How and why minimal residual disease studies are necessary in leukemia: a review from WP10 and WP12 of the European LeukaemiaNet

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ABSTRACT

Resistance to therapeutic agents is a major factor in the failure of cancer treatments. In leukemia, the resistant cells remaining in the bone marrow and/or peripheral blood constitute minimal residual disease and are detectable by highly sensitive assays when the patient appears to be in complete remission. Early detection of the expansion of residual cells permits clinical intervention with the aim of reversing the proliferation of resistant leukemic cells. Therefore, accurate and precise measurement of minimal residual disease can greatly enhance optimization of oncology patients' clinical management. This notion is supported by a large body of data among chronic myeloid leukemia patients, but minimal residual disease detection and monitoring is increasingly applied to other types of leukemia, and is starting to be a factor in decision-making for some therapeutic trials in childhood acute lymphoblastic leukemia. Here, from the solid ground of minimal residual disease detection in chronic myeloid leukemia, the current state of the art and development of molecular techniques in other leukemias and the growing field of multiparameter flow cytometry are reviewed in two separate parts reporting on the respective advances, advantages and pitfalls of these emerging methods.

Key words: minimal residual disease, leukemia, flow cytometry, standardization, monitoring.

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Introduction

Rapid progress in understanding the etiology of hematologic malignancies and technological advances have in recent years increased the specificity and sensitivity of detection of malignant cells in patients who appeared to be *cured or in remission* by conventional techniques.[1,2] Therefore patients' therapeutic response can now be assessed by monitoring minimal residual disease (MRD) i.e. detection of malignant cells at $\geq 1\times10^{-4}$ sensitivity, at sub-clinical levels.

However, MRD studies with sensitivities of 1×10^{-4} or higher have brought new challenges in differentiating malignant from normal cells and consequently, definition and clinical significance of remission and early relapse have become ambiguous. Thus, assigning prognostic value to MRD levels requires defined thresholds in selecting optimal therapeutic agent dose and the timing of hematopoietic stem cell transplantation (HSCT) or alternative drug; while conversely, treatment reduction could be considered in those predicted to have a favorable prognosis and thus minimize exposure to toxic agents.

Post induction therapy, one million or more leukemic cells may persist,[2] even when the residual cells are undetectable, i.e. the patient appears to be in complete molecular remission (CMR). CMR is defined as failure to detect cancer cells by the most sensitive molecular methodology available, with acceptable control gene transcript numbers, e.g 10,000 *ABL1* transcripts.[3] Definition of CMR could be further refined by being valid only when leukemic cells are undetectable in three sequential samples one month apart, in addition to the prerequisite of an adequate number of control gene transcripts. In addition, an internationally recognized reference material enabling inter-laboratory comparison and an accurate assessment of the level of sensitivity achievable by a myriad of methodologies applied would strengthen definition of CMR. It is also generally accepted that informative MRD studies are

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best achieved when the peripheral blood leukocyte count is within normal limits[4] with an adequate number of cells to achieve sensitivity of up to 1×10^{-4} to 1×10^{-5}. For patients who had undergone HSCT, Mughal *et al.* defined molecular relapse (MR) as either three sequential samples, tested one month apart, with a *BCR-ABL1/ABL* ratio of 0.02% or showing clearly rising levels with the last two higher than 0.02%, or two results over a minimum period of four weeks higher than 0.05%.[5] Thereby they were able to classify patients according to risk of progression. More generally, a confirmed one log increase in *BCR-ABL1/ABL* ratio or three consecutive increases is clinically significant. Timing and frequency of MRD is largely dependent upon clinical data and the aggressiveness of the leukemic clone, which is likely to vary between patients and diseases. A directive to perform regular close monitoring of peripheral and/or bone marrow at set time points may diminish the ambiguity and permit better inter-laboratory data comparison.

Tumor load, type of leukemia, whether disease specific marker is identifiable and technological limits will determine the optimum methodology for monitoring MRD. Whilst molecular monitoring targets disease-specific transcription of chimeric mRNA (e.g *AML1-ETO*) or utilizes somatic mutations, e.g. Nucleophosmin (*NPM1*) and/or B and T-cell clonal gene rearrangement, FCM detects the expression patterns of cell differentiation (CD) antigens thereby distinguishing leukemic cells from normal cells. Here we review the application of molecular and FCM methodologies, which are now also indispensible tools for diagnosis and monitoring chronic and acute leukemia.

Molecular studies

Polymerase chain reaction (PCR) has been eloquently exploited to detect and measure DNA sequences of interest. More recently, mRNA studies using reverse transcription PCR (RT-PCR) have become widespread. This approach brings junctional breakpoints separated by introns and exons into close proximity thereby enabling detection of different transcripts encoded by the same chromosomal translocation in a single multiplex PCR.[6] Furthermore, RT-PCR detects RNA from viable cells and thus targets genes expressed that are likely to have functional role, directly or indirectly, in cellular proliferation.

Quantification of specific sequences of DNA has been greatly simplified by real time quantitative polymerase chain reaction (RQ-PCR),[7] hereafter referred to as Q-PCR. In Q-PCR the rate of accumulation of amplicons is proportional to the number of target transcripts in the starting material during the exponential phase of the PCR. This technique also offers increased specificity with the inclusion of the third reporter labeled oligonucleotide probe using hydrolysis based technology, which anneals between forward and reverse primers.[1] Hydrolysis is one of many methods now available for detection and quantification of target sequences.[8]

A sensitivity of 1×10^{-5} is achievable by Q-PCR but contamination is a major concern and hence strict working practices must be adhered to, e.g RNA extraction, cDNA synthesis and post PCR analysis must be geographically separated. Equally, false negatives due to a lack of mRNA or sub-optimum integrity of mRNA and/or cDNA must be controlled for. This is achieved by concomitantly measuring one of the ubiquitously expressed housekeeping genes, such as *ABL1*, *BCR*, β_2-microglobulin, β-glucuronidase (*GUSB*) or glucose-6-phosphate dehydrogenase (*G6PD*).[9-12]

Gene rearrangement studies

The immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements during normal B and T-lymphocyte development, respectively, generate unique fusions of variable, diversity and joining (VDJ) segments, interspersed by random nucleotide (N) insertion and/or deletion (Figure 1).[13,14] These B and T-clonal recombinations generate patient-specific DNA length and sequences which represent ideal molecular markers for detection and quantification of leukemic cells among normal lymphocytes in remission samples. Whilst sensitive, the technology is susceptible to false negatives due to clonal evolution during natural history of the disease, thus some patients may relapse with a clone different to that observed at presentation. Furthermore, the sensitivity may be diminished through quenching by normal polyclonal B cells.[15] The risk of false negatives can be diminished by targeting two Ig/TCR gene rearrangements when conducting MRD-PCR studies.

Consensus primer PCR and allele specific oligonucleotide PCR (ASO-PCR) are the two immunoglobulin (Ig) PCR strategies for MRD studies (Figure 1). The principle of the former is to amplify the third complementary-determining region (CDRIII) of the *Ig* gene, using a standard set of universal primers, a primer that recognizes a consensus sequence in the JH region and a primer for family specific framework regions (Figure 1). This qualitative method has sensitivity of 1×10^{-2} to 1×10^{-4}. ASO-PCR utilizes primers designed to anneal to a unique patient specific Ig sequence and subsequently is used to monitor sequential samples in follow-up studies. This method overcomes the difficulty associated with the presence of normal polyclonal B cells and significantly improves the sensitivity of MRD studies. However, ASO-PCR is time consuming and expensive, despite the improvements following the introduction of *Genescan* which eliminates the need for polyacrylamide gels (Figure 1).[15-18] Combination of ASO primers and consensus oligonucleotide probes make it accessible to Q-PCR, permitting precise quantification of MRD with a sensitivity of 1×10^{-4} to 1×10^{-5}.

PCR-based studies have proved to be more sensitive than FCM, with around 10% of the cases having detectable clonal rearrangement in patients with disease below the FCM detection limit.[19,20] Screening at intervals by PCR, with closer monitoring by ASO-PCR and FCM in parallel would reduce the risk of false negative data. Despite the complexity with improved inter-laboratory standardization the Ig-TCR studies[15-18,21] are increasingly being seen as the gold standard for ALL MRD studies.

Standardization

Biggs and Denson in 1967, concerned with coumarin therapy, observed that *a single scale properly applied at dif-*

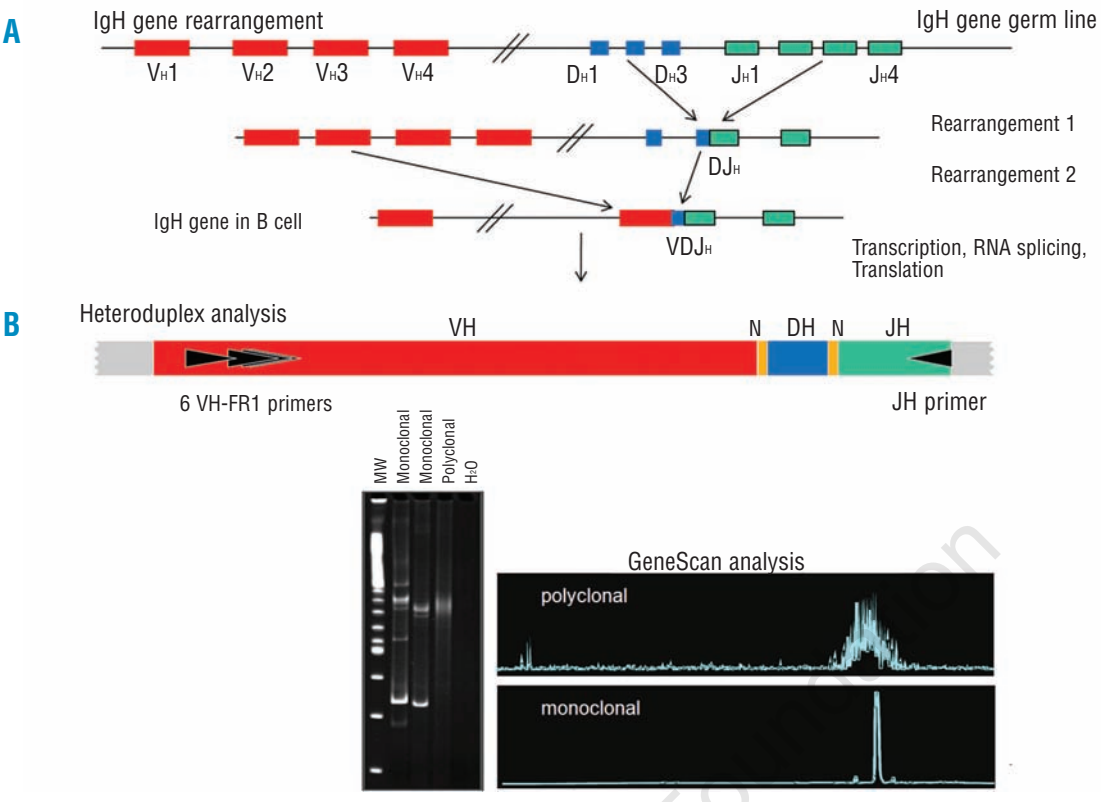


Figure 1. IgH rearrangement and heteroduplex clonality studies by polymerase chain reaction (PCR). (A) A schematic representation of the IgH gene rearrangement is shown. Double strand DNA breaks are made to enable the V, D and J heavy immunoglobulin genes rearrangement in cells that are destined to become B lymphocytes and define these cells from others. Clonal population is detected when a group of cells with a unique VDJ spliced sequence expands such that it is detectable above the normal background of B-cell populations that have undergone rearrangement. (B) Heteroduplex analysis. (i) Schematic representation of the IgH rearranged gene (VH-DH-JH) is shown along with position of VH–family specific and JH consensus primers. The yellow lines represent the position of insertion and/or deletion of nucleotides at junctional regions of IgH. (ii) Ethdium stained acrylamide gel with results of a heteroduplex analysis is illustrated. In brief, the junctional region heterogeneity of PCR products of rearranged IgH or TCR genes is exploited to distinguish between monoclonal and polyclonal lymphoid B or T expansion. In heteroduplex studies PCR amplicons are heat denatured and rapidly cooled to induce homo- or heteroduplex. Samples with clonal lymphoid cells the PCR products of rearranged IgH or TCR genes yield homoduplex. In contrast samples with polyclonal lymphoid expansion the single strand PCR fragments leads to formation of heteroduplexes upon re-annealing. In samples with polyclonal and monoclonal expansion, both homoduplex and heteroduplex arise. Thus, because of the conformation differences the homo and heteroduplexes forms can be separated from each other by gel electrophoresis through non-denaturing acrylamide gels as shown. Homoduplexes migrate through the gel faster than the heteroduplexes with imperfect complimentary pairing. The latter form a background smear of slow migrating fragments. The homoduplex yields a relatively sharp discrete amplicon band. (iii) Automated clonal studies (florescent Genescan analysis). Polyclonal VH-DH-JH products form peaks reflecting a Gaussian distribution of average junctional region sizes in normal B lymphocytes. Monoclonal VH-DH-JH gene rearrangements form a discrete fluorescence peak, representing products of identical size. Adapted with permission from Van Dongen *et al. Leukemia 2003;17:2257-17*.

ferent centers would ensure safety and uniform dosage for a patient moving from one place to another and would greatly improve the standard of clinical trials carried out at more than one center.[22] As a consequence, the International Normalised Ratio (INR) evolved. Similarly, in an era of multi-center clinical trials and wide accessibility of MRD studies by Q-PCR the need for standardization of MRD studies is essential. However, standardization of RNA based studies has proved to be complex because mRNA is labile and cDNA synthesis adds to the logistical difficulties in establishing inter-laboratory quality assurance. Such difficulties are negligible with the highly stable genomic DNA, as the latter can be made available easily and the quantity included in MRD assays can be measured with much greater accuracy than RNA. Hence, considerable progress has been made in standardization of Ig and TCR rearrangement studies.[15-19,21] Furthermore, since the amount of DNA per cell can be calculated, reporting in terms of number of malignant cells is feasible.

Because of the difficulties associated with standardization of RNA studies, it is described here in greater detail, illustrated by *BCR-ABL1*. A meeting convened in 2002 in Bethesda, Washington, made some key recommendations when monitoring CML patients in the era of tyrosine kinase inhibitors[9] and proposed an International Scale (IS) for *BCR-ABL1* measurement based on the use of *ABL1*, *BCR* or *GUSB* control genes.[9,23] This meeting, the Europe Against Cancer (EAC) consortium, as well as other investigators, highlighted the need for protocol standardization given inter-laboratory diversity of methodologies from collection of sample to final report.[9,24-25] Despite achieving a consensus with key factors, e.g. use of random hexam-

ers, processing of samples within 24 h, the lack of World Health Organisation (WHO) recognized primary reference material make inter-laboratory comparisons and accurate assessment of achievable sensitivity difficult. Thus, an urgent need for a WHO recognized External Quality Assurance (EQA) standard for the MRD targets soon became apparent. The establishment of EQA requires a suitable material that would enable all steps involved in MRD analysis to be monitored and one that is stable over a long period.[26,27] Furthermore, it must be non-toxic, non-infectious and readily available. A number of investigators have suggested biological materials that might be suitable for an EQA scheme such as lyophilized cell lines that express the target genes or synthesis of protected RNA that can be reverse transcribed once it is heated to the appropriate temperature.[19,20] Whilst these products mimic the starting material and therefore test the major aspects of MRD analysis, their long-term stability and suitability remain to be determined. The use of armored RNA as a reference material has enormous potential. In the absence of formally recognized WHO standard, consensus is emerging to use formulae applied in the landmark CML clinical phase II multi-center trial *International Randomised study of Interferon and STI571* (IRIS) molecular report.[23] Briefly, the international *BCR-ABL1* scale (corresponding to the IRIS baseline) baseline is set at 100%, and major molecular response (MMR), is defined as 3-log reduction, i.e. 0.1%. By exchanging samples with a reference laboratory conversion factor (CF) the International Standardised Ratio (ISR) is calculated per formulae shown below.[28] The baseline value must be defined by individual laboratories and this can vary between the centers. The ISR would probably benefit from the adoption of a recommended reference method and reagents, thereby further enhancing inter-laboratory comparisons.

Formulae for calculating the IS *BCR-ABL1* ratio.[28]
BCR-ABL1:100
BCR-ABL1 MMR = 0.1
CF=MMR-IS ÷MMR-LL
Then, LR = CF X Y
Where:
MMR:Major molecular response
IS:International standard
LL:Local lab BCR-ABL1 MMR
CF:Conversion factor
LR:Local international standardised BCR-ABL1 % ratio
Y:locally *BCR-ABL1* % ratio for a given patient

In the following section the reported MRD findings for different types of leukemia are summarized.

Chronic myeloid leukemia

The clinical utility of MRD studies was proven in CML patients who had undergone HSCT[3,29,30] and has been shown to be equally useful for tyrosine kinase inhibitors (TKI) treated patients.[23,31-33] Indeed Q-PCR has largely replaced cytogenetic or FISH for closely monitoring CML patients' response, particularly as a vast majority of patients achieve CCyR with TKI. Q-PCR also helps detect emerging TKI resistant clone much earlier than other methodologies.

Patients who do not achieve 1-log reduction at three months are said to have a reduced probability of achieving CCyR and/or MMR.[31-35] More precisely, those who achieve less than 1-log reduction three months post therapy have a 13% chance of ever achieving MMR compared with >70% patients who have a deeper depletion at three months.[31-36] Although long-term longitudinal studies are needed for confirmation, the published reports support the notion that early and close monitoring of patients assists in classifying patients likely to achieve MMR and permits the early detection of a resistant clone. A 2-fold increase in tumor load is reported to be suggestive of *BCR-ABL1* kinase (KD) mutation, diminishing TKI efficacy.[37] In addition, findings suggest that detection of KD mutation in patients who are in CCyR is associated with a significantly increased risk of cytogenetic relapse.[38] By inference, together these observations suggest patients who experience a 2-fold increase in *BCR-ABL1* transcripts have significantly increased risk of cytogenetic relapse. Furthermore, recent analysis implies patients in whom *BCR-ABL1/ABL1* ratio is ≥0.05% have a statistically significant risk of loss of CCyR and progression free survival.[39]

Acute myeloid leukemia

Molecular monitoring by Q-PCR in AML is largely limited to fusion genes resulting from chromosomal aberrations, and exemplified by t(15;17),[40] t(8;21)[41] and inv(16),[42] quantification of somatic mutations using mutation specific e.g *NPM1*[43] and aberrantly expressed genes e.g. ecotropic virus integration-1 (*EVI1*).[44] The accumulating AML MRD data support the notion that such studies are an essential tool for relapse risk stratification of patients during treatment.[45-47] A study among 70 APL patients showed that MRD levels after first consolidation therapy was a powerful predictor of relapse.[48] Patients with residual disease $\geq 1\times10^{-3}$ had a 10-fold increase of relapse at five years compared with those who had $<1\times10^{-3}$ (p=0.001).[48]

Rearrangements involving the core-binding factor *AML1* and *CBFβ* resulting from t(8;21) and inv(16) are considered to be associated with good prognosis, and account for 15-20% of adult and pediatric AML cases. However, monitoring MRD in *AML1-ETO* and *CBFβ-MYH1* patients is less than straightforward, as qualitative RT-PCR is often positive even when all other indicators are consistent with the patient being in long-term remission. This might be due to expression of *AML1-ETO* in non-leukemic stem cells, monocytes, and B cells in remission marrow, and/or in a fraction of B cells in leukemic marrow.[49] These authors conclude that chimeric fusion gene is acquired in the hematopoietic stem cell and it is the acquisition of additional genetic lesions that lead to transformation of the affected stem cell.[49] This implies the additional lesions arise downstream during differentiation or that *AML1-ETO* is only functional in more mature cells. Alternatively the persistence of *AML1-ETO* in long-term remission patients may reflect level of sensitivity and/or the number of cells that can be detected by RT-PCR. However, in either case, these observations highlight the need for quantification to assess the kinetics of the leukemic clone.[50,51] More generally, reports suggest that a <1.0% ($<1\times10^{-3}$)

MRD post-induction therapy correlates with good outcome.[52-56]

In the absence of disease-specific target molecular markers, the tumor suppressor gene expression levels, e.g *WT-1*, have been reported to be useful. *WT-1* expression, normally highly regulated, is reported to be overexpressed in approximately 80% of AML patients and is therefore considered to be a specific feature of AML.[57,58] There is evidence that all patients with higher levels of *WT-1* in peripheral blood post induction therapy subsequently relapsed, with a median of 12 months after diagnosis. But, the notion that expression level at diagnosis is prognostic could not be confirmed.[59] Furthermore, the significance of *normalized WT-1* expression levels post induction therapy was less clear, as 21 of 48 these patients relapsed. These findings are supported by Ommen *et al*.[60] The available studies indicate that *WT-1* levels above normal levels, which may be seen in normal regenerating marrow, are associated with subsequent relapse. These data are supported by finding them to correlate with disease-specific fusion gene transcript numbers.[57,58] Similarly, the overexpression of *EVI1*, mapping to 3q26, has been described in 8.0-20.0% of AML cases. The increased *EVI1* expression is reported to correlate with worse prognosis and is therefore a useful marker for evaluation at diagnosis and follow-up studies.[61-63] Furthermore, some AML patients express ME (*ME*$^+$) resulting from intragenic fusion between *MDS1* and *EVI1*, the former is 140 kb upstream of *EVI1*. Interestingly, patients who express *EVI1* but are ME negative are reported to have poorer treatment response.[44] Similarly, the preferentially expressed antigen of melanoma (PRAME) is overexpressed in 30-40% of cases and has been suggested as a possible marker too.[63] PCR has also been applied in AML patients to detect mutations reported to have prognostic value, e.g. *NPM1*[43] and *FLT3*.[64] An internal tandem duplication (ITD), that adds 5-100 base pairs to the juxtamembrane domain, is the most frequently observed *FLT3* mutation.[64-66] The presence of FLT3-ITD at diagnosis in AML is reported to be associated with a 8.5-fold higher frequency of MRD cells after the first course of chemotherapy compared to those with wild-type FLT3.[67] This correlates with overall survival (OS), relapse free survival (RFS) and disease free survival (DFS), and if confirmed early evaluation of FLT3 inhibitor efficacy would be feasible. However, although these mutations are relatively common in normal karyoptype AML, their potential as MRD markers is unclear due to the need to design patient specific assays and mutant alleles instability.[68-70]

Exon 12 *NPM1* mutations that displace the protein to the cytoplasm represent the most common genetic lesions observed in AML patients with normal karyotype. Three of the mutations account for 90% of all mutated cases.[71] Reported data imply that unlike *FLT3*, *NPM1* mutant alleles are stable and therefore a reliable MRD marker with prognostic value.[72-75] Moreover, sensitivities of $\geq 10^{-5}$ have been reported to be achievable by targeting *NPM1* mutations.[71-74] The CCAAT/enhancer binding protein α (*CEBPA*) mutations, observed in approximately 10% of AML patients associated with good prognosis, have also been proposed as markers to monitor AML.[75] In a study which included 149 patients' samples analyzed at diagnosis and relapse, the *CEBPA* mutations were found to be stable, raising the possibility of patient specific Q-PCR MRD studies.[75] Recently authors described the utility of *CEBPA*, *NPMI* and *FLT3* mutations at diagnosis in cytogentically normal AML patients under the age of 60 to define those who might benefit from HSCT.[76] Furthermore, FLT-3 mutation as predictor of relapse may be modified by NPM1.

Acute lymphoblastic leukemia

Philadelphia chromosome positive ALL patients' leukemic clone kinetics can be monitored accurately and precisely by Q-PCR using *BCR-ABL1* as the target.[77,78] By these studies 42 patients could be classified into two groups as follows: good molecular responders with >2-log and >3-log reduction after induction and consolidation therapy, respectively, and poor molecular responders, with higher MRD levels at both time points. The two year OS was determined to be 48% and 0% for good and bad molecular responders (p=0.0026), respectively.[78] The studies among Ph (+) ALL patients suggest that those expressing e1a2 *BCR-ABL1* transcripts, representing approximately 70% of Ph(+) ALL, have a higher risk of relapse.[79-81] The risk is estimated to be 8.7 compared to 2.2 for those expressing e13a2 and/or e14a2.[81] The relative risk of relapse is estimated to be 4.4 among those patients who have detectable MRD compared with those in whom the *BCR-ABL1* transcripts are undetectable at 4-6 months post transplant.[81] Available data imply that MRD levels are higher in the bone marrow than peripheral blood; therefore BM samples should be tested at regular intervals.[82] However, as PB based MRD studies are relatively non-invasive, patients are much more likely to acquiesce to them and therefore permit closer monitoring. This may offset the 1-log greater sensitivity achieved with BM samples. Thus, PB based analysis could be used as an indicator for timing of BM samples and confirmation of any minor changes. Furthermore, lymphocyte enrichment is essential to maximize sensitivity when analyzing PB samples from ALL patients.

Early clearance of leukemic cells is a favorable prognostic indicator in childhood ALL, whereas high levels of MRD at the end of induction therapy appear to be associated with a high risk of relapse.[83-86] Available data imply that low molecular MRD after induction is a good prognostic factor in pediatric ALL, independent of other clinically relevant risk factors, such as age, blast count, immunophenotype, chromosomal aberrations at diagnosis and response to prednisone. Furthermore, investigators report that patients who relapse after remission and are again subjected to re-induction therapy have event free survival rates of 86% and 0% among those determined to have MRD levels of $<1\times10^{-3}$ and $\geq1\times10^{-3}$ by RQ-PCR (p<0.0001), respectively.[87] The adult MRD ALL data is relatively limited but as in children, early depletion of tumor load after induction is prognostic of response to chemotherapy. A rapid decline in MRD levels, down to undetectable or $<1\times10^{-4}$ on days 11 and 24, during and after induction therapy, respectively, has

been reported to be associated with low risk with three year DFS and OS of 100%. Conversely, patients with a decline of 1×10^{-4} reached at week 16 were at high risk, with a 3-year DFS and 3-year OS at 45.1%.[88] In contrast Mortuza *et al.*, reported that MRD positivity was associated with increased risk of relapse which is more pronounced at three and five months post induction.[89] Significantly, the deletion of the *IKZF1* gene, that encodes IKAROS Krupple family zinc finger transcription factor in ALL, is associated with poor prognosis independent of age, sex, cytogenetic findings, leukocyte count at diagnosis and MRD data.[90]

Chronic lymphocytic leukemia

Although patient management decisions are largely based upon clinical data, Rai[91] and Binet[92] staging has in recent years been superseded in stratification of CLL patients at diagnosis into good and bad prognosis by a myriad of markers.[93] The most widely used molecular marker, the immunoglobulin variable region heavy-chain gene mutational status segregates CLL according to aggressiveness of the disease.[94,95] The complexity of this assay led to the description of alternative surrogate markers. The most commonly assessed include ζ-chain associated protein kinase (*ZAP-70*),[96] CD38[97] and the presence of chromosomal aberrations (e.g. 11q and 17p and deletions)[88,98] dysfunctional and/or mutated p53.[99]

Wierda *et al*. reported achieving 1×10^{-5} sensitivity when evaluating a chemotherapy regimen with fludarabine, cyclophosamide and rituximab (FCR) for CLL using patient specific PCR based assay.[100] These investigators showed 21% of the patients assessed had undetectable MRD, with median time of progression of 44 months compared with 27 months with detectable CLL cells.[100]

The European Research Initiative in CLL (ERIC) concluded that a sensitivity of 1×10^{-4} was achievable either by ASO-PCR or FCM[101] and defined MRD negativity as less than a single CLL cell in 10,000 leukocytes.[102] However, it is unclear if MRD negativity is to be based on a single sample analysis or sequential samples. Given that ASO-PCR is theoretically 1-log more sensitive, it might be preferable to report MRD negativity as defined by ERIC, as undetectable as there may be up to a million or more CLL cells in samples which are reported to be *negative* by ASO-PCR. Significantly, it is suggested that PB samples are acceptable for monitoring most therapeutic agents, except perhaps alemtuzumab or rituximab, where bone marrow aspirate samples could be preferred, as antibody clears PB faster than BM.[102]

MRD data support the notion that CLL patients with undetectable disease post HSCT or immunotherapy have a greatly improved overall survival. These observations are consistent with early identification of patients at risk of relapse by monitoring MRD. In CLL patients treated with anti-CD52 antibody based therapy (CAMPATH, alemtuzumab) or autologous transplant, positive MRD studies, FCM- or PCR-based, are highly indicative of subsequent relapse. Rawstron *et al.*, reported that for 19 of 25 cases who achieved CR when treated with CAMPATH-1H antibody or autologous transplant with undetectable MRD, the EFS was >90%,[103] whilst those with detectable MRD at time of CR subsequently relapsed.[103] Similarly, Esteve *et al.*,[104] reported that 4 of 5 (80%) patients with detectable MRD, while in CR following autologous transplant, eventually relapsed compared with 2 of 9 (22%) with undetectable MRD.[104] Whilst these studies support the emerging consensus of the critical need for MRD studies in CLL, they require confirmation given the small sample numbers.

Flow cytometry

The notion that minimal residual disease (MRD) could be monitored by using the increasingly versatile and specific capacities of flow cytometry (FCM) emerged as early as the late 80s, following studies of normal mouse bone marrow.[105]

FCM has evolved gradually over the last 20 years, with advances in technology and software making it increasingly accessible for MRD detection, such that it is now becoming an essential tool for monitoring malignant clones.[106] By contrast with molecular studies, it is of interest that FCM will explore viable cells. It is likely that this approach will develop increasingly in therapeutic trials, first for appraisal of the feasibility and informativity of the method, later probably as a therapeutic decision-indicator, as is already the case for molecular MRD in childhood ALL.[107,108]

This part of the review will first depict general considerations about the use of FCM in MRD detection, while recalling the major steps of the development of this technique, which sustain the rationale for its broader application.

Immunophenotypic patterns

From a strictly technological point of view, the construction of monoclonal antibody (Moab) combination panels applicable for the detection of MRD should consider four critical issues:

- lineage and maturation related molecular associations, in order to identify the coexpression of antigens normally mutually exclusive;
- cross-lineage expression, which may occur as part of the abnormal characteristics of the leukemic clone;
- differentiation antigens expression intensity, which has to be known from normal expression, in order to best choose the appropriate fluorochromes and avoid possible steric hindrance and quenching for antigens in close vicinity on the cell membrane.

These panels, may also behave differently when applied to normal versus leukemic cells and should be versatile enough to take these features into account.

Indeed, the challenge in FMC detection of MRD is to separate residual leukemic cells from non-malignant cells, including at stages when regenerating BM may contain more early maturation forms than normal samples at a steady stage of hematopoiesis. This presupposes both excellent immunophenotypic knowledge of the malignant clone and of normal bone marrow.

Leukemic cells can be similar to normal cells blocked in their differentiation and are recognized as such in FCM diagnosis by increased numbers compared to the scarcity of normal cells expressing the same immunophenotype. But, on closer examination, leukemic cells

also frequently express abnormal maturation patterns. These immunophenotypic alterations have been referred to as leukemia associated immunophenotypic patterns (LAIP).[109] They can be classified in four types: i) cross lineage or aberrant expression, ii) asynchronous expression, iii) overexpression and iv) lack of expression.[109] The immunophenotypic diagnosis of leukemias must therefore be meticulous, in order to identify such patterns, which may subsequently be used to track the residual leukemic cells. The earliest contributions to tracking abnormal patterns in flow cytometry can be traced back to the early 1990s.[110] Earlier attempts[111] had used cytospin smears to identify the combined expression of cytoplasmic CD3 and terminal deoxynucleotidic transferase (TdT) in ALL. In 1990, Campana *et al*.[112] reported on dilution experiments of AML cells coexpressing CD33 and CD7 in normal bone marrow, demonstrating the absence of such cells in normal BM and their retrieval in two-color FCM down to 1×10^{-3}. Gross *et al*. in 1993,[113] using dilutions of the cell line REH with 250 million of normal PBMC showed that such mixtures allowed retrieval of *abnormal* cells with a sensitivity of 1×10^{-6}. This work also usefully defined the notion of *empty spaces* not occupied by normally maturing cells. This notion of *empty spaces*, however, is only significant following detailed studies of normal bone marrow with the same antibody combinations as designed for MRD detection, and the latter are clearly scarce and are not standardized in the literature.[114-116] Table 1 provides an example of the large variety of proposals made in the literature to detect MRD in FCM.

Targeting blast cells

In 1997, Jennings and Foon,[127] reporting on the diagnosis and follow-up of leukemia, stressed the need to use CD45 to discriminate cell subsets and most notably the immature/blastic cells with low side scatter (SSC) and low CD45 expression.[128,129] Using this approach in a first gating for the selection of MRD within maturing cells in the BM is becoming increasingly common (Figure 2A). In the same paper,[127] these authors also reviewed the description of aberrant immunopheno-

Table 1. Variety in panels for minimal residual disease detection in flow cytometry as reported in the literature[106,109,112,117-126] figures refer to CDs.

Campana*	Orfao*	Baer	Sievers	Kern 2003	Feller	Kern 2004	Langebrake	Babusikova	Perea
TdT/5/3	7/5/3/34	45/14/DR	11b/DR/45	11b/117/34	3/4/45/34	11b/117/34	15/34/DR	DR/13/45/33	15/34/DR
34/5/3	7/2/3/34	3/4/8	19/5/45	14/13/4	15/13/45/34	14/3/34	10/20/19	11b/38/45/34	10/20/19
19/34/10/58	7/4/8/34	15/34/56	36/38/45	15/34/33	2/56/45/34	15/34/33	2/33/19	11b/117/45/34	2/33/19
19/34/10/38	7/13/3/34	33/13/DR	13/16/45	34/56/33	5/7/45/34	34/7.1/33	22/13/3	33/34/45/7	22/13/3
19/34/10/45	7/33/3/34	16/32/64	34/15/45	34/13/19	11c/11b/45/34	34/116/33	7/117/45	4/56/45/34	7/117/45
19/34/10/TdT	TdT/5/3/7	7/13/2	33/14/45	34/15/33	65/117/45/34	34/13/19	66/56/64	2/56/45/34	66/56/64
19/34//10/22	10/20/19/34	11b/13/33	56/7/45	34/2/33	61/33/45/34	34/135/117	36/GPA/45	14/13/45/19	36/GPA/DR
19/34/10/13	13/13/19/34	38/34/DR		34/7.1/33	71/19/45/34	34/15/33	3/4/45		34/41/45
19/34/TdT/M	10/33/19/34	33/13/19		34/116/33	22/90/45/34	34/19/13	34/11b/45		34/11b/45
19/34/10/66c	TdT/10/19/45			34/135/117	42b/34//45/14	34/2/33	4/123/DR		4/123/DR
19/34/10/33	15/10/19/45			36/235/45	DR/20//45/34	34/56/33	14/135/45		14/135/45
19/34/10/65	10/7.2/19/20			38/133/34	34/133/45/38	36/235a/45	5/16/45		5/16/45
19/34/10/15	**34/33/DR/45**			38/34/90		38/133/34	MPO/79a/c3		MPO/79a/3
19/34/10/21	**34/117/33/45**			4/64/45		38/34/90	TdT/MPO/c3		TdT/MPO LZ LF/3
19/34/10/7.1	**DR/117/33/34**			65/87/34		4/64/45			
13/117/34/33	**15/13/33/34**			7/33/34		64/4/45			
15/117/34/33	**15/33/DR/34**			90/117/34		65/87/34			
13/133/34/33	**15/117/33/3**			DR/33/34		7/33/34			
13/56/34/33	**34/56/33/45**			MPO/LF/c15		90/117/34			
DR/117/34/33	**7/33/34/45**			TdT/33/45		DR/33/34			
11b/13/34/33	**2/33/DR/34**					MPO/LF/c15			
38/13/34/33	**19/13/33/34**					TdT/c33/c45			
15/13/34/33	**34/11b/33/34**					TdT/c22/c3			
7/13/34/33	**15/7.1/33/34**					TdT/c79/c3			
45/13/34/33									
19/13/34/33									
11b/117/34/33									
DR/13/34/33									
13/7.1/34/33									

**Lineage dependent panels, boldface for AML.*

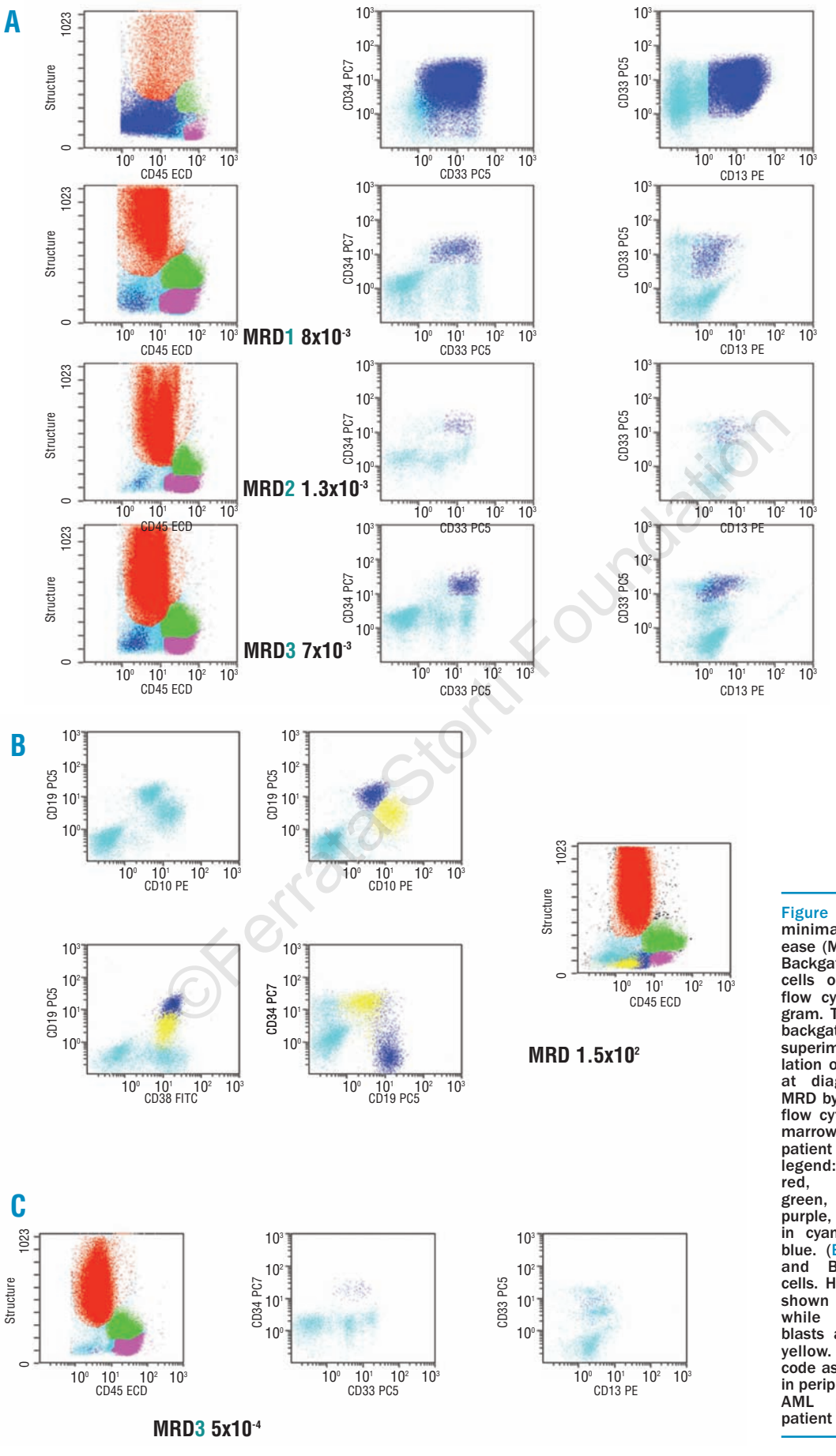


Figure 2. Monitoring minimal residual disease (MRD) in FCM. (A) Backgating of leukemic cells on a CD45/SSC flow cytometry scattergram. The procedure of backgating was used to superimpose the population of cells identified at diagnosis and as MRD by multiparameter flow cytometry in bone marrow samples of a patient with AML. Color legend:granulocytes in red, monocytes in green, lymphocytes in purple, immature cells in cyan, MRD in dark blue. (B) Hematogones and B-ALL leukemic cells. Hematogones are shown in dark blue while the remaining blasts are displayed in yellow. Other colour code as above. (C) MRD in peripheral blood of an AML patient. Same patient as in panel A.

types reported by a number of investigators, i.e. CD2 in t(15;17) AML or CD19 in CBF t(8;21). In 1999, Campana and Coustan Smith[117] estimated that the top four combinations for acute leukemia MRD detection in flow were (i) CD19/CD34/CD10; (ii) CD13/CD33/CD34, iii) CD13/CD33/CD117 and iv) CD13/CD34/CD117. It infers that these immunophenotypic features should be assessed at diagnosis, in order to retain information as to the specific characteristics of a given leukemic clone in terms of coexpression and fluorescence intensity of differentiation antigens. It should, however, be noted that in some cases, these features may be lost over time.[130-132] The subset of leukemic cells which persists will nevertheless retain a clonal quality, i.e. continue to present homogeneous features. These residual cells appear as a tight cluster of cells still with a *frozen* immunophenotype among maturing cells in FCM studies. Although no real consensus exists, it is commonly admitted that a cluster of between 10 and 100 MRD cells should be identified in a given sample to ensure that MRD cells have been seen.[132] Thus, to achieve a sensitivity of between 1×10^{-4} to 1×10^{-5}, approximately 10^5 to 10^6 leukocytes must be screened, stressing again the value of assessing MRD in samples with normalized cell counts.[4]

Data expression

Although not necessary, Ficoll enrichment in earlier studies was established as a standard procedure for samples from ALL patients. ALL MRD is thus often expressed as a proportion of mononuclear cells[132] rather than as a proportion of leukocytes, as recommended for AML. More significantly, density gradient centrifugation may lead to loss of the MRD cells in AML studies. Therefore, in AML MRD studies, total cellular analysis following red cell lysis, especially in no-wash procedures, will provide the most clinically relevant picture. For these reasons this strategy is increasingly being adopted for ALL patients.

Bone marrow or peripheral blood?

For acute leukemia, there is usually 1-log difference between BM and PB analysis, with the highest MRD in BM.[118,133,134] Thus, as with molecular based studies, PB could be exploited to establish MRD monitoring schedules with more frequent FCM investigations, with BM sampling being restricted to cases where MRD is repeatedly undetectable in PB samples.

Acute lymphoblastic leukemia

MRD detection in ALL, and especially childhood ALL, has been extensively explored with molecular tools derived from the specific rearrangements of the IgH or TCR, even leading to a new definition of remission.[135] Concomitantly, FCM studies also developed, albeit with very heterogeneous panels, illustrating the broad potential of FCM in identifying ALL residual cells. The absence of consensus, even with multiparametric FCM, has probably delayed full recognition of this method as a valuable decision-making tool, while molecular detection of MRD was more readily applied.

The first series using flow cytometry appeared in the early 90s.[110-112] Drach in 1991[136] developed a two-color indirect fluorescence FCM assay combining surface staining and intranuclear labeling of TdT, reporting a sensitivity of 2×10^{-3}. The same year, Imamura and Kuramoto[137] reported on CD10/TdT staining in FCM in a small series of 6 ALL patients. In one of these patients, while considered to be in morphological remission, FCM detected about 2% of aberrant cells predicting later relapse. A new two-color FCM study from Drach in 1992[138] included 24 ALL patients. Several authors then reported on two- then three-color approaches, using aberrant antigen expression on ALL blast cells.[130,139]

In 1998, a large study from Dario Campana's group[140] described data that correlated well with other reported predictive features associated with either good or poor prognosis.[141] The techniques used at this time were quite cumbersome yet began to consistently reach a sensitivity of 1×10^{-4}.

The increasing application of FCM was also soon reflected in T-ALL studies.[112,142]

A number of investigators[19,20,110,132,143-145] studied the correlation between FCM data and results obtained with PCR analyses, showing good correlation and emphasising the fact that FCM could be used for more patients.

Later on, the increasing sophistication of FCM led to more and more pertinent studies. Interestingly, Coustan-Smith *et al.*[145] reported in 2006 on a simplified assay based on a three-color combination (CD19/CD10/CD34), which when applied at the post induction timepoint was highly significant for predicting relapse in childhood B-lineage ALL. In ALL studies, three FCM approaches have been mostly used.

Mike Loken group's suggestion to identify patterns at variance with those expressed by normal bone marrow cells, was applied as early as 1998[146] in a three color approach with CD45, systematically present in all combinations of monoclonal antibodies.

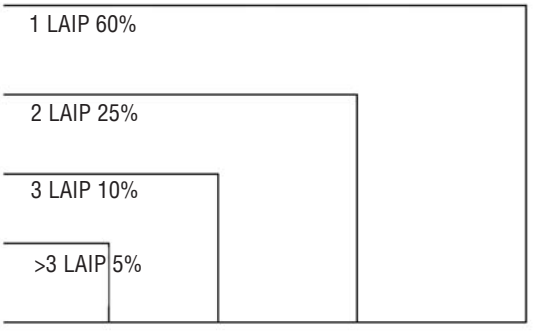
Another approach uses a minimal standardized panel for B-lineage ALL, also defined for normal cells such that it becomes possible to pinpoint abnormal events,[144,147,148] More recently, a large study from the Children's Oncology Group[149] confirmed the efficacy of a two-tube strategy in a series of over 2,000 children with a threshold of 1×10^{-4}.

A third approach, based on the detection of LAIP, was mostly developed by Dario Campana and Elaine Coustan-Smith. The strategy of this group, applicable to

Table 2. Combinations identifying LAIP with CD34 lack of expression.[122]

$CD11b^+/CD117^+/CD34^-$	n=9
$CD34^-/CD135^+/CD117^+$	n=5
$CD34^-/CD15^{++}/CD33^+$	n=1
$CD38^+/CD34^-/CD90^+$	n=1
$CD65^+/CD87^{++}/CD34^-$	n=1
$CD7^+/CD33^+/CD34^-$	n=1
$DR^-/CD33^+/CD34^-$	n=7
$CD34^-/CD56^+/CD33^+$	n=3
$CD34^-/7.1^+/CD33^+$	n=1

Table 3. Cumulative incidence of LAIP in acute myeloid leukemia.[123]



common B-lineage ALL, is based on the detection of aberrant expression of a number of antigens on cells homogeneously defined by the combination of CD19, CD34 and CD10. In a large study in 2000, Coustan-Smith *et al.*[150] reported that 204 of the 350 children who benefited from immunophenotyping at diagnosis exhibited an LAIP and 195 were included in MRD detection by FCM. The major difficulty in detecting MRD for patients with common B-II ALL ($CD10^+$) is to differentiate blast cells from hematogones, which can be quite abundant in regenerating bone marrow. The combination CD34/CD19/CD10/CD38 is quite pertinent to differentiate these cell types, based on the difference in fluorescence intensity displayed by hematogones and blasts (Figure 2B).

The value of CD58 detection for FCM MRD analysis was confirmed in two series,[151-152] supporting an earlier study by de Waele *et al.*[153]

The 2002 study by Coustan-Smith *et al.*[118] emphasized the detection of MRD in peripheral blood by FCM.

The NOPHO Scandinavian study published in 2003 used tailored panels issued from the Biomed I concerted action. In this series of 70 children, a threshold of 1×10^{-4} was established as of prognostic value.[154] Finally, a recent paper[155] reported on the reproducibility of MRD detection in FCM in a multi-center study.

Acute myeloid leukemia

In 1997, San Miguel *et al.*[156] published a landmark study carried out among 84 AML patients, demonstrating the prognostic value of thresholds of 5×10^{-3} MRD cells in the first remission BM and of 2×10^{-3} MRD cells at the end of intensification.

In a large study involving MRD assessments at a range of timepoints throughout treatment in 56 AML patients, Venditti *et al.*[157] tested 437 BM samples and reported that the most frequent aberrations observed were $CD33^+/CD7^+$ ($\pm CD34^+$) and $CD34^+/CD11b^+$ ($\pm CD117^+$). Baer *et al.*[120] and Langebrake *et al.*[124] showed, using three- and four-color approaches, respectively, that LAIP shifts do not affect MRD monitoring in AML. Kern *et al.*[122] endeavoured to define at least one LAIP per patient by comparison to 26 normal BM samples. LAIP were observed 140 times for 68 patients in 50 different patterns. Only one LAIP was seen in 28 cases. Among the 50 patterns reported, 10 included a lack of CD34 expression (Table 2). Feller *et al.*[123] studied samples from 72 patients, which allowed for the identification of 122 LAIP (Table 3). Two further studies by the Munich group[109,158] explored the possibility of shifting from an LAIP approach to a more comprehensive general strategy irrespective of the individual patient's features. This work confirmed the presence of LAIP patterns in normal bone marrow with a frequency of 7 to 3×10^{-4} cells. Feller *et al.*[159] in 2005, used the same 12 tube panel as reported by Kern *et al.*[122] in BM and mobilized PB samples from 54 patients, showing the presence of blasts in mobilized PB in AML. This was also reported in non-mobilized samples, by Maurillo *et al.*[160]

It is generally accepted that in AML the presence of myeloid cells of clearly immature immunophenotype in PB is aberrant, especially if they obey the criteria of low SSC/low CD45 (Figure 2C). These observations make the development of MRD detection in AML very attractive in easy-to-obtain PB samples, thus, making a rapid turnaround of data possible. Perea *et al.*[126] tested MRD in CBF AML and reported clinically discriminative thresholds of 1×10^{-3} for FCM and 10 copies for molecular studies. A less sensitive approach was reported after induction by Sievers *et al.*[121]

The impact of MRD assessment in AML is likely to evolve in the coming years. It might be of particular relevance in younger patients for whom allo-SCT is discussed, as suggested by the study from Laane *et al.*[161]

Chronic lymphocytic leukemia

In CLL, the recent development of aggressive but apparently successful therapies aroused a renewed interest in MRD detection. MRD studies in CLL patients are non-invasive as these can be performed using peripheral blood. However, normal B cells from the innate immune system, with immunophenotypic features akin to those of B-CLL exist physiologically. This theoretically decreases the sensitivity of MRD detection because the specificity threshold could be higher. However, little is known of the fate of these normal cells after CLL-directed chemotherapy. Among the first studies dealing with MRD in CLL, Rawstron *et al.*[103] studied PB and BM with the aim of distinguishing CLL cells from the early B-cell population of hematogones in BM. This phenotype was expressed by less than 2% of normal B cells, or 1×10^{-4} leukocytes. Caballero *et al.*[162] proposed a different four-color combination of CD23/CD79/CD19/CD5 which yielded a $1\times10^{-3}/1\times10^{-4}$ sensitivity in BM. In 2006, Moreno *et al.*[101] retained for a 10^{-4} threshold in both PB and BM the two combinations CD20/CD79b/CD19/CD5 and CD22/CD23/CD19/CD5.

Relying on clonality by assessing immunoglobulin light chains did not yield good results in their study. Maloum *et al.*[163,164] in two different studies also retained the CD19/CD5/CD20/CD79b combination. Interestingly, Kay *et al.*[165] published a clinically relevant study in which remaining CLL cells were solely identified by the two color combination CD5/CD19 on PBMC and applied a 1% threshold. More recently, however, Rawstron *et al.*[166] reported on an international standardization approach recommending three combinations, respectively associating CD5/CD19 with CD20/CD38, CD81/CD22 and CD79b/CD43.

Chronic myeloid leukemia

Very few studies have considered flow cytometry for the detection of MRD in CML. Mention can be made of the work of Lanza *et al.*[167] suggesting the interest of tracking CD56$^+$ CD34$^+$ cells.

Summary

The notion that monitoring MRD is highly useful in the stratification of patients according to risk of relapse by molecular and/or FCM is now widely accepted and increasingly incorporated in the follow-up design of multi-center trials, assessing novel therapeutic agents for rare disorders. Apart from this clinical application, MRD studies will undoubtedly also help in better understanding the kinetics of the leukemic clone and thereby the biology of the malignant cell. The value of reported prognostic markers, to be associated with aggressiveness of the malignant clone at diagnosis are open to conjecture. However, all indications are that they will prove to be useful. The identification of prognostic markers could be used to aid the timing and the frequency of MRD studies, although patients' age, type of sample and leukemia will also influence the frequency of MRD studies. RQ-PCR and improvements in FCM have made these techniques highly useful, there is now a need for defined reference methods and robust EQA schemes which will help to further strengthen the utility of MRD studies.

In conclusion, MRD studies, molecular and FCM, through early detection of relapse at sub-clinical levels permit early clinical intervention, perhaps before early progenitor cells, including CD34$^+$ cells, acquire genetic lesions that increase the aggressiveness of the clone. These methods are, therefore, highly useful in improving the prognosis of hematology-onclogy patients. Central to optimization of clinical management is pre-emptive modulation of therapy, for example, the need for more intensive therapy to overt the potential risk associated with MRD positivity post consolidation. Finally, genetic alterations at diagnosis or as the disease evolves or as a consequence of treatment, e.g *BCR-ABL1* kinase domain mutation, affect the function and signaling molecules, transcription factors, growth-factor receptors and influence the response to treatment. As these genetic lesions are identified it will become increasingly time consuming and inefficient to detect and quantify the numerous markers in separate assays. Therefore, micro-array may be needed to be developed. This has the potential to provide a patient specific disease footprint that could be used to tailor patient specific therapy and optimize clinical management in conjunction with MRD studies.

Authorship and Disclosures

Both authors contributed equally to this paper.

The authors reported no potential conflicts of interest.

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