Faculty of Medicine and Health Sciences

IMMUNOPHENOTYPIC CHARACTERISATION AND EVALUATION OF MINIMAL RESIDUAL DISEASE (MRD) IN ACUTE LEUKAEMIA PATIENTS IN SARAWAK BY FLOW CYTOMETRY

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ABSTRACT

Leukaemia is a malignant disease of the bone marrow and blood which usually affect children and adults. Minimal residual disease (MRD) is the definition given to a small numbers of leukaemic cells that remain in the patient when the patient is in remission, and a known cause of relapse in cancer and leukaemia. Using immunophenotypic multiparametric flow cytometry, sequential studies (diagnosis and follow-up) of the characterisation of the leukaemic blast and the association between the clinical parameters of 147 acute myeloid leukaemia (AML) patients and 122 acute lymphoblastic leukaemia (ALL) were investigated. Patients diagnosed with acute leukaemia were recruited from hospitals all over Sarawak within the duration of 3 years, from 2007 until 2010. Patients were studied at diagnosis with a panel of monoclonal antibodies in 3- and 4-colour antibody combinations for detections of common, aberrant or uncommon phenotypic features. Using the patient's immunophenotypic features during diagnosis, the identification of residual leukaemic cells were made possible after completion of induction chemotherapy and the frequency of antigen expression was determined for minimal residual detection. Other clinical data such as haemoglobin level, lymph node enlargement, liver, splenomegaly, platelet and white blood count were also analysed. The odds ratio between MRD and the relevant parameters involved was also calculated with statistical software. The data were analysed statistically and p value < 0.05 is considered as statistically significant. In the AML cases, female childhood patients were found to have higher total white count compared to males (p =0.008). Immunophenotypic results also showed similar expressions between childhood and adult group with high expression of CD33, CD13 and aberrant marker such as CD19, and may be established as diagnosis standard markers. In the ALL cases, for the Malay/Melanau ethnic

group, more adult males were diagnosed with B-ALL compared to adult females (p=0.043). Antigen expressions for both childhood and adult groups were similar to previous studies, with CyCD79a, CD19, CD22 for B-ALL, while CD7 and CyCD3 for T-ALL, thus suggesting the antibody panels used may be established as diagnostic markers. For the MRD analysis, only 28 AML cases (19.0%) were analysed for MRD and no clinical and immunophenotypic parameter was significantly related with MRD outcome. Multiple logistic regression (MLR) analysis for AML was also not significant. As for ALL, 77 cases (64.2%) were studied for MRD. It was found that low haemoglobin level was significantly associated with positive MRD ($p<0.001$*). In MLR analysis, haemoglobin level and splenomegaly were found to be significantly associated with MRD positivity, with odd risks of 1.68 and 37.98 respectively. Overall, from the study it can be concluded that clinical parameters and immunophenotypic expressions could be used as a predictive factors in MRD outcome. This study could be improved by a greater effort to coordinate leukaemia patients' treatment and follow up in Sarawak. A more comprehensive study regarding MRD in acute leukaemia patients is therefore recommended in order to provide the health providers more concrete data about the prognostic/predictive factors that may influence the disease outcome, and eventually assist in the future treatment and management of acute leukaemia in Sarawak.

ABSTRAK

Leukemia adalah penyakit malignant bagi tulang sumsum dan darah yang selalunya melibatkan golongan kanak-kanak dan dewasa. Penyakit residu minimal (MRD) boleh ditakrifkan sebagai sebahagian kecil sel leukemia yang masih tertinggal di dalam pesakit semasa pesakit berada dalam keadaan remisi, dan dikenalpasti sebagai punca utama leukemia dan kanser berulang. Menggunakan *immunophenotyping multiparameter flow cytometry*, kajian secara berterusan (diagnosis dan susulan) dilakukan terhadap ciri-ciri sel blast leukemia dan kaitannya dengan parameter klinikal bagi 147 pesakit leukemia myeloid akut dan 122 pesakit leukemia limfoblas akut. Nisbah risiko antara MRD dan parameter berkaitan juga dilakukan dengan menggunakan analisa statistik. Pesakit-pesakit yang didiagnosis dengan leukemia akut telah diperolehi dari seluruh hospital di Sarawak selama tempoh 3 tahun, iaitu dari 2007 sehingga 2010. Pesakit-pesakit ini telah dikaji semasa diagnosis dengan menggunakan panel antibodi monoklonal menggunakan 3 dan 4 kombinasi warna antibodi untuk mengesan ciri-ciri *phenotypic* yang biasa, luar biasa atau *aberrant*. Berdasarkan ciri-ciri *immunophenotypic* pesakit semasa diagnosis, pengenalpastian sel residu leukemia boleh dilakukan selepas selesainya rawatan kemoterapi induksi dan frekuensi ekspresi antigen juga telah ditentukan untuk pengesanan penyakit residu minimal. Analisis data klinikal pesakit yang lain seperti tahap hemoglobin, nodus limfa, hati, limpa, kiraan platelet, dan kiraan sel darah putih juga dilakukan. Kesemua data tersebut kemudiannya dianalisis dengan beberapa ujian statistik dan bacaan nilai *p* kurang daripada 0.005 dianggap sebagai signifikan secara statistik. Dalam kajian kes diagnosis AML, didapati pesakit kanak-kanak perempuan mempunyai kiraan sel darah putih yang tinggi berbanding kanak-kanak lelaki (p =0.008). Keputusan *immunophenotypic* juga menunjukkan ekspresi yang hampir sama

untuk kumpulan pesakit AML dewasa dan kanak-kanak, khasnya ekspresi yang tinggi bagi CD33, CD13 dan juga penanda *aberrant* seperti CD19, dan boleh digunakan sebagai penanda standard bagi kes diagnosis. Hasil kajian kes bagi diagnosis ALL pula mendapati bahawa dalam etnik Melayu/Melanau, lelaki dewasa lebih cenderung didiagnosis dengan sub-jenis ALL iaitu B-ALL berbanding wanita dewasa (p = 0.043). Ekspresi antigen untuk kedua kumpulan kanak-kanak dan dewasa juga menunjukkan persamaan ekspresi yang hampir sama, dengan ekspresi tinggi bagi CyCD79a, CD19, CD22 bagi B-ALL manakala CD7 dan CyCD3 untuk sub-jenis T-ALL. Bagi kajian MRD, hanya sebanyak 28 kes AML (19.0%) daripada kes-kes diagnosis telah dianalisis untuk MRD dan tiada parameter klinikal dan *immunophenotypic* yang dijumpai signifikan dengan hasil MRD. Analisis *multiple logistic regression* (MLR) bagi AML juga didapati tidak signifikan dengan MRD. Bagi ALL, sebanyak 77 kes (64.2%) daripada kes-kes diagnosis elah layak diteruskan untuk analisis MRD. Didapati bahawa tahap haemoglobin yang rendah bagi pesakit ALL adalah signifikan dengan MRD yang positif ($p<0.001$*). Bagi analisis MLR pula, tahap hemoglobin dan *splenomegaly* didapati mempunyai perkaitan yang signifikan dengan hasil MRD yang positif, dengan masing-masing mempunyai nisbah risiko sebanyak 1.68 dan 37.98. Keseluruhannya, daripada kajian ini dapat disimpulkan bahawa parameter-parameter klinikal dan ekspresi *immunophenotypic* boleh diambil kira sebagai faktor ramalan bagi hasil MRD. Kajian ini boleh ditambah baik dengan usaha yang lebih berkesan dan menyeluruh dalam mengkoordinasi rawatan pesakit leukemia serta rawatan susulan di Sarawak khasnya. Kajian yang lebih komprehensif berkenaan MRD di Malaysia pula adalah disyorkan untuk menyediakan data yang lebih konkrit berkenaan faktor prognostik/ramalan yang boleh mempengaruhi prognosis penyakit kepada penyedia penjagaan kesihatan, sekaligus membantu mereka dalam rawatan dan pengurusan pesakit yang lebih berkesan pada masa hadapan.

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ALL	Acute lymphocytic/lymphoblastic leukaemia
AML	Acute myeloid leukaemia
HSCs	Hematopoietic stem cells
%	Percentage
WHO	World Health Organization
FAB	French-American British
MDS	Myelodysplastic syndrome
WBC	White blood counts
CD	Cluster of differentiation
MPO	Myeloperoxidase
RARA	Retinoic acid receptor α
PML	Promyelocytic leukaemia
Cy	Cytoplasmic
Sm	Surface membrane
TdT	Terminal deoxynucleotidyl transferase
Gly A	Glychophorin A
Ig	Immunoglobulin
TCR αβ	T-cell receptor alpha beta
MRD	Minimal residual disease
LAIP	Leukaemia-associated phenotypes
BMA	Bone marrow aspirates
MoAbs	Monoclonal antibodies
OS	Overall survival
RFS	Relapse-free survival
FISH	Fluorescent in situ hybridization
RT-PCR	Real-time polymerase chain reaction
FCM	Flow cytometry
PCR	Polymerase chain reaction
RQ-PCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
LCR	Ligase chain reaction
NCR	National Cancer Registry
CR	Cancer incidence
CML	Chronic myeloid leukemia
HSCT	Hematopoietic stem cell transplantation
T-ALL	T-cell Acute lymphocytic leukaemia
B-ALL	B-cell Acute lymphocytic leukaemia
PB	Peripheral blood
FBC	Full blood count
PBS	Phosphate buffered saline
µm	Micro meter

µl	Micro litre
UV	Ultraviolet
nm	Nano meter
PMT	Photomultipliers
ml	mili liter
FSC	Forward Scatter
SSC	Side Scatter
FL	Fluorochrome
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
APC	Allophycocyanin
g	Gravity
RBC	Red blood cells
PBS	Phosphate buffered saline
Hb	Haemoglobin
Plt	Platelet
S.D	Standard deviation
L	Litre
TWC	Total white count
MLR	Multiple logistic regression
LN	Lymph node

CHAPTER 1: INTRODUCTION

1.1 Leukaemia

Leukaemia is a malignant disease of the bone marrow and blood. In the United States alone, a total of estimated 44,600 new cases of leukaemia would be expected in 2011, acute myeloid leukaemia with 12,950 cases and acute lymphoblastic leukaemia with 5,730 cases. It is also estimated 21,780 deaths would be expected to occur in 2011 due to leukaemia in the United States. (American Cancer Society, 2011) Bone marrow is the site in the body where most blood cells are produced, including the defective blast cells (Figure 1.1.1). When bone marrow produces an excessive number of blast cells, these cells may or may not become abnormal, and if it does, these abnormal cells are now known as leukaemic blasts. Leukaemia is one of the most common blood-related malignancies, and generally occurs when too many white blood cells remain as blast cells. Unlike normal cells, these mutated blast cells do not eventually mature and begin functioning within the body. They usually continue to be immature, and are more often formed at a rapid pace. Leukaemia is divided into two main categories; myeloid or lymphocytic cell lineages, and each of this-subtype can either present as acute or chronic. There is also biphenotypic leukaemia, the term used when the cells are showing both lymphoid and myeloid maturation.

1.1.1 Acute Leukaemia

In general, there are mainly two types of acute leukaemia: i.e acute lymphocytic leukaemia (ALL) and acute myeloid leukaemia (AML). The term lymphocytic indicate that the cancerous change takes place in the type of blood cells that forms lymphocytes, while the term myeloid indicate that the cell change takes place in a type of blood cell that normally

goes on to form red cells, some types of white cells, and platelets. These cells are each composed of blast cells, known as lymphoblasts or myeloblasts respectively. Acute leukaemia have the tendency to progress rapidly if compared to chronic leukaemia and do especially so when especially when left without treatment. Acute leukeamias, if left untreated may lead to death in weeks or months whereas in chronic leukaemias, if untreated may lead to death in months or years (Bain, 2003).

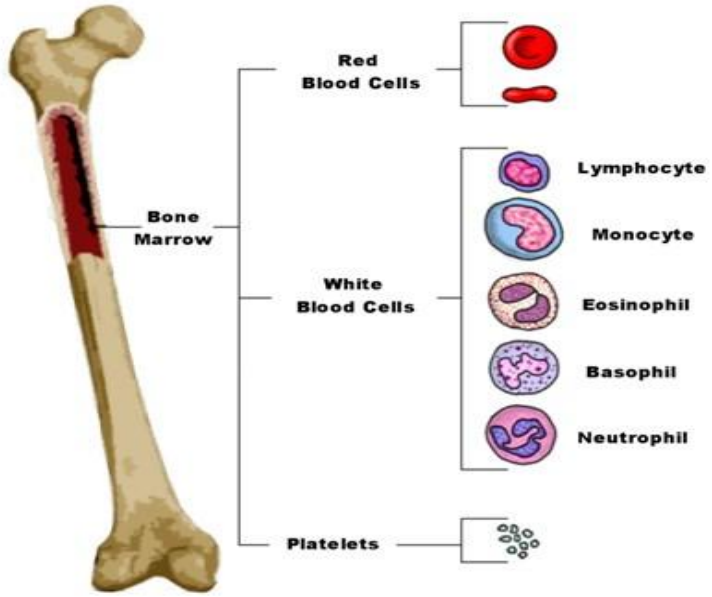


Figure 1.1.1 The formation of normal blood cells in bone marrow (Buccheri V et al., 1992).

1.1.2 Acute leukaemia pathogenesis

Haematopoiesis is the process by which all the different cell lineages that form the blood and immune system are generated from a common pluripotent stem cell. During the life of an individual, two separate hematopoietic systems exist, the primitive and definitive. Both arise during embryonic development but only one persists into adulthood. The primitive hematopoietic system is derived from the extra-embryonic yolk sac and consists mainly of nucleated erythroid cells, which carry oxygen to the developing embryonic tissues. As the

embryo increases in size, this early circulatory system is replaced by the more complex definitive hematopoietic system, which originates within the embryo itself and continues throughout life. The definitive hematopoietic system is made up of all adult blood cell types. All these cells are derived from pluripotent hematopoietic stem cells (HSCs) through a succession of precursors with progressively limited potential under the control of specific cytokines such as interleukins and granulocyte/monocyte-stimulating factors. HSCs are primarily found in the bone marrow, and are also present in a variety of other tissues including peripheral blood and umbilical cord blood, and at low numbers in the liver, spleen, and perhaps many organs (Holyoake TL, 1999).

The process of haematopoiesis involves a complex interplay between the intrinsic genetic processes of blood cells progenitors and their microenvironment. This relationship determines whether HSCs, progenitors, and mature blood cells remain inactive, proliferate, differentiate, self-renew, or undergo apoptosis (Orkin SH, 2002). HSCs are able to generate every lineage found in the haematopoietic system including red blood cells, platelets, and a variety of lymphoid and myeloid cells (Figure 1.1.2) (Eaves C, 1997).

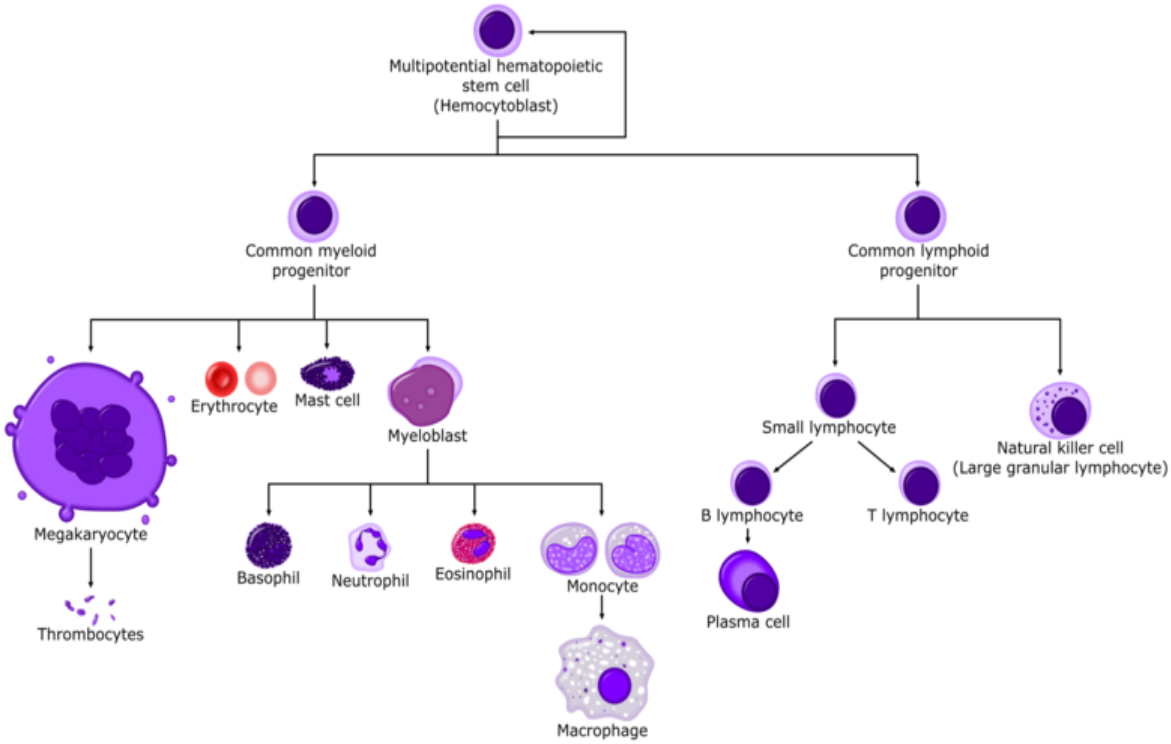


Figure 1.1.2 Normal haematopoeisis
Source: (Huh HY et al., 1996)

Leukaemia is postulated to be a consequence of mutation(s) in a single stem cell, the progeny of which will form a clone of leukaemic cells. Genetic event contributing to malignant transformation is hypothsised to be accumulative and these include inappropriate expression of oncogenes and loss of function of tumour suppressor genes (Bain, 2003). The main cause of the mutation still remains unknown, but the risk factors have been identified. Among the risk factors identified are exposure to radiation, smoking, benzene, and many more (Zhang, 1998). Generally, the four types of leukaemia, either lymphoid or myeloid (acute &chronic), - each begin in a mutated cell in the bone marrow. With these risk factors affecting the cells, it will undergo a series of leukaemic change and multiplies into many progeny cells. The

leukaemia cells population will grow and acquire better survival compared to normal cells and, over time, they overpopulate the normal cells.

In acute leukaemia, normal polyclonal haemopoietic cells are largely replaced by abnormal cells, which are the progeny of a single cell and are therefore, designated a clone. The rate at which leukaemia progresses and how the cells replace the normal blood and marrow cells are different with each type of leukaemia. In many instances, the abnormal clone has acquired a single or a few chromosomal abnormalities, which may be detectable by examination of the chromosomes of abnormal cells arrested at metaphase. When the karyotype of bone marrow cells is studied, some cells may show random abnormalities, which may further need to be distinguished from a non-random or consistent abnormality. Non-random or consistent chromosomal abnormality indicates the presence of an abnormal clone. According to the International System of Nomenclature (Nomenclature, 1978), a clone is considered to be present if two cells showed identical structural change or additional chromosomes or if three cells showed the exact same missing chromosome. The comparison between normal and leukaemic stem cell haematopoiesis is illustrated in Figure 1.1.3

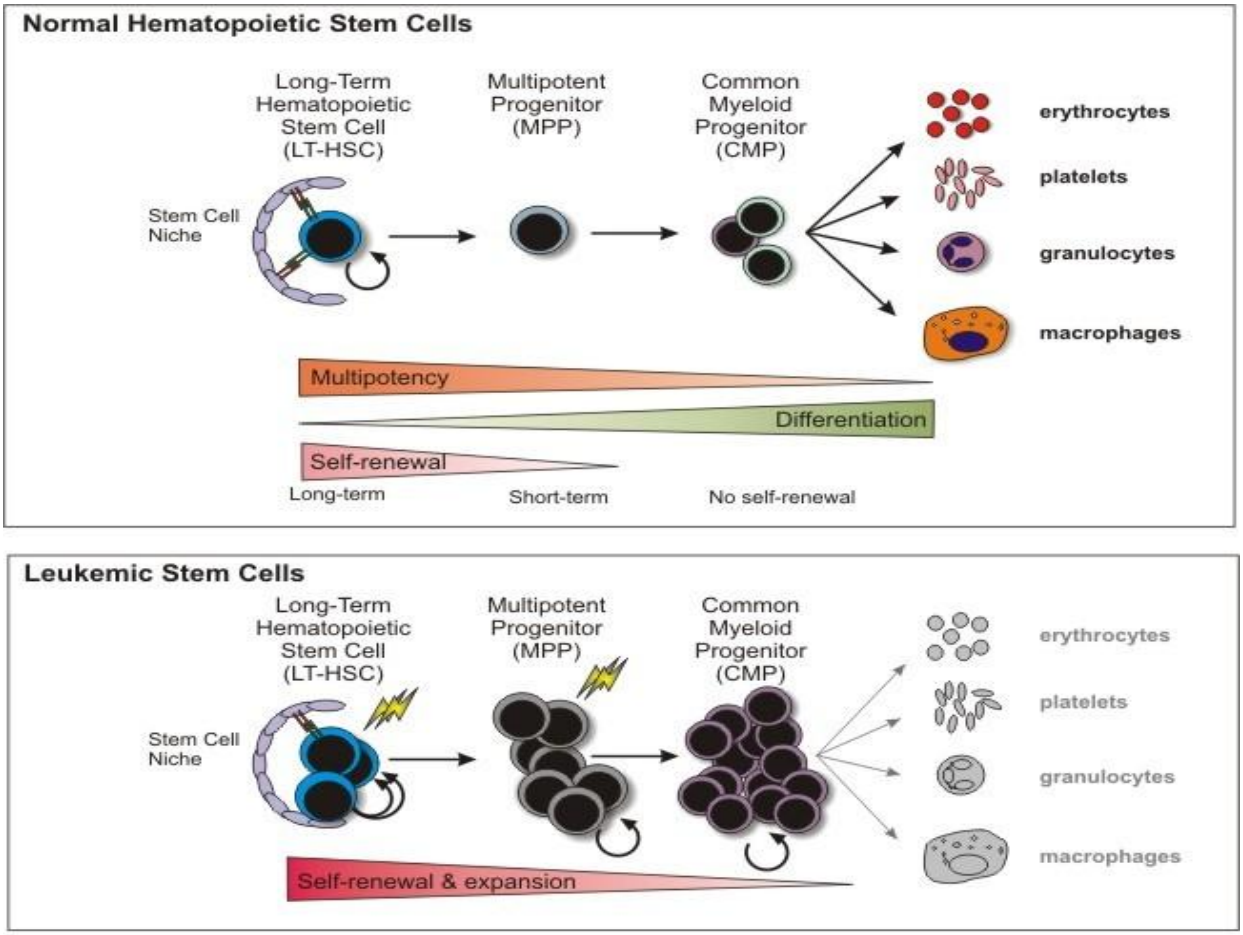


Figure 1.1.3 Self renewal and differentiation of normal and leukaemic stem cells (van Schravendijk MR et al., 1992)

1.2. Acute Myeloid Leukaemia (AML)

Acute myeloid leukaemia (AML) is a malignant disease of the bone marrow in which precursors of blood cells are arrested at various stages of early development and maturation. Most AML subtypes show more than 30% blasts of a myeloid lineage in the blood, bone marrow, or both (Bain, 2003). The maturational arrest of bone marrow cells is usually caused by genetic abnormalities. There are various sub-types of AML, depending on exactly what cell type of cell that becomes malignant and at which stage it is in the maturation process. Typically, AML develops quite quickly and rapidly becomes increasingly worse over a few weeks or so unless treated. It is the commonest type of leukaemia in adults, with low incidence in childhood (Bain, 2003). It is most often diagnosed in older people, aged over 65 years old (Bain, 2003). Based on the French-American-British (*FAB*) classification system, AML is divided into 8 subtypes, from M0 to M7, based on the type of cell from which the leukaemia developed and its degree of maturity (Table 1.1) (Bennett J, 1976).

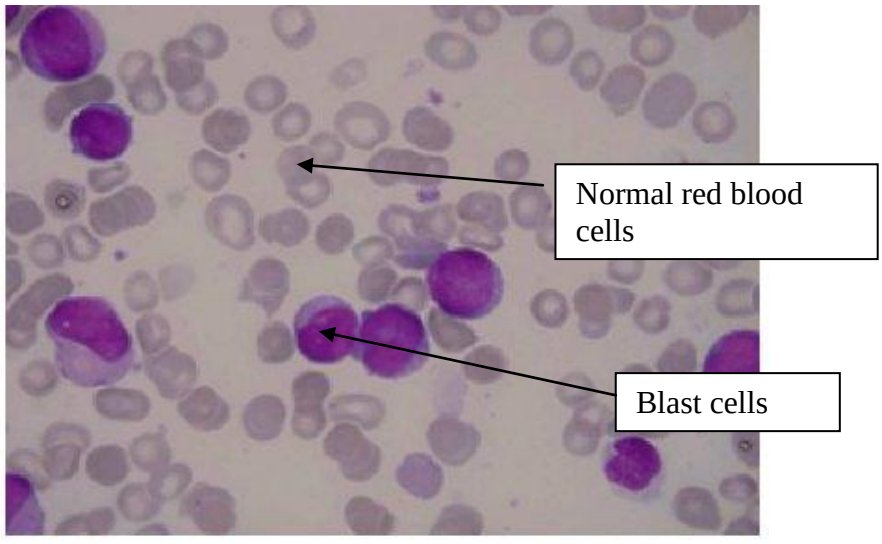


Figure 1.1.4 Typical morphology of bone marrow aspirate in acute myeloid leukaemia showing myeloblasts (R. Gudena, 2005)

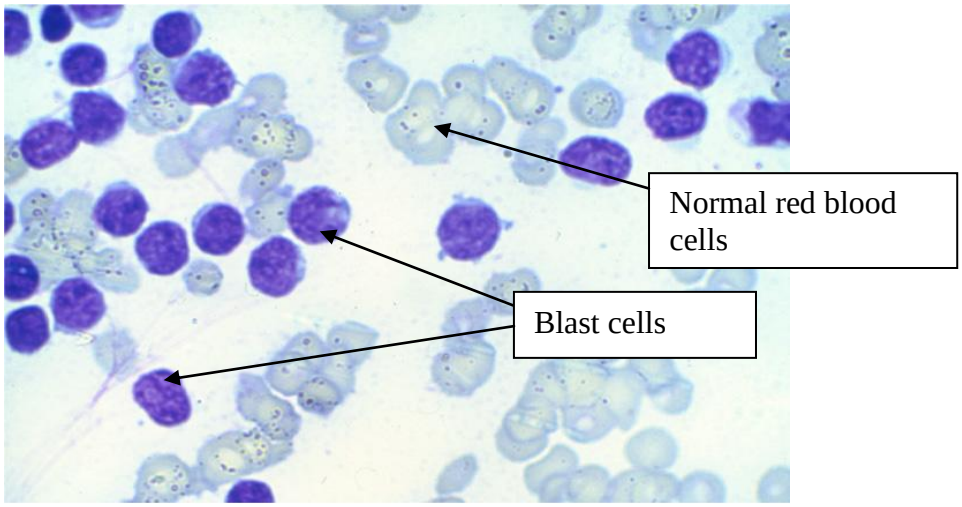


Figure 1.1.5 Another example of AML blasts morphology, as seen under a microscope. (Lim et al., 2008)

Table 1.1 FAB classification of AML

Subtypes	Morphology
M0	Minimal myeloid differentiation
M1	Poorly differentiated myeloid
M2	Myeloblastic with differentiation
M3	Promyelocytic
M4	Myeloblastic and monocytic
M5	Monoblastic
M6	Erythroleukaemic
M7	Megakaryoblastic

During the last decade, the classification of leukaemia has been revised and the 2001 World Health Organization (WHO) classification of acute myeloid leukaemia is gaining prominence. This classification was devised to be more clinically relevant and hence able to produce more

meaningful prognostic information in comparison to the FAB criteria. The AML classification by WHO is shown in Table 1.2 (Vardiman JW, 2002).

WHO classification divides AML into broader groups based on factors such as genetic abnormalities and the presence of other leukaemia-related blood disorders, for example myelodysplastic syndrome. A basic concept of the WHO system is that the classification of haematopoietic and lymphoid neoplasms should incorporate not only morphologic findings but also all available information, including genetic, immunophenotypic, biologic, and clinical features to define specific disease entities. The WHO classification also attempts to incorporate those disease characteristics that have proved to have clinical and biologic relevance into a useful, working nomenclature (Vardiman JW, 2002).

Over the years, clinicians and scientists realised that morphologic-genetic correlations are not always perfect, and the genetic findings may predict the prognosis and biologic properties of the leukaemia more consistently than does morphology. Although the FAB classification recognises the morphologic heterogeneity of AML, it does not always reflect the underlying genetic or clinical diversity of the disease. Although the WHO classification may be more useful, the FAB system is still widely used, most probably because its simplicity compared to the WHO classification, especially in less developed or developing countries.

Table 1.2 The new classification of AML by WHO

WHO classification of AML	
Subtype	**Description**
AML with characteristic genetic abnormalities	Includes: • AML with translocations between chromosome 8 and 21 [t(8;21)] • AML with inversions in chromosome 16 [inv(16)] • AML with translocations between chromosome 15 and 17 [t(15;17)] In general, these patients have a higher rate of remission and a better prognosis compared with other types of AML.
AML with multilineage dysplasia	Includes patients who have had a prior myelodysplastic syndrome (MDS) or myeloproliferative disease that transforms into AML. This occurs most often in elderly patients and often has a worse prognosis.
AML and MDS, therapy-related	Includes patients who have had prior chemotherapy and/or radiation and subsequently develop AML or MDS. These may have specific chromosomal abnormalities and often carry a worse prognosis. • Alkylating agent-related - alkylating agents are a certain type of drug used in chemotherapy for various cancers. Examples would include drugs like altretamine. • Epipodophyllotoxin-related - epipodophyllotoxins are also used in chemotherapy
AML not otherwise categorised	Includes other subtypes of AML that do not fit into the above categories.

1.2.1 Signs and symptoms of AML

The most common symptom of AML resulted from the shortage of normal blood cells due to the overcrowding of the bone marrow by abnormal leukaemic blast cells and its compromised ability to produce adequate numbers of normal red cells, white cells and platelets. Due to the rapid progression of AML, patients usually report feeling unwell for only a short period of time before they are clinically symptomatic and diagnosed. Some patients, particularly the younger ones, may present with acute symptoms over a few days to 1-2 weeks. The common symptoms of AML (Karen Seiter,2010) include:

- Bone marrow failure: Patient may present with anaemia, neutropaenia, and thrombocytopaenia. Anaemia usually presents as fatigue and patient may appear pale with or without cardiac flow murmur, whilst neutropaenia and thrombocytopaenia may lead to infection and bleeding tendencies. Patients may also in retrospective noted a decreased energy level months before presentation.
- Fever: This may occur with or without specific documentation of an infection.
- Organomegaly: Symptoms due to organomegaly may be the consequence of organ infiltration with leukaemic cells and manifested as hepatosplenomegaly and to a lesser degree, lymphadenopathy.
- High WBC counts (>100,000 cells/μL): can present with symptoms of leukostasis, in which among the symptoms are respiratory distress and altered mental status.
- Bone pain: This is usually caused by increased pressure in the bone marrow.

1.2.2 Genetic abnormalities and immunophenotyping of AML

Various techniques have been invented and improvised to demonstrate the presence of genetic abnormalities in malignant cells, especially in leukaemia. This is due to it being relatively easier to obtain leukaemic cells for genetic studies. The abnormal cells that had acquired a chromosomal abnormality can be detected by examination of the metaphase spread of the cancer cell chromosomes. Besides that, other molecular techniques including polymerase chain reaction, fluorescent *in situ* hybridisation and microarray technology are increasingly being used. In addition to molecular genetic studies, immunophenotyping by flow cytometry is also performed on cases of leukaemia and during patient follow up. Immunophenotyping is the analysis of heterogeneous populations of cells for the purpose of identifying the presence and proportions of the various population of interest. Antibodies are used to identify cells by detecting specific antigens expressed by these cells, which are known as markers. However, they are often expressed on more than one cell type. Generally in leukaemia, immunological markers that are expressed in myeoid lineage leukaemia differ from those exhibited by lymphoid leukaemia, and this characteristic has been exploited for leukaemia diagnosis and disease monitoring. Furthermore, immunophenotyping can yield results faster than most molecular techniques. As such, flow cytometric immunophenotyping has been used widely in the diagnosis of leukaemia. In AML, the common markers include CD13, CD33, CD65 and CD117 clusters and reactivity with myeloperoxidase (MPO) (Bain, 2003). The recommended immunophenotyping antibodies panels for AML is shown in Table 1.3 (Bene MC, 1995). In most centres, molecular studies, morphological examination and flow cytometric immunophenotyping of leukaemia are performed, as this information is part of the requirement for leukaemia diagnosis as recommended by WHO.

t(8;21)(q22;q22) is one of the two most common specific translocations in AML, the other being t(15:17) (Haferlach T, 1996). For t(8;21)(q22;q22), the molecular mechanism of leukaemogenesis is fusion part of the *AML1* or *RUNX1* gene at 8q22 with part of the *ETO*(eight twenty –one) gene from 21q22 (Nucifora G, 1993). The fusion gene, *AML1-ETO* or *CBFA-ETO* (CBFa-ETO), which is formed on the derivative chromosome 8 as a result of the translocation, encodes for a chimeric protein that is expressed in the leukaemic cells. The *AML-ETO* protein may exert its oncogenic effect by interfering with the transcription factor activity of normal *AML1* protein. For the same chromosome translocation (t(8;21)(q22;q22), immunophenotyping of the blast cells are characteristically positive for CD13, CD33, CD34, CD65, CD117, MPO and HLA-DR (Schmitz, 1996). Expression of CD34, HLA-DR and MPO is stronger than in other cases of AML whereas expression of CD13 and CD33 is more likely to be absent or weak (Foon, 1997).

The second most common specific translocation in AML is t(15;17)(q22;q21). t(15;17)(q22;q21) is present in the great majority of patients with M3 and M3v AML, and is confined to this category of leukaemia. The translocation is detected by conventional cytogenetic techniques in about 90% of cases. Other patients have a *PML-RARA* fusion gene formed by insertion. The molecular leukaemogenesis is fusion of part of the PML (promyelocytic leukaemia) gene at 15q22 with part of the RARA (retinoic acid receptor α, (also designated RARα) gene from 17q21 (Warrell RP, 1993) to form a fusion gene on the derivative chromosome 15. The PML-RARA fusion protein may be oncogenic because of its ability to sequester normal PML protein. Immunophenotyping of t(15;17)(q22;q21) shows that CD13, CD33 and MPO are characteristically positive but CD33 is more consistently positive than CD13 (Brunning RD, 2001). CD 33 expression is homogeneous whereas CD13 tends to be heterogenous (Richards SJ, 2002). Other known translocations include

t(11;17)(q23;q21)/*PLZF-RARA* fusion, t(16;16)(p13;q22)/*CBFB-MHT11* fusion, t(9;22)(q34;q11)/*BCL-ABL* fusion (Bain, 2003).

Table 1.3 Panel of antibodies recommended by the European Group for the Immunological Characterization of Leukaemias (EGIL) for the diagnosis and classification of AML

First panel	**Antibodies**
Myeloid	Anti-MPO,CD13,CD33,CD65,CD117
Second panel	**Antibodies**
If myeloid	Anti lysozyme, CD14,CD15,CD41,CD61,CD61, anti-glychophorin A (Gly A)
Non- lineage specific	TdT,CD34,HLA-DR

c, cytoplastic;CD,cluster of differentiation;MPO, myeloperoxidase;Sm,surface membrane;TdT, terminal deoxynucleotidyl transferase.

1.3 Acute Lymphoblastic Leukaemia (ALL)

Acute lymphoblastic leukaemia (ALL) is a condition where the bone marrow produces large numbers of abnormal immature lymphocytes. These cells crowd the bone marrow, preventing it from generating normal blood cells. They can spread out into the blood stream and circulate around the body. Due to their arrested development, they are unable to function properly to prevent or fight infection. There are various sub-types of ALL. For example, the abnormal lymphoblasts can be immature B or T lymphocytes (Figure 1.1.7 and 1.1.8). Typically, ALL progresses quite quickly and rapidly become worse unless treated. ALL is the most common type of childhood leukaemia, and the most common childhood cancer (Bain, 2003). ALL is subdivided into 3 subtypes according to the French-British-American (FAB) classification system (Table 1.4) (Bennett J, 1976).

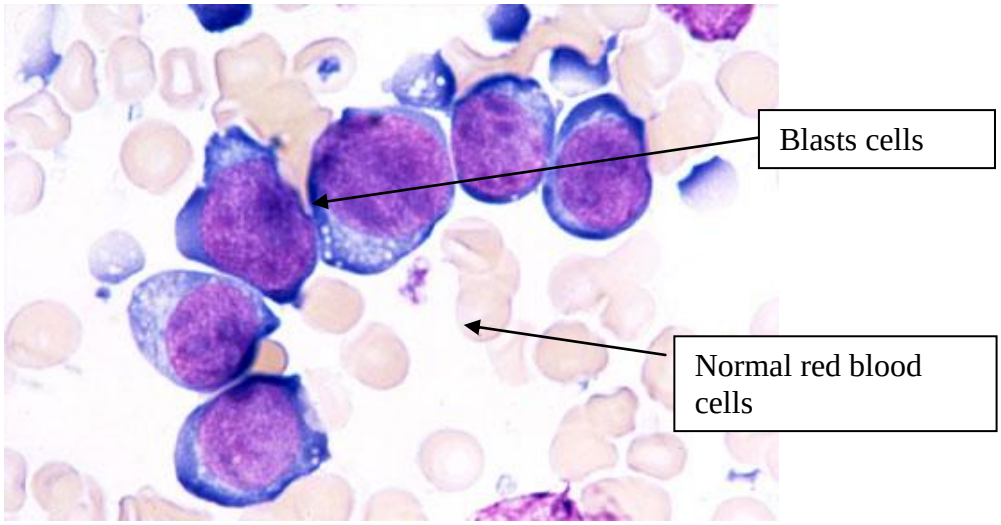


Figure 1.1.6 Morphology of B-cell acute lymphoblastic leukaemia (Zainal Ariffin and Saleha, 2011).

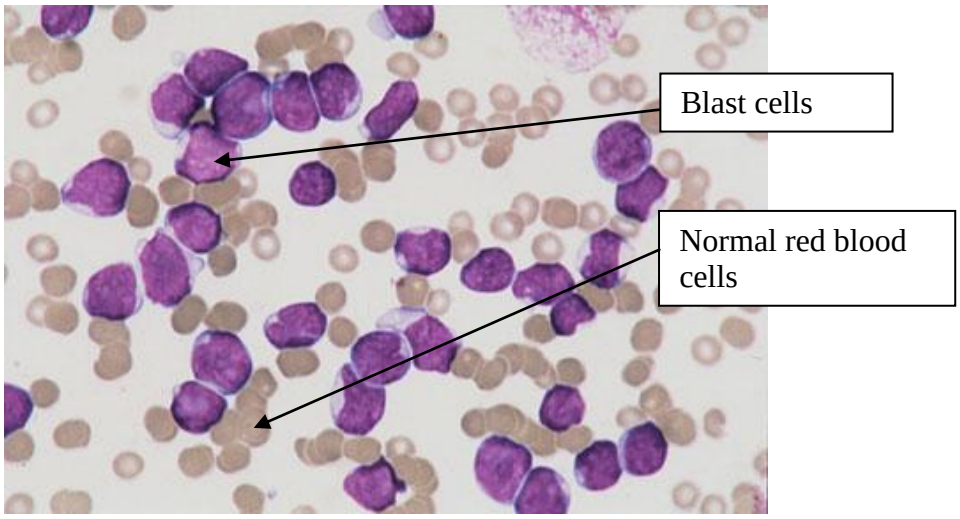


Figure 1.1.7 Bone marrow aspirate from a child with T-cell acute lymphoblastic leukaemia (Zainal Ariffin and Saleha, 2011).

Table 1.4 FAB classification of ALL

Subtypes	Morphology
L1	Small and homogenous
L2	Larger and heterogeneous
L3	Larger and homogenous

The WHO International panel on ALL however recommended that the FAB classification be abandoned, since the morphological classification has no clinical or prognostic relevance. It instead promotes the use of the immunophenotypic classification mentioned in Table 1.5 (Randolph, 2004). The WHO classification is as follows:

1) Acute lymphoblastic leukaemia/lymphoma, the equivalent of FAB L1/L2

i. Precursor B acute lymphoblastic leukaemia/lymphoma. Cytogenetic subtypes include:

- t(12;21)(p12,q22) *TEL/AML-1*
- t(1;19)(q23;p13) *PBX/E2A*
- t(9;22)(q34;q11) *ABL/BCR*
- T(V,11)(V;q23) *V/MLL*

ii. Precursor T acute lymphoblastic leukaemia/lymphoma

2) Burkitt's leukaemia/lymphoma, the equivalent of FAB L3

3) Biphenotypic acute leukaemia

Table 1.5 The use of a TdT assay and a panel of monoclonal antibodies to T cell and B cell associated antigens will identify almost all cases of ALL.

Types	FAB Class	Tdt	T cell associated antigen	B cell associated antigen	c Ig	s Ig
Precursor B	L1,L2	+	-	+	-/+	-
Precursor T	L1,L2	+	+	-	-	-
B-cell	L3	-	-	+	-	+

1.3.1 Signs and symptoms

The most common symptoms of ALL are mainly caused by the shortage of normal lymphocytes in the circulating blood. Patients with acute lymphoblastic leukaemia (ALL) present with either symptoms relating to direct infiltration of the marrow or other organs by leukemic cells or symptoms relating to the decreased production of normal marrow elements. Therefore, the symptoms are almost similar with AML. Among the signs and symptoms (Karen Seiter, 2010) are:

- Fever: It may be present without any other evidence of infection. However, in these patients, one must assume that all fevers are presumably infections until proven otherwise, because a failure to treat infections promptly and aggressively can be fatal. Infections are still the most common cause of death in patients undergoing treatment for ALL.
- Anaemia: It includes fatigue, dizziness, palpitations, and dysponea upon even mild exertion. Pallor and a cardiac flow murmur may be present.

- Bleeding tendency: Bleeding can be the result of thrombocytopaenia due to marrow replacement. Patients with thrombocytopaenia may present with petechiae, particularly on the lower extremities.
- Bone pain: Infiltration of the marrow by massive numbers of leukaemic cells frequently manifests as bone pain. This pain can be severe and is often atypical in distribution.
- Others: renal failure, shortness of breath and many more.

1.3.2 Genetic abnormalities and immunophenotyping of ALL

ALL is divided into two main subtypes which are B-cell ALL and T-cell ALL, and these two subtypes of ALL usually have different chromosomal abnormalities and immunophenotypic expression. In B-cell ALL, t(12;21)(p12;22)/ *ETV6-AML1* fusion is one of the commonest subtypes of chromosomal abnormalities in ALL. The translocation is difficult to detect by conventional cytogenetic analysis and may be misinterpreted. The molecular mechanism of leukaemogenesis is the fusion of two transcription factor genes, *ETV6* of the ETS family and *AML1* to form a fusion gene, *ETV6-AML1* on the derivative chromosome 21. The other chromosomal abnormality that is common in B-ALL is the t(1;19)(q23;p13)/E2A-PBX which resulted from the fusion of the PBX1 gene from 1q23 with part of the transcription activator gene, E2A, at 19p13 to form a hybrid gene E2A-PBX1. This gene encodes for an abnormal transcription factor (Hunger SP, 1991). The immunophenotype may be early precursor B, common or pre-B ALL with the relative frequencies varying considerably between different reported series. Myeloid antigens such as CD13 and CD33 are co-expressed in a quarter to a half of cases (Borkhardt A, 1997). In comparison with other precursor-B ALL, there is higher

expression of CD10, CD40, and HLA-DR and lower expression of CD9,CD20 and CD86 (Alessandri AJ, 2002).

Another genetic abnormality in B-cell ALL is the t(9;22)(q34;q11)/BCR-ABL fusion. The mechanism of leukaemogenesis is fusion of part of the *ABL* oncogene from chromosome 9 with part of *BCR* gene on the chromosome 22 to form a hybrid gene on chromosome 22 designated *BCR-ABL*. *BCR-ABL* gene encodes for a chimeric protein with aberrant tyrosine kinase activity, which functions in intracellular signalling pathways. The immunophenotype is usually that of common ALL but a minority have a pre-B immunophenotype and a small minority is precursor-B cell (Hématologique, 1996). 20-50% of cases express myeloid antigens (Chessels JM et al, 1997). Homogenous expression of CD10 and CD34 with low but heterogeneous expression of CD38 and expression of CD13 has been found to be reasonably sensitive and specific for *BCR-ABL*-positive ALL (Tabernero MD et al, 2001).

Other cytogenetic abnormalities concerning B-lineage ALL include t(4;11)(q21;q23)/MLL-AF4 fusion, t(2;8)(p12;q24)/*MYC* dysregulation and t(14;18)(q32;q21)/*MYC* dysregulation.

In T-lineage ALL, known cytogenetic abnormality is the t(10;14)(q24;q11) *HOX11* dysregulation. Dysregulation of *HOX11* a transcription gene, dysregulated by proximity αδ *TCR* gene cluster at 14q11. For immunophenotyping, majority of cases have an intermediate or common thymocyte phenotype, such as CD1, CD4 and CD8 and are expressed in addition to CD5 and CD7. About a quarter of cases express CD10 (Chessels JM *et al*, 1997).

TAL^{d} deletion/TAL dysregulation, is common among children and adolescents than adults. A small deletion leads to the fusion of the most of the sequences of transcription factor gene on chromosome 1, *TAL1* with the promoter of an upstream gene, *SIL*. This leads to dysregulation of the *TAL1* gene. No specific immunophenotype has been recognized except that this subtype of T-lineage ALL occurs preferentially among TCR αβ and CD10 is usually

expressed (Delabesse E, 1997). The recommended immunophenotyping antibodies panels for ALL is shown in Table 1.6 (Bene MC, 1995).

Table 1.6 Panel of antibodies recommended by the European Group for the Immunological Characterization of Leukaemias (EGIL) for the diagnosis and classification of ALL

First panel	**Antibodies**
B lymphoid	CD19, cCD22, CD79a,CD10
T lymphoid	cCD3,CD2,CD7
Second panel	**Antibodies**
If B lineage	cμ, λ, κ,CD20,CD24
If T lineage	CD1a, SmCD3,CD4,CD5,CD8, anti-TCR αβ, anti γσ

c, cytoplasmic;CD,cluster of differentiation;Sm,surface membrane;TCR,T-cell receptor

1.4 Acute leukaemia treatment

When the diagnosis of acute leukaemia is made, treatment will then be instituted based on the subtype of the acute leukaemia diagnosed. The main treatment for acute leukaemia is chemotherapy. Other treatments include radiotherapy, growth factors, and bone marrow or stem cell transplants. The treatment varies depending on the prognostic factors such as type of leukaemia (ALL or AML), general health, age, race, response to therapy and cytogenetics. Basically there are 3 stages of chemotherapy as summarised below:

- Induction phase: this is the first phase of treatment. The treatment with combination chemotherapy aims to destroy the leukaemic cells and achieve remission, which is defined when there are no detectable leukemic cells in the peripheral blood or bone marrow.
- Consolidation phase: this is the second phase and it is aimed at preventing the resurgence of the disease. The treatment may include high doses of multiple chemotherapy drugs, or more intensive treatment, like a bone marrow or stem cell transplant.
- Maintenance phase: The aim is to keep the leukaemia in remission for longer period. It usually involves having low dose chemotherapy drugs and short courses of steroids for up to 2 years.

Sometimes leukaemia cells may be left in the bone marrow after the treatment. This is called resistant leukaemia. To overcome this, more chemotherapy, or possibly a stem cell transplant is needed. If the leukaemia still comes back after a period of remission it is called a relapse.

1.4.1 Minimal residual disease (MRD)

Failure to achieve sustained remission in patients with acute leukaemia is caused by the survival of chemotherapy resistant neoplastic cells, which has been held responsible for the relapse. The presence of residual malignant cells among normal cells is termed minimal residual disease (MRD) (Faderl S, 1999). Minimal residual disease (MRD) is the definition given to a small numbers of leukaemic cells that remain in the patient when the patient is in remission. It is known as the main cause of relapse in cancer and leukaemia.

1.4.2 Incidence of MRD

The initial early weeks of induction treatment will usually kill most of leukaemia cells and the marrow may start to recover and the normal range of cell types reappears. Morphologically, immature white cells are still visible, and may resemble leukaemic cells, but more likely regenerating into normal cells. The patient is also free from symptoms of the disease. However, in most cases a few leukaemic cells survive the treatment, and may persist in the marrow for months or years. Due to the very small number of blast cells, better techniques with better sensitivity than morphological method is needed to detect and quantify these MRD cells. Therefore, molecular and immunological methods have been developed by researchers and are rapidly developing and used as a tool to detect MRD.

1.4.3 Techniques for measuring MRD in leukaemia

The current strategy for MRD studies are based on the following steps:

1) identification of the unique phenotype by a multicolour staining technique
2) definition of a patient-specific “immunophenotype” or leukaemia-associated phenotypes (LAIP)

3) tracking residual leukaemic cells after complete remission is achieved and during subsequent follow-up, using the specific patient LAIPs

For example, Figure 1.1.8 demonstrates the MRD algorithm used for identifying LAIP in patients with AML (Adhra Al-Mawali, 2009).

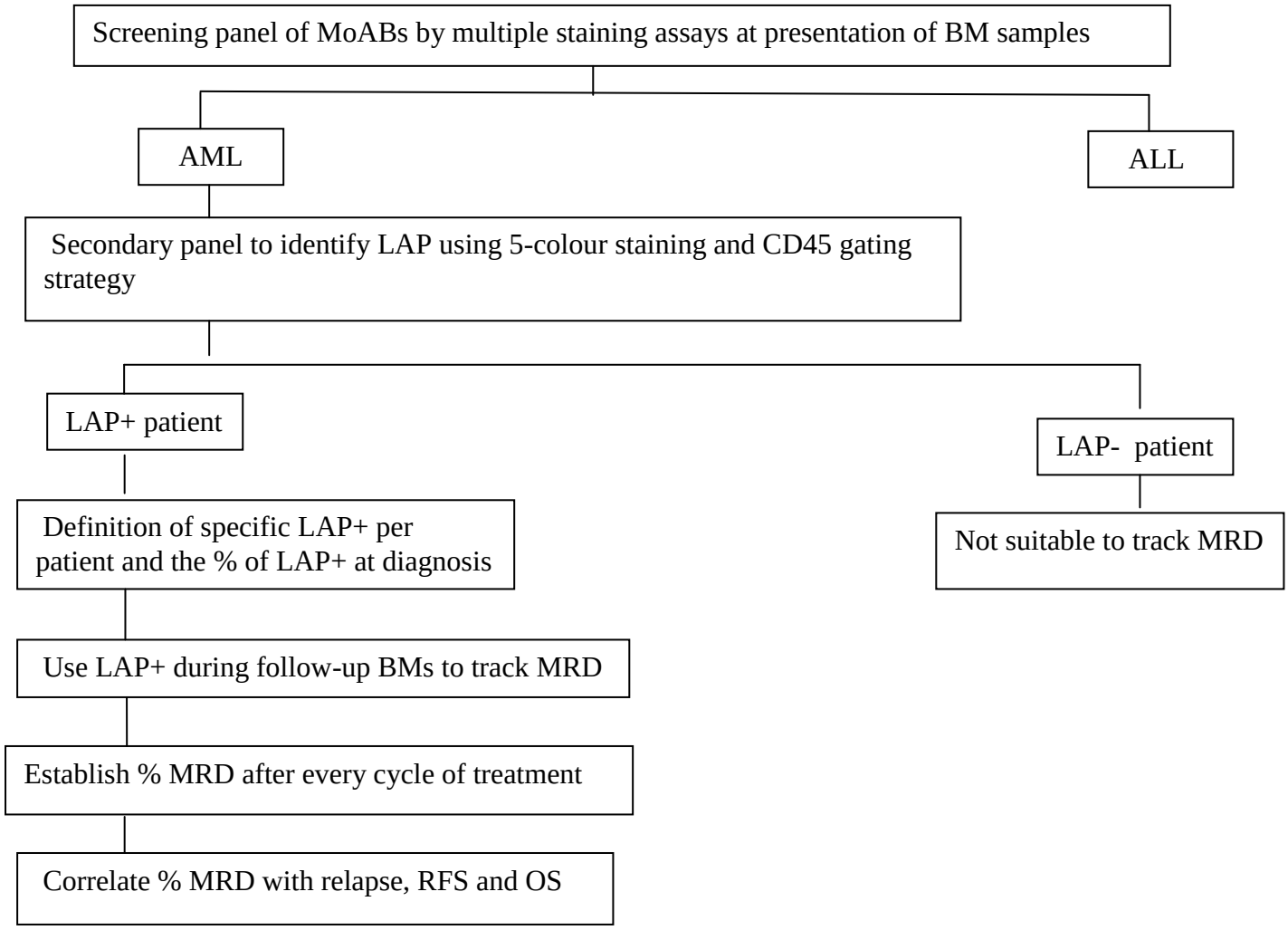


Figure 1.1.8 Algorithm used for identifying leukemia-associated phenotype (LAP) in patients with acute myeloid leukemia (AML) and for detection of minimal residual disease (MRD). Once LAP is identified, it will serve to establish a phenotype to trace residual leukemia after a complete remission is recognized morphologically. Adapted from Campana and Pui and Campana and Coustan-Smith and previous literature on MRD. ALL, acute lymphoblastic leukemia; BM, bone marrow; MoAbs, monoclonal antibodies; OS, overall survival; RFS, relapse-free survival; +, positive; –, negative.

There are various techniques applicable for MRD detection, which differ in specificity of markers used as well as detection levels. Some of them are limited by their low sensitivity, such as morphology of the cells, clonogenic assays, and conventional cytogenetics. Conventional cytogenetics had been successfully replaced by other techniques, like fluorescent *in situ* hybridization (FISH) or RT-PCR (Kaeda J, 2002). Nowadays, measurement of MRD by flow cytometry (FCM) or polymerase chain reaction (PCR) emerges as an attractive new tool for risk assessment in acute leukaemia (Campana, 2000). The most sensitive techniques of MRD detection could confirm the state of remission and are useful for appropriate selection of a treatment strategy for each particular patient, which can improve long-term survival (Schuler F, 2006). The ultimate goal of MRD assays is to guide therapeutic decisions by recognising patients who responded well to therapy, and thus should be spared from further therapy and distinguishing them from patients in whom therapy must be continued or intensified to minimise the likelihood of clinical relapse.

Optimal MRD techniques should be characterized by patient specificity (or at least leukaemia specificity), satisfactory sensitivity (at least 10^{-4}, for one malignant cell among 10,000 normal cells), applicability for the vast majority of patients under the study, laboratory reproducibility and feasibility, which in this case easy standardization and rapid collection of results for clinical application. Another requirement for reliable MRD technique is precise quantification of MRD levels. The stringent criteria described above are mostly met by three approaches, namely flow cytometric (FCM) immunophenotyping, real-time quantitative polymerase chain reaction (RQ-PCR)-based detection of fusion gene transcripts or breakpoints, and RQ-PCR-based detection of clonal immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements (Campana, 2003b, Munoz L, 2000).

1.4.3.1 Flow cytometry (FCM) immunophenotyping

FCM detection of MRD is based on the identification of immunophenotypic combinations expressed only in malignant cells but not on normal bone marrow or peripheral blood cells (Campana D, 1999). Immunological based testing of leukaemia utilises proteins on the surface of the cells. White blood cells (WBC) have a variety of proteins on the surface depending upon the type of WBC. Leukaemic cells often show unusual and unique combinations, also known as the leukaemic phenotype of these cell surface proteins. These proteins can be stained with fluorescent dye or fluorochromes labelled antibodies and detected using flow cytometry. Cells are incubated with monoclonal antibodies, which recognize only antigens expressed on the surface of the malignant cells.

The antibodies are labelled with various fluorochromes, crucial for detection, and then counted by a flow cytometer, a laser-based instrument. This method can detect 1 leukaemic cell per about 10 000 normal cells (Campana D, 1999). This methodology relies on tracing the leukemia-specific immunophenotypes as the result of cross-lineage antigen expression, maturational asynchronous expression of antigens, antigen overexpression, absence of antigen expression, ectopic antigen expression, and various combinations of above-mentioned features bringing the blasts cells into differentiation with the normal cells (Campana, 2003b). Leukaemia-associated immunophenotype, (LAIP) are absent or very infrequent in normal bone marrow cells. Presence of LAIPs on leukaemic cells is the basis of flow cytometric monitoring of leukaemia such as AML (Kern W, 2003).

Flow cytometry relies on the detection of qualitative and quantitative differences of antigen expression between leukaemic cells and their normal counterparts. Results from the flow cytometry analysis are available within a short time period and are quantitative. The simplest

method uses a limited panel of antibodies and three colours FCM. This technique was capable of a sensitivity of 10^{-3} (Dworzak MN, 2002).

In order to perform immunologic MRD monitoring, it is important to know the antigen expression pattern of the leukemic cells at disease presentation. Post induction or in remission, residual leukemic cells are detected based on these antigen characteristics. Two limitations should be at concern; one is the fact that recovering bone marrow cells may sometimes be misinterpreted as abnormal cells, because normal precursor cells share a significant number of antigens with leukemic blast cells in a given patient. The other limitation is that leukaemic antigen expression may change due to therapy. To overcome this problems, one must adopt a comprehensive antibody panels testing and applying the knowledge of normal hematopoietic antigen expression patterns to their interpretation (Paietta, 2002). In the post- induction phase, FCM, which optimally has the ability to detect as few as between one and five leukemic blasts among 10 000 normal cells, can be equally effective in the detection of residual leukemic cells in acute lymphocytic leukaemia (ALL), as is qualitative allele-specific oligonucleotide PCR (Malec M *et al*, 2001). FCM offers applicability with a clinically relevant sensitivity to most patients with AML. Some studies have showed that although RQ-PCR is more sensitive than flow cytometry, both techniques provide concordant prognostic results (Kerst G, 2005). Still the detection limit of this technique is not lower than $10^{-3} – 10^{-4}$, which nevertheless should be sufficient for identifying high-risk ALL patients (Gaipa G, 2005).

Concerns about the instability of MRD markers leading to false negative results were first raised more than a decade ago. There is now good evidence that if multiple targets are used, that is if two antigen receptor loci or several immunophenotypic combinations are used, the risk of false negative results can be reduced to less than 5% (Pongers-Willemse MJ, 1999).

Technical improvement such as the use of 5 or more colours in FCM will lead to further increase in the applicability and sensitivity of the FCM methods (Wolfgang Kern, 2008). Small single-centre studies even claimed that results of MRD detection by flow cytometry and quantitative PCR of patient-specific Ig/TCR gene rearrangements are largely comparable (Malec M *et al*, 2001)

1.4.3.2 PCR- based tests

PCR-based techniques for the monitoring of MRD rely on detection of two kinds of targets, breakpoint regions of leukaemia-specific chromosomal aberrations (RT-PCR analysis), and immunoglobulin or T-cell receptor gene rearrangements (RQ-PCR analysis) (Gemano G, 2003).

1.4.3.2.1 Chromosomal aberrations by RT-PCR analysis

Leukaemia-associated fusion genes resulting from chromosomal translocations are directly linked to the development of leukaemia and therefore represent very good and stable disease-specific markers. The protocols for detection and quantification of fusion gene transcripts based on RT-RQ-PCR employing TaqMan technology have been developed by European EAC network (Gabert J, 2003). These MRD assays are characterized by reproducibly high sensitivity of 10 plasmid molecules or 10^{-4} RNA cell line dilution for the majority of the targets. The limitation discovered with this technique is, because of the high sensitivity, cross contamination of RT-PCR products between patient samples has become a major problem in RT-PCR-mediated MRD studies, resulting in up to 20% of false-positive results (Gleissner B, 2001). Such cross-contamination is difficult to recognize, as fusion gene transcripts, although leukaemia-specific, are not patient-specific markers. The levels of fusion gene transcripts can vary significantly between patients. This is in contrast to PCR products obtained from

genomic breakpoint fusions, which can be identified by the use of patient-specific oligonucleotide probes in RQ-PCR assays.

1.4.3.2.2 *Ig/TCR* gene quantification by RQ-PCR analysis

RQ-PCR-based detection and quantification of junctional regions of clonal *Ig* and *TCR* gene rearrangements is by far the most widely employed strategy of MRD monitoring in ALL. Despite being laborious, expensive and time consuming, this MRD method is the most reproducible technique not only within the same laboratory but also between different laboratories. The junctional regions of clonal *Ig* and *TCR* gene rearrangements acts as fingerprint-like sequences for each lymphoid malignancy and can be identified in the vast majority of ALL patients using the standardized primer sets established through the European collaboration within the BIOMED-1 and -2 frameworks (Van Dongen JJM, 2003). MRD monitoring, based on quantification of MRD levels, is then performed with the use of real-time quantitative polymerase chain reaction (RQ-PCR) and designed primers/probes (Szczepañski T, 2001).

Unfortunately, sensitivity levels for PCR analysis vary, largely dependent upon the particular target gene sequence to be amplified, the primer combination chosen, and the quality of nucleic acid extractions. Additional technical aspects affect accurate quantitation, most importantly the choice of reference genes used for normalization, as recently exemplified in a large CML transplant study (Radich JP, 2001). The higher sensitivity of PCR enables detection of 1 leukaemic cell among 10^5 – 10^6 normal cells (Willemse MJ, 2002). Comparison between RQ-PCR analysis of immunoglobulin and TCR gene rearrangements and FCM shows that MRD levels may differ and these techniques are not exchangeable (Malec M, 2004). Table 1.7 demonstrates the characteristics of the flow cytometry and PCR techniques to quantify MRD (Campana, 2003a, Szczepan´ ski T, 2006).

Table 1.7 Characteristics of flow cytometry and PCR techniques practiced for MRD

	Flow cytometric immunophenotyping	**PCR analysis of chromosome aberrations (mainly detection of fusion gene transcripts)**	**PCR analysis of *Ig/TCR* genes (junctional region specific approach)**
Sensitivity	10^{-3}-10^{-4}	10^{-4}-10^{-6}	10^{-4}-10^{-5}
Applicability Precursor B-ALL T-ALL	 >95% >95%	 40-45% 15-35%	 90-95% 90-95%
Advantages	Applicable for most patients Relatively cheap Rapid 1-2 days	Relatively easy and cheap Sensitive and leukemia-specific Rapid 2-3 days Suitable for monitoring of uniform patient groups (e.g. *BCR-ABL* +ALL)	Applicable for virtually all patients, if *IGH*,*IGK*-Kde,*TCRG*, and *TCRD* gene rearrangements are used as targets Sensitive and patient specific Rapid during follow up:2-3 days (if junctional region is identified and RD-PCR is used)
Disadvantages	Limited sensitivity Need for preferably two aberrant immunophenotypes per patient, because of chance of immunophenotypic shifts Drug-induced modulation of the immunophenotype might influence the levels of antigenic expression	Useful in only a minority of patients. Not patient-specific-cross-contamination of PCR products might lead to false positive results (even at diagnosis) Differences in fusion transcript expression levels between the patients Stability of fusion gene transcripts decreases over time	Time consuming at diagnosis: identification of the junctional regions and sensitivity testing Relatively expensive Need for preferably two PCR targets per patient, because of chance of clonal evolution Two sensitive targets($<10^{-4}$) available in ~80% of patients
Recent developments and standardization in European networks	≥6 colour cytometry gives promises of increased sensitivity and specificity, currently under development in the European Euroflow Consortium	Largely standardized thanks to pan-European collaboration within the BIOMED-1 (fusion transcript detection) and EAC project (RQ-PCR).Methods for identification of fusion gene breakpoints at the DNA level provide patient-specific targets	Target identification standardized within the European BIOMED-1 and BIOMED-2 networks RQ-PCR for MRD detection standardized by the European Study Group for MRD detection in ALL (ESG-MRD-ALL)

1.4.3.3 Other techniques for MRD detection

Cytomorphology is still a standard technique employed for identification of complete remission but the detection limit is low, which is between $10^{-1} – 10^{-2.}$ This routine technique is based on assessment of morphology of bone marrow cells with the use of a light microscope (Toren A, 1996). The same sensitivity can also be seen in cytogenetic analysis based on an analysis of chromosomal morphology. This method strongly depends on a number of metaphases obtained from blood or marrow sample. Problems such as failure to detect chromosomal abnormality could happen, due to technical problems with culturing the cells or mostly because of a low quality of the material obtained from the hospital. Table 1.8 shows the most common cytogenetic abnormalities in acute lymphoblastic and acute myeloid leukaemias (Justyna Jólkowska et al., 2007).

Table 1.8 The most common cytogenetic abnormalities in ALL and AML

Acute Lymphoblastic leukaemia (ALL)		**Acute myeloid leukaemia (AML)**
B-line lymphocytes	**T-line lymphocytes**	
		t(9;22)(q34;q11)
t(9;22)(q34;q11)	t(1;7)(p32; q34)	t(8;21)(q22;q22)
t(4;11)(q21;q23)	t(1;14)(p32;q11)	t(15;17)(q22;q11-21)
t(1;19)(q23;p13)		t(16,16)(p13;q22)
		t(3;21)(q26;q22)

Fluorescence in-situ hybridisation (FISH) is another method that could be employed to detect the absence or presence of additional chromosomes and also to detect or confirm gene or chromosome changes (detection of fusion genes: *BCR-ABL, TEL-AML MLL-AF4, PML-RAR, AML1/ETO*) (Raanani P, 2004). FISH relies on hybridization of specific fluorescently labelled probes to the metaphase chromosomes or interphase nuclei. In a case of translocation, a specific molecular probe applied spans the site of breakage and is complementary to the known sequence. The fluorescently labelled probe hybridizes with the sample DNA at the target site and then signals can be seen under a fluorescent microscope.

For example, FISH is routinely used for detection of the *BCR-ABL* fusion product as a result of t(9;22). Detection limits of fluorescence in situ hybridization are between 10^{-2}–10^{-3}(Liang R, 1997). Although approximately 100 times more sensitive than standard cytogenetics, the sensitivity level achieved by FISH is noticeably below that of desired for MRD detection, which is one cell for every 10 000 cells. Another limitation is the possibility that genetic defects detected by interphase FISH may not be actually translated and thus reflect 'silent' and physiologically potentially meaningless disease, therefore causing a significant complication to the interpretation of FISH data (Paietta, 2002).

MRD analysis based on detection of clonality can be also performed with the use of ligase chain reaction (LCR). This is a DNA amplification technique based upon the ligation of oligonucleotide probes. The probes are designed to match exactly 2 adjacent sequences of a specific target DNA. The sensitivity of LCR is 1 malignant cell per 500 000 cells (Jilani I, 2006). Table 1.9 summarizes the comparison for the techniques used to detect MRD (Adhra Al-Mawali, 2009).

Table 1.9 Comparison of main techniques used for detecting MRD

Technique	Advantages	Disadvantages	Sensitivity
Standard morphologic studies	-	Low sensitivity; not suitable for low level of leukaemic cells	1%-5%
Cytogenetics	-	Labour intensive; slow; requires metaphase chromosome preparations	5%
FISH	Dividing cells not required; large number of cells can be analyzed in a short time;interphase FISH precludes need for high-quality metaphases (cf standard cytogenetics)	Labour-intensive;limited sensitivity	0.3%-5%
Gene rearrangements by 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 (1-3d);no for very low $<10^6$ background in normal cells;sensitive;patient-specific	False-positive results;applied in<50% of AML cases;relatively expensive	10^{-4} to 10^{-5}
Flow cytometric immunophenotyping	Applicable for most cases (>80%);quantification simple;single cell analysis;cell viability can be determined; information on normal cells relatively easy;cheap; rapid (1-2d);relatively patient-specific	Not as specific as PCR;presence of subpopulation in AML; immunophenotypic shifts can occur between diagnosis and relapse	10^{-4}

AML, acute myeloid leukaemia; FISH, fluorescence in situ hybridization; PCR, polymerase chian reaction.

1.5 Leukaemia prevalence in Malaysia

In the year 2006, National Cancer Registry (NCR) Malaysia reported a total of 21,773 cancer cases among Malaysians in Peninsular Malaysia (Registry, 2006). It comprises of 9,974 males and 11,799 females. For leukaemia, as shown in Table 2.0, it was reported that leukaemia cancer incidence by sex which showed the high number of cancer incidence (CR) among males with CR value 3.6 compared to females with 2.2 (Registry, 2006).

Table 2.0 Leukaemia cancer incidence (CR) by sex, Peninsular Malaysia 2006

Sex	**No.**	**%**	**CR**
Male	363	62.3	3.6
Female	220	37.7	2.2
Both	583	100	2.9

It was also reported that leukaemia incidence in Malaysia increases with age both in male and females. In males, based on the age-specific cancer incidence per 100 000 population graph, the highest CR value achieved was at 13.8, when the age reached over 70 years old. Meanwhile for females, the highest leukaemia incidence per population was reached at age 60-69 years old, with 15.5 of incidence value before falling drastically at age over 70 years old (Registry, 2006). This data is demonstrated in Figure 1.1.9 below.

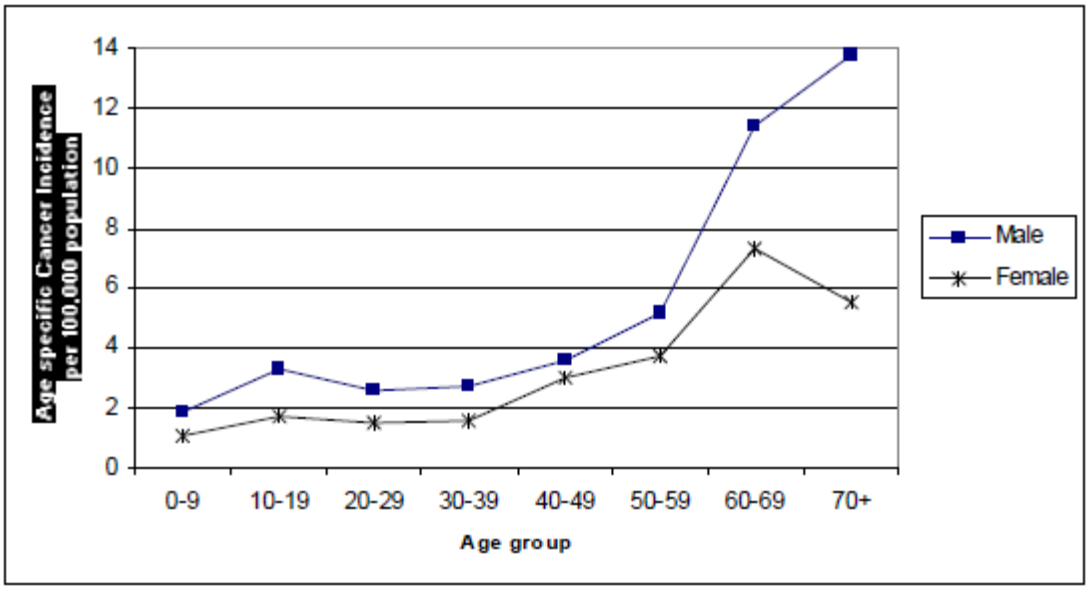


Figure 1.1.9 Leukaemia age specific cancer incidence per 100,000 population by sex, Peninsular Malaysia 2006

For the purpose of comparing leukaemia distribution among main races in Malaysia, the same report also reported that leukaemia incidence among Chinese males and females was the highest when compared to other races like Malay and Indians, with leukaemia incidence of 18.5 for male and 11.9 for female at age over 70 years old (Table 2.1) (Registry, 2006).

Table 2.1 Leukaemia Age specific Cancer Incidence per 100,000 population, by race and sex, Peninsular Malaysia 2006

		Age groups, year								
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	70+	CumR
Male	Malay	2	3.7	3	2.5	3.8	5.7	10.8	8.9	0.4
	Chinese	1.1	2.6	2.1	1.5	2.1	3.6	9.3	18.5	0.3
	Indian	0	0	1.9	2.2	4.1	2.4	10.7	5.5	0.3
Female	Malay	1.1	1.7	1.2	1.9	2.7	2	7.4	0.6	0.2
	Chinese	0.7	1.4	1.7	1	2.7	6.3	5.8	11.9	0.2
	Indian	0.6	3	0.6	0.7	3.2	3.5	0	8.3	0.1

1.5.1 Myeloid and Lymphatic leukaemia incidence in Malaysia

National Cancer Registry (NCR) Malaysia had produced two reports respectively in 2002 and 2004 regarding cancer incidence in Malaysia but the data covers only the Peninsular Malaysia. The data for Sabah and Sarawak however was not available in both of these reports. In the 2004 NCR report for leukaemia, a total of 539 cases of myeloid leukaemia were reported. Males predominate at a ratio of 1.1:1 for myeloid leukaemia. For lymphatic leukaemia, 433 cases were reported the same year, with higher incidence among males at a ratio of 1.7:1 (Lim and (Eds). 2004).

1.5.1.1 Sex incidence in myeloid and lymphatic cases

It was also reported that myeloid leukaemia cases among males were higher with cancer incidence per 100 000 population (CR) value at 3.0. As in myeloid leukaemia, lymphocytic leukaemia was also diagnosed mainly in males with CR value at 2.8, with both data are shown in Table 2.2 and Table 2.3 (Lim and (Eds). 2004).

Table 2.2 Myeloid Leukaemia Cancer Incidence per 100,000 population (CR) by sex, Peninsular Malaysia, 2003

Sex	No.	%	CR
Male	286	53.1	3
Female	253	46.9	2.7
Both	539	100	2.8

Table 2.3 Lymphocytic Leukaemia Cancer Incidence per 100,000 population (CR) by sex, Peninsular Malaysia, 2003

Sex	No.	%	CR
Male	274	63.3	2.8
Female	159	36.7	1.7
Both	433	100	2.3

1.5.1.2 Age incidence in myeloid and lymphatic cases

For myeloid leukaemia, the age-specific cancer incidence per 100 000 population was highest in males age 60-69 years old, with the highest incidence value 8.0. In lymphocytic leukaemia, the highest age-specific cancer incidence per 100 000 population was at age 0-9 years old, with highest incidence in male, CR value 6.6. (Lim and (Eds). 2004).

1.5.1.3 Ethnic incidence in myeloid and lymphatic cases

Myeloid leukaemia was distributed almost evenly among both sexes between the main ethnic in Malaysia, but with highest incidence value 12.7 per 100,000 population detected among Indian male aged 60-69 years old. Table 2.4 demonstrate the comparison of myeloid leukaemia incidence among the races (Lim and (Eds). 2004).

Table 2.4 Myeloid Leukaemia Age specific Cancer Incidence per 100,000 population, by ethnicity and sex, Peninsular Malaysia 2003

		Age groups, year								
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	70+	CumR
Male	Malay	3.2	2	1.9	2.8	3.8	2.8	8.4	2.6	0.3
	Chinese	0.6	1.3	1.7	4.7	3.5	2.6	5.7	8.7	0.3
	Indian	4.3	0.6	2	2.9	2.6	4.3	12.7	6.1	0.3
Female	Malay	2.3	1.5	2.5	1.9	2.9	4.4	6	2.8	0.3
	Chinese	1.4	1.7	2	3.8	2.8	2.8	6.5	6.5	0.3
	Indian	0.6	4.2	0	1.4	5.9	4.3	8.2	10.2	0.3

Lymphocytic leukaemia was also distributed almost evenly among the ethnics in Malaysia but with less incidence value compared to myeloid leukaemia. Indian showed the highest incidence of lymphocytic leukaemia for both sexes at age 0-9 years old. Table 2.5

demonstrate the comparison of lymphocytic leukaemia incidence among the races. (Lim and (Eds). 2004)

Table 2.5 Lymphocytic Leukaemia Age specific Cancer Incidence per 100,000 population, by ethnicity and sex, Peninsular Malaysia 2003

		Age groups, year								
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	70+	CumR
Male	Malay	6.6	3.9	1.3	0.7	1.1	1.5	2	0.9	0.2
	Chinese	6.3	0.9	1	0.7	1.1	1.1	1.9	1.2	0.1
	Indian	8.7	2.9	1.3	0.7	1.7	1.4	3.2	0	0.2
Female	Malay	5.2	1.8	0.3	0.4	0.2	1	0.9	0.7	0.1
	Chinese	2.1	1.4	1	0.8	1.1	0.8	0.6	0.9	0.1
	Indian	2.3	2.4	0.7	0	1.7	4.3	2.7	0	0.1

1.5.2 Incidence of leukaemia in Sarawak

Since both NCR reports did not provide the data for Sabah and Sarawak, the details on cancer incidence, especially on the status of leukaemia cases in Sarawak is still unclear. However, based on leukaemia samples sent to the lab for immunophenotyping, the numbers for leukaemia cases in Sarawak are raising concerns. As shown in Table 2.6 malignant neoplasms or cancer is the highest cause of deaths in government hospitals in the year 2008 (Sarawak State Health Department, 2008). However, there is still no available published data on leukaemia incidence in Sarawak.

Table 2.6 10 principal causes of deaths in Sarawak government hospitals, for the year 2008

	10 PRINCIPAL CAUSES OF DEATHS IN GOVERNMENT HOSPITALS, SARAWAK 2008		
Causes		**Number**	**%**
1.	Malignant neoplasms (cancer)	629	18.30
2.	Heart diseases and diseases of pulmonary circulation	517	15.04
3.	Cerebro-vascular diseases	363	10.56
4.	Septicaema	344	10.01
5.	Pneumonia	159	4.62
6.	Certain conditions originating in the perinatal period	151	4.39
7.	Chronic lower respiratory diseases	147	4.28
8.	Diseases of the digestive system	147	4.28
9.	Nephritis, Neprotic syndrome & Nephrosis	123	3.58
10.	Accidents	86	2.50
	Sub-total	2,666	77.55
	Others	772	22.45
	Total	3,438	100.00

1.6 MRD significance

The study of MRD has provided many useful information in leukaemia research and treatment. It is used mainly as a guide for prognosis, individualization of treatment and patient monitoring for signs of recurring leukaemia.

1.6.1 As a guide to prognosis or relapse risk

Detection of MRD has applicability in clinical practice due to its important prognostic value in a number of haematological diseases, such as ALL, AML, CML (van der Velden VH, 2006). The level of MRD at a certain time in treatment is a useful guide, to the patient's prognosis. For instance in childhood leukaemia, doctors traditionally take a bone marrow sample after 5 weeks, and assess the level of leukaemia in that sample. Even with microscopy, they were able to identify a few patients whose disease had not been cleared, and those patients received different treatment. With modern treatment, over 95% of children suffering from acute lymphoblastic leukaemia (ALL) achieved a remission defined by light microscopy as the presence of less than 5% blasts in the bone marrow. Unfortunately, approximately 25% of patients will subsequently relapse (Pui CH, 2001).

By standard procedure, when the patients were studied, patients with high levels of disease at this stage which often means leukaemia more than 1 cell in 1000, were at risk of relapse. Patients with levels below 1 in 100,000 were very unlikely to relapse. In ALL, for example, quantitation of MRD levels at stated time-points during therapy enables classification of patients into risk groups, such as identification of patients at high (MRD levels $>10^{-3}$), intermediate, and low risk (no MRD detection) of relapse (van der Velden VH, 2006). Other studies have also demonstrated feasibility of prognostication in AML based on

MRD. In one study done by Kern *et al*, in the prognostic impact of day 16 bone marrow blasts, MRD levels significantly correlated to complete remission rate (CRR), event-free survival (EFS), overall survival (OS), and relapse-free survival (RFS) (Kern W *et al*, 2004). Many other studies have shown that MRD levels significantly correlate with the patient's clinical outcome (Willemse MJ, 2002). MRD level detection also enables evaluation of treatment effectiveness in haematological disorders and also represents a powerful prognostic factor helping to predict the clinical outcome (Campana D, 1999).

1.6.2 Individualization of treatment

Individualization of treatment, is the identification of risk factors. Currently most patient receives the same treatment, but leukaemia is a very variable disease, and different patients probably have widely different treatment needs in order to eradicate the disease. For instance, the initial 5 week induction treatment might rapidly clear off the disease for some patients. For others, the same treatment might leave behind significant amount of disease. By measuring MRD level, this will help physicians to decide the patient's needs. For example, when patient's individual risk of relapse has been identified, then theoretically this will allow them to receive the right amount of treatment to prevent it. But without MRD information, there is not much doctors can do but give the same treatment to all patients. Therefore, some patients will receive inadequate treatment but for others it may be excessive, which is impractical. Thus, identification of risk factors, to help individualize treatment, is important. When MRD level is measured, the next step is, having identified a patient whom standard treatment leaves at high risk, whether there exist different treatments regimes that they could offer, in order to lower the risk.

One benefit of individualization of treatment is children with undetectable MRD at the end of induction have an excellent prognosis and are good candidates for treatment de-

intensification and should not be subjected to further treatment intensification, particularly to hematopoietic stem cell transplantation (HSCT). In contrast, children with high MRD levels at the end of induction treatment are in urgent need for treatment intensification or even for new treatment approaches, particularly when such high MRD levels persist into the consolidation treatment (Dworzak MN, 2002). It was also demonstrated that treatment stratification of standard risk adult ALL patients can be substantially improved when including MRD information (Bruggemann M, 2006). Detection of MRD identifies differences in treatment response between patients with T-ALL and precursor-B-ALL(Szczepañski T, 2001). The most important seems also individualization of treatment between T-ALL and pre B-ALL, on the basis of results of MRD analysis (Willemse MJ, 2002). Introduction of MRD-based protocols may also improve the therapy of AML (Goulden N, 2006). Knowledge about MRD levels at particular points of medical management enables early or more aggressive treatment intervention (Hillmen P, 2006).

1.6.3 Monitoring for early signs of recurring leukaemia

Another possible use of MRD is to identify if or when someone starts to relapse, early, before symptoms come back, by regular checking of blood or marrow samples. MRD molecular tests may show tumour levels starting to rise, very early, possibly months before symptoms recur. Starting treatment early might be useful, because the advantage is the patient will be fitter because of fewer leukaemic cells to deal with, and the cells may be responding well to treatment. Usually at clinical relapse the malignant cells have often become more resistant to drugs used. Sometimes, MRD results can also help physicians in making additional therapeutic decisions or in better monitoring of disease development, especially in patients after non-myeloablative allogeneic transplantation (Galimberti S, 2005).

1.7 MRD treatment

The standard treatment for eliminating MRD nowadays is to add to the initial treatment, several months of extended treatment, or conductiong a number of several treatment strategies, but the options are limited (Paietta, 2012). Relapse is a problem in leukaemia, and most approach taken by the doctors is to prevent relapse rather than treat it, because if the disease relapses, it is usually more resistant to treatment. Also if the patients have relapsed once, they are at risk of another relapse in the future. Generally the approach is to bring a leukaemia into remission first and then try to eradicate the remaining cells (MRD). Often the treatments needed to eradicate MRD, differ from those used initially. It seems a sensible idea to aim to reduce or eradicate MRD. Treatments which specifically target MRD can include the following:

- Intensive conventional treatment with more drugs, or a different combination of drugs
- Stem cell transplant, e.g. marrow transplant. This allows more intensive chemotherapy to be given, and in addition the transplanted bone marrow may help eradicate the minimal residual disease
- Immunotherapy
- Patient monitoring for early signs of relapse.
- Treatment with monoclonal antibodies which target cancer cells (f) cancer vaccines

1.8 Objectives of study

Currently there are not many MRD studies done using flow cytometry in Malaysia, especially in Sarawak. This is probably because of low number of FCM availability in every state hospital in Malaysia. The high cost of running the MRD service and also the lack of expertise contribute to the low number of leukaemic MRD flow cytometry research. Since Sarawak General Hospital (SGH) is the centre for providing leukaemia treatment and diagnostic service in Sarawak, it is advocated that MRD analysis is done for all leukaemia patients in Sarawak. The reason of choice of FCM over PCR for this study besides its availability is because PCR work for MRD is more tedious, laborious and may require longer time to achieve the results. As for FCM, reproducibility and faster results are the main reason for the choice. Therefore this study will help to give us more information regarding MRD which could help to individualise the treatment of acute leukaemia patients in Sarawak in the future.

The objectives of this study are stated as follows:

1. To characterize the immunophenotypes of leukaemic blasts in acute leukaemia patients in Sarawak by flow cytometry.
2. To establish a standard protocol for minimal residual disease analysis in acute leukaemia by flow cytometry.
3. To determine the association between clinical parameters, immunophenotypes and gender in acute leukaemias at diagnosis and during minimal residual disease.
4. To determine the association between the independant variables, haematological and immunophenotypes expression with the MRD status in AML and ALL.

In order to achieve these objectives, specific objective too has been determined for this research which is to evaluate the MRD parameters that cause relapse in leukaemia patients after the first MRD assessment.

Research hypothesis for each of the objectives are as follows:

1. Using flow cytometry, the characteristics of immunophenotypes for AML is shown with the expression of specific monoclonal antibodies, such as CyMPO, CD33, CD13 and CD 117 while for ALL such as CyCD79a, CD19, CD7, CyCD3 , CD10 and CD22.
2. The flow cytometry protocol used in this study will be able to identify leukaemia cells that may cause minimal residual disease after the first induction treatment.
3. Clinical parameters, immunophenotypes and gender at diagnosis may have association with minimal residual disease.
4. Independant variables, haematological and immunophenotypes expression may have association with the MRD status.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sample collection

Bone marrow aspirates (BMA) or peripheral blood (PB) samples were selected upon confirmation that the samples were acute leukaemia samples after morphology and immunophenotyping screening.

2.2 Patient samples

BMA or PB samples were collected from acute leukaemia patients from Sarawak General Hospital and Normah Medical Specialist Centre after informed consent was obtained from the patients or their guardians. All samples received were collected into the 3 ml EDTA-anticoagulated tubes (BD Vacutainer®, Beckton Dickinson) and were processed within 24 hours upon arrival. Samples that took more time to reach the lab were kept in ice packs to help preserve the samples.

2.3 Patient's clinical data

Ethical approval had been obtained prior to this study from the Faculty of Medicine and Health Sciences UNIMAS Medical Ethics Committee and Ministry of Health. The information of the patients such as age, sex, ethnic group, full blood count, leukaemia subtype, liver and spleen enlargement was collected from clinical data obtained from the clinical records at the hospitals. The acute leukaemia was categorised according to the French-American-British (FAB) classification. Permission was sought from the doctors responsible for the patients to access the patients' data. Besides that, informed consents were obtained from the patients or the parents/guardian of the patient for approval to participate in the study.

2.4 Sample preparation

To prepare the samples**,** all samples received were washed twice, filtered and full blood count (FBC) was performed, as detailed below:

2.4.1 Sample washing

For washing, the bone marrow aspirates (BMA) or peripheral blood (PB) samples from the 3ml EDTA-anticoagulated tube were transferred into 15 ml tube (Greiner Bio One, Cellstar®) and filled with phosphate buffered saline (PBS) (Sigma-Aldrich Inc, St Louis, MO) buffer until the tube was full. The blood samples were then centrifuged at 25000 rpm for 5 minutes with a multipurpose centrifuge model 5810 (Eppendorf, Hamburg, Germany). After centrifugation was done, the upper layer that consisted of PBS was discarded using a 3 ml graduated Pasteur pipette leaving behind sediment of the blood at the bottom of the tube.

2.4.2 Sample filtration

Filtration of samples was performed on BMA samples only by using 70 μm cell strainer (BD Falcon Cell Strainer™, BD Biosciences). The samples that had been filtered were transferred back into the 3 ml EDTA-anticoagulated using the Pasteur pipette in order to perform full blood count, particularly to acquire the white blood cell (WBC) count. However, it was later recommended that filtering of samples can also be done first before it was transferred into the 15 ml tube in order to obtain more pure samples.

2.4.3 Full Blood Count (FBC)

The final step for the sample preparation after sample filtration was to count the white blood cells using automated hematology analyzer (Sysmex KX 21™, Sysmex, Japan). When the reading of WBC was taken and in the appropriate range, the calculation was done. Occasionally, dilution was performed when the WBC was too high by adding PBS to the blood sample, then another WBC count was done. The standard calculation used to count the volume (*v*) of blood samples to be added to each tube in order to get the optimal binding with 10 µl monoclonal antibody (McAb), as recommended by BD Biosciences. The formula for the calculation was as follows:

For diagnosis samples:

$$v = 1000 / \text{WBC count (µl)}$$

Example of calculation is as follows**:**

WBC count = 20 X 10^6 µl, therefore volume of blood samples added:

$$\frac{1000}{20} = 50\mu l$$

For MRD samples:

$$v = 2000 / \text{WBC count (µl)}$$

Example of calculation is as follows**:**

WBC count = 20 X 10^6 µl, therefore volume of blood samples added:

$$\frac{2000}{20} = 100\mu l$$

2.5 Flow Cytometry (FCM)

Flow cytometry is the measurement of cells in a flow system, which delivers the cells past a point of measurement one at a time. The flow cytometer used in this study was BD FACSCalibur™ Flow Cytometer, manufactured by Becton Dickinson. The FACSCalibur™ has one laser with an excitation line at 488nm, allowing three colour fluorescence analysis. But later in the study the flow cytometer was upgraded to four colour system. In theory, FCM refers to instruments in which light is focused at the point of measurement. The scattered light and fluorescence of different wavelengths are then recorded. Typically, light scatter at two different angles and from one to six or more fluorescence will be measured. The three main components of a flow cytometry are the fluidics, optics and electronics.

Figure 1.2.0 A typical look of a flow cytometry system

2.5.1 Fluidics

Basically, the sheath fluid is driven through the flow chamber by air pressure supplied by a compressor. The same pressure is used to force the sample into the sheath. The rate of the sample flow is regulated by a pressure regulator. This is usually fixed to three settings: high, medium and low. The elements of the fluidics are shown in Figure 1.2.1

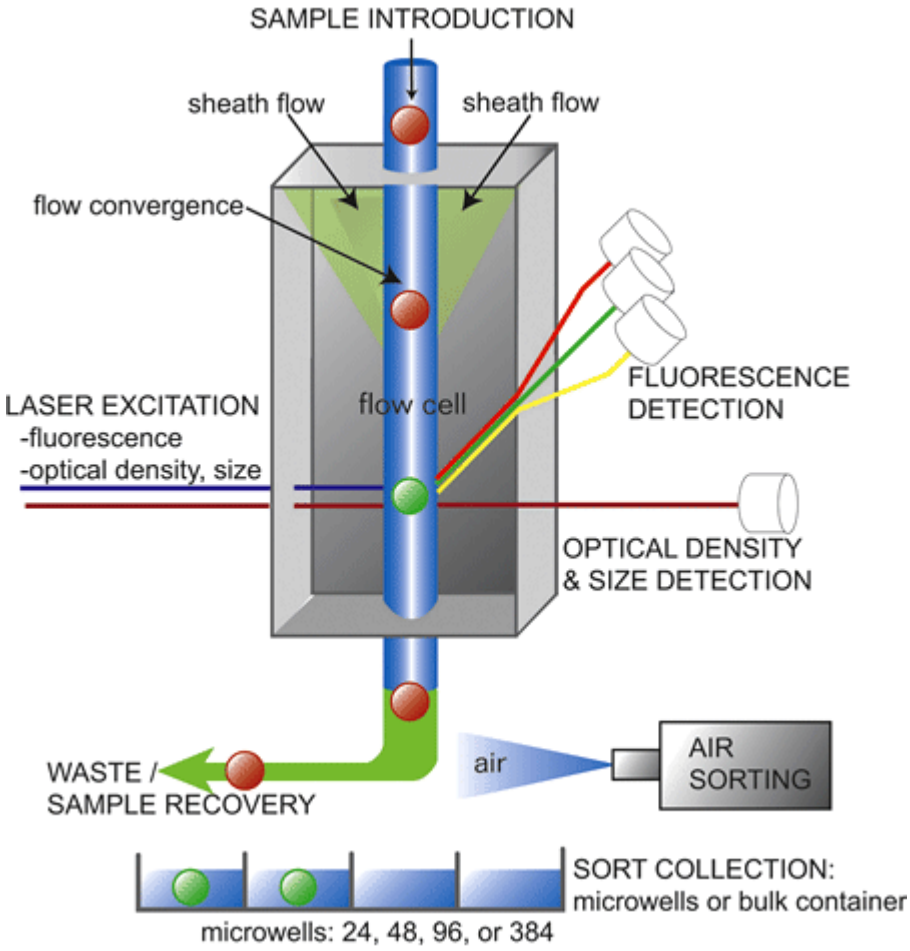


Figure 1.2.1 The fluidics system in flow cytometry

2.5.2 Optics

Nowadays, majority of FCM use laser as the light source. Lasers are chosen because they produce a high intensity beam of monochromatic light. They also have a small size, which is important since the light needs to be focused into a small volume to obtain maximum excitation of a single cell and to minimize the probability of there being more than one cell in the laser beam. The most common primary laser is an air-cooled argon-ion laser producing blue light at 488 nm. The argon laser is preferred, because it is the only coherent source of excitation satisfactory for the most used fluorochrome in the field—fluorescein. The argon-ion laser could produce lines in the UV (350 nm), deep blue (457 nm), blue (488 nm), and blue-green (514nm) regions, making it a very useful light source. The light source needs to be focussed to a spot and a desired shape. This is accomplished by using a beam-shaping optic to obtain the desired crossed-cylindrical beam shape.

2.5.3 Electronics

For measuring fluorescence and scatter at right angles to the laser beam, photomultipliers are used. The sensitivity of PMTs is dependent on the wavelength of the light. For positions used to detect wavelengths of 600 nm and over, red sensitive PMTs are selected. After (pre-) amplification, the signal from a photomultiplier undergoes further processing. The instrument must be set to respond to signals derived from the particle of interest and to ignore debris and electronic noise. A threshold level is set on one, or possibly two, parameters such that a cell is only detected when the signal rises above this level. As the cell passes through the laser beam, a signal pulse will be generated, which has a height, width and an integrated area. If the width of the laser beam is greater than that of the cell diameter, the peak (or height) of the signal will accurately reflect the total fluorescence of the cell. As this is faster and easier to

record, this parameter is usually recorded. To produce a signal proportional to the total fluorescence of the cell, the pulse is integrated. The width and peak of the pulse may also be recorded and this will give some information about the length of the cell passing through the beam.

2.5.4 Flow Cytometry Analysis

Before starting acquisitioning of any stained samples with FCM, there are several protocols that should be performed in order to obtain the correct results. Figure 1.2.2 showed the main steps involved:

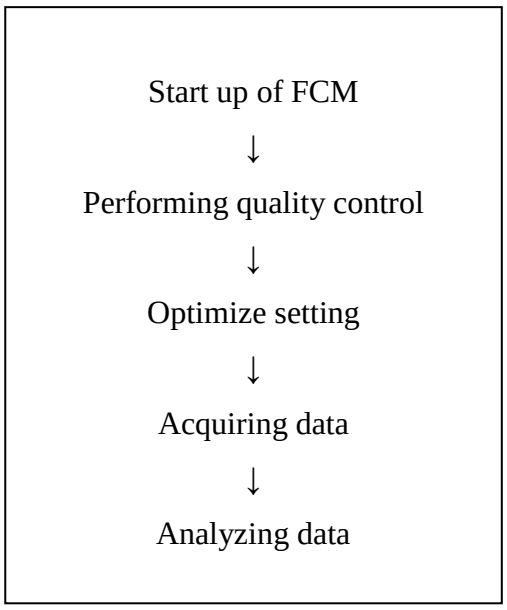


Figure 1.2.2 Multistep process of flow cytometry data acquisition

In the beginning of study, three colour FCM system was used and then was later replaced with four colour system. The shift in colour system had caused a few changes made from the original protocol to suit the new system, which will be mentioned in the next few sections.

2.5.5 Performing Quality Control

Quality control of the instrument was performed by running BD CaliBRITE™ beads (BD, San Jose, CA) with FACSComp™ software (BD San Jose, CA). The software monitored the instrument's performance and provided an automated instrument setup. This software, together with BD CaliBRITE beads would provide routine instrument setup for three/four - colour immunophenotyping applications of the cells.

2.5.5.1 Preparation of BD Calibrite Beads

Two tubes were labelled as tube A (unlabelled) and tube B (mixed). 1 ml of bead dilution buffer (BD, San Jose, CA) was added into tube A and 2 ml was added into tube B. Before adding the buffer into the tubes, the stock vials were mixed by either inverting or with gentle vortexing. One free-falling, complete drop of each calibration bead was added to the tubes as shown in Table 2.7.

Table 2.7 Preparing tubes for calibration

Setup	Tube A (unlabelled)	Tube B (mixed)
3-colour setup using PerCP- Cy5.5	1 drop of unlabelled bead added with 1ml of bead dilution buffer	1 drop of unlabelled, FITC, PE, PerCP-Cy5.5 beads and 2 ml of bead dilution buffer
4-colour setup using PerCP- Cy5.5	1 drop of unlabelled bead and APC bead added with 1ml of bead dilution buffer	1 drop of unlabelled, FITC, PE, PerCP-Cy5.5, APC beads and 2 ml of bead dilution buffer

When the flow cytometry was upgraded to 4-colour system, the calibration was also changed with the addition of the APC bead, into tube A and B.

When the beads were ready to be analysed, FACSComp software was launched to proceed with the calibration process. Once the tube was installed to the instrument (starting with tube A) instructions as stated in the software were done accordingly. When the lyse/wash assay setup run was completed, the calibration continued with the lyse/no wash assay. When these steps were completed, the summary report of the status of the flow cytometry was printed for reference.

2.5.5.2 Settings Optimisation

Before collecting the data, the electronics for the samples needed to be optimised, because different type of samples may have different settings. Emission spectra of different type of fluorochromes used are at different wavelength (Figure 1.2.3). It is noticed that some of these fluorochromes are almost at the same wavelength. Therefore this causes the spill over of the adjacent wavelengths into other detectors, which increases the background error (Figure 1.2.4). One way to minimise this error is to perform optimisation. The setting was optimised with cytometer controls accessed from BD CellQuest Pro™ software (BD, San Jose, CA). The optimisation sequence for immunophenotyping samples acquired was as follows:

- Forward Scatter (FSC) and Side Scatter (SSC) detectors adjustments
- FSC threshold setting adjustment
- Gating on the population of interest
- FL1, FL2 , FL3 and FL4 detector settings adjustments
- Fluorescence compensation adjustments

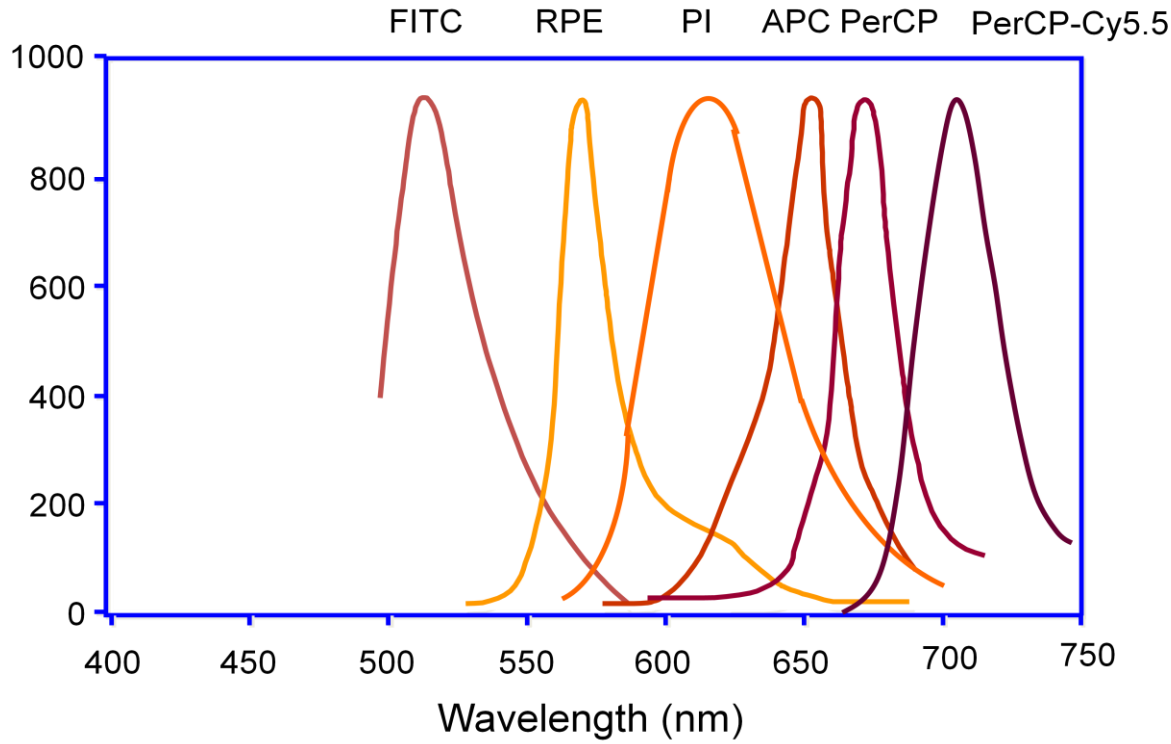


Figure 1.2.3 Emission spectra of different type of fluorochoromes

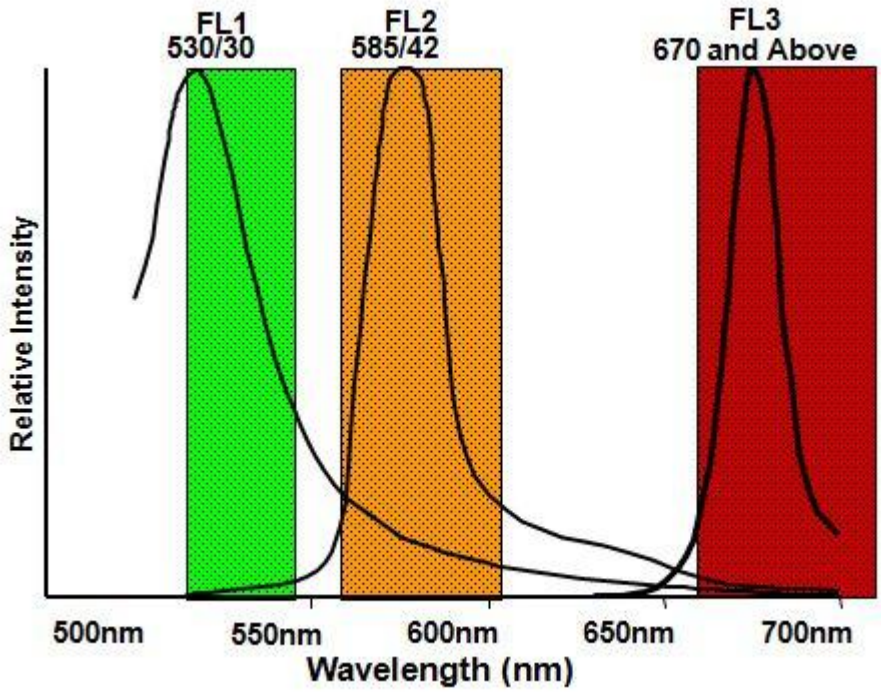


Figure 1.2.4 Spill over into other detectors causes background error

The samples used for this study were obtained from the peripheral blood and bone marrow aspirates of leukaemia patients; therefore for optimisation normal human blood was used and stained with McAb according to the antibody panel as mentioned in Table 2.8.

Table 2.8 3-colour optimisation panel for surface and cytoplasmic staining

	Surface optimization tube		**Cytoplasmic optimization tube**
1	Unstained	**1**	Unstained
2	CD4 FITC	**2**	CyMPO FITC
3	CD8 PE	**3**	Cy79a PE
4	CD19 PerCP Cy5.5	**4**	CD3 PerCPCy5.5
5	CD4 FITC /CD8 PE /CD19 PerCP Cy5.5	**5**	CyMPO FITC/Cy79a PE /CD3 PerCPCy5.5

When the flow cytometry was upgraded to 4-colour system, the optimisation panel was added with another fluorochrome, the APC (Table 2.9).

Table 2.9 4-colour optimisation panel for surface and cytoplasmic staining

	Surface optimization tube		**Cytoplasmic optimization tube**
1	Unstained	**1**	Unstained
2	CD4 FITC	**2**	CyMPO FITC
3	CD8 PE	**3**	Cy79a PE
4	CD19 PerCP Cy5.5	**4**	CD3 PerCPCy5.5
5	CD3 APC	**5**	CD 19 APC
6	CD4 FITC /CD8 PE /CD19 PerCP Cy5.5/CD3 APC	**6**	CyMPO FITC /Cy79a PE /CD3 PerCPCy5.5/CD 19 APC

The volume of normal human blood added to all tubes was determined at 200 µl, except for the unstained tube, which was 300 µl. The volume of all monoclonal antibodies that were added to each tube was determined at 20 µl. The stained tubes containing the normal human blood were stained according to their respective protocols (Figure 1.3.3 and 1.3.4). When the staining was done, it was then fixed with 1% paraformaldehyde and to be acquired in the flow cytometry for optimisation.

2.5.5.3 Side scatter (SSC) detectors adjustments

In flow cytometry, SSC determines the granularity of the cell. The already stained optimisation tubes were installed in the flow cytometry, one tube at a time according to the sequences in Table 2.8 and 2.9. Basically the protocols for optimization for both types of staining were similar as stated below. By using the unstained tube, the side scatter voltage was adjusted using the application software to place the cell population of interest on scale in the forward scatter versus the side scatter plot, until the following pattern as shown in Figure 1.2.5 (b) was achieved.

(a) **(b)**

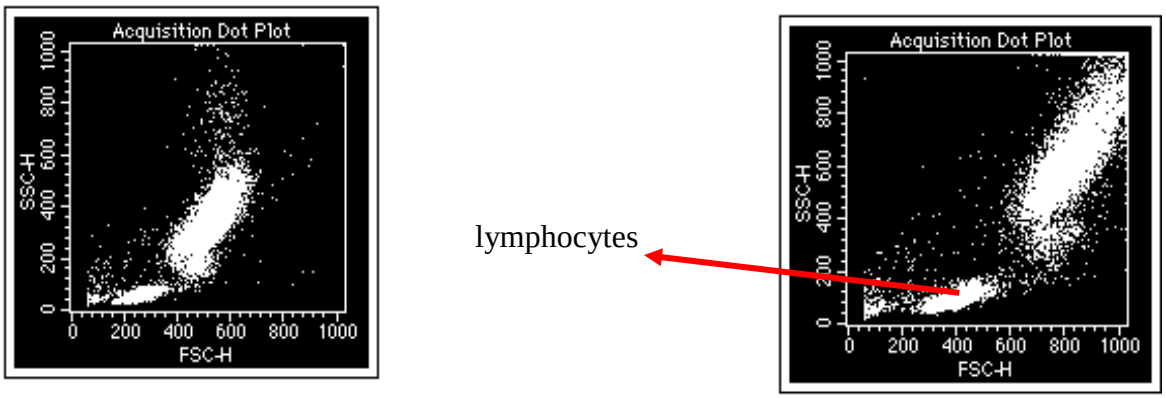


Figure 1.2.5 FSC versus SSC plot for the adjustment of SSC using application software **(a)** Before adjustment. (**b)** After adjustment (BD Biosciences)

2.5.5.4 Forward scatter (FSC) threshold adjustments

The purpose for adjusting the FSC threshold as shown in Figure 1.2.6 was to exclude unwanted events or debris. This was done in order to obtain data files that contained mainly events of interest. Usually the samples that had been run in the flow cytometry contained debris, which was usually small and appeared in the lower left end corner of the FSC scale, Figure 1.2.6 (c). Thus, increasing the FSC threshold would remove the unwanted debris, Figure 1.2.6 (d).

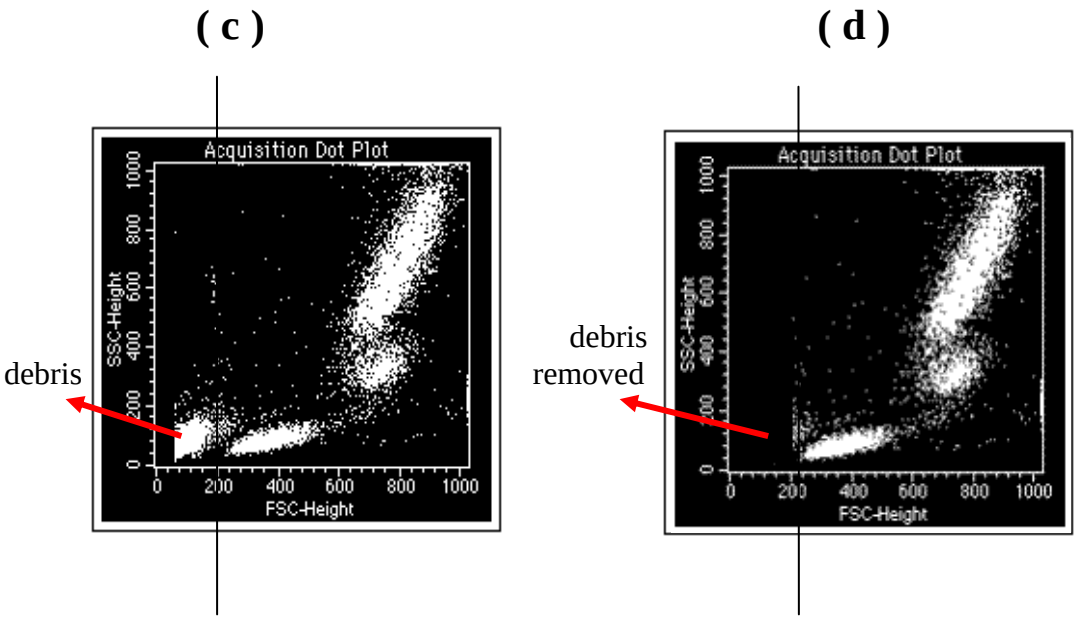


Figure 1.2.6 FCS versus SSC plot for the threshold adjustment of FSC using the application software**. (c)** Before adjustment **(d)** After adjustment and debris removal. (BD Biosciences)

2.5.5.5 Cell population of interest gating

In this study, flow cytometry immunophenotyping was done by gating the CD45/CD34^{+} population and in some scatter plots CD45/SSC population gating were used. Cell populations of interest usually had different light scatter and auto fluorescent characteristics. To ensure that fluorescence instrument settings were optimised for only the population of interest, a region was drawn around the lymphocytes population and only the gated events was viewed when optimising fluorescence photomultiplier tube (PMT) settings, as shown in Figure 1.2.7.

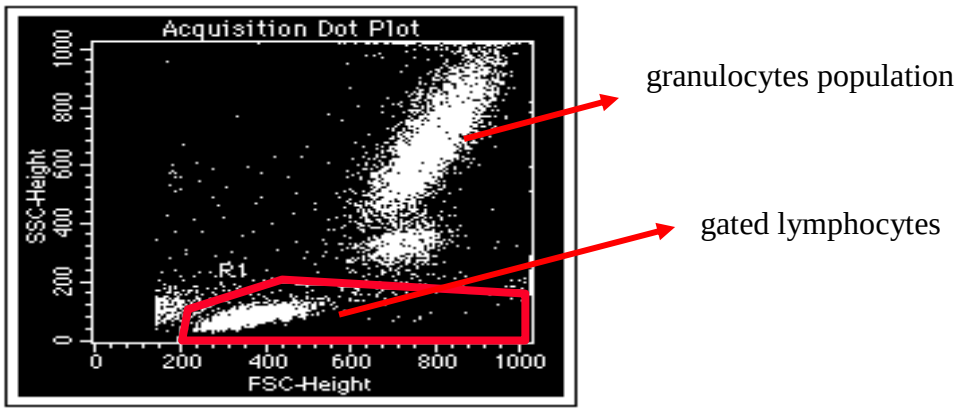
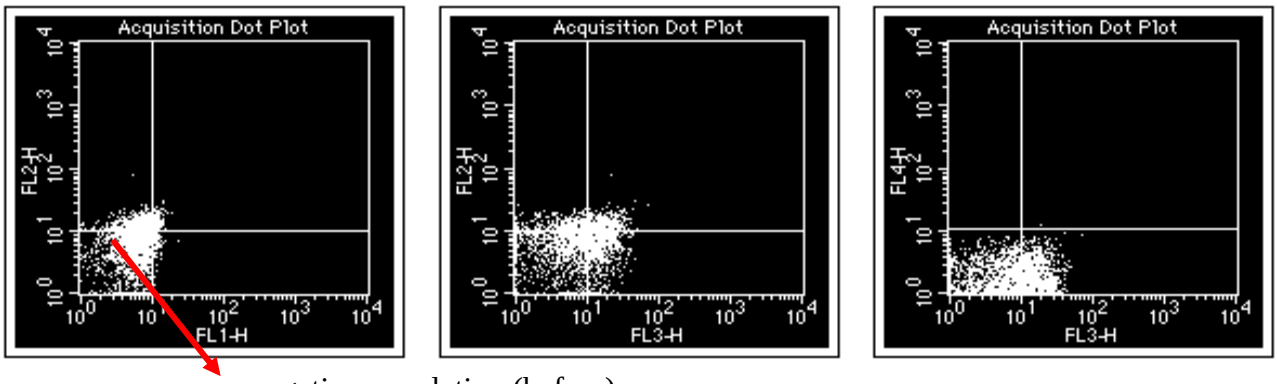


Figure 1.2.7 Lymphocyte population drawn in the R1 gate for PMT optimization. (Bio Sciences)

2.5.5.6 FL1, FL2, FL3 and FL4 detector settings adjustments

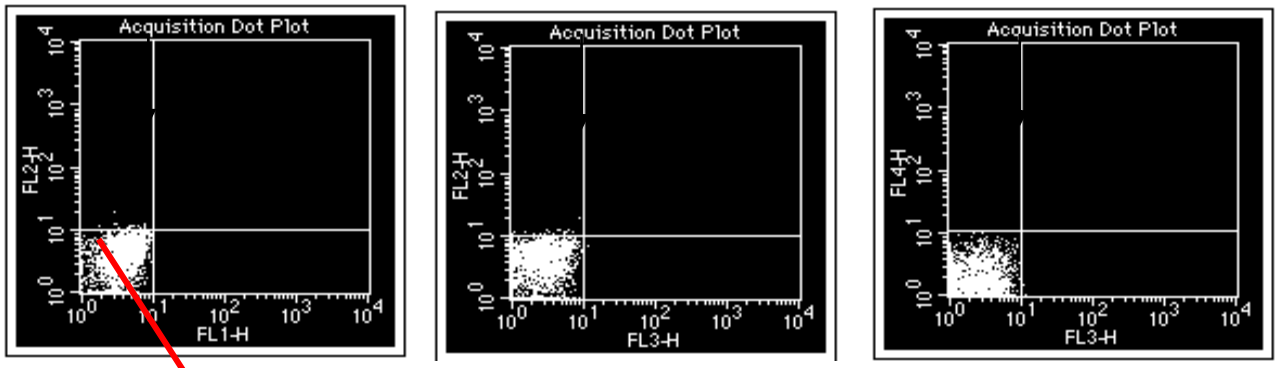
The FL1, FL2, FL3 and FL4 detector settings needed to be adjusted using the application software in order to place the negative population in the lower-left corner of the plot. The reason for placing the negative population in the lower left was to leave plenty of room for the positives on the x- and y-axes. This would make it easier to distinguish between negative and positive population, since the negative population was already at a fixed location on the lower-left corner of the plot, as shown in Figure 1.2.8 (f). Also, FL4 setting adjustment then was added when 4-colour system was upgraded to the flow cytometry system.

(e)



negative population (before)

(f)



negative population (after)

Figure 1.2.8 The negative population adjustment. **(e)** Before adjustment **(f)** After adjustment to the lower left quadrant (BD Biosciences).

2.5.5.7 Fluorescence compensation adjustments

The final step in optimization process was to adjust compensation to correct for spectral overlap. Compensation adjustments were dependent on the detector voltages established on the isotype control. Once the voltages were set compensation was adjusted on the fluorescence-positive cells. To adjust the compensation, the normal blood samples that were stained with monoclonal antibodies suggested in Table 6 were used, one at a time. The protocol for adjusting the compensation was the same for both surface and cytoplasmic staining. The following compensation are shown in Figure 1.2.9, 1.3.0 and 1.3.1.

(g) **(h)**

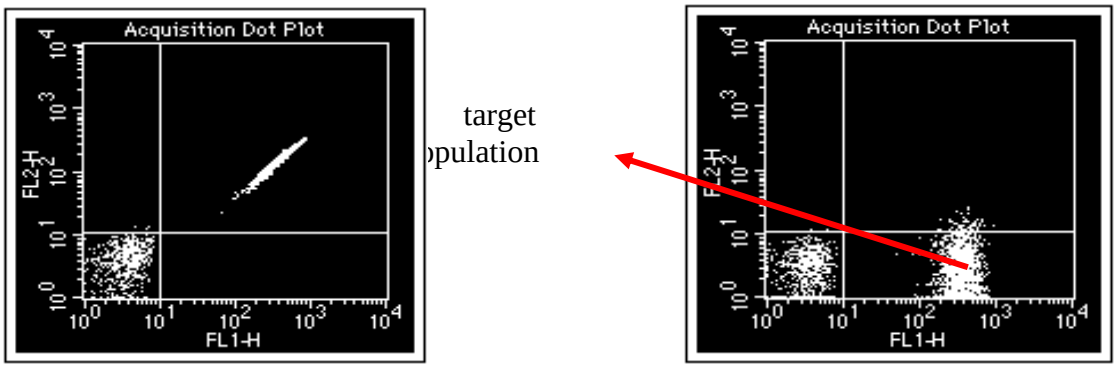


Figure 1.2.9 Compensation adjustment for FL2-FL1 plot from the software application.**(g)** Before adjustment **(h)** When the CD4 FITC (FL1-H) tube was run, the FL2 - FL1 plot was adjusted (BD Biosciences).

(i) **(j)**

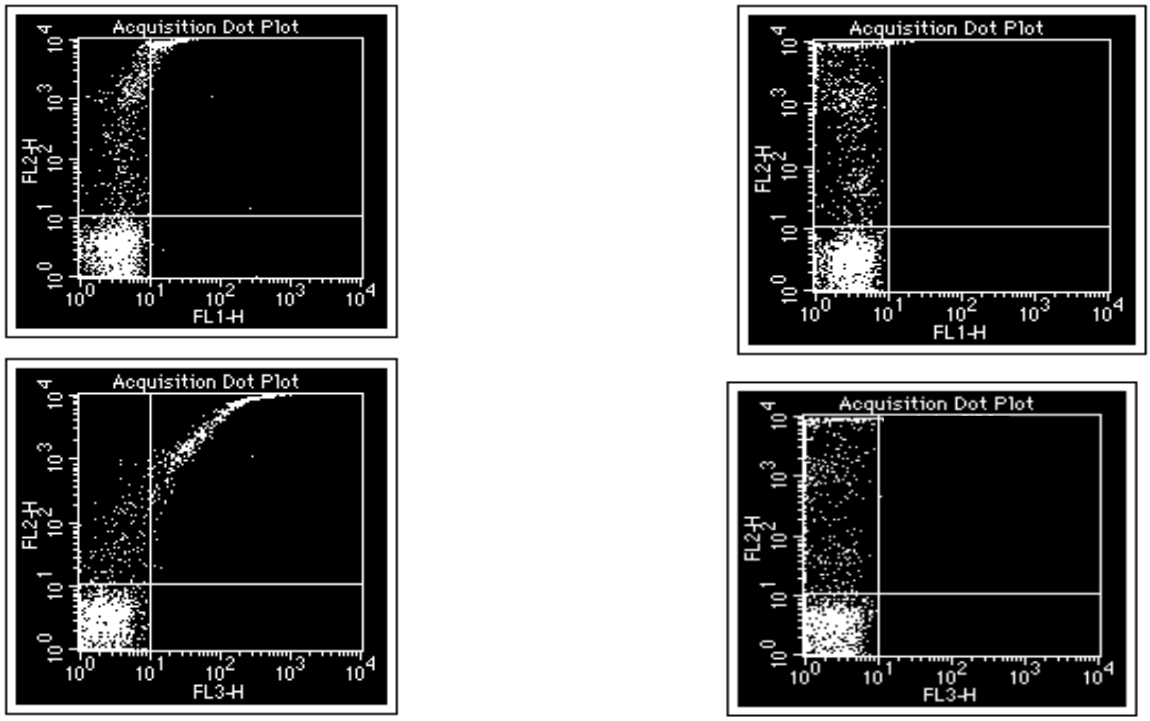


Figure 1.3.0 Compensation adjustment for FL1-FL2 and FL3-FL2 plots from the software application.**(i)** Before adjustment **(j)** When CD8 PE (FL2-H) tube was run, FL1 - FL2 and FL3 - FL2 were adjusted (BD Biosciences).

(k) **(l)**

Figure 1.3.1 Compensation adjustment for FL4-FL3 plot from the software application **(k)** Before adjustment **(l)** When CD19 PerCP-Cy5.5 (FL3-H) tube was run, FL4-FL3 was adjusted (BD Biosciences).

Additional tube was prepared for 4 colour system, which was the CD3 APC for surface and CD 19 APC for cytoplasmic (Figure 1.3.2).

(m) **(n)**

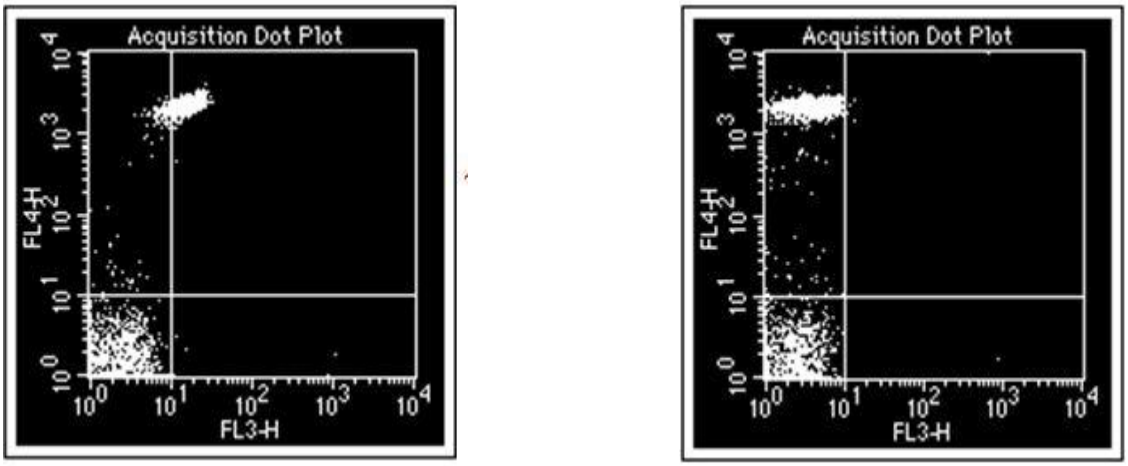


Figure 1.3.2 Compensation adjustment for FL3-FL4 plot from the software application **(m)** Before adjustment **(n)** When CD3 APC (FL4-H) tube was run, FL3-FL4 was adjusted (BD Biosciences).

When the above processes were completed, the final tube that consisted all of the antibodies was run to ensure that optimization was done correctly. The optimized instrument settings was saved and applied to the human leukaemia samples.

2.6 Immunophenotyping

Two types of staining that were used in immunophenotyping were surface and cytoplasmic antigen staining. Surface staining is also known as whole blood lysing method while cytoplasmic staining using fixing and permeabilisation method.

2.6.1 Surface staining

As shown in Figure 1.3.3, after cell count was done on the washed and filtered samples, appropriate volume of blood (10 μl McAb for 2 X 10^6 of cell dose) was added to each tube containing the combination panel of fluorochrome-conjugated monoclonal antibodies as shown in Table 3.0, 3.1, 3.2, and 3.4. All McAbs used were purchased from Becton Dickinson (BD, San Jose, CA) except for CD79a, Kappa/Lambda, Kappa/Lambda/CD19, Myeloperoxidase (anti-MPO) from DAKO (Hamburg, FRG) Terminal Deoxynucleotidyl Transferase (anti-TdT) from Supertechs, Inc. and CD11b, CD65 from Beckman Coulter (BC, Krefeld, FRG). The cells were incubated for 15 minutes in the dark at room temperature. Careful measure was taken to ensure that the pipette tip did not touch the side of the tube when adding in the blood. Immediately after 15 minutes incubation, 2 ml of 1X FACS lysing solution (BD, San Jose, CA) was added to each tube, and incubated for an additional 10 minutes in the dark at room temperature. FACS lysing solution contained formaldehyde that lysed the red blood cells (RBC). The samples were then centrifuged at 200 g for 5 minutes and washed twice with PBS. Finally the samples were resuspended with 300 μl of 1% paraformaldehyde (Merck, Darmstadt, Germany) to fix the stained cells.

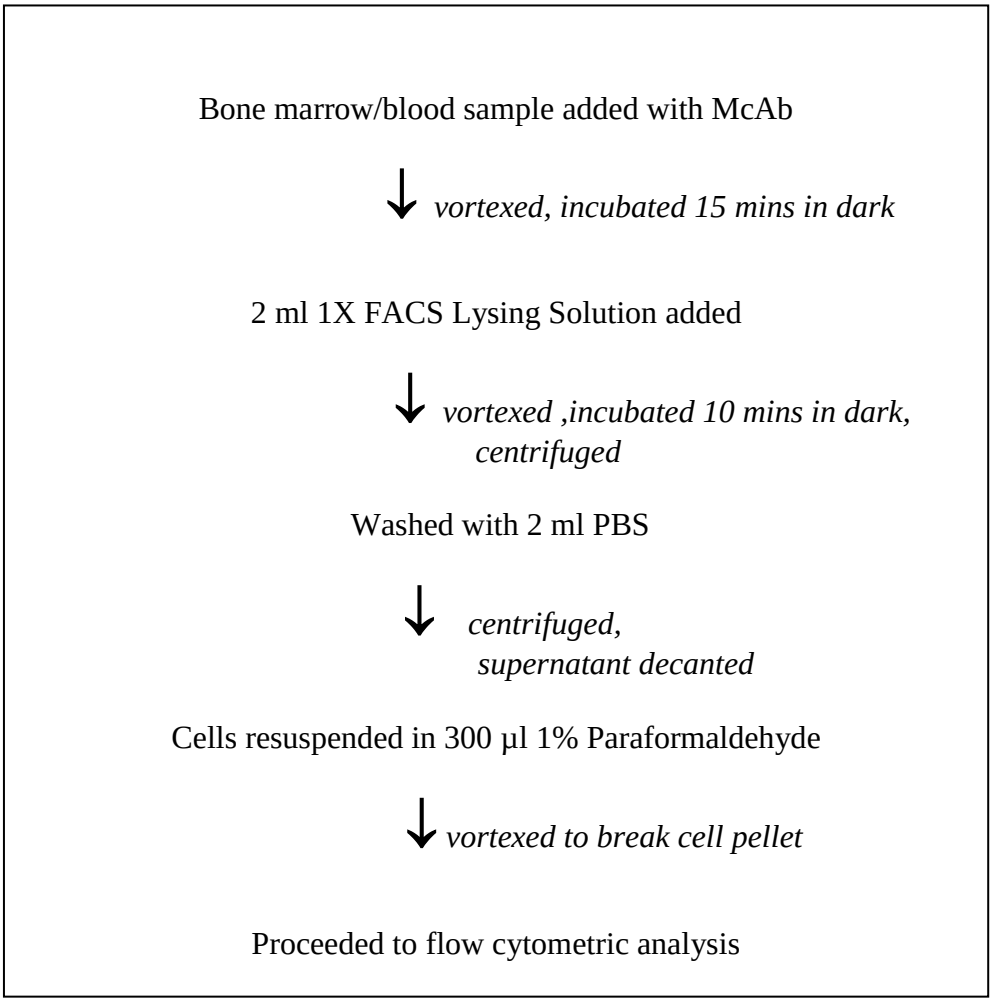


Figure 1.3.3 Flow chart of surface antigen staining.

2.6.2 Cytoplasmic staining

As shown in Figure 1.3.4, the first step of cytoplasmic staining was similar to the surface staining, which was 15 minutes incubation of samples with fluorochrome-conjugated McAb to stain the surface antigens. The next step used Fix and Perm® Cell Permeabilization Kit (Invitrogen™, CALTAG™ Laboratories, Austria) which consists of Reagent A, the fixation medium and Reagent B, the permeabilisation medium. First, 100 µl of Reagent A was added and left to incubate for 15 minutes in the dark at room temperature. Then the samples were centrifuged at 200 g for 5 minutes and washed once with PBS. Next, after the PBS

supernatant was decanted, red pellet could be seen formed at the bottom of the tube. The red pellet was resuspended by vortexing the tube. 100 μl Reagent B was added to the resuspended samples together with the respective McAb to stain the cytoplasmic antigens at room temperature. This time a longer incubation period of 20 minutes in the dark was needed for the monoclonal antibodies to permeabilise and bound with the cytoplasmic antigens. After incubation, the samples were washed with PBS by centrifugation twice at 200 g for 5 minutes. The supernatant was then decanted and the stained cells were finally fixed with 1% paraformaldehyde and ready for flow cytometry analysis.

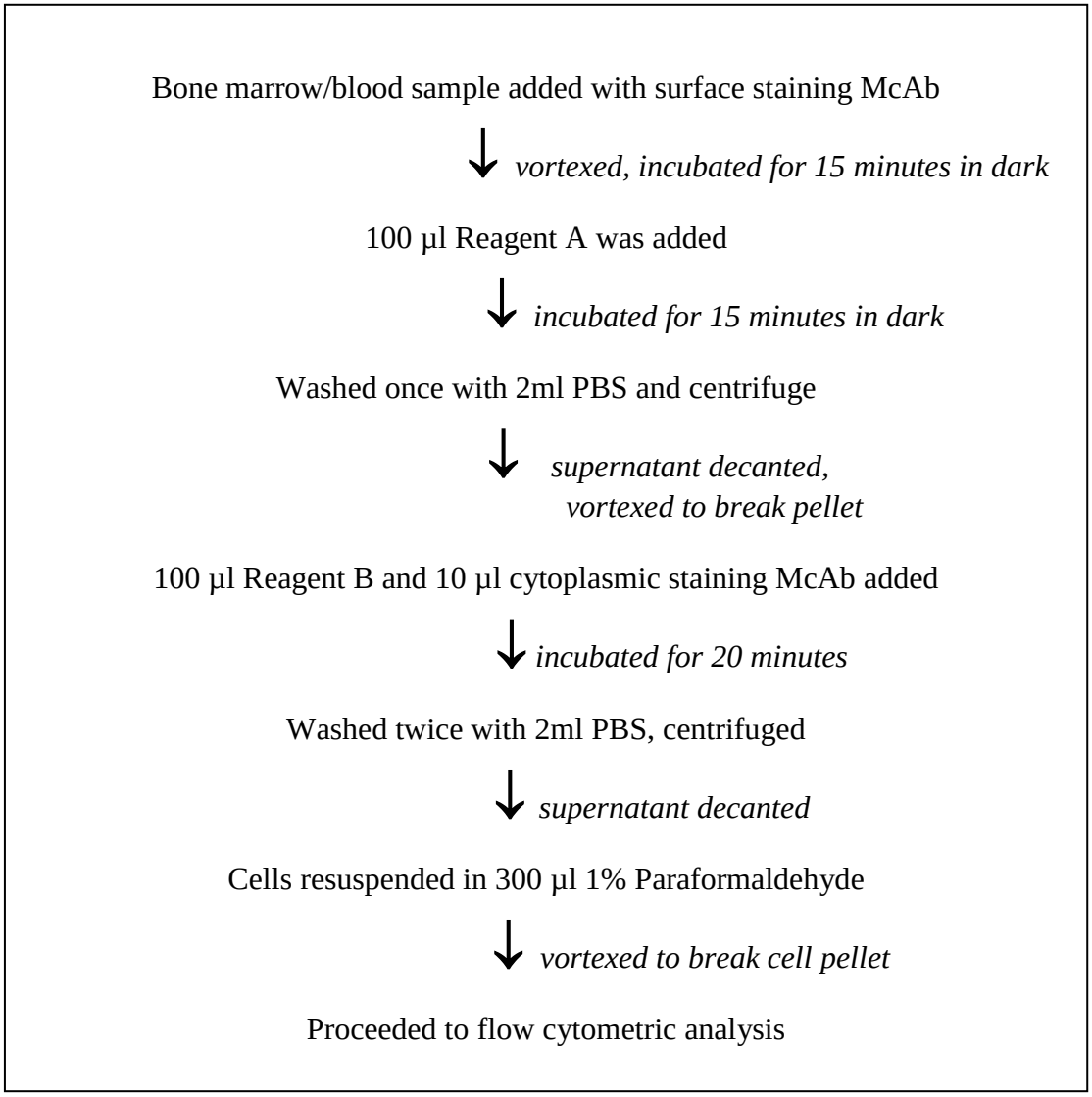


Figure 1.3.4 Flow chart shows the procedure of cytoplasmic antigens staining.

Every new case would be screened to identify the type of acute leukaemia for the patient. The acute leukaemia screening panel used in the laboratory is shown in Table 3.0. Among the respective markers for AML, B-ALL and T-ALL were MPO, CD19 and CD7. When all these markers and other additional markers were positive for FCM, the follow-up panel according to the result, AML (Table 3.1), B-ALL (Table 3.2) and T-ALL (Table 3.3) were used. Further classification of acute leukamias based on the immunophenotypic characteristics shown in Table 3.8 (AML) and Table 3.9 (B-ALL).

Table 3.0 Acute Leukaemia Screening Panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**Characterization**
1	-	CD34	CD45	Control/blasts
2	CyCDMPO	CD34	CD45	AML
3	CD19	CyCD79a	CD45	B-ALL
4	CD7	CyCD3	CD45	T-ALL

Table 3.1 Acute Myeloid Leukaemia (AML) panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**Characterization**
1	CD11b	CD13	CD45	Myeloid maturation pattern
2	CD15	CD10	CD45	Myeloid maturation pattern
3	HLA-DR	CD117	CD45	M3/myeloid blasts
4	CD64	CD14	CD45	M2/M4/M5
5	CD71	GlyA	CD45	M6
6	CD61	CD33	CD45	M7
7	CD65	CD123	CD45	M2/ Aberrant expression
8	CD2	CD56	CD45	Aberrant expression

Table 3.2 B-Acute Lymphoid Leukaemia (B-ALL) panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**Characterization**
1	nTdT	CD10	CD19	B-cell blasts
2	CD20	CD10	CD19	Blasts/haematogone
3	Kappa	Lambda	CD19	B-ALL
4	CyIgM	CD22	CD45	B-ALL
5	CD19	CD38	CD45	B-ALL
6	CD19	CD13	CD45	Aberrant expression
7	CD19	CD33	CD45	Aberrant expression

Table 3.3 T-Acute Lymphoid Leukaemia (T-ALL) panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**Characterization**
1	nTdT	CD3	CD45	T-cell blasts
2	CD4	CD8	CD45	T-ALL
3	HLA-DR	CD1a	CD45	T-ALL
4	CD5	CD10	CD45	T-ALL/B-ALL
5	CD2	CD56	CD45	Aberrant expression
6	CD7	CD13	CD45	Aberrant expression
7	CD7	CD33	CD45	Aberrant expression

Note: CD = cluster of differentiation
Cy = requires cytoplasmic staining (including nTdT)

During the duration of research, the flow cytometry system was upgraded from 3-colour to 4-colour and therefore there were slight changes to the antibody panels with the addition of one more fluorochrome, which is Allophycocyanin, also known as APC. The upgrading to 4-colour helped to increase the sensitivity of flow cytometry analysis and also allows more cellular parameters to be assessed simultaneously at one time. The 4-colour antibody panels used are shown below (Table 3.4-3.7).

Table 3.4 Acute Leukaemia Screening Panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**APC** *(Allophycocyanin)*	**Characterization**
1	CyMPO	-	CD45	CD34	Myeloid blasts (AML)
2	CD19	CyCD79a	CD45	CD34	B-cell blasts (B-ALL)
3	CD7	CyCD3	CD45	CD34	T-cell blasts (T-ALL

Table 3.5 Acute Myeloid Leukaemia (AML) panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**APC** *(Allophycocyanin)*	**Characterization**
1	CD11b	CD13	CD45	CD34	Myeloid maturation pattern
2	CD15	CD10	CD45	CD34	M2/Myeloid maturation pattern
3	HLA-DR	CD117	CD45	CD34	M3/Myeloid blasts
4	CD64	CD14	CD45	CD34	M4/M5/Monocytic series
5	CD71	Gly A	CD45	CD34	M6
6	CD61	CD33	CD45	CD34	M7
7	CD65	CD123	CD45	CD34	M2/Aberrant markers
8	CD2	CD56	CD45	CD34	Aberrant markers

Table 3.6 B-Acute Lymphoid Leukaemia (B-ALL) panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**APC** *(Allophycocyanin)*	**Characterization**
1	nTdT	CD38	CD19	CD34	B-cell blasts
2	CD20	CD10	CD19	CD34	B-cell maturation pattern/Common B-ALL
3	Kappa	Lambda	CD19	CD34	Mature B-ALL
4	CD22	CyIgM	CD19	CD34	Pre-B-ALL
5	CD15	CD13	CD45	CD19	Aberrant markers
6	CD65	CD33	CD45	CD19	Aberrant markers

Table 3.7 T-Acute Lymphoid Leukaemia (T-ALL) panel

Tube	**FITC** (*flourescein isothiocynate*)	**PE** (*phycoerythrin*)	**PerCP Cy5.5** (*peridinin chloroanphyll protein*)	**APC** *(Allophycocyanin)*	**Characterization**
1	nTdT	CD3	CD45	CD34	T-cell blasts
2	CD4	CD8	CD3	CD34	T-ALL
3	HLA-DR	CD1a	CD3	CD34	T-ALL
4	CD2	CD56	CD3	CD34	Aberrant expression
5	CD5	CD10	CD45	CD3	T-ALL
6	CD7	CD13	CD45	CD3	Aberrant expression
7	CD65	CD33	CD45	CD3	Aberrant expression

Note: CD = cluster of differentiation
Cy = requires cytoplasmic staining (including nTdT)

Table 3.8 Immunophenotypic Cell Markers in Acute Myeloid Leukaemia (AML)

Cell Markers	M0	M1	M2	M3	M4 / M5	M6	M7
CD34	+	+	+ / -	+ / -	+ / -	-	+ / -
CD1a (HLA-DR)	+	+	+	-	+	+ / -	+
CD13	+	+	+	+	+	+ / -	-
CD33	+	+	+	+	+	+ / -	+ / -
CD15	+ / -	+ / -	+	+	+	-	-
CD9	-	-	-	+	-	-	-
CD11b	+ / -	+ / -	+ / -	+ / -	+	-	-
CD36	-	-	-	-	-	+	+
CD14	-	-	-	-	+	-	-
Glycophorin A	-	-	-	-	-	+	-
CD41 / CD42	-	-	-	-	-	-	+
CD71	-	-	-	-	-	+	-
CD61	-	-	-	-	-	-	+
TdT	+ / -	+ / -	-	-	-	-	-

M0 is undifferentiated AML with negative light microscopic cytochemistry for AML, absence of lymphoid antigens and MPO+ by electron microscopy.
Detection of cytoplasmic MPO by monoclonal antibody appears more sensitive than CD13 and CD33 combined. M0 blasts may express CD7 or CD4 (both lymphoid markers).

The flow appearance of **M1** is similar to M0 and probably not separable (though it may not express as much CD34).

Expression of CD19 and less often CD56 in **M2** is associated with t(8;21) and a favourable prognosis.

M3 and **M3v** are CD34-, CD13+ and CD33+; CD9 is almost always positive (it is negative in most other FAB types) and HLA-DR is absent. CD2 in HLA-DR- AML is correlated with M3 and t(15;17). Myeloid/NK cell acute leukaemia, with morphology and immunophenotype similar to M3v but without RAR rearrangement has HLA-DR-, CD33+, CD13 weak, CD34 variable and CD56+ phenotype.

M4 and **M5** are similar phenotypically though M4 is more often CD34+ than M5. The combination of CD33 positivity with CD13- and CD34- is highly correlated with M5.
CD56 and weak CD7 or CD4 expression may be seen in some cases of M5. The presence of CD2 is correlated with M4Eo [associated with inv(16) and a better prognosis.
M6 usually has positive HLA-DR, CD34, CD13 and CD33. The order of appearance of markers in the megakaryocytic lineage: 1a, PPO, acid phosphatase, CD61 (GP IIIa), CD41 (GP IIb/IIIa), CD42 (GP IX/Ib), vW Ag and PAS. The megakaryoblastic nature of the blasts must be confirmed by ultrastructural demonstration of platelet peroxidase or by immunophenotyping

Table 3.9 Immunological Classification of Acute Lymphoblastic Leukaemia (ALL)

Reactivity against	Markers	B-lineage				T-lineage	
		Null / Pro-B / Pre-pre-B	cALL	Pre-B	B-ALL	Pre-T	T-ALL
Precursor Cells	HLA-DR	+	+	+	+	- / +	-
	TdT	+	+	- / +	- / +	+	+
	CD34	+	+	-	-	-	-
B-cell Antigens	CD19	+	+	+	+	-	-
	CD22	+	+	+	+	-	-
	CD24	+	+	+	+	-	-
	CD10 (CALLA)	-	+	+	- / +	- / +	-
	Cy μ chain	-	-	+	+	-	-
	CD20	-	- / +	- / +	+	-	-
	SmIg	-	-	-	+	-	-
T-cell Antigens	CD7	-	-	-	-	+	+
	CD3	-	-	-	-	+	+
	CD5	-	-	-	-	- / +	+
	CD2	-	-	-	-	-	+
	CD4 / CD8	-	-	-	-	-	+
	E rosettes	-	-	-	-	-	+

Referring to Table 3.9, all B-lineage cases show rearrangement of Ig heavy chain gene and all T-lineage cases show rearrangement of T-cell receptor β, γ and/or δ chain genes. TdT is positive in all types of ALL with exception of B-ALL. It is also positive in 15-20% of AML, particularly M0 and M1. In lymphoblastic crisis of CML, TdT is always positive; T-lymphoblastic lymphoma is the only NHL with positive TdT activity; in chronic T-cell proliferation, TdT activity is always absent. Infants with ALL that is CD19+, CD10- and express aberrant CD15 are likely to have 11q23 translocations in the chromosomes and a poor prognosis. t(1;19) in pre-B-cell ALL is always associated with CD19+, CD10+, CD9+, CD34- and variable expression of CD20. B-cell ALL is equivalent to Burkitt's lymphoma in leukaemic phase (FAB L3) and all patients have one of t(8;14), t(2;8) or t(8;22). T-cell ALL can be divided into early cortical phenotype (CD2+, CD5+, CD7+ and strong TdT), late cortical phenotype (CD1+, CD2+, CD5+, CD7+ and dual CD4/CD8 with minimal surface CD3) and medullary phenotype (CD2+, CD5+, CD7+ with segregated CD4 or CD8 and less common CD3 and TdT).

2.6.3 Data Acquisition

Samples that had been fixed by 1% paraformaldehyde in the immunophenotyping process were acquired using FACSCalibur™ (BD, San Jose, CA). An appropriate set up was used using BD Cell QuestPro including applying the instrument setting that had been optimized earlier. Instrument settings that had been optimized correctly would provide desirable plots on flow cytometry. A total of 20,000 events and 250,000 events were acquired in each sample at diagnosis and after completion of induction chemotherapy respectively. Higher events acquired after the induction chemotherapy was to determine the presence of blast cells in the patients' samples and to enable minimal residual disease determination of the status of the patients as well.

Live gating was performed in respective minimal residual disease (MRD) panels after the induction chemotherapy. A threshold of 1 leukaemic cell in 1000 cells (0.1%) was used to ensure a definition of the minimal residual disease, patients whose values were lower than the threshold were considered negative for MRD (Campana 2004). All samples that had been acquired were automatically saved as FCS files when the desired target events had been reached. BD Paint-A-Gate™ Pro software (BD, San Jose, CA) was used to analyze the FCS files by using the appropriate cell gating strategy and antibody reactivity of leukaemic cells. Examples of the acquisition plots for positive leukaemia case are shown in Figure 1.3.5 and Figure 1.3.6. The gated plots for MRD patient is shown in Figure 1.3.7.

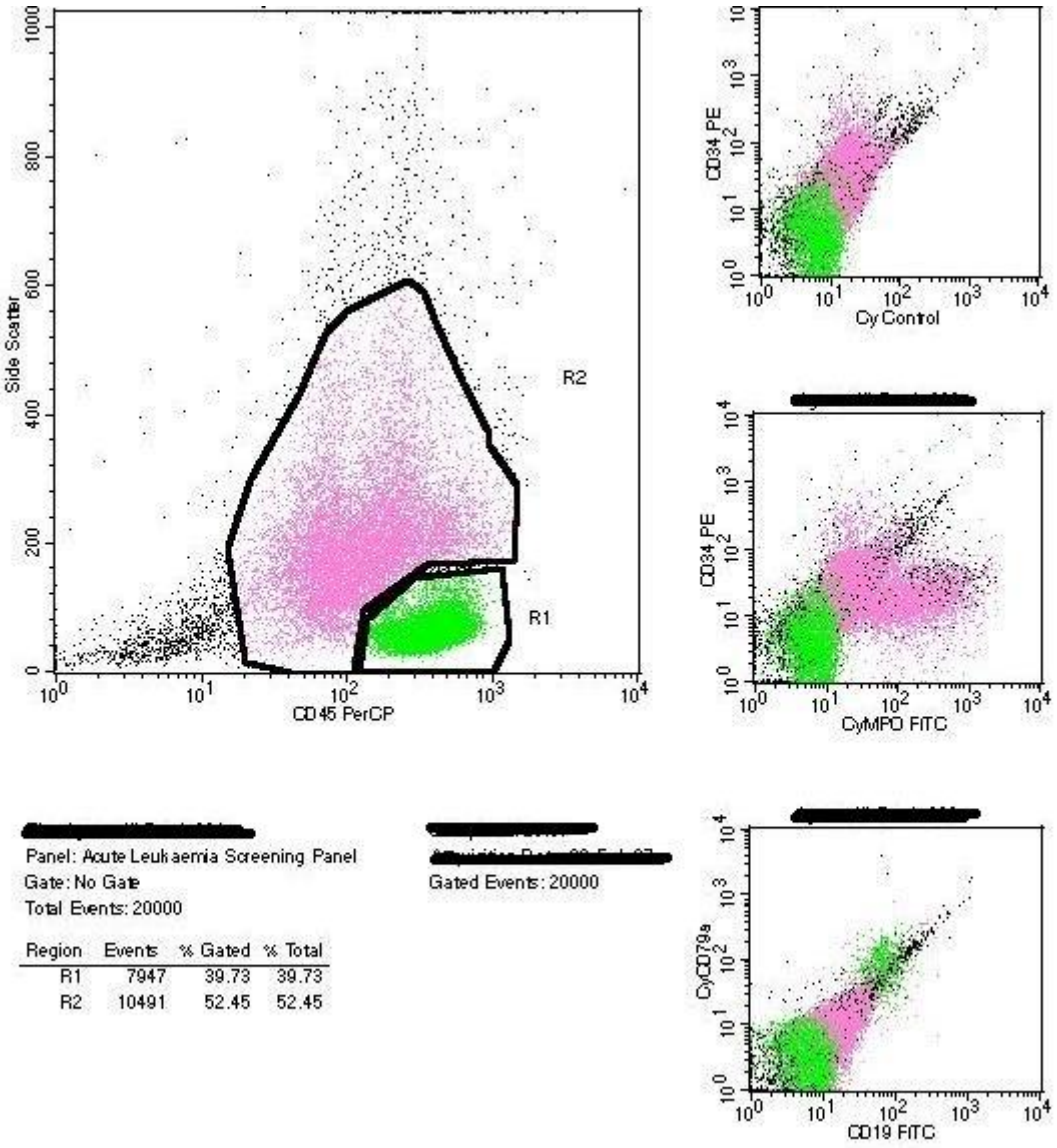


Figure 1.3.5 CD45 PerCP versus SSC plot was used as the main plot to gate the blast cells (R2) and to be used as reference for other plots with different CD.

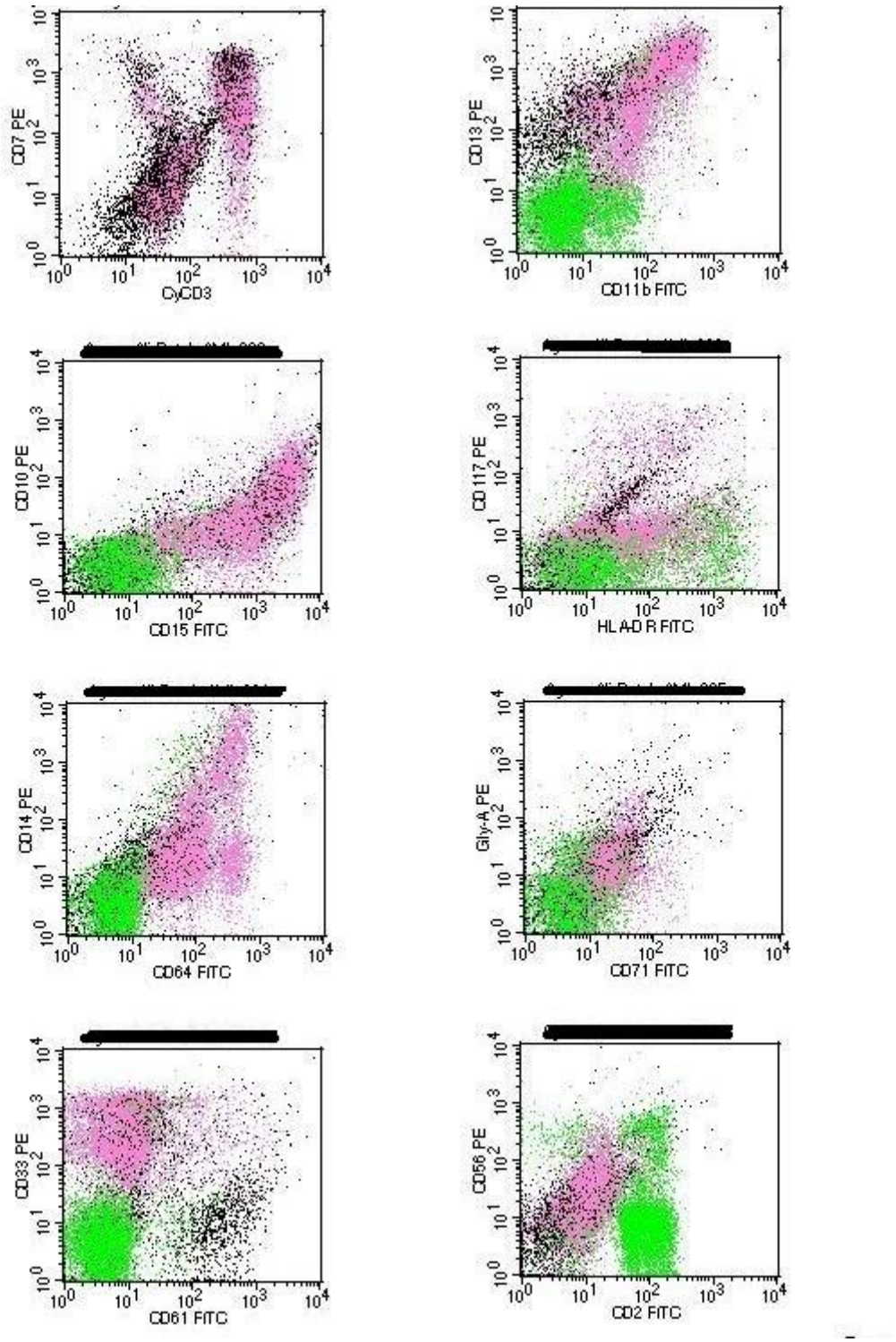
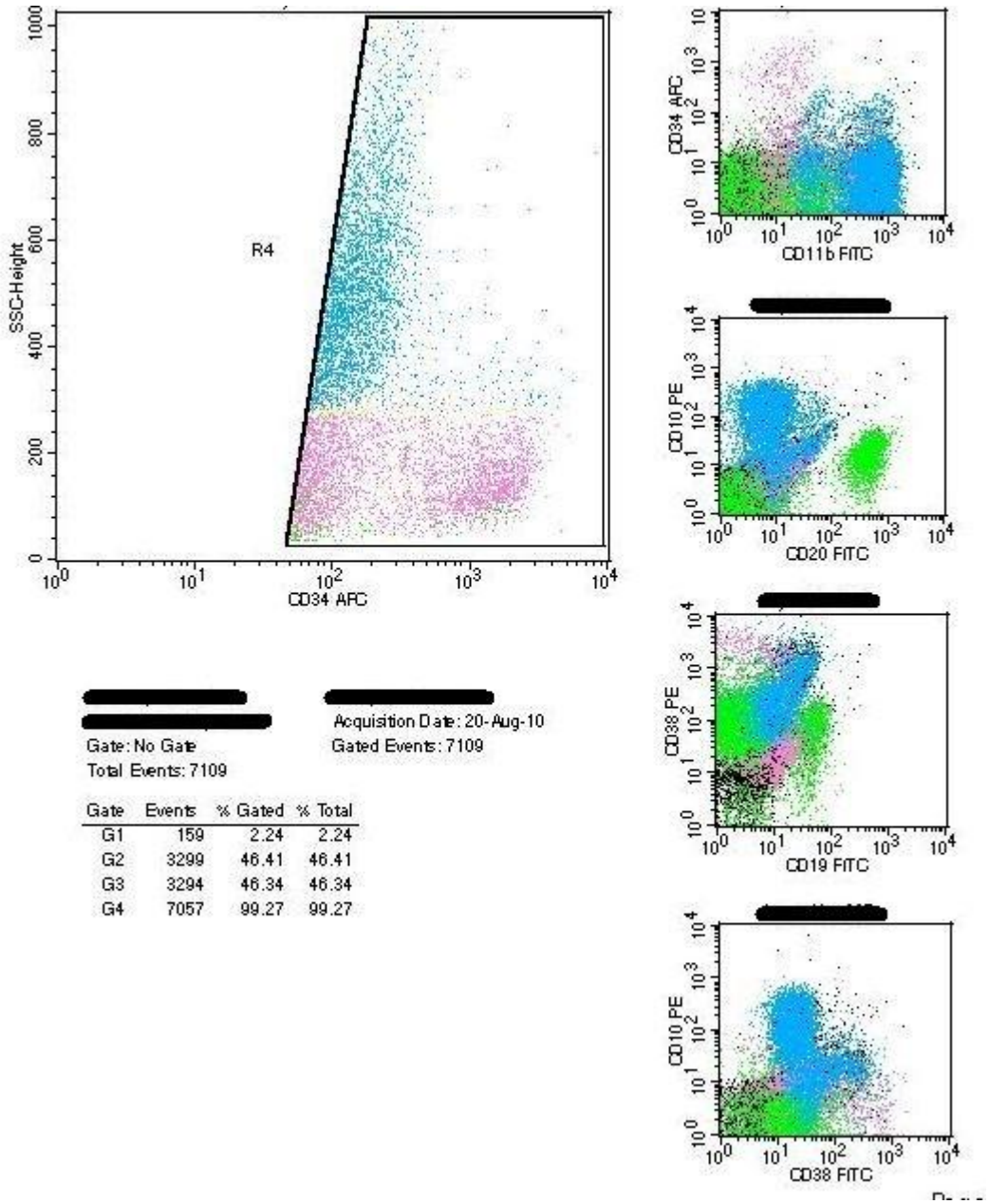


Figure 1.3.6 From the gated blast cells in CD45 vs SSC plot (Figure 2.2.6), the blast cells (in pink) was located in other plots with different CD used to determine the population whether it was positive, negative or heterogeneous.



Gate	Events	% Gated	% Total
G1	159	2.24	2.24
G2	3299	46.41	46.41
G3	3294	46.34	46.34
G4	7057	99.27	99.27

Figure 1.3.7 In this MRD sample, live gating was done for CD34 APC and only cells gated in R4 was collected when the 250 000 target cells were reached in acquisation.

2.6.4 Data analysis

Data obtained from flow cytometry analysis was analyzed statistically using Predictive Analytics SoftWare Statistics (PASW®) version 18.0 (SPSS Inc. USA). Clinical data such as immunophenotypic expression, blast percentage, full blood count (Hb, WBC, Plt) and hepatosplenomegaly were analysed. The comparison of complications between various subgroups by age, sex, ethnicity and FBC were analysed using T-independent test, Chi-square and Fisher's exact tests. For MRD, the odds ratio was analysed using multiple logistic regression (MLR) analysis. All statistical tests were two-tailed and p value < 0.05 was considered to be statistically significant.

CHAPTER 3: RESULTS

3.0 Introduction

About 245,000 people in the United States are affected with some form of leukemia, including those that have achieved remission or cure. Approximately 44,270 new cases of leukemia were diagnosed in the year of 2008 in the US. Children are most commonly affected by acute lymphoblastic leukemia. Only about 3% of cancer diagnoses among adults are for leukaemias, but because cancer is much more common among adults, more than 90% of all leukaemias are diagnosed in adults (Society, 2009). In peninsular Malaysia between 2003-2005, Malays had higher cancer incidences as compared to the Chinese and Indians for both males (64.4%) and females (62.8%). Age specific incidence per 100,000 populations of Indians in Malaysia (6.5) was higher than in Singapore (4.8) and India (4.4), whereas the age specific incidence for Singapore Chinese (6.2) was higher than Malaysian Chinese (5.6) (Lim et al., 2008).

Currently, leukaemias are diagnosed using a combination of morphology, immunophenotype, cytochemistry, and karyotype. Acute leukemia displays characteristic patterns of surface antigen expression (CD antigens), which facilitate their identification and proper classification. One way to detect these leukaemic antigen expressions is by flow cytometric immunophenotyping. There are a number of advantages of using flow cytometry, such as its sensitivity in detecting cells, in the range of 1 in 10^2 to 10^4, its rapidity, and its ability to analyse a large number of cells quantitatively. Also, the usage of multiple antigens simultaneously in immunophenotyping has improved the identification of malignant cells. Multiparameter flow cytometric analysis has

become common in most laboratories and an invaluable tool in the diagnosis, classification, and plays an important role in establishing proper treatment plans.

3.1 Aims of this chapter

The aims of this chapter are to study the distribution and demographics of acute leukaemias in Sarawak, which will include the distribution of the study population, clinical parameters and immunophenotypes.

3.1.1 Study population of acute leukaemia

Patients diagnosed with acute leukaemia were recruited from hospitals all over Sarawak via convenient sampling within the duration of 3 years, from 2006 until 2010. The patients' age, gender and ethnicity are among the demographics data included in the acute leukaemia population study. Clinical characteristics of the patients such as the haemoglobin level, total white cell count, presence of lymph node, liver, and spleen enlargement were also noted. The comparison of complications between various subgroups by age, sex, ethnicity and FBC were analysed using T -independent test, Chi-square and Fisher's exact tests. All hypothesis tests were based on two-sided tests and *p* value less than 0.05 was considered as statistically significant.

3.2 Acute Myeloid Leukaemia

A total of 147 AML cases were diagnosed and analysed during the duration of the study. In this study the analysis was divided into childhood and adult.

3.2.1 Childhood\Adolescent Acute Myeloid Leukaemia

AML usually affects adults, but childhood AML is also common. In this study, childhood AML were diagnosed in 49 cases (33.3%).

3.2.1.1 Age distribution

The age distribution for the childhood AML was divided into 2 categories, childhood (0-10 years) and adolescent (11-17 years). The mean values for respective category were as follows: Childhood 3.30±3.29 years and adolescent: 13.25±1.91 years. The distribution of childhood AML according to age is demonstrated in Table 4.0 and Figure 1.3.8.

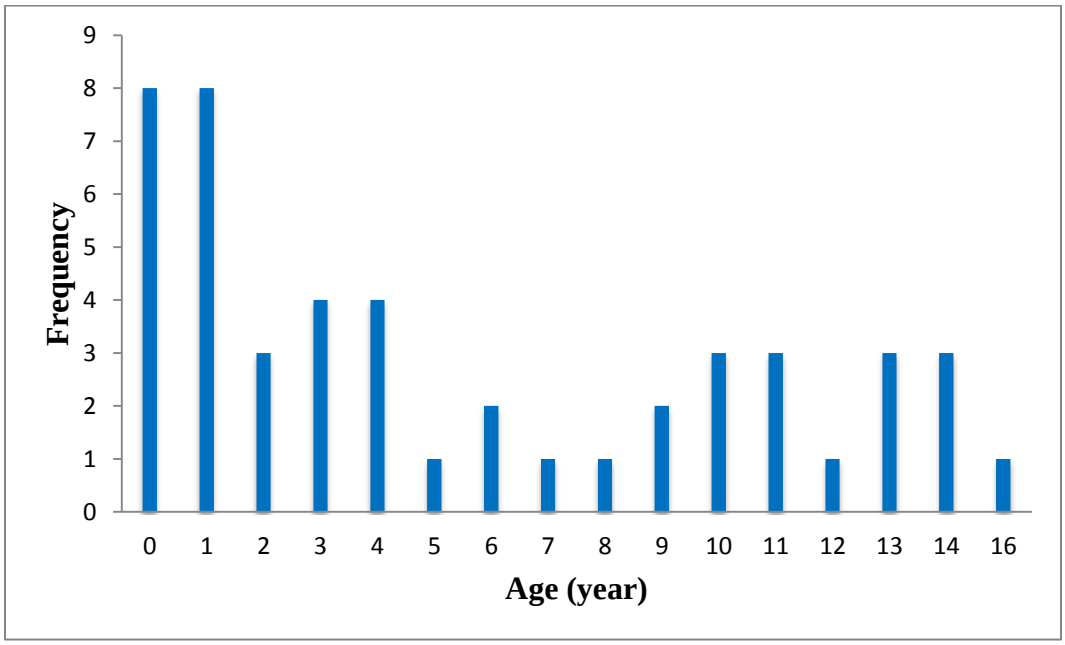


Figure 1.3.8 The age distribution of childhood AML cases.

3.2.1.2 Gender distribution

In childhood AML, from a total of 49 cases, 26 cases were males (53.1%) and 23 cases were females (46.9%). The gender distribution of the AML in childhood is demonstrated in the Figure 1.3.9.

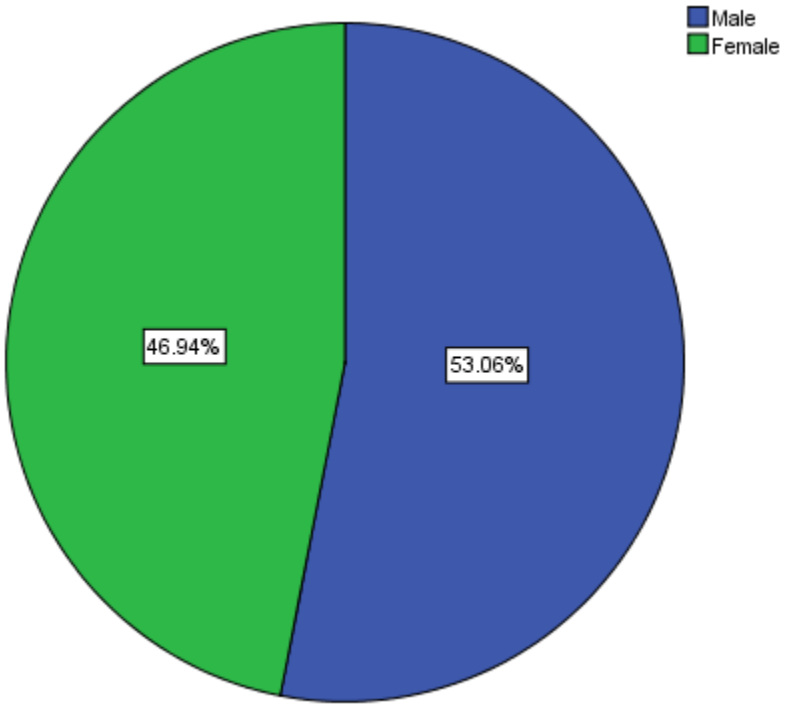


Figure 1.3.9 The sex distribution of childhood AML cases.

3.2.1.3 Ethnic distribution

Childhood AML was highest among the Sarawak Bumiputeras, with 20 cases (40.8%), followed by Malay/Melanau with 17 cases (34.7%), Chinese with 8 cases (16.3%) and others with 4 cases (8.2%). The ethnicity distribution is demonstrated in Figure 1.4.0.

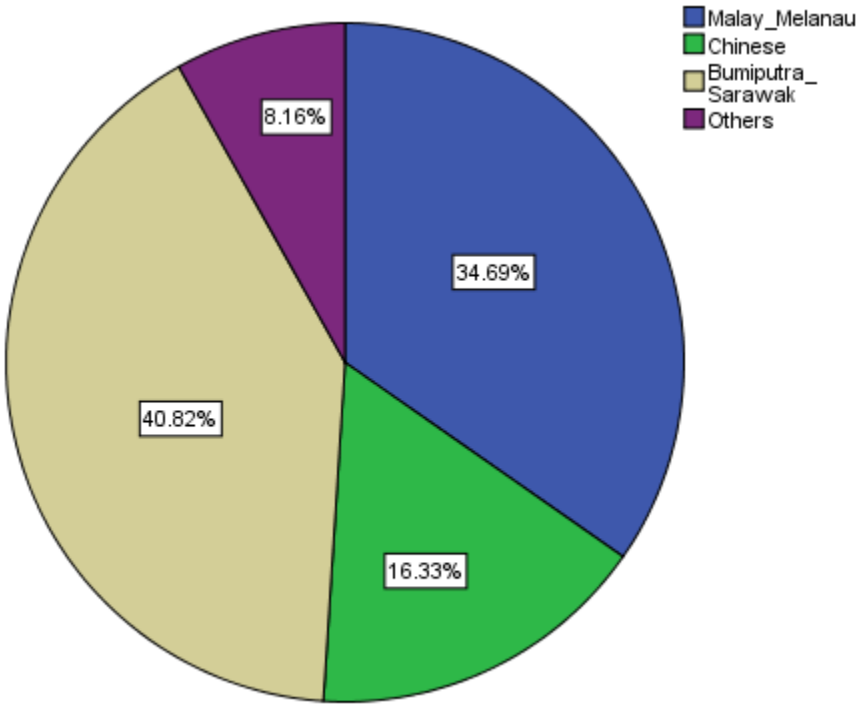


Figure 1.4.0 The distribution of ethnicity for childhood AML cases in Sarawak

3.2.1.4 Clinical characteristics of childhood and adolescent AML

The clinical characteristics analysis of FBC (haemoglobin, total white count and platelet) childhood AML is divided into childhood (0-9 years) and adolescent (10-17 years). For haemoglobin, the mean values were as follows: 9.22±3.59 g% for children while for adolescent: 8.74±2.48 g%.

For total white count, the mean values were as follows: mean value 77.16 ±82.52 ($X10^9$/L) for children group while for adolescent 61.26±69.58 ($X10^9$/L). For platelet, the mean values were as follows: mean value of 97.19±108.89 ($X10^9$/L) for the children group while for adolescent the mean value was 58.72±46.93($X10^9$/L). It was also noted that, 32.7% of childhood AML patients

had lymph nodes enlargement, 65.3% had liver enlargement and 42.9% had splenomegaly at time of diagnosis.

3.2.1.4. (a) Association between full blood count (FBC) and gender in childhood AML

There was no difference in the haemoglobin level and platelet count between male and female childhood AML patients at presentation. TWC at time of diagnosis was however found to be significantly higher in males compared to females (p = 0.008). The results of the analysis are demonstrated in Table 4.0 (a) and 4.0 (b) below.

Table 4.0 (a) Association between haemoglobin and platelet with gender in childhood AML

FBC	Male (n =22) Mean (SD)	Female (n =22) Mean (SD)	Mean Differ. (95% CI)	t statistic (df)[a]	p value[a]
Hb (g%)	9.1 (3.49)	9.1 (3.23)	0.9 (-2.0,2.1)	0.01 (42)	0.989
Plt (10^9/l)	86.2 (71.50)	88.9 (120.72)	-2.8 (-63.2, 57.5)	-0.09 (42)	0.925

[a]Independent t-test

Table 4.0 (b) Association between total white count and gender in childhood AML

FBC	Male (n =22) Mean (SD)	Female (n =23) Mean (SD)	Mean Differ. (95% CI)	t statistic (df)[a]	p value[a]
TWC (10^9/l)	104.4 (87.87)	43.5(57.02)	60.8 (16.5,105.2)	2.77 (43)	0.008*

[a]Independent t-test

3.2.1.4. (b) Association between age and gender in childhood AML

There was no significant difference in the age of disease presentation between male and female childhood AML cases (p=0.249). The result of the analysis is demonstrated in the Table 4.1 below.

Table 4.1 Association between age and gender in childhood AML

Variable	Male (n = 26) Mean (SD)	Female (n = 23) Mean (SD)	Mean Differ. (95% CI)	t statistic (df)[a]	p value[a]
Age	5.5 (5.67)	5.9 (4.87)	-0.42 (-3.5, 2.6)	-0.28 (47)	0.249

[a] Independent t- test

3.2.1.4. (c) Association between ethnicity and gender in childhood AML

There was no significant association found between gender and ethnicity in these cases. The result of the analysis is demonstrated in the Table 4.2 below.

Table 4.2 Association between ethnicity and gender in childhood AML

Variable (Ethnic)	N	Male Freq (%)	Female Freq (%)	χ^2 statistic[a] (df)	p value[a]
Malay/Melanau	17	10 (38.5)	7 (30.4)	0.88 (2)	0.643
Sarawak Bumi	20	9 (34.6)	11 (47.8)		
Others	12	7(26.9)	5(21.7)		

[a]Chi-Square test for independence

3.2.1.5 Immunophenotypes of childhood AML

Immunophenotyping was done with various types of monoclonal antibody markers and the results were visualised with flow cytometry. The aim was to tag the cells associated with acute leukaemia with the combination of these markers. In the childhood AML cases, the cases demonstrated the significant expression of CD33 (94.0%), CyMPO (87.8%), CD13 (87.8%), CD117 (85.7%), and HLA DR (81.6%). There were also expression of aberrant markers such as CD56 (61.2%) and CD7 (53.1%). (Table 4.3) CD33, CD13, and CyMPO, are among common antigens expressed in AML because these antigens are commonly expressed in AML from FAB subtype M0 until M7. CD117 is the first line AML marker and is commonly expressed in AML subtype M2. Known for its less expression in AML antigens, CD56 and CD7 were also found to be highly expressed. CD56 are usually found in subtype M2 (53.1%) AML while CD7 may be expressed in some M0 (16.3%) myeloblasts blast cells, although also regularly found in ALL.

Weak expressions of these antigens were also found: CD14 (81.6%), CD10 (81.6%), CyCD79a (73.5%), CD19 (67.3%), CD11b (67.3%), and CD61 (53.1%) (Table 4.4). Most of these are aberrant markers except for CD14, CD11b and CD61. CD11b is commonly expressed in AML subtype M4/M5 (2.0%) and may also be expressed in M0/ M1 (16.3%), and M2/M3 as well but weakly expressed. Weak expression of CD14 (also known as monocytoid marker) and CD61 (megakaryotic marker) were probably due to the fact that there were only expressed in AML subtype M4/M5 (2.0%) and M7 (6.1%) respectively. CD10, CD19 and Cy79a were aberrant antigens that were commonly expressed in ALL.

There were also a few atypical antigens that showed both expression and non expression in the population such as CD15, CD64 and CD65. CD15 was expressed in 42.9% cases but weakly

expressed in 51.0% cases. Another atypical antigen, CD64 also showed the same expression with positive expression in 51.0% of cases while negative expressions were detected in 46.9% of studied AML cases. CD65 antigen also showed dual expression, with positive expression found in 26.5% of cases, and weakly expressed in 14.3% studies AML cases. The subtype for childhood AML showed the highest incidence in M2 subtype with 26 cases (53.1%). Weak expression of CD15 can be associated myeloid cells and monocytes with the common expression in subtype M2 (53.1%), M3, (2.0%) M4/M5 (2.0%) and may be in M0/M1 (16.3%). CD64 and CD65 are also associated with monocytic and myeloid cells. Figure 1.4.1 and 1.4.2 displayed the flow cytometry dot plots for one of the AML positive childhood patient.

Table 4.3 Expression of monoclonal antibody markers gated in childhood AML CD34/CD45 population

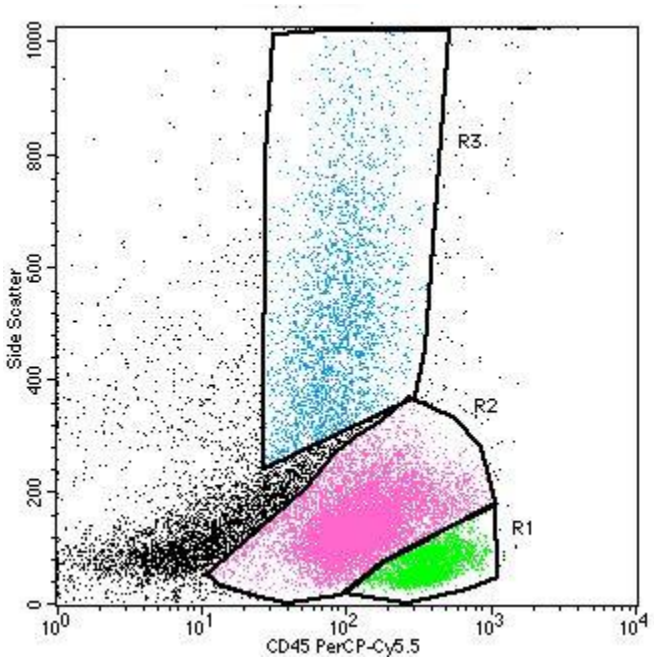
Antigen	Freq.(%)	Characterisation
CD45	48(98.0)	Control/blasts
CD33	46(93.9)	AML
CyMPO	43(87.8)	AML
CD13	43(87.8)	AML
CD117	42(85.7)	AML
HLADR	40(81.6)	AML
CD34	40(81.6)	Control/blasts
CD56	30(61.2)	NK, myeloma cells
CD7	26(53.1)	T-ALL, Myeloblast cells

Freq = Frequency

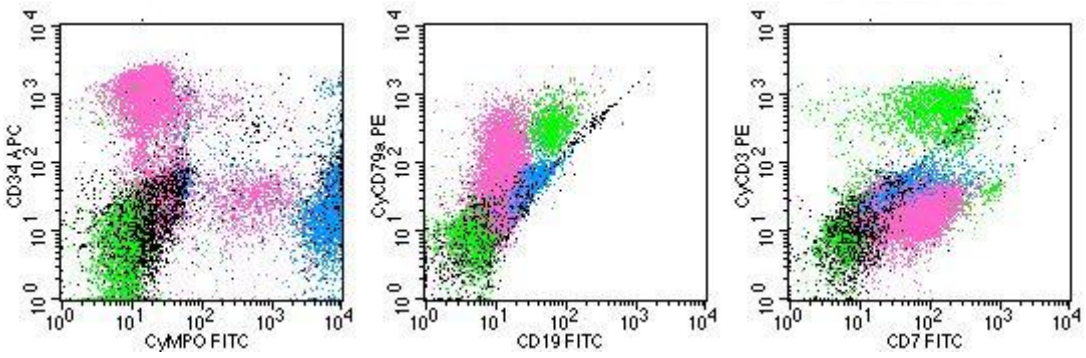
Table 4.4 Weak expressions of monoclonal antibody markers gated in childhood AML CD34/CD45 population

Antigen	Frequency (%)	Characterisation
CD14	40(81.6)	AML
CD10	40(81.6)	Aberrant expression
Cy79a	36(73.5)	Aberrant expression
CD19	33(67.3)	Aberrant expression
CD11b	33(67.3)	AML
CD61	26(53.1)	AML

Freq = Frequency

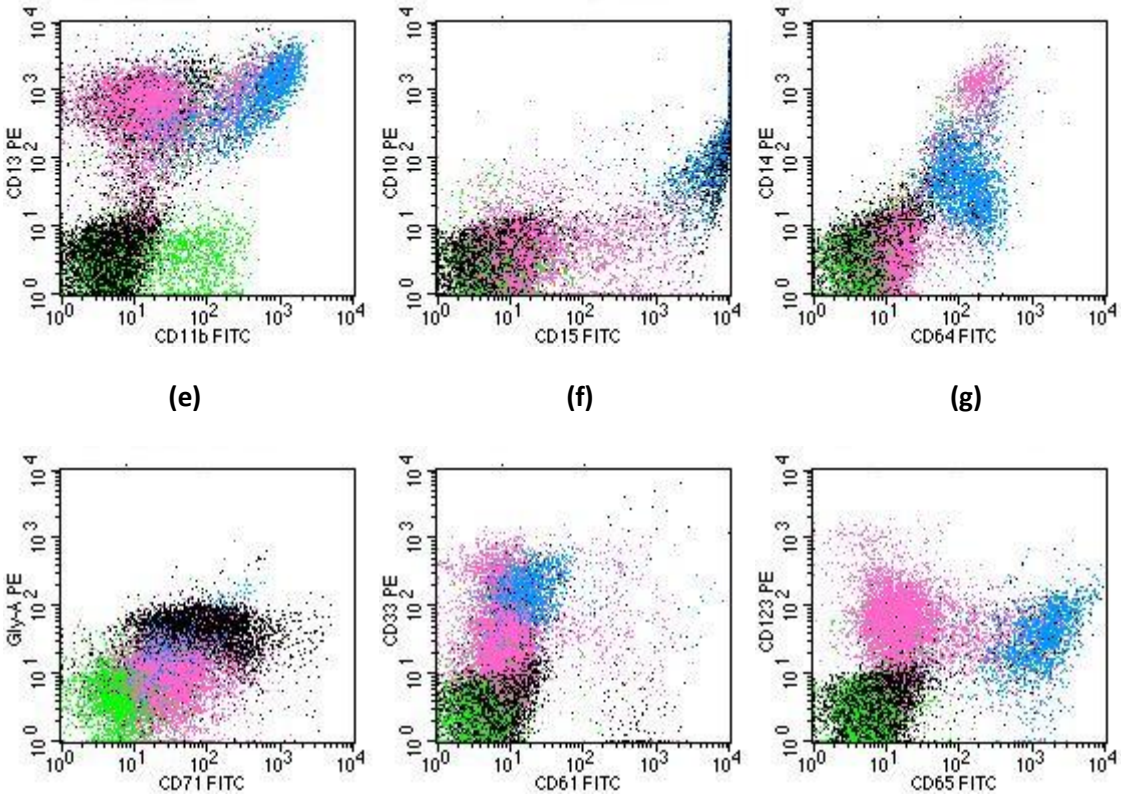


(a)



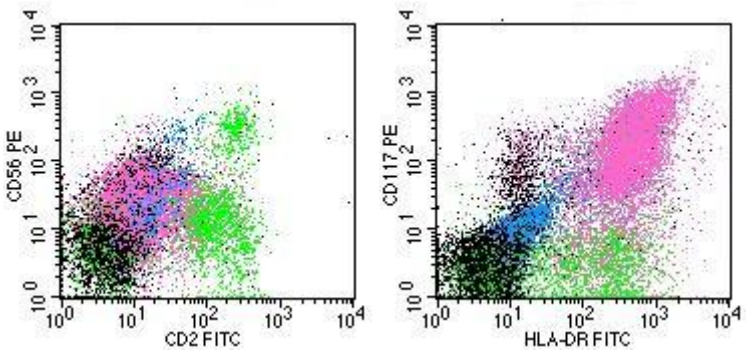
(b) (c) (d)

Figure 1.4.1 Plots (a) to (d) were taken from the acute leukaemia screening panel positive for childhood AML subtype M2. The blast cells population was marked with the colour pink. In this patient (b) – (d), the blast cell population was positive for the following antigens: CyMPO, CD34, CD79a and CD7.



(e) (f) (g)

(h) (i) (j)



(k) (l)

Figure 1.4.2 The plots (e) to (l) were the dot plots of the same patient positive for childhood AML in the previous plots. These plots were taken from the AML immunophenotyping panel performed on the sample. The following blast populations (in pink) were positive for the following antigens: CD13, CD33, CD123, CD56, CD117 and HLA-DR.

3.2.2 Adult Acute Myeloid Leukaemia

AML is known to be very common among adults. In this study, there were 98 cases of adult AML studied (66.7%).

3.2.2.1 Age distribution

The age distribution for the adult AML was divided into 2 categories, adult (18-49 years) and elderly (>50 years). The mean age for each respective category are as follows: Adult: 36.1±9.17 years and elderly: 61.0±7.27 years. The age distribution of adult AML cases is demonstrated in Figure 1.4.3. The age distribution in childhood and adult AML is shown in Table 4.5.

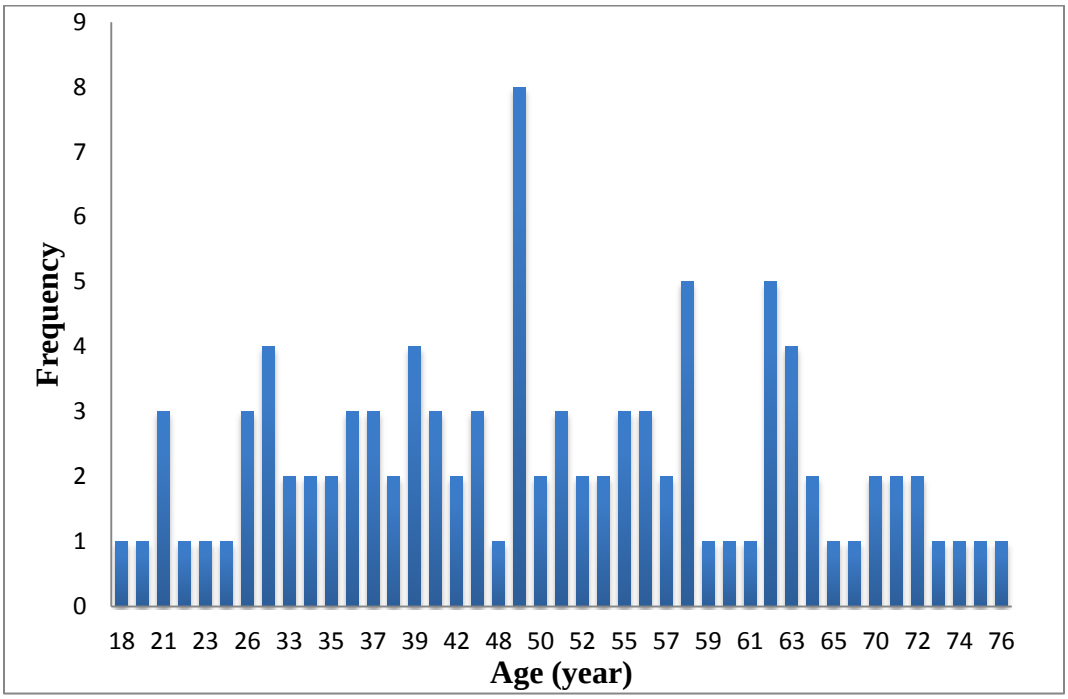


Figure 1.4.3 The age distribution of adult AML cases

Table 4.5 The age distribution of childhood and adult AML

Category	Mean age (years)
Childhood (0-10 years)	3.30
Adolescent (11-17 years	13.25
Adult (18-49 years)	36.1
Elderly (>50 years).	61.0

3.2.2.2 Gender distribution

From a total of 98 adult AML cases, 51 cases were males (52.0%) and 47 cases were females (48.0%). The distribution of the distribution of in adult AML is demonstrated in the Figure 1.4.4

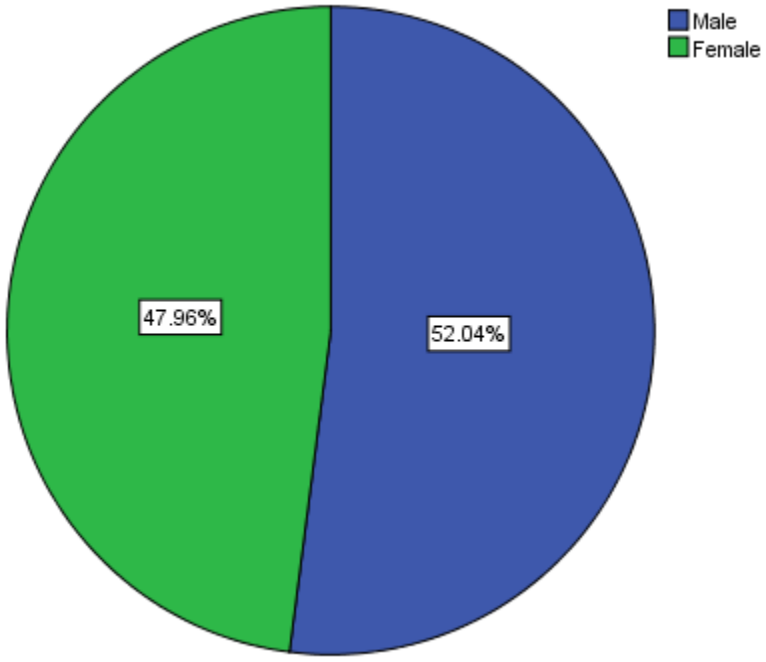


Figure 1.4.4 The sex distribution of adult AML cases.

3.2.2.3 Ethnic distribution

Adult AML incidence in Sarawak was seen highest among the Sarawak Bumiputeras, with 28 cases (28.6%), followed by Chinese with 27 cases (27.6%), Malay/Melanau with 23 cases (23.5%) and others with 20 cases (20.4%). The ethnicity distribution is demonstrated in Figure 1.4.5.

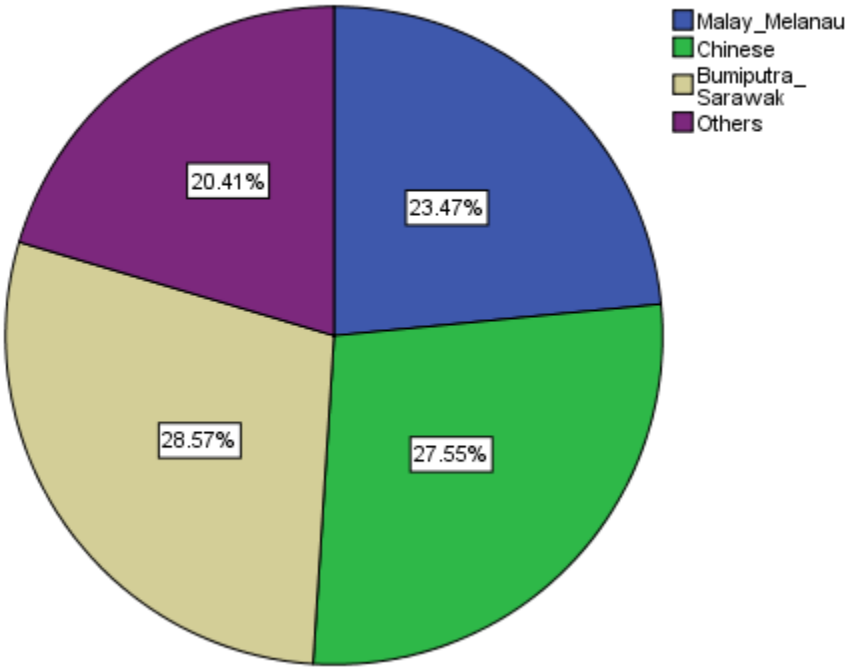


Figure 1.4.5 The distribution of ethnicity for adult AML cases in Sarawak

3.2.2.4 Clinical characteristics of adult AML

The clinical parameters analysis of FBC (haemoglobin, total white count and platelet) adult AML is divided into adult (18-50 years) and elderly (>50 years). For haemoglobin, the mean values were as follows: 7.92±2.15 g% for adult group while for elderly: 7.63±1.94 g%.

For total white count, the mean values were as follows: mean value 59.53 ±65.86 ($X10^9$/L) for adult group while for elderly 32.0±34.30 ($X10^9$/L). For platelet, the mean values were as follows: mean value of 61.63±59.0 ($X10^9$/L) for the adult group while for elderly the mean value was 54.70±43.94 ($X10^9$/L). It was also noted that, 9.7% of adult AML patients had lymph nodes enlargement, 18.4% had liver enlargement and 12.6% had splenomegaly at time of diagnosis.

3.2.2.4.(a) Association between FBC and gender in adult AML

From the analysis, it was found that there was no difference in haemoglobin level, total white count level and platelet count at presentation between male and female adult AML patients. The results of the analysis are demonstrated in Table 4.6 (a) and 4.6 (b) below.

Table 4.6 (a) Association between TWC and platelet with gender in adult AML

Variable	Male (*n* =46) Mean (SD)	Female (*n* =46) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
TWC (10^9/l)	49.6 (59.25)	42.6 (49.35)	7.0 (-15.6,29.6)	0.62 (90)	0.539
Plt (10^9/l)	56.4 (58.9)	59.8 (44.28)	-34 (-25.0,18.1)	-0.32 (90)	0.752

[a] Independent t-test

Table 4.6 (b) Association between haemoglobin and gender in adult AML

Variable	Male (*n* =45) Mean (SD)	Female (*n* =46) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
Hb (g%)	7.6 (2.01)	7.9 (7.94)	-0.3 (-1.1,0.5)	0.75(89)	0.434

[a] Independent t-test

3.2.2.4.(b) Association between age and gender in adult AML

There was no significant difference in the age of disease presentation between male and female adult AML cases (p=0.877). The result of the analysis is demonstrated in the Table 4.7 below.

Table 4.7 Association between age and gender in adult AML

Variable	Male (n = 51) Mean (SD)	Female (n = 47) Mean (SD)	Mean Differ. (95% CI)	t statistic (df)[a]	p value[a]
Age	48.0 (14.57)	48.5 (15.59)	-0.5 (-6.5, 5.6)	-0.16 (96)	0.877

[a] Independent t-test

3.2.2.4. (c) Association between ethnicity and gender in adult AML

No significant association was found between ethnicity and gender in these adult AML cases. The results of the analysis are demonstrated in the Table 4.8 below.

Table 4.8 Association between ethnicity and gender in adult AML

Variable (Ethnic)	N	Male Freq (%)	Female Freq (%)	χ^2 statistic[a] (df)	p value[a]
Malay/Melanau	23	9 (17.6)	14 (29.8)	2.03 (3)	0.566
Sarawak Bumi	28	16 (31.4)	12 (25.5)		
Chinese	27	15 (29.4)	12(25.5)		
Others	12	7(26.9)	5(21.7)		

[a]Chi-Square test for independence

3.2.2.5 Immunophenotypes of adult AML

The markers which were expressed and weakly expressed in the gating of CD34/CD45 population were as follows: All 98 cases of adult AML demonstrated significant expression of CD33 (92.9%), CyMPO (86.7%), CD13 (85.7%), CD117 (79.6%), HLA DR (75.5%) and CD64 (52.0%) antigens (Table 4.9). Aberrant markers such as CD56 (61.2%) and CD7 (55.1%) were also expressed. High expression of CD13, CD33, HLADR and CD34 were associated with the commonly expressed antigens in almost all AML subtypes. These were common AML markers. CD117 and CD56 were also commonly expressed in M2 subtype (31.1%). CD7 is an ALL marker but may also to be expressed in M0 (1.0%) blast cells, which might explained its expressions in these cases.

Weak expressions of the following antigens were also found: CD10 (90.8%), CD14 (83.7%), CD11b (68.4%), CD19 (68.4%), CD61 (70.4%), and CyCD79a (64.3%) (Table 5.0). CD11b is commonly expressed in AML subtype M4/M5 (21.4%) and may also be expressed in M0/M1 (1.0%), and M2/M3 (31.1%) as well. Weak expression of CD14 and CD61 were also detected due to the fact that there were mainly expressed in AML subtype M4/M5 (21.4%) and M7 (1.0%) respectively. CD10, CD19 and Cy79a were aberrant antigens that were commonly expressed in ALL.

There were also few atypical antigens that showed both positive and negative expressions such as CD15, with significant expression found in 40.8% and weak expression in 46.9% of studied cases. The another antigen, CD 65 also showed the same atypical expression with positive expression in 17.3% while weak expression demonstrated in 25.5% of cases. The subtype for adult AML also showed the highest incidence in M2 subtype with 32 cases (32.7%). It was found

that the expression of CD15 could be associated with the common expression in subtype M2(31.1%), M3(1.9%), M4/M5 (21.4%) and may also be in M0 (1.0%) and M1 (11.7%) AML subtypes. CD64 and CD65 are commonly associated with monocytic and myeloid cells. Figure 1.4.6 and 1.4.7 display the flow cytometry dot plots for one of the AML positive adult patient.

Table 4.9 Expression of monoclonal antibody markers gated in adult AML CD34/CD45 population

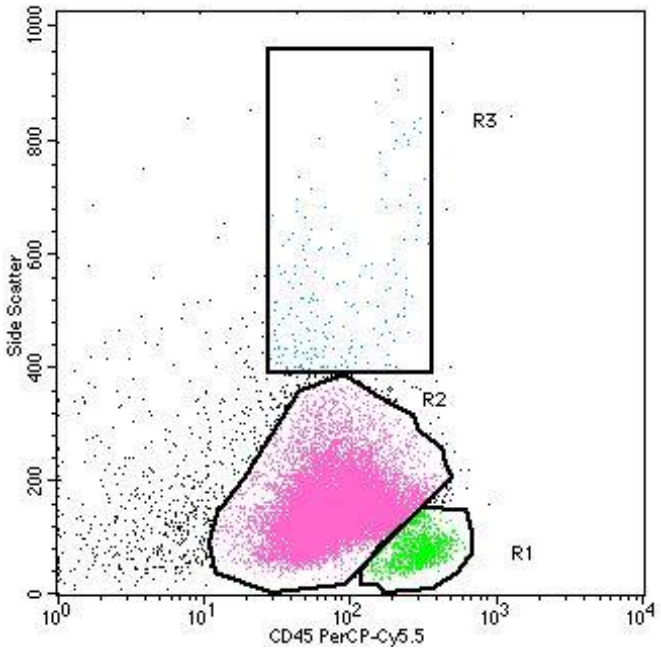
Antigen	Freq. (%)	Characterization
CD45	97 (99.0)	Blasts/Control
CD33	91(92.9)	AML
CyMPO	85(86.7)	AML
CD13	84 (85.7)	AML
CD117	78 (79.6)	AML
HLADR	74(75.5)	AML
CD34	65 (66.3)	Blasts/Control
CD56	60 (61.2)	AML
CD64	51(52.0)	AML
CD7	54 (55.1)	T-ALL

Freq. = Frequency

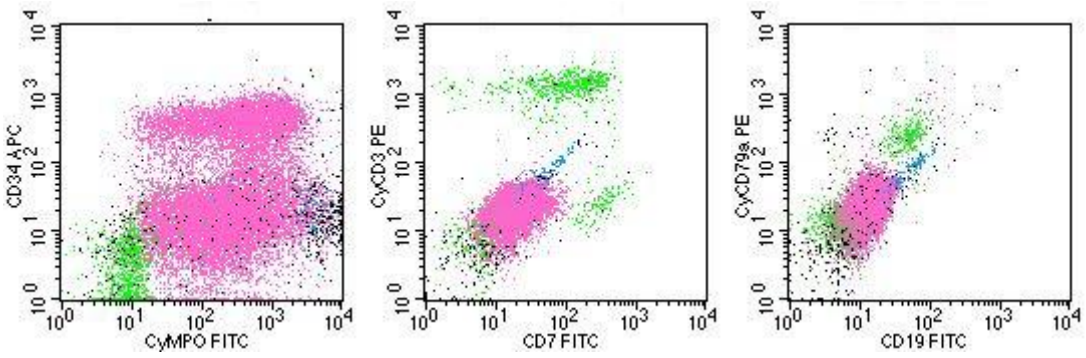
Table 5.0 Non expression of monoclonal antibody markers gated in adult AML CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CD10	89 (90.8)	Aberrant expression
CD14	82 (83.7)	AML
CD11b	67 (68.4)	AML
CD19	67 (68.4)	Aberrant expression
CD61	67 (70.4)	AML
Cy79a	63 (64.3)	Aberrant expression

Freq. = Frequency

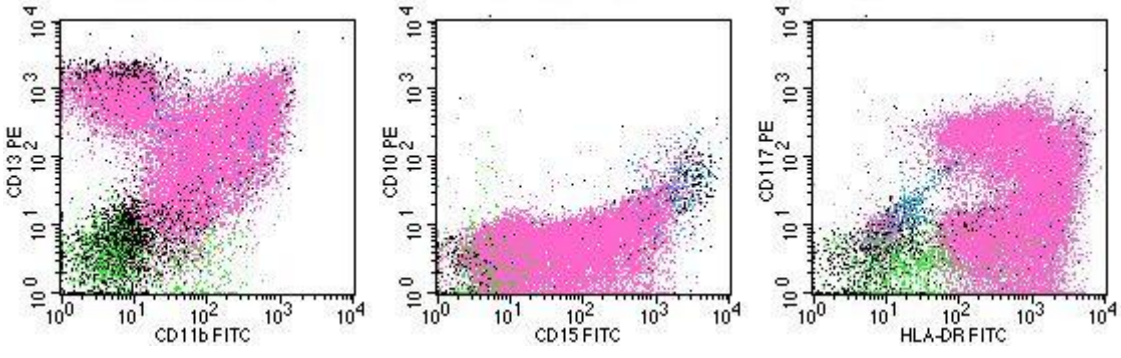


(a)

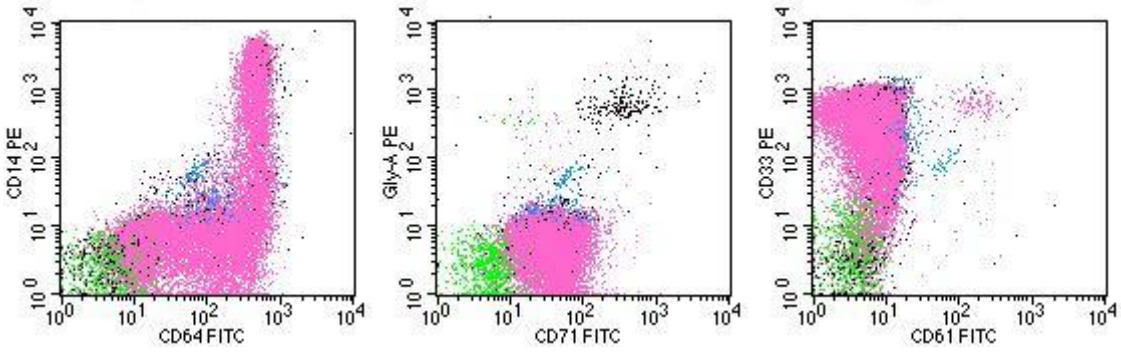


(b) **(c)** **(d)**

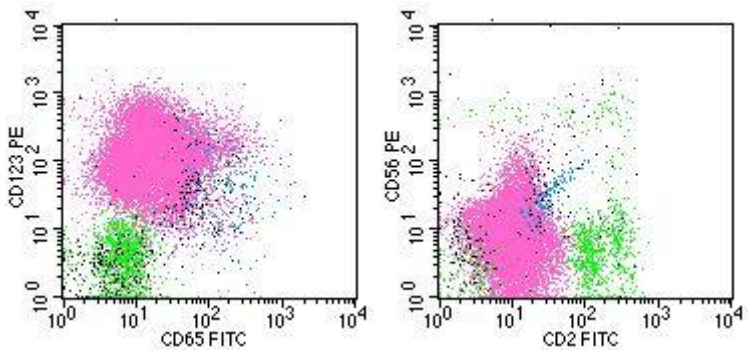
Figure 1.4.6 Plots (a) to (d) were the one of the dot plots for the acute leukaemia screening panel positive for adult AML subtype M5. In (a), the blast cells population was marked with the colour pink. In (b) and (c) the blast cell population was also detected positive for CyMPO, CD34 and CD7 antibodies. In (d) the blast cell population were negative for both CD19 and CyCD79a.



(e) **(f)** **(g)**



(h) **(i)** **(j)**



(k) **(l)**

Figure 1.4.7 The dot plots (e) to (l) were from the same patient positive for adult AML in the previous plots. These plots were taken from the AML immunophenotyping panel performed on the sample. The following blast populations (in pink) were positive for the following antigens: CD13, HLADR, CD117, CD33 and CD123.

3.3 Acute Lymphoid leukaemia

There were a total of 119 cases associated with ALL. From this number, 65.5% of cases were childhood ALL and 34.5% were adult ALL.

3.3.1 Childhood/Adolescent Acute Lymphoid Leukaemia

Ninety cases (73.8%) were diagnosed as childhood ALL. For ALL, the distribution of childhood leukaemia can be divided into B-ALL and T-ALL. A total of 81 cases (90.0%) were of childhood B-ALL, and only 9 cases were (10.0%) of T-ALL. The distribution of childhood B-ALL and T-ALL is demonstrated in Figure 1.4.8

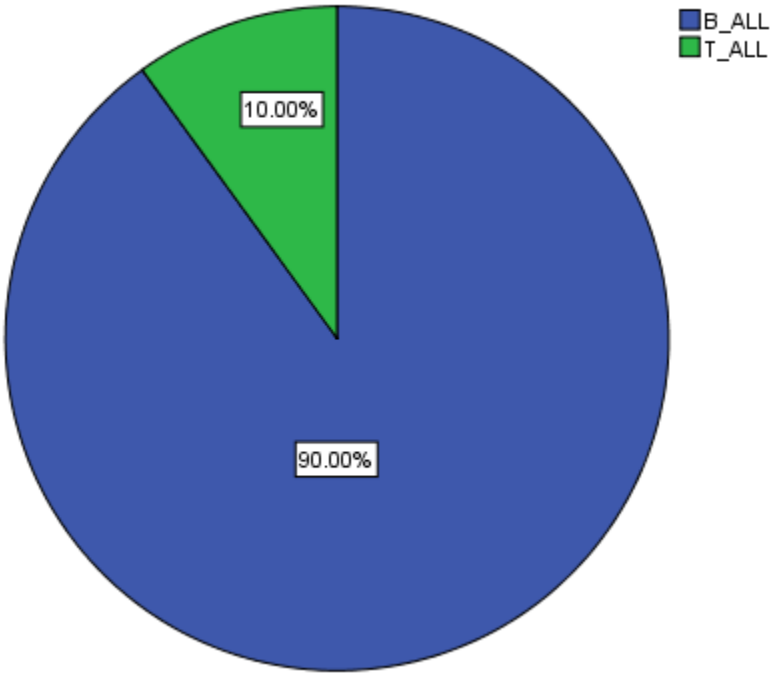


Figure 1.4.8 The distribution of B-ALL, and T-ALL in childhood ALL

3.3.1.1 Age distribution

The age distribution for the childhood ALL was divided into two categories, childhood (0-10 years) and adolescent (11-17 years). The mean age for each respective category are as follows: childhood: 3.7±2.21 years and adolescent: 12.2±1.98 years. The age distribution of childhood ALL is demonstrated in Figure 1.4.9

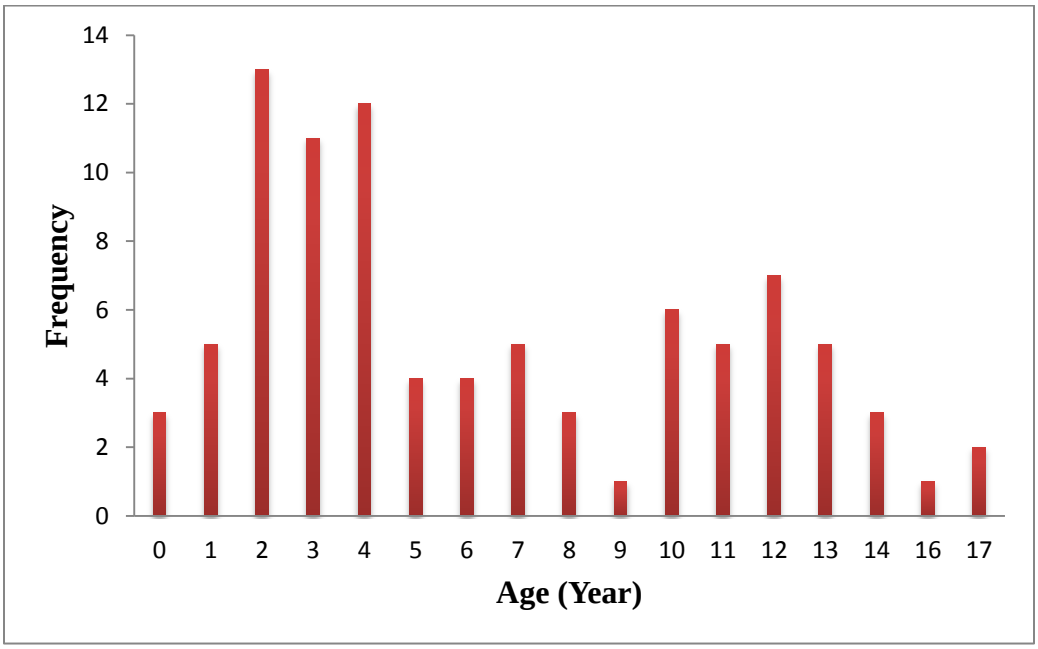


Figure 1.4.9 The age distribution of childhood ALL cases

3.3.1.2 Gender distribution

From a total of 90 cases, 49 cases were males (54.4%) and 41 cases (45.6%) were females. The sex distribution of the childhood ALL is demonstrated in the Figure 1.5.0

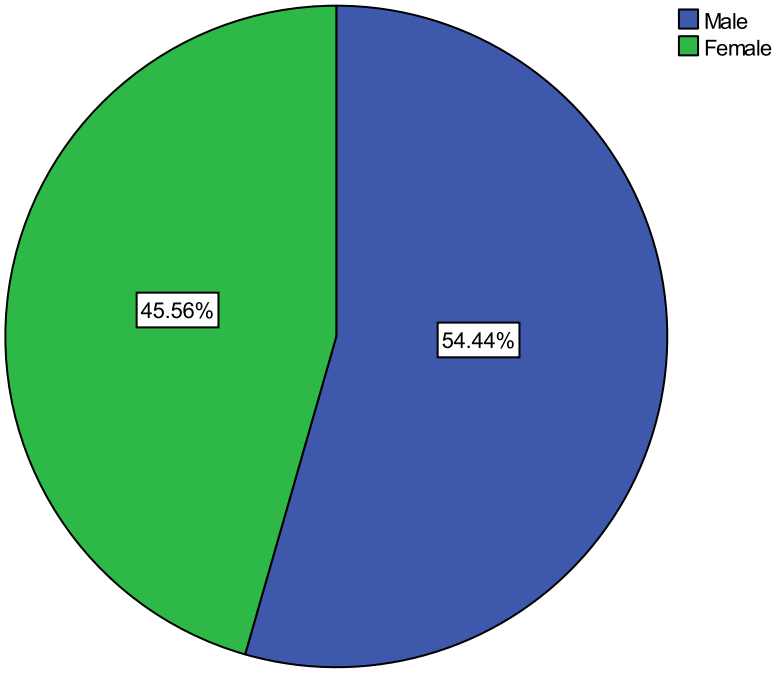


Figure 1.5.0 The sex distribution of childhood ALL cases.

3.3.1.3 Ethnic distribution

Childhood ALL cases in Sarawak was found highest among the Malay/ Melanau, with 29 cases (32.2%), followed by Sarawak Bumiputera with 28 cases (31.1%), Chinese with 27 cases (30.0%) and others with 4 cases (4.4%). The ethnicity distribution is demonstrated in Figure 1.5.1.

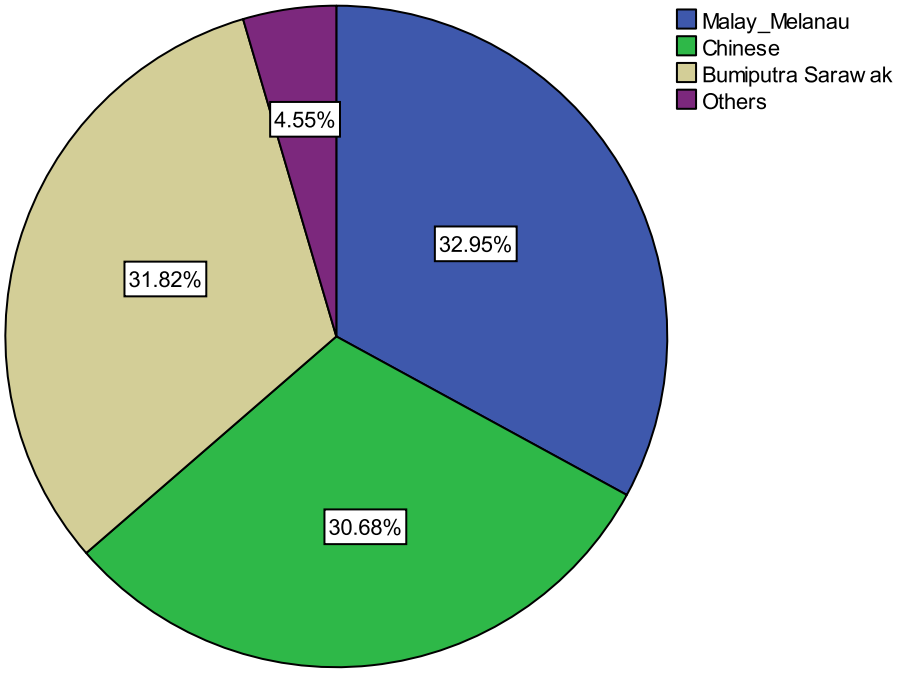


Figure 1.5.1 Ethnicity distribution of childhood ALL cases in Sarawak

3.3.1.4 Clinical characteristics of childhood and adolescent ALL

The clinical characteristics analysis of FBC (haemoglobin, total white count and platelet) childhood ALL is divided into childhood (0-9 years) and adolescent (10-17 years). For haemoglobin, the mean values were as follows: 6.76±2.56 g% for childhood while for adolescent: 7.97±2.89 g%.

For total white count, the mean values were as follows: mean value 43.0 ±61.96 ($X10^9$/L) for childhood while for adolescent 115.12±188.14 ($X10^9$/L). For platelet, the mean values were as follows: mean value of 77.6±129.47 ($X10^9$/L) for childhood while for adolescent the mean value was 64.25±50.31($X10^9$/L). It was also noted that, 45.1% of childhood ALL patients had lymph

nodes enlargement, 58.2% had liver enlargement and 48.4% had splenomegaly at time of diagnosis.

3.3.1.4. (a) Association between FBC and gender in childhood ALL

From the analysis, it was found that there was no difference in haemoglobin level, total white count level and platelet count at presentation between male and female childhood ALL patients. The results of the analysis are demonstrated in Table 5.1 (a), 5.1 (b) and 5.1 (c) below.

Table 5.1 (a) Association between haemoglobin and gender in childhood ALL

FBC	Male (*n* =48) Mean (SD)	Female (*n* =36) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
Hb (g%)	7.1 (2.92)	7.1 (2.44)	0.02 (-1.2, 1.2)	0.04(82)	0.966

[a] Independent t-test

Table 5.1 (b) Association between total white count and gender in childhood ALL

FBC	Male (*n* =49) Mean (SD)	Female (*n* =37) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
TWC (10^9/l)	73.9(151.0)	56.7 (69.1)	17.1 (-36.0,70.3)	0.6 (84)	0.523

[a] Independent t-test

Table 5.1 (c) Association between platelet and gender in childhood ALL

FBC	Male (*n* =48) Mean (SD)	Female (*n* =36) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
Plt (10^9/l)	73.9 (138.1)	72.9 (62.9)	1.0 (-47.6,49.7)	0.04 (83)	0.966

[a] Independent t-test

3.3.1.4. (b) Association between age, ethnicity and gender in childhood ALL

There was no significant association between the patients' age, ethnicity and gender. The results of the analyses are demonstrated in the Table 5.2 and 5.3 below.

Table 5.2 Association between age and gender in childhood ALL

Variable	Male (*n* = 49) Mean (SD)	Female (*n* = 40) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
Age	7.0 (4.69)	5.8 (4.39)	1.2 (-0.7, 3.1)	1.2 (87)	0.222

[a] Independent t-test

Table 5.3 Association between ethnicity and gender in childhood ALL

Variable (Ethnic)	*N*	Male Freq (%)	Female Freq (%)	χ^2 statistic[a] (*df*)	*p* value
Malay/Melanau	29	16 (34.0)	13 (32.5)	0.59(2)	0.743
Chinese	27	13 (27.7)	14 (35.0)		
Others	31	18 (38.3)	13(32.5)		

[a]Chi-Square test for independence

3.3.1.4. (c) Association between ethnicity and gender in childhood B-ALL and T-ALL

There was no significant association between the patients' age, ethnicity and gender for both ALL subtypes. The results B-ALL and T-ALL of the analysis are demonstrated in the Table 5.4 and 5.5 below respectively.

Table 5.4 Association between ethnicity and gender in childhood B-ALL

Variable (Ethnic)	***N***	**Male Freq (%)**	**Female Freq (%)**	χ^2 **statistic**[a] **(*df*)**	***p*** **value**
Malay/Melanau	25	13 (33.3)	12 (30.8)	0.54(2)	0.762
Chinese	25	11 (28.2)	14 (35.9)		
Others	28	15 (38.3)	13(33.3)		

[a]Chi-Square test for independence

Table 5.5 Association between ethnicity and gender in childhood T-ALL

Variable (Ethnic)	***N***	**Male Freq (%)**	**Female Freq (%)**	χ^2 **statistic**[a] **(*df*)**	***p*** **value**
Malay/Melanau	4	3 (37.5)	1 (100.0)	1.4(1)	0.444
Others	5	5 (62.5)	0 (0.0)		

[a]Chi-Square test for independence

3.3.1.5 Immunophenotypes of childhood ALL

Generally for ALL childhood cases, the following markers demonstrated significant expression in CD34/CD45 gated population: CyCD79a (93.3%), CD19 (90.0%), CD38 (84.4%), CD22 (84.4%), CD10 (84.4%), CD34 (63.3%), CD45 (60.0%) and nTdT (52.2%). CDCy79a and CD 22, CD19, nTdT and CD10 were the B-ALL markers. Weak expressions of the following antigens were also found in a number of studied cases: CyMPO (91.1%), CyIgM (84.4%), CD7 (81.1%), CD33 (64.4%), CyCD3 (65.6%), and CD20 (47.8%). (Table 5.6 and 5.7) Markers such as CyIgM, CD7, CyCD3 and CD20, however were known ALL markers. One antigen that showed atypical expression in ALL was CD13, its highly expressed in 40.0% of cases and its weak expression was demonstrated in 41.1% of cases studied. This marker is usually associated

with AML, thus indicative of aberrant expression in ALL. The aberrant antigen expression might be useful in further investigation of these cases.

Table 5.6 General expression of monoclonal antibody markers in childhood ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
Cy79a	84(93.3)	B-ALL
CD19	81(90.0)	B-ALL
CD38	76(84.4)	B-ALL
CD22	76(84.4)	B-ALL
CD10	76(84.4)	B-ALL
CD34	57(63.3)	Blasts/Control
CD45	54(60.0)	Blasts/Control
nTdT	47(52.2)	ALL

Freq. = Frequency

Table 5.7 Weak expression of monoclonal antibody markers in childhood ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CyMPO	82(91.1)	Aberrant markers
CyIgM	76(84.4)	B-ALL
CD7	73(81.1)	T-ALL
CD33	58(64.4)	Aberrant markers
CyCD3	59(65.6)	T-ALL
CD20	43(47.8)	B-ALL
CD45	36(40.0)	Blasts/Control

Freq. = Frequency

In 81 childhood cases of ALL subtype B-ALL, among the antigens that had displayed significant expression in CD34/CD45 population were CD19 (98.8%), CDCy79a (97.5%), CD22 (92.6%), CD38 (90.1%), CD10 (87.7%), CD45 (55.6%) and nTdT (50.6%). (Table 5.8) CDCy79a and CD 22 are the first line B-ALL markers while CD19 and nTdT are expressed in pro-B, common (cALL), Pre-B, and B-ALL cells. CD10 is expressed in common ALL (cALL), pre-B and B-ALL cells.

Antigens that were weakly expressed include CyMPO (91.4%), CyIgM (91.4%), CD7 (90.1%), CyCD3 (72.8%), CD33 (61.7%) and CD20 (50.6%). (Table 5.9). CyIgM is commonly expressed in pre-B and B-ALL cells and is the second line B-ALL marker. CD7 and CyCD3 are the T-ALL markers and commonly expressed in prothymocyte, immature thymocyte, common thymocyte, mature thymocyte and mature T cells. CD 20 may be expressed in pre-B and B-ALL cells. CD33 is an aberrant marker for ALL.

One antigen that displayed atypical expressions was CD13 with significant expression at 40.7% and weak expression at 39.5%. CD13 is associated with monocytes, neutrophils, eosinophils and basophils and sometimes can be expressed in B-ALL. Figure 1.5.2 demonstrates the flow cytometry dot plots for one of the B-ALL positive patient.

Table 5.8 Expression of monoclonal antibody markers in childhood B-ALL gated in CD34/CD45 population

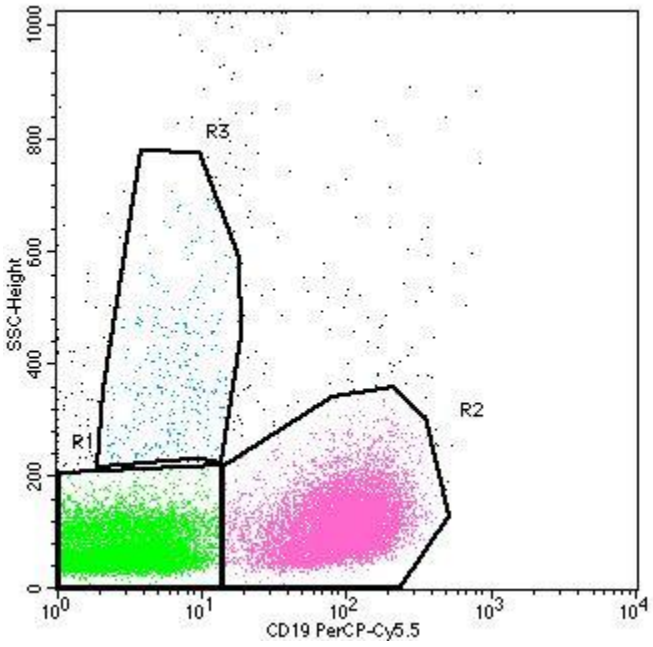
Antigen	Freq. (%)	Characterisation
CD19	80(98.8)	B-ALL
Cy79a	79(97.5)	B-ALL
CD22	75(92.6)	B-ALL
CD38	73(90.1)	B-ALL
CD10	71(87.7)	B-ALL
CD45	45(55.6)	Blast/Control
nTdT	41(50.6)	ALL

Freq. = Frequency

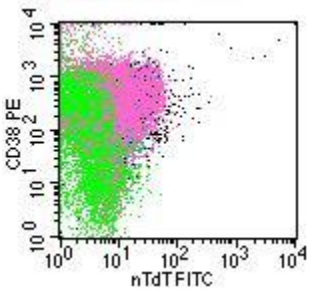
Table 5.9 Weak expression of monoclonal antibody markers in childhood B-ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CyMPO	74(91.4)	Aberrant expression
CyIgM	74(91.4)	B-ALL
CD7	73(90.1)	T-ALL
CyCD3	59(72.8)	T-ALL
CD33	50(61.7)	Aberrant expression
CD20	41(50.6)	B-ALL
CD45	36(44.4)	Blast/Control

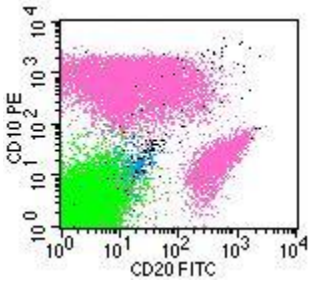
Freq. = Frequency


SSC-Height
CD19 PerCP-Cy5.5
R1
R2
R3

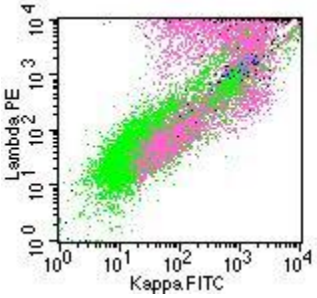
(a)


CD38 PE
nTdT FITC

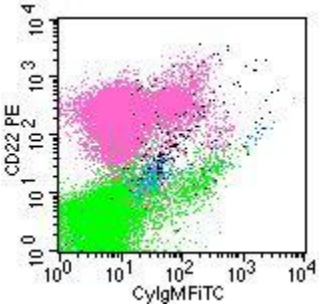
(b)


CD10 PE
CD20 FITC

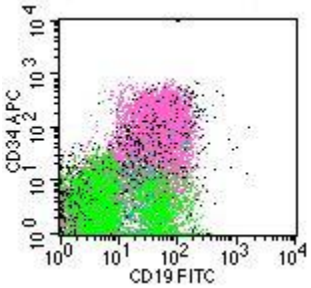
(c)


Lambda PE
Kappa FITC

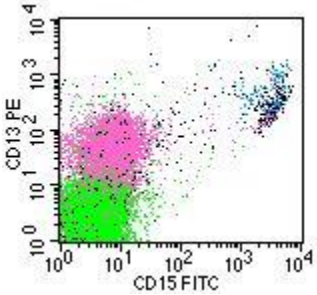
(d)


CD22 PE
CyIgMFITC

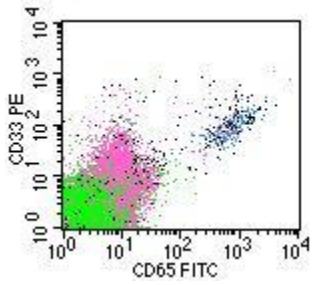
(e)


CD34 APC
CD19 FITC

(f)


CD13 PE
CD15 FITC

(g)



(h)

Figure 1.5.2 The dot plots (a)-(h) from a patient positive for childhood B-ALL. These plots were taken from the B-ALL immunophenotyping panel performed on the sample. The blast populations (in pink) were positive for the following antigens: CD19, CD10, CD22, CD38, CD34, CD13, and nTdT.

There were 9 cases of childhood ALL subtype T-ALL studied for the immunophenotyping. Among the antigens that had displayed significant expression in CD34/CD45 population were CD45 (100.0%), CD5 (100.0%), CyCD3 (88.9%), CD2 (77.8%), and CD34 (66.7%) (Table 6.0) CD34 is usually expressed in null/pro-B and common (cALL). CD5 is commonly expressed in immature thymocyte, common thymocyte, mature thymocyte and mature T cells. CyCD3 and CD2 are expressed in prothymocyte, immature thymocyte, common thymocyte, mature thymocyte and mature T cells. Antigens that were weakly expressed included CyMPO (88.9%), CD33 (88.9%), CD4 (66.7%), HLA DR (66.7%). CD8, CD10 and CyCD79 share a significant expression of 55.6% (Table 6.1). CD4/CD8 are commonly expressed in common thymocyte and either one of them is expressed in mature thymocyte and T-cell. CyMPO, CD33, HLA DR, CD10 and CyCD79a are aberrant markers for T-ALL. One antigen that displayed atypical expressions was CD56 with significant expression in (55.6%) of cases. This antigen is commonly associated with NK cells, myeloma cells and B cells. Figure 1.5.3 demonstrates the flow cytometry dot plots for one of the T-ALL positive patient.

Table 6.0 Expression of monoclonal antibody markers in childhood T-ALL gated in CD34/CD45 population

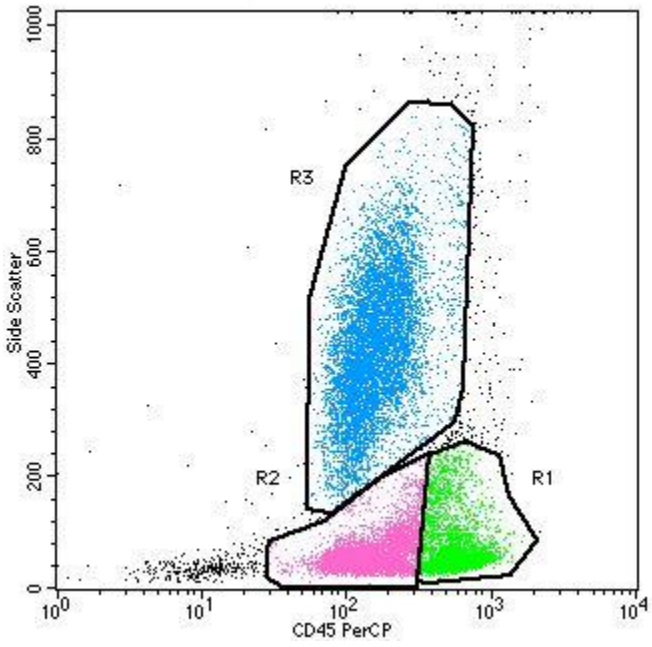
Antigen	Freq. (%)	Characterisation
CD45	9(100.0)	Blasts/Control
CD5	9(100.0)	B-ALL
CyCD3	8(88.9)	T-ALL
CD2	7(77.8)	T-ALL
CD34	6(66.7)	Blasts/Control

Freq. = Frequency

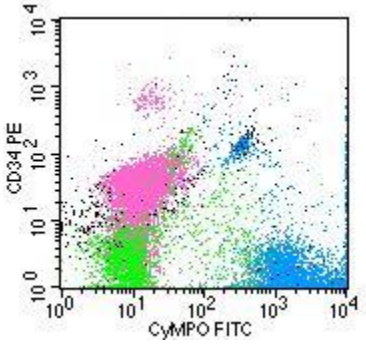
Table 6.1 Weak expression of monoclonal antibody markers in childhood T-ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CyMPO	8(88.9)	Aberrant expression
CD33	8(88.9)	Aberrant expression
CD4	6(66.7)	T-ALL
HLADR	6(66.7)	Aberrant expression
CD8	5(55.6)	T-ALL
CD10	5(55.6)	B-ALL
CyCD79a	5(55.6)	B-ALL

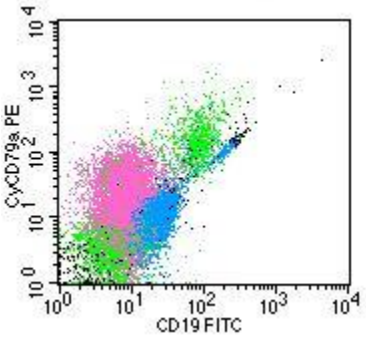
Freq. = Frequency


Side Scatter
CD45 PerCP
R1
R2
R3

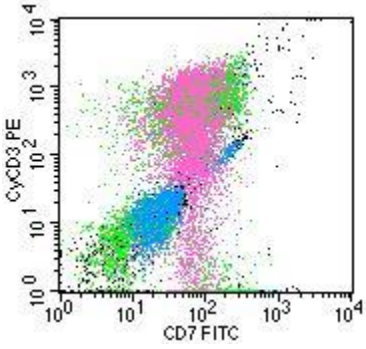
(a)


CD34 PE
CyMPO FITC

(b)


CyCD79a PE
CD19 FITC

(c)


CyCD3 PE
CD7 FITC

(d)

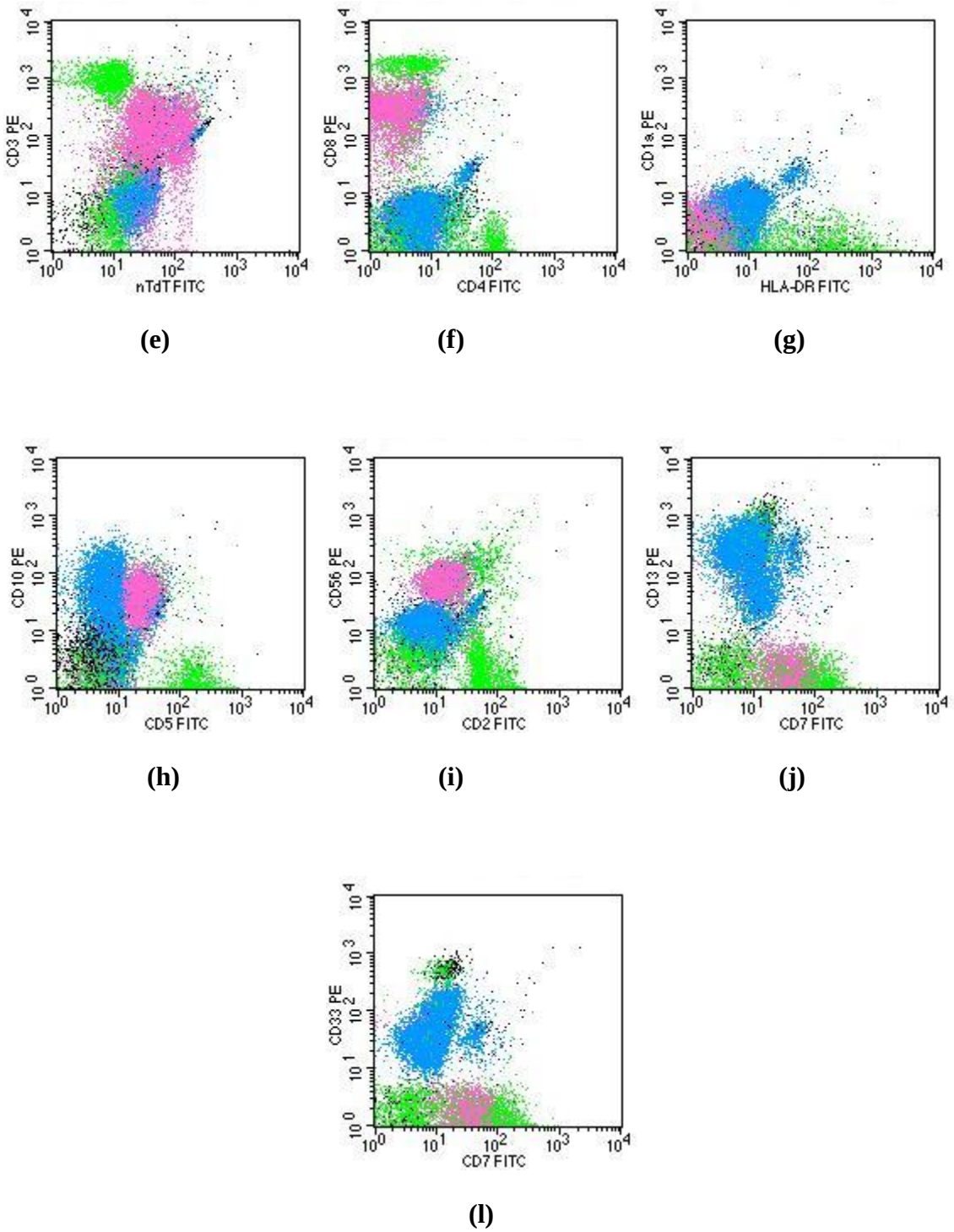


Figure 1.5.3 The dot plots (a)-(l) from a patient positive for childhood T-ALL. These plots were taken from the T-ALL immunophenotyping panel performed on the sample. The blast populations (in pink) were positive for the following antigens: CD34, CD45, CD56, CD10, CyCD79a, CD8, nTdT, CyCD3, CD3, CD7 and CD5.

3.3.2 Adult Acute Lymphoid Leukaemia

The cases of adult ALL were also categorized into B-ALL and T-ALL. For adult ALL, 23 cases (79.3%) were of B-ALL and 6 cases (20.7%) of T-ALL were diagnosed. The distribution of adult B-ALL, T-ALL is demonstrated in Figure 1.5.4.

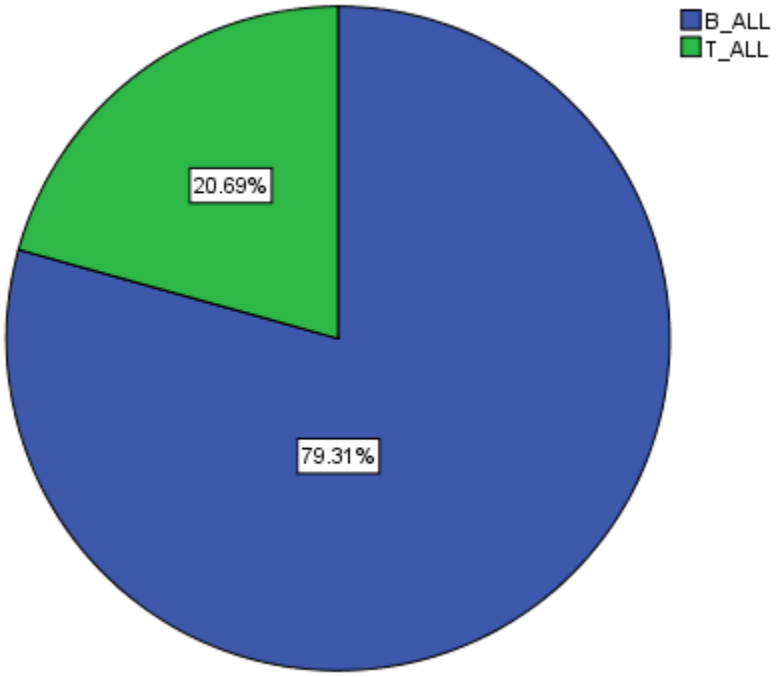


Figure 1.5.4 The distribution of B-ALL, T-ALL in adult ALL

3.3.2.1 Age distribution

The age distribution for the adult ALL was divided into 2 categories, adult (18-49 years) and elderly (>50 years). The mean age for each respective category are as follows: Adult: 30.2±7.92 years and elderly: 58.9±7.49 years. The distribution of adult ALL cases is demonstrated in Figure 1.5.5 while the age distribution in childhood and adult ALL is shown in Table 6.1.

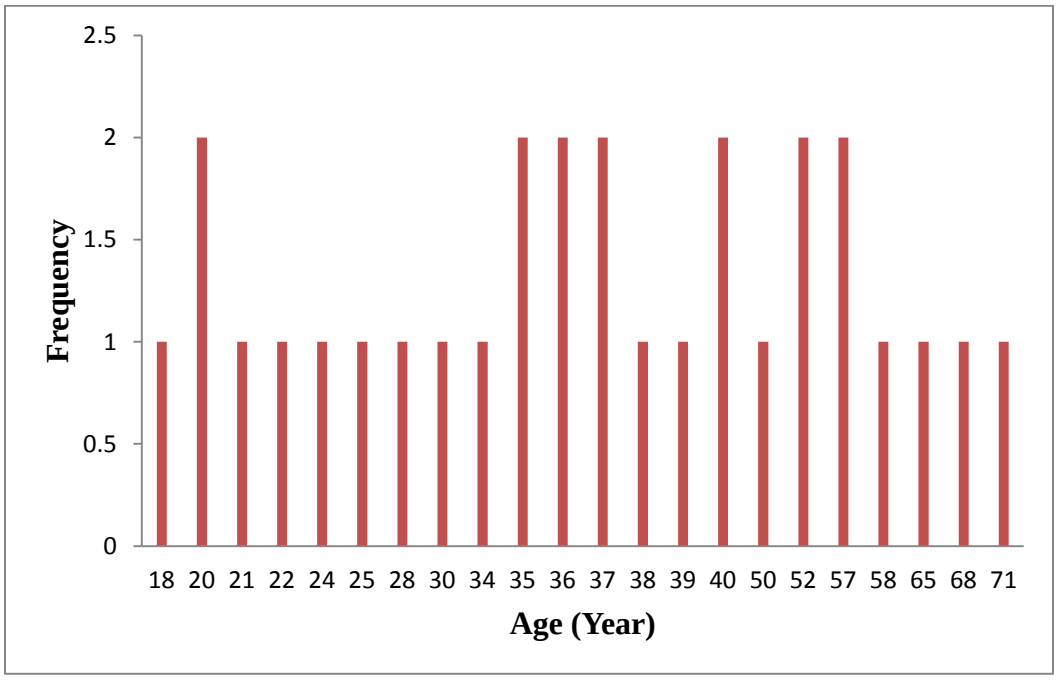


Figure 1.5.5 The age distribution of adult ALL cases

Table 6.2 The age distribution of childhood and adult ALL

Category	Mean age (years)
Childhood (0-10 years)	3.7
Adolescent (11-17 years	12.2
Adult (18-49 years)	30.2
Elderly (>50 years).	58.9

3.3.2.2 Gender distribution

For adult ALL, from a total of 29 cases, 18 cases were males (62.1%) and 11 cases (37.9%) were females. In B-ALL, the gender distribution were equal for both sexes (48.0% respectively), while for T-ALL all cases were males (100.0%). The sex distribution of the adult ALL is demonstrated in the Figure 1.5.6.

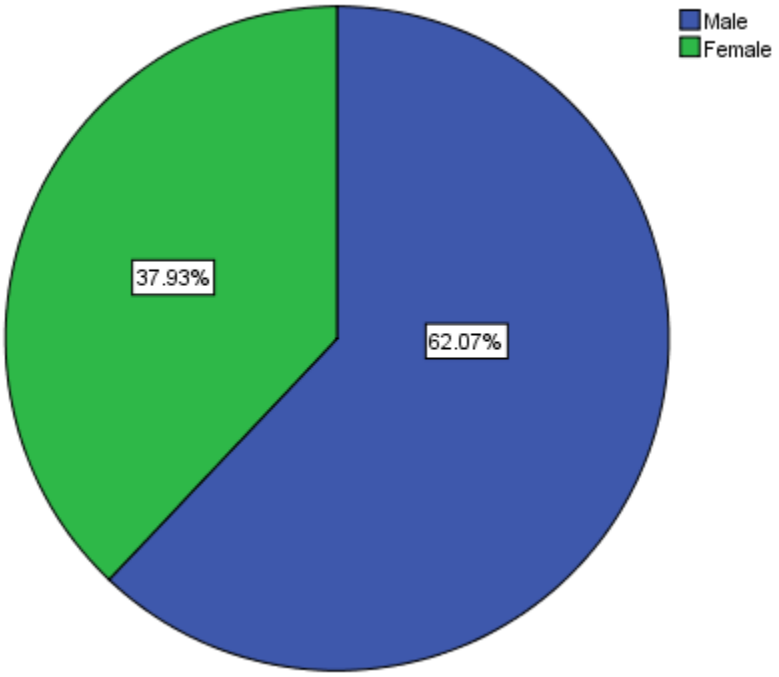


Figure 1.5.6 The sex distribution of adult ALL cases.

3.3.2.3 Ethnic distribution

It was observed that during the study period, adult ALL cases in Sarawak has the highest incidence among the Malay/Melanau group, with 13 cases (44.8%), followed by Chinese with 7

cases (24.1%), others with 5 cases (17.2%) and Sarawak Bumiputera with 3 cases (10.3%). The ethnicity distribution is demonstrated in Figure 1.5.7.

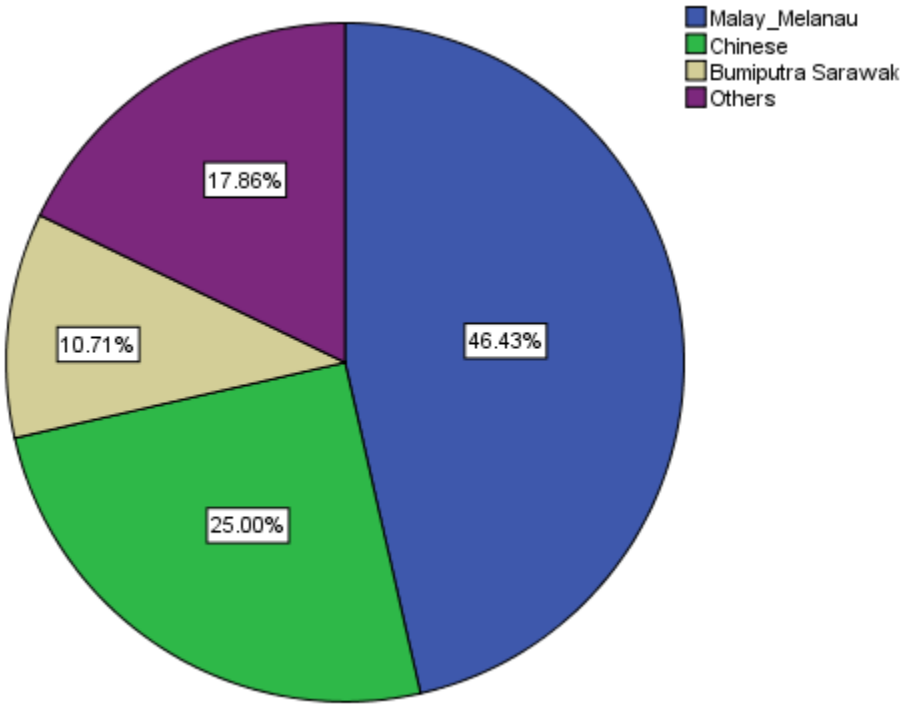


Figure 1.5.7 Ethnicity distribution of adult ALL cases in Sarawak

3.3.2.4 Clinical characteristics of adult ALL distribution

The clinical characteristics analysis of FBC (haemoglobin, total white count and platelet) adult ALL is divided into adult (18-49 years) and elderly (>50 years). For haemoglobin, the mean values were as follows: 7.54±3.43 g% for adult while for elderly: 10.11±1.86 g%.

For total white count, the mean values were as follows: mean value 210.74 ±469.89 ($X10^9$/L) for adult while for elderly 89.7±97.33 ($X10^9$/L). For platelet, the mean values were as follows: mean value of 76.56±113.15 ($X10^9$/L) for adult while for elderly the mean value was 102.0±78.45($X10^9$/L). It was also noted that, 32.3% of adult ALL patients had lymph nodes enlargement, 41.9% had liver enlargement and 45.2% had splenomegaly at time of diagnosis.

3.3.2.4. (a) Association between FBC and gender in adult ALL

Statistical test was done to find mean comparison and the significance difference value between the FBC (haemoglobin, total white count and platelet) values and gender. From the analysis, no significant association was found between Hb level, total white and platelet count and gender in these cases. The results of the analysis are demonstrated in the Table 6.3 (a) and (b) below.

Table 6.3 (a) Association between haemoglobin and platelet with gender in adult ALL

FBC	Male (*n* =16) Mean (SD)	Female (*n* =10) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
Hb (g%)	8.2 (3.30)	8.5 (3.27)	-0.3 (-3.0, 2.4)	-0.2(24)	0.822
Plt (10^9/l)	97.4 (123.96)	63.5 (54.53)	33.9 (-52.2,120.1)	0.8 (24)	0.966

[a] Independent t-test

Table 6.3 (b) Association between total white count relations and gender in adult ALL

FBC	Male (*n* =16) Mean (SD)	Female (*n* =10) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
TWC (10^9/l)	245.5 (475.9)	42.6 (61.47)	202.9 (-111.1,516.8)	1.3 (25)	0.195

[a] Independent t-test

3.3.2.4. (b) Association between between age and ethnicity with gender in adult ALL

There was no significant association between age and ethnicity when compared to gender. The results of the analysis are demonstrated in the Table 6.4 and 6.5 below.

Table 6.4 Association between age and gender in adult ALL

Variable	Male (*n* = 18) Mean (SD)	Female (*n* = 12) Mean (SD)	Mean Differ. (95% CI)	*t* statistic (*df*)[a]	*p* value[a]
Age (years)	38.6 (15.7)	39.1 (15.6)	-0.4 (-12.5, 11.5)	-0.1 (28)	0.936

[a] Independent t-test

Table 6.5 Association between ethnicity and gender in adult ALL

Variable (Ethnic)	*N*	Male Freq (%)	Female Freq (%)	χ^2 statistic[a] (*df*)	*p* value
Malay/Melanau	13	11 (61.1)	2 (18.2)	5.1(1)	0.052
Others	16	7 (38.9)	9 (81.8)		

[a]Chi-Square test for independence

3.3.2.4. (c) Association between ethnicity and gender in B-ALL and T-ALL

There were more B-ALL cases found in Malay/Melanau ethnic group compared to other ethnic groups (p =0.043). For T-ALL, the result could not be generated due to insufficient data. The result of ethnicity and gender in B-ALL analysis is demonstrated in the Table 6.6 below.

Table 6.6 Association between ethnicity and gender in adult B- ALL

Variable (Ethnic)	*N*	Male Freq (%)	Female Freq (%)	χ^2 statistic[a] (*df*)	*p* value
Malay/Melanau	10	8 (66.7)	2 (20.0)	4.8(1)	0.043*
Others	12	4 (33.3)	8 (80.0)		

[a]Chi-Square test for independence

3.3.2.5 Immunophenotypes of adult ALL

Generally for adult ALL cases, the cases demonstrated significant expression of these antigens: CyCD79a (82.8%), CD19 (79.3%), CD45 (72.4%), CD34 (58.6%), CD22 (58.6%), CD38 (51.7%), nTdT (58.6%) and CD10 (48.3%) (Table 6.7). CyCD79a, CD19, CD22, CD10 are the first line of B-ALL markers. CD38 expression is a marker of initial lineage commitment. nTdT and CD34 are markers for precursor cells.

Weak expressions of these antigens were also found in a number cases studied: CyMPO (86.2%), CyIgM (65.5%), CyCD3 (62.1%), CD7 (58.6%) and CD33 (48.3%) (Table 6.8) CyMPO and CD33 are the aberrant ALL markers, commonly expressed in AML. CyIgM is the second line of B-ALL marker, while CyCD3 is the first line of T-ALL marker. CD7 is commonly used as second line of T-cell antigens marker.

One antigen that showed mixed expression was CD13, with both significant and weak expression at 44.8%. This antigen is usually expressed in AML.

Table 6.7 Expression of monoclonal antibody markers in adult ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CyCD79a	24(82.8)	B-ALL
CD19	23(79.3)	B-ALL
CD45	21(72.4)	Blast/Control
CD34	17(58.6)	Blast/Control
CD22	17(58.6)	B-ALL
CD38	15(51.7)	B-ALL
nTdT	17(58.6)	ALL
CD10	14(48.3)	B-ALL

Freq. = Frequency

Table 6.8 Weak expression of monoclonal antibody markers in adult ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CyMPO	25(86.2)	Aberrant expression
CyIgM	19(65.5)	B-ALL
CyCD3	18(62.1)	T-ALL
CD7	17(58.6)	T-ALL
CD33	14(48.3)	Aberrant expression
CD45	8(27.6)	Blasts/Control

Freq. = Frequency

In 21 adult cases of ALL subtype B-ALL, among the antigens that had displayed significant expression were CD19 (100.0%), CDCy79a (100.0%), CD22 (71.4%), CD38 (66.7%), CD45 (66.7%), nTdT (66.7%), CD34 (61.9%), CD10 (57.1%), and CD13 (52.4%). (Table 6.9) CD19 commonly expressed in pro-B, common (cALL), pre-B and B-ALL cells. CDCy79a is highly

sensitive and specific for B-lineage cells, present from the earliest stages B cell malignancy. CD22 is common B cell marker, expressed in null/pro-b/pre-pre-B, cALL, pre-B, B-ALL. nTdT is the first line of non lineage restricted marker in acute leukaemia, expressed in null/pro-b/pre-pre-B, cALL, pre-T, T-ALL and may also be expressed in pre-B and B-ALL. CD38 is a plasma cell marker. CD34 is a common uncommitted haemopoietic progenitors, maybe expressed in both progenitor cells of ALL and AML. CD10 is commonly positive in ALL cells but negative in pre-pre/precursor B-ALL. CD13 is an aberrant markers for ALL since it is only expressed in AML. Antigens that were weakly expressed include CyIgM (85.7%) and CD33 (52.4%). (Table 7.0) CyIgM is expressed in B-ALL lineage while CD33 is an aberrant marker for ALL.

One antigen that displayed both expressions was CD33 with significant expression at 42.9% and weak expression at 52.4%. This antigen is an aberrant antigen for ALL. Figure 1.5.8 demonstrates the flow cytometry dot plots for one of the B-ALL positive patient.

Table 6.9 Expression of monoclonal antibody markers in adult B-ALL gated in CD34/CD45 population

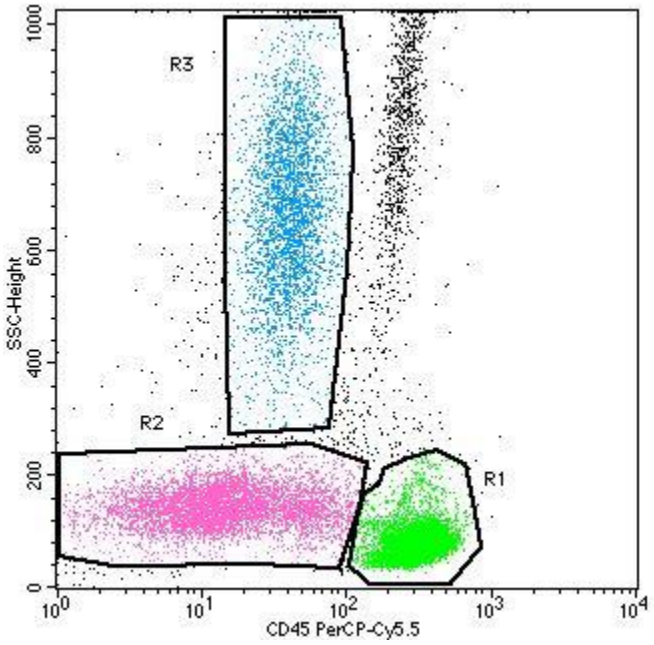
Antigen	Freq. (%)	Characterisation
CD19	21(100.0)	B-ALL
CyCD79a	21(100.0)	B-ALL
CD22	15(71.4)	B-ALL
CD38	14(66.7)	B-ALL
CD45	14(66.7)	Blast/Control
nTdT	14(66.7)	ALL
CD34	13(61.9)	Blast/Control
CD10	12(57.1)	B-ALL
CD13	11(52.4)	Aberrant expression

Freq. = Frequency

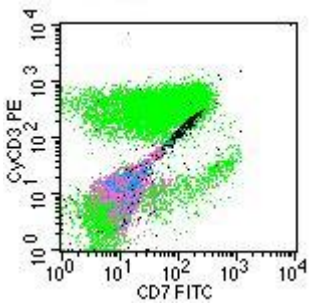
Table 7.0 Weak expression of monoclonal antibody markers in adult B-ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CyIgM	18(85.7)	B-ALL
CD33	11(52.4)	Aberrant expression
CD45	7(33.3)	Blast/Control

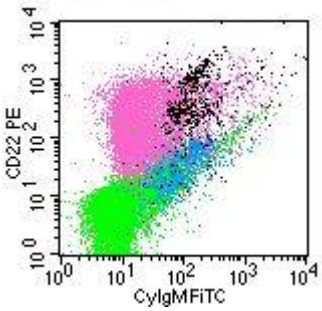
Freq. = Frequency



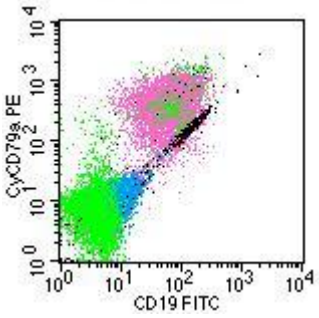
(a)



(b)



(c)



(d)

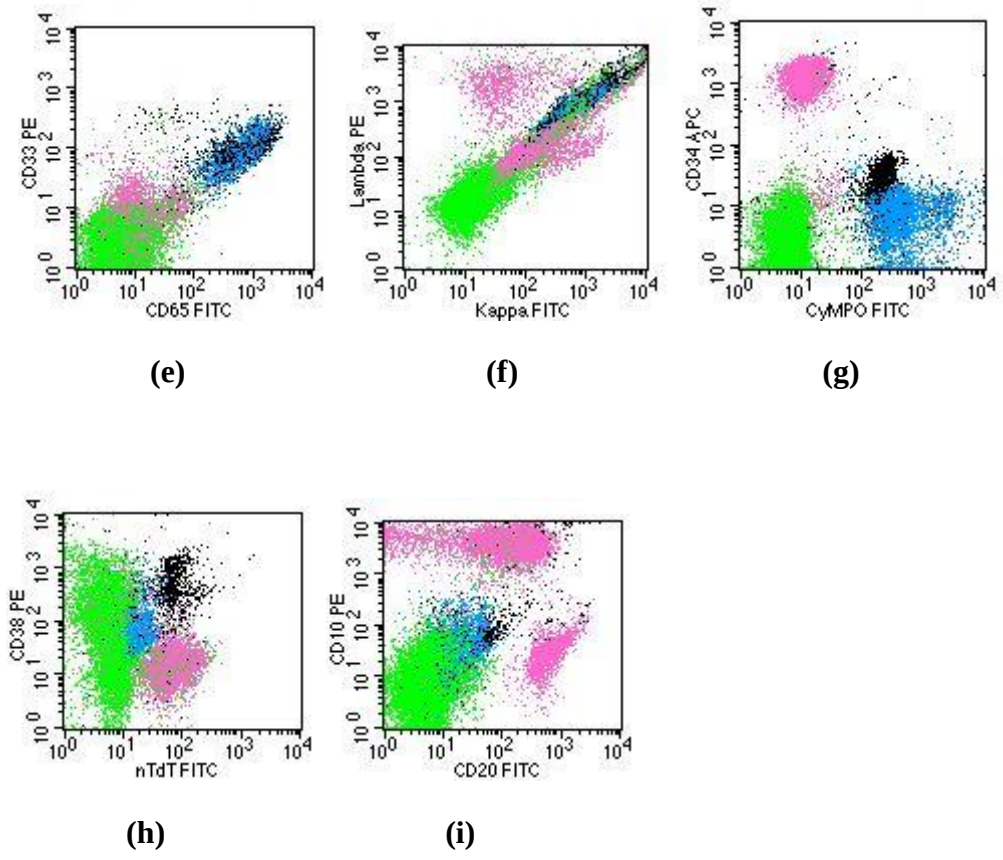


Figure 1.5.8 These were the dot plots of the patient positive for adult B-ALL. These plots were taken from the acute leukaemia screening and B-ALL immunophenotyping panel performed on the sample. The blast populations (in pink) were positive for the following antigens: CD34, CD20, CD19, CD10, CyCD79a, CD38, CyIgM, nTdT and CD22.

There were 5 cases of adult ALL subtype T-ALL studied. Among the antigens that had displayed significant expression were CD7 (100.0%), CyCD3 (100.0%), CD5 (80.0%) CD45 (80.0%), CD56 (80.0%), nTdT (60.0%) and CD2 (60.0%) (Table 7.1.). Antigens CD7, CD 2 and CyCD3 are commonly expressed in prothymocyte, immature thymocyte, common thymocyte, mature thymocyte and mature T cells. CD5 commonly expressed in immature thymocyte, common

thymocyte, mature thymocyte and mature T cells. nTdT expressed in prothymocyte, immature thymocyte, common thymocyte. CD56 is usually expressed in NK cells, myeloma and some B cells.

Antigens that were weakly expressed include CD34 (80.0%), CyMPO (100.0%), CD33 (80.0%), CD8 (80.0%), CD4 (80.0%), HLA DR (80.0%), CD56 (80.0%) and CD1a (60.0%) (Table 7.2). CD34 is commonly positive in haemopoietic progenitors. CD4/CD8 commonly expressed in common thymocyte and either one is expressed in mature thymocyte and mature T cells. HLA-DR expressed in null/pro-b/pre-pre-B, cALL, pre-B, B-ALL and may be expressed in pre-T and T-ALL. CD56 is usually expressed in NK cells, myeloma and some B cells. CD1a is associated with Langerhans cells and CyMPO and CD33 are aberrant markers for T-ALL. The aberrant expression of these antigens might proved useful in further immunophenotypic analysis of these cases. Figure 1.5.9 demonstrates the flow cytometry dot plots for one of the T-ALL positive patient.

Table 7.1 Expression of monoclonal antibody markers in adult T-ALL gated in CD34/CD45 population

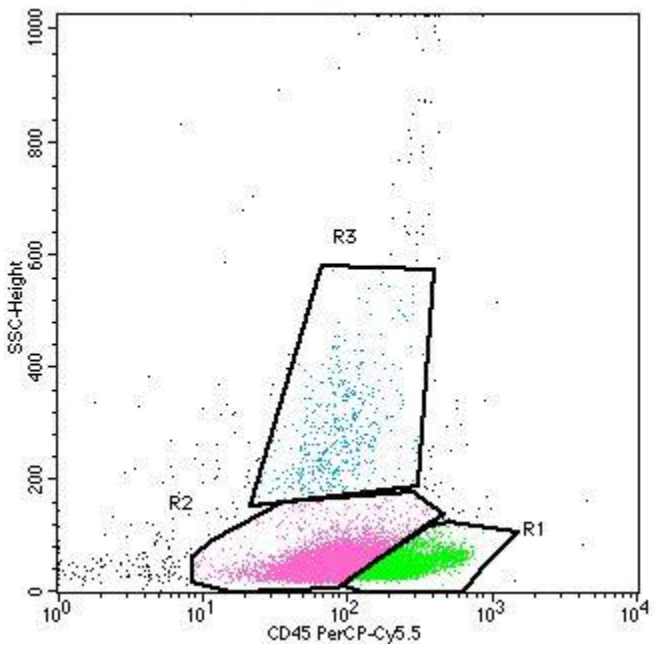
Antigen	Freq. (%)	Characterisation
CD7	5(100.0)	T-ALL
CyCD3	5(100.0)	T-ALL
CD5	4(80.0)	T-ALL
CD45	4(80.0)	Blast/Control
CD56	4(80.0)	Aberrant expression
nTdT	3(60.0)	ALL
CD2	3(60.0)	T-ALL

Freq. = Frequency

Table 7.2 Weak expression of monoclonal antibody markers in adult T-ALL gated in CD34/CD45 population

Antigen	Freq. (%)	Characterisation
CD34	4(80.0)	Blast/Control
CyMPO	5(100.0)	Aberrant expression
CD33	4(80.0)	Aberrant expression
CD8	4(80.0)	T-ALL
CD4	3(60.0)	T-ALL
HLADR	4(80.0)	Aberrant expression
CD56	4(80.0)	Aberrant expression
CD1a	3(60.0)	T-ALL
CD45	1(20.0)	Blast/Control

Freq. = Frequency



(a)

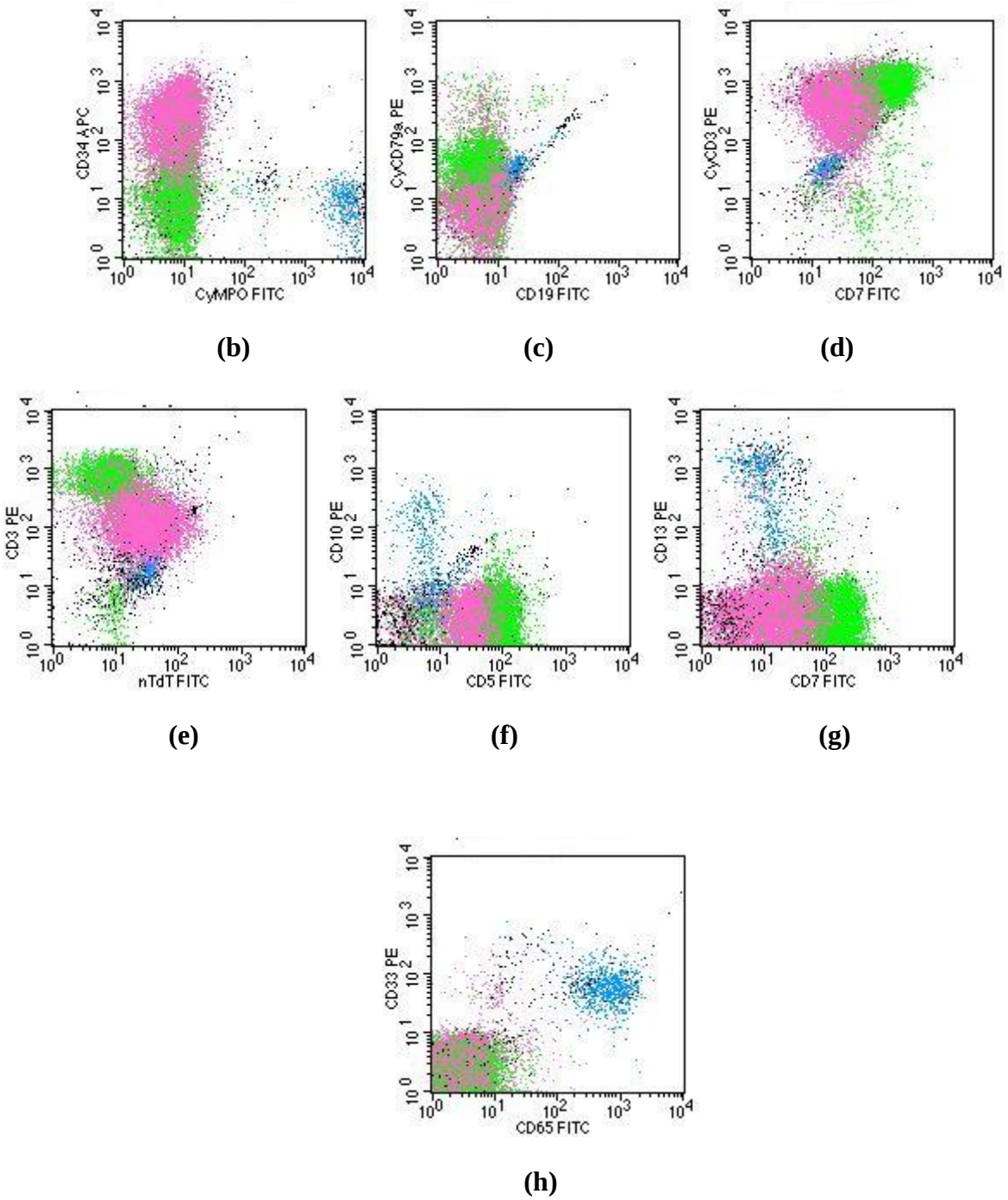


Figure 1.5.9 The plots (a) to (h) were taken from a patient positive for adult T-ALL. The plots were taken from the acute leukaemia screening and T-ALL immunophenotyping panel performed on the sample. The blast populations (in pink) were positive for the following antigens: CD45, CD34, nTdT, CD5, CyCD3 and CD7.

3.4 Minimal residual disease analysis

Increases in improvements in therapy derived from clinical trials have resulted in improvements in the clinical outcome for acute leukaemia patients. However, there are patients who still remain with poor outcome for the disease. In the last decade, the detection of minimal residual disease (MRD) by flow cytometry or molecular techniques has come to be recognized as one of the most important measures of clinical response to therapy and has become a routine in experimental clinical treatment protocols. In comparison with molecular techniques, the flow cytometric detection of MRD has the advantage of general applicability, speed and lower cost, and hence has been the preferred method used for MRD detection. The potential applications of MRD studies in the clinical management of acute leukemia include early identification of patients at a higher risk of relapse and detection of impending clinical relapse (Campana D, 1999). In addition, MRD studies provide a powerful tool for assessing bone marrow or peripheral blood that has been harvested for autologous hematopoietic stem cell transplantation, and for determining the efficacy of ‘‘purging’’ procedures (Rambaldi A et al., 1998). Also, they may serve to demonstrate leukemic involvement of the central nervous system (Bradstock KF et al., 1980, Hooijkaas H et al., 1989). Monitoring of MRD in patients could provide useful information on the biology of acute leukemia and its responsiveness to treatment. MRD measurements could be used as endpoints to rapidly compare the effectiveness of different chemotherapeutic regimens.

3.4.1 Aims

The aims of this section are to find the association between the variables and the odd ratios of both clinical presentation and immunophenotyphic expressions with the minimal residual disease cases.

3.4.2 Minimal residual disease in Acute Myeloid Leukaemia

From 147 AML cases involving both adult and children, only 28 cases (19.0%) were further investigated for minimal residual disease (MRD) study after completion of induction therapy or designated as MRD in this study. From these MRD cases, 13 cases (8.8%) were classified as in remission and 15 cases (10.2%) did not achieve remission. The small number of cases being investigated for MRD-1 were contributed by a number of reasons, among them patient refusal for further treatment, patient unable to be present for appointment due to geographical difficulty, like living in the rural areas and death.

From the total of MRD cases, 17 cases (60.7%) were childhood AML and 11 cases (39.3%) were adult AML. The comparison of clinical presentation and immuphenotyphic expressions variables with MRD were analysed using T-independent, Mann-Whitney and Chi-square Fisher's exact tests. The odds ratio was analysed using multiple logistic regression (MLR) analysis. All hypothesis tests were based on two-sided tests and *p* value less than 0.05 will be considered as statistically significant.

3.4.2.1 AML clinical presentation association with MRD

The association between clinical presentation for age, blast flow, blast morphology, full blood count (Table 7.3 a-f) and gender, ethnicity, lymph node (LN), spleen and liver enlargement (Table 7.4) with MRD were shown below:

Table 7.3 (a) Association between age and MRD cases

Variable	Remission (*n* =13) Median (IQR)	No remission (*n* = 15) Median (IQR)	Z statistic[a]	*p* value[a]
Age (year)	11.00(33)	12.00(44)	-0.485	0.628

[a]Mann-Whitney test

Table 7.3 (b) Association between blast (flow) and MRD cases

Variable	Remission (*n* =13) Median (IQR)	No remission (*n* = 15) Median (IQR)	Z statistic[a]	*p* value[a]
Blast F (%)	25.00(53)	21.60(41)	-3.24	0.746

[a]Mann-Whitney test

Table 7.3(c) Association between blast (morphology) and MRD cases

Variable	Remission (*n* = 13) Median (IQR)	No remission (*n* = 15) Median (IQR)	Z statistic[a]	*p* value[a]
Blast M (%)	62.00(57)	56.13(36)	-0.48	0.629

[a]Mann-Whitney test

Table 7.3 (d) Association between haemoglobin and MRD cases

Variable	Remission (*n* = 13) Median (IQR)	No remission (*n* = 15) Median (IQR)	Z statistic[a]	*p* value[a]
Hb (g%)	6.60(4)	9.09(4)	-1.22	0.222

Mann-Whitney test

Table 7.3 (e) Association between total white count and MRD cases

Variable	Remission (*n* =13) Median (IQR)	No remission (*n* = 15) Median (IQR)	Z statistic[a]	*p* value[a]
TWC (10^9/l)	35.9(196)	13.2(89)	-1.34	0.134

[a]Mann-Whitney test

Table 7.3 (f) Association between platelet and MRD cases

Variable	Remission (*n* = 13) Median (IQR)	No remission (*n* = 15) Median (IQR)	Z statistic[a]	*p* value[a]
Plt (10^9/l)	35.00(107)	43.00(101)	-0.21	0.836

[a]Mann-Whitney test

For AML clinical presentation that consisted of age, blasts, Hb level, total white count and platelets above, Mann-Whitney test was done because of the sample size of one of the group being less than 30. Therefore, from the results, all the median for age, blasts, Hb level, total white count and platelets at presentation of the two MRD groups (remission and no remission) were not significantly different.

Table 7.4 Clinical factors at presentation associated with AML minimal residual disease

Variable	*N*	Remission Freq (%)	No remission Freq (%)	χ^2 statistic[a] (*df*)	*p* value
Sex				0.48 (1)	0.488
Male	17	7 (41.2)	10 (58.8)		
Female	11	6 (54.5)	5 (45.5)		
Ethnicity				3.25 (3)	0.354
Malay/Melanau	9	2 (22.5)	7 (77.8)		
Chinese	4	2 (50.0)	2 (50.0)		
Bumi Swk	10	6 (60.0)	4 (40.0)		

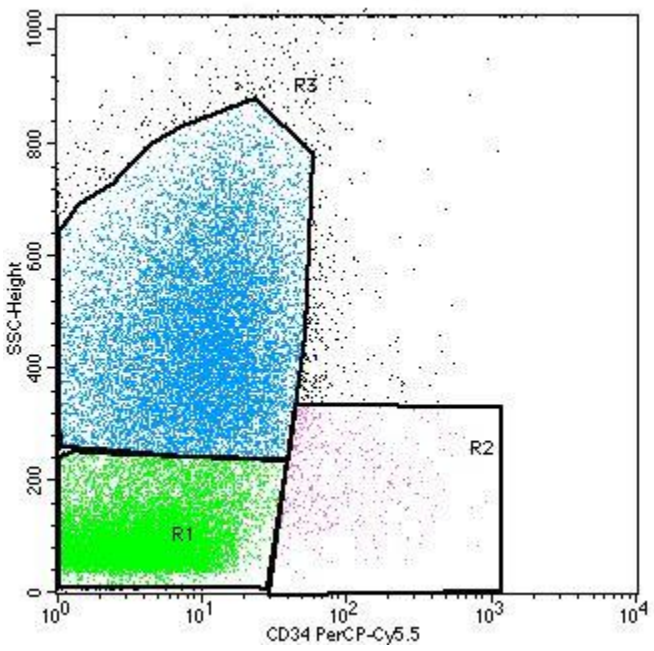
Others	5	3 (60.0)	2 (40.0)		
LN				0.221 (1)	0.150
No	8	2 (25.0)	6 (75.0)		
Yes	20	11 (55.0)	9 (45.0)		
Liver				0.526 (1)	0.655
No	6	2 (33.3)	4 (66.7)		
Yes	22	11 (50.0)	11 (50.0)		
Spleen				0.480 (1)	0.700
No	11	6 (54.5)	5 (45.5)		
Yes	17	7 (41.2)	10 (58.8)		

[a]Chi-Square test for independence

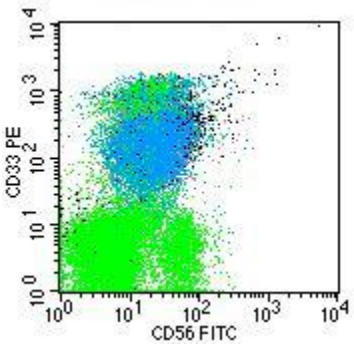
There was no significant association of the variables of gender, ethnicity, lymph node, spleen and liver enlargement with AML MRD. Fisher exact test was applied whenever expected cell assumptions were not met in this test.

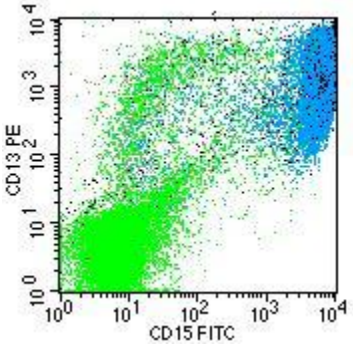
3.4.2.2 Immunophenotypic expression association with MRD

Besides clinical presentation, immunophenotyphic expressions of AML were also analysed in order to study their association with MRD. These antigens were analysed and compared with minimal residual disease result (Table 7.5). The MRD immunophenotyphic expressions were obtained by flow cytometry similar to that done for AML cases at diagnosis with introduction of live gating and higher events collection up until 100, 000 events. By combining information on cell size and granularity and intensity of expression of surface and intracellular molecules, flow cytometry can identify a phenotypic signature that distinguishes leukemic cells from their normal counterparts. Figure 1.6.0 and 1.6.1 demonstrate the flow cytometry dot plots for MRD and the live gating dot plots in one of the AML remission patient.



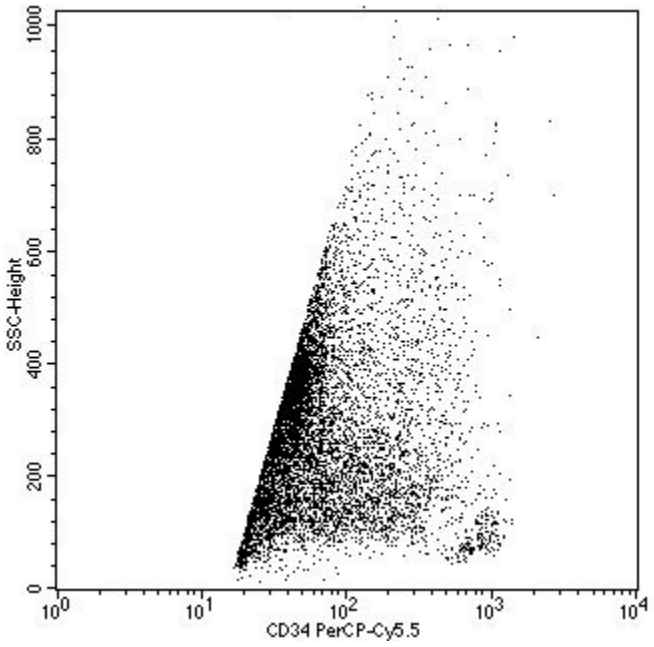
(a)





(b) **(c)**

Figure 1.6.0 Plots (a) to (c) were taken from the MRD remission AML patient subtype M2. The blast cells population was marked with the colour pink. A total of 30 000 events were acquired from this sample.



(e)

Figure 1.6.1 Plots (e) was taken from the same MRD remission AML patient. Live gating was performed on this sample. A total of 250 000 events were acquired from this sample.

Table 7.5 Association of **i**mmunophenotyphic expression with AML MRD

Variable	***N***	**Remission Freq (%)**	**No remission Freq (%)**	χ^2 **statistic**[a] ***(df)***	***p* value**
CD34				0.01 (1)	1.000
Neg	2	1 (50.0)	1 (50.0)		
Pos	26	12 (46.2)	14 (53.8)		
CD33				1.19 (1)	0.464
Neg	1	1 (100.0)	0 (50.0)		
Pos	27	12 (44.4)	15(55.6)		
CD13				2.49 (1)	0.206
Neg	2	2 (40.0)	0 (0.0)		
Pos	26	11(46.4)	15 (53.6)		
HLADR				0.10 (1)	1.000

Neg	5	2 (40.0)	3 (60.0)		
Pos	23	11 (47.8)	12 (52.0)		
CyMPO				1.87 (1)	0.484
Neg	2	0 (0.0)	2 (100.0)		
Pos	26	13 (50.0)	13 (50.0)		
CD117				0.19 (1)	0.662
Neg	12	5 (41.7)	7 (58.3)		
Pos	16	8 (50.0)	8 (50.0)		
CD10				1.87 (1)	0.484
Neg	26	13 (50.0)	13 (50.0)		
Pos	2	0 (0.0)	2 (100.0)		
CD 14				0.48 (1)	0.488
Neg	17	7 (41.2)	10 (58.8)		
Pos	11	6 (54.5)	5 (45.5)		
CD 15				2.67 (1)	0.137
Neg	11	3 (27.3)	8 (72.7)		
Pos	17	10 (58.8)	7 (41.2)		
CD7				0.48 (1)	0.488
Neg	11	6 (54.5)	5 (45.5)		
Pos	17	7 (41.2)	10 (58.8)		
CD64				0.14 (1)	0.705
Neg	14	6 (42.9)	8 (57.1)		
Pos	14	7 (50.0)	7 (50.0)		

[a]Chi-Square test for independence

In AML immunophenotyphic expression, no single antigen expression as demonstrated above showed a significant result association with AML MRD detection. Fisher's exact test was applied whenever expected cell assumptions were not met in this test.

3.4.2.3 Odds ratio of MRD AML

Data from the flow cytometry diagnosis, MRD as well as clinical data for the acute leukaemia were analysed using the multiple logistic regression (MLR) analysis in order to find the inter-relations and odds ratio value between the acute leukaemia variables and minimal residual disease. Result for MLR analysis on AML data for minimal residual disease is shown in Table 7.6.

Table 7.6 Odds ratio values of AML variables associated with MRD in AML

Variable	**Adj. *OR***	**(95% CI *OR*)**	***p* value[a]**
Blast F (%)	0.97	(0.86,1.09)	0.600
Hb (g%)	0.49	(0.13,1.84)	0.294
TWC ($X10^{9)}$	0.97	(0.92,1.01)	1.165
LN	0.00	(0.00,23.11)	0.172
CD15	0.00	(0.00,6.88)	0.146
CD11b	1.65	(0.11,25.54)	0.719

Adj. OR = Adjusted odds ratio [a]Likelihood Ratio (*LR*) test

From the analysis with AML MRD cases, the odds of AML MRD were not significantly different with all the variables tested using the multiple logistic regression.

3.4.3 Minimal residual disease in Acute Lymphoblastic Leukaemia

From 120 ALL cases involving both adult and childhood, 77 cases (64.2%) were further investigated for minimal residual disease (MRD) study after completion of induction therapy or designated as MRD-1 in this study. From these MRD cases, 63 cases (81.8%) were classified as in remission and 14 cases (18.2%) did not achieve remission. From the total of MRD cases, 67 cases (74.4%) were associated with childhood ALL and 10 cases (31.3%) associated with adult ALL. The comparison of clinical presentation and immuphenotyphic expressions variables with MRD were analysed using Mann-Whitney and Chi-square Fisher's exact tests. The odds ratio was analysed using multiple logistic regression (MLR) analysis. All hypothesis tests were based on two-sided tests and *p* value less than 0.05 will be considered as statistically significant.

3.4.3.1 ALL clinical presentation association with MRD

The association between clinical presentation for both numerical (Table 7.7 a-f) and categorical (Table 7.8) ALL variables with MRD were shown below:

Table 7.7 (a) Association between age and MRD cases

Variable	Remission (*n* =63) Median (IQR)	No remission (*n* = 14) Median (IQR)	Z statistic[a]	*p* value[a]
Age (year)	5.00(8)	8.50(13)	-1.192	0.233

[a]Mann-Whitney test

Table 7.7 (b) Association between blast (flow) and MRD cases

Variable	Remission (*n* =63) Median (IQR)	No remission (*n* = 14) Median (IQR)	Z statistic[a]	*p* value[a]
Blast F (%)	76.5(34)	76.4(35)	-0.495	0.620

[a]Mann-Whitney test

Table 7.7 (c) Association between haemoglobin and MRD cases

Variable	Remission (*n* =63) Median (IQR)	No remission (*n* = 14) Median (IQR)	Z statistic[a]	*p* value[a]
Hb (g%)	6.3(3)	8.9(4)	-3.20	<0.001*

[a]Mann-Whitney test

Table 7.7 (d) Association between total white count and MRD cases

Variable	Remission (*n* =63) Median (IQR)	No remission (*n* = 14) Median (IQR)	Z statistic[a]	*p* value[a]
TWC (10^9/l)	22.2(72)	42.5(78)	-0.78	0.436

[a]Mann-Whitney test

Table 7.7 (e) Association between platelet count and MRD cases

Variable	Remission (*n* =63) Median (IQR)	No remission (*n* = 14) Median (IQR)	Z statistic[a]	*p* value[a]
Platlet (10^9/l)	47.0(63)	63.0(96)	-1.25	0.212

[a]Mann-Whitney test

For ALL clinical presentation numerical variables, Mann-Whitney test was done because of the sample size of one of the group was less than 30. Therefore, from the results, all the median for numerical variables of the two MRD groups (remission and no remission) were not significantly different, except for haemoglobin ($p< 0.01$), which was the only parameter found to be significantly associated with presence of MRD in ALL.

Table 7.8 Association between ALL factors (categorical) with minimal residual disease

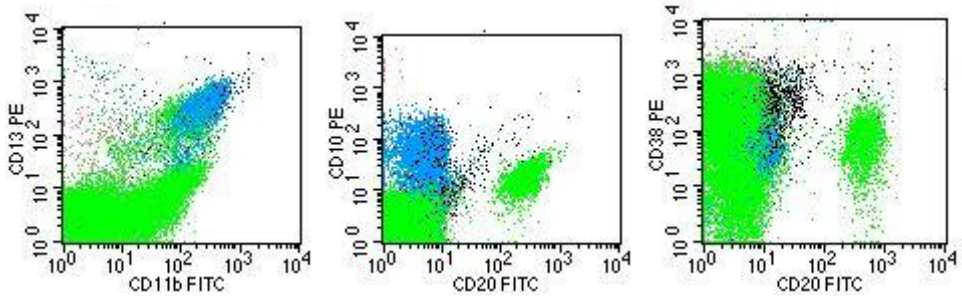
Variable	***n***	**Remission Freq (%)**	**No remission Freq (%)**	χ^2 **statistic**[a] (***df***)	***p* value**
Sex				2.86 (1)	0.091
Male	45	34 (75.6)	11 (24.4)		
Female	32	29 (90.6)	3 (9.4)		
Ethnicity				0.43 (2)	0.806
Malay/Melanau	25	21 (84.0)	4 (16.0)		
Chinese	30	25 (83.3)	5 (16.7)		
Others	22	17 (77.3)	5 (22.7)		
LN				0.93 (1)	0.684
No	12	11 (91.7)	1 (8.3)		
Yes	65	52 (80.0)	13 (20.0)		
Liver				0.94 (1)	1.000
No	4	4 (100.0)	0 (0.0)		
Yes	73	59 (80.8)	14 (19.2)		
Spleen				3.16 (1)	0.098
No	20	19 (95.0)	1 (5.0)		
Yes	57	44 (77.2)	13 (22.8)		

[a]Chi-Square test for independence

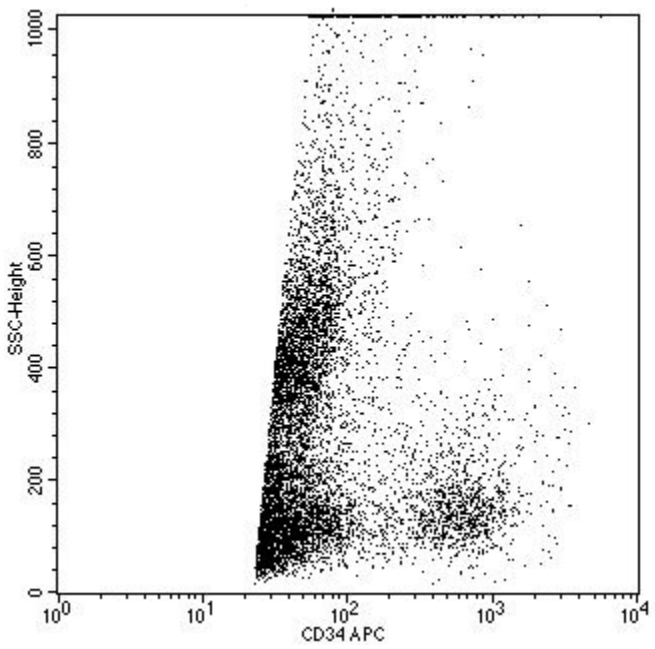
For ALL clinical presentation categorical variables, none of the variables demonstrated a significant result in association with MRD. Fisher exact test was applied whenever expected cell assumptions were not met in this test.

3.4.3.2 Immunophenotypic expression association with MRD

Besides clinical presentation, immunophenotyphic expressions of ALL were also analysed in order to find the association with MRD. These antigens were analysed and compared with minimal residual cases and to find their significant association if available (Table 7.9) The MRD immunophenotyphic expressions were obtained by flow cytometry as in ALL diagnosed cases but with designated antigens, introduction of live gating and higher events collection. By combining information on cell size and granularity and intensity of expression of surface and intracellular molecules, flow cytometry can identify a phenotypic signature that distinguishes leukemic cells from their normal counterparts. To identify immunophenotypes for effective MRD studies, the variations in the composition and immunophenotype of bone marrow cell populations that occur with age and exposure to drugs must be considered. Figure 1.6.2 demonstrated the flow cytometry dot plots for MRD and the live gating dot plots in one of the B-ALL remission patient. Figures 1.6.3 demonstrated the flow cytometry dot plots for MRD-and the live gating dot plots in one of the T-ALL remission patient.

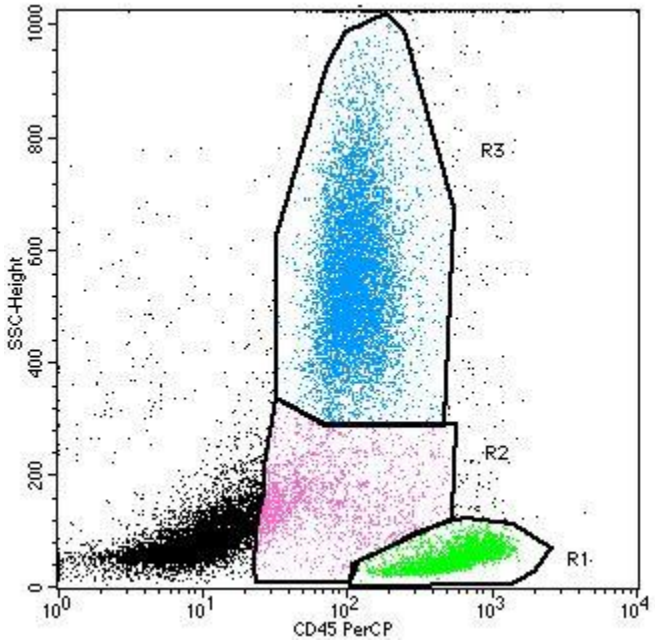


(a) **(b)** **(c)**



(d)

Figure 1.6.2 Plots (a) to (c) were taken from the MRD remission of B-ALL patient. The blast cells population was marked with the colour pink. A total of 30 000 events were acquired from this sample. In (d) live gating was performed acquiring 250 000 events from the sample.



(a)

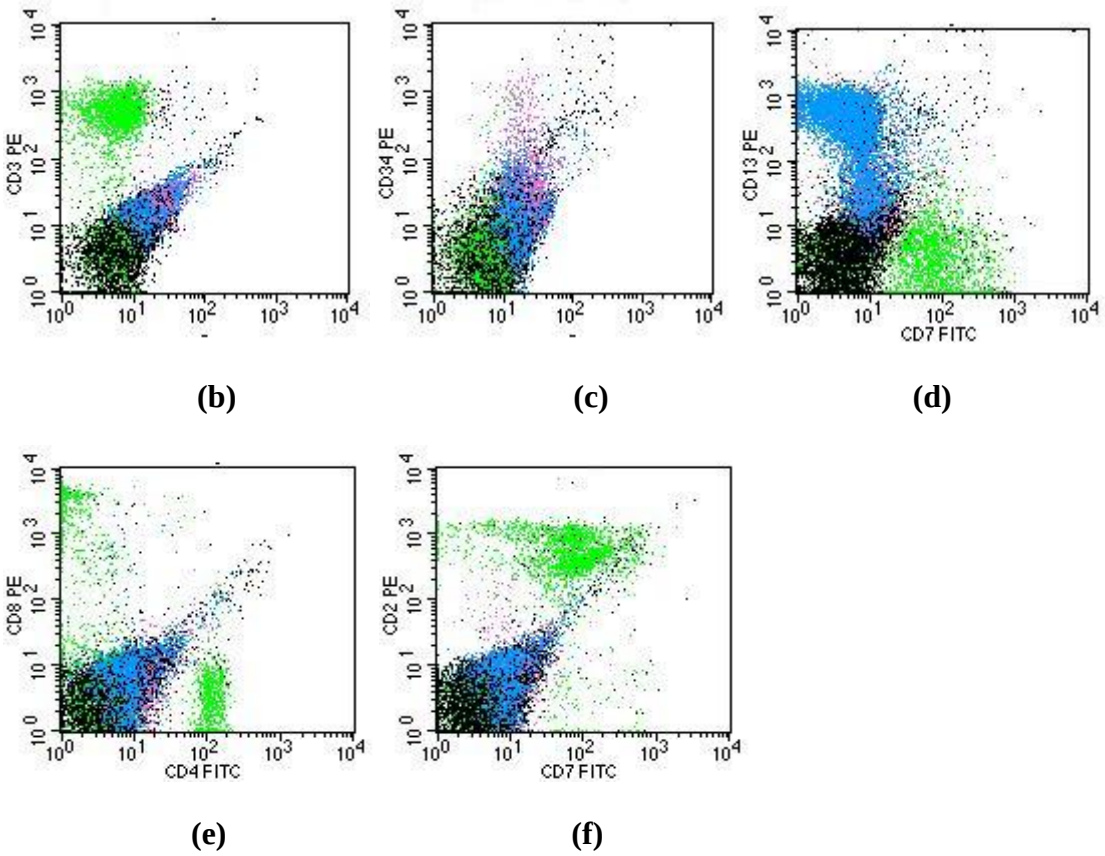


Figure 1.6.3 Plots (a) to (f) were taken from the MRD of T-ALL patient in remission. The blast cells population was marked with the colour pink. A total of 30 000 events were acquired from this MRD sample.

Table 7.9 ALL Immunophenotypic association with minimal residual disease

Variable	***n***	**Remission Freq (%)**	**No remission Freq (%)**	**χ^2 statistic[a] (*df*)**	***p* value**
CD34				0.00 (1)	1.000
Neg	16	13 (81.3)	3 (18.8)		
Pos	61	50 (82.0)	11 (18.0)		
CD45				2.85 (1)	0.091
Neg	32	29 (90.6)	3 (9.4)		
Pos	45	34 (75.6)	11 (24.4)		
CD20				0.29 (1)	0.591

Neg	39	31 (79.5)	8 (20.5)		
Pos	38	32 (84.2)	6 (15.8)		
CD19				1.00 (1)	0.298
Neg	6	4 (66.7)	2 (33.3)		
Pos	71	59 (83.1)	12 (16.9)		
CD10				9.16 (1)	0.018
Neg	4	1 (25.0)	3 (75.0)		
Pos	73	62 (84.9)	11 (15.1)		
Cy79a				1.71 (1)	0.222
Neg	5	3 (60.0)	2 (40.0)		
Pos	72	60 (83.3)	12 (16.7)		
CD38				0.46 (1)	1.000
Neg	2	2 (100.0)	0 (0.0)		
Pos	75	61 (81.3)	14 (18.7)		
CD13				0.06 (1)	0.803
Neg	31	26 (83.9)	5 (16.1)		
Pos	38	31 (81.6)	7 (18.4)		
CD33				0.00 (1)	1.000
Neg	50	41 (82.0)	9 (18.0)		
Pos	27	22 (81.5)	5 (18.5)		
Cy IgM				0.13 (1)	0.560
Neg	73	60 (82.2)	13 (17.8)		
Pos	4	3 (75.0)	1 (25.0)		
nTdT				0.16 (1)	0.352
Neg	9	6 (66.7)	3 (33.3)		
Pos	68	57 (83.8)	11 (16.2)		
CD22				0.46 (1)	1.000
Neg	2	2 (100.0)	0 (0.0)		
Pos	75	61 (81.3)	14 (18.7)		
CyMPO				0.46 (1)	1.000
Neg	75	61 (81.3)	14 (18.7)		
Pos	2	2 (100.0)	0 (0.0)		

CyCD3				2.54 (1)	0.174
Neg	57	49 (86.0)	8 (14.0)		
Pos	20	14 (70.0)	6 (30.0)		
CD7				2.24 (1)	0.154
Neg	69	58 (84.1)	11 (15.9)		
Pos	8	5 (62.5)	3 (37.5)		

[a]Chi-Square test for independence

In ALL immunophenotyphic expression, there were no significant association between the antigen expressions and MRD in ALL. Fisher exact test was applied whenever expected cell assumptions were not met in this test.

3.4.3.3 Odds ratio of MRD ALL

Data from the flow cytometry diagnosis, MRD-1 as well as clinical data for the acute leukaemia were analysed using the multiple logistic regression (MLR) analysis in order to find the inter-relations and odds ratio value between the acute leukaemia variables and minimal residual disease. Results for MLR analysis on ALL data for minimal residual disease are shown in Table 8.0.

Table 8.0 Odds ratio values of ALL variables associated with MRD ALL

Variable	**Adj. *OR***	**(95% CI *OR*)**	***p* value[a]**
Hb	1.62	(1.14,2.31)	0.007*
Spleen			
Yes	37.98	(1.02,1418.62)	0.049*
No	1.00		
CD45			
Yes	2.14	(0.36,12.84)	0.407

No	1.00		
CD10			
Yes	0.09	(0.01,1.59)	0.102
No	1.00		
CD79a			
Yes	10.19	(0.19,558.53)	0.256
No	1.00		
nTdT			
Yes	0.13	(0.01,2.81)	0.195
No	1.00		
CyCD3			
Yes	1.83	(0.29,11.23)	0.513
No	1.00		
CD7			
Yes	1.71	(0.15,18.94)	0.662
No	1.00		

Adj. OR = Adjusted odds [a]Likelihood Ratio (*LR*) test

From the analysis with ALL MRD cases, there were two variables, which were haemoglobin (p = 0.007) and spleen enlargement (p = 0.049) that demonstrated significant results when tested using multiple logistic regression. This showed that patients with low haemoglobin level have 1.62 times odds risk of having MRD, and patients with spleen enlargement were likely to have 37.98 times odds risk of having MRD compared to those with no spleen enlargement.

CHAPTER 4: DISCUSSIONS

4.0 Characteristics of acute leukaemia patients in Sarawak

From the year 2006-2010, a total of 266 cases of patients were diagnosed with two (2) major types of acute leukaemia. The two major types of leukaemia, AMLs and ALLs (B- and T-) were included in this study. This number also includes acute leukaemia patients who went for MRD assay after completion of their induction treatment. All samples were received from Sarawak General Hospital (SGH) in Kuching, which is the referral centre for haematological samples from leukaemia patients from all over Sarawak, including Sibu, Bintulu and Miri hospitals. There were also a minority of cases received from outside the general hospitals such as from a private hospital, namely Normah Specialist Medical Centre (NMSC). This however does not reflect the exact number of acute leukaemia cases in Sarawak in general because some cases were excluded due to various reasons. As there was no official published data on the incidence of acute leukaemia cases in Sarawak, thus it is difficult to get the actual incidence of acute leukaemia cases for the past years. The closest recorded data available are from the National Cancer Registry (NCR) and Sarawak Health Department report, but NCR registry data mainly focuses on incidence data from Peninsular Malaysia cancer patients. From the NCR, a total of 3328 cases of all types of leukaemias were reported in a 3-year period between 2003 and 2005 (Lim et al., 2008). In a published report by the Sarawak Health Department in 2008, the three commonest cancers that caused deaths in Sarawak government hospitals were cancer of the trachea, bronchus and lung (18.44%), followed by lip, oral and pharynx cancers (8.90%), and stomach (7.95%) (Department, 2008). Acute leukaemia,

however was not listed among the top three common cancers that contributed in high number of deaths in Sarawak in 2008.

There were 193 bone marrow (BMA) samples and 72 peripheral blood (PB) samples collected for this study. The type of sample preferred for use in this study was bone marrow aspirates (BMA). Peripheral blood (PB) was also used as the secondary preferred samples, in case the BMA was insufficient or difficulty in obtaining adequate BMA samples in very young patients. There was other sample that can be used for this study such as cerebrospinal fluid (CSF) but the number of cases was low. BMA was chosen as primary sample because it was the source of production for the blood cells in the body therefore more cells could be targeted.

Based on the immunophenotyphing results obtained for both ALL and AML, it can be summerized that the flow cytometry protocol in this study was successfully established, as it produced reproducible results. The immunophenotyping protocol applied to both three and four colour flow cytometry system with some modifications. Although only used for a short period of time, the four colour system proved to produce more extensive results as compared to the three colour system. Besides that, it is recommended to use monoclonal antibodies that were not exceeding the expiry date for better results.

According to 2007 NCR Report, female cancer incidence in Sarawak (53.9%) was much higher than male (46.1%). The report also stated that Chinese male (282) and female (278) recorded the highest number of new cancer cases compared to other races such as Malay, Melanau, Iban and Bidayuh (Zainal Ariffin and Saleha, 2011). However this number only reflects the cancer incidence in general, not specifically acute leukaemia.

Currently there are not many studies published particularly on acute leukaemias in Malaysia and especially in Sarawak itself. The main sources for comparison are only from the 1st and

2nd National Cancer Registry which contained the data produced by the Ministry of Health (MOH) Malaysia. Unfortunately, NCR data only focuses on leukaemia patients in peninsular Malaysia in 2003-2005.

4.1 Acute Myeloid Leukaemia (AML)

Of 266 cases, 147 cases were categorised as acute myeloid leukaemia (AML). Meanwhile the number of AML cases reported in Peninsular Malaysia from 2003 to 2005, 1640 were myeloid (Lim et al., 2008). The population size in Peninsular Malaysia was 22.6 millions, compared to Sarawak (2.4 millions) (Department of Statistics, 2011). Therefore, the incidence rate of AML in Sarawak was 6.13 cases per 100,000 population, lower in comparison to Peninsular Malaysia with 7.26 cases per 100,000 population.

AML is usually common among adults, as stated in one international study (Pui and W. E. Evans, 1998), with only about 15% of acute leukaemia involving childhood AML, indicating the infrequent incidence of this disease among childhood. About 13,410 people in the United States were diagnosed with AML in 2007 (Society, 2008). Geographic variations in the incidence and subtype of AML had been reported. The highest incidence of AML in adults was in North America, Western Europe and Oceania and the lowest in Asia and Latin America (Miller and PR, 2000).

4.1.1 Childhood Acute Myeloid Leukaemia (AML)

Fourty nine of 147 cases (33.3%) were categorised as childhood AML. For this study, the age range for childhood AML was from birth till 17 years old. The mean age for childhood AML was 3.30 ± 3.29 years whilst for adolescent AML was 13.25 ± 1.91 years.

This finding was in concurrence with an immunophenotypic study done in Indonesia where it was found that the peak age of childhood AML among childhood was found in amongst children aged of 3 years (Eddy Supriyadi et al., 2011b). In another study done in Hospital Medan, Indonesia, it was found that the median age for childhood AML was 9 years (Selvi Nafianti et al., 2008). In Peninsular Malaysia between 2003-2005, highest incidence of childhood AML were recorded among those aged 0-9 (2.5 incidence per 100,000 population) (Lim et al., 2008). Therefore, from these studies, it was demonstrated that a similar pattern affected age group (between 0-10 years) with high incidence of childhood AML in the South East Asia region. In another study, it was demonstrated that for childhood AML, peak incidence occurred in the first year of life, then decreased until age 4, and there after remained relatively constant until adulthood (Gurney et al., 1995).

In childhood AML, boys were more commonly affected (53.1%) compared to girls in Sarawak. This result was in concordance with the findings in the Medan (Selvi Nafianti et al., 2008) and Pakistan studies, (Zaki et al., 2002) where both studies found common disease preponderance in the male gender. This finding was also concurrent with Mexico childhood AML study, where 62.0% of the patients were boys (Rogelio Paredes-Aguilera et al., 2003). However, the result of this study contradicted with the Hong Kong childhood AML study, where in a total of 43 patients, 24 of them were girls, and in which the number was higher than boys (Natalie P.H. Chan et al., 2004).

In Peninsular Malaysia the scenario was similar whereby the incidence of childhood AML, was recorded more in males (2.5 incidence per 100,000 population) (Lim et al., 2008), as compared to females (1.9 incidence per population). This showed that in childhood AML was more common in males, which were further supported by similar findings of high incidence

of males even in different countries, such as in Indonesia and Pakistan studies as mentioned previously.

For ethnic distribution, the ethnicity distribution for acute leukaemia in Sarawak is different from that of Peninsular Malaysia due to the Sarawak's ethnic diversity and population size. Sarawak is roughly made up of 2.4 million people with 28 different ethnic groups, which comprises of Malay, Melanau, Iban, Chinese, Bidayuh, Orang Ulu and many more. Sarawak Bumiputera which consisted of many ethnics groups such as Iban, Bidayuh and Orang Ulu reported the highest incidence of AML (40.8%) compared to other ethnic groups. The highest incidence among Sarawak bumiputera in childhood AML was likely associated with the fact that the combination of the ethnics like Iban, Bidayuh and Orang Ulu that make up for 44.0% of the Sarawak population, (Bureau, 2012). Compared to the Peninsular statistics where the ethnicity consisted of predominant Malay with 63.1% of the population (Department of Statistics, 2011), besides Chinese and Indians. Being the majority residents in the Peninsular, the Malays also recorded highest incidence of childhood AML (2.5 incidence per 100,000 population) (Lim et al., 2008). The difference of ethnic incidence of childhood AML may be due to the size of the ethnic population in the certain region/country itself.

4.1.1.1 Clinical characteristics of childhood and adolescent AML

The analysis of each full blood count (FBC) at clinical presentation such as haemoglobin, total white count and platelet was analysed and divided into two groups, childhood (0-9 years) and adolescent (10-17 years) groups, respectively.

For haemoglobin (Hb), the mean value for childhood AML was 9.22 ± 3.59 g%, whilst for adolescent the mean value was 8.74 ± 2.48 g%. The values were similar compared to the mean haemoglobin presentation done in the AML study among children in Pakistan that was

7.7 ±2.6 g%, with 10 patients had less than 7.0 g% (Zaki et al., 2002). Similar result was also obtained in childhood AML Hong Kong study with Hb values ranging from 7.1-10.4 g% (Natalie P.H. Chan et al., 2004). In another study done among Mexico AML childhood cases demonstrated much lower Hb values for childhood AML (1.5 to 2.5 g%) compared to this study, suggesting severe anaemia condition for most of the patients (96.6%) (Rogelio Paredes-Aguilera et al., 2003).

For the total white count (TWC), the mean values for childhood AML in this study was 77.16 $X10^9$/L ± 82.52), and for adolescent was 61.26 $X10^9$/L ± 69.58. In Pakistan children, the TWC mean value was 48.0 $X10^9$/L ± 51.0 (Zaki et al., 2002), suggesting a similar clinical presentation. These results demonstrated that the wide variation in WBC among patients at presentation were common findings in any part of the globe. This result showed most of the AML patients had high count of TWC compared to normal range. A very high TWC count could also be significantly associated with poor outcome in acute leukaemia patients as reported in an Iranian study which found out that 13.0% deaths occurred in the group with initial WBC between 5.0-10.0×10^3/μL and most of deceased cases had an initial WBC more than 10.0×10^3/μL (Parvin Ayremlou et al., 2012). Therefore, one of the factors contributing to death and poor outcome could be associated with the high count of TWC at diagnosis.

For platelet, the mean value for AML patients in this study was 97.19 ± 108.89 X 10^9/L for childhood AML, while for adolescent AML was 58.72 ±46.93 $X10^9$/L. Other AML children study demonstrated a platelet mean value similar to this study, with 109.0 ± 165 X 10^9/L (Zaki et al., 2002). In a Hong Kong study, the platelet count range was between 15-121 X 10^9/L (Natalie P.H. Chan et al., 2004) and in the Mexico study, 44.8% of childhood AML had platelet readings less than 20 X 10^9/L (Rogelio Paredes-Aguilera et al., 2003). The platelet

count for childhood and adolescent AML in this another study showed similar pattern of presentation with other geographic regions.

In this study, it was also noted that, 32.7% of childhood AML patients had lymph nodes enlargement, 65.3% had liver enlargement and 42.9% had splenomegaly at time of diagnosis. As a comparison, study demonstrated much lower percentage of liver enlargement (10.34%), splenomegaly (6.9%) and lymph node enlargement (13.8%) in one childhood study (Rogelio Paredes-Aguilera et al., 2003). This could be attributed to the disease stage at presentation and could also be contributed by accessibility of patients to nearest medical facility in Sarawak.

4.1.1.1 (a) Association between full blood count (FBC) and gender in childhood AML

The association between FBC and gender in childhood AML was investigated to compare the mean of FBC variables with the patient's gender. In this study, the total white count (TWC) mean value for male was 104.4 X 10^9/l and female was 43.5 4 X 10^9/l, and the TWC mean value between male and female with childhood AML was found to be significantly different ($p = 0.008$). Therefore, this finding suggests that gender and TWC may be of prognostic importance that contributed to risk stratification of childhood AML. Results from the US studies reported that children with AML whose white blood count less than 100,000 cell per cubic mililitre at diagnosis were cured more often than those with higher counts (Society, 2012). As for Hb and platelet counts, the analysis showed that there were no significant differences between the males and females mean values for both variables, ($p = 0.989, 0.925$ respectively) thus suggesting no association between them.

4.1.1.1 (b) Association between age and gender in childhood AML

The association between age and gender in childhood AML was investigated to compare the mean age with the patient's gender. No significant correlation between age and gender in

childhood AML was found. The main reason that might lead to this result is the fact that AML more commonly diagnosed in adults than children (Pui and W. E. Evans, 1998). Also, age was also known as one of the prognostic factors for childhood AML, with children younger than age 2 seem to do better than older children (Society, 2012).

4.1.1.1 (c) Association between ethnicity and gender in childhood AML

The association between ethnicity and gender in childhood AML was investigated to compare ethnicity with the patient's gender. No association was found between ethnicity and gender in childhood AML. Although there was no local study investigating the relationship between ethnicity and gender childhood AML, one American study revealed that Hispanic and black children with AML had worse survival rates compared to white children (Richard Aplenc et al., 2006), suggesting ethnicity association with the outcome of AML. Another American study demonstrated that girls had better outcomes than boys, with African-American patients experience significantly inferior outcomes compared white patients (Woods et al., 2001).

4.1.1.2 Immunophenotyphes of childhood AML

Immunophenotypic analysis of childhood AML at diagnosis showed significant expressions of CD33+ with highest frequency of 93.9%, CyMPO+ (87.8%), CD13+(87.8%), CD117+ (85.7%) and HLADR+ (81.6%).

In a study on surface antigen expression in childhood AML, among the non-lineage-restricted markers, HLA-DR was the most commonly expressed antigen **(**80**%),** whereas CD34 and nTdT were expressed with intermediate or low frequency (45% and 21%, respectively). Surface expression of CD13 and CD34 was detected in only 21% to 25% of patients with FAB M5, whereas expression of HLA-DR, CD33, and CDw65 was found in the vast majority of the patients (Creutzig U et al., 1995). The strong expressions of CD13, CyMPO and HLA-

DR were associated with AML with t(8;21) and strong expression of CD117 associated with AML inv(16) or t(16;16) (Gert J. Ossenkoppele et al., 2011) . In another study, bright CD33 expression was observed in all AML-M0 samples (Patricia K. Kotylo et al., 2000b). The expression of CD13+, CD33+ and CD117+ were common because these antigens were known for their wide distribution in most of AML cases (RO Casasnovas et al., 2003, Buccheri V et al., 1992). These were the primary myeloid markers and were defined by the expression of two or more associated markers in the absence of specific lymphoid markers (Morilla, 1999).

CD117 for instance, was more specific for identifying myeloid lineage as compared to CD13 and CD33, as it was also a crucial marker for definition of biphenotypic leukaemia. (Kamps et al., 2010). It is also suggested that analysis of cytoplasmic CD13+ and CD33+ expressions can also be helpful in the definition of poorly differentiated AML (Stasi R et al., 1995). Expression of CD34+ is normally associated with AML M3 and M4 blasts (Solary E et al., 1992), which could be relevant with the finding in this study. Also the CD34/CD56 combination appears to be useful because they are simultaneously expressed in approximately 20% of childhood AML (Hurwitz CA et al., 1992), although CD56 was not frequently expressed in this study.

Meanwhile, antigens such as CD14, CD10, and CyCD79a were showing strong weak expressions, with frequency percentage value 81.6%, 81.6% and 73.5% respectively. This finding is in concordance for CD14, as seen in one AML childhood study, where the other myeloid-associated antigens (ie, CD14, CD15, CD41, glycophorin A) were detected less frequently and in varying proportions depending on the maturational stage and FAB-subtype of myeloid leukaemic cell. The same study also demonstrated the low expression of CD10 and co-expression of T-lymphoid features (CD2, CD4, CD7) occurred much more frequently

than co-expression of the B-lymphoid-associated antigen CD19 (2%) and CD10 (l%, data not shown). Expression of CyCD79a was associated with aberrant expression in AML with t(8;21) and weakly expressed CD14 commonly associated with mutated AML (Gert J. Ossenkoppele et al., 2011).

Besides that the lack of CD15 expression in this study could be suggesting most childhood AML were not associated with monoblastic AML populations (Lo SK et al., 1997). Finally CD11b that showed high negative expression for childhood AML in this study (67.3%) because as comparison in a study (Paietta E et al., 1998), it was also to be found only to be expressed in only 95 out of 382 newly diagnosed AML. Variable expression of CD11b was associated with acute promyelocytic leukaemia (APL) with t(15;17) (Benter et al., 2001).

Previous studies also suggested the common markers for AML patients according to the FAB subtypes: CD45, HLA-DR, CD19 in M2, CD45, HLA-DR and CD34 in M3, CD64 in M4 and CD34, CD45, CD41,CD61 and CD7 in M7 (Mirbehbahani NB et al., 2011). Also, other study also found that CD13 and CD33 in M1, CD33 in M2 and CD13 in M3 were most common (KermaniI, 2002). Furthermore, one study conducted in China observed that commonly expressed AML antigens were CD33, CD13, myeloperoxidase (MPO) and CD117 (Tong HX et al., 2009). Expect for a few antigens like CD19, CD41 and CD61, all these findings were in concurrence with the immunophenotypic analysis in this study. Expression of particular aberrant markers (CD19, CD56, nTdT) may serve as key flow cytometric clues to the diagnosis of AML (Chen et al., 2008, Hurwitz et al., 1992, Khalidi et al., 1998). Results from this study, indicated that the immunophenotypic findings of childhood and adolescent leukaemia in Sarawak was in concordance with other findings. These results could partly be due to the choice of markers that were chosen for the leukaemia panels in this study were mainly based on previous published studies.

4.1.2 Adult Acute Myeloid Leukaemia

Ninety-eight of 127 adult AML from the age range of 18 till 75 years old were studied. The mean age values for respective category were as follows: Adult was 36.1± 9.17 years and elderly 61.0 ± 7.27 years. The result in the elderly category was in concordance with the following studies which stated that adult AML usually diagnosed in elderly adult. Like in peninsular Malaysia between 2003 until 2005 for instance, highest incidence of adult AML was recorded among patients aged 60-69 (Lim et al., 2008). A study conducted in Sweden also reported median age of 65 years among 9729 AML patients between the year 1973-2005 (Åsa Rangert Derolf et al., 2009). In the United States, AML incidence began to increase significantly among people with 50 years and older (Society, 2011). One study from Pakistan reported that between 1988-1996, the mean age at time of diagnosis was 38 years (Kakepoto et al., 2002). The study demonstrated similar results, and the mean age was almost similar with the adult category value obtained in this study. The incidence of AML progressively increased with age, and in adults over the age of 65 years was approximately 30 times that in children (Bhatia and JP, 1995).

As in childhood AML, in this study, males (52.0%) were more commonly diagnosed with adult AML, compared to females, and this was similar with the findings in Peninsular Malaysia. In Peninsular Malaysia between 2003-2005, the incidence of adult AML was highest among male aged 60 and above (Lim et al., 2008). Males were also found to be more affected compared to females in adult AML patients in Sweden (Åsa Rangert Derolf et al., 2009). The above findings were also compareble with the United States (Society, 2011) and Pakistan studies (Kakepoto et al., 2002), which demonstrated that males were more likely to be diagnosed with AML. Many other studies also confirmed that incidence rates were usually higher in males (Petridou et al., 2008). Kobayashi et al reported 72.4% male dominancy in

AML (Kobayashi H et al., 2009) while Santos et al reported that 62.0% of AML patients were men (Santos et al., 2009). These gender consistencies findings supported the tendency of AML incidence to be more common among adult males than females in general.

Adult AML was found to be highest among the Sarawak Bumiputera compared to other ethnic groups with 28.6%, although overall it looked almost evenly distributed. The reason for high incidence of Sarawak Bumiputera was actually the same as childhood AML stated previously. In Peninsular Malaysia which had a different ethnic composition and a bigger population, Indians had the highest incidence of adult AML (14.1 incidence per 100,000 population) compared to the Malays and Chinese (Lim et al., 2008). Indians in Mumbai (1.9 incidence per 100,000 population) had lower incidence compared to Malaysian Indians (3.0 incidence per 100,000) (Lim et al., 2008). This demonstrated that the AML incidence in adults was not necessarily influenced by the size of the population in certain regions.

4.1.2.1 Clinical characteristics of adult and adolescent AML

The analysis of each full blood count (FBC) clinical presentation such as haemoglobin, total white count and platelet was divided into two groups, adult (18-50 years) and elderly (>50 years) respectively.

For haemoglobin, the mean values for adult AML was 7.92 g% ± 2.15 whilst for elderly the mean value was 7.63 g% ± 1.94. The mean haemoglobin values was slightly lower compared with the study conducted for Pakistan adult, which was 8.3 ± 2.4 g% (Kakepoto et al., 2002). An AML study involving 1795 adults demonstrated median Hb value of 9.2 g%, which correlates with the findings in this study (Byrd et al., 2002). These low values demonstrated

that most AML patients were anaemic at the time of diagnosis, and may need supportive treatment along with chemotherapy.

For total white count, the mean value for adult AML was 59.53 ± 65.86 X 10^9/L while for elderly was 32.0 ± 34.30 X10^9/L. The mean count obtained in Pakistan (49.7 X10^9/L), (Kakepoto et al., 2002) was much lower compared with the adult group in this study, but was higher compared to the elderly group, meanwhile in another study the mean TWC of adult AML varied, from 1.0-38.8 X10^9/L compared to childhood, 19.6 X10^9/L (Patricia K. Kotylo et al., 2000b). One of the factors for the variation of WBC values for different studies could be associated with the age factor, as elderly group demonstrated a much lower count compared to adult group as shown in this study.

For platelet, the mean values for adult AML was 61.63 ± 59.0 X10^9/L for adult while for elderly was 54.70 ± 43.94 X10^9/L. Thrombocytopenia is a common finding in acute leukaemia patients. The mean platelet count for adult Pakistani was 65.5 X10^9/L (Kakepoto et al., 2002), which is slightly higher compared to the elderly group in this study suggesting the close similarity of these values. However, an Iranian study demonstrated that 62.9% of AML patients had platelet count less than 50.0 X10^9/L (Parvin Ayremlou et al., 2012). Besides that, one American study (Byrd et al., 2002) on adult AML patients showed median value of platelet 57 X10^9/L, which is similar to the results in this study.

4.1.2.1 (a) Association between full blood count (FBC) and gender in adult AML

The association between FBC and gender in adult AML was investigated to compare the mean of FBC variables with the patient's gender. It was found that for FBC, (total white count, haemoglobin and platelet) there were no difference between the severity of anaemia, TWC and platelet count between the genders in adult AMLs, thus suggesting no association

between them. However it was reported in one study that female gender with leukocyte count less than $10.0X10^9$/L and haemoglobin greater than 10 gm/dl are associated with longer disease-free survival (Bennett et al., 1997).

4.1.2.1 (b) Association between age and gender in adult AML

The association between age and gender in adult AML was investigated to compare the mean age with the patient's gender. No significant difference was found between age and between genders in adult AMLs, thus suggesting no association between them.

4.1.2.1 (c) Association between ethnicity and gender in adult AML

The association between ethnicity and gender in adult AML was investigated to compare ethnicity with the patient's gender. In this study, no association was found between ethnicity and gender in adult AML. A study conducted in the USA demonstrated that the incidence of AML varied with gender and ethnicity, with male (4.6 per 100,000) and whites (3.8 per 100,000 persons) as the predominant gender and ethnic group in AML respectively (Deschler and Lübbert, 2006).

4.1.2.2 Immunophenotyphes of adult AML

Immunophenotypic analysis of adult AML diagnosis showed the high expression of CD33+ (92.9%), CyMPO+ (86.7%), CD13+ (85.7%), CD117+ (79.6%), HLADR+ (75.5%), CD34+ (66.3%), CD56+ (61.2%), CD64+ (52.0%) and CD7+ (55.1%). A study by Legrand et al, (2000) demonstrated the following antigen expressions in their immunophenotype AML study; CD13+ (95%), CD33+ (91%), MPO+ (73%), HLADR (87%), CD117(73%) and CD34 (68%). CD7+ expression was also found to be positive in 37% of the patients. With exception of a few antigens such as CD56, CD64 and CD7, the results were almost similar with the

expressions found in this study. An Australian study also demonstrated the expression of myeloid lineage antigens such as CD13 (71%) and CD33 (79%), with T-cell marker CD7 (28%) was also detected (K Bradstock et al., 1994). Another flow cytometry immunophenotypic study involving 11 AML adults showed expressions that were similar in the following antigens; CD34 (11/11), CD13 (10/11), CD33 (8/11), and HLADR (10/11). CD7 was also expressed in 4 out of 11 patients (Patricia K. Kotylo et al., 2000a).

The expression of CD7+, a T-cell lineage, in this study was similar to a study in Canada (Anurag Saxena et al., 1998) which demonstrated that 10% of AML patients studied also showed positivity for CD7 expression. According to the study, the expression of CD7+ was associated with early haemopoietic precursors in these patients. Expressions of non myeloid markers such as CD7 and CD56 on myeloid blast markers in this study were usually associated with aberrant markers (Campana, 2003). The primary myeloid markers such as CD 13+, CD33+ and CD 117+ were commonly expressed in adult AML, as these three markers were the primary AML markers (RO Casasnovas et al., 2003, Morilla, 1999, Schwartz S et al., 1999). Past study also acknowledged CD33 as the most important myeloid surface antigen (Scott CS et al., 1999, Khalidi HS et al., 1998). The expression of HLADR+ and CD34+ were common because these two markers were said to be having a close distance linkage with respect to myeloid antigens, despite the fact that these are non lineage markers (RO Casasnovas et al., 2003). High CyMPO+ expression was common due to the fact that this antigen was the myeloid lineage specific marker which was expressed in the cytoplasm (Marie C. Bene et al., 1999). Generally the immunophenotypic antigen expressions in adults were almost similar with childhood AML, only with the absence of CD33+, therefore suggesting there is not much difference between the expressions of childhood and adult AML antigens.

In addition to CD10 (98.0%) and CD14 (83.7%) that displayed high negative expressions in childhood and adult AML, CD11b (68.4%), CD19 (68.4%), CD61 (70.4%) and CD79a (64.3%) were also weakly expressed in adult AML. One similar study showed the low expressions of myeloid antigens such as CD14 and CD11b, with CD14 demonstrated in less than 20%, and CD11b was found higher, in 40% of the cases studied. The same study also stated the low expression of B-cell marker, the CD19 (7% of all the AML cases) (K Bradstock et al., 1994). Legrand *et al* (2000) discovered the low expression of B-cell markers, CD10 (10%) and CD19 (16%) in their study. The same study also found that CD14 were positively expressed in only 25% of the patients, thus rather similar to the results in this study.

The lack of expression in CD14 and CD11b could suggest immunophenotyping shift in AML from the time of diagnosis until MRD as detected in childhood AML. Also it has been shown before to give out false-negative MRD results (Coustan-Smith et al., 1998). Markers associated with lineage differentiation include CD14 and CD11b in AML-M4 and M5, glycophorin A (GlyA) in AML-M6 and platelet glycoproteins CD41, CD42 and CD61 in AML-M7 (Smith et al., 2004). Previous study also demonstrated that lymphoid-associated antigens were commonly expressed in AML patients, although not in high percentage, such as CD20, CD7, CD19, CD2 ,CD3, CD5, and CD10 (Khalidi HS et al., 1998).

4.2 Acute Lymphoid Leukaemia

From a total of 266 acute leukaemia cases, 122 (45.9%) were categorised as acute lymphoid leukaemia (ALL), with incidence in childhood age group (65.5%) higher compared to adult (34.5%). In comparison to Peninsular Malaysia in 2003-2005, the number in this study was relatively small since there were 1296 cases of lymphoid leukaemias reported during that particular period (Lim et al., 2008). A study in Thailand also reported that the most common

type of leukaemia among childhood from was ALL (65.7%) (Kamsa-ard et al., 2006). ALL is further divided into two subtypes, the B-ALL and T-ALL, and in this study, B-ALL was found to be more common (90.0%) compared to T-ALL (10.0 %). This result was concurrence with other studies done in the region, such as in Malaysia-Singapore (93.0%) (Ariffin H et al., 2007), Thailand (82.0%) (Tiensiwakul P et al., 1999), Hong Kong (82.0%) (Shing MM et al., 1999) and Indonesia (83.0%) (Eddy Supriyadi et al., 2011a). B-ALL subtype to be more common compared to T-ALL in the above mentioned studies.

4.2.1 Childhood Acute Lymphoid Leukaemia (ALL)

The mean age for the children group was 3.7± 2.21 years and for adolescent was 12.2 ± 1.98 years. In peninsular Malaysia (2003-2005), highest incidence of childhood ALL were recorded in age group 0-9 years (Lim et al., 2008). In a Thailand study it was reported that half of the childhood ALL cases were between 0-4 years (Kamsa-ard et al., 2006), while in the United States, ALL was common in children aged 1-7 years old, and in adolescents aged 14 and below (Society, 2011). All these findings further supported that in childhood ALL, the age (0-10 years) represents the majority of children diagnosed with ALL.

In Sarawak, the incidence of childhood ALL were found to be slightly higher in males (54.4%) compared to females (45.6%). Similar finding was also reported in the Peninsular Malaysia where males (9.7 incidence per 100,000 population) had higher incidence than females (7.1 incidence per 100,000 population) (Lim et al., 2008). This finding showed similar findings with the Thailand study (Kamsa-ard et al., 2006) and the American study (Society, 2011). Across Asia, the incidence rate of childhood leukemia was also reportedly higher among boys than girls (Parkin DM et al., 1996).

Highest ethnic distribution for childhood ALL was found among the Malay/Melanau (32.2%) compared to other ethnics such as Sarawak Bumiputera and Chinese. In Peninsular Malaysia, even though the Malays makes up the majority, the number of ALL cases among Malay children (6.0 incidence per 100,000) was lower compared to Indians (6.9 incidence per 100,000 population) (Lim et al., 2008).

4.2.1.1 Clinical characteristics of childhood and adolescent ALL

The analysis of each full blood count (FBC) such as haemoglobin, total white and platelet counts was divided into two groups, childhood (0-9 years) and adolescent (10-17 years) respectively. The mean haemoglobin value for childhood ALL was 6.76 ± 2.56 g% while for adolescent the mean value was 7.97 ± 2.89 g%. There were a number of studies that showed similar Hb results such as the Pakistan study, (7.8 ± 2.4g/dl) (Khalid et al., 2010), and a study conducted by Conter V et al in 2004, (7.0-11.0 g%). Patients who presented with higher Hb levels at diagnosis (Hb > 80 g/L) had a poorer outcome compared to patients with lower Hb levels (Hb <80 g/L) (Oliver Teuffel et al., 2008). In some studies, a higher Hb level was associated with a worse prognosis (Shuster et al., 1990, Steinherz et al., 1991) whilst in other studies, there was no association, or even an opposite association (Pui et al., 1990).

For total white count (TWC), the mean values for childhood AML was 43.0 ± 61.96 ($X10^9$/L) for childhood while for adolescent was 115.12 ± 188.14 $X10^9$/L. There was a wide range of TWC values between the childhood and adolescent groups, suggesting TWC count could be affected by age.

From this study, it was noted that adolescent TWC mean value was higher than childhood group, (>100.0 $X10^9$/L). Both TWC values for childhood and adolescent in this study were higher compared to a mean value obtained in Pakistan children, which was 32.7 ± 67.0 x

10^9/L (Khalid et al., 2010). Another study reported 53% of childhood ALL with TWC below 10,000 mm^3 (Conter V et al., 2004), a value which is lower than the finding in this study. Another study reported TWC count below 50,000 cells/cm was observed in 34% children ALL in Pakistan (Nuzhat Yasmeen and Ashraf, 2009).

For platelet, the mean value was 77.6 L ± 129.47 ($X10^9$/L) for childhood while for adolescent was 64.25 ± 50.31 ($X10^9$/L). These values were rather similar to a study in ALL Pakistani children, which was 64.0 ± 94 ($X10^9$/L) (Khalid et al., 2010). A study demonstrated that platelet count in 47% of childhood ALL patients in the range of 20,000-99,000 mm^3 (Conter V et al., 2004), which was similar to the range obtained in this study. Platelet count range in a childhood Pakistan study was in 20,000-100,000 mm^3, and found in 45% of the patients (Nuzhat Yasmeen and Ashraf, 2009).

It was also noted that, 45.1% of childhood ALL patients had lymph nodes enlargement, 58.2% had liver enlargement and 48.4% had splenomegaly at time of diagnosis. A collective study on childhood ALL reported that hepatosplenomegaly and lymphadenopathy occurred at diagnosis in 70% and 50% of children ALL respectively (Conter V et al., 2004). A Pakistan study concluded that lymphadenopathy, hepatomegaly and splenomegaly were common findings in children ALL at presentation occurred in 75%, 67% and 58% respectively (Nuzhat Yasmeen and Ashraf, 2009).

4.2.1.1. (a) Association between full blood count (FBC) and gender in childhood ALL

The association between FBC and gender in childhood ALL was investigated to compare the mean of FBC variables with the patient's gender. The analysis found out that there was no gender association with full blood count in childhood ALL. On the contrary, one study demonstrated that ALL children with high white blood counts at diagnosis and low

haemoglobin levels may have a better prognosis than predicted association by the white blood count alone (Hann et al., 1981). However the study did not attempt to study any association between FBC and gender. Another study found patients with lower Hb level (<8 gm/dl) and high WBC (>50,000/mm^3) showed better remission rates, especially amongst the males (Settin et al., 2007).

4.2.1.1 (b) Association between age, ethnicity and gender in childhood ALL

The association between age, ethnicity and gender in childhood ALL was investigated to compare the mean age with the patient's gender. In this analysis, gender was not associated with age and ethnicity. Other study reported that the peak incidence of childhood ALL was between 2 and 5 years old and usually slightly older among boys (Conter V et al., 2004). In one childhood ALL study conducted in the United States, it was shown that blacks, Hispanic and American Indian/Alaskan Native children had worse survival rates than the white and Asian/Pacific Islander children (Kadan-Lottick NS et al., 2003a). Also, when compared with white children, black and Hispanic children had worse outcomes and Asian children fared better after adjusting for known risk factors (Smita Bhatia et al., 2002).

4.2.1.1 (c) Association between ethnicity and gender in childhood B-ALL and T-ALL

The association between ethnicity and gender in childhood B-ALL and T-ALL was investigated to study the association between ethnicity and ALL subtype. No association was found between gender and ethnicity in both childhood B- and T-ALL, suggesting that no ethnic and gender was exclusive to a certain subtype of ALL. An American study, also showed an indifferent result when the association between the ethnic and subtype of ALL was studied. Gender association, however, was not studied, but the study demonstrated an association between ethnicity and ALL subtype, T-ALL in particular. The study showed that

survival rates among ALL children from the black and Hispanic background was lower compared to white children, and Asian children fared slightly better than white children. The reason for better outcomes in white and Asian children than in black and Hispanic children was at least partially explained by the different spectrum of ALL subtypes. For example, blacks had a higher incidence of T-cell ALL and lower rates of favourable genetic subtypes of ALL (Bhatia., 2004, Kadan-Lottick NS et al., 2003b).

4.2.1.2 Immunophenotypes of childhood ALL

Immunophenotypic analysis of childhood ALL involved the frequency analysis of immunophenotypes at presentation. General immunophenotypic analysis of childhood ALL at diagnosis displayed the expressions of CyD79a+ (93.3%), CD19+ (90.0%), CD38+ (84.4%), CD22+ (84.4%), CD10+ (84.4%), CD34+ (63.3%), CD45 (60.0%) and nTdT (52.2%). Similar result was also demonstrated in a Moroccan children study, where all cases expressed CD79a and CD22. Other antigens expressed include CD20 (54.2%), CD10 (91.2%), nTdT (89.8%), HLA-DR (98.1%), CD34 (75%) and CD45 (87%) (Fatima Bachir et al., 2009). An Iranian study also showed similar pattern in the antigen expressions for both CD19 (90.2%) and CD10 (84.4%), but with the exception for HLA-DR expression (70.58%), (Mirbehbahani NB et al., 2011) which was lowly expressed in this study. Another study also showed concurrence findings with these result, whereby the frequently expressed markers in ALL patients were CD7 (11-28%), CD2 (5-21%) and CD19 (3-14%). CD10 (1-5%) and CD20 (9%) (KermaniI, 2002).

Also, quantitative differences in antigen expression can also be used to distinguish these leukaemic cells from normal cells. For example, the expression of CD19+,CD10+ and CD34+ in B-lineage ALL can be 10-fold greater than that of normal B cell progenitors (Lavabre-Bertrand T et al., 1994). However, in this study quantitative differences analysis was not

done, and it was assumed that all the cells that expressed the markers were leukemic cells. HLA-DR and CD19 are also known as common markers in ALL, with high prevalence of HLA-DR expressed in L1 subtype, thus suggesting the association between both entities (Mirbehbahani NB et al., 2011). The HLA-DR antigen was commonly not expressed in T-cell ALL (Feller et al., 1986), which was another phenotypic feature that distinguishes T-ALL from Pre-B-cell ALL and B-cell ALL. Another study in China showed that most prevalent markers of B-cells included CD19, CD10, CD22 and CD20 (Tong et al., 2010).

Meanwhile, antigens such as CyMPO (91.1%), CyIgM (84.4%), CD7 (81.1%), CD33 (64.4%) CyCD3 (65.6&) and CD20 (47.8%) displayed high frequency of expressions, though having weak/negative (dim) level of expressions. Markers such as CyMPO, CD33 and CD7 were customarily identified as AML markers in most other studies (Tong HX et al., 2009, KermaniI, 2002, Mirbehbahani NB et al., 2011), hence the typical negative expressions in these ALL patients. There was one antigen that was lowly expressed, the CD13. CyMPO and CD13 can be considered as aberrant markers for ALL, since they are usually expressed in AML (Morilla, 1999).

For ALL, the markers used in immunophenotyping vary between B-ALL and T-ALL because of the different stages of blood cell maturation between the 2 subtypes. This explains the different immunophenotyping panels between the two subtypes. Therefore in childhood B-ALL, antigens that displayed significant expression were CD19+ (98.8%), CDCy79a+ (97.5%), CD22+ (92.6%), CD38+ (90.1%), and CD10+ (87.7%). These were all B-ALL markers, while weak expressions of CyMPO (91.4%), CyIgM (91.4%, B-ALL marker) and CD7 (90.1%, T-ALL marker) were also observed. CD13, an aberrant marker for B-ALL, was co-expressed, positively and negatively expressed in B-ALL. The expression of Cy79a+, CD19+, and CD22+ were usually associated with precursor B-cell ALLs. An Indonesian

study demonstrated similar results pattern, with CD19, CD10, CyCD79a and CD22 identified as frequent B-ALL markers, while CD7, CyCD3 and CD2 as T-ALL markers (Eddy Supriyadi et al., 2012). A study on 113 patients with ALL in China showed that the most prevalent markers were CD19 (99.0%), CD10 (82.5%), CD22 (74.8%) and CD20 (37.5%) (Tong et al., 2010).

For childhood T-ALL, CD45+ and CD5+ were expressed in all cases, and CyCD3+, CD5+, and CD10+ were expressed in 88.9%, 77.8% and 66.7% of the cases respectively. All of these were T-ALL markers except for CD5 which was a B-ALL marker. CD5 and CD7 were the most common markers in T-cell line (Wenxiu and Ch., 2005). CyMPO and CD33 were weakly expressed, and both were considered as aberrant markers for T-ALL. CD56, an aberrant marker, showed dual expressions (both positive and negative) in almost the same percentage of T-ALL cases studied.

4.2.2 Adult Acute Lymphoid Leukaemia

The age distribution for the adult ALL was divided into 2 categories, adult (18-49 years) and elderly (>50 years). The mean age for the adult category was 30.2 ± 7.92 years and for the elderly category was 58.9±7.49 years. In comparison, adult ALL incidence in Peninsular Malaysia showed the highest age incidence in those aged 40-49 years (1.4 incidence per 100,000 population) and for the elderly was highest in group aged over 70 years (4.6 incidence per 100,000 population) (Lim et al., 2008). Sixty-six adult ALL patients recruited for a study in Iran showed mean age of 33 years (Mohammad Ali Mashhadi et al., 2012), which showed similar age group distribution result with this study. The median age of Swedish adult ALL patients was 54 years for a 10-year period (1983-2010) (Gunnar Juliusson

et al., 2010). Another study in Iran reported a much lower mean age at diagnosis for ALL, which was 24.2 years (Ayremlou et al., 2012).

In adult ALL, males in Sarawak (62.1%) demonstrated higher incidence of ALL compared to females (37.9%). Similar result was also shown in the Peninsular Malaysia with adult males (4.6 incidence per 100,000 population) having higher incidence when compared to females (3.5 incidence per 100,000 population) during the year 2003-2005 (Lim et al., 2008). This finding was consistent with an Iranian study, where the incidences in males (63.5%) was higher compared to females (36.5%) (Ayremlou et al., 2012). The recent United States statistical survey also showed that adult ALL patients were more common among males than females (Society, 2011).

In terms of ethnic distribution, for adult ALL in Sarawak, Malay/Melanau (44.8%) made up the majority of patients affected compared to other ethnic groups. In Peninsular Malaysia, it was shown that the Chinese had the highest prevalence compared to other ethnic groups in year 2003-2005 (Lim et al., 2008). In Singapore it was reported that there was no racial differences in leukaemia incidence among the 3 ethnic races namely Chinese, Indians and Malay but the number of patients were higher among the Chinese (Suri, 1989).

4.2.2.1 Clinical characteristics of adult and elderly ALL

The analysis of the haematological parameters at clinical presentation such as haemoglobin level, total white count and platelet was divided into two groups, adult (18-49 years) and elderly (>50 years) respectively. For haemoglobin (Hb), the mean value for adult ALL was 7.54 ± 3.43 g% and for elderly the mean value was 10.11 ± 1.86 g%. A study conducted in Bangladesh which studied ALL adult patients demonstrated Hb mean level of 8.31 g%, (Islam et al., 2012) which is rather similar to the findings in this study. Another study which

comprised of 455 acute leukaemia patients found higher percentage of deaths (65.4%) amongst those with Hb count in the range of 5-10 g/dl (Parvin Ayremlou et al., 2012).

The mean total white count, for adult ALL in this study 210.74 ± 469.89 ($X10^9$/L) while for elderly the mean was 89.7 ± 97.33 ($X10^9$/L). The above findings demonstrated the wide range of TWC found (leucopenia to frank leukocytosis) among patients at presentation. A study on 609 recurring ALL among adults, demonstrated that at least 40.72% (248 cases) of patients with TWC of less than 10 $X10^9$/L, different with the result in this study (Adele K. Fielding et al., 2007). The mean values of white cell count for both groups in this study were more than 50 $X10^9$/L. An ALL study conducted in Bangladesh had a mean TWC value 52.78 $X10^9$/L (Islam et al., 2012), and was much lower than those obtained in this study. In several studies the values achieved for initial WBC count were $50\times10^3/\mu L$ (Gaynon PS et al., 1998, Chang H et al., 2004) and $10\times10^3/\mu L$ (JE, 2002, Bonilla M et al., 2010, Pulsoni A et al., 2008). The mean TWC obtained for both adult and elderly groups in this study were notably higher than the above values. It was demonstrated that leukocyte count of < 30 $\times10^3/\mu L$ had no significant effect in outcome, but leukocyte count of > 50 $\times10^3/\mu L$ was associated with a poor outcome (Mohammad Ali Mashhadi et al., 2012). Yanada et al (2007) found that in a series of T-ALL adults, patients with initial WBC count of 3-5 $\times10^3/\mu L$ had longer survival rates than others. It would be interesting to see if those with high count (>50 $\times10^3/\mu L$ leucocyte count) has a poorer outcome, but due to logistic difficulty in tracing the patients records (such as patient mobility: transfer from one hospital to another) during his/her care, this analysis was not performed.

For platelet, the mean value for adult ALL group was 76.56 ± 113.15 ($X10^9$/L) and for the elderly group the mean was 102.0 ± 78.45 ($X10^9$/L). The mean platelet count demonstrated in 30 adults with ALL was 76.8 $X10^9$/L (Islam et al., 2012), which was almost similar when

compared to the findings from the adult group in this study. Another ALL treatment study demonstrated 39% of the 204 ALL patients studied, had platelet count below 100 $X10^9$/L (By Hagop M. Kantarjian et al., 2000). The platelet count of less than 30,000 was identified as one of the poor prognostic factors in an adult ALL Iranian study (Mohammad Ali Mashhadi et al., 2012).

It was also noted that in this study, 32.3% of adult ALL patients had lymph nodes enlargement, 41.9% had liver enlargement and 45.2% had splenomegaly at time of diagnosis. Other reports also supported this finding such as in the Pakistan study which revealed that 35.2% and 18.5% of ALL patients had enlarged lymph nodes and liver, respectively, with 63.0% of patients had splenomegaly (Shaikh M U et al., 2011a). A Singapore study also reported that lymphadenopathy (35.5%), and organomegaly (18.5%) were common ALL clinical manifestation (Shaikh M U et al., 2011b). Another study conducted in Iran also showed concordant findings with this study with lymphadenopathy and organomegaly detected in 25.8% and 16.7% of patients, respectively (Mohammad Ali Mashhadi et al., 2012).

4.2.2.1 (a) Association between selected Hb level, Total white cell and platelet count and gender in adult ALL

The association between selected haematological parameters Hb level, Total white cell and platelet count and gender in adult ALL was investigated to compare their mean with the patient's gender. The analysis found that there was no association between these parameters and gender in adult ALL. In one study in Iran, although gender association was not analysed, it was discovered that there was no significant relationship between death and hemoglobin and platelet levels, though it was observed that most deaths occurred in patients who

presented with fever and high initial WBC count (more than $10.0\times10^3/\mu L$). Less number of deaths (13.0%) was reported in the group with initial WBC of $5.0\text{-}10.0\times10^3/\mu L$ (Ayremlou et al., 2012). Another study stated that having WBC count ($> 50.0\times10^9/L$) and the male gender were found to be influencing the survival outcomes in adult ALL studied (Shaikh M U et al., 2011a).

4.2.2.1 (b) Association between age, ethnicity and gender in adult ALL

The association between age, ethnicity and gender in adult ALL was investigated to compare the mean age of diagnosis with the patient's gender. In the analysis, there was no association found between gender, age and ethnicity. However, one study indicated that risk factors that could influence survival outcomes in adult ALL included age (>30 years) and the male gender (Shaikh M U et al., 2011a). There is no local study to compare the association between ethnicity, age and gender in adult ALL, though one American study demonstrated that the incidence of adult ALL in Hispanics males and females between the ages of 20-54 were higher compared to white males and females of the same group (Sheeja T. Pullarkat et al., 2009).

4.2.2.1 (c) Association between ethnicity and gender in adult B-ALL and T-ALL

The association between ethnicity and gender in adult B-ALL and T-ALL was also investigated. In T-ALL group, this analysis was not performed due to insufficient data. The occurrence of B-ALL was found to be diagnosed more often in adult male patients of Malay/Melanau ethnicity (p=0.043). Other ethnics (Sarawak bumiputera, Chinese, others) however did not show any significant association with B-ALL incidence. Similar local study associating adult male Malay/Melanau or any ethnicity with B-ALL however was not found, therefore no similar comparison can be made. Incidence of ALL in certain ethnic group was

common as indicated in the American study which claimed that hispanics/latinos had higher incidence and mortality rates for acute lymphocytic leukemia (ALL) than non-Hispanic/Latino whites (Ries LAG et al., 2006, Matasar MJ et al., 2006).

4.2.2.2 Immunophenotypes of adult ALL

Immunophenotypic analysis of childhood AML includes the frequency analysis of immunophenotypes at presentation. In general, expression of immunophenotypic antigens between childhood and adult patients were not much different. Frequency analysis of adult ALL diagnosis showed the significant expression of CyD79a+ (82.8%) (B-ALL marker), CD19+ (79.3%) (B-ALL marker), CD45+ (72.4%), CD34 (58.6%), CD22 (58.6%), nTdT (58.6%) CD38 (51.7%) and CD10 (48.3%). This result demonstrated similarity to several other immunophenotyping studies where high expression of the above mentioned antigens was found (Bayard L. Powell et al., 1999, Renate Thalhammer-Scherrer et al., 2012, Legrand et al., 2000).

Meanwhile, antigens such as CyMPO (86.2%) (aberrant marker), CyIgM (65.5%) , CyCD3 (62.1%), CD7 (58.6%) and CD33 (48.3%) were also expressed, though in most cases showed weak expressions. CyCD3 and CD7 were positively expressed in all T cell-lineage ALL cases, as shown in a study (Thalhammer-Scherrer et al., 2002). Another study demonstrated low expression of CD7 (2%) displayed in B-lineage ALL and CD33 (2%) in T-lineage ALL, in which both were aberrant markers for the respective lineages (MS et al., 1999). The low expression of CD7 could be caused by the low number of adult T-ALL cases recruited in this study. CyCD79a, CyCD3, CD10, and CD2, which were typical of either B - or T cell–lineage ALL were reported as significant immunologic markers for ALL (Thalhammer-Scherrer et al., 2002). There was one atypical antigen that had both strong and weak expressions, which was CD13, an aberrant marker. It was common for myeloid antigens to be expressed in ALL

patients, where there were significant differences of the myeloid antigen and CD13 between the older adults and younger adults (Ma et al., 2010). Co-expression of myeloid antigens in ALL was common, where 18.0% of the myeloid antigens was expressed in ALL patients (Thalhammer-Scherrer et al., 2002). Co-expression of myeloid markers were associated almost exclusively with early precursor ALLs (Drexler and WD, 1993).

In adult B-ALL, antigens that displayed significant expression were CD19+ (100%), CDCy79a+(100%), and CD22+ (71.4%), CD10+ (57.1%). These are all B-ALL markers, whilst CyIgM (B-ALL marker) and CD33 (aberrant marker) were weakly expressed. One study demonstrated myeloid antigen co-expression of CD13 and/or CD33 in 282 of these patients, where leukemic cells of 25 patients (8.9%) were positive for at least one of the two markers (C Boucheix et al., 1994).

In a China adult B-cell acute lymphoblastic leukaemia study, the positive rates of CD19 and HLA-DR in 178 cases were 100% and the positive rate of CD33 in young adults was significant higher than that in adolescent (Ma J et al., 2010). One study on B-ALL discovered the most common aberrancies detected were abnormal expression patterns of CD34 and/or TdT, overexpression or under expression of CD10, CD22, HLA-DR and under expression of CD38 and CD45 (Weina Chen et al., 2007). Immunophenotypic aberrancies were defined as patterns of antigen expression on neoplastic cells that are different from those seen on hematogones. Hematogones exhibit a well defined spectrum of antigen expression as they mature (McKenna RW et al., 2001, RW McKenna et al., 2004).

For adult T-ALL, antigens that displayed significant expression were CD7+ (100%), CyCD3+ (100%), and CD5+ (80%). All of these are T-ALL markers while among weak expressions were also noted in CyMPO (100%), CD33 (80%), HLADR, CD8 (both 80%) and

CD4 (60%). One study with T lineage ALL patients showed the percentage of expression of antigens in studied subjects were as follows: CD7 (93%), CD5 (84%), CD38 (78%), CD2 (70%), CD4 (61%) and CD8 (55%) (MS et al., 1999), where some antigens showed similar expressions with this study. The expression of antigens in T-ALL for this study however may not be significant because of the small number of T-ALL cases studied (only 5 cases in total). It was shown in a previous study that the immunophenotypes of adult T-ALL were evidently heterogeneous in different ages, and expression with more aberrant phenotypes indicates poor prognostic significance in patients older than 35 years (Ma J et al., 2010).

A poor prognosis has been coupled to a pre-T phenotype (CD7+ and CD5+ CD2-, TdT+ HLADR-) in adults (Rossi et al., 1987). In adult T-cell ALL, expression of CD5 has been observed more frequently than expression of CD2 (Thalhammer-Scherrer et al., 2002, MS et al., 1999).

4.3 Minimal residual disease (MRD)

Minimal residual disease (MRD) is defined as a small numbers of leukaemic cells that remain in the patient after the treatment when the patient is in remission. The presence of minimal residual disease in the bone marrow of patients with acute myeloid leukemia (AML) following chemotherapy has been established by many studies to be strongly associated with relapse of leukemia (Adhra Al-Mawali, 2009, Campana D, 1999). This study aimed to study the association between the clinical presentations, immunophenotypic expressions and the presence of MRD.

4.3.1 Minimal residual disease in Acute Myeloid Leukaemia

For MRD in AML patients, out of 147 AML diagnosed, only 28 MRD cases (19.0%) were investigated after completion of the induction therapy. This number includes both childhood

and adult cases. In this study, most of the cases were lost during the study due to a variety of factors, such as patient mobility (transfer to other district hospitals) during further follow up visits and other reasons. The number of AML MRD cases studied was low compared to similar studies such as Wolfgang et al, San Miguel et al and Reading et al (Wolfgang Kern, 2008). From the total cases, 17 cases (60.7%) childhood AML and 11 cases (39.3%) were adult AML. In normal circumstances, the number of childhood AML cases were less compared to adult, though in this MRD study childhood AML cases outnumbered adult cases. This is probably due to geographical location of patients, defaulters and disease outcome of patients recruited into this study. A few reasons have contributed to the lower number of adult MRD patients in this study, such as failure of them to continue with induction therapy, deaths before the therapy and incomplete clinical data which have resulted in their data to be excluded from the study. This could be improved if some of the above issues could be addressed (for example defaulter rate) and systematic follow up of all these patients from all over Sarawak.

Non remission AML MRD cases (10.2%) were found to be slightly higher compared to remission cases (8.8%). There were also other cases of AML MRD status that were not specified in the patient's data, therefore made up the rest of percentage (81.0%). The numbers only represent for the patient's MRD status after completion of induction therapy, therefore that was not the final status of the patient since their status may change after the first MRD, like relapse or even death after remission.

These AML MRD cases were investigated with statistical tests in order to compare the clinical presentations with the MRD cases. From the test, all the clinical presentation factors such as age, blast flow, full blood count (haemoglobin, total white count, platelet), gender, ethnicity, lymph node (LN) spleen and liver were found to be not significantly different

between patients with MRD and those who had achieved remission. This suggested no association between these variables with the presence of MRD. The limited number of AML cases who were investigated for MRD could have contributed to the above findings. In other study, univariate analysis of prognostic factors revealed that, in addition to MRD levels, low total white count (less than or greater than or equal to 50 X 10^9/L) was significantly associated with shorter relapse-free survival. The same study also found that patients with low TWC (< 50 X 10^9/L) counts also had lower levels of MRD than patients with higher absolute numbers of leukocytes (Jesús F. San Miguel et al., 2001).

Besides clinical presentation, immunophenotyphic expression was also investigated using the same statistical tests in order to find the association of antigens expressions with MRD status. None of the following antigen; CD34, CD33, CD13, HLADR, CyMPO, CD117, CD10, CD14, CD15, CD7 and CD64 were found to be significantly associated with MRD status. A study showed that CD2, CD56, CD7, CD19, and CD11b were the most useful markers for detection of MRD (Adhra Al-Mawali et al., 2008), and these antigens were usually aberrantly expressed in AML. Kern et al (2004) monitored MRD in AML patients in complete remission after induction and consolidation therapy and found that the degree of reduction in cells expressing aberrant immunophenotypes was significantly and independently related to treatment outcome. The immunophenotype of AML is generally more heterogeneous than ALL, and it is common to observe several immunophenotypic subsets at the time of diagnosis. This heterogeneity may complicate the selection of the most appropriate markers and gates to use for MRD studies (Campana D, 1999).

The clinical presentations and immunophenotyphic expressions variables were analysed with multiple logistic regression (MLR) in order to find the odds ratio between the variables or the interaction of variables with MRD status. From the analysis, six variables (blast,

haemoglobin, TWC, lymph node, CD15 and CD11b) were found to be potentially associated with presence of MRD, but none were significant. Therefore the odds ratio for these clinical variables association with the presence of MRD could not be determined. Also, there were no similar studies that could be compared with this finding.

4.3.2 Minimal residual disease in Acute Lymphoblastic Leukaemia

In ALL, a total of 77 cases were investigated for MRD after completion of induction therapy. This number includes both childhood and adult cases. The number of cases was higher than AML cases relatively, but overall the number was still low as compared to the diagnosed cases. The main factors that may have contributed to this, was believed to be the same as mentioned previously in AML. Other MRD studies demonstrated higher number of cases such as in a study by Vidriales et al (2003), where a total of 102 cases were recruited for ALL MRD study and 110 cases in another separate study (Forida Y. Mortuza et al., 2002).

From the total of ALL MRD cases in Sarawak, 67 cases (74.4%) were childhood ALL and 10 cases (31.3%) associated with adult ALL. Higher cases of childhood MRD cases correlated with the higher number of childhood ALL cases at diagnosis recorded in this study. Furthermore, the prevalence of ALL was more common in children than adults (Society, 2011). In the ALL, 81.8% of cases more were found to be in remission as compared to 18.2% which failed to achieve remission after completion of induction therapy. This is supported in other studies, as ALL cases did achieve good response to treatment which may be due to better treatment protocol and good supportive treatment, received by the patients during the treatment. Also, as in AML, the limitation for MRD status for this study was until the first MRD assessment only.

All ALL that were studied for MRD were analysed using the appropriate statistical tests in order to study the different factors that may influence the MRD status of these patients. There is no significant association found between the clinical presentation features such as age, blast count, full blood count (TWC and Plt), gender, ethnicity, lymph node, spleen, spleen and liver enlargement and MRD status (remission and no remission). Only haemoglobin level was found to have a strong significant association with MRD ($p<0.001$*). However, there was no similar study that could be compared to support this observation. The association of MRD status with low Hb level could imply a higher degree of marrow infiltration/involvement in these patients, which was then translated as low Hb level.

A careful selection of cases for long term MRD follow up and a more systematic follow up care may enable researchers to recruit more patients and further support this observation. Besides clinical presentation, immunophenotyphic expression was also investigated using the same statistical tests in order to find the association of antigens expression with MRD status. There is no significant association found between expressions of the 15 ALL antigens (CD34, CD45, CD20, CD19, CD10, Cy79a, CD38, CD13, CD33, CyIgM, nTdT, CD22, CyMPO, CyCD3 and CD7) with MRD status. However, a previous study demonstrated that the immunologic evaluation of MRD at day +35 was the most relevant independent prognostic parameter for adult patients with ALL, and together with age, white blood cell (WBC) count at diagnosis and presence of the Philadelphia (Ph) chromosome, represented the most informative combination of variables for predicting relapse-free survival (María-Belén Vidriales et al., 2003). Another cause of false-negative results during monitoring for residual leukaemia was clonal evolution during and after treatment, which may cause the disappearance of one or more of the markers detected at diagnosis, as suggested in several studies. For example, Pui et al described loss of CD10 and TdT in ALL cases studied (Pui CH

et al., 1991), while Raghavachar et al. (1987) reported loss of CD1, CD2, TdT and a decreased percentage of CD20 cells in their study.

The clinical presentations and immunophenotyphic expressions variables were analysed with statistical test in order to compare with the MRD cases. From the test, all the clinical factors and immunophotyping expressions such as Hb level, splenomegaly, CD45, CD10, CD79a, nTdT, CyCD3 and CD7 were found to be not significantly different, but only haemoglobin (p =0.007) and splenomegaly (p=0.049**) w**ere significantly associated with MRD status**.** The analysis suggests that patients with low haemoglobin would have 1.62 times of odds of having MRD, and patients with spleen enlargement would have the probability of 37.98 times of having MRD compared to those without spleen enlargement**.** There was no similar study that can be found to be compared with finding, but there were other factors which was associated with MRD as mentioned in several studies. For instance, one study conducted in China demonstrated that T-ALL was associated with a high TWC, increased age, more central nervous system involvement, a poor drug response, and high MRD (Gao C et al., 2012). A study by E. Coustan-Smith *et al* (2000), reported that the rates of MRD detection on completion of induction therapy were not significantly related to gender, race, leukocyte count, presence of a mediastinal mass, or central nervous system status. However, the residual disease was significantly more frequent found in infants and patients 10 years of age or older than in children of intermediate ages (E. Coustan-Smith et al., 2000).

CHAPTER 5: CONCLUSIONS

In this study, a total of 266 acute leukaemia that consisted of AML and ALL patients were recruited from all over Sarawak from 2007 till 2010. Only those samples that met the study criteria were chosen. The samples used were bone marrow aspirates and in a proportion of cases were peripheral blood. A 4-colour flow cytometric immunophenotyping was performed on all the samples and the results were analysed accordingly.

For AML, 147 cases were recruited, with 33.3% childhood and 67.0% adult cases respectively. The incidence of adults AML was more when compared to childhood AML, and this distribution correlated well with the previous AML studies that reported that AML was more commonly found in adults than children. A higher incidence of AML among the male gender as compared to females were also found in AML, both for childhood and adult patients, and in tandem with other published studies. In this study, it was found that the Sarawak Bumiputera had the highest incidence of AML in both adult and childhood groups compared to other ethnic groups in Sarawak such as Malay/Melanau, Chinese and others. The main factor that might have contributed to the outcome of this analysis was most likely due to the distribution of Sarawak Bumiputera that forms the majority of Sarawak population. High total white count (TWC) in AML patients in this series was found to be significantly associated with the female gender (p =0.008) in childhood AML, but no significant gender association was found in adult AML.

The above results suggested that gender in childhood AML may contribute to the prognostic outcome, and similar studies in a bigger Malaysian population would be more appropriate. For immunophenotyping results, all typical AML markers were expressed in high frequency (over

80.0%) in both age groups, such as CD33, CyMPO and CD13. The antigen expression pattern was almost similar in both adult and childhood groups, and a proportion of cases did express aberrant antigenic surface antigens like in CD56 and CD7 (over 50.0%). The AML markers chosen in this study may be established as standard markers since the immunophenotypic expressions were also supported by previous published studies.

As for the ALL series, 122 cases were recruited, which included both B cell and T cell subtypes. In Sarawak, childhood ALL (65.5%) was found to be more common compared to adult ALL in terms of incidence and similar to other published studies. B-ALL cases (90.0%) contributed to most of the ALL cases in this study, and T-ALL cases represented only the minority of cases (10.0%). In both childhood (54.4%) and adult (62.1%) ALL groups, males were found to be more commonly affected than females. More Malay/Melanau males are affected in both childhood and adult groups as compared to other ethnic groups in Sarawak, though the association was not statistically significant. There was no association found between gender and other clinical parameters at disease presentation. For the ethnic group, the Malay/Melanau adult males were found more likely to be diagnosed with B-ALL (p=0.043). For immunophenotyping results, the markers that were frequently expressed in majority of cases (over 70.0%), were CyCD79a, and CD19. The antigen expression pattern was almost similar in adult and childhood group. Most of antigenic expressions that were expressed in high frequency were B-ALL typical markers. The T-ALL markers were also expressed but in low frequency due to the lower incidence. As in AML, both the B-ALL and T-ALL markers used in this study may also be established as standard markers since the immunophenotypic expressions were supported by previous published studies.

Minimal residual disease analysis was also done to find the association between the independent variables (age, gender, race), some haematological parameters (Hb, level, TWC and platlet count) and immunophenotypic expression with the MRD status in both AML and ALL. For AML, twenty-eight cases fully investigated for MRD and consisted of both childhood and adults groups patients. All the clinical parameters of disease presentation and their immunophenotypic expressions were analysed using multiple logistic regression to find their association with MRD. None of the parameters analyzed was significantly associated with the MRD status at the end of study. The low number of samples recruited for AML MRD study may be one of the reasons for these findings. As mentioned previously, patients lost during follow up visits could be the main contributor to the above results.

As for ALL, 77 cases of ALL were recruited for MRD analysis and consisted of both the B and T subtypes after completion of the induction therapy. Most of the ALL MRD cases studied for MRD status were childhood cases (74.4%), with only a small percentage from the adult group (25.6%). In the analysis of clinical parameters in association with MRD status, it was found that only the haemoglobin level was shown to be significantly associated with MRD. It suggested before that a lower Hb level at presentation may signify possibility of a positive MRD after completion of induction therapy amongst ALL patients. This also may imply a higher degree of marrow infiltration in the patients which was translated into low Hb level and possibly poorer outcome. As for the immunophenotype expressions, all 15 antigens commonly expressed in ALL were found not to be significantly associated with ALL MRD status. In the multiple logistic regression test, of all the variables analysed, both haemoglobin level and splenomegaly were found to be significantly associated with ALL MRD outcome. It was shown that patients with

low haemoglobin levels had an odd risk of 1.68 of having minimal residual disease after completion of induction chemotherapy. It was also shown that, with the odd risk of splenomegaly associated with MRD positivity were 37.98 times more likely to have minimal residual disease in their bone marrow after completion of induction chemotheraphy, and also higher compared to haemoglobin level odd risk value (1.62 times). This finding implied that the advanced disease at first presentation may determine the minimal disease status after induction chemotherapy, which can be explained by probable greater degree of marrow infiltration.

5.1 Limitations of study

There were several limitations encountered in this study. The main limitation was the difficulty to obtain the patients data, due to many reasons stated previously. This also includes the inconsistencies encountered during follow up for obtaining the data for MRD patients, which contributed to low number of MRD cases, especially in ALL. These limitations affected the data analysis. The high percentage of non-specified MRD status was due to the incomplete data that was received from the hospitals. Other limitation was the usage of only three/four colour flow cytometry system instead of six colours, because higher cost needed to upgrade the system and also to purchase the monoclonal antibodies.

5.2 Recommendations of study

Greater effort to coordinate leukaemia patients treatment and follow up in Sarawak may yield better understanding of the factors that may influence the outcome of acute leukaemia patients in Sarawak, and Malaysia as a whole. It is recommended that a more comprehensive study regarding MRD in acute leukaemia patients be undertaken to provide the health providers with

more concrete and representative data about the prognostic factors that may influence acute leukaemia outcome in Malaysia. These data could be useful in disease and patient stratification in acute leukaemia and would help clinicians in patient treatment and management in the future. It would be desirable that a central database for all acute leukaemia patients and their follow up details could be created to enable valuable data regarding their disease outcome to be kept. This effort could benefit patient's treatment in the future as it would serve as a database of acute leukaemia outcome in Sarawak, and Malaysia as a whole.

Future study could also be extended to follow up relapse, the second and even third MRD assessment leukaemia patients. With better management and consistency in handling the patient's data, survival analysis study of the leukaemia patients especially the MRD patients with the usage of more than 4-colour flow cytometry system can also be considered.

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