THE UNIVERSITY OF ADELAIDE
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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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Research conducted at Institute of Medical and Veterinary Science

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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)*

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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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5. The Role of Multiparameter Flow Cytometry for Detection of Minimal Residual Disease in Acute Myeloid Leukaemia. **Al-Mawali A**, Gillis D, Lewis I. *(2008). Manuscript submitted.*

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

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"*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 presence of Leukaemia-Associated Phenotypes in adult Acute Myeloid Leukaemia at diagnosis is of prognostic significance"*

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Eleventh Adelaide Blood Club Meeting with HSA and TIGSA

Sunset Cove, Yankalilla, SA from 8-10 September 2006

"*The Presence of Leukaemia-Associated Phenotypes is an Independent Predictor of Poor Prognosis in Acute Myeloid Leukaemia"*

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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^{TM} 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.