RATIONALISING LABORATORY WORKFLOW TO IMPROVE THE EFFICIENCY OF DIAGNOSTIC SERVICE DELIVERY:

A CRITICAL REVIEW OF HAEMATOLOGICAL MALIGNANCIES BY FLOW CYTOMETRIC IMMUNOPHENOTYPING AT THE CHARLOTTE MAXEKE JOHANNESBURG ACADEMIC HOSPITAL

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Medicine in Haematological Pathology.

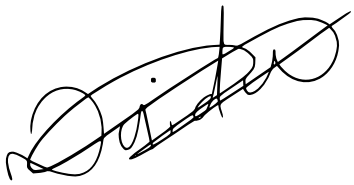
Johannesburg, 2023

Declaration

I, Dr Maynolia Naidoo, declare that this research report is my own, unaided work.

This current research report, in the form of a conventional monograph, is being submitted for the Degree of Master of Medicine in Haematological Pathology at the University of the Witwatersrand, Johannesburg.

This research report has not been submitted previously for any degree or examination at any other University.



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(Signature of candidate)

13th of May 2023

Dedication

To my husband and son,
Kinesh and Kiaan Reddy,
for their
support and understanding
during this challenging but rewarding degree.

Abstract

The 2006 Bethesda medical indications guideline provides concise indications for flow cytometric immunophenotyping (FCI), to enable rationalising a decision for sample processing. The local practice of processing every sample received for FCI places an enormous burden on the resources of the laboratory, and leads to unnecessary expenditure for state health. A 'triage' process based on the current Bethesda medical indications guideline may be beneficial in developing countries. The aim of this study was to determine how the implementation of a triage process would impact on the diagnostic service delivery, and the ability to detect or miss disease.

A retrospective review of 500 bone marrow aspirate (BMA) samples submitted for FCI analysis was performed from October to December 2019. The sensitivity, specificity, and predictive values of the BMA cytomorphology against the FCI outcomes ('test-all') were determined. Thereafter, the Bethesda medical indications guideline was retrospectively applied to the same data set ('triage'), to compare the decision to process or not to process samples, against objective evidence of disease in various BMA investigations.

After exclusion of inadequate quality samples that preclude comparison, 429 'test-all' and 455 triage cases were evaluated. The 'test-all' analysis revealed a 97.1% sensitivity and 89.8% specificity, with a 64.1% positive predictive value (PPV) but striking 99.4% negative predictive value (NPV). The triage was largely effective in identifying cases with disease, revealing a 100% sensitivity and 83.3% specificity, with a PPV of 32.5% and very high NPV of 93.8%. Without impacting clinical outcomes, the implementation of a triage process can reduce the burden of FCI testing by 18%. Preliminary cytomorphological review of the accompanying BMA is strongly recommended as an additional step to improve the overall PPV of the triage, while safely reducing unnecessary FCI sample processing in a further 56% of cases.

The implementation of a triage process with modifications for local use in flow cytometry laboratories, would enable the appropriate rationalisation of resources, improve the cost-effectiveness, and overall diagnostic service delivery in developing countries like Sub-Saharan Africa.

Acknowledgements

First and foremost, I would like to express my special gratitude to Professor Debbie Glencross as my supervisor for her unconditional assistance and support throughout this project.

A special thanks to Mr Innocent Maposa, senior biostatistician from the University of Witwatersrand, for statistical guidance.

I am sincerely grateful to Thamara Naidoo who continually assisted with research guidance.

Lastly, my heartfelt thanks to Kinesh and Kiaan Reddy for their support, patience and understanding.

The invaluable advice, motivation, and encouragement that I received during this degree has contributed tremendously to the successful completion of my MMed.

Table of Contents

List of Figures

CHAPTER THREE

List of Tables

CHAPTER TWO

CHAPTER THREE

Nomenclature

AHI	Aparticulate, haemodilute and/or inadequate
BLPD	B-cell lymphoproliferative disorder
BMA	Bone marrow aspirate
BMAT	Bone marrow aspirate and trephine biopsy
BMT	Bone marrow trephine
CD	Cluster of differentiation
CHBAH	Chris Hani Baragwanath Academic Hospital
CI	Confidence interval
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CSF	Cerebrospinal fluid
DIFF	Differential
DLBCL	Diffuse Large B-cell lymphoma
EDTA	Ethylenediaminetetraacetic acid
FBC	Full blood count
FCI	Flow cytometric immunophenotyping
FISH	Fluorescent in situ hybridisation
FN	False negative
FNA	Fine needle aspiration
FP	False positive
HIV	Human immunodeficiency virus
HJH	Helen Joseph Hospital
HL	Hodgkin lymphoma
HLH	Haemophagocytic lymphohistiocytosis
HRS	Hodgkin Reed-Sternberg
IgG	Immunoglobulin G
IgH	Immunoglobulin heavy chain
IHC	Immunohistochemical
LIS	Laboratory Information System
LN	Lymph node
LPD	Lymphoproliferative disorder
MDS	Myelodysplastic syndrome
NHLS	National Health Laboratory Service

NPV	Negative Predictive Value
PB	Peripheral blood
PCR	Polymerase chain reaction
PPV	Positive Predictive Value
TB	Tuberculosis
TCR	T-cell receptor
TN	True negative
TP	True positive

CHAPTER 1 - INTRODUCTION AND LITERATURE REVIEW

Flow cytometric immunophenotyping (FCI) is an objective and highly complex laboratory testing service that is utilised widely across the biosciences (1). Common indications for FCI include leukaemia and lymphoma diagnostics, classification, staging, prognostication and disease monitoring in patients with haematolymphoid neoplasms (2–4). Over several years, the advances in technology have enabled the development of modern flow cytometers with several basic components including fluidic, optical and electronic systems (1).

1.1 Principles of Flow Cytometry

Flow cytometric immunophenotyping has advanced immensely over decades since the very first attempt to measure or count cells as originally described by Moldavan in 1934 (5). Moldavan's experiments began by utilising a microscope directed on a capillary glass tube, where a forced suspension of cells were passed through the glass tube, and a photoelectric device located at the eyepiece was used to record the passage of each cell (5). In 1953, Crosland-Taylor created a device for enumerating erythrocytes suspended in sheath fluid through a capillary tube (6). Hydrodynamic focusing describes the passage of two fluids through the flow chamber at different velocities, with the sheath fluid located peripherally and the patient's sample maintained centrally. This is a key component in flow cytometers and results in a single passage of cells through the flow chamber (7). A laser beam is applied at a 90° angle to the flow chamber, leading to scattering of laser light when in contact with individual cells. The point of contact between the laser light and a single cell is termed the interrogation point (1). The scattered light enables two cell principles, forward scatter (size of the cell) and side scatter (internal complexity and granularity), and this was originally described by Loken et al. to enable identification of several haematological cell populations (1,8). Kohler et al. invented the hybridoma technology for the production of monoclonal antibodies, that ultimately enabled the detection of surface and cellular proteins, or antigens (9). Various cell types selectively express collections of antigens on their cell surface (10,11). Specific antigens, or surface markers, assist in identifying a particular cell lineage as well as different stages of differentiation (10,11). Each monoclonal antibody is referred to as a 'cluster of differentiation' (CD). These are determined by the antigenic specificity of the cluster, and named according to a particular function (as receptor or ligand) affected by the respective monoclonal antibody (10,11). Different CD's are used to define specific haematolymphoid cell populations (1,11).

The presence or absence of cells in suspension are immunophenotypically detected with fluorescently labelled monoclonal antibodies (1,12). These antibodies are bound to antigens on the cell surface, and pass through a series of filters that separate out various wavelengths of light. Optical detectors identify both laser-scattered light and different wavelengths of fluorescence emissions (1,13,14). Upon reaching the photomultiplier tubes, these signals are converted to voltages and subsequently, translated into a format that can be read with a computer software programme for analysis (1,13). Specifically designed computer software programmes enable precise data acquisition and analysis (1).

The FCI service is essential across multiple medical disciplines and involves testing of various patient samples from peripheral blood, bone marrow to different types of bodily fluids and disaggregated biopsy specimens (1,3,14). The medical indications for FCI testing are based on the 2006 Bethesda International Consensus recommendations (3).

1.2 '2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of haematolymphoid neoplasia: Medical indications'

The guidelines for FCI have evolved over the years since the first release of the U.S Canadian Consensus Recommendations in 1997 (2). The current 'Bethesda International Consensus Recommendations on the flow cytometric immunophenotypic analysis of haematolymphoid neoplasia: Medical indications', were formulated in 2006 by a group of international experts in the field of flow cytometry. These guidelines provide objective medical indications for FCI and are the most widely accepted guidelines globally (3).

The FCI is performed on various samples including peripheral blood (PB), bone marrow aspirate (BMA), bone marrow trephine (BMT) biopsies, fine needle aspirate (FNA) of tissue masses, cerebrospinal fluid (CSF) and serosal fluid samples including pleural and ascitic fluids (3,14).

The medical indications for FCI, as defined by the Bethesda group, include cytopenia's (pancytopenia, monocytopenia and a combination of anaemia, leucopenia and/or thrombocytopenia), leucocytosis (lymphocytosis, monocytosis, eosinophilia), monoclonal gammopathies or plasmacytosis, the presence of atypical cells or blasts in the PB, BMA or bodily fluids, the presence of organomegaly (hepatomegaly, splenomegaly), lymphadenopathy or tissue masses and infiltrates (3,12).

The FCI is indicated for the diagnosis, staging and prognostication of haematolymphoid neoplasms as well as detecting potential therapeutic targets e.g. CD20 and CD52 (3,12). Moreover, FCI is performed for monitoring of previously diagnosed haematolymphoid neoplasms for minimal residual disease, relapse, progression, and the evaluation of disease acceleration. The diagnosis of inter-current haematological malignancies, either therapy-related such as myelodysplastic syndromes (MDS) or coincidental, are included amongst the medical indications for FCI testing (3,12).

Haematolymphoid neoplasms can present with an anaemia, leucopenia and/or thrombocytopenia, however, an isolated anaemia is commonly associated with non-neoplastic diseases, and thus, not an immediate indication for FCI testing (3). The following conditions are exclusions for FCI testing: mature neutrophilia, polycythaemia, thrombocytosis, basophilia and polyclonal hypergammaglobulinemia (3,12). These exclusions are usually not associated with haematolymphoid neoplasms, nor detectable in the FCI analysis if concurrently identified with malignancies (3). Screening for lymphoproliferative neoplasms in the absence of a splenomegaly, absolute lymphocytosis and cytomorphological evidence of atypical lymphocytes or lymphoid cells are contra-indications for FCI testing (2).

1.3 Factors impacting successful flow cytometry outcomes

Several factors require consideration to enhance the successful outcomes of FCI specimens submitted for investigation of haematolymphoid disease. These include establishing if samples are submitted with valid medical indications for FCI, the specimen type and quality, sample viability, storage temperatures and the type and duration of the anticoagulant used for sample preservation (13,15). Relevant clinical features, prior definitive diagnoses, and the use of certain medications are other pertinent factors that may impact on sample processing and ultimately, the FCI outcomes (13,15). Furthermore, a sufficient proportion of blasts or abnormal cells is required prior to FCI, as a paucity or loss of neoplastic cells during processing can lead to unsuccessful outcomes (12,14). Poor quality BMA specimens secondary to poor technique, a ‘dry tap’, or samples that are aparticulate and haemodilute with a paucity or absence of tumour cells, may reveal a falsely normal immunophenotype, despite an abnormal BMA on cytomorphological review (14). Bone marrow aspirate specimens of haemodilute quality may mask bone marrow infiltration, and underestimate the percentage of disease (16) .

In addition to the recommended medical indications for FCI, many technical factors require consideration to determine the suitability of samples for FCI testing. Cellular viability is one of the most fundamental factors for samples with suspected haematolymphoid neoplasms, as intact cellular membranes are necessary to enable adequate monoclonal antibody binding and antigenic expression (15). The collection date of the sample is crucial as samples that are received more than 48-72 hours from the time of collection, may lead to non-specific binding of monoclonal antibodies, and incorrect interpretation of fluorescence expression (1,13). The presence of dead cells does not necessarily exclude sample analysis, as the United States Canadian consensus recommendations adopted less stringent viability restrictions, provided that dead cells are actively excluded from the overall analysis, using specific viability dyes or stains (14). Paucicellular samples may negatively impact clinical diagnostic outcomes, as sample preparation requires a suspension of viable cells with a concentration for optimal monoclonal staining of 0.5–1 x 10^7 cells/ml (100 µl per tube) (15). Furthermore, haemodilute or poor quality samples may not provide accurate results due to a paucity of viable cells.

Cell preservation with anticoagulants offer advantages and disadvantages with regard to cell viability. Heparin, ethylenediaminetetraacetic acid (EDTA), sodium citrate and acid citrate dextrose are suitable anticoagulants for the preservation of PB and BMA specimens for FCI testing (1,13). The most universally recommended anticoagulant is EDTA as it allows for cytomorphological assessment (13). Although anticoagulants enable the preservation of specimens, prolonged storage may negatively impact the cellular viability (13,14). The preservation of PB and BMA samples in EDTA and sodium heparin for 12-24 and 48-72 hours respectively, is recommended for haematology-oncology cases (14). Heparin is preferred for MDS samples (15) and EDTA, acid citrate dextrose or heparin is acceptable for paroxysmal nocturnal haemoglobinuria analysis (18).

The general recommendation for storage temperatures of PB and BMA samples is room temperature for a maximum of 48 hours, however, specimens submitted for investigation of B-cell clonality require a temperature of +4°C for up to 72 hours to ensure validity (13). Samples that are stored for a longer duration than recommended, or at inappropriate temperatures may interfere with sample viability and subsequently, the reliability of the FCI results (13).

The type of specimen submitted for FCI analysis may impact on the overall diagnostic outcome. Cerebrospinal fluid and other biological fluid specimens are submitted for FCI to investigate for neoplastic cells, either primary disease or central nervous system involvement in cases with known malignancies. A more pragmatic or cost-effective approach, as recommended by the US consensus guideline and the British Committee for Standards in Haematology, would involve an initial pre-screening of the CSF or fluid cytology for the identification of leucocytes or malignant cells, prior to deciding if the FCI sample requires processing (2,13).

Hodgkin lymphoma (HL) (2,3) and non-haemopoietic malignancies (19) are not medical indications for FCI testing. The FCI is not recommended as the most optimal method for definitively diagnosing HL in BMA specimens. This is because of very small quantities of malignant cells that exist in cell suspension, that are frequently lost during processing (20). Moreover, most laboratories are not equipped with the necessary resources or procedures for identification of HL cells (2,20,21). In these instances, the BMT biopsy with associated immunohistochemical (IHC) and special stains offer a greater diagnostic potential. In non-haemopoietic malignancies, the large-sized tumour cells are frequently lost during FCI processing owing to their tendency to form clumps (13,19). In many instances, the BMA cytomorphology may not detect disease due to the desmoplastic reaction of the tumour cells, however, the BMT biopsy is often floridly infiltrated (13,19). The FCI is classically indicated for haematolymphoid malignancies with the use of CD45 positivity to discern cells of leucocyte origin (12,22). Non-haemopoietic malignancies can be identified immunophenotypically by the absence of CD45 expression. The currently used monoclonal antibodies were manufactured for haemopoietic cells; thus, the specific combination of markers used in the current FCI practice are not helpful in characterising the type and origin of non-haemopoietic malignancies (23).

Cytomorphological and histological assessments of the BMA and BMT respectively, are highly subjective and observer dependent (4). Although FCI is an integral part of the overall objective diagnosis of haematolymphoid neoplasms, it is only one of many laboratory investigations required for a definitive diagnosis. A BMA biopsy is submitted for various laboratory investigations including BMA cytomorphology, FCI analysis, BMT biopsy and molecular investigations. The various molecular investigations include conventional cytogenetics, polymerase chain reaction (PCR) for immunoglobulin heavy chain (IgH) and T-cell receptor (TCR) gene rearrangement studies, and fluorescent in situ hybridisation (FISH).

The integrative assessment of the BMA cytomorphology, histological examination of the BMT biopsy, FCI analysis and molecular investigations enables an enhanced and objective diagnostic analysis, and eliminates inter-observer interpretative discrepancies (2,15). Furthermore, the concurrent submission of molecular tests with FCI samples offer a better diagnostic evaluation, than the outcomes solely reported in the FCI analysis (2,4).

1.4 Reasons supporting the implementation of the '2006 Bethesda International Consensus Recommendations on the flow cytometric immunophenotypic analysis of haematolymphoid neoplasia: Medical indications'

The Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) is a multidisciplinary tertiary centre and a busy referral facility. A high volume of samples for FCI are received from many of the hospitals in the Gauteng region, including CMJAH, Chris Hani Baragwanath Academic Hospital (CHBAH), Helen Joseph hospital (HJH), Rahima Moosa and other surrounding regional hospitals. Samples are also received from tertiary centres across the country, and several nearby academic centres requiring more detailed FCI analysis, if these services are not performed in their centres. All samples received for FCI at the National Health Laboratory Services (NHLS) flow cytometry department at CMJAH are processed, and analysed as per the request of the attending physician. This approach was designated as the 'test-all' approach for the purposes of this study, and has led to a high burden on the service delivery, without clear clinical benefit. Additionally, preventable expenses are incurred with such a testing approach, leading to unnecessary expenditure to the state for specialist pathology testing.

A pilot study was undertaken in the CMJAH unit to review this 'test all' FCI practice, and the analysis revealed that many samples had no valid medical indications or inappropriate clinical indications for FCI testing. The pilot study consisted of 70 PB and BMA samples, that were routinely referred for FCI analysis during January and February 2020. Samples were processed using limited screening FCI panels including kappa, lambda, CD19 and CD5, CD10 or CD34 as well as CD14 and CD45. The results revealed no evidence of invasive haematolymphoid disease, specifically a B-cell lymphoproliferative disorder (LPD) and non-haemopoietic malignancies, in 100% of cases investigated by FCI (Prof DK Glencross, personal communication). Importantly, morphologically worrying small populations of abnormal cells were reported in the BMA cytomorphology in 11 of the 70 cases, however, no disease was

found in the FCI analysis. Notably, only one case showed evidence of infiltration by malignant plasma cells in the corresponding BMT biopsy.

Another local study undertaken in the CMJAH flow cytometry laboratory investigated the diagnostic outcomes of FCI in all routinely referred CSF samples during 2019 (24). Among 422 CSF samples processed, malignant disease was found in only 19 cases in the FCI analysis, whilst conventional cytology identified malignant cells in 6 samples (24). These 19 cases comprised predominantly of precursor B-cell lymphoblastic leukaemia (14 cases), one case of T-cell lymphoblastic leukaemia/lymphoma, two cases of acute leukaemia that were unclassifiable, and two cases with B-cell lymphoma (one high-grade and one diffuse large B-cell lymphoma) (24). No disease was found in most cases despite 250 of the 422 samples with a medical indication of a suspected haematological malignancy (24).The results of this study showed that FCI offers a limited potential in detecting haematological malignancies, with no value as a screening tool for CSF samples (24).

The 2006 Bethesda International Consensus group reported similar experiences to the outcomes of these smaller studies performed at CMJAH, with many FCI specimens submitted with appropriate medical indications, however, approximately 50% revealed no evidence of haematolymphoid neoplasms (4). This can lead to an excessive workload and a burden on the service delivery, impacting negatively on workflow and depleting the limited resources in a budget-constraint setting. Recent work published by the CMJAH group showed that using a standardised multi-parameter approach can markedly reduce the service burden in the unit as the very high number of individual and duplicated FCI tests can be consolidated into substantially fewer testing tubes (25,26). However, these innovative and modern consolidated labour approaches only reduce the time spent by technologists by consolidating multiple tests into fewer tubes; the systems described do not address the fact that the majority of samples tested for FCI do not have disease (25,26).

To make maximal use of the available resources and reduce the financial burden in FCI laboratories, it seems plausible to decrease the amount of time and resources required to process all samples received for FCI testing. Furthermore, in developing countries with limited resources, FCI is costly in a diagnostic laboratory setting particularly with a ‘test-all’ approach (2). Based on the Bethesda medical indications guideline, more objective FCI rejection criteria are required. This will allow for a more directed and extensive focus on the evaluation of

samples with haematolymphoid neoplasms, that require a comprehensive FCI analysis (4). Ultimately, this will reduce unnecessary costs, improve laboratory workflow, and result in a more efficient service delivery. The implementation of the '2006 Bethesda International Consensus Recommendations on the flow cytometric immunophenotypic analysis of haematolymphoid neoplasia: Medical indications' (3) guideline can potentially alleviate the existing challenges faced in FCI laboratories, including the CMJAH FCI service. For the purposes of this study, the application of the aforementioned Bethesda medical indications guideline (3) to the retrospective case cohort was termed the 'triage process'.

1.5 Study aims and objectives

1.5.1 Aims

i. In the 'test-all' approach, we aimed to determine the relationship between the BMA cytomorphology and FCI outcomes, to establish if BMA cytomorphology reliably predicts disease by FCI.

ii. In the triage process, we aimed to determine if the Bethesda medical indications guideline is effective in reliably categorising samples for processing or not for processing, depending on the presence or absence of disease.

1.5.2 Objectives

i. In the 'test-all' approach, we compared the diagnostic sensitivity, specificity, and predictive values of the BMA cytomorphology outcomes to the corresponding FCI outcomes.

ii. In the triage process, we determined the diagnostic sensitivity, specificity, and predictive values of the Bethesda medical indications guideline against objective evidence of disease in various BMA laboratory investigations.

iii. In the triage process, we determined amongst the cases with and without Bethesda medical indications, the diagnostic sensitivity, specificity, and predictive values of the FCI outcomes against evidence of disease in the BMAT and associated molecular investigations.

CHAPTER 2 - METHODOLOGY

2.1 Research Design

A retrospective, descriptive laboratory-based study.

2.2 Study setting and population

This study was conducted at the NHLS Haematology and Flow Cytometry laboratory at CMJAH. A total of 500 consecutively received BMA samples submitted for FCI analysis were included in the study, from October 2019 to December 2019. The FCI was performed on all cases received, irrespective of the medical indication, from predominantly CMJAH and CHBAH. Samples were also received from HJH, Sebokeng, Thelle Mogoerane and Rahima Moosa in Gauteng, Frere Hospital in the Eastern Cape as well as other provincial and district hospitals.

2.3 Selection Criteria

2.3.1. Inclusion criteria

The first 500 consecutively received BMA samples submitted for FCI testing, from both adult and paediatric patients, were included in this study. For the 'test-all' approach, we included BMA samples submitted for FCI analysis with a corresponding BMA cytomorphology report on the laboratory information system (LIS). For the triage process, in addition to the FCI analysis, evidence of disease was required in at least one or more of the following investigations viz. BMA cytomorphology, BMT biopsy and/or BMA molecular studies.

2.3.2. Exclusion criteria

Samples without a corresponding BMA cytomorphology report on the LIS were excluded from this study. Additionally, the following samples were excluded: specimens with a prior diagnosis made in the FCI analysis, follow-up BMA samples or minimal residual disease monitoring, degenerate samples (older than 3 days), samples with too few cells for meaningful assessment, specimens cancelled by the clinician, and any cases with laboratory errors.

2.4 Ethical considerations

Approval to conduct this study was obtained from the University of the Witwatersrand Human Research Ethics Committee (clearance certificate number M2011157). Please refer to Appendix A. The data will be stored for 5-10 years from the time of publication.

2.5 Data Collection

All 500 cases were retrieved from the flow cytometry daily logbook. The data was extracted from the NHLS LIS by a thorough database search using the patients' hospital or specimen episode numbers. The data was captured onto a data collection sheet (Appendix B) using Microsoft Excel, 2022. Each specimen was allocated a case number from 1-500 and remained anonymised.

The following parameters were recorded for each case:

- Medical indication for FCI analysis.
- Age, gender, human immunodeficiency virus (HIV) status, clinical presentation, and examination findings (specifically the presence of hepatomegaly, splenomegaly, lymphadenopathy, and other masses).
- Full blood count (FBC), differential (DIFF) count and peripheral blood smear features.
- FCI results.
- Quality of the BMA and/or BMT biopsy specimens.
- Pertinent morphological features of the BMA cytomorphology and BMT biopsies, particularly related to the presence or absence of malignancies and/or an increase in blasts.
- Results of the BMA molecular investigations including PCR, FISH, and cytogenetics.
- Additional relevant investigations related or unrelated to the BMAT if available e.g., biopsy results, tuberculosis (TB) cultures, autoimmune and viral screens.

2.6 Data Analysis

The data was described and analysed according to the following sub-headings: descriptive statistics of the patients' characteristics and relevant laboratory findings, the 'test-all' approach and the triage process. The triage process was further categorised into two subgroups.

2.6.1 Descriptive statistics of patients' characteristics and relevant laboratory findings

The baseline characteristics and overall relevant laboratory findings of the case cohort were described including age, gender, HIV status and the number of cases with Bethesda medical indications as per the guideline (3). The presence or absence of disease in the BMA cytomorphology and the FCI outcomes were documented.

We analysed the quality of all BMA cytomorphology specimens, as per the findings reported by haematopathologists. In a subgroup of BMA specimens described as 'aparticulate', 'haemodilute' and/or 'inadequate' quality, the percentage of marrow lymphocytes were reviewed. In this study, these three subtypes were collectively denoted as 'AHI' quality. The lymphocyte percentages were categorised into the following subgroups: less than 10%, 10-20% and more than 20%. Furthermore, we recorded the number of specimens with no documentation of lymphocyte percentages or 'no myelogram'. Notably, some cases were diagnostic with evidence of disease in both or either the BMA cytomorphology and FCI analysis, despite the quality of the sample.

The definition of a bone marrow lymphocytosis is dependent on the age of the patient. Bain et al classified a bone marrow lymphocytosis as more than 15-25% lymphocytes, in the adult population (27). For the purposes of this study, a bone marrow lymphocytosis was defined as more than 20% lymphocytes in adult patients. In AHI specimens, a marrow lymphocytosis may often be secondary to haemodilution of the sample, by peripheral blood that has a higher lymphocyte count, rather than indicative of the presence of disease (27). The number of cases with a marrow lymphocytosis in AHI BMA cytomorphology specimens was correlated with disease in the FCI analysis, to determine the relationship between AHI quality samples with a higher proportion of lymphocytes.

In the paediatric population, the percentage of normal bone marrow lymphocytes in the BMA is variable according to the age of the child (27). In addition, paediatric patients have higher proportions and absolute count numbers of lymphocytes relative to other leucocytes in the peripheral blood, compared to the adult population (27). The lymphocyte to granulocyte ratio is typically known to decline with increasing age, and is ultimately reversed by the age of 16 years, with a predominance of neutrophils by adulthood (27). In view of the age-dependant percentages of a bone marrow lymphocytosis in the paediatric cohort, these cases were individualised and discussed separately.

In this sub-group of AHI BMA cytomorphology specimens, the adult and paediatric lymphocyte percentages were described separately. In those samples with a true bone marrow lymphocytosis, we described and correlated these cases with the presence or absence of disease in the FCI analysis.

The data was then analysed into two approaches: the 'test-all' and the triage process. The triage process was further categorised into two subgroups: cases with medical indications requiring FCI sample processing, and cases without medical indications that do not require FCI processing.

2.6.2 The 'test-all' approach

In the 'test-all' approach, the BMA cytomorphology outcomes were correlated with the FCI outcomes to determine the relationship between these two investigations, and to assess for the presence or absence of disease.

The BMA cytomorphology outcomes was the test and relatively subjective investigation, while the FCI outcomes was the index and more objective investigation. The analysis reviewed whether the outcomes of the BMA cytomorphology (disease or no disease) could reliably predict FCI outcomes.

In the 'test-all' approach, the diagnostic sensitivity, specificity, positive and negative likelihood ratios, disease prevalence, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the BMA cytomorphology outcomes to the FCI outcomes were calculated.

The following definitions were applied based on the 'test-all' approach:

- True positive (TP)'test-all': cases with disease reported in both the BMA cytomorphology and FCI outcomes.
- True negative (TN)'test-all': cases with no disease in both the BMA cytomorphology and FCI outcomes.
- False positive (FP)'test-all': cases with disease, or suspicious cells reported in the BMA cytomorphology, but no disease was documented in FCI outcomes.
- False negative (FN)'test-all': cases with no disease in the BMA cytomorphology, but disease detected in the FCI outcomes.

The BMA samples of poor quality and reported as 'AHI for meaningful assessment', with no myelogram or interpretative comment, were excluded from the 'test-all' analysis. In certain instances, cases were reported as aparticulate but not haemodilute, with evidence of disease. These cases were termed diagnostic due to evidence of disease, and were included in this cohort to ensure an accurate assessment of the analysis. Of the excluded cases, we determined the number of samples with and without Bethesda medical indications for FCI testing.

2.6.3 The triage process: Application of the Bethesda medical indications guideline for flow cytometric immunophenotyping

We applied the '2006 Bethesda International Consensus Recommendations on the flow cytometric immunophenotypic analysis of haematolymphoid neoplasia: Medical indications' (3) (Appendix E) to all 500 consecutively received BMA samples submitted for FCI analysis. The Bethesda medical indications guideline was applied to the case cohort to determine if disease would reliably be identified in those cases submitted for FCI, and subsequently, determine cases for FCI sample processing. Therefore, the triage process would reliably identify those samples for processing (with disease), or not for processing (without disease). The presence or absence of disease, as the standard against the triage to process or not to process, was confirmed by review of various BMA laboratory investigations. These investigations included the BMA cytomorphology, BMT biopsy, FCI outcomes, and various BMA molecular investigations.

In the triage process, we included the BMT and BMA molecular studies, to ensure that disease was fully excluded on several laboratory investigations. In cases where the BMA cytomorphology was AHI quality, we further reviewed the histological findings of the BMT biopsy to identify disease. If disease was excluded in the BMT, we then screened the molecular studies for evidence of malignancies. If the above-mentioned tests reliably confirmed or excluded disease, these cases were included in the case cohort. If any BMA cytomorphology case was AHI in quality, and the corresponding BMT biopsy was inadequate quality or not found on LIS, these cases were excluded from the analysis. Of the cases excluded, we determined the number of samples with and without Bethesda medical indications for FCI testing.

The diagnostic sensitivity, specificity, positive and negative likelihood ratios, disease prevalence, PPV, NPV and accuracy were calculated for the triage process.

The following definitions were applied based on the triage process:

- TP_{triage}: cases with medical indications for FCI sample processing, with disease detectable in various BMA laboratory investigations.
- TN_{triage}: cases without medical indications for FCI sample processing, with no disease in various BMA laboratory investigations.
- FP_{triage}: cases with medical indications for FCI sample processing, but no evidence of disease in various BMA laboratory investigations.
- FN_{triage}: cases without medical indications for FCI sample processing, with disease in one or more BMA laboratory investigations.

The data collected from the triage process case cohort was further categorised into two subgroups based on the guidelines: cases with Bethesda medical indications that were recommended for FCI sample processing, and cases without Bethesda medical indications that were not recommended for FCI sample processing. These two subgroups were then correlated with the presence or absence of disease in various BMA laboratory investigations. The laboratory investigations in this cohort were separated into the BMAT and BMA molecular studies collectively (test) versus the FCI outcomes (disease). The outcomes of the FCI analysis were contrasted with the outcomes in the BMAT and BMA molecular investigations, to determine if the FCI outcomes could predict disease in these associated investigations.

The triage process: cases with Bethesda medical indications that were recommended for FCI sample processing

In the triage process, cases with Bethesda medical indications were classified into cases that were recommended for FCI sample processing. This subgroup focused on the ability of FCI to detect disease, versus documented evidence of disease in the corresponding BMAT or BMA molecular investigations. The presence or absence of disease in the FCI analysis was correlated with the presence or absence of disease in the corresponding BMAT or BMA molecular investigations.

The diagnostic sensitivity, specificity, positive and negative likelihood ratios, disease prevalence, PPV, NPV and accuracy of the outcomes in the FCI analysis and corresponding BMAT and BMA molecular investigations were calculated.

The following definitions were applied based on the triage cases that were recommended for FCI sample processing:

- $TP_{triage\ (processed)}$: cases recommended for FCI sample processing, with confirmed evidence of disease in the FCI, BMAT and/or BMA molecular investigations.
- $TN_{triage\ (processed)}$: cases recommended for FCI sample processing, with no confirmed evidence of disease in the FCI, BMAT and/or BMA molecular investigations.
- $FP_{triage\ (processed)}$: cases recommended for FCI sample processing, with no evidence of disease in the FCI analysis, but disease was identified in the BMAT and/or BMA molecular investigations.
- $FN_{triage\ (processed)}$: cases recommended for FCI sample processing, with possible evidence of disease in the FCI analysis, but no identifiable disease in the BMAT and/or BMA molecular investigations.

The triage process: cases without Bethesda medical indications that were not recommended for FCI sample processing

In the triage process, cases without Bethesda medical indications were classified into cases not recommended for FCI sample processing. The presence or absence of disease in the FCI analysis was correlated with the presence or absence of disease in the corresponding BMAT or BMA molecular investigations.
The diagnostic sensitivity, specificity, positive and negative likelihood ratios, disease prevalence, PPV, NPV and accuracy of the outcomes in the FCI and corresponding BMAT and BMA molecular investigations were calculated.

The following definitions were applied based on the triage cases that were not recommended for FCI sample processing:

- $TP_{triage\ (not\ processed)}$: cases that were not recommended for FCI sample processing, with evidence of disease in the FCI, BMAT and/or BMA molecular investigations.
- $TN_{triage\ (not\ processed)}$: cases that were not recommended for FCI sample processing, with no evidence of disease in FCI, BMAT and/or BMA molecular investigations.
- $FP_{triage\ (not\ processed)}$: cases that were not recommended for FCI sample processing, with no evidence of disease in the FCI analysis, but disease was identified in the BMAT and/or BMA molecular investigations.
- $FN_{triage\ (not\ processed)}$: cases that were not recommended for FCI sample processing, with possible evidence of disease in the FCI analysis, but no definitive disease in the BMAT and/or BMA molecular investigations.

2.7 Statistical analysis

The retrospectively collected data was analysed using Microsoft Excel, 2022. Descriptive statistics were used for patient demographics, and the results were reported as numbers and percentages.

The diagnostic sensitivity, specificity, positive and negative likelihood ratios, disease prevalence, PPV, NPV and accuracy were calculated using two-way tables and the MedCalc Statistical Software version 20.112 (MedCalc Software Ltd, Ostend, Belgium) (28).

The following definitions and calculations were applied to the data set (28,29):

- Sensitivity: The probability that a test will be positive, in the presence of disease (True positive rate).
- Specificity: The probability that a test will be positive, in the absence of disease (True negative rate).
- PPV: The probability that the disease is present when a test is positive.
- NPV: The probability that the disease is absent when the test is negative.
- Disease prevalence: proportion of the population with the disease.
- Accuracy: the overall probability of correctly classifying a patient.
- Positive likelihood ratio: ratio between the probability of a positive test result in the presence of disease, and the probability of a positive test result in the absence of disease.
- Negative likelihood ratio: ratio between the probability of a negative test result in the presence of disease, and the probability of a negative test result in the absence of disease.

Sensitivity, specificity, disease prevalence, accuracy and positive and negative predictive values were expressed as percentages. Confidence intervals for sensitivity, specificity and accuracy are “exact” Clopper-Pearson confidence intervals. Confidence intervals for likelihood ratios are calculated using the “Log method” (28,30). Confidence intervals for predictive values are the standard logit confidence intervals (28,31).

The following formulas were applied for the sensitivity, specificity, PPV and NPV:

Sensitivity: $TP/(TP + FN) \times 100$

Specificity: $TN/(TN + FP) \times 100$

PPV: $TP/(TP + FP) \times 100$

NPV: $TN/(TN + FN) \times 100$

Table 2.1 Two-way table (29)

		Disease		
		Disease	No Disease	Total
Test	Positive	TP	FP	$T_{(Test\ Positive)}$
	Negative	FN	TN	$T_{(Test\ Negative)}$
		$T_{Disease}$	$T_{No\ Disease}$	Total

The results are represented in tables and diagrams in Chapter 3, and the two-way tables are depicted in Appendix F.

CHAPTER 3 - RESULTS

3.1 Descriptive statistics of patients' characteristics and relevant laboratory findings

The study population (n=500) consisted of 266 females (53.2%) and 234 males (46.8%). Overall, most patients were between 35-60 years of age and comprised 48% of the cohort. HIV positivity was documented in 50.8%, with an unknown or indeterminate HIV status in 90 patients (18%). Of the 500 cases in this cohort, 407 specimens were referred with Bethesda medical indications, and 204 (41%) were submitted with a history of a suspected malignancy or bone marrow infiltration. No disease was detected in 431 (86.2%) FCI samples and in 324 (64.8%) BMA cytomorphology cases. A total of 71 cases were AHI quality for a comprehensive assessment. The key aspects of the patients' characteristics and relevant laboratory findings are summarised in Table 3.1.

3.2 Quality of the BMA cytomorphology specimens

The quality of BMA samples submitted for cytomorphology were reviewed and correlated with disease in the FCI analysis. Of the 500 samples received, 257 (51.4%) were described as good or adequate quality, and only 38 (7.6%) of these cases had disease in the FCI analysis. Of the 24 suboptimal quality samples, only one case had definitive evidence of disease in the FCI analysis (Table 3.2). This case was submitted from a one year old child who was being investigated for a clonal haemopoietic disorder, after presenting with pallor and a splenomegaly. The BMA cytomorphology found 12% 'primitive mononuclear cells' and the FCI analysis identified 4-5% myeloid blasts with aberrant antigenic expression. A review of the BMT biopsy and molecular studies confirmed an acute myeloid leukaemia with a genetic aberration.

Table 3.1 Patients' characteristics and relevant laboratory findings

Characteristic	Value (n=500) n (%)
Age (years)	
<1	6 (1.2)
1-9	34 (6.8)
10-17	29 (5.8)
18-34	116 (23.2)
35-60	240 (48)
>60	75 (15)
Gender	
Female	266 (53.2)
Male	234 (46.8)
HIV status	
Positive	254 (50.8)
Negative	156 (31.2)
Unknown	89 (17.8)
Indeterminate	1 (0.2)
Medical indication for FCI as per Bethesda guidelines	
Yes	407 (81.4)
No	93 (18.6)
Flow cytometric immunophenotyping specimens	
Disease identified	69 (13.8)
No disease identified	431 (86.2)
BMA cytomorphology specimens	
Disease identified	105 (21)
No disease identified	324 (64.8)
AHI for meaningful assessment	71 (14.2)

Table 3.2 Quality of the BMA cytomorphology specimens versus FCI disease

Quality of BMA specimens n=500	FCI disease n (%)	FCI no disease n (%)	Total cases n (%)
Good or adequate	38 (7.6)	219 (43.8)	257 (51.4)
Suboptimal	1 (0.2)	23 (4.6)	24 (4.8)
Aparticulate, Haemodilute, Inadequate	28 (5.6)	185 (37)	213 (42.6)*
Not documented	2 (0.4)	4 (0.8)	6 (1.2)
	69 (13.8)	431 (86.2)	500 (100)

*Incudes 71 cases that were too AHI for meaningful assessment (see Table 3.3 for details).

Of the total case cohort, 213 cases (42.6%) were described as AHI quality in the BMA cytomorphology reports. In these cases, no disease was detected in 99 (46.5%) BMA cytomorphology, and 185 (86.9%) FCI samples. Only 43 of the 213 cases (20.2%) had disease in the BMA cytomorphology, in contrast to the FCI outcomes where disease was noted in only 28 cases (13.1%). Notably, amongst the AHI quality BMA specimens, 71 samples (33.3%) were reported as being too haemodilute or inadequate for a comprehensive assessment, with no myelogram or interpretative comment (Table 3.3). A review of the corresponding BMT biopsies in all AHI quality samples revealed 54 inadequate quality biopsies, and in a further 8 cases, no BMT biopsies were found on the LIS. This accounts for 29% (62/213) of all AHI quality samples being inadequate for assessment.

Table 3.3 Bone marrow aspirate and FCI outcomes in AHI quality samples

n = 213	BMA cytomorphology outcomes n (%)	FCI outcomes n (%)
Disease	43 (20.2)	28 (13.1)
No disease	99 (46.5)	185 (86.9)
AHI for meaningful assessment	71 (33.3)	-

3.3 Description of lymphocyte percentages in AHI quality BMA specimens

Of the 213 AHI quality BMA specimens, 195 and 18 cases were obtained from adult and paediatric patients, respectively. Importantly, of the 25 diagnostic cases, 23 had disease in the FCI analysis and two cases had disease only in the BMA cytomorphology. No myelogram or documentation of lymphocyte percentages were noted in 37.1% (79/213) of this sub-group. The lymphocyte percentages in both population groups are described in Table 3.4.

A lymphocyte percentage of more than 20% was found in 36 of the 213 samples (16.9%), 32 adult samples and four paediatric samples. Of the 36 cases, only three adult samples showed definitive disease in the FCI analysis, of which clonal plasma cells were found in two cases and one case revealed a CD19/10 co-expressing cell population favouring a high grade B-cell lymphoma or Diffuse Large B-cell Lymphoma (DLBCL). All three cases had valid medical indications for FCI analysis; the former two cases were submitted to investigate for a suspected plasma cell myeloma and the other case was to determine the cause of the pancytopenia.
An age-related, absolute marrow lymphocytosis was confirmed in all four paediatric cases that were categorised in the more than 20% lymphocyte group. Furthermore, none of these cases had concurrent disease in the FCI analysis.

Table 3.4 Lymphocyte percentages in AHI quality adult and paediatric BMA specimens

	Adult samples n = 195	Paediatric samples n = 18	Total n = 213 (%)
<10%	24	2	26 (12.2)
10-20%	44	3	47 (22.1)
>20%	32	4	36 (16.9)
No myelogram	77	2	79 (37.1)
Diagnostic	18	7	25 (11.7)

3.4 Hodgkin lymphoma and non-haemopoietic malignancies

3.4.1 Hodgkin lymphoma

Of the total 18 (3.6%) Hodgkin lymphoma cases in the overall cohort, 10 cases were submitted for staging of disease with a known histological diagnosis of HL made on the lymph node (LN) biopsy, and eight cases were incidentally diagnosed on the BMT biopsy. Notably, no evidence of HL was found in both the BMA cytomorphology and FCI analysis in all specimens. Of the 18 cases, only 12 (2.4%) had histological evidence of HL disease on the BMT biopsy that was confirmed with IHC and special stains. Of these 12 cases, eight specimens were reported as good or adequate quality in the BMA cytomorphology reports, one was suboptimal and three were reported as being AHI. Two of the 12 cases had 'primitive mononuclear cells' reported in the BMA cytomorphology; one adequate quality BMA specimen reported '2% primitive mononuclear cells' and one AHI quality BMA specimen identified '1% primitive mononuclear cells'. Disease could not be excluded or the specimen was too AHI for meaningful assessment in the remaining two AHI quality BMA samples. No disease was found in the BMA cytomorphology in the remaining cases (one suboptimal and seven good quality samples).

3.4.2 Non-haemopoietic malignancies

Non-haemopoietic malignancies comprised 4.8% (24/500) of this case cohort, with no disease detected in the FCI analysis in all 24 cases. Notably, the corresponding BMA and BMT revealed evidence of disease in seven and nine cases, respectively.

3.5 'Test-all' analysis

In the 'test-all' approach, 71 of the 500 cases were excluded from the analysis due to the AHI quality of the BMA cytomorphology slides. These samples offer no diagnostic potential or meaningful assessment to compare with the reported FCI outcomes (Figure 3.1). Of the excluded 71 cases, 53 had valid medical indications in accordance with the Bethesda medical indications guideline. A review of these 53 cases revealed 24 samples that were submitted for investigation of a bicytopenia and 14 were malignancy-associated for either disease staging or possible bone marrow infiltration. Nine cases were received for investigation of a pancytopenia and six cases to determine the cause of the organomegaly or lymphadenopathy.

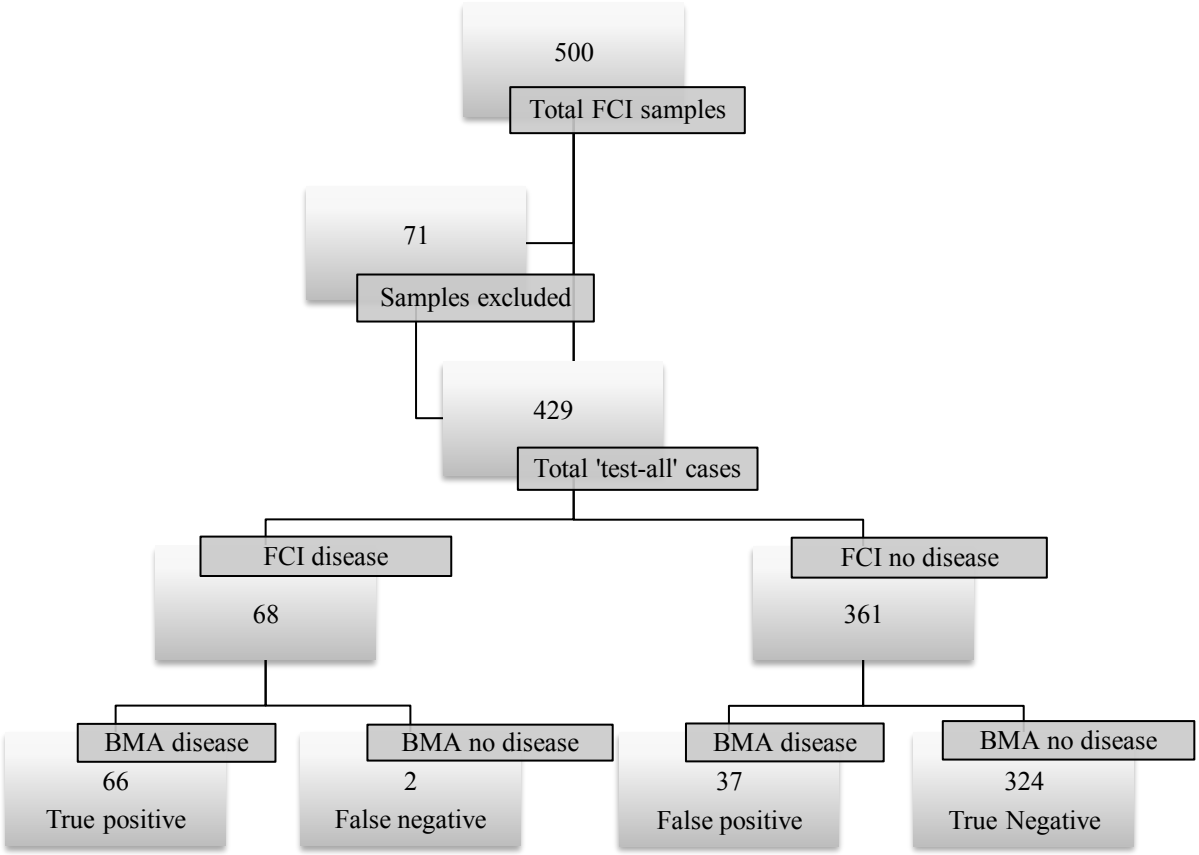

Figure 3.1 Diagrammatic representation of the 'test-all' samples

A total of 429 cases were available for review after exclusion of unsuitable samples as shown in Figure 3.1. The comparison of BMA cytomorphology to the FCI outcomes revealed a 97.1% sensitivity, 95% CI [89.8-99.6%] and specificity of 89.8 %, with a 95% CI [86.2 – 92.7%]. The NPV of the BMA cytomorphology outcomes to the FCI outcomes was calculated at 99.4% with a 95% CI [97.6-99.8%] and the 'test-all' PPV was 64.1 % with a 95% CI [56.7-70.8%] as depicted in Table 3.5. The accuracy of the BMA cytomorphology outcomes to identify disease in the FCI analysis was 90.9%.

Table 3.5 The 'test-all' and triage process diagnostic parameter results

	'Test-all' (BMA cytomorphology versus FCI outcomes)		**Triage process** (Triage versus bone marrow laboratory evidence of disease)		**Triage: Cases with Bethesda medical indications for processing** (FCI versus BMAT/molecular disease)		**Triage: Cases without Bethesda medical indications and not for processing** (FCI versus BMAT/molecular disease)	
Diagnostic parameter	Value (%)	95% CI	Value (%)	95% CI	Value (%)	95% CI	Value (%)	95% CI
Sensitivity	97.1	89.8 - 99.6	96	90.9 - 98.7	100	94.8 - 100	0	0 - 97.5
Specificity	89.8	86.2 - 92.7	22.7	18.3 - 27.6	83.3	78.7 - 87.3	94.9	87.5 - 98.6
Positive likelihood ratio	9.5	6.9 - 12.9	1.2	1.2 - 1.3	6	4.7 - 7.7	0	-
Negative likelihood ratio	0.03	0.01 - 0.1	0.2	0.1 - 0.4	0	-	1.1	1.0 - 1.1
Disease prevalence*	15.9	12.5 - 19.7	27.5	23.4 - 31.8	18.4	14.6 - 22.7	1.3	0.03 - 6.8
Positive Predictive value*	64.1	56.7 - 70.8	32	30.5 - 33.5	57.5	51.3 - 63.5	0	-
Negative Predictive value*	99.4	97.6 - 99.8	93.8	86.1 - 97.3	100	-	98.7	98.6 - 98.8
Accuracy*	90.9	87.8 - 93.5	42.9	38.3 - 47.6	86.4	82.5 - 89.7	93.8	86.0 - 97.9

* These values are dependent on disease prevalence.

The analysis revealed 66 cases with evidence of disease and 324 cases without disease in both BMA cytomorphology and the FCI outcomes. Sensitivity and specificity analysis reflected these findings with an overall disease prevalence of 15.9%. The 'test-all' false positive and false negative cases accounted for 37 and two cases, respectively.

The two false negative cases had disease in the FCI analysis, but no disease in the BMA cytomorphology. In both cases, a small population of clonal cells were identified in the FCI analysis as depicted in Table 3.6. In case 230, the FCI analysis showed a population of 1% small-sized B-cells with lambda light chain restriction, that was concluded as being of uncertain significance. This finding was not supported in the molecular investigations as reflected by the negative PCR for the IgH gene rearrangement study. Case 371 was performed to investigate for a plasma cell myeloma and the FCI analysis accurately detected 0.1% aberrant plasma cells, that were further supported by monoclonal IgH products.

Table 3.6 False negative cases in the 'test-all' approach

Case number	Medical indication for FCI testing	BMA Outcome	BMT Outcome	FCI Outcome	Molecular Investigations
230	To determine the cause of the anaemia	No increase in blasts or evidence of an infiltrate	No BMAT infiltration	1% small sized B-cells with lambda light chain restriction	Negative IgH
371	To investigate for plasma cell myeloma	No increase in plasma cells, blasts or evidence of an infiltrate	No BMAT infiltration	0.1% aberrant plasma cells	Monoclonal IgH

There were 37 false positive cases with features of suspected disease in the BMA cytomorphology, but no definitive evidence of disease in the corresponding FCI analysis. Of these 37 false positive cases, 17 cases were reported to have suspicious 'primitive mononuclear cells' in the BMA cytomorphology, and were described as occasional or reported as a percentage of the myelogram (enumerated from one to 18%). We further reviewed the BMT biopsy reports of the false positive cases, to determine if the FCI analysis failed to detect disease that was suspected cytomorphologically in the BMA. The BMT biopsies of these 17 cases

revealed one case with bone marrow infiltration with DLBCL and two cases with Hodgkin lymphoma. Of the remaining 14 cases, no disease was found in the corresponding BMT biopsy in eight cases, four cases were inadequate for assessment and a further two cases were reported as 'suboptimal for a definitive exclusion of disease'. A detailed description of the 37 false positive cases are described in Appendix C.

3.6 Triage process: Application of the Bethesda medical indications guideline for flow cytometric immunophenotyping

In the triage process, we applied the '2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotyping analysis of haematolymphoid neoplasia: medical indications' (3) to all 500 consecutively received BMA samples submitted for FCI, to categorise cases for 'processing' or 'not for processing'. Forty-five cases were excluded from this analysis as the BMT biopsies were reported as inadequate quality in 38 cases, and in seven cases, no BMT biopsies were found on the LIS. The latter finding suggests that only BMA cytomorphology slides were submitted for investigation. Of the 45 excluded cases, 37 cases had Bethesda medical indications for FCI testing. Of the 37 cases, 17 were submitted for investigation of a bicytopenia and 10 were malignancy-associated for staging of disease or possible marrow infiltration. A pancytopenia was being investigated in seven cases, and the cause for the lymphadenopathy and splenomegaly was queried in one and two cases, respectively.

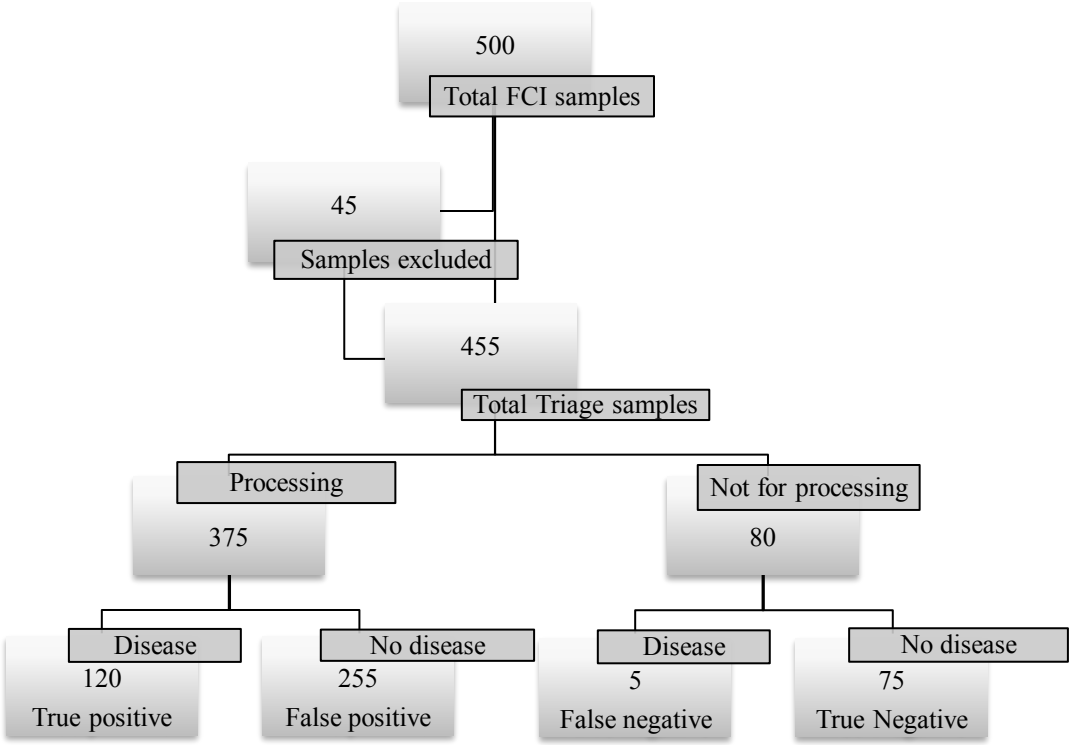

Figure 3.2 Diagrammatic representation of the overall triage process samples

The disease prevalence in the triage process was 27.5%, 95% CI [23.4-31.8%] and accuracy 42.9%, 95% CI [38.3-47.6%]. The triage process revealed a 96% sensitivity, 95% CI [90.9-98.7%] with a PPV of 32%, 95% CI [30.5-33.5%]. The specificity was 22.7%, 95% CI [18.3-27.6%] and NPV calculated at 93.8%, 95% CI [86.1-97.3%] as reflected in Table 3.5.

The application of the Bethesda medical indications guideline to the remaining 455 cases revealed 375 cases with medical indications for FCI sample processing, and 80 cases without medical indications, that are not recommended for FCI testing. Of the 375 cases requiring processing, laboratory evidence of disease was identified in 120 cases and no disease was found in 255 cases, despite all 375 cases having valid Bethesda medical indications.

A review of the medical indications in the 255 cases with no disease across all BMA laboratory investigations, revealed that just over one-third (95 cases) were submitted for investigation of a bicytopenia, 42 cases for pancytopenia, 49 for suspected bone marrow infiltration and 47 for staging of a recently diagnosed malignancy (Table 3.7).

Table 3.7 Triage process: cases with medical indications for FCI and no disease in bone marrow laboratory investigations

Indication for FCI testing	n=255 n (%)
Pancytopenia	42 (16.5)
Bicytopenia	95 (37.3)
Isolated cytopenia	4 (1.6)
Organomegaly or lymphadenopathy	18 (7.1)
Suspected bone marrow infiltration	49 (19.2)
Disease staging of known malignancies	47 (18.4)

Of the 80 cases with no medical indications for FCI sample processing, only five cases had evidence of disease in the bone marrow laboratory investigations and the remaining 75 cases had no disease. The five false negative cases are illustrated in Table 3.8. Notably, the BMA molecular investigations offered no diagnostic potential in all five cases. In case 230, no other laboratory investigation confirmed evidence of a B-cell LPD in this patient.

Table 3.8 Triage process: cases without medical indications for FCI, but various bone marrow laboratory investigations showed evidence of disease – five false negative cases

Case Number	Medical Indication	BMA Outcome	BMT Outcome	FCI Outcome	Molecular Investigations
156	Isolated anaemia	Aparticulate and haemodilute	Non-haemopoietic infiltration	No infiltrate	Negative IgH
192	Isolated anaemia	No infiltrate	Some primitive cells	No infiltrate	-
230	Isolated anaemia	No infiltrate	No infiltrate	1% B-cells with lambda light chain restriction	Negative IgH
271	Isolated anaemia	No infiltrate	HL infiltration	No infiltrate	-
343	Staging for HL	No infiltrate	HL infiltration	No infiltrate	Normal cytogenetics

The triage process case cohort comprising 455 samples were further analysed into two subgroups: 375 cases with Bethesda medical indications for FCI sample processing and 80 cases without Bethesda medical indications that are not recommended for FCI sample processing (Figure 3.3). In these two subgroups, the outcomes of the FCI analysis were correlated with the outcomes in the BMAT and BMA molecular investigations, to determine if FCI findings could predict disease in the aforementioned investigations.

3.6.1 Triage process: FCI samples with Bethesda medical indications, that were recommended for processing

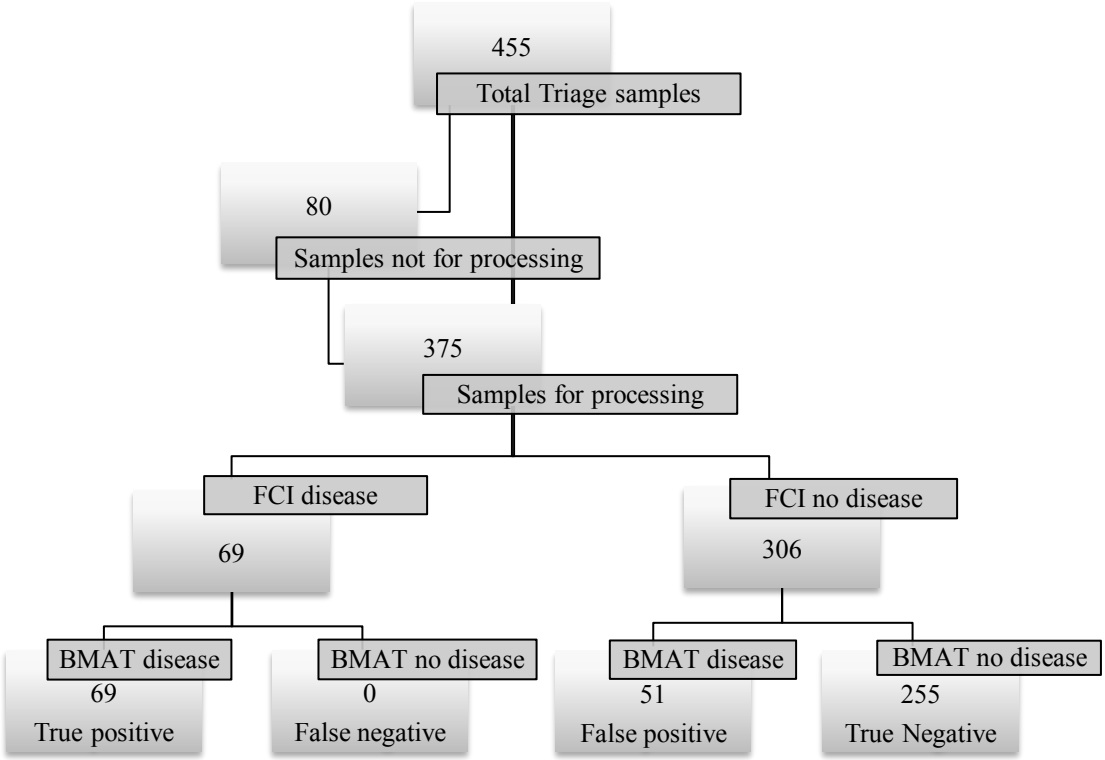

Figure 3.3 Diagrammatic representation of the triage process samples with medical indications for FCI sample processing

The triage process showed a sensitivity of 100%, 95% CI [94.8-100%] and specificity of 83.3%, 95% CI [78.7-87.3%] in predicting whether there was evidence of disease confirmed by FCI, with a PPV of 57.5 %, 95% CI [51.3-63.5%] and NPV of 100%. The disease prevalence in this data set was 18.4%, 95% CI [14.6-22.7%] and accuracy 86.4%, 95% CI [82.5-89.7%] as depicted in Table 3.5.

Of the 375 cases with valid medical indications for FCI processing, disease was reported in the FCI analysis in 69 cases and 306 cases had no evidence of disease. In all 69 cases with evidence of disease in the FCI analysis, the associated BMAT and BMA molecular studies confirmed disease. Of the 306 cases with no disease in the FCI analysis, 255 cases had no concurrent BMAT or molecular evidence of disease.

A review of the medical indications revealed a clinical suspicion of an underlying malignancy or bone marrow infiltration in 210 of the 306 cases. Other medical indications in this subgroup included patients referred for disease staging, pancytopenia, hepatosplenomegaly, lymphadenopathy, or other masses. A more detailed review of the medical information supplied on the FCI request form indicated that 89 of the 210 cases with a clinical suspicion of an underlying malignancy or bone marrow infiltration, had a concomitant bicytopenia. Amongst these, only six cases had evidence of disease in the corresponding BMAT or BMA molecular investigations. Three of the six cases were diagnosed with classical HL in the BMT biopsy, and the other three cases had non-specific findings (3% 'primitive mononuclear cells' in the BMA cytomorphology, 'occasional primitive cells' in the BMT biopsy and reported as worrying for Haemophagocytic Lymphohistiocytosis).

Importantly, no false negative cases were found in this subgroup, however, 51 false positive cases were detected. These cases had no disease in the FCI analysis, but the corresponding BMAT or BMA molecular investigations showed definite or possible evidence of disease. A more detailed review of these cases revealed definite disease in 27 samples and 24 with possible disease as evidenced by suspicious 'mononuclear/atypical cells' in the BMA cytomorphology or BMT biopsy. Of these 51 false positive cases, 10 cases were reported to have bone marrow infiltration with HL in the BMT biopsies. Seven of the 10 HL cases had disease in the BMT only, and three had disease in the BMT biopsy and the BMA cytomorphology. The BMA cytomorphology is these cases described 'variable primitive mononuclear cells' that were reported as occasional to 2%. A further eight of the 51 false positive cases had bone marrow infiltration with a non-haemopoietic malignancy. Of the eight cases, two had disease in the BMT only and six cases had marrow infiltration in both the BMA cytomorphology and the BMT biopsies.

There were 19 of the 51 cases with variable percentages of 'primitive mononuclear or abnormal cells' in the corresponding BMT biopsies. Amongst these 19 cases, three cases had BM infiltration with HL as discussed above, one case had myelofibrosis, another was infiltrated with DLBCL and one case with a possible diagnosis of myelodysplastic syndrome. Thirteen of the 19 cases had 'mononuclear cells' in the BMA cytomorphology but no disease in the BMT, and two cases had evidence of a plasma cell myeloma in the BMT biopsies. Additionally, there was one case with blastic infiltration by T-cells in the BMT, two cases with a marrow lymphocytosis, a single case with 17% plasma cells in the BMA cytomorphology only, one case with a monoclonal TCR, and one with a monoclonal IgH gene rearrangement study on PCR analysis. All 51 false positive cases are detailed in Appendix D.

3.6.2 Triage process: FCI samples without Bethesda medical indications, that were not recommended for processing

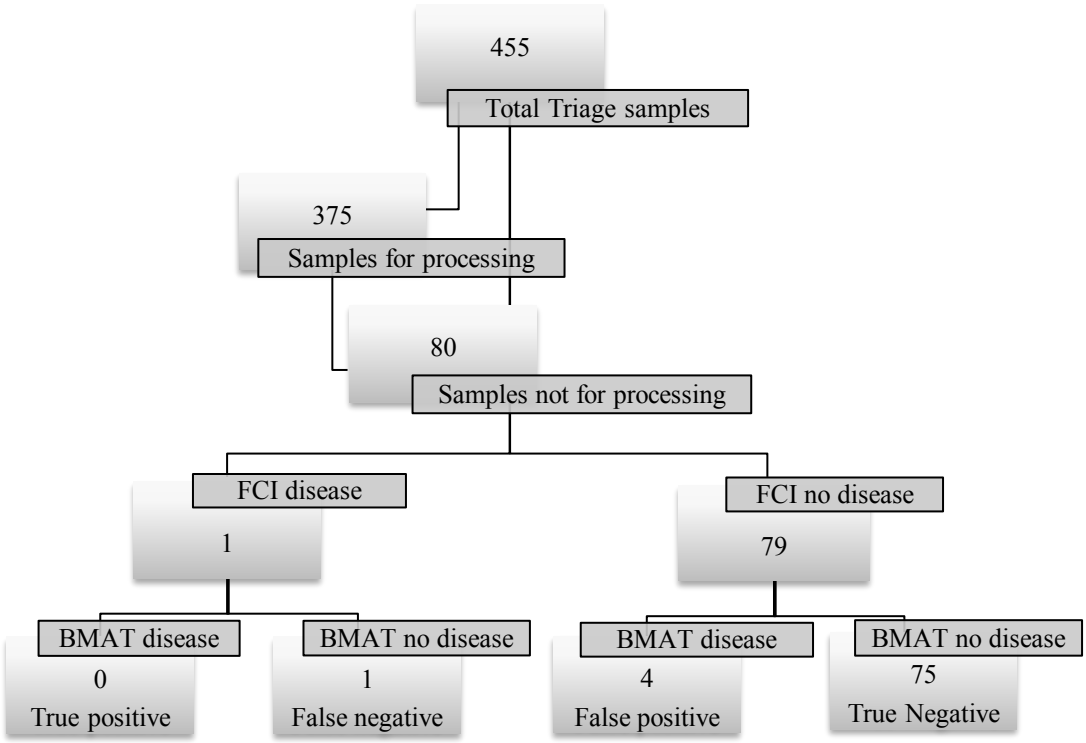

Figure 3.4 Diagrammatic representation of the triage process samples without medical indications for FCI sample processing

The triage process correctly excluded cases in this subgroup that had no disease by FCI, revealing a 0% sensitivity and 0% PPV (as no cases with disease were identified). However, the specificity (by contrast, and as expected as there was no confirmed disease in any of these cases) was high, and calculated at 94.9%, 95% CI [87.5-98.6%]; similarly, the NPV was 98.7%, 95% CI [98.6-98.8%]. In this group, the disease prevalence was 1.3%, 95% CI [0.03-6.8%] and accuracy of 93.8%, 95% CI [86.0-97.9%], as depicted in Table 3.5.

Of the 80 cases without Bethesda medical indications that are not recommended for FCI sample processing, 79 cases had no confirmed disease in the FCI analysis (Figure 3.4). Of these cases, 75 were true negative cases with no concurrent disease in the BMAT or BMA molecular investigations. Four false positive cases were detected with histological evidence of disease discovered in the corresponding BMT biopsies. Of the four false positive cases, two had evidence of HL, one case had a non-haemopoietic infiltration and another case reported 'some primitive cells'. A description of the four false positive cases is shown in Table 3.9.

Table 3.9 Triage process: cases without medical indications for FCI - four false positive cases

Case number	Medical Indication	BMA Outcome	BMT Outcome	FCI Outcome	Molecular investigations
156	Isolated anaemia	Aparticulate and haemodilute	Non-haemopoietic infiltration	No infiltrate	Negative IgH
192	Isolated anaemia	No infiltrate	Some primitive cells	No infiltrate	-
271	Isolated anaemia	No infiltrate	HL infiltration	No infiltrate	-
343	Staging for HL	No infiltrate	HL infiltration	No infiltrate	Normal cytogenetics

The single false negative case in this subgroup was described in the 'test-all' and overall triage analysis (case 230). This case revealed possible evidence of disease with approximately 1% small-sized B-cells with lambda light chain restriction in the FCI analysis only, however, additional investigations failed to confirm the presence of a malignancy.

CHAPTER 4 - DISCUSSION

In 2006, the 'Bethesda International Consensus recommendations on the Flow Cytometric Immunophenotypic analysis of Haematolymphoid Neoplasia: Medical indications' (3) were published to guide the practice of FCI, and provide concise medical indications for sample processing. The testing approach at the CMJAH flow cytometry laboratory has evolved over the years to a 'test-all' laboratory practice, where all samples were processed irrespective of the existing medical indication. This 'test-all' approach is impractical and depletes the limited resources in a public-sector flow cytometry laboratory. The results of this study revealed that the use of the Bethesda medical indications guideline can efficiently and safely reduce the workload in up to 18% (80/455) of cases submitted for testing (Figure 3.2 and 3.4). This outcome alone may have important implications for clinical diagnostics and local laboratory resources, by preventing unnecessary testing of FCI samples.

This is the first study known to the authors to evaluate the effectiveness of the Bethesda medical indications guideline in a sub-Saharan African flow cytometry laboratory.

4.1 Patients' characteristics and overall laboratory findings

The patients' characteristics and overall laboratory findings yielded some interesting but not unexpected findings. Of the 500 cases included in this study, HIV positivity was noted in more than half of the overall case cohort. In the specimens with Bethesda medical indications for FCI testing (81.4%), only half were submitted with a history of a suspected malignancy or bone marrow infiltration (41%). Notably, over a third of the above-mentioned cases were submitted for investigation of a bicytopenia. This is relevant in our local setting where a high prevalence of HIV infection has shown to be a cause of a bicytopenia (32). Disease in the FCI analysis was identified in only 14% of the overall case cohort, despite over 80% with valid Bethesda medical indications. A review of the BMA cytomorphology cases revealed only 21% with evidence of disease and interestingly, several local studies reported a similar diagnostic yield ranging from 27% to 47% (33–35). These outcomes are reflected in the sensitivity and specificity analysis (Table 3.5).

4.2 Quality of BMA cytomorphology specimens submitted for analysis

The quality of all BMA specimens submitted for cytomorphological assessment were reviewed. The analysis revealed good quality specimens in just over half of the cases, with 42.6% reported to be AHI. Amongst the AHI quality BMA specimens, 33% were reported as 'inadequate for meaningful assessment', with no accompanying myelogram or interpretative comment, and 47% revealed no morphological evidence of disease. Despite the reported absence of disease in the latter group, this cannot be excluded with certainty, as a sample of inadequate quality is not completely representative of marrow reserve. The findings of this study emphasize the need to distinguish cases with disease, that were often only reported as aparticulate, but not as haemodilute or inadequate. In aparticulate samples, the extent of disease identified may often not be an accurate reflection of the degree of marrow infiltration. Thus, the documented disease percentages are likely an underestimation of actual disease.

Furthermore, in AHI quality specimens, we noted a tendency amongst haematopathologists to record the presence of 'occasional worrying primitive mononuclear cells', with very low percentages or no reported percentages. In accordance with the Bethesda medical indications guideline, FCI testing is indicated for the presence of atypical cells. However, the results of this study showed that the FCI analysis frequently failed to detect very small populations of 'primitive mononuclear cells', that were described in the BMA cytomorphology reports.

The description of the quality of BMA cytomorphology samples was inconsistent and not standardized. Good and adequate quality specimens appeared to be optimal for a comprehensive assessment; in comparison to AHI quality specimens that were often not associated with a full assessment, unless easily identifiable normal or malignant cells were found. Samples described as suboptimal quality were often occupied by a full interpretative assessment, however, it was unclear what defined a specimen of this type of quality.

The quality of the BMA specimens vastly impacts the outcomes of all BMA investigations. The first step to ensuring accurate FCI and BMA cytomorphology results is dependent on the performance of good quality BMAT biopsies (36). Ideally, bone marrow biopsy procedures should be performed by experienced medical practitioners in a controlled and sterile environment, with adequate analgesia and clinical expertise. The considerable number of AHI quality specimens in this cohort (42.6%) is highly concerning, as poor-quality specimens

compromise meaningful outcomes (36). Moreover, the bone marrow biopsy procedure is an invasive investigation with associated morbidity and mortality (37). The overall quality of the BMA cytomorphology samples in this study, particularly specimens of AHI or suboptimal quality, may reflect a lack of experience in training and supervision in local clinical services. Furthermore, Yang et al showed that a lack of knowledge about BMA adequacy contributed to suboptimal samples, and the quality of BMA specimens improved significantly with educational sessions (36). We further reviewed the corresponding BMT biopsies in all AHI quality BMA samples, and the findings revealed inadequate quality specimens or no results in 62 cases. This accounts for a substantial 29% of the case cohort and reflects on the lack of clinical expertise in performing BMAT biopsies. The performance of BMA samples requires continuous training with supervision, and this may be beneficial to ensure that better quality specimens are received in the laboratory. Failure to obtain a bone marrow aspiration is termed a ‘dry tap’ and should not always be dismissed as poor technique. A ‘dry-tap’ is frequently observed in haematological conditions associated with fibrosis, and hypercellularity secondary to a predominance of immature or tumour cells (38–40). There were no BMA cases reported as a ‘dry tap’ in this case cohort, as these samples did not fulfil the inclusion criteria.

4.3 Bone marrow lymphocytosis in BMA samples described as AHI quality

According to the Bethesda medical indications guideline, a bone marrow lymphocytosis is a valid medical indication for FCI testing, that is often associated with lymphoid neoplasms (3). In the adult population, a bone marrow lymphocytosis is defined as 15-25% lymphocytes (27), however, in paediatric patients this percentage is variable and age dependent (27). In samples of AHI quality, a marrow lymphocytosis is likely secondary to haemodilution with peripheral blood that has a higher lymphocyte count. Subsequently, this may lead to lymphocyte populations appearing more prominent, but not truly reflective of disease. Among the group of AHI quality BMA samples, a marrow lymphocytosis was seen in 36 adult and paediatric patients, but only three adult cases had disease in the FCI analysis (two with clonal plasma cells and one with a CD19/10 co-expressing B-cell lymphoma). All three cases had clinically suspected features of a malignancy and medical indications necessitating FCI testing. The processing of AHI quality samples for FCI may indeed be beneficial, despite a degree of haemodilution, if physical examination features and/or abnormal peripheral blood smear findings are suggestive of malignancy. This is further supported by the 25 cases in this subgroup

that proved to be diagnostic by FCI testing, despite the AHI quality of the specimens (Table 3.4).

Conversely, as the outcomes of this study revealed in many cases, FCI testing was not helpful if the corresponding BMA cytomorphology samples were reported as AHI quality. In this context, the decision to undertake FCI testing should be made on an individualised basis and limited to cases where haematolymphoid disease is clinically suspected and/or identified cytomorphologically. The processing of AHI samples for FCI will incur preventable expense, in the absence of a screening process. This highlights the value of a triage process with a comprehensive review of each case submitted for FCI testing. The Bethesda medical indications guideline does not stipulate a review of the corresponding BMA cytomorphology slides prior to FCI testing. However, the data from this study suggests that implementing this additional step to the triage will identify samples that do not require processing, despite existing medical indications. Moreover, reviewing the BMA cytomorphology slides prior to rejection of the FCI sample, will ensure that disease is excluded even in cases without FCI medical indications. This could substantially reduce the burden on the overall diagnostic service delivery.

4.4 Hodgkin lymphoma

The tumour microenvironment in patients with HL is comprised predominantly of an inflammatory milieu, with a paucity of malignant mononuclear and Hodgkin Reed-Sternberg (HRS) cells (20). These malignant cells frequently represent 1% to less than 0.01% of the neoplastic cell population (20). The rarity and large size of the HRS cells as well as T-cell rosetting of the neoplastic cells, results in difficulties with isolation and identification during FCI processing and analysis (20). These large cells are frequently lost during processing of BMA samples, and as a result are not readily identified in the FCI analysis (20). The Bethesda medical indications guideline states that BMA samples submitted for staging of disease in Hodgkin lymphoma patients, is not a valid medical indication for FCI testing (3). This is further supported by the findings of this study, where no disease was found in both the BMA cytomorphology and the FCI analysis in all samples submitted for HL staging. Conversely, as extensively shown in the literature, LN biopsy specimens may successfully identify HRS cells in the FCI analysis (20,21,41–43). Furthermore, Di Gaetano et al. demonstrated that the tumour microenvironment in LN biopsies may be identified immunophenotypically, in cases with a known histological diagnosis of Hodgkin lymphoma (44). Cader et al. further described the

$CD4^+$ regulatory T-cell rich HL microenvironment using mass cytometry (45). The use of FCI has proven valuable for analysing LN biopsies for HL, however, its role in assessing BMA specimens currently offers no diagnostic potential.

4.5 Non-haemopoietic malignancies

Non-haemopoietic malignancies are typically large cells dispersed in clumps, that infiltrate the BMAT depending on the type of solid tumour and extent of disease (13,19). These abnormally large cells may be lost during FCI sample processing, and subsequently, not detected in the FCI analysis (13,19). This notion is further supported by the findings of this study, as the FCI analysis failed to identify non-haemopoietic infiltrations in all 24 cases. Samples with confirmed non-haemopoietic malignancies were identified in either or both the BMA cytomorphology and BMT biopsies. Although non-haemopoietic infiltrations can potentially be detected by their absence of leucocyte common antigen (CD45) expression in the FCI analysis (22,23); FCI provides a limited diagnostic potential in comparison to the BMT biopsy. The BMT biopsy can delineate the type of tumour and the degree of tumour infiltration with special and IHC stains, thus negating any value that FCI can bring to the overall diagnostic outcome. Furthermore, limited data exists to support the use of FCI in differentiating non-haemopoietic malignancies in paediatric patients (46,47). The use of FCI in non-haemopoietic malignancies remains controversial, due to its limited potential in identification and lineage characterisation in various non-haemopoietic tumours.

4.6 'Test-all' analysis: BMA cytomorphology predicts disease detected by FCI, and reliably excludes disease

This study aimed to assess the sensitivity, specificity, and predictive values of the BMA cytomorphology, in a cohort of samples that were all previously tested and analysed by FCI. This was primarily undertaken to establish the relationship between the relatively subjective BMA cytomorphology outcomes, with the more objective FCI results. We aimed to determine whether the BMA cytomorphology could reliably predict or confirm absence of disease established in the FCI outcomes. The 'test-all' analysis revealed a 97.1% sensitivity and slightly lesser specificity of 89.8%, with a PPV of 64.1% but striking 99.4% NPV. These findings correlate with the large number of cases where disease was reliably excluded in both the BMA cytomorphology and FCI outcomes. This important outcome suggests that the workload can be substantially reduced, by a detailed review of the BMA cytomorphology, prior to FCI testing.

This approach will result in a slight delay of a few hours in FCI testing, however, it will ensure that samples warranted for FCI are processed, even in cases without medical indications or clinically-suspected disease.
The absence of disease cytomorphologically can safely defer the decision to not process cases for FCI testing. These findings suggest that BMA cytomorphology can be used as a reliable arbiter in deciding whether to process, or not process cases, with and without medical indications for FCI testing.

The FCI is a highly sensitive investigation, as evident by the two cases with identifiable disease in the FCI analysis, but not in the corresponding BMA cytomorphology (denoted as false negatives, see Figure 3.1). The comparison between FCI and BMA cytomorphology was specifically undertaken to assess the relationship between these two testing modalities. This was performed to mimic the usual scenario, where other BMA investigations are not yet processed, or reported at the time of FCI sample processing. A BMAT is submitted for various investigations to exclude disease. The FCI is only one test of multiple BMA investigations and is not usually performed in isolation. To understand the role of FCI in the two false negative cases, it is important to understand the clinical context of each case. Upon review of these two cases, case 230 was submitted to determine the cause of the symptomatic anaemia in a patient with end-stage renal disease, and case 371 was performed to investigate for an underlying plasma cell myeloma. In case 230, no disease was identified in the BMA cytomorphology, but FCI found 1% small sized B-cells with lambda light chain restriction of uncertain significance. The IgH for B-cell gene rearrangement study performed on the same FCI sample was negative, thus excluding a clonal process. This suggests that the light chain expressing population detected in the FCI analysis, may have been a technical aberrancy or alternatively, the IgH study failed to identify PCR products. There were no additional investigations found on LIS to confirm a clonal cell population. A review of the raw FCI data, to assess this possible technical aberrancy was beyond the scope of this study. In case 371, FCI identified 0.1% aberrant plasma cells, but no morphological evidence of disease was found in the corresponding BMA cytomorphology and BMT biopsy. In contrast to the previous case, the IgH for B-cell gene rearrangement study, revealed a monoclonal band confirming a clonal condition. In this instance, FCI played a fundamental role, that led to the discovery of malignant disease. This case proves that FCI is more sensitive, than the BMA cytomorphological assessment.

The use of BMA cytomorphology to defer a decision to process, may result in some inaccuracy as evident by the 37 (9%) false positive cases. Disease was reported or suspected in the BMA cytomorphology, but FCI failed to identify clonal cell populations in all 37 false positive cases. In almost half of these cases, the haematopathologists reported 'primitive mononuclear cells' in the BMA cytomorphology, however, only three cases had disease in the BMT biopsies (Figure 3.1 and Appendix C). Among these cases, two samples were diagnosed with HL and one case with diffuse large B-cell lymphoma. The FCI analysis is unable to detect HL disease, and the Bethesda medical indications guideline, do not advocate FCI testing in these samples (3). In DLBCL, large cells may be lost during FCI sample processing, and may not be detected in the FCI analysis (19).

4.7 The triage process: The impact of the Bethesda medical indications for flow cytometric immunophenotyping

A secondary aim of this study was to assess the clinical impact of the Bethesda medical indications, by applying the guidelines for FCI testing, to the same 'test-all' cohort (i.e. subjecting all the cases to the triage process, but with the safety net of already established, and reported outcomes). This analysis was important to ascertain, whether the triage of samples was effective in rationalising a decision to process, or not process FCI specimens. Furthermore, it was important to establish that the use of the triage, would not miss samples with disease ie. samples without disease would be correctly categorised as 'not process'. This study objective was achieved by determining the sensitivity, specificity, and predictive values of the triage process against objective evidence of disease in various BMA investigations. In contrast to the 'test-all' approach, where only the BMA cytomorphology outcomes, and the FCI analysis were being correlated, in the triage process, we further assessed the BMT biopsy and BMA molecular investigations, to confirm or exclude any evidence of disease. We embarked on this approach for the triage process, to further enhance the efficacy of the results, by including various BMA investigations in which disease may be identified. For this part of the study, the number of excluded cases were less than in the 'test-all' approach, due to the inclusion of these additional investigations, where disease was confirmed. This resulted in an increase in the disease prevalence from approximately 16% in the 'test-all' approach, to 27% in the triage data set.

Of the 455 triage cases, 375 (82%) fulfilled the Bethesda medical indications to 'process', with disease noted in only 32% (120/375). This included 69 cases with disease in the FCI, BMAT and/or molecular studies, and another 51 cases with disease only in the BMAT and/or molecular studies, but not the FCI analysis (Figure 3.3). This led to a very high sensitivity (96%) in the triage, but reduced specificity of 22.7%, coupled with a PPV of 32%. This was attributable to the many cases without confirmed disease (68% or 255/375 cases), despite having valid medical indications for processing. This finding was echoed by the high NPV calculated at 94%, confirming that many 'processed' samples were unnecessary, despite all cases having valid medical indications.

A review of the medical indications in this subgroup, revealed that a substantial number of cases were referred for investigation of a bicytopenia (n=95). A bicytopenia is defined as any two of the following cytopenias: anaemia, thrombocytopenia or neutropenia (48). In our South African setting, a bicytopenia is commonly associated with various non-malignant, and infectious diseases, notably HIV and TB. Processing of these samples for FCI, would not add any value other than excluding an incidental, co-existing haematolymphoid malignancy (33,34,49). This is an important aspect to be considered in our local context, when establishing relevant and appropriate guidelines for FCI processing.

Eighty of the 455 triaged cases did not meet any criteria for FCI and were categorised as 'not processed' (Figure 3.2). No objective evidence of disease was identified in approximately 94% (75/80), with a very small proportion (6%) where disease was potentially 'missed', due to categorisation as 'not process' (Figure 3.2). A comprehensive review of these cases, showed that the absence of definitive disease in the FCI analysis, would not have impacted on the clinical outcomes in all five cases (Table 3.8). Among these cases, two patients were diagnosed with HL, one with a non-haemopoietic infiltration and 'primitive cells' were described in the BMT biopsy in one case. In the latter case, there were no further laboratory investigations confirming disease. Case 230 was the only case with objective evidence of disease in the FCI analysis, however, this finding proved to be insignificant as there was no confirmation of clonality by IgH gene rearrangement studies (Table 3.8, Figure 3.2 and 3.4). This case was also denoted as a false negative case in the 'test-all' analysis (Figure 3.1 and Table 3.6). These findings suggest that the triage was largely effective in eliminating unnecessary sample processing, in cases where FCI offers no diagnostic potential.

4.8 The value of FCI to detect disease in samples with Bethesda medical indications, categorised as 'process'

Overall, the triage process accurately categorised cases with disease in the FCI analysis, but also revealed a significant number of cases with no disease. In this subgroup of 375 cases categorised as 'process', FCI identified disease in only 69 cases, and this was confirmed by disease in the corresponding BMAT and BMA molecular investigations (Figure 3.3). Despite all the cases in this subgroup having valid Bethesda medical indications, 82% (306/375) had no disease in the FCI analysis. Importantly, the absence of disease in the FCI analysis, was confirmed in the corresponding BMAT and BMA molecular investigations in 83% (255/306) of cases (Figure 3.3). This could potentially reduce unnecessary FCI sample processing, in 56% (255/455) of cases submitted for routine testing.

There were 51 false positive cases, where FCI did not detect disease that was suspected in the corresponding BMA cytomorphology, BMT or molecular investigations (Figure 3.3). Among these cases, 10 were diagnosed with HL, and another 8 cases with non-haemopoietic malignancies. The FCI analysis does not usually identify disease in these cases, particularly with HL, and processing of these samples did not yield a meaningful outcome. Furthermore, 19 of the 51 cases had 'primitive or abnormal mononuclear cells' reported in the BMA cytomorphology, and only 6 (12%) of the 19 cases had definitive disease in the BMT biopsy. This finding was reiterated in the 'test-all' analysis (Figure 3.1), where the subjectivity of reporting 'suspicious or primitive cells' was evident. Identifying 'occasional primitive mononuclear cells' in the BMA cytomorphology is often subjective, and difficult to interpret, unless extensive disease is identified in other anatomical sites. In an ideal context, such cases would benefit from an overall individualised triangulation of the clinical presentation and examination findings, FBC and peripheral blood smear results, and the BMAT outcomes prior to FCI sample processing.

4.9 The FCI samples in the triage process that were not recommended for processing, due to the absence of Bethesda medical indications

Of the 80 samples categorised as 'not process', four false positive cases and one false negative case was identified (Figure 3.4). The latter case was also described in the 'test-all', and overall triage analysis, as a false negative case. In view of the non-specific findings of this case, with no valid Bethesda medical indications for FCI, and in the absence of conclusive disease in various laboratory investigations, this case was accurately categorised under samples not recommended for processing.

Of the four false positive cases in this subgroup, FCI does not usually detect disease in samples with HL or non-haemopoietic malignancies, or offers a limited diagnostic evaluation in comparison to the BMA and BMT biopsies. Furthermore, the presence of 'occasional primitive cells' is a non-diagnostic finding, as disease could not be confirmed on additional investigations.

The sensitivity and PPV of 0% in this subgroup was acceptable, as this analysis could not confirm any cases with definitive disease. The very low disease prevalence, very high NPV and accuracy is evident that the triage process is effective in rationalising cases that do not require FCI sample processing, without missing samples with definitive disease.

4.10 Limitations

A limitation of the study is that the leukaemia diagnostic panels used during 2019 for FCI analysis were not standardised, and were chosen according to patients' history and laboratory findings. The choice of FCI panel was dependent on the amount and quality of the sample received. Most of the cases analysed were screened with a limited panel, including a combination of the following monoclonal antibodies: kappa, lambda, CD19, CD5, CD34, CD10, CD14 and CD45, to exclude the presence of an underlying B-cell lymphoproliferative disorder, an acute leukaemia or non-haemopoietic malignancies. It is uncertain how the extent of variable FCI workup has impacted on FCI outcomes recorded. The reporting of the FCI, BMA cytomorphology and BMT biopsy outcomes is not fully standardised, as these are undertaken by several consultants employed in the department, and therefore, the outcomes recorded are the diagnostic opinion of the authorising consultants.

4.11 Recommendations

The findings of this study revealed that most samples had valid Bethesda medical indications for FCI testing, however, the FCI analysis showed no disease in most cases. Furthermore, the absence of disease in the FCI analysis, was confirmed in the corresponding BMAT and molecular investigations.

The results of the 'test-all' analysis showed a very high sensitivity, relatively high specificity, and strong NPV of BMA cytomorphology versus FCI outcomes, where BMA cytomorphology is distinctly able to definitively exclude disease. Moreover, the triage process revealed a high sensitivity and specificity of documented disease by FCI, versus disease in the BMAT and molecular investigations.

The evidence-based outcomes of this study, suggests a need for modification of the currently utilised Bethesda medical indications guideline. Thus, the following recommendations or modifications are proposed to the current 2006 Bethesda medical indications guideline, particularly for developing countries (Appendix E):

1. All flow cytometry laboratories should institute a triage-based laboratory approach using concise medical indications as described in the international Bethesda medical indications guideline (3). This practice is pertinent in developing countries around the world to minimise on expense and technical effort efficiency.
2. A comprehensive, individualised assessment of each patient's history, clinical examination findings and relevant laboratory results should be performed, prior to sample rejection (50).
3. A review of the clinical context is essential in cases with an isolated cytopenia, bicytopenia, non-massive hepatomegaly or splenomegaly and generalized lymphadenopathy, but no other malignancy associated features. In a HIV prevalent country, a combination of generalized lymphadenopathy and/or hepatomegaly and/or splenomegaly may well be a result of infective conditions and opportunistic infections such as tuberculosis. In these instances, flow cytometry does not offer any clinical value unless there is an obvious lymphocytosis, without sample haemodilution.
4. In state practice, all cases should be screened by registrars and overseen by a specialist haematopathologist, prior to FCI sample processing.

5. All FCI samples submitted for analysis should have a separate slide prepared, stained and microscopically reviewed for exclusion of disease, prior to sample rejection (50). These outcomes should be closely correlated with the corresponding BMA cytomorphology report.
6. In samples with no medical indication for FCI testing, and no disease is identified on the microscopic analysis of the FCI samples, and the BMA cytomorphology slides, the FCI sample may reliably be rejected and not processed. We recommend that a full case history be reported as part of the reasoning for rejection (not processing) of the submitted sample. This assists with consolidating the triage rationale and serves to educate clinicians on the medical indications for FCI testing. This process can be supplemented with information from the attending physicians in all clinical disciplines, who should be contacted if further clarification pertaining to difficult cases is required. Sample rejection, where the decision to not process a sample may not be justified, should be discussed with the attending physician who referred the sample for testing.
7. The processing of samples described as AHI quality in both the BMA cytomorphology and FCI slide, may not add any value, even in cases with valid medical indications. Processing of these samples is futile and results in preventable expense, particularly in developing countries.
8. Staging of disease in patients with HL is not an indication for FCI testing as highlighted in the current Bethesda medical indications guideline. The current FCI technology is unable to detect HRS cells in BMA specimens and this recommendation is supported by the data from this study.
9. Non-haemopoietic malignancies are occasionally identified in the FCI analysis, but with no diagnostic potential for further subtyping and characterisation of non-haemopoietic cells. Immunohistochemical and special stains performed on the BMT biopsy are more crucial for further delineation of the tumour type and origin. In developing countries, it seems plausible to not process these cases for FCI analysis, however, the final decision for sample processing should be made in consultant with the specialist haematopathologist.

To improve the overall efficiency of the FCI outcomes, pre-analytical, analytical and post-analytical laboratory variables also require addressing. The prevention of pre-analytical errors by the provision of clear guidelines on sample collection, labelling and transport is of utmost importance to all clinicians and laboratorians. All staff analysing and interpreting FCI samples

should be adequately trained and competent with the skills and standards of expected knowledge as per the Bethesda International Consensus Recommendations for Training and Education to Perform Clinical Flow Cytometry (51). A standard reporting template for the FCI outcomes may be beneficial to both clinicians and pathologists for ease of interpretation, if implemented nationally.

Without impacting clinical outcomes, the implementation of a triage process can modestly reduce the burden of FCI testing by 18% (80/455). Substantial resources can be saved, and triage made more effective and accurate, by taking advantage of the high local specificity and NPV of BMA cytomorphology to FCI, without missing disease. Preliminary cytomorphological review of the accompanying BMA is strongly recommended as an additional step to improve the overall PPV of the triage, whilst safely reducing unnecessary FCI sample processing in a further 56% (255/455) of cases submitted for routine testing.

CHAPTER 5 - CONCLUSION

The triage process used to categorise samples was based on the current 2006 Bethesda medical indications guideline, and was developed by a group of international experts in the field of FCI. The results of this study revealed that the triage process was effective in reliably categorizing samples for processing, or not for processing, depending on the presence or absence of disease in various BMA laboratory investigations. Furthermore, the BMA cytomorphology reliably predicted disease in the FCI analysis.

The Bethesda guidelines were established with a first world setting in mind, with a focus largely on non-communicable disease, and without restricted access to resources. A modified Bethesda medical indications guideline for haematolymphoid malignancies is essential in low-income countries, where access to resources is limited. Moreover, the implementation of these guidelines as a laboratory triage process, will enable identification of pertinent cases requiring FCI sample processing, and ultimately, reduce the existing financial burden in developing countries. Guidelines are implemented for appropriate clinical and laboratory practice. The stringent use of guidelines should be avoided, as the decision to process a sample should be individualised based on local disease prevalence.

A triage-based approach with modifications for local use, is universal to all specialist laboratory testing, and the principles can potentially be applied across all pathology disciplines.

In conclusion, the implementation of a triage process in flow cytometry laboratories would enable the appropriate rationalisation of resources, improve the cost-effectiveness and overall diagnostic service delivery in developing countries like those in Sub-Saharan Africa.

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APPENDICES

APPENDIX A: Ethics clearance certificate

APPENDIX B: Data Collection sheets

APPENDIX C: False positive cases in the 'test-all' approach

APPENDIX D: False positive cases in the triage process - samples with medical indications for FCI processing

APPENDIX E: '2006 Bethesda International Consensus Recommendation on the Flow Cytometric Immunophenotypic analysis of haematolymphoid neoplasia: Medical Indications' (3) and proposed recommendations or modifications in our local setting

APPENDIX F: Two-way tables

APPENDIX G: Plagiarism/Turn-it-in report

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Dr M Naidoo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M2011157

NAME: (Principal Investigator)	Dr M Naidoo
DEPARTMENT:	School of Pathology Department of Molecular Medicine and Haematology Medical School University
PROJECT TITLE:	*Rationalising laboratory workflow to improve the efficiency of diagnostic service delivery: a critical review of haematological malignancies by flow cytometric immunophenotyping at the Charlotte Maxeke* Johannesburg Academic Hospital Change of study title noted on 2023/01/20
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Laboratory study under M1704129
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Professor DK Clemcross
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/01/19

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **November** and therefore reports and re-certification will be due in the month of **November** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator _____ Date _____

APPENDIX B: Data collection sheets

Table 1: 'Test-all' analysis

Case number	Medical indication (reason for referral)	Medical indication (1) No medical indication (2)	Clinical presentation and examination findings (if available)	FBC	DIFF and peripheral blood smear findings	BMA quality and outcomes	FCI findings (diagnosis)	FCI Disease (1) No disease (0)

Table 2: Triage process

Case number	Medical indication (reason for referral)	Medical indication (1) No medical indication (2)	Clinical presentation and examination findings (if available)	FBC	DIFF and peripheral blood smear findings	BMA quality and outcomes	FCI findings (diagnosis)	FCI Disease (1) No disease (0)	BMT biopsy quality, outcomes and IHC, SS	Molecular & additional findings

APPENDIX C: False positive cases in the 'test-all' approach

Table 3: False positive cases in the 'test-all' approach

Case number	Medical indication for FCI	BMA cytomorphology outcomes	BMT biopsy outcomes	FCI outcomes	Additional investigations
15	Cause of the bicytopenia	Lymphocytosis ~ 64% Plasmacytosis ~ 17%	No BMT infiltration	No BLPD	TCRG: polyclonal
20	Non-Hodgkin lymphoma, in keeping with Sezary syndrome	Very occasional lymphoid cells with some nuclear folding	Inadequate	No mature LPD	TCRG: monoclonal
77	Cause of the bicytopenia	1% primitive mononuclear cells of uncertain significance.	Bone marrow infiltration by Classical HL	No infiltrate	IgH gene rearrangement studies: negative
83	Staging BMAT for Follicular lymphoma	Marrow lymphocytosis - 23%	No lymphomatous infiltrate	No BLPD	LN Biopsy: low grade Follicular lymphoma
87	Staging BMAT for Mycosis fungoides	Occasional large blasts.	No BMT infiltration	No BLPD or non-haemopoietic infiltrate	TCRG: polyclonal
89	Staging BMAT for DLBCL	2% atypical mononuclear cells	No BMT infiltration	No non-haemopoietic infiltrate	IgH: negative
91	Staging BMAT for Hodgkin lymphoma	2% primitive mononuclear cells	BMT infiltration by classical HL	No BLPD	LN Biopsy: classical HL

94	Cause of the thrombocytopenia	2% blasts or primitive mononuclear cells with uncertain significance	Inadequate	No BLPD or non-haemopoietic infiltrate	-
116	Generalised lymphadenopathy-Disseminated TB/Non-Hodgkin lymphoma queried	1% primitive mononuclear cells with uncertain origin/significance	Suboptimal	No BLPD or non-haemopoietic infiltrate	-
119	To investigate for multiple myeloma with a paraprotein	5% plasma cells (some larger primitive), 35% lymphocytes	Inadequate	No BLPD or non-haemopoietic infiltrate	-
146	Suspected acute leukaemia	Extensive infiltration by non-haemopoietic cells	BMT infiltration: non-haemopoietic infiltrate	No BLPD, non-haemopoietic infiltrate or increase in blasts	Biopsy of bone mass: synovial sarcoma
160	Staging BMAT for Plasmablastic lymphoma	Occasional larger worrisome cells	BMT infiltration	No BLPD	-
169	Staging BMAT for DLBCL	1-2% primitive mononuclear cells	BMT infiltration by DLBCL	No BLPD or non-haemopoietic infiltrate	LN Histology: DLBCL
208	Cause of the pancytopenia	3% large mononuclear cells	Suboptimal, no evidence of malignancy	No increase in blasts or an infiltrate	-
244	Staging BMAT: non-haemopoietic infiltrate	Extensive bone marrow infiltration by non-haemopoietic cells	BMT infiltration by non-haemopoietic infiltrate	No BLPD or non-haemopoietic infiltrate	-
247	Suspected bone marrow infiltration	7% primitive mononuclear cells	Inadequate	No BLPD, non-haemopoietic infiltrate or increase in plasma cells	-

249	Cause for the pancytopenia	16% primitive mononuclear cells	Possible MDS with excess blasts: 5-10% blasts	No BLPD	-
255	Suspected multiple myeloma with a paraprotein	4% plasma cells (well differentiated and occasional larger forms)	10-15% plasma cells	No BLPD or non-haemopoietic infiltrate	-
257	Staging BMAT for possible non-haemopoietic infiltrate	Extensive bone marrow infiltration by non-haemopoietic cells	Extensive BMT infiltration by metastatic neuroblastoma	No non-haemopoietic infiltrate	FISH for N-MYC gene: positive
293	Suspected malignancy	Numerous clumps of cohesive cells	BMT infiltration by non-haemopoietic infiltrate	No BLPD	-
298	Cause of the anaemia	Occasional mononuclear cells	No BMT infiltration	No BLPD or Increase in blasts	-
299	Suspected bone marrow infiltration	No overt infiltrate or increase in blasts - marrow lymphocytosis- 21%	No BMT infiltration	No infiltrate	-
304	Cause for the pancytopenia	Clumps of cells: suspected non-haemopoietic infiltrate	BMT infiltration by a non-haemopoietic infiltrate	No BLPD	-
324	Staging BMAT for Non-Hodgkin lymphoma	Occasional large primitive mononuclear cells	No BMAT infiltration	No BLPD or non-haemopoietic infiltrate	-
351	Staging BMAT for Rhabdomyosarcoma	Extensive BM infiltration by non-haemopoietic infiltrate	BMT infiltration by non-haemopoietic infiltrate	No non-haemopoietic infiltrate	FOXO1 gene positivity

359	Cause of the bicytopenia	Single clump of worrying cells - 3% primitive mononuclear cells	Inadequate	No BLPD, non-haemopoietic infiltrate or increase in blasts	-
367	Cause of the bicytopenia	No infiltrate or increase in blasts - few clumps of cells with uncertain significance	Granulomas - no malignant infiltrate	No BLPD	-
369	Cause for the splenomegaly	Occasional primitive mononuclear cells	No BMT infiltration	No BLPD or increase in blasts	
379	Suspected bone marrow infiltration - FNA showed T-cell Acute Lymphoblastic Leukaemia	7% primitive mononuclear cells	No BMT infiltration	No infiltrate	FNA showed T-cell Acute Lymphoblastic Leukaemia
389	Cause for the lymphadenopathy and hepatomegaly	9% blasts	No BMT infiltration	No infiltrate	-
403	Suspected multiple myeloma with a paraprotein	63% atypical plasma cells	No BMT infiltration	No BLPD	-
424	Suspected non-benign lesion	18% primitive mononuclear cells	Inadequate	No BLPD or non-haemopoietic infiltrate	-
447	Suspected acute leukaemia	6% primitive mononuclear cells	No BMT infiltration	No non-haemopoietic infiltrate or increase in blasts	-
465	Staging BMAT	1% abnormal cells	No BMT infiltration	No BLPD	-
472	Cause of the bicytopenia	6% atypical mononuclear cells	No BMT infiltration	No infiltrate	-

491	Massive hepatosplenomegaly - Haemophagocytic lymphohistiocytosis (HLH) suspected	5% primitive mononuclear cells	No BMT infiltration	No increase in blasts.	HLH seen on liver biopsy, IgH was monoclonal and cytogenetics: 49XX and t(6;8)
498	Cause of the bicytopenia	Occasional small clumps of degenerate cells of uncertain significance	Inadequate	No non-haemopoietic infiltrate	-

BLPD, B-cell lymphoproliferative disorder; BMA, bone marrow aspirate; BMT, bone marrow trephine; BMAT, bone marrow aspirate and trephine biopsy; DLBCL, diffuse large B-cell lymphoma; FCI, flow cytometric immunophenotyping; FNA, fine needle aspirate; HL, Hodgkin lymphoma; HLH, haemophagocytic lymphohistiocytosis; IgG, immunoglobulin G; IgH, immunoglobulin heavy chain; LN, lymph node; LPD; lymphoproliferative disorder; MDS, myelodysplastic syndrome; TB, tuberculosis; TCRG; T-cell gene rearrangement studies.

APPENDIX D: False positive cases in the triage process - samples with medical indications for FCI processing

Table 4. False positive cases in the triage process - samples with medical indications for FCI processing

Case number	Medical indication for FCI	BMA cytomorphology outcomes	BMT biopsy outcomes	FCI outcomes	Additional investigations
12	Bicytopenia and generalised LAD	Aparticulate and haemodilute, can't exclude an infiltrate	Extensive infiltration by HL	No BLPD	FNA: underlying malignancy suspected
15	Cause for the bicytopenia	Marrow lymphocytosis ~64%, Plasmacytosis 17%	No BMAT infiltration	No BLPD	TCRG rearrangement studies: polyclonal
20	Non-Hodgkin lymphoma in keeping with Sezary syndrome	Very occasional lymphoid cells with some nuclear folding	Inadequate	No mature LPD	TCRG rearrangement studies: monoclonal
21	Cause for the anaemia and thrombocytopenia, suspected disseminated TB	Aparticulate and haemodilute, no infiltrate	Occasional primitive mononuclear cells of uncertain significance	No non-haemopoietic infiltrate or increase in blasts	IGH gene rearrangement studies: polyclonal
31	Staging BMAT for HL	No infiltrate	Bone marrow infiltration by classical HL	No BLPD or non-haemopoietic infiltrate	Histology of FNA: classical HL

40	Suspected MDS with FISH positivity for isolated deletion of 5q.	Aparticulate, no infiltrate	No BMAT infiltration	No disease. ~3% B-cells with an increased kappa to lambda ratio. This significance of this is uncertain	IgH gene rearrangement studies: monoclonal products of uncertain significance
48	Cause for bicytopenia	Aparticulate and haemodilute, can't exclude an infiltrate – suspected secondary HLH	No evidence of disease	No BLPD or non-haemopoietic infiltrate	-
77	Cause for the bicytopenia	Aparticulate and haemodilute, 1% primitive mononuclear cells of uncertain significance. No increase in blasts	BM infiltration by Classical HL (confirmed on IHC stains)	No infiltrate	IgH gene rearrangement studies: negative
83	Staging BMAT for Follicular lymphoma	Aparticulate, marrow lymphocytosis - 23%	Poor quality- no lymphomatous infiltrate	No BLPD	Lymph node biopsy: low grade Follicular lymphoma
89	Staging BMAT for DLBCL	Adequate quality with 2% atypical mononuclear cells	No BMAT infiltration	No disease	IgHgene rearrangement studies: negative
91	Staging BMAT for Hodgkin lymphoma	Adequate quality with 2% primitive mononuclear cells	BMT infiltration by classical HL	No BLPD	Histology of excision lymph node biopsy: classical HL

94	Cause for the thrombocytopenia	Good quality with 2% blasts or primitive mononuclear cells of uncertain significance	Inadequate, can't exclude an infiltrate	No BLPD or non-haemopoietic infiltrate	-
111	Cause for the cytopenia(s)	Good quality with no infiltrate or increase in blasts	BMAT infiltration: Classic HL.	No infiltrate	-
116	Generalised lymphadenopathy suspected TB or NBL	Haemodilute with 1% of primitive mononuclear cells of uncertain significance	Suboptimal for diagnosis	No BLPD or non-haemopoietic infiltrate	-
146	Suspected acute leukaemia	Good quality with extensive infiltration by non-haemopoietic infiltrate	BMAT infiltration: non-haemopoietic infiltrate	No BLPD, non-haemopoietic infiltrate or increase in blasts	Biopsy of bone mass: synovial sarcoma with SS18 gene positivity
159	Staging BMAT for Breast adenocarcinoma	Aparticulate and haemodilute for meaningful assessment with no myelogram. Clumps of cells on myelogram	BMAT infiltration with possible non-haemopoietic infiltrate	No BLPD or non-haemopoietic infiltrate	-
160	Staging BMAT for Plasmablastic lymphoma	Good quality with occasional larger worrisome cells	BMAT infiltration	No BLPD	-
166	Suspected myeloproliferative neoplasm	Adequate quality with 1-2% primitive mononuclear cells	Myelofibrosis	No BLPD	CALR and JAK2 gene positivity
169	Staging for DLBCL	Adequate quality with 1-2% primitive mononuclear cells	BMAT infiltration	No BLPD or non-haemopoietic infiltrate	Histology: DLBCL

179	Staging HL	Good quality with no infiltrate/increase in blasts	BMAT infiltration	No BLPD, non-haemopoietic infiltrate or increase in blasts	Histology: classical HL
197	Plasma Cell Myeloma on tumour biopsy with a paraprotein	Good quality with no infiltrate or increase in plasma cells	BMAT infiltration with clonal plasma cells	No BLPD or increase in plasma cells	-
208	Cause for the pancytopenia	Adequate quality with no increase in blasts or infiltrate - 3% large mononuclear cells	Suboptimal, no evidence of malignancy	No increase in blasts or an infiltrate	-
211	Suspected malignancy	Inadequate quality with no myelogram	BMAT infiltration with plasma cell myeloma	No BLPD	-
214	Cause for the bicytopenia	Aparticulate and haemodilute for meaningful assessment , no myelogram	BMAT infiltration with classical HL	No BLPD or non-haemopoietic infiltrate	-
224	Suspected plasma cell myeloma with a paraprotein	Adequate quality with 1% atypical plasma cells	No BMAT infiltration	No BLPD or increase in plasma cells	-
244	Staging BMAT for non-haemopoietic infiltrate	Aparticulate and haemodilute with extensive non-haemopoietic infiltrate	BMAT infiltration by non-haemopoietic infiltrate	No BLPD or non-haemopoietic infiltrate	-
247	Suspected bone marrow infiltration	Aparticulate and haemodilute with 7% primitive mononuclear cells	Inadequate	No BLPD, non-haemopoietic infiltrate or increase in plasma cells	-
249	Cause for the pancytopenia	Aparticulate and haemodilute with 16% primitive mononuclear cells	Suspected MDS with excess blasts: 5-10% blasts	No BLPD	-
255	Suspected plasma cell myeloma with a paraprotein	Good quality with 4% plasma cells	10-15% plasma cells	No BLPD or non-haemopoietic infiltrate	-

257	Staging BMAT for non-haemopoietic infiltrate	Adequate quality with extensive non-haemopoietic infiltrate	Extensive BMAT infiltration by metastatic neuroblastoma	No non-haemopoietic infiltrate	N-MYC gene positivity
264	Suspected HLH	Adequate quality with no increase in blasts or infiltrate	BMAT infiltration by HL	No non-haemopoietic infiltrate	-
293	Suspected malignancy	Aparticulate and haemodilute with numerous clumps of cohesive cells	BMAT infiltration by non-haemopoietic infiltrate	No BLPD	-
299	Suspected bone marrow infiltration	Adequate quality with no overt infiltrate or increase in blasts, marrow lymphocytosis- 21%	No BMAT infiltration	No infiltrate	TCRG: monoclonal on peripheral blood sample
302	Cause for the pancytopenia	Adequate quality with no increase in blasts or infiltrate	Focal infiltrate: suspected HL	No BLPD	-
304	Cause for the pancytopenia	Aparticulate and haemodilute with clumps of cells - suspected non-haemopoietic cells	BMAT infiltration by non-haemopoietic infiltrate	No BLPD	-
324	Staging BMAT for NHL	Adequate quality with occasional large primitive mononuclear cells noted	No BMAT infiltration	No BLPD or non-haemopoietic infiltrate	-
351	Staging BMAT for Rhabdomyosarcoma	Good quality with extensive BM infiltration by non-haemopoietic infiltrate	BMAT infiltration by non-haemopoietic infiltrate	No non-haemopoietic infiltrate	FOXO1 gene was positive

359	Cause for the bicytopenia	Good quality with a single clump of worrying cells - 3% primitive mononuclear cells	Inadequate - can't exclude an infiltrate	No BLPD, non-haemopoietic infiltrate or increase in blasts	-
374	Suspected myeloproliferative neoplasm	Adequate quality with suspected myeloproliferative neoplasm	Not found on LIS	No BLPD	JAK2 mutation was positive
379	Suspected bone marrow infiltration	Good quality with 7% primitive mononuclear cells	No BMAT infiltration	No infiltrate	FNA showed T-cell acute lymphoblastic leukaemia
389	Cause for the lymphadenopathy and hepatomegaly	Haemodilute with 9% blasts	No BMAT infiltration	No infiltrate	-
403	Suspected plasma cell myeloma with a paraprotein	Adequate quality with 63% atypical plasma cells	No BMAT infiltration.	No BLPD	-
424	Suspected non-benign lesion	Aparticulate and haemodilute with 18% primitive mononuclear cells	Inadequate	No BLPD or non-haemopoietic infiltrate	-
447	Suspected acute leukaemia	Aparticulate and haemodilute with 6% primitive mononuclear cells	No BMAT infiltration	No non-haemopoietic infiltrate or increase in blasts	-
452	Suspected bone marrow infiltration	Good quality with no infiltrate or increase in blasts	No BMAT infiltration	0.1% T-cells with loss of CD7	TCRG: monoclonal. Histology revealed Anaplastic large cell lymphoma

465	Staging BMAT	Good quality with 1% abnormal cells	No BMAT infiltration	No BLPD	-
472	Cause for the bicytopenia	Aparticulate and haemodilute with 6% atypical mononuclear cells	No BMAT infiltration	No infiltrate	-
475	Hepatosplenomegaly with suspected plasma cell myeloma without a paraprotein	Good quality with no infiltrate, increase in blasts or plasma cells	BMAT infiltration by non-haemopoietic infiltrate	No BLPD	-
481	Cause of pancytopenia	Good quality with no infiltrate or increase in blasts	BMAT infiltration by HL	No infiltrate	-
486	Generalised LAD and splenomegaly	Inadequate quality with no myelogram	BMAT infiltration by blasts of T-cell origin	No BLPD	-
491	Suspected HLH with a massive hepatosplenomegaly	Good quality with 5% primitive mononuclear cells	No BMAT infiltration	No increase in blasts.	HLH seen on liver biopsy, IgH: monoclonal and cytogenetics: 49XX and t(6;8)

BLPD, B-cell lymphoproliferative disorder; BM, bone marrow; BMA, bone marrow aspirate; BMT, bone marrow trephine; BMAT, bone marrow aspirate and trephine biopsy; DLBCL, diffuse large B-cell lymphoma; FCI, flow cytometric immunophenotyping; FISH, fluorescent in situ hybridization; FNA, fine needle aspirate; HL, Hodgkin lymphoma; HLH, haemophagocytic lymphohistiocytosis; IgG, immunoglobulin G; IgH, immunoglobulin heavy chain; IHC, immunohistochemical; LAD, lymphadenopathy; LN, lymph node; LPD; lymphoproliferative disorder; MDS, myelodysplastic syndrome; NBL, non-benign lesion; NHL, Non-Hodgkin lymphoma; TB, tuberculosis; TCRG; T-cell gene rearrangement studies.

APPENDIX E: '2006 Bethesda International Consensus Recommendation on the Flow Cytometric Immunophenotypic analysis of haematolymphoid neoplasia: Medical Indications' (3) and proposed recommendations or modifications in our local setting

Bethesda Medical indications for FCI testing	Proposed recommendations based on the results of this study and recent studies (24) at the CMJAH flow cytometry laboratory
❖ **Cytopenias:** • Anaemia, leucopenia and/or thrombocytopenia • Pancytopenia • Monocytopenia ❖ **Observation of atypical cells or blasts:** • Blasts in the peripheral blood and bone marrow ❖ **Evaluation of body fluids:** • Atypical mononuclear cells in body fluids • Serous effusions and cerebrospinal fluids with a history of haematolymphoid neoplasms ❖ **Plasmacytosis or monoclonal gammopathy:** • Determination of clonal, aberrant, and reactive plasma cells ❖ **Organomegaly and tissue masses:** • Lymphadenopathy • Organomegaly (not limited to splenomegaly or hepatomegaly) • Tissue infiltrates (including but not limited to skin, mucosal sites, and bone) ❖ **Patient monitoring indications:** • Staging of disease. • Detection of potential therapeutic targets eg.CD20 and CD52 • Assessment of therapeutic response. • Identification of disease progression or relapse	❖ **We recommend that all FCI samples have a slide prepared, stained and microscopically reviewed to exclude cytomorphological evidence of disease. These findings should be correlated with the BMA cytomorphology reports.** ❖ **Samples without medical indications for FCI, the following is recommended prior to sample rejection:** • Review BMA cytomorphology report to ensure no evidence of disease • Review FCI slide for evidence of disease ❖ **BMA samples of AHI quality:** • Review BMA cytomorphology report to ensure no evidence of disease • Review FCI slide to exclude features of disease ❖ **CSF and biological fluid samples with a known malignancy:** • Process FCI sample ❖ **CSF and biological fluid samples without a known malignancy:** • Assess cytology results for evidence of malignant cells. If no abnormal cells, FCI sample processing may not be indicated.

• Diagnosis of additional concurrent haematolymphoid neoplasms (therapy-related or coincidental) • Identification of disease acceleration or transformation • Disease prognostication **Exclusions for FCI testing:** ❖ Isolated anaemia ❖ Neutrophilic leucocytosis in the absence of blasts (mature neutrophilia) ❖ Polycythaemia ❖ Thrombocytosis ❖ Basophilia ❖ Polyclonal hypergammaglobulinemia ❖ Hodgkin lymphoma	❖ **Bicytopenia without evidence of malignancy:** • Individualise all cases with a thorough review of the clinical and laboratory investigations • Review BMA cytomorphology report to ensure no evidence of disease ❖ **Bicytopenia with evidence of malignancy:** • Process all FCI samples ❖ **Non-haemopoietic malignancies:** • Not recommended for FCI sample processing ❖ **Hodgkin lymphoma staging of disease in BMA samples:** • Not recommended for FCI sample processing ❖ **Reporting:** • Standardised reporting templates are recommended nationally, for ease of interpretation by the clinicians

APPENDIX F: Two-way tables

Table 1. Two-way table of the 'test-all' approach

N = 429	FCI disease	FCI no disease	Total
BMA disease	66 (TP)	37 (FP)	103 (TP + FP)
BMA no disease	2 (FN)	324 (TN)	326 (FN + TN)
Total	68 (TP + FN)	361 (FP + TN)	429 (Total)

Table 2. Two-way table of the overall triage process

N = 455	Disease in bone marrow investigations	No disease in bone marrow investigations	Total
Triage process	120 (TP)	255 (FP)	375 (TP + FP)
Triage not process	5 (FN)	75 (TN)	80 (FN + TN)
	125 (TP + FN)	330 (FP + TN)	455 (Total)

Table 3. Two-way table of the triage process: cases with Bethesda medical indications

N = 375	FCI disease	FCI no disease	Total
BMAT disease	69 (TP)	51 (FP)	120 (TP + FP)
BMAT no disease	0 (FN)	255 (TN)	255 (FN + TN)
Total	69 (TP + FN)	306 (FP + TN)	375 (Total)

Table 4. Two-way table of the triage process: cases without Bethesda medical indications

N = 80	FCI disease	FCI no disease	Total
BMAT disease	0 (TP)	4 (FP)	4 (TP + FP)
BMAT no disease	1 (FN)	75 (TN)	76 (FN + TN)
Total	1 (TP + FN)	79 (FP + TN)	80 (Total)

APPENDIX G: Plagiarism/Turn-it-in report

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