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SERIES: XXX

CHRONIC MYELOID LEUKEMIA:

FROM THERAPY MONITORING TO PERSONALIZED MEDICINE. ASSESSMENT OF INDUSTRIAL PROCESS.

Thesis written with the financial contribution of DITTA DINO PALADIN

Coordinator: Prof. Piero Maestrelli

Supervisor: Prof. Pietro Giusti

Co-Supervisor: Dr. Dino Paladin

Ph.D. Student: Paola Gonzalez Ruiz

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1. ABSTRACT

Introduction:

Chronic myeloid leukemia (CML) is a white blood cells cancer, which is characterized by a BCR-ABL fusion gene. The disease is caused by a reciprocal translocation between chromosome 9 and 22, t(9; 22)(q34; 11) [1] commonly known as the Philadelphia chromosome (Ph), resulting in an abnormally BCR-ABL tyrosine kinase, which is responsible for the pathogenesis of CML. The high efficacy of the tyrosine kinase inhibitors (TKIs) in the treatment of CML has caused the need for sensitive methods to monitor the course of therapy. Quantification of BCR-ABL transcripts with qRT-PCR Real-Time has demonstrated to be the most accurate method available. [2] Following the European LeukemiaNet recommendations, [3] the lack of initial response can be detected only after 3-6 months from the diagnosis. The ability to understand how patients respond to the different TKIs available as first-line treatment at the moment of diagnosis would help clinicians to prescribe more patient-tailored therapy, decreasing the onset of future drug resistance and decreasing treatment cost.

Materials and Methods:

We have developed and validated two devices (RQ-BCR-ABL p210 One-Step and RQ-BCR-ABL p190 One-Step) for monitoring therapy of CML. The RQ-BCR-ABL p210 One-Step kit has undergone a further external validation in three reference laboratories, belonging to LabNet network.

For what concern the prevision of therapy's response the University of Verona has developed LeukoPredict, an *in-vitro* device to screen the inhibitory potential of several BCR-ABL-targeting drugs and to obtain the percentage of inhibition compared to the same non-treated samples. We took part to this project in the framework of industrial planning, performing a Freedom to Operate analysis and a Cost-Effectiveness analysis.

Results:

Both kits based in qRT-PCR Real-Time One-Step have showed high reproducibility and high sensitivity in quantification of BCR-ABL transcripts, proving to be suitable for CE-IVD mark. Moreover, RQ-BCR-ABL p210 One-Step kit has been verified by the LabNet network as a suitable device for the monitoring of CML, improving the reproducibility regarding the current system used in routine.

The Freedom to Operate analysis of LeukoPredict has found some close prior patents documents, but none could hinder entry into the market. The Cost-Effectiveness analysis has demonstrated that LeukoPredict is either cost saving or very cost-effective, depending on the scenario analyzed, generating significant savings for health systems.

Conclusions:

This project is able to connect actual principal issues in CML. On one side we have developed and validated two devices that completely satisfy actual request of therapeutic monitoring in pharmaceutical market making a complete panel to track CML. The develop of devices as LeukoPredict helps to decrease the risk of disease's progression to more aggressive phase, personalizing the therapy and obtaining the maximum effectiveness of therapeutical choices. This can help physicians in an evidence based decisional therapeutic process, avoiding potential conflict of interest and giving a rational explanation to other kind of treatment when the risk of failure is too high. Finally, it has been considered as a technology that can be affordable and that could contain the cost of healthcare.

2. INTRODUCTION

2.1. CHRONIC MYELOPROLIFERATIVE SYNDROMES

The term Chronic Myeloproliferative Syndromes refers to certain diseases that originate from the neoplastic transformation of the pluripotent stem cell and characterized by clonal proliferation of one or more hematopoietic progenitor stem cells in bone marrow and extramedullary sites. The proliferation is associated with a relative normal maturation and/or differentiation that lead to an increase of granulocytes, red blood cells and/or platelets in peripheral blood. Hepatomegaly and splenomegaly are often observed and they can be attributed to both the excess of elements in circulating blood and to extramedullary hematopoiesis.[4]

Biological characteristics, clinical presentations and natural history of the disease can be different in each pattern that can however transform from one syndrome to another during the course of the disease. Myeloproliferative neoplasms are a heterogeneous group of disorders characterized by altered cellular proliferation of one or more hematologic cell lines in the bone marrow.

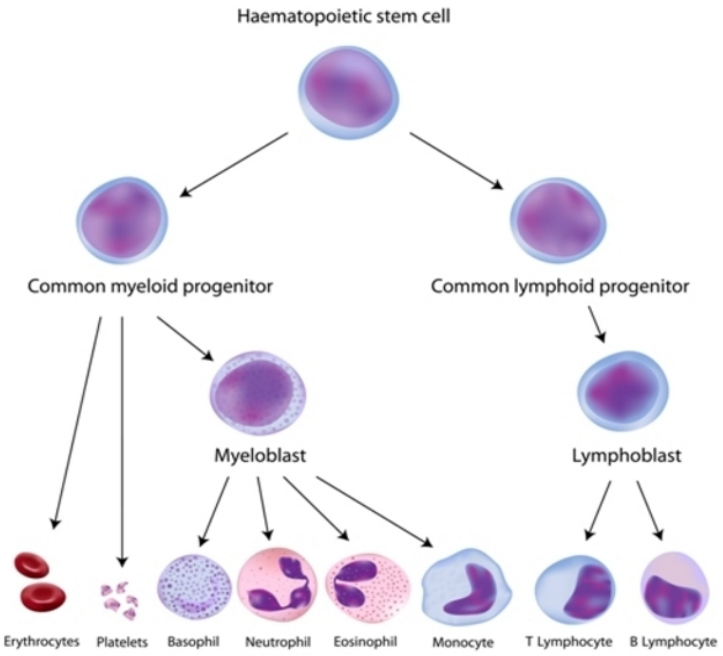

Figure 1. Scheme of haemotopoiesis process

2.2. CHRONIC MYELOID LEUKEMIA

Chronic Myeloid Leukemia (CML) represents the most biologically studied chronic myeloproliferative syndrome and constitutes a reference model for similar pathologies.
It is a clonal myeloproliferative disorder, a consequence from the neoplastic transformation of the primitive hemopoietic stem cell. The disease is originally monoclonal, involving myeloid, monocytic, erythroid, megakaryocytic, B-cell, and occasionally T-cell line-age.[5,6]

It is characterized by a constant cytogenetic alteration, called Philadelphia (Ph) chromosome. [1,7] It was the first disease in history in which a specific chromosomal abnormality was associated to the pathogenesis of the disease. The natural course of the CML foresees three phases: chronic phase (CP), accelerated phase (AP) and blastic phase (BP).

CML was probably the first leukemia recognized as a distinct nosological entity. [8] Some of the earliest case reports are highly suggestive of CML and date back to 1845. Two patients reported massive splenomegaly associated with leukocytosis. Neither of the two cases can be related to any of the common diseases known at the time that could give splenomegaly such as tuberculosis, already known clinically in the 1840s. [9]

The most important indication of its pathogenesis came a century late. Progress in the second half of the 20th century depended critically on the application to leukemia of cytogenetics and molecular biology. Advances in chromosome analysis led Nowell and Hungerforf in 1960 to the discovery of the cytogenetic abnormality that came to be known as the Philadelphia (Ph) chromosome, [10] involving chromosome 22 (Figure 2). In 1973, Rowley found out that the Ph chromosome alteration was the result of a translocation involving also chromosome 9. This chromosomal alteration was called t(9;22)(q34;q11). [11] Advances in molecular biology set the scene for the characterization in the early 1980s of the breakpoint cluster region of what was subsequently named the BCR gene, and led rapidly to the identification of the BCR-ABL fusion gene. [12] Researchers in the 1990s provided convincing evidence that this fusion gene really was the “initiating event” in the chronic phase of CML.

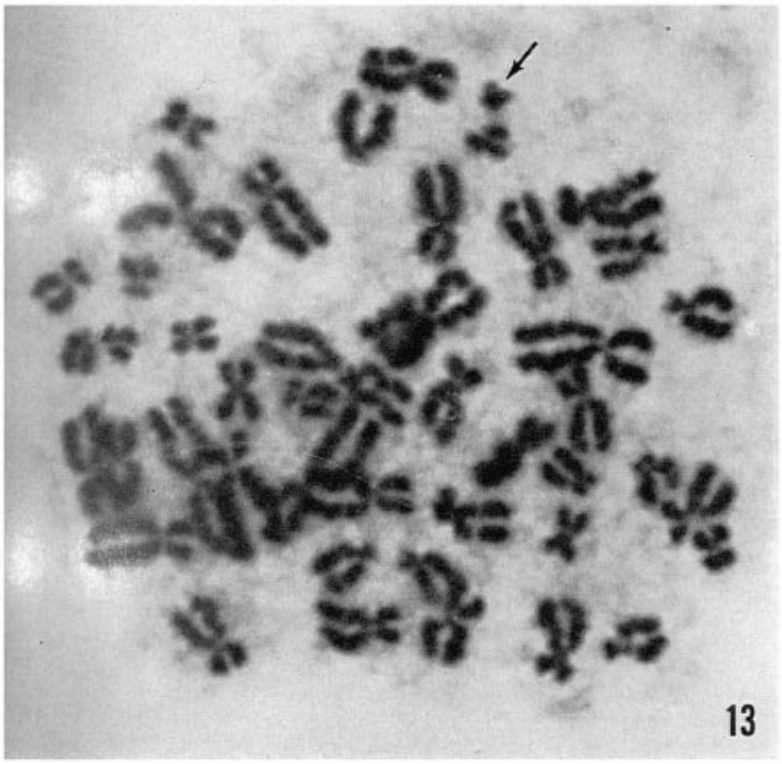

Figure 2. Detail from Nowell and Hungerford's paper in 1960 identifying the Philadelphia chromosome. (From the Journal of the National Cancer Institute)

2.2.1. EPIDEMIOLOGY

Although knowledge about clinical and molecular characteristics of chronic myeloid leukemia (CML) has increased, reliable epidemiological information on CML is limited.[13]

CML is relatively rare. CML is more common in older adults and amongst men. The number of new cases of chronic myeloid leukemia was 1.8 per 100000 people every year, and with a median age at diagnosis of around 64 years.[14]

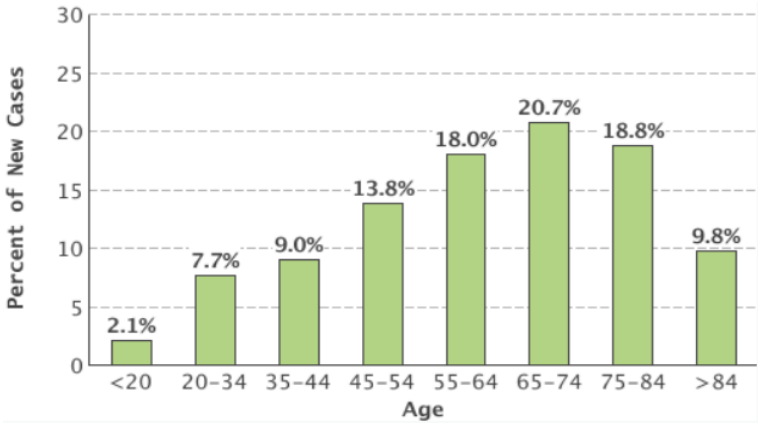

Figure 3. Percent of New Cases by Age Group. (From SEER, all races, both sexes)

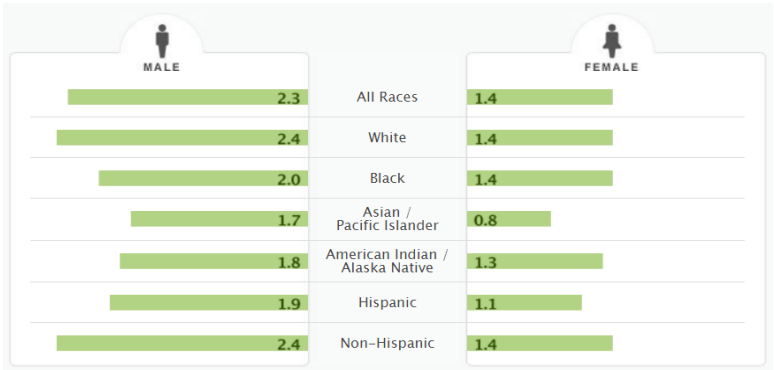

Figure 4. Number of New Cases per 100,000 Persons by Race/Ethnicity & Sex (From SEER, Age-Adjusted)

Disease incidence appears to be consistent across geography and ethnicity, although it is noted that survival rates in some countries are likely to be impacted by the availability of drugs and diagnostic technologies. [14] The increasing prevalence rate was mainly due to the use of TKIs therapy positively influencing survival and life expectancy of CML patients.[15]

2.2.2. PHYSIOPATHOLOGY

CML phases

Chronic phase

Chronic phase is the initial phase, if untreated, has a median duration of 3.5-5 years before evolving to a blastic phase, sometimes preceded by an intermediate or accelerated phase.
At this phase the disease is frequently asymptomatic. [16] Whenever the symptoms occur, the patients present a gradual onset of fatigue, weight loss, increasing sweating, anorexia, early satiety and left upper quadrant pain because of splenic enlargement.[17]
At this stage, there is a substantial increase in myeloid precursors and mature cells that reach prematurely blood flow, but are still able to differentiate.

Accelerated phase

This phase is characterized by an increase of blasts in peripheral blood (around 15%) and is often associated with variable degree of bone marrow fibrosis. In about 70% of CML cases, the accelerated phase evolves naturally in the blast crisis.[18]
Sometimes the accelerated phase may go unnoticed, in consequence of the absence of obvious clinical and laboratory signs and the shortness of the phase, so normally the chronic phase seems to evolve directly to the blastic phase. In the clinical practice it is not simple to identify the three phases of the disease, probably linked to the time of diagnosis, the therapy or the follow up.

Blast phase

This phase is also defined as 'terminal phase' or 'aggressive phase'. [4] The progressive stoppage of maturation and/or differentiation leads to an increase in peripheral and medullary blasts. Blast infiltration pattern mediates anemia and thrombocytopenia, increasingly accentuated by the course of this phase.
It is defined as a percentage of blasts greater than 30% detected in peripheral blood and/or in the marrow, or extramedullary disease.[19]
In this phase, additional chromosomal alterations of cases like trisomy 8, isocromosome 17, t(3;21)(q26;q22) are described in 50-80% cases.[20]
The duration of the blast crisis is short, it usually covers about 6 months and ends in 90% of the cases with the patient's death. While following therapy, reconversion of the blast crisis in the chronic phase is observed in some cases (10-30%) and especially in lymphoid blast-like crisis (40-70%).

The definitions of the CML's phases that are identified by European LeukemiaNet (ELN) [3] are not the same as those identified by the World Health Organization (WHO) [21] Table 1, but

are the ones that have been used globally, and applied in almost all recent and relevant studies of CML.

Accelerated phase	***Definition***
ELN criteria	Blast in blood or marrow 15-29%, or blasts lus promyelocytes in blood or marrow >30%, with blasts <30% Basophils in blood ≥20% Persistent thrombocytopenia (<100 x 10^9/L) unrelated to therapy Clonal chromosome abnormalities in Ph+ cells (CCA/Ph+), major route, on treatment
WHO criteria	Blasts in blood or marrow 10-19% Basophils in blood ≥20% Persistent thrombocytopenia (<100 x 10^9/L) unresponsive to therapy Increasing spleen size and increasing white blood cell count unresponsive to therapy
Blast phase	
ELN criteria	Blasts in blood or marrow ≥30% Extramedullary blast proliferation, apart from spleen
WHO criteria	Blasts in blood or marrow ≥20% Extramedullary blast proliferation, apart from spleen Large foci or clusters of blasts in the bone marrow biopsy

Table 1. List of the criteria for the definition of AP and BP, as recommended by ELN[3,22] and by the World Health Organization[21]

2.2.3. ETIOPHATOLOGY

CML is characterized by the presence of the Philadelphia chromosome.[11]

For many years, the Philadelphia chromosome has been the only known cytogenetic abnormality related to a specific human malignant disease. This chromosome is made from reciprocal translocation of genetic material between chromosomes 9 and 22, reported as t(9,22)(q34;q11). [11] The result is that a fusion gene is created by juxtaposing the 3'-end of a strand of *ABL1 (Abelson)* gene on chromosome 9 to a 5'-end of a strand part of the *BCR* (breakpoint cluster region) gene that stays on chromosome 22. Translocation results in an oncogenic BCR-ABL gene fusion.

The Ph chromosome is present in more than 95% of cases of CML. It also found in 5% of Acute Lymphoblastic Leukemia (ALL) cases in children, 10-25% of ALL cases in adults and 2% of Acute Myeloid Leukemia (AML). For ALL and CML presence of this translocation is a marker for a negative prognosis.[23]

In cases where the Philadelphia chromosome is not observed through conventional cytogenetic analysis, BCR-ABL translocation can be detected using FISH and/or molecular biology techniques. In these cases so-called CML Ph-negative (Ph-masked) more complex mechanisms can occur, such as the insertion of BCR-ABL arrangement from chromosome 22 to chromosome 9, successively to the initial t(9;22). [24] As a consequence of these mechanisms the alteration is not visible with conventional cytogenetic methods.

2.2.3.1. The physiologic function of the translocation partners

In order to understand the mechanisms through which the BCR-ABL oncogene plays a leading role in the pathogenesis of CML, and therefore the consequential formation of proteins, it is important to analyze the biological function of genes involved in translocation t(9;22): ABL and BCR.[25]

ABL gene

ABL is a tyrosine-kinase protein (TKP). TKPs regulate a multiple cellular processes, including cell growth, differentiation, migration and apoptosis. Their activity is normally closely controlled and regulated. Perturbation of TKPs signalling by mutations or other genetic alterations results in deregulated kinase activity and malignant transformation.[26]

ABL gene encodes a nonreceptor tyrosine kinase. [27] It is a 145-kd protein (p145), ubiquitously expressed. This protein presents two isoforms (type 1a or 1b) that originate from an alternative splicing in the first exon. [27] This protein has been well studied, identifying different structural domains within it (Figure 5).

The p145 contains:

- one of the N-terminal domains encoded respectively by exon 1a or 1b;
- three SRC-homology (*SH1-SH3*) near to the N-terminal. The SH1 domain possesses the tyrosine kinase function, while the SH2 and SH3 domains allow interaction with other proteins;[28]
- proline-rich sequences (*PPs*) in the center of the molecule, which may interact with SH3 domains of other proteins, like a Crk;[29]

Toward the 3' end the following are found:

- nuclear localization signals (*NLS*);[30]
- one nuclear exporting signal (*NES*);
- a DNA-binding domain (*DBD*);[31]
- an actin-binding domain (*ABD*); [32] this actin-binding domain contains binding sites for both monomeric (G) and filamentous (F) forms of actin.

Figure 5. ABL gene structure.

Thereby, the ABL protein is implicated in several cellular processes, such a regulation of the cell cycle, [31,33] the cellular response to genotoxic stress, [34] and in the transmission of information about the cellular environment through integrin systems.[35]

<u>*BCR* gene</u>

BCR gene encodes for a 160-kb protein (p160). As with ABL, it is ubiquitously expressed.[27] The 160-kDa protein has different domains (Figure 6):

- the first N-terminal exon encodes a serine/threonine (*S/T*) kinase;
- a coiled-coil (*CC*) oligomerization domain;[36]
- a Dbl/CDC24 guanine-nucleotide exchange factor homology (DH) domain and a pleckstrin homology (PH) domain in the center of the molecule, that activate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP);[37]
- a putative calcium-dependent lipid binding site (CaLB);

- and a RAC guanosine (p21rac) [38] triphosphatase-activating protein (RAC-GAP) domain.[39]

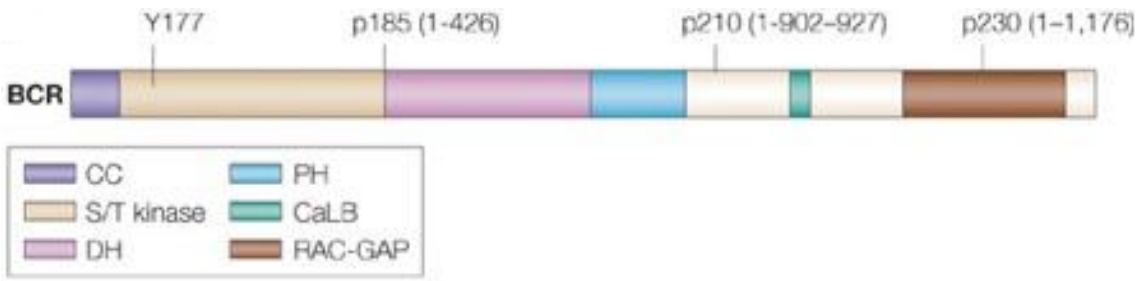

Figure 6. BCR gene structure

Moreover, *BCR* can be phosphorylated on many tyrosine residues, [40] particularly tyrosine 177, which interacts with Grb-2, an essential molecule implicated in the activation of the Ras pathway. [41] The true biologic relevance of BCR is still unknown.

2.2.3.2. Molecular anatomy of the BCR-ABL translocation

Breakpoints within the ABL gene may occur anywhere in the 300kb area of the 5' ends, either before the first alternative exon 1b, after the second alternative exon 1a or, as it more frequently occurs, between the two. [23]

In BCR gene, however, breakpoints occur in 1 of 3 so-called Breakpoint Cluster Regions (BCR), resulting formation of at least three different fusion genes coding proteins with different molecular weight. These proteins turn out to be associated with three different types of leukemia (Figure 7):[42]

- **P190:** chromosomal breakpoint on ch 22 between exons e1-e2 (Minor Breakpoint Cluster region, m-bcr) associated to 20-30% of acute lymphoblastic leukemias (ALL).
- **P210**: breakpoint between exons b1-b5 (Major Breakpoint Cluster region, M-bcr, almost 5.8 Kb) associated to 90% of CML.
- **P230**: breakpoint between exons e19-e20 (centromere breakpoint zone compared to M-bcr) associated to a subgroup of patients with chronic neutrophilic leukemia (CNL).

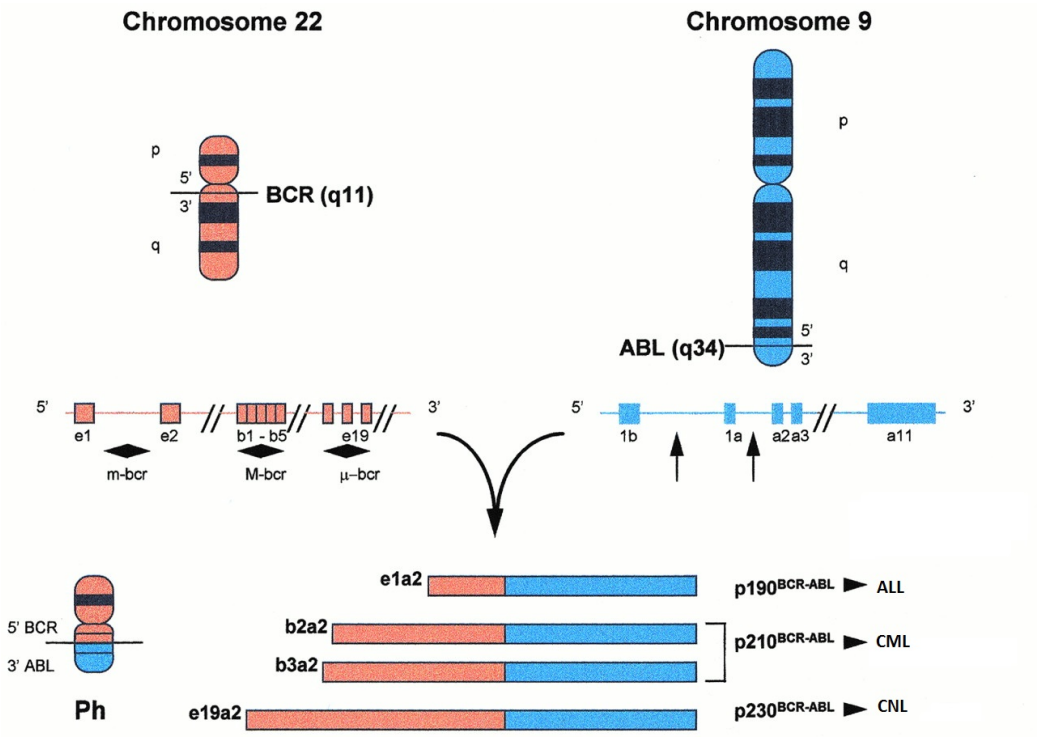

Figure 7. Translocation t(9,22)(q34;11) and structure of the chimeric mRNAs derived from the various breakpoints.

Although the e1-a2 transcript (that encodes for the $p190^{BCR-ABL}$) was primarily associated with ALL, cases characterized exclusively by this type of transcript have been described in sporadic CML, with the exclusive production of $p190^{BCR-ABL}$ protein. These types of patients

are associated with a worse outcome with the tyrosine kinase inhibitors (TKIs) therapy, and must be identified as a high-risk group. [43] Moreover, it has been verified that most of CML patients express at the diagnosis not only the typical $p210^{BCR-ABL}$ transcript, but also low levels of e1-a2 transcript (through an alternative splicing mechanism).[44]

It should be noted that the ABL part of the chimeric protein is almost invariable, whereas the portion of BCR varies considerably. It can be deduced that it is the portion of ABL that probably carries the principle of malignant transformation, whereas the different sizes of the BCR sequences may determine the disease phenotype.[42]
It is unclear whether differences in the activities of the three proteins make it possible to associate them with different phenotypes of the disease or whether the expression of each protein occurs in a distinct hematopoietic line. [23] It has been shown that larger size of the BCR portion in the fusion protein is related to a decrease in tyrosinkinase activity of ABL: consequently, p230 shows lower tyrosinkinase activity than p210 and p190, and in turn p210 has less activity than p190. [45-47]

The specific cause of chromosome translocation is unknown today. One of the proposed hypotheses foresees the presence of fragile sites in correspondence with the breakpoints in the interested DNA region.[48]

However, according to another hypothesis, [49] the proximity of the ABL and BCR genes could favor illegitimate recombination because of the spatial distribution of chromosomes 9 and 22 before the metaphase, could favor illegitimate recombination. Nevertheless, analysis of the flanking sequences at the breakpoints has not revealed the presence of repeated sequences that could favor translocations.

2.2.3.3. Mechanisms of BCR-ABL mediated malignant transformation

BCR-ABL oncogene formation leads to a structural alteration of some of the regulatory domains of the normal ABL and BCR proteins:

- The tyrosin-kinase activity of ABL, usually present at the nuclear level, is predominantly transferred to the cytoplasm and is constitutively activated, either because of the loss of the inhibitory action of SH3 domain, [50] or through oligomerization of the N-terminal domain of BCR, with consequent autophosphorylation in multiple sites;
- The spatial configuration of some BCR domains is modified, making them available to bond with proteins that activate different signal transduction pathways;[42]

These alterations give Ph-positive cells a high tyrosin-kinase activity, which plays a central role in the leukogenesis process.

BCR-ABL induces the progression of the disease through 4 principal mechanisms (Figure 8):

1. **Altered adhesion properties**

   By modifying the adhesive properties to bone marrow stroma cells and to extracellular matrixes, increasing the rate at which they are released into peripheral blood and to the extracellular matrix. [51,52] Ph-positive cells exhibit a dysregulation of signal transduction mediated by the integrin system and are characterized by the hyperphosphorylation of Crkl[53] a protein involved in cellular motility and cell adhesion through association with other factors including paxilin;[54]

2. **Activation of mitogenic signalling**

   By causing the activation of mitogenic signals. [42] The involvement of the JAK-STAT pathway had been demonstrated by studies in v-Abl-transformed B cells. [55] The RAS system is involved either directly by its phosphorylation, or indirectly by the phosphorylation of intermediate substrate (Shc, Crkl). [53,56] The involvement of the JAK-STAT system has been demonstrated in numerous Ph-positive cell lines[57] and in primary CML cells. [58] The two systems, RAS and JAK-STAT, make the BCR-ABL a number of growth factor-dependent cell lines factor independent. [59,60]On the other hand, PI3 kinase activity, like a Myc pathway, contributing for the proliferation activity.[61,62]

3. **Inhibition of apoptosis**

   By inhibiting apoptosis by activating some signal transduction pathways such RAS, [63,64] and involving several proteins like Bcl-2. [65] In particular, CML cells express a number of anti-apoptotic molecules that can contribute to the growth in survival rates of leukemic cells, such as the Bcl-2 family of proteins. The role and effects of Bcl-2

and other anti-apoptotic molecules may depend on the presence of endogenous inhibitors and antagonists expressed in leukemic cells, one of which is represented by the Bim protein. [66] The members of the Bcl-2 family act by regulating the translocation of cytochrome C from the mitochondria to the cytosol, an event that activated the caspase cascade. [67,68] Physiologically, Bim levels are regulated by cytokines such as IL-3, while phosphorylation and subsequent degradation of Bim by BCR-ABL is one of the major mechanisms through which leukemia cells become resistant to apoptosis.[66]

4. **Degradation of inhibitory proteins**.

A recent study shows that BCR-ABL induces the proteasome-mediated degradation of Abi-1 and Abi-2, two with inhibitory function, may be the first indication of yet another way by which BCR-ABL induces cellular transformation. [69]

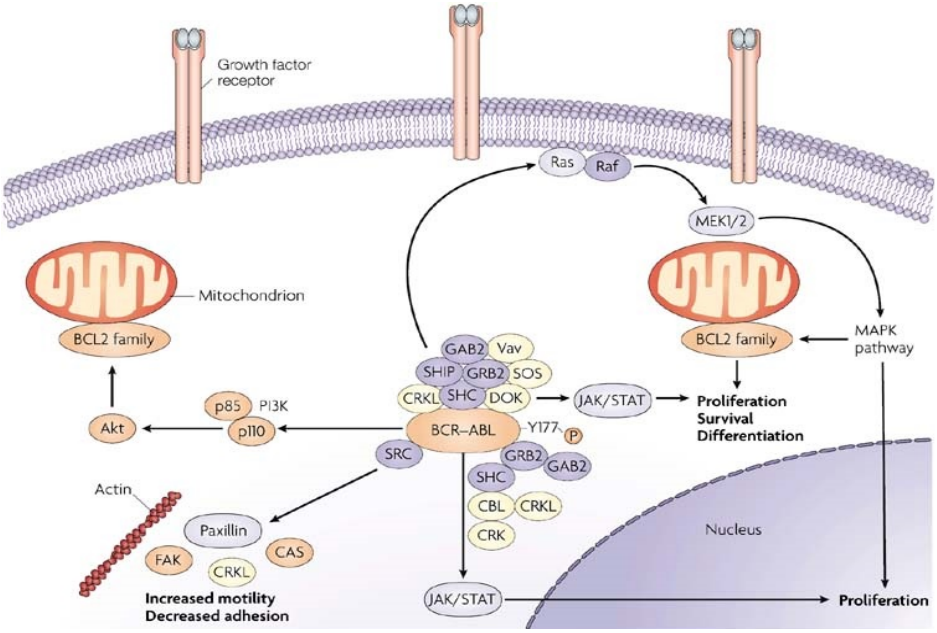

Figure 8. Signaling pathways activated in BCR-ABL positive cells.

2.2.4. MONITORING OF CHRONIC MYELOID LEUKEMIA

The detection of BCR-ABL positivity is crucial for the genotyping of the disease. Monitoring of the amount of BCR-ABL transcripts gained relevance for the assessment of treatment efficacy. An early reduction of transcript levels has been shown to be a prognostic factor in CML, predicting cytogenetic response and clinical outcome in treated patients with tyrosine kinase inhibitors (TKIs).[70-72]

In addition, the degree of molecular response correlated with progression-free survival (PFS) and event-free survival (EFS) in these patients. [73] For patients submitted to stem cell transplantation (SCT) reappearance or persistence of BCR-ABL transcripts indicates an increased risk of relapse. [74,75] Warning signs are given due to rising transcripts levels with a loss of Major Molecular Response (MMR, defined as BCR-ABL expression ≤ 0.1 according to the International Scale, explained in more detail below) as well a serial increase of transcript levels. [3] In cases in which patients do not get quickly significant reduction of transcript levels, a lower probability of achieving a MMR and higher risk of disease progression exists. [76,77]

ELN (European LeukemiaNet) and NCCN (National Comprehensive Cancer Network) recommend monitoring the treatment through hematologic, cytogenetic, and molecular tests, every 3-6 months from the start of therapy, in order to identify and optimize treatment for that minority of respondents who respond more slowly. [3,78] PCR-based tests are considered the "gold standard" methods for molecular monitoring, through the detection and quantification of BCR-ABL transcripts, since they show a high sensitivity (up to 0.001%).

Molecular response (MR) is measured through the fraction of the total amount of BCR-ABL transcript divided by the total amount of the *housekeeping* control gene.

$$\frac{BCR - ABL}{Control\ gene}\ x\ 100$$

There are different levels of MR (Figure 9):

- **MR^3 (≥3-log reduction from IRIS baseline)** = Detectable disease ≤0.1% BCR-ABLIS or absence of BCR-ABL transcripts in cDNA with 1000-10000 ABL transcripts.
- **MR^4 (≥4-log reduction from IRIS baseline)** = Detectable disease ≤0,01% BCR-ABLIS or absence of BCR-ABL transcripts in cDNA with 10000-31999 ABL transcripts.
- **$MR^{4.5}$ (≥4.5-log reduction from IRIS baseline)** = Detectable disease ≤0.0032% BCR-ABLIS or absence of BCR-ABL transcripts in cDNA with 32000-99999 ABL transcripts.

- **MR[5] (≥5-log reduction from IRIS baseline)** = Detectable disease ≤0.001% BCR-ABLIS or absence of BCR-ABL transcripts in cDNA with ≥100000 ABL transcripts.

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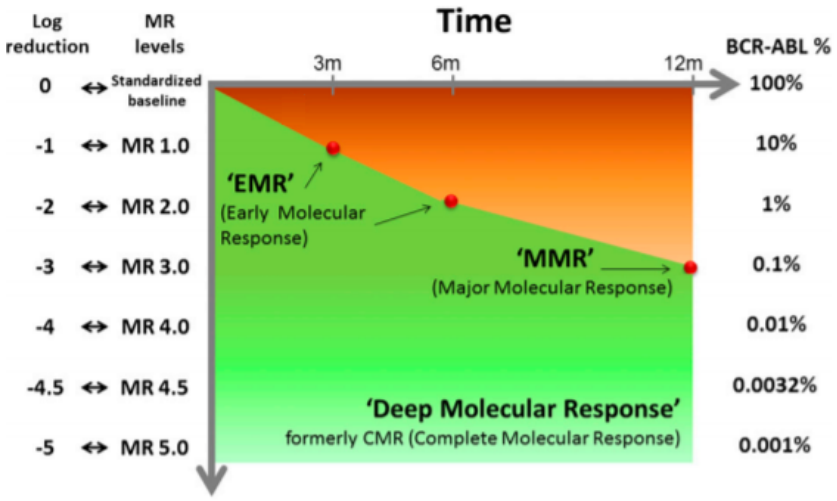


Figure 9. Scheme of different levels of Molecular Response
(From Baccarani, M., & Soverini, S. (2014) Blood, 124*(4), 469-471).*

Major Molecular Response (MMR)

A key indicator of molecular response is Major Molecular Response (MMR), the reduction of ≥ 3 logarithms in the amount of BCR-ABL transcripts over a standardized baseline. This baseline is determined in the IRIS study for untreated patients.[79]

Achieving MMR is strongly associated with an increased probability of long-term stable molecular response, and better progression-free survival (PFS) in patients receiving TKIs.[73,80-82]

Despite the proven prognostic value of MMR, a wide variation in the methods used to quantify BCR-ABL transcripts and the lack of universally accepted standards have led to significant variations in results, making comparisons between laboratories difficult. Therefore, a harmonization of BCR-ABL measurement is required for optimal clinical management. Thus, it will be possible to compare and group results from different laboratories and study groups.

Bearing in mind the clinical importance of MR evaluation, methods have been developed to harmonize the results of BCR-ABL quantification worldwide. Perhaps, one of the most important steps towards this standardization was the creation of the International Scale (IS) in 2005. [83] This International Scale has two standard values: an initial baseline value of

100%^{IS} (as determined in the IRIS study for untreated patients), [79] and the value of 0.1%^{IS} which corresponds to the MMR point (3 log reduction from the initial baseline).

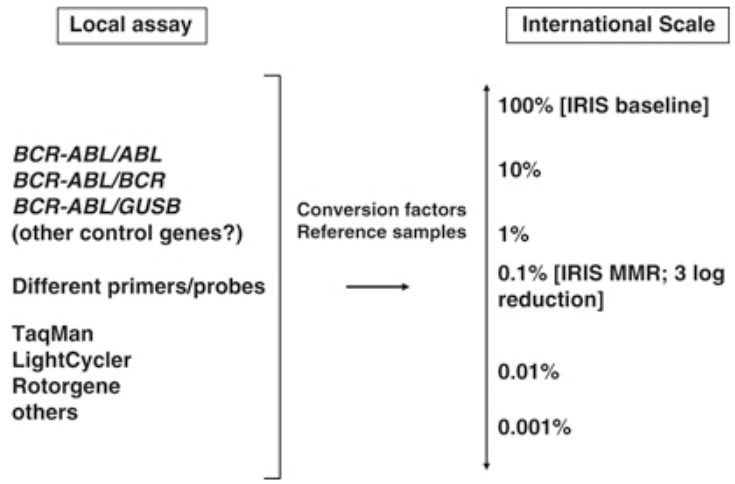


Figure 10. The international Scale (IS) for BCR-ABL real-time quantitative measurements. (From Müller, M. C., et al. Leukemia *23.11 (2009): 1957-1963.)*

The only mechanism currently available, by which laboratories can adopt the IS for their results is to establish a laboratory-specific conversion factor (CF). The CF is obtained by quantifying a certified reference material with its own method. [84]

The different laboratories can continue to use their routine system for BCR-ABL measurements, and generate comparable results with their historical data. With the CF application, this data can be converted to IS, making it directly traceable with the IRIS scale (Figure 10). In this way, the results of different center can be compared to each other.

In Italy it is the LabNet network that monitor this standardization. To date, 120 haematology centers and 56 laboratories have been standardized and are subject to quality controls.

Control Gene

The election of an adequate control gene is decisive for the success of molecular response monitoring. Choosing an appropriate control gene is crucial in order to have reliable data that can be reproduced. Comparison of the results obtained for the control gene helps in the identification of unsuitable RNA samples. [85-87]

Using an appropriate control gene is important for several reasons:[85,88-90]

- To compensate for variations in transcription levels that may occur due to sample

degradation after collection;

- To regulate the differences in the efficiency of the inverse transcription phase and the variations in the final amount of RNA;
- To help to evaluate the sensitivity of each sample measurement.

The control gene must fulfil the three following criteria:

1. It should have an expression level broadly similar to that of BCR-ABL at diagnosis of CML;
2. It should have stability similar to BCR-ABL;
3. Primers for the gene should be proven not to amplify sequences from genomic DNA such a pseudogenes.

Similar levels of RNA stability are essential since delays in sample processing are common and substantial changes in expression can occur very rapidly after blood collection. [90,91]
The most appropiate and frequently used three control genes for BCR-ABL quantification are BCR, ABL and β-glucuronidase (GUSB). Currently, the most used is ABL, although BCR and GUSB are equally suitable. [89]

2.2.4.1. Test Principle

The PCR method (Polymerase Chain Reaction) was the first method of DNA amplification described in literature. [92] It is a method for in vitro amplification of a specific part of DNA (target sequence) by use of a thermostable DNA polymerase. This technique was shown to be a valuable and versatile instrument of molecular biology: its application contributed to a more efficient study of new genes and their expression and has revolutionized for instance the fields of laboratory diagnostics and forensic medicine.

The Real-Time PCR represents an advancement of this basic research technology, providing the possibility to determine the number of amplified DNA molecules (amplicons) during the polymerase chain reaction (PCR).

In the system at hand, monitoring the amplification progress is based on primers/probes labeled with fluorescent molecules. These probes contain a reporter fluorophore and a molecule (quencher) that blocks the reporter's specific fluorescence. Its distance to the quencher determines the fluorescent emission of the reporter. As long as a probe is not bound to a target sequence, the reporter and quencher are in close proximity and the reporter's fluorescence is blocked. Upon binding to a target sequence, quencher and reporter become separated and the reporter can emit fluorescent light, which is detected.

Typically, the main part of a Real-Time PCR run consists of 30 to 50 amplification cycles. A

thermocycler equipped with an adequate detector can record the fluorescence events at each cycle and thus monitor the reaction in “real time”. The cycle at which the fluorescence of the amplification product becomes clearly distinguishable from the background is specific for each reaction and is correlated to the initial concentration of the target sequence. This cycle is called threshold cycle (Ct). The Ct value is used to determine the initial target concentration, with the help of a standard curve. A standard curve is created by amplification of solutions with known concentrations of the target sequence (Figure 11).

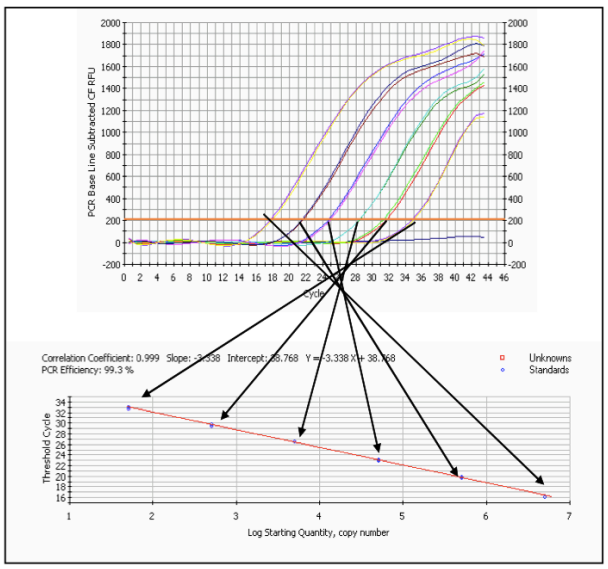


Figure 11. Creating a standard curve using points with known concentrations

The main advantage of the Real Time PCR compared to conventional techniques of amplification is the possibility to perform a semi-automated amplification, generating not only qualitative data but also quantitative data. This means, the extra steps necessary to visualize the amplification result are be avoided and the risk of contamination by post-PCR manipulation is reduced.

2.2.5. CHRONIC MYELOID LEUKEMIA THERAPY

The treatment of CML patients has undergone a great evolution in a relative short period of time. Initially, several treatments were tried in the hope of improving CML prognosis (arsenic, splenectomy, splenic irradiation, busulfan) without achieving good results. In 1963, hydroxyurea, a ribonucleotide reductase inhibitor was developed, and immediately afterwards it was tested in CML therapy[93] Hydroxyurea remains useful for short periods before tyrosine kinase inhibitors (TKIs) treatment, until confirmation of CML diagnosis. [3] The first therapy capable of inducing a considerable cytogenetic remission was interferon-alpha (IFN-α), today used only in rare cases where TKIs cannot be used. The combination IFN- α with TKIs seems to be potentially efficient, but is still in an experimental phase.[94]

Recently, CML therapy has radically improved with the introduction of tyrosine kinase inhibitors.[95]

2.2.5.1. Definition of the response

In each patient the response to therapy will be evaluated over time. European LeukemiaNet (ELN) distinguishes three grades of response. These definitions are clinically important because they provide a guide to therapy (Table 2). The response to treatment may be defined as “optimal”, “failed” or “warning”, according to ELN. [3]

	Optimal	Warning	Failure
Baseline	NA	High risk Or CCA/Ph+, major route	NA
3 mo	BCR-ABL1 ≤10% and/or Ph+ ≤35%	BCR-ABL1 >10% and/or Ph+ 36-95%	Non-CHR and/or Ph+ >95%
6 mo	BCR-ABL1 <1% and/or Ph+ 0	BCR-ABL1 1-10% and/or Ph+ 1-35%	BCR-ABL1 >10% and/or Ph+ >35%
12 mo	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 CHR Loss of CCyR Confirmed loss of MMR* Mutations CCA/Ph+

Table 2. Definition of the response to TKIs (any TKI) as first-line treatment (From Baccarani, Michele, et al. Blood *122.6 (2013): 872-884.)*

2.2.5.2. First Generation of Tyrosine Kinase Inhibitor

IMATINIB

Since 1999, CML therapy has undergone profound changes with the introduction of the first TKI, Imatinib (STI571 or Gleevec®/Glivec®), an antineoplastic agent developed with the aim of selectively inhibiting certain tyrosine kinases involved in oncogenesis. This became the first example of targeted therapy, as it targets and attacks the causative agent of CML. Imatinib is a 2-phenylamina pyrimidine class[96,97] (to which ABL tyrosine kinase belongs) and was created using the kinase ATP binding site structure (Figure 12)[98]

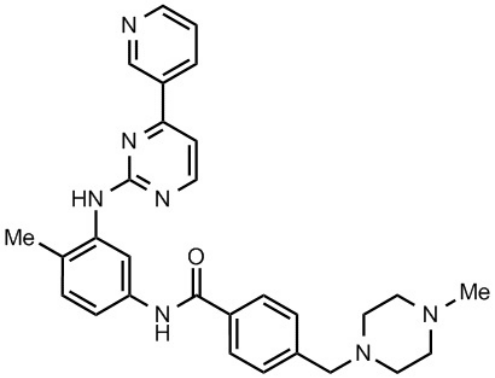


Figure 12. Imatinib (Glivec®, Novartis)

It was initially developed as a specific inhibitor of the platelet-derived growth factor (PDGF) signal transduction pathway, but was later discovered to inhibit the autophosphorylation of several tyrosine-kinase such v-Abl, c-Kit, and to block PDGF-induced inositol phosphate formation.[97,99]Additional analyses revealed that Imatinib binds to the inactive form of BCR-ABL on the normal ATP-binding site, thus preventing interaction with ATP and switching to active form (Figure 13).[100]

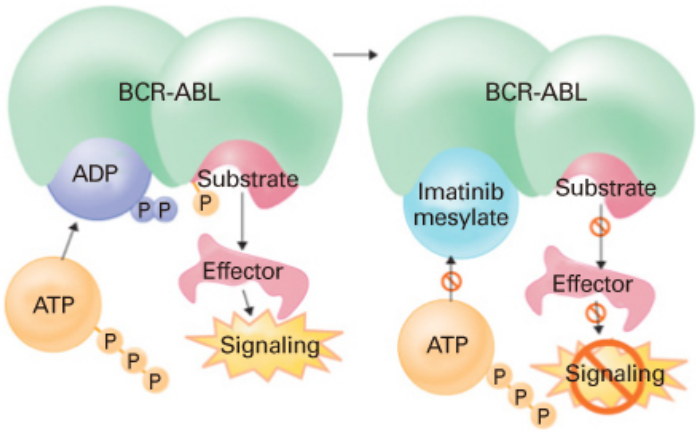


Figure 13. Imatinib interaction with tyrosine-kinase proteins

This results in a block of both the ability of BCR-ABL to autophosphorylation and its ability to phosphorylate other target proteins, essential to conferring and maintaining the Ph-positive leukemia phenotype, [96,97] thus inhibiting proliferation and inducing cellular apoptosis[98,101] (Figure 14).

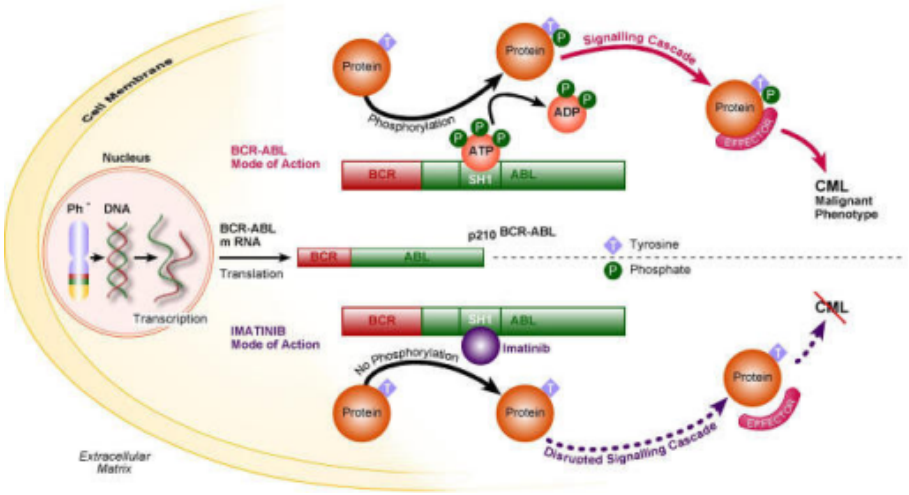


Figure 14. Imatinib mechanism of action

In 1998 Imatinib was introduced for clinical investigation and in 2001 was approved by FDA (Food and Drug Administration) and EMA (European Medicines Agency) for CML patients who had unsuccessful treatment with IFN. [102-105] In December 2002, treatment of patients with newly diagnosed CML (first-line treatment) was approved. [106] Since 2016, Novartis'composition of matter Imatinib was expire. The impact on health system spending levels for CML after generic Imatinib has become available is the subject of considerable interest among stakeholder including physicians, payers, and patients.

Recently the first-line therapy with Imatinib have been reported in many studies. [73,79,81,107-125] 49% to 77% of the patients who used 400 mg of Imatinib daily for 1 year achieved the complete cytogenetic response (CCyR), and 18% to 58% achieved the major molecular response (MMR). [111,112,114,121,125] Instead, using 600 mg or 800 mg the CCyR was achieved from 63 to 88% of the patients and from 43% to 47% for what concerns the MMR.

For high risk patient, [73,107,112,121-125] the CCyR at 1 year ranged from 48% to 64%, whereas the MMR ranged from 16% to 40%.

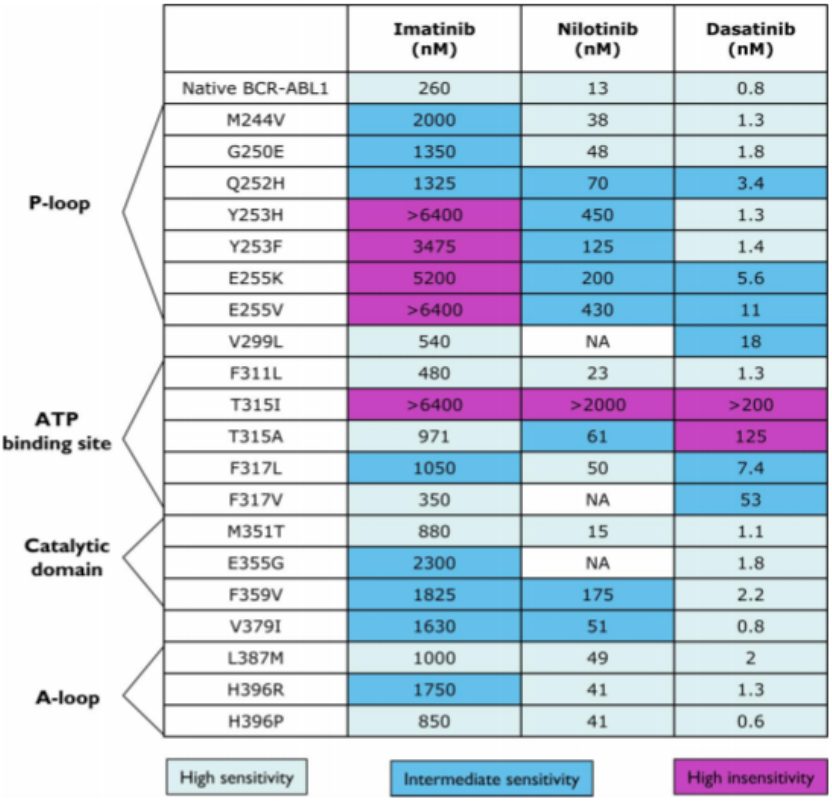
The progression-free survival (PFS), at least at 5 years of follow-up, was described growing

from 83% to 94%; the overall survival (OS) ranged between 83% and 97%. The patients still receiving Imatinib as initial treatment was reported at 63% to 79% after 3 to 5 years, and after 8 years only the 50%.[2,83,113,118,120] More than 800 mg daily was reported in one study. [118] To date no clinically relevant late onset side effect are described.

The selective inhibition of BCR-ABL by Imatinib has substantially changed the therapy and the natural history of the disease. However, despite the high percentage of hematologic and cytogenetic responses, resistance to treatment may develop in a portion of patients. In addition, there is a minority of patients who are intolerant to treatment. The resistance to Imatinib can be hematologic, cytogenetic and molecular. This resistance comprises the inability of the drug to induce a normalization of haematological values or to reduce the share of BCR-ABL transcripts.

Molecular resistance to treatment often occurs due to the existence of mutation in the kinase domain. Some of the mutations to the kinase domain reported so far have a detrimental effect on the affinity and efficacy of TKIs. [126] The mechanism by which some mutations disrupt the binding affinity has been studied. One of the mutations, T315I, substitutes a threonine with an isoleucine. The substitution between these two amino acids removes an important H-bond donor from the ATP binding site. To date, the result of this mutation yields one of the most resilient mutations to TKIs known. [127]

Several mutation points have been shown to be related with an increase of resistance to TKIs (Figure 15), thus showing how the region in which the mutation happens is a possible predictor of the clinical outcome.[128]



		Imatinib (nM)	Nilotinib (nM)	Dasatinib (nM)
	Native BCR-ABL1	260	13	0.8
P-loop	M244V	2000	38	1.3
	G250E	1350	48	1.8
	Q252H	1325	70	3.4
	Y253H	>6400	450	1.3
	Y253F	3475	125	1.4
	E255K	5200	200	5.6
	E255V	>6400	430	11
	V299L	540	NA	18
ATP binding site	F311L	480	23	1.3
	T315I	>6400	>2000	>200
	T315A	971	61	125
	F317L	1050	50	7.4
	F317V	350	NA	53
Catalytic domain	M351T	880	15	1.1
	E355G	2300	NA	1.8
	F359V	1825	175	2.2
	V379I	1630	51	0.8
A-loop	L387M	1000	49	2
	H396R	1750	41	1.3
	H396P	850	41	0.6

Figure 15. Scheme of the activity of TKIs against a selection of BCR-ABL1 mutants found in patients with CML. The concentrations are shown in nM/mL and represent IC50 values (from Quintàs-Cardama, Blood 113 2009)

New molecules and Imatinib analogs were developed to avoid resistance, giving rise to the second and third generation of TKIs.

2.2.5.3. Second Generation of Tyrosine Kinase Inhibitors

As stated above, a percentage of patients treated with Imatinib result resistant or intolerant to it. The resistance is due to mutations points in the kinase domain of the ABL portion in 70-90% of cases. These mutations are involved specifically in residues implicated in binding to Imatinib or, more frequently, residues important for BCR-ABL's ability to adopt a conformation favorable to binding Imatinib. [126,127,129-131] Based on this, new drugs have been developed: Nilotinib and Dasatinib.

NILOTINIB

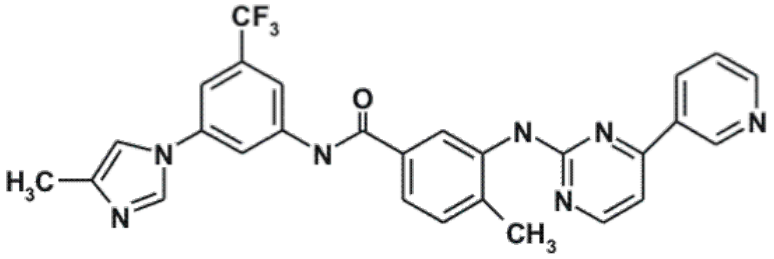


Figure 16. Nilotinib (Tasigna®, Novartis)

The binding of Nilotinib (Figure 16) to BCR-ABL is more favorable than the same bond with Imatinib. This results in a more selective action of Nilotinib on target cells. In fact, it has been demonstrated that Nilotinib has shown a 30-fold higher potency than Imatinib *in vitro*. [123] This feature is particularly important for older patients, because they tend to have more difficult tolerating treatment with Imatinib, especially if used in high doses.

The efficacy of Nilotinib in patients with Imatinib resistance has been demonstrated in several phase 2 trials applying a dosage of 400 mg twice daily. [132-134] In 2007, Nilotinib was approved for second-line treatment in blast phase and accelerated phase.

Today, Nilotinib can be used as a first-line treatment, [3] with significantly higher and faster rates of MMR and CCyR and lower progression rates compared with Imatinib.[121]
Furthermore, it has been shown that Nilotinib has pro-apoptotic properties resulting in an increase in Bim protein levels and thus acting as an oncosuppressor in leukemic cells. Because the BCR-ABL acts by degrading Bim and thus inhibiting the apoptotic process, Nilotinib inhibiting BCR-ABL activity, acts as antagonist.[135]

DASATINIB

Dasatinib (Figure 17) is a dual specific ABL and SRC gene kinase inhibitor, not structurally related with Imatinib. Dasatinib has demonstrated 325-fold higher potency than Imatinib *in vitro,* for unmuted BCR-ABL. [136] Dasatinib is able to inhibit other tyrosine kinases such SFK, c-kit and PDGFR-beta. SFK has an important role in the progression to blastic phase, thus a part of the therapeutic effect of Dasatinib might be related to inhibition of SFK. [137-139]

Many phase II trials in patients with resistance or intolerance to Imatinib demonstrated proof of efficacy within the START program (SRC/ABL Tyrosine Kinase Inhibition Activity: Research Trials of Dasatinib).[140-146]

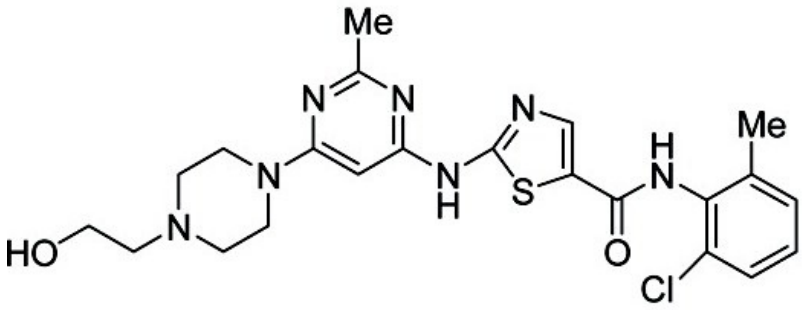


Figure 17. Dasatinib (Sprycel®, Bristol-Myers Squibb)

Dasatinib was approved in 2006 for the treatment of patients with resistance or intolerance to Imatinib. [147] A high efficacy as first-line therapy for CP has been proven. A comparative study that investigated Dasatinib 100 mg daily versus Imatinib 400 mg daily as first-line therapy, demonstrated higher and faster rates of CCyR and MMR after one year. [124]

The European LeukemiaNet (ELN) recommendations indicate that patients with new diagnosis of CML should be treated with Imatinib, Nilotinib or Dasatinib, and have a follow-up to identify the success or failure of therapy. [3] Even though it is known that Nilotinib and Dasatinib are more powerful and induce deeper and faster remissions and lower risks of accelerated phase/blast phase (AP/BP) transformation, there is not yet concrete proof of a better outcome, if a second-generation TKI is used first-line. [123,125] Second generation of TKIs are frequently preferred in patients with a high score in the Sokal[148] o Hasford[149] scale, or when the priority is to rapidly reach deep Molecular Responses (MRs). [150] However, the long-term safety profile of these drugs remains unclear to date. Even though serious adverse effects (AE) such as pulmonary toxicities[151,152] or vascular disease[153,154] occur infrequently with second-generation TKIs, these AEs lead to significant morbidity when they occur.

Despite the clear differences in AP/BP transformation rates, these AEs contribute to the lack of significant difference in overall survive rates (OS) between patients treated with Imatinib and those receiving Nilotinib or Dasatinib. [123,125]

In general, for most patients, the current treatment for CML is rendering it more of a chronic disease with a high median survival rate. [155] Quality of life issues require more attention since the overall survival of most patients has improved.

2.2.5.4. Stem Cell Transplantation

After the introduction of TKIs treatment the percentage of allogeneic Stem Cell Transplantation (SCT), the only treatment that can currently render patients molecularly negative, has significantly decreased. The associated procedural-related morbidity and mortality remain a major brake. [156] Indications for allogeneic SCT in fit patients below the age of 65 are today:[157]

i. A significative progression to advanced phases;
ii. Failure of first- or second-generation TKI treatment;
iii. Appearance of the T315I mutation with failure of TKI treatment;
iv. Patient's willingness.

2.2.6. PREDICTION OF THERAPEUTIC RESPONSE

To date there still are issues that remain pending regarding the long-term safety profile of these drugs. Although severe adverse effects, such as cardiovascular or pulmonary, do not commonly occur with 2nd generation of tyrosine kinase inhibitors (TKIs), when they appear they lead to a worse morbidity. This toxicity could partly contribute to the lack of significant overall survive differences among patients treated with Imatinib and those treated with Nilotinib or Dasatinib, despite the differences in the acute phase/blastic crisis transformation rate[158].

Approximately 1/3 of patients treated with Imatinib at first line and about 1/10 of those treated with Nilotinib and Dasatinib do not get early molecular response (EMR). The failure of early molecular response is not predictable on the basis of clinical features, so the identification of biological parameters that can define the response to treatment would be of great interest and clinical significance.

The European LeukemiaNet recommendations have considered that a single molecular test of BCR-ABL transcript quantification cannot be sufficient to take such an important decision as the change of treatment. At least two tests are required, at 3 and 6 months (Table 2), to provide a sounder basis for treatment decisions.[3]

The existence of several first-line therapeutic options and the inability to predict each individual response slow down the therapeutic course, not allowing full use of the potential for therapeutic efficacy due to the availability of more first-line therapeutic options.

As already mentioned there are several methods for CML diagnosis and monitoring. However, these analyses do not provide information on how drugs could work on patients in terms of efficacy and bioavailability.

Several factors may give some indication of TKIs response and outcome. There are three prognosis systems –Sokal[148], Euro[149], and EUTOS[159]- based on clinical and haematological data that have been shown to be still useful[160] (Table 3).

Study	Calculation	Risk definition by calculation
Sokal et al. 1984	Exp 0.0116 × (age − 43.4) + 0.0345 × (spleen − 7.51) + 0.188 × [(platelet count ÷ 700)^2 − 0.563] + 0.0887 × (blast cells − 2.10)	Low risk: <0.8 Intermediate risk: 0.8-1.2 High risk: >1.2
Euro Hasford et al. 1998	0.666 when age ≥50 y + (0.042 × spleen) + 1.0956 when platelet count >1500 × 10^{9}L + (0.0584 × blast cells) + 0.20399 when basophils >3% + (0.0413 × eosinophils) × 100	Low risk: ≤780 Intermediate risk: 781-1480 High risk: >1480
EUTOS Hasford et al. 2011	Spleen × 4 + basophils × 7	Low risk: ≤87 High risk: >87

Table 3. Calculation of the risk related to obtaing a poorer response to TKIs (From Baccarani, Michele, et al. Blood *122.6 (2013): 872-884.)*

To date, there is no evidence of which of the three prognosis system is superior or more convenient and there is no clear evidence that patients with intermediate risk behave differently than low-risk patients.

Prognostic implications of some basic factors such as gene expression profiles, the polymorphism of genes encoding TKI transmembrane transporter, TKI-mediated apoptosis, and detailed molecular dissection of the genome have been demonstrated. However, these data are not yet sufficiently mature to be used for treatment planning[3].

2.2.6.1. Tyrosine Kinase Assay

Tyrosine-kinase assays are well-established instrument in the modern biochemistry. In the last few decades, the importance of cell phosphorylation events has been increasingly recognized, based on the knowledge of the fundamental role of kinase proteins in all aspects of cellular physiology such as growth, differentiation, and metabolism[161]. Using HTS (High Throughput Screening) technology, new drugs have been developed that work as kinase enzymatic modulators in a wide range of diseases such as neoplasms, inflammatory diseases, neurological disorders, and metabolic disorders.[162,163]

The classification of the different kinase assays includes:

- Detection of the phospho-peptide
- ATP consumption
- Analysis of ADP production

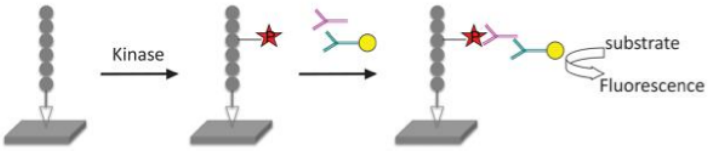


Figure 18. Schematic of the tyrosine kinase assay based on phospho-peptide. On the left the peptide bound to a solid phase is shown. After addition of specific kinase the phosphorylation that occurs between the interaction kinase-peptide can be detected using antibodies coupled with horseradish peroxidase (HRP) or other appropriate reporters.[162,163]

2.2.6.2. LeukoPredict™

LeukoPredict screens in vitro the inhibitory potential of several BCR-ABL-targeting drugs simultaneously and obtain the percentage of inhibition compared to the same non-treated samples. This system indicates the most effective first-line treatment before therapy initiation. It consists of a solid enzyme immunoassay, based on specific substrates that allow the measurement of BCR-ABL endogenous activity.

LeukoPredict works similarly to the phosphopeptide-based assay (Figure 18). The peptide is synthetic and interacts with a specific form with the enzyme domain ABL1 and the domain SH3 of the BCR-ABL enzyme[164-166].

The peptide is synthesized using solid phase Fmoc chemistry. A lysine has been functionalized with a biotin molecule to allow the peptide to be easily bonded to a solid substrate.

The domain named “reporter” (Figure 19), with the EAIYAAPFAKKK sequence, is an optimized substrate for ABL1[167]. The domain named "targeting" (Figure 19), with the

APTYSPPPPP sequence, is a sequence rich of proline that is recognized by the SH3 domain that mediates the interaction between peptides and proteins, greatly improving its specificity for the target.

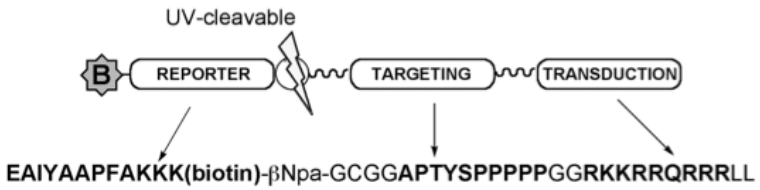


Figure 19. ABL peptide scheme. Complete sequence and domains.

The LeukoPredict's protocol foresees the following steps:

1. Peripheral blood collection of the patient before and after treatment.
2. Isolation of BCR-ABL enzyme presents in the patient's blood using a specific lysis buffer. This lysis buffer is a creation of the university of Verona and is under patent application.
3. Incubation of cell lysate in the ELISA plate coated with the peptide substrate.
4. Washing the cell lysate.
5. Detection of phosphorylation with biotin-labeled monoclonal antibody.
6. Washes.
7. Amplification of the signal by secondary and chromogenic antibodies.

The ABL peptide is immobilized at the bottom of the wells of ELISA plate. In the presence of high enzymatic activity (translated in poor action of the TKI), the BCR-ABL enzyme adds phosphate groups to the ABL. If instead the TKI inhibits the action of the BCR-ABL enzyme, the amount of such phospate groups will decrease. This difference in enzyme activity is quantifiable across the fluorescence difference of the monoclonal antibody.
The preliminary tests show the feasibility of this approach.

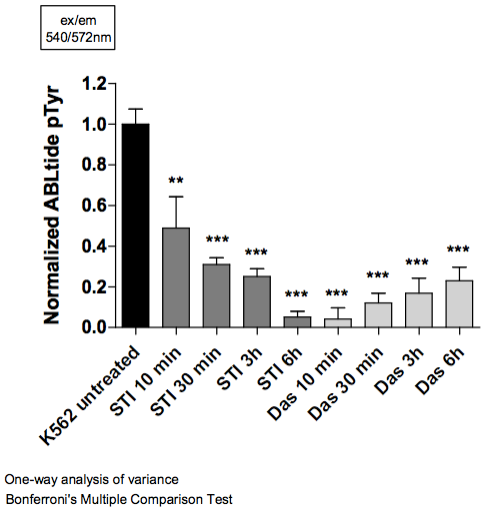


Figure 20. Time course analysis of BCR-ABL activity in K562 treated with the TKIs Imatinib (STI) and Dasatinib (Das).

The K562 CML cell line was treated in the times indicated with 5 μM Imatinib (STI) and 16 nM Dasatinib (Das) (Figure 20). The cells were lysed and 4 μg of the cell lysate was in contact with the immobilized ABL peptide. The data comes from three independent experiments.

LeukoPredict test was developed by the University of Verona, and the product optimization tests will be carried out by their researches. The company DITTA DINO PALADIN has conducted the activities related to the industrialization process of the product.

2.3. INDUSTRIALIZATION PROCESS

The assessment of an assay's potential as a commercial IVD includes many different steps, preveeincluding as preliminary steps of Freedom to Operate and a pharmaco-economic analysis. These steps may be repeated and updated as increasing degrees of evidence are collected.

Freedom to operate Analysis

This kind of analysis is aimed at defining the level of risk related to products launched on the market. It's important to look at the risk to interfere with third-party existing patents.

Pharmaco-economic analysis

With the current international situation, the disproportionate increase in costs of many new health technologies and the growing pressure to reduce the budget set aside for healthcare in most countries, it is becoming increasingly necessary to make and economic evaluation of health interventions.

Pharmaco-economy is a relatively recent world that refers to the application of economy to pharmaco-therapy. The concepto comes from developed countries and specifically from Australia, where was adopted in the 80s by the pharmaceutical giants.

The majority of these pharmaco-economic studies focus on three targets:[168]

a. Economic evaluations to define the convenience of a new product both in the early and developing stages.
b. Economic evaluations to justify the price of the product, identifying the key elements for fixing the prices in terms of production costs and market characteristics.
c. Economic analysis to calculate the product payback.

The pharmaco-economic departments of pharmaceutical companies are often under the supervision of the marketing department, [169] that is unlike the departments responsible for clinical studies that are autonomous.

There are many different economic analyses.
The **cost-benefit analysis (CBA)** is the most common and well known. It is an analysis of the expected balance of benefits and costs, including an account of previous alternatives and the *status quo*. In CBA, benefits and costs are expressed in monetary terms, and are adjusted for the time value of money.

Cost-effectiveness analysis (**CEA**) is a form of economic analysis that compares the relative costs and outcomes (effects) of different plans of action. Typically the CEA is used in the field of health services expressed in terms of a ratio where the denominator is a health gain from a measure (years of life, premature births averted, sight-years gained) and the numerator is the cost associated with the health gain.

The more complex is the **cost–utility analysis** (**CUA**). This is a form of financial analysis used to guide procurement decisions. The most common and well-known application of this analysis is in pharmacoeconomics, especially health technology assessment (HTA). In health economics the purpose of CUA is to estimate the ratio between the cost of a health-related intervention and the benefit it produces in terms of the number of years lived in full health by the beneficiaries. In the reference case analysis, the measure of utility must be a quality-adjusted life-year compatible health-related quality-of-life-score.

In the health field, cost-effectiveness and cost-utility are often used interchangeable.

In developed countries where it is possible to settle and control costs of health devices' production and where health insurance is basically absolute, pharmaco-economics can clearly lead to improve the clinical decision-making process, mainly between different medical treatments that looks to be fairly similar. The principal aim shouldn't be cost control but the improvement of efficiency to gain equity of access to health care.[170]In most developed countries the different healthcare systems defines who pays for medicine and how much. When there is a co-payment system there is a specific level of participation between parties: in developed countries the government covers more than the 60% of the cost.

In developing countries, where it is a large percentage of the population doesn't have free access to drugs and many people die from diseases for which there are preventive medicines, pharmacoeconomics should have a different approach and distinct characteristics. In addition to cost management, it's important to make the use of resources more efficient and to gain new sources of funding so as to ensure the medical coverage to everybody. Therefore, the priority has been given to the macroeconomic analysis compared to single medicaments. In many developing countries the consumer pays directly the 70% of treatment's cost. Furthermore, the Government is largely absent in defining mechanisms of treatment's funding to make it accessible to all citizens. In several parts of Africa, Asia and Latin America the issue isn't about choosing the most appropriate treatment but it's about having access to the only available resource. Quality of life is off the table, it's just about having the chance to survive to an infection treated with first generation antibiotics.

It is clear that if individual and social benefits of many medicines resulted in monetary terms and prices of medicine, this would lead to an uncontrollable increase in treatment prices. In this sense it the need to establish methodological compulsory and standard guidelines to encourage the governmental control and an objective comparison between different studies has been suggested. [171]

There are many different key elements to be taken into account when defining studyies's criteria: [172]

a. The typology of economic analysis that has to be considered.
b. Methods to be used in economic evaluation, procedures to develop them and needed criteria, social impact or economic outcomes resulting from specific therapeutic strategy.
c. Ethical criteria that should lead the economic evaluation of medicaments.

There has to be a total independence between the scientist and the investor.
In this field of study exists a specific terminology, which is important to understand before to facing this kind of analysis. In succession these are the most important terms:[173]

Quality-Adjusted Life-Year (QALY): a year of life lived in perfect health.
Quality-Adjusted Life Expectancy (QALE): the average number of QALYs adjusted of life expectancy.
Competing alternative: An alternative practice for diagnosing or treating the disease you are studying in a cost-effective analysis.
Average Cost-Effectiveness Ratio: the net cost of an intervention divided by its net benefit gain versus the no-intervention option.
Incremental Cost-Effectiveness Ratio (ICER): Between two competing alternatives, it is the ratio of the change in costs and the incremental gain in benefit.

$$\frac{(Cost\ of\ intervention\ 2 - Cost\ of\ intervention\ 1)}{(QALE\ 2 - QALE\ 1)}$$

The intervention less effective is usually positioned on the right-hand side of the equation, ensuring that the ratio will be positive if the intervention costs money but improves health and negative if the intervention saves money and improves health.

Health-related quality-of-life (HRLQ) score: a numerical evaluation of life in a given health state anchored between 0 (death) and 1 (perfect health). These scores are used to adjust

life-years to quality-adjusted life-years (QALYs). Thus, a year of life lived at an HRLQ of 0.7 is equivalent to 0.7 QALYs (or 0.7 years of perfect health).

Discounting Future Costs: medical interventions, especially preventive interventions, often result in decreased future medical costs that must be accounted for in present-day terms. Humans have a tendency to place a lower value on future events than on events that occur in the present. This phenomenon is called discounting. In cost-effectiveness analysis, all future cost and effectiveness values must be discounted into their net present value. [174] The Panel on Cost-Effectiveness in Health and Medicine bases the recommended discount rate on a valuation that is roughly equal to the real rate of return on government bonds. Because this rate varies over time and the reference case is concerned with making all cost-effectiveness analyses comparable, the panel has settled on a rate of 3 percent. However. Standards vary from country to country.
In Europe this value is established by NICE guidelines, which estimates a discount of 3,5%.
The general formula for discounting future costs is:

$$\textit{Discounted cost} = \frac{Cost\ of\ the\ future\ event}{(1+discount\ rate)^{years\ in\ the\ future}}$$

Decision analysis model: a model used to calculate the expected value of a given health strategy. Decision analysis models are the most frequently used means for calculating incremental cost-effectiveness ratios.

Markov model: a decision analysis model that incorporates an element of time.

Sensitivity analysis: an analysis that varies model inputs over their plausible range of real-world value in order to examine how they might influence model outputs.

Tornado diagram: a test of the effect of error on model outputs in which each variable of interest within the model is sequentially varied over a range of plausible values holding all other variables constant. Graphs of the variables are stacked according to their overall influence on the model, so the output assumes the appearance of a tornado.

3. AIMS

The lack of prognostic factors that could unbalance physicians towards a certain tyrosine kinase inhibitor compared with another one makes the therapeutic drug monitoring really essential to evaluate the therapeutic efficacy and potential cross-resistances.

This project has been focused on CML therapy, facing the subject from two totally different angles: the therapeutic drug monitoring and the prediction of response to treatment.

3.1. MONITORING THERAPY

As stated above, the therapeutic monitoring gold standard technoque is qRT-PCR Real Time through quantification of molecular response because of its ability of quantifying and identifying until few copies of BCR-ABL transcripts. Then, we developed two new kits based on qRT-PCR Real Time to identify and measure the translocation t(9;22)(q34;11) and its variations, $p210^{BCR\text{-}ABL}$ (RQ-BCR-ABL p210 One-Step) and $p190^{BCR\text{-}ABL}$ (RQ-BCR-ABL p190 One-Step), to have a full picture of disease's monitoring.

3.2. PREDICTION OF THERAPEUTIC RESPONSE

Currently there's no way to predict the therapy's effect with tyrosine kinase inhibitor on a each patient. For this purpose LeukoPredict has been developed LeukoPredict, an *in vitro* test to screen simultaneously the inhibitory potential of several BCR-ABL-targeting drugs, and to obtain the percentage of inhibition compared to the same non-treated samples. LeukoPredict test was developed by the University of Verona, and we have conducted some activities related to the industrialization process of the product.

4. METHODS

4.1. DEVELOPMENT AND VALIDATION OF THE NEW "RQ-BCR-ABL P190 ONE-STEP"

The choice of primers and probes was done following the European LeukemiaNet (ELN) recommendations[85]

We carried out validation experiments on the "Applied Biosystems 7500 Fast-Dx Real-Time PCR System (Applied Biosystems)" instrument (abbreviated as ABI 7500 Fast Dx).

As controls we used the standard 5-point curve of the "REALQUALITY RQ-BCR-ABL p190 STANDARD" kit (100-1,000,000 copies for 5 µL) for BCR-ABL p190 and for ABL, calibrated checking the ABL target using the ERM -AD623 (IRMM), certified reference material.

To determine the specificity and diagnostic sensitivity values on ABI 7500 Fast Dx, a total of 17 RNA (leukocyte pellets and cell lines) were analysed:

- 4 RNAs derived from serial dilutions of IVS-0032 Clonal Control RNA;
- 1 RNA derived from negative cell line for BCR-ABL p190 translocation;
- 6 RNAs of patients with ALL;
- 1 RNA of patient with CML (positive for BCR-ABL p210);
- 3 IVS-0032 Clonal Control RNA;
- 2 Control RNA: High and Low BCR-ABL1 p190 Control (ipsogen BCR-ABL1 mbcr Controls Kit)

The negativity / positivity of investigated translocation samples was verified by the supplier using another CE IVD method (ipsogen® BCR-ABL1 mbcr). IVS-0032 Clonal Control RNAs are commercial controls positive for the $p190^{BCR-ABL}$ translocation.

The rarity of investigated translocation has complicated the finding of more samples and the finding of collaborations in order to subject the product to external validation.

All the samples analysed during this validation were processed according to the buffy coat method[175] (with red blood cell lysis buffer) and then the RNA was extracted from leukocyte pellet using the following kits:

Automatic: Maxwell® 16 System (Promega)

Manual: RNeasy Mini Kit (QIAGEN)

Evaluation Criteria for Sample Analysis

Working in replicates, it is sufficient that there is amplification in one of the wells of the BCR-ABL p190 target to give a positive result; instead, to give a negative result, it is necessary that there is not any amplification in any of the wells.

In the case of the positivity of at least one of the replicates in the quantitative analysis, the use of the standard curve for BCR-ABL p190 and ABL allows to transform the Ct values of the investigated samples into ABL copy numbers $(ABL)_{CN}$ and BCR-ABL p190 $(BCR\text{-}ABL\ p190)_{CN}$.

The eligibility criterion required for sample analysis in which MMR is evaluated is that the number of ABL copies is ≥ 10000. [85] This requirement allows obtaining the optimum sensitivity level for the specific survey type.

4.2. DEVELOPMENT AND VALIDATION OF THE NEW "RQ-BCR-ABL P210 ONE-STEP"

The choice of primers and probes was done following the European LeukemiaNet (ELN) recommendations[85]

We conducted validation experiments on the "Applied Biosystems 7500 Fast-Dx Real-Time PCR System (Applied Biosystems)" tool (abbreviated as ABI 7500 Fast Dx).

As controls we used the standard 5-point curve of the "REALQUALITY RQ-BCR-ABL p210 STANDARD" kit (100-1,000,000 copies for 5 µL) for BCR-ABL p210, ABL and GUSB calibrated checking the ABL target using the ERM-AD623 (IRMM), a certified reference material of known titer, and 1st World Health Organization (WHO) International Genetic Reference Panel for quantitation of BCR-ABL.

To determine the specificity and diagnostic sensitivity values on ABI 7500 Fast Dx, a total of 78 RNA (leukocyte pellets and cell lines) were analysed:

- 50 RNAs of patients with CML;
- 10 RNAs of patients with AML (acute myeloid leukemia);
- 8 RNAs of patients with ALL (acute linfoblastic leukemia) and positives for p190 variant;
- 4 RNAs WHO Sensitivity Panel;
- 6 RNAs IVS-0011 Sensitivity Panel.

The negativity / positivity of the samples detected by the validated device was established by double-sample analysis with RQ-BCR-ABL p210 One-Step product. In case of disagreement between the methodology under examination and the reference test (ipsogen® BCR-ABL1 Mbcr IS-MMR kit), other CE IVD assays RS-BCR-ABL p210 kit (AB ANALITICA) and/or the RS-BCR-ABL p190 kit (AB ANALITICA) were used.

4.3. LABNET VALIDATION OF "RQ-BCR-ABL P210 ONE-STEP"

RQ-BCR-ABL p210 One-Step has undergone further clinical validation through Labnet network. Specifically, this validation has been conducted in three different national referece laboratories (Bologna, Torino, Napoli). In each center the same samples have been tested with our device and with the routine method in use.

Clinical samples

30 RNA samples, representative of the four levels of the disease (10%, 1%, 0.1%, 0.01%), were distributed to the three laboratories by the Haematologic center of Torino. They have been prepared with the Maxwell extractor (Promega), using the Maxwell® 16 LEV simplyRNA Blood kit.

Dilutions at 40ng/µL of the samples were prepared and a final amount of 200ng/5µL was tested together with a secondary reference material (*RNA Reference* of ELITech Group and RNA REFERENCE of AB ANALITICA).

A total of 6 runs per each center (3 plates with our kit, 3 plates with routine assay) have been performed. In all three centers the instrument used was the Applied Biosystems 7900HT Fast Real-Time PCR.

Methods used in each laboratory routine:
Napoli: 'in-house' reagent for retrotrascription/amplification with Ipsogen standard (BCR-ABL1 Mbcr IS-MMR).
Bologna: BCR-ABL1 Mbcr b3a2 & b2a2 kit (Ipsogen), which uses the two-step method. The standard controls are contained in the kit and are unique plasmids.
Torino: Kit BCR-ABL P210 ELITe MGB (ELITechGroup), kit that, like our device, is a One-Step method. The standard controls are those contained in the kit and are unique plasmids.

Calculation of Conversion Factors (CF):

In the last few years, the need arose to standardize molecular monitoring of the deep Molecular Response (deep MR) and to define guidelines for interpreting results.[2] Definition of the International Scale for *BCR-ABL* p210 detection and quantification has been helped by the recent development of the first reference panel accredited by the World Health Organization (*1st World Health Organization (WHO) International Genetic Reference Panel for quantitation of BCR-ABL translocation by RQ-PCR*[176]).

Therefore reference kits have been developed and marketed to calculate CF in each laboratory. These reference kits are composed of total RNA blends of a cell line containing BCR-ABL translocation (9.22) p210 b3a2 in a normal cell line RNA. Such mixtures correspond to a Reference Title that results from a calibration performed using the *1st World Health Organization (WHO) International Genetic Reference Panel for quantitation of BCR-ABL translocation by RQ-PCR* (NIBSC, UK, cod 09/138). This value corresponds, for each RNA mixture, to the transient mRNA t (9.22) p210 percentage compared to the ABL mRNA.

The conversion factors of the three centers were calculated in Napoli by analysing the data obtained with the PHILADELPHIA P210 RNA Reference Kit (ELITech Group) and using the Bland-Altman statistical method.

The CFs calculated for “RQ-BCR-ABL p210 One-Step” device in each center were:
Orbassano (Torino) = 0.8876
Napoli = 0.9932
Bologna = 0.8625

4.4. PREDICTION OF THERAPEUTIC RESPONSE: LEUKOPREDICT INDUSTRIALIZATION ACTIVITIES.

4.4.1. FREEDOM TO OPERATE ANALYSIS (FTO)

A freedom to operate (FTO) analysis is aimed at defining the level of risk related to the expected entry into market of a product or of a service, to the extent they might interfere with third parties' patent rights (or other intellectual property rights, as the case may be).

For this analysis, Esp@cenet and Fampat database (from Questel Orbit) have been used. It must be pointed out that the patent applications are published 18 months after filing. Before their publication, such documents cannot be accessed or consulted and so they do not appear in any database. Therefore, possible patent applications filed during the last 18 months are not considered in this analysis. It is generally advisable that a supplementary FTO analysis is conducted once the product is ready to go on the market, to make sure the 18-month window is uncovered and all risks are taken into account. The relevant patent landscape for the FTO has been identified within applicable International Patent Classification (IPC) classes, using keywords (that have been masked and truncated to have maximum coverage in the search).

For the assessment of risk profiles of interference between the technology and third party rights, the following conditions were applied:

- The freedom to operate is assessed only with reference to third party patents which (i) are pertinent with respect to the technology, (ii) have been actually granted at the date of the present analysis, or (iii), in case of patent applications, there is a chance such applications reach grant shortly, thus becoming a threat to practice the technology.
- The literal infringement occurs when the invention being considered in infringement repeats all the (characterizing and characterized) elements of the claim, so that even the absence of one only of said elements leads to a conclusion of non-infringement.
- Since the FTO analysis implies a comparison between the technology and currently valid and enforceable claims, it is assumed that the technology, will not change significantly and, to the purposes of the FTO, the present knowledge faithfully describes the main feature of such the technology.
- Because of the territorial nature of patents, the FTO risks must be always determined according to the actual validity and enforceability of third parties' patents in specific countries and markets, not in general.

For the purposes of the FTO analysis, the Technology can be segmented in different aspects:

4.4.1.1. Samples preparations

As described first, LeukoPredict is based on the measurement of BCR-ABL activity. In order to measure the activity of this kinase, it is crucial to maintain its stability during the preparation of the samples. Indeed, the enzyme seems to be very susceptible to degradation by lytic enzymes derived from degranulation activity of polymorphonucleated cells (PMN).

In order to preserve the best morphology of cells and non-lytic activity from PMN, a particular protocol has been developed. In particular, peripheral or bone marrow whole blood can be treated with TKIs for 2h at 37 °C. After TKIs treatments, blood samples are subjected to red blood cells (RBCs) lysis with RBCs lysis buffer.

Subsequently, white blood cells are counted and left on ice.

After treatments and RBC lysis, cells are lysed in the appropriate prechilled buffer: isotonic lysis + protease inhibitors.

The composition of this lysis buffer has been protected by a patent application.

Then, the supernatant is collected by centrifugation for 10-15 minutes at the maximum speed in a microfuge (13.000 rpm, 4°C). After the centrifugation step, it is possible to proceed with protein quantification by Bradford analysis using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

It must be pointed out that the protocol described above is not yet definitive and some modifications might still occur. This aspect was considered during the research.

The following landscaping strategy has been used to identify relevant patent documents and valid claims:

- Keywords in the title, abstract or independent claims:
  Lysate preparation, lysis buffers and/or solutions, lysis method, protein activity analysis, protease inhibitors, isolation, extraction, composition, EDTA, pepsatin, DDT/dithithreitol, benzamidine, PAO/Phenylarsine oxide, triton X, Tris-HCL, NaF, Sodium orthovanadate, supernatant, centrifugation, blood, protein, biological sample.
- International Patent Classification:
  - C12N 1/00: Micro-organisms, e.g. protozoa; Compositions thereof (medicinal preparations containing material from protozoa, bacteria or viruses A61K 35/66, from algae A61K 36/02, from fungi A61K 36/06; preparing medicinal bacterial antigen or antibody compositions, e.g. bacterial vaccines, A61K 39/00); Processes of propagating, maintaining or preserving micro-organisms or compositions thereof; Processes of preparing or isolating a composition containing a micro-organism; Culture media therefor [2006.01].

- C12N 9/00: Enzymes, e.g. ligases (6.); Proenzymes; Compositions thereof (preparations containing enzymes for cleaning teeth A61K 8/66, A61Q 11/00; medicinal preparations containing enzymes or proenzymes A61K 38/43; enzyme containing detergent compositions C11D); Processes for preparing, activating, inhibiting, separating, or purifying enzymes.
- G01N 1/00: Sampling; Preparing specimens for investigation.
- C12N 5/00: Undifferentiated human, animal or plant cells, e.g. cell lines; Tissues; Cultivation or maintenance thereof; Culture media therefor.
- C12N 15/00: Mutation or genetic engineering; DNA or RNA concerning genetic engineering, vectors, e.g. plasmids, or their isolation, preparation or purification; Use of hosts therefor (mutants or genetically engineered micro-organisms C12N 1/00, C12N 5/00, C12N 7/00; new plants A01H; plant reproduction by tissue culture techniques A01H 4/00; new animals A01K 67/00; use of medicinal preparations containing genetic material which is inserted into cells of the living body to treat genetic diseases, gene therapy A61K 48/00; peptides in general C07K).
- G01N 33/00: Investigating or analyzing materials by specific methods not covered by groups G01N 1/00-G01N 31/00.
- G01N 33/50: Chemical analysis of biological material, e.g. blood, urine; Testing involving biospecific ligand binding methods; Immunological testing (measuring or testing processes other than immunological involving enzymes or micro-organisms, compositions or test papers therefor; processes of forming such compositions, condition responsive control in microbiological or enzymological processes C12Q).
- C12Q 1/00: Measuring or testing processes involving enzymes or microorganisms (measuring or testing apparatus with condition measuring or sensing means, e.g. colony counters, C12M 1/34); Compositions thereof; Processes of preparing such compositions.

4.4.1.2. Samples analysis

The BCR-ABL activity is measured through a kinase assay. The peptide substrate (ABL-biosensor peptide, CEAIYAAPFAKKK[165] is immobilized on a neutravidin-coated surface. The phosphorylation that occurs after the interaction between the kinase present in the samples and the immobilized peptide can be detected with phospho-specific primary antibody and an enzyme-conjugated secondary antibody. Subsequently, upon addition of a suitable substrate for the conjugated enzyme, the chromogenic, luminescent, or fluorescent readout is quantified. However, other methods can be used for detecting the phosphorylation. These

methods can include the use of fluorescently labelled antibodies, as well as the use of biotinylated reagents and their recognition with fluorescently labelled streptavidin.

The following strategy has been used:

- Keywords in the title, abstract or independent claims:
  Kinase assay (and tyrosine kinase assay), chronic myeloid leukemia (CML), diagnosis tests, BCR-ABL, diagnosis kit, molecular diagnosis, screening, profiling, drug selection, monitoring response to treatment, measuring enzyme activity, blood, enzyme, enzyme assay, enzymatic activity, phosphorylation, peptide arrays, immunoassay, immobilized peptide, immobilized substrate, tyrosine kinase inhibitor (TKI), ABL peptide biosensor, biosensor peptide, biomarker, Imatinib, Dasatinib, ponatinib.
- International Patent Classification:
  - G01N 33/00: Investigating or analyzing materials by specific methods not covered by groups G01N 1/00-G01N 31/00.
  - G01N 33/50: Chemical analysis of biological material, e.g. blood, urine; Testing involving biospecific ligand binding methods; Immunological testing (measuring or testing processes other than immunological involving enzymes or micro-organisms, compositions or test papers therefor; processes of forming such compositions, condition responsive control in microbiological or enzymological processes C12Q).
  - G01N 33/53: Immunoassay; Biospecific binding assay; Materials therefor.
  - C12Q 1/00: Measuring or testing processes involving enzymes or micro-organisms (measuring or testing apparatus with condition measuring or sensing means, e.g. colony counters, C12M 1/34); Compositions therefor; Processes of preparing such compositions.
    In particular C12Q 1/48: involving transferase.
  - C40B 30/00: Methods of screening libraries (C40B 30/08: by measuring catalytic activity).

4.4.2. COST-EFFECTIVENESS ANALYSIS: HEALTH ECONOMIC (DECISION-ANALYTIC) MODEL FOR THE EARLY LEUKOPREDICT TEST IN CML

Decision-analytic modeling was used for the analysis. To ensure optimal methodological approach it was used a methodological checklist of modeling companion diagnostics in economic evaluations of targeted oncology therapies by Double et al. [177].

The key elements of model structure and rationale for the analysis are present in the following table:

Component	Summary
Time horizon	Eight years in the model base case equal to a median follow-up of eight years in the study Neelakantan et al.[178]
Outcomes	LYG and QALYs
Methods used to generate results	Markov model; cohort expected value analysis
Cycle length	Three months
Transition probabilities	Transition probabilities presented in Table 5
Discount rate	3.5% of costs and outcomes

Table 4. Summary of model structure and rationale. Legend: LYG – life years gained, QALY – quality adjusted life years

A state transition Markov model with a cycle length of three month's cycle length was used in combination with decision tree was used to evaluate the cost-effectiveness of management strategy with use of LeukoPredict. [179-182]

4.4.2.1. Strategies compared

Decision analytic model compared two strategies:

1) CML standard of care strategy
- In the base-case analysis patients receive first-line treatment in line with real-world settings, 33,33% per TKI (include Imatinib, Dasatinib and Nilotinib)
- In the scenario analysis 1 patients receive first-line treatment with Imatinib.
- In the scenario analysis 2 patients receive first-line treatment with Imatinib but costs decreases of Imatinib due to the generic entry was modeled, with prioritizing patients to receive Imatinib in the first line treatment due to the lower costs.

2) LeukoPredict test as a companion diagnostic test added to the standard of care strategy.

It is possible to triage patients based on their drug resistance potential and thus allowing selection of a drug which will have a best possible initial response measured by early

molecular response (EMR). The assumption used in the published CML economic evaluations that patients were taking only hydroxycarbamide after CML disease progression to accelerated phase and blast crisis was reused for the purpose of this analysis. [183-189]
In this analysis, in the both treatment arms, patients received stem cell transplant (SCT) or hydroxycarbamide, as a third-line treatment after TKI failure.

4.4.2.2. Model structure

A Markov model was developed to reflect the natural history of CML patients as suggested in the current guidelines recommendations[182] and based on previously published decision-analytic models for management of CML. [182-189] During each 3-month cycle, patients have the following possibilities: continuing on the first; switching to the second line therapy due to the non-responding measured by EMR after 6 months; switching to the third line due to the non-responding to the second-line therapy after 12 months; progression of disease to the accelerated phase and blast crisis; and death (Figure 21).

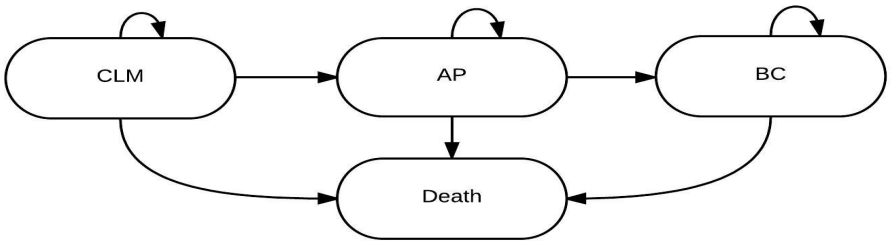


Figure 21.State-transition (Markov) model influence diagram of CML natural history of disease.
Legend: AP – accelerated phase; BC – blast phase

The clinical management algorithm is presented in Figure 22 and Figure 23. Patients will have a treatment switch after first-line treatment failure to Imatinib or 2nd generation TKIs Dasatinib and Nilotinib in the base-case analysis. In the scenario analysis, after receiving 100% Imatinib as first-line treatment patients can have a treatment switch to Dasatinib and Nilotinib in equal proportions.

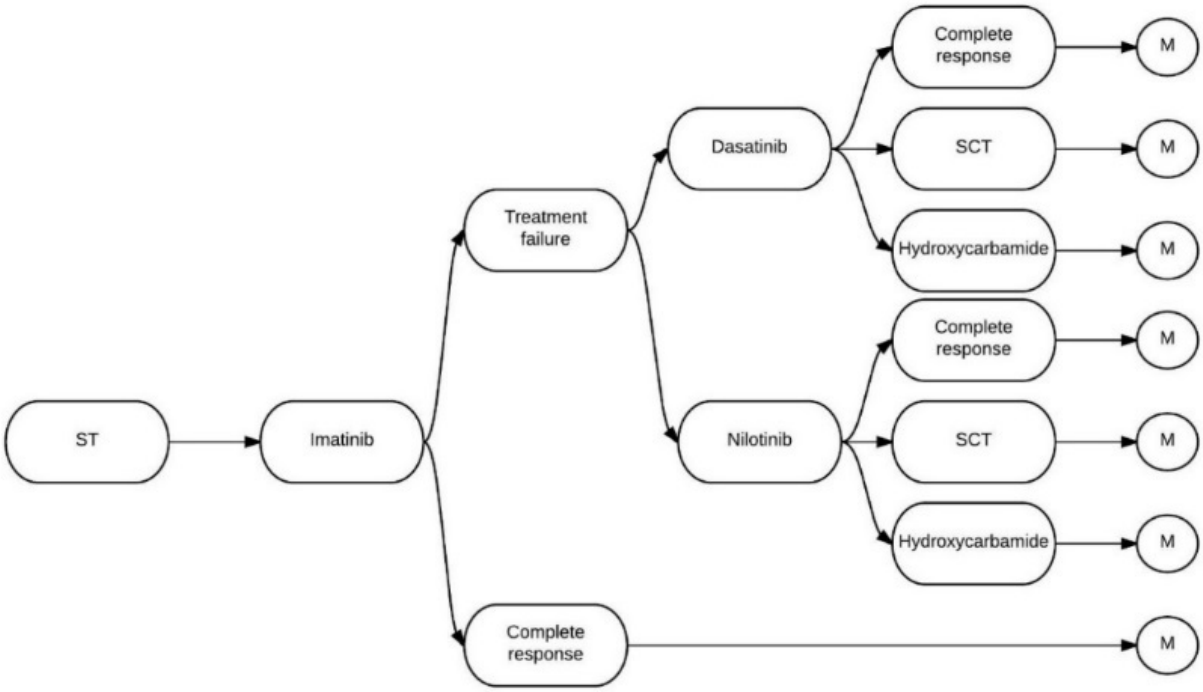


Figure 22.Treatment arm as scenario 1 and 2.
Legend: ST – standard treatment; SCT – stem cell transplantation; M – Marcov model

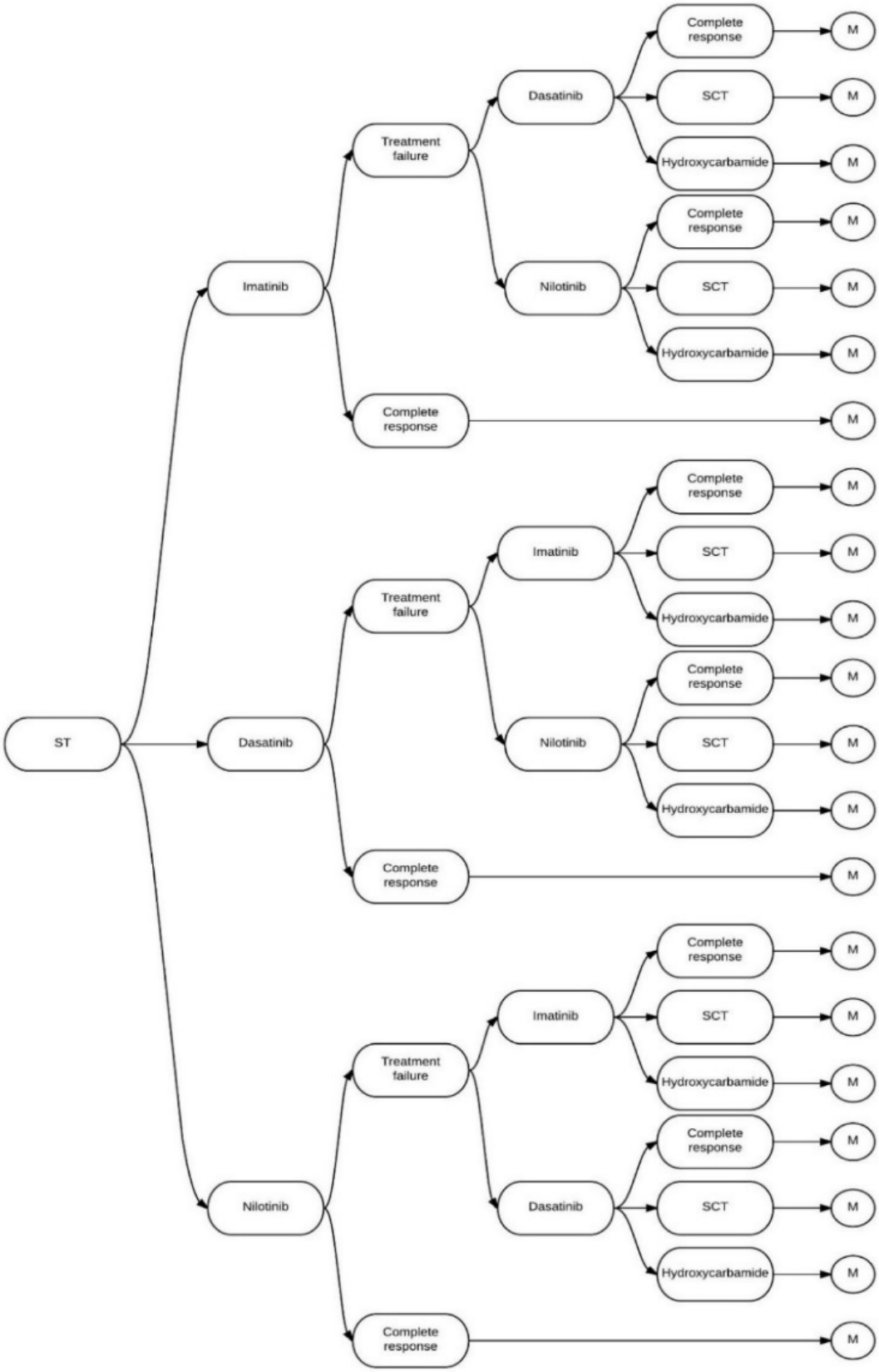


Figure 23. Standard treatment (ST) arm real-world practice.
Legend: SCT – stem cell transplantation; M – Marcov model

In modeling a diagnostic test, practical application of Bayes' theorem allows to incorporate the test's sensivity and specificity for the analysis. Prevalence of presence/absence of resistance to TKIs and diagnostic test sensitivity and specificity have been used to calculate various events probabilities employed Bayesian revision. [190] The proportions of patients testing positive or negative was based on the assumed accuracy of the LeukoPredict test in the following way:

True positive (TP) = Prevalence of resistance * sensitivity
False positive (FP) = (1 – Prevalence of resistance) * (1 – specificity)
False negative (FN) = Prevalence of resistance * (1 – sensitivity)
True negative (TN) = (1 - Prevalence of resistance) * specificity
Sensitivity = TP/(TP+FN)
Specificity = TN/(TN+FP)

Based on the proportion of those four groups patients will have three possible treatment paths as depicted in Figure 24. True negative and false negative patients will have the same treatment sequence as a standard of care arm and will not give any incremental value to the LeukoPredict test. True positive and false positive will have received treatment, which will be without treatment failure due to the primary resistance and those patients will represent a group of patients with biggest beneficial of using LeukoPredict.

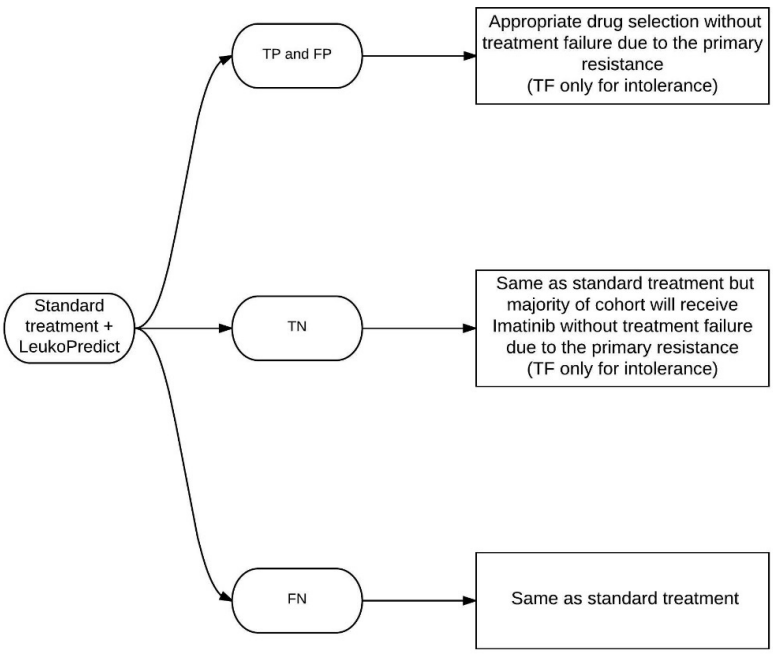


Figure 24. LeukoPredict arm decision tree as a companion diagnostic test added to the standard treatment

4.4.2.3. Decision analytic modelling methods and assumptions

The following methods and assumptions were used in the modeling:

- Sensitivity and specificity of the diagnostic tests cannot be considered separately due to the facts that they are negatively correlated[191]. Therefore, inputs parameters regarding diagnostic accuracy for the economic evaluation of the diagnostic test ideally should be linked to the positivity threshold. This allows identification of the "Optimal Operating Point," or test threshold with the sensitivity and specificity pair at which the test is most cost-effective.[192,193]In the future, identified optimal threshold should be used for the base-case analysis in economic evaluation and the optimization of future clinical trials.[194]
- The LeukoPredict test is modeled as a point-of-care diagnostic test, where human intervention is minimized. It assumed for the purpose of the analysis that one hour of a laboratory technician labor is needed when the test is done "on-the-fly" on blood samples in the first hours of the treatment. The costs for cell cultures and in vitro drug testing will be much higher, and they are not part of this analysis.
- Patients will be checked for treatment efficacy using EMR with two tests, at three and six months, and additional tests in between as recommended by guidelines.
- Patients who achieved a negative EMR (and the indicator of an unimproved health status) will switch from the initially selected TKI to an alternative TKI and evaluated for outcomes through the additional time in the model.
- Conversely, patients who achieved a positive EMR (an indicator of an improved health status) were assumed to continue on the initially selected TKI and evaluated for outcomes over the same time frame.
- After switching to the second line therapy, the patient can experience treatment failure again which will lead to third line treatment which was assumed to be allogenic transplantation.
- The models assume perfect patient adherence to TKI-based treatment across all treatment modalities.
- Mortality is based on statistics of overall survival (OS) stratified by treatment group.
- In additional scenario analysis, the price of Imatinib was assumed to follow the pre-specified decrease starting in 2016.

4.4.2.4. Data inputs

Transition probabilities

The foundation for analysis was the data from a study conducted in the UK.[178]Transition probabilities regarding treatment response defined by EMR as depicted in Table 5, as well as survival in relation to the EMR status at three and six months were derived from the source mentioned above. Some of the transition probabilities, as a progression to accelerated phase and blast phase were utilized from the economic model by Padula et al. [186], after checking primary source of data and adaptation for three months' cycle. Taking into account that study used in Padula et al. [186] were confirmed by a panel of CML experts, all membe rs of the European LeukemiaNet (ELN) CML committee, as well as that study, was non-industry sponsored this source can be considerate as a highly relevant. Also, this is the only published model that uses EMR as a surrogate endpoint for the survival, and treatment switch.

Probabilities	Proportion (95% CI)	Sources
Imatinib		
3 months EMR non-responders	0.24 (0.15-0.34)	178
3 months EMR re-responders	0,23 (0,18-0.28)	
6 months EMR non-responders	0.16 (0.08-0.27)	
Switch to Dasatinib	0.5 (fixed)	186
Switch to Nilotinib	0.5 (fixed)	186
Progress to AP/BP	0,004 (0.003-0.006)	195,196
Dasatinib		
3 months EMR non-responders	0.16 (0.11-0.21)	196
6 months EMR non-responders	0.11 (0.07-0.16)	196
Switch to Imatinib	0.15 (fixed)	186
Switch to Nilotinib	0.85 (fixed)	186
Progress to AP/BP	0.004 (0.003-0.005)	196
Nilotinib		
3 months EMR non-responders	0.09 (0.06-0.14)	195
6 months EMR non-responders	0.03 (0.01-0.06)	195
Switch to Imatinib	0.15 (fixed)	186
Switch to Dasatinib	0.85 (fixed)	186
Progress to AP/BP	0.001 (0.0006-0.003)	195
Common probabilities for all TKI		
Fraction of treatment failure due to the intolerance	0.23 (0.16-0.29)	Figure 24
Transition from AP to BP	0.27 (0.21-0.32)	187

Table 5. Base case transition probabilities for the standard treatment arm.
Legend: EMR – early molecular response, AP – accelerated phase, BP – blast phase.

A meta-analysis for treatment failure in the LeukoPredict arm (including non-responder and intolerant) from the cost-effectiveness analysis[186] was re-done to disaggregate transition probabilities to non-responders and intolerant.

To adequately triage patient switching to the third line treatment with a possibility to receive stem cell transplant (SCT) or hydroxycarbamide, age-related proportions of patients eligible for receiving a stem cell transplant was determined from the literature (Table 6) [187].

Age (years) at which TKIs fail	Patients receiving SCT
50–59	60%
60–64	40%
65–69	15%
70–74	5%
75+	0%

Table 6. Age-related proportions of patients receiving a stem cell transplant

4.4.2.5. Survival

Survival for the purpose of this analysis was based on an overall (OS) and progression-free survival (PFS), additionally adjusted for the differences of being in specific health state (chronic phase or advanced phases of the CML).

Overall survival (OS) was informed by study Neelakantan et al. [178] conducted in the UK. This study reports the 8-year probability of overall survival according to the BCR-ABL1 transcript level at three and six months as presented in Figure 25. The study confirms that initial EMR is a prognostic factor for overall survival already with the first measurement at three months and at six months as well. Also, this study allows modeling survival based on dynamic changes of the EMR at three and six months, dividing initial cohort into four groups as depicted in Figure 25. The prognostic value of the EMR assessment was confirmed being the independent prognostic factor and not affected by treatment switching.

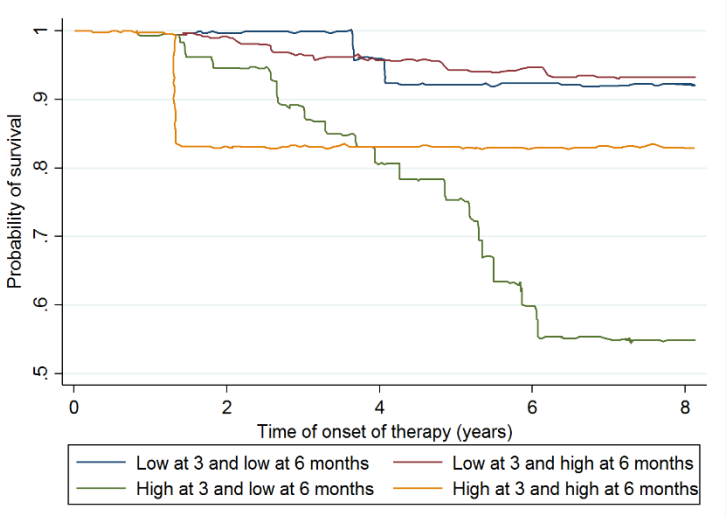


Figure 25. Overall survive according to the BCR-ABL transcript level at three and six months

Survival in the advanced phases of the CML was reported as the average life expectancy of 9.6 months for accelerated phase and life expectancy of 3–6 months for blast crisis, according to National Institute for Health and Clinical Excellence's guidance (NICE) final appraisal determination (FAD) for second-line, high-dose Imatinib, Dasatinib, and Nilotinib for CML. [185]

4.4.2.6. Costs

Two main categories of resource utilization and costs are be included in the analysis: LeukoPredict and drug acquisition costs and follow-up costs. The costing index year for the analysis was 2016, and all cost data were adjusted for inflation, using the Hospital and Community Health Services Index. [197]

LeukoPredict and drug acquisition costs

The expected costs of LeukoPredict test and resource use associated with cost were estimated in the communication with the client, as presented in Table 7.

Test/Resource use	Unit cost (£)
LeukoPredict (Point-of-care testing)	129
One additional blood sample withdrawal	12
1-hour labor of a laboratory technician	13

Table 7. LeukoPredict cost and resource use

The cost of drugs was divided into the three subcategories taking into account differences in dose and frequency of usage primarily between first- and second-line treatments. All costs for first-line (Table 8) and second-line (Table 9) treatments were informed by the NICE technology appraisal guidance.

Drug	Dose and frequency	Cost per pack (£)	3-month cost per patient (£)
Imatinib (400 mg 30 tablet pack)	400 mg once daily	1836.48	5509.44
Nilotinib (150 mg 112 tablet pack)	300 mg twice daily	2432.85	7785.12
Dasatinib (100 mg 30 tablet pack)	100 mg once daily	2504.96	7514.88

Table 8. First-line CML treatment drug cost

Drug	Dose and frequency	Cost per pack (£)	3-month cost per patient (£)
Dasatinib (100 mg 30 tablet pack)	100 mg once daily	2504.96	7514.88
Nilotinib (200 mg, 112-cap pack)	400 mg twice daily	2432.85	7785.12
Imatinib (400 mg 30 tablet pack)	400 mg twice daily	1836.48	11018.88

Table 9. Second-line CML treatment drug cost

The cost for the third-line treatments is presented in Table 10. Peninsula Assessment Technology group estimated ongoing drug and monitoring costs after SCT as a weighted mean cost per month of £113. [187]

Treatment	Dose and frequency	Cost per pack (£)	Cost per patient (£)
Hydroxycarbamide	20–30 mg/kg daily	10.47 per 500 mg, 100-capsule pack	36 (three-month cycle)
Stem cell transplant (SCT) for third line treatment	-	-	83247
Ongoing drug and monitoring cost after SCT	-	-	346

Table 10.Third-line CML treatment. Legend: SCT – stem cell transplantation

Imatinib price adjustment after generic entry in 2016 was introduced into scenario analysis. Study by Conti et al. [198] reported on the drug–specific cost trajectories after generic entry, as well as per patient, per month cost trajectory of Imatinib-based therapy utilized as follows: (i) 100% of the branded treatment cost prior to generic entry for the first six months, (ii) 60% to 80% for the second six months following generic entry and (iii) 10% to 30% thereafter.

4.4.2.7. Follow up costs

A range of medical management costs, including nurse treatment, consultant outpatient visits, bone marrow tests, and hospitalization (without drugs) was also included in the analysis. The cost of a routine appointment and their frequency, as well as resource use per drug and health state, are sourced from the Novartis industrial submission as presented in Table 11 and Table 12.

Month	No. of routine appointments	Cost of routine appointments (£) (first and second line therapy)
1	3	422
2	1	141
3	0	0
4	1	141
5	0	0
6	1	141
>7	2	282*

Table 11. Cost of routine appointments

Phase	Imatinib (£)	Dasatinib (£)	Nilotinib (£)
CP	(0, 422)	(0, 422)	(0, 422)
AP	94	94	94
BP	186	186	186

Table 12. Resource use, excluding drugs

4.4.2.8. Analysis

The analysis was performed from a perspective of the UK NHS over the eight years' time horizon. In line with NICE (National Institute for Health and Care Excellence) recommendations, all costs and outcomes beyond the first year were discounted at 3.5% annually.[199] LeukoPredict was considered cost-effective if the incremental cost-effectiveness ratio (ICER, which is calculated by dividing difference in cost between two arms by difference in quality-adjusted life years) was below the lower-bound willingness-to-pay threshold of £30,000/QALY.[199]

4.4.2.9. Threshold analysis

The laboratory data regarding Bcr/Abl activity variation after TKI treatment of cell lines (responders-K562 and resistant-BaF T315I), depending on the resistance to TKIs and presence of the Bcr-Abl mutations were used for estimation of the sensitivity and specificity of the test (Figure 26).

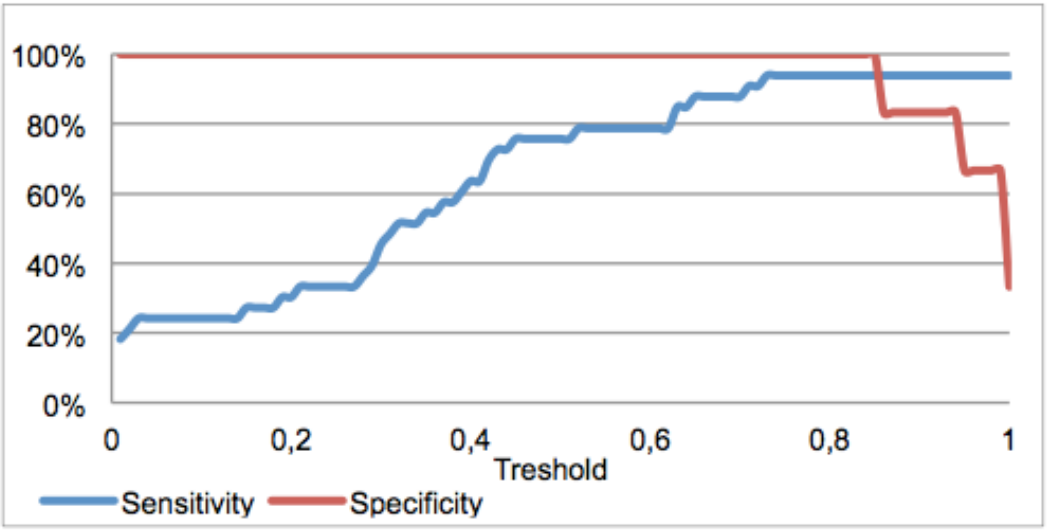


Figure 26. Sensitivity and specificity of LeukoPredict test as a function of the threshold on BCR/ABL activity variation.

In addition, threshold analysis was undertaken to identify "Optimal Operating Point" as a test threshold with the most cost-effective sensitivity and specificity profile. ICER was calculated with varying positivity thresholds linked to the sensitivity and specificity of LeukoPredict test.

4.4.2.10. Sensitivity analyses

Uncertainty about data inputs was evaluated in deterministic one-way and two-way sensitivity analysis. In the one-way analysis, each model's parameter was varied individually while holding other variables fixed at base-case values to evaluate the impact of each variable at ICER.

5. RESULTS

5.1. DEVELOPMENT AND VALIDATION OF THE NEW "REALQUALITY RQ-BCR-ABL P190 ONE-STEP"

5.1.1. DIAGNOSTIC SPECIFICITY AND SENSITIVITY

Because of $p190^{BCR-ABL}$ CML represents only 1% of patients with CML, a small number of samples was available for validation.

All 5 negative samples analysed were considered suitable for analysis. The assay was able to properly identify 5 out of 5 negative samples for BCR-ABL p190 translocation obtaining results that agree with the expected.

All of the 7 positive samples analysed were considered suitable for the analysis. The assay was able to correctly identify 7 out of 7 positive samples for the BCR-ABL p190 translocation.

Specificity and diagnostic sensitivity were defined as follows:

DIAGNOSTIC SPECIFICITY = Negative results / total negative samples:
6/6 = **100%**
DIAGNOSTIC SENSITIVITY = Positive results / total positive samples:
11/11 = **100%**

5.1.2. ACCURACY

The accuracy was calculated as the percentage of correct results in relation to the total number of tests. The total accuracy of RQ-BCR-ABL p190 One-Step is 100%

5.1.3. ANALYTICAL SPECIFICITY

The analytical specificity of the kit is guaranteed by an accurate and specific selection of primers and probes and the use of stringent amplification conditions.

Alignment of primers and probes in the most important databases showed no non-specific pairing.

5.1.4. REPRODUCIBILITY

In order to determine the assay variability (variability among replicates of the same sample in different analysis sessions), dilutions of extracted total RNA from positive human cell lines for BCR-ABL p190 translocation e1a2 were tested in five replicates in five consecutive analysis sessions.

The variability coefficient (CV) of the device was determinate using the cycle threshold value (Ct) of each point, dividing the mean value of the standard deviation of Ct (Ct SD) for the mean Ct value calculated by the instrument (Ct mean).

$$\mathbf{CV} = \frac{Ct\ SD\ x\ 100}{Ct\ mean}$$

Target	N	Ct Mean	Ct Standard deviation	CV
IVS-0032	25	19,95	0,09	0,46
1:10 IVS-0032	25	23,38	0,14	0,63
1:100 IVS-0032	25	26,81	0,25	0,95
1:1000 IVS-0032	25	30,21	0,35	1,16
1:10000 IVS-0032	25	33,91	0,47	1,39

Table 13. Reproducibility data

The variability coefficient on the ABI 7500 Fast Dx system is in the range of 0.46% to 1,39%. As expected, the variability increases as the concentration of the target decreases, although it always remains below 5% (Table 13).

5.1.5. ANALYTIC SENSITIVITY

5.1.5.1. LIMIT OF DETECTION

Five points (2.6, 1.3, 0.65, 0.26 and 0.13 target / reaction copies) of a serially diluted total RNA extracted from a positive human cell line of BCR-ABL p190 translocation were tested in eight replicates per dilution point in three consecutive analysis sessions.

Results, analysed by probit regression, allowed calculating the value of analytic sensitivity (with p = 0.05) indicated in the graph (Figure 27).

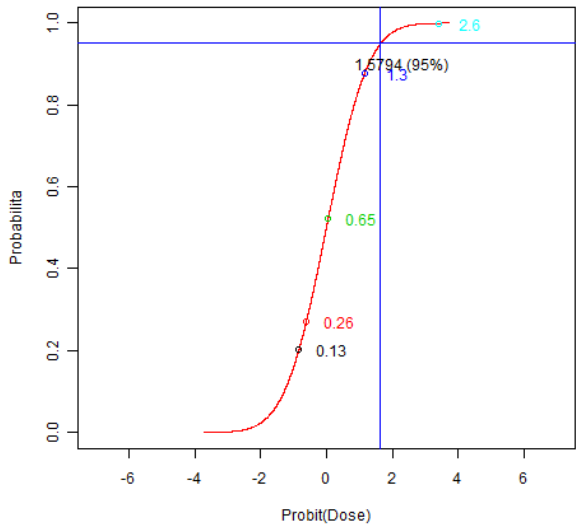


Figure 27.Probit graph

As can be seen from the chart (Figure 27), you have a 95% probability of detecting 1.58 copies of BCR-ABL p190 / reaction on ABI 7500 Fast Dx.

5.1.5.2. LINEAR RANGE

The linear range of this assay was determined using a panel of the quantification standard "REALQUALITY RQ-BCR-ABL p190 STANDARD". Specifically, for each target, the points corresponding to $5x10^6$, $5x10^5$, $5x10^4$, $5x10^3$, $5x10^2$, 5x10, 5, 2.5 and 1.6 copies of BCR-ABL p190 / reaction were amplified in duplicate.

Considering that a replication of the 1.6 copy / reaction dilution has produced a very different Ct result than the other two replicates, the analysis was done by omitting this dilution point.

	BCR-ABL p190 Real time mix	**ABL Real time mix**
Threshold	0.1	0.1
Baseline	3/15	3/15
Slope	-3.336	-3.3374
R2	0.997	0.994

Table 14. Linear range data

The analysis of the obtained data with linear regression showed that the assay has a linear response (R2> 0.99) in the range of $5x10^6$-2.5 copies of the translocation BCR-ABL p190/reaction.

5.2. DEVELOPMENT AND VALIDATION OF THE NEW "REALQUALITY RQ-BCR-ABL P210 ONE-STEP"

5.2.1. DIAGNOSTIC SPECIFICITY AND SENSITIVITY

All 26 negative samples analysed were considered suitable for analysis. The assay was able to properly identify 26 out of 26 negative samples for BCR-ABL p210 translocation obtaining results that agree with the expected.

All 52 positive samples analyzed were considered suitable for analysis. The assay was able to correctly identify 51 of 52 positive for $p210^{BCR-ABL}$ translocation. The device obtained ratio between BCR-ABL p210 variant / ABL with orders of magnitude comparable to the expected.

Specificity and diagnostic sensitivity were defined as follows:

DIAGNOSTIC SPECIFICITY = Negative results / total negative samples:
26/26 = **100%**
DIAGNOSTIC SENSITIVITY = Positive results / total positive samples:
51/52 = **98%**

5.2.2. ACCURACY

The accuracy was calculated as the percentage of correct results in relation to the total number of tested samples (n = 78). The total accuracy of RQ-BCR-ABL p210 One-Step is: 77/78 = 99%

5.2.3. ANALYTICAL SPECIFICITY

The analytical specificity of the kit is guaranteed by an accurate and specific selection of primers and probes and the use of stringent amplification conditions.

Alignment of primers and probes in the most important databanks showed no non-specific pairing.

5.2.4. REPRODUCIBILITY

In order to determine the assay variability (variability among replicates of the same sample in different analysis sessions), dilutions of extracted total RNA from positive human cell lines for BCR-ABL p210 translocation were tested in five replicates in five consecutive analysis sessions.

The variability coefficient (CV) of the device was determinate using the cycle threshold value (Ct) of each point, dividing the mean value of the standard deviation of Ct (Ct SD) for the mean Ct value calculated by the instrument (Ct mean).

$$\mathbf{CV} = \frac{Ct\ SD\ x\ 100}{Ct\ mean}$$

	N	Ct Mean	Ct Standard deviation	CV
WHO A1	25	31,72	0,34	**1,08**
WHO A2	25	29,70	0,14	**0,47**
WHO A3	25	26,50	0,08	**0,29**
WHO A4	**25**	**23,31**	**0,13**	**0,55**

Table 15. Reproducibility data

5.2.5. ANALYTIC SENSITIVITY

5.2.5.1. LIMIT OF DETECTION

For the calculation of analytical sensitivity, dilutions of WHO positive controls belonging to the 1st WHO International Genetic Reference Panel for quantification of BCR-ABL translocation by RQ-PCR have been used.

Five points (2.8, 1.4, 0.7, 0.28 and 0.14 target / reaction copies) of a serially diluted total RNA extracted from a WHO control were tested in eight replicates per dilution point in an analysis session.

Results, analysed by probit regression, allowed calculating the value of analytic sensitivity (with p = 0.05) indicated in the graph (Figure 28).

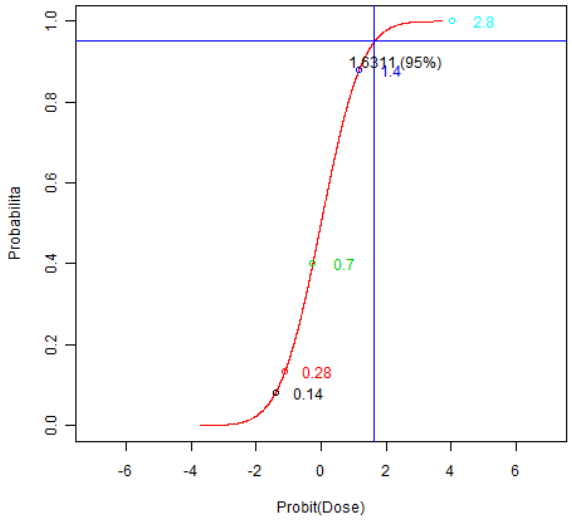


Figure 28. Probit graph

As can be seen from the chart (Figure 28), you have a 95% probability of detecting 1.63 copies of BCR-ABL p210 / reaction on ABI 7500 Fast Dx.

5.2.5.2. LINEAR RANGE

The linear range of this assay was determined using a panel of the quantification standard "REALQUALITY RQ-BCR-ABL p190 STANDARD". Specifically, for each target, the points corresponding to $5x10^6$, $5x10^5$, $5x10^4$, $5x10^3$, $5x10^2$, 5x10, 5, 2.5 and 1.6 copies of BCR-ABL p210 / reaction were amplified in duplicate.

Considering that a replication of the 1.6 copy / reaction dilution has produced a very different Ct result than the other two replicates, the analysis was done by omitting this dilution point.

	BCR-ABL p190 Real time mix	ABL Real time mix
Threshold	0.1	0.1
Baseline	3/15	3/15
Slope	-3.261	-3.329
R2	0.997	0.994

Table 16. Linear range data

The analysis of the obtained data with linear regression showed that the assay had a linear response (R2> 0.99) in the range of $5x10^6$-2.5 copies of the translocation BCR-ABL p210 / reaction.

5.3. LABNET: CLINICAL VALIDATION

5.3.1. COMPARISON OF FINAL DATA IN I.S. OBTAINED WITH "RQ-BCR-ABL P210 ONE-STEP" AND THE ROUTINELY KIT USED IN THE THREE CENTERS:

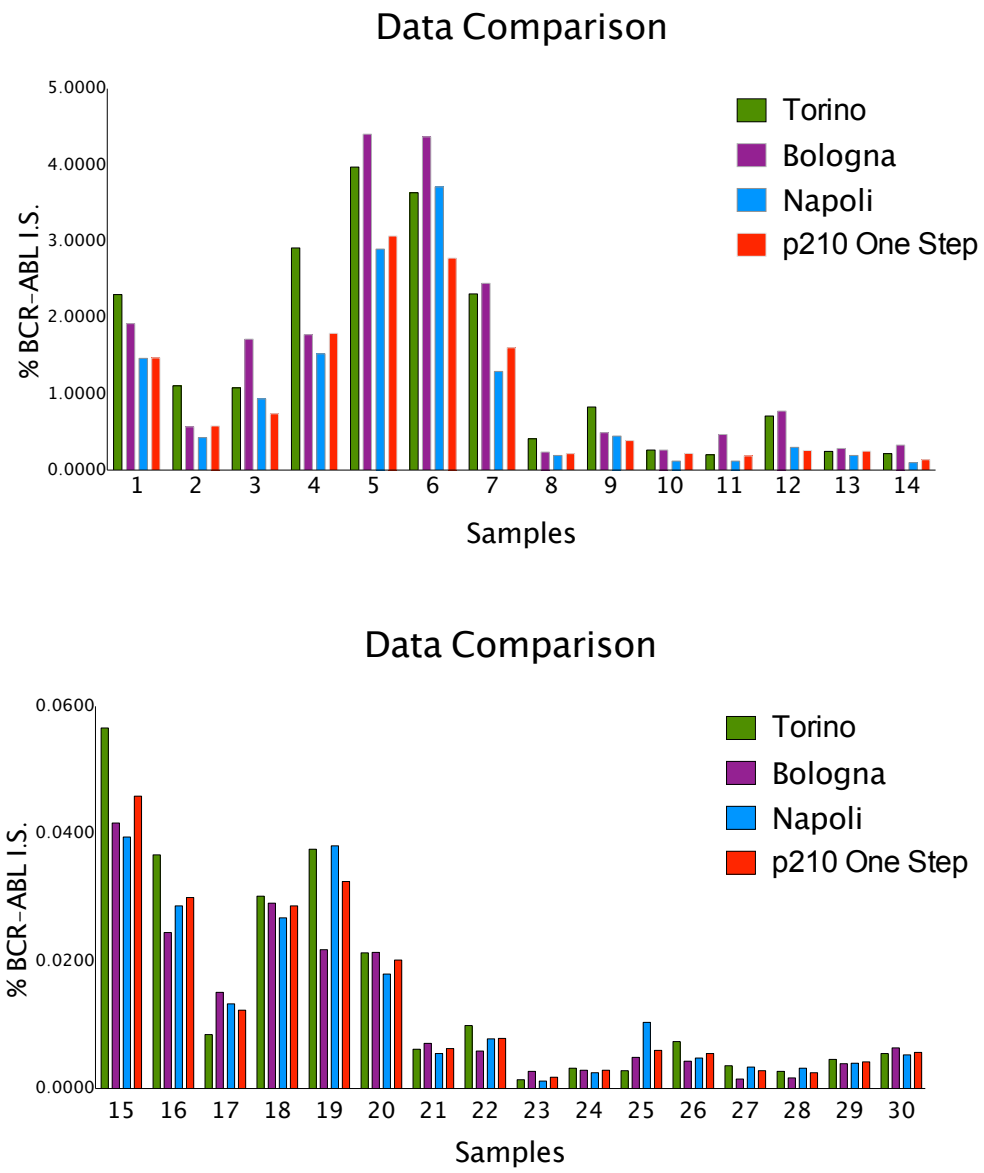


Figure 29. Comparison of final data expressed in International Scale (IS) obtained with the routinely kit used in the three centers (Torino, Bologna and Napoli) and "RQ-BCR-ABL p210 One-Step" kit.

The final results of the 30 samples were divided into two groups to fa cilitate its graphic representation (Figure 29). In this descriptive analysis there are no substantial differences between 'routine assays' and RQ-BCR-ABL p210 One-Step.

RQ-BCR-ABL p210 One Step kit was able to detect a greater number of samples with a $MR^{4.5}$ (detectable disease ≤0.0032% $BCR\text{-}ABL^{IS}$) disease level compared to the other routine methods, proving to have a high analytical sensitivity.

5.3.2. REPRODUCIBILIY OF FINAL DATA: DETERMINATION OF THE FINAL DATA VARIABILITY WITH ROUTINE METHODS COMPARED TO RQ-BCR-ABL P210 ONE-STEP:

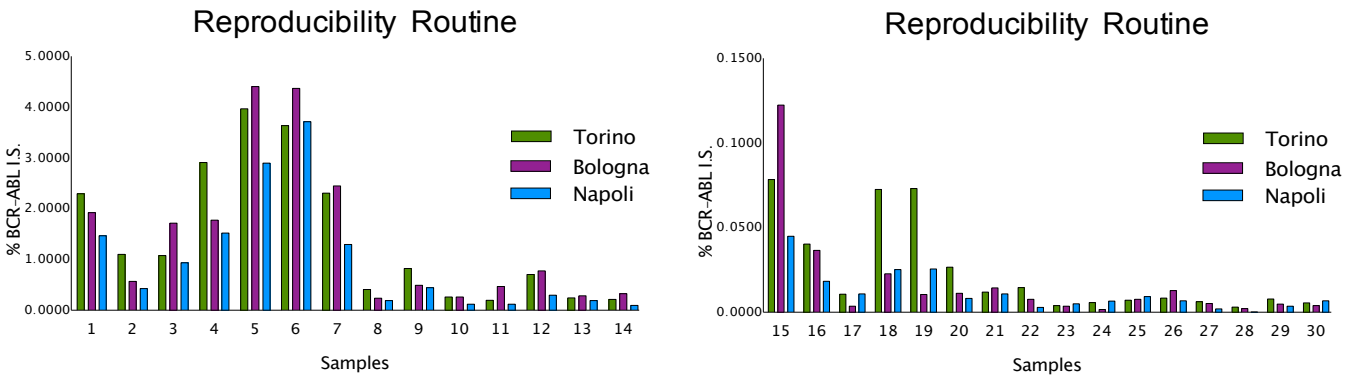


Figure 30. Comparison of final data obtained with routine methods.

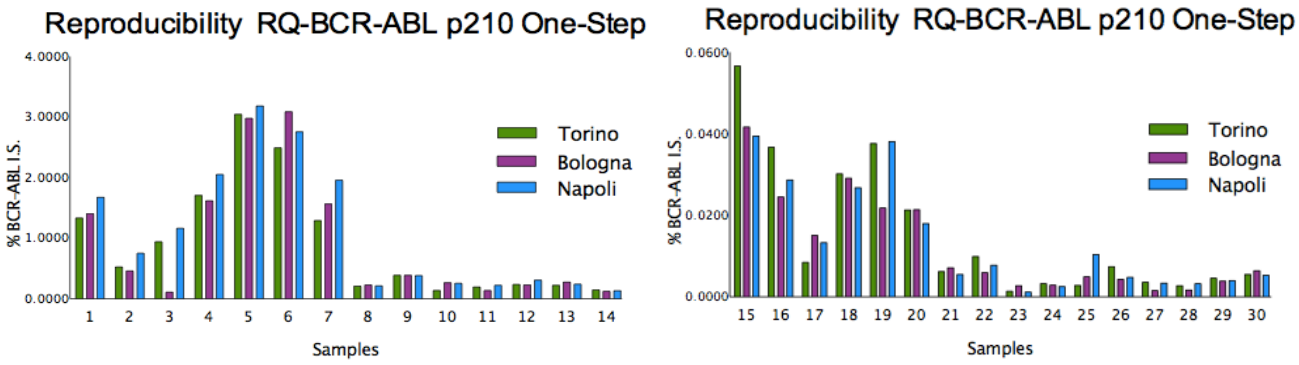


Figure 31. Comparison of final data obtained with "RQ-BCR-ABL p210 One-Step" in each center.

The reproducibility of the methods was compared by analysing the mean of the standard deviations of data obtained with the routine methods for each single sample with those obtained with p210 One Step in the three different laboratories (Figure 32).

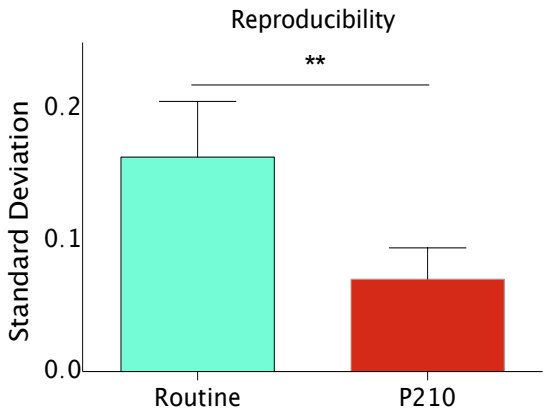


Figure 32. Analysis of reproducibility of the routine methods and "RQ-BCR-ABL p210 One-Step" method.

5.3.3. ANALYSIS OF AGREEMENT BETWEEN ROUTINE METHODS AND RQ-BCR-ABL P210 ONE-STEP DEVICE

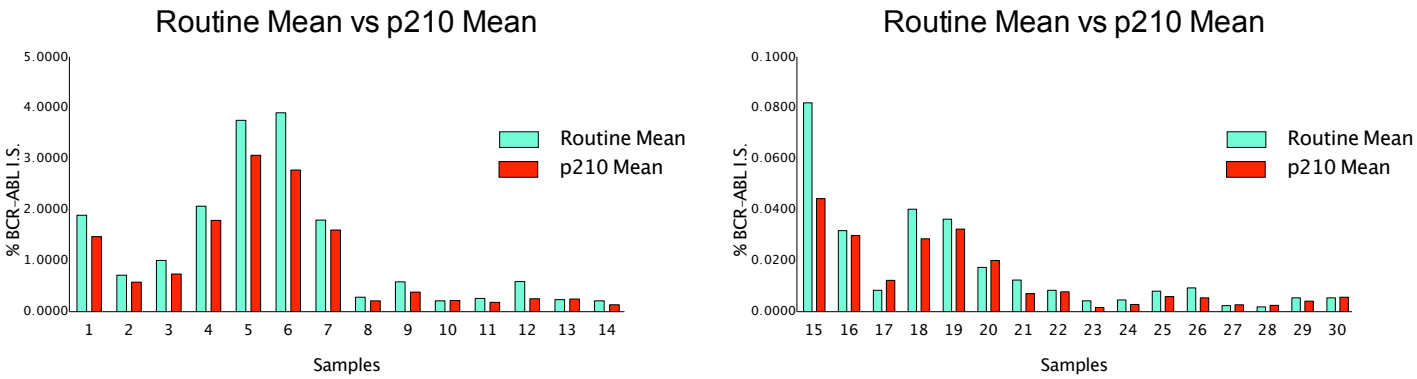


Figure 33. Comparison of the average result of each sample obtained with routine methods and with RQ-BCR-ABL p210 One-Step.

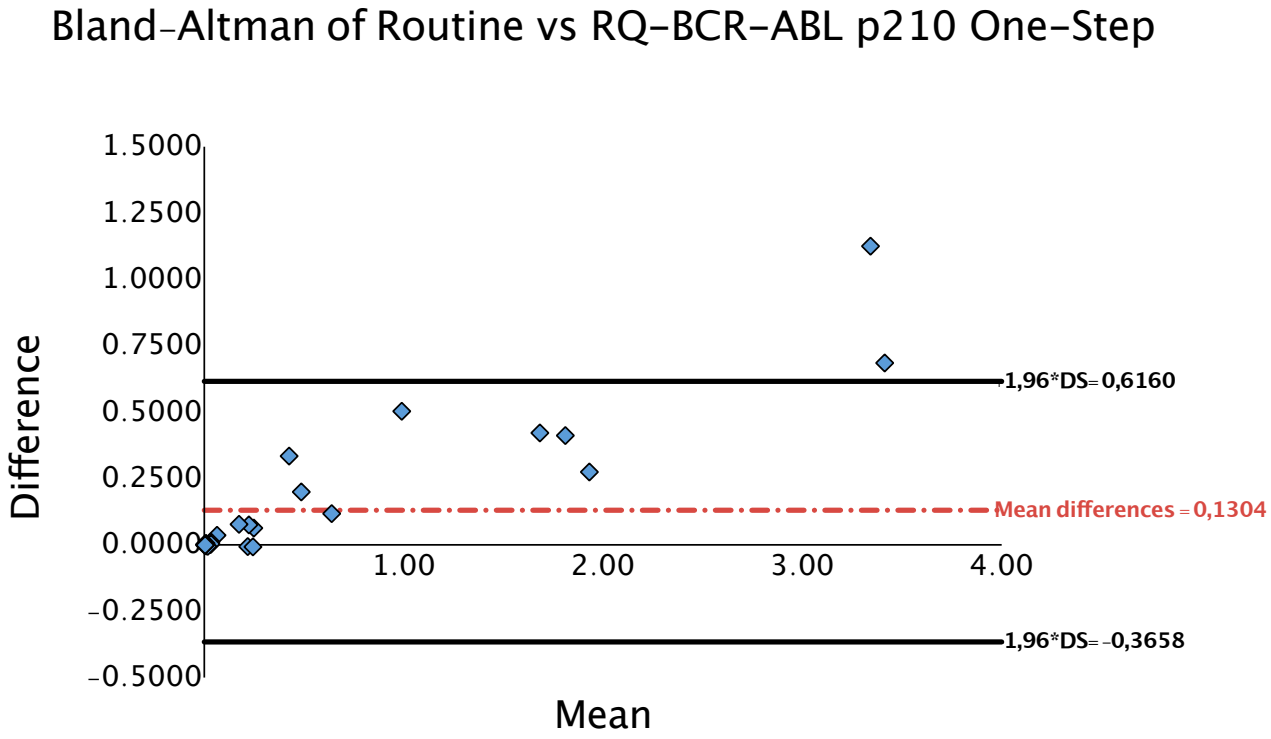


Figure 34. Bland-Altman plot analysis

This type of analysis allows us to compare a new quantification method with another reference, in our case the average of routine methods. The graph correlates the sample concentrations (expressed as the mean of the two methods) with the differences between the two methods.
Most of the values are around zero and within confidence limits, demonstrating that our method is comparable to those used in routine. No significant bias is observed since the value 0 is compressed to its 95% confidence interval (Figure 34).

5.3.4.QUANTIFICATION OF HOUSEKEEPING GENE: ABL GENE

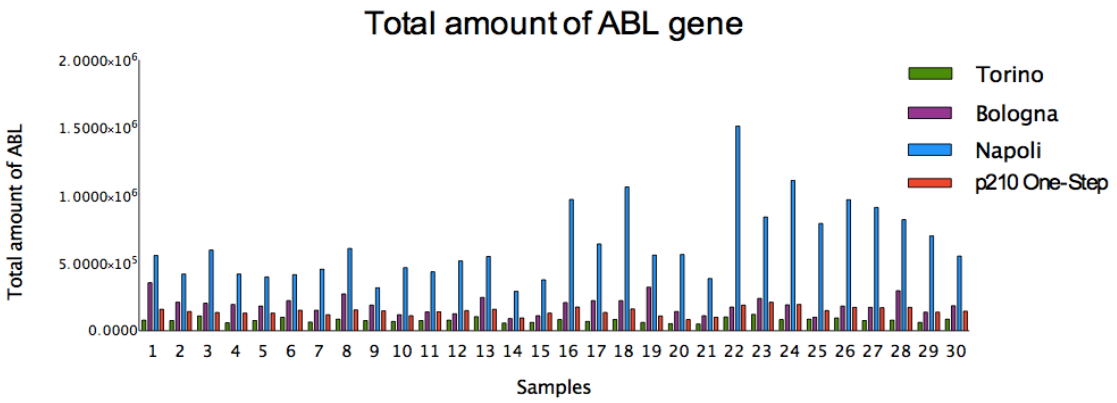


Figure 35. Comparison of the quantification of ABL gene with the different methods

Significant differences can be observed in quantification of the ABL gene. These differences directly affect inter-laboratory variability.

5.3.5.REPRODUCIBILITY OF HOUSEKEEPING GENE DETECTION: ABL GENE

Total ABL gene quantification for each sample was analysed with the different molecular methods.

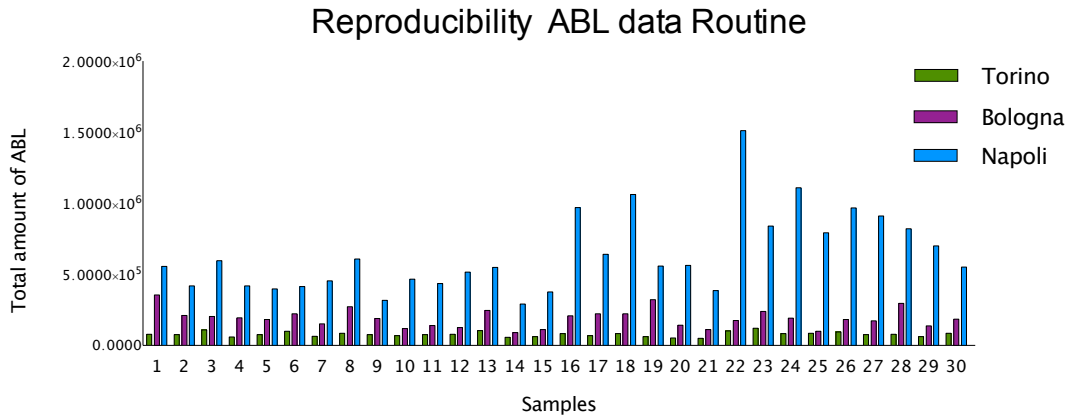


Figure 37. Analysis of quantification of ABL gene with routine methods

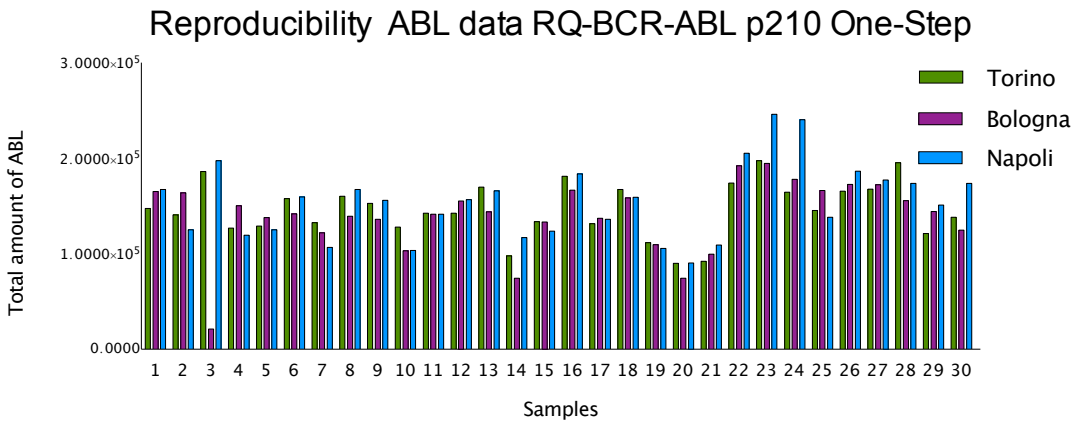


Figure 37. Analysis of quantification of ABL gene with RQ-BCR-ABL p210 One-Step

There were great differences in ABL quantification with the three routine methods. Homogeneous data were obtained with RQ-BCR-ABL p210 One-Step device in the three laboratories. The low number of ABL copies for samples 3 is due to a evaporation of the sample during the amplification phase at the Bologna center.

In addition, RQ-BCR-ABL p210 One-Step kit can detect a high average number of ABL copies, enabling the analysis of low-level samples such as MR^4 or $MR^{4.5}$. We can observe in the following figures (Figure 39 and Figure 39) how RQ-BCR-ABL p210 One-Step is capable of classificate a major number of samples in MR^4 or $MR^{4.5}$ than routine methods.

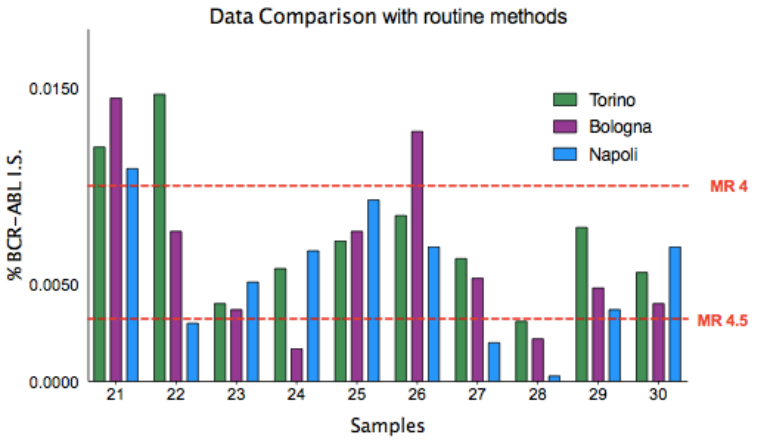


Figure 39. Quantification of low-level samples (from sample 21 to sample 30) with routine methods.

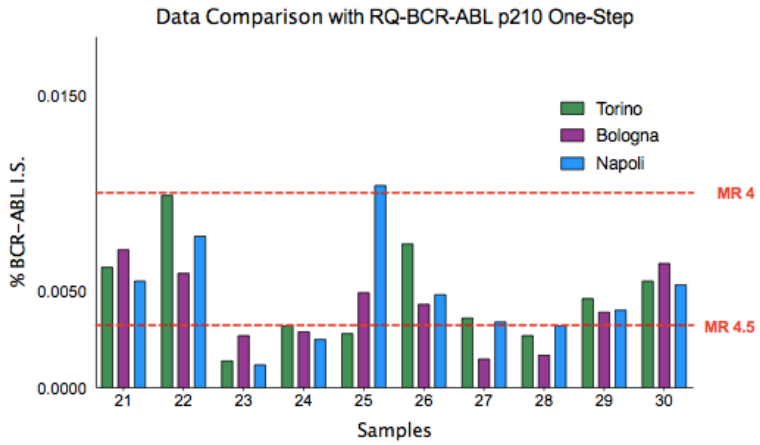


Figure 39. Quantification of low-level samples (from sample 21 to sample 30) with RQ-BCR-ABL p210 One-Step.

5.4. INDUSTRIALIZATION PROCESS OF LEUKOPREDICT

5.4.1. FREEDOM TO OPERATE ANALYSIS

For the purposes of the Freedom To Operate (FTO) analysis, the technology of LeukoPredict can be segmented in two different aspects:

1) Samples preparation
2) Samples analysis

For each of these aspects, we have performed a reconstruction of the patent scenario and a risk assessment connected with situations of interference.

5.4.1.1. Samples Preparation

RECONSTRUCTION OF THE PATENT SCENARIO

A widest patent scenario, in which it is likely there are third party patents that the technology could interfere with, is set on the basis of the International classes (IPC) identified above.

The patent scenario appears to be characterized by high density. At the date of the present analysis, there are not less than 70522 patent families.

The graph below illustrates (Figure 40), from a subjective point of view, who are the first twenty owners of technologies of this group of patents. As seen, there is a significant presence of large corporations. However, there is also an important number of public research centers.

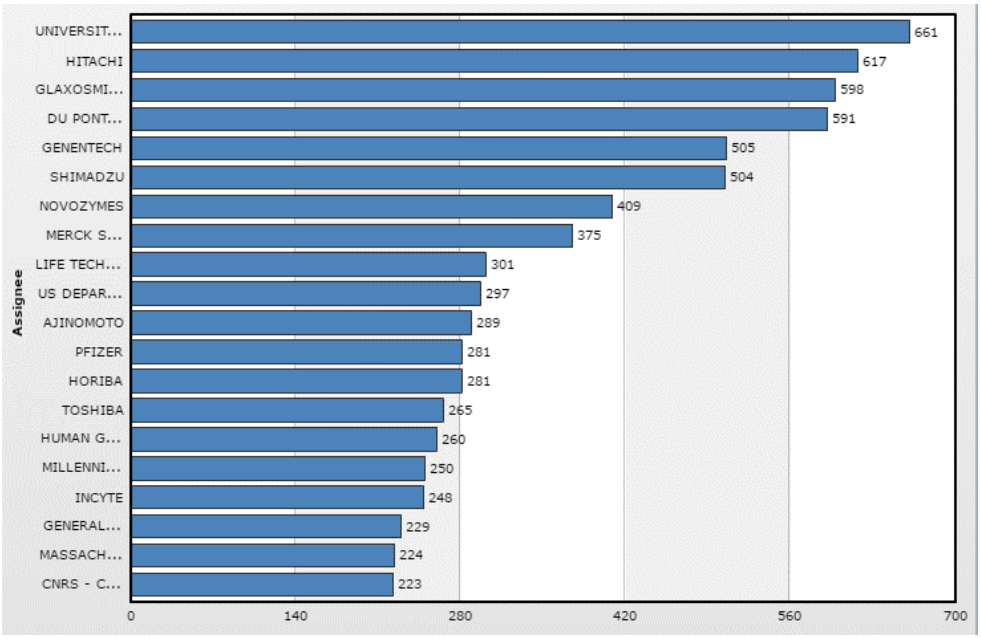


Figure 40. Distribution of search results by Assignee

The following graph (Figure 41) illustrates the patenting rate for the first twenty owners, starting from 1996 (intended as publication year of the applications). The selection of the year is made relying on the patent life-length, which is twenty years from priority filing. It is supposed that patents published in 1996 had a lifetime at most until 2016.

As apparent, the patent activity maintains on fair levels, although the years in which it is observed an increased acceleration are those comprised in the first decade of the current century. The slowdown of the subsequent years in the patenting activity can be explained with the reaching of a mature phase within the various patent categories. Furthermore, it must be pointed out that the patent applications are published 18 months after filing. Therefore, possible patent applications filed during the last 18 months do not appear in these graphs. Considering this, the data related to the last two years are inevitably incomplete.

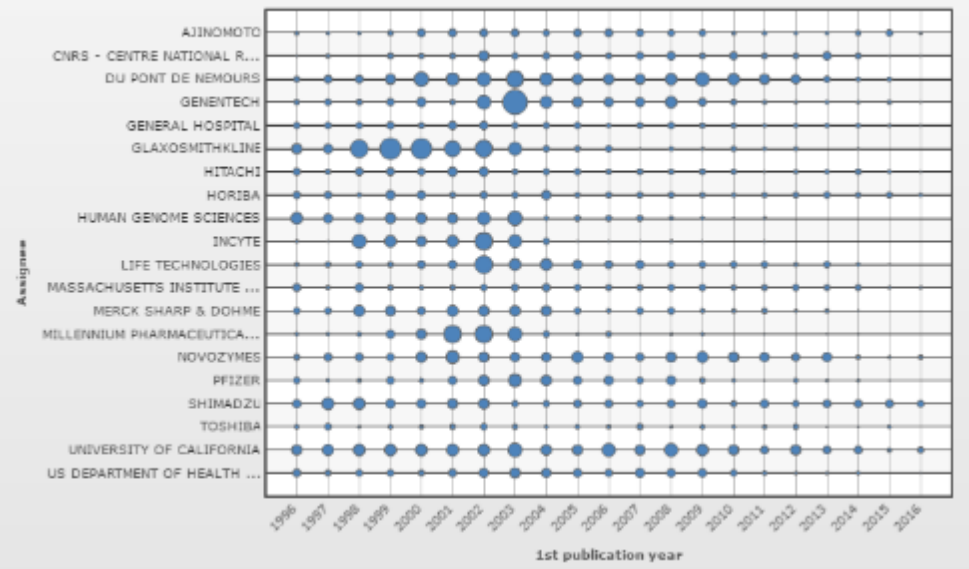


Figure 41. Distribution of search results by 1st publication year/Assignee

The following graph (Figure 42) illustrates the trend of the published applications starting from 1996. As seen, the graph confirms the decreasing trend noted above.

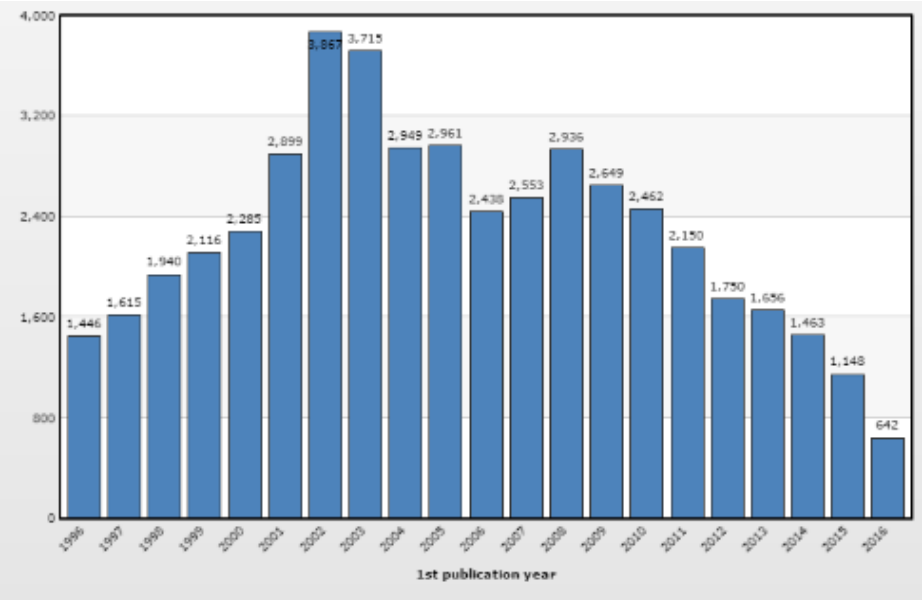


Figure 42. Distribution of search results by 1st publication year

The following image (Figure 43) illustrates the geographical distribution of patent publications. This image specifies, from a geographical point of view, which are the areas wherein the patent activity is greater by publication of applications. It deals with information that visually identifies the most important markets, both from a technological point of view and from that of business opportunities. In the countries in which the patenting activity is significant, the risk of identifying interfering patents is higher. Evidently, the most important markets are USA, Japan, Europe, Australia and Canada.

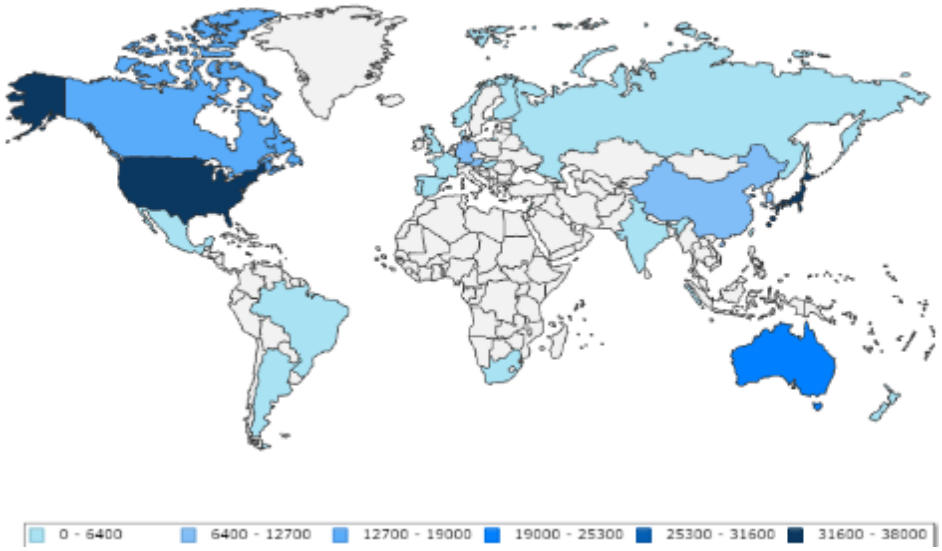


Figure 43. Distribution of search results by Publication country (without EP nad WO)

The graph below (Figure 44) provides a quick indication of the number of published applications in each country, in cumulative terms.

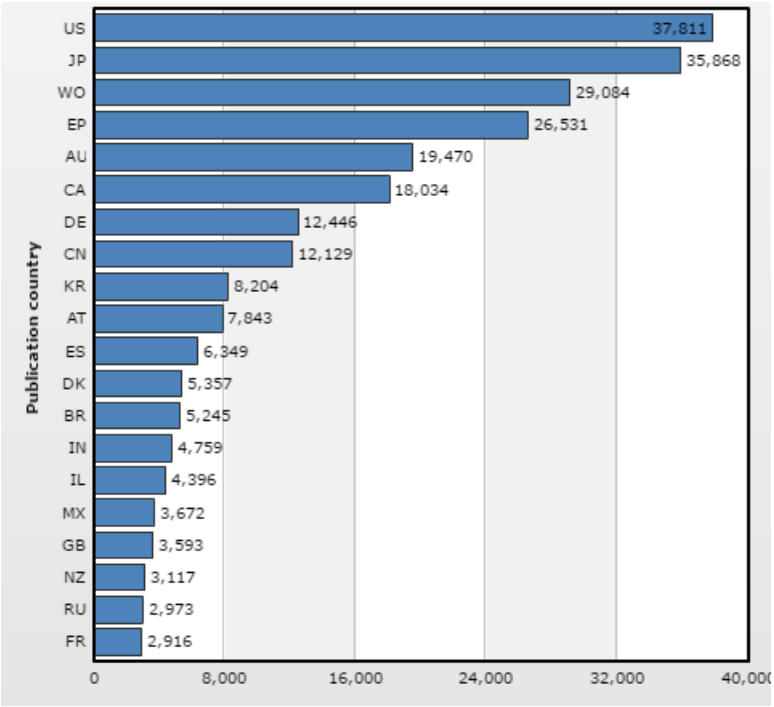


Figure 44. Distribution of search results by Publication country

LIST OF POTENTIALLY RELEVANT PATENT DOCUMENTS:

N.		Publication number	Title	Assignee
1	2013-05-24	CN104178424	**Red blood cell lysis solution and lysis method thereof.**	Surexam Biotech
2	2013-05-10	CN103627638	**Composition for lysing red cells, red cell lysing reagent and application of red cell lysing reagent**	BEIJING DONGFANG HUAHUI BIOMEDICAL TECHNOLOGY
3	2014-10-20	WO2016064887	**Red blood cell lysis solution**	GEN-PROBE
4	2012-04-18	WO2013158092	**Methods and compositions for dual extraction of protein and nucleic acid**	SYNGENTA PARTICIPATIONS
5	2013-12-30	CN103789298	**Erythrocyte lysis buffer and application thereof**	SANSURE BIOTECH
6	2010-05-17	WO2011144304	**Universally applicable lysis buffer and processing methods for the lysis of bodily samples**	CURETIS KURETISU

In details, 6 patent documents have been single out (not only issued patents, but also published application) that can be potentially relevant.

As already written, it must be pointed out that the protocol for preparing the samples for the assay is not yet definitive and some modifications might still occur. This aspect must be considered in ingredients of the RBCs lysis buffer might be modified, thus changing the risk of interference with the patent application CN104178424. More details of this patent application are shown below:

1. CN104178424 Red blood cell lysis solution and lysis method thereof.

- **Patent Assignee**

SUREXAM BIOTECH

- **Inventor**

XU JIASEN
LIU ZHIMING
WU SHIYANG
YANG WEIWEI

- **International Patent Classification**

C12N-001/06

- **Priority date:**

2013-05-24

Legal status:

CN pending

Abstract: The invention provides a red blood cell lysis solution and a method for lysing red blood cells by using the red blood cell lysis solution. The red blood cell lysis solution comprises the following components by concentration: 0.1 M to 0.5 M of ammonium chloride, 0.001 M to 0.2 M of potassium bicarbonate and 0.001 to 0.05 M of ethylenediamine tetraacetic acid or disodium ethylene diamine tetraacetate. The red blood cell lysis solution provided by the invention has the advantages of a good red blood cell lysis effect, safety to and no toxic and side effect on operators and high lysis efficiency. Formaldehyde is used as a fixing agent, and the red blood cell lysis solution is used together with other components to realize removal of red blood cells in blood; moreover, the solution can be used as a protection agent for a part of non-erythrocyte cells in a blood sample for sample transport, thereby overcoming the disadvantage that remaining cell components in purified blood cannot be stably preserved in the prior art.

Independent claims:

1. A red cell lysate, characterized in, comprising the following component concentrations: 0 . 1M-0.5M chloride, 0.001M-0.2M potassium bicarbonate, 0.001-0.05 M EDTA or disodium EDTA.

5.4.1.3. Samples Analysis

RECONSTRUCTION OF THE PATENT SCENARIO

The patent scenario in this case appears to be characterized by high density. At the date of the present analysis, there are not less than 110511 patent families.

The graph below (Figure 45) illustrates, from a subjective point of view, who are the first twenty owners of technologies of this group of patents. As seen, there are a lot of important companies. However, it is important to note the presence of different public research centers.

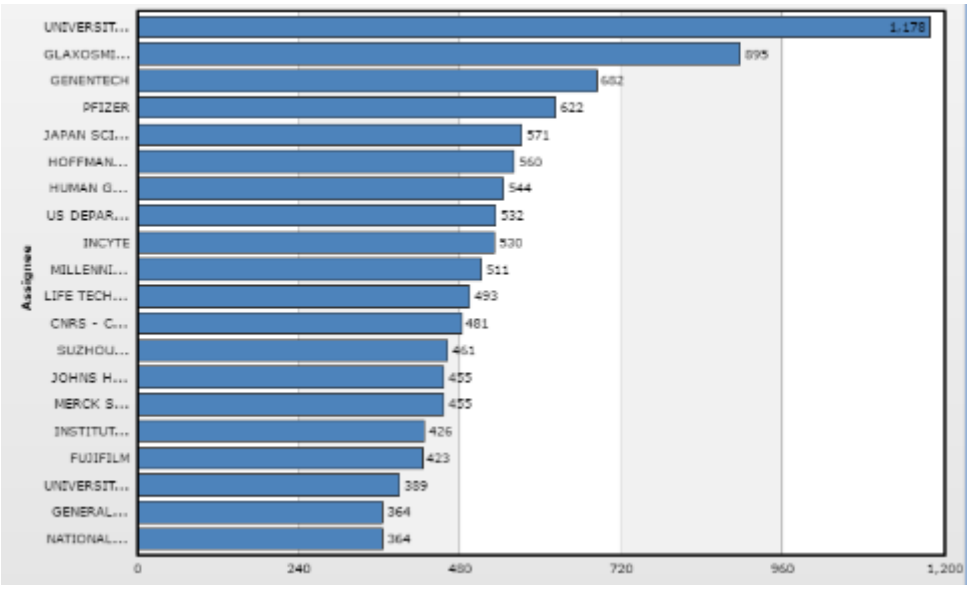


Figure 45. Distribution of search resuts by Assignee

The following graph (Figure 46) illustrates the patenting rate for the first twenty owners, starting from 1996 (intended as publication year of the applications). The selection of the year is made basing on the patent life-length, which is twenty years. It is supposed that patents published in 1996 had a life-time at most until 2016. As seen, the patent activity maintains on fair levels, although the years in which it is observed an increased acceleration are those comprised in the first five years of the current century. Also in this case, it must be pointed out that the patent applications are published 18 months after filing. Considering this, the data related to the last two years are incomplete.

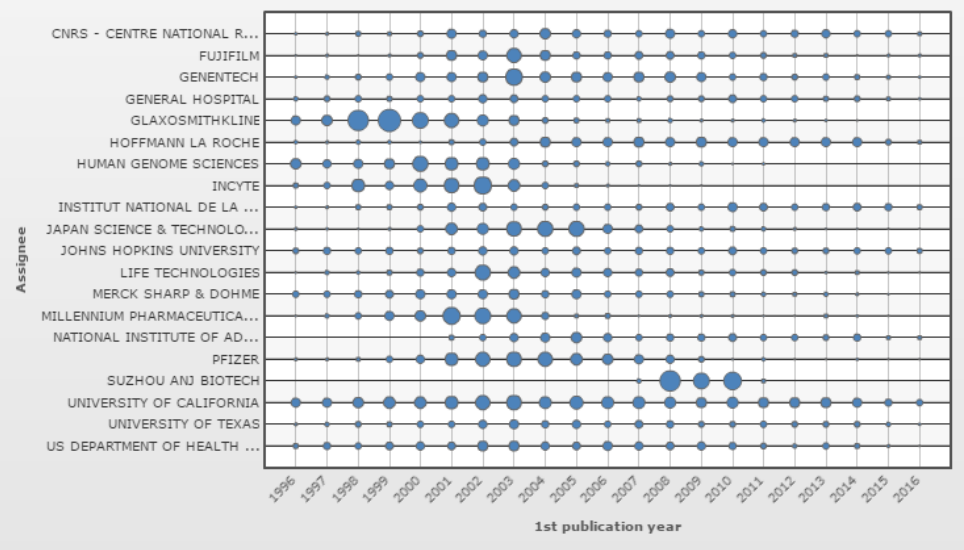


Figure 46. Distribution of search results by 1st publication year/Assignee

The following graph (Figure 47) illustrates the trend of the published applications starting from 1996. As seen, the graph confirms the weak decreasing trend noted above.

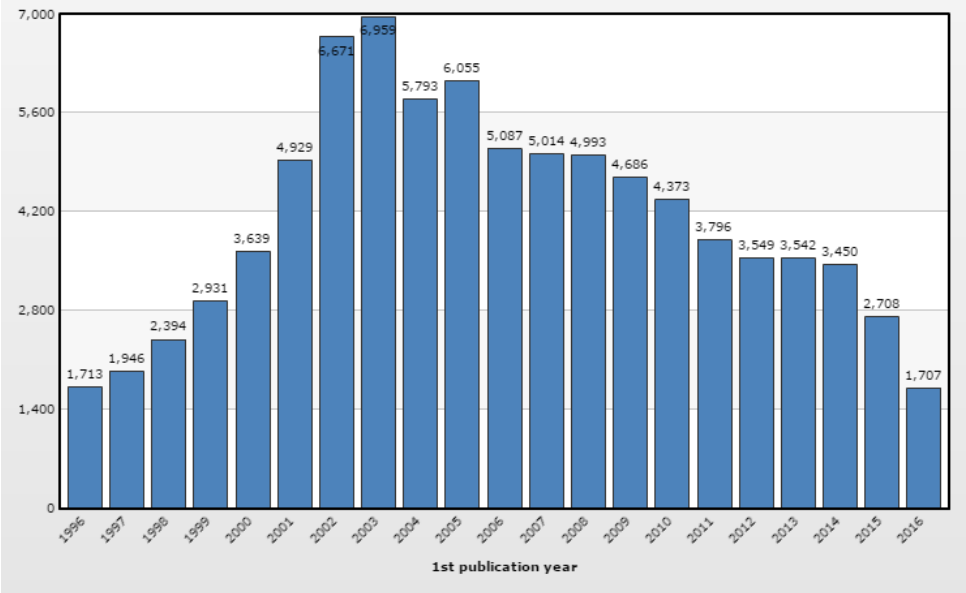


Figure 47. Distribution of search results by 1st publication year

The following image (Figure 48) illustrates the geographical distribution of patent publications. This image specifies, from a geographical point of view, which are the areas wherein the patent activity is greater by publication of applications. It deals with an information which visually identifies the most important markets, both from a technological point of view and from that of business opportunities. In the countries in which the patenting activity is significant, the risk of identifying interfering patents is higher. As seen, the most important markets are USA, Japan and Europe.

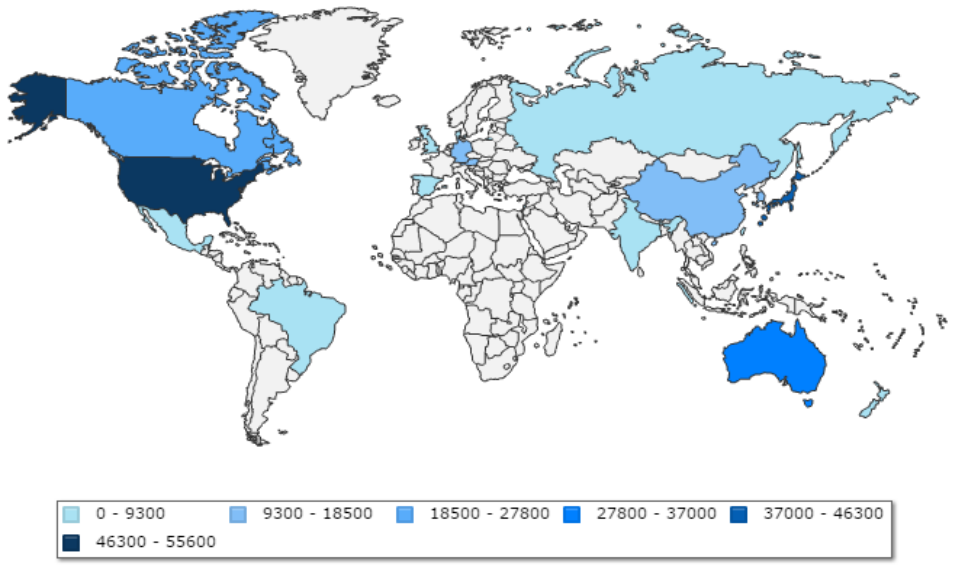


Figure 48. Distribution of search results by Publication country (without EP and WO)

The last graph (Figure 49) provides a quick indication of the number of published applications in each country, in cumulative terms.

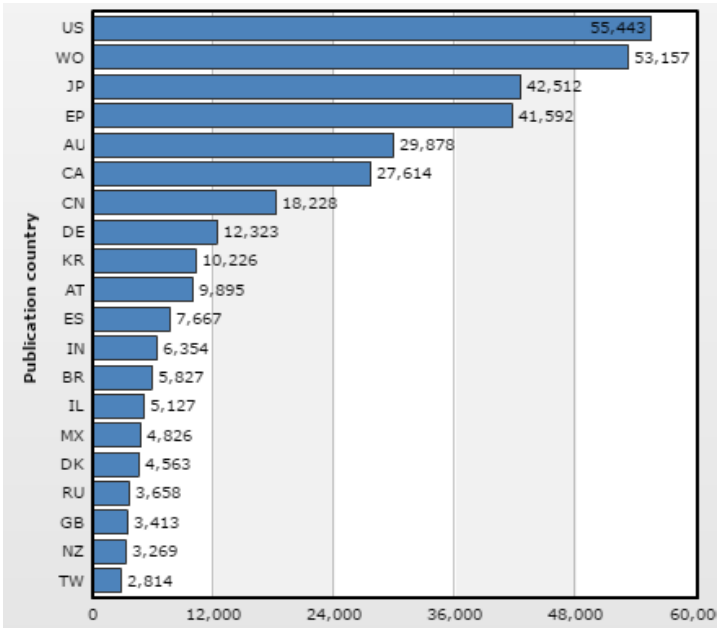


Figure 49. Distribution of search results by Publication country

LIST OF POTENTIALLY RELEVANT PATENT DOCUMENTS:

N.	Priority date	Publication number	Title	Assignee
7	2010-11-09	US8921062	**Diagnostic and prognostic assays based on circulating tyrosine kinase activity**	QUEST DIAGNOSTICS
8	2010-11-09	US2015184222	**Diagnostic and prognostic assays based on circulating tyrosine kinase activity**	QUEST DIAGNOSTICS
9	2010-10-15	WO2012049329	**Method for predicting tyrosine kinase inhibitor (tki) resistance in patients suffering from chronic myelogenous leukemia (cml)**	PAMGENE VERENIGING VOOR CHRISTELIJK HOGER ONDERWIJS WETEN VERENIGING VOOR CHRISTELIJK HOGER ONDERWIJS WETENSCHAPPELIJK ONDERZOEK EN PATIENTENZORG
10	2008-04-11	WO2009125020	**Method for profiling drug compounds**	PAMGENE
11	2006-10-27	WO2008049930	**A method for profiling kinase inhibitors**	PAMGENE
12	2009-04-10	WO2010116000	**Method for profiling drug compounds using protein kinase inhibitors**	PAMGENE
13	2006-03-17	WO2007109571	**Methods of predicting and monitoring tyrosine kinase inhibitor therapy**	NESTEC PROMETHEUS LAB
14	2012-09-13	US20140072516	**Methods for detecting enzyme activity using fluorescence lifetime imaging**	PURDUE RESEARCH FOUNDATION PURDUE UNIVERSITY
15	2012-02-07	US20130231265	**Tyrosine kinase biosensors and methods of use**	PURDUE RESEARCH FOUNDATION PURDUE UNIVERSITY
16	2014-05-05	WO2015171395	**Degradation Resistant Peptide Based Biosensors**	PURDUE RESEARCH FOUNDATION

In details, we singled out 10 patent documents (not only issued patents, but also published applications) that can be potentially relevant. In particular, the titles number 7, 8, 9 and 12 can be considered as the closest prior art. As far as the title 13 (WO2007109571, US granted) is concerned, the claims present in the PCT application could have been potentially relevant. Nevertheless, this application was filed on March 17th 2006, and therefore, it has lapsed and it cannot be infringed. Furthermore, we consider that the Technology does not infringe the granted patent US7908091 (which presents different claims).

In more details:

7. US8921062 Diagnostic and prognostic assays based on circulating tyrosine kinase activity.

• **Patent Assignee** QUEST DIAGNOSTICS • **Inventor** YEH CHEN-HSIUNG • **International Patent Classification** C12Q-001/46 C12Q-001/48 G01N-033/574 • **Priority date:** 2010-11-09
Legal status: **US granted**
Abstract: Provided herein are methods for the diagnosis, prognosis, or management of diseases, such as cancer, by measuring the tyrosine kinase activity in acellular body fluids. Further provided are methods of predicting response to therapy in certain populations of cancer patients by contacting an acellular body fluid sample from a patient with a test agent, such as a tyrosine kinase inhibitor, and then measuring the effect of the test agent on tyrosine kinase activity in the sample.
Independent claims:
1. A method for predicting the response of a patient with a BCR-ABL1-positive leukemia to therapy with a BCR-ABL1 kinase inhibitor, the method comprising: (a) measuring a first level of circulating tyrosine kinase (cTK) activity in a first sample of an acellular body fluid from a patient wherein the acellular body fluid is selected from the group consisting of plasma and serum, and wherein the measuring comprises contacting the plasma or serum with one or more tyrosine kinase (TK) substrates and measuring the phosphorylation of the one or more substrates by cTKs; (b) contacting a second sample of plasma or serum from the patient with an effective amount of one or more BCR-ABL1 kinase inhibitors; (c) measuring a second level of cTK activity in the second plasma or serum sample after it has been contacted with the one or more BCR-ABL1 kinase inhibitors, wherein the measuring comprises contacting the plasma or serum with one or more tyrosine kinase (TK) substrates and measuring the phosphorylation of the one or more substrates by cTKs; and (d) comparing the first level of cTK activity to the second level of cTK activity, wherein a decrease between the first level and the second level indicates that the patient is a potential responder to therapy with the one or more BCR-ABL1 kinase inhibitors, and wherein no change between the first level and the second level indicates that the patient is potentially not a responder to therapy with the one or more BCR-ABL1 kinase inhibitors.

8. US2015184222 Diagnostic and prognostic assays based on circulating tyrosine kinase activity.

• **Patent Assignee** QUEST DIAGNOSTICS • **Inventor** YEH CHEN-HSIUNG • **International Patent Classification**

C12Q-001/48

• **Priority date:**
2010-11-09

Legal status:
US pending

Abstract: Provided herein are methods for the diagnosis, prognosis, or management of diseases, such as cancer, by measuring the tyrosine kinase activity in acellular body fluids. Further provided are methods of predicting response to therapy in certain populations of cancer patients by contacting an acellular body fluid sample from a patient with a test agent, such as a tyrosine kinase inhibitor, and then measuring the effect of the test agent on tyrosine kinase activity in the sample.

Independent claims:
1. A method for identifying the presence or absence of a neoplastic disease in a subject, the method comprising: (a) measuring the level of circulating tyrosine kinase (cTK) activity in an acellular body fluid sample from a human subject suspected of having a neoplastic disease; and
(b) identifying the subject as: (i) having a neoplastic disease when the level of cTK activity in the sample differs from a reference cTK activity level; or
(ii) not having a neoplastic disease when the level of cTK activity in the sample does not differ from a reference cTK activity level.

21. A method for determining a prognosis for a patient having a proliferative hematological disorder, comprising: (a) measuring a first level of cTK activity in a first acellular body fluid sample collected at a first time point from a patient;
(b) measuring a second level of cTK activity in a second acellular body fluid sample collected at a second time point from the patient;
(c) comparing the first level to the second level; and
(d) identifying the subject as: (i) having a good prognosis when there is a decrease between the first level and the second level; and
(ii) having a poor prognosis when there is an increase between the first level and the second level or a lack of a decrease between the first level and the second level.

9 . WO2012049329 Method for predicting tyrosine kinase inhibitor (TKI) resistance in patients suffering from chronic myelogenous leukemia (CML).

• **Patent Assignee**
PAMGENE VERENIGING VOOR CHRISTELIJK HOGER ONDERWIJS WETEN VERENIGING VOOR CHRISTELIJK HOGER ONDERWIJS WETENSCHAPPELIJK ONDERZOEK EN PATIENTENZORG

• **Inventor**
BOENDER PIETER JACOB
VAN DEN BERG ADRIANA
RUIJTENBEEK ROBBY
JANSSEN JEROEN JOHANNES WILHELMUS MARIA
OSSENKOPPELE GERRIT JOHAN

• **International Patent Classification**
C12Q-001/48 G01N-033/574

• **Priority date:**
2010-10-15

Legal status:
US pending

EP lapsed

Abstract: The present invention relates to a method for determining or predicting the response of a patient diagnosed with chronic myelogenous leukaemia (CML) to treatment with a tyrosine kinase inhibitor. More specifically, the present invention provides methods which measure kinase activity by studying phosphorylation levels and profiles and inhibitions thereby diagnosing CML patients resistant to treatment with Imatinib.

Independent claims:

1. A method for predicting the response of a patient diagnosed with chronic myelogenous leukaemia (CML), to a tyrosine kinase inhibitor (TKI), comprising the steps of:
(a) measuring the kinase activity of a sample, obtained from said patient diagnosed with CML, by contacting said sample with immobilized protein kinase substrates, thereby providing a phosphorylation profile of said sample, said phosphorylation profile comprising the phosphorylation levels of phosphorylation sites present in at least 10 peptide markers as listed in Table 1 ; and,
(b) determining from said phosphorylation profile the response of said patient to said TKI.

10. A method for diagnosing CML for a patient, wherein the kinase activity of a sample, obtained from said patient, is measured, wherein said kinase activity measurement provides phosphorylation profiles of said sample thereby diagnosing CML for said patient.

14. A method for predicting the discontinuation of tyrosine kinase inhibitor treatment to patients diagnosed with CML but who are unware of being cured and continue to receive tyrosine kinase inhibitor treatment, comprising the steps of:
(a) measuring the kinase activity of a sample, obtained from said patient diagnosed with CML, who continues to receive tyrosine kinase inhibitor (TKI) treatment, by contacting said sample with immobilized protein kinase substrates, thereby providing a phosphorylation profile of said sample, said phosphorylation profile comprising the phosphorylation levels of phosphorylation sites present in at least 10 peptide markers as listed in Table 1 ; and, (b) determining from said phosphorylation profile the disease free status of said patient.

Table 1: SEQ ID NO Name Sequence (N terminus to C terminus)
1 ACHD 383 395 YISKAEEYFLLKSG
2 AMPE 5 17 EREGSKRYCIQTKG
3 ANXA1 14 26 IENEEQEYVQTVKG
4 ANXA2 17 29 HSTPPSAYGSVKAG
5 B3AT 39 51 TEATATDYHTTSHG
6 CALM 93 105 FDKDGNGYISAAEG
7 CALM 95 107 KDGNGYISAAELRG
8 CBL 693 705 EGEEDTEYMTPSSG
9 CD3Z 116 128 KDKMAEAYSEIGMG
10 CD79A 181 193 EYEDENLYEGLNLG
1 1 CDK2 8 20 EKIGEGTYGWYKG
12 CDK3 12 26 EGTYG WYKA KN R ETG
13 CTNB1 79 91 VADI DGQYAMTRA
14 DCX 109 121 GIVYAVSSDRFRSG
15 DDR1 506 518 LLLSNPAYRLLLAG
16 DYR1A 212 224 KHDTEMKYYIVHLG
17 DYR1A 312 324 CQLGQRIYQYIQSG
18 EFS 246 258 GGTDEGIYDVPLLG
19 EGFR 1062 1074 EDSFLQRYSSDPT
20 EGFR 1103 1 115 GSVQNPVYHNQPLG
21 EGFR 1 118 1130 APSRDPHYQDPHS
22 EGFR 1165 1 177 ISLDNPDYQQDFFG
23 EGFR 1190 1202 STAENAEYLRVAPG
24 EGFR 862 874 LGAEEKEYHAEGG
25 EGFR 908 920 MTFGSKPYDGIPA
26 ENOG 37 49 SGASTGIYEALELG
27 EPHA1 774 786 LDDFDGTYETQGGG
28 EPHA2 581 593 QLKPLKTYVDPHTG SEQ ID NO Name Sequence (N terminus to C

terminus)
29 EPHA2 765 777 EDDPEATYTTSGGG
30 EPHA4 589 601 LNQGVRTYVDPFTG
31 EPHA4 921 933 QAIKMDRYKDNFTG
32 EPHA7 607 619 TYIDPETYEDPNRG
33 EPHB1 771 783 DDTSDPTYTSSLGG
34 EPHB1 921 933 SAIKMVQYRDSFLG
35 EPHB4 583 595 IGHGTKVYIDPFTG
36 EPOR 361 373 SEHAQDTYLVLDKG
37 EPOR 419 431 ASAASFEYTI LDPG
38 ERBB2 1241 1253 PTAENPEYLGLDVG
39 ERBB2 870 882 LDIDETEYHADGG
40 ERBB4 1181 1 193 QALDNPEYHNASN
41 FABPH 13 25 DSKNFDDYMKSLGG
42 FAK1 569 581 RYMEDSTYYKASKG
43 FAK2 572 584 RYI EDEDYYKASVG
44 FER 707 719 RQEDGGVYSSSGLG
45 FES 706 718 REEADGVYAASGGG
46 FGFR2 762 774 TLTTNEEYLDLSQG
47 FGFR3 641 653 DVHNLDYYKKTTNG
48 FGFR3 753 765 TVTSTDEYLDLSAG
49 FRK 380 392 KVDNEDIYESRHEG
50 INSR 1348 1360 SLGFKRSYEEHIPG
51 INSR 992 1004 YASSN PEYLSASDG
52 JAK1 1015 1027 AIETDKEYYTVKDG
53 K2C6B 53 65 GAGFGSRSLYGLGG
54 KSYK 518 530 ALRADENYYKAQTG
55 LAT 194 206 MESIDDYVNVPESG
56 LAT 249 261 EEGAPDYENLQELG
57 LCK 387 399 RLIEDNEYTAREGG
58 MBP 198 210 ARTAHYGSLPQKSG
59 MBP 259 271 FGYGGRASDYKSAG
60 MBP 263 275 GRASDYKSAHKGFG
61 MET 1227 1239 RDMYDKEYYSVHNG
62 MK01 180 192 HTGFLTEYVATRW
63 MK01 198 210 IMLNSKGYTKSID
64 MK07 21 1 223 AEHQYFMTEYVATG
65 MK10 216 228 TSFMMTPYWTRYG
66 MK12 178 190 ADSEMTGYVVTRWG
67 MK14 173 185 RHTDDEMTGYVATG
68 NTRK1 489 501 HIIENPQYFSDACG
69 NTRK2 509 521 PVI EN PQYFGITNG
70 P85A 600 612 NENTEDQYSLVEDG
71 PAXI 1 11 123 VGEEEHVYSFPNKG
72 PAXI 24 36 FLSEETPYSYPTGG
73 PDPK1 2 14 ARTTSQLYDAVPI
74 PDPK1 369 381 DEDCYGNYDNLLS
75 PECA1 706 718 KKDTETVYSEVRKG
76 PERI 458 470 QRSELDKSSAHSYG
77 PGFRB 1002 1014 LDTSSVLYTAVQPG
78 PGFRB 572 584 VSSDGHEYIYVDPG
79 PGFRB 709 721 RPPSAELYSNALPG SEQ ID NO Name Sequence (N terminus to C terminus)
80 PGFRB 768 780 SSNYMAPYDNYVPG
81 PGFRB 771 783 YMAPYDNYVPSAPG
82 PLCG1 1246 1258 EGSFESRYQQPFEG

83 PLCG1 764 776 IGTAEPDYGALYEG
84 PLCG1 776 788 EGRNPGFYVEANP
85 PP2AB 297 309 EPHVTRRTPDYFLG
86 PRRX2 202 214 WTASSPYSTVPPYG
87 RASA1 453 465 TVDGKEI YNTI RRG
88 RBL2 99 11 1 VPTVSKGTVEGNYG
89 RET 1022 1034 TPSDSLIYDDGLSG
90 RET 680 692 AQAFPVSYSSSGAG
91 RON 1346 1358 SALLGDHYVQLPAG
92 RON 1353 1365 YVQLPATYMNLGPG
93 SRC8 CHICK 470 482 VSQREAEYEPETVG
94 SRC8 CHICK 476 488 EYEPETVYEVAGAG
95 SRC8 CHICK 492 504 YQAEENTYDEYENG
96 STA5A 687 699 LAKAVDGYVKPQIG
97 STAT1 694 706 DGPKGTGYIKTELG
98 STAT3 698 710 DPGSAAPYLKTKFG
99 STAT4 686 698 TERGDKGYVPSVFG
100 STAT4 714 726 PSDLLPMSPSVYAG
101 TEC 512 524 RYFLDDQYTSSSGG
102 TYR03 679 691 KI YSG DYYRQGCAG
103 VGFR1 1049 1061 KNPDYVRKGDTRL
104 VGFR2 1052 1064 DIYKDPDYVRKGD
105 VGFR3 1061 1073 DIYKDPDYVRKGSG
106 VINC 815 827 KSFLDSGYRI LGAG
107 ZAP70 485 497 ALGADDSYYTARSG
108 ZBT16 621 633 LRTHNGASPYQCTG

12. WO2010116000 Method for profiling drug compounds using protein kinase inhibitors.

- **Patent Assignee**

PAMGENE

- **Inventor**

RUIJTENBEEK ROBBY

- **International Patent Classification**

C40B-030/04 G01N-033/50

- **Priority date:**

2009-04-10

Legal status:
EP granted
US pending

WO2010116000

Abstract: The present invention relates to a method for determining the effect of a drug on the kinase activity in a sample or predicting the response of a patient to a drug, wherein the kinase activity of a sample in the presence of a protein kinase inhibitor is measured, said protein kinase inhibitor mimicking the effect of said drug compound on the kinase activity in said sample.

Independent claims:

1. A method for determining the effect of a drug on the kinase activity in a cell lysate sample, comprising the steps of:
(a) measuring the kinase activity of said cell lysate sample, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said cell lysate sample in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor inhibits the kinase domain of a surface receptor targeted by said drug thereby mimicking the effect of said drug on the kinase activity in said cell lysate sample.

2. A method for determining the effect of a drug on the kinase activity in a cell lysate sample, comprising the steps of:
(a) measuring the kinase activity of said cell lysate sample, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said sample cell lysate in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor inhibits proteins associated with the signal transduction pathway of a surface receptor targeted by said drug thereby mimicking the effect of said drug on the drug targeted signal transduction pathway in said cell lysate sample.

3. A method for predicting the resistance of a patient to a drug, comprising the steps of:
(a) measuring the kinase activity of a cell lysate sample, obtained from said patient, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said cell lysate sample in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor acts towards proteins of one or more alternative signal

transduction pathways causing tumor growth, thereby reporting on the activity of alternative signal transduction pathways causing tumor growth which are not affected by the drug, thereby causing resistance.

4. A method for predicting the resistance of a patient to a drug, comprising the steps of:
(a) measuring the kinase activity of a cell lysate sample, obtained from said patient, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said cell lysate sample in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor inhibits proteins of a signal transduction pathway not targeted by said drug, thereby reporting on the activity of a signal transduction pathway which is not affected by said drug, thereby reporting resistance.

EP2417452 B1

Abstract: The present invention relates to a method for determining the effect of a drug on the kinase activity in a sample or predicting the response of a patient to a drug, wherein the kinase activity of a sample in the presence of a protein kinase inhibitor is measured, said protein kinase inhibitor mimicking the effect of said drug compound on the kinase activity in said sample.

Independent claims:

1. A method for determining the effect of a drug on the kinase activity in a cell lysate sample, comprising the steps of:
(a) measuring the kinase activity of said cell lysate sample, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said cell lysate sample in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor inhibits the kinase domain of a surface receptor targeted by said drug thereby mimicking the effect of said drug on the kinase activity in said cell lysate sample.

2. A method for determining the effect of a drug on the kinase activity in a cell lysate sample, comprising the steps of:
(a) measuring the kinase activity of said cell lysate sample, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said sample cell lysate in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor inhibits proteins associated with the signal transduction pathway of a surface receptor targeted by said drug thereby mimicking the effect of said drug on the drug targeted signal transduction pathway in said cell lysate sample.

3. A method for predicting the resistance of a patient to a drug, comprising the steps of:
(a) measuring the kinase activity of a cell lysate sample, obtained from said patient, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said cell lysate sample in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor acts towards proteins of one or more alternative signal transduction pathways causing tumor growth, thereby reporting on the activity of alternative signal

transduction pathways causing tumor growth which are not affected by the drug, thereby causing resistance.

4. A method for predicting the resistance of a patient to a drug, comprising the steps of:
(a) measuring the kinase activity of a cell lysate sample, obtained from said patient, in the presence and in the absence of a protein kinase inhibitor, thereby providing a phosphorylation profile of said cell lysate sample in the presence of a protein kinase inhibitor and a phosphorylation profile of said cell lysate sample in the absence of a protein kinase inhibitor; and,
(b) determining from said phosphorylation profiles in the presence and in the absence of a protein kinase inhibitor the differential phosphorylation profile, said differential phosphorylation profile indicating the effect of said drug on the kinase activity in said cell lysate sample, wherein said protein kinase inhibitor inhibits proteins of a signal transduction pathway not targeted by said drug, thereby reporting on the activity of a signal transduction pathway which is not affected by said drug, thereby reporting resistance.

5.4.2. COST-EFFECTIVENESS ANALYSIS

5.4.2.1. Model validation

The model has been validated in line with the recommendation of good research practice in modeling. [200] Validation of the model was limited at internal and cross-validation.

5.4.2.2. Internal validation

Internal model validation was done in two steps: inputs verification procedure and extreme value analysis procedure. As a part of inputs verification procedure, each model input was checked with the primary source. Extreme value analysis procedure use sensitivity analysis by incorporating extreme or null values of parameters inputs to access whether the results are moving in the expected direction. All identified “bugs” were successfully corrected.

5.4.2.3. Cross-validation

Decision analytic model from this analysis was cross-validated with Padula et al. [186]model, as the only published model that uses EMR as a surrogate endpoint in the modeling of survival and transition probabilities. Cross-validation was limited to the QALY calculated by the models in the standard of care arm for the base-case analysis and scenario analysis. Results are presented in Table 13.

Table 17. Cross-model validation of 5-years results in standard treatments arms.

Model	Standard of care arm – five years’ results	
	Base-case* QALY gained	Scenario analysis** QALY gained
LeukoPredict model	4.02	3.80
Padula et al. [186]	3.98	3.82

Legend: *First-line treatment real-world settings, **First-line treatment, guidelines recommendations, QALY – quality adjusted life years

Results are presented at the five years, as a time horizon used in the Padula et al. [186] analysis. Results can be considered as a similar, and they were calculated by models using different methods. Namely, Padula et al. [186] were conducted the patient-level simulation, while this analysis is used cohort simulations. The main data sources for the EMR as a surrogate endpoint were different in the analyses, while this analysis used Neelakantan et al. [178]study, Padula et al. [186] were used a meta-analysis of several published clinical trial results.

5.4.2.4. Base-case results

Threshold analysis results are presented in Figure 50. Due to the evident cost-saving effect at any threshold the “Optimal Operating Point” was selected based on the threshold linked to the sensitivity/specificity pair leading to the highest QALY gained Figure 51.

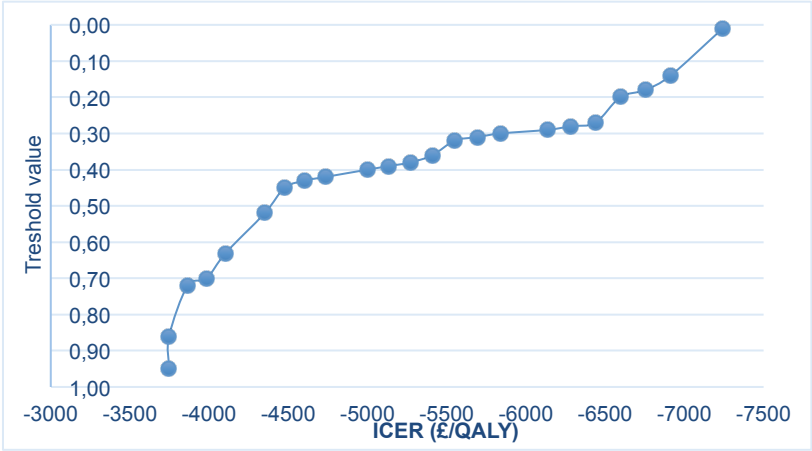


Figure 50. Changes in ICER by changing test positivity threshold

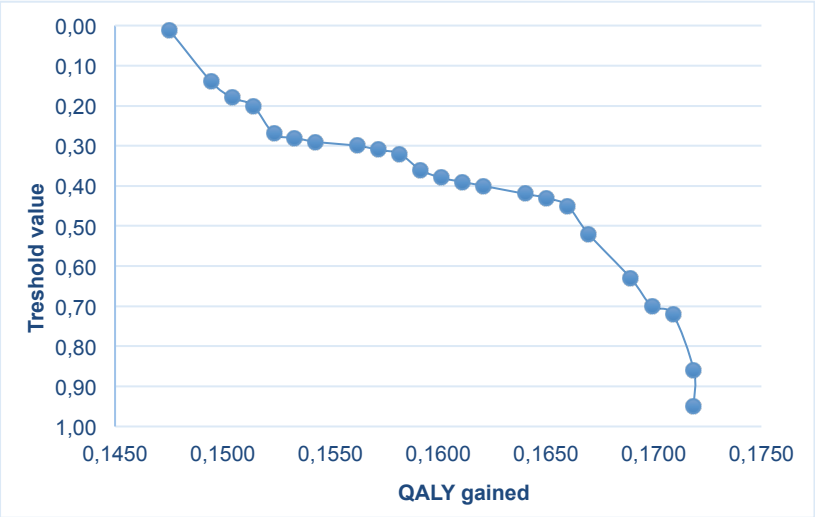


Figure 51. Changes in QALY gained by changing test positivity threshold

Therefore, the threshold at 0,86 linked to the 94% sensitivity and 83% specificity was selected as an “Optimal Operating Point” leading to the biggest cost savings and highest QALY gain.

As already explained in the Methods section in the base-case analysis patients receive first-line treatment in line with real-world settings (33.33% per tyrosine kinases inhibitor, including Imatinib, Dasatinib, and Nilotinib). Results are presented in Table 18.

Table 18. Base-case results of cost-utility analysis

	Cost, £	∆ cost, £	LYG, years	∆ LYG	QALY	∆ QALY	ICER, £/QALY
Standard of care	196577		6,53		5,72		
Standard of care plus LeukoPredict	196277	-300	6,63	0,10	5,89	0,17	**Dominates**

Legend: LYG – life years gained, QALY – quality adjusted life years. ICER – incremental cost-effectiveness ratio.

Within standard approach in quantifying uncertainty in decision analytic models, a one-way sensitivity analysis (Figure 52) was conducted to address parameters uncertainty. Parameters with the biggest impact on the ICER were drug cost for first and second line treatments.

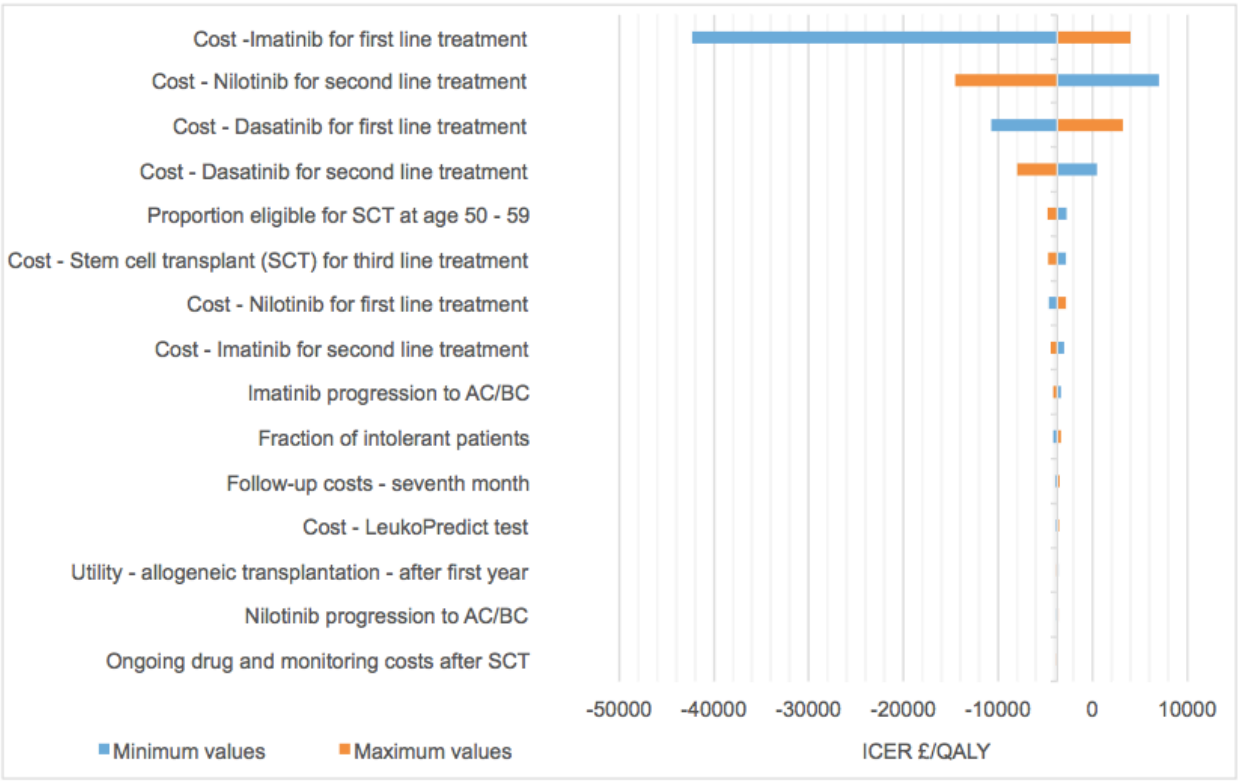


Figure 52. Tornado diagram -- base-case analysis

5.4.2.5. Scenario analysis results

To properly address different possible alternatives and relevant clinical scenarios which one-way sensitivity analyses were not addressed two additional scenario analysis were conducted.

Scenario analysis 1

In the scenario analysis, 1 patients receive first-line treatment with Imatinib (100% Imatinib). Results are presented in Table 19

Table 19. Cost-utility results scenario analysis 1

	Cost, £	Δ cost, £	LYG, years	Δ LYG	QALY	Δ QALY	ICER, £/QALY
Standard of care	159556		6,31		5,35		
Standard of care plus LeukoPredict	164486	4930	6,48	0,17	5,66	0,31	15865

Legend: LYG – life years gained, QALY – quality adjusted life years. ICER – incremental cost-effectiveness ratio.

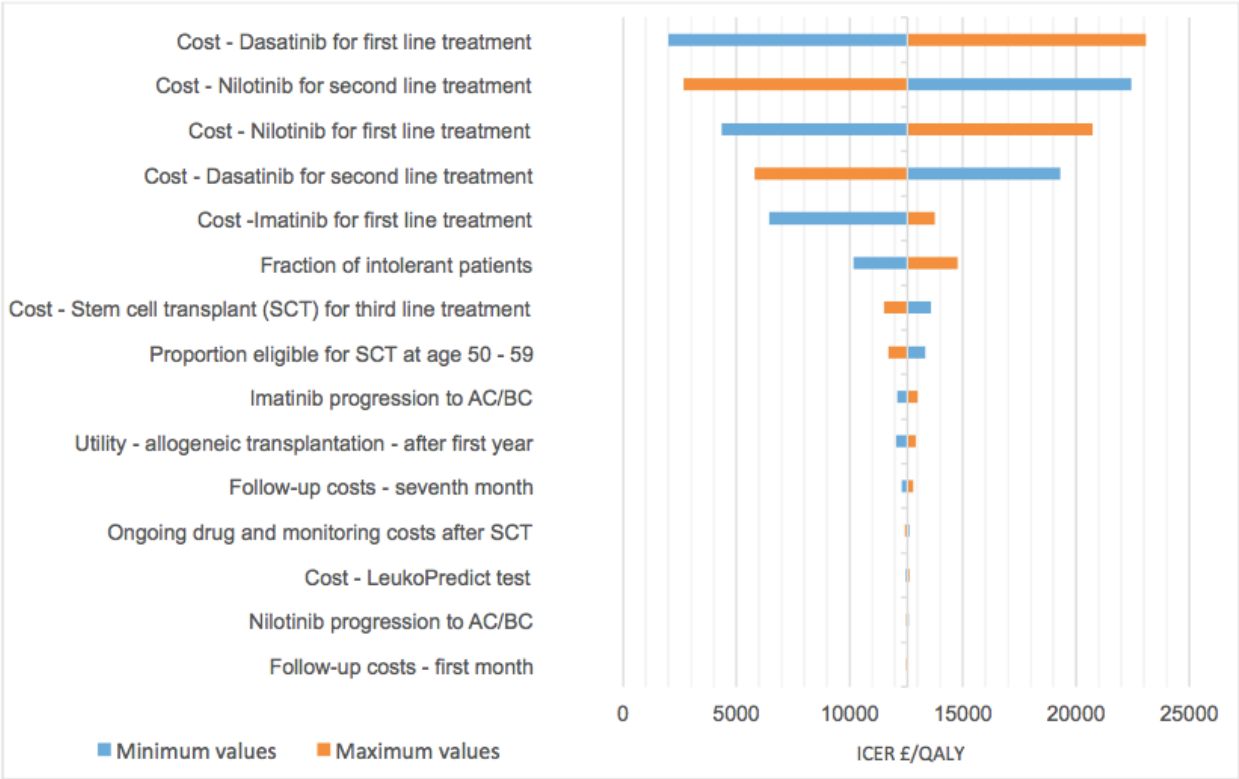


Figure 53. Tornado diagram -- scenario analysis 1

In the scenario analysis 1, the most sensitive parameters (Figure 53) are still drug costs and expectably drug cost for the first line treatment for Dasatinib and Nilotinib which will be used instead of the Imatinib in resistance patients in LeukoPredict arm. Consequently, a fraction of the intolerant patients starts to play a major role due to the biggest probability of drug resistance for the Imatinib.

Scenario analysis 2

In the scenario analysis 2 patients receive first-line treatment in line with European LeukemiaNet recommendations (100% Imatinib) but cost decreases of Imatinib due to the generic entry was modeled, with prioritizing patients to receive Imatinib in the first line treatment due to the lower costs. Results are presented in Table 20

Table 20. Cost-utility results analysis 2

	Cost, £	∆ cost, £	LYG, years	∆ LYG	QALY	∆ QALY	ICER, £/QALY
Standard of care	95794		6,31		5,35		
Standard of care plus LeukoPredict	98084	2290	6,48	0,17	5,66	0,31	7371

Legend: LYG – life years gained, QALY – quality adjusted life years. ICER – incremental cost-effectiveness ratio.

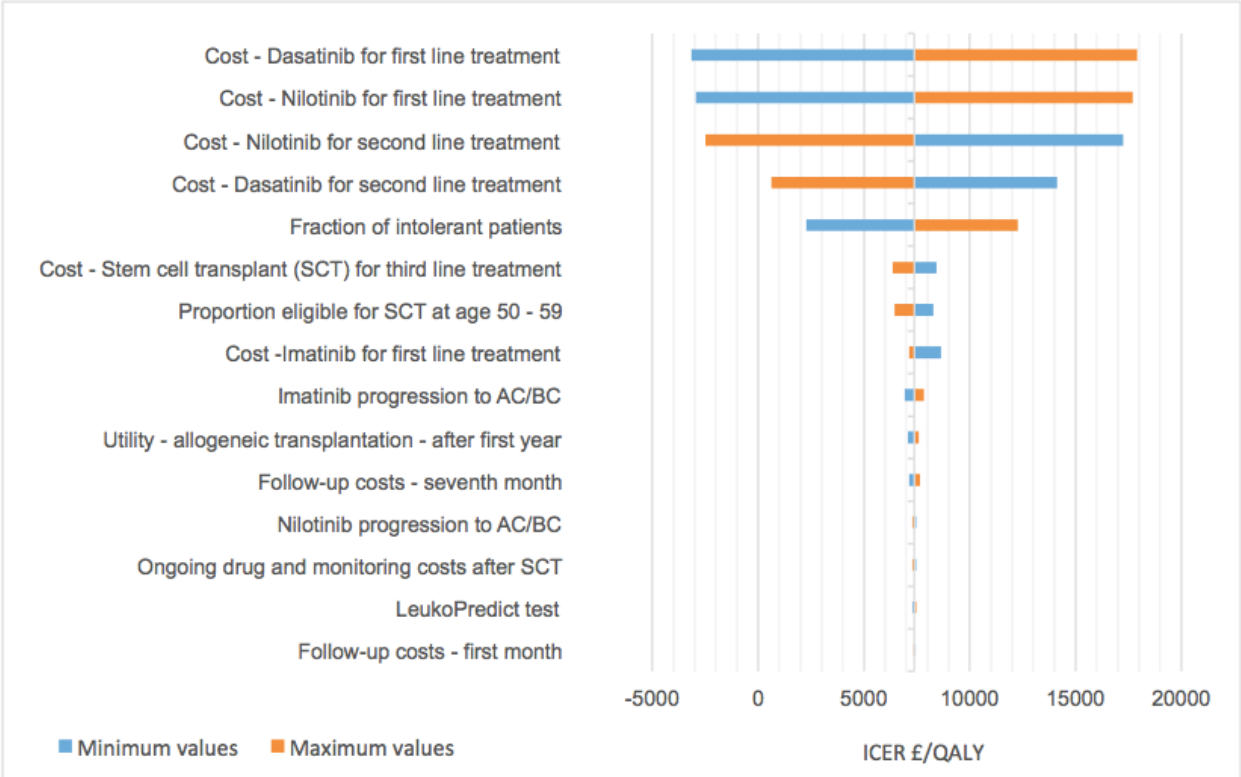


Figure 54. Tornado diagram -- scenario analysis 2

Results of the one-way sensitivity analysis presented at Figure 54 revealed same cost parameters as a most sensitive on cost variation in line with sensitivity analysis for base-case and scenario analysis 1.

5.4.2.6. Limitations of the analysis

Decision analytic modeling has an in-built limitation of simulating reality. In the analysis, multiple sources of information are collected together, and they might lack proper correspondence. In general, the main limitation of the early health technology assessment, including this analysis, is that empirical data to inform decision analytic model are limited or immature and incomplete, and therefore uncertainty regarding results is always of greater extent in comparison with the standard economic evaluations.

Specifically for this research question there are several important limitations, which should be considered when interpreting the results of this study

- The diagnostic accuracy data regarding LeukoPredict test was estimated from the preclinical studies which will are not necessarily correspond to clinical performance. The data cost of test itself as well as a resource used is based on the assumption in the personal communication with test developers.
- Cohort's starting age was 50 years of life according to the main available data source used in the analysis, but according to the data from Hematological Malignancy Research Network (HMRN) reported in the HTA report by Pavey et al. [187] mean age of the diagnosis of CML in the UK is a 57 years of life.
- Survival after stem cell transplantation was informed by the real-world data from Hammersmith Hospital, London, between 2000 and 2010 [187]with patients with median age of 60 years of life. This deviation from the starting age of modeled cohort should not affect overall modeled survival to a great extent due to the small fraction of the cohort receiving stem cell transplantation. On the other hand, those were the only available data regarding stem cell transplantation as a third line therapy in the treatment of the CML patients in the UK.
- The model was not including any patient categorizations by commonly used risk scores categories at the beginning of the treatment (e.g. Sokal, EUTOS, Hasford).

6. DISCUSSION

The role of BCR-ABL rearrangement in the development and pathogenesis of CML is well documented. In fact, the formation of BCR-ABL tyrosinkinase protein plays a major role in the pathogenesis of CML, and it is able to influence biological events such as uncontrolled cell proliferation, inhibition of apoptosis, and reduction of cellular adhesion.

Currently, TKI therapy has proven to be effective, with high overall survival rates and event-free survival rates in most patients. However, there is a percentage of patients who do not have an optimal response to therapy, or who, after an early effective period, develops resistance to treatment. The problem of non-responders should not to be underestimated, both regarding the patient's health and the healthcare costs involved.

The response to TKI is the most significant prognostic value. Defining the disease level through regular BCR-ABL transcript quantification is the most effective method for measuring the pharmacological effect of TKI, with the aim of maintaining or modifying the therapeutic plan according to these results. The monitoring of therapeutic response has become necessary and routinely. It can be performed at the molecular or cytogenetic level, or both.

Since disease levels are expressed as the ratio between BCR-ABL transcripts and control gene quantity, high sensitivity and measuring accuracy of these are required, thus as small variations could lead to the inclusion of a patient in a level of disease to which it doesn't really belong to.

The method used for BCR-ABL quantification has evolved over time. At first it was only possible to identify the presence or absence of transcripts, either with classical PCR or with 2-step 'nested' amplification with internal primers to improve sensitivity.

To date, thanks to the use of qRT-PCR Real-Time, not only it is possible to identify the BCR-ABL transcripts, but also its quantification is very fast and sensitive, capable of detecting up to 0.001% BCR-ABL / ABL. In addition, small steps towards the automation of the process, such as the introduction of One-Step technology, help to reduce the amount of time it takes to analyse it, eliminating steps and avoiding errors and contaminations of the sample.

In this direction, the company DOTT. DINO PALADIN has developed and validated two new kits (RQ-BCR-ABL p190 One-Step and RQ-BCR-ABL p210 One-Step) based on the qRT-PCR Real-Time One-Step method for CML monitoring. Both devices allow, through One-step technology, the retrotranscription and amplification directly from RNA of patient in a one unique step.

The RQ-BCR-ABL p210 One-Step device has demonstrated a diagnostic specificity of 100%. To calculate it, 26 negative RNA for M-bcr b2a2 and b3a2 ($p210^{BCR-ABL}$) were tested with the RQ-BCR-ABL p210 One-Step, without getting any sign of positivity for any of the samples. The kit has

obtained a diagnostic sensitivity of 98%, since it has detected 51 positives samples out of a total of 52.

The non-concordant sample in question was tested three times with the device, in a first session in double test, then in the second session in single test.

During these sessions, 2 out of 3 replicates were undetermined for the target while one replicate was positive, though with a very low signal. The percentage ratio of first session has been consistent, but the standard of ABL was unsuitable. In the second session, however, this sample has identified as negative, which is why it isn't in agreement with the expected result. There is the possibility of degradation of the sample or, most likely, the patient was in remission of the disease ($MR^{4.5}$).

The values obtained show a robust and reliable device for therapeutic monitoring according to reproducibility and linearity. In addition, an analytical sensitivity of 1.63 copies $p210^{BCR-ABL}$/ABL makes it possible to quantify those samples with lower levels of disease.

Moreover, the RQ-BCR-ABL p210 One-Step kit has demonstrated a high specificity at when identifying and quantifying only the M-bcr b2a2 e b3a2 ($p210^{BCR-ABL}$) translocation, as resulted by the analytical specificity tests performed during the kit validation. Such tests are carried out with the aim of detecting a possible cross-reactivity. A total of 10 ALL patients positives for $p190^{BCR-ABL}$ translocation were tested, finding no positivity result in the analysis with the RQ-BCR-ABL p210 One-Step kit.

The need to develop a kit for detection and quantification of $p190^{BCR-ABL}$ variant is related to the presence of a small percentage of patients with CML who present these transcripts[43,44], instead of the characteristic $p210^{BCR-ABL}$, and to its use in the therapeutic monitoring of acute lymphoblastic leukemia (ALL), where the presence of $p190^{BCR-ABL}$ is most characteristic. Identifying these patients as early as possible is very important as explained by Dushyant V. et al. "$p190^{BCR-ABL}$ CML is associated with an inferior outcome to therapy with TKI. These patients need to be identified as high-risk patients." [43]

RQ-BCR-ABL p190 One-Step kit has been shown to have a great high diagnostic sensitivity and specificity (100% in both cases) and to be very reproducible, with variability in results below 5%. The kit has shown a limit of detection of 1.58 copies $p190^{BCR-ABL}$/reaction, succeeding in identifying samples with a very low level of disease. In addition, the assay has been shown to be linear in a wide range, $5x10^6$-2,5 copies/reaction, providing robustness in the quantification of samples with different disease loads.

The values obtained for the performance of both devices fully meet the requirements for an *in vitro* diagnostic device. The assay was therefore suitable for commercialization and use in diagnostic practice such as CE-IVD.

As explained above in clinical practice, two measurements are performed for each test sample: an estimate of the amount of BCR-ABL transcript as a measure of leukemic load in the patient, and an estimate of the amount of control gene as a measure of the quantity and quality of cDNA contained in the sample. The number of control gene obtained for samples that are negative to translocation provides an indication of sensitivity that can excludes residual disease for that particular patient.

Studies have shown that the amplification of targets with highly differing concentrations in the same reaction may lead to a strong competition, which severely impedes amplification of the lower concentrated target and results in false-negatives. For this reason, the amplification of ABL and BCR-ABL p210 cDNA is performed in separate reactions.

Both kits have the design of standard controls for the quantification of the samples in common. A single plasmid, including both the target region (m-bcr region for RQ-BCR-ABL p190 One-Step kit and M-bcr region for RQ-BCR-ABL p210 One-Step kit) and the control gene region (ABL) was created. They were subsequently calibrated with certified material with a known concentration. For quantification standard calibration, IRMM ERM-AD623 BCR-ABL1 was used, that allows quantification of M-bcr transcripts in the sample and normalization to the expression of the housekeeping gene ABL. We also used IVS panel, which is a panel with different known concentrations of ABL.

The use of a single plasmid as a control for BCR-ABL transcript quantification is advantageous for several reasons. On one hand, it eliminates possible errors in the final data due to a possible degradation of one of the two plasmids, which would cause a sub-quantification of one of the two (BCR-ABL or ABL). With the use of a single plasmid, any plasmid degradation errors happen for both targets, minimizing the damage in the final quantification. In addition, its use speeds up and simplifies the procedure. Moreover, choosing plasmids that contain both target and control sequences are recommended by several studies[83,201] to limit the variability of the assay.

The existence of variations in the quantification of BCR-ABL transcripts according to the method used has led to standardization of results by applying the International Scale. (IS). To be able to express quantification data in IS, a conversion factor is required. This conversion factor can be obtained by testing reference material the 1st WHO International Genetic Reference Panel for quantification of BCR-ABL translocation (b3a2), which includes 4 points at different known concentrations of BCR-ABL/control gene (ABL, BCR or GUSB).

With the aim of harmonizing inter-center variations in mind, a European network has been created by laboratories and reference centers to evaluate and control the methods used in diagnostics. This organization performs periodic inspections in the various reference laboratories, in order to ensure the uniformity of all results.

In Italy these functions are carried out by LabNet network. This network links Italian haematology physicians who treat CML with laboratories where molecular tests are performed. Today, LabNet CML includes 56 laboratories, standardized and subjected to quality controls. Each of them works as a reference for one or more hematology centers.

The RQ-BCR-ABL p210 One-Step has undergone a clinical validation within this network, specifically in three different reference centers, with the aim of being validated as a certified method for CML monitoring.

The comparison between our method and the methods used in diagnostic routine in each laboratory was satisfactory. The statistical analysis used to compare the final results was the Bland-Altman method. This type of analysis allows us to juxtapose a new quantification method (RQ-BCR-ABL p210 One-Step) with another reference, in our case the average of routine methods. The graph (Figure 34) correlates the sample concentrations (expressed as the mean of the two methods) with the differences between the two methods.

Most of the values are around zero and within confidence limits, demonstrating that our method is comparable to those used in routine. No significant bias is observed since the value 0 is compressed to its 95% confidence interval.
The presence of two values outside the confidence limits (samples 5 and 6) is due to the data obtained with the routine method used in Bologna. The results obtained with Bologna's routine method for the two samples were completely different from those obtained with the other reference methods. Therefore, these two values do not depend on the data obtained with RQ-BCR-ABL p210 One-Step.

The reproducibility of methods was compared by analysing the mean of standard deviations of data obtained with the routine methods for each single sample with those obtained with RQ-BCR-ABL p210 One-Step in the three different laboratories.

The results show that the RQ-BCR-ABL p210 One-Step device produced very similar values for the same sample in different laboratories where the analyses were performed; whereas the results of routine systems of individual labs showed less homogeneous data with standard deviations more marked than those of RQ-BCR-ABL p210 One-Step device.

Quantification of the ABL transcript not only allows normalization of the BCR-ABL fusion transcript, but also permits the user to detect reaction inhibitors and to assess the quality of the RNA extract. This is a valuable tool for identifying false-negative results.

Given the importance of a correct measurement of the control gene, we have also compared the results of ABL quantification.

The average number of ABL copies obtained in three centers with the RQ-BCR-ABL p210 One-Step kit varies from 70,000 to 76,000, an average to high number that allows for low levels of disease such as $MR^{4.5}$.

The average number of ABL copies obtained with the reference method used in Napoli is $5x10^5$ copies/reaction, more than twice the average obtained with the other devices. This difference may be due to the fact that the routine method used in Napoli provides a non-calibrated ABL standard, which overestimates the number of ABL copies. This underlines how important is to use standards and controls calibrated with certified materials, in order to ensure reproducible data.
Furthermore, RQ-BCR-ABL p210 One-Step significantly improves the quantification of the ABL gene compared with the routine method used in Torino. This is a very significant result because it was the only center that uses a One-Step method similar to our own (BCR-ABL P210 ELITe MGB kit, ELITech Group).
In this type of analysis, the smaller differences are found in the comparison between RQ-BCR-ABL p210 One-Step and the routine method used in Bologna, obtaining a similar number of copies of ABL in the majority of samples.

Finally, the RQ-BCR-ABL p210 One-Step device has proved to be comparable to the other three systems currently used in the CML therapeutic care and monitoring centers. LabNet has certified that ours is an easy-to-use, reproducible and robust device, which in some cases improves ABL quantification and is able to shorten the time of the analysis procedure.

With the aim to minimise the variability of results, a conversion factor has been calculated and applied to all devices before quantifying 30 samples. Despite this, comparative data expressed in IS has detected the existence of main differences between different laboratories due to the method used.

So far guidelines have been based on cytogenetic and molecular values taken from monitoring. The guidelines suggest qRT-PCR Real Time for molecular monitoring, recommending primers and probes design, as well as the control gene to be used. There is a lack of information about sensitivity, specificity and reproducibility that a device, developed for this purpose, shall have.

Nowadays, a variability between different assays and centers, that leads to obtain different data of diseases for the same patient, in accordance to the device that has been used, is still present.

Looking at the data we can observe many differences. For example, the sample 15 has been classified by all the devices as MR^3,i.e. the achievement of MMR. In contrast Bologna's routine method obtained inferior molecular response data, reporting this patient as not reaching the MMR and to be submitted to further analyses, extending the uncertainty of the treatment success.

Other differences have been noticed in samples with deeper molecular responses such as sample 28. This sample has been detected by all the analyses as a disease's level $MR^{4.5}$. When a patient presents this molecular response for a prolonged period, it would be evaluated for a therapeutic suspension. Alternatively, the analysis made in Torino with their device has classified sample 28 as a MR^4, that could postpone this decision to a further deeper molecular analysis.

To avoid this kind of inconsistencies further efforts to align the results are needed, through standardization of design and validation of this kind of devices by guideline accepted performance.

The current challenge in the pharmaceutical area is finding the best drug for each patient before the beginning of treatment, so it can target the individual towards the best therapeutic plan to avoid relapses and obvious consequences on the patient's well-being and national welfare.

The personalized medicine in CML could have great steps forward with the developing and marketing of ground-breaking devices such as LeukoPredict. In particular, LeukoPredict measures the difference in enzyme activity of BCR-ABL in patients treated with a TKI, identifying if the patient will respond in an optimal way or not in a few hours from the beginning of treatment. In this way, it could be possible to know from the very first days of diagnosis which is the best drug for a patient, without waiting 6 months to do a therapeutic-switch towards a different and more effective TKI.

With this project the industrial process that implies the developing of an innovative method to have a prediction of therapeutic response as LeukoPredict has been analysed. In this case the term "industrialization" refers to an assessment of the assay's potential as a commercial IVD device, since it is a project that is in the early stages of development. This project, conceived and developed from by University of Verona, has man points of development from an industrial perspective.

The assessment of the Leukopredict's potential as a commercial IVD device includes many phases, first of all the Freedom to Operate analysis that provides information about the danger of copyright infringement of already existing patents. This kind of analysis is also made to identify how patents and patent applications are spread around the world, identifying in which countries it would be more convenient to start a technology or dispensing business.

In this case we divided the research in two sections. In the first part of the analysis we identified potential patents or patent applications that could compromise the technology concerning the sample's preparation, i.e. the enzyme BCR-ABL's isolation keeping its tyrosine-kinase activity. In the second part of the analysis we evaluated the potential copyright infringements in terms of the sample's analysis.

We found a total of 16 documents that could interfere with the exit the market launch of LeukoPredict. We carefully analysed each of them, finding some more controversial than others.

In the sample preparations search, the document number 1 - **CN104178424** *Red blood cell lysis solution and lysis method* seems to have the greatest resemblance to the LeukoPredict technology.

It can be considered that the technology of LeukoPredict may infringe this patent (if granted). The composition of the red blood cell lysis solution used in the protocol of the technology is similar but not identical to the one described by the independent claim 1. The composition of the RBCs lysis buffer used in the technology differs for the concentration of EDTANa2, which is lower than the range claimed in the patent application. This patent refer to red cell lysis with the purpose to eliminate the fraction of red blood cells present in blood, and not with the LeukoPredict's purpose of prevention of proteolysis necessary for protein detection. However, even if there is not a literal infringement, we consider that there is a risk of infringement by equivalence. The relevance of the risk of infringement by equivalence depends on the jurisdiction. The document refers to a Chinese patent application, in which case it would represent a limited source of risk, to the extent the application has not been extended abroad.

It is with regards to the sample analysis section that more relevant documents have been found. We singled out 10 patent documents (not only issued patents, but also published applications) that could be potentially relevant. In particular, the titles number 7, 8, 9 and 12 can be considered as the closest prior art. As far as the title 13 (WO2007109571, US granted) is concerned, the claims presented in the PCT application could have been potentially relevant. Nevertheless, this application was filed on March 17th 2006, and therefore, it has lapsed and it cannot be infringed.

Title 7 - **US8921062 -** *Diagnostic and prognostic assays based on circulating tyrosine kinase activity* it is possible that the technology infringes this patent. This is because, even though the method described by the independent Claim 1 is based on measuring the level of circulating tyrosine kinase activity in a sample of an acellular body of fluid (selected from the group consisting of plasma and serum), in the description of the patent it is written that the cTK activity can also be determined by using a cell-containing sample. Furthermore, the method includes the step of contacting a second sample with an effective amount of one or more BCR-ABL1 kinase inhibitors. This step is also included in the protocol of the LeukoPredict. Indeed, as described above, the

protocol includes the step of treating peripheral or bone marrow whole blood with TKIs. Considering this, it is possible to assert that there is a risk of infringement by equivalence.

Title 8 - **US2015184222 -** *Diagnostic and prognostic assays based on circulating tyrosine kinase activity* is correlated with Title 7. The method described in Claim 1 identifies the presence or absence of a neoplastic disease in a subject. Our device is based on screening the inhibitory potential of several Bcr-Abl-targeting drugs. Considering this, as far as the Claim 1 is concerned, the risk of infringement is low.

Another title that could hamper LeukoPredict's trade is document number 9 - **WO2012049329 -** *Method for predicting tyrosine kinase inhibitor resistance in patients suffering from chronic myelogenous leukemia*. We consider that our device may infringe this patent (if granted), particularly as far as the independent claim 10 is concerned. Indeed, this claim (which is written to have a broad scope) describes a method for diagnosing CML by measuring the kinase activity and providing phosphorylation profiles. However, the document refers to a patent application and, consequently, at the moment it only represents a limited source of risk.

As far as the independent claims 1 and 14 are concerned, it is important to point out that the method is based on a phosphorylation profile comprising the phosphorylation levels of phosphorylation sites present in at least 10 peptide markers that are listed in the table. Instead, LeukoPredict is based on only one kinase substrate, which is the ABL-biosensor peptide (Seq: CEAIYAAPFAKKK).

The last title that is relevant to our technology is number 12 - **WO2010116000 -** Method for profiling drug compounds using protein kinase inhibitors).

As far as the patent EP2417452 (B1) is concerned, the four independent claims describe methods for determining the effect of a drug on the kinase activity in a sample (claims 1 and 2) and for predicting the resistance of a patient to a drug (claims 3 and 4). All the methods comprise the step of measuring the kinase activity in a cell lysate sample in the presence and in the absence of a protein kinase inhibitor and the step of determining the differential phosphorylation profile. In particular, said protein kinase inhibitor:

- Inhibits the kinase domain of a surface receptor targeted by a drug thereby mimicking the effect of said drug on the kinase activity in said cell lysate sample (claim 1);
- Inhibits proteins associated with the signal transduction pathway of a surface receptor targeted by a drug thereby mimicking the effect of said drug on the drug targeted signal transduction pathway in said cell lysate sample (claim 2);
- Acts towards proteins of one or more alternative signal transduction pathways causing tumor growth, thereby reporting on the activity of alternative signal transduction pathways

causing tumor growth which is not affected by the drug, thereby causing resistance (claim 3);

- Inhibits proteins of a signal transduction pathway not targeted by the drug, thereby reporting on the activity of a signal transduction pathway which is not affected by said drug, thereby reporting resistance (claim 4).

As far as the patent application US2012021939 (derived from the patent application WO2010116000) is concerned, the four independent claims describe methods for determining the effect of a drug on the kinase activity in a sample (claims 1 and 2) and for predicting the resistance of a patient to a drug (claims 3 and 4). All the methods comprise the step of measuring the kinase activity in a cell lysate sample in the presence and in the absence of a protein kinase inhibitor and the step of determining the differential phosphorylation profile. In particular, said protein kinase inhibitor:

- Mimics the effect of a drug on the kinase activity in the sample (claim 1);
- Mimics the effect of a drug on the drug targeted signal transduction pathway in the sample (claim 2);
- Reports on the activity of alternative signal transduction pathways causing tumor growth, which are not affected by the drug, thereby causing resistance (claim 3).
- Reports on the activity of a signal transduction pathway which is not affected by the drug, thereby reporting resistance (claim 4).

The chimeric BCR-ABL oncoprotein formed from the aberrant gene is a constitutively active tyrosine kinase involved in several signal transduction pathways. Considering this, it is possible to assert that there is an actual risk of interference between our technology and these patents.

At a first level of analysis, it can be concluded that LeukoPredict presents a risk profile that is consistent with the plan to enter the market upon completion of its development and, as of the date of this analysis, risk are medium-to-moderate. In particular, a set of patent documents for each of the two aspects that characterize LeukoPredict, that might pose risks of infringement and that, at this stage, require increasing attention have been single out. Importantly, the identified documents are not necessarily yet patents. The majority refers to patent applications, which represent a limited source of risk. Moreover, some of these applications do not feature positive search reports and will be likely limited in their scope. Lastly, their geographic coverage is not yet defined and the correlated risk has to be assessed in relation to the expected market presence of the technology.

For prudential reasons, a second level FTO will be done once LeukoPredict approaches the market and its protocol becomes definite, particularly as far as the integration of the two different aspects of the method is concerned. Moreover, once the preparation of the sample and the analysis of this are further developed, we will protect any such characteristic of its technical

solution that appears as distinctive and inventive and that has not been yet disclosed or made available to the public with patent applications. Fence patent applications would reinforce this device's position on the market and allow for easier defence against potential third parties' threats or claims.

Another fundamental part of the assessment of the LeukoPredict's potential as a commercial IVD device is the pharmacoeconomics study of the economic impact of this treatment on society. These kinds of study are requested more and more by governments to evaluate the strength and efficacy of a new device or health strategy in order to have all the elements to therefore include it in a new standard care.

In this case we followed a decision-analytic model for the early LeukoPredict test in CML, integrating that in the cure for disease as a companion diagnostic.

In particular we compared three differents strategies. In the first analysis (base-case) patients receive first-line treatment in line with real-world settings (33.33% per TKI, including Imatinib, Dasatinib and Nilotinib). In the base-case analysis, LeukoPredict led to less cost and generated additional life years and quality-adjusted life year during eight years and dominated standard of care arm. Expected differences in QALYs and LYs gained are relatively small, but should be expected of being considerably larger at lifetime horizon.

On the other hand, in the scenario analysis 1, patients receive first-line treatment with Imatinib (100%) Imatinib. LeukoPredict to the standard treatment, in this case, is not any more cost-saving, but still is cost-effective bellow willingness to pay the threshold of £30,000/QALY.
Lastly, in the scenario analysis 2, patients receive first-line treatment with Imatinib (100% Imatinib) but the cost of Imatinib decreases due to the generic entry that was modeled, with the prioritizing of patients to receive Imatinib in the first-line treatment due to the lower cost. In this case, adding LeukoPredict to the standard treatment lead to the even more favorable cost-effectiveness ratio of 7371 £/QALY.

Results demonstrated that LeukoPredict is either cost saving in the base-case analysis or very cost-effective, in the scenario analysis 1 – 15865 £/QALY, and in the scenario analysis 2 – 7371 £/QALY under lower bound of the willingness to pay threshold of £30,000/QALY from the perspective of UK NHS over the eight years' time horizon.

In base-case analysis as well as in both scenario analysis LeukoPredict generated additional life years and quality-adjusted life year in comparison with the standard treatment.

In conclusion, this project was able to address actual principal issues in CML. On one side hand we have developed and validated two devices that completely satisfy actual resquest of therapeutic monitoring in the pharmaceutical market making a complete panel to track CML.

Currently the external validation of RQ-BCR-ABL p210 One-Step made by references centers brought to light the need to have clinical data standardized, at least in Italy, making guidelines for technical characteristics of devices to obtain standardized clinical data. The development of devices such LeukoPredict helps to decrease the risk of disease's progression to more aggressive phases, personalizing the therapy and obtaining the maximum effectiveness of therapeutical choices. This helps physicians in an evidence based decisional therapeutic process, avoiding potential conflicts of interest and providing a rationale to considering to other kinds of treatment when the risk of failure is too high. Finally it has been shown that this technology can be affordable and that could contain the cost of healthcare.

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