Automated Database and Pattern-guided Immunophenotypic Classification of Acute Leukaemia Using Next Generation Flow Cytometry

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Automated Database and Pattern-guided Immunophenotypic Classification of Acute Leukaemia Using Next Generation Flow Cytometry

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Abstract

Acute leukaemia is a heterogeneous group of disorders characterized by clonal expansion of abnormal haemopoietic cells arrested at early stages of maturation. Appropriate management of these disorders requires accurate diagnosis and classification based on the WHO classification of tumors of haemopoietic and lymphoid tissues. Despite several advances in molecular pathology, flow cytometry still remains an essential tool for diagnosis of this group of haematological disorders.

The aim of this study was to validate the EuroFlow acute leukaemia orientation tube (ALOT) and its corresponding database-guided interpretation tool using the FACS Canto II flow cytometer and following in-house cytometer settings, compensation and staining procedures. The study also aimed to use the principal component analysis (PCA) and automated population separator (APS) tools on the Infinicyt software to merge the standard flow cytometry files (FCS) from the in-house acute leukaemia panel in order to create and compare immunophenotypic profiles of acute leukaemia cases in this study.

A total of 24 specimens from 16 acute leukaemia cases were tested using both the in-house acute panel as well as the ALOT tube under validation. Results showed 100% agreement between the two methods as well as the ALOT automated database-guided interpretation tool compared to the conventional hematopathologist (expert-based) approach of results interpretation. Furthermore, the flow cytometry merged files and immunophenotypic profiles were successfully used to build in-house ALOT and acute leukaemia database-guided interpretation tools. Results of these newly developed tools showed 100% agreement

between the in-house database-guided tools and the conventional expert-based interpretation of flow cytometry results.

Unsupervised comparison of the immunophenotypic profiles of B- acute lymphoblastic leukaemia (B-ALL) cases in this study showed some overlap between some cases with similar immunophenotypic profile and subsequent cytogenetic translocations as well as cases presented with CNS involvement. Further analysis confirmed that all the cases with CNS involvement in this study were CD34 negative. Limitations of this new approach were discussed in more detail, including the additional costs of the ALOT tube and the automated database interpretation tool.

In conclusion, results of this study confirmed the validity of ALOT tube and its corresponding database using local cytometer settings and procedures. Results also showed that using next generation flow cytometry with automated database-guided interpretation tools is a promising approach that can facilitate standardised interpretation of flow cytometry results in future.

Declaration

I hereby declare that this thesis is a result of my own work and all experiments described herein were carried out at the department of haematology, Sidra Medicine under the supervision of Dr Nina Dempsey-Hibbert and Dr Sandra Sapia. Work other than my own is clearly referenced to the appropriate authors or their publication. No material contained herein has previously been submitted for a degree at this or any other university. The copyright of this thesis rests solely with the author. No quotation of it should be published in any format, including electronic/digital, or the internet, without the author's prior consent. All information derived from this thesis must be acknowledged correctly and in full.

Hani Bibawi

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Abbreviations

AF700	Alexa Fluor 700
ALL	Acute lymphoblastic leukaemia
ALOT	Acute leukaemia orientation tube
ALPS	Autoimmune lymphoproliferative syndrome
AmCyan	Anemonia majano Cyanine
AML	Acute myeloid leukaemia
APC	Allophycocyanin
APL	Acute promyelocytic leukaemia
APS	Automated population separator
APTT	Activated partially thromboplastin time
AUL	Acute undifferentiated leukaemia
B-ALL	B-cell acute lymphoblastic leukaemia
B-CLPD	B-cell chronic lymphoproliferative disorders
BCP	B-cell precursor
BCSH	British Committee for Standards in Haematology
BD	Becton Dickinson
BM	Bone marrow
BME	Biomedical Engineering

CAP	College of American Pathologists
CD	Cluster of differentiation
CLPD	Chronic lymphoproliferative disorder
CS&T	Cytometer setting and tracking beads
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CyMPO	Cytoplasmic myeloperoxidase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMA	Eosin-5-malemide
EU	European Union
FAB	French-American-British
FACS	Fluorescence-activated cell sorting
FCS	Flow cytometry standard files
FGFR1	Fibroblast growth factor receptor 1
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
FSC	Forward scatter light

Hb	Haemoglobin
ILSG	International lymphoma study group
IP	Immunophenotypic profile
IVD	In vitro diagnostic use
LST	Lymphoid screening tube
LWB	Lysed washed blood
MCL	Mantle cell lymphoma
MDS	Myelodysplastic syndrome
MFI	Mean fluorescence intensity
MLL	Mixed lineage leukaemia
MPAL	Mixed phenotypic acute leukaemia
MRD	Minimal residual disease
NICE	National Institute for Health and Clinical Excellence
NK	Natural killer
NOS	Not otherwise specified
Nu	Nuclear
PacB	Pacific blue
PB	Peripheral blood
PBS	Phosphate buffered saline

PCA Principal Component analysis

PCD Plasma cell disorder panel

PDGFRA Platelet derived growth factor A

PDGFRB Platelet derived growth factor B

PE Phycoerythrin

PECy7 Phycoerythrin Cyanine dye Cy7

PerCPCy5.5 Peridinin chlorophyll protein Cyanine dye Cy5.5

Plts Platelets

PMTVs Photomultiplier tube voltages

PT Prothrombin time

QC Quality control

REAL Revised European-American classification of lymphoid neoplasms

Rpm Rotation per minute

SOPs Standard operating procedures

SOVs Spill over values

SSC Side scatter light

SST Small sample tube

T-ALL T-cell acute lymphoblastic leukaemia

TAM Transient abnormal myelopoiesis

TDT Terminal deoxynucleotidyl transferase

Vol Volume

WBCs White blood cells

WHO World Health Organisation

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1. Introduction

1.1. Background and classification of acute leuaemia:

Acute leukaemias are heterogeneous group of diseases characterized by clonal expansion of abnormal haemopoietic cells arrested at very immature stages of differentiation (Swerdlow, 2008 and Swerdlow, 2017). Like all other diseases in medicine, acute leukaemias need to be described, defined and named before they can be diagnosed or treated (Swerdlow, 2008).

Early attempts to classify acute leukaemias included the widely used French-American-British (FAB) classification in 1976 (Bennett, 1976). FAB methodology provided a uniform system of acute leukaemias classification based on conventional morphological and cytochemical stains of peripheral blood and bone marrow samples. Despite its initial success and wide use, the classification lacked critical immunophenotypic, cytogenetic and molecular abnormalities in acute leukaemias which started to become more evident with advances in immunophenotyping and genetic technologies.

In 1994, the Revised European-American Classification of Lymphoid Neoplasms (REAL) proposed by the International Lymphoma Study Group (ILSG) set the path for a new classification of acute leukaemia to take place by including clinical, morphological, immunophenotypic, cytogenetic and molecular basis in lymphoma (Chan, 1995).

These principles presented the basis for the third edition of the WHO classification of Tumours of Haematopoietic and Lymphoid Tissues as the first true worldwide consensus classification

of haematological malignancies (Jaffe, 2001). When the 3rd edition of the WHO classification was published, previous proponents of other classifications of haematological neoplasms agreed to accept and use the new classification, ending decades of controversy over the classification of these tumours (Lukes, 1966; Bennett 1974; Bennett 1976; Rosenberg, 1982; Bennett, 1982; Chu, 1987; Bennett, 1994; Raphael, 1994 and Favara, 1997).

In 2008, the continued collaboration between the European Association for Haematopathology and the Society for Hematopathology led to the development of the 4th edition of the WHO classification of Tumours of Haematopoietic and Lymphoid Tissues (Swerdlow, 2008). In addition to over 130 pathologists and haematologists from around the world involved in writing its chapters, the advice of clinical haematologists and oncologists was also obtained to ensure that the classification was clinically useful (Swerdlow, 2008).

This process generated a new degree of cooperation and communication among pathologists and oncologists from around the world and facilitated better understanding and treatment approaches for haematological malignancies. The identification of antigens, genes, and pathways that could be targeted for therapies (e.g. rituximab as an anti-CD20 and imatinib as a tyrosine kinase inhibitor) were testament to this new approach (Swerdlow, 2008).

The most recent 2017 revised edition of WHO classification of Tumours of Haematopoietic and Lymphoid Tissues continued to categorize acute leukaemias on the basis of blast cells lineage in addition to morphological, clinical, cytogenetic/molecular lesions and, to less extent the detailed immunophenotype of tumour cells (Swerdlow, 2017). The main two

groups in this classification were acute myeloid leukaemia (AML) and related neoplasms and precursor lymphoid neoplasms. The latter was further subdivided into B- and T- cell precursor acute lymphoblastic leukaemia/lymphoma (BCP-ALL and T-ALL, respectively).

A small number of cases were categorised separately as acute leukaemias of ambiguous lineage as they do not fit any of the above two groups. This was either because they do not show clear evidence of differentiation to a single lineage (hence named acute undifferentiated leukaemia- AUL) or; because they express antigens highly specific to more than one lineage and therefore named as mixed phenotypic acute leukaemia (MPAL) (Swerdlow, 2008; van den Ancker, 2010 and Swerdlow, 2017). Table 1 below summarizes the major subtypes of acute leukaemias according to the revised 4th edition of the WHO classification of Tumours of Haematopoietic and Lymphoid Tissues (Swerdlow, 2017).

Table 1.1. WHO classification of Tumours of Haematopoietic and Lymphoid Tissues (Adapted from Swerdlow, 2017).

Acute myeloid leukemia (AML) and related neoplasms	**Precursor lymphoid neoplasms**
AML with recurrent genetic abnormalities	B-lymphoblastic leukemia/lymphoma, NOS
AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1	B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11	B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2);BCR-ABL1
APL with PML-RARA	B-lymphoblastic leukemia/lymphoma with t(v;11q23.3); KMT2A rearranged
AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A	B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); ETV6-RUNX1
AML with t(6;9)(p23;q34.1);DEK-NUP214	B-lymphoblastic leukemia/lymphoma with hyperdiploidy

Acute myeloid leukemia (AML) and related neoplasms	**Precursor lymphoid neoplasms**
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM	B-lymphoblastic leukemia/lymphoma with hypodiploidy
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1	B-lymphoblastic leukemia/lymphoma with t(5;14)(q31.1;q32.3) IL3-IGH
AML with BCR-ABL1	B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3);TCF3-PBX1
AML with mutated NPM1	Provisional entity: B-lymphoblastic leukemia/lymphoma, BCR-ABL1–like
AML with biallelic mutations of CEBPA	Provisional entity: B-lymphoblastic leukemia/lymphoma with iAMP21
Provisional entity: AML with mutated RUNX1	
AML with myelodysplasia-related changes	**T-lymphoblastic leukemia/lymphoma**
Therapy-related myeloid neoplasms	Early T-cell precursor lymphoblastic leukemia
AML, not otherwise specified	Natural killer (NK) cell lymphoblastic leukemia/lymphoma
AML with minimal differentiation	
AML without maturation	**Acute leukaemias of ambiguous lineage**
AML with maturation	Acute undifferentiated leukemia
Acute myelomonocytic leukemia	Mixed phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2); BCR-ABL1
Acute monoblastic and monocytic leukemia	MPAL with t(v;11q23.3); KMT2A rearranged
Pure erythroid leukemia	MPAL, B/myeloid, NOS
Acute megakaryoblastic leukemia	MPAL, T/myeloid, NOS
Acute basophilic leukemia	MPAL, NOS, rare types
Acute panmyelosis with myelofibrosis	Acute leukaemias of ambiguous lineage, NOS
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	
Transient abnormal myelopoiesis (TAM)	
Myeloid leukemia associated with Down syndrome	
Blastic plasmacytoid dendritic cell neoplasm	

Of important note, recent advances in high throughput genetic technologies such as gene expression profiling and next-generation sequencing have revealed new mechanisms and understanding of the tumorigenesis process as well as new potential therapeutic targets. It is likely that lineage plasticity will gradually start to give way to future tumorigenic and prognostic classifications similar to the current category of myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1.

1.2. The role of flow cytometry in haematopathology:

Despite advances in cytogenetic and molecular technologies, immunophenotyping of acute leukaemias using flow cytometry remains an essential diagnostic tool for many diseases in this group of malignancies (Davis, 2006; Stetler-Stevenson, 2007 and Swerdlow, 2017). Several immunophenotypic profiles have already been associated with unique cytogenetic and molecular abnormalities and their prognostic values (Orfao, 1999; De Zen, 2000; Tabernero, 2001 and Orfao, 2004). These have added more value to the diagnostic role of flow cytometry in haematological malignancies (Hrusak, 2002 and Vaskova, 2005).

Moreover, flow cytometry has increasingly been used for DNA aneuploidy and cell cycle studies. In haematological malignancies, particularly acute B-lymphoblastic leukaemia (B-ALL), this has a significant added value for differentiation of two distinct categories of B-ALL with hypodiploidy and its counterpart B-ALL with hyperdiploidy (Rachieru-Sourisseau, 2010).

In addition to its diagnostic role, immunophenotyping has also led to a better understanding of haematopoietic stages of maturation by comparing blast cells to the normal lymphoid, neutrophilic, monocytic, megakaryocytic and erythroid precursors (van Dongen, 2012). This has proven flow cytometry to be of great sensitivity for detection of minimal residual disease in a number of disorders including B-ALL, chronic lymphocytic leukaemia and some lymphomas (Szczepanski, 2001 and Theunissen, 2017).

With recent developments and expansions of antigen targeted therapy (e.g. CD20 by rituximab), flow cytometry is increasingly becoming a powerful tool for monitoring treatment for patients on antibody-based therapy (García-Marco, 2019). Immunophenotyping by flow cytometry also plays a key role in stem cell enumeration (CD34) and therefore remains an essential tool for all allogeneic and autologous stem cell transplantation procedures (Dauber, 2011 and Murugesan, 2019).

More recently, flow cytometric immunobead assays have successfully been developed for detection of fusion proteins in lysed leukaemia samples. These include assays for detection of fusion proteins for BCR-ABL, PML-RARA, TEL-AML1, E2A-PBX1, MLL-AF4, AML1-ETO and CBFB-MYH11 translocations (Weerkamp, 2009; Dekking, 2010 and Dekking, 2012). In these assays, immuobeads have been developed with capture-antibody for the specific fusion protein on one side and a fluorochrome-conjugated detection antibody on the other side. These assays can certainly be a very fast and easy tool for classification of acute leukaemia patients who express these fusion proteins (Dekking, 2012).

In addition to the above mentioned roles in the diagnosis of haematological malignancies, flow cytometry has also been used for non-malignant applications in haematopathology. These include but are not limited to; detection of red cell membrane defects (e.g. hereditary spherocytosis and elliptocytosis using Eosin 5 Malemide assay), immunological platelet counting, as well as platelet function/glycoprotein assays (e.g. for diagnosis of Bernard Soulier Syndrome and Glanzmann's disease) (Suemori, 2015 and Pasalic, 2017).

1.3. Standardization of Flow Cytometry in the diagnosis of haematological neoplasms:

Due to its significant role in diagnosis and follow-up of different disorders in haematopathology and the growing increase in clinically useful antibodies and fluorochromes, several studies have been published in the last two decades that have attempted to standardise panels, procedures and instrument settings for multicolor flow cytometry (Stewart, 1997; Wood, 2007; Kalina, 2012; van Dongen 2012 and Johansson, 2014). The main reasons behind those efforts were to provide some consensus between different laboratories performing assays based on different antibody panels as well as attempting to minimise subjectivity in results interpretation, which often depends on local knowledge and experience in different clinical diagnostic laboratories (Kalina, 2012). Additional challenges of standardisation included lack of specific recommendations on antibody clones, combination of fluorochromes, sample preparation and standard operating procedures as well as strategies for data analysis (Kalina, 2012 and van Dongen 2012).

In order to provide some consensus and to overcome these challenges, the British Committee for Standards in Haematology (BCSH) published the guidelines on the use of multicolor flow cytometry in the diagnosis of haematological malignancies in 2014 (Johansson, 2014). The guidelines provided key recommendations on instrument settings, multicolor tube design and validation, sample requirement and stability, staining procedure as well as data acquisition and analysis (Johansson, 2014). Two unique features of these guidelines were not included in other standardisation studies in such depth. Firstly, the integrated report approach highlighting the clinical context of the flow cytometry report following the National Institute for Health and Clinical Excellence (NICE) guidance (Johansson, 2014). These guidelines provided a clear template of the integrated report highlighting the value of clinical information, sample investigated, blood counts and morphology, cytochemical stains and immunophenotyping, cytogenetics and molecular findings as well as concluding notes.

A second feature of these guidelines was highlighting the training needs for both technical staff and laboratory scientists performing flow cytometry as well as recommendations for flow cytometry training resources (Johansson, 2014).

In another attempt to overcome the standardisation challenges in clinical flow cytometry, the European Union (EU) supported the EuroFlow Consortium to initiate a project aimed to design and evaluate panels for diagnosis and classification of the most frequent leukaemias and lymphomas (EU-FP6, LSHB-CT-2006-018708, Kalina, 2012). Following six years of extensive collaboration between multiple EuroFlow centers across Europe, the Consortium published

their recommendations on 8-color flow cytometry panels for normal and malignant leucocytes in blood and bone marrow. The EuroFlow protocols covered in extensive details the selection of appropriate fluorochromes combinations, laboratory standard operating procedures (SOP) for instrument settings and compensation, sample preparation and staining procedures as well as introduction of new tools for data analysis (Kalina, 2012).

As a result of this project, EuroFlow published a diagnostic algorithm including single-tube screening panels as well as multi-tube disease specific panels for final diagnosis and classification of various haematological malignancies (van Dongen 2012). The screening tubes included the acute leukaemia orientation tube (ALOT), lymphoid screening tube (LST), plasma cell screening tube (PCST) and small sample tubes (SST) for screening for lymphomas in small samples such as CSF and other body fluids (van Dongen 2012).

On the other hand, the multi-tube disease specific panels included the B- and T- cells precursor panels (BCP-ALL and T-ALL, respectively), acute myeloid leukaemia and myelodysplastic syndrome panel (AML/MDS), B-cells, T-cells and natural killer cells chronic lymphoproliferative panels (B-CLPD, T-CLPD and NK-CLPD respectively) as well as plasma cell disorder panel (PCD).

Figure one below summarises the proposed EuroFlow diagnostic flowchart for immunophenotypic characterisation of haematological malignancies (van Dongen 2012).

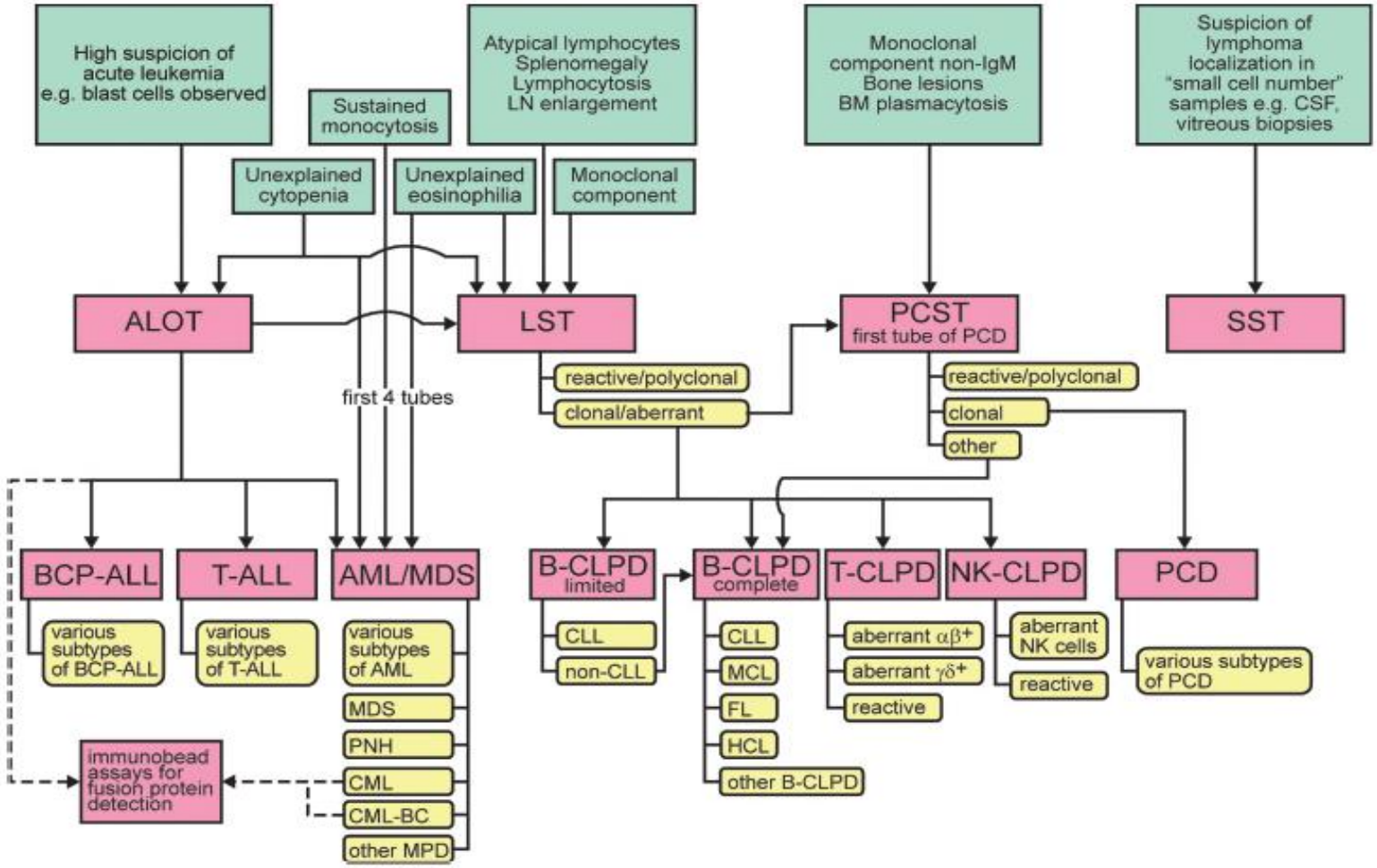


Figure 1.1. Flowchart diagram of the EuroFlow strategy for immunophenotypic characterization of hematological malignancies (Taken from van Dongen, 2012).

More recently in 2017, the EuroFlow Consortium published new guidelines for standardised flow cytometry for highly sensitive minimal residual disease (MRD) measurements in B-cell acute lymphoblastic leukaemia (Theunissen, 2017). The guidelines provided detailed laboratory procedures for an 8-color antibody panel to measure minimal residual disease (MRD) in B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) using two 8-color antibody tubes which allowed separation between normal and malignant BCP-ALL cells in 99% of studied patients. A new erythrocyte bulk-lysis protocol was described allowing acquisition of >4 million cells in B-ALL patients. Results from this study showed unprecedented sensitivity by flow cytometry of $\leq 10^{-5}$, comparable to the current real-time quantitative polymerase chain reaction (RQ-PCR) in 98% of samples with MRD <0.01%.

In this study, the development and validation of the acute leukaemia orientation tube (ALOT) will be discussed in more details.

1.4. Acute Leukaemia Orientation Tube (ALOT):

1.4.1. Background, Purpose and Development:

Acute leukaemia orientation tube (ALOT) was developed as part of the 6-year EuroFlow consortium project for standardisation of immunophenotyping in acute leukaemia panels (van Dongen, 2012). The purpose of this 8-color antibody panel tube is to provide the first screening step in a sample suspected of containing blast cells. This is to be followed by a more comprehensive multi-tube panel specifically designed for full characterisation of the malignancy depending on the ALOT tube results (namely, B- cell precursor panel for B–acute

lymphoblastic leukaemia (BCP-ALL), T- cell panel for T- acute lymphoblastic leukaemia (T-ALL) or acute myeloid leukaemia/myelodysplastic syndrome panel (AML/MDS) for non-lymphoid acute leukaemia) (van Dongen, 2012).

The ALOT (orientation) tube was therefore designed to provide a rapid and efficient initial assessment of the nature of the hematopoietic blast cells in a suspected acute leukaemia sample. Figure two below highlights the role of an ALOT tube in acute leukaemia diagnosis (van Dongen, 2012).

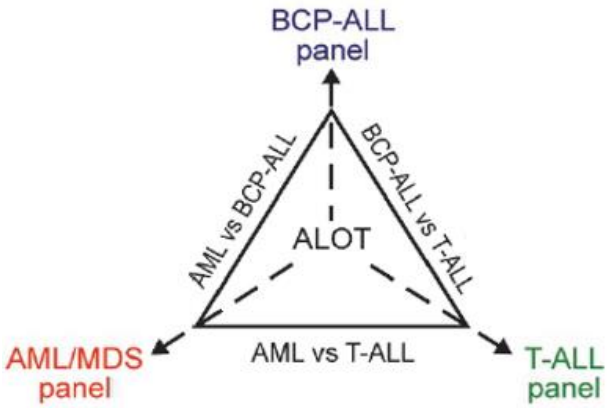


Figure 1.2. The role of an ALOT tube in acute leukaemia diagnosis (ALOT, acute leukaemia orientation tube; AML, acute myeloblastic leukaemia; BCP-ALL, B-cell precursor acute lymphoblastic leukaemia; MDS, myelodysplastic syndrome and T-ALL, T-cell acute lymphoblastic leukaemia (Taken from van Dongen, 2012).

1.4.2. Antibodies selection for ALOT tube:

In order to fulfil the orientation purpose of the ALOT tube above, antibody selection has to reflect specific cell lineages as well as sensitivity to recognise those antigens. An ideal specific

marker is an antigen constantly expressed by all cells of that lineage with no or minimal cross reactivity with other cell lineages (van Dongen, 2012).

Cytoplasmic CD3 (CyCD3) was therefore considered as the most specific marker for T-ALL (van Dongen, 1988). Exceptional cases of the relatively rare natural killer cell (NK) acute leukaemia also share this marker (van Grotel, 2008). When combined with surface membrane (Sm) CD3, natural killer cells (CyCD3+/SmCD3-) as well as T-ALL can easily be identified (van Dongen, 1988 and van Dongen, 2012). In addition to cytoplasmic and surface CD3, CD7 was also added to the ALOT tube as virtually positive in all cases of T-ALL despite being positive in a subset of AML (usually CyMPO negative AML cases).

Similarly, cytoplasmic myeloperoxidase (cyMPO) was selected as the most specific marker for myeloid lineage. Although other surface myeloid specific markers such as CD13 and CD33 were available, these were excluded as they can be found frequently in B-ALL or T-ALL (Khalidi, 1999; Hrusak, 2002 and Robert, 2007).

Surface CD19 was selected as a very sensitive B- cell marker as it is expressed in early B-cell lineage and virtually all BCP-ALL cases (van Dongen, 2012). Despite not being very specific for B-cell lineage as it is also co-expressed in a subset of AML cases, its expression on the myeloid lineage is at much lower and heterogeneous levels. More specific B-cell markers such as surface immunoglobulins were excluded as they are only expressed on a subset of B-cells at later stages of maturation and therefore lack sensitivity for B-ALL blast cells (van Dongen, 2012).

Consequently, cytoplasmic (Cy) CD79a was selected as a second B-cell marker to improve B-lineage assignment although it is also expressed in t(8;21) positive AML (Hurwitz, 1992; Kita, 1992; Tiacci, 2004 and Bhargava, 2007) and at low levels in some T-ALL cases (Pilozzi, 1998; Hashimoto, 2002 and Asnafi, 2004). CD22 was excluded from the ALOT panel selection as it is not lineage specific and expressed at high levels in basophils, mast cells and some dendritic cells (Escribano, 1998; Han, 1999 and Martin-Martin 2009).

In order to better isolate/ 'gate' the haemopoietic cells, CD45 was selected as the common leucocyte antigen marker based on its dim/low expression as well as its ability to exclude normal residual cells in the sample (Borowitz, 1993; Lacombe, 1997 and Vial, 2001).

Finally, options for immaturity markers included CD34, CD117 as well as nuclear (Nu) TdT. CD117 was excluded as it is more specific for the myeloid lineage and lacks sensitivity to B-ALL cases. The choice between CD34 and TdT was studied extensively in order to select the most appropriate immaturity marker. To achieve this, 34 cases of acute leukaemia (17 B-ALL, 11 AML and 6 T-ALL) were tested for both markers in parallel (van Dongen, 2012). Results showed no evidence in favour of one marker over the other (15% CD34 positive/TDT negative and 15% CD34 negative/TdT positive). Although this highlighted the complementary role of both markers, constraints of 8-color cytometry only allowed the option for one additional immaturity marker to be added. CD34 was selected over TdT as it is less specific for one cell lineage and therefore has the advantage of identifying immature cells across all cell lineages. A second advantage of CD34 was it only requires surface staining allowing it to be used as a

backbone in the subsequent BCP-ALL panel. On other hand, TdT staining requires cell permiabilization which was not possible for the subsequent confirmatory B-ALL panel.

Table 1.2. Summary of antibodies in the ALOT tube and their diagnostic values

Antibody	Key Cell lineage	Main Diagnostic Value
CD19	B-cell lineage	B-ALL and subsets of AML
CD79a	B-cell lineage	B-ALL and subsets of AML and T-ALL
CD45	Common leucocyte antigen	Separate blasts and residual normal cells
CD34	Immaturity marker	Blast cells identification
CyCD3	T-cell lineage	T-ALL and NK neoplasms
SmCD3	T-cell lineage	T-ALL and NK neoplasms
CD7	T-cell lineage	T-ALL and subsets of AML

Abbreviations: B-ALL, B-cell acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; T-ALL, T-cell acute lymphoblastic leukaemia.

1.4.3. Evaluation of ALOT antibodies combination:

The final design of the ALOT tube required five consecutive rounds of evaluation to ensure sufficient backbone markers were included for the subsequent EuroFlow acute leukaemia panels as well as appropriate fluorochrome labelling of the antibodies to allow optimal detection of antigen expression. This led to the ALOT tube being developed in close synergy with the subsequent EuroFlow confirmatory panels (BCP-ALL, T-ALL and AML/MDS) (van Dongen, 2012).

Accordingly, CD45, CD34 and CD19 were selected as backbones for the subsequent BCP-ALL panel, while CD45, CyCD3 and SmCD3 served as backbones for the T-ALL panel. Finally, CD45 and CD34 were considered the two backbones for the AML/MDS panel.

In total, 385 acute leukaemia samples were tested before the ALOT tube was considered to be in its final configuration. Table 2 below summarises the contents of the ALOT tube following evaluation as well as the specific backbones for subsequent EuroFlow panels. An additional, 158 acute leukaemia samples (89 B-ALL, 27 T-ALL, 37 AML and 5 AUL/MPAL) were further used to validate the final ALOT configuration above. Results demonstrated good correlation between the in-house panels and the ALOT tube across all EuroFlow laboratories (van Dongen, 2012).

Table 1.3. Final design of ALOT tube with inclusion of common backbone markers with the subsequent acute leukaemia panels. (Adapted from van Dongen, 2012).

Label	PacB	PacO	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7
Antibody	CyCD3^{T}	CD45^{B,T,M}	CyMPO	CyCD79a	CD34^{B,M}	CD19^{B}	CD7	SmCD3^{T}

Abbreviations: APC, allophycocyanin; B; B-ALL marker; APC-H7, APC-cyanine tandem dye; FITC, fluorescein isothiocyanate; M, AML/MDS marker; PacB, pacific blue; PacO, pacific orange; PE, phycoerythrin; PE-Cy7; PE cyanine tandem dye; T, T-ALL marker.

1.5. Automated database guided analysis and Next Generation Flow Cytometry:

1.5.1. Merging Flow Cytometry data files using Clinical Software:

One of the limitations of flow cytometry currently used in routine diagnostic laboratories is the increasing number of phenotypic criteria used in the WHO classifications with relatively limited technology to accommodate enough markers in the same tube to represent the overall phenotype of cells of interest (DiGiuseppe, 1998; Braylan, 2001; Matutes, 2002; Braylan, 2004 and Kaleem, 2006). This resulted in diagnostic panels mostly consisting of 15-25 markers (Sanchez, 2003; Gervasi, 2004 and Sanchez, 2006) with multiple markers repeated in several tubes (e.g. CD45, CD34 and/or CD19) to aid subsequent gating and interpretation of the results (Pedreira, 2008). In order to overcome this limitation in technology, several studies described the development and validation of different clinical software tools for merging and calculation of flow cytometric data files from several tubes into a single "*super*" multicolor file with all required markers (Robinson, 1991; Robinson, 1992; Costa, 2006 and Pedreira, 2008).

In one study, 60 cases diagnosed with B-cell chronic lymphoproliferative disorders (B-CLPD) were analysed using actual measured parameters from different tubes (including mean fluorescence intensity (MFI) and coefficient of variation -CV) versus calculated data produced from the merged file using the Infinicyt software program (Cytognos, Salamanca, Spain) (Pedreira, 2008). In this study, a total of 17 markers were used to identify abnormal populations as well as residual normal cells in each sample. Results showed a high degree of

correlation between measured and calculated data from the merged file (r^2 >0.91 to 1.00) allowing the opportunity for further significant expansions in this area of software development (Pedreira, 2008). Figure 3 below demonstrates the expression of one marker (CD19 in this case) in different tubes following merging of data files using Infinicyt software.

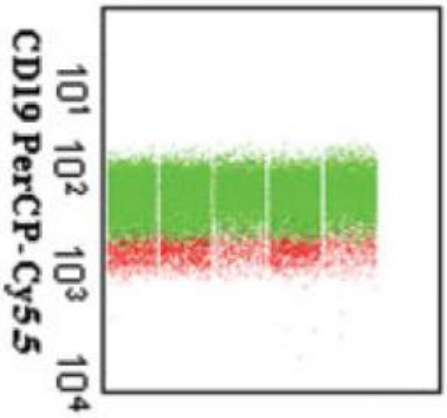


Figure 1.3. The expression of CD19 in five different tubes following data file merge. Each bar represents a different test tube. In red, CD19 expression in each tube (Taken from Pedreira, 2008).

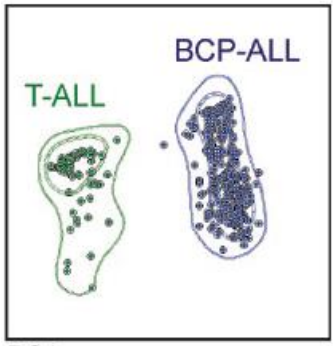
1.5.2. Principal Component Analysis (PCA) and Automated Population Separator (APS):

The ability to merge files has helped visualisation of all parameters (markers) in an experiment into a single automated population separator (APS) diagram (Costa, 2010).

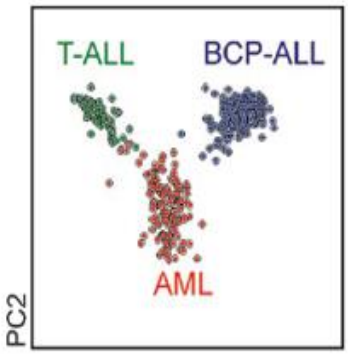
Unlike the widely used two dimensional (2D) dot plot diagrams in flow cytometry, the APS diagram uses a Principal Component Analysis (PCA) algorithm to display the best separation of all events into clusters based on "*all*" markers in the experiment (not two parameters at a

time) (Costa, 2010). For example, an eight color experiment will have ten parameters' properties displayed on one APS diagram (8 antibody markers plus FSC and SSC).

The horizontal axis (X-axis) of this APS diagram (also known as PC1) highlights the intergroup differences of the populations (e.g. B versus T-cells in figure 4a below). Whereas, the vertical axis (Y-axis) of the APS diagram (also known as PC2) displays the intragroup heterogeneity (e.g. AML vs T-ALL and BCP-ALL in figure 4b below (i.e. myeloid vs lymphoid).



a.



b.

c.

	PC1		PC2
CD19	24%	CyMPO	29%
CD7	24%	CD19	19%
CyCD3	19%	CD7	15%
CyCD79a	13%	CyCD79a	11%
CD34	11%	CyCD3	11%

Figure 1.4. Automated population separator diagram (APS) showing clear distinction between well-defined blast cells in different cases of acute leukaemia. Each dot in the diagram represents a separate case of acute leukaemia after merging all files using the Infinicyt software. The degree of separation (value) caused by each marker in the experiment can easily be seen as a percentage value in the APS diagram configuration (4c). AML= acute myeloblastic leukaemia; BCP-ALL= B-cell precursor acute lymphoblastic leukaemia and T-ALL=T-cell acute lymphoblastic leukaemia (Taken from van Dongen, 2012).

In summary, development of the principal component analysis (PCA) and the automated population separator diagram (APS) have contributed to better separation of different cell

populations in the experiment allowing identification and comparison of all cells in one diagram (Figure 1.5).

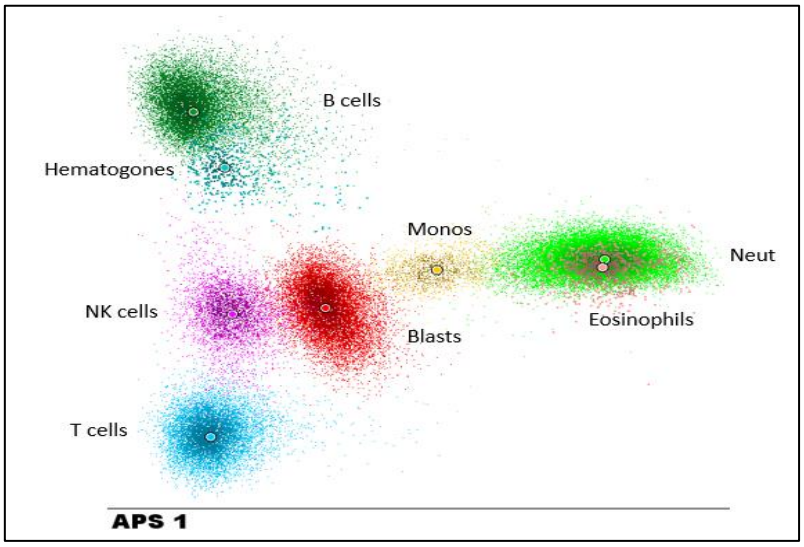


Figure 1.5. Automated population separator diagram showing blast cells in addition to normal residual populations in a bone marrow specimen with acute myeloid leukaemia.

1.5.3. Automated classification and interpretation of clinical flow cytometry using Principal Component Analysis (PCA):

Despite several advances in flow cytometry for standardisation of reagent composition, sample preparation, instrument set-up and data acquisition (Kalina, 2012; van Dongen, 2012), final interpretation of the results still relies on local individual expertise and relatively arbitrary criteria for identification of leukaemic cells and definition of positivity for individual markers (Lhermitte, 2018).

Development of a pattern-guided Principal Component Analysis (PCA) has led to further developments in automated classification of B-cell chronic lymphoproliferative disorders (Costa, 2010). This was mainly achieved by merging all antigen expression data files for each case to create a single immunophenotypic profile (IP) (i.e. merged file or "signature") of the malignant cells using the EuroFlow standardised panels and the Infinicyt software (Costa, 2010).

This was followed by creating a reference database (bank) for some of the common B cell chronic lymphoproliferative disorders (B-CLPD). In order to create the reference database, three groups of B-cell chronic lymphoproliferative disorders (B-CLPD) (namely; B-cell chronic lymphocytic leukaemia (B-CLL; n=10), mantle cell (MCL; n=10), follicular lymphoma (FL; n=10)) were randomly selected from a total of 205 B-CLPD cases and were used to build a reference phenotypic profile for each sub group using merged data files and PCA principle (Costa, 2010).

Subsequently, a total of 175 cases (testing set) were evaluated and electronically assigned (using the automated PCA-analysis tool) to one of the three reference groups above or, to a fourth group named as "other B-CLPD" based on their specific immunophenotypic profile (IP). Evaluation of the automated PCA-analysis against the established expert-based diagnosis and the WHO gold standard criteria showed that 89% of the cases were correctly assigned to the right group describing a new promising tool for automated interpretation of clinical flow cytometry for lymphoproliferative disorders (Costa, 2010).

1.5.4. Development and validation of automated database for orientation and diagnosis of acute leukaemia:

Similarly to the automated standardised interpretation of B-CLPD above, more recently the EuroFlow scientific consortium reported the design, construction and validation of an automated database-guided analytical algorithm for diagnosis and classification of acute leukaemia (Lhermitte, 2018). In order to build a reference database, results from 656 acute leukaemia patients stained with the EuroFlow acute leukaemia orientation tube (ALOT) were analysed and merged using the Infinicyt software (Cytognos, Salamanca, Spain) to create an individual immunophenotypic profile (IP) for each case. Depending on the final diagnosis, the data was sub-grouped to T, B, Myeloid groups or to other mixed-lineage groups (Lhermitte, 2018).

To validate the database, the principal component analysis (PCA) and automated population separator diagram (APS) were subsequently used to analyse a further 783 acute leukaemia cases in comparison to the reference database. Results showed that 781/783 cases (99.7%) correctly identified the cell orientation and the subsequent immunophenotyping panel required for final diagnosis in comparison to the traditional expert-based decision (Lhermitte, 2018). Furthermore, results showed that the correct cell lineage and final diagnosis were already established in >93% of the cases even without the subsequent full characterisation panels. These findings showed that the automated database-guided analysis can be used as

a powerful tool to standardise and automate the interpretation of the ALOT tube results (Lhermitte, 2018).

1.6. Purpose and justification:

Sidra medicine is a newly opened 400-bed women's and children's hospital in Qatar. The hospital opened officially for inpatients in January 2018 with roughly 3900 staff members (Sidra, 2020). Over the past two years, more than 40 new tests were validated and activated to support the patient needs at the department of haematopathology. The hospital developed very rapidly to obtain the full accreditation of the internationally recognised College of American Pathologists (CAP) in 2019. This was in addition to all other mandatory requirements of the local department of health as well as higher education institutions.

The next phase of expansion for the department of haematopathology was to introduce flow cytometry services. This was essential for acute leukaemia diagnosis; particularly in a paediatric setting; as well as lymphocytes subsets for primary immune-deficiencies. Other flow cytometry based tests included but not limited to: platelets glycoproteins for assessment of platelet function, eosin-5-malemide (EMA) assay for red cell membrane defects, DNA index, CD34/stem cell enumeration as well as neutrophil function test.

Several acute leukaemia panels were discussed based on previous experiences of the haematopathology team from Canada, UK, USA, Sweden and Argentina. These also included the standardised EuroFlow panels described above. The Euroflow panels had the advantage

of being extensively validated, ready-made commercial product that is almost ready to use with minimal local validation. This approach was supported by three consultant haematopathologists with previous experience in flow cytometry in North America. On the other hand, one haematopathologist with extensive flow cytometry experince both in Europe and Latin America; including Euroflow; was more fexible in which approach to be taken locally. The scientist conducting this study was trained in the UK with previous experience on in-house designed panels as well as limited exposure to the Euroflow panels in Salamanca, Spain.

1.7. Cost analysis:

In parallel to technical and scientific evaluation of the Euroflow and in-house proposed panels, a detailed cost analysis was performed to evaluate the cost implications of this new service. The annual expected workload was between 30-40 new paediatric acute leukaemia cases. The majority of these acute leukaemia cases were expected to be lymphoblastic leukaemia for this specific patient age group (0-16 years). A standard cost analysis excel spread sheet was used to include the initial cost of reagents, internal quality control material, staffing, external quality assurance schemes as well as any additional organization specific costs.

Using this template, the initial cost of the full in-house acute leukaemia panel was estimated to be around 200 pounds sterling. Two commercial products of the EuroFlow ALOT tube were available. The first was the BD OneFlow ALOT tube (BD Biosciences, reference 660228) with a local cost of approximately 280 pound sterling per test. Surprisingly, this single tube was more

expensive than the full in-house acute leukaemia panel including 29 different markers. The second ALOT product was from Cytognos solutions (Cytognos, Salamanca, reference CYT-ALOT) at a local cost of approximately 70 pounds per test.

The estimated total cost of the extended Euroflow tubes ranged from 434 to 750 pounds sterling per test (for acute lymphoblastic leukaemia and acute myeloid leukaemia; respectively). Therefore, the expected overall annual cost of the in-house acute leukaemia panel was 12,000 pounds sterling compared to approximately 45,000 pounds sterling for the Euroflow panel. The local cost analysis of the in-house acute leukaemia panel compared to the Euroflow panels is summarized in Table 1.4 below (ALOT, BCP, T-ALL and MDS/AML).

Table 1.4: Estimated local cost analysis of acute leukaemia panels using standerdised Euroflow panel compared to an in-house acute leukaemia panel (in pounds sterling).

	In-house Panel	**Euroflow acute leukaemia Panels**				
	A1 to A4	ALOT-BD	ALOT Cytognos	BCP	T-ALL	AML/MDS
Number of tubes	4	1	1	4	4	7
Cost per case	200	280	70	434	434	750
Annual cost (60 tests)	12,000	16,800	4,200	26,040	26,040	45,000

Abbreviations: AML, acute myeloid leukaemia; BCP, B-cell precursor acute lymphoblastic leukaemia; MDS, myelodysplastic syndrome; T-ALL, T-cell acute lymphoblastic leukaemia.

Due to the specific local paediatric patient needs as well as efficient use of the budget, the general consensus was to develop an in-house four tubes panel for all acute leukaemias (T, B and Myeloid) (table 1.5). This is to be supported by an additional tube with antibodies specifically designed to support the diagnosis of T-acute lymphoblastic leukaemia (T-ALL) or acute megakaryocytic leukaemia (AML-M7) based on the initial phenotype (Appendix 1).

Table 1.5: Original in-house acute leukaemia panel:

	BLUE				RED		VIOLET	
	FITC	PE	PerCpCy5.5/PC5.5	PECy7	APC	APCH7 or Cy7	BV 421	V500
Acute 1	CD20	CD13	CD9	CD34	CD10	CD11b	CD19	CD45
Acute 2	CD15	CD117	HLA-DR	CD34	CD33	CD3	CD7	CD45
Acute 3	CD2	CD56	CD4	CD34	CD64	CD14	CD36	CD45
Acute 4	nuTDT	CyMPO	CyCD79a	CD34	CyIgM	CyCD3	-	CD45

Additional tubes as needed:

A5: T-ALL	CD1a	CD99	CD4	CD34	CD8	CD3	CD7	CD45
A7: AML-M7	CD61	CD41a	-	CD34	CD42	-	CD19	CD45

1.8. Development and validation of an in-house acute leukaemia panel:

The in-house acute leukaemia panel was extensively validated for clinical use at Sidra Medicine (unpublished data, Sidra medicine 2020). In addition to shorter turn around time

for patients at Sidra Medicine, the project also aimed to establish a second diagnostic oncology flow cytometry laboratory in the country to support the only oncology flow cytometry diagnostic laboratory at the National Cancer Center. Moreover, localizing such services will have direct impact on training of local workforce, promote research and services expansion at Sidra medicine as well as reducing costs of send away tests.

1.8.1. The validation plan:

A detailed validation plan was prepared prior to commencing acute leukaemia diagnosis at Sidra Medicine. This included but not limited to; development of a business case for senior management, securing of appropriate space within the department, cost analysis, selection and procurement of suitable equipment, installation plan, panel development as well as technical valiation.

However, the main challenge to initiate this project was the lack of appropriately trained laboratory scientists or technologists with previous oncology flow cytometry experience. Only one clinical scientist with appropriate flow cytometry experience was available prior to commencing this project. Therefore, training was one of the key components for this service to be initiated and continued.

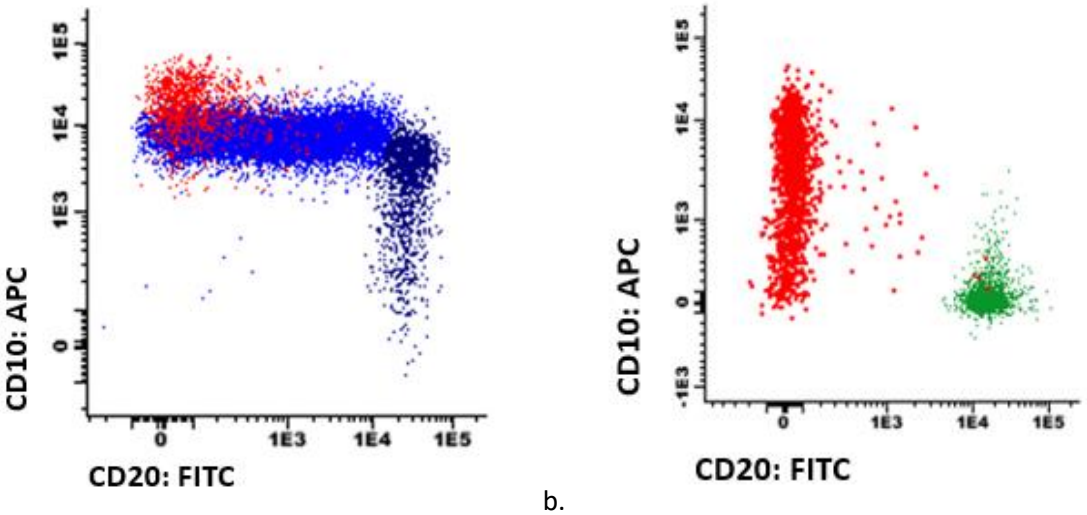
Following market assessment and local procurement procedures, a brand new; free of charge; FACSCanto II was acquired from a lacal supplier with appropriate service contract and a

minimum of 3 years warranty. Both installation and operational qualification were performed by the local vendor as well as the hospital biomedical engineering team.

1.8.2. Antibodies selection for the in-house acute leukaemia panel:

In order to fulfil the local pediatric patient needs, antibody selection had to reflect specific cell lineages as well as adequate combination of antibodies to monitor normal and abnormal cell maturation. The first tube in the in-house panel (table 1.5), was mainly B-cell focused with core back bone markers (i.e. CD19, CD45 and CD34), two B-cell maturation markers (CD10 and CD20) as well as two additional myeloid maturation markers (namely; CD13 and CD11b).

The first backbone marker to be selected inorder to isolate and gate haemopoietic cells was CD45 as the common leucocyte antigen (Borowitz, 1993; Lacombe, 1997 and Vial, 2001). CD19 was then selected as a very sensitive B-cell marker as it is expressed in early B-cell lineage and virtually all cases of B-ALL (van Dongen, 2012). This was followed by CD34 as the first immaturity marker in this panel (Borowitz, 1990). Additionally, CD10 and CD20 were selected as key maturation markers for B-cell lineage as normal B-cells mature through gradual loss of CD10 in parallel to acquisition of CD20 (figure 1.6).



a. b.

Figure 1.6: Normal B-cell maturation stages in a bone marrow sample. A: In red type I hematogones, in blue type II hematogones (CD34 negative with partial CD20), in dark blue type III hematogones (naïve B-cells, CD10/CD20 positive) and traces of mature B-cells (bottom right) CD20 positive and CD10 negative mature B-cells. B: Malignant B-cell precursors (blast cells) showing maturation arrest (i.e. discontinuation of maturation) (in red: malignant B-cells; in green: residual benign B-cells in this specimen).

Finally, CD13 and CD11b were also added to this tube in addition to CD10 inorder to monitor normal myeloid maturation; particularly in bone marrow specimens (figure 1.7). CD10 was also a useful marker to differentiate mature neutrophils (CD10 positive) from monocytes (CD10 negative) in this tube.

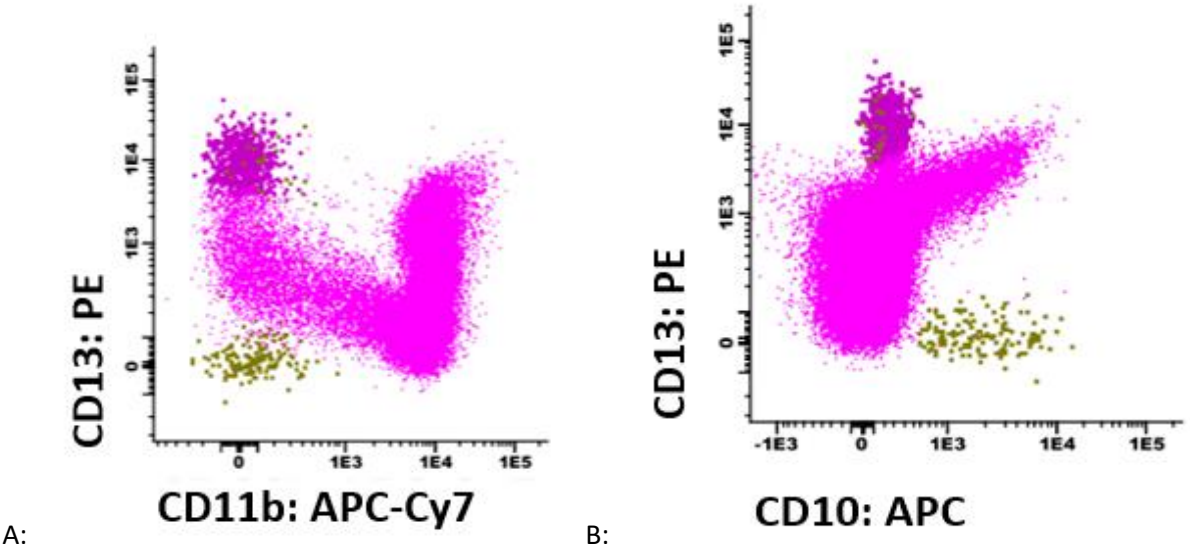


Figure 1.7: Normal myeloid maturation stages in a bone marrow sample. A: CD13 and CD11b showing normal gradual loss of CD13 in parallel to acquisition of CD11b and finally, reactivation of CD13 in mature granulocytes. B: CD13 and CD10 showing normal gradual loss of CD13 in parallel to acquisition of CD10 in mature neutrophils.

The second tube on this panel contained mainly myeloid and T-cell markers in addition to the previously mentioned back bones (CD45 and CD34). Three main myeloid markers were selected in this tube namely; CD33, CD117 and HLA-DR (Stewart, 1997). Although lacking myeloid specificity; HLA-DR provided an excellelnt myeloid maturation marker when combined with CD117 as a second myeloid immaturity antigen (figure 1.8). Likewise, combination of HLA-DR with CD33 also provided a good opportunity for monitoring monocytes, granulocytes and dendritic cells maturation. Moreover, HLA-DR also provided an additional value as a key marker present in almost all cases of B-ALL (Stewart, 1997).

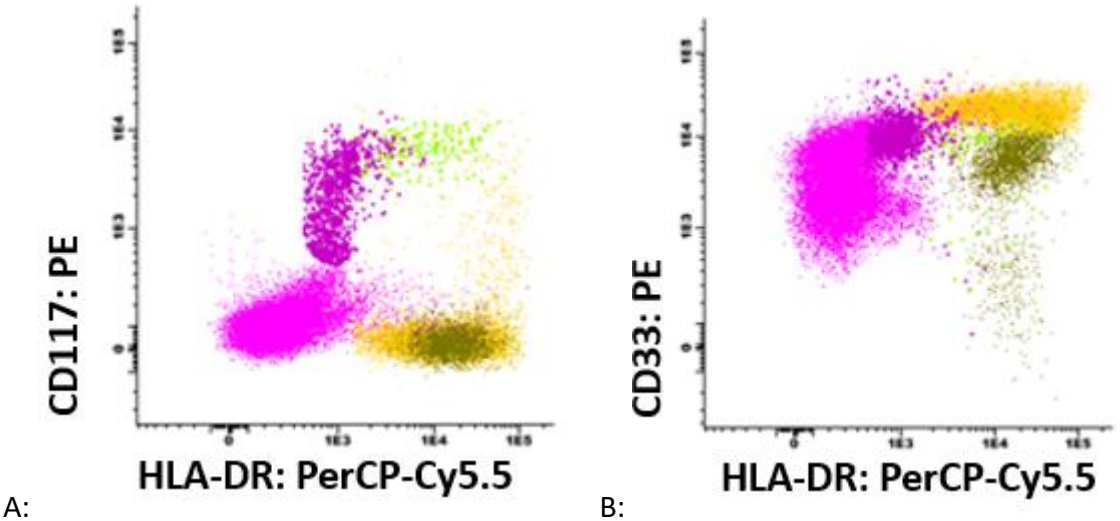


Figure 1.8: Normal myeloid maturation stages in a bone marrow sample. A: HLA-DR and CD117 showing normal myeloid maturation (light green: early blast cells; dark purple: promyelocytes and mylocytes; in pink: mature granulocytes; orange: monocytes and dark green: dendritic cells). B: CD33 and HLA-DR showing normal monocytes (orange), granulocytes (purple and pink) and dendritic cells maturation (dark green).

Finally, surface CD3 together with CD7 were also included in this tube in order to identify mature T-lymphocytes (CD3 and CD7 positive) as well as natural killer (NK) cells (CD7 positive and CD3 negative) (van Dongen, 1988).

The third tube in this panel was mainly focused on monocytes and nucleated red cells. Markers in this tube included CD4, CD64 and CD14 which can be used to isolate both monoblasts as well as mature monocytes (monoblasts CD4 and CD64 positive, while mature monocytes are CD4, CD64 positive in addition to CD14) (Stewart, 1997). CD36 was was added

to this tube to accurately enumerate nucleated red cells in addition to low FSC and SSC light properties of nucleated red cells.

The fourth tube in this panel (cytoplasmic tube) included both surface and cytoplasmic markers for diagnosis of acute myeloid and lymphoid neoplasms. In addition to the back bone markers (CD45 and CD34), nuclear TDT was added to this tube as an additional immaturity marker in this tube. Cytoplasmic myeloperoxidase (cyMPO) was selected as the most specific marker for myeloid lineage (Khalidi, 1999; Hrusak, 2002 and Robert, 2007).

Moreover, cytoplasmic (Cy) CD79a was also selected as a second B-cell marker to improve B-lineage assignment. It was important to consider that CyCD79a is also expressed in AML with t(8;21) (Hurwitz, 1992; Kita, 1992; Tiacci, 2004 and Bhargava, 2007) and at low levels in some T-ALL cases (Pilozzi, 1998; Hashimoto, 2002 and Asnafi, 2004). In addition to cytoplasmic CD79a, cytoplasmic (Cy) IgM was further added to this tube as an additional B-cell maturation marker. Finally, cytoplasmic (Cy) CD3 was included in this tube as the most specific T-cell marker in T-cell acute lymphoblastic leukaemia (van Dongen, 1988).

In addition to these four tubes, two optional acute leukaemia diagnostic tubes were also designed for sub-classification of acute leukaemias, namely; T-ALL and AML-M7 (AML with megakablastic differentiation).

The first of these was tube A5 (appendix 1) contained additional T-cell markers namely; CD1a, CD99 in addition to CD4, CD8, CD3, CD7 as well as the back bones of CD45 and CD34. On the

other hand, the second tube (A7) was specifically designed for identification of acute megakaryblasts with CD41a, CD42 and CD61 antibodies (Swerdlow, 2017).

1.8.3. Performance Qualification:

Following antibodies selection, detailed performance qualification criteria were included in the validation paln. This included voltage setup and compensation, antibodies titration, accuracy and precision studies, patient comparibility, sample stability, carry over studies, antibody specificity as well as tissues processing and viability testing (unpublished data Sidra medicine, 2020).

Manufacturer procedures were followed for voltage (PMTV) and compensation settings using the BD OneFlowTM setup beads (BD Biosciences, USA) and (BD™ CompBead) as highlighted above (section 2.1). Moreover, BD FACSDiva™ software and the cytometer setting and tracking (CS&T) beads were used to perform daily quality control of the instrument's optics, electronics and fluidics.

1.8.4. Accuracy and precision studies:

For accuracy studies, a total of 13 patient and control samples for lymphocyte subsets (TBNK) were performed on the FACS Canto II analyser in parallel to the Navios flow cytometer used routinely for lymphocyte subset assay. The mean, standard deviation as well as co-efficient of variation (CV %) were calculated for each parameter tested using the newly developed

acute leukaemia panel. This included CD3, CD4, CD8, CD19 as well as CD56 positive populations. A co-efficient of variation of < 20% was considered as acceptable.

All tested TBNK samples showed <10% co-efficient of variation (CV %) accept one sample which showed CV of 38% in B-cells count. Careful review of the data showed that this specimen had a very low B-cell population (only 3%) leading to high co-efficient of variation.

In order to establish the between-run reproducibility (inter-assy precision), a minimum of 10 repeats of whole blood contols as well as the CS&T beads were repeated on different days. Resutls showed co-efficient of variation (CV %) of less than 10% for all repeats.

Likewise, to establish the with-in run reproducibility (intra-assay precision), blast cell count was obtained independently from each tube in the newly developed acute leukamia panel. Results showed that all tubes in the experiment produced a comparable blast cells count (CV <20%) except tube A2. Repeat analysis of this tube after consultation with colleagues from the Euroflow group and exclusion of CD15 showed consistency of blast cells count among all tubes.

It was evident that the CD15 (FITC) was very bright resulting in erroneous low neutrophils count and subsequently a false high blast cell count in this tube. It was therefore decided to remove the CD15 from this tube ad replace it by CD2 in order to be able to provide a more accurate T-cell and NK-cell count from tube A2 (T-cells are CD2 and CD3 positive, while NK cells are CD2 positive and surface CD3 negative). For accurate neutrophils enumeration, CD45

together with CD10, CD13, CD33, CD64, CD11b as well as FSC and SSC scatter lights properties were used in different tubes instead of CD15.

1.8.5. Patient correlation and comparibility:

In order to ensure accurate final diagnosis, a total of 12 newly diagnosed acute leukaemia samples were tested in-house in parallel to an accredited reference laboratory (National Cancer Center, Qatar). A qualitative analysis (positive, negative or partial expression) was performed for all markers in the 12 specimens. Only one cytoplasmic IgM result was discrepant among all markers in the 12 testsed specimens. Unfortunately, the reference laboratory was not able to share the raw data files for independent analysis. However, this did not have an impact on the final diagnosis for this particular case (B-ALL).

1.8.6. Carry over and sample stability:

A total of 13 blank distilled water samples were acquired for a minimum of one minute following 10 different patient samples to ensure there was no carry over between specimens. All samples recorded less than 10 events during the one minute acquisition and was therefore considered as adequate evidence for carry over validation.

All blood or bone marrow samples were tested within 48 hours of collection in EDTA anticoagulat as per published recommendations (Ulrika 2014, European; Stelzer, 1997). All comparibility results were acceptable as highlighted above and therefore local procedures

considered 48 hours as the maximum allowable testing time for blood and bone marrow specimens.

1.8.7. Antibodies titration, tissue processing and viability assay:

Furthermore, antibody titration was performed for all antibodies in this panel in order to select the minimal acceptable concentration for each antibody. Antibodies with manufacturer recommendation of 20ul were tested subsequently using 10ul and 5ul and the corresponding mean fluorescent intensity (MFI) was compared for all volumes. Likewise, antibodies with manufacturer recommendation of 5ul were tested in parallel using 2ul (namely; CD19 and CD45).

Interestingly, only cytoplasmic MPO showed a significant drop in MFI with subsequent titration (MFI dropped by 74% compared to less than 25% in all other markers). It was therefore decided to continue with the manufacturer recommendation of 20ul for this particlualr antibody (MPO-PE).

Finally, two freshly collected lymph nodes in RPMI medium with new diagnosis of acute lymphoblastic leukaemia/lymphoma were disaggregated and tested by flow cytometry in parallel to the bone marrow specimens and immunohistochemistry (IHC) staining. Prior to staining for flow cytometry analysis, the samples were diaggregated, washed in PBS, filtered and finally a total WBC count as well as viability testing was performed using 7AAD dye. Both

samples showed >80% viability and were therefore processed by flowcytometry. Results showed full concordance between flow cytometry and IHC for all tested antibodies.

1.8.8. Standard operating procedures (SOPs), training and proficiency testing:

Prior to final test activation, detailed standard operating procedures were developed within the department. This was followed by intensive techinal staff training on sample preparation and acquisition. A simple trouble shooting guidance was also developed as part of the maintenance and quality control procedure. Inintial competency assessment was signed for all newly trained staff prior to any patient testing. This was followed by 6 months re-assement as well as annually afterwards as per the College of American Pathologists (CAP) requirements.

For proficiency testing, commercial external quality assurance samples were tested at least quarterly (i.e. CAP samples). For tests, were no external quality assurance scheme is available (e.g. DNA index) an alternative internal proficiency testing procedure was developed. Essentially, for this type of tests; three samples were tested independently by two users and results are compared by a senior staff member to ensure consistency among team members.

Finally, a detailed validation report was prepared to conclude all the validation steps highlighted above. This also included a final “new test activation check list” which covered other areas such as laboratory information system development, finance and revenue cycle as well as end users notification and activation date.

1.9. Validation of the ALOT tube:

In addition to this in-house panel, the acute leukaemia orientation tube (ALOT) was also considered as a potential screening tube. Considering that the laboratory is not following the EuroFlow instrument settings or standard operating procedures, it was not very clear if the ALOT tube will function as designed by the EuroFlow consortium in this independent evaluation. Another factor considered was the cost of purchasing the ALOT interpretation database from EuroFlow and the impact of this on the total cost per patient. It was also not very clear if the laboratory can apply the same principle component analysis (PCA) described above to develop their own local database for results interpretation instead of re-purchasing the EuroFlow database.

Finally, the haematopathology team was not sure if the PCA principle can be used to create an immunophenotypic profile of blast cells using the recently developed in-house panel. Can this be used to predict the final leukaemia diagnosis and/or cytogenetics abnormalities based on semi-automated database analysis? Will this semi-automated interpretation of flow cytometry data be comparable to the traditional expert-based interpretation? Will blast cells from different cases cluster in any specific pattern if allowed to cluster in an unsupervised manner using the new database PCA tool? This study was therefore designed to address the above questions in order to meet the specific local patient needs.

1.10. The aims of this study are:

- To validate the EuroFlow acute leukaemia orientation tube (ALOT) for the diagnosis of acute leukaemia using the 8-color FACS Canto flow cytometer and following in-house flow cytometer settings, compensation and staining procedures.
- To validate the use of EuroFlow automated database-guided interpretation for immunophenotypic diagnosis and classification of acute leukaemia.
- To develop and validate an in-house ALOT reference database based on local instrument settings and staining procedures.
- To use the Infinicyt software to analyse and merge the immunophenotypic files generated from the full in-house acute leukaemia panel for all cases included in the study as big data, allowing them to be grouped in an unsupervised manner using the Principal Component Analysis (PCA) and automated population separator (APS) tool.
- To compare the immunophenotypic profile (IP) of each group generated by the Principal Component Analysis (PCA) above with other biological, molecular, cytogenetics or clinical findings.
- Where possible, to develop in-house reference database(s) based on the above findings.

2. Materials and Methods:

2.1. Patients and specimens:

A total of 24 consecutive specimens from 16 patients with new diagnosis of acute leukaemia were included in this study. All samples were submitted for flow cytometry analysis as part of their clinical workup during the nine months period of this study (December 2019 to August 2020). These included 13 peripheral blood samples (54%) and 11 bone marrow aspirates (46%). Eight out of the sixteen patients had multiple specimens (peripheral blood and bone marrow). During the study period, three patients with established acute leukaemia diagnosis were subsequently referred for follow up during their treatment plan.

All new cases of acute leukaemia during the study period were included in the acute leukaemia orientation tube (ALOT) validation project. However, follow up samples with less than 1% blast cells or when there was no need to perform flow cytometry analysis for clinical use, samples were excluded from this project. Only excess of flow cytometry samples submitted for clinical workup were used. As this was a validation study of a new assay compared to an existing technique in use in the laboratory, ethical approval was not necessary.

2.2. Selection of flow cytometry instrument:

At the time of initiation of this study, the laboratory had two ≥ 8-color flow cytometer instruments from two different manufacturers. The first was a Navios flow cytometer (10

colors, 3 lasers) from Beckman Coulter; while the second was an 8-color, 3 lasers FACSCanto II flow cytometer from Becton Dickinson (BD). Both instruments had enough flexibility and compatible optical configurations with the EuroFlow settings to be used for the validation of the ALOT tube (Kalina, 2012). The Navios flow cytometer was mainly used for immunological assays (e.g. lymphocyte subsets, neutrophil function test and autoimmune lymphoproliferative syndrome -ALPS). It was purchased firstly, just prior to hospital opening and had the initial advantage of the TetraChrome software which was used for validation of lymphocyte subsets testing late in 2019.

On the other hand, the FACSCanto II was relatively newer (acquired towards the end of 2019) and was primarily used for the validation of the 8-color acute leukaemia diagnostic panel used in the department (Appendix 1). Additional tests undergoing validation on the FACSCanto II include platelet glycoprotein assay for assessment of platelet function, eosin 5-malemide assay for red cell membrane defects, DNA index assay as well as stem cell enumeration (CD34 count). It was decided to use the FACSCanto II for this ALOT validation project as an additional haematology test while keeping the Navios for “immunological” assays which presented the larger workload. Long term plans include cross validation of both instruments; particularly for lymphocyte subset and acute leukaemia panels; to act as a backup system during unpredicted down times.

2.3. Cytometer Settings:

BD FACSCanto II flow cytometer is a fairly flexible instrument designed to allow measurement of light scatter properties of various cells in a sample simultaneously. It was also built to be able measure the fluorescence emission of a wide range of fluorochromes (3 lasers, 8-colors, 4-2H-2V) that can be used to label different cells of interest in the test specimen (BD Biosciences, USA). Briefly, there were four main goals to be achieved by standardising the flow cytometer settings. Firstly, to ensure that all populations of interest (including nucleated red cells) are visible within the measuring scale limit. Secondly, there is enough scatter resolution between individual cell populations in order to be able to separate and identify them correctly. Thirdly, the auto fluorescent of some populations (e.g. eosinophils) is taken into account so as these events are distinguishable from "true positive" cells as well as from the background debris. Lastly, to ensure the accuracy and reproducibility (precision) of the photomultiplier tube voltages (PMT) and the corresponding detectors (Kalina, 2012).

In order to achieve these goals, the One Flow Setup beads, Cytometer Settings and Tracking (CS&T) beads as well as compensation beads (BD Biosciences, USA) were all used to standardise the FACSCanto II flow cytometer prior to patient testing.

2.3.1. BD OneFlow™ Setup Beads:

Unlike the EuroFlow instrument setting recommendations, the Eight-peak Rainbow bead calibration particles (Spherotech, USA) were not used to set up the initial photomultiplier tube

voltages (PMTVs) on the FACSCanto II cytometer in the department. Instead, the BD OneFlow™ setup beads (BD Biosciences, USA) were used for this purpose. These are fluorescent particles intended to set the cytometer photomultiplier tube voltages (PMTVs) for BD multicolor tube assays (3-lasers, 8-colors (4-2H-2V) BD default optical configuration). During the initial voltage setup, the PMTVs were manually adjusted to place the OneFlow setup beads within their lot specific median fluorescence intensity (MFI) target ranges.

This was subsequently followed by using lysed washed normal blood sample (LWB) to set up the cytometer forward and side scatter (FSC and SSC) voltages respectively. Red cells in the sample were lysed (10 mins) using 2 ml of 1/10 dilution of 10X BD Pharm Lyse ammonium chloride-based lysing reagent in distilled water (vol/vol). The sample was then centrifuged (2min at 3000 rpm) and the cell pellet was subsequently washed in 2 ml of phosphate buffer saline (PBS; PH 7.4) containing 0.1% bovine serum albumin. The cell pellet was finally diluted in a minimum of 250 µl of the PBS albumin mixture, vortexed and acquired on the FACSCanto within the first hour of preparation. Once more, the EuroFlow preparation procedure was not followed here.

2.3.2. Cytometer Setting and Tracking (CS&T) beads:

BD FACSDiva™ software and the cytometer setting and tracking (CS&T) beads were used to perform daily quality control of the instrument's optics, electronics and fluidics. The CS&T beads are a mixture of 3 dyed beads with variable stain intensity (bright, mid and dim) which are excited by the cytometer's lasers. The mean fluorescent intensity (MFI), robust coefficient

of variation (rCV) were measured for each bead intensity followed by calculation of various measurements to ensure the analyser stability. These include but not limited to; linearity, fluorescence detection efficiency (Qr), relative background (Br) and laser power. Initially, a defining baseline was established for each new CS&T new beads lot. This was followed by daily check of analyser performance based on the pre-determined acceptable criteria of the CS&T beads.

2.3.3. Compensation Settings:

Most fluorochromes used in flow cytometry have a broad fluorescence emission spectrum (Baumgarth, 2000 and Shapiro, 2004). This leads to spill over (overlap) of the primary fluorochrome of interest into a second detector channel causing a false positive signal of other fluorochromes. As the proportion of spectral overlap is constant for each fluorochrome, it can be mathematically calculated and subtracted (Baumgarth, 2000). Technically, the process of calculation and subtraction of the spectral overlap between different fluorochromes is defined as “fluorescence compensation”. In general, florescence compensation values depend on spectral properties of the dye in use, filters and mirrors (optics) mounted in the cytometer and finally, the voltage used in the photomultiplier tube (PMT) for that specific fluorochrome (Kalina, 2012).

Almost all conventional flow cytometers are supported by software compensation tools. On FACSCanto II flow cytometers, the FACSDiva software was used for compensation. In order to correctly calculate the compensation values, it was essential to run and collect light emissions

for each individual fluorochrome used in the experiment prior to using it in a multi-color assay (Roederer, 2002). Two options were available to calculate compensation values for this study. Firstly, an 8-Color Kit for BD OneFlow™ assays compensation kit (BD Biosciences, USA) and secondly; an in house single reagent stained cells or mouse immunoglobulin (Ig) capture beads (BD™ CompBead) (BD Biosciences, USA).

2.3.3.1. BD FC Beads 8-Color Kit for BD OneFlow™ Assays:

This is a standardised compensation kit used to calculate fluorescence compensation on the BD FACSCanto II flow cytometer and BD FACSDiva software using default 3-laser, 8-color (4-2H-2V) configuration settings (BD Biosciences, USA). Polystyrene beads (3-µm) were coupled with 8 single fluorochromes dried in eight 12 x 75 mm tubes. They were specially designed to compensate experiments with FITC, PE, PerCP-Cy5.5, PE-cy7, APC, APC-H7, BD Horizon V450 and BD Horizon V500.

In order to calculate the spill over values (SOVs) 0.5 ml of BD FC beads dilution buffer was added to each tube in addition to one drop of positive and negative compensation beads. Each tube was then vortexed vigorously and incubated in dark for 15 mins at room temperature. Following incubation, all tubes were immediately acquired using the FACSDiva software. At least 5,000 total events were acquired at medium flow rate. This was followed by gating the beads population in a forward/side scatter dot plot (FSC-A vs SSC-A), then positive and negative bead populations for each fluorochrome in the experiment (figure 2.1).

Finally, the FACSDiva software was used to calculate the compensation (spill over) values between the 8-colors in the experiment.

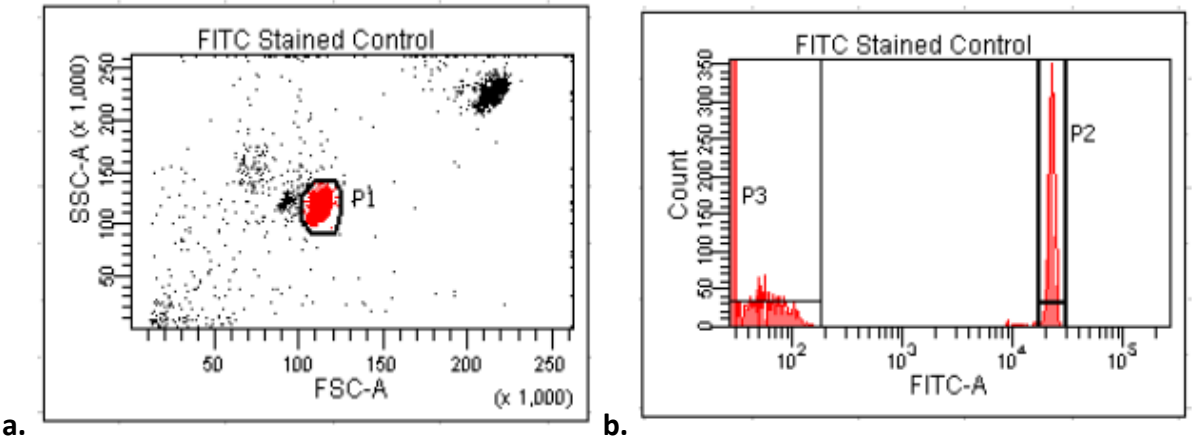


Figure 2.1: Cytometer compensation using BD FC Beads 8-Color Kit for BD OneFlow™ Assays (Taken from BD Biosciences, USA).

2.3.3.2. Anti-Mouse Ig, κ/Negative Control Compensation Particles Set:

Unlike the above mentioned 8-color kit with dried fluorochrome tubes, the compensation particles set contains two sets of unstained polystyrene beads with no fluorochromes provided with the kit. The first set of beads was used as a negative control population (unstained) while the second as a positive control for each fluorochrome in the in-house experiment. To calculate compensation using these beads, 100 µl of phosphate buffer saline (PBS) were added to one drop of each compensation beads (positive and negative) in 8 different 12 x 75 mm test tubes. This was followed by single staining of each tube by one fluorochrome as required for the experiment. To each test tube, the appropriate volume of antibody labelled fluorochrome was added as per manufacturer recommendations.

All tubes were then incubated in dark at room temperature for at least 15 minutes after vigorous vortex. The beads were then re-suspended in a total volume of 300 µl of phosphate buffer saline (PBS) and were acquired immediately using the FACSDiva software. An unstained tube (negative control) was acquired in parallel. Finally, the software was used to calculate the compensation (spill over) values between the 8-colors in the experiment (figure 2.2).

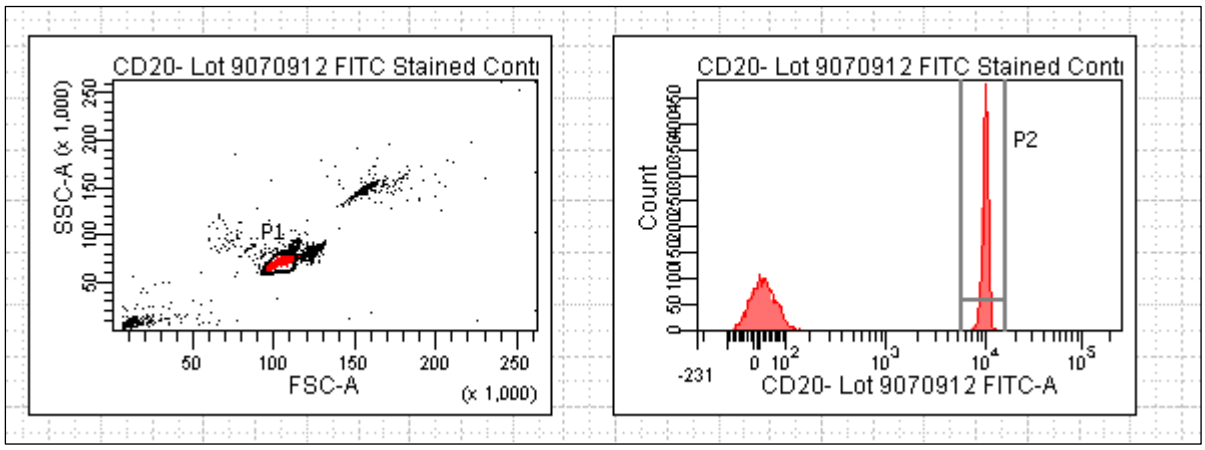


Figure 2.2: Example of cytometer compensation using positive and negative control compensation beads and FITC CD20 antibody.

2.4. Additional quality control procedures:

In addition to the above mentioned daily cytometer setting and tracking (CS&T) beads, several quality control measures were used in parallel to ensure the overall quality of flow cytometry results. Daily maintenance logs were completed including start up, CS&T, daily clean and shut down of the analyser. For each failed CS&T value, a comment was added defining the potential cause as well as corrective action taken immediately. No patient samples were performed in case of quality control failure. All reagents used for patient testing were

accurately recorded (including lot numbers and expiry dates) for audit purposes. Where possible, internal controls from normal populations in the samples were reviewed to ensure the integrity of all antibodies in use.

Weekly back up of the analyser data was performed by a senior scientist to ensure that all patient flow cytometry standard files (FCS) were saved for at least 10 years. Moreover, all reagent stock levels were checked on weekly basis to ensure all reagents were validated prior to clinical use. A new lot to lot validation procedure was used defining the acceptable criteria for all new reagents and quality control materials. Weekly stock control procedures were also essential to minimize reagents waste.

Monthly CS&T reports were prepared at the end of each month to ensure that the photomultiplier tube voltages (PMT) and lasers were within the pre-determined acceptable ranges. All errors, CS&T failures or troubles recorded on the trouble shooting log were reviewed on monthly basis by a senior staff member to identify any trends and potential root causes. An overall internal monthly quality control log was also used to ensure that all antibodies in use were able to identify sub populations of normal cells in various specimens. For rare antigens (e.g. CD1a or CD99), at least bi-annual positive and negative control beads were used to verify the integrity of the reagents.

The laboratory was also accredited by the college of American pathologists (CAP) and receives samples quarterly for independent assessment and peer comparisons. For tests were no CAP material was available (e.g. DNA index assay), at least three samples were repeated biannually

by two staff members to ensure reproducibility of the results between staff members. All staff members performing patient testing were initially trained by a senior staff member and their initial competency for a specific test was assessed and documented before commencing patient testing. Initial competency was then re-assessed with in the first 12 months (ideally at 6 months) of initial training and subsequently annually as per CAP requirements.

Finally, the analyser was serviced bi-annually by specialist biomedical engineer team (BME). Following each BME preventative maintenance visit, a new CS&T baseline was performed and compensation calculation was repeated. Compensation was also repeated whenever a new batch of tandem dyes (e.g. PE-Cy7 or APC-H7) was received and validated for patient testing.

2.5. Sample preparation and staining standard operating procedures (SOPs):

Samples for flow cytometry were stained using the in-house customised acute leukaemia standard operating procedures (SOPs). This included staining of both, surface and cytoplasmic antigens. All samples were processed at room temperature within 48 hours of venipuncture. Where needed, bone marrow and peripheral blood samples were pre-diluted to obtain white cells counts of 10-20 x 10^9/L using phosphate buffer saline (PBS; PH 7.4).

For surface antigens, appropriate amounts of antibodies were added to each test tube in the panel (appendix 1). This was followed by 50 µl of well mixed sample. All tubes were then vortexed and incubated in dark for 15 minutes at room temperature. A 2 ml volume of 1/10 diluted lysing solution (Pharm Lyse) was then added to each tube to lyse the red cells in the

specimen. Samples were vortexed and re-incubated for additional 10 minutes before washing twice in phosphate buffer saline solution (PBS) containing 0.1% bovine serum albumin (centrifugation at 3000rpm for 2 mins). The cell pellet was finally re-suspended in a minimum of 250 µl of the PBS/0.1% albumin mixture, vortexed and acquired on the FACSCanto II within the first hour of preparation.

For cytoplasmic antigen staining, only back bone antibodies were added initially to the test tube (i.e. CD19, CD34 and CD45). This was followed by 50 µl of well mixed sample. The cytoplasmic test tube was then vortexed and incubated in dark for 15 minutes at room temperature. Excess surface antibodies were then washed out in 2 ml of PBS solution containing 0.1% bovine serum albumin.

The stained cell pellet was then re-suspended in 100 µl of FIX & PERM Reagent A (fixing solution) (Nordic-MUbio, Netherlands) and incubated for additional 15 minutes in dark. The fixing solution was then washed and sample centrifuged in excess of PBS solution as above. Lastly, 100 µl of FIX & PERM Reagent B (permeabilisation solution) (Nordic-MUbio, Netherlands) were added to the cell pellet in addition to cytoplasmic antibodies (i.e. MPO, cyCD3, CD79a, IgM and TdT) and further incubated for additional 15 minutes in dark.

Finally, the excess amount of unbound cytoplasmic antibodies and the permeabilisation solution were washed in 2 ml PBS solution and the stained cell pellet was finally re-suspended in a minimum of 250 µl of the PBS/0.1% albumin solution. Stained sample was vortexed and acquired on the FACSCanto II within the first hour of preparation.

2.6. Workflow and interpretation of results:

All peripheral blood and bone marrow samples included in the study underwent an initial morphological assessment prior to flow cytometry. This included slides prepared from EDTA samples for flow cytometry in addition to the bed side bone marrow smears. Morphological assessment of the EDTA bone marrow samples was essential to assess the samples quality as well as the haemodilution frequently seen in subsequent bone marrow aspirates. Samples were then stained for flow cytometry using the previously mentioned eight color (25 markers) in-house acute leukaemia panel (Table 2.1) to obtain the diagnosis for clinical use.

Table 2.1. Original in-house acute leukaemia panel:

	BLUE				RED		VIOLET	
	FITC	PE	PerCpCy5.5/PC5.5	PECy7	APC	APCH7 or Cy7	BV 421	V500
Acute 1	CD20	CD13	CD9	CD34	CD10	CD11b	CD19	CD45
Acute 2	CD15	CD117	HLA-DR	CD34	CD33	CD3	CD7	CD45
Acute 3	CD2	CD56	CD4	CD34	CD64	CD14	CD36	CD45
Acute 4	nuTDT	CyMPO	CyCD79a	CD34	CyIgM	CyCD3	-	CD45

Additional tubes as needed:

A5: T-ALL	CD1a	CD99	CD4	CD34	CD8	CD3	CD7	CD45
A7: AML-M7	CD61	CD41a	-	CD34	CD42	-	CD19	CD45

Flow cytometry results were then analysed and initially reported by the principal clinical scientist conducting the study. At least one consultant haematopathologist then reviewed the results and verified the final flow cytometry report for clinical use. In addition to the full acute leukaemia panel, the new test under validation (ALOT tube) was performed and analysed in parallel using excess flow cytometry samples. A consultant haematopathologist then reviewed the analysis independently to provide an expert opinion on results. No results from the test under validation (ALOT tube) were released for clinical use during the validation process.

Where available, bone marrow biopsies were sent in parallel for histological assessment and additional samples were sent for cytogenetics analysis (FISH and Karyotyping) as well as molecular detection of minimal residual disease (MRD). Finally, an integrated report was released containing all available clinical information, blood count results, morphological assessment, immunophenotyping, cytogenetic results as well as any molecular findings. The final integrated report classified acute leukaemias according to the revised WHO classification of tumours of haematopoietic and lymphoid tissues (Swerdlow, 2017).

2.7. Validation of the EuroFlow acute leukaemia orientation tube (ALOT):

Two kits were commercially available for the EuroFlow acute leukaemia orientation tube (ALOT) at the time of conducting this study. The first was from Cytognos Flow Cytometry Solutions (Salamanca, Spain); while the second was the BD OneFlow™ ALOT (BD Biosciences, USA). Both kits followed the EuroFlow recommendations for antibodies selection (table 2.1).

However, minor differences occurred in the fluorochromes selection; particularly on the violet laser. Cytognos used Orange Cytognos 515 (OC515) to label CD45 and APC-C750 to label surface CD3 instead of the PacO and APCH7 recommended by EuroFlow respectively. On the other hand, BD OneFlow™ ALOT kit used V450 and V500 to replace PacB and PacO on CyCD3 and CD45 respectively. Despite the minor differences, both kits were approved for in vitro diagnostic use (IVD) and commercialised accordingly.

Table 2.2. Comparison between the EuroFlow, Cytognos and BD design of acute leukaemia orientation (ALOT) tube:

	CyCD3^{T}	CD45^{B,T,M}	CyMPO	CyCD79a	CD34^{B,M}	CD19^{B}	CD7	SmCD3^{T}
EuroFlow	PacB	PacO	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7
Cytognos	PacB	OC515	FITC	PE	PerCPCy5.5	PECy7	APC	APC-C750
BD	V450	V500	FITC	PE	PerCPCy5.5	PECy7	APC	APC-H7

In order to select the more appropriate product for clinical use in the department, both kits were trialed briefly in the laboratory. However, due to lack of PacB, OC515 and APC-C750 in the parallel in house acute leukaemia panel; it was preferred to continue with a trial BD OneFlow™ ALOT kit to avoid additional compensation and maintenance procedures related to the Cytognos product.

2.8. Sample preparation and staining procedures (SOPs) for the BD OneFlow™ ALOT tube:

For surface staining, 50 µL of unwashed specimen in addition 50 µL of wash buffer were added to the BD OneFlow ALOT surface (S) tube containing dried CD3, CD7, CD19, CD34, and CD45 antibodies. The mix was vortexed vigorously for 3-5 seconds and was subsequently incubated at room temperature for 15 minutes in dark. Following incubation, excess antibodies were washed out by adding 2 ml phosphate buffer saline (PBS) containing 0.1% albumin and centrifugation at 3000 rpm for 2 minutes. The supernatant was then discarded without disturbing the cell pellet and leaving approximately 50 µL of residual liquid in the tube. 100 µL of FIX & PERM Reagent A (fixation solution, Nordic-MUbio, Netherlands) were then added to the tube, vortexed and further incubated at room temperature for 15 minutes in dark.

The fixing solution was then washed and the sample centrifuged in excess of PBS solution as described above, leaving approximately 50 µL of residual liquid in the tube. The residual liquid and cell pellet were vortexed well and the stained cells were transferred to the second BD OneFlow cytoplasmic (C) tube containing dried CD3, CD79a and MPO antibodies. A further 100 µL of FIX & PERM Reagent B (permeabilisation solution, Nordic-MUbio, Netherlands) were added to the cytoplasmic tube, vortexed well and incubated at room temperature in dark for 15 minutes. The excess of unbound cytoplasmic antibodies and permeabilisation solution B were then washed in excess of PBS solution as described above. Finally, 300 µL of the PBS wash solution were added to the cell pellet and acquired immediately on the

FACSCanto II cytometer using FACSDiva software and default instrument configuration (4-2H-2V).

2.9. Manual analysis and interpretation of acute leukaemia orientation tube (ALOT):

Following acquisition of approximately 100,000 events, the ALOT tube was initially analysed on the FACSDiva software using an experiment template developed internally for this purpose. Subsequently, flow cytometry (FCS) files were analysed off the analyser using the Infinicyt software developed by Cytognos Flow Cytometry Solutions (Salamanca, Spain). This novel data analysis tool was progressively developed to incorporate the EuroFlow Consortium analysis strategies for flow cytometry data (Costa, 2006; Pedreira, 2008 and Costa, 2010).

Briefly, all duplicate events were firstly excluded using the area and height of the forward and side scatter light properties (FSCA vs FSCH and SSCA vs SSCH). Where needed, forward and side scatter width (W) were also used to exclude further duplets. CD45 was then used against side scatter to orient all events in the experiment (including nucleated red cells). Similarly, forward and side scatter plot (FSCA vs SSCA) was used to view all events in parallel. Finally, a combination of dot plots using two antibodies at a time were used to identify all populations in the specimen (e.g. CD3 vs CD7 to identify natural killer cells). Sequential gating was a key tool in this software, by which a sequence of hierarchical gates could easily by drawn using conventional computer mouse.

All analysis for this validation project was initially performed by the clinical scientist conducting the study. This was followed by an independent parallel analysis performed by a consultant hematopathologist to provide the expert-based phenotype of the acute leukaemia based on ALOT tube results only. The final results of the ALOT tube were then compared to the final results obtained by the full in-house acute leukaemia panel used for routine diagnosis.

2.10. Validation of the automated database-guided interpretation of ALOT tube:

The Infinicyt software is also equipped with an automated database-guided interpretation of the ALOT tube results. This feature was initially developed and validated by Cytognos Flow Cytometry Solutions as described earlier in section 1.5.4 (Lhermitte, 2018).

In order to validate the use of this automated database- guided interpretation tool in the laboratory, blast cells for each case were initially gated using the routine manual gating procedure described above. Cytognos database option was then used to interpret the orientation of blast cells (i.e. B vs T or myeloid lineages). The software was also equipped with a Compass tool to present the results of orientation. Finally, results of the "automated" database-guided interpretation were then compared to the initial "expert-supervised" interpretation for the ALOT tube as well as the full in-house acute leukaemia panel.

2.11. Development and validation of an in-house ALOT database:

As the laboratory was not following the EuroFlow standardised settings or sample preparation procedures, a database bank (library) of all cases in the study was developed in parallel. In order to achieve this, the 24 specimens already stained using the ALOT tube in this study were used. These included 13 peripheral blood samples and 11 bone marrow aspirates. The sample population was then divided into two groups, a database "build group" and a database "validation group".

Due to the relatively small number of cases under study, the two groups were not selected randomly. Instead, specimens were divided in a systemic manner to ensure adequate representation of different cases in each group (B-ALL, T-ALL and AML). Moreover, it was also important to consider that both types of specimens (peripheral blood and bone marrow) were represented in each group. Subsequently, this systemic approach of the sample population led to development of a database "build group" composed of 12 specimens (6 peripheral blood samples and 6 bone marrows) and; a validation group including 12 specimens (7 peripheral bloods and 5 bone marrows) (table 2.2).

To develop the in-house ALOT database, each case in the build group was manually analysed using the procedure described above (section 2.9). Blast cells for each case were then saved separately according to their corresponding known final diagnosis obtained from the in-house full acute leukaemia panel. Subsequently, the database "build group" was subdivided into B-ALL, T-ALL and AML subgroups based on their final diagnosis (table 2.2).

Table 2.3: Grouping of samples used for development and validation of the ALOT database-guided interpretation tool.

Category	Database Build Group	Database Validation Group	Total
B-ALL	9	7	16
T-ALL	1	2	3
AML	2	3	5
Total	12	12	24

Abbreviations: AML, acute myeloid leukaemia; B-ALL, B-acute lymphoblastic leukaemia; T-ALL, T-acute lymphoblastic leukaemia.

The Infinicyt database configuration tool was then used to build a diagnostic Compass with three categories (B-ALL, T-ALL and AML respectively). The results from each case in the "build group" were saved in the appropriate category of the compass to allow the software later to identify new cases based on the saved immunophenotypic signature in the database. Finally, the "validation group" was used to test the validity of the recently developed database-guided interpretation (Compass) tool.

2.12. Merging data files to obtain an immunophenotypic profile for each case:

The merge of flow cytometry data files feature was initially developed to overcome the technology limitations in flow cytometry (Kalina, 2012). The current WHO classification of haematological malignancies requires identification of up to 30 different markers in some neoplasms while the available flow cytometry technology used in most clinical laboratories

do not have the ability to acquire this number simultaneously (Braylan, 2001; Swerdlow, 2017). To overcome this, the current in-house acute leukaemia panel used in the laboratory contained four tubes (8-color each) (appendix 1). A fifth confirmatory tube was added with additional specific T-ALL markers (e.g. CD1a and CD99) or AML-M7 markers (e.g. CD41, CD42 and CD61) whenever needed to establish a final diagnosis of these neoplasms (Swerdlow, 2017).

In order to further improve the merging and gating of neoplastic and normal cells in different tubes, CD19 was further added to tubes 2-4 in the in-house acute leukaemia panel as an additional backbone (table 2.3). This way at least 5 markers were common among all tubes in the panel (namely; forward (FSC), side scatter (SSC), CD19, CD34 and CD45) while another five markers remained unique to each tube.

Table 2.4: Modified in house acute leukaemia panel

	BLUE				RED		VIOLET	
Fluorochrome	FITC	PE	PerCpCy5.5/PC5.5	PECy7	APC	APCH7	BV 421	V500
Acute 1	CD20	CD13	CD9	CD34	CD10	CD11b	CD19	CD45
Acute 2	CD2	CD117	HLA-DR	CD34	CD33	CD3	CD19	CD45
Acute 3	CD71	CD56	CD4	CD34	CD64	CD14	CD19	CD45
Acute 4	nuTdT	cyMPO	cyCD79a	CD34	cyIgM	cyCD3	CD19	CD45
Additional tubes as needed:								
A5: T-ALL	CD1a	CD99	CD4	CD34	CD8	CD3	CD7	CD45
A6: B-ALL	CD15	CD66c	-	CD34	CD123	CD38	CD19	CD45
A7: AML-M7	CD61	CD41a	-	CD34	CD42	-	CD19	CD45

Finally, to obtain the full immunophenotypic profile (signature) of malignant cells from all tubes in the experiment in a single data fine, the merge tool in the Infinicyt software (Cytognos, Salamanca, Spain) was used. All tube files for each patient were opened simultaneously. This was followed by gating the cells of interest (blasts) in all tubes using only the common backbone markers (i.e. FSC, SSC, CD19, CD34 and CD45). The calculation function was then used to merge all the blast populations from different tubes into a single blast cells population with all markers simultaneously. This way, all markers in the acute leukaemia panel across all tubes in the experiment were possible to visualise at the same time (e.g. CD13 from tube 1 vs CD33 from tube 2). A unique immunophenotypic profile (signature) was therefore created for each case in the study.

2.13. Comparison of the immunophenotypic profiles for all cases under study:

Once the full immunophenotypic profile (merge file) was created for all cases, the automated population separator (APS) and visualisation tools in Infinicyt software were used to visualise all blast cells for each case as one dot represented by the population median (figure 2.3). The blast cell population for each case was then saved as one merged file and named accordingly (e.g. Case - 1 blast cells).

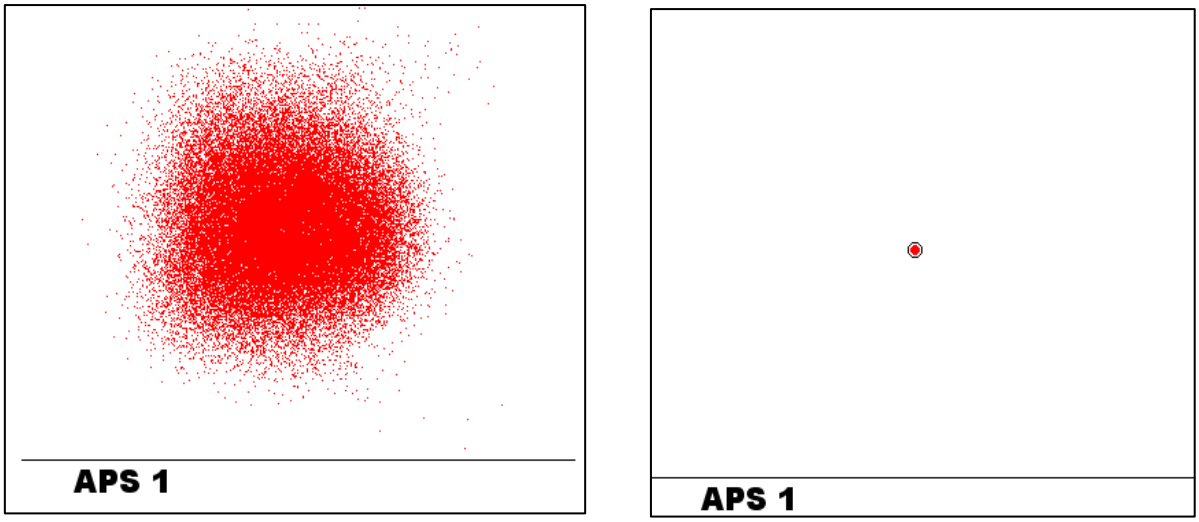


Figure 2.3: Automated population separator (APS) tool following blast cells merge as one file.

Lastly, all sixteen merged files representing all cases in the study were opened simultaneously using the analysis tool of the Infinicyt software. To analyse and gate the sixteen different blast populations from all cases in the study, one file was visualised at a time to show blast cells for one patient. A gate was drawn around the blast population and a new population was created in the analysis tree and named accordingly (e.g. Case 1 blast cells).

The gating was repeated for the sixteen cases in the study creating sixteen populations in one analysis each population represented the blast cells from one patient. Finally, blast cells from all cases in the study were visualised simultaneously to allow a direct comparison between all cases in one plot. The principal component analysis tool (PCA) and the automated population separator (APS) diagram were used for this purpose and all blast cells were allowed to group in an unsupervised manner.

2.14. Development and validation of an in-house acute leukaemia interpretation database:

The purpose of this database was to develop a potential automated database-guided interpretation tool for acute leukaemia diagnosis in comparison to the conventional expert-based results interpretation. The proposed tool would interpret the diagnosis solely on the immunophenotypic profile and categorise new cases of acute leukaemia into one of three categories, namely; B-ALL, T-ALL or AML. Previously recognised phenotypic signatures from cases in this study were used to build the reference database (library).

At this point, the final diagnosis of all cases in the study was already established using clinical, morphological, immunophenotypic, cytogenetic and where possible; molecular findings as described above (section 2.6). The sample population consisting of 15 diagnostic samples was then divided into two groups; a reference database "build group" and a database "validation group". Once more, due to the limited number of cases under study, the two groups were not selected randomly. Instead, specimens were divided in a systemic manner to ensure adequate representation of different cases in each group (B-ALL, T-ALL and AML) as well as different sample types (peripheral blood and bone marrow).

Accordingly, the first reference database "build group" contained seven diagnostic samples (four B-ALL, two AML and one T-ALL). On the other hand, the database "validation group" contained 8 samples (6 B-ALL, one T-ALL and one AML). One follow_up sample was not

included in either groups as the diagnostic flow cytometry was performed in a different hospital and the case was only referred to the department later on during treatment.

Table 2.5: Grouping of patients used for development and validation of the acute leukaemia database-guided interpretation tool.

Category	Database Build Group	Database Validation Group	Total
B-ALL	4	6	10
T-ALL	1	1	2
AML	2	1	3
Sample type	5 PB + 2 BM	6 PB + 2 BM	11 PB + 4 BM
Total	7	8	15

Abbreviations: AML, acute myeloid leukaemia; B-ALL, B-acute lymphoblastic leukaemia; BM, bone marrow; PB, peripheral blood; T-ALL, T-acute lymphoblastic leukaemia.

The new acute leukaemia database-guided interpretation tool was developed using the "merged files" generated from the analysed acute leukaemia panel for each case described above (section 2.12). Blast cells for each case of the reference database "build group" were then saved separately according to their corresponding known final diagnosis (B-ALL, T-ALL or AML). The Infinicyt database configuration tool was then used to build a new diagnostic Compass tool with three categories (B-ALL, T-ALL and AML respectively).

The results from the reference database "build group" were saved in each category of the Compass to allow the software later to identify new cases based on the saved reference

immunophenotypic signature. Finally, the “validation group” was used to test the validity of the newly developed acute leukaemia database-guided interpretation tool.

2.15. Development and validation of a cytogenetic database-guided prediction tool:

The purpose of this database was to develop a potential automated database-guided tool to predict recurrent cytogenetic abnormalities in B-acute lymphoblastic leukaemia (B-ALL). At this point, the final diagnosis of all 11 new B-ALL cases enrolled in the study was already established as described in section 2.6. In order to be able to predict any cytogenetic abnormalities, an additional cytogenetic-based tube (A6) was added to the diagnostic in-house acute leukaemia panel from cases 9 onwards (appendix 2). This tube contained antibodies of known cytogenetic prediction value (e.g. CD38, CD66c, CD123 and CD15) in addition to the core back bone markers (i.e. CD19, CD34 and CD45). CD38, CD66c, CD123 and CD15 are known to have a cytogenetic prediction value; specifically for t(12;21), t(9;22), hyperdiploidy as well as MLL rearrangements respectively (Hasan, 1999; De Zen, 2000; Tabernero, 2001 and Djokic, 2009).

To build and validate this cytogenetic prediction tool, 12 samples from 11 B-ALL cases in the study were divided into two groups: a reference database “build group” and a database “validation group”. As the number of cases in the study was limited, specimens were divided in a systemic manner to ensure adequate representation of different B-ALL sub categories in each group. Accordingly, the “build group” contained 8 samples (3 cases of B-ALL not otherwise specifies (B-ALL, NOS), two cases of B-ALL with hyperdiploidy, one case of B-ALL

with translocation t(12;21), one case with translocation t(1;19) and one case of B-ALL with BCR-ABL1 like translocation t(Y;14) (tables 2.5 and 2.6).

On the other hand, the "validation group" contained 4 samples of B-ALL. These included two cases of B-ALL not otherwise specifies (B-ALL, NOS), one case of B-ALL with hyperdiploidy and one case of B-ALL with translocation t(12;21) (tables 2.5 and 2.6). There were no adequate cases to verify the development of t(1;19) and t(Y;14) categories at the time of presenting these results.

Table 2.6: B-ALL samples used for development and validation of the cytogenetic database-guided predication tool.

Case	Sample type	Disease phase	Build Group	Validation Group	Final diagnosis
1	BM	Diagnostic	-	X	B-ALL with Hyperdiploidy
2	BM	Diagnostic	-	X	B-ALL-NOS
3	PB	Diagnostic	X	-	B-ALL with Hyperdiploidy
4	PB	Diagnostic	X	-	B-ALL-NOS
9	PB	Diagnostic	X	-	B-ALL-NOS
	BM	Diagnostic	-	X	B-ALL-NOS
10	BM	Diagnostic	X	-	B-ALL-NOS
11	PB	Diagnostic	-	X	B-ALL with t(12;21)
12	PB	Diagnostic	X	-	B-ALL with Hyperdiploidy
13	PB	Diagnostic	X	-	B-ALL with t(12;21)
14	BM	Diagnostic	X	-	B-ALL with t(1;19)
16	PB	Diagnostic	X	-	B-ALL with BCR ABL1 like

Table 2.7: Grouping of B-ALL sub categories for development and validation of the cytogenetic database-guided predication tool.

Category	Database Build Group	Database Validation Group	Total
B-ALL, NOS	3	2	5
B-ALL with hyperdiploidy	2	1	3
B-ALL with t(12;21)	1	1	2
B-ALL with t(1;19)	1	0	1
B-ALL with t(Y;14)	1	0	1
Total samples	8	4	12

Abbreviations: B-ALL, B-acute lymphoblastic leukaemia; NOS, not otherwise specified.

Similar to the development of previous in-house acute leukaemia interpretation database tool described in section 2.14; the cytogenetics prediction tool was developed based on the "merged files" generated from the full acute leukaemia panel (appendix 2).

The Infinicyt database configuration tool was used to build a cytogenetic predictive compass with five categories representing all B-ALL subgroups in the study (B-ALL, NOS, B-ALL with t(12;21), B-ALL with t(1;19), B-ALL with BCR ABL1 like translocation t(Y;14) and B-ALL with hyperdiploidy). The merged files from the "build group" were saved in each category of the compass to allow the software later to identify new cases based on the saved immunophenotypic signature in the database. Finally, the "validation group" was used to test the validity of the recently developed cytogenetic prediction tool in comparison to the actual cytogenetic results produced by FISH and karyotype analysis.

2.16. Statistical analysis:

Where applicable, MEDCALC® software (MedCalc, 2020) was used to perform comparison between categorical variables. The specificity, sensitivity, accuracy and prevalence of each antibody in the ALOT tube was individually assessed against the reference in-house acute leukaemia panel. Moreover, the results of database guided interpretation tools were compared to the expert-based interpretation of results to verify the accuracy of these new tools.

3. Results:

3.1. Flow Cytometer Settings:

3.1.1. Results of BD OneFlow™ Setup Beads:

The BD OneFlow setup beads were used to set the initial cytometer photomultiplier tube voltages (PMTVs). During this process, the PMTVs were manually adjusted to place the OneFlow setup beads within their lot specific median fluorescence intensity (MFI) target ranges. This was followed by adjusting forward and side scatter voltages using normal lysed washed blood sample (LWB). The results of the PMTVs are shown below (table 3.2).

Table 3.1: Cytometer voltage settings.

Parameter	Voltage
FSC	305
SSC	420
FITC	418
PE	418
PerCP-Cy5.5	547
PE-Cy7	535
APC	544
APC-H7	522
BD V450	369
BD V500	408

Abbreviations: APC, allophycocyanin; BD, Becton Dickinson; Cy7, cyanin 7; FITC, fluorescein isothiocyanate; H7, hilite 7; FSC, forward scatter; PE, phycoerythrin; PerCPCy5.5, peridinin-chlorophyll-protein-cyanin5.5; SSC, side scatter.

3.1.2. Results of Cytometer Setting and Tracking (CS&T) beads:

The Cytometer Setup and Tracking (CS&T) beads were used on daily basis to ensure the stability of baseline voltages (PMTVs), laser power as well as detectors efficiency. Acceptable criteria were defined by the manufacturer as delta PMTV < 50, bright Robust % CV < 6% and laser output of 46 – 69 mW, 16 – 24 mW and 12 – 18 mW for the violet, blue and red lasers; respectively. Any CS&T failure was repeated initially with a fresh preparation and, if needed, the manufacturer trouble shooting recommendations were followed. No patient testing was performed during CS&T failure.

3.1.3. Results of Compensation Settings:

Initially, the 8-Color Kit for BD OneFlow™ assays compensation kit (BD Biosciences, USA) and an in-house single reagent stained capture beads (BD™ CompBead) procedures were both tested. However, as there was no significant variation between the two calculated compensation values (tables 3.3 and 3.4), the department continued with the in-house single stain procedure to save the additional costs of the 8-Color BD OneFlow™ assays compensation kit.

Table 3.2: Compensation values using BD FC Beads 8-Color Kit for BD OneFlow™ Assays.

	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7	V450	V500
FITC	100	1.39	0.01	0.15	0.00	0.01	0.00	4.97
PE	10.58	100	0.00	1.24	0.00	0.01	0.00	0.88
PerCP-Cy5.5	3.55	43.40	100	7.85	1.04	0.13	0.00	0.49
PE-Cy7	0.30	3.43	16.62	100	0.12	1.35	0.00	0.06
APC	0.00	0.06	3.09	0.07	100	2.99	0.00	0.03
APC-H7	0.00	0.01	8.16	7.85	9.31	100	0.00	0.00
BD V450	0.00	0.00	0.00	0.00	0.00	0.07	100	8.62
BD V500	5.15	0.02	0.00	0.02	0.00	0.05	18.79	100

Abbreviations: APC, allophycocyanin; BD, Becton Dickinson; Cy7, cyanin 7; FITC, fluorescein isothiocyanate; H7, hilite 7; PE, phycoerythrin; PerCPCy5.5, peridinin-chlorophyll-protein-cyanin5.5.

Table 3.3: Compensation values using Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (beads).

	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7	V450	V500
FITC	100	1.37	0.00	0.09	0.00	0.00	0.00	2.44
PE	10.14	100	0.00	0.52	0.00	0.00	0.00	0.41
PerCP-Cy5.5	3.36	42.54	100	7.08	0.97	0.12	0.00	0.23
PE-Cy7	0.25	3.20	15.85	100	0.10	1.29	0.00	0.00
APC	0.02	0.08	2.92	0.02	100	2.69	0.00	0.00
APC-H7	0.00	0.00	5.43	10.48	8.39	100	0.01	0.00
BD V450	0.00	0.00	0.00	0.00	0.00	0.00	100	10.47
BD V500	4.50	0.00	0.00	0.00	0.00	0.00	19.64	100

Abbreviations: APC, allophycocyanin; BD, Becton Dickinson; Cy7, cyanin 7; FITC, fluorescein isothiocyanate; H7, hilite 7; PE, phycoerythrin; PerCPCy5.5, peridinin-chlorophyll-protein-cyanin5.5.

3.2. Patients and specimens:

A total of 24 consecutive specimens from 16 patients with new diagnosis of acute leukaemia were included in this study. These included 13 peripheral blood samples (54%) and 11 bone marrow aspirates (46%). Eight out of the sixteen patients had multiple specimens (peripheral blood and bone marrow) either at diagnosis or during their treatment plan (table 3.1).

Table 3.4: Summary of patients and specimens in the study.

Case	Gender	Age	Disease phase	Sample type	Diagnosis
1	M	7 years	Diagnostic	BM	B-ALL
2	F	5 years	Diagnostic	BM	B-ALL
3	M	2 years	Diagnostic	PB	B-ALL
4	M	2.5 years	Diagnostic	PB	B-ALL
5	F	6 years	9 months post treatment	BM	Recovering AML
6	M	13 months	Diagnostic	PB	AML
			Post Intensification- 1	BM	Relapse
7	F	11 months	Diagnostic	PB and BM	AML
8	M	14 years	Diagnostic	PB	T-ALL
			Post induction-2	PB	Recovering
9	M	6 years	Diagnostic	PB and BM	B-ALL
10	M	13 years	Diagnostic	PB and BM	B-ALL
11	F	6 years	Diagnostic	PB and BM	B-ALL
12	M	4 years	Diagnostic	PB and BM	B-ALL
13	F	2.5 years	Diagnostic	PB and BM	B-ALL
14	F	5 years	Diagnostic	BM	B-ALL
15	M	14 years	Diagnostic	PB	T-ALL
16	M	5 years	Diagnostic	PB	B-ALL

Abbreviations: BM, bone marrow; PB, peripheral blood; F, female; M, male.

The age of patients in the study ranged from 11 months to 14 years old with a median age of 5 years (figure 3.1). The majority of the cases were less than 10 years old (81%). Ten of cases in study were males (62%), while female patients represented 6 cases only (38%) (Figure 3.2).

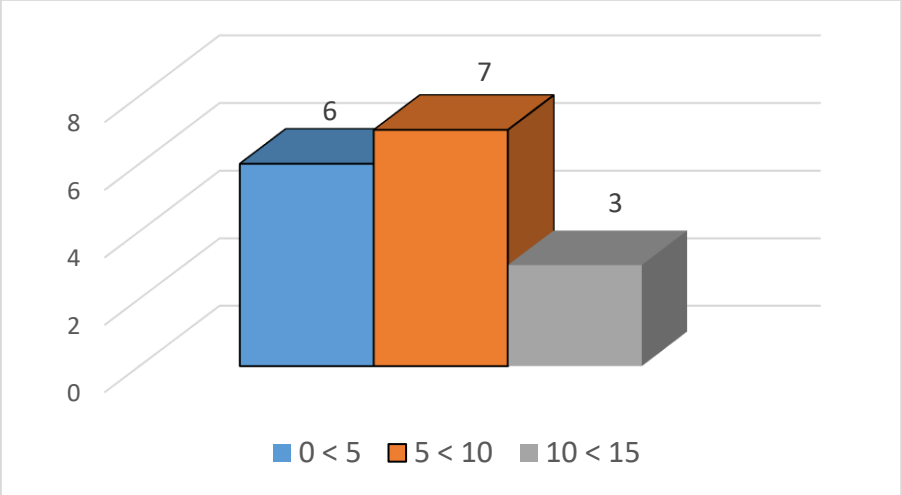


Figure 3.1: Age distribution of patients included in the study.

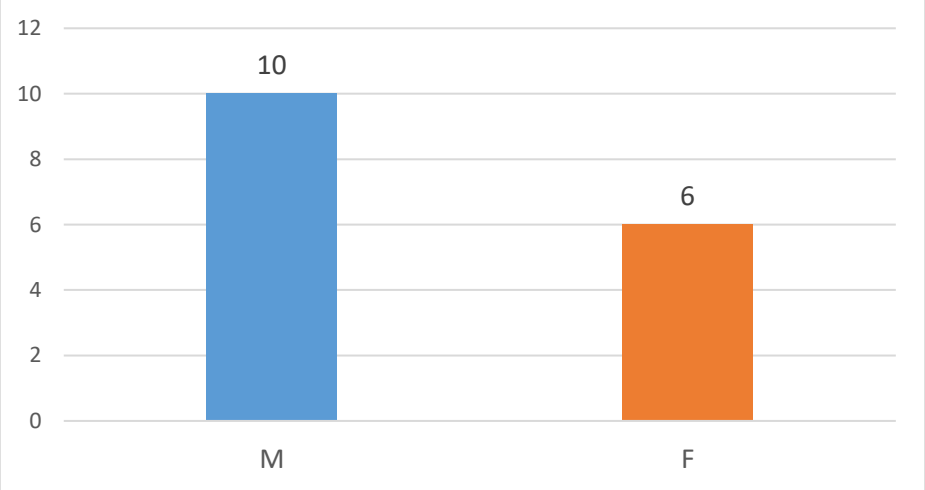
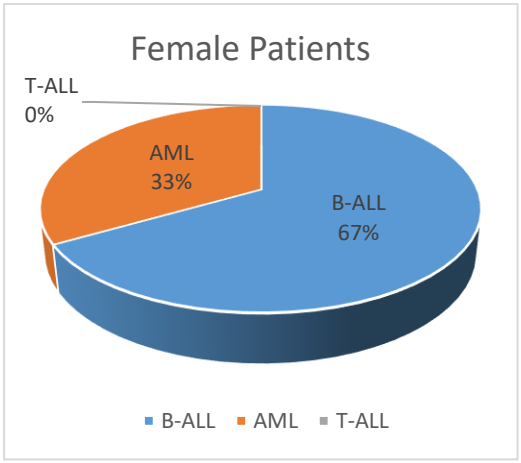


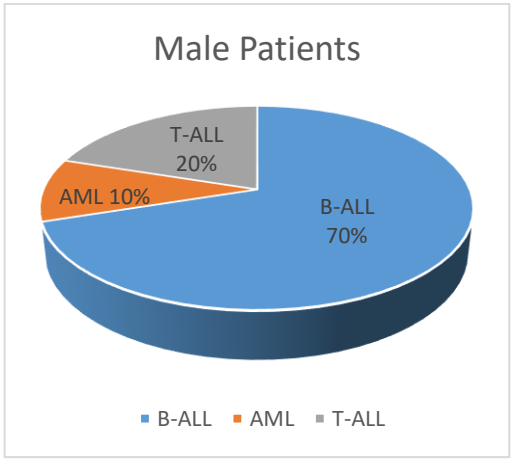
Figure 3.2: Gender distribution of patients included in the study.

The majority of cases in the study (11/16, 69%) had a final diagnosis of B- acute lymphoblastic leukaemia (B-ALL). Three cases had a diagnosis of acute myeloid leukaemia (AML, 19%), while only 2 cases had a diagnosis of T-acute lymphoblastic leukaemia (T-ALL, 12%) (Figure 3.3).

a.



b.



c.

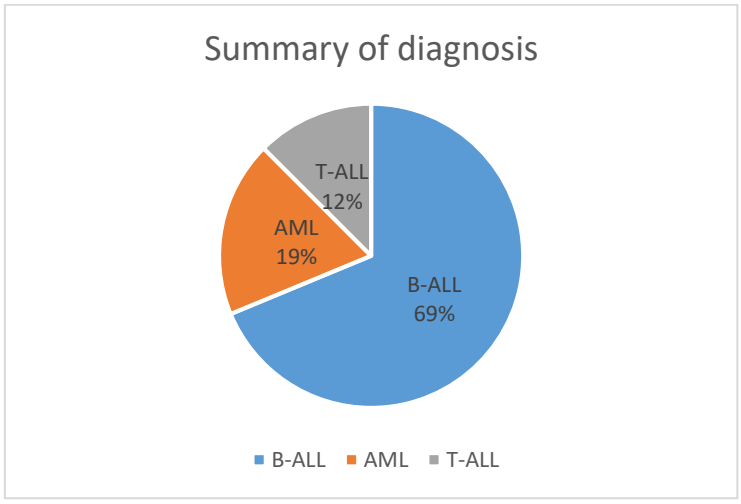


Figure 3.3: Summary of diagnosis for all patients included in the study. A: Distribution of different types of leukaemias among female patients, b: acute leukaemias among male patients and, c: overall distribution of all leukaemia cases.

3.3. Diagnosis:

In order to establish the final diagnosis for each case in the study, the clinical presentation, blood counts and morphology, coagulation screen studies, bone marrow aspirate and biopsy, cerebrospinal fluid examination, flow cytometry, cytogenetic anaylis as well as additional molecular studies were performed for all cases in the study as needed. Detailed results of all these findings for each case in this study are included in appendix 3.

3.3.1. Summary of clinical presentations:

Detailed patient history as well as clinical examination was performed by the clinical team for each case in the study. Results show that fever was the most common presentation for patients with acute leukaemia in this study (11/16; 69%). This was followed by bleeding and hepatosplenomegaly (56% and 50%; respectively), bone pain and polar (43% for each) and lymphadenopathy (31%). Other less frequent symptoms included night sweats, infection, abdominal discomfort as well as headache. The clinical presentations for all cases in the study were summarized in table 3.5 below.

Table 3.5: Summary of main symptoms and clinical presentations.

Case	Fever	Night sweat	Bleeding	hepatospl enomegaly	Lymphad enopathy	Diagnosis	Others
1	No	No	No	No	No	B-ALL	back pain
2	Yes	No	Bruises	No	No	B-ALL	N/A
3	Yes	Yes	No	Yes	Yes	B-ALL	Pale, trauma RT lower limb
4	Yes	No	No	Yes	No	B-ALL	Pale, Abdominal discomfort, tonsillitis
5	No	No	Petechiae	No	No	Follow up AML	N/A
6	Yes	No	Bruises	No	No	AML	URI
	No	No	No	No	No	Relapse	Skin lesions
7	Yes	No	No	Yes	No	AML	URI
8	Yes	No	No	No	Yes	T-ALL	Bone pain, headache, mediastinum mass
	No	No	No	Yes	No	Follow up	Abdominal pain (VOD)
9	Yes	No	Gingivitis	No	No	B-ALL	N/A
10	No	No	Epistaxis	Yes	No	B-ALL	pale, back pain, loss of appetite
11	No	No	Epistaxis	Yes	Yes	B-ALL	pale, loss of appetite
12	Yes	Yes	Petechiae	Yes	No	B-ALL	pale, bone pain
13	Yes	No	No	No	Yes	B-ALL	pale, bone pain, multiple vomits
14	Yes	Yes	Epistaxis	No	No	B-ALL	N/A
15	Yes	No	Sub-conjunctival hemorrhage	No	Yes	T-ALL	cough, nausea, vomiting and diarrhea
16	No	No	No	Yes	No	B-ALL	pale, limping from fall

Abbreviations: AML, acute myeloid leukaemia; B-ALL, B-acute lymphoblastic leukaemia; T-ALL, T-acute lymphoblastic leukaemia; VOD, veno-occlusive disease; URI, upper respiratory infection.

3.3.2. Blood counts, morphology, CSF and coagulation studies:

Full blood count was perfomed on all cases in this study. Fifteen out of the sixteen cases showed circulating blast cells at presentation (94%). The percentage of blast cells in peripheral blood varied from 8% to >90% in some cases. Interestingly, only one case in this study presented with completely normal blood count at presentation (case 1). The diagnosis of acute leukaemia was only established on this case after obtaining a bone marrow aspirate and trephine samples performed due to unexplained back pain.

The second major abnormality found on peripheral blood on these cases was thrombocytopenia. Reduced platelets count was found in 13 out of 16 cases at diagnosis (81%). The platelets count in this study ranged from 13 to 142 X 10^9/L. The following most common blood count abnormalities in this study were anemia and leukocytosis found in 11 out of 16 cases in this study (69%). Neutropenia was also noted in approximately 30% of the cases (6 out of 16).

Coagulation screening studies were mostly normal in patients with acute leukaemia in this study. Only three cases showing borderline prolonged prothrombin time (PT). Interestingly, one of these three cases had prolonged PT and APTT with significantly low fibrinogen level (0.9g/L). This patient also had severe thrombocytopenia (31 X 10^9/L) and presented with significantly high white blood cells count (231 X 10^9/L) and multiple bruises suggesting an early consumptive process and disiminated intravascular coagulation (DIC). The final diagnosis for this case was acute myeloid leukaemia with monoblastic differentiation (AML;

NOS). This patient was found later on to have a complex karyotype involving the MLL gene as described below in the cytognetic results section. Surprisingly, one case was found to have factor 7 (VII) deficiency incidently during coagulation screening.

Finally, only 4 cases in this study (25%) were found to have central nervous system involvement with blast cells (CNS-2). Three of these cases had a final diagnosis of B-ALL, while one case had a diagnosis of AML. Interestingly, all four cases with CNS involvement were CD34 negative by flow cytometry. The overall full blood count, coagulation screen and CSF findings for all 16 cases in the study were summarized below (tables 3.6)

Table 3.6: Summary of blood counts and coagulation screen.

Case	Phase	**WBC** (10^9/L)	**Hb** (g/L)	**Plts** (10^9/L)	**Neut.** (10^9/L)	**Blasts** (%)	**PT** (secs)	**APTT** (secs)	**Fib** (g/L)	**CNS**
1	Diagnosis	5.4	119	168	1.5	0	13	34	4	1
2	Diagnosis	5.7	110	19	0.7	8 %	13	20	2.7	1
3	Diagnosis	27.6	42	13	0.3	75 %	13	30	2.5	1
4	Diagnosis	43.5	92	40	9.1	70 %	11	24	2.5	2
5	Follow up	5.4	123	142	3.2	0	14	35	4	N/A
6	Diagnosis	231	90	31	3.9	77 %	19	35	0.9	2
	Relapse	6.4	106	294	2	< 1%	N/A	N/A	N/A	2
7	Diagnosis	24.6	55	14	2.2	35 %	13	32	3.2	1
8	Diagnosis	360	135	64	10.8	> 90 %	24	30	1.2	N/A
	Follow up	2	61	230	0.14	15 %	11	35	4.8	N/A
9	Diagnosis	1.8	71	261	0	10 %	15	31	4	1
10	Diagnosis	4.8	122	24	1.2	11 %	12	28	3.4	2
11	Diagnosis	43	34	12	0.9	> 90 %	15	25	2.9	2
12	Diagnosis	67	74	50	0.7	> 90 %	14	31	2.7	1
13	Diagnosis	25.8	74	145	1.6	> 80 %	23 (low FVII)	29	4.8	1
14	Diagnosis	6.7	97	185	1.8	7 %	13	27	4.4	1

Case	**Phase**	**WBC** (10^9/L)	**Hb** (g/L)	**Plts** (10^9/L)	**Neut.** (10^9/L)	**Blasts** (%)	**PT** (secs)	**APTT** (secs)	**Fib** (g/L)	**CNS**
15	Diagnosis	153	150	68	18.3	79 %	15	28	2.4	N/A
16	Diagnosis	17.3	61	18	1.2	51 %	14	34	N/A	1

Abbreviations: FVII, factor seven activity; N/A, not applicable.

3.3.3. Bone marrow aspirates and biopsies:

Bone marrow aspirates and biopsies were obtained from 14 out of 16 cases in this study (88%). The main reasons for bone marrow samples were to confirm the peripheral blood diagnosis, to perform cytogenetic analysis for final classification and prognosis and; to establish a molecular marker to allow future follow up (molecular minimal residual disease, MRD) where appilicable.

Only two cases in this study did not have bone marrow samples at diagnosis due to very high peripheral white blood cell count (>150 x 10^9/L) and the increased risk of tumor lysis syndrome (TLS). Therefore, both cytogentic and molecular analysis were performed from the peripheral blood on these two cases due to adequate number of circulating blast cells.

Diagnostic bone marrows confirmed the presence of excess blasts in all new cases in this study. The blast count ranged from 35-98% in the bone marrow aspirates. This variability is usually due to blood contamination during the aspiration procedure and therefore a diluted bone marrow aspirate can be obtained frequently. For this reason, bone marrow biopsies were obtained in parallel to aspirates for all cases undergoing the aspiration procedure under general anethesia. The bone marrow biospsies further confirmed the peripheral blood and bone marrow aspirate findings with cellularity mostly ranging 95 - 100% in almost all new cases in the study.

One follow up patient (case 5) had approximately 7% blast cells in her bone marrow aspirate. However, subsequent flow cytometry analysis on this bone marrow aspirate revealed approximately 18% precursor cells. The majority of these cells (15%) were found to be normal early B-cell precursors (type two hematogones); 2% less mature B-cell precursors (type one

hematogones) and only 1% early myeloid precursors. Differentiation of these early precursors and their maturation stage was only possible due to selectivity of specific markers in the in-house acute leukaemia panel as detailed in the flow cytometry section below.

3.3.4. Flow cytometry analysis:

3.3.4.1. Results of the in-house acute leukaemia panel:

The above mentioned in-house acute leukaemia panel was used to establish the diagnosis of acute leukaemia in all new cases in this study. The majority of the cases in the study (11/16, 69%) had a final diagnosis of B-acute lymphoblastic leukaemia (B-ALL). B-cell markers including CD10, CD19, cytoplasmic CD79a, HLA-DR s well as nuclear TdT and CD9 were present in all cases of B-ALL in this study (100%) (Figure 3.4).

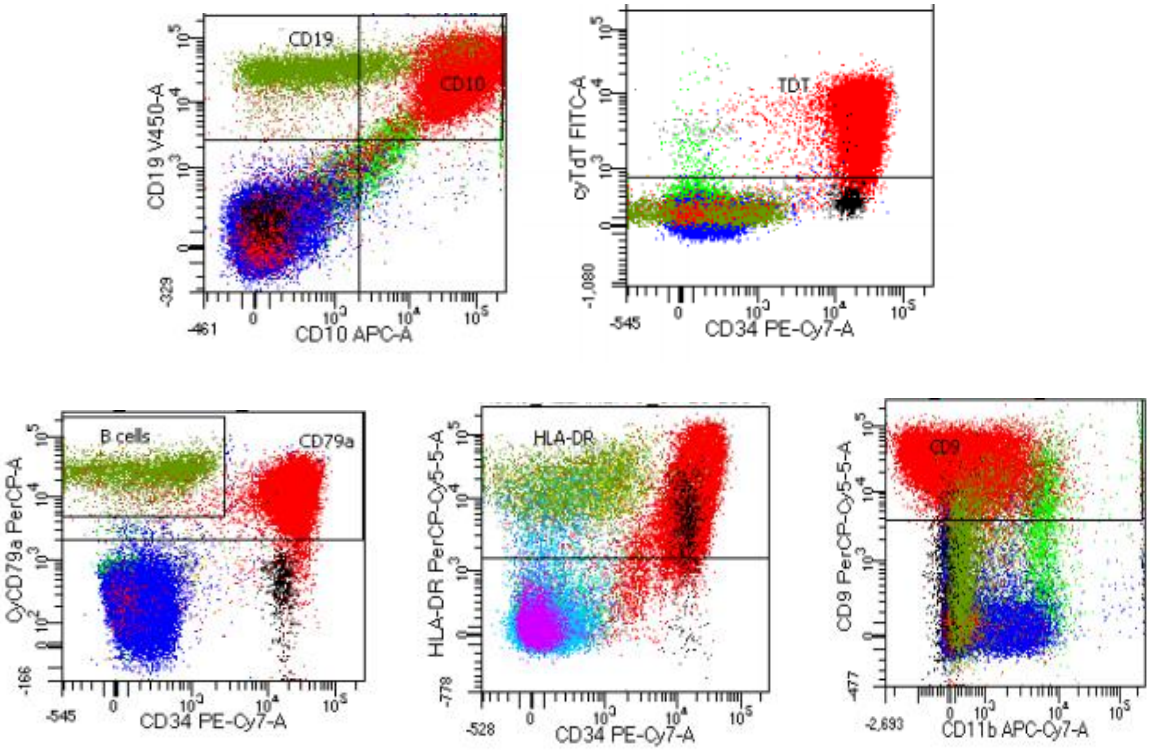


Figure 3.4: Most common markers found in B-cell acute lymphoblabstic leukaemia (B-ALL).

Interestingly, the other immaturity marker (CD34) was less common in B-ALL with a prevalence of 73% (8 out of 11 cases) compared to nuclear TdT which was positive in all 11 cases of B-ALL in this study (100%).

CD20 was another useful marker in identifying B-cells with at least partial expression in 8 out of 11 cases in this study (73%). Other myeloid markers including CD11b, CD13, CD33, CD117, CD14, CD64, CD7 and Cytoplasmic MPO were all negative in cases with B-ALL in this study. Likewise, T-cell specific markers (e.g. CD3, CD2, CD4, CD8, CD1a, CD5 and CD99) were also negative in cases with B-cell acute lymphoblastic leukaemia.

Three out of the sixteen cases in this study had a final diagnosis of AML (19%). Once more, CD9 was present in all cases with AML (100%). Interestingly, CD45 was positive in all three cases of AML (100%) compared to only in 9% of B-ALL cases. Other markers such as CD34, CD117, HLA-DR, CD33 and CD4 were positive in two out of the three cases of AML (67%), while other myeloid specific markers such as CD13, CD64 and cytoplasmic MPO were only present in one of the AML cases (33%).

Intersetingly, the immaturity marker (i.e. nuclear TDT) was negative in all AML cases in this study compared to (67%) positivity for CD34.

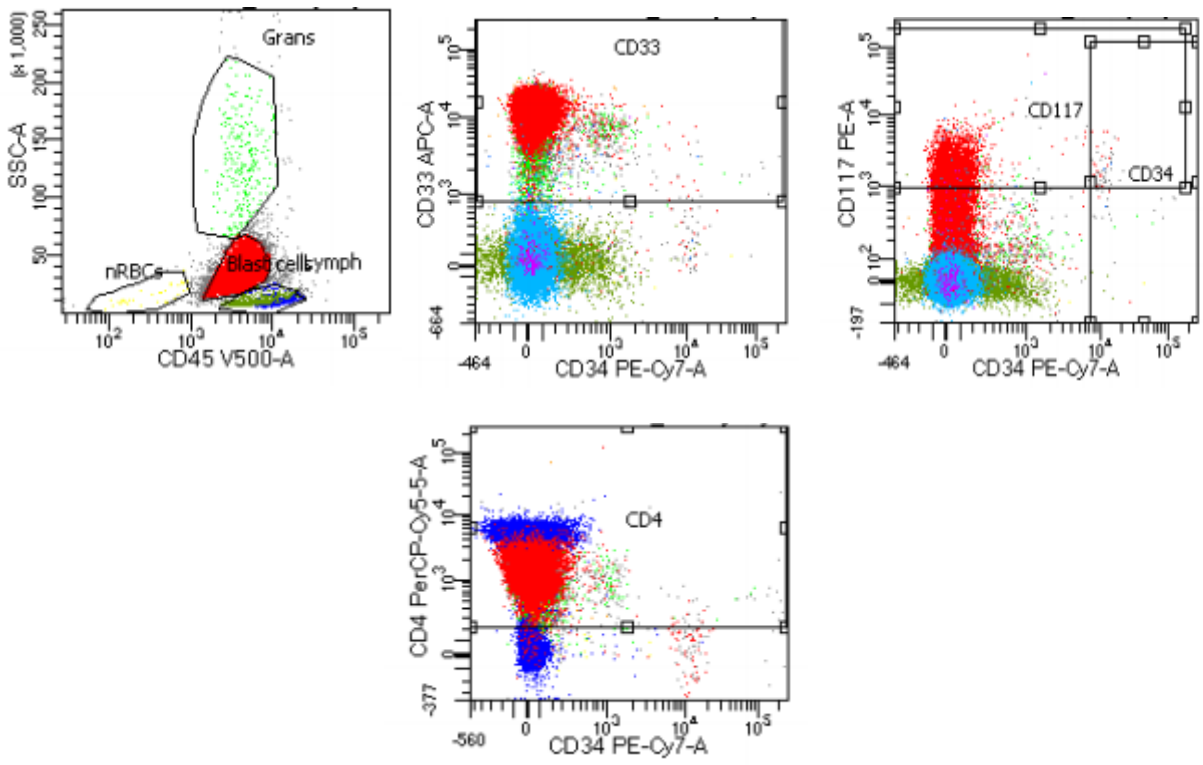


Figure 3.5: Common markers found in acute myeloid leukaemia leukaemia (AML).

Finally, only two of of 16 cases in this study had a final diagnosis of T-ALL (13%). Cytoplasmic CD3 was a key marker to identify T-lymphoblasts in both cases (100%). Other T-cell markers were also present in both cases in this study including CD2, CD4, CD7 and CD8 (100%). Futhermore, the immaturity marker (nuclear TDT) was also present in both T-ALL cases unlike CD34 which was negative in both cases. Similar to AML cases, both cases of T-ALL had a positive CD45 expression (100%) compared to only one case of B-ALL (9%).

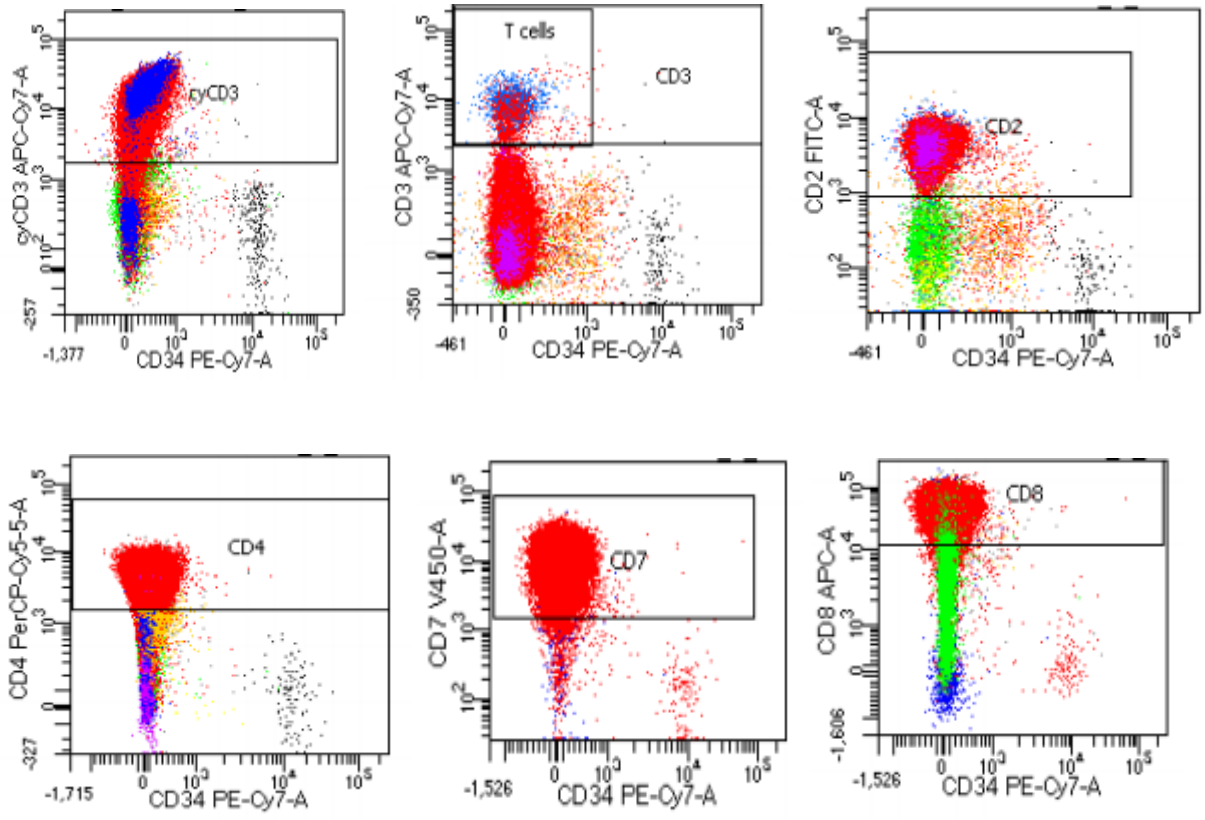


Figure 3.6: Common markers found in T-cell acute lymphoblabstic leukaemia (T-ALL).

3.3.4.2. Results of the (ALOT) tube compared to in-house acute panel:

In parallel to the in-house acute leukaemia panel, flow cytometry analysis was performed on all cases in the study using the Euroflow acute leukaemia orientation tube (ALOT). The specificity, sensitivity, accuracy and prevalence of each antibody in the ALOT tube was individually assessed against the reference in house acute leukaemia panel (table 3.7). Results showed 100% agreement with all antibodies in the ALOT tube compared to the in-house acute leukaemia panel.

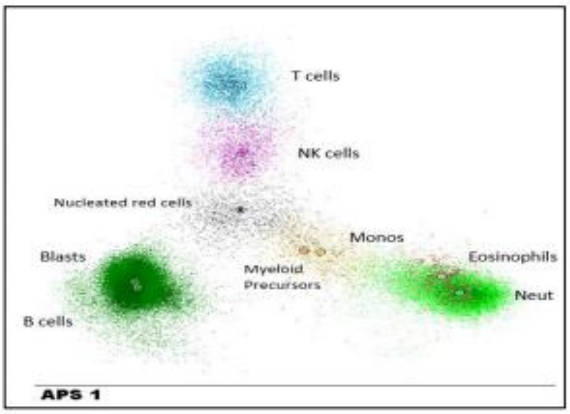
Table 3.7: Sensitivity and specificity results of antibodies in the ALOT tube.

	CyCD3	CD45	CyMPO	CyCD79a	CD34	CD19	CD7	SmCD3
Sensitivity:	100%	100%	N/A	100%	100%	100%	100%	100%
Specificity:	100%	100%	100%	100%	100%	100%	100%	100%
Prevalence	8%	71%	N/A	71%	54%	71%	17%	4%
Accuracy	100%	100%	N/A	100%	100%	100%	100%	100%

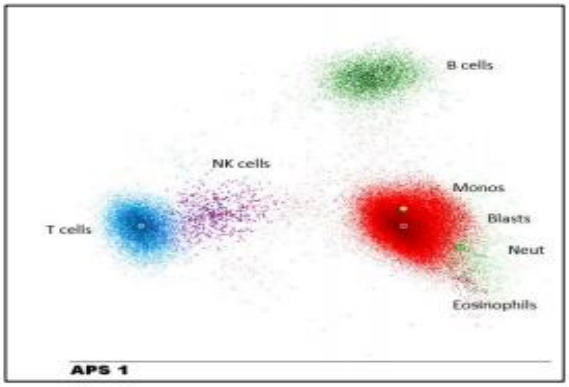
Abbreviations: N/A, not applicable.

Moreover, the results of the automated population separator (APS) and the principal component analysis (PCA) showed clear separation of the blast cells from residual normal cells in tested specimens (Figure 3.6).

a.



b.



c.

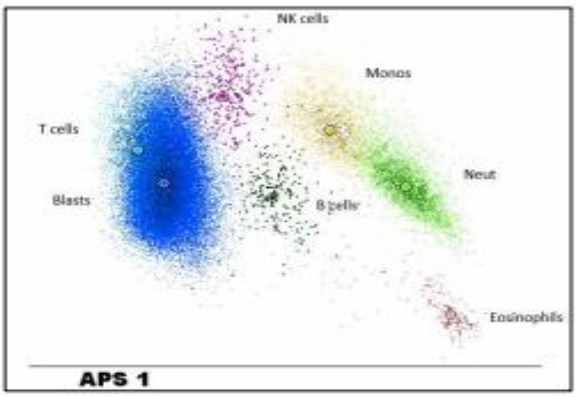
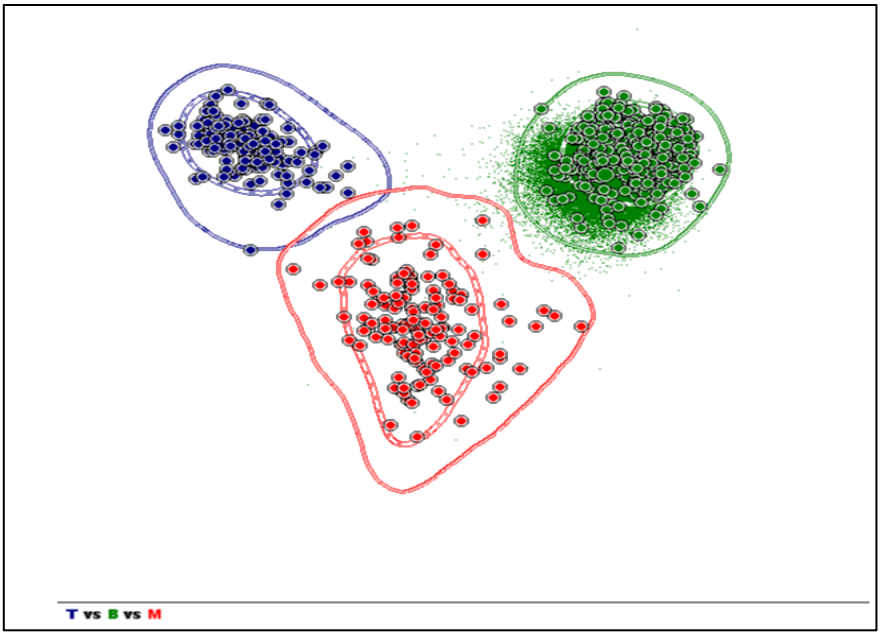


Figure 3.7: The automated population separator (APS) and the principal component analysis (PCA) with clear separation of the blast cells from residual normal cells in tested specimens. A. B-cell blast cells (in dark green), b. myeloid blast cells (in red) and c. T-cell blast cells (in blue).

3.3.4.3. Results of the EuroFlow automated database-guided interpretation tool for the (ALOT) tube:

The automated database-guided interpretation of the ALOT tube was compared to the manual (i.e. expert interpretation) of the ALOT tube in all 24 specimens in the study (table 3.8). Results showed 100% (24/24) agreement between the two interpretation approaches compared to the full in-house acute leukaemia panel. Moreover, the results of the ALOT tube alone in the 21 diagnostic specimens in the study were consistent with the final diagnosis of acute leukaemia without any additional markers (100% agreement with the full in-house panel results).

a.



b.

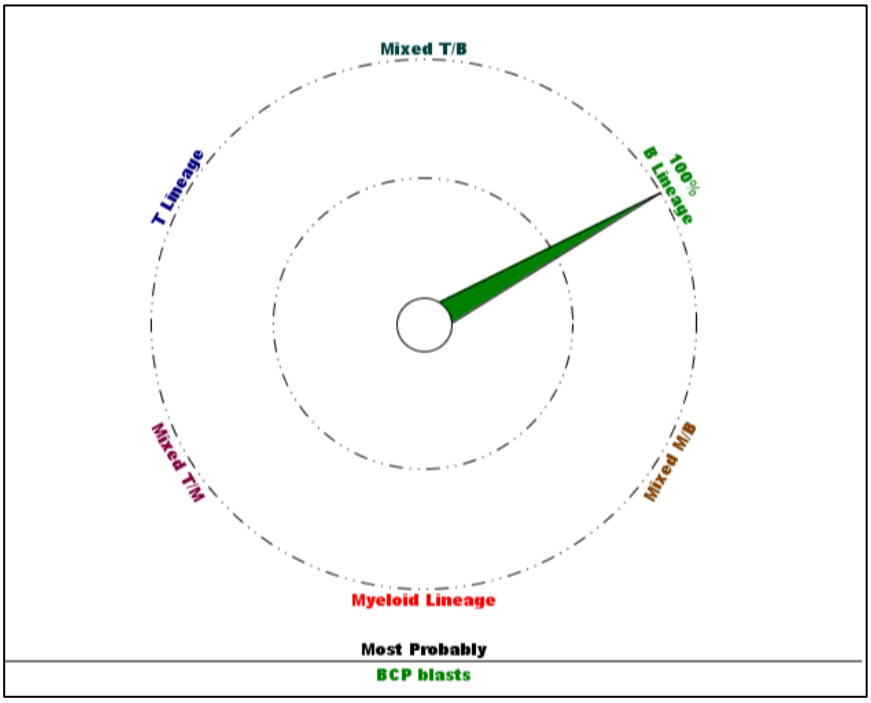
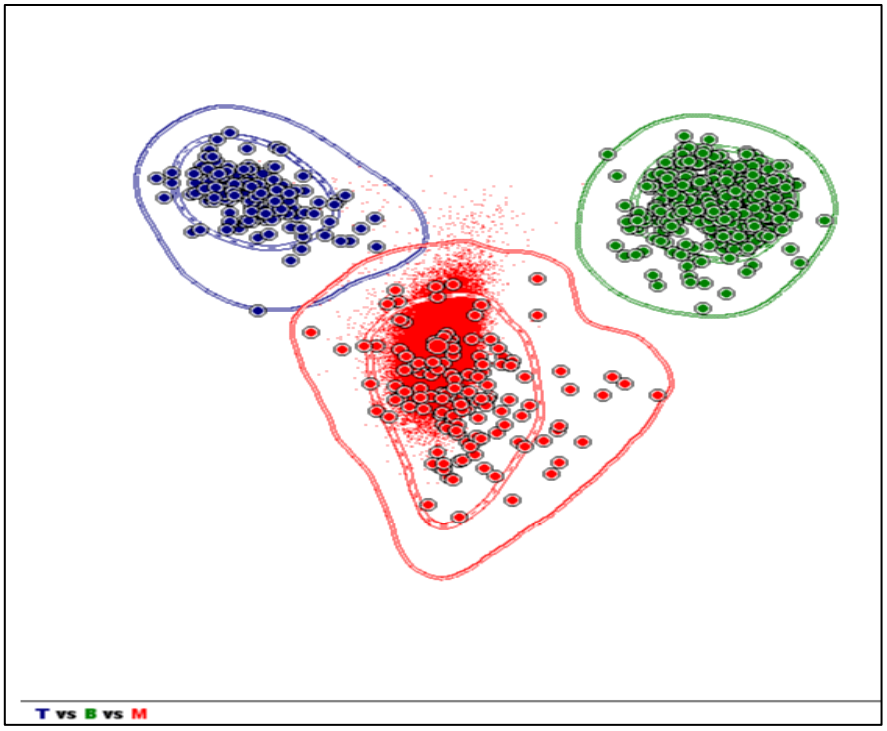


Figure 3.8: B-cell blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage (case 3).

a.



b.

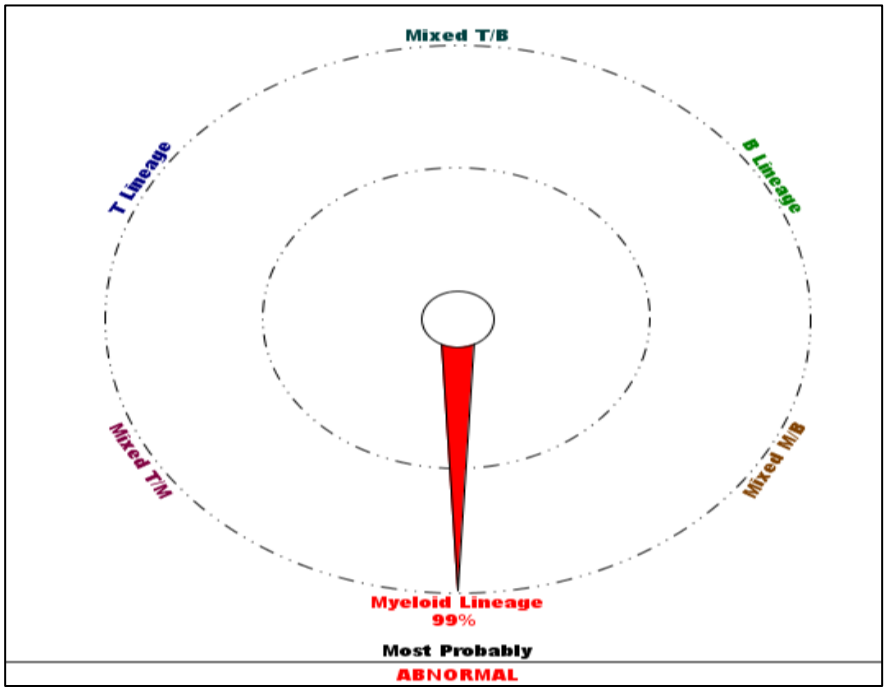
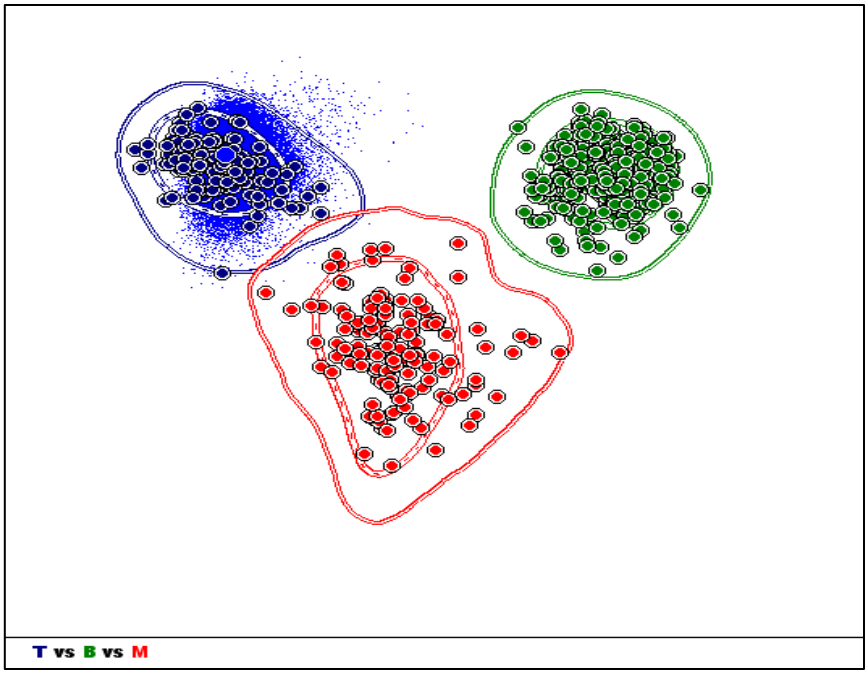


Figure 3.9: Peripheral blood myeloid blast cells orientation using automated database a. orientation among myeloid cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage (case 6).

a.



b.

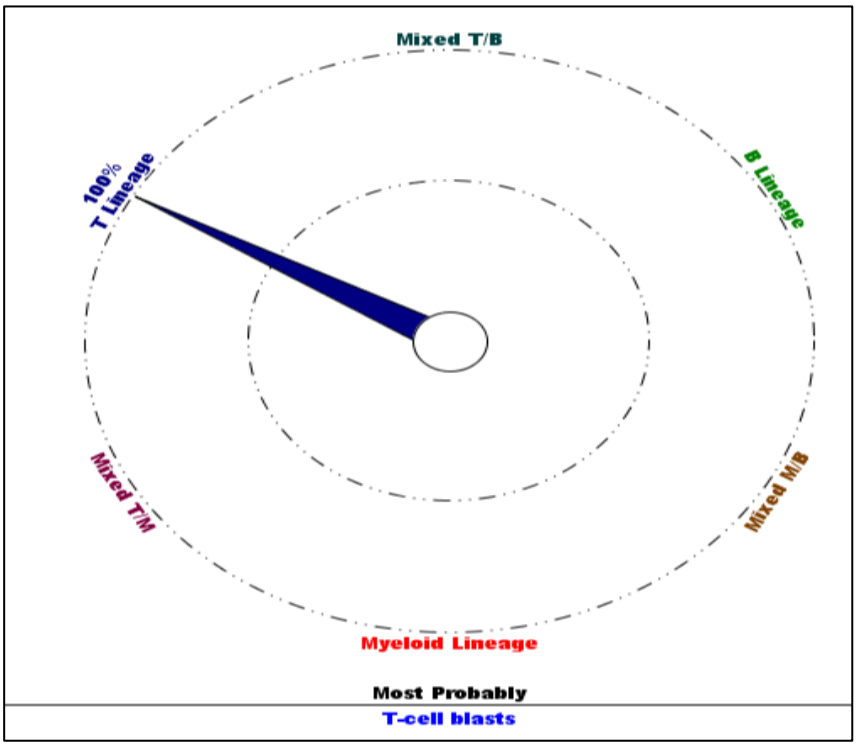


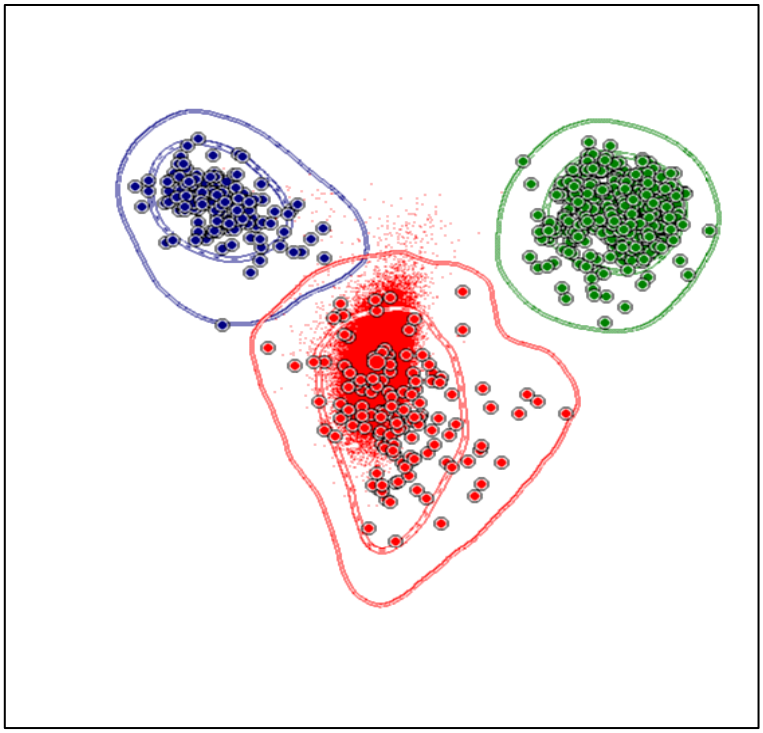
Figure 3.10: T-cell blast cells orientation using automated database a. orientation among T-cells (in blue) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of T-cell lineage (case 8).

Table 3.8: Comparison between expert-supervised and the automated database-guided interpretation of ALOT tube.

Case	Sample type	Disease phase	In-house acute panel	ALOT Expert-Supervised	ALOT Automated database	Final diagnosis
1	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
2	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
3	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
4	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
5	BM	9 months--post treatment	Mixed-lineages (B/M)	Mixed-lineages (B/M)	Mixed-lineages (B/M)	Increased hematogones
6	PB	Diagnostic	Myeloid	Myeloid	Myeloid	AML
	BM	Post Intensification-1	Myeloid	Myeloid	Myeloid	Relapsed AML
7	PB	Diagnostic	Myeloid	Myeloid	Myeloid	AML
	BM	Diagnostic	Myeloid	Myeloid	Myeloid	AML
8	PB	Diagnostic	T-lineage	T-lineage	T-lineage	T-ALL
	PB	Post induction-2	Myeloid	Myeloid	Myeloid	Recovering BM
9	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
10	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
11	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
12	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
13	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
14	BM	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL
15	PB	Diagnostic	T-lineage	T-lineage	T-lineage	T-ALL
16	PB	Diagnostic	B-lineage	B-lineage	B-lineage	B-ALL

Surprisingly, the ALOT tube and the automated database interpretation tool was successful in identifying the new AML cases (cases 6 and 7) despite lacking cytoplasmic MPO (figures 3.10 and 3.11). Case six was an acute myeloid leukaemia with monoblastic differentiation (AML-M5a) and was essentially negative for all markers in the ALOT tube (except CD45) with no expression of any B-cell, T-cell or myeloid specific markers. On the otherhand, the second case (case 7) was only positive for CD45 and CD7 markers in the ALOT tube. Despite lacking any myeloid specific markers in both cases in the ALOT tube, the automated database interpretation tool successfully identified both cases as myeloid lineage.

a.



b.

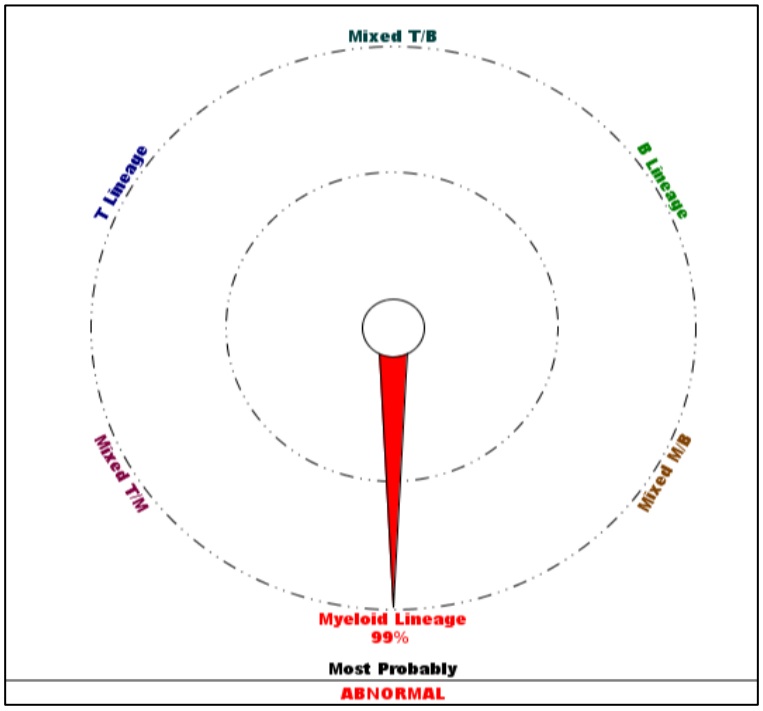
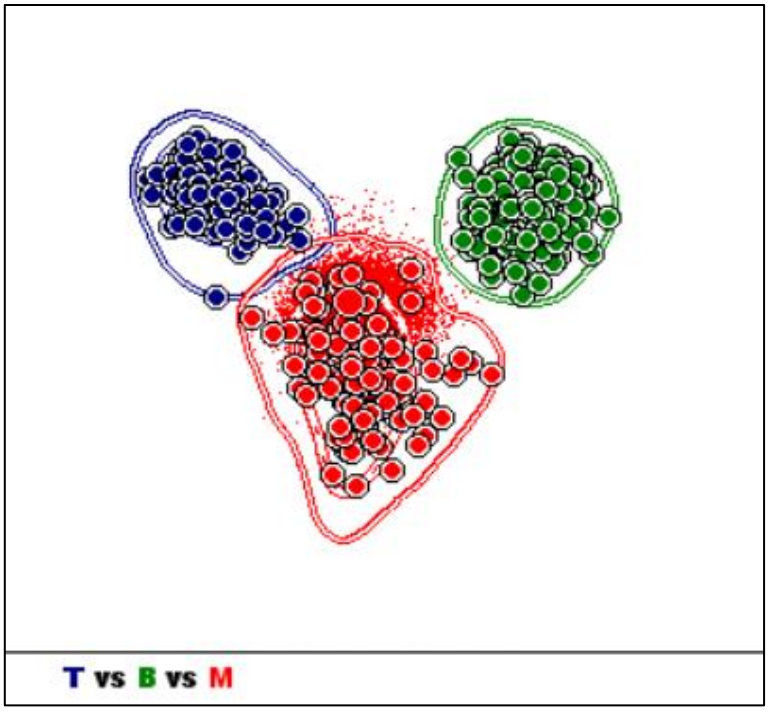


Figure 3.11: Case 6 peripheral blood blast cells orientation using automated database a. orientation among myeloid cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

a.



b.

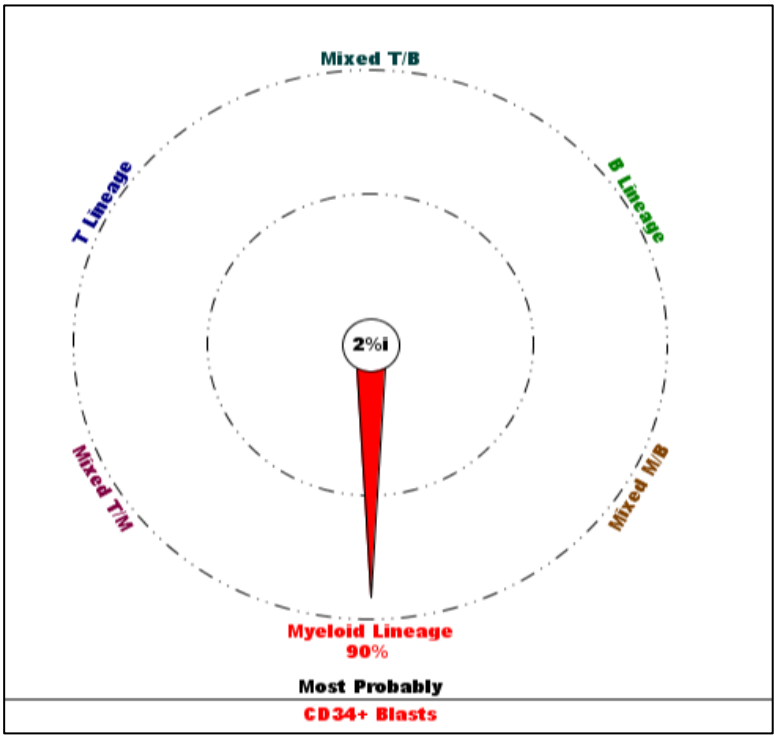


Figure 3.12: Case 7 peripheral blood blast cells orientation using automated database a. orientation among myeloid cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

In one of the followup bone marrow aspirates (case 5), flow cytometry was performed to determine the cell lineage of immature precursors noted by morphology. Gating on CD34 positive population showed a non-conclusive mixture of myeloid and B cell lineages (Figure 3.13). Subsequent gating of CD19/CD34 positive B-cell precursors (type I-haematogones) and CD19 negative/ CD34 positive precursor cells (myeloid precursors) showed clear distinction between the normal B-cell precursors and myeloid precursors using the Cytognos EuroFlow database and the compass tool (Figures 3.14).

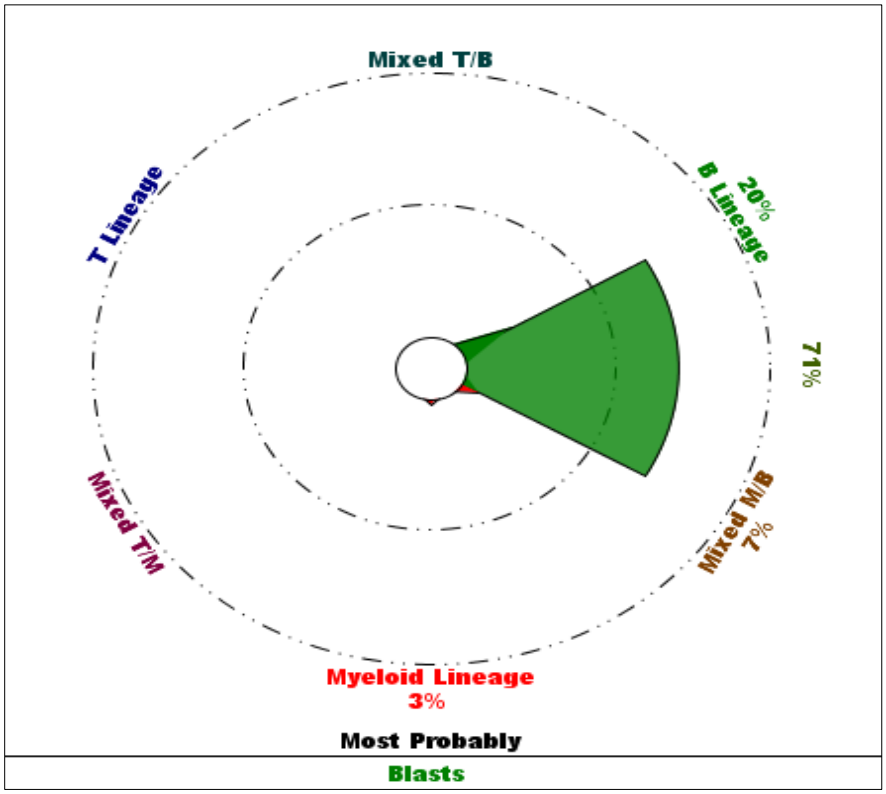
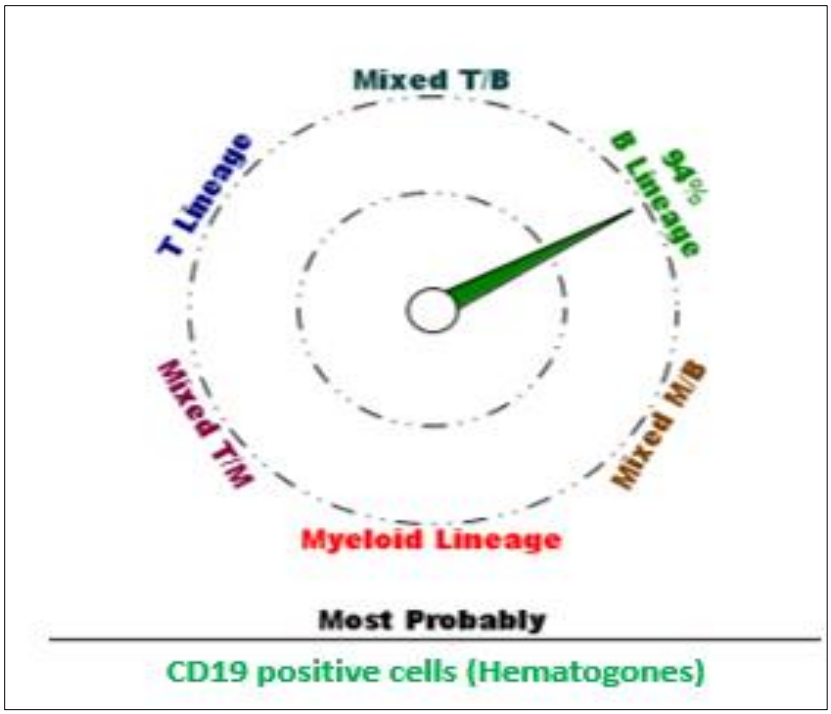


Figure 3.13: Case 5 CD34 positive precursor cells appearing to be of a mixed lineages.

a.



b.

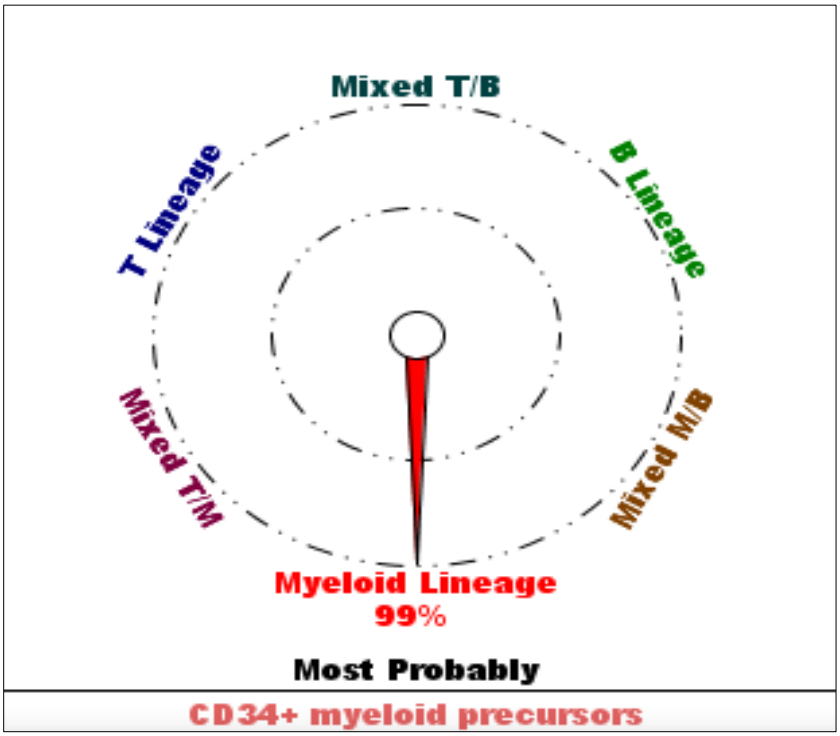
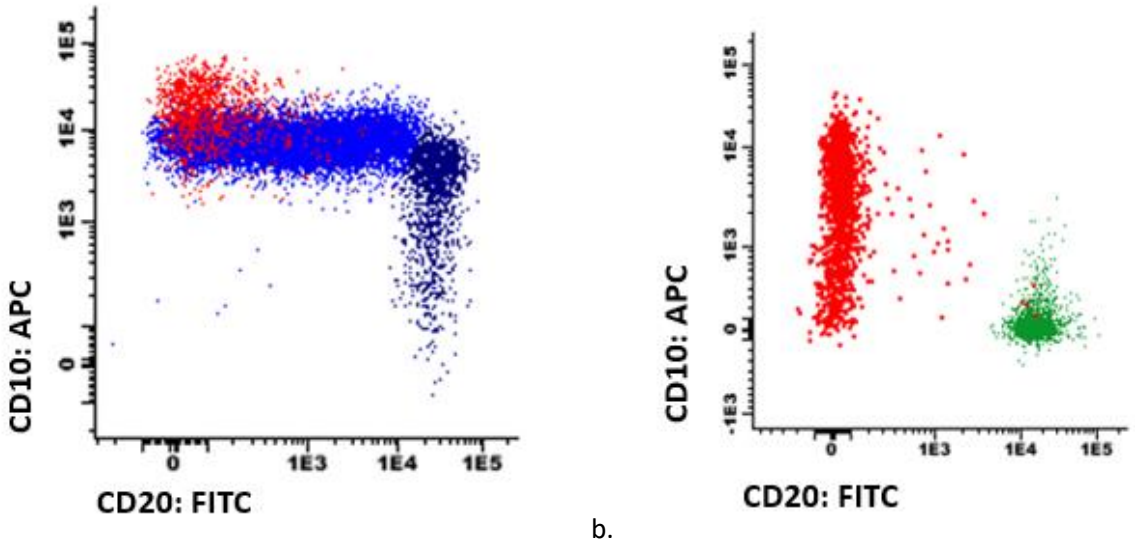


Figure 3.14: Case 5 database compass showing a. CD19/CD34 positive cells (hematogones) to be of a B-cell lineage and; b. CD34 positive and CD19 negative cells to be of myeloid origin.Of

important note, co-expression of CD10 and CD20 showing normal B-cell maturation was critical to identify these cells as benign B-cell precursors compared to malignant B-cell blast cells seen in acute leukaemia (Figure 3.15). This critical distinction between benign and malignant was only possible by using the combination of these antibodies in the in-house acute leukaemia panel (i.e. Tube A1 with CD19, CD45, CD34, CD10 and CD20) (appendix 1).



a. b.

Figure 3.15: a: Showing normal B-cell maturation stages (in red type I hematogones, in blue type II hematogones (CD34 negative with partial CD20), in dark blue type III hematogones (naïve B-cells, CD10/CD20 positive) and traces of mature B-cells (bottom right) CD20 positive and CD10 negative mature B-cells). B: Malignant B-cell precursors (blast cells) showing maturation arrest (i.e. discontinuation of maturation) (in red: malignant B-cells; in green: residual benign B-cells in this specimen).

3.3.5. Results of cytogenetic analysis:

Following the morphological and flow cytometry diagnosis of acute leukaemia, subsequent interphase fluorescence in-situ hybridization analysis (FISH) and karyotype analysis were performed on all new cases in this study. Table 3.9 below summarises the cytogenetic results for all cases.

Table 3.9: Summary of Cytogenetic results.

Case	Flow Cytometry Diagnosis	FISH Results	Karyotype	Final WHO Classification
1	B-ALL	Extra signals of multiple probes	60,XY	B-ALL with hyperdiploidy
2	B-ALL	Normal	46, XX	B-ALL; NOS
3	B-ALL	Extra signals of multiple probes	55,XY	B-ALL with hyperdiploidy
4	B-ALL	Extra copies of Chromosome 21	47, XY	B-ALL; NOS
5	AML	Abnormal RUNX1/RUNX1T1 robes	46,XX,t(8;21;13)	AML with t(8;21)
6	AML	Abnormal 11q probe	46,XY,t(11;19)	AML; NOS (M5a)
7	AML	Normal	46,XX	AML; NOS(M7)
8	T-ALL	Abnormal TRA/D probes	46,XY,t(11;14)	T-ALL
9	B-ALL	Extra copies of Chromosome 21	46,XY,Add(5),+21	B-ALL; NOS
10	B-ALL	Normal	46,XY	B-ALL; NOS
11	B-ALL	ETV6/RUNX1 fusion	46,XX	B-ALL with t(12;21)
12	B-ALL	Extra signals of multiple probes	58~68,XXY	B-ALL with hyperdiploidy
13	B-ALL	ETV6/RUNX1 fusion	46,XX,add(12)	B-ALL with t(12;21)
14	B-ALL	Normal	46,XX,t(1;19)	B-ALL with t(1;19)
15	T-ALL	Normal	46,XX,t(8;14)	T-ALL
16	B-ALL	Abnormal IGH rearrangement	46,YX,t(Y;14)	B-ALL with BCR-ABL1 like transloc.

Analysis of eleven B-ALL cases in this study identified four cases of B-ALL not otherwise specified (B-ALL; NOS), three cases of B-ALL with hyperdipoloidy, two cases of B-ALL with translocation t(12;21), one case of B-ALL with translocation t(1;19) as well as one case of B-ALL with BCR-ABL1 like translocation t(Y;14).

3.3.6. Correlating the results of cytogenetic and flow cytometry analysis:

The cytogenetic findings correlated well with the corresponding phenotype for each case in this study. For instance, in cases with B-ALL with hyperdilpoidy flowcytometry results showed higher forward scatter properties of blast cells compared to the residual normal T-cells in the background (Figure 3.16) suggesting a possible hyperdilpoidy.

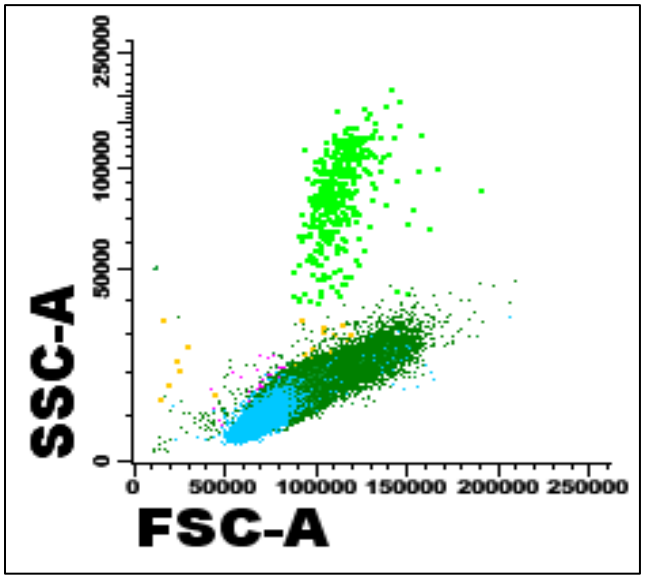


Figure 3.16: Forward and side scatter properties of B-cell blast cells (in green) and normal T-cells (blue) indicating a possible hyperdilpoidy based on forward scatter properties only.

Moreover, cases of B-ALL with hyperdilpoidy (e.g. case 12) showed positive expression of CD66c and CD123 showing the additional value of these markers in prediction of B-ALL with hyperdilpoidy (Figure 3.17).

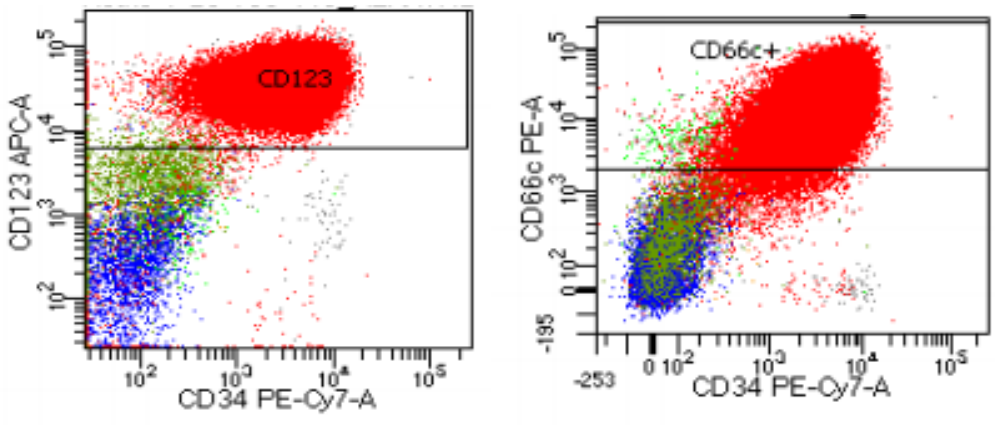


Figure 3.17: B-cell blast cells showing positive expression of CD66c and CD123 suggesting a possible hyperdiploidy.

Additionally, cases of B-ALL with t(12;21) ishowed moderate expression of CD38 in addition to variable expression with CD34 which correlates well with the phenotype expected for this cytogenetic abnormality (Figure 3.18).

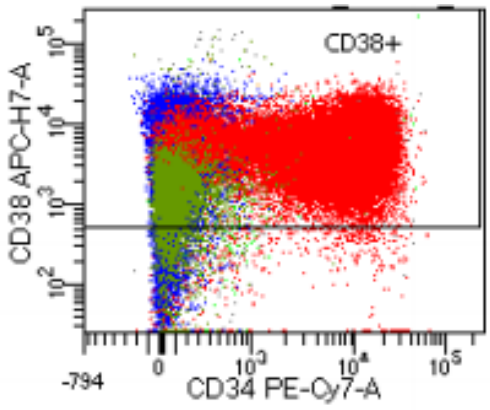


Figure 3.18: B-cell blast cells showing positive expression of CD38 suggesting a possible translocation between chromosome 12 and 21, t(12;21).

Finally, one case of B-ALL with t(1;19) showed a strong expression of CD9 as well as lack of CD34 expression (Figure 3.19) which corresponds well with the phenotype for this cytogenetic abnormality.

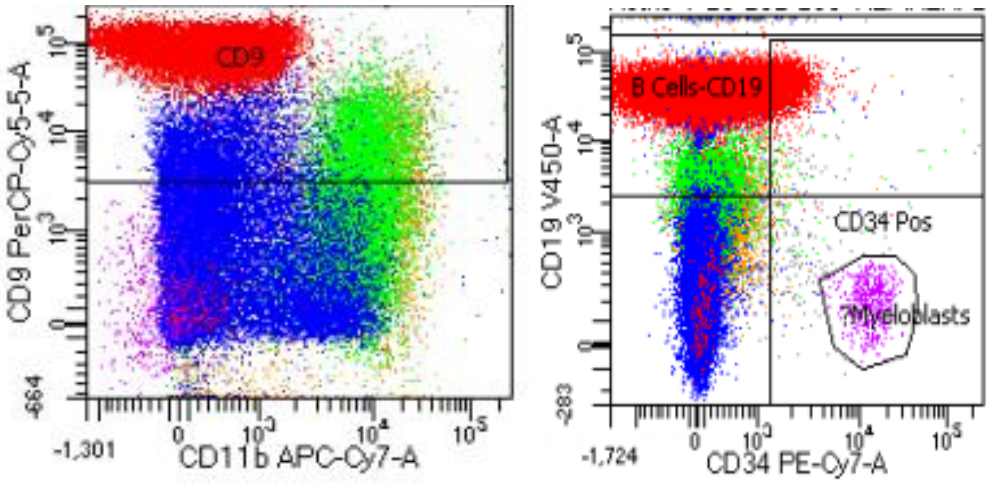


Figure 3.19: B-cell blast cells showing a strong positive expression of CD9 suggesting a possible translocation between chromosome 1 and 19, t(1;19).

Furthermore, cytogenetic analysis in this study further subclassified the three acute myeloid leukaemia into AML with t(8;21) and two cases of AML not otherwise classified (AML; NOS). Interestingly; one case of AML had an abnormal MLL gene rearrangement t(11;19) showed clear expression of CD15 by flow cytometry suggesting the powerful use of this marker in prediction of MLL rearrangement subsequently (Figure 3.20).

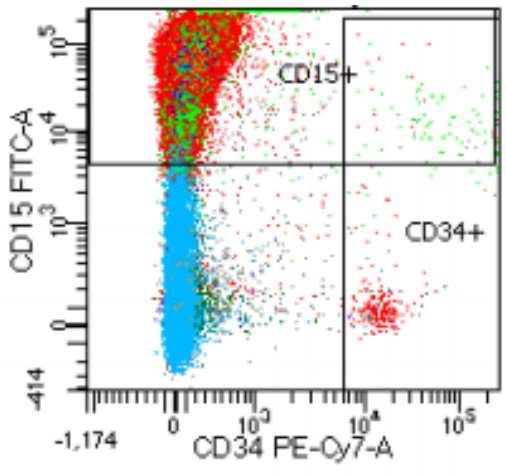


Figure 3.20: Blast cells showing a strong positive expression of CD15 suggesting a possible MLL rearrangement.

A second case of AML in this study with translocation t(8;21) showed an aberrant partial expression of CD19 (a B-cell marker) by flow cytometry which is frequently associated with acute myeloid leukaemia with this specific cytogenetic abnormality (Figure 3.21).

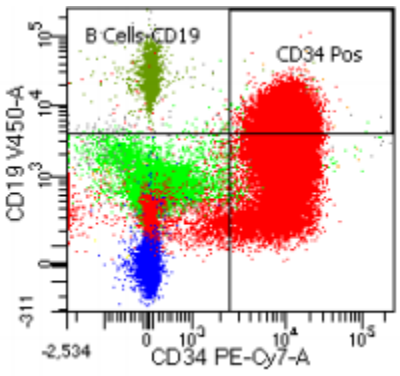


Figure 3.21: Blast cells (in red) showing an aberrant partial expression of CD19 on myeloid blast cells suggesting a presence of t(8;21).

In addition to its diagnostic and classification roles in acute leukaemia, cytogenetic and flow cytometry analysis also played a key role in disease monitoring in some cases in this study as highlighted below.

3.3.7. Disease monitoring and detection of minimal residual disease:

During this study, three cases were presented for repeat flow cytometry analysis due to clinical and morphological suspicion of relapse. At this point, the leukaemia associated phenotype (LAP) or diagnostic signature of blast cells was already established for each case in the study. Addtionally, full blood count, morphological assessment of blood and bone marrow, cytogenetic analysis as well as molecular testing were all performed for each patient, as needed. The choice of which technique or procedure to be used for disease monitoring relied heavily on the initial presentation of each case as well as the presence of suitable specific markers for follow up as highlighted below.

3.3.7.1. Case 5:

This case was a 6 years old girl with known history of AML with t(8;21). She presented 9 months later with petechial skin rash similar to her diagnostic presentation. Her mother was very concerned that the leukaemia is back. On examination, she looked well with clear throat, chest and abdomen. There was no organomegaly, fever, diarrhea, abdominal pain or bleeding symptoms apart from the petechial skin rash. Blood counts were requested for follow up.

Her blood count showed normal haemoglobin level for age (haemoglobin 123 g/L) and borderline thrombocytopenia (platelets count of 142 x 109/L). There was no evidence of leucocytosis with

a white blood cells count (WBCs) of 5.4 x 10^9/L and she had a normal neutrophil count of 3.2 x 109/L.

Her blood film examination showed macrocytosis with tear drop cells and occasional dysplastic (hypo granular) neutrophils. Occasional immature looking monocytes were present, however; no definite blast cells were identified.

Bone marrow aspirate was particulate with adequate quality for assessment. Differential count identified approximately 7% blast cells. These were variable in morphology with some small to medium-sized cells with high nuclear cytoplasmic (N/C) ratio, mildly irregular nuclear contours, fine chromatin and indistinct nucleoli. Other blast cells were larger with abundant cytoplasm showing limited granularity, fine chromatin, and variably prominent nucleoli.

Erythroid precursors showed mild megaloblastic changes while myeloid and megakaryocytic showed mild dysplasia (including hypo granularity and hypolobation). There was active trilineage haematopoiesis which was also confirmed by the bone marrow biopsy. Bone marrow cellularity was approximately 50% (low for age).

Her bone marrow aspirate was received for flow cytometry for assessment of blast cells. Additional bone marrow samples were also referred for cytogenetic analysis (FISH and karyotype) to detect the presence of the diagnostic clone with t(8;21). Flow cytometric immunophenotyping performed on the bone marrow aspirate identified an immature population comprising of approximately 18% of the total cell population.

A small population (approximately 2%) were expressing CD34, CD9, CD10, CD19, HLA-DR, strong CD38 in addition to cytoplasmic CD79a and TdT. This population showed no significant expression of other myeloid or T-cell markers likely indicating normal CD34 positive hematogones (type I hematogones).

In addition to this population, there was a second population of approximately 15% with CD9, CD10, CD19, HLA-DR, strong CD38, partial CD20 in addition to cytoplasmic CD79a and TdT. This population showed no significant expression of CD34, CD45, CD2, CD3, CD4, CD7, CD11b, CD13, CD33 or any other myloid markers indicating a more mature B-cell precursor population (type II hematogones). Co-expression of CD10 with partial CD20 was essential to identify these populations as benign (Figure 3.22).

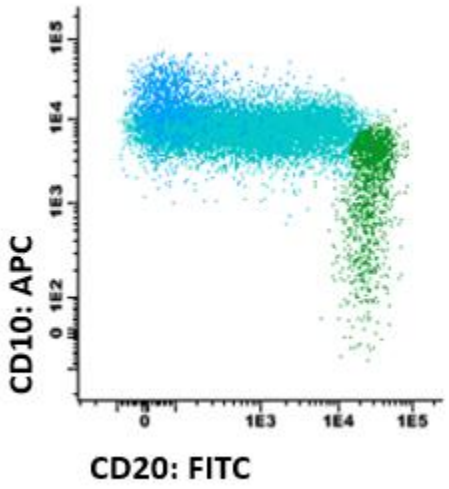


Figure 3.22: CD10 and CD20 showing normal B-cell maturation pattern (in dark blue type I hematogones (CD10 positive, CD20 negaive), in blue type II hematogones (CD10 positive with partial CD20), in green type III hematogones (naïve B-cells, CD10 and CD20 positive) as well as traces of mature B-cells (CD20 positive and CD10 negative).

Finally, a small population comprising approximately 1% of total cells was also identified. This showed positive CD34 expression with dim CD45, partial CD13, CD33, CD117 and HLADR representing early myeloid precursors. Importantly, there was no aberrant expression of CD19 on this myeloid population which was associated with the diagnostic phenotype.

Subsequent interphase fluorescence in-situ hybridization (FISH) and karyotype analysis performed on the bone marrow aspirate showed normal karyotype with 46 chromosomes (46,XX). There was no evidence of the diagnostic t(8;21) (RUNX1/RUNXT1) translocation. Furthermore, there was no evidence of any recurrent rearrangements of the BCR/ABL1, ETV6/RUNX1, MLL, IGH or CDKN2A associated with B-acute lymphoblastic leukaemia.

The overall morphologic, immunophenotypic and cytogenetic findings were therefore consistent with increased hematogones with peripheral macrocytosis. There was no immunophenotypic or cytogenetic evidence of leukemia or relapse.

3.3.7.2. Case 6:

This was a 13 months old boy referred from a primary healthcare center due to increased white blood cells count (WBC 231 x 109/L). The child had a history of fever and congested throat with upper respiratory symptoms for one week. Four days earlier, his mother noticed that he had bruises over his right knee. The bruises rapidly increased in size and number over both thighs and knees.

His intial workup established the diagnosis of AML with t(11;19) three months ago. His flow cytometry was performed on both, peripheral blood and bone marrow aspirate samples and the

leukaemia associated phenotype (LAP) was moderate CD45 expression, positive expression of CD9, CD33, CD4, CD64, and CD15 with partial expression of CD13, CD117, CD38 with an aberrant expression of CD56. There was no significant expression of CD34, HLA-DR, CD2, CD3, CD7, CD10, CD11b, CD19, CD20, CD66c, cytoplasmic MPO, cyCD79a, cyCD3, cyIgM or TdT.

The most critical finding in this case was the presence of strong expression of CD33 and CD117 in absence of HLA DR in blast cells (Figure 3.23). There were no FLT3 ITD or NPM1 detected by molecular at diagnosis.

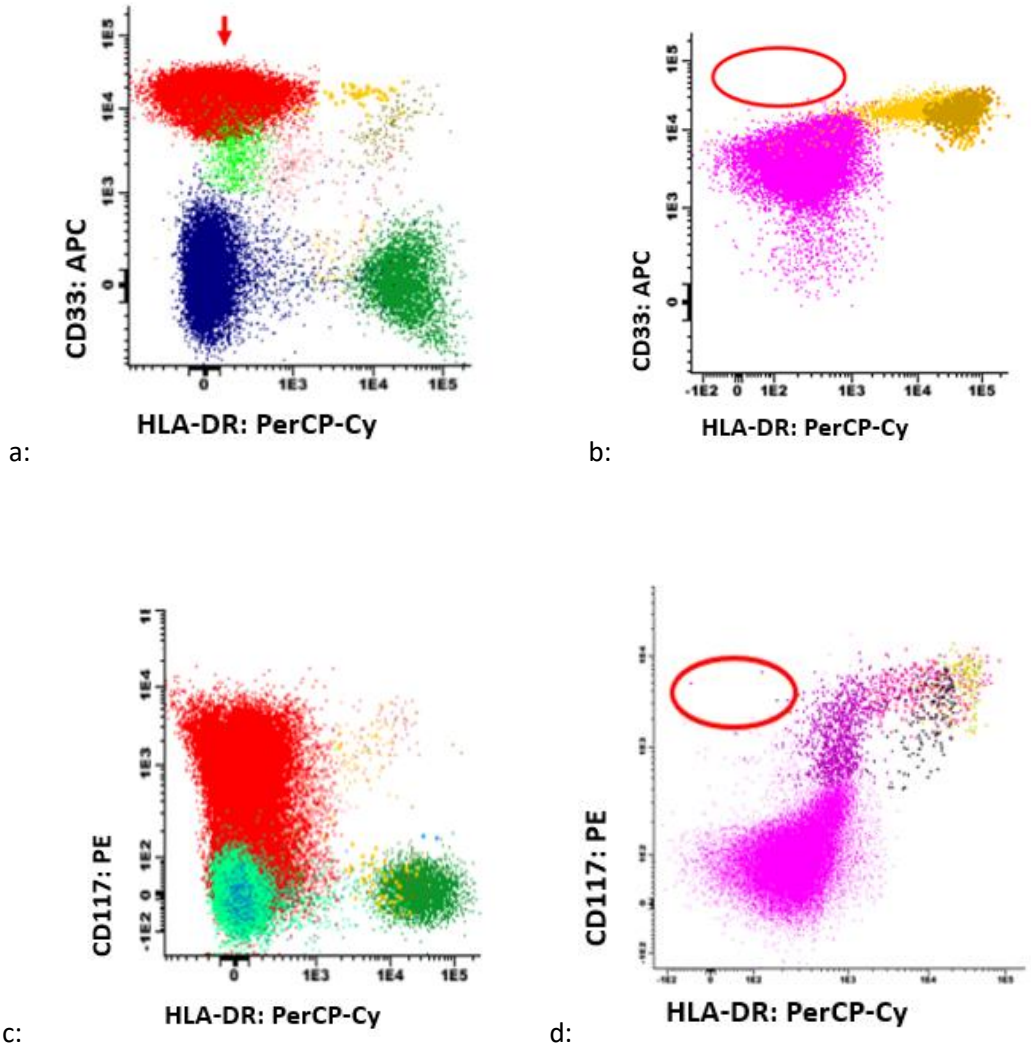


Figure 3.23: Blast cells showing abnormal positive expression of CD33 and CD117 in absence of HLADR. A: Arrow denotes abnormal blast cells with bright CD33 (in red) compared to normal maturation pattern in plot b. (empty circle) C: Abnormal blast cells expression of CD117 (in red) in absence of HLADR compared to the normal pattern in plot d.

Three months later the child was admitted for elective chemotherapy (post intensification I). He was afebrile, well hydrated with no respiratory or abdominal abnormalities during examination. However, he had small lesions in the face, hands and buttocks which were not vesicles and some were hard to touch. There was no hotness or tenderness.

His full blood count showed normal white blood cell count (WBC 6.4 x10^9/L) with normal haemoglobin for age (106 g/L) and normal platelets count (294 x 10^9/L). However, his blood smear showed left shifted, leucoerythroblastic picture with rare blasts with morphology consistent with leukemic population. These blasts were medium to large in size with abundant grey/blue cytoplasm, few fine granules and occasional vacuoles. The nuclei were irregular with mostly fine chromatin and occasional prominent nucleoli (Figure 3.24).

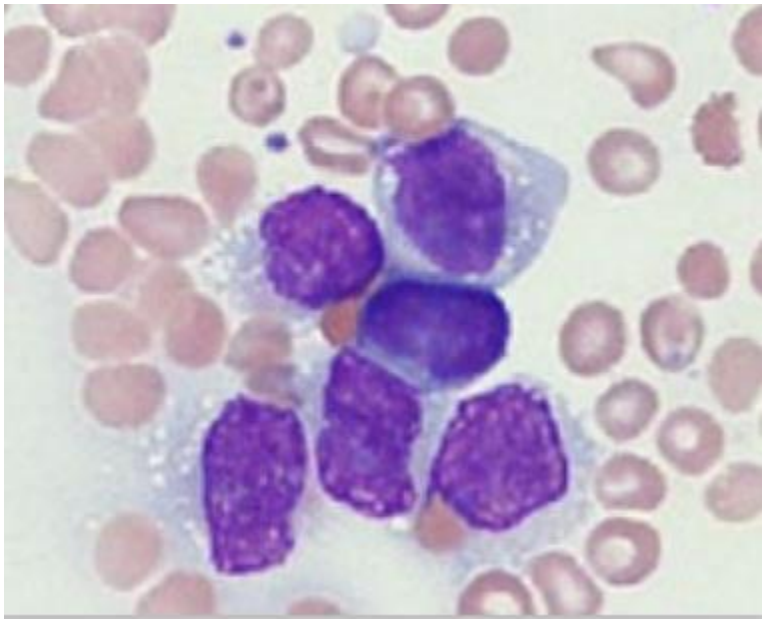


Figure 3.24: Abnormal blast cells with grey/blue cytoplasm and occasional vacuoles.

Bone marrow aspirate and biopsy were therefore indicated to confirm the presence of potential early relapse. The aspirate was particulate with excellent quality and cell release. An approximately 24% blast cell population was identified. These resembled the diagnostic morphology with large size, abundant cytoplasm, some having vacuoles and granules. No Auer rods were identified.

Flow cytometry on this bone marrow aspirate identified approximately 20% blast cells. These showed similar phenotypic expression to the diagnostic sample with positive CD33, CD64, CD4 and partial expression of CD117 and CD56. There was no significant expression of CD34, CD3, CD19, HLADR, CD2 or CD14 (Figure 3.25). The overall morphologic, immunophenotypic findings were consistent with relapsed acute myeloid leukaemia, not otherwise specified (acute monoblastic leukaemia).

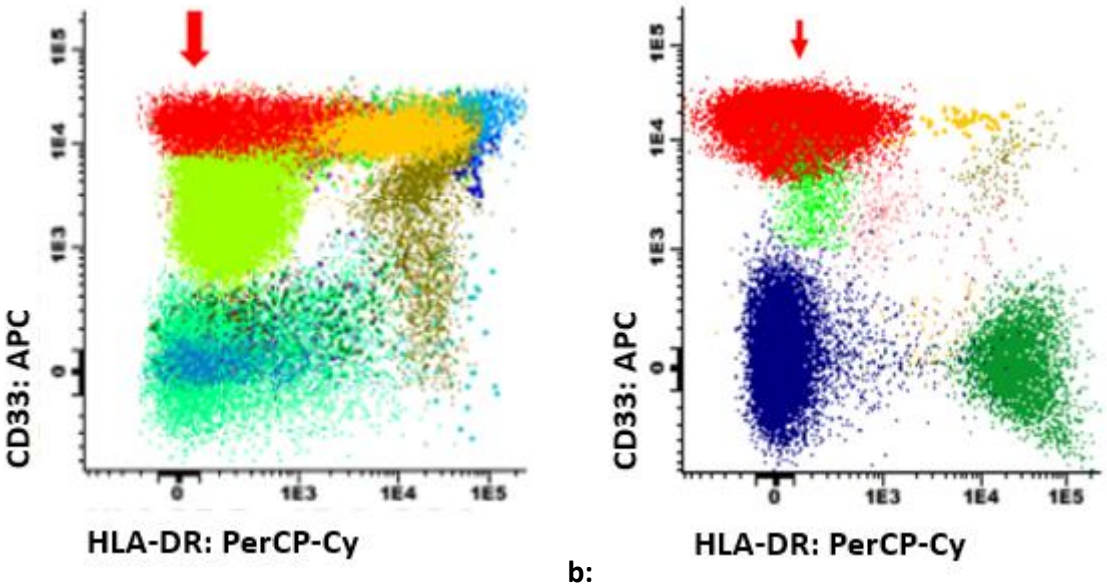


Figure 3.25: Follow up flow cytometry showing residual malignant cells A. Arrow denotes blast cells (in red) compared to the diagnostic leukaemia associated phenotype (LAP) in b.

3.3.7.3. Case 8:

This was a 14 years old male with 2 months history of lymphadenopathy. Three days before presentation, he complained of headache, fever and bone aches. Bilateral neck swelling was worsening. There was no cough, vomiting, diarrhea or bleeding history. On examination bilateral

scattered cervical and submandibular lymph nodes were enlarged as well as bilateral inguinal lymph nodes. Tonsils were also enlarged with no other organomegaly, abdominal or chest symptoms. Subsequent chest x-ray showed wide mediastinum mass.

Blood counts showed marked leucocytosis with a white blood cells count of 360 x 10^9/L and mild neutrophilia (neutrophils 10.8 x 10^9/L). His platelets count was reduced (64 x 10^9/L) while the haemoglobin result was normal for age (135 g/L). Blood smear examination revealed > 90% blast cells. These were small to medium in size with variable amount of basophilic agranular cytoplasm. No Auer rods were identified. Nuclear contours were irregular with fine chromatin and inconspicuous nucleoli.

Flow cytometry of the peripheral blood sample identified 87% blast cells population with moderate CD45 expression. These cells showed positive expression of CD1a, CD2, CD4, CD7, CD8, CD38, CD99 and cytoplasmic CD3 with dim expression of CD9, CD10 and nuclear TdT. There was no significant expression of CD34, surface CD3 or any other markers in this panel (Figure 3.26).

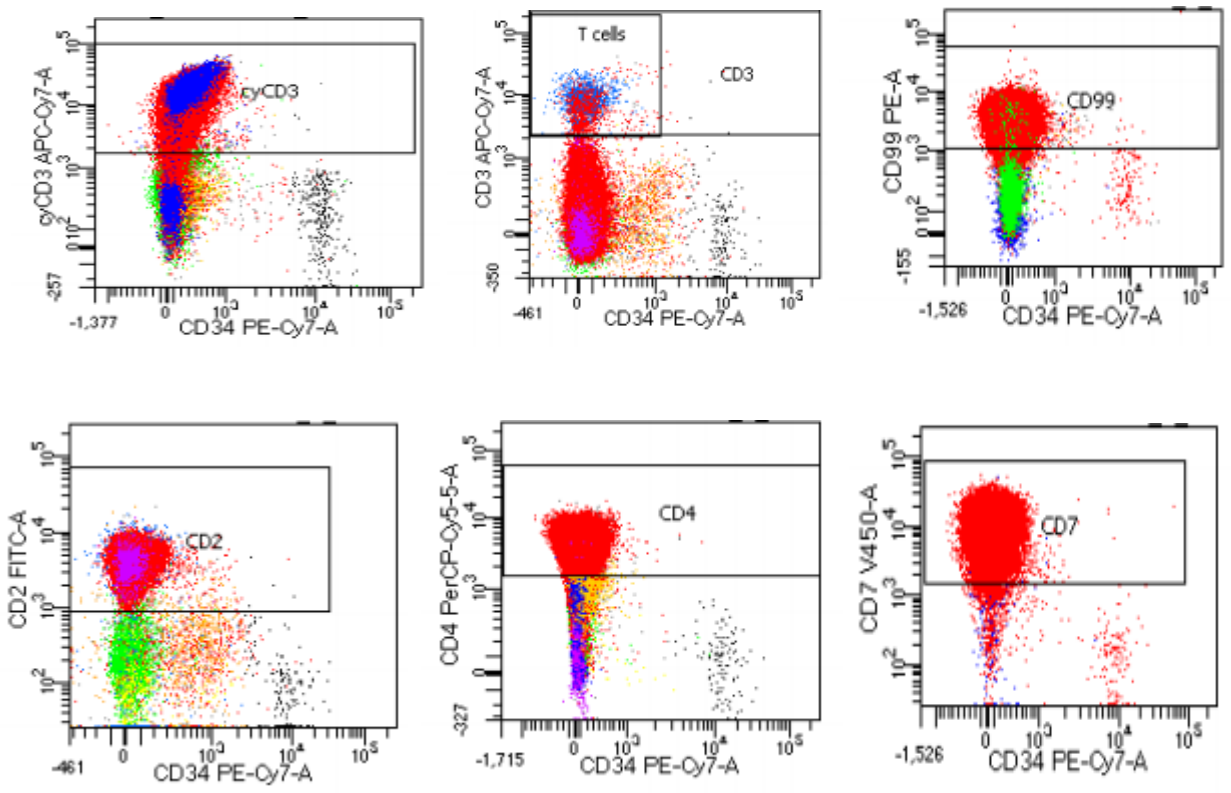


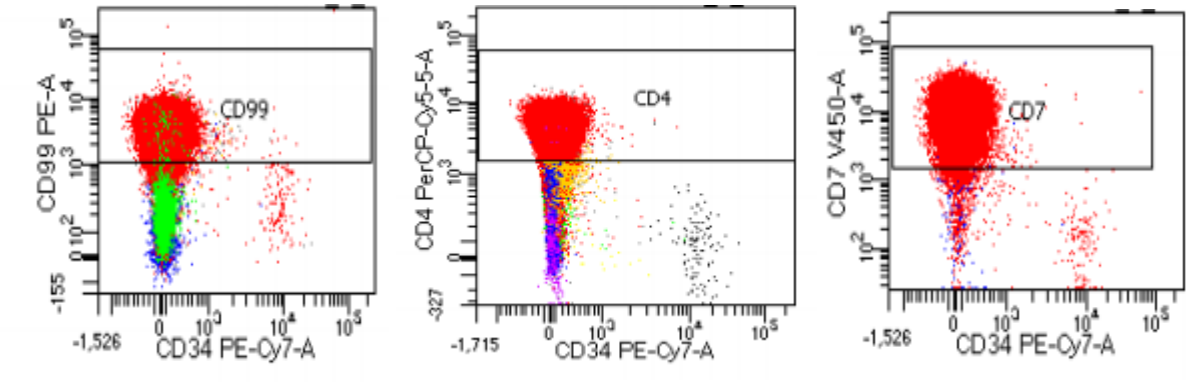
Figure 3.26: Showing T-blast cells (in red) with positive expression of cytoplasmic CD3, surface CD99, CD2, CD4 and CD7 consistent with acute T-cell lymphoblastic leukaemia. Of note, surface CD3 was negative on this occasion indicating immature T-cells.

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the peripheral blood sample revealed an abnormal hybridization pattern with TRA/D probe indicating T- cell receptor gene rearrangement in 81% of cells analysed. Karyotype analysis confirmed the FISH findings with translocation t(11;14) (46,XY, t(11;14)(p11.2;q11.2). The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of T-cell acute lymphoblastic leukemia. Final WHO subtype classification was T-Lymphoblastic leukaemia/lymphoma per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

The patient presented later on (post induction II) with abdominal pain and hepatomegaly. His blood counts confirmed the anaemia with haemoglobin of 61 g/L. White blood cells count was reduced (2.0 x 10^9/L) with severe neutropenia (0.14 x 10^9/L). Platelets count was normal with 230 x 10^9/L. However, blood smear examination was worrisome with 15% blast cells. These were small to medium in size with variable amount of basophilic agranular cytoplasm. No Auer rods were identified. Nuclear contours were irregular with fine chromatin and inconspicuous nucleoli. The sample was therefore referred for flow cytometry assessment of the blast cell population.

Flow cytometry analysis of the peripheral blood sample confirmed the presence of approximately 18% myeloid blast cells. These showed moderate CD45 expression with positive expression of CD34, CD13, CD33, HLADR, CD117, and CD123 with partial expression of CD9, CD38 and minimal expression of 11b and MPO. There was no significant expression of CD1a, CD2, CD4, CD7, CD99 or cytoplasmic CD3 assciated with the diagnostic phenotype (LAP) (Figure 3.27). No cytogenetic or molecular analysis were performed at this point of follow up.

A:



B:

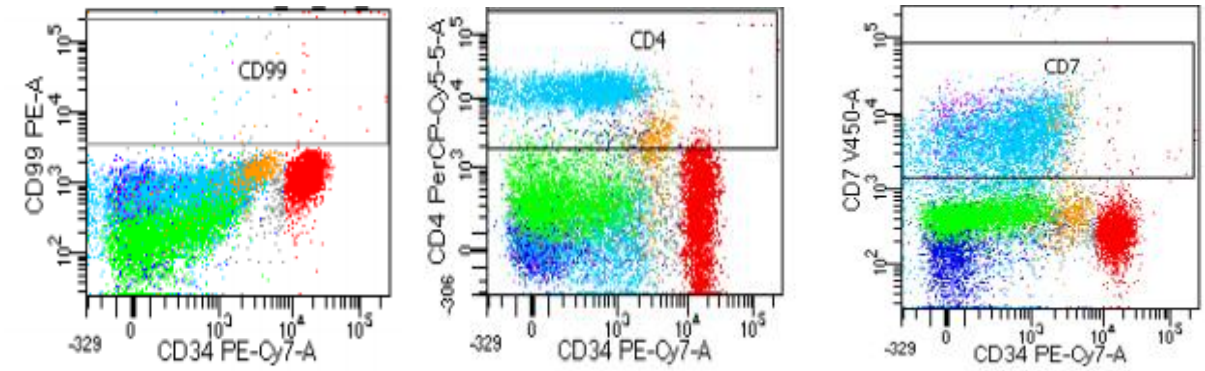


Figure 3.27: Leukaemia associated phenotype (LAP) A: in red, with positive expression of CD99, CD4 and CD7 compared to B: with no expression of CD99, CD4 or CD7 in the follow up sample.

The final clinical interpretation of these unusual myeloid looking precursors was recovering bone marrow with circulating myeloid precursor's likely secondary to bone marrow stress/recent infection. The patient was monitored closely with subsequent blood counts. Blast cells rapidly matured and disappeared from the blood circulation with subsequent counts.

3.3.8. Molecular analysis:

All B- and T-ALL cases in this study were sent to reference laboratory for molecular analysis of minimal residual disease (MRD). This was performed at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment according to the BFM 2017 treatment protocol.

Similarly, all cases of acute myeloid leukaemia in this study were tested for FLT3 ITD mutation as well as NPM1 mutation for prognostic and follow up purposes. Results of all three AML cases showed no evidence of FLT3 or NPM1 mutations rendering morphology, flow cytometry and cytogenetic analysis as the only available tools for disease moitoring in these cases.

3.3.9. Final diagnosis and integrated reports:

The final diagnosis for each case in this study was integrated in one report (the integrated report) including clinical presentation, blood counts, bone marrow aspirate and biopsy findings, flow cytometry, cytogentic analysis as well as any additional molecular testing as required. Classification of disease was following the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

3.4. Results of development and validation of an in-house (ALOT) database-guided interpretation tool:

An automated in-house database-guided software tool was developed to automatically classify new leukaemia cases. The main purpose for this development was to reduce the additional costs of purchasing the commercially available database from Cytognos (Cytognos, Salamanca) which charges per single use for each case. Twelve specimens (6 peripheral blood samples and 6 bone marrows) were used as a "build group" to develop the database compass (bank). Subsequently, a "validation group" including 12 different specimens (7 peripheral bloods and 5 bone marrows) were used to validate the newly developed software tool. Results of the "validation group" are shown below (table 3.10).

Table 3.10: Comparison between the newly developed in-house ALOT "automated" database-guided interpretation tool compared to the final diagnosis using the conventional expert-base interpretation of flow cytometry results.

Case	Sample type	Disease phase	ALOT In-house automated database tool	Final diagnosis
3	PB	Diagnostic	B-lineage	B-ALL
4	PB	Diagnostic	B-lineage	B-ALL
5	BM	9 months--post treatment	Mixed-lineages (B/M)	Increased hematogones
6	BM	Post Intensification-1	Myeloid	Relapsed AML
7	BM	Diagnostic	Myeloid	AML
8	PB	Post induction-2	Myeloid	Recovering BM
9	PB	Diagnostic	B-lineage	B-ALL
10	BM	Diagnostic	B-lineage	B-ALL
11	PB	Diagnostic	B-lineage	B-ALL
12	BM	Diagnostic	B-lineage	B-ALL
13	PB	Diagnostic	B-lineage	B-ALL
15	PB	Diagnostic	T-lineage	T-ALL

3.4.1. Identification of B-cell acute lymphoblastic leukaemia using the newly developed in-house (ALOT) database-guided interpretation tool:

Seven cases of known B-cell acute lymphoblastic were used to test this newly developed ALOT interpretation tool (namely; cases 3, 4, 9, 10, 11, 12 and 13). Results successfully identified all cases as B-cell lineage consistent with known diagnosis of B-ALL (figure 3.28).

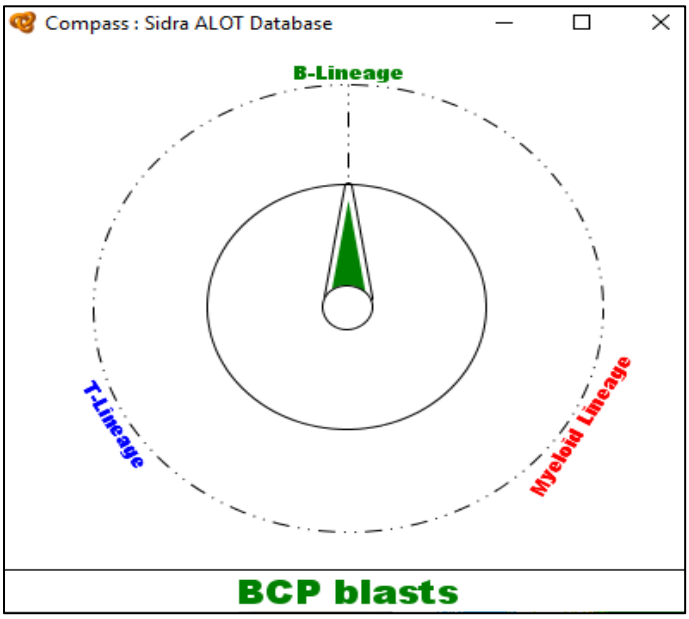


Figure 3.28. Case 3 peripheral blood blast cells orientation using the newly developed in-house automated database-guided interpretation tool. Database compass correctly identified blast cells of B-cell lineage.

3.4.2. Identification of myeloid blasts using the newly developed in-house (ALOT) database-guided interpretation tool:

Similarly, two cases of known acute myeloid leukaemia were used to test this newly developed ALOT interpretation tool (namely; cases 6 and 7). Once more, results successfully identified both

cases as myeloid lineage consistent with known diagnosis of acute myeloid leukaemia (figure 3.29).

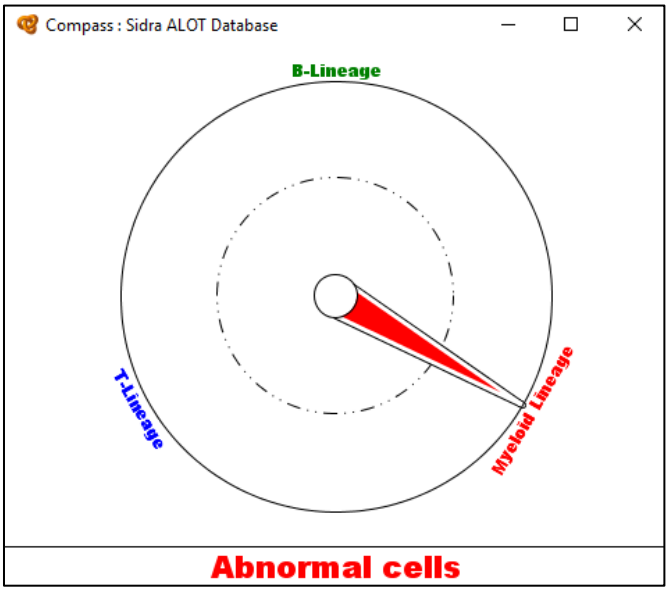


Figure 3.29. Case 6 bone marrow blast cells orientation using the newly developed in-house automated database-guided interpretation tool. Database compass correctly identified blast cells of myeloid lineage consistent with the diagnosis of acute myeloid leukaemia.

3.4.3. Identification of T-cell acute lymphoblastic leukaemia using the newly developed in-house (ALOT) database-guided interpretation tool:

One cases with known diagnosis of T-cell acute lymphoblastic was used to test this newly developed ALOT interpretation tool (case 15). Results successfully identified blast cells in this case as T-cell lineage consistent with known diagnosis of T-ALL (figure 3.30).

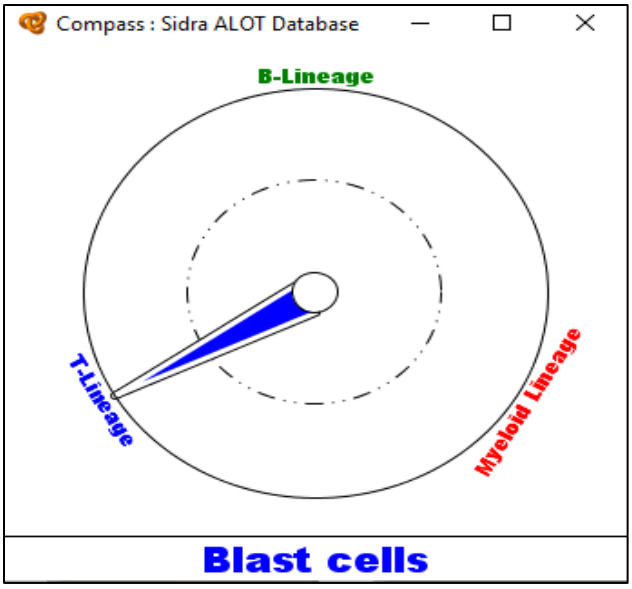
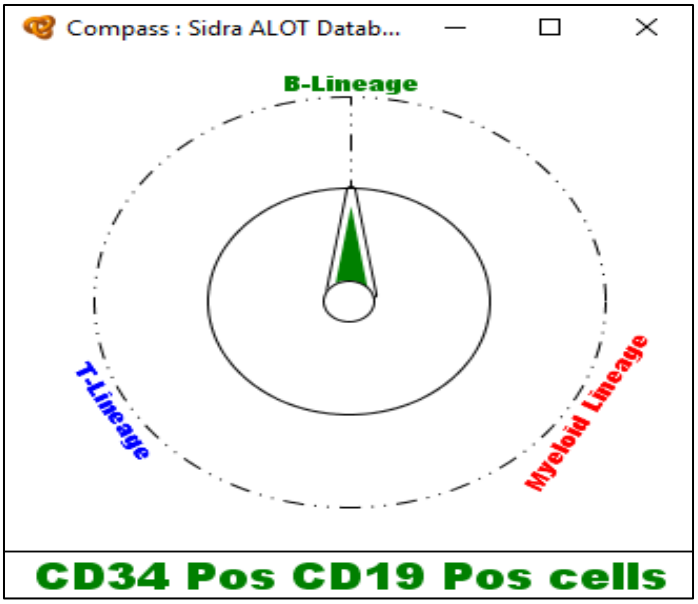


Figure 3.30. Case 15 peripheral blood blast cells orientation using newly developed in-house automated database-guided interpretation tool. Database compass indicating blast cells of T-cell lineage

3.4.4. Identification of mixed populations using the newly developed in-house (ALOT) database-guided interpretation tool:

One cases with mixed blast cell populations (case 5) was further used to validate this newly developed in-house interpretation tool. Once more, the data-base guided interpretation tool successfully identified both populations of blast cells (figure 3.31). However, it was very import to acknowledge that presence of two populations of precursor cells does not necessiarily mean a diagnosis of mixed phenotypic acute leukaemia. In this particular case, both populations were normal benign precursors in a recovering bone marrow. It is critical to highlight the value of integrated approach of leukaemia diagnosis were flow cytometry results are interpreted in light of clinical information provided, treatment stage, blood and bone marrow morphology as well as any molecular or cytogenetic findings.

a.



b.

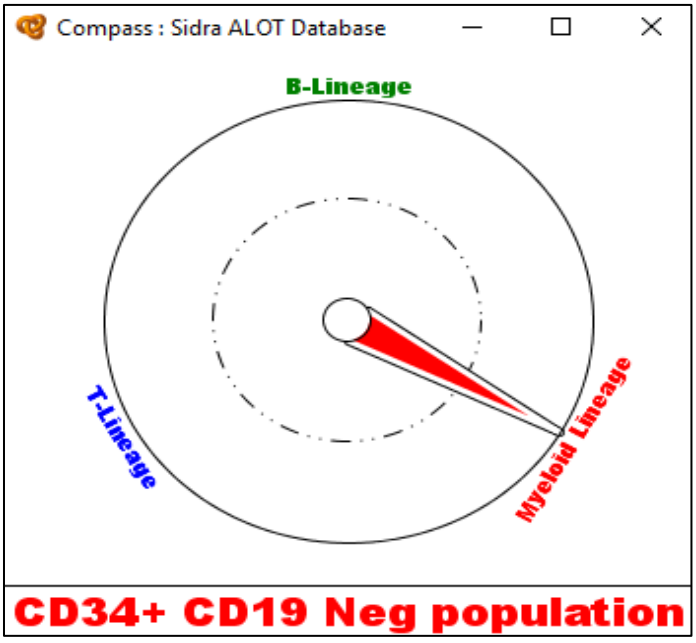


Figure 3.31. Case 5 bone marrow blast cells orientation using newly developed in-house automated database-guided interpretation tool. Database compass indicating two different precursor cell populations; B-cell lineage and a myeloid lineage.

Likewise, a second case with a known diagnosis of T-ALL was also used to test this newly developed interpretation tool (case 8). This was a peripheral blood sample following induction chemotherapy. Unfortunately, blood smear showed approximately 18% circulating blast cells. Flow cytometry analysis was performed to identify the nature of these blast cells. Surprisingly, both conventional flow cytometry in-house panel and the newly developed automated data-base guided interpretation tool identified these cells as myeloid precursors (figure 3.32).

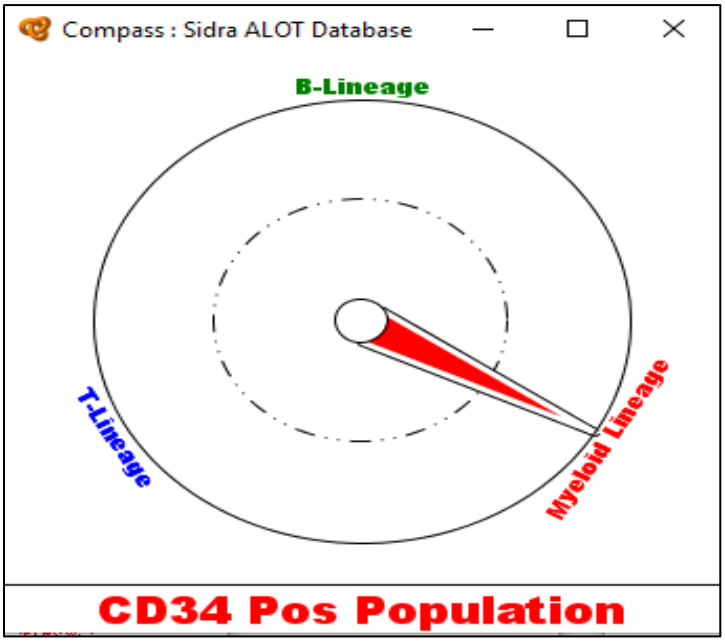


Figure 3.32. Case 8 follow up peripheral blood blast cells orientation using newly developed in-house automated database-guided interpretation tool. Database compass indicating blast cells of myeloid lineage.

Results of the in-house acute leukaemia panel showed moderate expression of CD45 together with CD34, CD13, CD33, HLADR, CD117, CD123, partial expression of CD9, CD38 and minimal expression of 11b and MPO on this population. There was no significant expression of CD1a, CD2, CD3, CD4, CD7, CD10, CD19, CD20, CD14, CD56, CD66c, CD99, cytoplasmic CD3, cyCD79a, cyIgM or TdT. This phenotype did not resemble the diagnostic phenotype of T-acute lymphoblastic leukaemia.

Parallel analysis of the ALOT tube under validation performed on the peripheral blood sample confirmed the immunophenotypic findings with moderate CD45 and positive CD34 expression. There was no significant expression of surface CD3, CD7, CD19, cytoplasmic MPO, cyCD79a or cyCD3 on this population. The principal component analysis (PCA) and automated population separator (APS) diagram were subsequently used to compare blast cells distribution to residual normal cell populations in the blood specimen (figure 3.33). Finally, the automated database orientation tool using Infinicyt software from Cytognos indicated blast cells to be of myeloid-cell lineage (figure 3.34).

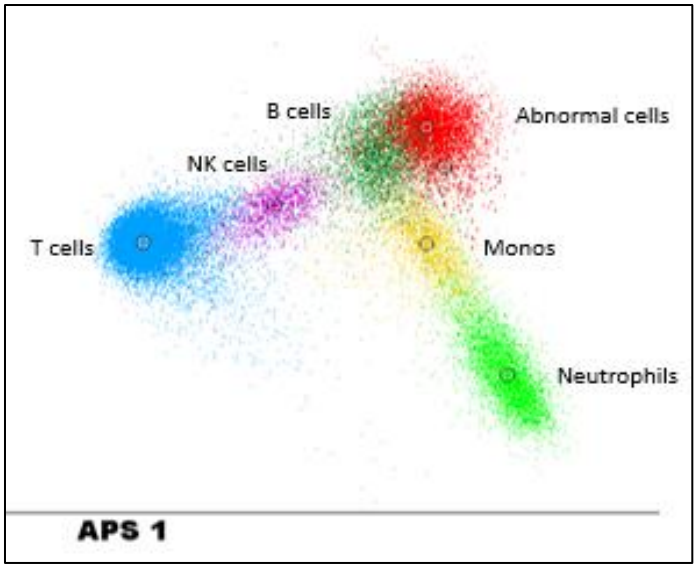


Figure 3.33. Case 8 follow up blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

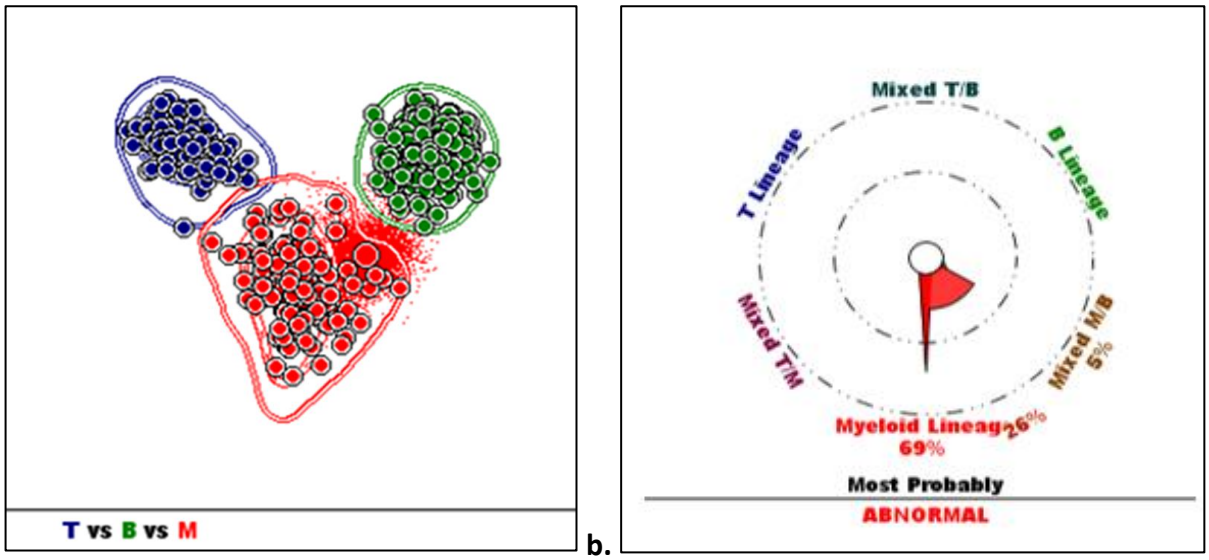


Figure 3.34. Case 8 follow up blast cells orientation using automated database a. orientation among myeloid-cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

The final clinical interpretation of these findings in light of flowcytoemtery and blood counts was recovering bone marrow with circulating myeloid precursors. The patient was closely followed with subsequent blood counts. Blast cells gradually matured and disappeared from the blood circulation.

Once more, the value of the integrated approach of flow cytometry results interpretation in leukaemia diagnosis was highlighted. Altough the newly developed data-base interpretation tool correctly identified the blast cells as myelpid lineage, this did not necessarily indicate the final interpretation of these findings. Integration of the clinical presenation as well as blood counts and treatment stage were essential for final interpretation of these findings.

3.5. Results of merging data files to obtain an immunophenotypic profile for each case:

The flow cytometry standard files (FCS) files from the in-house full acute leukaemia panel were merged to create a unique immunophenotypic profile (signature) for each case in the study. Following files merge it was possible to view all markers from different tubes on one diagram (figure 3.35).

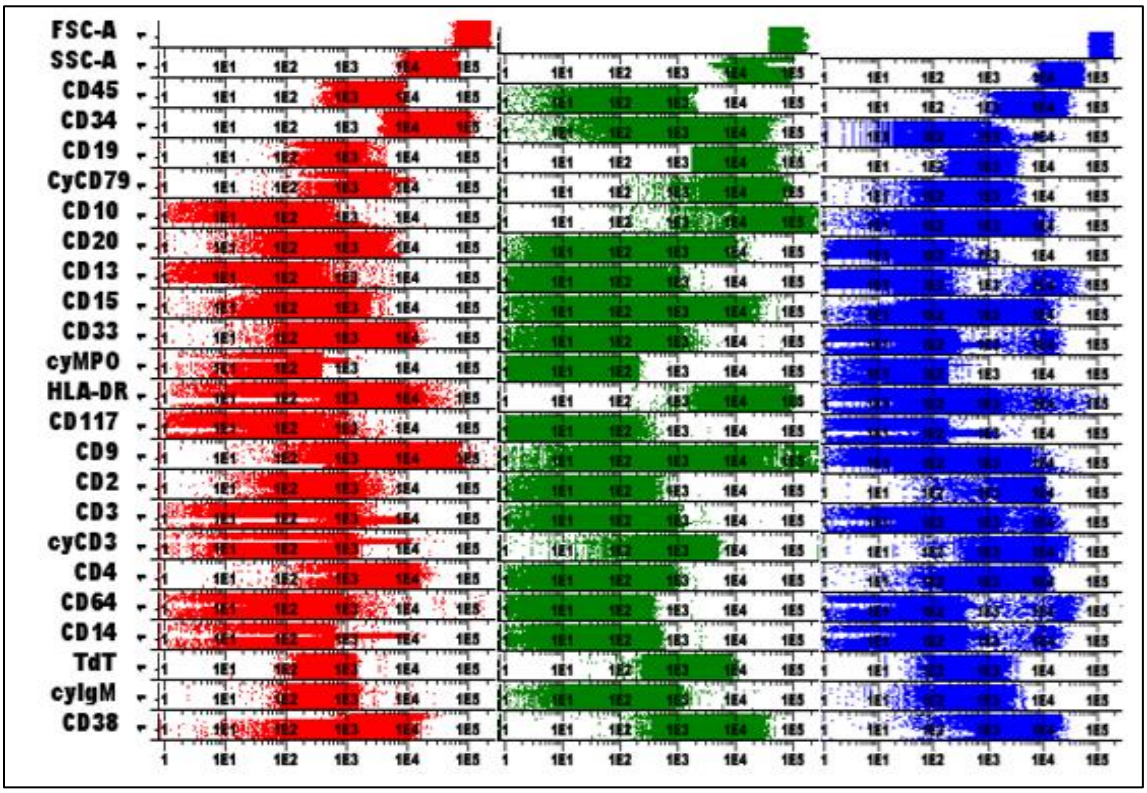


Figure 3.35. Examples of immunophenotypic profiles (signature) of different types of leukaemia (AML- red, B-ALL - green and T-ALL- blue).

3.6. Development and validation of an in-house acute leukaemia interpretation database:

A novel automated acute leukaemia interpretation tool was developed using the immunophenotypic profiles (signatures) created in section 2.5. In total, seven acute leukaemia samples were used to develop the reference database. This included four samples of known B-ALL, two samples of AML and one sample of T-ALL. In order to test this novel acute leukaemia database interpretation tool, additional eight samples (including 6 B-ALL, 1 AML and 1 T-ALL) were used (figures 3.36 and 3.37). Results of this validation showed 100% agreement between the conventional expert interpretation of flow cytometry results and the newly developed database interpretation tool (table 3.11).

Table 3.11: Comparison between the newly developed "automated" acute leukaemia database interpretation tool and the the conventional expert interpretation of flow cytometry results.

Case	Sample type	Disease phase	In-house automated database tool	Final diagnosis
3	PB	Diagnostic	B-lineage	B-ALL
4	PB	Diagnostic	B-lineage	B-ALL
7	BM	Diagnostic	Myeloid	AML
9	PB	Diagnostic	B-lineage	B-ALL
10	BM	Diagnostic	B-lineage	B-ALL
11	PB	Diagnostic	B-lineage	B-ALL
13	PB	Diagnostic	B-lineage	B-ALL
15	PB	Diagnostic	T-lineage	T-ALL

Abbreviations: AML, acute myeloid leukaemia; B-ALL, B-acute lymphoblastic leukaemia; BM, bone marrow; PB, peripheral blood; T-ALL, T-acute lymphoblastic leukaemia.

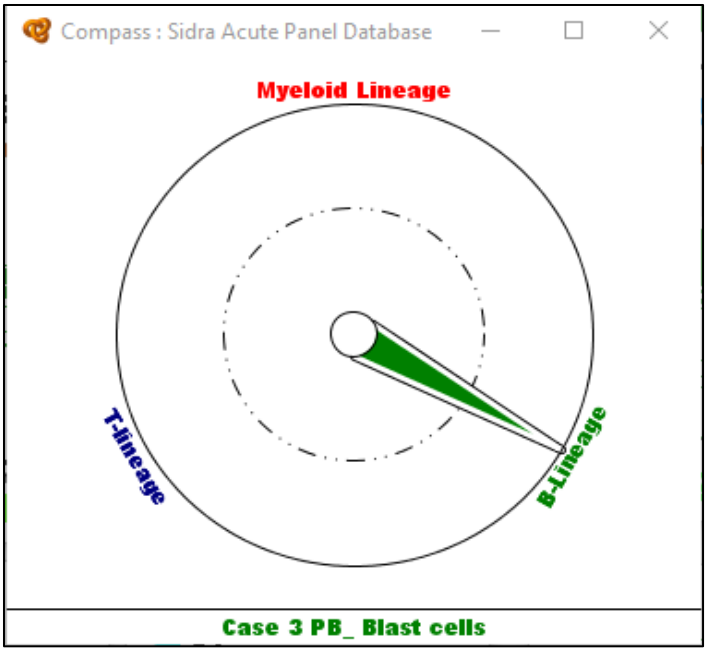


Figure 3.36. Case 3 peripheral blood blast cells orientation using the newly developed in-house automated database-guided interpretation tool. Database compass indicating blast cells of B-cell lineage.

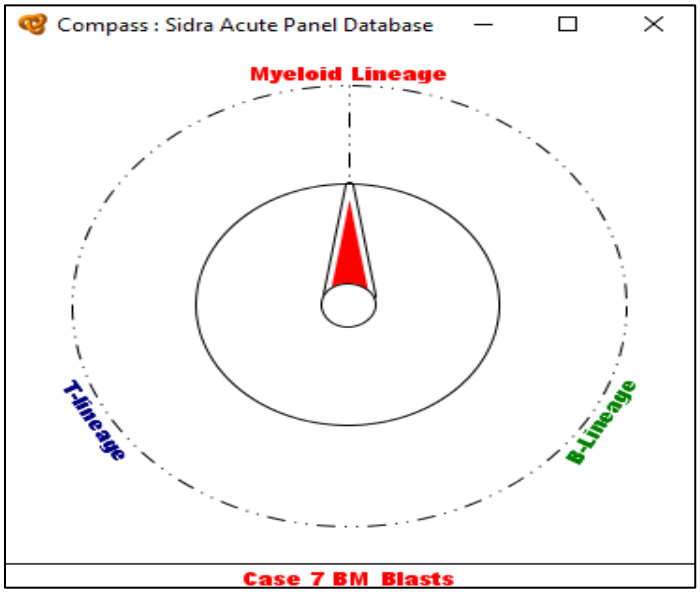


Figure 3.37. Case 7 bone marrow blast cells orientation using the newly developed in-house automated database-guided interpretation tool. Database compass indicating blast cells of myeloid lineage.

Initially, the newly developed database interpretation tool failed to classify two cases in any of the three cells lineages (T-cell, B-cell or myeloid) (figures 3.38a and 3.39a). Further investigation showed that blast cells in the first case (case 10) were all CD34 negative. This was unlike all other cases of B-ALL added to the relatively small database bank were CD34 was at least partially positive in all cases.

In order to improve the reference database, the merged files of this case was added to the recently developed database bank to enrich the variability of blast cells. Subsequent repeat analysis using the automated database interpretation tool correctly identified the case as B-ALL (figure 3.38).

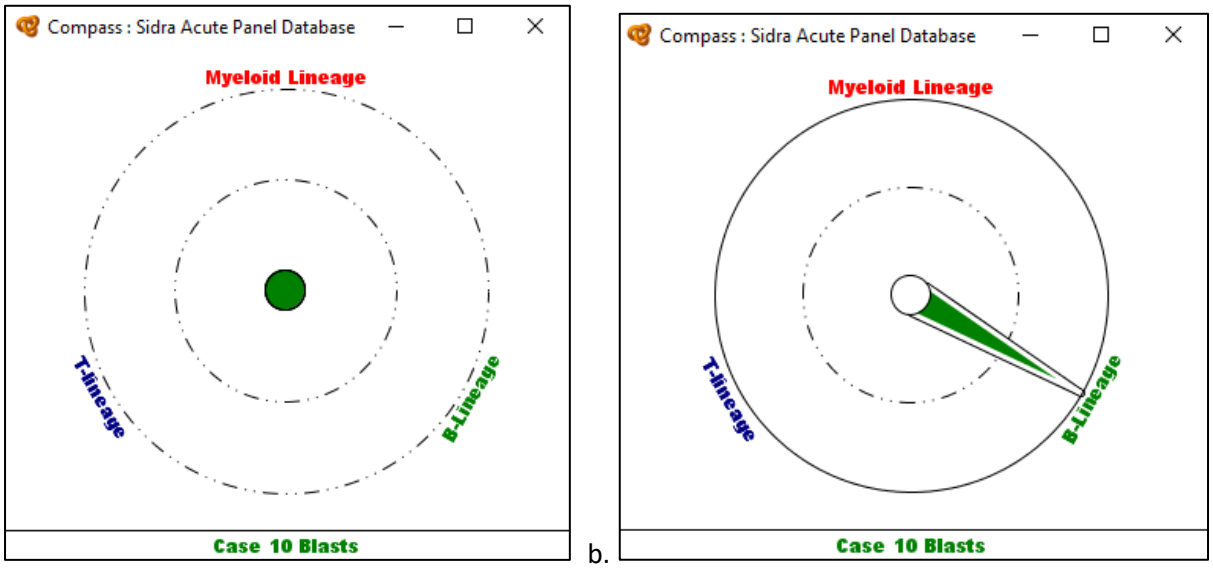


Figure 3.38. Case 10 bone marrow blast cells orientation using the newly developed in-house automated database-guided interpretation tool. A. Database failed to identify the blast cells lineage and b. database compass indicating blast cells of B-cell lineage.

Likewise, initial analysis of one T-ALL case (case 15) using the newly developed database tool failed to identify the cell lineage of this case (figure 3.39a). However, further investigation showed that blast cells in this case were different from case-8 T-ALL blast cells which were used in building the database. Unlike the reference case, this case showed no expression of CD1a with minimal expression of surface CD3. The merged files of this case were then added to the recently developed database to enrich the variability of T-ALL blast cells. Subsequent repeat analysis using the automated database interpretation tool correctly identified the case as T-ALL (figure 3.39b).

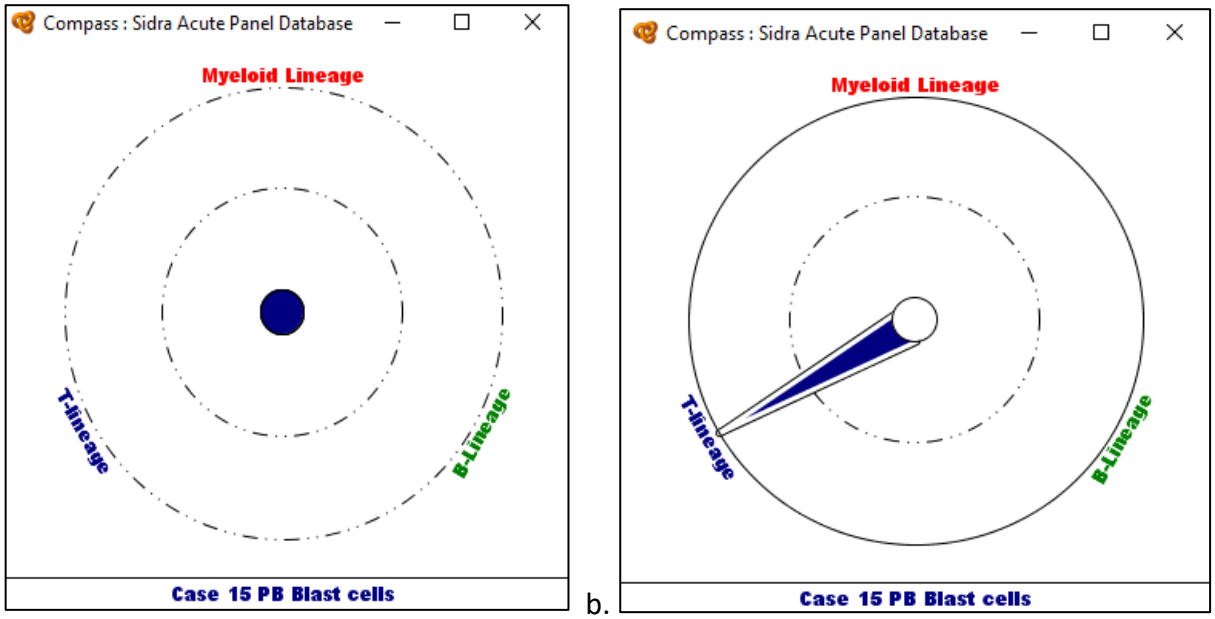


Figure 3.39. Case 15 peripheral blood blast cells orientation using newly developed in-house automated database-guided interpretation tool. A. Database failed to identify the blast cells lineage and b. database compass indicating blast cells of T-cell lineage.

3.7. Development and validation of an in-house cytogenetic database prediction tool:

Similarly, a novel cytogenetic database prediction tool was also developed using 12 samples from newly diagnosed B-ALL patients as described in section 2.15. Eight samples were used to build the database while 4 samples were used for its validation (tables 2.5 and 2.6). The results of the newly developed predication tool is summarized in table 3.12 below.

Table 3.12: Comparison between the newly developed in-house cytogenetic prediction tool and the actual final cytogenetic results.

Case	Sample type	Disease phase	In-house prediction tool	Final diagnosis
1	PB	Diagnostic	B-ALL with hyperdiploidy	B-ALL with hyperdiploidy
2	PB	Diagnostic	Failed, then B-ALL, NOS	B-ALL, NOS
9	BM	Diagnostic	B-ALL, NOS	B-ALL, NOS
11	PB	Diagnostic	Failed, then B-ALL with t(12;21)	B-ALL with t(12;21)

Abbreviations: AML, acute myeloid leukaemia; B-ALL, B-acute lymphoblastic leukaemia; BM, bone marrow; NOS, Not otherwise specified; PB, peripheral blood; T-ALL, T-acute lymphoblastic leukaemia.

3.7.1. Results of cytogenetic prediction tool:

The cytogenetics database predication tool correctly identified blast cells from case one as B-ALL with hyperdiploidy (figure 3.40). This finding correlates well with the literature and aberrant expression of CD123 and CD66c as well as high forward scatter (FSC) in B-ALL with hyperdiploidy (Theunissen, 2017). This particular phenotypes makes B-ALL with hyperdiploidy have a unique immunophenotypic profile (signature) that could easily be identified by comparison to a reference database.

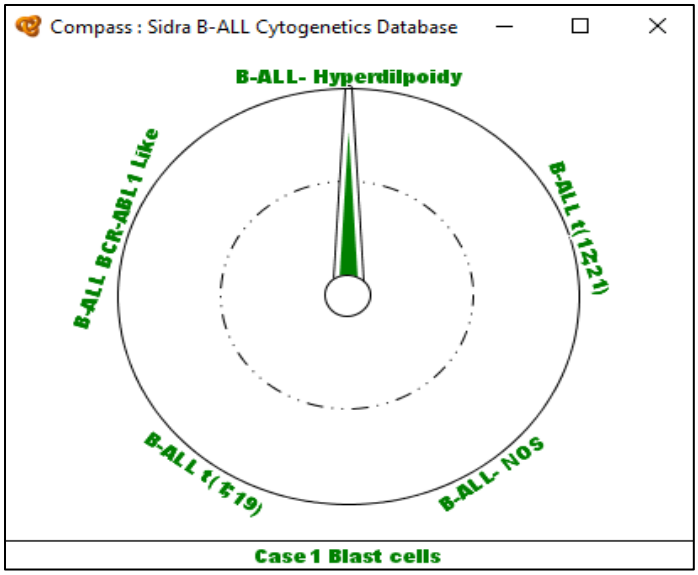


Figure 3.40. Case 1 blast cells orientation using newly developed cytogenetic database predication tool. Database compass predicting blast cells to be of B-ALL with hyperdiploidy.

Likewise, the newly developed database correctly predicted blast cells from case 9 as B-ALL not otherwise specified (NOS) (figure 3.41).

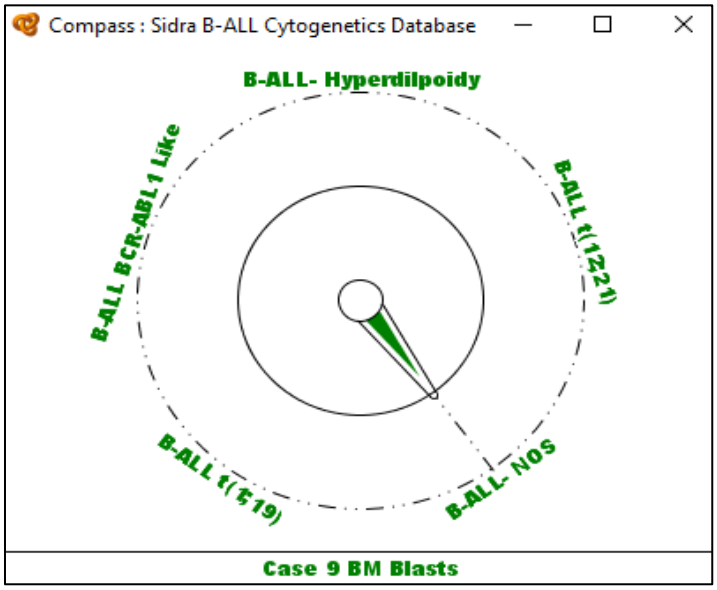


Figure 3.41. Case 9 blast cells orientation using the newly developed cytogenetic database prediction tool. Database compass correctly predicted blast cells to be of B-ALL NOS subgroup.

However, the cytogentic predication tool failed to identify a second case of B-ALL NOS and one case of B-ALL with t(12;21) despite examples of both categories included in the built reference database. Detailed analysis of these two cases showed that both cases had a slightly different immunophenotypic profile (IP) compared to the reference database.

The first case of B-ALL-NOS (case 2) had a strong positive CD9 and CD34 expression compared to the other B-ALL NOS cases used in developing the database with no CD34 expression. Moreover, the in-house panel was not fully modified when case 2 was tested and therefore did not include all the additional cytogenetic specific markers (i.e. CD38, CD66c, CD123 and CD15).

The second failed case was B-ALL with t(12;21) (case 11). Review of the detailed phenotype of this case showed that it had a negative CD34 expression compared to the case used in building the database (case 13) with positive expression of CD34. This has subsequently led to software failure in identifying the case as B-ALL with t(12;21).

Both failed cases (2 and 11) were subsequently added to the reference database in order to enrich this local database with diversity of blast cells for future identification. Subsequent re-analysis of both cases using the updated database has correctly identified both cases as B-ALL NOS and B-ALL with t(12;21) respectively (figures 3.42 and 3.43).

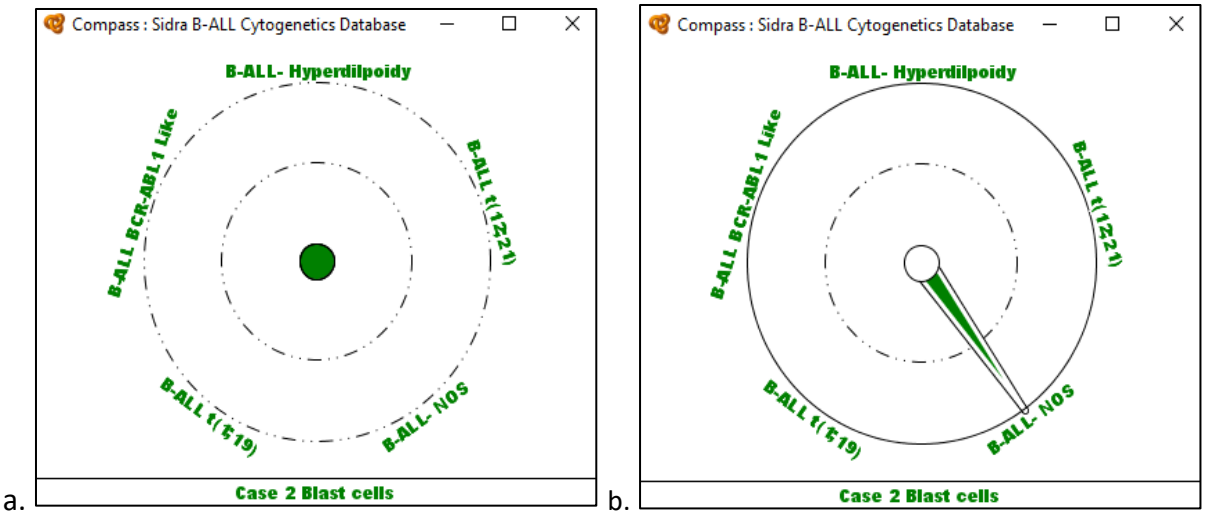


Figure 3.42. Case 2 bone marrow blast cells orientation using newly developed cytogenetic database predication tool. A. Database failed to predict the cytogenetic group and b. database compass predicted blast cells to be of B-ALL NOS subgroup.

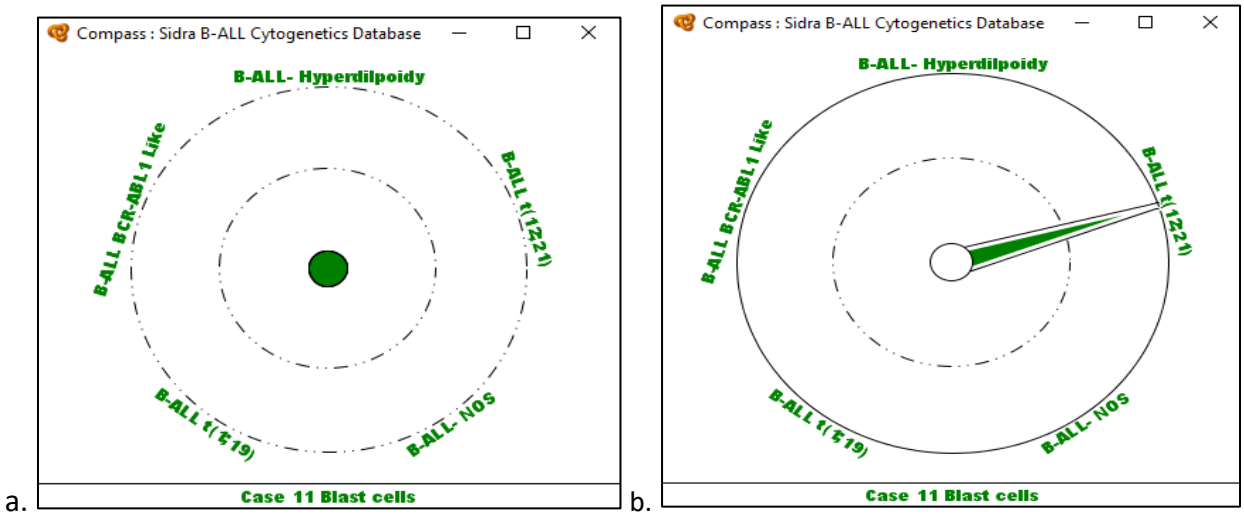
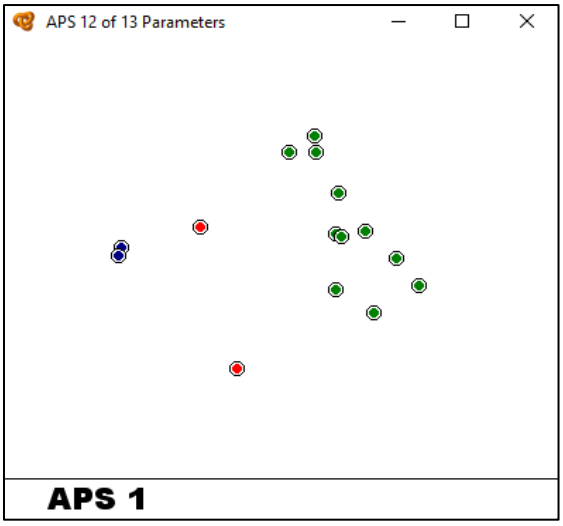


Figure 3.43. Case 11 bone marrow blast cells orientation using newly developed cytogenetic database predication tool. A. Database failed to predict the cytogenetic group and b. database compass predicted blast cells to be of B-ALL NOS subgroup.

3.8. Comparison of the immunophenotypic profiles of all cases in the study:

3.8.1. Comparison of ALOT immunophenotypic profiles:

Following the individual analysis of the ALOT tube of each case in the study (section 3.3), the blast cells of all 15 new acute leukaemia cases in the study were directly compared to each other using the principal component analysis tool (PCA) and the automated population separator diagram (figure 3.44). Results showed clear clustering of the 11 B-ALL cases (in green) and the two T-ALL cases (in blue). However, the remaining two AML cases (in red) despite being separate from both B-ALL and T-ALL clusters they were not actually overlapping in a clear AML cluster group. Finally, the four T-ALL and AML cases were removed from the APS comparison diagram allowing only the 11 B-ALL cases in the study to separate in an unsupervised manner (figure 3.45). Results showed no clear clustering of B-ALL cases based on the ALOT tube findings only.

a. 

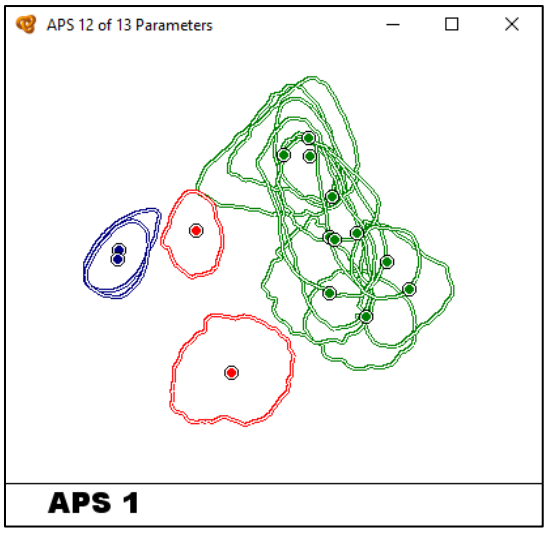
b. 

Figure 3.44. Comparison of ALOT tube of all cases in the study showing a. 11 B-ALL cases (green), 2 T-ALL cases (blue) and 2 AML cases (red). B. Adding 2SD deviation of each blast population.

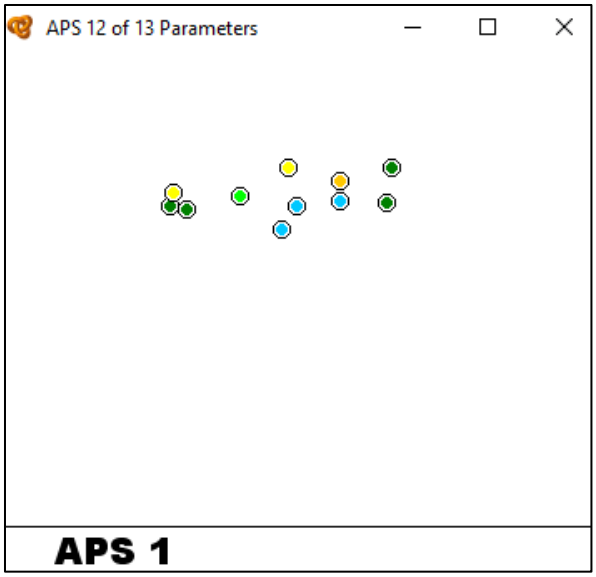


Figure 3.45. Comparison of ALOT tube of B-ALL cases only. Four cases of B-ALL (NOS) (dark green), 3 cases of B-ALL with hyperdiploidy (blue), 2 cases of B-ALL with t(12;21) (yellow), one case of B-ALL with t(1;19) (light green) and one case of B-ALL with BCR-ABL1 like (orange).

3.8.2. Comparison of the full acute leukaemia phenotypic profiles:

Similarly, the blast cells of B-ALL cases only were once more compared to each other using the merged files generated from the full acute leukaemia panel described in section 3.8. However, because the in-house acute leukaemia panel was modified during the study to include a cytogenetic predication tube (tube A6) it was only possible to compare the last 6 B-ALL cases analysed using the modified panel (figure 3.46). Results showed a clear overlap between case 11 and case 13 as they both shared the same cytogenetic finding with t(12;21).

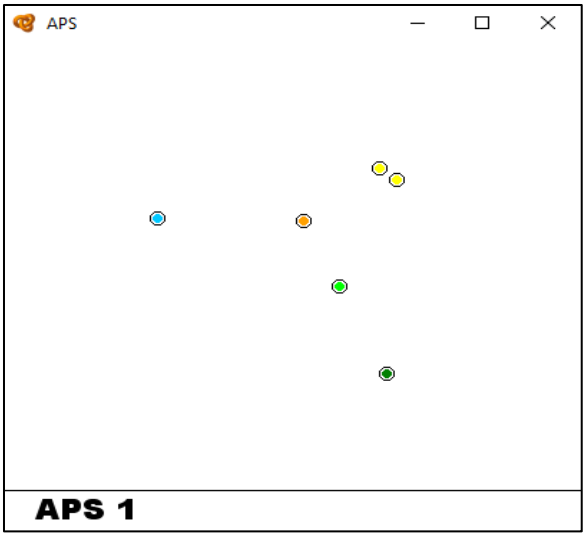


Figure 3.46. Comparison of the full immunophenotypic profile of B-ALL cases (10-16) only. Two cases of B-ALL with t(12;21) (yellow), one case of B-ALL (NOS) (dark green), 1 cases of B-ALL with hyperdiploidy (blue), , one case of B-ALL with t(1;19) (light green) and one case of B-ALL with BCR-ABL1 like (orange).

4. Discussion:

4.1. General Discussion:

Acute leukaemia is a heterogeneous group of disorders characterized by clonal expansion of abnormal haemopoietic cells arrested at early stages of maturation (Swerdlow, 2008 and Swerdlow, 2017). Appropriate management of these disorders requires accurate diagnosis and classification of acute leukaemia based on the latest WHO classification of tumors of haemopoietic and lymphoid tissues (Swerdlow, 2017).

Despite several advances in cytogenetic and molecular analysis techniques, flow cytometry remains an essential tool for diagnosis of this group of haematological disorders (Davis, 2006 and Stetler-Stevenson, 2007). Several studies have been published in the last two decades that have attempted to standardise panels, procedures and instrument settings for multicolor flow cytometry (Stewart, 1997; Wood, 2007; Kalina, 2012; van Dongen 2012 and Johansson, 2014).

More recently, the design and validation of novel standardised, highly sensitive EuroFlow-based next generation flow cytometry (NGF) have been reported for detection of minimal residual disease (MRD) in multiple myeloma (MM) and B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) (Flores-Montero, 2017 and Theunissen, 2017). In these assays, red cell bulk-lysis procedure was described allowing the acquisition of up to ≥ 10^7 cells/sample and increasing the flow cytometry sensitivity of these assays to at least real-time quantitative polymerase chain reaction levels (RQ-PCR) (≤ 10^{-5}). Moreover, the novel NGF-MRD assay took advantage of recent advances in analysis tools and identification of plasma cells using automatic identification against

reference databases of normal and patient bone marrows (Pedreira, 2008; Pedreira 2013 and Flores-Montero, 2017).

Despite these attempts, several laboratories around the world still perform acute leukaemia diagnosis based on a hybrid of international guidelines, standardised panels and in-house customised tubes (Kalina, 2012 and van Dongen 2012). This can be due to several reasons including limited availability of resources, relatively higher cost of standardised panels, challenges in supplies, specific local patient population or simply because of local expert preferences.

In this study, validation of the standardised EuroFlow acute leukaemia orientation tube (ALOT) and its corresponding database was performed independently using local staining and instrument settings procedures. With regards to sample preparation, 50 µl of EDTA blood was used as recommended by the EuroFlow standardised protocol (Kalina, 2012). However, subtle differences in the staining protocol included the use of 0.1% PBSA and 1X Pharm lyse lysing solution instead of 0.5% PBSA and 1X FACS lysing solution as recommended by EuroFlow consortium, respectively (Kalina, 2012). Moreover, the in house prepared PBSA mixture did not contain sodium azide (0.09% NaN_3) as recommended by EuroFlow. Finally, samples were centrifuged at 3000rpm for 2 minutes (2419 g) instead of EuroFlow recommendations of 540g for 5 mins.

For instrument settings, the Eight-peak Rainbow bead calibration particles (Spherotech, Lake Forest, IL, USA) were not used for initial PMT characterisation or for daily instrument checks (Kalina 2012). Instead, the OneFlow setup beads were used for initial PMT settings while the

cytometer setting and tracking beads (CS&T) were used for daily monitoring of the analyser (BD Bioscience, USA). Finally, the EuroFlow recommendations of forward (FSC) and side scatter (SSC) values of 55,000 +/- 5,000 and 13,000 +/- 2,000 respectively for unstained lymphocyte singlets were not also used. Instead, an arbitrary visual adjustment of FSC and SSC was established using unstained lymphocytes for the in house acute leukaemia panel.

It was not very clear whether these deviations in sample preparation and instrument settings from the EuroFlow recommendations would have an impact on the ALOT tube results or the automated database interpretation. Therefore, this study was primarily aimed to validate the ALOT tube using local staining procedures and instrument settings.

4.2. Development and validation of an in-house acute leukaemia panel:

Prior to the validation of the EuroFlow acute leukaemia orientation tube (ALOT) and its corresponding database, an in-house acute leukaemia panel was extensively validated for clinical use at Sidra Medicine (unpublished data Sidra medicine, 2019-2020). The main purpose of the project was to perform acute leukaemia diagnosis at Sidra Medicine instead of sending flow cytometry samples to the national reference laboratory at the Cancer Center, Qatar or Mayo Clinic, USA.

In addition to shorter turn around time for patients at Sidra Medicine, the project also aimed to establish a second diagnostic oncology flow cytometry laboratory in the country to support the only diagnostic flow cytometry laboratory at the National Cancer Center. Moreover, localizing

such services will have direct impact on training of local workforce, promote research and services expansion at Sidra medicine as well as reducing costs of send away tests.

A detailed validation plan was prepared prior to commencing acute leukaemia diagnosis at Sidra Medicine. This included but not limited to; development of a business case for senior management, securing of appropriate space within the department, cost analysis, selection and procurement of suitable equipment, installation plan, panel development as well as technical valiation and staff training (section 1.8).

In order to fulfil the local pediatric patient needs, antibody selection had to reflect specific cell lineages as well as adequate combination of antibodies to monitor normal and abnormal cell maturation (appendix 1). CD45 was the first backbone marker to be selected inorder to isolate and gate haemopoietic cells as the common leucocyte antigen (Borowitz, 1993; Lacombe, 1997 and Vial, 2001). CD19 was then selected as a very sensitive B-cell marker as it is expressed in early B-cell lineage and virtually all cases of B-ALL (van Dongen, 2012). This was followed by CD34 as the first immaturity marker in this panel (Borowitz, 1990). Additionally, CD10 and CD20 were selected as key maturation markers for B-cell lineage (Stewart, 1997).

CD13 and CD11b were further added to this panel in addition to CD10 inorder to monitor normal myeloid maturation; particularly in bone marrow specimens. CD10 was also a useful marker to differentiate mature neutrophils (CD10 positive) from monocytes (CD10 negative) in this tube.

The second tube on this panel contained mainly myeloid and T-cell markers in addition to the previously mentioned back bones (CD45 and CD34). Three main myeloid markers were selected in this tube namely; CD33, CD117 and HLA-DR (Stewart, 1997). Although lacking myeloid specificity; HLA-DR provided an excellelnt myeloid maturation marker when combined with CD117 as a second myeloid immaturity antigen. Likewise, combination of HLA-DR with CD33 also provided a good opportunity for monitoring monocytes, granulocytes and dendritic cells maturation (Stewart, 1997). Moreover, HLA-DR also provided an additional value as a key marker present in almost all cases of B-ALL (van Dongen, 2012).

Finally, surface CD3 together with CD7 were also included in this tube in order to identify mature T-lymphocytes (CD3 and CD7 positive) as well as natural killer (NK) cells (CD7 positive and CD3 negative) (van Dongen, 1988).

The third tube in this panel was mainly focused on monocytes and nucleated red cells. Markers in this tube included CD4, CD64 and CD14 which can be used to isolate both monoblasts as well as mature monocytes (monoblasts CD4 and CD64 positive, while mature monocytes are CD4, CD64 positive in addition to CD14) (Stewart, 1997). CD36 was was added to this tube to accurately enumerate nucleated red cells in addition to low FSC and SSC light properties of nucleated red cells.

The fourth tube in this panel (cytoplasmic tube) included both surface and cytoplasmic markers for diagnosis of acute myeloid and lymphoid neoplasms. In addition to the back bone markers (CD45 and CD34), nuclear TDT was added to this tube as an additional immaturity marker in this

tube. Cytoplasmic myeloperoxidase (cyMPO) was selected as the most specific marker for myeloid lineage (Khalidi, 1999; Hrusak, 2002 and Robert, 2007).

Moreover, cytoplasmic (Cy) CD79a was also selected as a second B-cell marker to improve B-lineage assignment. It was important to consider that CyCD79a is also expressed in AML with t(8;21) (Hurwitz, 1992; Kita, 1992; Tiacci, 2004 and Bhargava, 2007) and at low levels in some T-ALL cases (Pilozzi, 1998; Hashimoto, 2002 and Asnafi, 2004). In addition to cytoplasmic CD79a, cytoplasmic (Cy) IgM was further added to this tube as an additional B-cell maturation marker. Finally, cytoplasmic (Cy) CD3 was included in this tube as the most specific T-cell marker in T-cell acute lymphoblastic leukaemia (van Dongen, 1988).

In addition to these four tubes, two optional acute leukaemia diagnostic tubes were also designed for sub-classification of acute leukaemias, namely; T-ALL and AML-M7 (AML with megakablastic differentiation). The first of these was tube A5 (appendix 1) contained additional T-cell markers namely; CD1a, CD99 in addition to CD4, CD8, CD3, CD7 as well as the back bones of CD45 and CD34. On the other hand, the second tube (A7) was specifically designed for identification of acute megakaryblasts with CD41a, CD42 and CD61 antibodies (Swerdlow, 2017).

4.3. Limitation of the in-house acute leukemia panel:

Despite the several merits for this panel and its rule in improving patient care at Sidra Medicine, few limitations started to become more evident with time. The first limitation of this panel was the absence of a screening tube. Unlike the EuroFlow sequential testing strategy (van Dongen, 2012), all four tubes in this panel were tested simultaneously regardless to the type of acute

leukaemia. Only tubes A5 and A7 were added selectively based on the initial results from the four first tubes (namely; A5 for T-ALL and A7 for AML-M7 subsequently).

Moreover, the in-house panel was validated based on a relatively limited number of acute leukaemia cases (12 new leukaemia cases in total). Although several normal blood and bone marrow samples were tested during validation; particularly for normal maturation patterns; the spectrum of diseases remained very limited. For instance, no cases with mixed phenotypic, erythroblastic leukaemia or dendritic cell neoplasms were included in this validation exercise.

Moreover, no other body fluids (e.g. CSF) were included in this in-house validation project. Only one case of known T-ALL with CNS relapse was tested using this panel. It was difficult to perform direct comparibility on this occasion maily due to sample volume as well as stability. Instead, correlation with clinical, morphological, CSF cells count, diagnostic phenotype and viability testing was used for a final diagnosis of CNS relapse.

Another limitation of this panel was lack of CD5 which could be particularly useful in diagnosis and follow up of T-ALL (van Dongen, 1988). The duplication of CD4 on tubes A3 and A5 could have easily been replaced by CD5 on tube A5 using the same florochrome PerCpCy5.5. Furthermore, cytoplasmic IgM was tested for all cases regardless to the type of acute leukaemia or CD20 expression status in B-ALL. Early B-cell precursors (CD20 negative) are negative for cytoplasmic IgM and therefore testing for cyIgM did not really add an additional value to this panel. In fact, all cases in the validation project had a negative cytoplasmic IgM (except one case which was non- conclusive). Likewise, cytoplasmic IgM did not add any diagnostic value to T-acute lymphoblastic leukaemia or acute myeloid leukaemia cases in this study.

Replacement of cytoplasmic IgM with CD7 in the cytoplasmic tube (A4) would have added more value to this panel. In addition to its diagnostic and follow up value in T-ALL, CD7 is frequently expressed in AML (CD7 positive, MPO negative acute myeloid leukaemia) (Venditti, 1998). Further more, combination of CD7 and CD3 in this tube (A4) would have provided an accurate natural killer cell (NK) count in this panel in addition to B, T and myeloid cells differentiation. In fact, applying these further developments to this particular tube (A4) would create a reasonable acute leukaemia screening tube including T-cell, B-cell, NK-cells, myeloid markers as well as immaturity markers.

A further limitation to this in-house panel was the lack of specific B-ALL minimal residual disease markers (namely; CD66c, CD123, CD73 and CD304) (Lhermitte, 2018). Likewise, this panel also lacked several antibodies with cytogenetic predication value. These include but not limited to CD15, CD38, CD66c as well as CD123 (Hurwitz, 1992; Hasan, 1999; De Zen, 2000; Tabernero, 2001; Hrusak 2002; Vaskova, 2005 and Djokic, 2009). For this reason, a specific cytogenetic prediction tube was futher developed and added to this panel (tube 6, appendix 2).

Finally, due to relatively low number of cases expected annually (30-60 cases per annum); no ready made antibodies cocktails were prepared for each tube in this panel. The stability of such cocktail was not validated and therefore each sample required a relatively longer processing time (45-60 minutes per case). Although time was not critical due to relatively small sample size, frequent pipetting of approximately 25-30 antibodies for each case carried an increased risk of wrong pipetting as well as antibodies contamination. Development and validation on antibody

cocktails is likely going to take place once the workload approaches one hundred samples per annum.

4.4. Acute Leukaemia Orientation Tube (ALOT):

Acute leukaemia orientation tube (ALOT) was developed as part of the 6-year EuroFlow consortium project for standardisation of immunophenotyping in normal, reactive and malignant leucocytes (van Dongen, 2012). The initial purpose of this 8-color antibody tube was to provide the first screening step in a sample suspected of containing blast cells. This screening tube is to be followed by a more comprehensive multi-tube panel as recommended by the EuroFlow consortium for diagnosis of BCP-ALL, T-ALL or AML/MDS respectively (van Dongen, 2012).

Eight markers were carefully selected for this tube in order to be able to identify blast cells from different cell lineages. Following five rounds of evaluation, the final ALOT design included surface and cytoplasmic CD3, surface CD7, CD19, CD34, CD45 as well as cytoplasmic MPO and CD79a (van Dongen, 2012). CD22 was excluded as it is not specific for B-cells and normally expressed in mast cells, basophils and some dendritic cells. Surface immunoglobulins (Ig) were also excluded despite being very specific to B-cells as they are only expressed at later stages of maturation and not sensitive for B-cell precursors. Other myeloid markers such as CD13 and CD33 were also excluded as less specific to myeloid cells and can be found frequently in B-ALL or T-ALL (Khalidi, 1999; Hrusak, 2002 and Robert, 2007).

Options for immaturity markers included CD34, CD117 as well as nuclear (Nu) TdT. CD117 was primarily excluded as it is more specific for the myeloid lineage and lacks sensitivity to B-ALL

cases. The choice between CD34 and TdT was studied extensively in order to select the most appropriate immaturity marker. CD34 was selected over TdT as it is less specific for one cell lineage and therefore has the advantage of identifying immature cells across all cell lineages. A second advantage of CD34 was it only requires surface staining allowing it to be used as a backbone in the subsequent BCP-ALL panel. On other hand, TdT staining requires cell permeabilization which was not possible for the subsequent confirmatory B-ALL panel.

A total of 385 acute leukaemia cases were tested to configure and evaluate the contents of this screening tube. Finally, 158 acute leukaemia samples (89 B-ALL, 27 T-ALL, 37 AML and 5 AUL/MPAL) were used to validate the final ALOT tube configuration (van Dongen, 2012).

In this study, 24 specimens from 16 acute leukaemia cases were tested using both the acute leukaemia in-house panel as well as the ALOT tube under validation. Results showed 100% agreement between the two methods in all cases in the study. Interestingly, the results of the ALOT tube alone in all the diagnostic specimens in this study (21 samples) were consistent with the final diagnosis of acute leukaemia using the full in-house acute leukaemia panel (100% agreement). Moreover, the specificity, sensitivity, accuracy and prevalence of each antibody in the ALOT tube was individually assessed against the reference in-house acute leukaemia panel. Once more, results showed 100% agreement with all antibodies in the ALOT tube compared to the in-house panel.

Despite these findings, the ALOT tube was not actually designed or recommended to use alone as a diagnostic tube for acute leukaemia, particularly where minimal number of blast cells are present (van Dongen, 2012). A major limitation of the ALOT tube is the lack of any specific

monocytic and dendritic cell markers in this tube. However, when combining the ALOT tube with the lymphoid screening tube (LST) and following the strategy suggested in the proposed flowchart diagram (Fig.1.1), virtually all haematological malignancies can be detected according to the EuroFlow recommendations (van Dongen, 2012).

In this study, one case of acute monoblastic leukaemia (case 6) was only successfully categorised as monoblastic after performing the full in-house acute leukaemia panel. Interestingly, the ALOT automated database-guided interpretation tool correctly identified the case as “myeloid” despite lack of any specific markers on the ALOT tube (negative for CD3, cyCD3, CD7, CD19, CD34, cyMPO and cyCD79a).

Similarly, the ALOT tube lacks specific markers for acute megakaryoblastic leukaemia. A second case in this study (case 7) was only categorised in this group following the full in-house acute leukaemia panel with additional megakaryocytic markers (namely; CD41, CD42 and CD61). Once more, despite lacking these specific markers as well as cyMPO, the ALOT automated database interpretation tool correctly identified this case as “myeloid” lineage. Of important note, this case showed dim CD45 expression with positive CD34 and CD7. The absence of both surface and cytoplasmic CD3 has correctly led to classification of the case as myeloid instead of T-cell lineage (CD7 positive, MPO negative acute myeloid leukaemia) (Venditti, 1998).

Likewise, the ALOT tube lack specific markers for erythroid, basophilic or plasmacytoid dendritic cell lineages. In absence of such cases as well as cases of acute leukaemia of ambiguous lineage in this study (including mixed phenotypic acute leukaemia (MPAL) and acute undifferentiated

leukaemia -AUL), it was not possible to comment independently on the validity of the ALOT tube in such cases.

To evaluate the use of ALOT tube in acute leukaemia of ambiguous lineage, a study of 17 cases of acute leukaemia of ambiguous lineage was performed by the EuroFlow consortium group. Results showed that cases of MPAL from T/myeloid and B/T lineages correctly clustered as expected between T-ALL and AML and between B-ALL and T-ALL respectively (van Dongen 2012). However, cases with B/myeloid phenotype were more heterogeneous. Only two out of five cases studied correctly clustered between B-ALL and myeloid groups as expected while two other cases were incorrectly identified as B-ALL. Interestingly, the fifth case was close to T-ALL group due to a strong CD7 expression. Finally, three out of four AUL cases were falsely categorised as myeloid origin. The study concluded that further extended multicenter studies are required to collect additional numbers of these rare cases in order to improve their identification using the ALOT tube (van Dongen, 2012).

Another limitation of the ALOT tube was the absence of nuclear TdT immaturity marker. Results in this study showed that all new cases of lymphoid leukaemias (B-ALL and T-ALL) had positive nuclear TdT expression (100% sensitivity). On the other hand, all new AML cases were negative for nuclear TdT (0% sensitivity). Overall, 13 out of the 16 new leukaemia cases in this study had a positive nuclear TdT expression (81%). The addition of TdT to the ALOT tube could have increased its sensitivity for both B- and T-cell acute leukaemias as well as early differentiation between lymphoid and myeloid malignancies.

On the other hand, the second immaturity marker of choice (i.e. CD34) was positive in 10 out of 16 new cases in this study (62% sensitivity). The expression of CD34 was not lineage specific. However, it was noticed that all T-ALL cases in this study were negative for CD34. Of interest, the only 3 B-ALL cases (3/11) which were negative for CD34 all had a positive CNS (CSF) involvement with blast cells as described below.

Finally, it is well documented that the ALOT tube alone cannot distinguish between CD34 negative B-ALL and mature B-cell lymphoproliferative disorders with positive expression of CD45, cyCD79a, CD19 and negative CD34 (van Dongen, 2012).

4.5. Automated database-guided interpretation of ALOT tube:

Despite several advances in flow cytometry for standardisation of reagent composition, sample preparation, instrument set-up and data acquisition (Kalina, 2012; van Dongen, 2012); final interpretation of the results still relies on local individual expertise and relatively arbitrary criteria for identification of leukaemic cells and definition of positivity for individual markers (Lhermitte, 2018).

In 2010, a study described a novel approach of interpretation of B-cell chronic lymphoproliferative disorders (B-CLPD) using a semi-automated classification tool (Costa, 2010). In this study, 30 cases of known (B-CLPD; namely CLL, MCL and FL) were used to build a reference database using merged files from multiple tubes for each case. The reference immunophenotypic profiles were subsequently used to electronically assign 175 cases to one of the three reference groups or; to a fourth group names as “other B-CLPD” based solely on their immunophenotypic

profile (IP). Results showed that 89% of the cases were correctly assigned to the right group when compared to the expert-based interpretation of results (Costa, 2010).

This automated database-guided interpretation approach of B-CLPD paved the path for a similar approach for an automated database-guided interpretation of acute leukaemia using results of the EuroFlow ALOT tube (Lhermitte, 2018). In this latter study, a much larger group consisting of 656 acute leukaemia cases were used to build the ALOT tube reference database. Subsequently, 783 cases were evaluated and electronically assigned to one of the database groups. Results showed that in 781/783 cases the right EuroFlow acute leukaemia panel was selected (99.7%). Moreover, in > 93% of cases, the final lineage diagnosis was correctly established even without using the subsequent full EuroFlow diagnostic panels (Lhermitte, 2018).

In this study, an independent evaluation of the ALOT automated database-guided interpretation tool (Salamanca, Spain) was performed in our laboratory. A total of 21 diagnostic specimens from 15 new acute leukaemia cases were used to evaluate the automated database-guided interpretation of ALOT tube compared to the conventional hematopathologist (expert) approach of results interpretation. Results showed 100% agreement in 21/21 acute leukaemia samples between the two interpretation methods. Moreover, the correct lineage specific diagnosis was already established in all cases (21/21) even without the use of the additional 27 markers in the full in-house acute leukaemia panel (100% agreement). Interestingly, the software correctly identified two the cases of acute myeloid leukaemia (monoblastic and megakaryoblastic) even without any additional markers as described above.

Three additional follow up samples were referred during this study and were further evaluated using the automated database-guided interpretation tool. The software once more correctly identified the cell lineages of all the three cases (one case of relapsed acute myeloid leukaemia, and two cases of recovering marrow with myeloid and B-cell precursors). However, despite the correct identification of the cell lineage in all the three cases, the software could not identify whether these cells were of benign or malignant origin. This could only be achieved by analysis of additional markers and the maturation pattern; particularly CD10 and CD20; from the subsequent in-house acute panel, as well as correlation with clinical, morphological, cytogenetic and molecular findings (Johansson, 2014).

Another limitation of the database-guided interpretation was also noticed in one of these three follow up samples (case 5). A mixture of myeloid and B-cell CD34 positive precursors were identified in this follow up bone marrow specimen as “mixed lineages” when gated based on CD34 positive population only. However, when the gates were subsequently modified the correct percentage of each population was accurately identified as CD19/CD34 positive precursor cells (hematogones) and CD34 positive/CD19 negative myeloid precursors.

The significance of correct gating of all different populations in the specimen was recognised by the EuroFlow consortium and led to further development of a reference databases for all normal populations allowing automated gating of bone marrow and peripheral blood samples using highly sensitive next generation flow cytometry analysis tool (Flores-Montero, 2017). This reference auto-gating database was recently commercialised and made available via Cytognos

solutions and Infinicyt Software (Cytognos, 2020). Unfortunately, the laboratory did not have access to this new database and was not able to verify its use independently in this study.

Finally, access to the ALOT database-guided interpretation tool added additional costs to the in-house acute leukaemia investigation panel. These databases charge per each analysed sample and do not provide unlimited access once subscribed. As all results still need to be verified by a qualified haematopathology team member, the database cost was not justifiable. Therefore the laboratory opted to develop their own local database based on the results of 24 specimens analysed in this study.

4.6. Merging and comparing the immunophenotypic profiles for all cases in the study:

One of the current limitations of current flow cytometry used in clinical diagnostic laboratories is the increasing number of phenotypic criteria used in the WHO classifications with relatively limited technology to accommodate enough markers in the same tube to represent the overall phenotype of cells of interest (DiGiuseppe, 1998; Braylan, 2001; Matutes, 2002; Braylan, 2004 and Kaleem, 2006). In order to overcome this limitation in technology, several studies described the development and validation of different clinical software tools for merging and calculation of flow cytometric data files from several tubes into a single "*super*" multicolor file with all required markers (Robinson, 1991; Robinson, 1992; Costa, 2006 and Pedreira, 2008).

In this study, the Infinicyt software (Cytognos, Salamanca, Spain) was used to merge blast cells for each case from all tubes in the acute leukaemia panel into a single standard flow cytometry file (FCS). For this purpose, the in-house panel was slightly modified during this study to include

CD19 as an additional marker in all tubes of the panel (appendix 2). This way, blast cells could easily be identified in all tubes using the backbone markers (CD19, CD34, CD45 in addition to FSC and SSC). Following gating of blast cells, the software was used to merge and calculate the full immunophenotypic profile of blast cells to show the full phenotype of all antibodies in the experiment in one automated population separator (APS) diagram.

Unlike the 2 dimensional dot plot diagrams, the APS diagram applies the principles of principal component analysis (PCA) to cluster different blast cells based on all 29 antibodies in the experiment as well as forward and side scatter properties simultaneously. This allows direct comparison of the immunophenotypic profile of all blasts from different cases at the same time. File merging has also allowed subsequent development of in-house reference databases of acute leukaemia as described below.

4.7. Development of in-house acute leukaemia, ALOT and cytogenetic prediction databases:

The principles of principal component analysis (PCA) and automated population separator (APS) were used to develop an in-house acute leukaemia interpretation tool, ALOT database interpretation tool and finally; an automated cytogenetic predication tool.

Despite the limited number of specimens in this study and time constraints, preliminary results showed the possibility of developing an automated database-guided interpretation tool using the 29 markers from the in-house acute leukaemia panel. The diagnostic samples from 15 new acute leukaemia cases in the study were used for this purpose. Seven samples were used to develop an in-house reference database of acute leukaemias (4-B-ALL, 2 AML and 1 T-ALL). Subsequently,

the newly developed database tool was tested using 8 samples of new acute leukaemia (6 B-ALL, 1 AML and 1 T-ALL). Results showed 100% agreement between the newly developed acute leukaemia database interpretation tool and the conventional expert-based interpretation of flow cytometry results indicating a promising approach of automated database-guided interpretation of acute leukaemia.

However, this study did not include the full spectrum of acute leukaemia sub-groups according to the revised 4th edition of the WHO classification of tumours of haematopoietic and lymphoid tissues (Swerdlow, 2017). It is not clear whether cases of acute leukaemia with ambiguous lineage (MPAL and AUL), erythroid or basophilic leukaemia can be correctly identified using this approach. There is a need for extended studies to include these relatively rare sub-types of acute leukaemia to enrich the local reference database.

Likewise, an in-house ALOT reference database and an automated interpretation tool was also developed. The main reason for developing this tool was to reduce the additional costs of purchasing the reference ALOT database as well as having an in-house reference database which was produced from local analyser settings and laboratory staining procedures. In order to develop this database, 12 specimens (6 peripheral blood samples and 6 bone marrows) were used as a “build group” to develop the reference database compass. Subsequently, a “validation group” including 12 different specimens (7 peripheral bloods and 5 bone marrows) was used to validate the newly developed in-house ALOT database interpretation tool.

Once more, results showed 100% agreement between the new database-guided interpretation tool and the final expert interpretation of ALOT tube. Results confirmed that the development of

local database-guided interpretation tool was successful and there was no need to continue purchasing the ALOT database access for results interpretation. However, it is important to note that the successful identification of the cell lineage using database-guided tools does not by itself provide the clinical interpretation of flow cytometry results.

For instance, a follow up sample from one case in this study (case 8, T-ALL) showed 18% precursor cells in peripheral blood which was correctly identified by both the ALOT database and the full in-house acute panel as myeloid lineage. However, further analysis of the maturation pattern as well as correlation with clinical and morphological findings confirmed that the patient is not in a relapse or developing a subsequent therapy related AML following recent treatment of T-ALL. Instead, this was an unusual reactive/recovering bone marrow with circulating myeloid precursors due to a recent bone marrow stress.

The findings were confirmed by spontaneous recovery of blood counts and complete maturation of myeloid precursors from peripheral blood within 48 hours of presentation. This was an example were the integrated clinical, morphological, phenotypic as well as any cytogenetic or molecular findings were collectively needed for final interpretation of flow cytometry results (Johansson, 2014).

Finally, the merged files from the full acute leukaemia in-house panel were used once more used in an attempt to build an automated cytogenetic predication tool using the immunophenotypic profile (IP) of blast cells. Several antibodies were already reported in the literature to have a cytogenetic predication value. These included but not limited to CD10, CD13, CD15, CD19, CD38,

CD66c as well as CD123 (Hurwitz, 1992; Hasan, 1999; De Zen, 2000; Tabernero, 2001; Hrusak 2002; Vaskova, 2005 and Djokic, 2009).

In order to be able to build this cytogenetic predication tool, the in-house acute leukaemia panel was modified to include an additional cytogenetic prediction tube (tube 6, appendix 2). This tube included CD15, CD38, CD66c, and CD123 in addition to the backbone markers (CD19, CD34 and CD45) to facilitate subsequent merging of blast cells. In order to develop this tool, 12 samples from 11 newly diagnosed B-ALL cases in this study were divided into two groups: a reference database "build group" and a database "validation group".

As the number of cases was limited, specimens were divided in a systemic manner to ensure adequate representation of different B-ALL sub-categories in each group. Accordingly, the "build group" contained 8 samples (3 cases of B-ALL not otherwise specifies (B-ALL, NOS), two cases of B-ALL with hyperdiploidy, one case of B-ALL with translocation t(12;21), one case with translocation t(1;19) and one case of B-ALL with BCR-ABL1 like translocation t(Y;14). The Infinicyt database configuration tool was then used to build a cytogenetic predictive compass with five categories representing all available B-ALL subgroups in this study (B-ALL NOS, B-ALL with hyperdiploidy, B-ALL with translocation t(12;21), B-ALL with t(1;19) and B-ALL with BCR-ABL1 like translocation).

On the other hand, the database "validation group" contained 4 samples of B-ALL. These included two cases of B-ALL not otherwise specifies (B-ALL, NOS), one case of B-ALL with hyperdiploidy and one case of B-ALL with translocation t(12;21). Unfortunately, there were no adequate cases

to verify the recently developed categories of B-ALL with t(1;19) and B-ALL with t(Y;14) at the time of presenting these results.

Results of this validation exercise showed that the newly developed cytogenetic prediction database tool was successfully able to predict the actual cytogenetic results in 50% of the cases (2/4). This included once case of B-ALL with hyperdiploidy and once case of B-ALL NOS. The aberrant expression of CD123 and CD66c as well as CD38 and high forward scatter (FSC) in B-ALL with hyperdiploidy (Theunissen, 2017) makes B-ALL with hyperdiploidy have a unique immunophenotypic profile (signature) that could easily be identified by the newly developed cytogenetic prediction tool.

In contrast, the database predication tool failed to identify one case of B-ALL NOS and one case of B-ALL with t(12;21) despite examples of both categories included in the built reference database.

Detailed analysis of these two cases showed that both cases had a slightly different immunophenotypic profile (IP) compared to the reference database. The first case of B-ALL-NOS (case 2) had a strong positive CD9 and CD34 positive expression compared to the other B-ALL NOS cases used in developing the database with no CD34 expression. Moreover, the in-house panel was not fully modified when case 2 was tested and therefore did not include all the additional cytogenetic specific markers (i.e. CD123).

The second failed case was B-ALL with t(12;21) (case 11). Review of the detailed phenotype of this case showed that it had a negative CD34 expression compared to the case used in building

the database (case 13) with positive expression of CD34. This has subsequently led to software failure in identifying the case as B-ALL with t(12;21). Both failed cases (2 and 11) were subsequently added to the reference database in order to enrich this local database with diversity of blast cells for future identification. Subsequent re-analysis of both cases using the updated database has correctly identified both cases as B-ALL NOS and B-ALL with t(12;21) respectively. These findings show the need of a larger reference database population before the success rate of this predication tool starts to become higher.

Of important note, that results of this cytogenetic prediction tool remains under development and are not shared with the clinical team for management decisions. It is very unlikely, that this tool will replace the conventional FISH and karyotype analysis of acute leukaemia cases even after development of larger database. However, these additional markers can have a significant value in follow up as well as further expansions in measurement of minimal residual disease (MRD) particularly CD38, CD66c and CD123 (Theunissen, 2017).

4.8. Comparison of ALOT immunophenotypic profile of all cases in the study:

The principal component analysis tool (PCA) and the automated population separator diagram (APS) tools in Infinicyt software (Salamanca, Spain) were once more used to compare the blast cells of all 15 new leukaemia cases in this study using results of the ALOT tube only. Results of this analysis showed a clear separation between B-cell, T-cell and myeloid leukaemic blast cells consistent with the EuroFlow validation results (Figure 4.1) (van Dongen, 2012 and Lhermitte, 2018).

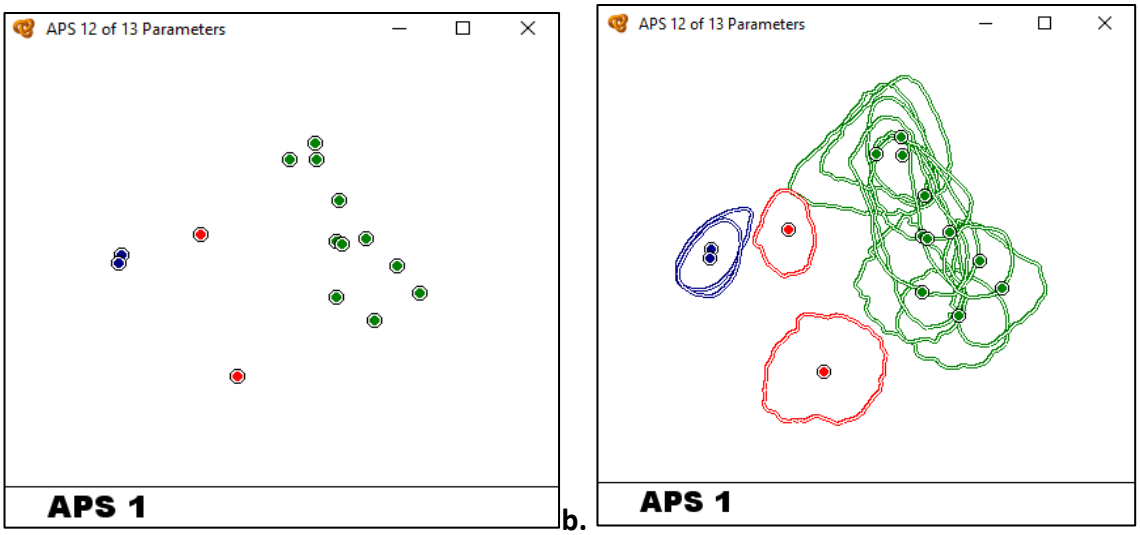


Figure 4.1. Seperation of B-cell, T-cell and myeloid leukaemic blast cells using PCA.

Detailed analysis of the x-axis (PC1) of the APS diagram showed that CD19 antibody had the highest significance value in separating the blast cells of all cases (24%). This was followed by CD7 (15%) and CD79a (14%) respectively. On the other hand, analysis of the y-axis data (PC2) of the APS diagram showed that CD34 had the highest significance in separating different blast cells vertically (38%). This was followed by CD79a (10%) and CD7 (9.8%) respectively.

These findings further confirmed the prevalence findings performed earlier in this study in parallel to sensitivity and specificity of individual antibodies in the ALOT tube (section 4.2). CD9, CD10, CD19, nuclear TdT and cyCD79a were among the highest prevalence antigens among all leukaemia cases in the study (100% of B-ALL cases). This was followed by CD45 (71%), CD34 (54%) and CD7 (17%) respectively. Interestingly, CD9 despite being among the highest prevalence antibodies in all cases in the study (100% of B-ALL and AML cases and 50% of T-ALL); it played a

very little role in differentiating the type of blast cells. Therefore, it was not included as one of the key markers in the ALOT tube (van Dongen, 2012).

In contrast to CD9, CD45 despite having a much lower sensistity (71%), it played a major role in identifying blast cells in the ALOT tube. In fact, CD45 showed 100% sensitivity in myeloid and T-ALL leukaemias in this study. However, it was only positive in 9% of B-ALL cases. The critical value of CD45 comes from its ability to separate the residual normal populations in tested specimen rather than lineage classification (Borowitz, 1993; Lacombe, 1997 and Vial, 2001).

Surface staining of CD3 despite being a very specific T-cell marker (van Dongen, 1988), showed very little contribution in separating various blast cells in the ALOT tube (4% and 1% respectively). On the other hand, cytoplasmic staining of CD3 had a much better value in separating blast cells with 14% and 10% on the x-axis (PC1) and y-axis (PC2) respectively. Interestingly, results showed that both T-ALL cases in this study had a negative surface CD3 with a positive expression of cytoplasmic cyCD3. Likewise, both T-ALL cases had a positive CD7 expression showing a higher sensitivity of CD7 (100%) in T-ALL compared to surface CD3 in the ALOT tube. However, CD7 lacks specificity and can be expressed in AML cases particularly MPO negative acute myeloid leukaemias (van Dongen 2012).

Furthermore, CD34 in addition to CD7 played a key role in separating the AML blast cells vertically on the APS diagram (38% and 9.8%, respectively). The two AML cases in this study despite being separated from both B-ALL and T-ALL clusters they were not actually overlapping in one clear AML group (figure 4.1. above). This was mainly due to positive expression of CD34 and CD7 on one case (case 7) while the second case was negative for both markers (case 6). These findings

were further confirmed by the full in-house acute leukaemia panel as one case showed positive expression of CD41, CD42 and CD61 confirming a diagnosis of acute megakaryoblastic leukaemia (Gassmann, 1995 and Swerdlow, 2017); while the second case showed positive expression of CD64 and CD4 indicating a monoblastic origin of blast cells (Dunphy, 2007).

Comparison of the all B-ALL cases using the PCA and APS showed minimal differentiation of B-ALL subtypes in this study using the ALOT tube only (figure 4.2). Detailed analysis of the x-axis (PC1) showed that CD34 had the highest significance value in separating the blast cell populations (55%). As all B-ALL cases were positive for CD19, CD79a and negative for CD3, CD7, cyCD3 and cyMPO; there were no adequate markers to separate these B-cells from each other based on the ALOT tube only.

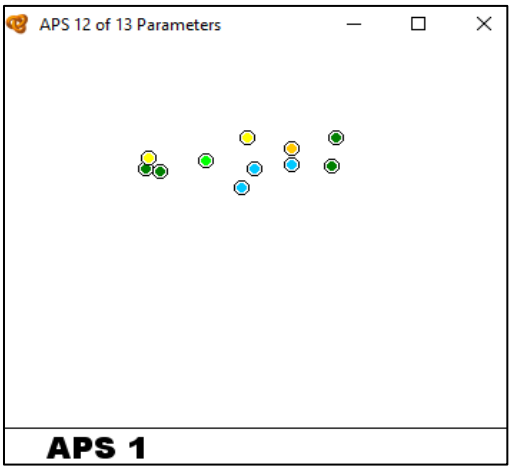


Figure 4.2. Comparison of ALOT tube of B-ALL cases only.

Surprisingly, analysis of the y-axis data (PC2) of the APS diagram showed that the forward and side scatter properties had the highest significance in separating different B-ALL blast cells vertically (13% and 11% respectively). All three cases of B-ALL with hyper diploidy in this study

(in blue) were relatively on the same side of the y-axis despite not fitting in one cluster. These interesting findings confirms the value of forward scatter properties (FSC) in predicting the cell size as well as its DNA content in B-ALL (Olcay, 1999 and Yurinskaya, 2017).

Furthermore, using this unsupervised APS analysis approach three more B-ALL cases were relatively adjacent to each other compared to the rest of the cases (namely, case 4, 10 and 11 on the left side of diagram 4.2). The three cases did not show similarity in their final cytogenetic diagnosis (2 B-ALL NOS and 1 B-ALL with t(12;21). However, detailed review of the clinical presentation, blood counts and the phenotypic profile of these three cases showed that the all cases presented with hepatosplenomegaly, negative CD34 expression, platelets count of <50 x 10^9/L and interestingly; they were the only 3 B-ALL cases with CNS involvement (CNS 2).

It is clear that CD34 negativity was the main contributor in this clustering of blast cells (55%) as shown by the detailed x-axis analysis (PC1). This interesting APS independent finding correlates well with the increased incidence of CNS involvement with CD34 negative blast cells in acute lymphoblastic leukaemia described in the literature (Borowitz, 1990; Pui, 1993 and Thomas, 1995).

4.9. Comparison of the full acute leukaemia phenotypic profile for B-ALL cases:

Finally, the blast cells of all B-ALL cases were once more compared to each other using the merged files generated from the full in-house acute leukaemia panel described in section 3.8. As the in-house acute leukaemia panel was modified during the study to include a cytogenetic predication tube (tube A6, appendix 2) it was only possible to compare the last 6 B-ALL cases.

Results showed a clear overlap between two cases (11 and 13, in yellow) as they shared the same cytogenetic finding with t(12;21) (figure 4.3).

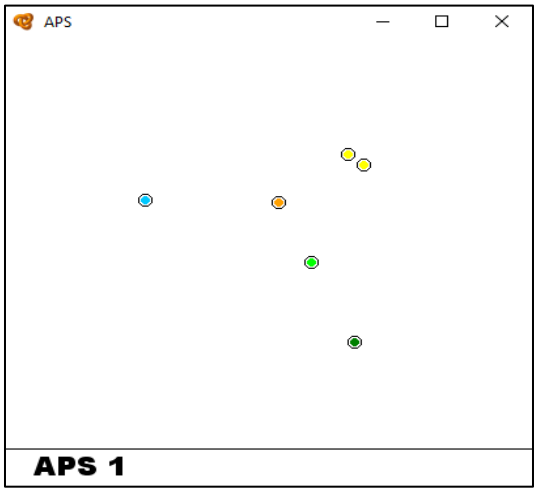


Figure 4.3. Comparison of immunophenotypic profile of B-ALL cases (10-16) only. Two cases of B-ALL with t(12;21) (yellow), one case of B-ALL (NOS) (dark green), 1 cases of B-ALL with hyperdiploidy (blue), , one case of B-ALL with t(1;19) (light green) and one case of B-ALL with BCR-ABL1 like (orange).

Detailed analysis of the x-axis (PC1) of the APS diagram showed that CD66c had the highest contribution of separating blast cells in this diagram among the 29 antibodies in this panel (21%). This was followed by CD123 with approximately 14% significance. On the other hand, analysis of the y-axis (PC2) showed that CD9 was the most significant antibody in separating the blast cells vertically (18%) despite being positive in all cases. This separation is likely attributed to the intensity of CD9 in various types of B-ALL. HLADR and CD34 also showed a contribution of this separation with a significance of 13% and 11% respectively. Overall, these findings are consistent with the well recognised cytogenetic prediction value of CD66c and CD123 in B-ALL with hyperdiploidy (Kiyokawa, 2014 and Bras; 2019).

4.10. Cost analysis:

Lastly, following the technical and scientific evaluation of the ALOT tube, a detailed cost analysis was performed to evaluate the cost implications of adding the EuroFlow ALOT tube to the current in-house acute leukaemia panel. For this purpose, a standard cost analysis excel spread sheet was used to include the initial cost of reagents, internal quality control material, staffing, external quality assurance schemes as well as any additional organization specific costs. Using this template, the initial cost of the full in-house acute leukaemia panel was estimated to be around 200 pounds sterling (section 1.7).

Two commercial products of the EuroFlow ALOT tube were available. The first was the BD OneFlow ALOT tube (BD Biosciences, reference 660228) with a local cost of approximately 280 pound sterling per test. Surprisingly, this single tube was more expensive than the full in-house acute leukaemia panel including 29 different markers. The second ALOT product was from Cytognos solutions (Cytognos, Salamanca, reference CYT-ALOT) at a local cost of approximately 70 pounds per test.

The estimated total cost of the additional Euroflow tubes ranged from 434 to 750 pounds sterling per test (for acute lymphoblastic leukaemia and acute myeloid leukaemia; respectively). Therefore, the expected overall annual cost of the in-house acute leukaemia panel was 12,000 pounds sterling compared to approximately 45,000 pounds sterling for the Euroflow panel.

Considering the added clinical value of the ALOT tube, it was not justifiable to proceed with either products. Firstly, ALOT tube was primarily designed as an orientation tube specifically aimed to

aid the selection of appropriate T-ALL, BCP-ALL or AML/MDS EuroFlow panels (van Dongen, 2012). As the laboratory uses only one in-house panel to classify all types of leukaemia, ALOT tube will not provide any additional clinical value apart from its automated database guided interpretation tool which also needed to be purchased separately (Cytognos, Salamanca).

Secondly, as shown in this study, the laboratory was able to develop their own local automated database guided interpretation tool not only for ALOT tube but as well for the full in-house acute leukaemia panel as well as a potential future cytogenetic predication tool. A more pragmatic approach was to further modify the in-house panel (namely tube A4) by removing the cytoplasmic IgM and replacing it by surface CD7. Analysis of 16 acute leukaemia cases in this study showed that cytoplasmic IgM was negative in all cases. In contrast, CD7 had a significant value in the diagnosis of the two T-ALL cases as well as one of the AML cases in this study (Venditti, 1998 and Swerdlow, 2017). Indeed, cytoplasmic IgM can always be added as an add-on marker in a separate tube only in a subgroup of CD20 positive B-ALL cases.

This way, the in-house tube A4 will practically have an equal value of the EuroFlow ALOT tube; if not more. Both tubes will share 7 key antibodies namely; CD45, CD19, CD34, CD7, cyMPO, cyCD79a as well as cyCD3. Despite that the in-house A4 tube lacks surface CD3, the marker is already tested in tube A2 in the in-house panel. Practically, testing surface CD3 in parallel to CD2 in tube A2 presents and added value of CD3 by identifying NK cells in addition to mature T-cells (van Dongen, 1988). It was evident from this study that surface CD3 is not as sensitive as cytoplasmic CD3 as shown in the two T-ALL cases. In fact, the proposed modified tube A4 is likely going to be of more value locally compared to the EuroFlow ALOT tube as it contains two

immaturity markers (nuclear TDT and CD34). If needed, the modified A4 tube can always be used as a frontline orientation tube equivalent to the ALOT followed by the rest of the full in-house panel at no additional cost to the department.

5. Conclusion and future plans:

In this study, the validation of EuroFlow acute leukaemia orientation tube and its corresponding database-guided interpretation tool was studied using 8-color FACS Canto II Flow cytometer and local settings and staining procedures. Results showed that the ALOT tube and database interpretation tool can be used as powerful tools for diagnosis of acute leukaemia even without following the exact EuroFlow staining procedures or analyser settings. Limitations of this approach were discussed in more detail, including the additional costs of the ALOT tube and the automated database interpretation tool as well as challenges related to acute leukaemia of ambiguous lineage.

The study concluded that there was no additional value of adding the ALOT tube to the current in-house acute leukaemia panel. However, results led to further modifications of the in-house acute leukaemia panel by adding the back bone markers to all tubes (CD19, CD34 and CD45), inclusion of the cytogenetic prediction tube (CD15, CD38, CD66c and CD123) as well as modification of the cytoplasmic tube to include surface CD7 instead of cytoplasmic IgM. Such modifications will provide equivalent results to the ALOT tube as well as added benefit to local future expansion plans and validation of minimal residual disease (MRD) using flow cytometry.

These modifications as well as application of the principal component analysis (PCA) and automated population separator (APS) tools have further led to development and validation of in-house ALOT and acute leukaemia automated database interpretation tools at no additional costs to the department. The results have also shown a potential use of the PCA and file merging

techniques to build a cytogenetic prediction tool based on the immunophenotypic profile (IP) of blast cells.

Due to the limited number of cases in this study, it was not possible to accurately verify whether the unsupervised clustering of blast cells using the automated population separator (APS) tool correlates with specific clinical, haematological or cytogenetic findings, although some overlap was noticed between cases with similar cytogenetic findings. Furthermore, results of the unsupervised automated population separator (APS) tool showed clustering of CD34 negative B-ALL cases which presented with hepatosplenomegaly, thrombocytopenia and CNS involvement. There is a need for further studies with larger sample volume to verify such findings.

Futher expansion plans in the department include the validation of minimal residual disease (MRD) using flow cytometry in parallel to molecular testing currently performed on day 33 and day 78. The addition of the cytogenetic prediction tube with CD38, CD66c and CD123 will have a significant value of detection of MRD by flow cytometry as reported in the literature (Theunissen, 2017).

Moreover, detection of MRD using flow cytometry will provide a much better turn around time compared to molecular MRD currently performed in reference laboratory. Indeed, flow cytometry can be performed at a significantly lower cost compared to molecular MRD. However, the sensitivity of such assay together with its alignment to treatment protocols need to be validated carefully before this can be applied for clinical use.

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6.2. Appendices:

Appendix 1: Original in-house acute Leukaemia Panel:

	BLUE				RED		VIOLET	
	FITC	PE	PerCpCy5.5/PC5.5	PECy7	APC	APCH7 or Cy7	BV 421	V500
Acute 1	CD20	CD13	CD9	CD34	CD10	CD11b	CD19	CD45
Acute 2	CD15	CD117	HLA-DR	CD34	CD33	CD3	CD7	CD45
Acute 3	CD2	CD56	CD4	CD34	CD64	CD14	CD36	CD45
Acute 4	nuTDT	CyMPO	CyCD79a	CD34	CyIgM	CyCD3	-	CD45

Additional tubes as needed:

A5: T-ALL	CD1a	CD99	CD4	CD34	CD8	CD3	CD7	CD45
A7: AML-M7	CD61	CD41a	-	CD34	CD42	-	CD19	CD45

Appendix 2: Modified in-house acute leukaemia panel:

	BLUE				RED		VIOLET	
Fluorochrome	FITC	PE	PerCpCy5.5/PC5.5	PECy7	APC	APCH7	BV 421	V500
Acute 1	CD20	CD13	CD9	CD34	CD10	CD11b	CD19	CD45
Acute 2	CD2	CD117	HLA-DR	CD34	CD33	CD3	CD19	CD45
Acute 3	CD71	CD56	CD4	CD34	CD64	CD14	CD19	CD45
Acute 4	nuTdT	cyMPO	cyCD79a	CD34	cyIgM	cyCD3	CD19	CD45

Additional tubes as needed:

A5: T-ALL	CD1a	CD99	CD4	CD34	CD8	CD3	CD7	CD45
A6: B-ALL	CD15	CD66c	-	CD34	CD123	CD38	CD19	CD45
A7: AML-M7	CD61	CD41a	-	CD34	CD42	-	CD19	CD45

Appendix 3: Detailed results for all cases in the study:

1. Case 1:

3.9. Clinical presentation:

This was a 7 years old previously healthy and fit boy with no history of chronic diseases. He presented with six weeks history of severe back pain disturbing his sleep, mobility and school attendance. There was no history of weight loss, night sweats or bleeding. On examination, there was no fever, gastrointestinal tract (GIT), respiratory, neurological or cord compression symptoms. His main complaint was mid-thoracic back pains.

3.10. Blood counts, morphology and coagulation studies:

Full blood count was essentially normal with haemoglobin of 119 g/L, white blood cells count of 5.4 x 10^9/L and a platelets count of 168 x 10^9/L. Neutrophil count was also normal for age (1.5 x 10^9/L) and no blast cells were identified on peripheral blood film examination. Coagulation studies were also normal for age with prothrombin time (PT) of 13 seconds, activated partial prothrombin time (APTT) 34 seconds and fibrinogen of 4.0 g/L. Bone marrow aspirate and biopsy were therefore indicated to confirm or rule out the presence of haematological malignancy.

3.11. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate and hypercellular with approximately 35% medium to large-sized cells with high nuclear cytoplasmic ratios, slightly irregular nuclear contours mostly

inconspicuous nucleoli (rarely prominent) and scant amounts of agranular cytoplasm indicating blast cells. There was progressive maturation of myeloid precursors, mild megaloblastic and dyserythropoietic changes with unremarkable megakaryocytic and lymphoid morphology.

Review of the touch preparation of the trephine biopsy reveals a markedly hypercellular marrow with sheets (>90% of cellularity) of monomorphic cells with a similar appearance to blast cells described above. Bone Marrow biopsy was adequate and confirmed hypercellularity (approximately 95-100%). Hematopoiesis was sub-totally replaced by sheets of mononuclear cells, consistent with blast cells. Few mitotic and apoptotic bodies were also noted. Erythroid, myeloid and megakaryocytic precursors were all markedly decreased. CSF examination showed no definite blast cells. This was considered as CNS-1 classification per the BFM 2017 protocol.

3.12. Flow cytometry:

Flow cytometric immunophenotyping performed on the bone marrow aspirate identified a blast population (comprising approximately 32% of total cells). These expressed CD9, CD10, CD19, CD20 (partial), CD34, HLA-DR, cytoplasmic CD79a and TdT. There was no significant expression of CD45, CD2, CD3, CD4, CD7, CD11b, CD13, CD14, CD15, CD33, CD36, CD56, CD64, CD117, cytoplasmic MPO, CD3 or cytoplasmic IgM. Blast cells showed a much higher forward scatted light (FSC) indicating a possible hyperdiploidy.

3.13. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization analysis (FISH) performed on the bone marrow aspirate revealed an abnormal hybridization pattern with extra signals for multiple

probes (BCR/ABL1, ETV6/RUNX1, IGH and CDKN2A) indicating hyperdiploidy of the cells analyzed. However, no recurrent rearrangements of the BCR/ABL1, ETV6/RUNX1, and MLL, IGH or CDKN2A probes were identified. Karyotype analysis confirmed the FISH findings with multiple chromosomes having extra copies. These findings were consistent with a high hyperdiploid karyotype.

(60,XY,+X,+Y,+2,+4,+5,+6,+9,+10,+14,+17,+18,+20,+21,+21(3)/46,XY(62).

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

3.14. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of B-cell acute lymphoblastic leukemia. Final WHO subtype classification was **B-Lymphoblastic leukaemia/lymphoma with hyperdiploidy** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

3.15. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation confirmed the initial immunophenotypic findings with positive CD34, CD19 and cytoplasmic CD79a. There was no significant expression of CD45, surface CD3, CD7, cytoplasmic MPO or cytoplasmic CD3. The principal component analysis (PCA) and automated population separator (APS) diagram (figure 3.4) showed blast cell

distribution compared to residual normal cell populations in the sample. The automated database orientation tool using Infinicyt software from indicated blast cells to be of B-cell lineage (figure 3.5).

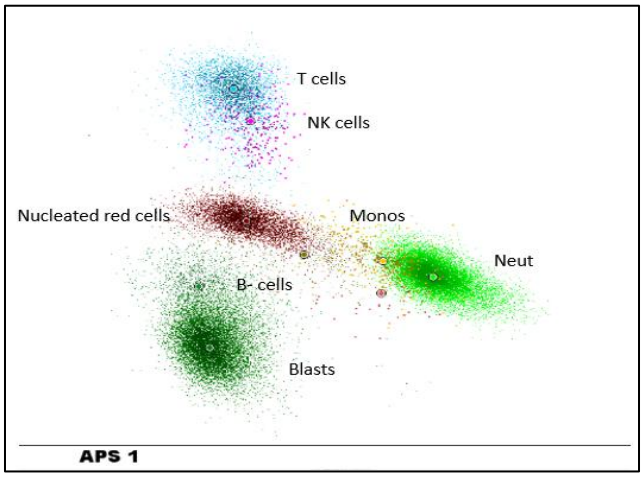


Figure 6.1: Blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

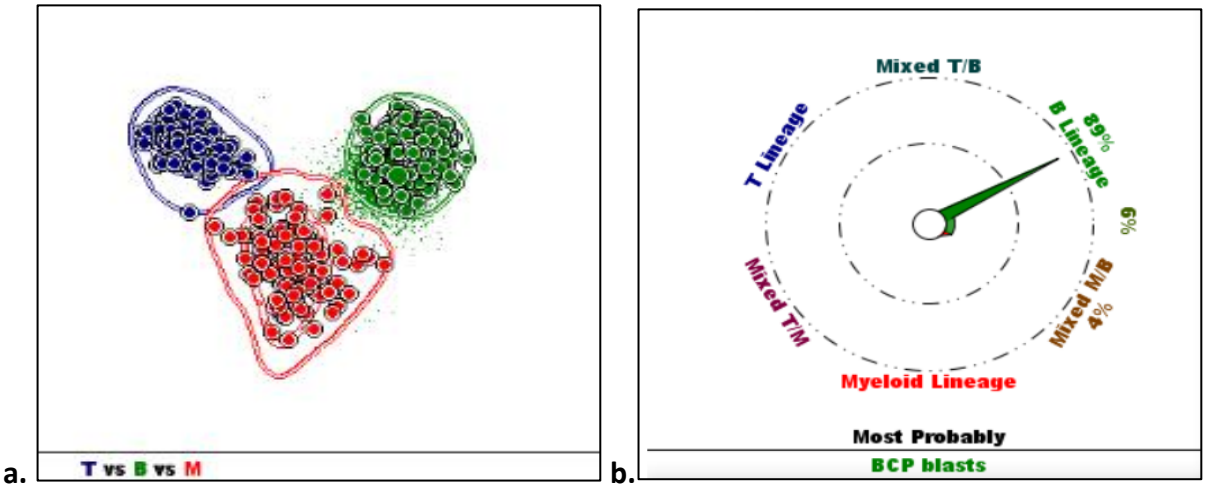


Figure 6.2: Case 1 blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

4. Case 2:

4.1. Clinical presentation:

This was a 5 years old girl with approximately 15 days history of fever. Two bruises were noticed by parents four days ago. She was found to be thrombocytopenic and was recently treated for urinary tract infection (UTI) in a primary healthcare center. Clinical examination showed a bruise on her forehead otherwise she looked well. There was no petechiae, organomegaly, other chest or abdominal abnormalities. A second small bruise was also seen in the right iliac fossa.

4.2. Blood counts morphology and coagulation studies:

Blood count confirmed severe thrombocytopenia with a platelets count of 19 x 10^9/L only. There was no leucopenia or anaemia at presentation (WBC 5.7 x 10^9/L and haemoglobin 110 g/L respectively). However, blood film examination showed neutropenia (neutrophils count was 0.7 x 10^9/L) with approximately 8% blast cells. Coagulation screen was normal for age with prothrombin time (PT) of 13 seconds, activated partial prothrombin time (APTT) 20 seconds (likely pre-analysis activation) and fibrinogen level of 2.7 g/L. Bone marrow aspirate and biopsy were therefore indicated to confirm the presence of a potential haematological malignancy.

4.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate and hypercellular with approximately 96% blast cells. These were mostly intermediate to large-sized cells with high nuclear cytoplasmic ratios, slightly irregular nuclear contours, finely dispersed chromatin, occasionally prominent nucleoli

with minimal to moderate amounts of agranular basophilic cytoplasm and occasional vaccuoles. There were rare erythroid, myeloid and megakaryocytic precursors as well as rare small mature-looking lymphocytes.

Bone Marrow biopsy was suboptimal (fragmented and crushed), however; confirmed hypercellularity (approximately 95-100%). Hematopoiesis was sub-totally replaced by sheets of mononuclear cells, consistent with blast cells (>90% of overall cellularity). Few mitotic and apoptotic bodies were also noted. Erythroid, myeloid and megakaryocytic precursors were all markedly decreased. CSF examination showed no definite blast cells. This was considered as CNS-1 classification per the BFM 2017 protocol.

4.4. Flow cytometry:

Flow cytometric immunophenotyping performed on the bone marrow aspirate identified a blast population (comprising approximately 64% of total cells) expressing CD9, CD10, CD19, CD34, HLA-DR, partial CD20, CD33 and CD56 in addition to cytoplasmic CD79a and TdT. There was no significant expression of CD45, CD2, CD3, CD4, CD7, CD11b, CD13, CD14, CD15, CD36, CD64, CD117 and cytoplasmic MPO, cyCD3 or cytoplasmic IgM.

4.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate did not identify any recurrent rearrangements of the BCR/ABL1, ETV6/RUNX1, MLL, IGH or CDKN2A probes. Karyotype analysis showed normal (46,XX) chromosomes in the 30 metaphase cells examined.

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

4.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia (B-ALL), not otherwise specified (NOS)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

4.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation confirmed the initial immunophenotypic findings with positive CD34, CD19 and cytoplasmic CD79a. There was no significant expression of CD45, surface CD3, CD7, cytoplasmic MPO or cytoplasmic CD3. The principal component analysis (PCA) and automated population separator (APS) diagram (figure 3.6) showed blast cells distribution compared to residual normal cell populations in the specimen. The automated database orientation tool using Infinicyt software from Cytognos indicated blast cells to be of B-cell lineage (figure 3.7).

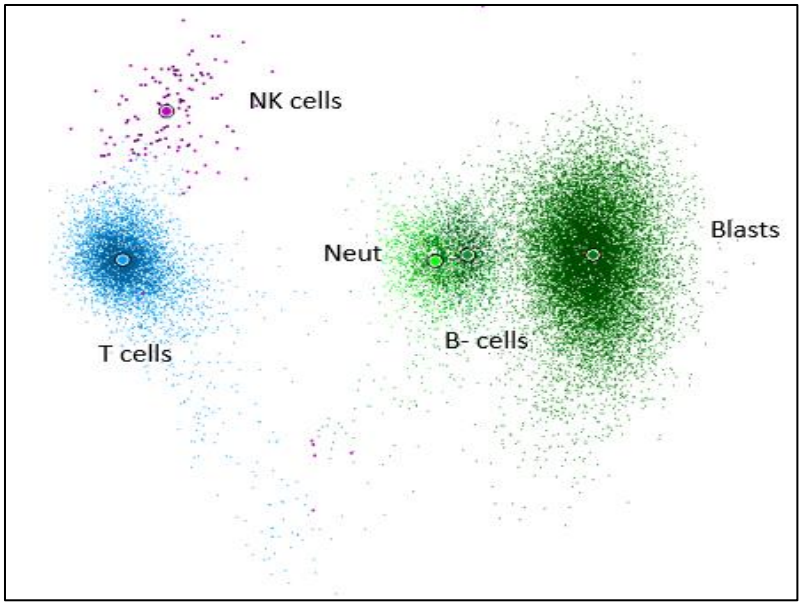


Figure 6.3: Case 2 blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

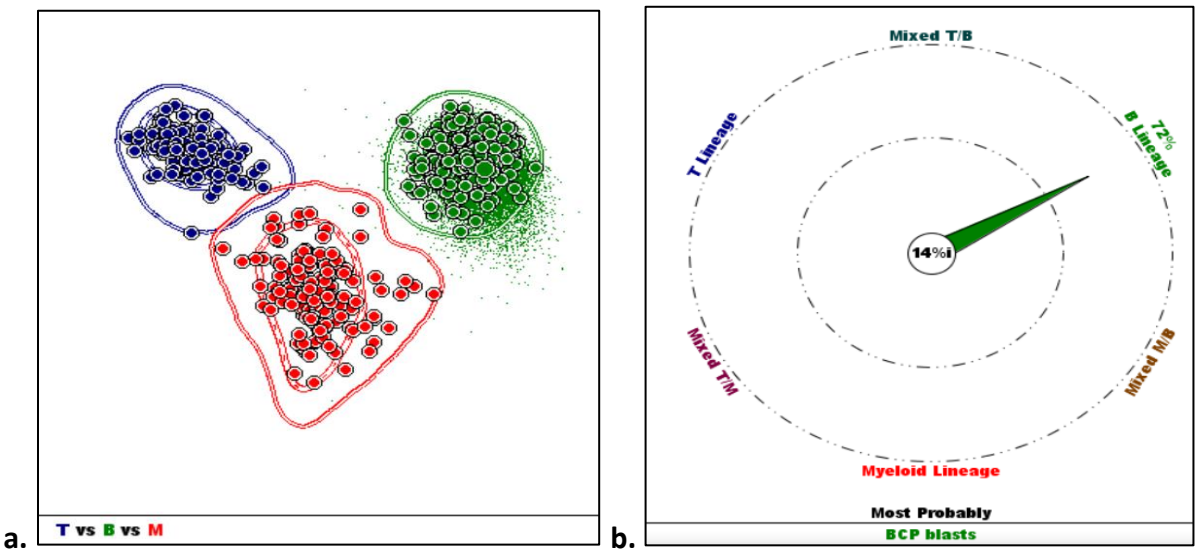


Figure 6.4. Case 2 blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

5. Case 3:

5.1. Clinical presentation:

This was a 2 years old boy with history of 2 days fever, body weakness and trauma in right lower limb with swelling. He tended to crawl to avoid walking and a sudden forehead swelling was noticed by mother, although same side of fall. He had night sweats for 2-3 night needing to change clothes for that. At presentation, he was pale and clinical examination revealed hepatosplenomegaly with palpable lymph nodes. There was no fever, bruises, gum bleeding or epistaxis. Her chest was clear with no abdominal discomfort (vomiting or diarrhea).

5.2. Blood counts morphology and coagulation studies:

Blood count confirmed severe anaemia (haemoglobin 42 g/L) with severe thrombocytopenia (platelets count of 13 x 109/L). There was leucocytosis with a white blood cells count (WBCs) of 27.6 x 109/L. Blood film examination showed neutropenia (neutrophils count 0.3 x 10^9/L) with approximately 75% blast cells. Coagulation screen was normal for age with prothrombin time (PT) of 13 seconds, activated partial prothrombin time (APTT) of 30 seconds and fibrinogen level of 2.5 g/L. Bone marrow aspirate and biopsy were therefore indicated to confirm the new diagnosis of acute leukaemia.

5.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was aparticulate, however; with adequate quality for assessment. Differential count identified approximately 98% blast cells. These were mostly small to medium-sized cells with high nuclear cytoplasmic ratios, finely dispersed chromatin with inconspicuous

nucleoli and scanty agranular basophilic cytoplasm. Occasional cytoplasmic vacuolation was also noticed. There were rare erythroid and myeloid precursors while no megakaryocytic precursors were identified in this suboptimal specimen.

Bone Marrow biopsy was suboptimal, however; confirmed hypercellularity (virtually 100%). Hematopoiesis was almost totally replaced by sheets of mononuclear cells, consistent with blast cells. Erythroid, myeloid and megakaryocytic precursors were not possible to identify from the background of blast cells. CSF examination showed no definite blast cells. This was considered as CNS-1 classification per the BFM 2017 protocol.

5.4. Flow cytometry:

Flow cytometric immunophenotyping performed on the bone marrow aspirate identified a blast population (comprising approximately 91% of total cells) and expressing CD34, CD9, CD10, CD19, HLA-DR, partial CD20 in addition to cytoplasmic CD79a and TdT. There was no significant expression of CD45, CD2, CD3, CD4, CD7, CD11b, CD13, CD14, CD15, CD33, CD56, CD36, CD64, CD117 and cytoplasmic MPO, cyCD3 or cytoplasmic IgM.

5.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate showed additional 3 copies of IGH probe on chromosome 14 and 3 copies of RUNX1 probe on chromosome 21 in 89% of cells analysed. Karyotype analysis revealed an abnormal clone (13/20) with extra copies of multiple chromosomes. A normal clone was also

present (55,XY,+X,+4,+8,+10,+14,+17,+18,+21,+21(13)/46,XY(7). These findings were consistent with the presence of a high hyperdiploid karyotype (HeH).

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

5.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of B-cell acute lymphoblastic leukemia. Final WHO subtype classification was **B-Lymphoblastic leukaemia/lymphoma with hyperdiploidy** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

5.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on the diagnostic blood sample and confirmed the initial immunophenotypic findings with positive CD34, CD19 and cytoplasmic CD79a. There was no significant expression of CD45, surface CD3, CD7, cytoplasmic MPO or cytoplasmic CD3. The principal component analysis (PCA) and automated population separator (APS) diagram (figure 3.8) showed blast cells distribution compared to residual normal cell populations in the specimen. The automated database orientation tool using Infinicyt software from Cytognos indicated blast cells to be of B-cell lineage (figure 3.9).

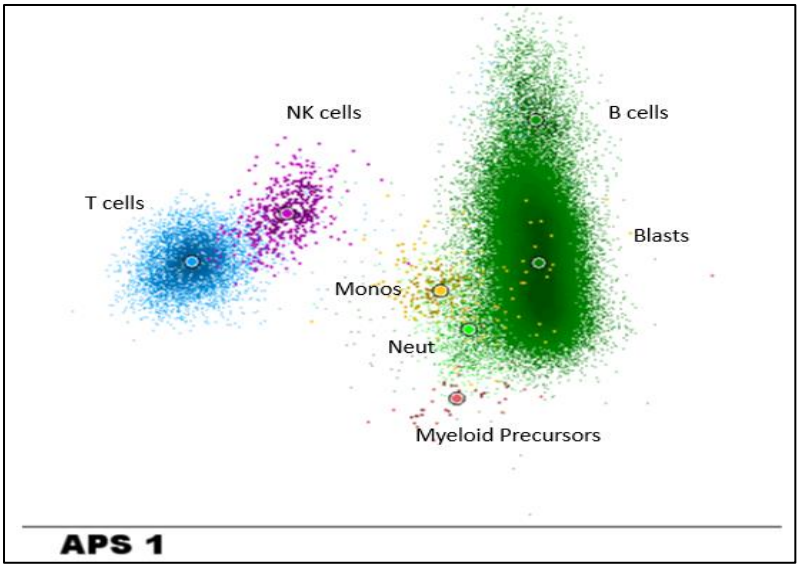


Figure 6.5: Case 3 blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

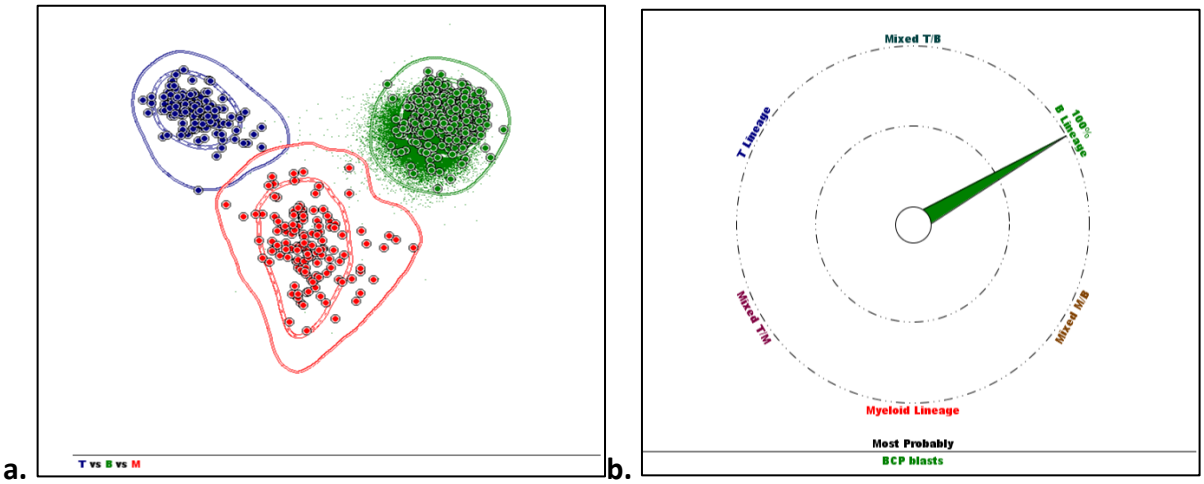


Figure 6.6. Case 3 blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

6. Case 4:

6.1. Clinical presentation:

This was a two and a half years old boy with known trisomy 21 and an atrioventricular septal heart defect (AVSD) which was repaired earlier. More recently, he had been treated for acute bronchitis. He presented with history of on and off fever, vomiting and abdominal distension for one 1 week. Clinical examination showed yellowish skin and sclera. Tonsillitis was also noticed. There was no fever, bruising, weight loss or history of night sweats. Mild hepatosplenomegaly was also noticed during examination.

6.2. Blood counts morphology and coagulation studies:

His blood count showed mild anaemia (haemoglobin 92 g/L) with severe thrombocytopenia (platelets count of 40 x 10^9/L). There was leucocytosis with a white blood cells count (WBCs) of 43.5 x 109/L. Blood film examination showed approximately 70% blast cells with mild neutrophilia (neutrophils count of 9.1 x 10^9/L). Coagulation screen was normal for age with prothrombin time (PT) of 11 seconds, activated partial prothrombin time (APTT) 24 seconds and fibrinogen level of 2.5 g/L. Bone marrow aspirate and biopsy were therefore indicated to confirm the new diagnosis of acute leukaemia.

6.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was aparticulate, however; with adequate quality for assessment. Differential count identified approximately 76% blast cells. These were mostly small to medium-sized cells with high nuclear cytoplasmic ratios, finely dispersed chromatin with inconspicuous

nucleoli and scanty agranular basophilic cytoplasm. Occasional cytoplasmic vacuolation was also noticed while no Auer rods were identified. There were rare megaloblastic erythroid precursors with many dysplastic features including multinucleation and nuclear budding. Myeloid precursors were markedly reduced with only few precursors identified. Megakaryocytic precursors were identified with few dysplastic features including small and hypolobated forms.

Bone Marrow biopsy was of sufficient length and adequate quality. This showed hypercellularity (virtually 100%) with normal hematopoiesis almost totally replaced by sheets of mononuclear cells, consistent with blast cells. Only few erythroid, myeloid and megakaryocytic precursors were identifiable.

CSF examination showed rare blast cells (< 5.0 cells/µl). This was considered as CNS-2 classification per the BFM 2017 protocol.

6.4. Flow cytometry:

Flow cytometric immunophenotyping performed on the peripheral blood sample identified a blast population (comprising approximately 68% of total cells) and expressing CD9, CD10, CD19, HLA-DR, strong CD38 in addition to cytoplasmic CD79a and TdT. There was no significant expression of CD45, CD34, CD2, CD3, CD4, CD7, CD11b, CD13, CD14, CD15, CD33, CD56, CD66c, CD64, CD117 and cytoplasmic MPO, cyCD3 or cytoplasmic IgM.

6.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization analysis (FISH) performed on the bone marrow aspirate showed four copies of chromosome 21 (RUNX1) consistent with the known diagnosis of Trisomy 21. Karyotype analysis showed 47 chromosomes with an additional chromosome 21 (47,XY, +21) consistent with the patient history. There was no evidence of any recurrent rearrangements of the BCR/ABL1, ETV6/RUNX1, MLL, IGH or CDKN2A, respectively.

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

6.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of B-cell acute lymphoblastic leukemia. Final WHO subtype classification was **B-Lymphoblastic leukaemia/lymphoma not otherwise specified** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

6.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on the diagnostic blood sample and confirmed the initial immunophenotypic findings with positive CD19 and cytoplasmic CD79a. There was no significant expression of CD34, CD45, surface CD3, CD7, cytoplasmic MPO or cytoplasmic CD3. The principal component analysis (PCA) and automated population separator (APS) diagram (Figure 3.10) showed blast cells distribution compared to

residual normal cell populations in the specimen. The automated database orientation tool using Infinicyt software from Cytognos indicated blast cells to be of B-cell lineage (figure.11).

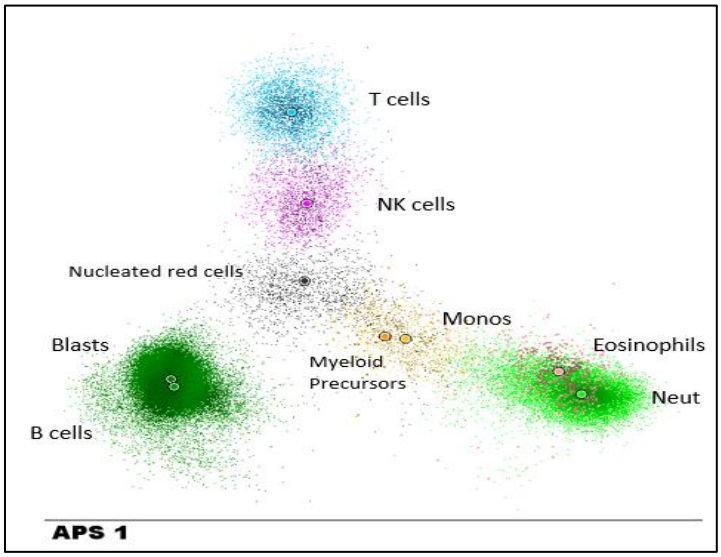


Figure 6.7: Case 4 blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

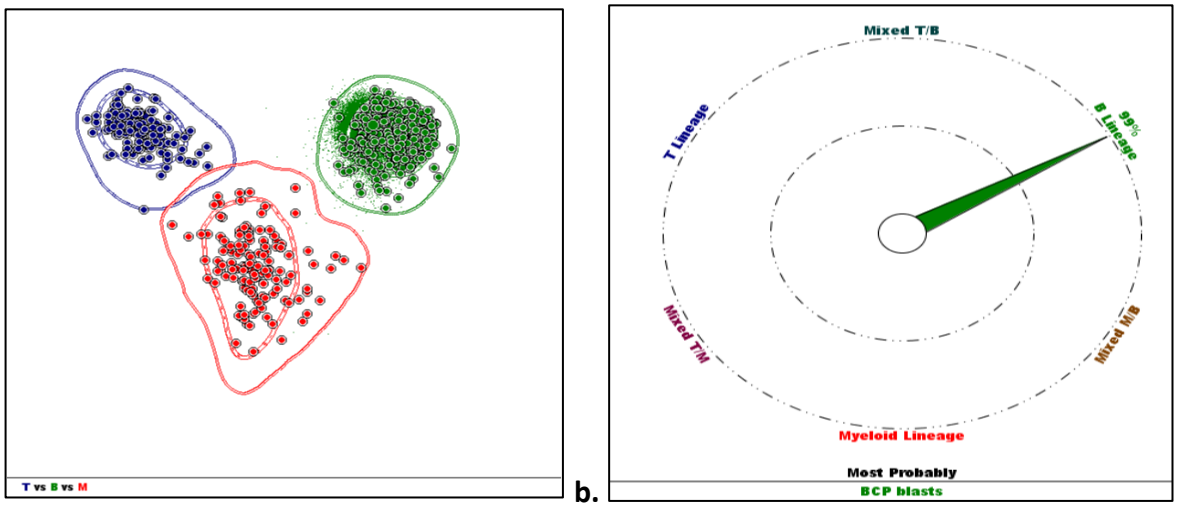


Figure 6.8: Case 4 blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

7. Case 5:

7.1. Clinical presentation:

This was a 6 years old girl with known history of acute myeloid leukemia with t(8;21). Her initial diagnostic flow cytometry workup was performed 9 months ago at a local sister hospital (detailed diagnostic report was obtained). Initial cytogenetic FISH and karyotype analysis confirmed the t(8;21) (RUNX1/RUNXT1) translocation at diagnosis. There was no evidence of FLT3 ITD or TKD mutations performed at diagnosis. She presented 9 months later with petechial skin rash similar to her diagnostic presentation. Her mother was very concerned that the leukaemia is back.

On examination, she looked well with clear throat, chest and abdomen. There was no organomegaly, fever, diarrhea, abdominal pain or bleeding symptoms apart from the petechial skin rash. Blood counts were requested for follow up.

7.2. Blood counts morphology and coagulation studies:

Her blood count showed normal haemoglobin level for age (haemoglobin 123 g/L) and borderline thrombocytopenia (platelets count of 142 x 109/L). There was no evidence of leucocytosis with a white blood cells count (WBCs) of 5.4 x 109/L. She had a normal neutrophil count of 3.2 x 109/L. Blood film examination showed macrocytosis with tear drop cells and occasional dysplastic (hypo granular) neutrophils. Occasional immature looking monocytes were present, however; no definite blast cells were identified. Bone marrow aspirate and biopsy were performed to exclude the presence of any early relapse.

7.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate with adequate quality for assessment. Differential count identified approximately 7% blast cells. These were variable in morphology with some small to medium-sized cells with high nuclear cytoplasmic (N/C) ratio, mildly irregular nuclear contours, fine chromatin and indistinct nucleoli. Other blast cells were larger with abundant cytoplasm showing limited granularity, fine chromatin, and variably prominent nucleoli.

Erythroid precursors showed mild megaloblastic changes while myeloid and megakaryocytic showed mild dysplasia (including hypo granularity and hypolobation). There was active trilineage haematopoiesis which was also confirmed by the bone marrow biopsy. Bone marrow cellularity was approximately 50% (low for age).

Her Bone marrow aspirate was received for flow cytometry for assessment of blast cells. Additional bone marrow samples were also referred for cytogenetic analysis (FISH and karyotype). No CSF was performed at this point.

7.4. Flow cytometry:

Flow cytometric immunophenotyping performed on the bone marrow aspirate identified an immature population comprising approximately 2% of total cells. These were expressing CD34, CD9, CD10, CD19, HLA-DR, strong CD38 in addition to cytoplasmic CD79a and TdT. This population showed no significant expression of CD45, CD2, CD3, CD4, CD7, CD11b, CD13, CD14, CD15, CD33, CD56, CD66c, CD64, CD117, and cytoplasmic MPO, cyCD3 or cytoplasmic IgM indicating likely normal CD34 positive hematogones (type I hematogones)..

In addition to this population, there was a second population of approximately 15% with CD9, CD10, CD19, HLA-DR, strong CD38, partial CD20 in addition to cytoplasmic CD79a and TdT. This population showed no significant expression of CD34, CD45, CD2, CD3, CD4, CD7, CD11b, CD13, CD14, CD15, CD33, CD56, CD66c, CD64, CD117, cytoplasmic MPO, cyCD3 or cytoplasmic IgM indicating a more mature B-cell precursor population (type II hematogones). Finally, a small population comprising approximately 1% of total cells was also identified. This showed positive CD34 expression with dim CD45, partial CD13, CD33, CD117 and HLADR representing early myeloid precursors.

7.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) and karyotype analysis performed on the bone marrow aspirate showed normal karyotype with 46 chromosomes (46,XX). There was no evidence of the diagnostic t(8;21) (RUNX1/RUNXT1) translocation.

Furthermore, there was no evidence of any recurrent rearrangements of the BCR/ABL1, ETV6/RUNX1, MLL, IGH or CDKN2A associated with B-acute lymphoblastic leukaemia. Additional EDTA samples were sent for DNA extraction and storage for any potential future testing; if needed.

7.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were consistent with increased hematogones with peripheral macrocytosis. There was no immunophenotypic evidence of leukemia or relapse.

7.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on the follow up bone marrow aspirate to determine the cell lineage of immature precursors. Gating on CD34 positive population showed a mixture of myeloid and B cell lineage (figure 12). However, subsequent gating of CD19 positive precursors (haematogones only) and CD19 negative, CD34 positive precursor cells (myeloid precursors only) showed clear distinction between B- and myeloid lineages using the Cytognos EuroFlow database and the compass tool (figures 3.13). The principal component analysis (PCA) and automated population separator (APS) diagram (figure 3.14) showed blast cells distribution compared to residual normal cell populations in the specimen.

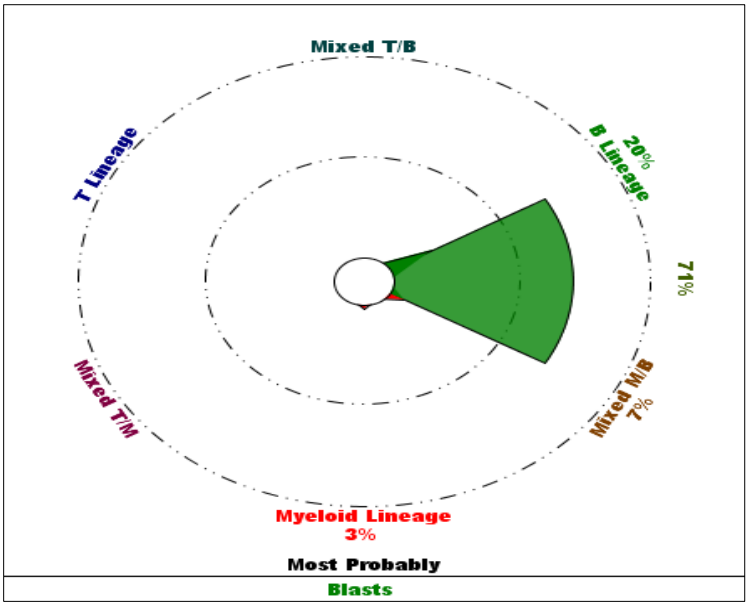
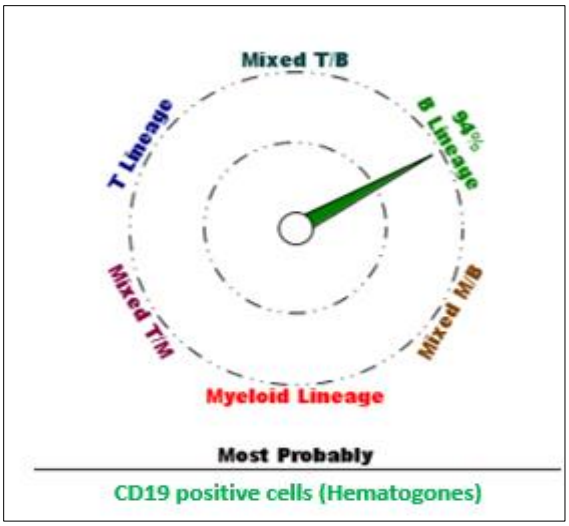


Figure 6.9: Case 5 CD34 positive precursor cells appearing to be of a mixed lineages (B and Myeloid/B).

c.



b.

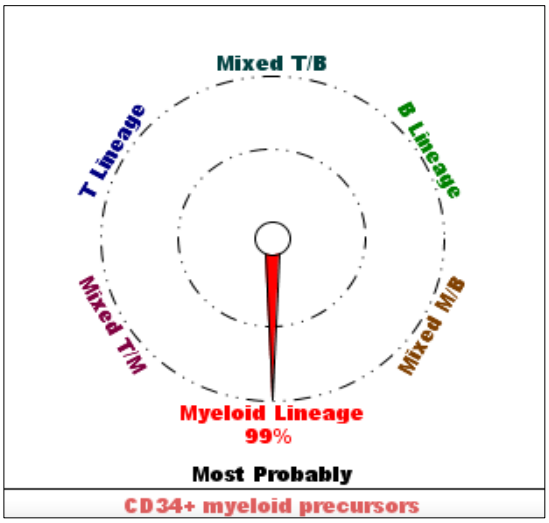


Figure 6.10: Case 5 database compass showing a. CD19 positive cells (hematogones) to be of a B-cell lineage and; b. CD34 positive and CD19 negative cells to be of myeloid origin.

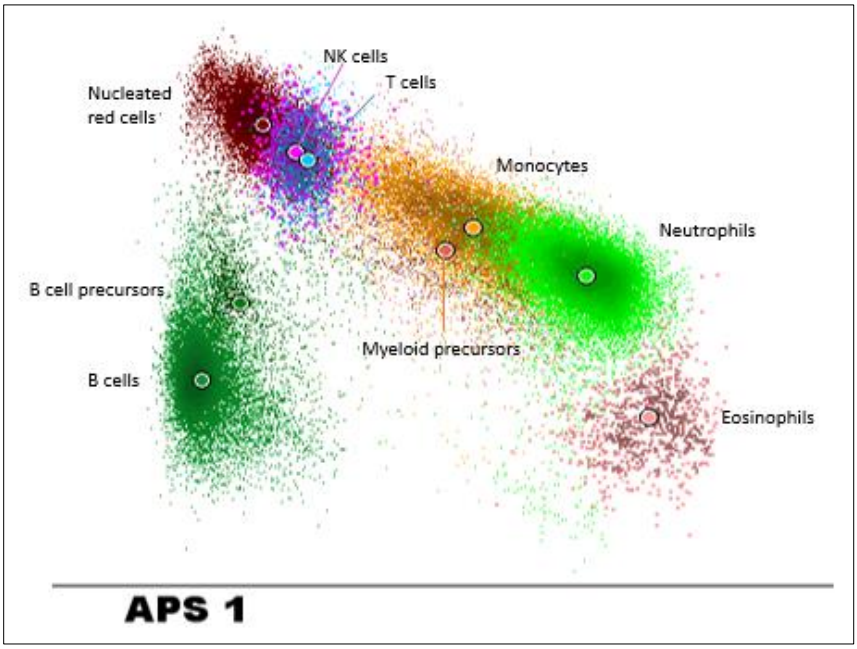


Figure 6.11: Case 5 precursor cells distribution compared to mature cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

8. Case 6:

8.1. Diagnostic clinical presentation:

This was a 13 months old boy referred from a primary healthcare center due to increased white blood cells count (WBC 231 x 109/L). The child had a history of fever and congested throat with upper respiratory symptoms for one week. Four days ago, the mother noticed that he had bruises over his right knee. The bruises rapidly increased in size and number over both thighs and knees. Earlier on presentation day, he also had blood in his stools. He also had a history of few days of poor appetite. No history of hematuria, weight loss or night sweats.

The mother stated he had recurrent viral infections with fever. He was admitted previously at 40 days of age due to sepsis and low WBC. On examination, he was sleeping and mother did not want to disturb him. He did not look pale, jaundiced or cyanosed. There were skin rashes over the face as well as bruises on both lower limbs. Emergency department confirmed leucocytosis and was transferred for haematology/oncology care.

8.2. Blood counts morphology and coagulation studies:

Full blood count performed in the laboratory confirmed leucocytosis with 77% blast cells on blood film. Blasts were highly suggestive of myeloid/monocytic lineage with very large blue grey cytoplasm, regular to convoluted and some bilobed nuclei, fine chromatin and prominent nucleoli. Cytoplasmic vacuolation was also noticed. No Auer rods were seen. Neutrophil count was normal for age (3.9 x 10^9/L) with marker lymphocytosis (31 x 10^9/L) confirmed by manual differential.

His haemoglobin was low for age (90 g/L). Severe thrombocytopenia (platelets count of 31 x 10^9/L) was also notice. Coagulation screen was also abnormal for age with prothrombin time (PT) of 19 seconds, activated partial prothrombin time (APTT) 35 seconds and fibrinogen level of 0.9 g/L. A baseline bone marrow aspirate and biopsy were therefore indicated to confirm the new diagnosis of acute leukaemia.

8.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate showed 82% blast cells (including promonocytes with lighter gray/blue cytoplasm, fine vacuolation and some fine granules). Other blasts were slightly smaller in size with more basophilic cytoplasm. No Auer rods seen on both populations. No megakaryocytes were seen with only rare erythroid precursors and virtually absent myeloid maturation. Bone marrow biopsy confirmed the findings with cellularity of almost 100%.

CSF was cellular with occasional blast cells seen (< 5 blast cells/µl). For a diagnostic sample, this qualified for a CNS-2 classification per the BFM 2017 protocol.

8.4. Flow cytometry:

Flow cytometry was performed on both, peripheral blood and bone marrow aspirate samples. Peripheral blood showed 78% blast cells with moderate CD45 expression, positive expression of CD9, CD33, CD4, CD64, and CD15 with partial expression of CD13, CD117, CD38 and aberrant expression of CD56. There was no significant expression of CD34, HLA-DR, CD2, CD3, CD7, CD10, CD11b, CD19, CD20, CD66c, cytoplasmic MPO, cyCD79a, cyCD3, cyIgM or TdT. Bone marrow flow cytometry conformed the findings on peripheral blood sample with 83% blast cells.

8.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate revealed an abnormal hybridization pattern with 11q23 probe indicating MLL rearrangement in 84% of the analysed cells. Karyotype/metaphase analysis confirmed the recurrent translocation involving chromosomes 11 and 19 at bands 11q23 and 19p13.3. In addition to the t(11;19), a derivative chromosome 2 as a result of translocation between long arms of chromosomes 1 and 2 at cytogenetic bands 1q32 and 2q37 was also detected. Final karyotype was 46,XY, der(2)t(1;2)(q32;q37),t(11;19)(q23;p13.3).

DNA samples were sent to reference laboratory for molecular analysis. Results showed no evidence of FLT3-ITD or exon 12 insertion/duplication mutation within NPM1.

8.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **acute myeloid leukaemia, not otherwise specified (acute monoblastic leukaemia)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues. The specific MLL rearrangement t(11;19)(q23;p13.3) had been reported to have an intermediate to poor prognosis. The high WBC count at diagnosis and the presence of an additional cytogenetic abnormality were additional poor prognostic features.

8.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation confirmed the initial immunophenotypic findings with only moderate CD45 expression. There was no significant expression of CD3, CD7, CD19, CD34 or cytoplasmic MPO, cyCD3 or CD79a. Blast cell distribution compared to residual normal cell populations in the sample was shown using the principal component analysis (PCA) and automated population separator (APS) diagram (figures 3.15). The automated database orientation tool using Infinicyt software from Cytognos indicated blast cells to be of a myeloid lineage (figure 3.16).

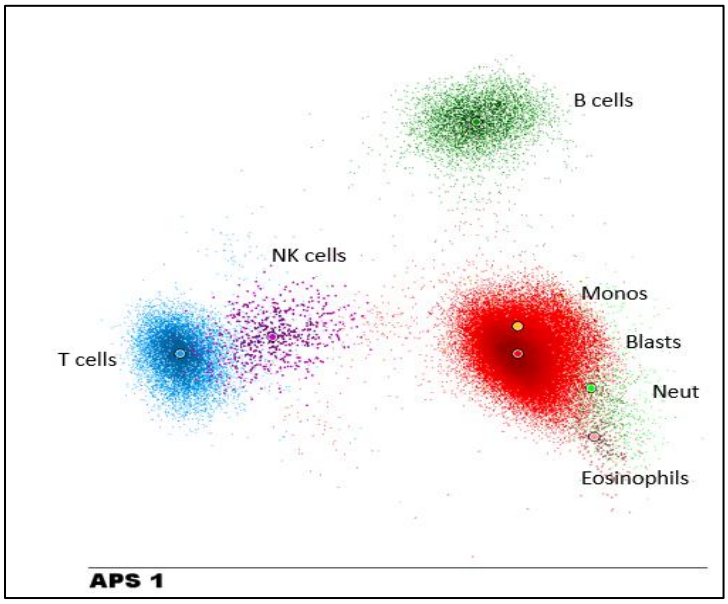


Figure 6.12. Case 6 peripheral blood blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

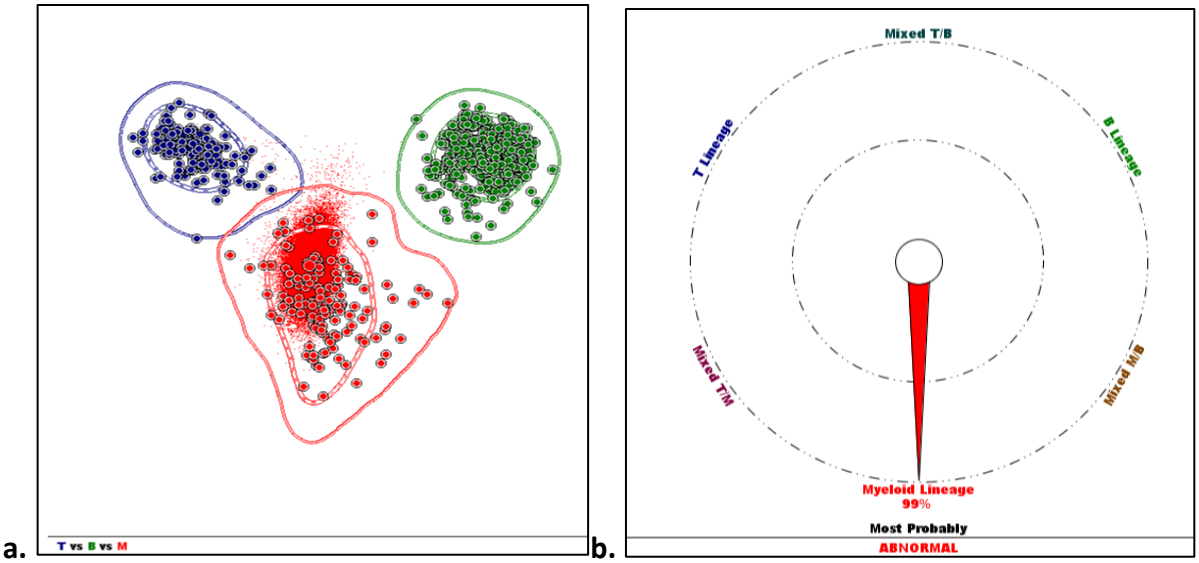


Figure 6.13. Case 6 peripheral blood blast cells orientation using automated database a. orientation among myeloid cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

8.8. Follow up clinical presentation:

Three month later the child was admitted for elective chemotherapy (post intensification I). He was afebrile, well hydrated with no respiratory or abdominal abnormalities during examination. However, he had small lesions in the face, hands and buttocks which were not vesicles and some were hard to touch. There was no hotness or tenderness.

8.9. Follow up blood counts, morphology and coagulation studies:

His full blood count showed normal white blood cell count (WBC 6.4 x10^9/L) with normal haemoglobin for age (106 g/L) and normal platelets count (294 x 10^9/L). However, his blood smear showed left shifted, leucoerythroblastic picture with rare blasts with morphology consistent with leukemic population. These blasts were medium to large in size with abundant

grey/blue cytoplasm, few fine granules and occasional vacuoles. The nuclei were irregular with fine chromatin and prominent nucleoli. Bone marrow aspirate and biopsy were therefore indicated to confirm the presence of potential early relapse.

8.10. Follow up bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate with excellent quality and cell release. An approximately 24% blast cell population was identified. These resembled the diagnostic morphology with large size, abundant cytoplasm, some having vacuoles and granules. No Auer rods were identified. Many nuclei were irregular with fine chromatin and prominent nucleoli. Some of the cells counted as blasts (approximately 5% of total cells) were slightly more mature and would be consistent with promonocytes. Complete maturation was noticed in myeloid precursors as well as erythroid cells despite being reduced.

Bone marrow biopsy confirmed the aspirate findings with cellularity approaching 100% with trilineage haematopoiesis almost replaced by a predominance of immature looking mononuclear cells. A skin punch biopsy taken from the left forearm showed an extensive infiltrate uniform cells with often bean-shaped nuclei and a small amount of cytoplasm having a monocytoid appearance. The infiltrate involved all through the dermis and extend into the subcutaneous fat which was also infiltrated.

The appearances were of a malignant hematopoietic infiltrate consistent with relapsed AML. CSF was cellular with occasional blast cells.

8.11. Follow up flow cytometry:

Flow cytometry on this bone marrow aspirate identified approximately 20% blast cells. These showed similar phenotypic expression to the diagnostic sample with positive CD33, CD64, CD4 and partial expression of CD117 and CD56. There was no significant expression of CD34, CD3, CD19, HLADR, CD2 or CD14. No cytogenetic or molecular analysis were performed at relapse.

8.12. Follow up final diagnosis and integrated report:

The overall morphologic, immunophenotypic findings were consistent with **relapsed acute myeloid leukaemia, not otherwise specified (acute monoblastic leukaemia)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues. The case was discussed in the weekly MDT meeting and was referred for bone marrow transplant. A search for a compatible donor was initiated and a salvage chemotherapy was planned (FLAG-Ida).

8.13. Follow up acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation confirmed the relapse phenotype findings with only moderate CD45 expression. There was no significant expression of CD3, CD7, CD19, CD34 or cytoplasmic MPO, cyCD3 or CD79a. Blast cell distribution compared to residual normal cell populations in the sample was shown using the principal component analysis (PCA) and automated population separator (APS) diagram (figures 3.17). The automated database

orientation tool using Infinicyt software from Cytognos confirmed blast cells to be of myeloid lineage (figure 3.18).

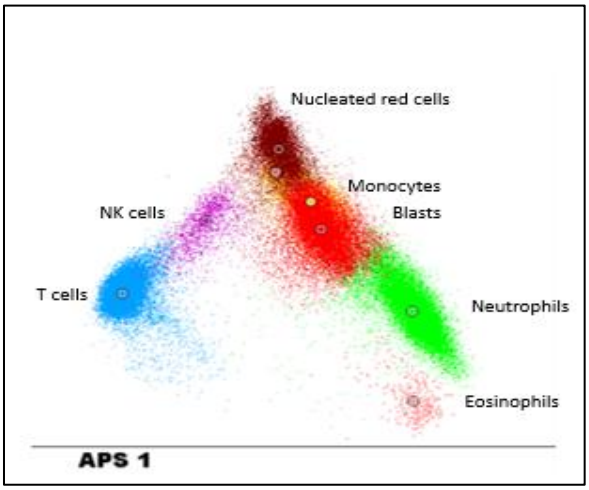


Figure 6.14. Case 6 bone marrow blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

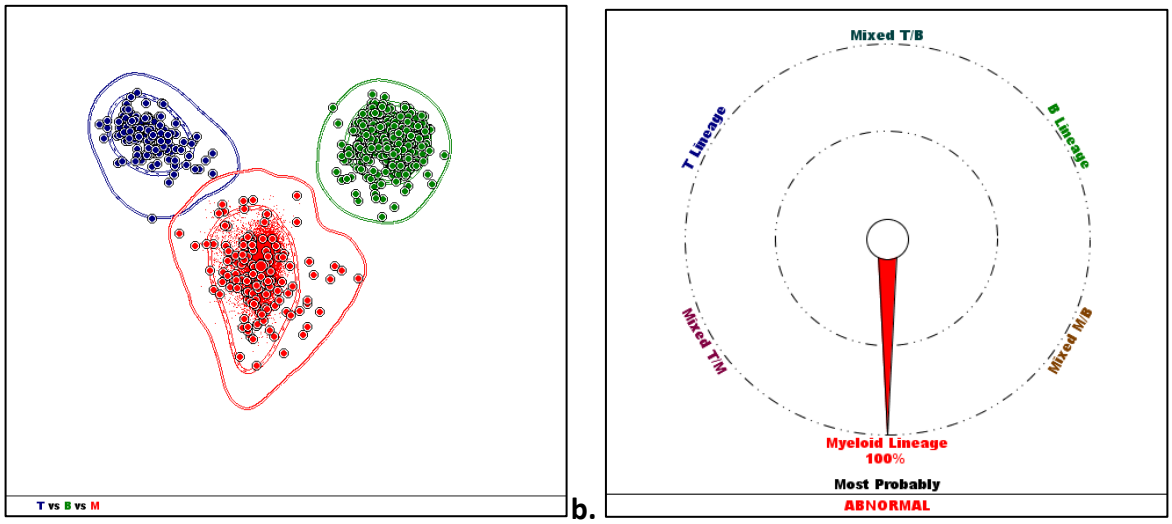


Figure 6.15. Case 6 bone marrow blast cells orientation using automated database a. orientation among myeloid cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

9. Case 7:

9.1. Clinical presentation:

This was an 11 months girl presented with a history of approximately 1 months fever. She had been treated for an upper respiratory tract infection however the fever did not resolve. There was no cough, runny nose, vomiting, bleeding or diarrhea. At examination, she was well hydrated with clear ears, nose and throat. There was no lymphadenopathy, jaundice, bruise or skin rash. Hepatosplenomegaly was noticed.

9.2. Blood counts morphology and coagulation studies:

Blood count results confirmed the anaemia with a haemoglobin of 55 g/L only. Severe thrombocytopenia was also noticed with a platelets-count of 14 x 10^9/L. White blood cells count was moderately elevated (24.6 x 10^9/L) with a normal neutrophil count of 2.2 x 10^9/L. Blood film examination revealed an abnormal population of blast cells (approximately 35%). These were mostly large in size with abundant basophilic cytoplasm with a ground-glass granularity. No Auer rods were seen. Some blast cells showed cytoplasmic blebbing. Nuclear margins were mildly irregular with fine chromatin and frequent multiple nucleoli.

Coagulation screen was normal for age with prothrombin time (PT) of 13 seconds, activated partial prothrombin time (APTT) 32 seconds and fibrinogen of 3.2 g/L. Bone marrow aspirate and biopsy were therefore indicated to confirm the presence of new acute leukaemia.

9.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate with adequate quality for assessment. This showed approximately 28% blast cells population with similar morphology to peripheral blood blast cells although cytoplasmic blebbing was more obvious in the bone marrow.

Erythroid precursors showed unremarkable morphology. Progressive maturation of myeloid precursors was seen as well as small mature looking lymphocytes. Megakaryocytes were not seen. Bone marrow biopsy confirmed the aspirate findings with cellularity of approximately 100%. Hematopoiesis was sub-totally replaced by patchy and interstitial sheets of mononuclear cells representing the blasts population. CSF examination showed no definite blast cells. This was considered as CNS-1 classification per the BFM 2017 protocol.

9.4. Flow cytometry:

Flow cytometric immunophenotyping performed on the peripheral blood sample identified 29% blast cell population. These showed dim CD45 expression with positive expression of CD34, CD4, CD7, CD9, CD33, CD41, CD42, CD61, CD71 partial expression of HLADR and CD38. There was no significant expression of CD2, CD3, CD10, CD19, CD13, CD11b, CD20, CD14, CD15, CD56, CD66c, CD117 or cytoplasmic MPO, cyCD3, cyCD79a, cyIgM or TdT. Flow cytometry was also performed on the bone marrow aspirate and confirmed the phenotype on peripheral blood sample.

9.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization analysis (FISH) performed on the bone marrow aspirate revealed normal hybridization pattern for all AML FISH probes including PML/RARA, RUNX1/RUNXT1, CBFB, MLL as well as BCR/ABL1. Karyotype analysis of metaphases showed normal (46,XX) chromosomes.

9.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of acute myeloid leukemia. Final WHO subtype classification was **acute myeloid leukaemia, not otherwise specified AML-NOS (Acute Megakaryoblastic Leukaemia)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

9.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation performed on both peripheral blood and bone marrow samples confirmed the initial immunophenotypic findings with dim CD45 expression, positive CD34 and CD7. There was no significant expression of surface CD3, CD19, cytoplasmic MPO, cyCD3 or cyCD79a. The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in both specimens (figure 3.19 and 3.21). Finally, the automated

database orientation tool using Infinicyt software from Cytognos confirmed blast cells to be of myeloid lineage (figure 3.20 and 3.22).

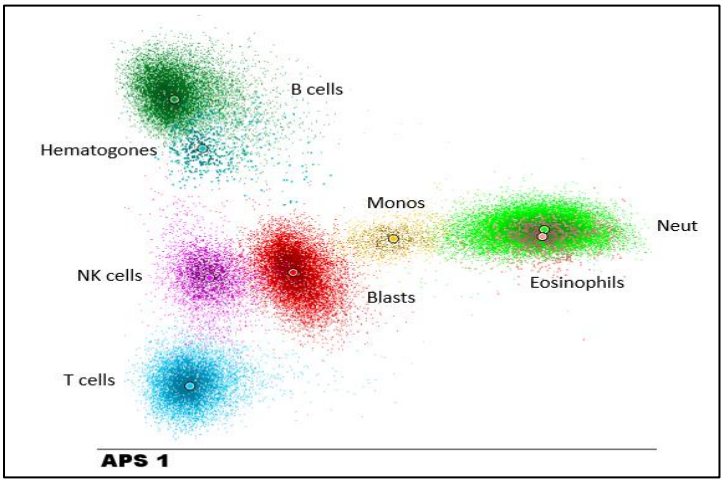


Figure 6.16. Case 7 peripheral blood blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

a. b.

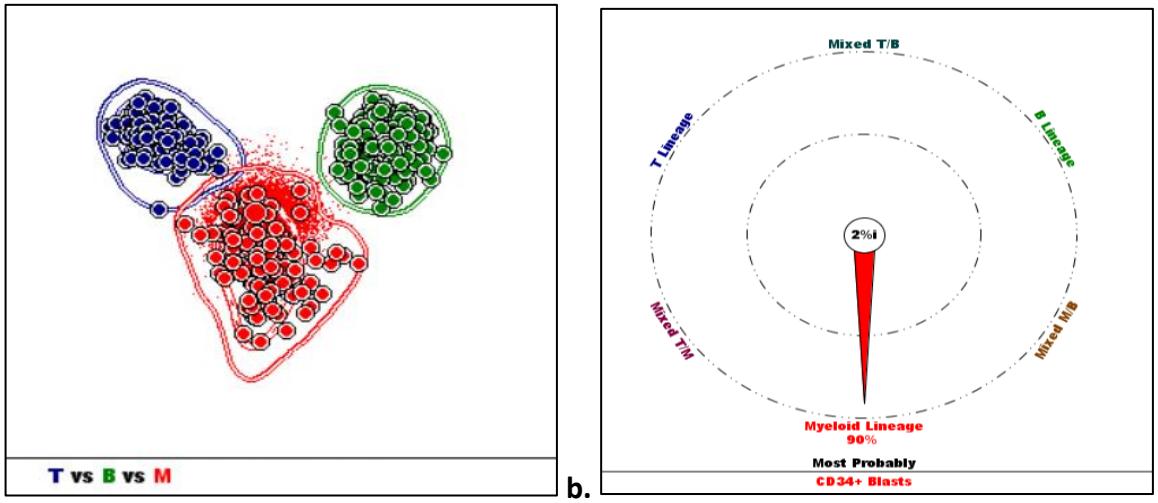


Figure 6.17. Case 7 peripheral blood blast cells orientation using automated database a. orientation among myeloid cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

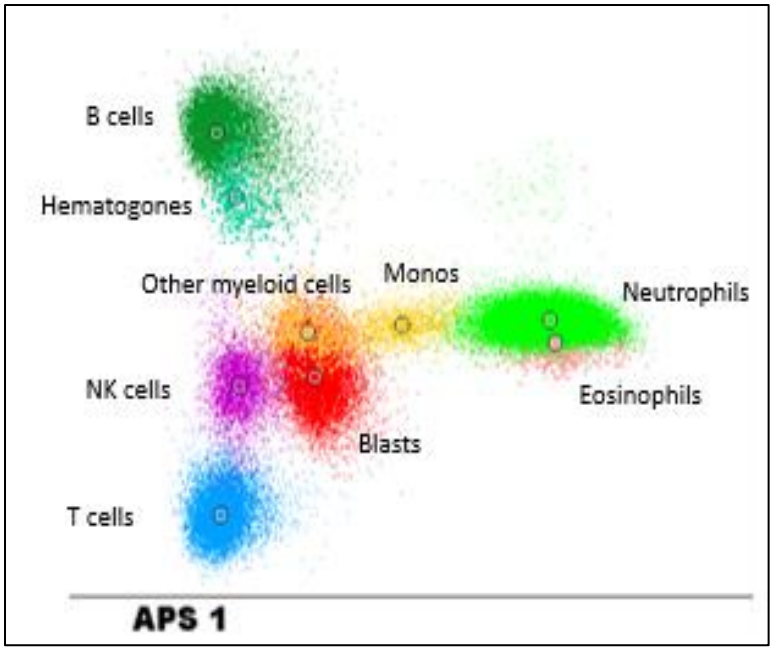


Figure 6.18. Case 7 bone marrow blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

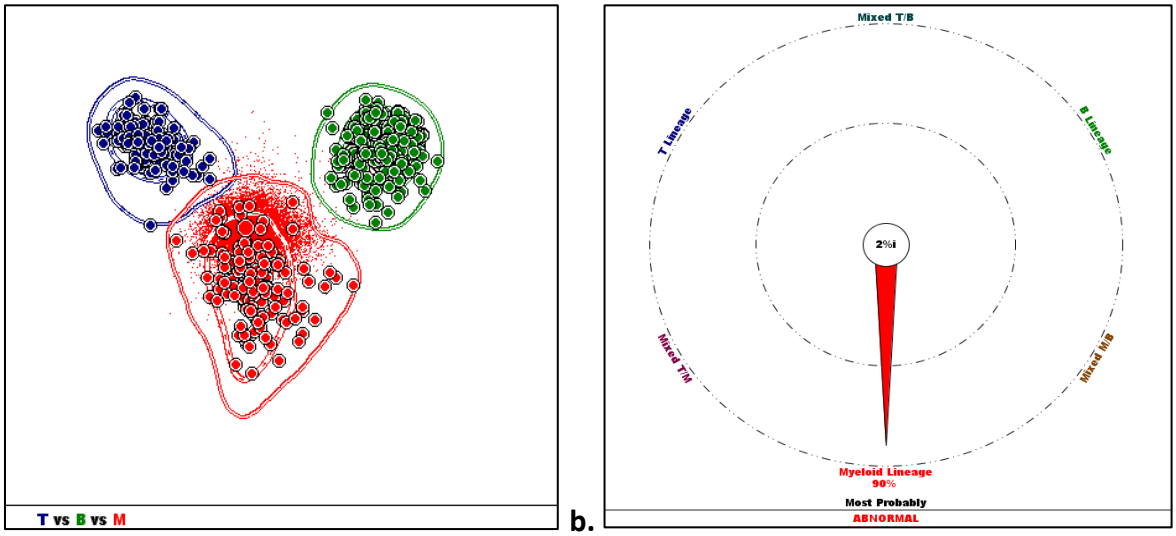


Figure 6.19. Case 7 bone marrow blast cells orientation using automated database a. orientation among myeloid cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

10. Case 8:

10.1. Clinical presentation:

This was a 14 years old male with 2 months history of lymphadenopathy. Three days ago he complained of headache, fever and bone aches. Bilateral neck swelling was worsening. There was no cough, vomiting, diarrhea or bleeding history. On examination bilateral scattered cervical and submandibular lymph nodes were enlarged as well as bilateral inguinal lymph nodes. Tonsils were also enlarged with no other organomegaly, abdominal or chest symptoms. Subsequent chest x-ray showed wide mediastinum mass.

10.2. Blood counts morphology and coagulation studies:

Blood counts showed marked leucocytosis with a white blood cells count of 360 x 10^9/L and mild neutrophilia (neutrophils 10.8 x 10^9/L). His platelets count was also reduced (64 x 10^9/L) while the haemoglobin result was normal for age (135 g/L). Blood smear examination revealed > 90% blast cells. These were small to medium in size with variable amount of basophilic agranular cytoplasm. No Auer rods were identified. Nuclear contours were irregular with fine chromatin and inconspicuous nucleoli.

Coagulation studies were abnormal with PT of 24 seconds and a low fibrinogen level of 1.2 g/L. APTT was normal with 30 seconds clotting time. Findings were consistent with a new acute leukaemia. No bone marrow aspirate was performed at this point due to high risk of tumor lysis syndrome.

10.3. Flow cytometry:

Flow cytometry of the peripheral blood sample identified 87% blast cells population with moderate CD45 expression. These cells showed positive expression of CD1a, CD2, CD4, CD7, CD8, CD38, CD99 and cytoplasmic CD3 with dim expression of CD9, CD10 and nuclear TdT. There was no significant expression of CD34, CD3, CD11b, CD13, CD14, CD15, CD19, CD20, CD33, CD56, CD64, CD66c, CD117, HLADR or cytoplasmic IgM, CD79a or MPO.

10.4. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the peripheral blood sample revealed an abnormal hybridization pattern with TRA/D probe indicating T- cell receptor gene rearrangement in 81% of cells analysed. Karyotype analysis confirmed the FISH findings with translocation t(11;14) (46,XY, t(11;14)(p11.2;q11.2).

10.5. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of T-cell acute lymphoblastic leukemia. Final WHO subtype classification was **T-Lymphoblastic leukaemia/lymphoma** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

10.6. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation performed on the peripheral blood sample confirmed the initial immunophenotypic findings with moderate CD45 expression, positive CD7 and cytoplasmic CD3. There was no significant expression of surface CD3, CD19, CD34, cytoplasmic MPO or cyCD79a. The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood specimen (figure 3.23). Finally, the automated database orientation tool using Infinicyt software from Cytognos indicated blast cells to be of T-cell lineage (figure 3.24).

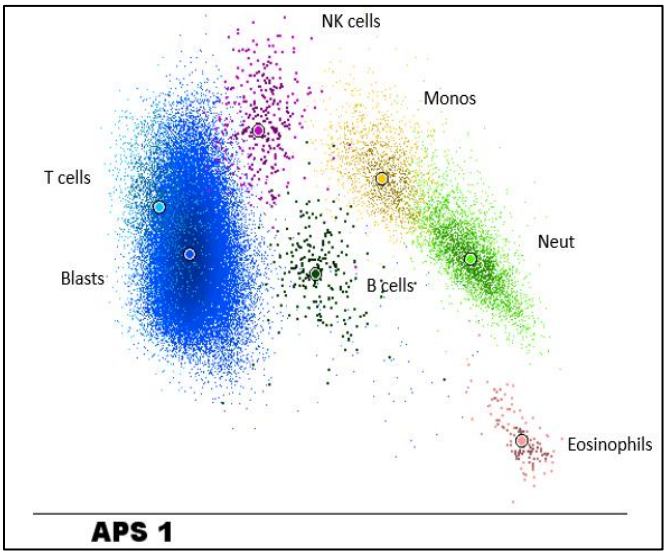


Figure 6.20. Case 8 blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

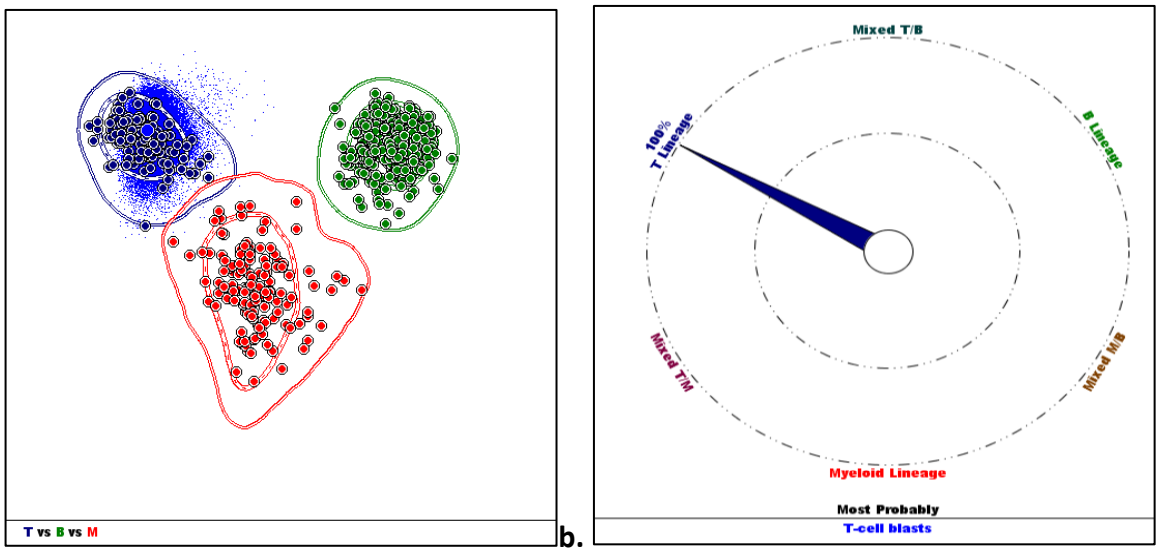


Figure 6.21. Case 8 blast cells orientation using automated database a. orientation among T-cells (in blue) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of T-cell lineage.

10.7. Follow up Clinical presentation:

The patient presented later on (post induction II) with abdominal pain and hepatomegaly. There were no additional, GI, chest, or CNS symptoms. He was managed as veno-occlusive disease (VOD) and the pain was improving.

10.8. Blood counts morphology and coagulation studies:

His blood counts confirmed the anaemia with haemoglobin of 61 g/L. White blood cells count was reduced (2.0 x 10^9/L) with severe neutropenia (0.14 x 10^9/L). Platelets count was normal with 230 x 10^9/L. Coagulation screen was normal with PT of 11 seconds, APTT of 35 seconds and fibrinogen level of 4.8g/L. However, blood smear examination was worrisome with 15% blast cells. These were small to medium in size with variable amount of basophilic agranular

cytoplasm. No Auer rods were identified. Nuclear contours were irregular with fine chromatin and inconspicuous nucleoli. The sample was therefore referred for flow cytometry assessment of the blast cell population.

10.9. Flow cytometry:

Flow cytometry analysis of the peripheral blood sample confirmed the presence of approximately 18% myeloid blast cells. These showed moderate CD45 expression with positive expression of CD34, CD13, CD33, HLADR, CD117, and CD123 with partial expression of CD9, CD38 and minimal expression of 11b and MPO. There was no significant expression of CD1a, CD2, CD3, CD4, CD7, CD10, CD19, CD20, CD14, CD56, CD66c, CD99, cytoplasmic CD3, cyCD79a, cyIgM or TdT. This phenotype did not resemble the diagnostic phenotype of T-acute lymphoblastic leukaemia. No cytogenetic or molecular analysis were performed at this point of follow up.

10.10. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation performed on the peripheral blood sample confirmed the immunophenotypic findings with moderate CD45 and positive CD34 expression. There was no significant expression of surface CD3, CD7, CD19, cytoplasmic MPO, cyCD79a or cyCD3 on this population. The principal component analysis (PCA) and automated population separator (APS) diagram were subsequently used to compare blast cells distribution to residual normal cell populations in the blood specimen (figure 3.25). Finally, the automated database

orientation tool using Infinicyt software from Cytognos indicated blast cells to be of myeloid-cell lineage (figure 3.26).

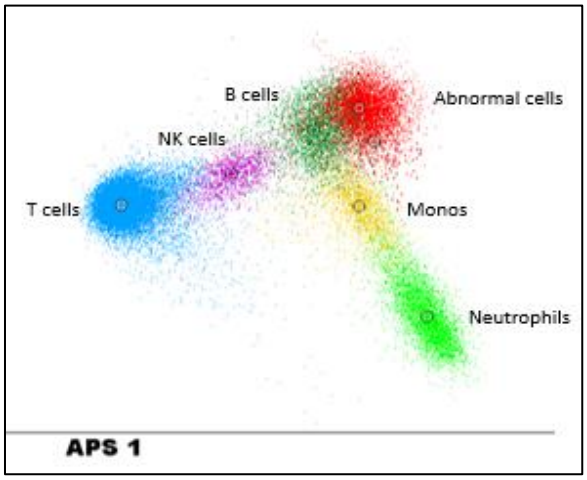


Figure 6.22. Case 8 follow up blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

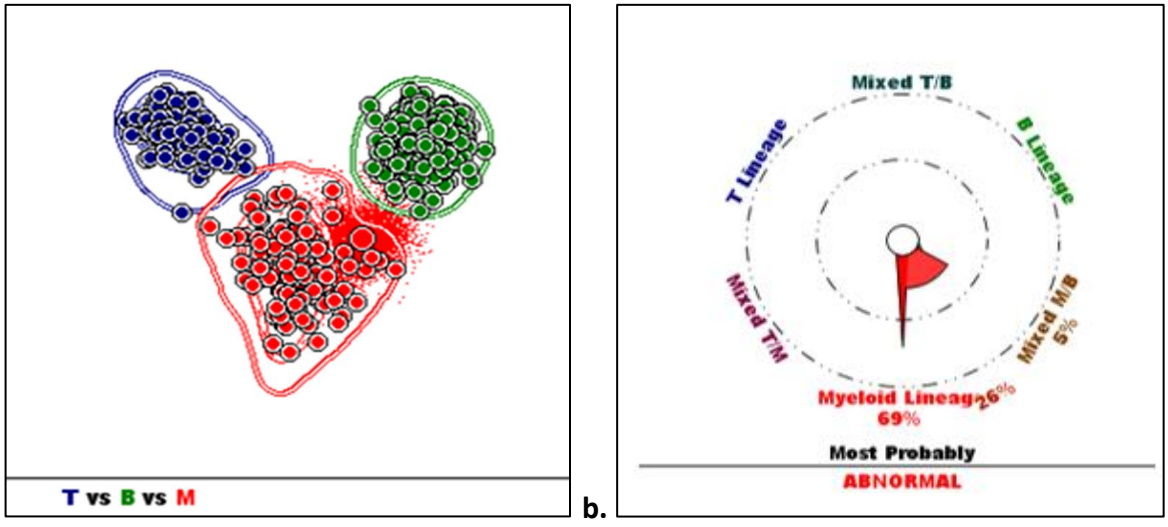


Figure 6.23. Case 8 follow up blast cells orientation using automated database a. orientation among myeloid-cells (in red) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of myeloid lineage.

10.11. Final diagnosis and integrated report:

Final clinical interpretation was recovering bone marrow with circulating myeloid precursors. The patient was closely followed with subsequent blood counts. Blast cells gradually matured and disappeared from the blood circulation.

11. Case 9:

11.1. Clinical presentation:

This was a 6 years old boy presented to accident and emergency with history of fever for 10 days. He had gum swelling (erythema) for the last 5 days and was on antibiotics treatment. His temperature was improving and there was no cough or runny nose. On examination he looked pale. Gingivitis was confirmed in the right lower molar area with no abscess. There was no lymphadenopathy or organomegaly.

11.2. Blood counts morphology and coagulation studies:

His full blood count confirmed the anaemia with haemoglobin level of 71 g/L. He was also leucopenic with a white cell count of 1.8 x 109/L. There was severe neutropenia with no detectable neutrophils using the auto-analyser. Platelets count was normal (261 x 10^9/L) as well as his coagulation studies (PT= 15 seconds, APTT= 31 seconds and fibrinogen of 4.0g/L).

Blood film examination confirmed the severe neutropenia and showed approximately 10% blast cells. There were predominantly small-sized with high nuclear to cytoplasmic ratios, variably condensed chromatin, indistinct nucleoli and scant amounts of agranular cytoplasm. No Auer rods were seen. Bone marrow diagnosis was therefore indicated to confirm the presence of a haematological neoplasia.

11.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was of adequate quality and showed 98% blast cells. These composed of two populations with frequent overlapping features. The first (major) population was composed of intermediate to large-sized blasts with irregular (and occasionally convoluted) nuclear contours, finely dispersed chromatin, mostly inconspicuous nucleoli and minimal to moderate amounts of agranular, basophilic cytoplasm. The second population consisted of small-sized blast cells with high nuclear to cytoplasmic ratio, round to slightly irregular nuclear contours, variably condensed chromatin, indistinct nucleoli and scanty amounts of cytoplasm. Other myeloid, erythroid and megakaryocytic precursors were almost absent.

Bone marrow biopsy confirmed the aspirate findings with almost 100% cellularity and sheets of the abnormal mononuclear cells. No blast cells were identified in the CSF and was therefore considered as CNS-1 per BFM 2017 protocol.

11.4. Flow cytometry:

Flow cytometry analysis was initially performed on the peripheral blood sample as the bone marrow aspirate was delayed to the following day. This confirmed the presence of circulating blast cells (approximately 6%). Blast cells showed dim to moderate CD45 expression with positive CD34, CD19, HLADR, and cytoplasmic CD79a with partial expression of CD9 and CD56 as well as dim expression of CD10 and TdT. There was no significant expression of CD2, CD3, CD4, CD11b, CD13, CD14, CD15, CD20, CD33, CD64, CD117 or cytoplasmic IgM or MPO.

Subsequent analysis of the bone marrow aspirate on the following day confirmed the peripheral blood findings with 90% blast cells.

11.5. Cytogenetic and molecular analysis:

Interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate showed 3 abnormal signals of RUNX1 on chromosome 21 in 48% of cells examined suggesting extra copies of chromosome 21 or 21q rearrangement. Subsequent karyotype analysis showed trisomy 21 in 21 out of 41 metaphases examined. An additional derivative chromosome 13 and 21 along with additional material of unknown origin on the long arm of chromosome 5 were also identified (46,XY,Add(5)(q35),der(13;21)(q10;q10),+21(21)/46,XY(20).

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

11.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia (B-ALL), not otherwise specified (NOS)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

11.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation performed on the peripheral blood and bone marrow samples confirmed the initial immunophenotypic findings with moderate CD45 expression, positive CD34, CD19 and cytoplasmic CD79a. There was no significant expression of CD7, surface or cytoplasmic CD3 or cytoplasmic MPO.

The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood and bone marrow specimens. Finally, the automated database orientation tool using Infinicyt software from Cytognos confirmed blast cells to be of B-cell lineage (figure 3.27 to 3.30).

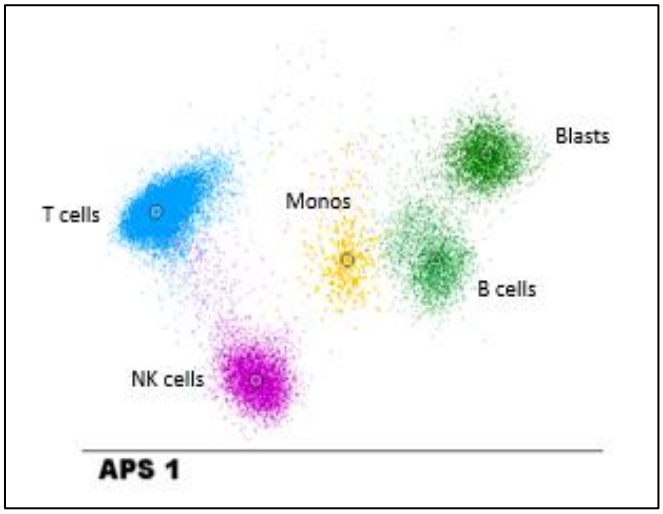


Figure 6.24. Case 9 peripheral blood blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

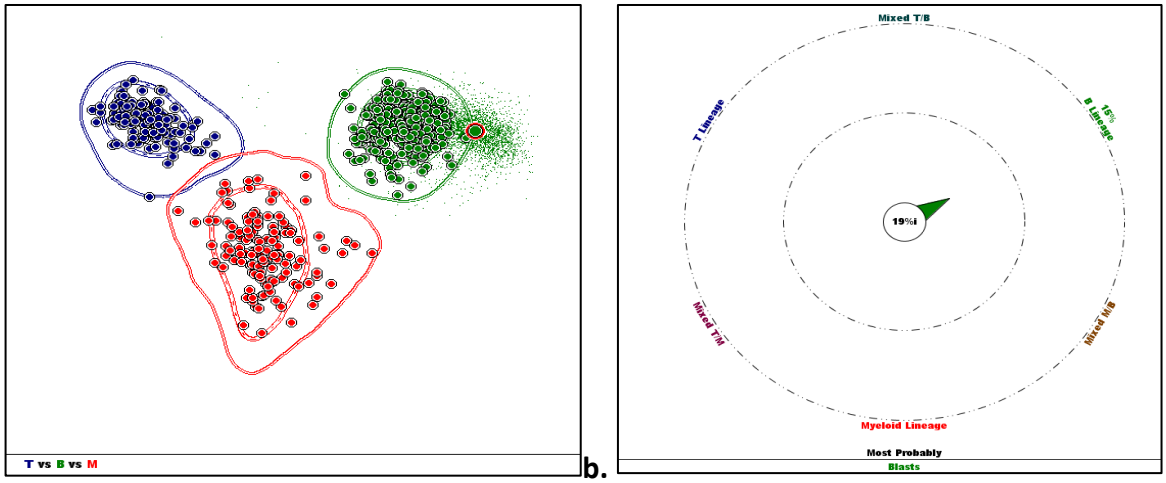


Figure 6.25. Case 9 peripheral blood blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

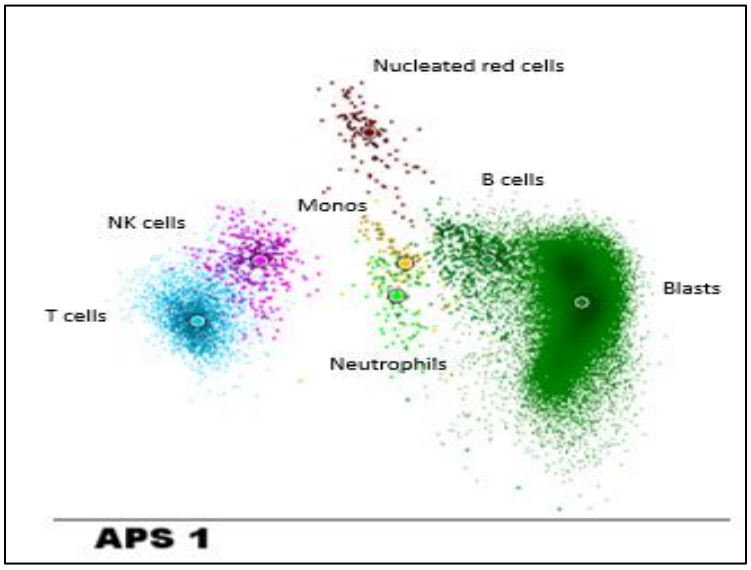


Figure 6.26. Case 9 bone marrow blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

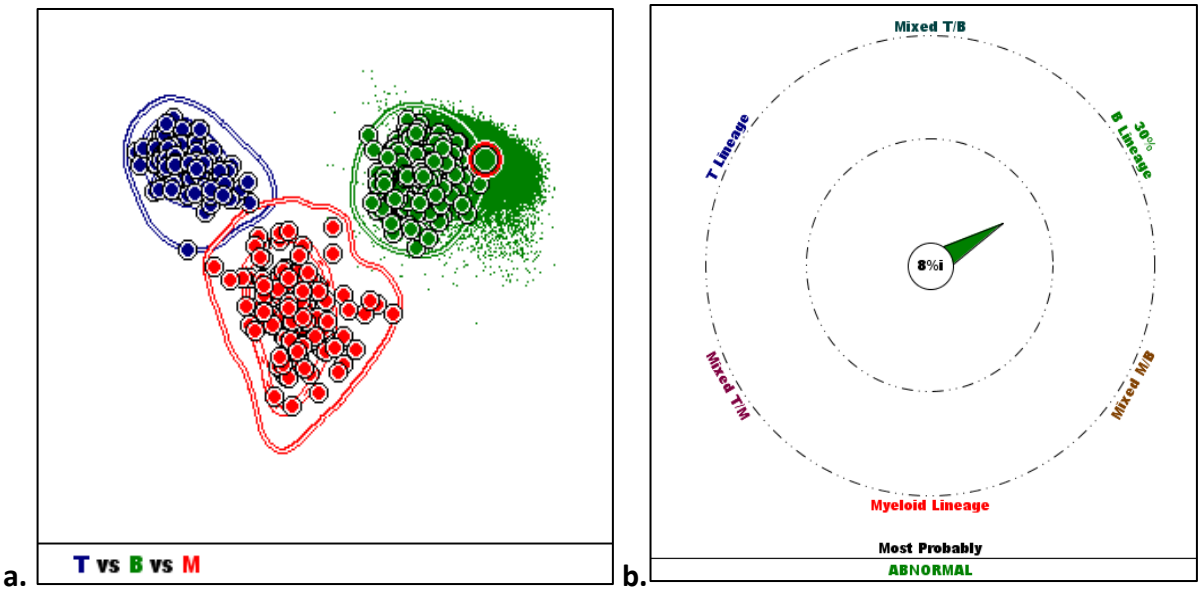


Figure 6.27. Case 9 bone marrow blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

12. Case 10:

12.1. Clinical presentation:

This was a 13 years old male previously healthy and athletic. He presented to accident and emergency with left side epistaxis for almost 4 hours. He also complained from body aches. There was no history fever, night sweats or lymphadenopathy. He experienced back pain, fatigue and loss of appetite lately. On examination, he looked slightly pale with palpable liver. No other abdominal, respiratory or CNS symptoms were detected.

12.2. Blood counts morphology and coagulation studies:

His blood count showed severe thrombocytopenia with a platelets count of 24 x 10^9/L. White blood cells count was within normal limits (4.8 x 10^9/L with mild neutropenia (1.15 x 10^9/L). Blood count also showed borderline anaemia with haemoglobin level of 122 g/L. All coagulation studies were normal with PT of 12 seconds, APTT 28 seconds and a fibrinogen of 3.4g/L. Examination of his blood smear showed an abnormal population of blast cells representing 11% of the total cells. These were predominantly small to medium in size with high nuclear to cytoplasmic ratio, round to slightly irregular nuclear contour with finely dispersed chromatin. Cytoplasm was scanty, basophilic with no granules or Auer rods.

12.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was paucispicular with adequate quality for assessment. This showed an approximately 86% blast cell population with similar morphological features to the peripheral blood. Other myeloid, erythroid and megakaryocytic precursors were rare with unremarkable

morphology apart from mild dyserythropoiesis. Few small mature-looking lymphocytes were also seen.

Bone marrow biopsy confirmed the aspirate findings with 95-100% cellularity and sheets of abnormal mononuclear cells representing blasts. CSF sample was contaminated with blood (traumatic sample) and showed rare blast cells (<5 cells/µl). This was considered as CNS-2 according to the BFM-2017 protocol.

12.4. Flow cytometry:

Flow cytometry analysis of the bone marrow aspirate sample showed 50% blasts cells (hemodiluted sample) with positive expression of CD9, CD10, CD19, and cytoplasmic CD79a, nuclear TdT with partial expression of HLADR, CD38 and minimal expression of CD33. There was no significant expression of CD45, CD2, CD3, CD4, Cd11b, CD13, CD14, CD15, CD20, CD34, CD64, CD66c, CD123 or cytoplasmic MPO and cyIgM.

12.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate did not identify any recurrent rearrangements of the BCR/ABL1, ETV6/RUNX1, MLL, IGH or CDKN2A probes. Karyotype analysis showed normal (46,XY) chromosomes in the 30 metaphase cells examined.

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

12.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia (B-ALL), not otherwise specified (NOS)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

12.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation confirmed the initial immunophenotypic findings on both peripheral and bone marrow samples. These showed positive expression of CD19 and cytoplasmic CD79a. There was no significant expression of CD45, surface CD3, CD7, CD34, cytoplasmic MPO or cyCD3.

The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood and bone marrow specimens. Finally, the automated database orientation tool using Infinicyt software confirmed blast cells to be of B-cell lineage (figure 3.31 to 3.34).

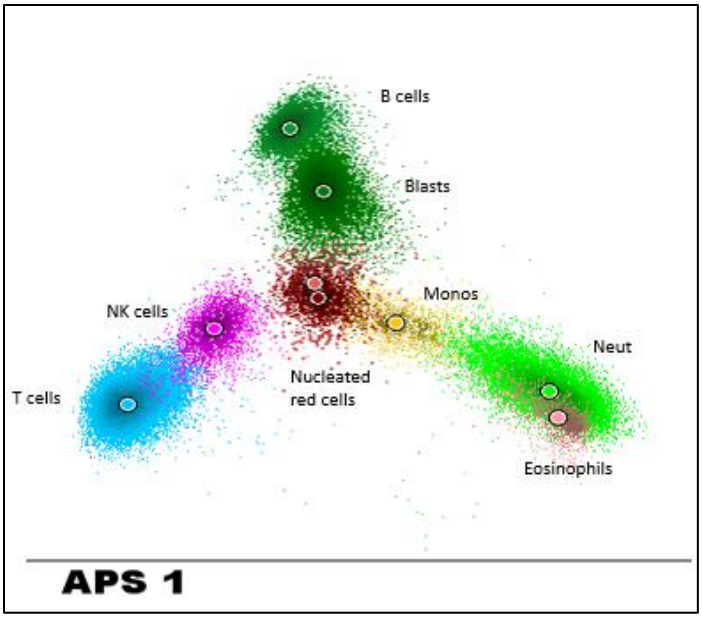


Figure 6.28. Case 10 blast cells distribution compared to residual normal cells in the blood specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

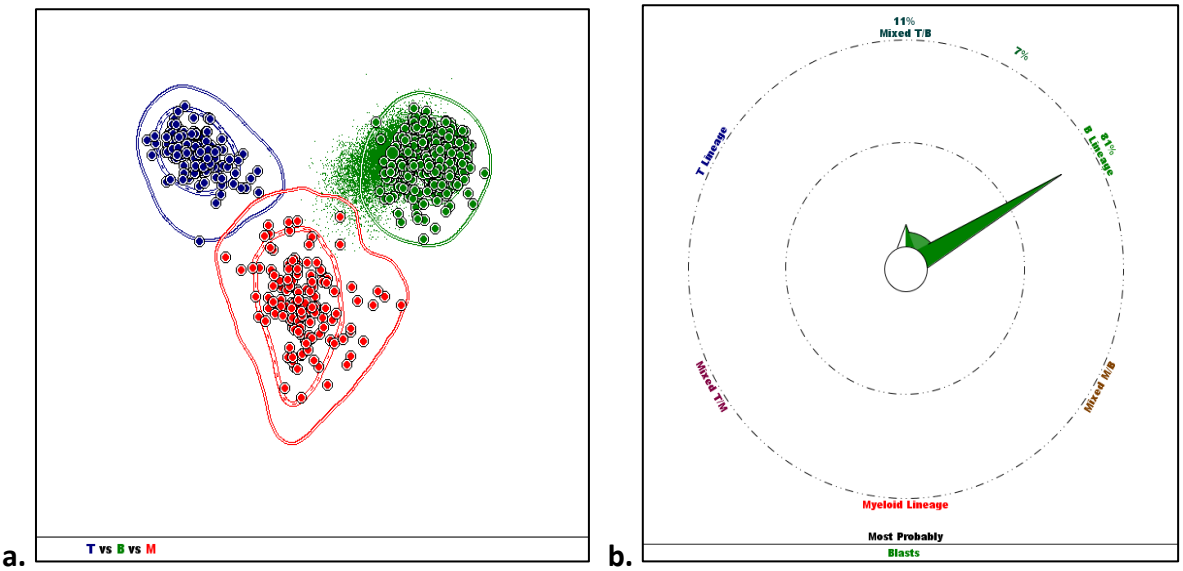


Figure 6.29. Case 10 peripheral blood blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

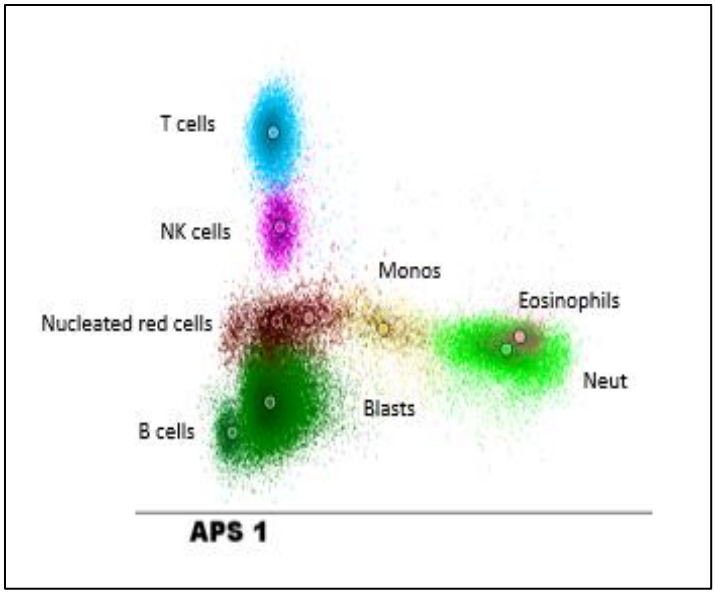


Figure 6.30. Case 10 blast cells distribution compared to residual normal cells in the bone marrow specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

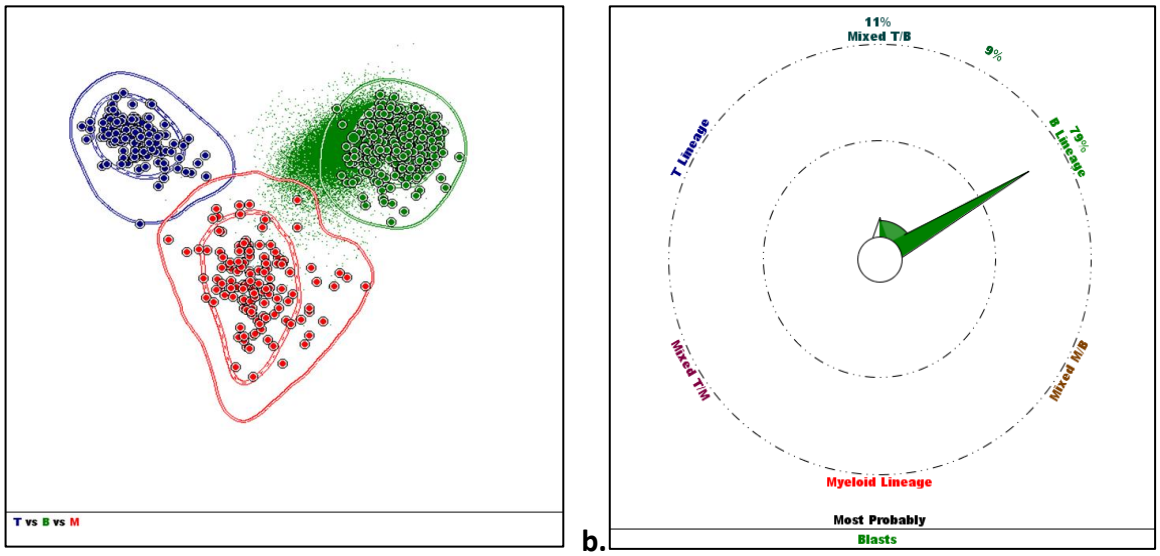


Figure 6.31. Case 10 bone marrow blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

13. Case 11:

13.1. Clinical presentation:

This was 6 years old girl presented to accident and emergency with pale and general weakness for 5 days. She complained of poor oral intake despite no fever or vomiting. Four days ago she had a mild nasal bleed which stopped spontaneously. There was no skin rash. One month ago she visited a private clinic due to abdominal pain. Her white blood cells count then was 33 x 10^9/L with predominant lymphocytes (30 x 10^9/L). Parents were advised to take her to a hospital for further investigations.

On examination, she showed a palpable liver with mild bilateral submandibular lymphadenopathy. There was mild mouth ulceration with no throat congestion. No other abnormal chest, abdominal or CNS symptoms were detected.

13.2. Blood counts morphology and coagulation studies:

Blood counts showed marked anaemia with haemoglobin of 34 g/L only. The patient also had severe thrombocytopenia with a platelet count of 12 x 10^9/L. On the other hand, white blood cells count was elevated (43 x 10^9/L) with neutropenia (0.87 x 10^9/L) and predominant blast cell population (approximately 93% on manual differential). Her coagulation studies were normal with PT of 15 seconds, APTT of 25 seconds and fibrinogen level of 2.9 g/L.

13.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate with adequate quality for assessment. This showed hypercellularity with predominant blast cells (97%). Blast cells were variable in size (mixture of small, intermediate and large-sized forms), with high nuclear to cytoplasmic ratios, slightly irregular to convoluted nuclear contours, variably condensed to finely dispersed chromatin, inconspicuous nucleoli (rarely prominent) and minimal to moderate amounts of agranular cytoplasm. No Auer rods were identified. Other myeloid, erythroid and megakaryocytic precursors were rarely seen and difficult to comment on. Bone marrow biopsy confirmed the aspirate findings with sheets of mononuclear (blast) cells.

13.4. Flow cytometry:

Flow cytometry analysis was performed on the peripheral blood sample. This showed 72% blast cells with negative to dim CD45 expression. Blast cells were positive for CD10, CD19, HLADR, CD15, CD123, cytoplasmic CD79a, and TdT with minimal expression of CD20 and CD33. There was also partial expression of CD66c and dim expression of CD38. Blast cells showed no significant expression of CD34, CD2, CD3, CD4, CD11b, CD13, CD14, CD56, CD64 or cytoplasmic MPO, cyCD3 or cyIgM.

13.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate showed abnormal hybridization pattern with 2-5 fusion for ETV6/RUNX1 probes indicating t(12;21) in 93% of cells along with 2 extra signals for all probes indicating

tetraploidy. Karyotype analysis of metaphases showed additional chromosome 16 (47,XX,+16 (16/46), 46,XX(14) in 16 out of 46 cells examined. There was no evidence of the t(12;21) initially detected by FISH analysis indicating a cryptic translocation (not detectable by karyotyping).

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

13.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1) (ETV6-RUNX1)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

13.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on both peripheral blood and bone marrow samples. Results confirmed the initial immunophenotypic with positive expression of CD19 and cytoplasmic CD79a. There was no significant expression of CD45, CD34, surface CD3, CD7, cytoplasmic MPO or cyCD3.

The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood

and bone marrow specimens. Finally, the automated database orientation tool using Infinicyt software from Cytognos confirmed blast cells to be of B-cell lineage (figures 3.35 to 3.38).

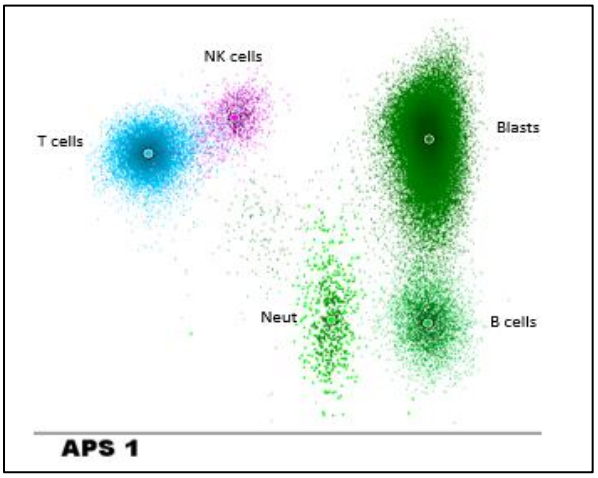


Figure 6.32. Case 11 blast cells distribution compared to residual normal cells in the blood specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

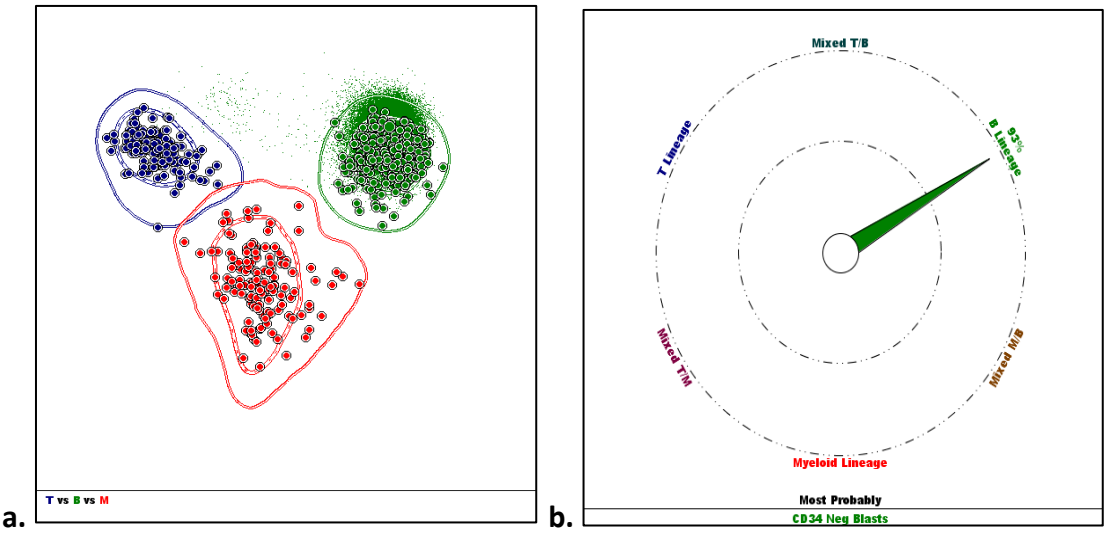


Figure 6.33. Case 11 peripheral blood blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

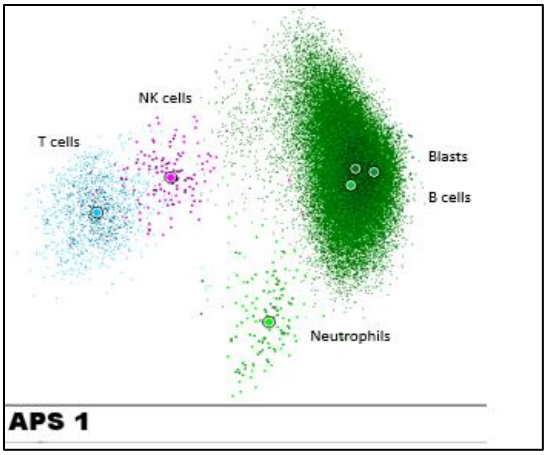


Figure 6.34. Case 11 bone marrow blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

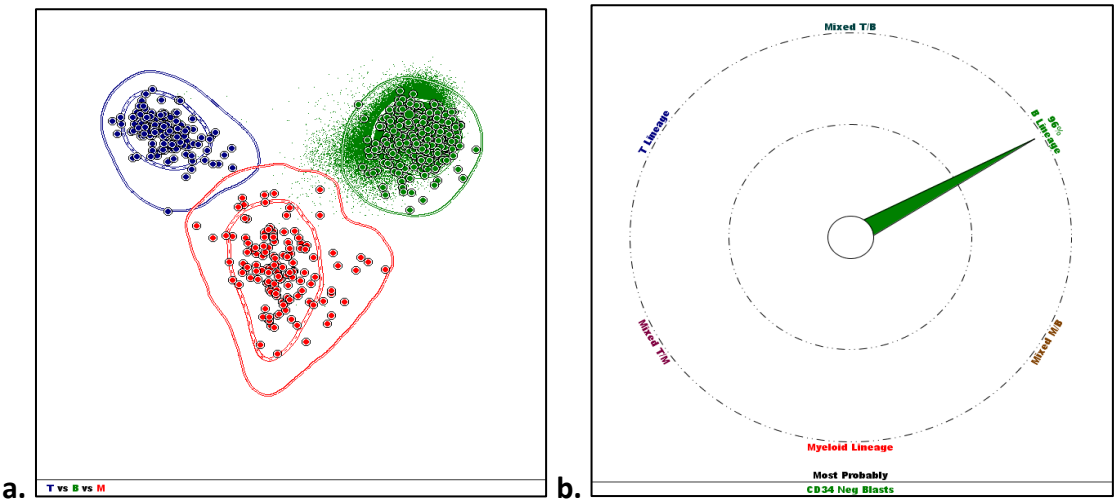


Figure 6.35. Case 11 bone marrow blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

14. Case 12:

14.1. Clinical presentation:

This was a 4 years old boy who was referred from a primary healthcare center. He was generally unwell with changes to polar, bone pain and mild fever for the last 2 days. Symptoms were worse during the night. At presentation, he had feet pain and petechial rash on the face. There was no gum bleeding or epistaxis history. On examination he had hepatosplenomegaly and skin rash. ENT (ear, nose and throat), chest, abdomen and CNS were all otherwise normal.

14.2. Blood counts morphology and coagulation studies:

His full blood count showed marked leukocytosis with a WBC count of 67 x 109/L and severe neutropenia (0.67 x 109/L). He also had severe thrombocytopenia (platelets count 50 x 109/L) and anaemia with a haemoglobin of 74g/L. Examination of blood film revealed predominant blast cells (approximately 95%). These were predominantly small to intermediate-sized with high nuclear to cytoplasmic ratio, irregular nuclear contours, variably condensed chromatin, inconspicuous nucleoli and scant amounts of cytoplasm. No Auer rods were identified. Coagulation studies were normal with PT of 14 seconds, APTT of 31 seconds and fibrinogen level of 2.7 g/L.

14.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was paucispicular with adequate quality for assessment. This showed hypercellularity with predominant blast cells (98%). Blast cells were predominantly small to intermediate -sized with morphology similar to peripheral blood blast cells. No Auer rods were

identified. Other myeloid, erythroid and megakaryocytic precursors were rarely seen and difficult to comment on. Bone marrow biopsy confirmed the aspirate findings with cellularity of 95-100% and sheets of mononuclear (blast) cells. No blast cells were identified in the CSF specimen. This was classified as CNS1 per BFM 2017 protocol.

14.4. Flow cytometry:

Flow cytometry analysis was performed on the peripheral blood sample. This showed 90% blast cells with negative CD45 expression and low side scatter. Blast cells were positive for CD9, CD10, CD19, HLADR, CD15, CD123, cytoplasmic CD79a, and TdT with partial expression of CD34, CD38, CD66c and minimal expression of CD20 and CD33. Blast cells showed no significant expression of CD2, CD3, CD4, CD11b, CD13, CD14, CD15, CD56, CD64, CD71 or cytoplasmic MPO, cyCD3 or cyIgM. The forward scatter (FSC) for blast cells was much higher than the normal T-cells in sample the background (figure 39) suggesting a possible hyperdiploidy.

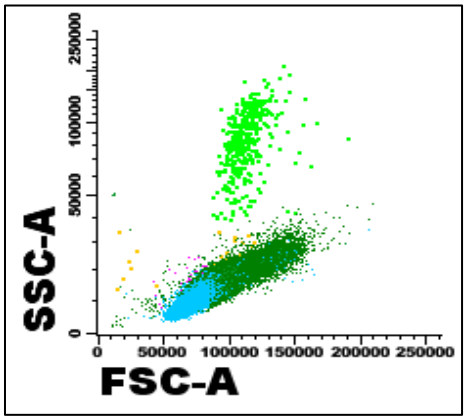


Figure 6.36. Forward and side scatter properties of blast cells (in green) and normal T-cells (blue) indicating a possible hyperdiploidy.

14.5. Cytogenetic and molecular analysis:

Interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate showed abnormal hybridization pattern with extra signals for RUNX1, MLL and IGH probes on chromosomes 21, 11 and 14 respectively. Karyotype/metaphase cytogenetics analysis performed on the bone marrow aspirate confirmed the FISH findings with an abnormal clonal proliferation in 3 of 23 metaphases. A clone with 58-68 chromosomes was detected, however; it was not possible to recognize each chromosome due to poor morphology/technical artifacts. The remaining 20 metaphase have a normal male karyotype, 46,XY. Full karyotype was

(58~68,XXY,.....[3]/46,XY[20].nuc ish(ETV6x2,RUNX1x3~4)[189/200]/nuc ish(MLLx3)[184/200]/nuc ish(IGHx3)[184/200]).

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment.

14.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia/lymphoma with hyperdiploidy** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

14.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on both peripheral blood and bone marrow samples. Results confirmed the initial immunophenotypic with positive expression of CD19, CD34 and cytoplasmic CD79a. There was no significant expression of CD45, surface CD3, CD7, cytoplasmic MPO or cyCD3.

The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood and bone marrow specimens. Finally, the automated database orientation tool using Infinicyt software confirmed blast cells to be of B-cell lineage (figures 40 to 43).

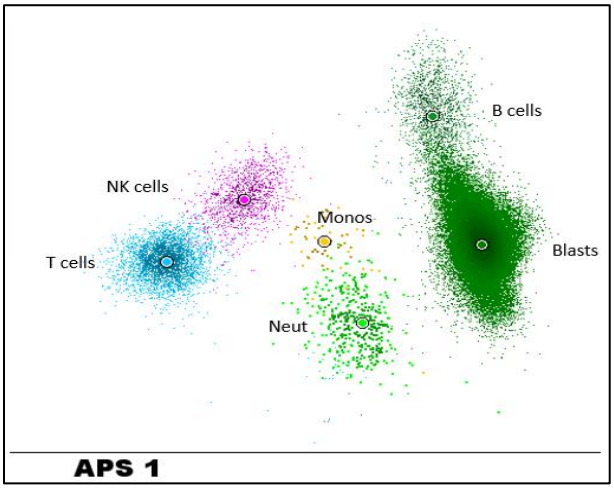


Figure 6.37. Case 12 peripheral blood blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

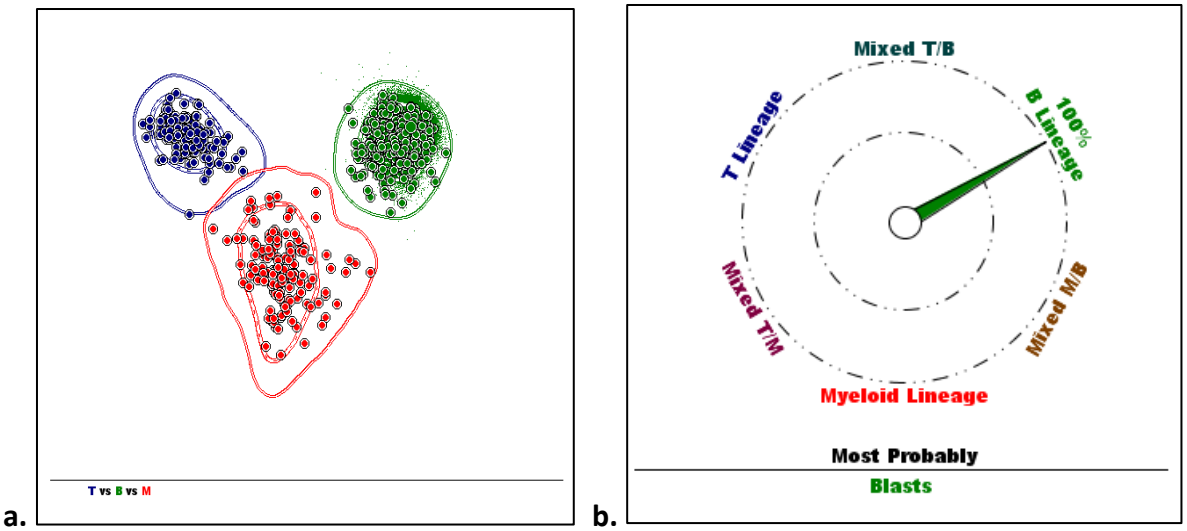


Figure 6.38. Case 12 peripheral blood blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

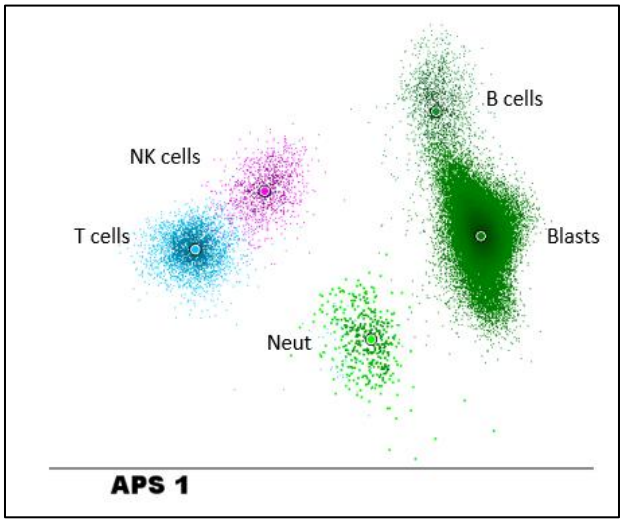


Figure 6.39. Case 12 bone marrow blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

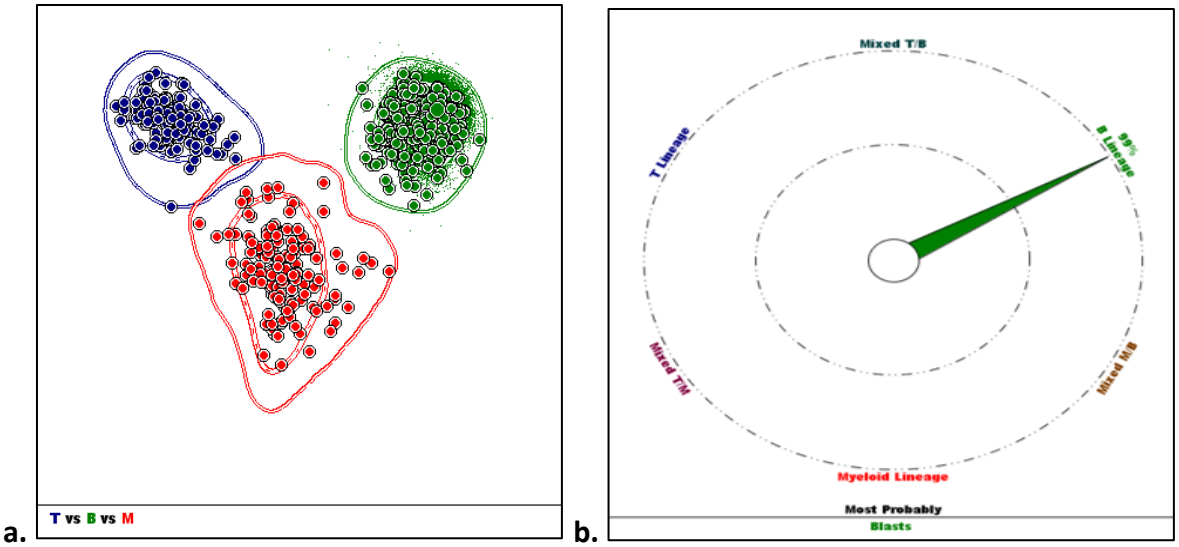


Figure 6.40. Case 12 bone marrow blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

15. Case 13:

15.1. Clinical presentation:

This was a 2 and a half years old girl who was referred from a primary healthcare center with preliminary diagnosis of acute leukaemia. She had a two weeks history of fatigue, fever and occasional cough for which she was given some antibiotics. One day ago she was back to the center with multiple vomits and loss of appetite, fast breathing and bone pain on chest, head and limbs. Her mother noted a small nodule on the left neck. Clinical examination revealed three palpable lymph nodes on the left anterior neck triangle (each ~ 1cm) as well as few left inguinal lymph nodes. She looked pale and unwell. No additional ENT, skin, respiratory, abdominal or CNS abnormalities were detected during examination.

15.2. Blood counts morphology and coagulation studies:

Her full blood count showed leukocytosis with a WBC count of 25.8 x 10^9/L and a normal neutrophil count of 1.55 x 10^9/L. She also had a borderline thrombocytopenia (platelets count 145 x 10^9/L) and anaemia with a haemoglobin of 74g/L. Examination of blood film showed predominant blast cell population representing > 80% of total cells. Her coagulation studies showed a prolonged PT of 23 seconds, normal APTT of 29 seconds and a slightly increased fibrinogen level (4.8g/L). Subsequent PT mixing studies with normal plasma showed immediate correction which was confirmed by a low factor 7 level (FVII 13%).

15.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate with adequate quality for assessment. This showed hypercellularity with predominant blast cells (96%). Blast cells were variable in size (small, intermediate to large-sized forms with high nuclear to cytoplasmic ratios, smooth to slightly irregular nuclear contours, finely dispersed to variably condensed chromatin, inconspicuous nucleoli and minimal amounts of agranular and occasionally vacuolated cytoplasm. No Auer rods are identified. Other myeloid, erythroid and megakaryocytic precursors were rarely seen and difficult to comment on. Bone marrow biopsy confirmed the aspirate findings with cellularity of 95-100% and sheets of mononuclear (blast) cells.

CSF showed no blast cells. This was considered as CNS-1 per the BFM 2017 protocol.

15.4. Flow cytometry:

Flow cytometry analysis was performed on the peripheral blood sample. This showed 66% blast cells with negative to dim CD45 expression and low side scatter. Blast cells were positive for CD34, CD10, CD19, CD38, HLADR, cytoplasmic CD79a, and TdT with partial expression of CD9, CD15 and minimal expression of CD20. There was no significant expression of CD2, CD3, CD4, CD11b, CD13, CD14, CD15, CD33, CD56, CD64, CD71 or cytoplasmic MPO, cyCD3 or cyIgM.

15.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate showed abnormal hybridization pattern with dual fusion for ETV6/RUNX1

probes indicating t(12;21) in 94% of cells along with homozygous deletion of CDKN2A probes on chromosomes 9p in 10% of the cells analysed. Karyotype analysis of metaphases showed an abnormal clone with additional material of unknown origin short arm of chromosome 12 at cytogenetic band 12p13. Full karyotype was 46,XX,add(12)(p13)[17]/46,XX[43].nuc ish(ETV6x2,

RUNX1x3)(ETV6 con RUNX1x2)[189/200]/nuc ish(CEP9x2,CDKN2Ax0)[21/200].

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment

15.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia/lymphoma with t(12;21))(p13.2;q22.1) (ETV6-RUNX1)** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

15.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on both peripheral blood and bone marrow samples. Results confirmed the initial immunophenotypic with positive expression of CD19, CD34 and cytoplasmic CD79a. There was no significant expression of CD45, surface CD3, CD7, cytoplasmic MPO or cyCD3.

The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood and bone marrow specimens (figures 44 and 46). Finally, the automated database orientation tool using Infinicyt software confirmed blast cells to be of B-cell lineage (figures 45 and 47).

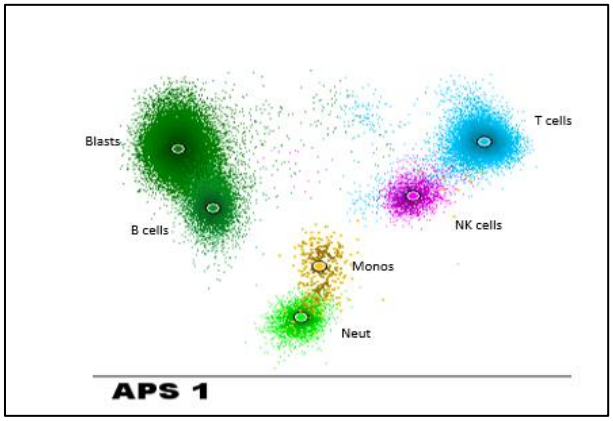


Figure 6.41. Case 13 peripheral blood blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

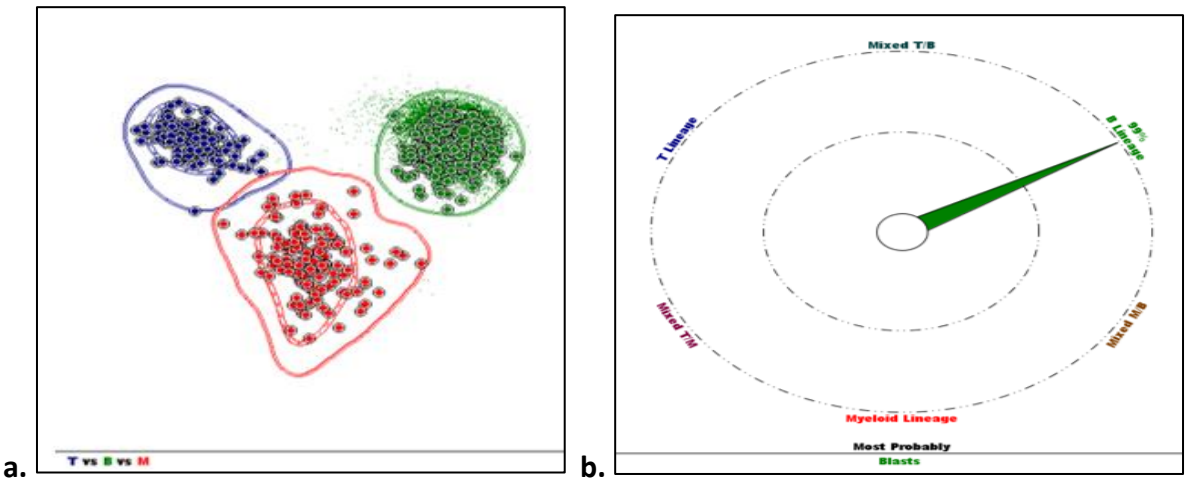


Figure 6.42. Case 13 peripheral blood blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

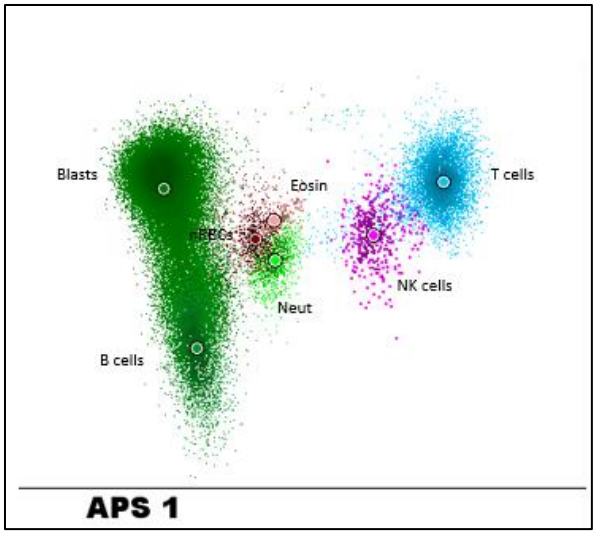


Figure 6.43. Case 13 bone marrow blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

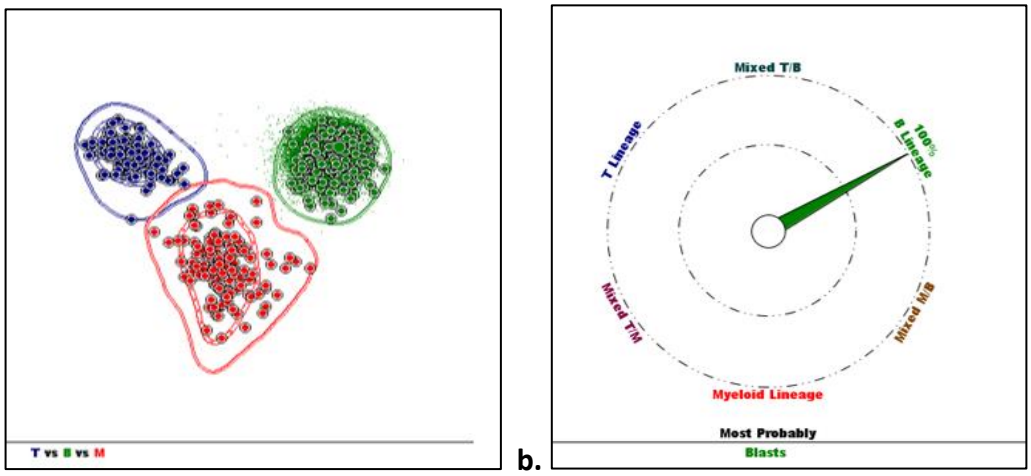


Figure 6.44. Case 13 bone marrow blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

16. Case 14:

16.1. Clinical presentation:

This was a 5 years old previously healthy girl. She had a history of 3 weeks of intermitted fever every 3 to 5 days with night sweats. She visited accident and emergency before and was prescribed antibiotics for congested throat. However, there was no significant improvement. She had few epistaxis episodes in the last 3-4 months according to her mother. Clinical examination revealed no additional ENT, skin, respiratory, abdominal or CNS abnormalities.

16.2. Blood counts morphology and coagulation studies:

Her full blood count showed a normal WBC count of 6.7 x 10^9/L with a normal neutrophil count of 1.79 x 109/L. Platelets count was also normal (185 x 10^9/L) with mild anaemia with a haemoglobin of 97g/L. Examination of blood film showed a small blast cell population representing only 7% of total cells. Her PT and APTT studies were both normal (13 and 27 seconds; respectively) with a slightly increased fibrinogen level (4.4g/L).

16.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate with adequate quality for assessment. This showed hypercellularity with predominant blast cells (81%). Blast cells were variable in size (small, intermediate to large-sized forms with high nuclear to cytoplasmic ratios, smooth to slightly irregular nuclear contours, finely dispersed to variably condensed chromatin, inconspicuous nucleoli and minimal amounts of agranular and occasionally vacuolated cytoplasm. No Auer

rods are identified. Other myeloid, erythroid and megakaryocytic precursors were rarely seen with unremarkable morphology for those were present.

Bone marrow biopsy confirmed the aspirate findings with approximate cellularity of 75% mostly representing mononuclear (blast) cells. CSF showed no blast cells. This was considered as CNS-1 per the BFM 2017 protocol.

16.4. Flow cytometry:

Flow cytometry analysis was performed on the bone marrow sample. This showed 23% blast cells with negative CD45 expression and low side scatter. Blast cells were positive for CD9 (bright), CD10, CD19, CD38, HLADR, cytoplasmic CD79a, and dim TdT with minimal expression of CD20. There was no significant expression of CD2, CD3, CD4, CD11b, CD13, CD14, CD15, CD33, CD56, CD64, CD71, CD123, cytoplasmic MPO, cyCD3 or cyIgM.

16.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate showed normal hybridization pattern for all probes in the panel (ETV6/RUNX1, BCR/ABL1, MLL, IGH and CDKN2A/CEP9. However, karyotype analysis of metaphases identified an apparently balanced translocation involving chromosomes 1 and 19 with breakpoints estimated at q23 and p13.3, respectively. The remaining 30 metaphases showed a normal female (46,XX) karyotype. Final karyotype was 46,XX,t(1;19)(q23;p13.3)[15]/46,XX[30]

DNA samples were sent to reference laboratory for molecular analysis of minimal residual disease (MRD) at diagnosis (day 0) as well as follow up bone marrows on day 33 and day 78 post treatment

16.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); TCF3-PBX1** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

16.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on the bone marrow specimen. Results confirmed the initial immunophenotypic with positive expression of CD19 and cytoplasmic CD79a. There was no significant expression of CD45, CD34, surface CD3, CD7, cytoplasmic MPO or cyCD3. The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the bone marrow specimen (figures 48). Finally, the automated database orientation tool using Infinicyt software confirmed blast cells to be of B-cell lineage (figures 49).

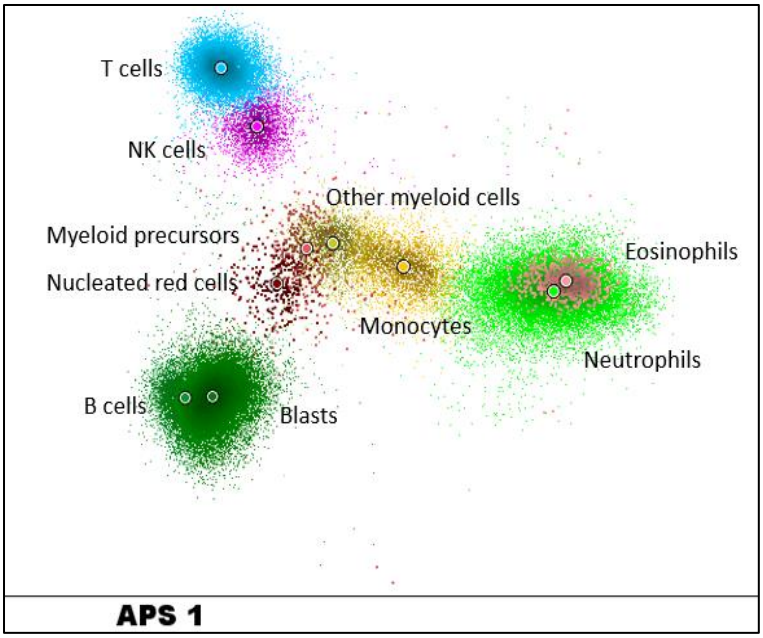


Figure 6.45. Case 14 bone marrow blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

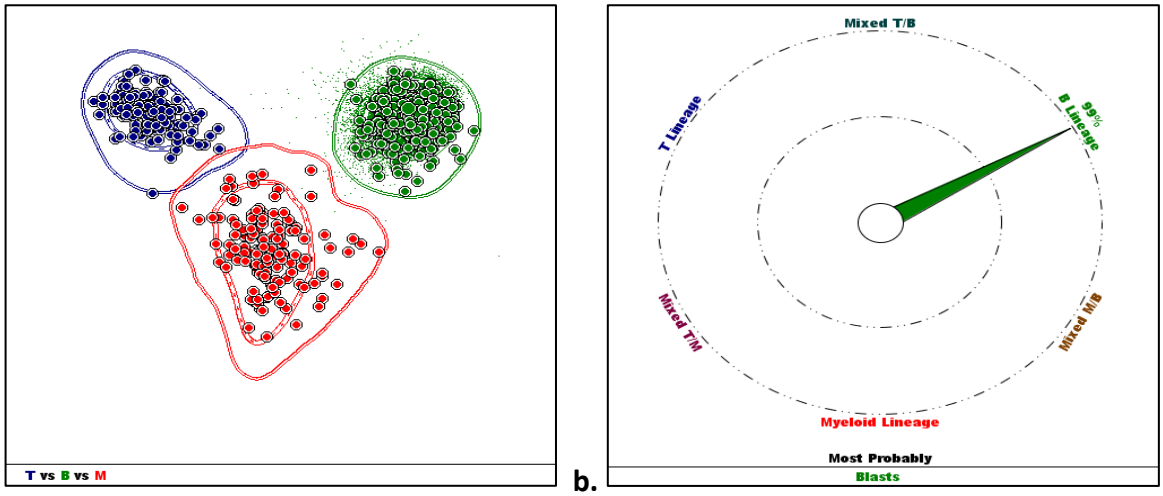


Figure 6.46. Case 14 bone marrow blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.

17. Case 15:

17.1. Clinical presentation:

This was a 14 years old male with a previous history of uncontrolled asthma. He presented with one week history of fever, cough, nausea, vomiting and diarrhea. There were no history night sweats, loss of appetite, weight loss, bone pain, skin rash or bleeding. Clinical examination revealed eye swelling with sub conjunctival hemorrhage on both eyes and multiple cervical lymph nodes bilaterally. There were no additional, chest, abdominal or CNS symptoms.

17.2. Blood counts morphology and coagulation studies:

His full blood count showed marked leukocytosis with a WBC count of 153 x 10^9/L, neutrophilia (neutrophil count of 18.3 x 10^9/L), moderate thrombocytopenia (68 x 10^9/L) and normal haemoglobin level of 150 g/L. Examination of blood film showed a predominant blast cell population representing 79% of total cells. These were intermediate to large in size with variable amount of agranular, basophilic cytoplasm. No Auer rods are identified. Nuclei were generally irregular with variably condensed chromatin and inconspicuous nucleoli.

Coagulation studies were normal with PT of 15 seconds, APTT of 28 seconds and fibrinogen level of 2.4g/L. No bone marrow was collected and flow cytometry was performed directly on the peripheral blood sample.

17.3. Flow cytometry:

This showed 84% blast cells with moderate CD45 expression and low side scatter. Blast cells were positive for CD2, cytoplasmic CD3, CD7, CD8, and CD99 with partial expression of CD4, CD38 and TdT. There was minimal expression of surface CD3 (<10%) and no significant expression of CD1a, CD10, CD13, CD15, CD33, CD34, CD56, CD66c, CD117, CD123, HLADR, cytoplasmic IgM, cyCD79a or MPO.

17.4. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the peripheral blood sample. Did not identify extra signals or recurrent rearrangements of the BCR/ABL1, TRA/D, MLL and CDKN2A/CEP9 probes. However, karyotype analysis identified an abnormal clone (2 of 76 cells) with a reciprocal translocation between the long arms of chromosomes 8 and 14 at cytogenetic bands 8q24 and 14q11.2, which may result in a TRA/MYC rearrangement. The remaining cells analyzed showed a normal male karyotype. Final karyotype was 46,XY,t(8;14)(q24;q11.2)[2]/46,XY[74].

17.5. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic **T-Lymphoblastic leukaemia/lymphoma** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

17.6. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on both peripheral blood sample. Results confirmed the initial immunophenotypic with positive expression of moderate CD45, cytoplasmic CD3, surface CD7 and minimal surface CD3 (10%). There was no significant expression of CD19, CD34, cytoplasmic MPO or cyCD79a. The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood and bone marrow specimens (figures 50). Finally, the automated database orientation tool using Infinicyt software confirmed blast cells to be of T-cell lineage (figures 51).

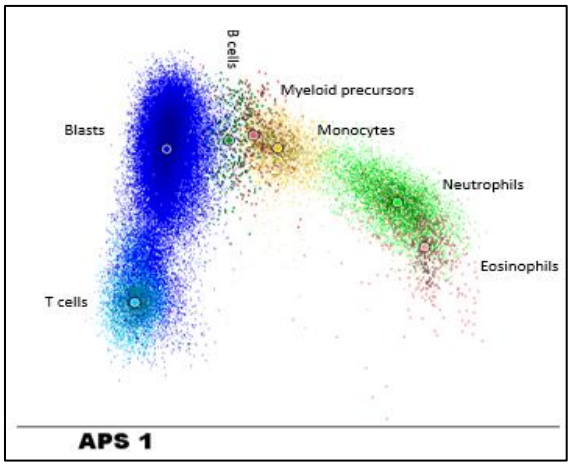


Figure 6.47. Case 15 peripheral blood blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

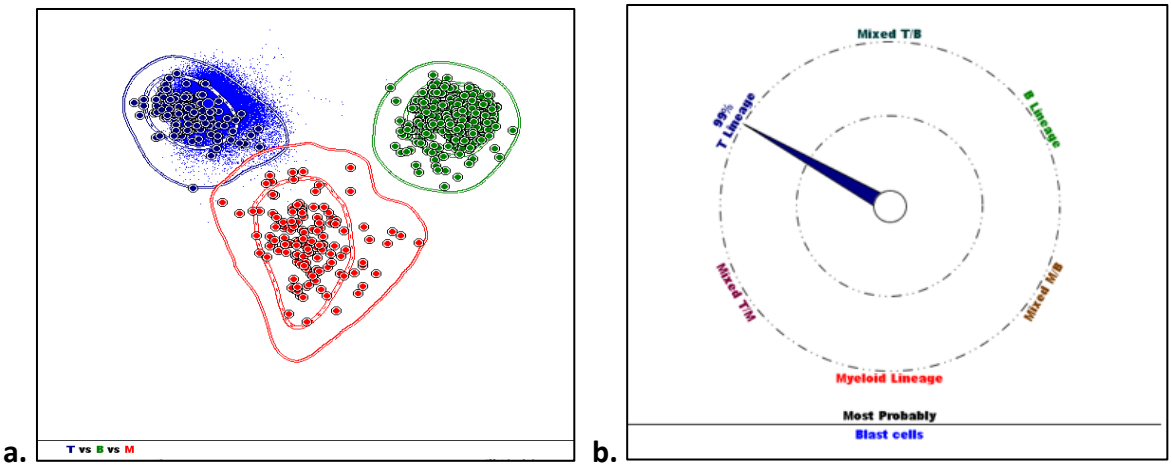


Figure 6.48. Case 15 peripheral blood blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of T-cell lineage.

18. Case 16:

18.1. Clinical presentation:

This was a 5 years old boy with a history of head trauma 6 weeks ago. He has had an x-ray following his head injury in a sister hospital and was re-assured it was clear. However, he was limping and not able to run since then, there was no history of fever, weight loss, bleeding, lethargy or loss of appetite. During examination he looked pale as well as tired with hepatosplenomegaly. There were no other ENT, CNS, abdominal or chest abnormalities during examination.

18.2. Blood counts morphology and coagulation studies:

His full blood count showed leukocytosis with a WBC count of 17.3 x 10^9/L and a normal neutrophil count of (1.2 x 10^9/L) for age. He also had severe thrombocytopenia (platelets count 18 x 10^9/L) and anaemia with a haemoglobin of 61g/L. Examination of blood film showed predominant blast cell population representing 51% of total cells. These were small in size with very high N/C ratio, scant, agranular, basophilic cytoplasm. No Auer rods were identified. A minority of blasts had small cytoplasmic vacuoles. Nuclei were mostly smooth in contour with fine to variably condensed chromatin and few showed small nucleoli. Parallel coagulation studies were normal with PT of 13.9 seconds and APTT of 34 seconds. No fibrinogen level was performed at diagnosis.

18.3. Bone marrow aspirate, biopsy and cerebrospinal fluid (CSF):

Bone marrow aspirate was particulate with adequate quality for assessment. This showed hypercellularity with predominant blast cells (96%). Blast cells had similar morphology to blood film described above. Other myeloid, erythroid and megakaryocytic precursors were rarely seen and difficult to comment on. Bone marrow biopsy confirmed the aspirate findings with cellularity of 95-100% and sheets of mononuclear (blast) cells.

CSF showed no blast cells. This was considered as CNS-1 per the BFM 2017 protocol.

18.4. Flow cytometry:

Flow cytometry analysis was performed on the peripheral blood sample. This showed 59% blast cells with negative to dim CD45 expression and low side scatter. Blast cells were positive for CD34, CD10, CD19, HLADR, cytoplasmic CD79a, and TdT with partial expression of CD123 and minimal expression of CD20. There was no significant expression of CD2, CD3, CD4, CD11b, CD13, CD14, CD15, CD33, CD56, CD64, CD66c, CD71, CD117 or cytoplasmic MPO, cyCD3 or cyIgM.

18.5. Cytogenetic and molecular analysis:

Subsequent interphase fluorescence in-situ hybridization (FISH) analysis performed on the bone marrow aspirate showed abnormal hybridization pattern with an IGH rearrangement in approximately 90% of the cells analysed. Karyotype analysis of metaphases showed no structural abnormality because of low cytogenetic resolution. However, interphase FISH

analysis of directly harvested cells revealed IGH rearrangement. Metaphase FISH analysis identified 3 cells with translocation between short chromosome Y and long arm of chromosome 14 at cytogenetic bands Yp11.2(?) and 14q32. The translocation may result in IGH/CRLF2 rearrangement. Final karyotype was 46,XY(40).ish t(Y;14)(p11.?2;q32)(5`IGH+3`IGH+)(3/44).

DNA samples were sent to reference laboratory for confirmation of the IGH/CRLF2 as well as molecular analysis of minimal residual disease (MRD) at diagnosis (day 0), day 33 and day 78 post treatment.

18.6. Final diagnosis and integrated report:

The overall morphologic, immunophenotypic and cytogenetic findings were diagnostic of **B-lymphoblastic leukemia/lymphoma with BCR-ABL1-like translocation involving IGH/CRLF2** per the revised 4th edition (2017) of the World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues.

18.7. Acute leukaemia orientation tube (ALOT) and automated database-guided orientation:

Parallel analysis of the ALOT tube under validation was performed on the peripheral blood sample. Results confirmed the initial immunophenotypic with positive expression of CD19, CD34 and cytoplasmic CD79a. There was no significant expression of CD45, surface CD3, CD7, cytoplasmic MPO or cyCD3. The principal component analysis (PCA) and automated population separator (APS) diagram were used to compare blast cells distribution to residual normal cell populations in the blood and bone marrow specimens (figures 52). Finally, the automated

database orientation tool using Infinicyt software confirmed blast cells to be of B-cell lineage (figures 53).

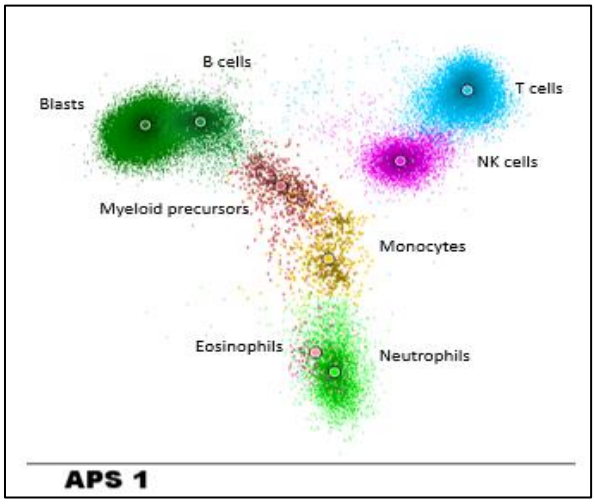


Figure 6.49. Case 16 peripheral blood blast cells distribution compared to residual normal cells in the specimen using the principal component analysis (PCA) and automated population separator (APS) diagram.

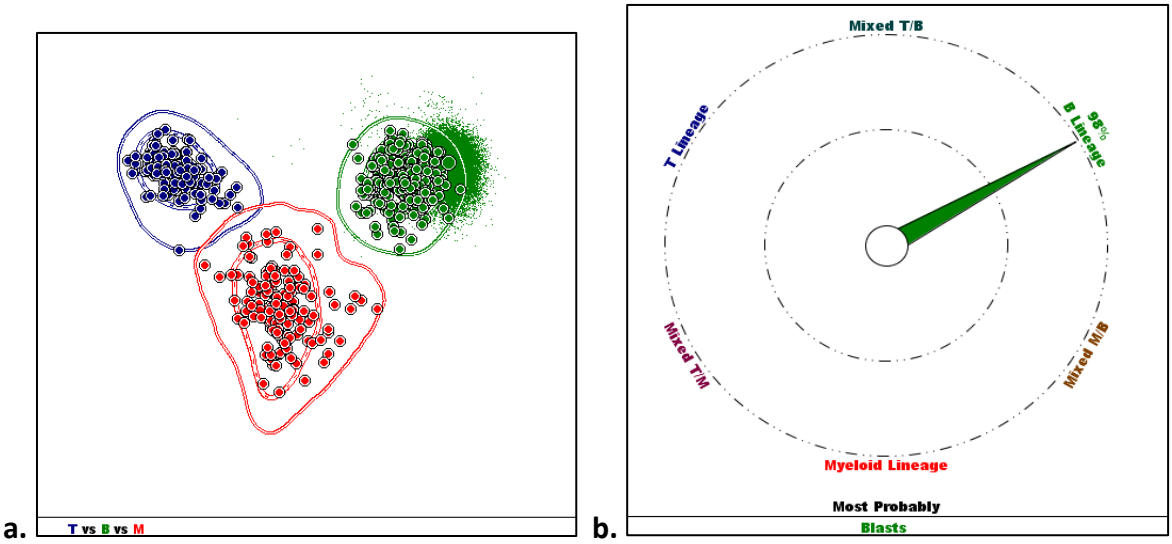


Figure 6.50. Case 16 peripheral blood blast cells orientation using automated database a. orientation among B-cells (in green) compared to the EuroFlow (Cytognos) database. B. Database compass indicating blast cells of B-cell lineage.