DIAGNOSIS OF HAEMATOLOGICAL MALIGNANCIES IN THE ERA OF TOTAL LABORATORY AUTOMATION: COMPARISON OF THE ADVIA 2120 TO IMMUNOPHENOTYPING AND MORPHOLOGY

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Haematology.

Johannesburg, March 2015

Declaration

I, Dashini Pillay, declare that this research report is my own unaided work. It is being submitted to the University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Haematology. It has not been submitted for any other degree or examination at this or any other University.

Dashini Pillay 13th day of March 2015

Dedication

This research report is dedicated to my late father, Mr Kandasamy Pillay, who gave me the opportunity to further my studies after school and taught me amongst many other things that knowledge and hard work are the key to success.

Abstract

The incidence of leukaemia in South Africa is 2.5 per 100 000 and has increased due to HIV. Accurate and timeous diagnosis of leukaemia directly impacts success of patient treatment and consequent survival. Usually the Full Blood count (FBC), white blood cell (WBC) differential count and review of the peripheral blood smear alerts the clinician to the possibility of leukaemia. However the number of qualified and skilled technologists in peripheral and central laboratories is on a continual decline making the performance of the critical function of peripheral blood review a challenge.

The Advia 2120 haematology analyser performs a WBC and differential count using principles of flow cytometry and the cytograms generated can be used to classify haematological malignancies through the Peroxidase and nuclear density analysis (PANDA) classification system. The presence of myeloperoxidase (MPO) activity in 3% or more leukaemic blasts confirms acute myeloid leukaemia, and enzyme activity can be detected by immunophenotypic analysis or conventional cytochemistry . Research on the comparison of the Advia 2120 and manual morphologic assessment in the classification of leukaemias is limited in the South African setting, where leukaemia often coincides with infection. The aim of this study was to determine if the FBC, differential count and cytogram assessment by the Advia 2120 using the PANDA classification is as reliable as morphologic assessment in the initial classification of haematological malignancies from peripheral blood samples when using flow cytometry as the gold standard..

150 cases of confirmed leukaemia were collected. The diagnosis obtained from either PANDA analysis and/or morphological assessment was compared to the diagnosis obtained by immunophenotypic analysis. Secondly, the MPO activity obtained by the Advia peroxidase cytogram was compared to the MPO obtained by conventional methods of immunophenotypic analysis and/or cytochemistry.

Using the PANDA analysis system, only 48% (72/150) of cases overall were accurately classified. The inaccuracy was 9.3% (14/150) and 42.7% of cases could not be classified. The positive predictive value (PPV) was 88%. The most significant finding was all of the acute

promyelocytic leukaemia (APL) cases (8/8) had a distinct pattern and were accurately classified on cytogram analysis alone. Accurate sub-classification of other types of acute myeloid leukaemia using PANDA analysis alone was inconsistent. However, the accuracy in classifying leukaemia was improved when the Advia cytogram was used in conjunction with morphological analysis, as 90% (135/150) of cases were accurately classified.

The sensitivity and specificity of the peroxidase cytogram in evaluating myeloperoxidase (MPO) activity was 85% and 88.6% respectively. The agreement between cytogram peroxidase activity and the reference methods was 89.1% and the Cohen's kappa was 76.9%. To the best of our knowledge, there is no data comparing peroxidase activity on the cytogram to other methods.

In conclusion, it was shown that the routine use of the Advia cytograms in conjunction with the morphology findings provides invaluable information in the initial screening of leukaemia. In cases with indistinct morphology, the cytograms have the potential to aid in a provisional classification. The peroxidase activity from the cytogram could be used as a surrogate marker for myeloperoxidase activity in leukaemia. Moreover, a tentative diagnosis of an APL is possible by simple analysis of the cytogram resulting in earlier diagnosis which could be life-saving.

Acknowledgements

I would like to acknowledge the following individuals and institutions for their assistance in the completion of this thesis:

* My supervisors, Professor Johnny Mahlangu and Dr. Suvarna Buldeo, for their guidance, patience and encouragement. I am truly grateful.

*The routine haematology laboratory staff at the Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Services. A special thanks to Mr Ndwankulu Nemuthengame and Godwin Obijiiaku for teaching me about the Advia 2120 analyser.

*Dr Jenifer Leigh Vaughan, haematopathologist at Baragwanath Hospital for helping me to collect the cases needed for this study. Her continued support and friendship is much appreciated.

* Professor Wendy Stevens, head of the Department of Molecular Medicine and Haematology at the University of the Witwatersrand for encouraging research in the department

*National Health Laboratory Services for providing facilities for research

*The registrars and consultants in the routine haematology laboratory who informed me of any leukaemia cases that were received for routine analysis.

*The paediatric and adult oncologists for cases who submitted samples to the laboratory for the work-up of leukaemias.

*Dr Elise Schakpkaitz for selflessly sharing her advice in all aspects of haematology.

*Karen Scheepers for making time to assist me with formatting my document and sharing her suggestions with me.

*My family, in particular my mum, Sheila Pillay and sister, Ruvani, for their unconditional love and continued support. Ravendren, Navandran and Thavendren Naicker (my cousins) for their patience in helping me with computer related issues and data analysis.

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Abbreviations

ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APL	Acute promyelocytic leukaemia
ATRA	Al trans retinoic acid
CC	Conventional cytochemistry
CD	Cluster of differentiation
CHBAH	Chris Hani Baragwanath Academic Hospital
CLL	Chronic lymphocytic leukaemia
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CML	Chronic myeloid leukaemia
CML- BP	Chronic myeloid leukaemia blast phase
EDTA	Ethylene diamine tetra acetic acid
EP	Evaluation protocol
FBC	Full blood count
FAB	French American British
FISH	Fluorescent in situ hybridisation
FN	False negative
FP	False positive
HCL	Hairy cell leukaemia
HIV	Human immunodeficiency virus
LPD	Lymphoproliferative disorder
LUC	Large unstained cells
MPO	Myeloperoxidase
NHLS	National Health Laboratory Services
NHL	Non hodgkin lymphoma
ND	Nuclear density
NK	Not known
PA	Peroxidase activity
PANDA	Peroxidase activity and nuclear density analysis
PCR	Polymerase chain reaction

PPV	Positive predictive value
PLL	Prolymphocytic leukaemia
SOPS	Standard operating procedures
TN	True negative
TP	True positive
WBC	White blood cell
WHO	World health organisation

Chapter 1: BACKGROUND

1.1 Introduction

Leukaemia is characterized by the uncontrolled proliferation of haematopoietic cells and displacement of normal precursors in the bone marrow. In South Africa the incidence of leukaemia in adults is estimated to be 2.5 per 100 000 (1). Leukaemia is the most common cause of childhood cancers representing approximately 19-31% of all cancers worldwide. (1). However in Africa approximately 3.6% of all childhood cancers are due to leukaemia. Some haematological malignancies have a strong association with Human Immunodeficiency Virus (HIV) infection and in South Africa approximately 80% of patients diagnosed with Burkitt lymphoma/leukaemia and Diffuse large cell B lymphoma/leukaemia are HIV positive (2).

Despite advances in therapeutic technology, mortality associated with leukaemia remains high. In Africa, 10,600 females and 11,200 males demised as a result of leukaemia in 2008 (1). The significant mortality rate has been attributed in part to delayed diagnosis which is the direct result of the lack of screening and diagnostic services in many areas in Africa (3), in particular rural healthcare facilities. Early detection and classification of leukaemia allows the clinician to promptly initiate the most appropriate treatment plan, as the prompt initiation of chemotherapy has been shown to confer a better prognosis(4).

The Full Blood Count (FBC), differential count and review of peripheral blood smear usually are the first line investigations that alert the clinician to a possible underlying leukaemia, but further investigations are required to confirm the diagnosis and classify the leukaemia before appropriate therapy can be commenced.

Acute promyelocytic leukaemia (APL) is a haematological emergency which requires prompt initiation of Al-trans retinoic acid (ATRA) to reduce the associated coagulopathy and reduce mortality(5, 6). A quick and simple method that can be used to confirm and classify leukaemia could therefore be lifesaving. The aim of this study was to determine if the FBC, differential count and white blood cell (WBC) cytogram assessment from an automated haematology

analyser was as reliable as morphological assessment in the classification of leukaemia when compared to flow cytometry.

1.2 Classification of leukaemia

The World Health Organization (WHO) and French American British (FAB) classifications of leukaemia are widely used for leukaemia diagnosis and classification. Accurate classification is needed as it influences management decisions and hence the overall prognosis of the patients (7).

Leukaemia is classified as acute or chronic depending on the presence and number of blasts in the peripheral blood and/or bone marrow. In addition the clinical course of the disease can suggest whether the leukaemia is acute/chronic. Morphologically acute leukaemia is defined as the presence of >/= 20% blasts in the peripheral blood or bone marrow, while chronic leukaemia is a clonal haematological disorder whereby the blast count represents less than 20% in the peripheral blood or bone marrow and runs a chronic clinical course (7).

The second step in the classification is to determine whether the leukaemia arises from a myeloid or lymphoid lineage, as the treatment regimens are different for myeloid and lymphoid leukaemia (8-10). Adults and children diagnosed with acute lymphoblastic leukaemia (ALL) require adjunctive steroids in addition to chemotherapy(11). Morphological characteristics such as the presence of Auer rods and specific immunophenotypic surface markers in the blasts confirm the myeloid lineage of the leukaemia (7). The FAB classification, developed in 1976 (12) further sub classifies acute myeloid (AML) (**Table 1.1**) and lymphoid leukaemias (**Table 1.2**). Conventionally this is performed by morphological and cytochemical findings.

Table 1.1:FAB classification of acute myeloid leukaemia(12)

Type	Name
M0	Minimally differentiated acute myeloblastic leukaemia
M1	Acute myeloblastic leukaemia, without maturation
M2	Acute myeloblastic leukaemia, with abnormal maturation
M3	Acute promyelocytic leukaemia
M4	Acute myelomonocytic leukaemia
M5	Acute monoblastic leukaemia (M5a) or Acute monocytic leukaemia (M5b)
M6	Acute erythroid leukaemia
M7	Acute megakaryoblastic leukaemia

Table 1.2: FAB classification of acute lymphoblastic leukaemia(12)

Type	
ALL-L1	Small uniform cells
ALL-L2	Large varied cells
ALL-L3	Large varied cells with vacuolation

In newly diagnosed paediatric Precursor B-cell ALL, the day 8 blast count after a seven day induction pre-phase with corticosteroids influences prognosis and further treatment stratification of these patients. Patients with a reduction in the peripheral blood blast count to <1x10^9/l have a more favourable prognosis (8). In most academic centres, the day 8 blast count is determined by immunophenotypic analysis.

1.3 Diagnosis of leukaemia

The diagnosis and classification of leukaemia currently requires a combination of approaches and these include assessment of the FBC, differential WBC count, bone marrow aspirate and trephine biopsy examination, cytochemical stain evaluation, immunophenotyping, Fluorescence in situ hybridisation (FISH), as well as molecular and cytogenetic analyses(7).

1.3.1 The Full Blood Count (FBC)

The FBC is an investigation that quantifies leucocytes, red cells and platelet parameters. FBC analytes can be increased or decreased when compared to the reference ranges and this suggests underlying disease. In leukaemia the white cell count can be normal, reduced or elevated. Causes of cytopenias in the background of leukaemia include displacement of normal haemopoiesis by an infiltrate or ineffective haemopoiesis when an acute leukaemia transforms from an underlying clonal myeloid disorder. Some lymphoproliferative disorders, for example chronic lymphocytic leukaemia, can be complicated by auto-immune haemolytic anaemia and these patients can present with anaemia and features of haemolysis.

The differential count and microscopy identifies quantitative and qualitative abnormalities of the leucocyte population. Traditionally microscopic review based on the manual count of one hundred white blood cells on a peripheral smear was the gold standard for evaluation of the differential count. However concerns have been raised regarding the precision and accuracy of this method. Three types of errors have been identified: statistical error as only 100 cells are being analysed, distribution error due to the inconsistent distribution of cells on the smear and subjective error as the result obtained is dependent on the experience and skill of the morphologist (13). Automated haematology analysers employ a number of principles to produce accurate and precise results with a significant improvement in turnaround time when compared to manual methods (13). The instruments that have been developed thus far utilise the following principles: impedance measurement, scattered laser light, high frequency measurement and fluorescence measurement via flow cytometry (13). Additionally, automated analysis of peripheral blood samples include the use of morphology flags to alert one to the need for manual review of the peripheral smear which may be required when there is an immature population of cells (14, 15).

1.3.2 Morphological assessment of bone marrow and peripheral blood smear

One of the indications for a bone marrow procedure is to confirm and diagnose a leukaemia and to assess residual haemapoietic activity, as the WHO favours the use of bone marrow material in the diagnosis of leukaemia. Analysis of the marrow involves assessing if the cellularity is age appropriate and if normal haemapoiesis is displaced by an underlying

malignancy. However, the diagnosis of leukaemia is sometimes made from the morphological assessment of a peripheral smear.

The presence of Auer rods in leukaemic blasts is in keeping with the diagnosis of a myeloid leukaemia. Additionally, residual haemapoiesis is assessed for dysplasia as this can be used to determine the lineage of the leukaemic population. An example of this is AML with abnormal myeloid maturation (FAB M2) whereby the presence of >/= 20% blasts and morphological features of dysplasia in the background maturing and mature granulocytes (pelgerised, hypogranular neutrophils and disordered cell nucleation) suggest a myeloid origin. Leucocyte cytochemistry staining (myeloperoxidase (MPO), specific esterase and non-specific esterase) may also be required to sub classify AML,but this requires the expertise of a trained and experienced technologist to prepare the cytochemistry and pathologist to interpret the results (7, 16).

1.3.3 Flow cytometry

Flow cytometry is a technique that uses hydrodynamic focusing to analyse single cells as they pass through the light emitted from a laser. Fluorescent monoclonal antibodies are used to detect specific membrane, cytoplasmic or nuclear antigens, which are used to detect the presence of blasts, immature precursors or mature cells and the lineage of the abnormal population. It allows for the rapid analysis of up to 10000 events. Flow cytometry is used to confirm the diagnosis and to classify the leukaemia (7).

Flow cytometry is expensive, time consuming and requires a skilled technologist to process samples. Appropriate knowledge and skills are required to interpret the results. In many academic centres analysis of samples for flow cytometry does not occur after working hours which results in a delay in diagnosis. Simpler alternatives to confirm the diagnosis of a haematological malignancy are therefore required.

1.3.4 Molecular diagnostics

Over the years, molecular diagnostic assays have influenced the classification of leukaemias. These tests include cytogenetic, FISH, Polymerase Chain Reactions (PCR) and microarray

analyses. The aim of these tests is to detect genetic aberrations which help to confirm the diagnosis, prognosticate and direct management of leukaemia (7).

1.3.5 Limitations of the current diagnostic strategy

Owing to a combination of high volumes of morphologic assessments required as a result of the HIV epidemic(16), decline in staff numbers due to natural attrition, current high demand for experienced technologists/pathologists overseas and poor output of training institutions, there is a critical shortage of morphologists in South Africa (14, 17). This together with the manual nature of morphologic assessment has a negative impact on the turnaround time for the diagnosis of haematological malignancies(18). Furthermore molecular assays are expensive, labour intensive, time consuming and again require skilled medical technologists/pathologists.

The grave shortage of morphologists in rural areas further delays diagnosis, as the FBC and differential count have to be referred to central laboratories. This delays the transfer of patients to oncology units, further work-up and management which has a negative impact on prognosis. Hence there is a need to improve the diagnostic approach so that treatment can be accessed earlier. The use of automated haematology analysers in the morphologic evaluation of haematological malignancies is a possible solution (14, 19, 20) as the FBC and differential count are the initial alarm of an undiagnosed leukaemia.

1.4 The value of automated haematology analysers in classifying leukaemia

In addition to generating the FBC and differential count, most current haematology analysers produce morphology flags to highlight the presence of blasts and other abnormal cells. Many studies have evaluated the sensitivity and specificity of automated haematology analysers by comparing the differential counts, and detection of abnormal white blood cells in the peripheral blood to manual review of the peripheral smear(19, 21, 22). The results of these studies are conflicting. The differential counts obtained from the ABX Pentra 120, Abbot CELL-DYN Ruby, the Sysmex XT-2000i and Coulter STKS showed good correlation to the manual differential count(19, 23). The Advia 2120 haematology analysers used in our laboratory also show good correlation with the manual differential count (24). In contrast, review of the Beckman Coulter LH750 reported slightly higher monocyte counts and the

Advia 120 reported lower monocyte counts than the reference method (25). The overall sensitivity of automated haematology analysers in detecting blasts is variable (25).

The Coulter VCS system uses volume, conductivity and light scatter to analyse FBC and differential counts. The predictive value of this analyser to identify leukaemic samples is greater than 90%. However differentiating the subtypes of AML using the FAB classification was difficult as the blasts from the cases that were classified as M4/M5 FAB cases clustered in the monocyte region of the scatterplot(21).

Performance evaluation of the five part differential patterns of blasts from the DXH 800 (Beckman Coulter) and XE-2100 (Sysmex) in samples with more than 10% blasts revealed that both analysers counted most lymphoblasts as lymphocytes (85% of cases) and myeloblasts as monocytes (74.3% of cases). Although the blast flag was raised in 91.2% of the cases, the blasts were counted as monocytes or lymphocytes in the differential count. The two cases of T-cell acute lymphoblastic leukaemia that did not generate a blast flag morphologically had a high nuclear cytoplasmic ratio and coarse chromatin, which probably resulted in the blasts being analysed as lymphocytes(21).

A new haematology analyser, Haematoflow (Beckman Coulter, USA), uses principles of flow cytometry and a panel of monoclonal antibodies to determine a 16 part WBC differential count. There was a good correlation in the blast counts detected by the Haematoflow, however the analyser was unable to confirm T-cell lineage in ALL (26).

1.5 The Advia 2120i haematology analyser

The Siemens Advia 2120 (Siemens Healthcare Diagnostics, Deerfield, Illinois, USA) is another example of an automated haematology analyser (24). The Advia 2120 measures the total and differential WBC count using principles of flow cytometry and a combination of reactions that occur within the Peroxidase and the Basophil/nuclear lobularity channels. Cluster analysis of the cells within each channel is used to generate a cytogram and the pattern that emerges has been shown to assist with the subtyping of haematological malignancies (27).

a. Peroxidase cytogram

Reagents are used to stain intracellular myeloperoxidase. As the cells pass through a flow cell the absorbance of light is used to measure the intensity of myeloperoxidase activity and the light scatter measures the size and complexity of cells. This generates a scatter plot with the x-axis representing increasing intensity of peroxidase staining and the y-axis representing increasing cell size.

b. Basophil cytogram

The Baso reagent lyses the red blood cells, platelets and the cytoplasm of all WBCs except the basophils. The WBCs pass through a laser flow cell and the size and complexity of cells are detected and recorded on the basophil cytogram. The x-axis records the high angle scatter which detects the nuclear complexity and the y-axis reflects cell size.

The patterns that emerge on the Peroxidase and Basophil cytograms of a normal blood sample are illustrated in **Figures 1.1** and **1.2** respectively.

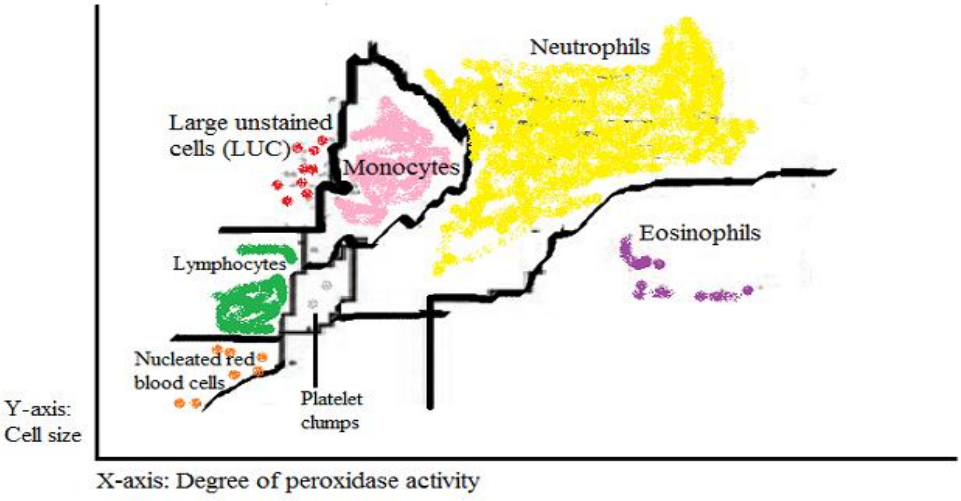

Figure 1.1: Peroxidase cytogram (27)

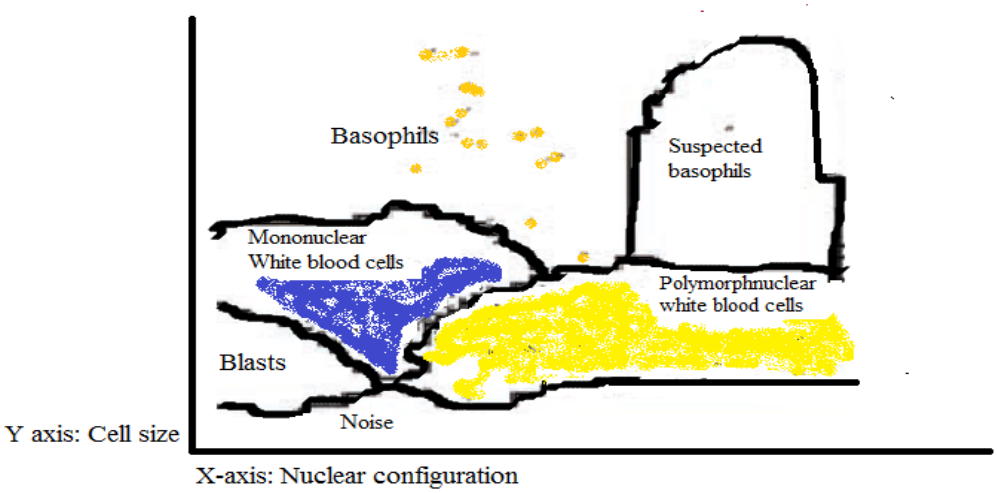

Figure 1.2: Basophil cytogram(27)

1.5.1 Peroxidase and nuclear density analysis (PANDA) system

Over the years an analysis system to detect and classify haematological malignancies according to pattern recognition of the cytograms obtained from the Advia analyser has emerged (28, 29). This system, referred to as Peroxidase activity and nuclear density analysis (PANDA), could potentially allow for faster recognition of leukaemias even before a peripheral smear is reviewed by a morphologist. The PANDA system was initiated in 2001 by G.Onofrio (29) and further explored by G.J.Gibbs in 2005(28).

This analysis system relies on the PANDA grid that integrates the patterns that are produced from the peroxidase and basophil cytograms **(Table1.3).** The different patterns are explained in section 3.2 of the results.

Table1.3:PANDA classification grid (29)

	P0	P1	P2	P3	P4	P5	P6
D0	CLL,PLL, HCL,ALL(L1), Total MPOdef	Severe MPO def	Partial MPOdef	CML	CML	AIDS MDS	
D1	ALL(L1-L3), NHL,M0, M5a, M6, M7	M1, M5a, M2, M4	M2, M4, M5a, M5b, M1	M2, M4, CML-BC	M2, M3v, M4	M3v,CML (at)	M3
D2	viral disease						

CLL, Chronic Lymphocytic Leukaemia; PLL, Prolymphocytic leukaemia; HCL, Hairy cell leukaemia; MPO, Myeloperoxidase; def, deficiency; ALL, Acute lymphoblastic leukaemia; NHL, Non Hodgkin lymphoma;CML,Chronic Myeloid Leukaemia; BC, blast crisis; V,Variant ; At, atypical; AIDS, Acquired immunodeficiency syndrome; MDS, Myelodysplastic syndrome

The advantage of this approach is that less skill is required and an initial screening and classification of a haematological malignancy is possible as soon as the FBC sample is analysed(28-31). The other advantage is improved laboratory turn around time. The current system requires a slide to be produced, stained, and reviewed by a technologist who then refers to the pathologist for final assessment and communication with the clinician. The use of the PANDA system will eliminate many of these steps.

The Advia cytograms have the potential to classify a leukaemia as acute or chronic (refer to section 3.2), MPO positive or negative and to sub classify Acute myeloid leukaemias (AML) according to the FAB classification. Some automated analysers have shown variable results in detecting blasts as lymphoblasts were counted as lymphocytes and myeloid blasts were counted as monocytes(21). Accurate blast detection is essential to differentiate acute and chronic leukaemias. However, the Advia 2120 demonstrated 100% sensitivity in detecting blasts and flagged samples that had only 2% blasts present(24). Using the PANDA approach, the Advia analyser displayed a 94.4% and 95.2% specificity for AML cases and a specificity of 100% for ALL (24, 32).

The results from studies conducted in the developed world cannot be extrapolated to the local setting as patients often have concomitant chronic infections such as HIV which may alter the

morphology. Our study explores the value of the Advia 2120 in leukaemic subtyping in a high throughput laboratory.

1.6 Myeloperoxidase activity in leukaemic blasts

Enzyme cytochemistry on peripheral blood smears or bone marrow aspirate slides is traditionally regarded as the gold standard for assessment of MPO activity in leukaemic blasts in order to confirm an AML and identify subtypes of AML (16). The development of lineage specific monoclonal antibodies, including cytoplasmic MPO, has established the role of immunophenotypic analysis in the classification of AML (7). There are varied results on the performance of conventional cytochemistry (CC) and immunophenotypic analysis in the assessment of MPO activity in leukaemia. Some studies suggest that CC is superior to immunophenotypic analysis (33) and others highlight that immunophenotypic analysis is the more sensitive technique (34, 35).

The accuracy of the two methods depends largely on the technical ability of staff to prepare reagents and samples, the presence of successful positive and negative controls, and a diagnostician that can confidently interpret the findings. In addition monoclonal antibodies are expensive. Within the National Health Laboratory Services (NHLS) CC and immunophenotypic analysis are usually performed in the academic setting.

The peroxidase cytogram on the Advia printout could potentially provide information on the MPO activity in leukaemias prior to a patient sample reaching a central laboratory. To date, a literature search reveals no studies that have compared peroxidase activity on the Advia printout to immunophenotypic analysis and CC.

1.7 Research Hypothesis

We hypothesised that in the initial classification of haematological malignancies from peripheral blood samples, the FBC, differential count and cytograms generated by the Advia 2120 are as accurate as manual morphologic assessment.

1.8 Aim and Objectives

The aim of this study was to determine the accuracy of the FBC, differential count and WBC cytogram assessment by the Advia 2120 using the PANDA classification in the initial classification of haematological malignancies.

The study had the following objectives:

1. To evaluate the performance of the Advia 2120 in the classification of haematological malignancies. This consisted of:
    a. Comparing the diagnosis obtained by either the Advia PANDA classification system and/or morphological assessment to the final diagnosis obtained on flow cytometry.
    b. Comparing the myeloperoxidase activity obtained by the Advia Peroxidase cytogram to cytochemical and flow cytometry MPO analyses.
2. To correlate the Day 8 blast count in paediatric acute lymphoblastic leukaemia obtained by flow cytometry to that from the Advia 2120.

Chapter 2: MATERIALS AND METHODS

2.1 Study design and study population

The flow cytometry records at the National Health Laboratory Services (NHLS), Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) were reviewed from November 2009 to May 2012, to identify cases of leukaemia. These included adult (>/= 15 years) and paediatric (<15 years) patients who were admitted either to CMJAH or Chris Hani Baragwanath Academic hospital (CHBAH). Leukaemia cases confirmed by flow cytometry that did not have an FBC analysed by the Advia haematology analyser at the haematology laboratory at CMJAH were excluded. The FBC and differential count of all cases recruited from CHBAH were re-analysed on the Advia haematology analyser at CMJAH.

Ethical clearance was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg prior to data collection. The clearance certificate number is M110323 (Appendix 1).

2.2 Sample collection

The investigations (flow cytometry, full blood count with differential and morphological assessments) for each case were requested by the treating physician as part of the diagnostic work up. No new samples were obtained from the patients specifically for this study.

Peripheral blood sample collected in an Etheylenediamine tetra acetic acid (EDTA) anticoagulated tube and the routine FBC and differential count were analysed on the ADVIA 2120 (Siemens healthcare diagnostics, Illinois, USA). A printout of the result, including the WBC cytograms, for each case was collected. Cases prior to April 2011 were retrieved from the laboratory data storage system , as the laboratory backs up data to prevent data overload, while the remaining cases were retrieved directly from the instrument database.

Samples for flow cytometry were collected in lithium heparin tubes and submitted for analysis. The complete immunophenotypic analysis report was authorised on the laboratory information system, WxDisa. The report included the final diagnosis, as well as the MPO result and Day 8 blast count if applicable.

The peripheral smears of the retrospective leukaemia cases, which were filed with the bone marrow aspirate samples in the laboratory in alphabetical order, were collected. A peripheral smear was made from the FBC samples received from CHBH that were reanalysed on the Advia 2120.

2.3 Laboratory procedures for sample analysis

Laboratory specific standard operating procedures were followed for the analysis of the flow cytometry, FBC, cytochemistry samples and the manual review of peripheral smears (36-42).

2.3.1 Immunophenotypic analysis

Peripheral blood mononuclear cells were separated by density gradient centrifugation using the Ficoll Separation Technique (Ficoll/Histopaque), washed with phosphate buffer solution and incubated with monoclonal antibodies conjugated with fluorescein isothyocynanate (FITC), phycoerthrin (PE), peridinin chlorophyll protein complex (PerCP) and allophycocyanin (APC) (36). The monoclonal antibodies are specific for cellular antigens known as cluster of differentiation (CD). Morphological assessment and standardised diagnostic panels were used to guide the choice of monoclonal antibodies (37). According to the laboratory standard operating procedure, if an acute leukaemia is suspected morphologically, an acute screen comprising of blast surface and lineage specific markers (myeloid, B and T-cell markers) are chosen (**Table 2.1**). If the morphological features are in keeping with a chronic lymphoproliferative disorder a chronic screen (B, T-cell surface markers and disease specific markers) is used (**Table 2.1**).

Table 2.1 Immunophenotypic markers used in flow cytometry

	Blast surface markers	Myeloid antigens	Lymphoid Antigens	Other antigens
Acute leukaemia	CD34, CD117	CD13, CD33, CD11b, CD13, CD14, CD15, cytoplasmic myeloperoxidase	*B-cells* CD19, CD20, CD22, kappa, lambda *Tcells* CD3, CD4, CD8, CD2, CD7, CD5, CD1a cytoplasmic CD3	CD45 HLA-DR Glycophorin A CD10 CD5, CD10
Chronic leukaemia			*B-cell* CD19, CD20, CD23, kappa lambda *T-cells* *CD3, CD4, CD8, CD7, CD2*	CD11c, CD103 CD25 CD16, CD56
Myeloma panel	CD38, CD56, CD45, CD19, CD138, CD20, kappa, lambda			

CD, clusters of differentiation

Analysis was performed on a dual-laser FACS Calibur (Becton Dickinson,San Jose, CA,USA) equipped with Cell Quest Pro and PAINT-A-GATE Pro Software, after gating on 10 000 events per sample. All samples were analysed in the presence of appropriate isotypic controls.

2.3.2 Morphological analysis of cases

A Nikon ECLIPSE E400 microscope (Nikon, Tokyo, Japan) was used to analyse the peripheral smears. The slides were stained with the May-Grünwald-Giemsa stain and a manual count was performed on 100 WBCs at the 50x magnification (38). If ≥ 20% blasts were seen, a diagnosis of acute leukaemia was made. The absence of blasts or a blast count of <20% were indicative of chronic leukaemia. The presence of Auer rods in the blasts favoured a leukaemia of myeloid lineage. The morphology was then reviewed in conjunction with the cytograms and a provisional diagnosis made.

The initial morphological assessment was made by an independent medically qualified morphologist (registrar or pathologist), a provisional diagnosis was made and this was used to to guide immunophenotypic analysis. The morphological assessment for each case was repeated by the investigator to ensure that she remained blinded to the final diagnosis. At study completion, the morphology findings obtained by the investigator were compared to the previous findings noted by the initial morphologist.

2.3.3 Advia WBC cytogram analysis

The Advia 2120 has two independent channels which are used to generate a WBC and differential count using peroxidase and Baso reagents. The Basophil method generates the total WBC and the peroxidase method provides the differential count and a secondary WBC. Commercial controls were analysed twice daily on the Advia 2120 and patient samples are only processed if controls are within two standard deviations of the known target value (39).

Myeloperoxidase activity was assessed from the peroxidase chamber using the patterns described by G. Onofrio(32). Cells that scattered near the y-axis have no myeloperoxidase while cells that scattered further away from the y-axis have increasing myeloperoxidase activity. The results of the Advia peroxidase cytogram were compared to the myeloperoxidase result obtained by flow cytometry and/or cytochemical staining. The basophil cytogram was used to assess the pattern of the mononuclear cell cluster (**Refer to section 3.2**).

The Advia triggers a blast flag when the % blasts is between 1.5% to 5.0% and %LUC

≥ 4.5%; or % blasts > 5.0% of the total WBC, or % BASO + %BASO Suspect + % BASO Saturation ≥ 10% (39).

a. Pilot study to define the criteria to be used in the analysis of the Advia 2120 White blood cell cytograms

Ten randomly selected cases were analysed to identify emerging patterns that would assist in the classification of cases and to develop a standardised and objective method of reviewing cases in this study. The analysis of the cytograms of each case was based on information obtained from the Advia 2120 user manual and PANDA analysis guidelines. We also looked for additional parameters from the Advia printout that assisted in classification of leukaemia. These included: patient age, WBC, haemoglobin, platelet count and blast flag.

The following approach was used to analyse each cytogram:

If the blast flag was triggered by the analyser, an acute leukaemia was suspected. The patterns seen on the basophil cytogram were used to classify the sample as D0 **(Figure 3.2)**, D1**(Figure 3.3)** or D2 **(Figure 3.4),** and the peroxidase cytogram pattern was used to classify the sample as P0-P6 (**Figure 3.1**) (27).The baso and peroxidase pattern classification of each case were integrated and the PANDA classification grid **(Table 1.3)** was used to determine the differential diagnosis. The age of the patient and the severity of cytopenias were used to determine the most likely diagnosis.

2.3.4 Myeloperoxidase activity

Intracellular MPO was performed by cytochemical staining or by flow cytometry. Conventional cytochemical staining was performed by fixing the slide for sixty seconds, washing with tap water and air drying. The slide was then dissolved in 3-amino-9 ethylcarbazole, dimethylsulfoxide and acetic acid sodium hydroxide. The slides were incubated, washed again, counterstained with Mayer's/Gill haematoxylin, rinsed and air dried. The reaction product is brown granular cytoplasmic staining. The presence of staining in at least 3% of the blasts is in keeping with an Acute Myeloid Leukaemia (40).

Detection of MPO activity by flow cytometry involved the use of Intrastain to fix and permaeabilise the peripheral blood mononuclear cells followed by the addition of the myeloperoxidase monoclonal antibody. The sample was incubated and then analysed by the flow cytometer. A second tube that was negative for MPO antibody was used as a control (41). On flow cytometry positive MPO staining in >/= 10% blasts is in keeping with a myeloid leukaemia. Assessment of MPO activity from the Advia printout is outlined in section 2.3.3.

2.3.5 Day 8 blast count

A whole blood sample is used for the analysis of the Day 8 blast count. The sample was first analysed on the Advia 2120 to obtain the total WBC. Then, 100ul of whole blood was added to a tube with monoclonal antibodies, incubated for 15 minutes at room temperature, lysed with ammonium chloride and washed with phosphate buffer solution. The choice of monoclonal antibodies was dependent on the presentation immunophenotype. The sample was analysed on the FACSCalibur (42). The Day 8 blast count was correlated with morphological assessment and was reported as an absolute value of the total White cell count.

The results of %LUC and % blast suspect(reflecting the percentage of abnormal cells in the D8 sample) generated by the Advia were used to calculate an estimated day 8 blast count. The absolute Day 8 blast count was calculated using the following calculation:

$$[(\%LUC + \%\ blast\ suspect)/100]\ x\ WCC(x10\text{^}9/l) = D8\ blast\ count\ (x10\text{^}9/l)$$

The result of the above calculation was recorded as <1x10^9/l or >1.0x10^9/l, and was compared to the value obtained on flow cytometry.

2.4 Data analysis

All of the results were tabulated on Microsoft excel spreadsheets. The final diagnosis obtained from analysis of the WBC cytograms, morphology and the combination of both analyses were compared to flow cytometry.

For the purpose of this study, the following definitions were used:

Diagnosis: the final classification of leukaemia

Inaccuracy: discrepant result obtained on cytogram/morphology or combination when compared to the final diagnosis obtained by flow cytometry

True positive (TP): diagnosis made on cytogram/morphology or combination correlated with the final diagnosis obtained by flow cytometry

False negative (FN): a diagnosis was not made on the cytogram/morphology or combination and a diagnosis was made by flow cytometry

False positive (FP): the diagnosis made on the cytogram/morphology or combination did not correlate with the diagnosis made by flow cytometry.

The results of each analysis were classified as TP, FN, FP when compared to the final diagnosis obtained by flow cytometry. True negatives could not be calculated as only cases of leukaemia were included. Refer to **Appendix B** which includes the raw data of the FN and FP cases that were classified using PANDA analysis.

True negatives were only calculated for specific classes of leukaemia and this enabled the calculation of sensitivity and specificity for these classes. The positive predictive value (PPV) and accuracy were calculated for each category using the following formulas:

$$PPV = \frac{true\ positives}{true\ positives + false\ positives} x100\%$$

$$\text{Accuracy} = \frac{true\ positives + true\ negatives}{true\ positives + false\ positives + false\ negatives + true\ negatives} x100\%$$

Similarly the myeloperoxidase activity assessed on the peroxidase cytogram was compared to flow cytometry and/or cytochemistry. A true positive (TP) was defined as positive peroxidase activity on all methods. A true negative (TN) was defined as absence of peroxidase activity on all methods. A false positive (FP) was defined as positive peroxidase activity on cytogram analysis and negative MPO on the reference methods and a false negative (FN) was defined as negative MPO on peroxidase cytogram and positive MPO on the reference method. The Cohen's kappa, Mcnemar test for symmetry, sensitivity and specificity were calculated using

the evaluation protocol (EP) evaluator (Data innovations, Vermont, USA). The EP evaluator is a software programme designed to evaluate and measure clinical laboratory performance of testing methods.

Microsoft excel 2010 was used to determine the correlation between the D8 blast counts obtained from the Advia printout and the reference method.

Chapter 3:RESULTS

3.1 Study population

206 cases of immunophenotypically confirmed leukaemias were identified from review of the flow cytometry records. The Advia printouts of 56 cases could not be retrieved due to technical issues with data storage. This study was therefore conducted on 150 cases, of which 50 cases were paediatric (<15 years old) and 100 cases were adults (>/= 15 years old). The patients' ages ranged from nine months to 82 years and the overall male: female ratio was 1.3:1.

The spectrum of haematological malignancies **(Table 3.1)** included ALL (49/150; 32.1%), AML (48/150; 32.1%), CML (15/150; 10.1%), CLL (20/150; 13%), other chronic lymphoproliferative disorders (3/150; 2%), Non-Hodgkin lymphoma (NHL) (9/150; 6%) and <1% plasma cell leukaemia. There were two cases of Acute Leukaemia of ambiguous lineage. Classification of leukaemia was not possible in one case due to insufficient sample volume for immunophenotypic analysis and the patient demised before further investigations.

Table 3.1: Spectrum of haematological malignancies in patient cohort

Haematological malignancy	Number of patients (n=150)
Acute lymphoblastic leukaemia	49
Acute myeloid leukaemia	48
Chronic myeloid leukaemia	15
Chronic lymphocytic leukaemia	20
Non- Hodgkin lymphoma	9(8/9 Burkitt lymphoma)
Plasma cell leukaemia	1
Other Chronic Lymphoproliferative disorders (mantle cell lymphoma, follicular lymphoma)	5
Acute leukaemia (ambiguous lineage)	2
Acute leukaemia (no lineage)	1 (insufficient sample, patient demised)

The provisional morphology diagnoses obtained by the investigator and the initial morphologist assessment were the same in all the cases (150/150; 100%).

3.2 Analysis of the white blood cell cytograms

The pilot study included the following spectrum of leukaemia cases: ALL (4/10), AML (2/10), CLL(1/10), APL (2/10) and NHL (1/10). The lymphoid cases showed a true downward left lateral shift of the mononuclear cell cluster pattern on the basophil cytogram and the myeloid cases showed a right shift towards the polymorphonuclear cell gate (**Figure 1.2**).

The peroxidase cytograms were classified as P0-P6 (**Figure 3.1 and Table 3.2**) and baso cytograms as D0-D2 **(Figures 3.2, 3.3 and 3.4).**

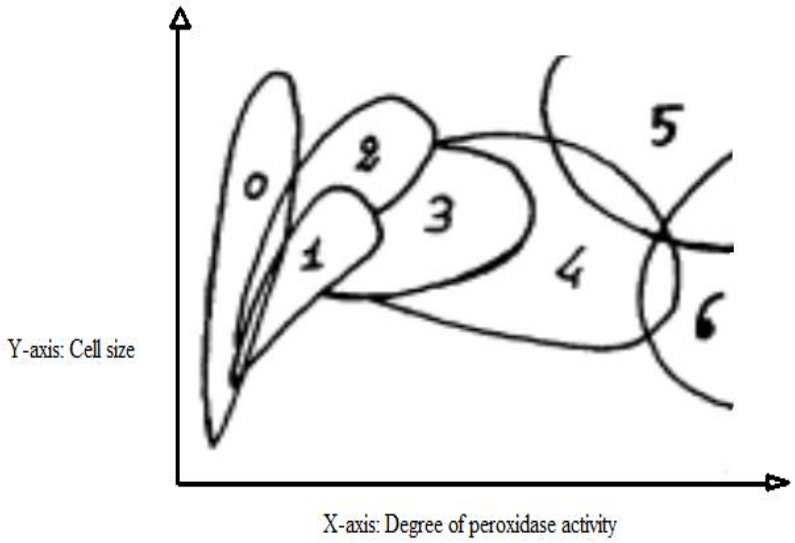

Figure 3.1: Approximate areas corresponding to leukaemia cell differentiation according to degree of peroxidase activity (27)

Table 3.2: Description of peroxidase patterns on cytogram P0-P6 (27)

Category	Pattern Description
P0	Peroxidase negative with no myeloid differentiation
P1	Scattering at the top of the main cell cluster indicating the presence of some cells with MPO activity and either early or partial myeloid differentiation
P2	Homogenous cluster of cells separated from the LUC area continuing across the monocyte area and about to enter the neutrophil area of the cytogram
P3	Moderate to strong peroxidase activity and homogenous cell size
P4	Strong heterogenous myeloperoxidase activity
P5	Strong peroxidase activity in very large cells
P6	Extremely intense peroxidase activity

In the D0 pattern the shape of the mononuclear cell cluster is noted as normal **(Figure 3.2)**. There is a down/leftward shift of the mononuclear cluster indicating the presence of cells with immature chromatin and the blast flag is positive in the D1 pattern **(Figure 3.3)**. In the D2 pattern there is an upward shift of the mononuclear cluster suggesting the presence of large cells with heterogeneous chromatin and is usually blast flag negative **(Figure 3.4)**.

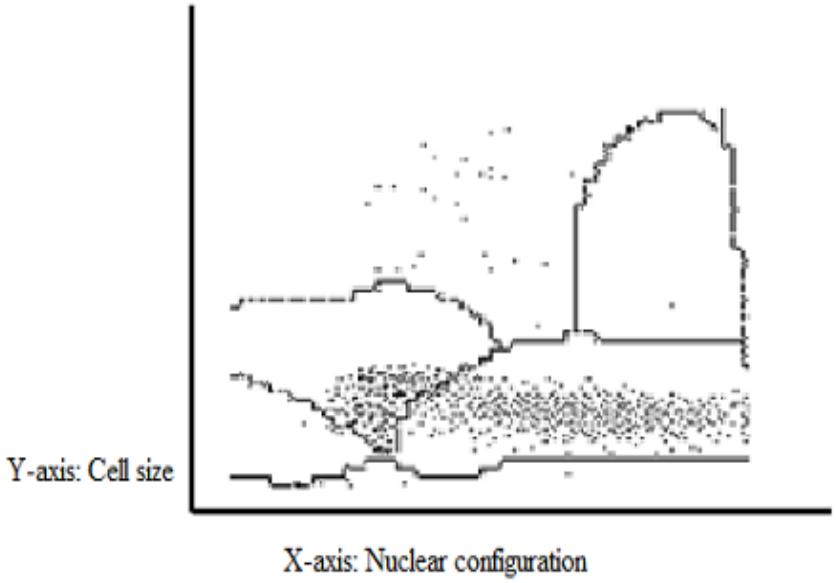

Figure 3.2: Basophil cytogram illustrating D0 (27)

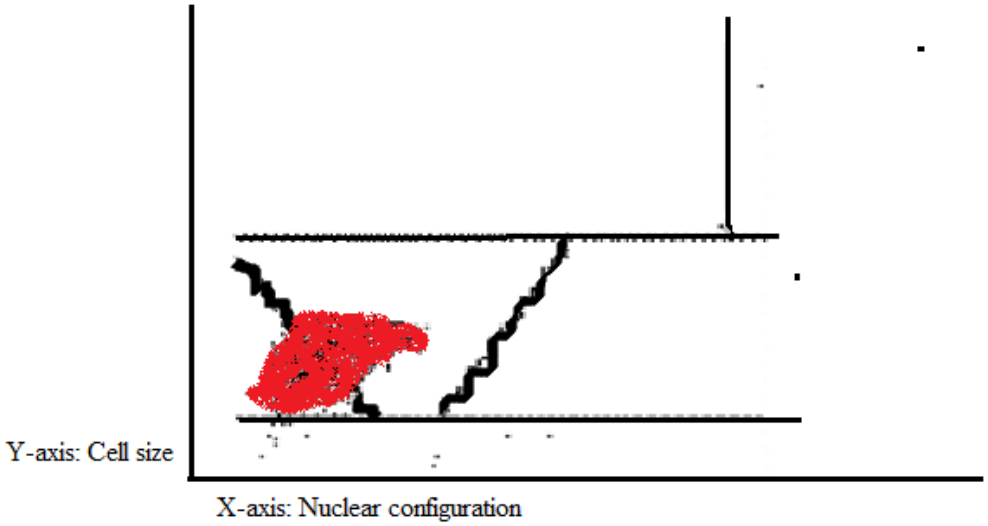

Figure 3.3: Basophil cytogram illustrating D1(27)

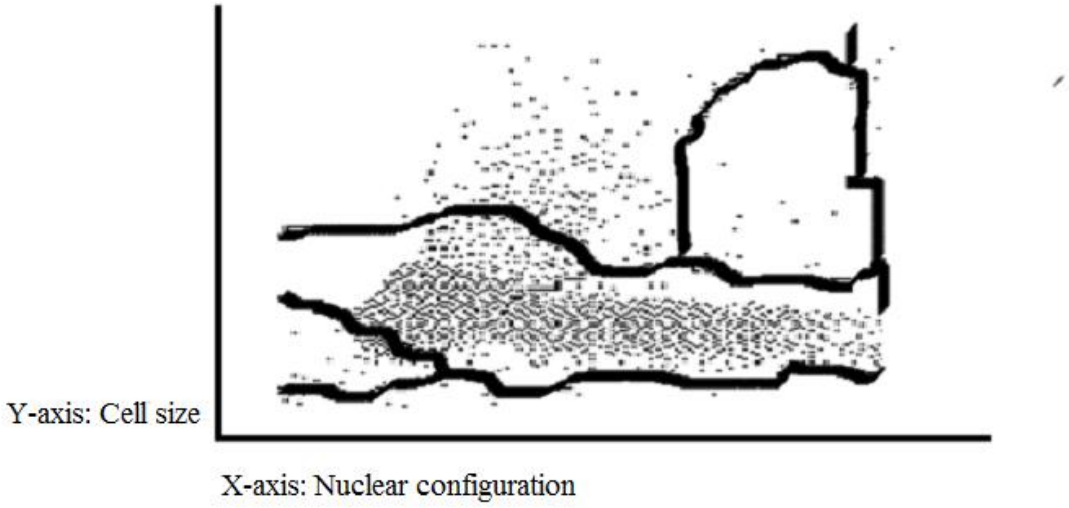

Figure 3.4: Basophil cytogram illustrating D2 (27)

Analysis of the peroxidase cytogram revealed that the lymphoid malignancies, including ALL, CLL and NHL, produced a P0 pattern. However, Acute Myeloid Leukaemia FAB M0 could not be excluded from this group as the blasts are negative for peroxidase activity. A P6 pattern was observed in APL and combined P2/P3 patterns were observed in other AML cases. Cases with a P0 pattern were defined as MPO negative and cases that had a P1-P6 pattern were MPO positive.

After identification of the D and P patterns from the basophil and peroxidase cytograms respectively, the leukaemia could be classified using The PANDA classification grid **(Table 1.3)**, which aided in establishing a differential diagnosis for most cases. The two cases of AML, for example, produced a D1 pattern which identified them as an Acute Leukaemia, and peroxidase activity was easily demonstrated with P3/P4 patterns in keeping with myeloid origin. Conversely, CLL, which is a chronic lymphoid malignancy produced a D0P0 pattern. APL is the only leukaemia with a unique pattern, which is classified according to the PANDA system as D1P6. The results of the pilot study suggested that the P and D patterns that were described by d'Onofrio and colleagues may be applicable in our setting.

3.3 The PANDA analysis system and morphology in the overall classification of leukaemia

The final diagnosis obtained by analysis of the PANDA system, peripheral smear morphology and combination of the two was compared to the diagnosis obtained by immunophenotypic analysis. Using the PANDA analysis system, 48% (72/150) of cases were accurately classified. The inaccuracy was 9.3% (14/150) and 42.7% of cases could not be classified. The positive predictive value (PPV) was 88%. The raw data of cases which had discrepant results on PANDA analysis are included in **Appendix B**. One of the main reasons for the inaccuracy was the difficulty in differentiating between acute lymphoblastic and acute myeloid leukaemia with the sub type FAB-M0, both of which produce a D1P0 pattern. There were 30 cases with a D1P0 pattern. Only 53.3% (16/30) cases that displayed a true downward left lateral shift of the mononuclear cell cluster on the basophil cytogram were correctly diagnosed as ALL. An incorrect diagnosis was made in 26.7% (8/30) cases based on this pattern. A lateral shift of the mononuclear cell cluster towards the polymorphonuclear gate was observed in 6.7% (2/30) and these cases were in keeping with a diagnosis of AML. In 13.3% (4/30) cases it was difficult to describe the pattern of the mononuclear gate.

Comparison of morphological assessment alone to immunophenotypic analysis accurately classified 54% (82/150) of the leukaemia cases with only one inaccurate diagnosis. A final diagnosis could not be made in 44% (67/150). The positive predictive value of morphology was 99% **(Table3.3)**. However, the accuracy was improved when the ADVIA cytogram was used in conjunction with morphological analysis, as 90% (135/150) of cases were accurately classified. 5.3% (8/150) were classified incorrectly and a diagnosis was not possible in 7/150 (4.7%). The positive predictive value using morphology and the ADVIA printout was 94% **(Table 3.3).**

Table 3.3: Summary of final diagnosis obtained from PANDA analysis, morphology and PANDA analysis plus morphology

Investigations used for the analysis of cases	TP N=150	FP N=150	FN N=150	Accuracy %	PPV %
PANDA ANALYSIS (CYTOGRAM)	72	14	64	48	88
MORPHOLOGY	82	1	67	54	99
PANDA ANALYSIS+ MORPHOLOGY	135	8	7	90	94

True positive (TP): PANDA system or morphology or PANDA system plus morphology correlates with the final diagnosis
False positive (FP): PANDA system or morphology or PANDA system plus morphology does not correlate with the final diagnosis
False negative (FN): No diagnosis obtained from PANDA system or morphology or PANDA system plus morphology
Positive predictive value (PPV)

3.4 PANDA system and morphology in the classification of specific sub types of leukaemia

As PANDA plus morphology produced the highest accuracy, the sensitivity and specificity for ALL, chronic myeloid leukaemia and chronic lymphocytic leukaemia were calculated for this group. The absence of that specific class of leukaemia was used to define the true negative group. The sub-classification of AML using PANDA and morphology was reviewed.

a. Acute lymphoblastic leukaemia

   The sensitivity of using PANDA analysis in conjunction with the morphology to diagnose ALL was 89.6% and the specificity was 99%.

b. Acute myeloid leukaemia

   The sub-classification of the AML cases is illustrated in **(Table 3.4)**. Of the 48 AML cases, flow cytometry was unable to provide a provisional FAB classification in 19 of the cases. 73.7% (14/19) were correctly diagnosed on analysis of the cytogram in combination with morphology as AML. 3/19 (15.8%) were incorrectly classified.

All 8 cases diagnosed as FAB M3, APL, were accurately classified on cytogram analysis based on the distinct D1P6 pattern on the cytograms. All cases with FAB M2 and AML transformed from underlying CML were correctly classified and 85% (6/7) of the M5b cases **(Table3.4)**.

Table 3.4: Classification of the acute myeloid leukaemia cases

Type of myeloid leukaemia	**Number of Patients**	**Number classified correctly on cytogram and morphology**
M1	2	0
M2	3	3
M3	8	8
M4	1	0
M5a	4	2
M5b	7	6
M7	1	0
AML not classified as FAB	19	14
AML from CML	2	2
Total number	48	35

c. Chronic myeloid leukaemia

The sensitivity of the PANDA system in diagnosing CML was 100% and the specificity was 97.8%.

d. Chronic lymphocytic leukaemia

The sensitivity and specificity of the PANDA system and morphology to diagnose CLL the sensitivity was 100% and specificity was 100%.

3.5 Comparison of the Myeloperoxidase (MPO) activity obtained from the ADVIA peroxidase cytogram to flow cytometry/cytochemistry

36.7 % (55/150) had an MPO done on flow and/or cytochemistry. When these results were compared to the peroxidase cytogram result, 30.9 % (17/55) yielded a true positive result, 7.3 % (4/55) yielded a false positive result, 5.4% (3/55) yielded a false negative result and 56.4% (31/55) yielded a true negative result. The sensitivity and specificity of the peroxidase cytogram in evaluating the presence or absence of MPO are 85% and 88.6% respectively. The negative predictive value (NPV) was 91.2% and the positive predictive value was 85.7% The

agreement between cytogram peroxidase activity and the reference methods was 89.1%, the McNemar test for symmetry passed with a p value > 0.999 and the Cohen's kappa was 76.9%.

3.6 Comparison of the Day 8 blast count obtained on the Advia to flow cytometry

The Day 8 blast count was evaluated in 25 newly diagnosed ALL cases and revealed an r value of 0.377 ($p<0.05$), slope = 0.055 ($p<0.05$) and y intercept value of 0.354 ($p<0.05$). In 88% (22/25) of these cases the day 8 blast count calculated from the Advia printout correlated with the value obtained from flow cytometry (i.e.<1x10^9/l or >/=1x10^9/l). In the remaining 3 cases flow cytometry detected a blast population of >1.0 x10^9/l while the ADVIA detected <1.0x10^9/l of % blast suspect and LUC.

Chapter 4: DISCUSSION

4.1 The efficacy of the Advia WBC cytograms in the classification of leukaemia when using the PANDA classification system

In an era where the use of automated haematology analysers is growing at a rapid pace, a review article by a South African scientist explored the value of the PANDA system in the classification of leukaemia, in particular APL (43). To the best of our knowledge there are no previous studies from South Africa assessing the PANDA system. In South Africa there is a delay in diagnosing and classifying leukaemia owing to the critical shortage of experienced morphologists in the peripheral laboratories. Peripheral blood smears are often requested by clinicians because cytopenias are common in a population burdened by HIV, Tuberculosis and malaria (44, 45). Innovative strategies to improve diagnostic services in laboratories outside of the academic centres are necessary to improve patient care (46, 47). Therefore assessment of the PANDA system in all leukaemic subtypes was important in our setting.

In our study males were more affected with leukaemia than females, and this finding concurs with the reported increased incidence of leukaemia in males (7). The majority of the samples were from the adult population and possible reasons for this finding include transformation to an acute leukaemia from underlying chronic leukaemia, concomittant HIV infection, increased chronic leukaemias in adults and increasing genetic mutations with increasing age.

The accuracy of the Advia cytograms to classify leukaemia was only 48% and the accuracy of morphology was 54%. The PPV of all cases using the PANDA system was 88% and this shows that the ability to classify a leukaemia is high when a pattern can be identified. In our study, the accuracy of PANDA was much lower than that obtained by Gibbs (77.8%) and G. Onofrio (91.1%) in previous studies. Gibbs correctly classified 100% of the ALL and CLL cases on cytogram pattern recognition which improved his overall accuracy. The exact reason for the difference in the overall prediction accuracy of the cytograms between this study and Gibbs is uncertain , but it is noted that Gibbs only evaluated four cases of ALL compared to the 49 cases analysed in this study (28, 29). In the pilot study a true downward left lateral shift of the mononuclear cell cluster was observed on the baso cytogram in the ALL cases and there

appeared to be a right shift of the mononuclear cells towards the polymorphonuclear gate in the AML cases. The hypothesis was that a shift toward the mononuclear cell gate may be an indication of a myeloid differentiation. However when this pattern was further evaluated, only a little more than 50% of the cases were correctly classified. The conclusion is that shifts of the mononuclear cell gate is non-specific and cannot be used as a lineage indicator.

In myeloid leukaemia, the presence of a D1 pattern and distinct peroxidase activity were used to identify the AML cases. The CML cases showed a specific pattern which included a D0 pattern (absence of blasts) and a spectrum of peroxidase activity suggesting varying stages of granulocytic maturation. In 40 % of AML cases it was not possible to classify these cases according to the FAB classification on cytogram analysis alone. In all the cases of APL, a correct diagnosis was made on evaluation of the cytogram alone due to the distinct D1P6 pattern. The advantage is that there is no other differential diagnosis for this pattern on the classification grid and this was confirmed in a previous study (48). The importance of this finding is the impact it has on the immediate management of the patient in terms of reducing the bleeding risk and the initiation of Al trans retinoic acid. However none of the cases included in this study were of variant APL, and the pattern of the typical APL may be different from the variant. In Gibbs's retrospective review of AML, four cases of variant APL had variable PANDA patterns which included one case with a P5D1 profile, another case with a P4D1 and the remaining two cases with D1P6 pattern (48).

Other than APL, all the other AML subtypes are duplicated across the PANDA classification grid (**Table 1.3**), which makes the classification difficult. This reflects the heterogenous nature of the disease, a finding that was noted previously by Gibbs (28). Interesting observations were noted in the AML M5a and M5b cases: although most of the cases had a D1 pattern with varying amounts of peroxidase activity, two of the AML cases with monocytoid differentiation had atypical patterns: D0P0 and D2P3. The reason for this is uncertain. It is possible that because of the spectrum of disease, in some cases the blasts are more mature and the ADVIA analyser identifies these cells as more mature cells, hence the D0 pattern. The reason for a D2 pattern is not clear, but could be related to the leucocytosis. Most AML M5a cases produced the typical D1P0 pattern, except for one which had a D2P3

pattern. Again, this may be due to the leucocytosis or the atypical morphology of the monoblasts. As the remaining AML subtypes were not well represented it was not possible to determine any further emerging patterns. The data reaffirmed previous findings that the PANDA classification is a good indicator of AML, but poor at sub-classification except for the M3 subtype (28).

In lymphoid leukaemia, the majority of cases (64%) could not be classified after Advia WBC cytogram analysis. On review of the PANDA analysis grid, it was noted that the differential diagnosis for a D1P0 and D0P0 patterns is very heterogenous. It is very difficult to commit to a single diagnosis based solely on cytogram analysis. The D0P0 pattern includes ALL, CLL, PLL and HCL and unless patient demographics and severity of cytopenias are also taken into account, it is near impossible to narrow the differential diagnosis. In the paediatric cases that presented with a marked leucocytosis and a DOP0 pattern a diagnosis of ALL was favoured as CLL, HCL and PLL is unlikely in a child. In addition the absence of peroxidase activity does not exclude the possibility of an AML as M0, M5a, M6 and M7 which are classically MPO negative. The remaining cases that could not be classified included the following patterns: D2P1, D2P3 and D2P1. The limitation of the PANDA classification grid is that there is no differential diagnosis for D2P1 and D2P3 cases. The D2 pattern was associated with a marked leucocytosis, suggesting that the distribution of cells on the basophil cytogram is influenced by a high WCC. Some of the ALL cases had a D2 pattern which excluded the presence of blasts. This could be attributed to varying blast morphology with small cells that had heterogenous chromatin and the Advia identifying these cells as lymphocytes.

4.2 Role of the Advia WBC cytograms in combination with morphology in the classification of leukaemia

Morphological analysis accurately identified 54% of cases. The cases that were diagnosed on morphology alone included those with features in keeping with chronic myeloproliferative and lymphoproliferative disorders overall favouring CML and CLL respectively, APL, typical L3 morphology favouring a Non-Hodgkin's lymphoma, Auer rods present confirming the presence of myeloblasts in AML and a case with plasma cell leukaemia. Manual review of

peripheral smear remains valuable as subtle morphological features can be missed on automated haematology analysers (19).

The diagnosis could not be made on morphology alone mainly in cases of acute leukaemia whereby the morphology of the blasts was non-specific making differentiation between myeloid and lymphoid blasts very difficult. These included cases of AML FAB M1, M5a, M5b, M7 and 2 cases of acute leukaemia of ambiguous lineage in which the morphology was not distinctive to confirm the diagnosis.

However when the Advia printout was used in combination with the morphology findings, there was a significant improvement in being able to make a provisional diagnosis with 90% of cases being correctly diagnosed. This supports that information obtained from the cytograms aids morphology and vice versa. In all except 2 cases with a D0P0 pattern, with morphological features of acute leukaemia the correct diagnosis of ALL was made using the PANDA classification grid. The 2 cases that were incorrectly diagnosed had a final diagnosis of AML, a differential diagnosis that does not feature on the PANDA D0P0 grid. Again this supports the heterogeneity of AML, and morphologically these blasts were small and had no appreciable features to suggest myeloid origin. Review of flow cytometry and cytochemistry results for these two cases suggest that these cases could be classified as AML- FAB M0.

The PPV for morphology and Advia cytogram analysis decreased from morphologic analysis alone due to the increased number of false positives (FP=8). Four of the false positive cases were AML cases, where the morphology was non-specific and on PANDA analysis a D1P0 pattern was noted with a downward lateral shift of the mononuclear cell gate resulting in these cases being classified as ALL. Cases of Acute leukaemia of ambiguous lineage accounted for two of the false positive cases. The remaining cases were an ALL case with a D2P1 pattern and an AML case with a D2P3 pattern, which do not form part of the differential diagnosis for these categories on the PANDA classification grid. The case with a D2P1 pattern had morphology that was in keeping with an acute leukaemia and because of the apparent presence of peroxidase activity a diagnosis of AML was made. This did not correlate with the final diagnosis of ALL. A possible reason for this discrepancy could be that the background

immature granulocytes were displaying peroxidase activity and on cytogram review these populations could not be separated from the large unstained cells, so it appeared that the tumour population had a small amount of peroxidase activity.

The remainder of cases that had a D0P0 pattern had morphological features of a chronic lymphoproliferative disorders favouring CLL. On review of the PANDA grid, the other differential diagnosis included PLL and HCL which usually have distinct morphological features. Hence a diagnosis of CLL was favoured and this was confirmed on immunophenotypic analysis.

The results show that the combined used of the cytograms with morphology definitely aid in the diagnosis of certain leukaemias, in particular cases that have a D0P0 and D1P0 patterns.

4.3 The Advia myeloperoxidase activity

The sensitivity and specificity of the peroxidase cytogram in predicting the MPO activity was 85% and 88.6% respectively. The sensitivity was reduced because of the presence of false negative results which included the cases of acute leukaemia of ambiguous lineage which had a very small population of ~3-5% blasts that were MPO positive on flow cytometry and a case of AML with very dim MPO positivity. This indicates that although the Advia MPO correlates well with conventional methods, it is limited when the target population with peroxidase activity is small. Four cases yielded a false positive result: The case of Non-hodgkin lymphoma had a background leucoerythroblastic reaction and the immature granulocytes that showed myeloperoxidase activity could not be isolated from the LUC and a diagnosis of AML was made. Additionally, two cases with a P1 and P2 pattern on the peroxidise cytogram analysis were MPO negative on flow cytometry and this could again reflect contamination from the background granulocyte precursors where as flow cytometry specifically focuses on the blast population.

The above findings highlight the potential use of the peroxidase cytogram to screen for MPO activity. Conventional MPO cytochemistry is time consuming, requires a skilled technologist and expensive control materials. In addition flow cytometry is expensive, time consuming and

again needs an experienced technologist. The EP evaluator (Data innovations) used by the NHLS compared the Advia MPO to the reference investigations for MPO: the McNemar test for symmetry passed with a p>0.999 and the Cohen's Kappa was 76.9% suggesting that there is high agreement between the methods, above what is expected by chance. To save time and reduce laboratory costs assessment of MPO activity from the perox cytogram can be used as a screening tool if the blast population is greater than 5%.

The disadvantage of the Advia is its inability to quantify the number of blasts that are MPO positive which is important in classifying myelomonocytic leukaemia and acute leukaemias of ambiguous lineage.

4.4 Day 8 blast count

Analysis of the Day 8 blast count revealed that there is poor correlation between the absolute values of the blast counts obtained on flow cytometry and the Advia analyser with an R value of 0.33. However when using the clinical significant value of <1.0 x10^9/l (9, 11), 22/25 (88%) day 8 blast counts calculated from the Advia printout paralleled the value obtained on flow cytometry (i.e.<1x10^9/l or >/=1x10^9/l). The Advia under-estimated the Day 8 blast count in 3 cases , that flow cytometry detected >1x10^9/l blasts. A possible reason for this difference included the presence of small lymphoid blasts that were counted by the FBC analyser as lymphocytes rather than blasts.

The findings suggest that the Day 8 blast count from ADVIA printout could be used as surrogate for flow cytometry as a predictor of prognosis in childhood leukaemia in resource constrained areas that do not have access to flow cytometry. However accurate absolute quantification of blasts was affected as in many of the cases the lymphoid blasts were counted as lymphocytes by the FBC analyser thereby underestimating the count. In view of this, it is unreliable to use the Day 8 blast count from the ADVIA machine and flow cytometry remains the investigation of choice.

4.5 Study limitations

The following limitations apply to this study:

- Although the patient cohort was relatively small, the cases reflect the spectrum of leukaemia seen at a tertiary hospital in Johannesburg and are similar to previous studies that explored a similar concept (31, 32, 48) . In the smaller subgroups, in particular the AML group there were too few cases from the different FAB subgroups and this made statistically significant findings difficult.
- The cytograms and morphology were analysed by one person and this introduced a potential bias to the study. To reduce the degree of bias, patient identifiers on the Advia printout were erased and assigned a study number. The cytograms were analysed separately and independent of morphology and the final diagnosis. The morphology was reviewed at a different time, in isolation of the Advia printout and final diagnosis. The morphology results were compared to the findings of the morphologist at the time of diagnosis. All the results were only correlated at the end of the individual analyses.
- The PANDA analysis grid also includes cases of MPO deficiency, AIDS, MDS and viral disease. Patients with these diseases were not included in the patient cohort as the focus of the study was the value of PANDA analysis in the classification of leukaemia.
- This study did not explore whether the Advia printout can be used to distinguish normal peripheral blood samples from leukaemic samples, as the aim of the current study was to establish if leukaemia can be classified on analysis of the cytograms.
- This study also illuminated some of the limitations of the current PANDA classification which includes the difficulties of sub classifying AML according to the FAB classification and differentiating ALL from AML without peroxidase activity. A revised cytogram classification grid is proposed **(Table 5.1).**

Chapter 5: CONCLUSION

The timeous identification and classification of leukaemia is crucial for patient survival, and is essential to prompt further investigations to confirm the diagnosis and to initiate treatment as soon as possible. The current system requires that a highly trained technologist identify the leukaemic infiltrate and then refer the smear to a pathologist for confirmation. The shortage of skilled and experienced morphologists in our setting especially in remote areas where pathologists are not available means that slides have to be transported to central laboratories for review. This leads to unnecessary diagnostic delays and may compromise patient outcome. The WBC cytograms from automated analysers can offer an effective, simple tool that can be used by less skilled morphologists to aid in the classification of leukaemia.

In this study it has been shown that the analysis of WBC cytograms in conjunction with morphology can improve the overall assessment of leukaemic cases by providing a provisional classification to the clinicians. Furthermore cytogram analysis alone has the potential to differentiate myeloid from lymphoid malignancies, provide a surrogate for the Day 8 blast count and to identify specific malignancies such as CML, APL and AML with definite peroxidase activity. In routine laboratory practice, amidst the many samples that are received, a simple review of cytograms of suspected leukaemia even before a slide is stained can alert the technologist/pathologist to a provisional diagnosis. Nonetheless it must be remembered that morphology is not a lost art as microscopic analysis of cases is essential as subtle morphology features are not appreciated by an automated analyser. Adequate training of both technologists and pathologists in the interpretation of cytograms in conjunction with morphology is strongly advocated.

Recommendations

We propose a revised PANDA classification grid to classify leukaemias (**Table 5.1**).

Table 5.1: Revised PANDA classification grid for the classification of leukaemias

	P0	P1/ P2/P3	P3/P4/P5	P6
D0	• Acute lymphoblastic leukaemia • Chronic lymphoid • Leukaemia		• Chronic myeloid leukaemia	
D1	• Acute lymphoblastic leukaemia • Non-Hodgkin lymphoma • Peroxidase negative acute myeloid leukaemia	Acute myeloid leukaemia with peroxidase activity	• Acute myeloid leukaemia with peroxidase activity • Chronic myeloid leukaemia-blast crisis	Acute promyelocytic leukaemia
D2	• Consider acute leukaemia			

The proposed classification is simpler and is not aimed at specifying different subtypes of leukaemia, but is implied rather as a screening tool. The limitation of the revised grid is that the focus is only on leukaemias while the differential diagnosis on the original grid included MPO deficiency, viral disease, myelodysplastic syndrome and AIDS. This was not explored in this study, and provides opportunity for future studies. In addition, the cytogram pattern for AML M3 variant was not assessed as no cases of this nature were collected during the study period, however a recent study that included four cases of FAB M3 variant revealed varying degrees of peroxidase activity. This study also confirmed the finding that cytograms can be used to identify a possible case of APL. Nonetheless, this proposed classification promptly alerts one to the provisional diagnosis of certain myeloid leukaemias and acute leukaemia.

The sensitivity and specificity of the peroxidase cytogram in evaluating the presence or absence of MPO are 85% and 88.6% respectively. It was shown that there is a high agreement between the ADVIA peroxidase cytogram interpretation and the reference methods (flow cytometry and cytochemistry). The findings suggest that in order to save time and reduce laboratory costs assessment of MPO activity from the peroxidase cytogram can be used as a screening tool if the blast population is greater than 5%.

The Day 8 blast count obtained from the ADVIA printout can possibly be used as a surrogate marker for prognosis in paediatric ALL especially in under-resourced health care facilities.

APPENDIX A: Ethics Clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Dashini Pillay

CLEARANCE CERTIFICATE	**M110323**
PROJECT	Diagnosis of Haematological Malignancies In The Era of Total Laboratory Automation: Comparison of The ADVIA 2120 To Immunophenotyping and Morphology Analyses
INVESTIGATORS	Dr Dashini Pillay.
DEPARTMENT	Department of Molecular Medicine & Haematology
DATE CONSIDERED	25/03/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 25/03/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Professor Johnny Mahlangu

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX B: Raw data of discrepant results using PANDA analysis

PANDA pattern	Favoured PANDA diagnosis	Analysis	Morphology	Analysis	PANDA and morphology	Analysis	Final diagnosis flow cytometry
D1P0	ALL	FP	Acute Leukaemia	FN	ALL	FP	AML
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D1P0	ALL	FP	Acute Leukaemia	FN	ALL	FP	AML
D0P0	NK	FN	Acute Leukaemia	TP	Acute leukaemia	TP	Acute Leukaemia
D2P0	NK	FN	Acute Leukaemia	FN	Acute leukaemia	FN	ALL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D2P0	NK	FN	Chronic LPD	TP	Chronic LPD	TP	Chronic LPD Mantle cell
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D1P0	ALL	FP	Plasma cell leuk	TP	Plasma cell leuk	TP	Plasma cell leuk
D2P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D1P0	ALL	FP	Acute Leukaemia	FN	ALL	FP	AML
D0P0	NK	FN	Acute Leukaemia	FN	AML monocytoid	TP	AML
D1P0	NK	FN	NHL	TP	NHL	TP	NHL- Burkitts
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D1P2	AML	FP	NHL	TP	NHL	TP	Burkitts
D0P0	NK	FN	NHL	TP	NHL	TP	Burkitts
D1P0	ALL	FP	Acute Leukaemia	FN	ALL	FP	AML
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D2P3	NK	FN	AML	TP	AML (M5b)	TP	AML (M5b)
D2P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D2P1	NK	FN	Acute Leukaemia	FN	AML	FP	ALL
D2P0	NK	FN	Acute leukaemia	FN	NK	FN	ALL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D1P0	ALL	FP	Chronic LPD	TP	Chronic LPD	TP	Chronic LPD- Mantle cell
D2P0	NK	FN	Chronic LPD	TP	Chronic LPD	TP	Chronic LPD Follicular lymphoma
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D1P4	AML	FP	CML	TP	CML	TP	CML
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	NHL	TP	NHL	TP	Burkitts
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL

D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D2P0	NK	FN	NHL	TP	NHL	TP	NHL
D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D1P1	AML	FP	NHL	TP	NHL	TP	Burkitts
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D1P0	ALL	FP	AML	TP	AML M5a	TP	AML M5a
D1P0	ALL	FP	Acute Leukaemia	FN	ALL	FP	Acute leukaemia ambiguous lineage
D0P0	NK	FN	Acute Leukaemia	FN	NK	FN	AML
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	NHL	TP	NHL	TP	Burkitts
D1P3	AML	FP	CML	TP	CML BP	TP	CML
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D2P3	NK	FN	Acute leukaemia	FN	AML M4/M5	TP	AML M5
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D2P0	NK	FN	Acute leukaemia	FN	Acute leukaemia	FN	ALL
D2P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D1P4	AML	FP	CML	TP	CML	TP	CML
D0P0	NK	FN	Acute leukaemia	FN	Acute leukaemia	FN	AML
D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D1P1	AML	FP	Acute leukaemia	FN	AML	FP	Acute leukaemia ambiguous lineage
D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D1P0	NK	FN	NK	FN	NK	FN	ALL
D0P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D1P0	NK	FN	AML	TP	AML	TP	AML (M5a)
D0P0	NK	FN	NHL	TP	NHL	TP	Burkitts
D0P0	NK	FN	Acute Leukaemia	FN	NK	FN	ALL
D1P0	NK	FN	Acute leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL

D0P0	NK	FN	NHL	TP	NHL	TP	NHL
D0P0	NK	FN	Acute Leukaemia	FN	ALL	TP	ALL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D0P0	NK	FN	CLL	TP	CLL	TP	CLL
D1P0	NK	FN	Acute Leukaemia	FN	AML	FP	ALL

Abbreviations

ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APL	Acute promyelocytic leukaemia
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CML- BP	Chronic myeloid leukaemia- blast phase
FN	False negative
FP	False positive
LPD	Lymphoproliferative disorder
NHL	Non Hodgkin lymphoma
NK	Not known
TP	True positive

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