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DEPARTMENT OF PHARMACY

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Contribution of RT-PCR in The Diagnosis and Therapeutic Monitoring of Leukaemias

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﴿ **They (angels) said: "Glory be to You, we have no knowledge except what you have taught us. Verily, it is You, the All-Knower, the All-Wise."**﴾

Surat Al-Baqarah [2:32]

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DEDICATION

MERKANTIA Hiba

My loving parents, caring brothers and sisters, tender sister-in-law, nice brothers-in-law, adorable nephews and nieces, lovely aunts and cousins, amazing friends, and courageous partner in this dissertation, I dedicate this work to you. Thank you very much for everything.

May Allah bless you all.

LIST OF ABBREVIATIONS

ABI 7900	**Applied Biosystems**
ABL/ABL1	**Abelson**
AFLP	**Amplified fragment length polymorphism**
Akt (PKB)	**Protein kinase B**
ALL	**Acute lymphoblastic leukaemia**
AML	**Acute myeloid leukaemia**
AP	**Accelerated phase**
ARMS	**Amplification of refractory mutation system**
ATP	**Adenosine triphosphate**
BC	**Blast crisis**
BCR	**Breakpoint cluster region**
BFU_E	**Erythroid burst colony-forming unit**
BM	**Bone marrow**
CALR	**Calreticulin**
CCyR	**Complete cytogenetic response**
cDNA	**Complementary**
CFU	**Colony-forming unit**
CFU_E	**Erythroid-colony forming unit**
CML	**Chronic myeloid leukaemia**
CMML	**Chronic myelomonocytic leukaemia**
CP	**Chronic phase**
C_T	**Threshold cycle**
CYP	**Cytochrome P450**
Cy5	**Cyanin Dye 5**
Dbl	**Diffuse B-cell lymphoma**
DEPC	**Diethyl pyrocarbonate**
dl	**decilitre**
DNA	**Deoxyribonucleic acid**
DNAse	**Deoxyribonuclease**
dNTP	**Deoxynucleotide triphosphate**
dsDNA	**Double-stranded DNA**
EDTA	**Ethylenediaminetetraacetic acid**
ELN	**European LeukemiaNet**
ELTS	**European treatment and outcome study long-term survival**
EPO-R	**Erythropoietin receptor**
ET	**Essential thrombocythaemia**
EURO	**Hasford score**
EUTOS	**European treatment and outcome study**
6-FAM	**Carboxyfluorescein**
FERM	**Four point one, Ezrin, Radixin, Moesin**
FISH	**Fluorescence in situ hybridisation**
FLT3	**Fms-like Tyrosine kinase 3**
FRET	**Fluorescence resonance energy transfer**

LIST OF ABBREVIATIONS

G	**Guanosine**
GAP	**GTPase activating protein**
G-CSF	**Granulocyte-Colony stimulating factor**
G-CSF-R	**Granulocyte-Colony stimulating factor receptor**
GDP	**Guanosine diphosphate**
GEMM	**Granulocyte Erythrocyte Macrophage Megakaryocte**
GTPase	**Guanosine triphosphatase**
GTP	**Guanosine triphosphate**
HEG	**Hexaethylene glycol**
HEX	**Hexachloro-fluoroscein**
HSC	**Haematopoietic stem cell**
HybProbes	**Hybridisation probes**
ITD	**Reccurent internal tandem duplication**
JAK	**Janus Kinase**
JH	**JAK Homology**
JMML	**Juvenile myelomonocytic leukaemia**
JOE	**5'-Dichloro-dimethoxy-fluorescein dye**
kb	**Kilobase**
kd	**Kilodalton**
l	**litre**
LED	**Light emitting diode**
M-BCR	**Major-Breakpoint cluster region**
m-BCR	**Minor- Breakpoint cluster region**
ml	**Millilitre**
MMR	**Major molecular response**
MR	**Molecular response**
MRD	**Minimal residual disease**
mRNA	**Messenger Ribonucleic acid**
MYC	**Master regulator of cell cycle entry and proliferative metabolism**
MYCN	**Master regulator of cell cycle entry and proliferative metabolism oncogene**
NCCN	**National Comprehensive Cancer Network**
nm	**nanometre**
PBS	**Phosphate-buffered saline**
PCR	**Polymerase Chain Reaction**
PCV	**Packed cell volume**
Ph+	**Philadelphia positive**
Ph-	**Philadelphia negative**
PI3K	**Phosphoinosytol 3 Kinase**
PMF	**Primary myelofibrosis**
PPC	**Production possibilities curve**
PPF	**Production possibility frontiers**
PV	**Polycythaemia vera**
P190BCR-ABL	**190 kd BCR-ABL protein**

LIST OF ABBREVIATIONS

P210BCR-ABL	**210 kd BCR-ABL protein**
P230BCRABL	**230 kd BCR-ABL protein**
qPCR	**Quantitative PCR**
RAS	**Rat sarcoma virus**
RNA	**Ribonucleic acid**
ROX	**Carboxyrhodamine**
RQ-PCR	**Reverse quantitative PCR**
RT	**Reverse transcriptase**
RT-qPCR	**Quantitative reverse transcriptase PCR**
RT-PCR	**Reverse Transcriptase PCR**
SARS-Cov-2	**Severe Acute respiratory syndrome coronavirus-2**
Scr	**Scarecrow**
SDS	**Sodium Dodecyl Sulphate**
SH	**Scr Homologous**
SNP	**Single nucleotide polymorphism**
SRSF2	**Serine and arginine rich splicing factor 2**
ssDNA	**Single-stranded DNA**
SSP	**Single specific oligonucleotide**
SSO	**Single specific primer**
STAT	**Signal transducers and activators of transcription**
T	**Thymine**
Taq	**Thermus aquaticus**
TAMRA	**5-Carboxytetramethylrhodamine**
TET	**Tetrachlorofluorescein**
TET2	**Tet methylcytosine dioxygenase**
TKI	**Tyrosine kinase inhibitor**
T_m	**Melting point**
TPO-R	**Thrombopoietin receptor**
tRNA	**Transfer RNA**
VIC	**Victoria dye**
V617F	**Valine 617 Phenylalanine**
WHO	**World Health Organisation**
°C	**Celsius degree**
µ-BCR	**Micro- Breakpoint cluster region**
µl	**Microlitre**

Table of Figures

TABLE OF FIGURES

Table of Tables

Table of Contents

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

INTRODUCTION

Since the invention of the PCR technique in 1983, molecular biology has not stopped evolving. While many hemobiology techniques such as blood smears, immunophenotyping, and marrow smears remain conventional, a new tool, molecular biology, has been able to prove its usefulness in a large number of routine medical analyses. The study of mutant genes using molecular biology has thus become essential in certain indications.

Leukemias with genetic mutations are becoming more common in Algeria, with BCR-ABL mutation being the most prominent. In the molecular diagnosis and follow-up, only conventional and molecular cytogenetics, represented by karyotyping and fluorescence in situ hybridization (FISH) respectively, were utilized. However, multiple subsequent studies showed the limitations of these tests and recommended the use of RT-PCR technology for better results.

On the other hand, molecular biology techniques are gradually being integrated alongside conventional methods and tend to generalize in laboratories specializing in the diagnosis and monitoring of leukaemias.

OBJECTIVE

The aim of our dissertation is to establish RT-PCR research techniques and Real-Time PCR quantification of BCR-ABL fusion transcripts and apply them in the context of the diagnosis and molecular monitoring of patients with CML and ALL Ph+. We conducted our experimental study in the haematology department of Blida's Anticancer Centre to establish the qualitative and quantitative diagnosis of CML and ALL Ph+ and assess the molecular response to treatment. Moreover, we aimed to discover early signs of nonresponse to treatment in order to provide the physician with the necessary information to decide whether to maintain, adjust, or change the treatment.

I. Haematopoiesis

1.1 Definition

Haematopoiesis means the process of blood cell production. **[1]**

1.2 Sites of haematopoiesis

Haematopoiesis occurs in different sites in the organism. Each stage of life has one or many sites **(Table 1)**

Table 1. Sites of haematopoiesis **[2]**

Fetus	0–2 months (yolk sac)
	2–7 months (liver, spleen)
	5–9 months (bone marrow)
Infants	Bone marrow (practically all bones)
Adults	Vertebrae, ribs, sternum, skull, sacrum and pelvis, proximal ends of femur

1.3 Physiology of haematopoiesis

Haematopoiesis starts with a pluripotent stem cell called "**H**aematopoietic **S**tem **C**ell" (**HSC**) which gives rise to separate cell lineages. **[2]**

The haematopoietic stem cell can self-renew by asymmetrical cell division, and differentiate *via* haematopoietic progenitors. The CFU (colony-forming unit) - GEMM, a common myeloid progenitor cell, gives rise to granulocytes, erythrocytes, monocytes and megakaryocytes. The Common lymphoid progenitor cell gives rise to lymphocytes. **[2] (Figure 1)**

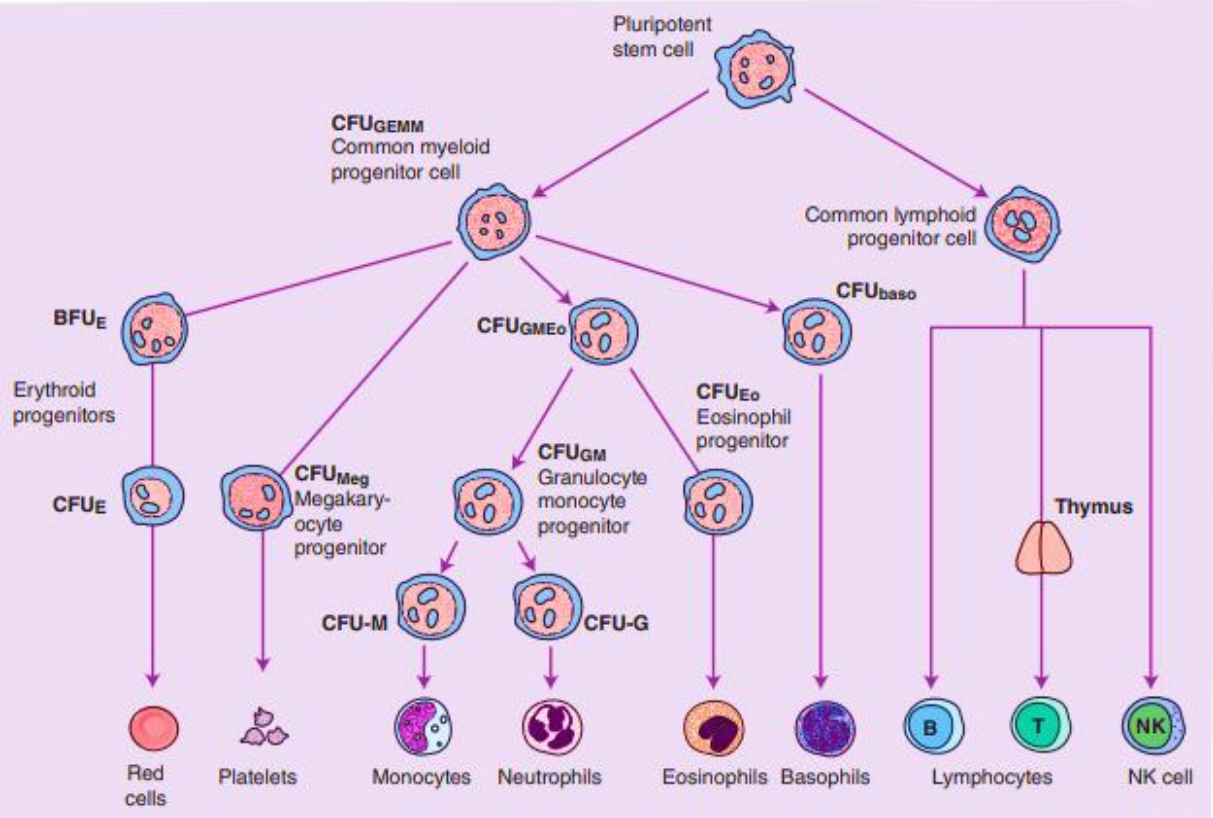

Figure 1. Diagrammatic representation of the bone marrow pluripotent stem cell and the lines that arise from it **[2]**

II. Haematological malignancies

2.1 Definition

Haematopoietic malignancies are clonal diseases that derive from a cell in the bone marrow or peripheral lymphoid tissue which has undergone genetic alteration. **[3]**

2.2 Physiopathology

Malignancies happen as a result of the accumulation of mutations in cellular genes which leads to the development of cancers. The genes involved are oncogenes and tumour-suppressor genes. **[3]**

Oncogenes acquire gain-of-function mutations, whereas tumour-suppressor genes acquire loss-of-function mutations. **[3]**

In normal cell proliferation, there is a balance of activity between the proto-oncogene and the tumour suppressor gene. The disruption of this balance leads to loss of control over cell division, engendering malignant cells. **(Figure 2)**

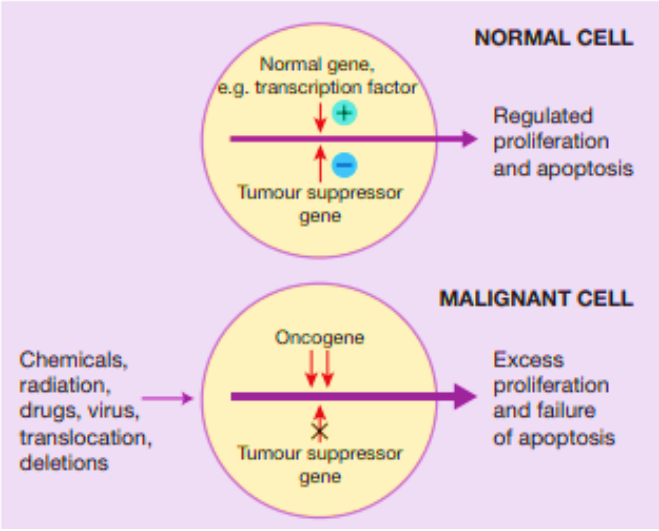

Figure 2. Proliferation of normal cells depends on a balance between the action of proto-oncogenes and tumour-suppressor genes. in a malignant cell, this balance is disrupted leading to uncontrolled cell division. **[3]**

2.3 Genetic abnormalities within haematological malignancies

Genetic defects play an important role in the pathogenesis of most haematological malignancies, including cytogenetic abnormalities, gene mutations, and abnormal gene expression. **[4]**

Many genetic abnormalities associated with haematological malignancies have been discovered, the most common of which are listed in **Table 2**.

Table 2. Some of the most frequent genetic abnormalities within haematological tumours **[3]**

Disease	Genetic abnormality	Genes involved
AML	t(8;21) translocation t(15;17) translocation Nucleotide insertion Mutation (internal tandem duplication) Mutation	RUNX1-*RUNX1T1* (*CBFα*) *PML*, *RARA* *NPM* *FLT3* *DNMT3A*
Secondary AML	11q23 translocations	*MLL*
Myelodysplasia	–5, del (5q) –7, del (7q)	*RPS 14* *N RAS*
CML	t(9;22) translocation	*BCR-ABL1*
Myeloproliferative disease	Point mutation	*JAK-2* or *CALR*
B-ALL	t(12;21) translocation t(9;22) translocation 11q23 translocations	*ETV6-RUNX1* *BCR-ABL1* *AF4/MLL*
T-ALL	Mutation	*NOTCH*
Follicular lymphoma Lymphoplasmacytic lymphoma Burkitt lymphoma Hairy cell leukaemia	t(14;18) translocation Mutation t(8;14) translocation Mutation	*BCL2* *MYD88* *MYC* *BRAF*
CLL	17p deletion Mutations	*P53* *NOTCH*, *SF3B1*, *ATM*

AML, acute myeloid leukaemia; B-ALL, B-acute lymphoblastic leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia;

2.4 Classification of haematological malignancies

Haematological malignancies have traditionally been classified by location based on whether the cancer was first found in the blood (leukaemia), lymph nodes (lymphomas: Hodgkin and non-Hodgkin), or bone (myelomas). **(Figure 3)**

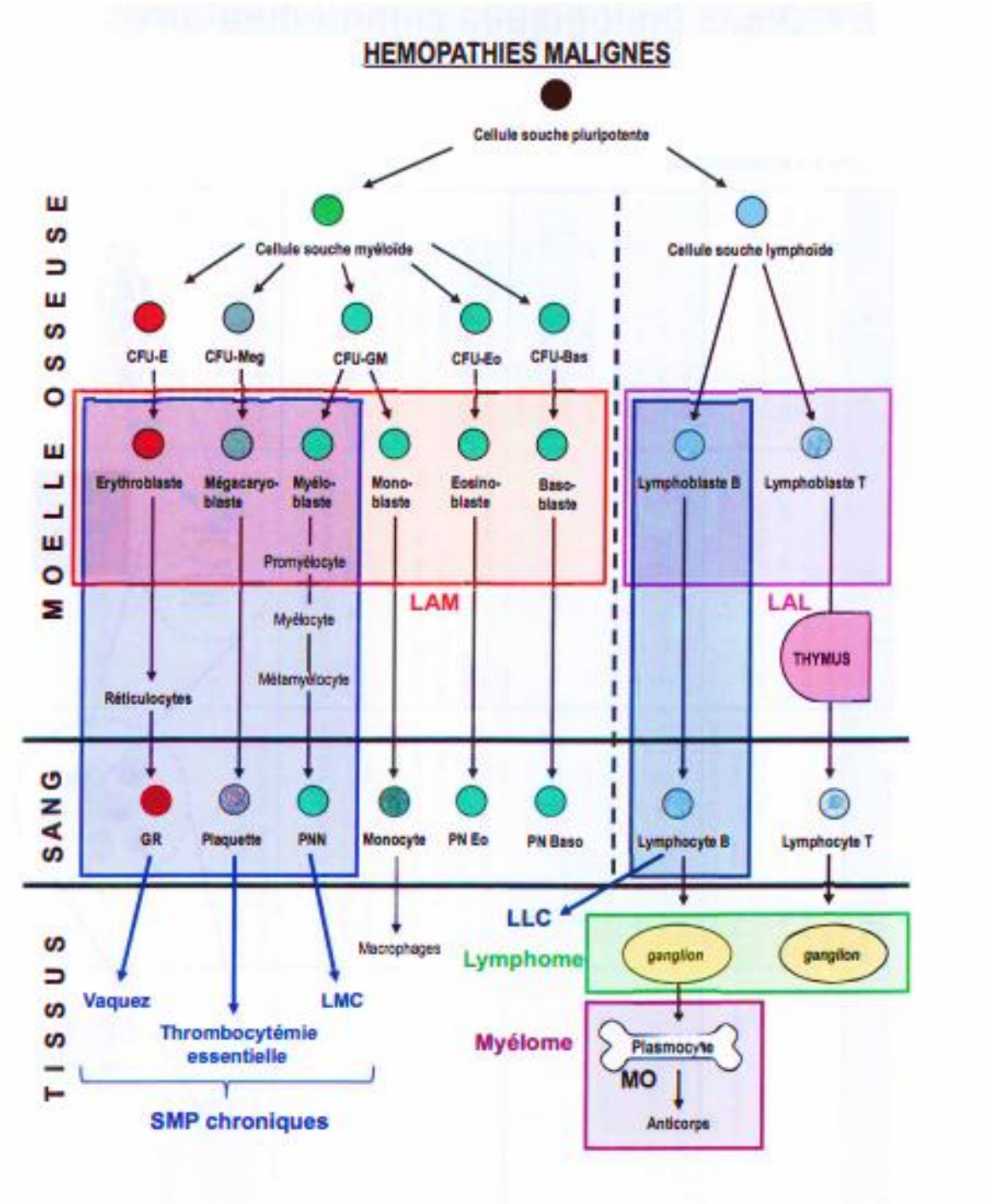

Figure 3. Haematological malignancies **[5]**

2.5 The WHO classification of malignant diseases of the haematopoietic and lymphoid tissues

2008 WHO classification of tumours of the haematopoietic and lymphoid tissues is a revision and update of the 3rd Edition, the first global consensus classification of haematological malignancies, and has been used by many experts after agreeing on the classification and diagnostic criteria upon which are based the definitions of the classified diseases. **[6]**

This classification uses all available information (morphology, cytochemistry, immune phenotype, genetics, and clinical features) to define the substance of clinically significant diseases. **[7]**

Significant contributions have been made through the application of high-throughput gene technologies such as gene expression profiling and next-generation sequencing. These techniques led to new diagnostic tools, revealing new mechanisms of tumorigenesis and new potential therapeutic targets. Due to these advances, the classification was updated and published in 2016 **[6] (See annexe 1)**

2.6 Diagnosis of haematological malignancies

2.6.1 Leukaemias

2.6.1.1 Definition

Leukaemias are diseases characterised by the accumulation of malignant leukocytes in the bone marrow and the blood. **[8]**

2.6.1.2 Epidemiology

According to global leukaemia statistics for 2020, the incidence is 2.5%, the mortality rate is 3.1%, and the prevalence for 5 years is 17.20 per 100,000 people. **[9]**

In Algeria, according to the 2020 leukaemia statistics, the incidence is 2.9%, the mortality rate is 3.4%, and the 5-year prevalence is 11.23 per 100,000 people. **[9]**

The overall proportion of new leukaemia cases in Algeria is estimated to be 5.2%, with males predominant at 5.4% and new female cases at 5.1%. **[9] (Figures 4, 5, and 6)**

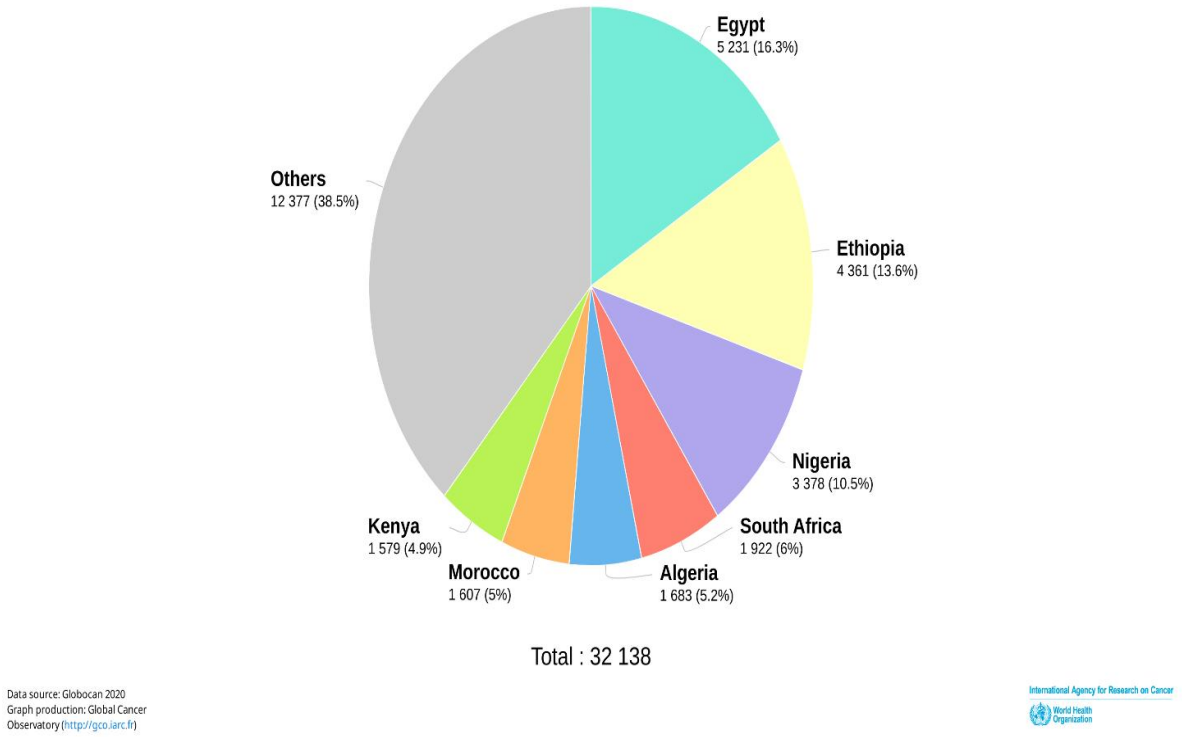

Figure 4. Estimated number of new cases in 2020, leukaemia, both sexes, all ages **[9]**

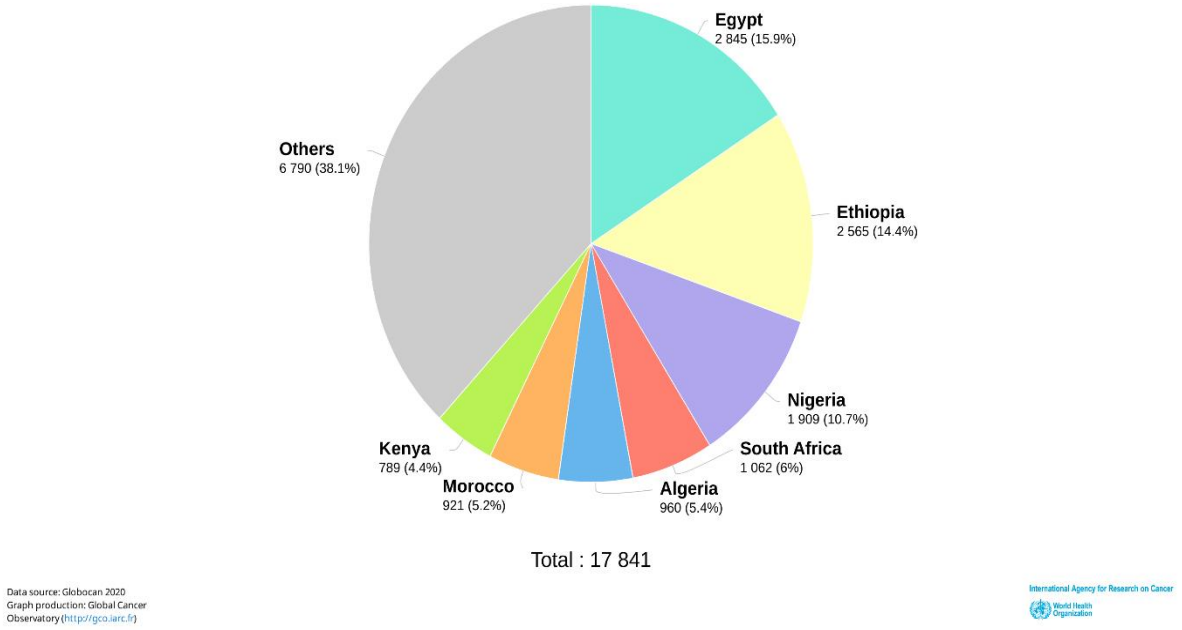

Figure 5. Estimated number of new cases in 2020, leukaemia, males, all ages **[9]**

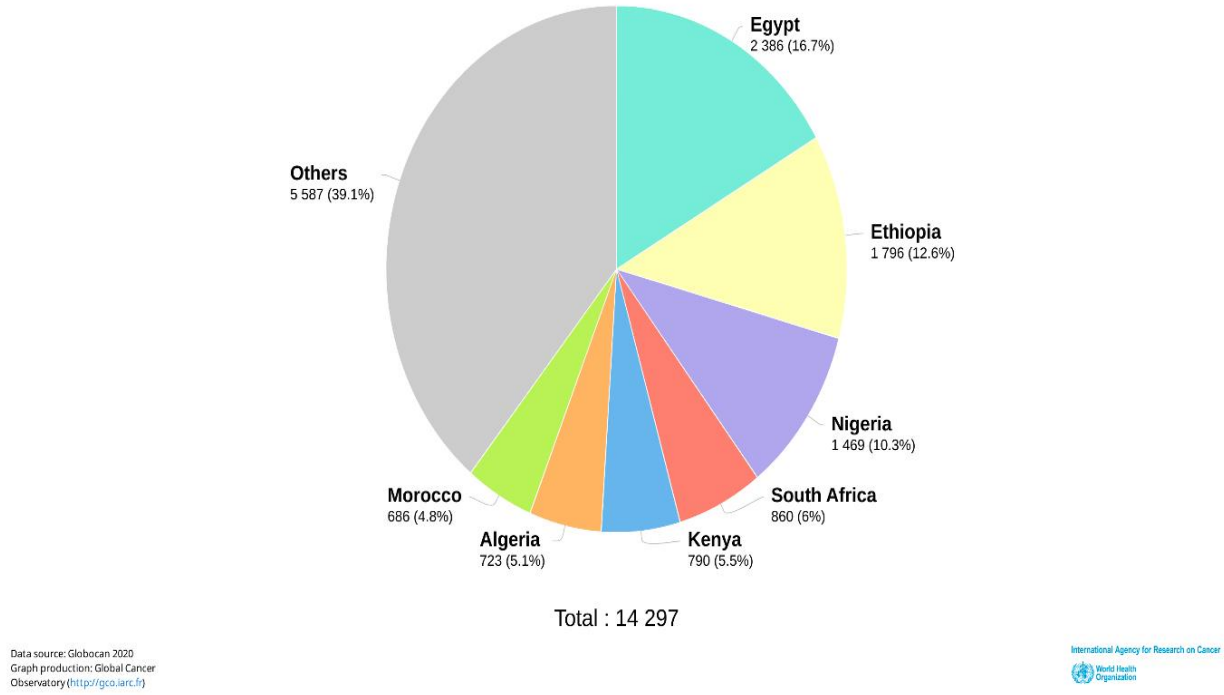

Figure 6. Estimated number of new cases in 2020, leukaemia, females, all ages **[9]**

2.6.1.3 Classification

The main classification is into four types: acute and chronic leukaemias, which are subdivided into lymphoid or myeloid. **[8]**

2.6.1.4 Acute leukaemias

Acute leukaemias are diseases in which a malignant transformation happens in the hematopoietic stem cell or early progenitors. It is the result of genetic damages that lead to:

- Increase the rate of proliferation
- Reduce apoptosis
- Block cell differentiation

Acute leukaemia is defined as the presence of over 20% of blast cells in the bone marrow. The lineage of the blast cells is defined by the examination of their morphology under the microscope, immunophenotypic (flow cytometry), cytogenetic and molecular analysis. **[8]**

2.6.1.4.1 Acute myeloid leukaemia

2.6.1.4.1.1 Definition

Acute myeloid leukaemia is a malignant clonal disease of hematopoietic progenitor cells. Malignant cells undergo genetic transformations that disrupt their normal mechanisms of self-renewal, proliferation, and differentiation. This process eventually leads to bone marrow failure. Acute myeloid leukaemia is rare in childhood, and its incidence increases with age; two-thirds of cases occur in people over the age of 60. **[10]**

2.6.1.4.1.2 Classification

The classification of AML Ph+ is shown in **Table 3**

Table 3. WHO 2016 classification of AML Ph+ **[6]**

AML with recurrent genetic abnormalities	
AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*	9896/3
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*	9871/3
Acute promyelocytic leukaemia with *PML-RARA*	9866/3
AML with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*	9897/3
AML with t(6;9)(p23;q34.1); *DEK-NUP214*	9865/3
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*	9869/3
AML (megakaryoblastic) with t(1;22)(p13.3;q13.1); *RBM15-MKL1*	9911/3
AML with BCR-ABL1	9912/3*
AML with mutated *NPM1*	9877/3*
AML with biallelic mutation of *CEBPA*	9878/3*
AML with mutated RUNX1	9879/3*

2.6.1.4.1.3 Clinical features **[8]**

- Frequent infections
- Signs of anaemia
- Signs of thrombocytopaenia
- Signs of intravascular disseminated coagulation
- Gum hypertrophy

2.6.1.4.1.4 Biological features [10]

- **Peripheral blood**

- Increased white cell count **[10]** (≥20% myeloblasts) **[11]**
- Anaemia
- Thrombocytopaenia

- **Bone marrow examination**

- Blasts infiltrating the bone marrow **[10]** (≥20%) **[11]**
- The myeloblasts may be found either in the bone marrow or the peripheral blood. **[11]**

- **Cytogenetics and molecular genetics**

- Detection of BCR-ABL1 gene by karyotyping and FISH. Also, RT-PCR is used to quantify the number of copies.

2.6.1.4.1.5 Treatment [10]

- Chemotherapy
- Stem cell transplantation

2.6.1.4.2 Acute lymphoblastic leukaemia

2.6.1.4.2.1 Definition

Acute lymphoblastic leukaemia (ALL) is a clonal malignancy caused by the accumulation of over 20% of malignant lymphoblasts in the bone marrow. In 80% of the cases, the malignant cells are primitive precursors of B-lymphocytes, and the 20% remaining cases are T-cell leukaemias. ALL has a peak incidence in childhood. **[10]**

2.6.1.4.2.2 Classification

The ALL Ph+ classification is shown in **Table 4**

Table 4. WHO 2016 classification of ALL Ph+ **[6]**

Precursor lymphoid neoplasms	
B-lymphoblastic leukaemia/lymphoma, NOS	9811/3
B-lymphoblastic leukaemia/lymphoma with t(9;22)(q34.1;q11.2); *BCR-ABL1*	9812/3
B-lymphoblastic leukaemia/lymphoma with t(v;11q23.3); *KMT2A*-rearranged	9813/3
B-lymphoblastic leukaemia/lymphoma with t(12;21)(p13.2;q22.1); *ETV6-RUNX1*	9814/3
B-lymphoblastic leukaemia/lymphoma with hyperdiploidy	9815/3
B-lymphoblastic leukaemia/lymphoma with hypodiploidy (hypodiploid ALL)	9816/3
B-lymphoblastic leukaemia/lymphoma with t(5;14)(q31.1;q32.1); IGH/*IL3*	9817/3
B-lymphoblastic leukaemia/lymphoma with t(1;19)(q23;p13.3); *TCF3-PBX1*	9818/3
B-lymphoblastic leukaemia/lymphoma, *BCR-ABL1*–like	9819/3*
B-lymphoblastic leukaemia/lymphoma with iAMP21	9811/3
T-lymphoblastic leukaemia/lymphoma	9837/3
Early T-cell precursor lymphoblastic leukaemia	9837/3
NK-lymphoblastic leukaemia/lymphoma	

2.6.1.4.2.3 Clinical features [10] [12]

- Pallor, lethargy, and dyspnoea
- Fever, malaise, features of mouth, throat, skin, respiratory, perianal, or other infections
- Spontaneous bruises, purpura, bleeding gums, and menorrhagia
- Tender bones
- Moderate hepatosplenomegaly
- Meningeal syndrome: nausea, headache, vomiting, blurring of vision…
- Lymphadenopathy

2.6.1.4.2.4 Biological features [10] [12]

- **Peripheral blood**

- Anaemia: normochromic normocytic
- Thrombocytopenia
- Total white cell count: decreased, normal, or increased

- Blood film: variable numbers of blast cells
  - **Bone marrow examination**
- Bone marrow aspirate and trephine: bone marrow hypercellular with >20% leukemic blast cells.
- Microscopic examination of the blast cells' morphology **(Figure 7)**

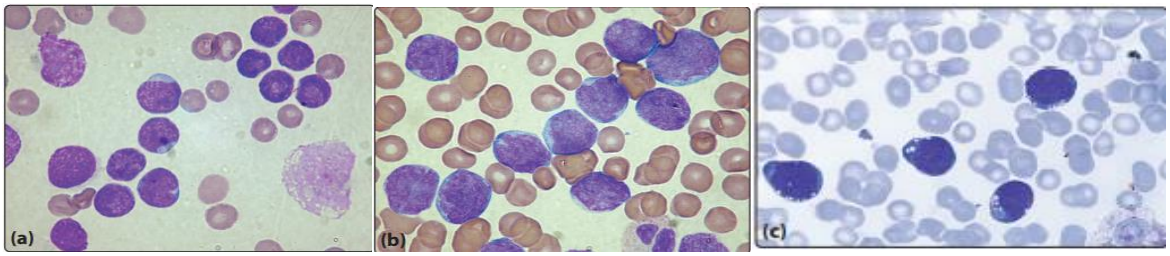

Figure 7. Morphology of ALL blast cells **[10]**

(a): Small uniform blast cells with scanty cytoplasm

(b): Large heterogeneous blast cells with nucleoli and low nuclear-cytoplasmic ratio

(c): Basophilic vacuolated blast cells.

- **Cytogenetics and molecular genetics**

Like CML, karyotyping and FISH may be used to detect BCR-ABL gene (if it is present, the ALL is Ph+. If it is absent, the disease is Ph-) **[10]**

RT-PCR is routinely performed to obtain a rapid diagnosis of Ph + ALL **[13]**

- **Immunophenotyping**

Immunophenotyping enables the detection of characteristic immunological surface and cytoplasmic markers for T-acute lymphoblastic leukaemia and B-lymphoblastic leukaemia. **[12] (Table 5)**

Table 5. Immunological markers for the classification of ALL **[12]**

	ALL	
Marker	**B**	**T**
B lineage		
CD19	+	–
cCD22	+	–
cCD79a	+	–
CD10	+ or –	–
clg	+ (pre-B)	–
slg	–	–
TdT	+	+
T lineage		
CD7	–	+
cCD3	–	+
CD2	–	+
TdT	+	+

c, cytoplasmic; s, surface.

2.6.1.4.2.5 Treatment [10] [12]

- **BCR-ABL negative ALL**

- Chemotherapy
- Radiotherapy
- Autologous or allogeneic stem cell transplantation

- **BCR-ABL positive ALL**

Imatinib is used in combination with chemotherapy and can achieve disease remission in most patients. Second-generation TKIs are also currently in use. However, recurrence is more common with the emergence of resistant subclones containing mutations in the BCR-ABL1 gene. Therefore, once remission is achieved, allogeneic stem cell transplantation is recommended whenever possible. **[12]**

- **Monitoring the response to treatment in ALL Ph+**

The existence of minimal residual disease (MRD) in children at day 29 or adults after 3 months of treatment has prognostic value in ALL and is increasingly considered in therapy planning. An MRD negative at one month in children and three months in adults indicates a favourable prognosis, whereas a positive MRD at 3 to 6 months is indicative of a bad prognosis. **[12]**

2.6.2 Chronic myeloproliferative diseases

2.6.2.1 Definition

Chronic myeloproliferative diseases are defined by the clonal proliferation of an abnormal hematopoietic stem cell. **[14]**

2.6.2.2 Classification

These disorders are subdivided into subcategories: **[15]**

- Chronic Myeloid Leukaemia (CML)
- Classical Philadelphia-negative myeloproliferative neoplasms: Polycythaemia Vera (PV); Essential Thrombocythemia (ET); Primary Myelofibrosis (PMF)
- Non-classical Philadelphia-negative myeloproliferative neoplasms: Chronic Neutrophilic Leukaemia and Chronic Eosinophilic Leukaemia.
- Unclassifiable myeloproliferative neoplasms

2.6.2.3 Chronic myeloid leukaemia

2.6.2.3.1 Definition

Chronic myeloid leukaemia is a clonal disease of a pluripotent stem cell, characterised by the presence of the Philadelphia (Ph) chromosome which is the result of the t(9;22) (q34;q11) translocation between chromosomes 9 and 22. **[8]**

2.6.2.3.2 Classification

The classification of CML Ph+ is displayed in **Table 6**

Table 6. WHO 2016 classification of CML Ph+ **[6]**

Myeloproliferative neoplasms	
Chronic myeloid leukaemia, *BCR-ABL1*–positive	9875/3
Chronic neutrophilic leukaemia	9963/3
Polycythaemia vera	9950/3
Primary myelofibrosis	9961/3
Essential thrombocythaemia	9962/3
Chronic eosinophilic leukaemia, NOS	9964/3
Myeloproliferative neoplasm, unclassifiable	9975/3

2.6.2.3.3 Phases of CML

CML is divided into three main phases: chronic phase (CP), accelerated phase (AP), and blast crisis (BC). **[16]**

Chronic phase: Blasts in blood or bone marrow are less than 10% **[17]**

Accelerated phase: ≥15% blasts in the peripheral blood or bone marrow **[18]**

Blast crisis: is characterised by the presence of over 20% blasts in the peripheral blood or bone marrow. **[19]**

2.6.2.3.4 Genetics

2.6.2.3.4.1 ABL gene

2.6.2.3.4.1.1 ABL Structure

The Abelson oncogene (c-ABL) is located at position 9q34 on chromosome 9. The name comes from the Abelson (v-ABL) gene, a viral homolog that causes leukaemia in mice. **[20]**

The ABL gene encodes a 145 kd non-receptor tyrosine kinase protein that plays a major role in signal transduction and regulation of cell proliferation. **[21]**

It consists of domains:

In the N-terminal region: the Scr Homologous (SH1) domain carries tyrosine kinase function, the SH2 domain binds to the phosphotyrosine-containing consensus site, and the SH3 domain binds to the proline-rich consensus sequence of the protein. The C-terminal region contains actin and DNA binding domains, three nuclear localization signals, and nuclear export signals. **[22]**

Abl's SH2 and SH3 domains are necessary for autoinhibition. **[22]**

The SH3 domain is a negative regulator of the SH2 domain, and the SH2 domain is a positive regulator of the SH1 domain that supports the tyrosine kinase activity of the Abl protein. **[20]**

The tyrosine kinase or SH1 domain is the basic functional domain of the carcinogenic activity of the BCR-ABL1 fusion protein. **[23]**

The Abl gene has 11 exons, spanning 230 kilobases (kb). It is transcribed into two different transcripts with the first alternative exons called 1a and 1b, respectively. **[21] [24]**

The main difference in the proteins encoded by these transcripts is that only the Abl protein from the Abl 1b transcript contains a myristoylation site. **[24]**

Myristoylation is a modification by adding a saturated fatty acid lipid group to glycine residues. **[20]**

This modification allows the protein derived from the 1b transcript to be anchored to the plasma membrane. **[24]**

The absence of this glycine residue in the 1a form (majority) is the reason for the predominant nuclear localisation. **[24]**

Abl protein is expressed in most somatic cells and is evenly distributed throughout the cell (nucleus and cytoplasm). **[25]**

Although ABL is primarily expressed in the nucleus, it can continuously reciprocate between these two compartments and perform several different functions. **[22] [24]**

The ABL structure is shown in **Figure 8**

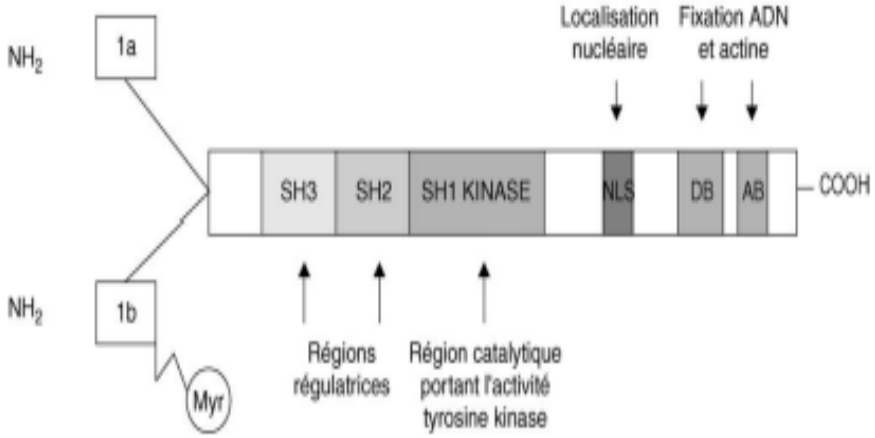

Figure 8. Schematic representation of Abl protein **[20]**

2.6.2.3.4.1.2 ABL Functions

The functions of the ABL protein are complex. **[22]**

In the nuclear compartment, ABL acts as a negative regulator of the cell cycle. When located in the cytoplasm, the ABLprotein plays an important role in cell growth and proliferation, participating in the transduction of the signal initiated by certain receptors to growth factors. **[20]**

Positive and negative regulations of cell growth by ABL protein depend essentially on its subcellular localisation (cytoplasmic or nuclear), its phosphorylation state, and its level of expression. **[24]**

Other functions of ABL protein include cellular response to genotoxic stress, **[22]** apoptosis, and control of cell adhesion. **[24]**

2.6.2.3.4.2 BCR gene

2.6.2.3.4.2.1 BCR Structure

The BCR gene, located on the long arm of chromosome 22, spans 135 kb and 23 exons, allowing transcription of two messenger RNAs that encode ubiquitously expressed proteins. **[20]**

The BCR protein consists of several domains: **(Figure 9)**

In the N-terminal part, the 1B domain is an important region since it allows the dimerization of the BCR-ABL protein leading to the opening of the kinase activity. **[20]** It is called the coiled-coil domain. **[24]**

The 2B domain includes two binding sites to the SH2 domains like those carried by the ABL protein. **[20]**

The central region has a homology domain with diffuse B-cell lymphoma (Dbl) proteins (guanosine triphosphate exchange factor [GTP]/guanosine diphosphate [GDP]). **[20]**

The C-terminal part of BCR, absent in the BCR-ABL fusion protein, has a GTPase activating protein (GAP) function. **[20]**

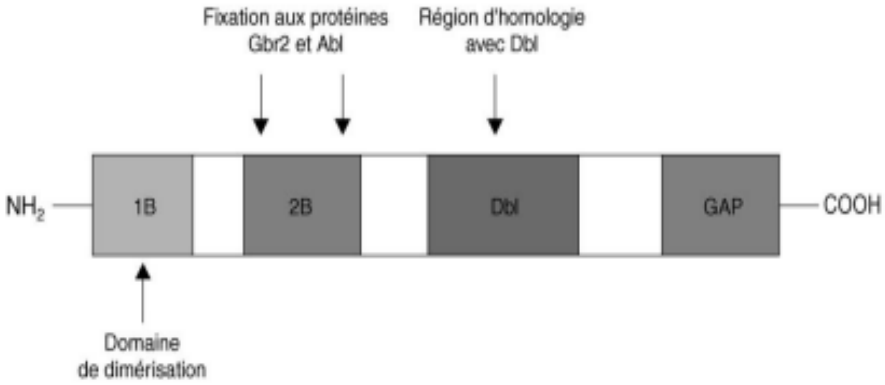

Figure 9. Schematic representation of BCR protein **[20]**

2.6.2.3.4.2.2 BCR Functions

The current functions of the BCR protein are little known. **[20]**

2.6.2.3.4.3 Philadelphia chromosome BCR-ABL

2.6.2.3.4.3.1 BCR-ABL fusion

The Philadelphia chromosome was the first recurrent chromosomal abnormality identified in hematologic disease. **[26]**

This chromosome is characteristic of CML but is also found in 5% of childhood acute lymphoblastic leukaemia (ALL) and 15-30% of adult ALL, and less frequently in *de novo* acute myelogenous leukaemia (<1%) **[23]**

It is a shortened chromosome 22 resulting from the reciprocal translocation t (9; 22) -(q34; q11) between the long arms of chromosomes 9 and 22. **[21]**

Part of the oncogene ABL1 is moved to the BCR gene on chromosome 22, and another part of chromosome 22 moves to chromosome 9. The chimeric BCR-ABL1 gene codes for a fusion protein that presents enhanced tyrosine kinase activity. **[8] (Figure 10)**

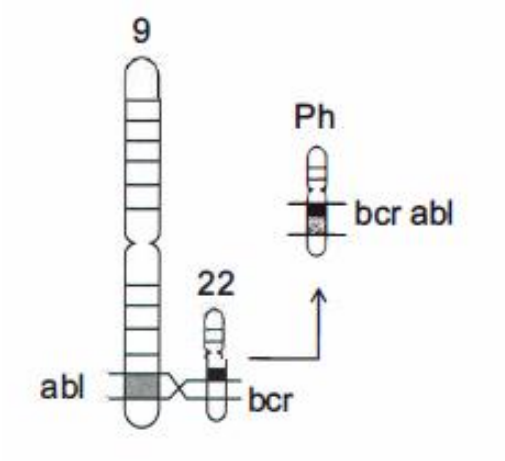

Figure 10. Philadelphia chromosome **[27]**

The mechanisms leading to the t (9;22) (q34; q11) translocation can be facilitated by the close proximity between chromosomes 9 and 22 in interphase cells or the presence of homologous sequences on the two chromosomes. **[25]**

Different sized segments of the BCR gene are fused to the ABL segment to generate fusion genes that encode different-sized mRNAs. **[23]**

Breakpoints within ABL occur upstream of exon 1b, downstream of exon 1a, or more commonly between exon 1b and 1a. **[28]**

There are three main breakpoints in the BCR.

- **Major-Breakpoint Cluster Region (M-BCR)**

This breakpoint contains five exons originally designated b1 to b5, corresponding to BCR exons e10 to e14. There may be two different types of BCR-ABL transcript junctions. In the first case, the BCR exon 13 is spliced to ABL exon 2 (e13a2 junction, formerly b2a2) and the second BCR exon 14 is spliced to ABL exon 2 (e14a2 junction, formerly b3a2). **[24]**

The M-BCR hybrid mRNA transcript is an 8.5 kb sequence encoding the 210 kd fusion protein p210BCR-ABL. **[22]**

The protein encoded by b3a2 is more common and contains 25 more amino acids than the protein encoded by b2a2. **[20]**

However, there is no significant difference in disease phenotype between either protein isoform. **[22]**

P210BCR-ABL is found in most patients with CML and about one-third of patients with Ph-positive acute lymphoblastic leukaemia (ALL). **[29]**

- **Minor-BCR (m-BCR)**

This rearrangement binds the sequence of the first BCR exon to ABL exon 2 (e1a2 junction). **[24]**

The m-BCR hybrid mRNA transcript is a 7 kb transcript in which exon e1 in BCR is linked to exon a2 in ABL. Transcripts of e1a2 junctions can be detected at very low levels in patients with M-BCR. **[22]**

The translated product is a 190 kd fusion protein, p190BCR-ABL (also known as p185BCR-ABL). **[22]**

P190BCR-ABL is found in the remaining patients with ALL and rarely in patients with CML. It is clinically characterised by predominant monocytosis, **[29]** usually a characteristic of myelomonocytic leukaemia (CMML). **[22]**

1% of patients with acute myeloid leukaemia (AML) also carry the p190 (m-BCR) or p210 (m-BCR) BCR-ABL fusion gene. **[30]**

- **Micro-BCR (µ-BCR):**

µ-BCR joins the BCR exon 19 to the ABL exon 2 (e19a2). It is found in a small proportion of CML patients. This transcript yields a fusion protein with a molecular weight of 230 kd (P230). **[24]**

P230BCR-ABL is the largest BCR-ABL fusion protein and was first described in the context of neutrophilic CML (also known as chronic neutrophil leukemia). **[22]**

It was also discovered in classical CML and AML. **[30]**

Rare cases of other junctions such as b2a3, b3a3, e1a3, e6a2, and e2a2 have been reported in patients with ALL and CML. **[29]**

Different BCR-ABL gene junctions are shown in **Figure 11**

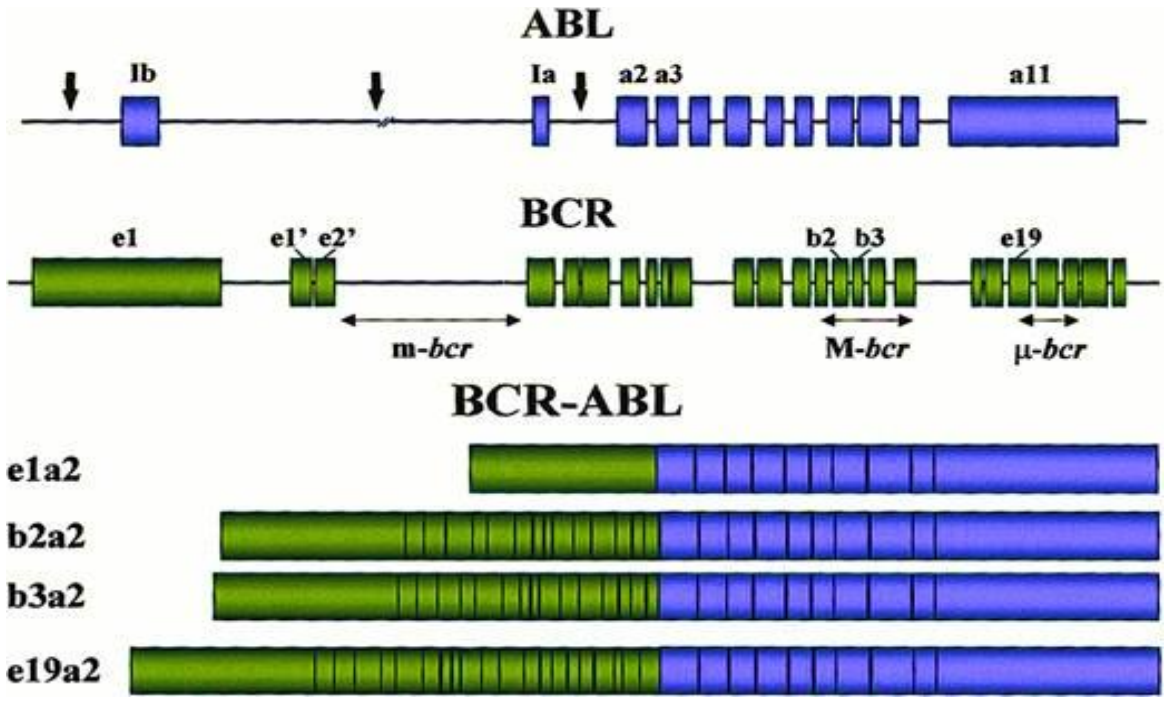

Figure 11. BCR-ABL chimeric genes **[29]**

2.6.2.3.4.3.2 BCR-ABL oncogene structure and functions

The 210 kd BCR-ABL protein is composed of three domains, SH1, SH2, and SH3, and all other domains of ABL. On the BCR side, the dimerization pattern is the most important part. This part of the BCR leads to the dimerization of the BCR-ABL protein and its autoactivation by transphosphorylation. In addition, the loss of the N-terminal portion of ABL suppresses its auto-inhibition. Changes in these two proteins explain the sustained activation of BCR-ABL tyrosine kinases. **[20]**

Since the ABL component of the gene is largely unchanged, changes in disease phenotype may be due to the protein sequence encoded by the translocation partner BCR. **[22]**

The BCR-ABL fusion protein has constitutive and deregulated tyrosine kinase activity that is critical for hematopoietic cell transformation. **[24]**

Tyrosine kinase is an enzyme that transfers phosphate from ATP to tyrosine residues in certain cellular proteins. They help control important cellular functions, including proliferation and differentiation. **[24]**

Tyrosine kinases can be activated by chromosomal translocations. As a result, changes in their function are one of the most common findings in human cancer. **[24]**

2.6.2.3.4.3.3 BCR-ABL oncogenic mechanisms

BCR-ABL plays a causal role in the aetiology of CML. BCR-ABL-transformed cells exhibit disordered cell proliferation, growth factor independence, **[28]** activation of the mitogenic signalling pathways, inhibition of apoptosis, and altered cell adhesion. **[24]**

- Activation of mitogenic signalling pathways

BCR-ABL leads to the activation of multiple signalling pathways that give hematopoietic cells malignant properties. **[31]**

Among these pathways: RAS, MYC, STAT, and PI3K/Akt pathways.

- RAS Pathway

Ras (rat sarcoma virus) proteins represent a group of a large family of membrane-localized small GTPases. They regulate cell proliferation, differentiation, migration, and apoptosis. **[32]**

One of the key signalling pathways constitutively activated in CML haematopoiesis is regulated by the Ras protein and its relatives. Ras activation is due to the interaction of P210 with other cytoplasmic proteins. These cytoplasmic proteins act as adapter molecules and form multiprotein signalling complexes. **[24]**

The importance of Ras signalling in CML is primarily associated with increased proliferation induced by BCR-ABL activation. **[24]**

- MYC pathway

The activation of the protooncogene MYC, which is expressed at high levels in CML cells, appears to be independent of the activation of the Ras signalling pathway. **[24]**

This transcription factor is known to induce transcription of specific genes involved in the cell cycle and apoptosis in response to mitogenic signals. **[24]**

- STAT pathway

The signal transducers and activators of transcription (STATs) proteins are a family of seven cytoplasmic transcription factors that contribute to signalling through cytokines, hormones, and growth factors. STAT proteins control basic cellular processes such as survival, proliferation, and differentiation. **[33]**

STAT1 and STAT5 are constitutively activated in BCR-ABL-positive cell lines and primary cells of CML patients. P190BCR-ABL can also activate STAT6. **[24]**

In normal cells, STAT nuclear translocation occurs only after the binding of cytokines to the receptor and is mediated by the activation of the receptor-related Janus Kinase (JAK). However, the activation of STAT by the BCR-ABL protein is direct. **[24]**

- PI3K/AKT pathway

Phosphoinosytol-3-kinase (PI3K) is an enzyme that converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (PIP3), an important second messenger in the growth and survival pathways. **[34]**

Protein kinase B (PKB also known as Akt) is a serine / threonine-specific protein kinase that plays an important role in controlling the balance between cells' survival and death pathways. **[35]**

Akt acts as a master switch for PI3K signalling and targets several important downstream signalling pathways. **[34]**

BCR-ABL can inhibit apoptosis at the cytoplasmic level, primarily by activating the PI3K / Akt pathway. **[24]**

In PI3K/Akt pathway, PI3K signalling is essential for the proliferation of Ph chromosome-positive CML cells, but not for normal hematopoietic precursors. In addition, Akt promotes the anti-apoptotic signalling required for leukaemia transformation and final onset. **[36]**

- Inhibition of apoptosis

The BCR-ABL protein induces the degradation of proteins involved in DNA repair. **[20]**. Therefore, BCR-ABL-positive cell lines are resistant to DNA damage-induced apoptosis, which leads to the initiation of multiple proliferative and anti-apoptotic signals that are difficult to isolate. BCR-ABL then shifts the balance towards inhibition of apoptosis while simultaneously providing growth stimuli. **[29]**

- Alteration of cellular adhesion

In normal haematopoiesis, progenitor cells attach to bone marrow stromal cells and their associated extracellular matrix. The latter contains proteins such as fibronectin that act as adhesive ligands for receptors expressed on the surface of hematopoietic progenitor cells **[22]**

The process of adhesion is essential for the regulation of haematopoiesis because it immobilizes progenitor cells near cytokine-secreting cells and provides a means of exposing them to specific fate-determining signals. Depending on the cytokine message they receive (or do not receive), the progenitors can be induced to survive or undergo apoptosis. Surviving progenitors can be stimulated to stop proliferating or growing. Proliferation may include replication and self-renewal of pluripotent progenitor cells, or it may include differentiation and commitment to the lymphoid or myeloid lineages. **[22]**

In CML, Ph-positive progenitor cells exhibit reduced adhesion to stromal cells and extracellular matrix, releasing them from regulatory signals delivered to normally adhering hematopoietic progenitor cells. **[22]**

The expression of adhesion molecules remains unchanged, but their function and the signals they induce are deregulated. **[20]**

2.6.2.3.4.4 Genetic instability in CML

BCR-ABL-positive leukaemia cells exhibit a genetic instability, probably due to the acquisition of additional genetic alterations. **[20] [24]**

In the case of CML, this genetic instability is recognised and increases as the chronic phase progresses to the advanced phase of the disease (accelerated and blast phases). **[20]**

Continuous unlimited expression and activity of BCR-ABL1 kinase are paramount to the maintenance as well as the progression of CML. BCR-ABL mRNA and protein levels are higher in BP than in CP. **[28]**

In addition, many carcinogenic proteins work with BCR-ABL and may be involved in disease progression. **[20]**

2.6.2.3.5 Clinical features [18]

- Hypermetabolism
- Splenomegaly
- Pallor, dyspnoea and tachycardia
- Bruising, epistaxis, menorrhagia or haemorrhage
- Gout
- Rare symptoms: visual disturbances and priapism

2.6.2.3.6 Biological features [18]

- **Peripheral blood**

- Leucocytosis
- Increased circulating basophils
- Normochromic normocytic anaemia
- Decreased platelet count, normal, or increased

- **Bone marrow examination**

- Hypercellular bone marrow with mainly granulocytes

- **Cytogenetics and molecular genetics**

- Karyotyping: Presence of BCR-ABL1 t (9;22) (q34; q11) translocation in 98% of the cases (**Figure 12**)

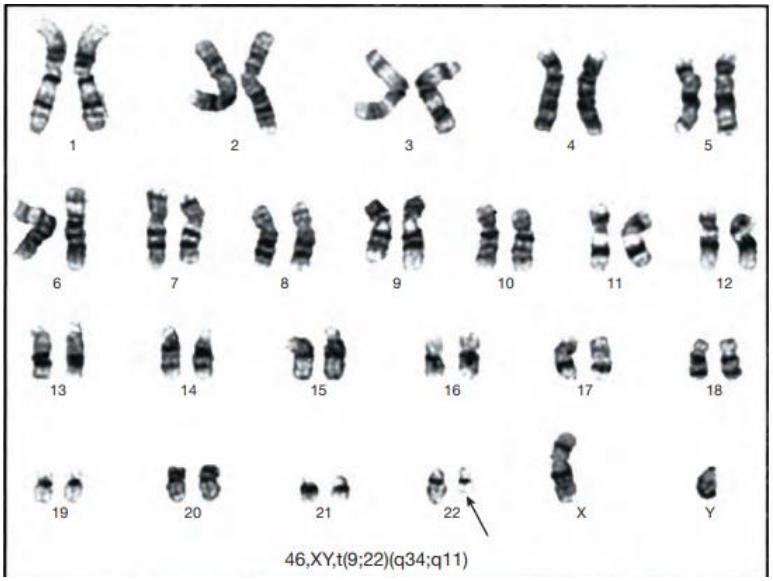

Figure 12. Karyotype showing the t (9;22) (q34; q11) translocation **[18]**

- Fluorescence *In Situ* Hybridisation (FISH) is performed on cytological or cytogenetic preparations, by hybridising fluorescent genomic probes complementary to the BCR and ABL1 genes. Hybridisation sites are detected by the microscopic examination of the fluorescent entities. The co-localisation of the BCR and ABL1 probes indicates the fusion of the genes. **[23] (Figure 13)**

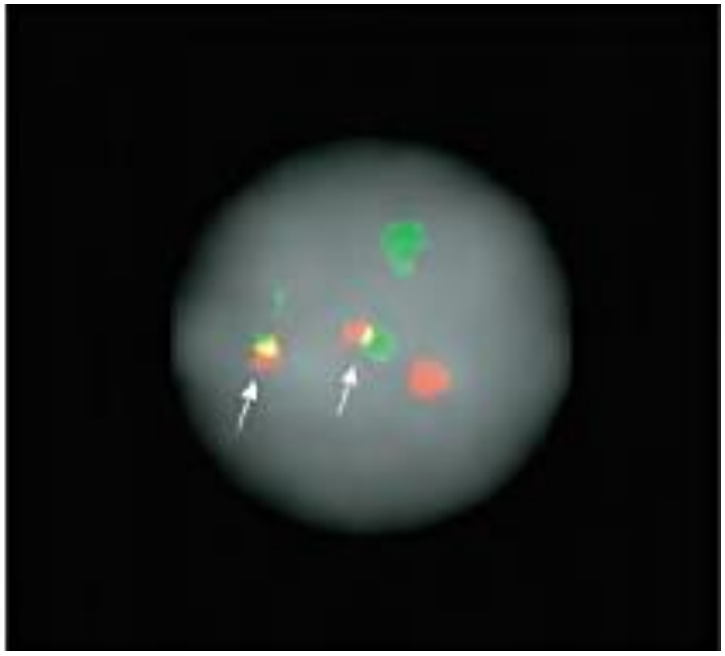

Figure 13. Visualization of the Ph chromosome on interphase cells by fluorescence *in situ* hybridization (FISH) analysis (ABL probe in red and BCR probe in green) with fusion signals (red/green which shows as yellow) on the Ph (BCR-ABL1) **[14]**

- RT-PCR: RT-PCR has become the gold standard for diagnosis to detect the BCR-ABL fusion gene. Reverse transcriptase-polymerase chain reaction (RT-PCR) highlights BCR-ABL fusion transcripts from bone marrow cells or more simply blood samples. This allows the detection of BCR-ABL transcripts at the RNA level and defines the molecular subtypes produced. **[20] [21]**

This test is currently essential for the diagnosis of CML. **[21]**

2.6.2.3.7 Differential diagnosis:

Chronic myelomonocytic leukaemia (CMML) is defined by monocytosis of more than 1.0 $\times 10^9$/L and blasts <20% in the bone marrow, dysplasia in other lineages, and negative BCR-ABL1 translocation **[37]**

Atypical CML is characterised by an increased white cell count with predominantly granulocytes and granulocyte precursors in the blood, hypercellular bone marrow, absence of BCR-ABL1 fusion gene, and features of myelodysplasia in the bone marrow or the blood. **[37]**

Chronic neutrophilic leukaemia: rare cases of CML with a p230 BCR-ABL transcript might be mistaken for this subtype. **[18]**

Essential thrombocythaemia:

Essential thrombocythaemia is a chronic myeloproliferative neoplasm characterised by a persistent increase in platelet count due to megakaryocyte proliferation and overproduction of platelets. **[38] [39]**

Polycythaemia vera:

Polycythaemia vera is a myeloproliferative neoplasm in which a pluripotential stem cell undergoes a mutation. polycythaemia is an increase in red blood cell count, haemoglobin, and Packed Cell Volume (PCV) above the normally accepted levels. **[38] [40]**

2.6.2.3.8 Prognostic scores and prognosis

- **Sokal score:**

Sokal score was introduced to classify patients treated with hydroxyurea into three risk groups: low risk (Sokal score <0.8), medium risk (Sokal score 0.8-1.2), and high risk (> 1.2). It uses in its calculation these baseline variables: age, spleen size, platelet count, and the percentage of blasts. The formula of the score is as follows: exp (0.0116 x (age [years] − 43.4)) + (0.0345 x (spleen size [cm] − 7.51) + (0.188 x ((platelets [109/L]/700)^2 − 0.563)) + (0.0887 x (blasts [%] − 2.10)) **[41]**

- **Hasford index (EURO score):**

The Hasford Index or EURO score was introduced to classify patients treated with interferon into three risk groups: low risk (score ≤ 780), medium risk (score 781-1480) and high risk (score ≥ 1481). Its calculation is based on these variables: age, spleen size, percentages of basophils, eosinophils and blasts, and platelet count. **[41]**

- **EUTOS score:**

The European Treatment and Outcome Study (EUTOS) scoring system stratifies patients treated with imatinib. It uses the percentages of basophils and spleen size as baseline variables in the calculation to distinguish between two risk groups: low risk (score <87) and high risk (score ≥87). **[41]**

ELTS score:

The European Treatment and Outcome Study (EUTOS) long-term survival (ELTS) score predicts disease-specific mortality in chronic myelogenous leukaemia (CML) patients treated with imatinib during the chronic phase (CP) of the disease **[42]**. Its formula is as follows: $0.0025 \times (\text{age}/10)^3 + 0.0615 \times \text{spleen} + 0.1052 \times \text{blasts} + 0.4104 \times (\text{platelet count}/1000)^{-0.5}$ **[43]**

It classifies the patients into three risk groups: low risk: score (≤1.5680), intermediate risk: (score 1.5680-2.2185), and high risk: (score >2.2185) **[43]**

RT-PCR analysis shows that TKI therapy is very effective, with progression-free survival of 85–90% after 5 years of treatment and overall survival of over 90%. Approximately 60% of patients who become negative for the BCR-ABL1 transcript remain negative or are in remission with stable, low transcript levels after stopping TKI therapy. For those who become BCR-ABL1 positive again with increased transcript levels, treatment with TKIs usually results in a negative BCR-ABL1 status. Therefore, TKIs may be able to treat some patients with CML. **[18]**

2.6.2.3.9 Treatment [18]

- Chemotherapy: Hydroxycarbamide

Mechanism of action of hydroxycarbamide:

Hydroxycarbamide, also known as hydroxyurea inhibits ribonucleotide reductase, which is an enzyme that catalyses the reduction of ribonucleoside diphosphate to deoxyribonucleotide precursors for *de novo* DNA replication and DNA repair. **[44] (Figure 14)**

Adult patients with chronic myelogenous leukaemia treated with hydroxyurea present an immediate decrease in white blood cell count, a decrease in splenomegaly, elimination of precursor cells from the bone marrow and peripheral blood, and an increase in haemoglobin level. **[45]**

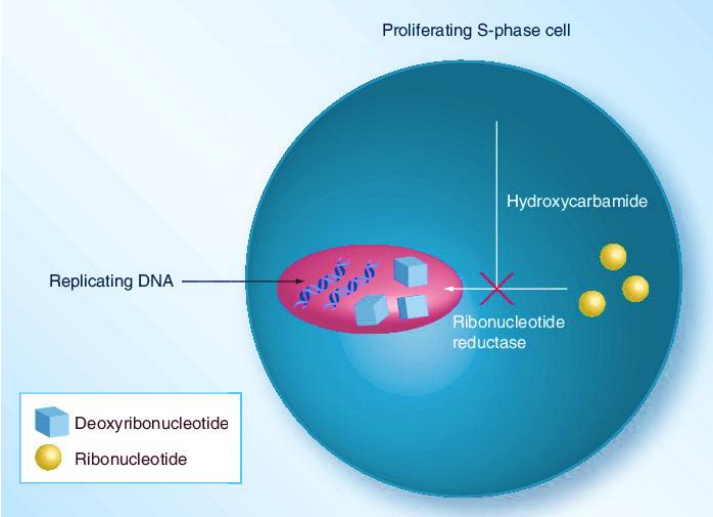

Figure 14. Hydroxycarbamide-induced inhibition of ribonucleotide diphosphate reductase in proliferating cells to inhibit *de novo* DNA synthesis **[46]**

❖ **Pharmacokinetics of hydroxycarbamide**

- **Absorption**

Oral absorption of the drug is almost complete

In cancer patients, the bioavailability of hydroxyurea has reached 79% in systemic circulation after oral administration. **[47]**

- **Distribution**

Hydroxyurea diffuses rapidly into tissues such as the brain. **[47]**

- **Metabolism**

The metabolism is little known. However, some studies have shown that the most efficient biotransformation happens in the liver and kidneys. **[47]**

In the liver, the conversion of hydroxyurea to urea is mediated by the monooxygenase system in the mitochondria. In addition, the degradation of hydroxyurea by urease present in the intestine might produce hydroxylamine. **[47]**

- **Excretion**

Elimination occurs through both renal and non-renal mechanisms. **[47]**

Hydroxyurea is eliminated by metabolism to urea and other products through the nonrenal mechanism. **[47]**

By renal mechanism, a metabolised fraction of hydroxyurea is recovered in the urine as urea. Another fraction is recovered unchanged in the urine. **[47]**

- α-Interferon
- Tyrosine kinase inhibitors (TKI)

First-generation TKI

Imatinib was the first TKI developed for CML, resulting in a high rate of complete cytogenetic responses and improved survival in patients with this disease. However, about 35% of CML patients in the chronic phase treated with imatinib have developed resistance or intolerance to this drug. Recognising the problem of imatinib failure has led to the development of second-generation TKIs. **[48]**

Second-generation TKI

Two second-generation TKIs, dasatinib and nilotinib, were approved as second-line treatment for patients who are resistant or intolerant to previous treatments (including first-generation TKI: imatinib): dasatinib is approved at all stages of CML and nilotinib was approved only in the CP or AP. Both were later approved as first-line treatments for the newly diagnosed Ph+ CP-CML in adults. **[48]**

Another second-generation TKI, bosutinib, was approved for the treatment of adults with CP, AP, or BP-Ph + CML who are resistant or intolerant to previous treatment with one or more TKIs. The indication was later expanded to include newly diagnosed Ph+ CP-CML in adults. **[48]**

Third-generation TKI:

Ponatinib, a third-generation TKI, is approved for the treatment of adults with CP, AP, or BP Ph + CML that is resistant or intolerant to other TKIs. It is also approved for patients with CP, AP, or BP-Ph + CML and the T315I mutation, which is known to be involved in imatinib resistance. **[48]**

❖ **Mechanism of action of imatinib**

Imatinib binds to the BCR-ABL kinase domain and prevents the transfer of phosphate groups to tyrosine on protein substrates and the subsequent activation of phosphorylated proteins. It

blocks the transmission of proliferation signals to the cell nucleus and induces apoptosis in leukemic cells. **[49] (Figure 15)**

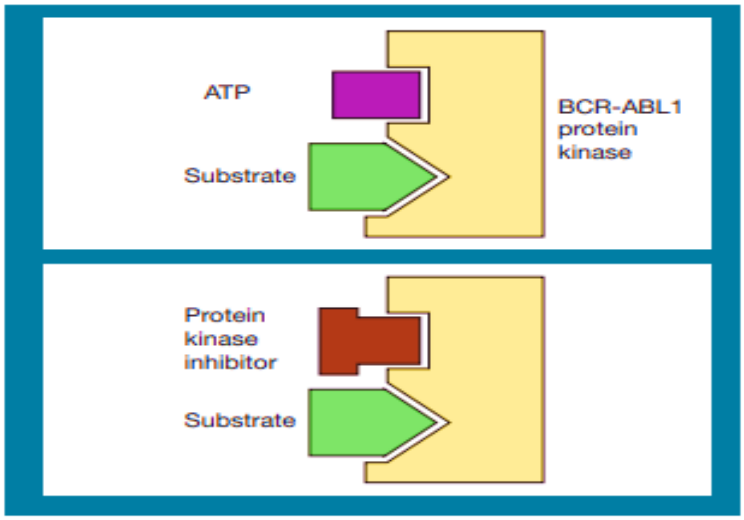

Figure 15. Mode of action of the tyrosine kinase inhibitor Imatinib **[18]**

❖ **Pharmacokinetics of imatinib**

- **Absorption**

Oral imatinib is well absorbed, with a bioavailability of 98% regardless of oral administration form or dosage. Maximum plasma concentrations are achieved within 1-2 hours. **[50]**

- **Distribution**

Approximately 95% of imatinib binds to human plasma proteins, especially albumin and α1-acid glycoprotein. It is characterized by widespread distribution in the organism. **[50]**

- **Metabolism**

Imatinib is primarily metabolized in the liver by cytochrome P450 (CYP) 3A4 or CYP3A5. **[50]**

- **Excretion**

The active substance is mainly excreted in the bile as metabolites, such as (CGP 74588) which has the same pharmacological activity as the parent substance. The elimination half-life is approximately 18 hours. **[50]**

➢ Stem cell transplantation

- **Monitoring the response to treatment**

RQ-PCR provides a reliable, high-throughput method for accurate assessment of therapeutic response and provides early signs of new drug resistance. **[51]**

According to NCCN, there are four types of cytogenetic response**. (Figure 16)**

Cytogenetic (Philadelphia chromosome)	• Complete cytogenetic response (CCyR): No Philadelphia chromosomes (Ph-) • Major cytogenetic response (MCyR): Ph+ are between 0% and 35% • Partial cytogenetic response (PCyR): Ph+ are between 1% and 35% • Minor cytogenetic response: Ph+ are between 36% and 65%

Figure 16. Types of cytogenetic response according to NCCN 2021 **[52]**

According to ELN 2020 recommendations for treating CML, TKIs are usually monitored at 3, 6, and 12 months by RT-PCR analysis of BCR-ABL1 transcripts. The molecular response is evaluated as the ratio of the BCR-ABL1 transcript to the ABL1 transcript, which must be expressed and reported as BCR-ABL1 % on a log scale, where 1%, 0.1%, 0.01%, 0.0032%, and 0.001% correspond to a decrease of 2, 3, 4, 4.5, and 5 logs, respectively, below the standard baseline. **[53]**

- BCR-ABL1 ≤ 1% is equivalent to complete cytogenetic remission (CCyR)
- BCR-ABL1 transcript level ≤0.1% is defined as major molecular response (MMR)/ MR3.
- A BCR-ABL1 transcript level ≤0.01% is defined as MR4.
- A BCR-ABL1 transcript level ≤0.0032% is defined as MR4.5.
- A BCR-ABL1 transcript level ≤0.001% is defined as MR5.

The term “complete molecular response” should be avoided and replaced with the term "molecularly undetectable leukaemia" with indication of the number of the control-gene transcripts. **[53]**

The evaluation of the response to TKIs is shown in **Table 7**

Table 7. The response to TKIs as first-line treatment in CML **[18]**

	Optimal	Warning	Failure
3 months	BCR-ABL1 ≤10% *and/or* Ph+ ≤35%	BCR-ABL1 >10% *and/or* Ph+ 36–95%	Non-CHR *and/or* Ph+ >95%
6 months	BCR-ABL1 <1% *and/or* Ph+ 0	BCR-ABL1 1–10% *and/or* Ph+ 1-35%	BCR-ABL1 >10% *and/or* Ph+ >35%
12 months	BCR-ABL1 ≤0.1%	BCR-ABL1 >0.1–1%	BCR-ABL1 >1% and/or Ph+ >0
Then, and at any time	BCR-ABL1 ≤0.1%	CCA/Ph– (–7, or 7q–)	Loss of complete haematological response Loss of complete cytogenetic response Loss of major molecular response (BCR-ABL1 expression of ≤0.1%) Mutations Clonal chromosome abnormalities in Ph+ cells

CCA, complex cytogenetic abnormalities; CHR, complete haematological response.

III. Molecular biology

Molecular biology is the result of the combination of genetics and biochemistry, two branches of biology that were developed in the early twentieth century, each with a clear object of study: genes for genetics, and proteins and enzymes for biochemistry. It emerged when the relationship between these two objects became clearer. Scientists identified the gene as a macromolecule (DNA), Its structure was determined and its role in protein synthesis was described. **[54]**

Molecular biology consists of techniques and discoveries that facilitate the performance of molecular analyses of the most fundamental biological processes, especially those involved in the stability, survival, and reproduction of organisms. **[54]**

History

The development of the techniques necessary for the study of macromolecules has taken place between **1920** and **1940**, and the new tools destined for biological analysis were created between **1940** and **1965**. Relevant operational management was achieved between **1972** and **1980** due to the development of genetic engineering. **[54]**

In **1965**, Robert Holley and his team managed to produce the first complete nucleic acid sequence, the alanine tRNA from *Saccharomyces cerevisiae*. **[55]**

During the mid-**1970s**, many techniques were discovered. The major advances that have remarkably contributed to the evolution of DNA sequencing appeared in **1977** with the development of Sanger's "chain-terminating inhibitors." **[55]**

Before the development of PCR in the **1980s**, the main method of making multiple copies of a gene was a time-consuming process called DNA cloning. This method is based on the insertion of the gene of interest into bacterial cells, which then replicate the gene along with their DNA during the processes of division and replication. **[56]**

In **1985**, the American chemist MULLIS Kary invented the **P**olymerase **C**hain **R**eaction (**PCR**). This technique was used for the first time to amplify the DNA fragment of the beta-globin to study the genetic profile of sickle cell anaemia. **[57]**

In **1992**, HIGUCHI Russell proposed to detect the accumulated products of PCR with Ethidium Bromide (EtBr): a fluorescent intercalator. **[58]** In the following year, Molecular Probes filed a patent for the innovation and development of an intercalator: SYBR® Green I capable of binding non-specifically with the DNA. Also, in the same year, the Nobel Prize in Chemistry was awarded to MULLIS Kary for his invention of the PCR which proved its usefulness in biology. **[58]**

Many thermocyclers were developed and their performance was gradually enhanced through time. In **1996**: Idaho Technology commercialized the first fluorometer*:* Idaho Technology LC 24 for micro-samples which presented a rapid control of temperature. **[58]** A year later, Roche Molecular Biochemicals produced the first LightCycler^{TM} **[58]**

3 Polymerase chain reaction

The fusion of the discovery of the thermostable DNA polymerase and the technique of repeated rounds of DNA synthesis has given scientists a very powerful technique known as the **P**olymerase **C**hain **R**eaction (**PCR**). **[59]**

3.1.1 Definition

Polymerase **C**hain **R**eaction (**PCR**) is a highly sensitive technique that allows rapid amplification of specific DNA segments. It makes billions of copies of a particular DNA fragment or gene, enabling the detection and identification of gene sequences using size- and charge-based visual techniques. **[60]**

3.1.2 Types of PCR

3.1.2.1 Amplification of Refractory Mutation System ARMS PCR

Amplification of Refractory Mutation System (ARMS) PCR is a technique used to detect changes in a single nucleotide in a sequence. It is used to detect only known mutations. **[61]**

3.1.2.2 Hot-start PCR

The purpose of the hot-start polymerase chain reaction (PCR) is to optimize the yield of the desired amplification product in PCR while suppressing non-specific amplification and primer dimer formation. **[62]**

3.1.2.3 Colony PCR

Colony PCR is a technique used to detect the presence or absence of DNA segments inserted into bacterial plasmid colonies. **[61]**

3.1.2.4 Nested PCR

This type of PCR is used to increase the specifications of the PCR to amplify the required regions of the DNA template and eliminate non-specific amplified regions. **[61]**

3.1.2.5 Asymmetric PCR

Asymmetric PCR is used to amplify only one strand of the DNA molecule with an unequal amount of primer concentration. **[63]**

3.1.2.6 Inverse PCR

This technique is used to amplify DNA sequences that are adjacent to one end of a known DNA sequence and for which primers are not available. **[64]**

It is an excellent tool to introduce the desired mutation at the desired position in the circular double-stranded DNA sequence. **[65]**

3.1.2.7 Degenerate PCR

Degenerate PCR is a technique that uses degenerate primers whose design is based on sequenced gene homologs, to amplify unknown DNA sequences. **[66]**

3.1.2.8 Multiplex PCR

Multiplex PCR is a technique that amplifies many targets using multiple initiator pairs in a single PCR experiment. **[61]**

3.1.2.9 Touchdown PCR

Touchdown polymerase chain reaction is a method used to reduce off-target priming and augment PCR specificity. **[67]**

3.1.2.10 AFLP PCR

Amplified **F**ragment **L**ength **P**olymorphism (**AFLP**) technology is based on the use of PCR to amplify a subset of genomic restriction fragments. **[68]**

3.1.2.11 SSP PCR

Single **S**pecific **P**rimer (**SSP**) PCR is a method that enables the amplification of genes for which only partial sequence information is available, allowing one-way genome walking from known to unknown regions of the chromosome. **[69]**

3.1.2.12 PCR-SSO

PCR-**S**equence-**S**pecific **O**ligonucleotide (**PCR-SSO**) is a technique based on sequence-specific oligonucleotide methods that fuse a series of synthetic probes designed to bind to the complementary strand of target exon DNA from the cell under evaluation. These probes are combined with primers, buffers, and Taq polymerase for PCR amplification. **[70]**

3.1.2.13 Digital PCR

Digital PCR is a highly sensitive and specific technique that accurately quantifies nucleic acids. **[71]**

3.1.2.14 Reverse Transcription PCR

The reverse transcription-polymerase chain reaction is a sensitive molecular in vitro technique and has an important role in the medical field. **[72]**

It is a modified version of conventional PCR in which RNA molecules are first converted to complementary DNA (cDNA) molecules and then amplified by PCR. **[73]**

3.1.2.15 Real-Time PCR

Real-Time or Quantitative PCR is the continuous collection of fluorescent signals from one or multiple polymerase chain reactions over a range of cycles. **[74]**

3.1.2.16 Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (RT-qPCR) is a technique that combines four steps: **[75]**

- Isolation of RNA
- reverse transcription of RNA and cDNA synthesis
- Amplification of cDNA by PCR
- Detection and quantification in real-time of amplification products

3.1.2.17 GeneXpert PCR

GeneXpert-based assays are used to identify leukaemia cells containing the BCR-ABL gene fusion from blood samples. This automated instrument is based on RT-qPCR technology and detects only major breakpoint (M-BCR) **[76]**

IV. Real-Time PCR

4.1 Genetic materials

Small amounts of DNA are directly quantified by quantitative PCR (real-time PCR) **[77]**

For qualitative detection of RNA expression, the reverse transcription-polymerase chain reaction (RT-PCR) technique is used to convert RNA templates into cDNA, and both Reverse Transcription-PCR and real-time PCR techniques are integrated for quantitative detection of RNA expression. **[78]**

Reverse transcription PCR is used to amplify, isolate, or identify RNA sequences. In this technique, the reverse transcriptase enzyme converts the RNA sequence into its complementary DNA (cDNA) sequence. A second enzyme, DNA polymerase, synthesises a second strand of DNA that forms, along with the cDNA, a double-strand DNA (dsDNA) molecule which can be amplified by traditional PCR. **[79] (Figure 17)**

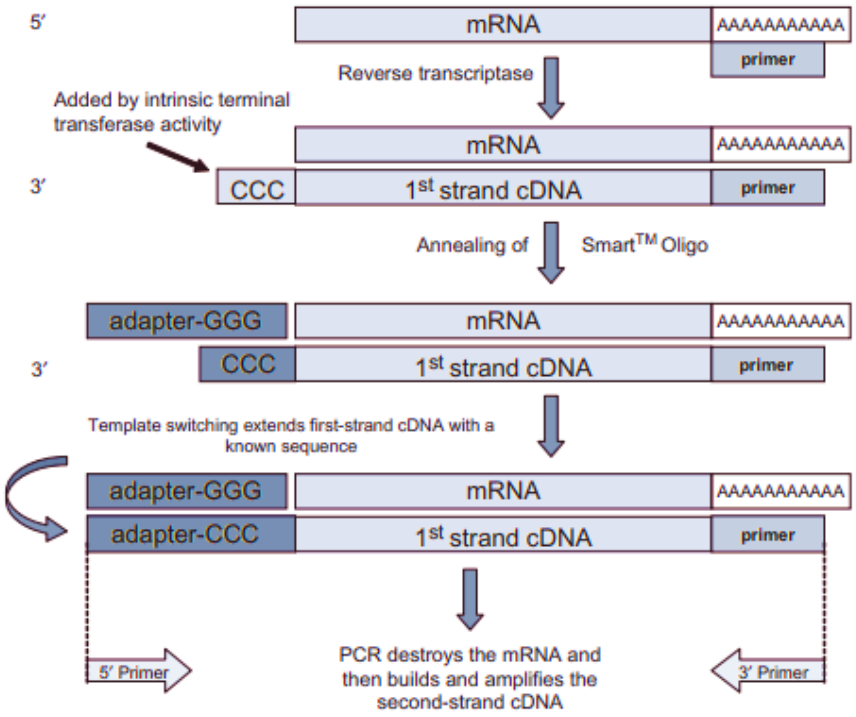

Figure 17. Template-switching cDNA synthesis using SMART™ (Clontech) technology **[80]**

- **Applications of reverse transcription PCR [21] [81]**

- Diagnosis and quantification of RNA of viruses causing infections (e.g., hepatitis C)
- Analysis of mRNA transcripts (e.g., mRNA transcripts produced by translocations generating non-Hodgkin's lymphomas, BCR-ABL1 gene…)
- Gene expression profiling

4.2 Principle of PCR

The principle of PCR is based on the replication in vitro of specific DNA sequences using DNA Polymerase. The DNA sample extracted from an organism can't be directly analysed because it contains many nucleotide sequences. Therefore, only the DNA sequences of interest are to be isolated and purified. The purified DNA fragments are then amplified, which generates a large number of copies. The amplification process is subdivided into three major phases: denaturation, hybridization with primers, and elongation. **[80] (Figure 18)**

4.2.1 Denaturation

The temperature must be high (optimal temperature is approximately 94 °C) to destroy the hydrogen bonds and transform double-stranded DNA into single-stranded DNA. **[82]**

4.2.2 Hybridisation

This phase requires primers, which are oligonucleotides chemically produced in a way that assures the best complementarity with both ends of the DNA sequence of interest. For that purpose, it is mandatory to have at least one pair of oligonucleotides, so that one identifies and binds complementarily with the sequence positioned upstream the fragment 5'-3' of the DNA sequence of interest, and the other one also binds complementarily with the sequence upstream the fragment 3'-5'. **[82]**

The temperature is decreased and maintained between 40 °C and 70 °C to allow the hydrogen bonds of the single-stranded DNA to reform and bind to complementary strands (primers). The higher the temperature, the more specific the process is. **[82]**

4.2.3 Elongation

In this phase, the Taq polymerase enzyme is of high importance. Its main role is to allow replication *in vitro*. It is extracted and purified from a microorganism called *Thermus aquaticus* that lives in hot regions. This explains the capacity of this enzyme to resist temperatures around 100 °C. Its optimal temperature of activity is around 72 °C. **[82]**

The temperature, therefore, is maintained at 72 °C. At this temperature, the synthesis of complementary strands happens with the contribution of Taq polymerase that binds to the primed single-stranded DNAs and catalyses the replication by consuming the deoxyribonucleoside triphosphate in the process. The whole operation takes 20-40 cycles to obtain an analysable quantity of DNA. **[82]**

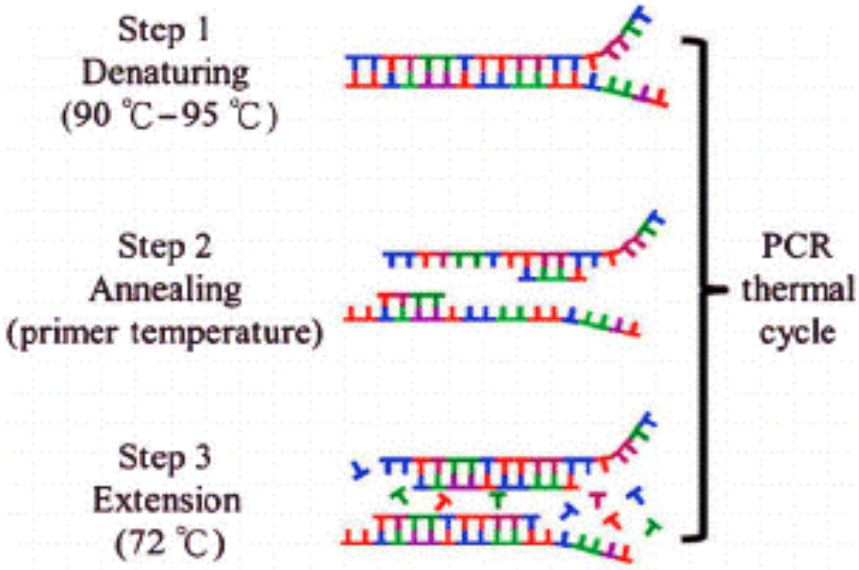

Figure 18. PCR was developed to exponentially amplify short segments of a longer DNA chain. A single thermal cycle of PCR includes the three steps of denaturing, annealing, and extension **[83]**

4.3 Real-Time PCR Instrumentation

There exist many well formats: 48-well plates, 96-well plates, 384-well plates **[84],** or 1536-well plates. In the pharmaceutical industry, the 1536-well plates represent the standard throughput screening. In individual laboratories, the 384-well plates are placed to augment the sample throughput. However, 96-well plates are the highest limit for hand pipetting. **[85]** The high throughput of the system enables the processing of a large number of samples with minimal effort. **[86]**

Real-time PCR instruments are composed of three main constituents: **[85]**

- Light sources: determine the range of reporter dyes used by the instrument
- Detection systems: determine the spectral range and the sensitivity of the assay
- Thermal cycling mechanism: Determines the rate at which the assay can be run, the uniformity of the temperature variations from sample to sample, and the number of samples taken simultaneously

4.3.1 Light sources

- **Argon-Ion Laser**: available only on the ABI 7900, emits strong light primarily at 488 nm. This is the ideal excitation wavelength for reporters such as 6-FAM. However, moving towards the red spectrum, the excitation energy of the 488 nm light source weakens the stimulation of the reporter dye and at the same time produces a weak emission signal. Weak signals can limit the usefulness of reporter dyes **[85]**
- **LED (light emitting diode) lasers**: emit light in the spectral range of 30-40 nm. The energy output of these lasers is not as high as that of argon ion lasers, but the power consumption and heat generated are significantly reduced at a lower cost per unit **[85]**
- **Quartz tungsten halogen lamps**: emit constant light from 360nm to over 1,000nm. They are called "white light sources" because they emit light over the entire visible spectrum. To select the desired wavelengths for multiple reporter dyes, two sets of excitation and emission filters are required **[85]**
- **Xenon lamps** are brighter than quartz-halogen-tungsten lamps and cover a similar spectral range. Five excitation filters coupled with six emission filters may allow six different assays in a single multiplex reaction **[85]**

4.3.2 Detection systems

The Real-Time PCR converts the fluorescent signals from each reaction into a numerical value for each sample. **[75]**

There are two main principles for the quantitative detection of amplicons: agents binding to double-stranded DNA (like SYBR® Green I) and fluorescent probes. In the second category, four major technologies exist: probe hydrolysis (TaqMan® assay), hybridization probes (HybProbes), molecular beacons, and scorpion primers. **[87]**

- **SYBR® Green I assay:** is a method in which the dye (intercalating agent) emits fluorescence after binding to the minor groove of double-stranded DNA molecules. **(Figure 19)**
- The advantages of this assay are the cost-effectiveness and the low risk of contamination of the tubes **[88]**
- The disadvantage, on the other hand, is the base pair of any double-stranded DNA, which reduces the specificity of the assay **[88]**

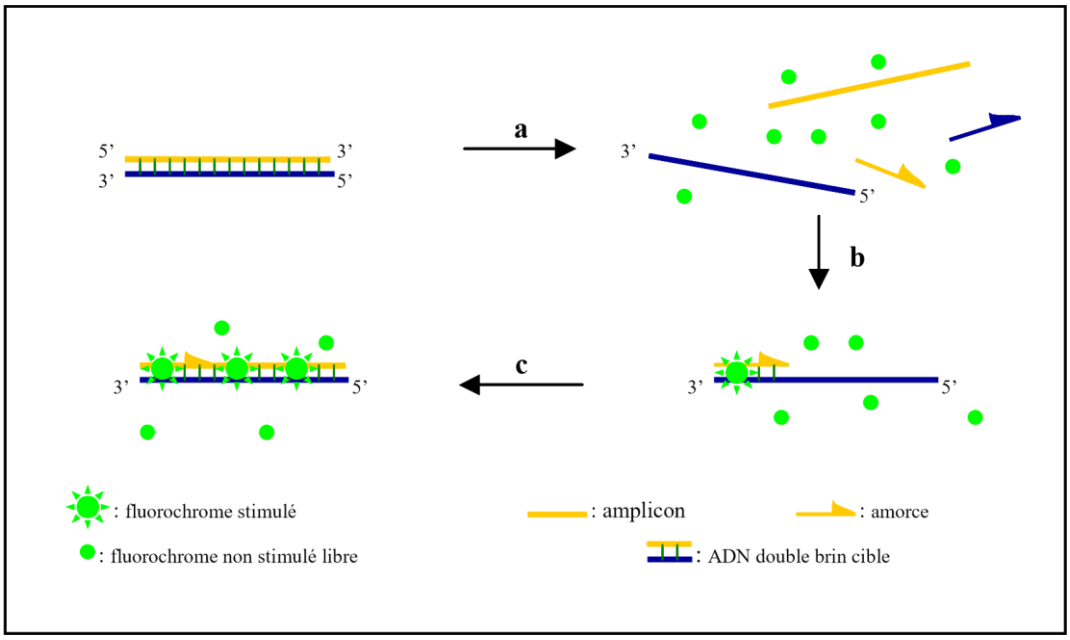

Figure 19. Agents binding to double-stranded DNA binding dyes (LightCycler™ assay)

(a) During denaturation, free SYBR® Green I dyes exhibit little fluorescence**. (b)** At pairing temperature, some molecules bind to the double strand of nascent DNA resulting in fluorescence emission during excitation. **(c)** During the polymerisation phase, an increasing number of molecules bind to the nascent strand and the increase in fluorescence can be monitored in real-time. **[87]**

Many dyes are used in real-time PCR instruments. The frequent ones are displayed in **Figure 20**

Free dyes	Max. ab (nM)	Max. em (nM)
SYBR® Green I	497	525
EvaGreen™	497	525
BOXTO™	515	552
Reporter dyes	**Max. ab (nM)**	**Max. em (nM)**
Pulsar® 650	460	650
Fluorescein™	492	520
6-FAM	494	518
Alexa 488™	495	519
JOE™	520	548
TET™	521	536
Cal Fluor Gold 540™	522	544
Yakima Yellow™	530	549
HEX™	535	556
Cal Fluor Orange 560™	538	559
VIC™	538	554
Quasar® 570	548	566
Cy3™	552	570
TAMRA™	565	580
Cal Fluor Red 590™	569	591
Redmond Red™	579	595
ROX™	580	605
Cal Fluor Red 635™	618	637
LightCycler®640	625	640
Cy5™	643	667
Quasar® 670	647	667
LightCycler®705	685	705
Dark dyes	**Max. ab (nM)**	**Max. em (nM)**
DABCYL	453	None
BHQ0™	495	None
Eclipse™	522	None
Iowa Black™ FQ	531	None
BHQ1™	534	None
BHQ2™	579	None
Iowa Black™ RQ	656	None
BHQ3™	680	None

Figure 20. Dyes used in real-time PCR and their wavelengths of absorption and emission **[85]**

- **TaqMan® assay:** is a fluorescent detection method. The actual form of this assay is a double-labelled fluorescent dye oligonucleotide probe (usually 18-22 bases) designed to anneal the 5' primer and 3' primer to the template between adjacent regions. A reporter fluorochrome is linked at the 5' end of the probe, most commonly 6carboxyfluorescein (6FAM), and a non-fluorescent quencher is attached to the 3' end. The spatial proximity of the reporter to the quencher quenches the reporter's fluorescence via fluorescence resonance energy transfer also known as FRET. **[88]**

  At a temperature between 68 and 72 °C, the 5' exonuclease linked to the Taq polymerase displaces and cleaves the probe, which anneals to the target strand when the upstream primer is elongated. The fluorescent reporter dye is then emitted from the probe and begins to fluoresce upon laser excitation. 5' → 3' Taq polymerase activity propagates through the region previously blocked by the currently displaced probe, resulting in a PCR product. As reporter fluorochromes are released from the probe and diffused from the quencher, more fluorescence is detected through the fibre optic system. **[88] (Figure 21)**

- The advantages of this method are its high specificity and the possibility of labelling the probe with several different reporter dyes (FAM, VIC, TET, TAMRA, HEX, JOE, ROX, Cy5, Texas Red…). This allows multiple detections of two or more different pathogens in the same reaction, improving throughput **[89]**
- The downside of the TaqMan® probe is its expensive price **[89]**

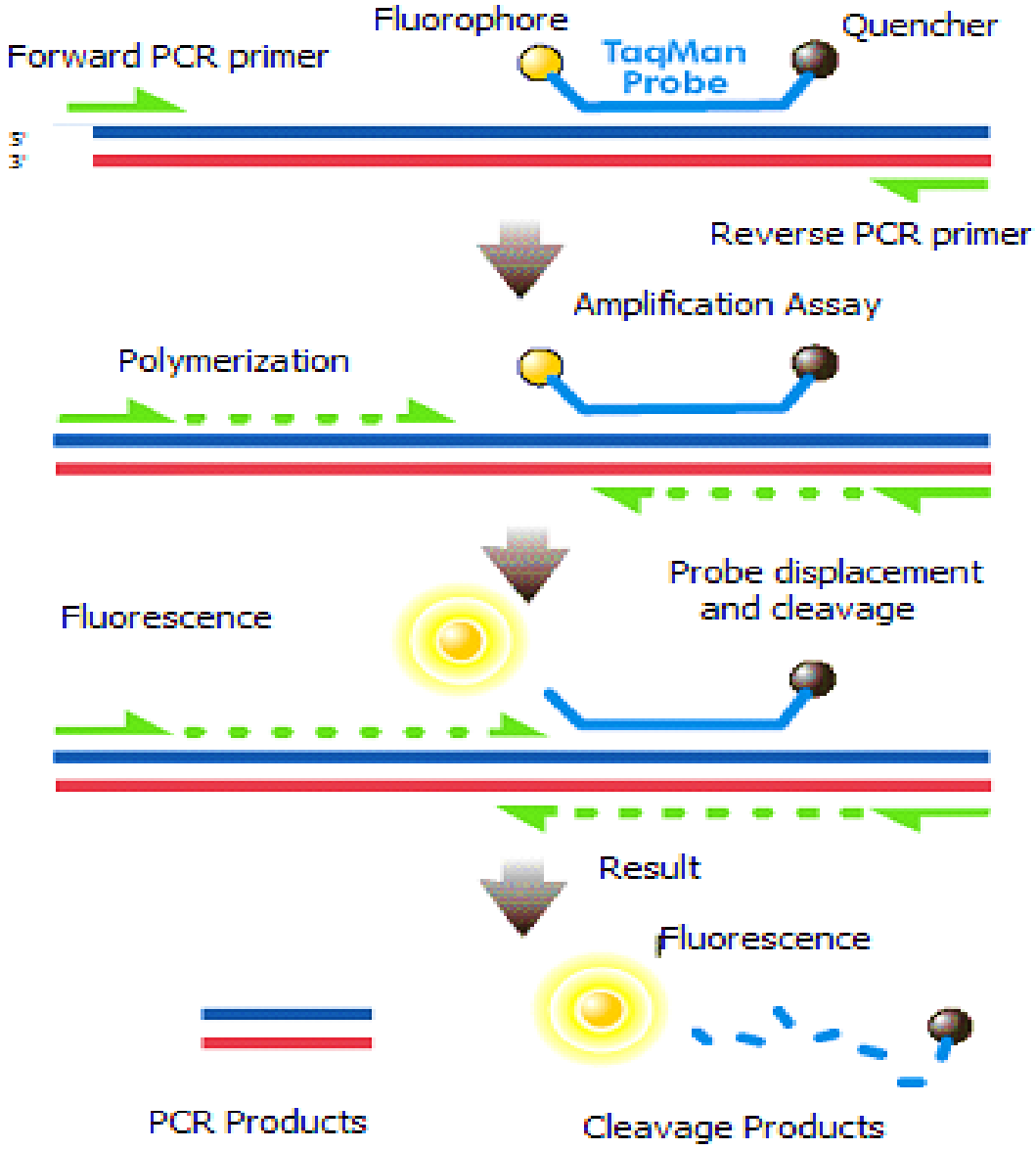

Figure 21. Real-time RT-PCR with TaqMan® fluorescent probes **[90]**

- **Hybridisation probes (HybProbes):**

Hybridisation probes are RNA or DNA oligonucleotides that bind to specific nucleotide sequences to form a probe-analyte hybrid. The formation of stable hybrids indicates the presence of DNA or RNA fragments that are complementary to known probe sequences. **[91]**

The first probe carries a donor fluorochrome that is blocked at the 3' end to prevent extension during the elongation step and produces green fluorescence when excited by a light source. The acceptor fluorochrome is attached to the 5' end of the second probe. Both probes are free and separated in solution. During the hybridisation step, the probe binds to its target

sequences. The close proximity of the two fluorochromes permits energy transfer from green fluorescein to the red acceptor fluorochrome following the FRET principle, which triggers the emission of fluorescence. During the polymerization step, the two probes independently return to the solution, eliminating red fluorescence. **[87] (Figure 22)**

- The advantages of these probes are: **[87]**
  - High specificity
  - High flexibility, especially in their design
  - Ability to use them more than once
  - General principles of the design of TaqMan® probes are applied to these probes as well.
- The disadvantages are: **[92]**
  - RNA probes produce higher background signals
  - The double-stranded DNA (dsDNA) probe must be transformed into single-stranded DNA (ssDNA) before the hybridisation and can be reannealed, which weakens the hybridisation signals
  - Hybrids that form DNA probes are less stable than hybrids that form RNA probes, and assays that use DNA probes are less sensitive than assays that use RNA probes

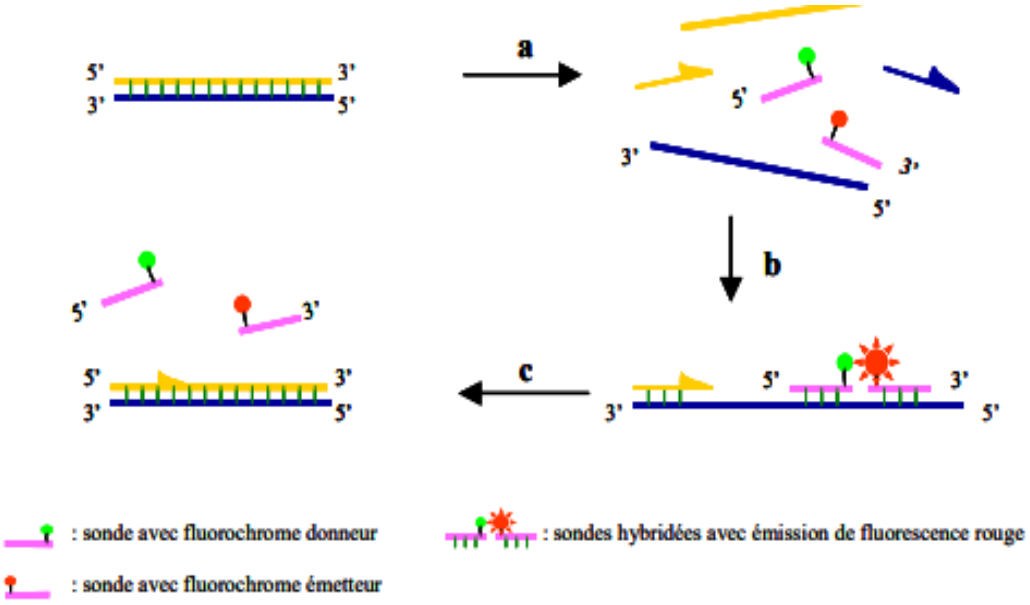

Figure 22. Hybridisation probes **[87]**

(a) During the denaturation step, the two probes remain separate and in solution. **(b)** At the pairing temperature, the probes hybridize to their respective target sequences and the proximity of the fluorochromes allows red fluorescence to be released according to the FRET principle. **(c)** The probes are liberated in solution. **[87]**

- **Molecular beacons** are specific oligonucleotide probes with a covalently linked fluorescent dye at the 5' end and a quencher dye at the 3' end. They have a characteristic stem-loop structure which keeps the 5' and 3' ends close together. **[88] [93] (Figure 23)**

- The advantages of these probes are the following
  - Highly efficient quenching of the reporter signals due to the close proximity of the two dyes **[93]**
  - Simultaneous targets can be detected using different coloured fluorophores **[94]**
  - Ability to distinguish different targets with a single base **[94]**
  - Usefulness in diagnostic assays **[94]**
- The disadvantage of these probes is that they require a large complementary target sequence to guarantee the stability of the complex **[95]**

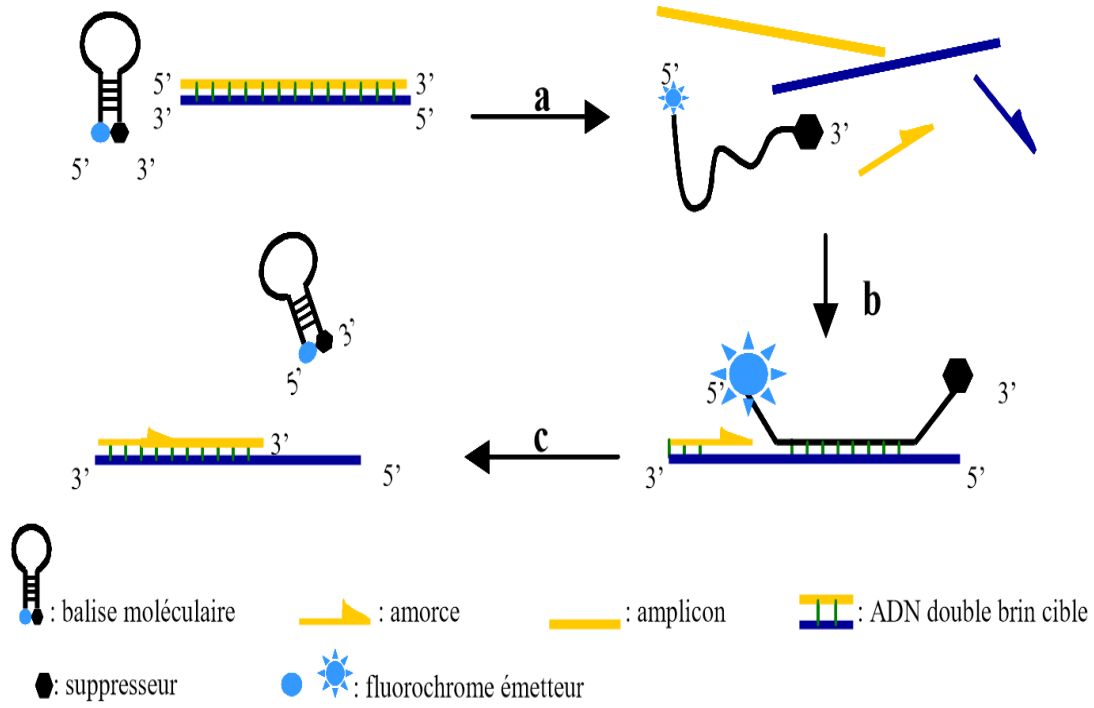

Figure 23. Molecular beacons **[87]**

(a) During the denaturation step, the molecular beacon is relaxed and free in solution but the proximity of fluorochromes allows inhibition of fluorescence. **(b)** When the probe hybridizes to its target sequence, the released fluorochrome is sufficiently distant from its quencher to allow the emission of fluorescence. **(c)** At the polymerization stage, the molecular beacon returns to its original form of a hairpin. **[87]**

- **Scorpions® primers:** are a variant of molecular beacon technology, where fluorochromes and the probe are irreversibly incorporated into the amplicon during PCR amplification. The addition of hexaethylene glycol (HEG) molecules, also known as blockers (stoppers), is mandatory to prevent the replication of molecular beacons by DNA polymerase. The 5' fluorescent dye emitter is FAM or ROX and the suppressor is usually methyl red. The primer region of the scorpion allows the molecular beacon to be incorporated into a new amplicon during the PCR reaction. Hairpin loops permit the hybridization of the probe to the complementary target sequences on the amplicon. **[87] (Figure 24)**
- The advantage of this technology is its high efficiency compared to TaqMan® technologies and molecular beacons, especially in a PCR programme with very short cycles. **[87]**

- The disadvantages are: **[96]**
  - Length and complexity of the probes
  - Reliance on secondary structure for their performance

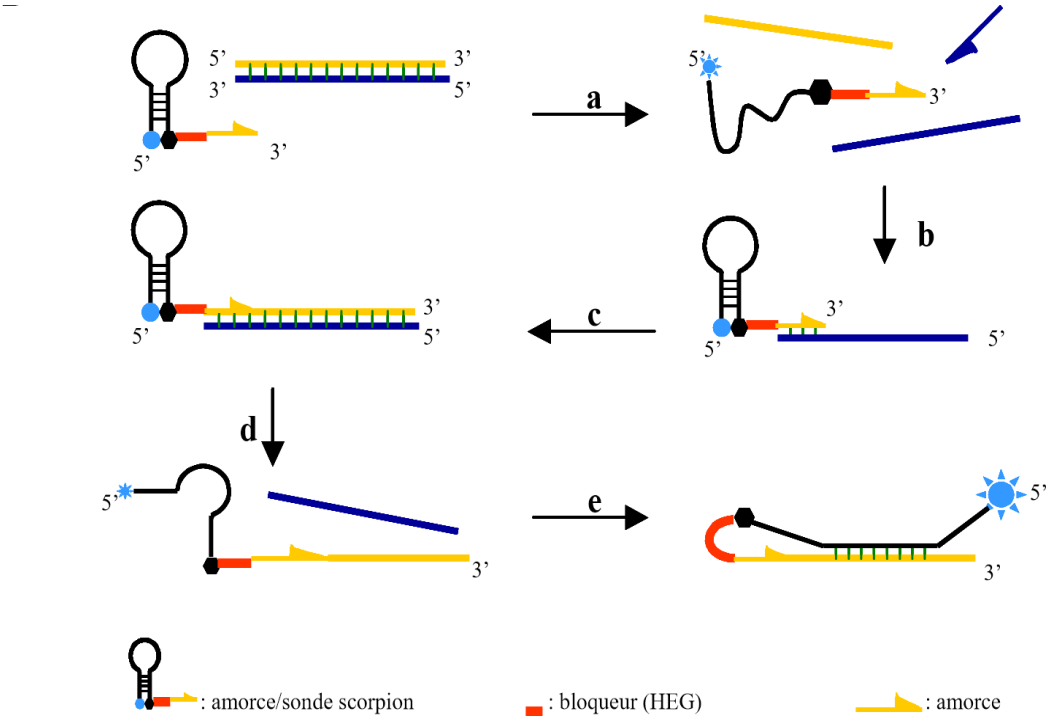

Figure 24. Scorpion primers **[87]**

(a) During the denaturation phase, the molecular beacon is in its free form in the solution but the proximity of fluorochromes allows inhibition of fluorescence. **(b)** Attachment of the scorpion primer to the target complementary sequence. **(c)** Polymerization of the complementary strand. **(d)** DNA strand denaturation. **(e)** The hybridisation of the complementary sequence of the molecular beacon part to its target sequence allows the emission of fluorescence. **[87]**

4.3.3 Thermal cycling mechanism

The thermal cycler is an automated system that is used in the PCR to perform the periodic temperature variations required for enzymatic amplification of specific DNA segments. The microcomputer automatically performs numerous thermal cycles and is flexible enough to change the temperature profile from cycle to cycle. **[97] (Figure 25)**

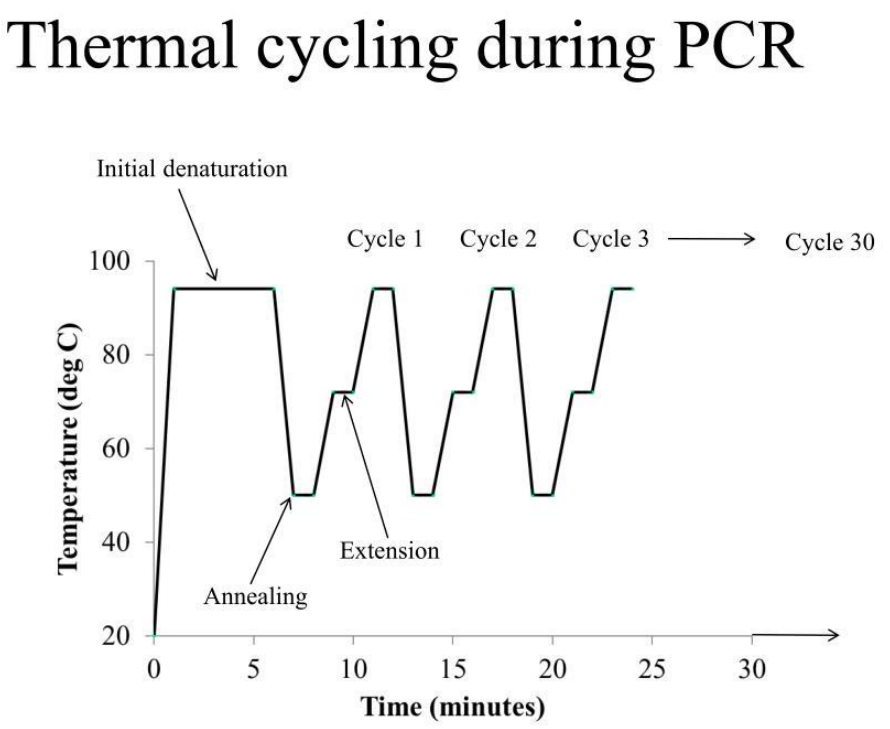

Figure 25. Thermal cycling during PCR **[98]**

4.4 Data collection and analysis

4.4.1 Data collection

Fluorescent data collected from real-time PCR should be processed by a data analysis method. There are multiple techniques used for real-time PCR data analysis. **[99]**

Software tools are usually used for automatic data analysis. The software evaluates the recorded data, applies new algorithms for adaptive background correction of signal trends, calculates the valid signal noise, and detects exponential phases. Furthermore, it verifies the validity of the results. **[100]**

Although many software packages provide different levels of automated data analysis, the results may not be as good as the manual manipulation of baseline and threshold settings. Most programmes offer a way to export metadata to a file that can be opened in Microsoft Excel® and export the graphic view to one or more graphic formats. These exports are extremely useful for further data analysis in other programmes and the integration of data or graphics in publications and presentations. **[85]**

4.4.2 Data analysis

There are two ways to work on data analysis in a real-time PCR application.

The first is a relative quantification method that compares the amplification of the experimental template with the amplification of the control sample. **[88]**

The second method, called absolute quantification, is used to statistically calculate the precise number of copies of a template molecule. This involves comparing the amplification behaviour of the experimental sample to the standard curve, called the standard curve method. **[88]**

4.4.2.1 Relative quantification

In relative quantification, the reference can be any transcript so long as its sequence is known, and the determination of its concentration is not required. **[101]**

Fluorescence values are recorded for each cycle, representing the number of amplicons produced at a given point in the reaction. This point is known as the threshold cycle (C_T). The relative quantification method is also known as the $\Delta\Delta C_T$ method because the C_T value of an unknown sample is compared with the amplification behaviour of the reference sample. **[88]**

The "threshold cycle" is the basis for accurate and repeatable quantification. It appears in the exponential phase of amplification, and its value can be converted into a quantitative result by comparing it to the C_T value generated by a known quantification matrix. **[87] [102]**

The higher the amount of template at the beginning of the reaction, the more likely it is that a significant increase in fluorescence will be detected. **[88]**

Two important points to note: **[88]**

(1) Low C_T value ➔ High template abundance

(2) Early detection is best to avoid quantifying during the variable plateau phase.

Amplification for each template occurs in four phases: **(Figure 26)**

1- Baseline: the baseline phase includes amplification below the limit of detection of the real-time instrument. There are no detectable signals, but exponential amplification of the template takes place during these cycles **[103]**

2- Exponential: The exponential phase consists of the earliest detectable signal of the polymerase chain reaction, with amplification proceeding at a maximum exponential rate. The length of this phase depends essentially on the template concentration and the quality of the real-time assay **[103]**

3- Linear: in the linear phase, the amplification efficiency decreases **[103]**

4- Plateau: in the plateau phase, there is little, if any, accumulation of product for each passing cycle. **[88]**

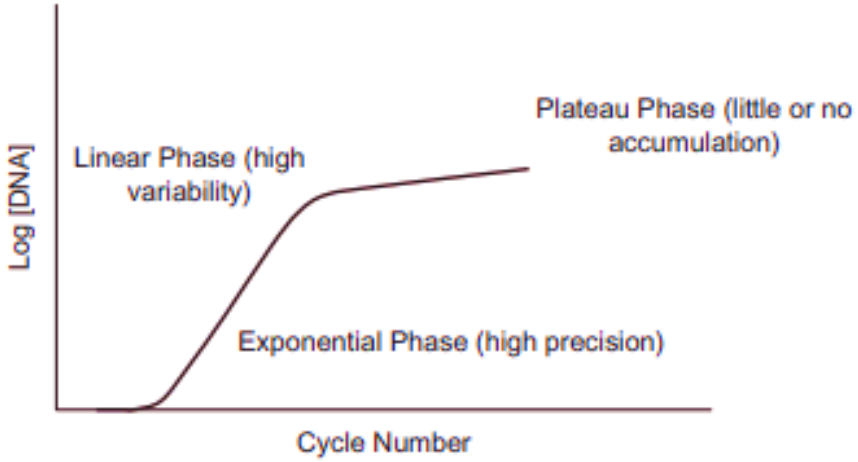

Figure 26. PCR plateau effect **[88]**

- **Advantages [104] [105]**

➢ No calibration curve required

➢ Ability to increase throughput because wells no longer need to be used for the standard curve samples.

➢ Absence of dilution errors made in creating the standard curve samples

➢ Ability to amplify the target and endogenous control in the same tube, which increases throughput and reduces pipetting errors.

- **Disadvantages [105]**

➢ The time required for DNase treatment and optimization of reverse transcription can affect cDNA conversion efficiency.

➢ All steps of the workflow have to be standardized

➢ Reference genes need to be carefully selected and validated

4.4.2.2 Absolute quantification

Absolute quantification is the quantification using a standard curve. **[106] (Figure 27)**

In this method, it is necessary to know the absolute quantity of the standard to determine the accurate number of target molecules present in the sample. Moreover, absolute quantification facilitates the comparison of data from different assays. **[107]**

The calibration curve used in absolute quantification is mainly based on the known concentration of the DNA standard molecule. **[108]**

No matter how precisely the concentration of the standard material is known, the result is always reported relatively compared to a defined unit of interest, e.g., copies per defined nanograms of total RNA **[108]**

- **Advantage**

➢ Performing target and endogenous control amplifications in separate tubes and using calibration curve analysis requires minimal optimization and validation **[104]**

- **Disadvantages [105]**

➢ Standard stability during long-term storage is difficult and can be problematic

➢ Absolute quantitative models require careful optimization of accuracy

➢ Special attention should be paid to the presence of inhibitors in biological samples

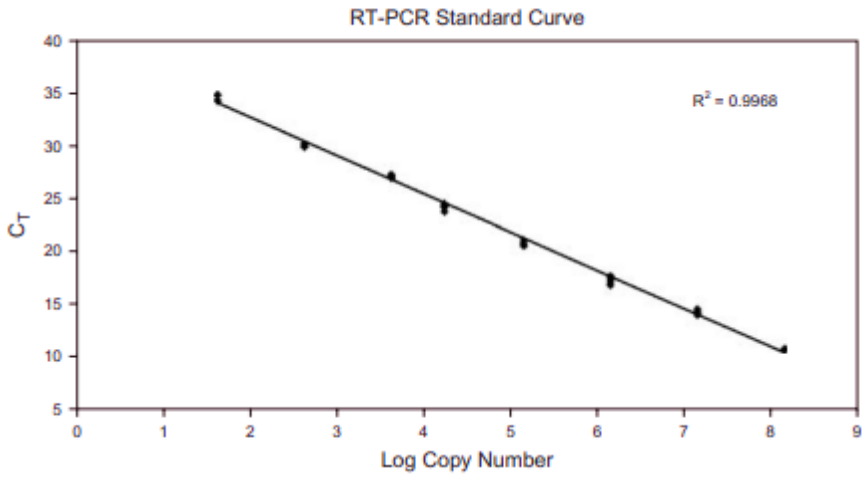

Figure 27. RT-PCR standard curve **[88]**

4.4.2.2.1 Melting curve analysis

Melting curve analysis is performed to identify products and detect non-specific products. **[94]**

This method is based on the conversion of double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) by heat denaturation which leads to DNA strand dissociation and dye release. It, therefore, monitors the decrease in fluorescence of the DNA-binding dye during temperature changes. Each part of dsDNA has a melting point (T_m) at which temperature 50% of the DNA is single-stranded. **[109] [110]**

- **Advantages**
- Applied on known or unknown mutations **[109]**
- Ability to distinguish mutations. **[111]**
- **Disadvantages [112]**
- Elevated T_m with broadening of the melting transition
- Compression of the Tm difference among genotypes.
- **Example of applications**
- Detection and provision of relative quantitative information regarding both the wild-type and the mutant alleles of JAK2 V617F mutation. **[111]**

4.4.2.2.2 High-resolution melting curve analysis

High-resolution melting curve analysis (HRM) is a post-PCR analysis method performed on double-stranded (ds) DNA samples and is used to identify genetic variation in the appropriate region of interest for a candidate gene. **[113]**

4.5 Advantages, disadvantages, and applications of Real-Time PCR

4.5.1 Advantages [86]

- Great potential across many disciplines, including pathogen detection, molecular diagnostics, mutation detection, an assay of alternative splicing, the analysis of tissue samples that may have been archived long ago, for long-term in-house studies, and in high-throughput laboratories.
- Great speed because it is an automated process
- Wide dynamic range
- The ability to recognise subtle variations in gene expression

- The closed-tube system reduces the risk of laboratory-wide amplicon contamination and data variations
- The accompanying software is capable of making sophisticated measurements

4.5.2 Disadvantages [88]

- The cost of purchasing the instrumentation
- The cost of the consumables needed for each experiment
- Not suitable for the simultaneous assay of more than a few genes

4.5.3 Some Applications of Real-Time PCR

- Real-time PCR applications in microbiology are more sensitive, time and cost-effective for both detection and quantification of pathogens and identification of the specific genes or mutations in microorganisms. **[107]**

  Moreover, Real-Time PCR allows the correction of non-specific serological false positive results, the identification of the genes that encode virulence factors or antimicrobial resistance, and the recognition of the mutations of genes that are associated with antimicrobial resistance. **[107]**

- Detects and quantifies nucleic acids **[114]**
- Detects **S**evere **A**cute **R**espiratory **S**yndrome **Co**rona**v**irus 2 (SARS-CoV-2) **[115]**
- Diagnoses cancers **[116]**

EXPERIMENTAL

STUDY

1 Material and methods

1.1 Material

In this perspective study conducted in the Anti-Cancer Centre's central laboratory from June 13th to July 14th, we examined 11 patients: 7 women and 4 men aged 22 to 63 years. These candidates were recruited from the Haematology Department of the same centre.

Among the 11 patients, 7 patients were studied at first diagnosis and the remaining 4 patients were monitored for minimal residual disease (MRD).

Several tests, including clinical and biological tests, were provided to all patients. In addition, an RT-PCR test was effected to confirm the diagnosis of CML or ALL Ph+.

Patient follow-up was performed by RT-PCR, and the response to treatment was evaluated in accordance with the ELN 2020 criteria.

Patient diagnostic and socio-demographic data were collected from medical records.

1.1.1 Laboratory material:

- 2 Ethylenediaminetetraacetic acid (EDTA) tubes of 9ml
- 25X Deoxynucleotide triphosphate (dNTPs) mix
- Buffer
- Centrifuge
- Dry tubes
- Elution solution
- Eppendorf
- Free nuclease water
- Freezer (-40 °C)
- Gene expression assay
- Genetic material doser
- Gloves
- Invisible film
- Isopropanol
- Kidney dish

- Laboratory coat
- Laboratory hood
- LEUKOLOCK^{TM} Filter
- Lysis binding solution
- Lysis solution
- Mask
- Micropipette
- Micropipette tips
- Phosphate-buffered saline (PBS) pH 7.4 rinse solution
- Production possibilities curve (PPC) ABL
- Production possibility frontiers (PPF) BCR-ABL
- Proteinase
- QUICDRA dispenser
- Random primers
- RNA binding beats
- RNAlater solution
- Scribe reverse
- StepOne 48 well plate
- Syringe of 5ml
- Vortex
- Wash solution

1.1.2 Instrumentation

In our experiment, we used Applied Biosystems™ StepOne™ Real-Time PCR System.

1.1.2.1 StepOne™ Real-Time PCR System

The StepOne™ real-time PCR system is a 48-well, low-throughput, real-time PCR device suitable for both beginners and experienced users. The StepOne ™ real-time PCR system can be set up in a variety of configurations and is ready to use with intuitive data analysis and instrument control software. The StepOne™ real-time PCR system utilizes robust LED-based three-color optical recordings and is designed to provide accurate and quantitative real-time PCR results for a wide range of genomic research applications.

1.1.2.2 StepOne™ Software

The StepOne ™ software included in the StepOne ™ real-time PCR system runs on Windows® XP, Windows Vista® and Windows® 7 operating systems and provides instrument control, data acquisition, and data analysis capabilities. This latest version includes the ability to collect melting curve data for **H**igh **R**esolution **M**elt (**HRM**) applications and the option to export to **R**eal-time PCR **D**ata **M**arkup **L**anguage (**RDML**) for compatibility with **M**inimum **I**nformation for publication of **Q**uantitative real-time PCR **E**xperiments (**MIQE**) recommendations.

1.1.2.3 High-sensitivity LED-based 3-colour fluorescence measurement

The StepOne™ real-time PCR system uses a long-life LED-based optical system that can record the fluorescence of FAM™ / SYBR® Green, VIC® / JOE™, and ROX™ dyes. This cost-effective 3-colour 48-well system provides accurate, real-time quantitative PCR results and stores data from all filters in each run, independent of computer or plate configuration. Two populations of 5,000 and 10,000 template copies of the TaqMan® assay can be distinguished with 99.7% confidence.

Free dyes	Max. ab (nM)	Max. em (nM)
SYBR® Green I	497	525
Reporter dyes	Max. ab (nM)	Max. em (nM)
6-FAM	494	518
JOE™	520	548
VIC™	538	554
ROX™	580	605

Dyes used in StepOne™ real-time PCR and their wavelengths of absorption and emission

1.1.3 Population:

The population of our experimental study is composed of 11 patients: 7 females and 4 males. The age range of this population is between 22 and 63 years old.

1.1.3.1 Inclusion/exclusion criteria

1.1.3.1.1 Inclusion criteria

- Chronic myeloid leukaemia Ph+
- Acute lymphoblastic leukaemia Ph+
- Different ages
- Both sexes

1.1.3.1.2 Exclusion criteria

- Atypical chronic myeloid leukaemia
- Acute lymphoblastic leukaemia Ph-
- Other haematological malignancies Ph-

1.2 Methods:

1.2.1 DNA/RNA extraction:

The genetic material (RNA or DNA) can be extracted from various types of samples:

- Different tissues (blood, skin, ...)
- Cell pellets (eukaryotic or prokaryotic)
- environmental samples (water, land…)
- faeces...

To guarantee an optimal extraction, we use the most suitable method according to the type of sample provided.

Whichever method is used, the major preparation steps are as follows:

- Lysis: mechanical, chemical or enzymatic depending on the nature of the sample
- Isolation of DNA or RNA: on a silica column or by differential centrifugation
- Purification: column elution after removal of proteins and other contaminants
- Quality control (optional): assay by spectrophotometry or visualization of the electrophoretic profile on the agarose gel.

RNA extraction steps

The extracted DNA or RNA is taken up in ultrapure water or elution buffer (usually Tris-EDTA). The quantity and quality of the extracted nucleic acids are evaluated by spectrophotometric assay on Implen NP80.

1.2.2 Extraction of nucleic acids

1.2.2.1 Precautions

- ✓ The preparation of RNA is more delicate than that of DNA because ribonucleases (RNAses) are very widespread (e.g., on the fingers) and are frequently able to renature after many treatments (even heat denaturation).
- ✓ Contamination is minimized by wearing gloves, using sterile dishes and solutions and/or treating with Diethyl pyrocarbonate DEPC (RNAse inhibiting agent).

Extraction of total RNAs

The safest method is the method of Chirgwin (extraction of RNA from the pancreas, a tissue very rich with RNAses).

- ✓ Grind the tissue in a Potter homogenizer with an adequate solution (Sodium Dodecyl Sulphate (SDS) or Sarcosyl detergent + a dissociating agent + a buffer solution + a reducing agent)
- ✓ Centrifuge the homogenate to remove cell debris.
- ✓ RNA extraction is done: either by differential precipitation of RNA and DNA / or by ultracentrifugation on a caesium chloride cushion (only RNA can, given its density, cross this cushion and be recovered in the nerve
- ✓ Wash RNA in sodium acetate and precipitate with alcohol

✓ The extracted RNA can be stored for more than a year either in the form precipitated in ethanol, or in the frozen form at -80 °C.

RNA extraction:

- Take 2 EDTA tubes (9ml) of blood for each patient
- Place the end of the LEUKOLOCK filter on a tube of blood and the second end of the filter on a dry tube under a vacuum which will suck out all the blood.
- The filter keeps the white blood cells (the same filter for the second tube of blood)
- Insert a syringe filled with PBS pH 7.4 3ml rinse solution into the filter
- Expel residues and keep a filter only with white blood cells
- Inject a syringe filled with 3ml RNAlater solution into the filter to stabilize the RNA
- Inject air into the filter to expel residue
- Prepare a lysis solution: 2.5 ml of lysis binding solution and 70 µl pH adjusted
- Inject lysis solution into the filter with the syringe
- Recover the filtrate which contains free RNA
- With an empty syringe, inject air into the filter to expel the rest of the RNA to be recovered
- Add 2.5 ml of free nuclease water to the free RNA tube
- Cover the tube and mix in a vortex
- Add 25 µl of proteinase to our RNA tube and mix manually
- Add (50 µl RNA binding beats + 2.5 ml isopropanol) to free RNA
- Incubate at room temperature for 5 minutes
- Centrifuge 3000 rotations per minute for 3 minutes
- Discard the supernatant
- Wash the RNA pellet with 600 µl of wash solution 1 concentrate
- Mix in vortex then centrifuge
- Discard supernatant
- Wash with 600 µl of wash solution 2/3 concentrate then centrifuge
- Discard the supernatant
- Add 50ml of elution solution then centrifuge

- Determination of RNA quantity in the tube:

- Launch a white tube first
- Place a drop of RNA solution in the QUICDRA dispenser

Reverse transcription

Prepare a reagent mixture: 10 µl in total (quantity x number of patients)

- 10X RT (reverse transcriptase) buffer 2 µl
- 25X dNTP mix (10MM) 0.8 µl
- 10X RT random primers 2 µl
- MultiScribe reverse transcriptase 1 µl
- Nuclease-free H_2O 4.2 µl
- Pour 10 µl of the mixture into a well
- Pour 10 µl of the free RNA solution into the mixture
- Cover the wells with an invisible film
- Place the plate in the StepOne™ automaton and launch the reverse transcriptase for 2h20

Amplification of complementary DNA (mutation screening / qualitative result)

- In an Eppendorf tube put the primers corresponding to our mutation (1 µl per mutation: M-BCR/ m-BCR)
- 1 µl Gene expression assay
- 10 µl master mix
- 5 µl RNase-free water
- Put the mixture into the wells
- Add 4µl of complementary DNA
- Positive control (replace: Gene expression assay by endogenous control)
- Negative control (replace: Gene expression assay by RNase-free water)
- Cover the plate with a transparent film
- Place the plate in the StepOne™ automaton and start the amplification for 1h16
- Read the graph in case of mutation detection

Quantification of mutations

Prepare a mixture of:

- 12.5 µl master mix
- 6.5µl distilled water
- 1 µl PPF BCR-ABL

- In a PCR plate put 4 DNA standard wells
- Take 4 µl of the complementary DNA with positive mutations mix
- Put the plate in the StepOne^{TM} automaton for 1h16
- Read the results

Quantification of healthy genes for the same patients

Prepare a mixture of:

- 12.5 µl master mix
- 6.5 µl distilled water
- 1 µl PPC ABL

- In a PCR plate put 4 DNA standard wells
- Take 4 µl of the complementary DNA wells and positive mutations mix
- Put the plate in the StepOne^{TM} automaton for 1h16
- Read the results
- Calculate the ratio: number of healthy genes/numbers of mutant genes

1.3 Results

Table 8. General results of the experimental study

Populations	ALL	CML
Number (patients)	03	08
Age (years)	22,28,54	41_63
Sex (female:male)	2F:1M	5F:3M

CML patients

Case 1

- Gender: Female
- Age: 53 years old
- State: Djelfa
- Occupation: None
- Date of diagnosis: June 8th, 2022 (newly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

-Type 3 splenomegaly measuring 11 cm on ultrasound

-Bone pain

- Biological features:

Blood film:

- ✓ Haemoglobin: 11g/dl
- ✓ White blood cells: 246950/ml
- ✓ Myelaemia: 36%
- ✓ Blasts: 2%
- ✓ Platelets: 248000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 253.11/µl of M-BCR copies.

- SOKAL score: 1.1 (intermediate risk)
- ELTS score: 2.083 (intermediate risk)
- Treatment:

HYDROXYUREA from 8th to 30th of June, 2022 at a dose of 5 capsules /day, then IMATINIB 400mg/day until today

- Evaluation: the response to treatment is being explored.

Case 2

- Gender: Female
- Age: 46 years old
- State: Djelfa
- Occupation: None
- Date of diagnosis: December 16th, 2021 (formerly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

No particular clinical features

- Biological features:

Blood film:

  - ✓ Haemoglobin 14.1 g/dl
  - ✓ White blood cells 60770 /ml
  - ✓ Myelaemia 28%
  - ✓ Blasts: 0%
  - ✓ Platelets 253000 G/L
  - ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 1.36/µl of M-BCR/ABL copies. (low)
- SOKAL score: 0.5 (low risk)
- ELTS score: 1.059 (low risk)
- Treatment:

HYDROXYUREA from December 16th, 2021 to January 15th, 2022 at a dose of 5 capsules/day, then IMATINIB 400mg/day until today

- Evaluation: Good response to treatment (in remission)

Case 3

- Gender: Female
- Age: 41 years old
- State: Medea
- Occupation: None
- Date of diagnosis: May 4th, 2022 (newly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

-Type 3 splenomegaly measuring 12 cm on ultrasound

-Signs of anaemia (pallor, fatigue, tachycardia…)

- Biological features:

Blood film:

- ✓ Haemoglobin 9.9 g/dl
- ✓ White blood cells 548800 /ml
- ✓ Myelaemia 41%
- ✓ Blasts 4%
- ✓ Platelets 156000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 563.69 /µl of M-BCR/ABL copies.

- SOKAL score:1.2 (intermediate risk)
- ELTS score: 2.370 (high risk)
- Treatment:

HYDROXYUREA from 4th to 27th of May, 2022 at a dose of 5 capsules/day, then

IMATINIB 400mg/day until today

- Evaluation: the response to treatment is being explored.

Case 4

- Gender: Male
- Age: 55 years old
- State: Djelfa
- Occupation: Day labourer
- Date of diagnosis: September 19th, 2017 (formerly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

Type 3 splenomegaly measuring 8 cm on ultrasound

- Biological features:

Blood film:

- ✓ Haemoglobin 12.6 g/dl
- ✓ White blood cells 119640 /ml
- ✓ Myelaemia 45%
- ✓ Blasts 0%
- ✓ Platelets 117000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 4269.08 /µl of M-BCR/ABL copies (a large number of copies), and a positive m-BCR/ABL screen.

- SOKAL score: 0.9 (intermediate risk)
- ELTS score: 2.108 (intermediate risk)
- Treatment:

HYDROXYUREA until October 19th, 2017 at a dose of 5 capsules/day, then IMATINIB 400mg/day for 3 months, then NILOTINIB 800 mg/day until today.

- Evaluation: No response to treatment (refractory patient)

Case 5

- Gender: Female
- Age: 60 years old
- State: Blida
- Occupation: None
- Date of diagnosis: May 19th, 2022 (newly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

Type 3 splenomegaly measuring 12 cm on ultrasound

Signs of anaemia (pallor, fatigue, tachycardia…)

- Biological features:

Blood film:

- ✓ Haemoglobin 7 g/dl
- ✓ White blood cells 499610 /ml
- ✓ Myelaemia 52%
- ✓ Blasts 3%
- ✓ Platelets 303000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 754.33 /µl of M-BCR/ABL copies.

- SOKAL score: 1.4 (high risk)
- ELTS score: 2.339 (high risk)
- Treatment:

HYDROXYUREA until June 8th, 2022 at a dose of 5 capsules/day, then IMATINIB 400mg/day until today

- Evaluation: the response to treatment is being explored.

Case 6

- Gender: Female
- Age: 63 years old
- State: Djelfa
- Occupation: None
- Date of diagnosis April 22nd, 2022 (newly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

No particular clinical features

- Biological features:

Blood film:

- ✓ Haemoglobin 11 g/dl
- ✓ White blood cells 519300/ml
- ✓ Myelaemia 23%
- ✓ Blasts 0%
- ✓ Platelets 193000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 1087.26 /µl of M-BCR/ABL copies.

- SOKAL score: 0.73 (low risk)
- ELTS score: 1.559 (low risk)
- Treatment:

HYDROXYUREA until June 8th, 2022 at a dose of 5 capsules/day, then IMATINIB 400mg/day until today

- Evaluation: the response to treatment is being explored.

Case 7

- Gender: Male
- Age: 53 years old
- State: Chlef
- Occupation: Builder
- Date of diagnosis: June 2nd, 2014 (formerly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

-Type 3 splenomegaly measuring 15 cm on ultrasound

-Signs of anaemia (pallor, fatigue, tachycardia…)

- Biological features:

Blood film:

- ✓ Haemoglobin 9.3 g/dl
- ✓ White blood cells 407120 /ml
- ✓ Myelaemia 55%
- ✓ Blasts 5%
- ✓ Platelets 387000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 2075.19 /µl of M-BCR/ABL copies. And positive screening for m-BCR/ABL.

- SOKAL score: 1.5 (high risk)
- ELTS score: 2.480 (high risk)
- Treatment:

HYDROXYUREA until June 26th, 2014 at a dose of 5 capsules/day, then IMATINIB 400mg/day for 3 months, until February 8th, 2015 (treatment failure) then DASATINIB until today.

- Evaluation: No response to treatment (refractory patient)

Case 8

- Gender: Male
- Age :59 years old
- State: Medea
- Occupation: Builder
- Date of diagnosis: April 7th, 2008 (formerly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

Type 3 splenomegaly measuring 14 cm on ultrasound

- Biological features:

Blood film:

- ✓ Haemoglobin 11.4 g/dl
- ✓ White blood cells 640600 /ml
- ✓ Myelaemia 24%
- ✓ Blasts 0%
- ✓ Platelets 150000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 2075.59 /µl of M-BCR/ABL copies and positive screening for m-BCR/ABL.

- SOKAL score: 1.1 (intermediate risk)
- ELTS score: 2.742 (high risk)
- Treatment:

HYDROXYUREA until March 13th, 2009 at a dose of 5 capsules/day, then IMATINIB 400mg/day until July 24th, 2014 (failure) then on NILOTINIB 800mg/day until today

- Evaluation: No response to treatment (refractory patient)

ALL patients

Case 9

- Gender: Female
- Age :22 years old
- State: Medea
- Occupation: None
- Date of diagnosis: February 3rd, 2022 (newly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

-Type 3 splenomegaly measuring 12 cm on ultrasound

-Signs of anaemia (pallor, fatigue, tachycardia…)

- Biological features:

Blood film:

- ✓ Haemoglobin 9.7 g/dl
- ✓ White blood cells 69720 /ml
- ✓ Blasts 12%
- ✓ Platelets 90000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 234.38 /µl of m-BCR/ABL copies and a positive screening for M-BCR/ABL.

- Treatment:

HYDROXYUREA until March 1st, 2022 at a dose of 5 capsules/day, then IMATINIB 400mg/day until May, 2022 (failure) then DASATINIB until today.

- Evaluation: the response to treatment is being explored.

Case 10

- Gender: Female
- Age :28 years old
- State: Blida
- Occupation: None
- Date of diagnosis: April 12th, 2022 (newly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

-Type 2 splenomegaly measuring 4 cm on ultrasound, bilateral cervical lymphadenopathy, and gingival hypertrophy.

-Signs of anaemia (pallor, fatigue, tachycardia…)

- Biological features:

Blood film:

- ✓ Haemoglobin 7.6 g/dl
- ✓ White blood cells 234370 /ml
- ✓ Blasts 100%
- ✓ Platelets 22000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 1.57 /µl of M-BCR/ABL copies. And 0.67 /µl of m-BCR/ABL copies

- Treatment:

HYDROXYUREA until May 10th, 2022 at a dose of 5 capsules/day, then IMATINIB 400mg/day until today.

- Evaluation: the response to treatment is being explored.

Case 11

- Gender: Male
- Age :56 years old
- State: Chlef
- Occupation: Merchant
- Date of diagnosis June 8th, 2022 (newly diagnosed)
- Department: haematology - Anticancer centre of Blida
- Clinical features:

-Type 3 splenomegaly measuring 18 cm on ultrasound

-Signs of anaemia (pallor, fatigue, tachycardia…)

- Biological features:

Blood film:

- ✓ Haemoglobin 7.7 g/dl
- ✓ White blood cells 8635 /ml
- ✓ Myelaemia 6%
- ✓ Blasts 16%
- ✓ Platelets 22000 G/L
- ✓ Quantification of BCR-ABL transcripts using the RT-PCR technique: 467.08 /µl of m-BCR/ABL copies.

- Treatment:

IMATINIB 800mg/day until today.

- Evaluation: the response to treatment is being explored.

1.4 Discussion

Based on the results of this study, we divide patients into three main categories:

1st category: patients who present **clinical features of CML/ALL Ph+** and undergo RT-PCR test to detect the BCR-ABL mutation and **confirm the diagnosis**. (CML population: patients 1 and 3 present only M-BCR breakpoint. ALL population: patient 11 presents both M-BCR and m-BCR breakpoints). The qualitative and quantitative results provided by this test allowed the physicians to confirm their diagnosis.

In a study conducted by Maria de Lourdes L.F. Chauffaille **[117]** to evaluate the reliability of karyotyping in the diagnosis of CML, the results showed that karyotyping was time-consuming (15 days), expensive, and was performed only on bone marrow aspirates. In our study, we obtained the results in 7 days and the test was performed on peripheral blood samples, which makes RT-PCR easier to perform and faster than karyotyping. In addition, the same study **[117]** demonstrated that less than 2% of CML patients have a normal karyotype at diagnosis (false-negative results). In these patients, the BCR-ABL1 fusion gene is detectable by RT-PCR. Cox et al **[118]** study also mentioned that several reports displayed the importance of RT-PCR in correcting false-negative cytogenetic analyses. Moreover, the same study **[118]** described the RT-PCR as the most sensitive tool for the diagnosis of BCR-ABL-positive leukaemias and proved that FISH provided little information about chimeric transcripts.

Flynn et al's **[119]** report revealed that GeneXpert technique has not currently been approved and validated for the diagnosis of CML.

2nd category: patients diagnosed with CML/ALL Ph+ **and already started the treatment**. This category undergoes follow-up to evaluate their response to treatment. (CML population: patients 2, 9 and 10 presented a good response to treatment and this was shown in the decreased number of M-BCR copies in comparison with the number of copies of the previous assessment)

Maria de Lourdes L.F. Chauffaille **[117]** indicated that the sensitivity of karyotype analysis is remarkably less than the sensitivity of RT-PCR, especially in the monitoring of minimal residual disease.

Besides, Cox et al **[118]** study results confirmed that FISH sensitivity for detection of minimal residual disease is low; Thus, this technique does not provide sufficient data about patients in remission in contrast to RT-PCR. Similarly, Bao et al **[120]** discovered in their study that FISH is not a suitable technique for the monitoring of minimal residual disease.

Furthermore, a study conducted by Jobbagy et al **[76]** revealed that the GeneXpert technique detects only M-BCR breakpoint and its sensitivity considerably decreases at an extremely low number of copies; therefore, it cannot replace conventional methods. On the other hand, the RT-PCR instrument that we used managed to detect three types of breakpoints (M-BCR, m-BCR, and µ-BCR) even at a low number of copies.

3rd category: refractory patients who do **not respond to treatment**. This category is divided into two subcategories: Refractory patients: 2, 4, 7 and 8. We noticed that the number of BCR-ABL transcripts was increasing during the monitoring of the response to different treatments: TKIs of first and second generations (nonresponse to imatinib, dasatinib, and nilotinib). These results make us think of two possibilities:

1- Patients have **T315I mutation**, and this requires changing the treatment and prescribing the TKI of the third generation: ponatinib.

2- Patients with **natural resistance** to TKIs, and the only treatment option is an allogeneic stem cell transplantation.

In order to prevent the problem of nonresponse to treatment, we highly recommend a sequencing before starting the treatment. It allows an early detection of the mutations responsible for the nonresponse to certain treatments, therefore, helps the physician select the best therapeutic option.

1.5 Contribution of RT-PCR :

According to the results of this study, we discovered that the RT-PCR presents a:

• Prognostic value: case of regular patients 2 /4 / 7/ 8

• Follow-up value: case of regular patients 2 / 9 / 10

• Diagnostic value: case of new patients 1/ 3/ 5/ 6/ 11

Consequently, the RT-PCR technique is, indeed, essential for the establishment of qualitative and quantitative diagnosis and the follow-up of CML and ALL Ph+.

CONCLUSION

With the evolution of science, molecular biology proved its utility as a powerful tool in the medical field. Different PCR techniques have remarkably contributed to several research experiments.

The study of the molecular structures and their associated abnormalities, especially mutations, facilitated the diagnosis, allowed to target the treatment, and made the monitoring more effective. These advancements were noticed in many cases of leukaemia.

In the light of our findings, RT-PCR contributes significantly to the detection of BCR-ABL molecular rearrangements, as well as other leukemia-related alterations, in order to create an appropriate molecular follow-up and identify patients at risk of treatment failure.

This method provides a better understanding of prognosis and facilitates the selection of optimal therapeutic options. As a result, its incorporation, with sequencing, into Algerian laboratories specializing in the diagnosis and monitoring of leukemias is necessary for the improvement of the diagnostic and therapeutic procedures, and therefore the management of patients with CML and ALL Ph+.

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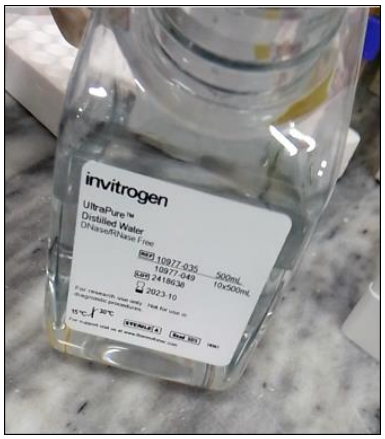
Annexes

Annexe 1: WHO 2016 classification of malignant diseases of the haematopoietic and lymphoid tissues

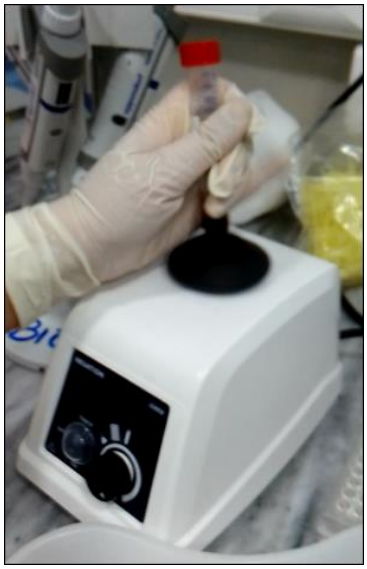
Myeloproliferative neoplasms
Mastocytosis
Myeloid/lymphoid neoplasms with eosinophilia and gene rearrangement
Myelodysplastic/myeloproliferative neoplasms
Myelodysplastic syndromes
Myeloid neoplasms with germline predisposition
Acute myeloid leukaemia (AML) and related precursor neoplasms
AML with recurrent genetic abnormalities
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukaemia, Not Otherwise Specified (NOS)
Myeloid sarcoma
Myeloid proliferations associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukaemias of ambiguous lineage
Precursor lymphoid neoplasms
Mature B-cell neoplasms
Mature T- and Natural Killer (NK)-cell neoplasms
Hodgkin lymphomas
Immunodeficiency-associated lymphoproliferative disorders
Histiocytic and dendritic cell neoplasms

Annexe 2:



RNAse/DNAse free distilled ultrapure water

Annexe 3:



Vortex

Annexe 4:

Proteinase K

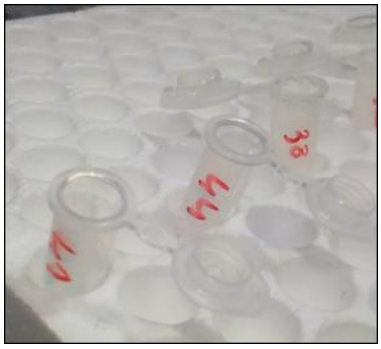
Annexe 5:

Centrifuge

Annexe 7:

Wash solution 1 concentrate

Annexe 8:

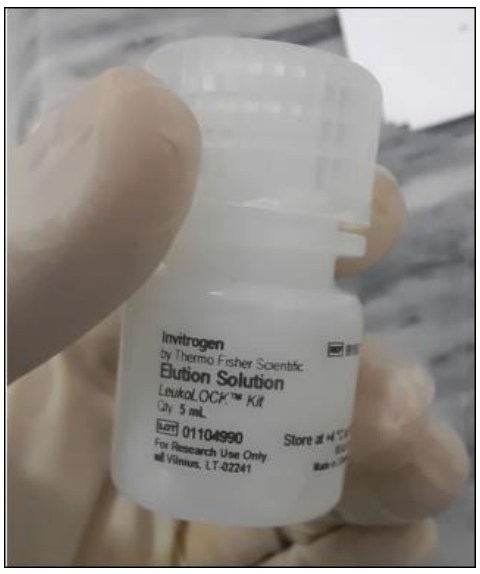


Eppendorf

Annexe 9:

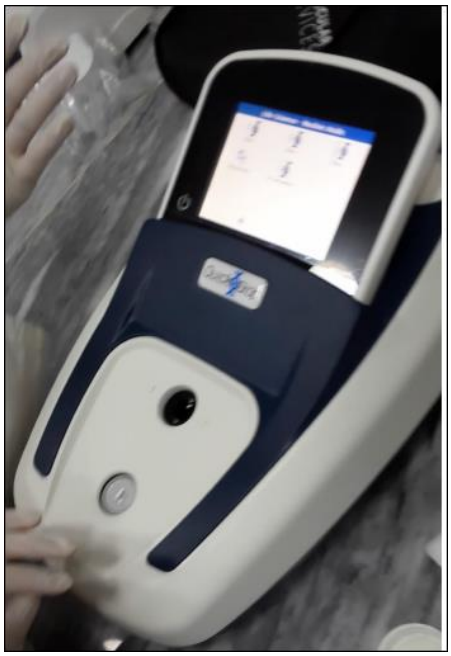
Master mix

Annexe 10:



Elution solution

Annexe 11:



Genetic material doser

Annexe 12:

dNTP mix

Annexe 13:

Syringe of 5ml

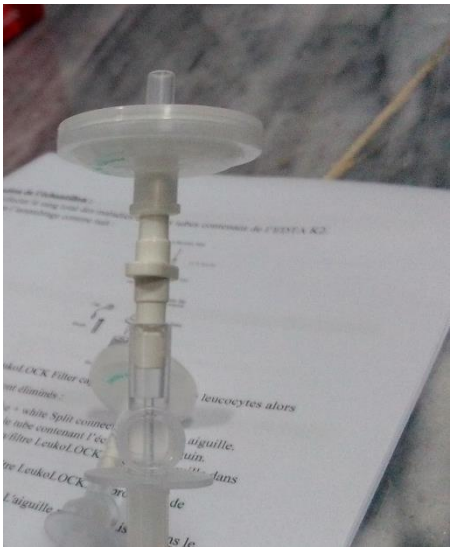
Annexe 14:

Lysis/binding solution

Annexe 15:

Extraction of RNA

Annexe 16:

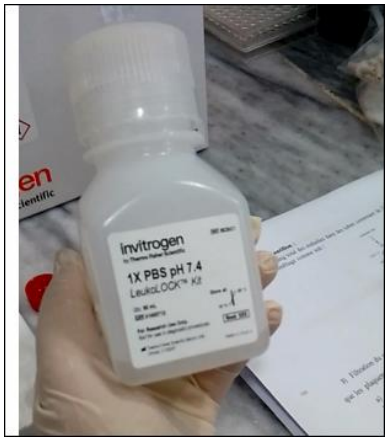


Filter

Annexe 17:

48- well plate

Annexe 18:



PBS pH 7.4

ABSTRACT

Haematopoiesis begins with a pluripotent stem cell, which produces multiple cell lines. The accumulation of genetic alterations in the bone marrow or peripheral lymphoid tissue cells causes haematological malignancies. Previously, cancer was classified by location according to whether the cancer was found in the blood (leukaemia), lymph nodes (Hodgkin lymphoma and non-Hodgkin lymphoma), or bone (myeloma). Leukaemias are clonal diseases that lead to an excess of malignant white blood cells in the bone marrow and blood.

The Philadelphia chromosome (BCR-ABL) is a feature of CML and is a shortened chromosome 22 resulting from the reciprocal translocation t (9; 22)(q34; q11) between the long arms of chromosomes 9 and 22. There are three major mutations in the Philadelphia chromosome: M-BCR, m-BCR, and µ-BCR. This chromosome might also be associated with ALL, AML, and other haematological malignancies.

Detecting mutations associated with leukaemia and other haematological malignancies and monitoring their response to treatment are based on PCR techniques.

Polymerase chain reaction (PCR) is a sensitive technique that allows rapid amplification of specific DNA segments. The amplification process is divided into three main phases: denaturation, hybridization with primers, and elongation.

Real-time or quantitative PCR is a type of PCR based on the continuous collection of fluorescent signals from one or more polymerase chain reactions over a series of cycles. Fluorescence data should be processed by a data analysis method.

RT-qPCR is the gold standard for the detection of the BCR-ABL fusion gene. In addition, it is a reliable method for the assessment of treatment response.

The experimental study shows that the RT-PCR technique gives reliable results and is useful in prognosis, diagnosis, and treatment monitoring.

Keywords: RT-PCR, diagnosis, monitoring, follow-up, leukaemias, CML, BCR-ABL, ALL Ph+, treatment.