

# Minimal Residual Disease Studies by Flow Cytometry in Acute Leukemia

Dario Campana<sup>a,b</sup> Elaine Coustan-Smith<sup>a</sup>

<sup>a</sup>Departments of Hematology-Oncology and Pathology, St. Jude Children's Research Hospital, and

<sup>b</sup>Department of Pediatrics, University of Tennessee College of Medicine, Memphis, Tenn., USA

## Key Words

Acute lymphoblastic leukemia · Acute myeloid leukemia · Flow cytometry · Minimal residual disease

## Abstract

Minimal residual disease (MRD) assays are increasingly important in the clinical management of patients with acute leukemia. Among the methods available for monitoring MRD, flow cytometry holds great promise for clinical application because of its simplicity and wide availability. Several studies have demonstrated strong correlations between MRD levels by flow cytometry during clinical remission and treatment outcome, lending support to the reliability of this approach. Flow-cytometric detection of MRD is based on the identification of immunophenotypic combinations expressed on leukemic cells but not on normal hematopoietic cells. Its sensitivity depends on the specificity of the immunophenotypes used to track leukemic cells and on the number of cells available for study. Immunophenotypes that allow detection of 1 leukemic cell in 10,000 normal cells can be identified in at least 90% of patients with acute lymphoblastic leukemia; immunophenotypes that allow detection of 1 leukemic cell in 1,000–10,000 normal cells can be identified in at least 85% of patients with acute

myeloid leukemia. Identification of new markers of leukemia by gene array technology should lead to the design of simple and reliable antibody panels for universal monitoring of MRD. Here we review the relative advantages and disadvantages of flow cytometry for MRD studies, as well as results obtained in correlative studies with treatment outcome.

Copyright © 2004 S. Karger AG, Basel

## Introduction

Studies of minimal residual disease (MRD) are becoming central to the clinical management of patients with acute leukemia. The most established methods for detecting MRD are polymerase chain reaction (PCR) amplification of antigen receptor genes and of fusion transcripts, and flow-cytometric detection of ectopic or aberrant immunophenotypes [1–6].

The discovery that leukemic cells expressed immunophenotypes not expressed by normal bone marrow and peripheral blood cells provided one of the first opportunities to study MRD [7–9]. Over the years, MRD assays based on immunophenotyping have been improved by consistent advances in the quality and variety of antibodies, by the refinement of flow cytometers, and by the

## KARGER

Fax +41 61 306 12 34  
E-Mail [karger@karger.ch](mailto:karger@karger.ch)  
[www.karger.com](http://www.karger.com)

© 2004 S. Karger AG, Basel

Accessible online at:  
[www.karger.com/aha](http://www.karger.com/aha)

Dario Campana, MD, PhD  
Department of Hematology-Oncology  
St. Jude Children's Research Hospital, 332 North Lauderdale  
Memphis, TN 38105 (USA)  
Tel. +1 901 495 2528, Fax +1 901 495 3749, E-Mail [dario.campana@stjude.org](mailto:dario.campana@stjude.org)

enormous progress in informatics that has occurred during the last decade. The striking correlations of MRD results obtained by flow cytometry with clinical features and treatment outcome provided much credibility to this approach [10–16].

Because of its wide availability and conceptual straightforwardness, flow cytometry is the most accessible method for MRD detection. In this article, we summarize technical issues that we deem important for the productive detection of MRD by flow cytometry, and the relation between results obtained with this method and treatment outcome in patients with acute leukemia.

### **Advantages and Disadvantages of Flow Cytometry**

One specific advantage of flow cytometry over PCR-based assays is that it allows direct quantitation of MRD, rather than extrapolating it from amounts of PCR product. This feature makes quantitation easier and, typically, more accurate [17]. In addition, flow cytometry allows the identification of dying cells and cellular debris. Therefore, leukemic cells irreversibly damaged by chemotherapy and unable to further expand (but capable of producing positive PCR signals) can be excluded from the counts. Obviously, this feature is useful only when analyzing samples freshly collected (e.g., 4 h or less), in which spontaneous apoptosis due to deprivation of survival factors has not yet affected a substantial number of cells.

Flow cytometry also has some specific limitations. Extreme sensitivity, such as detection of 1 leukemic cell among  $10^5$  or more normal cells, is difficult to achieve consistently by flow cytometry but well within the range of PCR. Such high sensitivity may be desirable, for example, in studies seeking MRD in patients who have a patchy distribution of the disease, or in cell harvests for autografting. Another limitation is that the immunophenotype of leukemic cells may change during the progression of the disease. If these changes affect markers used for monitoring MRD, a false-negative finding may result [10, 18, 19]. The potential adverse effect of this phenomenon is inversely related to the number of marker combinations that can be applied to each patient. That is, if cells express more than one suitable phenotype, the effects of losing one phenotypic pattern may be offset by the persistence of other aberrant patterns. Finally, a general limitation of flow-cytometric assays is that results may not appear as 'black and white' as those of PCR. This is because the distinguishing immunophenotypic features of leukemia are

often, although not always, the result of quantitative differences in antigen expression between leukemic and normal cells. Nevertheless, objective MRD estimates are possible if one determines the limits of normal antigenic expression using a variety of normal samples, and avoids the use of immunophenotypes that partially overlap that of normal cells.

### **Markers of Leukemia**

#### *Immunophenotypes for MRD Studies*

To be useful for MRD studies, immunophenotypes must be expressed on leukemic cells and not expressed on normal bone marrow and peripheral blood cells. Proteins that are produced or dysregulated by gene fusions, such as *BCR-ABL*, *AML1-ETO*, or *PBX-1* in *E2A-PBX1* should contribute to such immunophenotypes, but antibodies that allow reliable detection of these proteins by flow cytometry are scarce [20].

The identification of immunophenotypes for effective MRD studies is complicated by variations in the cellular composition and immunophenotype of normal bone marrow that occur with age and exposure to drugs. For example, proportions of early lymphoid progenitors (or 'hematogones') are low in the bone marrow of healthy adults and especially low in patients receiving corticosteroids or chemotherapy [21]. By contrast, proportions are high in the bone marrow of young children [22–24], or of patients with malignancies after transplantation or cessation of chemotherapy [25–28]. These conditions may uncover normal cells expressing phenotypes that are undetectable in samples obtained from healthy individuals.

#### *Markers for MRD Studies in Acute Lymphoblastic Leukemia*

Table 1 summarizes combinations of markers used in our laboratory to study MRD in children with acute lymphoblastic leukemia (ALL) and their applicability in consecutive cases studied from January 1999 to July 2002. The reader should refer to the listed references for markers used by other investigators [11, 29–33].

The normal equivalents of T lineage ALL cells are immature T cells. Since these are confined to the thymus whereas leukemic T lymphoblasts can circulate, MRD studies in patients with T lineage ALL consist of searching for cells with the phenotype of immature T cells in the bone marrow or in the peripheral blood. The most useful immunophenotypes for this task are coexpression of T cell markers such as CD3 and CD5 with TdT or CD34 (ta-

**Table 1.** Marker combinations used to study MRD in childhood ALL

Leukemia cell lineage	Marker combination	Applicability, % <sup>a</sup>
T lineage ALL (n = 39)	TdT/CD5/CD3/(CD19/CD33/HLA-Dr)	92
	CD34/CD5/CD3/(CD19/CD33/HLA-Dr)	21
B lineage ALL (n = 169)	CD19/CD34/CD10/CD38	52
	CD19/CD34/CD10/CD58	49
	CD19/CD34/CD10/CD45	47
	CD19/CD34/CD10/TdT	43
	CD19/CD34/CD10/CD66c	31
	CD19/CD34/TdT/IgM	17
	CD19/CD34/CD10/CD22	11
	CD19/CD34/CD10/CD13	10
	CD19/CD34/CD10/CD15	10
	CD19/CD34/CD10/CD21	6
	CD19/CD34/CD10/CD33	6
	CD19/CD34/CD10/NG-2	5
	CD19/CD34/CD10/CD65	4

n = Number of cases studied.

<sup>a</sup> Percentage of patients within each type of leukemia in whom MRD could be studied with the listed antibody combination. Percentages were calculated by including only cases in which intensity of antigen expression was sufficiently different from that of normal bone marrow cells to afford a sensitivity of detection of 1 in 10<sup>4</sup>.

ble 1) [34]. Other authors indicated that antibody combinations including CD7 and CD3 with CD2 or CD5 may also be aberrantly expressed in a proportion of T-ALL patients [29, 30].

The normal equivalents of B lineage ALL cells are B cell progenitors which normally reside in the bone marrow, and can also be found in low proportions in the peripheral blood. Therefore, MRD studies in B lineage ALL must distinguish leukemic cells from their normal counterparts. This is possible because several molecules can be expressed at abnormally high or low levels in leukemic cells (fig. 1) [26, 31, 33–36]. For example, myeloid-associated markers CD13, CD15, CD33 and CD65, and the mature B cell-associated marker CD21 can be expressed by CD19+CD34+ B lineage ALL cells, whereas normal CD19+CD34+ B cell progenitors do not express these markers or express them very weakly [34]. Expression of CD19, CD10, TdT and CD34 in B lineage ALL can be significantly different (higher or lower) than that of their normal counterparts [11, 34, 37] and CD38 and CD45 (or CD45RA) are often underexpressed in leukemic cells [33, 34]. In efforts to prevent false-negative MRD findings due to immunphenotypic shifts, we use antibody panels that are somewhat more extensive than the ones used in other laboratories.

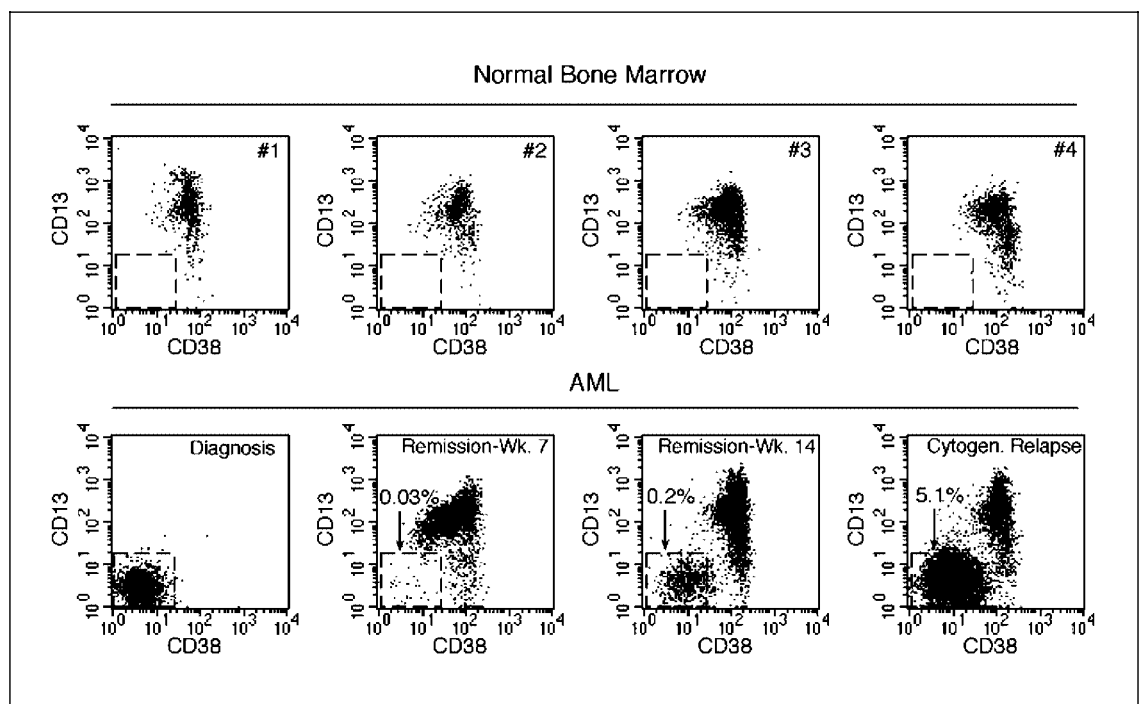
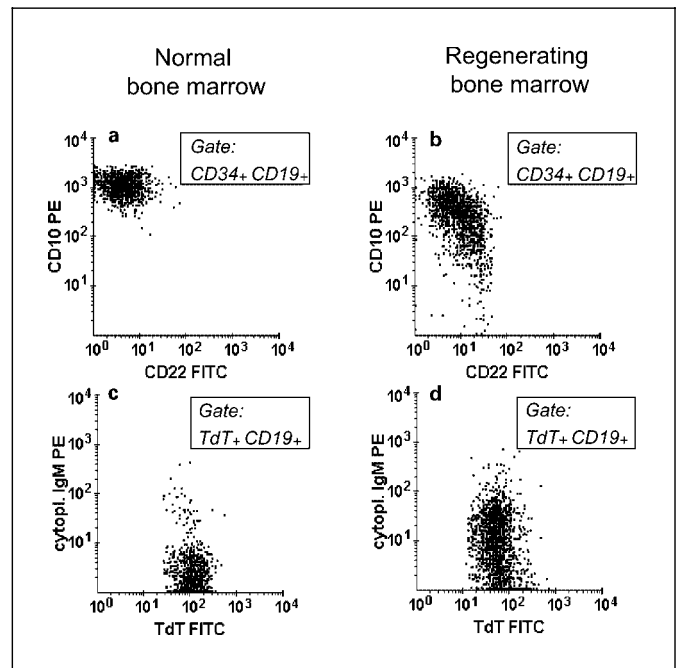
#### *Markers for MRD Studies in Acute Myeloid Leukemia*

Detection of MRD in acute myeloid leukemia (AML) requires the identification of immunophenotypic features that can distinguish leukemic myeloblasts from normal bone marrow myeloid cells. Phenotypic abnormalities in AML include expression of markers normally not expressed on myeloid cells, coexpression of markers normally expressed at different stages of maturation, as well as overexpression and under expression of myeloid markers [38].

Detection of MRD by flow cytometry in AML presents some specific difficulties. Due to their immunophenotypic heterogeneity [39], AML cells usually spread across many areas of each dot plot instead of forming the tight cluster typical of ALL cells. Therefore, with any given marker combination, only a fraction of cells may appear to be phenotypically abnormal. In addition, AML cells often have light scattering properties similar to that of normal cells with high autofluorescence. These features introduce complexity in the analysis, and may reduce the sensitivity of the assay. Nevertheless, sensitive MRD detection in AML is feasible (fig. 2). In recent studies with four-color flow cytometry, we have identified immunophenotypic combinations that allow measurement of MRD with a sensitivity of 1 leukemic cell among 10,000 or more normal cells in 48% of children, and 1 leukemic

cell among 1,000 cells in an additional 37% [39]. San Miguel et al. [13] found that 175 of 233 adult patients with AML expressed leukemia-associated immunophenotypes, while Venditti et al. [40] detected them in 65 of 93 patients. A recent study by Sievers et al. [41] indicated that residual disease could be studied by flow cytometry

**Fig. 1.** Immunophenotypic differences between immature B cells in normal and regenerating bone marrow (BM). Bone marrow mononucleated cells were collected from healthy individuals (**a, c**) and patients with leukemia recovering from chemotherapy but MRD negative by flow cytometry and PCR (**b, d**). Shown are expression of CD22 and CD10 on selectively gated CD34+CD19+ lymphoid cells (**a, b**), and expression of TdT and cytoplasmic  $\mu$  heavy chains on selectively gated TdT+CD19+ lymphoid cells (**c, d**). Each dot plot is the overlay of two samples; all dot plots have a similar number of events, ranging from 1,223 to 1,288. Dot plot overlaying was done with the FCS Express software (DeNovo Software, Thornhill, Canada).



**Fig. 2.** Detection of MRD in AML by four-color flow cytometry. Bone marrow mononuclear cells from 4 healthy donors (top) and 1 patient with AML at different stages of treatment (bottom) were labeled with antibodies to CD38, CD13, CD34 and CD33. The same number of mononuclear cells were studied in all samples. CD13 and CD38 expression in CD34+CD33+ cells is shown. In normal samples, these cells also express CD13 and/or CD38. In the AML patient, most leukemic cells at diagnosis were CD34+CD33+ but lacked CD13 and CD38 (dashed square). Cells with this phenotype were detectable at weeks 7 and 14 of therapy, a finding that was followed by cytogenetic relapse first and then by clinical relapse.

in all patients, although this was an assumption because the immunophenotype at diagnosis was not determined. Therefore, the actual prevalence of leukemia-associated immunophenotypes in this series is unclear.

#### *Identification of New Markers*

The advent of arrays that allow genome-wide analysis of gene expression has opened new possibilities to identify markers for MRD studies [42]. To test the validity of this concept, we compared the gene profile of ALL cells to that of purified normal B cell progenitors [43]. Among the ~4,000 genes studied, we found over 250 that were over-expressed in more than one leukemic sample. We selected 9 of these genes for which antibodies were easily available and measured expression of the encoded proteins by flow cytometry. Seven proteins (CD58, creatine kinase B, nin-jurin1, Ref1, calpastatin, HDJ-2 and annexin VI) were expressed in B lineage ALL cells at higher levels than in normal CD19+CD10+ B cell progenitors.

The results with CD58 were in line with a previous report indicating overexpression of this molecule in leukemic cells [44]. CD58 is now one of the most useful markers for the study of MRD in B lineage ALL (table 1). These results suggest that a comparison of the gene profiles of normal and leukemic cells will identify new, widely applicable markers for MRD studies in ALL and in AML, and should ultimately allow the design of simple antibody panels for practical, reliable, and universal monitoring of MRD.

#### *Sensitivity and Measurement of MRD*

Two main variables influence rare cell detection by flow cytometry: (1) the degree of morphologic and phenotypic difference between target cells and the remaining cells, and (2) the number of cells that can be analyzed. Under ideal conditions, i.e. very distinct target cells and a large number of cells ( $10^7$  or more) available for analysis, the sensitivity of flow cytometry is similar to that of PCR [45]. During analysis of MRD in clinical samples, however, the number of cells that can be analyzed for each set of markers in children is usually less than  $1 \times 10^6$ . Because a distinct cluster of at least 10–20 dots is necessary to interpret suspect flow-cytometric events, the maximum sensitivity achievable in these circumstances would be 1 in  $10^5$  cells. The phenotype of primary leukemic cells may not be as distinct as that of cell lines used in an experimental setting and, in the case of B lineage ALL, sensitivity of detection may be influenced by the treatment interval at which the sample is taken, because of the variable proportion of normal B cell precursors. Therefore, a con-

sistent sensitivity of 1 in  $10^4$  is probably the maximum that can be expected during routine MRD testing.

Flow cytometry has the potential for a very accurate quantitation of MRD. In experiments with serial dilutions of KOPN-57bi pro-B ALL cells admixed with normal peripheral blood mononuclear cells at ratios of 3 in  $10^5$  and higher, the estimate of the leukemic cell content in each mixture was extremely accurate ( $r^2 = 0.999$ ) [17]. In other experiments, we compared the results of multiple measurements of residual leukemia in the same cell mixture [10]. In 23 tests of mixtures containing 1 leukemic cell in  $10^4$  normal cells, results were remarkably similar (coefficient of variation = 15%); in 22 tests of mixtures containing 1 leukemic cell in  $10^3$  cells, the coefficient of variation was 10%.

### **Clinical Applications**

#### *Prognostic Value of MRD in ALL*

We used flow cytometry to prospectively study MRD in 195 children with newly diagnosed ALL enrolled in a single-institution chemotherapy program (TOTAL XIII) [10, 12, 15]. We found that detectable MRD (i.e.,  $\geq 0.01\%$  leukemic mononuclear cells) at each time point (day 19 of remission induction therapy, end of remission induction and weeks 14, 32 and 56 of continuation) was significantly associated with a higher rate of relapse. Patients with high levels of MRD at the end of the remission induction therapy ( $\geq 1\%$ ) or at week 14 of continuation therapy ( $\geq 0.1\%$ ) had a particularly poor outcome. The incidence of relapse among patients with MRD at the end of induction was  $7 \pm 7\%$  if MRD became undetectable at week 14 of continuation therapy, compared with  $68 \pm 16\%$  (SE) if MRD persisted. Notably, 53 of the 112 patients studied at day 19 of remission induction therapy had achieved MRD negativity ( $< 0.01\%$ ) despite the short chemotherapy period [15]. The 3-year cumulative incidence of relapse was  $1.9 \pm 1.9\%$ , as compared to  $28.4 \pm 6.4\%$  for patients who were MRD positive at this time point. At all time points, the prognostic value of MRD was independent from that of other known clinical and biologic prognosticators of outcome.

Other investigators have used flow cytometry to study MRD in patients with ALL undergoing chemotherapy and found a good correlation with outcome [11, 14, 30]. MRD detected by flow cytometry in bone marrow samples taken prospectively from 24 patients with ALL undergoing stem cell transplantation before starting the conditioning regimen was a significant predictor of outcome [46].

### *Detection of MRD in Peripheral Blood*

We recently compared MRD measurements in 747 pairs of bone marrow and peripheral blood samples collected from 231 children during treatment for newly diagnosed ALL [6, 16]. MRD was detected in both marrow and blood in 78 pairs and in marrow but not in blood in 67 pairs; it was undetectable in the remaining 602 pairs. Findings in marrow and blood were completely concordant in the 179 paired samples from patients with T lineage ALL: for each of the 41 positive marrow samples, the corresponding blood sample was positive. In B lineage ALL, however, only 37 of the 104 positive marrow samples had a corresponding positive blood sample. Results of a recently reported study by another group of investigators are in agreement with the remarkable concordance of MRD results in marrow and blood of patients with T-ALL [47]. We also observed that peripheral blood MRD in B lineage ALL patients was associated with a very high risk of relapse [16].

### *MRD Studies in AML*

A recent study found immunophenotypic abnormalities in 41 of 252 children with AML who responded to initial therapy, a finding that was associated with a poorer outcome [41]. In studies performed in adult patients with AML, the first bone marrow in morphologic remission obtained after induction treatment was found to be very informative [13]. Of the 126 patients studied, 8 had <0.01% leukemic cells and none had relapsed at the time of the report; 37 had 0.01–0.1% leukemic cells and a 3-year cumulative relapse rate of 14%; 64 had 0.1–1% leukemic cells and a relapse rate of 50%; 17 had more than 1% residual cells and a relapse rate of 84%. In another study of 51 patients in whom MRD was examined after consolidation, the most predictive MRD cutoff value determined retrospectively was 0.035%: 17 of 22 patients with that level of MRD or higher levels relapsed compared with 5 of 29 patients with lower MRD levels [40]. We recently reported that detection of MRD in children with AML is prognostically important [39]. In patients with AML receiving autologous bone marrow transplantation, levels of MRD measured by flow cytometry in the autograft correlated with disease recurrence [48].

### **Future Outlook**

In some forms of acute leukemia, e.g. childhood ALL, a central issue is the identification of patients who require more aggressive therapy to avert relapse and of those who

can be spared unnecessarily intense and toxic treatment. By precisely measuring early treatment response *in vivo*, MRD studies have great potential in this context. In other forms of leukemia, e.g. ALL in adults and AML, novel treatments are urgently needed if one hopes to improve cure rates substantially. Thus, the highest value of MRD assays in these diseases may possibly lie in the rapid measurement of the effect of novel therapies on the leukemic clone.

The discovery of a small set of new leukemia-specific markers that can be detected in most cases would extend the benefits of MRD detection to a larger number of patients. Because flow-cytometric analysis of leukemic cell phenotype is performed in nearly all pediatric and adult cancer centers worldwide, newly developed methods of MRD detection can realistically be accessible to most patients. Internet-based methods of rapid file transfer between flow cytometers at remote centers [49] should allow rapid quality control and distance learning and help to set up this methodology in laboratories with experience in leukemia phenotyping but novice at MRD detection. However, many of these centers cannot support the use of complex, costly antibody panels and a reduced panel of antibodies may not allow optimal MRD monitoring. Gene expression analysis with microarrays hold the potential to identify new markers of leukemia for MRD studies. New markers should allow the simplification of the current immunophenotypic panels and the wide applicability of flow-cytometric studies of MRD.

### **Acknowledgment**

This work was supported by grants CA60419 and CA21765 from the National Cancer Institute, by the Rizzo Memorial Grant from the Leukemia Research Foundation, and by the American Lebanese Syrian Associated Charities (ALSAC).

## References

- Campana D, Pui CH: Detection of minimal residual disease in acute leukemia: Methodologic advances and clinical significance. *Blood* 1995;85:1416-1434.
- van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, Gottardi E, Rambaldi A, Dotti G, Griesinger F, Parreira A, Gameiro P, Diaz MG, Malec M, Langerak AW, San Miguel JF, Biondi A: Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: Investigation of minimal residual disease in acute leukemia. *Leukemia* 1999;13:1901-1928.
- Pui CH, Campana D: New definition of remission in childhood acute lymphoblastic leukemia. *Leukemia* 2000;14:783-785.
- Faroni L, Harrison CJ, Hoffbrand AV, Potter MN: Investigation of minimal residual disease in childhood and adult acute lymphoblastic leukaemia by molecular analysis. *Br J Haematol* 1999;105:7-24.
- Szczepanski T, Orfao A, van der Velden VH, San Miguel JF, van Dongen JJ: Minimal residual disease in leukaemia patients. *Lancet Oncol* 2001;2:409-417.
- Campana D: Determination of minimal residual disease in leukemia patients. *Br J Haematol* 2003;121:823-838.
- Bradstock KF, Janossy G, Tidman N, Papa-georgiou ES, Prentice HG, Willoughby M, Hoffbrand AV: Immunological monitoring of residual disease in treated thymic acute lymphoblastic leukaemia. *Leuk Res* 1981;5:301-309.
- van Dongen JJ, Breit TM, Adriaansen HJ, Beishuizen A, Hooijkaas H: Detection of minimal residual disease in acute leukemia by immunological marker analysis and polymerase chain reaction. *Leukemia* 1992;6(suppl 1):47-59.
- Campana D, Coustan-Smith E, Janossy G: The immunologic detection of minimal residual disease in acute leukemia. *Blood* 1990;76/1: 163-171.
- Coustan-Smith E, Behm FG, Sanchez J, Boyett JM, Hancock ML, Raimondi SC, Rubnitz JE, Rivera GK, Sandlund JT, Pui CH, Campana D: Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. *Lancet* 1998;351:550-554.
- Farahat N, Morilla A, Owusu-Ankomah K, Morilla R, Pinkerton CR, Treleaven JG, Matutes E, Powles RL, Catovsky D: Detection of minimal residual disease in B-lineage acute lymphoblastic leukaemia by quantitative flow cytometry. *Br J Haematol* 1998;101/1:158-164.
- Coustan-Smith E, Sancho J, Hancock ML, Boyett JM, Behm FG, Raimondi SC, Sandlund JT, Rivera GK, Rubnitz JE, Ribeiro RC, Pui CH, Campana D: Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. *Blood* 2000;96:2691-2696.
- San Miguel JF, Vidriales MB, Lopez-Berges C, Diaz-Mediavilla J, Gutierrez N, Canizo C, Ramos F, Calmuntia MJ, Perez JJ, Gonzalez M, Orfao A: Early immunophenotypic evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. *Blood* 2001;98:1746-1751.
- Dworzak MN, Froschl G, Printz D, Mann G, Potschger U, Muhlegger N, Fritsch G, Gadner H: Prognostic significance and modalities of flow cytometric minimal residual disease detection in childhood acute lymphoblastic leukemia. *Blood* 2002;99:1952-1958.
- Coustan-Smith E, Sancho J, Behm FG, Hancock ML, Razzouk BI, Ribeiro RC, Rivera GK, Rubnitz JE, Sandlund JT, Pui CH, Campana D: Prognostic importance of measuring early clearance of leukemic cells by flow cytometry in childhood acute lymphoblastic leukemia. *Blood* 2002;100:52-58.
- Coustan-Smith E, Sancho J, Hancock ML, Razzouk BI, Ribeiro RC, Rivera GK, Rubnitz JE, Sandlund JT, Pui CH, Campana D: Use of peripheral blood instead of bone marrow to monitor residual disease in children with acute lymphoblastic leukemia. *Blood* 2002;100: 2399-2402.
- Neale GA, Coustan-Smith E, Pan Q, Chen X, Gruhn B, Stow P, Behm FG, Pui CH, Campana D: Tandem application of flow cytometry and polymerase chain reaction for comprehensive detection of minimal residual disease in childhood acute lymphoblastic leukemia. *Leukemia* 1999;13:1221-1226.
- Oelschlagel U, Nowak R, Schaub A, Koppel C, Herbst R, Mohr B, Loffler C, Range U, Gunther H, Assmann M, Siegert E, Wendt E, Huhn R, Brautigam E, Ehninger G: Shift of aberrant antigen expression at relapse or at treatment failure in acute leukemia. *Cytometry* 2000;42/ 4:247-253.
- Baer MR, Stewart CC, Dodge RK, Leget G, Sule N, Mrozek K, Schiffer CA, Powell BL, Kolitz JE, Moore JO, Stone RM, Davey FR, Carroll AJ, Larson RA, Bloomfield CD: High frequency of immunophenotype changes in acute myeloid leukemia at relapse: Implications for residual disease detection (Cancer and Leukemia Group B Study 8361). *Blood* 2001; 97:3574-3580.
- Sang BC, Shi L, Dias P, Liu L, Wei J, Wang ZX, Monell CR, Behm F, Gruenwald S: Monoclonal antibodies specific to the acute lymphoblastic leukemia t(1;19)-associated E2A/pbx1 chimeric protein: Characterization and diagnostic utility. *Blood* 1997;89:2909-2914.
- Paolucci P, Hayward AR, Rapson NT: Pre-B and B cells in children on leukaemia remission maintenance treatment. *Clin Exp Immunol* 1979;37/2:259-266.
- Longacre TA, Foucar K, Crago S, Chen IM, Griffith B, Dressler L, McConnell TS, Duncan M, Gribble J: Hematogones: A multiparameter analysis of bone marrow precursor cells. *Blood* 1989;73:543-552.
- Caldwell CW, Poje E, Helikson MA: B-cell precursors in normal pediatric bone marrow. *Am J Clin Pathol* 1991;95:816-823.
- Lucio P, Parreira A, van den Beemd MW, van Lochem EG, Van Wering ER, Baars E, Porwit-MacDonald A, Bjorklund E, Gaipa G, Biondi A, Orfao A, Janossy G, van Dongen JJ, San Miguel JF: Flow cytometric analysis of normal B cell differentiation: A frame of reference for the detection of minimal residual disease in precursor-B-ALL. *Leukemia* 1999;13:419-427.
- Asma GE, van den Bergh RL, Vossen JM: Regeneration of TdT+, pre-B, and B cells in bone marrow after allogeneic bone marrow transplantation. *Transplantation* 1987;43: 865-870.
- Ciudad J, San Miguel JF, Lopez-Berges MC, Garcia MM, Gonzalez M, Vazquez L, del Canizo MC, Lopez A, van Dongen JJ, Orfao A: Detection of abnormalities in B-cell differentiation pattern is a useful tool to predict relapse in precursor-B-ALL. *Br J Haematol* 1999;104: 695-705.
- van Lochem EG, Wiegers YM, van den BR, Hahlen K, van Dongen JJ, Hooijkaas H: Regeneration pattern of precursor-B-cells in bone marrow of acute lymphoblastic leukemia patients depends on the type of preceding chemotherapy. *Leukemia* 2000;14:688-695.
- McKenna RW, Washington LT, Aquino DB, Picker LJ, Kroft SH: Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. *Blood* 2001;98:2498-2507.
- Porwit-MacDonald A, Bjorklund E, Lucio P, van Lochem EG, Mazur J, Parreira A, van den Beemd MW, Van Wering ER, Baars E, Gaipa G, Biondi A, Ciudad J, van Dongen JJ, San Miguel JF, Orfao A: BIOMED-1 concerted action report: Flow cytometric characterization of CD7+ cell subsets in normal bone marrow as a basis for the diagnosis and follow-up of T cell acute lymphoblastic leukemia (T-ALL). *Leukemia* 2000;14:816-825.
- Ciudad J, San Miguel JF, Lopez-Berges MC, Vidriales B, Valverde B, Ocqueteau M, Mateos G, Caballero MD, Hernandez J, Moro MJ, Mateos MV, Orfao A: Prognostic value of immunophenotypic detection of minimal residual disease in acute lymphoblastic leukemia. *J Clin Oncol* 1998;16:3774-3781.
- Lucio P, Gaipa G, van Lochem EG, Van Wering ER, Porwit-MacDonald A, Faria T, Bjorklund E, Biondi A, van den Beemd MW, Baars E, Vidriales B, Parreira A, van Dongen JJ, San Miguel JF, Orfao A: BIOMED-I concerted action report: Flow cytometric immunophenotyping of precursor B-ALL with standardized triple-stainings. BIOMED-1 Concerted Action Investigation of Minimal Residual Disease in Acute Leukemia: International Standardization and Clinical Evaluation. *Leukemia* 2001; 15:1185-1192.

- 32 Weir EG, Cowan K, LeBeau P, Borowitz MJ: A limited antibody panel can distinguish B-precursor acute lymphoblastic leukemia from normal B precursors with four color flow cytometry: Implications for residual disease detection. *Leukemia* 1999;13:558–567.
- 33 Dworzak MN, Fritsch G, Fleischer C, Printz D, Froschl G, Buchinger P, Mann G, Gadner H: Comparative phenotype mapping of normal vs. malignant pediatric B-lymphopoiesis unveils leukemia-associated aberrations. *Exp Hematol* 1998;26:305–313.
- 34 Campana D, Coustan-Smith E: Advances in the immunological monitoring of childhood acute lymphoblastic leukaemia. *Best Pract Res Clin Haematol* 2002;15/1:1–19.
- 35 Hurwitz CA, Loken MR, Graham ML, Karp JE, Borowitz MJ, Pullen DJ, Civin CI: Asynchronous antigen expression in B lineage acute lymphoblastic leukemia. *Blood* 1988;72/1:299–307.
- 36 Wells DA, Hall MC, Shulman HM, Loken MR: Occult B cell malignancies can be detected by three-color flow cytometry in patients with cytopenias. *Leukemia* 1998;12:2015–2023.
- 37 Lavabre-Bertrand T, Janossy G, Ivory K, Peters R, Secker-Walker L, Porwit-MacDonald A: Leukemia-associated changes identified by quantitative flow cytometry. I. CD10 expression. *Cytometry* 1994;18/4:209–217.
- 38 Terstappen LW, Loken MR: Myeloid cell differentiation in normal bone marrow and acute myeloid leukemia assessed by multi-dimensional flow cytometry. *Anal Cell Pathol* 1990;2/4:229–240.
- 39 Coustan-Smith E, Ribeiro RC, Rubnitz JE, Razzouk BI, Pui CH, Pounds S, Andreansky M, Behm FG, Raimondi SC, Shurtleff SA, Downing JR, Campana D: Clinical significance of residual disease during treatment in childhood acute myeloid leukemia. *Br J Haematol* 2003;123:243–252.
- 40 Venditti A, Buccisano F, Del Poeta G, Maurillo L, Tamburini A, Cox C, Battaglia A, Catalano G, Del Moro B, Cudillo L, Postorino M, Masi M, Amadori S: Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood* 2000;96:3948–3952.
- 41 Sievers EL, Lange BJ, Alonzo TA, Gerbing RB, Bernstein ID, Smith FO, Arceci RJ, Woods WG, Loken MR: Immunophenotypic evidence of leukemia after induction therapy predicts relapse: Results from a prospective Children's Cancer Group study of 252 patients with acute myeloid leukemia. *Blood* 2003;101:3398–3408.
- 42 Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Behm FG, Raimondi SC, Relling MV, Patel A, Cheng C, Campana D, Wilkins D, Zhou X, Li J, Pui CH, Evans WE, Wong L, Downing JR: Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell* 2002;1:133–143.
- 43 Chen JS, Coustan-Smith E, Suzuki T, Neale GA, Mihara K, Pui CH, Campana D: Identification of novel markers for monitoring minimal residual disease in acute lymphoblastic leukemia. *Blood* 2001;97:2115–2120.
- 44 De Waele M, Renmans W, Jochmans K, Schots R, Lacor P, Trullemans F, Otten J, Balduck N, Vander GK, Van Camp B, van Schie RC, Van Riet I: Different expression of adhesion molecules on CD34+ cells in AML and B-lineage ALL and their normal bone marrow counterparts. *Eur J Haematol* 1999;63/3:192–201.
- 45 Gross HJ, Verwer B, Houck D, Recktenwald D: Detection of rare cells at a frequency of one per million by flow cytometry. *Cytometry* 1993;14:519–526.
- 46 Sanchez J, Serrano J, Gomez P, Martinez F, Martin C, Madero L, Herrera C, Garcia JM, Casano J, Torres A: Clinical value of immunological monitoring of minimal residual disease in acute lymphoblastic leukaemia after allogeneic transplantation. *Br J Haematol* 2002;116:686–694.
- 47 van der Velden V, Jacobs DC, Wijkhuijs AJ, Comans-Bitter WM, Willemse MJ, Hahlen K, Kamps WA, Van Wering ER, van Dongen JJ: Minimal residual disease levels in bone marrow and peripheral blood are comparable in children with T cell acute lymphoblastic leukemia (ALL), but not in precursor-B-ALL. *Leukemia* 2002;16:1432–1436.
- 48 Reichle A, Rothe G, Krause S, Zaiss M, Ullrich H, Schmitz G, Andreesen R: Transplant characteristics: Minimal residual disease and impaired megakaryocytic colony growth as sensitive parameters for predicting relapse in acute myeloid leukemia. *Leukemia* 1999;13:1227–1234.
- 49 Lorenzana R, Coustan-Smith E, Antillon F, Ribeiro RC, Campana D: Simple methods for the rapid exchange of flow cytometric data between remote centers. *Leukemia* 1999;14:336–337.