Novel Multiparameter Flow Cytometry Techniques for the Detection of Leukaemia Associated Phenotypes and Minimal Residual Disease Monitoring In Acute Myeloid Leukaemia

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TABLE OF CONTENTS

DECLARATION I

ACKNOWLEDGMENTS II

PUBLICATIONS ARISING FROM THIS THESIS VII

ABBREVIATIONS IX

CONFERENCE PRESENTATIONS XII

AWARD ARISING FROM THIS THESIS XIV

DEDICATION XV

ABSTRACT XVI

CHAPTER 1: GENERAL INTRODUCTION 1

1.1 SIGNIFICANCE OF THE RESEARCH 1

1.2 INTRODUCTION 2

1.3 LEUKAEMIA 2

1.4 WHAT IS AML? 2

1.5 EPIDEMIOLOGY 3
1.5.1 Genetic Predisposition 3
1.5.2 Environmental Exposure 3
1.5.3 Prior Therapy 4
1.5.4 Prior Bone Marrow Disorders 4
1.5.5 Age 4

1.6 PATHOPHYSIOLOGY 4

1.7 DIAGNOSIS 6
1.7.1 Classification according to biological and clinical features 6
1.7.2 Classification according to prognosis 7

1.8 CHEMOTHERAPEUTIC TREATMENT 7

1.9 OUTCOMES 10

1.10 PROGNOSTIC FACTORS IN AML AT DIAGNOSIS 11
1.10.1 Age and Gender 11

1.10.2 Cytogenetics and molecular genetics ... 12
1.10.3 Molecular markers ... 13
1.10.3.1 FLT3 ... 13
1.10.3.2 CCAAT/ enhancer binding protein a ... 14
1.10.3.3 Nucleophosmin (NPM1) ... 14
1.10.3.4 Mixed lineage leukaemia gene ... 15
1.10.3.5 Wilms tumor gene ... 15
1.10.3.6 Multidrug resistance genes ... 15
1.10.4 Secondary leukaemia and advanced disease ... 16
1.10.5 White blood cell count ... 16
1.10.6 Immunophenotyping ... 17
1.10.7 FAB Classification ... 17
1.10.8 Gene expression profiles ... 18

1.11 PROGNOSTIC FACTORS IN AML AFTER INDUCTION THERAPY ... 18
1.11.1 Response to first induction ... 19
1.11.2 Minimal residual disease as a predictive factor for relapse ... 19

1.12 WHAT IS MINIMAL RESIDUAL DISEASE? ... 20

1.13 METHODS AVAILABLE FOR THE DETECTION OF MRD ... 21
1.13.1 Morphology ... 21
1.13.2 Cell culture assays ... 21
1.13.3 Cytogenetic analysis ... 22
1.13.4 Fluorescence in situ hybridization techniques (FISH) ... 22
1.13.5 Polymerase Chain Reaction (PCR) ... 22
1.13.6 Flow cytometry and immunophenotyping ... 23

1.14 FACTORS THAT INFLUENCE FLOW CYTOMETRY FOR DETECTING MRD . 26
1.14.1 Frequency of LAPs ... 26
1.14.1.1 The quality of LAP for MRD detection ... 27
1.14.1.2 Points for consideration for LAPs detection ... 28
1.14.2 Level of sensitivity: ... 29

1.15 ADVANTAGES AND DRAWBACKS OF MFC FOR DETECTING MRD ... 29

1.16 CLINICAL STUDIES IN MRD DETECTION ... 31

1.17 NEWER APPROACHES: LEUKAEMIC STEM CELLS AND MARKERS ... 32
1.17.1 Understanding AML stem cells will lead to more effective therapies ... 32

1.18 FUNCTIONAL AND PHENOTYPIC CHARACTERIZATION OF LEUKAEMIC STEM CELLS ... 33
1.18.1 Functional characterization of LSC ... 33
1.18.2 Phenotypic characterization of LSC ... 34

1.19 TARGETS OF AML THERAPY DETECTABLE IN LEUKAEMIC STEM CELLS 38
1.19.1 Targets expressed on the surface of AML cells ... 38
1.19.2 Intracellular targets of AML therapy ... 39

1.20 HYPOTHESES AND AIMS OF THE RESEARCH ... 41
1.20.1 Hypotheses of the research: ... 41
1.20.2 Aims of the research ... 41

1.21 HOW THE THESIS AIMS CONTRIBUTE TO THE CURRENT KNOWLEDGE? . 41

CHAPTER 2: MATERIALS & METHODS ... 44

2.1 MATERIALS ... 44

2.1.1 PATIENT POPULATION ... 44
2.1.1.1 Sample size calculation ... 44
2.1.1.1.1 Understanding the Sample Size Table ... 45
2.1.1.1.2 Choosing the sample size ... 46
2.1.1.2 Patient samples ... 47
2.1.1.2.1 Selection criteria ... 47
2.1.1.2.2 Study conduct ... 47
2.1.1.2.3 Study parameters ... 48
2.1.1.3 Normal bone marrow samples ... 48
2.1.1.4 Regenerating bone marrow samples ... 48
2.1.1.5 Chemotherapeutic treatment ... 48
2.1.2 SUPPLIERS OF COMMONLY USED REAGENTS ... 50
2.1.3 IMMUNOFLUORESCENCE REAGENTS ... 51
2.1.3.1 FACS Fix ... 51
2.1.3.2 FCS - Foetal Calf Serum ... 51
2.1.3.3 Lysis Solution ... 51
2.1.3.4 Reagent A - Erythrocyte Lysis Reagent for Q-Prep System ... 52
2.1.3.5 Reagent B - Leukocyte Stabiliser Reagent for Q-Prep System ... 52
2.1.3.6 Reagent C - Leukocyte Fixative Reagent for Q-Prep System ... 52
2.1.3.7 1% Sodium Hypochlorite (W/V) ... 52
2.1.3.8 White Cell Fluid (WCF) ... 53
2.1.3.9 MACS $CD34^+$ Buffer (PBS/0.5% BSA/2mM EDTA) ... 53
2.1.3.10 Thawing solution ... 53
2.1.3.11 Monoclonal Antibodies (MoAbs) ... 54
2.1.4 DNA MANIPULATIONS REAGENTS ... 60
2.1.4.1 TBE Buffer ... 60
2.1.4.1.1 5 x Stock Tris-Borate (TBE) Buffer ... 60
2.1.4.1.2 TBE Buffer - Working Solution ... 60
2.1.4.2 Agarose gel mix ... 60
2.1.4.2.1 2% Agarose Gel ... 60
2.1.4.3 Loading Buffer ... 60
2.1.4.4 Molecular Weight Marker pUC19 ... 61
2.1.4.4.1 Working Solution for pUC19 ... 61
2.1.4.5 Ethidium Bromide ... 62
2.1.4.5.1 Ethidium Bromide Stock Solution ... 62
2.1.4.5.2 Ethidium Bromide Working Solution ... 62
2.1.4.6 dNTP Reagent ... 63
2.1.4.6.1 dNTP Stock Reagent 100 mM ... 63
2.1.4.6.2 dNTP Working Solution 2 mM of each dNTP ... 63
2.1.4.7 Taq DNA polymerase kits ... 63

2.2 METHODS ... 63
2.2.1 MORPHOLOGY AND CYTOCHEMISTRY ... 63
2.2.2 CYTOGENETICS ... 63
2.2.3 IMMUNOPHENOTYPING ... 64
2.2.3.1 Cryopreservation of cells ... 64
2.2.3.2 Thawing cryopreserved cells ... 64
2.2.3.3 Specimen processing ... 64
2.2.3.3.1 Cell count ... 64
2.2.3.3.1.1 Cell count using Haematocytometer ... 65
2.2.3.3.1.2 Cell count using Epics® - XL-MCL and Cytomics® FC500 flow cytometer 65
2.2.3.3.1.3 Cell count data interpretation ... 65
2.2.3.3.2 Specimen dilution ... 68
2.2.3.4 Staining technique ... 68
2.2.3.4.1 Manual staining ... 68
2.2.3.4.2 Lysis Method ... 69
2.2.3.4.3 Surface and cytoplasmic staining ... 69
2.2.3.4.4 Blocking techniques ... 70
2.2.3.4.5 Viability staining ... 70

2.2.3.5 Method of Antibody evaluation ... 70
2.2.3.5.1 Antibody Selection: ... 70
2.2.3.5.2 Patient Sample Selection for Antibody evaluation: ... 71
2.2.3.5.3 Initial Investigation – Determination of Working Titration: ... 71
2.2.3.5.4 Patient Comparison: ... 73
2.2.3.5.5 Uncommon Markers: ... 73
2.2.3.5.6 Method comparison-Mean Difference ... 73
2.2.3.6 Antibody dilution list ... 73
2.2.3.7 Antibody panels ... 74
2.2.3.8 Compensation ... 75
2.2.3.8.1 Manual compensation ... 76
2.2.3.8.2 Five-colour compensation for FITC, PE, ECD, PC5 and PC7 ... 78
2.2.3.8.3 BD CompBeads ... 80
2.2.3.9 Gating strategy for LAPs ... 81
2.2.3.9.1 Staining and analysing marker expression in a 1st run ... 81
2.2.3.9.2 Defining LAPs and verifying these in a 2nd run ... 81
2.2.3.9.3 After 1st run: Analyses of marker expressions on the leukaemia blast population 83
2.2.3.9.4 After a 2nd run: Establishment of a LAP ... 84
2.2.3.9.5 Data reporting and interpretation ... 84
2.2.3.10 Gating strategy for MRD ... 84
2.2.3.10.1 Data analysis ... 85
2.2.3.10.2 Analyses of MRD ... 85
2.2.3.10.3 Data reporting and interpretation ... 85
2.2.3.11 Fluorescence activated cell sorting ... 92
2.2.4 IMMUNO-FLUORESCENCE CONFOCAL MICROSCOPY ASSAYS ... 92
2.2.5 MOLECULAR TECHNIQUES ... 93
2.2.5.1 Purification of DNA ... 93
2.2.5.1.1 Salt extraction ... 93
2.2.5.1.2 QIAmp® Kits ... 94
2.2.5.2 Calculation of DNA Concentration and Purity by Spectrophotometer ... 94
2.2.5.3 Electrophoresis of DNA ... 95
2.2.5.4 Size determination and quantitation of DNA fragments ... 96
2.2.5.5 Primers used for PCR and sequencing ... 96
2.2.5.6 Polemerase chain reaction ... 96
2.2.5.7 Sequencing the DNA ... 97
2.2.6 IMMUNOMAGNETIC ASSAYS ... 98
2.2.6.1 Isolation of $CD34^+$ Cells: MACS Separation ... 98
2.2.7 STATISTICAL ANALYSIS ... 99

CHAPTER 3: THE PRESENCE OF LEUKAEMIA-ASSOCIATED PHENOTYPES IS AN INDEPENDENT PREDICTOR OF INDUCTION FAILURE IN ACUTE MYELOID LEUKAEMIA ... 102

3.1 ABSTRACT ... 102

3.2 INTRODUCTION ... 104

3.3 RESULTS ... 106
3.3.1 AML samples ... 106
3.3.2 Leukaemia-associated phenotypes ... 108
3.3.2.1 Lineage infidelity ... 108
3.3.2.2 Asynchronous antigen expression ... 108
3.3.2.3 Absence of lineage specific antigens ... 108
3.3.3 The presence of leukaemia-associated phenotypes is associated with a poor response to induction therapy ... 110
3.3.4 Leukaemia-associated phenotypes may predict relapse of AML ... 114

3.4 DISCUSSION ... 119

CHAPTER 4: INCIDENCE OF LEUKAEMIA ASSOCIATED PHENOTYPES IN ACUTE MYELOID LEUKAEMIA PATIENTS: A BASIS FOR THE DESIGN OF SPECIFIC FIVE-COLOUR STAINING TO BE USED FOR MINIMAL RESIDUAL DISEASE INVESTIGATION 124

4.1 ABSTRACT 124

4.2 INTRODUCTION 126

4.3 RESULTS 128
4.3.1 Clustering of bone marrow lineages using CD45 gating strategy 128
4.3.2 Utility of CD45/SSC gating in frozen samples 135
4.3.3 Expression of different MoAbs 135
4.3.3.1 CD45 135
4.3.3.2 HLA-DR 136
4.3.3.3 CD34/CD38 136
4.3.3.4 CD117 143
4.3.3.5 CD33 143
4.3.3.6 CD13 143
4.3.3.7 CD14 144
4.3.3.8 CD15 144
4.3.3.9 CD56 144
4.3.3.10 CD2 144
4.3.3.11 CD7 144
4.3.3.12 CD19 145
4.3.3.13 CD10 145
4.3.3.14 CD11b 145
4.3.3.15 CD64 145
4.3.3.16 CD65 145
4.3.3.17 CD123 146
4.3.3.18 Cytoplasmic MPO 146
4.3.3.19 Nuclear TdT 146
4.3.4 Identification of leukaemia-associated phenotypes 151
4.3.4.1 Lineage infidelity 152
4.3.4.2 Asynchronous antigen expression 152
4.3.4.3 Absence of lineage specific antigens 153
4.3.5 Aberrant markers expression in normal and regenerating bone marrow cells 155
4.3.6 Quantification of differences in cells carrying aberrant immunophenotypes between AML samples, and normal and regenerating bone marrows 160
4.3.7 Investigation of the 'in vitro' sensitivity of flow cytometry immunophenotyping for detection of MRD 163
4.3.7.1 Dilution of AML samples in normal and regenerating bone marrow 163
4.3.7.2 Assessment of linearity 163
4.3.7.3 Assessment of reproducibility 168
4.3.8 Appearance of LAPs using Immuno-Fluorescence Confocal Microscopy 172

4.4 DISCUSSION 175

CHAPTER 5: DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKAEMIA IDENTIFIES PATIENTS WITH HIGH RISK OF RELAPSE 179

5.1 ABSTRACT 179

5.2 INTRODUCTION 180

5.3 RESULTS 182
5.3.1 Clinical Characteristics 182

5.3.2 Establishing the optimal threshold that can split patients into 2 groups with residual leukaemic cells in terms of relapse status ... 186
5.3.3 Detection of MRD in bone marrow post induction ... 192
5.3.4 Detection of MRD in bone marrow post consolidation ... 196
5.3.5 Relation between level of MRD post induction and consolidation chemotherapy ... 199
5.3.6 Prognostic impact of conventional parameters in univariate analysis ... 203
5.3.7 Prognostic determinants in multivariate analysis ... 203
5.3.8 Prognostic impact of log difference in RFS ... 206
5.3.9 Prognostic impact of log difference in OS ... 206

5.4 DISCUSSION ... 210

CHAPTER 6: CHARACTERISTICS AND PROGNOSIS OF ADULT ACUTE MYELOID LEUKAEMIA WITH INTERNAL TANDEM DUPLICATIONS IN THE *FLT3* GENE ... 216

6.1 ABSTRACT ... 216

6.2 INTRODUCTION ... 217

6.3 RESULTS ... 220
6.3.1 Incidence of FLT3/ITD in our cohort ... 220
6.3.2 Characterisation of patients ... 220
6.3.3 Clinical response and prognosis of AML patients with and without FLT3/ITD ... 223
6.3.4 Evaluation of prognostic factors ... 227
6.3.4.1 Correlation to cytogenetics ... 227
6.3.4.2 Correlation with leukocyte count in FAB subgroups ... 228
6.3.4.3 Correlation with percentage of blasts at diagnosis ... 228
6.3.4.4 Correlation with FAB subtype ... 228
6.3.4.5 Correlation with Age and sex ... 228

6.4 DISCUSSION ... 229
6.4.1 Clinical implications of FLT3 mutations in AML ... 229
6.4.1.1 Incidence of FLT3/ITD ... 229
6.4.1.2 The impact of FLT3/ITDs on patient prognosis ... 230

CHAPTER 7: THE ONCOGENIC EVENTS OF FLT3 INTERNAL TANDEM DUPLICATION HAPPEN AT A STAGE OF STEM CELLS THAT POSSESS IL-3 ALPHA RECEPTOR (CD123) ... 237

7.1 ABSTRACT ... 237

7.2 INTRODUCTION ... 239

7.3 RESULTS ... 242
7.3.1 Expression of CD123 (IL-3 α receptor) in AML blast cells ... 242
7.3.2 Expression of CD123 (IL-3 α receptor) in AML stem cells $CD34^+/CD38^-$ ... 242
7.3.3 Expression of CD123 (IL-3 α receptor) in normal bone marrow $CD34^+/CD38^-$ fraction 242
7.3.4 Characterization and phenotype of leukaemic stem cells ... 244
7.3.5 Sorting AML stem cells ... 249
7.3.6 Optimisation of the PCR technique for low DNA concentrations ... 249
7.3.7 Detection of FLT3/ITD in the sorted AML stem cells ... 253
7.3.8 Sequencing the FLT3/ITD in two sorted AML stem cells ... 254
7.3.9 Detection of FLT3/ITD in the sorted population of LAP positive AML patient ... 254

7.4 DISCUSSION ... 258

CHAPTER 8: SUMMARY, GENERAL DISCUSSION, AND PERSPECTIVES...263

8.1 SUMMARY...263

8.2 GENERAL DISCUSSION...267
8.2.1 Clinical applications of MRD in AML...267
8.2.1.1 Comparison with previous clinical studies...267
8.2.1.2 Future directions for MRD detection...271
8.2.1.3 MRD detection using five-colour MFC...271
8.2.2 Potential limitations for MRD detection using immunophenotyping...272
8.2.3 Points for consideration...274
8.2.4 CD123 as a marker for LSC and a single standardised marker for MRD studies...276
8.2.5 Prognostic value of FLT3/ITD in AML...277
8.2.6 Does FLT3/ITD mutation occur at the level of the leukaemic stem cells?...278

8.3 CONCLUDING REMARKS...279

CHAPTER 9: BIBLIOGRAPHY...283

PUBLICATIONS...324

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Adhra Al-Mawali

Signed:----------------------------- Date:-----------------------------

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"Obstacles cannot crush me; every obstacle yields to stern resolve"

Leonardo da Vinci

"I don't know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Sir Isaac Newton (1642- 1727)

PUBLICATIONS ARISING FROM THIS THESIS

1. The Presence Of Leukaemia-Associated Phenotypes Is An Independent Predictor Of Induction Failure In Acute Myeloid Leukaemia. **Al-Mawali A**, L Bik T, Gillis D, Hissaria P, Mundy J, Lewis I. *International Journal of Laboratory Hematology (2008). In Press. Early online published article (accepted 17/9/07)*

2. Incidence, Sensitivity and Specificity of Leukaemia Associated Phenotypes in Acute Myeloid Leukaemia Patients using Specific Five-Colour Multiparameter Flow Cytometry. **Al-Mawali A**, Hissaria P, Gillis D, Lewis I. *American Journal of Clinical Pathology (2008). In Press. (accepted 21/12/07).*

3. The Oncogenic Events Of FLT3 Internal Tandem Duplication Happen At A Stage Of Stem Cells That Possess IL-3 Alpha Receptor. **Al-Mawali A**, Gillis D, Thomas D, Ramshaw H, Lopez A, Lewis I. (*2008*). *Manuscript in preparation.*

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ABBREVIATIONS

Ab	Antibody
ALL	Acute lymphoid leukaemia
AlloSCT	Allogeneic stem cell transplants
AML	Acute myeloid leukaemia
APL	Acute promyelocytic leukaemia
AutoSCT	Autologous stem cell transplants
BM	bone marrow
BSA	bovine serum albumin
CBF	core binding factor
CD	Cluster of differentiation
CD34	(sialomucin = haemopoietic progenitor cell antigen-1, HPCA-1)
CLL-1	C-type lectin–like molecule 1
CML	Chronic myeloid leukaemia
CR	Complete remission
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
ECD	Phycoerythrin-Texas Red (PE-TR)
EDTA	Ethylenediaminetetra acetic acid
FAB	French- American- British
FACS	Flouresence activating cell sorter
FC	Flow cytometry
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate

FLT3	Fms like tyrosine kinase3
FLT3/ITD	Fms like tyrosine kinase3 – internal tandem duplication
HSC	Haemopoietic stem cell
IL-3α	Interleukin 3 alpha receptor
IMDM	Iscove's modification of dulbecco's medium
LAP	leukaemia associated phenotype
Lin–	lineage specific marker negative
LSC	Leukaemic stem cells
MACS	Magnetic activated cell sorting
MCF	Mean cell fluorescence
MDR1	Multidrug resistance protein
MFC	Multiparametric flow cytometry
MFI	Mean fluorescence Intensity
MNC	Mononuclear cells
MoAb	Monoclonal antibody
MRD	Minimal residual disease
MUD	Matched unrelated donor
ND	Not done
NK	Natural killer
NOD/SCID	Non obese diabetic –severe combined immunodeficient
OS	Overall survival
PB	Peripheral blood
PBS	Phosphate-buffered saline
PC5	Cy5 coupled to R-Phycoerythrin.
PC7	Cy7 coupled to R-Phycoerythrin.
PCR	Polymerase chain reaction

PE	Phycoerythrin
PFA	Paraformaldehyde
RFS	Relapse free survival
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
RT	Room temperature
SCID	Severe combined immunodeficiency
WBC	White blood cell
WHO	World health organisation
WT	Wildtype

CONFERENCE PRESENTATIONS

New Direction in Leukaemia Research NDLR 2006 Conference

Sunshine Coast, Brisbane from 2 – 5 April 2006

"*Leukaemia-Associated Phenotypes in adult Acute Myeloid Leukaemia at diagnosis: their characteristics, clinical significance and usefulness as prognostic markers*".

***A. Al-Mawali*, J. Mundy, D. Gillis, L. B. To, I. Lewis**

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"*The Oncogenic Events Of FLT3 Internal Tandem Duplication Happen At A Stage Of Stem Cells That Possess IL-3 Alpha Receptor"*

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"*Incidence Of Leukaemia Associated Phenotypes In Acute Myeloid Leukaemia Patients: A Basis For The Design Of Specific Five-Colour Staining To Be Used For Minimal Residual Disease Investigation"* **AND**

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DEDICATION

To my grandmother: Thuraya

Who passed away when I was in the second year
of my PhD program. She was always very kind, encouraging, supportive and
praying for me all the time.

To my father: Hilal

You have taught me so much throughout my life. Your examples of
determination and ambition have encouraged me to always strive to be the
very best I can be.
Father: the best is yet to come!

To my mother: Zayana

You never stopped believing in me and
encouraging me to always give of my best.
Mum: This is a small gift.

To my son: Ezzaldeen

You have grown so much during the time in which I have been studying for
this PhD, and I feel that I have missed so many special moments of your
childhood while doing my PhD. I promise to be there for you always from
now on.

Primarily to my husband: Essa

For your endless support, love and encouragement through the
hardest days of my PhD, especially for your patience during the most difficult
times when I had to work on weekends and remain at work for very long
hours. *Essa: I dedicate this PhD to you*

ABSTRACT

Despite high remission rate in acute myeloid leukaemia (AML) after chemotherapy, relapse of the underlying disease remains a major challenge and one of the most frequent causes of treatment failure. In this study, the presence of leukaemia-associated phenotypes (LAPs) was first studied retrospectively using our standard diagnostic protocol with 3-colour flow cytometry. LAPs were present in 54 (64%) of 84 AML patients analysed between 2002 to 2004. The presence of LAPs was correlated with failure to respond to induction chemotherapy ($p < 0.05$) in univariate analysis. Presence of LAPs was shown to be an independent predictor for failure to respond to induction chemotherapy with a relative risk ratio of 1.6 ($p < 0.05$, 95% CI, 1.0-2.6) in multivariate analysis.

Subsequently, in a prospective study, we used 5-colour multiparametric flow cytometry (MFC) for detection of LAPs to determine if LAPs could be detected in a greater proportion of leukaemic patients and minimal residual disease (MRD) detection could therefore be applied in more patients. In 54 consecutive, newly diagnosed AML patients from 2005 to 2007, LAPs were identified in 51 (94%). Thus, MRD studies were potentially applicable to virtually all patients. The sensitivity and specificity of MFC technique was improved by analysing 10 normal and 5 regenerating bone marrows (BM) for the presence of these LAPs and by determining maximum log difference (LD). CD7, CD19, CD2, CD11b and CD56 were the most sensitive and reliable markers for MRD studies. LAPs were rarely detected in either normal or regenerating BMs. Through dilutional experiments from 50% LAPs to 0.001%, it was determined that 1 leukaemic in 10^4 and 10^5 normal cells could be detected using the improved techniques.

Of the 54 patients, 31 received chemotherapy, with 27 achieving complete remission (CR). Two were LAP negative and thus 25 were evaluable for MRD post induction and 22-post consolidation chemotherapy. Detection of MRD >0.15% was able to distinguish between two groups of patients according to relapse status. Although, the number of patients was small, detection of MRD post induction > 0.15% was shown to be an independent predictor of adverse prognosis for both relapse free survival (RFS) and overall survival (OS) in a multivariate analysis [p = 0.037 and 0.026, 95% CI (1.1-20.5 and 1.2-22.2), hazard ratio 4.7 and 5.2 respectively]. Post consolidation, there was a trend for patients with higher MRD values to show shorter RFS (p = 0.06).

MFC using 5-colour allows us to detect LAPs in virtually all AML patients and our preliminary results suggest the technique is a suitable approach for MRD analysis. However, 5-colour MFC is technically challenging, resource intensive, and may not be feasible in a routine diagnostic laboratory. This led us to assess whether we could identify other potential markers for LAPs.

Interleukin-3 alpha receptor- chain IL-3α (CD123) has been suggested to be a marker of leukaemic stem cells (LSC). These cells are thought to be responsible for initiating and maintaining leukaemic cell growth post chemotherapy and hence to give rise to relapse of the disease. Therefore, we analysed 34 AML patients for expression of CD123 in the blast population and defined a population containing leukaemic stem cells using the immunophenotypic markers $CD123^{+}/CD34^{+}/CD38^{-}$. Thirty-two (94%) of AML patients expressed CD123. We then used a molecular marker to determine whether CD123 expression was confined to the LSC. Thirty-nine patients were screened for the presence of FMS-like tyrosine kinase 3 - internal tandem duplication (FLT3/ITD) as the most common molecular abnormality in

AML patients. Of those, 12 (31%) were FLT3/ITD positive. In seven of them, $CD34^+/CD38^-/CD123^+$ and $CD34^+/CD38^-/CD123^-$ populations were sorted to homogeneity by Fluorescence Activated Cell Sorting (BD FACSAria™ Cell Sorter) and tested for FLT3/ITD. In six of seven patients with FLT3/ITD positive AML, we could not detect the mutation in the $CD34^+/CD38^-/CD123^-$ fraction, but the mutation was detected in the $CD34^+/CD38^-/CD123^+$ fraction in all seven patients.

This novel finding demonstrates that, the oncogenic event occurs in CD123 positive cells, thus supporting the concept that CD123 is a marker of the LSC in CD123 positive AML. This observation suggests novel treatment approaches employing surface marker CD123-targeting antibodies may be of use in the treatment of AML.

In conclusion, we demonstrate that using five-colour MFC improves LAP detection in AML and enables MRD studies using immunophenotyping to be applied to virtually all AML patients. Additionally, it increases the sensitivity of the technique for detecting LAP populations. Moreover, evaluation of MRD post induction chemotherapy is the most sensitive time point for detection of MRD, with MRD levels >0.15% predicting relapse and worse prognosis. As an alternative to using individualised LAPs specific to each patient, $CD34^+/CD38^-/CD123^+$ cells may in the future serve as a better marker for MRD studies. This marker identifies the putative LSC, which is responsible for regrowth of leukaemia and relapse of the disease. Thus, instead of looking at whole "blast" population which results in huge data analysis and interpretation for the different LAPs which may have different underlying biology, it may be more informative to look at the frequency of LSC after achieving CR using $CD34^+/CD38^-/CD123^+$ as the single LAP for MRD studies.

CHAPTER 1

General Introduction

CHAPTER 1: GENERAL INTRODUCTION

1.1 SIGNIFICANCE OF THE RESEARCH

AML accounts for approximately 25% of all leukaemias in adults in the Western world, and therefore is one of the most frequent forms of leukaemia (Greenlee *et al.* 2001; Wingo *et al.* 2003). Despite important advances in therapy of AML, the majority of patients will relapse and die from their disease. Progress in therapy and supportive care over the past three decades has led to gradual improvement in the overall results, especially in adults up to age 55-60 years. However, very little progress has been made in the long-term survival of older adults with AML. Studying the factors that cause relapse in AML patients will aid in understanding and predicting impending relapses and may lead to therapeutic intervention. In addition, awareness of the limitations of the current chemotherapeutic treatments will help us to develop newer approaches that will improve the survival rate. Furthermore, understanding the immunophenotyping and molecular mutations associated with the disease and correlate these with prognosis of AML and the mechanisms how these factors work together will lead to newer chemotherapeutic treatment that will target these specifically and ultimately cure the disease.

The literature review in this chapter will discuss AML starting from diagnosis to prognosis and the factors that predict prognosis with particular focus on MRD as one of the most important factors for predicting relapse using MFC approach.

The second part of this review will concentrate on LSC based on the current knowledge that LSC are acting as AML-initiating and -maintaining cells and therefore maybe the key elements for regrowth of leukaemic clone post chemotherapeutic treatments and thus responsible for relapse of the disease. Hence, LSCs should be the most important targets for chemotherapy in AML.

The last part will highlight on future perspectives and the objectives of this research as well as how these objectives might add to the current knowledge.

1.2 INTRODUCTION

1.3 LEUKAEMIA

The term "leukaemia" is derived from the Greek and means "white blood". Leukaemia is a malignant disease (cancer) of the BM and blood (Piller 2001). Leukaemia accounts for approximately 2% of all cancer cases and affects all ages and sexes (Appelbaum *et al.* 2001) (Zeeb and Blettner 1998). As leukaemia progresses, the function of the BM becomes impaired and if the disease is unchecked, the abnormal cells become dominant and are carried throughout the body by the bloodstream. Uncontrolled, leukaemia leads to infections, due to a decrease of normal white blood cells (WBC) ; severe anaemia, due to the lack of red blood cells; and bruising and haemorrhage, due to lack of platelets. The aim of leukaemia treatment is to bring about a CR, which may result in cure. Relapse indicates a return of the cancer cells and return of other signs and symptoms of the disease. For leukaemia, a CR that lasts five year after treatment often indicates cure. Leukaemia is broadly divided into four categories: myeloid or lymphoblastic, each of which can be acute or chronic. Acute leukaemia affects immature WBCs, progresses rapidly, and is the type most often seen in children and adults. Chronic leukaemia occurs most often in adults and elderly and progresses slowly, often over a period of many years.

1.4 WHAT IS AML?

AML is a life threatening neoplasm characterised by uncontrolled proliferation of myeloblasts. It represents a group of clonal haemopoietic stem cell (HSC) disorders

in which both failure to differentiate and overproliferation into the stem cell compartment results in the accumulation of myeloblasts (Stone *et al.* 2004).

1.5 EPIDEMIOLOGY

Each year in Australia around 715 people are diagnosed with AML. The overall incidence is 3.7 cases per 100,000 of the population (Leukaemia Foundation website: http://www.leukaemia.org.au/web/aboutdiseases/leukaemias_aml.php (online, accessed 12 July 2007). The median age at diagnosis is approximately 70 years (Tallman 2005).

Risk factors for the development of AML include the following:

1.5.1 Genetic Predisposition

Chromosomal instability in a number of autosomal-dominant conditions such as Bloom's syndrome, ataxia-telangiectasia, neurofibromatosis, and Fanconi's anemia, as well as germline mutations in the *AML-1* gene are associated with a higher risk of the development of AML (Appelbaum *et al.* 2001). In addition, congenital immunodeficiency disorders including infantile X-linked agammaglobulinemia and Down's syndrome have also been associated with an increased prevalence of AML (Stone *et al.* 2004).

1.5.2 Environmental Exposure

Ionizing radiation (Levine and Bloomfield 1992) and organic solvents such as benzene and other petroleum products have been associated with an increased risk of developing AML (Austin *et al.* 1988). Both *ras* mutations and polymorphisms resulting in inactivation of NAD(P)H: quinone oxidoreductase have been found in patients with these exposures (Smith *et al.* 2001).

1.5.3 Prior Therapy

Therapy-related AML usually develops after alkylating agent-induced damage at a median of 5 to 7 years after therapy for the primary malignancy, and is generally associated with an antecedent myelodysplastic disorder (Le Beau *et al.* 1986). DNA-topoisomerase II agents may also generate gene rearrangements leading to AML (Albain *et al.* 1990).

1.5.4 Prior Bone Marrow Disorders

Secondary AML can develop in patients with different haematological disorders such as aplastic anaemia and severe congenital neutropenia. Myelodysplastic and myeloproliferative syndromes can also progress to AML (Lowenberg *et al.* 1999).

1.5.5 Age

The incidence of AML increases with age. Age-adjusted population incidence is 17.6 per 100,000 for people 65 years old or older, compared with 1.8 per 100,000 for those younger than 65 years in the United States (Cancer Facts and Figures-1991. Atlanta, American Cancer Society Inc, 1991, pp 1-31). Likewise, chromosomal abnormalities occur with larger frequency among this older population of patients (Stone *et al.* 1995) .

1.6 PATHOPHYSIOLOGY

Haemopoiesis in normal cells involves the differentiation of a HSC into all blood cell lineages including granulocytes, monocytes, erythrocytes, lymphocytes, and megakaryocytes. In AML, this process of cell differentiation is interrupted in those cells committed to the myeloid lineage. Some literature supports the concept of a single transformed HSC, whereas others argue that transformation can occur at any point from stem cell to lineage-committed progenitor cell (Gilliland 2002; Passegue

et al. 2003; Gilliland *et al.* 2004; Hiddemann *et al.* 2005). This transformation can occur either as a *de novo* event or associated with previous therapy.

Several molecular and genetic lesions have been identified in AML, leading to advances in defining its pathogenesis. Most cases of AML are sporadic, characterised by acquisition of somatic mutations in haemopoietic progenitors that confer a proliferative and/or survival advantage, impair haemopoietic differentiation, and confer properties of limitless self-renewal (Gilliland *et al.* 2004). Most cases of AML can be attributed to acquired somatic mutations that occur in haemopoietic progenitors. Many AML disease alleles were identified by molecular cloning of recurring chromosomal translocations, which often pinpoint the location of leukaemogenic fusion genes. Examples include fusions involving core binding factor (CBF), retinoic acid receptor-α (RARα), HOX family members, the MLL protein, and transcriptional co-activators such as CBP, MOZ, and TIF2. More recently, it has been appreciated that point mutations also contribute to a significant proportion of cases, including activating mutations in receptor tyrosine kinases such as FLT3 and KIT, and in RAS family members. In addition, loss-of-function point mutations have been identified in haemopoietic transcription factors such as AML1, C/EBPα, and GATA-1 (Table 1-1).

Table 1-1 Correlation between Morphologic Subtypes of AML and Recurring Chromosomal Translocations (adapted from Hoffman: Hematology: Basic Principles and Practice: 4th edition)

FAB Subtype	Cytogenetic Aberration	Molecular Aberration
M0, M1	t(10:11)	CALM-AF10
M1	Trisomy 11	Partial duplication of MLL
M2	t(8;21)	AML1-ETO
M3	t(15;17), t(11;17), t(5;17)	PML-RARα, PLZF-RARα, NPM-RARα
M4eo	inv(16)	CBFβ-MYH11
M4 with erythrophagocytosis	t(8;16)	MOZ-CBP
M5	11q23 translocations	MLL fused to one of its partner genes
M6	t(3;5)	NPM-MLF1
M1, M2, M4, and MDS	t(6;9), ?t(9;9)	DEK-CAN, SET-CAN
Multiple FAB subtypes and MDS	t(16;21)	TLS/FUS-ERG
M2, M4	t(7;11)	NUP98-HOXA9
MDS, *de novo*/T-AML	inv(11)	NUP98-DDX10
MDS, T-AML	t(3;21)	AML 1-EAP/MDS1/EVI1

1.7 DIAGNOSIS

The diagnosis of AML involves the identification of greater than 20% leukaemic blasts in the BM. Additional analysis then must separate AML from acute lymphoblastic leukaemia (ALL) by demonstrating commitment to the myeloid lineage, which is usually achieved by immunophenotyping. The leukaemic clone giving rise to AML can occur at any point in the differentiation of the myeloid cell, creating heterogeneity among patients.

1.7.1 Classification according to biological and clinical features

The subtypes of AML were previously described as M0 through M7 by the French-American-British (FAB) system. However, in 2001, the World Health Organization

(WHO) reclassified AML into four categories, in an attempt to more precisely predict the prognosis and biologic properties of AML subcategories and improve the clinical significance of the system (Vardiman *et al.* 2002). This new classification reflected those entities with similar biological and clinical features. It also takes into account the morphologic, genetic, and immunophenotypic features of the disease entities. The four categories are AML with recurrent genetic abnormalities, AML with multilineage dysplasia, therapy-related AML and myelodysplastic syndromes, and AML not otherwise categorized, which is smilar to the FAB classification. The WHO classification system differs from the FAB system in that the previous blast cell threshold of 30% for diagnosis of AML has been reduced to 20%, and patients with recurring cytogenetic abnormalities are now categorized as having AML regardless of blast percentage (Vardiman *et al.* 2002).

1.7.2 Classification according to prognosis

The British Medical Research Council (MRC) AML 10 trial (Grimwade *et al.* 1998) and a Cancer and Leukaemia Group B (CALGB) trial (Mayer *et al.* 1994) found that patients could then be classified prognostically based on their pre-treatment cytogenetics. Patients could be separated into three categories based upon response to induction treatment, relapse risk, and OS: favourable, intermediate, and adverse cytogenetic groups (Grimwade *et al.* 1998). Favourable cytogenetics include t(15;17), t(8;21) and inv(16). Poor cytogenetics include complex ($\geq$3) abnormalities, deletion of 5q, abnormal 3q, and deletion of chromosome 7 (Bullinger *et al.* 2004).

1.8 CHEMOTHERAPEUTIC TREATMENT

Therapy for AML includes remission induction followed by post-remission chemotherapy. For some patients, this is followed by haemopoietic stem-cell

transplantation. Treatment recommendations for AML differ, taking into account patient age, cytogenetics, and prognostic factors. The recommendations are often divided into those for patients younger than 60 years and those older than 60 (Table 1-2).

The purpose of *induction chemotherapy* is to induce a CR by eliminating leukaemic cells enabling proper function of the BM to recover. Induction regimens vary but usually include an anthracycline and cytarabine, with or without etoposide. In most Australian centres, induction chemotherapy consists of idarubicin 9 mg/m^2 for 3 days, cytarabine 3 g/m^2 bd on days 1, 3, 5 and 7, and etoposide 75 mg/m^2 for 7 days. Using this regimen, 80% of patients achieve CR (Bradstock *et al.* 2005).

Post-remission chemotherapy then aims to eradicate any residual disease in an attempt at cure. Post-remission chemotherapy regimens also vary between centres. Approaches include 5 + 2 + 5 regimen of cytarabine, an anthracycline and etoposide. The number of consolidation courses to be given is usually two but some centres give up to four courses.

Table 1-2 Treatment-Related Statistics for AML Stratified by Age (Adapted from reference Stone RM, O'Donnell MR, Sekeres MA. Washington, DC: *American Society of Hematology Education Book*, 2004).

NOTE: This table is included on page 9 of the print copy of the thesis held in the University of Adelaide Library.

BM transplantation is an additional option for post-remission therapy in patients with an HLA-matched sibling or matched-unrelated donor.

The treatment of acute promyelocytic leukaemia (APL) varies from the recommendations for AML. As an alternative, ATRA, which promotes differentiation of leukaemic promyelocytes into mature cells, has been shown to improve both disease-free survival and OS compared with chemotherapy alone (Tallman *et al.* 1997). ATRA, in combination with an anthracycline, is currently the standard of care for patients with APL.

1.9 OUTCOMES

The outcome for adults with AML varies based on a variety of well-defined factors including age of the patient, intensity of post–remission therapy (in younger adults), and biologic characteristics of the disease. The most important of which is the karyotype at diagnosis (Tallman 2005).

A number of specific molecular mutations have been described in AML. These are of emerging importance in the risk stratification, particularly in patients with normal cytogenetics (Tallman 2005). The over expression of FLT3/ITD, the commonest molecular abnormality identified in AML, confers an adverse prognosis.

These proteins and genes provide targets for which new agents have been developed, and these agents are being incorporated into new therapeutic strategies. Over the past few decades, success in the treatment of AML has improved only for patients under the age of 60. In 1966, the median survival of adult patients with AML was 40 days (Council 1966). Today, AML patients under the age of 60 have complete response rates of 70% to 80% after induction chemotherapy (Mayer *et al.* 1994). However, OS remains at only about 50% for those who achieve a complete response, or 30% overall (Appelbaum *et al.* 2001). In 1998, the MRC AML 10 trial found that patients could be separated into three prognostic groups: favourable, intermediate, and adverse, defined by pre-treatment cytogenetics. Overall survival at 5 years was found to be 65, 41, and 14%, respectively (Grimwade *et al.* 1998). If a patient undergoes an allogeneic HSCT while in first remission, the complete-response rate ranges from 45% to 65%, although patient selection influences these numbers (Edenfield and Gore 1999). However, in relapsed AML, complete response after allogeneic HSCT is 35% or less (Grigg *et al.* 1999). Nevertheless, the prognosis for older patients remains poor. In the MRC AML 8 trial, the remission

rate was 70% for patients under age 50 years old, 52% for those 60 to 69 years old, but only 26% for those older than 70 years (Rees *et al.* 1986).

1.10 PROGNOSTIC FACTORS IN AML AT DIAGNOSIS

Over the years, a number of prognostic markers have been identified in AML. Understanding of the individual prognostic factors has become of significant importance for treatment choice in today's clinical practice. In addition to cytogenetics, in recent years new leukaemia-related prognostic factors (e.g. *FLT-3* mutations) have been recognised, which have an important impact on prognosis.

1.10.1 Age and Gender

Age has a strong prognostic impact on the outcome of AML (Schoch *et al.* 2004). There is a decline in the prognosis for patients with AML with increasing age. There is a progressive decrease in treatment response and survival as a function of decade of age (Oxford Collaborative AML Intergroup, pers. comm.). Patients 60 years of age or younger have response rates to remission induction chemotherapy of approximately 75% and approximately 35-40% of them survive following diagnosis for 5 years or more (Mayer *et al.* 1994; Burnett *et al.* 1998). In contrast, patients with AML who are more than 60 years old have an overall response to induction therapy of 45-55%, and fewer than 10% of these survive for a minimum of 5 years (Lowenberg *et al.* 1998). While the results of treatment have improved steadily over the last 20 years in younger adults, due to more intensive cytotoxic treatment, the use of stem cell grafts and improvements in supportive care, no significant change in outcome has been noted among individuals of 60 years of age. The reasons for the poorer outcome of patients of higher age most probably relate to the increased frequency of unfavourable cytogenetics among older patients with AML, a greater

frequency of antecedent myelodysplasia, as well as a greater frequency of drug resistance phenotypes. Also, because of the reduced general health of older patients, they cannot withstand intensive chemotherapy as well as younger individuals do (Leith *et al.* 1997).

Several large databases of phase III studies in untreated patients with AML would also suggest that female sex is an independent favourable prognostic parameter for disease-free survival, although of moderate impact only (Lowenberg 2001).

1.10.2 Cytogenetics and molecular genetics

Cytogenetic abnormalities are seen in approximately 60% of cases in AML and are highly predictive of response to therapy as well as the probability of relapse (Table 1-3) (Ferrara *et al.* 2004; Avivi and Rowe 2005). The translocations t(8;21), inv16 and t(15;17) normally carry a relatively favourable prognosis and these are more commonly seen among younger patients with AML.

Among patients with these chromosomal abnormalities or the corresponding molecular abnormalities AML1-ETO, CBFb-MYH11 (core binding factor beta gene-smooth muscle myosin heavy chain gene), PML-RARa the response to induction therapy is 80% or greater and for those entering remission the probability of relapse is 30-40% resulting in 5 years survival rates of 60-70% (Wheatley *et al.* 1999).

In contrast patients with t(6;9), 11q23 (MLL-gene) abnormalities (Dimartino and Cleary 1999), monosomies of chromosomes 7 (-7) or 5 (-5) and complex cytogenetic abnormalities (three different cytogenetic aberrations or more) represent a group with adverse cytogenetics and generally have a distinctively poor prognosis. In these individuals (when 60 years or younger), the average complete response to induce treatment is 60% with a majority (approximately 80%) relapsing within 2

years so that their survival at 5 years is approximately 15% only (Wheatley *et al.* 1999).

Table 1-3 Cytogenetic Risk stratification (Adapted from Grimwade *et al* paper) (Grimwade *et al.* 2001)

NOTE: This table is included on page 13 of the print copy of the thesis held in the University of Adelaide Library.

1.10.3 Molecular markers

Many molecular markers have been suggested to predict the prognosis in AML; however, only a few seem to have a significant independent prognostic impact.

1.10.3.1 FLT3

Several studies have shown that mutations in the FLT3 tyrosine kinase receptor, resulting in its overexpression, are strongly associated with an increased relapse risk and reduced OS in patients younger than 60, irrespective of cytogenetics (Kottaridis *et al.* 2001; Meshinchi *et al.* 2001; Spiekermann *et al.* 2003; Wang *et al.* 2004; Chen *et al.* 2005; Yanada *et al.* 2005). The presence of FLT3/ITD may be even

more important than cytogenetics in predicting relapse risk and disease-free survival (Kottaridis *et al.* 2001).

FLT3/ITD mutations are most frequent in patients with APL (37%) and those with a normal karyotype (34%) (Kottaridis *et al.* 2001). Indeed, standard-risk patients bearing this mutation have a significantly higher relapse rate compared with patients without the mutation (74 vs. 48%). However, the prognostic impact of FLT/ITD positivity in APL patients is still unclear (Shih *et al.* 2003).

1.10.3.2 CCAAT/ enhancer binding protein a

CCAAT/enhancer binding protein *a* (CEBPA) is a transcription factor involved in the regulation of myelopoiesis. Mutant CEBPA has recently been suggested to predict for a favourable prognosis in patients with normal karyotype, irrespective of FLT3 mutations and MLL PTDs (Frohling *et al.* 2004).

1.10.3.3 Nucleophosmin (NPM1)

NPM1 is mutated in around half of cases with a normal karyotype (Falini *et al.* 2005). Although the functions of NPM1 are not completely understood, it is thought to play an important role in centrosome assembly, and has RNA binding and chaperone activity (Falini *et al.* 2006). As an alternative to sequence analysis, the presence of an underlying NPM1 mutation can be inferred by immunohistochemistry, which shows an abnormal cytoplasmic localization of the protein in leukaemic blasts (Falini *et al.* 2005; Falini *et al.* 2006).

NPM1 mutations positively correlate with AML with high WBCs, normal karyotypes, and FLT3/ITD mutations. NPM1 mutations associate inversely with the occurrence of CEBPA and NRAS mutations. Roel G *et al* (Verhaak *et al.* 2005) demonstrate that patients with intermediate cytogenetic risk AML without

FLT3/ITD mutations but with NPM1 mutations have a significantly better OS and event free survival (EFS) than those without NPM1 mutations. They were able to demonstrate that NPM1 mutations express independent favourable prognostic value with regard to OS and EFS in multivariable analysis.

1.10.3.4 Mixed lineage leukaemia gene

This molecular rearrangement, identified in patients with normal cytogenetics, is a tandem duplication of the internal portion of the *MLL* gene that spans exons 2 to 6 or exons 2 to 8. This partial tandem duplication (PTD) is detected in approximately 10% of all AML patients with normal cytogenetics and in 90% of AML patients with trisomy 11 as the only abnormality. Some studies have suggested MLL PTD status to be one of the most significant independent prognostic factors predicting for survival in patients with a normal karyotype (Dohner *et al.* 2002). However, other studies have failed to confirm these results; thus, the significance of this mutation as an independent prognostic factor remains uncertain (Steudel *et al.* 2003).

1.10.3.5 Wilms tumor gene

The Wilms tumour gene is overexpressed in patients with AML. A high level of expression of this gene has recently been reported to be the most significant adverse independent prognostic factor affecting disease-free survival and relapse risk in patients younger than 60, stronger than leukocyte count at presentation, FLT3 mutations, and cytogenetics (Ogawa *et al.* 2003; Barragan *et al.* 2004; Cilloni *et al.* 2006).

1.10.3.6 Multidrug resistance genes

One of the most important mechanisms of resistance to chemotherapy is the rapid drug efflux mediated by various members of the ATP-binding cassette transporter

superfamily such as the multidrug resistance gene 1(MDR1) encoding for P-glycoprotein, multidrug resistance-associated protein (MRP1), and lung resistance protein (LRP) as well as the soluble drug resistance–related calcium-binding protein (sorcin). These genes are mostly expressed in elderly patients, which may partly explain these patients' inferior response to chemotherapy. However, it is still unclear whether MDR positivity has a similarly independent adverse prognostic impact on CR rate and long-term outcome of younger AML patients (Galimberti *et al.* 2003; Galimberti *et al.* 2004; Schaich *et al.* 2004). Furthermore, a significant number of patients may relapse despite a low MDR1/MRP1 expression, suggesting the involvement of other intracellular resistance mechanisms.

1.10.4 Secondary leukaemia and advanced disease

Secondary leukaemia has traditionally been considered to be an independent prognostic factor associated with reduced remission rate and shorter survival (Schoch *et al.* 2004). However, recent studies suggest that despite being generally associated with a poor prognosis, it has almost no independent impact on survival but tends to correlate with unfavourable karyotype. Patients with therapy-related AML who have favourable cytogenetics at presentation have been reported by some groups to have a prognosis similar to those with *de novo* AML (Schoch *et al.* 2004). It is impossible to determine whether other factors, related to prior therapy, have contributed to the adverse prognosis (Wheatley *et al.* 1999; Schoch *et al.* 2004).

1.10.5 White blood cell count

In several large prospective studies of previously untreated patients with AML, the WBC count stands out as an independent prognostic factor. There is a subgroup of patients presenting at diagnosis with hyperleucocytosis (WBC more than 100 X

10^9/l) who generally have a significantly reduced complete response rate and a greater rate of relapse (Kiyoi *et al.* 1999). The WBC count also provides a practically useful parameter for distinguishing the prognostic heterogeneity among patients with favourable cytogenetics. Thus those with t(8;21), inv(16) and t(15;17) as well as a WBC of 20 X 10^9/l or greater do significantly worse than those with the same cytogenetic subtypes and low WBC counts.

1.10.6 Immunophenotyping

Cell stage and cell lineage specific markers have been used for immunophenotyping of AML. Despite the fact that the cytogenetic and molecular genetic markers hold a strong prognostic impact, the value of immunophenotyping for the assessment of prognosis has not been firmly established. This is true for the myeloid markers CD13, CD14, CD15, CD11b, HLA-DR, CD117 (c-kit) as well as CD34 for which controversial results regarding the prognostic value for response have been obtained in a variety of studies (Bradstock *et al.* 1994; Kanda *et al.* 2000; Basso *et al.* 2001).

1.10.7 FAB Classification

In multivariate analysis, the cytomorphological classification also appears to add some prognostic information, independent of cytogenetics and molecular genotyping. In particular, AMLs with the FAB types M0, M6 and M7 appear to correlate with an inferior outcome in most of the large phase III prospective trials. Since patients with M0, M6 and M7 represent a minority of AML, the prognostic value of FAB classification remains limited. In clinical practice, FAB classification is not generally applied to risk assessment in AML. In small series of patients with minimally differentiated AML (FAB M0), AML-M0 was associated with a poor

prognosis (Bennett *et al.* 1991; Stasi *et al.* 1994; Cuneo *et al.* 1995; Kaleem and White 2001).

1.10.8 Gene expression profiles

Specific behaviour of an individual gene is likely to be of less significance than the product of clusters of genes. Therefore, gene expression profiles, determined by gene microarrays, are likely to have greater prognostic significance (Bullinger *et al.* 2004; Valk *et al.* 2004). Gene microarrays contain tens of thousands of short DNA probes of specified sequences arrayed in an orderly fashion. Fluorescently labelled RNAs enable the detection of the corresponding genes, expressed by leukaemic cells. It is likely that a specific gene expression profile is independently predictive of outcome. Indeed, patients expressing t(15;17) tend to have a specific pattern of gene expression profiling, whereas patients expressing t(8;21)/inv16 seem to be divided into newly defined "gene cluster" subgroups, indicating the heterogeneity of these "cytogenetic favourable risk group" patients (Bullinger *et al.* 2004). Furthermore, patients presenting with a normal karyotype do separate into two major gene cluster groups, significantly differing in their long-term OS.

It is anticipated that gene expression profiling will lead to a more accurate molecular classification of adult AML, enabling the development of specific targeted therapies for different AML subtypes.

1.11 PROGNOSTIC FACTORS IN AML AFTER INDUCTION THERAPY

Several prognostic factors after induction therapy have been reported to affect long-term survival as discussed below. However, the precise impact of these parameters on the outcome of various types of leukaemia has still to be proven.

1.11.1 Response to first induction

Different groups have reported that time to achievement of complete morphologic remission is an independent post induction prognostic factor in AML (Wheatley *et al.* 1999; Estey *et al.* 2000; Barragan *et al.* 2004). Response to initial induction therapy has been suggested to be an independent prognostic factor, irrespective of the cytogenetics at presentation (Wheatley *et al.* 1999; Estey *et al.* 2000; Kern *et al.* 2003). Whereas patients with resistant disease (more than 15% blasts after one induction) have a very poor prognosis (5-year OS = 21%), patients who achieve a partial remission (5 to 15% blasts) have slightly worse prognosis compared with those who achieve CR (less than 5% blasts) (OS = 44 vs. 53%) (Wheatley *et al.* 1999).

In contrast to these previous studies, a recent report by the Eastern Cooperative Oncology Group concluded that time to CR is not a significant prognostic factor in AML. Five-year OS was 36% in patients with disease remission after one cycle (n = 406) vs. 35.7% in patients with disease remission after second induction (n = 805) (Rowe *et al.* 2004). These differences may arise from different criteria used to define response to first induction.

1.11.2 Minimal residual disease as a predictive factor for relapse

Despite a high remission rate, approaching 80% in younger adults, most patients with AML eventually relapse, indicating the existence of residual leukaemic cells below the threshold of morphologic detection. This cell population is defined as minimal residual disease and can be detected by several techniques: polymerase chain reaction (PCR) (Raanani and Ben-Bassat 2004), multiparametric flow cytometry (Vidriales *et al.* 2003), and fluorescence *in situ* hybridization (FISH) (Schmidt *et al.* 2004). The exact time following induction at which MRD positivity

defines a higher risk for relapse is unclear. It seems to be dependent on leukaemic subtype and MRD technique.

While the detection of MRD may predict relapse, it is important to emphasise that much of the administered post remission therapy is directed at eradicating a leukaemia cell population below the level of detection by even the most sensitive MRD techniques. The precise value of serial MRD determinations to predict for response has not been as clear-cut as in ALL.

1.12 WHAT IS MINIMAL RESIDUAL DISEASE?

Despite advances in the treatment of AML, a significant proportion of patients relapse, usually with the same malignant clone found at diagnosis.

MRD describes the lowest level of disease detectable using available methods. MRD is a term used when there is evidence (either immunophenotypic, molecular or cytogenetic) that leukaemic cells remain in the BM, but there are insufficient cells to be detected by routine examination under the microscope. Previously, light microscopy and cytogenetic analysis were standard techniques used for the detection of residual leukaemic cells in the blood and marrow of patients after treatment. However, the sensitivity of these methods does not allow identification of low levels of disease, nor do they allow accurate quantification of leukaemic cell numbers. Since these residual leukaemic cells may be the source of ultimate relapse, there has been great interest in developing techniques able to detect very low level of leukaemic cells.

For many years, Southern blot hybridisation has been the gold standard for the detection of DNA sequence alteration at specific genetic loci, but it has been largely superseded by PCR amplification of DNA sequences. However, PCR can only be applied to less than half of AML patients who express specific marker detectable by

real-time PCR (Raanani and Ben-Bassat 2004) and thus another technique with a similar level of sensitivity is necessary for MRD detection.

1.13 METHODS AVAILABLE FOR THE DETECTION OF MRD

To date, several methods have been used to determine the presence of residual leukaemic cells in blood and BM (Table 1-4).

Methods include: morphology, cell culture assays, cytogenetics analysis, fluorescence *in situ* hybridization techniques, molecular analyses including Southern blotting and PCR, and flow cytometry and immunophenotypic analyses.

1.13.1 Morphology

In AML, remission is the term used to describe a BM containing fewer than 5% blast cells using conventional light microscopy. However, this may still represent a considerable tumour burden since, at diagnosis, the leukaemic cell number may be 10^{12} and, following therapy, the leukaemic cell number may drop only by 2 logs to 10^{10} even in the presence of fewer than 5% marrow blasts (Kern *et al.* 2005). Standard morphology alone is not a sensitive method for determining low level of disease and is not a useful technique for predicting impending relapse.

1.13.2 Cell culture assays

These involve growing T-cell- depleted marrow in culture after the patient has undergone treatment, followed by subsequent morphological, immunophenotypic and karyotypic analyses on the colonies produced. Due to variability of culture techniques between and within laboratories, this method has proved unreliable and insensitive for detecting persisting blasts. In addition, culture techniques do not provide any estimate of cell number and hence provide little information about leukaemic cell burden (Provan and Gribben 2005).

1.13.3 Cytogenetic analysis

Detection of non-random chromosomal translocation is of great value in the diagnosis of leukaemia. Chromosomal abnormalities are present in at least 60% of patients with AML (Keating *et al.* 1988). However, karyotypic analysis is of limited value following therapy, with a sensitivity level of around 5%, making it little better than standard morphological analysis. In addition, cytogenetics is a laborious procedure and its success relies on obtaining adequate numbers of suitable metaphases (Mrozek *et al.* 1997; Mrozek *et al.* 2004).

1.13.4 Fluorescence in situ hybridization techniques (FISH)

FISH can detect smaller chromosomal abnormalities than standard karyotyping and allows analysis of interphase nuclei. FISH permits karyotypic analyses of non-dividing cells and can recognize numeric and structural chromosomal abnormalities (Anastasi *et al.* 1990; Bentz *et al.* 1994).

The technique is useful in the diagnosis of trisomies and monosomies. The sensitivity of the technique is around 1%, making it more useful than standard karyotyping for follow-up marrows in patients with leukaemia, but is still of limited value for MRD detection because of the limited sensitivity and only a proportion of leukaemias will have abnormalities detected by FISH (Bentz *et al.* 1994; Arkesteijn *et al.* 1996).

1.13.5 Polymerase Chain Reaction (PCR)

PCR has emerged as a promising approach for the detection of MRD. It is a technique that involves amplification of specific DNA sequence. PCR offers the advantage of very high sensitivity with a detection limit of approximately 10^{-4} to 10^{-6} (Miller *et al.* 1993) (Provan and Gribben 2005). Although PCR techniques

appear to be promising for monitoring MRD in AML, they are currently applicable only on leukaemias that bear specific DNA markers such as fused genes. The commonest chromosomal translocations, i.e. t(8;21), t(15;17) and inv(16), comprise only about 30% of patients with AML (Yin and Tobal 1999).

1.13.6 Flow cytometry and immunophenotyping

Immunophenotypic analysis using single monoclonal antibodies (MoAbs) to cell membrane or cytoplasmic proteins lack absolute specificity for leukaemia and is therefore of limited value. However, using accurate combination of MoAbs allows more specific detection of leukaemic cells and can be used for quantification of MRD. Using combination of MoAbs / multi-colour flow cytometric analysis, the sensitivity of this technique can be greatly enhanced.

The MFC approach to the detection of MRD in patients with AML is based on the premise that leukaemic cells display aberrant phenotypic features that allow their distinction from normal cells at diagnosis. These are called leukaemia-associated phenotypes (LAPs) (Reading *et al.* 1993; Macedo *et al.* 1995; Macedo *et al.* 1995; Kern *et al.* 2003). These LAPs are either not present or only very infrequently present on normal blood or BM cells.

For AML, the most relevant type of aberrations include (1) asynchronous antigen expression (simultaneous expression of early and late markers in one cell such as the co-expression of the CD34 and CD15 antigens), (2) lineage infidelity (expression of the lymphoid-associated markers i.e. CD2, CD3, CD5, CD7, CD10, and CD19 on myeloid blast cells), (3) antigen over-expression (abnormally increased expression of a certain antigen per cell), (4) aberrant light-scatter properties (the expression of the lymphoid-associated antigens in blast cells displaying a relatively high forward scatter (FSC) and side scatter (SSC),

Figure 1-1 Leukaemia treatment outcomes. The red line indicates a sequential reduction of the leukaemic cell mass with the exception of a slight increase before the second consolidation therapy. Eventually, cure is achieved. The green lines indicate cases with relapses of AML in which increasing MRD levels are present before relapse. The light blue area refers to the cytomorphologic finding of 1% to 5% BM blasts, which is compatible with CR. The intermediate blue area refers to a 0% BM blasts count with MRD levels detectable by MFC or QRT-PCR. The dark blue area refers to a 0% BM blasts count with MRD levels below the sensitivity of MFC and QRT-PCR (adapted from Kern *et al* (Kern *et al.* 2005).

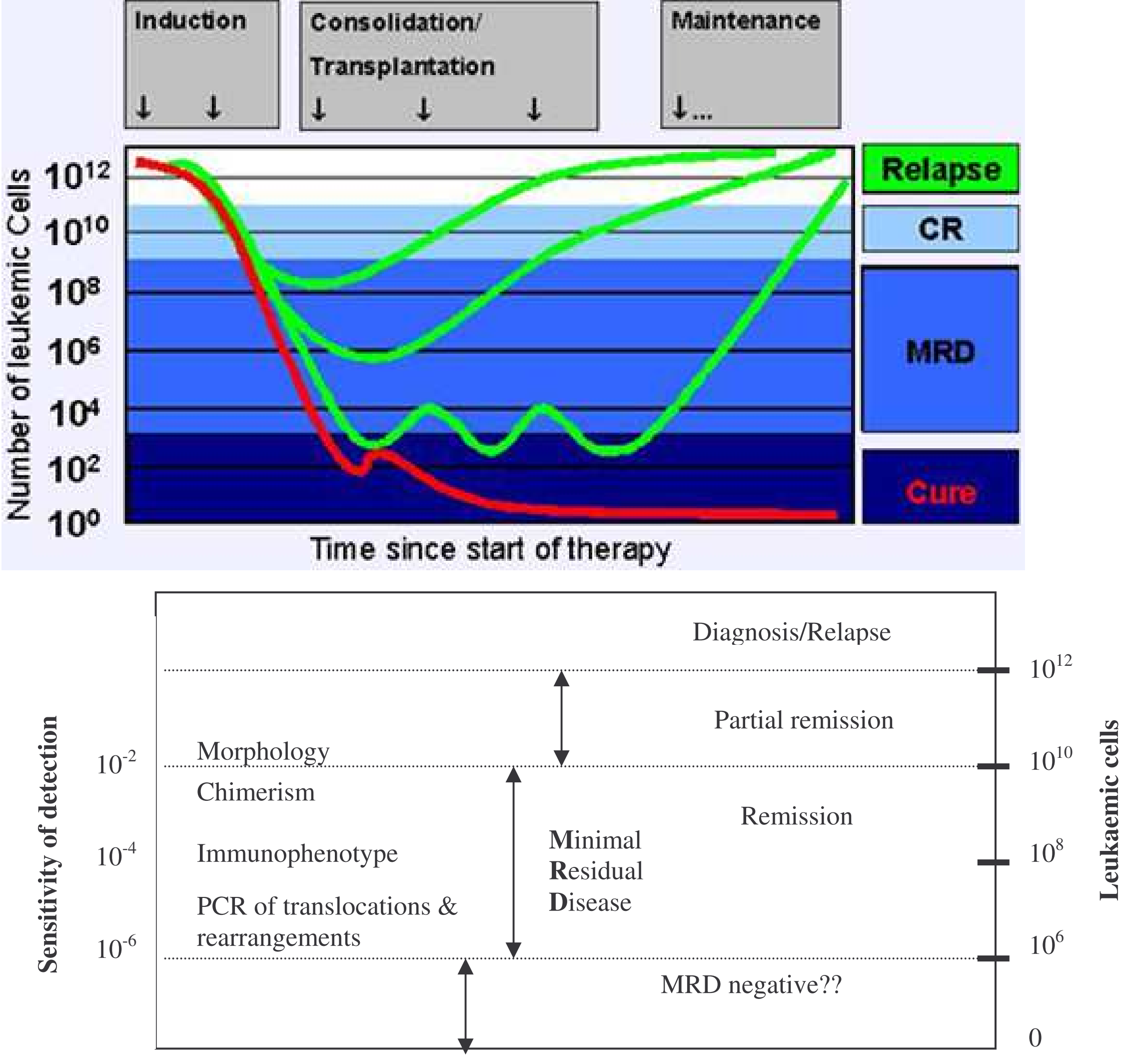


Table 1-4: Methods for detection of MRD in AML

Technique	Advantages	Disadvantages	Sensitivity
Standard Morphology		-Low sensitivity - Not suitable for low level of leukaemic cells.	1-5%
Cytogenetics		-Labour intensive -Slow -Requires metaphase chromosome preparations	5%
Fluorescence *in situ*	- Dividing cells are not required - Large number of cells can be analysed in a short time - Interphase FISH precludes need for high quality metaphases (cf. standard cytogenetics)	-Labour intensive -Limited sensitivity	0.3-5%
Gene rearrangements Southern blotting	-Low risk of contamination -Patient specific	-Labour intensive -Slow -Limited sensitivity	1-5%
PCR analysis	- Can be identified with limited set of primers - High stability of DNA - Relatively easy - Rapid (1-3 days) - No (or very low $<10^{-6}$) background in normal cells - Sensitive - Patient specific	-False positive results -Applied in < 50% of AML patients - Relatively expensive	10^{-4} to 10^{-5}
Flow cytometric Immunophenotyping	- Applicable for most patients (>80%) - Quantification simple - Single cell analysis - Cell viability can be determined - Information on normal cells -Relatively easy - Cheap - Rapid (1-2 days) - Relatively patient-specific	- Not as specific as PCR - Presence of subpopulation in AML - Immunophenotypic shifts can occur between diagnosis and relapse	10^{-4}

corresponding to normal myeloid cells) (Campana and Pui 1995; Macedo *et al.* 1995; San Miguel *et al.* 1997) and (5) absence of lineage specific antigens (absence of antigen expression such as CD13 and CD33 on myeloid blasts) (Voskova *et al.*

2003). These LAPs can be identified by triple- or quadruple-colour staining techniques performed with antibodies conjugated to different fluorochromes. In reality, using more than two colour staining allows a more precise detection of LAPs. Based on this, the current strategy for MRD studies relies on the following steps:

(1) Identification of the aberrant phenotypes by a multiple staining technique,

(2) Definition of a patient "fingerprint" or aberrant immunophenotype (LAP) ,

(3)Tracking residual leukaemic cells after achieving CR and during subsequent follow-up, using the specific patient LAPs (Campana and Pui 1995) (Figure 1.2).

1.14 FACTORS THAT INFLUENCE FLOW CYTOMETRY FOR DETECTING MRD

The applicability and feasibility of MFC approach depends on two factors. Firstly, the frequency of LAPs that can be identified in AML, and hence the number of patients that can be monitored for MRD by MFC. Secondly, the level of sensitivity which can be reached using MFC.

1.14.1 Frequency of LAPs

The proportion of leukaemia cases that can currently be monitored for MRD by FC varies from laboratory to laboratory. Factors influence this variability include: (a) the number of markers tested, (b) the inclusion of normal and regenerating BM samples post chemotherapy in determining normal versus abnormal phenotypes and levels, (c) the rigour with which the laboratory defines LAPs and MRD (Campana and Coustan-Smith 1999).

The literature indicates that in AML, 60–88% of the patients display an aberrant phenotype at diagnosis. Adriaansen *et al.*, identified subsets of myeloblasts, which

expressed TdT in 75% of 45 AML cases; however in most of these cases TdT was positive in less than 20% of blasts (Adriaansen *et al.* 1990). Reading *et al.*, detected LAPs in 85% of 272 cases of AML (Reading *et al.* 1993). Macedo *et al.*, found that 29 (73%) of 40 AML patients analysed, displayed the existence of at least one aberrant phenotypes (Macedo *et al.* 1995); San Miguel *et al.*, reported that 46 out of 53 (85%) AML cases had an aberrant phenotype (San Miguel *et al.* 1997); this figure was confirmed by the same authors in another report on 126 AML cases (San Miguel *et al.* 2001). In the experience of Venditti. *et al.*, 70% of 113 newly diagnosed AML carried an aberrant phenotype (Venditti *et al.* 2000; Venditti *et al.* 2002). In addition, incidence as high as 88% have been also reported, (Bahia *et al.* 2001) possibly because of the use of a large variety of MoAbs. The lowest incidence of aberrant immunophenotypes was reported by Drach *et al.*, who observed LAPs in 35 (51%) of 68 AML patients (Drach *et al.* 1992). The use of double, but not triple/quadruple staining assays and a limited panel of antibodies are possible explanations for this lower frequency. Altogether, these results demonstrate LAP detection by MFC is a feasible approach for MRD detection in a significant proportion of AML cases. In this respect, further development is needed with the increasing knowledge about the antigenic composition of the leukaemic cells.

1.14.1.1 The quality of LAP for MRD detection

Analytical Sensitivity refers to how good LAP detection by MFC is at correctly identifying LAPs. Specificity, on the other hand, refers to how good the test is at correctly identifying cells that are normal.

Thus, the quality of LAP for MRD detection depends on:

1. Specificity (depends on the percentage of LAP expression on normal BM cells, a high specificity can be achieved by including primitive markers (CD34,

CD133 and CD117) if present on AML. The specificity of LAPs further depends on many other factors. In most cases, LAP expression on normal BM cells is < 0.1%.

2. Sensitivity: the sensitivity of MRD detection depends among others on the percentage of LAP expression on the leukaemic blast population at diagnosis and the number of cells analysed. For this reason only LAPs which are expressed >10% on the leukaemic blast population should be considered.

3. Stability: LAPs may undergo phenotypic shifts. During the disease, marker expression on AML may disappear, resulting in false-negativity. Especially dim expression of markers is susceptible for changes. Furthermore, CD19 expression has been shown to disappear on several occasions in the course of disease (these definitions adapted from Dutch/Belgium task force for MRD detection in AML in cooperation with the European working group on clinical cell analysis).

1.14.1.2 Points for consideration for LAPs detection

Several issues need to be considered for LAPs detection: (1) the need for testing wide and comprehensive antibody panels with progenitor markers included (i.e. CD34 and/or CD117, CD133); (2) the correct interpretation of antigen expression patterns as compared to normal versus regenerating haemopoietic pathway; (3) the concept that the leukaemic blasts are certainly composed by distinct phenotypic clones (Drach *et al.* 1992; Campana and Pui 1995; Venditti *et al.* 2002). In this regard, Macedo *et al*., (Macedo *et al.* 1995) have found that most AML patients with a LAP have more than one aberration. Although this finding needs to be interpreted with caution, multiple staining assays to confirm the co-existence of more than one aberrant phenotype may be a tool to identify the population of interest in a more appropriate way and then to avoid the phenomenon of phenotypic switch at relapse.

1.14.2 Level of sensitivity:

In theory, MFC may allow a maximum level of sensitivity of one leukaemic cell in 10^6 after an accurate and prolonged cleansing of the fluidics system (Campana and Coustan-Smith 1999). However, a more realistic figure for practical applications is one target cell in 10^4 normal cells (Campana and Coustan-Smith 1999). Using serial dilution of leukaemic cells in normal peripheral blood and BM cells, it was shown that MFC is able reliably to detect cells displaying LAPs up to a level of 10^{-4}. However, it should be noted that the level of sensitivity varies (from 10^{-3} to 10^{-5}) depending on the type of phenotypes being analysed, the combination of MoAbs reagents used for their detection (Campana and Coustan-Smith 1999) and the sample under study.

The introduction of Multiparameter FC analysis has improved on the sensitivity and specificity of leukaemic cell detection (Terstappen and Loken 1990; Terstappen *et al.* 1990; Terstappen *et al.* 1990; Terstappen *et al.* 1992). In the experience of some groups, serial dilution experiments of leukaemic cells with normal BM cells determined the detection limit of MFC ranges between 10^{-4} and 10^{-5} (Venditti *et al.* 2002; Kern and Schnittger 2003; Kern *et al.* 2005).

1.15 ADVANTAGES AND DRAWBACKS OF MFC FOR DETECTING MRD

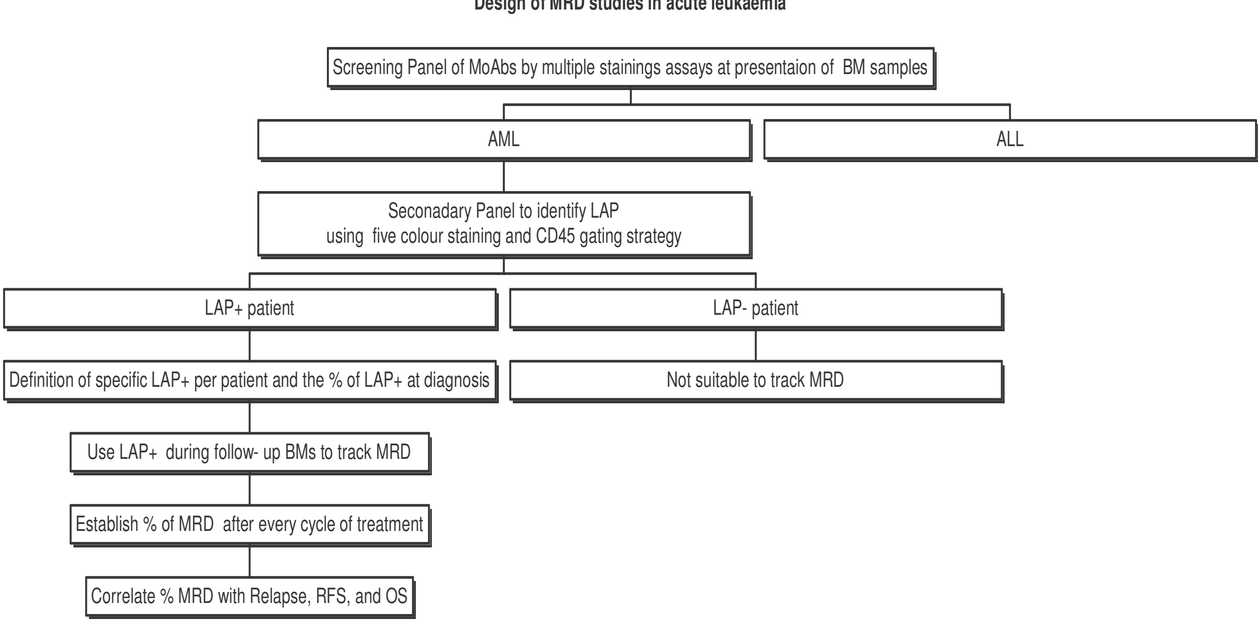
PCR-based quantification of MRD has high sensitivity, and the proportion of AML cases amenable to PCR detection may be significantly increased by targeting length mutations of the FLT3 gene and partial tandem duplications within the MLL gene in addition to the fusion transcripts AML1-ETO, CBFB-MYH11, and PML-RARA (Kern *et al.* 2004). However, this still results in approximately 50% of cases not having an identifiable leukaemia-specific genetic alteration, and these patients

therefore are not subject to PCR-based monitoring of MRD. In fact, in practical terms, MRD detection by PCR is only routinely applied in APL patients. In contrast, recent data indicate that immunologic monitoring may be applicable to >80% patients with AML when pursuing a comprehensive approach (Kern *et al.* 2003; Kern *et al.* 2004). Additionally, sensitivity of MRD detection can range as high as 1 leukaemic cell per 10^4 to 10^5 normal cells. Furthermore, rapid and accurate quantification can be achieved.

Factors that reduce the sensitivity of immunophenotyping include: (1) the lack of antigen specificity for malignant cells as these cells represent the counterparts of normal cells with, in many cases, identical or similar antigen profiles, (2) the existence of several subpopulations, some of them as minor clones, that are difficult to identify, (3) the incapability to identify phenotypic switch, a phenomenon that may occur at relapse, although at a low rate in AML. It can also give false negative results and complex analysis (San Miguel *et al.* 1997; San Miguel *et al.* 2001), and (4) a high number of cells need to be counted and some degree of technical expertise is required making this procedure not accessible to every routine laboratory (Paietta 2002).

In addition, detection of MRD by flow cytometry in AML presents some specific difficulties due to immunophenotypic heterogeneity (Campana 2003). Further, AML cells usually spread across many areas of the dot plot instead of forming a tight cluster when compared with the homogenous ALL.

Figure 1-2 illustrates the algorithm based on which a Leukaemia associated phenotype is identified in AML patient and used for detection of MRD. Once LAP is identified it will serve to establish a phenotype to trace residual leukaemia after a morphologic CR is recognised (Adapted from (Campana and Pui 1995; Campana and Coustan-Smith 1999) papers and previous literature in MRD).



1.16 CLINICAL STUDIES IN MRD DETECTION

Although studies of MRD detection by MFC in AML are still limited as compared to ALL (Ciudad *et al.* 1998; Coustan-Smith *et al.* 1998; Coustan-Smith *et al.* 2002; Coustan-Smith *et al.* 2003), several reports have been published providing evidence that study of MRD are a useful tool for predicting relapse (Drach *et al.* 1992; Sievers *et al.* 1996; San Miguel *et al.* 1997; Plata *et al.* 2000; Venditti *et al.* 2000; San Miguel *et al.* 2001; Venditti *et al.* 2002; Venditti *et al.* 2003; Feller *et al.* 2004). The findings of the current studies reveal that the precise evaluation of MRD by MFC has a prognostic significance in predicting relapse and may also have a major impact in the clinical management of patients with AML. In particular, the

understanding of the clinical significance of MRD at different stages of treatment may help at designing modified therapeutic programs according to patient risk category. Above all, the main challenge for expert MRD investigators is to simplify methods while maintaining or increasing their reliability, therefore propagating the potential benefits of MRD monitoring to all patients.

1.17 NEWER APPROACHES: LEUKAEMIC STEM CELLS AND MARKERS

1.17.1 Understanding AML stem cells will lead to more effective therapies

The precise cellular and molecular factors predisposing for (or suppressing) the development of relapses in patients in CR, remain to be determined. It is becoming evident that relapses occur in patients in whom the leukaemic cells survive chemotherapy, which show evidence of functional properties of stem cells (Bonnet 2005). A number of observations proposed that AML clones in each patient represent a heterogeneous mixture of cells with varying phenotypic and functional properties (Sutherland *et al.* 1996; Blair *et al.* 1997; Bonnet and Dick 1997; Blair *et al.* 1998; Blair and Sutherland 2000; Appelbaum *et al.* 2001; Bonnet 2005). In particular, despite differentiation and maturation arrest, AML clones are organized in a hierarchical manner similar to normal haemopoietic cells (Sutherland *et al.* 1996; Bonnet and Dick 1997; Appelbaum *et al.* 2001; Stone 2001). On the top of this hierarchy, LSCs are acting as AML-initiating and maintaining cells, whereas their more mature progeny-cells are unable to maintain the long-term growth of leukaemias (Sutherland *et al.* 1996; Bonnet and Dick 1997; Appelbaum *et al.* 2001). This hypothesis has been confirmed for various subtypes of AML by utilising repopulation assays (Sutherland *et al.* 1996; Appelbaum *et al.* 2001).

LSCs obtained from patients with AML are defined by their AML repopulating capacity *in vivo*, i.e. their ability to give rise to leukaemias in immunodeficient mice (Sutherland *et al.* 1996; Bonnet and Dick 1997; Appelbaum *et al.* 2001).

Over the last few years, these cells have been characterised in terms of their frequency in AML clones, their phenotype, and their functional properties. Similar to normal HSCs, LSC represent only a small fraction of cells within a given AML clone (0·2–100 cells in 10^6 cells) (Bonnet and Dick 1997). In common with their daughter cells, most LSC express CD34 (Bhatia *et al.* 1997; Bhatia *et al.* 1999; Bonnet *et al.* 1999; Appelbaum *et al.* 2001).

As above, LSC despite being present in low numbers may be responsible for the relapses in AML and therefore are considered to contribute essentially to the pathology and clinical outcome in these patients. So rather than monitoring the whole blasts cell population, it may be more relevant to search for residual LSCs.

1.18 FUNCTIONAL AND PHENOTYPIC CHARACTERIZATION OF LEUKAEMIC STEM CELLS

1.18.1 Functional characterization of LSC

Analogous to the repopulating potential of HSC, LSC have the potential to repopulate haemopoietic tissues in severe combined immunodeficient (SCID) mice (Sutherland *et al.* 1996; Bonnet and Dick 1997; Appelbaum *et al.* 2001; Stone 2001). Though, only a small sub fraction of cells within leukaemic clones represent LSC, whereas the vast majority of AML cells in a given clone are unable to repopulate SCID mice with leukaemia (Bonnet and Dick 1997; Appelbaum *et al.* 2001). The capacity of LSC to self-renew *in vivo* is further supported by the fact that the leukaemic cells that can be generated from these cells in SCID mice can

again give rise to leukaemia when serially transplanted into 'secondary' cohorts of SCID mice (Bonnet and Dick 1997). The morphology and the immunophenotype of the LSC-derived AML cells in these SCID mice are identical to the original AML clone (Bonnet and Dick 1997). These results demonstrate the potential of LSC to initiate the re-growth of AML cells *in vivo*. Based on this finding, it is tempting to speculate that LSC represent those residual AML cells (MRD) that can regrow to overt relapsing leukaemias after chemotherapy or BM transplantation.

An important aspect of LSC in patients with AML is that a high percentage of them appear to be quiescent (Ki67-negative), thus contrasting with the high cell burden and potentially aggressive clinical course of these leukaemias (Jordan *et al.* 2000). This observation may explain why these LSC are often less responsive against cell cycle-active chemotherapeutic agents compared to their more mature progeny. Even though LSC reportedly express receptors for IL-3 and other cytokines, it remains unknown whether such cytokines can induce the growth or/and differentiation of LSC.

In addition, although Jordan *et al* (Jordan *et al.* 2000) have shown that IL-3 α receptor (CD123) is detectable on LSC and not HSC, little is known about their exact functional role in LSC.

1.18.2 Phenotypic characterization of LSC

Previous studies demonstrate that LSC are mainly found within the $CD34^+$, $CD38^-$, Lin^- subset of the leukaemic clone (Figure 1-3) (Sutherland *et al.* 1996; Bonnet and Dick 1997). Other clonal leukaemic cells with a more mature phenotype are unable to initiate AML *in vivo* (Sutherland *et al.* 1996; Appelbaum *et al.* 2001). These data are similar to the repopulation characteristics of normal human HSC in SCID mice (Sutherland *et al.* 1996; Bonnet and Dick 1997; Bonnet *et al.* 1999; Appelbaum *et*

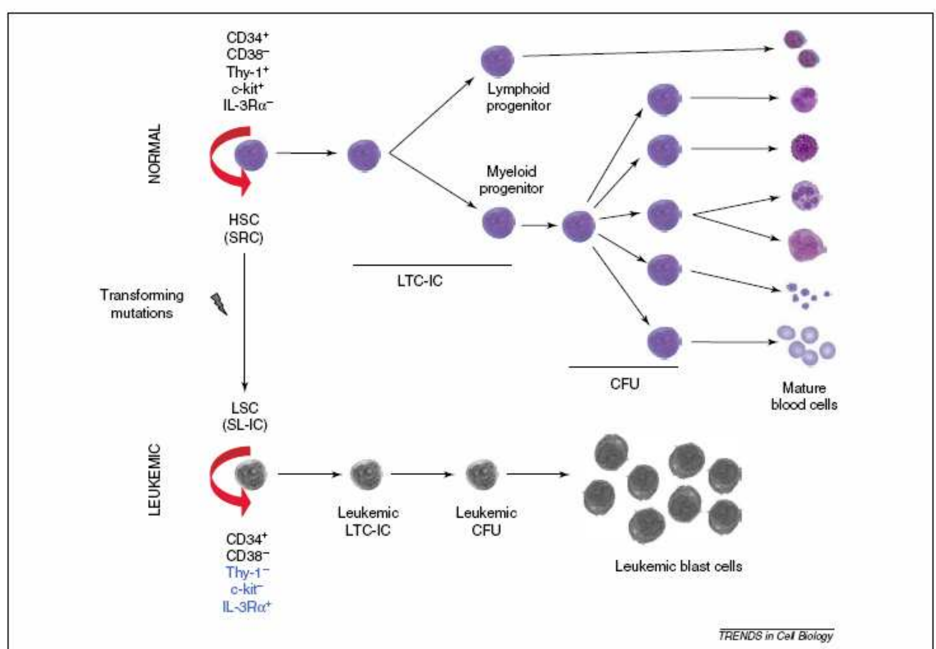
al. 2001; Guenechea *et al.* 2001; Bonnet 2005; Bonnet 2005). However, despite phenotypic and functional similarities, it has to be highlighted that slight differences in the cell surface phenotype are found when comparing normal HSC with LSC. Similarly, LSC, but not HSC, express the IL-3 receptor α chain (CD123) (Jordan *et al.* 2000; Moretti *et al.* 2001). Another marker that is detectable on LSC in (a subset of) patients with AML, is Siglec-3 (CD33) (Hauswirth *et al.* 2007) (Krauth *et al.* 2007). In addition, LSC may also show immunophenotypical heterogeneity concerning other surface molecules such as CD116 (GM-CSFRα), CD117 (SCFR), or CD71 (Blair *et al.* 1997; Blair *et al.* 1998; Blair and Sutherland 2000; van Der Velden *et al.* 2001).

C-type lectin-like molecule-1 (CLL-1) has also been shown to be expressed in LSC and not HSC in a very recent report by Anna van Rhenen *et al*, (van Rhenen *et al.* 2007). $CD34^+/CD38^-/CLL\text{-}1^+$ cells were found to engraft NOD/SCID mice and high $CLL\text{-}1^+$ fraction was associated with quick relapse. In this regards, CLL-1 may serve as a marker for quantification of minimal residual stem cell disease. Further, CD44 has also recently been described as a target on $CD34^+/CD38^-$ cells (Jin *et al.* 2006). It was shown that the activating antibody H90 results in differentiation of cells and in a major reduction of engraftment in NOD/SCID mice. However, CD44 is also weakly expressed on normal $CD34^+/CD38^-$ cells and on more differentiated haemopoietic cells.

Phenotypic differences between LSC and HSC may be of great value. These differences allow discrimination of these cells in flow cytometry assays and to separate LSC from HSC for genetic analyses. Markers specific for LSC may prove suitable targets for the development of novel therapies; because otherwise normal HSC would also be eradicated, and such an approach would then only be possible

when combining with a transplantation strategy. As LSCs are responsible for relapse, they may also be used for MRD detection.

Figure 1-3. Schematic illustration of the normal and leukaemic human haemopoietic hierarchies. Human haemopoietic cells are organized in a hierarchy that is sustained by a small population of self-renewing HSCs. HSCs give rise to progressively more lineage-restricted, differentiated progenitors with reduced self renewal capacity (LTC-ICs, long-term culture-initiating cells; CFU, colony-forming units), which in turn produce functionally mature blood cells. Disruption of pathways regulating self-renewal and differentiation through the acquisition of transforming mutations generates LSCs capable of sustaining growth of the leukaemic clone in vivo. LSCs possess an altered differentiation program, as demonstrated by aberrant expression of some cell-surface markers (indicated in purple) and give rise to an aberrant developmental hierarchy that retains aspects of its normal counterpart. In vivo, reconstitution assays using immune-deficient mouse recipients enable detection of HSCs and LSCs as SCID-repopulating cells (SRCs) and SCID leukaemia-initiating cells (SL-ICs), respectively (Wang and Dick 2005).



1.19 TARGETS OF AML THERAPY DETECTABLE IN LEUKAEMIC STEM CELLS

Molecular constructions in AML cells that may serve as targets of specific therapy are located in various compartments of the leukaemic cell. Generally, such targets are either detectable on the cell surface or within the cytoplasm.

1.19.1 Targets expressed on the surface of AML cells

Based on target molecules expressed on the surface of leukaemic cells, a number of new treatment strategies have recently been established (Matthews *et al.* 1999; Matthews *et al.* 1999; Treish 2000; Hamann *et al.* 2002; Hamann *et al.* 2002; Krauth *et al.* 2007). One of these concepts employs humanized antibodies (Ab) conjugated with a cytostatic drug. These conjugates bind to leukaemic cells through an interaction of the Ab with the target structure on the surface of AML blasts. Consecutively, the Ab–drug conjugate is internalized by the leukaemic cells. After internalization, the drug is released from the Ab and inhibits critical cell functions (depending on the nature of the cytostatic drug) and eventually leads to cell death (Sievers 2001; Hamann *et al.* 2002; Hamann *et al.* 2002).

An important example for such conjugates is Mylotarg (gemtuzumab/ozogamicin), which consists of a humanized anti-Siglec-3 Ab (CD33) and the highly potent (toxic) cytostatic drug calicheamicin (Sievers 2001; Hamann *et al.* 2002; Hamann *et al.* 2002).

Another treatment concept for surface antigen targeted anti-leukaemic therapy is based on conjugates consisting of (humanized) antibodies and radio-isotopes (Terpstra *et al.* 1997; Matthews *et al.* 1999; Guzman *et al.* 2002; Pagel *et al.* 2002). One example is ^{131}I-anti-CD45 (Guzman *et al.* 2002; Pagel *et al.* 2002). However, it

remains unclear whether this therapy will result in an improvement in the disease-free survival of patients with AML.

1.19.2 Intracellular targets of AML therapy

Over the past few years, a large number of cytoplasmic and nuclear target structures in AML cells have been identified (Dash and Gilliland 2001; Guzman *et al.* 2002; Stirewalt *et al.* 2003; Gilliland *et al.* 2004). Among these are DNA-methylating enzymes, histone deacetylases, leukaemia-specific fusion gene-products (such as PML/RARα), pro-oncogenic transcription factors (STAT-family, Ets, c-Myb, HOX, NFκB, others) and critical elements in pro-oncogenic signal transduction cascades (RTKs, mutated oncogenic forms of Ras, others) (Gilliland *et al.* 2004).

Recent reports suggest that nuclear factor κB (NF-κB) is constitutively expressed in blast cells in a majority of patients with AML (Guzman *et al.* 2001; Guzman *et al.* 2002; Guzman and Jordan 2004). Additionally, NF-κB-activity is detectable in the (quiescent) LSC population in these patients, whereas normal unstimulated HSC do not express NF-κB activity (Guzman *et al.* 2001; Guzman *et al.* 2002).

A number of different tyrosine kinase inhibitors have recently been identified and applied in clinical trials in leukaemic patients (Ruggeri *et al.* 1999; Karp *et al.* 2001; Druker *et al.* 2002; Ebihara *et al.* 2002; Levis *et al.* 2002; Reilly 2002; Weisberg *et al.* 2002; Zheng *et al.* 2002; O'Farrell *et al.* 2003; O'Farrell *et al.* 2003; Spiekermann *et al.* 2003; Smith *et al.* 2004). Important stem cell receptor tyrosine kinases (RTK) expressed in AML cells are the SCF receptor KIT, M-CSF receptor FMS, PDGFRβ, FLT1 and FLT3 (Gilliland and Griffin 2002; Reilly 2002; Gilliland *et al.* 2004). At least some of these tyrosine kinases are also expressed in LSC (Ebihara *et al.* 2002). Several previous and more recent observations suggest that these molecules do play an important role in leukaemogenesis (Gilliland and Griffin

2002; Gilliland and Griffin 2002; Reilly 2002; Galimberti *et al.* 2004; Gilliland *et al.* 2004). Similarly, the *FLT3* gene is the most frequently mutated gene in patients with AML (Gilliland and Griffin 2002; Gilliland and Griffin 2002; Kelly *et al.* 2002; Gilliland *et al.* 2004). These mutations lead to ligand-independent dimerisation of the receptor and its auto-phosphorylation with consecutive activation of multiple signal transduction pathways including the STAT5-, RAS/MAPK- and PI3K/AKT-pathway (Orlowski *et al.* 2002). Since these mutations apparently act pro-oncogenically, it is appealing to speculate that they all take place and are detectable at the stem cell level in patients with AML.

A number of drugs targeting RTKs have recently been applied to AML cells in clinical and/or pre-clinical trials. Likewise, the inhibition of *FLT3* by AG1296 or Herbimycin A in AML cells in mice was found to counteract the progression of leukaemia (Gilliland and Griffin 2002; Gilliland *et al.* 2004). In addition, a number of targeting drugs directed against RTKs have been developed in recent years, including CEP701, CEP751, SU5614, SU5416, SU11248 and PKC412 (Karp *et al.* 2001; Mesters *et al.* 2001; Ebihara *et al.* 2002; Reilly 2002; Weisberg *et al.* 2002; O'Farrell *et al.* 2003; O'Farrell *et al.* 2003; Spiekermann *et al.* 2003; Smith *et al.* 2004). These inhibitors may act on several RTKs including FLT3, thus inhibiting proliferation of leukaemic cells. Additionally, some of these inhibitors have already been evaluated *in vivo*. Likewise, CEP701 has been reported to induce responses in AML patients refractory to conventional chemotherapy (Smith *et al.* 2004). Interestingly, at least some of these RTK-type receptors are known to be expressed in LSC.

1.20 HYPOTHESES AND AIMS OF THE RESEARCH

1.20.1 Hypotheses of the research:

1. LAPs can be detected in the majority of AML patients at diagnosis with high sensitivity and specificity using improved techniques of 5-colour MFC, an extensive panel of MoAbs, and CD45 gating strategy.
2. Presence of MRD post chemotherapy predicts worse prognosis.
3. FLT3/ITD mutation is present at leukaemic stem cell level and not secondary event in leukaemogenesis and it is confined to $CD34^+/CD38^-$ fraction that possess IL-3 α receptor (CD123) in AML.

1.20.2 Aims of the research

1. To detect LAPs using five-colour MFC from consecutive, newly diagnosed AML patients using the improved techniques with an extensive panel of MoAbs and CD45 gating strategy.
2. To assess the sensitivity of MFC technique for MRD detection.
3. To evaluate the role of MRD in predicting relapse and prognosis in AML.
4. To demonstrate whether FLT3/ITD is confined to the population of LSC as defined by $CD34^+/CD38^-/CD123^+$.

1.21 HOW THE THESIS AIMS CONTRIBUTE TO THE CURRENT KNOWLEDGE?

The ideal method for the detection of MRD in a marrow or blood samples should have several criteria. One of them is that the method should be applicable to most of the patients. In addition, the method should be specific for leukaemic cell type and sensitive enough to detect very low level of residual leukaemic cells. Moreover, the

method should allow quantitation of leukaemic burden for prognostic purposes (Provan and Gribben 2005).

The proposed design of five-colour MFC, extensive MoAbs and CD45 gating strategy will enable us to cover all the criteria required for a successful MRD study using only the most aberrant phenotypes and thus more sensitive markers for MRD. It will also highlight on the usefulness of LAPs as an additional prognostic marker in AML, which should be taken into account when dealing with this aggressive disease.

Further, this study highlights on the prognostic value of MRD post induction chemotherapy, which may play a role to post induction treatment stratification.

Importantly, from the study we gained some insight on the oncogenic event in FLT3/ITD positive AML patients, which might lead to novel treatment concepts, employing surface marker CD123-targeting antibodies combined with intracellular FLT3 inhibitors against AML stem cells.

Finally, this study enhances our knowledge for the utility of $CD34^{+}/CD38^{-}/CD123^{+}$ as a standardised one single LAP. This phenotype can be found in the majority of myeloid and may be lymphoid leukaemia patients. Thus, it is may be more informative looking for LSCs rather than the whole blast population.

CHAPTER 2

Materials & Methods

CHAPTER 2: MATERIALS & METHODS

2.1 MATERIALS

2.1.1 PATIENT POPULATION

Fresh and cryopreserved BM samples were used in all of our studies in this thesis from patients diagnosed with AML in Division of Haematology, Royal Adelaide Hospital (RAH) and Institute of Medical and Veterinary Science (IMVS).

2.1.1.1 Sample size calculation

The first study was conducted as a pilot study (Chapter 3) with the aim to identify the incidence of LAPs in AML patients and correlate their presence with response to induction chemotherapy and disease outcome. For that purpose, different sample size scenarios were calculated with a power of 80% and test significance of 0.05 as shown in Table 2-1.

This sample size calculation was done for the first hypothesis, which is: "*LAPs can be detected in more than half of AML patients at diagnosis using the current standard diagnostic panel*".

Table 2-1 Sample Size Calculation for Hypothesis 1 for the Pilot Study

Information Required	*Scenario 1*	*Scenario 2*	*Scenario 3*	*Scenario 4*	*Scenario 5*
Test significance level	0.05	0.05	0.05	0.05	0.05
1 or 2 sided test?	1	1	1	1	1
Null hypothesis proportion	0.5	0.5	0.5	0.5	0.5
Expected proportion	0.65	0.7	0.75	0.8	0.85
Power (%)	80	80	80	80	80
Sample size required	67	37	23	15	11

Scenario 1 was interpreted as follows: a one-sample z-test with a one-sided significance level of 0.05 will have 80% power to detect the difference between the null hypothesis proportion of 0.5 and the expected proportion of 0.65 when the sample size is 67. The other scenarios were interpreted similarly. Therefore, a bigger sample size was taken, 84 patients from 2002 to 2004 to demonstrate this hypothesis.

The second study (Chapter 4) was designed to test the applicability of five-colour staining approach to identify a higher incidence of LAPs using extensive panel of MoAbs in order to follow these patients for MRD detection. Sample size scenarios were calculated for the following hypotheses to be tested:

Hypothesis 1: "*LAPs can be detected in the majority of AML patients at diagnosis using 5- colour staining approach*".

Hypothesis 2: "*The presence of MRD after chemotherapy predicts worse prognosis*".

Table 2-2 Sample Size Scenarios for Prospective Study

Sample Size Calculations	*Hypothesis 1*			*Hypothesis 2*		
Information Required	*Scenario 1*	*Scenario 2*	*Scenario 3*	*Scenario 4*	*Scenario 5*	*Scenario 6*
Confidence level	0.95	0.95	0.95	0.95	0.95	0.95
1 or 2 sided interval?	2	2	2	2	2	2
Expected proportion	0.8	0.8	0.8	0.9	0.9	0.9
Distance from proportion to limit	0.05	0.10	0.15	0.05	0.10	0.15
Sample size required	246	62	28	139	35	16

2.1.1.1.1 Understanding the Sample Size Table

Scenarios 1-3 relate to hypothesis 1 and scenarios 4-6 relate to hypothesis 2. The confidence level was chosen to be 0.95 since a 95% confidence interval was calculated.

It was a 2-sided confidence interval. We expected the proportion of LAP positive patients to be at least 0.8 (i.e. 80%) using the 5-colour approach and extensive panel of MoAbs. In addition, based on previous literature we anticipated the proportion of MRD positive patients who might relapse is 90 (i.e. 90%). We have considered 3 different widths for the 95% confidence interval for each hypothesis (± 0.05, ± 0.10 and ± 0.15) and these were the distances from the expected proportion to the limit in the Table 2-2.

Scenario 1 was interpreted as follows. When the sample size is 246, a two-sided 95% confidence interval for a single proportion using the large sample normal approximation will extend 0.05 from the observed proportion for an expected proportion of 0.80. In practical terms, this means that if the proportion of LAP positive patients in our cohort is 0.8, then a 95% confidence interval for the true proportion of LAP positive people will be given by 0.8 ± 0.05 = (0.75, 0.85). The other scenarios were interpreted similarly.

2.1.1.1.2 Choosing the sample size

We have chosen the width of the confidence interval, which we were interested in for each hypothesis that gave us a sample size for both hypothesis 1 and hypothesis 2. Based on the limited time of my PhD candidature and the minimum follow-up period required for MRD studies, sample size of 246 patients (with a distance from proportion to limit of 0.05) was not feasible for this project. We have taken a distance from the expected proportion to limit of 0.15 that included 28 and 16 patients for hypotheses 1 and 2 respectively. Thus, we aimed for a sample size greater than 30 to cover both hypotheses.

2.1.1.2 Patient samples

Fresh BM obtained from 84 newly diagnosed AML patients from 2002 to 2004 for the pilot study (Chapter 3) and 54 AML patients for the prospective study of 5-colour MFC from 2005 -2007(Chapter 4).

2.1.1.2.1 Selection criteria

Routine diagnostic flow cytometry was performed on fresh BM samples. We analysed the results of flow cytometry from 84 consecutive, unselected, newly diagnosed, *de novo* or secondary and untreated AML patients at diagnosis between 2002-2004 for the pilot study. For the prospective study, 54 consecutive, unselected, newly diagnosed and untreated AML patients were analysed at diagnosis using two panels of MoAbs. Diagnosis of patients was based on morphology, immunophenotyping, and cytogenetics (Lowenberg *et al.* 1999). The WBC count, FAB diagnosis, gender, age, clinical and outcome data were collected for each patient.

Criteria for inclusion in the MRD study were (1) diagnosis of AML, (2) expression of LAPs, (3) eligibility of intensive chemotherapy, (4) achievement of morphologic CR after induction chemotherapy, and (5) at least 2 follow-up samples available.

2.1.1.2.2 Study conduct

Prior to therapy, all patients gave their informed consent for participation in the current evaluation after having been advised about the purpose and investigational nature of the study as well as potential risks. The study design was approved by the Research Ethics Committee of the RAH prior to its initiation.

2.1.1.2.3 Study parameters

Follow-up BM examinations were carried out after regeneration of BM following both induction and consolidation therapies. Response to therapy was assessed according to standardised criteria.

2.1.1.3 Normal bone marrow samples

Normal BM as a control for the study was obtained from 10 healthy volunteers and analysed by same panels of MoAbs as AML patients for investigating the presence of LAPs.

2.1.1.4 Regenerating bone marrow samples

Regenerating BMs from non-myeloproliferative disorders patients as another control for the study were obtained from 5 patients and analysed by same panels of MoAbs as AML patients for investigating the presence of LAPs.

2.1.1.5 Chemotherapeutic treatment

All AML-M3 cases were treated with all-trans-retinoic acid (ATRA) 45 mg/m^2/day until CR plus idarubicin 12 mg/m^2/day on day 2, 4, 6 and 8. Non- M3 patients were treated with induction chemotherapy consisting of idarubicin 9 mg/ m^2/day on days 1-3, etoposide 75 mg/ m^2 on days 1-7 and cytarabine. Cytarabine was given as either low dose (100 mg/ m^2/day on days 1-7) or high dose (3 g/ m^2/day on days 1, 3, 5 and 7). Consolidation therapies consisted of idarubicin 9 mg/ m^2/day on days 1-2, etoposide 75 mg/ m^2 on days 1-5 and cytarabine (100 mg/ m^2/day on days 1-5).

The unit protocol for elderly AML (age> 60) include induction chemotherapy consisted of idarubicin 9 mg/ m^2/day on days 1-3 and cytarabine (100 mg/ m^2/day on days 1-7) of total 14 doses. HiDAC Consolidation therapies consisted of cytarabine (2 g/ m^2/over 3 hours, 12 hourly on days 1, 3 and 5) total of 6 doses for 2-4 cycles.

CR was defined according to criteria reported by Cheson *et al*. (<5% BM blast cells and recovery of haematological parameters) (Cheson *et al.* 1990). Patients were classified in three risk groups for cytogenetic abnormalities according to Grimwade *et al*. (Grimwade *et al.* 2001). Favourable risk was defined by the presence of t(15;17) or t(8;21) or inv(16), and poor risk was defined by the presence of either five unrelated abnormalities, monosomy 5 or 7, abnormalities of the long arm of chromosome 5, or inv(3) (q21:q26). Patients who did not meet the criteria for poor or favourable risk were classified as intermediate risk.

2.1.2 SUPPLIERS OF COMMONLY USED REAGENTS

Table 2-3: Commonly Used Reagents and their Suppliers

Reagent	Supplier	Catalogue Number
Bovine serum albumin (BSA) powder (Fraction V)	Sigma®	A-9418
DNase	Sigma®	D-5025
Dimethyl sulphoxide (DMSO)	APS Fine Chemicals	10323
Ethanol (analytical grade)	AnalaR®, BDH	10107.2500P
Formaldehyde	AnalaR®, BDH	10113
Formic acid	AnalaR®, BDH	94318
Iscove's modified Dulbecco's medium (IMDM)	JRH Biosciences	51471-500 M
Potassium dihydrogen orthophosphate (KH2PO4)	AnalaR®, BDH	10203.4B
Sodium chloride	APS Fine Chemicals	465-2.5Kg
Dulbecco Phosphate Buffered Saline (DPBS)	JRH Biosciences	51444-500MA
D Glucose	Sigma®	G-7021
EDTA	APS Fine Chemicals	180/500g
Boric Acid	AnalaR®, BDH	10058
Potassium Chloride	AnalaR®, BDH	10198
Sodium Chloride	AnalaR®, BDH	10241.4J
Ammonium Chloride	AnalaR®, BDH	10017
Sodium azide	AnalaR®, BDH	103692K
Potassium Bicarbonate	AnalaR®, BDH	10206
Sodium Carbonate(anhydrous)	AnalaR®, BDH	10240
Sodium Hypochlorite	J&J	1917
Acetic acid, glacial	AnalaR®, BDH	10001
Methyl Violet	Gurr	23169

Reagent	Supplier	Catalogue Number
Sodium Hydroxide	M&B	L740
Agarose	NuSieve	50084
Agarose LE	Promega	V3125
Bromophenol blue	Sigma®	B5525
Xylene cyanole	Sigma®	X4126
Glycerol	AnalaR®, BDH	10118
Ethidium bromide	Sigma®	E-1510
dNTP	Amersham Biosciences	27-2035-02

2.1.3 IMMUNOFLUORESCENCE REAGENTS

2.1.3.1 FACS Fix

Reagents:

D-Glucose	$C_6H_{12}O_6$	20 g
Formaldehyde Solution 37 - 40 % W/V	HCHO	10 ml
Sodium Azide	NaN_3	0.2 g
DPBS		

Approximately 800 ml DPBS was added to a 1 litre volumetric flask and then glucose and sodium azide were added. Formaldehyde was added. The volumetric flask mixed, and then it was made up to 1 litre.

2.1.3.2 FCS - Foetal Calf Serum

Commercial foetal calf serum is stored in 500 ml bottles at > -20°C.

2.1.3.3 Lysis Solution

Reagents:

Ammonium chloride	NH_4Cl	8.26 g
Potassium Bicarbonate	$KHCO_3$	1.00 g
Tetrasodium EDTA	$C_{10}H_{14}N_2Na_2O_8.3H_2O$	0.037 g
Or		
Disodium EDTA	$C_{10}H_{14}N_2Na_2O_8.2H_2O$	0.036 g
Milli Q water		

2.1.3.4 Reagent A - Erythrocyte Lysis Reagent for Q-Prep System

Reagents:

Formic Acid	HCOOH	2.4 ml
Deionised or Milli Q water		

2.1.3.5 Reagent B - Leukocyte Stabiliser Reagent for Q-Prep System

Reagents:

Sodium Carbonate (anhydrous)	Na_2CO_3	12 g
Sodium Chloride	NaCl	29 g
Sodium Sulphate (anhydrous)	Na_2SO_4	62.6 g
Deionised or Milli Q water		

2.1.3.6 Reagent C - Leukocyte Fixative Reagent for Q-Prep System

FACS FIX was used as a substitute for Reagent C.

2.1.3.7 1% Sodium Hypochlorite (W/V)

Reagents:

Sodium Hypochlorite solution (12.5% W/V)	8 ml
Deionised or Milli Q water	92 ml

Alternative Reagent:

Milton Anti-bacterial Solution 1L Bottle 1% Sodium Hypochlorite

2.1.3.8 White Cell Fluid (WCF)

Reagents:

Acetic acid	2.0 ml
Milli-Q® Water	98 ml
Methyl Violet	Few crystals

Acetic acid was added slowly to Milli-Q® Water. The methyl violet (Gurr®, BDH) was then added, and the solution mixed well to ensure that the crystals completely dissolved. The solution was filtered using a 0.2 μm bottle top filter, and stored indefinitely at room temperature.

2.1.3.9 MACS CD34$^+$ Buffer (PBS/0.5% BSA/2mM EDTA)

Reagents:

PBS	50 ml
10% BSA	25 ml
1% ACD-A	5 ml
EDTA (100 mM stock)	10 ml

BSA was dissolved slowly in PBS using a magnetic stirrer in 500 ml volumetric flask. Then, ACD-A was added. The EDTA was added to the buffer, and the flask topped up with 410 ml Milli-Q® Water. The solution sterilised using a 0.2 μm bottle top filter. The buffer was stored at 4°C.

2.1.3.10 Thawing solution

Reagents:

IMDM	375 ml
FCS	100 ml
ACD (5%)	25 ml

DNase (50 units/ml)	25000 units

IMDM was added into 500 ml bottle. The remaining constituents FCS, ACD and DNase were then added and the solution sterilised using a 0.2 μm bottle top filter. The solution was stored at 4°C, and allowed to warm up prior to use.

2.1.3.11 Monoclonal Antibodies (MoAbs)

The antibodies used for immunophenotyping of cell suspensions by flow cytometry are shown in Table 2-6.

Table 2-4 Monoclonal antibodies used for immunostaining of AML Patients

Antibody	Clone	Isotype	Specificity	Cat No	Dilution	Volume	Conjugate	Source
CD2	SFCI3Pt2H 9	IgG1 κ	Pan T cell, prothymocytes, subpopulation of NK cells	6603863	1/5	10 µl	FITC	Beckman Coulter
CD7	3A1E-12H7	IgG2b κ	haemopoietic progenitors, thymocytes, T, NK	6603824	1/5	10 µl	FITC	Beckman Coulter
CD10	SS2/36	IgG1 κ	B precursors , T precursors and mature neutrophils	R0848	1/10	10 µl	PE	Dako
CD11b	Bear1	IgG1 κ	NK Cells, neutrophils, monocytes, and macrophages	IM2581	Neat	10 µl	PE	ImmunoTech
CD13	L138	IgG1 κ	Granulocytic and monocytic cells, mast cells, GM-progenitor cells	347837	Neat	5 µl	PE	Beckton Dickinson
CD14	MfP9	IgG2b κ	Monocytes and Macrophages	347493	Neat	5 µl	FITC	Beckton Dickinson
CD15	MMA	IgM κ	Granulocytes and monocytes	347423	1/10	10 µl	FITC	Beckton Dickinson
CD19	HD37	IgG1 κ	B cells,	F0768	1/5	10 µl	FITC	Dako

Antibody	Clone	Isotype	Specificity	Cat No	Dilution	Volume	Conjugate	Source
CD33	P67.6	IgG1 κ	Monocytes, activated T cells, myeloid progenitors, mast cells	347787	Neat	10 µl	PE	Beckton Dickinson
CD33	P67.6	IgG1 κ	Monocytes, activated T cells, myeloid progenitors, mast cells	340533	Neat	10 µl	FITC	Beckton Dickinson
CD34	8G12	IgG1 κ	Haemopoietic progenitor cells, vascular endothelium, some tissue fibroblasts	348053	Neat	20 µl	FITC	Beckton Dickinson
CD34	581	IgG1	Haemopoietic progenitor cells, vascular endothelium, some tissue fibroblasts	IM2648	Neat	5 µl	PC5	ImmunoTech
CD38	LS198-4-3	IgG1	variable levels on majority of haemopoietic cells, high expression on plasma cells, early B and thymocytes	6604928	Neat	10 µl	PE	Beckman Coulter
CD38	T16	IgG1	variable levels on majority of haemopoietic cells, high expression on plasma cells, B and T cells	IM0775	Neat	5 µl	FITC	Beckman Coulter
CD45	Immu19.2	IgG1 κ	Haemopoietic cells, on the surface of all leukocytes	IM2652	1/2	5 µl	PC5	ImmunoTech

Antibody	Clone	Isotype	Specificity	Cat No	Dilution	Volume	Conjugate	Source
CD45	J33	IgG1	Haemopoietic cells, on the surface of all leukocytes	IM2710	Neat	5 µl	ECD	ImmunoTech
CD56	N901 (NKH-1)	IgG1 κ	NK, T cells subset	6603067	1/20	10 µl	PE	Beckman Coulter
CD64	22	IgG1	monocytes, macrophages, dendritic cells	IM1604	Neat	10 µl	FITC	ImmunoTech
CD65	88H7	IgM	granulocytes, subset of monocytes , myeloid leukaemias	IM1654	Neat	10 µl	FITC	ImmunoTech
CD117	104D2D1	IgG1	haemopoietic progenitors, mast cells	IM2732	Neat	20 µl	PE	ImmunoTech
CD117	104D2D1	IgG1	haemopoietic progenitors, mast cells	IM3698	Neat	5 µl	PC7	Beckman Coulter
CD123	9F5	IgG1 κ	lymphocytes subset, basophils, haemopoietic progenitors, macrophages, dendritic cells, megakaryocytes	340545	Neat	5 µl	PE	Beckton Dickinson
CD235a (Glyco A)	JC159	IgG1 κ	erythrocytes	F0870	Neat	10 µl	FITC	Dako
HAL-DR	L243	IgG2a	APC, B cells, monocytes, macrophages, thymic epithelial cells, activated T cells	P01133F	1/10	10 µl	FITC	BioDesign

Antibody	Clone	Isotype	Specificity	Cat No	Dilution	Volume	Conjugate	Source
MPO	MPO-7	IgG1 κ	Neutrophil granulocytes	F0714	Neat	10 µl	FITC	Dako
TdT	HT-6	IgG1 κ	immature, pre-B, pre-T lymphoid cells, and ALL/lymphoma cells	F7139	Neat	10 µl	FITC	Dako
SNeg*		IgG1 κ IgG2b κ	Setting fluorescence markers to establish a boundary between negatively and positively stained leukocytes events and assessing non specific staining	340041	Neat	10 µl	FITC/PE	Beckton Dickinson
Control ECD	679.1MC7	Mouse IgG1	Induces non specific immunolabeling on haemopoietic cells and therefore constitutes an isotype control, devoid of any relevant specificities with regard to the studied cell population	IM2714	Neat	10 µl	ECD	ImmunoTech
Control PC-5	679.1Mc7	Mouse IgG1	Induces non specific immunolabeling on haemopoietic cells and therefore constitutes an isotype control, devoid of any relevant specificities with regard to the studied cell population	IM2663	Neat	10 µl	PC5	ImmunoTech

Antibody	Clone	Isotype	Specificity	Cat No	Dilution	Volume	Conjugate	Source
Control PC-7	679.1Mc7	Mouse IgG1	Induces non specific immunolabeling on haemopoietic cells and therefore constitutes an isotype control, devoid of any relevant specificities with regard to the studied cell population	6607099	Neat	10 µl	PC7	Beckman Coulter

*** S neg which is a combination of FITC IgG1 and PE IgG2b were used for all the antibodies. BD simultest™ control γ_1/γ_{2a} (IgG_1 FITC/ IgG_{2b} PE) is a 2 colour direct immunofluorescent for use as *a negative control.***

2.1.4 DNA MANIPULATIONS REAGENTS

2.1.4.1 TBE Buffer

Reagents:

2.1.4.1.1 5 x Stock Tris-Borate (TBE) Buffer

	For 1 litre	**For 4 litres**
Tris 7-9	54 g	216 g
Boric acid (H_3BO_3)	27.5 g	110 g
0.5M EDTA, pH 8.0	20 ml	80 ml

The above constituents were dissolved in deionised water to total volume of 1 litre or 4 litres as appropriate. The buffer was stored at RT.

2.1.4.1.2 TBE Buffer - Working Solution

Stock TBE 1 in 5 with deionised water was diluted. The buffer was stored at RT.

2.1.4.2 Agarose gel mix

2.1.4.2.1 2% Agarose Gel

Reagents:

Agarose	4 g
TBE buffer	200 ml

NuSieve and Agarose were placed in a 500 ml Pyrex bottle. Two hundred millilitres (200 ml) TBE buffer was added. The mixture was boiled. The bottle was swirled briefly to mix. The solution was allowed to cool to approximately 45°C; left at RT.

2.1.4.3 Loading Buffer

Loading buffer containing bromophenol blue & xylene cyanol

Reagents:

Bromophenol Blue	0.25 g
Xylene Cyanol	0.25 g
Glycerol	30 ml
Deionised water	70 ml

All reagents were combined in a beaker. The bulk solution was stored at 4°C.

2.1.4.4 Molecular Weight Marker pUC19

pUC19 DNA restricted with HPA II DNA marker

Bresatec Cat.No. DMW-P1 100 μg (500 ng/μl) or pUC19 DNA restricted with HPA II DNA marker, Biotech Cat.No. PUC-H2 100 μg

2.1.4.4.1 Working Solution for pUC19

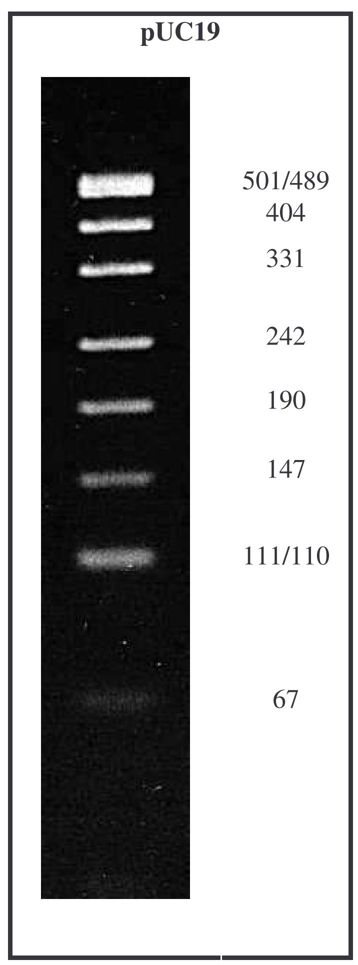
Reagents:

Milli Q water	40 µl
Load Buffer (as used for routine samples)	40 µl
DNA marker	20 µl

Reagents were combined in an eppindorf tube. The marker was stored at 4°C.

pUC19: Fragment sizes in base pairs

501 : 489 : 404 : 331 :242: 190 : 147 : 111 : 110 : 67 :34 :34 : 26



2.1.4.5 Ethidium Bromide

2.1.4.5.1 Ethidium Bromide Stock Solution

Ethidium bromide, aqueous solution, 10 mg/ml, Sigma Cat # E-1510 10 ml

2.1.4.5.2 Ethidium Bromide Working Solution

Reagents:

Ethidium Bromide Stock Solution	75 µl
Deionised water	500 ml

2.1.4.6 dNTP Reagent

2.1.4.6.1 dNTP Stock Reagent 100 mM

(Amersham Biosciences dNTP set (4x100 μmol): Cat No: 27-2035-02

2.1.4.6.2 dNTP Working Solution 2 mM of each dNTP

A mix of dATP:dCTP:dGTP:dTTP was prepared in autoclaved milliQ water with 200 μl dATP, 200 μl dCTP, 200 μl dGTP, 200 μl dTTP and 9.2 ml autoclaved purified water. The solution was stored in 500 μl aliquots at ≤ -20ºC. The working solution was stored at 2-8ºC.

2.1.4.7 Taq DNA polymerase kits

(Perkin Elmer AmpliTaq Gold: 250 units, 5 U/μl, IMVS stock code #13866). The AmpliTaq Gold was supplied with 10 x PCR Buffer II and $MgCl_2$ Solution 25 mM. The working solution was stored at 2-8°C.

2.2 METHODS

2.2.1 MORPHOLOGY AND CYTOCHEMISTRY

All morphological and cytochemistry analysis was done in Division of Haematology, IMVS. The AML patients were classified according to FAB classification (>20% blasts in BM as established with morphologic techniques).

2.2.2 CYTOGENETICS

Cytogenetics analysis was performed in Division of Molecular Pathology, IMVS. Patients were classified in three risk for cytogenetic abnormalities according to Grimwade *et al*. (Grimwade *et al.* 2001).

2.2.3 IMMUNOPHENOTYPING

2.2.3.1 Cryopreservation of cells

Cells were cryopreserved in a FCS solution containing the cryoprotectant, dimethyl sulphoxide (DMSO) (10% (v/v) final concentration). Cells were suspended in FCS (neat) and, immediately prior to freezing, an equal volume of ice-cold 20% (v/v) DMSO in FCS was added to the cells drop-wise with constant mixing. The cell suspension was transferred quickly to cryoampoules (Nelagene®) and the samples stored in liquid nitrogen (-196°C). The final concentration was a maximum of 1x10^{7}/ml.

2.2.3.2 Thawing cryopreserved cells

Cells were removed from liquid nitrogen and thawed rapidly in a 37°C water bath. The thawed cell suspension was transferred quickly to a 50 ml polypropylene conical tube (Falcon), and approximately 5 ml of thaw solution (warmed to 37°C) was added drop wise with constant mixing. The sample volume was then increased to 50 ml with thaw solution and the cells pelleted by centrifugation at 1400 rpm for 10 minutes with no brakes in a Heraeus multifuge 3S-R bench top centrifuge. The supernatant was aspirated and the cells washed a further 2 times with PBS to remove any residual DMSO.

2.2.3.3 Specimen processing

2.2.3.3.1 Cell count

Processing of all samples for cell count, cell transfer steps, removing supernatant, washing steps, mixing of uncapped specimens and preparing specimen dilutions were performed in safety biohazard hood (Class II Biological Safety Cabinets

(BSC-II) to protect from possible aerosols and then all cells were capped before vortexing.

2.2.3.3.1.1 Cell count using Haematocytometer

Cell concentration was determined by diluting the cell suspension in WCF, while cell viability was assessed by diluting samples with 0.4% trypan blue solution. Ten microlitres (10 µl) of these suspensions were transferred to haematocytometer counting chamber (Neubauer Improved, Assistant, Germany) and cell concentration and viability calculated accordingly.

2.2.3.3.1.2 Cell count using Epics® - XL-MCL and Cytomics® FC500 flow cytometer

All specimen aliquots were taken from primary tubes to be sampled using autoclaved filter pipette tips. Prior to staining specimens were prepared to produce a cell suspension of approximately 10^6 cells/100 µl. This was done by adding 100 µl of BCS to labelled tube. Then, 10 µl of specimen was added and the mixture was processed through Multi Q-Prep system (Beckman Coulter). After that, 10 µl of Flow CountBeads (Beckman Coulter, Flow count fluorospheres PN 7547053-200 test (1x20 ml) was added. The tube was capped and vortexed. The tube was processed on flow cytometer within 2 hours and cell count was written on specimen. The proper specimen dilution was prepared.

2.2.3.3.1.3 Cell count data interpretation

Total WBC count and percent lymphocyte values were obtained using the number of lymphocytes counted on an automated haematology instrument multiplied by the percentage of positively- stained cells measured on a flow cytometer. However, this

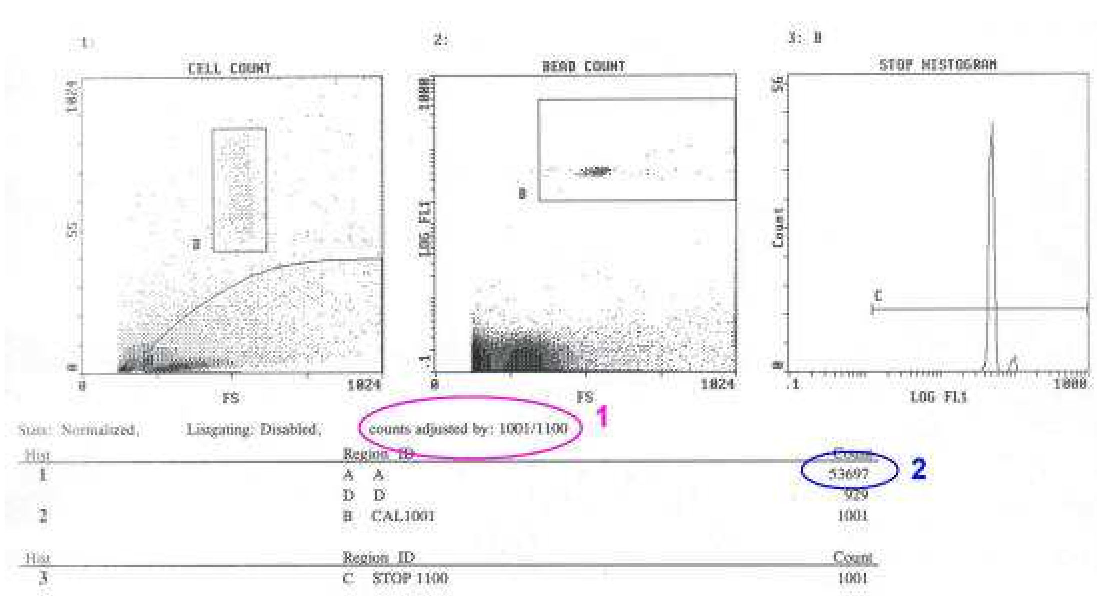
standard (indirect) method is associated with wide inter-laboratory variation (Rumke 1985; Rumke 1985; Koepke and Landay 1989).

Flow-Count Fluorospheres (FCF) is an efficient alternative to the standard (indirect) method for absolute counts. When identical volumes of a biological specimen and FCF were present, a ratio of cells in the specimen to fluorospheres was established. The cells of interest and the fluorospheres were then counted on the EPICS XL/XL-MCL® or Cytomics FC 500® flow cytometer. Since the concentration of fluorospheres was known, the absolute count of the cells was determined using the following formula:

$$\text{Absolute Count (cells/µl)} = \frac{\text{Total Number of Cells Counted}}{\text{Total Number of Fluorospheres Counted}} \times \text{FCF assayed concentration}$$

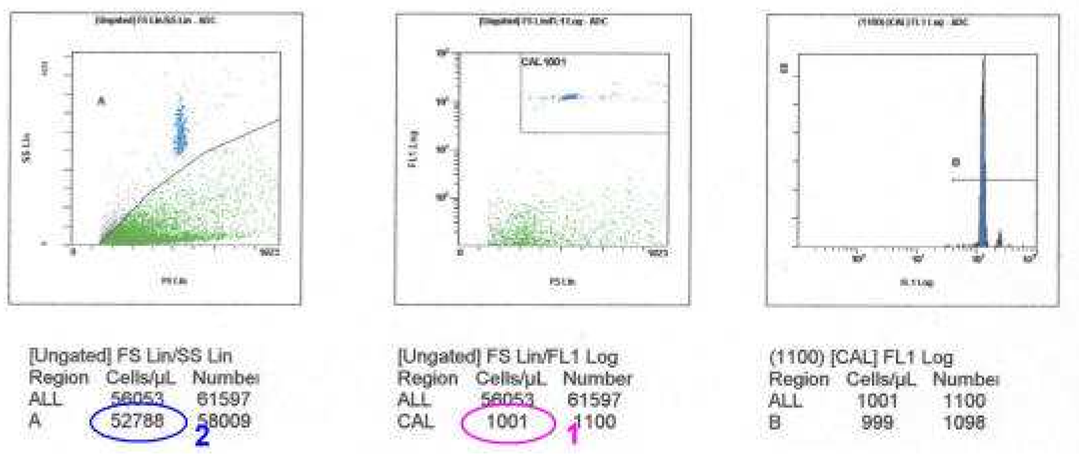
The FCF have a pre-determined concentration of spheres per µl (the CAL factor), this was noted on the ‘Assay Sheet’ included as a package insert. Each lot number was different; therefore, the value was updated on each flow cytometer at change of lot number. An example of cell count is in Figure 2.1.

Figure 2-1 Cell Count Calculation using Epics® - XL-MCLFlow Cytometer



There must be a" counts adjusted by xxxx/1100" value - note 1. The cell count, in cells/µl is shown in Figure 2.1 region A, EDTA, note 2.

Figure 2-2 Cell Count Calculation using FC500 Flow Cytometer



The CAL region on Figure 2.2 must be equivalent to the concentration of the Flow-Count lot - note 1. The cell count, in cells/µl is shown in Figure 2.2 region A, note 2.

2.2.3.3.2 Specimen dilution

Once the cell count was done, the dilution of samples was prepared according to the following table to give a cell suspension of approximately 10^6 cells/100 µl.

Table 2-5 Specimen dilution

WCC/uL*	Volume of Sample	Volume of BCS
≤ 10,000	Used Neat	Nil
10,001 - 20,000	1000 µl	1000 µl
20,001 - 30,000	600 µl	1400 µl
30,001 - 40,000	500 µl	1500 µl
40,001 - 50,000	400 µl	1600 µl
50,001 - 1000,000	200 µl	1800 µl
> 100,000 -	The lowest dilution was taken (200µl:1800 µl) and 2nd cell count was done and depending on the 2nd cell count, the dilution was prepared.	

**** A WCC of 10,000/µl ≡ 10.0 x 10^9/L***

2.2.3.4 Staining technique

The volume of each Antibody added was indicated in Table 2-4. The final stained tubes were stored at 2-8°C.

2.2.3.4.1 Manual staining

Appropriate antibodies (see Table 2-4) were added to labelled tubes. Then, 100 µl of sample was added to each tube according to the dilution prepared so that approximately 10^6 cells/ tube. The tubes were mixed and incubated in the dark at RT for 15 minutes. The tubes then were processed through TQ-Prep (Beckman Coulter). Cells were then washed once prior to fixation in FACS Fix. Fixed samples were stored at 4°C in the dark until analysis was performed. The fluorescence intensity of cell suspensions was examined using an Epics® - XL-MCL and Cytomics® FC500 flow cytometer and Cytomics™ CXP Analysis Version 1

Software (Beckman Coulter). Control tubes stained with an isotype-matched control were included in all experiments, and were used to define the cut-off point for positive/negative staining along with internal controls.

2.2.3.4.2 Lysis Method

To lyse erythrocytes and leave the nucleated elements intact, 2 ml lysing solution (ammonium chloride-based, NH_4Cl) was added to the 100 µl through Multi Q-Prep. The tube was centrifuged and the supernatant was decanted. Then, 2 ml wash solution was added, centrifuged and again the supernatant was decanted. This step was repeated one more time. After that, the antibody was added. The tube mixed and incubated at 2-8°C for 20 minutes. Finally, 500 µl FACS Fix was added.

2.2.3.4.3 Surface and cytoplasmic staining

Appropriate antibody was added for surface staining to labelled tubes (2 times normal volume). Then, 200 µl of sample was added. The tube was mixed and incubated at RT for 10 minutes and processed through Multi Q-Prep. The tube was centrifuged and the supernatant decanted. Then, 2 ml wash solution was added. The tubes centrifuged again and the supernatant decanted. After that, 100 µl of Fix and Perm Medium A to each tube was added and mixed well. The tube was mixed and incubated at RT for 15 minutes. Another 2 ml wash solution was added. The tubes centrifuged and the supernatant decanted. After that, 100 µl of Fix and Perm Medium B to each tube was added and mixed well. Then, antibody for cytoplasmic staining was added to labelled tube/s (2 times normal volume). The tube was mixed and incubated at RT for 15 minutes. Finally, 500 µl FACS Fix was added. Fixed samples were stored at 4°C in the dark until analysis was performed.

2.2.3.4.4 Blocking techniques

Monocytes and macrophages in particular have many Fc-receptors on the cell surface and therefore, blocking Fc- receptor is advisable to block nonspecific binding of MoAbs by pre-incubation of cells with human AB serum (HAB). However, since we are mainly dealing with blasts population in this study, blocking non-specific binding of MoAbs was not needed in our staining assays. Blocking techniques is not necessary for staining BM samples, because serum in high concentration is present during staining.

2.2.3.4.5 Viability staining

Cell viability was assessed by diluting samples with 0.4% trypan blue solution. Ten microlitres (10 μl) of these suspensions were transferred to a haemocytometer counting chamber (Neubauer Improved, Assistant, Germany) and cell viability calculated accordingly.

2.2.3.5 Method of Antibody evaluation

2.2.3.5.1 Antibody Selection:

Antibodies were chosen based on performance, cost and availability. Performance was determined by the greater separation of positive vs. negative populations. Thus, every new antibody was evaluated prior to use. Antibodies for evaluation were all directly conjugated. Antibody conjugate, FITC, PE, ECD, PC5 or PC7 was dependent upon possible antibody combinations. ECD fluorochrome was reserved for CD45 as well as PC5 for CD34 and PC7 for CD117. The conjugate PE was chosen where cell surface antigen density was expected to be low. Antibody selection was based on availability. Wherever possible we attempted to evaluate a minimum of two antibody sources, preferably three. Background marker

information was obtained from source Protein Reviews on the Internet at http://sucasaweb.nlm.nih.gov/prow/.

2.2.3.5.2 Patient Sample Selection for Antibody evaluation:

Patient selection was dependent upon the antibody under investigation. Sample selection i.e. blood or BM was also dependent upon the antibody under investigation. The cell count of sample was measured as per procedure mentioned above (see section 2.2.3.3.1). Aliquot of sample was used to obtain approximately 10^6 cells in 100 µl according to the following table:

Table 2-6 Specimen dilution for Antibody evaluation

WCC	*Volume of sample (µl)*	*Volume of FCS (µl)*
< 10,000	100	-
10,001 - 20,000	50	-
20,001 - 30,000	30	70
30,001 - 40,000	25	75
40,001 - 50,000	20	80
50,001 - 100,000	10	90

2.2.3.5.3 Initial Investigation – Determination of Working Titration:

Initially, several samples were obtained and stained with the antibody under evaluation. Subsequently, 10 samples were chosen, five with a high percentage of cells positive for the antigen and five with a low percentage of positive cells. Cells were stained performing an effective titration using decreasing volumes of antibody per 10^6 cells i.e. neat, 1/2, 1/4, 1/5, 1/10, and 1/20 in 0.4% azide in DPBS. The neat dilution was the manufacturer's recommended working volume. Some dilutions were not suitable (i.e. not able to separate the positive from the negative population), identified as the evaluation proceeded and therefore was removed from the evaluation study. Samples were stained with appropriate negative control

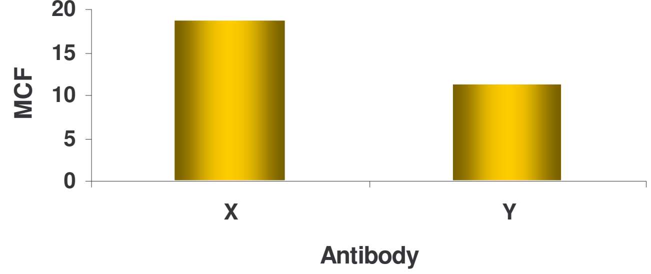
reagent by using the current Q-Prep method. An isotype control was run concurrently to exclude the negative cell population from the positive cell population. The negative cell population was always set at the first quadrant. The mean negative and positive cell fluorescence (MCF) was tabulated as well as percentage of positive cells (See Table 2-7).

Table 2-7 A representatative example for evaluation of antibody using two different brands.

Antibody	*MCF Negative Cell Population*	*MCF Positive Cell Population*	*% Positive Cells*
Negative control	0.195		
Brand X	0.460	28.3	45
Brand Y	0.855	25.9	32

In addition, the fluorescent intensity for the antigen was determined. The optimal working dilution of the antibody was selected based on: MCF of the negative cell population, which was equivalent to isotype control and good separation of positive and negative cells.

Figure 2-3 A representative example for evaluation of two antibodies using mean of the positive cell population MCF.



2.2.3.5.4 Patient Comparison:

This process was only performed when project involved the investigation of a replacement antibody for one currently in use. This was a double-blinded study and was performed in parallel with the current antibody marker. It consisted of 20 patients and this was done only for evaluation of CD45 ECD and compared it with CD45 PC5, which is the currently used antibody in the standard diagnostic panel.
The percentage of positive cells for each antibody was recorded and data compared using "Method Comparison- Mean Difference of methods" (Hollis 1996).

2.2.3.5.5 Uncommon Markers:

When the antibody was not used routinely, or if the number of positive cell populations was infrequent, the antibody was introduced into routine use without a comparison study. All the new antibodies used in this project were evaluated using the above methods.

2.2.3.5.6 Method comparison-Mean Difference

This procedure was used for all parallel method comparisons conducted for this thesis, as part of the evaluation protocol. This was done for examination of the bias, the new method against the existing method especially when we used new antibody conjugated to different fluorochrome than the one used in our laboratory or from different source. The standard error of the mean difference is a measure of the bias of the new method against the existing method.

2.2.3.6 Antibody dilution list

Unless otherwise specified in the Table 2-4, most of the antibodies used in this thesis were diluted after being evaluated for their usage. The following table shows antibody dilution preparation list.

Table 2-8 Antibody dilution preparation list

Antibody	Dilution	Expiry
CD2	420 µl CD2 + 1680 µl 0.4% azide in DPBS	21 days
CD7	190 µl CD7 + 760 µl 0.4% azide in DPBS	21 days
CD10	80 µl CD10 + 720 µl 0.4% azide in DPBS	7 days
CD15	120 µl CD15 + 1080 µl 0.4% azide in DPBS	21 days
CD19	180 µl CD19 FITC + 720 µl 0.4% azide in DPBS	7 days
CD45	825 µl CD45 + 825 µl 0.4% azide in DPBS	7 days
CD56	20 µl CD56 + 380 µl 0.4% azide in DPBS	7 days
HLA-DR	30 µl HLA-DR + 270 µl 0.4% azide in DPBS	7 days

2.2.3.7 Antibody panels

Antigen expression was analysed using five combinations of the following MoAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-TR (ECD), phycoerythrin-cyanin 5 (PC-5) and phycoerythrin-cyanin 7 (PC-7) at diagnosis. Two MoAb panels were used in the study, screening (the standard panel) and secondary panels. The screening panel was used to differentiae between AML and ALL (Table 2-9). The secondary panel was used to define LAPs. (Table 2-10).

Table 2-9 The Screening Panel

FITC	PE	PC-5
CD7	CD13	CD45
CD15	CD117	CD45
CD19	CD10	CD45
CD34	CD33	CD45
CD14	CD56	CD45
HLA-DR		CD45
MPO		CD45
TdT		CD45
S NEG		CD45

Table 2-10 The Secondary Panel

FITC	PE	ECD	PC-5	PC-7
Control FITC	Control PE	CD45	Control PC-5	Control PC-7
CD2	CD56	CD45	CD34	CD117
CD7	CD33	CD45	CD34	CD117
CD14	CD11b	CD45	CD34	CD117
CD15	CD33	CD45	CD34	CD117
CD65	CD33	CD45	CD34	CD117
CD33	CD13	CD45	CD34	CD117
CD64	CD33	CD45	CD34	CD117
CD19	CD10	CD45	CD34	CD117
CD38	CD123	CD45	CD34	CD117

2.2.3.8 Compensation

Below are overlapping emission spectra of fluorochromes used in this study (Figure 2-4 and 2-5).

2.2.3.8.1 Manual compensation

All dyes that were bound to antibodies were illustrated below (Figure 2-5). All dyes were excited by the Argon 488 nm laser.

FITC fluorescein isothiocyanate - a 488 nm excitable dye fluorescing in the green end of the spectrum (peak detected at 525 nm) used primarily for cell surface marker applications.

PE phycoerythrin - a dye exciting at 488 nm and emitting in the yellow end of the spectrum primarily used in cell surface marker applications.

ECD energy coupled dye - a tandem dye exciting at 488 nm and emitting at the orange end of the spectrum (peak detected at 620 nm) used with cell surface markers.

Some important facts were taken into account when applying compensation to an experiment and these were:

- Dyes that minimise the spectral overlap were chosen in the collection area of the filters being used (Roederer 2001).
- Cells stained with a single colour as a control was used to determine the amount of compensation. Studies have shown that setting up flow cytometry systems with cells stained with single colour immunofluorescent markers is the optimal method for calculation of the colour compensation required for the experiment (Diamond RA, De Maggio S. In Living Color – Protocols in Flow Cytometry and Cell Sorting. 1st Ed. Springer, Berlin 1999; 92-97) (Tung *et al.* 2004). Other methods using mixtures of coloured beads may not be as satisfactory as they do not represent real cells stained with real antibody-based fluorochromes.

- High PMT voltage was not applied to one of the detectors as this may make it difficult to compensate out overlap into that detector.
- Only the fluorochromes used in the experiment were used for colour compensation setup.

Compensation settings were tied to the sample preparation and instrument configuration. Any adjustment to the gain, PMT voltage, cells, dyes, or filters required that the amount of compensation be re-determined for the experiment.

Figure 2-4 The Emission spectra shown are for equal concentrations (mg antibody/ml) of mouse anti-human IgG directly conjugated with fluorescein (FITC), phycoerythrin (PE), and tandem conjugates of phycoerythrin with Texas red (PE-TR or 613) and Cy5 (PE-Cy5 or Tricolour), with excitation at 490nm. Boxes demarcate the passband of the green (525nm), yellow (575nm), orange (620nm), and red (660nm long pass) filters used in front of the fluorescence detectors (From Practical Flow Cytometry Third Edition, Howard M. Shapiro p.164).

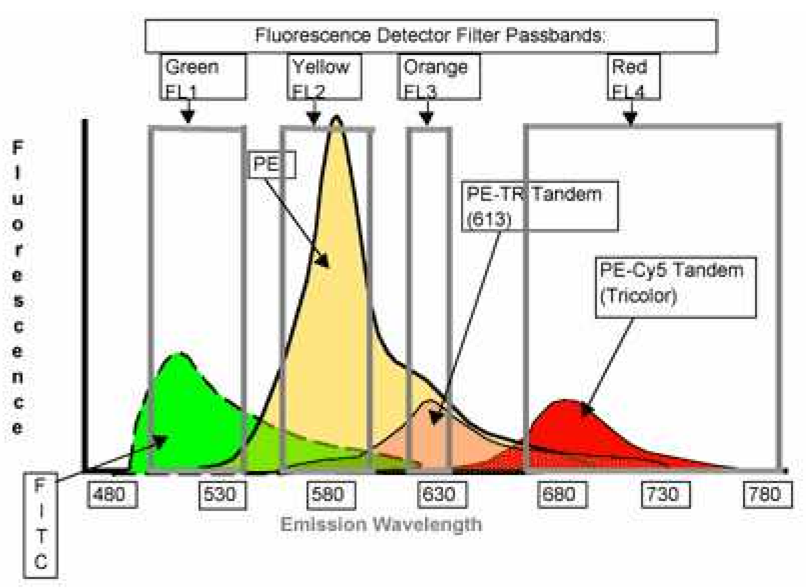
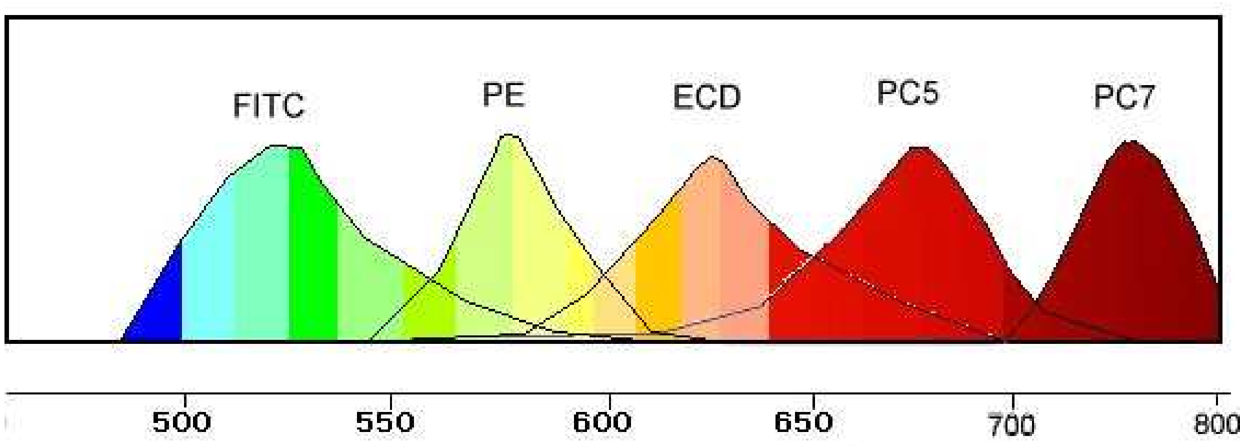


Figure 2-5 Spectral Overlap of Fluorochromes used in this study as excited by Argon 488 nm laser.



2.2.3.8.2 Five-colour compensation for FITC, PE, ECD, PC5 and PC7

MoAbs against the Leukocyte Common Antigen (CD45) were preferred because CD45 has the highest expression, which produces a high intensity signal in all the fluorescent detectors.

The compensation was done by staining seven tubes according to Table 2-11 using the staining procedure described in section 2.3.3.4. Tube 1): negative control tube, a five-colour Isotype controls was used to adjust the PMT voltages and to place the negative populations in the first decade, tube 2): FITC CD3 only stained cells, tube 3): PE CD3 only stained cells, tube 4): ECD CD45 only stained cells, tube 5): PC5 CD45 only stained cells, tube 6): PC7 CD45 only stained cells, and tube 7): compensation verification control consisting of cells stained with five-colour MoAbs with different specificity, used to verify compensation.

Table 2-11 Compensation Panel of MoAbs

Tube	FITC	PE	ECD	PC-5	PC-7
1	S neg	S neg	Control ECD	Control PC-5	Control PC-7
2	CD3	S neg	Control ECD	Control PC-5	Control PC-7
3	S neg	CD3	Control ECD	Control PC-5	Control PC-7
4	S neg	S neg	CD45	Control PC-5	Control PC-7
5	S neg	S neg	Control ECD	CD45	Control PC-7
6	S neg	S neg	Control ECD	Control PC-5	CD45
7	CD8	CD4	CD19	CD45	CD2

A proper system start up was performed and the instrument was meeting expectation of HPCVs and channel means by using Flow-Check ™ Fluorospheres. For a full description of colour compensation, please refer to the instructions for use, Cytomics FC500 training modules. Briefly, the compensation protocol for the application was loaded. Negative control was run to adjust the PMT values in order to have all the negatives in the first decade and adjusted the quadrants. The blast region was selected based on CD45 gating strategy. Each of the single stained samples (tubes 2, 3, 4, 5 and 6) was run one at a time started with FITC stained sample and any overlap between the dye of interest into the other dyes was subtracted.

2.2.3.8.3 BD CompBeads

The introduction of the FC500 and CXP software has seen improvements in the performance of colour compensation. However, there were still issues with the performance of such, finding cells that were positive for markers that were attached to all fluorochromes that require compensation especially when looking at blast population (characterised in most of the instances by CD34 or CD117). For that purpose, compensation particles, 'CompBeads' had been sourced from BD [Compensation Particles Set (BD Cat # 552843) with Bead set 1 - CompBeads Anti-mouse Ig, k capture bead and Bead set 2 - CompBeads Negative Control].

'CompBeads' are a set of two non-fluorescent polystyrene beads. The first bead in the set was able to capture the mouse kappa light chain of any fluorescently tagged antibody composed of such a light chain. The second bead had no binding capacity. When mixed together the beads provided distinctive positive and negative populations that were used to set compensation levels.

Antibody/fluorochrome combinations were selected to perform colour compensation. CD3-FITC, CD5-PE, CD45-ECD, CD45-PC5, CD45-PC7. The beads were stained as per package insert. Briefly, 100 µl DPBS to each tube was added. Appropriate volume of antibody was added to each tube. One drop of each bead, Anti-mouse Ig, κ capture bead and negative bead was added to each tube. The tubes were mixed and incubated for 20 minutes at RT. After that, 2 ml wash solution was added and the tubes were centrifuged. The supernatant decanted and 500 µl DPBS was added. Appropriate PMT voltage and gains for FS, SS linear, LFL1 – FITC, LFL2 – PE, LFL3 – ECD, LFL4 – PC5 and LFL5 – PC7 was determined. The compensation was set to zero. The compensation tubes were run

one at a time. The visual inspection of compensation was acceptable, all populations were well defined (Figure 2-6).

Once the PMT and gains had been determined and the 'Flow-Set' and 'Settings' protocols optimised, the procedure proceeded smoothly. The beads displayed high SSC but were easily identifiable on the SS/FS plot. The scatter plot was SS v FS gating strategy. Each of the single stained beads was run one at a time started with FITC stained beads and same procedure followed as in section 2.2.3.8.2.

2.2.3.9 Gating strategy for LAPs

2.2.3.9.1 Staining and analysing marker expression in a 1st run

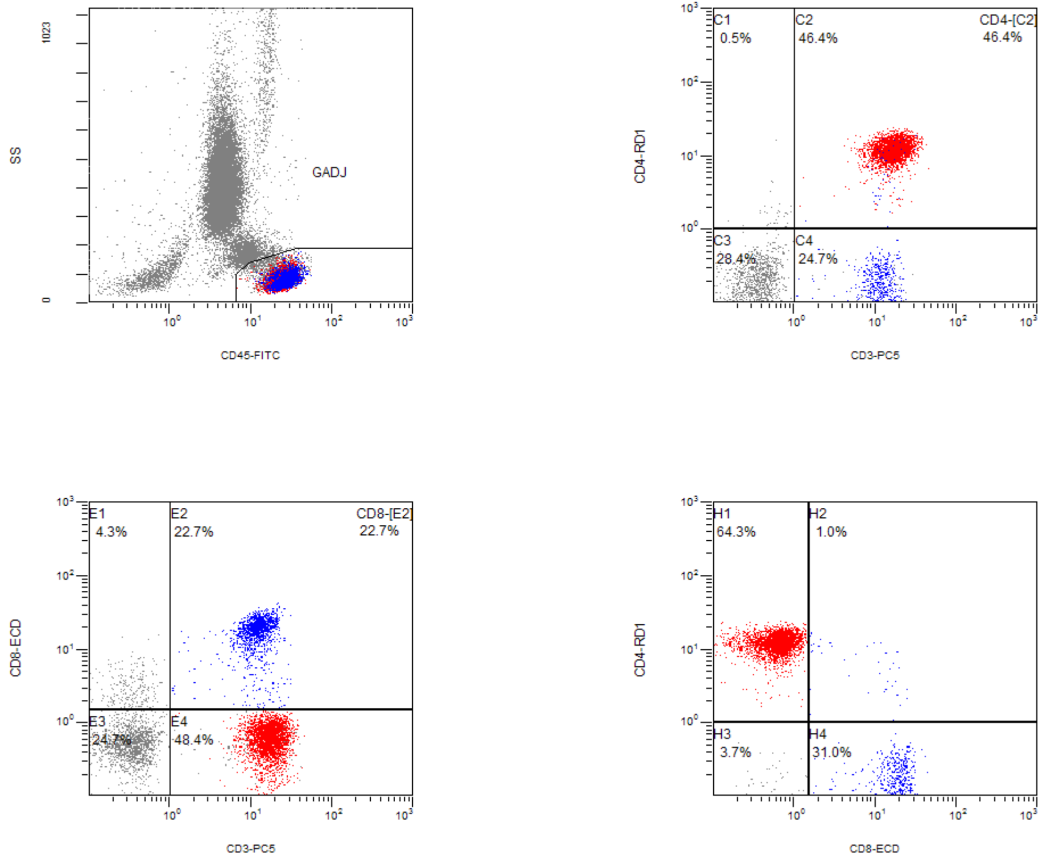
For each tube 20,000-50,000 WBC events (characterised by CD45 expression and SS Log, see Figure 2-7) was acquired on flow cytometer. The marker expression was analysed on the leukaemic blast population(s) (See section 2.2.3.9.5 Data reporting and interpretation).

2.2.3.9.2 Defining LAPs and verifying these in a 2nd run

LAPs based on the marker expressions as measured and analysed in the first run were defined according to the instructions (Figure 2-8). At least two LAPs with different aberrant marker expressions were defined. If only one aberrant marker was present, a second LAP with the same aberrant marker was defined but with a different myeloid or primitive marker. The putative LAPs was verified in a second run by staining in a FACS tube the MoAbs which make up the LAP and acquiring 20,000-50,000 WBC events.

Data analysis was performed with Beckman Coulter CXP® software. The gating strategy both for the percentage of different cell populations (blasts, lymphocytes,

Figure 2-6 Compensation using BD CompBeads



monocytes and granulocytes) and for marker expression in AML blasts are represented with CXP® software.

The gating steps as described below were illustrated in Figure 2-8.

1 Leukaemic blasts (characterised by CD45 dim-SS Log low (Lacombe *et al.* 1997)),

2 Lymphocytes (characterised by CD45 high-SS Log low),

3 Monocytes (characterised by CD45 high-SS Log intermediate),

4 Granulocytes (characterised by CD45 low-SS Log high),

These different cell populations were illustrated in Figure 2-7.

The region on blast population was fine tuned by back gating those blasts showing expression of a primitive marker (CD34, CD117). The blasts were further distinguished from monocytes by the presence of an aberrant phenotype or lack of CD14 expression. The gate on the monocytes was fine tuned by back gating those cells showing CD14 expression (see Figure 2-9).

2.2.3.9.3 After 1st run: Analyses of marker expressions on the leukaemia blast population

In all defined leukaemia blast gates (immature, mature and aberrant, depending on their presence), the marker expressions of the labelled cells was established. The intensity of marker expression (no, dim or positive expression) was determined by comparing the expression of these markers with the autofluorescence of the unlabelled cells (negative controls). Only markers expressed on >10% of the total leukaemia blast population were used for LAP identifcation. Back gating was done to check where the LAP was located in FS vs SS. These back gating steps were necessary:

1) To recognise apoptotic cells (these cells were located low in the FSC scale) (Figure 2-9). These cells were ignored.

2) To establish the maturity and/or type of these cells by:

   i. Assessing the location of the cells in the CD45-SS Log. When cells were located outside the area of the blasts, these cells belong to another cell population and;

   ii. Assessing the expression of other immature/mature markers on cells, which were simultaneously labelled in the same tube.

With this approach, we confirmed whether the marker expression of cells of interest originated from another (leukaemia) cell population.

2.2.3.9.4 After a 2nd run: Establishment of a LAP

The gating steps described below were illustrated in Figure 2-8. To determine whether the combination of marker expressions on a leukaemia blast population formed a reliable LAP, we checked the FSC-SSC and CD45-SS Log by back gating the localisation of leukaemia cell population that expressed the LAP. When the LAP included the presence of an aberrant marker combined with a primitive marker (CD34, CD117), the percentage of the aberrant marker on blasts showing the primitive marker was exceeded by 20% expression (normal blasts may also show ~ 5%-10% aberrant marker expression).

2.2.3.9.5 Data reporting and interpretation

The percentage of CD45 aberrant leukaemic blasts, the aberrant marker and its percentage of the blast compartment and the LAP and its percentage of the blast compartment, which determines the LAP, was reported. The sensitivity, specificity, stability of LAPs determined the quality of the LAP for the detection of MRD in follow-up samples.

2.2.3.10 Gating strategy for MRD

BM of AML patients in CR was processed for MRD studies. The amount of cells per tube was dependent on the number of required antibody staining. For 1 staining about $1x10^6$ cells was taken. When the total available number of WBC was too low, only the most reliable LAP(s) was determined with as many cells as possible. Preferably, 2 independent LAPs were used to exclude false-negative MRD measurements due to phenotypic shifts. The MoAbs that define the LAP expression as established in diagnosis material were pipetted in FACS tubes according to

secondary panel combinations. When possible, $0.5x10^6$-$1x10^6$ WBC events were acquired for each tube or until tube was empty on flow cytometer.

2.2.3.10.1 Data analysis

Data analysis was performed with Beckman Coulter CXP® software. The percentage of CD45 dim blasts was analysed as described in section 2.2.3.9.

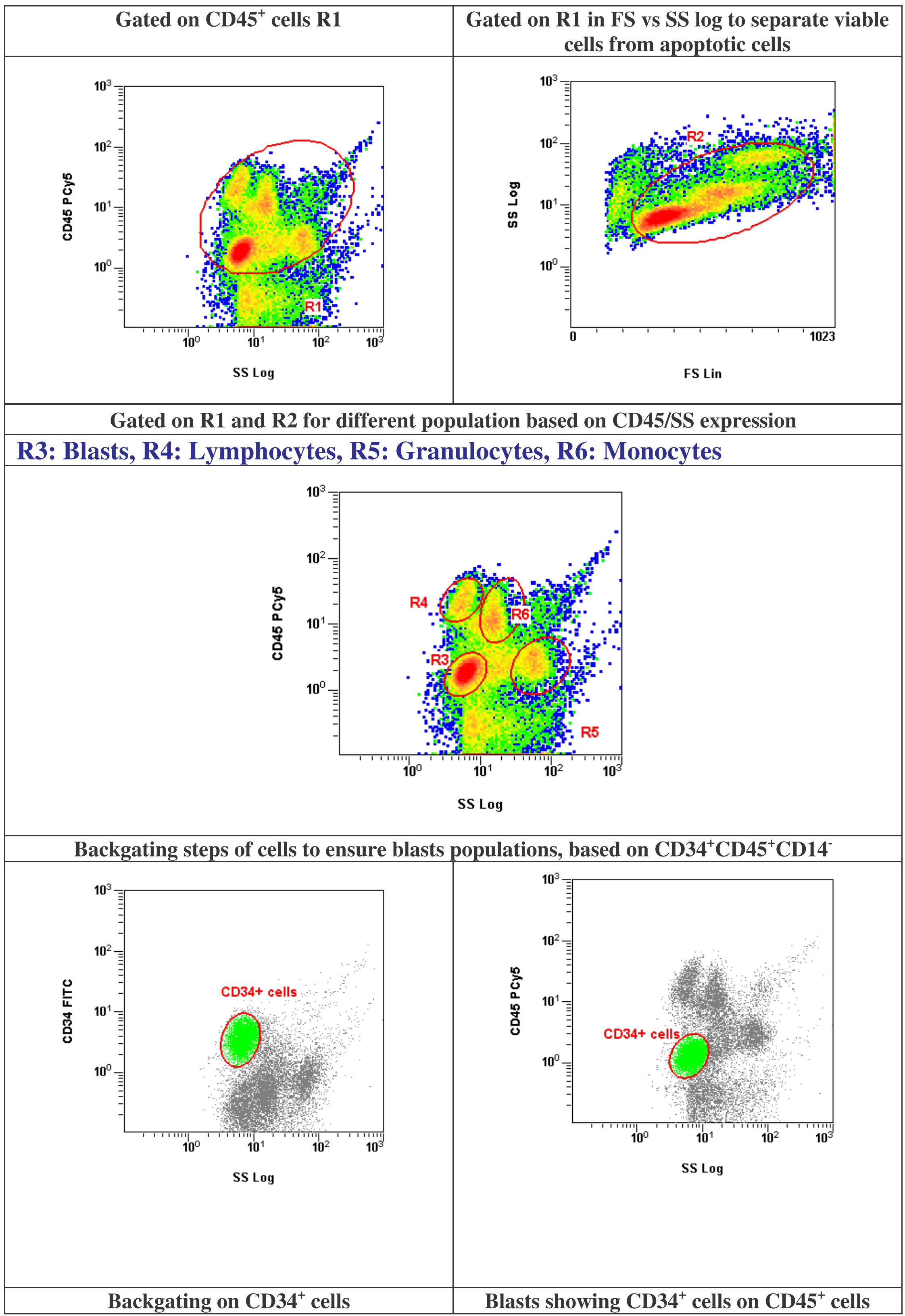
2.2.3.10.2 Analyses of MRD

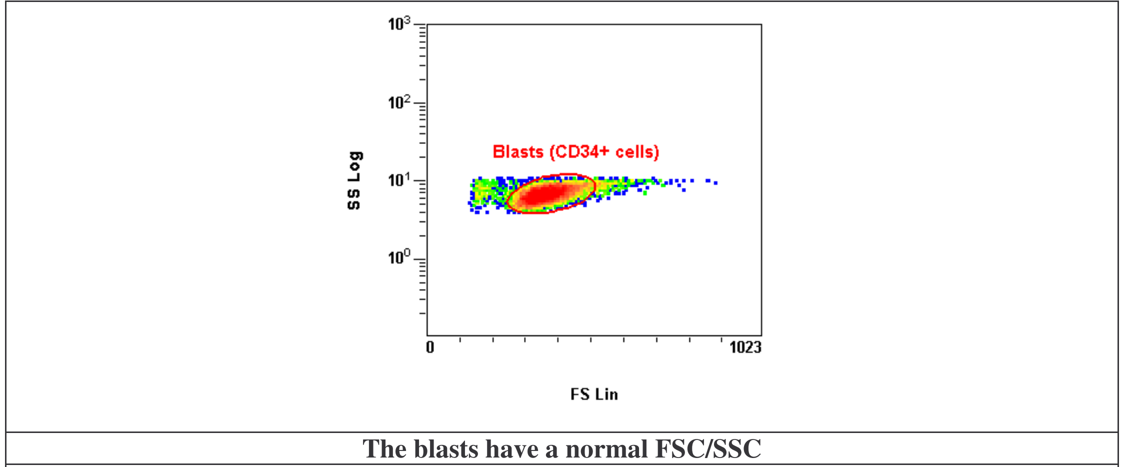
The percentage of LAP^+ cells in the WBC cell compartment, characterised by CD45-SS Log was analysed, in which dead cells and cell fragments were excluded. LAP^+ cells based on the LAP expression in newly diagnosed material was gated taking into account that: 1) the intensity of markers in the LAP might have been undergone some changes compared to diagnosis material and 2) normal cells might contaminate this LAP gate. Therefore, the gate on the LAP^+ cells were adjusted taking into account that most AML LAP^+ cells were included but with minimal contamination of normal cells. The characteristics of the LAP^+ cells were verified by backgating the LAP^+ cells in the density plots FSC/SSC and CD45/SS Log. LAP^+ cells in these plots were clustered similar to as at diagnosis. The cells with other characteristics were excluded when necessary. The percentage of the LAP^+ cells in the WBC compartment was noted.

2.2.3.10.3 Data reporting and interpretation

The percentage of blasts, the combination of markers that defined the LAP and the percentage of LAP as established in diagnosis material, the percentage of LAP and MRD in the WBC compartment was reported.

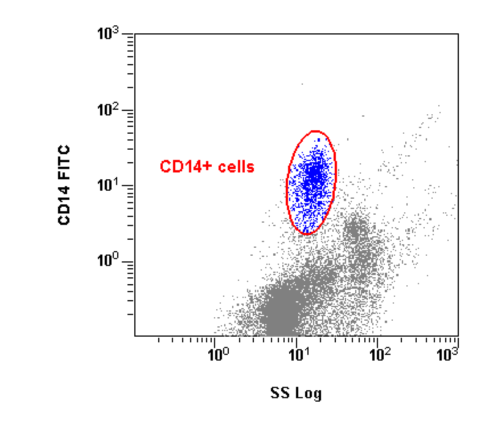
Figure 2-7 Analysis of Different Cell Populations



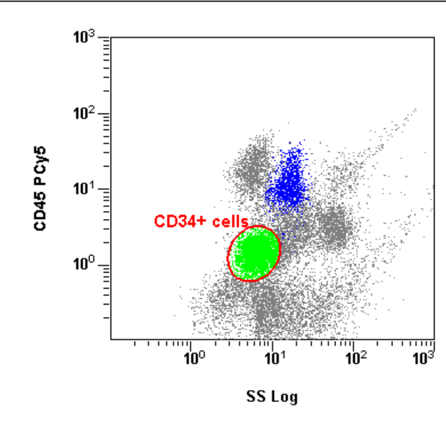


The blasts have a normal FSC/SSC

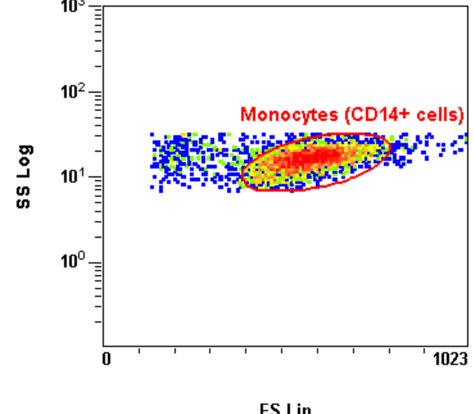
Differentiation status of cells



Backgating on CD14+ cells, to ensure that the blasts cells are not contaminated with monocytes



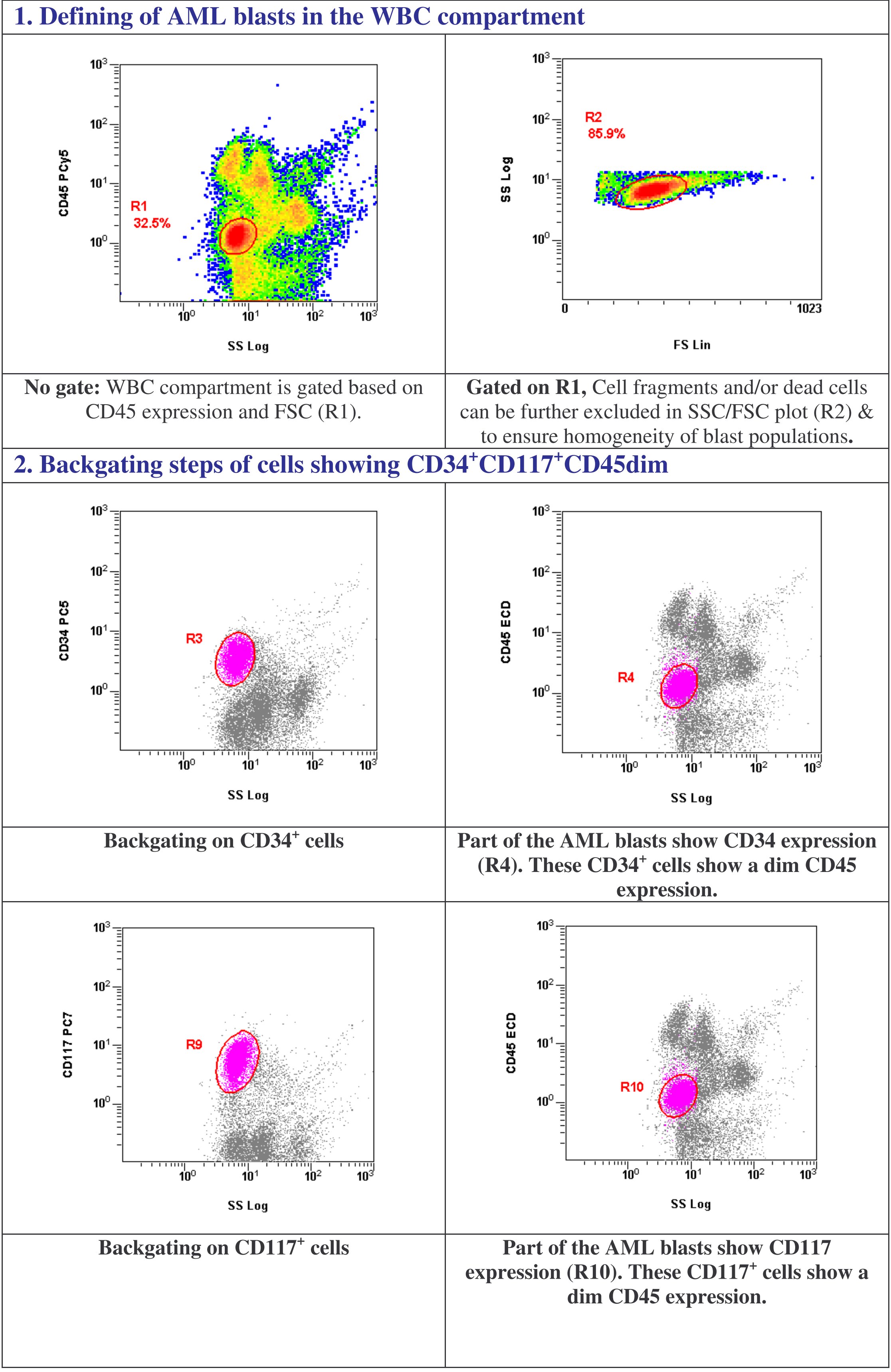
Gated on blasts showing CD34+/CD14- on CD45+ cells, and CD14+ cells on Monocytes only

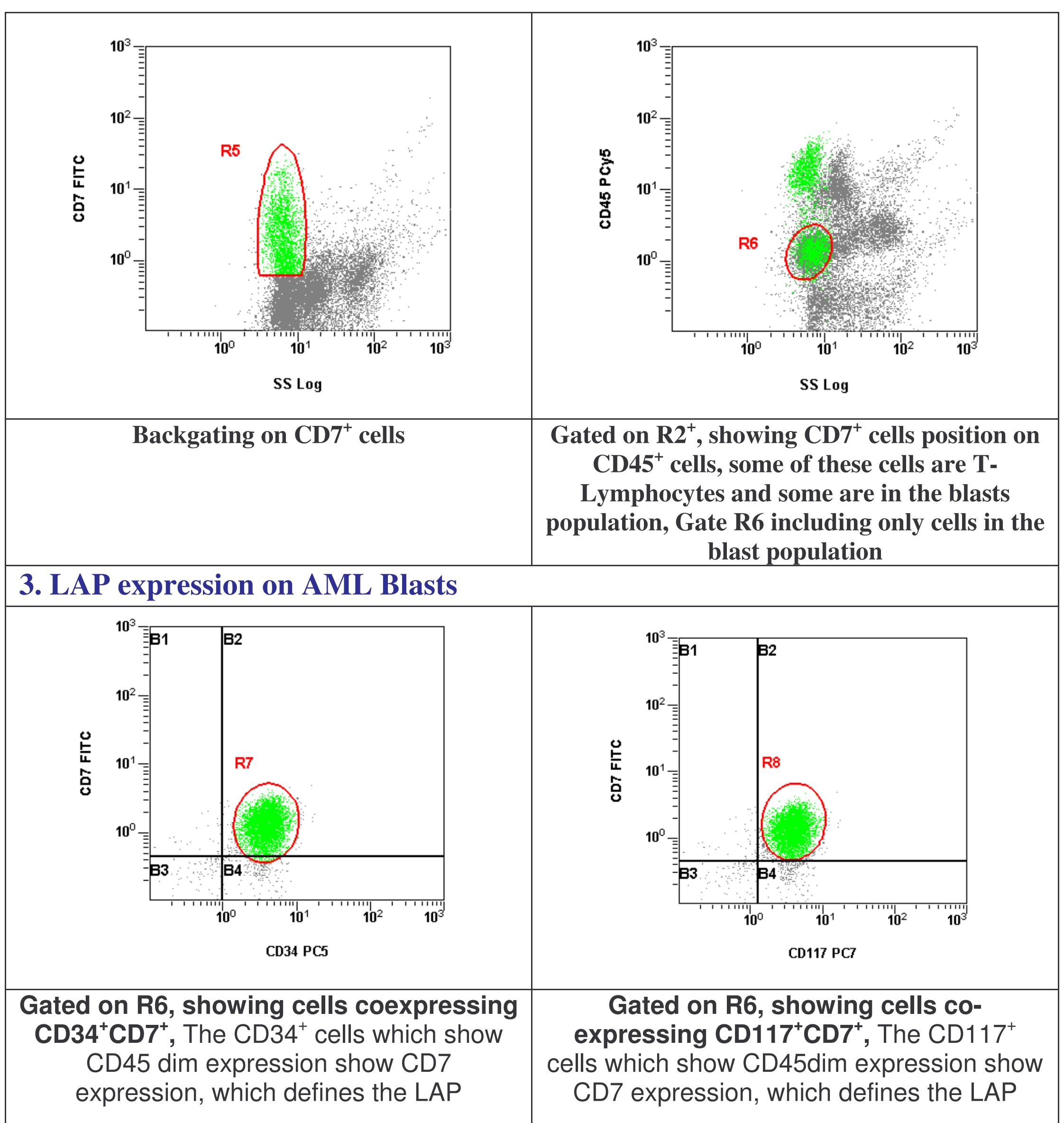


Summary:

AML blasts are identified by CD45dim/SSC (R3) (Lacombe *et al.* 1997) . The AML blasts show CD34 expression. The CD34+ cells are located in the centre of the CD45 gate and have a normal FSC/SSC pattern. However, the AML blasts with CD14 expression are located in the outer range of the CD45 gate. These cells are located somewhat lower in the FSC. In conclusion, these CD14+ cells are originated form another cell population (monocytes).

Figure 2-8 Establishment of a LAP





Backgating on CD7$^+$ cells

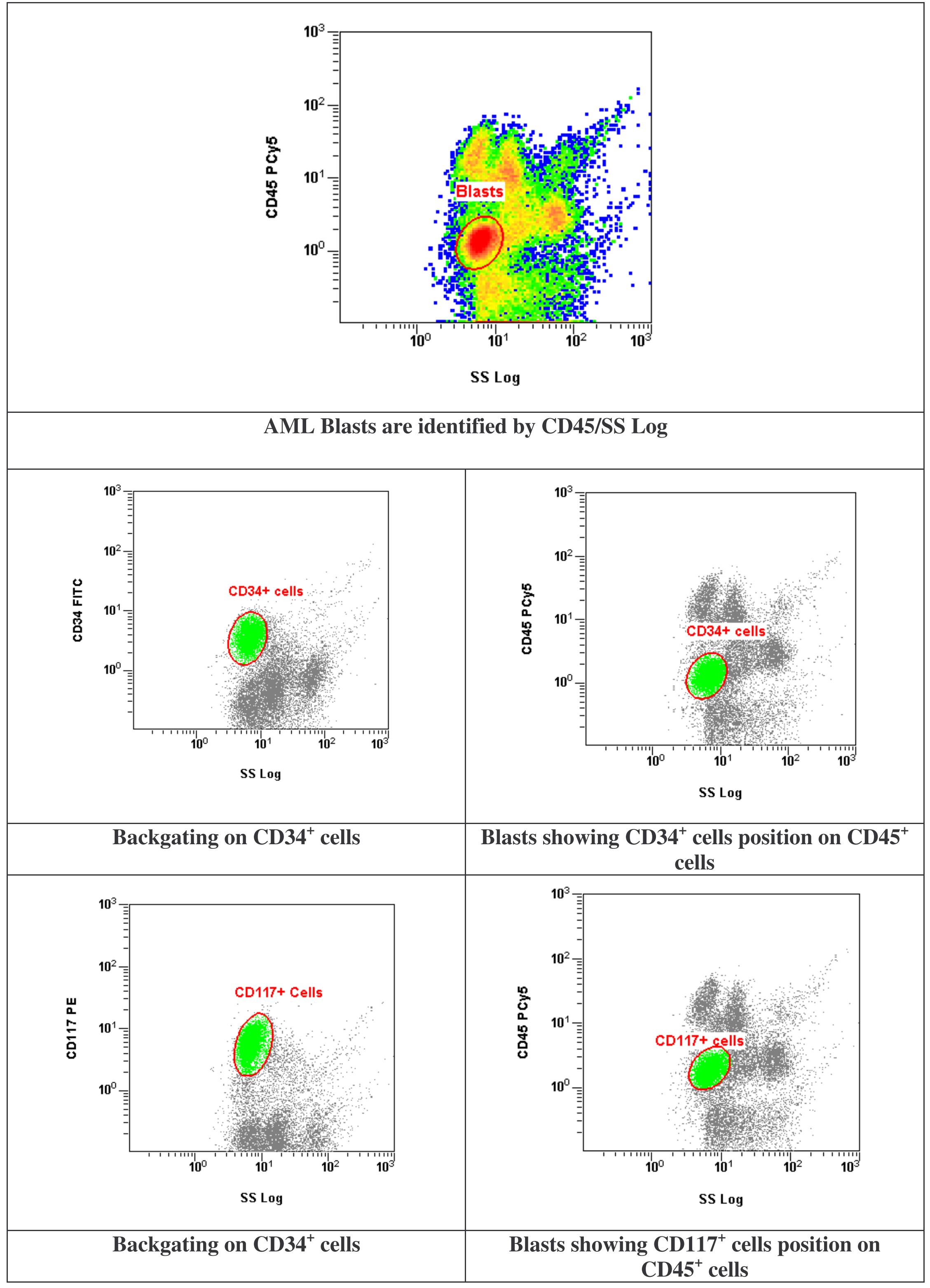
Gated on R2$^+$, showing CD7$^+$ cells position on CD45$^+$ cells, some of these cells are T-Lymphocytes and some are in the blasts population, Gate R6 including only cells in the blast population

3. LAP expression on AML Blasts

Gated on R6, showing cells coexpressing CD34$^+$CD7$^+$, The CD34$^+$ cells which show CD45 dim expression show CD7 expression, which defines the LAP

Gated on R6, showing cells co-expressing CD117$^+$CD7$^+$, The CD117$^+$ cells which show CD45dim expression show CD7 expression, which defines the LAP

Figure 2-9 Apoptotic / Viable Status of Cells



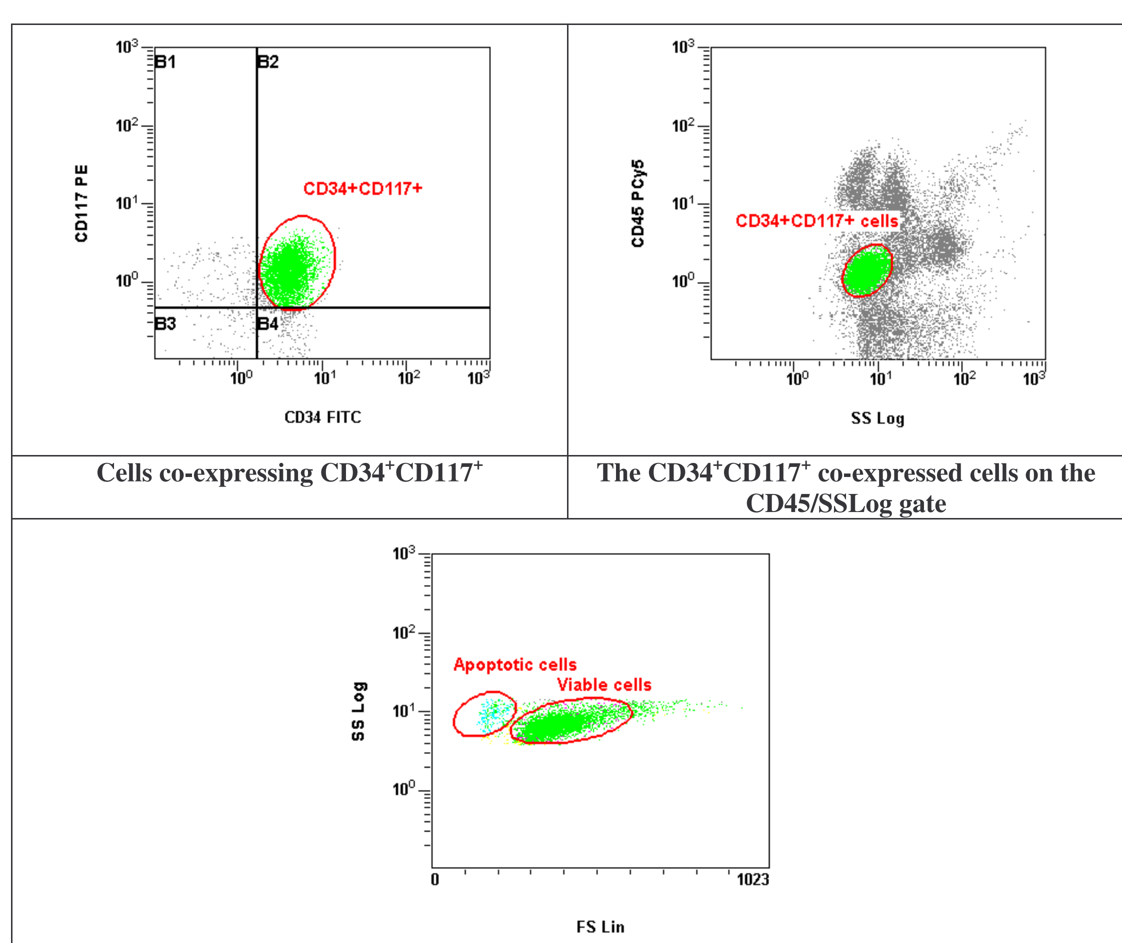
AML Blasts are identified by CD45/SS Log

Backgating on $CD34^+$ cells

Blasts showing $CD34^+$ cells position on $CD45^+$ cells

Backgating on $CD34^+$ cells

Blasts showing $CD117^+$ cells position on $CD45^+$ cells



Cells co-expressing CD34^{+}CD117^{+}

The CD34^{+}CD117^{+} co-expressed cells on the CD45/SSLog gate

Gated on the CD34^{+}CD117^{+} AML Blasts, these cells have a population of normal FSC/SS Log pattern (viable cells) and a population with low FSC, these cells are apoptotic cells.

2.2.3.11 Fluorescence activated cell sorting

Cells were thawed using the above method (See section 2.2.3.2) using IMDM thawing medium, washed twice in PBS containing 3% FCS and stained with the desired combination of antibodies CD34 PC5, CD38 FITC ,CD123 PE, and CD45 ECD and incubated for 20 minutes in dark at RT. Cells were run through BD FACSARIA™ Cell Sorter (BD Biosciences, San Jose, CA95131USA). Pools of cells expressing similar level of $CD34^+/CD38^-/CD123^+$ and $CD34^+/CD38^-/CD123^-$ cells were collected in FACS tubes with 2/3 IMDM medium without DNase, after applying CD45 gating strategy. Cells were centrifuged using a Heraeus multifuge 3S-R bench top centrifuge and resuspended in 200 μl PBS. After that, DNA was purified from these cells and PCR was done.

2.2.4 IMMUNO-FLUORESCENCE CONFOCAL MICROSCOPY ASSAYS

Immuno-Fluorecence Confocal Microscopy method was performed using Laser Scanning Confocal Microscopy at Detmold Family Imaging Centre, Hanson Institute/IMVS, Adelaide SA. The cells were mounted on the slide by adding a drop of anti-fade mounting medium for fluorescence confocal microscopy after being stained with FITC, PE, PC5 fluorochromes as for Flow cytometry staining. The images were produced using the BioRad Radiance 2100 confocal microscope (at Detmold Imaging Core Facility, Hanson Institute) equipped with three lasers, Argon ion 488nm; Green HeNe 543nm; Red Diode 637nm and Olympus IX70 inverted microscope. The objective used was a 20x UAPOW (NA= 0.70) or 40x UPLAPO with NA=1.15 water, or a 60X UPLAPO with NA=1.4 water. The single, dual or triple labelled samples were imaged with three separate channels (PMT tubes) in a sequential setting. The Green fluorescence (FITC) was excited with Ar 488 nm laser

line and the emission was viewed through a HQ515/30 nm narrow band barrier filter in PMT1. The red fluorescence (PE) was excited with Green HeNe 543 nm laser line and the emission was viewed through a long pass barrier filter (570LP) to allow only red light wavelengths pass through PMT2. The far red fluorescence (PC5) was excited with Red Diode 637nm laser line and the emission was viewed through a long pass barrier filter (660LP). Automatically all signals from PMTs 1, 2 and 3 were merged. The image data were stored on a CD for further analysis using a Confocal Assistant software program for the Microsoft® Windows™ (Todd Clark Brelje. USA).

2.2.5 MOLECULAR TECHNIQUES

2.2.5.1 Purification of DNA

Two techniques were used in the purification of DNA. These are Qiagen kits and salt extraction. After purification of the target DNA, samples were electrophoresed and stained with ethidium bromide as in section 2.2.5.3. The gel was then scanned on the Geldoc system and a printout the same size as the gel was obtained.

2.2.5.1.1 Salt extraction

DNA was extracted directly from the sorted cells when we had a low number of $CD34^+/CD38^-/CD123^+$ or $CD34^+/CD38^-/CD123^-$ cells. Cells were digested using Proteinase K. DNA was then extracted from the cell digest with sodium chloride. Ethanol was used to precipitate the DNA, which was then washed to produce a product suitable for PCR analysis.

Briefly, to each eppendorf, 80 µl of 6M NaCl was added and vortexed for 15 seconds. The tube was centrifuged on High using Sorvall microcentrifuge for 10 minutes. The supernatant was pipetted into a 1.5 ml eppendorf tube and the pellet

was discarded. Then, 700 µl of 100% ethanol were added and the tube inverted for 1 minute or until visible strands of DNA was seen. The tube was centrifuged on High using Sorvall microcentrifuge for 10 minutes again. The supernatant was discarded and the pellet was tipped off. One millilitre (1 ml) of 70% ethanol was added and the tube was vortexed. The tube was centrifuged on High using Sorvall microcentrifuge for 10 minutes. The supernatant was discarded and the pellet was tipped off. The pellet was dried in heat block. Then, 20 µl autoclaved water was added, mixed and stored at 2-8°C. DNA concentration was measured as detailed later. DNA preparation was made to a concentration of 100 ng/µl.

2.2.5.1.2 QIAmp® Kits

The QIAmp® Kit (QIAamp® Blood Kit - QIAGEN Cat No. 51106 (250 test) or Cat No. 51104 (50 test) was designed for the rapid purification of DNA from free cell suspensions. Cells were initially lysed and treated with a protease to release nuclear contents. DNA was precipitated using ethanol and was then subsequently trapped on a membrane enclosed in a spin column. The DNA precipitate was washed to remove contaminants, specifically haemoglobin. DNA was eluted from the membrane using water. This procedure was done exactly as QIAamp Blood Kit and QIAamp Tissue Kit Handbook QIAGEN 1999. L, 500 µl .

2.2.5.2 Calculation of DNA Concentration and Purity by Spectrophotometer

DNA concentration was determined by measuring the absorbency of a DNA preparation at 260 nm. The purity of the solution was assessed using the ratio of absorbance at 260 nm to an absorbance at 280 nm. Pure DNA had as A260/280 ratio of 1.7 - 1.9 on a Nanaodrop ND-1000 spectrophotometer. For genomic DNA

samples below desired concentration, samples were dried in speedivac and the final volume was calculated as follows:

$$\text{Final volume} = \frac{\text{Current concentration (in ng/µl) x current volume}}{\text{Desired concentration (in ng/µl)}}$$

Volume of water to add = Final volume – current volume

Calculations of DNA concentration and purity were based on the following:

One OD unit at A260 is equivalent to 50 ng/µl of genomic (1 cm light path) DNA and 20-30 ng/µl of oligonucleotide DNA. Alternatively, DNA was electrophoresed and the intensity of the ethidium bromide stained bands was compared to those with pUC19.

2.2.5.3 Electrophoresis of DNA

DNA was separated by electrophoresis on agarose gel (DNA grade, Progen) made in 1x Tris-Borate-EDTA (TBE) with the percentage of the gel dependent on the size of the DNA products to be detected. Generally, 2% agarose gels were used. Gels were immersed into a horizontal electrophoresis tank containing 1x TBE. DNA samples (10 µl) were combined with loading buffer (2 µl) and loaded into the wells. Gels were electrophoresed using BioRad Power Pac 300 at 130V until the bromophenol blue dye front was three quarters along the gel. Gels were then removed, stained with ethidium bromide for 10 minutes and destained in water for 10 minutes. The Geldoc system was used to visualise DNA bands and the image was captured using UV/Photo.

2.2.5.4 Size determination and quantitation of DNA fragments

The size of DNA fragments was calculated by comparing their relative mobilities in agarose with DNA of known size. The commercially available molecular weight markers used were pUC19 DNA.

2.2.5.5 Primers used for PCR and sequencing

Table 2-12 summarises the location of the oligonucleotide primers used to sequence or amplify *FLT3*. The location of these oligonucleotide primers is based on the *FLT3* sequence published by Nakao *et al* (Nakao *et al.* 1996).

Table 2-12 Oligonucleotide Primers

Name	*Oligonucleotide Sequence*	*Melting temperature*	*Location*	*MW*
11F	5'-GCAATTTAGGTATGAAAGCCAGC-3'	66°C	Exon 14	7097
12R	5'-CTTTCAGCATTTTGACGGCAACC -3'	68°C	Exon 15	6975

2.2.5.6 Polemerase chain reaction

All reagents were aliquoted using non-aerosol tips in order to reduce contamination. Reactions were prepared in 0.5 ml microcentrifuge tubes to a final volume of 25 µl. For amplification of DNA, reactions containing 1.25 ng DNA (10 µl), 2 ng/µl each of forward and reverse primers (0.5 µl), 0.2 mM dNTPs (2.5 µl), 1.5 mM MgCl2 (1.5 µl), Taq buffer (2.5 µl), Taq Gold polymerase (0.25 µl) made to the final volume with sterile Milli-Q water (7.25 µl) were prepared. DNA was amplified using a DNA Thermal Cycler (Eppendorf Mastercycler) with an initial 7 minutes denaturation step at 94°C followed by 35 cycles (1 minute) of denaturation, primer annealing (62°C), and elongation (72°C), finishing with a final extension at 72°C for 7 minutes. Completed reactions were stored at 4°C. The temperature selected for the

annealing of primers was 1-5°C lower than the average melting temperature for the two-oligonucleotide primers. The melting temperature was calculated using the formula:

Melting Temperature (^{o}C) = 2(A+T) +4(C+G)

Optimised PCR conditions for our set of primers are detailed in Table 2-13 below.

Table 2-13 Optimised PCR conditions for the primers

Process	*Time (mins)*	*Temperature (°C)*	*Number of cycles*
Denaturation	7	94	
Denaturation	1	94	35
Annealing	1	62	35
Elongation	1	72	35
Elongation	7	72	
end	4		

2.2.5.7 Sequencing the DNA

The Bigdye Terminator ready reaction mix and 5x Sequencing Buffer (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, Version 2.0 (P/N 4314414-6) was used to sequence DNA purified using the Qiaquick PCR purification kit (Cat No: 28106 or 28104). For a sequencing reaction, 1 µl of Terminator Ready Reaction Mix (A, G, C, T-Dye Terminator, dGTP, dATP, dCTP, dTTP, Tris-HCL, pH 9; $MgCl_2$, thermostable pyrophosphatase AmpliTaq polymerase was added to 20 ng of Template DNA (2 µl), 3.2 pmol of sequencing primer (2.4 µl) and 5x Sequencing Buffer (3.5 µl). The final volume was made up to 20 µl with Milli-Q water. The reaction was cycled using a Thermal Cycler (Eppendorf Mastercycler) using the following program: [96°C 10 seconds, 50°C 5 seconds, 60°C 4 minutes] x 25 cycles, followed by a 4°C hold step. Following cycle

sequencing, the DNA was precipitated with 80 µl 75% isopropanol and incubated at RT for 15 minutes. The DNA was pelleted by spinning at 13,200 rpm for 30 minutes, washed with 200 µl of 75% isopropanol by spinning at 13,200 rpm for 15 minutes and dried in 65°C heat block for 5 minutes. Samples were then submitted to the IMVS sequencing facility, Molecular Pathology, for analysis on an automated sequencing system (a Perkin Elmer automated sequencer), which uses capillary electrophoresis system to separate the oligonucleotide products and fluorescence was monitored.

2.2.6 IMMUNOMAGNETIC ASSAYS

2.2.6.1 Isolation of CD34^{+} Cells: MACS Separation

The isolation of CD34^{+} cells was performed on mononuclear cells (MNC) from the BM of AML patients. MNC were washed twice with MACS CD34^{+} buffer, and CD34^{+} progenitor cells were purified using a MACS CD34^{+} progenitor cell selection isolation kit (Miltenlyi Biotech, Germany) according to the manufacturers instructions. Briefly, cells were suspended in 300 µl MACS buffer per 10^{8} cells, and 100 µl of both FcR Blocking reagent and hapten-Antibody added per 10^{8} cells. The suspension was mixed well and incubated for 45 minutes at 4°C. Cells were washed twice in MACS CD34^{+} buffer using a Heraeus multifuge 3S-R bench top centrifuge and resuspended in 400 µl MACS buffer per 10^{8} cells. MACS Anti-Hapten MicroBeads (100 µl per 10^{8} cells) were added to magnetically label the cells, and the suspension was mixed well and incubated at 4°C for 45 minutes. The cells were washed twice with MACS CD34^{+} buffer using a Heraeus multifuge 3S-R bench top centrifuge, and then resuspended in 500 µl degassed MACS buffer per 10^{8} cells (for less than 10^{8} cells, a 500 µl volume was used).

A MACS LS+ column was placed in a mini-MACS separator magnet and pre-washed with 3 ml of degassed MACS $CD34^+$ buffer. The cell suspension was then placed onto the column and the column was washed with cold buffer until no cells were detected in the effluent. Labelled cells within the column were removed by adding 3 ml buffer to the column, removing the column from the magnet, and plunging. The eluate was collected in a 10 ml polypropylene conical tube (Falcon) and centrifuged for 5 minutes a in a Heraeus multifuge 3S-R bench top centrifuge. The cells were resuspended in 500 µl of buffer and reloaded onto fresh MACS LS+ column that had been pre-washed with 3 ml of degassed MACS $CD34^+$ buffer. The column was washed with cold buffer until no cells were detected in the effluent. Labelled cells within the column were removed by adding 3 ml buffer to the column, removing the column from the magnet, and plunging. The eluate containing the $CD34^+$ cells was collected in a 10 ml polypropylene conical tube and centrifuged for 5 minutes in a Heraeus multifuge 3S-R bench top centrifuge. The purity of $CD34^+$ cells following the isolation procedure was calculated by staining with an anti-CD34-PC5.

2.2.7 STATISTICAL ANALYSIS

CR was defined as an absolute neutrophil count of ≥1,500/µl, platelet count of ≥ 100,000/µl, no leukaemic blasts in the blood nor evidence of extramedullary leukaemia, BM with a cellularity of more than 20%, maturation of all three-cell lineages, no Auer rods, and less than 5% BM blast cells with persistence for at least 1 month. Relapse was defined as the reappearance of circulating blasts not attributable to "overshoot" following recovery from myelosuppressive therapy, or ≥ 5% blasts in the BM not attributable to another cause, or development of extramedullary leukaemia. RFS was defined only for patients who achieved CR and

was measured from the documented date of CR until date of relapse or last follow-up date available or death regardless of cause. OS was measured from date of diagnosis until date of death, regardless of cause of death or last follow-up date available.

Kaplan-Meier curves were constructed for RFS and OS comparing MRD positive and MRD negative groups. The log-rank test was performed to determine whether there was a significant difference between the survival curves of the two groups. Finally, to adjust for potential confounding covariates, a Cox proportional hazards model was built using a backwards variable selection procedure to determine whether the two groups, as an indicator variable, remained significant as a prognostic factor once other covariates adjusted the model. Firstly, univariate models for each clinical characteristic at baseline were fit. Secondly, univariate models incorporating an artificial time-dependent covariate expressed as the product of the covariate and the log of the time variable were fit to assess whether the proportional hazards assumption was met. If the proportional hazards assumption was not met for a particular variable, then the artificial time-dependent covariate was included in all of the subsequent models containing that variable. Thereafter, variables reflecting a P from the likelihood ratio test in the univariate models of <0.25 were incorporated together in a full model. Variables reporting a P of >0.05 from the corresponding Wald statistic in the full model were subsequently dropped one at a time in determining the final model. Statistical analyses were performed using SAS version 9.1 (Cary, NC, USA) in Discipline of Public Health, University of Adelaide.

CHAPTER

3

The Presence of Leukaemia-Associated Phenotypes is an Independent Predictor of Induction failure in Acute Myeloid Leukaemia

CHAPTER 3: THE PRESENCE OF LEUKAEMIA-ASSOCIATED PHENOTYPES IS AN INDEPENDENT PREDICTOR OF INDUCTION FAILURE IN ACUTE MYELOID LEUKAEMIA

3.1 ABSTRACT

Historically, prognosis in AML was based on morphology and cytochemistry. While morphology remains the initial diagnostic tool in AML, it does not have independent prognostic significance. Cytogenetics, molecular and some immunophenotypic markers have become important in therapeutic decision-making and prognosis. Many clinical features and laboratory parameters have been reported to have prognostic significance in AML. However, on multivariate analyses there are very few independent prognostic factors. Studies of prognosis in leukaemia using immunophenotyping have yielded conflicting results.

The aims of this study were to determine the frequency of LAPs in AML using the standard diagnostic panel in our institution and to correlate their presence with response to induction chemotherapy and relapse following induction chemotherapy. We investigated the presence of LAPs in 84 AML patients at diagnosis using the standard panel of MoAbs with triple staining flow cytometry and stringent criteria for scoring, and assessed the association of LAPs with response to induction chemotherapy and risk of relapse.

LAPs were observed in 54 (64%) patients: lineage infidelity in 19 (35%), asynchronous antigen expression in 29 (54%), and lack of lineage specific antigens in 19 (35%). Significant correlations were found between LAPs and both responses to induction therapy and risks of relapse. Response to induction chemotherapy was more frequent in the absence of LAP [$p = 0.01$, estimated risk ratio of 1.7 (95% CI, 1.1-2.5)], and [$p = 0.04$, estimated risk ratio of 1.5 (95% CI, 1.0-2.2)] after adjusting for potential

confounders in a multivariate analysis. Furthermore, risk of relapse was lower in LAP negative patients ($p = 0.03$). After exclusion of patients with APL, the association remained significant for response to induction therapy ($p = 0.04$) with an estimated risk ratio of 1.6 (95% CI, 1.0 - 2.6). However, the association disappeared for risk of relapse ($p > 0.05$).

In conclusion, our data show the presence of LAPs in AML is an independent predictor for response to induction chemotherapy and may need to be considered in counselling of patients and planning therapy.

3.2 INTRODUCTION

AML is a disease in which outcome can be partly predicted by a number of clinical, morphological and molecular markers. The immunophenotype of the leukaemic cells provides another parameter that reveals their biology but its role as a predictor of outcome has not been clearly defined.

Immunophenotyping using MFC (Campana and Coustan-Smith 1999; San Miguel *et al.* 1999) enables the frequency and distribution of aberrant antigen expression on AML cells to be identified (San Miguel *et al.* 1986; Campana and Pui 1995). Early reports identified the presence of antigens not associated with normal myeloid differentiation (Andrews *et al.* 1983; Griffin *et al.* 1983). These aberrant marker expressions which are called leukaemia-associated phenotypes (LAPs) in most studies (Reading *et al.* 1993; San Miguel *et al.* 1997; Kern *et al.* 2003) may reflect inherent biological characteristics of the malignancy or an underlying dysplastic process predisposing to the malignancy. Hence, LAPs may have an association with prognosis and response to treatment. There are many studies looking at the prevalence of LAPs in AML and their role in clinical management (Griffin *et al.* 1986; Macedo *et al.* 1995; Bahia *et al.* 2001). Most focus on aberrant expression of individual antigens like CD7, CD14 and CD56 (Del Poeta *et al.* 1995; Norgaard *et al.* 2001; Raspadori *et al.* 2001). We investigated the usefulness of flow cytometry in detecting these aberrant expressions in a routine diagnostic settings and their clinical relevance in assessing prognosis.

In a preliminary study, 84 AML patients were analysed by flow cytometry to assess presence of LAPs: lineage infidelity, asynchronous antigen expression or absence of antigen expression using the current panel of 13 MoAbs at our institution (Chapter 2, section 2.2.3.7). Antigen over-expression and aberrant light scatter properties were shown to have insufficient reproducibility and so were not examined in this study. A

multivariate approach was used to evaluate the correlation between the presence of LAPs and the clinical response to induction chemotherapy.

Statistical methods specific to this chapter

A log binomial model was used to analyse the data. The outcomes of interest were response after induction and relapse after achieving CR. Whether the patient was LAP positive or negative was included in each model as a predictor. A number of potential confounders were identified (age at diagnosis, gender, cytogenetics risk group, *de novo* or secondary AML and WBC count) and only those with a p-value of less than 0.25 in univariate analyses were included in the model (Lengfelder *et al.* 2000). Both unadjusted and adjusted risk ratios were calculated to compare LAP positive with negative patients.

OS was measured from the date of diagnosis until date of death or last date available and RFS for patients who achieved CR was measured from the date of CR to relapse. OS and RFS were plotted by Kaplan-Meier method; differences between curves were analysed by the log-rank test. The log-rank test was used to validate equality of the survival distributions. Cox-regression was also used to obtain the hazard ratio.

This analysis was performed in SAS version 9.1 (Cary, NC, USA). A p-value ≤ 0.05 was required for statistical significance.

3.3 RESULTS

3.3.1 AML samples

Routine diagnostic flow cytometry was performed by Division of Human Immunology /IMVS. The immunophenotyping was carried out by the diagnostic staff. We analysed the results of 84 consecutive unselected patients with newly diagnosed and untreated AML from January 2002 to December 2004 (Table 3-1). The study design was approved by the Research Ethics Committee of the RAH prior to its initiation. Diagnosis of patients was based on morphology, immunophenotyping, and cytogenetics. Patients with APL have a different biology and received different therapy as compared to other AML subtypes and therefore data was also analysed after exclusion of APL patients from analysis (n = 10) for both response to induction chemotherapy and risk of relapse.

Table 3-1 Patient characteristics stratified by LAP^+ and LAP^- patients

Patient characteristics	**Total**	**LAP^+**	**LAP^-**
No. patients	84	54	30
Male/ female	54/30	35/19	19/11
Age at diagnosis, mean, (range)	60 (18-88)	61 (18-88)	58 (28-82)
WBC count at diagnosis × 10^9 /L, median (range)	4.5 (0.25-166)	4.8 (0.25-166)	3.6 (0.36-95)
AML *de novo*/ secondary n (%)	63 (75)/21 (25)	43 (51)/11 (13)	20 (24)/10 (12)
FAB classification, n (%)			
Mo	1 (1)	1	0
M1	14 (17)	10	4
M2	21 (25)	15	6
M3	10 (12)	1	9
M4	9 (11)	6	3
M5	6 (7)	4	2
M6	4 (5)	3	1
M7	1 (1)	0	1
Not classified	18 (21)	14	4
Cytogenetic risk group (Grimwade *et al.* 2001), n (%)			
Favourable	11 (13)	3	8
Intermediate	51 (61)	34	17
Poor	22 (26)	17	5

3.3.2 Leukaemia-associated phenotypes

In 84 AML patients at diagnosis, LAPs were observed in 54 (64%) [95% CI, 0.53-0.74]. We investigated the following antigen aberrations: Lineage infidelity or co-expression of lymphoid-associated antigens (CD7, CD10, CD19) on myeloid blast cells, and asynchronous antigen expression based on reactivity on the blast cells for both mature and early myeloid cell associated antigens [CD117^{+} CD34^{+} and CD15^{+} (or CD14^{+}); CD117^{+} CD34^{-} and CD15^{+} (or CD14^{+}); CD117^{-} CD34^{+} and CD15^{+} (or CD14^{+}); CD34^{+} CD56^{+}, as well as lack of one of the expected myeloid specific markers i.e. CD13 or CD33] as detailed in Table 3-2.

3.3.2.1 Lineage infidelity

Lineage infidelity was detected in 19 (35%) of 54 LAP positive patients. The most frequent lymphoid antigen detected was CD7 (26%) followed by CD10 (4%), and CD19 in a single case (Table 3-2).

3.3.2.2 Asynchronous antigen expression

Asynchronous antigen expression was present in 29 (54%) of patients displaying LAPs (Table 3-2). Eighteen were due to the co-expression of early markers either CD34 or CD117 or both with markers associated with advanced maturation stages of myeloid differentiation (CD15) and 11 with CD56. A representative example of asynchronous marker expression in an AML patient is given in Figure 3-1.

3.3.2.3 Absence of lineage specific antigens

In 19 patients, at least one myeloid marker was absent (Table 3-2).

Furthermore, of all 54 LAP positive patients, 20 (37%) showed the presence of more than one LAP while 34 (63%) showed only one LAP. Additionally, 15, 4 and 1 patient/s had 2, 3 and 5 LAPs respectively.

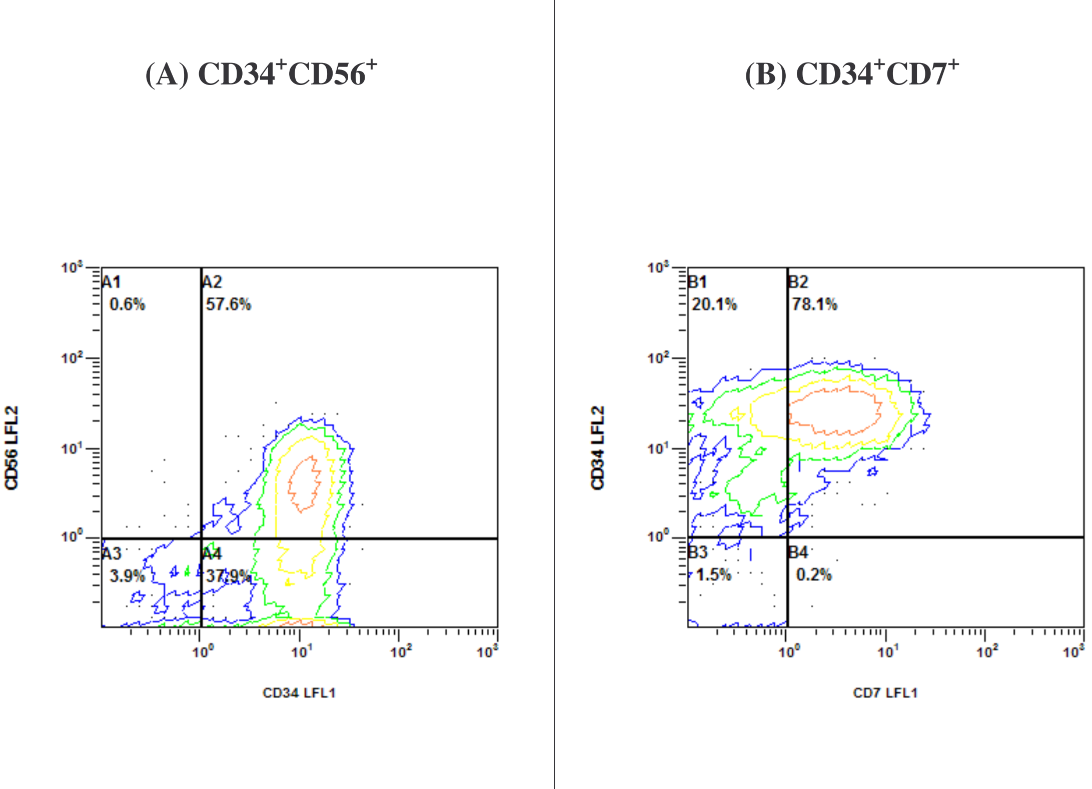
Table 3-2 Aberrant phenotypes in AML patients (n=54)

Aberrant Phenotype	No. of cases (%)
Lineage infidelity	
CD7	14 (26)
CD10	2 (4)
CD2*	1 (2)
CD4*	1 (2)
CD19	1 (2)
total	19 (36)
Asynchronous antigen expression	
$CD117^{+}/CD15^{+}$	$7^{¥}$ (13)
$CD34^{+}/CD56^{+}$	11 (20)
$CD34^{+}/CD15^{+}$	7 (13)
$CD34^{+}/CD117^{+}/CD15^{+}$	4 (7)
total	29 (54)
Lack of lineage specific antigen	
$CD33^{+}/CD13^{-}$	9 (17)
$CD13^{+}/CD33^{-}$	10 (19)
total	19 (36)

*** Only done on one case.**

¥ One out of ten APL patients was LAP^{+} for asynchronous antigen expression $CD117^{+}CD15^{+}$.

Figure 3-1 Dual parameter contour plot histograms of an AML patient with asynchronous antigen expression and lineage infidelity.(**A**) Double staining for CD34 FITC/CD56 PE showing co-expression of 57.6%, (**B**) Double staining for CD34 PE/CD7 FITC showing co-expression of 78.1%.



3.3.3 The presence of leukaemia-associated phenotypes is associated with a poor response to induction therapy

We initially analysed the response to induction chemotherapy in all 59 treated patients, including those with M3. Fifty eight were evaluated for response after first induction (1 died during induction therapy because of refractory disease). There were 37 LAP positive and 21 LAP negative patients (Figure 3-2). Nineteen (51%) of the 37 LAP positive patients did not achieve CR after one cycle of induction chemotherapy and 18 (49%) achieved CR. Four (19%) of the 21 LAP negative patients did not go into remission after induction chemotherapy whereas 17 (81%)

achieved CR. The difference between LAP positive and LAP negative patients in their response to induction chemotherapy was statistically significant (p = 0.01) with an estimated risk ratio of 1.7 (95% CI, 1.1 - 2.5). Thus, the absence of LAPs at diagnosis was strongly predictive for the achievement of CR whereas the presence of LAPs was associated with failure to achieve CR after induction chemotherapy.

When univariate analyses were performed to determine the effect of potential confounders on whether patients achieve CR; cytogenetic risk group and *de novo/* secondary AML were found to have p-values of 0.020 and 0.047 respectively and were included in a multivariate analysis. The other confounding factors (age, gender, WBC count at diagnosis) had p values > 0.25 and were not included (Table 3-3). This is consistent with the known importance of cytogenetics and secondary AML as prognostic markers in AML. In our series, we did not detect LAPs in 9 of 11 (82%) patients who had favourable cytogenetics. Moreover, patients with secondary AML had LAPs detected in 11 of 21 (52%) cases compared to 43 of 63 (68%) patients with *de novo* AML. Despite this, using a multivariate approach, we were able to demonstrate that the presence of LAP is an independent risk factor for failure to achieve CR after adjusting for cytogenetics and *de novo* or secondary AML (p = 0.04 with an estimated risk ratio (adjusted) of 1.5 (95% CI, 1.0-2.2). Additionally, CR rate was significantly lower in $CD7^+$ cases (29% vs. 43%, p = 0.04), and $CD34^+CD56^+$ (9% vs. 55%, p = 0.003).

APL is a unique subtype of AML with targeted therapy and good prognosis. We analysed the results after excluding the ten M3 patients in the series to see whether the association persisted. Of 74 patients (excluding ten APL patients), 51 received induction chemotherapy and 23 patients did not because of the following reasons: age > 70 (n =17), secondary AML or considered biologically unfit for induction (n

= 5) and one patient was visitor and returned to his home state for treatment. Of the 51 patients who received induction chemotherapy, 28 (55%) achieved CR (Figure 3-3).

Figure 3-2 Flow chart illustrating treatment outcomes of the 84 patients

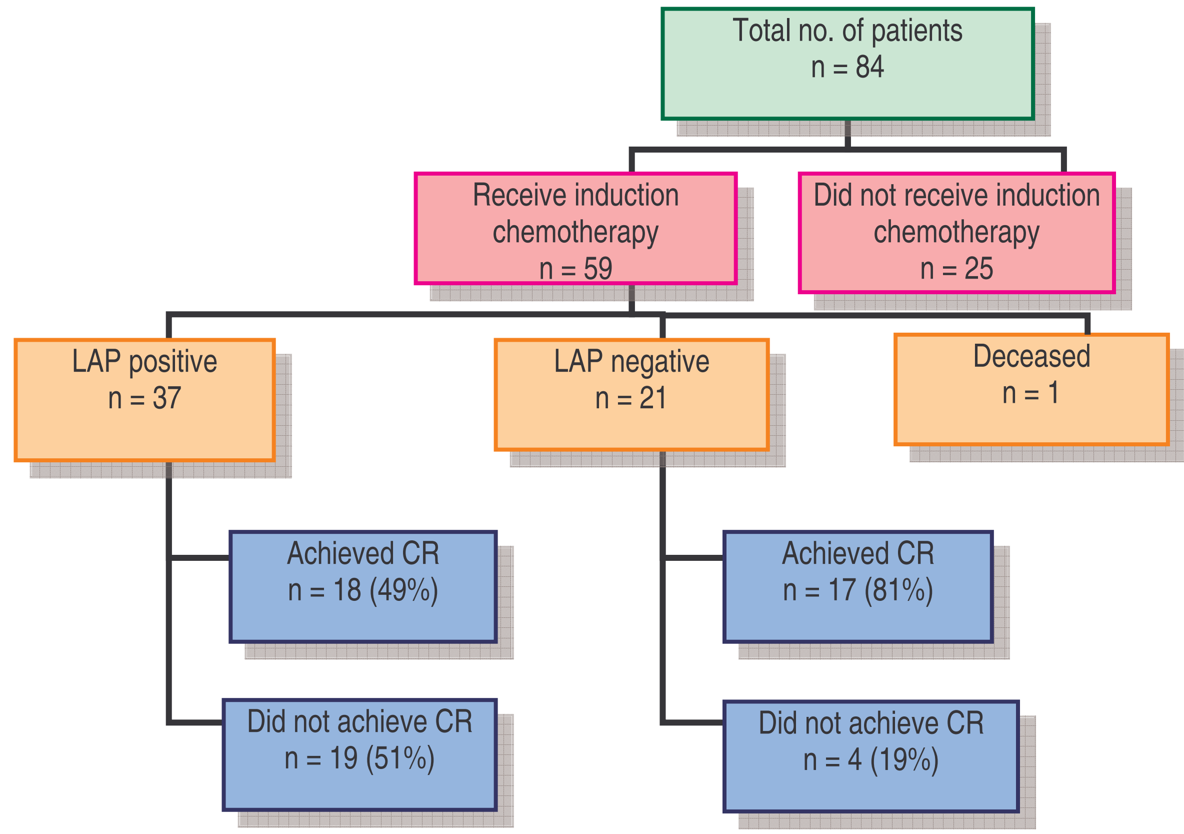
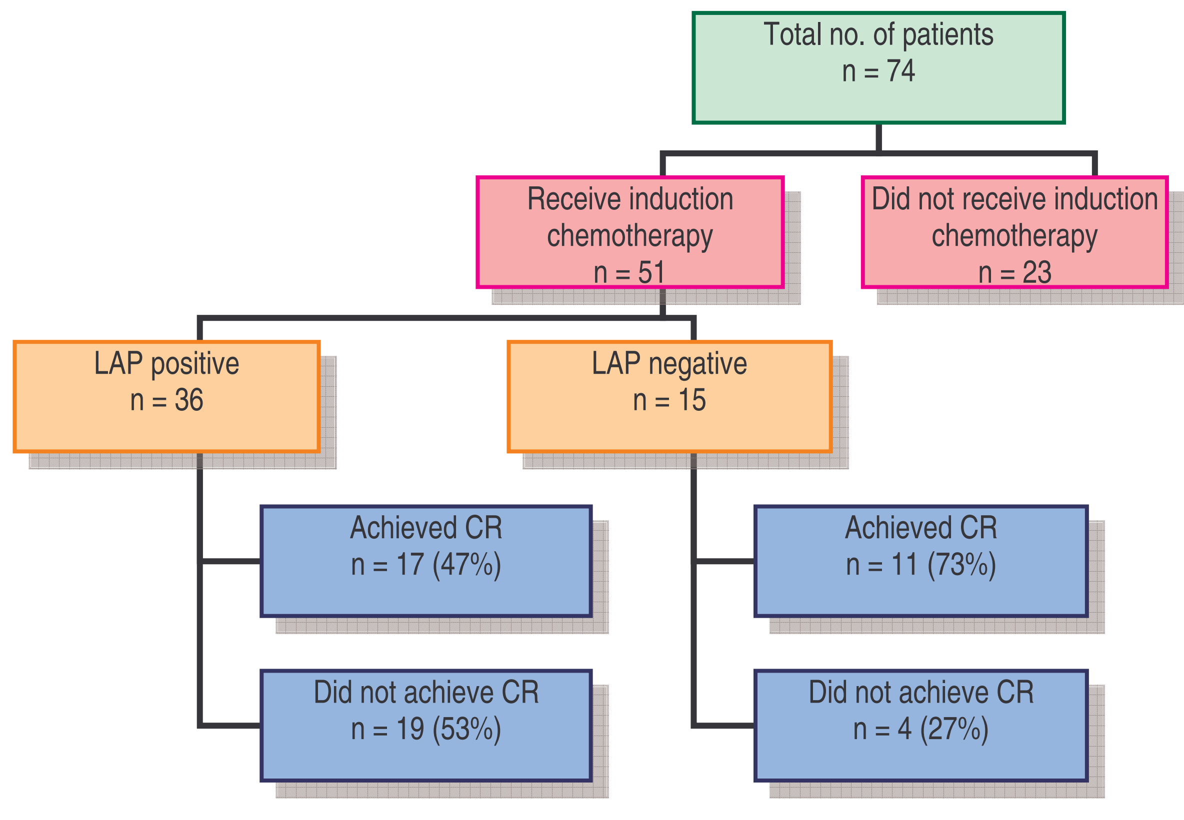


Table 3-3 Univariate analysis of the association between response after first induction and LAPs.

Variable	p-value
LAPs	0.010*
Cytogenetics Risk Group	0.020 *
De novo/ secondary AML	0.047 *
Age at diagnosis	>0.25
Gender	>0.25
WBC count at diagnosis	>0.25

*** p-value < 0.25 so predictor was included in the log binomial model**

Figure 3-3 Flow chart illustrating treatment outcomes of the 74 patients without M3 patients



In the 74 AML patients at diagnosis, LAPs were observed in 53 (72%) [95% CI, 0.61- 0.82] (Table 3-2) (only one out of the ten APL patients was LAP positive with asynchronous antigen expression $CD117^{+}CD15^{+}$). Of 51 patients who received induction chemotherapy, there were 36 LAP positive and 15 LAP negative (Figure 3-3). Nineteen of the 36 (53%) LAP positive patients did not achieve CR after one cycle of induction chemotherapy and 17 (47%) achieved CR. Four (27%) of the 15 LAP negative patients did not go into remission after induction chemotherapy and 11 (73%) achieved CR. Univariate analyses once again revealed cytogenetic risk group and *de novo/* secondary AML as potential confounding factors with p-values of 0.175 and 0.086 respectively and were included in the log binomial model (Table 3-4). The other confounding factors (age, gender, WBC count at diagnosis) had p values > 0.25 and were not included. Multivariate analysis (using cytogenetics and secondary AML as potential confounding variables) demonstrated the relationship between LAPs and response to induction chemotherapy remained statistically significant ($p = 0.04$). Thus, this finding confirmed that the absence of LAP at diagnosis was associated with a good response to induction chemotherapy in terms of achieving CR as compared with LAP positive patients, with an estimated risk ratio of 1.6 (95% CI, 1.0 - 2.6).

3.3.4 Leukaemia-associated phenotypes may predict relapse of AML

There were 29 patients who completed induction and consolidation chemotherapy and were evaluable for relapse (Figure 3-4), 17 (59%) were LAP positive and 12 (41%) were LAP negative. The median follow-up for patients was 36 months (range: 2.5-45). LAP positive patients were at higher risk of relapse with 8/17 (47%) having relapsed and 9/17 (53%) remaining in CR. Only 1/12 LAP negative patient relapsed and this patient was in the intermediate cytogenetic risk group (trisomy 8).

Figure 3-5 shows results for LAP positive versus LAP negative patients and RFS in a Kaplan-Meier plot. The difference was significant using log-rank statistics (p = 0.03) with an estimated hazard ratio of 7.01 using Cox-regression model. When univariate analyses were performed, none of the confounding factors was found to have a p-value of <0.25 and therefore the adjusted analysis was not performed.

When the data analysed excluding M3 patients, 23 patients were evaluable for relapse (Figure 3-6). Sixteen of them (70%) were LAP positive and 7 (30%) were LAP negative. While there was a trend for LAP positive patients to relapse, this was not statistically significant (p> 0.05).

Table 3-4 Univariate analysis of the association between response after first induction and LAPs.

Variable	**p-value**
LAPs	0.04*
Cytogenetics Risk Group	0.175*
De novo/ secondary AML	0.086*
Age at diagnosis	> 0.25
Gender	> 0.25
WBC count at diagnosis	> 0.25

*** p-value < 0.25 so predictor was included in the log binomial model**

Figure 3-4 Flow chart illustrating the outcome of 29 patients evaluated for relapse according to LAP status.

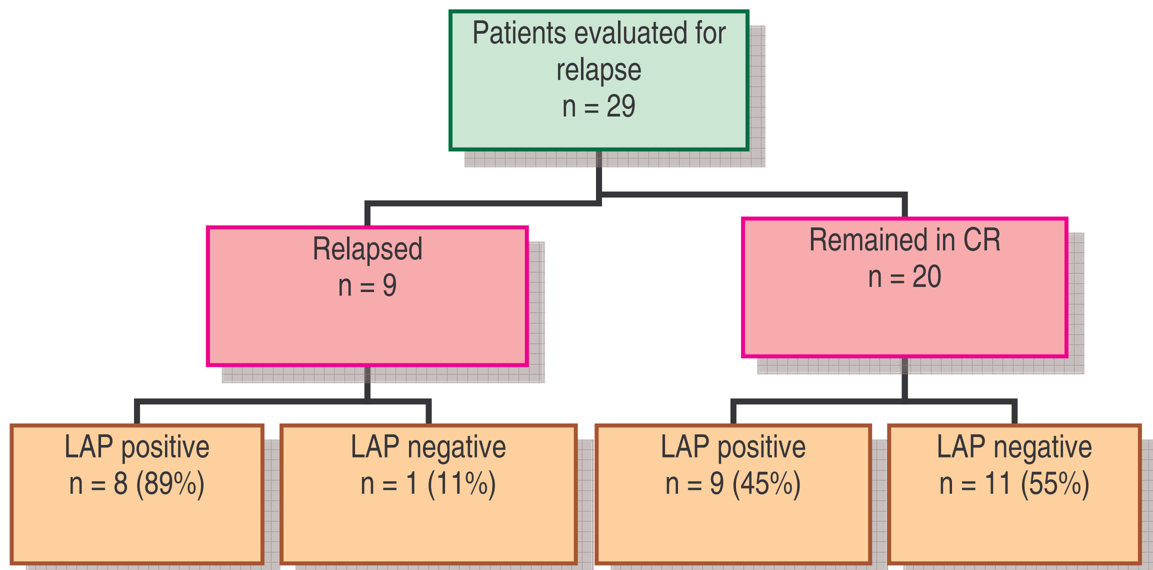


Figure 3-5 Correlation between LAP frequency and relapse-free survival. Kaplan-Meier plot of 29 patients evaluated for relapse. The difference between LAP^+ and LAP^- patients was significant using log-rank statistics (p = 0.03).

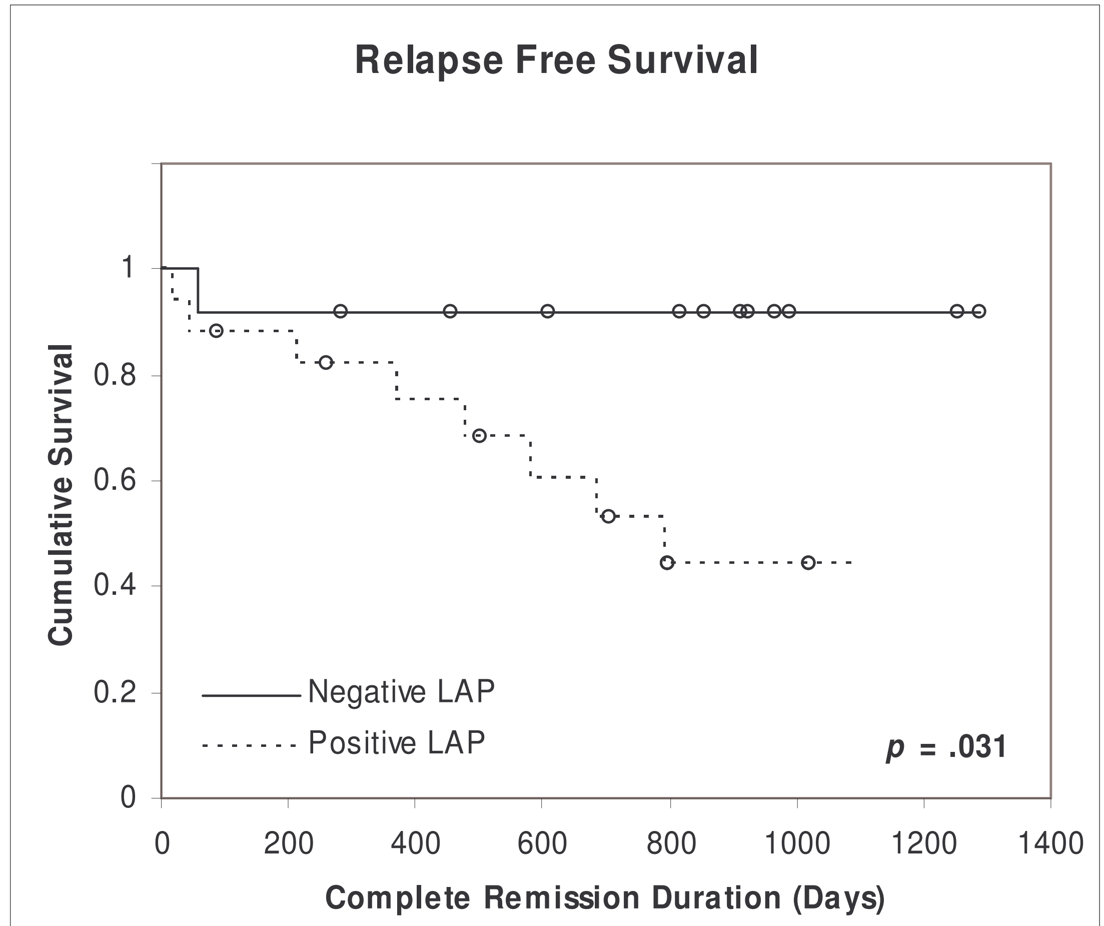
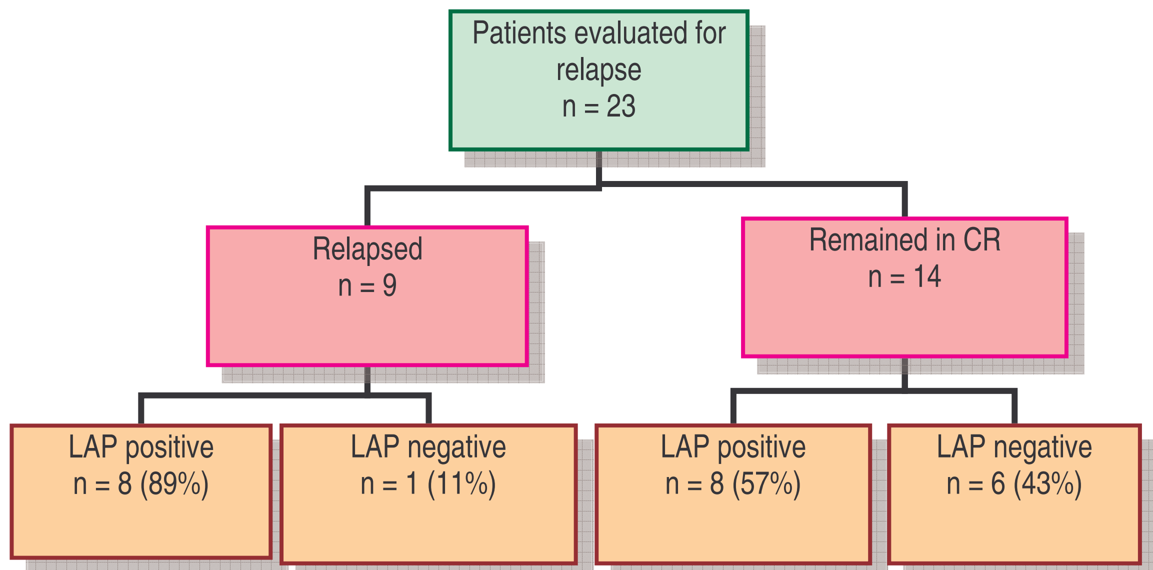


Figure 3-6 Flow chart illustrating the outcome of 23 patients evaluated for relapse according to LAP status (excluding M3 patients).



3.4 DISCUSSION

It is increasingly evident that the treatment outcome of tumours is based on a conglomeration of biological, disease related and treatment variables. We postulated that the expression of LAPs is a reflection of grossly disturbed cell function. Consequently, we have studied co-expression of markers rarely or never appearing simultaneously in normal haemopoietic differentiation and the relationship between these markers and response to induction chemotherapy and disease outcome.

Sixty-four percent (including M3) and 72% (excluding M3) of the patients in the present study displayed aberrant phenotypes, in accordance with previously published data (Terstappen *et al.* 1991; Macedo *et al.* 1995). Nevertheless, our incidence was slightly lower (Terstappen *et al.* 1991; Macedo *et al.* 1995), which could, at least partially, be explained by the fact that only three types of aberrations were investigated in our study and limited MoAbs panel used. Lower incidences of AML aberrant phenotypes reported by others (Campana *et al.* 1990; Drach *et al.* 1991; Drach *et al.* 1992) are due to both the use of a limited panel of MoAbs and only some types of aberrations were investigated (Terstappen *et al.* 1991; Macedo *et al.* 1995). In our study, lineage infidelity occurred in 35% of cases, in agreement with other reports, which showed incidences of 37.5, and 40% in all AML cases (Reading *et al.* 1993; Macedo *et al.* 1995). Asynchronous antigen expression was the most frequent phenotypic aberration (54%) in our study, similar to other reports 62.5%, 50%, and 82.4% respectively (Macedo *et al.* 1995; Bahia *et al.* 2001; Voskova *et al.* 2003). The presence of early antigens (i.e. CD117 and CD34) in association with CD15 was slightly more frequent 18/29 (62%).

Furthermore, we show that the presence of LAPs in AML is of prognostic significance. LAP positive cases enter CR less frequently than those who are LAP

negative. This is an interesting finding since the utility of LAPs has been considered in the context of MRD monitoring. LAPs correlated significantly with a higher chance of failure to achieve CR after induction chemotherapy (p = 0.04) with an estimated risk ratio of 1.5 (95% CI, 1.0 - 2.2) when M3 patients were included and with an estimated risk ratio of 1.6 (95% CI, 1.0 - 2.6) when M3 patients were excluded in multivariate analysis. Del Poeta *et al* (Del Poeta *et al.* 1995) found that CR rate was significantly lower in $CD7^+$ cases (32% vs. 74%, p = 0.001), similar to our findings in this study (29% vs. 43%, p = 0.04). Rigolin *et al* (Rigolin *et al.* 1993) reported the expression of the CD14 and CD34 was correlated with a worse prognosis, while the $CD34^+$ patients further showed a lower probability to achieve a CR. We also noted that patients with a $CD34^+CD56^+$ phenotype did worse than those without (9% vs. 55%, P= 0.003). Even though we demonstrated that some specific LAPs are associated with lower CR rate, we also show conclusively that the presence of any LAP is an adverse risk factor for achievement of CR independent of other confounding factors (p = 0.04).

We also show that the presence of LAP may be an adverse risk factor for predicting relapse in univariate analysis (p = 0.03 when M3 included). However, the association did not reach statistical significance when M3 patients excluded. This may be explained by the small sample size of patients evaluable for relapse (n = 23). There have been a few previous reports of the prognostic significance of aberrant phenotypes in AML (Smith *et al.* 1992; Kanda *et al.* 2000; Bahia *et al.* 2001). Smith *et a1* (Smith *et al.* 1992) reported that expression of lymphoid associated antigens in AML cells lacked prognostic significance. However, most of the patients included in their series were children, while all cases analysed in our study were adults, which may explain the discrepancy as the disease biology might be different in

adults when compared with children. We found expression of lymphoid associated antigens in AML blast cells was also associated with a significantly lower CR (P= 0.05).

Our findings would favour LAPs being reflective of the malignant transformation of the leukaemic cells as opposed to them being epiphenomenon related to the dysregulated cell machinery. Furthermore, we showed that LAP is independent of the cytogenetic subtype. This is not surprising because the LAP is more a functional readout of the transformation process, subject to both oncogenic as well as epigenetic events.

The significance of LAPs is further highlighted by the fact that the presence of LAPs was associated with poor treatment outcome even after adjusting for cytogenetics and secondary AML types, which stood out as confounding variables on univariate analysis. We noted that traditional prognostic markers like age and total WBC count were not found to be significantly associated with treatment outcome. This is due to the elderly patients (17/23) in the group not receiving induction chemotherapy. Nevertheless, this is an important finding, which needs to be studied further to elucidate the biological role of LAPs in pathophysiology of leukaemia. This would require standardisation of the immunophenotyping reporting of all AML to include LAPs and incorporating them in the disease stratification algorithms.

Future technical efforts in this area should focus on techniques to identify more LAPs so that MRD studies can be applied to the majority of patients. In addition, the relationship established between genetic abnormalities and certain phenotypes within different FAB subtypes suggests that, in future, immunophenotypical studies could be used for the screening of AML cases carrying specific genetic aberrations.

Ultimately, it may be useful to compare treatment strategies for patients in different studies to see if particular phenotypic classes respond differently to different therapies. Lymphoid directed therapies in patients with aberrant expression of lymphoid antigens might benefit these patients.

In conclusion, our results show that using the standard panel of MoAbs for immunophenotypical characterisation of AML, more than half of the patients will display aberrant phenotypes and the co-existence of more than one aberrant antigen frequently could be detected. Importantly, LAP can be used as markers for predicting response to induction chemotherapy, independent from other known risk factors.

CHAPTER 4

Incidence of Leukaemia Associated Phenotypes in Acute Myeloid Leukaemia Patients: A Basis for the Design of Specific Five-Colour Staining to Be Used For Minimal Residual Disease Investigation

CHAPTER 4: INCIDENCE OF LEUKAEMIA ASSOCIATED PHENOTYPES IN ACUTE MYELOID LEUKAEMIA PATIENTS: A BASIS FOR THE DESIGN OF SPECIFIC FIVE-COLOUR STAINING TO BE USED FOR MINIMAL RESIDUAL DISEASE INVESTIGATION

4.1 ABSTRACT

Multiparameter immunophenotypic analysis of leukaemic cells has proven to be of a great help for the investigation of MRD in ALL. However, its utility has not been systematically explored in AML. Most of the studies published so far on the detection of MRD used MFC applied three or four fluorochromes with different triple- quadruple combinations of MoAbs, implying that the basic parameters used in all combinations for the identification of the blast population are the light scatter characteristics, i.e. FSC and SSC. A significant step forward has been the introduction of the simultaneous detection of five or more fluorochromes and the use of CD45 gating.

In this study, we assessed the advantage of using 5-colour MFC to detect LAPs in AML at diagnosis and determined its utility in MRD detection. To achieve this we: i) analysed the sensitivity of MFC, ii) assessed specificity by determining immunophenotypic profiles of LAPs in AML versus regenerating and normal BMs and, iii) determined the incidence of these LAPs in a series of 54 consecutive, newly diagnosed AML patients.

Overall, 94% (n = 51) of the AML patients displayed LAPs at diagnosis. The frequency of leukaemic BM /median frequency of LAP in normal or regenerating BMs using maximum LD statistics revealed that CD2, CD56, CD11b, CD7 and CD19 expression on AML blasts represented the most aberrant immunophenotypes and therefore are the most sensitive and reliable markers for MRD detection. Serial

dilutional experiments showed that the sensitivity level of immunophenotyping ranges between 10^{-4} and 10^{-5} and the approach was highly reproducible.

In summary, the present study shows that immunophenotyping analysis using CD45 gating strategy, five-colour staining and extensive panel of MoAbs allows the identification of LAPs in 94% of AML and these phenotypes can be used for MRD monitoring with a sensitivity limit of 10^{-4} to 10^{-5}.

4.2 INTRODUCTION

Immunophenotyping of haematological malignancies is one of the most important clinical applications of flow cytometry. In recent years, its use has extended from clinical research to diagnostic laboratories. In spite of the well-established utility of immunophenotyping for the diagnosis, classification, prognostic stratification, and monitoring of MRD in ALL, limited and controversial data are found for AML due to disease heterogeneity. Until now no systematic study has been carried out with five-colour flow cytometry to assess the applicability, specificity and sensitivity of MFC immunophenotyping for detection of MRD.

Previous reports on MFC-based monitoring of MRD in AML have focused on cases displaying highly aberrant phenotypes (Sievers *et al.* 1996; San Miguel *et al.* 1997; Venditti *et al.* 2000; San Miguel *et al.* 2001; Sievers *et al.* 2003). Some of these reports used four-colour staining, however, without implementing CD45 as a basic parameter (Coustan-Smith *et al.* 2003).

The existence of LAPs may be a valuable tool for the detection of MRD (Campana and Coustan-Smith 1999; San Miguel *et al.* 1999; San Miguel *et al.* 2001). In recent years there have been an increasing number of reports on the existence of either lineage infidelity or unusual immunophenotypic combinations in AML (Macedo *et al.* 1995; Babusikova *et al.* 1996; Venditti *et al.* 2003; Voskova *et al.* 2003). However, most of these studies have only analysed a limited number of MoAbs and thus the real incidence of LAPs as well as the most sensitive and reliable markers for MRD has not been firmly established.

In addition, preliminary reports suggest that high levels of sensitivity can be reached by using MFC when appropriate data acquisition and multidimensional analytical procedures are combined (Orfao *et al.* 1999; Kern and Schnittger 2003; Kern *et al.*

2005). Recently, the sensitivity of AML immunophenotyping was markedly improved by the availability of a great spectrum of MoAbs and by improvement of gating strategies (Basso *et al.* 2001; Kern *et al.* 2004).

We have designed and implemented a 5-colour MFC to improve the sensitivity, specificity and applicability of this approach. All antibody labellings, cell processing, staining and analysess were nalaysed by us. The aim of the present study was to explore the applicability and sensitivity of five-colour MFC immunophenotyping for the investigation of MRD in AML patients using a wide spectrum of MoAbs, CD45 gating and five-colour staining for the investigation of MRD levels in AML.

4.3 RESULTS

4.3.1 Clustering of bone marrow lineages using CD45 gating strategy

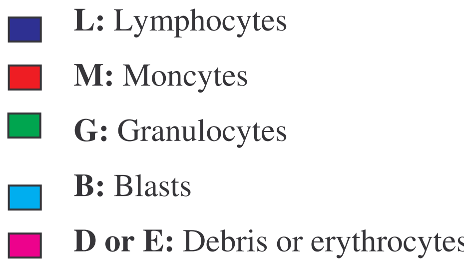
Firstly, the recommended use of CD45/SSC as against FSC/SSC (as the preferred gating strategy for leukaemia) was validated. Figure 4-1 shows the advantage of the CD45/SSC log gating procedure over FSC/SSC for identifying leukaemic cells. When the FSC/SSC gate is used (Figure 4-1 b, d) without immunological marker, the blast cells in AML partially overlap with the gate of normal lymphocytes and monocytes. On the other hand, CD45/SSC gating clearly separates the four cell categories of lymphocytes, monocytes, granulocytes and blast cells (Figure 4-1 a, c). Mature lymphocytes show the highest CD45 fluorescence intensity and the lowest SSC signal. Mature monocytes express slightly lower but still high amounts of CD45 and they easily distinguished from lymphocytes by their higher SSC signal. Granulocytic lineage expresses low CD45 and very high SSC. In few samples , two populations were seen: the CD45 low population representing immature granulocyte and the CD45 medium population exhibiting mature features (Figure 4-2) (Stelzer *et al.* 1993). Blast cells had the lowest CD45 intensity and a low SSC, reflecting features of normal immature BM precursor cells. Erythrocytes and platelets, which do not express CD45 antigen, were excluded.

In 54 consecutive samples, the morphological BM blasts percent were compared with the results of the blasts from CD45/SSC gating procedure. A strong positive correlation was found for the blast population (r = 0.8) (Figure 4-3). The gating strategy used to identify LAPs is explained in detail in Chapter 2.

It was confirmed that the cells classified as lymphocytes, monocytes, granulocytes and blast cells did indeed express the appropriate immunological markers (Figure 4-

4). The contamination of the blast cell population by T lymphocytes and B lymphocytes is obvious if blasts were gated on FSC/SSC. The sensitivity of the method is documented with a study of CD7 antigen. T lymphocytes were $CD7^+$, as expected. The blast cells had two populations: one CD7 negative and one CD7 positive. The analysis of CD7 positivity based on FCS/SSC gating alone would have led to the erroneous interpretation that CD7 positivity is due to lymphocytes contamination.

Figure 4-1 Improvement of separation of populations by CD45 gating. (**A**) normal BM CD45-SS Log plot, (**B**) normal BM FS-SS log plot), (**C**) AML BM CD45-SS Log plot, (**D**) AML BM FS-SS log plot. CD45-SS Log gating allows isolating BM blasts from all other populations, which is not possible by FS-SS gating.



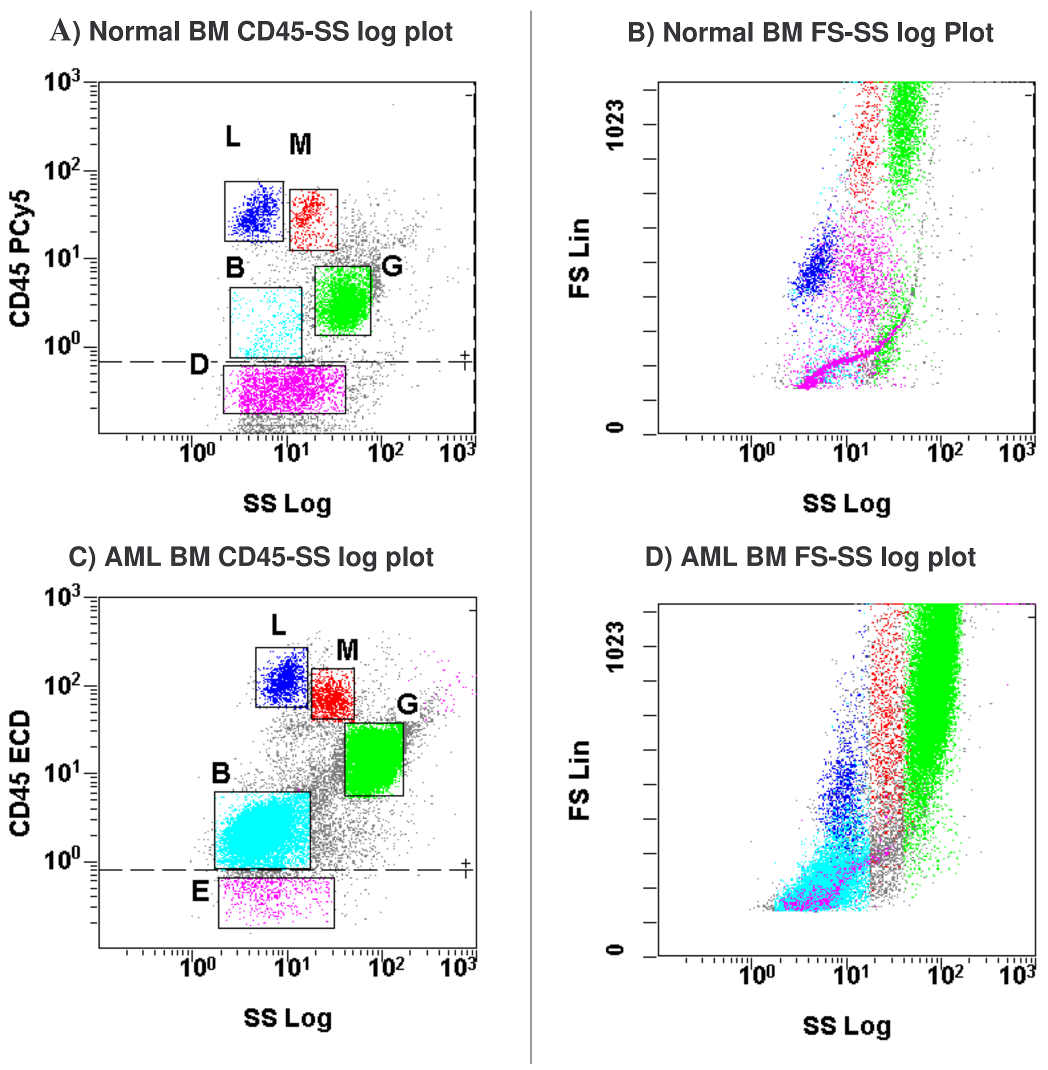
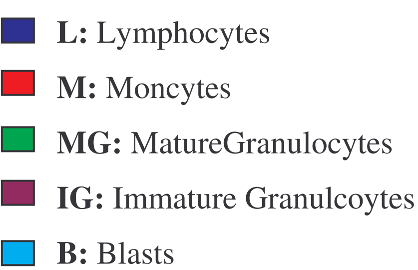


Figure 4-2 Granulocytic lineage expresses low CD45 and very high SSC., Two populations were seen, in density plots (**A & C**) and dot plots (**B & D**): the CD45 low population representing immature granulocyte and the CD45 medium population showing mature features



A) Density Plot

B) Dot Plot

C) Density Plot

D) Dot Plot

Figure 4-3 Correlation between a light microscopic BM blasts percentage and the flow cytometry blasts percentage based on CD45/SSC log gating strategy.

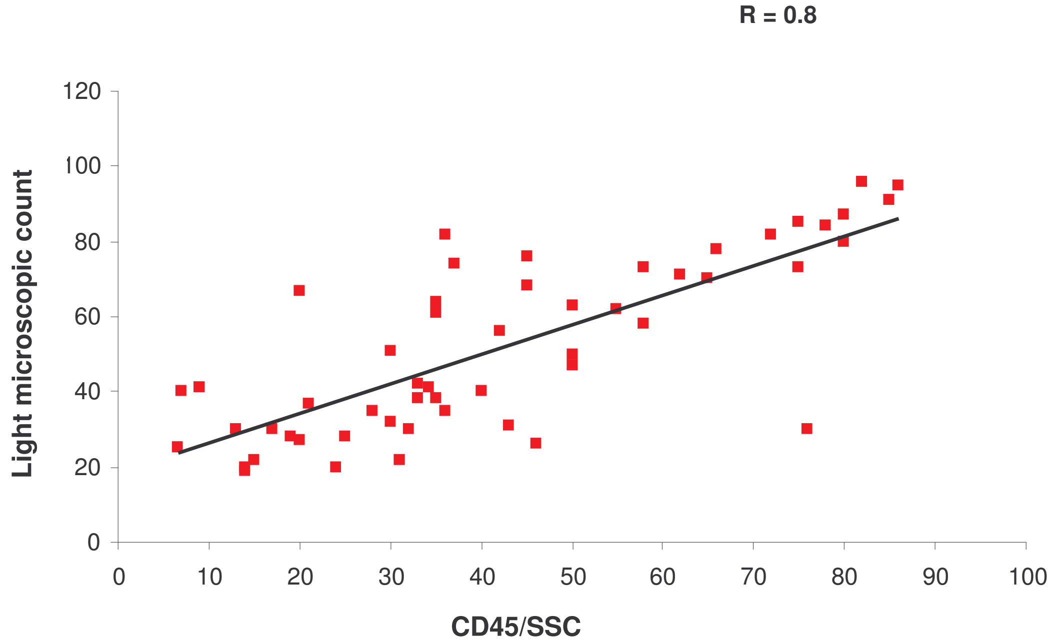
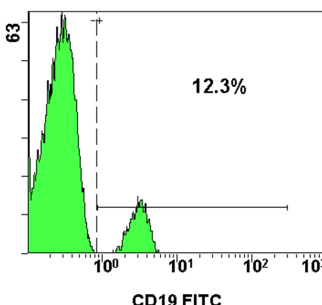


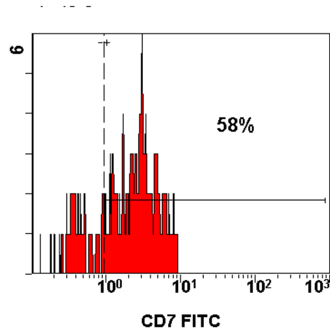
Figure 4-4 Univariate histograms of CD expression in lymphocytes, monocytes, granulocytes and their expression in blast cells in the same AML patient demonstrating the difference in expression of these markers in blasts versus other residual normal cells. Histograms are gated on each specified subpopulation. From top to bottom, the distributions of fluorescence are shown for CD19, CD7, CD14 and CD15. Histograms (**A-D**) are conditioned on gate L or M or G of Figure 4-1. Histograms (**E-H**) are conditioned on gate B of Figure 4-1 on the same patient.

Specific CD expression on WBC subpopulations

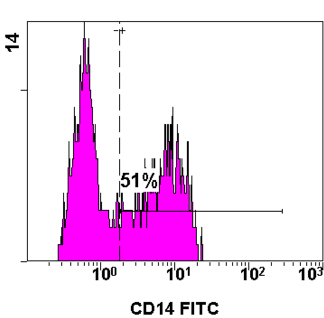
A) *B Lymphocytes*



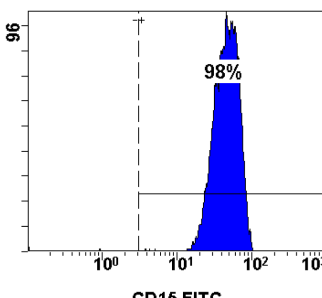
B) *T Lymphocytes*



C) *Monocytes*

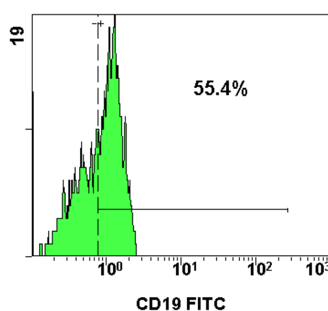


D) *Granulocytes*

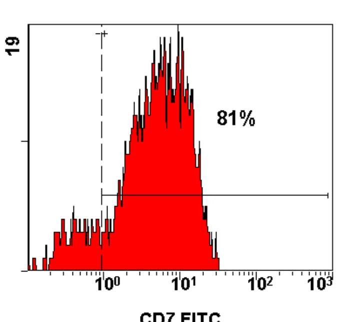


CD expression on blast cells using CD45/SSC gating

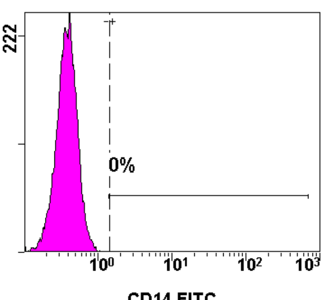
E) *Blasts*



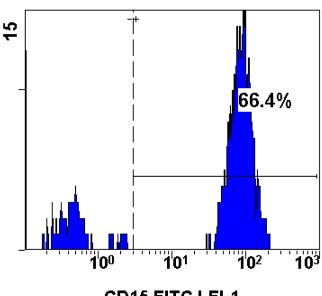
F) *Blasts*



G) *Blasts*



H) *Blasts*



4.3.2 Utility of CD45/SSC gating in frozen samples

When frozen samples were used, CD45/SSC gating remained a valuable and important step to identify blast cells. It was clear that after freezing, the conservation of CD45 antigen expression was excellent. Table 4-1 indicates that the percentages of CD45 low blast cells in the fresh sample and the corresponding frozen one were very similar; showing the CD45/SSC log gating approach can be applied in both settings.

4.3.3 Expression of different MoAbs

MoAbs against T-cell antigens CD7, CD2 and B-cell antigens CD19, CD10 served as individual, internal controls of setup of gates and for compensation in the addition of isotype matching controls. The leukaemic samples frequently displayed more than one subpopulation based on CD45 gating strategy with combinations of MoAbs. Samples were considered positive for the respective antigen if ≥ 20% leukaemic cells expressed the antigen. Quadrants were set based on autofluorescence and staining of the isotype controls as well as internal controls.

Results of antibody staining were listed according to incidence of positive samples. Because the study was designed to be performed prospectively as AML patients present, in some cases all the markers were not analysed, as some patients were initially not suspected as having AML.

4.3.3.1 CD45

CD45 was expressed in all samples included in this study. The expression was usually high and rather homogenous. One patient with rapidly progressive pancytopenia was also initially phenotyped under the clinical diagnosis of AML with significant population large

CD45-negative cells with intermediate SSC log. Most of these CD45 negative cells were megakaryoblasts, which were identified by CD61.

4.3.3.2 HLA-DR

HLA-DR was expressed in the leukaemic samples of all but seven patients, the expression was high 46/54 (87%), median expression = 90%, mean = 83%) (Figure 4-5: I). In one case it was not done. One of the seven HLA-DR^{-} was cytogenetically classified as M3.

4.3.3.3 CD34/CD38

The combination of CD34 and CD38 was chosen as leukaemic cell differentiation is thought to correspond in part to normal haemopoietic cell differentiation (Terstappen *et al.* 1991). CD34 allows the identification of leukaemic cells based on the aberrant over-expression of CD34 (Terstappen *et al.* 1991), which was observed in 72% of cases (n = 39, median expression = 89%, mean = 83%). In addition, the distinction of less and more mature leukaemic cells may be of prognostic significance (Terstappen *et al.* 1992). While some leukaemic samples are homogenous, with all blasts clustering at one stage of differentiation, others show heterogeneity ranging from very immature, stem-like cells to more mature progenitor cells (Figure 4-6). CD38 was present on at least one subpopulation in all AML aspirates.

Table 4-1 The percentages of CD45 low blast cells in stem, myeloid and lymphoid population markers. The common progenitor markers (CD34% and CD117%), lymphoid marker (CD7%) when present in blast subpopulation, myeloid marker (CD13% and CD33%) in the fresh sample and the corresponding frozen one are shown in three AML patients, showing the CD45/SSC log gating approach can be applied to both fresh and frozen samples. R-value for correlation was calculated for each sample: frozen vs. fresh sample.

Patient no.	AML1		AML2		AML3	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
% blast	85	80	24	41	32	30
CD34	99	96	86	80	77	78
CD117	99	90	77	77	72	78
CD7	98	98	19	33	70	86
CD13	97	85	82	22	79	53
CD33	0.3	0.1	92	53	3.0	3.1
r		0.993		0.969		0.906

Table 4-2 Patient characteristics

Patient characteristics	**Total**
No. patients	54
Male/ female	37/17
Age at diagnosis, mean, (range)	58 (18-85)
WBC count at diagnosis × 10^9 /L, median (range)	5.6 (0.26-179)
Haemoglobin at diagnosis g/L, median (range)	94 (52-143)
Platelets at diagnosis × 10^9 /L, median (range)	61 (11-241)
BM blasts % by morphology, median (range)	42 (19-96)
BM blasts % by MFC, median (range)	36 (7-86)
AML de novo/secondary n (%)	42 (78)/12 (22)
FAB classification, n (%)	
Mo	0 (0)
M1	9 (17)
*M2**	11 (21)
M3	2 (4)
M4	9 (17)
M5	5 (9)
M6	1 (2)
M7	1 (2)
Not classified	16 (30)
Cytogenetic risk group, n (%)	
Favourable	9 (17)
Intermediate	25 (46)
Poor	17 (31)
No metaphases	3 (6)

*** Of the 11 M2 cases, only one was with t(8;21) and it was $CD19^+$.**

Figure 4-5: Representative example of CD expression in the same AML patient with comparison to isotypes matched controls. **A**) Density plot of CD45/SSC log gating strategy for identification of blast population based on CD45 dim and low SSC. **B**) Gated on the blast cells to identify the viable cells in FSC/SSC and therefore the reliable cells for further analysis. **C**) Dot plot of CD45/SSC log gating strategy for identification of blast population gated on "blast" gate with the blue colour and **D**) to identify the blue viable cells in **C**).Histograms (**E, F, G, H, I, J, K, L**) demonstrate some positive and negative markers in this patient all gated on **B** or **C**. The dotted line represents the isotype matched controls for each fluorochrome utilised. It is clear that CD14 is negative in the blast population. The rest of the markers shown in this figure were >20% and therefore defined as positive markers. CD7 and CD10 are lymphoid associated markers; CD56 is in the group of asynchronous markers and CD34 and CD117 are progenitor markers.

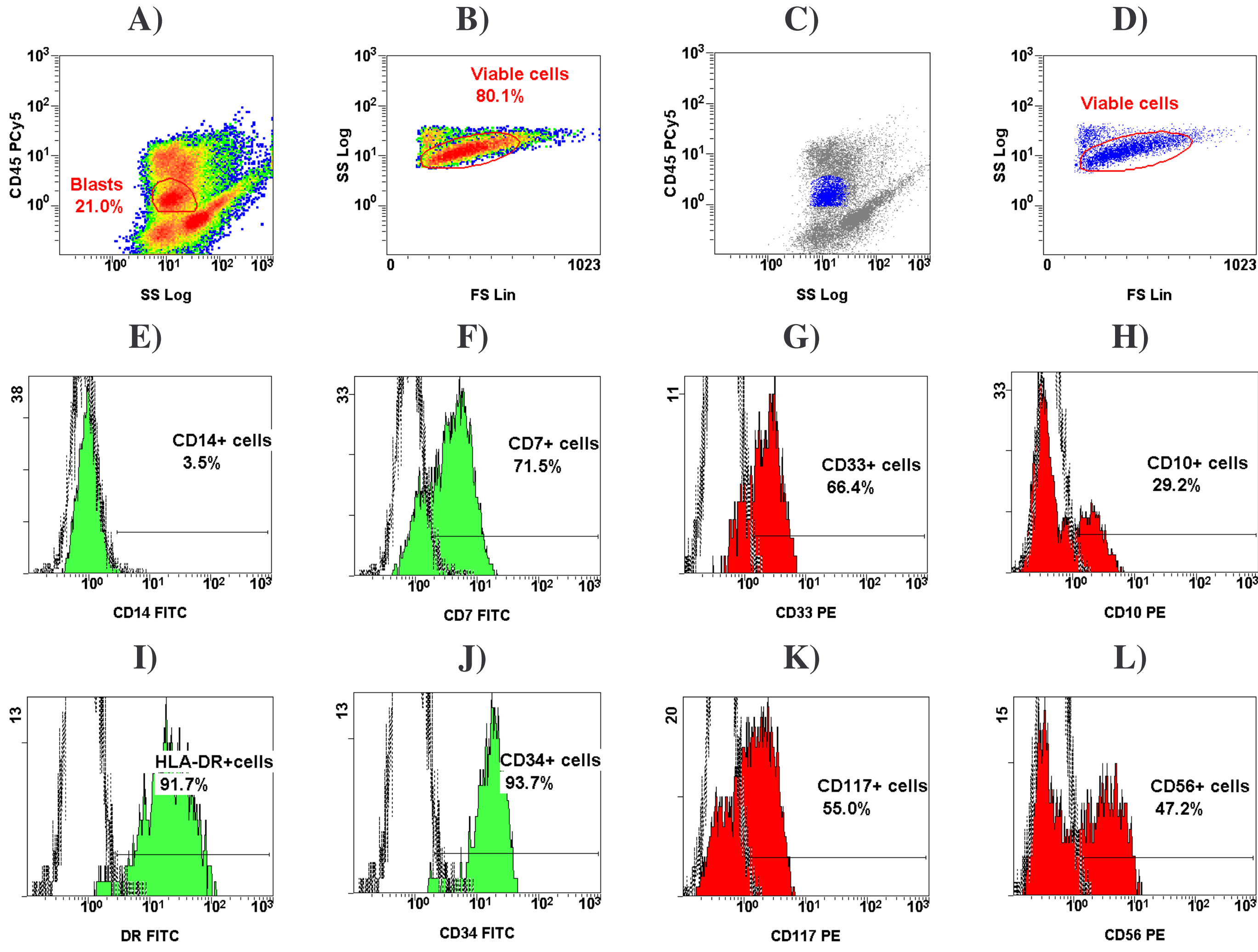
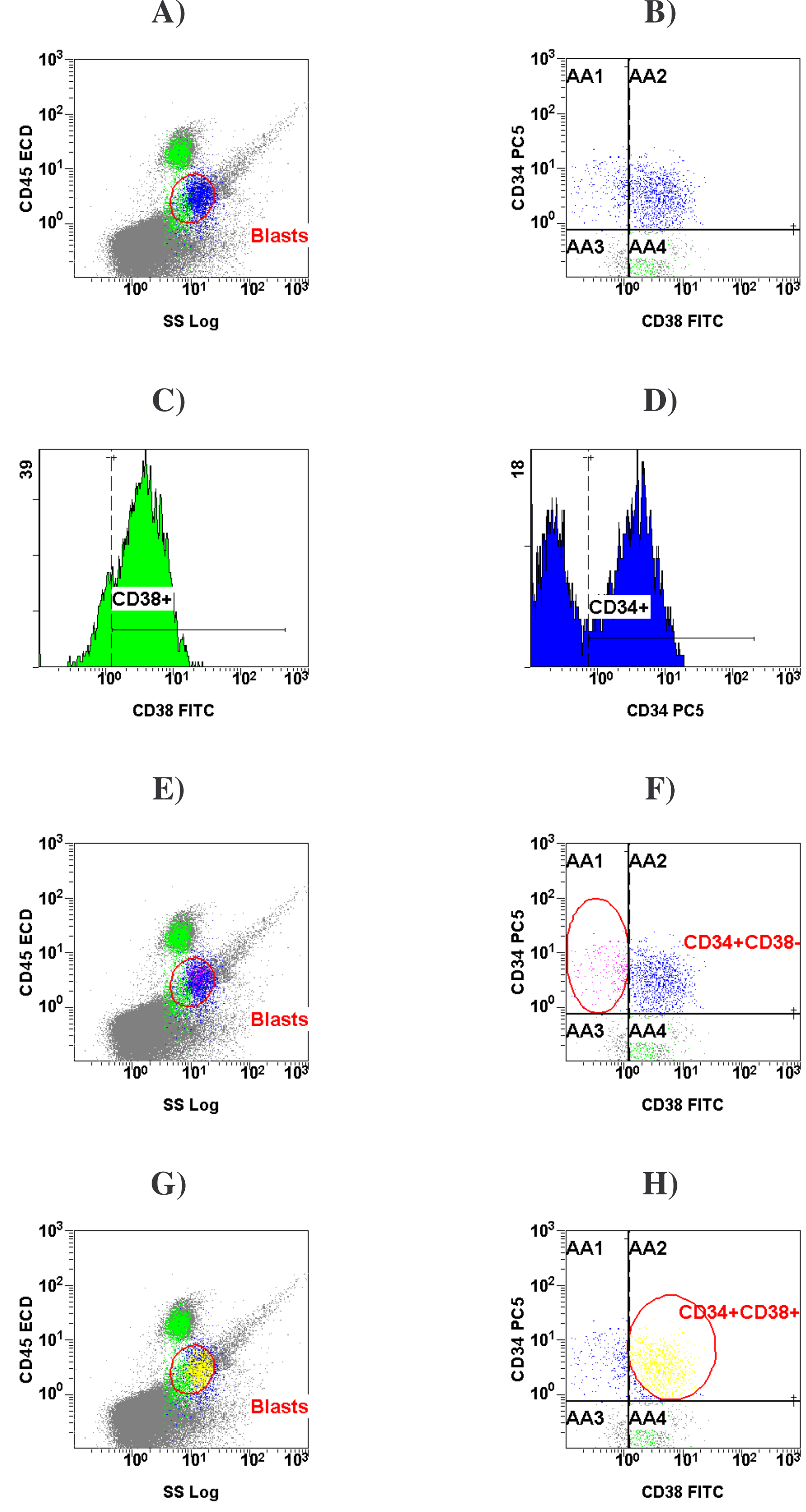

A)
B)
C)
D)
CD45 PCy5
Blasts
21.0%
SS Log
SS Log
Viable cells
80.1%
FS Lin
CD45 PCy5
SS Log
SS Log
Viable cells
FS Lin
E)
F)
G)
H)
CD14+ cells
3.5%
CD14 FITC
CD7+ cells
71.5%
CD7 FITC
CD33+ cells
66.4%
CD33 PE
CD10+ cells
29.2%
CD10 PE
I)
J)
K)
L)
HLA-DR+cells
91.7%
DR FITC
CD34+ cells
93.7%
CD34 FITC
CD117+ cells
55.0%
CD117 PE
CD56+ cells
47.2%
CD56 PE

Figure 4-6 Coexpression of CD34 and CD38 on myeloid leukaemic blasts. Five subpopulation can be distinguished: $CD34^{++}/CD38^{-}$ (AA1 in **B**, **F**, **H**) coloured pink in (**F**), $CD34^{++}/CD38^{+}$ (AA2 in **B**, **F**, **H**) coloured blue in (**B**, **F**), and yellow (**H**), $CD34^{+}/CD38^{++}$ (between AA2 and AA4 in **B**, **F**, **H**), $CD34^{-}/CD38^{+}$ coloured green (AA4 in **B**, **F**, **H**) and $CD34^{-}/CD38^{-}$ cells coloured grey (AA3 in **B**, **F**, **H**). Histograms in **C**, **D** show the $CD34^{+}$ and $CD38^{+}$ cells based on internal control and isotype matched controls. Dot plot (**A**) shows the localisation of $CD34^{+}$ cells (blue) and $CD38^{+}$ cells (green) on CD45/SSC. Dot plot (**E**) shows the localisation of CD34+/$CD38^{-}$ cells (pink) on CD45/SSC within the "blast" gate. $CD34^{+}/CD38^{-}$ cells demonstrate higher intensity for CD45 compared with that of $CD34^{+}/CD38^{+}$ (yellow) in dot plot (**G**) with the "blast" cells.


A)
CD45 ECD
SS Log
Blasts
B)
AA1
AA2
AA3
AA4
CD34 PC5
CD38 FITC
C)
39
CD38+
CD38 FITC
D)
18
CD34+
CD34 PC5
E)
CD45 ECD
SS Log
Blasts
F)
AA1
AA2
AA3
AA4
CD34+CD38-
CD34 PC5
CD38 FITC
G)
CD45 ECD
SS Log
Blasts
H)
AA1
AA2
AA3
AA4
CD34+CD38+
CD34 PC5
CD38 FITC

The example in (Figure 4-6) was chosen to outline further insights into leukaemia cell biology that can be gained from MFC. Five subpopulations can be distinguished: $CD34^{++}/CD38^{-}$, $CD34^{++}/CD38^{+}$, $CD34^{+}/CD38^{++}$, $CD34^{-}/CD38^{+}$ and $CD34^{-}/CD38^{-}$ cells. Normal haemopoietic progenitor cell differentiation proceeds from the stage of $CD34^{+}/CD38^{-}$ to $CD34^{-}/CD38^{++}$ cells. The population defined as $CD34^{+}/CD38^{-}$ represents an immature population and this may be of prognostic significance.

4.3.3.4 CD117

CD117 is a haemopoietic progenitor marker. The majority of the patients expressed CD117 (n = 47, 90%, median expression = 83%, mean = 78%). CD117 was used to further confirm the blast population with CD45 dim population when CD34 was negative in AML (Figure 4-7, D, J, and P).

4.3.3.5 CD33

CD33 is normally expressed on myeloid progenitors and monocytes. CD33 was present in at least one subpopulation in 43 (80%) of the 54 patients (median expression = 82%, mean = 76%). The expression was very heterogeneous. It differed even between the subpopulation from one patient. (Figure 4-7: M).

4.3.3.6 CD13

CD13 normally expressed on granulocytic and monocytic cells. CD13 was a reliable marker of myeloid leukaemic cells and was detected in 50 (93%) patients, median expression = 79%, mean = 75%. When present, expression was moderate to intense. In the vast majority of cases, it was coexpressed with CD33. We did not observe any case in our series that lacked both CD13 and CD33 on at least one

subpopulation. The combination of CD13 and CD33 was always combined in the same tube to allow us to study the lack of lineage specific antigen (Figure 4-7: N).

4.3.3.7 CD14

Expression was tested on all samples but one. CD14 (a marker for monocytes and macrophages) was positive only in 2 cases. In one case, the expression was strong (64%) and the other one was weak (25%).

4.3.3.8 CD15

CD15 (a marker for granulocytes) was tested on all patients but one. CD15 was positive almost on half of the patients 26/53 (49%, median expression = 49%, mean = 51%).

4.3.3.9 CD56

CD56 (a marker for NK cells and T cell subset) was investigated in all patients except one. CD56 was expressed in 8 patients (15%), median expression = 49%, mean = 53 % (Figure 4-7: B).

4.3.3.10 CD2

CD2 (pan T cell marker) was tested on all patients but 6. It was present in 5 patients. The median expression was 68%, mean = 72%. Eighty percent of them (4 patients) were M1 and M2 (Figure 4-7: A).

4.3.3.11 CD7

CD7 (haemopoietic progenitor and T cell marker) was found on myeloid cells in 14/54 cases (26%), median expression = 59%, mean = 59%. The expression was heterogeneous. The staining was moderate in the blast compared with the high

intensity of lymphocytes. The $CD7^+$ T-cells were clearly distinguishable based on homogenously strong expression of the antigen and well separated when using CD45/SSC gating (Figure 4-8: E).

4.3.3.12 CD19

CD19 (B cell marker) on myeloid leukaemic cells was expressed only in two samples. Expression was weak in one sample and strong in the other case. Both cases demonstrate t(8;21) as expected.

4.3.3.13 CD10

CD10 (B precursors and neutrophils marker) was expressed only in one case in our series. The expression was weak 29% of the leukaemic blasts, and clearly distinct from homogenously intensive staining in B-Lineage ALL using CD45/SSC gating (Figure 4-5: H).

4.3.3.14 CD11b

CD11b (neutrophils and monocytes marker) was performed in all patients but 3. CD11b was expressed on 13 (25%) with a median expression of 51% and mean = 49%.

4.3.3.15 CD64

CD64 (monocytes and macrophages marker) was not performed in 11 cases. CD64 was present in 19 (26%), median expression = 54% and mean = 58%.

4.3.3.16 CD65

CD65 (a marker for granulocytes and subset of monocytes) was performed in all patients but 5. CD65 was expressed in more than half of the patients 27 (55%) with

a median expression of 47% and mean = 56%. The intensity of expression was moderate to high in all the patients. Using Wilcoxon sign rank tests we found that there was no significant statistical relationship between aberrant phenotypes and cytogenetic risk category except for $CD34^{+}CD65^{+}$ ($p = 0.013$) and $CD117^{+}CD65^{+}$ ($p = 0.023$). Those in the favourable group showed higher values for these two LAPs than those in the intermediate and poor groups.

4.3.3.17 CD123

CD123 was added later in the project as it has been shown to be a unique marker for LSCs (Jordan *et al.* 2000). Therefore, 20 cases were not analysed for CD123. Of the remaining 34 cases, only 2 did not express CD123. The intensity of staining was dim to moderate in the vast majority of cases. The median expression of the 32/34 (94%) positive cases was 86% with a mean of 72%.

4.3.3.18 Cytoplasmic MPO

MPO was not tested in 10 cases. In the remaining ones, more than half (59%, n = 26) expressed cytoplasmic MPO. The median expression was 97% and mean = 83%.

4.3.3.19 Nuclear TdT

Fourteen cases were not tested for TdT. As expected, all the remained tested 40 cases showed clear negativity for TdT.

Figure 4-7 Representative example of co-expression of some LAPs in an AML patient with early progenitor markers: CD34 and CD117 demonstrating the advantage of using five-colour in increasing the specificity of LAPs by further identifying and characterising the stem and blast populations. The dotted line represents the isotypes matched controls for each fluorochrome. From left to right in each lane shows one tube, which has five-colour including CD45 (not shown). (**C** & **D**), (**I** & **J**), (**O** & **P**) show CD34 PC5 and CD117 PC7 in each of the 3 tubes, the coexpression of which was analysed with CD expression in FITC and PE (**A**& **B**), (**G** & **H**), (**M** & **N**) respectively and thus each combination of 5-colour can give 4 LAPs if both CD antigen in FITC and PE are positive as the case in (**G** & **H**) and (**M** & **N**). Although, CD11b expression in histogram **H** is positive, however, there is no coexpression with CD34 (**L**) or CD117 (not shown). The myeloid markers CD13 and CD33 are both positive in this patient and both coexpressing with CD34 (**Q**) and CD117 (**R**) [2 combinations shown in the figure $CD34^{+}CD13^{+}$ and $CD117^{+}CD33^{+}$].

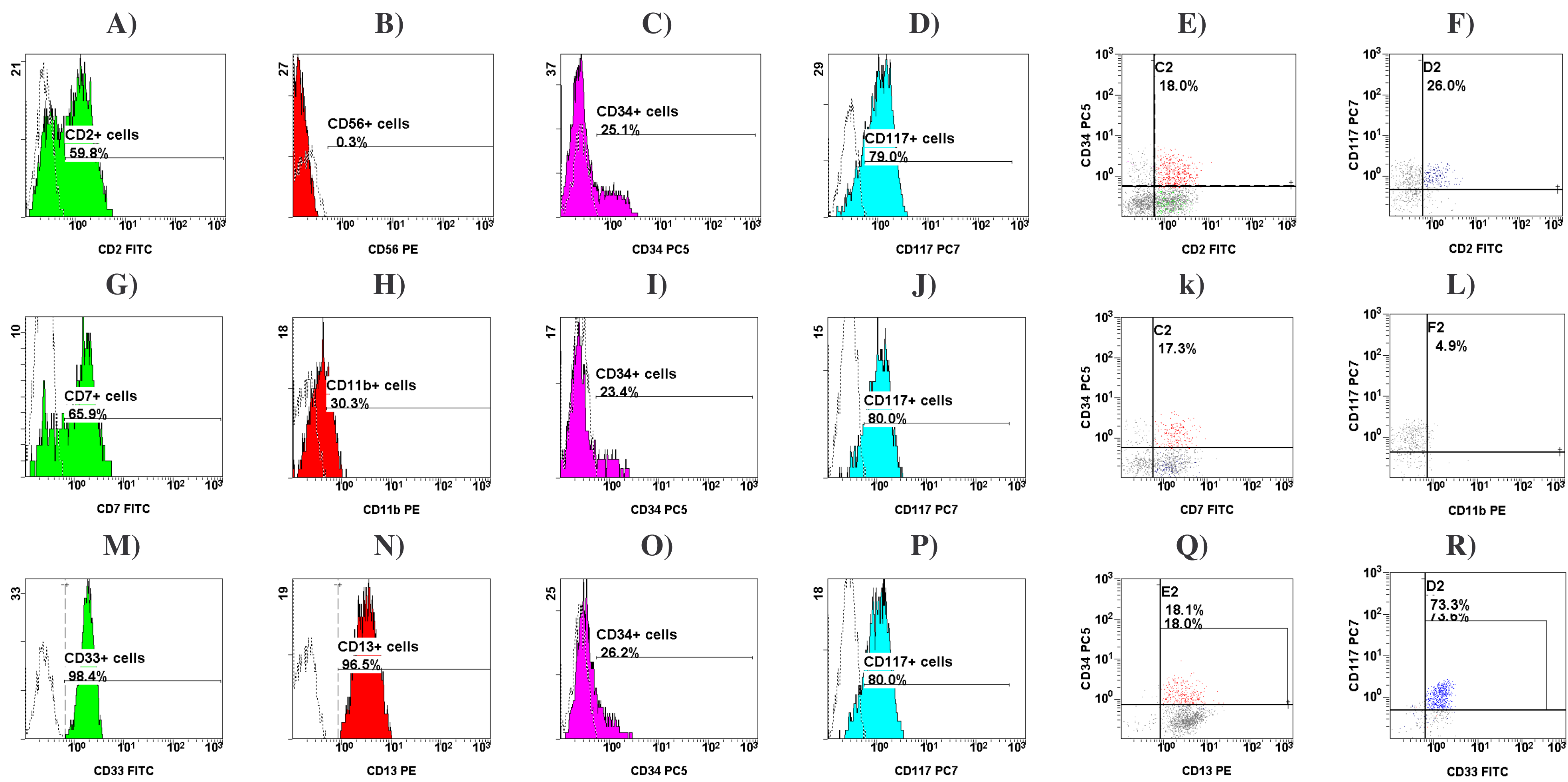
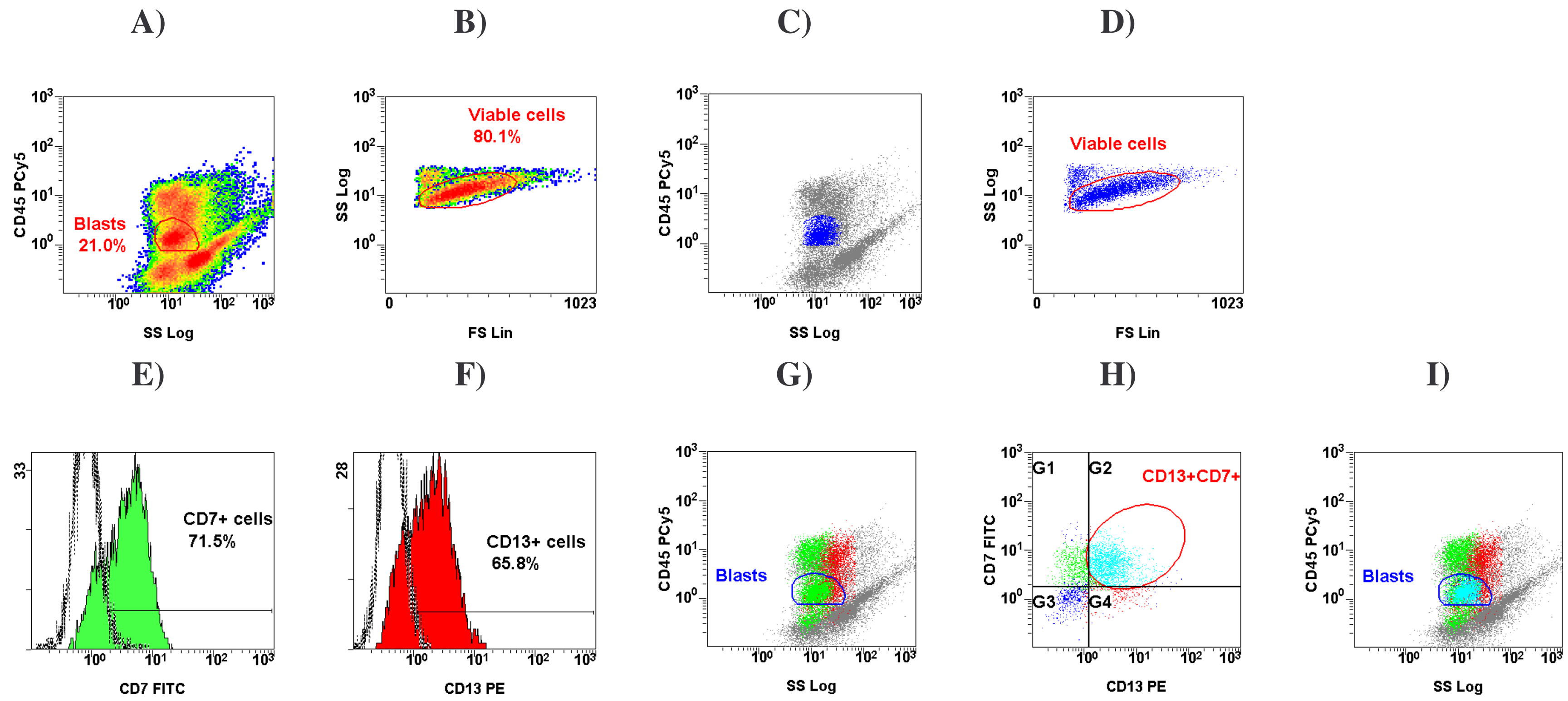

A)
B)
C)
D)
E)
F)
CD2+ cells
59.8%
CD2 FITC
CD56+ cells
0.3%
CD56 PE
CD34+ cells
25.1%
CD34 PC5
CD117+ cells
79.0%
CD117 PC7
C2
18.0%
CD34 PC5
CD2 FITC
D2
26.0%
CD117 PC7
CD2 FITC
G)
H)
I)
J)
k)
L)
CD7+ cells
65.9%
CD7 FITC
CD11b+ cells
30.3%
CD11b PE
CD34+ cells
23.4%
CD34 PC5
CD117+ cells
80.0%
CD117 PC7
C2
17.3%
CD34 PC5
CD7 FITC
F2
4.9%
CD117 PC7
CD11b PE
M)
N)
O)
P)
Q)
R)
CD33+ cells
98.4%
CD33 FITC
CD13+ cells
96.5%
CD13 PE
CD34+ cells
26.2%
CD34 PC5
CD117+ cells
80.0%
CD117 PC7
E2
18.1%
18.0%
CD34 PC5
CD13 PE
D2
73.3%
CD117 PC7
CD33 FITC

Figure 4-8 A representative AML case with CD7 positivity and the higher sensitivity achieved by using CD45 gating strategy to discriminate CD7 positive T-lymphocytes from CD7 blast population. From **A-D**: left to right, identification of blast population based on CD45 dim /SSC low (in density plot: **A**; and dot plot: **C**) and **B**, **D** shows the separation of dead cells from viable cells. The green histogram (**E**) shows CD7 positive cells in blast gate. The red histogram (**F**) shows CD13 positive cells in the blast gate. The dot plot in (**G**) shows the green dots: $CD7^+$ cells in blasts as well as lymphocytes, and CD13: red dots shows $CD13^+$ cells in blasts as well as monocytes. The quad region (**H**) demonstrates $CD13^+CD7^+$ only in the blast cells (aqua) which shows very clearly in (**I**) that these cells are not contaminating with lymphocytes or monocytes.


A)
B)
C)
D)
CD45 PCy5
SS Log
Blasts
21.0%
SS Log
FS Lin
Viable cells
80.1%
CD45 PCy5
SS Log
SS Log
FS Lin
Viable cells
E)
F)
G)
H)
I)
CD7 FITC
CD7+ cells
71.5%
CD13 PE
CD13+ cells
65.8%
CD45 PCy5
SS Log
Blasts
CD7 FITC
CD13 PE
G1
G2
G3
G4
CD13+CD7+
CD45 PCy5
SS Log
Blasts

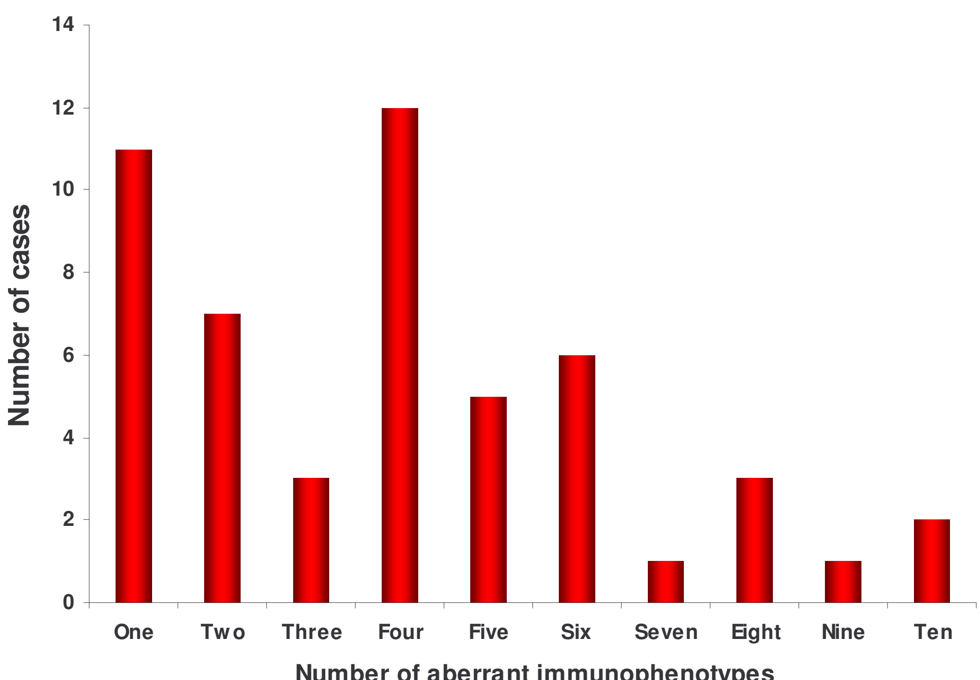
4.3.4 Identification of leukaemia-associated phenotypes

Fifty-four AML patients entered the study between January 2005 to April 2007 and were analysed for the expression of an aberrant immunophenotype by MFC, applying the two panels of MoAbs described in chapter 2 (Table 4-2). In 54 AML patients at diagnosis, LAPs were observed in 51 (94%) cases. On the other hand, looking at the data using the standard panel used at our institution by three-colour staining, LAPs were only observed in 79.6 % ($p < 0.05$).

Application of the extensive panel of MoAbs resulted in the identification of 200 LAPs within the 51 LAP positive patients. Only one aberrant immunophenotype was identified in 11 (22%) patients, but in the other 40 (78%) patients 2 to 10 aberrant immunophenotypes were identified (2 LAPs: n = 7; 3 LAPs: n = 3; 4 LAPs: n = 12; 5 LAPs: n = 5; 6 LAPs: n = 6; 7 LAPs: n = 1, 8 LAPs: n = 3; 9 LAPs: 1; 10 LAPs: n = 2) (Figure 4-9). The most frequent LAP identified was asynchronous antigen expression of progenitor cell markers and differentiation markers (LAPs = 146) and then lineage infidelity (LAPs = 40). The number of aberrant immunophenotypes identified by the application of the various combinations of antibodies is detailed in Table 4-3.

The percentage of AML cells carrying specific LAPs was assessed in each case and found to range from 10% to 99% (median, 52%). Only the most discriminating aberrant immunophenotype per patient was considered, i.e. the one yielding the highest log difference [*frequency in leukaemic BM / median frequency of LAP in normal or regenerating BM*] (Kern *et al.* 2003).

Figure 4-9 Incidence of aberrant immunophenotypes in AML (51 patients).



The distributions of these frequencies among the different classes of aberrant immunophenotypes as well as among the respective combinations of antibodies are shown in Table 4-4.

4.3.4.1 Lineage infidelity

Lineage infidelity was detected 40 times. The most frequent lymphoid antigen detected was CD7 (24%), followed by CD2 (10%), CD19 (4%), and CD10 (2%) in one case (Table 4-3).

4.3.4.2 Asynchronous antigen expression

Asynchronous antigen expression was detected 146 times. The most common asynchronous antigen expression in our cohort was $CD117^{+}CD15^{+}$ (49%) (Table 4-3).

4.3.4.3 Absence of lineage specific antigens

In 14 times at least one myeloid marker was absent with CD33 being the most frequent marker absent, 9 cases (18%) (Table 4-3).

The most common LAPs identified were: $CD117^+CD15^+$, $CD117^+CD65^+$, $CD34^+CD15^+$, and $CD34^+CD65^+$ and these were present in 49%, 43%, 39% and 29% respectively.

Table 4-3 Frequencies of LAPs in AML patients (n=51)

LAPs, n = 200	**No. of cases**	**% Positive cells in AML BM (range)**
Lineage infidelity (n = 40)		
$CD34^{+}CD2^{+}$	5	26-92
$CD34^{+}CD7^{+}$	12	11-88
$CD34^{+}CD10^{+}$	1	22
$CD34^{+}CD19^{+}$	2	24-65
$CD117^{+}CD2^{+}$	5	13-91
$CD117^{+}CD7^{+}$	11	18-90
$CD117^{+}CD10^{+}$	1	17
$CD117^{+}CD19^{+}$	2	15-53
$CD34^{+}GlycoA^{+}$	0	0
$CD117^{+}GlycoA^{+}$	1	30
Asynchronous Antigen expression (n = 146)		
$CD34^{+}CD11b^{+}$	11	11-80
$CD34^{+}CD14^{+}$	0	0
$CD34^{+}CD15^{+}$	20	10-36
$CD34^{+}CD56^{+}$	5	29-84
$CD34^{+}CD64^{+}$	6	10-49
$CD34^{+}CD65^{+}$	15	11-63
$CD117^{+}CD11b^{+}$	10	12-62
$CD117^{+}CD14^{+}$	0	0
$CD117^{+}CD15^{+}$	25	10-55
$CD117^{+}CD56^{+}$	6	11-80
$CD117^{+}CD64^{+}$	9	11-70
$CD117^{+}CD65^{+}$	22	10-62
$CD33^{+}CD15^{+}$	2	80-94
$CD33^{+}CD64^{+}$	5	18-97
$CD33^{+}CD65^{+}$	5	10-99
$CD33^{+}CD11b^{+}$	3	20-26
$CD33^{+}CD56^{+}$	2	10-78
Lack of lineage specific antigen (n = 14)		
$CD33^{++}CD13^{-}$	5	93-99
$CD33^{-}CD13^{++}$	9	25-98

4.3.5 Aberrant markers expression in normal and regenerating bone marrow cells

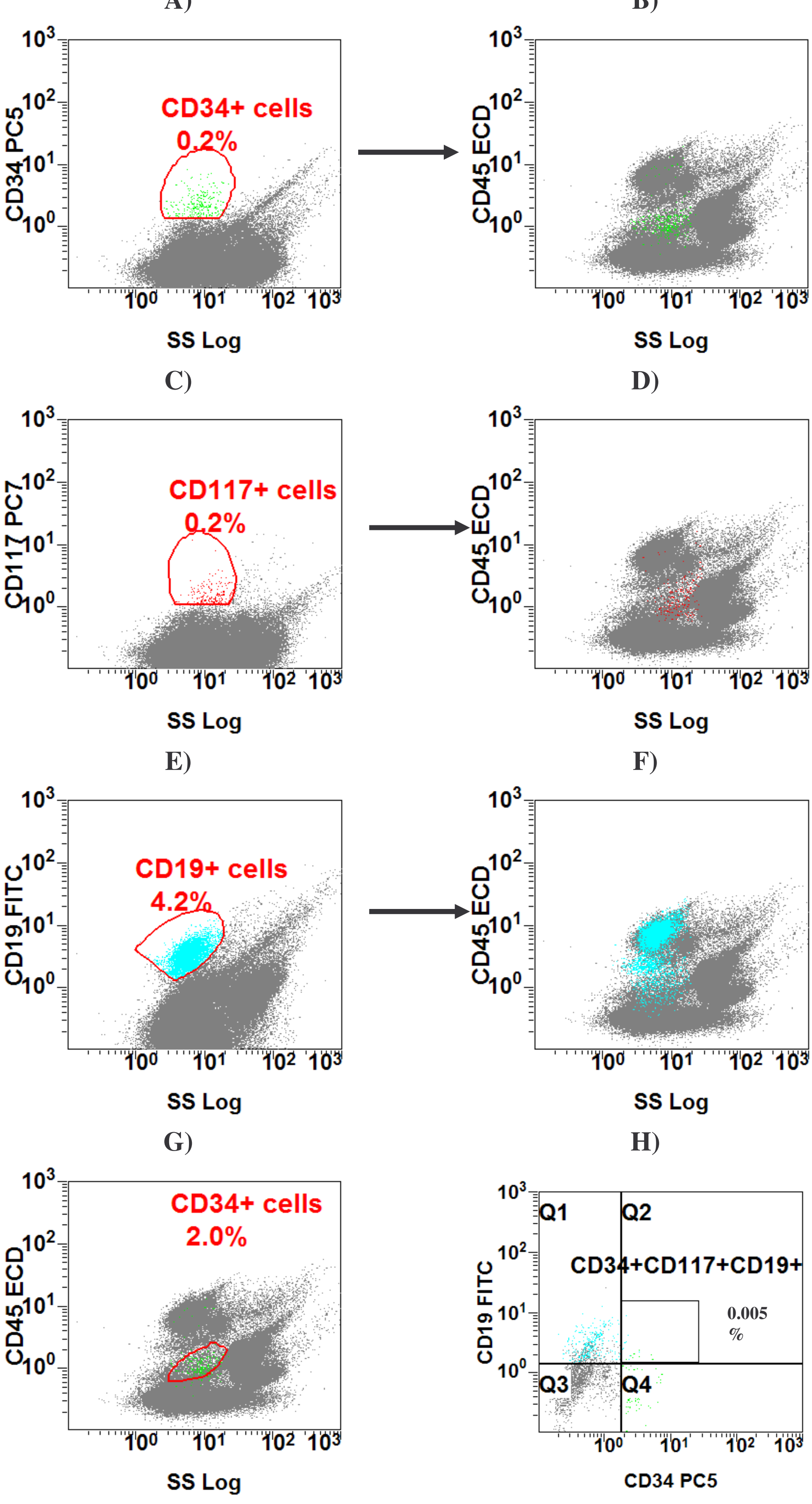
Ten samples of normal BM and five regenerating BM were analysed by the same panel of antibody combinations. The frequencies of LAP characteristic cells within these normal BM samples and regenerating BM were quantified using the gating strategy described in Chapter 2. Figure 4-10 is an example of the frequency of $CD34^{+}CD117^{+}CD19^{+}$ cells in normal BM. We have chosen this example because it is known that CD34+CD19+ are potentially frequent in normal precursors of B-cells.

The median percentage of LAP positive cells within the normal and regenerating BM samples was calculated for each LAP (Table 4-4). This median percentage ranged from 0.002 to 0.067 for normal BM and 0.004 to 0.086 in regenerating BM.

Restricting these analyses to the most sensitive LAP in each patient, resulted in a range from 0.000% to 0.0672% for the median percentage of LAP positive cells in the normal BM (median, 0.002%) and from 0.0005% to 0.0864% in regenerating BM (median, 0.00386%). The distributions of these frequencies among the different classes of LAP as well as among the respective combinations of antibodies in normal and regenerating BM are shown in Table 4-4.

MFC analysis showed no differences in expression of these LAP characteristic cells in normal and regenerating BM ($p = 0.2$) and that LAPs were extremely rare.

Figure 4-10 A representative example of the frequency of CD34$^+$CD117$^+$CD19$^+$ in normal BM. (**A**) backgating step to identify total CD34+ cells, (**B**) CD34$^+$ cells are shown in CD45/SS log (green). (**C**) Backgating step to identify total CD117$^+$ cells for more accurate detection of the blast population, (**D**) CD117$^+$cells are shown at the centre of CD45/SS log (red). (**E**) Backgating of CD19$^+$ cells, (**F**) CD19$^+$ cells are shown in in CD45/SS log (blue), as represented these cells are positive on B-lymphocytes, haematogones show characteristic and reproducible patterns of antigen expression representing the B-cell maturation sequence and and these are shown just below the lymphoctes as small population, and very few CD19$^+$ also shown in the blast population. (**G**) Shows the CD34$^+$% in CD45/SS log. Combination of three backgating steps are shown in (**H**) for coexpression of CD34+CD117+CD19+ cells in normal BM.


A)
B)
C)
D)
E)
F)
G)
H)
CD34+ cells
0.2%
CD117+ cells
0.2%
CD19+ cells
4.2%
CD34+ cells
2.0%
CD34 PC5
CD45 ECD
CD117 PC7
CD19 FITC
SS Log
Q1
Q2
Q3
Q4
CD34+CD117+CD19+
0.005 %

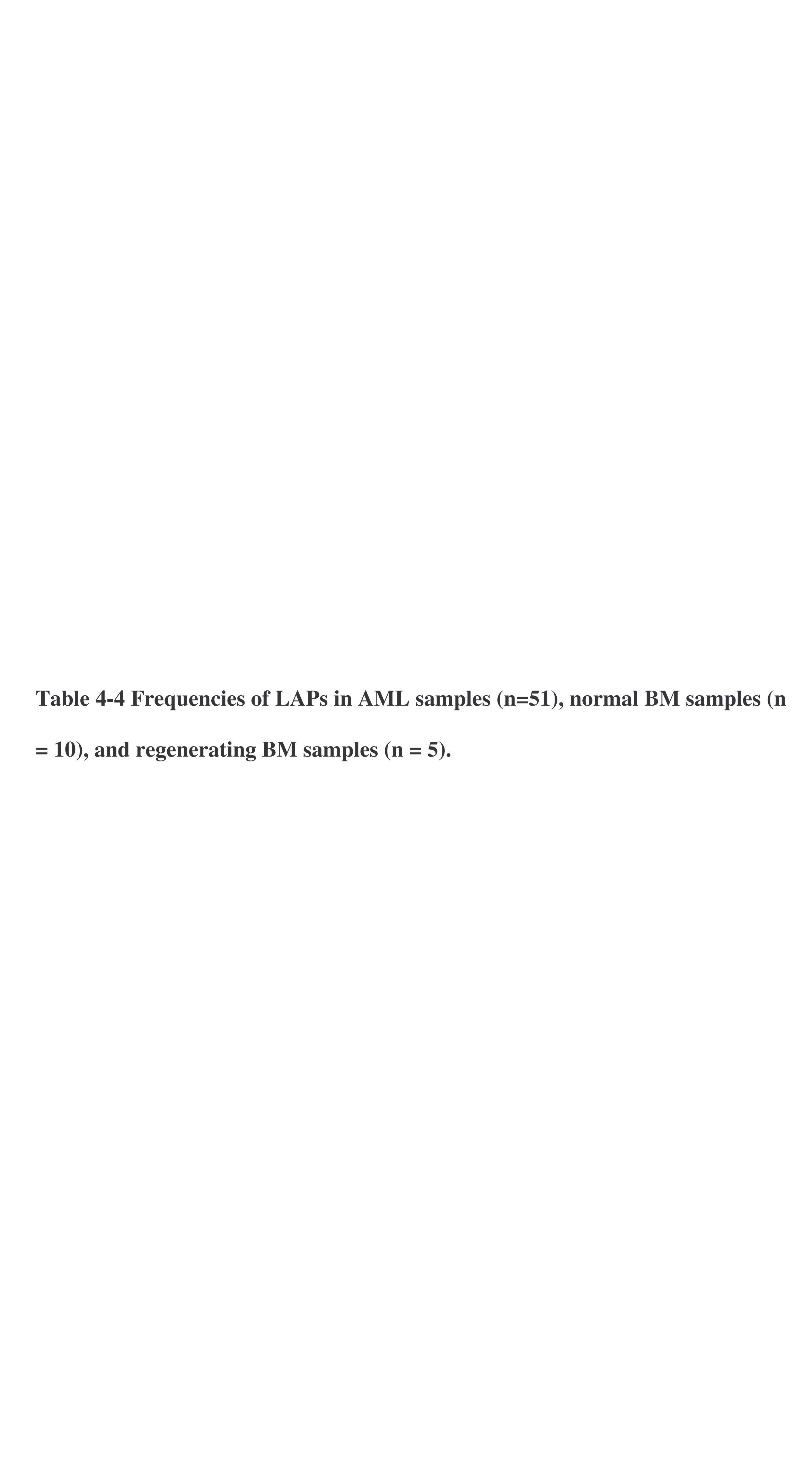


Table 4-4 Frequencies of LAPs in AML samples (n=51), normal BM samples (n = 10), and regenerating BM samples (n = 5).

LAPs	No. of cases	% LAPs in AML		% positive cells in normal BM (n=10)			Log diff.		% positive cells in regenerating BM (n=5)			Log diff.	
		min	*max*	*median*	*min*	*max*	*min*	*max*	*median*	*min*	*max*	*min*	*max*
CD34+CD2+	5	26	92	0.001	0.000	0.030	4.41	4.96	0.004	0.000	0.030	3.81	4.36
CD34+CD7+	12	11	88	0.002	0.000	0.009	3.74	4.64	0.004	0.000	0.005	3.49	4.39
CD34+CD10+	1	22	22	0.012	0.000	0.185	3.26	3.26	0.035	0.002	0.072	2.79	2.79
CD34+CD19+	2	24	65	0.002	0.000	0.007	4.08	4.51	0.003	0.000	0.015	3.96	4.39
CD117+CD2	5	13	91	0.003	0.000	0.010	3.64	4.48	0.003	0.000	0.019	3.70	4.54
CD117+CD7+	11	18	90	0.003	0.000	0.033	3.78	4.48	0.006	0.001	0.017	3.48	4.18
CD117+CD10+	1	17	17	0.001	0.000	0.014	4.23	4.23	0.003	0.001	0.050	3.75	3.75
CD117+CD19+	2	15	53	0.002	0.000	0.011	3.88	4.42	0.004	0.000	0.004	3.63	4.18
CD34+GlycoA+	0	0	0	0.000	0.000	0.006	NC§	NC§	0.053	0.002	0.237	NC§	NC§
CD117+GlycoA+	1	30	30	0.001	0.000	0.008	4.48	4.48	0.015	0.003	0.443	3.31	3.31
CD34+CD11b+	11	11	80	0.002	0.000	0.022	3.74	4.60	0.002	0.001	0.095	3.74	4.60
CD34+CD14+	0	0	0	0.000	0.000	0.001	NC§	NC§	0.002	0.001	0.005	NC§	NC§
CD34+CD15+	20	10	36	0.004	0.000	0.024	3.40	3.95	0.012	0.002	0.014	2.92	3.48
CD34+CD56+	5	29	84	0.001	0.000	0.039	4.46	4.92	0.001	0.000	0.024	4.35	4.81
CD34+CD64+	6	10	49	0.051	0.000	0.160	2.29	2.98	0.086	0.000	0.105	2.06	2.75
CD34+CD65+	15	11	63	0.013	0.005	0.047	2.93	3.69	0.057	0.003	0.106	2.29	3.05
CD117+CD11B+	10	12	62	0.002	0.000	0.048	3.78	4.49	0.001	0.000	0.026	4.08	4.79
CD117+CD14+	0	0	0	0.000	0.000	0.010	NC§	NC§	0.001	0.000	0.011	NC§	NC§
CD117+CD15+	25	10	55	0.010	0.000	0.039	3.00	3.74	0.017	0.005	0.093	2.76	3.50
CD117+CD56+	6	11	80	0.003	0.000	0.037	3.56	4.43	0.002	0.000	0.025	3.74	4.60
CD117+CD64+	9	11	70	0.067	0.001	0.127	2.22	3.02	0.040	0.002	0.247	2.44	3.24
CD117+CD65+	22	10	62	0.007	0.001	0.042	3.15	3.95	0.028	0.023	0.266	2.55	3.34
Median		***11***	***62***	***0.002***	***0.000***	***0.027***	***3.74***	***4.43***	***0.004***	***0.001***	***0.028***	***3.48***	***4.18***
Min		***0***	***0***	***0***	***0.000***	***0.001***	***2.22***	***2.98***	***0.001***	***0.000***	***0.004***	***2.06***	***2.75***
Max		***30***	***92***	***0.067***	***0.005***	***0.185***	***4.48***	***4.96***	***0.086***	***0.023***	***0.443***	***4.35***	***4.81***

NC§ No Case were found for that defined aberrant immunophenotype.

4.3.6 Quantification of differences in cells carrying aberrant immunophenotypes between AML samples, and normal and regenerating bone marrows

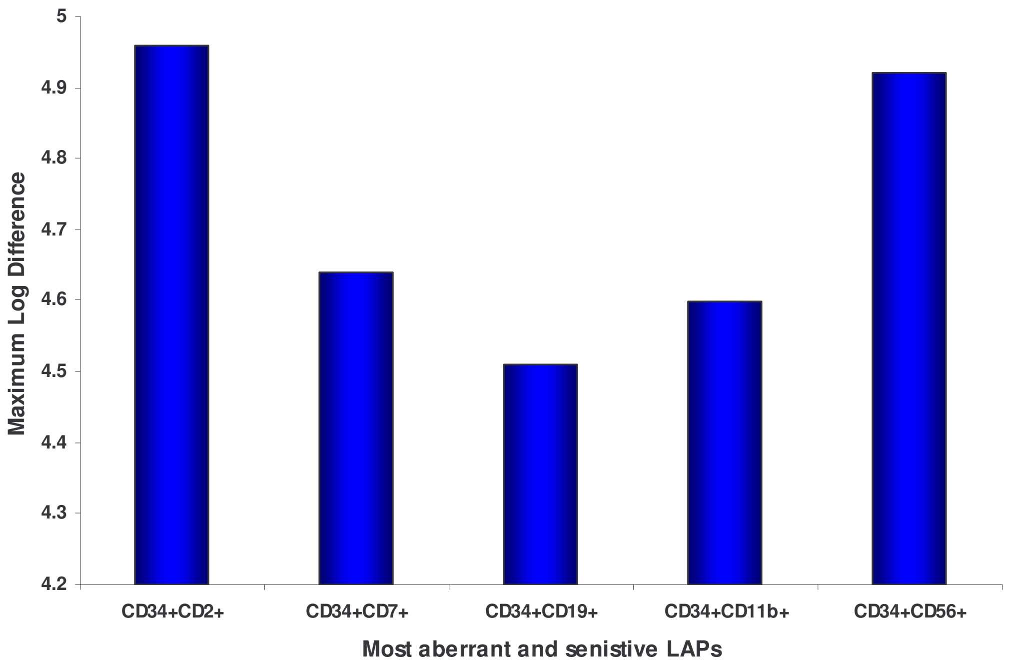
The frequencies of cells within the normal and regenerating BM samples carrying the respective LAPs were determined for each individual LAP as defined in cases with AML. To estimate the degree of the reduction in leukaemic cell mass that is detectable by the present approach for each individual LAP, the LD was calculated. For example, the LD would have been 3.00 in a case in which LAP positive cells were present in 50% in the leukaemic BM and in a median of 0.05% in normal BM. In case of a median frequency of 0.00% of LAP positive cells in normal or regenerating BM, this frequency was set to 0.001% in order to allow the calculation of LD (0.001% is the highest frequency displayed as 0.00% and was chosen as worst case possible). If more than one LAP was defined in one patient, the LAP that allowed maximum analytical sensitivity for MRD study was selected for the respective evaluations as indicated on the basis of the maximum LD in comparison to other LAPs in the same patient.

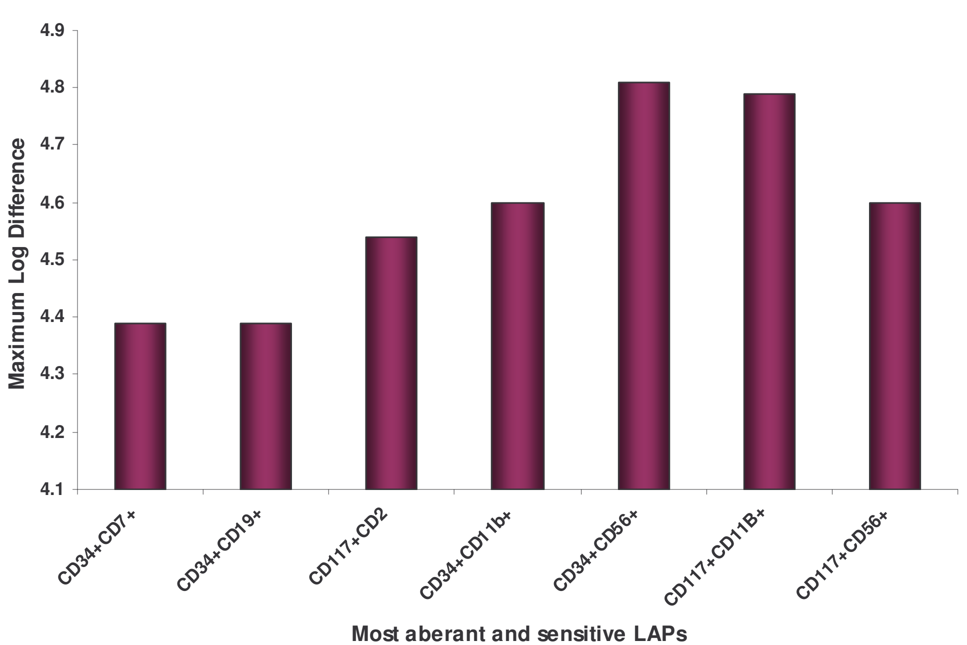
In normal BM, the median of this difference was 3.92 and its range from 2.22 to 4.96. In regenerating BM, the median of difference was 3.67 and its range from 2.06 to 4.81 (Table 4-4).

Restricting the analysis to the maximum LD resulted in five most sensitive LAPs in our series and these are CD2, CD56, CD7, CD11b and CD19 when compared to normal BM and CD56, CD11b, CD2, CD7 and CD19 in regenerating BM respectively in order of the level of maximum LD (Figure 4-11). This means that these LAPs are the most sensitive markers for tracking MRD when present in AML samples. These markers represent a reasonable percentage of our cohort: CD2 (9%),

CD56 (17%), CD7 (28%), CD11b (14%) and CD19 (4%) and thus should be included in any panel for MRD monitoring.

Figure 4-11 The most aberrant immunophenotypes and most sensitive ones based on maximum LD in percentage positive cells of LAPs in AML samples from normal BM (top figure, blue) and regenerating BM (bottom figure, red)





4.3.7 Investigation of the 'in vitro' sensitivity of flow cytometry immunophenotyping for detection of MRD

4.3.7.1 Dilution of AML samples in normal and regenerating bone marrow

In order to determinate the level of sensitivity of MFC immunophenotyping for the detection of leukaemic cells when present at very low frequencies, serial dilutional experiments were performed. This was done by adding increasingly low numbers of LAP positive cells obtained from selected samples of AML patients (n = 8), normal BM (n=5), and regenerating BM (n=3). The aberrant immunophenotypes analysed were $CD34^{+}CD7^{+}$, $CD117^{+}CD7^{+}$, $CD34^{+}CD56^{+}$ and $CD117^{+}CD56^{+}$. The AML samples were selected with these LAPs based on two reasons: 1) these LAPs have very high analytical sensitivity when compared to their expression level on normal and regenerating BMs, and 2) these LAPs represent two major groups of LAPs: lineage infidelity and asynchronous antigen expression. For each experiment, the dilutions covered a range from 50% to 0.001%.

4.3.7.2 Assessment of linearity

Two aspects of sensitivity were investigated: linearity and reproducibility. As shown in Table 4-5 and Figure 4-12, the immunophenotypic approach allowed the detection of at least one aberrant leukaemic cell among 10^4 and 10^6 normal haemopoietic cells. Accordingly, the *in vitro* sensitivity level of this approach would be of at least 10^{-4}. Dilutions covering range from 50% to 0.001% resulted in coefficients of correlation (CC) of >0.9 (p = 0.002) in all eight cases. Two cases are shown in Figure 4-13 with $CD34^{+}CD56^{+}$ and $CD34^{+}CD7^{+}$.

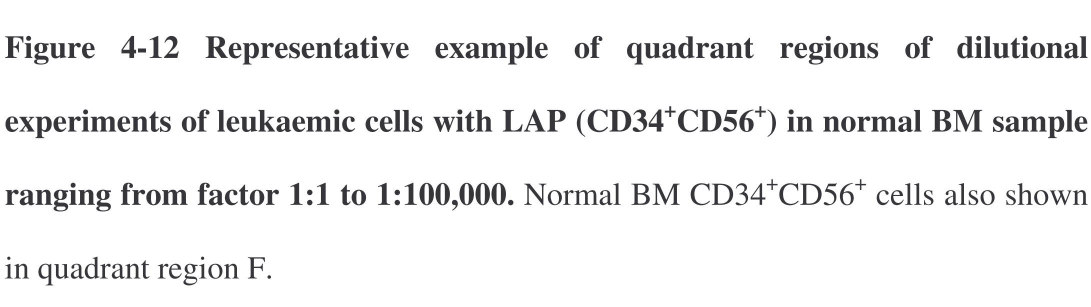


Figure 4-12 Representative example of quadrant regions of dilutional experiments of leukaemic cells with LAP (CD34$^+$CD56$^+$) in normal BM sample ranging from factor 1:1 to 1:100,000. Normal BM CD34$^+$CD56$^+$ cells also shown in quadrant region F.

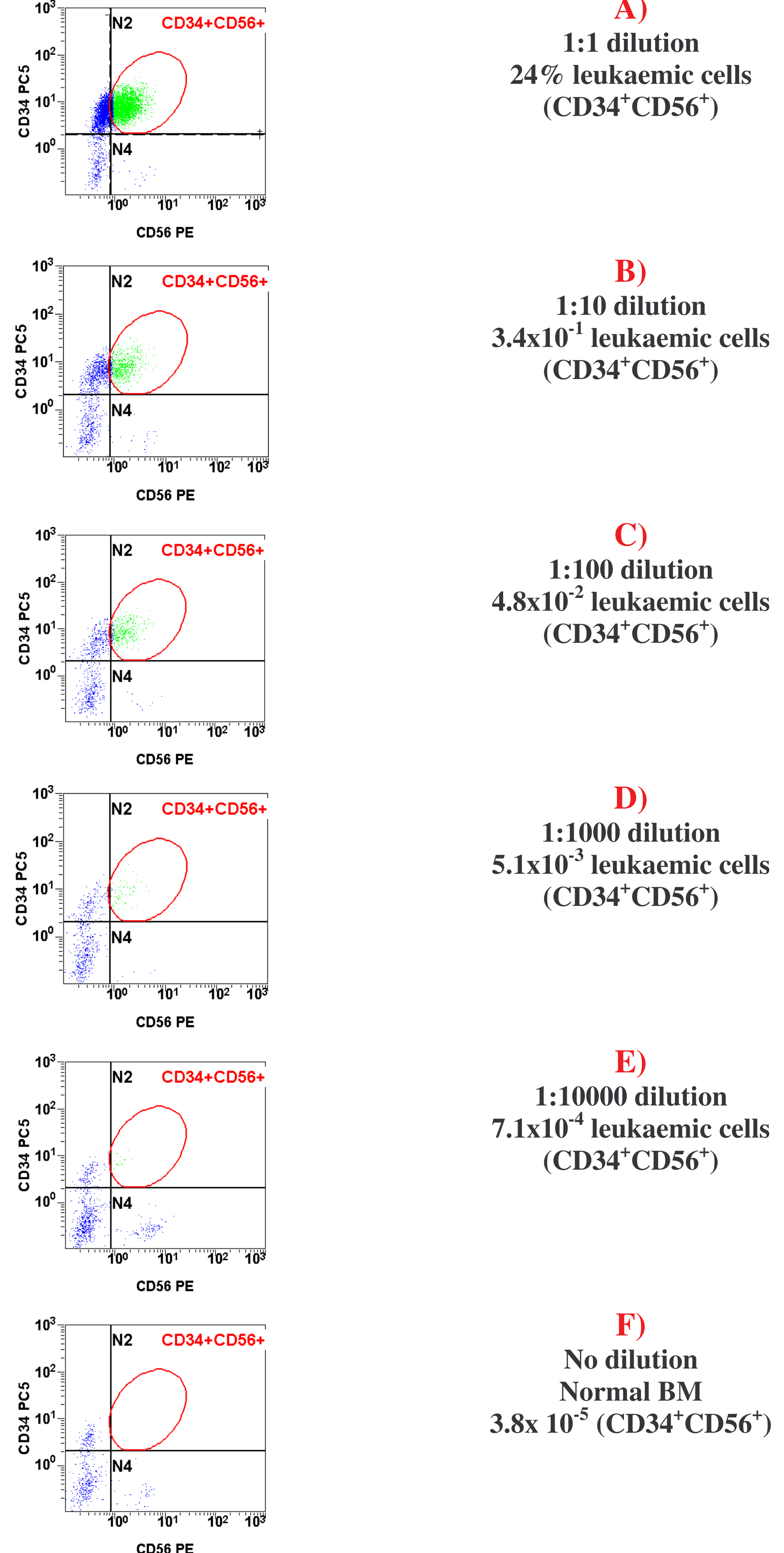
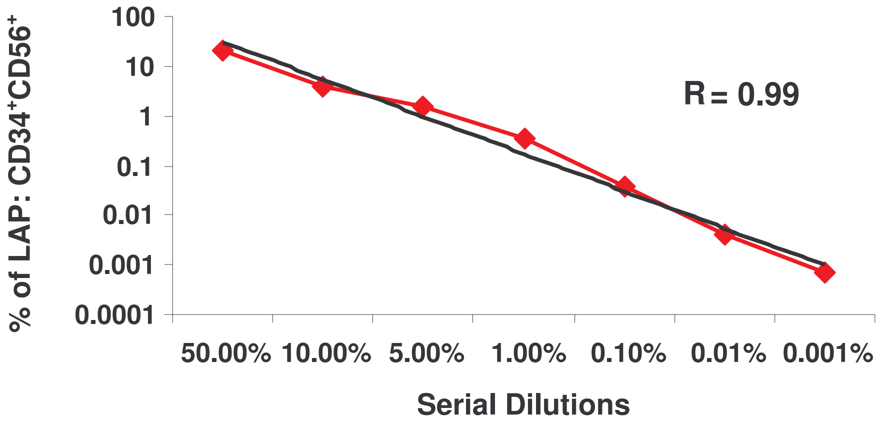

CD34 PC5
CD56 PE
N2
N4
CD34+CD56+
A)
1:1 dilution
24% leukaemic cells
(CD34+CD56+)
B)
1:10 dilution
3.4x10-1 leukaemic cells
(CD34+CD56+)
C)
1:100 dilution
4.8x10-2 leukaemic cells
(CD34+CD56+)
D)
1:1000 dilution
5.1x10-3 leukaemic cells
(CD34+CD56+)
E)
1:10000 dilution
7.1x10-4 leukaemic cells
(CD34+CD56+)
F)
No dilution
Normal BM
3.8x 10-5 (CD34+CD56+)

Figure 4-13 Assessment of linearity in sensitivity experiments. Two dilutional experiments are shown. **A**) Dilution from 50% to 0.001% of AML sample with $CD34^{+}CD56^{+}$, **B**) Dilution from 50% to 0.001% of AML sample with $CD34^{+}CD7^{+}$. Both cases resulted in coefficients of correlation (CC) of 0.99 (**A**) and 0.97 (**B**).

A)



B)

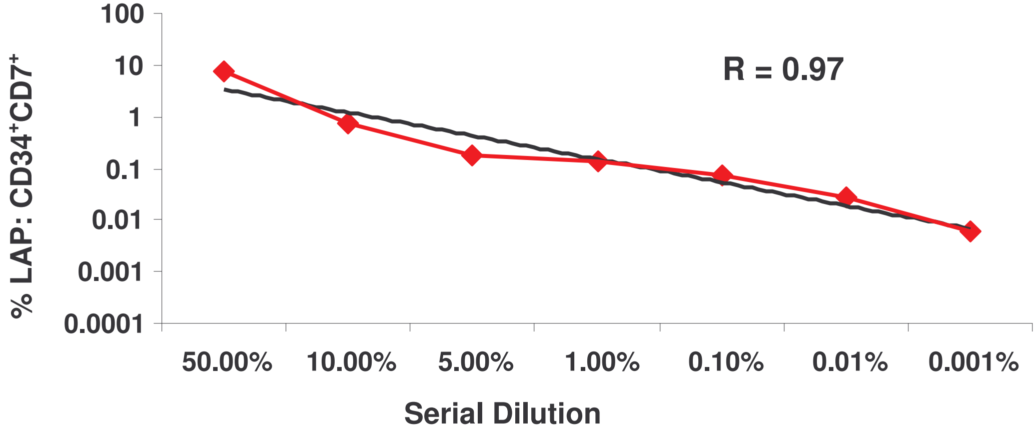


Table 4-5 Sensitivity level of MFC immunophenotyping based on dilutional experiments of leukaemic cells in either normal BM (cases 1 to 5) or regenerating BM (case 6-8)

LAPs	Case	% of LAPs*	Sensitivity level
$CD34^+CD7^+$	1	0.0700	10^{-4}
$CD117^+CD7^+$		0.0600	10^{-4}
$CD34^+CD56^+$	2	0.0007	10^{-6}
$CD117^+CD56^+$		0.0005	10^{-6}
$CD34^+CD7^+$	3	0.0060	10^{-5}
$CD117^+CD7^+$		0.0060	10^{-5}
$CD34^+CD56^+$	3	0.0304	10^{-4}
$CD117^+CD56^+$		0.0199	10^{-4}
$CD34^+CD7^+$	4	0.0032	10^{-5}
$CD117^+CD7^+$		0.0022	10^{-5}
$CD34^+CD7^+$	5	0.0012	10^{-5}
$CD117^+CD7^+$		0.0025	10^{-5}
$CD34^+CD56^+$	5	0.0360	10^{-4}
$CD117^+CD56^+$		0.0540	10^{-4}
$CD34^+CD56^+$	6	0.0176	10^{-4}
$CD117^+CD56^+$		0.0040	10^{-5}
$CD34^+CD7^+$	7	0.0016	10^{-5}
$CD117^+CD7^+$		0.0028	10^{-5}
$CD34^+CD7^+$	8	0.0148	10^{-4}
$CD117^+CD7^+$		0.0020	10^{-5}

*** Results expressed as the percentage of LAPs detected in the tube corresponding to the 1:100,000 dilutional experiments.**

4.3.7.3 Assessment of reproducibility

To assess the precision and reproducibility as another aspect of the sensitivity, experiments were repeated 5 times under the same conditions and dilutions using the same normal BM and AML sample on the same day. The results were calculated by measuring the coefficient of variation (CV).

We linked CV and $p(\kappa)$ (where κ can be any number greater than one and arbitrarily close to one) (Reed *et al.* 2002) in order to: 1) assess whether or not the difference between five different measurements is due to random variation, and 2) assess whether the variation in a set of replicates is larger than that implied by the assumed CV (Table 4-6).

The results revealed that sensitivity down from level 0.01% to 0.001% dilutions is reproducible and precise using four LAPs mentioned above for each LAP from 6 samples. As shown in Table 4-7, $p(\kappa)$ in the six samples performed were higher than 0.05, indicating that there was no statistical significant difference in the 5 trials except when highlighted in sample 1, 2 and 5 at levels 0.01%, 0.001%, and 0.001% respectively. These experiments concluded that detection of one leukaemic cell among 10^4 is achievable and possibly 10^5 is also feasible in most of the cases. Linearity was also established in all samples in the five replicate trials as shown in Figure 4-14.

Table 4-6 Reproducibility and precisions in sensitivity experiments. An example of CD117$^+$CD56$^+$ showing the results of dilutions from the five trials covering the range from 1.00 % to 0.001%. Results from BM without any dilutions are also shown. The largest and lowest values within each dilution were tabulated, then the difference was taken and the percent difference for which was calculated and the probability p(κ) was determined. K is 1+ the percent increase between the two values. Assuming that the actual CV is the mean of the five trials, allowed us to calculate the p(κ) which is the probability that you would obtain a difference in CVs just by random error. p(κ) >0.05 indicates that it is random error, and a value < 0.05 is an indication that the difference is significant. As it is clear from the p(κ) values, all of them are >0.05 indicating that nothing else happening in the data other than random error (Reed *et al.* 2002). Thus, this approach is reproducible.

Dilutions / Trials	1.00%	0.10%	0.01%	0.001%	BM only
Trial 1	0.19	0.08	0.02	0.020	0.004
Trial 2	0.20	0.04	0.03	0.028	0.003
Trial 3	0.18	0.03	0.02	0.022	0.003
Trial 4	0.22	0.03	0.01	0.009	0.005
Trial 5	0.20	0.04	0.02	0.001	0.006
Lowest value	0.18	0.03	0.01	0.009	0.003
Highest value	0.22	0.08	0.03	0.028	0.006
Difference	0.05	0.05	0.02	0.018	0.003
%increase	0.27	1.67	1.53	2.000	1.000
k	1.27	2.67	2.53	3.000	2.000
CV	0.20	0.05	0.02	0.020	0.004
p(k)	**0.95**	**0.69**	**0.62**	**0.52**	**0.210**

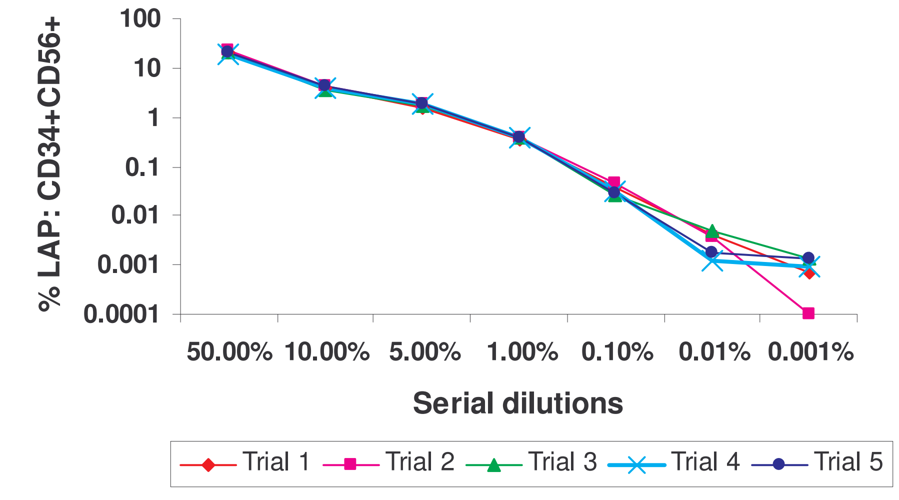
Table 4-7 Summary of reproducibility for sensitivity experiments (p values obtained using six samples in 5 trials per sample). The grey highlight indicates that likely the difference between the 5 trials is significant and not due to random error. The table demonstrates that in all samples, detection of one leukaemic cell among 10^4 normal cells is achievable, except in sample 1. In three samples, detection of 1 leukaemic in 10^5 normal cells was also feasible**.**

Sample No	LAP	p(k)	LAP (No dil)%	50.000%	10.000%	5.000%	1.000%	0.100%	0.010%	0.001%	BM only
Sample 1	$CD34^+CD56^+$	p(k)	0.991	0.983	0.969	0.971	0.974	0.8	0.001	0	ND*
Sample 2	$CD117^+CD56^+$	p(k)	0.991	0.985	0.966	0.894	0.977	0.845	0.243	0.027	ND*
Sample 3	$CD34^+CD56^+$	p(k)	ND	ND	ND	ND	0.893	0.732	0.62	0.449	0.033
Sample 4	$CD117^+CD56^+$	p(k)	ND	ND	ND	ND	0.945	0.688	0.619	0.515	0.205
Sample 5	$CD34^+CD7^+$	p(k)	ND	ND	ND	ND	0.945	0.717	0.74	0.018	0
Sample 6	$CD117^+CD7^+$	p(k)	ND	ND	ND	ND	0.945	0.695	0.719	0.059	0.001

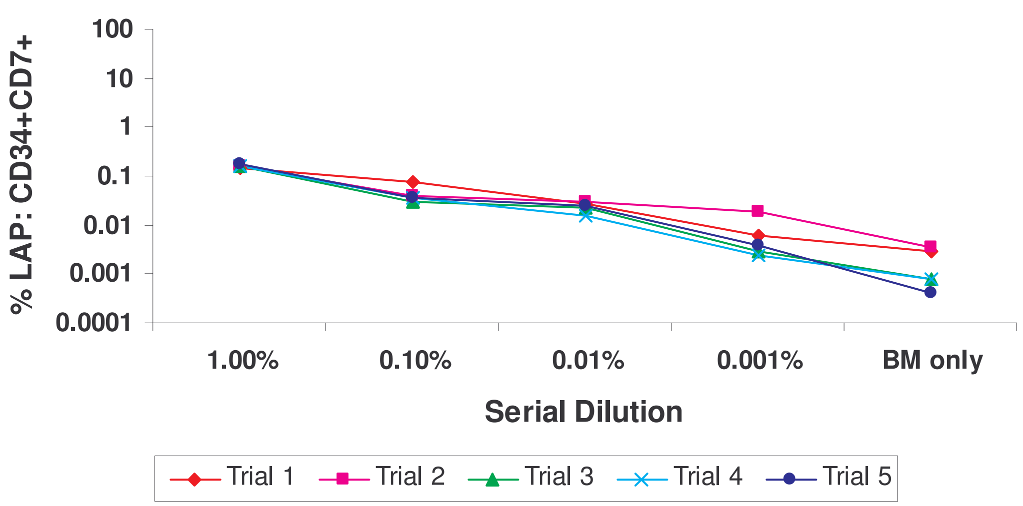
* **ND: not done**

Figure 4-14 Assessment of linearity in sensitivity experiments for the five replicates of the same samples analysed. Two dilutional experiments are shown. **A**) Five replicates of dilution from 50% to 0.001% of AML sample with CD34^{+}CD56^{+}, **B**) Five r of dilution from 1.00% to 0.001% of AML sample with CD34^{+}CD7^{+} (BM only results also shown).

A)



B)

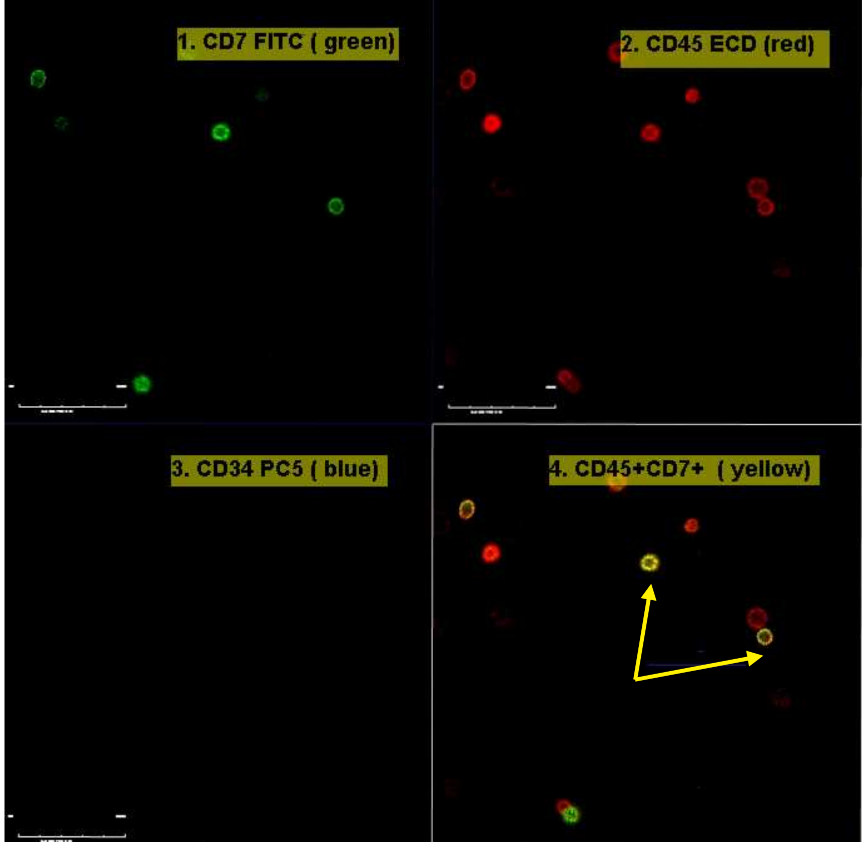


4.3.8 Appearance of LAPs using Immuno-Fluorescence Confocal Microscopy

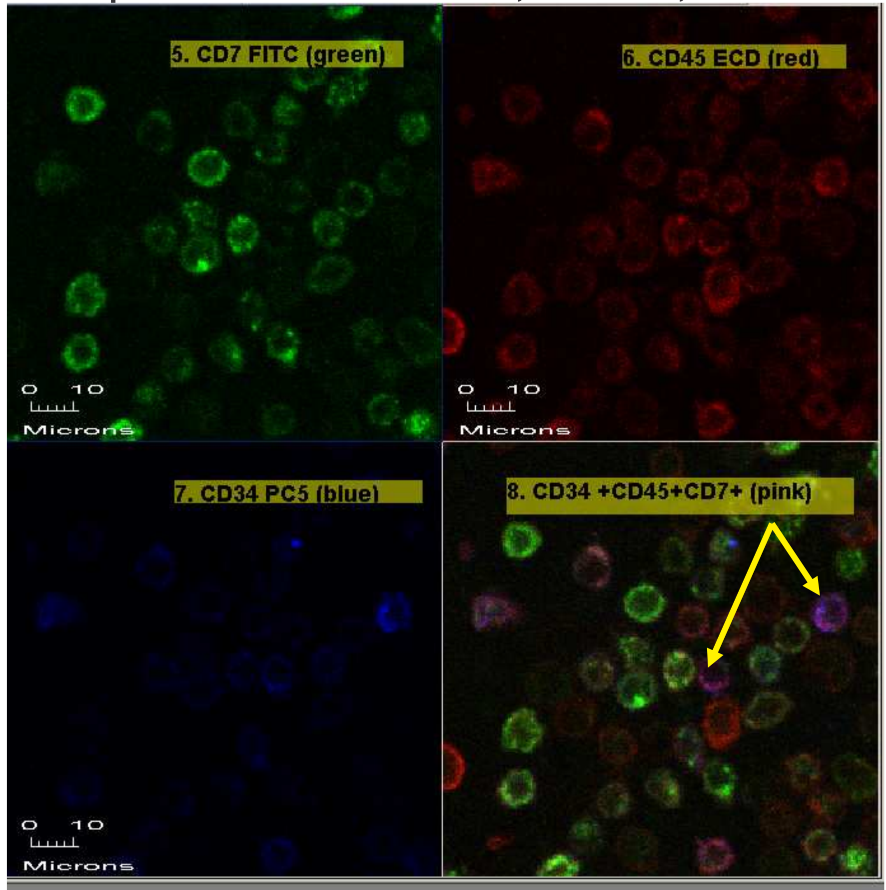
Confocal microscopy was used to see the difference between normal BM and AML sample positive for LAP morphologically. The single, dual and triple labelled samples were imaged with three separate channels (PMT tubes) in a sequential setting. Figure 4-15 shows the merged photo for co-localisation of $CD45^{+}CD34^{+}CD7^{+}$ (pink colour) in some cells which demonstrate that these cells are blasts with an aberrant phenotypes $CD34^{+}CD7^{+}$ (Figure 4-15, B), whereas some cells in the same sample are $CD45^{+}CD7^{+}$ but not coexpressing $CD34^{+}$ and these are the T lymphocytes (yellow colour). It should be noted here that some cells are not coexpressing either CD7 or CD34 (shown with their original colour). Normal BM (Figure 4-15, A) shows no CD34 at all in this section. Coexpression of $CD45^{+}CD7^{+}$ is indicating the T lymphocytes with the yellow colour.

Figure 4-15 Appearance of aberrant immunophenotype ($CD34^+CD7^+$) under confocal microscopy using three-colour FITC, ECD and PC5 with comparison to normal BM. Figure **A** (1-4) demonstrates the normal BM, **1**: shows $CD7^+$ cells (green), **2**: shows $CD45^+$ cells (red), **3**: shows no $CD34^+$ cells in this section in comparison to AML sample in Figure **B**: **7**. **4**: shows the localisation of $CD45^+CD7^+$ cells (yellow) demonstrated by the arrows. These are the T- lymphocytes. Figure **B** (**5-8**) demonstrates the AML sample with aberrant immunophenotypes $CD34^+CD7^+$ cells. **5**: shows $CD7^+$ cells (green), **6**: shows $CD45^+$ cells (red), **7**: shows $CD34^+$ cells (blue), **8**: shows the co-localisation of $CD45^+CD34^+CD7^+$ cells (pink) demonstrated by the arrows which represent the LAP. There are some cells coexpressing only $CD45^+CD7^+$ (faint yellow) and these are T lymphocytes easily distinguished from the LAP cells. There are also $CD45^+$ cells and $CD7^+$ cells.

A) Normal BM stained with CD45 ECD, CD7 FITC, CD34 PC5



B) AML sample stained with CD45 ECD, CD7 FITC, CD34 PC5



4.4 DISCUSSION

Monitoring of MRD may become increasingly important to guide therapy in patients with AML. Current methods used to quantify MRD are hampered by the lack of providing a high sensitivity and applicability for each patient with AML.

The present study using five-colour MFC, CD45 gating and an extensive panel of MoAbs in AML patients shows that LAPs are present in virtually all patients at diagnosis suggesting an improvement in LAP detection compared to previous approaches. Moreover, in 78% of cases, leukaemic cells simultaneously displayed more than one LAP. This is important when immunophenotypic shifts occur during treatment (Chen *et al.* 2007).

We confirmed earlier reports suggesting that the blast cell population can be clearly identified by virtue of their low antigen membrane CD45 antigen expression and their light scatter properties (Terstappen *et al.* 1991; Borowitz *et al.* 1993; Stelzer *et al.* 1993; Kern *et al.* 2005). Other groups have recommended CD45/SSC gating to provide a better definition of all cell-subpopulations of the samples in normal BM analysis (Stelzer *et al.* 1993) as well as in acute leukaemia (Borowitz *et al.* 1993). We found that CD45/SSC gating is the most reliable and easy way to perform AML immunophenotyping especially for samples with low proportions of blast cells.

Based on our results, we propose specific five-colour combinations of MoAbs that would identify aberrant phenotypes in virtually all cases. Further, our data shows that CD2, CD56, CD7, CD19 and CD11b are potentially the most useful markers for MRD detection as these markers allowed the maximum analytical sensitivity when normal and regenerating bone marrows were taken into account as a baseline. Twenty-four (44%) of the cases express at least one of these markers and thus they should be included in any diagnostic panel for AML for MRD monitoring. In

Chapter 3, we have shown that presence of LAPs was an adverse prognostic indicator after induction chemotherapy. In this chapter, using 5-colour MFC, 94% of AML patients displayed LAPs. This finding needs to be re-evaluated. The finding of Chapter 3 is from a retrospective analysis and may be not widely applicable. It may be that specific LAPs confer an adverse prognosis after induction chemotherapy but this can only be addressed on large prospective studies.

The high incidence of aberrant phenotypes detected in the present study and the marker combinations defined for their identification would represent ideal probes for detection of MRD. However, an additional prerequisite of MRD techniques is to be sensitive enough to detect low level of residual leukaemic cells. The dilutional experiments are rarely performed in these types of studies and as an important aspect of quantitative assay studies is to be precise and reproducible Our results show that, based on a two-step acquisition procedure in which specific selection of LAPs is used in the latter step, MFC immunophenotyping is a highly sensitive technique for the detection of minimal numbers of myeloid blasts displaying aberrant phenotypes. In all experiments, a minimum sensitivity level of 10^{-4} (one leukaemic cell among 10^4 normal cells) was reached, independently of the type of aberration explored. Moreover, it should be noted that in a significant number of cases, an even higher (10^{-5}) sensitivity level was reached by this approach. In addition, this approach is highly reproducible.

Based on the applicability and sensitivity of the MFC immunophenotyping approach shown in the present study, it could be concluded that this is a well-suited approach for the specific detection of minimal numbers of leukaemic cells and hence, could help us to obtain a more precise and early evaluation of the effectiveness of new treatment strategies and to better assess the CR status in AML

patients (Borowitz *et al.* 1993; Rainer *et al.* 1995; Gratama *et al.* 1998; Zwick *et al.* 2006).

Future longitudinal prospective studies with bigger sample size of AML patients to determine MRD frequency is necessary. Based on these findings, it can be concluded that because of the clinical utility of comparing the results obtained from immunophenotypic characterisation of healthy and leukaemic cells, a major challenge for the near future is the possibility of performing stable, calibrated, and standardized measurements in such a way that identical cells provide identical phenotypic patterns whenever they are analysed at different times and in different laboratories.

In summary, we show the high applicability and sensitivity of multiparameter immunophenotyping by flow cytometry for the detection of leukaemic cells in AML patients. For that purpose, unique five-colour staining combinations of MoAbs are specifically proposed.

CHAPTER 5

Detection of minimal residual disease in acute myeloid leukaemia identifies patients with high risk of relapse

CHAPTER 5: DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKAEMIA IDENTIFIES PATIENTS WITH HIGH RISK OF RELAPSE

5.1 ABSTRACT

Early response to therapy is one of the most important prognostic factors in acute leukaemia. We hypothesised that early immunophenotypical evaluation may help identifying patients at high risk for relapse from those who may remain in CR. The aim of the study was to determine the impact of MRD on clinical outcome.

Using MFC with five-colour, the level of MRD was evaluated in the BM in morphologic CR obtained after induction and post consolidation treatments from 25 and 22 patients respectively for AML patients who displayed LAPs at diagnosis. The threshold discriminating MRD^- from MRD^+ cases was set at 0.15% residual leukaemic cells, a level that allowed the identification of distinct subgroups of patients, both at post induction ($p = 0.05$) and post consolidation ($p = 0.009$) time points using receiver operating characteristics (ROC) analysis with optimal sensitivity and specificity. MRD level post induction not only influenced RFS ($p = 0.004$) but also OS ($p = 0.003$). Multivariate analysis showed that MRD level post induction was the most powerful independent prognostic factor for both RFS and OS ($p = 0.037$, 0.026) followed by MRD post consolidation ($p = 0.06$) for RFS. In conclusion, the threshold of 0.15% is valid in discriminating risk categories in adult AML and post induction MRD assessment is critical to predict disease outcome.

5.2 INTRODUCTION

The management of AML is a therapeutic challenge due to resistant or relapsing disease in a significant number of patients despite intensive chemotherapeutic induction and post remission approaches as well as autologous and allogeneic stem cell transplantation (Appelbaum 2002; Burnett 2002; Buchner *et al.* 2003). LAPs has been used previously for MRD detection. However, it has not been widely applied and few studies have been reported so far (San Miguel *et al.* 1997; Venditti *et al.* 2000; San Miguel *et al.* 2001; Feller *et al.* 2004). This may be related to the technical difficulties associated with the immunologic characterisation of myeloid leukaemia given that large panels of MoAbs are needed to cover all myeloid lineages and that several blast cell subpopulations may coexist at diagnosis. Altogether, these technical problems complicate the identification of phenotypic aberrancies in AML to be used as leukaemia-associated markers for MRD detection. Thus, there is a general agreement that more reliable tools are needed to distinguish patients at higher risk of relapse from those who are not; and then may be possible to modify post induction and consolidation chemotherapy according to the risk of relapse based on MRD level.

Initially, studies in immunophenotypical detection of MRD have been based on double antigen staining analysed by fluorescence microscopy (Campana *et al.* 1990; Adriaansen *et al.* 1993). However, more recent data show that MFC allows a more precise detection of phenotypic aberrancies (Campana 2003) as we described in chapter 4. Moreover, previous MRD studies have been based on the phenotypic characteristics of the predominant blast population, ignoring the existence of two or more phenotypically different blast cell subsets at diagnosis in a high proportion of AML cases and not taking into account that relapse could be due to a minor blast

cell subset that in turn could be highly resistant to chemotherapy (Campana *et al.* 1990; Drach *et al.* 1992; Reading *et al.* 1993).

Accordingly, in the present study, we used five-colour MFC for a more sensitive and accurate quantification of MRD in AML. Five-colour MFC was used to investigate the level of MRD after induction and consolidation chemotherapy in AML patients in first CR using a CD45 gating strategy and extensive panel of MoAbs. This method was able to detect LAPs in 94% of the patients and was therefore applicable for MRD monitoring to virtually all patients. Our aim was to determine if patient specific LAPs could be used for MRD analysis. The secondary aim was to determine the optimal threshold value, in terms of residual leukaemic cells and relapse status that can split patients into 2 groups, and finally to establish the time point of choice, that is, post induction or post consolidation, able to better predict outcome. To our knowledge, this is the first MRD study using five-colour MFC.

5.3 RESULTS

5.3.1 Clinical Characteristics

Fifty-four AML BM samples were analysed from consecutive, newly diagnosed patients using a comprehensive panel of MoAbs with five-colour staining for expression of LAPs. These LAPs were identified in (51) 94% patients as demonstrated in chapter 4. Thirty one (57%) received chemotherapy, of these 27(87%) achieved CR, two of them were LAP negative and therefore 25 were followed-up for MRD monitoring post induction therapy. One patient died after induction and 2 did not receive consolidation chemotherapy, and hence 22 patients were evaluable for MRD post consolidation therapy (I, II, III, IV, V) as summarised in Figure 5-1.

Furthermore, 72 follow-up BM samples in 25 patients were analysed at different time points during therapy, applying only the LAP specific combination defined at diagnosis. In cases where more than one LAP was present in the same patient, the most sensitive LAP was chosen based on the maximum LD explained in detail in chapter 4. The patients characteristics are shown in Table 5-1.

For samples at diagnosis, 20,000 events were acquired, whereas for follow-up samples and normal as well as regenerating BM samples, at least 500,000 events were acquired. For the follow-up samples, only the combinations for the respective LAPs from the time of the first diagnosis were applied. The median follow-up duration for patients was 18.3 months (range, 1.4-51.1).

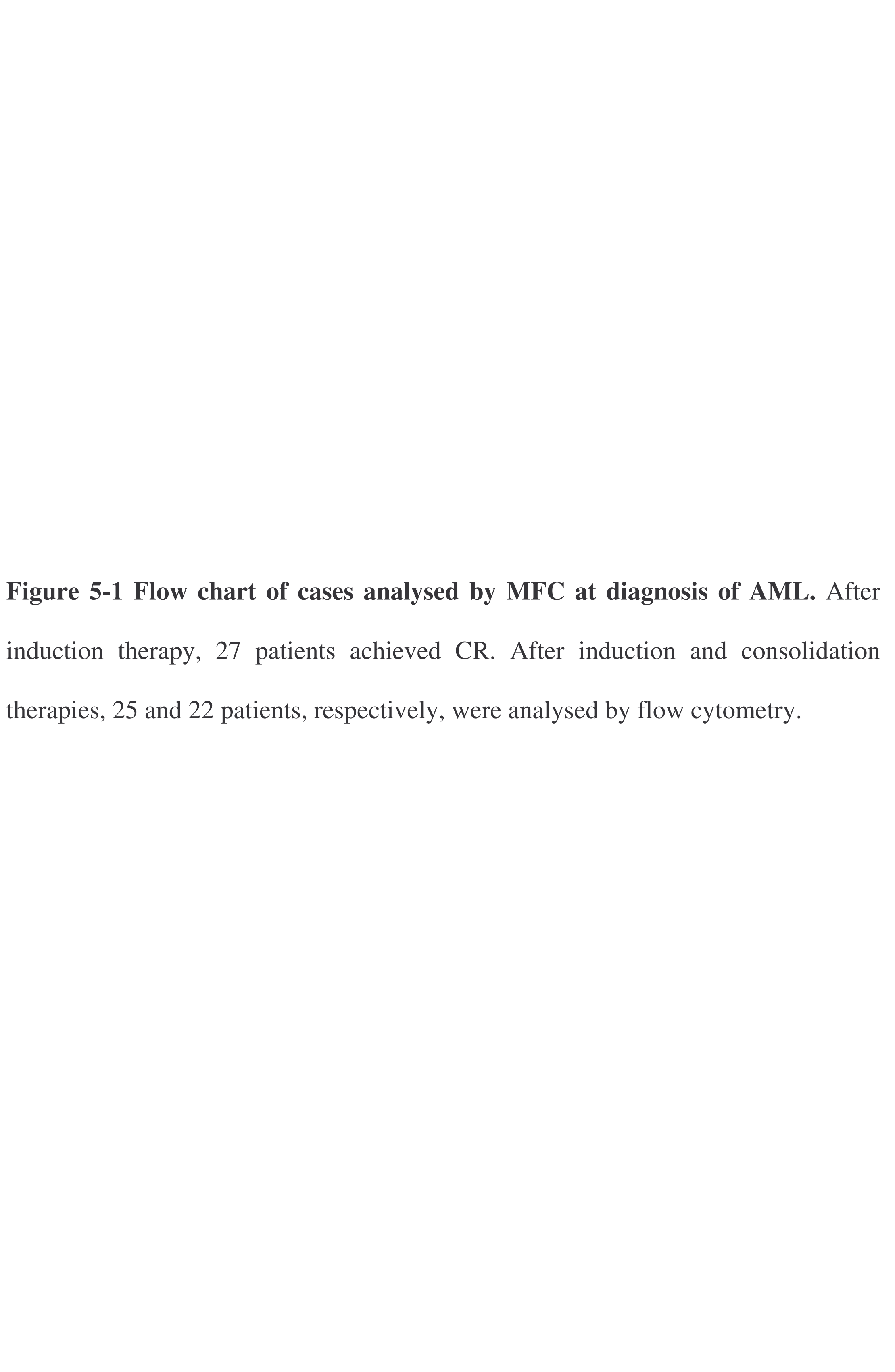


Figure 5-1 Flow chart of cases analysed by MFC at diagnosis of AML. After induction therapy, 27 patients achieved CR. After induction and consolidation therapies, 25 and 22 patients, respectively, were analysed by flow cytometry.

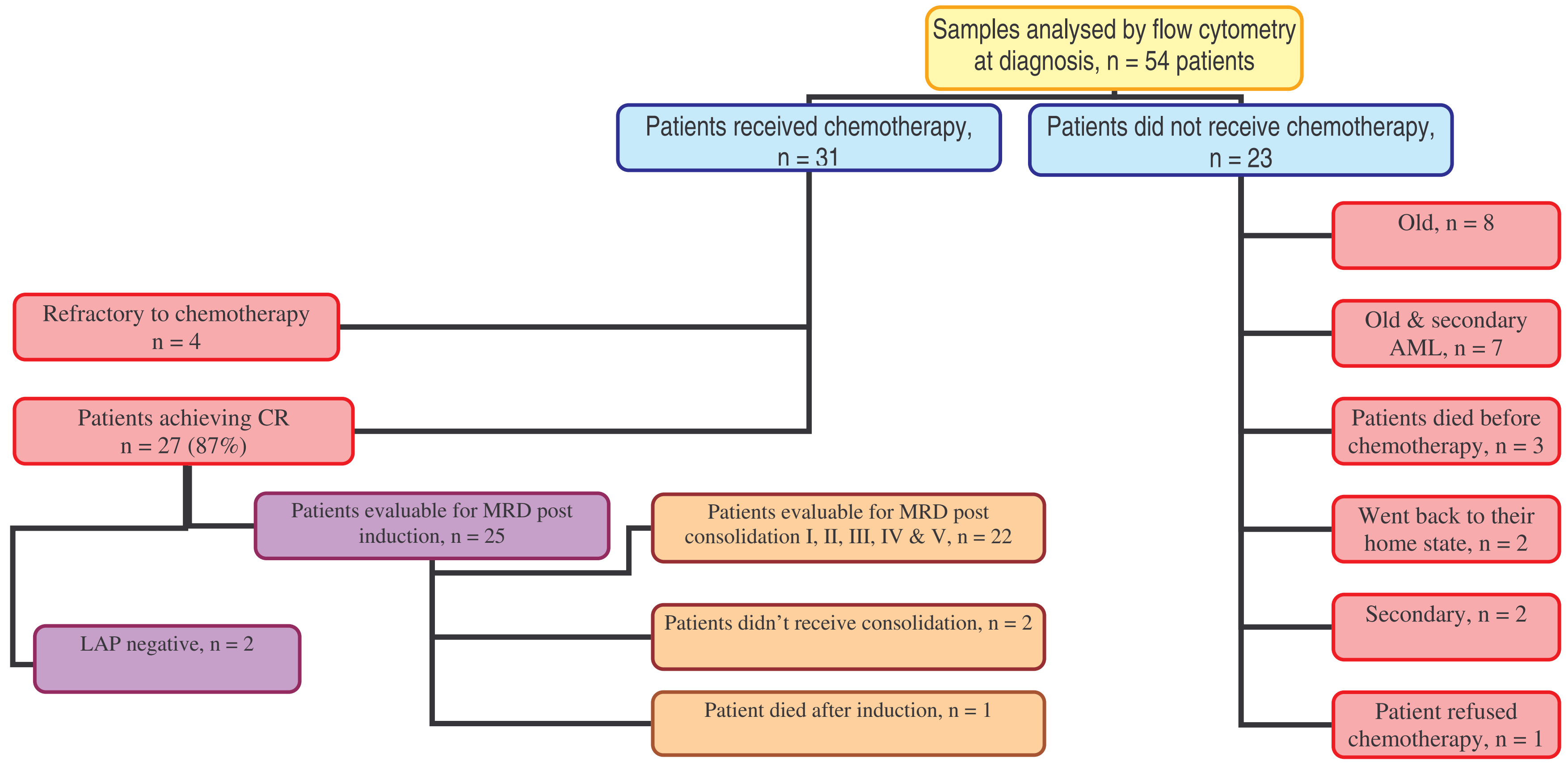

Samples analysed by flow cytometry at diagnosis, n = 54 patients
Patients received chemotherapy, n = 31
Patients did not receive chemotherapy, n = 23
Old, n = 8
Old & secondary AML, n = 7
Patients died before chemotherapy, n = 3
Went back to their home state, n = 2
Secondary, n = 2
Patient refused chemotherapy, n = 1
Refractory to chemotherapy n = 4
Patients achieving CR n = 27 (87%)
Patients evaluable for MRD post induction, n = 25
Patients evaluable for MRD post consolidation I, II, III, IV & V, n = 22
Patients didn't receive consolidation, n = 2
Patient died after induction, n = 1
LAP negative, n = 2

Table 5-1 Patient clinical characteristics

Patient characteristics	**Total (%)**
No. patients	25
Male/ female	7/18
Age at diagnosis, mean, (range)	48 (20-75)
WBC count at diagnosis × 10^9 /L, median (range)	5.1 (0.86-179)
BM blasts % by morphology, median (range)	50 (6.10-85)
AML de novo/secondary n (%)	42 (78)/12 (22)
FAB classification, n (%)	
M1	8 (32)
M2	7 (28)
M3	2 (8)
M4	3 (12)
M5	4 (16)
M6	1 (4)
Cytogenetic risk group	
Favourable	7 (28)
Intermediate	12 (48)
Poor	6 (24)
Induction	25
Consolidation I	21
Consolidation II	13
Consolidation III	4
Consolidation IV	2
Consolidation V	2
AuSCT/AlloSCT	3 (12)/6 (24)

5.3.2 Establishing the optimal threshold that can split patients into 2 groups with residual leukaemic cells in terms of relapse status

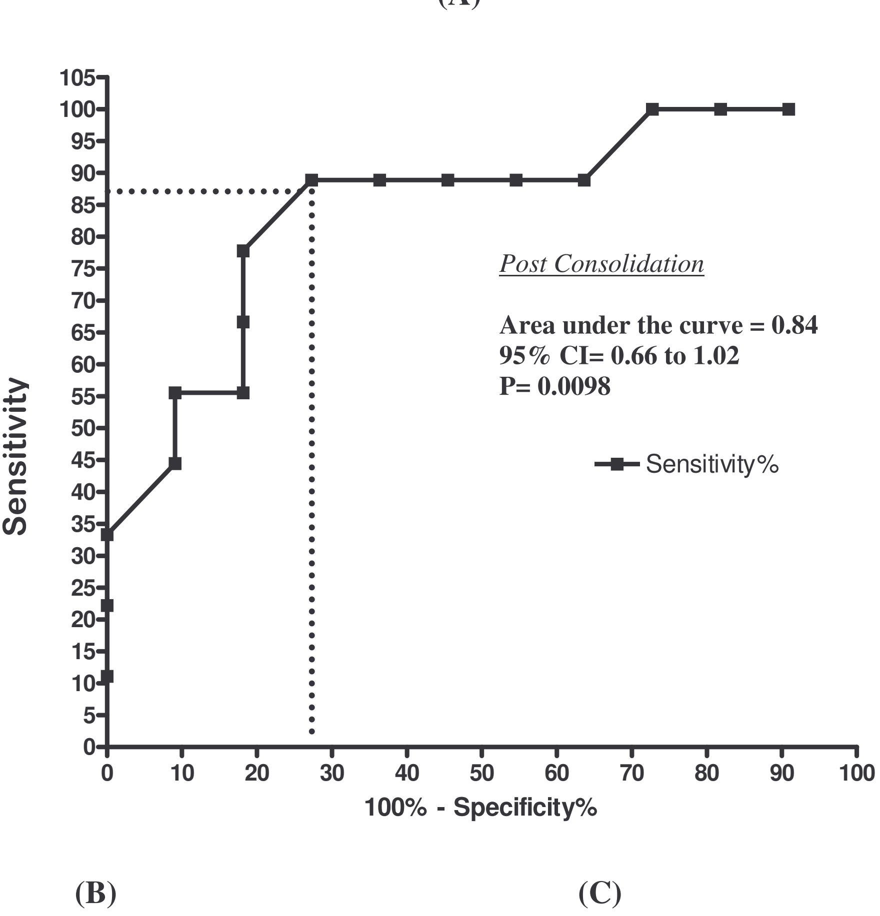
ROC analysis was carried out using Prism™ software (GraphPad software Inc, San Diego, CA, USA) to determine the optimal threshold yielding the best separation of AML patients into two groups for MRD and relapse status with optimal sensitivity and specificity.

Choosing the cutoff value closer to an area of (0, 1) from the ROC curve revealed that the optimal threshold is 0.145 for post consolidation and 0.155 for post induction (Figure 5-2). These threshold values represent the optimal sensitivity (89%) and specificity (73%) with a likelihood ratio of 3.26 for post consolidation and a sensitivity of 66% and specificity of 38% with a likelihood ratio of 1.63 for post induction. The likelihood ratio of 1.63 represents the highest ratio among the other cutoff points.

Therefore, we decided to utilise the MRD level of 0.15% residual leukaemic cells as a threshold value to discriminate MRD^- from MRD^+ cases both after induction and consolidation (Figure 5-3). Consequently, patients with a residual leukaemic cells ≤0.15% were categorised as MRD^-, whereas those with residual leukaemic cells >0.15% were grouped as MRD^+.

Figure 5-2 ROC analysis from 11 relapsed patients and 9 who had no relapse (Post consolidation) and 13 relapsed versus 9 who had no relapse (post induction). **A**: a ROC curve of sensitivity (Y-axis) versus its false positive specificity (X-axis) obtained at each cut-off level. The discrete points on the empirical ROC curve and its 95% confidence interval is 0.66 to 1.02 (Post Consolidation) and 0.33 to 0.82 (Post Induction). **B**: ROC curve table representing 100%- specificity vs. sensitivity %, **C**: the sensitivity and specificity table of the test at various cut-offs values between MRD levels for patients who did not relapse (as a normal) and MRD levels for patients who underwent relapse (abnormal). As indicated from both ROC curves and tables that the optimal cut-off point for both optimal sensitivity and specificity is MRD level of ~ 0.15% with a likelihood ratio of 3.26 (post consolidation) and 1.63 (post induction).

(A)



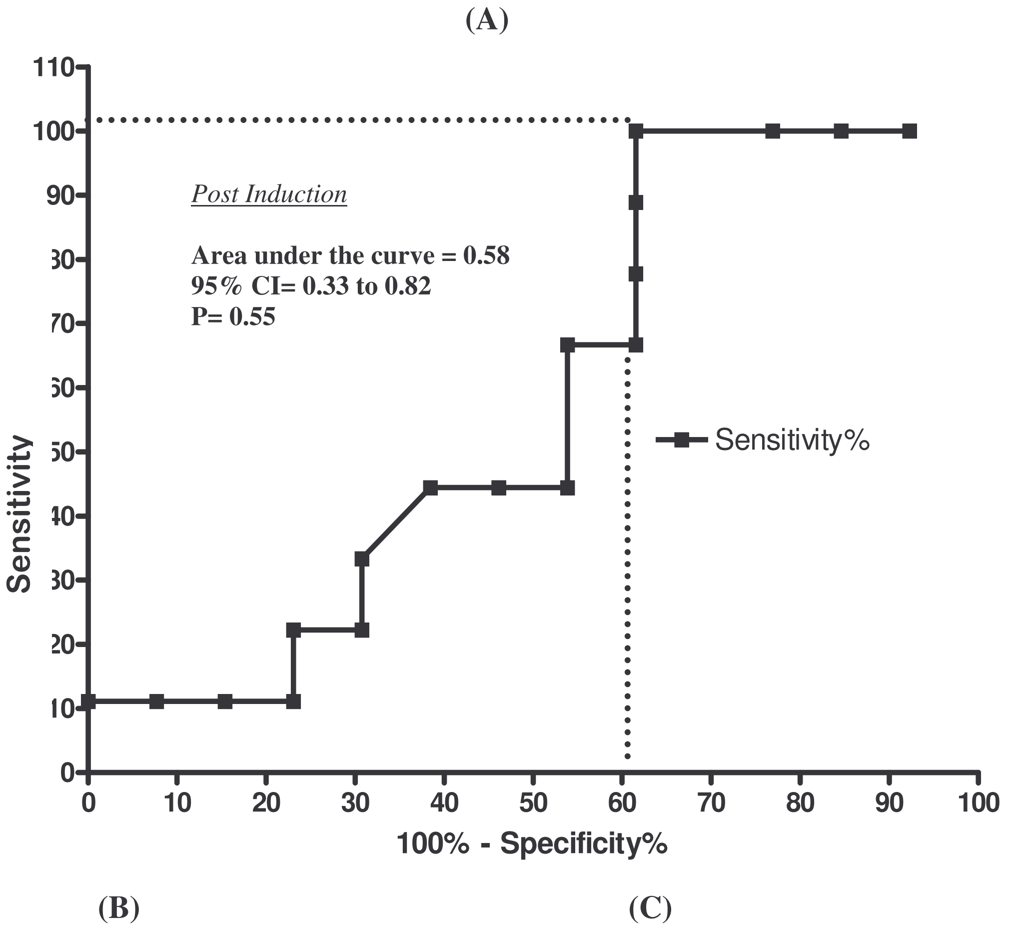
(B)

100% - Specificity%	Sensitivity%
0.000	11.111
0.000	22.222
0.000	33.333
9.090	44.444
9.090	55.555
18.181	55.555
18.181	66.666
18.181	77.777
27.272	**88.888**
36.363	88.888
45.454	88.888
54.545	88.888
63.636	88.888
72.727	100.000
81.818	100.000
90.909	100.000

(C)

Cutoff	Sensitivity%	Specificity%	Likelihood ratio
< 0.0005	11.110	100.000	
< 0.0055	22.220	100.000	
< 0.0350	33.330	100.000	
< 0.0650	44.440	90.910	4.89
< 0.0750	55.560	90.910	6.11
< 0.0850	55.560	81.820	3.06
< 0.0950	66.670	81.820	3.67
< 0.1150	77.780	81.820	4.28
< 0.1450	**88.890**	**72.730**	**3.26**
< 0.2600	88.890	63.640	2.44
< 0.3750	88.890	54.550	1.96
< 0.4000	88.890	45.450	1.63
< 0.4200	88.890	36.360	1.40
< 0.4550	100.000	27.270	1.38
< 0.5000	100.000	18.180	1.22
< 0.5400	100.000	9.091	1.10

(A)



(B)

100% - Specificity%	Sensitivity%
0.000	11.111
7.692	11.111
15.384	11.111
23.076	11.111
23.076	22.222
30.769	22.222
30.769	33.333
38.461	44.444
46.153	44.444
53.846	44.444
53.846	66.666
61.538	66.666
61.538	77.777
61.538	88.888
61.538	**100.000**
76.923	100.000
84.615	100.000
92.307	100.000

(C)

Cutoff	Sensitivity%	Specificity%	Likelihood ratio
< 0.0030	11.110	100.000	
< 0.0060	11.110	92.310	1.44
< 0.009500	11.110	84.620	0.72
< 0.0125	11.110	76.920	0.48
< 0.0175	22.220	76.920	0.96
< 0.0245	22.220	69.230	0.72
< 0.0285	33.330	69.230	1.08
< 0.0405	44.440	61.540	1.16
< 0.0540	44.440	53.850	0.96
< 0.0635	44.440	46.150	0.83
< 0.0715	66.670	46.150	1.24
< 0.09650	66.670	38.460	1.08
< 0.1250	77.780	38.460	1.26
< 0.1400	88.890	38.460	1.44
< 0.1550	**100.000**	**38.460**	**1.63**
< 0.2400	100.000	23.080	1.30
< 0.3700	100.000	15.380	1.18
< 0.6000	100.000	7.692	1.08

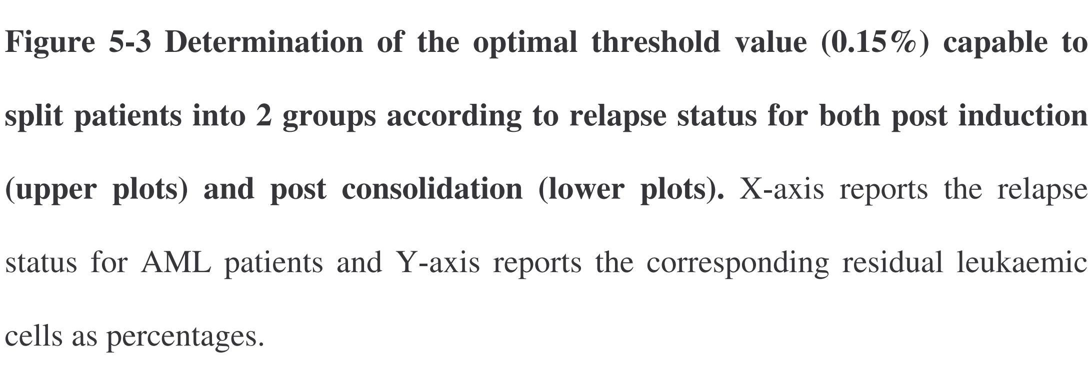
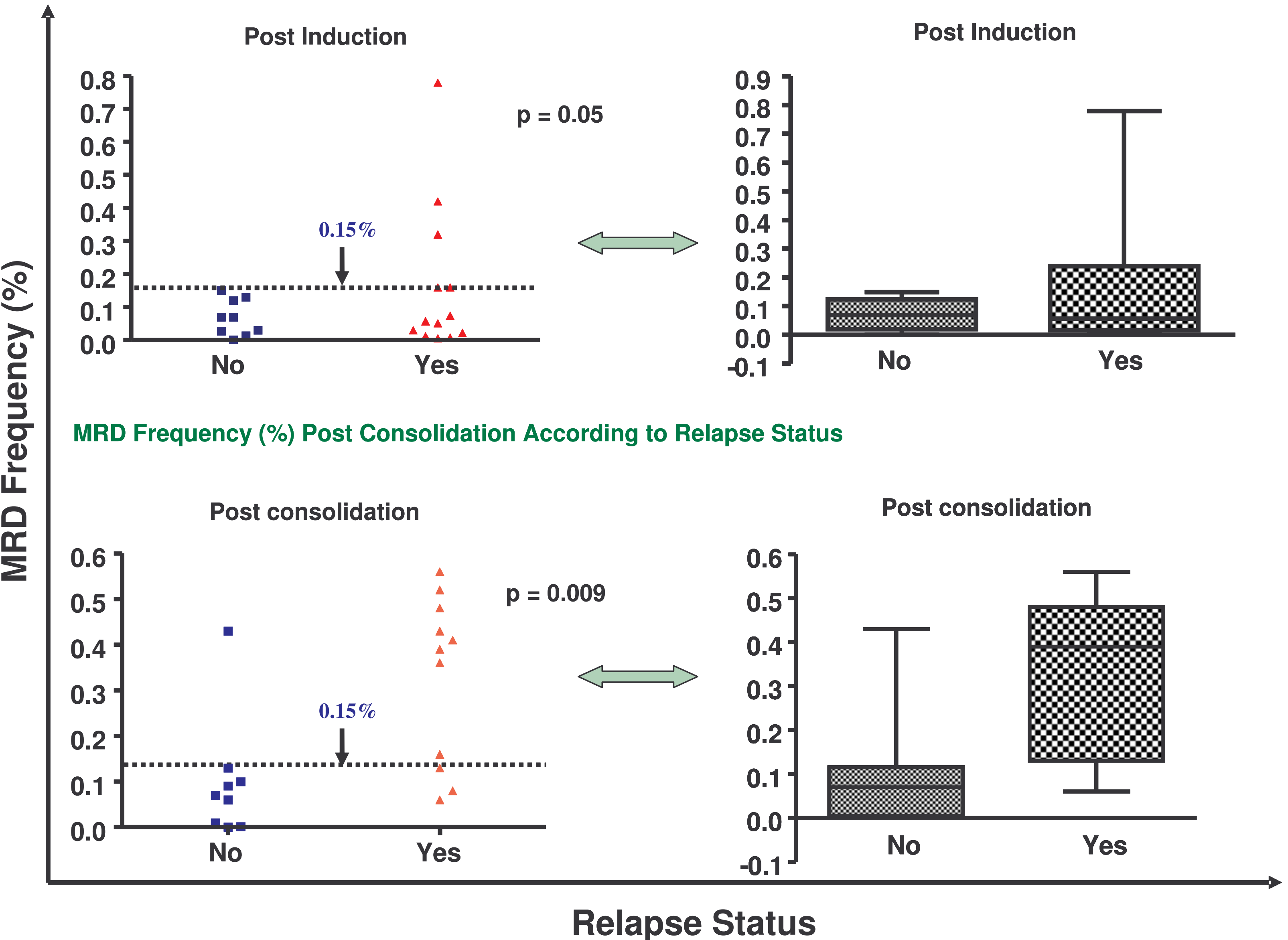


Figure 5-3 Determination of the optimal threshold value (0.15%) capable to split patients into 2 groups according to relapse status for both post induction (upper plots) and post consolidation (lower plots). X-axis reports the relapse status for AML patients and Y-axis reports the corresponding residual leukaemic cells as percentages.



MRD Frequency (%) Post Iinduction According to Relapse Status
Post Induction
p = 0.05
0.15%
No
Yes
Post Induction
No
Yes
MRD Frequency (%) Post Consolidation According to Relapse Status
Post consolidation
p = 0.009
0.15%
No
Yes
Post consolidation
No
Yes
MRD Frequency (%)
Relapse Status

5.3.3 Detection of MRD in bone marrow post induction

After induction, the median level of the residual leukaemic cells was 0.07% (range 0.001-0.78%). At this time point, 72% of the patients (18/25) were MRD^- and 28% (7/25) were MRD^+ as shown in Figure 5-4. Five of seven (71%) patients in the MRD^+ group had disease relapse at a median time of 12.9 months (range 1.4-18.2) and 2 died post induction chemotherapy, whereas in the MRD^- group 8/18 (44%) relapsed at a median time of 24.7 months (range 9.5-51.1) ($p = 0.048$), 9/18 (50%) patients remained in CR at a median time of 18.5 months (range 1.8-25.1) and 1 patient died after induction chemotherapy. The probability of RFS and OS was illustrated in (Figure 5-6) for patients in the MRD^- group, compared to those in the MRD^+ group ($p = 0.004$ and 0.003, respectively). Figure 5-5 demonstrates a representative example for MRD detection in consecutive BM samples of a relapsing patient and of a patient still in remission.

Figure 5-4 Flow chart of cases analysed by flow cytometry after induction

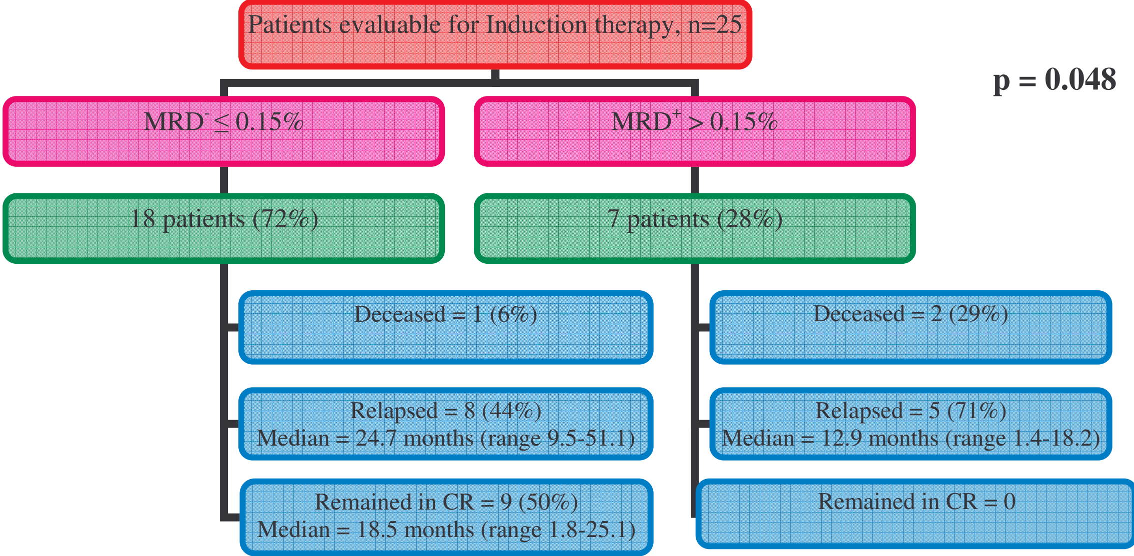


Figure 5-5 MRD detection in consecutive BM samples of a relapsing patient and of a patient still in remission. Cells from AML at diagnosis and follow-up BM samples are stained with a combination of MoAbs, which identifies a LAP. (**A**) The gating strategy starts by defining the WBC compartment characterized by CD45 expression and SS Log. On these selected cells, the cell population with a primitive marker expression, in this case CD117, and low SSC is subsequently gated for detection of the cells with aberrant phenotypes which is the example shown in **C**, and on (**B**) these selected cells, the cell population with a primitive marker expression, in this case CD34, and low SSC is subsequently gated for detection of the cells with aberrant phenotypes which is the example shown in **D**. (**C**) Example of a patient who relapsed within 6 months after achieving CR. The aberrant phenotype was $CD117^{+}$ $CD15^{+}$ expression on the $CD34^{+}$ $CD117^{+}$ cells. The MRD% was 0.78, 0.42, and 0.64 after Induction, consolidation I and consolidation II chemotherapy, respectively. Dot plot at the extreme right of the figure shows relapsed material with LAP expression similar to at diagnosis material. (**D**) Example of a patient who is still in CR after 24 months. The LAP includes $CD34^{+}$ $CD7^{+}$ expression. The MRD% was 0.12, 0.15, 0.13 and 0.09 after induction, consolidation I and consolidation II chemotherapy, respectively.

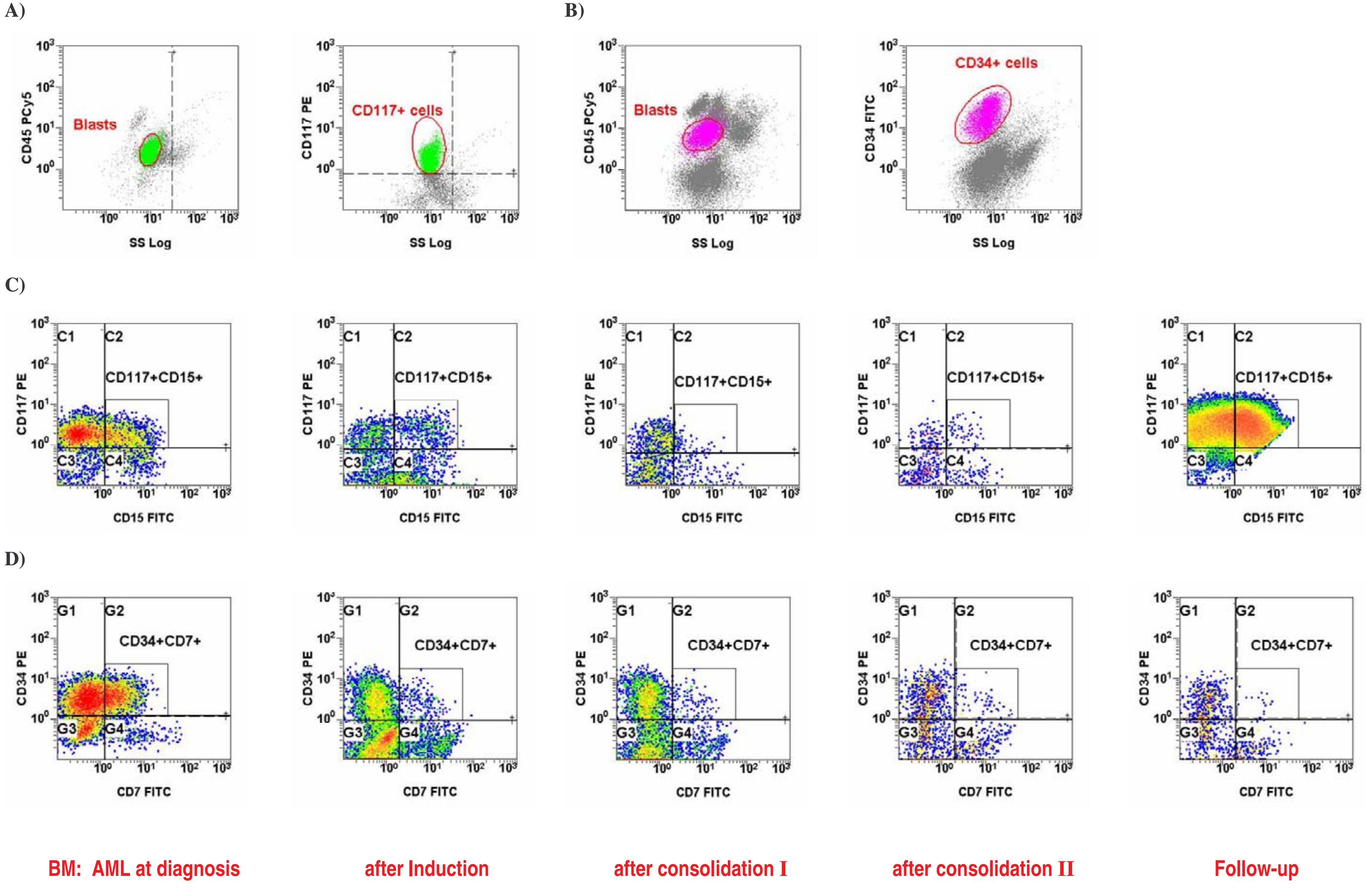
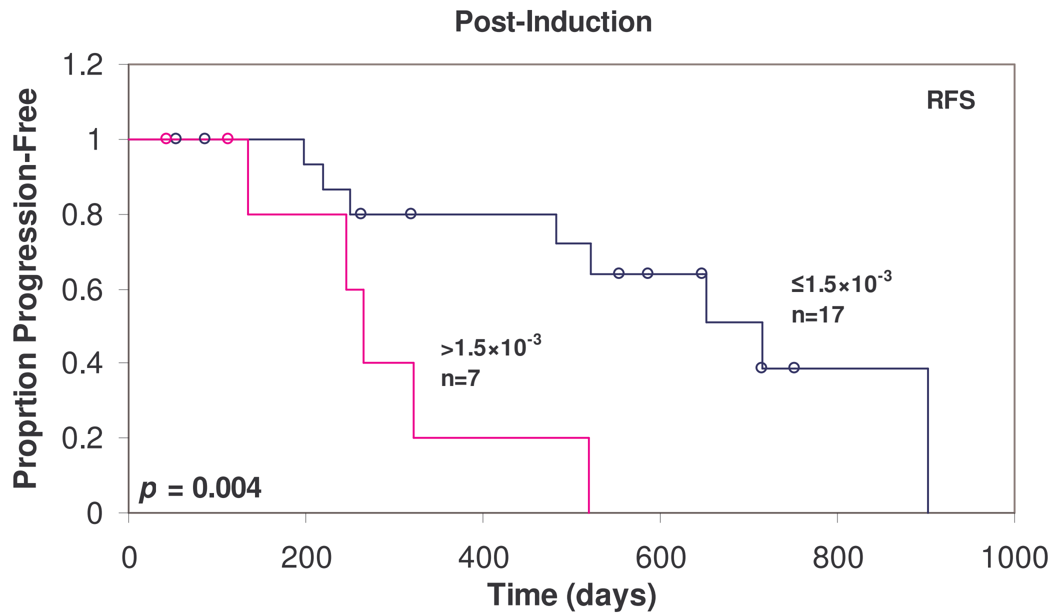
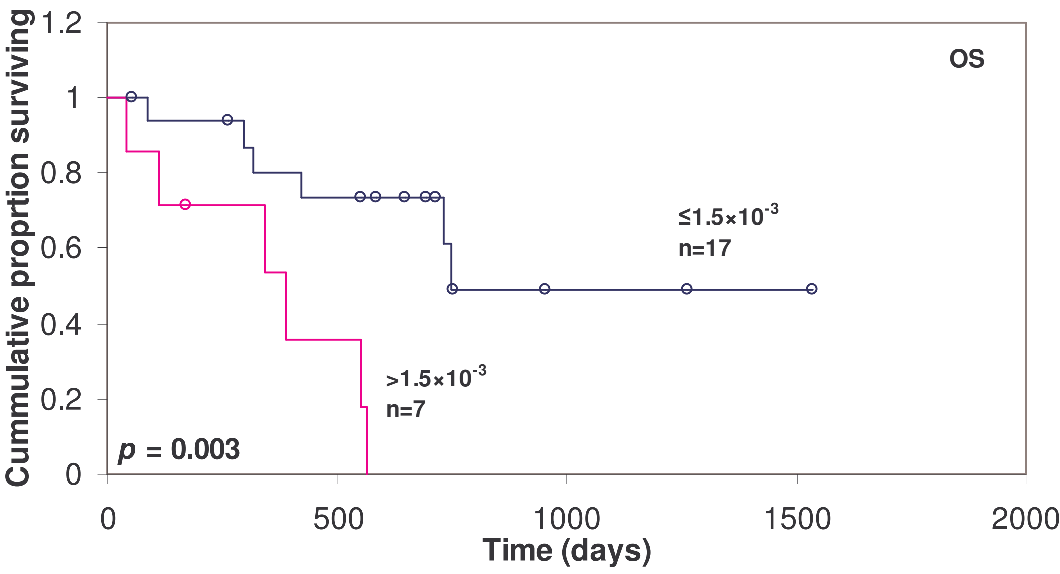

A)
B)
C)
D)
Blasts
CD117+ cells
CD34+ cells
CD45 PCy5
CD117 PE
CD34 FITC
CD34 PE
SS Log
CD15 FITC
CD7 FITC
C1
C2
C3
C4
CD117+CD15+
G1
G2
G3
G4
CD34+CD7+
BM: AML at diagnosis
after Induction
after consolidation I
after consolidation II
Follow-up

Figure 5-6 RFS and OS of AML patients according to MRD levels after induction chemotherapy. Patients were grouped according MRD threshold value of 1.5×10^{-3} residual leukaemic cells, as determined at post-induction and evaluated for RFS (**upper plot**) and OS (**lower plot**).





5.3.4 Detection of MRD in bone marrow post consolidation

The MRD value for each patient after consolidation was taken for consolidation I, however, whenever the patient received more than one consolidation therapy, the MRD value for the final consolidation was taken in the analysis for RFS and OS due to the small sample size available to analyse them separately.

One patient died after induction therapy (was MRD^+), 2 patients did not receive consolidation and 22 proceeded to receive consolidation; of these, 2 patients died after consolidation (were MRD^-) and 20 were evaluable for consecutive MRD evaluation. At post consolidation analysis, the median level of residual leukaemic cells in the whole series was 0.115 (range 0.0-0.56). Thirteen (59%) of the patients were MRD^- and 9 (41%) were MRD^+. Three of 13 (23%) and 8 of 9 (89%) patients in MRD^- and in MRD^+ group underwent a relapse at a median time of 24.5 months (range 18.2-31.8) and 15.5 months (range 5.7-51.1), respectively ($p = 0.009$) (Figure 5-7). The probability of RFS and OS was depicted in (Figure 5-8) for patients in the MRD^- group, compared to those in the MRD^+ group ($p = 0.06$ and 0.98 respectively).

Of 25 patients evaluable for MRD in our series, three patients underwent allogeneic stem cell transplantation (AlloSCT) because of medical decision after achieving the first CR. Of these, 2 were MRD^+ post induction and became MRD^- post consolidation and the other one was already MRD^- post induction and remained MRD^- post consolidation.

Figure 5-7 Flow chart of cases analysed by flow cytometry after consolidation

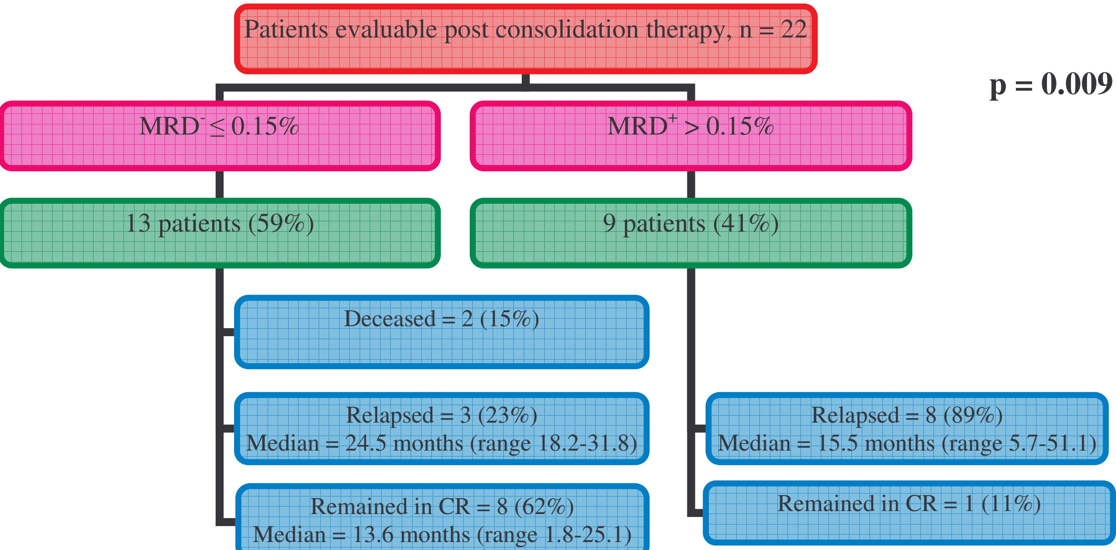
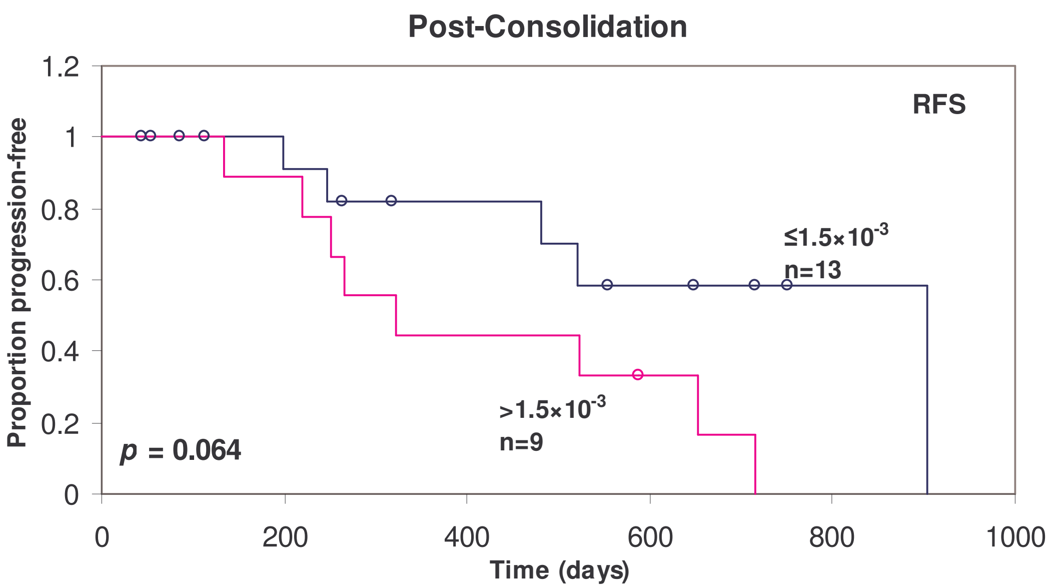
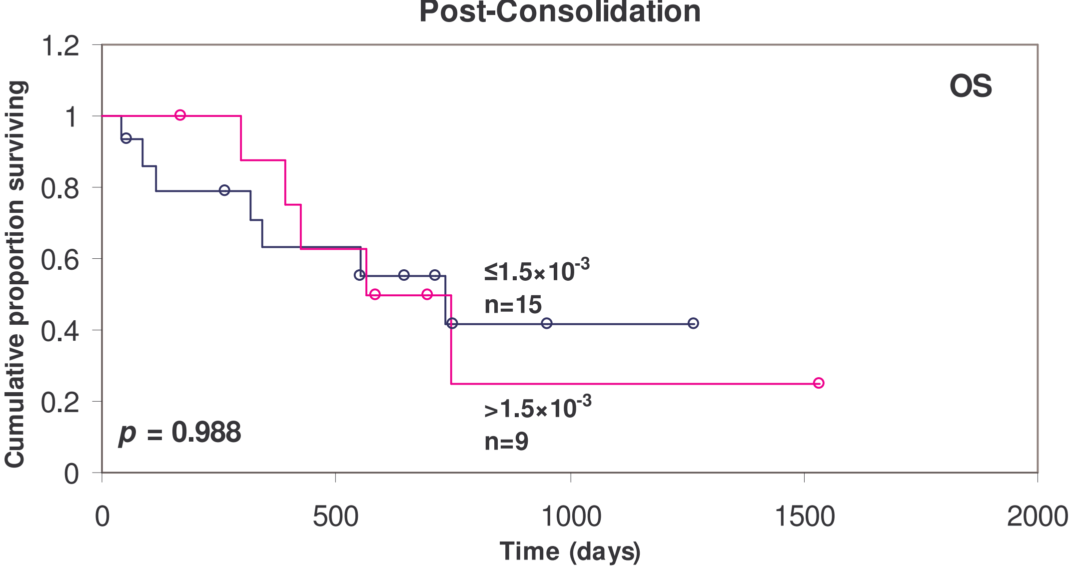


Figure 5-8 RFS and OS of AML patients according to MRD levels after consolidation chemotherapy. Patients were grouped according MRD threshold value of 1.5×10^{-3} residual leukaemic cells, as determined at post-consolidation and evaluated for RFS (**upper plot**) and OS (**lower plot**).





5.3.5 Relation between level of MRD post induction and consolidation chemotherapy

Figure 5-9 shows the dynamics of MRD fluctuations after induction and consolidation therapy in the 22 patients completing both treatment phases. Based on the comparative analysis of MRD levels detected at the two time points, we separated the series into four groups: (1) $Ind^{+}Cons^{+}$ [3 patients], MRD^{+} both after induction and consolidation; (2) $Ind^{+}Cons^{-}$ [2 patients], MRD^{+} after induction converted into MRD^{-} after consolidation; (3) $Ind^{-}Cons^{+}$ [6 patients], MRD^{-} after induction converted into MRD^{+} at the end of consolidation; and (4) $Ind^{-}Cons^{-}$ [11 patients] who were MRD^{-} at both time points. The analysis of RFS and OS rates showed that the MRD^{-} status at the end of induction was the most significant predictor of outcome, regardless of the levels of MRD after consolidation. Comparison of the four groups was significant for RFS ($p = 0.025$). In addition, patients with an $Ind^{+}Cons^{-}$ status had different probability of RFS ($p = 0.408$) and border line significance for OS ($p = 0.054$) than those never exceeding the threshold of 0.15% residual leukaemic cells ($Ind^{-}Cons^{-}$) [Figure 5-10]. These results suggest a good treatment outcome in patients with an early response in terms of MRD clearance analogous to the findings in ALL.

Figure 5-9 Fluctuations of the MRD levels after induction and consolidation cycles. Twenty-two patients were evaluable in terms of comparison between induction and consolidation therapy; Thirteen (59%) were MRD$^-$ at the end of consolidation; 11 of them were already negative after induction and 2 became MRD$^-$ only after consolidation. On the contrary, 9 patients were MRD$^+$ at the end of consolidation; six of them, MRD$^-$ after induction, progressed into an MRD$^+$ status in spite of administration of the consolidation cycle. The remaining 3 patients were in a MRD$^+$ status throughout the induction and consolidation cycles.

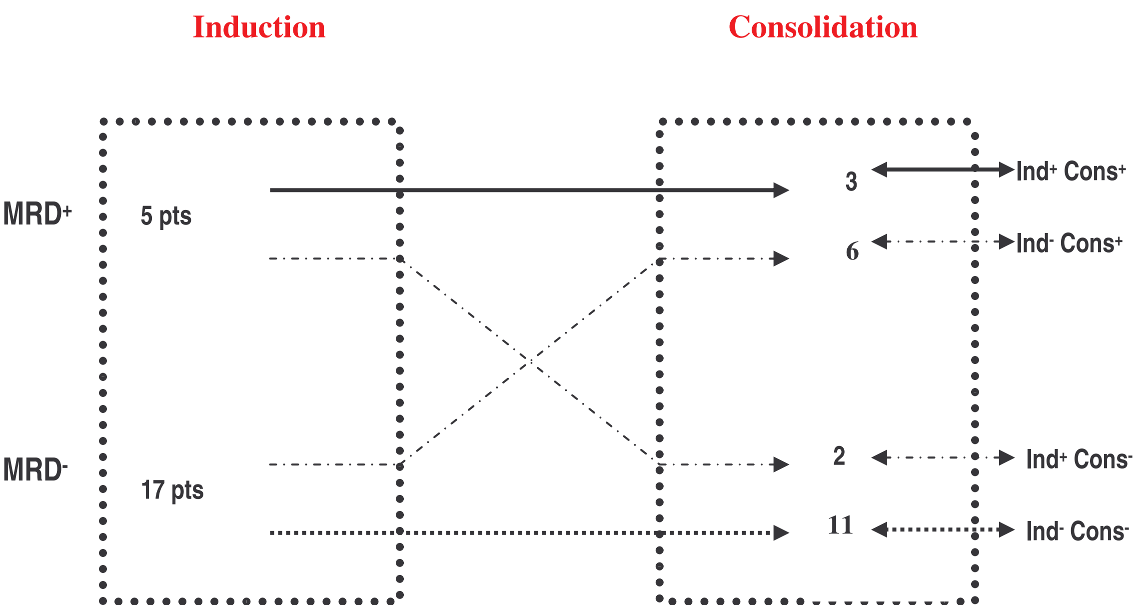
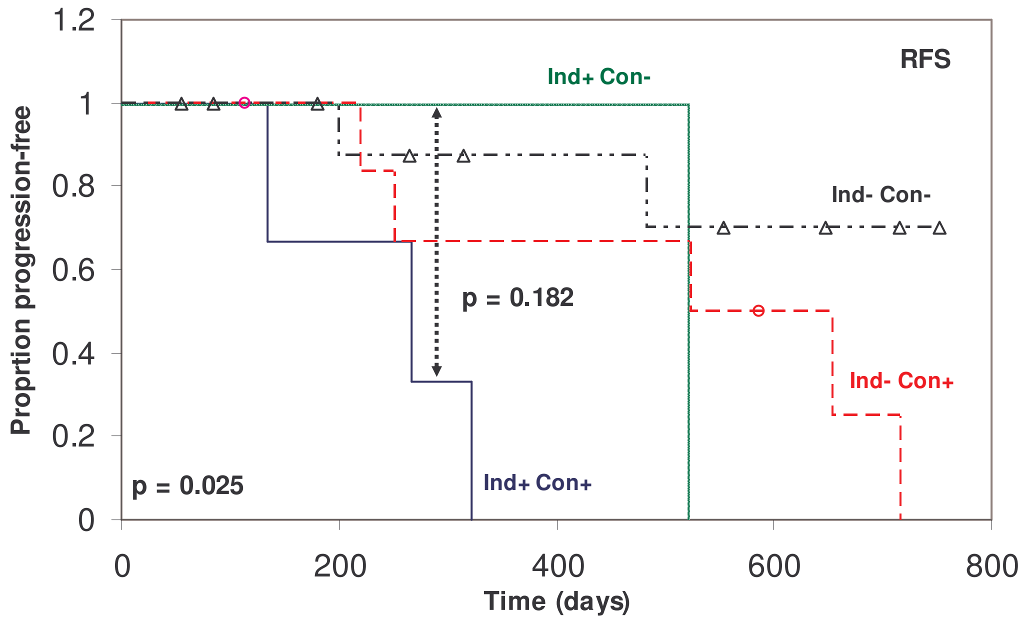
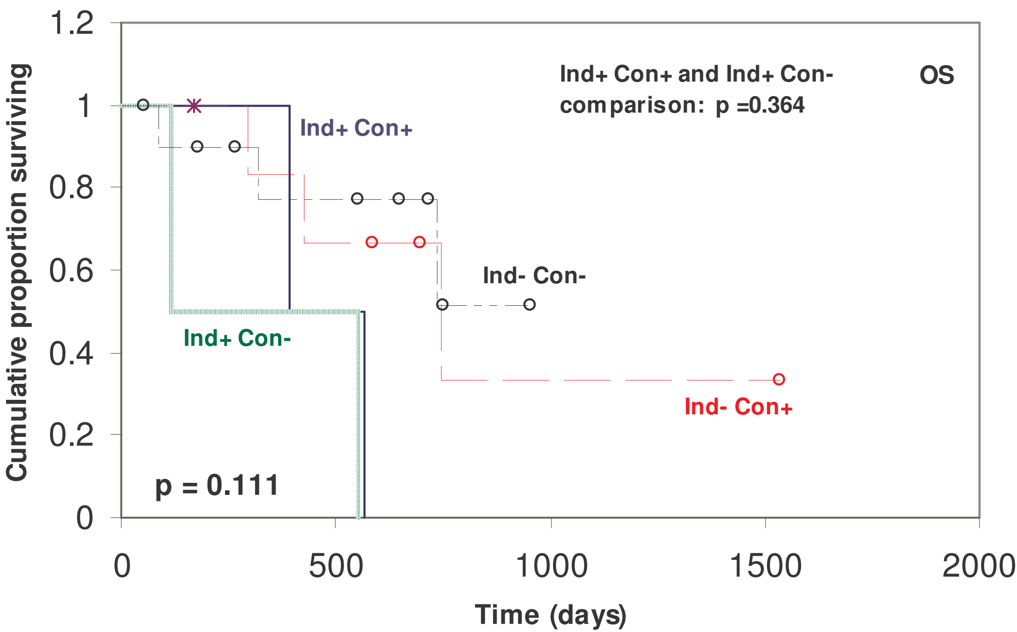


Figure 5-10 RFS and OS of AML patients according to the comparison of the MRD status after induction and consolidation cycle. RFS (**upper panel**) and OS (**lower panel**) of 22 patients evaluable after consolidation is illustrated. Four different categories have been identified based on the combination of the MRD status after induction and consolidation time point: (1) Ind^+Con^+ (3 patients), MRD^+ both after induction and consolidation; (2) Ind^+Con^- (2 patients), MRD^+ after induction converted into MRD^- after consolidation; (3) Ind^-Cons^+ (6 patients), MRD^- after induction converted into MRD^+ at the end of consolidation; and (4) Ind^-Cons^- (11 patients) who were MRD^- at both time points. For each panel, p values obtained by comparing the four Kaplan-Meier curves (log-rank test) are reported at the bottom; double headed arrow indicates Ind^+Con^- and Ind^+Con^+ Kaplan-Meier curves with the p value obtained by comparing these latter curves alone are also reported.


1.2
1
0.8
0.6
0.4
0.2
0
Proprtion progression-free
RFS
Ind+ Con-
Ind- Con-
p = 0.182
Ind- Con+
p = 0.025
Ind+ Con+
0
200
400
600
800
Time (days)


1.2
1
0.8
0.6
0.4
0.2
0
Cumulative proportion surviving
Ind+ Con+ and Ind+ Con-
comparison: p =0.364
OS
Ind+ Con+
Ind- Con-
Ind+ Con-
Ind- Con+
p = 0.111
0
500
1000
1500
2000
Time (days)

5.3.6 Prognostic impact of conventional parameters in univariate analysis

The prognostic impact of poor cytogenetics, gender, FAB subtypes (M0-M2) vs. (M4-M5) and transplant as dichotomous variables as well as age, WBC count at diagnosis, percentage of BM blasts at diagnosis as continuous variables was analysed using RFS and OS as dependent variables.

For both analyses, after induction and consolidation therapy, none of the variables were significant as shown in Table 5-2. However, FAB subtype group (M0-M2) in RFS and OS, and WBC count at diagnosis were close to significance ($p < 0.25$) and thus were pooled in multivariate model.

5.3.7 Prognostic determinants in multivariate analysis

Finally, all the relevant prognostic variables with a statistical significance >0.25 in univariate analysis (Hosmer and Lemeshow 2000) being WBC count, FAB subtype and MRD status after induction and consolidation were pooled into a multivariate model to determine to what extent they affected independently the outcome of treatment. In this analysis, post-ind MRD^+ status was found to be an independent variable significantly associated with a higher frequency of relapse ($p <0.05$) and a shorter duration of OS ($p = 0.026$) and RFS ($p = 0.037$) with an estimated hazard ratio of 4.7 (95% CI, 1.1-20.5) for RFS and 5.2 (95% CI, 1.2- 22.2) for OS . The prognostic impact of post-induction MRD status remained significant even after adjustment for WBC, MRD post-consolidation and FAB subtype (group M0-M2 and M4-M5) ($p <0.05$).

Thus after induction therapy, the MRD frequency correlated with the clinical outcome, as measured in the 25 evaluable patients. In the total group again MRD frequency inversely correlated both with RFS and OS ($p < 0.05$) (Table 5-3).

Table 5-2 Correlation between clinical variables and MRD status defined by the threshold value of 0.15%.

Variables	RFS (p-value)	OS (p-value)
Cytogenetics adverse risk group	0.3	0.7
Age	0.3	0.5
% blast at diagnosis	0.4	0.8
FAB classification (M0-M2) vs. (M4-M5)	0.09	0.06
MRD post Induction	0.004	0.0026
MRD post Consolidation	0.06	0.98
WBC count at diagnosis	0.07	0.4
Gender	0.5	0.17
Transplant	0.6	0.9

Table 5-3 Relative risk of a relapse defined by MRD frequency in BM. In multivariate analysis, Post-Induction MRD status was independently associated with shorter duration of OS and RFS.

	Threshold value (%)	n	% of patients with MRD > threshold value	Univariate analysis for RFS , OS (p- values)	Relative risk of relapse (95% CI)	Multivariate analysis for RFS (p-value)	Relative risk of death (95% CI)	Multivariate analysis for OS (p-value)
BM Post Induction	0.15	25	28%	(0.0041 , 0.0026)	4.7 (1.10-20.48)	0.037	5.2 (1.22-22.16)	0.029
BM Post Consolidation	0.15	22	41%	(0.06 , 0.98)	3.1 (0.80- 12.18)	0.10	1.6 (0.33-7.67)	0.57

5.3.8 Prognostic impact of log difference in RFS

To account for the baseline of LAPs expression in normal and regenerating BMs when compared to the expression of LAPs in AML patients, LD as a continuous variable was correlated to RFS by running another model without MRD post induction and consolidation to see wether or not by its own can predict relapse taking into account the other covariables in multivariable analysis. We found that LD does not predict RFS ($p > 0.05$) in univariate and multivariate analyses.

5.3.9 Prognostic impact of log difference in OS

LD was also correlated to OS without including MRD post induction and consolidation into the model, LD in a univariate analysis correlated with shorter OS ($p = 0.03$). In a multivariate setting including age, WBC count at diagnosis, gender, blast percentage at diagnosis , LD and gender stood out as statistically significant parameters for OS (p values = 0.0029, 0.011) respectively with an estimated hazard ratio of 9.7 (95% CI, 2.2-43.5) for LD and 13.0 (95% CI, 1.8-95.2) for gender. Although, the 95% CI is very wide, the data still indicate that higher risk of death is associated with patients greater than 3 LD (in LAP expression as described in chapter 4) in addition to males. When the model was only run with these 2 variables, LD and gender were again significantly associated with OS with an estimated relative risk to death [6.2 (95% CI, 1.6-24.2), $p = 0.008$] and [7.7 (95% CI, 1.5-40.5), $p = 0.015$] respectively.

Figure 5-11 illustrates Kaplan Meier curves for RFS and OS when LD was categorised according to 25% percentile (3.5) as dichotomous variable. Patients with an LD lower than the median (3.5) tended to have longer OS but not RFS;

however, due to the limited number of patients, this difference was not statistically significant.

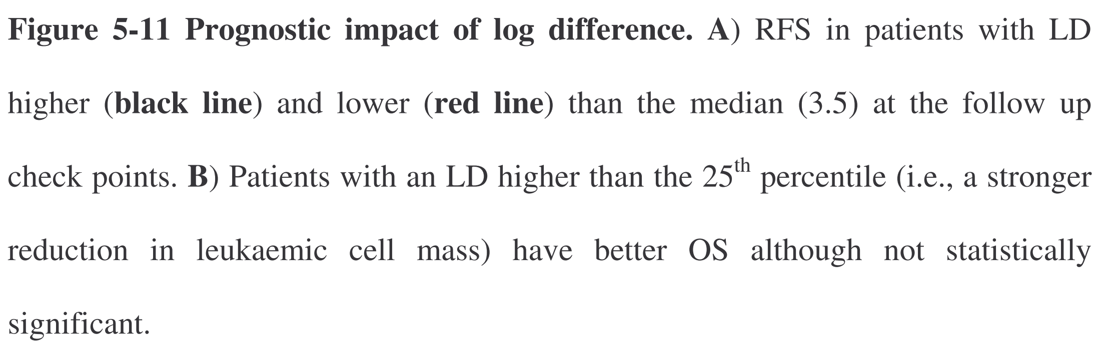
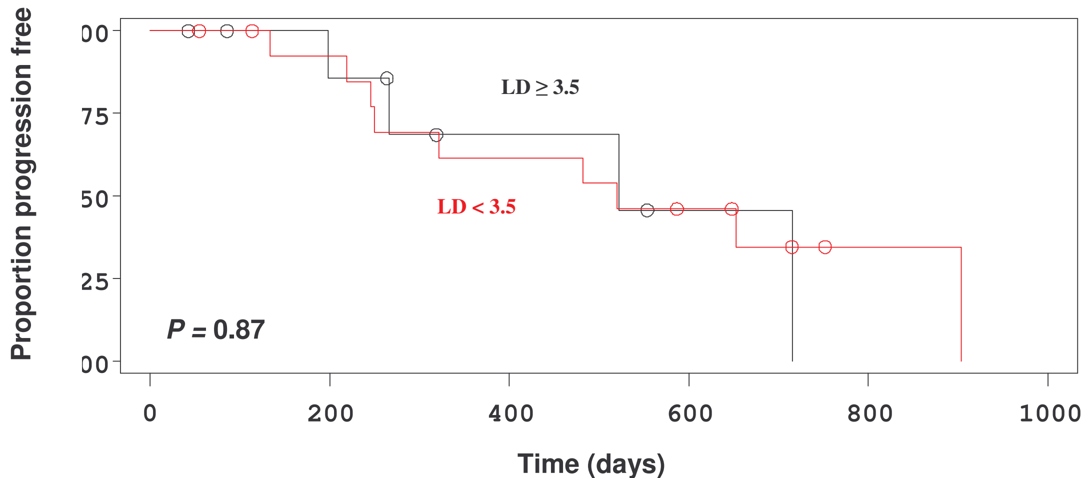


Figure 5-11 Prognostic impact of log difference. A) RFS in patients with LD higher (**black line**) and lower (**red line**) than the median (3.5) at the follow up check points. **B**) Patients with an LD higher than the 25th percentile (i.e., a stronger reduction in leukaemic cell mass) have better OS although not statistically significant.

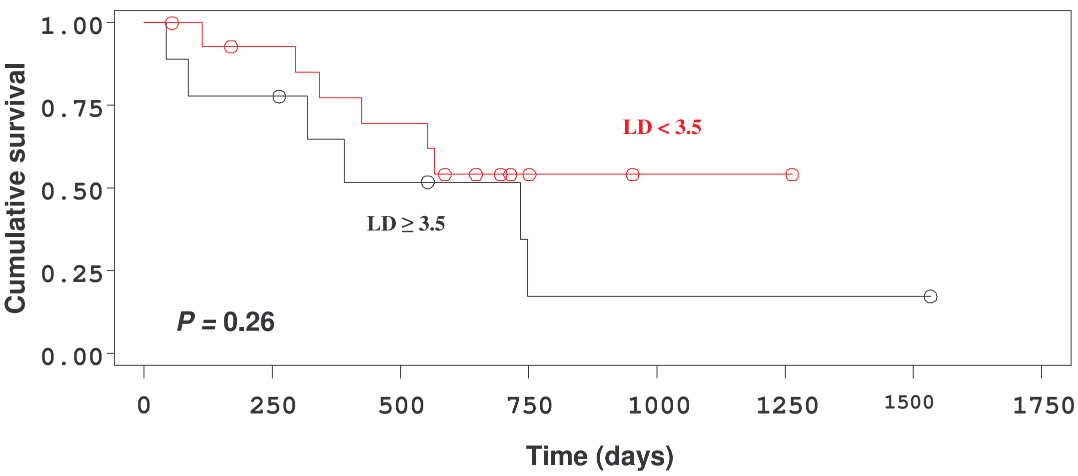
(A) RFS based on LD Category

25%ile of LD was 3.5



(B) OS based on LD Catogery

25%ile of LD was 3.5



5.4 DISCUSSION

The present study demonstrates the implementation of five-colour flow cytometry significantly improves MFC-based MRD quantification by allowing detection of patient specific LAPs in virtually all cases of AML. Determining the LD for each LAP studied based on the baseline of normal and regenerating BM and taking only the most sensitive LAP for each patient for MRD study enabled better MRD quantification. The five-colour approach allowed us to detect more than 2 LAPs in the vast majority of patients as shown in Chapter 4. The applicability of this technique for MRD quantification in virtually all AML patients provides an important basis for refined and individualized therapeutic management approaches (e.g. the designing of individual post remission therapy strategies).

We have shown that MRD level of 0.15% is the most important threshold value that can discriminate patients in terms of residual leukaemic cells and their relapse risk for both post induction and consolidation therapies. Although our series of patients was limited, our data show that patients in morphological CR who have detectable MRD after induction chemotherapy have a worse prognosis. Moreover, AML patients in whom MRD is not detectable after induction treatment and who remain MRD negative at the end of post-consolidation chemotherapy have superior RFS and OS. Therefore, these patients may not require transplantation procedures that are associated with relatively high treatment-related morbidity and mortality.

In selecting a strategy of obtaining the optimal threshold value in MRD, it is of particular importance to test cut-off values with optimum specificity because our aim should be to prevent over treating patients with low MRD level as the treatment is potentially toxic. Yet, we do not know the level at which MRD negative patients are not going to relapse. This needs to be further explored, may be by using six- or

seven-colour MFC with increasing more stem cell markers i.e. CD133 to better identify the blast population or further investigating and tracing leukaemic stem cells instead of the bulk whole blast cell population.

Thus, post induction MRD^{-} patients had a superior outcome in terms of relapse rate, OS and RFS ($p < 0.05$, for all comparisons) in multivariate analysis. If the MRD frequency was above the threshold value, the relative risk of relapse was a factor of 4.7 and 5.2 fold higher for RFS and OS , respectively, compared to the group of patients lower than that threshold level.

To take full advantage of the present data, the approach should be used and validated in the context of clinical trials evaluating individualised post remission therapies because an accurate assessment of the tumour burden during the post-remission phase has the potential to individualise post remission treatment approaches, avoiding over or under treatment.

We have compared our MRD data with those of three other groups, which reported prognostic impact of MRD frequency using immunophenotypic detection methods (Table 5-4) (San Miguel *et al.* 1997) (Venditti *et al.* 2000; San Miguel *et al.* 2001; Sievers *et al.* 2003). Considering the threshold levels for distinguishing two patient groups with different clinical outcome, Venditti and co-workers were not able to establish such a threshold level after one cycle of induction chemotherapy (Venditti *et al.* 2002). On the other hand, San Miguel *et al* (San Miguel *et al.* 2001) and Sievers *et al* (Sievers *et al.* 2003) established a threshold level of 0.2% after one or two cycles of induction chemotherapy, which is similar to our threshold level of 0.15% established after one cycle of induction chemotherapy. Furthermore, after consolidation chemotherapy two patient groups with different RFS were defined at a threshold level of 0.035% in the study of Venditti *et al* and 0.2% in the study of

San Miguel *et al*. We found a trend towards significance using a threshold level of the median MRD% of 0.12 after consolidation therapy. Remarkably, despite the fact that both groups in our study were small, we found that in most subgroups threshold levels could be defined that identified two groups of patients with a significant difference in RFS.

Therefore, MRD analysis by flow-cytometry may be used for refining the selection of therapeutic strategies and improving clinical outcome in individual patients. Data from previously published studies, as well as our study, indicate that MRD levels above 0.15% define the population of patients with the highest risk of relapse (San Miguel *et al.* 2001; Coustan-Smith *et al.* 2003; Sievers *et al.* 2003; Feller *et al.* 2004). However, MRD levels between 0.01-0.1% may also define AML patients with an increased risk of relapse (San Miguel *et al.* 2001).

By improving the panel of antibodies and the application of more fluorochromes, flow cytometry could be used for all AML patients (Kern *et al.* 2003). The limitation of this approach may be a lower sensitivity due to the increased application of less aberrant LAP. We improved the sensitivity and applicability of the flow cytometric method by adding CD45 into a five-colour MRD assessment and determining the LD for each LAP. To confirm these single institution results and to further improve response-adapted management of patients with AML, large prospective multi-centre studies are needed in which MRD levels are thoroughly followed and used to allocate patients to different predefined therapies.

Table 5-4 Prognostic impact of MRD in AML

Study	No. patients	No. patients evaluable for MRD P Ind	No. patients evaluable for MRD P cons	AML patients		CR rate	Median follow-up	Relapse Rate in MRD^+ vs MRD^- (p cons)	MFC	Univariate analysis				
				Adult AML	Elderly AML					Age	FAB	MRD P cons	Cytogenetics	MDR1
Venditti *et al* (Venditti *et al.* 2000)	93	56	51	18-60 AML 10	>60 AML-13	56(60%)		77% vs 17%	2-3	NS	NS	P<0.05	P<0.05	P<0.05
Venditti *et al* 2002(Venditti *et al.* 2002)	113	63	57	18-60 AML 10	>60 AML-13	73(65%)		81% vs 27%	3			P<0.05	P<0.05	P<0.05
Buccisano *et al* 2006(Buccisano *et al.* 2006)	120	100	92	18-60 AML 10	>60 AML-13	100(83%)	5 yrs	84% vs 25%	3-4	P<0.05	NS	P<0.05	P<0.05	P<0.05
Feller *et al* 2004(Feller *et al.* 2004)	72	51	52	HOVON 29 <60	HOVON 32 >60	51(71%)	2.5 yrs		4	P<0.05	NS	P<0.05	P<0.05	
San Miguel *et al* 1997(San Miguel *et al.* 1997)	89	53	42	AML 87<60	AML 91 <60	53(60%)	2 yrs	69% vs 32%	3	-	-	-	-	P<0.05
San Miguel *et al* 2001(San Miguel *et al.* 2001)	233	126	-	AML 87<60	AML 91 <60	126(54%)	3 yrs	84%	3	-	-	P<0.05	P<0.05	P<0.05
Kern *et al* 2003(Kern *et al.* 2003)	68		18	-	-	-			3	-			-	-

Kern *et al* 2004(Kern *et al.* 2004)		58	62	AMLCG trial		304/486 (63%)	2 yrs		3	NS	NS	P<0.05	P<0.05	-
Edward *et al* 2006(Laane *et al.* 2006)	62	45	31			53(85%)	5 yrs	85% vs 42%	3	NS	NS			
Our Study	**54**	**25**	**22**	**-**	**-**	**27/31 (87%)**	**17 Mo.**	**89% vs 23%**	**5**	**NS**	**NS**	**0.06**	**NS**	**-**

Study	**Multivariate analysis (RFS)**				**MRD post Ind (median)**	**MRD Post Cons (cut-off)**	**MRD post ASCT**
	MDR1 phenotype	**MRD P Ind**	**MRD P Cons**	**Cytogenetics**			
Venditti *et al* (Venditti *et al.* 2000)	P<0.05	NS	P<0.05	NS	0.035%	0.035%	
Venditti *et al* (Venditti *et al.* 2002)	NS	NS	P<0.05	NS	0.018%	0.035%	No effect
Buccisano *et al* (Buccisano *et al.* 2006)	NS	NS	P<0.05	NS	0.1%	0.035%	No effect
Feller *et al* (Feller *et al.* 2004)	-	P<0.05	P<0.05	NS	1%	0.11%	
San Miguel *et al* (San Miguel *et al.* 1997)		P<0.05	P<0.05	P<0.05	0.5%	0.2%	-
San Miguel *et al* (San Miguel *et al.* 2001)		P<0.05	P<0.05	P<0.05			
Kern *et al* (Kern *et al.* 2003)	-	-	-	-	-	-	-
Kern *et al* (Kern *et al.* 2004)		-	-	P<0.05	LD>25%ile	LD>75%ile	-
Edward *et al* (Laane *et al.* 2006)	-	-	-	-	0.13%	0.06%	AutoSCT may help
Our Study	**-**	**P<0.05**	**NS**	**NS**	**0.07%**	**0.15%**	**No effect but small # to draw any significant conclusion**

CHAPTER

6

Characteristics and prognosis of adult acute myeloid leukaemia with internal tandem duplications in the *FLT3* gene

CHAPTER 6: CHARACTERISTICS AND PROGNOSIS OF ADULT ACUTE MYELOID LEUKAEMIA WITH INTERNAL TANDEM DUPLICATIONS IN THE *FLT3* GENE

6.1 ABSTRACT

Constitutive activation of the FLT3 receptor tyrosine kinase by internal tandem duplication (ITD) of the juxtamembrane (JM) region has been described in patients with AML. FLT3/ITDs are present in about 20-30% of all AML cases. We analysed the prevalence and the potential prognostic impact of FLT3 mutations in 39 AML patients. We then compared 12 samples with FLT3/ITDs and 27 AML samples without these mutations with respect to clinical prognosis. Results were correlated with cytogenetic data and the clinical response. FLT3/ITD mutations were found in 31%. FLT3/ITD was associated with similar clinical characteristics and was more prevalent in patients with normal karyotype. More FLT3/ITD aberrations were found in patients with FAB M1, and fewer were found in patients with FAB M2, M4, and M5. Although less frequent in patients with cytogenetic aberrations, FLT3/ITDs were found in 2 of 4 patients with t(15;17). Although patients with FLT3/ITD mutation showed a tendency towards shorter CR duration and higher relapse rate, this was not statistically significant most likely because of the limited sample size. Taken together, these data confirm that FLT3/ITD mutations represent a common alteration in adult AML and may be an important prognostic marker.

6.2 INTRODUCTION

The fms-like tyrosine kinase 3 (FLT3), also known as stem cell tyrosine kinase-1 (STK1) or foetal liver tyrosine kinase-2 (FLK-2), belongs to the group of class III receptor tyrosine kinases (RTKs), which also include the receptors c-kit and c-fms (Gilliland *et al.* 2004). Signals generated by ligand-induced dimerisation of these receptors involve tyrosine phosphorylation of certain regions of the receptor and activation of cellular tyrosine kinases. Whilst a large proportion of AML have been found to express class III RTKs (Schnittger *et al.* 2002; Thiede *et al.* 2002; Schnittger *et al.* 2004; Wang *et al.* 2005), their potential role in leukaemia is still unclear.

In 1996, Nakao *et al*. (Nakao *et al.* 1996) were the first to report a novel mutation in the *FLT3* gene in a small number of AML patients. These mutations were shown to be ITDs, mainly involving a tyrosine rich stretch at the end of exon 14 (587-NEYFYVDFREYEYD-560), coding for the JM domain of the receptor (Figure 6-1, overview of the *FLT3* gene). In addition, some ITDs involving exon 15 have been published. All ITDs were in frame and resulted in an elongated JM domain.

In 17-20% of AML patients in the Japanese (Yokota *et al.* 1997) and West-European (Horiike *et al.* 1997) population, as well as in patients with leukaemic transformation of myelodysplasia (Kiyoi *et al.* 1997; Kiyoi and Naoe 2006), ITDs have been reported in the part of the *FLT3* gene coding for the JM through first tyrosine kinase (TK-1) domains of the protein. This type of mutation has not been found in ALL, CML, blast crisis CML, or in normal individuals. Kiyoi *et al*. (Kiyoi and Naoe 2006) have shown that in COS-cells, transduced with cDNA encoding for FLT3/ITDs, elongation of the JM domain and ligand-independent dimerisation of the mutant receptor occurs,

resulting in a constitutive activation. This effect was seen in both mutant/mutant and in mutant/wildtype (WT) dimers of the receptor.

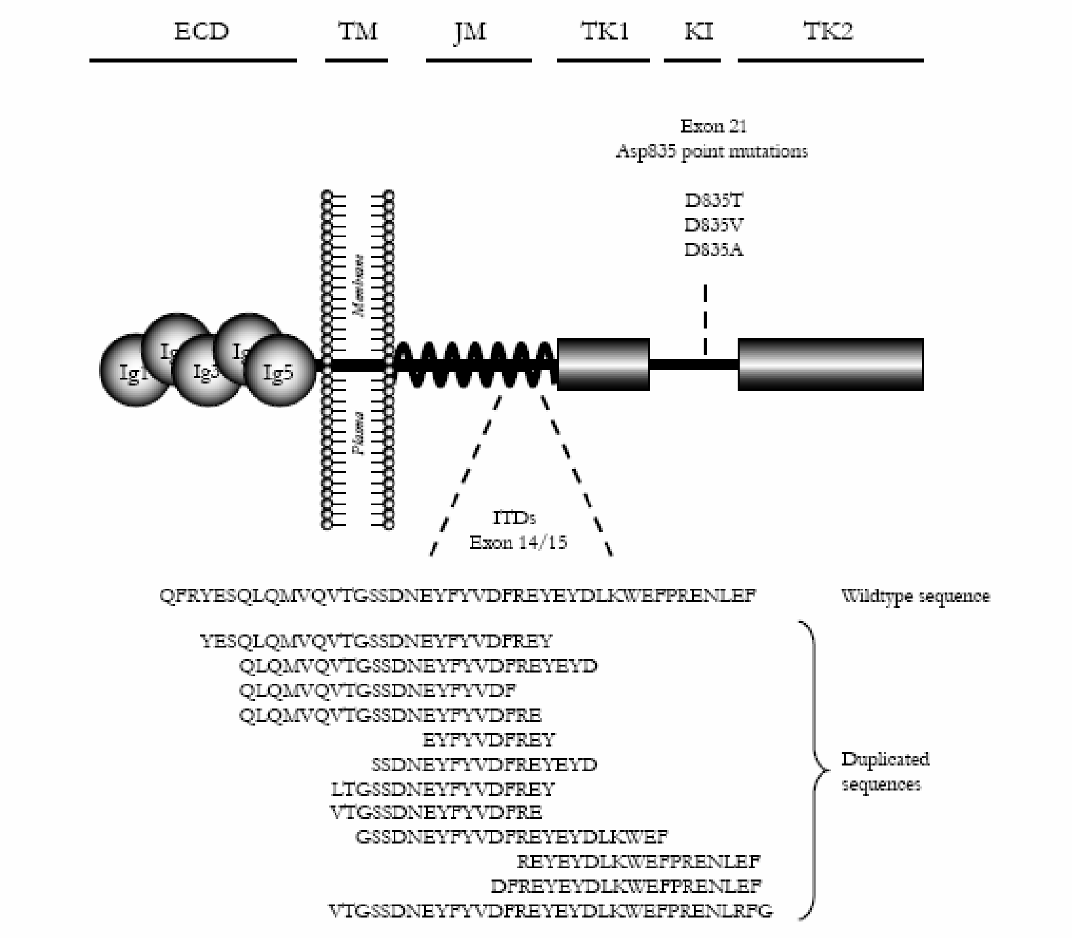
Kelly *et al*. (Kelly *et al.* 2002) showed that introduction of FLT3/ITDs into a murine BM transplant model resulted in a myeloproliferative phenotype characterised by leukocytosis and splenomegaly with extramedullary haemopoiesis in spleen and liver. As these ITDs were insufficient to induce leukaemia in this model, these data suggest that other co-operating mutations are necessary for the development of a leukaemic phenotype.

In this study, we determined the incidence of FLT3/ITD mutation in AML patients and assessed its prognostic significance.

Statistical method specific to this chapter:

RFS was measured from the date of CR to the date of relapse or else from the date of CR to the last follow-up date if relapse had not occurred. OS was measured from the date of diagnosis to the date of death or else from the date of diagnosis to the last follow-up date if death had not occurred. All analyses were done using R, R Development Core Team (2007). R: A language and environment for statistical computing. R Foundation for statistical Computing, Vienna, Austria.(Core 2007
) ISBN 3-900051-07-0, URL http://www.R-project.org.

Figure 6-1 Overview of the FLT3 monomer spanning the plasma membrane. This figure represents the 5 extracellular Ig-like domains (Ig1-5) in the ligand binding domain of the extracellular part of the receptor (ECD), the transmembrane domain (TM), the JM and the two intracellular tyrosine kinase domains (TK1 and TK2) separated by the kinase insert (KI). The positions of the two known types of mutations involved in AML, i.e. internal tandem duplications (ITDs) in the JM domain and point mutations in the KI, are indicated. Of each type of mutation, some examples are presented.



6.3 RESULTS

6.3.1 Incidence of FLT3/ITD in our cohort

Genomic DNA was obtained from the BM of 39 patients at diagnosis. In addition, follow-up samples were obtained at 2 to 5 time points from 10 patients carrying the FLT3 mutation. Exons 14 to 15 of the *FLT3* gene were amplified by genomic PCR. The FLT3/ITD amplification yielded a higher molecular weight product on a 2% agarose gel stained with ethidium bromide (Figure 6-2). Of the 39 samples analysed, 12 (31%) revealed FLT3/ITD. The sizes of the FLT3/ITD varied from approximately 21 base pairs (bp) to more than 150 bp and comprised different parts of the JM domain.

6.3.2 Characterisation of patients

Of the 12 patients with FLT3/ITD, 10 (83%) had a normal karyotype, similar to other reports (Rombouts *et al.* 1999). In most of the FLT3/ITD positive patients, the mutation was present as a heterozygous mutation but in 1 case the ITD appeared as a single aberrant band on gel, indicating the presence of either as a homozygous mutation or, more likely, a hemizygous deletion of the WT gene. The characteristics of 12 AML patients with the mutation and 27 AML patients without such a mutation included in this study are shown in Table 6-1. Sequence analysis from two patients of the FLT3 mutant samples (Table 6-2) confirmed the presence of tandem duplication of different sizes.

Table 6-1 Patient clinical characteristics

Patient characteristics	**AML pos for FLT3/ITD**	**AML neg for FLT3/ITD**
No. patients	12	27
Male/ female	8/4	17/10
Age at diagnosis, median, (range)	62 (23-73)	69 (23-88)
WBC count at diagnosis × 10^9 /L, median (range)	6.6 (0.9-179)	6.3 (0.7-227)
BM blasts % by morphology, median (range)	72 (26-85)	35 (20-87)
AML *de novo*/secondary n (%)	9 (75)/3 (25)	23 (85)/4 (15)
FAB classification, n (%)		
Mo	0 (0)	0 (0)
M1	6 (50)	2 (7)
M2	1 (8)	10 (37)
M3	2 (17)	2 (7)
M4	1 (8)	3 (11)
M5	1 (8)	4 (15)
M6	0 (0)	0 (0)
M7	0 (0)	0 (0)
Not classified	1 (8)	6 (22)
Cytogenetic risk group, n (%)		
Favourable	2 (17)	4 (15)
Intermediate	10 (83)	13 (48)
Poor	0 (0)	9 (33)
Insufficient sample	0 (0)	1 (4)
Induction therapy response		
CR	7 (70)	15 (88)
Failure	3 (30)	2 (12)

Table 6-2 Sequence analysis of the FLT3/ITDs in 2 patients. The bold base pairs (bp) are not part of *FLT3* gene.

Patient No.	**Duplicated bp sequence**	**Length**
AML 1	**catcccccc**gatttcagagaatatgaatatgat	33 bp
AML 2	gtacaggtgaccggctcctcagataatgagtacttctacgttgatttcagagaatatgaatatgatctcaaatgggag	78 bp

6.3.3 Clinical response and prognosis of AML patients with and without FLT3/ITD

Twenty seven patients were evaluated for their initial response to therapy. There was a trend for patients with FLT3/ITD to not achieve CR when compared to patients in the population without FLT3/ITDs (7/10 versus 15/17) [Table 6-2]. A larger sample size may have lead to a greater difference in remission rates. At a median follow-up period of 10 months in the group with ITDs, 3 of the 7 (43%) patients who had achieved CR had relapsed, whilst 8 out of 15 (53%) patients without ITDs had relapsed ($p = 0.6$).

The group with FLT3/ITD as compared to the group without the mutation showed no statistically significant difference ($p > 0.05$) in our series with respect to RFS (Figure 6-3 A) and OS (Figure 6-3 B). Details for the RFS and OS of the population with FLT3 mutations are shown in Table 6-3. As the heterogeneity of our AML population have impacted on our analysis , we also looked at the RFS and OS for the two largest sub-populations that could be distinguished within our dataset, i.e. patients with *de novo* AML (Figure 6-3 D) and patients with an intermediate-risk karyotype. Although, secondary AML showed shorter OS, this was not statistically significant ($p = 0.07$) neither did the group of patients with an intermediate cytogenetic risk and FLT3 mutation. Age is known to be an important prognostic factor and we divided the samples into two groups according to age (Figure 6-3 C). In both age groups (under 60 years and over 60 years), the difference with respect to OS was significant ($p = 0.043$) in univariate analysis. Other known potential confounders i.e. (WBC, FAB subtypes, cytogenetics, percentage of blasts at diagnosis) were also investigated and all were found not to be statistically significant with respect to OS ($p > 0.05$).

Figure 6-2 Genomic PCR for the FLT3/ITD. Genomic DNA prepared from diagnostic marrow specimens were PCR amplified and resolved on 2% agarose gel electrophoresis and visualised under UV light. **A**) Lane **12**: no DNA, Lanes **13** and **14**: positive and negative controls, **M**: marker pUC19, Lanes **1-11**, samples from **11** different adult AML patients. High-molecular weight bands in lanes **4, 6, 7, 9** and **10** represent FLT3/ITD. The solid arrow points to the normal *FLT3* gene product while the dotted arrow points to the FLT3/ITD mutation which varied in length (number of bp inserted) between the five positive patients. **B**) Lane **1-5**, samples from 5 different adult AML patients. Patients sample in lane **3** does not have a normal FLT3 band.

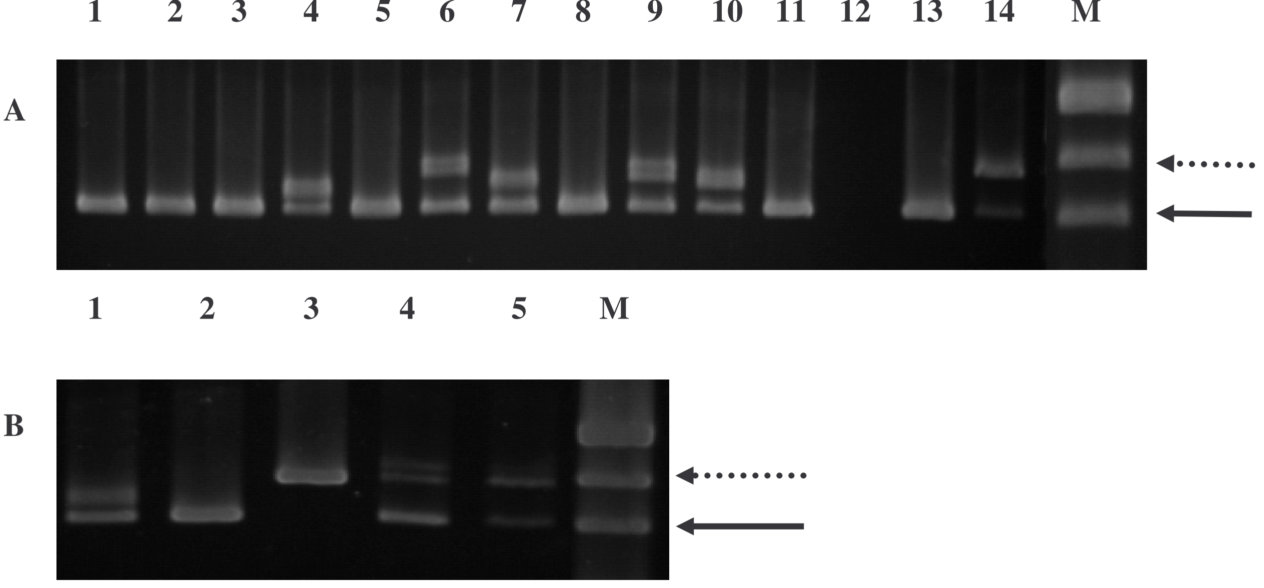
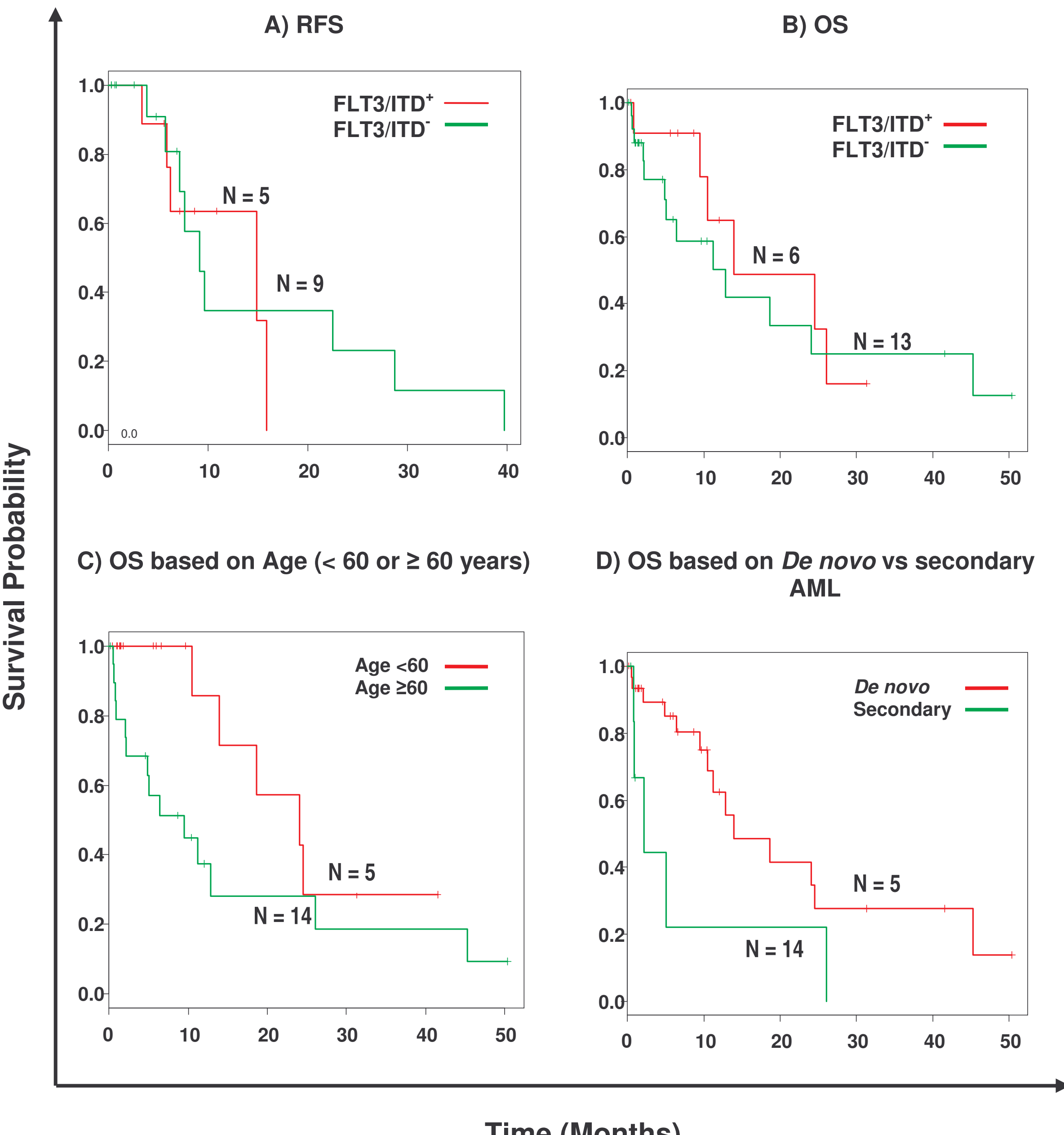


Table 6-3 Kaplan-Meier survival table of the RFS and OS of the AML patients with FLT3/ITDs.

Patient No.	CR rate (Months)	RFS (Months)	Survival probability for RFS	Standard Error of RFS	95% CI for RFS	OS (Months)	Survival probability for OS	Standard Error of OS	95% CI for OS	Cause of death	FAB subtype
AML1	No treatment	NA*				0.4				Alive	M4
AML2	No treatment	NA				0.8	0.909	0.087	0.754-1		Prior MDS
AML3	1.0	6.2	0.635	0.169	0.238-0.866	13.9	0.487	0.188	0.228-1	Relapse	M5a
AML4	1.3	15.9	0.000	NA	NA	24.6	0.325	0.183	0.108-0.978	Relapse	M1
AML5	1.8	8.6				10.5	0.649	0.167	0.392-1	Transplant AlloSCT	M1
AML6	1.2	10.8				12.1				Alive	M1
AML7	1.0	14.9	0.317	0.240	0.015-0.729	31.3				Alive	M1
AML8	1.6	7.1				8.7				Alive	M3
AML9	NA	NA				26.1	0.162	0.147	0.028-0.954	Induction failure	M2
AML10	2.1	3.4	0.889	0.105	0.433-0.984	5.6				Alive	M1
AML11	1.1	5.6				6.6				Alive	M3
AML12	2.3	5.8	0.762	0.148	0.332-0.935	9.5	0.779	0.141	0.546-1	Relapse	M1

*** NA = not applicable**

Figure 6-3 Kaplan-Meier survival curves of the effect of FLT3/ITDs in AML. Green lines, FLT3/ITD negative samples, red lines FLT3/ITD positive population. Censored cases are marked by a dash. (**A**) Cumulative RFS of all cases investigated, 13 cases were deleted from the analysis due to "no treatment"; (**B**) cumulative OS of all cases investigated. (**C**) OS in patients with and without FLT3/ITD in patients with an age below 60 years and those older or equal to 60 years. (**D**) OS in patients with and without FLT3/ITD in patients with *de novo* vs. secondary AML.



6.3.4 Evaluation of prognostic factors

Multivariate analysis including cytogenetics, age, WBC count at diagnosis, percentage of blasts at diagnosis, and secondary etiology of AML as covariates showed that the FLT3/ITD state is not an independent prognostic factor for OS or RFS. This probably relates to small sample size in our study.

6.3.4.1 Correlation to cytogenetics

Cytogenetic analyses were available from 38 analysed patients. They were grouped into 3 categories according to cytogenetics: intermediate group (n = 23); favourable group (n = 6); and poor group (n = 9) and 1 patient was not analysed due to insufficient sample received.

Ordinal χ^2 analysis shows that FLT3/ITD is not randomly distributed within cytogenetic subgroups ($p < 0.0001$). Of the 12 patients with FLT3/ITD, 10 (83%) had a normal karyotype (Table 6-2). This was more than in the group without the mutation, in whom only 13 of 27 (48%) were cytogenetically normal ($p = 0.07$). The mutation is most common in the normal karyotype group and in the t(15;17) group (17%). In contrast, compared with the total cohort, FLT3/ITD was not found in patients with t(8;21), 11q23 translocations , −5/5q−/7q− and complex rearrangements. The mutation also was not detected in the 2 patients with inv (16)/ t(16;16).

FLT3/ITD was significantly more common in patients with *de novo* AML (75%) as compared to patients with secondary AML (25%) ($p = <0.001$) (Table 6-2). The incidence of FLT3/ITD according to karyotype was ranked as follows: normal karyotype (83%) > t(15;17) (17%) > complex karyotype (0%).

6.3.4.2 Correlation with leukocyte count in FAB subgroups

For 39 patients, the leukocyte count was higher in the group with the FLT3/ITD than in the group without the mutation (median WBC, 6.6 vs. 6.3) respectively.

6.3.4.3 Correlation with percentage of blasts at diagnosis

For 39 patients, the blast percentage count was higher in the group with the FLT3/ITD than in the group without the mutation (median blasts %, 72 vs. 35) respectively.

6.3.4.4 Correlation with FAB subtype

For 32 patients, FAB classification was available. Fifty percent of FLT3/ITD positive was M1 (Table 6-2). The incidence of FLT3/ITD was ranked as follows: M1 (50%) > M3 (17%) > M2 (8%), M4 (8%), M5 (8%) > M0 (0%), M6 (0%), (0%). No patient was positive for the ITD in M0, M6, or M7. However, patients with M6 or M7 were encountered only as small groups in the prospective study and no definite conclusion is possible for these subtypes.

6.3.4.5 Correlation with Age and sex

The median age of patients with FLT3/ITD was 62 years versus 69 years in the group without the mutation. As shown in Table 6-2, the mutation was more frequent in men than in woman, with a 2:1 ratio.

6.4 DISCUSSION

6.4.1 Clinical implications of FLT3 mutations in AML

6.4.1.1 Incidence of FLT3/ITD

To date over 20 studies, in total comprising over 5000 patients, have been published investigating the incidence and clinical implications of FLT3/ITDs in adult or paediatric AML. As is clear from the data summarised in Table 6-4 many studies involved a limited number of patients or only a subset of AML, which may explain the variations in incidence rates. Even more important, many of the studies represent retrospective studies using banked leukaemia samples. Using stored samples one would expect to induce a bias for samples with high WBC counts or BM cellularity. As most studies indicate FLT3/ITDs to be linked to high WBC counts, one expects the incidence rates as illustrated in this table to be somewhat over estimated.

Incidence rates in the studies can also be influenced by source material (peripheral blood, BM or plasma DNA (Jilani *et al.* 2003) or the techniques used to amplify and detect the FLT3 mutations. Most of the studies are based on PCR amplification of genomic DNA or RNA followed by simple agarose gel electrophoresis, whilst others use a single strand conformation polymorphism analysis to detect mutations not detected in the standard gel electrophoresis.

So far, with regard to incidence of FLT3/ITDs several features are evident from the studies described in this table. First, the incidence of FLT3/ITDs is increased in patients with FAB-M3 and patients with intermediate risk cytogenetics. On the other hand, the incidence is decreased in patients with t(8;21) or inv(16), the so called core binding factor (CBF) leukaemias. Second, the incidence of the mutations

in juvenile AML is reduced when compared to adult AML. However, 4 of the 6 studies involving juvenile AML have reported FLT3/ITD mutations to increase with age 8-11. This is in contrast to adult AML where only one study reported an increasing incidence of FLT3/ITD with age (Schnittger *et al.* 2002). Though FLT3 mutations are clearly more common in FAB-M3, they otherwise appear to be very evenly distributed over all other FAB classes. Nevertheless, some studies do report significant differences in the incidence within certain FAB classes (Schnittger *et al.* 2002; Thiede *et al.* 2002; Zwaan *et al.* 2003). However, as these studies indicate different FAB classes to be involved they are most likely caused by biases in the sampled populations. Taken together, from the data in Table 6-4 the occurrence of FLT3/ITDs can be estimated to be around 20% in adult AML (30% for the intermediate risk population) and around 14% in childhood leukaemia. The overall incidence of FLT3/ITD mutations in our study was 31% (12 out of 39), which is consistent with numbers reported by other groups (Nakao *et al.* 1996). This high incidence indicates that FLT3 is an important target of mutational activation in adult AML.

6.4.1.2 The impact of FLT3/ITDs on patient prognosis

Of the 15 studies involving adult AML presented in Table 6-4, two exclusively studied the impact of FLT3/ITDs in APL and both studies observed no effects of FLT3/ITDs on patient prognosis (Yokota *et al.* 1997; Noguera *et al.* 2002). However, the numbers of these APL series were relatively small and thus the statistical power of these analyses was limited. Of the remaining 13 studies, 12 provided an analysis regarding the impact of FLT3/ITDs on the OS. Seven of these studies observed a reduced OS in FLT3/ITD AML in general, whilst three others only found a reduced OS in patients with either a hemizygous deletion of the WT

allele (Whitman *et al.* 2001) or patients with a high mutant/WT ratio (Thiede *et al.* 2002);(Steudel *et al.* 2003). When only patients of cytogenetic intermediate prognostic risk are analysed, FLT3/ITDs has a clearly negative impact on OS (Kainz *et al.* 2002). The two remaining studies, which failed to detect any effect of FLT3/ITDs on OS both involved an intensified conditioning regimen (Boissel *et al.* 2002; Schnittger *et al.* 2002). It should be noted that while the median follow-up in the study by Boissel *et al*. is unknown, the study by Schnittger *et al*. only had a median follow-up 11.1 months, which might have been too early for detecting differences in OS. This is undoubtedly true for our study with a median follow-up of 6 months as we could not find a negative impact for OS or RFS with FLT3/ITD. We only had a small sample size in comparison with most other studies. Differences might also be attributed to the assessment of FLT3/ITDs. In our study, we regarded any additional FLT3 signal as ITD^+.

The major factor attributing to the lower survival of FLT3/ITD AML appears to be the increased relapse rates. Only in two studies involving adult AML, FLT3/ITD AML showed a reduction in the remission rate (Rombouts *et al.* 2000; Kottaridis *et al.* 2001).

Only the study of Stirewalt *et al*. (Stirewalt *et al.* 2001) specifically investigated the prognostic impact of FLT3/ITDs in elderly AML. Most other studies have involved paediatric patients. This one study failed to detect an effect of FLT3/ITDs in elderly patients. However, the median OS of seven months in the older age population may have obscured a possible negative impact of these mutations in elderly patients.

The majority of the studies published so far indicate the presence FLT3/ITDs to be a significant and independent predictor of an unfavourable clinical response. Yet, as many studies have focussed on specific subgroups of AML patients and the duration

of the follow-up is quite heterogeneous, it is difficult to directly compare the overall prognostic impact.

Comparison of FLT3/ITD data and the clinical and cytogenetic findings revealed that FLT3/ITD was associated with higher WBC count and higher numbers of BM blasts compared to patients without such a mutation. As an interesting finding and in contrast to others, our data indicate that mutational FLT3 activation was increased in patients with AML FAB M1, among whom approximately 50% carried a mutated *FLT3* gene. In contrast, FAB subtypes M2 and M4 and M5 were less frequently associated with FLT3 activation, which is also in line with the expression patterns of this protein during normal haemopoietic differentiation. With respect to the cytogenetic data, ITD mutations were approximately 4 times more prevalent in patients with normal karyotype than in patients with cytogenetic alterations. Most patients with cytogenetic aberrations showed a low number of FLT3/ITD mutations (Kiyoi *et al.* 1997; Zwaan *et al.* 2003).

In conclusion, with an occurrence of around 20% in adult AML and 30% in the normal karyotype, FLT3-mutations are to date the most frequent genetic mutations observed in AML. Although the mechanism by which mutations in the *FLT3* gene influence patient prognosis is unresolved, it is clear that AML with FLT3/ITDs represent a subset of leukaemia with a poor prognosis. In our limited sample size and short follow-up, we were not able to demonstrate this.

Table 6-4 Occurrence and prognostic impact of FLT3/ITDs in AML.

Study	FLT3/ITD present							Therapy	Median follow-up	Overall survival	CR rate	Relapse rate
	Adult AML	Elderly AML	Juvenile AML	2nd AML	FAB-M3	t(8;21) inv(16)	Int. risk					
Rombouts(Rombouts *et al.* 2000)	23.7% (14/59)	22.9% (8/35)		18.2% (2/11)	33.3% (2/6)		24.2% (16/66)	Conv.	23 Mo.	↓	↓	↑
Kyoi(Kiyoi *et al.* 1997)					20.3% (15/74)			ATRA	26 Mo.	↔	↔	↔
Yamamoto (Yamamoto *et al.* 2001)	18.9% (81/429)					11.8% (2/11)	28.0% (23/82)	Conv.	50 Mo.	↓		↑
Abu-Duhier(Abu-Duhier *et al.* 2000)					10% (1/10)	5.8% (3/52)	22.7% (10/44)	Conv.		↓		
Stirewalt (Stirewalt *et al.* 2001)		33.6% (47/140)						Conv.		↔		
Kottaridis (Kottaridis *et al.* 2001)	26.6% (227/854)			27.4% (17/62)	36.8% (49/133)	8.3% (9/109)	34.2% (96/281)	Conv.	52 Mo.	↓	↓	↑
Whitman (Whitman *et al.* 2001)							28.0% (23/82)	Conv.	20.4 Mo.	↔[a]	↔	↑
Thiede (Thiede *et al.* 2002)	22.2% (217/979)			8.8% (3/34)	33.3% (13/39)	4.5% (4/88)	29.7% (134/451)	Intens.	12.2 Mo.	↔[b]		↑

Study	**FLT3/ITD present**							**Therapy**	**Median follow-up**	**Overall survival**	**CR rate**	**Relapse rate**
	Adult AML	**Elderly AML**	**Juvenile AML**	**2nd AML**	**FAB-M3**	**t(8;21) inv(16)**	**Int. risk**					
Schnittger (Schnittger *et al.* 2002)	23.3% (234/1003)			15.6% (12/77)	35.8% (24/67)	5.4% (6/111)	39.3% (149/379)	Intens.	11.1 Mo	↔	↔	↑
Boissel (Boissel *et al.* 2002)	25.2% (40/159)					0.0% (0/23)	34.5% (28/79)	Intens.		↔	↔	↔
Noguera (Noguera *et al.* 2002)					36.7% (33/90)			AIDA	> 8 yrs	↔	↔	↔
Frohling (Frohling *et al.* 2002)	22.8% (119/523)			9.2% (7/76)	39.2% (20/51)	5.7% (4/70)	31.7% (71/224)	Intens.	34 Mo.	↓	↔	↑
Kainz (Kainz *et al.* 2002)					38.1% (8/21)	7.7% (2/26)	30.2% (16/53)	Conv.		↓[c]		
Moreno (Moreno *et al.* 2003)	15.3% (32/208)			17.4% (4/23)	31.5% (6/19)	0.0% (0/14)	27.6% (31/112)	Conv.			↔	↑
Jilani (Jilani *et al.* 2003)	21.2% (18/85)				25.0% (1/4)		25.4% (14/55)				↔	
Steudel (Steudel *et al.* 2003)	26.1% (55/211)							Conv.		↔[d]		↑
Iwai (Iwai *et al.* 1999)			5.3% (5/94)		11.1% (1/9)			Conv.		↓		

Study	FLT3/ITD present							Therapy	Median follow-up	Overall survival	CR rate	Relapse rate
	Adult AML	Elderly AML	Juvenile AML	2nd AML	FAB-M3	t(8;21) inv(16)	Int. risk					
Xu (Xu *et al.* 1999)			13.8% (12/87)					Conv.		↓		
Kondo (Kondo *et al.* 1999)			10.9% (7/64)		66.6% (2/3)			Conv.		↓	↔	↑
Meschinchi (Meshinchi *et al.* 2001)			16.5% (15/91)					Conv.+ Intens.		↓	↓	↑
Liang (Liang *et al.* 2002)			11.3% (9/80)		25.0% (3/12)			Conv.		↔	↔	
Zwaan (Zwaan *et al.* 2003)			11.5% (27/234)		22.2% (2/9)	2.7% (1/37)		Conv.	39 Mo.	↓	↓	↔[e]
Arrigoni (Arrigoni *et al.* 2003)			27.0% (20/74)		34.5% (10/29)			Conv.		↓	↔	
Our Study	**30.76% (12/39)**			**42.9% (3/7)**	**50.0% (2/4)**	**0.0% (0/1)**	**43.48% (10/23)**	**Conv.+ Intens**	**6 Mo.**	↔	↔	↔

[a] OS is significantly reduced in patients with deletions of the wildtype gene.
[b] OS is significantly reduced in patients with a high mutant to wildtype ratio.
[c] OS is significantly reduced in patients with intermediate risk cytogenetics.
[d] OS is significantly reduced in patients with a high mutant to wildtype ratio.
[e] relapse rates are increased in patients with a high mutant to wildtype ratio.

↓ = Decreased
↔ = No effect
↑ = Increased

CHAPTER

7

The oncogenic events of FLT3 Internal Tandem Duplication happen at a stage of stem cells that possess IL-3 alpha receptor (CD123).

CHAPTER 7: THE ONCOGENIC EVENTS OF FLT3 INTERNAL TANDEM DUPLICATION HAPPEN AT A STAGE OF STEM CELLS THAT POSSESS IL-3 ALPHA RECEPTOR (CD123)

7.1 ABSTRACT

In CD34 positive AML, the leukaemia-initiating event likely takes place in $CD34^+/CD38^-$ cells (Blair and Pamphilon 2003). Survival of these cells after chemotherapy hypothetically leads to MRD and relapse. Recently, the IL-3 alpha receptor (CD123) has been shown as a unique marker of LSCs within the $CD34^+/CD38^-$ compartment (Jordan *et al.* 2000). ITD mutations of the *FLT3* gene are the most frequent molecular abnormality in AML. While the normal FLT3 receptor is expressed in early haemopoietic progenitor cells, it has not been clearly determined whether FLT3 mutations are present at the LSC level. The aims of this study were to investigate whether or not FLT3/ITD are present at LSC level and to demonstrate whether or not FLT3/ITD mutation is confined to the population of LSC as defined by $CD34^+/CD38^-/CD123^+$ and not $CD34^+/CD38^-/CD123^-$ cells.

We investigated 34 patients for CD123 expression using five-colour flow cytometry. We tested 25 (74%) of 34 patients for FLT3/ITD, of those 10 were positive for FLT3/ITD. Seven positive FLT3/ITD primary AML samples were sorted to homogeneity into stem cell-enriched $CD34^+/CD38^-/CD123^+$ and $CD34^+/CD38^-/CD123^-$ fractions. Both fractions were analysed for the presence of FLT3/ITD.

We found that anti-CD123 homogenously stained the leukaemic blast population in 94% (32/34) cases [median expression = 86%, range (20-99%)]. CD123 was also strongly expressed in the $CD34^+/CD38^-$ cells (96 ± 2% positive) from 28 (87.5%) of 32 primary specimens. CD123 was not expressed on normal BMs analysed (n = 5). In 7 of the ten FLT3/ITD positive patients sorted for stem cell-enriched fraction,

FLT3/ITD was present in $CD34^+/CD38^-/CD123^+$ and absent in 6 patients with $CD34^+/CD38^-/CD123^-$ cells. Taken together, these experiments establish that the presence of FLT3/ITD mutation in $CD34^+/CD38^-/CD123^+$ and not $CD34^+/CD38^-/CD123^-$ cells support the hypothesis that the mutation occurs at a stem cell level and may not be a secondary event in leukaemogenesis. Furthermore, we provide evidence that in AML, the oncogenic events happen at a stage of precursor cells that possess IL-3α receptor.

7.2 INTRODUCTION

Relapse is thought to occur because of the failure of chemotherapy to eradicate LSC (van Rhenen *et al.* 2005). Human AML stem cells have been defined as $CD34^+/CD38^-$ cells with the ability to reconstitute marrow of mice with SCID-repopulating ability, which is a reflection of their capacity to self-renew (Bonnet and Dick 1997; Blair *et al.* 1998).

In order for any AML therapy to be curative, it needs to be effective against the cells that propagate and sustain the disease, the so called LSCs. However, previous studies suggest that LSCs are biologically distinct from more mature leukaemic blasts and may not be responsive to conventional chemotherapeutic regimens (Terpstra *et al.* 1996; Terpstra *et al.* 1996). In 1997, Bonnet and Dick described the phenotype for LSCs as $CD34^+/CD38^-$ (Bonnet and Dick 1997). Subsequent studies showed that LSCs are also $CD34^+$/HLA-$DR^-/CD71^-$ and fail to express Thy-1 (Blair *et al.* 1997; Blair *et al.* 1998). Numerous studies have attempted to distinguish leukaemic from normal stem cells. One potential difference between normal and leukaemic cells lies in their response to haemopoietic growth factors. Several studies have examined the cytokine response of primary leukaemia cells and demonstrated mitogenic activity for IL-3, G-CSF, GM-CSF, SCF, FL, TPO and other factors (Smith *et al.* 1996; Ailles *et al.* 1997; Murayama *et al.* 1998). Although these studies have focused on bulk populations of leukaemia cells, it could be speculated that differential sensitivity to cytokines may also exist at the stem cell level. Jordan *et al.* (Jordan *et al.* 2000) show that interleukin-3 receptor alpha chain (CD123) is highly expressed on leukaemic but not normal $CD34^+/CD38^-$ haemopoietic cells.

The biological role of CD123 is unclear. However, the presence of CD123 on AML $CD34^+/CD38^-$ cells demonstrates that LSCs are biologically distinct from their normal

stem cell counterparts. In addition, because CD123 is not found on normal HSCs, it provides a unique marker that can be used to identify the malignant clone. This feature may be very useful in MRD studies as a single and standardised maker when compared to five-colour MFC and the extensive MoAbs required. Furthermore, the CD123 epitope represents a target to which therapeutic strategies may be directed.

FLT3 is preferentially expressed on haemopoietic stem/progenitor cells and plays a role in both differentiation and proliferation (Lyman and Jacobsen 1998). FLT3 is also expressed on the leukaemic blasts in the majority of cases of acute leukaemia, even in CD34 negative cases (Meierhoff *et al.* 1995; Drexler 1996; Rosnet *et al.* 1996). Somatic mutation of FLT3 involving ITDs of the JM domain have been identified in approximately 17% - 34% of AML cases as shown in chapter 6 (Nakao *et al.* 1996; Abu-Duhier *et al.* 2001; Kottaridis *et al.* 2001; Meshinchi *et al.* 2001; Gilliland and Griffin 2002; Schnittger *et al.* 2002; Thiede *et al.* 2002; Levis and Small 2003).

At the present time, there is only one study demonstrating the presence of FLT3 mutations at LSC level. Levis *et al* group (Levis *et al.* 2005) sorted primary AML samples harbouring FLT3/ITD mutations into stem cell-enriched $CD34^+/CD38^-$ fractions and then analysed the sorted and unsorted cells for the FLT3 mutant-WT ratio. In each case, the FLT3 mutant-WT ratio was not changed by selection of $CD34^+/CD38^-$ cells, implying that the mutations are present in the LSCs. The authors used the stem cell-enriched fraction to engraft non-obese diabetic-severe combined immunodeficient (NOD/SCID) mice and then confirmed that the FLT3/ITD mutation was present in the resultant engrafted marrow. However, in this paper they did not take into account that some of $CD34^+/CD38^-$ cells might be normal HSCs and another marker is therefore needed to define the LSCs.

In addition, the finding that BM cells from patients with AML harbouring FLT3/ITD mutations had a greater capacity to engraft NOD/SCID mice than cells from patients lacking such mutations is also supporting the hypothesis that FLT3/ITD is present at LSC level and hence more likely to engraft the NOD/SCID mice (Rombouts *et al.* 2000; Lumkul *et al.* 2002).

Therefore, we explored the issue of whether or not FLT3/ITD mutations are present at LSC level as defined by the phenotype $CD34^+/CD38^-/CD123^+$. Seven primary AML samples harbouring FLT3/ITD mutations were sorted into stem cell-enriched fractions $CD34^+/CD38^-/CD123^+$ and stem cell-enriched fractions lacking CD123, and FLT3/ITD was analysed in the two sorted fractions. Our data provide the first definitive evidence that FLT3/ITD mutations occur at LSC level at a stage of cells that possess IL-3 alpha receptor.

7.3 RESULTS

7.3.1 Expression of CD123 (IL-3 α receptor) in AML blast cells

Thirty-four AML patients at diagnosis were tested for the expression of CD123 (Table 7-1) in the total blast population and at the stem cell level.

CD123 expression was found in the majority of AML patients 32/34 (94%) with a median expression in the whole blast population of 86%, (range, 20-99%). In 24 (75%) patients, the majority of blasts (> 60%) expressed substantial amounts of CD123 from $CD123^+$ AML and in the remaining 8 (25%) patients, only a subset of blasts expressed CD123.

7.3.2 Expression of CD123 (IL-3 α receptor) in AML stem cells $CD34^+/CD38^-$

The expression of CD123 in the stem cells fraction as defined by $CD34^+/CD38^-$ was tested using the strategy outlined in Figure 7-1. Four patients were totally CD34 negative and therefore the estimation of CD123 expression in the $CD34^+/CD38^-$ compartment was not possible. Two of these patients were M5a, one M1 and one M3 FAB classification. CD123 was strongly expressed in the $CD34^+/CD38^-$ cells (96 ± 2% positive) from 28 (87.5%) of 32 primary specimens.

7.3.3 Expression of CD123 (IL-3 α receptor) in normal bone marrow $CD34^+/CD38^-$ fraction

Five normal BMs were tested for the expression of CD123 on $CD34^+/CD38^-$ cells and they were all CD123 negative. The median level of CD123 in normal $CD34^+/CD38^-$ stem cells (0.119%), range (0.004% - 1.43%) in the 5 normal BMs Figure (7-2).

Table 7-1 Patient characteristics

Patient characteristics	**Total (%)**
No. patients	34
Male/female	24/10
Age at diagnosis, y, mean (range)	63 (23-86)
% blasts at diagnosis (morphology)	41.5 (20-96)
% blasts at diagnosis (Flow)	42.5 (9-86)
WBC count at diagnosis, 10^9/L, median (range)	4.5 (0.71-179)
De novo/Secondary AML	27 (79)/7 (21)
FAB classification, *n* (%)	
M_0	0 (0)
M_1	8 (24)
M_2	10 (29)
M_3	1 (3)
M_4	2 (6)
M_5	4 (12)
M_6	1 (3)
M_7	0 (0)
Not classified	8 (24)
Cytogenetic risk group, *n* (%)	
Favourable	2 (6)
Intermediate	19 (56)
Poor	12 (35)
No metaphases	1 (3)
FLT3/ITD, *n* (%)	
Present	10 (29)
Absent	15 (44)
Not analysed	9 (26)
CD123	
Present	32 (94%)
Absent	2 (6%)

7.3.4 Characterization and phenotype of leukaemic stem cells

To establish the nature (leukaemic or normal) of the $CD34^+/CD38^-$ cells, CD123 expression was detected in 28 cases. In 28 of 34 cases, greater than 94% of $CD34^+/CD38^-$ cells were $CD123^+$ confirming the leukaemic nature of these cells. In four patients, CD34 was totally negative, so we could not investigate the leukaemic nature of $CD34^+/CD38^-$ cells. In the remaining two $CD123^-$ negative cases, the leukaemic nature of the $CD34^+/CD38^-$ cells was confirmed using phenotypical characteristics (i.e., the presence of a LAP on the $CD34^+/CD38^-$ cells). The LAPs of these 2 patients were aberrant expression of CD2 and CD7 in one patient and CD7 in the other. These LAPs were also present on $CD34^+/CD38^-$ cells. To conclude, phenotypical characteristics confirmed the malignant nature of the $CD34^+/CD38^-$ cells in these two patients.

Figure 7-1 Gating strategy in newly diagnosed AML to identify $CD34^+/CD38^-/CD123^+$ cells. **A**, after labelling of AML cells with the appropriate antibody combinations, the $CD34^+/CD38^-$ cells were identified by a CD45 dim/SS low strategy. Gating on blasts characterized by CD45 dim/low SSC (Lacombe *et al.* 1997). **B**, gating of the blasts within the gate defined by FSC and SSC to identify a population that is roughly homogeneous for scatter properties. **C**, cells from the FSC/SSC plot defined in (**B**) are shown in (**C**) in a plot defined by CD34 and CD38 expression. The $CD38^-$ population was defined using isotype matching as a negative control. **D**, the $CD34^+/CD38^-$ population defined in (**C**) is back gated in a FSC/SSC plot to identify a $CD34^+/CD38^-$ population with homogeneous scatter properties. The frequency of the thus determined $CD34^+/CD38^-$ population was used in this study. **E**, cells from the FSC/SSC plot defined in (**D**) are shown in (**E**) in a plot defined by CD34 and CD123 expression. The $CD123^-$ population was defined using isotype matching as a negative control. **F**, the $CD34^+/CD38^-/CD123^+$ population defined in (**E**) is back gated in a FSC/SSC plot to identify a $CD34^+/CD38^-/CD123^+$ population with homogeneous scatter properties.

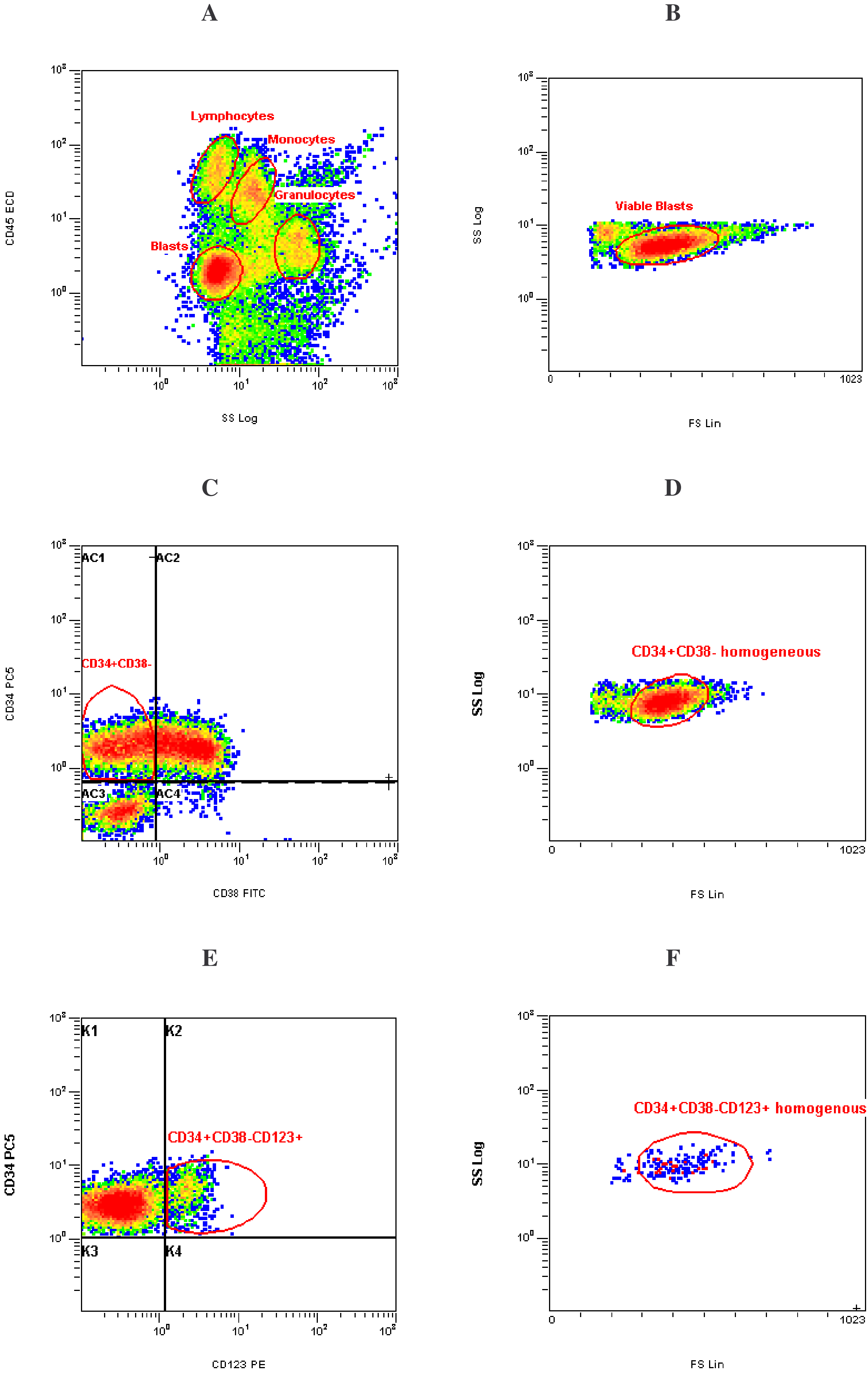
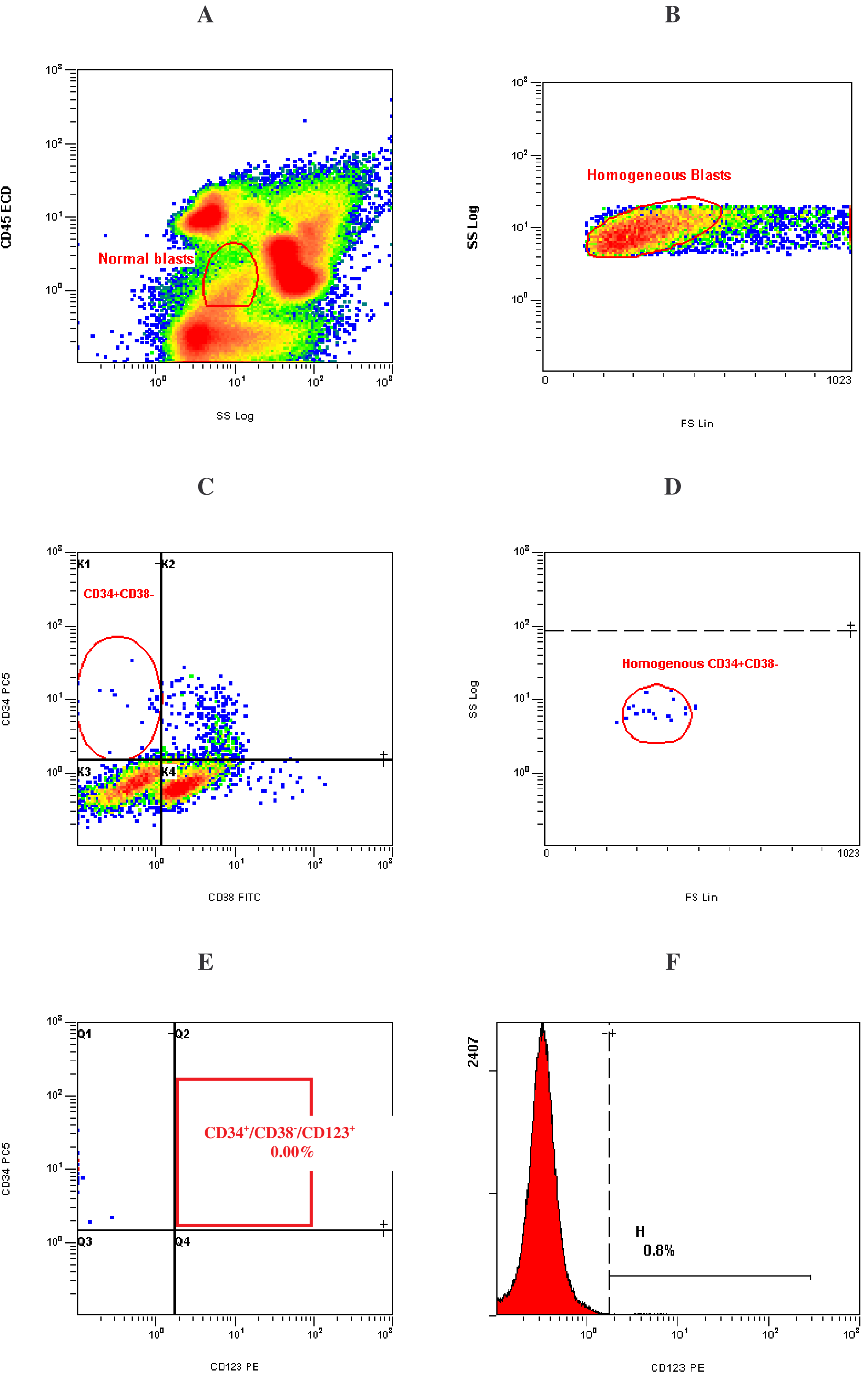

A
B
C
D
E
F
Lymphocytes
Monocytes
Granulocytes
Blasts
CD45 ECD
SS Log
Viable Blasts
FS Lin
AC1
AC2
AC3
AC4
CD34+CD38-
CD34 PC5
CD38 FITC
CD34+CD38- homogeneous
K1
K2
K3
K4
CD34+CD38-CD123+
CD123 PE
CD34+CD38-CD123+ homogenous

Figure 7-2 Gating strategy in normal BM to identify CD34$^+$/CD38$^-$/CD123$^+$ cells. **A**, after labelling of normal BM cells with the appropriate antibody combinations, the CD34$^+$/CD38$^-$ cells were identified by a CD45 dim/SS low strategy. Gating on blasts characterized by CD45 dim/low SSC (Lacombe *et al.* 1997). **B**, gating of the blasts within the gate defined by FSC and SSC to identify a population that is roughly homogeneous for scatter properties. **C**, cells from the FSC/SSC plot defined in (**B**) are shown in (**C**) in a plot defined by CD34 and CD38 expression. The CD38$^-$ population was defined using isotype matching as a negative control. **D**, the CD34$^+$/CD38$^-$ population defined in (**C**) is back gated in a FSC/SSC plot to identify a CD34$^+$/CD38$^-$ population with homogeneous scatter properties. The frequency of thus determined CD34$^+$/CD38$^-$ population was used in this study. **E**, cells from the FSC/SSC plot defined in (**D**) are shown in (**E**) in a plot defined by CD34 and CD123 expression. **F**, the CD123$^-$ population was defined using isotype matching as a negative control**.**


A
B
C
D
E
F
CD45 ECD
SS Log
Normal blasts
SS Log
FS Lin
Homogeneous Blasts
CD34 PC5
CD38 FITC
K1
K2
K3
K4
CD34+CD38-
SS Log
FS Lin
Homogenous CD34+CD38-
CD34 PC5
CD123 PE
Q1
Q2
Q3
Q4
CD34+/CD38-/CD123+
0.00%
2407
CD123 PE
H
0.8%

7.3.5 Sorting AML stem cells

Of the 32 patients positive for the expression of CD123, 10 were FLT3/ITD positive, 15 were WT and 9 were not analysed for the mutation.

To determine expression of FLT3/ITD in AML stem cells, highly purified (purity >95%) $CD34^+/CD38^-/CD123^+$ and $CD34^+/CD38^-/CD123^-$ cells were examined for FLT3/ITD mutation in seven patients with FLT3/ITD positive AML as demonstrated in Figure 7-4. We were unable to perform analysis in the remaining three patients because of lack of material.

The numbers of $CD34^+/CD38^-/CD123^+$ cells sorted ranged from 150 to 300,000 cells and 16 to 148396 cells for $CD34^+/CD38^-/CD123^-$ cells (Table 7-2). Immunomagnetic cell selection (MACS) was used to enrich CD34 cells from patient no. 4, as CD34 in this patient was only 2.3% (Table 7-3). Flow cytometric analysis after selection showed 67% $CD34^+$ cells.

All of $CD34^+/CD38^-/CD123^+$ fractions amounted to less than 1% of the total cells except sample no. 6. In addition, with the exception of sample no. 3, all of $CD34^+/CD38^-/CD123^-$ fractions also amounted to less than 1% of the total cells (Table 7-3).

7.3.6 Optimisation of the PCR technique for low DNA concentrations

To optimise the PCR method for very low DNA concentrations obtained from very few numbers of cells, a study was conducted using different DNA concentrations 5 ng/µl , 2.5 ng/µl, 1.25 ng/µl in 15 µl PCR reaction mix, with 10 µl of DNA added in each tube in duplicate from an AML patient positive for FLT3/ITD. The results of this experiment revealed that FLT3/ITD could be detected at 5, 2.5 and 1.25 ng/µl DNA as shown in Figure 7-3: A. Thus, we used 1.25 ng/µl DNA in sorting experiments for PCR.

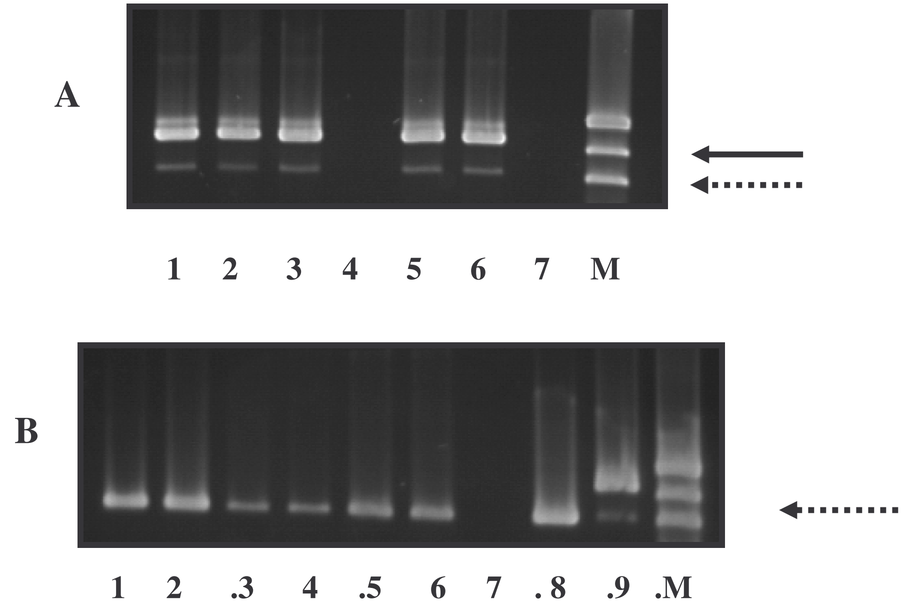
Additionally, another experiment was conducted to optimise the method based on the number of cells obtained from sorting (Figure 7-3: B). Two different methods were undertaken for DNA preparation in the first sample sorted. This was done in order to obtain the maximum yield of DNA. These were salt extraction and QIAmp® Kits. There was no significant difference in the DNA concentration obtained by both methods and hence we proceeded with QIAmp® for the rest of the samples.

Table 7-2: Summary of results from sorting and immunomagnetic cell selection MACS. AML samples were stained with anti-CD34, anti-CD38 and anti-CD123 antibodies and subjected to immunomagnetic selection MACS (patient no. 4) and sorting. Total unsorted cells represents the starting sample size, whereas total $CD34^+/CD38^-/CD123^+$ and $CD34^+/CD38^-/CD123^-$ cells refers to the total number of $CD34^+/CD38^-/CD123^{+/-}$ cells obtained from a given sample after sorting. The cells were also stained with anti-CD123 to analyse for cell surface expression of IL-3α receptor. Genomic DNA from sorted cells was isolated, PCR amplified, and analysed for the FLT3/ITD mutation. Genomic DNA was sequenced for FLT3/ITD for sample no. 3 and 4. ND indicates not done.

Sample No.	Total Unsorted cells	CD34% Unsorted cells	%CD123 Unsorted cells	Total $CD34^+/CD38^-/CD123^+$ cells from the sorter	Total $CD34^+/CD38^-/CD123^-$ cells from the sorter	Sequencing FLT3/ITD
1	$10X10^6$	96	86	150	1606	ND
2	$18X10^6$	89	99	76000	418	ND
3	$12X10^6$	91	86	12674	148396	78 bp
4	$27X10^6$	73[¥]	93	1839	16	33 bp
5	$30X10^6$	10	64	559	164	ND
6	$17X10^6$	15	99	300,000	69	ND
7	$40X10^6$	79	68	640	3602	ND

[¥] after $CD34^+$ selection by MACS, the total yield of $CD34^+$ = $2.4X10^5$ cells

Figure 7-3 Optimisation of PCR technique using FLT3/ITD positive and FLT3/ITD negative patient. A) Using 3 different DNA concentrations 5, 2.5, 1.25 ng/µl in FLT3/ITD positive AML patient. Lanes **1** & **2** (duplicate): FLT3/ITD detected with 50 ng DNA added, Lanes **3** & **4** (duplicate): FLT3/ITD detected with 25 ng DNA added and the PCR failed in lane **4**, lanes **5** & **6** (duplicate): FLT3/ITD detected with 12.5 ng DNA added, **M**: pUC19 molecular marker. The solid line points to FLT3/ITD bp inserted while the dotted arrow points to the WT *FLT3* gene. **B**) Using DNA obtained from different number of cells ($2X10^6$, 150, 1606 cells in duplicates) on WT FLT3 AML patient based on the number of cells obtained from the first sorted sample (sample no.1 in Table 7-2). Lane **1** & **2** (duplicate) DNA obtained from $2X10^6$ cells: FLT3 WT detected, Lane **3** & **4** (duplicate) DNA obtained from 150 cells: FLT3 WT detected, Lane **5** & **6** (duplicate) DNA obtained from 1606 cells: FLT3 WT detected, Lane **7**, no DNA so the PCR was specific, Lanes **8** & **9** are the negative and positive controls and M was pUC19 marker.



7.3.7 Detection of FLT3/ITD in the sorted AML stem cells

Genomic DNA from unsorted and sorted cells was isolated and PCR was performed using primers flanking exons 14 and 15 of the *FLT3* gene (Abu-Duhier *et al.* 2001; Murphy *et al.* 2003). In the 7 positive FLT3/ITD samples analysed, the mutation was clearly present in the LSC-enriched fraction $CD34^+/CD38^-/CD123^+$ confirming that FLT3/ITD mutation arises at the stem cell level (Figure 7-4: A). Interestingly, $CD34^+/CD38^-/CD123^-$ cells in 6 patients were FLT3/ITD negative. In the remaining patient, it is possible that no true $CD123^-$ cells were obtained as CD123 was expressed on 99.2% of $CD34^+/CD38^-$ cells and very low numbers of cells were collected in $CD34^+/CD38^-/CD123^-$ fraction (69 cells only).

In sample 1, the mutation in LSC-enriched fraction $CD34^+/CD38^-/CD123^+$ was most likely present in homozygous form, as less than 1% of the PCR product represented the WT *FLT3* gene. In samples 2 ,3 ,4 ,5 ,6 and 7, the mutations were present in heterozygous form, as approximately 50% of the PCR product represented the WT *FLT3* gene, demonstrated by thickness of the band (Figure 7-4:A). In sample 1, after sorting of the $CD34^+/CD38^-/CD123^+$ fraction, no WT signal was detectable, probably as a result of eliminating the small percent of normal haemopoietic cells in the sample. In all other 6 cases, the FLT3/ITD were very similar between sorted and unsorted fractions. In no case was the mutant allele depleted by sorting for LSCs, which would have occurred if the mutations were not present in those cells. It is interesting to note that 6 samples were heterozygous for FLT3/ITD, even in the stem cell-sorted fractions. It is possible that this reflects a PCR bias for the shorter WT molecule, and that this ratio actually represents 100% of cells with a heterozygous mutation. Alternately, these ratios may suggest the presence of either

additional subclones of leukaemic $CD34^+/CD38^-/CD123^+$ cells that lacked the mutation, or residual normal (i.e., non-leukaemic) stem cells within these samples.

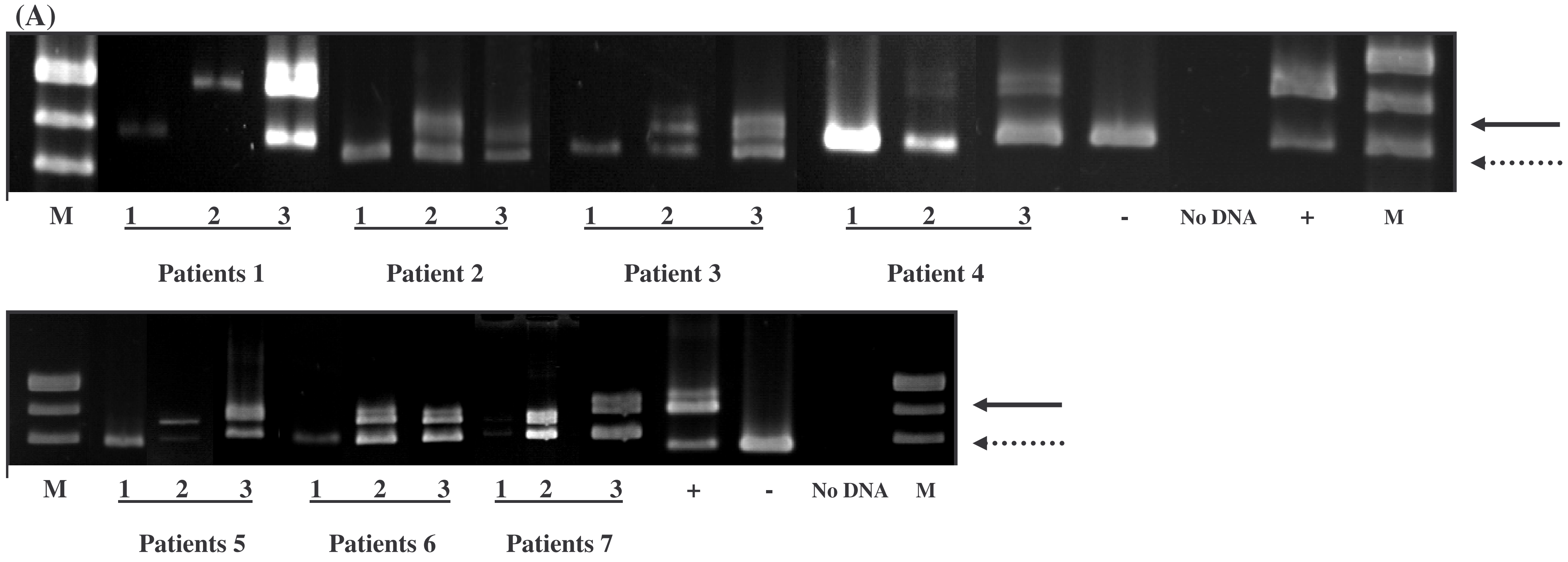
7.3.8 Sequencing the FLT3/ITD in two sorted AML stem cells

The FLT3/ITD mutations in the $CD34^+/CD38^-/CD123^+$ cells were sequenced in 2 patients (patient 3 and 4)(Figure 7-4: B) to confirm that they represented the identical mutations present in the sorted and unsorted original samples. In one patient, 33 bp inserted and the other one was 78 bp inserted. The fact that this expanded population of LSC contained the identical FLT3/ITD mutation as was observed in the original unsorted samples constitutes direct evidence that the FLT3/ITD mutations were present in LSC.

7.3.9 Detection of FLT3/ITD in the sorted population of LAP positive AML patient

In one patient positive for FLT3/ITD, aberrant phenotypes $CD34^-/CD38^-/CD2^+$ & $CD34^+/CD38^-/CD2^-$ as well as $CD34^+/CD38^-/CD7^+$ & $CD34^-/CD38^-/CD7^-$ populations were sorted to homogeneity by BD FACSAria™ cell sorting system and then the genomic DNA from the purified cells were analysed for FLT3/ITD (Figure 7-5). The results from this experiment demonstrated two things: 1) at least for this patient, LAP is arising from LSC clone, and 2) FLT3/ITD in the LAP was in the homogenous form, and the WT allele initially identified in the diagnosed unsorted sample was depleted. This means that all the cells in that LAP are mutated. However, these results cannot be generalised as this experiment was done only for one patient.

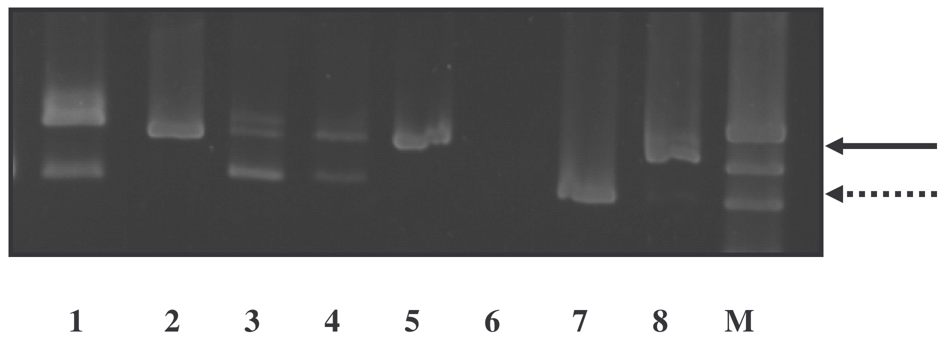
Figure 7-4 FLT3/ITD mutations in the BM cells of 7 AML patients. (**A**) $CD34^+/CD38^-/CD123^+$ and $CD34^+/CD38^-/CD123^-$ cells from different patient samples were isolated by sorting (sorting results displayed in Table 7-2). For each of the 7 samples, genomic DNA was isolated from the (**1**) $CD34^+/CD38^-/CD123^-$, (**2**) $CD34^+/CD38^-/CD123^+$ cells, and (**3**) unsorted cells at diagnosis. PCR was performed using primers flanking the JM region of FLT3 (along with positive and negative controls). The characteristic doublet of the FLT3/ITD mutation is easily visualized after electrophoresis in 2% agarose with ethidium bromide staining. Genomic DNA from known positive and negative cases was used as controls. (**B**) The sequences of the FLT3/ITD mutations from the 2 samples (patients 3 and 4) are shown. These sequences, obtained from the DNA of $CD34^+/CD38^-/CD123^+$ cells, matched the sequences of the mutations present in the pre-sorted samples. bp indicates base pair. Boldface sequence refers to the inserted, duplicated material.



M Molecular marker pUC19
1 $CD34^+$/$CD38^-$/$CD123^-$
2 $CD34^+$/$CD38^-$/$CD123^+$
3 Unsorted sample at diagnosis
- Negative control
+ Positive control
(B)

Patient 3: (33bp): catcccccc**gatttcagagaatatgaatatgat**
Patient 4: (78bp): **gtacaggtgaccggctcctcagataatgagtacttctacgttgatttcagagaatatgaatatgatctcaaatgggag**

Figure 7-5 FLT3/ITD in sorted LAP positive population of an AML Patient. Lane **1** demonstrates the unsorted diagnosed AML with about 420 bps insertion detected, lane **2** & **5** show the sorted population for LAPs $CD34^+/CD38^-/CD2^+$ & $CD34^+/CD38^-/CD7^+$: ITD with ~ 100 bps insertion detected (homozygous form indicating the all the cells sorted for LAPs are mutated) and the WT allele was lost. Lanes **3** & **4** demonstrate $CD34^-/CD38^-/CD2^-$ & $CD34^-/CD38^-/CD7^-$ populations: FLT3/ITD detected with the same size but with the WT band as well. Lanes **6**, **7** & **8**: no DNA, negative and positive controls, respectively. **M** indicates the molecular marker pUC19. The dotted arrow points to the WT allele while the solid arrow points to the FLT3/ITD.



7.4 DISCUSSION

In this study, we hypothesised that the FLT3/ITD mutation occurs at a stage of stem cells defined by $CD34^+/CD38^-$ and IL-3 α receptor in AML. In addition, FLT3/ITD is not a secondary event in leukaemogenesis.

We have identified that expression of CD123, which facilitate LSCs discrimination from normal HSCs, is found on virtually almost all AML specimens examined similar to Jordan *et al* (Jordan *et al.* 2000). The high levels of expression observed could simply be indicative of some other conserved event in leukaemogenesis. Furthermore, CD123 expression was also demonstrated on the primitive subpopulation of $CD34^+/CD38^-$ cells.

We demonstrate FLT3/ITD mutations are found in a primitive fraction of cells defined as $CD34^+/CD38^-/CD123^+$. In addition, there was evidence to suggest that the FLT3/ITD mutations were present within purified enriched LSC defined by CD123 and absent within stem cells without CD123. The specimens derived from several different FAB subtypes M1, M2, M4, M5a and therefore represent a broad cross-section of commonly detected AML types.

Although the sorted cells in most of the samples comprised less than 1% of the total population, it is certainly possible that the true LSCs represent a smaller fraction of the total $CD34^+/CD38^-$ group. If $CD34^+/CD38^-/CD123^+$ subset contains a significant fraction of LSCs, then the FLT3/ITD imply that most of these mutations are in cells capable of self-renewal. Ideally, these enriched leukaemic cells should be injected into NOD/SCID mice but this is technically challenging with such small number of cells. This issue is important because several lines of evidence have previously suggested that these mutations can occur relatively late in the development of leukaemia. Three separate studies have demonstrated that in a small proportion of cases, FLT3/ITD

mutations are lost at relapse (Nakano *et al.* 1999; Kottaridis *et al.* 2002; Shih *et al.* 2002). This suggests that, in these few cases, the mutations occurred in a subclone that was eliminated by treatment. Given that numerous small molecule FLT3 inhibitors are in development, it is important to establish that the target of these new agents is a target within the cell responsible for initiating and maintaining the disease.

One could hypothesise because HSC already possess the ability for enhanced self-renewal (a necessity for LSC), fewer mutations would be required to subvert this cell into an LSC. Our results are not consistent with the previous findings of occasional loss of FLT3/ITD mutations at relapse. It still seems likely that in at least a subset of AML, the FLT3/ITD mutations are present only in subclones derived from the original LSC, subclones that lack the capacity for self-renewal. It may be that our sample size was simply not large enough to uncover such cases. In particular, the group of leukaemia samples we studied did not include any with a low mutant-WT ratio. These may represent the cases in which the mutation arose as a relatively late hit in leukaemogenesis, and may be the cases in which the mutation is lost at relapse. Alternately, FLT3 mutations could always be present in LSC, but occasionally chemotherapy succeeds in eradicating the FLT3/ITD samples, whereas other LSCs that lack the mutation are resistant.

In the study of Masao *et al* (Mizuki *et al.* 2003), they identified target genes of the FLT3/ITD by microarray expression profiling. ITD mutations induced transcriptional programs that partially mimicked IL-3 activity with many genes being specifically regulated by the mutations but not by ligand- activated WT FLT3. They also have shown that FLT3/ITD mutations induce a transcriptional program that is fundamentally different from the program induced by FLT3-WT. The FLT3/ITD mutations mimic IL-3 activation and activate genes involved in cellular growth and proliferation. In addition, FLT3/ITD regulates transcription factors independent of the

IL-3-mimicking effects and antagonizes the activity of WT FLT3 on myeloid differentiation programs. Therefore, the combination of both mechanisms may be the basis for the strong oncogenic function of ITD mutations.

A number of recent studies suggest that targeting of CD123/IL-3 alpha receptor may be a novel promising treatment approach in patients with $CD123^+$ AML (Jordan *et al.* 2000; Moretti *et al.* 2001; Munoz *et al.* 2001). This concept is based on the notion that in most patients with AML, myeloblasts express CD123 as shown in our study. Considering the use of CD123-targeted drugs in AML, it is of particular importance to identify that these cells express leukaemia specific molecular abnormality such as FLT3 mutations.

In the future, it would be of great interest to study the frequency of $CD34^+$/$CD38^-$ $CD123^+$ cells during follow-up as these cells may represent the highly resistant cells to chemotherapy and might be the cells that cause regrowth of leukaemia and thus relapse of the disease. In principle, this would allow us to correlate LSC frequency at diagnosis with LSC frequency in CR after chemotherapy. It would be of value to study if the prognostic impact of the MRD stem cell frequency might improve the already strong impact of total MRD frequency in LAPs on outcome. Apart from LAP expression, the LSC selective expression of CD123 and of the recently described CLL-1 (Bakker *et al.* 2004; van Rhenen *et al.* 2007) should be very helpful in this respect.

In CD34 negative AML, the compartmentalization of stem cells is still unknown. As ~ 28% of AML samples in our series were CD34 negative, more effort is needed to identify stem cells in CD34 negative AML.

Eradication of malignant stem cells can probably be reached in part of the patients, using conventional chemotherapy through the application of repetitive cycles of consolidation therapy (Mayer *et al.* 1994). However, given that LSCs are contained

within the CD34$^+$/CD38$^-$/CD123$^+$ population in AML and the apparent difficulties in eradicating malignant cells from the patient, it will be necessary to develop therapies that specifically target these malignant stem cells of this phenotype after reaching CR. The possibility to detect and characterize stem cells will help to reach this goal.

CHAPTER 8

Summary, General Discussion, And Perspectives

CHAPTER 8:SUMMARY, GENERAL DISCUSSION, AND PERSPECTIVES

8.1 SUMMARY

AML is an aggressive haematological malignancy that currently is managed with intensive chemotherapy. Cytogenetic and molecular genetic markers are well-established prognostic markers. The role of immunophenotyping in assessing prognosis has been previously studied in two ways. The presence of individual markers has been correlated with rate of relapse and OS. Alternatively, MRD has been detected following chemotherapy and persistence of MRD has been correlated with relapse. In spite of a number of previous studies, the role of immunophenotyping in assessing prognosis in AML remains unclear.

The choice of antibodies, the threshold values for positivity (e.g. percentage positive cells) and varying patient population selection may explain some of the discrepancy between different studies. Of concern, most studies demonstrating positive correlations between immunophenotyping or the presence of MRD as detected by immunophenotyping techniques and prognosis do not hold up in multivariate analysis indicating that other associated determinants exert a greater effect on prognosis.

The ideal assay system for the detection of small numbers of leukaemic cells (MRD) in a marrow or blood samples should fulfil the following criteria:

1. The method should be applicable in most cases of the disease under investigation,
2. The method should be specific for neoplastic cell type,
3. The method should be sensitive,
4. The method should allow quantitation of tumour burden for prognostic purposes (Provan and Gribben 2005).

In **Chapter 3,** we have shown that using the standard diagnostic panel at our institution, LAPs were detected in 54 (64%) of 84 patients analysed for AML in three years. The presence of LAPs was an independent predictor of failure to achieve CR after induction chemotherapy in multivariate analysis considering all the known confounding factors ($p < 0.05$, 95% CI, 1.0 - 2.6) with an estimated risk ratio of 1.6. The relapse rate was also higher in patients who were LAP positive, although this did not reach statistical significance ($p > 0.05$) when APL patients excluded from the analysis. This study was recently accepted for publication in *International Journal of Laboratory Hematology (IJLH).*

In **Chapter 4,** using a five-colour MFC based approach and extensive MoAbs combinations, LAPs were detected in the vast majority of patients 51/54 (94%). In contrast, when the same cohort of patients were studied with the routine standard diagnostic panel at our institution (for comparison purpose), LAPs were only detected in 43/54 (79.6%) ($p < 0.05$). In addition, this approach was highly reproducible and sensitive with a detection limit of one leukaemic cell in approximately 10^5 normal cells. We demonstrated by determining the maximum LD that CD7, CD19, CD2, CD11b and CD56 are the most sensitive and reliable markers for MRD studies. Furthermore, CD45 gating strategy in addition to inclusion of progenitor stem cells markers such as CD34 and CD117 in immunophenotyping increased the specificity of this assay for the detection of malignant cells by accurate identification of the blast and stem cells. Analysis of normal and regenerating BMs showed the incidence of LAPs in these populations was extremely rare, further justifying the use of LAPs as markers for MRD detection. There was no significant difference in the incidence of LAPs between normal or regenerating BMs as controls for MRD studies.

In **Chapter 5,** detection of MRD using five-colour MFC was able to predict relapse with a threshold of 0.15% residual leukaemic cells with optimal sensitivity and specificity for both after induction and consolidation chemotherapy. With the limited sample size analysed, we were surprised to find that the presence of MRD post induction was an independent predictor of poorer prognosis as indicated by both decreased RFS and OS (hazard ratio = 4.7 and 5.2, $p = 0.037$ and 0.026, 95% CI, 1.1-20.5 and 1.2-22.2, respectively). The presence of MRD post consolidation showed a trend towards poor prognosis with decreased RFS ($p = 0.06$).

In **Chapter 6,** we studied the incidence of FLT3/ITD in AML. FLT3/ITD was detected in 31% of our cohort. FLT3/ITD patients had higher WBC count and blast percentage at diagnosis. The presence of the mutation did not correlate with decreased OS or RFS as demonstrated in previous studies. However only a small sample size was analysed and patient follow up was short.

In **Chapter 7,** we presented evidence that the FLT3/ITD mutation is present at the LSC level and may be a primary and not secondary event in leukaemogenesis. Our findings demonstrated that FLT3/ITD mutation was confined to a population of $CD34^+/CD38^-/CD123^+$ but not $CD34^+/CD38^-/CD123^-$ cells in the 7 AML patients. We have shown that the oncogenic events of FLT3/ITD happen at cell stage possessing the alpha chain of the IL-3 receptor. These novel findings provide a rationale for treatment involving CD123-targeting antibodies combined with intracellular FLT3 inhibitors directed against AML stem cells. We are currently developing techniques to further investigate the nature/function of $CD34^+/CD38^-/CD123^+$ and $CD34^+/CD38^-/CD123^-$ cells.

The results from this thesis indicate that the use of five-colour MFC improves detection of aberrant immunophenotypes in AML and extends the applicability of

MRD studies to virtually all AML patients with high sensitivity and specificity for cells possessing LAPs. This study is (to the best of our knowledge) the first MRD analysis to use five-colour. Additionally, it shows that detection of LAPs at diagnosis and at follow-up after achieving CR using immunophenotyping techniques to detect MRD are independent predictors for poor prognosis in multivariate analysis.

However, as an alternative, the detection of $CD34^+/CD38^-/CD123^+$ cells, which include the LSC population, may serve as a better approach for MRD studies in the future. This marker set characterises cells that are responsible for initiation and maintenance of AML and regrowth of the leukaemia and thus relapse of the disease. It might also be a useful prognostic indicator at diagnosis, as the current chemotherapeutic approaches do not target these cells, so it is tempting to speculate that the frequency of these cells at diagnosis is the same as their frequency after achieving CR. This needs to be further explored.

8.2 GENERAL DISCUSSION

8.2.1 Clinical applications of MRD in AML

8.2.1.1 Comparison with previous clinical studies

In the past two decades, tremendous effort has been made to describe and characterise residual leukaemic cells and relate their presence to prognosis and therapy. The goal of such studies of MRD is to optimise the clinical management of the post-remission phase in patients with acute leukaemia, offering the opportunity to guide the therapeutic decisions based on specific biologic findings. The ultimate purpose of these types of studies is to distinguish patients who respond successfully to standard treatment, and those who are likely not to need additional therapy to reduce risk of relapse.

Drach *et al* (Drach *et al.* 1992) evaluated MRD in 68 patients with AML. In follow-up assessment of BM samples collected consecutively after induction therapy, they observed that only patients in whom LAP could continue to be detected suffered from early relapse. On the other hand, patients who became MRD negative remained in CR with median remission duration of 52 weeks after the first negative finding. They concluded that the persistence of MRD was indicative of impending relapse. In a paediatric series of 39 AML patients, Sievers *et al*. (Sievers *et al.* 1996) detected MRD in more than 50% of patients in morphologic CR; the estimated risk of relapse during the MRD positive period was 2.8 times greater than during time in which MRD was negative ($p = 0.02$).

In another study (Feller *et al.* 2004), AML patients in a trial setting were evaluated for residual leukaemic cells. MRD was monitored in follow-up samples taken from BM of 72 patients after three different cycles of chemotherapy and from autologous

peripheral blood stem cell (PBSC) products. The MRD % in BM after the first cycle (n = 51), second cycle (n = 52) and third cycle (n = 30), as well as in PBSC products (n = 39) strongly correlated with RFS. At a cut-off level of 1% after the first cycle and median cut-off 0.14% after the second, 0.11% after the third cycle and 0.13% for PBSC products, the relative risk of relapse was higher by a factor of 6.1, 3.4, 7.2, and 5.7, respectively, for patients in the higher MRD groups. In addition, absolute MRD cell number/ml was highly predictive of the clinical outcome. After the treatment had ended, an increase of MRD % predicted forthcoming relapses, with MRD assessment intervals of ≤ 3 months. Thus, Feller *et al*. concluded that MRD assessment at different stages of disease is highly reliable in predicting survival and impending relapses and even after the completion of chemotherapy, sequential evaluation of MRD frequencies can be used to predict impending relapses.

San Miguel *et al*. (San Miguel *et al.* 1997) examined the prognostic role of MRD detection in 53 AML patients with LAPs at presentation. The patients had achieved morphological CR after standard AML regimens and the levels of MRD were investigated serially at the end of induction and intensification therapy. The authors established that the amount of MRD at the end of induction and intensification was directly correlated with the relapse and RFS in line with our findings. Specifically, they found that the patients with MRD cells ≥ 0.5% after induction therapy had a higher rate of relapse (67%) compared to those with < 0.5% residual cells (20%) ($p = 0.002$). Similarly, these patients had a shorter RFS ($p = 0.01$). At the end of intensification, the value of 0.2% residual leukaemic cells grouped patients in two different categories with a relapse rates of 69% and 32%, respectively ($p = 0.02$); the patients with ≥ 0.2% residual leukaemic cells also showed a lower RFS (p =

0.04). Detection of MRD ≥ 0.5% after induction and 0.2% after intensification was also correlated with a shorter duration of OS. In addition, the authors investigated whether the levels of MRD were correlated with the expression of multidrug resistance protein (MDR1) function. In the cases where MRD was > 0.5% residual leukaemic cells after induction therapy, a significantly higher rhodamine-123 efflux at diagnosis was observed. Finally, the impact of the level of MRD on RFS was established in multivariate analysis.

However, Venditti *et al*, in their study of 56 AML patients with LAPs (Venditti *et al.* 2000) found that the level of MRD after consolidation therapy was the best predictor of outcome. In fact, MRD level of 0.035% after consolidation was significantly correlated with a high relapse rate (77% vs. 17%) ($p < 0.001$). This MRD level was also significantly correlated with poor or intermediate cytogenetics, MDR1 phenotype, short duration of RFS and OS ($p = 0.014$; 0.031, 0.00022 and 0.00014, respectively). The prognostic role of MRD positivity after consolidation therapy was confirmed in multivariate analysis. Importantly, in their study, autologous stem cell transplantation was shown not to play a role in changing the negative effect of high MRD levels after consolidation. Indeed, the relapse rate after transplantation was 70% vs. 28% in those patients with a negative result at the end of consolidation ($p = 0.031$).

Although the concluding message is the same, Venditti *et al* and our study are discrepant. In fact, we and San Miguel *et al*. demonstrated a correlation with relapse for level of MRD after induction; on the contrary, Venditti *et al* found that the level of MRD after consolidation, but not after induction, was the important prognostic factor. The different therapeutic regimens used in the two studies may explain this variance. In San Miguel study, the induction therapy consisted of one or two

courses of an anthracycline and cytosine arabinoside (3 + 7 regimen), followed by an identical consolidation course. This was followed by one or two intensification courses consisting of intermediate/high dose cytosine arabinoside and either daunorubicin or idaraubicin. This is similar to the treatment in our centre. However, in Venditti at al study, the patients were treated on the EORTC/GIMEMA AML-10 and AML-13 protocols, which are three drug-based regimens, associating an anthracycline with cytosine arabinoside and etoposide. In addition, in the AML-10 protocol, cytosine arabinoside administration is prolonged through 10 days instead of the conventional seven, during the induction phase. Thus, one would believe that in the study of San Miguel and our study, less intensive therapy was associated with a milder purging effect, which in turn may account for different levels of MRD associated with increased risk of relapse. This may also explain why the number of residual leukaemic cells after induction correlated with the rate of relapses and RFS, whereas, in Venditti experience such a correlation was found only after consolidation.

San Miguel *et al*. in a further publication (San Miguel *et al.* 2001) confirmed the importance of the level of MRD after induction therapy. The authors found that early response to chemotherapy, as determined by immunophenotyping in the first BM in morphologic CR after induction therapy, might identify patients at low risk of relapse. The study included 126 patients with AML who had aberrant phenotypes at diagnosis. Based on the level of MRD after induction therapy, four categories of risk were identified: (1) very low risk ($<10^{-4}$ cells), (2) low risk (10^{-4} to 10^{-3} cells), (3) intermediate risk (10^{-3} to 10^{-2} cells), and (4) high risk ($>10^{-2}$ cells). The authors concluded that MRD detection after first cycle of chemotherapy and achieving morphologic CR is an important tool for risk assessment in patients with AML.

Even with a smaller cohort of patients, we were able to show that MRD detection greater than 0.15% is an independent predictor of poor prognosis after induction chemotherapy and our studies support MRD detection as a prognostic indicator of use in clinical practice.

8.2.1.2 Future directions for MRD detection

Precise information on MRD may in future provide the biological basis for therapeutic decision making (e.g. pre-emptive treatment in cases of molecular relapse), and thus allow specific treatment to be tailored to the needs of the individual patient. Ultimately, the clinical usefulness of MRD monitoring in the routine management of patients, will need to be confirmed in prospective trials involving large numbers of patients, uniformly treated and monitored within well-defined protocols. Quantitation of MRD, especially with sensitive PCR and MFC assays, has not only enabled the study of the kinetic behavior of leukaemic cells during chemotherapy and remission, but has also increased our understanding of the biology of AML and leukaemia in general and opened up new areas of scientific interest which will need further enquiry.

In AML, novel treatments are needed if one hopes to improve cure rates substantially. Thus, the highest value of MRD assays may possibly lie in the rapid measurement of the effect of novel therapies on the leukaemic clone.

8.2.1.3 MRD detection using five-colour MFC

Previous studies reporting on the prognostic impact of MRD level in patients with AML in CR mainly focused on only a proportion of patients with AML expressing the highly aberrant immunophenotypes (Venditti *et al.* 2000; San Miguel *et al.* 2001; Coustan-Smith *et al.* 2003; Feller *et al.* 2004). In our study, LAPs were able

to be detected in almost every patient with AML when the comprehensive panel of MoAbs was applied. The introduction of four-colour flow cytometry and CD45 gating has been shown to improve detection of leukaemic cells among normal BM cells with a log increase of sensitivity for MRD quantification over three-colour flow cytometry (Kern *et al.* 2004).

In our study, we demonstrate that the detection of LAPs by five-colour flow cytometry with CD45 gating can be further improved by determining the maximum LD between % LAPs at presentation and at the baseline normal and regenerating BMs. The maximum LD and hence, the most sensitive LAPs were found in the groups of LAPs with lineage infidelity: CD2, CD7, CD19 and asynchronous antigen expression: CD11b and CD56.

The percentage of LAP positive cells in AML samples was the same for both five-colour and three-colour flow cytometry. However, the frequency of LAP positive cells in normal and regenerating BM was reduced by applying five-colour staining flow cytometry and hence the specificity for MRD detection increased. This finding is most important with respect to post-therapy evaluations, as regenerating BM samples have not been used in previous reports as controls. In addition, the number of LAPs identified using five-colour was more frequent when compared to three-colour. The sensitivity of MFC increases in parallel to the number of aberrantly expressed antigens.

8.2.2 Potential limitations for MRD detection using immunophenotyping

The literature varies in the reported incidence of LAPs expressed in leukaemic samples, and a clear example of which is the described incidence for cross-lineage and asynchronous antigen expression in AML (Campana *et al.* 1990; Reading *et al.* 1993; Campana and Pui 1995; Macedo *et al.* 1995; Orfao *et al.* 1999; Vidriales *et*

al. 2003; Voskova *et al.* 2003; Chang *et al.* 2004) and its potential prognostic value. A critical analysis of these reports shows that there are a wide range of different reagents and methods used for both sample preparation and data acquisition and analysis. In addition, a lack of standardised criteria for data interpretation is a common finding that introduces variability. Hence, morphology continues to be the standard of reference for the immunophenotypic characterisation of leukaemia, which according to Paietta *et al*. (Paietta *et al.* 1996) should be considered as "a relic of the past".

A significant number of factors that may affect the results of MRD analysis have already been identified, at least to a large extent (Bene *et al.* 1995; Lanza 1996; Borowitz *et al.* 1997; Davis *et al.* 1997; Jennings and Foon 1997; Jennings and Foon 1997; Bain *et al.* 2002). Among them, technical aspects such as the type and quality of the sample, the reagents and the sample preparation protocols, instrument set-up and calibration, and the potential component of bias introduced during data analysis or with the interpretation of the results correspond to the most common sources of variability. Also, the inability to identify immunophenotypic switch as well as lineage switch, a phenomenon that may occur at relapse, although at a low rate in AML patients (San Miguel *et al.* 1997; San Miguel *et al.* 2001).

Based on this knowledge, several scientific groups (Bene *et al.* 1995; Lanza 1996; Borowitz *et al.* 1997; Davis *et al.* 1997; Jennings and Foon 1997; Jennings and Foon 1997; Bain *et al.* 2002) have discussed these aspects in detail and have reported their consensus opinions and recommendations. However, in many of these reports, the type of information provided by the flow cytometry immunophenotyping of AML is not analysed in depth. Such a detailed analysis would certainly contribute to understanding the answers provided by flow

cytometry to the specific questions posed in current practice that are related to the MRD in AML, include: detection of MRD in CD34 and CD117 negative leukaemic cells, and accurate quantification of over-expression of myeloid or stem cell markers as it not clear from the literature what is the true definition/level of over-expression of an antigen. Furthermore, true threshold of MRD positivity or negativity. Moreover, although in few cases, however, the differentiation of leukaemic blasts and monocytes in M4 and M5 using MFC is not clear, as usually in these cases the blast cells form a continuum and merge with a large monocytoid population. Therefore, more efforts are needed in these areas.

In general, it may be considered that, at least to a certain extent, the type of information requested influences the analytical procedure to be used. In other words, one of the most relevant aspects when performing or requesting flow cytometric immunophenotyping of AML is to have a clear idea of the type of information provided by this test that could help in answering a specific clinical question.

Other potential limitation of this research was the small sample size, which has been reduced from 54 AML patients analysed at diagnosis to 22 patients followed for MRD investigation after achieving CR. Indeed, this represents the reality when studies done on prospective fashion from consecutive, unselected patients and may be more informative. Additionally, the short follow-up period might be another limitation of this study due to the time constraints of the PhD candidature.

8.2.3 Points for consideration

A number of important points for consideration and questions, which have biological and clinical relevance, remain unanswered:

(1) The need for changing the current definition of CR, which is still, based on the morphologic appearance of BM whatever the method used; investigation of MRD identifies patients who will relapse regardless of a standard morphologic CR. Following the proposal of Pui and Campana in paediatric ALL (Pui and Campana 2000) and the paradigmatic experience of the GIMEMA group in the treatment of APL (Lo Coco *et al.* 1999), it may be useful that in AML, CR is re-defined according to immunologic and molecular criteria;

(2) Which level of MRD is well matched with cure?

(3) The value of immunophenotyping as compared to molecular monitoring of MRD. The two techniques may be able to be efficiently integrated since approximately 30% of AML carry distinguishable molecular markers in contrast to the high incidence of LAPs. The combined use of FISH and MFC for MRD detection established that only 25–30% of the residual leukaemic cells as identified by an aberrant phenotype, had the same cytogenetic markers as observed at diagnosis in at least 90% of the blasts (Venditti *et al.* 2002). Theoretically, molecular defects and chromosomal abnormalities may take place at precise time-points during the development of the leukaemic phenotype, as established in APL (Turhan *et al.* 1995);

(4) The role of additional therapy in the case of high level of MRD after induction and/or consolidation therapy;

(5) Analysis of peripheral blood samples to determine whether MRD may be successfully monitored in this source as already demonstrated in paediatric ALL (Coustan-Smith *et al.* 2002; Coustan-Smith *et al.* 2003),

(6) The use of CD45-gating strategy. A significant step forward has been the introduction of the simultaneous detection of more fluorochromes and the use of

CD45-gating, since CD45 is differentially expressed not only between different lineages of haemopoietic differentiation but also within these lineages during the process of maturation, it is capable in combination with SS (Kern *et al.* 2004) of clearly differentiating blasts from other cells. Future clinical trials have to validate the translation of these data into improved prognostic parameters.

(7) The value of studying MRD in CD34 negative AML, as approximately 20% of AML samples are completely negative for CD34 or CD117. More effort is needed to identify stem cells in CD34 negative AML.

(8) One of the main causes of false-positive MRD results by flow cytometry is the use of inappropriate markers to distinguish leukaemic cells from normal cells. The range of normality needs to be established by extensive studies of BM collected not only from healthy individuals but also from regenerating BM i.e. patients at various stages of treatment or post transplantation.

8.2.4 CD123 as a marker for LSC and a single standardised marker for MRD studies

It is now generally accepted that relapse starts with leukaemic cell expansion arising from the AML stem cell. In AML, the stem cell are contained within a population of CD34 positive, CD38 negative cells or, in the absence of such a fraction, within a population defined by specific staining with the vital dye Hoechst 33342, the so-called side population (SP). However, these markers do not discriminate between normal and LSCs. Recently, it was found that the antigen CD123 and CLL-1 (van Rhenen *et al.* 2007) are present on leukaemic but not normal stem cells. It may be possible to define MRD using these markers (i.e. $CD34^{+}/CD38^{-}/CD123^{+}$), which detect populations containing the LSC. This approach would use a much smaller number of antigens and antigen combinations, resulting in relatively simple data

interpretation. Lastly, the approach enables MRD detection in LAP negative AML, not accessible for the classic "whole blast MRD" detection.

The approach of tracking LSC cells through following the $CD34^{+}/CD38^{-}/CD123^{+}$ population post chemotherapy could be used in the following ways:

1. That detection of residual LSC can replace MRD follow-up using LAPs.
2. Its relative simplicity will make the technique accessible for routine use. The experience already acquired during implementation of "whole blast MRD" in large clinical studies, will serve as a matrix.
3. The possibility to predict relapse for all individual patients will open the way for individualised therapy. Similar to "whole blast MRD", detection of residual LSC could guide clinical intervention.
4. Hopefully, the above approach will also allow LSC enriched populations for any characteristic that may serve to develop new therapies or improve existing therapies.

8.2.5 Prognostic value of FLT3/ITD in AML

Clinical data suggest that poorer prognosis is related to the presence of FLT3/ITDs. We could not show such a correlation most likely due to the reasons discussed in chapter 6. However, patients with FLT3/ITDs belong to a distinct AML sub-population that seems to poorly respond to current treatment protocols and thus may need risk stratification with treatment tailored to these prognostic factors.

Present studies do not exclude that the poor prognosis of FLT3/ITD AML is an epiphenomenon, i.e. the observed poor prognosis is not due to the mutations themselves but by some other feature of the LSCs. Such a mechanism could for example be a genetic instability that induces a tendency to result in the mutation of

both FLT3 (Libura *et al.* 2003) and other genes, which in turn are the true cause of the poor prognosis.

8.2.6 Does FLT3/ITD mutation occur at the level of the leukaemic stem cells?

Recently the concept of LSC (Reya *et al.* 2001) has gained increasing support. If the leukaemia within a patient arises from such a malignant stem cell, the treatment should ideally be directed against these stem cells.

Most of the studies carried out to reveal the biological characteristics of FLT3/ITD AML have been performed using cell line models, such as 32D and BAF/3. These studies do not resolve the question of whether or not FLT3/ITDs occur at the level of the LSCs or at a later stage. However, certain observations in our patients allow us to address this question. The presence of FLT3/ITD in $CD34^{+}/CD38^{-}/CD123^{+}$ and not in $CD34^{+}/CD38^{-}/CD123^{-}$ populations in 6 AML patients supports the hypothesis that this mutation is present at LSC level and may not be a secondary event. Ideally, engraftment of these cells in NOD/SCID mice should be the best evidence. This approach was not able to be carried out due to technical reasons.

However, several independent studies of paired diagnostic and relapse AML samples have revealed that a small but consistent portion of patients with AML initially harbouring FLT3/ITD mutations lack these mutations at relapse (Nakano *et al.* 1999; Kottaridis *et al.* 2002; Shih *et al.* 2002). This would suggest that at least in some cases, the mutations occurred at a later stage of leukaemic transformation, and that chemotherapy was successful in eradicating the clones expressing the FLT3 mutations. Furthermore, some samples were actually found to contain multiple different FLT3/ITD mutations, again suggesting that they are present in subclones of cells (Lowenberg *et al.* 1999).

Until now, the observation of an increased outgrowth of FLT3/ITD AML in NOD/SCID mice suggests that in a large part of the FLT3/ITD positive leukaemias, the mutation is present at the level of the malignant stem cell. Nevertheless, it is not clear if the observed outgrowth is solely dependent on the transplanted LSCs or that also other, more committed, cells are able to expand in this model. The occurrence of an enhanced survival of FLT3/ITD AML cells is further supported by the observations of Schnitgger *et al*. (Schnittger *et al.* 2002) and Kottaridis (Kottaridis *et al.* 2002) who observed that FLT3/ITD patients who relapsed, for the greater part showed an increased mutant to WT ratio at relapse.

8.3 CONCLUDING REMARKS

Based on published data and our study, it is expected that the quantification of MRD will significantly improve the evaluation of the prognosis in patients with AML and ultimately play a major role as a stratification parameter to guide the risk-adapted therapy of the disease.

Therefore, it seems reasonable to intensify therapy for those patients who have a slow early response to treatment and have detectable MRD during clinical remission (Campana and Coustan-Smith 1999). Alternatively, the excellent clinical outcome of MRD-negative cases raises the possibility of using MRD assays to identify candidates for evaluating reductions in treatment intensity. Moreover, MRD monitoring during the follow-up after the end of therapy is useful, providing an adequate observation in patients who demonstrate a tendency toward MRD growth.

We still do not know whether early detection of relapse and subsequent changes in therapeutic strategies will improve cure rates, but there are reasons to believe that this might be the case. First, it is well established that the residual leukaemic cell burden and the curability of cancer are related. Second, the likelihood of the

emergence of drug-resistant leukaemic cells by mutation increases as the number of cell divisions increases, and hence relates to the total leukaemic cell burden (Goldie and Coldman 1986). Thus, timely detection of MRD would identify patients who need more intensive therapy to remain in remission. However, known prognostic factors are not 100% predictive and MRD studies might well complement and enhance their informative value.

Our experience in the use of MFC for detection of MRD in AML has shown that the method is feasible, rapid and sensitive. To ensure the reliability of MRD testing, the procedures for cell collection, separation, staining and analysis must be followed carefully. Most importantly, the sequence in which antibodies are added to cells and the times of incubation must be rigorously standardised, because variations in these procedures can alter the intensity of cell labelling (Campana and Coustan-Smith 1999). Moreover, the reproducibility of MRD analysis can be affected by changes between different batches of antibodies and by instrument instability as well as variations in fluorochromes overlapping into different channels. These variations must be monitored by frequent staining of normal samples and by regular testing of the instrument settings and the need for correct fluorochromes compensation. In addition to the above technical limitations specific to flow cytometric detection of MRD, other factors may affect any method of MRD detection. For example, the anatomic distribution of leukaemic cells during clinical remission may be uneven (Mathe *et al.* 1966; Martens *et al.* 1987), leading to sampling variability and false-negative results.

Over the last few years, the functional properties and phenotype of human LSCs have begun to be explored. Today, AML stem cells can be discriminated and separated from normal stem cells and from more mature AML cells using MoAbs.

Due to their repopulating and disease-maintaining effects, these cells appear to be a most important target cell population in the context of curative therapies. Therefore, a number of studies are presently focusing on AML stem cells and on effective drugs that can attack these cells in a target-dependent manner. The currently available data are promising and point to the potent anti-leukaemic effects of 'stem cell-targeted' drug therapy. However, these data also show that many targets and pathways still need to be explored. The most promising approach for future concepts may be to target multiple extracellular and intracellular targets and unrelated downstream signalling pathways in AML stem cells by the co-administration of various targeted drugs (advanced targeted drug therapy). Whether these strategies will help in the eradication of AML clones and the management of minimal residual AML remains to be determined in future clinical trials.

CHAPTER 9

Bibliography

CHAPTER 9: BIBLIOGRAPHY

Abu-Duhier, F. M., A. C. Goodeve, G. A. Wilson, R. S. Care, I. R. Peake and J. T. Reilly (2001). Genomic structure of human FLT3: implications for mutational analysis. *Br J Haematol* **113**(4): 1076-7.

Abu-Duhier, F. M., A. C. Goodeve, G. A. Wilson, R. S. Care, I. R. Peake and J. T. Reilly (2001). Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. *Br J Haematol* **113**(4): 983-8.

Abu-Duhier, F. M., A. C. Goodeve, G. A. Wilson, M. A. Gari, I. R. Peake, D. C. Rees, E. A. Vandenberghe, P. R. Winship and J. T. Reilly (2000). FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. *Br J Haematol* **111**(1): 190-5.

Adriaansen, H. J., B. C. Jacobs, M. C. Kappers-Klunne, K. Hahlen, H. Hooijkaas and J. J. van Dongen (1993). Detection of residual disease in AML patients by use of double immunological marker analysis for terminal deoxynucleotidyl transferase and myeloid markers. *Leukemia* **7**(3): 472-81.

Adriaansen, H. J., J. J. van Dongen, M. C. Kappers-Klunne, K. Hahlen, M. B. van 't Veer, J. H. Wijdenes-de Bresser, A. C. Holdrinet, E. J. Harthoorn-Lasthuizen, J. Abels and H. Hooijkaas (1990). Terminal deoxynucleotidyl transferase positive subpopulations occur in the majority of ANLL: implications for the detection of minimal disease. *Leukemia* **4**(6): 404-10.

Ailles, L. E., B. Gerhard and D. E. Hogge (1997). Detection and characterization of primitive malignant and normal progenitors in patients with acute myelogenous leukemia using long-term coculture with supportive feeder layers and cytokines. *Blood* **90**(7): 2555-64.

Albain, K. S., M. M. Le Beau, R. Ullirsch and H. Schumacher (1990). Implication of prior treatment with drug combinations including inhibitors of

topoisomerase II in therapy-related monocytic leukemia with a 9;11 translocation. *Genes Chromosomes Cancer* **2**(1): 53-8.

Anastasi, J., M. M. Le Beau, J. W. Vardiman and C. A. Westbrook (1990). Detection of numerical chromosomal abnormalities in neoplastic hematopoietic cells by in situ hybridization with a chromosome-specific probe. *Am J Pathol* **136**(1): 131-9.

Andrews, R. G., B. Torok-Storb and I. D. Bernstein (1983). Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. *Blood* **62**(1): 124-32.

Appelbaum, F. R. (2002). Hematopoietic cell transplantation beyond first remission. *Leukemia* **16**(2): 157-9.

Appelbaum, F. R., J. M. Rowe, J. Radich and J. E. Dick (2001). Acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*: 62-86.

Arkesteijn, G. J., S. L. Erpelinck, A. C. Martens, A. Hagemeijer and A. Hagenbeek (1996). The use of FISH with chromosome-specific repetitive DNA probes for the follow-up of leukemia patients. Correlations and discrepancies with bone marrow cytology. *Cancer Genet Cytogenet* **88**(1): 69-75.

Arrigoni, P., C. Beretta, D. Silvestri, V. Rossi, C. Rizzari, M. G. Valsecchi, G. Cazzaniga and A. Biondi (2003). FLT3 internal tandem duplication in childhood acute myeloid leukaemia: association with hyperleucocytosis in acute promyelocytic leukaemia. *Br J Haematol* **120**(1): 89-92.

Austin, H., E. Delzell and P. Cole (1988). Benzene and leukemia. A review of the literature and a risk assessment. *Am J Epidemiol* **127**(3): 419-39.

Avivi, I. and J. M. Rowe (2005). Prognostic factors in acute myeloid leukemia. *Curr Opin Hematol* **12**(1): 62-7.

Babusikova, O., M. Glasova, E. Konikova and J. Kusenda (1996). Leukemia-associated phenotypes: their characteristics and incidence in acute leukemia. *Neoplasma* **43**(6): 367-72.

Bahia, D. M., M. Yamamoto, L. Chauffaille Mde, E. Y. Kimura, J. O. Bordin, M. A. Filgueiras and J. Kerbauy (2001). Aberrant phenotypes in acute myeloid leukemia: a high frequency and its clinical significance. *Haematologica* **86**(8): 801-6.

Bain, B. J., D. Barnett, D. Linch, E. Matutes and J. T. Reilly (2002). Revised guideline on immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders. *Clin Lab Haematol* **24**(1): 1-13.

Bakker, A. B., S. van den Oudenrijn, A. Q. Bakker, N. Feller, M. van Meijer, J. A. Bia, M. A. Jongeneelen, T. J. Visser, N. Bijl, C. A. Geuijen, W. E. Marissen, K. Radosevic, M. Throsby, G. J. Schuurhuis, G. J. Ossenkoppele, J. de Kruif, J. Goudsmit and A. M. Kruisbeek (2004). C-type lectin-like molecule-1: a novel myeloid cell surface marker associated with acute myeloid leukemia. *Cancer Res* **64**(22): 8443-50.

Barragan, E., J. Cervera, P. Bolufer, S. Ballester, G. Martin, P. Fernandez, R. Collado, M. J. Sayas and M. A. Sanz (2004). Prognostic implications of Wilms' tumor gene (WT1) expression in patients with de novo acute myeloid leukemia. *Haematologica* **89**(8): 926-33.

Basso, G., B. Buldini, L. De Zen and A. Orfao (2001). New methodologic approaches for immunophenotyping acute leukemias. *Haematologica* **86**(7): 675-92.

Basso, G., F. Lanza, A. Orfao, S. Moretti and G. Castoldi (2001). Clinical and biological significance of CD34 expression in acute leukemia. *J Biol Regul Homeost Agents* **15**(1): 68-78.

Bene, M. C., G. Castoldi, W. Knapp, W. D. Ludwig, E. Matutes, A. Orfao and M. B. van't Veer (1995). Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* **9**(10): 1783-6.

Bennett, J. M., D. Catovsky, M. T. Daniel, G. Flandrin, D. A. Galton, H. R. Gralnick and C. Sultan (1991). Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-MO). *Br J Haematol* **78**(3): 325-9.

Bentz, M., H. Dohner, G. Cabot and P. Lichter (1994). Fluorescence in situ hybridization in leukemias: 'the FISH are spawning!' *Leukemia* **8**(9): 1447-52.

Bhatia, M., D. Bonnet, D. Wu, B. Murdoch, J. Wrana, L. Gallacher and J. E. Dick (1999). Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J Exp Med* **189**(7): 1139-48.

Bhatia, M., J. C. Wang, U. Kapp, D. Bonnet and J. E. Dick (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A* **94**(10): 5320-5.

Blair, A., D. E. Hogge, L. E. Ailles, P. M. Lansdorp and H. J. Sutherland (1997). Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood* **89**(9): 3104-12.

Blair, A., D. E. Hogge and H. J. Sutherland (1998). Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34(+)/CD71(-)/HLA-DR. *Blood* **92**(11): 4325-35.

Blair, A. and D. H. Pamphilon (2003). Leukaemic stem cells. *Transfus Med* **13**(6): 363-75.

Blair, A. and H. J. Sutherland (2000). Primitive acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo lack surface expression of c-kit (CD117). *Exp Hematol* **28**(6): 660-71.

Boissel, N., J. M. Cayuela, C. Preudhomme, X. Thomas, N. Grardel, X. Fund, I. Tigaud, E. Raffoux, P. Rousselot, F. Sigaux, L. Degos, S. Castaigne, P. Fenaux and H. Dombret (2002). Prognostic significance of FLT3 internal tandem repeat in patients with de novo acute myeloid leukemia treated with reinforced courses of chemotherapy. *Leukemia* **16**(9): 1699-704.

Bonnet, D. (2005). Cancer stem cells: lessons from leukaemia. *Cell Prolif* **38**(6): 357-61.

Bonnet, D. (2005). Leukemic stem cells show the way. *Folia Histochem Cytobiol* **43**(4): 183-6.

Bonnet, D., M. Bhatia, J. C. Wang, U. Kapp and J. E. Dick (1999). Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive human hematopoietic cells transplanted at limiting doses into NOD/SCID mice. *Bone Marrow Transplant* **23**(3): 203-9.

Bonnet, D. and J. E. Dick (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**(7): 730-7.

Borowitz, M. J., R. Bray, R. Gascoyne, S. Melnick, J. W. Parker, L. Picker and M. Stetler-Stevenson (1997). U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: data analysis and interpretation. *Cytometry* **30**(5): 236-44.

Borowitz, M. J., K. L. Guenther, K. E. Shults and G. T. Stelzer (1993). Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis. *Am J Clin Pathol* **100**(5): 534-40.

Bradstock, K., J. Matthews, E. Benson, F. Page and J. Bishop (1994). Prognostic value of immunophenotyping in acute myeloid leukemia. Australian Leukaemia Study Group. *Blood* **84**(4): 1220-5.

Bradstock, K. F., J. P. Matthews, R. M. Lowenthal, H. Baxter, J. Catalano, T. Brighton, D. Gill, P. Eliadis, D. Joshua, P. Cannell, A. P. Schwarer, S. Durrant, A. Gillett, J. Koutts, K. Taylor, J. Bashford, C. Arthur, A. Enno, L. Dunlop, J. Szer, M. Leahy, S. Juneja and G. A. Young (2005). A randomized trial of high-versus conventional-dose cytarabine in consolidation chemotherapy for adult de novo acute myeloid leukemia in first remission after induction therapy containing high-dose cytarabine. *Blood* **105**(2): 481-8.

Buccisano, F., L. Maurillo, V. Gattei, G. Del Poeta, M. I. Del Principe, M. C. Cox, P. Panetta, M. I. Consalvo, C. Mazzone, B. Neri, L. Ottaviani, D. Fraboni, A. Tamburini, F. Lo-Coco, S. Amadori and A. Venditti (2006). The kinetics of reduction of minimal residual disease impacts on duration of response and survival of patients with acute myeloid leukemia. *Leukemia* **20**(10): 1783-9.

Buchner, T., W. Hiddemann, W. E. Berdel, B. Wormann, C. Schoch, C. Fonatsch, H. Loffler, T. Haferlach, W. D. Ludwig, G. Maschmeyer, P. Staib, C. Aul, A. Gruneisen, E. Lengfelder, N. Frickhofen, W. Kern, H. L. Serve, R. M. Mesters, M. C. Sauerland and A. Heinecke (2003). 6-Thioguanine,

cytarabine, and daunorubicin (TAD) and high-dose cytarabine and mitoxantrone (HAM) for induction, TAD for consolidation, and either prolonged maintenance by reduced monthly TAD or TAD-HAM-TAD and one course of intensive consolidation by sequential HAM in adult patients at all ages with de novo acute myeloid leukemia (AML): a randomized trial of the German AML Cooperative Group. *J Clin Oncol* **21**(24): 4496-504.

Bullinger, L., K. Dohner, E. Bair, S. Frohling, R. F. Schlenk, R. Tibshirani, H. Dohner and J. R. Pollack (2004). Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med* **350**(16): 1605-16.

Burnett, A. K. (2002). Acute myeloid leukemia: treatment of adults under 60 years. *Rev Clin Exp Hematol* **6**(1): 26-45; discussion 86-7.

Burnett, A. K., A. H. Goldstone, R. M. Stevens, I. M. Hann, J. K. Rees, R. G. Gray and K. Wheatley (1998). Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of MRC AML 10 trial. UK Medical Research Council Adult and Children's Leukaemia Working Parties. *Lancet* **351**(9104): 700-8.

Campana, D. (2003). Determination of minimal residual disease in leukaemia patients. *Br J Haematol* **121**(6): 823-38.

Campana, D. and E. Coustan-Smith (1999). Detection of minimal residual disease in acute leukemia by flow cytometry. *Cytometry* **38**(4): 139-52.

Campana, D., E. Coustan-Smith and G. Janossy (1990). The immunologic detection of minimal residual disease in acute leukemia. *Blood* **76**(1): 163-71.

Campana, D. and C. H. Pui (1995). Detection of minimal residual disease in acute leukemia: methodologic advances and clinical significance. *Blood* **85**(6): 1416-34.

Chang, H., F. Salma, Q. L. Yi, B. Patterson, B. Brien, M. D. Minden, M. B. Vidriales, J. F. San-Miguel, A. Orfao, E. Coustan-Smith, D. Campana, B. Xu, C. Hu, X. D. Miao, Y. H. Wu, Z. S. Yi, Y. Yang, F. Y. Meng, R. Feng, Q. F. Liu, X. L. Liu, W. Y. Zheng, I. V. Gal'tseva, V. G. Savchenko, S. M. Kulikov, E. N. Parovichnikova, G. Miterev, E. R. Maslova and V. G. Isaev (2004). Prognostic relevance of immunophenotyping in 379 patients with acute myeloid leukemia. *Leuk Res* **28**(1): 43-8.

Chen, W., D. Jones, L. J. Medeiros, R. Luthra and P. Lin (2005). Acute myeloid leukaemia with FLT3 gene mutations of both internal tandem duplication and point mutation type. *Br J Haematol* **130**(5): 726-8.

Chen, W., N. J. Karandikar, R. W. McKenna and S. H. Kroft (2007). Stability of leukemia-associated immunophenotypes in precursor B-lymphoblastic leukemia/lymphoma: a single institution experience. *Am J Clin Pathol* **127**(1): 39-46.

Cheson, B. D., P. A. Cassileth, D. R. Head, C. A. Schiffer, J. M. Bennett, C. D. Bloomfield, R. Brunning, R. P. Gale, M. R. Grever, M. J. Keating and et al. (1990). Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. *J Clin Oncol* **8**(5): 813-9.

Cilloni, D., E. Gottardi and G. Saglio (2006). WT1 overexpression in acute myeloid leukemia and myelodysplastic syndromes. *Methods Mol Med* **125**: 199-211.

Ciudad, J., J. F. San Miguel, M. C. Lopez-Berges, B. Vidriales, B. Valverde, M. Ocqueteau, G. Mateos, M. D. Caballero, J. Hernandez, M. J. Moro, M. V. Mateos and A. Orfao (1998). Prognostic value of immunophenotypic

detection of minimal residual disease in acute lymphoblastic leukemia. *J Clin Oncol* **16**(12): 3774-81.

Core, T. R. D. (2007). R: A language and environment for statistical computing. . *R Foundation for Statistical Computing* **Vienna, Austria**.

Council, T. M. R. (1966). Treatment of acute leukaemia in adults: comparison of steroid and mercaptopurine therapy, alone and in conjunction. Second report to the Medical Research Council of the Working Party on the evaluation of different methods of therapy in leukaemia. *Br Med J* **1**(5500): 1383-9.

Coustan-Smith, E., F. G. Behm, J. Sanchez, J. M. Boyett, M. L. Hancock, S. C. Raimondi, J. E. Rubnitz, G. K. Rivera, J. T. Sandlund, C. H. Pui and D. Campana (1998). Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. *Lancet* **351**(9102): 550-4.

Coustan-Smith, E., R. C. Ribeiro, J. E. Rubnitz, B. I. Razzouk, C. H. Pui, S. Pounds, M. Andreansky, F. G. Behm, S. C. Raimondi, S. A. Shurtleff, J. R. Downing and D. Campana (2003). Clinical significance of residual disease during treatment in childhood acute myeloid leukaemia. *Br J Haematol* **123**(2): 243-52.

Coustan-Smith, E., J. Sancho, M. L. Hancock, B. I. Razzouk, R. C. Ribeiro, G. K. Rivera, J. E. Rubnitz, J. T. Sandlund, C. H. Pui and D. Campana (2002). Use of peripheral blood instead of bone marrow to monitor residual disease in children with acute lymphoblastic leukemia. *Blood* **100**(7): 2399-402.

Cuneo, A., A. Ferrant, J. L. Michaux, M. Boogaerts, H. Demuynck, A. Van Orshoven, A. Criel, M. Stul, P. Dal Cin, J. Hernandez and et al. (1995). Cytogenetic profile of minimally differentiated (FAB M0) acute myeloid leukemia: correlation with clinicobiologic findings. *Blood* **85**(12): 3688-94.

Dash, A. and D. G. Gilliland (2001). Molecular genetics of acute myeloid leukaemia. *Best Pract Res Clin Haematol* **14**(1): 49-64.

Davis, B. H., K. Foucar, W. Szczarkowski, E. Ball, T. Witzig, K. A. Foon, D. Wells, P. Kotylo, R. Johnson, C. Hanson and D. Bessman (1997). U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: medical indications. *Cytometry* **30**(5): 249-63.

Del Poeta, G., R. Stasi, A. Venditti, C. Cox, G. Aronica, M. Masi, A. Bruno, M. D. Simone, F. Buccisano and G. Papa (1995). CD7 expression in acute myeloid leukemia. *Leuk Lymphoma* **17**(1-2): 111-9.

Dimartino, J. F. and M. L. Cleary (1999). Mll rearrangements in haematological malignancies: lessons from clinical and biological studies. *Br J Haematol* **106**(3): 614-26.

Dohner, K., K. Tobis, R. Ulrich, S. Frohling, A. Benner, R. F. Schlenk and H. Dohner (2002). Prognostic significance of partial tandem duplications of the MLL gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: a study of the Acute Myeloid Leukemia Study Group Ulm. *J Clin Oncol* **20**(15): 3254-61.

Drach, J., D. Drach, H. Glassl, C. Gattringer and H. Huber (1992). Flow cytometric determination of atypical antigen expression in acute leukemia for the study of minimal residual disease. *Cytometry* **13**(8): 893-901.

Drach, J., C. Gattringer and H. Huber (1991). Combined flow cytometric assessment of cell surface antigens and nuclear TdT for the detection of minimal residual disease in acute leukaemia. *Br J Haematol* **77**(1): 37-42.

Drexler, H. G. (1996). Expression of FLT3 receptor and response to FLT3 ligand by leukemic cells. *Leukemia* **10**(4): 588-99.

Druker, B. J., S. G. O'Brien, J. Cortes and J. Radich (2002). Chronic myelogenous leukemia. *Hematology Am Soc Hematol Educ Program*: 111-35.

Ebihara, Y., M. Wada, T. Ueda, M. J. Xu, A. Manabe, R. Tanaka, M. Ito, H. Mugishima, S. Asano, T. Nakahata and K. Tsuji (2002). Reconstitution of human haematopoiesis in non-obese diabetic/severe combined immunodeficient mice by clonal cells expanded from single CD34+CD38- cells expressing Flk2/Flt3. *Br J Haematol* **119**(2): 525-34.

Edenfield, W. J. and S. D. Gore (1999). Stage-specific application of allogeneic and autologous marrow transplantation in the management of acute myeloid leukemia. *Semin Oncol* **26**(1): 21-34.

Estey, E. H., Y. Shen and P. F. Thall (2000). Effect of time to complete remission on subsequent survival and disease-free survival time in AML, RAEB-t, and RAEB. *Blood* **95**(1): 72-7.

Falini, B., N. Bolli, J. Shan, M. P. Martelli, A. Liso, A. Pucciarini, B. Bigerna, L. Pasqualucci, R. Mannucci, R. Rosati, P. Gorello, D. Diverio, G. Roti, E. Tiacci, G. Cazzaniga, A. Biondi, S. Schnittger, T. Haferlach, W. Hiddemann, M. F. Martelli, W. Gu, C. Mecucci and I. Nicoletti (2006). Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc+ AML. *Blood* **107**(11): 4514-23.

Falini, B., C. Mecucci, E. Tiacci, M. Alcalay, R. Rosati, L. Pasqualucci, R. La Starza, D. Diverio, E. Colombo, A. Santucci, B. Bigerna, R. Pacini, A. Pucciarini, A. Liso, M. Vignetti, P. Fazi, N. Meani, V. Pettirossi, G. Saglio, F. Mandelli, F. Lo-Coco, P. G. Pelicci and M. F. Martelli (2005).

Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* **352**(3): 254-66.

Feller, N., M. A. van der Pol, A. van Stijn, G. W. Weijers, A. H. Westra, B. W. Evertse, G. J. Ossenkoppele and G. J. Schuurhuis (2004). MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. *Leukemia* **18**(8): 1380-90.

Ferrara, F., S. Palmieri and G. Mele (2004). Prognostic factors and therapeutic options for relapsed or refractory acute myeloid leukemia. *Haematologica* **89**(8): 998-1008.

Frohling, S., R. F. Schlenk, J. Breitruck, A. Benner, S. Kreitmeier, K. Tobis, H. Dohner and K. Dohner (2002). Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. *Blood* **100**(13): 4372-80.

Frohling, S., R. F. Schlenk, I. Stolze, J. Bihlmayr, A. Benner, S. Kreitmeier, K. Tobis, H. Dohner and K. Dohner (2004). CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. *J Clin Oncol* **22**(4): 624-33.

Galimberti, S., F. Guerrini, G. Carulli, R. Fazzi, G. A. Palumbo, F. Morabito and M. Petrini (2004). Significant co-expression of WT1 and MDR1 genes in acute myeloid leukemia patients at diagnosis. *Eur J Haematol* **72**(1): 45-51.

Galimberti, S., R. Testi, F. Guerrini, R. Fazzi and M. Petrini (2003). The clinical relevance of the expression of several multidrug-resistant-related genes in patients with primary acute myeloid leukemia. *J Chemother* **15**(4): 374-9.

Gilliland, D. G. (2002). Molecular genetics of human leukemias: new insights into therapy. *Semin Hematol* **39**(4 Suppl 3): 6-11.

Gilliland, D. G. and J. D. Griffin (2002). Role of FLT3 in leukemia. *Curr Opin Hematol* **9**(4): 274-81.

Gilliland, D. G. and J. D. Griffin (2002). The roles of FLT3 in hematopoiesis and leukemia. *Blood* **100**(5): 1532-42.

Gilliland, D. G., C. T. Jordan and C. A. Felix (2004). The molecular basis of leukemia. *Hematology Am Soc Hematol Educ Program*: 80-97.

Goldie, J. H. and A. J. Coldman (1986). Application of theoretical models to chemotherapy protocol design. *Cancer Treat Rep* **70**(1): 127-31.

Gratama, J. W., A. Orfao, D. Barnett, B. Brando, A. Huber, G. Janossy, H. E. Johnsen, M. Keeney, G. E. Marti, F. Preijers, G. Rothe, S. Serke, D. R. Sutherland, C. E. Van der Schoot, G. Schmitz and S. Papa (1998). Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells. European Working Group on Clinical Cell Analysis. *Cytometry* **34**(3): 128-42.

Greenlee, R. T., M. B. Hill-Harmon, T. Murray and M. Thun (2001). Cancer statistics, 2001. *CA Cancer J Clin* **51**(1): 15-36.

Griffin, J. D., R. Davis, D. A. Nelson, F. R. Davey, R. J. Mayer, C. Schiffer, O. R. McIntyre and C. D. Bloomfield (1986). Use of surface marker analysis to predict outcome of adult acute myeloblastic leukemia. *Blood* **68**(6): 1232-41.

Griffin, J. D., R. J. Mayer, H. J. Weinstein, D. S. Rosenthal, F. S. Coral, R. P. Beveridge and S. F. Schlossman (1983). Surface marker analysis of acute

myeloblastic leukemia: identification of differentiation-associated phenotypes. *Blood* **62**(3): 557-63.

Grigg, A. P., J. Szer, J. Beresford, A. Dodds, K. Bradstock, S. Durrant, A. P. Schwarer, T. Hughes, R. Herrmann, J. Gibson, C. Arthur and J. Matthews (1999). Factors affecting the outcome of allogeneic bone marrow transplantation for adult patients with refractory or relapsed acute leukaemia. *Br J Haematol* **107**(2): 409-18.

Grimwade, D., H. Walker, G. Harrison, F. Oliver, S. Chatters, C. J. Harrison, K. Wheatley, A. K. Burnett and A. H. Goldstone (2001). The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood* **98**(5): 1312-20.

Grimwade, D., H. Walker, F. Oliver, K. Wheatley, C. Harrison, G. Harrison, J. Rees, I. Hann, R. Stevens, A. Burnett and A. Goldstone (1998). The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* **92**(7): 2322-33.

Guenechea, G., O. I. Gan, C. Dorrell and J. E. Dick (2001). Distinct classes of human stem cells that differ in proliferative and self-renewal potential. *Nat Immunol* **2**(1): 75-82.

Guzman, M. L. and C. T. Jordan (2004). Considerations for targeting malignant stem cells in leukemia. *Cancer Control* **11**(2): 97-104.

Guzman, M. L., S. J. Neering, D. Upchurch, B. Grimes, D. S. Howard, D. A. Rizzieri, S. M. Luger and C. T. Jordan (2001). Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* **98**(8): 2301-7.

Guzman, M. L., C. F. Swiderski, D. S. Howard, B. A. Grimes, R. M. Rossi, S. J. Szilvassy and C. T. Jordan (2002). Preferential induction of apoptosis for primary human leukemic stem cells. *Proc Natl Acad Sci U S A* **99**(25): 16220-5.

Hamann, P. R., L. M. Hinman, C. F. Beyer, D. Lindh, J. Upeslacis, D. A. Flowers and I. Bernstein (2002). An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker. *Bioconjug Chem* **13**(1): 40-6.

Hamann, P. R., L. M. Hinman, I. Hollander, C. F. Beyer, D. Lindh, R. Holcomb, W. Hallett, H. R. Tsou, J. Upeslacis, D. Shochat, A. Mountain, D. A. Flowers and I. Bernstein (2002). Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug Chem* **13**(1): 47-58.

Hauswirth, A. W., S. Florian, D. Printz, K. Sotlar, M. T. Krauth, G. Fritsch, G. H. Schernthaner, V. Wacheck, E. Selzer, W. R. Sperr and P. Valent (2007). Expression of the target receptor CD33 in CD34+/CD38-/CD123+ AML stem cells. *Eur J Clin Invest* **37**(1): 73-82.

Hiddemann, W., K. Spiekermann, C. Buske, M. Feuring-Buske, J. Braess, T. Haferlach, C. Schoch, W. Kern, S. Schnittger, W. Berdel, B. Wormann, A. Heinecke, C. Sauerland and T. Buchner (2005). Towards a pathogenesis-oriented therapy of acute myeloid leukemia. *Crit Rev Oncol Hematol* **56**(2): 235-45.

Hollis, S. (1996). Analysis of method comparison studies. *Ann Clin Biochem* **33 (Pt 1)**: 1-4.

Horiike, S., S. Yokota, M. Nakao, T. Iwai, Y. Sasai, H. Kaneko, M. Taniwaki, K. Kashima, H. Fujii, T. Abe and S. Misawa (1997). Tandem duplications of

the FLT3 receptor gene are associated with leukemic transformation of myelodysplasia. *Leukemia* **11**(9): 1442-6.

Hosmer, D. W. and S. Lemeshow, Eds. (2000). Applied Logistic Regression. Wiley Series in Probability and Statistics. New York, John Wiley & Sons, Inc.

Iwai, T., S. Yokota, M. Nakao, T. Okamoto, M. Taniwaki, N. Onodera, A. Watanabe, A. Kikuta, A. Tanaka, K. Asami, I. Sekine, H. Mugishima, Y. Nishimura, S. Koizumi, Y. Horikoshi, J. Mimaya, S. Ohta, K. Nishikawa, A. Iwai, T. Shimokawa, M. Nakayama, K. Kawakami, T. Gushiken, N. Hyakuna, T. Fujimoto and et al. (1999). Internal tandem duplication of the FLT3 gene and clinical evaluation in childhood acute myeloid leukemia. The Children's Cancer and Leukemia Study Group, Japan. *Leukemia* **13**(1): 38-43.

Jennings, C. D. and K. A. Foon (1997). Flow cytometry: recent advances in diagnosis and monitoring of leukemia. *Cancer Invest* **15**(4): 384-99.

Jennings, C. D. and K. A. Foon (1997). Recent advances in flow cytometry: application to the diagnosis of hematologic malignancy. *Blood* **90**(8): 2863-92.

Jilani, I., E. Estey, T. Manshuri, M. Caligiuri, M. Keating, F. Giles, D. Thomas, H. Kantarjian and M. Albitar (2003). Better detection of FLT3 internal tandem duplication using peripheral blood plasma DNA. *Leukemia* **17**(1): 114-9.

Jin, L., K. J. Hope, Q. Zhai, F. Smadja-Joffe and J. E. Dick (2006). Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* **12**(10): 1167-74.

Jordan, C. T., D. Upchurch, S. J. Szilvassy, M. L. Guzman, D. S. Howard, A. L. Pettigrew, T. Meyerrose, R. Rossi, B. Grimes, D. A. Rizzieri, S. M. Luger

and G. L. Phillips (2000). The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* **14**(10): 1777-84.

Kainz, B., D. Heintel, R. Marculescu, I. Schwarzinger, W. Sperr, T. Le, A. Weltermann, C. Fonatsch, O. A. Haas, C. Mannhalter, K. Lechner and U. Jaeger (2002). Variable prognostic value of FLT3 internal tandem duplications in patients with de novo AML and a normal karyotype, t(15;17), t(8;21) or inv(16). *Hematol J* **3**(6): 283-9.

Kaleem, Z. and G. White (2001). Diagnostic criteria for minimally differentiated acute myeloid leukemia (AML-M0). Evaluation and a proposal. *Am J Clin Pathol* **115**(6): 876-84.

Kanda, Y., T. Hamaki, R. Yamamoto, A. Chizuka, M. Suguro, T. Matsuyama, N. Takezako, A. Miwa, M. Kami, H. Hirai and A. Togawa (2000). The clinical significance of CD34 expression in response to therapy of patients with acute myeloid leukemia: an overview of 2483 patients from 22 studies. *Cancer* **88**(11): 2529-33.

Karp, J. E., J. E. Lancet, S. H. Kaufmann, D. W. End, J. J. Wright, K. Bol, I. Horak, M. L. Tidwell, J. Liesveld, T. J. Kottke, D. Ange, L. Buddharaju, I. Gojo, W. E. Highsmith, R. T. Belly, R. J. Hohl, M. E. Rybak, A. Thibault and J. Rosenblatt (2001). Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase 1 clinical-laboratory correlative trial. *Blood* **97**(11): 3361-9.

Keating, M. J., T. L. Smith, H. Kantarjian, A. Cork, R. Walters, J. M. Trujillo, K. B. McCredie, E. A. Gehan and E. J. Freireich (1988). Cytogenetic pattern in acute myelogenous leukemia: a major reproducible determinant of outcome. *Leukemia* **2**(7): 403-12.

Kelly, L. M., Q. Liu, J. L. Kutok, I. R. Williams, C. L. Boulton and D. G. Gilliland (2002). FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood* **99**(1): 310-8.

Kern, W., S. Danhauser-Riedl, R. Ratei, S. Schnittger, C. Schoch, H. J. Kolb, W. D. Ludwig, W. Hiddemann and T. Haferlach (2003). Detection of minimal residual disease in unselected patients with acute myeloid leukemia using multiparameter flow cytometry for definition of leukemia-associated immunophenotypes and determination of their frequencies in normal bone marrow. *Haematologica* **88**(6): 646-53.

Kern, W., T. Haferlach, C. Schoch, H. Loffler, W. Gassmann, A. Heinecke, M. C. Sauerland, W. Berdel, T. Buchner and W. Hiddemann (2003). Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: data from the German AML Cooperative Group (AMLCG) 1992 Trial. *Blood* **101**(1): 64-70.

Kern, W. and S. Schnittger (2003). Monitoring of acute myeloid leukemia by flow cytometry. *Curr Oncol Rep* **5**(5): 405-12.

Kern, W., C. Schoch, T. Haferlach and S. Schnittger (2005). Monitoring of minimal residual disease in acute myeloid leukemia. *Crit Rev Oncol Hematol* **56**(2): 283-309.

Kern, W., D. Voskova, S. Schnittger, C. Schoch, W. Hiddemann and T. Haferlach (2004). Four-fold staining including CD45 gating improves the sensitivity of multiparameter flow cytometric assessment of minimal residual disease in patients with acute myeloid leukemia. *Hematol J* **5**(5): 410-8.

Kern, W., D. Voskova, C. Schoch, W. Hiddemann, S. Schnittger and T. Haferlach (2004). Determination of relapse risk based on assessment of minimal

residual disease during complete remission by multiparameter flow cytometry in unselected patients with acute myeloid leukemia. *Blood* **104**(10): 3078-85.

Kiyoi, H. and T. Naoe (2006). FLT3 mutations in acute myeloid leukemia. *Methods Mol Med* **125**: 189-97.

Kiyoi, H., T. Naoe, Y. Nakano, S. Yokota, S. Minami, S. Miyawaki, N. Asou, K. Kuriyama, I. Jinnai, C. Shimazaki, H. Akiyama, K. Saito, H. Oh, T. Motoji, E. Omoto, H. Saito, R. Ohno and R. Ueda (1999). Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood* **93**(9): 3074-80.

Kiyoi, H., T. Naoe, S. Yokota, M. Nakao, S. Minami, K. Kuriyama, A. Takeshita, K. Saito, S. Hasegawa, S. Shimodaira, J. Tamura, C. Shimazaki, K. Matsue, H. Kobayashi, N. Arima, R. Suzuki, H. Morishita, H. Saito, R. Ueda and R. Ohno (1997). Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). *Leukemia* **11**(9): 1447-52.

Koepke, J. A. and A. L. Landay (1989). Precision and accuracy of absolute lymphocyte counts. *Clin Immunol Immunopathol* **52**(1): 19-27.

Kondo, M., K. Horibe, Y. Takahashi, K. Matsumoto, M. Fukuda, J. Inaba, K. Kato, S. Kojima and T. Matsuyama (1999). Prognostic value of internal tandem duplication of the FLT3 gene in childhood acute myelogenous leukemia. *Med Pediatr Oncol* **33**(6): 525-9.

Kottaridis, P. D., R. E. Gale, M. E. Frew, G. Harrison, S. E. Langabeer, A. A. Belton, H. Walker, K. Wheatley, D. T. Bowen, A. K. Burnett, A. H. Goldstone and D. C. Linch (2001). The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first

cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* **98**(6): 1752-9.

Kottaridis, P. D., R. E. Gale, S. E. Langabeer, M. E. Frew, D. T. Bowen and D. C. Linch (2002). Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood* **100**(7): 2393-8.

Krauth, M. T., A. Bohm, H. Agis, K. Sonneck, P. Samorapoompichit, S. Florian, K. Sotlar and P. Valent (2007). Effects of the CD33-targeted drug gemtuzumab ozogamicin (Mylotarg) on growth and mediator secretion in human mast cells and blood basophils. *Exp Hematol* **35**(1): 108-16.

Laane, E., A. R. Derolf, E. Bjorklund, J. Mazur, H. Everaus, S. Soderhall, M. Bjorkholm and A. Porwit-MacDonald (2006). The effect of allogeneic stem cell transplantation on outcome in younger acute myeloid leukemia patients with minimal residual disease detected by flow cytometry at the end of post-remission chemotherapy. *Haematologica* **91**(6): 833-6.

Lacombe, F., F. Durrieu, A. Briais, P. Dumain, F. Belloc, E. Bascans, J. Reiffers, M. R. Boisseau and P. Bernard (1997). Flow cytometry CD45 gating for immunophenotyping of acute myeloid leukemia. *Leukemia* **11**(11): 1878-86.

Lanza, F. (1996). Towards standardization in immunophenotyping hematological malignancies. How can we improve the reproducibility and comparability of flow cytometric results? Working Group on Leukemia Immunophenotyping. *Eur J Histochem* **40 Suppl 1**: 7-14.

Le Beau, M. M., K. S. Albain, R. A. Larson, J. W. Vardiman, E. M. Davis, R. R. Blough, H. M. Golomb and J. D. Rowley (1986). Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes

and acute nonlymphocytic leukemia: further evidence for characteristic abnormalities of chromosomes no. 5 and 7. *J Clin Oncol* **4**(3): 325-45.

Leith, C. P., K. J. Kopecky, J. Godwin, T. McConnell, M. L. Slovak, I. M. Chen, D. R. Head, F. R. Appelbaum and C. L. Willman (1997). Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. *Blood* **89**(9): 3323-9.

Lengfelder, E., A. Reichert, C. Schoch, D. Haase, T. Haferlach, H. Loffler, P. Staib, A. Heyll, W. Seifarth, S. Saussele, C. Fonatsch, W. Gassmann, W. D. Ludwig, A. Hochhaus, D. Beelen, C. Aul, M. C. Sauerland, A. Heinecke, R. Hehlmann, B. Wormann, W. Hiddemann and T. Buchner (2000). Double induction strategy including high dose cytarabine in combination with all-trans retinoic acid: effects in patients with newly diagnosed acute promyelocytic leukemia. German AML Cooperative Group. *Leukemia* **14**(8): 1362-70.

Levine, E. G. and C. D. Bloomfield (1992). Leukemias and myelodysplastic syndromes secondary to drug, radiation, and environmental exposure. *Semin Oncol* **19**(1): 47-84.

Levis, M., J. Allebach, K. F. Tse, R. Zheng, B. R. Baldwin, B. D. Smith, S. Jones-Bolin, B. Ruggeri, C. Dionne and D. Small (2002). A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. *Blood* **99**(11): 3885-91.

Levis, M., K. M. Murphy, R. Pham, K. T. Kim, A. Stine, L. Li, I. McNiece, B. D. Smith and D. Small (2005). Internal tandem duplications of the FLT3 gene are present in leukemia stem cells. *Blood* **106**(2): 673-80.

Levis, M. and D. Small (2003). FLT3: ITDoes matter in leukemia. *Leukemia* **17**(9): 1738-52.

Liang, D. C., L. Y. Shih, I. J. Hung, C. P. Yang, S. H. Chen, T. H. Jaing, H. C. Liu and W. H. Chang (2002). Clinical relevance of internal tandem duplication of the FLT3 gene in childhood acute myeloid leukemia. *Cancer* **94**(12): 3292-8.

Libura, M., V. Asnafi, A. Tu, E. Delabesse, I. Tigaud, F. Cymbalista, A. Bennaceur-Griscelli, P. Villarese, G. Solbu, A. Hagemeijer, K. Beldjord, O. Hermine and E. Macintyre (2003). FLT3 and MLL intragenic abnormalities in AML reflect a common category of genotoxic stress. *Blood* **102**(6): 2198-204.

Lo Coco, F., D. Diverio, B. Falini, A. Biondi, C. Nervi and P. G. Pelicci (1999). Genetic diagnosis and molecular monitoring in the management of acute promyelocytic leukemia. *Blood* **94**(1): 12-22.

Lowenberg, B. (2001). Prognostic factors in acute myeloid leukaemia. *Best Pract Res Clin Haematol* **14**(1): 65-75.

Lowenberg, B., J. R. Downing and A. Burnett (1999). Acute myeloid leukemia. *N Engl J Med* **341**(14): 1051-62.

Lowenberg, B., S. Suciu, E. Archimbaud, H. Haak, P. Stryckmans, R. de Cataldo, A. W. Dekker, Z. N. Berneman, A. Thyss, J. van der Lelie, P. Sonneveld, G. Visani, G. Fillet, M. Hayat, A. Hagemeijer, G. Solbu and R. Zittoun (1998). Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy--the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. *J Clin Oncol* **16**(3): 872-81.

Lumkul, R., N. C. Gorin, M. T. Malehorn, G. T. Hoehn, R. Zheng, B. Baldwin, D. Small, S. Gore, D. Smith, P. S. Meltzer and C. I. Civin (2002). Human AML cells in NOD/SCID mice: engraftment potential and gene expression. *Leukemia* **16**(9): 1818-26.

Lyman, S. D. and S. E. Jacobsen (1998). c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood* **91**(4): 1101-34.

Macedo, A., A. Orfao, M. Gonzalez, M. B. Vidriales, M. C. Lopez-Berges, A. Martinez and J. F. San Miguel (1995). Immunological detection of blast cell subpopulations in acute myeloblastic leukemia at diagnosis: implications for minimal residual disease studies. *Leukemia* **9**(6): 993-8.

Macedo, A., A. Orfao, M. B. Vidriales, M. C. Lopez-Berges, B. Valverde, M. Gonzalez, M. D. Caballero, F. Ramos, M. Martinez, J. Fernandez-Calvo and et al. (1995). Characterization of aberrant phenotypes in acute myeloblastic leukemia. *Ann Hematol* **70**(4): 189-94.

Martens, A. C., F. W. Schultz and A. Hagenbeek (1987). Nonhomogeneous distribution of leukemia in the bone marrow during minimal residual disease. *Blood* **70**(4): 1073-8.

Mathe, G., L. Schwarzenberg, A. M. Mery, A. Cattan, M. Schneider, J. L. Amiel, J. R. Schlumberger, J. Poisson and G. Wajcner (1966). Extensive histological and cytological survey of patients with acute leukaemia in "complete remission". *Br Med J* **5488**: 640-2.

Matthews, D. C., F. R. Appelbaum, J. F. Eary, D. R. Fisher, L. D. Durack, T. E. Hui, P. J. Martin, D. Mitchell, O. W. Press, R. Storb and I. D. Bernstein (1999). Phase I study of (131)I-anti-CD45 antibody plus cyclophosphamide and total body irradiation for advanced acute leukemia and myelodysplastic syndrome. *Blood* **94**(4): 1237-47.

Matthews, D. C., P. J. Martin, C. Nourigat, F. R. Appelbaum, D. R. Fisher and I. D. Bernstein (1999). Marrow ablative and immunosuppressive effects of 131I-anti-CD45 antibody in congenic and H2-mismatched murine transplant models. *Blood* **93**(2): 737-45.

Mayer, R. J., R. B. Davis, C. A. Schiffer, D. T. Berg, B. L. Powell, P. Schulman, G. A. Omura, J. O. Moore, O. R. McIntyre and E. Frei, 3rd (1994). Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. *N Engl J Med* **331**(14): 896-903.

Meierhoff, G., U. Dehmel, H. J. Gruss, O. Rosnet, D. Birnbaum, H. Quentmeier, W. Dirks and H. G. Drexler (1995). Expression of FLT3 receptor and FLT3-ligand in human leukemia-lymphoma cell lines. *Leukemia* **9**(8): 1368-72.

Meshinchi, S., W. G. Woods, D. L. Stirewalt, D. A. Sweetser, J. D. Buckley, T. K. Tjoa, I. D. Bernstein and J. P. Radich (2001). Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. *Blood* **97**(1): 89-94.

Mesters, R. M., T. Padro, R. Bieker, M. Steins, M. Kreuter, M. Goner, S. Kelsey, P. Scigalla, W. Fiedler, T. Buchner and W. E. Berdel (2001). Stable remission after administration of the receptor tyrosine kinase inhibitor SU5416 in a patient with refractory acute myeloid leukemia. *Blood* **98**(1): 241-3.

Miller, W. H., Jr., K. Levine, A. DeBlasio, S. R. Frankel, E. Dmitrovsky and R. P. Warrell, Jr. (1993). Detection of minimal residual disease in acute promyelocytic leukemia by a reverse transcription polymerase chain reaction assay for the PML/RAR-alpha fusion mRNA. *Blood* **82**(6): 1689-94.

Mizuki, M., J. Schwable, C. Steur, C. Choudhary, S. Agrawal, B. Sargin, B. Steffen, I. Matsumura, Y. Kanakura, F. D. Bohmer, C. Muller-Tidow, W. E. Berdel and H. Serve (2003). Suppression of myeloid transcription factors and

induction of STAT response genes by AML-specific Flt3 mutations. *Blood* **101**(8): 3164-73.

Moreno, I., G. Martin, P. Bolufer, E. Barragan, E. Rueda, J. Roman, P. Fernandez, P. Leon, A. Mena, J. Cervera, A. Torres and M. A. Sanz (2003). Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica* **88**(1): 19-24.

Moretti, S., F. Lanza, M. Dabusti, A. Tieghi, D. Campioni, M. Dominici and G. L. Castoldi (2001). CD123 (interleukin 3 receptor alpha chain). *J Biol Regul Homeost Agents* **15**(1): 98-100.

Mrozek, K., N. A. Heerema and C. D. Bloomfield (2004). Cytogenetics in acute leukemia. *Blood Rev* **18**(2): 115-36.

Mrozek, K., K. Heinonen, A. de la Chapelle and C. D. Bloomfield (1997). Clinical significance of cytogenetics in acute myeloid leukemia. *Semin Oncol* **24**(1): 17-31.

Munoz, L., J. F. Nomdedeu, O. Lopez, M. J. Carnicer, M. Bellido, A. Aventin, S. Brunet and J. Sierra (2001). Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies. *Haematologica* **86**(12): 1261-9.

Murayama, T., S. Imoto, T. Natazuka, K. Chihara and T. Matsui (1998). Proliferative reaction of myelogenous leukemia cells with cytokines G-CSF, GM-CSF, M-CSF, SCF and TPO. *Leuk Res* **22**(6): 557-60.

Murphy, K. M., M. Levis, M. J. Hafez, T. Geiger, L. C. Cooper, B. D. Smith, D. Small and K. D. Berg (2003). Detection of FLT3 internal tandem duplication and D835 mutations by a multiplex polymerase chain reaction and capillary electrophoresis assay. *J Mol Diagn* **5**(2): 96-102.

Nakano, Y., H. Kiyoi, S. Miyawaki, N. Asou, R. Ohno, H. Saito and T. Naoe (1999). Molecular evolution of acute myeloid leukaemia in relapse: unstable N-ras and FLT3 genes compared with p53 gene. *Br J Haematol* **104**(4): 659-64.

Nakao, M., S. Yokota, T. Iwai, H. Kaneko, S. Horiike, K. Kashima, Y. Sonoda, T. Fujimoto and S. Misawa (1996). Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia* **10**(12): 1911-8.

Noguera, N. I., M. Breccia, M. Divona, D. Diverio, V. Costa, S. De Santis, G. Avvisati, M. B. Pinazzi, M. C. Petti, F. Mandelli and F. Lo Coco (2002). Alterations of the FLT3 gene in acute promyelocytic leukemia: association with diagnostic characteristics and analysis of clinical outcome in patients treated with the Italian AIDA protocol. *Leukemia* **16**(11): 2185-9.

Norgaard, J. M., L. H. Olesen, G. Olesen, K. Meyer, J. S. Kristensen, K. Bendix, B. Pedersen, E. Kjeldsen and P. Hokland (2001). FAB M4 and high CD14 surface expression is associated with high cellular resistance to Ara-C and daunorubicin: implications for clinical outcome in acute myeloid leukaemia. *Eur J Haematol* **67**(4): 221-9.

O'Farrell, A. M., T. J. Abrams, H. A. Yuen, T. J. Ngai, S. G. Louie, K. W. Yee, L. M. Wong, W. Hong, L. B. Lee, A. Town, B. D. Smolich, W. C. Manning, L. J. Murray, M. C. Heinrich and J. M. Cherrington (2003). SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. *Blood* **101**(9): 3597-605.

O'Farrell, A. M., J. M. Foran, W. Fiedler, H. Serve, R. L. Paquette, M. A. Cooper, H. A. Yuen, S. G. Louie, H. Kim, S. Nicholas, M. C. Heinrich, W. E. Berdel, C. Bello, M. Jacobs, P. Scigalla, W. C. Manning, S. Kelsey and J. M. Cherrington (2003). An innovative phase I clinical study demonstrates inhibition of FLT3 phosphorylation by SU11248 in acute myeloid leukemia patients. *Clin Cancer Res* **9**(15): 5465-76.

Ogawa, H., H. Tamaki, K. Ikegame, T. Soma, M. Kawakami, A. Tsuboi, E. H. Kim, N. Hosen, M. Murakami, T. Fujioka, T. Masuda, Y. Taniguchi, S. Nishida, Y. Oji, Y. Oka and H. Sugiyama (2003). The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. *Blood* **101**(5): 1698-704.

Orfao, A., G. Schmitz, B. Brando, A. Ruiz-Arguelles, G. Basso, R. Braylan, G. Rothe, F. Lacombe, F. Lanza, S. Papa, P. Lucio and J. F. San Miguel (1999). Clinically useful information provided by the flow cytometric immunophenotyping of hematological malignancies: current status and future directions. *Clin Chem* **45**(10): 1708-17.

Orlowski, R. Z., T. E. Stinchcombe, B. S. Mitchell, T. C. Shea, A. S. Baldwin, S. Stahl, J. Adams, D. L. Esseltine, P. J. Elliott, C. S. Pien, R. Guerciolini, J. K. Anderson, N. D. Depcik-Smith, R. Bhagat, M. J. Lehman, S. C. Novick, O. A. O'Connor and S. L. Soignet (2002). Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies. *J Clin Oncol* **20**(22): 4420-7.

Pagel, J. M., D. C. Matthews, F. R. Appelbaum, I. D. Bernstein and O. W. Press (2002). The use of radioimmunoconjugates in stem cell transplantation. *Bone Marrow Transplant* **29**(10): 807-16.

Paietta, E. (2002). Assessing minimal residual disease (MRD) in leukemia: a changing definition and concept? *Bone Marrow Transplant* **29**(6): 459-65.

Paietta, E., J. Andersen and P. H. Wiernik (1996). A new approach to analyzing the utility of immunophenotyping for predicting clinical outcome in acute leukemia. Eastern Cooperative Oncology Group. *Leukemia* **10**(1): 1-4.

Passegue, E., C. H. Jamieson, L. E. Ailles and I. L. Weissman (2003). Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a

reacquisition of stem cell characteristics? *Proc Natl Acad Sci U S A* **100 Suppl 1**: 11842-9.

Piller, G. (2001). Leukaemia - a brief historical review from ancient times to 1950. *Br J Haematol* **112**(2): 282-92.

Plata, E., H. Choremi-Papadopoulou, V. Viglis and X. Yataganas (2000). Flow-cytometric detection of minimal residual disease with atypical antigen combinations in patients with de novo acute myeloid leukemia. *Ann Hematol* **79**(10): 543-6.

Provan, D. and J. G. Gribben (2005). Detection of minimal residual disease in hematological malignancies. Molecular Hematology. USA, Blackwell Publishing Inc**:** 53-71.

Pui, C. H. and D. Campana (2000). New definition of remission in childhood acute lymphoblastic leukemia. *Leukemia* **14**(5): 783-5.

Raanani, P. and I. Ben-Bassat (2004). Detection of minimal residual disease in acute myelogenous leukemia. *Acta Haematol* **112**(1-2): 40-54.

Rainer, R. O., L. Hodges and G. T. Seltzer (1995). CD 45 gating correlates with bone marrow differential. *Cytometry* **22**(2): 139-45.

Raspadori, D., D. Damiani, M. Lenoci, D. Rondelli, N. Testoni, G. Nardi, C. Sestigiani, C. Mariotti, S. Birtolo, M. Tozzi and F. Lauria (2001). CD56 antigenic expression in acute myeloid leukemia identifies patients with poor clinical prognosis. *Leukemia* **15**(8): 1161-4.

Reading, C. L., E. H. Estey, Y. O. Huh, D. F. Claxton, G. Sanchez, L. W. Terstappen, M. C. O'Brien, S. Baron and A. B. Deisseroth (1993).

Expression of unusual immunophenotype combinations in acute myelogenous leukemia. *Blood* **81**(11): 3083-90.

Reed, G. F., F. Lynn and B. D. Meade (2002). Use of coefficient of variation in assessing variability of quantitative assays. *Clin Diagn Lab Immunol* **9**(6): 1235-9.

Rees, J. K., R. G. Gray, D. Swirsky and F. G. Hayhoe (1986). Principal results of the Medical Research Council's 8th acute myeloid leukaemia trial. *Lancet* **2**(8518): 1236-41.

Reilly, J. T. (2002). Class III receptor tyrosine kinases: role in leukaemogenesis. *Br J Haematol* **116**(4): 744-57.

Reya, T., S. J. Morrison, M. F. Clarke and I. L. Weissman (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**(6859): 105-11.

Rigolin, G. M., A. Latorraca, S. Moretti, R. Previati and F. Lanza (1993). [Immunophenotypic study of acute myeloid leukemia (AML): biological significance and prognostic implications]. *Boll Soc Ital Biol Sper* **69**(5): 301-5.

Roederer, M. (2001). Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry* **45**(3): 194-205.

Rombouts, W. J., I. Blokland, B. Lowenberg and R. E. Ploemacher (2000). Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the Flt3 gene. *Leukemia* **14**(4): 675-83.

Rombouts, W. J., A. Broyl, A. C. Martens, R. Slater and R. E. Ploemacher (1999). Human acute myeloid leukemia cells with internal tandem duplications in

the Flt3 gene show reduced proliferative ability in stroma supported long-term cultures. *Leukemia* **13**(7): 1071-8.

Rosnet, O., H. J. Buhring, S. Marchetto, I. Rappold, C. Lavagna, D. Sainty, C. Arnoulet, C. Chabannon, L. Kanz, C. Hannum and D. Birnbaum (1996). Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells. *Leukemia* **10**(2): 238-48.

Rowe, J. M., H. T. Kim, P. A. Cassileth, M. R. LITZOW and P. H. Wiernik (2004). Time To Complete Remission Is Not a Significant Prognostic Factor in AML: A Report on 1,959 Consecutive Patients Registered to 6 Studies of the Eastern Cooperative Oncology Group (ECOG). *Blood* **104**(862a).

Ruggeri, B. A., S. J. Miknyoczki, J. Singh and R. L. Hudkins (1999). Role of neurotrophin-trk interactions in oncology: the anti-tumor efficacy of potent and selective trk tyrosine kinase inhibitors in pre-clinical tumor models. *Curr Med Chem* **6**(9): 845-57.

Rumke, C. L. (1985). Imprecision of ratio-derived differential leukocyte counts. *Blood Cells* **11**(2): 311-4, 315.

Rumke, C. L. (1985). The imprecision of the ratio of two percentages observed in differential white blood cell counts: a warning. *Blood Cells* **11**(1): 137-40.

San Miguel, J. F., J. Ciudad, M. B. Vidriales, A. Orfao, P. Lucio, A. Porwit-MacDonald, G. Gaipa, E. van Wering and J. J. van Dongen (1999). Immunophenotypical detection of minimal residual disease in acute leukemia. *Crit Rev Oncol Hematol* **32**(3): 175-85.

San Miguel, J. F., M. Gonzalez, M. C. Canizo, J. P. Anta, H. Zola and A. Lopez Borrasca (1986). Surface marker analysis in acute myeloid leukaemia and correlation with FAB classification. *Br J Haematol* **64**(3): 547-60.

San Miguel, J. F., A. Martinez, A. Macedo, M. B. Vidriales, C. Lopez-Berges, M. Gonzalez, D. Caballero, M. A. Garcia-Marcos, F. Ramos, J. Fernandez-Calvo, M. J. Calmuntia, J. Diaz-Mediavilla and A. Orfao (1997). Immunophenotyping investigation of minimal residual disease is a useful approach for predicting relapse in acute myeloid leukemia patients. *Blood* **90**(6): 2465-70.

San Miguel, J. F., M. B. Vidriales, C. Lopez-Berges, J. Diaz-Mediavilla, N. Gutierrez, C. Canizo, F. Ramos, M. J. Calmuntia, J. J. Perez, M. Gonzalez and A. Orfao (2001). Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. *Blood* **98**(6): 1746-51.

Schaich, M., R. Koch, S. Soucek, R. Repp, G. Ehninger and T. Illmer (2004). A sensitive model for prediction of relapse in adult acute myeloid leukaemia with t(8;21) using white blood cell count, CD56 and MDR1 gene expression at diagnosis. *Br J Haematol* **125**(4): 477-9.

Schmidt, H. H., S. Strehl, D. Thaler, D. Strunk, H. Sill, W. Linkesch, U. Jager, W. Sperr, H. T. Greinix, M. Konig, W. Emberger and O. A. Haas (2004). RT-PCR and FISH analysis of acute myeloid leukemia with t(8;16)(p11;p13) and chimeric MOZ and CBP transcripts: breakpoint cluster region and clinical implications. *Leukemia* **18**(6): 1115-21.

Schnittger, S., C. Schoch, M. Dugas, W. Kern, P. Staib, C. Wuchter, H. Loffler, C. M. Sauerland, H. Serve, T. Buchner, T. Haferlach and W. Hiddemann (2002). Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* **100**(1): 59-66.

Schnittger, S., C. Schoch, W. Kern, W. Hiddemann and T. Haferlach (2004). FLT3 length mutations as marker for follow-up studies in acute myeloid leukaemia. *Acta Haematol* **112**(1-2): 68-78.

Schoch, C., W. Kern, S. Schnittger, T. Buchner, W. Hiddemann and T. Haferlach (2004). The influence of age on prognosis of de novo acute myeloid leukemia differs according to cytogenetic subgroups. *Haematologica* **89**(9): 1082-90.

Schoch, C., W. Kern, S. Schnittger, W. Hiddemann and T. Haferlach (2004). Karyotype is an independent prognostic parameter in therapy-related acute myeloid leukemia (t-AML): an analysis of 93 patients with t-AML in comparison to 1091 patients with de novo AML. *Leukemia* **18**(1): 120-5.

Shih, L. Y., C. F. Huang, J. H. Wu, T. L. Lin, P. Dunn, P. N. Wang, M. C. Kuo, C. L. Lai and H. C. Hsu (2002). Internal tandem duplication of FLT3 in relapsed acute myeloid leukemia: a comparative analysis of bone marrow samples from 108 adult patients at diagnosis and relapse. *Blood* **100**(7): 2387-92.

Shih, L. Y., M. C. Kuo, D. C. Liang, C. F. Huang, T. L. Lin, J. H. Wu, P. N. Wang, P. Dunn and C. L. Lai (2003). Internal tandem duplication and Asp835 mutations of the FMS-like tyrosine kinase 3 (FLT3) gene in acute promyelocytic leukemia. *Cancer* **98**(6): 1206-16.

Sievers, E. L. (2001). Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukaemia in first relapse. *Expert Opin Biol Ther* **1**(5): 893-901.

Sievers, E. L., B. J. Lange, T. A. Alonzo, R. B. Gerbing, I. D. Bernstein, F. O. Smith, R. J. Arceci, W. G. Woods and M. R. Loken (2003). 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* **101**(9): 3398-406.

Sievers, E. L., B. J. Lange, J. D. Buckley, F. O. Smith, D. A. Wells, C. A. Daigneault-Creech, K. E. Shults, I. D. Bernstein and M. R. Loken (1996). Prediction of relapse of pediatric acute myeloid leukemia by use of multidimensional flow cytometry. *J Natl Cancer Inst* **88**(20): 1483-8.

Smith, B. D., M. Levis, M. Beran, F. Giles, H. Kantarjian, K. Berg, K. M. Murphy, T. Dauses, J. Allebach and D. Small (2004). Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood* **103**(10): 3669-76.

Smith, F. O., B. C. Lampkin, C. Versteeg, D. A. Flowers, P. A. Dinndorf, J. D. Buckley, W. G. Woods, G. D. Hammond and I. D. Bernstein (1992). Expression of lymphoid-associated cell surface antigens by childhood acute myeloid leukemia cells lacks prognostic significance. *Blood* **79**(9): 2415-22.

Smith, M. A., J. G. Smith, C. J. Pallister and C. R. Singer (1996). Haemopoietic growth factors, the cell cycle of acute myeloblastic leukaemia progenitors and sensitivity to cytosine arabinoside. *Leuk Lymphoma* **23**(5-6): 467-72.

Smith, M. T., Y. Wang, E. Kane, S. Rollinson, J. L. Wiemels, E. Roman, P. Roddam, R. Cartwright and G. Morgan (2001). Low NAD(P)H:quinone oxidoreductase 1 activity is associated with increased risk of acute leukemia in adults. *Blood* **97**(5): 1422-6.

Spiekermann, K., K. Bagrintseva, R. Schwab, K. Schmieja and W. Hiddemann (2003). Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. *Clin Cancer Res* **9**(6): 2140-50.

Spiekermann, K., R. J. Dirschinger, R. Schwab, K. Bagrintseva, F. Faber, C. Buske, S. Schnittger, L. M. Kelly, D. G. Gilliland and W. Hiddemann (2003). The protein tyrosine kinase inhibitor SU5614 inhibits FLT3 and induces growth arrest and apoptosis in AML-derived cell lines expressing a constitutively activated FLT3. *Blood* **101**(4): 1494-504.

Stasi, R., G. Del Poeta, A. Venditti, M. Masi, E. Stipa, T. Dentamaro, C. Cox, B. Dallapiccola and G. Papa (1994). Analysis of treatment failure in patients with minimally differentiated acute myeloid leukemia (AML-M0). *Blood* **83**(6): 1619-25.

Stelzer, G. T., K. E. Shults and M. R. Loken (1993). CD45 gating for routine flow cytometric analysis of human bone marrow specimens. *Ann N Y Acad Sci* **677**: 265-80.

Steudel, C., M. Wermke, M. Schaich, U. Schakel, T. Illmer, G. Ehninger and C. Thiede (2003). Comparative analysis of MLL partial tandem duplication and FLT3 internal tandem duplication mutations in 956 adult patients with acute myeloid leukemia. *Genes Chromosomes Cancer* **37**(3): 237-51.

Stirewalt, D. L., K. J. Kopecky, S. Meshinchi, F. R. Appelbaum, M. L. Slovak, C. L. Willman and J. P. Radich (2001). FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood* **97**(11): 3589-95.

Stirewalt, D. L., S. Meshinchi and J. P. Radich (2003). Molecular targets in acute myelogenous leukemia. *Blood Rev* **17**(1): 15-23.

Stone, R. M. (2001). Postremission therapy in adults with acute myeloid leukemia. *Semin Hematol* **38**(3 Suppl 6): 17-23.

Stone, R. M., D. T. Berg, S. L. George, R. K. Dodge, P. A. Paciucci, P. Schulman, E. J. Lee, J. O. Moore, B. L. Powell and C. A. Schiffer (1995). Granulocyte-

macrophage colony-stimulating factor after initial chemotherapy for elderly patients with primary acute myelogenous leukemia. Cancer and Leukemia Group B. *N Engl J Med* **332**(25): 1671-7.

Stone, R. M., M. R. O'Donnell and M. A. Sekeres (2004). Acute myeloid leukemia. *Hematology (Am Soc Hematol Educ Program)*: 98-117.

Stone, R. M., M. R. O'Donnell and M. A. Sekeres (2004). Acute myeloid leukemia. *Hematology Am Soc Hematol Educ Program*: 98-117.

Sutherland, H. J., A. Blair and R. W. Zapf (1996). Characterization of a hierarchy in human acute myeloid leukemia progenitor cells. *Blood* **87**(11): 4754-61.

Tallman, M. S. (2005). New strategies for the treatment of acute myeloid leukemia including antibodies and other novel agents. *Hematology Am Soc Hematol Educ Program*: 143-50.

Tallman, M. S., J. W. Andersen, C. A. Schiffer, F. R. Appelbaum, J. H. Feusner, A. Ogden, L. Shepherd, C. Willman, C. D. Bloomfield, J. M. Rowe and P. H. Wiernik (1997). All-trans-retinoic acid in acute promyelocytic leukemia. *N Engl J Med* **337**(15): 1021-8.

Terpstra, W., R. E. Ploemacher, A. Prins, K. van Lom, K. Pouwels, A. W. Wognum, G. Wagemaker, B. Lowenberg and J. J. Wielenga (1996). Fluorouracil selectively spares acute myeloid leukemia cells with long-term growth abilities in immunodeficient mice and in culture. *Blood* **88**(6): 1944-50.

Terpstra, W., A. Prins, R. E. Ploemacher, B. W. Wognum, G. Wagemaker, B. Lowenberg and J. J. Wielenga (1996). Long-term leukemia-initiating capacity of a CD34-subpopulation of acute myeloid leukemia. *Blood* **87**(6): 2187-94.

Terpstra, W., H. Rozemuller, D. A. Breems, E. J. Rombouts, A. Prins, D. J. FitzGerald, R. J. Kreitman, J. J. Wielenga, R. E. Ploemacher, B. Lowenberg, A. Hagenbeek and A. C. Martens (1997). Diphtheria toxin fused to granulocyte-macrophage colony-stimulating factor eliminates acute myeloid leukemia cells with the potential to initiate leukemia in immunodeficient mice, but spares normal hemopoietic stem cells. *Blood* **90**(9): 3735-42.

Terstappen, L. W., S. Konemann, M. Safford, M. R. Loken, K. Zurlutter, T. Buchner, W. Hiddemann and B. Wormann (1991). Flow cytometric characterization of acute myeloid leukemia. Part 1. Significance of light scattering properties. *Leukemia* **5**(4): 315-21.

Terstappen, L. W. and M. R. Loken (1990). Myeloid cell differentiation in normal bone marrow and acute myeloid leukemia assessed by multi-dimensional flow cytometry. *Anal Cell Pathol* **2**(4): 229-40.

Terstappen, L. W., R. A. Mickaels, R. Dost and M. R. Loken (1990). Increased light scattering resolution facilitates multidimensional flow cytometric analysis. *Cytometry* **11**(4): 506-12.

Terstappen, L. W., M. Safford, S. Konemann, M. R. Loken, K. Zurlutter, T. Buchner, W. Hiddemann and B. Wormann (1992). Flow cytometric characterization of acute myeloid leukemia. Part II. Phenotypic heterogeneity at diagnosis. *Leukemia* **6**(1): 70-80.

Terstappen, L. W., M. Safford and M. R. Loken (1990). Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. *Leukemia* **4**(9): 657-63.

Thiede, C., C. Steudel, B. Mohr, M. Schaich, U. Schakel, U. Platzbecker, M. Wermke, M. Bornhauser, M. Ritter, A. Neubauer, G. Ehninger and T. Illmer (2002). Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* **99**(12): 4326-35.

Treish, I. M. (2000). Targeting leukemia cells with gemtuzumab ozogamicin. *Cancer Pract* **8**(5): 254-7.

Tung, J. W., D. R. Parks, W. A. Moore and L. A. Herzenberg (2004). New approaches to fluorescence compensation and visualization of FACS data. *Clin Immunol* **110**(3): 277-83.

Turhan, A. G., F. M. Lemoine, C. Debert, M. L. Bonnet, C. Baillou, F. Picard, E. A. Macintyre and B. Varet (1995). Highly purified primitive hematopoietic stem cells are PML-RARA negative and generate nonclonal progenitors in acute promyelocytic leukemia. *Blood* **85**(8): 2154-61.

Valk, P. J., R. G. Verhaak, M. A. Beijen, C. A. Erpelinck, S. Barjesteh van Waalwijk van Doorn-Khosrovani, J. M. Boer, H. B. Beverloo, M. J. Moorhouse, P. J. van der Spek, B. Lowenberg and R. Delwel (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* **350**(16): 1617-28.

van Der Velden, V. H., J. G. te Marvelde, P. G. Hoogeveen, I. D. Bernstein, A. B. Houtsmuller, M. S. Berger and J. J. van Dongen (2001). Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: in vivo and in vitro saturation and internalization by leukemic and normal myeloid cells. *Blood* **97**(10): 3197-204.

van Rhenen, A., N. Feller, A. Kelder, A. H. Westra, E. Rombouts, S. Zweegman, M. A. van der Pol, Q. Waisfisz, G. J. Ossenkoppele and G. J. Schuurhuis (2005). High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. *Clin Cancer Res* **11**(18): 6520-7.

van Rhenen, A., G. A. van Dongen, A. Kelder, E. J. Rombouts, N. Feller, B. Moshaver, M. S. Walsum, S. Zweegman, G. J. Ossenkoppele and G. Jan Schuurhuis (2007). The novel AML stem cell associated antigen CLL-1 aids

in discrimination between normal and leukemic stem cells. *Blood* **110**(7): 2659-66.

Vardiman, J. W., N. L. Harris and R. D. Brunning (2002). The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood* **100**(7): 2292-302.

Venditti, A., F. Buccisano, G. Del Poeta, L. Maurillo, A. Tamburini, C. Cox, A. Battaglia, G. Catalano, B. Del Moro, L. Cudillo, M. Postorino, M. Masi and S. Amadori (2000). Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood* **96**(12): 3948-52.

Venditti, A., L. Maurillo, F. Buccisano, A. Tamburini, G. Del Poeta, M. I. Del Principe, P. Panetta, M. I. Consalvo, C. Mazzone, A. Tendas, M. Trawinska, V. Forte and S. Amadori (2003). Multidimensional flow cytometry for detection of minimal residual disease in acute myeloid leukemia. *Leuk Lymphoma* **44**(3): 445-50.

Venditti, A., A. Tamburini, F. Buccisano, G. Del Poeta, L. Maurillo, P. Panetta, K. A. Scornajenghi, C. Cox and S. Amadori (2002). Clinical relevance of minimal residual disease detection in adult acute myeloid leukemia. *J Hematother Stem Cell Res* **11**(2): 349-57.

Verhaak, R. G., C. S. Goudswaard, W. van Putten, M. A. Bijl, M. A. Sanders, W. Hugens, A. G. Uitterlinden, C. A. Erpelinck, R. Delwel, B. Lowenberg and P. J. Valk (2005). Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood* **106**(12): 3747-54.

Vidriales, M. B., J. F. San-Miguel, A. Orfao, E. Coustan-Smith and D. Campana (2003). Minimal residual disease monitoring by flow cytometry. *Best Pract Res Clin Haematol* **16**(4): 599-612.

Voskova, D., L. Valekova, J. Fedorova, J. Hudecek and P. Kubisz (2003). Leukemic cells and aberrant phenotypes in acute leukemia patients: a flow cytometry analysis. *Neoplasma* **50**(6): 422-7.

Wang, J. C. and J. E. Dick (2005). Cancer stem cells: lessons from leukemia. *Trends Cell Biol* **15**(9): 494-501.

Wang, L., D. Lin, X. Zhang, S. Chen, M. Wang and J. Wang (2005). Analysis of FLT3 internal tandem duplication and D835 mutations in Chinese acute leukemia patients. *Leuk Res* **29**(12): 1393-8.

Wang, L. H., C. L. Zhou, X. W. Zhang, S. Chen, M. Wang and J. X. Wang (2004). [Prevalence and clinical significance of FLT3 internal tandem duplication mutation in acute leukemia]. *Zhonghua Xue Ye Xue Za Zhi* **25**(7): 393-6.

Weisberg, E., C. Boulton, L. M. Kelly, P. Manley, D. Fabbro, T. Meyer, D. G. Gilliland and J. D. Griffin (2002). Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell* **1**(5): 433-43.

Wheatley, K., A. K. Burnett, A. H. Goldstone, R. G. Gray, I. M. Hann, C. J. Harrison, J. K. Rees, R. F. Stevens and H. Walker (1999). A simple, robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukaemia derived from the MRC AML 10 trial. United Kingdom Medical Research Council's Adult and Childhood Leukaemia Working Parties. *Br J Haematol* **107**(1): 69-79.

Whitman, S. P., K. J. Archer, L. Feng, C. Baldus, B. Becknell, B. D. Carlson, A. J. Carroll, K. Mrozek, J. W. Vardiman, S. L. George, J. E. Kolitz, R. A. Larson, C. D. Bloomfield and M. A. Caligiuri (2001). Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a cancer and leukemia group B study. *Cancer Res* **61**(19): 7233-9.

Wingo, P. A., C. J. Cardinez, S. H. Landis, R. T. Greenlee, L. A. Ries, R. N. Anderson and M. J. Thun (2003). Long-term trends in cancer mortality in the United States, 1930-1998. *Cancer* **97**(12 Suppl): 3133-275.

Xu, F., T. Taki, H. W. Yang, R. Hanada, T. Hongo, H. Ohnishi, M. Kobayashi, F. Bessho, M. Yanagisawa and Y. Hayashi (1999). Tandem duplication of the FLT3 gene is found in acute lymphoblastic leukaemia as well as acute myeloid leukaemia but not in myelodysplastic syndrome or juvenile chronic myelogenous leukaemia in children. *Br J Haematol* **105**(1): 155-62.

Yamamoto, Y., H. Kiyoi, Y. Nakano, R. Suzuki, Y. Kodera, S. Miyawaki, N. Asou, K. Kuriyama, F. Yagasaki, C. Shimazaki, H. Akiyama, K. Saito, M. Nishimura, T. Motoji, K. Shinagawa, A. Takeshita, H. Saito, R. Ueda, R. Ohno and T. Naoe (2001). Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* **97**(8): 2434-9.

Yanada, M., K. Matsuo, T. Suzuki, H. Kiyoi and T. Naoe (2005). Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations for acute myeloid leukemia: a meta-analysis. *Leukemia* **19**(8): 1345-9.

Yin, J. A. and K. Tobal (1999). Detection of minimal residual disease in acute myeloid leukaemia: methodologies, clinical and biological significance. *Br J Haematol* **106**(3): 578-90.

Yokota, S., H. Kiyoi, M. Nakao, T. Iwai, S. Misawa, T. Okuda, Y. Sonoda, T. Abe, K. Kahsima, Y. Matsuo and T. Naoe (1997). Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. *Leukemia* **11**(10): 1605-9.

Zeeb, H. and M. Blettner (1998). Adult leukaemia: what is the role of currently known risk factors? *Radiat Environ Biophys* **36**(4): 217-28.

Zheng, R., A. D. Friedman and D. Small (2002). Targeted inhibition of FLT3 overcomes the block to myeloid differentiation in 32Dcl3 cells caused by expression of FLT3/ITD mutations. *Blood* **100**(12): 4154-61.

Zwaan, C. M., S. Meshinchi, J. P. Radich, A. J. Veerman, D. R. Huismans, L. Munske, M. Podleschny, K. Hahlen, R. Pieters, M. Zimmermann, D. Reinhardt, J. Harbott, U. Creutzig, G. J. Kaspers and F. Griesinger (2003). FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. *Blood* **102**(7): 2387-94.

Zwick, D., L. Cooley and M. Hetherington (2006). Minimal residual disease testing of acute leukemia by flow cytometry immunophenotyping: a retrospective comparison of detection rates with flow cytometry DNA ploidy or FISH-based methods. *Lab Hematol* **12**(2): 75-81.

PUBLICATIONS

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