

# **Molecular Monitoring of Chronic Myeloid Leukemia Treated with Imatinib Mesylate**

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君子之道，辟如行远，必自迩；辟如登高，必自卑。

——孔子 《中庸》

*To travel far away begins with one footstep; to climb to the top of the mountain starts from the bottom.*

----- by Confucius

*To my parents and Meng*

## MOLECULAR MONITORING OF CHRONIC MYELOID LEUKEMIA TREATED WITH IMATINIB MESYLATE

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**Abstract:** The *BCR-ABL* fusion gene product is a constitutively activated tyrosine kinase, which is fundamental in the pathogenesis of chronic myeloid leukemia (CML). Imatinib mesylate (imatinib, Glivec® or Gleevec®), a small molecule inhibitor of the BCR-ABL tyrosine kinase, is now the first-line treatment for all newly diagnosed chronic phase CML patients. Imatinib treatment results in a high frequency of complete cytogenetic response (CCgR). Patients in CCgR can be further stratified by the degree of minimal residual disease, measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The present thesis deals with different aspects on molecular monitoring of imatinib treated CML patients. By serially analyzing peripheral blood and bone marrow *BCR-ABL* transcript levels using qRT-PCR in CML patients commencing imatinib therapy, we found that the major decline in *BCR-ABL* transcripts occurred within 6 months after start of imatinib treatment. An apparent plateau in *BCR-ABL* transcript level seems to have been reached after 12-15 months of imatinib treatment, which indicates a stable number of remaining *BCR-ABL* positive cells. To search for markers associated with molecular response in CML patients treated with imatinib, we studied the mRNA expression of apoptosis-related genes in peripheral blood nucleated cells from chronic phase CML patients commencing imatinib treatment. We found that a lower *BAD* expression at diagnosis correlates with a better molecular response at 12 months of imatinib therapy. Studies of *BCR-ABL* kinase domain mutations in imatinib treated CML patients revealed that point mutations were mainly associated with acquired resistance, but not with cytogenetic or molecular disease persistence in CML patients without signs of increasing leukemia burden. Finally we studied “off-target” effects of imatinib on peripheral blood on T-lymphocytes. We found that therapeutic doses of imatinib alter the expression of apoptosis related genes in CD3+ lymphocytes and change the phenotype of CD4+CD28+ T-helper cells.

**Key words:** CML, imatinib, *BCR-ABL*, apoptosis, mutation, T-lymphocyte

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## LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

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- II. Wei Y, Stockelberg D, Hullberg S, Ricksten A, Wadenvik H. Changes in expression of apoptosis-related genes are linked to the molecular response to imatinib treatment in chronic-phase chronic myeloid leukemia patients. *Acta Haematol.* 2007;117(2):83-90.
- III. Wei Y, Hardling M, Olsson B, Hezaveh R, Ricksten A, Stockelberg D, Wadenvik H. Not all imatinib resistance in CML are BCR-ABL kinase domain mutations. *Ann Hematol.* 2006 Dec;85(12):841-7.
- IV. Wei Y, Olsson B, Jönsson S, Hezaveh R, Stockelberg D, Jacobsson S, Ricksten A, Wadenvik H. Therapeutic doses of imatinib are associated with changes in T-cell phenotype and expression of apoptosis-related genes. *Manuscript*

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## ABBREVIATIONS

ABL	Abelson murine leukemia viral oncogene
ACAD	Activated T-cell autonomous death
AICD	Activation-induced cell death
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP	Accelerated phase
ARG	Abelson-related gene
BAD	BCL-2-antagonist of cell death
BAX	BCL-2-associated X protein
BC	Blast crisis
BCL-2	B-cell CLL/lymphoma 2
BCL-X <sub>L</sub>	BCL-X long isoform
BCL-X <sub>S</sub>	BCL-X short isoform
BCR	Breakpoint cluster region
CBL	Casitas B-lineage lymphoma pro-oncogene
CCgR	Complete cytogenetic response
CHR	Complete hematologic response
CML	Chronic myeloid leukemia
CP	Chronic phase
Ct	Cycle threshold
GRB2	Growth factor receptor-bound protein 2
D-HPLC	Denaturing-high performance liquid chromatography
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FISH	Fluorescence <i>in situ</i> hybridization
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPK1	Hematopoietic progenitor kinase 1
HPK1-C	HPK1 C-terminal cleavage fragment
JAK	Janus family of tyrosine kinases
JUK	JUN kinase
LCK	Lymphocyte cell-specific protein-tyrosine kinase
LDA	Low density array
MAPK	Mitogen-activated protein kinase
MCgR	Major cytogenetic response
MFI	Median fluorescence intensity
MoAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
NK	Natural killer
PDGFR	Platelet derived growth factor receptor
Ph	Philadelphia
PI-3K	Phosphatidylinositol-3 kinase
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
SHC	SRC homology 2-containing protein
STAT5	Signal transducer and activator of transcription 5
TCR	T-cell receptor

## INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal disorder of hematopoietic stem cells [1]. The disease arises as a consequence of a rare gene abnormality. The course of the disease is characteristically triphasic: a chronic phase (CP) lasting three to six years is followed by transformation to an accelerated phase (AP) and then a terminal blast phase of short duration [2, 3].

Initially described in 1845 [4, 5], CML is one of the best understood diseases from the aspect of its cytogenetic abnormalities and the molecular mechanisms involved. CML was the first human disease in which a specific abnormality of the karyotype, the Philadelphia (Ph) chromosome, could be linked to a malignant disease [6]. Later on, it was shown that the Ph chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22, which produces the *BCR-ABL* fusion oncogene [7]. The BCR-ABL oncoprotein, a constitutively activated tyrosine kinase, recruits and activates several pathways transducing intracellular signals, which ultimately lead to abnormal cellular adhesion, enhanced proliferation and inhibition of apoptosis [8, 9].

The treatment of CML has long been in the frontier of cancer therapy. It was among the first neoplastic diseases in which therapy with a biologic agent, interferon alpha (INF- $\alpha$ ), was found to suppress the leukemic clone and prolong survival [10]. CML was also among the first neoplastic diseases treated with a specific molecular target agent, imatinib mesylate (STI571, imatinib, Glivec® or Gleevec®, Novartis, Basel, Switzerland). The remarkable therapeutic efficacy of imatinib led treatment of CML into a new era.

### **Clinical characteristics of CML**

CML has an incidence of 1 case per 100,000 people per year and accounts for 15 percent of leukemias in adults [11]. The median age of patients at presentation is 45 to 55 years. Up to one third of the patients are over 60 years old, which is an important consideration for therapeutic strategies such as stem cell transplantation and INF- $\alpha$  treatment. In



general, the cause of CML is unclear but high doses of ionizing radiation may be an etiologic factor [12].

Most cases (85 percent) of CML are diagnosed in CP. The typical symptoms at presentation are fatigue, anorexia, and weight loss. The most common abnormality on physical examination is splenomegaly, which is present in up to half of the patients. About 40 percent of patients are asymptomatic, and in these patients, the diagnosis is suspected because of accidental detection of abnormal blood counts [3]. The main laboratory findings are peripheral blood neutrophilia with a left shift of the differential count, and basophilia. Bone marrow examination shows myeloid predominance, left shift and megakaryocytic abnormalities.

Without curative intervention, CP CML will invariably transform through an AP, often heralded by the appearance of increased number of immature myeloid cells in the bone marrow and peripheral blood, as well as new cytogenetic changes in addition to the Ph chromosome. AP often manifests itself by unexplained fever, bone pain, weight loss and a general loss of well-being. However, the laboratory definition of the AP is vague [2]. After a short period of 3-18 months, progression then proceeds to blast crisis (BC), which is defined by the presence of 30 percent or more blast cells in peripheral blood or bone marrow, or the presence of extramedullary infiltrates of blast cells [13, 14]. In two thirds of cases, the blasts belong to the myeloid lineage, with a phenotype similar to that of acute myeloblastic leukemia. The remaining one third of cases has blasts with a phenotype which is similar to acute lymphoblastic leukemia (ALL). In addition, few cases are biphenotypic, or have megakaryoblastic or erythroblastic phenotype [15]. BC is highly resistant to treatment, with death generally occurring from infection and bleeding complications. The median survival of patients in BC receiving chemotherapy, is 4-6 months for myeloid blast transformation and 12 months for lymphoid blast transformation [2].

## Pathology of CML

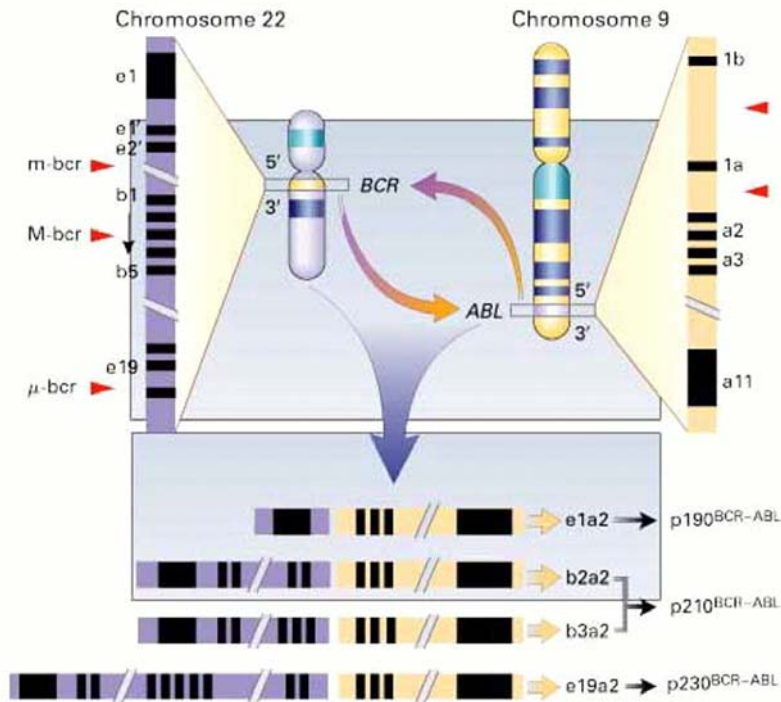
### *The Ph chromosome*

In 1960, a major clue to the pathogenesis of CML was provided by Nowell & Hungerford's landmark discovery of the Ph chromosome and its association with the disease [6]. Using quinacrine fluorescence and Giemsa banding, Rowley and colleagues showed that the Ph chromosome resulted from a reciprocal translocation between the long arms of chromosomes 9 and 22; t(9;22)(q34;q11) [7]. This is the hallmark of CML and is found in more than 95 percent of CML patients [16]. It is also found in 5 percent of children and in 15 to 30 percent of adults with ALL, and in 2 percent of patients with newly diagnosed acute myeloblastic leukemia [17, 18].

The t(9;22)(q34;q11) adds a 3' segment of the *ABL* gene from chromosome 9q34 to the 5' part of the *BCR* gene on chromosome 22q11, creating a hybrid *BCR-ABL* gene that is transcribed into a chimeric *BCR-ABL* messenger RNA (mRNA) (Figure 1). The *ABL* gene contains 11 exons among which the first exon has two variants: 1a and 1b [17]. The *ABL* gene encodes a ubiquitously expressed, non-receptor tyrosine kinase with a molecular mass of 145 kD (p145<sup>ABL</sup>). The isoforms of ABL, i.e. 1a and 1b, derive from alternative splicing of the first exon. The breakpoint in the *ABL* gene may occur within a region longer than 300 kilobases (kb), but usually before exon 2. The *ABL* exons 2 to 11 (also called a2 to a11) are juxtaposed to the 5' part of *BCR*. The major breakpoint cluster region (M-bcr) of the *BCR* gene on chromosome 22 is located between exon 12 and 16 (referred to as b1 to b5) and extends over 5.8 kb [19]. Two fusion transcripts, e13a2 and e14a2 (b2a2 and b3a2, respectively), are created, and both translate into a chimeric protein of 210 kD named p210<sup>BCR-ABL</sup> [17] (Figure1). In 95% of *BCR-ABL* positive CML, the leukemic cells have either b2a2 or b3a2 transcripts, but in 5 percent of cases, alternative splicing events cause the expression of both fusion products [20]. The clinical features, response to treatment and prognosis are similar in patients with b2a2 and b3a2 transcripts, except for a higher platelet count in patients with b3a2 transcripts [16].

The breakpoint in the minor breakpoint cluster region (m-bcr) results in a fusion transcript named e1a2, which gives rise to a 190 kD protein, p190<sup>BCR-ABL</sup> [21, 22] (Figure

1). P190<sup>BCR-ABL</sup> is rare in CML and is mainly seen in adults and children with Ph-positive ALL [23] (Figure 1). Breakpoints in the micro breakpoint cluster region ( $\mu$ -bcr) create another fusion transcript (e19a2) which is translated into a 230 kD protein, p230<sup>BCR-ABL</sup>. P230<sup>BCR-ABL</sup> has been identified to associate with neutropenic CML [24] as well as some rare cases of CML [25].



**Figure 1. The translocation of t(9;22)(q34;q11) in CML.**

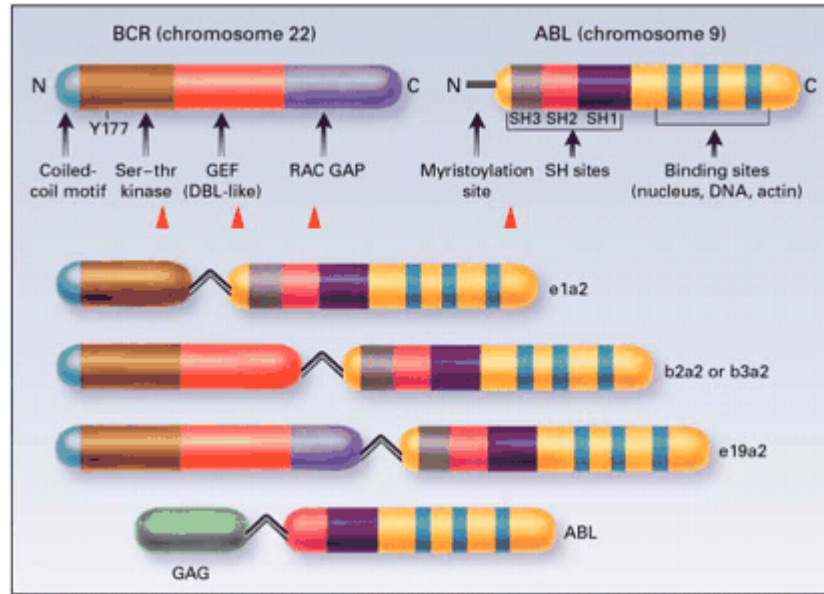
The Ph chromosome is a shortened chromosome 22 that results from the translocation of 3' (toward the telomere) ABL segments on chromosome 9 to 5' BCR segments on chromosome 22. In most cases breakpoints (arrowheads) in the ABL gene are located in the 5' end (toward the centromere) of exon a2. Various breakpoint locations have been identified along the BCR gene on chromosome 22. Depending on which breakpoints are involved, differently sized segments from BCR are fused with the 3' sequences of the ABL gene. This results in fusion messenger RNA molecules (e1a2, b2a2, b3a2, and e19a2) of different lengths that are translated into different chimeric protein products (p190, p210 and p230) with variable molecular weights. m-bcr: minor breakpoint cluster region, M-bcr: major breakpoint cluster region, and  $\mu$ -bcr: micro breakpoint cluster region. (reproduced and modified from [2])

### **The BCR-ABL protein**

The leukemogenic potential of p210<sup>BCR-ABL</sup> resides in the fact that the normally regulated tyrosine kinase activity of the ABL protein is constitutively activated in the fusion

oncoprotein. ABL proteins are non-receptor tyrosine kinases that have important roles in signal transduction and the regulation of cell growth [26]. There are two isoforms of ABL, isoform 1a and isoform 1b. Isoform 1b, which is expressed at higher levels in early hematopoietic progenitor cells, is myristoylated on its second glycine residue at the N-terminal [27]. Loss of myristoylation in ABL dramatically enhances its tyrosine kinase activity [28]. Downstream to the myristoylation site, at the N-terminal segment of ABL, there are three SRC homology domains (SH3, SH2 and SH1). SH2 and SH3 regulate the tyrosine kinase function of ABL and SH1 harbors the tyrosine kinase activity of ABL. SH3 has a negative regulatory effect on the tyrosine kinase function. Deletion of SH3 or mutation in SH3 facilitates tyrosine kinase activity of ABL [27, 29, 30]. Defects in the functional integrity of SH2 decrease phosphotyrosine binding and reduce the transforming capacities of ABL [31]. The C-terminal part of ABL contains a DNA-binding domain, nuclear localization signals, and a binding site for actin [32] (Figure 2). The disruption of ABL protein by genetic fusion is responsible for the up-regulated tyrosine kinase activity.

The uncontrolled tyrosine kinase activity of BCR-ABL is also caused by the juxtaposition of alien BCR sequences. The N-terminal coiled-coil motif of BCR promotes dimerization and increases BCR-ABL tyrosine kinase activity and enables binding of F-actin to ABL [33]. The serine-threonine kinase domain of BCR activates signaling pathways mediated by BCR-ABL tyrosine kinase [34]. BCR which also contains SH2 binding sites fusion to ABL adds a large amino acid sequence to the SH2 segment of ABL [35] (Figure 2). BCR interferes with the adjacent SH3 and SH2 kinase regulatory domain, which in turn causes ABL to become constitutively active as a tyrosine phosphokinase.



**Figure 2. Functional domains in  $p160^{BCR}$ ,  $p145^{ABL}$  and  $p210^{BCR-ABL}$ .**

Important functional domains of the BCR and ABL gene products as well as different fusion-protein products are shown ( $p190^{BCR-ABL}$ ,  $p210^{BCR-ABL}$  and  $p230^{BCR-ABL}$ ). Breakpoints are indicated by arrowheads. N: N-terminal amino acid sequence, C: C-terminal amino acid sequence, Ser-thr: serine-threonine, GDP: guanosine diphosphate, GTP: guanosine triphosphate, GEF: GDP-GTP exchange factor, DBL: diffuse B-cell lymphoma oncogene, RAC: a RAS-like GTPase, GAP: guanosine triphosphatase-activating function protein, and SH: SRC homology domain. (Reproduced and modified from [2])

### **Signaling pathways of BCR-ABL**

The structure of  $p210^{BCR-ABL}$  allows multiple protein-protein interactions which involves diverse intracellular signaling pathways. Several domains in BCR-ABL serve to bind adapter proteins such as growth factor receptor-bound protein 2 (GRB2), CRK-like protein (CRKL), casitas B-lineage lymphoma pro-oncogene protein (CBL), and SRC homology 2-containing protein (SHC) [36]. The SH2 domain of GRB2 binds to a conserved tyrosine residue (Y177) of BCR in  $p210^{BCR-ABL}$ , which links  $p210^{BCR-ABL}$  to RAS, a guanosine triphosphate (GTP) –binding protein involved in the regulation of cell proliferation and differentiation, and located at the core of the most prominent signaling pathway in the pathogenesis of CML [37] (Figure 3). Signaling events downstream of RAS are not well characterized and may involve mitogen-activated protein kinases



Signal transducer and activator of transcription 5 (STAT5) of the JAK/STAT5 pathway can be phosphorylated directly by BCR-ABL, independent of the RAS signaling pathway [41, 42], which leads to the up-regulation of the anti-apoptotic protein BCL-X<sub>L</sub> [43, 44]. Mediated by CRKL and CRK, phosphorylation of the phosphatidylinositol-3 kinase (PI-3K) pathway is activated by BCR-ABL, which results in enhanced pro-mitogenic and anti-apoptotic signals [45]. C-Myc has also been identified to be involved in the BCR-ABL signaling pathway [46].

Although the different signaling pathways of BCR-ABL have been intensively studied, none has been identified to explain all phenotypic features described in CML. It has to be remembered that most interactions and activation processes have been studied only in cell lines *in vitro* and under conditions of forced overexpression. Therefore, their existence in primary leukemic cells and their contribution to the CML phenotype *in vivo* remain uncertain. However, as an end result, the uncontrolled kinase activity of BCR-ABL gives rise to deregulated cell proliferation, decreased adherence of leukemic cells to the bone marrow stroma, and inhibition of apoptosis.

## **Diagnosis and monitoring of CML**

### ***Morphologic findings***

The typical laboratory findings in CP CML are leukocytosis with a remarkable left shift of the differential count, basophilia and eosinophilia. Platelet count may be either high or low, and mild anemia is commonly observed. The leukocyte alkaline phosphatase activity is reduced, although phagocytic function remains essentially normal [47]. Blasts can be present in peripheral blood. Eosinophils, mature or immature, are also present, but their count may not be significantly increased. The peripheral blood findings change with disease progression. Quantifying the proportion of basophils, circulating blasts and platelets in peripheral blood is important because they can serve as prognostic predictors [48-50].

The bone marrow of patients with CML is notoriously hypercellular and devoid of fat. All stages of myeloid maturation are present, with predominance of myelocytes [51, 52].

In CP, the sum of myeloblasts and promyelocytes usually accounts for less than 15% of the marrow cellularity. Megakaryocytes may be increased, and Gaucher-like cells can be observed in 10% of cases [53-55]. Bone marrow basophilia and eosinophilia are frequently encountered. Blasts are scant, with morphologic characteristics indistinct from those of normal myeloblasts. Varying degree of fibrosis in bone marrow may be present [56]. As the disease progresses, the proportion of blasts increases, morphologic alterations of the myeloid cells can become noticeable, and the fibrosis of bone marrow may increase [57].

### ***Cytogenetic analysis***

As the *BCR-ABL* fusion is the hallmark for CML, a conclusive diagnosis of CML relies on cytogenetic and/or molecular testing to identify this specific genetic abnormality. The G-banding karyotyping is utilized for cytogenetic analysis and usually 25-30 metaphase cells are examined. Cytogenetic analysis detects the Ph chromosome in approximately 95% of patients with CML at the time of diagnosis. The rest CML cases carry “masked” translocations that can be detected only by molecular techniques, such as fluorescence *in situ* hybridization (FISH) or reverse transcriptase-polymerase chain reaction (RT-PCR) for the *BCR-ABL* fusion [58, 59]. Occasionally, additional chromosome abnormalities occur in CML, e.g. trisomy 8, i(17q), an extra Ph chromosome and trisomy 19. Less common chromosomal abnormalities found in CML include -7, -17, +17, +21, -Y and t(3;21)(q26.2;q22) [60].

Because of the limited number of cells being examined, the sensitivity of cytogenetics in detection of residual disease post treatment is only 1 leukemic cell in 25-30 normal cells. In other words, the sensitivity of detection is 3-4% Ph-positive cells. However, its ability to quantify makes cytogenetics a useful test for monitoring therapeutic responses. Nevertheless, the accuracy of quantification is poor, especially when Ph-positive cells constitute less than 10% of the total. Moreover, cytogenetics requires dividing cells for analysis of metaphase chromosomes, and the best specimen is bone marrow, which contains more proliferating cells than blood does.



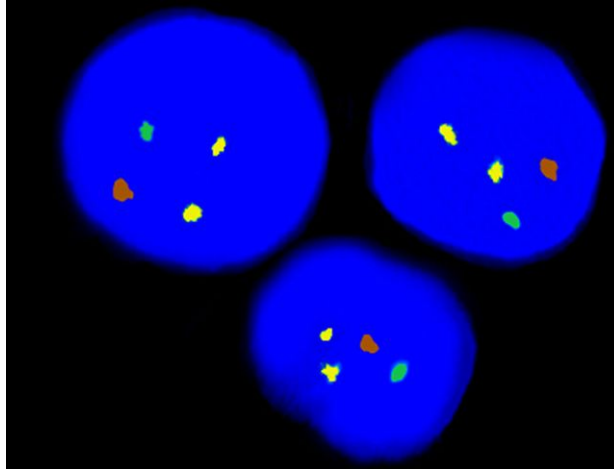
In spite of its low sensitivity for detecting minimal residual disease, cytogenetics is currently a standard tool for monitoring CML patients. Also, cytogenetics can reveal karyotypic abnormalities in addition to the Ph chromosome that can arise during disease progression.

### ***Fluorescence in situ hybridization (FISH)***

FISH analysis is typically performed by co-hybridization of a *BCR* and an *ABL* probe to denatured metaphase chromosomes or interphase nuclei. Traditional FISH (also known as S-FISH or dual-FISH) is a two-colour technique in which a 5' *BCR* fluorescent probe as well as a second 3' *ABL* fluorescent probe are utilized with contrasting colours to detect the position of the respective genes [61]. The random superimposition of fluorescent probes in normal interphase nuclei can lead to a false-positive result. The frequency of false positivity can be as high as 3-10%, making quantification below 10% unreliable [62-64].

Triple probe FISH (or three-colour FISH) increases the sensitivity of the two-probe S-FISH technique by introducing a third probe that spans the breakpoints in either *BCR* or *ABL*. Each probe is labelled with a separate, distinct fluorochrome. In the Ph-positive cells, in addition to the *BCR/ABL* fusion signal, the signal from the third probe is lost. This two-step verification process allows for an increased sensitivity in the detection of Ph-positive cells, with a false-positive rate of 0.065-0.27% [65, 66].

Double FISH (or D-FISH) utilizes four probes. Except those two used in S-FISH, that bind to the 3' *BCR* and the 5' *ABL*, respectively. The additional two probes span the breakpoints of both chromosomes 9 and 22. In the presence of the *BCR-ABL* translocation, D-FISH yields a double fusion signal because the four probes bind to their respective *BCR-ABL* and *ABL-BCR* loci (Figure 4). This further reduces the frequency of both false-positive and false-negative results compared to S-FISH [62, 67].

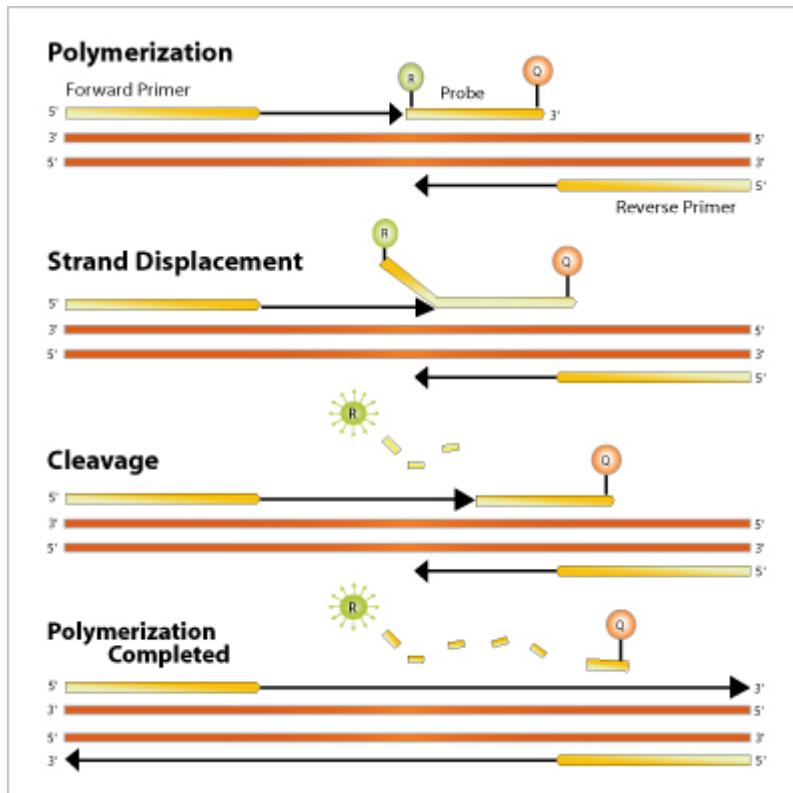


***Figure 4. D-FISH for detection of BCR-ABL fusion in interphase cells.***

FISH detects *BCR-ABL* in about 95% of CML cases and it detects the approximately 5% of cases with “masked” translocations that are missed by conventional cytogenetics [68, 69]. It also detects rare cases with variant breakpoints falling outside the regions covered by PCR primers [70-72]. In addition, a FISH study usually analyzes 200 to 500 nuclei. Thus, quantification generated by FISH might be more accurate than conventional cytogenetics. FISH can also be performed on interphase cells from both peripheral blood and bone marrow. Therefore, FISH is another tool that can be routinely used for quantification of residual leukemic cells [73-75].

#### ***Quantitative RT-PCR***

A method with high sensitivity is required to monitor patients treated with regimens inducing a marked reduction in tumor burden. PCR techniques, such as RT-PCR, multiplex PCR and nested-PCR, targeting the *BCR-ABL* oncogene or onco-mRNA have been shown to detect the CML disease with high sensitivity [76]. Over the past decade, quantitative RT-PCR (qRT-PCR) assays have been established to measure *BCR-ABL* transcript levels in peripheral blood and bone marrow, which enables monitoring the dynamics of residual disease over time [77-81].



**Figure 5. Real-time PCR with TaqMan® technique.**

The probe consists of two types of fluorophores and is complementary to the template. Before the polymerase acts, the quencher (Q) fluorophore (usually a long wavelength colored dye, such as red) reduces the fluorescence from the reporter (R) fluorophore (usually a short wavelength colored dye, such as green). This procedure that one dye is inhibited by another without emission of a proton terms fluorescence resonance energy transfer (FRET). After denaturation, the primers and the probe anneal to the single strand template. During the elongation, Taq polymerase reaches the probe and its exonuclease activity separates the quencher from the reporter, which allows the reporter to emit its light that can be quantified.

The most common qRT-PCR technique for monitoring CML makes use of sequence-specific probes, e.g. TaqMan® single exonuclease hydrolysis probe. The probe, with sequence complementary to *BCR-ABL* cDNA, is dual-labeled. One fluorophore serves as reporter and its emission spectra is inhibited by the second fluorophore (quencher). Specifically binding to the *BCR-ABL* template, the probe is cleaved by the exonuclease activity of *Taq* polymerase during PCR reaction, which separates the reporter and the quencher, and results in a detectable fluorescent emission (Figure 5). During the ongoing

PCR reaction, the fluorescence intensity increases parallel with the amount of the PCR product and can be detected in real time [81].

The transcript level reflects the number of leukemic cells in the blood and marrow, and can be used as a reliable measure of the response to therapies. The clinical usefulness of *BCR-ABL* quantitation by qRT-PCR has been demonstrated in several studies. Early reduction of *BCR-ABL* transcript levels predicts cytogenetic response in CP CML patients treated with imatinib and the degree of reduction of *BCR-ABL* correlates with prognosis [82-84].

## **Treatment of CML**

### ***Conventional treatment before the imatinib era***

Standard treatment options for CML patients in CP, before the imatinib era were cytoreductive chemotherapies, IFN- $\alpha$  and allogeneic stem cell transplantation. Chemotherapies, as hydroxyurea and busulfan, can effectively reduce the tumor burden. However, cytogenetic response is rare, and these drugs can hardly modify the natural history of CML [85].

IFN- $\alpha$  is a member of glycoprotein family, which has antiviral and antiproliferative properties. IFN- $\alpha$  was first shown to be an active agent in CML in the early 1980s, and it became the non-transplant treatment of choice for CP CML patients [86]. IFN- $\alpha$  has been shown to increase survival when administered in CP. While hematologic responses are seen in the majority (80%) of patients, cytogenetic responses are seen in only 30-50% of patients, with complete cytogenetic responses (CCgR) in only 10-20% of IFN-treated patients [87-89]. Unfortunately, many patients tolerate IFN- $\alpha$  poorly, necessitating dose reduction or discontinuation of treatment.

Currently, the only curative approach for CML is allogeneic stem cell transplantation. The outcome of this procedure depends on a series of risk factors, the most important of which are the patient's age and the phase of the disease [57]. For young (age <40 years) CP patients undergoing HLA-matched transplants within 1 years from diagnosis, long-

term survival rates are reported to be 70-80% [90]. With advances in molecular HLA-typing, improvements in infection control and graft versus host disease prophylaxis, outcomes for related and unrelated donor transplants appear similar [91]. Unfortunately, up to one third of CML patients are over the age of 60, for whom the allogeneic stem cell transplantation usually is not feasible, because of the high risk of treatment-related mortality. Donor availability is another obstacle. Therefore, for many patients with CML, stem cell transplantation is not an option.

### ***Imatinib and its advantages***

As the BCR-ABL tyrosine kinase plays a key roll in CML pathogenesis, attempts to target the BCR-ABL tyrosine kinase evolved as new therapeutic strategies. The antecessor of imatinib was initially developed, by scientists at Ciba-Geigy (currently Novartis, Basel, Switzerland), as a specific platelet-derived growth factor receptor (PDGFR) inhibitor. It was also found to be a potent ABL tyrosine kinase inhibitor [92]. Further optimized for v-ABL tyrosine kinase inhibition, imatinib mesylate was generated [93, 94]. Imatinib selectively inhibits ABL tyrosine kinase, including BCR-ABL [94, 95]. Further studies revealed that a limited number of other tyrosine kinases are also targeted by imatinib, including PDGFR [96], c-KIT [97] and ARG [98]. Preclinical studies showed that imatinib selectively inhibits the proliferation of cell lines holding p210<sup>BCR-ABL</sup> and the clonal growth of myeloid cells from CML patients [99, 100]. It was also shown in mice models that imatinib had *in vivo* activity against *BCR-ABL* positive cells and that continuous exposure to imatinib was necessary to eradicate the tumors, suggesting this would be important for an optimal antileukemic effect [99, 101]. Prior to clinical testing, imatinib was shown to have an acceptable toxicology profile in animal models.

A phase I clinical trial with imatinib started in June 1998, in CP CML patients refractory or resistant to IFN-based therapies. Targeting a dose of 300 mg or greater, 53 of 54 patients on treatment for at least 4 weeks had complete hematologic response (CHR); cytogenetic responses were seen within 5 months in 29 patients including 17 with major cytogenetic response (MCgR; <35% Ph-positive metaphases) and 7 with CCgR (no Ph-

positive metaphases). Side-effects, such as nausea, myalgias and edema, were manageable. Thrombocytopenia and neutropenia occurred in 16% and 14% of the patients treated, respectively. Pharmacokinetic studies showed that the half-life of imatinib was 13-16 hours, which is sufficiently long to permit one daily dosing [102]. Although the follow-up on this group of patients was relatively short, these data indicated that an ABL specific tyrosine kinase inhibitor had significant activity in CML, even in IFN refractory patients. This trial also demonstrated the essential role of BCR-ABL tyrosine kinase activity in CML and provided an example of successful drug development based on a specific molecular abnormality present in a human malignancy.

Based on these extremely promising results, the phase I study was expanded to patients with myeloid and lymphoid blast crisis of CML and patients with relapsed or refractory Ph-positive ALL [103]. Although a satisfying frequency of hematologic responses was seen, most patients relapsed within weeks to months. Thus, imatinib had remarkable single agent activity in CML BC and Ph-positive ALL, but responses were not durable. However, these studies demonstrated that in the majority of cases, the leukemic clone in *BCR-ABL* positive acute leukemias, including CML blast crisis, remains at least partially dependent on BCR-ABL kinase activity for survival.

The success of imatinib treatment in CP CML patients in phase I studies led to large-scale phase II and phase III studies. Phase II studies began in late 1999 using imatinib as a single agent for all stages of CML. For patients in BC and with Ph-positive ALL, these studies confirmed the results of the phase I trials [104, 105]. Patients in CP who had failed IFN- $\alpha$  therapy did much better than expected: 95% of those patients treated with 400 mg imatinib daily achieved a CHR and 60% achieved a MCgR [106]. As expected, the efficacy in patients with AP was intermediate between CP and BC [107].

Imatinib and the combination of IFN- $\alpha$  plus cytarabine were compared in the international randomised study of interferon versus STI-571 (IRIS), which rapidly showed that imatinib was superior compared to IFN- $\alpha$  [108]. The five-year follow-up data of the IRIS trial showed that, at 60 months of imatinib treatment, 98% have achieved

CHR and 87% CCgR. The event-free survival was 83%, and the overall survival is 89% at five years [109]. These good responses could be further stratified by qRT-PCR and those with the best molecular response, i.e. at least a 3 log reduction in the *BCR-ABL* transcript number at 12 months, had negligible risk of disease progression [110].

### ***Some concerns with imatinib remain***

#### *(i) Molecular persistence*

Although approximately 80% of previously untreated CP patients can be expected to achieve CCgR, a majority of patients remain durably positive when tested by qRT-PCR for *BCR-ABL* transcripts [110], i.e. they have a persisting minimal residual disease. Even those who have undetectable *BCR-ABL* transcripts, may still harbor as many as  $10^7$  leukemic cells in their bodies [111], and there is a high likelihood for relapse if the drug is stopped [112, 113]. Bone marrow studies have shown that the residual Ph-positive cells are part of the leukemic stem cell compartment [114, 115]. Studies performed *in vitro* suggest that many primitive Philadelphia-positive progenitor or stem cells are relatively insensitive to imatinib [116]. These different lines of evidence suggest that imatinib, although being highly active against the differentiated mass of CML cells, probably fails to eradicate leukemic stem cells.

The cancer stem cell hypothesis postulates that a very rare population of cells within tumors have the capacity for limitless self-renewal [117]. This theory is exemplified by the following model in CML [118]. In CML, the leukemic stem cells can be considered quiescent, spending most of their time in  $G_0$ . Under certain circumstances, leukemic stem cells can enter cell cycle and give rise to progenitors, which produce differentiated leukemic cells. Thus, the disease relapses [117, 119, 120]. Expansion of Ph-positive progenitors is inhibited by imatinib [99] and life-long imatinib therapy is likely required to continuously suppress the remaining leukemic cells in CML patients, even in the best responders. Development of treatment targeting these quiescent stem cells, e.g. immunotherapy, is a challenge in CML.

*(ii) Resistance*

Although imatinib induces a high frequency of satisfactory responses, primary and acquired resistance can be seen in all stages of CML. Resistance to imatinib is multifaceted. Various definitions of resistance have been used in previous published studies. Generally, there are two types of resistance, primary and acquired. Primary resistance may be defined at the hematologic, cytogenetic or molecular levels. Acquired resistance can be defined as: (i) progression into AP or BC; (ii) loss of a sustained CHR or cytogenetic response; (iii) a 5- to 10-fold rise in *BCR-ABL* transcript number [121].

The mechanism of resistance has been intensively studied in the recent five years. However, the mechanism of primary resistance is still mainly unsolved. In general, there are two possible categories of the molecular mechanisms of imatinib resistance, i.e. BCR-ABL independent and BCR-ABL dependent [122]. In the first category, secondary oncogenic changes can occur in the leukemic cells and render the cell proliferation independent of BCR-ABL. In this scenario, BCR-ABL is no longer a relevant target and even the most ideal BCR-ABL inhibitor would be ineffective in this setting. However, this BCR-ABL-independent mechanisms may be rare events, especially in CP CML [123].

In the second, BCR-ABL-dependent category, something has changed in either the patient (host-mediated) or the leukemic clone (cell-intrinsic) that prevents the drug from effectively shutting down the target BCR-ABL protein. Host-mediated resistance can occur through enzymatic modification of imatinib by a P450 enzyme in the liver or by production of a protein that neutralizes drug activity, such as alpha-1 acid glycoprotein [124-126]. Cell-intrinsic resistance could occur by modification of the target BCR-ABL tyrosine kinase through gene amplification or *BCR-ABL* kinase domain mutations, or by a reduction of intracellular drug concentration through overexpression of multidrug resistance genes. Among these mechanisms, the *BCR-ABL* kinase domain mutations are the most studied.



The first reported mutation mediating resistance was T334I (T315I in the type 1a numbering scheme originally used) [127]. To date, more than 50 different *BCR-ABL* kinase domain mutations have been found to be associated with imatinib resistance [128-131]. A point mutation in *BCR-ABL* kinase domain can cause an amino acid change, which impairs the critical contact points of imatinib binding or alters the conformation of the protein. Regarding the three dimensional distribution, the *BCR-ABL* kinase mutations cluster into four main groups. The first group (G250E, Q252H, Y253F and E255K) includes the corresponding amino acids in the nucleotide-binding loop for ATP, also known as the p-loop [128, 132, 133]. The second group of mutations locates in the imatinib-binding site and directly interacts with the drug *via* a hydrogen bond (T315I) and Van der Waals' interactions (V289A, T315I and F317L) [127, 128]. The third group of mutations (M388L and H396P) is found in the activation loop (A-loop) [132]. These mutations result in a transition of the protein from inactive conformation to active conformation to which imatinib can not bind [134]. The fourth group includes amino acids distant from the imatinib binding site [128, 131], which form a hydrophobic patch between helices E, F and I in the C-terminal lobe of the enzyme, highly conserved region within the tyrosine kinase family [128, 135]. The kinase activity is not abrogated by these mutations. However, some mutants have enzymatic activity lower than that of wild-type *BCR-ABL* [136]. Also, the p-loop mutations are considered to bear a poor prognosis [128]. Despite their different locations, several mutants, such as T315I and E255K, are completely insensitive to imatinib at clinically achievable doses, whereas others, such as M351T and Y253F retain intermediate levels of sensitivity to imatinib [130].

### ***“Off-target” effects of imatinib***

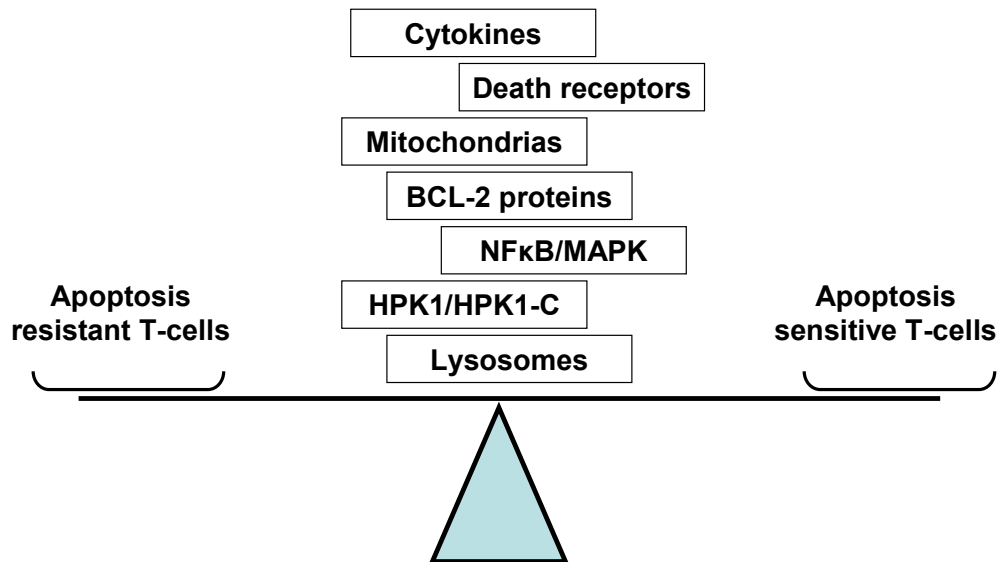
Besides the *BCR-ABL* protein, imatinib also target *ABL*, *ARG*, *PDGFR $\alpha$*  and  $\beta$ , and *c-KIT*. These tyrosine kinases are involved in many signaling pathways crucial for basic cellular processes. For instance, the transmembrane receptor tyrosine kinase *c-KIT* plays a crucial role in the development of various cell types including hematopoietic cells, germ cells, neuronal cells, melanocytes and intestinal pacemaker cells [137, 138]. It also regulates the proliferation and differentiation of early T-cell progenitors [139]. *ABL* and *ARG* (also known as *ABL1* and *ABL2*) are indispensable in the regulation of cell

proliferation and survival, cytoskeletal reorganization, cell migration, and the response to oxidative stress and DNA damage [140]. PDGFR has been shown to be important for the *ex vivo* expansion of normal early stem and progenitor cells [141]. Thus, inhibition of these tyrosine kinases may lead to alterations of the normal cell function.

Recently “off-target” effects of imatinib have been recognized, such as a reversible and dose-dependent lymphocytopenia and hypogammaglobulinemia [142], changes of serum phosphate levels [143], immunosuppression [102, 106, 144] and inducing apoptosis of cardiomyocytes [145]. Moreover, inhibitory and antiproliferative effects of imatinib on different compartments of the normal hematopoietic hierarchy have been reported. Imatinib impairs the function of hematopoietic stem cells [104, 106, 146] and CD34+ peripheral blood progenitor cells [147], which might in part explain the mild myelosuppression that is seen during imatinib treatment. Imatinib has also been shown to deregulate the function of monocyte-derived dendritic cells [148] and monocytes [149], and to inhibit both CD4+ and CD8+ T-cell proliferation and activation [150-152]. However, most of these results have been obtained from *in vitro* studies or animal models. The effect on the immune system of therapeutic doses of imatinib and its mechanism *in vivo* is still unclear.

Regulation of T-cell apoptosis is critical for lymphocyte homeostasis and immune functions. During an adaptive immune response, naïve and memory T-cells proliferate and fulfill their effector function. This expansion is followed by a contraction phase in which the number of T-cells declines and reaches normal levels. This process is highly regulated and requires a switch from an apoptosis-resistant towards an apoptosis-sensitive state [153]. T-cell homeostasis is basically controlled by two separate apoptosis pathways: activation-induced cell death (AICD) [154] and activated T-cell autonomous death (ACAD) [155]. Preactivated and expanded T-lymphocytes, which receive a restimulation *via* their T-cell receptor (TCR) undergo AICD involving death receptors, e.g. CD95 (Apo-1/Fas) [156, 157]. This extrinsic death pathway is crucial for AICD. In ACAD, where T-cells undergo apoptosis without TCR restimulation, cell death is determined by the ratio between anti- and pro-apoptotic BCL-2 family members [158,

159]. This intrinsic death pathway is critical for ACAD. Too little cell death of activated lymphocytes can result in autoimmune disorders and too much cell death can lead to immunodeficiency. Several factors control the shift between apoptosis resistance and sensitivity in T-cells: (i) cytokines, (ii) death receptors, (iii) mitochondria and BCL-2 proteins, (iv) NF- $\kappa$ B and MAPK signaling, (v) hematopoietic progenitor kinase 1 (HPK1)/HPK1 C-terminal cleavage fragment (HPK1-C), and (vi) lysosomes (Figure 6). Recently, an immunosuppressive effect of imatinib has been suggested. However, if this effect is mediated by imatinib induced alteration of apoptosis is unresolved.



**Figure 6. T-lymphocytes can switch between life and death.**  
*Apoptosis resistance and apoptosis sensitivity of T-cells is controlled at several levels of cellular decision making.*

## AIMS OF THE STUDY

The objective of the present thesis was to study the following aspects of CML:

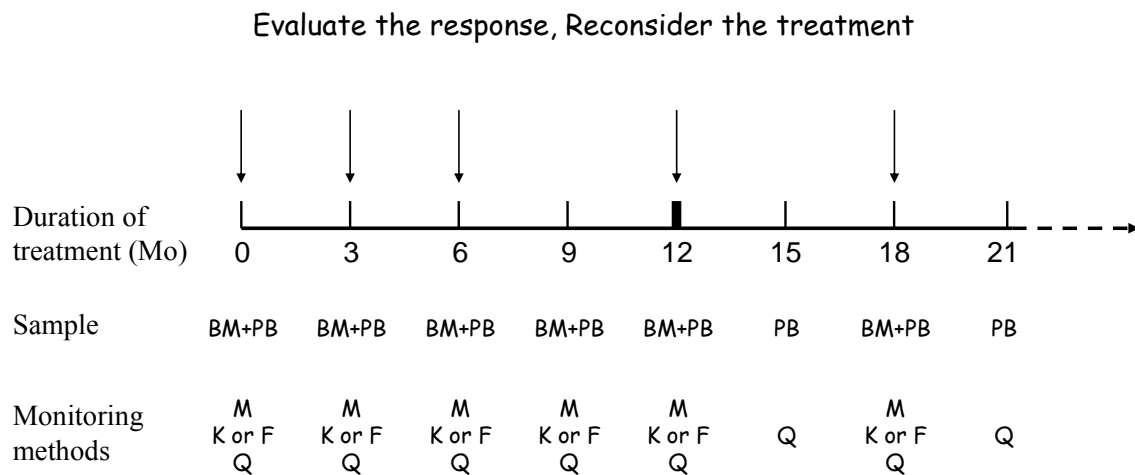
- (i) To compare the results of qRT-PCR for *BCR-ABL* transcript quantification and conventional cytogenetics in CML patients commencing imatinib treatment.
- (ii) To evaluate the prognostic value of measuring the expression of five different members of the BCL-2 family in CML.
- (iii) To study the frequency of *BCR-ABL* kinase domain mutations in CML.
- (iv) To study the effect of imatinib on “normal” T-lymphocytes *in vivo*.

## MATERIALS AND METHODS

### Statement of official approval

All studies were approved by the Regional Ethics Committee, Göteborg University, Göteborg, Sweden. Informed consents were obtained from all patients and healthy volunteers.

### Patients and controls



**Figure 7. Monitoring of CML patients treated with imatinib.**

*BM: bone marrow, PB: peripheral blood, M: morphologic evaluation of bone marrow smears, K: metaphase karyotyping, F: FISH and Q: qRT-PCR.*

The diagnosis of CML was confirmed prior to imatinib treatment by morphologic review of peripheral blood and bone marrow, and by documentation of the presence of the *BCR-ABL* fusion gene using conventional metaphase cytogenetic analysis or molecular studies.

The patients were treated with imatinib targeting a dose of 400 mg/day for CP, 600 mg/day for AP and 800 mg/day for BC. The patients were routinely monitored every three months by morphologic evaluation of bone marrow smears, metaphase karyotyping and/or FISH and qRT-PCR analysis of *BCR-ABL* transcripts on peripheral blood and/or bone marrow specimens (Figure 7).

Healthy volunteers were included in Paper II and IV. In paper IV, the healthy individuals were age and gender matched to the CML patients.

### **Morphologic review**

Peripheral blood smears were evaluated for basophilia, the presence of myeloid precursors, and immature cells. Wright-stained bone marrow smears were used to calculate a myeloid-to-erythroid cellular ratio, and to evaluate the total myeloid and erythroid cell compartments.

### **Cytogenetic analysis**

Cytogenetic studies were performed on 24 hours or 48 hours bone marrow cell culture, using standard methods for preparation and G-banding. Chromosome identification and karyotype designation were made according to the International System for Human Cytogenetic Nomenclature [160]. The number of metaphases analyzed on each specimen varied from 16 to 30. Cytogenetic responses were defined using standard criteria [103, 161]: no Ph-positive metaphases = complete cytogenetic response (CCgR), 1-35% Ph-positive metaphases = partial cytogenetic response, 36-65% positive metaphases = minor cytogenetic response, 66%-95% Ph-positive metaphases = minimal cytogenetic response and above 95% Ph-positive metaphases= no cytogenetic response.

### **Cell preparation**

The nucleated cell fraction was used in Paper I-IV and purified CD3+ T-cells were used in Paper IV. The nucleated cell fraction was isolated from EDTA-anticoagulated whole blood and/or bone marrow aspirates, by lysis of erythrocytes and pelleting the remaining cells by centrifugation.

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated whole blood by Ficoll™ (GE Healthcare Bio-Sciences, Uppsala, Sweden) density gradient centrifugation. After removal of the CD14<sup>+</sup> monocytes, CD3<sup>+</sup> T-cells were purified from the isolated PBMC using immunomagnetic cell sorting (MACS™, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated T-cells was confirmed by fluorescence-activated cell sorting (FACS) analysis and was in mean 96.2% (Paper IV).

### **RNA isolation and reverse transcription**

Messenger RNA was used in Paper I-III and total RNA was used in Paper IV. Messenger RNA was extracted from 10<sup>5</sup> nucleated cells using an automated poly-A RNA purification method, GenoM-48 Robotic Workstation (GenoVision, Oslo, Norway), according to the manufacturer's standard protocol. Total RNA was isolated from 2-4×10<sup>6</sup> purified CD3<sup>+</sup> T-cells using RNeasy® Mini Kit (Qiagen, Solna, Sweden). The RNA quality and concentration were determined using the NanoDrop spectrophotometer (NanoDrop, Wilmington, USA). Complementary DNA (cDNA) was generated by reverse transcriptase (RT) with random primers (Hexanucleotidemix, Roche, Sweden) using the Superscript II enzyme (Invitrogen, Stockholm, Sweden). The RT was optimized to obtain the maximal and parallel reaction efficiency. The cDNA was stored at -20°C.

### **Quantitative PCR**

Generally, the real-time PCR to quantify the target gene expression was based on TaqMan® technique. All samples were analyzed in duplicate. Absolute quantification was used in Paper I-III. *BCR-ABL* cDNA (Paper I-III) was quantified using self-designed primers and TaqMan® probe. Both *GAPDH* and *ABL* served as reference genes. In paper II, self-designed assays were used to quantify the apoptosis-related genes, *BCL-2*, *BAX*, *BCL-X<sub>L</sub>*, *BAD* and *BCL-X<sub>S</sub>*, together with *GAPDH* as reference gene. For these assays, plasmids containing the target genes in serial dilutions were used to construct the respective calibration curves. Using the cycle threshold (Ct) value obtained from the real-time PCR, the copy numbers of individual genes were calculated from the respective calibration curves. The estimated amount of target gene was the quote between target

gene copies and reference gene copies. Duplicate samples of calibrators were included in every reaction to measure the reliability and variability of the assays.

Relative quantification was used in Paper IV. Commercial available TaqMan® Gene Expression Assay for *GAPDH* (Applied Biosystems, Stockholm, Sweden) was used to optimize and validate the efficiency of RT and real-time PCR. Apoptosis related gene expression was determined by TaqMan® Low Density Array (LDA) human apoptosis panel (Applied Biosystems, Stockholm, Sweden). This array was designed to quantify the expression of 93 genes involved in the apoptosis pathways and three reference genes (*18S*, *actin-β* and *GAPDH*). Complementary DNA from one patient and the age and gender matched control was loaded in duplicate on one array card and was analyzed by the Applied Biosystems ABI prism 7900HT real-time PCR system equipped with a TaqMan® LDA upgrade (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instruction. Gene expression levels were calculated based on the  $\Delta\Delta C_t$  method. Briefly, every target gene expression in every patient or control was calculated as  $\Delta C_t = C_{t \text{ target gene}} - C_{t \text{ reference gene}}$ . For an individual target gene, the difference in  $\Delta C_t$  between the patient and his paired control was calculated as  $\Delta\Delta C_t = \Delta C_{t \text{ patient}} - \Delta C_{t \text{ control}}$ . Setting the target gene expression level as one in the control, the relative quantities in patients were determined using the equation:  $\text{relative quantity} = 2^{-\Delta\Delta C_t}$ , i.e. fold change in patients compared to controls.

### **Sequencing of *BCR-ABL* kinase domain**

Analysis of mutations was performed using a method modified from Shah *et al* [131]. The *BCR-ABL* kinase domain was amplified using a two-step RT-PCR procedure. With cDNA as template, and forward and reverse primers located in *BCR* exon b2 and *ABL* exon 9, respectively, the first PCR step generated a 1.3 kb fragment containing *BCR-ABL* junction and *ABL* kinase domain. In the second PCR step, using a forward primer annealing in *ABL* exon 4 and the same reverse primer as in the first step, an 858 bp fragment was generated. After purification, the 858 bp fragment was sequenced in the forward and reverse direction using BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Stockholm, Sweden) and ABI Prism 3100



Genetic Analyzer system (Applied Biosystems, Foster City, CA, USA). Using GenBank accession No. M14752 as reference, sequences were aligned and analyzed with the CodonCode sequence analysis software (CodonCode, Corporation, Dedham, MA, USA). From subcloning experiment it was estimated that this assay will reveal mutant clones once they represent more than 20%-30% of the leukemic clones.

### **Flow cytometry**

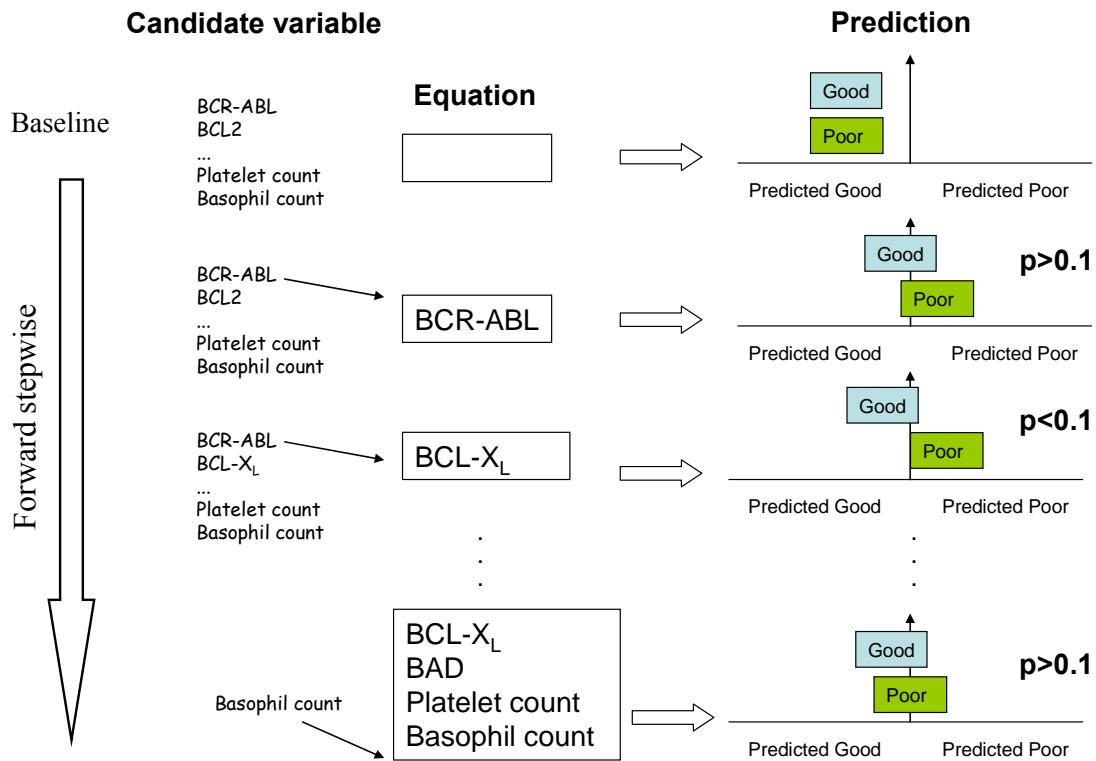
A 6-colour FACS analysis was used to study the surface expression of receptors and activation markers on T-lymphocyte (Paper IV). The following fluorochrome conjugated monoclonal antibodies (MoAbs): CD3-PerCP, CD4-FITC, CD16-PE-Cy7, CD28-APC and CD8-APC-Cy7 (all from BD Bioscience, Stockholm, Sweden), were used to quantify lymphocyte subsets, i.e. T-lymphocytes (CD3+), T-helper lymphocytes (CD3+CD4+CD28+), cytotoxic T-lymphocytes (CD3+CD8+CD28+) [162], suppressor T-lymphocytes (CD3+CD8+CD28-) [163] and natural killer cells (NK; CD3-CD16+). The lymphocyte phenotype was studied using the following MoAbs: CD140-PE (PDGFR $\beta$ ), CD95-PE (FAS; TNF receptor superfamily, member 6), CD69-PE (T-cell activation antigen), CD49d-PE (VLA-4; integrin alpha 4 chain of the adhesion receptor), CD25-PE (IL-2 receptor  $\alpha$ ), CD158a-PE (KIR2DL1; killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 1), CD158b-PE (KIR2DL2/DL3), NKB1-PE (KIR3DL1; killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1), CD94-PE (NK cell receptors for MHC class I), CD184-PE (CXCR4; chemokine (C-X-C motif) receptor 4), CCR4-PE (chemokine (C-C motif) receptor 4), CCR6-PE (chemokine (C-C motif) receptor 6) (all from BD Bioscience, Stockholm, Sweden) and CX3CR1-PE (chemokine (C-X3-C motif) receptor1) (Nordic Bio-Site, Stockholm, Sweden).

Peripheral blood nucleated cells were prepared from EDTA-anticoagulated whole blood after removal of red cells by lysis.  $5\text{-}10 \times 10^5$  nucleated cells, in a volume of 100  $\mu\text{l}$ , were incubated with 6 different fluorochrome-conjugated MoAbs at 4°C in dark for 15 min. Thereafter, the cells were washed once and resuspended in 0.5 ml 0.01M phosphate buffered saline. The expression of cell surface antigens was assessed using flow

cytometry (FACSCanto, BD Bioscience, San Jose, CA, USA) and the BD FACSDiva™ software (BD Bioscience, San Jose, CA, USA). The quantity of lymphocyte subsets was presented as percentage and the expression of cell surface antigens was presented as median fluorescence intensity (MFI).

### **Statistical analysis**

The statistical analysis was performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA). Differences between groups were evaluated by Student's t-test, Mann-Whitney U-test and Wilcoxon signed rank test, where appropriate. Logistic regression analysis was used in Paper II. The aim of the regression model was to establish an equation which can predict the molecular response. Briefly, the analysis was undertaken by including candidate variables one by one into the equation. The accuracy of the prediction was determined after each step, i.e. forward stepwise. If inclusion of a variable in the equation can change the accuracy of the prediction significantly, the variable was kept in the equation in the following steps; otherwise, it was removed from the equation. The statistical model is demonstrated in Figure 8.



**Figure 8. Flow chart of the logistic regression model used in Paper II.**

The aim of the regression model is to establish an equation which can predict the molecular response at 12 months of imatinib therapy. The dependent which is the molecular response at 12 months of imatinib treatment, is a binary categorical variable, i.e. good or poor responders. The covariates are the candidate variables. The analysis will identify which of the candidate variables that can be included in the equation of prediction. The first step is to set the baseline by including no covariates in the model and set all patients as good responders. The regression starts with the BCR-ABL in the equation but there is no significant difference in the accuracy of prediction. Thus, BCR-ABL is removed from the equation. In the next step, BCL-X<sub>L</sub> was included, which significantly changes the accuracy of prediction. Therefore, BCL-X<sub>L</sub> is kept in the equation. In this way, the factors having significant impact on the molecular response at 12 months of imatinib therapy were identified.

## RESULTS AND DISCUSSION

### 1. Quantitative RT-PCR is a useful and reliable method to monitor CML patients treated with imatinib (Paper I)

We established a qRT-PCR method to detect *BCR-ABL* transcripts in CP CML patients treated with imatinib. The detection limit of the method corresponded to five copies of the *BCR-ABL* plasmid or one K562 cell. After CCgR was obtained, *BCR-ABL* transcript could still be detected by qRT-PCR. The *BCR-ABL* transcript number analyzed by qRT-PCR showed a significant correlation with the number of Ph-positive metaphases detected by cytogenetic analysis ( $r=0.7$ ;  $p<0.001$ ). There was also a highly significant correlation between the *BCR-ABL/GAPDH* ratios obtained in peripheral blood and bone marrow ( $r=0.9$ ;  $p<0.001$ ), implying that peripheral blood specimens are as reliable as bone marrow specimens, for quantification of the leukemia burden. Therefore, unlike conventional cytogenetics which requires “bone marrow” cells, it is an easier and more convenient technique for frequent monitoring.

Several other qualitative or semi-quantitative RT-PCR assays for *BCR-ABL* transcripts quantification have been shown to be equally sensitive in peripheral blood and bone marrow [75, 164, 165]. However, these techniques are more labor intensive compared to the real-time RT-PCR assays. Although, the reagents for qRT-PCR are more expensive than for cytogenetics and the other RT-PCR assays, the total cost is lower if the labor cost is accounted for [166].

A generally accepted qRT-PCR protocol, for quantification of *BCR-ABL* transcripts, has not been agreed on, and there is a variation in results reported from different laboratories using these assays. Efforts have been made by European Against Cancer program [167, 168] and by Hughes *et al* [169], to standardize the protocol. In these reports *ABL* was recommended as a reference gene. Also, the validity of *GAPDH* as a reference gene has been questioned because of inconsistent mRNA levels [170] and the existence of pseudogenes [168]. In our study, we analyzed the *GAPDH* and *ABL* expression in 40 blood samples from healthy individuals and 22 blood samples from CML patients. No

significant differences were seen between the healthy individuals and the CML patients as regards *ABL/GAPDH* and *GAPDH/ABL* quotes. A significant correlation was found between the *GAPDH* Ct value and the *ABL* Ct value obtained on the same sample ( $r=0.84$ ;  $p<0.01$ ), suggesting that these genes are expressed in parallel and that *GAPDH* should perform as well as *ABL* as reference gene.

Although the sensitivity of cytogenetic analysis is not as good as that of qRT-PCR, cytogenetics can reveal other cytogenetic abnormalities in addition to the Ph chromosome and warn for disease progression. In our cytogenetic analyses, we found losses of chromosomes both before and during imatinib therapy. Most interestingly, a Ph-negative clone with an extra chromosome 8 (trisomy 8) evolved in two patients, after 9 and 15 months of imatinib therapy, respectively. The clinical significance of this additional cytogenetic abnormality, in Ph-negative cells, is still unresolved. Thus, cytogenetics still plays an important role in diagnosis and monitoring of CML patients and can not be fully substituted by qRT-PCR.

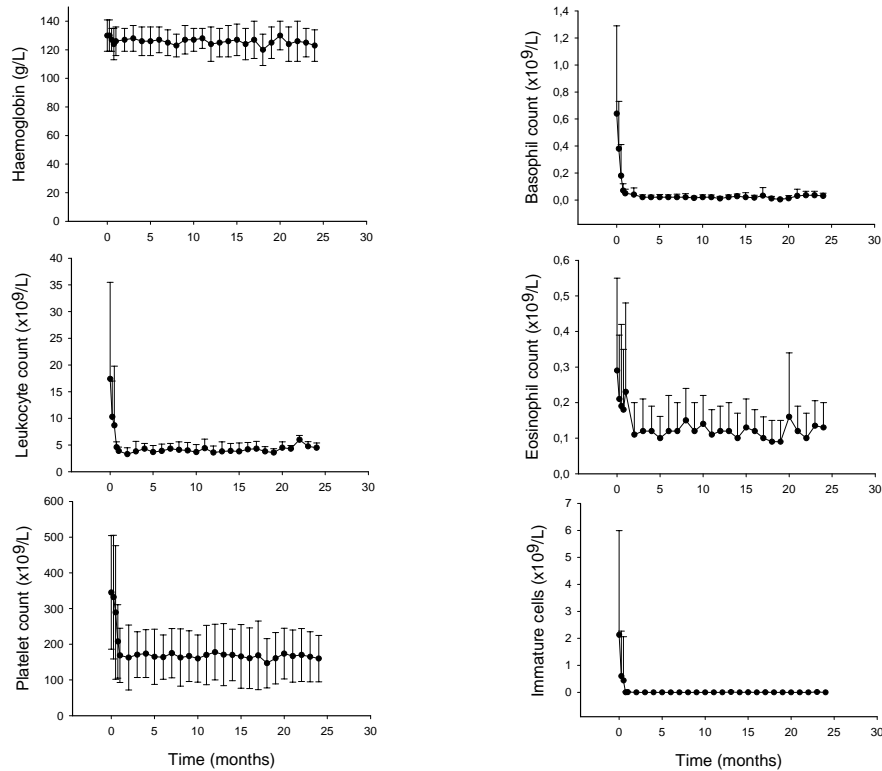
## **2. Dynamics of *BCR-ABL* in CML patients treated with imatinib (Paper I)**

After 3 months of imatinib treatment, 16 out of the 17 CML patients studied in Paper I had obtained CHR (Figure 9) and there was no morphologic evidence of CML, with normal or slightly decreased cellularity in bone marrow.

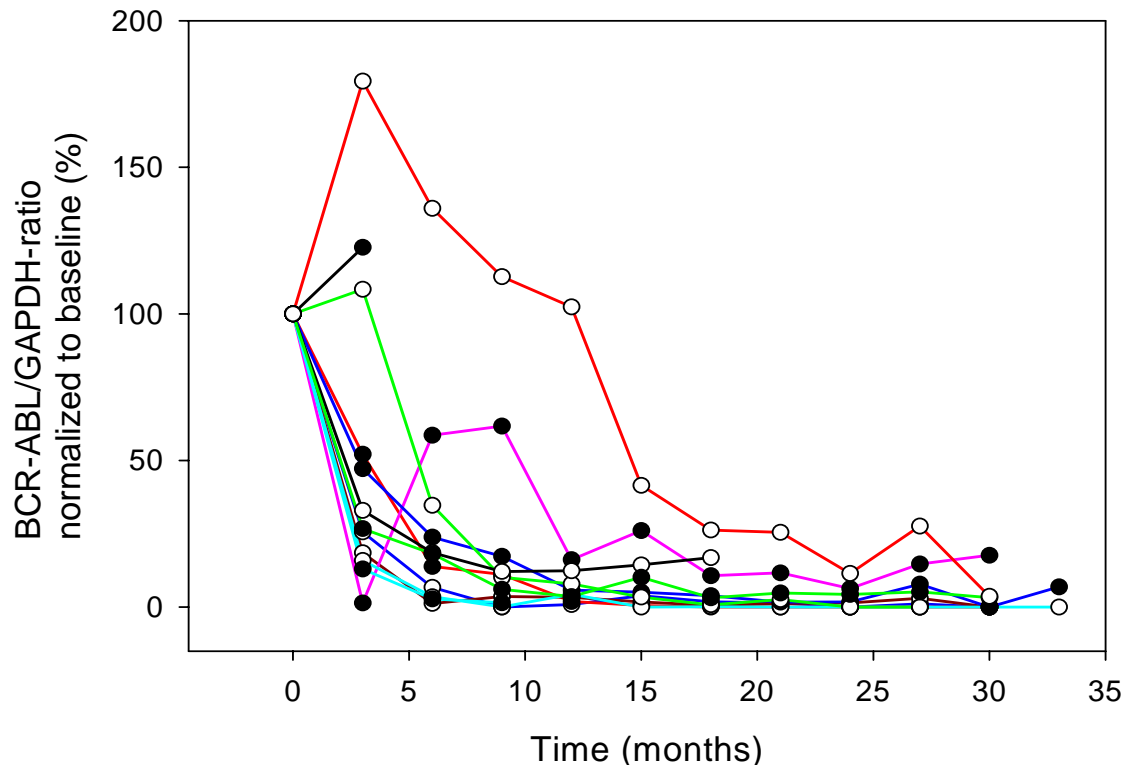
The serial *BCR-ABL/GAPDH* ratios obtained by qRT-PCR analysis on peripheral blood samples collected every three months are presented in Figure 10. The major decline in *BCR-ABL/GAPDH* ratio occurred within 6 months after start of imatinib therapy. Thereafter, the decline in *BCR-ABL/GAPDH* ratio levelled off and an apparent plateau was obtained after 12-15 months. There was no significant difference ( $p=0.1$ ) between the mean *BCR-ABL/GAPDH* ratios obtained at 15 and 30 months of imatinib therapy,  $19.1\pm 14.5$  and  $11.2\pm 10.0$ , respectively (Figure 10).

The molecular plateau represents the residual *BCR-ABL* positive cells. According to the stem cell theory, the plateau might represent the leukemic stem cell compartment [118]. It

can be hypothesized that these cells are not in the cell cycles and will not be eliminated by imatinib treatment. Therefore, they might be the origin of relapse if imatinib treatment is discontinued.



**Figure 9. Peripheral blood counts in CP CML patients treated with imatinib**



**Figure 10. The results for serial measurements of BCR-ABL/GAPDH ratio in peripheral blood for 17 CML patients treated with imatinib.**

The results for individual patients were normalized to their baseline value, i.e., the value recorded immediately before start of imatinib therapy. The main reduction in BCR-ABL transcripts occurred within the first 6 months of imatinib treatment. After 12 to 15 months of imatinib treatment, an obvious plateau was achieved with small fluctuations.

### 3. Clinical significance of monitoring CML by qRT-PCR (Paper I)

A high frequency of CCgR is obtained in imatinib treated CML patients, but most of them remain qRT-PCR positive. Our data showed that the *BCR-ABL/GAPDH* ratio can vary with 3-log (base 10) magnitude when a CCgR is obtained, indicating that the patients should be further stratified by qRT-PCR. The ultimate objective of CML treatment must be to eradicate the disease and to achieve a sustained “molecular remission”. A major molecular response means  $\geq 3$ -log reduction in *BCR-ABL* transcript number compared to a standardized pre-treatment baseline value [110]. Obtaining a major molecular response appears to bear prognostic information. In the IRIS study, in patients who had a CCgR and a reduction in transcript number of at least 3 log (base 10)

at 12 months, the probability of remaining progression-free was 100 percent at 24 months, as compared to 95 percent for patients with a reduction less than 3 log and 85 percent for patients who were not in CCgR at 12 months ( $P < 0.001$ ) [110].

Also, in our patients with an “apparent molecular plateau” small changes in *BCR-ABL* transcript numbers, but without other signs of disease relapse, were frequently observed upon serial measurements. This finding corroborates Goldman and co-workers criteria for loss of response to imatinib [171]. They proposed that an increase in *BCR-ABL/ABL* ratio of 1 log or more on serial testing, or a *BCR-ABL/ABL* ratio that rises into the range associated with Ph-positivity, should be considered as a loss of response. However, Branford *et al* reported that a rise in *BCR-ABL* of more than 2-fold can be used as an indicator to test patients for *BCR-ABL* kinase domain mutations [172]. These diverging results are most likely explained by differences in laboratory practice, and emphasize the need for standardization.

A baseline value for *BCR-ABL* transcript number is required for adequate monitoring of the disease and for evaluation of the response to imatinib. In the IRIS study, a standardized baseline value was applied to all patients, and the log (base 10) reduction in *BCR-ABL* transcripts was calculated at different time points. Unfortunately, this standardized baseline reference is not universally available, and most laboratories can not express the results in a way that is fully comparable to the IRIS data. While waiting for an international standard, we suggest that the values should be normalized to the individual baseline value. However, it has to be taken into account that at baseline, i.e. before treatment, there is a 1-log variation in *BCR-ABL/GAPDH* ratio.

#### **4. Lower *BAD* expression at diagnosis correlates to better molecular response after 12 months of imatinib therapy (Paper II)**

We performed a landmark analysis at 12 months of imatinib treatment and divided the patients into good and poor molecular responders, based on whether they had a reduction of the *BCR-ABL* transcript number above or below 3 log (base 10) compared to their individual baseline value.



The good responders had significantly lower *BAD* expression before commencing imatinib therapy compared to patients with a poor molecular response ( $p=0.02$ ). It appears unlikely that differences in cell subsets, i.e. percentage of lymphoid and myeloid cells, in the samples from which mRNA was prepared, can account for the difference in *BAD* expression; at diagnosis there were no statistical differences in the mean peripheral blood hemoglobin level, total leukocyte count, lymphocyte count, number of immature cells and platelet count between the good and poor molecular responders.

To evaluate the association between the apoptosis-related gene expressions before start of imatinib therapy, and molecular response at 12 months of imatinib treatment, *BCR-ABL* transcript levels, expression of the BCL-2 family members, baseline clinical features, i.e. age, hemoglobin levels, white blood cell counts, platelet counts, and the proportion of peripheral basophiles were introduced as continuous variables into a logistic regression model. Variables with a  $p$ -value  $<0.1$  in the univariate analysis were included in a multivariate ‘Wald forward’ regression model. In the univariate model, expression of *BCL-X<sub>L</sub>* and *BAD* as well as platelet counts were correlated with the molecular response. However, in the multivariate model, only *BAD* expression had a significant impact on the molecular response at 12 months (Table 1). We also introduced the continuous variables as categorical variables in a logistic model described by Lange *et al* [173]. Same result was found, i.e. *BAD* expression level at baseline related to the molecular response seen at 12 months ( $p=0.018$ ).

Apoptosis-related genes have previously been successfully used for identification of prognostic factors in acute myeloid leukemia (AML) [174] and CML in myeloid BC [173]. *BAD* is a pro-apoptotic protein and the survival function of *BCR-ABL* oncogene is mediated partly by *BAD*-dependent pathways [175]. Our finding that lower *BAD* expression correlated with a good molecular response is unexpected since one might assume the opposite. However, similar results were seen in an AML study, that higher *BAD* expression correlated with negative outcome in AML [174]. These findings can not be easily interpreted by the current paradigm. However, the net apoptotic signals

delivered by the BCL-2 family depend not only on the relative ratios of pro- and anti-apoptotic members but also on the degree of phosphorylation of BCL-2 family members as well. Further preclinical and clinical studies are required to determine whether apoptotic alterations can serve as prognostic markers and if additional targeting of apoptotic pathways will be a successful strategy for improving the efficacy of CML treatment.

<b>Variables</b>	<b>Univariate</b>	<b>Multivariate</b>	<b>Odds ratio</b>
<i>BCR-ABL</i>	0.157		
<i>BCL-2</i>	0.146		
<i>BCL-X<sub>L</sub></i>	0.085	0.936	
<i>BAX</i>	0.68		
<i>BAD</i>	0.017	0.042	353.5
<i>BCL-X<sub>S</sub></i>	0.238		
Age	0.143		
White blood cell count	0.764		
Platelet count	0.098	0.102	
Peripheral basophil count	0.448		

**Table 1. The association between the apoptosis-related gene expressions prior to imatinib therapy and molecular response at 12 months of imatinib treatment.**

*BCR-ABL* transcript levels and expression of the BCL-2 family members were introduced as continuous variables into a logistic regression model. Variables with a *p*-value <0.1 in the univariate analysis were included in a multivariate ‘Wald forward’ regression model. In the univariate and multivariate models, expression of *BAD* correlated with the molecular response at 12 months.

### **5. Not all resistance is *BCR-ABL* kinase domain mutations (Paper III)**

In Paper III, we screened for *BCR-ABL* kinase domain mutations every 3-6 months from start of imatinib treatment in 40 CML patients. Irrespective of disease phase, no *BCR-ABL* kinase domain mutation was detected in any sample collected before start of imatinib therapy. During imatinib treatment, mutations in *BCR-ABL* kinase domain were found in 2 (7%) of the 30 early-CP patients, 4 (57%) of 7 late-CP patients, and in all 3 (100%) AP patients. Six different point mutations were detected. Three mutations (G250E, Y253H and E255K) clustered in the p-loop, one mutation (T315I) was located in the imatinib binding site, one mutation (E355K) was located in the activation loop, and

one mutation (E450G) was located in the C-terminal part of the kinase domain. The E450G was the most frequently observed mutation, detected in 4 of our 40 patients.

Four of the 30 early-CP patients had a primary cytogenetic resistance, defined as failure to achieve at least a minimal cytogenetic response at 6 months or a MCgR at 12 months, and none of them displayed any *BCR-ABL* kinase domain mutation up to 12 months after start of imatinib therapy. Also, none of the patients with molecular disease persistence, detectable only by qRT-PCR but without other signs of imatinib resistance, had any kinase domain mutation at 12 months. These patients have been followed with mutation screening every six months and no mutations have evolved during a median follow-up of 31 months (range from 12 to 59 months). Conversely, acquired imatinib resistance is frequently associated by mutations. Three of our 30 patients treated in early-CP lost an earlier obtained MCgR or CHR. Two of these patients were found to bear kinase domain mutations at the time of resistance detection; in one them the mutation was discernible in a sample collected 3 months prior to clinical signs for imatinib resistance. Furthermore, 8 out of our 40 patients developed an acquired imatinib resistance, either hematologic resistance (n=6), defined as loss of CHR or transformation into BC, or cytogenetic resistance (n=2), defined as loss of MCgR or CCgR. *BCR-ABL* kinase domain mutations were found in 6 of them.

The degree of response to imatinib therapy in CML seems to be the best predictor of prognosis [110]. Monitoring for unsatisfactory response has therefore become routine to identify patients at risk of disease progression. Such imatinib resistance can either be primary or acquired. Our results suggest that primary cytogenetic resistance or molecular persistence, in an otherwise stable disease, is rarely caused by point mutations in the *BCR-ABL* kinase domain. In contrast, we found point mutations in the majority of cases with acquired resistance.

## **6. Clinical significance of mutation screening**

There is evidence in the majority of patients with acquired resistance of either increased expression of *BCR-ABL* or, more frequently, mutations in the kinase domain of *BCR-ABL*. The resistance caused by some mutants, such as M351T or Y253F, can be overcome by dose increase of imatinib, whereas other mutants, such as T315I and E255K, are insensitive to imatinib at clinically achievable doses [130]. Today, alternative ABL inhibitors that have higher potency or capture additional conformations of the ABL kinase have become available. Two of these compounds, Sprycel® (BMS-354825, dasatinib, Bristol-Myers Squibb, New York City, NY, USA) and Tasigna® (AMN107, Nilotinib, Novartis, Basel, Switzerland), have passed phase I trials and phase II trials are still ongoing [176-180]. Both compounds demonstrate very encouraging clinical activity, even against the most imatinib-resistant *BCR-ABL* kinase domain mutants, with the notable exception of the T315I mutant, which is completely resistant to imatinib, nilotinib and dasatinib [181, 182]. Thus, evaluating imatinib-resistant patients for *BCR-ABL* kinase domain mutations provides a guide for clinical management.

The sensitivity of the sequencing method is about 20-30%. Other methods, e.g. allele-specific oligonucleotide PCR and denaturing-high performance liquid chromatography (D-HPLC), will detect *BCR-ABL* kinase domain mutations with better sensitivity. However, it can be argued that mutant clones at low levels may not have the same clinical significance as clones that are detected in the context of rising disease burden. Even using direct sequencing, we found in our study two patients treated in late CP with temporary mutations. These patients have been followed for 23 and 54 months, respectively, and the mutations have not reappeared and no imatinib resistance has evolved. Thus, a mutation out of the context of imatinib resistance should be interpreted with caution.

In conclusion, monitoring all patients for mutations at regular time points is not cost-effective. Patients with stable or decreasing *BCR-ABL* levels do not require mutation screening. Conversely, in patients with signs of an expanding disease burden, a search for *BCR-ABL* kinase domain mutations are warranted.

## **7. Therapeutic doses of imatinib alter the expression of apoptosis-related genes in T-lymphocytes**

Using TaqMan® Low Density Array, 93 genes within the apoptosis pathways were quantitatively analyzed in T-cells from CP CML patients in CCgR, and age and gender matched controls. Among these genes, 12 had significantly different expression levels in the patients compared to the controls. All these 12 genes had lower expression levels in the patients, with 1.2- to 12- fold differences compared to the controls.

Apoptosis of T-lymphocytes is a fundamental process regulating antigen receptor repertoire selection during T-cell maturation and homeostasis of the immune system. Resting mature T-cells are activated by an antigen to elicit an appropriate immune response. In contrast, pre-activated T-cells undergo AICD in response to TCR triggering alone [183, 184]. Thus, death by apoptosis is essential for function, growth and differentiation of T-lymphocytes. However, our gene expression results can not tell the ultimate effect of imatinib on apoptosis in T-cells, since both anti-apoptotic and pro-apoptotic genes were found among the 12 genes which showed significantly lower expression levels in T-cells from imatinib treated CML patients. It has to be kept in mind that apoptosis is the end result of the interplay between different pathways mediating death signals and the ultimate fate of the cell can not in a simple way be predicted by the relative ratios of anti- and pro-apoptotic members.

## **8. Therapeutic doses of imatinib alter the T-cell phenotype**

Fourteen different antigens, representing PDGFR $\beta$ , FAS, adhesion receptors, activation markers, KIRs and chemokine receptors, were analyzed on three subsets of T-cells, i.e. T-helper lymphocytes, cytotoxic T-lymphocytes, suppressor T-lymphocytes, and NK-cells. Differences in surface expression of these antigens were only observed for CD4+CD28+ T-helper lymphocytes. For these cells, CD140 (PDGFR $\beta$ ), CD158b (KIR2DL2/DL3) and NKB1 (KIR3DL1) had significantly lower surface expression in the CML patients compared to controls, whereas CD49 (VLA-4, integrin alpha 4 chain of the adhesion receptor) showed significantly higher median fluorescence intensity in the CML patients

( $p \leq 0.05$ ). No statistically significant changes were seen for cytotoxic T-lymphocytes, suppressor T-lymphocytes or NK-cells between CML patients and controls.

CD4<sup>+</sup> T-cells are not required for the development of efficient primary CD8<sup>+</sup> T-cell responses against infectious agents. However, without CD4<sup>+</sup> T-cells, memory CD8<sup>+</sup> T-cells will produce a poorer secondary response [185, 186]. Thus, it is possible that the inhibitory effect of imatinib on the expansion of memory CD8<sup>+</sup> T-cells, reported by other investigators [187], is partly mediated through its effect on T-helper cell activity. It has been reported that imatinib suppress the phosphorylation of LCK and ERK1/2. These two proteins are associated with TCR-mediated signaling [188], and may account for the previously reported suppression of cytokine synthesis by CD4<sup>+</sup> T-cells from CML patients treated with imatinib. In addition, since imatinib affects functions of dendritic cells, this effect on antigen presenting cells may compromise CD4<sup>+</sup> T-helper cell activity [148, 189]. The effects of imatinib on CD4<sup>+</sup> helper T-cells responses *in vivo* merit further investigation and could shed light on the depressed memory CD8<sup>+</sup> T-cell response, previously reported by other investigators.

## CONCLUSIONS

1. Quantitative RT-PCR is a sensitive and reliable method to monitor CML patients treated with imatinib. Using this method, the CML patients in complete cytogenetic response can be further stratified, based on their *BCR-ABL* transcript numbers. The *BCR-ABL* transcripts level reaches an apparent plateau after 12-15 months of imatinib treatment, which indicates a stable number of remaining *BCR-ABL* positive cells.
2. The expression of the apoptosis related gene *BAD* at diagnosis correlates with the molecular response seen at 12 months of imatinib therapy. Further studies are required to confirm this finding.
3. *BCR-ABL* kinase domain mutations are linked to acquired imatinib resistance in CML. The primary imatinib resistance and molecular persistence can not be explained by *BCR-ABL* kinase domain mutations.
4. Therapeutic doses of imatinib alter the expression of apoptosis related genes in CD3+ lymphocytes and change the phenotype of CD4+CD28+ T-helper cells. The effect of imatinib on T-helper cell responses *in vivo* merits further investigation and could shed light on the suppressed memory CD8+ T-cell response reported by other investigators.



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