSM12369 COSM23571		
LEADING THREE REPORTED PRIMARY TISSUES	lung (32 confirmed somatic of 98 total)		

Figure 39. Description of the Pathogenic variant in the EGFR gene.

Interpretation: The report was given with the identification of a pathogenic variant in the *EGFR* gene, c.2240_2254delTAAGAGAAGCAACAT.

4.3. Clinical case recurring to different technologies

Clinical case 1

A sample came into the laboratory with a referral of AML, for cytogenetics. In the analysis of the karyotype, an inversion of the chromosome 16 was detected (Figure 40). After that, the case was referred to molecular genetics for the identification and quantification of the transcript present in this patient.

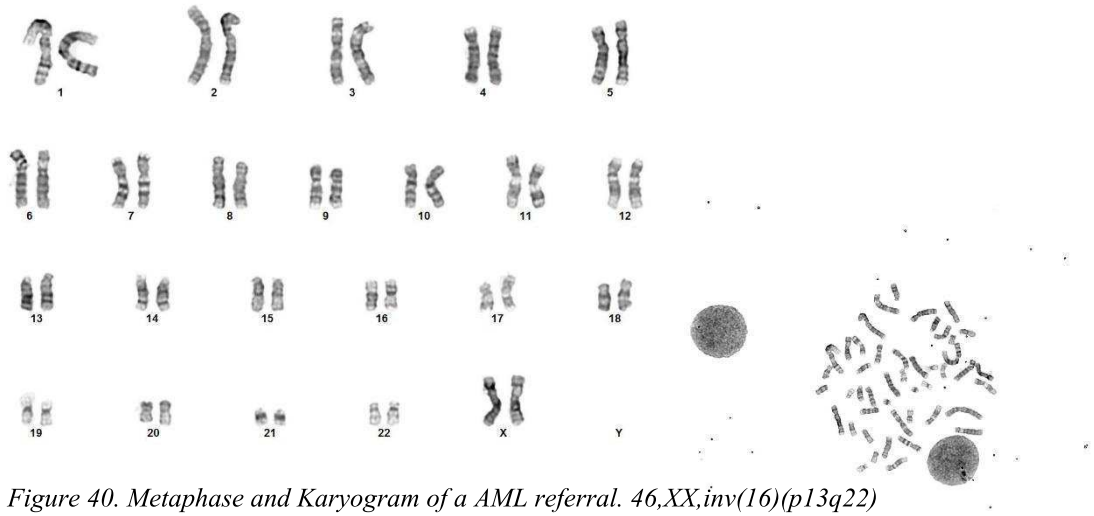


Figure 40. Metaphase and Karyogram of a AML referral. 46,XX,inv(16)(p13q22)

RNA was extracted and quantified. It was done a qualitative PCR with two sets of primers that identify different transcripts. Results: In Figure 41, it is possible to identify that the transcript present in the patient was the transcript one. It was done a RT-qPCR only for the transcript present. Although, in this specific case, the two PCRs were done almost simultaneously and therefore in the quantitative PCR it was used the two sets of primers.

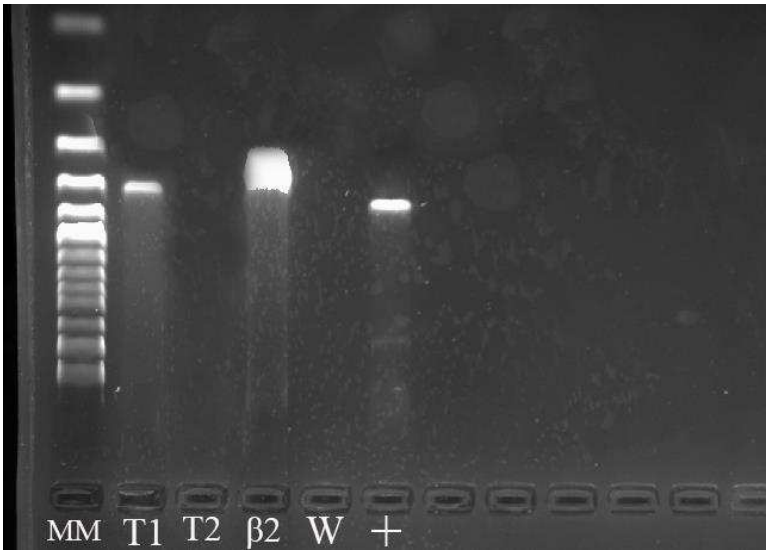


Figure 41. Electrophoresis of the qualitative PCR for identification of the transcript. MM- molecular marker; T1- transcript 1; T2- transcript 2; β2- housekeeping gene; W- NTC; +- positive.

In Figure 42, it is possible to observe the amplification curves of the samples and controls and compare it to one another. In Figure 43, it is possible to observe the screen capture from the program that analysis the data from the RT-qPCR, this gives the data of the amplification cycle threshold, Cts. It is possible to observe the amplification curves of the replicas corresponding to the first transcripts, in the cycle of 20, and the residual fluorescence due to not having amplification of the second transcripts. This is in concordance with the qualitative PCR, that detected the first transcript.
In Figure 44, it is represented a screen capture of the excel sheet that is used for the calculations of the quantity of transcript present in the sample. First, the quantities of the fusion transcript are summed as well as the housekeeping gene, second it is done a ratio of these quantities and after it is multiplied by 100. Thus, giving the quantity of transcript present in that sample.

Figure 42. Graphical displays of the quantification of the transcript.

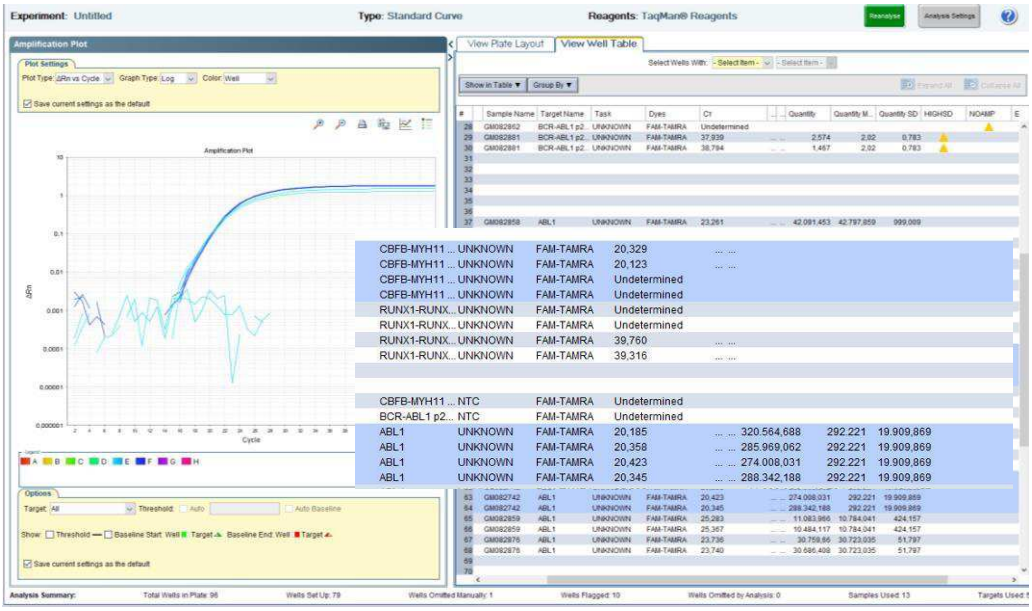


Figure 43. Display of the cycles in the RT-qPCR.

Sample Name	Sum Quantity CBFB-MYH11	Sum Quantity ABL1	CBFB-MYH11/ABL1	CBFB-MYH11/ABL1 * 100	*CF
	1017128,14	606533,75	1,6770	167,6952	167,6952

Figure 44. Treatment of the data withdrawn from the RT-qPCR.

Interpretation: The cytogenetics report of this case mentioned an inversion of the chromosome 16. The molecular genetics reported the identification of the transcript and the quantification of the same.

5. Final Considerations

The presence of genetic alterations in Oncology is a useful tool that allows to define the diagnosis, risk stratification, therapeutic individualization, definition of response and resistance to specific treatments, as well as, monitoring the therapeutic response (MRD), onco-immunological response. This type of stratification is very important in modern medicine for the best treatment of the patients and their prognosis.

The Genetics Service of the IPO-Porto is a reference laboratory at a national level. During this internship I was able to consolidate the knowledge related to the available techniques of cytogenetics and molecular genetics in the study of neoplasias, and also genetic aberrations associated with hereditary predisposition to cancer.

This internship was essential in my training, because by observing and executing the techniques that are implemented in the laboratory, I was able to acquire work experience in the laboratory and learn with complex cases, complementing theoretical training.

I was able to become autonomous in cell culture and cytogenetic processing of the samples and obtain practice in general laboratory management. I was able to become autonomous in extraction of nucleic acids, performing all the routine techniques of the service, during almost the eight months of my internship.

I was able to understand that with the evolution of these techniques, it is now possible to provide better care to the patient. I believe that it was only possible to acquire this knowledge because of the variety of cases referenced to an institution in the area of oncology, that is a national and international reference in this field.

Thus, I thank the whole team of the Genetics Service of the IPO-Porto for the warmth with which I was received, the time dedicated and availability in the transmission of knowledge throughout the internship period.

6. Bibliography

© 1999 - 2018 Roche Molecular Systems, Inc.
© 2022 Biocartis
© 2022 Illumina
© ISO, International Standard Organization
© MRC Holland
©2022 F. Hoffmann-La Roche Ltd

Agersborg, S., Mixon, C., Nguyen, T., Aithal, S., Sudarsanam, S., Blocker, F., Weiss, L., Gasparini, R., Jiang, S., Chen, W., Hess, G. and Albitar, M., 2018. Immunohistochemistry and alternative FISH testing in breast cancer with HER2 equivocal amplification. *Breast Cancer Research and Treatment*, 170(2), pp.321-328.

Alenezi, W., Fierheller, C., Recio, N. and Tonin, P., 2020. Literature Review of BARD1 as a Cancer Predisposing Gene with a Focus on Breast and Ovarian Cancers. Genes, 11(8), p.856.

Alexander, M., Kim, S. Y., and Cheng, H., 2020. Update 2020: Management of Non-Small Cell Lung Cancer. Lung, 198(6), 897–907. https://doi.org/10.1007/s00408-020-00407-5

Arceci, R., 2012. TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. *Yearbook of Oncology*, 2012, pp.150-151.

Asif, A., Mushtaq, S., Hassan, U., Akhtar, N., Hussain, M., Azam, M., and Qazi, R., 2018. Fluorescence in Situ Hybridization (FISH) for Differential Diagnosis of Soft Tissue Sarcomas. *Asian Pacific journal of cancer prevention : APJCP*, *19*(3), 655–660. https://doi.org/10.22034/APJCP.2018.19.3.655

Avery A., 2009. Molecular diagnostics of hematologic malignancies. *Top Companion Anim Med.* 24(3):144-50. doi: 10.1053/j.tcam.2009.03.005. PMID: 19732733.

Bahakeem, E., and Qadah, T., 2020. Current Diagnostic Methods for Hematological Malignancies: A Mini-Review, *Pharmacophore*, 11(3), 63-68.

Balajee, A. and Hande, M., 2018. History and evolution of cytogenetic techniques: Current and future applications in basic and clinical research. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 836, pp.3-12.

Barzaman, K., Karami, J., Zarei, Z., Hosseinzadeh, A., Kazemi, M. H., Moradi-Kalbolandi, S., Safari, E., and Farahmand, L., 2020. Breast cancer: Biology, biomarkers, and treatments. International immunopharmacology, 84, 106535. https://doi.org/10.1016/j.intimp.2020.106535

Baskota, S., Dabbs, D., Clark, B. and Bhargava, R., 2020. Prosigna® breast cancer assay: histopathologic correlation, development, and assessment of size, nodal status, Ki-67 (SiNK™) index for breast cancer prognosis. *Modern Pathology*, 34(1), pp.70-76.

Beck, T., Mullikin, J. and Biesecker, L., 2016. Systematic Evaluation of Sanger Validation of Next-Generation Sequencing Variants. Clinical Chemistry, 62(4), pp.647-654.

Branford, S., and Hughes, T., 2006. Detection of BCR-ABL mutations and resistance to imatinib mesylate. *Methods in molecular medicine*, *125*, 93–106. https://doi.org/10.1385/1-59745-017-0:93

Bridgford, J., Lee, S., Lee, C., Guglielmelli, P., Rumi, E., Pietra, D., Wilcox, S., Chhabra, Y., Rubin, A., Cazzola, M., Vannucchi, A., Brooks, A., Call, M. and Call, M., 2020. Novel drivers and modifiers of MPL-dependent oncogenic transformation identified by deep mutational scanning. *Blood*, 135(4), pp.287-292.

Bunz, F., 2018. *Principles of cancer genetics*. Springer.

Castellanos, E., Feld, E., and Horn, L., 2017. Driven by Mutations: The Predictive Value of Mutation Subtype in EGFR-Mutated Non-Small Cell Lung Cancer. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer, 12(4), 612–623. https://doi.org/10.1016/j.jtho.2016.12.014

Castilla, L., Perrat, P., Martinez, N., Landrette, S., Keys, R., Oikemus, S., Flanegan, J., Heilman, S., Garrett, L., Dutra, A., Anderson, S., Pihan, G., Wolff, L. and Liu, P., 2004. Identification of genes that synergize with Cbfb-MYH11 in the pathogenesis of acute myeloid leukemia. *Proceedings of the National Academy of Sciences*, 101(14), pp.4924-4929.

Cerveira, N., Bizarro, S., Teixeira, M. and Mariz, J., 2021. When to Stop TKIs in Patients with Chronic Myeloid Leukemia and How to Follow Them Subsequently. Current Treatment Options in Oncology, 22(6).

Cerveira, N., Lisboa, S., Correia, C., Bizarro, S., Santos, J., Torres, L., Vieira, J., Barros-Silva, J., Pereia, D., Moreira, C., Meyer, C., Oliva, T., Moreira, I., Martins, A., Viterbo, L., Costa, V., Marschalek, R., Pinto, A., Mariz, J. and Teixeira, M., 2012. Genetic and Clinical Characterization of 45 Acute Leukemia Patients with MLL Gene Rearrangements From a Single Institution. *Blood*, 120(21), pp.2477-2477.

Christopoulos, P., Dietz, S., Angeles, A., Rheinheimer, S., Kazdal, D., Volckmar, A., Janke, F., Endris, V., Meister, M., Kriegsmann, M., Zemojtel, T., Reck, M., Stenzinger, A., Thomas, M. and Sültmann, H., 2021. Earlier extracranial progression and shorter survival in ALK- rearranged lung cancer with positive liquid rebiopsies. Translational Lung Cancer Research, 10(5), pp.2118-2131.

Chrzanowska, N., Kowalewski, J. and Lewandowska, M., 2020. Use of Fluorescence In Situ Hybridization (FISH) in Diagnosis and Tailored Therapies in Solid Tumors. *Molecules*, 25(8), p.1864.

Ciccolini, J., Milano, G. and Guchelaar, H., 2021. Detecting DPD deficiency: when perfect is the enemy of good. Cancer Chemotherapy and Pharmacology, 87(5), pp.717-719.

cobas® EGFR Mutation Test v2, Roche Molecular Systems, Inc., Pleasanton, CA, USA

Daver, N., Schlenk, R., Russell, N. and Levis, M., 2019. Targeting FLT3 mutations in AML: review of current knowledge and evidence. *Leukemia*, 33(2), pp.299-312.

De Braekeleer, E., Douet-Guilbert, N., and De Braekeleer, M., 2014. RARA fusion genes in acute promyelocytic leukemia: a review. *Expert review of hematology*, *7*(3), 347–357. https://doi.org/10.1586/17474086.2014.903794

De Braekeleer, E., Douet-Guilbert, N., Morel, F., Le Bris, M., Basinko, A. and De Braekeleer, M., 2012. ETV6 fusion genes in hematological malignancies: A review. *Leukemia Research*, 36(8), pp.945-961.

De Braekeleer, E., Douet-Guilbert, N., Rowe, D., Bown, N., Morel, F., and Berthou, C., 2011. ABL1 fusion genes in hematological malignancies: a review. *European Journal Of Haematology*, *86*(5), 361-371. doi: 10.1111/j.1600-0609.2011.01586.x

Etienne-Grimaldi, M. C., Boyer, J. C., Thomas, F., Quaranta, S., Picard, N., Loriot, M. A., Narjoz, C., Poncet, D., Gagnieu, M. C., Ged, C., Broly, F., Le Morvan, V., Bouquié, R., Gaub, M. P., Philibert, L., Ghiringhelli, F., Le Guellec, C., Collective work by Groupe de Pharmacologie Clinique Oncologique (GPCO-Unicancer), and French Réseau National de Pharmacogénétique Hospitalière (RNPGx), 2015. UGT1A1 genotype and irinotecan therapy: general review and implementation in routine practice. Fundamental & clinical pharmacology, 29(3), 219–237. https://doi.org/10.1111/fcp.12117

Falini, B., Brunetti, L. and Martelli, M., 2021. How I diagnose and treat NPM1-mutated AML. *Blood*, 137(5), pp.589-599.

Falini, B., Brunetti, L., Sportoletti, P. and Martelli, M., 2020. NPM1-mutated acute myeloid leukemia: from bench to bedside. *Blood*, 136(15), pp.1707-1721.

Finaz, C. and Grouchy, J., 1972. Identification of individual chromosomes in the human karyotype by their banding pattern after proteolytic digestion. *Human Genetics*, 15(3), pp.249-252.

Fouad, Y. A., and Aanei, C., 2017. Revisiting the hallmarks of cancer. *American journal of cancer research*, *7*(5), 1016–1036.

Gabert, J., Beillard, E., van der Velden, V., Bi, W., Grimwade, D., and Pallisgaard, N., 2003. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – A Europe Against Cancer Program. *Leukemia*, *17*(12), 2318-2357. doi: 10.1038/sj.leu.2403135

Goldberg, J. and Borgen, P., 2006. Breast cancer susceptibility testing: past, present and future. Expert Review of Anticancer Therapy, 6(8), pp.1205-1214.

H. Vogel, MSN, FNP, AOCNP®, W., Minhas, MD, A. and Baumrucker, MD, S., 2020. Dihydropyrimidine Dehydrogenase Deficiency: To Screen or Not to Screen?. Journal of the Advanced Practitioner in Oncology, 11(1).

Hahn, W. C., Bader, J. S., Braun, T. P., Califano, A., Clemons, P. A., Druker, B. J., Ewald, A. J., Fu, H., Jagu, S., Kemp, C. J., Kim, W., Kuo, C. J., McManus, M., B Mills, G., Mo, X., Sahni, N., Schreiber, S. L., Talamas, J. A., Tamayo, P., Tyner, J. W., … Cancer Target Discovery and Development Network (2021). An expanded universe of cancer targets. Cell, 184(5), 1142–1155. https://doi.org/10.1016/j.cell.2021.02.020

Hanahan D., 2022. Hallmarks of Cancer: New Dimensions. Cancer discovery, 12(1), 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059

He, R., Devine, D., Tu, Z., Mai, M., Chen, D., Nguyen, P., Oliveira, J., Hoyer, J., Reichard, K., Ollila, P., Al-Kali, A., Tefferi, A., Begna, K., Patnaik, M., Alkhateeb, H. and Viswanatha, D., 2019. Hybridization capture-based next generation sequencing reliably detects FLT3 mutations and classifies FLT3-internal tandem duplication allelic ratio in acute myeloid leukemia: a comparative study to standard fragment analysis. *Modern Pathology*, 33(3), pp.334-343.

Heather, J. and Chain, B., 2016. The sequence of sequencers: The history of sequencing DNA. *Genomics*, 107(1), pp.1-8.

Heikamp, E. and Pui, C., 2018. Next-Generation Evaluation and Treatment of Pediatric Acute Lymphoblastic Leukemia. The Journal of Pediatrics, 203, pp.14-24.e2.

Heim, S. and Mitelman, F., 2015. *Cancer Cytogenetics*. 4th ed. Wiley.

Hochhaus, A., Baccarani, M., Silver, R. T., Schiffer, C., Apperley, J. F., Cervantes, F., Clark, R. E., Cortes, J. E., Deininger, M. W., Guilhot, F., Hjorth-Hansen, H., Hughes, T. P., Janssen, J., Kantarjian, H. M., Kim, D. W., Larson, R. A., Lipton, J. H., Mahon, F. X., Mayer, J., Nicolini, F., and Hehlmann, R., 2020. European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. *Leukemia*, *34*(4), 966–984. https://doi.org/10.1038/s41375-020-0776-2

Hömig-Hölzel, C. and Savola, S., 2012. Multiplex Ligation-dependent Probe Amplification (MLPA) in Tumor Diagnostics and Prognostics. *Diagnostic Molecular Pathology*, 21(4), pp.189-206.

Hong, L., Zhang, J., Heymach, J. V., and Le, X., 2021. Current and future treatment options for MET exon 14 skipping alterations in non-small cell lung cancer. Therapeutic advances in medical oncology, 13, 1758835921992976. https://doi.org/10.1177/1758835921992976

Hu, L., Ru, K., Zhang, L., Huang, Y., Zhu, X., Liu, H., Zetterberg, A., Cheng, T. and Miao, W., 2014. Fluorescence in situ hybridization (FISH): an increasingly demanded tool for biomarker research and personalized medicine. *Biomarker Research*, 2(1).

Hutchison, C., 2007. DNA sequencing: bench to bedside and beyond. *Nucleic Acids Research*, 35(18), pp.6227-6237.

Intedya.pt. 2021. *Sistema de Gestão da Qualidade ISO 15189.*.

IPO-PORTO. 2021. *Institucional - IPO-PORTO*.

IPO-PORTO. 2021. *Serviço de Genética - IPO-PORTO*.

Iwamoto, T., Kajiwara, Y., Zhu, Y. and Iha, S., 2020. Biomarkers of neoadjuvant/adjuvant chemotherapy for breast cancer. Chinese Clinical Oncology, 9(3), pp.27-27.

Jain A., Jain A., Gulbake A., Hurkat P. and Jain S. K.. 2011. Solid tumors: a review. International Journal of Pharmacy and Pharmaceutical Sciences. ISSN- 0975-1491

Jensen, M., Lænkholm, A., Nielsen, T., Eriksen, J., Wehn, P., Hood, T., Ram, N., Buckingham, W., Ferree, S. and Ejlertsen, B., 2018. The Prosigna gene expression assay and responsiveness to adjuvant cyclophosphamide-based chemotherapy in premenopausal high-risk patients with breast cancer. *Breast Cancer Research*, 20(1).

Jorde, L., Carey, J. and Bamshad, M., 2015. *Medical genetics*. 5th ed. Elsevier. ISBN 978-0-323-18835-7

Kashork, C., Theisen, A. and Shaffer, L., 2010. Diagnosis of Cryptic Chromosomal Syndromes by Fluorescence In Situ Hybridization (FISH). *Current Protocols in Human Genetics*, 67(1).

Kentsis, A., 2020. Why do young people get cancer?. Pediatric Blood & Cancer, 67(7).

Kim, Y., Lee, G., Park, J., Yoon, J., Kim, H., Min, W. and Kim, M., 2015. Quantitative fragment analysis of FLT3-ITD efficiently identifying poor prognostic group with high mutant allele burden or long ITD length. *Blood Cancer Journal*, 5(8), pp.e336-e336.

Kreatech, Leica Biosystems.

Kutny, M., Moser, B., Laumann, K., Feusner, J., Gamis, A., Gregory, J., Larson, R., Powell, B., Stock, W., Willman, C., Woods, W. and Meshinchi, S., 2012. FLT3 mutation status is a predictor of early death in pediatric acute promyelocytic leukemia: A report from the Children's Oncology Group. *Pediatric Blood & Cancer*, 59(4), pp.662-667.

Laboratory Quality Management System: Handbook., 2012. World Health Organization. Paperback. ISBN: 978-92-4-154827-4

Lao, E., García, Á., Figuerola, M., Moreno, E. and Paraire, A., 2017. Errors of Clinical Laboratory and Its Impact on Patient Safety. *Open Journal of Social Sciences*, 05(03), pp.243-253.

Leisch, M., Jansko, B., Zaborsky, N., Greil, R. and Pleyer, L., 2019. Next Generation Sequencing in AML—On the Way to Becoming a New Standard for Treatment Initiation and/or Modulation?. Cancers, 11(2), p.252.

Levy, G., Carillo, S., Papoular, B., Cassinat, B., Zini, J., Leroy, E., Varghese, L., Chachoua, I., Defour, J., Smith, S. and Constantinescu, S., 2020. MPL mutations in essential thrombocythemia uncover a common path of activation with eltrombopag dependent on W491. *Blood*, 135(12), pp.948-953.

Lewandowska, A., Rudzki, M., Rudzki, S., Lewandowski, T. and Laskowska, B., 2019. Environmental risk factors for cancer – review paper. Annals of Agricultural and Environmental Medicine, 26(1), pp.1-7.

Litton, J. K., Burstein, H. J., and Turner, N. C., 2019. Molecular Testing in Breast Cancer. American Society of Clinical Oncology educational book. American Society of Clinical Oncology. Annual Meeting, 39, e1–e7. https://doi.org/10.1200/EDBK_237715

Lunenburg, C., van der Wouden, C., Nijenhuis, M., Crommentuijn-van Rhenen, M., de Boer-Veger, N., Buunk, A., Houwink, E., Mulder, H., Rongen, G., van Schaik, R., van der Weide, J., Wilffert, B., Deneer, V., Swen, J. and Guchelaar, H., 2019. Dutch Pharmacogenetics Working Group (DPWG) guideline for the gene–drug interaction of DPYD and fluoropyrimidines. European Journal of Human Genetics, 28(4), pp.508-517.

MagNa Pure Versatile Nucleic Acid Purification, Roche Molecular Systems, Inc., Pleasanton, CA, USA

Maillard, M., Eche-Gass, A., Ung, M., Brice, A., Marsili, S., Montastruc, M., Puisset, F. and Thomas, F., 2021. Severe toxicity of capecitabine in a patient with DPD deficiency after a safe FEC-100 experience: why we should test DPD deficiency in all patients before high-dose fluoropyrimidines. Cancer Chemotherapy and Pharmacology, 87(4), pp.579-583.

Mansouri A, Hachem LD, Mansouri S, Nassiri F, Laperriere NJ, Xia D, Lindeman NI, Wen PY, Chakravarti A, Mehta MP, Hegi ME, Stupp R, Aldape KD, Zadeh G., 2019. MGMT promoter methylation status testing to guide therapy for glioblastoma: refining the approach based on emerging evidence and current challenges. Neuro Oncol. 14;21(2):167-178. doi: 10.1093/neuonc/noy132. PMID: 30189035; PMCID: PMC6374759.

Mardekian, S. K., Solomides, C. C., Gong, J. Z., Peiper, S. C., Wang, Z. X., and Bajaj, R., 2015. Comparison of Chromogenic In Situ Hybridization and Fluorescence In Situ Hybridization for the Evaluation of MDM2 Amplification in Adipocytic Tumors. *Journal of clinical laboratory analysis*, *29*(6), 462–468. https://doi.org/10.1002/jcla.21794

Marmolejo, D., Wong, M., Bajalica-Lagercrantz, S., Tischkowitz, M., Balmaña, J., Patócs, A., Chappuis, P., Colas, C., Genuardi, M., Haanpää, M., Vetti, H., Hoogerbrugge, N., Irmejs, A., Kahre, T., Klink, B., Krajc, M., Milagre, T., de Putter, R., Steinke-Lange, V., Wadt, K. and Wimmer, K., 2021. Overview of hereditary breast and ovarian cancer (HBOC) guidelines across Europe. European Journal of Medical Genetics, 64(12), p.104350.

Martelli, M., Rossi, R., Venanzi, A., Meggendorfer, M., Perriello, V., Martino, G., Spinelli, O., Ciurnelli, R., Varasano, E., Brunetti, L., Ascani, S., Quadalti, C., Cardinali, V., Mezzasoma, F., Gionfriddo, I., Milano, F., Pacini, R., Tabarrini, A., Bigerna, B., Albano, F., Specchia, G., Vetro, C., Di Raimondo, F., Annibali, O., Avvisati, G., Rambaldi, A., Falzetti, F.,

Tiacci, E., Sportoletti, P., Haferlach, T., Haferlach, C. and Falini, B., 2021. Novel NPM1 exon 5 mutations and gene fusions leading to aberrant cytoplasmic nucleophosmin in AML. *Blood*, 138(25), pp.2696-2701.

Mesri, E., Feitelson, M. and Munger, K., 2014. Human Viral Oncogenesis: A Cancer Hallmarks Analysis. *Cell Host & Microbe*, 15(3), pp.266-282.

Mocellin, S., Rossi, C., Pilati, P., Nitti, D. and Marincola, F., 2003. Quantitative real-time PCR: a powerful ally in cancer research. *Trends in Molecular Medicine*, 9(5), pp.189-195.

Morgan, K. and Gilliland, D., 2008. A Role for JAK2 Mutations in Myeloproliferative Diseases. *Annual Review of Medicine*, 59(1), pp.213-222.

NCCN. 2022. *National Comprehensive Cancer Network - Home*.

Nebral, K., Denk, D., Attarbaschi, A., König, M., Mann, G., Haas, O., and Strehl, S., 2009. Incidence and diversity of PAX5 fusion genes in childhood acute lymphoblastic leukemia. *Klinische Pädiatrie*, *221*(03). doi: 10.1055/s-0029-1222663

Nowell, P., 1976. The Clonal Evolution of Tumor Cell Populations. *Science*, 194(4260), pp.23-28.

Parker, B. and Zhang, W., 2013. Fusion genes in solid tumors: an emerging target for cancer diagnosis and treatment. Chinese Journal of Cancer, 32(11), pp.594-603.

Pastorino, R., Basile, M., Tognetto, A., Di Marco, M., Grossi, A., Lucci-Cordisco, E., Scaldaferri, F., De Censi, A., Federici, A., Villari, P., Genuardi, M., Ricciardi, W., and Boccia, S., 2020. Cost-effectiveness analysis of genetic diagnostic strategies for Lynch syndrome in Italy. PloS one, 15(7), e0235038. https://doi.org/10.1371/journal.pone.0235038

Patel, A., O'Hare, T. and Deininger, M., 2017. Mechanisms of Resistance to ABL Kinase Inhibition in Chronic Myeloid Leukemia and the Development of Next Generation ABL Kinase Inhibitors. *Hematology/Oncology Clinics of North America*, 31(4), pp.589-612.

Pestinger, V., Smith, M., Sillo, T., Findlay, J., Laes, J., Martin, G., Middleton, G., Taniere, P. and Beggs, A., 2020. Use of an Integrated Pan-Cancer Oncology Enrichment Next-Generation Sequencing Assay to Measure Tumour Mutational Burden and Detect Clinically Actionable Variants. Molecular Diagnosis & Therapy, 24(3), pp.339-349.

Pettersson, E., Lundeberg, J., and Ahmadian, A., 2009. Generations of sequencing technologies. Genomics, 93(2), 105–111. https://doi.org/10.1016/j.ygeno.2008.10.003

Plebani M., 2015. Diagnostic Errors and Laboratory Medicine - Causes and Strategies. *EJIFCC*, *26*(1), 7–14.

Ponder, B., 2001. Cancer genetics. *Nature*, 411(6835), pp.336-341.

Pu, M., Messer, K., Davies, S., Vickery, T., Pittman, E., Parker, B., Ellis, M., Flatt, S., Marinac, C., Nelson, S., Mardis, E., Pierce, J. and Natarajan, L., 2019. Research-based PAM50 signature and long-term breast cancer survival. *Breast Cancer Research and Treatment*, 179(1), pp.197-206.

Rack, K., van den Berg, E., Haferlach, C., Beverloo, H., Costa, D., Espinet, B., Foot, N., Jeffries, S., Martin, K., O'Connor, S., Schoumans, J., Talley, P., Telford, N., Stioui, S., Zemanova, Z. and Hastings, R., 2019. European recommendations and quality assurance for cytogenomic analysis of haematological neoplasms. *Leukemia*, 33(8), pp.1851-1867.

Rao, A. M., Quddusi, A., Shamim, M. S., 2018. The significance of MGMT methylation in Glioblastoma Multiforme prognosis. J Pak Med Assoc. 68(7):1137-1139. PMID: 30317322.

Ratan, Z., Zaman, S., Mehta, V., Haidere, M., Runa, N. and Akter, N., 2017. Application of Fluorescence In Situ Hybridization (FISH) Technique for the Detection of Genetic Aberration in Medical Science. *Cureus*, 9(6): e1325. DOI 10.7759/cureus.1325.

Rautenstrauß, B., and Liehr, T., 2012. *FISH Technology*. Springer.

Sarwar, S., Mushtaq, S., Hassan, U., Maqbool, H., Qazi, R., 2021 Diagnostic utility of FISH for MDM2 in adipocytic neoplasms. *J Ayub Med Coll Abbottabad*;33(4):563–7.

Schouten, R., Vessies, D., Bosch, L., Barlo, N., van Lindert, A., Cillessen, S., van den Broek, D., van den Heuvel, M. and Monkhorst, K., 2021. Clinical Utility of Plasma-Based Comprehensive Molecular Profiling in Advanced Non–Small-Cell Lung Cancer. JCO Precision Oncology, (5), pp.1112-1121.

Schulz, C., Boeck, S., Heinemann, V., Stemmler, H. J., 2009. UGT1A1 genotyping: a predictor of irinotecan-associated side effects and drug efficacy? Anticancer Drugs. 20(10):867-79. doi: 10.1097/CAD.0b013e328330c7d2. PMID: 19770637.

Simundic, A., Nikolac, N., Vukasovic, I. and Vrkic, N., 2010. The prevalence of preanalytical errors in a Croatian ISO 15189 accredited laboratory. *Clinical Chemistry and Laboratory Medicine*, 48(7).

Singh, J., Birbian, N., Sinha, S., and Goswami, A., 2014. A critical review on PCR, its types and applications, *Int. J. Adv. Res. Biol.Sci*. 1(7): (2014): 65–80, ISSN: 2348-8069

Small, D., 2006. FLT3 Mutations: Biology and Treatment. *Hematology*, 2006(1), pp.178-184.

Smith, C. and Fan, G., 2008. The saga of JAK2 mutations and translocations in hematologic disorders: pathogenesis, diagnostic and therapeutic prospects, and revised World Health Organization diagnostic criteria for myeloproliferative neoplasms. *Human Pathology*, 39(6), pp.795-810.

Song, H., Cicek, M. S., Dicks, E., Harrington, P., Ramus, S. J., Cunningham, J. M., Fridley, B. L., Tyrer, J. P., Alsop, J., Jimenez-Linan, M., Gayther, S. A., Goode, E. L., and Pharoah, P. D., 2014. The contribution of deleterious germline mutations in BRCA1, BRCA2 and the mismatch repair genes to ovarian cancer in the population. Human molecular genetics, 23(17), 4703–4709. https://doi.org/10.1093/hmg/ddu172

Suster, D., Ronen, S., Peterson, J., Mackinnon, A., Hes, O., Suster, S. and Lin, D., 2019. MDM2 amplification and immunohistochemical expression in sarcomatoid renal cell carcinoma. *Human Pathology*, 87, pp.28-36.

Swerdlow, S., 2017. *WHO classification of tumours of haematopoietic and lymphoid tissues*. Lyon: International Agency for Research on Cancer.

Syngal, S., Brand, R. E., Church, J. M., Giardiello, F. M., Hampel, H. L., Burt, R. W., & American College of Gastroenterology (2015). ACG clinical guideline: Genetic testing and management of hereditary gastrointestinal cancer syndromes. The American journal of gastroenterology, 110(2), 223–263. https://doi.org/10.1038/ajg.2014.435

Takano, M, Sugiyama, T., 2017. UGT1A1 polymorphisms in cancer: impact on irinotecan treatment. Pharmgenomics Pers Med. 28;10:61-68. doi: 10.2147/PGPM.S108656. PMID: 28280378; PMCID: PMC5338934.

Turnbull, C., and Hodgson, S., 2005. Genetic predisposition to cancer. Clinical medicine (London, England), 5(5), 491–498. https://doi.org/10.7861/clinmedicine.5-5-491

Uicc.org. 2022. *GLOBOCAN 2020: New Global Cancer Data | UICC*.

Valencia, O. M., Samuel, S. E., Viscusi, R. K., Riall, T. S., Neumayer, L. A., and Aziz, H., 2017. The Role of Genetic Testing in Patients With Breast Cancer: A Review. JAMA surgery, 152(6), 589–594. https://doi.org/10.1001/jamasurg.2017.0552

van Dongen, J., Macintyre, E., Gabert, J., Delabesse, E., Rossi, V., Saglio, G., Gottardi, E., Rambaldi, A., Dotti, G., Griesinger, F., Parreira, A., Gameiro, P., Diáz, M., Malec, M., Langerak, A., San Miguel, J. and Biondi, A., 1999. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. *Leukemia*, 13(12), pp.1901-1928.

Wakai, T., Prasoon, P., Hirose, Y., Shimada, Y., Ichikawa, H. and Nagahashi, M., 2018. Next-generation sequencing-based clinical sequencing: toward precision medicine in solid tumors. International Journal of Clinical Oncology, 24(2), pp.115-122.

Walker-Smith, T. L., and Peck, J., 2019. Genetic and Genomic Advances in Breast Cancer Diagnosis and Treatment. Nursing for women's health, 23(6), 518–525. https://doi.org/10.1016/j.nwh.2019.09.003

Wallden, B., Storhoff, J., Nielsen, T., Dowidar, N., Schaper, C., Ferree, S., Liu, S., Leung, S., Geiss, G., Snider, J., Vickery, T., Davies, S., Mardis, E., Gnant, M., Sestak, I., Ellis, M., Perou, C., Bernard, P. and Parker, J., 2015. Development and verification of the PAM50-based Prosigna breast cancer gene signature assay. *BMC Medical Genomics*, 8(1).

Walsh, M. F., Nathanson, K. L., Couch, F. J., and Offit, K., 2016. Genomic Biomarkers for Breast Cancer Risk. Advances in experimental medicine and biology, 882, 1–32. https://doi.org/10.1007/978-3-319-22909-6_1

Wang, Y., Wu, N., Liu, D. and Jin, Y., 2017. Recurrent Fusion Genes in Leukemia: An Attractive Target for Diagnosis and Treatment. *Current Genomics*, 18(5).

Waters, J., Barlow, A. and Gould, C., 1998. Demystified ... FISH. *Molecular Pathology*, 51(2), pp.62-70.

Wegner, R., 1999. *Diagnostic Cytogenetics*. New York: Springer-Verlag Berlin Heidelberg.

Weissman, S. M., Burt, R., Church, J., Erdman, S., Hampel, H., Holter, S., Jasperson, K., Kalady, M. F., Haidle, J. L., Lynch, H. T., Palaniappan, S., Wise, P. E., and Senter, L., 2012. Identification of individuals at risk for Lynch syndrome using targeted evaluations and genetic testing: National Society of Genetic Counselors and the Collaborative Group of the Americas on Inherited Colorectal Cancer joint practice guideline. Journal of genetic counseling, 21(4), 484–493. https://doi.org/10.1007/s10897-011-9465-7

Wendt, C. and Margolin, S., 2019. Identifying breast cancer susceptibility genes – a review of the genetic background in familial breast cancer. Acta Oncologica, 58(2), pp.135-146.

Wesoła, M. and Jeleń, M., 2015. A Comparison of IHC and FISH Cytogenetic Methods in the Evaluation of HER2 Status in Breast Cancer. *Advances in Clinical and Experimental Medicine*, 24(5), pp.899-904.

White, C., Scott, R., Paul, C., Ziolkowski, A., Mossman, D. and Ackland, S., 2021. Ethnic Diversity of DPD Activity and the DPYD Gene: Review of the Literature. Pharmacogenomics and Personalized Medicine, Volume 14, pp.1603-1617.

WHO, World Health Organization

Willey, J., Morrison, T., Austermiller, B., Crawford, E., Craig, D., Blomquist, T., Jones, W., Wali, A., Lococo, J., Haseley, N., Richmond, T., Novoradovskaya, N., Kusko, R., Chen, G., Li, Q., Johann, D., Deveson, I., Mercer, T., Wu, L. and Xu, J., 2021. Advancing NGS quality control to enable measurement of actionable mutations in circulating tumor DNA. Cell Reports Methods, 1(7), p.100106.

Yadav, S., and Couch, F. J., 2019. Germline Genetic Testing for Breast Cancer Risk: The Past, Present, and Future. American Society of Clinical Oncology educational book. American Society of Clinical Oncology. Annual Meeting, 39, 61–74. https://doi.org/10.1200/EDBK_238987

Yoon, N., Do, I. and Cho, E., 2013. Analysis of HER2 status in breast carcinoma by fully automated HER2 fluorescence in situ hybridization (FISH): comparison of two immunohistochemical tests and manual FISH. *APMIS*, 122(9), pp.755-760.

Yoshida, R., 2020. Hereditary breast and ovarian cancer (HBOC): review of its molecular characteristics, screening, treatment, and prognosis. Breast Cancer, 28(6), pp.1167-1180.

Yurgelun, M. B., and Hampel, H., 2018. Recent Advances in Lynch Syndrome: Diagnosis, Treatment, and Cancer Prevention. American Society of Clinical Oncology educational book. American Society of Clinical Oncology. Annual Meeting, 38, 101–109. https://doi.org/10.1200/EDBK_208341

Zhao, C., Jiang, T., Hyun Ju, J., Zhang, S., Tao, J., Fu, Y., Lococo, J., Dockter, J., Pawlowski, T. and Bilke, S., 2020. TruSight Oncology 500: Enabling Comprehensive Genomic Profiling and Biomarker Reporting with Targeted Sequencing.