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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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in the School of Medicine at the University of Adelaide

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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)


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoid leukaemia</td>
</tr>
<tr>
<td>AlloSCT</td>
<td>Allogeneic stem cell transplants</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukaemia</td>
</tr>
<tr>
<td>AutoSCT</td>
<td>Autologous stem cell transplants</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CBF</td>
<td>Core binding factor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD34</td>
<td>(sialomucin = haemopoietic progenitor cell antigen-1, HPCA-1)</td>
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<td>CLL-1</td>
<td>C-type lectin–like molecule 1</td>
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<td>CML</td>
<td>Chronic myeloid leukaemia</td>
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<td>CR</td>
<td>Complete remission</td>
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<td>DMSO</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ECD</td>
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<td>EDTA</td>
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<tr>
<td>FAB</td>
<td>French- American- British</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activating cell sorter</td>
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<tr>
<td>FC</td>
<td>Flow cytometry</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>Acronym</td>
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<tr>
<td>FLT3</td>
<td>Fms like tyrosine kinase3</td>
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<td>FLT3/ITD</td>
<td>Fms like tyrosine kinase3 – internal tandem duplication</td>
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<td>HSC</td>
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<tr>
<td>IL-3α</td>
<td>Interleukin 3 alpha receptor</td>
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<td>IMDM</td>
<td>Iscove’s modification of dulbecco’s medium</td>
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<td>Matched unrelated donor</td>
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<td>PB</td>
<td>Peripheral blood</td>
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<td>Paraformaldehyde</td>
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<td>RFS</td>
<td>Relapse free survival</td>
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<td>Ribonucleic acid</td>
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<tr>
<td>ROC</td>
<td>Receiver operating characteristics</td>
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<td>RT</td>
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<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>WBC</td>
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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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Adelaide, South Australia 2-June 2006

“The presence of Leukaemia-Associated Phenotypes in adult Acute Myeloid Leukaemia at diagnosis is of prognostic significance”

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

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The Australian Society for Medical Research, ASMR, SA Scientific Meeting 2007
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A. Al-Mawali, D. Gillis, I. Lewis

30th Annual Meeting of the Australasian Flow Cytometry Group AFCG 2007

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A. Al-Mawali, D. Gillis, I. Lewis


“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” ,

“The Oncogenic Events of FLT3 Internal Tandem Duplication Happen At A Stage Of Stem Cells That Possess IL-3 Alpha Receptor” AND

“Detection of Minimal Residual Disease in Acute Myeloid Leukaemia Identifies Patients with High Risk of Relapse and May Play A Role in Post Induction Treatment Stratification”.
A. Al-Mawali, D. Gillis, I. Lewis
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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
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 FACS\textsuperscript{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.