MYELOID LEUKEMIA – CLINICAL DIAGNOSIS AND TREATMENT

Edited by **Steffen Koschmieder** and **Utz Krug**

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Edited by Steffen Koschmieder and Utz Krug

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Preface

Myeloid leukemias have been studied for decades, and considerable progress has been made in the elucidation of critical pathogenetic factors including transcription factor networks and signaling pathways and in the diagnosis and treatment of these leukemias. However, while the prognosis of a fraction of patients (particularly those with chronic myeloid leukemia in chronic phase) has improved dramatically with the advent of novel rationally designed therapies, the prognosis of many other patients (i.e. with most subtypes of acute myeloid leukemia) has not improved to the same degree. Futhermore, molecular targeted therapies are expensive and are not readily available in all parts of the world.

The intention of this book is to provide a global scope on these issues. Following an open call, authors were invited to propose topics and send in an abstract of the chapter they wanted to contribute. After selection of appropriate abstracts, full chapters were provided and reviewed. Revised chapters were again reviewed and final chapters selected for publication.

The topics of the present book focus on clinical aspects of myeloid leukemias and cover the following:

- Diagnosis and treatment of chronic myeloid leukemia (CML), including standard and high dose imatinib as well as second generation inhibitors
- Response Monitoring in CML with a special focus on current molecular monitoring
- Rare adverse events during TKI therapy (drug-induced pneumonitis)
- Novel therapeutic approaches towards a potential cure of CML
- Diagnosis and treatment of acute myeloid leukemia (AML), in line with the WHO 2008 classification
- Gene Expression Profiling in AML
- Potential ethnic differences in the clinical characteristics of AML
- Acute Promyelocytc Leukemia lacking the classic translocation (15;17)
- Treatment of Elderly Patents with AML
- Prognosis and Survival in AML
- Complications of Treatment in AML, including Bacillus cereus sepsis

Each chapter is a sole-standing publication that reflects each author´s interpretation of the data. However, the unifying theme is myeloid leukemia. Thus, the book displays a multi-facetted picture of our current understanding of the clinical implications for diagnosing and treating patients with myeloid leukemias. In addition, the open access structure of the book will guarantee wide-spread access even in cases where resources required for subscription to more expensive scientific journals or books are limited. We encourage the readers to send their comments. This is an exciting new way of discussing science and to support the effort of increasing the alertness and education of patients and physicians all around the globe.

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Treatment of Chronic Myeloid Leukaemia: Current Practice and Future Prospects

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1. Introduction

Chronic myeloid leukaemia (CML) is a cancer of the hematopoietic system that arises from the Philadelphia chromosome (Ph[1]). This results from the reciprocal translocation of chromosomes 9 and 22 which generates a *Bcr-Abl* fusion gene encoding a 210kDa protein with constitutive tyrosine kinase activity (Ben-Neriah et al., 1986; Kuzrock et al., 1988). This constitutively active tyrosine kinase drives proliferation and survival through multiple downstream pathways (Reviewed in Cowan-Jacob et al., 2004; Ren et al., 2005). The disease is characterised by three stages; the chronic phase marked by an accumulation of mature granulocytes and myeloid precursors in the bone marrow and peripheral blood; the accelerated phase characterised by a rise in myeloid precursors and a blast crisis stage which is characterised by a marked accumulation of differentiation-arrested blast cells of either myeloid or lymphoid lineage (Calabretta & Perotti, 2004; Savage et al., 1997). The generation and clinical use of the Bcr-Abl tyrosine kinase inhibitor (TKI) imatinib mesylate (IM) has revolutionised the treatment of CML patients (Druker et al., 1996; Druker et al., 2006) and has become the standard line of therapy for CML patients. Following treatment with imatinib, over 90% of patients obtain a complete haematologic response and more than 80% achieve a complete cytogenetic response. However, there are limitations associated with IM therapy. The drug is highly effective in the chronic phase of the disease but the response of patients in blast crisis is limited (Hehlman & Saussele, 2008). Furthermore, in approximately 40% of patients, resistance develops i.e. resistance in 100 patient years (Gorre et al., 2001). Great progress has been made over the last ten years in elucidating the molecular mechanisms of IM-resistance *in vitro* but correlating any of these individual resistance mechanisms in a clinical sample does not always indicate that it alone drives clinical progression as additional modes of resistance may be at work. The mechanisms by which patients become resistant to IM therapy include Bcr-Abl dependent mechanisms such as an increase in the levels of Bcr-Abl mRNA expression and corresponding upregulation of protein levels and amplification of the Bcr-Abl gene (Mahon et al. 2000). Bcr-Abl independent mechanisms include activation of signalling pathways downstream of Bcr-Abl including the phosphatidylinositol 3-kinase (PI3K)/Akt cell survival pathway or activation of signalling pathways separate to that of the *Bcr-Abl* gene and an efflux of IM via multidrug resistant proteins such as p-glycoprotein (Capdeville et al., 2002). The most well-characterised cooperating pathway involves the Src Family Kinases (SFKs) which have

been demonstrated to play a role in altering responsiveness to TKIs as well as promoting disease progression (Danhauser-Riedl et al., 1996; Wilson et al., 2002).
However, the major factor influencing IM resistance is due to mutations at critical points in the kinase domain of the *Bcr-Abl* gene which interferes with the ability of IM to interact with the enzyme. To date over 60 amino acid substitutions in the kinase domain have been found. Of these T315I is one of the most common Bcr-Abl mutations identified in patients and importantly this is also associated with the highest degree of IM-resistance, preventing the formation of the critical hydrogen bond and changing the conformation of the Bcr-Abl protein in such a way to render the protein completely resistant to imatinib. The frequency of the T315I mutation in IM-resistant patients is reported to range between 2% and 20% with variability related to detection methods along with patient cohort characteristics and treatment (Nicolini et al., 2009). Recent data suggests that the survival rate of patients harbouring a T315I mutation is dependent on disease phase at the time of mutation detection, with chronic phase patients responding to some investigational compounds (Nicolini et al., 2009). For example, Legros et al, (2007) reported T315I transcript disappearance in an IM-resistant CML patient treated with homoharringtonine and Giles et al, (2007) reported that 3 patients harbouring the T315I mutation achieved clinical responses with the aurora kinase inhibitor MK-0457. A greater understanding of the molecular basis of IM-resistance has provided the molecular rationale for the development of second and now third generation therapies for patients with CML. Such therapies will play a key role in the control of CML over the next decade.

2. Second generation tyrosine kinase inhibitors

Shortly after the introduction of IM in the clinic, reports of primary and secondary resistance cases began to emerge which led to the search for agents that might overcome this problem. The first second-generation TKI that was clinically evaluated was dasatinib (BMS-354825) and it was approved by the FDA for treatment of all phases of IM-resistant CML in June 2006. Dasatinib is able to bind to both Bcr-Abl and Src family kinases and it was originally identified in a screen of compounds that demonstrated potent Src/Abl kinase inhibition with antiproliferative activity in CML cell lines and xenograft models systems (Lombardo et al., 2004). Nilotinib (AMN107) was subsequently developed by rational drug design based on the crystal structure of an Abl/imatinib complex, allowing researchers to optimise the potency and selectivity of the compound (Weisberg et al., 2005).
Dasatinib and nilotinib were initially evaluated in patients with IM-resistant or intolerant CML. In phase II clinical trials with a 24 month follow up, both dasatinib and nilotinib were shown to have efficacy in patients in the chronic phase of CML (see Table 1). In the nilotinib trial, intolerance to imatinib was defined as having intolerance with no major cytogenetic response whereas in the dasatinib trial, imatinib-intolerant patients included patients who had a major cytogenetic response. These results were very promising and led to further Phase 3 trials where these second-generation TKIs were compared directly with imatinib as front-line therapies. It would be of interest in the future to determine the optimal time point for switching to second line treatment.
Saglio et al., (2010), recently reported results from the Evaluating Nilotinib Efficacy and Safety in Clinical Trials-Newly Diagnosed Patients (ENESTnd) trial. In this phase 3, randomized, multicenter study, 846 patients with chronic phase CML received either nilotinib (at a dose of either 300 mg or 400 mg twice daily) or imatinib (at a dose of 400 mg

once daily). At 12 months, the rates of major molecular response for nilotinib (44% for the 300-mg dose and 43% for the 400-mg dose) were nearly twice that for imatinib (22%). The rates of complete cytogenetic response by 12 months were significantly higher for nilotinib (80% for the 300-mg dose and 78% for the 400-mg dose) than for imatinib (65%). Patients receiving nilotinib also had a significant improvement in the time to progression to the accelerated phase or blast crisis, as compared with those receiving imatinib. Based on these results the US FDA has granted accelerated approval of nilotinib for the treatment of patients with newly diagnosed CML in chronic phase.

Response	**Dasatinib**	**Nilotinib**
No. of patients	387	321
Percent imatinib-resistant	74	70
Percent imatinib-intolerant	26	30
Percent CHR	91	95
Percent McyR	62	59
Percent CCyR	53	44

Table 1. Summary of results of phase 2 studies of dasatinib and nilotinib in patients with chronic phase CML demonstrating either IM-resistance or intolerance. CHR. Complete hematologic response; McyR, major cytogenetic response; CCyR, complete cytogenetic response.

Similarly, Kantarjian et al., (2010a) has reported the results of a trial comparing dasatinib versus imatinib in treatment naïve CML patients (DASISION trial). 519 patients with newly diagnosed chronic phase CML were randomly assigned to receive dasatinib at a dose of 100 mg once daily (259 patients) or imatinib at a dose of 400 mg once daily (260 patients). After a follow-up of 12 months, the rate of complete cytogenetic response was higher with dasatinib than with imatinib (77% vs. 66%). The rate of major molecular response was higher with dasatinib than with imatinib (46% vs. 28%), and responses were achieved in a shorter time with dasatinib. Progression to the accelerated or blastic phase of CML occurred in 5 patients who were receiving dasatinib (1.9%) and in 9 patients who were receiving imatinib (3.5%) while the safety profiles of the two treatments were similar. These results also led to accelerated FDA approval in October 2010 for this second generation TKI for initial therapy of CML. Taken together, the results of these recent trials suggest that the best treatment for resistance may be preventing the emergence of resistance in the first place by using these alternative frontline therapies. In addition to second generation TKIs, modified imatinib-based regimes (e.g. increasing the dose of imatinib to 800mg/day) are also currently under evaluation. It would also be of interest to determine whether administration of two or more TKIs together or consecutively would improve disease control as compared to single-agent therapy but such a course of study would present many difficulties in terms of clinical trial design.

3. Combination approaches and investigational compounds

The currently available TKIs do not demonstrate efficacy against the T315I mutation suggesting the need for additional strategies such as combination approaches with alternative classes of agents. Prior to the introduction of imatinib, interferon (IFN) alpha-

based regimens were the gold standard for treatment of early chronic phase CML patients. The combination of IFN-alpha with imatinib was recently investigated in two large clinical trials, the French SPIRIT trial and the German CML Study IV. In the SPIRIT trial 636 patients were randomised 1:1:1:1 to receive either imatinib 400mg/day, 600mg/day, 400mg/day plus cytarabine or 400mg/day plus INF-alpha. After 18 months MMR rates were 41% versus 52% versus 53% versus 62% respectively (Preudhomme et al., 2010). In the German study CML IV, patients were given imaitinib 400mg/day versus 800mg/day versus 400mg/day plus IFN-alpha. Response rates were higher in the imatinib (800mg/day) cohort (CCyR 65%, MMR 54%) when compared to the imatinib (400mg/day) cohort with or without IFN-alpha (CCyR 52% and 51%; MMR 30% and 35%, respectively) suggesting in this case that high dose imatinib increases the rate of MMR at 12 months (Hehlmann et al., 2011). Furthermore, they demonstrated that achievement of MMR by month 12 is directly associated with improved survival (Hehlmann et al., 2011).

A number of investigational compounds, many of which are active against T315I mutants have also been identified and many are currently undergoing clinical trials and are summarised in Table 2. These can be subdivided into four classes; third generation TKIs, aurora kinase inhibitors, switch pocket inhibitors and apoptosis modulators.

3.1 Third generation TKIs

Despite the very promising results with dasatinib and nilotinib there is still room for improvement. Due to the fact that the currently available TKIs have no activity against T315I mutants, many investigational compounds are currently being clinically evaluated in this cohort of patients. Ponatinib (AP24534) is an orally bioavailable multi-targeted compound with activity against many kinases including native Bcr-Abl, the T315I mutant and other mutants (O'Hare et al., 2009). Ponatinib does not need to make a hydrogen bond with T315 therefore it can accommodate the side chain of the isoleucine residue in the T315I mutation (O'Hare et al., 2009). In kinase-based assays, ponatinib potently inhibited the activity of wild-type Bcr-Abl and the T315I mutant in the nanomolar range. Ponatinib also exhibited nanomolar activity against other kinases such as SRC, FGFR1, FLT3, KIT and VEGFR. A phase I trial of ponatinib has recently been completed and a phase II trial is currently underway. If ponatinib can be demonstrated to be a pan-Bcr-Abl inhibitor in the clinic and it is proven to be safe and effective it may be a future frontline therapeutic for CML.

Bosutinib, is a third generation TKI that is currently being developed by Pfizer. It inhibits Bcr-Abl with higher potency than imatinib but it also demonstrates activity against a number of other kinases including SFKs, c-Kit and PDGF receptors (Remsing-Rixet et al., 2009). Bosutinib is currently undergoing frontline testing against imatinib with promising results. This third generation TKI may shortly win FDA approval for initial therapy of CML (Bixby & Talpaz, 2011).

INNO-406 is an orally bioavailable dual Abl/Lyn kinase inhibitor which is up to 50 times more potent than imatinib against Bcr-Abl. Results of a Phase I trial with this TKI have recently been reported (Kantarjian et al., 2010b). INNO-406 was administered to 56 patients with imatinib resistance (n = 40) or intolerance (n = 16). Other previous treatments included nilotinib (n = 20 patients), dasatinib (n = 26 patients), and dasatinib/nilotinib (n = 9 patients). Of 31 patients with CML in chronic phase who received INNO-406, the major cytogenetic response rate was 19%. No responses were observed in patients who had CML in accelerated phase or in blastic phase. The maximium tolerated dose was identified at 240mg twice daily and further phase II studies are planned.

XL228 is an intravenously available multi-targeted TKI that has significant *in vitro* activity against the T315I mutant. It is currently been investigated in a small clinical trial of 27 patients where 20 of the patients harbour the highly resistant T315I (10 patients), F317L (7 patients) and V299L (3 patients) mutations. Preliminary clinical activity has been reported in a poster session at the 50th Annual American Society of Haematology in December 2008 (Abstract 3232) and looks promising. XL228 is being currently tested in two Phase I clinical trials, one for the treatment of CML or Ph+ acute lymphoblastic leukaemia (ALL), and the second for the treatment of advanced malignancies including lymphoma.

3.2 Aurora kinase inhibitors

Aurora kinases A and B are a group of serine/threonine kinases also known as mitotic kinases, that regulate the transition from G2 through cytokinesis. Aurora kinases are overexpressed in several types of solid tumours and some haematological malignancies including acute myeloid leukaemia (AML) (Ye et al., 2009). Inhibition of these kinases in leukaemia cells has been shown to result in aberrant mitosis which in turn can lead to mitotic catastrophe. Aurora kinase inhibitors are being developed as potential targeted therapies for cancer patients. There is much similarity between the ATP binding sites of aurora kinases and other kinases including Bcr-Abl.

Danusertib is an intravenously administered multi-targeted kinase inhibitor which demonstrates significant activity against various aurora kinases (Gontarewicz & Brummendorf, 2010). It has also demonstrated *in vitro* efficacy against native Bcr-Abl and the T315I mutant. A phase I clinical trial is currently underway with advanced phase CML patients resistant or intolerant to imatinib and/or a second generation TKI.

AT9283 is an Aurora kinase A and B inhibitor which is administered intravenously. It exhibits efficacy in the nanomolar range against Abl and the T315I mutant along with a range of other kinases including JAK 2 and 3 and FGF4 (Howard et al., 2009). Phase I trials are currently underway in the United States.

One potential problem associated with aurora kinase inhibitors is that they all require prolonged intravenous administration and response have frequently been associated with the periods at which the drug is administered.

3.3 Switch pocket inhibitors

Recently a series of non-ATP competitive multi-kinase inhibitors have been developed. Switch pocket inhibitors bind to amino acid residues that kinases use to undergo the conformational change from the inactive(closed) to the active(open) state and therefore they keep the kinase in the inactive conformation (Chan et al., 2011; Eide et al., 2011). An important structural feature of the Abl kinase is the presence of a series of hydrophobic residues that are stacked in a layer and help to stabilise the active conformation. Indeed the T315I mutant further stabilises the active conformation possibly leading to increased activity of the enzyme. DCC-2036 is one of the lead switch pocket inhibitors. It is an orally bioavailable compound that has demonstrated activity against both native Abl and the T315I mutant and a number of other kinases such as VEGFR2 (Chan et al., 2011; Eide et al., 2011). The compound is currently been evaluated in a phase 1 clinical trial for use in imatinib-resistant CML including patients with T315I mutation.

3.4 Apoptosis modulators

Certain compounds that function independently of kinase inhibition activity are also being developed. Omacetaxine is one such compound. Omacetaxine is a semisynthetic formulation of the alkaloid homoharringtone which can be administered subcutaneously. Homoharringtone has been shown to exhibit anti-tumoural effects by disrupting protein synthesis and downregulating the anti-apoptotic protein myeloid cell leukaemia-1 (MCL-1) (Tang et al., 2006). This leads to disruption of the mitochondrial membrane with release of cytochrome c, caspase activation resulting in apoptotic cell death (Tang et al., 2006). Omacetaxine is currently been evaluated in two multicentre Phase III clinical trials for patients with CML who have failed two or more TKIs or for patients with the T315I mutation. It may become the first drug to be approved for third-line therapy in CML.

Drug Class	Company	Targets	Activity against T315I mutant	References
TKIs				
Ponatinib	Ariad U.S.A.	ABL, FGFR1, FLT3, KIT, VEGFR	Yes	O'Hare et al., 2009
Bosutinib	Pfizer U.S.A.	ABL,CAMK2G, STE20, TEC	No	Bixby & Talpaz, 2011; Remsing Rix et al., 2009
INNO-406	CytRx U.S.A	ABL, KIT, LYN, PDGFR	No	Kantarjian et al., 2010b
XL228	Exelixis U.S.A	ABL, Aurora A, FGFR1-3, IGF1R, SRC	Yes	Not applicable
Aurora kinase inhibitors				
AT9283	Astex, U.K.	ABL, Aurora A & B, FLT3, JAK2, JAK3	Yes	Howard et al., 2009
Danusertib	Nerivano medical sciences, Italy	ABL, Aurora A & B,FGFR1, RET, TRK	Yes	Gontarewicz & Brummendorf , 2010
Switch pocket inhibitors				
DCC-2036	Deciphera, U.S.A.	ABL, FLT3, KDR, SFK, TIE2	Yes	Chan et al., 2011; Eide et al., 2011
Apoptosis modulators				
Omacetaxine	ChemGenex, Australia	Cytochrome c, MCL-1	Yes	Tang et al., 2006
Hsp90 inhibitor KOS-1022	Kosan, U.S.A.	Cytochrome c	Yes	Gorre et al., 2002

Table 2. Investigational compounds in chronic myeloid leukaemia

Heat shock protein 90 (Hsp90) is a chaperone protein that assists client proteins in folding and prevents protein misfolding and degradation by the proteasome. The Hsp90 antagonist, 17-allyamino-17-demethoxygeldanamycin (17-AAG), has been shown to cause release of

cytochrome c, caspase activation and apoptosis in native Bcr-Abl cells and those expressing the T315I mutant (Gorre et al., 2002). Clinical trials with the more soluble analogue of 17-AAG, 17-DMAG (KOS-1022) are currently ongoing.

Our group have recently reported the effects of representative members of the novel pro-apoptotic microtubule depolymerising pyrrolo-1,5-benzoxazepines or PBOX compounds on chemotherapy-refractory CML cells using a series of Bcr-Abl mutant cell lines, clinical *ex vivo* patient samples and an *in vivo* mouse model (Bright et al., 2010). The PBOX compounds potently reduced cell viability in cells expressing the E225K and H396P mutants as well as the highly resistant T315I mutant. The PBOX compounds also induced apoptosis in primary CML samples including those resistant to imatinib. In addition we have shown that the PBOXs enhance the apoptotic efficacy of imatinib in CML cell lines (Bright et al., 2009; Greene et al., 2007). Furthermore we have demonstrated the *in vivo* efficacy of a representative pro-apoptotic PBOX compound, PBOX-6, in a CML mouse model of the T315I Bcr-Abl mutant. Results from this study highlight the potential of these novel series of PBOX compounds as potential therapy against CML.

4. Stem cell transplantation

Prior to the advent of imatinib and other TKIs, allogeneic hematopoietic stem cell transplantation (HSCT) was the main therapeutic option for CML patients and indeed is the only known curative treatment for CML to date. After the initial results with imatinib were published, allogeneic transplantation began to decline as a frontline treatment for CML. However to date there is no prospective study that compares imatinib and HSCT as frontline treatments. A retrospective review of over 1000 patients who received an allogeneic transplant in the pre-imatinib era reported an overall survival rate of 47% after 8 years and a relapse rate of 33% after 5 years (Gratwohl et al., 1993). More recently, Saussele et al., reported the results of an analysis of a subgroup of the randomized German CML study IV. These patients received a transplant after imatinib failure and demonstrated a 91% survival after 3 years (Saussele et al., 2010). Imatinib does not appear to impair engraftment and the incidence of graft versus host disease and survival was the same as for patients in the same stage of the disease who were not treated with imatinib. Since TKIs have no harmful effect on the transplant outcome they can be used until a suitable donor is found and the transplant procedure is performed. On the recommendation of the European LeukemiaNet, allogeneic transplant is now considered for those patients who have failed treatment with a second-generation TKI, patients in the advanced or blastic phase of CML at the time of diagnosis (as these patients are not responsive to TKIs) or those with the T315I mutation (see Table 3). Transplantation may also be an option for those patients that develop mutations while undergoing second line therapy (Baccarani et al., 2009). Finally transplantation may possibly be an option for pediatric or young patients with a suitable donor as the long term effects of TKIs such as drug toxicity (Kerkala et al., 2006) and immune dysfunction (Dietz et al., 2004) have not been clearly identified to date. Finally the capacity to combine novel TKIs with allogeneic transplantation in high-risk patients will potentially improve survival but further studies are required. Unfortunately only a low percentage of patients receive a transplant for a variety of reasons such as age and lack of appropriate donors. In the German CML study IV, of the 1,242 CML patients involved, 84

patients underwent allogeneic HSCT, with a relatively young age of patients reported (median age of 36 years) (Saussele et al., 2010).

Chronic phase, frontline therapy	Imatinib (400mg daily)
Chronic phase, second-line therapy *IM-intolerance* *IM-failure*	Nilotinib (400mg twice daily) or Dasatinib (100mg daily) Nilotinib (400mg twice daily) or Dasatinib (100mg daily) or HSCT in low transplantation risk and high risk disease (e.g. T315I mutation)
Chronic Phase, third-line therapy *In case of dasatinib or nilotinib failure*	HSCT
Accelerated and blastic phase *Frontline* *Second-line*	Imatinib followed by HSCT wherever possible Nilotinib or Dasatinib followed by HSCT wherever possible

Table 3. Current recommendations for treatment of CML patients (modified from Baccarani et al., 2009)

5. Chronic myeloid leukaemia stem cells

There is also a mounting body of evidence suggesting that in many cancers, including CML, cancer stem cells (CSCs) evolve as a result of both genetic and epigenetic events that alter hematopoietic progenitor differentiation, survival and self-renewal. Hematopoietic stem cells (HSCs) are defined by their capacity for self-renewal and their ability to give rise to all mature haematopoietic cell lineages throughout an individual's lifetime. There is accumulating evidence to suggest that CML cells emerge due to expression of Bcr-Abl in normal HSCs. Transplantation of multipotent murine HSCs expressing Bcr-Abl into recipient mice induces a CML-like myeloproliferative disorder (Pear et al., 1998) whereas CML is not induced in committed murine haematopoietic progenitor cells expressing Bcr-Abl (Huntly et al., 2004). There is also accumulating evidence that the signalling pathways that control normal HSC fate also determine maintenance of stem cell function. Recently signalling pathways or molecules such as Wnt/β-catenin, hedgehog (Hh), promyelocytic leukaemia (PML) and forkhead box class O of transcription factors (FOXO) have been shown to control stem cell fate in both normal hematopoiesis and in CML.

5.1 Signalling pathways underlying maintenance of CML stem cells

The wnt/β-catenin signalling pathway is thought to play a role in maintenance of CML stem cells. There are numerous reports demonstrating that β-catenin regulates normal mouse HSC renewal (Reya et al., 2003; Zhao et al., 2007). Furthermore, Zhao et al., 2007 performed a series of mouse genetic studies demonstrating that conditional deletion of β-catenin reduced maintenance of CML stem cells in the chronic phase. Loss of β-catenin also suppressed infiltration of CML cells into the lung and liver of mice injected with CML stem cells (Zhao et al., 2007).

The hedgehog signalling pathway is also though to underlie stem cell fate in both normal hematopoiesis and CML. In the absence of Hh ligands, Patched (Ptch) a twelve-

transmembrane receptor inhibits smo, a seven-transmembrane receptor. The binding of Hh ligands such as Indian hedgehog, Desert hedgehog or Sonic hedgehog to the Patched receptor in turn activates Smo and this receptor activates downstream signalling events mediated through activation of Gli transcriptional effectors. Two recent studies have demonstrated that expansion of Bcr-Abl leukaemic stem cells is dependent on the hedgehog pathway. Conditional *Smo* deletion caused CML stem cell suppression and impaired CML progression (Dierks et al., 2008; Zhao et al., 2009). Furthermore expression of constitutively active Smo increased the frequency of CML stem cells and accelerated CML development (Zhao et al., 2009) demonstrating an essential requirement for the Hh signalling pathway in maintenance of CML stem cells.

The promyelocytic leukaemia (PML) protein is a tumour suppressor protein localising to PML nuclear bodies. It plays a role in a wide array of biological activities including apoptosis, senescence and the DNA damage response pathway. Ito et al., 2008 reported high expression of PML in normal HSCs and demonstrated that conditional deletion of *Pml* resulted in intensive cell cycling which in turn resulted in impaired self-renewal capacity. They also demonstrated the defective ability of $Pml^{-/-}$ CML stem cells to develop CML at the 3rd serial transplantation.

Forkhead box class O of transcription factors (FOXO) have been shown to control stem cell fate in both normal hematopoiesis and in CML. The FOXO family of transcription factors include FOXO1, FOXO3a, FOXO4 and FOXO6 and they are all downstream targets of the cell survival phosphatidylinositol-3-kinase-AKT signalling pathway. When a ligand such as a growth factor or insulin binds to its receptor and activates the PI3-K-AKT pathway, AKT phosporylates FOXOs preventing their translocation to the nucleus and causing their degradation. It is widely believed that Bcr-Abl activates AKT signalling and suppresses FOXOs which in turn enhances the proliferation or inhibits the apoptosis of CML cells. However, Naka et al., (2010) have recently shown that FOXO3a plays an essential role in the maintenance of CML stem cells through the use of a syngeneic transplantation system and a CML-like myeloproliferative disease mouse model. They demonstrated that cells with a nuclear localisation of FOXO3a and decreased AKT phosphorylation are enriched in CML stem cell population, despite expression of Bcr-Abl. They also found that the ability of CML stem cells to promote malignancy at the 3rd transplantation is significantly decreased by Foxo3a deficiency *in vivo*. In addition, they have shown that TGF-beta is a critical regulator of AKT activation in CML stem cells and control the localisation of FOXO3a. This suggests the potential of TGF-beta-FOXO signalling inhibitors in eradicating CML stem cells.

The transcription factor JunB has been shown to protect against myeloid malignancies including CML by limiting hematopoietic stem cell proliferation and differentiation. Inactivation of JunB deregulates the cell-cycle machinery and increases the proliferation of HSCs without impairing their self-renewal or regenerative potential *in vivo* (Santaguida et al., 2009). Such data increases our understanding of how defects in signalling pathways that control the proliferation of stem cells leads to an increase in their transformation ability.

5.2 Mechanisms of tyrosine kinase inhibitor resistance of CML stem cells

Many studies have shown that TKIs such as imatinib, dasatinib and nilotinib potently inhibit TKI in differentiated CML stem cells but are not as effective in quiescent CML stem cells. For example, the presence of detectable primitive leukaemic progenitor cells in CML patients with an established complete cytogenetic response after 5 years on imatinib

treatment has been demonstrated (Bhatia et al., 2003). Furthermore, patients with an apparent molecular remission of CML following cessation of imatinib treatment quickly relapse (Cortes et al., 2004). It has been suggested therefore that these quiescent stem cells may be a reservoir for relapse (Holyoake, 1999; Wang et al., 1998). Drugs that are capable of eradicating the CML stem cells would provide much improved treatment for CML patients. To date, a number of potential mechanisms mediating TKI-resistance of CML stem cells have been postulated.

Firstly FOXO has been suggested to contribute to resistance to TKI therapy. Komatsu et al., (2003), has previously reported that FOXO3a is a downstream effector of imatinib induced cell cycle arrest in Bcr-Abl expressing cells and that FOXO inactivation sensitises cells to imatinib treatment suggesting that FOXO contributes to resistance to TKI treatment. To study this further in CML stem cells, Naka et al., (2010) investigated the roles of Foxo3a in response to TKI therapy using a CML mouse model. They showed that Foxo3a deficiency sensitised CML stem cells to TKI treatment and suggested that Foxo3a plays diverse roles in CML stem cell and non-stem cells. In their model, FOXO activation protects CML stem cells against TKI treatment while in non-CML stem cells it induces apoptosis or cell cycle arrest. In this same paper, they provided both *in vitro* and *in vivo* data which demonstrates a role for the TGF-beta/FOXO signalling pathway in maintaining imatinib-resistant CML stem cells. Treatment of CML stem cells with a TGF-beta inhibitor, Ly364947, impaired their colony forming ability *in vitro* and a combination of TGF-beta inhibition, Foxo3a deficiency and imatinib treatment resulted in efficient depletion of CML *in vivo*. Thus inhibition of TGF-beta signalling may result in eradication of the reservoir of CML stem cells.

There have also been recent reports demonstrating that Bcr-Abl stimulates the proteasome mediated degradation of certain FOXO family members in an animal model and in samples taken from CML patients (Jagani et al., 2009). Treatment with the proteasome inhibitor, bortezomib, resulted in an inhibition of Bcr-Abl mediated downregulation of FOXO and a regression of leukaemia suggesting that bortezomib is a candidate therapeutic in the treatment of Bcr-Abl-induced leukaemia. Furthermore, recent data demonstrate that bortezomib has significant activity against CML stem cells and synergises with imatinib in a CML murine model (Heaney et al., 2010; Hu et al., 2009). Bortezomib has also been shown to inhibit proteosomal degradation of protein phosphatase 2A (PP2A). This in turn reactivates PP2A which is an important negative regulator of Bcr-Abl (Hu et al., 2009). However, due to the known toxicities of bortezomib, including myelosuppression, the likely initial clinical application of bortezomib in CML would be in resistant and advanced disease.

Other work has demonstrated that Hh signalling contributes to TKI resistance. Dierks et al., (2008) demonstrated that inhibition of the Hh signalling pathway with cyclopamine, which maintains Smo in its inactive form, impairs development of CML by CML stem cells. In addition, a combination of cyclopamine with nilotinib delayed the recurrence of the disease compared to treatment with nilotinib alone (Dierks et al., 2008).

Another key molecule that may control TKI resistance of CML stem cells is reported to be the arachidonate 5-lipoxygenase (*Alox5*) gene which encodes a lipoxygenase 5-LO (Chen et al., 2009). Gene expression profiling demonstrated that *Alox5* expression is up-regulated by Bcr-Abl. In the absence of Alox5, Bcr-Abl failed to induce CML in mice. This *Alox5* deficiency caused impairment of the function of CML stem cells but not normal hematopoietic stem cells by affecting their differentiation and cell division. This in turn caused a depletion of CML stem cells and a failure of CML development. Treatment of CML

mice with a 5-LO inhibitor, zileuton, also impaired the function of CML stem cells and prolonged survival of CML affected mice. These results demonstrate that a specific target gene can be found in CML stem cells and its inhibition can inhibit the function of these stem cells. It is of interest to note that upregulation of *Alox5* was not inhibited by treatment with TKIs which may go some way to explaining why imatinib does not affect CML stem cells.

PML as described above is also an important target of CML stem cell therapy. Ito et al., (2008) demonstrated the critical role of this tumour suppressor in CML stem cell maintenance, and presented a new therapeutic approach for targeting quiescent CML stem cells by pharmacological inhibition of PML. Treatment of mice with arsenic trioxide, which downregulates PML expression, completely eradicated CML stem cells when used in combination with the chemotherapeutic drug Ara-C. This suggests that targeting PML for degradation could be an attractive therapeutic approach for targeting CML stem cells.

Autophagy is a genetically controlled process whereby organelles and long lived proteins are sequestered and engulfed into vacuoles called autophagosomes. These autophagosomes then fuse with lysosomes to produce autolysosomes which are targeted for either destruction or recycling (Kroemer & Levine, 2008). In certain situations autophagy serves as an alternative to apoptosis and is thus called type II cell death whereas in other cellular contexts, such as starvation induced by growth factor withdrawal/metabolic stress, it serves as a cell survival mechanism allowing tumour cells to become metabolically dormant. It has recently been reported that imatinib not only induces apoptotic cell death in CML cells but also induces autophagy following the induction of ER stress (Bellodi et al., 2009). In addition, inhibition of autophagic cell death using pharmacological inhibitors of autophagosome-lysosome fusion (chloroquine and bafilomycin) enhanced imatinib-induced cell death in CML cell lines and primary CML cells including those expressing partially IM-resistant Bcr-Abl mutants. Furthermore and of even greater importance, CML stem cells were shown to be extremely sensitive to the combination treatment. Knockdown of the autophagy genes Atg5 and Atg7 in CML cells also enhanced TKI-induced cell death. These workers therefore postulated that TKI-induced autophagy may antagonise TKI-induced cell death through apoptosis and inhibition of autophagy may eliminate this survival mechanism by restoring sensitivity of CML stem cells to TKI therapy (Bellodi et al., 2009). This approach would avoid the necessity of targeting CML stem cells through Bcr-Abl-indpendent approaches. In addition normal stem cells would not be targeted as these autophagic inhibitors had little or no effect on normal progenitors. The results of the Bellodi study have recently led to a randomised phase II clinical trial of IM versus IM/hydrochloroquine in CML patients which is being initiated at a number of centres in the U.K. This is known as the CHOICES (chloroquine and imatinib combination to eliminate stem cells) trial.

Histone deacetylase inhibitors (HDACIs) are drugs that target histone deacetylase complexes which modulate chromatin acetylation resulting in changes in gene expression. These inhibitors have a wide variety of effects as they also inhibit deacetylation of chaperone proteins such as Hsp90, transcription factors and a variety of other signalling mediators. It has previously been shown that treatment of CML cells with HDACIs such as LBH589 resulted in a downregulation of Bcr-Abl and an induction of apoptosis (Fiskus et al., 2006). In addition synergistic effects were observed with HDACIs in combination with a variety of TKIs (dasatinib, nilotinib and imatinib). The HDACIs are thought to target Hsp90 which results in decreased chaperone activity of Hsp90 leading to increased proteosomal degradation of Bcr-Abl. A recent report has demonstrated that the HDACI LBH589 when

used in combination with imatinib induced apoptosis of quiescent CML stem cells with a subsequent lack of engraftment in immunodeficient mice (Zhang et al., 2010). Thus a further possibility for eradication of CML stem cells may lie in combining TKIs with HDACIs.
Table 4 below summarises the key signalling pathways/molecules that are thought to play a role in mediating resistance to TKI therapy and drugs that target these pathways/molecules that may have potential either alone or in combination for CML therapy.

Drug	**Drug target in CML stem cells**	**Combination therapy**	**Reference**
Ly364947	TGF-beta-FOXO signalling pathway	Ly364947 and imatinib improved survival of CML mice	Komatso et al., 2003
Cyclopamine	Smo in the hedgehog signalling pathway	Cyclopamine and nilotinib improved survival of CML mice	Dierks et al., 2008
Zileuton	5-lipoxygenase	Zileuton and imatinib prolonged survival of CML mice	Chen et al., 2009
Arsenic trioxide	PML	Arsenic trioxide and AraC prolonged survival of CML mice	Ito et al., 2008
Chloroquine	Autophagy	Chloroquine sensitised primary CML stem cells to imatinib-induced cell death	Bellodi et al., 2009
LBH589	HDACs	LBH589 and imatinib co-treatment induced apoptosis of CML stem cells and prevented subsequent engraftment in immunodeficient mice	Zhang et al., 2010

Table 4. Signalling pathway/key molecules underlying TKI-resistance in CML stem cells as potential drug targets

6. Immunotherapy for the treatment of CML

Clinical interest in immunotherapy still remains as allogeneic stem cell transplantation, which relys on a graft versus leukaemia effect, provides the only long-term eradication of CML. The differences in minor histocompatibility antigens between recipient and donor along with effector cells specifically targeted at leukaemic antigens contributes to the cure of the disease (Rezvani & Barret., 2008). Additional evidence that CML is a disease susceptible to immunotherapy is provided by reports demonstrating the benefit of allogeneic donor lymphocyte infusions following transplantation (Drobyski & Keever, 1993; Kolb et al., 1995).

As mentioned above, CML is a clonal disorder of pluripotent haematopoietic stem cells which is characterised by the Bcr-Abl fusion protein. This results from the reciprocal translocation of chromosomes 9 and 22 which generates a *Bcr-Abl* fusion gene (Ben-Neriah et al., 1986). The t(9;22) mRNA is translated to a chimeric Bcr-Abl protein of molecular weight 210kDa often referred to as the p210 protein. However different breakpoint areas in the bcr gene have been identified resulting in slight variations in fusion transcripts. The most commonly expressed transcripts are the b3a2 and b2a2 transcripts (Deininger & Goldman, 2000). This generates a neo-antigen which is tumour specific because it contains a new sequence of amino acids in the junctional region of p210 that are not present in normal hematopoietic stem cells. This in turn provides a unique target for immunotherapeutic intervention using a vaccine-based approach.

6.1 Antigen-specific targets in CML-Bcr-Abl junctional peptides

The junctional regions of p210 contain not only a unique sequence of amino acids but additionally a new amino acid is formed due to codon split during translocation. Thus a lysine in b3a2 and a glutamic acid in b2a2 is generated (Shtivelman et al., 2006). There have been many reports of immunogenicity of the fusion region derived peptides of p210 with respect to the major histocompatibility complex (MHC) class I and II. For example, the p210/b3a2-derived fusion protein amino acid sequences have been shown to bind to various class I HLA antigen molecules including A0201, A3, A11 and B8 (Berke et al., 2000) supporting the potential of these peptides as target for class I HLA-restricted T-cell cytotoxicity. However, presentation of other Bcr-Abl junctional peptides has not been established in other HLA types which somewhat limits the clinical potential of class I peptides to subpopulations with specific HLA alleles. Strategies have been implemented to improve the binding of HLA class I molecules by amino acid substitutions at key binding residues of Bcr-Abl peptides to try and overcome their somewhat poor immunogenicity (Pinilla-Ibarz et al., 2005). Interest has also developed in class II Bcr-Abl specific peptides although less is known regarding the interaction of Bcr-Abl peptides with HLA class II molecules (Mannering et al., 1997; Yasukawaet al., 1998). In addition several clinical trials have been initiated using peptide based vaccines to treat CML, often with concomitant treatment of interferon-alpha or imatinib (Bocchia et al., 2005; Cathcart et al., 2004; Pinilla-Ibarz et al., 2000; Rojas et al., 2007). Results of these trials are reviewed by Pinilla-Ibarz et al., (2009).

6.2 Selectively expressed and over-expressed antigens in CML

Another potential target for immunotherapy are antigens that are selectively expressed or over-expressed. Wilms' tumour antigen 1 (WT1) is a transcription factor that is over-expressed in many human leukaemias including CML and also in solid malignancies and several class I restricted epitopes have been identified to date (Ariyaratana & Loeb, 2007). The expression of WT1 in CML has been shown to correlate with disease progression. Many peptides have been designed and cytotoxic T-lymphocytes generated in the presence of some of these peptides were able to specifically target WT1-expressing leukemic cells while sparing normal progenitors (Oka et al., 2000).

The efficacy of WT1-based vaccines has been the study of a number of trials with patients with AML, breast cancer, lung cancer, myelodysplastic syndrome and mesothelioma with promising results (Chaise et al., 2008; Li Z et al., 2005; Oka et al., 2004).

Another promising target in immunotherapy is PR3, a serine protease which is stored in neutrophils and is over-expressed in 75% of CML patients. CD8+ T cells specific for PR3 have been identified in patients in remission following HSCT and correlated with cytogenetic remission (Moldrem et al., 2006).
Several other antigens have been reported as being over-expressed in CML including preferentially expressed antigen of melanoma (PRAME) (Rezvani et al., 2009) and human telomerase reverse transcriptase (hTERT) (Gannage et al., 2005) and these may also be useful for immunotherapy in leukaemia.
It is also important to note the differential and sequential expression of several tumour antigens in different phases of CML suggesting the importance of combining several antigens in the design of future vaccines. The safety and immunogenicity of a combined vaccine of two antigenic peptides, PR1 and WT1, has recently been described and supports further studies of immunisation strategies in CML patients (Rezvani et al., 2008).

6.3 Immunomodulatory effects of TKIs

It has been hypothesised that imatinib reduces the efficacy of graft versus leukemia effect or other T-cell-based immunotherapies. This is based on several studies reporting impaired T-cell specific proliferation and responses as well as the inhibition of antigen-specific memory T cells (Boissel et al., 2006; Mumprecht et al., 2006). Conversely, imatinib has also been demonstrated to initiate an increase in IFN-gamma-producing T cells following 3 months of treatment and it may restore the function of Th1 helper T cells (Aswald et al., 2002).
In vivo antitumour T-cell immunity has been observed in several clinical trials using both Bcr-Abl peptide vaccines and other cellular vaccines (Maslak et al., 2008 & Smith et al., 2006). The use of imatinib in conjunction with donor lymphocyte infusion for relapsed CML patients following HSCT has also been shown to be efficacious suggesting that the clinical effect of imatinib may actually be beneficial (Olavarria et al., 2007; Savani et al., 2005).
Second generation TKIs have also been shown to have immunomodulatory effects. For example, nilotinib has been shown to inhibit the expansion of CD8+ T lymphocytes specific for viral or leukemia antigens much more potently that the same inhibitory effect elicited by imatinib. These effects are thought to mediated through inhibition of phosphorylation of the Src family kinase Lck (Blake et al., 2008). Furthermore, dasatinib was found to inhibit T-cell receptor mediated signal transduction, cytokine production and *in vivo* T cell responses (Blake et al., 2008; Fei et al., 2008). Again the effect is thought to be mediated by the inhibition of Lck.

7. New application of old therapies

Interferon was the most efficacious drug in the treatment of patients in the chronic phase of the disease prior to the advent of TKIs. There is now evidence that interferon-alpha may interfere with stem cell retention in the microenvironment and that it activates dormant haematopoietic stem cells (Essers et al., 2009). In response to treatment of mice with interferon-alpha, HSCs efficiently exited the dormant G(o) and entered an active cell cycle. In addition, HSCs pretreated with interferon-alpha were eliminated by 5-fluorouracil treatment, which raises the possibility for new applications of type I interferons to target CML stem cells. Two large randomized studies show improved outcome when pegylated IFN-alpha is combined with imatinib (Hughes et al., 2010). It could be suggested that IFN-

alpha stimulates the quiescent stem cells to proliferate thereby increasing sensitivity to imatinib. Although imatinib and other TKIs are very efficient, they are rarely curative. IFN-alpha could be included in combination treatment protocols aimed at curing patients and thus could still be an important drug in CML treatment.

8. Concluding remarks

The understanding of the biology underlying CML has rapidly advanced in the last fifty years. From initially identifying a cytogenetic abnormality, we have gone on to translating this finding into treatment strategies for this disease. Imatinib has revolutionised the treatment of CML and for patients who fail this treatment, nilotinib and dasatinib may reduce the rate of progression of the disease. Indeed some of these second generation tyrosine kinase inhibitors may represent a better first-line treatment option for some patients with possible benefits including an improvement in side-effects and tolerability profiles, the ability to suppress a wider range of mutant clones and reaching a response milestone sooner thus avoiding or reducing the risk of relapse. Furthermore third generation drugs are in development that show activity against the T315I mutant, which has emerged as a common Bcr-Abl mutation based resistance mechanism. However, despite the enormous therapeutic benefits of TKIs these drugs do not eradicate leukaemia-initiating stem cells allowing the persistence of a reservoir of Bcr-Abl positive stem cells that are potentially responsible for disease progression. There is therefore a requirement to elucidate why CML stem cells are insensitive to TKIs and to define differences in quiescent versus proliferating CML stem cells. Thus current research should lead to development of novel therapeutic strategies that may eradicate the stem cell population and finally lead to a cure for CML. It is likely though that any new therapeutics for CML will be administered either following or in combination with a tyrosine kinase inhibitor.

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2

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)*.

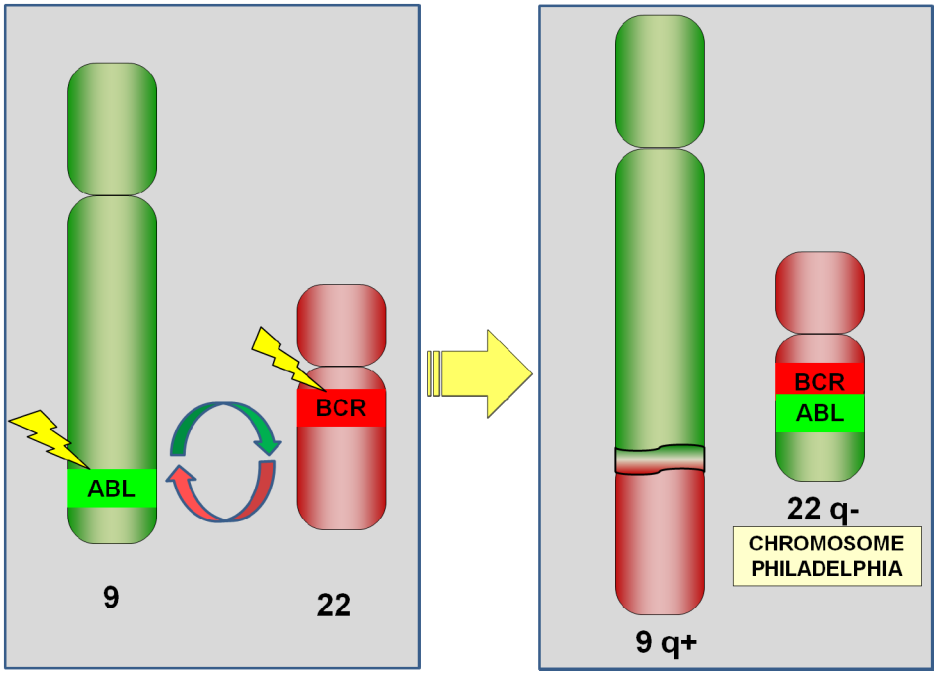

Fig. 1. Schematic diagram of the translocation that results in Philadephia chromosome. The *ABL* gene resides on the long arm of chromosome 9, the *BCR* gene resides on the long arm of chromosome 22. As a result of the (9;22) translocation, a *BCR-ABL* gene is formed on the derivative chromosome 22 (Philadelphia chromosome)

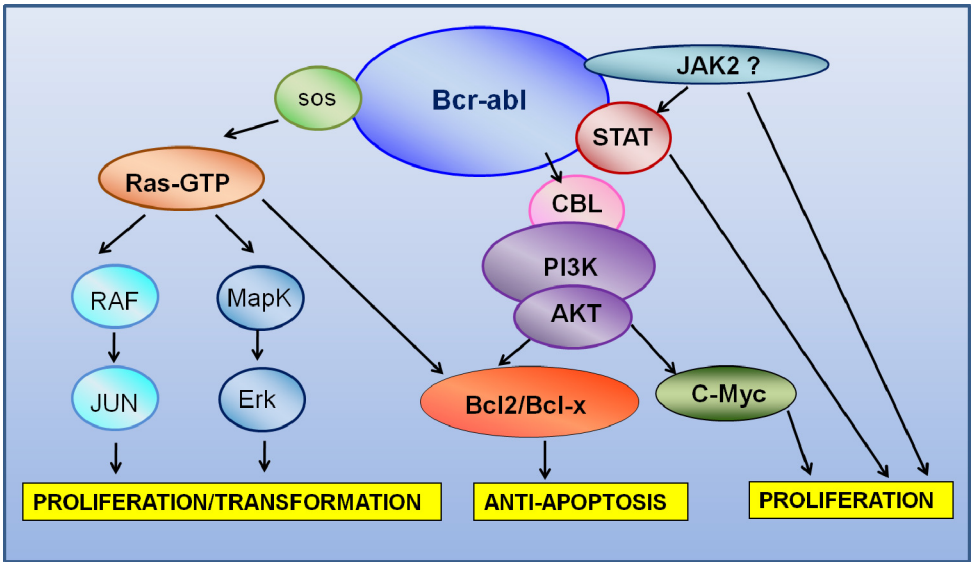

Fig. 2. Schematic signalling pathways activated by BCR-ABL that contribute to growth and survival

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)*.

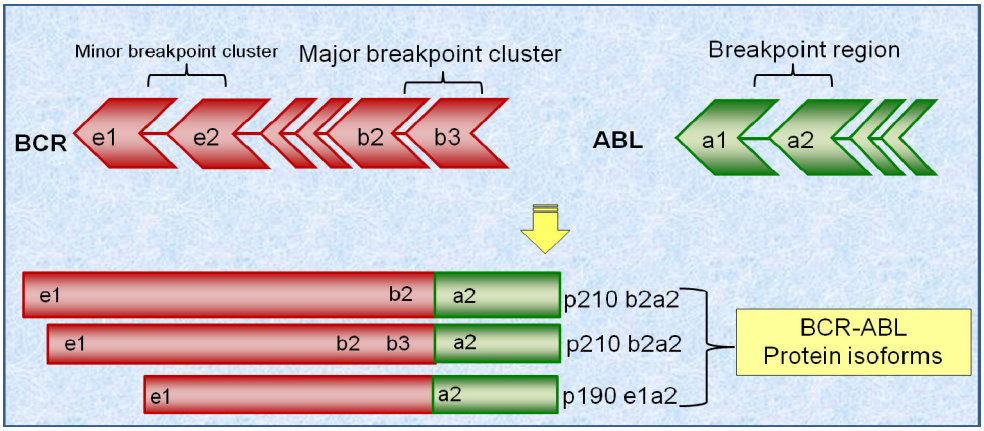

Fig. 3. Breakpoints within the *BCR* and *ABL* genes and corriponding proteins

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*, *β_2-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).*

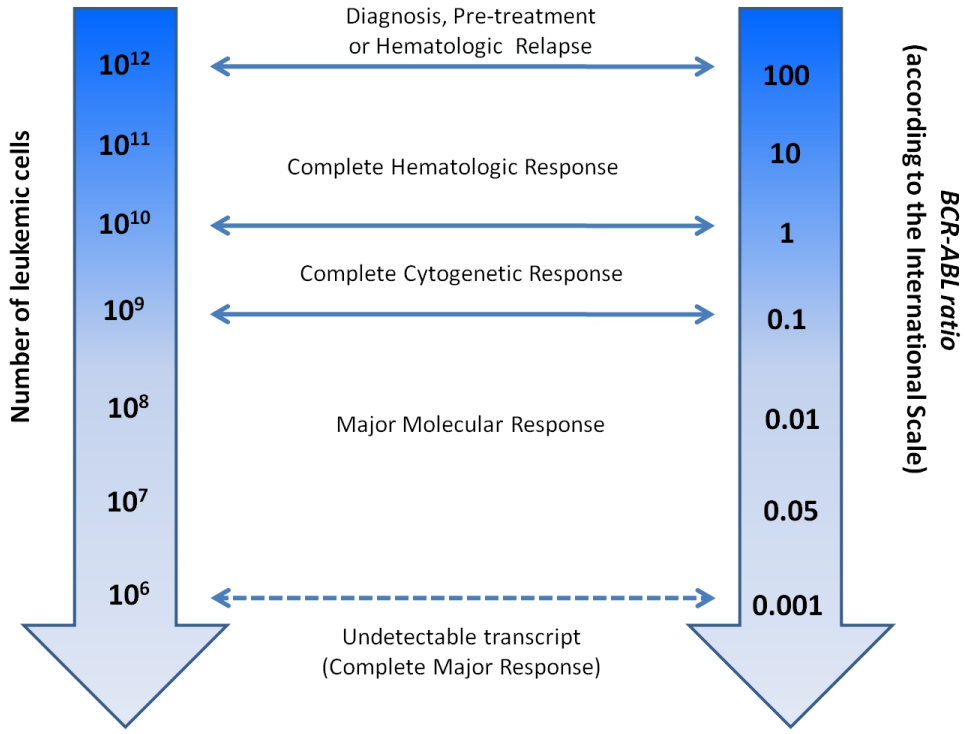

Fig. 4. Relationship between response, the number of leukemic cells and the level of *BCR-ABL* transcript. Reproduced and adapted with permission; Baccarani et al., *Blood* 2006: Sep 15;108(6):1809-20.

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 ≤ 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.

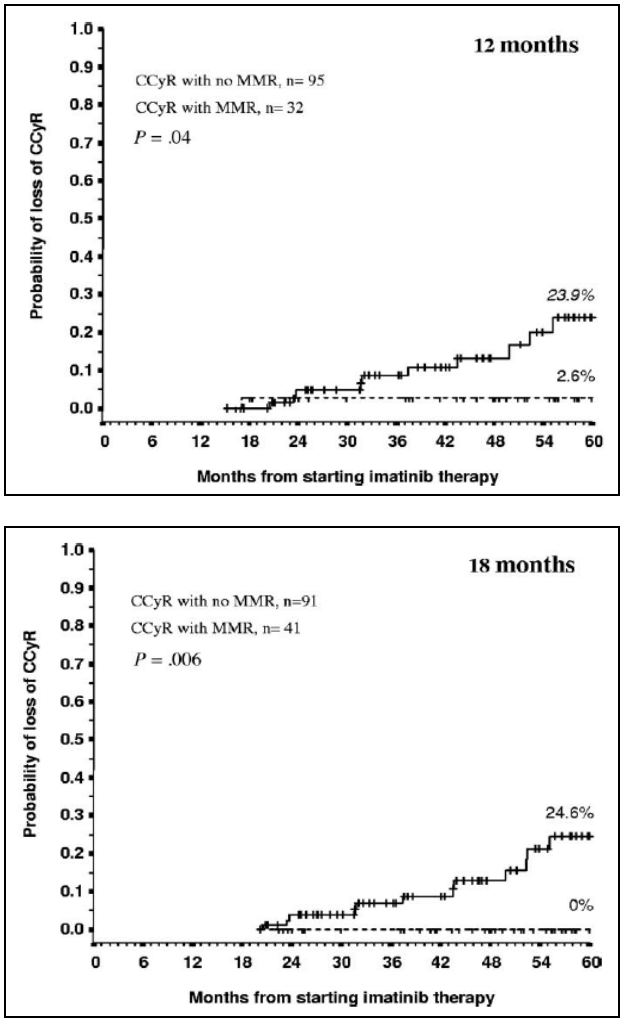

Fig. 5. Twelve- and 18-months landmark analyses for loss of CCyR according to the level of molecular response. Vertical lines represent censored patients. Reprinted with permission; Marin et al., *Blood* 2008;112(12):4437-44.

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.

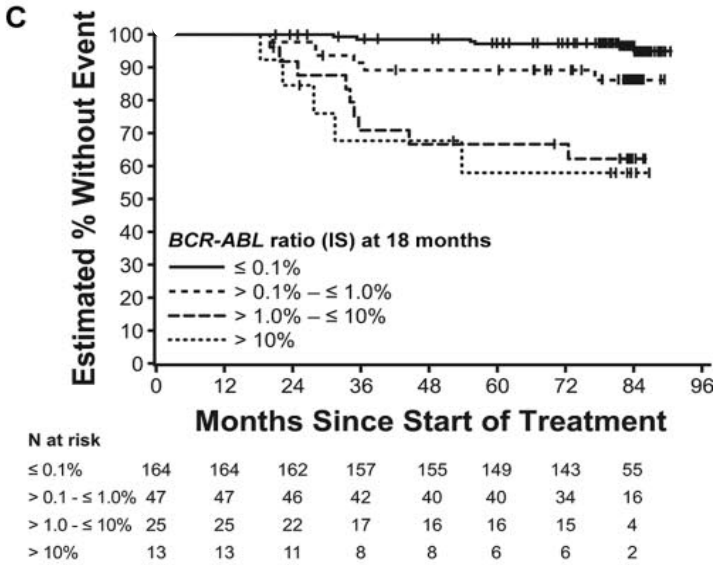

Fig. 6. EFS at the 18-month landmark by molecular response. EFS definition does not include loss of CCyR. Reprinted with permission; Hughes et al., *Blood* 2010, Nov 11;116(19):3758-65.

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.

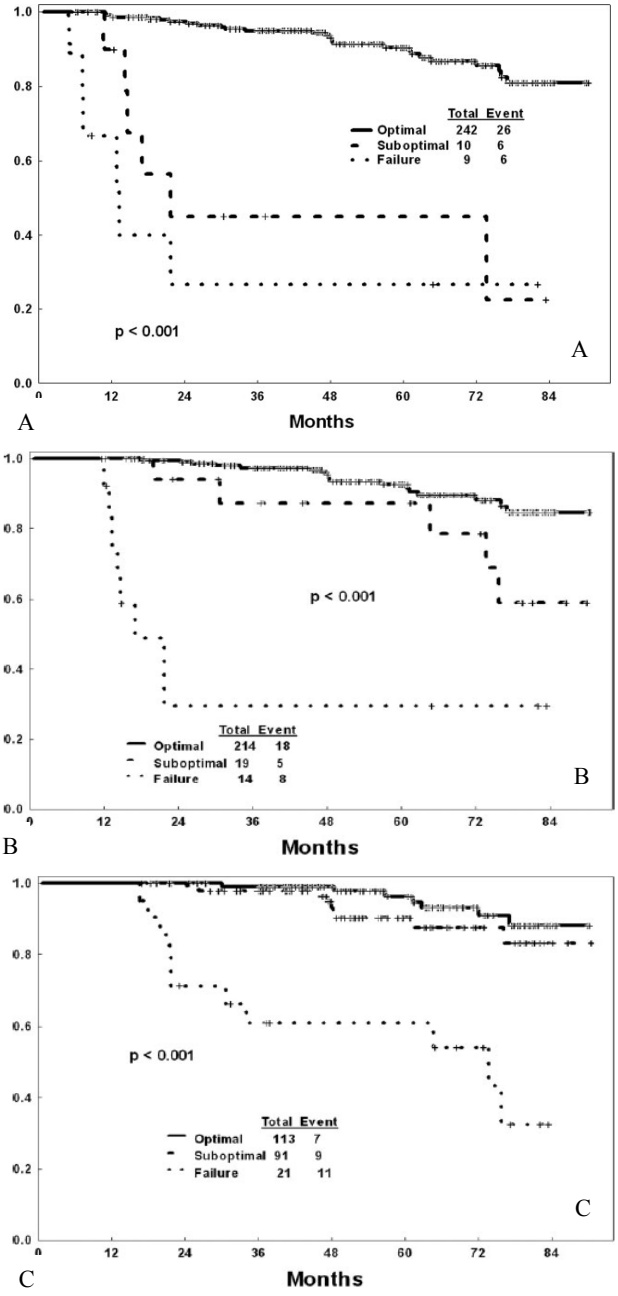

Fig. 7. Event-free survival according to response at 6 (A), 12 (B), and 18 (C) months in patients treated with imatinib in early CP. Reprinted with permission; Alvarado et al., *Cancer* 2009, Aug 15;115(16):3709-18.

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.

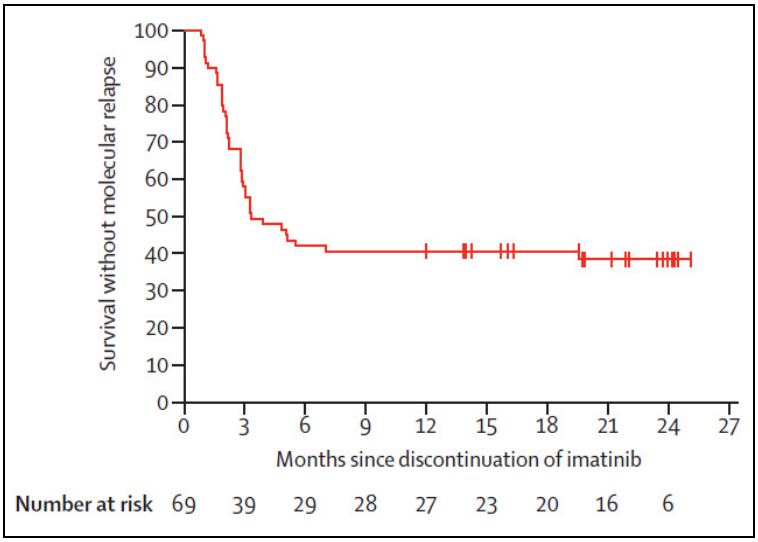

Fig. 8. Kaplan-Meier estimates of complete molecular remission after discontinuation of imatinib in patients with chronic myeloid leukemia for the 69 patients at least 12 months of follow-up after discontinuation of imatinib. The estimated molecular relapse-free survival was 41% (29-52) at 12 months 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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3

Role of High Dose Imatinib in BCR/ABL^{pos}/Ph^{pos} CML

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1. Introduction

The therapy with imatinib became the standard of care for the initial treatment of chronic myeloid leukemia (CML) regardless of age, disease status or prognostic scores. Based on the most recent update of the International Randomized Interferon versus STI571 (IRIS) study comparing imatinib 400 mg once daily with interferon alpha plus low doses of Ara C the overall survival (OS) of CML patients at 8 years has improved dramatically to 85% (Deininger et al., 2009) and the estimated median survival is estimated to improve to more than 20 years. In consequence, this will increase the prevalence of CML patients in Europe dramatically from about 70.000 to 120.000 in 2011 to 160.000 and more than 300.000 in the year 2050, assuming a stable incidence of CML between 1 to 2 per 100.000 persons. Interestingly, despite this dramatic success in the therapy of CML with imatinib 400 mg once daily the maximum tolerated dose of imatinib has not been identified in the phase I study (Druker et al., 2001). Nevertheless, there is clear evidence of a dose response relationship from pre-clinical models as well as from the phase I study (Druker et al., 2001; Druker et al., 1996; Deininger et al., 1997; Cambacorti-Passerini et al., 1997). The main reason for choosing a dose of 400mg as starting dose for subsequent clinical trials was the fact that early and significant rates of hematologic and even more important, major and complete cytogenetic responses were achieved with the 400 mg dose that were not further increased with higher doses in these early times (Druker et al., 2001). In contrast, drug related adverse events (AEs), especially WHO grade 3 or 4 AEs that were not seen with imatinib doses up to 300 mg slightly increased with higher doses ranging from 600 mg up to 1000 mg per day. In general, however, imatinib was well tolerated even at higher doses and demonstrated a clear benefit over interferon alpha plus low doses of Ara C in terms of both, efficacy and tolerability (Druker et al., 2001).

2. Phase II studies on High Dose (HD) imatinib in Chronic Phase (CP)

The rational for the use of higher imatinib doses frontline in CP CML patients is that despite the impressive results with 400 mg once daily a substantial number of patients experiences only suboptimal responses according to the criteria of the European Leukemia Net (ELN) (Baccaranai, JCO 2006, 2009) or a minority of patients even fails to reach a response. Early good responses are known to be associated with a favorable long-term outcome as shown

previously in patients from the IRIS study where none of the patients that achieved a complete cytogenetic response (CCyR) and a major molecular response (MMR) at 18 months progressed during the follow up phase (Druker et al., 2006). The first non-randomized study using high doses of imatinib front-line was performed at the M. D. Anderson Cancer Center with CML patients in chronic phase (CP) of their disease after failure to interferon alpha (IFN alpha) (Cortes et al, 2003). Cortes and colleagues treated 36 CP CML patients after failure to interferon alpha with 400 mg of imatinib twice daily (total daily dose of imatinib: 800mg) and 90% of the evaluable patients achieved a major cytogenetic response (MCyR) and 89% a CCyR. Moreover, this treatment with HD imatinib was also associated with a high rate of molecular remissions. In 50% and 56%, however, the dose of imatinib had to be reduced to 600 mg or 400 mg after 3 months and 6 months, respectively. The most common cause for dose reduction in this pre-treated patient population was myelosuppression including thrombocytopenia and neutropenia (Cortes et al, 2003). Nevertheless, 71% of the patients were at least capable to continue with an imatinib dose of ≥ 600 mg. In a subsequently performed phase II study in 114 newly diagnosed CP CML patients, high imatinib doses of 800 mg per day were capable to induce a MCyR in 96% and a CCyR in 90% of the patients. The estimated 2 year survival rate was 94% (Kantarjian et al., 2004). In terms of toxicity the higher doses of imatinib were comparable to the standard imatinib dose of 400 mg in regard to non-hematologic toxicities. Hematologic toxicities were again higher with the imatinib 800 mg dose compared to historical controls. In this trial on newly diagnosed CML patients in CP the dose of 800 mg of imatinib could be maintained in 64% of the evaluable patients at 6 months and in 66% of the evaluable patients at 12 months (Kantarjian et al., 2004). The data from both studies suggested that higher doses of imatinib were capable to induce higher rates of cytogenetic (both MCyR and CCyR) and molecular responses with the price of more frequent myelosuppression. The Italian GIMEMA CML working party prospectively investigated efficacy and tolerability of high dose imatinib (800 mg per day) in a multi-institutional trial (Castagnetti et al., 2009) focusing on a particular subgroup of newly diagnosed CML patients in CP, namely on 78 patients with intermediate SOKAL risk score. (Sokal et al., 1984). They found high rates of CCyR (88%) at 12 months and at 24 months (91%), respectively. Furthermore 56% and 73% of the patients having achieved a CCyR also achieved a major molecular remission (MMR) at 12 and 24 months. They reported on slightly more frequent non-hematologic toxicities like skin rash, myalgia, bone pain, gastrointestinal intolerance, fluid retention and asthenia compared to reported studies with standard dose imatinib. Moreover, they also found WHO grade 3 and 4 hematologic toxicities in terms of leukopenia (18%), thrombocytopenia (17%) and anemia (9%). Between month 3 and 6 (second quarter of therapy) 44% of the patients received the full scheduled dose of 400 mg twice daily (Castagnetti et al., 2009). Another multicenter phase II study, the "Rationale and Insight for Gleevec High – Dose Therapy (RIGHT) trial was conducted in 115 newly diagnosed CML patients in CP (Cortes et al., 2009). This study has again suggested that imatinib 800 mg per day leads to a more rapid reduction in tumor burden with higher rates of MCyR, CCyR and major molecular responses (MMR^{IS}) according to the international scale (IS) compared to historical controls for the price of a slightly increased toxicity including myelosuppression, rash, fatigue and musculoskeletal symptoms (Cortes et al., 2009). In this multicenter trial, 64% of the

patients attained 90% or more of the planned dose. The dose intensity was similar in patients younger than 65 years or in patients ≥ 65 years. The authors reported that the patients with ≥ 90% dose intensity had a significantly higher chance to obtain a MMR^{IS} and a complete molecular remission (CMR) at 18 months: 79% of the patients who received ≥ 90% dose intensity achieved a MMR^{IS} compared to only 42% of the patients who received < 90% dose intensity (p=0.015). Similarly, a CMR (defined as a ≥ 4.5 log reduction from a standardized baseline) was achieved at 18 months by 67% of the patients that received ≥ 90% dose intensity compared to 29% of patients that received < 90% dose intensity (p=0.029).

A slightly different approach was chosen by the Australasian Leukemia and Lymphoma group (ALLG) that conducted a "Therapeutic Intensification in DE-novo Leukemia" (TIDEL) phase II study (Hughes et al., 2008). Compared to all other phase II studies they did not start with 800 mg imatinib per day but with a slightly lower dose of imatinib with 600 mg per day in newly diagnosed CP CML patients and allowed an early dose intensification to 800 mg imatinib if specific response criteria were not met. All patients had an intense response monitoring of marrow cytogenetics and blood for RT-PCR of BCR-ABL mRNA levels every 3 months. The rationale for this design was the assumption that many patients would receive excellent responses with only 600 mg imatinib instead of 800 mg and that the 800 mg imatinib dose could be limited to those patients not achieving an optimal response with the 600 mg dose. The criteria for increasing the imatinib dose were as follows: failure to achieve a complete hematological response (CHR) at 3 months, a MCyR at 6 months, a CCyR at 9 months and a MMR^{IS} (defined as less than 0.01% BCR-ABL by RQ-PCR on the international scale) at 12 months. Within the first year a dose escalation from 600 mg to 800 mg imatinib was indicated in 17 out of 103 patients but only possible in 8 patients (47%) primarily due to ongoing toxicity or subsequent trial withdrawal. These patients failed to achieve or failed to maintain a MCyR at 6 months or a CCyR at 9 months. After the first year a dose escalation was indicated in 73 patients because these patients did not achieve MMR^{IS}. Dose escalation was possible in 62% of the cases. Using this two-step design, the rates of CCyR were 88% at 12 months and 90% at 24 months, respectively. These CCyR-rates were significantly better than those obtained in the IRIS trial (CCyR was 69% at 12 months and 80% at 24 months, respectively) where dose escalations were not allowed at early time points. Similarly were MMR^{IS} rates superior with 47% at 12 months and 73% at 24 months in patients receiving a daily average dose of 600 mg imatinib compared to the IRIS trial (40% MMR at 12 months and 55% at 24 months).

In summary, all these phase II studies suggest earlier and higher rates of cytogenetic and molecular remissions with higher imatinib doses for the price of slightly higher non-hematologic toxicities and higher rates of hematologic toxicities. There was hope and enthusiasm – based on these studies - that earlier achievement of cytogenetic remissions, especially CCyR and molecular remissions would result in lower rates of treatment failures and subsequently translate in superior overall survival rates.

3. Phase III studies on High Dose imatinib in Chronic Phase (CP)

Based on the superior rates of cytogenetic and molecular responses that occur faster with doses > 400 mg imatinib, several phase III studies were initiated on this issue (Table 1).

Study	Author	Design	Primary end point Comparison HD* vs SD**
TOPS	Cortes et al., 2010	newly diagnosed CP patients, n=476 800 mg imatinib/day vs 400 mg imatinib/day	MMR^{IS} at 12 months: 46% vs 40% p=0.2035
ELN	Baccarani et al., 2009b	newly diagnosed SOKAL high risk CP patients, n=216 800 mg imatinib/day vs 400 mg/day	CCR at 12 months: 64% vs 58% p=0.435
ISTAHIT	Petzer et al., 2010a	non-TKI pre-treated patients in late CP, n=227; 800 mg imatinib/day for 6 months, 400 mg imatinib thereafter as maintenance vs 400 mg imatinib/day	MCyR at 12 months: 64.4% vs 56.8% p=0.354
GERMAN CML SG and SAKK	Hehlmann et al., 2011	newly diagnosed CML patients, tolerability adapted 800 mg imatinib/day vs 400 imatinib mg/day	MMR^{IS} at 12 months: 59% vs 44% p<0.001

*HD: imatinib high dose (>400 mg/day)
**SD: imatinib standard dose (≤400 mg/day)

Table 1. Randomized phase III Studies comparing HD imatinib (600 mg - 800 mg per day) to SD imatinib (400 mg per day)

3.1 The tyrosine kinase inhibitor optimization and selectivity study (TOPS)

The tyrosine kinase inhibitor optimization and selectivity study (TOPS) evaluated the safety and efficacy of the initial treatment with imatinib 800 mg (400 mg twice daily) versus the regular 400 mg once daily dosing in newly diagnosed CML patients in CP (Cortes et al., 2010). In this study all CP CML patients were included regardless of their Sokal risk status (Sokal et al., 1984). In spite of the fact that molecular (MMR^{IS}) and cytogenetic responses (CCyR) occurred faster in patients assigned to the 800 mg dose these parameters were not significantly different at 12 months. This included the primary endpoint, the MMR^{IS} rate at 12 months which was only slightly, but not statistically significantly different with an MMR^{IS} rate of 46% in the 800 mg and 40% in the 400 mg group (p=0.2035), respectively. Moreover, the progression free survival (PFS) and overall survival (OS) was also only slightly, but not statistically significantly improved with a PFS of 97.4% and an OS of 98.2% at 18 months in the imatinib 800 mg arm compared to a PFS of 95.0% and an OS of 98.7% at 18 months in the 400 mg arm. Progression to AP or BC occurred in 1.9% in the 800 mg arm and in 3.2% in the 400 mg arm. Adverse events (AE) were generally reported to be mild or moderate in both arms. Rates of all-grade and WHO grade 3 or 4 AEs, however, were higher in the imatinib HD group (98.1% all grades and 63.6% WHO grade 3 or 4 AEs in the 800 mg group versus 93.6% all grades and 33.1% in the 400 mg group, respectively). Hematologic AEs including leukopenia, neutropenia, thrombocytopenia and anaemia were more

common in the 800 mg arm. Interestingly, biochemical abnormalities like hypophosphatemia, hypocalcaemia or transaminase elevations were not different between the 2 arms and were generally low except hypophosphatemia (12% in the 800 mg and 14.6% in the 400 mg imatinib arm, respectively). AEs in general led to a higher discontinuation rate of 9.4% in the imatinib HD group compared to 3.8% the 400 mg group. Half of the patients in the high dose arm required a dose reduction to < 600 mg at some point during the study. Nevertheless, the average daily doses of imatinib were 662 mg in the HD arm and 388 mg in the standard dose arm. At 12 months, 61% of the patients in the HD arm were still treated with their assigned dose and 78% of the patients in the HD arm were capable to take an imatinib dose of at least 600 mg per day.

3.2 High dose imatinib for Sokal high risk patients?

Potential candidates that were thought to benefit particularly from HD imatinib were patients with a high Sokal risk score (Sokal et al., 1984). A retrospective subgroup analysis of 115 patients in the TOPS trial, however, reveals an almost identical overall CCyR rate by 12 months for both groups (61.9% CCyR with 400mg/day and 63.0% with HD imatinib, p=1.0) and a slightly improved MMR^{IS} rate at 12 months for the HD group (26.2% MMR^{IS} with imatinib 400mg/day and 39.7% MMR^{IS} with HD imatinib, p=0.16). The number of patients in the high Sokal risk group was, however, too small to draw definitive conclusions (n=68). A study on this particular patient population (i.e. patients with high Sokal risk score) was initiated by the ELN with a prospective trial that compared imatinib 400 mg and 800 mg daily in the front-line treatment of 216 Sokal high risk CML CP patients (Baccarani et al., 2009a). This study, however, failed to demonstrate any benefit for HD imatinib over standard dose for the Sokal high risk population. The CCyR rate at 12 months (the primary endpoint of the study) was similar with 64% CCyR in the HD arm and 58% CCyR in the imatinib standard dose arm (p=0.435). CCyR rates, however, appeared to be related to the actual dose as 96% of the patients that were capable to take the intended dose of 800 mg imatinib in fact achieved a CCyR. In contrast, CCyR was lowest with 20% in patients assigned to the HD arm with an average daily dose of less than 400 mg imatinib per day. Furthermore, no significant differences could be detected in cytogenetic or molecular responses at any time and OS, PFS and event free survival (EFS) were also not different. The authors of this study therefore have suggested that HD imatinib (800 mg per day) cannot be recommended as front-line therapy in CML Sokal high risk patients (Baccarani et al., 2009a). In an additional study that compares a tolerability-adapted 800 mg imatinib dose per day with the standard 400 mg dose in newly diagnosed CML patients from the German CML study group in cooperation with the Swiss group for clinical research (SAKK) and which is described in detail below, the authors report that the rapidly occurring MMR^{IS} that they noticed with the tolerability-adapted 800 mg imatinib dose were only observed in low- and intermediate-risk but not in high-risk patients according to the Sokal and the EuroScore (Hasford et al., 1998), another prognostic score that was initially developed to predict the survival of CML patients treated with interferons. For unknown reasons high-risk patients seem to be less responsive to any therapy including HD imatinib, although nowadays even Sokal high-risk CML patients benefit significantly from imatinib therapy compared to treatments from the pre-imatinib era and the survival has improved dramatically even in this high-risk cohort.

3.3 Imatinib standard dose versus high dose induction trial ("ISTAHIT" study)

A different approach was tested by the Central European Leukemia Study Group (CELSG) in the international multicentre Imatinib STAndard dose versus High dose Induction Trial ("ISTAHIT") (Petzer et al., 2010a). Different from the other phase III trials that tested HD imatinib versus the standard dose they tested *pre-treated* CML patients in CP. These patients were - although pre-treated - tyrosine kinase inhibitor (TKI) naïve but pre-treated with drugs from the pre-imatinib era, such as hydroxyurea, interferons, busulfan or Ara-C. The medium number of pre-treatments before study entry was 2. Overall, this patient population was in later CP of their disease and subsequently at a higher risk for disease progression. In addition, HD imatinib (800 mg per day) was limited to the first 6 months of therapy and then reduced to 400 mg as "maintenance" therapy. The reasons for choosing this strategy were a "hit hard and early" concept in order to achieve rapid and deep cytogenetic and molecular responses on the one hand and concerns on reported hematotoxicity on the long term on the other hand, especially in regard to this pre-treated patient population in later chronic phase (Cortes et al, 2003). A report on a planned interim analysis (after half of the patients had been treated for 12 months) demonstrated significant improvements in the rates of MCyR and CCyR at early time points such as 3 and 6 months as well as in MMR^{IS} at 6 months in favour of HD imatinib compared to the standard dose (400 mg once daily) (Petzer et al., 2010a). As expected for this heavily pre-treated patient population, WHO grade 3 and 4 hematotoxicity was significantly increased during the first 6 months in HD imatinib arm, whereas WHO grade 3 and 4 non-hematotoxic AEs were comparable. Notably, severe infections were not improved in spite of the higher rates of leukopenias and neutropenias. In a first report on the final analyses of this study the authors have reported that in spite of the fact that significantly higher and more rapid cytogenetic responses occurred not only at early time points but even at later time points when HD imatinib was already reduced to standard dose after 6 months (e.g. CCyR at 12 months was superior with 52.9% in the experimental HD arm compared to 31.8% in standard dose imatinib arm; p=0.006; MMR^{IS} at 24 months was 42.5% compared to 26.5% in favour of the experimental HD arm; p=0.034) the strategy of using high doses of imatinib as induction therapy again did not improve OS and PFS (Petzer et al., 2010b). In general, in terms of the biologic effects (i.e. the achievement of cytogenetic and molecular responses) the data are similar to other imatinib HD trials, where imatinib was at least intended to be given throughout the entire study period.

3.4 Tolerability-adapted 800 mg imatinib: Experience form the German CML-SG and the Swiss group for clinical research (SAKK)

Another slightly different approach was chosen by the German CML study group in cooperation with the Swiss group for clinical research (SAKK) (Hehlmann et al). They tried to optimize the therapy for newly diagnosed CML patients in CP by comparing a tolerability-adapted 800 mg imatinib dose per day with the 400 mg dose in order to avoid higher grade toxicity. In the imatinib 800 mg/day arm the full 800 mg dose was given after a 6-week-run-in period with 400 mg imatinib per day to avoid excessive cytopenias. The median dose of imatinib in the tolerability-adapted 800 mg arm was 628 mg per day compared to 400 mg in the 400 mg per day arm. The highest median dose of imatinib was reached in the second 3-month period (month 3 to 6) with 737 mg per day. Thereafter, the

dosage decreased to around 600 mg/day due to adaptation of the dose to tolerability according to the protocol. Again, significantly higher rates of CCyR and MMR^{IS} were achieved with the higher imatinib dosage compared to the 400 mg per day dose at earlier time points (e.g. at 6 and 12 months, respectively) but these differences again subsequently decreased at later time points (e.g. MMR^{IS} at 36 months was 81.6% for the tolerability-adapted 800 mg imatinib dose and 79.3% for the imatinib standard dose arm with 400 mg per day). AEs were more frequent with 800mg imatinib per day, especially oedemas and gastrointestinal problems, but WHO grade 3 and 4 AEs were reported to be rare and not more frequent in the tolerability-adapted 800 mg imatinib dose arm. Although the rates of MMR^{IS} by 12 months were superior in the tolerability-adapted 800 mg imatinib dose arm and the achievement of MMR^{IS} by 12 months was directly associated with an improved survival no differences were reported by the authors for OS (2-year survival was 96.0% in the tolerability-adapted 800 mg dose arm and 96.9% in the imatinib 400mg dose arm, respectively) and PFS when comparing the 2 different treatment arms. This also includes progressions and numbers and causes of death (Hehlmann et al).

Taking all these phase III studies on early dose intensification of imatinib into consideration one has to state that so far none of these studies has demonstrated a substantial benefit over the standard dose of 400 mg/day, especially in terms of OS or PFS. This is somehow surprising as the majority of the studies showed a significantly higher rate of cytogenetic and molecular responses occurring at earlier time points. An improved early response was recently linked to a prolonged survival in CML-patients (Hughes et al.,2010; Iacobucci et al., 2006). These data were, however, collected from CP CML patients that were all treated upfront with a daily imatinib dose of 400 mg. It very much looks as if, over time, the inhibitory effect of imatinib reaches a plateau that is accomplished earlier with higher doses of imatinib and later with the standard 400 mg dose and no clear evidence has been available so far that under these circumstances these earlier responses will translate into an improved survival. Maybe a longer follow-up will give us the appropriate answer. For the moment, based on the data available so far, an initial high dose imatinib treatment with 800 mg per day cannot be recommended at the present time outside of clinical studies. A longer follow-up will show whether initial higher imatinib doses will possibly translate into improved long-term outcomes.

4. Dose escalation after imatinib failure

As outlined above, up front dose escalation with imatinib leads to improved and earlier cytogenetic and molecular responses in CML patients in CP but this has so far not led to an improved survival advantage. However, in the very beginning of the imatinib era the optimal dosing schedule has not been investigated extensively. This is reflected by the fact that the maximum tolerated dose has not been reached in the phase I trial (Druker et al., 2001) that comprised 83 patients. Beyond a dose of 300 mg per day toxicity and efficacy was not different in a subset of patients which led to the use of 400 mg imatinib per day in the following phase II studies. Among these trials the option to increase the imatinib dose to 2 x 400 mg per day after insufficient response to standard dose imatinib suggested that dose escalation might be a possible strategy in patients with imatinib resistance (Kantarjian et al., 2002).

4.1 Primary and secondary resistance to imatinib

Patients resistant to imatinib may experience primary (intrinsic) or secondary (acquired) resistance (Jabbour et al., 2009a). Primary resistance has been defined as the lack of a distinct level of response at various time points during treatment (landmark response). By the NCCN guidelines, primary resistance is defined as the failure to achieve CHR within 3 to 6 months of treatment initiation, lack of any level of cytogenetic response at 6 months or the lack of a MCyR at month 12 or a CCyR at month 18 (National Comprehensive Cancer Network (NCCN). Clinical Practice Guidelines in Oncology. Chronic Myelogenous Leukemia. Version 2.2010. Jenkintown, Pa: NCCN; 2009.
http://www.nccn.org/professionals/physician_gls/PDF/cml.pdf).
According to recommendations from the ELN treatment failure is defined as lack of CHR at 3 months, no CHR or lack of any cytogenetic response (CyR) at 6 months, less than partial CyR (PCyR) at 12 months or less than a CCyR at 18 months (Baccarani et al., 2009b).
Secondary resistance is a disease progression and the loss of any therapeutic effect during the treatment with imatinib. This occurs in approximately 24% of patients, mostly within the first three years, as has been shown in the IRIS-trial (Druker et al., 2006).
The reasons for resistance include point mutations of BCR-ABL, amplification of BCR-ABL, low Oct-1 activity resulting in low influx of imatinib, high MDR-1 activity resulting in high imatinib efflux, and additional clonal aberrations. According to the ELN-guidelines these patients might be candidates for dose escalation of imatinib. However, some point mutations like T315I, G250K, E255K, F486S and E255V cause absolute imatinib resistance and therefore are contraindications for a dose escalation strategy. With the exception of T315I the use of second generation TKIs like nilotinib or dasatinib is recommended in these cases. Patients with T315I are resistant to all currently available TKIs and should be treated within clinical trials and the option of stem cell transplantation should be evaluated. Other mutations like M315T, V299L etc., however, cause relative imatinib resistance, which makes dose escalation of imatinib feasible. A prerequisite for imatinib dose escalation is the absence of relevant side effects with the 400 mg dose once daily. Other reasons for relative imatinib resistance that potentially might be overcome by dose escalation are additional genetic abberations, a high efflux of the drug due to high MDR1-activity, low influx of the drug by low Oct-1 activity, and BCR-ABL amplification (figure 1).

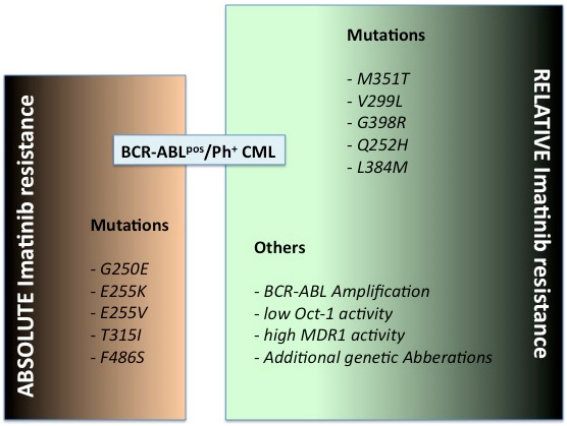

Fig. 1. Overview on causes leading to absolute or relative imatinib resistance in CML (adapted from Rudzki et al., 2011)

4.2 Clinical studies showing effect of dose escalation after imatinib failure

There are several clinical trials supporting dose escalation in patients already receiving standard dose imatinib. First, a study performed by Kantarjian et al. included patients of the phase II studies 110 and 113 and represents a single center study at the MD Anderson Cancer Center. Dose escalation was allowed from 400 to 800 mg per day if standard dose imatinib was well tolerated. If the starting dose was 300 mg or if the dose had to be decreased from 400 mg to 300 mg due to initial side effects, the dose was escalated to 600 mg per day. The indication for dose escalation was defined as not achieving a CHR (defined as haematological resistance) after 3 months, failure to achieve a MCyR at 12 months (defined as cytogenetic resistance), if CHR was lost at any time (defined as hematologic relapse) or if Ph-positive cells increased by 30% at two occasions (defined as cytogenetic relapse). From 261 included patients, 47 were escalated to 800 mg per day and 7 were escalated to 600 mg per day. Among 34 patients that were escalated due to cytogenetic resistance or relapse, 56% achieved a cytogenetic response again. CCyR, however, was only reached in 18%. Success rates were higher among patients treated for haematological resistance or relapse as from 20 dose escalated patients 65% regained a hematologic response. With this study the authors demonstrated that some effect could be achieved by dose escalation after primary imatinib failure. However, the depth of response achieved with this procedure might be insufficient as only a minority of patients reached CCyR. This, however, is the goal of treatment as CCyR or MMR (however, this study does not report on molecular response) is known to be of high relevance for the long-term outcome in CML patients (Kantarjian et al., 2003).

Zonder et al. performed a dose escalation study in 12 CP CML and four CML patients in AP with signs of disease progression (Zonder et al., 2003) Again, some responses could be achieved but the majority of patients did not benefit from this treatment. Such a transient response has also been shown by Marin and colleagues (Marin et al., 2003). An Italian study (Breccia et al., 2010), however, was capable to demonstrate a prolonged response in patients with cytogenetic relapse, especially in patients with acquired cytogenetic resistance. According to the current ELN guidelines, patients with progressive disease or with cytogenetic resistance are currently considered as treatment failures and qualify for second generation TKIs or an allogeneic stem cell transplantation and not for dose escalation (Baccarani et al., 2009b).

In patients with suboptimal molecular response the benefit also seems to be limited, whereas dose escalation in patients with suboptimal cytogenetic response is more promising (Rea et al., 2009). However, data obtained from these studies have to be interpreted with caution as the patient numbers are rather low. A larger study was again presented by the MD Anderson Cancer Center. Out of 626 patients, 84 patients were dose escalated due to treatment failure on standard dose imatinib (Jabbour et al., 2009b). In 72 out of these patients the dose was increased from 400 mg to 800 mg per day and in 12 patients from 300 mg to 600 mg per day. 40% achieved a CCyR with a minority of patients reaching deep and prognostic meaningful responses. Patients that already had a previous CCyR seemed to especially benefit from dose escalation. Other patients showed disappointing low and insufficient response rates with a significantly worse EFS. A point of criticism with this study is that only 25 out of the 84 patients were tested for BCR-ABL mutations and mutations that are associated with high imatinib resistance have not been detected. In contrast to the patient cohort of the MD Anderson Cancer Center, the patient population of the IRIS trial was not previously treated with IFN-alpha. Within the latter, a

dose escalation was performed in a two-step manner. The first step was an escalation to 600 mg per day. If no sufficient response was noted two weeks later a further escalation to 800 mg per day was allowed. 39 patients were treated by dose escalation, but only a small proportion of patients reached a CCyR (Kantarjian et al., 2009). PFS for these patients was 84% and OS 89% three years after dose escalation. The criteria for resistance, however, were slightly different to the current ELN-guidelines (Baccarani et al. 2009b). Retrospectively, 48 patients would have fulfilled the criteria of imatinib resistance. Significantly different results, however, cannot be expected due to this fact. The Korean CML study group evaluated the efficacy of dose escalation in patients with suboptimal response or treatment failure according to the ELN-guidelines. In total they included 64 CML patients in CP, AP or BC and reported a CCyR rate of 23,9% at 12 months and a clear correlation between early molecular response and time to treatment failure (Koh et al., 2010).

4.3 Dose escalation versus second-generation Tyrosine Kinase Inhibitor (TKI) or IFN-alpha

The START trial compared the efficacy of imatinib dose escalation with the use of the second generation TKI dasatinib. Patients harbouring mutations associated with a high degree of imatinib resistance were excluded (Kantarjian et al., 2009). Patients previously treated with 400 to 600 mg imatinib per day were randomized to receive either 800 mg imatinib per day or dasatinib 70 mg twice a day. Treatment with dasatinib resulted in higher rates of CHR (93 vs 82%), MCyR (53 vs 33%), CCyR (44 vs 18%), MMR (29 vs 12%) and in a significantly prolonged PFS. A significant proportion of 70% of patients in this study, however, already were dose escalated to 500 or 600 mg of imatinib before the inclusion into the study, making the interpretation of these results difficult. If only patients are compared that were either dose escalated from 400 mg to 800 mg imatinib per day or received dasatinib, the results are less impressive in favour of dasatinib with almost identical rates in MCyR and CCyR.

The Spanish PETHEMA and the Australasian CML study group also compared high dose imatinib to alternative treatments in patients not achieving an optimal response (Cervantes et al., 2010). If patients did not achieve a CHR at 3 months they were randomized to continuation of standard dose imatinib or to high dose imatinib (800 mg/day). Patients not achieving a CCyR at 6 months were randomized to high dose imatinib or standard dose imatinib in combination with IFN-alpha and patients not achieving a MMR^{IS} at 18 months were dose escalated as well. 210 patients were included. At month 6, 17 patients had the dose of imatinib increased, 16 out of them reached a CCyR at month 18. 9 patients were dose escalated at month 18 and 8 achieved an MMR a few months later (Cervantes et al., 2010).

4.4 Early dose escalation

A very interesting approach has been investigated by the Australasian CML study group with the TIDEL II study (Yeung et al., 2010). This is a single arm study that allowed dose escalation at a very early time point based on the levels of the imatinib plasma levels that had to be above 1000 ng/ml. BCR-ABL levels of >10% at 3 months, >1% at 6 months or less than a MMR (i.e. >0.1%) at 12 months were indications for a switch to nilotinib. The MMR achieved by this approach was 66% at 12 months which is far better than published for

nilotinib in the ENESTnd and dasatinib in the Daisision trial (43 and 46%, respectively). The early escalation of imatinib upon suboptimal response, therefore, seems an interesting approach for future studies.

5. Accelerated phase and blast crisis

High dose imatinib in accelerated phase (AP) and blast crisis (BC) has been studied in phase II studies only (table 2). Talpaz et al. firstly reported the results on 181 patients with CML-AP. Initially, patients were enrolled in the STI 571 0109 study and were treated with 400 mg imatinib. In 119 patients the starting dose was increased to 600 mg per day after the final results of the phase I study confirmed the safety and efficacy of high dose imatinib. Analysis at 48 months revealed that 18% of patients remained on imatinib while 82% discontinued imatinib. The primary reasons for that included progression or lack of efficacy. Best observed responses were CHR in 40%, PCyR in 7% and CCyR in 20% of patients. The median OS was 43 months for CML-AP patients treated with 600 mg imatinib per day. The major prognostic factor was response: 72% of the patients with a MCyR at month three were alive at 48 months compared to 42% of patients without MCyR at month three (Talpaz et al., 2002). In a long term follow up report the same authors stated that 23% of the patients remained on study follow up with 9% still taking the study drug (Silver et al., 2009). The circumstance that initially a part of the patients was treated with 400 mg imatinib enabled a retrospective analysis comparing these patients with the other part of the patients treated with 600 mg upfront. These analyses revealed that CML patients in AP that started with 600 mg imatinib had a favourable OS and PFS compared to patients starting with 400 mg imatinib once daily. These facts led to the recommendation to use 600 mg imatinib per day as starting dose for the treatment of advanced CML. These results were confirmed by a similar phase II study, which was performed by Palandri et al. They treated 111 patients in CML-AP with 600 mg imatinib per day. After a median long term follow up of 82 months they have reported that 96% of the patients converted to CML-CP and 71% of patients achieved a CHR. 30% of patients reached a MCyR and 21% a CCyR. These responses were maintained for at least 4 weeks. After the prolonged follow-up 14% of the patients received a second-generation tyrosine kinase inhibitor and 19% of the patients were still alive on imatinib therapy. The median OS was 37 months, and was significantly associated with CHR or CCyR (Palandri et al., 2009).

Author	n	CML-phase	Dose imatinib [mg/d]	median OS
Talpaz et al., 2002	119	AP	600	43 months
Palandri et al., 2009	111	AP	600	37 months
Sawyers et al., 2002	229	BC	600	7 months
Palandri et al., 2008	92	BC	600	7 months

Table 2. Phase II studies investigating high dose imatinib in CML-AP and -BC

Efficacy of high dose imatinib in BC was investigated in the STI571 0102 study. 229 patients were enrolled in this phase II trial and treated with 400 mg or 600 mg imatinib per day (Sawyers et al., 2002). At 48 months, only 3% of patients still were on imatinib therapy while the remaining patients were off treatment due to progression or lack of efficacy. CHR was achieved in 9%, MCyR in 16%, and CCyR in 7% of patients. The estimated OS was 7 months for patients treated with 600 mg imatinib per day. Similar results were obtained in an Italian phase II study. Palandri et al. treated 92 patients in BC with 600 mg imatinib per day. The results are very similar to the STI571 0102 study by showing a median OS of 7 months and only a minority of patients reaching sufficient cytogenetic responses. These were maintained for at least 4 weeks and after a median follow-up of 66 months, 8% of the patients were alive (Palandri et al., 2008).

6. Conclusion

For the moment, sufficient data are not available to recommend an initial high dose imatinib therapy in CP CML patients in spite of well documented superior cytogenetic and molecular remissions that are obtained earlier by using higher imatinib doses. In contrast, a dose of 600 mg per day is recommended for the treatment of advanced phases (i.e. AP and BC). A dose increase as a consequence of suboptimal response or failure according to ELN or NCCN criteria is a valid option if no imatinib resistant point mutation on the one hand and no significant side effects with the 400 mg imatinib dose on the other hand are present at that time. Alternative options at that time are also already commercially available second generation TKIs like dasatinib or nilotinib. These latter two TKIs are meanwhile also registered for the first line treatment of CP CML and may diminish the need of further investigations on the use of high imatinib doses in the future.

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8. References

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4

Therapeutic Drug Monitoring of Imatinib for Chronic Myeloid Leukemia Patients

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1. Introduction

Imatinib mesylate (Glivec®; Novartis, Basel, Switzerland), a protein kinase inhibitor of the BCR–ABL fusion protein, has demonstrated significant clinical efficacy in the treatment of Philadelphia (Ph) chromosome-positive chronic myeloid leukemia (CML). Imatinib mesylate (hereinafter shortly referred to as imatinib) produces durable responses and prolonged survival; therefore, it has become the standard of care for this disease (Goldman 2007; O'Brien, et al. 2003a). Notwithstanding the positive effects of imatinib, nearly 20% of the patients who take imatinib fail to achieve a complete cytogenetic response (CCyR); others may develop intolerable side effects or drug resistance overtime. Factors that might be associated with suboptimal responses and failure to treatment include (i) biological factors, such as the baseline presence or later emergence of BCR–ABL mutations or other genetic variants (Gorre, et al. 2001; Radich, et al. 2006), or organic cation transporter-1 (OCT1)-mediated drug influx (White, et al. 2010); (ii) clinical features, such as the disease status of the patients or the Sokal risk score at baseline (Crossman and O'Brien 2004); (iii) pharmacokinetic (PK) factors, such as PK-related interindividual variation affecting imatinib metabolism and drug–drug interactions (Cortes, et al. 2009; Peng, et al. 2004b); and (iv) the patient's compliance with therapy (Marin, et al. 2010).

In this chapter, we review the factors that affect imatinib pharmacokinetics, including the daily dose of imatinib, polymorphisms of imatinib-associated drug transporters, and the currently available methods for quantitative determination of imatinib. Moreover, we discuss the clinical significance of therapeutic drug monitoring (TDM) of imatinib.

2. Relationship between daily dose of imatinib and clinical response

The standard daily dose of imatinib—established by the International Randomized Study of Interferon and STI571 (IRIS)—is 400 mg for patients with chronic phase CML (Druker, et al. 2006; Hochhaus, et al. 2009). However, several studies have suggested that the administration of doses higher than 400 mg improves the response in some patients. Indeed, a better response was observed in accelerated and blast phases of CML with a dose of 600 mg/day (Talpaz, et al. 2002). In another study of 107 Japanese patients with chronic phase CML, patients given higher average daily doses of imatinib (more than 350 mg) not only achieved higher CCyR rate at 12 and 30 months but also had longer CCyR duration than

those given lower average daily doses (Nagai, et al. 2010). Collectively, these results suggest a clear dose-response relationship between daily dose of imatinib and treatment results.

3. Clinical significance of trough imatinib plasma concentrations

The imatinib plasma trough concentration (C_0) appears to affect the clinical response of patients (Table 1) (Ishikawa, et al. 2010; Larson, et al. 2008; Picard, et al. 2007; Singh, et al. 2009; Takahashi, et al. 2010b; Forrest, et al. 2009; Sakai, et al. 2009). Picard et al. reported that a steady-state imatinib C_0 measured after at least 12 months of treatment with a standard imatinib dose correlated with both cytogenetic and molecular responses (Picard, et al. 2007). Takahashi et al. have reported that in multiple analyses, the major molecular response (MMR) is significantly associated with the age of patients and imatinib C_0, whereas CCyR is associated only with daily dose (Takahashi, et al. 2010b). In addition, Picard et al. suggested that the threshold for the imatinib C_0 should be set above 1002 ng/mL, as this level was significantly associated with an MMR based on a concentration-dependent receiver-operating characteristic curve analysis with best sensitivity (77%) and specificity (71%) (Picard, et al. 2007). According to this threshold C_0 of imatinib, clinical responses were evaluated in several reports (Table 2). Takahashi et al. and Marin et al. reported that patients with imatinib C_0 less than 1000 ng/mL have a significantly lower success rate in achieving improved MMR (P = 0.012 and 0.02, respectively) but not CCyR (Marin, et al. 2010; Takahashi, et al. 2010b). Thus, the efficacy threshold C_0 of imatinib should be set above 1000 ng/mL for CML patients.

Reference	N	Response	Responders		Nonresponders		P value
			N	Mean C_0 (ng/mL)[1]	N	Mean C_0 (ng/mL)[1]	
Larson et al.	351	CCyR	297	1,009 ± 544	54	812 ± 409	0.01
Takahashi et al.	254	CCyR	218	1,057 ± 585	36	835 ± 524	0.033
		MMR	166	1,107 ± 594	88	873 ± 528	0.002
Picard et al.	68	CCyR	56	1,123 ± 617	12	694 ± 556	0.03
		MMR	34	1,452 ± 649	34	869 ± 427	0.001
Singh et al.	40	Clinical response	20	2,340 ± 520	20	690 ± 150	0.002
Ishikawa et al.	60	MMR	38	1,093 (median)	22	853 (median)	0.002
Sakai et al.	33	Optimal	25	1,242	8	736	0.0087
Forrest et al.	78	CCyR	53	1,010 ± 469	24	1,175 ± 656	0.29
		MMR	51	1,067 ± 473	27	1,063 ± 643	0.74

[1]All values, except those belonging to the studies by Ishikawa et al. and Sakai et al., are presented as the mean ± standard error.
Abbreviations: C_0, plasma trough concentration; CCyR, complete cytogenetic response; MMR, major molecular response

Table 1. Correlation of imatinib pharmacokinetics with clinical response

Reference	N	Response	C_0 (ng/mL)				*P* value
			N	≤1,000	N	>1,000	
Marin et al.	84	CCR	43	23.3%	41	44.4%	0.14
		MMR		60.1%		83.2%	0.02
Takahashi et al.	254	CCyR	146	83.6%	108	88.9%	0.276
		MMR		58.9%		74.1%	0.012
Picard et al.	68	MMR	32	25.0%	36	72.2%	0.03
Ishikawa et al.	60	MMR	29	48.3%	31	77.4%	0.019

Table 2. Clinical response and target plasma trough concentration (C_0)

4. Reported methods for the quantitative determination of imatinib

Table 3 summarizes the available methods, including the internal standard used, for the quantitative determination of imatinib (Bakhtiar, et al. 2002; Chahbouni, et al. 2009; Davies,

Reference	Analyte(s)	IS	Method
Miura et al. (2011)	Imatinib	IS: Dasatinib	HPLC–UV (265 nm)
Roth et al. (2010)	Imatinib	IS: None	HPLC–UV–Diode Array (265 nm)
Davies et al. (2010)	Imatinib, *N*-desmethylimatinib, Nilotinib	IS: Clozapine	HPLC–UV (260 nm)
Chahbouni et al. (2009)	Imatinib (Erlotinib, Gefitinib)	IS: D8-Imatinib	LC–MS/MS
De Francia et al. (2009)	Imatinib (Dasatinib, Nilotinib)	IS: Quinoxaline	HPLC–MS
Rochat et al. (2008)	Imatinib	IS: D8-Imatinib	LC–MS/MS
Oostendorp et al. (2007)	Imatinib, *N*-desmethylimatinib	IS: 4-Hydroxybenzophenone	HPLC–UV (265 nm)
Titier et al. (2005)	Imatinib	IS: D8-Imatinib	LC–MS/MS
Widmer et al. (2004)	Imatinib	IS: Clozapine	HPLC–UV–Diode Array (261 nm)
Velpandian et al. (2004)	Imatinib	IS: None	HPLC–UV (265 nm)
Schleyer et al. (2004)	Imatinib, *N*-desmethylimatinib	IS: None	HPLC–UV (260 nm)
Parise et al. (2003)	Imatinib, *N*-desmethylimatinib	IS: D8-Imatinib	LC–MS
Bakhtiar et al. (2002)	Imatinib, *N*-desmethylimatinib	IS: D8-Imatinib	LC–MS/MS

Abbreviations: IS, internal standard; LC–MS, liquid chromatography with mass spectrometry; LC–MS/MS, liquid chromatography with tandem mass spectrometry; HPLC–UV, high-performance liquid chromatography with ultraviolet detector

Table 3. Analytical methods for the quantitation of imatinib in human plasma

et al. 2010; De Francia, et al. 2009; Miura, et al. 2011; Oostendorp, et al. 2007; Parise, et al. 2003; Rochat, et al. 2008; Roth, et al. 2010; Schleyer, et al. 2004; Titier, et al. 2005; Velpandian, et al. 2004; Widmer, et al. 2004). High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, liquid chromatography with mass spectrometry (LC–MS), and liquid chromatography with tandem mass spectrometry (LC–MS/MS) have been used in clinical studies to measure the plasma concentration of imatinib. HPLC–UV is less expensive than LC–MS or LC–MS/MS detection and requires equipment that is widely available in hospital laboratories. As such, a validated HPLC–UV assay provides the most practical platform to measure imatinib plasma concentration in actual clinical practice.

5. Interpatient variability of trough imatinib plasma concentration

Despite the linear relationship between imatinib C_0 and its daily dose, substantial interpatient variability is observed (Takahashi, et al. 2010b). Even among patients taking the same 400 mg/day dose, the imatinib C_0 ranges widely (140–3910 ng/mL) (Table 4) (Forrest, et al. 2009; Ishikawa, et al. 2010; Larson, et al. 2008; Marin, et al. 2010; Picard, et al. 2007; Takahashi, et al. 2010b). Factors that could underlie this interpatient variability include body size, age, gender, liver function, renal function, interaction with other medications given concomitantly, adherence to medication regimens, and polymorphisms of enzymes or transporters related to imatinib pharmacokinetics and/or pharmacodynamics.

Reference	N	C_0 (ng/mL)		
		Mean	Minimum	Maximum
Larson et al.	351	979	153	3,910
Picard et al.	68	1,058	181	2,947
Marin et al.	84	900	400	1,600
Forrest et al.	70	1,065	203	2,910
Takahashi et al.	190	1,392	140	2,457
Ishikawa et al.	46	1,005 (median)	450	1,875

Table 4. Steady-state plasma trough concentration (C_0) range at 400 mg of imatinib daily

6. Pharmacokinetics of imatinib

Imatinib is rapidly and completely absorbed because of an oral bioavailability of 98.3% (Peng, et al. 2004a). Moreover, it is extensively metabolized, with up to 80% of the administered dose recovered in feces as metabolites or unchanged drug (Gschwind, et al. 2005). The mean plasma half-life of imatinib is 13.5–18.2 h (Gschwind, et al. 2005; le Coutre, et al. 2004; Peng, et al. 2004b; Wang, et al. 2009). The cytochrome P450 (CYP) system is involved in the oxidative metabolism of imatinib, the major reaction being catalyzed by CYP3A4/5 (O'Brien, et al. 2003b; Peng, et al. 2005; van Erp, et al. 2007). Indeed, the main metabolite of imatinib, the *N*-desmethyl derivative CGP74588, is primarily formed in the liver by cytochrome CYP3A4, whereas a number of other enzymes such as CYP1A2, CYP2D6, CYP2C9, and CYP2C19 are involved in the formation of minor metabolites (O'Brien, et al. 2003b; van Erp, et al. 2007). CGP74588 represents approximately 20% of the parent drug plasma level in patients, and it has similar biological activity but a longer terminal half-life (85–95 h) than imatinib, as measured after discontinuation of therapy

Transporter	Polymorphism	N	Effects on PK	Effects on clinical response	Reference
P-glycoprotein *(ABCB1)*	3435 T	82	CL/F =	-	Gardner et al.
	3435 T	90	C_0 =	MMR =	Dulucq et al.
	3435 T	34	CL/F ↓	-	Yamakawa et al.
	3435 T	67	C_0 =	MMR =	Takahashi et al.
	3435 T	22	CL/F ↑	-	Gurney et al.
	3435 T	229	-	OS ↓	Kim et al.
	3435 T	52	-	Resistance ↑	Ni et al.
	3435 T	46	-	MMR, CMR ↓	Deenik et al.
	3435 CC	65	-	Failure ↑	Maffoli et al.
	1236 T	90	C_0 =	MMR ↑	Dulucq et al.
	1236 T	34	CL/F =	-	Yamakawa et al.
	1236 T	67	C_0 =	MMR =	Takahashi et al.
	1236 T	22	CL/F ↑	-	Gurney et al.
	1236 T	229	-	CCyR, MMR =	Kim et al.
	1236 T	52	-	Resistance ↑	Ni et al.
	1236 T	46	-	MMR, CMR ↓	Deenik et al.
	2677 T/A	90	C_0 =	MMR ↑	Dulucq et al.
	2677 T/A	34	CL/F =	-	Yamakawa et al.
	2677 T/A	67	C_0 =	MMR =	Takahashi et al.
	2677 T/A	22	CL/F ↑	-	Gurney et al.
	2677 T/A	229	-	CCyR, MMR =	Kim et al.
	2677 A	52	-	CCyR ↑	Ni et al.
	2677 T	46	-	CMR ↓	Deenik et al.
	TTT haplotype	90	C_0 ↑	MMR ↑	Dulucq et al.
	TTT haplotype	22	CL/F ↑	-	Gurney et al.
BCRP *(ABCG2)*	421 A	82	CL/F =	-	Gardner et al.
	421 A	34	CL/F =	-	Yamakawa et al.
	421 A	67	C_0 ↑	MMR =	Takahashi et al.
	421 A	46	CL/F ↓	-	Petain et al.
	421 A	229	-	MMR, CMR ↑	Kim et al.
OCT1 *(SLC22A1)*	480 G	229	-	Loss of response	Kim et al.
	480 G	67	C_0 =	MMR =	Takahashi et al.
	1022 T	67	C_0 =	MMR =	Takahashi et al.
	1022 T	34	CL/F =	-	Yamakawa et al.
	1222 G	67	C_0 =	MMR ↑	Takahashi et al.

Abbreviations: C_0, plasma trough concentration; CCyR, complete cytogenetic response; CL/F, clearance; CMR, complete molecular response; MMR, major molecular response; PK, pharmacokinetics

Table 5. Transporter polymorphism and effects on pharmacokinetics and the clinical response

(Gschwind, et al. 2005; le Coutre, et al. 2004). Imatinib is a substrate for P-glycoprotein, which is encoded by the *ABCB1* gene, and breast cancer-resistance protein (BCRP), which is encoded by the *ABCG2* gene (Burger and Nooter 2004; Burger, et al. 2004; Dohse, et al. 2010; Ozvegy-Laczka, et al. 2004). P-Glycoprotein is a membrane efflux transporter normally

expressed in the small intestine, biliary canalicular front of hepatocytes, and renal proximal tubules (Thiebaut, et al. 1987). BCRP is widely expressed in the small intestine, liver, and placenta (Hirano, et al. 2005; Zhang, et al. 2006). Imatinib and its metabolites are excreted predominantly via the biliary–fecal route by these ATP-binding cassette (ABC) efflux transporters, P-glycoprotein and BCRP. Imatinib is also a substrate of the uptake transporter OCT1, which is encoded by *SLC22A1* (Choi and Song 2008; White, et al. 2006). Because OCT1 is a highly expressed solute carrier in the basolateral membrane of hepatocytes, it facilitates the hepatocellular accumulation of imatinib before metabolism and biliary secretion. Further, it may play an important role in governing drug disposition and hepatotoxicity (Zhang, et al. 1998a; Zhang, et al. 1997; Zhang, et al. 1998b). One of the factors affecting interpatient variability could be polymorphism of drug transporters. However, the involvement of multiple transporters in imatinib pharmacokinetics hampers the investigation of imatinib transport mechanisms. Moreover, the level of drug transporter expression likely correlates with the intracellular imatinib concentration, because primary CML cells express the transporters on the cell surface (Burger, et al. 2005; White, et al. 2006).

7. Impact of pharmacogenetic variation of drug transporters

Pharmacogenetic research has focused on the interaction of imatinib with enzymes such as CYP3A4/5 and transporters such as P-glycoprotein, BCRP, and OCT1 (Table 5) (Deenik, et al. 2010; Dulucq and Krajinovic 2010; Gardner, et al. 2006; Kim, et al. 2009; Maffioli, et al. 2010; Ni, et al. 2011; Petain, et al. 2008; Takahashi, et al. 2010a; Yamakawa, et al. 2011).

7.1 CYP3A4/5

CYP3A4/5 expression is strongly correlated with a single-nucleotide polymorphism (SNP) in the gene (Hustert, et al. 2001; Rodriguez-Antona, et al. 2005). Nonetheless, *CYP3A4*1B* (-392A>G) and *CYP3A5*3* (6986A>G) had no significant influence on the plasma concentration of imatinib (Gardner, et al. 2006; Gurney, et al. 2007; Takahashi, et al. 2010a). A drug interaction occurs upon coadministration of imatinib and rifampicin or St. John's wort's CYP3A inducers, resulting in a decrease in the plasma concentration of imatinib (Bolton, et al. 2004; Smith, et al. 2004). In contrast, ketoconazole, a potent CYP3A4 inhibitor, significantly increased the C_{max} and AUC_{0-24} of imatinib (Dutreix, et al. 2004). However, the effects of *CYP3A4* and *CYP3A5* polymorphisms are less likely to be clinically significant in imatinib exposure.

7.2 P-Glycoprotein (*ABCB1*)

Gurney et al. (sample size = 22) reported that oral clearance of imatinib in patients receiving 600 mg of imatinib daily was significantly lower in those with the *ABCB1* 1236C/C, 2677G/G or 3435C/C genotypes than in those with the corresponding *ABCB1* 1236T/T, 2677T/T or 3435T/T genotypes (Gurney, et al. 2007). However, Gardner et al. (sample size = 82) reported that the *ABCB1* 3435C>T polymorphism had no significant effect on oral clearance of imatinib (Gardner, et al. 2006). In another study, Takahashi et al. (sample size = 62) reported that 1236C>T, 2677G>T/A, and 3435C>T polymorphisms had no significant effect on dose-adjusted imatinib C_0 (Takahashi, et al. 2010a). Although other studies have

reported the relationship between *ABCB1* polymorphisms and imatinib pharmacokinetics, or between *ABCB1* polymorphisms and clinical response, the results are still controversial (Table 5). However, the 3435T polymorphism, which is associated with low expression of P-glycoprotein, tends to correlate with poor clinical response. This finding suggests that P-glycoprotein is involved in imatinib pharmacokinetics to a greater extent than the intracellular imatinib concentration in primary CML cells.

7.3 BCRP (*ABCG2*)

Five studies have reported the *ABCG2* 421 polymorphism and imatinib pharmacokinetics or clinical response. Takahashi et al. (sample size = 62) reported that the dose-adjusted imatinib C_0 was significantly lower in Japanese patients with *ABCG2* 421C/C than in patients with C/A+A/A genotypes (Takahashi, et al. 2010a). In agreement, Petain *et al.* (sample size = 46) reported that imatinib clearance in patients carrying the *ABCG2* 421C/A genotype was significantly lower than in those with the 421C/C genotype (Petain, et al. 2008). Moreover, *ABCG2* 421A/A has a significant effect on achieving MMR/CCyR (sample size = 229) (Kim, et al. 2009). Because the 421C>A SNP of the *ABCG2* gene is associated with a higher imatinib exposure than is the wild-type genotype, CML patients with this SNP might more efficiently achieve molecular responses much more than their wild-type counterparts.

7.4 OCT1 (*SLC22A1*)

SLC22A1 (OCT1) expression levels likely correlate with the intracellular imatinib concentration, as primary CML cells expressing high levels of OCT1 have a greater drug uptake than those exhibiting more modest OCT1 expression (Thomas, et al. 2004; Wang, et al. 2008; White, et al. 2006). On the other hand, Kim et al. reported that the *SLC22A1* 480G/G genotype correlated with high rate of loss of response or treatment failure to imatinib therapy (Kim, et al. 2009). However, no association between dose-adjusted imatinib C_0 and *SLC22A1*156T>C, 480G>C, 1022C>T, or 1222A>G polymorphisms has been observed (Takahashi, et al. 2010a). The *SLC22A1* polymorphisms analyzed to date are therefore not important for imatinib exposure. OCT1 may contribute to the cellular uptake of imatinib rather than to imatinib exposure.

8. Pharmacokinetics of second-generation BCR-ABL inhibitors

Second-generation inhibitors, including nilotinib, dasatinib, and bosutinib, have been developed to counter imatinib resistances associated with BCR-ABL mutations, BCR-ABL gene amplification, increased efflux via ABC pump activation, or decreased influx via OCT1 activation. Nilotinib is a close structural analogue of imatinib with greater binding affinity and selectivity for the BCR-ABL kinase than imatinib. Dasatinib and bosutinib are dual ABL-SRC kinase inhibitors. All these second-generation inhibitors have been evaluated in clinical trials (Kantarjian, et al. 2010; Keller, et al. 2009; Saglio, et al. 2010), and nilotinib and dasatinib have already been approved in many countries for the treatment of patients with CML.

In pharmacokinetics studies with dasatinib (Christopher, et al. 2008), nilotinib (Tanaka, et al. 2010), or bosutinib (Abbas, et al. 2011), exposures (C_{max} and AUC) were shown to be linear and the dose proportional. C_{max} was observed at 0.5, 3, and 6 h after single oral

administration of each inhibitor, and a mean terminal elimination half-life ($t_{1/2}$) was <4, 17, and 32–39 h, respectively. Absorption was rapid for dasatinib and relatively slow for nilotinib and bosutinib. Similarly to imatinib, they are metabolized primarily by CYP3A4. However, unlike imatinib, nilotinib and dasatinib are not substrates for OCT1 transporter (Clark, et al. 2008; Giannoudis, et al. 2008; Hiwase, et al. 2008). Nilotinib and dasatinib are high-affinity substrates of BCRP and also interact with P-glycoprotein (Hiwase, et al. 2008). However, neither P-glycoprotein nor BCRP induce resistance to bosutinib (Hegedus, et al. 2009).

There are no published data on the relationship between drug plasma concentration and outcome or adverse events, and no clinically relevant data to suggest that dose changes are necessary based on sex, age, or pharmacokinetic differences that depend on the pharmacogenetic variation of drug transporters for second-generation inhibitors.

9. Therapeutic drug monitoring of imatinib for CML patients

Patients are more likely to achieve higher response rates with a satisfactory level of response if the 1,000 ng/mL drug plasma threshold considered as an adequate imatinib C_0 is achieved and maintained. Because the interpatient variation of imatinib levels is influenced by multiple factors, including genetic polymorphisms or coadministered drugs, a routine therapeutic drug monitoring (TDM) service for CML patients taking imatinib might be useful. According to the European Leukemia Net (ELN) recommendations (Baccarani, et al. 2009), the clinical response for CML patients receiving imatinib therapy should be evaluated at 3, 6, 12, and 18 months. In addition to *BCR–ABL* mutation analysis for CML patients, TDM could be also useful when making decisions related to imatinib therapy for patients not achieving CCyR or MMR at the above time points. If the target C_0 is not reached and no intolerance is found, dose escalation of imatinib is recommended. On the other hand, if the target is achieved but the patients lack a sufficient clinical response, imatinib could be withdrawn and replaced by a second-line tyrosine kinase inhibitor. Moreover, among the above-mentioned drug transporters, BCRP seems to most strongly influence imatinib exposure. We have reported that the daily dose of imatinib for patients with *ABCG2* 421C/C and 421C/A or 421A/A should be 400 mg and 300 mg, respectively, to attain the 1000 ng/mL drug plasma threshold (Takahashi and Miura 2011). If the ABCG2 421C>A polymorphism is detected before initiating therapy, dosing decisions may be improved to achieve optimal imatinib exposure immediately after intake. Further study is necessary to prospectively confirm the benefit of TDM of imatinib in the treatment and management of CML patients.

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5

Drug- Induced Pneumonitis: A Rare Complication of Imatinib Mesylate Therapy in Patients with Chronic Myeloid Leukemia

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1. Introduction

Therapy by drugs that block the activity of the protein Bcr-Abl, specific inhibitors of Bcr-Abl tyrosine kinase (TKI), significantly changed the prognosis of chronic myeloid leukemia (CML). Bcr-Abl gene is located on the Philadelphia chromosome (Ph'-chromosome), resulting from t(9;22) translocation, plays a key role in the onset and progression of CML. To date, the standard in the treatment of CML patients is imatinib mesylate (Gleevec, "Novartis Pharma AG", Switzerland). In addition, TKI 2nd generation, nilotinib and dasatinib, which differ in activity and impact points, also show encouraging results as first-line therapy of CML. According to an international multicenter study of IRIS (after 60 months of imatinib therapy) is shown that a complete hematologic remission was achieved in 96% of patients, major cytogenetic response - at 92%, complete cytogenetic response - 86% [1]. Imatinib treatment is well tolerated; treatment withdrawal because of intolerance is noted only in 5% of patients [2, 3]. The most frequent side effects are edema (peripheral edema, pleural or pericardial effusion, ascites, and pulmonary edema), rapid increase of body weight (independently from peripheral edema), nausea, vomiting, myalgia, muscle cramps, diarrhea, skin rash [4, 5].

Respiratory side effects of imatinib are rare. The most frequent among them are cough (9—22%), dyspnea (5—16%), flu-like syndrome (11,1%), upper respiratory tract infections (16,5%), pneumonia (1—10%) [4, 5]. Quite infrequent complications are pulmonary fibrosis and drug-induced pneumonitis [6].

We have some cases of such complications in available literature [7, 8, 9, 10, 11, 12]. Signs and symptoms of pneumonitis are similar: constitutional symptoms, malaise, low-grade fever, dyspnea (both exertional and at rest), cough, interstitial pulmonary infiltrates [13]. These symptoms are nonspecific and are often seen in other disorders. Rosado M.F. et al. have published one of the first case reports of imatinib-induced pneumonitis in 63 year-old woman with CML. At month 2 of imatinib treatment she has experienced dry cough and moderate exertional dyspnea. At 5th month of imatinib treatment both cough and dyspnea have worsened and hypoxemia was found (SaO_2 88%). The diagnosis was confirmed by results of CT scan and bronchoscopy with transbronchial needle aspiration, excluding bacterial, viral and fungal etiology of pneumonitis [8]. J.Rajda et al. have described drug-induced pneumonitis in 77 year-old woman with CML during first 4 weeks of imatinib

treatment. The progressive exertional dyspnea has led to nearly complete disability, where she could feel comfortable only at rest; later on a low-grade fever occurred. SaO_2 was 85% [13]. In other patients the disease manifestations, diagnostic approach and treatment were quite similar.

Although most cases of imatinib-induced pulmonary adverse events have been reported in patients with early chronic phase CML (from 0.2 to 1.3%). Dyspnea during imatinib therapy is most often related to fluid retention and pulmonary edema. Fluid retention may be due to prolonged platelet-derived growth factor inhibition by imatinib. Platelet-derived growth factor pathways are involved in the regulation of interstitial fluid homeostasis [16]. Imatinib pneumonitis develops in the period from 10 to 282 days (median time, 49 days) after treatment with imatinib (range, 200 to 600 mg daily). Dyspnea, hypoxemia and fever are usually seen. The chest CT scan shows diffuse or patchy ground-glass opacity, consolidation, or fine nodular opacity. The lung pathology may show interstitial pneumonitis and fibrosis, destruction of alveolar septa, lymphocytic alveolitis, plasma cell infiltrates, or type II pneumocyte hyperplasia. The resolution of pneumonitis after corticosteroid therapy has been reported. Ohnishi et al [16] reported that pneumonitis developed in 4 of 11 patients with a history of imatinib-induced pneumonitis after reexposure to imatinib [7, 13]. Diagnose lung disease caused by taking drugs is not always easy due to lack of specific clinical and morphological manifestations.

2. Clinical observations

In 2007—2008 in Hematology Research Center (Moscow, Russia) we have observed (including retrospective) in CML patients 4 cases of suspected imatinib-induced pneumonitis (Table 1). Three female and 1 male patients aged 13, 64, 66, and 40 correspondingly have initial diagnosis of chronic phase CML high-risk group, according to Sokal. The CML duration before imatinib treatment was 24, 11, 2, and 2.5 months and imatinib treatment duration – 73, 48, 7, and 13 months correspondingly. Pneumonitis has occurred at months 2, 13, 47 and 48 of imatinib treatment.

Patient	Gender	Age at diagnosis, yrs	CML diagnosis	Start of imatinib	Date of pneumonitis	Imatinib termination	Imatinib treatment duration before pneumonitis, months	Imatinib treatment duration, months
#1	F	13	XI/99	XI/2001	XII/2005	IX/2008	47	73
#2	F	64	XII/02	XI/2004	XI/2008	XI/2008	48	48
#3	F	66	I/08	III/2008	V/2008	XI/2008	2	7
#4	M	40	IX/06	XI/2008	III/2008	III/2008	13	13

Table 1. Characteristics of drug-induced pneumonitis patients.

Here are the case reports of our patients with imatinib-induced pneumonitis. In 2 patients it was revealed after a short, while in another two it was associated with prolonged imatinib treatment. In each case, describes the stages of diagnosis lung disease that emerged while taking imatinib (including retrospective), and treatment.

The first case of drug-induced pneumonitis, a 20 year-old woman, admitted to our center in November, 2007. At age of 13 (in November 1999) the patient was diagnosed with chronic

phase of CML, high-risk group by Sokal on the basis of leukocytosis (WBC 286×10^9/L) with prominent left shift of differential count with 15% blasts, hyperthrombocytosis 2279×10^9/L, spleen +8 cm below costal margin, liver +1 cm below costal margin. It should be noted that under the new WHO classification of 2008, the patient was in accelerated phase CML. The Ph'-chromosome was found in 100% metaphases. Within two years she was treated with combinations of low-doses ARA-C with hydroxyurea or alpha-interferon (IFN-α), and ARA-C + doxorubicin (7+3). The only result was temporary hematological response without a cytogenetic one. Moreover, the treatment was complicated by avascular necrosis of femoral head. Spleen began to increase gradually, up to 10% of blasts and basophiles (25% or more) was determined in peripheral blood . Since November, 2001 she was taking imatinib (400 mg daily) with restoration of complete hematological response. Despite the absence of cytogenetic response the imatinib dosage was increased to 600 mg daily only at month 32 of treatment. Since December, 2005 (after 11 months at imatinib, 600 mg qd) for another 11 months she also received treatment for suggested disseminated *tuberculosis* with infiltration and destruction, though it was not confirmed bacteriologically. At the same time the daily imatinib dosage was increased to 800 mg daily because of increasing platelet count (up to 1800×10^9/L). After that she became doing worse with prominent weakness and exertional dyspnea. She has undergone an additional evaluation at Institute of tuberculosis. The CT scan has found multiple confluent areas of alveolar consolidation. The revision of lung biopsy, performed during antituberculosis treatment, revealed mild lymphoid and histiocytic infiltration of bronchioles. She was supposed to have exogenic allergic alveolitis of unknown origin and treated with methylprednisolon (8 mg daily) since May, 2007 along with imatinib. Four months of such treatment has led to further deterioration. The development of pulmonary complications during prolonged high-dose imatinib treatment, ineffectiveness of both antituberculosis drugs and methylprednisolon has allowed suggesting imatinib-induced alveolitis. At September, 2007 imatinib treatment was stopped and the patient has lost hematological response (hyperthrombocytosis, elevating WBC count).

At admission to our center she was complaining of weakness, palpitations, dyspnea at minimal exertion, episodes of chest pain, sense of epigastral pressure, fever (up to $38,5^o$) during last 1—2 months, productive cough, pruritis in legs. At physical examination there was only low-grade fever and pigmentation. The chest was deformed because of scoliosis. Lung margins were normal, respiratory rate — 18 breaths per minute, on auscultation the expiratory prolongation (predominantly left-sided) with basilar rales were heard. Heart rate was about 120 bpm, liver and spleen were not enlarged.

The hematological and cytogenetic resistance to imatinib treatment along with suggested non-hematological toxicity (alveolitis) necessitated the additional evaluation and moving the patient to 2^{nd} line TKIs.

The CT scan revealed prominent diffuse bilateral interstitial lung infiltration with honeycomb appearance in upper and middle parts of lungs, along with the focus of lung consolidation in paravertebral part of right S10 without effusion (Figure 1). This was considered as non-specific interstitial pneumonia with supervening infection at right S10.

The bronchoscopy data was normal. The study of bronchoalveolar lavage excluded bacterial overgrowth, PCR analysis has revealed the cytomegalovirus DNA (DNA-CMV). The lavage sediment contained 49% alveolar macrophages, 33% segmented neutrophils, 7% eosinophils, and 11% lymphocytes.

She has undergone the chemotherapy cycle (5+2) along with antibacterial and antiviral drugs; corticosteroids were stopped. The tachycardia (up to 150 bpm), probably due to steroid cardiomyopathy and febrile neutropenia episodes, necessitated the usage of beta-1 blockers (atenolol).

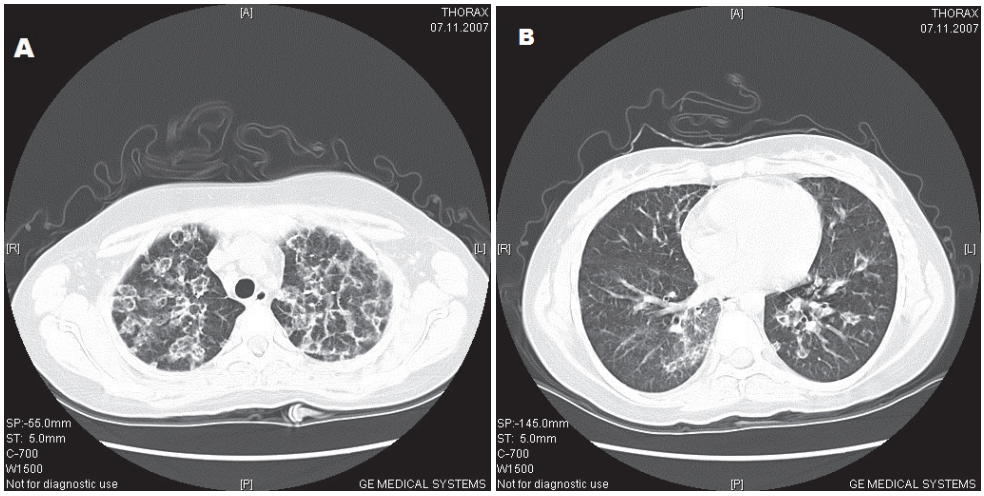

Fig. 1. A, B. Chest CT scan of patient #1 (A — middle, B — lower parts): non-specific interstitial pneumonia with supervened infection.

The serial CT scan showed some regression of interstital pneumonia with retraction of alveolar infiltration area (against the withdrawal of imatinib). But the infiltrative focus in right lower lobe has enlarged and pleural involvement appeared. The serum galactomannan level was increased to 2,55 ng/ml (normal < 0,5 ng/ml), permitting to diagnose invasive aspergillosis. In addition, the repetitive lavage evaluation has revealed Enterococcus spp. growth and HSV-1,2 DNA. We have added antifungal drugs (Amphotericin-B) and modified both antibacterial and antiviral therapy. Thus, the patient noted the multiple pulmonary pathology, which complicates the course of the underlying disease. Therapy has improved the medical condition of the patient: the fever became sub febrile and only few rales could be heard. Since February, 2008 she started treatment with 2nd generation TKI, dasatinib.

At control evaluation (December, 2008) the medical condition of patient was good and stable without any fever. Dasatinib therapy allowed achieving not only complete hematological, but also minor cytogenetic response. The control CT scan picture has shown major improvement with virtually complete regression of interstitial lung infiltration (Fig. 2).

This case demonstrates the development of complex pulmonary disease: **a retrospectively** revealed imatinib-induced pneumonitis after prolonged therapy with imatinib mesylate. Rapid worsening at increased dose of imatinib prompted to reevaluate the previous diagnosis of tuberculosis and to stop imatinib because of suggested exogenic allergic alveolitis. After the resolution of supervened severe pleuropneumonia mixed etiology and lung aspergillosis we became able to start dasatinib treatment with stabilization and improvement of both lung pathology and CML response.

In patient #2, 64 year-old woman, CML chronic phase (high-risk group) was diagnosed in December, 2002. At that moment she was complaining of malaise. There was splenomegaly (+6 cm below costal margin), liver size was normal. The peripheral blood analysis presented hyperleukocytosis (117×10^9/L), moderate thrombocytosis (669×10^9/L), left shift of differential count. Bone marrow aspirate was hypercellular, karyological examination revealed Ph-chromosome in 100% metaphases. In December, 2002 — November, 2004 she was treated with IFN-α, but achieved only hematological response without cytogenetic one. The next 2 years she was receiving imatinib, 400 mg daily, but didn't achieve major cytogenetic response. Since January, 2007 its dose was increased to 600 mg daily. In August, 2008 she was evaluated for dry cough and dyspnea. There were no rales, or prominent tachypnea (respiration rate 20 per minute) and X-ray didn't find any abnormalities, but persistent complaints have prompted to suspect an imatinib side effect. In November, 2008 the TKI treatment was interrupted.

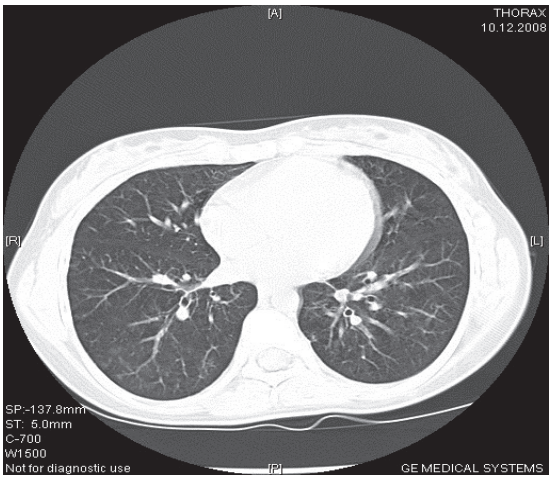

Fig. 2. Control CT scan of patient #1. Nearly complete regression interstitial lung infiltration.

The patient was reevaluated in specialized pulmonologic center. The auscultation revealed basilar crepitation with respiratory rate 22 per minute and SaO_2 97%. Pulmonary function tests showed airflow obstruction (isolated decrease of expiratory flow in distant airways). Static lung volumes were normal and diffusion capacity was moderately decreased. The lung biopsy was not performed. The chest CT scan found interstitial abnormalities with "ground glass" appearance. This data allowed to suggesting drug-induced pneumonitis.

An 8-week prednisolone therapy (25 mg daily) and imatinib interruption has led to significant improvement with resolution of both complaints and CT scan abnormalities.

Taking into account the imatinib intolerance (non-hematological toxicity grade 3) and primary cytogenetic resistance, we decided to begin treatment with 2nd generation TKI, nilotinib, 800 mg daily. The nilotinib treatment duration has reached now 24 months. She has achieved a major cytogenetic response (14% bcr/abl-positive cells by FISH). The complaints are absent.

The previous experience helped us to suspect the association of drug-induced pulmonitis with imatinib treatment. Prompt patient evaluation, interruption of imatinib, and quick

response to prednisolone allowed suggesting the development of such a rare complication. Notably, the response to nilotinib underlines the importance of early beginning of 2nd generation TKI treatment in case of primary cytogenetic resistance and imatinib intolerance.
Patient #3, 66 year-old woman, was admitted to Hematological Research Center in June, 2008. The diagnosis of high-risk chronic phase CML was established in January, 2008. At diagnosis peripheral blood analysis showed high WBC count (113×10^9/L) with left-shifted differential count (blasts — 1%, myelocytes — 11%, metamyelocytes — 6,5%, bands — 24%, segmented — 33,5%, basophils — 8%, eosinophils — 5%, platelets — 849×10^9/L, Hb 103 g/L). Bone marrow smear showed granulocytic predominance with Ph-chromosome in 98% metaphases. The spleen was +5 cm below costal margin. She also suffered from arterial hypertension, treated with lisinopril. Two months after diagnosis (since 01.03.2008) she began receiving imatinib at standard dose of 400 mg qd. But 2 weeks later she began doing worse and malaise, low-grade fever and progressive dyspnea appeared. Lung auscultation revealed expiratory prolongation without rales. Hemodinamically she was stable. Chest X-ray revealed diffuse interstitial process, confirmed later by CT scan.
The CT scan (Fig. 3) shows diffuse increase of pulmonary vascularity with its deformation by infiltration of intralobular paraseptal interstitium. There were also symmetrical areas of decreased pneumatization with "ground glass" appearance, predominantly in central parts of both lungs. Pleural and pericardial effusions were absent.

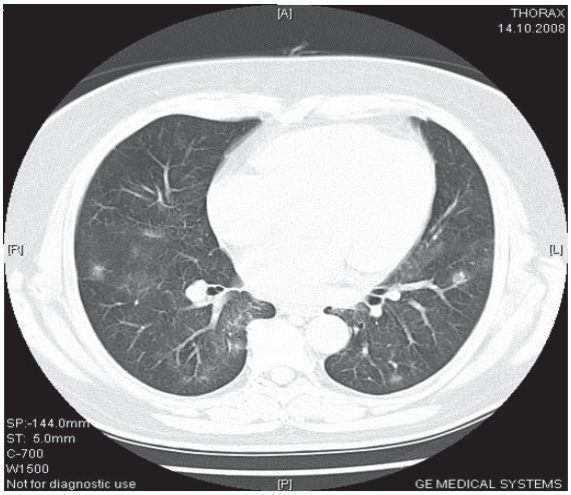

Fig. 3. Chest CT scan with signs of interstitial lung disease (patient #3).

The clinical presentation and CT scan data permitted to suspect these abnormalities to be an imatinib-induced pneumonitis (non-hematological toxicity grade 2). Imatinib was discontinued (since 01.05.2008), and corticosteroids (methylprednisolone, 40 mg daily for 20 days with gradual dose tapering) were started. This therapy has led to significant dyspnea improvement with normalization of temperature and auscultatory findings. CT scan showed the same "ground glass" focuses, but their intensity has decreased. The complete resolution of signs and symptoms with delayed resolution of radiological findings after imatinib discontinuation and corticosteroid treatment confirmed the

suggested association of pneumonitis with imatinib treatment. During the treatment interruption (40 days) she has lost the hematological response (WBC — 25×10^9/L, platelets — 742×10^9/L). The patient continued treatment with imatinib in decreased dose (300 mg daily) and hypotensive treatment with lisinopril. Two weeks later the hematological response restored. The control caryological examination after 3 months of treatment has confirmed a major cytogenetic response (25% Ph+ metaphases). But 2 weeks after the treatment was resumed the dyspnea had relapsed, while control CT scan revealed deterioration (marked increase in size and intensity of previously seen "ground glass" focuses of interstitial infiltration). The increased vascularity was still remaining, along with paraseptal and interlobular interstitial infiltration. These findings once more confirmed the association of pneumonitis with CML treatment. After the imatinib discontinuation she has undergone 16-day methylprednisolone therapy (40 mg daily with gradual tapering). The treatment was discontinued and the hematological response was lost again, necessitating the hydroxyurea treatment (3 g daily).

During 6 months of imatinib treatment this patient achieved optimal response, but it was lost shortly after the treatment interruption (due to toxicity). The non-hematological toxicity (imatinib-induced pneumonitis grade 2), necessitated the treatment swapping to 2nd generation TKIs — nilotinib or dasatinib.

Nilotinib (Tasigna, AMN107, Novartis Pharma AG) is a structural analogue of imatinib. As well as imatinib, it binds ABL-tyrosine kinase in inactive conformation, but is 25—30 times more potent in vitro and active against the majority of its mutated forms (except T315I). Notably it has no cross-resistance with imatinib. Dasatinib (Sprycel, BMS-354825, Bristol Myers Squibb) structurally differs from imatinib. It binds ABL-tyrosine kinase in active conformation and also inhibits SRC-kinases superfamily; by vitro activity it is 300 times more potent, than imatinib and is active against all known mutated forms, except T315I. However, dasatinib should be used cautiously in hypertensive patients. The coexistent arterial hypertension in our patient led us to prefer nilotinib.

At the moment of nilotinib treatment Ph'-chromosome was found in 100% metaphases and hematological response was absent too. Now the nilotinib treatment duration is 24 months; she has achieved stable hematological, complete cytogenetic and major molecular response. The control CT scan revealed nearly complete resolution of interstitial lung infiltration and the lung vascularity merely returned to normal pattern. No treatment interruptions needed and the patient noted good drug tolerability.

Concerning the last case (#4) of 40 year-old male we have only a brief information. The diagnosis of chronic phase CML, high-risk group by Sokal was established in September, 2006 (neutrophilic leukocytosis with left-shifted of differential count, hyperthrombocytosis up to 2400×10^9/L and Ph'-chromosome by karyological examination). Two months later he began successfully taking imatinib (400 mg daily) for a year without side effects. However, in February, 2008 (13th month of treatment) low-grade fever and exertional dyspnea occurred. The evaluation allowed excluding infections. The CT scan has revealed "ground glass" lung abnormalities, with increased and deformed lung vascularity. The patient refused from further evaluation and treatment in specialized center.

This case report is not representative for imatinib-induced pneumonitis. But our goal is to attract attention of physicians to be aware of typical complaints of patients with this pathology and diagnose it earlier.

3. Discussion

The above-mentioned rare disease belongs to a group of interstitial lung diseases, including now more than 150 entries. Despite the etiological differences, including the diseases of unknown origin, all of them involve lung interstitium and its vasculature. This feature underlies their clinical, radiological and pathological similarity.

According to CTC, both interstitial pneumonia and pneumonitis have 5 grades of severity. Grade 1 is asymptomatic and is revealed only radiologically (X-ray, CT scan). Grade 2 is characterized by signs and symptoms, not interfering activities of daily living. Typical clinical presentation along with disturbances of gaseous exchange and activities of daily living indicates a grade 3 pneumonitis. Grade 4 is a life-threatening condition with a need for respiratory support, and death from pneumonitis is considered as 5 grade of toxicity [14].

The pathological examination typically reveals both inflammatory infiltrates and areas of fibrosis. Radiological findings can't be used for differential diagnosis, because they lack specificity and are not only shared within this group, but also could be seen in non-related diseases. **The high-resolution CT scan is more specific and is most useful in serial examinations**. **Equally important in differential diagnosis are arterial blood gas examination and bronchoscopy with transbronchial biopsies (4—6 specimens) and lavage.** The obtained material should undergo bacteriological, virological and immunological examination.

The mechanism of interstitial lung diseases is complex and has a number of distinctive features. It is thought to be a result of immune complex-mediated reaction with the principal role of T cells and cytokines. The alteration of alveolocytes leads to acute alveolitis. If it doesn't resolve, the inflammation extends to interstitium and its capillaries, leading to pneumosclerosis, alveolar deformation and disturbances of lung diffusion capacity [15].

The treatment of drug-induced pneumonitis consists of avoidance of allergen (here — imatinib) and prednisolone, 1 mg/kg body weight daily, for 2—4 weeks. The dose is then tapered to minimal, sufficient for good results of pulmonary function tests. If contact with allergen is avoided there is no need for continuous corticosteroid treatment [15].

Drug-induced pneumonitis is a rare complication of imatinib treatment in CML patients. In two cases it has developed more than 2 year after the beginning of treatment, despite its good tolerance earlier. In other cases it was revealed at first months of therapy, when good compliance is especially needed for achieving of optimal response. Unfortunately, the complete evaluation (bronchoscopy with transbronchial biopsy and lavage, pulmonary function tests, arterial blood gas examination) was not done in all patients at the time of pneumonitis which is associated with the rare detection of such cases, and often the unwillingness of patients carrying invasive research methods. For example, bronchoscopy with transbronchial biopsy and lavage were performed only in patient #1, the study of blood gases and pulmonary function tests in patient #2. Patients receiving outpatient treatment are often reluctant to conduct invasive research methods. However, the problem of doctors in clinical practice is, in particular, in explaining the importance and need for a comprehensive survey of every obscure case. Its data could elucidate the mechanism of drug-induced pneumonitis development. All of our patients had serial CT scan examination (with the identification of the characteristic CT picture of "ground glass"), the association of interstitial lung disease with imatinib treatment is followed up, and the effectiveness of corticosteroids is estimated.

4. Conclusion

These case reports illustrate the importance of search for signs of toxicity at different phases of treatment. Careful searching for adverse events of imatinib in CML patients and differential diagnosis with similar diseases allows prompt diagnosis and treatment of both frequent and rare side effects, including those not in onco- hematology specialist clinics, but also in clinical practice. Proper changing of treatment strategy can help to avoid frequent and prolonged interruptions of treatment due to toxicity and increase the efficacy of treatment, permitting to achieve optimal results.

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6

Towards the Cure of CML by the Molecular Approach Strategy

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1. Introduction

Chronic myeloid leukaemia (CML) is a hematopoietic stem cell (HSC) disorder accounting for about 15-20% of all leukemias of the adult (Goldman & Melo, 2003; Black et al., 1997). The main haematological features are represented by an increase in the number of circulating mature granulocytes and their precursors and, subsequently, by a secondary evolution in acute leukaemia.

In 1960, a major clue to the cause of CML was provided by Nowell and Hungerford who for the first time described an unusual small chromosome present in leukocytes from patients with this hematologic malignance (Nowell & Hungerford, 1960). This "minute chromosome" abnormality, designed as the Philadelphia (Ph) chromosome, after the city in which it was discovered, was found in all malignant cells of CML patients and is now considered the hallmark of this neoplasia (Nowell & Hungerford, 1960). Importantly this discovery was the first demonstration of a chromosomal rearrangement linked to a specific cancer, and had sparked searches for associations of additional chromosomal aberrations with specific forms of cancer. In 1973, Rowley demonstrated that the Ph chromosome resulted from a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9:22)(q34;q11) (Rowley, 1973). Later it was shown that this process fuses the c-ABL (human homologue of the Abelson Murine Leukaemia virus), a tyrosine kinase encoding oncogene on chromosome 9, and BCR (Breakpoint Cluster Region), on chromosome 22, the function of which is still not clear (Groffen et al., 1984). This balanced translocation leads to a fusion gene, the product of which is a chimeric BCR-ABL protein equipped with cellular transforming ability which is ascribed to the elevated tyrosine kinase (TK) activity of the molecule compared to the native c-ABL (Konopka et al., 1984; Daley et al., 1990).

The biochemical signal transduction pathways stimulated by BCR-ABL kinase activity are responsible for Ph+ CML oncogenesis (Ren, 2005; Calabretta & Perrotti, 2004; Krebs & Hilton, 2001; Neshat et al., 2000; Sattler et al., 2002; Sattler et al., 1999).

Further studies have established BCR-ABL as a leukaemogenic oncogene since both mouse models and in vitro assays have shown that BCR-ABL, is able to induce leukaemia (Daley & Baltimore, 1988).

2. Molecular mechanisms of BCR-ABL

Several BCR-ABL isoforms with different molecular weights have been reported (Melo & Deininger, 2004). Accordingly, while in all chimeric proteins the breakpoint within ABL gene is consistently located upstream of exon 2 (a2), the breakpoint in the BCR gene varies in its localization (Melo, 1996). A major breakpoint cluster region (M-bcr) and a minor breakpoint cluster region (m-bcr) have been defined (Kurzrock et al., 1988).
The M-bcr maps to a 5.8 Kilobase (Kb) area spanning exons 12 through 16. The resulting fusion transcripts with ABL generate a 210-kDa protein named p210 which is the most common BCR-ABL form, being observed in 99% of the CML patients and in one-third of Ph-positive B cell acute lymphoblastic leukaemia (Ph+ B-ALL) (Faderl et al., 1999). m-bcr localizes to a 54.4-kb area sited downstream of exon 1. It gives rise to a fusion transcript with ABL named p190. It is rarely observed in CML, but is the most frequent BCR-ABL isoform in Ph+ B-ALL. Finally, 3' breakpoints downstream of BCR exon 19 have also been described and they give rise to a 230-kDa fusion protein (p230 BCR-ABL), which is typically found in the rare chronic neutrophilic leukaemia (CNL) (Pane et al., 1996).
All three BCR-ABL fusion protein variants induce a similar CML-like syndrome in mice, but differ in their ability to induce lymphoid leukaemia (Li et al., 1999).

3. Cellular pathways involved in oncogenic BCR-ABL signalling

The oncogenic potential of BCR-ABL derives from its capacity to activate intracellular signalling cascades that lead to uncontrolled cell proliferation, altered cell adhesion, and apoptosis inhibition (Daley et al., 1990; Kelliher et al., 1990). To date several signalling pathways affected by the constitutively active BCR-ABL have been identified, as well as numerous binding partners and substrates that provide a link between this pathways and the defects that characterize CML. Increased susceptibility to proliferate derives from BCR-ABL's capacity to activate the RAS-mitogen activated protein (MAP) kinase signalling cascade and JAK/STAT signalling; the interaction with SRC is responsible for increased cell motility; resistance to apoptosis is thought to result from BCR-ABL-mediated activation of phosphatidylinositol- 3-phosphate kinase (PI3K) and thereby of AKT. In summary, the net effects of these molecular alterations include inhibition of apoptosis, increased cell proliferation, aberrant interaction with the bone marrow stroma and genetic instability. Importantly all these events drive disease progression (Deninger et al., 2000).
Consistent with these molecular sequelae, BCR-ABL was shown to transform hematopoietic progenitor cells in vitro and in vivo studies (Kantarjian et al., 2006; Hehlmann et al., 2007). Recent reports identified a role for other signalling cascades in CML biology, including Hedgehog, Wnt and Ikaros, suggesting that pharmacological inhibitors of these pathways may find application in the treatment of CML (Chen Zhao et al., 2009; Dierks et al., 2008; Mullighan & Dowing, 2008; Dierks et al., 2008). Finally, also micro RNA (miRNA) regulation appears to apply to CML biology since miR-203, which would normally suppress BCR-ABL expression, is either mutated or epigenetically silenced in CML. In the latter type of condition, demethylating drugs such as 5-azacytidine and 4-phenylbutyrate were shown to restore miR-203 and to thereby decrease BCR-ABL expression and proliferation rate of Ph+ human CML cell lines (Faber et al., 2008; Croce & Calin, 2005).

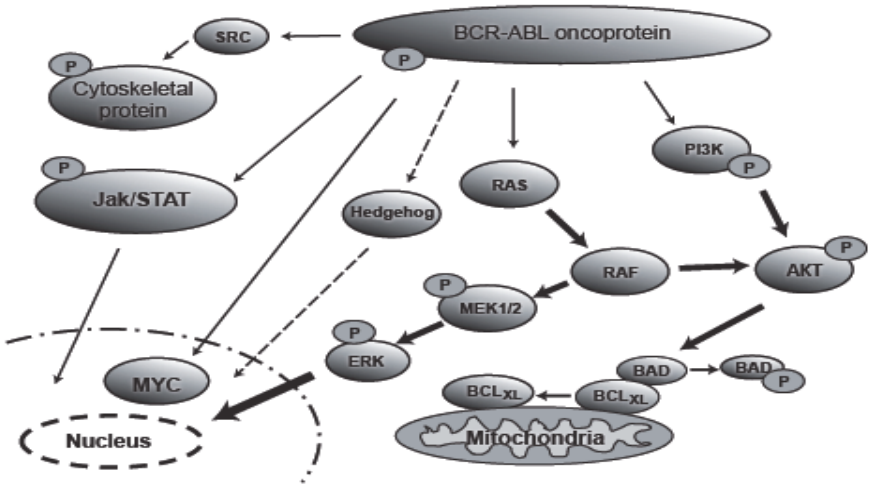

Fig. 2. Schematic view of the signal transduction pathways in cells transformed by BCR-ABL.

4. The CML leukaemia stem cell

Increasing evidence suggests that only a rare subset of immature cells within the tumor, named "leukaemia stem cells" (LSC), are able to propagate the CML (Reya et al, 2001). This cell has many common features with the hematopoietic stem cells - such as self-renewal and pluripotency pensions- unlike these, however, are refractory to conventional chemotherapy. Despite the remarkable improvements in the treatment of CML, the TKIs treatment is not curative, suppresses the disease but is not able to eradicate the CML Achilles hell, the leukaemia stem cell, causing recurrence of disease (Graham et al., 2002; Copland et al., 2006) The relapses in CML are thought to result from the outgrowth of quiescent LSC therapy-resistant, as the majority of leukemic cells in relapses represent (sub-) clones already present at diagnosis. To date the only long-term, sustainable remission derives from allogeneic bone marrow/peripheral blood stem cell transplantation which successfully restores normal hematopoiesis (Michor et al., 2005; Ljungman et al., 2009).

Recent data suggest that aberrant self-renewal is one of the central mechanisms underlying the pathogenesis of chronic myeloid leukaemia - acting either at the level of the BCR-ABL positive pluripotential stem cell in chronic phase or at the level of a more differentiated progenitor to cause blastic transformation, or most probably at both levels. Excessive self-renewal of LSCs may be mediated via several developmental pathways, including the Wnt/Frizzled/beta-catenin and Musashi-Numb pathway, or TWIST-1 oncogene and Polycomb-group protein BMI-1 (Hu et al., 2009; Ito et al., 2010; Cosset et al., 2011). An additional candidate is the Smoothened (SMO)/Sonic Hedgehog (sHH) signalling pathway, which is reasonably well characterised in solid tumours but is less well studied in leukaemia (Dierks et al., 2008; Chen Zhao et al., 2009;). Particularly it is essential during embryonic development, and might play a key role in human malignancies when aberrantly activated.

5. CML treatment options

The definition of the molecular structure of BCR-ABL tyrosine kinase domain has led to development of potent and specific tyrosine kinase inhibitor (TKIs) (Druker, 2008; Johnson et al., 2003). TKIs such as imatinib mesylate (Gleevec™, Novartis), nilotinib (Tasigna™, Novartis) and dasatinib (Sprycell™, Bristol-Myers Squibb) induce apoptosis in CML but not in healthy tissues, which is thought to result from addiction of CML cells to BCR-ABL signalling. The use of TKIs has led to remarkable improvements in disease outcome, in turn making TKIs the gold standard front line CML therapeutics. Importantly, although TKIs do induce disease remissions in most CML patients, they are not curative because of their incapacity to eradicate CML-LSC. Moreover, acquired resistance to TKIs is commonly observed and requires the prompt introduction of other TKIs that retain activity against BCR-ABL (Talpaz et al., 2002; Sawyers et al., 2002). Therefore, a timely and accurate follow-up is crucial for the management of CML and for effective therapeutic decisions (Druker et al., 2006; Kantarjian et al., 2008; O`Brien et al., 2003; Lahaye et al., 2005; Cervantes et al., 2003; Branford et al., 2003; Hughes & Branford, 2006). Additionally, such relapses are thought to result from the activation and proliferation of otherwise quiescent and therapy-resistant LSCs (Graham et al., 2002; Copland et al., 2006). Newer molecular therapies are being developed to eradicate the LSC pool by targeting critical signaling molecules that are essential for LSC maintenance.

6. CML monitoring

The remarkable progress in the treatment of CML over the past decade has been accompanied by steady improvements in our ability to accurately and sensitively monitor the status of the disease with the use of molecular markers, aimed at recognizing the depth of remission, and by use of readings to guide the choice of strategy for therapeutic interventions (Hughes et al., 2006).

However, the identification of patients that will experience a failure of TKI treatment, and appropriately altering the therapeutic strategy based on such monitoring, remains a challenge.

Routine CML diagnostics largely relies nowadays on traditional blood cell count, cytogenetic analysis (standard karyotype with or without fluorescence in situ hybridization-FISH), and real time quantitative polymerase chain reaction (RT-Q-PCR) for BCR-ABL messenger RNA (mRNA). These tests allow defining the haematological, cytogenetic, and molecular response to treatment, respectively (Kantarjian et al., 2008; Hughes et al., 2006). The haematological response to treatment is assessed by peripheral blood cell counts and by spleen size, and is classified as:

1. *Complete haematological response (CHR):* normalization of peripheral blood counts with no immature blood cells and with disappearance of any sign of disease
2. *Partial haematological response (PHR):* presence of immature blood cells and/or persistent splenomegaly. The next level of response is the cytogenetic one (CyR), defined as a decrease in the number of Ph+metaphases in a bone marrow aspirate (using ≥ 20 metaphases). This is categorized as:

1. Complete cytogenetic response (CCyR): 0% Ph+ metaphases
2. Partial cytogenetic response (PCyR): 1-35% Ph+ metaphases
3. *Minor cytogenetic response*: 36-65% Ph+ metaphases

4. Minimal cytogenetic response: 66-95% Ph+ metaphases

CCyR or PCyR configure a major cytogenetic response (MCyR). Finally, residual leukaemia cells (minimal residual disease, MRD) can be detected using RT-Q-PCR. Particularly, the molecular response is defined as a decrease of the BCR-ABL to control gene transcript ratio according to the International Scale (IS) (see below):

1. *Complete molecular response (CMR):* undetectable level of chimeric transcript
2. *Major molecular response (MMR):* reduction in transcript levels of at least 3-log from standard baseline level (which represent 100% on the International Scale) or ≤1%.

6.1 Cytogenetic and FISH

The Ph chromosome can be detected by standard cytogenetic techniques in the vast majority of patients (Osarogiagbon, 1999). In patients who are cytogenetically Ph chromosome negative (Ph–), molecular techniques such as FISH and RT-Q-PCR may be useful in detecting BCR-ABL. Cytogenetic analysis is typically performed by chromosome banding of at least 20 bone marrow cells in metaphase allowing to identify the t(9:22) translocation (Haferlach et al., 2007). In addition, cytogenetic also allows to define any additional chromosomal abnormality (i.e. additional Ph chromosome, isochromosome 17q, trisomy 8, or trisomy 19), thereby providing additional prognostic information. Baccarani et al. recommend that, at diagnosis, two cytogenetic analyses are performed in order to increase the sensitivity of the method. Furthermore, if less than 20 metaphases are visualized, the cytogenetic analysis should be validated by FISH or by RT-Q-PCR (see below) (Baccarani et al., 2008). Importantly, in 5% of CML cases no cytogenetically-detectable Ph chromosome can be demonstrated, since the BCR-ABL fusion oncogene derives from a submicroscopic genetic fusion. In these cases, FISH or RT-Q-PCR will demonstrate the presence of the specific genetic abnormality. Traditional FISH uses 5′ BCR and 3′ ABL fluorescent probes of different colours while more recent FISH reagents use 3-4 probes (D-FISH). Such probes can detect the variant translocations leading to Ph chromosome formation and are also associated with low false positive rates (Dewald et al., 1998; Wang et al., 2001; Landstrom & Tefferi, 2006; Sinclair et al., 1997; Seong et al., 1995). Interphase or hypermetaphase FISH can be performed on peripheral blood specimen or bone marrow aspirates, respectively. Interphase FISH is applicable to a larger population of cells since does not require cycling cells. On the other hand, this technique is associated with a background signal greater than 1-5% (depending on the specific probe used in the assay) (Cuneo et al., 1998; Le Gouill et al., 2000; Lesser et al., 2002; Raanani et al., 2004). Hypermetaphase FISH is applicable only to dividing bone marrow cells (Schoch et al., 2002). This approach is more sensitive and can analyze up to 500 metaphases at a time. Usually, FISH results correlate with traditional cytogenetic analysis and with RT-Q-PCR results, thus remaining a convenient and sensitive diagnostic tool (see below).

6.2 PCR-based approaches to CML monitoring

Nested reverse transcriptase PCR can detect one CML cell in a background of ≥ 100.000 normal cells (Martinelli et al., 2006). However, it remains a purely qualitative assay which is only capable of demonstrating the presence or absence of CML cells. Nested-PCR is normally only used to confirm the achievement of CMR. RT-Q-PCR methods are less sensitive than qualitative PCR (by 0.5-1 order of magnitude) but they have the advantage of determining the actual percentage of BCR-ABL transcripts and can therefore be used to

track changes in the number of leukemic cells over time (Lowemberg, 2003; Hughes et al., 2003; Merante et al., 2005; Mauro et al., 2004; Cortes et al., 2004). Currently, RT-QPCR for BCR-ABL is the recommended approach for routine follow-up of CML patients and is considered the gold standard test for routine therapeutics decision. The BCR-ABL transcript levels are expressed as a percentage ratio of BCR-ABL compared to ABL transcripts. ABL acts as control gene to compensate for variations in the quality of the RNA and for differences in the efficiency of the reverse transcription reaction. The last years have seen numerous efforts to standardize the molecular approaches to CML monitoring as well as their interpretation criteria. In order to harmonize the results across laboratories worldwide, a standard pre-treatment baseline value for each laboratory was established. Thus, a molecular response is defined by reductions from an absolute baseline (common to all) rather than a relative baseline (individualized). This ensures that patients with the same level of response have the same degree of residual disease. Additionally, under- or over-estimation of the extent of response due to individual variations is avoided by using a common standard baseline. According to the international reporting scale (IS) the absolute BCR-ABL value to define major molecular response is standardized at 0.1% (or 3 log) reduction from the laboratory-specific pretreatment standard baseline (Hochhaus & Dreyling, 2008; Hochhaus et al., 1996). A value of 1.0% is approximately equivalent to the achievement of a CCyR and a CMR is achieved when transcripts are undetectable (Branford et al., 2006; Muller et al., 2007, 2008). Because of its high sensitivity, CML monitoring by RT-Q-PCR enables to define an early loss of response once CCyR has been achieved (Wang, 2000, Press et al., 2006). Additionally, early molecular monitoring after initiation of treatment helps to identify patients at higher risk of relapse after pharmacological treatment onset as well as after allogeneic bone marrow transplantation (Olavarria et al. 2002; Lange et al., 2004; Asnafi et al., 2006). Finally, another advantage of CML monitoring by RT-Q-PCR is the feasibility of this method on peripheral blood samples. In a large cohort of patients monitored to BCR-ABL mRNA levels after allogeneic bone marrow transplantation, we found that peripheral blood and bone marrow samples perform equally well in terms of sensitivity in relapse detection and show a very good correlation of results. Thus, molecular monitoring of CML with RT-Q-PCR can be performed using peripheral blood samples instead of bone marrow (Ballestrero et al., 2009). The drawbacks of this method include a substantial incidence of false negative tests, which on the other hand, is strongly reduced when serial evaluations are performed. Nowadays, RT-Q-PCR monitoring is included as integral part of the management of CML patient treated with TKIs and must be performed every 3 months even in patients in MMR. An increase in BCR-ABL levels of 2 to 5 fold is an early sign of relapse, and suggests the need to switch to another type of treatment as soon as possible.

6.3 Mechanisms of resistance

A growing problem in the treatment of CML is resistance to treatment since most patients in chronic phase initially respond to TKIs but subsequently relapse and/or progress to accelerated phase or blast crisis (Talpaz et al., 2002; Sawyers et al., 2002). Primary resistance or, perhaps more appropriately, primary refractoriness (typically BCR-ABL independent), is defined as the failure to achieve initial response to therapy and is only seen in approximately 5% of newly diagnosed patients in chronic phase of CML. (Apperley, 2007) Acquired resistance, defined as the loss of previous response, is more common. About 10-

15% of patients in TKIs treatment develop treatment failure at a rate of approximately of 1-4%/year). Resistance to TKIs may be primary or secondary and is usually classified in BCR-ABL-dependent or -independent. The BCR-ABL-dependent mechanisms include reactivation of BCR-ABL signaling through mutations in the ABL kinase domain (KD), and increased production of BCR-ABL at the genomic (gene amplification) or transcript (overexpression) levels (Campbel et al., 2002, Morel et al., 2003; Hochhaus et al., 2002). Conversely, BCR-ABL independent resistance mechanisms involve: i) a drop in the intracellular drug concentration through expression of drug efflux (such as multidrug-resistant P-glycoprotein MDR-1) (Mahon et al., 2000; le Coutre et al., 2000) or drug influx (such as hOCT1 that affects intracellular drug availability) (Thomas et al., 2004) genes; ii) activation of Src family of kinases (SFKs); and iii) acquisition of additional chromosomal abnormalities with Ph-chromosome (O'Dwyer et al., 2002, 2004; Schoch et al., 2003). Although gene amplification occurs more frequently than point mutations (10^{-4} per cell division vs. 10^{-9}(Hochhaus A et al., 2002) clinical resistance is much more likely to be due to a point mutation in the BCR-ABL TK domain than to BCR-ABL amplification (Willis et al., 2005). To date more than 50 mutations have been identified, each of which arises at variable frequencies and with different consequences (Jabbour et al., 2006; Shah et al., 2002; Branford et al., 2002; Hofmann et al., 2002; Roche-Lestienne et al., 2002; Deninger et al., 2000; Soverini et al., 2004, 2005; Chu et al., 2005; Nicolini et al., 2006; Barthe et al., 2002; Irving et al., 2004; Wei et al., 2006; Wang et al., 2006). Mutations may occur in various ATP-binding sites, such as the phosphate-binding loop (P-loop), activation site, catalytic site, or other areas in the BCR-ABL structure. Depending on the mutation site, resistance to imatinib will either be absolute or relative, or it will be clinically irrelevant. Earlier studies have associated P-loop mutations and the T315I mutation with the worst outcomes (Cortes et al., 2007). Mutations within the P-loop site are found in 30-40% of the resistant cases and reduce susceptibility to imatinib by 70 to 100 folds. The T315I mutation in BCR-ABL occurs in 0.16-0.32% of newly diagnosed patients in chronic phase, leading to substitution of threonine 315 with isoleucine. This "gatekeeper" mutation also affects the response to the currently existing second-generation TKIs. Therefore, upon its identification, patients should be considered for alternative pharmacological treatments or for allogeneic bone marrow transplantation.

6.4 Mutational analysis

A careful mutational screening allows the timely identification of potential mutant clones and suggests the most suitable second-line treatment based on the in vitro sensitivity of the specific mutation. The technologies used to identify and quantify the ABL KD mutations include: direct sequencing (Branford et al., 2003), subcloning and sequencing, denaturing-high performance liquid chromatography analysis (DHPLC), pyrosequencing and allele specific oligonucleotide PCR. Direct sequencing represents the most widespread method used for routine monitoring. Its main drawback is the low detection limit (20%) which is responsible for false negative results. Fluorescent-based allele-specific oligonucleotide PCR (ASO-PCR) assays have higher detection limit (0.1%), although their main drawback is that the search for specific mutations does not include screening of the entire KD region of the BCR-ABL gene. Nowadays, numerous groups perform DHPLC to monitor CML patients, followed by a sequence analysis to confirm the data. DHPLC has a detection limit of 1-5% (Deininger et al., 2004). Mutation studies might be performed on peripheral blood or bone

marrow although a direct comparison of these two types of samples has not been done yet. The search for BCR-ABL mutations should be performed, according to NCCN CML guidelines (NCCN Clinical Practice Guidelines in Oncology, 2010), in the following conditions:

1. Progression to accelerated or blast phase
2. Treatment failure
3. Suboptimal therapeutic responses
4. Increasing BCR-ABL levels (5 to 10 fold in mRNA)

6.5 Scheduling CML diagnostics and monitoring

An effective CML monitoring entails an appropriate follow up-schedule (Baccarani et al., 2006). Evidence obtained in clinical trials has prompted experts to formulate consensus recommendations to assess the response to treatment in patients with Ph+ CML (Quintas-Cardama & Cortes, 2005). In the diagnostic setting, bone marrow cytogenetics is recommended before initiation of treatment. Additionally, a nested PCR confirms the diagnosis of CML and establishes the type of BCR-ABL fusion transcript present. Bone marrow cytogenetics is able to detect chromosomal abnormalities that FISH is not able to detect. However, if bone marrow collection is not feasible, FISH on peripheral blood specimen with dual probe (BCR and ABL genes) is a suitable tool to confirm the diagnosis. Subsequently, the cytogenetic evaluation is recommended at 6 and 12 months from the beginning of treatment. If a CCyR is achieved at 6 months, it is not necessary to repeat the cytogenetic evaluation at 12 months. If patients is not in a CCyR at 12 months, a cytogenetic evaluation should be repeated at 18 months. Once cytogenetic remission is achieved, residual disease should be monitored using BCR-ABL transcript levels by RT-Q-PCR, which is the most sensitive technique to monitor BCR-ABL. The hybrid transcript levels should be measured every 3 months at the beginning of treatment and then every 3-6 months since a CCyR is achieved. A steady decline in BCR-ABL transcripts indicates an ideal response to therapy. Rising level of BCR-ABL transcript (1 log increase) following the achievement of a MMR, mandates to repeat the molecular analysis after 1 month (Baccarani et al., 2006). If the result is confirmed, bone marrow cytogenetics should be performed, BCR-ABL quantifications by RT-Q-PCR should be scheduled every month, and a kinase domain mutational analysis should also be done (Wang et al., 2003). The evaluation of the hematologic response foresees that, starting from treatment onset, blood cell counts are performed every 2 weeks until a stable CHR is achieved, then every 3 months (Deininger, 2005). If the patient fails to achieve CHR by 3 months, the treatment is generally regarded as a failure, indicating the need to consider alternative therapeutic strategies.

In summary, the international guidelines recommend the following testing schedule when monitoring treatment of CML patients:

1. Hematologic responses should be assessed at diagnosis, then every 2 weeks until a CHR has been achieved and confirmed, then every 3 months or as required.
2. Cytogenetic responses should be assessed at diagnosis, and every 6 months until a CCyR is achieved and confirmed, then every 12 to 36 months as long as MMR is stable
3. Molecular responses should be assessed every 3 months, or monthly if an increasing BCR-ABL transcript level is detected.
4. Mutational analysis in occurrences of suboptimal response or failure; recommended before changing to other TKIs or other therapies

FISH may be preferred over conventional cytogenetics as it can evaluate more cells and peripheral blood can be used instead of bone marrow. However it is only recommended prior to treatment to identify cases of Ph-, BCR-ABL CML and those with variant translocations, Ph amplification, or del9q+.

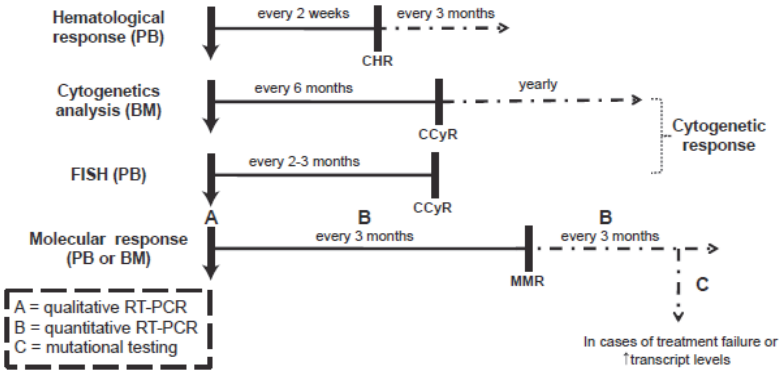

Fig. 1. Proposed algorithm for CML monitoring according to the National Comprehensive Cancer Network guidelines.

7. Conclusions

Chronic myeloid leukaemia is a biological model of how the molecular understanding of a disease is able to provide the substrate for therapy and diagnostics. The recent molecular analysis of the leukaemia cell has generated an extraordinary range of discoveries about the anomalies developed during the cell growth, promoting the development of innovative therapeutic approaches for this type of hematopoietic neoplasia. In particular with the introduction of TKIs we have embarked on a journey aiming to reduce disease burden and prolonging survival.

Additionally the molecular tools to monitor disease and characterize resistance are remarkably effective not only in the diagnostic evaluation but even in the management of CML patients. While traditional cytogenetics with or without FISH and qualitative nested-PCR are essential for the diagnosis of CML, serial RT-Q-PCRs are the mainstay of therapeutic monitoring and MDR assessment (Kantarjian et al., 2008). In cases of treatment failure, highlighted by increasing BCR-ABL levels and/or by loss of hematologic and cytogenetic responses, mutational analysis to identify KD mutations should be considered in order to meet the better treatment decisions (i.e. use alternative TKIs or stem cell transplantation) (Hughes et al., 2006). Additionally, an early identification of treatment failure increases the chance that alternative treatments will be effective (Jabbour et al., 2009).

However the major current impediment to cure for CML patients resides in the cancer stem cell population that is neither oncogene addicted nor sensitive to TKIs. Thus, one of the major challenges is to recognize as early as possible the patient destined to fail TKIs to revise the therapeutic strategy. Additionally, an early identification of treatment failure increases the chance that alternative treatments will be effective.

Hence the need for increasingly sophisticated technologies for an early detection of molecular relapse. In this field the comprehensive analysis of the CML genome, by the

single nucleotide polymorphism arrays, will provide the basis for a molecular approach to guide therapeutic decisions. (Boultwood et al., 2010)In summary the CML represents one of the best examples of tumour malignancies and despite the numerous advantages of modern technologies, it is important to continue interpreting laboratory data within the clinical context of the patient in order to effectively and inexpensively utilize current and nascent laboratory tools.

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7

Therapy of Acute Myeloid Leukemia

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1. Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults[1]. Over the past twenty years, the studies on the pathogenesis and prognosis of AML have made considerable progress.

Clinically, patients with AML typically present with signs or symptoms of bone marrow failure, although sometimes they can present with symptoms of leukostasis with pulmonary or neurological dysfunction. Rarely, patients will present with primary extramedullary disease, which should be approached in the same way as systemic AML.

A certain number of factors can be involved in the etiology of AML: as an example, exposure to ionizing radiation and long-term exposure to benzene are known risk factors. AML could be part of the natural history of patients with congenital disorders of DNA repair, such as the Fanconi's anemias; also the myeloproliferative disorders (MPD) and myelodysplastic syndromes (MDS).

AML is a heterogeneous disease; standard treatments may be applied to biologically distinct subgroups, resulting in different treatment outcomes. However, less than one-third of all adult patients with AML can be cured even to this date. The treatment of refractory, relapsed and elderly AML remains a major challenge. In recent years, new regimens and novel agents are being studied in an effort to improve complete remission (CR) rate and overall survival. The concept of risk-adapted therapy allows for recognition of this biologic diversity by incorporating key biologic features, such as cytogenetic and molecular markers, when formulating treatment regimens and investigating emerging targeted therapies based on disease characteristics. Although AML has been the focus of significant laboratory and clinical investigation, it remains difficult to treat, perhaps partly because of the fundamental nature of the disorder, which requires substantial institutional resources to adequately deal with the complications of bone marrow failure and sustain patients through periods of intensive therapy. Several large studies have helped categorize chromosomal abnormalities into good-, intermediate-, and poor-risk groups[2-5]. This hierarchical system of karyotype classification is predictive value across different age groups in de novo and secondary AML. It was also found to retain prognostic significance across the different treatment modalities of chemotherapy and autologous and allogeneic bone marrow transplantation. Generally, the poor-risk or unfavorable group includes those with complex karyotypes (> 3-5 abnormalities), chromosome 5 or 7 abnormalities, or chromosome 3q abnormalities. The results for these patients are dismal, with standard chemotherapy causing some to advocate

patients undergoing stem cell transplantation in first remission[3,5]. In contrast, such an "aggressive" approach as allogeneic stem cell transplantation in first complete remission (CR) is generally not recommended in patients with good-risk or favorable cytogenetics. Included in this category are those with t(15;17), t(8;21), t(16;16), and inv(16) translocations[6]. Acute promyelocytic leukemia (APL), defined by the t(15;17) translocation, has a distinct biology, and combinations of dose-intensive anthracyclines, all-*trans*-retinoic acid (ATRA), and arsenic trioxide may be curative for most patients.

Age was not, however, found to be the only significant factor affecting treatment outcomes. Although supportive measures have generally improved within the past 20 years, the drugs that form the backbone of standard AML chemotherapy remain essentially unchanged.

2. Therapy

2.1 High dose daunorubicin

The standard induction regimen for newly diagnosed AML consists of daunorubicin (DNR) 45 mg/m^2 intravenously for 3 days and cytarabine (AraC) 100 mg/m^2 by continuous infusion for 7 days. With this regimen 60% to 80% of young adults and 40% to 60% of older adults can achieve a CR[7].

Several major studies, particularly Cancer and Leukemia Group B (CALGB) 9621 and the French ALFA 9801 studies, have shown that higher doses of DNR (80 or 90 mg/m^2) can be administered safely[8,9]. Recently, there are two major prospective studies compared DNR 90 mg/m^2 with 45 mg/m^2 in the induction regimen[10,11]. Eastern Cooperative Oncology Group (ECOG) studied 657 AML patients between the age of 17 to 60[10]. The study showed significantly higher CR rate for patients receiving 90 mg/m^2 (70% versus 57%). More importantly, overall survival (OS) was significantly prolonged (23.7 vs 15.7 months). The Dutch-Belgium Hemato-Oncology Cooperative Group (HOVON)/Swiss Group for Clinical Cancer Research (SAKK) compared DNR 90 mg/m^2 versus 45 mg/m^2 in 813 patients older than 60 years[11]. The results showed that CR rate was 64% and 54% respectively, while CR rate after only one course of treatment was 52% and 35% respectively. The OS rate was not significantly different for the whole group. However, for the patients between the age of 60 to 65, the OS rate was significantly better in the high dose group (38% vs 23%). The rates of serious adverse events were similar in the two treatment groups in both studies.

Based on historic trials and the most recent prospective studies, the 45 mg/m^2 of DNR should no longer be the standard-dose for induction therapy. Instead, for induction therapy of all age groups, DNR dose should be between 60 mg/m^2 to 90 mg/m^2 for 3 days, but the exact optimal dosage remains to be established[12].

2.2 New formulations of old agents

Liposomal encapsulation of drugs can reduce the toxicity and decrease drug doses with controlled-release effect. CPX-351 is a liposomal formulation that encapsulates cytarabine and daunorubicin at a 5:1 molar ratio. A recently completed phase 1 study[13] recommended that 90-minute infusions of 101 u/m^2 be given on days 1, 3, and 5 (1 u = 1 mg Ara-C + 0.44 mg DNR). The results showed that liposomal encapsulation of this chemotherapy changed the safety profile by reducing non-hematologic toxicities including hair loss, gastrointestinal toxicities and hepatic toxicity, while retaining hematopoietic cytotoxicity.

2.3 Targeted therapy regimens

In recent years, encouraging results have been achieved by using monoclonal antibodies for targeted therapy of the solid and hematologic malignancies. CD33 antigen is expressed in more than 90% of AML cells, while expression in normal tissue is very weak. Gemtuzumab ozogamycin (GO) is chemoimmunotherapy agent consisting of a monoclonal antibody against CD33 conjugated to calichemycin. GO triggers apoptosis when hydrolyzed in the leukemic blasts. GO has been approved by the U.S. FDA for the treatment of the elderly (> 60 years) with AML in first relapse. Standard induction regimen with or with out GO were compared in a randomized study which enrolled 1115 younger adults with AML. The preliminary analysis showed a similar CR rate in both arms, but a significantly improved DFS among patients receiving GO--51% versus 40% at 3 years (P = .008)[14]. However, due to toxicity concern and the lack of definite survival benefit after longer follow-up, FDA has recently withdrawn its approval.

A phase II study of My-FLAI aiming to assess toxicity and efficacy was done in patients with newly diagnosed AML aged more than 60 years[15]. The results showed that the four drug regimen My-FLAI was well tolerated in an elderly AML population, but its efficacy did not appear to be superior to that of standard "3+7" regimen[15].

2.4 New agents nucleoside analogues

Nucleoside analogues transform into active metabolites (triphosphate nucleoside analogues) in the cells and inhibit DNA synthesis. Clofarabine is a new nucleoside analogue, a potent inhibitor of both ribonucleotide reductase and DNA polymerase. At the 2009 ASH meeting, a few studies on Clofarabine were reported[16-18], either clofarabine alone or in combination with low-dose Ara-C, or high-dose Ara-C with the monoclonal antibody GO in the treatment of elderly AML or relapsed AML. Two novel nucleoside analogues, sapacitabine and elacytarabine, were also reported for the therapy of the elderly with refractory or relapsed AML[19,20].

2.5 FLT3 inhibitors (Fms-like tyrosine kinase 3 inhibitors)

The Flt3-internal tandem duplication (ITD) can be found in approximately 30% of all AML patients and confers a poor risk status characterized by an increased relapse rate and poor overall survival[21,22]. Moreover, Flt3-ITD-positive AML patients relapsing after allogeneic stem cell transplantation (SCT) have very limited therapeutic options. Sorafenib is a multikinase inhibitor that is approved for the treatment of metastatic renal cell and hepatocellular carcinoma. Sorafenib monotherapy has significant clinical activity in Flt3-ITD positive relapsed and refractory AML[23,24].

In addition, combination therapy with sorafenib was shown to be effective in reducing mutant clones in patients with FLT3 mutations but was not able to completely eradicate them[25]. These data suggest that sorafenib can achieve temporary disease control, but should be integrated into induction and consolidation regimens to achieve maximal outcome

2.6 Farnesyl-Transferase Inhibitor (FTI)

In recent years, studies have shown that Ras gene mutation plays an important role in leukemogenesis[26]. By inhibiting farnesyl protein transferase, FTI prohibits the Ras protein farnesylation, schizolysis and carboxyl methylation, thus disrupting the critical Ras

signaling pathway. Tipifarnib (± bortezomib) may represent an important option in a subset of high risk/frail AML patients[27].

2.7 Histone deacetylase inhibitors

Vorinostat is a new anti-cancer agent inhibiting histone deacetylase and has been shown to have some efficacy in treatment of AML[28]. Vorinostat in combination with idarubicin and ara-C has synergistic antileukemia activity in a sequence dependent fashion. Therefore, the combination of vorinostat, idarubicin and cytarabine is safe and active in AML. CR or CRi was achieved by 18% pts with MDS, 8% with relapsed/refractory AML, and 36% with untreated AML[29]. The combination of vorinostat with decitabine either concurrently or sequentially is possible without significant toxicity and shows activity in MDS and untreated AML[30].

2.8 DNA Methyl-transferase inhibitors

The demethylating agents 5-azacytidine and Decitabine are remarkably active, even at low doses with mild hematologic toxicity, in patients with high-risk MDS. This disease shares many poor prognostic features with AML of the elderly.

Decitabine: Decitabine inhibits DNA methyltransferase, leading to DNA hypomethylation and cell differentiation or apoptosis. A combination of decitabine and GO was found to be effective with low side effects in previously untreated or refractory/relapsed AML patients, especially in elderly patients[31]. The toxicities were minimal and the regimen can be safely delivered to older patients. The pioneering study by Pinto and coworkers[32] described 12 patients with AML who received Decitabine (90-120 mg/m^2 as a 4-h infusion 3 times daily for 3 days, repeated every 4–6 weeks). Three patients achieved a complete remission (CR) and one a partial remission (PR) ; extra-hematological toxicity was generally mild. Preliminary results of a trial using low-dose decitabine in older patients with AML were reported more recently[33]: Cashen and coworkers gave the drug over 1 h on 5 consecutive days (20 mg/m^2 per day), repeated every 28 days. Fifty-five patients with a median age of 74 years old were enrolled and treated with a median of three cycles : overall response rate was 25% (complete response rate, 24%) and median survival was 7.7 months.

5-Azacytidine: despite the fact that more than 100 trials of high dose 5-azacytidine were performed in AML in the 1970s and 1980s (mostly in combination with other chemotherapies), a very limited number of trials using this drug in AML have been published. When the CALGB compared the use of 5-Azacytidine to best supportive care in people with all risk groups of MDS, there was a trend toward a survival benefit in those patients who received the 5-Azacytidine[34]. There was a decrease in the P15 methylation associated with response. 5-azacytidine was approved by the FDA in 2004 for the treatment of MDS (all subtypes). Phase II studies of 5-azacytidine in MDS had been initiated by Silverman and the CALGB[35] (7 daily administrations of 75 mg/m^2, total dose 525 mg/m^2, repeated every four weeks); overall response rate of 49% was obtained, with 12% CRs and median response duration of 14.7 months. A pivotal phase III study of the CALGB[34] compared subcutaneous 5-azacytidine randomized against best supportive care (BSC), with the possibility of "cross-over" from BSC in case of progressive disease. An overall response rate of 60%, with 7% CRs and 16% PRs and median response duration of 15 months was achieved in the experimental arm. Quality of life was also significantly improved in 5-

azacytidine treated patients. Side effects included mainly myelosuppression and associated effects, particularly during the first cycles. Non-hematological toxicties, e.g. nausea and vomiting, were rare, but skin reactions occurred more frequently. The French ATU program[36] performed a retrospective analysis of 184 patients with refractory or relapsed AML who received azacytidine. 11% of the patients responded (7%CR, 3%CRi, 1% PR). It appears that single agent azacytidine has only limited activity in AML patients relapsed or refractory to intensive frontline therapy. Combination of azacitidine with bortezomib or low-dose GO was also studied in relapsed or refractory AML patients[37,38]. In a large confirmatory trial[39], 5-azacytidine was compared to conventional treatment as determined prior to randomization by the treating physician (either BSC, low-dose ara-C, or induction chemotherapy). Of 358 patients included, 179 were randomized to 5-azacytidine, 179 to conventional care (105 to BSC, 49 to low-dose ara-C, 25 to standard induction chemotherapy). Study drug was administered for a median of 9 cycles. 28.5% of patients in the experimental arm achieved CR or PR. Median survival was 24.4 months in the 5-azacytidine group compared to 15 months in the conventional care group (P=0.0001), with a doubling of the 2-year survival (50.8 vs. 26%, P<0.0001).

3. Valproic acid, an inhibitor of Histone Deacetylases (HDACs)

Valproic acid (VPA) is an inhibitor of class I HDACs. Over the last 5 years, the drug has been studied as either a single agent or in combination with various drugs including ATRA. VPA has provided a 50% overall response rate in low-risk MDS and a lower rate of response in high-risk MDS[40] and AML[41,42]. The contribution of ATRA probably was modest. Thus, the role of single-agent VPA may be rather limited in AML. Nevertheless, the drug in combination with an active drug such as decitabine, may have enhanced activity, as demonstrated in vitro[43]. A large phase II study of AML and MDS performed at the MD Anderson Cancer Center demonstrated the feasibility of DAC combined with 10 days of intravenous VPA[44].

3.1 Other agents in early clinical development

Voreloxin: is a first-in-class anticancer quinolone derivative that intercalates DNA, inhibits topoisomerase II, and induces apoptosis. A preliminary report on a voreloxin trial revealed clinical activity in previously untreated elderly (age ≥ 60) AML patients who are unlikely to benefit from standard chemotherapy[45]

Amonafide L-malate (AS1413): is a unique DNA intercalator, in combination with cytarabine produced a high complete remission rate and durable responses in both older and younger patients with secondary AML[46].

Ezatiostat hydrochloride: is a glutathione S-transferase P1-1 inhibitor, evaluated in myelodysplastic syndrome. In a phase I/II study[47], trilineage responses were observed in 4 of 16 patients (25%) with trilineage cytopenia. These responses were accompanied by improvement in clinical symptoms and reductions in transfusion requirements.

Lenalidomide: (LEN) is one of the three new drugs approved by the U.S. FDA to treat 5q-low-risk MDS. Lenalidomide has demonstrated multiple mechanisms of action[48]. In a recent phase II study[49] of LEN in combination with Ara-C and daunorubicin in high risk MDS/AML with del 5q, 28% responded. The results show that LEN combined with chemotherapy in AML treatment is feasible, without significant additional toxicity.

Ribavirin: The eukaryotic translation factor, eIF4E, is over expressed in AML, and is associated with poor prognosis. Ribavirin is clinically used as an antiviral molecule, and its structure is similar to the m(7)G cap of mRNA, thus inhibiting eIF4E-induced export and translation of sensitive transcripts[50].
ARRY-520: The kinesin spindle protein (KSP) plays a major role for the assembly of a normal bipolar spindle and is also required for cell cycle progression through mitosis. ARRY-520 is a potent, selective inhibitor of KSP[51].
AZD1152: Aurora B kinase plays a major role in regulating mitosis and is over expressed in AML. Also it's a highly potent and selective inhibitor of aurora B kinase. It has been shown to inhibit tumor growth in vivo[52].
AZD6244: is one of the orally bioavailable small molecule inhibitors of MEK kinase[53-55].
Terameprocol: The inhibitor of apoptosis protein (IAP), survivin, is a key regulator of cell cycles. In leukemic cells, survivin is involved in leukemia cell survival and resistance to chemotherapeutics and Flt-3 inhibitors[56].

3.2 Allogeneic Stem Cell Transplant (allo-SCT)

In patients with AML, published guidelines and treatment recommendations are usually the basis for starting the work-up process for allo-SCT[57]. However, only consistent recommendations would allow a standardized clinical practice. A comprehensive systematic literature search could allow to evaluate the best available evidence from controlled clinical trials. The following aspects were selected for systematic comparison: factors for risk assessment and categorization, role of type of donor, significance of allo-SCT in first or second complete remission and in relapse/progressive disease; and role of reduced intensity conditioning (RIC) regimens. The use of myeloablative and non-myeloablative allogeneic stem cell transplant represents a potentially curative approach for patients suffering from acute and chronic leukemias such as AML. Thousands of patients have been treated worldwide by the transplant of hematopoietic stem cells from a related or unrelated donor (available at: http://www.bmdw.org). However, it is obvious that not all patients diagnosed with AML will benefit from allogeneic stem cell transplant. Therefore, the establishment of definitive, clear, evidence-based recommendations as to which patients are likely to benefit from transplant is needed. Several interesting findings emerge when comparing recommendations for transplant in key guidelines: (i) for patients with relapsed or refractory disease, donor availability should be explored and patients should receive transplant, though this is not based on reliable evidence from genetically randomized studies; (ii) patients in CR1 with intermediate-or high-risk disease and an available matched related donor should receive allogeneic stem cell transplant (intermediate-risk: allo-SCT, reasonable option); (iii) for patients who lack a family donor the recommendations are not consistent; (iv) allogeneic transplant with reduced conditioning in patients with AML is feasible, but the superiority over standard therapeutic regimens is not yet proven. At this point in time, there is no doubt that hematopoietic allogeneic stem cell transplant is an effective clinical procedure with a curative ability, but intensive induction and consolidation chemotherapy may also be sufficient for many patients. But it is likely that only well-defined subgroups of patients with AML will benefit from stem cell transplant. The delineation of these specific patient groups will be a major objective of future clinical trials.
Transplant of patients with AML in first CR: in patients who achieved CR, the role of allo-SCT is still under discussion. A variety of clinical studies have tried to evaluate the benefit

of transplant in this situation, but most studies were non-randomized, non-controlled trials[3,5,6]. Only a few trials that were analyzed based on donor availability ('genetic' or 'biological' assignment) were useful as supporting evidence in the guideline recommendations, which were mainly based on cytogenetic risk factors. These methods of patient assignment often introduce biases. Therefore, some guidelines regard these trials as level II (cohort study) and not as level I evidence (randomized clinical trials). Cassileth et al.[58] reported a study of patients with AML aged 16–55 years with complete remission after induction therapy who were offered allogeneic transplant if a genotypically or phenotypically human leukocyte antigen (HLA)-matched or single-antigen mismatched family donor was available (n=113). Remaining patients were randomized to autologous transplant (n=116) or a single course of high-dose cytarabine (n=117). The distribution of karyotypes did not differ significantly among treatment groups. After a median follow-up time of 4 years, the authors found no evidence for significant differences in disease-free survival (DFS) between the chemotherapy group (35%), autologous transplant group (35%), and allogeneic transplant group (43%). Overall survival (OS) was marginally better after chemotherapy than following autologous or allogeneic transplant (52% vs. 43% vs. 46%). Twenty five percent of patients died after allogeneic transplant as compared to 14% following autologous transplant and 3% after chemotherapy. The subset analysis based on cytogenetic risk groups showed many methodological limitations of the study: a high proportion of patients did not receive the assigned therapy, which tends to reduce the measurable treatment effect in an intention-to-treat analysis.

The EORTC–GIMEMA (European Organization for Research and Treatment of Cancer–Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto) AML 8A trial[59] showed no significant difference in overall survival after a median follow-up of 6 years among patients receiving allogeneic transplant from a sibling donor (overall survival: 48%) versus patients without a donor (40%, p=0.24; patients received autologous transplant or conventional chemotherapy).

A higher incidence of early mortality after allogeneic transplant was counterbalanced by a lower incidence of late mortality. In the EORTC AML 10 trial[60] these results were confirmed, with the exception of the high-risk group. In total, the survival rate at 4 years was 58.3% in the allogeneic, sibling donor group versus 50.8% in the autologous transplant group (p=0.18). In the high-risk group, in patients receiving allogeneic transplant the overall survival at 4 years (50.2%) was significantly better than after chemotherapy or autologous transplant (29.4%). In the standard-risk group and the low-risk group the results for overall survival were similar.

However, the GOELAM (Groupe Ouest Est Leucemies Aigues Myeloblastiques) trial[61] reported no survival benefit for patients receiving an allogeneic transplant regardless of cytogenetic risk group. Interestingly, a trend toward better survival at 4 years following allogeneic transplant was more pronounced in the low-risk group (71.4% vs. 66.5%; p=0.6) and the high-risk group (41% vs. 30%, p=0.97) compared to the intermediate-risk group (40.5% vs.56.5%, p=0.08). The BGMT 87 trial[62] reported overall survival only for the entire patient population and not stratified for risk groups. In patients receiving allogeneic transplant, 3-year survival was 65% in the donor group (n=36) and 50.9% in the no-donor group (n=60) receiving chemotherapy or autologous transplant. An analysis from all trials undertaken by the BGMT shows an improved survival only for the Intermediate and high-risk population[62]. The MRC AML 10 trial by Burnett et al.[63] evaluated 1063 patients on a

donor versus no-donor basis. All patients received four courses of induction chemotherapy followed by consolidation chemotherapy, after which patients with an HLA-matched sibling donor and in appropriate condition were scheduled to receive an allogeneic transplant. The remainder was randomized between autologous transplant and no further therapy. Sixty-one percent of patients with a donor underwent transplant in first remission. In a donor versus no-donor analysis, significant benefit in disease-free survival and overall survival was seen only in the standard-risk cytogenetic group (DFS: 50% vs. 39%, p=0.001; OS: 55% vs. 44%, p=0.01). There were twice as many deaths in first remission among patients with a donor than among patients with no donor (19% vs. 9%; p<0.001).

Recently, the results of three trials from the Dutch-Belgian Hemato-Oncology Cooperative Group and the Swiss Group for Clinical Cancer Research (HOVON/SAKK) based on a donor versus no-donor analysis in patients with AML in first remission were published as an individual patient data (IPD) analysis[64]. Based on the available IPD, the study included a total of 1032 patients from the trials. An HLA-identical sibling donor was available for 32% of patients, whereas 58% of patients lacked such a donor. Following risk-group analysis, disease free survival was significantly better for patients with available donor and standard- or high-risk profile (p=0.01 and p=0.003) and also better for patients younger than 40 years (p<0.001). However, the improved disease-free survival in the donor group did not translate into significantly better overall survival (p=0.07). Treatment-related mortality was significantly higher in the donor group (21% vs. 4%; p<0.001).

Three meta-analyses investigated the apparent heterogeneity of clinical study results. Cornelissen et al. [64] performed a meta-analysis based on published data including more than 4000 patients with AML in first remission enrolled in the HOVON/SAKK studies, the Medical Research Council (MRC) studies, the trials of the EORTC, and the BGMT studies. Disease-free survival and overall survival were analyzed, stratified for cytogenetic risk profile and age. Overall, transplant from a family donor statistically significantly prolonged disease-free survival in patients with intermediate- and high-risk profiles, but not in patients with a low-risk profile. This effect was pronounced but not restricted to patients below the age of 35 years. However, a benefit for overall survival in patients receiving stem cell transplant from a related donor was seen only in patients with intermediate- and low-risk disease up to the age of 35 years. In this analysis there was no evidence for improved outcomes in the favorable-risk group. These results are in accordance with the meta-analysis published by Koreth et al.[6] in 3638 patients with AML in first CR. However, Yanada et al.[65] identified a beneficial effect of allogeneic transplant in these patients limited to the high-risk group.

In summary, there is only a limited number of phase III trials evaluating the role of allo-SCT in patients with AML in first remission. The results of these trials are heterogeneous, and often limited to matched-related donor transplant.

Transplant in relapse/progressive disease: it is generally accepted that the overall prognosis of patients with relapsed or progressive disease is poor. Especially in patients with initial duration of remission below 1 year, the success with standard regimens for inducing second remission is rare. Therefore, it is not surprising that in this situation most treatment guidelines recommend allogeneic transplant once a patient has achieved second remission. However, we did not identify any randomized trial in literature search addressing this clinical situation. In a phase II study, Schmid et al.[66] treated 103 patients with refractory AML using dose-reduced chemotherapy followed by allogeneic transplant

from a related or unrelated donor. Refractory disease was defined as primary induction failure, relapse within 6 months after induction, or second relapse. Overall, 1 year after transplant the authors reported a disease free survival of 47% and an overall survival of 54%. After a follow-up of 4 years, disease-free survival and overall survival declined to 30% and 32%. The risk for treatment failure was significantly increased in patients who received more than two cycles of chemotherapy before stem cell transplant, in patients with primary induction failure, bone marrow infiltration of more than 50% blasts, and more than a median of 215 days from diagnosis to transplant, or in patients with low CD34 cell count in the graft and if stem cells from a related donor were used. While the risk for non-relapse mortality increased when using an unrelated donor, age, sex, and underlying karyotype were not predictive for outcome. Minimal residual disease (MRD), mostly defined as the positive detection of genetic AML-specific mutations during microscopically diagnosed complete remission, is often examined in the follow-up period after induction therapy for leukemia. In small cohort of 45 patients, Laane et al.[67] claimed a benefit by allogeneic transplant for those patients who were identified with detectable MRD after standard therapy.

Reduced intensity conditioning: reduced intensity conditioning has broadened the use of allogeneic stem cell transplant to elderly patients and patients with comorbidities. Allogeneic transplant is no longer restricted to younger, fitter patients who are better able to tolerate the toxicities of a myeloablative regimen. The use of a less intensive approach potentially results in a reduced leukemia cell kill, which in turn increases the risk for relapse and a higher incidence of engraftment failure. Uncertainty remains regarding the frequency of graft-versus-host disease (GvHD) after reduced intensity allografts. Usually, a higher dose of immunosuppressive agents or the use of long-acting agents is recommended to reduce the rate of GvHD after transplant, thereby increasing the risk of relapse. In conclusion, there is an urgent need to define the role of allogeneic transplant in the mixed chimérisme setting. Unfortunately, published literature is often based on single centre experience with small numbers of patients. The Seattle experience is derived from large multicenter studies including a high number of patients. These studies have refined the optimal non-myeloablative treatment procedure, but they do not compare reduced intensity allografts with other treatment strategies[68].

At our knowledge, two randomized controlled studies, evaluating the role of reduced conditioning followed by allogeneic stem cell transplant for AML, have been published. Mothy et al.[69] assigned 95 newly diagnosed adult patients with AML in first complete remission on a donor versus no-donor basis. All patients had a high-risk karyotype or clinical profile. Based on 'biological' randomization, patients with a matched sibling donor (n=35) were assigned to a transplant arm using reduced conditioning, whereas patients lacking such a donor (n=60) were assigned to standard treatment procedures. In an intention-to-treat analysis, 4-year disease-free survival was significantly higher in the donor group (54%) as compared to the no-donor group (30%; p=0.01). Furthermore, overall survival was significantly higher in the donor group as compared to the no-donor group (p=0.04). Transplant-related mortality was 12%.

Estey and colleagues[70] prospectively assessed the applicability of reduced intensity conditioning in patients with AML or MDS. Of 99 patients who entered complete remission after induction chemotherapy, 14 received an allogeneic transplant (13 siblings). The authors conducted a matched-pair analysis, comparing patients receiving chemotherapy with

patients who underwent transplant. Matching criteria were age, cytogenetics, and time between achieving CR and transplant. There was a significantly longer disease free survival in the transplanted patients, but the results for overall survival were similar between patients receiving transplant and patients receiving chemotherapy.
In a large retrospective trial performed by the EBMT[71], 315 patients with AML or MDS who received reduced intensity conditioning were compared with 407 patients who received a Myeloablative approach. In multivariate analysis, acute GvHD and transplant-related mortality were significantly decreased, and relapse incidence was significantly higher after reduced intensity conditioning.

3.3 Autologous Stem Cell Transplantation (auto-SCT)

For patients with AML who are unable to secure an acceptable HLA donor, the role of autologous stem cell transplantation (auto-SCT) has remained controversial. Its effectiveness remains unclear as, when analyzed on intention-to-treat strategies, a significant number do not undergo the procedure, whereas others seem to fail therapy from pre transplant recurrences. Recently, Novitzky et al.[72] compared the outcome of patients in first remission of AML who actually underwent autologous or allogeneic transplantation. The choice for the type of graft was based on availability of HLA identical siblings. Patients received myeloablative conditioning followed by allogeneic or autologous cytokine mobilized peripheral blood stem cell transplantation. For prophylaxis of graft-versus-host disease (GVHD), grafts were incubated ex vivo with anti-CD52 antibodies and patients were prescribed cyclosporin until day 90. Patients were stratified by clinical and laboratory factors as well as cytogenetic risk. The endpoints were treatment-related mortality (TRM), disease-free survival (DFS), and overall survival (OS). The median presentation age for both transplant groups was 35 (14-60) years. Of the 112 consecutive patients achieving remission, autologous or allogeneic grafts were transplanted to 43 and 32 patients, respectively. There was no significant difference in the presentation clinical features, laboratory parameters, marrow morphology, or proportion of low and intermediate cytogenetic risk for both transplant options. Treatment mortality as well as relapse rate was similar (14% and 15%; 39% and 27%, respectively). At a median of 1609 and 1819 post transplant days, 56% and 63% in each group survived. In univariate analysis performance status, cytogenetic risk, morphologic features of dysplasia, blast count, and lactate dehydrogenase (LDH) were significant factors for survival. Although for the entire group there was no difference in survival between both modalities, all patients with unfavorable cytogenetics receiving an autologous graft died of disease recurrence (3-year survival 35% versus 0%; P = 0.05). They conclude that patients with AML who have low or intermediate cytogenetic risk undergoing myeloablative conditioning followed by autologous or allogeneic T cell-depleted stem cell transplantation appeared to have similar outcome. However, those with unfavorable karyotype are unlikely to be cured with autologous grafts and are candidates for experimental modalities.

3.4 Other regimens for refractory /relapsed AML

High-dose cytarabine (HiDAC) is commonly used for re-induction of relapsed or refractory AML. Recently, Thomas et al.[73] et al reported a novel, timed-sequential regimen that takes advantage of synergy when mitoxantrone is given after cytarabine. Those patients received HiDAC/mitoxantrone regimen, with cytarabine at 3 g/m^2 over four hours on days 1 and 5

plus mitoxantrone at 30 mg/m^2 over one hour immediately following the HiDAC on days 1 and 5. HiDAC/mitoxantrone induction was well tolerated and complete remission was achieved in 89%. of patients.

To further enhance the CR rate in refractory/relapsed AML, the Japanese Adult Leukemia Study Group (JALSG) reported a phase II study of FLAGM (Fludarabine + High-Dose Ara-C + G-CSF + mitoxantrone) in 41 patients with relapsed or refractory AML[74]. FLAGM yielded a 70% response rate in either relapsed or refractory AML patients. Although randomized studies are still needed, FLAGM appears to be a good option for the treatment of either relapsed or refractory AML patients.

4. Conclusions: Future directions

Achieving a cure for AML, even for younger adult patients with de novo AML, remains a challenge. While more than 70% of such patients will enter a first CR1 after induction chemotherapy, a substantial number experience disease relapse. Allo-SCT is a curative treatment option for younger patients with AML in CR1. However, concerns regarding allo-SCT–related toxicity, and questions regarding its benefit, limit its use for patients who have attained an initial remission. Alternative therapies include intensive consolidation chemotherapy or auto-SCT. The current consensus, reflected in treatment guidelines of the National Comprehensive Cancer Network (V1.2009: available at http://www.nccn.org), is based on cytogenetic stratification into good-, intermediate-, and poor-risk AML. Compared with non-allo-SCT therapies, allo-SCT has significant relapse-free survival and overall survival benefit for intermediate- and poor-risk AML but not for good-risk AML in CR1.

Prognostic markers, such as NPM1, Flt3-ITD, and cytogenetic abnormalities have made it possible to prospectively formulate aggressive treatment plans for unfavorable AML. If no Flt3-ITD mutation is present, *CEBP*α and *NPM1* are generally associated with a favorable prognosis, and testing will be important in defining biologic subtypes that require less therapy. The presence of some of these mutations may modify the effect of others, so the establishment of a panel of significant markers may be needed to adequately assess risk and plan care. However, the long-term survival of AML with unfavorable factors remains unsatisfactory. Prolonged survival without curing high risk MDS/AML patients suggests that disease modification instead of cure of AML patients may be an alternative goal of treating elderly patients not suitable for aggressive therapy. New regimens and novel agents targeting specific pathways reviewed in this report may bring AML treatment into a new era.

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8

Diagnosis of Acute Myeloid Leukaemia

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1. Introduction

Acute myeloid leukaemia (AML) is a clonal, malignant disease of hematopoietic tissue characterized by accumulation of abnormal cells, mainly leukaemic blasts in bone marrow and impaired production of normal hematopoietic cells. Leukaemia was discovered in 1845, roughly at the same time by two doctors, Rudolph Virchow, a young Berlin pathologist and a Scottish pathologist, John Hughes Bennett. The term leukaemia was first used by Rudolph Virchow to describe the blood appearance of his female patient after she died. She was a 50-year-old cook, admitted to hospital, complaining of fatigue, frequent nosebleeds and swelling of the legs and abdomen. They were the first who begun to understand what exactly goes wrong in this unusual disease.
The aim of this chapter is to present a step by step approach in diagnosing acute myeloid leukaemia and also to identify potential diagnostic pitfalls.

2. Signs and symptoms

The clinical course of AML, treated or untreated, is complex, so care for these patients requires the experience of a specialist physician. Signs and symptoms usually seen in AML are associated with complications, meaning signs and symptoms associated with anaemia, thrombocytopenia, and leukopenia and also signs of organ involvement. Without being specific, they reflect the anaemia development, but there is no direct proportionality between the severity of anaemia and the manifestation of these signs and symptoms: fatigue, asthenia, weakness, pallor, dizziness, irritability, dyspnea, tachycardia, palpitations, and general lack of wellness. Petechial, nosebleeds, gum bleeding, conjunctivas haemorrhage, prolonged bleeding from mild skin lesions, these all reflect the thrombocytopenia and are common early manifestations of the disease. Bleeding of the gastrointestinal tract, genital-urinary, lung or central nervous system (CNS) may occur infrequently.
According to Hu et al. the frequencies of the most important presenting features in AML are presented below in Table 1 (Hu et al., 2011).
Blood transfusions may be necessary but can – in the case of hyperleukocyosis - also lead to a rapid increase in blood viscosity and compromise blood flow. In addition, coagulation abnormalities, including disseminated intravascular coagulation, increase the risk of local haemorrhage. The use of platelet transfusion is recommended, especially since the number of platelets may be overestimated due to the presence of fragments of blasts that are wrongly considered by the automated haematology analyzers. Acute promyelocytic

leukaemia is most commonly associated with intravascular coagulation and bleeding (Tallman & Kwaan, 2004). Platelets interact with normal peripheral blood (PB) cells via soluble mediators of adhesion molecules and these, once released, may affect hematopoietic stem and progenitor cells. Recently the interactions between platelets and AML cells has been characterized in detail (Foss & Bruserud, 2008): blasts and platelets can affect each other's functions, drugs used to treat AML can alter some platelet functions, systemic levels of cytokines are increased during chemotherapy, including cytokines known to affect platelet activation and blasts, platelet secretion of growth factors is clearly detected in peripheral blood of persons with stem cell autografts.

Acute Myeloid Leukaemia		
Initial symptom	Younger Group (age < 60) %	Elderly Group (age ≥ 60) %
Hypodynamia	57.8	68.3
Pale face	60.3	55.5
Fever	40.5	33.5
Dizziness	14.7	10.4
Hemorrhagic dermatologic mucosa	15.5	14.0
WBC infiltration	6.9	12.2
Myalgias	4.3	3.0
White cell count (10^9/L)		
< 4	24.1	40.2
4 - 10	16.4	9.8
> 10	59.5	50.0
Haemoglobin (g/L)[2]		
Abnormal	93.1	95.7
Normal	6.9	4.3
Platelets (10^9/L)		
≤10	29.3	21.3
>10	70.7	78.7
Bone marrow cellularity		
Severely hypercellular	53.4	30.5
Moderately hypercellular	21.6	34.8
Normocellular	13.8	20.7
Hypocellular	11.2	14.0

Table 1. The frequencies of the most important presenting features in AML, according to Hu et al.

Infections remain a major cause of morbidity and mortality associated with therapy in both adults and children with AML. Pustules or other skin infections and various skin lesions are the most common minor infections encountered. More serious infections like sinusitis, pneumonia, pyelonephritis and meningitis occur rarely in the beginning. After starting chemotherapy, with aggravation of neutropenia and monocytopenia, serious infections occur more frequently with various bacterial, fungal or viral agents. Progressive decline in immune function makes aged patients with AML theoretically more susceptible

to nosocomial infection than younger ones. However, a study in this regard indicated that there is no significant difference in the overall incidence of infections, such as febrile episodes, the pattern of nosocomial infection sites, the average duration of antimicrobial therapy and overall survival (Fanci et al., 2008). Gram-negative bacteria were more common in patients with severe sepsis (Hämäläinen et al., 2008). A case was reported in the literature of AML with marked hyponatremia and impaired consciousness probably due to treatment with linezolid, but sodium supplementation restored the natremia. Viridans streptococci in children with AML are a major cause of infections and pneumonia in cases with neutropenia. Viridans streptococci sepsis developed at different times after chemotherapy was initiated and patients were febrile for a median of 15 days. 33% of the 172 children with AML included in this study had hypotension, 28% had acute respiratory distress syndrome and 17% had fungal infections (Okamoto et al., 2003). Neutropenic enterocolitis and acute appendicitis are also complications that occur in children with severe or prolonged neutropenia, and may endanger their lives (Alioglu et al., 2007). Hepatosplenic fungal infections are important infectious complications in adults with AML being diagnosed with computerized tomography and high levels of alkaline phosphatase. Mortality related to these infections is low if treatment is appropriate (Masood & Sallah, 2005).

Fever is present at diagnosis in approximately 40-50% of the patients. Anorexia and weight reduction are also common, percentages of 25% being reported (Burns et al., 1981).

Splenomegaly and hepatomegaly are seen in approximately one third of patients, especially in those with a monocytic or monoblastic morphologic subtype. Adenopathy is rare, the exception being the monocytic variant of AML where a frequency of more than 30% can be found (Burns et al., 1981; Hu et al., 2011).

2.1 Specific organ involvement

Blast cells are circulating and infiltrating various tissues. Occasionally, a biopsy or autopsy reveals infiltration with leukaemic cells at different levels. They cause disruption to the affected structures. Extramedullary involvement is more frequent in monocytic and myelomonocytic leukaemia. Recently it was demonstrated that haematopoietic progenitors and leukaemic cells are retained in the bone marrow (BM) microenvironment through chemokine receptors, such as CXCR4. A prospective study evaluated by flow cytometry the prognostic involvement of CXCR4 in AML. The study showed that low expression of CXCR4 on leukaemic cells is correlated with a better prognosis than high expression (Spoo et al., 2007). Different sites may be involved (Liesveld & Lichtman, 2006):

Skin involvement. Skin injury prior to BM and blood involvement is rare and can be of three types: nonspecific lesions (maculae, papules, vesicles, pyoderma gangrenosum, vasculitis, neutrophilic dermatitis, erythema multiform or nodosa), skin leukaemia and granulocytic sarcomas (myeloid sarcomas), being the result of skin infiltration by blastic cells. The most common sites of infiltration are the scalp, trunk and extremities.

Sensory organ involvement. Sensory organ involvement is very rare, however, infiltration of retina, choroids, iris and optic nerve may occur. Otitis external and internal bleeding, infiltration of the mastoid with VII nerve damage may be other signs of disease presentation.

Gastrointestinal tract involvement. May be affected at any level, but functional disorders are rare. Involvement of the oral cavity, colon and anal canal most often lead to symptoms. Involvement of the oral cavity often means the patient goes to the dentist. Infiltration of

gums, periodontal abscess can cause prolonged bleeding following extractions. Enterocolitis may be a way of disease presentation or can occur during treatment. Fever, abdominal pain, bloody diarrhoea or ileus may be present and sometimes mimic appendicitis. Isolated gastrointestinal tract involvement is rare. Proctitis usually occurs in the monocytic variant of leukaemia and is a difficult problem to solve during the period with severe granulocytopenia.

Respiratory tract involvement. Infiltrates may lead to laryngeal obstruction, functional disorders, severe symptoms and radiological changes in the case of parenchymal, alveolar or pleural infiltrates.

Heart involvement. Cardiac involvement is common, but rarely causes symptoms. Pericardial infiltrates, ventricular transmural endocarditis can occasionally cause severe cardiac arrhythmias or even death. Infiltrations of the excitoconductor system and myocardial infarction were also cited.

Urogenital tract involvement. The kidneys are infiltrated with leukaemic cells in a large number of cases, but functional disorders are rare. Bleeding into the collector is common. Cases have been cited with vulvar, prostatic and testicular penetration.

Osteoarticular system involvement. Its involvement is accompanied by various degrees of bone and joint pain. Bone necrosis may also occur. Pseudo-gout arthritis (calcium pyrophosphate dehydrate) or gout arthritis (uric acid) is sometimes responsible for synovitis.

Central nervous system involvement (CNS). CNS involvement is rare, although meningeal infiltration is seen in the monocytic type of AML. An association between CNS involvement and diabetes insipidus has been reported in AML with monosomy 7 and inv16 (Castagnola et al., 1995).

Granulocytic sarcomas. Also known as sarcomas or chloromas, myeloid sarcomas are tumours composed of myeloblasts, monoblasts or megakaryocytes. These tumours can occur as extramedullary masses without evidence of leukaemia in BM or peripheral blood (nonleukaemic myeloid sarcomas) or in combination with leukaemia. When they appear as isolated lesions they are usually chloromas and considered extranodal lymphoma, because of the biopsy appearance similar to lymphoid cells. When myeloid sarcoma is the first manifestation of AML, the involvement of BM and blood appears after a few months. Theoretically myeloid sarcomas can have any location: skin, orbit, paranasal sinuses, bone, chest wall, breast, gastrointestinal tract, respiratory, genital-urinary, central nervous system, peripheral nervous system or lymph node (Hernández et al., 2002; Liesveld & Lichtman, 2006; Paţiu et al., 2008). Abnormalities involving chromosome 8 are the most common cytogenetic abnormalities in nonleukaemic sarcomas (Tsimberidou et al., 2003). Initially, these tumours were termed chloromas because of the green colour due to the presence of myeloperoxidase in myeloid leukaemic cells. Patients with AML with t(8;21) have a propensity to develop extramedullary leukaemia with poor outcomes after treatment.

2.2 Neonatal and childhood AML

A study conducted between May 1988 and June 2000 that included 698 children with AML tried to find the relationship between the age of onset of the disease, clinical characteristics and evolution. AML onset was observed at a very young age and is accompanied by intermediate risk cytogenetics (high incidence of 11q23 translocations). French-American-British (FAB) distribution is also based on age: types M5 and M7 are more common at

younger ages and types M0, M2, M3 more common in older children. In terms of clinical diagnosis, CNS involvement and digestive tract involvement is more common in very young children. Very young children develop severe diarrhoea, nausea and vomiting after chemotherapy (Webb et al., 2001). A recent study shows the impact of the undifferentiated form of AML-M0 on disease progression in children with AML with and without Down syndrome (DS), analyzing two clinical trials (Children's Cancer Group Clinical Trials AML). The main issues pursued were morphology and cytogenetics. Children with AML-M0 without DS had a lower number of leukocytes than patients with non-M0 AML and a higher incidence of del5, non constitutive trisomy 21 and hypoploidy. The analysis of AML in children without DS showed no differences between cases and non-M0 M0. Also there was no difference in evolution between children with DS and M0 and those with DS and non-M0 AML (Barbaric et al., 2007).

Four AML related syndromes are described in newborns:

- Transient myeloproliferative disease that may be present at birth or immediately after in about 10% of children with DS. The syndrome is followed shortly by acute leukaemia, usually myeloid (less common lymphoblastic).
- Transient leukaemia. 25% of children with DS and transient leukaemia develop AML - M7 in the first 4 years of life.
- Congenital Leukaemia.
- Neonatal leukaemia. Children who develop leukaemia during the first weeks of life are often pale, with insufficient increase in weight, diarrhoea and lethargy. The presence of cytogenetic abnormalities involving chromosome 11 has an extremely unfavourable prognosis.

The last two syndromes can occur in children without DS, but 10 times less frequently than in those with DS. Leukocytosis, bone marrow and blood infiltration with blasts, hepato-splenomegaly, thrombocytopenia, anaemia, purpura and skin infiltrates are common manifestations. Unfortunately, these children do not survive more than a few weeks or months. The disease can be highlighted during the prenatal period because cytogenetic abnormalities appear and mark the leukaemic clone. Monocytic differentiation of leukaemia and t(4;11) are common features. A case was even reported of transplacental transmission of acute monocytic leukaemia (Liesveld & Lichtman, 2006).

2.3 Elderly AML

AML in the elderly is a biologically distinct clinical entity. AML is generally a disease of old age, because the diagnosis is usually made in the decade of 60-70 years. The unfavourable course of the disease is due to biological characteristics at this age and various associated co-morbidities. In the United States the elderly population is the fastest growing segment, the average age at diagnosis of AML being 67 years (Melchert, 2006). Some data indicate that the disease develops from haematopoietic precursors that are in an early stage of maturation and may thus involve more than one haematopoietic line. This could explain the clinical and biological behaviour of the disease and prolonged neutropenia after chemotherapy. In addition, a large number of blasts express drug resistance glycoprotein - MDR1 and the incidence of unfavourable cytogenetics is high (7-, 5-). These factors, rather than age itself, are responsible for the unfavourable evolution of the disease. Compared to younger ages AML, AML in the elderly often derives from a previous haematological disease or after treatment for another malignancy. The morphological signs of dysplasia are frequently

observed (Ferrara & Pinto, 2007; Hiddemann et al., 1999). Many of these patients cannot cope with intense chemotherapy and its complications. The acute toxicity of chemotherapy is greater in patients with chronic heart, lung, liver or kidney disease. For example, age-related reduction of left ventricular ejection fraction limits the use of anthracyclines or mitoxantrone. Cardiotoxicity can occur at any time during mitoxantrone therapy and the risk increases with cumulative dose. Congestive heart failure, potentially fatal, may occur either during therapy with mitoxantrone or months to years after termination of therapy. The risk of symptomatic congestive heart failure was estimated to be 2.6% for patients receiving up to a cumulative dose of 140 mg/m^2. Elderly patients also have reduced regenerative capacity of BM, even if the cytoreduction treatment was successful. Their inability to tolerate long periods of pancytopenia, malnutrition and toxicity of aminoglycosides or amphotericin are major barriers to successful treatment (Rathnasabapathy & Lancet, 2003).

3. Laboratory investigations

3.1 Complete Blood Count (CBC)

BM infiltration by leukaemic cells is almost invariably accompanied by anaemia and thrombocytopenia, absolute neutrophil count being low or normal depending on the total number of leukocytes.

Although CBC is a routine investigation, it has not lost its relevance in the diagnosis of haematological diseases and hence in AML. CBC helps to highlight the three major complications in AML: infections (due to neutropenia), anaemia (low value of haemoglobin, low red blood cells count) and bleeding (due to thrombocytopenia).

The first clue for the diagnosis of AML is an anomalous result of the total number of leukocytes. Between 5-20% of patients may present with a very large number of cells (> 100 x 10^9/L). Although leukocytosis is a frequent feature, AML may also present with a normal leukocyte count and only a low number of platelets and erythrocytes, or even leukopenia (aleukaemic forms of AML).

The blast cells may be counted as lymphocytes or monocytes by the automated haematology analyzers and frequently not counted at all. Another important issue is that the analyzers cannot differentiate between myeloblasts and lymphoblasts. Eosinophilia and basophilia may be present in some subtypes of AML.

CBC is used after the diagnosis to monitor disease progression and also has prognostic impact. It is known that the increased number of leukocytes (> 30000/microL or number of blasts > 15000/microL) and a very low number of platelets (< 30000/microL) are factors of poor prognosis in AML.

3.1.1 Hyperleukocytosis and leukostasis

Leukaemic cells are considerably less deformable than mature myeloid cells. With the increasing number of blasts in PB, leukocytosis (total leukocyte count > 10000/microL) or hyperleukocytosis (total leukocyte count > 100000/microL) appears and microcirculation is threatened by the formation of caps from these rigid cells. Local hypoxemia may be exacerbated by increased metabolic activity of blasts and by the production of various cytokines. These events lead to impaired endothelial integrity and haemorrhage occurring in the existing fund of hypoxia. 5% of patients with AML develop signs and symptoms due

hyperleukocytosis. Circulation of the central nervous system and of the lungs is the most susceptible to the effect of leukostasis. Cerebral haemorrhage due to vascular occlusion is the most aggressive manifestation.

3.1.2 Hypoplastic leukaemia

10% of AML patients have pancytopenia, often with no blasts in peripheral blood, without hepato-splenomegaly. Three quarters of these patients are men aged over 50 years. A BM biopsy shows a hypocellular aspect, but blasts present in a ratio of 15-90%. Hypoplastic leukaemia must be distinguished from aplastic anaemia and hypoplastic myelodysplastic syndrome. Diagnosis is made on the presence of ≥ 20% blasts in the hypocellular marrow. A history of toxic exposure (chemicals, alcohol or chemotherapy for another malignancy) was demonstrated in approximately 20% of these cases (Gladson & Naeim, 1986).

3.1.3 Oligoblastic leukaemia

In 10% of cases, usually in patients aged over 50 years, AML manifests with anaemia and thrombocytopenia, white blood cell count is low, normal or increased. The proportion of blasts present in peripheral blood varies from 0-19% and between 3-20% in the BM. These cases are classified either as oligoblastic myeloid leukaemia or myelodysplastic syndrome, especially refractory anaemia with blasts excess. The disease has high morbidity and mortality through infections and bleedings.

3.1.4 Red blood cell count

Anaemia is almost always present in AML, because of inadequate production of RBC in the BM and shortened lifespan. Anisocytosis and poikilocytosis are variously reflected in erythrocyte indices and Price Jones curve. A vicious chain develops when bleedings occur, but the BM is not able to produce new erythrocytes. The reticulocyte number is usually low.

3.1.5 Platelet count

The platelet count is usually low, with different degrees of thrombocytopenia. In rare cases the platelet number can be normal. The mechanisms of thrombocytopenia are inadequate production of platelets in the BM and shortened lifespan. The platelets are usually big in size as shown by increased medium platelet volume on CBC.

3.2 Blood smear

Diagnosis and classification of AML is becoming increasingly complex. Current classifications refer to morphological features, immunophenotype and genetics in order to classify the different subtypes of leukaemia. Still, a competent and rigorous microscopic examination remains essential for diagnosis of AML.

Presumptive diagnosis of AML can be made by examining the peripheral smear, where leukaemic blasts are circulating in peripheral blood, but definitive diagnosis is made by examination of the aspirate or biopsy of BM. Classic May-Grunwald-Giemsa staining of peripheral blood and BM are used. Currently the diagnosis of AML is based on the evidence of 20% blasts in BM. In some cases, if the condition of patient does not allow the puncture of BM or biopsy and if there is evidence of 20% blasts in peripheral blood, the diagnosis can be made. On the other hand, we must not forget that some patients do not have blasts in their blood, so we could erroneously conclude it is not leukaemia. In such cases, if there is a

suspicion of haematologic malignancy (anaemia, thrombocytopenia) the examination of BM is obligatory.

In the past 30 years the classification of AML has been done after the FAB system (French-American-British Cooperative Group classifications of AML), based on morphological and cytochemical criteria and includes eight types of AML: M0-M7.

- M0 (AML with minimal differentiation)
- M1 (AML without maturation)
- M2 (AML with maturation granulocyte)
- M3 (promyelocytic) or acute promyelocytic leukaemia
- M4 (acute myelomonocytic leukaemia)
- M4Eo (with BM eosinophilia)
- M5 monoblastic acute leukaemia (M5a) or acute monocytic leukaemia (M5b)
- M6 (acute erythroid leukaemia) that includes erythroleukemia (M6a) and very rarely pure erythroblast leukaemia (M6b)
- M7 (acute megakaryoblastic leukaemia)

Blast morphology is considered of three types based on the cytoplasmic content in azurophilic granules: type I myeloblasts with no cytoplasmic granules, type II myeloblasts with less than 20 azurophilic granules and type III myeloblasts with more than 20 azurophilic granules in their cytoplasm. Type II and III may also contain Auer rods (Naeim & Rao 2008). The percentage of Auer rods recognized by Wright-Giemsa (WG) staining was 20.8%, but three times higher by peroxidase staining techniques (Jain et al., 1987). In peripheral blood a variable number of blasts are present and not related with the number of myeloblasts in the BM. Sometimes the BM is highly infiltrated, even if we have few blasts in the blood. Usually, blast morphology in the peripheral blood is in concordance with the BM, although sometimes differences may occur (differential diagnosis with acute lymphoblastic leukaemia, some lymphomas). So, both attentive examination of blood smear and BM are needed.

Red blood cells morphology is variously affected, with large and small erythrocytes (anisocytosis) and different shapes especially if the leukaemia developed from a myelodysplastic syndrome (ovalocytes, tear drop erythrocytes). Erythroblasts and stippled erythrocytes may also be present.

Thrombocytes may be giant or with granulation abnormalities (usually hypogranulated).

Different dysplastic changes may be present if AML has undergone transformation from myelodysplastic syndrome: hyper/hypo granulation or hyper/hypo segmentation of granulocytes.

3.3 Bone marrow examination

Currently, the diagnosis of acute myeloid leukaemia is based on the presence of a minimum of 20% blasts in the BM.

By the term blasts we understand myeloblasts, promonoblasts, monoblasts, megakarioblasts or promyelocytes. According to the World Health Organization (WHO) 2008, an exception from the 20% rule is possible, if there is evidence of AML with recurrent abnormalities: AML with t(8;21)(q22;q22), inv(16)(p13.1q22) or t(16;16)(p13.1;q22) and APL with t(15;17)(q22;q12) are considered as acute leukaemia regardless of blast count in the blood or BM, but in contrast to the previous edition, for AML with t(9;11)(p22;q23) or other 11q23 abnormalities, as well as for all other subgroups (except the rare instance of some cases of

erythroleukemia) blasts of 20% or more of white blood cells in peripheral blood (PB) or of all nucleated BM cells is required for the diagnosis of AML.
The former FAB morphologic entities are now included as acute myeloid leukaemia not otherwise specified:

AML with minimal differentiation (FAB M0)

Medium-sized blasts with no signs of differentiation, with fine nuclear chromatin and agranular cytoplasm. Sometimes blasts are small, resembling lymphoblasts. The BM is usually hypercellular and survival is poor.

AML without maturation (FAB M1)

In the BM we usually find above 90% myeloblasts from nonerytroid cells with few signs of differentiation. Blast may have azurophilic granules and/or Auer rods, but most are agranular blasts. Sometimes blasts look like lymphoblasts and BM is typically hypercellular. The literature cites cases of AML - M1 with mirror cells (although they are most commonly found in acute lymphoblastic leukaemia) and even with Auer rods (Casasnovas et al., 2003).

AML with maturation (FAB M2)

This category represents 30-45% of AML. It is also the most frequent AML in children. We find 20% or more myeloblasts in the blood or BM and 10% or more neutrophils in various stages of maturation (promyelocytes, myelocytes and metamyelocytes). Monocytes represent less than 20% of BM cells. Myeloblasts can be with or without azurophilic granules and Auer rods. Abnormal nuclear segmentation of neutrophils and increased number of eosinophilic precursors are frequent. The BM is usually hypercellular. In some cases the immature cells have abundant cytoplasm and basophilia, with a variable number of granules, sometimes indistinguishably, sometimes coalescent, making difficult the difference between M1 and M2.

Acute promyelocytic leukaemia (FAB M3)

In the classic form the predominant cells are abnormal promyelocytes with many primary granules. Auer rods are frequent and often occur in bundles. In the mycrogranular version (M3v), leukaemic cells have monocytic aspect with cleaved nuclei and abundant cytoplasm with indistinguishable granules.

Acute myelomonocytic leukaemia (FAB M4)

This category accounts for between 15-25% of AML. Some patients have a history of chronic myelomonocytic leukaemia. It is characterized by the proliferation of neutrophilic and monocytic precursors, 20% or more myeloblasts, monoblasts and promonocytes being needed in the BM nucleated cells to distinguish between chronic myelomonocytic leukaemia and AML, and 5 x 10^9/L or more blood monocytes.
Monoblasts are large size, with round nuclei, abundant cytoplasm and prominent nucleoli, and sometimes have fine azurophilic granules. There is the eosinophilic variant (M4Eo), in which eosinophils are increased in number (>5%) and this variant is associated with chromosome 16 abnormalities.

Monoblastic acute leukaemia (FAB M5a) or acute monocytic leukaemia (FAB M5b)

This is characterized by a percentage exceeding 80% of leukaemia cells of monocytic type: monoblasts, promonocytes and monocytes. The two differ in the relative proportions of

monoblasts and promonocytes. If most cells are monoblasts, it is M5a type (usually over 80%), and if most cells are promonocytes, it is M5b type.
Monoblastic acute leukaemia is characterized by large basophilic monoblasts, with abundant cytoplasm, with formation of pseudopods, round nuclei with one or more prominent nucleoli. Rarely Auer rods are observed. BM is hypercellular with an increased number of monoblasts.
Monocytic acute leukaemia is characterized by the presence of promonocytes with irregular nuclei, with moderate basophilic cytoplasm and azurophilic granules. The erytrofagocitosis phenomenon can be seen.
From a clinical point of view, M4 and M5 subtypes are accompanied with signs of medullary and extramedullary involvement: fever, fatigue, haemorrhage, gingival hyperplasia, hepato-splenomegaly, lymphadenopathy and involvement of the nervous system. Because the involvement of the central nervous system is frequent, the examination of cerebrospinal fluid is recommended, even if no clinical signs of involvement are present.

Acute erythroid leukaemia (FAB M6)

This includes erythroleukemia (M6a) and very rarely pure erythroblast leukaemia (M6b). The two types are characterized by the presence of a predominant erythroid population and in the case of M6a by the presence of an important myeloid component. M6 may be present de novo or evolve from a myelodysplastic syndrome. Occasionally, some cases of chronic myeloid leukaemia may develop into M6. Pancytopenia is a common feature.
Over 50% of nucleated cells of the MO are abnormal erythroblast. Displastic changes in erythroblasts may be important: giant forms, multinucleated, cytoplasmic vacuolation and megaloblastoid change.
Erythroleukemia (M6a) is characterized by 50% or more erythroid precursors from nucleated cells in the BM, 20% or more myeloblasts from non erythroid population in the BM, displastic erythroid precursors, with megaloblastoid nuclei and multinucleated erythroid cells. Dysplasia is also seen on the megakaryocytic line. Myeloblasts are of medium size, occasionally with Auer rods. Ringed sideroblasts can be present and the BM is usually hypercellular. This morphologic type represents the majority of acute erythroid leukaemias. Pure erythroblast leukaemia (M6b) is characterized by medium and large sized erythroblast with round nuclei, fine chromatin, one or more nucleoli, intense basophilic cytoplasm and occasionally coalescent vacuoles.

Acute megakaryoblastic leukaemia (FAB M7)

This represents 3-5% of AML, blast cells appertaining to the megakaryocytic line. It is characterized by cytopenia, displastic changes of neutrophils and platelets.
Megakarioblasts are of medium to large size, with incised or round nuclei and with one or more nucleoli. Cytoplasm is basophilic, agranular with pseudopods. In some cases lymphoblast-like morphology (increased nuclear/cytoplasmic ratio) has been reported. We can frequently see in blood micromegakaryocytes circulating, fragments of megakaryocytes, large and displastic platelets and hypogranulated neutrophils. BM is often fibrous and BM puncture may be white (blinded). It presents with two peaks according to age: in children 1-3 years old related with Down syndrome and in adults. According to 2008 WHO classification, myelodysplastic syndrome and AML related to Down syndrome are biologically identical and considered as myeloid leukaemia associated with Down syndrome.

Acute basophilic leukaemia

This is a form of AML with primary differentiation to basophils. It is a rare, representing 1% of AML. It is accompanied by secondary signs of hyperhistaminemia, circulating blasts, organomegalia and BM failure. Morphologically it is characterized by medium-sized blasts with high nuclear/cytoplasmic ratio, round, oval, bilobate nucleus with one or more nucleoli, moderate basophilic cytoplasm containing a variable number of basophilic granules, red cell dysplasia and hypercellular BM.

Acute panmyelosis with myelofibrosis

This condition can occur at any age, de novo or after treatment with alkylating agents and/or radiation. There is an acute proliferation associated with fibrosis of BM. It is characterized by pancytopenia, marked anisocytosis, displastic change of myeloid line, hypercellular BM on the osteomedulary biopsy, varying degrees of hyperplasia of erythroid granulocytic, megakaryocytic precursors in BM, increased number of megakaryocytes with displastic changes and a marked increase in the number of reticulin fibres in BM.
There are even some overlapping features with M7 subtype, the distinction is that in M7 the predominant population of blasts is of megakaryocytic origin and in acute panmyelosis with myelofibrosis it is of non-megakaryocytic origin. The prognosis is unfavourable.

3.3.1 Particular morphological forms of acute myeloid leukaemia

Acute myeloid leukaemia with cup-like morphology

Various authors have attempted to characterize the morphology of AML with this special morphological appearance, raising questions as to whether this is a new disease entity or an artificial phenomenon (Barbaric et al., 2007; Benderra et al., 2005). Investigating the 'cup-like' morphology of 266 randomly selected patients with AML and association with haematological, immunological and prognostic parameters, it was found that this morphology was present in 21% of cases, was associated with the female sex, increased numbers of leukocytes and blast, normal karyotype, low expression of CD34 and HLA-DR. With regard to FLT3 mutations, NPM1 were found in 84.9% of cases, compared to 58.1% in cases without this morphology. Response to treatment and survival were not influenced in this study. Electronic microscopy showed that the cups contain lots of organelles. This particular form does not appear as a distinct category in any classification. It is sometimes difficult to do the differential diagnosis with acute promyelocytic leukaemia, the mycrogranular variant, in which neoplastic cells with prominent, bilobed nuclei can partially resemble blasts with cup-like nuclei. Immunophenotyping cannot identify the difference because both acute myeloid leukaemia with cup-like morphology and acute promyelocytic leukaemia are CD34- and HLA-DR-, with strong myeloperoxidase reaction.

3.4 Biochemistry

No specific biochemical pattern characterizes AML. Usually high activity of serum lactate dehydrogenase (LDH) is present. LDH is a biochemical marker reflecting tumour load and anaerobic glycolysis. When tissues are shifted from aerobic to the anaerobic glycolysis, LDH activity increases to accelerate the conversion of pyruvate to lactate, with the release of energy. At the molecular level, hypoxia induces expression of vascular-endothelial growth factor (VEGF) and fibroblasts growth factor (bFGF), and thus angiogenesis. Enhancing

angiogenesis is a phenomenon which was observed in AML. Studies have shown that the activity of serum LDH and not the concentrations of two growth factors (VEGF, bFGF) may be used as a parameter predictor for BM angiogenesis in AML (Teng et al., 2006). Another value of this marker is the prediction of tumour lysis syndrome (TLS), which usually occurs in patients with hyperleukocytosis. Despite the prophylactic use of allopurinol, morbidity and mortality related to tumour lysis syndrome (TLS) still occurs in some patients with AML. The criteria for tumour lysis syndrome are serum creatinine level over 1.4 mg/dL (normal range 0.5 to 1.4 mg/dL) and an increase of at least one of the following parameters: potassium > 5 mEq/L (normal range 3.7 to 5.0 mEq/L.), uric acid > 7.5 mg/dL (normal range 3.0 to 7.0 mg/dL), phosphate > 5 mg/dL (normal range 2.4 to 4.1 mg/dL) and calcium < 8 mg/dL (normal range 8.4 to 10.2 mg/dL). TLS can be a life-threatening complication in patients during induction therapy in AML. Characteristic laboratory changes may occur spontaneously before initiation of induction therapy due to catabolism and to increased turnover of leukaemic cells, but is most commonly induced by intensive chemotherapy. Yet few studies have focused upon TLS in AML, so the incidence and development of TLS in these patients are not well defined. A study that included 772 patients with AML treated with allopurinol and intense hydration showed that 17% of patients developed TLS. Multivariate analysis showed that pretreatment levels of LDH above the normal range, creatinine over 1.4 mg/dl, uric acid over 7.5 mg/dl and WBC count $25x10^9$/L, were independent prognostic factors for TLS (Montesinos et al., 2008). In children with AML, life-threatening pulmonary complications were cited in combination with TLS and mimic systemic inflammatory response syndrome (SIRS). Severe SRSI is more common in association with monocytic and myelomonocytic AML (M4, M4Eo, M5), especially in M4Eo.
A mild or moderate increase of uric acid plasma level is frequent, especially in monocytic and myelo-monocytic AML.
Changes of ions concentrations (sodium, potassium, calcium, hydrogen) are mild or moderate and infrequent. Hypokalemia is the most frequent finding at presentation, related to renal tubular dysfunction, and artifactual increase of potassium is associated in vitro in patients with hyperleukocytosis. Hypocalcaemia can appear as a result of multiple mechanisms, as direct skeletal invasion by malignant cells, ectopic parathyroid hormone (PTH) production or bone-resorbing cytokines. Hypophosphataemia as a result of leukaemic cell up-take, also can occur. Hyponatremia and lactic acidosis as presenting features of AML are rare. Hyponatremia is proposed to be due to inappropriate production of antidiuretic hormone by the leukaemic cells. There has been no well defined cause for lactic acidosis due to leukaemia per se, but probable explanations are due to anaerobic glycolysis by leukaemic cells and due to increased blast count with its attendant leukostasis (Udayakumar et al., 2006).
Various abnormalities of coagulation are met in AML: decrease in α2 antiplasmin, antithrombin III and fibrinogen. Especially in promyelocytic acute leukaemia, there is a high risk for disseminated intravascular coagulation, because of procoagulants released from the cytoplasmic granules.

3.5 Cytochemical stains

Cytochemical stains with full analysis of blood and careful morphological examination of peripheral smear and BM help to classify most cases of AML. Research into signs of dysplasia is important work and it is hard to quantify cell dysplasia when the line is poorly represented (Braham-Jmili et al., 2006).

Cytochemical stains that characterize each morphological subtype are the following:
M0 (AML with minimal differentiation)
- Myeloperoxidase staining is negative (MPO)
- Sudan Black staining is negative (SBB)
- Naphthol chloroacetate esterase staining is negative (<3% positive blasts)
- Alpha naphthyl acetate and butyrate esterase staining are negative
- Periodic acid Schiff (PAS) staining is negative
- This subtype can only be diagnosed using flow cytometry

M1 (AML without maturation)
- 3% or more of the blasts positive for MPO and SSB
- PAS is usually negative

M2 (AML with maturation granulocyte)
- A large number of blasts are MPO positive
- PAS is usually negative

M3 (promyelocytic) or acute promyelocytic leukaemia
- Blasts are MPO and chloroacetate esterase positive
- The hypogranular variant behave similarly regarding the cytochemical stains

M4 (acute myelomonocytic leukaemia)
- MPO positive in at least 3% of blasts
- Monoblasts, promonocytes and monocytes are typically nonspecific esterase (NSE) positive

Monoblastic M5 acute leukaemia (M5a) or acute leukaemia monocytic (M5b)
- Typically NSE is strongly positive
- MPO is negative, but the MPO may occasionally be positive in M5b
- Lysozyme is positive

M6 (erythroid acute leukaemia)
- Red cell precursors are PAS positive
- Blasts are MPO, SSB negative, but may be positive for NSE

M7 (acute megakaryoblastic leukaemia) (23)
- Stains are negative for MPO and SSB
- Blasts can be PAS and NSE positive
- This subtype can only be diagnosed using flow cytometry

Acute basophilic leukaemia (26)
- Blasts are acid phosphatase positive
- MPO, SBB, NSE are negative

Acute panmyelosis with myelofibrosis
- In some cases blasts may be MPO positive

Granulocytic sarcoma
- Tumour cells may express myeloid associated molecules in the biopsies, such as MPO, NSE or lysozyme.

3.6 Immunophenotyping

Flow cytometry is a technique used for counting, examining and sorting microscopic particles suspended in fluid. It also allows multiparametric analysis of the physical and/or chemical characteristics of a single cell passing through an optical and/or electronic detection device. Immunophenotyping is an essential technique for the diagnosis,

classification, staging and monitoring of leukaemia. In the last 10 years, expanding monoclonal antibodies (MoAb) and flurochromes allow differentiation of normal cell populations, of leukaemic cells, defining the state of maturation and recognition of aberrant phenotypes.

Despite recommendations for standardization for multiparametric flow cytometry (Bene et al., 1995; Rothe & Schmitz, 1996; Stewart et al., 1997; Bain et al., 2002), the number, specificity and combination of reagents used for diagnosis and classification of acute leukaemia in different laboratories are varied. A study showed that using combinations of four MoAb from a minimum panel of 13 MoAb and CD45/sideward scatter gating strategy, achieved the correct classification in 97.2% of cases of acute leukaemia (155 patients), of which 79 were AML (Ratei et al., 2007).

To monitor minimal residual disease (MRD), application of five-colour flow cytometry is more appropriate, enhancing sensitivity and precision of the method (Bacârea et al., 2007; Voskova et al., 2007). Another study also demonstrated that multiparametric flow cytometry in five colours is useful for the detection of leukaemia associated phenotypes in BM of patients with de novo AML and detection of MRD. Another study showed that the six-colour cytometry allowed for the identification of leukaemia associated phenotypes that are not expressed in normal BM or postchimiotherapy and can be used successfully to monitor the MRD. The practical relevance of the multicolour approach is the ability to detect patients at high risk of relapse (Olaru et al., 2008).

Immunophenotyping of acute leukaemia cells after density gradient separation is currently the gold standard, but the destruction of red blood cells after whole blood lysis and direct marking is a widespread and used procedure. In addition, data show that for both methods, the mean expression of antigens being tested was similar: CD4, CD7, CD11b, CD11c, CD13, CD14, CD15, CD33, CD34, CD65s, glycophorin A, HLA-DR (Schwonzen et al., 2007). For manual counting, blasts represent the percentage of total nucleated cells. For flow-cytometric studies, the lysis step for removing erythrocytes, removes a variable number of red cell precursors. So, the obtained values determined by flow cytometry are a percentage of all analyzed cells or all nonerytroid cells. These differences also affect the use of flow cytometry to assess erythroleukemia, the erythroid/myeloid type, where the criteria require more than 50% red cell precursors of the total population of nucleated cells and above 20% myeloblasts from the nonerytroid population. Using cell separation with Ficol also leads to alteration of cell proportions and is strongly discouraged. On the other hand, blasts can be difficult to recognize on morphological examination, or are destroyed during preparation of blood or BM smears. So it is prudent to perform both immunophenotyping and morphological blast count (Craig & Foon, 2008).

Usually, the expression of an antigen is considered positive if 20% or more blasts react with a specific antibody. Blast cells can be distinguished from myeloid precursors through the expression of immature markers CD34, CD117 and lack of maturity markers CD11b, CD15, CD16. Some blasts are negative for CD34 and CD117, and are difficult to distinguish from more mature cells. For example, it is difficult to distinguish mature monocytes from CD34 negative monoblasts. Therefore, even if it is tempting, it is preferable not to make the selection of blasts according to CD34. CD117 antigen is expressed on the blasts, but also on mast cells. Mature myeloid cells when hypogranulated may fall below on the side scatter and may fall into the blasts window on CD45/side blasts scatter plot.

The advantages of flow cytometry are given by the possibility of quick analysis of several thousand cells, multiparametric analysis. It also allows the assessment of aberrant markers and mixed phenotypes and investigation of MRD. Disadvantages are the costs (an expensive device and antibodies), the panels that can change, problems of interpretation and the fact that the technique does not allow diagnosis of acute leukaemia, which is cytological.
To standardize the work in specialized laboratories it is recommended an initial assessment of the line and then a secondary assessment. In the attempt to define the optimal number of markers to determine the immunophenotype in acute leukaemia with a sensitivity of 95%, some recommended markers for AML are: myeloperoxidase (MPO), CD33, CD13, CD14, CD15, CD117, CD34 (Lee et al., 2006). Other authors recommend wider panels with specific antibodies: CD13, CD14, CD15, CD33, CD64, CD117, CD36, MPO and antigens associated with haematopoietic cell maturation (CD34, CD38, TDT) and myeloid antigens (CD16, CD66). In addition, it is recommended to use other auxiliary markers in determining non-specific antigens: CD7, CD19 and CD56 are very useful to monitor residual disease (Woźniak & Kopeć-Szlęzak, 2008).
AML is regarded as a stem cell disease. In AML CD34 + leukaemic stem cells are recognized as CD38-. This CD34 + CD38- population survives chemotherapy and is most likely the cause of residual disease (MRD - with poor prognosis), which will then lead to relapse. Thus, by showing CD34 + CD38- malignant cells after chemotherapy, detection of MRD at stem cell level is possible (van Rhenen et al., 2007).
Based on antigen positivity we can establish different immunological profiles:

- Myeloblastic – CD13, CD33, CD117, CD15, HLA-DR usually positive
- Myelomonocytic – CD11, CD13, CD33, CD14, HLA-DR usually positive
- Erythroblastic – Glycophorin, spectrin, carbonic anhidrase I, HLA-DR usually positive
- Promyelocytic – CD11, CD13, CD33, CD15 usually positive
- Monocytic – CD11, CD13, CD33, CD14, HLA-DR usually positive
- Megakaryoblastic – CD34, CD41, CD42, CD61, von Willebrand factor

For practical reasons it is necessary that FAB classification and immunological profile correspond.
M0 (AML with minimal differentiation)

- CD 34 and HLA-DR usually positive, but CD38 is negative in most cases
- Myeloid associated antigens often positive – CD13, CD33, CD117
- About half of the cases express TdT and/or CD7
- Monocytic markers are usually negative
- Occasionally the blasts may aberrantly express CD10, CD19, CD2, CD56
- Lack of lymphoid antigen expression: cyCD3 for T line, cyCD79 and cyCD22 for B line

M1 (AML without maturation)

- Myeloid associated antigens often positive – CD13, CD33 and CD117
- CD34, HLA-DR. cyMPO are often positive

M2 (AML with maturation granulocyte)

- Myeloid associated antigens often positive – CD13, CD33 and CD117
- CD34, HLA-DR, cyMPO are often positive
- Occasionally the blasts may aberrantly express CD56, CD19
- Monocytic markers are usually negative

M3 (promyelocytic) or acute promyelocytic leukaemia

- Leukaemic promyelocytes express strongly for MPO and SSB, and also for CD9, CD13, CD33

- CD34, HLA-DR are negative
- Sometimes it is difficult to make a differential diagnosis between acute promyelocytic leukaemia, the hypogranular variant and acute myeloid leukaemia with cup-like morphology, because it is also characterized by the immunophenotype: CD34-, HLA-DR-. Also, CD7 is usually negative and myeloid markers are often positive (CD13, CD33, CD117, myeloperoxidase).

M4 (acute myelomonocytic leukaemia)

- Myeloid antigens CD13, CD33 are often positive
- Partial expression of CD34, MPO, CD11c, CD36, CD64, CD117, HLA-DR
- Aberrant expression of CD2, CD7, CD56
- CD14 may have partial expression or sometimes may be negative

M5 (monoblastic acute leukaemia - M5a or acute monocytic leukaemia - M5b)

- Monocytic markers are usually positive: CD11c, CD36, CD64, CD14
- HLA-DR is positive
- A small proportion of blasts express CD13, CD33, CD117, MPO (weak)
- CD34 is usually negative
- Aberrant expression of CD56
- High levels of CD64 expression distinguish AML subtype - M5, but low expression of CD64 by itself does not distinguish between subtypes of AML M4 and M5. Some authors consider the association of CD68 and CD11b useful to differentiate M5a and M5b FAB subtypes, given that CD68 and CD11b expression are much higher in M5a than in M5b (Pagano et al., 2005).
- When immuphenotyping cannot be done successfully (inadequate smears, white puncture) immunohistochemistry can identify the monocytoid component. The usefulness of CD163 (scavenger for haemoglobin molecule present on monocytes/macrophages) is well established (Garcia et al., 2008).

M6 (erythroid acute leukaemia)

- Erythroid precursors are usually positive: glycophorin, CD71
- CD34, HLA-DR are negative
- Myeloid, monocytic markers are negative

M7 (acute megakaryoblastic leukaemia)

- Platelet glycoproteins are positive both on the surface and intracytoplamatic – CD41, CD42, CD62
- Sometimes megakaryoblasts may express CD36, CD7
- HLA-DR is often negative

Acute basophilic leukaemia

- Some myeloid markers may be positive – CD13, CD33
- CD34, HLA-DR are usually positive

Acute panmyelosis with myelofibrosis

- CD34 and myeloid antigens CD13, CD33, CD117, MPO are often positive
- A small proportion of blasts express platelets associated antigens

Granulocytic sarcoma

- Sarcomas are a mixture of immature and mature myeloid cells. The marker's presence is based on the balance between the two.

Phenotype in myeloid leukaemia associated with Down syndrome

- Usually Down syndrome is associated with megakaryoblastic acute leukaemia, being CD41, CD42, CD62 positive.

Different studies have tried to correlate immunophenotype with cytogenetic profile and clinical manifestations, showing that karyotype abnormalities and clinical manifestations are closely related to abnormal antigen expression in AML (Plesa et al., 2008; Thalhammer-Scherrer et al., 2002; Zheng et al., 2008):

- Co-expression of CD19 was found in subtypes M0, M1 and M2.
- The expression of CD14 is associated with subtypes M4, M4Eo, M5b, accompanied by poor outcome, low complete remission rate and shorter survival.
- Expression of CD7 was found in subtypes M0, M1, M2, M4 and most frequently in M5a.
- Expressions of CD22, CD56, TDT were correlated with the presence of abnormal karyotype.
- t(8;21) was present in M2 and strongly associated with expression CD15/CD19/CD34/CD56.
- In the M3, although lymphoid markers were detected in a considerable number of cases, they were not highlighted in any patient with t(15;17).
- In M4, CD2 and CD34 expression was associated with abnormal karyotype. CD2 expression was higher in the M4Eo version, but had no correlation with inv(16). Other studies indicate the presence of CD2 in M4Eo and M3variant.
- In M5 there was a higher expression of CD14 and CD56.
- The expressions of CD4, CD7, CD14, CD56, TDT were correlated with clinical features: increased numbers of leukocytes, platelets and patient age.
- The few studies investigating AML-M7 confirm the high heterogeneity of this subtype. Cytogenetic abnormalities in adults are frequently those of secondary leukaemia and few of them have a history and morphology with dishematopoesis. In children, besides the famous Down syndrome (DS) associated M7, t(1;21) is characterized by young age of onset, female sex, tumour presentation and low percentage of blasts in BM, sometimes without megakaryoblastic marrow involvement, but always with dismegakaryopoesis associated with micromegakaryocytes. It appears that these children generally respond well to intensive chemotherapy (Duchayne et al., 2003).

3.6.1 Flow cytometry and minimal residual disease (BMR)

It is known that flow cytometry can be used not only for diagnosis of AML, but also to monitor the BMR. Two highly sensitive methods, multiparametric flow cytometry and real-time quantitative PCR (RQ-PCR), are widely used to monitor the BMR and disease management. Multiparametric flow cytometry is particularly useful for investigating the early clearance of blasts, and blast count after consolidation therapy. Later, BMR levels quantified by RQ-PCR in cases of AML with fusion gene had the highest prognostic power, the sensitivity of RQ-PCR being between 10^{-4} - 10^{-7}. Both methods are able to detect early disease relapse. Multiparametric flow cytometry may be used for most patients, however, to be successfully applied, two concepts have emerged which should be carefully weighed up: to include only those leukaemia associated phenotypes that are absent in normal BM, respectively do not consider cases with less aberrant immunophenotypes for MRD monitoring. In most cases the phenotype at diagnosis is the same at relapse. But, this may be true for only a part of the leukaemic cells and the intensity of expression and aberrantly expressed antigens may change (Kern et al., 2008):

- Lymphoid antigen expression (e.g. CD33+/CD2+/CD34+, CD34+/CD13+/CD19+)
- Antigenic overexpression

(e.g. HLA-DR++/CD33++/CD34++, CD64++/CD4++/CD45++)
- Lack of antigens (e.g. HLA-DR-/CD33+/CD34+)
- Asynchronous antigen expression
(e.g. CD15+/CD33+/CD34+, CD65+/CD33+/CD34+)

3.7 Secondary acute myeloid leukaemia

Secondary AML is a poorly defined term that usually refers to the AML that develops after a history of myelodysplastic syndrome (MDS), myeloproliferative neoplasm or myelodysplastic/myeloproliferative neoplasm (MDS/MPN). The 2008 WHO classification defined the cases with a history of MDS or MDS/MPN and have evolved to AML, or cases that have a myelodysplasia-related cytogenetic abnormality, or at least 50% of cells in two or more myeloid lineages that are dysplastic as myelodysplasia-related changes. Some cases previously assigned to the subcategory of AML not otherwise specified as acute erythroid leukaemia or acute megakaryoblastic leukaemia may be reclassified as AML with myelodysplasia-related changes (Vardiman et al., 2009). Secondary AML may occur after chemotherapy with alkylating agents or topoisomerase II inhibitors, after radiation or exposure to environmental carcinogens. The 2008 WHO classification classifies cases after use of alkylating agents or topoisomerase II inhibitors as therapy-related myeloid neoplasms. The question is whether secondary AML itself is associated with poor prognosis or whether this is due to association with some morphological and biological characteristics. Dysplasia in de novo AML is related to unfavourable prognosis, but has no prognostic relevance under intensive therapy. Since there is no correlation between cytogenetic risk subgroups and dysplasia, cytogenetics continues to have proven impact in both de novo AML and secondary AML. Cytogenetic abnormalities spectrum in secondary AML is similar to de novo AML, but the frequency of unfavourable cytogenetic abnormalities associated with high risk and intermediate risk (complex karyotype, trisomy 8, monosomy 7 and others) is higher in secondary AML. Survival of patients with therapy-induced AML is shorter than those with de novo AML within the same cytogenetic risk group. Genetic and molecular differences that determine the phenotype and prognosis of secondary AML still require several additional studies (Larson, 2007).

3.8 Acute leukaemias of ambiguous lineage

According to WHO 2008, the classification encompasses the following entities:
- Acute undifferentiated leukaemia
- Mixed phenotype acute leukaemia with t(9;22)(q34;q11.2); *BCR-ABL1*
- Mixed phenotype acute leukaemia with t(v;11q23); *MLL* rearranged
- Mixed phenotype acute leukaemia, B-myeloid, NOS
- Mixed phenotype acute leukaemia, T-myeloid, NOS
- Provisional entity: natural killer (NK) cell lymphoblastic leukaemia/lymphoma:

This is a rare subtype of acute leukaemia, which is much debated. Although the nature of NK cells is questionable, is a variant of leukaemia with distinct morphological features and immunophenotype (blasts express CD56, CD2, CD7 and are negative for B or myeloid antigens). The cases previously classified as 'blastic natural killer cell leukaemia/lymphoma' are now 'myeloid related blastic plasmacytoid dendritic neoplasm' (Vardiman et al., 2009).
The requirements for assigning more than one lineage to a single blast population in mixed phenotype acute leukaemia (MPAL) are presented in Table 2.

For myeloid lineage	Myeloperoxidase (flow cytometry, immunohistochemistry or cytochemistry) or monocytic differentiation (at least two of the following: nonspecific esterase, CD11c, CD14, CD64, lysozyme)
For T lineage	Cytoplasmic CD3 (flow cytometry with antibodies to CD3 epsilon chain; immunohistochemistry using polyclonal anti-CD3 antibody may detect CD3 zeta chain which is not T cell–specific) or surface CD3 (rare in mixed phenotype acute leukaemia)
For B lineage (multiple antigens required)	Strong CD19 with at least one of the following strongly expressed: CD79a, cytoplasmic CD22, CD10 or weak CD19 with at least two of the following strongly expressed: CD79a, cytoplasmic CD22, CD10

Table 2. The requirements for assigning more than one lineage to a single blast population in mixed phenotype acute leukaemia (MPAL).

The former European Group of Immunological Markers for Leukaemia (EGIL) scoring system to evaluate biphenotypic acute leukaemia (BAL) had limitations because of over-diagnosis of BAL, plus it ignored the cytogenetic data. Because of this, well defined genetic abnormalities could be classified as BAL. The new classification includes cytogenetics in the evaluation of MPAL. A lot of studies showed that when applying the 2008 WHO classification the number of MPAL decreased (BAL became ALL with aberrant myeloid markers or AML with aberrant lymphoid markers). The pitfall still remains the over-diagnosis of MPAL, because of misinterpretation of immunological studies (e.g. immunophenotyping for MPO). Regarding MPO, it is preferable, if possible, to have both immunophenotyping and cytochemistry to consider it positive. Care must be taken, for example, not to consider as MPAL the patients with t(9;22) in blast crisis with former chronic myeloid leukaemia (CML). Cases of BCR-ABL1 positive and MLL positive acute leukaemias may meet the criteria for MPAL (Vardiman et al., 2009).

3.9 Cytogenetics

Compared with the 2001 WHO classification, the number of recognized recurrent genetic abnormalities has grown. The current 2008 WHO classification recognizes the importance of recurrent genetic abnormalities, which are crucial for correct diagnosis and treatment of AML:

- AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
- APL with t(15;17)(q22;q12); *PML-RARA*
- AML with t(9;11)(p22;q23); *MLLT3-MLL*
- AML with t(6;9)(p23;q34); *DEK-NUP214*
- AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
- AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
- Provisional entity: AML with mutated NPM1
- Provisional entity: AML with mutated CEBPA

The detection of recurrent genetic abnormalities requires cytogenetics - karyotyping, fluorescence in situ hybridization (FISH) and molecular analysis - reverse transcriptase polymerase chain reaction (RT-PCR).

The new classification is helpful for clinicians because data can provide more prognostic significance. Cytogenetics currently provides the most important prognostic information

both at diagnosis and at relapse. Given the progress made in recent years on understanding disease pathogenesis, given the profile of the genes, new therapeutic targets will develop, with the hope that this new agents potentially will improve the disease (Avivi & Rowe, 2005). Cytogenetic abnormalities have a frequency of 85% in de novo AML and 95-100% in secondary AML, consistent abnormalities being classified as specific (constant) and nonspecific (random). They can be balanced (translocations or chromosomal rearrangements) and unbalanced (chromosome loss or acquisitions). The specific, balanced abnormalities are present with a higher frequency in a given morphological FAB subtype. There are evidences for more than 80 balanced chromosomal rearrangements: [t(8:21) in AML2, inv16 in AML4 eosinophilic variant, t(9:11) in AML5, t(11:19) in AML4]. Constant unbalanced abnormalities (-7, -5) appear in all morphological subtypes. Some of these abnormalities are good prognostic factors [t(8; 21) (Q22, Q22 - fusion AML1-ETO), t(15; 17) (Q22, Q12 ~ 21 - fusion PML-RARA), inv (16)(p13q22) / t (16; 16) (p13; Q22 - CBFB-MYH11 fusion)], and others poor prognostic factors (5 -, 7 -, 5q-, 7q-, trisomy 8, trisomy 11, t (6, 9) or combinations). AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1* is a common leukaemia usually associated with AML with maturation morphology and sometimes with myelomonocytic morphology. Auer rods are frequent. Eosinophilia and basophilia are common features. Displastic changes and association with myeloid sarcomas may also been seen. AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* may be associated with other cytogenetic abnormalities like trisomy 21, 8, loss or deletion of Y chromosome. It is associated with BM eosinophilia and lymphadenopathy. APL is one variant of AML associated with t(15;17). It is the retinoic acid receptor α gene (RARα) that is affected. In acute promyelocytic leukaemia, the variant RARA translocations with other partner genes should be considered separately, because of different features and different responses to treatment. Generating chimerical fusion proteins from chromosomal translocations results in blocking cell differentiation and contributes to the biological characteristics of different subtypes of leukaemia, representing the primary event in the pathogenesis of AML. In recent years it has become clear that other mutations have a role in pathogenesis:

- Mutations of genes encoding transcription factors involved in haematopoiesis (often in AML1-M0, CEPBA - common in M1, M2)
- Mutations of genes that encode components of the signal translation pathways, mutations in the tyrosine kinase receptor (FLT3 gene - confers proliferative advantage)
- Mutations in genes that encode nucleophosmin (NPM1 - nucleocytoplasmatic protein involved in regulating gene expression - 35% of cases of AML) (Dohner et al., 2005; Falini et al., 2005; Schnittger et al., 2005)

Extensive studies have proposed a system of risk stratification based on cytogenetics in AML patients who fall into three risk categories: favourable, intermediate and poor. The information obtained from diagnosis is used to further stratify therapy. For patients with abnormal karyotype before treatment, cytogenetic analysis has been recommended to document complete remission (CR), based on data showing that the persistence of even a single metaphase with an abnormality present at diagnosis leads to significantly higher incidence of relapse (Cheson et al., 2003). Because multilineage dysplasia is not associated with independent prognostic significance when cytogenetics is taken into account, the 2008 WHO classification named this group 'AML with myelodysplasia-related changes'. The cytogenetic abnormalities sufficient for diagnosis of AML with myelodysplasia-related changes when 20% or more PB or BM blasts are present are shown in Table 3:

Complex karyotype	
Unbalanced abnormalities	Balanced abnormalities
-7 or del(7q) -5 or i(17q) or t(17p) -13 or del(13q) del (11q) del (12p) or t(12p) del(9q) Idic(X)(q13)	t(11;16)(q23;p13.3) t(3;21)(q26.2;q22.1) t(1;3)(p36.3;q21.1) t(2;11)(p21;q23) t(5;12)(q33;p12) t(5;7)(q33;q11.2) t(5;17)(q33;p13) t(5;10)(q33;q21) t(3;5)(q25;q34)

Table 3. Cytogenetic abnormalities sufficient for diagnosis of AML with myelodysplasia-related changes

Even if we have patients with therapy-related AML or with myelodysplasia-related changes, it is better to classify them according to their cytogenetic abnormalities. Still, because the outcome in patients with therapy related AML is worse than in those with de novo AML, it is important to correctly classify the therapy related AML, because it could bring important pathogenetic information and as it well known that not all patients taking such treatment develop AML (Vardiman at al., 2009).

As is known, patients with AML with normal karyotype are the largest group and are classified as intermediate risk. They should be analyzed for *FLT3*, *NPM1* and *CEBPA* mutations, and, if present, the abnormality should be noted in the diagnosis. FLT3 gene encodes a member of the class III receptor tyrosine - kinase, which is expressed on the surface of haematopoietic progenitors from BM, with a role in the survival and differentiation of pluripotent stem cells. FLT3 - ITD encodes a protein that causes abnormal activation and stimulates autophosphorylation of the receptor, activating the pathway below. Results of studies show the FLT3-ITD as a strongly independent negative prognostic factor influencing remission duration and survival in the group with normal karyotype, located in the intermediate cytogenetic risk group. Mutation is found in 28-33% of cases. Additional mutations that occur in the signal transduction molecules (receptor tyrosine kinase - c-kit and FLT3, NRAS and KRAS) are required to generate the disease. FLT3 is overexpressed in most cases of AML and is mutated in approximately 35% of cases of AML. These mutations lead to activation of FLT3 with activation of anti–apoptotic pathways. In addition, it activates MAPK (mitogen activated protein kinase), AKT and Stat5 (signalling molecules) leading to activation of Pim-1 (proto-oncogene which encodes a cytoplasmic serine - threonine kinase) and overexpression of Bcl-XL (protein inhibiting cell death and inhibiting caspase activation, thereby inhibiting apoptosis). Simultaneous blockage of both caspase pathways predict poor response to chemotherapy and is prognostic for decreased overall patient survival (Schimmer et al., 2003).

Nucleophosmin mutations have been reported in 46-62% of cases of AML with normal karyotype, as the most frequent gene alteration in this group of AML cytogenetics. NPM1 mutations have been associated with pre-treatment characteristics such as female sex, higher percentage of blasts in MO, elevated levels of lactate dehydrogenase (LDH), increased number of leukocytes, platelets and low or absent expression of CD34 marker.

Approximately 40% of patients with NPM1 mutations also harbour FLT3-ITDs, which together with the tyrosine kinase domain mutations (FLT3-TKD), are twice as common in NPM1 positive patients as those with wild-type NPM1. CEBPA mutations occur with similar frequency in patients with and without NPM1 mutations. Many studies have shown that NPM1 mutation is associated with clinical outcome. Cytoplasmic localization of nucleophosmin is a favourable prognostic factor for achieving CR (Schnittger et al., 2005).
CEBPA mutations (enhancer binding protein - the gene encodes a myeloid transcription factor and plays an important role in normal granulopoesis) were detected in 15-20% of cases of AML with normal karyotype. These cases, compared to wild-type mutation, have a higher percentage of blasts in peripheral blood and decreased numbers of platelets, but lymph and extramedullary involvement is much rarer and less likely to carry FLT3-ITD, FLT3-TKD and MLL-PTD. Regarding MLL, although the t(9;11)(p22;q23) is clearly named in the classification, it is recommended that variant *MLL* translocation also be specified in the diagnosis, for example, AML with t(11;19)(q23;p13.3); *MLL-ENL*. MLL-PTD should not be classified in this category (Gaidzik & Döhner, 2008; Marcucci et al., 2008; Mrózek et al., 2007; Vardiman et al., 2009). Cytogenetically normal AML with CEBPA mutations is associated with favourable prognosis. Some recent studies suggest that there is a heterogeneity among mutated CEBPA AML and just the cases with double, biallelic mutations have a favourable outcome (Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Fröhling et al., 2004; Preudhomme et al., 2002; Schlenk et al., 2008). The question of whether the presence of a FLT3-ITD has an impact on prognosis in patients with CEBPA mutations remains open (Renneville et al., 2009; Schlenk et al., 2008).
Down related AML harbour the same molecular abnormality – GATA1 mutation and also other features (clinical, morphological and immunophenotypical) and this was the reason for their separation in the myeloid proliferations related with the Down syndrome category.

4. Differential diagnosis

Regarding the distinction between subtypes of AML, the most important and with consequences for treatment is that between APL and other AML subtypes. APL, especially the hypogranular variant, may look like AML with monocytic differentiation. Leukaemic promonocytes often have Auer rods and are MPO strongly positive. The immunophenotype is different, leukaemic promonocytes being negative for CD34, HLA-DR and monocytic markers.
AML with multilineage dysplasia must be distinguished from refractory anaemia with blasts excess (RAEB). The presence of 20% blasts in peripheral blood or BM makes the difference. In order to have the diagnosis of AML with multilineage dysplasia, patients must have 20% or more blasts in the PB or BM and evolve from previously MDS or MDS/MPN, specific myelodysplasia-related cytogenetic abnormalities (see Table 3), or present dysplasia in 50% or more of the cells in two or more myeloid lineages.
The differential diagnosis of acute megakaryoblastic leukaemia includes AML without differentiation, idiopathic myelofibrosis, acute panmyelosis with myelofibrosis and metastases of BM. The presence of Down syndrome, megakarioblasts in peripheral blood, the positivity of CD41, CD42 and CD61 pleads for the diagnosis of acute megakarioblastic leukaemia. In acute panmyelosis with myelofibrosis, the blasts are of non megakaryocytic origin. Care must be taken in patients with hypoplastic and oligoblastic leukaemia when examining specimens of BM, where the diagnosis is made on the presence of ≥ 20% blasts in the hypocellular marrow.

The difference between AML and ALL is easy to assess using immunophenotyping.
Chronic myeloid leukaemia (CML) in myeloid blast crisis can mimic AML, but the presence of the Philadelphia chromosome, splenomegaly and myeloid cells at all levels of differentiation distinguish CML from AML. Acute basophilic leukaemia is another entity to consider, knowing the characteristic basophilia. Care must be provided for example not to consider as MPAL the patients with t(9;22) in blast crisis with former CML. The 2008 WHO classification shows that there is the atypical CML category, that is *BCR-ABL1*–negative, and it is not a variant of CML, *BCR-ABL1*–positive. On the other hand, cases of *BCR-ABL1*–positive AML have been reported, but because it is difficult to distinguish it from CML in blastic crisis, the 2008 classification does not recognize it (Vardiman et al., 2009).
Leukaemoid reactions and nonleukaemic pancytopenia can be differentiated from AML because the blasts are missing in blood or BM. Sometimes in infections, such as tuberculosis, the proportion of blasts in the marrow may increase, but it does not reach the proportion of blasts required for a diagnosis of AML.
Pseudoleukaemia is a condition usually met after administration of granulocyte colony stimulating factor. Attentive observation of patients clarifies the problem, because in a short time the morphological appearance of BM will normalize. In the beginning it is manifested with severe leukopenia and usually normal thrombocytes.
Agranulocytosis is an acute condition involving severe leukopenia most commonly neutropenia in the circulating blood. The concentration of granulocytes falls below 100 cells/mm^3 of blood. When infection and bleeding are present the diagnosis is more complicated. With examination of BM, a history of drug use helps in giving the correct diagnosis.

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9

Diagnostic Approach in Acute Myeloid Leukemias in Line with WHO 2008 Classification

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1. Introduction

The last four decades have witnessed major transformations in the approach to the diagnostic work-up and therapeutics in the field of hematology. The identification of the Philadelphia chromosome in Chronic Myelogenous Leukemia has served as a prototype for diagnosis and subsequent monitoring of response. This discovery has led to the understanding of the pathogenesis and subsequent developments in therapeutic targeting the pathways. These principles have helped evolve therapeutic strategies aimed at molecular pathways in several disorders. Acute leukemias were classified based on morphology and cytochemistry supplemented by immunophenotyping, as proposed by the French–American–British (FAB) group. Following advances and greater access to immunophenotyping techniques and simultaneous refinements in cytogenetic methods, the MIC groups proposed the classification of acute leukemias incorporating morphology, immunologic typing and cytogenetic analysis. MIC–M classification granted recognition to molecular genetic information by formally incorporating it into the classification (Bain BJ, 1998).

In 2001, the World Health Organization (WHO), in collaboration with the Society for Hematopathology and the European Association of Haematopathology, published a Classification of Tumors of the Hematopoietic and Lymphoid Tissues as part of its 3rd edition of the series, WHO Classification of Tumors (Jaffe ES et al, 2001). This classification system was a worldwide consensus classification system for hematological malignancies. It stratified neoplasms according to the lineage. Within each category distinct entities are defined based on morphology, immunophenotype, genetic features and clinical syndromes. The classification reflected a paradigm shift from previous schemes as for the first time, genetic information was incorporated. A revised classification was published in 2008 as the 4th edition of the WHO monograph series (Swerdlow SH et al, 2008). The revision incorporates new scientific and clinical information. Refined diagnostic criteria for previously described neoplasms and newly recognized distinct entities have been defined. The new classification defines 108 new diagnostic entities in hematopathology, including 50 new or provisional leukemia entries and also recognizes provisional entities that have a definite prognostic significance (Arber DA, 2010; Betz BL & Hess JL, 2010; Swerdlow SH et al, 2008).

2. WHO 2008 recommendations for work-up of AML

WHO 2008 is one of the most scientifically devised systems to diagnose, prognosticate and also accordingly treat the haematological malignancies. The objectives are to work-up a case to obtain the information on all variables, which affect the outcome. However, for much of the world population where funding options are restricted, stringent diagnostic algorithms are a major deterrent in the management of acute leukemias. Resources are spread between diagnostic and therapeutic needs. We present here a workable and practical approach to address the need for important diagnostic parameters in AML with the focus on identifying potentially curable ones in the resource-constrained areas.

3. Diagnostic work-up

There is no single "gold standard" protocol for the diagnosis and classification as per the WHO 2008 system which broadly categorises AML as follows -

a. AML with recurrent genetic abnormalities
b. AML with myelodysplasia-related changes
c. Therapy related myeloid neoplasms
d. AML (not otherwise categorized)
e. Myeloid sarcoma
f. Myeloid proliferations related to Down syndrome
g. Blastic plasmacytoid dendritic cell neoplasm (BPDC)

The categories A, B, C, and F require genetic studies and/or clinical history to classify.

Morphology is always essential and sometimes it is diagnostic. Common ancillary studies relevant to bone marrow diagnosis are: cytogenetics, FISH studies, molecular studies (typically PCR or RT-PCR) for antigen receptor gene rearrangements and/or to detect specific translocations, immunophenotyping and immunohistochemistry. These tests will confirm the diagnosis of AML, subcategorize them, add to prognostication and more importantly differentiate from the related malignancies. The latter include acute leukemias of ambiguous lineage – acute undifferentiated leukemia (AUL) and mixed phenotypic acute leukemia (MPAL); non-Hodgkin lymphomas, round cell tumors and other metastases. Such information derived at the time of diagnosis is at the discretion of the treating physician and the pathologist subject to availability of expertise and affordability of the patient.

3.1 Morphology

The starting point for diagnosis of leukemia is morphologic examination of bone marrow or blood to document the presence of at least 20% blasts. Rarely the diagnosis is based on trephine biopsy or tissue biopsy.

3.1.1 Peripheral blood and bone marrow aspirate

The blasts were earlier categorized as type I and II based on criteria proposed by the FAB group (Bain BJ, 2003; Mufti GJ et al, 2008). Type I blasts lack granules and have uncondensed chromatin, a high nucleocytoplasmic (N:C) ratio and usually prominent nucleoli (**Figure 1a**). Type II blasts resemble type I blasts except for the presence of a few azurophilic granules and a slightly lower N:C ratio (**Figure 1b**). Goasguen et al defined type III blasts, which had more than 20 azurophilic granules, otherwise with typical blast

morphology (Goasguen JE et al, 1991; Mufti GJ et al, 2008). The WHO 2001 classification lacked specific definition of blasts. However, in the WHO 2008 classification, the blasts are

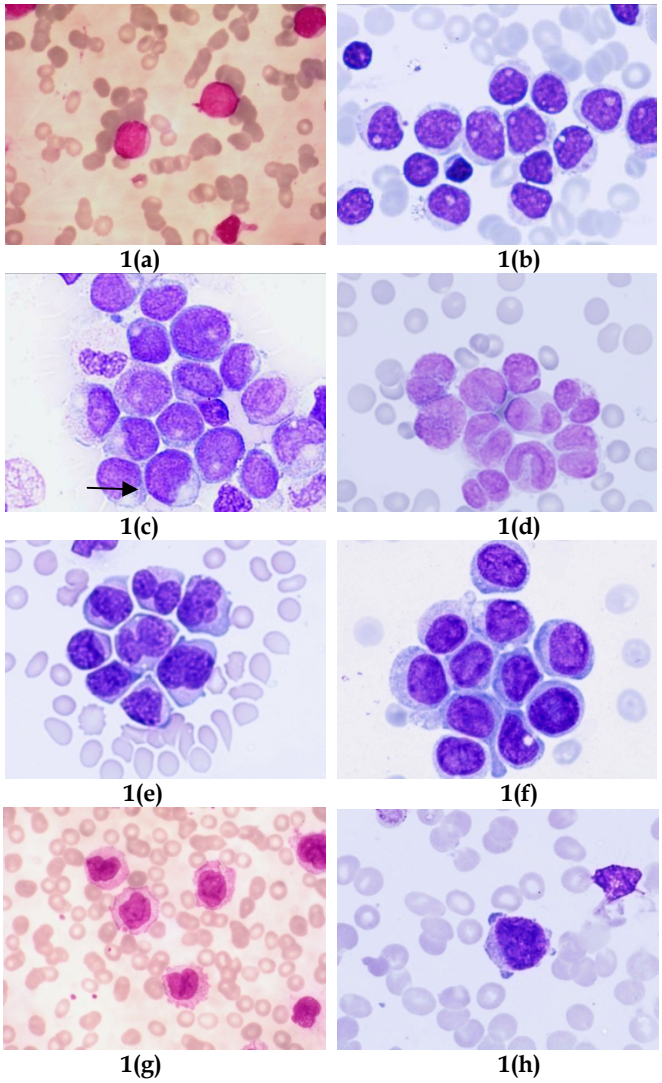
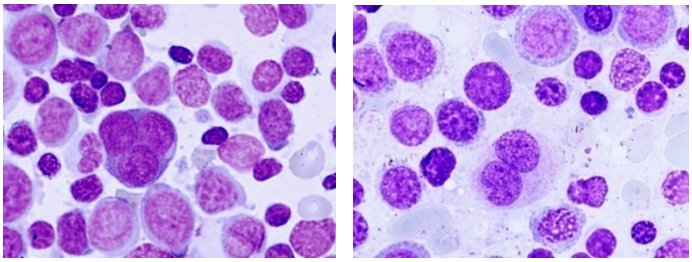


Fig. 1. Different types of blasts in AML (1000x; Giemsa)
(a) Type 1 Blasts with scant agranular cytoplasm (b)Type 2 blasts showing moderate granular cytoplasm (c) Type 2 blasts with perinuclear hof, characteristic of t(8;21) , a single blast shows Auer rod [arrow] (d) Abnormal promyelocytes with hypergranular cytoplasm and convoluted nucleus (e) Abnormal promyelocytes of Microgranular variant type of APL (f) Monoblasts with abundant blue-grey cytoplasm (g) Promonocytes with convoluted nuclei (h) Megakaryoblasts with characteristic cytoplasmic blebs

defined according to the criteria proposed by the International Working Group on Morphology of Myelodysplastic Syndrome (Mufti GJ et al, 2008; Swerdlow SH et al, 2008; Vardiman JW et al, 2009).

Myeloblasts were defined as the cells with high N:C ratio, easily visible nucleoli and usually fine nuclear chromatin, however, with a variable nuclear shape. Cytoplasmic features are variable in terms of basophilia, granules and Auer rods. Golgi zones are not detected except in cases of AML with t(8;21), where these are prominent and seen as perinuclear clearing or hofs **(Figure 1c)**. The agranular blasts corresponded to FAB type I blasts and the granular blasts included both type II blasts of FAB and type III blasts of Goasguen JE et al (Mufti GJ et al, 2008; Goasguen JE et al 1991; Swerdlow SH et al, 2008)**.** The promyelocytes of acute promyelocytic leukemia (APL) with PML-RARA are the blast equivalents and these are morphologically of two types – the classical or hypergranular and the microgranular or hypogranular types (Vardiman JW et al, 2002). The hypergranular promyelocytes are characterized by kidney-shaped or bilobed nuclei, although the shape may greatly vary (Liso V & Bennett J, 2003; Sainty D et al, 2000)**.** The cytoplasm is marked by densely-packed granules, sometimes may obscure nuclear margins, and variable presence of Auer rods **(Figure 1d)**. Some cells may be characterized by bundles of Auer rods (faggot cells). The promyelocytes of microgranular variant have bilobed, multilobed, or reniform nucleus and under usual staining are devoid of granules or contain fine azurophilic granules **(Figure 1e)** (Golomb HM et al, 1980; Sainty D et al, 2000). There are few cases of variant RARA translocations; of these those associated with ZBTB16 fusion partner at 11q23 have a characteristic morphology. These cells have regular nuclei, many granules, usual absence of Auer rods, and an increased number of Pelgeroid neutrophils (Corey SJ et al, 1994; Melnick A & Licht JD, 1999; Sainty D et al, 2000) . Monoblasts are large cells with abundant cytoplasm, which is light grey to deeply blue and may show pseudopod formation **(Figure 1f)**. The nuclei are round to oval with delicate lacy chromatin and prominent nucleoli. Promonocytes are counted as monoblast equivalents (Vardiman JW et al, 2002). These cells have a delicate convoluted, folded or grooved nucleus with finely dispersed chromatin, a small indistinct or absent nucleolus, and finely granulated cytoplasm **(Figure 1g)**. Distinction of promonocytes from abnormal monocytes is essential but very difficult on morphological basis as the diagnosis of acute monocytic or acute myelomonocytic leukemia versus chronic myelomonocytic leukemia depends on this distinction; therefore, flow cytometry and other methods are needed to improve specificity. The abnormal monocytes are characterized by more clumped chromatin, variably indented, folded nuclei and grey cytoplasm with more abundant lilac colored granules.

Megakaryoblasts are usually medium to large in size with a round, indented or irregular nucleus with finely reticular chromatin and one to three nucleoli. Cytoplasm is basophilic, agranular, and may show cytoplasmic blebs **(Figure 1h)** (Bennett JM, 1985)**.** Small dysplastic megakaryocytes and micromegakaryocytes, seen in various myeloid neoplasms, are not blasts. Erythroid precursors (erythroblasts) are not counted as blasts, except in cases of pure erythroleukemia (a variant of AML-M6), where these are considered as blast equivalents. The cells are basically proerythroblasts, which are medium to large-sized, with round nuclei, fine chromatin and one or more nucleoli. The cytoplasm is deeply basophilic, agranular and frequently contains poorly demarcated vacuoles (Swerdlow SH et al, 2008). Acute leukemias with FLT3 mutations have characteristic blasts with nuclear invaginations spanning more than 25% of the nuclear diameter or a prominent ''fishmouth'' nucleus (Chen W et al, 2006; Kussick SJ et al, 2004; McCormick et al 2010)**.**



2(a) **2(b)**

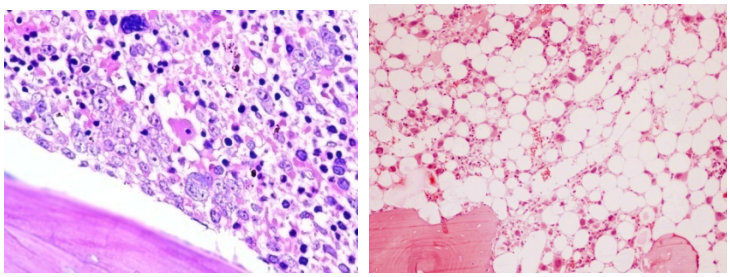
Fig. 2. AML with myelodysplasia related changes (Giemsa stain,1000x) (a) Erythropoiesis is megaloblastoid with multinucleate erythroblasts and blasts in the background (b) Dyspoietic dwarf megakaryocytes with megaloblastoid erythropoiesis.

The blasts are expressed as the percentage of nucleated cells and the count is typically based on a 200-cell count in peripheral blood and 500-cell count in the bone marrow. If there are more than 50% erythroid precursors, the erythroid progenitors are also excluded from the blast count. This is quite important in the diagnosis of acute erythroleukemia, where the erythroid precursors are $\geq$ 50% of the total nucleated cells and the myeloblasts are $\geq$ 20% of the non-erythroid marrow nucleated cells (Swerdlow SH et al, 2008). Pure erythroid leukemia consists of precursors committed exclusively to erythroid lineage, which are $\geq$ 80% of marrow nucleated cells without evidence of a significant myeloblast component. In rare cases, the diagnosis of acute leukemia can be made with low marrow blast count (< 20%) when associated with recurring genetic abnormalities as t(8;21)(q22;q22), inv(16)(p13.1q22), or t(16;16)(p13.1;q22) or t(15;17)(q22;q12) (Vardiman JW et al, 2002). These entities not only define unique disease with characteristic morphology, clinical features and biology but also have a significant prognostic implications. In AML with t(8;21), many neoplastic cells have abundant granules that may be mistaken as promyelocytes.

Relevance of non-blast myeloid precursors: The evaluation of other precursors may give important information. The presence of immature eosinophilic granules in the promyelocyte and myelocyte stages is an important diagnostic feature of cases of AML with inv(16)(p13.1q22), or t(16;16)(p13.1;q22). These granules are often larger than those normally present in immature eosinophils, purple-violet in color, and in some cases are so dense that they obscure the cell morphology. It is important to assess the degree of dysplasia in the different lineages. Dysplasia in at least 50% of the cells in 2 or more hematopoietic lineages is essential for the morphological diagnosis of AML with myelodysplasia related changes, which has adverse prognostic implications **(Figures 2a and 2b)** (Arber DA et al, 2003; Vardiman JW et al, 2009; Weinberg OK et al, 2009; Yanada M et al, 2005). The dysplastic features are also seen in cases of therapy-related myeloid neoplasms, AML with t(6;9)(p23;q34), and AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) (Swerdlow SH et al, 2008).

Bone Marrow Biopsy

It is done when the aspirate is a dry tap to evaluate for the presence of blasts **(Figure 3a)**, especially in AML-M7 and in situations where there are significant stromal changes **(Figure 3b)** (Bennett JM, & Orazi A, 2009; Lorand-Metze I et al, 1991).



3(a) 3(b)

Fig. 3. Bone marrow biopsy in AML: (a) Paratrabecular collection of blasts with large vesicular nucleus (H&E stain, 400x) (b) Hypoplastic AML with prominence of fat spaces, interstitial blast prominence and dyspoietic megakaryocytes in the background (H&E stain, 100x)

3.1.3 Myeloid sarcomas

These may sometimes be preceeding or associated acute leukemias. The differentiation from lymphoblastic leukemia and round cell tumors is essential. These can involve almost any site of the body (**Figure 4**). These need to be differentiated from other malignancies – as lymphoblastic leukemia, lymphomas, round cell tumor, carcinomas, round cell melanomas, etc. Immunohistochemistry is done to resolve these issues. Molecular studies may be performed– FISH or PCR to further look for specific genetic abnormalities. These are a common occurence in AML with t(8;21). Usually these patients need allogeneic /autologous transplantation and have better survival rates as compared to other modalities as high dose chemotherapy, radiation or surgery (Pileri SA et al, 2007).

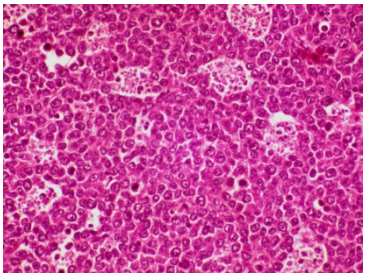
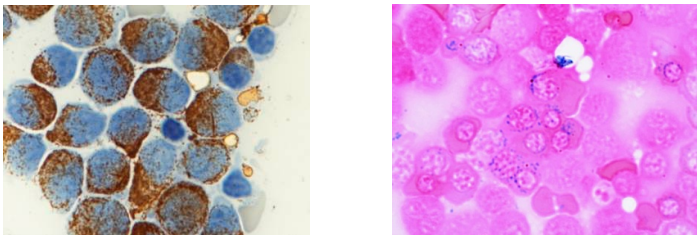


Fig. 4. Lymph node section shows sheets of large cells. These have granular cytoplasm and large convoluted nuclei. (H&E, 400x)

3.2 Cytochemistry

The role of cytochemistry has become redundant in WHO 2008 with the regular use of flow cytometry for the lineage determination (Arber DA, 2010; Betz BL & Hess JL, 2010). The stains generally used for identifying lineage type are myeloperoxidase (MPO), Sudan black B, nonspecific esterases (NSE), chloro-acetate esterase and periodic acid- Schiff. The MPO stain is most specific indicator of myeloid differentiation (**Figure 5a**), however, negativity does not rule out myeloblasts. NSE is still used as one of the identifiers for monocytic differentiation. The stain that still has a definite role is the Perl's stain not only to evaluate

iron stores, but also for identification of ringed sideroblasts (**Figure 5b)** (Mufti GJ et al, 2008). These stains are adjunct to morphology and useful for defining the subtypes of AML-NOS. There is a need for these stains in places where access to flow cytometry is difficult and rational decisions may still be taken through diligent practices (Scott C.S et al, 1993).



5(a) **5(b)**

Fig. 5. Cytochemistry in AML: (a)Myeloperoxidase stain shows golden brown granules in myeloblasts (Hematoxylin counterstain, 1000x) (b) Perl's stain demonstrates ring sideroblasts, characteristically showing Prussian blue dots around the nucleus (Eosin counterstain, 1000x)

3.3 Flow cytometry

Flow cytometry in hematological malignancies is based on the principle that neoplastic cells frequently show nonrandom expression of antigens in a manner that deviates from the tightly regulated patterns of antigen expression seen in normal maturation (Wood BL, 2007). Flow cytometric immunophenotyping (FCI) plays a well-established role in the diagnosis of acute leukemia, including AML, principally for blast enumeration, lineage assignment, and identification of immunophenotypic abnormalities suitable for post-therapeutic disease monitoring (Casasnovas RO et al, 1998; Orfao A et al, 2004; Peters JM & Ansari MQ, 2011; Weir EG & Borowitz MJ, 2001; Wood BL, 2007)**.** It is mandatory to perform FCI to diagnose AML-M0, AML-M6, AML-M7, and acute leukemias of ambiguous lineage that include acute undifferentiated leukemia and mixed phenotypic leukemia. A three- or 4-color flow cytometer is good enough for routine diagnostic work-up, although there are some centers using 9- to 10- color flow cytometers (Kussick SJ & Wood BL, 2003; Wood BL, 2006). Various panels have been recommended according to the type of flow cytometer, regional requirements, available resources, and personal preferences (Bene MC et al, 1995; Gujral S et al, 2008; Nguyen D et al, 2003). There is no universal consensus on the panel design. Each has its merits and limitations, undoubtedly the panels with more number of antibodies yields better results. Either bone marrow aspirate or peripheral blood containing good number of blasts can be processed for lineage typing. However, bone marrow aspirate is recommended for subtyping. In special situations, when the aspirate is a dry tap, BM core scraping suspensions can be utilized for FCI. However, because of lack of preservation of architectural features and the potential for artifactual alterations of the relative frequency of abnormal cells, the FCI data must always be correlated with histologic sections of the BM biopsy.

Recognizing a Hematopoietic Origin: The blasts express CD45, albeit have a weak expression as compared to lymphocytes, thus favoring an immature process and the differential diagnosis includes lymphoblasts, or myeloblasts. It is important to note that

CD45 negativity does not exclude AL as some cases of B-ALL/B-LBL and all cases of AML with erythroid and megakaryocytic lineages are CD45 negative (Nguyen D et al, 2003). This feature is of vital importance.

Markers of Immaturity: CD34 is the most commonly used marker to identify a precursor stage **(Table 1).** CD117 demonstrates a similar expression pattern and is more sensitive than CD34 in AML (Rizzatti EG et al, 2002). Flow cytometry should not be taken as an alternative to morphology for blast enumeration as blast is a morphological definition. The percentages of CD34 positive population equivalent to blasts can vary; may be falsely decreased due to hemodilution or falsely increased due to loss of erythroid precursors (the denominator for morphologic counts includes nucleated erythroid cells). CD117 strongly favors a myeloid blast lineage because it is not seen in B-ALL and is reported only very rarely in T-ALLs (<2%) (Paietta E et al, 2005). TdT is expressed in 20% of AML cases, especially those with t(8;21) (Porwit-MacDonald A et al, 1996; Wood BL, 2007). CD133 and CD38 are useful markers whenever CD34 and CD117 are non-contributory.

Lineage	Markers Positive
Precursor stage	CD34, CD117, CD133, HLA-DR, CD38, TdT
Granulocytic markers	CD13, CD15, CD16, CD33, CD65, cytoplasmic myeloperoxidase (cMPO)
Monocytic markers	Nonspecific esterase (NSE), CD11c, CD14, CD64, lysozyme, CD4, CD11b, CD36
Megakaryocytic markers	CD41 (glycoprotein IIb/IIIa), CD61 (glycoprotein IIIa), CD42 (glycoprotein 1b)
Erythroid markers	CD235a (glycophorin A), CD71
B-lymphoid markers	CD19, CD10, CD22
T-lymphoid markers	cytoplasmic CD3, CD2, CD5, CD7
NK cell markers	CD16, CD56

Table 1. Usual antigens associated with stage and lineages of blasts

HLA-DR in AML: HLA-DR is expressed in most AML and is characteristically negative in APL and AML-M6 and up to half of AML-M7. HLA-DR negativity once thought to be characteristically associated with APL has now been found to be present in a subset of AML with cup-shaped nuclei and FLT-3 gene internal tandem duplication (Bain BJ et al, 2002; Craig FE & Foon KA, 2008; Kussick SJ et al, 2004; Nguyen D et al, 2003).

Myeloid lineage Antigens: Myeloblasts are well recognized for demonstrating marked immunophenotypic heterogeneity. Thus, multiple lineage-specific antibodies may be necessary to confirm the AML classification. CD13 and CD33 are the most sensitive myeloid markers. The assigning of myeloid lineage relies on identifying the expression of antigens characteristic of early myelomonocytic differentiation, including CD13, CD15, CD33, CD64, CD117, and cytoplasmic myeloperoxidase (Bain BJ et al, 2002; Chang CC et al, 2000; Cohen PL et al 1998; Craig FE & Foon KA, 2008; Wood BL, 2007). CD64 is expressed in AML subtypes M0 to M5 in varying intensities: strong expression characterizes AML M5, whereas heterogeneous, dim, or moderate expression is seen in M0 through M4 subtypes. However, the pattern of any CD64 expression when associated with strong CD15 expression distinguishes AML-M4 or M5, from other AML subtypes (Dunphy CH & Tang W, 2007). Promonocytes are characterized by the expression of high CD64, low CD13, intermediate

CD15 and CD36, and high HLA-DR without significant CD34, minimal CD117, and, importantly, with low to absent CD14 (Wood BL, 2007). These are distinguished from more mature monocytes by more uniform high expression of HLA-DR, lower CD13 and CD36, higher CD15, and low to absent CD14. This demarcation is important when one objectively needs to differentiate AML-M5 from CMML. The current WHO 2008 recommendation is the expression of myeloperoxidase for assigning the cells as myeloblasts and presence of at least two of the following parameters to assign as monoblasts – NSE, CD11c, CD14, CD64, and lysozyme (Swerdlow SH et al, 2008). Erythroid lineage is identified by the expression of Glycophorin A, CD71, CD36 with CD117 in absence of CD64. Megakaryocytic lineage is characterized by the expression of CD41 and CD61 (Bain BJ et al, 2002; Craig FE & Foon KA, 2008; Wood BL, 2007).

Lymphoid Antigens in AML: Aberrant expression of lymphoid antigens, such as CD2, CD5, CD7, CD19, and CD56, is common and generally does not indicate bilineal or mixed lineage differentiation (Auger MJ et al, 1992; Baer MR et al, 1997; Khalidi HS et al, 1998; Kita K et al,1992; Wood BL, 2007). The presence of cytoplasmic or surface CD3 is essential to designate blasts as that of T-lineage. For categorizing B-lineage blasts, when these cells express strong CD19 with one of the following- CD79a, cytoplasmic CD22 or CD10 and when the CD19 is weak, then these should express two of the above antibodies.

3.3.1 Immunoprofiles in AMLs

AML, Not otherwise specified: The characteristic immunoprofile of various entities (AML-M0 to AML-M7) is described above. In **acute basophilic leukemia, t**he blasts usually express CD13 and/or CD33 with CD123, CD203c, CD11b, CD9, CD34, and HLA-DR. These are usually negative for CD117 and CD25 (Swerdlow SH et al, 2008).

AML with recurrent genetic abnormalities: There is a strong correlation of certain immunophenotypes in AML with specific cytogenetic and molecular abnormalities (Hrusak O & Porwit-MacDonald A, 2002; Wood BL, 2007). AML with t(8;21) has a high incidence of aberrant expression of CD19, high CD34, CD56, and TdT (**Figure 6**) (Porwit-MacDonald A et al, 1996; Wood BL, 2007). t(15;17) AML demonstrates an immunophenotype typical of promyelocytes, including a variable increase in side scatter, lack of significant CD34, expression of variable CD13 and CD117, aberrantly high CD33, and aberrantly low to absent CD15 (Orfao A et al, 1999; Wood BL, 2007). AML with inv (16) or t(16;16) generally displays myelomonocytic differentiation and sometimes is associated with CD2 expression (Adriaansen HJ et al, 1993).

AML with myelodysplasia-related changes: The immunophenotyping results vary according to the cytogenetic abnormality. Those with abnormalities of chromosomes 5 and 7 show a high incidence of CD34, TdT and CD7 expression. CD56 and / or CD7 are seen aberrantly in cases of antecedent MDS. Most noticeable is a decrease in side scatter on mature neutrophils, the flow cytometric equivalent of morphologic hypogranularity (Wells DA et al, 2003; Wood BL, 2007). However, one has to keep in mind that aged samples also give rise to hypogranularity (Wood BL, 2007).

Myeloid leukemia associated with Down's syndrome: The blasts usually are of megakaryocytic lineage with a phenotype showing positivity for CD117, CD13, CD33, CD7, CD4 (dim), CD42, CD36, CD41, CD61, CD71 and negative for MPO, CD15, CD14 and glycophorin A (Swerdlow SH et al, 2008; Xavier AC & Taub JW, 2009). CD34 is seen in 50% cases only.

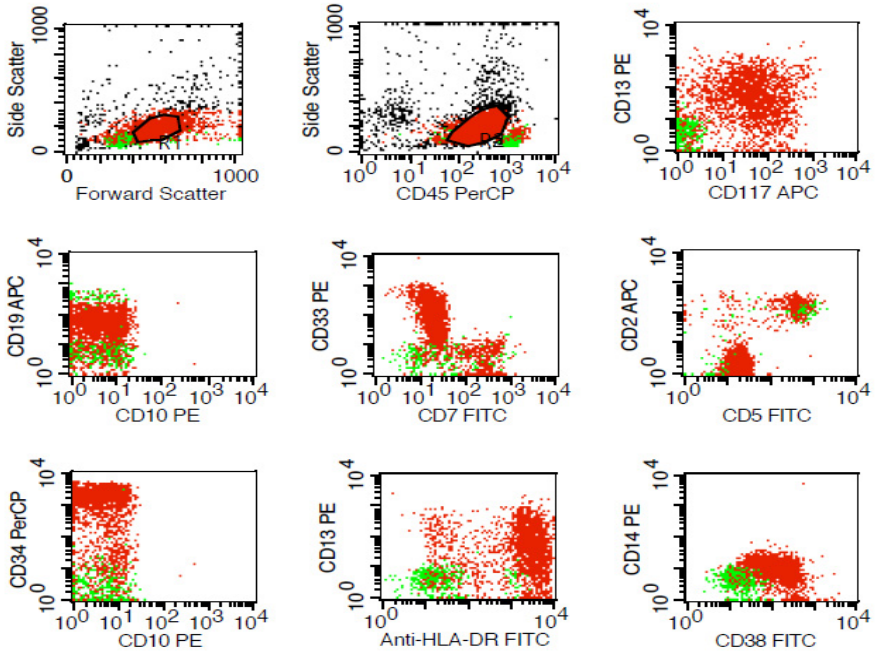
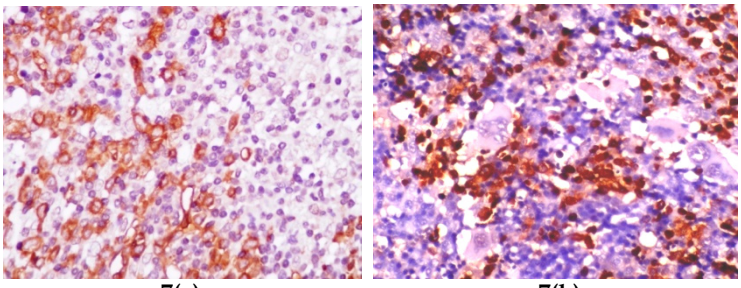


Fig. 6. Flow cytometry on the peripheral blood showed blasts (painted red) in the blast region in the CD45 /side scatter plot. These cells are CD45 dim and express CD13, CD117, CD33, CD19, CD38, CD34, and HLA-DR. These were negative for CD10, CD2, CD5, CD14 and CD7. Normal lymphocytes are painted green.

Blastic plasmacytoid dendritic cell neoplasm: Earlier known as agranular CD4+/CD56+ hematodermic neoplasm or blastic NK cell lymphoma, is characterized by the expression of CD4, CD43, CD56 and CD45RA by the blasts (Miwa H et al, 1998). These express CD123 and may sometimes express CD68, CD7, and CD33. These are negative for CD34, CD117, MPO, T-lineage and other monocytic lineage markers.

3.3.2 Acute leukemia of ambiguous lineage

This group includes the acute undifferentiated leukemia (AUL) and mixed phenotypic leukemias (MPAL) (Swerdlow SH et al, 2008). AUL is characterized by the absence of T- and myeloid lineage specific markers, i.e. cytoplasmic CD3 and MPO as well as cCD22, cCD79a or strong CD19. These leukemias lack erythroid, megakaryocytic and plasmacytoid dendritic cell lineage markers. These cells may express CD34, HLA-DR, and /or TdT. MPAL can show combinations of myeloid with B- or T- lineage specific antigens. Sometimes these are associated with specific chromosomal abnormalities as MPAL with t(9;22)(q34;q11.2); BCR-ABL1 and MPAL with t(v;11q23); MLL rearranged, where these blasts are commonly categorized as B-lymphoblasts with a high frequency of myeloid lineage antigen expression.



7(a) 7(b)

Fig. 7. IHC on trephine biopsy section (a) Blasts show strong membranous CD34 positivity (Hematoxylin counterstain, 400x) (b) Myeloperoxidase positivity in blasts (Hematoxylin counterstain, 400x)

3.4 Immunohistochemistry (IHC)

Although various studies have shown that FCI is the preferred method of immunophenotyping acute leukemias, certain situations where FCI is unavailable, immunohistochemistry (IHC) is an alternate or sometimes adjunct to flow cytometry. In situations where an appropriate specimen with adequate cellularity is not available, as in a ''dry tap'', the diagnostic cells are low in yield, FCI is usually less informative. FCI may not be routinely requested if leukemia is not an initial diagnostic consideration, especially in extramedullary or extranodal site biopsies. Similarly, fresh cells may not be consistently submitted for consultation cases, and the technology may not be immediately accessible in community settings (Olsen RJ, 2008). The main objective of IHC is to confirm a hematologic malignancy, differentiating ALs from high grade NHLs, round cell tumors and other non-hematologic malignancies. These help categorize ALs into B-ALL, T-ALL and AML. To an extent these can also subtype AMLs (Dunphy CH, 2004). Comparison of IHC results with FCI suggests that there is significant concordance in the results for markers that can be used with both techniques, indicating that the sensitivity and specificity of both methods is comparable (Manaloor EJ, 2000).

IHC is useful in confirming the blast lineage and in categorizing the following AML groups - AML-NOS, which is subdivided based on the traditional FAB classification, myeloid sarcoma and BDPC neoplasm. AML may not be definitively classified with IHC. However, differentiation toward myeloid, monocytic, erythroid or megakaryocytic lineages can be demonstrated with appropriate staining panels. Certain staining characteristics may guide genetic testing such as fluorescence in situ hybridization studies on the paraffin-embedded tissue according to the type of blasts present (Olsen RJ, 2008). The commonly available antibodies for AML include CD45 (LCA) (marker for hemopoietic origin), CD117, CD34, TdT, HLA-DR (markers of precursor stage), MPO (specific myeloid marker), CD68, lysozyme, CD163 (markers for monocytic lineage), CD41, CD61, factor VIII (FVIII) (markers for megakaryocytic lineage), hemoglobin A1, glycophorin A (markers for erythroid lineage), and CD15 (marker for myeloid maturation). . The fact that various antibodies have variable reactivity in FC and IHC has to be kept in mind while interpreting the results. Although most studies found a better detection of CD34 by flow, some did not find any difference. CD15 and CD117 are better detected by FC analysis and MPO is better detected by IHC

analysis (Dunphy CH et al, 2001). Some of the antibodies as CD13 and CD33 are not available or not standardized well by IHC.

LCA positivity recognizes a hemopoietic malignancy; however, it can be negative in AML-M6 and M7. CD34 (QBEND10) is less sensitive by IHC than by FC and is detected in only 50% of cases (**Figure 7a**) (Manaloor EJ, 2000; Olsen RJ, 2008). CD117 (c-Kit) is a much more sensitive marker of immaturity than CD34 and is also a marker for myeloid lineage (Rizzatti EG et al, 2002). TdT is expressed in cases of AML with t(8;21). Myeloperoxidase is the most specific marker for assigning myeloid lineage **(Figure 7b)**; however, is negative in AML-M0 and blasts of monocytic, erythroid, and megakaryocytic lineages. Hemoglobin A1 and glycophorin A are positive in 90% to 100% of erythroid lineage cells, and FVIII is positive in 90% of megakaryocytic cells, but rare cases demonstrating inadequate lineage maturation (early megakaryoblasts) may be negative (Chuang SS & Li CY, 1997; Manaloor EJ, 2000). CD41 and CD61 expression favor megakaryoblastic lineage; however, CD41 expression can be sometimes observed in other subtypes of AML. As in FCI, IHC can also demonstrate lymphoid lineage reactivity – as with CD2, CD7, CD4 and PAX-5. The expression of PAX-5 correlates highly with AML showing the t(8;21) abnormality.

The results of IHC should be evaluated carefully keeping in mind the limitations of the technique. Where there is unequivocal demonstration of immaturity, i.e. CD34 and/or CD117 expression, with MPO staining in the blasts, a diagnosis of AML can be made confidently. However, it is a challenge to interpret MPO negative AL cases. In such cases of AL, one has to first ensure that the B- and T- lymphoblastic lineages have been ruled out by a negative staining for CD79a, PAX-5, CD20, and CD3 (should detect CD3 epsilon chain and not zeta chain by a polyclonal antibody, which is non-specific) (Swerdlow SH et al, 2008). Monocytic lineage can be established using the CD68 (both KP-1 and PG-M1 epitopes) and lysozyme. Possibilities of AML-M6 or AML-M7 should be ruled out; these may be more challenging as they may be LCA negative. If the blasts express CD117 and TdT without CD79a, PAX-5, CD79a, MPO, and CD3 possibility of AML-minimally differentiated may be suggested. In the LCA negative cases, work-up towards other possibilities should be done before making a diagnosis of AL. Ancillary techniques should be appropriately used before a final conclusion. The role of IHC in the diagnosis of **AL of ambiguous lineage** is questionable. The possibility of AUL can be suggested when the blasts fail to express the immunophenotypic features of either lymphoid or myeloid differentiation. It is important to consider non-hemopoietic malignancies. **BDPC neoplasm** is a diagnosis usually based on tissue biopsy, most often a skin lesion (Petrella T et al, 1999**).** Morphologically suspected as leukemia cutis, the primary panel is usually inconclusive - weakly positive for LCA/CD45, variably and focally positive for CD68. The pattern may be confusing because of the absence of lineage-specific markers. The diagnosis should be suspected and a further panel should be done for a conclusive opinion. The cells are positive for CD4, CD43, CD56, and CD123 (plasmacytoid dendritic cell marker) and the expression of CD2 and CD7 is variable. This pattern may be seen in myeloid sarcoma (AML- M4 or M5). These entities are distinguished by the clinical presentation, and more importantly by CD13, and CD33 expression, which are readily available by FC. CD13 and CD33 are present in AMLs and are usually absent in BDPC **(**Jacob MC et al, 2003).

3.5 Cytogenetics

Conventional cytogenetic analysis is now an integral component of the diagnostic evaluation of a patient with suspected acute leukemia. This is done best at the time of

diagnosis before initiating therapy. Chromosome abnormalities are detected in approximately 55% of adult AML (Döhner H et al, 2010; Grimwade D et al, 1998). There are seven recurrent balanced translocations and inversions, and their variants recognized in the WHO category – AML with recurrent genetic abnormalities. Several cytogenetic abnormalities are considered sufficient to establish the WHO diagnosis of AML with myelodysplasia-related features, when 20% or more blood or marrow blasts are present. A minimum of 20 metaphase cells analyzed from bone marrow is considered mandatory to establish the diagnosis of a normal karyotype, and also to define an abnormal karyotype. Abnormal karyotypes may be diagnosed from blood specimens, or the biopsy core scrapings, when the marrow aspirate is scanty or insufficient. Leukemic blasts carrying AML-associated chromosome aberrations can constitute only a fraction of cells dividing in vitro. Moreover, a blood specimen can sometimes be cytogenetically normal when the marrow is abnormal. In the CALGB database, this was found in approximately 5% of AML patients whose marrow and blood specimens were studied simultaneously (Grimwade D et al, 1998; Mrózek K et al, 2001, 2007).

Acquired genetic alterations, both those detectable microscopically as structural and numerical chromosome aberrations, and those detected as submicroscopic gene mutations and changes in gene expression, are commonly seen in AML. At present, cytogenetic aberrations detected at the time of AML diagnosis constitute the most common basis for predicting clinical outcome (Byrd JC et al, 2002; Mrózek K &Bloomfield CD, 2006; Slovak M L et al, 2000). Acquired clonal chromosome abnormalities are defined as a structural aberration or a trisomy observed in at least 2 and monosomy found in at least 3 metaphase cells. These are detected in the pretreatment marrow of 50% to 60% of adults with de novo AML. In 10% to 20% of patients, the abnormal karyotype is complex, defined as the presence of more than 3 abnormalities in karyotypes not including the abnormalities seen in the recurrent genetic abnormalities group, i.e. t(8;21), inv(16), t(16;16), t(15;17) or t(9;11) (Swerdlow SH et al, 2008). In around 40% to 50% of patients no cytogenetic abnormality can be detected using standard banding methods (Byrd JC et al, 2002; Farag SS et al, 2006; Grimwade D et al, 1998; Mrózek K et al, 2001, 2007; Slovak M L et al, 2000). The role of cytogenetics is of paramount importance in the diagnosis of AML with recurrent genetic abnormalities – those associated with balanced translocations and inversions, and AML with myelodysplasia-related changes.

3.5.1 AML with balanced translocations/ inversions

This group is composed of ALs with detection of balanced translocations between chromosomes and are usually associated with a specific prognosis. All large cytogenetic studies of AML have shown that patients with t(15;17)(q22;q12- 21) have an excellent outcome and those with t(8;21)(q22;q22) or inv(16)(p13q22)/ t(16;16)(p13;q22) a relatively favorable prognosis. Those with inv(3)(q21q26)/ t(3;3)(q21;q26), –7 and a complex karyotype have an unfavourable outcome (Mrózek K &Bloomfield CD, 2006) .

3.5.1.1 Core-Binding Factor (CBF) AML

CBF-AML is a relatively frequent subtype of adult *de novo* AML, with t(8;21) being detected in 7% and inv(16)/t(16;16) in 8% of patients (Byrd JC et al, 2002; Marcucci G et al, 2005; Mrózek K & Bloomfield CD, 2006). As in APL both these leukemias have a characteristic morphology based on which these cytogenetic abnormalities are predicted and specifically

looked for. Both t(8;21) and inv(16) are related at the molecular level as they disrupt the α and β subunits of CBF, respectively.

AML with t(8;21)(q22;q22); RUNX1-RUNX1T1: This abnormality detected in 20% adult and 40% children de novo AML cases is associated usually with FAB AML-M2, rarely with other subtypes (**Figure 8**). Over 70% are associated with secondary chromosome aberrations – as loss of a sex chromosome (–Y in men and –X in women) and del(9q) with loss of 9q22 being the most frequent. Despite good prognosis relapse is a major problem, especially in first 2 years of remission (Marcucci G et al, 2005; Mrózek K & Bloomfield CD, 2006; Schlenk RF et al, 2004**)**.

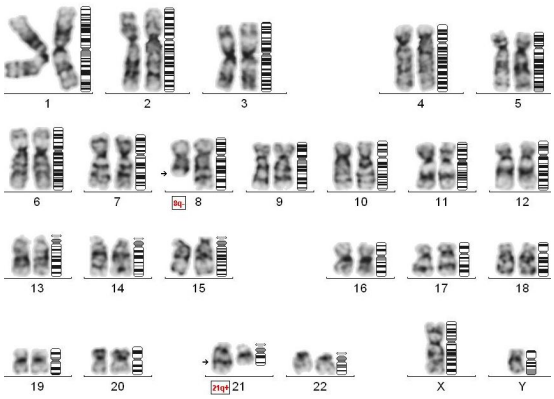


Fig. 8. Karyotype showing balanced translocation of 46,XY, t(8;21)(q22;q22)

AML with inv(16)(p13.1q22) / t(16;16) (p13.1q22); CBFB-MYH11: These are associated with characteristic FAB M4Eo morphology, higher WBCs, percentages of PB and BM blasts, more often showing extramedullary involvement, lymphadenopathy, splenomegaly, gingival hypertrophy and skin/mucosa involvement and characteristic cytogenetic features. Approximately two thirds of patients with inv(16)/t(16;16) have this rearrangement as a sole chromosome abnormality. Most frequent secondary chromosome aberrations in inv(16)/t(16;16)-positive patients are +22, +8, del(7q) and +21. These studies identified additional cytogenetic prognostic factors differentiating the two cytogenetic subsets of CBF AML. Among patients with inv(16)/t(16;16), those who harbored +22 as a secondary abnormality were found to have a significantly lower cumulative incidence of relapse compared with patients with inv(16)/t(16;16) as a sole abnormality in the CALGB study and longer relapse free survival than patients without +22 in the German Acute Myeloid Leukemia Intergroup study(Marcucci G et al, 2005; Mrózek K & Bloomfield CD, 2006; Schlenk RF et al, 2004).

3.5.1.2 AML associated with RARA translocation including variant translocations

APL constitutes 5-8% of AML (Swerdlow SH et al, 2008). In 1977 Rowley and colleagues identified the t(15;17) balanced reciprocal chromosomal translocation as the karyotypic hallmark of the disease (Rowley J et al, 1997 as cited in Sirulnik A et al, 2003). In the early 1990s it was discovered that in classical APL this reciprocal translocation involves a fusion between the RARA gene on chromosome 17 and a previously unknown locus named promyelocytic leukemia (PML) on chromosome 15 (Kakizuka A et al, 1991 as cited in Sirulnik A et al, 2003). Other additional chromosomal abnormalities can be found in 30 to

40% of patients with APL in addition to t(15;17). The most common of these are trisomy 8 and isochromosome 17. Additional chromosomal abnormalities do not have a negative effect on the overall prognosis (Johannson B et al, 1994; Schoch C et al. 1996; Slack JL, 1997). However, there have been cases morphologically reported as APL without a detectable t(15;17) on a conventional karyotype. Some of these have a cryptic PML/RARA translocation, i.e. these are submicroscopic and not detected by conventional method and require ancillary techniques as the FISH or PCR for a confirmation. Others have translocations not involving the t(15;17) (Goyal M et al, 2010; Grimwade DF et al, 1997).

AML with Variant RARA translocations: The current WHO categorizes morphologically diagnosed cases of APL into those associated with t(15;17)(q22;q21)/ PML-RARA rearrangement, and those lacking PML/RARA rearrangements based on the cytogenetic and molecular studies (Swerdlow SH et al, 2008). The latter group is separately categorized as variant RARA translocations due to refractory / variable response to ATRA. Instead of PML the partner genes in this group could be ZBTB16/ PLZF at 11q23, NUMA1 at 11q13, NPM1 at 5q35 and STAT5B at17q11.2.

3.5.1.3 AML with t(9;11)(p22;q23); MLLT3-MLL and variant MLL translocations in AL

The MLL gene on chromosome 11 band q23 is frequently involved in chromosome translocations in acute lymphoblastic leukemia and acute myeloid leukemia. The MLL gene located at 11q23 has been described as a 'promiscuous' gene due its involvement with a large number of genetic partners (Moorman AV et al, 1998). More than 80 different partner chromosome regions have been described till date. The translocation results in the formation of a fusion gene on the derivative 11 chromosome consisting of the 5' part of the MLL gene and the 3' part of another gene. MLL gene rearrangements generally correlate with a poor prognosis; however AML with t(9;11)(p22;q23) is associated with intermediate prognosis. Therefore, the presence of 11q23 aberration has direct implications for treatment stratification, making early and rapid detection of utmost importance (van der Burg et al, 1999). AML with t(9;11) are associated with acute monocytic and myelomonocytic leukemias (Baer MR et al, 1998; Sorensen PHB et al, 1994; Swansbury GJ et al, 1998). This entity involves MLLT3 (AF9), which is the most common MLL translocation in AML. Secondary chromosomal abnormalities as +8 are commonly seen, however, these do not affect the prognosis.

AML with variant MLL translocations: Various other partner chromosomes are known to be associated with the MLL gene (Moorman AV et al, 1998). 19p13.1 is involved almost only with AML, others can be seen both in ALL and AML, and all have been categorized in variant MLL translocations in acute leukemia. The WHO 2001 encompassed all MLL related translocations into the category of AML with 11q abnormalities. However, the WHO 2008 now separates AML with t(9;11) from other MLL related translocations, which are placed in the category variant MLL translocations in acute leukemia. It is imperative to mention the specific abnormality associated with MLL to place in the latter category. Cases of AML with specific MLL translocations as t(11;16)(q23;p13.3) and t(2;11)(p21;q23) if not associated with cytotoxic chemotherapy should be considered as AML with myelodysplasia-related changes and not variant translocation of 11q23 (Swerdlow SH et al, 2008).

3.5.1.4 AML with t(6;9)(p23;q34); DEK-NUP214

Morphologically these are AML with or without monocytic features, usually associated with basophilia and multilineage dysplasia. The t(6;9)(p23;q34) results in fusion of DEK on

chromosome 6 with NUP214 on chromosome 9. Usually it is an isolated abnormality. Very occasional reports of complex karyotype are known **(**Chi Y et al, 2008; Slovak ML et al, 2006). These are frequently associated with FLT3-ITD mutations and a poor prognosis.

3.5.1.5 AML with inv(3)(q21q26.2) or t(3;3)(q21q26.2); RPN1-EVI1

These are morphologically AML with multilineage dysplasia and characterized by the translocation involving EVI1 or MDS-1EVI1 located at 3q26.2 and RPN1 at 3q21 respectively. Other abnormalities involving the 3q26.2, seen in therapy related AML are not included in this category. This group is frequently associated with secondary karyotypic abnormalities – monosomy 7, 5q deletions, and complex karyotypes. AML with inv(3)(q21q26.2) or t(3;3)(q21q26.2) is an aggressive disease with a short survival (Lugthart S et al, 2008).

Karyotypic abnormalities	**AML with myelodysplasia related changes**	**Therapy related AML (t-AML)**
	Complex karyotype	Complex karyotype
Unbalanced abnormalities	-7 /del(7q); -5/ del(5q); i(17q)/ t(17p);-13/ del(13q); del(11q); del(12p)/t(12p); del(9q); idic (X)(q13)	-7 /del(7q); -5/ del(5q); del(13q); del (20q); del(11q); del(3p); -17; -18; -21; +8
Balanced abnormalities	t(11;16)(q23:p13.3); t(3;21)(q26.2;q22.1); t(1;3)(p36.3;q21.1); t(2;11)(p21;q23); t(5;12)(q33;p12); t(5;7)(q33;q11.2); t(5;17)(q33;p13); t(5:10)(q33;q21); t(3;5)(q25;q34)	**t(11;16)(q23:p13.3); t(3;21)(q26.2;q22.1); t(2;11)(p21;q23);** t(9;11)(p22;q23); t(11;19)(q23;p13); t(8;21)(q22;q22); t(15;17); t(3;21)(q26.2;q22.1)); inv(16)(p13q22)

Table 2. Types of cytogenetic abnormalities defining AML with myelodysplasia related changes and Therapy related AML (t-AML)

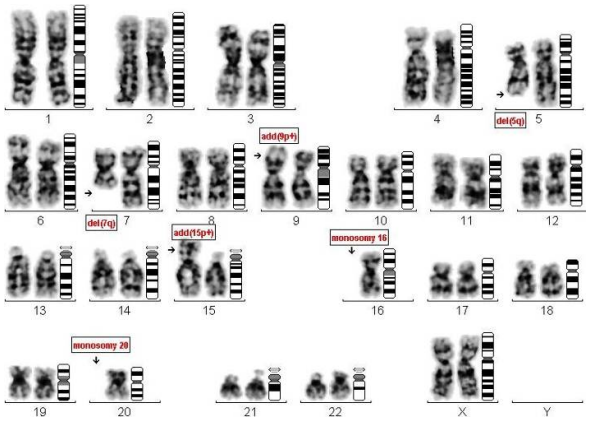


Fig. 9. Case of a t-AML, post treatment for carcinoma breast showing a complex karyotype: 44,XX,del(5q),del(7q),der(9)add(9p),der(15)add(15p),-16,-20

3.5.1.6 AML with t(1;22)(p13;q13); RBM15-MKL1

These are morphologically acute megakaryoblastic leukemia associated with a very poor prognosis. Usually t(1;22)(p13;q13) is the sole abnormality. However, cytogenetics may not always be successful to depict this abnormality due to poor bone marrow aspirate yield and one may need to resort to molecular analysis as well. When a morphological diagnosis of AML-M7 is suspected, this chromosomal/ molecular abnormality should be looked for (Swerdlow SH et al, 2008).

3.5.2 AML with myelodysplasia related changes and therapy related AML

The chromosomal abnormalities are similar to those found in MDS and often involve gain or loss of major segments of certain chromosomes with complex chromosomal abnormalities. Various balanced and unbalanced abnormalities are known to occur **(Table 2)**. Some abnormalities as t(11;16)(q23;p13.3) and t(3;21)(q26.2;q22.1) seen in AML with myelodysplasia-related changes, also occur commonly in t-AML and clinical details should be evaluated to differentiate between the two (Secker-Walker LM et al, 1998). Trisomy 8, del 20q and loss of Y are common in MDS, however, are not considered disease-specific. Hence, their isolated presence is not sufficient to consider a case as AML with myelodysplasia-related changes. Cases of AML with myelodysplastic changes in bone marrow may show t(6;9)(p23;q34), inv(3)(q21q26.2) or t(3;3)(q21q26.2) on a karyotype and should be categorized as such and not in AML with myelodysplasia-related changes. In t-AML unbalanced chromosomal aberrations are seen in 70% cases **(Figure 9**). These are associated with longer latent period, myelodysplastic changes and alkylating agent and/ or radiation therapy. Balanced translocations seen in 20-30% are associated with shorter latency, absence of myelodysplasia and prior therapy with topoisomerase inhibitors. The prognosis for t-AML is dependent on the karyotype – is generally poor, except in cases associated with balanced translocations as t(15;17) and inv(16)(p13q22), which is also poorer as compared to de novo cases (Swerdlow SH et al, 2008).

3.5.3 AML- not otherwise specified

There are no specific chromosomal abnormalities associated with different subtypes. However, higher frequency of few abnormalities is seen in certain subtypes. Cuneo A et al, 1995 compared cases of AML-M0 and AML-M1 and showed that abnormal karyotypes, complex karyotypes, unbalanced chromosome changes (-5/5q- and/or -7/7q- and +l3) were more frequent in AML-M0 than in AML-M1. However, many cases were regrouped in the AML with myelodysplasia-related changes. Trisomy 8 may be seen in acute myelomonocytic leukemia and t(8;16)(p11.2;p13.3) may be seen in acute monocytic or myelomonocytic leukemia. Cases of AML-M7 associated with mediastinal germ cell tumors have shown several cytogenetic abnormalities of which i(12p) is the most characteristic. There are no specific abnormalities documented in other subtypes.

3.5.4 Down's syndrome related AML

In addition to trisomy 21, trisomy 8 is a common cytogenetic abnormality seen in DS-AML (13-44%). More importantly the focus should be on detecting GATA1 mutations, which are commonly seen in children below 5 years (Swerdlow SH et al, 2008; Xavier AC & Taub JW, 2009).

3.5.5 Blastic Plasmacytoid Dendritic Cell neoplasm

Chromosomal abnormalities are found in two-thirds of BPDC patients, although a specific karyotype is lacking. Complex karyotypes are common and six major recurrent chromosomal abnormalities are found: 5q21 or 5q34, 12p13, 13q13-21, 6q23-qter, 15q and -9.

3.5.6 Cytogenetically normal AML (CN-AML)

The proportion of adults with de novo CN-AML has varied between 40% and 49% in the largest cytogenetic studies (Byrd JC et al, 2002; Grimwade D et al, 2001; Mrózek K et al, 2007; Slovak ML et al, 2000). A patient is defined karyotypically normal when full analysis of at least 20 metaphase cells originating from a marrow sample cultured in vitro for 24 to 48 hours is performed (Mrózek K et al, 2007). There are patients who, despite having a normal karyotype on standard cytogenetic investigation, carry 1 of the fusion genes identical to those generated by recurrent translocations (eg, *PML-RARA*/t(15;17), *RUNX1-RUNX1T1 (AML1-ETO)*/ t(8;21)) or inversions (*CBFB-MYH11*/inv(16)) and categorized in AML with recurrent genetic abnormalities. In most instances, these fusion genes are created by cryptic insertions of very small chromosome segments that do not alter the chromosome morphology (Grimwade D et al, 2000; Mrózek K et al, 2007; Rowe D et al, 2000). Both RT-PCR and FISH can be used to detect the presence of the aforementioned hidden rearrangements. Such testing is definitely warranted in CN-AML patients with FAB M2, M3, M3v, and M4Eo marrow morphology but is otherwise not routinely recommended outside of a clinical trial (Mrózek K et al, 2007; National Comprehensive Cancer Network (NCCN), 2006).

3.5.7 Prognosis associated with chromosomal abnormalities

The risk stratification with regards to cytogenetics is based on studies performed on patients below 60 years of age (Byrd JC et al, 2002; Grimwade D et al, 1998; Mrózek K & Bloomfield CD, 2006; Slovak ML et al, 2000). The favourable risk group have only balanced translocations – include t(15;17)(q22;q12-21), t(8;21)(q22;q22) and with inv(16)(p13.1q22) / t(16;16) (p13.1q22) **(Table 3)**. The CN-AML is included in the intermediate-risk group. The unfavourable risk group includes complex karyotype, various balanced translocations, unbalanced translocations and numerical abnormalities. An MRC study found the outcome

Karyotypic abnormality	**Favourable**	**Intermediate**	**Poor**
		CN-AML	**Complex karyotype**
Balanced Structural Rearrangements	**t(15;17)(q22;q12-21)** **t(8:21)(q22;q22)** **inv(16)(p13q22)/ t(16;16)(p13;q22)**	**t(9;11)(p22;q23)**	**inv(3)(q21q26)/ t(3;3)(q21;q26)** **t(6;9)(p23;q34)** **t(6;11)(q27;q23)** **t(11;19)(q23;p13.1)**
Unbalanced Structural Rearrangements	**None**	**del(7q)** **del(9q)** **del(11q)** **del(20q)**	**Del (5q)**
Numerical aberrations:	**None**	**-Y ; +8 ; +11; +13; +21**	**-5** **-7**

Table 3. Known cytogenetic abnormalities with associated favourable, intermediate and unfavourable prognosis.

of patients above 60 years with fewer than 5 abnormalities, regardless of the presence of abnormalities involving chromosomes 5, 7 and 3q, to be comparable to the intermediate-risk category. They included only those patients with a complex karyotype with 5 or more aberrations in the adverse risk category (Grimwade D et al, 2001). A CALGB study confirmed that older AML patients with a complex karyotype with 5 or more aberrations have particularly poor disease free survival (DFS) and overall survival (OS), with no patient surviving 5 years after diagnosis (Farag SS et al, 2006).

3.6 Fluorescent-In-Situ Hybridisation (FISH)

FISH is an improvisation of cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. Karyotype analysis has an advantage that the entire genome can be analyzed however, is applicable to actively dividing cells, and the resolution is limited to chromosomal rearrangements that are >3Mb in size. In addition technical aspects of sample collection, storage, transport, and culture may lead to suboptimal results. Poorly spread or contracted metaphases, low mitotic index and highly complex cytogenetic abnormalities may also lead to faulty results. This technique is labour-intensive and time-consuming. FISH is capable of detecting aberrations of sizes between 10kb to 5Mb. These are accurate, rapid, however, targeted analysis of the genomes. FISH provides increased resolution, thus elucidating submicroscopic deletions, cryptic or subtle duplications and translocations, complex rearrangements, involving many chromosomes and marker chromosomes. Interphase FISH has advantages of screening more number of cells, and also that both proliferating and not proliferating cells can be analyzed. The test can be performed on fixed bone marrow suspensions, paraffin-embedded tissue sections, bone marrow or blood smears, and touch preparations of cells from tissues. The test can be reliably used for routine diagnostic screening and whenever the patient's material is not sufficient or suitable for cytogenetic/RT-PCR analysis.

Role of FISH in AML: The main applications in AML are detection of recurrent cytogenetic abnormalities, whenever the cytogenetic analysis fails or in a CN-AML case, where morphology is suggestive of AML with recurring cytogenetic abnormalities. Dual color dual- fusion probes specific for the abnormality are used when a reciprocal translocations are suspected, e.g. t(8;21), t(15;17), etc **(Figure 10)**. The presence of a translocation is

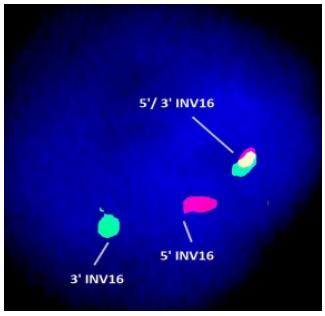


Fig. 10. Interphase FISH analysis showing 1red– 1green– 1yellow fusion signal pattern; as compared to the normal cells which show a pattern of 0red– 0green– 2yellow (not shown). The splitting of yellow signal into 1red and 1green indicates translocation involving chromosome 16 in the region of CBFB (DAPI counterstain, ×1000)

detected by the formation of a red-green or yellow fusion signal. *MLL* gene is involved in more than 80 different translocations. To detect AL with MLL gene involvement break-apart probes complimentary to MLL gene are useful. This approach has an advantage that it detects all types of *MLL* gene translocations, independent of the partner gene **(**van der Burg et al, 1999**).** However, for detecting specific abnormality, dual color dual-fusion specific probes are used to detect the balanced translocations. In cases of AML with myelodysplasia associated changes, where numerical aberrations are more common CEPs are used to detect +8, -5, -7, etc.

3.7 PML nuclear bodies immunofluorescence test

An immunofluorescence based test is developed for rapid diagnosis of APL, using antibodies directed against the amino terminal portion of the human PML gene product, PG-M3 monoclonal antibody (Falini B et al, 1997). The wild type PML produces a characteristic nuclear speckled pattern that is due to localization of the protein into discrete dots (5 to 20 per nucleus), named PML nuclear bodies. The architecture of PML nuclear bodies appears to be disrupted in APL cells that bear the t(15; 17), thus resulting in abnormal (micropunctate) pattern of the PML/RARA fusion protein (usually ≥50 small granules/per nucleus). These are characteristically seen in APL with PML/RARA translocation and not in PLZF/RARA APL and other AMLs. Immunocytochemical labeling with PG-M3 represents a rapid, sensitive, and highly-specific test for the diagnosis of APL that bears the t(15; 17) and allows an easy and correct diagnosis of this subtype of acute leukemia to any laboratory provided with a minimal equipment for immunocytochemistry work.

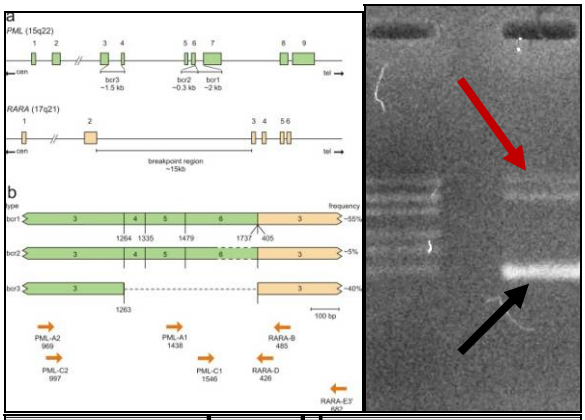
3.8 Polymerase chain reaction

Nucleic acid amplification studies have become an integral part of diagnostic and prognostic work-up in the field of hematology. These include detection of DNA or the RNA by a process known as polymerase chain reaction (PCR). A marrow or peripheral blood specimen is routinely taken for molecular diagnostics. Ideally, DNA and RNA should be extracted and viable cells stored; if sample quantity is limited, RNA extraction should be a priority, because RNA is suitable for molecular screening for fusion genes and leukemia-associated mutations.

3.8.1 PCR in the diagnosis of recurrent genetic abnormalities

Molecular diagnosis by RT-PCR for the recurring gene fusions, such as *RUNX1-RUNX1T1*, *CBFB-MYH11*, *MLLT3-MLL*, *DEK-NUP214*, can be useful in certain circumstances. RT-PCR is an option to detect these rearrangements, if chromosome morphology is of poor quality, or if there is typical marrow morphology but the suspected cytogenetic abnormality is not present and for a rapid diagnosis (Mrózek K et al, 2001). The standardized protocols are published by the BIOMED-1 group (van Dongen JJM et al, 1999).

Acute Promyelocytic Leukemia with PML/RARA translocation: Five different chromosomal translocation partners have been identified in patients with APL, and all involve the RARA gene on chromosome 17q21 fused to one of the partners, PML on chromosome 15q22 being the most common. This results in fusion PML/RARA, mRNA transcription and a chimeric protein. RT- PCR amplification of the PML/RARA fusion transcript is now widely used for both diagnostic and monitoring studies (Sirulnik A et al, 2003).



PM1110Z	Size (bp)
Internal control	312
bcr2 variant (ins intron6)	954
bcr1	867
bcr2 variant (-exon5, ins intron6)	809
bcr1 variant (-exon5)	724
bcr1 variant (-exon6)	607
bcr1 variant (-exon5&6)	464
bcr3	393
bcr3 variant	>312, <393

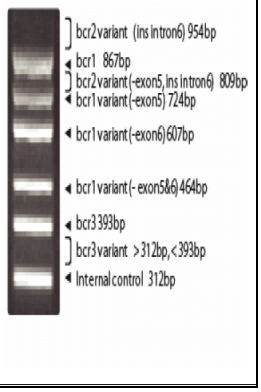


Fig. 11. The upper left panel shows a schematic representation of possible break-points in the PML and RARA genes, thus generating the isoforms – bcr1, bcr2 or bcr3 of PML-RARA fusion transcript. (Idea adapted from van Dongen JJM et al, 1998). The bcr1 and bcr2 breakpoint regions are juxtaposed in intron 6 and exon 6, respectively. The upper right panel shows presence of bcr1 form of PML-RARA transcript (red arrow) with an internal control (black arrow). The lower panel gives a reference chart for the location of various PML-RARA transcripts and their relative sizes based on the number of base pairs

The exact type of breakpoint on the PML gene can be determined. RT-PCR allows the detection of minimal residual disease at high sensitivity levels. Some pitfalls include poor RNA yield and stability, as well as the low expression of the hybrid PML/RARA gene. The chromosome 17 breakpoints are localized within a 15 kb DNA fragment of the RARA intron 2. The PML gene spans 35 kb of genomic DNA and contains nine exons (Chen Z & Chen SJ, 1992; Sirulnik A et al, 2003). Three regions of the PML locus are involved in the translocation breakpoints: intron 6 (bcr1; 55% of cases), exon 6 (bcr2; 5%), and intron 3 (bcr3; 40%). Bcr1 and bcr2 are considered as long (L) forms and bcr3 is considered as short (S) form (**Figure 11).** Because bcr2 (also referred to as 'variant' or V form) and bcr1 are located in PML exon 6 and intron 6, respectively, sequencing of all L transcript cases would be needed to clearly distinguish these two isoforms. There is no difference in the clinical features of various

isoforms (Lo-Coco F et al, 1999, 2003; van Dongen JJM et al, 1999**).** Recognition of the infrequent PLZF/RARA subtype is clinically relevant in the light of its presumed unresponsiveness to ATRA and other agents such as arsenic trioxide (Lo-Coco F et al, 2003).

Acute myeloid leukemia with RUNX1-RUNX1T1 or AML-ETO: The t(8;21) fuses the RUNX1 or AML1 or CBFA2 gene on chromosome 21 to the RUNX1T1 or ETO gene on chromosome 8. RUNX1-RUNX1T1/ AML1-ETO fusion transcripts are found by RT-PCR in virtually all cases of t(8;21)-positive AML, including those with complex translocations and also in a significant proportion of t(8;21)-negative AML (van Dongen JJM et al, 1999). Transcripts of this fusion gene can be specifically and sensitively detected by RT-PCR**.** They generate predominant PCR products of a constant size, corresponding to an in-frame fusion of AML1 exon 5 to ETO exon 2 **(**Downing JR et al, 1993; Liu Yin JA, 2002**)**.

Acute myeloid leukemia with CBFB-MYH11: This translocation involves fusion of CBFB gene located on16q22 with MYH11 gene located on 16p13. There is marked heterogeneity in the fusion transcripts, arising from variable genomic breakpoints in both CBFB and MYH11 genes and alternative splicing. Ten different CBFB-MYH11 fusion transcripts have been reported and have been designated as types A to J (Liu Yin JA, 2002; van Dongen JJM et al, 1999). More than 85% of the positive patients have type A transcript; two other transcripts (D and E) represent nearly 5% each, whereas all others represent unique cases (Liu PP et al, 1995; Liu Yin JA, 2002**)**.

Other cytogenetic abnormalities: There are primers directed to diagnose other recurring genetic abnormalities, especially those involving MLL gene, the partners being MLLT3 /AF9, AF6, AF10, ENL, etc. In addition PCR can be used to diagnose DEK-CAN related to t(6;9)(q23;q34), EVI-1 associated with inv 3(q21;q26)/ t(3;3) (q21;q26), AML1-EVI-1, t(3;21)(q26;q22) and rarely the BCR-ABL1, i.e. t(9;22)(q24;q11) (Swerdlow SH et al, 2008; Vardiman JW et al, 2009). Although BCR-ABL1–positive AML has been reported, criteria for its distinction from CML initially manifesting in a blast phase are not entirely convincing, and for this reason, BCR-ABL1–positive AML is not recognized in this classification. Many cases of BCR-ABL1– related AL will meet the criteria for ALL or MPAL, provided that a blast phase of a previously unrecognized CML can be excluded **(**Vardiman JW et al, 2009). GATA 1 mutations are detected in children less than 5 years in cases of AML associated with Down's syndrome. (Swerdlow SH et al, 2008; Xavier AC & Taub JW, 2009)

3.8.2 Cytogenetically Normal AML

According to the various cytogenetic classifications mentioned above around 50% to 70% of AML patients are considered to be a part of an intermediate-risk group. Most of these patients have a normal karyotype (40-50% of all AML patients), but the heterogeneity is most pronounced in this group (Schlenk RF et al, 2008). Somatically acquired mutations have been identified in several genes, the notable ones are the NPM 1, CEBPA, and FLT3, which have been proven to have prognostic implications. AML with mutations in NPM1 or CEBPA have been incorporated in the WHO classification as provisional entities. The FLT3 internal tandem duplication (ITD) mutation was detected and found to be the most common gene mutation in AML. Subsequent research shows that mutations of the NPM1 gene can occur in up to 60% of patients with AML and are most common in patients with a normal karyotype. The European Leukemia Net panel recommends that mutations of NPM1, CEBPA and FLT3 be analyzed at least in patients with CN-AML who will receive treatment other than low-dose chemotherapy or best supportive care (Döhner H et al, 2010; Döhner K

& Döhner H, 2008). There are few more as MLL, BAALC, WT1, etc which have also an impact on the prognosis.

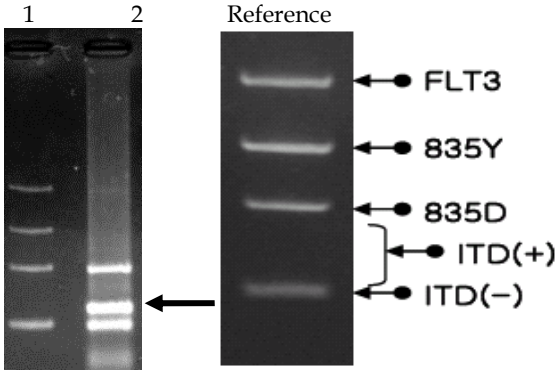


Fig. 12. A patient showing FLT3 – ITD in lane 2 (arrow) as a distinct band from the wild type of FLT-3. Lane 1 shows 100bp markers. The reference gel on the right panel shows the various locations of the wild and mutant products.

Mutations of the nucleophosmin, member 1 (NPM1) gene: Mutations of NPM1 gene, which codes for a nuclear/cytoplasmic shuttling protein, are found in 50–60% of CN-AML cases (Schiffer CA, 2008). Heterozygous mutations in exon 12 of the NPM1 gene, results in abnormal cytoplasmic expression of its protein product, nucleophosmin. The presence of NPM1 mutations has been associated with pretreatment features as female sex, increased bone marrow blast percentages, LDH levels, WBC and platelet counts, and low or absent CD34 expression. In many studies, the presence of NPM1 mutation in CN-AML has been associated with good prognosis. 40% of patients with NPM1 mutations also harbor FLT3-ITDs (Döhner K et al, 2005; Falini B et al, 2006; Schnittger S et al, 2005; Thiede C et al, 2006).

Mutations of the CCAAT/enhancer-binding protein alpha (CEBPA) gene: CEBPA protein is critical for normal hematopoietic differentiation and loss of activity either by mutation or epigenetic silencing can result in a block in normal differentiation. The incidence varies between 7% and 20% in various studies (Schiffer CA, 2008). Those with CEBPA mutations present with higher percentages of peripheral blood blasts, lower platelet counts, less lymphadenopathy and extramedullary involvement, and are less likely to also carry FLT3-ITD, FLT3-TKD and MLL-PTD. CEBPA mutations confer favorable prognosis. CEBPA mutations are best studied by DNA sequencing, and hence are not available on a routine basis.

FLT 3 mutations: FLT3 is a transmembrane tyrosine kinase receptor with important roles in hematopoietic stem/progenitor cell survival and proliferation. FLT3 is the most frequently mutated gene in AML. Different mutations of the gene exist. Most common are the internal tandem duplications (ITDs) in the juxta membrane domain (JMD) and found in 23% of AML patients (Figure 12). FLT3-ITD can be detected in all subtypes of AML but contradictory results have been published concerning its relationship with FAB type (Bacher U et al, 2008; Boissel N et al, 2006; Cairoli R et al, 2006; Frohling S et al, 2002; Gale RE et al, 2008; Kottaridis PD et al, 2001; Schnittger S et al, 2002). Its incidence is associated with hyperleukocytosis and age (Cairoli R et al, 2006). The frequency is higher in elderly patients

and lower in children. FLT3 is highly expressed in infant MLL gene re-arranged ALL and offers an interesting target for this high-risk group of patients (Döhner H et al, 2010). In addition to FTL3-ITD, point mutations in the FLT3 gene involving aspartic acid 835 of the kinase domain (KD), may also lead to constitutive activation of the receptor. FLT3-KD point mutations in other sites are found less frequently. FLT3-KD point mutations are seen in 8-12% of AML patients. Both types of mutation constitutively activate FLT3. Many studies in AML have shown that the presence of ITD mutations portends a poor prognosis. Thiede et al showed that the outcome of AML patients is dependent on the ratio of mutant and wild-type FLT3 (Thiede C et al, 2002). In most studies the KD point mutants do not seem to have the same unfavorable prognostic effect. FLT3 mutations can also be detected in other types of AML including those with t (6;9) and APL (Schiffer CA. 2008). Testing for FLT3 mutations in younger patients, i.e. less than 60 years of age, with de novo AML is now recommended by the NCCN Practice Guidelines in Oncology. Testing for FLT3-ITD and for the other molecular markers is available mostly at only the large university centers and is performed as part of clinical trials **(**NCCN, 2006).

Overexpression of WT1 (Wilm's Tumor 1) gene: The levels of WT1 were found to be 10^2 – 10^3 times higher in AML than in normal bone marrow, where it is either undetectable or expressed at very low levels. WT1 is over-expressed in approximately 90% of AML patients, except in FAB AML-M5, where its expression is lower **(**Gaidzik VI et al, 2009; Inoue K et al, 1994; King-Underwood L et al, 1996; Liu Yin JA, 2002; Paschka P et al, 2008). Many studies show that levels of WT1 transcript are prognostically valuable and can predict early relapse in AML.

Miscellaneous

Partial tandem duplication (PTD) of the MLL (mixed lineage leukemia) gene was the first molecular alteration shown to impact on clinical outcome of CN-AML patients. It is detected in approximately 5% to 10% of these patients. Patients with MLL-PTD have a poorer prognosis than patients without the MLL-PTD (Mrózek K, & Bloomfield CD, 2006). Overexpression of the BAALC gene in PB at diagnosis was detected in adults under the age of 60 years. These are associated with lower WBC, less frequent diagnosis of FAB M5 AML and an unfavourable prognosis (Mrózek K, & Bloomfield CD, 2006). ERG overexpression is a recently identified molecular marker predicting adverse outcome (Mrózek K, & Bloomfield CD, 2006). Mutations of the C- KIT proto-oncogene, a tyrosine kinase receptor, result in a constitutive proliferative signal, have been described in patients with CBF- AML, with data suggesting a poorer outcome in patients with this additional mutation **(**Mrózek K, & Bloomfield CD, 2006; Schiffer CA, 2008).

3.9 Electron microscopy

The role of electron microscopy has diminished ever since the introduction of flow cytometry in the diagnostic work-up. Currently the WHO recognizes its role in the diagnosis of acute basophilic leukemia, which is characterized by the presence of granules containing structures characteristic of basophil precursors. These structures are electron dense particulate substance, are internally bisected, or contain crystalline material arranged in pattern of scrolls or lamellae (Swerdlow SH et al, 2008). The demonstration of metachromatic granules with toluidine blue stain and flow cytometry are enough to make the diagnosis.

3.10 Role of clinical history

The role of clinical evaluation cannot be undermined for any diagnosis and evaluation of etiology and other prognostic factors. However, in the diagnostic algorithm of AML, clinical history is a vital component. A past history of receiving chemotherapy or radiation is of paramount importance to categorize therapy related AML. Similarly past history of myelodysplasia is important for the diagnosis of AML with myelodysplasia related changes. In a known scenario of Down's syndrome AML should be separately grouped. Usually all these entities require cytogenetics supplementation.

Factors	Good	Poor
Clinical Parameters	ECOG $\leq$ 1 No CNS or extramedullary tumors	Age < 2 and > 60 years ECOG > 1 AML with prior chemotherapy or MDS CNS involvement Extramedullary tumors
Laboratory Parameters	TLC < 25000/ cu.mm	TLC > 100,000/ cu.mm Elevated LDH
Morphology	FAB AML-M3, M2, M4Eo	FAB AML-M0, M6, M7
Immunophenotype	CD19, CD2 expression	CD56, CD7 expression
Cytogenetics	t(15;17); t(8;21) inv(16)/ t(16;16)	Complex karyotype inv (3) or t (3;3), t(6;9), t(6;11), t(11;19), monosomy 5, or 7
Molecular	Presence of fusion PML/RARA; RUNX1-RUNX1T1; CBFB-MYH11; Presence of NPM-1 mutation without FLT3-ITD; CEBPA mutations	FLT3-Internal tandem duplication; MLL-Partial tandem duplication; BAALC, WT-1, ERG-2 over-expression; mutations of C-KIT;
Response to treatment	MRD negative	MRD positive

Table 4. Factors that influence prognosis

4. Prognostic work-up

There is a marked heterogeneity in the behavior of AML patients in terms of their response to the treatment and their survival rates. Various factors were found to have an effect on the prognosis in AML (**Table 4**) (Frohling S et al, 2006; Reinhardt D et al, 2000; Saxena A et al,1998). Several groups have published studies using cytogenetics to stratify patients into different risk groups (Byrd JC et al, 2002; Slovak ML et al, 2000). AML cytogenetic subgroups can be identified using molecular profiling with the potential for further subdividing patients to begin to explain the heterogeneity in outcome among patients of the same cytogenetic type. Cytogenetics and molecular studies are very important in the prognostication of acute leukemias. In cytogenetically favorable CBF-AML, the presence of a *KIT* mutation has been shown to have an unfavorable influence on outcome in retrospective studies (Boissel N et al, 2006; Cairoli R et al, 2006; Schnittger S et al, 2006). Numerous molecular markers are known to have impact on prognosis (Preudhomme C et al, 2002;

Renneville A et al, 2009; Wouters BJ et al, 2009b). However, one needs to remember that it is the interaction of these factors, which are responsible for the ultimate prognosis, including the post-therapy remission status (Preisler H, 1993).

5. Assessing the status of the therapeutic targets

Ever since the introduction of all-trans retinoic acid and the Arsenic trioxide in the treatment of APL, imatinib in CML and rituximab in non-Hodgkin lymphomas of mature B-cell type, focus has been on developing specific drugs that would target those molecules and proteins that are specific to the leukemic cells and not affecting the normal hemopoietic cells. The various targets identified and worked upon in cases of AML are CD33, FLT3-ITD, enzymes as farnesyl transferase, histone deacetylase, P-glycoprotein, bcl-2 protein, and vascular endothelial growth factor (Stone RM, 2007). Of these the most widely are evaluated are CD33 and FLT3-ITD and its downstream pathway. Gemtuzumab ozogamicin (Mylotarg, CMA 676) is a monoclonal humanized anti-CD33 antibody chemically linked to the cytotoxic agent calicheamicin that inhibits DNA synthesis and induces apoptosis (Döhner H et al, 2010). It has shown significant activity in patients with relapsed acute myeloid leukemia, in elimination of minimal residual disease and in patients with APL who had evidence of disease only at the molecular level. Several FLT3-selective tyrosine kinase inhibitors (e.g., midostaurin, lestaurtinib, sunitinib) have in vitro cytotoxicity to leukemia cells. A number of FLT3 inhibitors have reached clinical trials as monotherapy in relapsed or refractory AML patients, some or all of whom had FLT3 mutations (Döhner H et al, 2010; Small D, 2006). Keeping these facts in mind assessment of the potential targets should be undertaken before starting these drugs, best at the time of diagnosis.

6. Evaluation of the baseline parameters useful during follow-up

Post-treatment assessment of residual disease is an important prognostic marker. Conventional morphology, karyotyping and FISH have not proven to be of any practical utility. Currently the best parameters at the time of diagnosis which can be used as follow-up markers of disease are molecular transcripts and antigenic profile of the blasts. The practical guidelines are that: for patients with t(15;17), t(8;21) and inv(16), which are about 30% of AML cases, it is recommended to quantify MRD by real-time RT-PCR (Liu Yin JA, 2002; Lo-Coco F et al, 1999, 2003). For patients without a molecular marker, the options are multiparameter flow-cytometry or assessment of the WT1, whichever is appropriate for a particular patient (Inoue K et al, 1994; Wood BL, 2007).

7. Parameters to assess baseline general health and detect comorbidities

The general health and comorbidities should be assessed at the time of diagnosis before the patient undertakes the treatment. These will be baseline results based on which the complications will be monitored during the treatment. The following tests should be performed - complete blood counts, biochemical analysis, coagulation tests (especially in APL), urine analysis, serum pregnancy test in women with child bearing potential, screening for Hepatitis A, B, C virus and HIV-1 and 2, chest radiograph and 12-lead EKG, ECHO cardiography and lumbar puncture, whenever indicated.

8. Planning for future

Although not recommended on a routine basis, however, these tests should be planned in advance to avoid subsequent confounding results. If the patient is an ideal subject for allogeneic stem cell transplant, HLA typing and cross-matching of the patient and potential donors should be performed at the outset and the results be sent to the Bone marrow transplant registry. The patients' pretreatment leukemic marrow and blood should be stored within a biobank.

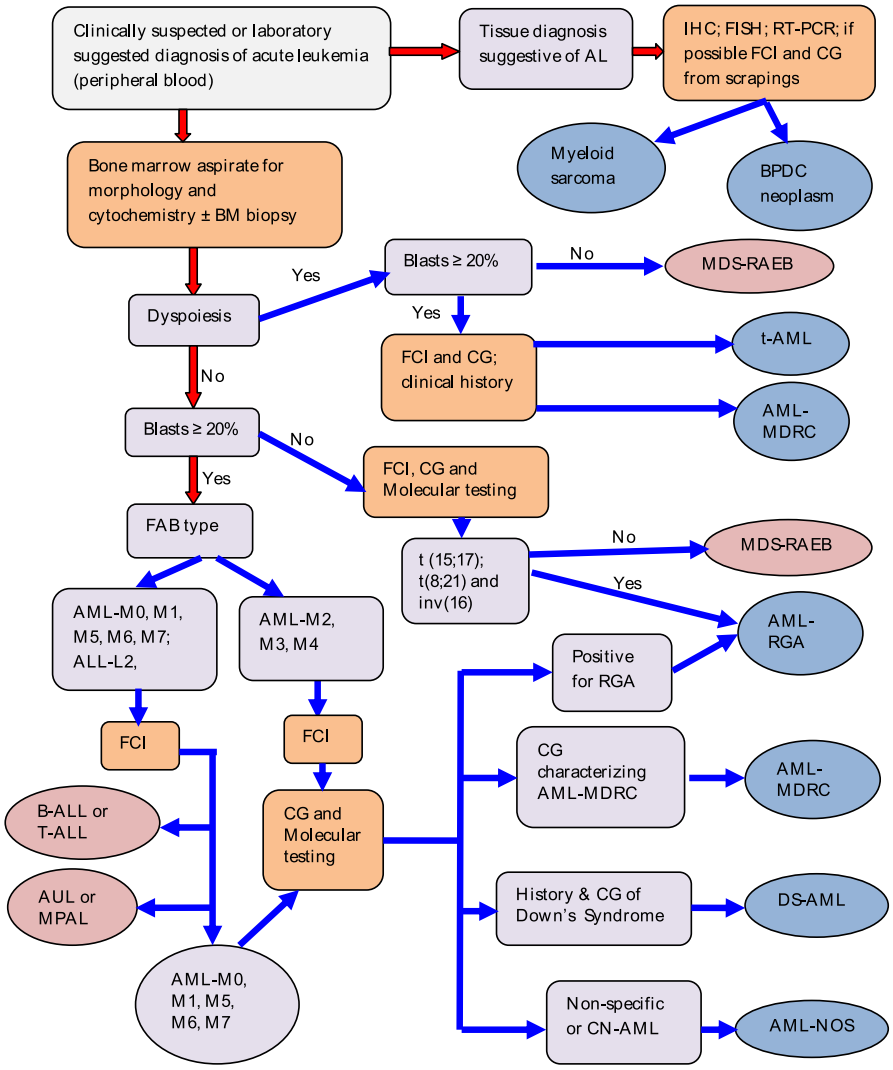
Genome-wide studies - The Probable Future: In recent years, DNA microarrays (complementary DNA (cDNA) and oligonucleotide), together with the availability of the complete nucleotide sequence of the human genome, have spurred the search for abnormalities in cancer, including AML (Wouters BJ et al, 2009a). There is enough data to support the fact that there is heterogeneity within established AML subtypes. The studies pertaining to the CBF-AMLs, each could be split into subgroups merely based on the GEP data (Wouters BJ et al, 2009a). Further validation of the generated data is necessary for assessing biologic significance.

9. Approach in an ideal set-up

The ideal work-up contains all elements described above with the aim of planning for future. The focus is to adopt whatever is clinically significant and proven in terms of diagnosis, prognosis, therapy, and disease monitoring at that point of time. It also involves archiving the necessary samples for future research and the data obtained thereafter may be available to incorporate newer information into clinical practice. The algorithms for this may be as illustrated in **Figures 13, 14 & 15.**

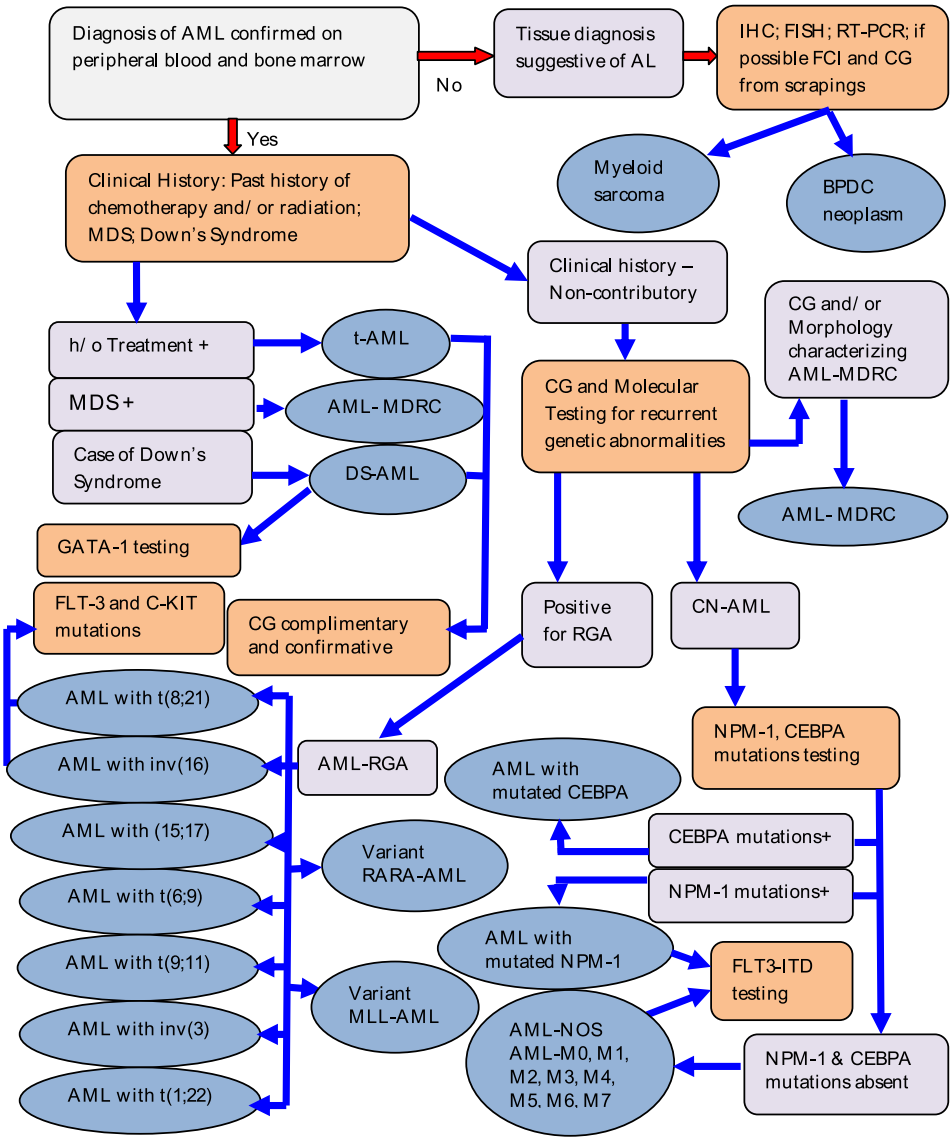
Approach in resource limited areas

WHO 2008 is the most appropriate classification in terms of prognostication and pathogenesis. This is however, a resource intensive process and liable to deviations in large parts of the world. The constraints on men, machine and material required to adhere to the current WHO classification are very real. It becomes necessary to devise methods that simplify the steps of diagnosis, prognosis, and monitoring treatment response. We find in our experience that it is possible to provide meaningful laboratory support for our under-resourced patient population. Morphology combined with cytochemistry forms the basis of identifying entities that are potentially curable [ALL, APL, AML with t(8;21) and AML with inv(16)] and those which are less likely to yield good response to treatment (AML-M0, AML with dyspoiesis, AML-NOS). With this objective work-up is planned. If treatment is a definite choice, baseline markers for monitoring response are necessary. The lack of resources including finances, infrastructure, expertise and socio-cultural factors that hinder treatment options are considerable. In such a situation diagnosing acute leukemia and recognizing AML in itself is an important step in patient management. Hence, the approach needs to be tailored to individual patient. The work-up designed in these circumstances may not always be in accordance with WHO 2008 guidelines. However, the information derived from this classification has improved our approach. The important end-points in this approach are to distinguish AML from ALL, identify the good-prognostic categories among AML. The algorithm for the same is proposed here **(Figure 16).**



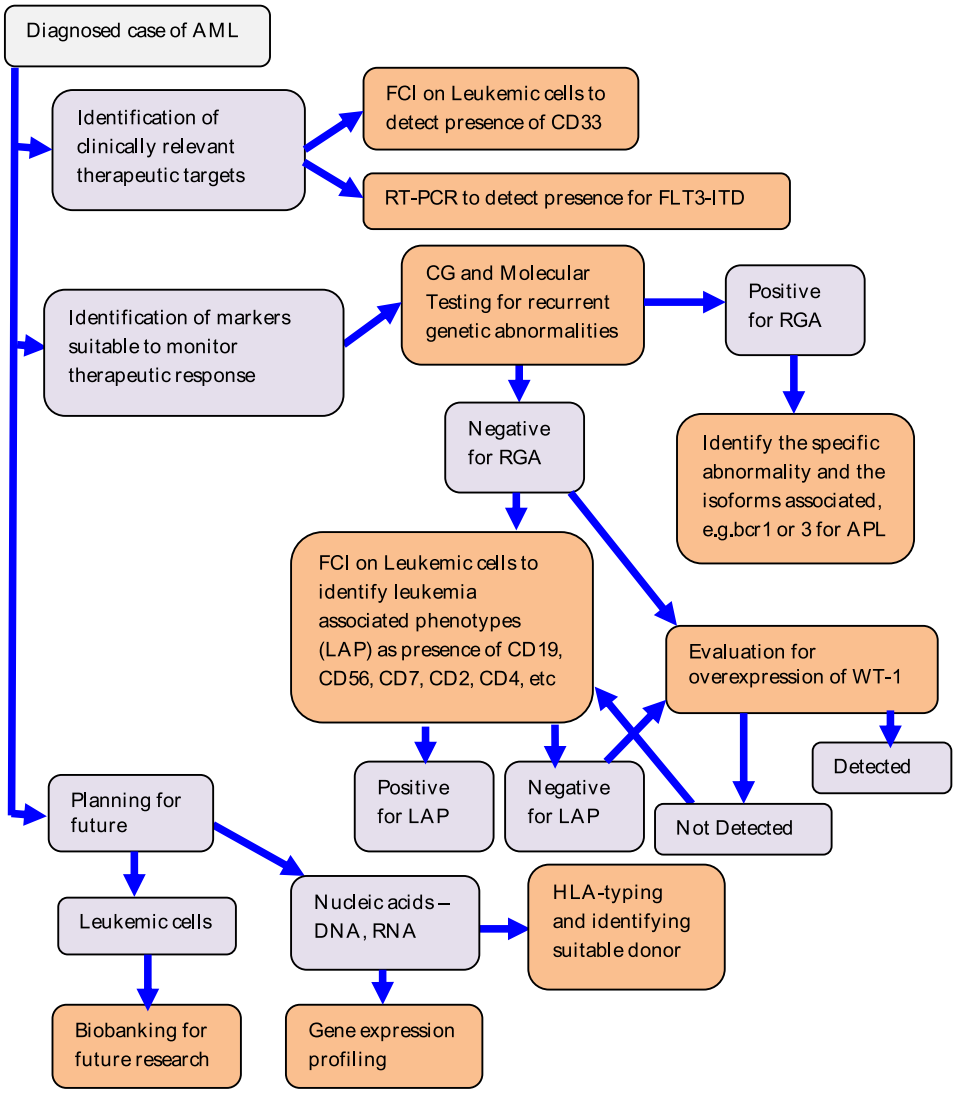
Abbreviations used (in alphabetical order) :AL-Acute leukemia; AML-Acute myeloid leukemia; AML-MDRC- AML with myelodysplasia related changes; ; AML-NOS- AML-Not otherwise Specified;AML-RGA- AML with recurrent cytogenetic abnormalities; AUL-Acute undifferentiated leukemia; B-ALL- B-Acute Lymphoblastic Leukemia; BPDC-Blastic plasmacytoid dendritic cell; CG- Cytogenetics; CN-AML-Cytogenetically normal AML; DS-AML- Down's Syndrome related AML; FAB-French-American-British; FCI-Flow cytometry Immunophenotyping; FISH-Fluorescent-in-situ hybridisation; IHC-Immunohistochemistry;MDS-RAEB-Myelodysplastic syndrome-Refractory anemia with excess blasts; MPAL- Mixed Phenotypic acute leukemia; RT-PCR-Reverse Transcriptase Polymerase chain reaction; T-ALL- T-Acute Lymphoblastic Leukemia; t-AML-therapy related AML

Fig. 13. Algorithm for establishing the diagnosis of AML. The important testing points are highlighted in "Beige". The end-points related to AML are highlighted in blue and the differential diagnoses are highlighted in pink.



Abbreviations used (in alphabetical order) :AL-Acute leukemia; AML-Acute myeloid leukemia; AML-MDRC- AML with myelodysplasia related changes; AML-RGA- AML with recurrent cytogenetic abnormalities; BPDC-Blastic plasmacytoid dendritic cell; CG- Cytogenetics; CN-AML-Cytogenetically normal AML; DS-AML- Down's Syndrome related AML; FCI-Flow cytometry Immunophenotyping; FISH-Fluorescent-in-situ hybridisation; IHC-Immunohistochemistry; MDS-Myelodysplastic syndrome; RGA- Recurrent Genetic Abnormalities; RT-PCR-Reverse Transcriptase Polymerase chain reaction; t-AML-therapy related AML

Fig. 14. Algorithm for Molecular Characterization of AML. The important testing points are highlighted in "Beige". The end-points related to AML are highlighted in blue.



Abbreviations used (in alphabetical order) : AML-Acute myeloid leukemia; CG- Cytogenetics; FCI-Flow cytometry Immunophenotyping; RGA- Recurrent Genetic Abnormalities; RT-PCR-Reverse Transcriptase Polymerase chain reaction

Fig. 15. Algorithm for Identification of Therapeutic Targets, Markers for Disease Monitoring and Planning for future: The important interventions are highlighted in "Beige".

Abbreviations used (in alphabetical order) :AL-Acute leukemia; AML-Acute myeloid leukemia; AML-MDRC- AML with myelodysplasia related changes; AML-NOS- AML-Not otherwise Specified; AUL-Acute undifferentiated leukemia; B-ALL- B-Acute Lymphoblastic Leukemia; BPDC-Blastic plasmacytoid dendritic cell; CG- Cytogenetics; FAB-French-American-British; FCI-Flow cytometry Immunophenotyping; FISH-Fluorescent-in-situ hybridisation; IHC-Immunohistochemistry; MDS-RAEB-Myelodysplastic syndrome-Refractory anemia with excess blasts; MPAL- Mixed Phenotypic acute leukemia; RT-PCR-Reverse Transcriptase Polymerase chain reaction; T-ALL- T-Acute Lymphoblastic Leukemia; t-AML-therapy related AML

Fig. 16. Diagnostic algorithm in resource constrained situations. The important testing points are highlighted in "Beige". The end-points related to AML are highlighted in blue and the differential diagnoses are highlighted in pink.

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10

Clinical and Biological Relevance of Gene Expression Profiling in Acute Myeloid Leukemia

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1. Introduction

Over the last decade, considerable effort has gone into defining global gene expression profiles (GEP) in many different types of malignancies. There is a dual aim behind these studies: on the one hand, to identify molecular signatures that correlate with clinically useful parameters and, on the other hand, to increase knowledge concerning the biology of the respective diseases. Some of these studies yielded molecular classifications of specific cancer types that better correlate with disease progression and/or response to therapy, whereas others revealed yet unknown biological properties of cancer cells that may represent the starting point for novel therapeutic approaches.

Acute myeloid leukemias (AML) represent a highly heterogeneous set of malignancies whose pathogenesis is linked to specific genetic abnormalities, including chromosome translocations and point mutations that involve genes encoding for key regulators of hematopoiesis (Marcucci, Haferlach, & Dohner 2011). Genetic information is the most relevant parameter for the correct classification of AML patients at diagnosis into three prognostic risk groups (favorable, intermediate and adverse) and, consequently, for directing therapeutic choices (Lo-Coco et al. 2008; Dohner et al. 2010). In fact, current diagnostic approaches include cytogenetic and molecular analyses for correct stratification of AML patients according to the World Health Organization (WHO) recommendations (Vardiman et al. 2009). However, these approaches are not fully satisfactory, particularly within the significant group of cytogenetically normal AML (CN-AML), where known prognostic markers are lacking. This likely reflects the genetic heterogeneity of the CN-AML group, the existence of yet unidentified genetic lesions and the co-existence of different genetic mutations in a significant number of cases.

AML was the first type of malignancy to be studied with a GEP approach, and hundreds of reports addressing specific clinical issues (classification, prognosis, response to therapy) have been published. An equally significant number of studies have addressed the molecular pathogenesis of AML by analyzing GEP in functionally characterized AML model systems, including transgenic mice, primary or established cell lines expressing leukemogenic oncogenes. The analysis of their specific target genes has been exploited to

unravel the functional consequences of AML-associated oncogene expression, including the arrest of myeloid differentiation and enhanced cell survival.

In recent years, the analysis of AML has been extended to other genomic approaches, including microRNA profiling and epigenetic studies. The rapid technological advancement, and in particular the advent of next-generation sequencing has imposed a dramatic change of outlook to the molecular basis of cancer, including AML, and it is likely that GEP approaches so far used will become obsolete, favoring more focused, clinically relevant expression studies.

What have GEP studies taught us about AML? We here propose an overview of the progress that has been made through GEP both in terms of clinical utility and of insight to the biology of the disease, and discuss future perspectives (Figure 1).

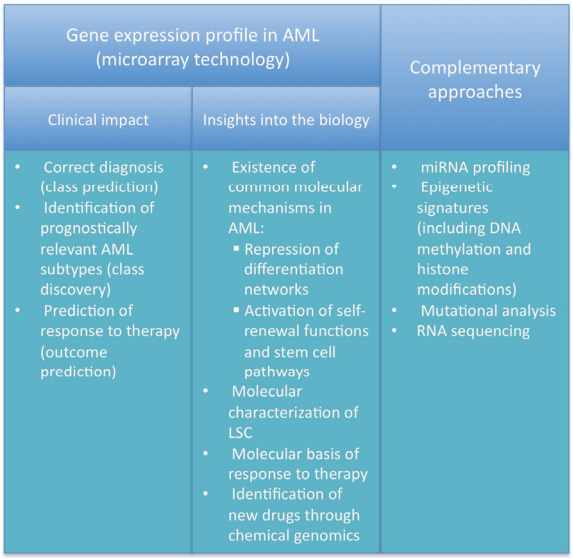

Fig. 1. Main points covered in this review.

2. Clinical utility of GEP analysis in AML

The first evidence that GEP could be employed as a tool for the correct classification of cancer was reported by Golub and collaborators in 1999, using acute leukemias as a test case (Golub et al. 1999). The authors were able to discriminate AML samples from acute lymphoblastic leukemia (ALL) samples without prior knowledge concerning the respective diagnosis, and suggested two important applications for GEP: "class discovery", which refers to the identification of new prognostically relevant tumor subtypes, and "class prediction", which assigns tumor samples to already known subtypes on the basis of their specific gene expression signature. Successive studies introduced the possibility to exploit GEP for predicting response to therapy ("outcome prediction") (Theilgaard-Monch et al. 2011). Technically, class discovery implies the search for significant similarities and differences in a cohort of samples, assuming that similar gene expression signatures will correspond to the same disease subtype, and relies on an unsupervised approach (i.e. no

prior knowledge of patient characteristics, such as age, cytogenetics, molecular abnormalities, etc.). Class prediction, instead, takes into account patient information to derive gene expression signatures that are specific for given parameters, which can then be used for predicting disease subtypes in samples of unknown status (i.e. supervised approach).

In *de novo* AML, chromosomal abnormalities can be detected at diagnosis in approximately 55% of cases by cytogenetics analysis, and specific genetic mutations can be identified in 85% of the remaining CN-AML. The most frequent chromosomal rearrangements include reciprocal translocations and inversions, such as t(8;21), which fuses the *AML1* and *ETO* genes, inv(16), which results in the *CBFβ/MYH11* chimeric gene, t(15;17), which generates the *PML/RARa* fusion specific of acute promyelocytic leukemia (APL), and a variety of translocations involving the *MLL* gene on chromosome 11q23, the most frequent being t(9;11) (Look 1997). The resulting fusion proteins possess oncogenic properties and frequently function as transcriptional regulators (Alcalay et al. 2001). It is therefore perhaps not surprising that AML blasts bearing such rearrangements display specific gene expression signatures, as demonstrated by several studies (Bullinger et al. 2004; Debernardi et al. 2003; Schoch et al. 2002; Valk et al. 2004). In fact, GEP can actually predict favorable cytogenetic AML subtypes, i.e. t(8;21), t(15;17) and inv(16), with 100% accuracy, and with >90% accuracy for AML with *MLL* rearrangements (Haferlach et al. 2005b; Ross et al. 2004), whereas the correlation is less stringent for other molecular subtypes (Verhaak et al. 2009).

The function of group-specific genes often reflects characteristics of the corresponding disease: for example, the t(15;17) signature of APL, which is clinically characterized by a hemorrhagic diathesis and a response to treatment with Retinoic Acid (RA), includes genes involved in hemostasis and suggests an impairment in the response to RA-induced differentiation (Bullinger et al. 2004). Interestingly, GEP can segregate APL cases (M3 subtype) from variant cases (M3v), which are characterized by a specific morphology of blasts and by a more severe prognosis (Haferlach et al. 2005a). The genes that are differentially expressed between APL-M3 and APL-M3v encode for functions such as granulation and maturation of blood cells, which are coherent with the morphological and clinical observations.

Specific somatic mutations are frequent events in AML, particularly in CN-AML, which are classified in the group with intermediate risk even though there are important differences. Currently, only mutations of the *NPM1*, *CEBPA* and *FLT3* genes have an impact on the clinics because of their correlation with prognosis. Other recurrent mutations in AML include *N-RAS*, *KIT*, *IDH1*, *IDH2*, *WT1*, *RUNX1* and *MLL*, but their clinical significance is either controversial or unknown, and their identification is at the moment not used for guiding therapeutic choices. Although these mutations are prevalent in CN-AML, they can also be found in association to other cytogenetic abnormalities (Marcucci, Haferlach, & Dohner 2011).

Specific GEP signatures have been described for AML that carry mutations, but their predictive accuracy appears to be lower than the one described for the recurrent cytogenetic rearrangements described above (Verhaak et al. 2009), likely due to the frequent co-occurrence of different genetic abnormalities or to the presence of other yet unknown mutations. Mutations in the *NPM1* gene represent the most frequent genetic abnormality in CN-AML (Falini et al. 2005), and are associated to mutations of the *FLT3* gene in a significant proportion of cases. AML with mutated *NPM1* without concurrent *FLT3*

mutations are characterized by a better response to induction therapy and a favorable prognosis (Falini et al. 2007). These cases present a gene expression signature characterized by over-expression of *HOX* and *TALE* genes (Alcalay et al. 2005; Verhaak et al. 2005). Other genes involved in maintenance of hematopoietic stem cells, such as the NOTCH ligand *JAG1*, are also over-expressed, suggesting that the cell of origin of AML with mutated *NPM1* may be an early hematopoietic progenitor, as further indicated by frequent multilineage involvement (Alcalay et al. 2005; Pasqualucci et al. 2006).

AML with *CEBPA* mutations are also associated with a favorable outcome. Generally, AML in this group carry mutations in both *CEBPA* alleles, whereas heterozygous mutations are less frequent. A specific GEP signature has been described for AML with biallelic *CEBPA* mutations, while no discriminating gene expression pattern was detectable in AML carrying single mutations (Wouters et al. 2009). Of note, in this study, only AML with biallelic *CEBPA* mutations correlated with a favorable outcome, whereas single mutations did not, suggesting a prognostic value for the specific gene expression signature. Interestingly, in a group of CN-AML patients displaying a GEP signature resembling that of AML with mutant *CEBPA*, but lacking such mutations, the *CEBPA* gene was found to be silenced as a consequence of promoter hypermethylation (Wouters et al. 2007). This result suggests that GEP may actually be more efficient than mutational analysis in identifying functional pathways that are perturbed in specific AML cases, and may be a useful tool for correct molecular classification of AML .

Two types of mutations involving *FLT3* can be found in AML: internal tandem duplications (*FLT3-ITD*), which are present in 20% of AML, and point mutations within the tyrosine kinase domain (*FLT3-TKD*) that can be detected in an additional 5-10% of cases. *FLT3-ITD* are associated with a poor prognosis in CN-AML, whereas the prognostic relevance of *FLT3-TKD* is not clear (Marcucci, Haferlach, & Dohner 2011; Mrozek et al. 2007). GEP analyses in AML with mutated *FLT3* have yielded controversial results. One study described an accurate separation of samples with *FLT3-ITD* from those with *FLT3-TKD* (Neben et al. 2005), while another study reported a specific gene expression signature that discriminates between *FLT3-TKD* and *FLT3*-wild type CN-AML (Whitman et al. 2008). On the other hand, other studies reported difficulties in predicting *FLT3* mutations from GEP results (Valk et al. 2004; Verhaak et al. 2009). Such conflicting results may reflect the frequent coincidence of *FLT3* mutations with other mutations in CN-AML (Dohner et al. 2010), where the co-occurrence of several genetic abnormalities is likely to impact on the phenotype and the specific GEP. Interestingly, however, a specific gene expression signature derived from *FLT3-ITD* CN-AML, although not highly accurate in predicting the *FLT3-ITD* genotype, proved to be extremely accurate in predicting clinical outcome (Bullinger et al. 2008), suggesting that activation of the *FLT3* pathway may be mediated by other yet unidentified genetic alterations.

In summary, GEP has proven to be extremely reliable in identifying AML cases with recurrent chromosomal abnormalities, but less predictive in identifying specific gene mutations in CN-AML (Verhaak et al. 2009), and this raises doubts as to its applicability in a clinical setting. However, a recent multicenter study involving 11 laboratories that use different microarray platforms (MILE – Miocrarray Innovations in Leukemia) demonstrated that GEP is a robust technology for the diagnosis of hematologic malignancies with high accuracy (Haferlach et al. 2010), and that in some cases GEP outperformed routine diagnostic procedures. The analysis of larger cohorts of AML cases, in particular of the less

frequent molecular subtypes, will likely be necessary for the identification of reliable gene expression signatures of clinical utility. Whether GEP-derived predictors can be of use for prognosis, and in particular whether GEP presents a concrete advantage over standard cytogenetic or molecular markers in terms of prognostic value, remains an open question. Different studies have identified specific gene expression signatures that correlate with clinical outcome in AML (Bullinger et al. 2004; Metzeler et al. 2008; Radmacher et al. 2006), suggesting that GEP may, in fact, yield diagnostic and prognostic information simultaneously.

3. GEP and the biology of AML

As discussed above, GEP results derived from AML patients have been exploited to derive information concerning the biology underlying the disease. For the purpose of identifying specific functional pathways relevant to leukemogenesis, this approach is, however, partially hampered by properties that are intrinsic to patient-derived material, including individual genetic variability and the presence of heterogeneous cellular populations in each sample. It is, therefore, possible to exploit different experimental model systems, including purified cellular subpopulations, cell lines or animal models, with the aim of unraveling the molecular mechanisms underlying leukemic transformation. Such approaches have been widely proved to be reliable by extensive validation through a variety of independent methods. A significant number of reports have identified transcriptional targets deregulated in specific types of AML, and it is not possible to discuss all the data and their implications in due detail. We will instead briefly review specific aspects that emerged from these studies, focusing on their possible relevance to AML pathogenesis and management.

3.1 Common functions: Myeloid differentiation and stem cell maintenance

A general feature of AML-associated oncogenes is the capacity to block the process of myeloid differentiation and to promote self-renewal of hematopoietic precursor/stem cells. A variety of model systems have been employed for GEP analyses with the aim of identifying specific genes and pathways underlying these properties. Some of these studies have highlighted the existence of target genes that are common to several AML-associated oncogenes, suggesting that diverse genetic mutations can lead to the deregulation of overlapping downstream functional pathways. This observation is of potential clinical importance, since it suggests the existence of common therapeutic targets, regardless of the specific initiating oncogenic lesion. For example, expression of AML fusion proteins such as AML1/ETO, PML/RAR, and PLZF/RAR in the U937 hematopoietic cell line resulted in deregulation of a large set of common targets (Alcalay et al. 2003). These included a decreased expression of genes involved in myeloid differentiation, such as *GFI1*, *CSF3R*, *STAT5A* and others, and the activation of pathways leading to increased stem cell renewal (in particular, the Jagged1/Notch pathway). A similar approach led to the identification of Wnt signaling activation by diverse AML oncogenes, through upregulation of *plakoglobin* expression (Muller-Tidow et al. 2004). The existence of common leukemogenic functions is further suggested by the observation that a specific cellular subpopulation derived from *CEBPA*-deficient leukemia, which is capable of transferring AML to recipient mice, revealed a GEP signature shared with MLL-AF9-transformed AML (Kirstetter et al. 2008).

The identification of overlapping functions deregulated by AML oncogenes is perhaps to be expected. In fact, the genetic lesions underlying AML pathogenesis mostly involve transcriptional regulators that function during myeloid differentiation in an orchestrated manner, and are physiologically cross-regulating each other's expression. For example, CEBPA is a crucial factor in myeloid differentiation, and its expression is often attenuated or repressed by oncogenic transcription factors such as AML1/ETO (Pabst et al. 2001). Therefore, part of the transcriptional response elicited by AML1/ETO may be due to a decrease in CEBPA activity. Overexpression of CEBPA in human CD34+ hematopoietic precursors induces the expression of genes involved in myeloid differentiation (Cammenga et al. 2003), which are presumably targets of deregulation not only in AML with mutated *CEBPA*, but also in all AML that present decreased levels of *CEBPA* expression.
Interestingly, mutations involving genes that do not directly regulate gene expression, such as *FLT3* or *NPM1*, are associated to alterations in global gene expression that partially overlap with those described for oncogenic transcription factors. Expression of *FLT3* mutants in murine 32Dcl3 cells resulted in repression of genes involved in myeloid differentiation, including *CEBPA*, and in the activation of a transcriptional program that partially overlaps with that induced by IL-3, a potent hematopoietic cytokine (Mizuki et al. 2003). Recently, the expression of an *NPM1* mutant allele in mouse HSC was shown to result in *HOX* gene overexpression, reproducing the situation of primary AML with mutated *NPM1* (Vassiliou et al. 2011). The molecular mechanisms through which these mutants elicit a transcriptional response remain to be elucidated.

3.2 Characterization of Leukemic Stem Cells

AML derives from the transformation of a single hematopoietic progenitor/stem cell, known as leukemic stem cell (LSC), which shares important properties with normal hematopoietic stem cells (HSC), including unlimited self-renewal and the capacity to give origin to a hierarchy of hematopoietic cells. The molecular characterization of LSC and the identification of functions that are specific of LSC with respect to normal HSC are clearly instrumental for designing novel therapeutic strategies aimed at eradicating AML.
The transforming genetic event does not necessarily occur in HSC, but may take place in more differentiated progenitors that reacquire stem cell characteristics (Passegue et al. 2003). In favor of the latter possibility, Krivtsov et al. isolated leukemic stem cells (LSC) from a mouse model of AML generated by the MLL-AF9 fusion protein, which revealed a GEP that was reminiscent of normal granulocyte/macrophage progenitors (Krivtsov et al. 2006). However, a subset of genes that is highly expressed in normal HSC appeared to be re-activated in LSC, including several *HOXA* genes, *STAT1* and *CD44*. Another study conducted on CD34+ AML revealed two distinct subpopulations of LSC, the more mature resembling normal granulocyte-macrophage progenitors in terms of GEP, and the immature LSC population reminiscent of lymphoid-primed multipotent progenitors (Goardon et al.). Taken together, these studies and the ones discussed in the previous section suggest that AML initiates in progenitor cells that re-acquire specific stem cell characteristics, such as activation of Notch and/or Wnt signaling and/or over-expression of *HOX* genes, which are tightly linked to the acquisition of an unlimited self-renewal capacity and cause an arrest in the differentiation program.
However, the identification of functions that are specific of LSC with respect to normal HSC is clearly of importance for the identification of novel therapeutic strategies aimed at

eradicating AML. In terms of global gene expression, LSCs are not simply characterized by the re-activation of stem cell pathways and maintenance of self-renewal. A direct comparison of the GEP of highly enriched normal human HSC and LSC from AML of diverse subtypes revealed differences in relevant functional pathways, including Wnt signaling, MAP Kinase signaling, and Adherens Junction (Majeti et al. 2009). The latter is particularly intriguing, since it suggests specific abnormalities in the relationship between LSCs and the microenvironment ("niche").

3.3 Response to therapy

AML are characterized by a heterogeneous response to therapy, and although there has been notable progress in the past decades, most patients still succumb to the disease. The search for new therapeutic strategies is therefore of paramount importance, and GEP studies have also been exploited to dissect the molecular basis underlying the response to AML therapy.

One way to identify genes/pathways that may determine response to therapy is to compare GEP of treated versus untreated AML cells. Among AML, APL represents an exception in that its exquisite sensitivity to RA and arsenic trioxide treatment has dramatically changed its prognosis to a 5-year survival rate of 90%. Several studies have described specific transcriptional programs that are modulated by RA in APL cells, with the aim of identifying targets that may be of wider use in AML treatment. GEP analysis of APL blasts and PML/RAR-expressing U937 cells treated with RA *in vitro* revealed that the transcriptional response to RA is characterized by regulation of genes involved in the control of differentiation, stem cell self-renewal and chromatin remodeling, suggesting that specific structural changes in local chromatin domains may be required to promote RA-mediated differentiation (Meani et al. 2005).

Another possible approach is to compare the GEP of sensitive versus resistant AML cells after treatment with drugs. Tagliafico et al. derived a molecular signature that predicts the resistance or sensitivity to differentiation induced by RA or vitamin D in six myeloid cell lines, and proved its validity in a set of primary AML blasts using an *in vitro* differentiation assay (Tagliafico et al. 2006). Similarly, Zuber at al., described the differences between a chemosensitive and a chemoresistant AML model: murine AML expressing the AML1/ETO fusion protein, which show a dramatic response to chemotherapy, displayed activation of the p53 tumor suppressor function. Murine AML expressing MLL fusion proteins are instead drug-resistant and present an attenuated p53 response. It appears, therefore, that the p53 network has a central role in the response to chemotherapy in AML (Zuber et al. 2009).

Importantly, GEP information can also be exploited for the identification of new therapeutic options. Corsello et al. defined an AML1/ETO GEP signature by comparing a t(8;21) bearing cell line before and after siRNA-mediated inhibition of the fusion protein, and used the resulting signature to screen a set of drug-induced expression profiles (Corsello et al. 2009). In a recent study, publicly available GEP data sets derived from APL patients were exploited for the identification of an "APL signature", which was then compared to a collection of expression profiles for more than 1300 bioactive compounds for the discovery of relevant drug candidates (Marstrand et al. 2010). Although these studies have not yet been transferred to the clinics, they open a concrete possibility for exploiting GEP data alongside chemical genomics approaches for the *in silico* identification of molecularly targeted drugs.

4. Conclusion and perspectives

Have GEP studies truly had an impact on the management of AML? Currently, no GEP-based diagnostic/prognostic tests are available for AML in clinical practice. However, tests that reliably predict the outcome for cancer patients based on the expression pattern of a selected subset of genes identified through GEP are available for other malignancies, such as breast cancer (van't Veer&Bernards 2008), and available evidence suggests that a reliable set of prognostic predictors could be established for AML as well. Furthermore, GEP can accurately sub-classify most AML according to the underlying genetic abnormality even when histopatholgical data are ambiguous, and outperforms routine diagnostic tests in certain cases, raising the possibility to introduce GEP-derived approaches in a diagnostic setting. A particularly exciting perspective is to exploit GEP data in combination with chemical genomics for the design of novel therapeutic strategies aimed at molecular targets.

Many factors concur in determining the AML phenotype, and in recent years there has been a growing interest in high-throughput approaches other than GEP to analyze microRNA (miRNA) expression, epigenetic modifications and whole-genome DNA sequencing in AML. MiRNAs are small non coding RNAs that can regulate the expression levels of numerous target mRNAs both at the transcriptional and post-transcriptional level. Similarly to what has been observed for mRNAs, specific miRNA signatures have been associated with AML subtypes (Garzon et al. 2008; Li et al. 2008; Marcucci et al. 2008), and may therefore represent an additional option for the development of clinically useful tools.

Epigenetic modifications including DNA methylation and covalent histone modifications, such as acetylation, methylation and ubiquitination, are known to play a crucial role in the regulation of gene expression, and different epigenetic alterations have been described in leukemias (Plass et al. 2008). Recently, specific DNA methylation profiles have been described for distinct cytogenetic and molecular AML subtypes, and a 15-gene methylation classifier was found to be predictive of overall patient survival (Figueroa et al. 2010). Interestingly, the integration of DNA methylation data with GEP was shown to further improve prognostication in AML, suggesting that integration of genomic approaches may prove of clinical importance (Bullinger et al. 2010).

The advent of next-generation sequencing technology has opened the possibility to investigate the complexity of cancer genomes, and the first complete sequence of a human malignancy reported was that of an AML genome (Ley et al. 2008). The authors identified ten somatic mutations, two of which had already been described (*NPM1* and *FLT3*), while the other eight were novel. None of the latter were, however, detected in a cohort of 187 AML cases, casting serious doubts as to their relevance in determining the leukemic phenotype. Successive studies using the same approach identified novel mutations in the *IDH1* and *DNMT3* genes that are instead recurrent in AML, underlining the power of this approach for the discovery of genetic alterations in cancer (Ley et al. 2010; Mardis et al. 2009). However, these studies also highlighted the relevant genetic heterogeneity among AML patients within the same subtype, since most of the mutations described were actually specific to the single patient under analysis, and their contribution to AML progression remains to be defined. One distinct possibility is that they represent "passenger" mutations that arise as a consequence of cancer-associated genomic instability, and bear no functional relevance to the malignant phenotype. On the other hand, such mutations may instead involve different players within complex functions (for example, regulators of proliferation or differentiation), and although the mutated genes are different, the functional

consequence(s) may be the same. In any case, the co-existence of several genetic alterations within the same cell is bound to have an impact on gene expression, and it will therefore be necessary to integrate GEP data with the corresponding results from mutational analyses.

Finally, microarray technology has inherent limitations, and with the advancement of current sequencing approaches may rapidly become obsolete. The possibility to sequence entire transcriptomes (RNA-seq) has several advantages over microarray-based GEP, including transcription start site mapping, gene fusion detection, small RNA identification and detection of alternative splicing events (Ozsolak & Milos 2011). The first RNA-seq analysis of an AML model reported an unexpected level of transcriptome variation between phenotypically similar LSC, including a large number of structural differences such as alternative splicing and promoter usage (Wilhelm et al. 2011). These results suggest a broad transcriptional heterogeneity in AML that is not limited to differences in mRNA levels.

In the next few years there will inevitably be an explosion of genomic data in AML, describing yet unknown molecular mechanisms underlying the disease. The large amount of already available GEP data will have to be integrated with the new findings to increase its value in generating knowledge that can ultimately be translated into clinically useful tools.

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11

Clinical Characteristics of Acute Myeloid Leukemia with t(8;21) in Japan and Western Countries

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1. Introduction

Acute myeloid leukemia (AML) with t(8;21) (q22;q22)translocation (t(8;21)AML) is one of the major disease group of AML, accounting for 7 to 8% of adult AML, with most classified as M2 by FAB classification (Ferrara & Del Vecchio, 2002). In the reports from Western countries to date, it has been reported that the survival rate of patients with t8;21-associated AML can be improved by employing consolidation therapy with high-dose cytarabine, which has a good remission and survival rate compared to other AML (Byrd *et al*, 1999) (Grimwade *et al*, 1998).

According to recent research by the Group B Study of Cancer and Leukemia, it has been suggested that the treatment outcome of t(8;21)AML may differ depending on race (Marcucci *et al*, 2005). This indicates that the knowledge from past research in Western countries cannot necessarily be directly transferred to Japanese patients, but the reality is that research related to the clinical features of Japanese t(8;21)AML is very limited. Of these publications, the best summarized report is the analysis of patients participating in a clinical study of Japan adult leukemia study group (JALSG) by Nishii et al. in 2003 (Nishii *et al*, 2003). This report reveals that the overall 5-year survival rate was 52%. However, this report mainly focuses on the additional chromosome abnormality of t(8;21)AML. Many clinicians felt the need for new research in order to clarify the clinical features of t(8;21)AML in Japanese patients, such as prognosis and treatment outcomes.

The research group of the authors appealed to clinicians and researchers aware of such issues, arranged participation of institutes for which approval was obtained, and conducted a retrospective study with the purpose of clarifying clinical features in Japanese patients. (Narimatsu *et al*, 2008b) This article will compare reports from Western countries to date focused on the outcome, and explain the clinical characteristics of t(8;21)AML in Japanese patients.

2. Japanese t(8;21)AML patients have a favorable survival rate

The authors retrospectively investigated clinical features of 46 adult t(8;21)AML patients newly diagnosed at facilities participating in the research from 2000 to 2005 as the subjects (Narimatsu et al, 2008b). The comparison of its outcome with prior researches (Appelbaum *et al*, 2006; Marcucci *et al*, 2005; Nguyen *et al*, 2002; Schlenk *et al*, 2004) is shown in Table 1. First off, the 3-year overall survival rate of Japanese t(8;21)AML patients reported by the authors was 70%. A definite conclusion cannot be drawn due to the short follow-up period, but close to 70% were estimated to have a 5-year survival rate. This is a good number compared to past reports from Western countries, in which 5-year survival rate ranged from 45% to 59% (Appelbaum *et al*, 2006; Marcucci *et al*, 2005; Nguyen *et al*, 2002). On the other hand, age, which is the greatest risk factor for the survival of leukemia patients, was older in reports from Japan compared to reports from Western countries. Moreover, white blood cell counts and blood platelet counts at the initial visit, which are also believed to be risk factors, were the same in reports from Japan and reports from Western countries. From these comparisons, it was suggested that the prognosis of Japanese t(8;21)AML patients was better than the prognosis of Westerners, and a possibility that racial differences may be involved as its cause, rather than the inclusion of background factors such as age and white blood cell count at initial visit, was suggested.

The authors also compared the survival rate of t(8;21)AML patients and leukemia patients that were diagnosed in each institute at the same time as AML(M2) having no t(8;21) abnormality(Narimatsu *et al*, 2008b). As a result, the overall survival rate of t(8;21)AML patients was significantly better than AML(M2) patients without t(8;21) (70% vs. 43% for 3-year overall survival rate). However, the age of AML(M2) patients without t(8;21) was significantly higher compared to t(8;21)AML, so the overall survival rate was compared by limiting patients to those 60-years old or younger. As a result, the 3-year survival rate was 71%(n=35, patients with t(8;21)) and 58% (n=49, patients without t(8;21)), respectively, narrowing the difference, with no significant difference observed between the two groups. Furthermore, when prognosis factor analysis was conducted with all these patients as subjects, the presence of t(8;21) was not a significant prognosis factor(Narimatsu *et al*, 2008b). These results suggest that young age is the main reason for good prognosis in t(8;21)AML patients, which is very interesting.

3. Prognosis factor

It was revealed from the investigation of Japanese t(8;21)AML patients by the authors that the older the age, the higher the white blood cell count at the initial visit, and the worse the survival rate. This, as shown in Table 1, can be said to be almost the same as the outcome of the research in Western countries. However, chromosome abnormalities additionally occurring in t(8;21), such as the deficiency of a sex chromosome and/or chromosome-9-abnormality that have been pointed out in much prior research, was not an apparent prognosis factor. This is the same finding as the research by Nishii et al. from JALSG, and there is a possibility that this may be the difference with Western patients (Nishii *et al*, 2003).

Moreover, although not included in the research by the authors, the report by Nishi et al. showed that the prognosis of patients with an addition of trisomy 4 is extremely poor, and

	Narimatsu et al	Nguyen et al	Schlenk et al	Marcucci et al	Appelbaum et al
Number of patients	46	161	191	144	174
Age (median (range))	49.5 (18-86)	28 (3-63)	43 (16-60)	37 (17-75)	36 (16-77)
White blood cell count at the initial visit (median (range))	9.3 (0.9-54.9)	12.0 (1.4-68.0)	10.8 (1.2-152)	10.5 (0.9-252)	10.6 (0.3-107)
White blood cell count at the initial visit (median (range))	34 (6-99)	-	30 (3-470)	40 (6-311)	37 (3-658)
Extramedullary infiltration	0.18	0.08	0.09	0.22	-
Remission rate	0.91	0.96	0.87	0.89	0.89
Overall survival rate	70% (3 years)	59% (5 years)	65% (3 years)	46% (5 years)	45% (5 years)
Prognosticator	Age White blood cell count	White blood cell count index	White blood cell count Blood platelet count Sex chromosome abnormality	Age Blood platelet count	Age † Percentage of peripheral blood blasts† Complex karyotypic abnormality†

† Analysis result combining t (8;21) and inv (16) AML

Table 1. Research on the recent clinical characteristics of t(8;l21)AML within Japan and other overseas countries

clinicians should make note of this fact. In one of those patients, Nishii et al showed c-kit mutation of the leukemia cells (Langabeer *et al*, 2003). KIT mutation may related to this unfavorable outcome, which needs to be clarified in further large study.

4. Is high dose cytarabine therapy effective?

It has been reported by prior research in Western countries that high dose cytarabine therapy can improve the treatment outcome of t(8;21)AML patients(Byrd *et al*, 1999; Ferrara & Del Vecchio, 2002). The authors also investigated whether or not the same can be said of Japanese patients. As a result, the survival rate of 14 patients who underwent consolidation therapy with a standard dose of cytarabine and that of 19 patients who underwent consolidation therapy with a high-dose cytarabine was approximately the same. However, numbers of those patients are small and the consolidation regimen of these patients covered various grounds, and it is difficult to discuss the efficacy of high dose cytarabine therapy from this result. Increased fatal side effects such as infectious diseases are a concern with high dose cytarabine therapy, but from this result, it is suggested that high-dose cytarabine can also be conducted in Japanese patients.

Recently, the outcome of a randomized controlled trial of consolidation therapy with high dose cytarabine therapy and multi-drug therapy was reported by a JALSG group. According to their report, although high dose cytarabine therapy shows a better outcome for disease free survival and overall survival compared to multi-drug therapy at a standard dosage, the results did not have any apparent significant differences (Miyawaki *et al*, 2011). This differs from the research report accounting for the usefulness of high-dose cytarabine in Western countries represented by the research by Bloomfield et al (Bloomfield *et al*, 1998). On the other hand, documented severe infections are common in high-dose cytarabine groups. This difference is presumed to be related to the difference in the incidence of tyrosine kinase such as KIT, etc., which is believed to affect the prognosis, and there is a need to clarify this in future research.

Taking the above outcomes into account, at this point, the decision regarding whether or not to apply high-dose cytarabine to Japanese t(8;21)AML patients must be determined by the on-site clinician.

5. The significance of measuring minimal residual disease

RUNX1 (AML1)/MTG8 (ETO) transcript occurs as a result of t(8;21)(q22;q22) translocation. The minimal residual disease can be evaluated by qualitatively and quantitatively measuring this transcript by the PCR method. In some small scale research from Western countries, it has been reported that patients with a high risk for relapse can be determined by evaluating minimal residual disease. (Krauter *et al*, 2003; Leroy *et al*, 2005; Perea *et al*, 2006; Tobal *et al*, 2000) (Weisser *et al*, 2007) Therefore, in the same way, the authors also investigated the clinical significance of minimal residual disease in Japanese t(8;21)AML patients by collecting the outcome of RUNX1/MTG8 quantitative tests from the clinical records of 26 t(8;21)AML patients that reached complete remission (Narimatsu *et al*, 2008a). As a result, between the group that reached less than 1,000 copies of RUNX1/MTG8 transcript when remission was reached (n=13) and the group that did

not (n=7), the relapse-free survival rate was better in the latter group. This shows that in contrast to reports from Western countries, in Japanese patients, the number of copies of RUNX1/MTG8 transcript when remission is reached does not necessarily reflect prognosis; however, number of study patients are small and it is difficult to make a definite conclusion. On the other hand, relapse is expected in patients in whom RUNX1/MTG8 transcript increased during the remission period, and monitoring RUNX1/MTG8 transcript during the remission period was suggested to have significance in terms of early prediction of relapse.

6. Conclusion - Issues to be solved in the future

The motivation for the group of the authors to initiate research was the hypothesis of the authors, "Reports on t(8;21)AML from Western countries do not match the feeling of actual clinical practice." Outcomes actually investigated also suggested a possibility of clinical features differing between Western t(8;21)AML patients and Japanese t(8;21)AML patients. It will be necessary to conduct large-scale research and/or a prospective study on Japanese patients as well in the future, in order to create evidence for Japanese t(8;21)AML patients. The following are listed in concrete terms.

1. It is necessary to conduct a large-scale retrospective study to compare the survival rate of t(8;21)AML to that of AML with other chromosome abnormalities or AML without any. Furthermore, it is necessary to clarify if t(8;21) translocation is a significant prognosis factor of AML (as in the research by the authors, if the young age of t(8;21)AML patients is responsible for good prognosis).
2. It is necessary to clarify the molecular biological characteristics of t(8;21)AML in Japanese patients. Particularly, investigation into whether or not the frequency of tyrosine kinase mutation, N-Ras mutation,,which are believed to have an effect on prognosis, is different between t(8;21)AML in Japan and Western countries, should be useful.
3. It is also necessary to reinvestigate the clinical significance of minimal residual disease by research designed so as to unify when specimens were retrieved and the method of examination with patients treated, using the same regimen as the subject.

The clarification of clinical features of t(8;21)AML in Japanese and Western patients and the establishment of optimum therapy customized for every ethnicity is hoped for in the near future.

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12

Acute Promyelocytic Leukemia Lacking the Classic Translocation t(15;17)

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1. Introduction

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) characterized by the reciprocal translocation t(15;17)(q22;q12) resulting in the fusion gene *PML-RARA* and an oncoprotein that impairs myeloid differentiation (Arber et al., 2008; de The et al., 1990; Rowley et al., 1977). Morphological and clinical characteristics include hypergranular leukemic promyelocytes, Auer rods, and coagulopathy. The use of all-trans retinoic acid (ATRA) has revolutionized the management of this disease that has become the most curable form of AML in adults (Castaigne et al., 1990; Tallman et al., 1997). In relapsed APL, arsenic trioxide can induce complete morphological, cytogenetic and molecular remission (Douer and Tallman, 2005; Soignet et al., 1998).
Cases lacking the classic t(15;17) are divided into two separate groups that behave differently and are now considered different disease entities (Arber et al., 2008). The first group represents cryptic and complex APL where t(15;17) is absent on routine cytogenetic studies but *PML-RARA* is present on molecular studies (Grimwade et al., 2000). This group shares the same phenotype, prognosis, and sensitivity to ATRA as classic APL, and is thus managed similarly. The second group, "AML with a variant *RARA* translocation", is no longer considered part of APL and includes acute myeloid leukemias with translocations involving *RARA* and a variety of partner genes other than *PML* (Arber et al., 2008). Compared to classic APL, these leukemias often exhibit significant differences in malignant phenotype and sensitivity to ATRA which will be further explored in this chapter.

2. Clinical characteristics

APL represents less than 10% of all AML, but seems to be over-represented in Hispanics (Yamamoto and Goodman, 2008). The median age of presentation is approximately 40 years (Vickers et al., 2000). Leukocytosis is only seen in about 25% of patients, and organomegaly is rarely found on diagnosis. The most common presenting signs are pancytopenia, fever, anemia, and bleeding. The latter can be fatal especially if occurring in the central nervous system (CNS), and is due to the combination of thrombocytopenia and the dreaded coagulopathy of APL (Warrell et al., 1993).

3. Morphology

Abnormal promyelocytes are larger than their normal counterparts, with a nucleus that is often bilobed or kidney-shaped. 75% of APL cases are hypergranular (M3) with densely-packed cytoplasmic granules that are bright pink, red, or purple, in addition to Auer rods in bundles called "faggot cells". The remaining 25% of cases are microgranular or hypogranular (M3v), the granules being visualized by electron microscopy but not light microscopy, and the cytoplasm may contain a few fine azurophilic granules.
In APL, myeloperoxidase (MPO) is strongly positive in all leukemic promyelocytes, and this can be especially helpful in microgranular APL which is sometimes confused with acute monocytic leukemia (Arber et al., 2008).

4. Immunophenotype

APL cells are usually CD13 positive and especially CD33 positive, but are characterized by low or absent expression of HLA-DR, CD34, CD11a, CD11b, CD18, and CD117 (Paietta et al., 2004). Hypogranular APL frequently coexpresses CD34 and CD2 (Exner et al., 2000). Expression of CD56 has been observed in about 20% of cases and confers a worse outcome (Ferrara et al., 2000).

5. Pathogenesis

APL is caused by the reciprocal translocation t(15;17)(q22;q12) that results in the fusion gene *PML-RARA* and an oncoprotein that impairs myeloid differentiation (Grignani et al., 1993). *PML* and *RARA* are both involved in normal hematopoiesis, and disruption of their physiologic roles by the formation of *PML-RARA* is essential to leukemogenesis.
PML possesses physiologic growth suppressor and proapoptotic properties that are disrupted by *PML-RARA*, possibly by the abnormal positioning of *PML* away from the nuclear body structure, thus contributing to leukemic transformation (Wang et al., 1998). Following this logic, treatment with ATRA restores the normal localization of *PML*, allowing the resumption of its physiologic functions.
On the other hand, *RARA* normally binds to response elements at the promoter region of target genes through heterodimerization with the retinoid X receptor (*RXR*). *RARA-RXR* results in the recruitment of nuclear corepressors (N-CoR) and histone deacetylase (HDAC) that repress transcription and inhibit differentiation (Grignani et al., 1993). This is thought to take place through epigenomic changes including histone deacetylation or methylation (Licht, 2009) and could have therapeutic implications in the future, especially as to the efficacy of histone deacetylase inhibitor in APL refractory to conventional treatment with ATRA. Physiologic amounts of retinoid acid (RA) unbind the N-CoR from *RAR-RXR*, allowing for activation of transcription of *RARA* target genes and myeloid differentiation. In the presence of *PML-RARA*, normal concentrations of RA are not enough for that separation and pharmacologic doses of ATRA are needed to allow myeloid differentiation (Warrell et al., 1993). Arsenic trioxide (ATO) can also lead to differentiation, but it does so by inducing degradation of the *PML-RARA* fusion transcript. Both drugs have recently been shown to also work on an entirely different level in APL by eradicating "leukemia-initiating cells" or "leukemic stem cells" (Nasr et al., 2009), leading to think that their combination in induction regimens could result in higher rates of prolonged remissions and cure.

6. Genetics

6.1 Classic t(15;17) APL

Around 92% of APL patients have the balanced t(15;17), leading to the fusion of the retinoic acid receptor-alpha (*RARA*) gene on chromosome 17 and the promyelocytic leukemia (*PML*) gene on chromosome 15 (Grimwade et al., 2000) (Fig. 1). FISH uses a dual color dual fusion probe to detect *PML-RARA* rearrangements. The typical normal FISH pattern for the dual color, dual fusion probe is 2 red signals (2R) and 2 green signals (2G) for the *PML* and *RARA* loci respectively. When t(15;17) is present, the characteristic FISH pattern is one red, one green and two fusion signals (Fig. 2).

Whereas the breakpoints in *RARA* are invariably at intron 2, those in *PML* can occur at any one of three breakpoint cluster regions (Bcr): intron 6 (Bcr1), exon 6 (Bcr2), and intron 3 (Bcr3) (Pandolfi et al., 1992). The 3 respective ensuing mRNA types, long (L)-form, variable (V)-form, and short (S)-form, can exhibit different phenotypes but do not affect complete remission (CR) rate or disease-free survival (DFS). The S-form, for example, is associated with increased leukocytosis which by itself is an adverse risk factor in APL, but after adjusting for that, does not independently influence CR rate and OS (Gallagher et al., 1997). The V form, originally thought to be less sensitive to ATRA, was later shown to be as equally sensitive to it as the other two types (Slack et al., 2000).

6.2 Cryptic and complex APL

As mentioned before, t(15;17) is absent in around 8% of patients diagnosed with APL (Grimwade et al., 2000), which should lead to the adoption of *PML-RARA* as the hallmark of APL. Cases lacking t(15;17) are divided into two separate disease entities: on one hand, cryptic and complex APL that share the same phenotype, prognosis, and sensitivity to ATRA as classic APL; and on the other hand, AML with a variant *RARA* translocation (Arber et al., 2008) which will be discussed later in this chapter.

In cryptic and complex APL, the classic t(15;17) is absent on routine cytogenetic studies but *PML-RARA* is present on molecular studies; the leukemia is morphologically and clinically similar to t(15;17) positive APL and is treated as such. The European working party was crucial in characterizing the rare APL cases lacking the classic t(15;17) on routine cytogenetic studies. 4% of the cases represented cryptic/masked APL with submicroscopic insertion of *RARA* into *PML* leading to the expression of the *PML-RARA* transcript, while 2% had complex variant translocations involving chromosomes 15, 17 and an additional chromosome, and were sub-classified as: (a) complex variant t(15;17) due to a 3-way balanced translocation involving 15q22, 17q21, and another chromosome; (b) simple variant t(15;17) involving 15q22 or 17q21 with another chromosome; and (c) very complex cases (Grimwade et al., 2000).

In these unusual cases, the diagnosis can be missed by conventional cytogenetic studies, and molecular methods are needed such as fluorescence in situ hybridization (FISH) (Fig. 2), reverse transcriptase polymerase chain reaction (RT-PCR) and direct sequencing. FISH is often not sensitive enough to detect small cryptic insertions (Han et al., 2007; Kim et al., 2008; Wang et al., 2009), while RT-PCR can also face technical challenges such as atypical *PML-RARA* rearrangement with new breakpoints in the *PML* gene that cannot be amplified with conventional primers (Barragan et al., 2002; Park et al., 2009), insertions of the *PML* gene to the *RARA* but too far apart to permit elongation and amplification of the *PML-RARA* sequence (Tchinda et al., 2004), or submicroscopic deletions of the 3′ *RARA* (Han et al., 2009).

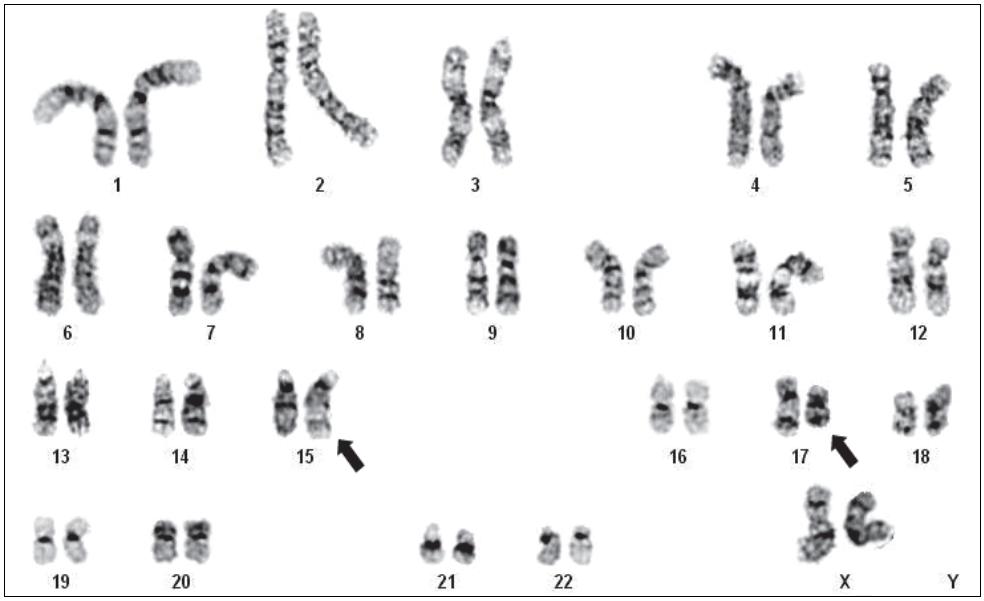

Fig. 1. G-banded karyotype with t(15;17)(q22;q21) at arrows.

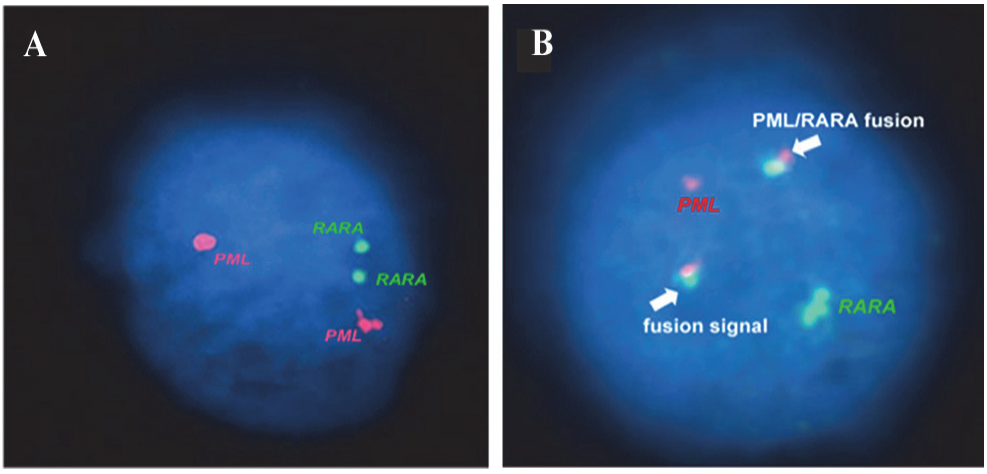

Fig. 2. Dual color dual fusion break apart probe for detection of *PML-RARA* rearrangement. Panel A shows a normal FISH pattern (2R,2G), whereas panel B reveals fusion of the *PML* and *RARA* loci at arrows.

6.3 AML with a variant *RARA* translocation

This term is now used by the WHO (World Health Organization) to designate a subset of acute myeloid leukemias morphologically similar to APL, but lacking both t(15;17) by cytogenetics and *PML-RARA* by FISH and RT-PCR (Arber et al., 2008). They do, however,

show different variant translocations involving *RARA* and 1 of 7 partner genes: *ZBTB16* (previously known as promyelocytic leukemia zinc finger gene or *PLZF*) on chromosome 11q23 (Licht et al., 1995), *NUMA1* (nuclear matrix-mitotic apparatus protein 1 gene) on chromosome 11q13 (Wells et al., 1996), *NPM1* (nucleophosmin gene) on chromosome 5q35 (Corey et al., 1994; Hummel et al., 1999), *STAT5B* (signal transducer and activator of transcription 5 beta) on chromosome 17q21.1-21.2 (Zelent et al., 2001), *PRKAR1A* (protein kinase, cAMP-dependent, regulatory, type I, alpha) on chromosome 17q24 (Catalano et al., 2007), *FIP1L1* (factor interacting with PAP 1-like 1) on chromosome 4q12 (Buijs and Bruin, 2007), and *BCOR* (BCL6 corepressor gene) on chromosome X (Yamamoto et al., 2010). Of the partner genes, the first 4 were included in the latest WHO classification, while the last 3 have been described since. As with other hematological malignancies, partner genes affect both neoplastic phenotype and response to treatment including ATRA, making their identification crucial in the evaluation of these patients.

6.3.1 ZBTB16-RARA

The *ZBTB16* or *PLZF* gene encodes for a zinc finger transcription factor of 673 amino acids (Chen et al., 1993). Its expression may play a role in the life of hematopoietic stem cells and seems to be down-regulated with differentiation (Shaknovich et al., 1998). Like *PML*, it possesses tumor suppressor activity that seems to be disturbed by t(11;17)(q23;q21) (Zelent et al., 2001). The European working party on APL found the t(11;17)(q23;q21) translocation in 0.8% of APL patients (Grimwade et al., 2000). The first case was identified in a Chinese patient from Shanghai (Chen et al., 1993), and more than 16 cases have been described since. The clinical presentation is usually indistinguishable from APL, with a low peripheral WBC count and a preponderance of promyelocytes in the bone marrow. The leukemic cells are usually microgranular, have a regular nucleus instead of bilobed, no Faggot cells, and there is often an increased number of Pelger-Huet-like cells (Sainty et al., 2000). The blasts are typically HLA-DR and CD34 negative, CD13 and CD33 positive. Several cases were strongly positive for the CD56 NK cell antigen.

The tumor suppressor properties of *ZBTB16* are thought to be inhibited by the *ZBTB16-RARA* fusion protein in t(11;17)(q23;q21). Except for anecdotal reports, patients with *ZBTB16-RARA* are resistant to ATRA since pharmacological doses of the drug fail to dissociate *ZBTB16* from the co-repressors (Licht et al., 1995).

6.3.2 NUMA1-RARA

The nuclear matrix-mitotic apparatus protein 1 gene (*NUMA1*) on chromosome 11q13 is a 236 kDa protein that serves in the completion of mitosis, is thought to be involved in the regulation of transcription and is affected by post-translational changes (Harborth et al., 2000; Saredi et al., 1996). So far, there's only been a single report of a patient with *NUMA1-RARA,* a 6 month-old boy who was diagnosed with APL with atypical features, received ATRA and was in complete remission (CR) more than 24 months following a bone marrow transplant (Wells et al., 1997; Wells et al., 1996). The pathogenesis of this leukemia is not well understood, but is thought to share several features with *PML-RARA* APL.

6.3.3 NPM1-RARA

The nucleophosmin gene *(NPM1)* plays a role in several important cell functions from the transportation of ribosomal precursors between cytoplasm and nucleolus (Szebeni et al.,

1997), to cell growth control (Zelent et al., 2001) and activation of transcription (Shi et al., 1997). It had been implicated in hematological malignancies including anaplastic lymphoma (Morris et al., 1994) and myelodysplastic syndrome (Yoneda-Kato et al., 1996). The *NPM1-RARA* fusion is a rare variant translocation (less than 0.5%) and has so far been reported in pediatric patients, with absent Auer rods but otherwise variable morphology. In contrast to classic APL, CD13 is negative, but the rest of the immunophenotype is similar to classic APL including absence of CD56. The reported cases have been very sensitive to treatment with ATRA (Corey et al., 1994; Grimwade et al., 2000; Hummel et al., 1999; Redner et al., 1996).

6.3.4 STAT5B-RARA

STAT5B is one of many latent cytosolic transcription factors to be activated by janus kinase (JAK) tyrosine kinases, allowing it to move to the nucleus where it regulates gene transcription (Arnould et al., 1999). To date, only 4 cases of AML with *STAT5B-RARA* have been reported, all men in their fourth to sixth decade of life, with a predilection for disseminated intravascular coagulation (DIC) but otherwise heterogeneous clinical, morphologic and immunophenotypic characteristics. Finally, S*TAT5B-RARA* is resistant to ATRA, similarly to *ZBTB16-RARA.* (Arnould et al., 1999; Iwanaga et al., 2009; Kusakabe et al., 2008).

6.3.5 PRKAR1A-RARA

PRKAR1A refers to protein kinase, cAMP-dependent, regulatory, type I, alpha.
Protein kinase A (PKA) is a multimeric protein which activity is dependent on cyclic adenosine monophosphate (cAMP). Downregulation of PKA occurs when phosphodiesterase, one of the substrates activated by the kinase, converts cAMP to AMP, effectively decreasing cAMP that can activate PKA. There's only one reported case of AML with *PRKAR1A-RARA* in a 66 year-old man. He presented with a normal WBC count, had a hypercellular marrow with 88% hypergranular promyelocytes, regular nuclei, and absent Auer rods and faggot cells. MPO was strongly positive, but expression of CD13, CD33, and CD11b was weak. The cells were negative for CD2, CD19, CD34, CD56, CD117, and HLA-DR (Catalano et al., 2007).

6.3.6 FIP1L1-RARA

Human FIP1 is an integral subunit of cleavage and polyadenylation specificity factor (CPSF), and plays a significant role in poly(A) site recognition and cooperative recruitment of poly(A) polymerase to the RNA (Kaufmann et al., 2004). Only 2 cases of *FIP1L1-RARA* have been described, and the entity seems to be sensitive to ATRA. The first case involved a 90 year-old woman who was clinically diagnosed with APL and achieved a complete remission by oral administration of ATRA alone. No further details were described in the paper as to clinical presentation, morphology, or immunophenotypic analysis (Kondo et al., 2008).
The second case involved a 20 month-old boy who was diagnosed with juvenile myelomonocytic leukemia after presenting with leukocytosis and anemia. Bone marrow aspirate showed hypercellularity including 11% promyelocytes, 25% myelocytes, 12% metamyelocytes, and 8% myelomonoblasts. These cells were hypergranular but had regular nuclei and no Auer rods. Immunophenotypic analysis was not published. Unfortunately, the patient did not receive ATRA, had an allogeneic stem cell transplant but died from relapse a few months later.

6.3.7 BCOR-RARA

As its name implies, *BCOR* is a corepressor of transcription through the oncoprotein BCL6, and its activity could be disrupted by the formation of *BCOR-RARA* (Huynh et al., 2000).
There's only one such case reported in the literature of a 45 year-old male patient who presented with leukocytosis and coagulopathy. Leukemic cells were MPO positive and less granular than classic APL. Interestingly, the cytoplasm contained periodic acid–Schiff rectangular and round cytoplasmic inclusion bodies and lacked Auer bodies and faggot cells. Immunophenotypic analysis showed HLA-DR negativity but positivity for CD33, CD13 and CD56. The patient was clinically responsive to ATRA but had several relapses with chemotherapy and ATRA (Yamamoto et al., 2010).

7. Treatment

In the previous section, we depicted the reported cases of AML with a variant *RARA* translocation, their response to treatment, and their varying sensitivity to ATRA depending on the partner gene. We will now discuss the management of classic APL, and cryptic and complex APL; these all share the same phenotype, prognosis, and sensitivity to ATRA, and therefore are treated similarly.

7.1 Induction therapy

When left untreated, APL is the deadliest form of AML with a median survival of less than 30 days (Hillestad, 1957). The introduction of ATRA in 1980 (Breitman et al., 1980) completely revolutionized the management of this disease that now boosts complete remission rates of 80 to 95% and cure rates of around 80% (Sanz and Lo-Coco, 2011). ATRA sets off the differentiation of malignant promyelocytes into mature granulocytes, improves homeostasis and shortens the duration of the dreaded coagulation syndrome of APL. It also generates the eradication of "leukemia-initiating cells" or "leukemic stem cells", a property shared by arsenic trioxide (ATO). In mice, a combination of both drugs can actually result in the elimination of leukemia-initiating cells and effectively "cure" APL (Nasr et al., 2009), opening the door to future trials combining ATRA and ATO without the use of chemotherapy. As mentioned before, if APL is suspected clinically and cytologically, ATRA should be promptly started even if cytogenetic and molecular confirmations of the diagnosis are pending.
Because of the short duration of CR with ATRA alone, and the known sensitivity of APL to anthracyclines (Head et al., 1995), the current standard induction regimen in APL is the administration of ATRA with anthracycline-based chemotherapy. This combined approach has been shown to be superior to a previously adopted sequential treatment of ATRA followed by chemotherapy (Fenaux et al., 1999). The median time to CR ranges from 38 to 44 days but could be as long as 90 days. In addition to its effect on CR, chemotherapy controls leukocytosis that is common when ATRA is used alone. In patients who have contraindications to anthracycline chemotherapy, the combination of ATRA and arsenic trioxide (ATO) for induction treatment should be considered (Sanz et al., 2009). The current standard chemotherapy regimens use daunorubicin with cytarabine or idarubicin alone, while there's a lack of experience and data with other anthracyclines. These 2 regimens have indirectly yielded comparable CR rates (Fenaux et al., 1999; Mandelli et al., 1997). When daunorubicin was used without cytarabine in one randomized prospective trial of young

patients with APL, the CR rates were similar but there were more relapses and lower overall survival in patients who did not get cytarabine (Ades et al., 2006). The additional benefit conferred by cytarabine, however, did not apply to all patients and was only observed in those with WBC > $10x10^9$/L (Ades et al., 2008) who are high-risk patients by Sanz's risk stratification (Table 1) (Sanz et al., 2000; Sanz et al., 2004). Based on these results and the findings of other trials suggesting a similar role for cytarabine in consolidation (Sanz and Lo-Coco, 2011), we recommend that APL patients younger than 60 years old with WBC > $10x10^9$/L receive cytarabine in addition to ATRA and an anthracycline. Other indicators of relapse, such as CD56 positivity, do not currently alter treatment decisions (Ferrara et al., 2000).

7.2 Consolidation therapy

Five to six weeks following induction, patients should be re-evaluated with bone marrow aspirate/biopsy and cytogenetics, while RTC-PCR for *PML-RARA* is not required since the transcript will still be detectable in about half of patients. Those in remission (> 90% of patients) will proceed with consolidation treatment to prevent relapse. This involves the use of an anthracycline (± cytarabine in high-risk patients), in addition to ATRA (Sanz et al., 2004; Sanz et al., 2008), but different regimens are still being prospectively studied.

7.3 Maintenance therapy

Molecular remission is required at the end of consolidation treatment, after which maintenance ATRA will increase disease-free survival and improve the 10-year cumulative incidence of relapse (Ades et al., 2010; Tallman et al., 2002). The most commonly used maintenance regimen lasts for 1 year and encompasses ATRA 45 mg/m2 orally daily for 15 days every 3 months or 7 days every 2 weeks, 6-mercaptopurine 60 mg/m2 orally every evening, and methotrexate 20 mg/m2 orally every 7 days (Avvisati G, 2003). Patients require close surveillance for toxicities, myelosuppression, and abnormal liver function tests, in addition to RTC-PCR every 3 months to monitor for disease relapse.

Risk stratification		3-year DFS
Low risk	WBC ≤ $10x10^9$/L, PLT > $40x10^9$/L	97%
Intermediate risk	WBC ≤ $10x10^9$/L, PLT ≤ $40x10^9$/L	97%
High risk	WBC > $10x10^9$/L	77%

Table 1. Risk stratification of APL patients based on WBC and Platelet (PLT) counts, and corresponding 3-year disease-free survival (DFS) following induction and consolidation therapies with ATRA + anthracycline-based chemotherapy, followed by standard maintenance (Sanz et al., 2000; Sanz et al., 2004)

8. Refractory and relapsed disease

8.1 Arsenic trioxide

Patients who do not achieve cytogenetic remission after induction therapy and/or molecular remission after consolidation are considered to have refractory disease, while those in remission who suddenly have detectable *PML-RARA* by RTC-PCR have relapsed APL. In both situations, salvage treatment is needed and arsenic trioxide (ATO) can induce CR in 85 to 88% of patients, and this can be followed by stem cell transplantation (Soignet et

al., 2001; Soignet et al., 1998). ATO not only induces degradation of the *PML-RARA* fusion transcript, leading to differentiation of malignant promyelocytes, but also leads to the death of "leukemia-initiating cells" (Nasr et al., 2009).
So far reserved for the treatment of refractory or relapsed disease, in addition to some use in patients with contraindications to anthracyclines (Sanz et al., 2009), ATO has and is currently being studied for use in first-line induction therapy alone or in combination with ATRA without any chemotherapy (Hu et al., 2009; Mathews et al., 2006). This, however, has not yet become standard of care.
ATO is usually given at 0.15 mg/kg/day intravenously until hematologic remission or for a maximum of 60 days. The major side-effects of this drug are fluid retention, differentiation syndrome and QT prolongation (Unnikrishnan et al., 2004).

8.2 Other agents

Repeat treatment with ATRA and chemotherapy in refractory and relapsed APL has had disparate success, and other agents that might be of benefit in this setting are still under investigation including gemtuzumab, Hum195 which is an anti-CD33 antibody, sodium phenylbutyrate, and calcitriol.
Of special note, tamibarotene, a synthetic retinoid synthesized by the University of Tokyo in 1984 and 10 times more potent than ATRA, seems to be especially promising. Tamibarotene is approved in Japan for use in relapsed and refractory acute APL, and was successfully used at our institution (University of Texas Southwestern Medical Center) in a patient with relapsed and refractory extra-medullary APL (Naina et al., 2011). Tamibarotene is currently being compared to ATRA for maintenance therapy in the ongoing APL204, a randomized phase III trial of the Japan Adult Leukemia Study Group.

9. Other considerations

9.1 Coagulopathy

Within the first 10 days of treatment, 5-10% of APL patients will develop fatal hemorrhage, especially in the central nervous system (CNS) and lungs (Rodeghiero et al., 1990). This is secondary to a characteristic coagulation disorder combining disseminated intravascular coagulation (DIC) and fibrinolysis that is not well understood. Platelets and cryoprecipitate should be transfused to maintain platelet counts more than 30-50x10^9/L, and fibrinogen level more than 150 mg/dL, respectively (Tallman et al., 2005). ATO and ATRA have both been shown to quickly correct this coagulation disorder, and the initiation of the latter has become a true emergency in any new APL patient. ATRA should be promptly started when APL is clinically and cytologically suspected even if cytogenetic and molecular confirmations of the diagnosis are pending (Sanz et al., 2009).

9.2 Central Nervous System (CNS) prophylaxis

The CNS is the most common site of extramedullary disease and relapse in APL (Evans and Grimwade, 1999), with elevated WBC count > 10x10^9/L being the only significant risk factor in a multivariate analysis (de Botton et al., 2006). There are no guidelines as to the systematic CNS prophylaxis of APL patients with leukocytosis. Groups who include intrathecal chemotherapy in their regimens administer it during consolidation, not during induction when the risk of fatal bleeding is high. ATO crosses the blood-brain barrier and is being

evaluated for use in first-line induction therapy; it is conceivable that such induction regimens will result in lower rates of CNS relapse.

9.3 Differentiation syndrome

Also known as the retinoic acid syndrome or cytokine storm, it is seen in around 25% of APL patients in the first 3 weeks following treatment with ATRA or arsenic trioxide (Vahdat et al., 1994). The differentiation syndrome is caused by the release of cytokines from neoplastic promyelocytes as they differentiate in response to treatment. Usual symptoms include fever, shortness of breath, peripheral edema, pulmonary infiltrates, hypoxemia, respiratory distress and hypotension. Patients can also develop renal and hepatic dysfunction, in addition to pleural and pericardial effusions. The syndrome can be fatal and prompt recognition is vital, leading to the initiation of intravenous dexamethasone 10 mg twice daily until clinical resolution, followed by slow steroid taper. Patients with WBC > $10x10^9$/L are suspected to be at increased risk, and some recommend treating this group prophylactically with steroids (Wiley and Firkin, 1995).

10. Conclusion

Over the last 2 decades, we have witnessed a change in acute promyelocytic leukemia from the most malignant form of AML to the most curable one; a remarkable medical achievement that did not rely on advances in chemotherapy, but rather on molecular targeted therapy in the form of differentiation agents. This innovative approach to the treatment of malignant neoplasms was later emulated by the use of tyrosine kinase inhibitors in chronic myeloid leukemia. The latest scientific breakthrough in APL is the discovery that ATRA and ATO not only induce differentiation but also eradicate "leukemia-initiating cells" or "leukemic stem cells" (Nasr et al., 2009), leading to think that their combination in induction regimens could result in higher rates of prolonged remission and cure. This has opened the door to new clinical trials in APL and a rational that might prove one day applicable in other hematologic malignancies.

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12. References

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13

Treating the Elderly Patient with Acute Myelogenous Leukemia

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1. Introduction

Among patients with acute myeloid leukemia (AML), treatment regimens and outcomes may differ among younger and older adults. Although there is no clear dividing line when considering age in AML, in most studies, "older adults" was defined as over age 60.The management of older patients with AML is a difficult challenge [1]. Older adults are more likely to have comorbidities that can limit treatment options; the disease tends to be more aggressive biologically; and outcomes are worse than in younger patients.

Decisions regarding the optimal treatment of acute myelogenous leukemia in the elderly patient requires the consideration of multiple factors. Population-based studies have demonstrated that, for all age groups, aggressive therapy results in improved survival and quality of life when compared with palliative care. The optimal induction and post remission regimen for older patients has yet to be determined. Furthermore, not all patients are candidates for such therapy. Consideration of patient and disease-related factors can help to determine the appropriateness of intensive therapy in a given patient. For those patients for whom aggressive induction therapy does not seem to be in their best interest, novel agents are being investigated that will hopefully address the issues of induction death and early relapse associated with these patient populations. This topic review will discuss the treatment of older adults with AML.

Most question that must be answer.

1. How Is Acute Myeloid Leukemia in the Elderly Different?
2. What Is the Standard Therapy for the Older Patient With AML?
3. Who Should Not Receive Intensive Therapy?
4. What Treatment Options Are Available for Patients Who Are Not Candidates for Intensive Induction Therapy?

Acute myeloid leukemia (AML) presents at all ages, but is mainly a disease of the elderly, with a median age of 69 years in the white US population**[93].** In the Swedish Acute Leukemia Registry, 68% of patients diagnosed with AML since 1973 were over age 60 years; between 1997 and 2005, 75% was aged 60 years or more**[94].** Prognosis worsens every decade beginning at age 30 to 40 **[93,95].** A report by the German Acute Myeloid Leukemia Cooperative Group looked at patients 16 to 85 years of age enrolled in two consecutive trials

in 1992 and 1999 with no upper age limit who had AML**[96].** In a multivariate analysis of prognostic factors, age ≥ 60 years was a statistically significant poor prognostic factor for complete remission (CR), overall survival (OS), remission duration, and relapse-free survival (RFS). Population-based studies have reported 3- and 5-year survival rates of only 9% to 10% and 3% to 8%, respectively, in patients over age 60, compared with 5-year survival rates of up to 50% for younger patients**[94,96,97].** Poorer outcome has traditionally been considered to be the result of less intensive therapy in this population, concurrent comorbidities, a higher likelihood of underlying hematopoietic disorders, and biologically poor risk disease. Moreover, because of the perception that older adults are less likely to do well with standard therapy, clinicians are less likely to treat these patients aggressively or refer them to centers that do so. As such, lower levels of aggressive treatment may compound underlying prognostic differences associated with patient factors and disease biology.

2. Pretreatment evaluation

General — The assessment of an older adult with AML includes those studies used for the pretreatment evaluation of younger adults with AML in addition to more specific investigations of physical functioning, nutrition, and comorbid conditions. Testing specific for older adults is presented in the following sections. The detailed pretreatment evaluation of all patients with AML is presented separately, as is an overview of the comprehensive geriatric assessment of cancer patients.

Physical functioning — The patient's performance status and ability to perform activities of daily living are measures of physical function that can help to predict the ability to withstand rigorous chemotherapy regimens.

Performance status — Several studies have supported the use of the Eastern Cooperative Oncology Group (ECOG) and Karnofsky performance status as measures of physical functioning and prognosis in patients with AML (table-1)

A retrospective study of data from five Southwestern Oncology Group (SWOG) trials that included 968 patients with AML found that the mortality rate within 30 days of initiation of induction therapy is dependent upon both the patient's age and ECOG performance status (PS) at diagnosis.

Thirty-day mortality rates were 2 to 3 percent for patients under the age of 55 years regardless of the PS.For patients over age 55 years, mortality rates ranged from 5 to 18 percent for patients with a PS of zero or 1. Patients 55 to 65 years old with a PS of 2 had a similar mortality rate (18 percent).Patients over age 55 years with a PS of 3 and those over age 65 with a PS of 2 or 3 had much higher mortality rates that ranged from 29 to 82 percent.The proportion of patients with poorer performance status increased with age. PS of 2 or 3 was observed in 15, 24, 26, and 32 percent in those under age 56, 56 to 65, 66 to 75, and >75 years of age, respectively.

Activities of daily living — Geriatricians commonly measure functional status by evaluating basic activities of daily living (ADLs) and instrumental activities of daily living (IADLs). ADLs are the skills that are necessary for basic living, and include feeding, grooming, transferring, and toileting. IADLs are required to live independently in the community and include activities such as shopping, managing finances, housekeeping, preparing meals, and taking medications. Assessment of ADLs and IADLs may add to the functional status obtained from the ECOG or Karnofsky performance status.

Karnofsky performance scale

Rating	Definition
100 percent	No evidence of disease
90 percent	Normal activity with minor signs of disease
80 percent	Normal activity with effort; signs of disease
70 percent	Cannot do normal activity but cares for self
60 percent	Requires occasional assistance
50 percent	Requires considerable assistance; frequent medical care
40 percent	Disabled, requires special care
30 percent	Severely disabled; hospitalization may be indicated
20 percent	Very sick; hospitalization necessary for supportive treatment
10 percent	Moribund
0 percent	Death

Table 1. Karnofsky performance status scale

Comorbid conditions — Comorbid conditions are poor prognostic factors in older patients with AML [8-10]. Patients with age-related chronic cardiac, pulmonary, hepatic or renal disorders or diabetes suffer greater acute toxicity from chemotherapy.

Older patients may also have decreased bone marrow regenerative capacity, even after successful leukemia cytoreduction. Inability to tolerate long periods of pancytopenia and malnutrition or the nephrotoxicity of drugs such as aminoglycosides or amphotericin remains a major barrier to successful treatment.

Frequently used measures of comorbidity include a modified Charlson comorbidity index (CCI) and the hematopoietic cell transplantation-specific comorbidity index (HCT-CI), neither of which was originally designed for older adults with AML.

Other comorbidity scores have incorporated information on infections prior to treatment and antecedent hematologic disorders. Assessment of other patient-related variables (eg, advanced age, performance status, organ function, karyotype, leukocytosis, CD34 expression) with or without a modified comorbidity index may be helpful for predicting such outcomes as attainment of complete remission, early mortality, and overall survival [3,4,11,12].

Charlson comorbidity index — The original Charlson comorbidity index (CCI) was devised as a measure of comorbidities in older adults. A revised version has been developed for use in older adults with AML with mixed results. (table 2).

A retrospective study evaluated the use of this modified CCI in 133 patients age 70 or older who received induction chemotherapy for AML [11]. CCI scores of zero, 1, and more than 1 were seen in 68, 13, and 19 percent of patients, respectively. When compared with those

with a CCI score of 1 or less, patients with a CCI score greater than 1 had a significantly lower rate of obtaining a complete response (35 versus 63 percent) and had a nonsignificant trend towards higher eight-week mortality rates (30 versus 19 percent) and lower two-year overall survival (24 versus 30 percent).

Modified Charlson comorbidity index

Comorbid condition	Point
Myocardial infarction	1
Heart failure	1
Cerebrovascular disease	1
Ulcer	1
Hepatic disease (mild)	1
Diabetes (mild or moderate)	1
Pulmonary disease (moderate or severe)	1
Connective tissue disease	1
Diabetes (severe with end-organ damage)	2
Renal disease (moderate or severe)	2
Solid tumor (without metastases)	2
Hepatic disease (moderate or severe)	3
Solid tumor (with metastases)	6
Total score	

Etienne, A, Esterni, B, Charbonnier, A, et al. Comorbidity is an independent predictor of complete remission in elderly patients receiving induction chemotherapy for acute myeloid leukemia. Cancer 2007; 109:1376.

Table 2. Charlson comorbidity index (CCI)

HCT comorbidity index — The hematopoietic cell transplantation specific comorbidity index (HCT-CI) was designed to predict outcomes in patients undergoing hematopoietic cell transplantation (HCT) (table 3). It has had mixed results in predicting outcome in older adults with AML.

Comparison of Charlson and HCTCI scoring systems

Comorbidity	CCI score	CCI definition	HCTCI score	HCTCI-definition
Mild pulmonary	1	Dyspnea on moderate activity (or with attacks, eg asthma)	0	Dyspnea on moderate activity or DLco or FEV_1 90-80 percent
Moderate pulmonary	1	Dyspnea on slight activity	2	Dyspnea on slight activity or DLco or FEV_1 >65 <80 percent
Severe pulmonary	1	Dyspnea at rest or requires oxygen	3	Dyspnea at rest or requires oxygen or DLco or FEV_1 ≤65 percent
Cardiac	1	HF (symptomatic and requiring tx) MI	1	CAD (one or more vessel-coronary artery stenosis requiring medical treatment, stent, or bypass graft), HF, MI, or EF <50 percent
Mild hepatic	1	Chronic hepatitis or cirrhosis	1	Chronic hepatitis or bilirubin >ULN-1.5 x ULN, or AST/ALT >ULN-2.5 x ULN
Moderate/severe hepatic	3	Cirrhosis with portal hypertension ± bleeding varices	3	Cirrhosis or fibrosis or bilirubin >1.5 x ULN, or AST/ALT >2.5 x ULN
Moderate/severe renal	2	Serum creatinine >265.2 µmol/l, renal dialysis, or renal transplant	2	Serum creatinine >176.8 µmol/l, renal dialysis, or renal transplant
Prior solid tumour	2	Initially treated in the last 5 years	3	Treated at any time point in the patient's past history, excluding non-melanoma skin cancer
Metastatic cancer	6	Metastatic cancer		
Psychiatric disturbance*	N/A	N/A	1	Depression/anxiety requiring psychiatric consult or treatment
Infection*	N/A	N/A	1	Requiring continuation of anti-microbial treatment after day 0
Obesity*	N/A	N/A	1	Patients with a body mass index >35 kg/m^2

CCI: Charlson comorbidity index; HCTCI: haematopoietic cell transplantation comorbidity index; FEV_1: forced expiratory volume in one second; DLco: lung diffusion capacity of carbon monoxide; HF: heart failure; MI: myocardial infarction; CAD: coronary artery disease; EF: ejection fraction; ULN: upper limit of normal; AST/ALT: aspartate transaminase/alanine transaminase.
* Added and validated HCTCI scored conditions.
Reproduced with permission from: Giles, FJ, Borthakur, G, Ravandi, F, et al. The haematopoietic cell transplantation comorbidity index score is predictive of early death and survival in patients over 60 years of age receiving induction therapy for acute myeloid leukaemia. Br J Haematol 2007; 136:624.

Table 3. Hematopoietic cell transplantation specific comorbidity index

A retrospective study of 177 patients over 60 years of age receiving induction chemotherapy for AML reported HCT-CI scores of zero, 1 to 2, and greater than 2 in 22, 30, and 48 percent

of patients, respectively [14]. Corresponding early death rates were 3, 11, and 29 percent, respectively. The same groups had median overall survival times of 45, 31, and 19 weeks, respectively.A second retrospective study evaluated the use of the HCT-CI in 92 patients age 80 or above with newly diagnosed AML offered induction chemotherapy [13]. Intensive therapy was given to 64 percent while the remainder elected supportive care. HCT-CI scores of zero to 1, 2 to 3, and 4 or greater were seen in 20, 35, and 45 percent, respectively. Patients with a HCT-CI score greater than 4 had a similar median survival when compared to those with a HCT-CI score of zero or 1 whether they received supportive care (1.9 versus 1.4 months) or intensive chemotherapy (3.5 versus 4.2 months).

Family discussions — A discussion with the patient and family members should include a review of the following

Prognostic information allowing them to make informed decisions on the type of treatment to be pursued [12]. Regardless of treatment choice, patients and their family members often report not being offered alternative treatment options and tend to overestimate the chance of cure [15]. Written consent forms required for clinical trials may serve an educational role, even for those who do not desire to enter into a formal study. Who has durable power of attorney for health issues if the patient becomes unable to make decisions? Does the patient have an updated will? Do other members of the family know where this information is kept? Will the family have access to adequate funds while the patient is hospitalized?A discussion concerning "code" status and the possibility that the patient might need to be transferred to an intensive care unit, with its attendant morbidity and mortality [16]. This should include issues related to "do not resuscitate" and "do not intubate" orders, such that the patient and family can make properly informed decisions on these matters. (See "Ethics in the intensive care unit: Informed consent; withholding and withdrawal of life support; and requests for futile therapies".)The effect on the patient's employment. Most patients will not be able to return to even part-time work until the completion of induction and consolidation chemotherapy.

3. Outcomes in older compared to younger patients

Overview — Overall survival rates for AML decrease as age increases (figure 1). Most series of older patients with newly diagnosed AML have noted CR rates between 40 and 60 percent [2,4,5,8,12,17-21]. While suitably selected older patients given aggressive induction therapy may achieve CR at a rate approximating that of younger patients [12], others may spend a significant proportion of their remaining life in a hospital setting receiving treatment.

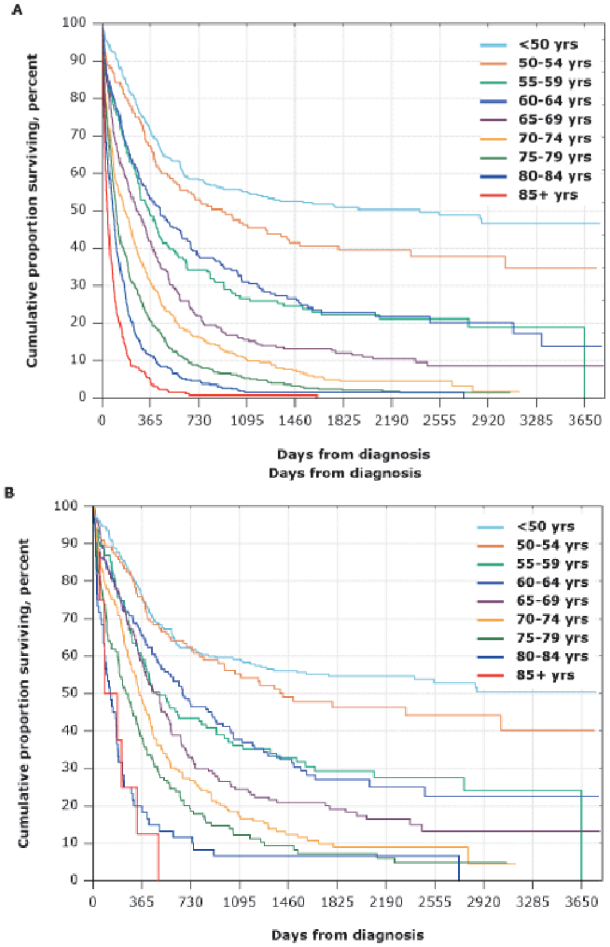
Older age, defined in most studies as over age 55, 60, or 65, is an independent poor prognostic factor in AML. Such patients have, in comparison with younger patients.

Poorer performance status Higher incidence of multidrug resistance Lower percentage of favorable cytogenetics Higher percentage of unfavorable cytogenetics Higher treatment-related morbidity and mortality Higher incidence of treatment-resistant disease Lower complete remission rates, shorter remission durations, and shorter median overall survival Fewer opportunities for allogeneic hematopoietic cell transplantation.

An analysis of Medicare claims for 2657 older patients with AML diagnosed between 1991 and 1996 underscored the grim prognosis for AML in the older patient.

Median survival for all patients was two months, with a two-year overall survival of 6 percent. For patients ≥85 years of age, median survival was only one month.Only 30 percent of patients received chemotherapy; when compared with those not receiving chemotherapy,

they tended to be younger (average age 73 versus 78 years) and live longer (median survival seven versus one month).Of those older patients dying from AML during the follow-up period of the study (94 percent of the sample), 31 percent of their remaining days had been spent in an inpatient facility.



Overall survival according to age irrespective of management (Panel A, n = 2767), and patients with de novo AML, fit for intensive treatment, and with WHO/ECOG performance status 0 to II (Panel B, n = 1229)

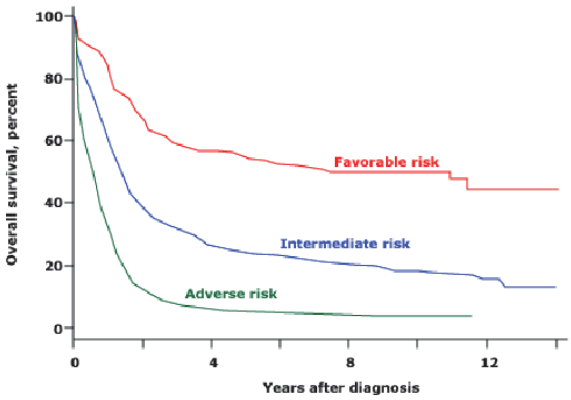
Fig. 1. Overall survival rates for AML decrease as age increases.

The outcomes might be better if more patients were offered induction chemotherapy. A retrospective analysis of 2767 patients with non-APL AML from the Swedish acute leukemia registry reported that early death rates (ie, death within 30 days of diagnosis) were considerably lower in patients receiving intensive induction chemotherapy when compared with those who received palliative therapy, even when stratified for performance status, however it remains possible that patients with a better prognosis were more likely to be

offered induction chemotherapy [4]. The difference between 30-day mortality rates for the two groups ranged from 16 to 35 percent. Patients who had de novo AML, were "fit" for intensive chemotherapy, had an ECOG performance status of zero to 2, and were age 16 to 55, 56 to 65, 66 to 75, and 76 to 89 had median overall survival times of 7 years, 18 months, 14 months, and 6 months, respectively.

Prognostic factors — A number of risk factors have been identified which occur more frequently in the older patient and appear to contribute to the worse outcome. The major independent prognostic factors in older adults with AML are Age Cytogenetics Performance status Secondary leukemia White blood cell count at diagnosis Multidrug resistance-1 (P-glycoprotein) expression.

The cytogenetic abnormalities most often associated with treatment failure in young patients with AML (eg, abnormalities of chromosomes 5 or 7 or complex karyotypes) are considerably more common in older patients, occurring in 32 to 57 percent of patients in two series [25,27-30]. Conversely, all of the "favorable" cytogenetic abnormalities, such as t(8;21), t(15;17), or inv(16), are more common in younger subjects and are responsible in part for their better disease-free survival, Figure 2.



This figure illustrates overall survival in adult subjects with acute myeloid leukemia (AML), according to the following cytogenetic risk categories: Favorable risk (median survival 7.6 years): t(8;21); inv(16) or t(16;16); del(9q). Intermediate risk (median survival 1.3 years): normal karyotype; -Y; del(5q); loss of 7q; t(9;11); +11; del(11q); abn(12p); +13; del(20q); +21. Adverse risk (median survival 0.5 years): complex karyotype (≥3 abnormalities); inv(3) or t(3;3); t(6;9); t(6;11); -7; +8 (sole abnormality); +8 with one other abnormality other than t(8;21), t(9;11), inv(16), or t(16;16); t(11;19)(q23;p13.1).

Fig. 2. Overall survival in aml according to the cytogenetic study.

4. Overview of treatment

Goals — The goal of remission induction chemotherapy is the rapid restoration of normal bone marrow function and attainment of complete remission.

Induction therapy aims to reduce the total body leukemia cell population from approximately 10^{12} to below the cytologically detectable level of about 10^{9} cells. It is generally assumed, however, that a substantial burden of leukemia cells persists undetected (ie, minimal residual disease), leading to relapse within a few weeks or months if no further therapy were administered.

Postinduction or "remission consolidation" therapy usually comprises one or more courses of chemotherapy or hematopoietic cell transplantation (HCT). It is designed to eradicate residual leukemia, allowing the possibility of cure. Rates of relapse and death are quite low after three to four years in remission, and most such patients are long-term disease-free survivors.

Decision to treat — After the diagnosis of AML has been established, the physician and staff must present the goals of therapy, as well as the side effects of treatment, to the patient and family. For almost all patients, this discussion can emphasize the potential benefits of intensive treatment with regard to both the short and long term outcome. Remission induction, even if short-lived, is an appropriate goal for most patients with AML.

Patients who achieve a remission have an improved quality of life compared with those patients who receive palliative therapy likely because they require fewer hospitalizations, transfusions, and antibiotics [4].Attainment of CR following intensive chemotherapy is required in order to assure meaningful prolongation of life.

Occasionally, intensive treatment with the intent to achieve CR may be less advisable because of advanced patient age, debility, presence of significant co-existing medical problems, and/or prior chemotherapy. Patients unlikely to survive treatment can be identified by their poor performance status using the Karnofsky or ECOG (Zubrod) performance.

In addition, there are a few patients with "acute leukemia" by the usual quantitative criteria of >20 percent bone marrow blast cells whose disease has a much more smoldering course. These patients suffer from bone marrow failure and pancytopenia more than hyperleukocytosis. Their survival may be equally long and their quality of life better, using transfusion support and antibiotics rather than intensive chemotherapy. This may be particularly true for the "hypoplastic/hypocellular" variant of AML. Supportive care may also be beneficial in acutely infected patients with advanced myelodysplastic syndromes. Occasionally, the clinical picture mimics AML, but resolves following treatment of the infection.

For otherwise healthy (ECOG performance status of two or less and few comorbidities) older adults with newly diagnosed AML, we suggest remission induction treatment, ideally on a clinical trial.For older patients with indolent AML, severe comorbidity, or high risk disease, we suggest the use of supportive care rather than standard induction chemotherapy.

It is frequently appropriate and necessary to repeat this discussion and counseling later during the patient's course, as a diagnosis of acute leukemia often leaves the patient and family unable to cope with the longer term consequences of this diagnosis until the patient has successfully passed through the initial weeks of chemotherapy and recovery.

INDUCTION — The best treatment strategy for older patients with AML remains controversial . Among the treatment options that have been evaluated are various forms of intensive or less-intensive chemotherapy, the administration of colony-stimulating factors to enhance neutrophil recovery, supportive therapy, low-dose cytarabine, high- or intermediate-dose cytarabine-based consolidation therapy, prolonged consolidation therapy, and maintenance treatment with interferon. Most of these studies have been disappointing.

Intensive chemotherapy — The best induction chemotherapy for older patients with AML remains to be identified . Intensive chemotherapy may be appropriate for selected patients with low or intermediate risk disease in whom the complete remission (CR) rate can be as

high as 70 to 80 percent . With this approach, median survival is approximately eight months, but 9 to 12 percent of patients will be alive at five years. Although pilot studies have used more intensive initial chemotherapy, a reasonable standard regimen for many older patients who are medically fit is seven days of continuous infusion cytarabine (ara-C, 100 mg/m2 per day) plus three days of daunorubicin (60 or 90 mg/m2 per day).

Randomized trials have investigated various modifications of cytarabine plus an anthracycline for the treatment of older adults with AML. In general, the choice of anthracycline (eg, daunorubicin, mitoxantrone, or idarubicin) does not appear to affect overall outcome. However, higher doses of anthracyclines may result in superior rates of complete remission (CR) without an apparent increase in toxicity.

For most older adults with favorable or intermediate risk AML and an ECOG performance status of two or less and few comorbidities, we suggest remission induction treatment with a combination of an anthracycline such as daunorubicin for three days and "standard" dose cytarabine for seven days rather than other chemotherapy regimens or supportive care alone. When induction treatment is chosen, it should be applied at sufficient dose intensity to provide the best chance of success. Further details on the administration of this regimen are presented separately as are recommendations for evaluation after completion of induction therapy.

Use of growth factors — Several groups have evaluated the effects of colony-stimulating factors (eg, GM-CSF, G-CSF, and glycosylated G-CSF) as an adjunct to intensive chemotherapy with largely disappointing results. The rationale for this approach is that older patients are particularly susceptible to infection and experience a higher infectious mortality rate during episodes of neutropenia. Shortening the duration of neutropenia might have a beneficial effect and improve the rate of complete remission.

What Treatment Options Are Available for Patients Who Are Not Candidates for Intensive Induction Therapy?

For those patients who are not considered to be candidates for intensive induction therapy, one would hope to identify agents and regimens that are more effective and less toxic to address the concerns regarding early induction death, inadequate response rate, and high risk of relapse. The NCRI AML 14 study was designed to allow for randomization of patients between intensive and nonintensive therapy, but only eight patients agreed to randomization[117,147]. As such, data available on novel agents comes from a variety of pilot and phase II studies with differing eligibility criteria. When evaluating the outcomes, it is important to also look at the characteristics of patients who were ultimately enrolled.

reviews available data from some of these studies .

As part of the NCRI AML 14 study, 212 patients who were deemed unfit for intensive treatment options by the local investigator were randomized to receive supportive care alone with hydroxyurea or cytarabine 20 mg twice daily by subcutaneous injection for 10 days every 4 to 6 weeks [95]. Outcome was improved for the low-dose (LD) cytarabine arm when compared with supportive care with hydroxyurea alone. CR was 18% versus 1%, and median survival was 575 days for those who achieved CR, compared with 66 days in nonresponders. DFS for responders was 8 months. Survival benefit was seen in all age groups, even those over age 75. As none of the patients with adverse cytogenetics achieved a CR, no survival benefit was, however, seen in that group. The early death rate was

39% at 8 weeks. Although no criteria were used to define unfit patients, 78% were over age 70, 27% had secondary AML, 30% had PS _ 2, 27% had heart disease, 49% had other

comorbidities, and 59% had a poor risk score by the Wheatley Risk Index [90]. Based on this study, LD cytarabine became the standard of care for the treatment of patients felt to be unfit for intensive chemotherapy, although one could argue that it should not be given to those with poor risk cytogenetics.

The DNA methyltransferase inhibitors have been the subject of several recent studies. In a multicenter phase II study of 55 patients over age 60 with untreated AML, decitabine was administered for 5 days monthly until disease progression. [96]. With a median of three cycles, the overall response rate was 24%, median survival was 7.7 months, and 30-day mortality was 7%. Responses were seen in all cytogenetic risk groups, as well as in those patients with prior MDS. An alternate schedule of decitabine was reported by Blum et al[97,98]. Patients received an initial one to two courses of 10 days of decitabine, followed by a course over 3 to 5 days every 4 weeks for 1 year. Of the 53 patients with a median age of 74, 36% had secondary AML, and 34% had a complex karyotype.

Eighteen patients had a HCTCI score of _ 3. There was a 64% response rate after a median of three cycles of therapy. CR occurred in all subsets, regardless of age, karyotype, presenting WBC, and prior AHD. One-year survival of poor risk patients was 30% (compared with 10% in patients with a similar Wheatley risk score in the AML 11 trial) [90].

In a study of azacitidine in AML with 20% to 30% blasts, patients who were deemed unfit for standard induction chemotherapy were randomized against either supportive care or LD cytarabine[99]. OS survival was superior in the azacitidine arm. There was a statistically significant difference seen in OS for patients with poor risk cytogenetics in favor of azacitidine, compared with conventional care regimens (12.3 vs 5.3 months, respectively, with 2-year OS of 38% vs 0%).

Gemtuzumab ozogamicin (GO) has been the subject of a recent study by the EORTC and GIMEMA leukemia groups (AML 19) [100].

In this randomized multicenter study, 84 patients were randomizedto receive one of two schedules of GO at attenuated doses or best supportive care. The proportion of patients either achieving a response or maintaining stable disease was greater in patients who receive GO at a dose of 6 mg/m2 on day 1 and 3 mg/m2 on day 8, when compared with a schedule of GO 3 mg/m2 on days 1, 3, and 5 (63% vs 38%, respectively). Results of the comparison with patients who were randomized to standard care are not yet available, and a phase III trial is ongoing.

Clofarabine has been studied as an agent in elderly patients with AML. In a phase II study of the agent in 112 patients over age 60 with untreated AML with at least one unfavorable baseline prognostic factor, there was a 46% response rate[101]. The median age of the patients was 71. Twenty-two percent of patients had a baseline PS of 2, 47% had a prior hematologic disorder (AHD) or secondary AML,55% had an unfavorable karyotype, and 62% were _ age 70.

Overall response rate (ORR) was 39 % for patients _ 70, 32% for PS 2, 51% for patients with AHD, 54% for intermediate karyotype and 42% for unfavorable karyotype, and 38% for patients with three risk factors. Median DFS was 37 weeks, and median OS was 41 weeks for all patients, 59 weeks for patients with CR/complete remission with incomplete platelet recovery (CRp), and 72 weeks for patients with CR. Early death rate (within 60 days) was 16%.

In two consecutive European studies of 106 untreated older patients with AML who were considered unfit for chemotherapy, participants were given four to six 5-day courses of

clofarabine[70,102]. In the UWCM (University of Wales College of Medicine)-001 study, patients who were either over age 70 (68%) or over age 60, with a PS of 2 or cardiac comorbidity, were treated with clofarabine for 5 days every 28 days for 2 to 4 courses. In the BIOV-121 study, patients were treated for 5 days every 4 to 6 weeks for up to six courses. All patients were age _ 65 and deemed unfit for chemotherapy.

Overall, 36% of patients had a PS _ 2, 30% had adverse risk cytogenetics, 46% had Wheatley poor risk disease, and 65% were age _ 70. The ORR was 48%, and the median OS was 19 weeks for all and 45 weeks for those who attained a CR/completeremission with incomplete blood count recovery (CRi). Responses were seen in patients with adverse cytogenetics (44% ORR), patients with secondary AML (31%), and patients age _ 70 (49%).

The death rate within 30 days was 18%. A novel agent, laromustine (VNP40101M), a sulfonylhydrazine alkylatingagent, has been studied in 85 patients with poor risk AML age_60 years.51 Patients received one to two cycles of laromustine at a doseof 600 mg/m2, followed by one cycle of cytarabine. Seventy-eightpercent of patients were age _ 70, 47% had an adverse karytype, 41%had a PS of 2, 77% had pulmonary disease, 73% had cardiac disease,and 3% had hepatic disease. All patients with unfavorable karyotype orECOG PS had at least one other risk factor at the time of enrollment.Seventy-five percent of patients had _ 3 risk factors. The ORR was32% and was similar in patients over age 70 (32%), with a PS of 2 (32%), with baseline pulmonary or cardiac dysfunction (27%–34%).

There was a 14% 30-day mortality. OS was 3.2 months (12.4 months for those with CR/CRp), and 1-year survival was 21% (52% for those with CR/CRp).

These phase II studies are encouraging, in that responses are seen in all poor risk categories, and early death rates are acceptable.Randomized trials are needed. Although randomized trials ofintensive versus nonintensive therapy have not been successful, theongoing AML 16 trial was designed to randomize patients who areconsidered not fit for intensive treatment to LD cytarabine versusLD cytarabine with GO, LD cytarabine with arsenic trioxide or tipifarnib, or LD clofarabine.52 The arsenic arm has been closed because of ineffectiveness with CR/CRi of 29%, compared with 24% and a 12-month OS of 27%, compared with 41%. The other arms continue to accrue patients.

POST REMISSION THERAPY — While a substantial percentage of older adults will attain a complete remission (CR) with induction chemotherapy, virtually all of these patients will relapse within a median of four to eight months unless given additional cytotoxic therapy. Even with post-remission therapy, relapses are common. Only about 10 percent of older adults, and generally only those with favorable or intermediate risk disease, attain long-term survival after the administration of post-remission therapy.

Post-remission therapy aims to destroy leukemia cells that survived induction chemotherapy but are undetectable by conventional studies. There are two generally accepted options for post-remission therapy: consolidation chemotherapy and allogeneic hematopoietic cell transplantation (HCT). Consolidation chemotherapy is less intensive and has a lower early mortality rate, but allogeneic HCT provides a graft-versus-tumor effect that decreases relapse rates. In younger patients, consolidation chemotherapy is usually given to patients with favorable risk disease while HCT is used for patients with unfavorable risk disease. The optimal treatment for intermediate risk disease is unknown. Evidence regarding the therapeutic benefit of any consolidation therapy in older patients with AML is limited and its value has remained uncertain. Newly discovered genetic

markers are helping to refine the risk stratification. A detailed description of these options in younger adults is presented separately. Post-remission therapy in older adults is complicated by high rates of treatment related toxicity. Older adults are generally not candidates for a fully myeloablative allogeneic HCT, but a subset may be able to undergo nonmyeloablative HCT after reduced intensity conditioning regimens. A choice among these strategies is generally made based upon the risk stratification of the patient's tumor and the patient's performance status and comorbidities that might affect tolerance of intensive therapy. A phase III trial demonstrated that post-remission therapy with single agent gemtuzumab ozogamicin did not improve clinical outcomes (probability of relapse, overall survival, or disease free survival), but added toxicities[75].

Consolidation chemotherapy — High dose cytarabine (HiDAC) is the standard consolidation chemotherapy for younger adults with AML of a favorable risk, but is associated with unacceptably high rates of severe toxicity and early death in older adults that counteract any improvement in efficacy over standard dose cytarabine. Instead, consolidation therapy with two cycles of daunorubicin (30 to 45 mg/m2 for two days) and cytarabine (ara-C, 100 mg/m2 per day for five days) for older adults is preferred. The use of consolidation chemotherapy in younger adults is presented separately.

Nonmyeloablative transplantation — Allogeneic hematopoietic cell transplantation (allo-HCT) is the preferred treatment for younger adults with unfavorable risk AML because of its graft-versus-leukemia effect. However, allo-HCT is associated with a very high treatment-related mortality rate in older patients that precludes its general use. Instead, various reduced intensity or nonmyeloablative [85] allo-HCT regimens have been employed in fit older adults. However, the comparable efficacy of this approach remains to be proven and a randomized, multinational trial by the European Group for Blood and Marrow Transplantation evaluating alloSCT versus conventional consolidation therapy in elderly patients is currently accruing patients. The use of allo-HCT in younger adults is presented separately, as is additional information on nonmyeloablative allo-HCT.

The development of less toxic and better tolerated nonmyeloablative regimens capable of inducing a state of mixed chimerism may allow allo-HCT to be performed in patients with AML and advanced age or co-morbidity, with the hope that such regimens would result in lower rates of treatment-related mortality without sacrificing relapse-free and overall survival, and with a reasonable balance between GVHD and the graft-versus-tumor effect. Additional experience with this approach is awaited.

SUPPORTIVE CARE — For older patients with indolent AML, severe comorbidity, or high risk disease, we suggest the use of supportive care rather than induction chemotherapy. Supportive care can include the use of red blood cell and platelet transfusions, antibiotics, and control of leukocytosis with agents such as low-dose cytarabine or hydroxyurea.

Low-dose cytarabine — While not curative, many committees, including the British Committee for Standards in Hematology, consider low-dose cytarabine to be the standard against which other palliative treatments for AML in the older patient should be evaluated.

A number of trials have investigated the use of low-dose cytarabine in older subjects with AML, both for induction and later for maintenance of remission .As an example, investigators in France randomly assigned 87 patients >65 to receive either intensive chemotherapy with cytarabine and rubidazone (a daunorubicin analogue) or low-dose subcutaneous cytarabine (10 mg/m2 every 12 hours for 21 days) .Although the number of complete remissions was greater with intensive chemotherapy, the early death rate was also higher.

Other supportive measures — Other measures of supportive care include the use of leukocyte-depleted, irradiated red blood cell and platelet transfusions as needed and the use of antibiotics to treat infections. As described above, patients treated with supportive care alone spend a similar amount of time in the hospital compared with those who receive intensive chemotherapy.

5. Approach to the elderly patient with AML

AML is a disease of the elderly, with the majority of patients over age 60. As our population ages, that percentage will only increase.

Unfortunately, the standard regimens that are successful in treating younger patients with AML are not as beneficial in the majority of older patients with the disease. Figure 3 outlines my approach to the elderly patient with AML. Understanding of the disease biology, as well as the prognostic factors associated with the host, allows us to better determine which patients are likely to benefit from standard therapy and which require alternative approaches. Objective scoring systems are being developed that allow us to define patients unfit for intensive chemotherapy on the basis of increased risk of induction death, low response rate, and/or low long-term DFS. Optimal induction and postremission therapy for patients appropriate for intensive therapy have yet to be defined, again, because results are not satisfactory with our current regimens, even in those patients who do not have definable poor prognostic factors. When compared with young patients with similar disease-related features, outcomes are inferior. For patients who are not candidates for intensive therapy because of comorbid conditions, low-intensity therapies appear to be superior to palliative care alone. Whenever possible, patients should be enrolled in clinical trials that will allow us to address these issues.

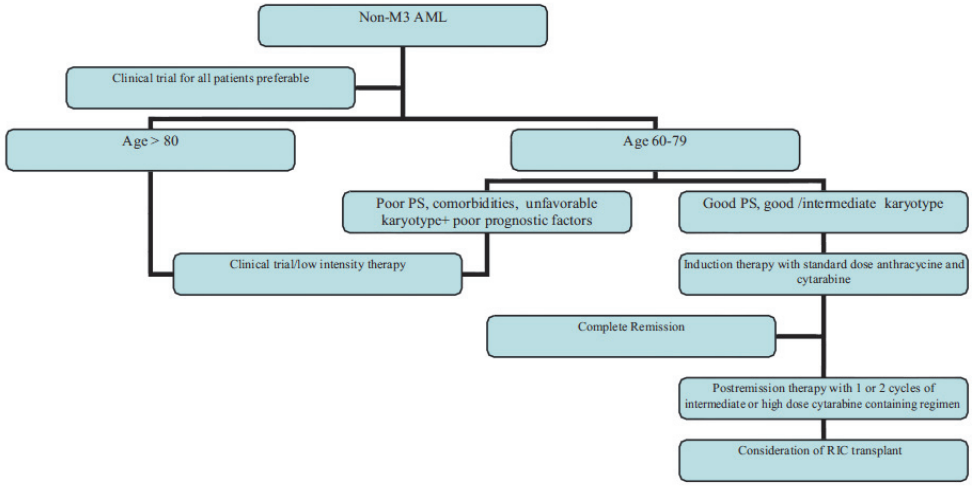


Fig. 3. Outlines my approach to the elderly patient with AML.

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Prognosis and Survival in Acute Myelogenous Leukemia

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1. Introduction

Progress in understanding the prognosis and survival in acute myelogenous leukemia (AML) has been dramatic over the last few decades. Traditionally, clinical risk factors such as age and performance status have been the main prognostic factors in AML. However, recent advances in cytogenetic studies and molecular markers in AML have revolutionized our approach to this disease. These have changed our understanding of AML as a heterogeneous group of diseases rather than a single disease, provided greater insight not only in understanding disease biology but also into predicting response to therapy and helped in the development of risk stratification-based treatment approach.

In 2010, there are about 12,330 new cases in the United States which represent about 0.8% and 29% of all new cancer and leukemia cases respectively. With about 8,950 estimated deaths related to AML, this represents about 1.6% of cancer related deaths in 2010. (American Cancer Society, 2010)

Although there has been some improvement in survival for AML patients over the last few decades, mainly in younger age groups as shown in figure 1, AML long term survival is still a big challenge. In the United States, data from Surveillance Epidemiology and End Results (SEER) dataset for 2001 to 2007 showed 5-year overall survival (OS) of 22.6% for all AML patients. There is still a lot to be done especially in the oldest age group (>65 years), that is showing a dismal 5-year OS of less than 5%, See Figure 2. This is of particular concern as more than half of the patients diagnosed in 2000-2004 were over 65 years old. (Howlader et al., 2011).

2. Clinical prognostic factors

2.1 Age

AML is seen more commonly in the elderly with median age at diagnosis of 66 with incidence increases dramatically after age of 55, See figure 3. Data from SEER (see figure 2) as well as from major studies of the largest cooperative groups including the Medical Research Council (MRC), the Southwest Oncology Group/Eastern Cooperative Oncology Group (SWOG/ECOG), AML cooperative group (AMLCG) and the Cancer and Leukemia Group B (CALGB) that included elderly patients have shown consistently worse outcome in this patient population, See figure 4. (Slovak et al., 2000; Byrd et al., 2002; Schoch et al., 2004a; Grimwade & Hill., 2009)

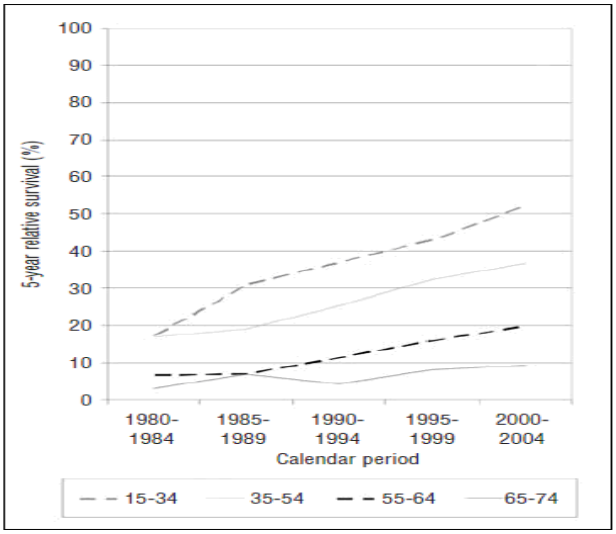


Fig. 1. Period estimates of 5-year relative survival of patients with AML by major age groups in defined calendar periods from 1980-1984 to 2000-2004. (Pulte et al., 2008).

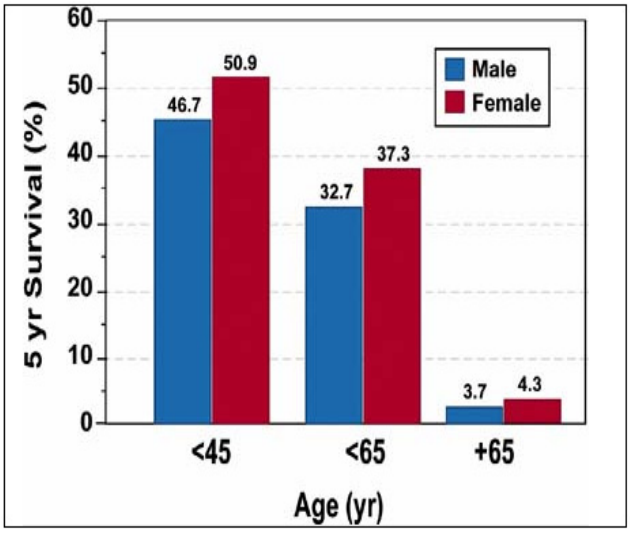


Fig. 2. Age and sex-associated with 5-year relative survival in patients with AML in the United States, 1996–2003 (From SEER cancer statistics, National Cancer Institute, 2007.)

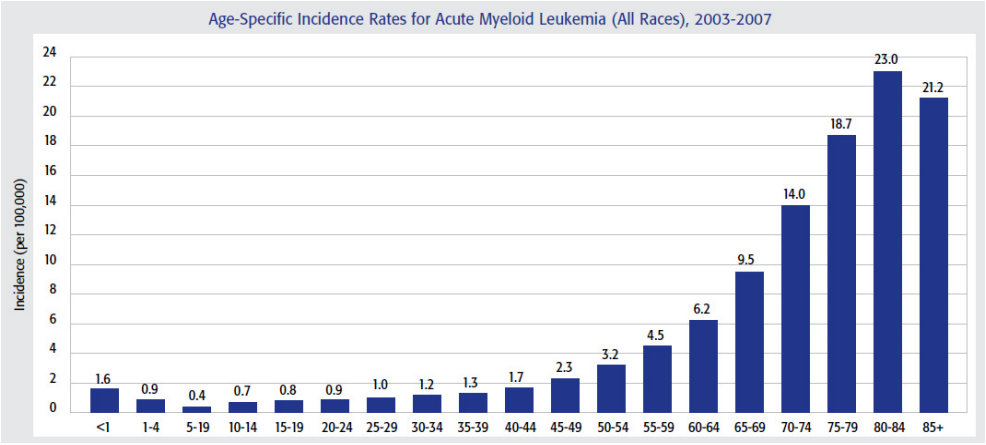
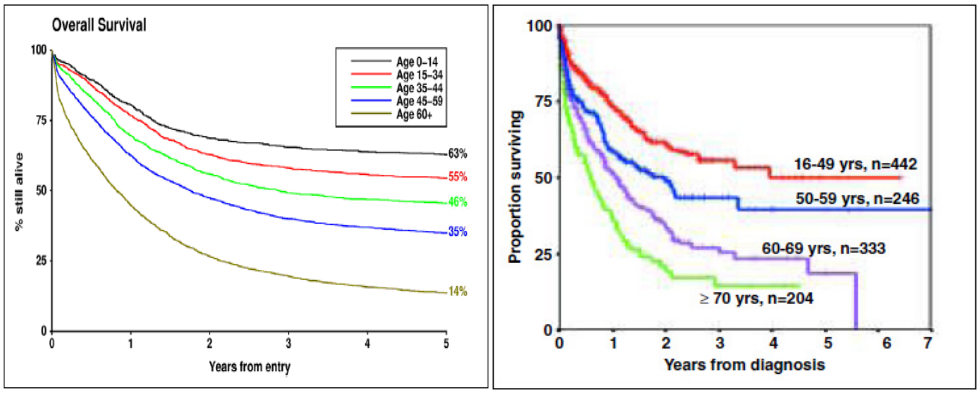


Fig. 3. Age-Specific incidence rates for AML from 2003 to 2007. (Altekruse et al., 2010).



(a) (b)

Fig. 4. Survival curves according to age groups. a: Patients treated in MRC AML trials (AML10, 11, 12, 14 and 15) (Smith et al. , 2011). b: Patients treated in AMLCG trials (AMLCG 1992, AMLCG 1999 and AMLCG APL trials) (Schoch et al., 2004a)

The worse outcome in elderly population is related to two components: resistance to treatment and treatment-related death. It is believed that most of treatment failure in elderly is related to the first component. This is mainly related to distinct biological and clinical features such as higher percentage of poor cytogentics, higher incidence of multidrug resistance protein (MDR) and preceding hematological disease, all of which are associated independently with worse prognosis in AML. (Lieth et al., 1997; Estey 2007). For example, in retrospective analysis from five SWOG clinical trials more than 50% of patients >75 years old had poor cytogenetics which translated into complete remission (CR) rate of 33%, See figure 5, table 1. (Appelbaum et al., 2006a)

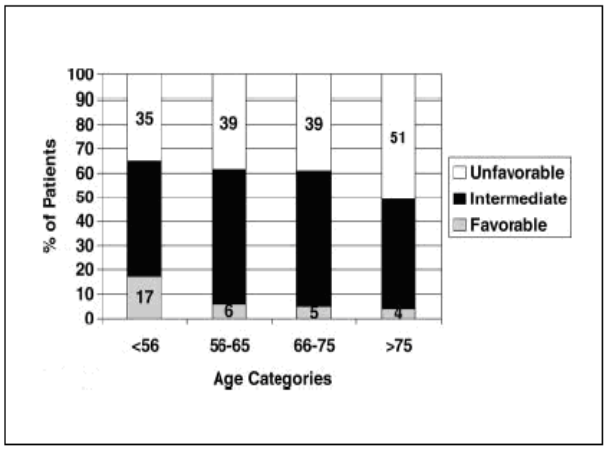


Fig. 5. Percentage of patients in the different cytogenetic risk groups by age category in five SWOG clinical trials (Appelbaum et al., 2006a).

	Younger than 56 y	56-65 y	66-75 y	Older than 75 y
No. patients	368	246	274	80
Response, no. (%)				
CR	235 (64)	113 (46)	108 (39)	26 (33)
Resistant disease	99 (27)	91 (37)	101 (37)	29 (36)
Median overall survival, no. (95% CI)	18.8 (14.9-22.6)	9.0 (8.1-10.2)	6.9 (5.4-7.7)	3.5 (1.4-6.1)
No. patients with CR	235	113	108	26
Median disease-free survival, no. (95% CI)	21.6 (15.8-25.5)	7.4 (6.5-8.8)	8.3 (6.3-10.2)	8.9 (5.8-10.8)

Table 1. CR rates in different age groups in the same patient population (Appelbaum et al., 2006a).

The second component of treatment failure is treatment-related death. This is mainly related to the worse performance status and organ function in this age group. Multiple studies have shown age along with poor performance status as very strong predictors of early post-induction mortality in AML, See table 2, Figure 6. (Appelbaum et al., 2006a; Juliusson et al., 2009).

This has motivated researchers to develop different prognostic and predictive models including clinical and laboratory variables that can help physicians deciding treatment in this challenging patient population. (Krug et al., 2010; Kantarjian et al., 2010)

Even after accounting for the above factors, elderly patients tend to have worse outcome with less CR rate and higher mortality rate. In two different reports from SWOG and AMLCG, elderly patients with favorable cytogenetic have worse outcome compared to younger patients. (Schoch et al,. 2004a; Appelbaum et al., 2006a)

The dismal prognosis in elderly population has another component which is undertreatment. While AML is more common in elderly, a large number of these patients do not receive intensive chemotherapy. This is because they are more likely to have poor

performance status and comorbidities at diagnosis and therefore less frequently judged to be fit for induction therapy. Menzin et al,. reviewed SEER data of AML in elderly patients. Among 2657 patients age > 65 years reviewed, only 30% of patients underwent intensive chemotherapy. Juliusson et al reported similar numbers from the Swedish Acute Leukemia Registry with only 45% of patients in age group 70-74 offered treatment as compared to 92% in 60-64 age group and 98% in <50 age group (Juliusson et al., 2009).

	Younger than 56 y	56-65 y	66-75 y	Older than 75 y
No. patients	364	242	270	79
Early deaths* by performance status, no./no. total patients (%)				
0	3/129 (2)	8/72 (11)	9/73 (12)	2/14 (14)
1	6/180 (3)	6/112 (5)	20/126 (16)	7/40 (18)
2	1/46 (2)	6/34 (18)	16/52 (31)	7/14 (50)
3	0/9 (0)	7/24 (29)	9/19 (47)	9/11 (82)

Table 2. Mortality within 30 days of induction treatment according to age group and performance status in 5 clinical SWOG trials (Appelbaum et al., 2006a).

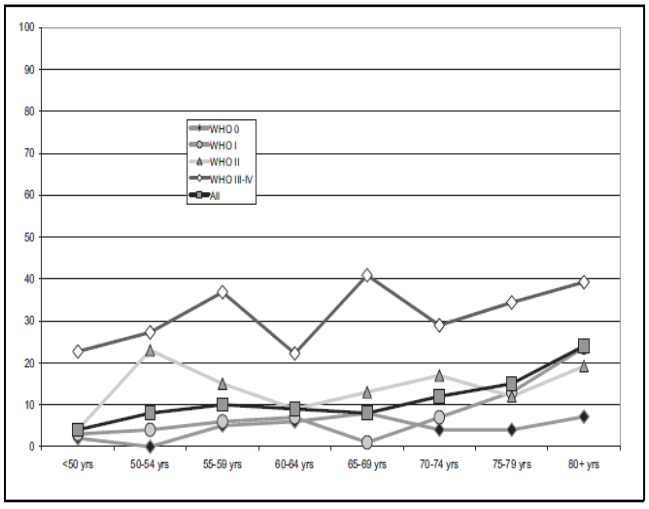


Fig. 6. Mortality within 30 days of induction treatment according to age group and performance status the Swedish acute leukemia registry (Juliusson et al., 2009).

So when interpreting data from various clinical trials we have to keep in our minds that the patient population in clinical trials includes only a subset of elderly patients with AML and survival numbers achieved could be an overestimate in this patient population. In the same report from Menzin et al including treated and untreated AML patients, patients older than 65 years had a median survival of two months with two-year OS of 6% (Menzin et al., 2002).

2.2 Performance status

Different clinical trials have consistently showed worse outcome in patients with poor performance. It is considered the strongest predictor of 30-day mortality after induction therapy, See table 2, Figure 6. (Appelbaum et al., 2006a; Juliusson et al., 2009). Poor performance usually reflects comorbidities and organ dysfunction. Assessing this parameter in elderly patients can be difficult. Acute infection or decompensation can easily change performance status and confuse our assessment of baseline performance status. Clinical trials exclude patients with poor performance status, so when reviewing data from any clinical trial we have to keep in our minds that it excludes a major part of patients who are rendered ineligible. This selection bias is more pronounced in elderly population as fit elderly are more likely to receive treatment.

2.3 Prior hematological disease

The prior diagnosis of myelodysplastic (MDS) or myeloproliferative (MPD) disease is well established as a poor prognostic factor in AML patients. While the poor survival is more associated with high prevalence of advanced age and poor cytogenetics in this patient population, it is still an independent prognostic factor after adjusting for both variables. Longer interval from onset of MDS or MPD disease to AML negatively affected outcomes in this patient population. One explanation is that a protracted history of prior hematological disease may select for higher rates of chemotherapy resistance after AML develops. Prior treatment for MDS is another poor prognostic factor in this patient population. (Bello et al., 2011).

2.4 Therapy-related AML

People exposed to cytotoxic agents are at higher risk of developing AML among other myeloid neoplasms. Therapy-related AML (t-AML) represents about 10-15% of all cases of AML (Schoch et al., 2004b). it is considered a poor prognostic factor. Goldstone et al. reported OS of 30% compared to 44% in de novo AML (Goldstone et al., 2002). In another report from Kayser et al, Outcome of patients with t-AML was significantly inferior with 4-year OS of 25.5% compared to 37.9% in de novo AML. (Kayser et al., 2011)

The risk is highest after exposure to two classes of cytotoxic agents: topoisomerase II inhibitors and alkylating agents. The current WHO classification does not subcategorize t-AML based on agents involved. This is mainly due the fact that most patients developing t-AML have been exposed to both types and it is not feasible to discriminate according to the previous therapy. (Swerdlow et al., 2008)

Each class related-AML has certain characteristics. While alkylating agents related-AML frequently is preceded by myelodysplastic phase and a long interval between exposure and development of AML (36-72 months), topoisomerase II related-AML usually presents without myelodysplastic phase and has an interval of usually 6 to 36 months. While alkyalting agents are usually associated with unbalanced cytogenetic abnormalities involving chromosome 5 and 7 as well as complex karyotype, patients with topoisomerase II inhibitors related t-AML are more likely to have balanced translocations involving MLL at 11q23, NUP98 at 11p15, RUNX1 at 21q22 and RARA at 17q21.

t-AML is commonly associated with abnormal karyotype ranging between 69 to 96%. Cytogenetic abnormalities in t-AML are the same described in de novo AML but with different frequencies. In one report, 46% of t-AML patients had unfavorable cytogenetic

profile as compared to 20% in de novo AML and only 10% had normal cytogenetics versus 40% in de novo AML. Similar distribution has been observed in other trials as well. (Schoch et al., 2004b ; Grimwade & Hill 2009; Kayser et al., 2011)
Patients with t-AML tend to be older than de novo AML patients. In one report , median age of t-AML was 57.8 years versus 53.2 years in de novo AML. (Kayser et al., 2011)
While the above factors contribute to the worse outcome seen in t-AML, inferior survival and response rate has been observed in all age and cytogenetic subgroups (Grimwade & Hill 2009; Borthakur et al., 2009), See figure 7.

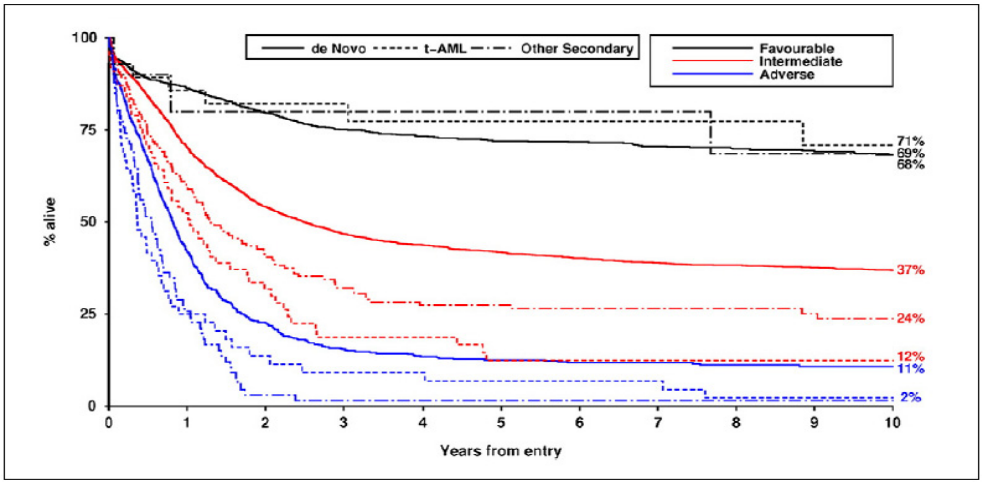


Fig. 7. Survival curves according to cytogenetics subgroups for patients treated in MRC AML trials (AML10, 11, 12, 14 and 15) with t-AML and de novo AML (Grimwade & Hill 2009).

2.5 Others

Clinical markers of high tumor burden like high LDH , high peripheral white blood cell (WBC) count and need for cytoreduction therapy are reported to be of adverse impact on prognosis. As will be discussed later in details, certain molecular abnormalities (FLT3 or KIT mutations) are more associated with high WBC count which could be the actual factor contributing to the prognosis. So much of the prognostic impact of leukocytosis may reflect the molecular abnormalities driving the proliferation. (Dalley et al., 2001; Martin et al., 2000; Burnett et al., 1999) Extramedullary involvement has been associated with worse outcome as well. (Change et al., 2004)

3. Karyotype

50 to 60 % of adult patients with de novo AML have karyotype abnormalities. Cytogenetics is the most powerful prognostic factor in AML. This has been illustrated in several analyses from small single institution studies as well as large multi-institutional trials from various research groups. Its importance has exceeded other variables by consistently showing strong prognostic value in predicting CR, risk of relapse as well as survival, See figure 7

(Grimwade & Hill 2009; Byrd et al., 2002; Slovak et al., 2000). Therefore it is the single most important factor that provides a framework for the current risk-stratified treatment approach in AML. This is clearly reflected on the current WHO classification of AML in which different groups are specified according to the cytogenetic abnormalities. (Swerdlow et al., 2008)

While there is agreement among different groups on defining the favorable cytogenetics group, there is variation on assigning the rest of karyotype abnormalities in the other two groups (i.e. intermediate and adverse). This could be related to variation in patient characteristics, treatment protocols among various trials, as well as the relatively small number of patients having a certain cytogenetic abnormalities in each trial. Table 3 is showing different cytogenetics risk groups in major cooperative groups clinical trials.

	Original MRC	SWOG/ECOG	CALGB	GIMEMA/ AML10	German AMLCG	HOVON/ SAKK	Refined MRC
Favorable	t(15;17) t(8;21) inv(16)/t(16;16)	t(15;17) t(8;21) [lacking del(9q), complex, ie, ≥ 3 unrel abn] inv(16)/t(16;16)/del(16q)	t(15;17) t(8;21) inv(16)/t(16;16)	t(15;17) t(8;21) inv(16)/t(16;16)	t(15;17) t(8;21) inv(16)/t(16;16)	t(15;17) t(8;21) alone inv/del(16) and lacking unfav abn	t(15;17) t(8;21) inv(16)/t(16;16)
Intermediate	Normal Other non-complex	Normal +6, +8, -Y, del(12p)	Normal Other non-complex	Normal -Y	Normal Other non-complex	Normal Other non-complex	Normal Other non-complex
Adverse	abn(3q) -5/del(5q) -7 complex [≥ 5 unrel abn] Excluding those with favorable changes	abn(3q),(9q),(11q),(21q) abn(17p) -5/del(5q) -7/del(7q) t(6;9) t(9;22) complex [≥3 unrel abn]	inv(3)/t(3;3) -7 t(6;9) t(6;11) t(11;19) +8 complex (≥ 3 unrel abn) Excluding those with favorable changes	Other	inv(3)/t(3;3) -5/del(5q) -7/del(7q) abn(11q23) del(12p) abn(17p) complex (≥ 3 unrel abn)	abn(3q) -5/del(5q) -7/del(7q) abn(11q23) t(6;9) t(9;22) complex (≥ 3 unrel abn)	abn(3q) [excluding t(3;5)] inv(3)/t(3;3) add(5q)/del(5q)/ -5,-7/add(7q) t(6;11) t(10;11) t(9;22) -17 abn(17p) with other changes Complex (> 3 unrel abn) Excluding those with favorable changes

Unrel abn indicates unrelated abnormality; abn, abnormal.

Table 3. Classification of different cytogenetics risk groups in major cooperative groups clinical trials. (Grimwade & Hill , 2009).

3.1 Favorable risk

Acute promyelocytic leukemia (APL) with t(15;17) translocation as well as AML with core binding factor (CBF) abnormalities [t(8;21) and inv(16)/t(16;16)] fall in the favorable risk group. They represent around 15% of all AML cases in adults. The favorable outcome in this group has been consistently reported by different research group trials. See Figures 7 and 8.

3.1.1 t(15;17)

APL represents a distinct subtype of AML with characterstic morphological features, clinical presentation, and treatment regimen that incorporates all trans retinoic acid (ATRA). Different clinical trials have reported excellent outcomes with CR rates of more than 90 %. If an ATRA-based regimen of induction, consolidation, and maintenance is used, rates of 3-year OS exceed 85 %. In one report from European APL group 10-year OS rate was 77%. (Ades et al. 2010; Lo-Coco et al. 2010; Sanz et al. 2010) While it carries a good prognosis in general, it is important to

notice that patients with age less than 30 years and WBC count less than 10,000/microL at presentation have superior event-free survival. (Asou et al., 1998)
About 40% of patient with APL have associated chromosomal abnormalities. These additional abnormalities have no impact on treatment outcome. (De Botton et al., 2000; Slack et al., 1997)

3.1.2 t(8;21)

It has been consistently reported to be of favorable prognosis with with CR rates exceeding 87-90% and a 5-year survival of at least 40-65% (Grimwade et al., 2010; Appelbaum et al., 2006a). Along with AML with Inv(16)/ t(16;16), AML with t(8;21) comprise CBF leukemias. In addition to sharing similar pathogenesis, the CBF leukaemias share the characteristics of sensitivity to high-dose cytarabine (HDAC) (Grimwade et al., 1998; Slovak et al., 2000; Byrd et al., 2002). Furthermore, the outcome can be improved substantially by post-remission therapy with HDAC. (Byrd et al., 1999; Palmieri et al., 2002)
While there is agreement on prognosis in AML with isolated t(8;21), there has been inconsistently when defining the prognostic significance of additional cytogenetic abnormalities. Three different small trials have showed poor prognosis with the presence of deletions of the long arm of chromosome 9 (del(9q)) (Schoch et al., 1996) and karyotype complexity (Appelbaum et al., 2006b). On the other side, one large cohort showed no negative impact on prognosis; on the contrary, loss of the Y chromosome in male subjects was associated with a trend for better overall survival (Grimwade et al., 2010).
On the other hand, adverse prognostic significance has been linked to high WBC or absolute granulocyte count, the presence of granulocytic sarcomas, expression of the neural cell adhesion molecule CD56 on leukemic blasts and high WBC index. (Nguyen et al., 2002)

3.1.3 Inv(16)/ t(16;16)

While it is commonly grouped with AML associated with t(8;21) due to similar pathogenesis and outcome, there are few differences. AML associated with inv(16) has different morphological features usually of FAB M4Eo morphology and is less likely to have secondary cytogenetic changes (Byrd et al., 1999, 2004; Nguyen et al., 2002; Delaunay et al., 2003). The presence of such abnormalities, particularly +22, predicted a better outcome in AML associated with inv(16), t(16;16). (Schlenk et al., 2004; Marcucci et al., 2005)
As with t(8;21), the outcome of adults with AML with Inv(16)/ t(16;16) can be improved substantially by intensive post-remission therapy with HDAC. Byrd et al reported the 5-year relapse rate was significantly decreased in patients with inv(16)/t(16;16) receiving 3–4 cycles of HDAC as compared with those receiving one HDAC course (43% versus 70%) (Byrd et al., 2004)
Inferior outcome has been reported in patients presenting with high WBC counts (Martin et al., 2000) and older age. (Delaunay et al., 2003)

3.2 Intermediate risk

This comprises the largest cytogenetics group of AML patients. This is because it includes patients excluded from favorable and adverse groups. This translates in wide variation of CR and survival rates. It is believed to be molecularly heterogeneous and advances in molecular analyses of leukemic cell helped identifying subgroups in this large

heterogeneous group. This is particularly important in the largest subset of this group, patient with normal cytogentics AML.

3.2.1 Normal karyotype

The proportion of adults with de novo AML with normal cytogenetics (AML-NC) has varied between 40% and 49% in various clinical trials which makes the largest cytogenetically defined group of patients.

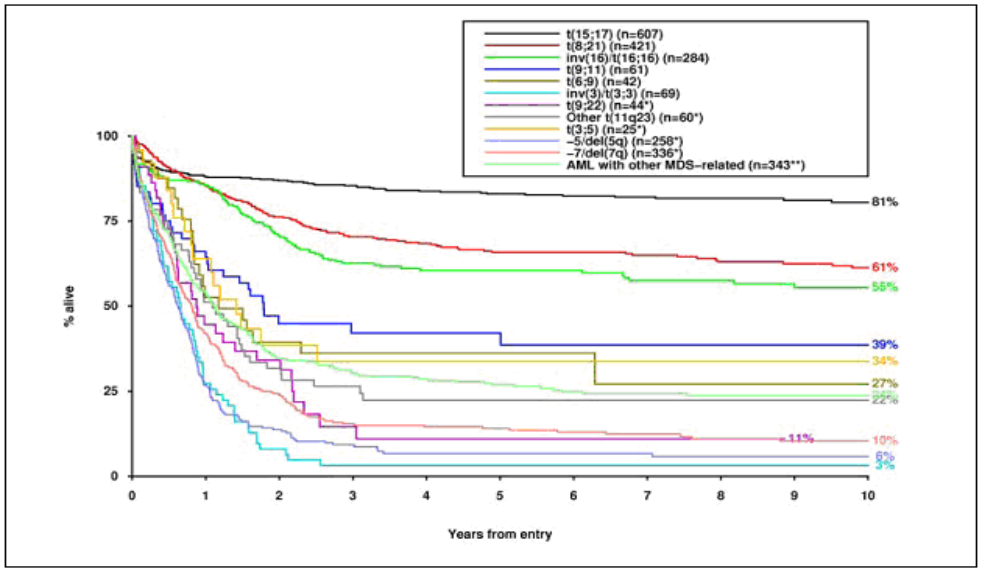


Fig. 8. Survival curves according to different cytogenetic aberrations for patients treated in MRC AML trials (AML10, 11, 12, 14 and 15) with t-AML and de novo AML (Grimwade & Hill , 2009).

While it is considered as one category in the intermediate risk group, AML-NC represents a heterogeneous group of patients as evident by the wide range of OS rates from 24% to 42%. (Gregory et al., 2009). While certain molecular abnormalities have been identified in AML-NC with prognostic significance that identify distinct subgroups of patients, further efforts are needed to subcategorize the rest of the patients in this heterogeneous group.

3.2.2 Trisomy 8

The prognosis of AML patients with trisomy 8 alone or with other aberrations is still a controversial issue. CR rates of patients with trisomy 8 have differed widely, from 29% to 91% (Schiffer et al., 1989; Dastugue et al., 1995). As a result, some groups such as the MRC and SWOG have assigned these patients to a intermediate risk group whereas the GALGB group consider trisomy 8 in the an unfavorable risk group, See table 3. The differences in prognosis of patients with trisomy 8 reported indicate that this population of patients is heterogeneous and identification of additional prognostic factors are needed.

3.2.3 Others

AML with other non-complex aberrations has been categorized in intermediate risk group due to CR and survival rates that fall between the other two major risk groups. MRC data showed a 10 year survival rate of 37% in patients with less than three aberrations not classified in other risk groups as compared to 38% in patients with normal karyotype, see figure 9b.(Grimwade et al.,2010)

3.3 Adverse risk

10-20% of AML patient have adverse risk cytogenetics. These patients tend to be older, often with a prior history of MDS or exposure to chemotherapy . Different trials have reported CR rates of less 60% and a 5-year survival of around 10%. (Grimwade et al., 2010; Byrd et al., 2002; Slovak et al., 2000)

While there is some variability in additional karyotypes defining unfavorable cytogenetics among different cooperative groups (See table 3), there is agreement on abnormalities of chromosomes 5 and 7 (monosomies of 5 and/or 7 (−5/−7) and deletions of 5q and 7q) ,chromosome 3 abnormalities (inv(3)/t(3;3) and 3q abnormalities except t(3;5)), and complex karyotype.

3.3.1 Chromosome 3 abnormalities

AML with inv(3)/t(3;3) represents approximately 1% to 2% of AML. CR rate has been reported to be < 50% with long term OS < 10%. (Grimwade et al., 2010; Byrd et al., 2002; Slovak et al., 2000). Advanced age and high WBC counts at diagnosis seem to confer an even worse outcome (Weisser et al., 2007).

As part of MDS-related cytogenetic abnormalities per 2008 WHO classification (Swerdlow et al., 2008), all 3q abnormalities have been associated with poor prognosis except for t(3;5). t(3;5) is a rare translocation associated with formation of the NPM1-MLF1 fusion gene. Clinically it occurs mainly occur in younger patients with a median age of 30 years and has a favorable outcome with CR rate exceeding 95%.(Grimwade et al., 2010)

3.3.2 Chromosome 5, 7 abnormalities

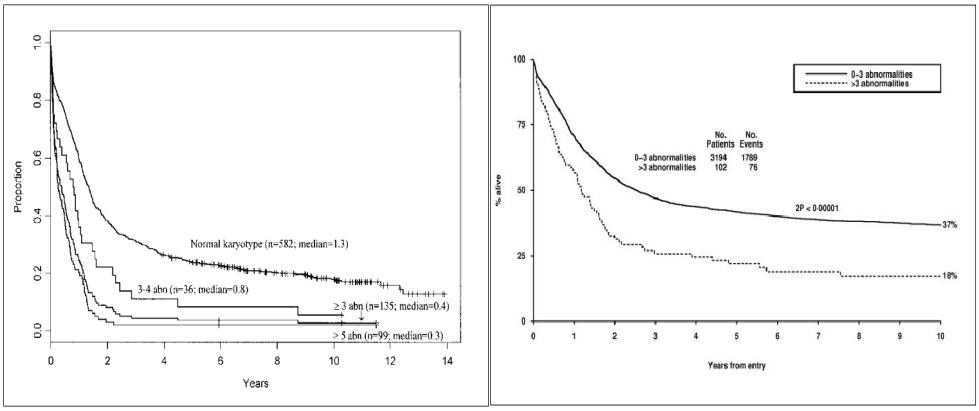
Aberrations of chromosomes 5 and 7 (-7/-5, 5q-, 7q-) are seen in 5% and 10% of cytogenetically abnormal AML respectively. There are usually associated with complex karyotype and rarely occur as a sole aberration. There are associated with MDS as well as t-AML related to alkylating agents and radiation. Prognosis is poor especially when part of complex karyotype, see Figure 8. On exception to that if these abnormalities are associated with favorable cytogenetic changes (t(15;17), t(8;21 and inv(16)/t(16;16)). (Heim & Mitelman, 2009)

3.3.3 Complex karyotype

The definition of complex karyotype differs between major cooperative groups while MRC defines it as the presence of a clone with at least five unrelated cytogenetic abnormalities , SWOG/ECOG, CALGB and AMLCG all go with three or more abnormalities. Although the outcome of patients with three or four abnormalities [other than t(8;21), inv(16)/t(16;16) or t(9;11)(p22;q23)] was better when compared to that of patients with five or more abnormal ties, both were grouped together due to the dismal prognosis in both (See figure 9).

3.3.4 11q23

Aberrations of chromosome band 11q23 occur in approximately 5% to 10% of adults with AML. In the current WHO classification, AML with these aberrations are regarded as a distinct entity. These aberrations occur in de novo as well as in therapy-related AML especially after treatment with topoisomerase II inhibitors. Aberrations of 11q23 commonly affect the MLL gene (also called HTRX, HRX, TRX1,and ALL-1). A special feature of the MLL translocations in AML is the large diversity of fusion partners. More than 50 different partner genes on various chromosomes have been described. The most common of those are *AF9* in the t(9;11) and *AF6* in the t(6;11). (Krauter et al. , 2009).



(a) (b)

Fig. 9. Survival curves according to the complexity of cytogenetics. a: Patients treated in CALGB 8461, tria l(Byrd et al., 2002) b: Patients treated in MRC AML trials (AML10, 12, and 15) (Grimwade et al., 2010).

While initially regarded of poor prognosis as a whole group, outcome of AML with 11q23 band aberrations differs according to the fusion partner. While t(6;11)(q27;q23) and t(10;11)(p12;q23) are associated with a poor prognosis in a number of studies (Martineau et al., 1998; Grimwade et al., 2010; Blum et al., 2004), t(9;11)(p22;q23) is considered of intermediate prognosis. Different trials have shown CR rates of of 79-84% and 10-year survival of about 39%. (Grimwade et al., 2010; Byrd et al., 2002)

4. Gene mutations

As previously stated, AML is a heterogeneous disease with variable outcome in each subgroup. Recent advances in molecular technology have revolutionized our understanding of AML biology and prognosis. It has been of great help in defining biological and clinically discrete subgroups especially in the heterogeneous group of AML-NC. It also provides new insight on new possible therapeutic targets. The impact of newly recognized gene mutations on the understanding of AML biology is evident by adding provisionally new subtypes of AML in the new WHO classification of myeloid neoplasms (i.e. AML with mutated NPM1

and AML with mutated CEBPA) (Swerdlow et al., 2008). The prognostic significance of certain mutation is evident by the new genetic risk grouping proposed by European LeukemiaNet (ELN) which divide AML-NC to subgroups according to associated mutations and put them in different risk groups. (Dohner et al., 2010)

4.1 FLT3

FMS-like tyrosine kinase 3 (*FLT3)* gene encodes a member of the class III receptor tyrosine kinase family that is normally expressed on the surface of hematopoietic progenitor cells and plays an important role in the survival and differentiation of multipotent stem. First described by Nakao et al in 1996, mutations in FLT3 are among the most common genetic mutations in AML with prevalence of 30 - 40% (Nakao et al, 1996; Gregory et al., 2009).

Mutations affect one of two functional domains of the receptor, the juxtamembrane domain (JMD) and the activation loop of the tyrosine kinase domain (TKD). The most common mutation in the JMD of the FLT3 gene is internal tandem duplications (FLT3-ITD) involving JMD with a prevalence of about 25% of adult AML patients. It is particularly more common in AML-NC and AML with t(15;17) where it is reported to in 28-38% and 20-35% respectively. Point mutations affecting TKD and JMD have been reported in about 5-10% and 2% of all AML patients respectively. (Marcucci et al., 2011; Thiede et al., 2002; Schnittger et al., 2002, Mrozek et al., 2007) Clinically, *FLT3*-ITD–positive patients present with increased WBC counts and are more often diagnosed

with de novo than secondary AML. While CR rates are comparable to unmutated AML-CN, prognosis is poor due to high relapse risk. The adverse outcome seen is related to the size of ITD. The longer the duplication the worse the prognosis. (Gregory et al., 2009)

In contrast to FLT3-ITD mutations, the prognostic significance of FLT3-TKD mutation is still controversial with conflicting conclusions from various studies (Mead et al., 2007; Whitman et al., 2008). In another report from Bacher et al., a neutral impact was seen when looking at all patients with TKD mutation. However in the presence of NPM1 or CEBPA mutation a favorable impact was observed and a negative impact was seen if a TKD mutation occurred in conjunction with MLL-PTD, t (15;17) or FLT3-ITD. (Schlenk et al. 2008, Bacher et al., 2008)

In addition to being a prognostic marker, FLT3-ITD is a potential therapeutic target. Several small-molecule inhibitors of FLT3 tyrosine kinase activity in combination with chemotherapy as a frontline therapy for patients with FLT3 mutation are currently evaluated in phase III clinical trials (Marcucci et al., 2011)

4.2 NPM1

Nucleophosmin (NPM1) is nucleocytoplasmic shuttling protein mainly localized in the nucleolus that has multiple functions involved in cell proliferation, apoptosis, DNA repair and ribosome biogenesis. The NPM1 gene belongs to a new category that functions both as an oncogene and tumor-suppressor gene, depending on gene dosage, expression levels, interacting partners, and compartmentalization. First reported by Falini et al in 2005, NPM1 mutations are very common as they are present in 50% to 60% of patients with AML-NC. (Falini et al, 2005; Gregory et al., 2009; Foran 2010)

Clinically, NPM1 mutations are associated with specific features, including predominance of female sex, higher bone marrow blast percentages, LDH levels, WBC and platelet counts, and high CD33 but low or absent CD34 antigen expression. NPM1 mutations tend to be stable over the disease course, supporting their role as primary lesions in leukemogenesis

and accordingly is recognized as a provisional entity in the 2008 revision of the WHO classification of myeloid neoplasms and acute leukemia. (Swerdlow et al., 2008).
Of notice, FLT3-ITD mutation is detected in approximately 40% of patients with NPM1 mutations. Mutated NPM1 without concurrent FLT3-ITD has been associated consistently with achievement of CR and favorable outcome comparable to CBF AML. (Smith et al., 2011). On the basis of this observation, AML with mutated NPM1 without FLT3-ITD has then recently been allocated to the genetic favorable-risk category of AML together with CBF AML in the new classification suggested by ELN. On the other hand, NPM1 mutations did not impact the poor outcome of patients with FLT3-ITD mutation. (Marcucci et al., 2011; Foran 2010)

4.3 CEBPA

The transcription factor CCAAT enhancer-binding protein alpha (CEBPA) is a key molecule in the mediation of lineage specification and differentiation of multipotent myeloid progenitors into mature neutrophils. Mutations in CEBPA were first identified in AML in a report from Pabst et al in 2001 . Reports following indicate 5% to 10% of de novo AML have this mutation with higher prevalence in AML-NC (15-20%). (Pabst et al., 2001; Fröhling et al., 2004; Foran 2010)
AML-NC patients carrying a CEBPA mutation are characterized by distinct clinical features such as higher peripheral blood blast counts, lower platelet counts, less lymphadenopathy, or extramedullary leukemia. As compared to NPM1 mutations, CEBPA mutations are less frequently associated with FLT3-ITD or TKD mutations. (Schlenk et al., 2008)
In the absence of a FLT3-ITD, CEBPA mutation has a favorable prognosis in patients with AML-NC with approximately 60% long-term survival. Prognosis is better if the mutation is biallelic, where it is categorized in the favorable risk group. (Dufour et al., 2010; Foran 2010; Smith et al., 2011)

4.4 KIT

KIT is the receptor for stem cell factor (KIT ligand) and is expressed on less than 5% of marrow cells. KIT mutation is frequently noted in CBF leukemia with prevalence of 30-40% and 20-30% in inv(16)/t(16;16) and t(8;21) leukemia respectively.
Clinically, Patients affected appear to have higher WBC counts and higher frequency of extramedullary disease such as paraspinal masses. (Foran 2010; Smith et al., 2011). Recent trials have reported significantly higher incidence of relapse and significantly lower survival in CBF leukemia harboring KIT mutation (Schnittger et al., 2006; Baschka et al., 2006). Clinical trials are currently underway evaluating KIT inhibitors in CBF leukemias. (Marcucci et al., 2011)

5. Future perspectives

Advances in molecular studies have changed our understanding of AML as a single disease. As discussed in previous section , certain gene mutations has fragmented previously known risk groups into smaller and more homogenous groups. Identification of new mutations and understanding their prognostic and predictive value is a major goal in AML research. In addition, gene and microRNA expression profiling is a very active area of research in AML with interesting recent observations. We hope that such advances will provide us with more

information that will help in systematic characterization of cancer genomes. We will review briefly few areas of active research showing promising results that need further efforts before it has its practical implications as prognostic and predictive tools.

5.1 Gene mutations

Further gene mutations have been identified in recent trials which are still waiting further clinical data to support their prognostic implications.

5.1.1 IDH1/IDH2 mutations

IDH1/IDH2 gene mutations, which were first reported in gliomas with good prognostic impact, have been recognized recently in AML with aggregate frequency of these two mutations of about 15% to 20% of all patients with AML and 25% to 30% of patients with AML-NC. There are conflicting data concerning the prognostic significance of IDH mutations in AML, with some studies suggesting that they are associated with a poorer outcome especially in NPM1 mutated AML, while others have found no evidence of that (Marcucci et al., 2010; Thol et al., 2010). Further studies with larger number of patients harboring this mutation are needed to further characterize the prognostic significance of this rare mutation.

5.1.2 CBL mutation

The Casitas B-cell lymphoma (CBL) gene on chromosome 11q23.3 contains several functional domains. One of these domains, the C-terminal domain, gives rise to the CBL protein which has ubiquitin ligase activity that targets a variety of tyrosine kinases for degradation by ubiquitination. Heterozygous CBL mutations have been recognized in 0.6% to 33% of AML patient (Sargin et al., 2007; Bacher et al., 2010; Ghassemifar et al., 2011). Interestingly, CBF AML patients represented a significant proportion of patients who have this mutation. (Abbas et al., 2008; Reindel et al., 2009). In one retrospective review including more than two hundred AML patients along with similar number of MDS and MDS/MPD diseases, the presence of CBL mutation was an independent adverse prognostic factor for OS. (Makishima et al., 2009)

5.2 Gene expression profiling

In addition to structural genetic aberrations, changes in expression of specific genes seem to impact prognosis of molecular subsets of patients with AML. Increased or decreased expression of specific genes (typically those involved in hematopoiesis, myeloid differentiation, or immune response) has been associated with response to therapy as well as survival.

5.2.1 BAALC

The brain and acute leukemia cytoplasmic (BAALC) gene is localized on chromosome band 8q22.3. It has been postulated to function in the cytoskeleton network due to its cellular location. It is most commonly seen in AML with trisomy 8 and AML-NC. Several studies have demonstrated that high BAALC expression is a poor prognostic indicator in AML-NC for such factors as OS, DFS, and resistant disease. BAALC expression appears to be particularly useful as a prognostic marker in AML-NC patients lacking FLT3-ITD and CEBPA mutations. (Mrozek et al., 2007; Gregory et al., 2009)

5.2.2 MN1

The meningioma 1 (MN1) gene encodes a protein that participates in a gene transcription regulator complex involving retinoid receptors. Recent studies have shown MN1 overexpression is associated with poor prognosis in AML in terms of response to induction chemotherapy, relapse rate and therefore OS. Interestingly, one study has shown low MN1 expression was correlated with better response therapy in AML. Together, both observations suggest that MN1 expression is not only a prognostic but also a predictive marker for response to treatment. (Foran 2010; Marcucci et al., 2011)

5.2.3 ERG

The ETS-related gene (ERG) is a member of the ETS family of transcription factors. High ERG expression is associated with the upregulation of many genes which are involved in cell proliferation, differentiation, and apoptosis. ERG overexpression mostly impacted outcome of low molecular risk AML-NC (mutated NPM1 without FLT3-ITD) and AML with low BAALC expression. (Gregory et al., 2009; Marcucci et al., 2011)

5.3 MicroRNA expression

MicroRNAs are noncoding RNAs of 19 to 25 nucleotides in length that regulate gene expression. They perform critical functions in cell development, differentiation, proliferation, and apoptosis. They have been shown to play a role in malignant transformation in solid malignancies. Recent studies in AML have shown that specific patterns of microRNA expression are closely associated with certain cytogenetics and molecular changes like FLT3-ITD. Results are reproduced and such patterns are considered like signatures. For example in two separate studies, upregulation of microRNAs expression from genes localized at chromosome band 14q32 has been found in APL with t(15;17) while the downregulation of certain mircoRNAs (*miR-133a)* was observed in patients with t(8;21). In AML-NC, specific microRNAs expression signature (*miR-155)* was associated with the presence of high risk features (lack of NPM1 mutation or the presence of FLT3-ITD), while upregulation of microRNAs (*miR-181)* was identified in CEBPA mutated AML. (Foran 2010; Marcucci et al., 2011)

6. Conclusion

AML is markedly heterogeneous disease with variable response to therapy and survival. While advances in AML therapy have been moving slowly over last few decades, there have been dramatic breakthroughs in the identification of reproducible prognostic variables in AML. In particular, advances in molecular biology as well as genomics technology have revolutionized our approach to AML and have added substantially to our understanding of biology and prognosis of this disease through identification of novel prognostic markers. This is particularly important in AML-NC which comprises a large heterogeneous group of patients. While such advances help separating AML patients into smaller homogenous groups, we hope to look for a day where individualized therapy for patients AML can be tailored to achieve the best outcome. Such breakthroughs facilitate risk-stratified approach to therapy in AML where more groups are separated into favorable or poor risk groups rather staying the large grey intermediate group. They also provide us with insight into potential therapeutic targets that can be assessed in clinical trials on which we largely depend to achieve breakthroughs.

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15

Bacillus cereus Sepsis in the Treatment of Acute Myeloid Leukemia

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1. Introduction

Fatal sepsis during chemotherapy-induced neutropenia is the most severe complication of which physicians must be keenly aware. Common bacterial pathogens in neutropenic patients usually include gram-positive cocci such as coagulase-negative staphylococci, *Staphylococcus aureus*, *Enterococcus* species, and gram-negative rods such as *Escherichia coli*, *Klebsiella* species, *Enterobacter* species, and *Pseudomonas aeruginosa* (Wisplinghoff, *et al* 2003). Thus, clinical practice guidelines for the use of antibiotics are likely to be aimed at targeting these pathogens including antibiotic-resistant strains (Freifeld, *et al* 2011). In the absence of effector cells for these pathogens, the rapid progression of invasive bacterial infections may occur; therefore, antibiotics are a life-saving measure during severe neutropenia.

Bacillus cereus (*B. cereus*) is an aerobic gram-positive, spore-forming, and rod-shaped bacterium that is widely distributed in the environment. Although *B. cereus* is a common cause of food-poisoning, abdominal distress such as vomiting and diarrhea is usually mild and self-limiting unless the host is immunocompromised. Some patients that undergo prolonged hospitalization have *Bacillus* species as a part of the normal flora in their intestine (Drobniewski 1993). Therefore, identification of this microorganism in clinical cultures has usually been considered to be due to contamination. For example, 78 patients were found to have cultures positive for *B. cereus* in a single center in the United States; however, only 6% of them resulted in clinically significant infections (Weber, *et al* 1989). On the other hand, *B. cereus* is a growing concern as a cause of life-threatening infections in patients with hematologic malignancies, including septic shock, brain abscess, meningitis, colitis, respiratory infections, endocarditis, and infection-related coagulopathy and hemolysis. The risk factors for patients with unfavorable outcomes, however, have not been totally elucidated. In addition, *B. cereus* sepsis generally does not respond to any antibiotics in spite of their *in vitro* efficacy (Drobniewski 1993). Akiyama et al. reviewed 16 case reports of *B. cereus* sepsis in patients with leukemia, and consequently reported only 3 survivors (Akiyama, *et al* 1997). Therefore, physicians should identify specific risk factors of *B. cereus* sepsis during chemotherapy for leukemia patients and establish a proper strategy to overcome this life-threatening sepsis.

2. *B. cereus* sepsis in patients with hematologic malignancies

In recent years, we encountered several cases of *B. cereus* sepsis including 4 fatal cases with acute leukemia in our hospital. These episodes prompted us to review all cases of *B. cereus* sepsis especially in hematologic malignancies. In the present study, we collected the data and the clinical features of these patients with *B. cereus* sepsis in a retrospective fashion, and identified risk factors for a fatal prognosis in these patients (Inoue, *et al* 2010). Based on these data, we also put forward a proposal for the rapid diagnosis of *B. cereus* sepsis and earlier therapeutic intervention for this infection.

2.1 Patients and methods

We reviewed the microbiology records of all patients who produced a positive blood culture for *B. cereus* from September 2002 to November 2009 in our hospital. We routinely took at least two sets of blood culture samples from all patients with hematologic malignancies who developed a high-grade fever of over 38℃. Each set consisted of two blood culture vials for both aerobic and anaerobic cultures. Identification of *B. cereus* was made on the basis of Gram-staining, colony morphology, and analysis with NGKG agar (Nissui, Tokyo, Japan). Antimicrobial disk susceptibility tests were performed using Sensi-Disc (Beckton Dickinson).

We defined a case as sepsis when more than two blood culture sets were positive for *B. cereus* or only a single set was positive in the absence of other microorganisms in patients who had definite infectious lesions, such as brain or liver abscesses. Instead, febrile cases that did not satisfy the above criteria were defined as an unknown pathogen or contaminated culture.

With regard to sepsis patients, we also reviewed their charts to obtain clinical information, including the underlying disease, insertion of a central venous (CV) catheter, nutrition route, neutrophil count, and prior chemotherapy or steroid treatment. Oral nutrition was defined only when patients were eating a regular diet without high-calorie parenteral nutrition support. We also documented clinical signs at febrile events, such as gastrointestinal (GI) and central nervous system (CNS) symptoms, antibiotic use, and the drug sensitivity of *B. cereus*. Then, we assessed the risk factors for a fatal prognosis; i.e., whether the underlying disease was acute leukemia, whether a CV catheter was inserted, whether the patient was receiving oral or parenteral nutrition, whether their neutrophil count was 0/mm^3 or above 0/mm^3, and whether characteristic clinical signs were present at the time of febrile events. We also reviewed the charts of patients without hematologic malignancies who had cultures positive for *B. cereus* in the same period. Furthermore, we assessed the above data in conjunction with those from previously reported patients with *B. cereus* sepsis, who had hematologic malignancies.

Statistical tests included χ^2 and Fisher's exact tests. All calculations were made using the program JMP 8.0 (SAS Institute, Cary, NC, US). All P-values of <0.05 were considered significant.

2.2 Results

2.2.1 Characteristics of *B. cereus* sepsis patients

A total of 68 febrile patients that produced positive blood cultures for *B. cereus* were identified from September 2002 to November 2009 in our institute. Twenty-three of these patients had hematologic malignancies, including 4 patients who died of fatal sepsis.

Although 11 of the 23 patients showed signs of infection such as a high-grade fever, we classified them with an unknown pathogen or contaminated culture, since other causes of fever could not be totally excluded. With respect to underlying diseases, 2 of 5 cases of non-Hodgkin lymphoma (NHL), 3 of 5 cases of acute lymphoblastic leukemia (ALL), 5 of 6 cases of acute myeloid leukemia (AML), 1 of 4 cases of myelodysplastic syndrome (MDS), and 1 of 3 cases of multiple myeloma (MM) were diagnosed with *B. cereus* sepsis. Thus, we determined as many as 12 (patients 1 to 12) of 23 patients with hematologic malignancies as having *B. cereus* sepsis; whereas, only 10 of 45 patients without hematologic malignancies were similarly diagnosed on the basis of the same criteria (P=0.012). All of these 10 patients recovered from *B. cereus* sepsis after treatment with appropriate antimicrobials including carbapenems, vancomycin, or fluoroquinolones. None of the 10 patients received chemotherapy. Their underlying diseases were as follows: chronic obstructive pulmonary disease, congestive heart failure, bronchial asthma, acute hepatitis, malnutrition, subarachnoid hemorrhage, ovarian cancer, gastric cancer, and cerebral infarction in 2 patients.

As shown in Table 1, we analyzed the profiles of the 12 patients with hematologic malignancies: 6 men and 6 women with a median age of 53.5 ranging from 20 to 85 years; 8 patients with acute leukemia, 5 who were treated with a CV catheter and 12 who received oral nutrition; 5 patients with a neutrophil count of 0/mm^3; all patients, except for patients 5, 10, and 12, had undergone prior steroid treatment within 2 weeks; and 8 patients exhibited GI symptoms including nausea, vomiting, diarrhea, and abdominal pain, and 6 patients displayed CNS symptoms ranging from disorientation to deep coma at the time of febrile episodes. Although CV catheters were removed in patients 2, 4, 6, and 12 as a part of the management for their febrile status, none of these catheters were found to be positive for *B. cereus*. In one patient (patient 6), postmortem cultures from CSF samples were performed, with positive results for *B. cereus*. Among 5 patients with CNS symptoms, lumbar puncture was only performed in patient 8, without *B. cereus* isolation. Lumbar puncture was not conducted for the remaining 4 patients because of their unstable conditions. No patient demonstrated other organisms as co-isolates in their initial blood cultures.

Patients 1 and 2, who had ALL, and patients 6 and 12, who had AML, developed consciousness disturbance, which resulted in a deep coma and brain stem dysfunction 3 days, 6 hours, 18 hours, and 8 hours after their febrile episode and they died 12 days, 7 days, 20 hours, and 15 hours after their febrile event, respectively, despite intensive antimicrobial therapy and supportive care (Table 1). All 4 patients had received intensive chemotherapy for acute leukemia, and febrile events occurred on day 13 after re-induction chemotherapy in patient 1; on day 18 after induction therapy in patient 2; on day 14 after consolidation in patient 6; and day 13 after induction therapy in patient 12. On the other hand, patient 7, who had received high-dose etoposide for the collection of peripheral blood stem cells, similarly developed a deep coma but recovered without sequela 28 hours after the onset of consciousness disturbance. Patients 8, 9, 10, and 11 also received intensive chemotherapy prior to *B. cereus* sepsis, as shown in Table 1. Patient 4 received methylprednisolone treatment (20 mg/day) for chronic graft-versus-host disease when the sepsis developed. The characteristics of the remaining patients are also shown in Table 1. In addition to patients 1, 2, 6, and 12, patient 8 died of underlying refractory AML 6 months after the onset of *B. cereus* brain abscesses despite successful treatment of the abscesses with long-term vancomycin administration, and patient 4 died of multiple organ failure caused by another bacterial infection 11 months later. No sequela or death occurred in the remaining patients, including

Patient	Age, y	Sex	Month Year	Primary diagnosis	CV catheter	Oral nutrition	ANC, cells/μl	Chemotherapy	Corticosteroid within 14 days	GI symptoms	CNS symptoms	Administered antibiotics	Outcome	Antibiotics *B. cereus* sensitive to (in vitro)
1	40	F	Jun–03	ALL	(+)	(+)	0	Reinduction (doxorubicin, methotrexate, and vindesine)	(+)	Vomiting	(+)	CFPM, ISP	Death	IPM, GM, ABK, EM, MINO, LVFX, VCM
2	42	F	May–07	ALL	(+)	(+)	0	Induction	(+)	Abdominal pain, diarrhea	(+)	MEPM, VCM, CLDM	Death	IPM, LVFX, EM, GM, CPFX, MEPM
3	58	F	May–07	MM	(–)	(+)	15200	(–)	(+)	Vomiting	(–)	IPM, MINO, LVFX	Recovery	IPM, LVFX, ABK, GM, VCM, MINO
4	49	M	Jun–07	MDS	(+)	(+)	4200	Allogeneic BMT 11 months before	(+)	Diarrhea	(–)	CAZ	Recovery	IPM, GM, ABK, EM, MINO, LVFX, VCM
5	85	F	Sep–07	NHL	(–)	(+)	8340	(–)	(–)	(–)	(–)	CLDM	Recovery	ABPC, IPM, GM, ABK, EM, MINO, LVFX, VCM
6	67	M	Apr–08	AML	(+)	(+)	0	Consolidation (cytarabine and mitoxantrone)	(+)	Vomiting, diarrhea	(+)	MEPM, VCM	Death	IPM, LVFX, EM, ABK, GM, FOM, VCM, MINO
7	67	M	Jun–08	NHL	(–)	(+)	2	High-dose etoposide	(+)	Abdominal pain, vomiting	(+)	MEPM, AMK	Recovery	IPM, GM, ABK, EM, MINO, LVFX, VCM
8	45	M	Oct–08	AML	(–)	(+)	8	Consolidation (high-dose cytarabine)	(+)	(–)	(+)	MEPM, VCM	Recovery	FMOX, IPM, GM, LVFX, VCM
9	31	F	Dec–08	ALL	(–)	(+)	0	Consolidation (cyclophosphamide, vincristine, and dexamethasone)	(+)	(–)	(–)	MEPM, VCM	Recovery	IPM, LVFX, EM, GM, FMOX, VCM
10	61	F	May–09	AML	(–)	(+)	6	Reinduction (cytarabine and idarubicin)	(–)	Diarrhea	(–)	MEPM, VCM	Recovery	IPM, GM, ABK, LVFX, VCM
11	20	M	Aug–09	AML	(–)	(+)	1	Consolidation (high-dose cytarabine)	(+)	Abdominal pain	(–)	DRPM, VCM	Recovery	IPM, GM, ABK, LVFX, VCM
12	74	M	Nov–09	AML	(+)	(+)	0	Induction (cytarabine, etoposide, and mitoxantrone)	(–)	(–)	(+)	DRPM, VCM	Death	IPM, GM, ABK, LVFX, VCM

CV catheter, central venous catheter; ANC, absolute neutrophil count; GI symptoms, gastrointestinal symptoms; CNS, central nervous system; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; MDS, myelodysplastic syndrome; JALSG, Japan Adult Leukemia Study Group; BMT, bone marrow transplantation; MEPM, meropenem; VCM, vancomycin; CLDM, clindamycin; DRPM, doripenem; IPM, imipenem; LVFX, levofloxacin; EM, erythromycin; GM, gentamicin; CPFX, ciprofloxacin; ABK, arbekacin; FOM, fosfomycin; MINO, minocycline; ABPC, ampicillin; FMOX, flomoxef; CAZ, ceftazidime; CFPM, cefepime; ISP, isepamicin.

Table 1. Clinical features of patients with *Bacillus cereus* sepsis in our cohort

patient 9, in whom the long-term administration of vancomycin was required for liver abscesses. Patients 9 and 10 successfully received allogeneic bone marrow transplantation (BMT) after recovering from severe *B. cereus* sepsis.

2.2.2 Results of autopsies

Of the 4 fatal cases, we performed autopsy in 3 patients. Autopsy of patient 2 demonstrated the presence of a small number of *B. cereus* in the subarachnoid space and venous thrombosis in the Vein of Galen and the superior sagittal sinus. In contrast, coagulation necrosis with bacterial infiltration in the liver and necrotizing leptomeningitis with subarachnoid hemorrhage (SAH) were observed in patient 6, and coagulation necrosis accompanied by *B. cereus* infiltration in the colon could be seen in patient 12. Histologic analyses of organs obtained in the autopsies of patients 2 and 6 are shown in Figure 1. Large venous thromboses in the vein of Gallen and superior sagittal sinus can be seen in patient 2 (A and B, H.E. staining, ×40). On the other hand, in patient 6, numerous gram-positive rods are present in the subarachnoid space (D, Gram staining, ×400) and outside of the subarachnoid membrane (E, H.E. staining, ×100, in the circle), which may have caused the coagulation necrosis of the vessels in the subarachnoid membrane (arrows). The coagulation necrosis is also seen without the infiltration of inflammatory cells in the surface area of the cerebrum (arrowheads), which is distant from the *B. cereus* clusters. Extensive coagulation necrosis with bacterial infiltration stands out without an inflammatory response in the liver of patient 6 (C, H.E. staining, ×100). A number of gram-positive rods can be seen clustering in the circle. In patient 12, coagulation necrosis with bacterial infiltration could be similarly seen in the liver in addition to *B. cereus* infiltration in the colon, although we could not obtain pathological analysis in CNS.

2.2.3 Risk factors for a fatal prognosis, which were identified in patients in our institution

As shown in Table 1, all 4 fatal cases shared common factors, that is, acute leukemia, insertion of a CV catheter, an extremely low neutrophil count, and CNS symptoms at febrile episodes. We then statistically analyzed clinical parameters of 12 patients listed in Table 1, and identified the following risk factors for death due to *B. cereus* sepsis: CV catheter insertion (P=0.010), a neutrophil count of 0/mm^3 (P=0.010), and CNS symptoms at the time of febrile events (P=0.010). While acute leukemia (P=0.141), GI symptoms (P=0.594), and prior steroid treatment within 2 weeks (P=0.764) did not show a close relationship with a fatal course of *B. cereus* sepsis.

2.2.4 Antibiotic susceptibility

The antibiotics employed in the present study included meropenem or doripenem for 8 patients (patients 2, 6-12) and vancomycin for 7 patients (patients 2, 6, 8-12). All of the isolated *B. cereus* strains were susceptible to imipenem, vancomycin, levofloxacin, and gentamicin; whereas, no isolated *B. cereus* strains, except for that from patient 5, were sensitive to penicillins or cephalosporins *in vitro.*

2.2.5 Risk factors for a fatal prognosis in previously reported patients and ours

To our knowledge, 46 *B. cereus* sepsis patients with hematologic malignancies have been previously reported (Akiyama, *et al* 1997, Arnaout, *et al* 1999, Christenson, *et al* 1999, Colpin,

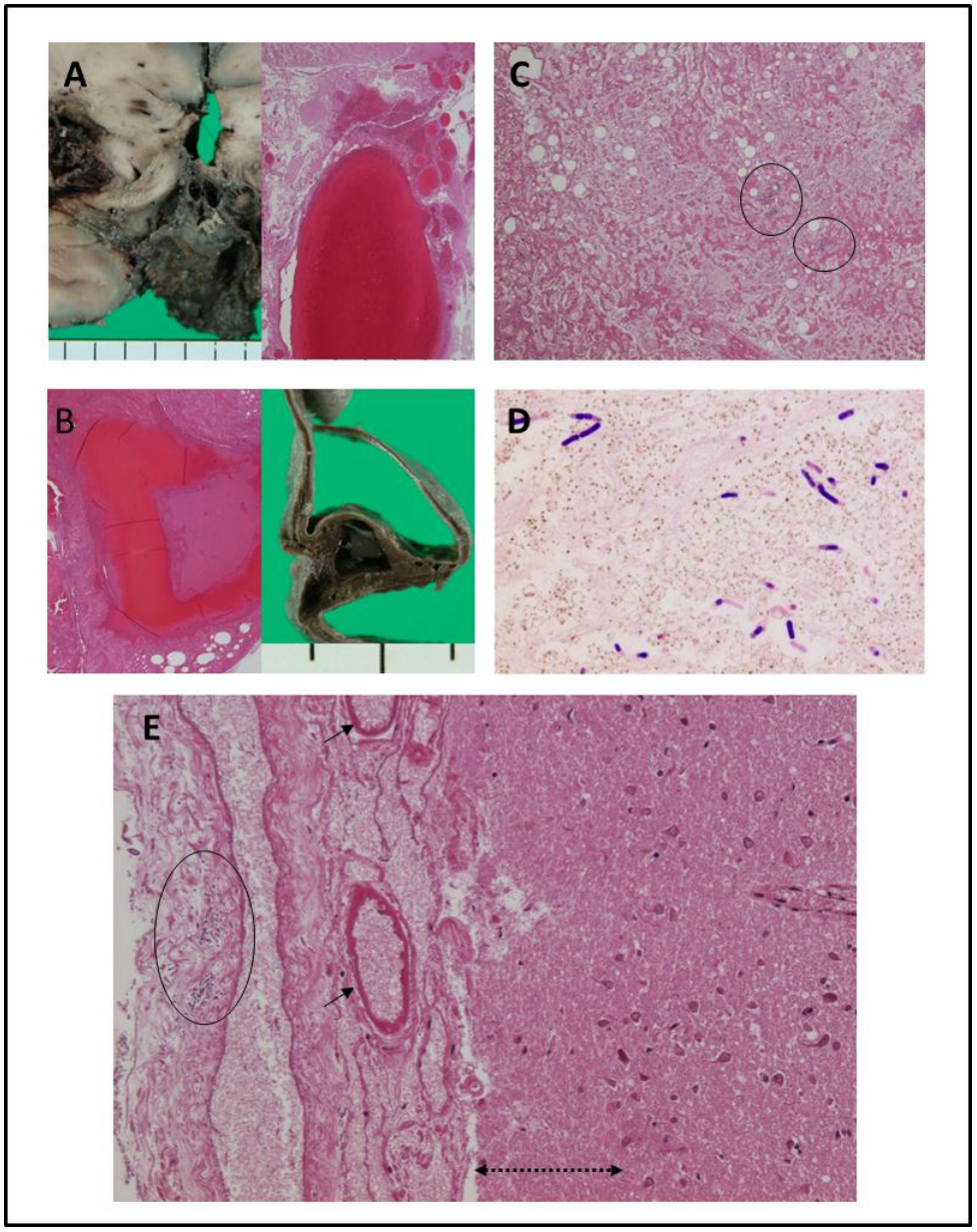

Fig. 1. Histologic analyses of organ specimens obtained in the autopsies of patients 2 and 6

et al 1981, Cone, *et al* 2005, Coonrod, *et al* 1971, Dohmae, *et al* 2008, Feldman and Pearson 1974, Frankard, *et al* 2004, Funada, *et al* 1988, Garcia, *et al* 1984, Gaur, *et al* 2001, Ginsburg, *et al* 2003, Ihde and Armstrong 1973, Jenson, *et al* 1989, Katsuya, *et al* 2009, Kawatani, *et al* 2009, Kiyomizu, *et al* 2008, Kobayashi, *et al* 2005, Kuwabara, *et al* 2006, Le Scanff, *et al* 2006, Leff, *et*

al 1977, Marley, *et al* 1995, Motoi, *et al* 1997, Musa, *et al* 1999, Nishikawa, *et al* 2009, Ozkocaman, *et al* 2006, Sakai, *et al* 2001, Strittmatter, *et al* 1995, Tomiyama, *et al* 1989, Trager and Panwalker 1979, Yoshida, *et al* 1993). On analyses of the clinical parameters of these patients, as in shown in Table 2, patients with acute leukemia, a neutrophil count of 0/mm^3 or below the lower limit of each institute, or CNS symptoms at febrile episodes were identified as risk factors closely correlated with a fatal prognosis (P=0.044, 0.004, and 0.002, respectively). Patients younger than 15 years old had a tendency to show a more favorable prognosis in comparison with older patients. (P=0.063). Male, GI symptom, corticosteroid administration, CV catheter insertion, and antimicrobial therapy except for that with vancomycin did not have a significant impact on the prognosis.

2.3 Discussion and proposal

2.3.1 How do we efficiently select high-risk patients?

Our report contains 12 adult *B. cereus* sepsis cases of hematologic malignancy, which is, to our knowledge, the largest cohort of *B. cereus* sepsis in adult patients from a single center. Because of the serious outcomes of these patients with hematologic malignancies, the detection of *B. cereus* from blood culture samples at febrile events from these patients should not be regarded as contamination. In our cohort, patients with a neutrophil count of 0/mm^3, with CNS symptoms, or who had undergone CV catheter insertion definitely had a poor prognosis. However, we had difficulties in identifying further precise prognostic factors because of the small number of *B. cereus* infection cases in our institution. Therefore, we assessed the data in conjunction with those from our 12 patients and from 46 previously reported patients, giving a total of 42 patients with acute leukemia, although reporting bias may have existed because severe cases with peculiar clinical features tend to be selectively reported and some reports did not refer all factors which we consider to be important. Consequently, patients who had acute leukemia, a neutrophil count of 0/mm^3 or a count below the lower limit of each institute, or CNS symptoms at febrile episodes were identified as being associated with a fatal prognosis. Interestingly, the relatively more favorable prognosis in younger patients implies the importance of appropriate evaluation in adult patients (Table 2).

Regarding the neutrophil count, patients 7, 8, 10, and 11 fully recovered from *B. cereus* sepsis complicated with coma, in clear contrast to patients 1, 2, 6, and 12 who had a neutrophil count of 0/mm^3 (Table 1), suggesting that both immediate therapeutic intervention and even a small number of neutrophils can effectively work against *B. cereus* sepsis. The poor outcomes in acute leukemia patients may have been an indirect consequence because of the greater immunosuppression following intensive chemotherapy, rather than due to the underlying disease. Regarding the relationship between *B. cereus* sepsis and the treatment process of acute leukemia in the combined clinical parameters, 35 patients developed sepsis during remission induction or reinduction therapy, 9 consolidation therapy, 4 post-transplantation, and 1 maintenance therapy in a total of 49 acute leukemia patients whose clinical data were available (Akiyama, *et al* 1997, Arnaout, *et al* 1999, Christenson, *et al* 1999, Colpin, *et al* 1981, Cone, *et al* 2005, Coonrod, *et al* 1971, Dohmae, *et al* 2008, Feldman and Pearson 1974, Frankard, *et al* 2004, Funada, *et al* 1988, Garcia, *et al* 1984, Gaur, *et al* 2001, Ginsburg, *et al* 2003, Ihde and Armstrong 1973, Jenson, *et al* 1989, Katsuya, *et al* 2009, Kawatani, *et al* 2009, Kiyomizu, *et al* 2008, Kobayashi, *et al* 2005, Kuwabara, *et al* 2006, Le Scanff, *et al* 2006, Leff, *et al* 1977, Marley, *et al* 1995, Motoi, *et al* 1997, Musa, *et al* 1999, Nishikawa, *et al* 2009, Ozkocaman, *et al* 2006, Sakai, *et al* 2001, Strittmatter, *et al* 1995,

			Survival	
		Number (Deaths)	Odds Ratio	*P*
Age, y	≧15	48(28)	5.600	0.063
	＜15	10(2)		
Sex	Male	34(21)	2.692	0.069
	Female	24(9)		
limited to ≧ 15 years old	Male	29(20)	3.056	0.122
	Female	19(8)		
GI symptom at febrile episodes	(+)	34(19)	1.497	0.451
	(−)	24(11)		
limited to ≧ 15 years old	(+)	27(18)	2.200	0.184
	(−)	21(10)		
CNS lesion or CNS symptoms	(+)	32(23)	6.937	**0.002**
	(−)	26(7)		
limited to ≧ 15 years old	(+)	27(21)	7.000	**0.005**
	(−)	21(7)		
Corticosteroid use within 14 days	(+)	31(17)	0.552	0.357
	(−)	16(11)		
limited to ≧ 15 years old	(+)	25(16)	0.808	0.754
	(−)	16(11)		
CV catheter	(+)	26(15)	1.273	0.863
	(−)	29(15)		
limited to ≧ 15 years old	(+)	18(14)	3.250	0.149
	(−)	27(14)		
VCM therapy	(−)	29(18)	1.964	0.238
	(+)	22(10)		
limited to ≧ 15 years old	(−)	26(17)	2.099	0.367
	(+)	19(9)		
Neutrophil count of 0 or less than the lower limit	(+)	22(18)	8.000	**0.004**
	(−)	25(9)		
limited to ≧ 15 years old	(+)	19(17)	13.222	**0.002**
	(−)	23(9)		
Underlying disease	Acute leukemia	50(29)	9.667	**0.044**
	Others	8(1)		
limited to ≧ 15 years old	Acute leukemia	41(27)	11.571	**0.032**
	Others	7(1)		

These data include both previous reports and our 12 sepsis patients. P-values were calculated using χ^2 and Fisher's exact tests. Odds ratios predict the possibility of death from *Bacillus cereus* sepsis. GI, gastrointestinal. CNS, central nervous system. CV, central vein. VCM, vancomycin.

Table 2. Univariate analysis of prognostic factors of *B. cereus* sepsis

Tomiyama, *et al* 1989, Trager and Panwalker 1979, Yoshida, *et al* 1993). Therefore, patients under induction or reinduction therapy may be more likely to be susceptible to *B. cereus* sepsis. Also, previous studies have shown that variations in toxins and enzymes, which were produced by *B. cereus*, such as cereolysin, enterotoxin, emetic toxin, phospholipase C, and sphingomyelinase, between isolates of *B. cereus* were correlated with the reversibility of clinical courses (Turnbull, *et al* 1979, Turnbull and Kramer 1983). With respect to clinical symptoms related to *B. cereus* sepsis, patients with CNS disturbance mostly had a fatal outcome (P=0.005, in adult patients) (Table 2). Gaur et al. reported that patients with possible CNS involvement had a tendency to exhibit severe neutropenia at the onset of sepsis and to have an unfavorable outcome, although their study was conducted in a children's hospital (Gaur, *et al* 2001). Given that most of the patients with a fatal prognosis had GI symptoms at the time of febrile episodes (Table 2), clinicians must be cautious of the early signs of CNS in addition to GI symptoms. Although GI symptoms were not significantly correlated with a fatal prognosis, we consider that the symptoms are very important in terms of early clues to the diagnosis of *B. cereus* sepsis. CV catheter insertion did not have a significant impact on the prognosis (P=0.149, in adult patients), although the result was opposite to that found in our cohort.

2.3.2 We have a very limited time to avoid CNS damage in the face of *B. cereus* sepsis

With respect to the results of autopsy, the findings observed in patient 2 have not been reported elsewhere, although coagulation necrosis with *B. cereus* infiltration of the liver and the GI tract may not be rare in *B. cereus* sepsis, as demonstrated in patients 6 and 12, respectively. In any case, the patients' condition rapidly deteriorated in spite of intensive antibiotic coverage, including carbapenems and vancomycin, which were effective against *B. cereus in vitro*, although these agents (especially meropenem and vancomycin) are still recommended because of the inherent ability of *B. cereus* to produce β lactamases and the presence of the blood brain barrier (Hasbun, *et al* 1999, Zinner 1999). The failure of apparently adequate therapy may have been due to inadequate tissue concentrations of antibiotics. However, we emphasize that delays in therapeutic intervention must be avoided even if the CNS may have already been damaged by *B. cereus* before the administration of adequate antibiotics, as seen in our fatal cases. Patient 7 (Table 1), with a neutrophil count of near 0, had consciousness disturbance at the febrile event. We started to treat this patient very quickly based on information from Patient 2 and 6 with antibiotics effective for *B. cereus*, with the successful recovery from sepsis including CNS symptoms. This experience may be very important in terms of the necessity of very early therapeutic intervention.

2.3.3 Proposal: Initial management of fever and neutropenia in AML patients in view of fatal *B. cereus* sepsis

According to the Infectious Diseases Society of America (IDSA) guideline for neutropenic patients with cancer, 'high-risk' patients are considered to be those with anticipated sustaining (>7-day duration) and profound neutropenia (absolute neutrophil count (ANC) <100 cells/mm^3) and/or significant medical co-morbid conditions, including hypotension, pneumonia, new-onset abdominal pain, or neurologic changes (Freifeld, *et al* 2011). It is generally assumed that all AML patients during intensive chemotherapy meet the high-risk criteria.

In the face of febrile AML patients, physicians should evaluate a complete blood count including a differential leukocyte count, although therapeutic intervention must be performed without delay in cases when the neutrophil count is expected to be 0/mm^3 or below the lower limit of each institute. At least 2 sets of blood culture are recommended, with a set collected simultaneously from each lumen of an existing CV catheter and from a peripheral vein. Without a CV catheter, 2 sets of blood culture should be obtained from different peripheral sites. The number of blood cultures has been described as correlated with the detectability of circulating pathogens, that is, only a single blood culture may cause misevaluation regarding underlying pathogens (Lee, *et al* 2007).

In the IDSA guideline, high-risk patients require initial antibiotic therapy that covers *Pseudomonas aeruginosa* and other serious gram-negative pathogens (Freifeld, *et al* 2011). Although the isolation of gram-positive organisms, such as coagulase-negative staphylococci, is more common than that of gram-negative pathogens, gram-negative bacteremias, especially those caused by *Pseudomonas aeruginosa*, are generally associated with greater mortality (Schimpff 1986). Thus, empirical monotherapy with an anti-pseudomonal β-lactam agent, such as cefepime, carbapenem (meropenem or imipenem-cilastatin), or piperacillin-tazobactam, is recommended and vancomycin should be considered only for clinically special indications, including suspected catheter-related infection, skin or soft tissue infection, pneumonia or hemodynamic instability (Freifeld, *et al* 2011). Coagulase-negative staphylococci, the most commonly identified microorganisms in septic patients with neutropenia, are clinically weak pathogens that rarely cause rapid deterioration; therefore, for many physicians, there is no urgent need to treat such infections with vancomycin at the time of a febrile event.

However, such a strategy as described above does not sufficiently satisfy appropriate treatment for fatal *B. cereus* sepsis, since *B. cereus* has an inherent ability to produce β lactamases (Hasbun, *et al* 1999, Zinner 1999). If neutropenic patients really suffer from *B. cereus* sepsis, it takes at least a few days to determine bacterial strains and, meanwhile, the patients' condition rapidly deteriorates. Although physicians should avoid the unnecessary administration of broad-spectrum antibiotics to prevent widely distributing resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococcus (VRE), extended-spectrum β lactamase (ESBL)-producing gram-negative bacteria, and *Klebsiella pneumonia* carbapenemase (KPC), therapeutic delays for *B. cereus* sepsis would result in a fatal outcome.

Therefore, as shown in Figure 2, at the first febrile event, we propose the prompt administration of both carbapenems and vancomycin for the following neutropenic AML patients with possible *B. cereus* sepsis, especially for patients with a neutrophil count of 0/mm^3 or below the lower limit of each institute, and CNS symptoms at febrile episodes. These 2 antibiotics are also desirable for febrile and neutropenic AML patients with CV catheter insertion or GI symptoms (Inoue, *et al* 2010). We consider that both agents are necessary as an initial management because of the presence of fulminant sepsis with *B. cereus* resistant to carbapenem (Kiyomizu, *et al* 2008). CV catheter removal is recommended if clinically possible. In patients with clinically and microbiologically documented infections other than *B. cereus*, appropriate agents should be started instead of carbapenems and vancomycin, and the duration of therapy depends on the species of pathogen and their infection site.

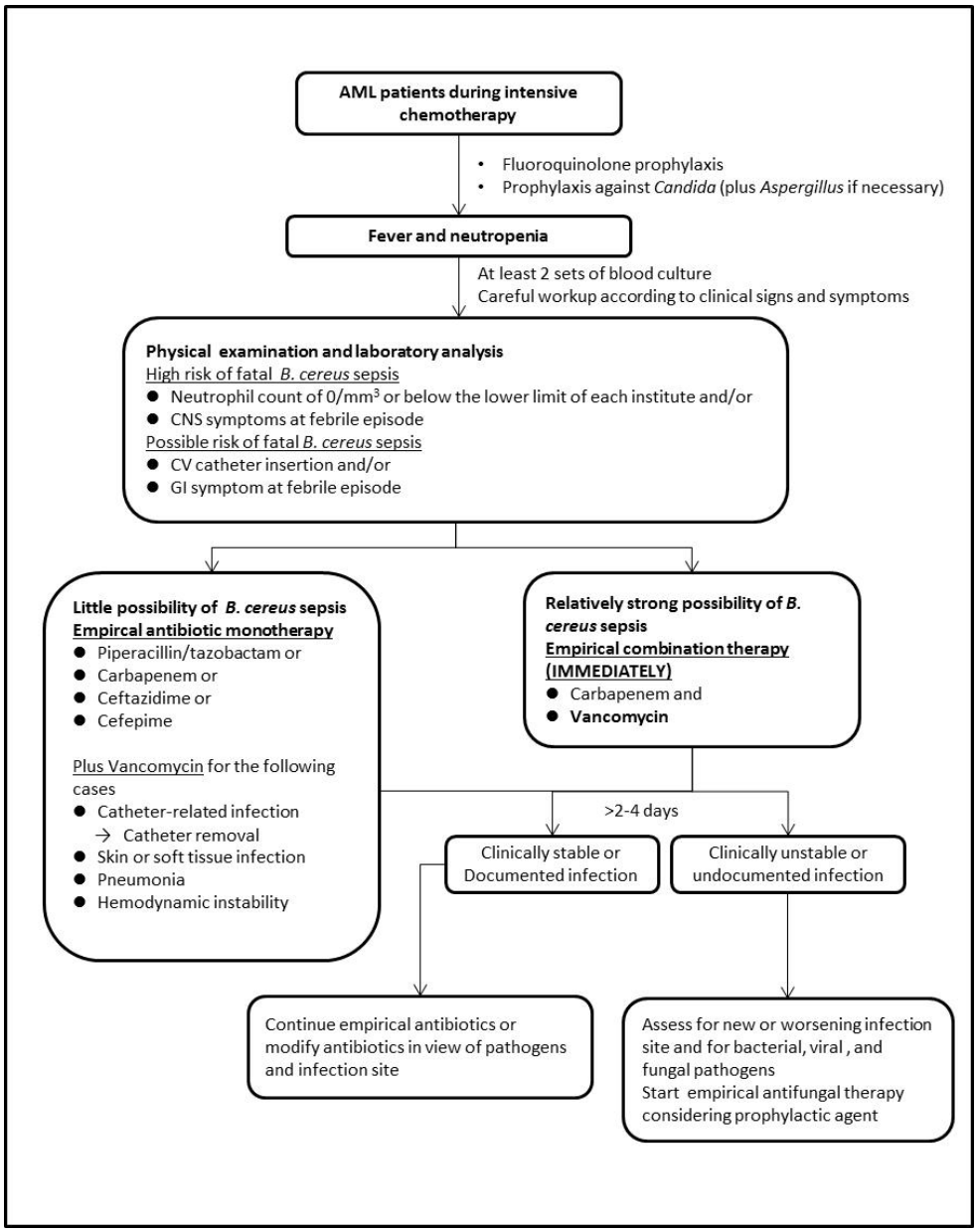

Fig. 2. Initial and urgent management for fever and severe neutropenia in AML patients in view of fatal *B. cereus* sepsis

The IDSA guideline recommends fluoroquinolone prophylaxis for high-risk patients with expected durations of prolonged and marked neutropenia (ANC≦100/mm^3 for >7 days) to reduce febrile events, documented infections, and infections involving the blood stream due to gram-positive or -negative bacteria (Bucaneve, *et al* 2005). Although fluoroquinolones, such as levofloxacin and ciprofloxacin, are usually efficacious against *B. cereus in vitro* and may prevent the rapid production of a large amount of bacterial toxins, there has been no report concerning the prophylactic efficacy of antibiotics against *B. cereus* sepsis (Bucaneve, *et al* 2005, Freifeld, *et al* 2011, Gafter-Gvili, *et al* 2005). The question of whether gut decontamination with oral fluoroquinolones can contribute to the reduction of *B. cereus*-related mortality remains to be addressed.

Fungal infections are encountered after the first week of prolonged neutropenia and empirical antibiotic therapy in the early phase of neutropenia, so that empirical antifungal therapy and investigation for invasive fungal infections should be considered for patients with persistent or recurrent fever after 2-4 days of antibiotics, including cases receiving prophylactic agents against *Candida* infections or invasive *Aspergillus* infection (Freifeld, *et al* 2011). Also, physicians should recurrently monitor possible fungal infection using the β-(1-3)-D glucan test, the galactomannan test, and high-resolution CT, leading to pre-emptive therapy if necessary.

2.3.4 What kind of environmental precautions should be taken?

It is reasonable to assume that *B. cereus*, which forms spores and is heat-resistant, in the environment or food passes through the GI tract or a CV catheter and enters into the circulation based on the results and information from our cases and previously reported patients (Banerjee, *et al* 1988, Terranova and Blake 1978). Especially, GI symptoms were present prior to the development of *B. cereus* sepsis in 8 cases, while no organism was grown from the tip of a CV catheter in any case (patients 2, 4, and 6) (Table 1). We regarded bananas, strawberries, and fried noodles as possibly causative foods in patients 1, 2, and 6, respectively. In these patients, the impairment of mucosal barriers due to intensive chemotherapy may have been an important factor; therefore, clinicians should pay strict attention to the foods consumed by such patients and prepared luncheon meats should be avoided, although Gardner et al. reported that avoidance of raw fruits and vegetables did not prevent major infection that led to death among AML patients in a randomized trial where cooked and noncooked food diets were compared (Gardner, *et al* 2008).

In previous reports, the inadequate sterilization of respiratory circuits (Bryce, *et al* 1993) and bacterial contamination of hospital linen (Barrie, *et al* 1994, Dohmae, *et al* 2008) were also considered to be major sources of nosocomial infection. *B. cereus* sepsis in patients 2 and 3 occurred in the same room and the same period (May, 2007). These facts prompted us to compare each *B. cereus* strain cultured from the blood samples of the 2 patients with *B. cereus* detected from hand towels, pajamas, a shared sink, and so on. However, each train proved distinct from the other strains detected, suggesting little possibility of nosocomial infection. Although *B. cereus* is widely distributed in the environment, the bacterial burden should be minimized because the threshold of the burden might determine the frequency of *B. cereus* sepsis. From this point of view, the regular surveillance of *B. cereus* strains in the environment may also be important.

3. Conclusion

We encountered fatal *B. cereus* sepsis in patients with acute leukemia, in whom apparently appropriate antibiotics were not effective, while we also encountered reversible cases. This report has provided risk factors for a fatal prognosis in combination with previous data. It may be highly instructive for clinicians treating leukemia patients with several prognostic factors identified in this study for *B. cereus* sepsis with special relevance to patients with acute leukemia, and we strongly recommend the immediate initiation of treatment with carbapenems and vancomycin in such situations. Similar studies with a larger cohort are necessary to establish successful therapeutic interventions.

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