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The Value of Molecular Response in Chronic Myeloid Leukemia: The Present and the Future

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1. Introduction

1.1 Historical notes

The last decade has witnessed profound changes in the treatment of chronic myeloid leukemia (CML). Previously, therapeutic options were restricted to the use of conventional chemotherapeutic agents such as hydroxyurea (*Goldman, & Marin, 2003*) and busulfan (*Brodsky, 1993*). These were essentially cosmetic treatments, offering only palliative care, and not substantially altering the natural history of the disease. Later in the 90s interferon alpha (IFNα), was introduced in the therapeutic armamentarium for CML patients (*Goldman, 2003*). When used at high doses, this agent proved to be superior to conventional chemotherapy in terms of hematological and cytogenetic response rates. In particular, 9- or 10-year overall survival (OS) rates in the range of 27% to 53% (*Bonifazi, 2001*) have been reported. However, residual leukemia was still detectable at the molecular level in the vast majority of patients (*Baccarani, 2003*). Overall, these observations indicated that none of these treatment options were curative for CML and allogeneic bone marrow transplantation remained the only disease-eradicating therapy, albeit at the price of substantial treatment-related mortality, especially for the higher EBMT risk score patients (*Gratwohl, 1998 ; Baccarani, 2006;* Passweg , 2004*).*

1.2 The modern era of CML treatment: the TKi revolution

In 1960 Nowell and Hungerford, working in Philadelphia, noticed the consistent presence of a small abnormal chromosome in the leukemic cells of CML patients (*Nowell & Hungerford, 1960*). Strikingly, this abnormality was present in nearly all cases and in all leukemic cells of a single patient, indicating that it could represent a disease marker and, possibly, a tumorigenic alteration. The abnormal chromosome was named "Philadelphia". Since then, the development of more sophisticated and reliable diagnostic technologies has led to precise characterization of the Philadelphia chromosome (Ph) as the result of the reciprocal translocation t(9;22), as well as the corresponding molecular defect, consisting in the formation of a chimeric oncogene, *BCR-ABL*, from the juxtaposition of the broken ends of chromosomes 9 (*ABL*) and 22 (*BCR*) (*Rowley, 1973)* (Fig.1). Molecular biology studies suggested that the product of *BCR-ABL* was an oncoprotein, provided with constitutive

phosphorylating activity (*Gale & Canaani 1984*; *Sefton, 1981*; *Witte, 1980*). This was shown to promote escape from apoptosis, uncontrollable proliferation, diminished adherence to the marrow stroma, and significant genetic instability (*Lugo, 1990*; *Melo,V 2004*) (Fig.2). Most importantly, when expressing *BCR-ABL* in animal models, the investigators demonstrated that BCR-ABL, as the sole oncogenic event, was able to induce leukemia (*Lugo, 1990*; *Melo, 2004*).

In the late 80s the tyrosine kinase inhibitor (TKi) program started and one leading compound of the 2-phenylaminopyrimidine class capable of inhibiting the ABL kinase was identified: STI571, or imatinib (*Zimmermann, 1996*; *Druke, 1996*; *Buchdunge, 1996*; *Druker & Lydon 2000*; *Carrol, 1997*). Since then, the drug has rapidly undergone preclinical and clinical development until FDA approval only 3 years after the initiation of the phase I study (*Druker, 2001a*; *Druker, 2001b*; *Kantarjian, 2002*; *Sawyers, 2002*). In June 2000 the landmark International Randomized Study of Interferon and STI571 (IRIS) was initiated. More than 1000 previously untreated CML patients in chronic phase (CP) were randomly allocated to either IFN+cytosine arabinoside (ARA-C) or imatinib. The remarkable superiority of the latter in terms of complete hematologic, major and complete cytogenetic responses, as well as rates of progression to advanced disease phases, namely accelerated phase (AP) or blastic phase (BP), led to early closure of the study and most of IFN+ARA-C patients being crossed over to imatinib (*Druker, 2006*; *O'Brien, 2003*).
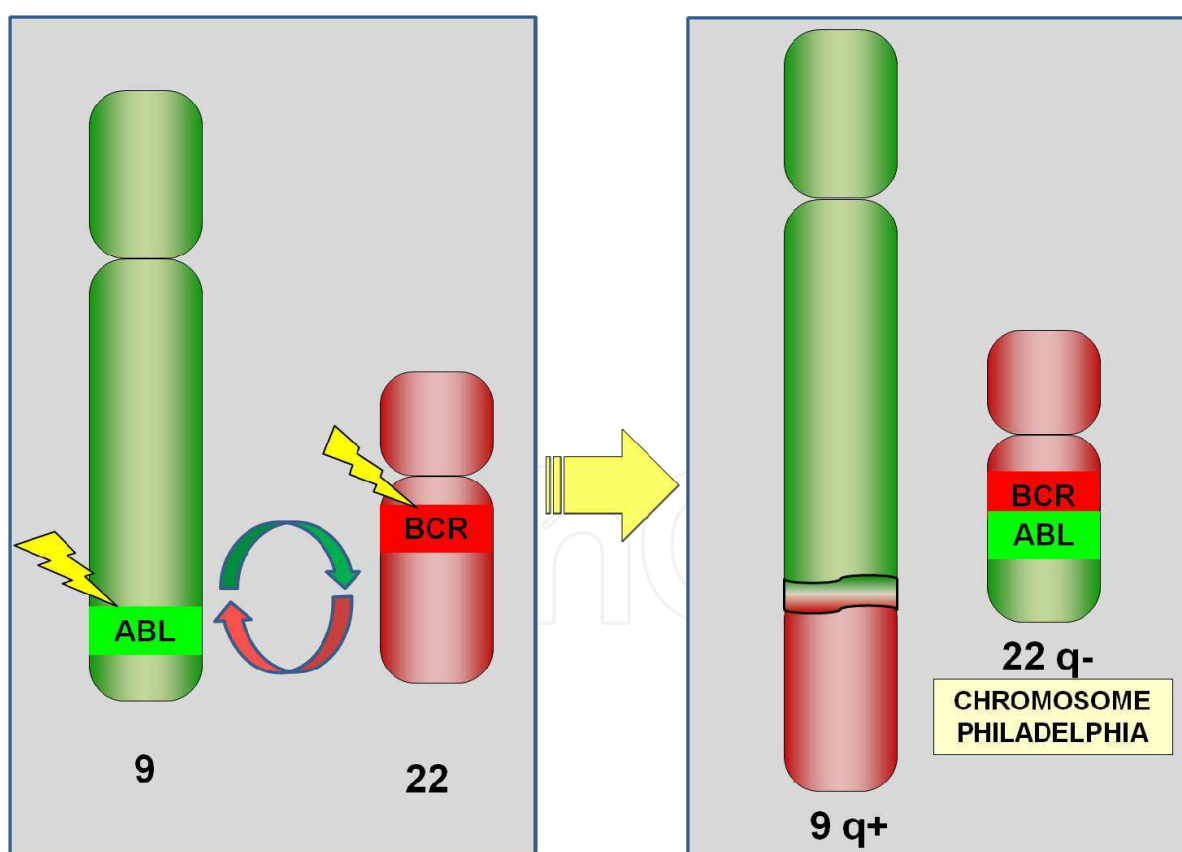

Fig. 1. Schematic diagram of the translocation that results in Philadephia chromosome. The *ABL* gene resides on the long arm of chromosome 9, the *BCR* gene resides on the long arm of chromosome 22. As a result of the (9;22) translocation, a *BCR-ABL* gene is formed on the derivative chromosome 22 (Philadelphia chromosome)
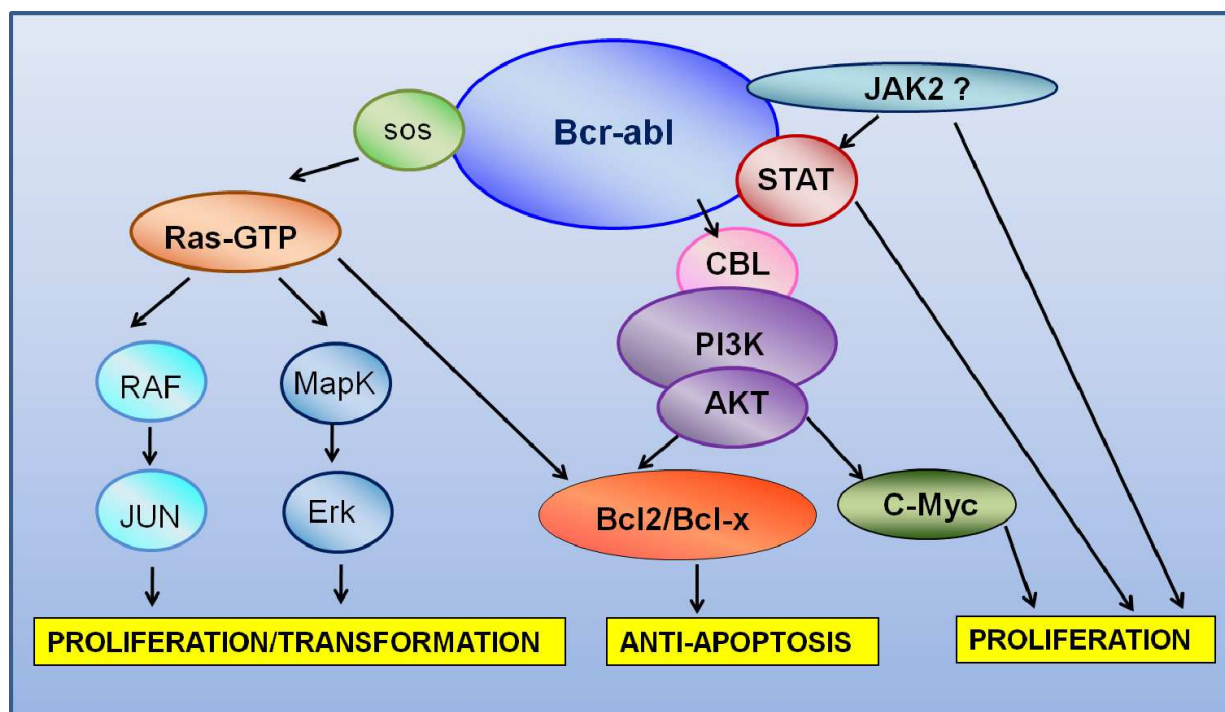
Fig. 2. Schematic signalling pathways activated by BCR-ABL that contribute to growth and survival

Overall, the introduction of TKis in clinical practice has brought dramatic improvement in the rates and quality of hematologic and cytogenetic responses and has led to a paradigm shift in the treatment approach of CML patients. These drugs have also defined our current view of desirable cancer therapy as a targeted tumor cell killing using agents that directly interfere with oncogenetic mechanisms. Finally, CML is a significant example of the usefulness of molecular. These techniques may also be used to predict early treatment failure and to direct therapeutic choices accordingly. This chapter covers various aspects of CML patient molecular monitoring, including the use of well-established diagnostic techniques, past and ongoing standardization practices, and the role of molecular diagnostics in clinical practice. Some as-yet open issues and unanswered questions in the field will also be pointed out.

2. Assessment and monitoring of CML: Molecular tools

As most patients treated with TKis achieve a complete cytogenetic remission (CCyR), highly sensitive molecular diagnostic techniques have been implemented in parallel with the clinical development of these drugs. This has allowed to fully appreciate the potency of these compounds and, more importantly, to evaluate leukemic cell clearance and quantify residual disease at a much deeper level. These techniques encompass fluorescent *in-situ* hybridization (FISH), reverse transcriptase (RT)-polymerase chain reaction (PCR) and real-time quantitative (RQ)-PCR, and will be briefly discussed.

2.1 FISH

This technique can be used to ascertain the presence of the *BCR-ABL* fusion gene in a given cell sample. FISH allows analysis of both dividing (metaphase) and non-dividing

(interphase) cells (*Faderl, 1999*), and can be performed on either peripheral blood or bone marrow samples. A minimum of 200 cells should be analyzed in order for the test to be informative (*Kantarjian, 2008*). Its sensitivity is high and the upper limit of false positivity is 1% to 5% (*Dewald, 1998*). The hypermetaphase FISH allows analysis of up 500 cells in metaphase per sample and produces no false positive results but cannot be performed on peripheral blood samples (*Seong, 1995*). The dual-color FISH (D-FISH) uses double-color probes that allow detection of a fusion signal. In CML, modern D-FISH strategies use a "green" probe to identify *BCR* and a "red" one to highlight *ABL*. A yellow signal indicates the presence of a *BCR-ABL* fusion sequence (*Tefferi, 2005*). This technique allows to detect not only the presence, but also the copy number of the fusion gene on the Ph, as well as the number of any additional *BCR-ABL*-bearing chromosomes, such as the ones resulting from variant translocations, cryptic translocations or insertions (*Dewald, 1998*). D-FISH has very low false-positive rates (≤0.8%) (*Wolff, 2007*). It should be remembered that D-FISH does not substitute for conventional cytogenetics because it will not detect additional cytogenetic abnormalities, unless specifically requested.

CML patients show 85 to 99% of *BCR-ABL*-positive nuclei in bone marrow before treatment, which decrease to less than 1% when therapy is successful (*Tefferi, 2005*). FISH has been evaluated as an alternative to routine marrow cytogenetics for monitoring purposes (*Testoni, 2006*). However, up to 18% of patients in CCyR by standard cytogenetics has 1% to more than 5% FISH-positive cells (*Kantarjian, 2008a*). The GIMEMA (Gruppo Italiano Malattie Ematologiche Adulto) CML working party has reported that as much as 83% of patients having a CCyR by conventional testing, also had <1% of *BCR-ABL* positive nuclei at interphase FISH. Conversely, among patients who had <1% positive nuclei by interphase FISH, 98% had a CCyR using conventional cytogentic analysis. Moreover, major molecular response rates were significantly higher in patients with <1% positivity by interphase FISH compared with patients with positivity rates of 1% to 5% (*Testoni, 2009*). This data show that interphase FISH is more sensitive than conventional karyotyping, and can be used as a monitoring tool in patients who are in CCyR as per classical cytogenetics (*Quintás-Cardama, 2011*).

2.2 RT-PCR

The *ABL* gene encodes a 145kd non-receptor tyrosine kinase. The breakpoint in *ABL* occurs usually at 5′ (toward centromere) of exon 2 of *ABL*. The *ABL* exons 2 (a2), are translocated and joined to the major breakpoint cluster region (M-bcr) of the *BCR* gene on chromosome 22 between exons 12 and 16 (b1 to b5). The breakpoint locations within *BCR* fall either 5′ between exons b2 and b3 or 3′ between b3 and b4. A *BCR-ABL* fusion gene with a b2a2 (40%) or b3a2 (55%) junction is created and transcribed into a 8.5 kb mRNA that encodes for a 210 kd fusion protein termed BCR-ABL (*Faderl, 1999*; *Sawyers 1999,Quintás-Cardama* & *Cortes* 2006). A second breakpoint involves a minor cluster region on chromosome 22, which is located upstream at the e1a2 junction, and gives rise to an mRNA translated into 190kDa protein (*Okamoto, 1997*)(Fig.3). In 5% of cases, alternative splicing produces an e1a2 fusion transcript. This encodes a p230 oncoprotein, which appears to be provided with less pronounced oncogenic potential.

PCR is used to detect and measure the amount of specific DNA sequences. For practical reasons it is easier to amplify a *BCR-ABL* mRNA that includes b2a2, b3a2 or e1a2 fusion sequences (*Hughes, 1990a; Hughes, 1990b*). In reverse transcriptase (RT)-PCR disease-specific

mRNA is first converted to complementary DNA and subsequently subjected to standard PCR (*Sawyers,1990;Kawasaki, 1988*). The resulting amplified product is then assessed by gel electrophoresis. Assay specificity and sensitivity in RT-PCR can be enhanced by the use of nested primers (nested RT-PCR) (*Biernaux, 1995*). Nested RT-PCR, is a two-step process. A first pair of PCR primers amplifies the target sequence in a standard RT-PCR. A second pair of primers (nested primers) then bind within the primary amplified PCR product to produce a second PCR product that is shorter in length. This technique is capable of detecting 1 leukemic cell in 10^6 to 10^7 (*Roth, 1992, Lion, 1999; Lee M 1992,Dhingra K 1992*) normal cells. Since CML patients in hematologic and cytogenetic remission may still show residual leukemic cells at RT-PCR, this technique has extensively been used to assess and monitor minimal residual disease in these cases (*Cross, 1993a*). However, because of the lack of quantitative information, positive detection of *BCR-ABL* transcript provides uncertain information and does not allow tracing disease level trends over time. Indeed, some PCR-positive patients could maintain their minimal disease state and eventually become PCR-negative while on therapy (*Hochhaus, 2000,Hughes, 1991).*
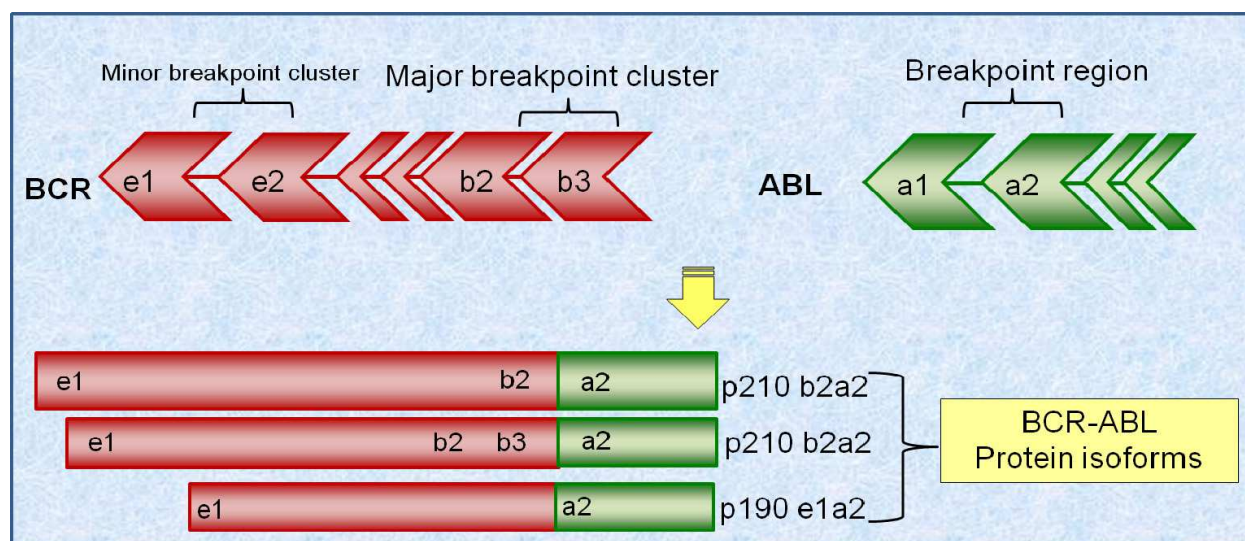

Fig. 3. Breakpoints within the *BCR* and *ABL* genes and corriponding proteins

2.3 RQ-PCR

Quantification of specific sequences of DNA has been made possible by the use of RQ-PCR (or Q-PCR) (*Mensink, 1998*). Compared to RT-PCR , RQ-PCR enables accurate quantification of gene expression during the exponential phase of the PCR amplification process. This is achieved by concomitantly measuring one ubiquitously expressed housekeeping gene, such as *ABL1*, *BCR*, *β₂-microglobulin*, *β-glucuronidase* or *glucose-6-phosphate dehydrogenase*. (*Hughes, 2006*) (*Guo, 2002*) (*Beillard, 2003*). Real-time PCR is based on the measurement of fluorescence emission during the PCR reaction. The detected fluorescence is proportional to the amount of target in the sample. Currently, three different RQ-PCR techniques are available: RQ-PCR using SYBR Green I Die, RQ-PCR using hydrolysis probes, RQ-PCR using hybridization probes (*Gabert, 2003*). The Europe Against Cancer (EAC) program standardized the RQ-PCR for the detection of residual disease in leukemia. This protocol uses the ABI 7700 platform with TaqMan probes that permit analysis of a large number of samples in a single run (96-well plate format). The TaqMan technology uses a single internal

oligonucleotide probe bearing a 5' reporter fluorophore and 3' quencher fluorophore. As long as the two fluorochromes are in each other's close vicinity (probe is intact), the fluorescence emitted by the reporter fluorochrome will be "adsorbed" by the quencher fluorochrome. During the amplification of the target sequence, the probe is hydrolyzed by the nuclease activity of the Taq polymerase, resulting in separation of the reporter and quencher fluorochromes and consequently in an increase in fluorescence. During each consecutive PCR cycle, this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes. In the TaqMan technology, the number of PCR cycles necessary to detect a signal above the threshold is called the cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the reaction. Using standards or calibrators with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample (Gabert ,2003; *Mocellin, 2003*; *Beillard, 2003*; *van der Velden, 2003* ; *White, 2010* ;*Cross, 1993b*). A sensitivity of 1 leukemic cell in up to 10^5 normal elements is achievable with RQ-PCR. False negatives (lack or sub-optimal integrity of mRNA and/or cDNA) must be considered and controlled (*Béné & Kaeda, 2009*). Although less sensitive than nested RT-PCR, RQ-PCR has gained an important role in CML molecular monitoring, especially to identify earlier patients not optimally responding to or at high risk of relapse on TKi therapy (*Lange, 2004*; *Serrano, 2000*; *Martinelli, 2000*).

3. The international scale

Molecular diagnostics have been tested as a means of assessing patient prognosis beyond the predictive power of cytogenetic tools. However, there has been considerable variability in the results of such analyses depending on the particular testing laboratory. Harmonization of critical pre-analytical and procedural steps in the PCR technique has proven feasible and was the first significant step towards full reproducibility and comparability of the quantitative results provided by different laboratories using different RQ-PCR platforms around the world (*Müller, 2009*).

One turning point in the process of harmonization has been represented by a consensus meeting held in Bethesda, MD, USA in 2005. An internationally recognized panel of experts aimed at providing recommendations to standardize the measurement of *BCR-ABL* RNA levels in any given laboratory worldwide by means of a reference scale, now known as the International Scale (IS) (*White, 2010*). The IS relies upon two specific concepts: the standardized baseline, or IS 100%, which is, by definition, the median pre-treatment level of *BCR-ABL* RNA in early chronic phase CML (as defined in IRIS imatinib trial), and major molecular response (MMR), or IS 0.1%, or a 3-log (1,000-fold) drop from the baseline value (*Hughes, 2003*; *Branford, 2006)*. A level of IS 1-2% roughly corresponds to the threshold for karyotypic CCyR. Following this line, a "complete molecular remission" was defined as undetectable *BCR-ABL* transcripts, that is, below the sensitivity of the assay. A comparison between cytogenetic and molecular response milestones is depicted in Fig 4. The panel recommended a desirable test sensitivity of at least IS 0.01% (= 4-log reduction from baseline) (*Quintás-Cardama, 2011*). It is to be noted that original material from the IRIS study was limited and therefore is no longer available as primary reference. However, traceability to the IRIS scale is provided by the extensive quality control data generated by the Adelaide laboratory over a period of several years (*Branford, 2008)*.
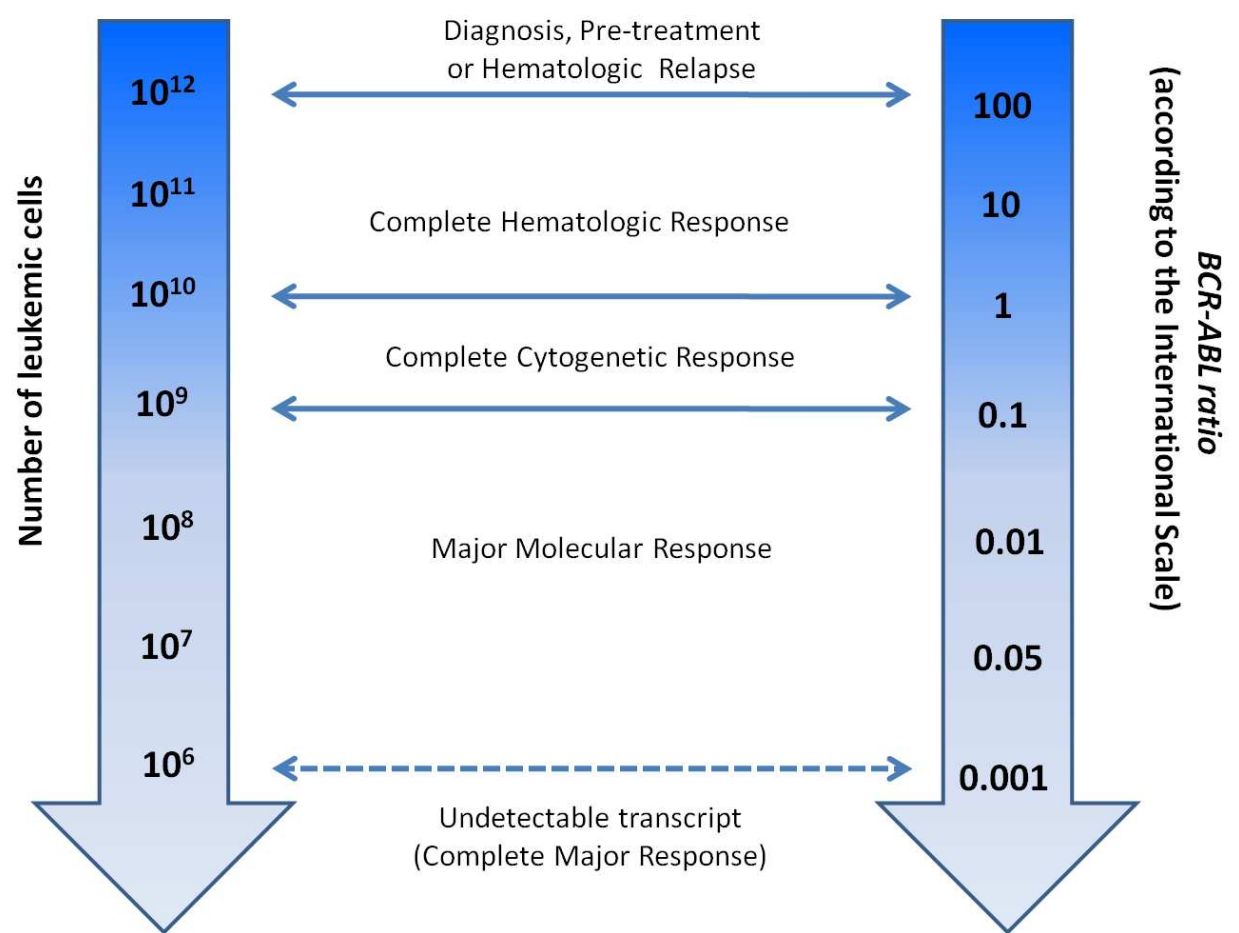
Fig. 4. Relationship between response, the number of leukemic cells and the level of *BCR-ABL* transcript. Reproduced and adapted with permission; Baccarani et al., *Blood* 2006: Sep 15;108(6):1809-20.

3.1 Generation of the IS

The standardized baseline value was determined by an exchange of reference standards with values established in reference labs. Reference and quality control samples would have to be widely available for any peripheral laboratory to standardize its internal protocol. The easiest way to achieve such standardization is by a laboratory-specific conversion factor (CF), established using the Adelaide laboratory process as the initial reference (*Branford, 2008*). In order for a certain laboratory to establish its own CF, typically 20-30 samples are exchanged with the reference laboratory that span at least 3 logs of detectable transcript levels, not exceeding IS 10%, to avoid distortions resulting from different control genes at higher disease levels. These samples are then analyzed by both laboratories over a certain period of time (to avoid intralab biases) and then compared. The results are plotted on a log scale for comparison. Lastly, they are validated through a second material exchange (*Branford, 2008*).

Currently, there is an ongoing collaborative effort to harmonize 57 different laboratories across Europe in the context of the European Treatment and Outcome Study (EUTOS) for CML project. The European reference laboratory is in Mannheim, Germany, by direct

alignment with results obtained in Adelaide (*Müller, 2009*). In the first step, samples are prepared by the reference lab to specifically reflect 10, 1, 0.1, and 0.01% disease levels. These are then shipped to the local laboratory for analysis. The local laboratory in turn sends patient samples covering approximately the same transcript levels, using internal protocols, as well as duplicate results for the calculation of the CF. This is generated by comparing reference and local laboratory values by linear regression. For labs with linear results a preliminary CF is calculated, and then validated using the method published by Branford et al. (*Branford, 2008*). Patient samples from the peripheral laboratory are analyzed by the reference one. Preliminary CFs are then used to compare the patient sample results from the reference laboratory (each multiplied by the Mannheim CF which is 0.878) and local laboratories (each multiplied by their respective preliminary CF). Concordance is recorded, and calculation adjustments to take bias into account are made (*Bland & Altman 1986, 1999, Müller, 2009*).

3.2 Beyond conversion factors: independent laboratory access to the IS

Despite the above mentioned efforts to standardize local protocols for *BCR-ABL* mRNA quantitation, (*Gabert, 2003*) there is still substantial variation among the various laboratories worldwide in the way RQ-PCR is performed and results are reported. (*Cross, 2009*, *Müller, 2007*). Such variability is evident even among laboratories that use the same commercially available kit. Reasons for this variability include the fact that there is no universally accepted control gene and the absence of independent reference materials. The use of CFs as a means of harmonization has undoubtedly allowed testing centers to continue use their internal protocols and express results according to local preferences in addition to the IS percentage values. Nevertheless, the establishment of CFs is a time-consuming, complex, and expensive procedure. Moreover, the timing with which a certain CF needs to be revalidated is not defined (*Müller, 2009*). For these reasons, a collaborative project has been undertaken among 11 reference laboratories worldwide with the aim of developing calibrated, accredited primary reference reagents for *BCR-ABL* RQ-PCR analysis. The experts chose freeze-dried K562 cells as a source of *BCR-ABL* and HL60 cells, known to be *BCR-ABL*-negative, as a source of control genes, including *ABL*, *BCR* and *GUSB*, as recommended by the Bethesda group. They then created a cell line mixture consisting of K562 cells and HL60 cells and corresponding to %*BCR-ABL/ABL* values of 10%, 1%, 0.1%, and 0.01%. Cell mixtures proved to be stable over time at temperatures below 37°C and homogeneous in terms of material distribution at each %*BCR-ABL*/control gene. This work has produced 3500 vials for each dilution level. Since this would be insufficient for the worldwide annual demand, a decision has been made to use these primary reagents as reference for calibration of secondary reagents that could be produced on a larger scale by laboratories, companies or other agencies and provided to single testing centers (*White, 2010*). Although of no immediate use, the development of primary reference reagents will be of great importance in the future to facilitate the production of more readily available IS calibrated reagents worldwide.

4. Prognostic role of molecular remission

The attainment of a CCyR has uniformly been shown to improve event free survival (EFS) and OS in CML patients receiving imatinib regardless of the baseline Sokal risk score, and

thus has been established as a robust endpoint for CML patients treated with imatinib in CP. However, conflicting data exist regarding similar prognostic value of MMR in this patient population. There is some evidence that the achievement of an MMR at 12 or 18 months after imatinib initiation, or at any time after CCyR predicts superior long-term clinical outcomes, as well as a significantly decreased risk of disease progression to more advanced disease stages (AP/BP). (*Baccarani, 2009b; Deininger, 2009, Hughes 2010*) It is true that, although OS should be considered the final endpoint in such clinical trials, sustained survival of CML patients in CP on TKi therapy implies that very long term follow-up may be needed for statistically significant differences in outcome to become apparent. For this reason, EFS, progression free survival (PFS), and transformation-free survival (TFS) are often used as surrogate endpoints.

4.1 The prognostic role of cytogenetic remission

Data from studies on CML patients treated upfront with imatinib 400 mg daily indicate a CCyR rate of 45 to 59% at 6 months, 57 to 72% at 12 months and 76% at 18 months (*O'Brien,2 003; Druker, 2006; Kantarjian, 2003, 2010; Saglio, 2010; Cortes, 2010a)*. Patients attaining a CCyR were protected from disease transformation, compared with those who did not achieve such response degree *(Deininger, 2009)*. Several subsequent landmark analyses confirmed a shorter survival free of progression to AP/BP for patients not achieving a CCyR both at 12 and 18 months of therapy (*O'Brien, 2003*). However an OS advantage was seen only in patients who achieved at least a partial response at 6 and 12 months versus patients who did not (*Kantarjian, 2008b*). Increasing upfront the dose of imatinib to 800 mg daily could not provide any survival advantage (*Baccarani, 2009a)*.

4.2 CCyR duration is improved in patients who achieve an MMR

Information on the prognostic implications of molecular response in CML patients in CCyR on imatinib therapy was provided by the IRIS study. In that trial, 39% of patients in CCyR on the imatinib arm achieved a 3-log reduction in *BCR-ABL* values. Landmark analysis at 12 months in patients on imatinib without disease progression revealed that PFS was 100%, 95% and 85% for patients with CCyR and 3-log *BCR-ABL* reduction (proposed as the definition of MMR), CCyR but no such reduction, and no CCyR, respectively (*Hughes, 2003, 2006)*. A synopsis of relevant studies analyzing the relationship between CCyR duration and level of molecular response is shown in Table 1.

Paschka et al. analyzed 323 samples from 48 Ph-positive IFNα-pretreated CML patients receiving imatinib. CCyR was obtained in 41 cases. At the time of best response, overall median *BCR-ABL/ABL* ratio in peripheral blood was 0.086%, but best responses of patients destined to relapsed were significantly higher than those of patients in continuous CCyR, either globally considered or in CP only (1.4% vs 0.071%, p .0017 and 2.1% vs 0.075%, p .0011, respectively). More importantly, whereas all 16 patients who achieved a *BCR-ABL/ABL* ratio of <0.1%were still in continuous CCyR at the time of writing, 6 (46%) patients with ratios ≥0.1%did lose their cytogenetic response, and this was the only significantly different parameter between the two groups. One possible weakness of this study is the shortness of follow-up, of only 13 (0-35) months, especially in light of the extremely sustained response durations seen with TKi therapy (*Paschka, 2003)*.

In line with these results, Iacobucci et al. assessed 97 CML patients in late CP for the duration of cytogenetic response according to the level of molecular response. *BCR-ABL*

transcript levels were significantly lower in patients maintaining their cytogenetic response, compared with those who subsequently relapsed. Moreover, with a median follow up time of 36 (12-54) months, CCyR duration was significantly longer in patients with MMR (defined as either an absolute BCR-ABL/β2 microglobulin % value $\leq$ 0.0005 or a 3-log reduction from pre-treatment median population or individual BCR-ABL value) both at the time of first CCyR and at 12 months from the start of imatinib treatment. Patients with loss of CCyR also showed a significantly reduced 4-year OS compared with stable CCyR patients (60% vs 95%, p .0004) (*Iacobucci, 2006*).

Study	No.	Length of follow-up in months (median)	% losing CCyR	
			Pts with MMR at 12 months (%)	Pts without MMR at 12 months (%)
Paschka 2003	29	13	0	46
Cortes 2005	280	31	5	37
Iacobucci 2006	97	36	8	30
Marin 2008	224	46.1	2.6	23.9
Marin 2008*	224	46.1	0	24.6
Press 2007	90	49	16	57

Table 1. Selected studies of the impact of molecular response on the duration of CCyR; *analysis at 18 months

Prognostic relevance of MMR with imatinib as first line has also been investigated (*Cortes, 2005*). Two hundred eighty previously untreated CML patients in CCyR on imatinib therapy with at least 1 PCR test done for follow-up were observed for a median of 31 (3-52) months at the M.D. Anderson Cancer Center (MDACC). MMR and complete molecular response (CMR) rates were 62% and 34%, respectively. CCyR was lost by 9 (5%) and 25 (37%) patients who did or did not achieve MMR defined as a 0.05% value, respectively (p .0001). The percentage of patients losing their CCyR was not significantly different between MMR and CMR patients (*Cortes, 2005*). Press et al. reached similar conclusions. They evaluated 90 CML patients, using a 3-log drop in BCR-ABL values from baseline as a definition of MMR. With a median follow-up of 49 months after the initiation of imatinib, 20 (22%) patients relapsed. Once again, the median *BCR-ABL* level as detected by RQ-PCR was significantly lower in patients with future stable cytogenetic response compared with those who subsequently relapsed at every time point from 12 to 36 months. Relapse rate was 16% in patients who attained MMR and 57% in patients who never did. Accordingly, relapse-free survival was significantly shorter in patients who did not achieve an MMR (median 46 months) versus patients that did (median not reached at the time of writing; p.0008), and the hazard ratio for relapse was 4.1 (95% confidence interval, 1.7-10; p.002) (*Press, 2007*). The Hammersmith group also has published their data from a series of 224 consecutive CML patients, with

particular attention to patients failing or sub optimally responding to first-line imatinib according to the 2006 version of the recommendations of the European LeukemiaNet (ELN). When analyzing the effect of molecular response on the probability of losing a CCyR, they found that patients in CCyR who had failed to achieve MMR at 12 or 18 months had a higher CCyR loss rate than patients who did achieve MMR, (23.6% versus 2.6%, p .04 and 24.6% versus 0%, p<.006, respectively (Fig.5) (*Marin, 2008*). It is to be noted that these recommendations have been updated in 2009, but long-term considerations on these changes cannot be made as of yet.
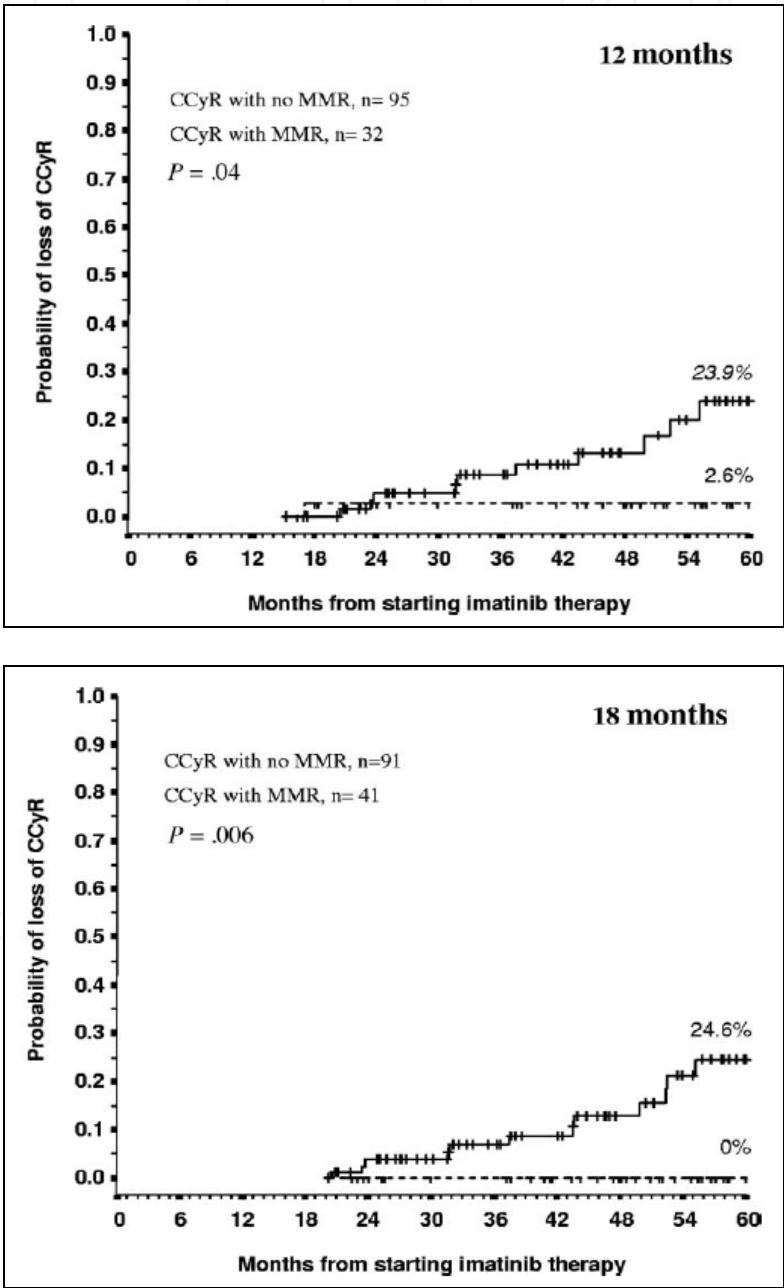

Fig. 5. Twelve- and 18-months landmark analyses for loss of CCyR according to the level of molecular response. Vertical lines represent censored patients. Reprinted with permission; Marin et al., *Blood* 2008;112(12):4437-44.

4.3 Impact of molecular remission on long-term patient outcome

In CML patients treated upfront with standard 400 mg imatinib daily, cumulative rates of MMR ranged from 12 to 40% at 12 months, and from 50 to 52% at 18 months in published studies. As previously mentioned, initial report from the IRIS trial on 370 patients (337 receiving imatinib as first-line) showed a 12-month MMR rate of 39% across all Sokal risk groups, with PFS 100% for patients achieving both CCyR and MMR at a median follow-up of 25 months (*O'Brien,2003; Hughes, 2010*). Seven-year follow-up analysis of the same trial highlighted some important points: first, rates of molecular responses tend to increase with continuous imatinib therapy over time. At 84 months, MMR rates were 87-92% and *BCR-ABL/ABL* ratio was 0.003-0.004% according to the IS (*Hughes, 2010*). Second, the virtually all MMR patients were also in CCyR at several timepoints. Third, and most importantly, at 12 and 18 months, but not at 6 months, there was a statistically significant advantage in EFS and TFS for MMR versus non-MMR patients (EFS rate: 91 vs 79.4% and 94.9 vs 75.3%, respectively, at 12 months; TFS: 99 vs 89.9% and 99.1 vs 90.1%, respectively, at 18 months). However, when comparing MMR patients to those with *BCR-ABL* ratios of >0.1 to ≤1%, an advantage of EFS for the former was evident only at the 18 month time point (Fig.6), and TFS was only marginally significant. Moreover, in each comparison, OS did not differ significantly at every time point.
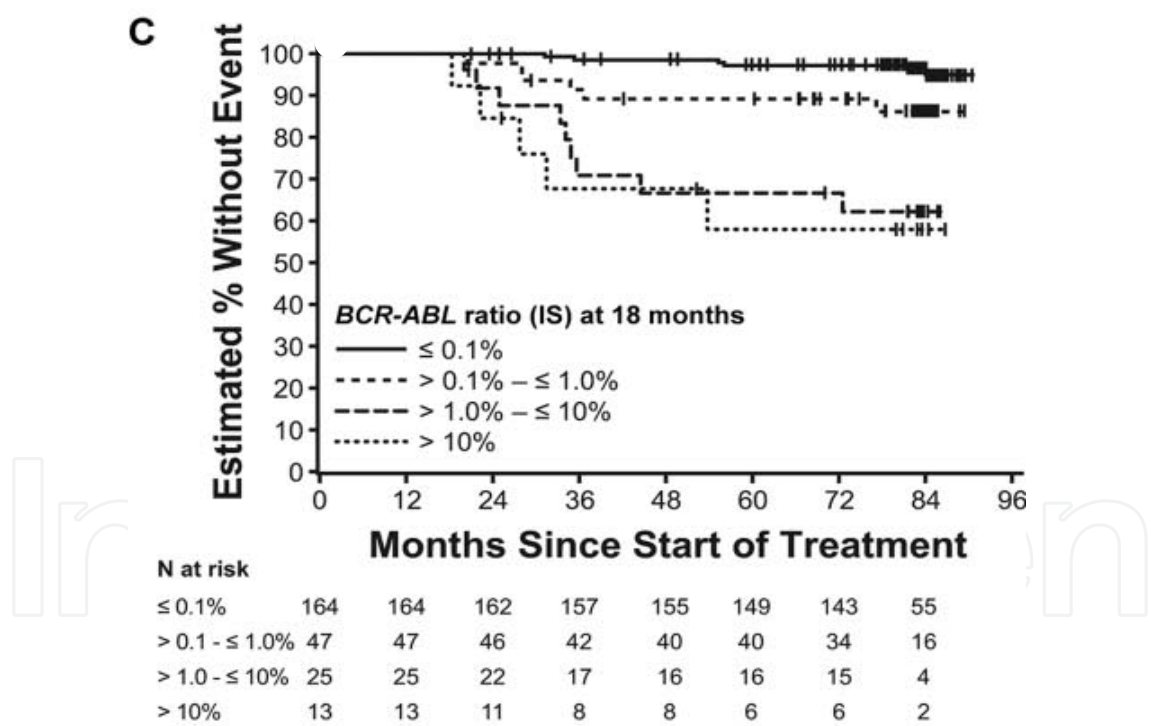

Fig. 6. EFS at the 18-month landmark by molecular response. EFS definition does not include loss of CCyR. Reprinted with permission; Hughes et al., *Blood* 2010, Nov 11;116(19):3758-65.

Similar conclusions were reached by several other single center analyses. For example, when looking at molecular responses in their published series, the Hammersmith group found that, either considering the whole patient population or only patients in CCyR after 12

months of imatinib therapy, the achievement of MMR at 12 or 18 months did not translate into a 5-year PFS or OS advantage (*Marin, 2008)*. Similarly, in the MDACC series of 269 patients treated with imatinib upfront with more than 1 molecular evaluation available, molecular response at various timepoints did predict from survival (mainly PFS), but this was not independent from the degree of cytogenetic response, PFS only somewhat differing in MMR-CCyR patients *(Kantarjian, 2008b)*. Taken together, these data led the ELN to express specific recommendations: that failure to achieve an MMR after 12 months of imatinib therapy be considered a "warning sign" (patients may require more frequent monitoring); that failure to achieve an MMR after 18 months of imatinib therapy be a criterion for defining "suboptimal" response (consider possible change in therapy). However, failure to achieve a MMR at any timepoint is never considered a treatment failure in the last version of the ELN guidelines (*Baccarani, 2009)*.

It is important to consider that landmark analyses are used to study patients achieving a certain level of response by a specific time point, different from treatment start and, by definition, they consider only patients who are on treatment and evaluable at that time. By doing so, a "better performing" population is always selected for the analysis. On the other hand, the definitions of cumulative CCyR, MMR, and CMR also include patients that meet such milestones only once over a long course of therapy with multiple serial evaluations. This way of reporting data may also provide a better than real picture of treatment efficacy, a bias avoided by landmark analyses.

4.4 The role of early MMR

Early identification of cancer patients failing on a certain therapy has become increasingly important in order to make potentially beneficial therapeutic adjustments before the end of treatment, and improve prognosis of patients otherwise destined to fare poorly. One such brilliant example is Hodgkin disease, in which a positive post-II cycle positron emission tomography (PET) can identify patients with dismal outcome and allow PET-oriented differential therapeutic strategies. In CML, several studies suggested that the degree of molecular response at early time points may predict later achievement of an MMR and, possibly, improved rates of PFS and EFS. Overall survival advantage for these patients, however, is little, if any, within the available follow up. Merx et al. demonstrated that a reduction of the *BCR-ABL/ABL* ratio to at least 20% of baseline after 2 months of treatment confers a significantly higher probability of major cytogenetic response (MCyR) later at 6 months (*Merx, 2002)*. In a separate analysis, although on a smaller number of patients, a *BCR-ABL/ABL* ratio reduction of 50% at 4 weeks, or a reduction of 90% at 3 months significantly predicted for the attainment of a MCyR at the 6 month time point. Also, with a median follow up of 16.5 months, there was suggestion that these achievements could predict better PFS. Branford et al. performed early and serial molecular follow up studies on 55 evaluable CML patients treated with imatinib either upfront or after failure of IFNα+ARA-C. The authors found a median 1.6-log reduction after 3 months of first-line TKi therapy, not significantly different from second-line imatinib. They used the 2-log reduction cutpoint (grossly equivalent to a CCyR in the study) at 3 months to distinguish rapid from slow responders and observed that the former had a higher likelihood of achieving an MMR by 24 months (100% vs 54.2% p .001) (*Branford, 2003a)*. Further confirmation of these findings came from an analysis conducted at the MDACC, showing that at time points progressively farther (ie, 3, 6, 12 months) from imatinib start, the probability of attaining a

CCyR for patients not yet at that point decreases, while in parallel the event rate increases. The probability of achieving a CCyR and a MMR by the degree of *BCR-ABL/ABL* ratio reduction showed that a reduction to at least 10% conferred a significantly higher likelihood of achieving such goal either at 3, 6 or 12 months. Moreover, when considering the 3 month cut point, 3 prognostic categories were distinguishable, ie, patients with a ratio of 1% or less, over 1% to 10%, and greater than 10%, with distinct probabilities of achieving CCyR and MMR (*Quintás-Cardama, 2009*)

5. Early switch to second generation TKis

As newer and more potent therapeutic options are being made available for CML patients, the need for early prediction of treatment failure is becoming more and more urgent, and the issue of early therapeutic switch in view of a non-satisfactory response has recently emerged as a crucial one. In fact, the above mentioned data suggest that treatment should not be continued indefinitely in patients not adequately responding to first-line imatinib as their likelihood of later response becomes progressively narrower, and especially early failure should prompt a change in the strategy. This concept is further reinforced by an analysis of the significance of suboptimal response to imatinib at different time points after the start of therapy. Such analysis was conducted in 281 CML patients mostly in early CP. Outcome of suboptimal responders in term of EFS tended to be more similar to that of failing patients at 6 months, whereas it was closer to the optimally responders thereafter (12 and 18 months). Likewise, the likelihood of achieving a MMR varied over time and tended to behave similarly to that of cytogenetic response (Fig. 7) (*Alvarado, 2009*).

Options for patients failing on imatinib include switch to a second generation TKi, namely nilotinib or dasatinib, and, for those who are candidates, allogeneic bone marrow transplantation. There is data to suggest that waiting until clinical or hematological CML relapse may be too late for a switch. Patients who failed INFα therapy could obtain high response rates and survival times if they were treated with imatinib at the time of cytogenetic, rather than hematologic relapse (*Kantarjian, 2002, 2004*). In a subsequent analysis from MDACC, the 3-year survival rates were 72%, 30%, and 7% for patients who remained in CP, progressed to AP, or to BP after imatinib failure, respectively. Moreover, 3-year survival rates were 92% and 57% for patients treated with second generation TKis at cytogenetic and hematologic relapse, respectively, with hematologic relapse, but not (yet?) therapy, being an independent poor prognostic factor (*Kantarjian, 2007*). A subsequent cumulative analysis of three, relatively homogeneous, dasatinib trials showed that patients treated with this second generation TKi at the time of loss of MCyR fared significantly better than those who received the drug when they had lost both MCyR and complete hematologic remission (CHR), or lost CHR having never attained an MCyR. For the three groups, CCyR rates were 72%, 42%, and 26% and MMR rates were 60%, 29%, and 26%, respectively. Twenty four month EFS, TFS, and OS were 89%, 98%, and 98%; 29%, 93%, and 93%; 64%, 79%, and 86% for the same three patient groups, respectively (*Quintás-Cardama & Cortes, 2009*). Whether adjusting treatment strategy in patients categorized as suboptimal responders at various time points could be beneficial in terms of survival has not been established as yet and very long-term follow-up studies may be needed to demonstrate a statistically significant survival advantage applying this strategy.
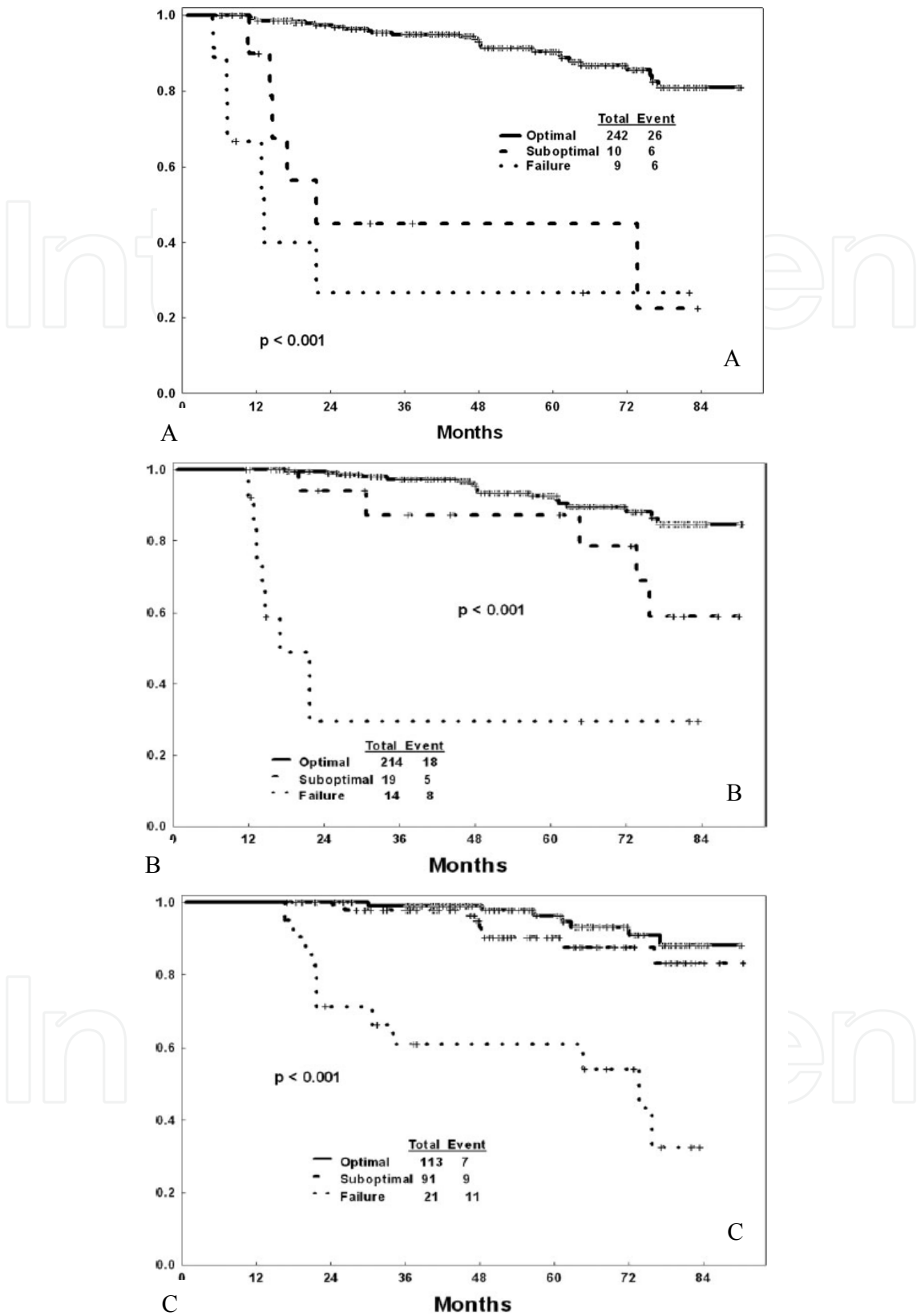
Fig. 7. Event-free survival according to response at 6 (A), 12 (B), and 18 (C) months in patients treated with imatinib in early CP. Reprinted with permission; Alvarado et al., *Cancer* 2009, Aug 15;115(16):3709-18.

6. Role of molecular remission with second generation TKis

As previously discussed, early molecular response on imatinib therapy predicts the probability of later achievement of an MMR, improved rates of PFS and EFS and lower the risk of disease transformation. Likewise, attainment of a precocious MMR may be a positive prognostic indicator for patients treated with the second-generation TKIs. Indeed, these agents have proven to work quicker than imatinib and to induce higher cytogenetic and molecular remission rates. This has provided the clinical rationale for successfully testing these agents as frontline treatment options for CML patients in chronic phase. Dasatinib (formerly BMS-354825) is 325-fold more potent than imatinib at inhibiting the unmutated form of BCR-ABL *in vitro* (*O'Hare, 2005)*. This drug is chemically unrelated to imatinib and binds to BCR-ABL protein at a different but overlapping site (*Tokarski, 2006).* Nilotinib (formerly AMN-107) is 20-fold more potent than imatinib *in vitro*. It was developed through a modification of the chemical structure of imatinib and therefore binds to a very similar binding site on the BCR-ABL. However, it fits much better into the tertiary structure of the oncoprotein, and this enhances its biological activity (*O'Hare, 2005; Weisberg, 2005).* These two drugs were evaluated in single-arm phase I and II studies, first in patients with resistance or intolerance to imatinib (*Quintás-Cardama, 2009a)*, and subsequently in the frontline setting. The trials showed that first-line treatment with dasatinib or nilotinib resulted in higher rates of CCyR and MMR earlier compared to what historically observed with imatinib (*Cortes, 2010, 2010c).* Results from two important phase III trials have been recently published comparing either dasatinib or nilotinib with standard dose imatinib as first-line treatment for patients with newly diagnosed CML in chronic phase.

In the ENESTnd trial (Evaluating Nilotinib Efficacy and Safety in clinical Trials of Newly Diagnosed Ph CML patients), nilotinib was employed at two different dosages, ie 300 mg or 400 mg twice daily. The primary endpoint of the study, MMR rate at 12 months, was largely met for both nilotinib dosages with MMR rates of 44%, 43% and 22% for nilotinib 300 mg twice daily, nilotinib 400 mg twice daily and imatinib, respectively (p<.001). CCyR rates by the same time point also significantly favored both nilotinib arms (80% for nilotinib 300 mg twice daily, 78% for nilotinib 400 mg twice daily, 65% for imatinib; both p<.001), and a similar trend for CCyR was evident at 6 months. Median time to MMR was 8.6 months with nilotinib 300 mg, 11.0 months with nilotinib 400 mg, and not reached with imatinib. Rates of *BCR-ABL* transcript reduction to or below the sensitivity limit of the PCR assay (set at ratio 0.0032%, CMR) were 13%, 12%, and 4%, respectively (*Saglio, 2010).* A 24 month follow up of the study showed MMR and CMR rates of 71% and 26% vs 67% and 21%, vs 44% and 10%, respectively, for nilotinib 400 mg BID vs nilotinib 300mg BID vs imatinib. Estimated freedom from progression to AP/BC and PFS at 24 months were also significantly superior for both nilotinib arms (99.3 (p .0059) and 98.1(p .0196)) versus imatinib (95.2), although estimated OS rate advantage at 24 month did not reach statistical significance (*Larson, 2011).*

In the DASISION trial (Dasatinib vs Imatinib Study in Treatment-Naive CML Patients), dasatinib 100 mg once daily was tested against imatinib 400 mg daily. Both CCyR and MMR rates at 12 months were significantly higher with dasatinib (CCyR: 83% vs 72%, p. 0011; MMR: 46% vs 28%, p. 0001). Rate of confirmed (c) CCyR by 12 months (the primary endpoint of the study) was also significantly increased (77% vs 66%, respectively; p. 0067). Importantly, CCyR rates at 3 and 6 months for dasatinib and imatinib were 54% vs 31% and 73% vs 59%, respectively. Median time to MMR for patients who achieved this goal was 6.3 months for dasatinib and 9.2 months for imatinib(*Kantarjian, 2010*). Recent update of this

study showed that 18-mo response rates for dasatinib versus imatinib were: cCCyR 78% vs 70%, p .0366; CCyR 84% vs 78%, p.0932; and MMR 56% vs 37%, p<.0001. CMR rates for dasatinib and imatinib were 13% and 7%, respectively. Six (2.3%) vs 9 (3.5%) patients, respectively, transformed to AP or BP on study (*Kantarjian, 2011a*).

The results of the trials described above clearly indicate that second generation TKis are able to achieve higher cytogenetic response rates and a substantially deeper leukemia clearance as demonstrated by the higher MMR rates and roughly doubled CMR rates in comparison with imatinib. Moreover, patients treated with nilotinib or dasatinib can achieve these important therapeutic milestones relatively early in the course of treatment, and, if the prognostic relevance of molecular remission will be confirmed, be possibly protected against the risk of loss of response and/or disease transformation later on.

7. Treatment failure prediction

Loss of molecular response at any time during therapy, as measured by confirmed rising *BCR-ABL* RNA levels, is considered a reliable criterion for prognosticating early treatment failure, and might influence an early treatment strategy change (*Press, 2010)*. However, at this time, there is no uniform definition of what should be considered a "significant" rise in *BCR-ABL* transcript level *(Kantarjian, 2009; Press, 2007)*. In their study, Press et al. found half-log *BCR-ABL* increase (after adjusting for a 0.5-log interassay variability) as a threshold to predict for subsequent relapse as well as for shortened relapse-free survival. This retained its prognostic value even with imatinib dose escalation as a therapeutic response. Moreover, such an increase remained predictive of shortened relapse-free survival when considering only MMR patients (*Press, 2007*).

A major cause of TKi treatment failure is the appearance of CML clones bearing *BCR-ABL* mutations that confer variable degree of insensitivity to the drug. For this reason, once rising *BCR-ABL* transcript levels are documented, screening for ABL kinase domain mutations is reasonable and recommended. A large number of different point mutations have been described over the last few years, affecting different spots in various domains of the BCR-ABL oncoprotein. Mutations can be categorized into four groups, based upon the crystallographic structure of ABL: a) those which directly impair imatinib binding to the catalytic domain of oncogenic protein; b) those within the ATP binding site; c) those within the activation loop, which prevent the kinase from inactivating, required for imatinib binding; d) those within the catalytic domain (*Baccarani, 2008*). Mutations in the P-loop (G250E; Q252H, Y253F; E255K) may impart a particularly poor prognosis (*Soverini, 2005, 2011, Quintás-Cardama, 2006*). Several technologies are available for the identification of *BCR-ABL* mutations. These include direct sequencing, subcloning and sequencing, denaturing high-performance liquid chromatography (D-HPLC), pyrosequencing, double-gradient denaturing electrophoresis, allele-specific oligonucleotide PCR. Direct sequencing is the most widely applied. Briefly, the total RNA from whole blood leukocytes is reverse transcribed with random primers and the cDNA product is amplified with *BCR/ABL* – specific primer set. The PCR product is then sequenced with *ABL*-specific primers. Standard dideoxy chain-termination DNA sequencing is performed and then analyzed using a specific software (*Jones, 2008*). The assay detects mutations in the *ABL* kinase domain between amino-acids 50 and 510. Direct sequencing has a detection limit of a mutation frequency of 20% (*Branford, 2002; Hochhaus, 2002; Roche-Lestienne, 2002*). Newly identified mutations should be confirmed by amplifying the normal ABL alleles to exclude

polymorphisms (*Hughes, 2006)*. Various groups use higher sensitivity D-HPLC to routinely screen for kinase domain mutations, and then characterize them by sequence analysis (*Soverini, 2004; Deininger, 2004*). Strategies to circumvent mutation-induced imatinib resistance include imatinib dose escalation (*Branford, 2003b*) and switch to a second generation TKi. These drugs have proven particularly effective in this regard, since most *BCR-ABL*-mutated patient can still achieve a quick and high-quality cytogenetic and molecular response when crossed over nilotinib (*Kantarjian, 2011b*) or dasatinib (*Quintás-Cardama, 2009*) after imatinib failure. The T315I mutation confers resistance to most TKis including nilotinib, dasatinib, and bosutinib (*Shah, 2004*).

8. Complete Molecular Remission

A proportion of patients in MMR on imatinib therapy, eventually achieve CMR, defined as undetectable *BCR-ABL* mRNA transcripts by real-time QPCR and/or NESTED/RT-PCR in 2 consecutive high-quality samples (with sensitivity $>10^4$) (*Baccarani, 2009b)*. CMR rates ranged from 4 to 41% in published studies. Such wide variability may be due to heterogeneity in treatment duration and dose, as well as in detection techniques employed. In the study by Press et al. 28 MMR patients on imatinib therapy eventually achieved CMR (3% and 18% of the entire cohort of patients at 12 and 24 months, respectively). Relapses occurred in 4% of CMR patients compared with 23% in MMR patients who failed to achieve CMR, with a median relapse-free survival of 44 versus not reached at the time of writing (p .0052). The achievement of a CMR, thus appears to define an excellent long-term prognosis and may be regarded as an optimal therapeutic goal (*Press, 2007*). Furthermore, with the use of second-generation TKIs (nilotinib and dasatinib) as frontline therapy for CML CP patients, even increased numbers of patients will ultimately achieve this level of response (*Saglio, 2010 ; Cortes, 2009 Shah, 2004; Quintás-Cardama & Cortes 2008*).

There is ongoing effort to better define CMR from a quantitative standpoint in order to use and validate it as a surrogate survival endpoint. Indeed, the definition of PCR negativity is poorly standardized among laboratories and certainly the phrase "below the sensitivity of the assay" cannot be used as a reference standard because such sensitivity is laboratory-specific by nature. Moreover, evidence that achievement of a CMR has an impact on long-term EFS, PFS, or OS is limited, and more follow up is needed to draw any conclusion in this regard. In conclusion, the concept of CMR is still an evolving one, and the consistency of its prognostic value remains to be proved.

9. Cure for CML?

The absence of detectable *BCR-ABL* transcripts in a CML patient does not appear to indicate disease eradication. Ross et al. analyzed by DNA PCR 18 CML patients in sustained CMR after imatinib, who stopped therapy as part of a clinical trial (*Ross*, 2010). DNA PCR has the advantage of being a genomic test (RQ-PCR detects *BCR-ABL* mRNA in up to 30% of normal individuals), of being patient-specific, and of eliminating the risk of cross-contamination between samples. It has a sensitivity of around $1/10^6$ (*Biernaux, 1995*). Seventeen of 18 studied patients in CMR had a positive DNA PCR result at least once. Ten patients did relapse and these had an exponential increase in the levels of *BCR-ABL* DNA. However, the test had positive and negative predictive values of 62 and 75%, respectively, and thus limited value as a predictor of relapse (*Ross, 2010*).

Several studies have explored the possibility of discontinuing imatinib therapy in patients with long lasting CMR, but high relapse rates have been observed in these study populations. In the Australian CML8 study, of 18 early or late CP-CML patients, 5 did relapse after stopping imatinib, all within 5 months. CMR was regained in every instance upon resuming treatment (*Ross, 2008*). Recently, a prospective multicenter Stop Imatinib (STIM) study has been published by Mahon et al (*Mahon, 2010*), in which 100 CP or AP CML patients treated with imatinib for at least 3 years, in sustained CMR for at least 2 years, were asked to stop therapy and were molecularly monitored monthly for the first year and bimonthly for the second. Combination of imatinib with other agents such as IFNα or ARA-C was permitted. Sixty nine of them had at least 12 months of follow-up. At a median follow up of 14 months, 42 of these patients relapsed molecularly after treatment discontinuation, mostly within 6 months, for a molecular relapse-free survival of 41% at 1 year, and 38% at 2 years (Fig. 8). Only two patients had a fluctuation of their *BCR-ABL* levels and remained in CMR. Sex, Sokal score and imatinib treatment duration, but not previous IFNα therapy, were independent prognostic factors for the risk of molecular relapse. Taken together, these data indicate that relapse after imatinib cessation occurs relatively early in a substantial proportion of CML patients in sustained CMR, and argue against the discontinuation of imatinib therapy in responding CML patients outside the context of a clinical trial. Better characterization of patients with sustained molecular responses after stopping imatinib is needed, especially in comparison with those who subsequently relapse having the same baseline level of *BCR-ABL* transcript. The issue has not yet been addressed with the upfront use of second generation TKis and no comment can be made in this regard.
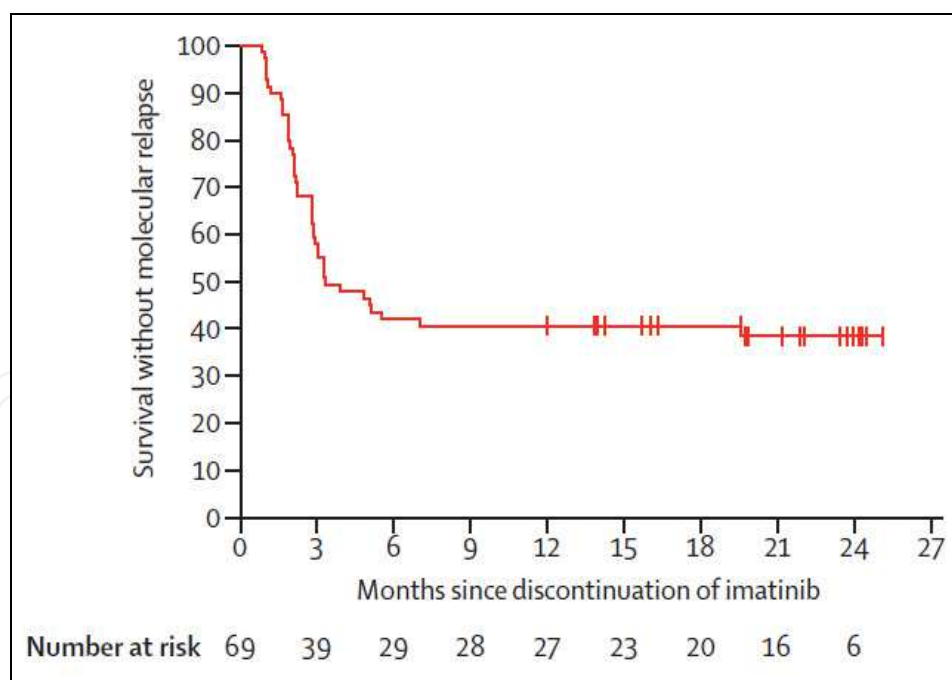

Fig. 8. Kaplan-Meier estimates of complete molecular remission after discontinuation of imatinib in patients with chronic myeloid leukemia for the 69 patients at least 12 months of follow-up after discontinuation of imatinib. The estimated molecular relapse-free survival was 41% (29-52) at 12 months and 38% (27-50) at 24 months. Reprinted with permission. Mahon et al. *Lancet Oncol*, 2010. Nov;11(11):1029-35.

10. Conclusions

Molecular monitoring of CML patients in CP receiving TKis as first-line treatment or after IFNα failure has emerged in recent years as a reliable, non-invasive diagnostic tool for assessing disease burden and treatment efficacy, and has replaced serial cytogenetic studies for these purposes. TKi therapy has dramatically increased the rates of high-quality response (ie, CCyR, MMR or CMR) compared to historical treatment options. Molecular studies, ie, RT- or RQ-PCR, allow appreciating this potency well under the threshold of classical cytogenetics. PCR protocols vary among different testing laboratories worldwide and may not provide fully comparable results and, ultimately, homogeneous measurement of treatment outcomes. The International Scale has represented a milestone in the achievement of harmonization of molecular monitoring and comparability of the test results. The first World Health Organization International Genetic Reference Panel for quantitation of *BCR-ABL* mRNA has been a major step forward in the standardization program allowing laboratory independent access to the IS.

The achievement of a MMR has consistently been shown to predict for sustained CCyR in TKi treated CML patients, and is a marker of long-term EFS and PFS versus CCyR patients that do not reach this milestone. Whether this will translate into benefit remains to be seen and longer patient follow-up will be needed. In addition, early attainment of a significant *BCR-ABL* transcript level drop or a formal MMR may signify long-term protection from disease transformation into the AP/BP, and, possibly, survival benefit. Second generation TKis, namely dasatinib and nilotinib have recently been approved for first-line use in CML patients in chronic phase. These agents allow an even higher fraction of patients to enter MMR within 6 months of therapy, providing an additional strong rationale for their upfront employment.

Confirmed rising *BCR-ABL* RNA levels, usually precedes cytogenetic and, eventually, clinical CML relapse, thus early predicting treatment failure, and prompting precocious strategy change. However, there is no consensus on when rise in *BCR-ABL* transcript level should be considered "significant". BCR-ABL point mutation screening is recommended whenever a consistent transcript rise is documented and appropriate therapeutic action should be taken accordingly.

Complete molecular remission may indicate the achievement of an even greater leukemic burden breakdown, but probably not yet disease eradication. Moreover, the definition of CMR has not been standardized yet, and its value as a positive prognosticator in CML patients treated with TKis in CP remains to be demonstrated. Finally, discontinuation of imatinib in patients with sustained CMR has been followed by disease relapse in more than 50% of patients, and cannot be recommended at this time.

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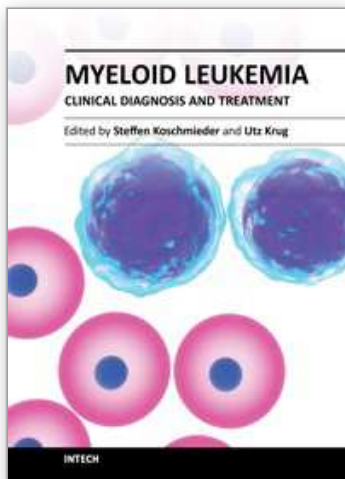
Myeloid Leukemia - Clinical Diagnosis and Treatment

Edited by Dr Steffen Koschmieder

This book comprises a series of chapters from experts in the field of diagnosis and treatment of myeloid leukemias from all over the world, including America, Europe, Africa and Asia. It contains both reviews on clinical aspects of acute (AML) and chronic myeloid leukemias (CML) and original publications covering specific clinical aspects of these important diseases. Covering the specifics of myeloid leukemia epidemiology, diagnosis, risk stratification and management by authors from different parts of the world, this book will be of interest to experienced hematologists as well as physicians in training and students from all around the globe.

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