

**Technical and clinical evaluation of the
BCR-ABL1 Tyrosine Kinase domain
mutation analysis in patients with
Chronic Myeloid Leukaemia**

MPhil thesis

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Contributions from others

BCR-ABL1 amplification was done by me following the protocol designed by Dr Jamshid Khorashad.

I designed the experiments and optimised the techniques that were used in this thesis unless otherwise stated in the text.

The cDNA for analysis of kinase domain mutations in chapter one was prepared by MRD group in the Department of Haematology, Hammersmith Hospital and I prepared some of materials as a member of this group. The methodology for preparation of the cDNA and measurement of BCR-ABL1 transcript levels was according to the protocols developed by Prof. Letizia Foroni who was in charge of MRD lab.

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List of Abbreviations

μl	Micro litre
μM	Micro molar
A- loop	Activation loop
ABL1	Human homologue of the Abelson Leukemia Virus Oncogene
AP	Accelerated phase
ASO-PCR	Allele-specific oligonucleotide-polymerase chain reaction
ATP	Adenosine triphosphate
BC	Blast crisis of chronic myeloid leukaemia
BCR	Breakpoint cluster region
CCyR	Complete cytogenetic response
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative genomic hybridisation
CHR	Complete haematological response
CML	Chronic myeloid leukaemia
CMR	Complete molecular response
DASA	Dasatinib
DNA	Deoxyribonucleic acid

HHT	Homoharringtonine (Omacetaxine)
h-OCT1	human organic cation transporter 1
IM	Imatinib
α -IFN	Interferon alpha
KD	Kinase domain
MCyR	Major cytogenetic response
MDM2	Murine double minute 2 gene Major molecular
MMR	response
MNC	Mononuclear cells
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
NILO	Nilotinib
P- loop	ATP phosphate-binding loop
PCR	Polymerase chain reaction
PCyR	Partial cytogenetic response
PFS	Progression free survival
Ph	Philadelphia
PMN	Polymorphonuclear leukocyte
RNA	Ribonucleic acid
RR	Relative risk
SCT	Stem cell transplant
SH1	SRC-homology region 1
SH2	SRC-homology region 2
SH3	SRC-homology region 3
SNP	Single nucleotide polymorphism
t-AML	Therapy related Acute Myeloid Leukaemia

TKI	Tyrosine kinase inhibitor
WBC	White blood cell

Abstract

Chronic Myeloid Leukaemia (CML) is a myeloproliferative disorder whose hallmark is represented by the *BCR-ABL1* fusion gene. The mechanisms associated with disease progression and development of resistance to treatment are still little understood and so far the best characterised mechanism of resistance is the expansion of a CML clone bearing an amino-acid substitution in the BCR-ABL1 kinase domain (KD). Screening by direct sequencing a large cohort of patients of over 260 CML individuals at various stages of disease, I assessed the incidence of BCR-ABL1 KD mutations and found that, in accordance with previous works, the detection of mutations was strongly linked to clinical resistance. Furthermore, patients with amino acid substitutions within the P-loop region had commonly presented with features of advanced stages at some point during disease course, accounting for the vast majority of all resistance-associated mutations. To evaluate the impact of the diverse BCR-ABL1 mutations on individual prognosis I stratified the whole cohort of patients on the grounds of disease phase at time of mutation detection and found that events of progression from chronic to advanced phase were mostly represented by patients harbouring KD mutations within the *P-loop* and *gatekeeper* domains. Detection of BCR-ABL1 mutations, however, does not always explain resistance to tyrosine-kinase inhibitors (TKIs). With several studies having indicated a role of certain Src family kinases (SFKs) in the disease progression, we have hypothesised that the ineffectiveness of treatment might be due to the introduction of point mutations within the TK domain of one or other of the three SFKs being implicated in the development and progression of CML, namely Hck, Lyn, and Fyn. Systematic evaluation of the mutations arising after treatment with 2G-TKIs has been performed over a subgroup of the initial cohort of CML patients, constituted by 24 non-responder CML patients described as negative for BCR-ABL1 mutations. Screening these patients for SFK Hck, Lyn and Fyn KD mutations, however, could not reveal the presence of any abnormality, suggesting that the clinical heterogeneity of CML requires therefore further investigation of alternative mechanisms responsible of the multifactorial resistance and relapse of CML patients.

I. Background

Chronic myeloid leukaemia (CML) is one of the most extensively studied human malignancies. CML is a myeloproliferative disorder characterised by the malignant expansion of hemopoietic stem cells (HSC) in the bone marrow. Underlying the aetiology of this disease is a balanced reciprocal chromosomal translocation between the long arms of chromosome 9 and 22, $t(9;22)(q34;q11)$, generating the Philadelphia (Ph⁺) chromosome¹, named after the city where it was first discovered, and whose presence usually confirms the diagnosis of CML, occurring in 95% of the patients with CML. This chromosomal aberration leads to the formation of the chimeric *BCR-ABL1* oncogene, characterised by the juxtaposition of parts of two genes: the *ABL1* (human homologue of the Abelson murine virus) proto-oncogene, present on chromosome 9, and *BCR* (breakpoint cluster region) gene, on chromosome 22. As a result, a 5' end of *BCR* and a 3' end of *ABL1* are fused together on a shortened chromosome 22. The leukaemogenic potential of the *BCR-ABL1* fusion gene resides in the constitutively activated tyrosine kinase of its *ABL1* portion.² The normal *ABL1* protein has a physiologically controlled kinase activity and is found in both the nucleus and the cytoplasm, shuttling between these two domains under the influence of nuclear localisation export signals whereas *BCR-ABL1* is essentially cytoplasmic.³ Nuclear *ABL1* has predominantly pro-apoptotic functions, playing an essential role in the cellular response to genotoxic stress; in contrast, *BCR-ABL1* is mainly anti-apoptotic and despite maintaining the *ABL1*-derived nuclear export and nuclear localisation domains, it seems unable to penetrate the nucleus.³ The main reason why the *BCR-ABL1* protein is trapped in the cytoplasm

is its aberrantly active tyrosine kinase. Furthermore, the hybrid *BCR-ABL1* oncogene leads to the transformation of a HSC to a leukaemic stem cell (LSC). Proof that a CML LSC derives from transformation of a HSC is provided by the observation that BCR-ABL1 is found in all myeloid lineages, and some lymphoid cells, including a small fraction of T and NK cells⁴, but clinically it only affects granulocytes. BCR-ABL fusion protein occurs in the majority of patients with CML and in about 15-25% of the adults with acute lymphoblastic leukaemia (ALL).⁵ CML is a triphasic disease: more than 90% of patients are diagnosed in an indolent chronic phase (CP), where mature granulocytes are still produced, but there is an increased number of myeloid progenitor cells in the peripheral blood. The natural history of CML is progression from CP to an intermediate accelerated phase (AP) characterised by 15%–29% blasts in blood or bone marrow, >20% basophils in blood, thrombocytosis or thrombocytopenia unrelated to therapy, or clonal cytogenetic evolution. AP within a year transforms into a terminal blastic phase/blast crisis (BP/BC), where haemopoietic differentiation has become arrested and immature blasts accumulate in the bone marrow and spill into the circulation, with over >30% blasts in bone marrow and >50% in peripheral blood. BP is characterized by extra-medullary blastic infiltration, defined as leukemic infiltration in organs other than liver, spleen, and lymph nodes and that is represented by a population of myeloid or lymphoid differentiation-arrested progenitor cells. These cells show enhanced proliferation and survival associated with resistance to chemotherapy and poor prognosis.⁶ Before the advent of tyrosine kinase inhibitors the entire continuum from CP to BP lasted on average 3 to 5 years, but the range was wide. Patients may present in any of these three phases and some of them progress directly to BP without an intermediate AP. The chapters below describe more in depth the triphasic nature of CML and the clinical

features of its phases.

I.I Pathogenesis of Chronic Myeloid Leukaemia

I.I.A. The *BCR-ABL1* Fusion Gene

Several experimental models have established a causal relationship between *BCR-ABL1* and CML. *BCR-ABL1*-expressing CD34 + cells in culture demonstrated several features characteristic of primary CML progenitors including increased proliferation in committed and primitive progenitor culture, reduced adhesion to fibronectin, and reduced chemotaxis to stroma-derived factor-1.⁷ Retrovirally transduced *BCR-ABL1*- positive murine cells generated mice that developed several hematologic malignancies: prominent among them was a myeloproliferative syndrome closely resembling the chronic phase of human CML.⁸ Inactivation of *ABL1* kinase activity by substituting a lysine with arginine at residue 1176 (K1176R) in the ATP-binding pocket, confers a non-leukaemic status to *BCR-ABL1*- expressing mice, even when the mutant is expressed in hemopoietic stem cells (HSCs).⁹

The breakpoints within the *ABL* gene take place either upstream of exon 1b, downstream of exon 1a, or, more frequently, between exons 1b and 1a (fig1.1).¹⁰

Variable breakpoints take place also within the *BCR* gene, all occurring within introns, leading to the formation of different *BCR-ABL1* fusion gene variants. In most patients with CML and in one-third of those with Ph-positive B-cell acute

lymphoblastic leukaemia (Ph⁺ B-ALL), the breakpoints within *BCR* map to a 5.8-kilobase (kb) area spanning exons e13-e14 (formerly called *b2-b3*) or can affect the intron downstream of exon 19.¹¹ The former is referred to as the major breakpoint cluster region (M-*BCR*). In CML patients, alternative splicing gives rise to different fusion transcripts, which encode for distinct proteins: p190^{BCR-ABL1} (M_r 190,000) (less than 5% of cases), p210^{BCR-ABL1} (M_r 210,000) (in 95% of cases), and p230^{BCR-ABL1} (M_r 230,000) (in granulocytic leukaemia, less than 1%). In about 95% of the CML patients the *BCR-ABL1* fusion transcripts *e14a2* (former *b3a2*) and *e13a2* (former *b2a2*) can be detected (reviewed in Barnes *et al.*, 2002). The translated product is in each case a p210 kD BCR-ABL1 protein.¹² In patients with Ph⁺ ALL a shorter transcript version, called BCR-ABL-e1a2, predominates, leading to the production of a p190^{BCR-ABL} protein.

Both BCR-ABL1 proteins p190 BCR-ABL1 and p210 BCR-ABL1 are characterized by a dramatically increased tyrosine-kinase activity, as compared to that of normal ABL protein, leading to aberrant phosphorylation of downstream target molecules. This abnormal BCR-ABL1 kinase activity is assumed to be sufficient and necessary to initiate CML, leading to stimulation of mitosis, increased genetic instability, decreased stromal regulation and finally inhibition of apoptosis.¹³

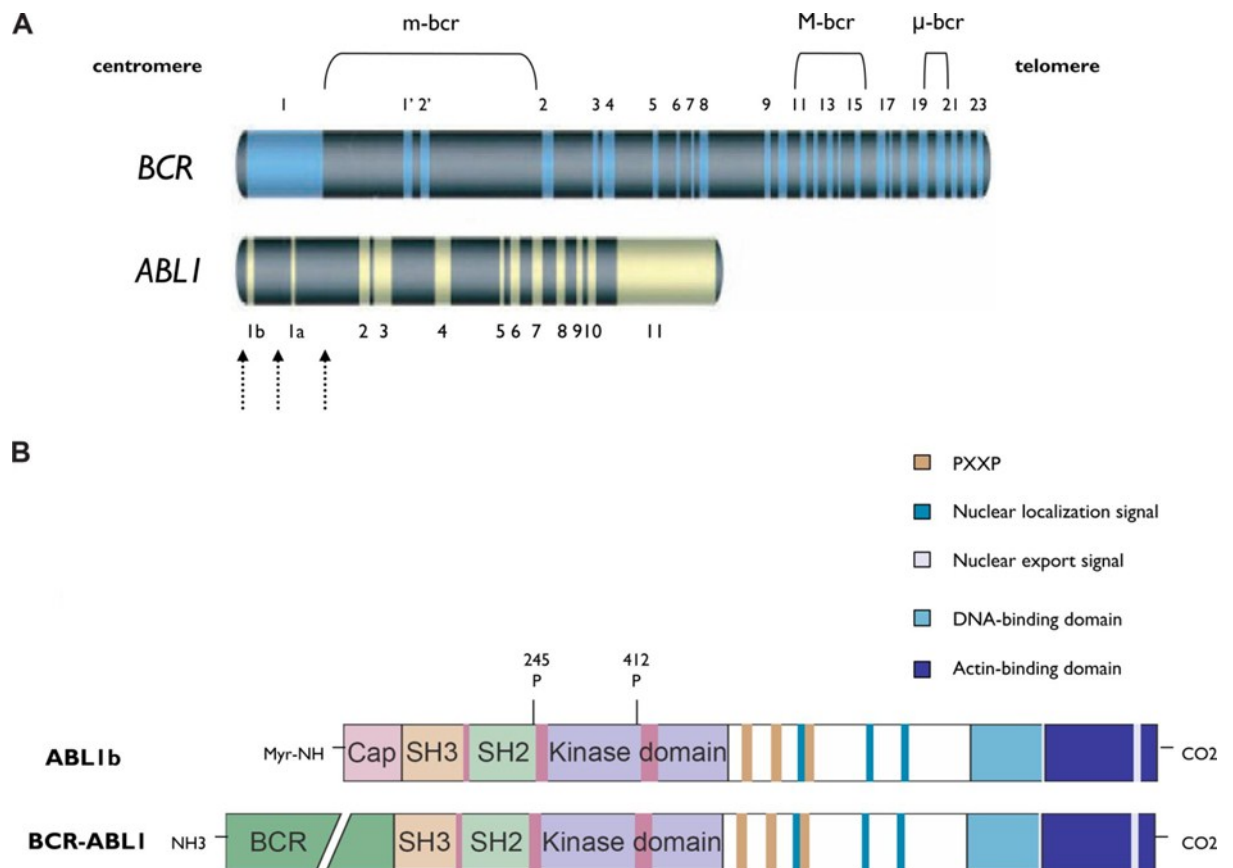


Fig1.1. Schematic representation of the ABL1 and BCR genes and the BCR-ABL1 kinase (from Quintas-Cardama, Blood 2009). (A) BCR contains 23 exons. The 3 breakpoint cluster regions (m- BCR, M-BCR and μ -BCR) are represented. *ABL1* contains 2 alternative first exons (1b and 1a) and the arrows represent the three different breakpoints in *ABL1*. The combination of different breakpoints in the 2 genes creates different fusion transcripts with distinct molecular weight (P190, P210, P230). (B) Structural modularity of ABL1b and BCR-ABL1 kinases. ABL1 kinase is constituted by a central core composed of a tyrosine kinase domain, SH2 and SH3 domains and, at the NH₂-terminus a “cap” region. Two isoforms of ABL1 (human types 1a and 1b) are generated by alternative splicing of the first *ABL1* exon. ABL1b contains a myristate site (Myr-NH) at the extreme end of the NH₂ terminal, which binds to the KD and keeps the SH2-SH3 autoinhibitory structure in place (i.e. in the “off state”).

I.I.B. Anatomy and autoregulation of the BCR-ABL protein

BCR-ABL1 kinase contains a series of functionally distinct domains (Fig.1.1). The NH2-terminus of BCR-ABL1 includes the “Cap” region, which is present in two different isoforms created by alternative splicing of the first exon, named 1a and 1b. ABL1b, differently from ABL1a, is expressed at higher levels and myristoylated at its extreme end of the amino-terminal region. In addition, ABL1 contains a tyrosine kinase domain, preceded by highly conserved Src homology motifs 2 and 3 (SH2 and SH3),¹⁴ a DNA-binding domain, an actin-domain, as well as 3 nuclear localisation signals and 1 nuclear export signal which determines ABL1 subcellular localisation in response to environmental stimuli.

I.I.C. Signalling pathways downstream of BCR-ABL1 kinase

BCR-ABL1 induces activation of numerous downstream signalling pathways activated by BCR-ABL1, all contributing to the oncogenic transformation of leukocytes in CML.¹⁵ Phosphorylation of BCR Tyr 177 serves as a docking site for the SH2 domain of GRB2, which binds GRB2 and SOS, recruiting in turn RAS and GAB2. Because the former is essential for the mitogenic RAS-MAPK pathway activation, this results in transcription of *BCL-2* gene.¹⁶ In turns, GAB2 causes constitutive activation of the PI3K, which through a signalling pathway increased transcription of MYC gene and stabilizes MYC protein. BCR-ABL also activates the SRC family kinases HCK and LYN that recruit STAT5, which modulates the antiapoptotic *BCL-X* gene transcription.¹⁷ Therefore, the net effect of BCR-ABL1 kinase activation is the promotion of both cell proliferation and survival.

I.I.D. Epidemiology

Chronic myeloid leukaemia represents approximately 15% of all types of adult leukaemias, although the ratio is higher in East Asia and Japan, where chronic lymphocytic leukaemia is more infrequent. Its incidence is of about 2 cases every 100,000 people per year, with a median age at presentation of 45 to 55 years. From 12 to 30% of patients are 60 years of age or older. Although the mechanisms of disease progression remain largely unknown, the risk of developing CML is higher following exposure to radiation. This is evident from studies of survivors of the atom bomb explosions in Japan in 1945 and from the follow-up of patients treated with radiation for ankylosing spondylitis and cervical cancer.

There is no known association of CML with any infectious agent or chemical exposures. No familial predisposition has been implicated in CML but there are isolated reports of CML occurring in first degree relatives of affected individuals. Relapse of CML originating in donor cells following a related donor allogeneic stem cell transplant (SCT) has been recorded.

I.I.E. Clinical manifestations

Chronic myeloid leukaemia if left untreated leads to death within a few years. The most consistent clinical laboratory manifestation is hyperleukocytosis. CML is a triphasic disease. Patients are typically in the chronic phase when CML is diagnosed, whose duration is variable and depends time of diagnosis as well as the therapies used. Invariably the disease progresses eventually to a more advanced phase which is usually

sub-classified as either *accelerated phase* – an intermediate phase, characterised by a more insidious transformation that can last for month or years - or *blastic transformation*, with the latter being especially resistant to therapy, resembling an acute leukaemia and leading to death within 6 to 8 months.

Chronic phase

90% of CML patients present in chronic phase and in recent years up to 50% of them have been diagnosed before the onset of symptoms thanks to the increasing awareness of today's population undergoing routine medical check-ups. When present, symptoms may include fatigue, increased sweating, fever, or bone pain. 50-70% of individuals at CML diagnosis show splenomegaly. The spleen extends considerably in length, resulting in the occupation of all the left side of the abdomen and occasionally becoming palpable in the right iliac fossa.

Accelerated and blastic phases

In contrast to the patients in chronic phase, patients in advanced phases nearly always will present some of or all the features described above, with fever and bone pain being the most frequent. In addition there may be episodes of infection and the liver and spleen are usually highly enlarged in size.

I.I. F. Diagnosis

The diagnosis of CML is generally straightforward and is obtained from the typical appearances of the peripheral blood and bone marrow. The most obvious and common features of chronic myeloid leukaemia are neutrophil leukocytosis basophilia. The detection of the Ph⁺ chromosome through cytogenetic analysis is confirmatory. About 5% of patients do not present the Ph⁺ chromosome and

confirmation of diagnosis is dependent on finding the *BCR-ABL1* rearrangements by either reverse transcription polymerase chain reaction (RT-PCR) or fluorescent-in-situ hybridisation assays. In a lower minority of cases, however, no Ph+ chromosome or *BCR-ABL1* transcripts can be detected, and they are referred to as Philadelphia negative (Ph-) and BCR-ABL negative, or also atypical CML, and they are considered as a separate disease entity.

Quantitative real-time PCR (qRT-PCR) thanks to its high sensitivity represents a suitable assay to monitor residual disease after cytogenetic remission. Considerable efforts have been pursued to standardise PCR techniques by definition of an internal standard to quantify *BCR-ABL1* levels and of an international set of recommendations from the European LeukemiaNet (table 1A).¹⁸

Table 1A. Current recommendations for monitoring chronic-phase CML during imatinib therapy, modified according to the recommendations from the European LeukemiaNet.

TABLE 1		
Definitions of response^{*1}		
	Definition	Monitoring (CP CML, IM therapy)
CHR (complete hematological response)	– WBC <10 G/L – Platelets <450 G/L – No granulocyte precursors in differential blood count – <5% basophils (all parameters are for peripheral blood) – Spleen not palpable	Every 2 weeks until CHR, then every 3 months
Cytogenetic response (bone marrow)	– Complete (CCyR): 0% Ph+ – Partial (PCyR): 1–35% Ph+ – Minor CyR: 36–65% Ph+ – Minimal CyR: 66–95% Ph+ – No CyR: >95% Ph+	Months 3 and 6, then every 6 months until CCyR achieved and confirmed, then every 12 months ^{*2}
Molecular response (qRT-PCR, peripheral blood)	– Complete (CMR) not detectable – Major (MMR) bcr-abl/control gene – ≤0.10	Every 3 months, every 6 months with CCyR + MMR

I.I.G. Prognostic factors for non-transplant therapy

The disease phase is the strongest prognostic factor in chronic myeloid leukaemia, with diagnosis in accelerated or blast crisis phases showing a dramatically worse outlook. In regard to CML in chronic phase, various scoring systems have been devised in order to predict the prognosis of individual patients. The most widely used is the final model arising from the Sokal score (developed in 1984), whose data were analysed from 813 patients, most of which received busulphan. According to four weighted clinical and laboratory values (age, spleen size, platelet count, and peripheral blood blasts cell percentage), patients can be classified into three prognostic groups for a 4-year survival; with a survival probability up to 62%, 43% and 33%, patients were segregated respectively into low-risk, intermediate-risk and high-risk groups. The Sokal score in an individual patient is no more than the relative risk (RR) for survival and it is based on the Cox proportional hazards equation using four patient variables (namely platelet count, age, blast count and spleen size). This model has been recently revised with a new prognostic classification, the Hasford score, which derived from the study of over 900 CML patients on interferon (IFN) and calculated using the classic four Sokal variables together with two others, namely blood percentage of eosinophils and basophils (Table 1B). In the new scoring system, patients were classified into the low-, intermediate- and high-risk groups according to a 5-year survival probability of 76%, 55% and 25% respectively. More recently, a new scoring system (European Treatment and Outcome Study [EUTOS] score) was developed by the European Leukemia-Net using data from 2,060 patients who were treated with imatinib-based regimens. The EUTOS score is similar to

the Sokal score, and patients are classified into two different prognostic groups (i.e. high and low risk) on the basis of the percentage of basophils in the peripheral blood and the spleen size at diagnosis.¹⁹ It has been suggested that the EUTOS score has superior prognostic power when compared with the Sokal and Hasford scores.¹⁹ However, a Swedish study reported that the Sokal but not the EUTOS score predicted relative survival in CML patients.²⁰

Ideally, a prognostic classification score should be able to identify patients with poor risk independently of whether patients are treated in the community or part of clinical trials. The Sokal score has been used for nearly 30 years and has been validated extensively in different clinical settings and therapies; clinicians need to be cautious before adopting a new prognostic system that has not yet been validated in a truly independent manner.

Table 1B. Main prognostic risk scores in chronic phase CML

Age is expressed in years, spleen in cm below costal margin, platelets in $10^9/\text{litre}$, and blasts (peripheral blood) as a percentage. The relative risk (RR) according to Sokal is based on patients treated with conventional chemotherapy and it is expressed as exponential of the total. The RR according to Hasford is defined based on patients treated with INF-based regimes and it is calculated as total x 1000.

	Calculation by Sokal et al	Calculation by Hasford et al
Age	$0.116 \times (\text{age} - 43.4)$	0.666 when age ≥ 50 yrs
Spleen	$0.0345 \times (\text{spleen} - 7.51)$	$0.042 \times \text{spleen}$
Platelet count, $\times 10^9/\text{L}$	$0.188 \times [(\text{platelet count} / 700)^2 - 0.563]$	1.0956 when platelet count $\geq 1500 \times 10^9/\text{L}$
Blood myeloblasts, %	$0.0887 \times (\text{myeloblasts} - 2.10)$	0.20399 when basophils $>3\%$
Blood basophils, %	NA	$0.0413 \times \text{eosinophils} >3\%$
Blood eosinophils, %	NA	
Relative risk (RR)		
Low	<0.8	≤ 780
Intermediate	$0.8 - 1.2$	$781 - 1480$
High	>1.2	>1480

I.I.H. Disease progression and transformation

The mechanisms of progression and transformation in CML to blast phase are complex and not entirely understood, partially still due to the lack of adequate animal models of BCR-ABL-induced leukaemia with a long, human-resembling chronic phase, necessary for right evaluation of the mechanisms involved in disease evolution. Numerous factors have been implicated as critical in the transformation from chronic to blastic phase CML.

A) ***BCR-ABL1 expression***: the constitutive expression of the *BCR-ABL1* fusion gene is paramount in almost certainly causing CML and for its evolution. In fact, *BCR-ABL1* expression is considerably higher in BP than in CP.²¹ Although the reason for the increment of *BCR-ABL1* transcript levels is not well understood, this phenomenon may result from selective pressure favouring the expansion of highly proliferative/poorly differentiated leukaemic clones.

B) ***Differentiation arrest***: it has been demonstrated that *BCR-ABL1* suppresses the expression of *CEPB α* , member of the basic leucine zipper family of transcription factors that is essential for normal granulocytes differentiation. Although *CEPB α* is normally expressed in CML-CP samples, its expression is undetectable in CML BP cells,²² suggesting that a lack of *CEPB α* activity reflects the emergence of a more primitive population.

C) ***Genomic instability***: still to be resolved is the dilemma of whether the accumulation of additional chromosomal abnormalities in CML is driven by BCR-ABL1 and/or by pre-existing mechanisms responsible for the t(9;22) itself. However,

what we know so far is that the mechanisms of genome surveillance and DNA repair are impaired in CML, which could explain the occurrence of the chromosomal aberrations that characterise the blastic transformation process. At the heart of the genome surveillance system are the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) nuclear protein kinases that function as DNA damage “sensors”. In CML only ATR has been documented to be inhibited.²³

Following exposure to cytogenetic agents, such as etoposide, BCR-ABL1 from its cytoplasmic localisation has been shown to translocate to the nucleus and bind ATR.²³ ATR is inhibited leading to a reduced activation of its substrate, checkpoint kinase 1 (CHK1), hence the abrogation of the intra-S-phase cell cycle checkpoint.²⁴

Moreover, it has been demonstrated that the BCR-ABL1 induces endogenous reactive oxygen species (ROS), which results in chronic oxidative damage, double-strand breaks (DSBs) and mutagenesis. The repair of DNA DSBs is taken over in mammalian cells by two mechanisms: non-homologous end-joining (NHEJ) and homologous recombination (HR). Both of these pathways have been indicated to be faulty in CML,²⁵ suggesting that BCR-ABL1-mediated ROS generation in combination with aberrant genome repair leads to a mutator phenotype in CML cells and results in genomic instability.²⁶ However, we cannot exclude the possibility that pre-existing conditions, which lead to t(9;22), may work in concert with BCR-ABL to accumulate chromosomal errors.

D) ***Telomere shortening***: typical of many other solid tumours and leukaemias, in CML the rate of telomere loss increases with disease progression from CP to BP. The degree and rate of shortening has also been found to be prognostic for the

length of time to disease evolution.²⁷ It just remains an open question whether telomere shortening is a cause of disease evolution in cancer (for an as yet unknown mechanism), or whether it is merely a symptom caused by the turnover of malignant cells.²⁸

E) ***Loss of tumour suppressor function:*** TP53 plays a major role in chronic myeloid leukaemia. In particular, isochromosome 17, resulting from the loss of chromosome 17 short arm where TP53 maps, is one of the most frequent alterations detected during disease progression from chronic phase to the blast crisis.^{29,30} Another tumour suppressor gene that is associated with CML progression is the serine/threonine phosphatase 2A (PP2A), a major cellular serine/threonine phosphatase working to down-regulate activation of mitogen-activated protein kinase cascade and the PI3K pathway. The activity of this gene in BP is negligible, while only moderately impaired in CML CP CD34+ cells. BCR-ABL1 kinase inhibits PP2A by up-regulation of SET (I2PP2A), a nucleus/cytoplasm-localised phosphoprotein that inhibits PP2A, activity that is mediated via the heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1). SET expression has been found to be markedly high in solid tumours and leukaemias.³¹ It is possible that the expression of active PP2A is incompatible with disease progression in CML, as this phosphatase recruits and activates the Src homology region 2 domain containing protein tyrosine phosphatase 1 (SHP1), which catalyses the dephosphorylation of BCR-ABL1. This, in turn, promotes the downregulation of the latter through proteasomal degradation.³¹

Although not a universal requirement for disease progression, abnormalities of other tumour-suppressor genes are associated with blastic transformation in subgroups of patients with CML.

I.II. Management of Chronic Myeloid Leukaemia

I.II.A Assessment of response to therapy

As for any disease, the ultimate measure of the efficacy for each therapy is judged by its individual impact on overall survival (OS) and event-free survival (EFS). Nevertheless, for a tumour with a relatively long disease course such as CML, an earlier assessment of efficacy is generally evaluated through the use of surrogate markers. Without receiving any treatment, a CML patient generally survives between approximately 3 to 5 years. It is possible to define three levels of disease control in CML. Complete haematological response (CHR) defines the normalisation in count and differential of the white cells, together with the disappearance of all disease symptoms and the resolution of prior splenomegaly. Complete cytogenetic response (CCyR) classifies the absence of Ph⁺ metaphases by using classical cytogenetics, with at least 20 metaphases available for analysis, whereas major cytogenetic response (MCR) specifies the presence of less than 35% Ph⁺ metaphases. Partial cytogenetic response (partial CyR) is defined as the absence of detectable BCR-ABL1 mRNA in real-time reverse transcriptase quantitative PCR (RT-PCR) and is not equivalent to eradication of leukemia since there may be minimal residual disease (MRD) below the lower limit of detection of the test.³² The paramount treatment goals for CML include the eradication of the leukaemic clone from the bone marrow, or prolongation of CP with sustained remission and minimal drug toxicity. To date, however, the only proven therapy resulting in a complete eradication of the malignant clone from

the bone marrow is allogeneic stem cell transplantation, while first- or second-generation tyrosine kinase inhibitors obtain primarily results in the control of the CP and in only 5% of patients we have evidence of eradication of the disease by the most sensitive technique used to date, molecular monitoring.

I.II.B CML treatment before imatinib

The first arguably effective treatment of CML is represented by radiotherapy, introduced in clinical practice at the beginning of 1900s and remaining standard therapy for over five decades. By the 1960s, radiotherapy was replaced by oral alkylating agents, whose rapid development led to the introduction of busulphan followed by interferon, which together with the introduction of cytarabine in the late 1990s was part of the conventional cytoreductive therapy, with which it became possible to produce sustained cytogenetic responses in up to one-third of patients.³³

Although one randomised study indicated that the combination of interferon and cytarabine is more effective than interferon alone,³⁴ it was not confirmed by a subsequent clinical trial, which showed no improvement in overall survival.³⁵ Using RT-PCR, BCR-ABL1 mRNA is still detectable in patients treated with INF, indicating that these long-term remissions amount only to a clinical cure, resulting in durable hematologic and cytogenetic remissions, rather than a biological cure of the disease (resulting in molecular evidence absent of very limited residual disease).

Hematopoietic stem cell transplant (HSCT), is so far the only treatment capable of disease eradication, with the majority of patients achieving RT-PCR negativity. Long-term disease free survival is in a range of 50 to 80% in most studies.³⁶ However, allografting has its limitations, due to significant transplant-related morbidity and mortality.

I.II.C ATP-competitive BCR-ABL1 inhibitors

Current established first-line therapy for all phases of CML is imatinib mesylate (Glivec/Gleevec/IM/STI-571; Novartis), a 2-aminophenylpyrimidine compound that can specifically target and inhibit the kinase activity of both normal ABL1 and oncogenic BCR-ABL1. IM is able to inhibit ABL1, platelet derived growth factor receptor α and β (PDGFR α , PDGFR β), and Kit. Therefore, IM may be effective in targeting all the fusion proteins containing these activated kinases, including BCR-ABL1, Ph+ ALL, FIP1L1-PDGFR α , and ETV6-PDGFR β respectively in CML, eosinophilic leukaemia, chronic myelomonocytic leukaemia and some infrequent forms of CML.³⁷ In regard to the mechanism of action, IM binds to the inactive, closed conformation of ABL1 kinase domain. IM is wedged between the N-lobe and C-lobe of the ABL1 kinase. Part of the IM molecule occludes the ATP binding site with the remaining part further penetrating into the hydrophobic region of the kinase and jamming itself between the activation loop and helix- α C (fig. 1.2). ATP is thus prevented from reaching its binding site, holding the kinase in an inactive status. The link between IM and BCR-ABL1 is

stabilised by the formation of six hydrogen bonds involving the aminoacid (AA) residues Met318, Thr-315, Glu-285, Asp-381, Ile-360 and His-361. The bond is further stabilised by the formation of a number of van der Waals interactions, through the AA residues Ile-293. A hydrophobic cage is completed with AA residues Leu-370, Leu-298, Leu-354, Val-379, and Phe-382 around the phenyl ring adjacent to the piperazinyl-methyl group of imatinib.

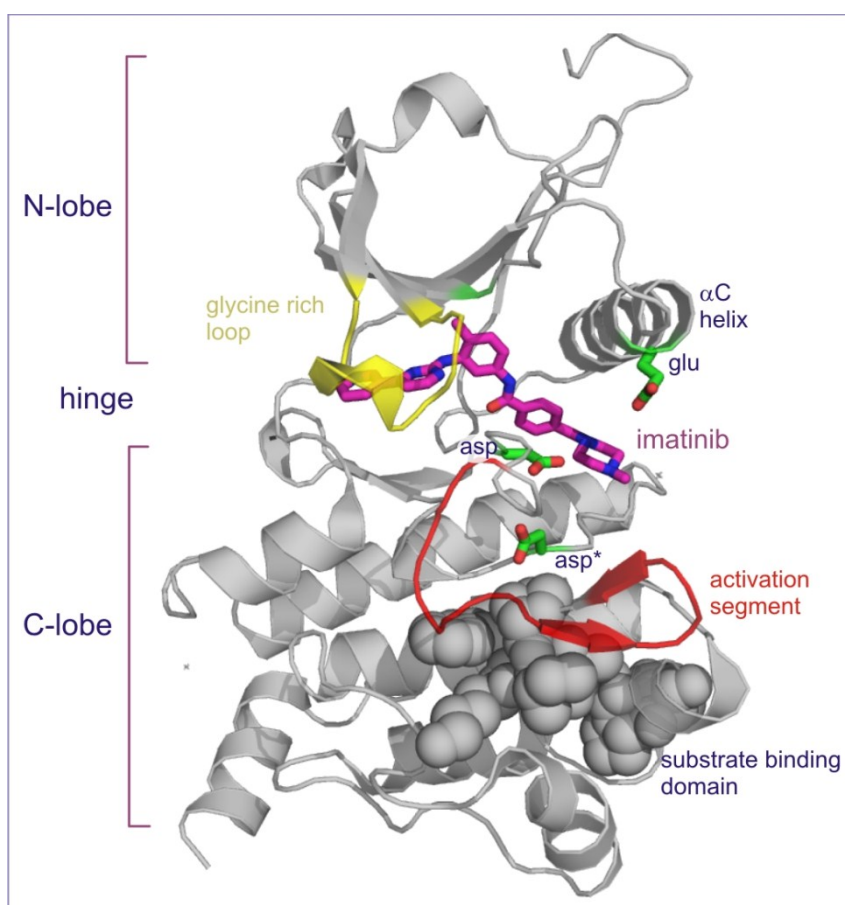


Fig. 1.2: Binding of Imatinib to the tyrosine kinase Abl (from <http://www.cellbiol.net/stp/rpimages.php>). Only the catalytic domain of the tyrosine protein kinase Abl is shown. Imatinib binds the ATP binding pocket of the kinase in its inactive conformation. The activation segment is not phosphorylated and covers the substrate binding site. In fact, imatinib prevents outward movement of the activation segment.

Thanks to its remarkable efficacy, since its introduction it has revolutionised the management of CML, regardless of disease phase. Recent studies have identified the presence of small numbers of primitive “quiescent” cells that are refractory to the pro-apoptotic effect of both Glivec and conventional chemotherapy agents. The insensitivity of these non-dividing stem cells has also important implications on CML management regarding the relapse following imatinib-induced response.³⁸

Nilotinib (AMN107/Tasigna; Novartis) is a phenylamino-pyrimidine derivative that is structurally related to imatinib. Nilotinib is 10-30 fold more potent than imatinib in inhibiting activity of the Bcr-Abl tyrosine kinase and proliferation of Bcr-Abl expressing cells. The drug effectively inhibits the auto phosphorylation of Bcr-Abl on Tyr-177 that is involved in CML pathogenesis. Synergistic activity of imatinib and nilotinib has been reported following coadministration. This might be a result of the fact that the drugs are taken up in cells by different mechanisms: imatinib influx is dependent on OCT1 but nilotinib is not. Nilotinib is also not a substrate for the efflux transporter P-glycoprotein pump, unlike imatinib. Resistance to nilotinib is associated with a limited spectrum of Bcr-Abl kinase mutations that mostly affect the P-loop and T315I.³⁹

Dasatinib (BMS-354825, Sprycel; Bristol-Myers Squibb) is a potent, oral inhibitor of BCR-ABL1, which in addition to bosutinib binds to SRC-family kinases (SFKs), which may contribute to CML disease progression and treatment resistance.⁴⁰ Dasatinib is 300 times more potent than imatinib and is effective against the imatinib-resistant active conformation of the kinase domain.

Haematological and cytogenetic responses are long-term in patients with chronic phase disease, but response durability decreases in the advanced phases.^{41,42,43} Despite causing a significant reduction in the total number of CD34⁺CD38⁻CML blasts compared to imatinib, dasatinib still fails to eliminate the primitive quiescent SC population.⁴⁴ Bosutinib (SKI-606; Wyeth) is another dual Src-family kinase/abl inhibitor, with a potent antiproliferative effect against imatinib-sensitive and-resistant BCR-ABL1⁺ neoplastic cells and effective against both inactive and intermediate conformations of BCR-ABL1.⁴⁵ SKI-606 is effective in suppressing the proliferation of CML progenitors, but is moderately capable in inducing apoptosis of the primitive, quiescent fraction. Furthermore, unlike dasatinib and nilotinib, bosutinib does not have effect on Kit and PDGFR and has more favourable toxicity profile.⁴⁶ Several other compounds have been developed, but will not be described here as they are not yet in routine clinical practice.

I.III Mechanisms of resistance to TK inhibitors in CML

Several studies have showed that a long-term favourable outcome of CML is more likely to be achieved when IM is given in newly diagnosed patients in chronic-phase disease. Patients passed to IM as second-line therapy after IFN failure, showed less convincing sustained remission rates. Nonetheless, even a consistent number of newly diagnosed patients experience treatment failure. Out of the patients included in the International Randomised Study on IFN and IM

(IRIS), circa 25% failed because of refractoriness (primary resistance), or loss of response after attainment of initial response and progression of the leukaemic clone (secondary resistance), or intolerance. In 2009 expert panels of the European LeukemiaNet updated the definition of treatment failure as based on haematological, cytogenetic and molecular responses, taking into consideration the time on therapy.¹⁸ The initial goal is represented by the normalisation of peripheral blood (PB) counts in association with less than 5% of blasts in PB, ideally after three months or less of treatment, named complete haematological response (CHR), which is the earliest monitoring point. Response to treatment is then monitored by bone marrow metaphase chromosome analysis (using at least 20 metaphases), which determines a cytogenetic response, whose level is based on the number of Ph⁺ metaphases. Absence of Ph⁺ metaphases defines a complete cytogenetic response (CCyR), which should be attained by 12 months. After CCyR achievement, further monitoring of BCR-ABL1 is then done by either by fluorescence-in-situ hybridisation, with a sensitivity of up to 10^{-3} or, preferably, by a quantitative real-time PCR assay, with a detection sensitivity of 10^{-5} .¹⁸

Finally, patients should achieve a three- or more log reduction in the concentration of BCR-ABL1 transcripts compared to a standardised baseline (being by definition a value representing the initial level of a measurable quantity, e.g BCR-ABL1 transcript initial level), which is now become synonymous with major molecular response (MMR). MMR is generally regarded as a prognostically favourable milestone for the response to IM, whereas failure to achieve it within 18 months is commonly considered as suboptimal response or treatment failure, meeting the definition of drug resistance. PCR-negativity indicates the ultimate milestone of response to treatment and it is defined as complete molecular response (CMR). Unfortunately even PCR negativity cannot be a guarantee of disease eradication:

treatment interruption in patients with minimal residual disease has led to disease recurrence and onset of resistance in approximately 60% of patients.⁴⁹

The existence of patients resistant to TK inhibitors has been evident since the introduction of the drugs into clinical practice. In contrast to chronic phase, in blastic phase disease primary resistance is common and durable responses are the exception.^{47,48,40,49} These findings led to considerable efforts to generate a model of the development of resistance. Resistant cell lines were created through exposure to progressively higher concentrations of imatinib. The mechanisms of clinical resistance to IM are generally complex and likely to be a result of extrinsic causes (i.e. increased plasma concentrations of α 1 acid glycoprotein, which binds the drug and decreases its effective concentration), and/or intrinsic resistance to the treatment. Intrinsic resistance to IM is likely to be a multifactorial process and can be postulated in two distinct types: a) the leukaemic cells survive and proliferate independently of BCR-ABL1; b) the leukaemic cells still need BCR-ABL1 but somehow elude the inhibitory effect of the drug. As every TKI is given orally, mechanisms identified include variations in oral bioavailability, as well as: plasma-protein binding; changes in intracellular drug availability; enzymatic inactivation; increased expression of BCR-ABL1; presence of the refractory quiescent stem cells;⁵⁰ and changes in expression or mutations of target molecule.

I.III The problem of resistance to TK inhibitors in CML

Several studies have shown that outcome of CML is best when IM is given in newly diagnosed patients in chronic-phase disease.^{51,52} Patients transferred to IM as

second-line therapy after IFN failure, showed less convincing sustained remission rates. Nonetheless, a number of newly diagnosed patients experience treatment failure. Out of the patients included in the International Randomised IFN and IM Study (IRIS), circa 25% ‘failed’ because of refractoriness (primary resistance), or lost response after attainment of initial response and progressed (secondary resistance), or showed intolerance.

In contrast to chronic phase, in blastic phase disease, primary resistance is common and durable responses are the exception.^{47,48,41,49} These findings led to concerted efforts to generate a model of the development of resistance. Resistant cell lines were created through exposure to progressively higher concentrations of imatinib. The mechanisms of clinical resistance to IM are generally complex and likely to be a multifactorial process and can be described in two distinct types: a) the leukaemic cells survive and proliferate independently of BCR-ABL1; b) the leukaemic cells still need BCR-ABL1 but somehow elude the inhibitory effect of the drug. As every TKI is given orally, mechanisms identified include variations in oral bioavailability, as well as: plasma-protein binding; changes in intracellular drug availability; enzymatic inactivation; increased expression of BCR-ABL1; presence of the refractory quiescent stem cells;⁵⁰ and changes in expression or mutations of the target molecule. Each of these mechanisms of resistance to IM are distinguished and examined in detail below.

I.III.A Increased expression of *BCR-ABL1*

Mahon and colleagues⁵⁴ showed that as the most frequent mechanism of resistance

in vitro is increased expression of BCR-ABL1. This was achieved by creating a resistant cell line, LAMA-84R, derived from a blast crisis CML patient and subsequently exposed to increasing concentrations of IM.

In clinical practice increased mRNA and protein expression of BCR-ABL1 appear to be a less recurrent phenomenon. The chance of gene amplification is likely to be the result of genetic instability with a rate of occurrence of 10^{-4} per cell division (against 10^{-9} per base per cell division attributed to the point mutations). This rate, however, is only followed in cell lines and point mutations appear in practice as a more common cause of resistance. In a large study of 66 patients with primary and secondary resistance, only 2 of them were found presenting *BCR-ABL1* gene amplification. Increased levels of BCR-ABL1 protein expression usually correlate with progressive disease and dose escalation of IM has been shown to help restore responsiveness in such patients.⁵⁵

I.III.B Drug efflux and influx

The role of some membrane pumps among various patients has been thought as an aetiology for varying IC₅₀ of IM, resulting in heterogeneous rates of response. It has been quite well established that IM is a substrate for the efflux channel P-glycoprotein (Pgp), also called ABCB1 (ATP-binding cassette sub-family B member 1) and MDR1 (multidrug resistance 1).⁵⁶ Making again use of the cell line LAMA 84-R, Mahon *et al.* reported an increase in ABCB1 expression. Subsequently, by exploiting two different inhibitors of this pump, Che and Kotaki

managed to restore sensitivity to IM in BP samples.⁵⁷ However, in follow-up studies, failure of attempts to demonstrate a causative role of Pgp expression levels in determining specific rates of clinical response to IM,⁵⁸ raised the suspicion that Pgp expression may not be a clinically relevant marker of resistance in CML patients. The major group of uptake transporters belong to the solute carrier (SLC) super family, which includes 12 organic cation transporters (OCT). Thomas and colleagues⁵⁸ showed that the transport of IM into the cells is an active rather than a passive process and seems to be mediated by hOCT1 protein, an ATP-dependant membrane protein channel. Subsequently Crossman *et al.*⁵⁶, analysing a group of patients who failed to achieve a minor CyR after 10 months of IM therapy, demonstrated reduced levels of hOCT1 in the bone marrow. Furthermore, it was reported that patients presenting the lowest hOCT1 activity were characterised by a markedly lower overall survival and progression-free survival compared with all the other cohorts. However, whether hOCT1 levels have a role in meaningful differences in the rate of clinical resistance is currently unknown.

I.III.C Drug binding

The serum protein α 1-acid glycoprotein (AGP) has been implicated as responsible for IM sequestration in the plasma, this preventing the drug from reaching its target BCR-ABL1 kinase, as demonstrated in mouse models.⁵⁷ However, although it was found that most of the IM-treated CML patients were characterised by elevated plasma AGP levels and these were slower to respond to treatment, yet binding of

IM to AGP has not been demonstrated in CML patients' samples⁵⁸ and AGP is now generally considered being an unlikely mechanism as an aetiology of IM resistance.

I.III.D Clonal cytogenetic evolution

Often related to disease progression, clonal cytogenetic evolution includes the acquisition of additional cytogenetic abnormalities in the Ph⁺ chromosome cell population.⁵⁰ Moreover, the frequency of developing clonal evolution appears to be higher than developing ABL-kinase domain mutations, as supported by a study led by Lahaye and colleagues in 300 patients in different CML phases on IM.⁵⁰

The CD34⁺/CD38⁻ population also seem the most inherently resistant of the stem cells to IM. Several possible explanations exist for this insensitivity, including over-expression of BCR- ABL1 mRNA, protein and kinase activity, as well as an increased expression of Pgp and a highly suppressed expression of hOCT1. Despite being deemed as an attractive explanation for the failure to completely eliminate all leukaemic cells even in the best CML responders, some contradictions remain with this hypothesis. The amount of these primitive cells represent still a relatively low number; CD34⁺/CD38⁻ are unlikely to develop point mutations, since these occur during proliferation. Ultimately, quiescence cannot justify the significantly rapid evolution to advanced phases after molecular response. A possible strategy to eradicate this quiescent compartment could be the combination of a tyrosine-kinase inhibitor with another signal- transduction

inhibitor with the ability to trigger the cell cycle.

I.III.F Epigenetic modification

Gene expression and protein function can be regulated by a balance of epigenetic acetylation and methylation, respectively. Disruption of this balance can promote abnormal cell proliferation and resistance to apoptosis. In a recent study Lee and co-workers⁶¹ documented IM resistance of a CML cell line subsequent to upregulation of Class I and III histone deacetylases (HDACs) and down-regulation of several histone acetyltransferases (HATs). Following this, these cells were treated with the histone deacetylase inhibitor SAHA (Suberoylanilide Hydroxamic Acid), restoring the apoptotic threshold. Although HDAC inhibitors have yet to be translated into clinical therapies for the treatment of IM-resistant patients, they are currently being studied in association with TKIs.

Epigenetic methylation can regulate the expression of genes with tumour-suppression and apoptotic functions. In a recent study it was shown that cell lines with reduced expression of the pro-apoptotic B-cell lymphoma 2 (BCL-2)-interacting mediator, when co-treated with the hypomethylating agent decitabine, showed increased susceptibility to IM. Yet, whether the therapeutic strategy to combine a TKI with a hypomethylating agent in patients with earlier phase disease, can increase resistance rates, has still to be unveiled.⁶¹

I.III.G. Low activity of some tyrosine phosphatases

Down-regulation of some phosphatases is another mechanism of resistance being identified in CML cell lines and documented in patients. As previously discussed in this manuscript, STAT5 is an important modulator of the anti-apoptotic BCL-X gene transcription and its constitutive phosphorylation is critical for proliferation of CML cells. The marked down-regulation of T cell protein tyrosine phosphatase (TC-PTP) in the IM resistant KTR cell line, which by having i(18)(q10) abnormal fusion isochromosomes lacking 18p11.2-11.3, the location of TC-PTP gene, lead to increased phosphorylation levels of STAT5 and increased resistance to IM.⁶³ The forced expression of T cell protein tyrosine phosphatase could restore susceptibility to IM. In another study, Neviani and co-workers described in p210-CML the inactivation of PP2A (the tumour suppressor protein phosphatase 2A), resulting into increased and sustained BCR-ABL1 activity. Restoring PP2A activity, achieved by SET downregulation, affected *in vitro* and *in vivo* wild-type and T315I BCR/ABL leukaemogenesis by antagonising the effects of BCR/ABL on its downstream signal transducers and promoting BCR/ABL inactivation and proteasome-dependent degradation, causing growth inhibition, apoptosis stimulation, and an *in vivo* leukaemogenesis reduction of both IM-sensitive and -resistant CML cell lines and blast crisis patient cells.⁶⁴

I.III.H. SFK Dependent Mechanisms

Although BCR-ABL1 mutations play a major role in IM resistance, treatment failure has also been observed in patients who do not harbour BCR-ABL1 mutations or amplification.⁶⁵ Furthermore, data from expression profiling and in vitro analysis predict the involvement of additional genes in IM resistance and progression to blast crisis, although most of these genes have yet to be thoroughly studied or described in clinical samples of CML patients.

The Src family kinases (SFKs) are non-receptor, intracellular tyrosine kinases that regulate multiple signal-transduction pathways involved in cell growth, differentiation, survival, cytoskeletal organisation, migration and adhesion, as a result of their ability to cross-talk with a number of various classes of cellular receptors and many distinct cellular targets.⁶⁶ SFKs comprise nine highly homologous members expressed in a tissue-specific manner with some of them been strongly linked to BCR-ABL1 signalling and leukaemogenesis.^{67,68} Multiple domains of BCR-ABL1 interact with and activate SFKs independently of BCR-ABL1 kinase activity^{69,70} and increasing evidence suggests that SFKs are involved in BCR-ABL-independent forms of imatinib resistance. Several SFK members including Hck, Lyn and Fyn, are targets of BCR-ABL1. Hck and Lyn are expressed and activated in the acute phase of CML patients, and their up-regulation correlates with disease progression and resistance in patients treated with imatinib.^{71,72} Dai and co-workers showed the role of the SRC kinase Lyn⁷³ in resistance to imatinib in a resistant cell line expressing low levels of BCR-ABL1 protein and mRNA. BCR-ABL1 independent imatinib resistance was

associated with marked activation of Lyn, while there was increased expression of Bcl1 and reduced expression of p-Crkl. Lyn is a potentially important downstream target of BCR-ABL1. Activation of Lyn is associated with a marked increase in the levels of the anti-apoptotic protein Bcl2.⁷³ Pronounced increase in mitochondrial-associated Bcl2 in these resistant cells may contribute to protection against IM mediated mitochondrial dysfunction.⁷³ Fyn is ubiquitously expressed and its expression is higher in blastic crisis than in chronic phase, and occurs in BCR-ABL1-expressing cells in vivo as well as in vitro at the mRNA ^{74,75}and protein level.

Thus, to overcome resistance to imatinib, which targets mainly BCR-ABL1, other compounds have been formulated to target activation of SFKs that is independent of BCR-ABL1. Dasatinib and bosutinib are dual SFK/BCR-ABL1 inhibitors for the treatment of patients that should be useful for imatinib-resistant CML. The introduction of dasatinib or bosutinib has indeed proved effective and the majority of patients achieve a durable CHR. Nevertheless, some subjects do not respond to these second-generation TKIs (2G-TKIs). T315I is the main mutation in the BCR-ABL1 kinase domain that causes pan-resistance to all currently employed TKIs. However, the mechanism of resistance acting in the non-responding patients who lack identifiable BCR-ABL1 mutations is still essentially unknown.

I.III.I BCR-ABL1 Kinase Domain Mutations

One of the most prevalent mechanisms leading to reactivation of BCR-ABL1 is point mutations throughout the ABL1 kinase domain and this might be the cause of resistance in some cases. The association between BCR-ABL1 kinase domain mutations and resistance to TKIs will be thoroughly discussed in the next chapter.

I.IV. Mutations within the ABL kinase domain

As earlier described in this thesis, the mechanism of acquired resistance has been attributed to various possible mechanisms, but certainly the best understood and most commonly documented in clinical practice is the selection of a Ph⁺ clone bearing a BCR-ABL1 kinase domain mutation.⁷⁶ Up to now, more than 50 mutants have been reported,^{77,78,79,80} with some at a higher frequency than others: 15 amino- acid changes account for over 85% of the detected mutations.

Most of the characterised mutations affect specific BCR-ABL1 KD residues that form hydrogen bonds or hydrophobic interactions with IM, hence leading to structural changes such that IM can no longer displace ATP. These kinds

of mutations, hitting amino acid positions involved in the direct bond with IM, such as Y253, T315 and F317, lead usually to strong IM resistance. A second group of mutations is represented by the capacity to shift the equilibrium of the KD toward an active state, thus obstructing IM access to the ATP-binding site; this group includes amino acid exchanges at H396, M388, S348 and M351. Alternatively, mutations can further be clustered according to their localisation among different functional domains. The recognised classification for the kinase domain motifs describes 5 domains: the P loop (also called ATP phosphate-binding loop, including amino acids 248 to 255, the gatekeeper region (occupying residues 299-317), the catalytic domain (spanning 351-359 AA residues), the activation loop (A loop, including AA 384-396), and the C-loop (also called C-terminal lobe, 453-486) (fig.1.3). It has been documented⁸¹ that mutations affecting P-loop and gatekeeper regions (with the T315I in spotlight) develop more frequently (fig.1.4) This categorisation, however, does not allow an estimate of the degree of resistance, and in fact the class of P loop mutations clusters amino acidic exchanges with moderate (G250A, Q252H, and E255D) to high IM resistance (G250E, Y253H, E255V/K). Even mutations occurring in the same residue – e.g. E255, with E255D, E255K, and E255V – can confer different TKI sensitivity.^{82,83} It seems that some AA substitutions may occur in particular CML stages; for example mutations M244, L248, F317, H396, and S417 are more likely to be detected in chronic-phase disease, whereas Q252, Y253, E255, T315, E459 and F486 are associated with advanced phases.⁵⁰ The probability of reporting mutations, however, increases with disease progression and with advanced phase disease. In vitro studies have reported that some mutants (e.g. T315I and E255K) cause one- or two-log increase to their IC50 for IM, compared with wild type BCR-ABL1, hence identification of the

precise mutation may have major clinical relevance. Some mutants, such as T315I and E255K, retain insensitivity to IM at clinically available doses, whereas others, such as M351T or Y253F show partial sensitivity to IM. Specifically, the mutation at position 315 (T315I) is notorious for conferring nearly complete clinical resistance to all currently approved ABL-kinase inhibitors and is mostly detected in advanced phases. Threonine 315 is located at the “gatekeeper” residue in the centre of the ATP-binding cleft, forming a crucial hydrogen bond with IM; the T315 side chain controls the binding of IM to the hydrophobic cage that is close to ATP-binding domain. The absence of an oxygen atom in the substituted isoleucine alters the three-dimensional structure of the hydrophobic cage, hence preventing bond formation with IM and the more recent kinase inhibitors nilotinib and dasatinib.^{76,84} Tyr 253 makes only a weak van der Waals contact with IM, but forms a water-mediated hydrogen bond with the side chain of Asp 322, folding the P loop down and increasing surface complementarity; substitution of Tyr with Phe would not be described as interfering directly with drug binding, but might decrease the affinity of an induced-fit interaction of IM with the ATP-binding site. Mutations of other residues in the ATP-binding site including conversions of Glu 255 to Lys or Val lead to enzymes that are insensitive to IM and have been reported in patients with advanced CML. (Sergei Roumiantsev, 2002) A strong association of the P loop mutations with a poor prognosis has been documented by Branford and colleagues in both chronic and accelerated phases’ CML patients. (Brandford, 2004)

The functional relevance of all these findings is still unclear, especially in terms of whether some amino acid substitutions are responsible for disease evolution, by conferring, for example, a proliferation advantage to the mutated cells, or whether

they simply act as a surrogate marker of the increased genomic instability typical of CML advanced phases. Several laboratories have demonstrated the occurrence of mutations even in untreated patients, and it has been suggested that the selective pressure applied by IM favours the outgrowth of pre-existing resistant clones.⁸⁵ Therefore, early detection of mutations in pre-TKI treatment patients could help to identify cases at high-risk of resistance and to select beforehand the optimal treatment. Biochemical and biological assays conducted by Griswold and colleagues⁸⁶ studied five common KD mutations in distinct KD regions, including Y253F and E255K (within the P loop), T315I (belonging to the gatekeeper region), M351T (within the catalytic domain), and H396P (found in the activation loop), showing that different mutations not only are characterised by distinctive resistance to IM, but also feature specific growth and proliferative capability. Exploiting the BaF3 cell line, these assays described the proliferative ability of the different clones in the following order: Y253H>E225K>WT>T315I>H396P>M351T. These findings may represent an important clarification in understanding the role of some IM-resistant mutations in disease progression from chronic to accelerated phase or blast crisis. Transforming capabilities correlated with kinase activity only for some mutations. Compared with the wild-type BCR-ABL1, Y253F was noted to produce enhanced kinase activity (gain of function) with E255K causing comparable activity. On the other hand, the remaining three point mutations lead to loss-of-function variants, by affecting the enzymatic function, maybe due to a specific selection exerted by the drug.⁸⁷ These data have however found discrepancy with the results published by Skaggs et al,(2006) following investigation of the correlation of oncogenicity and kinase activity in regard to seven frequent and clinically relevant KD mutants T315I/A, E255K/V,

Y253H, M351T, and F317L, together with wild-type p210-BCR-ABL1. In most cases oncogenic potency was associated with kinase activity. An interesting exception was represented by T315I, which showed greater potency capacity but reduced kinase activity in comparison with wild-type BCR-ABL1. Through a global phosphoproteome analysis, revealing the tyrosine phosphorylation profiles, it was possible to identify a unique phosphosubstrate signature associated with each drug-resistant point mutation. This indicates that a single aminoacid conversion in the kinase domain can produce specific effects on substrate recognition. Three groups appeared from this analysis. T315I and E255K systematically showed a 10–20% increase in oncogenicity relative to wild-type BCR-ABL1, demonstrating that they are indeed gain-of-function mutants as suggested by clinical evidence of pre-treatment detection. The second group emerging from this analysis contained Y253F and E255V, which displayed transforming activity similar to wild-type p210. The final group contained four point KD mutations (Y253H, M351T, F317L, and T315A) with consistently reduced potency. Examination of the respective kinase activity of these mutants showed that with the exception of M351T and T315A, having substantial reduced kinase activity, and the two mutants E255K and Y253F, with stronger activity, most kinase inhibitor-resistant mutants exert comparable kinase activity to wild-type BCR-ABL1. To determine the correlation between kinase activity and oncogenicity, $V_{\max}$ (maximum reaction rate) was plotted versus the oncogenicity score that resulted in the bone marrow assays. These two variables were closely related for most of the TKI-resistant mutants with one notable exception: the T315I mutant showed a 20% greater potency than wild-type p210 in bone marrow transformation despite a nearly 50% reduction in $V_{\max}$.

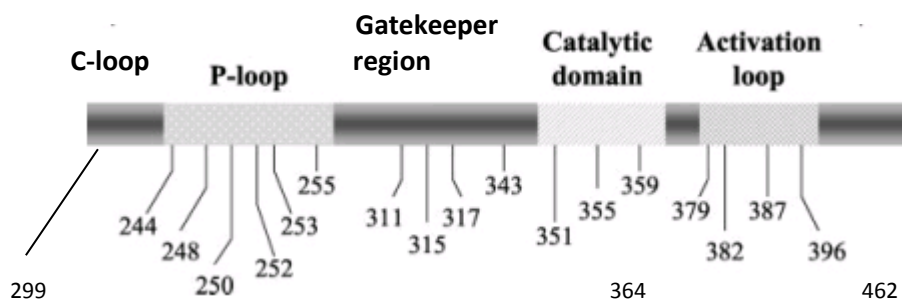
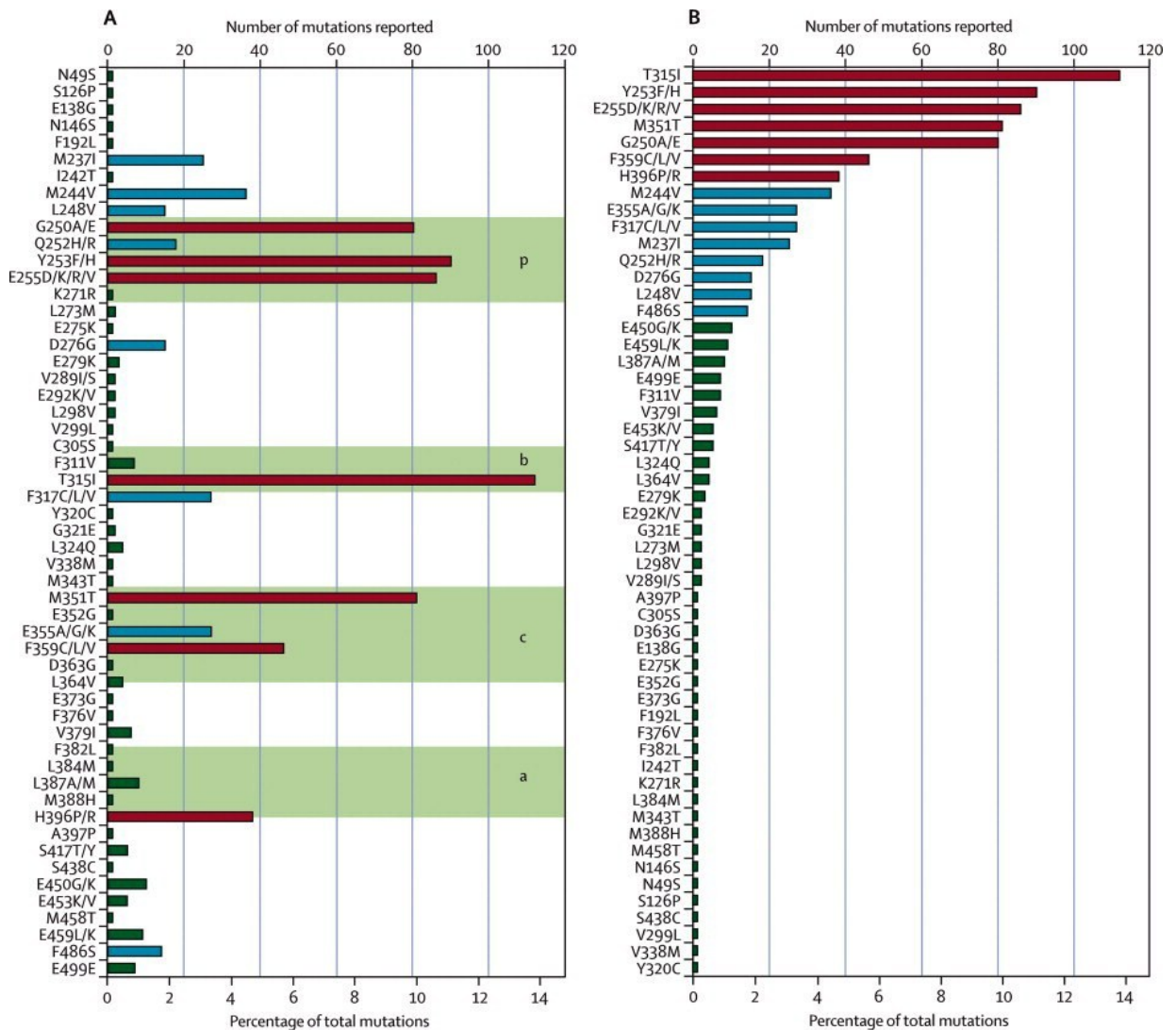


Fig.1.3 Schematic representation of the BCR-ABL1 KD, with the P-loop, Catalytic domain, Activation loop and C-loop. The AA changes identified so far are shown. Fig. adapted from Soverini *et al.*, Clinical Chemistry, 2004.



J Apperley, The Lancet, 2007

Fig.1.4 Incidence of mutations in relation to their domain clustering and their absolute frequency. (A) Incidence of detected tyrosine-kinase mutations by absolute number and by percentage of total. The seven most frequent aminoacid substitutions of BCR-ABL1 KD are depicted in red. Specific domains of the kinase domain are indicated as P-loop (p), gatekeeper region (b), catalytic domain (c) and activation loop (a). (B) Incidence of the same mutations in order of frequency.

II. Aims

The overall aims of this thesis have been:

- 1) Establishment of novel methodologies for the detection of mutations that satisfies the criteria of specificity, sensitivity and rapidity in relation to the data from existing literature, carrying out a comparative test with the well-known direct sequencing technique.
- 2) Systematic evaluation of the mutations arising after treatment with imatinib and second- generation tyrosine kinase inhibitors (2G-TKIs) dasatinib, nilotinib and bosutinib.
- 3) I have hypothesised that the ineffectiveness of treatment with dual SFK/BCR-ABL inhibitors dasatinib and bosutinib might be due to the introduction of point mutations within the tyrosine-kinase domain of one or other of the three Src kinases that have been implicated in the development and progression of CML: Hck, Lyn, and Fyn. Point mutations in the kinase domain of these Src kinases might result in a change of structure that impeded the contact of the drug with the Src kinases in a manner analogous to BCR-ABL1 kinase domain mutations.

Consequently, the aims of this thesis have been the identification of the patients who did not respond to dual SFK/BCR-ABL inhibitors dasatinib and bosutinib and did not present BCR-ABL1 kinase domain mutations, and an investigation into whether SFK KD mutations played a causal role for resistance.

II. Patients and Methods

Patients characteristics and subgroups

II.I Patients and Samples

The samples used in this study were acquired from routine blood samples provided by patients with CML. The majority were obtained during hospital visits to the Hammersmith Hospital and a small proportion was sent from the patients' local hospitals. All patients gave informed consent for the use for research of material remaining from routine blood testing.

Bone marrow morphology and cytogenetics were assessed by other members of the Department at diagnosis and then every 3 months until patients achieved complete cytogenetic response (CCyR). Thereafter patients were monitored by real-time quantitative PCR and annual bone marrow examinations.

Response to therapy was assessed according to the recommendations from the European LeukemiaNet. Complete haematologic response (CHR) was defined as blast count of less than 10% in the bone marrow, no blasts or promyelocytes in peripheral blood, neutrophil count of at least $1.5 \times 10^9/l$ with <5% immature forms (excluding promyelocytes and blasts), platelet count up to $450 \times 10^9/l$, and no evidence of extramedullary involvement. Partial haematologic response (PHR) was defined as blast count on blood and bone marrow less than 15%, neutrophil count, at least $1 \times 10^9/l$ and platelet count at least $20 \times 10^9 /l$ (without platelet transfusion and without evidence of bleeding), and no evidence of extramedullary involvement. CCyR was defined either by

the failure to detect any Ph-chromosome-positive metaphases in two sequential bone marrow aspirates with at least 20 metaphases examined, or by a 2-log reduction in BCR-ABL1 transcript levels from diagnosis or the laboratory-specific standard baseline. Major cytogenetic response (MCyR) was defined by combining the number of complete and partial cytogenetic responses (1 - 35% Ph-positive metaphases). Cytogenetic relapse (loss of CCyR) was defined by the detection of one or more Ph-positive marrow metaphases, also confirmed by a subsequent study. Bone marrow examination was triggered by a rise in BCR-ABL1 transcript numbers to a level consistent with cytogenetic relapse.

Molecular response was evaluated using RQ-PCR methodology. BCR-ABL1 transcript levels were expressed as a percentage ratio of BCR-ABL1 to ABL. A reduction in BCR-ABL transcript levels of >3 log (1000- fold reduction) from diagnosis or the laboratory-specific standard baseline was defined as a major molecular response (MMR). A complete molecular response (CMR) was defined as an undetectable BCR-ABL transcript level by RQ-PCR confirmed by a negative nested PCR whose lowest limit of detectability is 10^{-6} and that I carried out myself. At 3 months of treatment optimal response, suboptimal response and failure were defined as follows: optimal response featured a CHR with at least a minor cytogenetic response (36-65% Ph-positive metaphases). Suboptimal response was defined as a CHR associated with failure in the cytogenetic response (meaning that more than 95% of metaphases are Ph+). Therapeutic failure was considered when CHR response had not been achieved.

Between November 2007 and December 2010, 269 adult patients with BCR-ABL1 positive CML were treated at the Hammersmith Hospital London (HH)

or referred at the latter from external institutions. Imatinib was used as front-line therapy for 256 patients, while it was administered as second-line therapy to 8 patients post failure with interferon or hydroxyurea (table 2A). Dasatinib was chosen as front-line treatment for 5 patients. Treatment with at least one of the three second-generation tyrosine kinase inhibitors (dasatinib, nilotinib, bosutinib) as second- or third-line therapy was received by 154 patients. Two patients received homoharringtonine as second- or third-line treatment. Details of chosen treatment for the 269 patients and their clinical characteristics are shown in table 2A and 2B. Bone marrow morphology and cytogenetics were assessed at diagnosis and then every 3 months until patients achieved CCyR. Cytogenetic response was defined as complete by the failure to detect any Ph-positive metaphases and it was confirmed when noted on two consecutive bone marrow assessments at least 28 days apart; major cytogenetic response (McyR) was defined by including complete and partial cytogenetic responses ($\leq 35\%$ Ph-positive metaphases). Cytogenetic relapse (loss of CcyR) was defined by the detection of one or more Ph-positive marrow metaphases, also confirmed by a subsequent study.

Further object of study was a second group, originated from the cohort of the 269 patients, which included patients on 2G-TKI dasatinib and/or bosutinib showing resistance to treatment without presenting BCR-ABL1 mutations. This group was enrolled in the investigation for the presence of mutations hitting the kinase domain of any of the 3 SFK genes *Fyn*, *Lyn* and *Hck* as a cause of treatment resistance. Combination of the results from the two groups of patients have been further used to establish new methodologies for mutation detection (i.e. pyrosequencing and d- HPLC) and compare them with the current gold standard (direct sequencing).

Table 2A: list of different therapies used on the whole cohort of 269 patients. List of the various treatments (each specified with alphabetical letter) and numbers of respective treated patients.

Treatment group (269 pts)	No. of pts
A: IM	106
B: IM + DASA	59
C: IM + NILO	37
D: IM + NILO + HHT	1
E: IM + DASA + NILO	31
F: IM + NILO + DASA	11
G: IM + DASA + HHT	1
H: IM + HHT + NILO + DASA	1
I: IM + DASA + BOSU	1
J: IM + DASA + BOSU + NILO	1
K: IM + NILO + BOSU	1
L: IM + BOSU	3
IM: IM + INF	2
N: INF + IM	1
O: INF + IM + DASA	2
P: INF + IM + HU + DASA + NILO	2
Q: HU + IM	1
R: HU + IM + NILO	1
S: HU + INF + IM + DASA + NILO	1
T: IM + DASA + HU	1
U: DASA	5

Table 2B. Summary of characteristics, dosing and response to treatment for the cohort of 269 patients. Patients have been clustered only accordingly to the type of TKI treatment received (independently of the type of administered non-TKI treatment). Patients who were administered single or multiple TKI-agent treatment were clustered together with patients who received the same TKI treatment combined with non-TKI agents (INF, HHT, HU).

	no. (%)	Male, no. (%)	Median age, y (range)	Median disease duration, mo (range)	Best response achieved	IM intolerant or resistant? no. (%)	Stem cell transplants? no. (%)	INF and/or HU received prior IM? no. (%)	HU or HHT or INF received post IM? no. (%)
TOTAL	269 (100)	158 (59)	55 (18-91)	58 (3-241)	MMR	I: 39 (14) R: 103 (38)	301 (11)	6 (4)	7 (3)
IM	110 (41)	69 (63)	55 (18-91)	67 (4-92)	MMR	I: 2 (2) R: 35 (32)	8 (7)	2 (2)	3 (3)
IM + DASA	61 (23)	37 (60)	56 (21-88)	49 (3-125)	CCyR	I: 20 (33) R: 18 (30)	7 (11)	2 (3)	2 (3)
IM + NILO	33 (12)	19 (58)	52 (24-80)	40 (4-198)	CCyR	I: 5 (15) R: 19 (58)	2 (6)	1 (3)	1 (3)
IM+ BOSU	3 (1)	2 (67)	62 (35-80)	38 (9-41)	MCyR	I: 0 (0) R: 1 (33)	1 (33)	0 (0)	0 (0)
IM + DASA + NILO	37 (14)	21 (57)	60 (38-77)	54 (4-241)	MCyR	I: 9 (24) R: 23 (62)	9 (24)	3 (8)	0 (0)

Continued (table 2B.)

	no. (%)	Male, no. (%)	Median age, y (range)	Median disease duration, mo (range)	Best response achieved	IM intolerant or resistant? no. (%)	Stem cell transplants? no. (%)	INF and/or HU received prior IM? no. (%)	HU or HHT or INF received post IM? no. (%)
IM + NILO + DASA	13 (5)	7 (54)	40 (34-79)	36 (5-56)	MCyR	I: 3 (23) R: 7 (54)	3 (23)	0 (0)	1 (8)
IM + DASA + BOSU	1 (<1)	1 (100)	35	71	MCyR	R: 0 (0)	0 (0)	0(0)	0 (0)
IM + DASA + BOSU + NILO	1 (<1)	1 (100)	79	112	CHR	R: 0 (0)	0 (0)	0 (0)	0 (0)
IM + NILO + BOSU	2 (1)	1 (50)	51 (44-58)	13 (9-17)	MCyR	R: 0 (0)	0 (0)	0 (0)	0 (0)
DASA	5 (2)	4 (80)	68 (63-73)	22 (5-68)	MMR	NA	0 (0)	NA	NA

Abbreviations: IM, DASA, BOSU and NILO indicate respectively imatinib, dasatinib, bosutinib and nilotinib; CP indicates chronic phase; AP, accelerated phase; BC, blastic crisis; CHR, complete haematological response; MCyR, major cytogenetic response; CCyR, complete cytogenetic response; MMR major molecular response. NA: not applicable. Percentages have been referred to the total cohort of 269 patients.

II.I.I Patients in the first and second generation tyrosine kinase inhibitors study

Object of this study was a group of 269 patients that included:

1. 110 patients that received imatinib as single tyrosine kinase inhibitor.
2. 156 patients who experienced imatinib resistance or intolerance and were therefore administered at least one second-generation TKI.
3. 5 patients who received dasatinib as frontline.

Depending on patients' toxicity levels and disease progression, imatinib was administered at a dose between 400 and 800 mg once daily; dasatinib was administered at a dose between 70 and 100 mg twice daily, nilotinib between 300 and 500 mg twice daily and bosutinib between 400 and 600mg once daily. Dosage was however reduced in case of presence of myelosuppression or other toxic effects. Of the 110 patients receiving IM as single TKI, two had been treated with IM as first line treatment followed by INF, and two had received IM as second-line therapy after failure with INF or HU. Patients' characteristics are summarised in Table 2B.

Each of the 296 patients had sequential samples for BCR-ABL1 kinase domain (KD) mutations analysis and were systematically screened for KD mutation by direct sequencing. When a mutation was detected and there was availability of three or more previous sequential samples (range 3–10) collected during treatment administration, pyrosequencing was carried out to track back mutation appearance and quantify its levels. D-HLPC was also conducted on a limited number (40) of both

on mutated and un-mutated samples to test sensitivity and specificity of this methodology.

Additionally, patients on 2G-TKIs dasatinib and bosutinib who had been found being resistant to therapy without harbouring BCR-ABL1 KD mutations, were selected for mutation analysis within the SFK genes Fyn, Lyn and Hck as reported in details in chapter II.

II.II RNA extraction

A white cell pellet was prepared from 10 to 20 ml peripheral blood and/or from 2 to 3 ml of marrow aspirate, both of which had been collected into EDTA or preservative- free heparin and treated with an ammonium chloride based red cell lysis solution according to standard operating procedures in the laboratory. The white cells were then suspended in 1 ml of guanidine isothiocyanate (GTC) buffer and homogenised using blunt-nosed needle sheering. Total RNA from peripheral blood, bone marrow and cell harvest samples was extracted using the RNeasy (Qiagen, Crawley, UK) mini kit in accordance with the manufacturer's instructions. One third of the GTC solution was used for RNA extraction by an ion exchange column chromatography and centrifugal force. The step by step procedure is described below.

1. GTC lysates were thawed (stored at -20°C) for RNA extraction.
2. 350µl of 70% Ethanol was transferred into appropriately labelled 1.5 ml micro-centrifuge tubes using 1000 ml plugged tip.

3. On thawing, GTC was vigorously vortexed one at a time.
4. 350µl of GTC lysate was transferred to appropriately labelled 1.5 ml micro-centrifuge tubes.
5. 700µl suspension was transferred to an appropriately labelled RNeasy spin column.
6. In addition to the samples, ethanol was included as blank control, i.e. add 700µl of 70% ethanol to a separate spin column and proceed as per patient sample.
7. The mixture was incubated at room temperature for 30 min.
8. The columns were centrifuged for 15 sec at 8000 x g.
9. The flow-through was discarded from the collection tubes.
10. 650µl RW1 Buffer (Qiagen RNeasy Kit) was transferred to the spin columns and centrifuged for 15 seconds at 8000 x g and the flow-through was discarded.
11. 500µl RPE Wash Buffer (Qiagen RNeasy Kit) was transferred to the spin column and centrifuged for 15 seconds at 8000 x g and the flow-through was discarded and the columns placed into a new collection tube.
12. 500µl RPE Wash Buffer was added to the spin columns and centrifuged for 2 minutes at 15000 x g.
13. The flow-through and the collection tubes were discarded.
14. The columns were placed upside down onto tissue paper in the hood, and the lids were kept closed for 2 minutes. This allowed any residual 70% ethanol at the bottom of the column to evaporate.
15. The columns were transferred to new labelled, 1.5ml micro-centrifuge tubes. The lids of the spin columns were opened slightly, and were left for approximately 20 min. This will allow any remaining 70% ethanol present within the spin columns to evaporate.

16. 25µl of RNase-free water was applied to the centre of each column. 25µl of water was also set aside into a 1.5ml micro-centrifuge tube which was used later as a control for subsequent cDNA synthesis.
17. The RNA was eluted from the membrane by centrifuging for 2 minutes at 15000 x g.
18. Each spin column was removed and discarded.
19. Before discarding the pink Qiagen columns, a RNA sample for the reverse transcriptase (RT) negative control was extracted. RT negative sample is prepared by eluting with 25 µl of RNase free water through one of the patients' Qiagen column, selected randomly, from which RNA had already been eluted once. This "RT-Negative control" was then treated as per other RNA samples. However, cDNA mix added to this "sample" was void of RT enzyme.
20. In addition to the samples, cDNA synthesis should also include the following blank controls:
 - (a) 70% Ethanol control from the RNA extraction protocol.
 - (b) Sterile water control (25µl), an aliquot of sterile water set aside from the RNA Extraction protocol.
 - (c) GTC control saved from the sample processing.
 - (d) RT Negative control.
21. The RNA was incubated at 65°C for 10 minutes. This is to ensure the RNA is linear, and any secondary structure is denatured.
22. The tubes were transferred to a micro-centrifuge and pulse spun to collect the contents to the bottom of the tube. Then the tubes were transferred to ice immediately, to avoid formation of RNA secondary structures.
23. One cDNA mix (see appendix 1) was thawed per 10 samples (including the blank

controls) at room temperature. For more than 10 samples, the aliquots of cDNA mix were thawed into a single 1.5ml micro-centrifuge tube.

24. 6µl RNAsin (Ribonuclease Inhibitor) (per 10 samples) was added to the cDNA mix and vortexed briefly.

25. 21µl of cDNA mix was taken and added to the non-template control (NTC) only.

26. 12µl Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV) RT (per 10 samples) was added to the remaining cDNA mix and vortexed briefly.

27. 21µl of cDNA mix was transferred to each of the other samples/controls (ie. not NTC tube).

28. Each tube was incubated at 37°C for 2 hours.

29. The mixture was incubated at 65°C for 10 minutes to inactivate the reverse transcriptase.

30. The cDNA was stored at -70°C.

All BCR-ABL1 transcript samples were tested by real-time quantitative polymerase chain reaction for ABL and BCR-ABL amplification as part of the MRD monitoring procedure.

II.III PCR amplification

II.III.A. *BCR-ABL1* KD PCR amplification

A two-step PCR reaction to amplify *BCR-ABL1* kinase domain was carried out on at least one sample of all the 269 patients. In the first round, the normal *ABL* allele was excluded from mutational analysis by amplifying a *BCR-ABL1* amplicon from exon 13 of the *BCR* gene to exon 10 of the *ABL* gene. This was obtained by adding 4µl of cDNA to a 20 µl master mix including forward primer B₂A (600nM), which anneals in *BCR* exon b2/e13, and reverse primer JamR (600nM) which anneals at the junction of *ABL* exon 9 and 10 (fig.2.1) (Primers sequences are shown in table 2C). The resultant amplicon was then subjected to nested primer PCR by adding 2µl of first step PCR product to a 60µl reaction containing primers NTPB+ and NTPE-, in order to amplify an 863bp fragment containing the entire *ABL1* kinase domain for direct sequencing (see appendix 2). To enable pyrosequencing, aliquots of the same 1st round PCR amplicon were also subjected to amplification using biotinylated primers to generate PCR products containing the putative point mutation identified in previous samples by direct sequencing. Specificity and efficiency of the amplification were assessed by electrophoresis of a 7µl PCR product aliquot on a SYBR Safe-stained 1.7% agarose gel in TBE buffer. The biotinilated products were tested for mutation by pyrosequencing, described in detail later in this chapter.

II.III.B. *SFK* KD PCR amplification

In order to amplify *FYN*-, *LYN*-, and *HCK*-KD, a single-step PCR was carried out on at least one sample of all the patients refractory to 2G-TKIs dasatinib and/or bosutinib and assessed as *BCR-ABL1* mutation negative. Amplification of *FYN*- kinase domain was obtained by using forward Fyn_F (400nM) and reverse Fyn_R (400nM) primers, respectively annealing in *FYN* exon 6 and *FYN* exon 11. Amplification of *LYN*- kinase domain was obtained by using primers Lyn_F (400nM) and Lyn_R (400nM), respectively, annealing in *LYN* exon 7 and *LYN* exon 12. Amplification of *HCK*- kinase domain was obtained by using primers Hck_F (400nM) and Hck_R (400nM), respectively, annealing in *HCK* exon 8 and *HCK* exon 11 (primer sequences in table 2D; details of PCR reaction in Appendix 3). The resultant amplicons containing the kinase domain had lengths of 902 bp for *FYN*, 894 pb for *LYN* and 895 bp for *HCK*, which were then subjected to direct sequencing.

II.IV Direct Sequencing

The *BCR-ABL1*-KD and *FYN*-, *LYN*- and *HCK*-KD PCR amplified products from each individual were analysed and if a single amplicon was observed the remaining PCR products were sequenced directly in a Sanger's dideoxy chain-termination reaction using a Big-Dye ABI 3130 sequencer (Applied Biosystems, Foster City, USA). However, if multiple bands were observed following the gel electrophoresis, the PCR fragment containing the *ABL1*-, *FYN*-, *LYN*- or *HCK*-KD, respectively for *BCR-ABL1*,

FYN, *LYN* and *HCK* genes, was isolated by excising the specific band from the gel and purified using commercially available gel purification kit (QIAquick PCR Purification Kit; Qiagen, UK) according to manufacturer's instructions. A 30µl aliquot of each reaction was primed with specific pairs of oligonucleotides spanning the domain of interest, whose sequences are reported on tables 2C and 2D. All PCR amplicons, before being subjected to direct sequencing, were purified using BigDye XTerminator Purification kit (Applied Biosystem, Foster City, USA) in accordance with manufacturers' instructions. The Sanger's dideoxy-chain terminator reaction was then obtained using a Big-Dye ABI 310 sequencer (Applied Biosystems, Foster City, USA).

In each case the sequences obtained were respectively compared with the published ABL1 type 1a sequence (GenBank M14752), *FYN* (Ensembl ENST00000354650), *LYN* (Ensembl ENST00000519728) and *HCK* (Ensembl ENST00000375852), using SEQMAN software (DNASTAR, Inc., Brighton, MA). All observed base substitutions were confirmed both by running in duplicate the PCR product and performing sequence analysis on both strands for each fragment.

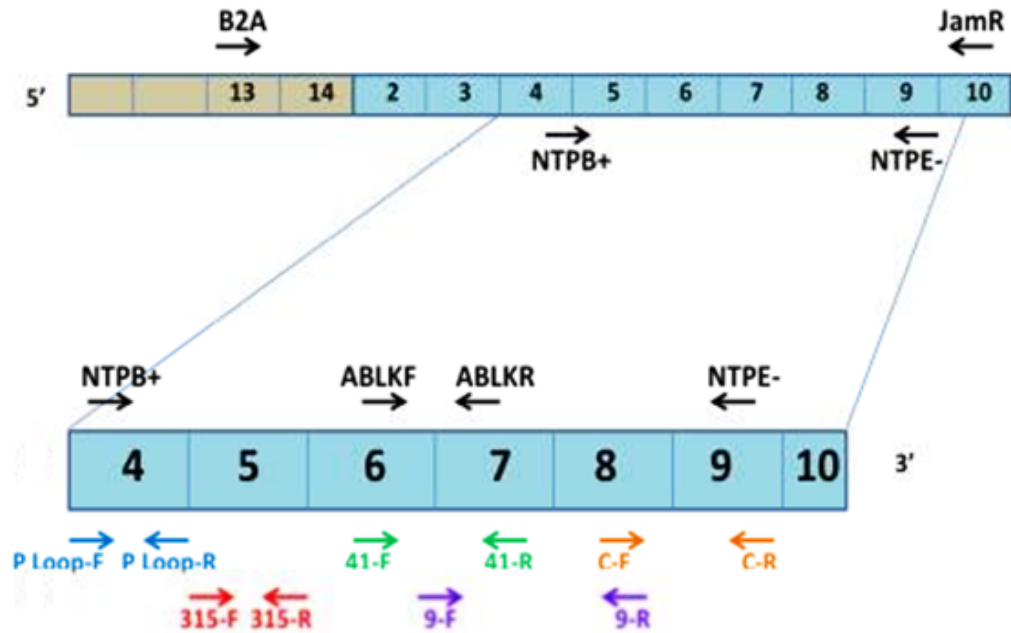


Fig 2.1. Schematic representation of the *BCR-ABL1* region studied by direct sequencing and pyrosequencing

The green and blue blocks represent *BCR* and *ABL1* genes and their respective exons joined together and constituting the BCR-ABL1 fusion gene. Exterior primers B2A and JamR were used to amplify through PCR reaction the kinase domain of BCR-ABL1 and to exclude the normal *ABL1* from mutation analysis. Interior primers NTPB+ and NTPE- were used for amplification of the kinase domain in the second round of the nested PCR to produce an 863 bp fragment. To sequence the kinase domain ABLKF, ABLKR, NTPB+ and NTPE- primers were used. The primer pairs of P Loop, 315, 41, 9 and C here represented in different colour, were used to amplify various fragments of the KD in different second steps of nested PCR reactions which were used for pyrosequencing.

Table 2C: Sequence of the primers used for first-step PCR, nested PCR, direct sequencing and pyrosequencing of BCR-ABL1.

Primer name	5'-to-3' sequence
B2A	5'-TTCAGAAGCTTCTCCCTGACAT-3'
JAMR	5' GTACTCACAGCCCCACGGA-3'
NTPB+	5'-AAGCGCAACAAGCCCACTGTCTAT-3'
NTPE-	5'-CTTCGTCTGAGATACTGGATTCTTG--3'
ABLKF	5'-AACGCCGTGGTGCTGCTGTAC-3'
ABLKR	5'-CAGTTTCGGGCAGCAAGATC-3'
ITKE	5'-CTGGAACATTGTTTCAAAGGCTTGGT-3'
P Loop-F1	5'-TACGACAAGTGGGAGATGGA-3'
P Loop-R1	5'-GCCACCGTCAGGCTGTATTT-3'
253+315-F	5'-CGCAACAAGCCCACTGTC -3'
41-F	5'-GTTGTACAAGGCCACTCAGATCTCGT-3' (Biotinylated)
41-R	5'-TCTCGGTGCAGTCCATTT-3'
315F	5'-GCCGTGAAGACCTTGAAGGA-3' (Biotinylated)
315R	5'-CCAGGAGGTTCCCGTAGGTC-3'
C-F	5'-TTACCCGGGAATTGACCTGT-3' (Biotinylated)
C-R	5'-TGTTTCAAAGGCTTGGTGA-3'
F9	5'-CTAGAGATCTTGCTGCCCCGAAACT-3' (Biotinylated)
R9	5'-GACAGGTCAATTCCTGGGTAA-3'
SNP-1-2	5'-CACCTGAAGCACAA-3'
SNP-2-9	5'-GAACGCACGGACATC -3'
SNP-231	5'-CATCTCCCACTTGTCG -3'
SNP-253/255	5'-CTGGGCGGGGGCCAG -3'
SNP-276	5'-TGAAGACCTTGAAGGAG -3'
SNP-299	5'-TTCTCTGGGCAGCCT -3'
SNP-311	5'-GAACTCAGTGATGATATAGA -3'
SNP-315/317	5'-CCCCCGTTCTATATCA -3'
SNP-351	5'-CCAAGATCTCTGTGGATGA-3'
SNP-359	5'-CAAGATCTCTGTGGATGA -3'
SNP-364	5'-AGTTTCGGGCAGCAA -3'
SNP-384	5'-TGTCCCCTGTCA -3'
SNP-396	5'-AACTTGGCTCCAGCA -3'
SNP-417	5'-GACGTCCGACTTGATG -3'
SNP-438	5'-CCAATTCCTGGGTAAAG-3'
SNP-453	5'-CCATGCGGTAGTCCT-3'
SNP-459	5'-CCTTCTGGGCGCT -3'
SNP-462	5'-TTCTCTGGGCAGCCT -3'
SNP-486	5'-CTTGGTGGATTTCAGC-3'

Table 2D: Sequences of primers used for PCR and Direct Sequencing of SFK genes *HCK*, *LYN* and *FYN*.

Primer name	5'-to-3' sequence
H_F	5'-GAA CGA CGG GCT CTG CCA GA -3'
H_R	5'-TCA TGG CTG CTG TTG GTA CT -3'
s_HF	5'-AAA AGT GAT GAG GGC AGC AA -3'
s_HR	5'-CAA AGG ACC AGA CGT CTG ACT -3'
L_R	5'-CCA ATC TTC TGC ACA AGC CAT C -3'
L_F	5'-GAT GGC TTG TGC AGA AGA TTG G -3'
s_LF	5'-GCT GGA TTT CCT GAA GAG CGA -3'
s_LR	5'-AGT GAA ACA TCC AAA GTT GAT T -3'
F_F	5'-TCT GCT GCC GCC TAG TAG TTC -3'
F_R	5'-CCA ACC TGG TGA AAA CCT GTA A -3'
s_FF	5'-CTC CAT CTT TTA AGA AAT CCA G -3'
s_FR	5'-TGA ACC TCC CGT ACA GGG CT -3'

II.V Denaturing high-performance liquid chromatography (d-HPLC)

D-HPLC WAVE (denaturing-high performance liquid chromatography) is one of the most frequently used methods for the diagnosis of DNA mutations. Highly sensitive and utilising recently developed methodology, d-HPLC is based on heteroduplex formation of PCR products amplified from both wild type and mutant alleles. Under optimised semi-denaturing conditions, these heteroduplexes can be distinguished from homoduplexes.

As with direct sequencing, to increase the sensitivity and specificity of ABL1 kinase domain amplification, a nested-PCR approach methodology was utilised. The first round of amplification was carried out using 2µl of cDNA in a 50µl final reaction, using primers Fwd-BCR-P210 (positioned between exons e12 and e13 on *BCR* gene) and Rev-ABL-B (positioned on exon 10 on *ABL* gene) (Fig.2.2). Re-amplification of a 2µl aliquot from first step PCR was then performed using three internal primer pairs: Fwd-ABL-A/Rev-ABL-A, Fwd-ABL-B/Rev-ABL-B and Fwd-ABL-C/Rev-ABL-C. As a result, the entire ABL kinase domain is divided into three partially overlapping fragments: ABL-A (393 bp, spanning codons 206-335), ABL-B (482 bp, codons 262-421), ABL-C (465 bp, codons 371-524) (fig.2.2). Primer sequences and their position on ABL are shown in Table 2E.

This strategy produces PCR products of optimal length for D-HPLC Analysis (recommended length, 200-500 bp). Using WAVE 4500HT D-HPLC (Transgenomic Ltd.) melting curves were calculated as described to select optimal temperatures for the three ABL mRNA fragments. In order to allow the heteroduplexes to form, and depending on the intensity of the band obtained by gel electrophoresis, aliquots of 6-10µl of the PCR product, was incubated for 10 minutes at 96°C and then allowed to

cool for 10 minutes at room temperature. The samples were then loaded onto the WAVE and run at the following selected temperatures: ABL-A at 61.3, 62.3, and 63.1°C; ABL-B at 60.3, 61.1, and 61.9°C; ABL-C at 60.2, 60.9 and 61.2°C. DNA was eluted from the column by a linear acetonitrile gradient in 0.1mM triethylamine acetate buffer (TEAA; Transgenomic) at a constant flow rate (1.5ml/min). Gradient was formed by mixing buffer A (0.1 mM TEAA) and buffer B (0.1mM TEAA, 25% acetonitrile). Eluted amplicons were detected by 260nm UV absorbance. A wild-type p210 BCR-ABL1 cDNA sample, derived from a BaF3 cell line transfected with a BCR-ABL1 fusion construct, was also used as wild-type.

The chromatogram from each tested patient was overlaid with the wild-type profile, and samples with extra peak(s) in the elution profiles were scored as positive. To ensure that mutations present in >85% of BCR-ABL1 positive cells could not escape D-HPLC detection, a mixture of all the amplicons described as mutated by direct sequencing and wild-type transcripts was also run, in order to allow formation of heteroduplexes in the presence of a mutant homoduplex.

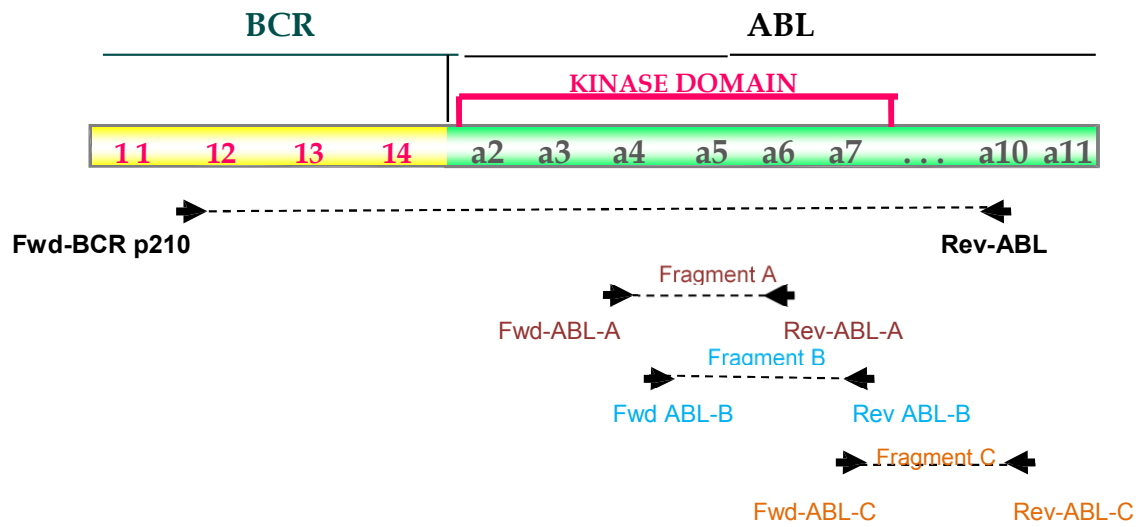


Fig.2.2. Position of amplified fragments on the *BCR-ABL1* transcript.

For all patients, the second round PCR was performed using three primers pair, in order to subdivide the entire ABL KD into three partially overlapping fragments (A, B, and C) of optimal recommended length of 200-500 bp for WAVE 4500HT D-HPLC analysis.

Table 2E: Sequence and position of primers used for the D-HPLC PCR of BCR-ABL1.

Primer name	5' to 3' sequence	Position
Fwd-BCR p210	GAGCAGCAGAAGAAGTGTTCAGA	BCR, exon 12
Rev-ABL	CTTGGAGTGAGGCATCTCAG	ABL, exon 10
Fwd-ABL-A	CATCATTCAACGGTGGCCGACGG	ABL, exon 4
Rev-ABL-A	GTTGCACTCCCTCAGGTAGTC	ABL, exon 6
Fwd-ABL-B	GAAGAAATACAGCCTGACGGGTG	ABL, exon 4
Rev-ABL-B	CGTCGGACTTGATGGAGAA	ABL, exon 7

II.VI Pyrosequencing

When a BCR-ABL1 mutation was detected by direct sequencing in a patient for whom we had at least three consecutive cDNA samples, these were subjected to pyrosequencing in accordance with the manufacturer's instructions (Pyrosequencing AB, Uppsala, Sweden). Pyrosequencing is a real-time, sequencing-by-synthesis

technique, catalysed by four kinetically titrated enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase. It principally differs from direct sequencing in the order the nucleotides are incorporated. Each nucleotide is dispensed and tested individually for its incorporation into a nascent DNA fragment. After each nucleotide incorporation, pyrophosphate (PPi) is released in a quantity equimolar to the amount of nucleotide incorporated. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulphate. ATP is then used by luciferase in converting luciferin into oxyluciferin, which generates visible light with an intensity that is proportional to the amount of ATP. The light generated is detected by a charge couple device (CCD) camera and displayed as a peak in the software, where each peak is proportional to the number of nucleotides incorporated. Unincorporated dNTPs and excess of ATP are continuously degraded by apyrase. After the degradation is completed, the next dNTP is added in a new pyrosequencing cycle, contributing to building up the complementary DNA strand. To pyrosequence an unknown DNA sequence, a cyclic nucleotide dispensation order (NDO) is generally used. As a result of each cycle per nucleotide, one of the four dNTPs is incorporated into the DNA growing strand, whereas the other dNTPs will be degraded by apyrase (fig.2.3.). On the other hand, if the DNA sequence to be pyrosequenced is known, non-cyclic NDOs can be programmed with predictable pyrograms. Nucleotide sequence is determined from the order of dNTPs dispensation and peak intensity using a pyrosequencing software.

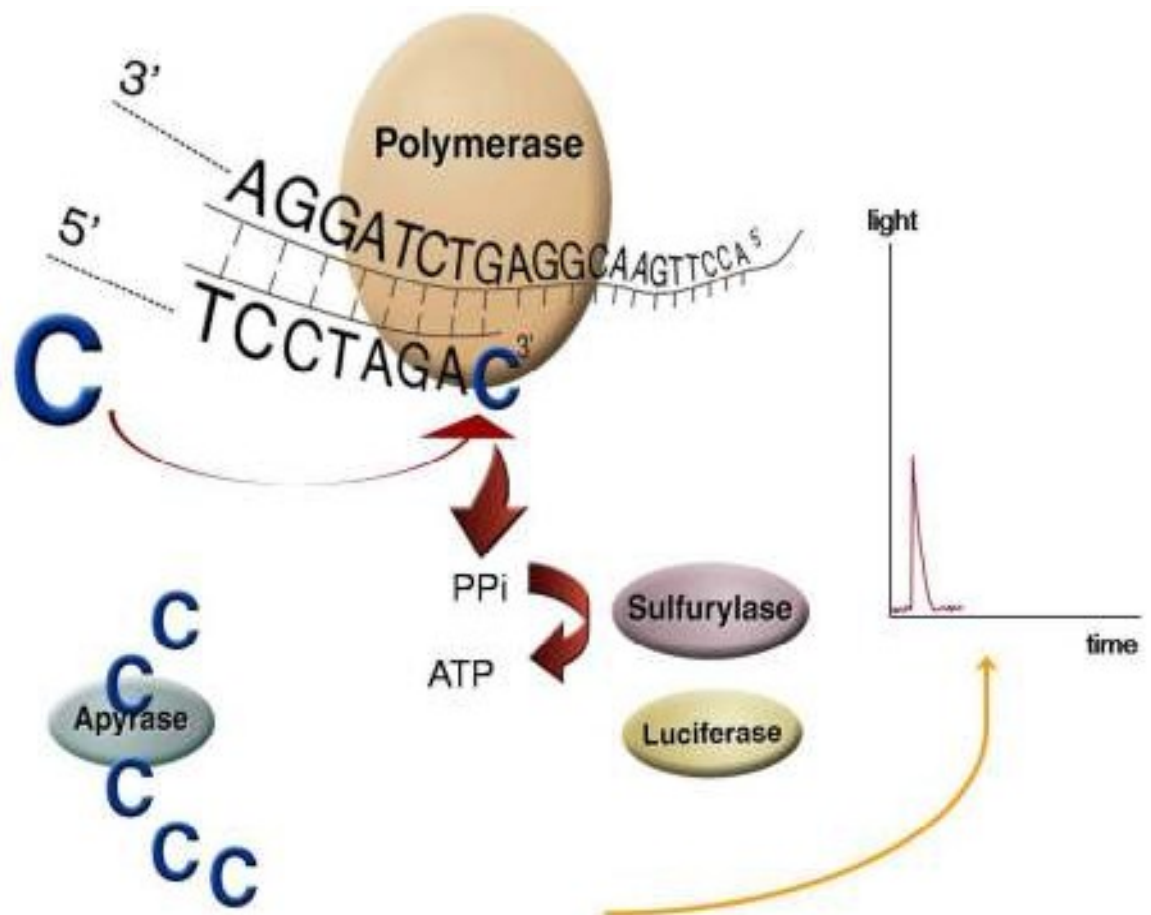


Fig.2.3. Principle of pyrosequencing. As clarified in the procedure section at <http://www.qiagen.com/products/pyromarkq24.aspx>.

The *BCR-ABL1* amplicon was used as a template for nested PCR using primers (with a 1 μ M concentration) that flanked the region containing the putative mutation. One of the 2 primers for each reaction was biotinylated. Sequence and position of some of the primers used for pyrosequencing reactions are shown in table 2A and fig.2.1.

Each PCR product was tested in duplicate. In brief, a single-stranded cDNA was isolated by combination of alkali denaturation buffer (NaOH) and mixing biotinylated PCR products with streptavidin-coated sepharose beads (GE Healthcare, Amersham, UK). The percentages of mutant and non-mutant were determined by the Allele Quantitation Algorithm using the PSQTM MA software (Pyrosequencing AB, Uppsala, Sweden).

Purification of target complementary strand of PCR product was done as described briefly below:

1. 20 μ l PCR product were transferred to a 24-well microtitre plate to which 70 μ l Binding buffer had already been added. The plate was sealed with Micro Amp clear adhesive film (Applied Biosystem, USA).
2. The microtitre plate was spun on the Monoshaker at maximum settings for 20 min.
3. The adhesive film was disposed of and the microtitre plate was placed on to the template preparation platform for pyrosequencing.
4. The vacuum pump connected to the hand held 24-pin grid was started.
5. The grid was then transferred to the microtitre plate making sure that each pin went to the bottom of the well till fluid was completely sucked out.
6. The grid was then transferred to the trough containing the 70% ethanol for 10-20 seconds to eliminate the bead residues.
7. The grid was then quickly transferred to the trough containing the denaturation buffer (NaOH) for about 5 seconds to denature the double stranded PCR product.

8. The grid was transferred into the trough containing the Wash Buffer (Biotage) and after the entire buffer went through the grid the pump was switched off.
9. The grid was transferred to the 24-well pyrosequencing plate containing the annealing buffer and primer mix (see appendix 5). The grid was shaken properly to release the beads from the tips into the respective wells.
10. To anneal the sequencing primer to the single stranded template, the plate was heated at 80°C for 2 min using the pyrosequencing iron, followed by slow cooling to RT.
11. The plate was placed in the slot of the pyrosequencing instrument.
12. The cartridge was filled with the enzyme and substrate mix and the 4 dNTPs in the respective wells as per instructions. The cartridge was then loaded into the pyrosequencer head and the reaction started.
13. The pyrosequencing results were analysed using PyroMark Q24 software (Biotage, Uppsala Sweden).

The linearity of Q-SNP by pyrosequencing was tested by subjecting cDNA generated from graded mixes of Ba/F3 cell lines transfected with BCR-ABL1 cDNAs containing either the wild-type ABL1 sequence (BaF3/BCR-ABL1) or the E255K mutation (BaF3/BCR-ABL1 E255K). The mixes ranged from 10 to 100% BCR-ABL1 E255K (Figure 1-3). To ensure reproducibility, the cDNA synthesised from each mix was tested in triplicate by Q-SNP. A similar experiment with lower percentage of mutations -including 1, 2 and 5% mutation- was repeated using BaF3/BCR-ABL T315I cells diluted against non-mutant BaF3/BCR-ABL1 cells.

For both D-HPLC and pyrosequencing, in presence of uncertain cases the samples were run together with positive control from vector pMIG210 carrying wild-type BCR-ABL1 (kindly provided by Dr S.Wagner, Imperial College).

II.VII. Transformation into competent bacteria

The pMIG210 vector contains the cDNA encoding the 210KDa form of BCR-ABL1, and the gene for the enhanced green fluorescent protein (EGFP), preceded by an internal ribosome entry site (IRES) and an ampicillin resistance cassette.

For propagation of pMIG210 plasmid, an aliquot of 10pg to 100ng of the vector was transfected into chemically competent cells (One Shot TOP10 vector kit, Invitrogen, CA, USA). The cells were then left on ice for 30min, subjected to heat shock at 42°C for 30 seconds and transferred back on ice for 1 min. SOC medium for bacterial growth (250µl) was then added to the cells, which were incubated at 37°C for 1h. After that, the content of each vial was plated on a selective agar plate (LB agar containing 100µg/ml ampicillin) and the plates were incubated overnight at 37°C to allow colony formation. 6 colonies were picked the next day, using the tip of a P200 Gilson pipette, and transferred each to a 15ml Falcon containing 5ml of L-Broth and 100µg/ml ampicillin. Liquid colonies were allowed to grow for 18 hours followed by plasmid DNA extraction using the Qiagen Miniprep Plasmid Isolation kit (Qiagen, Hilden, Germany).

II.VIII. Statistical Analysis

The graphs have been generated by using SPSS v19 software (SPSS, Inc., Chicago, IL). All statistical analyses were two-sided.

III. Results

III.I Estimation of the sensitivity of different conventional methods for BCR-ABL1 kinase domain mutation analysis

Six serial dilutions were created using wild-type cDNA and E255K BCR-ABL1 cDNA, both extracted from BCR-ABL1 transfected BaF3 cell lines. Mutant cDNA was diluted in WT cDNA to create the following serial dilutions: 40%, 20%, 10%, 5% and 1%. Dilutions were amplified for kinase domain of ABL1 in a nested PCR and the final product was tested for detection of mutation E255K by direct sequencing, pyrosequencing and d-HLPC.

Direct sequencing. DS was able to detect mutation E255K up to the 20% dilution, while could not pick up any abnormality in 10%, 5% or 1% dilution samples. No other mutations were detected in either of these dilutions, confirming the absence of contamination. The level of sensitivity of direct sequencing has been therefore considered to be above 20%, in accordance with previous studies from Baccarani⁸⁷ and Ernst.⁸⁸

Pyrosequencing. Pyrosequencing was able to detect the mutation up to 5% dilution, while could not pick up any abnormality in 1% dilution samples. Thus, the level of sensitivity for pyrosequencing from this study has been described as equal to or above 5%, in accordance with previous studies from Baccarani and colleagues.⁸⁷

Denaturing-High Performance Liquid Chromatography. In order to assess d-HPLC sensitivity, in addition to the E255K-mutant dilutions, I investigated another 5 serial dilutions of T315I-mutant cDNAs, already mixed and kindly provided by

Junia Melo (Institute of Medical and Veterinary Science, Adelaide). First and second-step PCRs were prepared and run by myself, as was the loading of the PCR products onto the d-HPLC apparatus. E255K mutation is located on exon 4 of ABL, at 109 nucleotide position of the amplicon and it is expected to appear on fragment A. However, this mutation did not show a consistent signal on the d-HPLC chromatogram for any of the dilutions and it was not therefore possible to interpret the results. Further work is needed to optimise the assay to enable reliable detection.

The results for the T315I-mutant dilutions were different. This mutation is located on exon 6 of ABL, at 290 nucleotide position of the amplicon. Therefore, fragments expected to show mutant pattern were A and B (as previously shown in table 2C). D- HPLC was able to detect mutation T315I up to 1% dilution in all the fragments B, as show in fig.3.3. Conversely, T315I was not identified in fragment A and this is likely to be due to its location in proximity of the end of the fragment A. Therefore, d- HPLC from this study has been described as having a sensitivity around 1% for the E255K mutation in accordance with previous studies from Baccarani⁸⁷ and Ernst⁸⁸.

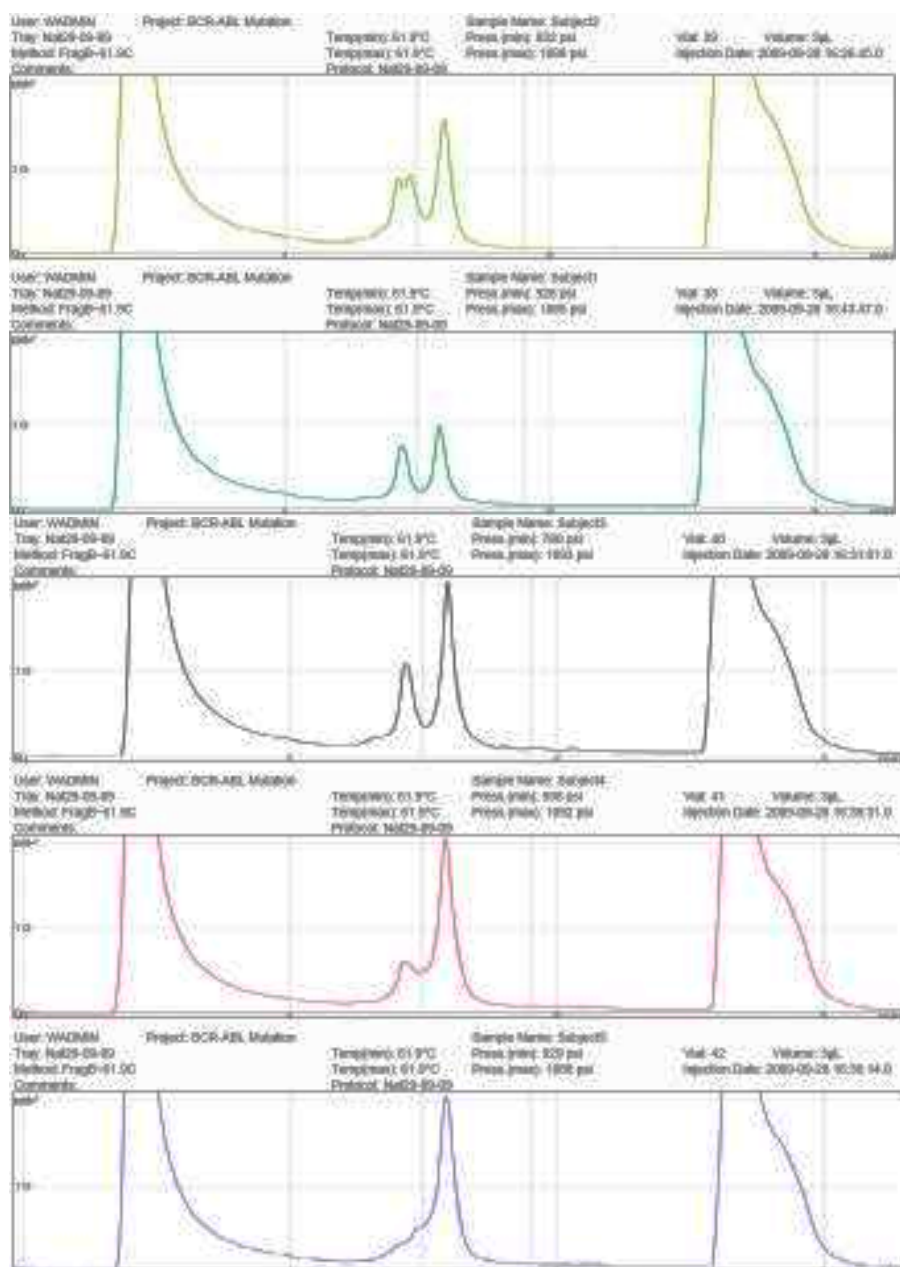


Fig 3.1: Results from serial dilution experiments to assess the sensitivity of the d-HPLC assay.

PCR products amplified from wild-type ABL1 and 5 serial dilutions of T315I mutant were mixed as indicated and subjected to d-HPLC. Each of the 5 chromatograms represents d-HPLC profiles of fragment B for T315I mutation from 40%, 20%, 10%, 5% and 1% dilutions respectively, following graphs from top to bottom. First of the 2 peaks represents the mutant fragments, which in the 40% dilution is still visible as double peak; the second peak represents the wild-type fragment. The degree of difference varied from an obvious heteroduplex peak (40%) dilution to a homoduplex peak (1% dilution) depending on the amount of the mutant cDNA.

III.II Results of mutation analysis in patients treated with IM and 2G-TKIs

III.II.A Patients' clinical features

This section includes the results from the study of the whole cohort of 269 patients treated with imatinib followed by second-generation TKIs, whose characteristics were presented in Chapter II. At the start of treatment, 193 (72%) patients were in chronic phase, 61 (23%) in accelerated phase and 16 (6%) in blastic crisis. During treatment, 163 (84%) of the 193 CP patients remained in CP, while 29 (14%) progressed to advanced phase (accelerated phase and blastic crisis). Of the 61 patients initially in AP, 30 (49%) went into CP and 6 (10%) progressed to BC. Of the 16 patients who started treatment in BC two (13%) went into AP and 4 (25%) into CP. 164 of the 269 (61%) patients achieved complete cytogenetic response (CCyR) during treatment with imatinib (n=85, 52%) or one of the three second-generation TKIs (dasatinib: n=45, 27%; nilotinib: n=29, 17%; bosutinib: n=4, 2%). All patients' characteristics are shown in table of Appendix 6.

III.II. B Acquisition and loss of mutations during treatment

Results from mutation analysis by direct sequencing showed 63 mutated patients (out of the 269 total patients, 23%). Of these 63 patients 44 developed mutations during treatment with IM. Most of these patients (48 out of 63, 76%) had a single mutation. However, eleven patients developed 2 different mutations, three

patients had 3 mutations and one patient harboured 4 different mutations. 23 of the 154 (15%) patients who received both imatinib and second-generation tyrosine-kinase inhibitors developed mutations on imatinib; of these, two (9%) developed new mutations during treatment with 2G-TKIs with loss of the mutation present at baseline, six (26%) maintained the same mutation from imatinib to end of therapy, seven (30%) lost their mutation and seven (30%) acquired new mutations in addition to the mutation occurred during imatinib. Five of the 269 (2%) patients had more than one mutation during treatment with imatinib, with the following genotypes: Y253H/M351T, E459K/T315I, L384M/V338F, M351T/H396R, G250E/T315I.

III.II.C Frequency and relative distribution of mutations

Overall, out of the 269 patients, mutational analysis by direct sequencing detected 63 (23%) patients harbouring 77 mutations at 23 different amino acids, with a total of 30 different mutations. Eleven (18%) of the 63 mutated patients harboured multiple mutations. The residues affected by amino acid substitutions spanned the whole BCR-ABL1 kinase domain; in detail, the group of AA substitutions occurring with the highest frequency were within the *gatekeeper domain*, with 4 different amino acidic substitutions detected (V299L, T315I, T315A, F317L) occurring in 23 (37%) patients. A second group of frequently detected mutations was found to fall within the *P-loop* region, with 6 different substitutions (L248V, G250E, Q252H, Y253H, E255K, E255V) all together affecting 21 (33%) of the mutated patients. The *catalytic domain* represents another region of BCR-ABL1 KD frequently mutated in CML, and was found to be involved in 12 (19%) cases with 5 distinct

nucleotide changes (M351T, E355G, F359C, F359I, F359V). Four different mutations (L384M, L387M, H396R) were clustered within the *activation loop*, with 4 cases (6%). Finally, mutations falling within the *C-loop* (E450K, E450V, E453K, E453V, E459K, E462K, F486S) were detected in 7 (11%) patients. Some other detected mutations (M244V – which used to be considered as part of the P-Loop - D276G, V338F, S417F) do not belong to any of the 5 recognised functional regions mentioned above.

T315I and G250E were the two most common single mutations with the *gatekeeper domain* being the most frequently affected in BCR-ABL1 KD. Frequency of detected KD is shown in fig 3.2.

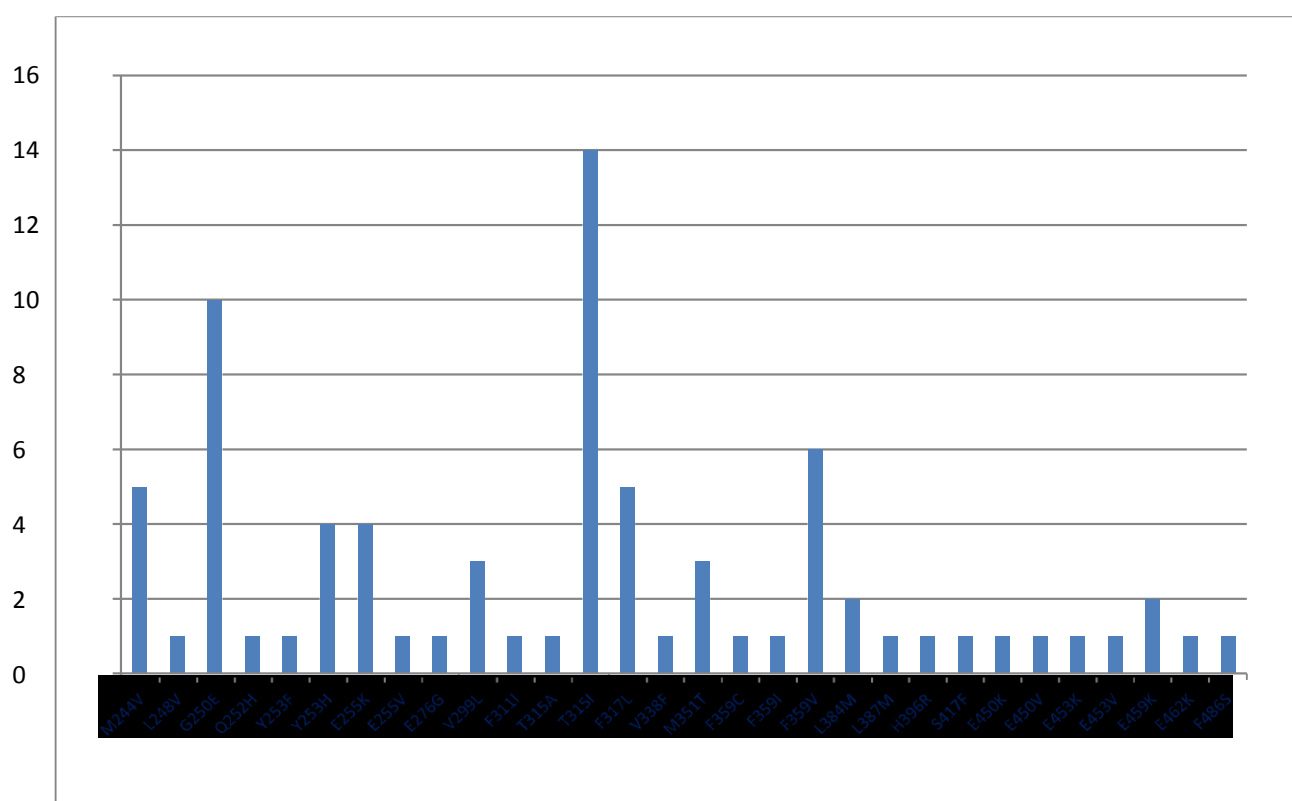


Fig.3.2. Distribution and relative frequency of KD mutations found in my study. Incidence of mutations within the kinase domain (x-axis) by absolute number of patients (y-axis).

III.II.D Emergence of KD mutations and their frequency according to disease phase

Mutations were found in: 32 out of 198 (16%) CP patients; 24 of 87 (28%) AP patients; 6 out of 17 (35%) BC patients (table 3B). Difference in mutation occurrence rate was therefore not substantial for patients in accelerated phase or blastic crisis. However, when examination of the position and relative frequency of mutations by disease phase was conducted, we observed highly resistant *gatekeeper* mutations T315I and *P-loop* mutations showing a preferential trend toward the advanced stages of disease (fig. 3.3).

III.II. E Mutations occurrence and disease progression

In our cohort most of the patients remained in CP (61%). However, most of the patients who progressed from chronic to advanced phase harboured KD mutations; of the 24 patients who progressed from CP to AP, 16 (67%) were mutation-positive. 3 out of 5 (60%) of the patients who progressed from CP to BC carried a mutation. Some patients developed more than one mutation before progressing to advanced phase, with a total number of 19 mutations. Five of these mutations occurred in more than one patient during their disease progression: T315I and G250E both occurred in 3 different patients; M351T, E255K and F317L were detected each in 2 patients. When

the 19 KD mutations involved in the progression to advanced phase were categorised on the grounds of their AA position, it was found that 11 out of the 19 (85%) were *gatekeeper* and *P-loop* KD mutations, characterised by high levels of resistance (i.e. G250E, T315I, E255K, F317L, Y253H). Patients harbouring these mutations showed a substantially worse outcome than the patients carrying mutations with low or intermediate levels of resistance during disease progression.

Table 3A. Mutated patients and their occurrence within different stages of the disease

	Total pts n	MUT n, (%)
CP	164	20 (12)
CP --> AP	24	16 (67)
CP --> BC	5	3 (60)
AP	25	6 (24)
AP--> CP	30	10 (33)
AP --> BC	6	2 (33)
BC	10	2 (20)
BC --> AP	2	2 (100)
BC --> CP	4	2 (50)

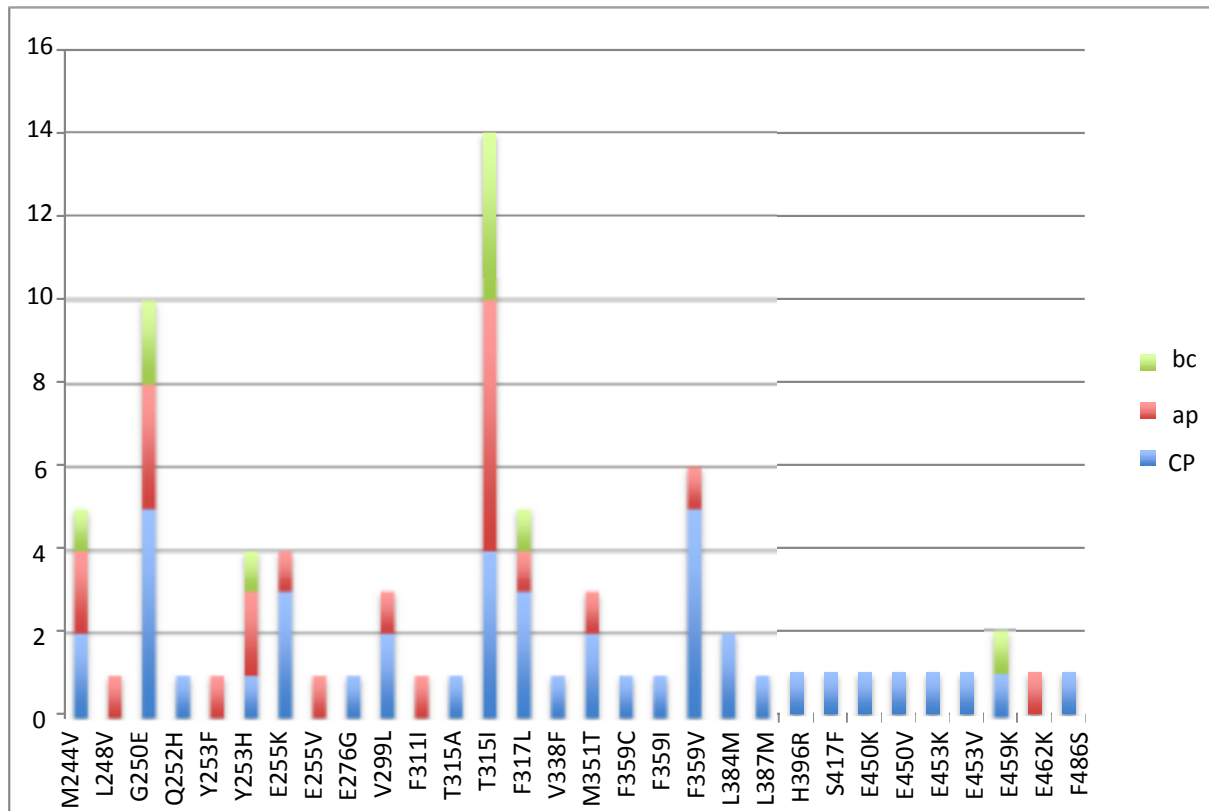


Fig.3.3. Distribution and relative frequency of KD mutations according to disease phase. CP, chronic phase; AP, accelerated phase; BC, blast crisis. Gatekeeper mutation region T315I and mutations part of the P-loop domain (AA248 – 255) occurred in advanced phases at higher frequency.

III.II. F Development of KD mutations and loss of CCyR

In 20 (12%) of the 164 patients who achieved CCyR, mutational analysis showed the emergence of BCR-ABL1 mutations. Out of these, five patients (25%) had one mutation detected before they achieved CCyR (H396R, E276G, Q252H and V299L, with the latter being harboured by two patients); in the same patients despite the above listed mutations were still detectable, BCR-ABL1 levels continued to decrease and CCyR was maintained during treatment. CCyR was achieved during treatment with dasatinib (2 patients) and nilotinib (3 patients).

In 15 patients mutations (T315I n=2, N231S, F359I, L384M/V338F, S417F, E453V, M351T/H396R, Y253H, V299L, F359V, G250E, F359V, E450V and F317L) were detected while in CCyR. Of these, eleven patients (representing 55% out of the total patients who achieved CCyR) lost response, with one individual progressing eventually to advanced phase. Patients with mutation showed to be more likely to experience loss of response. Eighteen (10%) of the 164 patients who achieved CCyR lost their response during follow up without showing presence of mutation (data shown in table 3B).

Table 3B: Correlation between development of KD mutations, disease phase and achievement of response.

GROUP	no. of pts	Achievement of CCyR n. (%)	Loss of response
ALL PATIENTS	269	164 (61)	29 (18)
BCR-ABL1 MUTATION			
Yes	63	20 (12)	11 (55)
No	206	144 (88)	18 (12)
DISEASE PHASE			
CP	198	143 (72)	24 (17)
AP	87	20 (23)	4 (20)
BC	17	1 (6)	1 (50)
MUTATIONS IN CP			
Mutation	32 (16)	20 (63)	9 (35)
No mutation	166 (84)	123 (74)	15 (12)
MUTATIONS IN AP			
Mutation	24 (28)	4 (17)	1 (28)
No mutation	63 (72)	17 (27)	3 (18)
MUTATIONS IN BC			
Mutation	6 (35)	1 (16)	1 (100)
No mutation	11 (65)	1 (9)	0 (0)

Abbreviations: CCyR indicates complete cytogenetic response.

“Loss of response” column represents patients who lost response after achievement of CCyR and its percentages are referred to “Achievement of CCyR” column.

III.II.G. Assessment of mutation emergence “specificity” during dasatinib and nilotinib treatment

As already reported in this study, results from mutation analysis by direct sequencing found 30 different mutations occurring in patients during treatment with imatinib and second generation TKIs. Some of these AA substitutions appeared to have a high frequency of occurrence, such as T315I (n=14), G250E (n=10), M244V (n=5), F317L (n=5) and Y253H. Emergence of T315I was detected during IM, dasatinib, nilotinib and bosutinib; G250 and M244V both developed during IM, dasatinib and nilotinib. Conversely, Y253H was only detected on IM (n=1) and nilotinib (n=3); F317L only occurred on dasatinib (n=5) (fig.3.4).

Soverini and colleagues⁸⁹ in a recent study on acute lymphoblastic leukaemia reported that treatment with second-line TKI dasatinib (following resistance to imatinib) may select TKI-specific BCR-ABL1 mutants.

Because direct sequencing sensitivity is over 20%, there was the possibility that in my samples mutations F317L and Y253H had in reality appeared during previous treatment with imatinib, when their levels were not sufficient to be detected by direct sequencing. Thus in order to assess whether Soverini's theory could be applied to my patients' cohort, samples from the five patients were analysed by pyrosequencing to assess F317L emergence only after introduction of dasatinib. Furthermore, following the same procedure, samples from the three patients bearing mutation Y253H were also tested to assess development of the mutation only following start of treatment with nilotinib.

In order to track back the appearance of these two mutations and assess their actual point in time of occurrence, analysis by pyrosequencing was carried out on at least 4 cDNAs of each of the 5 patients with F317L and 3 patients with Y253H.

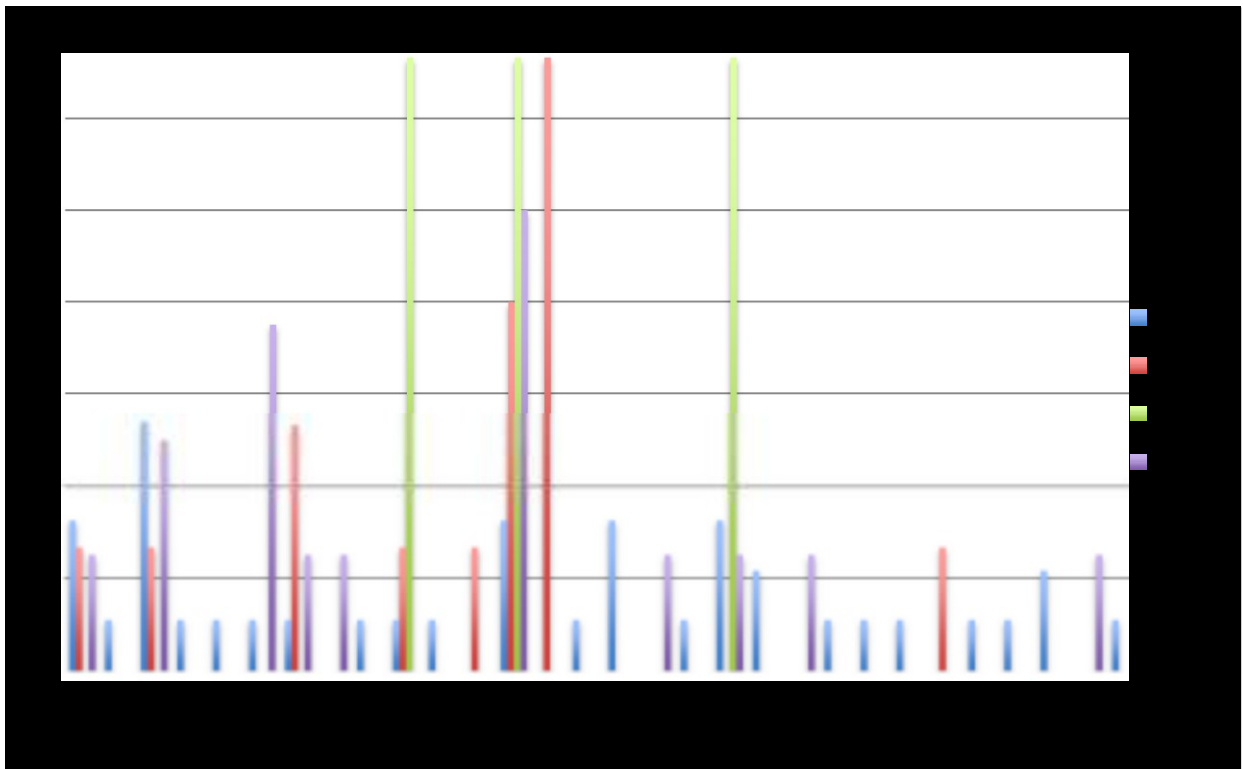


Fig.3.4 Distribution and relative frequency of KD mutations according to treatment with TKIs.

Dasatinib and F317L emergence study

At the time of F317L mutation detection, out of the five patients 3 were in chronic phase and two in accelerated. Three patients had concomitant mutations in addition to F317L (with M351T and H396R, M244V, M351T and F311L; the latter eventually also acquired G250E and T315I). After a median time of 6 months (range 5-8) from F317L detection, two of the CP patients progressed to accelerated phase, and one of the AP patients went into BC (patients' characteristics are reported in table 3C). Results from pyrosequencing confirmed for all the five patients that F317L mutation had emerged only after dasatinib was administered, with the median time from start of dasatinib to detection of the F317L mutations being 6 months (range 2-9 months).

Reported in Fig.3.4. are the monitored kinetics of the mutations in relation to treatment variation over periods ranging from 20 to 40 months respectively for 2 of the patients who developed F317L.

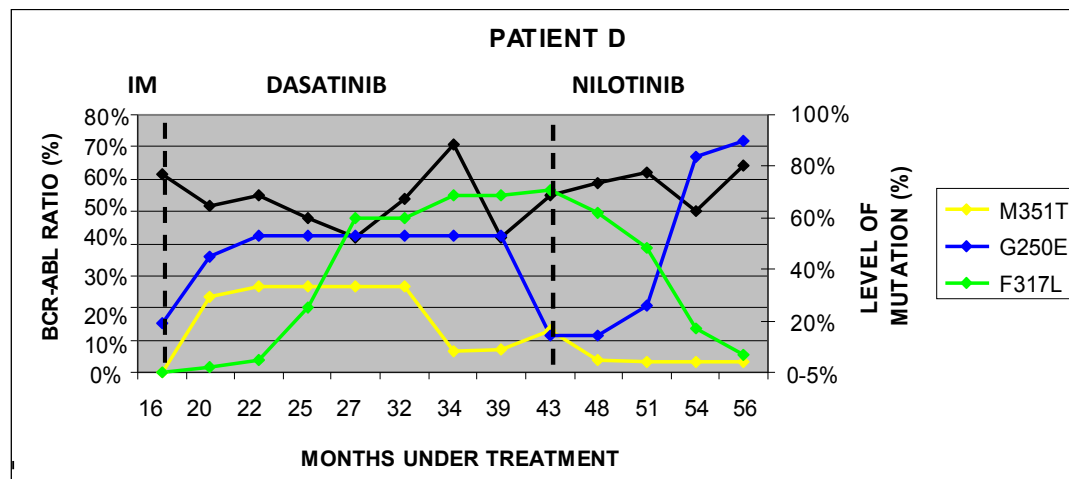
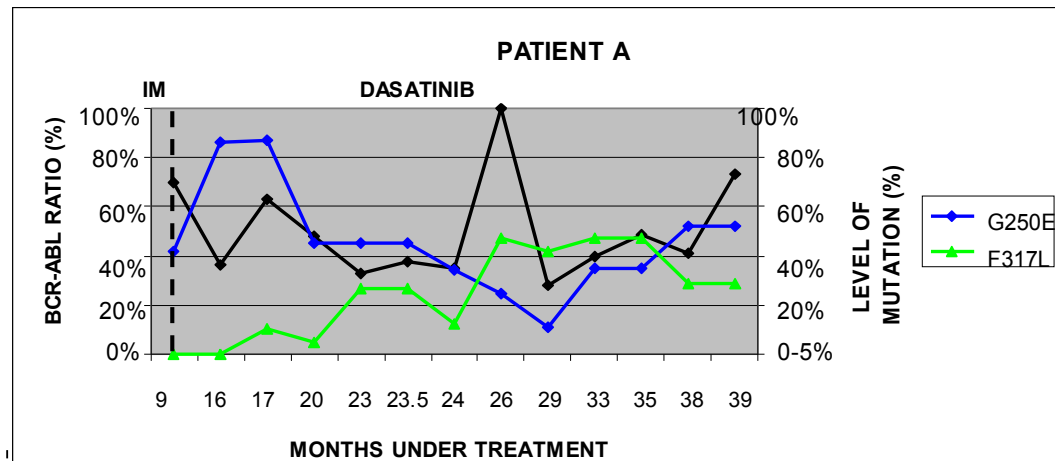


Fig 3.5. Results from pyrosequencing analysis show emergence of F317L BCR-ABL1 kinase domain mutation following dasatinib therapy in two representative patients.

The kinetics of the BCR-ABL1 transcripts measured by RQ-PCR is represented by the bold black line in all 4 panels (expressed as a ratio to the ABL control transcripts x 100%). The coloured lines represent the proportions of the mutant subclones measured at different time points. Months under treatment represent the time from imatinib start.

PATIENT A: A 64-year-old male was started on 400mg daily IM. After 9 months of treatment mutation G250E was identified. Due to lack of response treatment was changed to

dasatinib, and after 4 months reduction in both G250E mutant clone and BCR-ABL1 ratio could be seen. However, mutant F317L levels started to increase and after 10 months from the start of dasatinib both BCR-ABL1 transcript levels and F317L clone increased, while G250E continued to decrease. At that time patients was receiving 90mg of dasatinib daily. Standard dosage of dasatinib (100mg) resumed 1 week after and BCR-ABL1 levels drastically decreased, together with G250E levels, while F317L remained stable. This suggests the 2 mutations, evolving discordantly, belonged to 2 different subclones.

PATIENT D: A 60-year-old male who relapsed after BMT, underwent treatment with IM for 3 months: BCR-ABL1 ratio decreased, but 2 mutations were detected: M351T and G250E. One month later, treatment was replaced with dasatinib, which after 2 months caused a decrease in BCR-ABL1 ratio. The 2 mutant M351T and G250E levels increased, and an additional mutation, F317L appeared. After over 2 years of treatment with dasatinib, nilotinib was introduced and BCR-ABL1 levels in the first instance decreased, only to then increase to their previous % level. Mutation G250E rapidly increased, while F317L drastically decreased. M351T mutant levels became undetectable. As a similar trend was observed for F317L and M351T mutants during the final disease phase, the possibility that the 2 mutations belong to the same subclone cannot be excluded.

Table 3C. Characteristics of the five patients showing emergence of F317L mutation on dasatinib.

	DISEASE PHASE ON IMATINIB	MUTATIONS ON IMATINIB	DISEASE PHASE START OF DASATINIB	TIME ON DASATINIB BEFORE F317L (months)	DISEASE PHASE AFTER F317L DETECTION	DISEASE PHASE ON NILOTINB	MUTATIONS ON NILOTINB	TYPE OF RESPONSE AFTER F317L
Patient A	CP	G250E	CP	4	CP	CP	/	NR
Patient B	CP	M351T; H396R	CP	6	CP	CP	/	NR
Patient C	CP	M244V	CP	3	AP	CP	/	LOR
Patient D	CP	M351T	CP	9	AP	CP	G250E; T315I	NR
Patient E	CP	/	AP	2	AP	CP	/	NR

CP, chronic phase. AP, accelerated phase. NR, no response. LOR, loss of response

Nilotinib and Y253H emergence study

Of the 63 mutation-positive patients, four were found by direct sequencing to harbour mutation Y253H. Since one patient showed such mutation during imatinib treatment, it was excluded from the study. Samples of the three other three patients for which T253H was detected by direct sequencing from nilotinib start were further analysed by pyrosequencing. At start with nilotinib, two of the three patients were in chronic phase and one in accelerated phase. One patient had concomitant mutations in addition to Y253H (E459K and T315I). None of the three patients showed a progression of disease phase.

Results from pyrosequencing confirmed emergence of Y253H from administration of nilotinib only in one patient. In the other two patients the mutation was picked up during treatment with imatinib after a median time of 9 months from its start. For the only patient in which pyrosequencing confirmed Y253H as arisen after start of therapy with nilotinib, mutation kinetics were monitored in relation to treatment variation over a period of 15 to 56 months (median 35.5 months) (Fig.3.6).

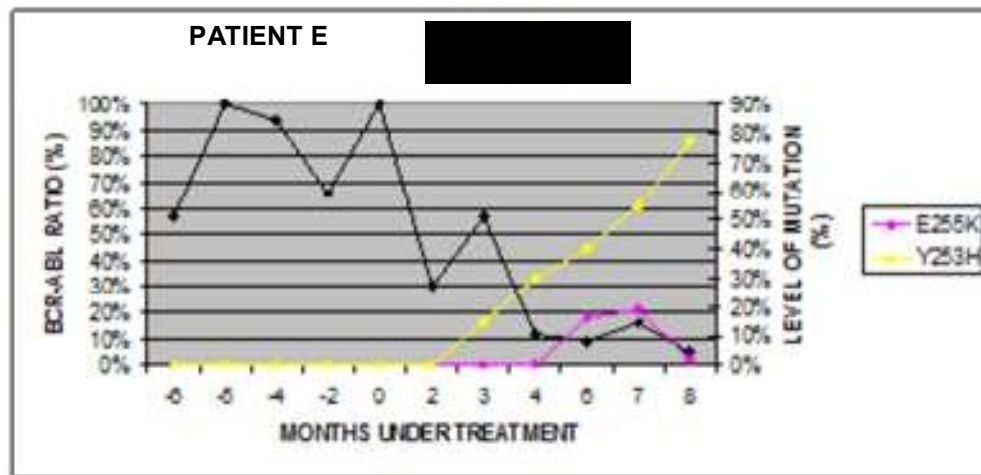


Fig 3.6. Emergence of Y253H BCR-ABL1 kinase domain mutation following nilotinib therapy in one patient. The kinetics of the *BCR-ABL1* transcripts measured by RQ-PCR are represented by the bold black line in all 4 panels (expressed as a percentage ratio over the ABL1 control). The proportions of the mutant subclones measured at different time points are represented by the coloured lines.

Under imatinib treatment the patient developed resistance and was therefore moved to second-line treatment nilotinib. *BCR-ABL1* ratio dropped rapidly and with the exception of a short increase in its levels, the patient achieved a reduction greater than 1 log. Emergence of Y253H mutation was identified at the third month after introduction of nilotinib and its levels continued to increase during the whole treatment duration. Emergence of E255K mutation was also detected, but it became undetectable after a two-month period.

III.III. Mutation analysis of kinase domain SFKs

Previous analysis from this work characterised 63 (23%) of the initial cohort of 269 patients as BCR-ABL1 mutation positive. Out of the 206 patients not harbouring a BCR-ABL1 mutation, 24 had been selected on the basis of treatment with either dual SFK/BCR-ABL1 inhibitors dasatinib or bostinib and when resistance to treatment was observed.

Patients' characteristics of the latter subset are reported in table 3D. Kinase domains of Hck, Lyn and Fyn were amplified for each of the patients and subjected to direct sequencing for screening of SFK KDs.

None of the 24 patients showed presence of such mutations. More recently, however, it was pointed out that, at the time of the SFK analysis, 6 patients out of the 24 originally selected presented a BCR-ABL1/ABL1 percent ratio less than 49%, leading to possible false-negative results and therefore calling the analysis invalid for this subset of patients presenting background normal cells as the predominant population.

Table 3D. Patients' characteristics for the subgroup of 24 patients selected for the dual SFK/BCR-ABL1 study. The only patient treated with bosutinib was in AP. Of the 23 patients on dasatinib, 10 were in CP, 7 in AP and 6 BC. None of them was a responder or harboured a BCR-ABL1 mutation. At the time of analysis 6 patients showed a BCR-ABL1/ABL1 percent ratio inferior to 50%, with the remaining 18 patients showing a BCR-ABL1/ABL1 percent ratio greater than 50%. The analysis has been therefore called invalid for this subset of 6 patients.

	Pts, n	Age, yrs	Sex		Disease phase on dasa/bosu			Previous T	BCR-ABL1/ABL1, %	
			F	M	CP	AP	BC		10 - 49	50 - 100
Tot pts, n	24	57	13	11	10	8	6	2	6	18
Dasa	23	57 (31-82)	12	11	10	7	6	2	6	17
Bosu	1	80	1	0	0	1	0	0	0	1

CP, Chronic phase. AP, accelerated phase. BC, blastic crisis.

T, transplant.

IV. Discussion

The aim of this study was to conduct a systematic evaluation of the appearance, trend and contribution to clinical resistance, of the various BCR-ABL1 mutations arising after treatment with imatinib and the second-generation tyrosine kinase inhibitors dasatinib, nilotinib and bosutinib.

The introduction of selective tyrosine inhibitors for the treatment of CML has revolutionised patient outcome and management of this disease. Imatinib is now the treatment of choice for virtually all newly diagnosed patients. Good responses, in terms of symptom control and haematological parameters, are usually obtained. However, failure to eradicate completely malignant cells (primary resistance) or the difficulty in maintaining a stable response to treatment (acquired resistance), characterising a subset of patients, has led to an intensive search for the mechanisms responsible for resistance development and subsequent treatments through which this refractoriness can be overcome. Resistance has been associated with various mechanisms, such as *BCR-ABL1* amplification, low TKI bioavailability and the acquisition of additional chromosomal abnormalities, but in the vast majority of patients, the decreased response to treatment is due to point mutations in the tyrosine kinase domain of BCR-ABL1.^{50;90}

In this study I have screened a large series of CML patients who had been treated at or referred to the Hammersmith Hospital and had shown resistance to IM or second-generation tyrosine kinase inhibitors. Patients that were intolerant to or failed imatinib treatment became candidates for second-line generation tyrosine kinase

inhibitors, such as dasatinib, nilotinib, and bosutinib. By screening BCR-ABL1 kinase domain for the presence of mutations we detected 69 (23%) patients harbouring a total of over 20 codons hit by mutations. Stratifying the mutation appearance by disease phase I did not observed a distinctive difference in the frequency of mutation emergence in patients in chronic and advanced-stage disease. However, despite the high number of these mutations and the consequently low relative frequencies of each single AA substitution, I observed that *gatekeeper* mutation T315I and the majority of *P-loop* domain mutations were more recurrent in the advanced stages of CML. We also observe a recurrent association between the emergence of these mutations and the progression of the patient to more advanced disease phases. Predictably, patients harbouring T315I experienced resistance to all changes in TKI treatment, as previously reported by others.⁸¹ The reason for this mutation's pan-resistance is that the 'gatekeeper' threonine 315 forms a crucial hydrogen bond with all three TKIs, and the subsequent substitution with a bulkier and more hydrophobic isoleucine abolishes this bond formation, determining a steric clash that prevents the inhibitory action of the imatinib and other TKIs. The P-loop is a highly conserved glycine-rich region occupying residues 248-256 of ABL1, responsible for ATP phosphate binding.⁹¹ Of note, the findings of this study paralleled the work carried out in other centres by different investigators,^{92,80,78} especially the incidence of certain gatekeeper and P-loop mutations prevalently in advanced phases, conferring a faster disease progression and refractoriness to second line treatment.

Second-generation tyrosine kinase inhibitors nilotinib and dasatinib are active against most of imatinib-resistant mutations, but some confer clinical resistance to nilotinib (i.e. Y253H, E255K)⁹³, to dasatinib (i.e. F317, V299L)^{94,95} or both (like the pan-

resistant T315I).⁹⁵ Detection of these mutations at imatinib failure may help selection of the most appropriate alternative treatment. Thus, mutation analysis should be always necessary before start of treatment and changing to other tyrosine kinase inhibitors.¹⁸ Conventional direct sequencing is the golden standard for kinase domain mutation screening,⁹⁶ but it has a detection limit around 20%, with the risk of failing detection of resistant clones present below this level. We therefore assessed sensitivity and specificity of newer available techniques, such as pyrosequencing and d-HPLC and compared them with direct sequencing. Results from this study showed that, despite being endowed with higher sensitivity and being suitable for a large-scale screening, D-HPLC still presents discordant results with those from direct sequencing. This tendency for false positive results was reported in my case for nearly 30% of the samples, rendering the setting conditions unreliable for analysis of diagnostic specimens. This would suggest that direct sequencing remains the method of choice at present for the detection of TKD mutations. Further study aiming to improve detection specificity and reliability of D-HPLC assay and will be resumed in the nearest future. Through serial dilution experiments pyrosequencing proved to be a considerably sensitive methodology (higher than 5%) for detecting and measuring mutation levels. In the four representative patient cases, with a total average of 11 samples initially screened by direct sequencing followed by pyrosequencing, sensitivity of conventional sequencing was confirmed being of only 20%. In patients with a known or suspected mutation history, even when classified as wild-type by direct sequencing, it was possible to detect up to 5% level of mutation. On this basis, pyrosequencing allows to “track back” a mutation emergence, resulting in the construction of patients’ kinetics and providing information concerning mutational patterns. The application of pyrosequencing can therefore allow for an early detection of mutations that confer resistance and this

study at present suggests that this methodology should be applied to all patients in whom a mutation has been detected by conventional sequencing. Samples prior to and following the point at which mutation was detected should be tested. Because pyrosequencing shows a greater sensitivity than direct sequencing, this would allow for therapy interventions to switch to different and more effective treatment when the level of mutations shows a rise in repeated samples.

In this project we additionally investigated the recurrent emergence of mutation F317L in patients on dasatinib, as previously reported by Soverini and colleagues.⁹⁴

Direct sequencing identified F317L in five cases during treatment with dasatinib; pyrosequencing confirmed F317L emergence being subsequent to the introduction of dasatinib. In our patients the dominance of the mutant clone seemed to be the result of a selective effect exerted by dasatinib rather than of a proliferative advantage over the non-mutant BCR-ABL1 clone. We followed the kinetics of 2 of the five patients reporting F317L emergence after introduction of dasatinib post treatment with imatinib. By pyrosequencing it was possible to quantify F317L levels, which had increased during dasatinib administration. A fall in the level of the mutation was observed in both patients when dasatinib treatment was withdrawn to be replaced by nilotinib. However, in both the patients the reduction in F317L level was accompanied by an increase in the level of a clone carrying G250E mutation, which eventually dominated the leukaemic population. To date there are no data about the proliferative activity of G250E but being part of the P-loop, whose mutations Y253F and E255K have been reported by Griswold and colleagues as having greater transforming potential over non-mutant BCR-ABL1 positive BaF3 cells,⁹⁷ it can be hypothesised that G250E might also have higher proliferative potential and it did not dominate on dasatinib therapy because F317L had a higher level of resistance to dasatinib. As soon as the selective pressure from the kinase

inhibitor was removed, the cells carrying G250E started to proliferate and became the dominant clone. It also proposes that G250E has proliferative advantage over the non-mutant BCR-ABL1 clone although this has not experimental confirmation yet. With dasatinib suspension M351T mutation levels also fell and became undetectable. Having M351T been described by Griswold et al.⁹⁷ to have a reduced transforming potential than BCR-ABL1 wild-type, it additionally supports the theory that once the selective pressure exerted by the treatment is removed, mutations that had developed during its administration will either further develop or disappear in correlation to their proliferative advantage. This theory stresses the need for early detection of an emerging resistant clone in order to draw a more stringent follow-up schedule allowing therapeutic choices to be made in a condition of low tumour load.

Mutations within BCR-ABL1 kinase domain, however, account for many but not all cases of resistance. More concerning are a growing number of imatinib and second-generation TKI-resistant CML patients who do not exhibit mutations in the Abl kinase domain but instead show a correlation with overexpression of SFKs, which are not direct targets for imatinib action. Endowed with a dual target of action, SFK/BCR-ABL1 inhibitors have been shown effective in overcoming resistance due to Src family kinase overexpression. Yet, some patients of my cohort who were not harbouring BCR-ABL1 mutations did not respond to such treatment.

The final aim of my thesis has been focused therefore on the investigation of the existence of point mutations within the kinase domain of the three member proteins of SFKs, namely Hck, Lyn and Fyn, as a potential factor contributing to treatment resistance. The study was carried out over a subset of patients that were resistant to SFK/BCR-ABL1 inhibitors dasatinib and bosutinib and were not presenting BCR-ABL1

mutations. To the best of our knowledge, ours was the first study to assess the presence of point mutations within the tyrosine kinase domain of Fyn and Hck in CML BCR-ABL1 patients. Wu and colleagues⁹⁸ investigated the presence of mutations in KD Hck on a relatively small (n=12) subset of CML resistant patients and could not identify any mutations. Although our field of study was larger (n=24) and each patient was screened for the presence of mutations in 3 distinct SFK members, we were not able to detect any mutation. With these results we feel therefore the right to speculate that the emergence of mutations within the KD of the SFKs Hck, Fyn and Lyn might be a highly unlikely (if not completely absent) mechanism of resistance exerted by CML and that further investigation should be continued taking these findings into account.

Future Work

The results reported in my thesis raise some new issues which could well be addressed in future work.

Further assessment and analysis of the application of d-HPLC for mutation analysis in CML could be carried out. Validation of this methodology can be pursued by following protocols already assessed in previous studies and adapted within our laboratory, on samples characterised as mutation-positive by pyrosequencing, which is of a greater sensitivity than direct sequencing.

With the first part of this project focused on the clinical significance of BCR-ABL1 kinase domain mutations and the problem of resistance, and the last one dedicated to the investigation of a possible cause of this resistance, further investigation can continue by screening for BCR-ABL1 mutations and possible genomic aberrations at diagnosis, in order to limit the expansion of potential mutant subclones and formulate a “more-tailored” and effective therapeutic strategy.

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Appendix

Appendix 1. Reagents used for cDNA synthesis

5mg/ml random hexamer primers: Reconstitute 50U pdN6 (Pharmacia) with 539 μ l of sterile water. Then add 21 μ l 0.5mol/L KCl.

5X RT Buffer (usually supplied) with M-MLV reverse transcriptase (RT):

0.25mol/L Tris-HCl, pH 8.3, 0.375mmol/l KCl, 15mmol/l MgCl₂.

25 mM dNTP stock: Mix an equal volume of ultrapure 100mmol/L dATP, dCTP, dGTP and dTTP

0.1M dithiothreitol (DTT) usually supplied with M-MLV RT.

cDNA mix: to 428 μ l of 5X RT-buffer add the following, 21.5 μ l of DTT, 85.5 μ l of 25 mmol/l dNTPs, 45 μ l of 5mg/ml random hexamers and make up to 1000 μ l with sterile water.

Appendix 2. Nested PCR

The first step: to amplify across the BCR-ABL1

First-round PCR mix without Taq polymerase

- Total volume- 10ml
- 10Xbuffer (–MgCl₂): 1ml
- dNTP: 0.25mM
- MgCl₂: 1.8mM
- Primer (Forward) B₂A: 600nM
- Primer (Reverse) JamR: 600nM
- Water: add to total volume of 10ml

Make aliquots of 200µl and store at -70°C.

First-round PCR to amplify BCR-ABL1 transcript:

- Add 1ml of Taq polymerase (5unit/µl) to 200µl of the PCR mix.
- For each reaction, take 20µl of PCR mix; add 5µl of cDNA.
- Run tubes in the PCR machine under Nick 60 program (Appendix 4)
- Store PCR products at 4°C till the second-round PCR is run.

The second step: to amplify the ABL1 kinase domain from the first round PCR products in order to exclude normal ABL1-kinase.

Second round PCR mix to amplify the ABL1- kinase domain

- Total volume: 10ml
- 10X buffer (-MgCl₂): 1ml
- dNTPs: 0.25mM
- MgCl₂: 1.8mM
- Primer (Forward) NTPB+: 600nM
- Primer (Reverse) NTPE-: 600nM
- Water: add to total volume of 10ml
- Make aliquots of 500μl and store at -70°C.

Second round PCR to amplify ABL1-kinase domain:

- To 500μl of the PCR mix add 2μl Taq polymerase
- For each reaction, take 50μl of the mix and add 1μl of the first step PCR product (amplified BCR-ABL transcript).
- Run on Nick-60 protocol (same as first round PCR).
- Store the PCR products at 4°C.

Appendix 3. PCR conditions for Hck, Fyn and Lyn

Hck-KD PCR amplification

PCR-mix without Taq polymerase

- Total volume: 10ml
- 10Xbuffer (–MgCl₂): 1ml
- dNTP: 0.25mM
- MgCl₂: 1.8mM
- Primer (Forward) H_F: 400nM
- Primer (Reverse) H_R: 400nM
- Water: add to total volume of 10ml

Make aliquots of 200ml and store at -70°C.

PCR to amplify Fyn_KD transcript:

- Add 1µl of Taq polymerase (5unit/µl) to 200µl of the PCR mix.
- For each reaction, take 20µl of PCR mix; add 5µl of cDNA.

- Run tubes in the PCR machine under Nick 60 program (Appendix 4).
- Store the PCR products at 4°C.

Fyn-KD PCR amplification

PCR-mix without Taq polymerase

- Total volume: 10ml
- 10Xbuffer (–MgCl₂): 1ml
- dNTP: 0.25mM
- MgCl₂: 1.8mM
- Primer (Forward) F_F: 400nM
- Primer (Reverse) F_R: 400nM
- Water: add to total volume of 10ml

Make aliquots of 200µl and store at -70°C.

PCR to amplify Fyn_KD transcript:

- Add 1µl of Taq polymerase (5unit/µl) to 200µl of the PCR mix.
- For each reaction, take 20ml of PCR mix; add 5µl of cDNA.

- Run tubes in the PCR machine under Nick 60 program (Appendix 4).
- Store the PCR products at 4°C.

Lyn-KD PCR amplification

PCR-mix without Taq polymerase

- Total volume: 10ml
- 10Xbuffer (–MgCl₂): 1ml
- dNTP: 0.25mM
- MgCl₂: 1.8mM
- Primer (Forward) L_F: 400nM
- Primer (Reverse) L_R: 400nM
- Water: add to total volume of 10ml

Make aliquots of 200µl and store at -70°C. PCR to amplify Lyn_KD transcript:

- Add 1µl of Taq polymerase (5unit/µl) to 200µl of the PCR mix.
- For each reaction, take 20µl of PCR mix; add 5µl of cDNA.
- Run tubes in the PCR machine under Nick 60 program (Appendix 4).
- Store the PCR products at 4°C.

Appendix 4: Nick_60 program

95°C 3 min

94°C 40 sec

61°C 40 sec

72°C 1 min

} 32 cycles

72°C 10 min

4°C

Appendix 5: Buffer and mixtures for pyrosequencing

Binding mixture: (for each sample)

- 38µl Binding Buffer (Qiagen)
- 2µl Cepharose beads
- 20µl water
- 20µl PCR product

Annealing mixture: (for each sample)

- 24.2 μ l Annealing Buffer
- 0.8 μ l sequencing primer 10 μ

Appendix 6. Clinical characteristics of the cohort of 269 patients.

Before CCyR	During CCyR	Before Disease progression
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Alphabetical letter of Treatment Group: to be referred to the above table. Mutation boxes have been coloured in green or yellow if the mutation was detected before or after achievement of CCyR respectively. Red-coloured mutations were detected before disease progression.

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
1	44	M	A							1	IM		
2	40	M	A							1	IM		
3	90	F	A							1	IM		
4	80	M	A							1	IM		
5	47	F	A							1	IM		
6	65	M	A	T						1	IM		
7	38	F	A							1	IM		
8	57	M	A							1	IM		
9	82	F	A							1	IM		
10	47	M	A							1	IM		
11	64	M	A							1	IM		
12	32	F	A	T						1	IM		
13	68	M	A							1	IM		
14	36	M	A							1	IM		
15	54	F	A							1	IM		
16	56	M	A							1	IM		

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
17	61	M	A							1	IM		
18	56	M	A							1	IM		
19	56	M	A							1	IM		
20	73	F	A							1	IM		
21	40	M	A							1	IM		
22	72	F	A							1	IM		
23	58	F	A							1	IM		
24	38	M	A							1	IM		
25	38	F	A							1	IM		
26	67	M	A							1	IM		
27	55	M	A	T						1	IM		
28	46	M	A							1	IM		
29	20	M	A							1	IM		
30	69	M	A							1	IM		
31	43	F	A							1	IM		
32	33	M	A							1	IM		
33	46	M	A							1	IM		
34	77	M	A							1	IM		
35	68	M	A							1	IM		
36	48	M	A							1	IM		
37	42	F	A							1	IM		
38	57	M	A		T315I					1	IM		
39	53	F	A							1	IM		
40	51	M	A							1	IM		

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
41	76	M	A							1	IM		
42	42	M	A							1	IM		
43	53	F	A							1	IM		
44	47	M	A							1	IM		
45	56	F	A							1	IM		
46	68	F	A							1	IM		
47	64	M	A							1	IM		
48	33	F	A							1	IM		
49	53	M	A							1	IM		
50	79	M	A							1	IM		
51	73	F	A							1	IM		
52	51	M	A							1	IM		
53	77	F	A		H396R					1	IM		
54	69	F	A							1	IM		
55	64	M	A							1	IM		
56	91	M	A							1	IM		
57	34	F	A							1	IM		
58	54	M	A							1	IM		
59	66	M	A							1	IM		
60	71	M	A							1	IM		
61	61	F	A							1	IM		
62	46	M	A							1	IM		
63	43	F	A							1	IM		
64	55	M	A							1	IM		

65	32	F	A							1	IM		
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Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
66	49	F	A	T						1	IM		
67	29	M	A							1	IM		
68	75	M	A							1	IM		
69	43	F	A							1	IM		
70	55	F	A							1	IM		
71	53	M	A							1	IM		
72	60	M	A							1	IM		
73	48	M	A	T	N231S					1		IM	
74	82	M	B							1	DASA	IM	
75	42	F	B							1	DASA	IM	
76	61	M	B	T						1	DASA	IM	
77	33	M	B	T						1	DASA		IM
78	33	F	B							1	IM	DASA	
79	62	M	B		F359I					1	IM; DASA		
80	62	M	B							1	IM; DASA		
81	36	M	B							1	IM; DASA		
82	21	M	B							1	IM; DASA		
83	52	F	B	T						1	IM; DASA		
84	35	M	B	T						1	IM; DASA		
85	38	M	B							1	IM; DASA		
86	71	M	B							1	IM; DASA		
87	61	M	B							1	IM; DASA		
88	61	M	B							1	IM; DASA		
89	59	F	B							1	IM; DASA		

90	60	F	B							1	IM; DASA		
91	38	F	B							1	IM; DASA		

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
92	66	M	B							1	IM; DASA		
93	62	F	B							1	IM; DASA		
94	66	F	B							1	IM; DASA		
95	65	F	B							1	IM; DASA		
96	52	M	B							1	IM; DASA		
97	51	M	B							1	IM; DASA		
98	40	M	B							1	IM; DASA		
99	64	M	B							1	IM; DASA		
100	41	M	B		L384M	V338F				1	IM; DASA		
101	50	F	B							1	IM; DASA		
102	57	F	B							1	IM; DASA		
103	69	F	B							1	IM; DASA		
104	54	F	B							1	IM; DASA		
105	64	F	B							1	IM; DASA		
106	73	M	B							1	IM; DASA		
107	68	F	B							1	IM; DASA		
108	88	F	B		E276G					1	IM; DASA		
109	27	F	B							1	IM; DASA		
110	69	F	B							1	IM; DASA		
111	53	M	B							1	IM; DASA		
112	45	F	B		S417F					1	IM; DASA		
113	63	M	B							1	IM; DASA		
114	48	F	B							1	IM; DASA		
115	37	M	B		E453V					1	IM; DASA		

116	55	M	B							1	IM; DASA		
117	32	M	B							1		IM	

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
118	54	F	C		F359V					1	IM		
119	33	M	C		Q252H					1	IM; NILO		
120	38	M	C							1	IM; NILO		
121	41	F	C							1	IM; NILO		
122	52	F	C							1	IM; NILO		
123	78	M	C							1	IM; NILO		
124	38	F	C							1	IM; NILO		
125	48	M	C							1	IM; NILO		
126	25	F	C							1	IM; NILO		
127	35	M	C							1	IM; NILO		
128	24	M	C							1	IM; NILO		
129	65	F	C	T						1	IM; NILO		
130	57	F	C							1	IM; NILO		
131	45	F	C							1	NILO	IM	
132	64	F	C							1	NILO	IM	
133	45	F	C		V299L					1	NILO	IM	
134	55	F	C	T						1	NILO	IM	
135	67	F	C							1	NILO	IM	
136	56	F	C							1	NILO		IM
137	66	M	E							1	DASA; NILO	IM	

138	75	M	E							1	IM; DASA; NILO		
139	60	M	E							1	IM; DASA; NILO		
140	52	F	E							1	IM; DASA; NILO		
141	70	M	E							1	IM; DASA; NILO		

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
142	69	M	E				E450V (DASA)			1	IM; DASA; NILO		
143	65	F	E		M351T	H396R	F317L (DASA)			1	IM; DASA; NILO		
144	67	M	E	T						1	NILO	IM; DASA	
145	63	M	E		L248V					1	NILO	IM; DASA	
146	41	M	E							1	NILO	IM; DASA	
147	58	M	E							1	NILO	IM; DASA	
148	34	M	F	T						1	IM; NILO; DASA		
149	50	M	F							1	IM; NILO; DASA		
150	73	F	F				Y253H (NILO)			1	IM; NILO; DASA		
151	57	F	F							1	NILO; DASA	IM	
152	42	F	F							1	NILO; DASA	IM	
153	35	M	I							1	IM; DASA; BOSU		
154	79	M	J				V299L (BOSU)			1	DASA; BOSU; NILO	IM	
155	58	F	K							1	BOSU	IM; NILO	
156	35	M	L	T			T315I (BOSU)			1	BOSU	IM	
157	62	M	L				F359V (BOSU)			1	IM; BOSU		
158	65	F	N							1	INF; IM		

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
159	54	M	O		G250E					1	INF; IM; DASA		
160	62	M	Q							1	HU; IM		
161	75	M	R							1	IM; NILO	HU	
162	63	M	U							1	DASA		
163	66	M	U							1	DASA		
164	65	F	U							1	DASA		
165	65	M	A		F359V					2	IM		
166	63	M	A							2	IM		
167	49	M	A							2	IM		
168	54	F	A							2	IM		
169	61	M	A							2	IM		
170	58	M	A							2	IM		
171	61	F	A							2	IM		
172	40	F	A							2	IM		
173	61	F	A							2	IM		
174	39	M	A							2	IM		
175	52	M	A							2	IM		
176	71	M	A							2	IM		
177	69	M	A		E255K					2	IM		
178	80	M	A							2	IM		
179	64	M	A							2	IM		
180	54	M	A							2	IM		
181	76	M	A							2	IM		
182	80	M	A							2		IM	

183	46	F	A							2		IM	
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Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
184	64	M	A	T	L364I					2		IM	
185	66	F	A							2		IM	
186	70	F	A							2		IM	
187	46	M	A							2		IM	
188	63	M	A							2		IM	
189	55	M	A							2		IM	
190	18	M	A		G250E					2		IM	
191	44	F	A		H396R					2			IM
192	47	M	A	T						2			IM
193	80	F	A							2			IM
194	46	F	A							2			IM
195	90	F	A		E355G					2			IM
196	57	F	A							2			IM
197	88	F	A		T315I	G250E				2			IM
198	63	F	B		F311I		T315I (DASA)			2	DASA	IM	
199	58	M	B	T			E255K (DASA)			2	IM	IM; DASA	
200	72	M	B							2	IM		DASA
201	53	M	B		G250E		T315I (DASA)			2	IM		DASA
202	76	M	B							2	IM		DASA
203	43	M	B							2	IM		DASA
204	82	F	B							2	IM; DASA		
205	41	F	B							2	IM; DASA		

206	51	M	B							2	IM; DASA		
207	75	F	B							2	IM; DASA		

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
208	51	M	B	T	E459K		V299L			2	IM; DASA		
209	58	F	B				M244V (DASA)			2		IM	DASA
210	81	M	B							2		IM;	
211	53	M	B	T	M244V					2		IM;	
212	31	M	B							2			IM; DASA
213	78	M	C				Y253H (NILO)			2	IM	IM; NILO	
214	77	M	C							2	IM	NILO	
215	51	F	C							2	IM	NILO	
216	49	M	C				T315I (NILO)			2	IM	NILO	
217	37	F	C							2	IM	NILO	
218	74	F	C							2	IM	NILO	
219	50	M	C		E255K					2	IM		NILO
220	50	M	C							2	IM; NILO		
221	63	F	C				F359C (NILO)			2	IM; NILO		
222	52	F	C							2	IM; NILO		
223	73	M	C		E450K					2	IM; NILO		
224	64	M	C							2	NILO	IM	
225	69	F	C				L387M (NILO)			2	NILO	IM	
226	80	F	C							2		IM; NIL	

227	45	M	C				E255V (NILO)			2		IM; NILO	
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Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
228	44	M	C				T315I (NILO)			2		IM; NILO	
229	44	M	C							2		IM; NILO	
230	59	M	C							2			IM; NILO
231	67	M	D		E459K	T315I	Y253H (NILO)			2		NILO; HHT	IM
232	48	M	E	T(2)	G250E		T315A (DASA)			2	DASA; NILO		IM
233	61	M	E	T			F359V (NILO)			2	DASA; NILO		IM
234	62	M	E	T	M351T		F317L (DASA)	G250E (NILO)	T315I (NILO)	2	IM	DASA	NILO
235	38	M	E	T			T315I (NILO)			2	IM	DASA; NILO	
236	69	F	E							2	IM		DASA; NILO
237	60	F	E	T			F317L (DASA)			2	IM		DASA; NILO
238	67	M	E		M244V					2	IM, DASA; NILO		
239	74	F	E				E255K (DASA)			2	IM; DASA; NILO		
240	69	F	E							2	IM; DASA; NILO		
241	70	F	E		F359V					2	IM; DASA	NILO	

242	62	F	E	T	M244V	F317L (DASA)		2	IM; DASA	NILO	
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Pt no. Group	Age	Sex	Treatment T (green when successful)		MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCvR?		CP	AP	BC
										Yes =1	No = 2			
243	69	F	E		F486S		E462K (NILO)			2		IM; DASA	NILO	
244	41	F	E							2		IM; DASA; NILO		
245	68	F	E							2		IM; DASA; NILO		
246	67	F	E							2		IM; DASA; NILO		
247	65	F	E							2		IM; DASA; NILO		
248	76	M	E							2		NILO	IM; DASA	
249	67	F	E							2			IM	DASA; NILO
250	77	F	E							2			IM; DASA	NILO
251	64	M	E	T						2			IM; DASA; NILO	
252	59	M	F		F359V					2		DASA	IM; NILO	
253	79	M	F							2		IM	IM; NIL; DASA	
254	64	F	F		L384M					2		IM; NILO	DASA	
255	54	F	F	T						2		IM; NILO; DASA		
256	65	M	F		G250E					2		NIL; DASA	IM	
257	71	M	F		Y253H					2			DASA	IM; NILO
258	46	F	G		T315I		G250E (DASA)			2			IM; DASA	HHT
259	41	M	H	T	Y253F	M351T	E255K (NILO)			2		NILO	IM; HHT	DASA

Pt no	Age	Sex	Treatment Group	T (green when successful)	MUT IM1	MUT IM2	MUT 2	MUT 3	MUT 4	CCyR? Yes = 1 No = 2	CP	AP	BC
260	80	F	L							2		IM; BOSU	
261	31	F	M	T						2	IM; INF		
262	81	M	M		T315I					2	IM; INF		
263	65	M	O		E453K					2	IM	DASA	INF
264	66	M	P		G250E		F317L (DASA)			2	INF; IM; HU; DAS; NIL		
265	65	F	P	T	T315I		M244V (NILO)			2	INF; IM; HU; DASA	NILO	
266	74	M	S				G250E (NILO)			2	IM	HU; INF; DASA; NILO	
267	71	M	T				T315I (DASA)			2	IM; HU	DASA	
268	70	M	U							2		DASA	
269	73	M	U							2		DASA	