INTRANUCLEAR TERMINAL DEOXYRIBONUCLEOTIDYL TRANSFERASE (TdT) EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIA: A FLOW CYTOMETRIC ANALYSIS IN BONE MARROW ASPIRATE FIXED-PERMEABILIZED CELLS.

A dissertation submitted in part fulfillment for the award of the degree of Master of Medicine in Human Pathology.

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DECLARATION

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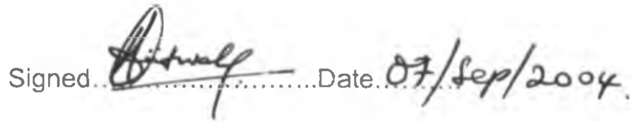

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DEDICATION

To my parents Mr. and Mrs. John Earnest Gachau, my siblings Mucheru, Wangari, Makenye and Kabue, my wife Josephine Wanja and my daughter Njeri for the support, inspiration, patience and guidance.

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LIST OF ABBREVIATIONS

TdT	Terminal deoxyribonucleotidyl Transferase
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AUL	Acute Undifferentiated leukemia
BMAs	Bone marrow aspirates
D	Dalton
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
FAB	French American British co-operative group
FACS	Fluorescence activated cell sorter
FCM	Flowcytometric measurements/analysis
FSC	Forward Scatter
KAVI	Kenya AIDS Vaccine Initiative
KD	Kilo Dalton
KNH	Kenyatta National Hospital
Mabs	Monoclonal antibodies
MIC	Morphology, Immunophenotype, Cytogenetic
MRD	Minimal residual disease

PAS	Periodic Acid Schiff
PBMCs	Peripheral blood mononuclear cells
SSC	Side Scatter

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ABSTRACT

BACKGROUND. Despite advances in the understanding of the biology and characterization of acute lymphoblastic leukemia (ALL), evidence of utility of novel diagnostic techniques based on these developments is lacking in our setup. This is against a background of increased case reporting and rising incidence of this disease.

OBJECTIVE. The aim of this study was to qualitatively and quantitatively detect the presence and level of TdT expression in cell isolates obtained from bone marrow aspirates in patients with ALL.

DESIGN. This was a cross sectional descriptive study that was carried out for three months between June and August 2003. Consecutive cases of qualifying patients were recruited. They were stratified into cases (for those with ALL) and controls for other diagnostic entities.

SETTING. This was a laboratory-based study carried out in the departments of Haematology and Blood Transfusion and KAVI laboratories at the University of Nairobi's Faculty of Medicine. Patients were recruited from the selected health care delivery points within the KNH. Two cases were recruited from two private hospitals (one case each) within Nairobi.

SUBJECTS. Thirty-two patients (eleven ALL and twenty one controls) were recruited over the study period.

INTERVENTIONS. Peripheral blood and a bone marrow aspirate were obtained from each patient selected to participate in the study. These were used for the morphological diagnosis of ALL and other pathologies. BMAs were further used for flow cytometric analysis of TdT.

MAIN OUTCOME MEASURES. For cases and controls, positivity or negativity for TdT expression was analyzed. Levels of expression were presented as a percentage of the total acquired events and the absolute numbers of positive events. Comparisons between the cases and controls were made.

RESULTS. The cases to controls ratio were 1:1.9. There were more males than females (1.8:1) in the ALL cluster. The median age for this group was nine years (range 3-13). Only three of these eleven had not received chemotherapy at the time of recruitment. Of the nine cases on treatment, only one was in morphologic complete remission. There was a strong positive correlation between the visual blasts cell counts and the absolute number of acquired events by FCM (r=0.99) a weaker association was exhibited by the controls (r=0.474)

The frequency of TdT expression was higher in ALL (63.6%) than in controls (38.1%). The cases of ALL on treatment had a TdT frequency of 66%.

CONCLUSION. This study has demonstrated that TdT may be useful in the characterization of ALL. The revelation that the number of positive events has significance at either spectrum of blast cell counts suggests that TdT is useful in diagnosis and follow up of patients who are on chemotherapy. However, more work needs to be done with a larger sample size and more markers for instance CD 19, CD 10, CD 13, surface membrane immunoglobulin and cytoplasmic immunoglobulin.

1.0 INTRODUCTION AND LITERATURE REVIEW

The acute leukemias are defined pathologically as blast cell malignancies of immature haemopoietic cells which exhibit clonal proliferation of either the lymphoid or myeloid lineage; hence the division into two main groups, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). In most cases, these have definable morphologic, cytogenetic and immunological characteristics (1, 2).

In ALL, abnormalities of lymphoid cell differentiation, proliferation or both lead to excessive infiltration of leukemic lymphoblasts in multiple organs with resultant organomegally and bone marrow failure. Its biological heterogeneity is linked to the fact that the leukemogenic-transforming event may develop at any point of the multistage lymphoid cell differentiation and will consist of an expanding clone of neoplastic cells that are frozen at an otherwise transient level of differentiation (2, 3, 4).

1.1 Epidemiology

Acute lymphoblastic leukemia is a worldwide entity that is commoner in children than in adults. In the United States of America (U.S.A), three thousand to four thousand new cases are diagnosed annually. Two thirds of these cases are in children making it the commonest childhood cancer (3). The annual incidence of ALL in children aged fifteen years and younger is 29.4 cases per one million population in whites and 13.7 cases per million among the blacks (2). Melvyn and Kasili have provided comparable statistics for age-matched population in Europe and U.S.A, and Kenya respectively (5, 6).

The peak age prevalence of ALL in developed countries occurs between 1 and 5 years with a median of 4.9 years and is commoner by twenty to thirty percent in males than in females in all age groups and immunophenotypes. Macharia, in a referral-hospital based study in Kenya, and Kasili documented a wide interval of peak age prevalence ranging between 2 to

10 years, (89.3 % of the cases studied fell within this age range) with a median age of 6 years (6,7). Twenty three and a half percent of the cases studied were below 5 years of age. The age-adjusted incidence of ALL in adults (over fifteen years of age) was seven cases per one million population. No obvious peak incidence of ALL was observed during adulthood. However, it has been documented that the incidence decreases with increasing age.

ALL accounts for between 65 and 80% of childhood leukemia in the U.S.A while in African children it accounts for about 40-60% (1, 2, 3, 5, 8). In Kenya Leukemia is the second commonest childhood neoplasia after Lymphoma with a relative frequency of 21.3% (7). Macharia in 1996 reviewed data on cancer related admissions between 1990-1995 at the KNH pediatric units and the pediatric cancer ward from where he documented that 67% of acute leukemias found in children less than twelve years of age were ALL (7). Data obtained from a national survey in 1979 showed that ALL accounted for 3.7% and 4% of all cases admitted in both medical and pediatric wards respectively (6,9). Recent data obtained from the medical records department of the KNH showed a sharp contrast from these previous findings (table 1). It can be hypothesized that the downgrading of these rates is probably due to increased admissions occasioned by HIV related morbidity most of whose pathologies have higher prevalence rates than ALL.

There is documented evidence that ALL has shown a rising trend in annual admissions over the years. There has been an increase of an average of 12 cases for the period 1975-1981 to 16 cases between 1990 and 1995 (7). Figures obtained from the medical records department of KNH showed even higher patient attendance as shown in the following table.

Table 1.

Number of cases admitted to KNH wards (year 2000-2002)

Year	No. of cases of ALL seen	Total admissions	Proportion of ALL to total admissions
2000	43	81930	0.05%
2001	50	80775	0.06 %
2002	58	77629	0.07 %
2003	Not compiled by the time of data collection		
Mean	50	80111	0.06 %

1.2 Diagnosis of ALL

A bone marrow aspirate is necessary for the diagnosis and precise classification of ALL. Being a heterogeneous disorder, an integration of the morphology with the immunology and karyotype of the neoplastic cells is currently being used (MIC Classification of 1986 and 1988).

1.2.1 Morphology

Morphological evaluation is done on freshly prepared, air-dried unfixed glass slide smears specimens. The May-Grunwald-Giemsa (MGG) and Leishman staining techniques are commonly used (12) The smears are fixed in methanol, stained in MGG and then transferred to a buffer solution after which they are left to air dry before studying them under a microscope. A diagnosis of ALL is made based on the presence of more than 30% blast cells in the bone marrow aspirate smear. Sub-classification into morphological sub-types namely ALL-L1, L2 and L3 is done simultaneously based on simple criteria (ALL score) that take into account:

- Nuclear to cytoplasmic ratio (high in L1)
- The presence of a prominent nucleolus in >25% of blasts (criterion for L2)
- The irregularity of the nuclear membrane (criterion for L2)

- Cell size (if more than 50% of blast are large, L2 is indicated)

The sum of the score, positive for L1 (0 to 2) and negative for L2 (-1 to –3) facilitates the diagnosis of L1 or L2 and it forms the basis for the French-American Co-operative Group (FAB) criteria for ALL.

Blasts cells of ALL-L3 when compared to those of L1 are homogenous and larger. they bear a loose arrangement of nuclear chromatin with an inconspicuous nucleolus and they have a deeply stained basophilic cytoplasm that is often vacuolated.

The diagnosis of acute leukemia at KNH is morphological in most cases. Where cases are not clearly defined this way, cytochemical evaluation of the blast cells is undertaken as an ancillary procedure.

Between the period of January and August 2000 and June and December of 2001 the Department of Hematology and Blood Transfusion (KNH) processed 34 and 22 samples respectively, of bone marrow aspirate smears (BMAs) for cytological diagnosis (using morphology) of ALL translating to an average of three to four cases per month. Between January and May 2003, thirty-nine BMAs from patients with ALL were evaluated. However, these included both initial and re-evaluation marrows aspirates to monitor disease responsiveness to treatment initiated (10,11).

On basis of morphology alone, leukemic lymphoblasts may be indistinguishable from immature normal lymphoid cells. Attempts to differentiate leukemic lymphoblasts on basis of size, nuclear-cytoplasmic ratio, presence of cytoplasmic granules among other features have been largely unsuccessful (2). A morphologic classification devised by the French American British cooperative group (FAB) has gained wide acceptance. Following the criterion, ALL has been divided into three subtypes namely L1, L2 and L3.

Based on Western statistics for these ALL subtypes. L1 accounts for about 90% of the childhood cases, L2 between 5 and 15% while L3 accounts for less than 10%. In adults however, L2 accounts for most of the cases. In contrast to AML, which can be readily

identified in most instances by morphologic features such as the presence of Auer rods, cytochemically by staining positive for myeloperoxidase or Sudan B Black or monocyte associated esterases, leukemic lymphoblasts may lack specific morphologic or cytochemical features. Further evaluation for assurance of the final diagnosis of ALL depends on immunophenotyping (3,12).

1.2.2 Leukocyte cytochemistry

Leukocyte cytochemistry encompasses the techniques used to identify diagnostically useful enzymes or other substances in the cytoplasm of haemopoietic cells.

The most important application of cytochemistry is in the separation of ALL from AML. This is particularly important in some cases of AML (M1, M2, M3 and M4) that may be diagnosed as ALL. These always show some degree of positivity with Sudan-Black stain unlike ALL, which is negative. There is a minority of cases, particularly AML Mo, which are negative for most cytochemical stains for AML. Although up to 40% of the AML Mo cases may be TdT positive, they lack reactivity with any of the specific Mabs for B- or T-lineage ALL though they react with specific anti-myeloid Mabs.

The use of cytochemistry to characterize lymphoproliferative disorders has been largely superseded by immunological tecniques (12). The results of cytochemical tests should always be interpreted in relation to Romanowsky stains and immunological tests.

1.2.3 Immunological analysis (immunophenotyping)

Application of monoclonal antibodies and other biologic probes in the analysis of leukemic lymphoblasts and their normal counterparts have greatly improved the precision with which particular cell types can be identified, isolated and characterized hence supplementing morphological findings in making the final diagnosis (13).

A panel of monospecific antibodies combined with fluorescent microscopy, or more commonly, flow cytometric (FCM) analysis makes possible the identification of specific antigens and/or enzymes on the membrane and/or in the cytoplasm or the nucleus. These help to identify the blast cells to be of lymphoid or myeloid lineage. In regard to ALL this allows a separation between B and T lineage ALL

The advent of FCM techniques, which allows simultaneous demonstration of cytoplasmic, membrane and nuclear antigens, has significantly improved the diagnostic potential of Mabs. Differences in surface membrane, cytoplasmic or intracellular antigenic components of lymphocytes can be assessed to identify and classify lymphoproliferative disease as to their cell of origin and stage of maturation. This provides information useful in the reproducible diagnosis and classification of acute leukemias (2,13,15)

For both ALL and AML these markers show that the blast cells resemble very closely rare cells in the normal bone marrow, or, for T-ALL, the thymus from which the leukemia appear to be derived by clonal expansion. In ALL, and lymphoid progenitors, TdT occurs on early non-differentiated cells and is routinely demonstrated in the nucleus of B- and T- lineage lymphoblasts.

During ontogeny, lymphocytic progenitors undergo clonal expansion and diversification. This process is achieved by a complex sequence of alteration in their cellular DNA and it involves enzyme mediated recombination events, deletions and somatic mutations to produce functional immunoglobulins or T cell receptor genes (5).

The modern diagnosis and further classification beyond the FAB criteria of all forms of ALL is by immunophenotyping (13). Monoclonal antibodies directed against various specific lymphoid cell antigens help detect lineage specificity in these cells and further reveal restriction of maturation as well. It is important to understand the antigenic patterns of

normal lymphopoietic differentiation. This becomes paramount in the context of evaluating bone marrow specimens in which leukemic cells have not completely replaced normal haemopoiesis. Familiarity with these maturation patterns of normal marrow elements makes it possible to recognize the presence of a leukemic cell population. Detailed immunophenotypic and molecular studies on ALL reflect the pattern of antigenic acquisition seen in normal haemopoietic differentiation of both B and T cells, yet invariably demonstrate distinct aberrant immunophenotypic features (14). Only a few monoclonal antibodies react with the most immature lymphoblasts. With maturation however, more monoclonal antibodies become reactive. Some aspects one needs to consider in selecting monoclonal antibodies for this purpose are the degree of lineage specificity and whether the expression is either membrane, cytoplasmic or intranuclear.

1.2.3.1 Principles of Flow Cytometry

Flow cytometry is the measurement of numerous cell properties (cytometry) as the cells move in a single file (flow) in a fluid column and interrupt a beam of laser light. It is one of the techniques in use for detecting cellular antigens using monoclonal antibodies (Mabs) in either viable or fixed test cell suspensions. It is a rapid and dynamic method of correlated multi-parameter, single cell analysis. It was designed to augment microscopic study of individual cells using fluorescent substrates and probes (16,17). It is widely used to phenotype cases of acute leukemia and is now clearly important in classification.
Advances in hybridoma technology, biotechnology and immunobiology associated with progresses in fluorochrome chemistry, computer hardware and software have all contributed to phenomenal and evolutionary developments in flow cytometry.
The basic instrumentation consists of a light source, usually a monochromatic single beam light from an air cooled argon laser, a single file jet stream of suspended test cells and photo multiplier tubes (fluorescence detectors). These are connected to an analytic system, usually a computer, fitted with responsive software for data interpretation and management.

Except for the computer system, the rest are aligned orthogonally at right angles to one another.

A suspension of cells, either directly or indirectly labeled with one or more fluorescent probes is hydrodynamically focused in a single file surrounded by sheath fluid (coaxial jet stream). When this jet stream intersects the laser beam, fluorescent excitation occurs. Each cell is measured for light scatter signals and the presence or absence of emitted fluorescence.

These scattered and emitted light signals are collected, collimated and directed through appropriately arranged filters to photo detectors. These are amplified and digitized prior to processing. They can be stored within the computer system and subsequently retrieved for subsequent analysis (16).

Cellular samples commonly analyzed may be obtained from peripheral blood, BMAs and lymph node suspensions. The interpretation of clinical data is based on appropriate reference ranges.

In regard to quality assurance, the combination of a unified instrumentation set up and reference micro-bead standards as calibrants, allows the establishment of primary performance characteristics and leads to confidence in test sample results. Routinely 10 000 to 20 000 cells (events) are collected (acquired). Nevertheless, this number may need to be increased in cases where rare cell populations are being analyzed. For instance, in cases of stem cells 50 000 to 100 000 events are usually acquired. Results of the acquired cellular events are usually displayed on a scatter plot when a combination of two or more Mabs is used to label the cells of interest (multi- parameter or multicolor analysis).

The display allows a visual recognition of various constituent subsets of cells from a cellular sample discriminated on basis of size, granularity of cytoplasm and fluorescence intensity. Gating strategies are used to isolate and enhance a selected cell population based on light scatter and fluorescence properties with various Mabs. Gating is the process whereby

subsets of interest for instance blasts in acute leukemia or lymphocytes for CD 4 analysis, are selected by the cytometer operator for analysis while excluding those events not of interest during the analysis. A gate region (a.k.a gate) is the area in the scatter plot delineated and "fenced off" for this purpose. All events within the gate are recognized by the data processing unit of the cytometer while those outside it are omitted.

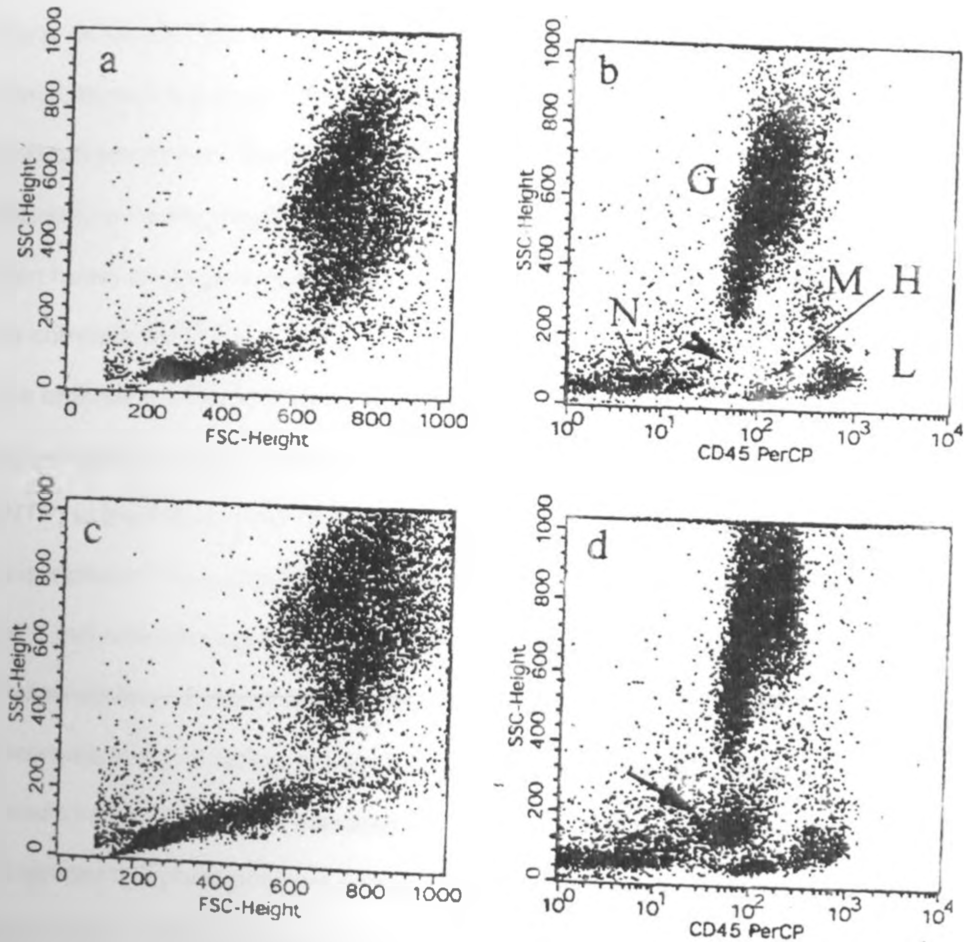
The use of gating regents to further ascertain the integrity of the scatter-based lymphocyte and blast cell gate has been developed. Use of anti-CD 45 Mab (the common leukocyte antigen) has elucidated this concept owing to the fluorescent properties of the cells that stain positive for it. The combination of anti-CD 45 intensity and right angle light scatter (RALS), also called side scatter (SSC) distinguishes leukemic cells from their mature normal counterparts (17,18). CD 45 is expressed at different intensities on the surface of various cell populations of haemopoietic origin. When combined with SCC, the pattern of CD 45 expression reveals several distinct clusters of events, each representing a constituent population of the bone marrow cells. In multicolor analysis, combinations of fluorescent conjugated antibodies allows further characterization of cell populations isolated on CD45/ SCC display.

This gating procedure has been found to be more useful, superior and informative compared to the traditional forward angle scatter (FSC) versus RALS (14). It is usually easier to separate the blast population from residual mature granulocytes and monocytes owing to a comparably minimal events overlap. On this display, leukemic cells occupy a unique blast region characterized by intermediate CD45 density and low RALS. This is called the "blast window" and is normally or nearly empty in both normal peripheral blood and BMAs respectively. Even small increases in blasts will appear as a distinct cluster of events within the blast window. By establishing analysis gates on the CD 45 versus SCC display, the combination of CD45 (with TdT in this case) produces a more detailed lineage specific and

maturation dependent immunophenotypic data. Analysis gates established on this blast window will form the basis for lymphoblasts analysis in subsequent chapter of this dissertation.

Figure 1.

Distribution of acquired BMAs events as seen on a dot scatter plot. Plots **a** and **c** are FSC/SCC while **b** and **d** are CD45/SCC plots. The light zone with a thick arrow in **b** and d is the blast zone. Analysis window was set around the zone during acquisition. Note the increased number of events corresponding to blasts in **d** (ALL) as opposed to those in **b** (normal BMAs)



Although flow cytometry is a valuable tool in the diagnostic evaluation of patients with haemo-lymphoid malignancies when applied in isolation, it is most effective as an ancillary technique in a combined modality approach (14). Immunophenotypic studies have the advantage of alleviating the limitations of light microscopy in classifying borderline cytological changes. These include observer subjectivity, sub-optimal quantifications owing to the small amounts of test samples usually used, relatively few cells quantified and the tiresome nature of the processes involved. Flowcytometry in contrast to light microscopy is more objective, provides both qualitative and quantitative results and a permanent record of the data as well (19).

1.2.3.2 Terminal deoxyribonucleotidyl transferase (TdT)

This is an intranuclear enzyme protein characteristic of certain primitive lymphocytes in normal thymus and bone marrow during their development (20). TdT is expressed in early lymphoid progenitors (early pro-B, pre-B cells and T-cells) for only a short period during the assembly of heavy chains. In adult humans it ceases to be expressed, at the pre-B stage, when heavy chain gene re arrangements is complete and light chain gene re arrangement has commenced (21).

The enzyme is a Deoxy ribonucleic acid (DNA) polymerase that catalyzes the addition of non-template encoded deoxynucleotides (N-Nucleotides) of deoxynucleotide triphosphate (dNTP) to the 3'-OH group of single stranded DNA. It is involved in the regulation and transcription of DNA and immunoglobulin heavy chain gene rearrangement during normal T and B cell development (8,20). In effect therefore, it functions as a somatic mutagen by random addition of nucleotides to DNA. It is expressed by both B and T lineage lymphocyte precursors and in a variety of hematological malignancies (22). Early TdT-positive B cell precursors have been characterized extensively in BMAs, where they have long been recognized morphologically as benign immature lymphocytes (21). High levels of this enzyme and increased numbers of TdT expressing cells are found in some lymphoblastic

leukemias and lymphomas. It has been shown that up to 95 % of ALL, 15% of AML and 3 % of AUL express TdT.

Of historic interest, this enzyme was discovered about forty years ago in extracts of calf thymus glands as part of a search for DNA replication enzymes in mammalian cells. It has a native molecular weight of 32000 Daltons (D) and consists of two peptides, α and β, estimated at 8 KiloDaltons (KD) and 24 KD respectively by sodium dodecyl sulfate electrophoresis.

It is not exclusively related to leukemia or other malignancies. It is a normal constituent of a limited population of cells in the adult haemopoietic systems namely the spleen, reactive tonsils, mucosal associated lymphoid tissue (MALT), liver, lung and peripheral blood of normal individuals. For example, normal bone marrow consists of less than 2% TdT positive cells. It is present in transient numbers in early postnatal life as well. It is considered a very selective marker for immature lymphoid cells (21,23). Since it distinguishes most lymphoblastic leukemia from myeloid leukemias, it is useful for diagnostic purposes and for accurate detection of residual disease, early relapses and the emergence of new leukemic clones post-treatment (15).

The behavior of Tdt in ALL has been documented. The different levels of TdT enzyme observed in leukemia are in line with a maturation-linked expression. The values seen in Thymic ALL and in T cell leukemia seem to correspond to the decrease of TdT levels seen during maturation of cortical thymocytes to medullary Thymic and peripheral T lymphocytes. To support this it has been shown that using maturational stage as a T-ALL prognostic factor, a significant difference in outcome was demonstrated between the three maturational stages of T cell ALL (24). Other studies have shown that the highest levels of TdT were found in cells classified as immature lymphoblasts or in their precursors (23). Thus, the presence of TdT has been interpreted as indicating the primitive stage of the cells in the

differentiation sequence, with no respect to cell lineage and hence a role as a useful differential marker in those leukemias not categorized by morphology alone (25,26).

As regards the response of TdT to chemotherapy, it has been observed that the qualitative attributes of this enzyme do not change. As long as there are leukemic cells present in the specimen, TdT will be detected. Quantitatively however, the detectable levels of expression will diminish with successful therapy as demonstrated by reducing blast cell counts (17).

The sensitivity of FCM in detecting malignant cells is appreciably high. The technique can detect as low as 20 events as demonstrated in mixing experiments. Indeed, this feature has formed the basis for evaluation of minimal residual disease (MRD) in cases of ALL (B and T) whereby the sensitivity matches that of polymerase chain reaction (the commonly used technique for ALL-associated MRD) whose sensitivity has been documented as $1:10^3$-$1:10^5$ cells.

The intensity of staining with TdT also varies with the treatment phase. For example, ALL blasts have a lower TdT fluorescence intensity than those of normal bone marrow B-cell precursors. Common-ALL (c ALL) blasts have low TdT and high CD10 and CD 19 expression in contrast with normal B-cells precursors that in contrast have high TdT and low CD 10 and CD 19. This difference can help to distinguish normal from leukemic cells after remission.

Following the recommendations of the US-Canadian consensus conference on the use of flow cytometry immunophenotyping in leukemia and lymphoma in 1997, the need for "homegrown" solution to antibody panel selection was expressed. No final recommendations were however made as to which monoclonal antibody combination constitutes the ideal panel. As regards ALL a minimum panel employed should at the least

- Discriminate between the myeloid and the lymphoid cells,
- Establish the maturity stage of the abnormal cells, and

- Identify the major category of the leukemia phenotype as either B or T cell (13).

1.2.4 Cytogenetics

This relates to the study of chromosomal abnormalities in different types of leukemias and lymphomas (1, 12). Its main purpose is to determine whether the chromosomal make-up (karyotype) of the affected cells is abnormal and, if so, in what ways. Clonal abnormalities are found in up to 90% of patients with ALL. Cytogenetic findings in ALL are important for:

- Diagnosis and classification of the disorder. The MIC classification of ALL is based on the demonstration of highly specific chromosome translocations in cases defined by both morphology and monoclonal antibody cell markers.
- Evaluation of prognosis. In acute leukemias, the karyotype may be an important may be an important and independent prognostic factor in predicting both remission achievement and the length of remission, and in distinguishing potential log-term survivors from those likely to fail on standard therapy.
- Prediction of transformation. Cytogenetic evaluation during the course of an indolent leukemia may be used to predict transformation to a more aggressive phase.

There are a number of factors that make cytogenetic studies in ALL particularly difficult. For instance, BMAs may have low cell counts. In some cases, the leukemic blasts are inert in culture while in others chromosomal morphology and response to band staining are poor.

2.0 JUSTIFICATION OF THE STUDY

Leukemia in Kenya is now a prevalent problem than previously thought. The number of cases being attended to has been rising annually (table 1; page 5). Two decades ago, Kasili had estimated that the Kenyan overall national crude incidence was 0.5 cases per 100,000 people in a population and that the maximum tribe specific incidence was 1.2 cases per 100,000, (6). Since then, no comprehensive review of the epidemiology, neither national nor regional, has been carried except for a hospital-based study by Macharia in 1996 (7) where he assessed the contribution of childhood malignancies including ALL, to the admissions load at the KNH. Between the years 2000 and 2002, the total number of cases of ALL seen at KNH rose from 43 to 58. These numbers accounted for 0.05% and 0.06% of the total annual admissions at the hospital. Part of the increase in the incidence of leukemia is now thought to be because of improved diagnosis that currently exists.

In the western setting, the disease has a peak occurrence in the first decade of life and a good remission induction rate of 90% for the childhood variety (2). In addition, it is associated with a low mortality rate compared to Acute Myeloblastic Leukemia (AML) and ALL in adults. This therefore underscores the importance of prompt, early and accurate diagnosis To stress this point, about four decades ago the disease was uniformly fatal and the extra-ordinary achievements in cure rates resulted from a series of studies that helped to clarify its pathobiology (2).

Although routine cytological and histological examinations are cheap and reliable for the diagnosis of pre-malignant and malignant conditions, morphology alone is an imperfect reflection of biologic potential (19). The FAB system criteria for instance, often fails to discriminate reproducibly between the various immunologic subtypes of leukemia arising from the same haemopoietic lineage, and at times even between leukemias of different

lineages (14). Use of TdT therefore has an advantage over morphology alone owing to its higher sensitivity in detecting abnormal cells. It is still useful even in absence of secondary monoclonal antibodies in discriminating between ALL and other lymphoproliferative disorders and AML Its use alone in this study was carefully considered as it gave an indication on the potential use of FCM in this setting in future.

The technique of flow-cytometric analysis (FCM) of leukemias is not at present available in our country. It is a user friendly, reliable and reproducible technique in diagnosis and management of leukemia in our setting. The main limitation in setting up the system would be the initial costs. The flow-cytometers are expensive machines to purchase and specialized training of technical personnel is not locally available. However, technical service back up and reagents sourcing are accessible locally and are reasonably priced.

This study aimed to determine the potential role and practicality of FCM as ancillary diagnosis and in clinical-laboratory management of ALL. Early response to therapy is one of the most important prognostic indicators in acute leukemia. Studies to asses complete morphological remission against markers of minimal residual disease have also shown that certain markers for instance CD10, CD 24 or myeloid cell surface antigen (CD 45) are associated with post intervention outcomes; namely event free survival. However, the independent prognostic importance of these markers is less well established. (17, 27, 28).

Although similar studies have been done elsewhere there is none that has been carried out in our set up. Therefore, this study will set pace for such evaluations studies in future.

Standardization, validation and improvement of reproducibility in diagnostic techniques are essential in laboratory practice. In addition, the understanding of leukemia cell biology and clinical risk factors have made it possible to recognize a spectrum of ALL subtypes and to predict treatment outcome with greater precision.

With the changing disease structure owing to improving healthcare, which provides accessibility to early detection of cases and successful control of communicable diseases, the importance of chronic diseases, cancer and leukemia inclusive cannot be ignored. Despite the scarcity of literature in regard to local and regional epidemiology of leukemia, the changing environmental patterns characterized by an increase in the use of agricultural chemicals, pesticides, therapeutic irradiation amongst others can by extrapolation, be said to contribute to the morbidity (1, 5, 6).

Research questions;

- Does the level of TdT expression by ALL correlate well with morpnologic blast cells counts?
- If so, can patterns of TdT expression be utilized to asses treatment response in ALL?

Hypothesis

TdT is useful in diagnosis and follow-up of patients with ALL.

2.2 Objectives of the study

2.2.1 Main objective:

The main objective of the study was to qualitatively and quantitatively detect and establish the levels of TdT expression in ALL using FCM in order to evaluate its role in the diagnosis and eventual follow up of patients with this disease in our setup.

2.2.2 Specific objectives

1. To determine the proportion of TdT positive ALL in the BMAs of the study population.
2. To compare patterns of TdT expression between the different stages of ALL cases on treatment and control BMAs.
3. To determine the correlation between microscopic percentage blast cell counts and TdT expressing events.
4. To find out the rate of TdT phenotype expression in morphologically unclassifiable acute leukemia (AUL).
5. To make recommendations based on the study findings.

3.0 METHODOLOGY

3.1 Study design and data collection

This study was a descriptive cross sectional study carried out between the months of June and August 2003. Data was obtained from the recruited cases using a directly administered pre-designed data collection form. Clinical and laboratory-based data relevant to the study was entered as guided.

3.2 Study areas and population

The study was conducted at the KNH hematology laboratory from where sixty-two patients initially enrolled into the study had their BMAs assed for pathology. They were categorized into untreated ALL, treated ALL and control cases. KNH is located within the City of Nairobi, Kenya and is a teaching and a National referral health care facility. Most cases of childhood and adult cancers are referred from all parts of the country for confirmation of diagnosis and /or treatment. In this regard, the hospital has become a focal point and a relevant entry point into clinical and epidemiological studies that have been widely published.

The subjects were recruited from the hospital's medical and pediatric wards, pediatric and adult hematology clinics and the oncology wards. These units serve as treatment and diagnostic workup points for cases referred from other units within the hospital and from other hospitals as well. Other hospitals within and around Nairobi Province had been considered as additional recruitment areas. Nevertheless, owing to logistics failure, only two cases each from The Nairobi Hospital and Gertrude's Garden Children's Hospital were recruited outside KNH. Patients recruitment followed investigation consultations (laboratory requests from treatment points for ALL workup) to the Department of Haematology and

Blood Transfusion. This served as a convenient focal point for case identification and thus optimized their selection as they presented. This is the only laboratory within the KNH that handles hematological work. In addition, the haematologic pathologists working in the hospital are stationed within this unit.

Once the specimens were obtained from the subject patients, they were processed and examined for morphology and cytochemistry within the Department of Hematology and Blood Transfusion's laboratory while FCM was carried out at the Kenya AIDS Vaccine Initiative's (KAVI) Laboratory located within KNH.

3.3 Sampling of study subjects

This was by stratified sampling. Consecutive cases were selected from the Haematology laboratory upon reception of consultation request forms for ALL work-up. Those that showed morphological features of ALL were recruited. They were stratified into two groups that were designated as per the treatment phase category thus, untreated and treatment phases. For each of these groups consecutive cases were selected as study subjects

3.3.1 Criteria for inclusion into the study

Patients who fulfilled the following criteria were selected for participation in the study.

1. Newly diagnosed cases of acute lymphoblastic leukemia according to cell morphology and expression of more than 30% lymphoblasts in the bone marrow specimen.
2. Those cases previously diagnosed as ALL and who were on treatment during the study period irrespective of the level of blast cell expression in their bone marrow specimens

3. Cases reported as undifferentiated acute leukemia whose histogenesis was uncertain using morphological examination alone.
4. Those who by own self or by legal proxy (that is parent/guardian) gave informed consent to be included in the study as per the official consent form (see appendix 1)
5. Those whose BMAs were considered suitable and satisfactory for morphological evaluation.
6. Cases that were selected as controls (see below).

3.3.2 Criteria for exclusion from the study

Patients who had any one of the following criterions were excluded altogether from participating in this study.

1. New cases of suspected acute lymphoblastic leukemia not yet on treatment and who exhibited less than 30% lymphoblasts in their bone marrow aspirates using morphological examination.
2. Failure to consent to inclusion into the study.
3. Poor quality BMAs unsuitable for a conclusive diagnosis.

3.3.3 Selection of control cases

These consisted of a group of patients who's BMAs showed morphological features other than those of acute lymphoblastic leukemia, for instance, megaloblastic anemia, iron deficiency anemia, and reactive marrow among others. A single case of AML was recruited as well.

Those who consented to inclusion into the study had their BMAs processed in line with the study's standard operating procedure (part 4.3.4 below). In addition, only BMAs considered suitable and satisfactory for morphological evaluation were included in the study.

3.3.4 Sample size

The known epidemiological and hospital prevalence of ALL is low. It is less than 1% in both situations (table 1). Whereas the expected outcome of TdT expression is predictable (it approaches 100% on well preserved lymphoblasts), use of formulae based on the above prevalence yielded a smaller sample size compared to consecutive case recruitment (appendix 3). A calculated minimum sample size of eight was obtained using the mean hospital prevalence of 0.06%, which was higher than the published community prevalence obtained from western literature which has documented 29.4 and 13.4 cases per million people among the white and black children respectively (section 1.1). A confidence interval of 95% was assumed.

To determine an optimal working sample size for the three months working period, two retrospective surveys were carried out by the investigator. Data on BMAs received and processed in the Department of Haematology and Blood Transfusion was obtained from the laboratory register. The number of ALL cases reported and signed out per month was obtained and three month moving averages calculated (10, 11). A survey by the investigator covering the five months preceding this study showed a total ALL load of 39 cases (adult and pediatric) with a fluctuating three month moving average of 11,8.66 and 11 (11). This however included, besides new cases, marrows on re-evaluation for among other things remission status, relapses and inadequate previous aspirates. In addition, summary data on annual hospital admissions for three years preceding the study was used (table 1)

This being a pilot study that was evaluating a long used diagnostic procedure and its potential pitfalls, the author found it prudent to recruit all patients presenting during the study period in order to optimize the sample size.

3.3.5 Patient evaluation, specimen collection and processing

The following steps were taken

(i) History taking and physical examination

History was taken and a complete physical examination performed on patients who met the requirements for inclusion in to the study. Biographic information was also obtained.

(ii) Specimen collection

The procedure for specimen collection and processing are given in detail in appendices 4 and 5.

In summary however, five milliliters of venous blood were aspirated by the investigator or a trained phlebotomist from each patient via a peripheral vein, preserved in EDTA, and transported to the laboratory where blood films were made on glass slides. These were left to air dry before staining.

In the same setting the investigator or the house officer aspirated two milliliters of bone marrow from each patient. Bedside smears on clean glass slides were immediately prepared and allowed to air dry. These were the transported to the laboratory where staining was done alongside that of the blood films.

The sample remaining in the syringe was transported to the laboratory in 0.25ml of EDTA for phenotypic studies.

(iii) Specimen analysis

All peripheral blood and bone marrow smears made were stained using May Grunwald Giemsa technique. These were reported by the hematologist on duty. The findings were transcribed to the data collection sheet and subsequently analyzed statistically.

Those exhibiting features of ALL were further subjected to phenotypic evaluation for TdT using flow cytometry. Mononuclear cells containing the blasts fraction were obtaining using Ficcol Histopaque ®- 1077 (Sigma diagnostics Inc) separation technique. These were washed twice in PBS and stained using anti-TdT and anti CD-45 monoclonal antibodies. The labeled cells were analyzed by use of a FACSCalibur flow cytometer (model E4583; BD Immunocytometry systems USA). The data obtained was analyzed alongside that of the peripheral blood films and BMAs smears. All immunophenotypic studies were done within 72 hours of collecting the peripheral blood and bone marrow aspirates. Those specimens that could not be analyzed within 24 hours were refrigerated at 2-4^{0}c.

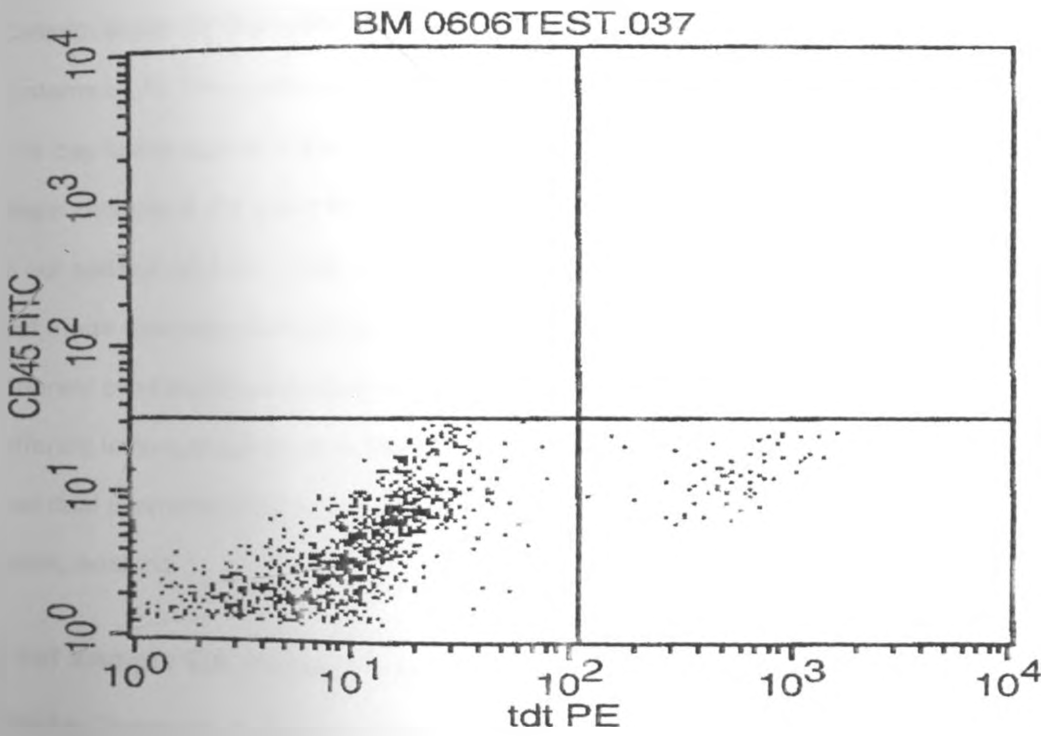
A dot plot was used for events acquisition while analysis quadrants were used for estimation of reactive events. All events to the right of a blank control threshold for the same sample were considered TdT positive while those that fell to the left were taken as negative. TdT positivity exhibits a log fluorescence of 10^{2} to 10[illegible]. Fluorescence below this range was considered negative. Positive events were further classified into diffuse and clustered depending on how close they were to each other. Each of these was sub classified into

- Dim; implying those events overlapping the threshold gate (borderline positivity).
- Bright, implying those distinctly clustered positive events away from threshold gate. These mainly corresponded to 10^{3} intensity threshold for fluorescence.

- Weak; implying a positive reaction nearer the threshold limit. These corresponded to intensity threshold between 10^2 and 10^3

In this study. CD-45 was used for gating purposes and its reactivity was not analyzed. In this regard, variability of events on either side of the horizontal threshold limit for CD45 (y-axis) was not considered.

Figure 2: Dot plot showing positive and negative events in a TdT positive ALL



3.4 Quality assurance (QA) for flow cytometry

Morphologic QA: A cytospin preparation was obtained from the mononuclear cell suspension from each of the bone marrow and peripheral blood samples. A cell count was performed to ensure there was no significant loss of the cell of interest. However, cell viability studies were not undertaken

Instrument QA: Samples were analyzed on a FACSCalibur four-colour flow cytometer (model E4583; BD Immunocytometry systems USA). The instrument was set up daily with Calibrite beads (BD Biosciences, USA) and FACSComp 4.2 software (BD Immunocytometry systems USA). The system was ready for use when time-delayed calibration was passed. The day-to-day stability of the set up was verified. Over the time of this study, there were no major changes in the optics of our instrument, so there was little day-to-day change needed in our settings, and the cluster of events generally occupied very similar positions over time. List mode data were analyzed with CellQuest software (Becton Dickinson, USA). Several different combinations of scatter were used in attempt to maximize the separation among different leukocyte populations. The CD 45-SSC-fluorescence display was used for gating, and dual parameter (TdT PE VS CD 45 FITC) graphs of the gated population were used during analysis.

Test sample QA: No test cells or calibrants for TdT positivity have been developed yet, (Becton Dickinson). In this study, blasts that reacted positive with the anti- TdT Mab were taken as truly positive. CD-45 single antibody stain (without anti TdT co-stain) was used to label peripheral blood cells from normal individuals suspended in phosphate buffered saline (PBS). This was treated as the TdT antibody blank. This was in reference to section 4.3.4.iii above All samples in this study were gauged against the reactivity of this peripheral blood blank for positivity and negativity.

3.3

3.5 Statistical analysis

Statistical analyses were performed with the use of SPSS for Windows™ version 11.0. The analysis in both the ALL and control groups were done using descriptive statistics. Non-parametric tests were used to compare two sets of data and inferences were thereby made. To test for significance and correlation respectively, Fischer's Exact Test, Chi square and Pearson's correlation were selected where appropriate. All significance tests were two-tailed with the confidence level (α) of 0.05.

4.0 ETHICAL CONSIDERATIONS

The study commenced following approval by the KNH-Ethics and Research Committee (Appendix 7).

Samples from the patients were collected following informed consent from the patients, their parents or guardians (Appendix 1).

To minimize patients discomfort owing to the traumatic and invasive nature of phlebotomy and bone marrow aspiration, each recruited case was subjected to the procedure only once in the course of the study and this was determined purely by the clinical indications. Predetermined volumes of the aspirates were used as the bedside benchmarks of the specimen adequacy (Appendix 4). Repeat aspirates prompted by uniterpretable smears for any reason were at the behest of the attending clinician. In such circumstances, another experienced clinician was sought to perform the procedure or recommend alternatives in regard to further worthwhile work up.

New cases clinically suspected to be suffering from ALL and whose diagnosis was yet to be established were recruited and evaluation performed as above. (See section 4.3.4). Only when the morphologic tests revealed ALL were immunophenotyping studies carried out and the data obtained used for the purpose of the study.

The turn-around time for hematological tests was not negatively affected by the study. The results of preliminary hematological tests obtained were communicated promptly to the House Officer on duty for the timely and continued management of the patients.

Although the study questionnaire bore the patients names and hospital unit number, these were used for clinical purposes where eventual follow up was necessitated by the study

findings. These patient identity factors were concealed at the time of publishing these study findings. Strict confidentiality of the patients' identity was maintained.

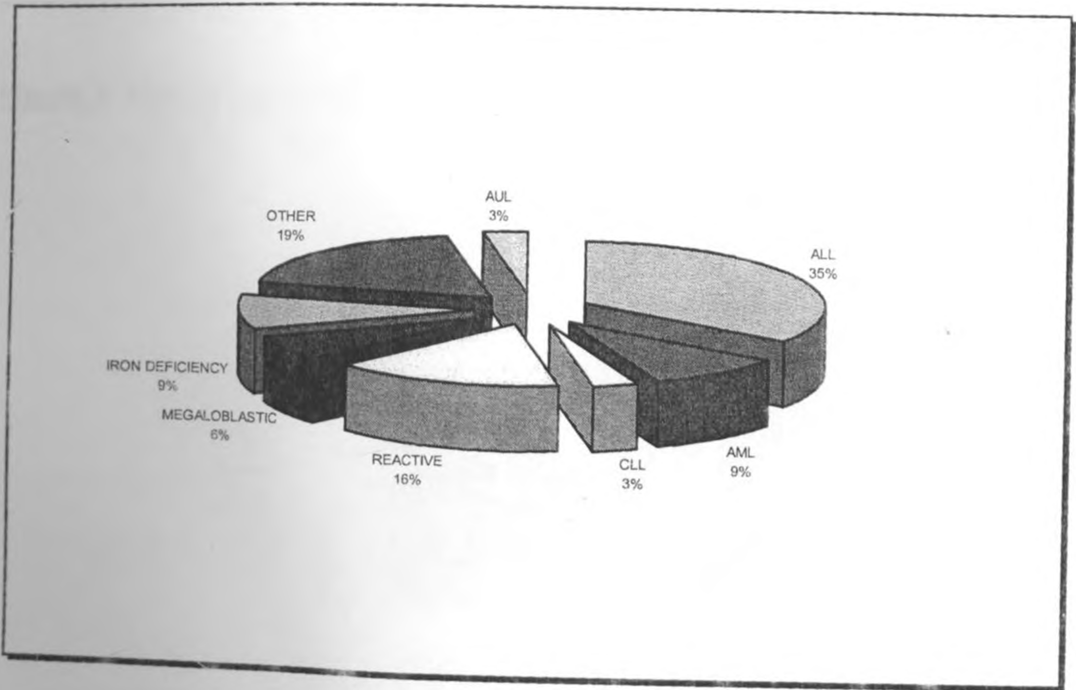
Relevant information gained about the TdT positivity that may have impacted on the diagnosis or follow-up of the patients was given to the attending clinicians.

5.0 RESULTS

Sixty-two BMAs from different patients were collected and analyzed using morphology and flow cytometry for TdT. However, owing to a computer data storage error, only data from thirty-two patients could be retrieved and analyzed. Of the missing cases, thirty were controls and only one was ALL.

Out of the thirty-two cases analyzed, eleven were ALL while the remaining twenty-one were non-ALL controls. This yielded cases to controls ratio of 1:1.9.

Figure 3: Morphological categories of the 32 BMAs analyzed.



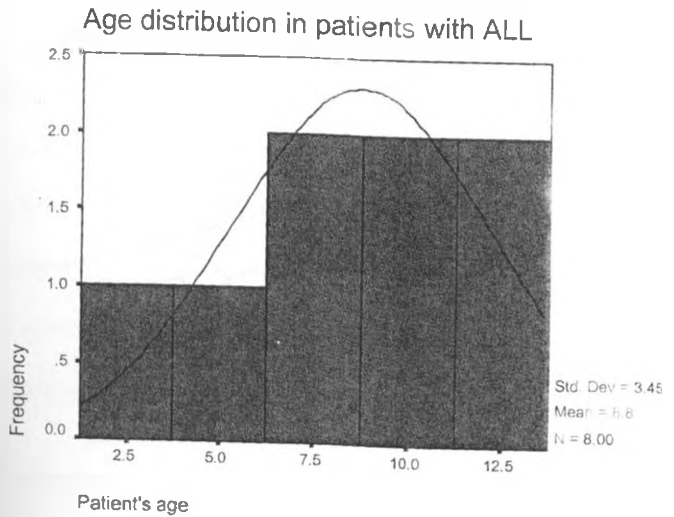
The control cases labeled as other consisted of aplastic marrow (n=1), plasma cell dyscrasia (n=1), hypoplastic BMAs with dysplasia (n=1) and congenital dyserythropoietic anemia

(n=1). A single case each from a morphologically normal BMAs and peripheral blood were included as well.

Since the study was aimed at assessing the flow-cytometric reactivity of TdT in ALL the controls were used purely in setting up the assay and for comparison of TdT reactivity between the two categories of BMAs. Therefore, descriptive inferences in their respect will be alluded to only where significant.

Of the eleven cases of ALL, seven were male while four were females. The male to females ratio was 1.8:1. Sex differences in various parameters were not evaluated owing to the small numbers of cases involved. The median age at recruitment was 9 years with a range of three to thirteen years.

Figure 4: Age distribution in ALL patients studied.



In reference to the treatment category for ALL, nine cases were on chemotherapy while two were newly confirmed cases and had not been started on chemotherapy at the time of recruitment. Of the nine patients on treatment, three exhibited morphologic complete

remission while the rest were cases in impending remission. Some of the hematological attributes are as shown below.

Table 2

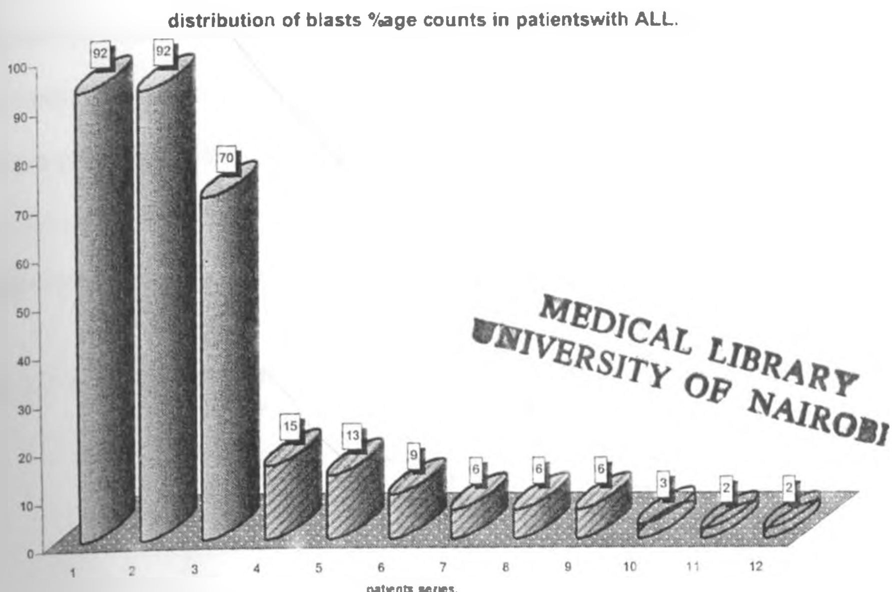
Table showing the means of peripheral blood parameters in patients with ALL

Parameter	Treated	Untreated	p-values
Hemoglobin (g/dl)	11.7	7.7	0.041
WBC ($x10^9$/l)	7.2	10.1	0.639
Neutrophils ($x10^9$/l)	1.7	2.6	0.162
Lymphocytes ($x10^9$/l)	1.8	1.4	0.06
Monocytes ($x10^9$/l)	0.4	0.8	0.1
Platelets ($x10^9$/l)	262	45	0.181
Blasts %	17.2	93	0.035

A comparison of means of some peripheral blood parameters between the treated ALL and the two untreated ALLs showed a significantly lower Hemoglobin level (p=0.041). The mean BMAs blasts percentage counts for the latter disease category was significantly higher (p=0.035). Other parameters showed observable differences between the individual cases but did not show statistical significance.

A single case of AUL was identified by morphological evaluation. It was not possible to classify its lineage using cytochemistry. TdT reactivity was positive. ? ALL

Figure 5:



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Key 3 dark bars (left)=untreated ALL; hatched bars=impending remission, 3 clear bars (right)=ALL in remission status

For the cases of ALL, a quantitative assessment of the association between the visual percentage BMAs blasts counts and the absolute number of acquired events in the blast window using FCM was done Both showed a strong positive correlation (r=0.99). The correlation was significant at the 0.01 confidence level in the two-tailed statistical test.

A weaker positive correlation was exhibited by the controls (r=0.474 which was significant at 0.05 confidence level.) This means that the number of positive events have more significance in regard to diagnosis at higher blast cell counts. It also implies that the test is sensitive to fluctuations and differences in target cell counts analyzed. This confirms published observations that correlation of TdT with blast cell counts are positive over a wide range (Table 5). This trend however weakens at very high blast counts (29, 30).

Qualitatively, the frequency of TdT expression was higher in ALL (63.6% of the cases) than in controls (38.1%). The antigen frequency for the ALL cases on treatment was 66.6%. Only one of the two cases of untreated ALL was TdT positive. There was no significant difference in the qualitative expression of TdT (positivity) between treatment cases of ALL and controls. This is because the blast cell counts of cases on treatment were approaching remission status thus approximating those of normal BMAs. This is a positive finding in regard to management of patients, which is a true reflection of the desired outcome of chemotherapy.

Table 3: Table showing the clusters of cases in this study and their TdT status.

Treatment status for BMAs		TdT status		
		Positive	Negative	Total
ALL on treatment	On remission	2	1	**3**
	Not on remission	3	3	**6**
ALL not on treatment		1	1	**2**
Controls (N/A)		8	13	**21**
Total		**14**	**18**	**32**

Table 4. Mean gated events in TdT and isotype control compartments of the assays

	Assay tube label			
	Isotype control		Anti-TdT test	
	Mean value*	Range	Mean value*	Range
No. Gated events	1381.9	5171	1599.3	6970

*[Pearson's correlation (r)=0.981,p value =0.01]

As a quality control measure, comparison of the number of gated events in the isotype control and the test samples run in parallel for both ALL and Non ALL control cases was also done. The isotype control was supplied by the Mab manufacturer as a staining control for fluorescence. It was designed to give an equal reaction compared to the anti-TdT antibody. In the staining procedure, both showed a strong positive correlation. (r=0.981; significant at the 0.01 level) This confirms that the specimens were treated equally at all levels (from collection to the data analysis phase) in order to arrive at our outcome measures.

The percentage count of blasts in BMAs was correlated against the gated events. An assessment of the correlation between the density of gated events (percentage proportion of gated events as a function of the total acquired events) and their TdT reactivity was evaluated as well. This was an attempt to evaluate the power of the position of the gate at the blast window. Compared to other positions that gave lower blast densities during acquisition, events at the blast gate were identified and used for our analysis.

Once assured of the strength of the gating procedure, an evaluation of TdT activity versus percentage blasts count in BMAs was evaluated. It yielded a positive correlation (r= 0.448, p=0.015). The clinical inferences made were that these findings could be applied at both diagnosis and follow-up of patients on treatment. For instance,

- Newly diagnosed cases of ALL can be followed up during treatment where response to therapy is monitored using both morphology and TdT reactivity.
- In MRD detection, residual malignant cells can be picked by FCM where morphologic counts may be within normal limits.

- Suspected relapses can be detected early before they become manifest morphologically. This can be aided through making comparisons with levels of Tdt recorded at attainment of remission status.

All these measurements of association gave positive correlations as shown in the table below.

Table 5

Table of correlation between various indices and parameters assessed in the blast window by flow cytometry.

Parameter	Pearson correlation (r)	P value
Gated events density Vs.% blasts in BMAs	0.610	0.001
Gated events density Vs. TdT activity	0.693	0.001
%Blasts in BMAs Vs. TdT activity	0.448	0.015

An assessment of the fluorescence intensity for TdT in both cases and controls was assessed and classified. There was a variable expression of fluorescence intensity between the cases of ALL. Majority showed borderline (dim) fluorescence. Qualitative comparisons of fluorescence intensity were made between dim events against the distinctly positive (bright and weak). Indeed none of the cases on chemotherapy showed bright positivity. A comparison of fluorescence intensity between cases and controls showed significant differences in fluorescence intensity (p=0.007). It would have been desirable to perform quantitative studies (fluorescence definitions) on our samples. However, the investigator and his technical team were not conversant with these settings of the flow cytometer at the time of this study.

The discrimination of fluorescence intensity between bright events versus the others was comparably stronger compared to that between dim positivity against distinctly positive and intermediate and bright TdT ($p=1.00$ vs $p=0.125$). Of interest to note was that all non-leukemia controls showed a dim TdT positivity ($p<0.05$) unlike the mixed picture in AML and ALL ($p=0.467$). The features of the latter may signify heterogeneity of phenotypes of variable biological behavior in the malignant clones as opposed to their benign counterparts.

6.0 DISCUSSION

Sixty-two patients were enrolled into this study. Data relevant to the study was obtained and entered into a computer data bank. Unfortunately, data from thirty patients could not be retrieved because of a data bank error that resulted from a computer system failure. Information for analysis was therefore available for only thirty-two patients. Morphologic diagnosis for ALL was the basis for case definition, patient inclusion and data analysis. The case to controls ratio was established at 1:1.9.

Two out of the twelve cases of ALL although having been assigned a study number did not have socio-demographic information available owing to logistic limitations. These were the only patients that were recruited from outside KNH. However, laboratory data including FCM were available for analysis.

The male to female ratio for ALL was established at 1.8:1. The figure approximates those obtained in other studies and all show a male preponderance. Macharia and Kasili, in separate studies obtained a figure of 1.5:1. Western studies have cited figures ranging from 1.2 to 1.3:1 (5, 6, 7).

The median age at recruitment was 9 years (range 3-13 years). Macharia in a hospital based study (KNH) showed varying statistics as shown in the table below. His retrospective study was aimed at assessing the admissions load contributed by childhood malignancies over a period of eight years (January 1990 to April 1998). Admission records for children with malignant conditions were analyzed. Those with a diagnosis of ALL were younger compared to those in this study. The higher median age in this study could have been contributed by an extreme age that formed the upper limit of the range (thirteen years). Furthermore, 89.9% of Macharia's cases were of ages two to ten years whereas in this study the same age bracket consisted of 75 % of the recruited cases of ALL.

Table 6.

Comparison of characteristics in patients with ALL in three studies.

Reference study	Age		% < 5yrs age	Sample size	M: F ratio
	Range	Median			
This study (2003), KNH	3-13	9	12.5%	12	1.8:1
Macharia (1996) KNH	2-10	6	23.5%	105	1.5:1
Melvyn (1996) USA	1-5	4.9	*** 1	*** 2	1.3:1

*** 1 2 Figures are based on national vital statistics. Ages had not been categorized as per our age groups.

The higher figures in median of age alluded to in this study may be attributed to the small sample size and also that Macharia's study design involved children less than twelve years of age. As opposed to the two studies, which were retrospective, this study was a cross-section one in design. Moreover, the former have reviewed records kept over a long time and therefore had little or no chance to encounter missed cases- a situation that was the converse in this study. In this study, only one patient was of age less than five years. In addition, Macharia's and Melvyn's studies were mainly epidemiological as opposed to the current one, which is laboratory, based. The implications are that the latter had exclusion criteria that were applied to filter non-cases of interest and hence the inherent bias in sampling and analysis of epidemiological variables.

Anaemia and neutropenia were observed as the only peripheral blood deficiencies in both treated and untreated cases of ALL based on sample means. A single case of untreated ALL had thrombocytopenia (45 x 10^9 per Liter). The mean blast cell count for untreated and treated ALL was 93% and 17% respectively. Other parameters showed observable

differences between the individual cases but did not show statistical significance. Three patients amongst those on treatment were in morphological remission at the time of recruitment. The rest of the cases showed marrow morphologic features of impending remission. Henceforth it was imprudent to compare the two clusters since only two cases of untreated ALL were included in this study and only one of them was TdT positive.

A Quality Assurance measure aimed at eliminating bias and improve consistency during FCM was instituted. The isotvpe control and TdT test sample were set in two different test tubes and run in parallel. Prior to analysis a visual inspection on the power of the position of the gate was performed. The position yielding the highest concentration of events was selected. An evaluation of the number of gated events for both samples during acquisition and analysis was compared using Pearson's correlation coefficient. The correlation was significant ($p<0.05$). which confirmed that the two samples were treated equally at the staining stage and subsequently.

All BMA s obtained from cases and controls were evaluated for expression of TdT. Seven (63.6%) of the eleven cases of ALL reacted positive while only 38.1% of the control BMAs turned positive. Studies from the west have shown case expression rates of up to 92% for newly diagnosed untreated cases of ALL (20). The differences between the two studies are probably due to treatment status and the study design. Most of these larger studies are collaborative multicenter projects, a situation that is a complete departure from this study. Moreover, Western researchers have phenotypically sub-classified the ALLs prevalent in their set up whereby mature B-cell ALL (a TdT negative entity) accounts for a small proportion of ALL. In our set up FCM studies on hematological malignancies are not done.

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All BMA s obtained from cases and controls were evaluated for expression of TdT. Seven (63.6%) of the eleven cases of ALL reacted positive while only 38.1% of the control BMAs turned positive. Studies from the west have shown case expression rates of up to 92% for newly diagnosed untreated cases of ALL (20). The differences between the two studies are probably due to treatment status and the study design. Most of these larger studies are collaborative multicenter projects, a situation that is a complete departure from this study. Moreover, Western researchers have phenotypically sub-classified the ALLs prevalent in their set up whereby mature B-cell ALL (a TdT negative entity) accounts for a small proportion of ALL. In our set up FCM studies on hematological malignancies are not done.

A single case of AUL was identified by morphological evaluation. It was not possible to classify its lineage using cytochemistry. However, it turned out to be a TdT-positive entity.

Immunophenotyping helped in this case which was thought to be a likely ALL based on the knowledge of distribution patterns of TdT.

TdT positivity by controls was contributed by BMAs exhibiting features of megaloblastic changes (n=1), AML (n=1), reactive changes (n=3), iron deficiency (n=2), and a single case of a normal BMA. The BMAs with marrow aplasia and the normal peripheral blood sample analyzed exhibited no TdT activity. All the positives cited had scattered events upon inspection of the respective blast windows in the acquisition dot plots and analysis quadrants. This finding is in keeping with earlier observations that TdT is a marker of lymphoid cell immaturity that is not restricted to malignant cells. That most of these marrows exhibited variable degrees of lymphoid cell proliferation was evident during morphological evaluation. Correlations in this regard however were not part of the study objectives despite this being a notable observation. Past publications have shown that TdT activity parallels that of HLA-DR, which is another proven marker of cell immaturity. Studies correlating morphological features with TdT positive cells in both BMAs and peripheral blood mononuclear cells (PBMCs) have shown that the positive cells are morphologically benign immature lymphoid cells. This feature is the immunological replica of precursor cells during their maturation (21, 29). In this respect, TdT expression is transient and it ceases when heavy chain gene rearrangements are complete and light chain gene rearrangements have commenced. Normal BMAs also express TdT in small amounts (15).

Our recruitment of controls was not structured to exclude any disease categories, except, ALL. The "blind" combinations of BMAs with variable TdT reactivity may have contributed to the insignificant disparity when compared with cases of ALL on chemotherapy ($p>0.05$). However, the strong positive correlations between blast cell count and TdT activity in this study adds power to the discriminative ability of FCM when used quantitatively. Bhatavdekar and Welte, using an enzymatic technique on peripheral blood had explored this feature (30,

31) They showed that peripheral blood exhibits TdT activity of less than 0.2U per 10^8 PBMCs. Patients with as low as ten percent blasts in blood had significantly higher levels of TdT when compared with blast negative patients. Nidal in a separate review cited that FCM can detect as few as two percent blasts in mixing experiments (20). This has found applications in evaluating patients for minimal residual disease. A positive correlation was also demonstrated between gated events and the absolute counts of TdT expressing events within the gate.

A comparison of visual blast counts against TdT percentage expression in the gated cells of treated ALL showed a strong positive correlation (r=0.99, p<0.05). The relationship was linear for both cases and controls. Borowitz had equally demonstrated that the correlation between morphologic blast cell counts and FCM estimates was positive and stronger at narrow ranges of counts (17). This concept may be refined in future when proposed analysis of antigen densities and knowledge-based software are developed (32).

In most studies cited, including this one, isolation of leukemic cells from BMAs has used density gradient separated mononuclear cells for phenotyping. This enriches for leukemia cells. However, it fails to account for relative compositions of BMAs unlike erythrocyte lysing techniques. Borowitz in a study performed a post recovery differential count following erythrocyte lysis technique (33). He reported a generally lower percentage of abnormal cells than morphologic counts of such cells had revealed prior to separation. However, these were correlated over a wide range. Significant discrepancies between morphologic counts and FCM estimates were related most often to poor aspirates or selective loss of cells during sample processing.

An obvious omission in this study was failure to perform a pre-analytic and a post-cell recovery BMAs automated differential count owing protocols for machine use in KAVI

laboratories. However, a technique for this procedure has been described (34, 35). Nevertheless, during the assay set up, serial morphological cell recovery assessments were done on each specimen after cell separation. Acceptable recovery rates were demonstrated in BMAs specimens as old as 84 hours. In this study, analysis of TdT was done within 72 hours of collecting the BMAs specimens from the recruited patients.

Flow cytometry is used widely to phenotype cases of acute leukemia and is now clearly important in classification (17, 36, 37). Although many laboratories have considerable experience with this technology there is no single accepted method for analyzing, interpreting or reporting data (17). When peripheral blood or bone marrow is replaced largely by blasts, it is generally no problem to determine the phenotype of the leukemic cells; however, cases in which leukemic and normal cells are present, routine methods of analysis make it difficult to interpret phenotypic information. This is particularly true if data are reported as percentage of cells marking with a particular antibody. This aspect was demonstrated by specimens analyzed in this study. None consisted purely of blasts, majority of the leukemic cases were impending morphological remission and some of the controls showed lymphoid cell hyperplasia of variable degrees. In FCM, such cells have individual variable characteristics that facilitate them to occupy variable positions in the scatter plots. Compounded by absence of a secondary cell marker, it became difficult to control the location of the gate to maximize on the position of blasts and exclude most of the outliers. Despite the low percentage positives displayed by the selected cases, our findings have been reinforced by the stronger correlations of cells gated around the blast window unlike in other positions.

Previous reports have indicated arbitrary thresholds for positivity at 20% of the isolated cells as opposed to our study where recommendations were that proportions of positivity be gauged against gated events. Moreover, the investigator used an antibody blank to set

threshold limits. Any degree of positivity against the blank was considered as so. An attempt was made to quantify numerically the absolute numbers of positives, which were then converted to a percentage. Both values were used during the data analysis.

Single marker studies are not inviolate from limitations. For instance, one case of AML by morphology was TdT positive (see appendix 6, case no. BMA 0605) but its true phenotype could not be established. Similarly, a few ALL cases failed to express the TdT phenotype. This limits the probabilities of observations in small studies. Phenotypic and genotypic re-arrangements may alter the specificity of the Mab. In leukemia biology, gene rearrangements owing to mutations, deletions and recombinant events are central pathobiologic events. (1, 2, 5, 13, 14). In their studies Adriansen and Drasch respectively showed that fifteen (13%) and ten (19%) of AMLs in their series exhibited an aberrant expression of ALL antigens namely TdT and/or CD 13 (38,39).

Lack of a standardization criterion for data interpretation is a common finding that introduces disturbing variability (40). Different analysis kits, procedures and instrumentation from as varied manufactures and commercial agents yield incomparable results. This predicament has been confirmed in several different reports on external quality assessment programmes on immunophenotyping procedures (41, 42, 43, 44). Such situations have led to enormous efforts by several groups to obtain specific consensus protocols for leukemia immunophenotyping and common criteria for data interpretation and reporting. These however have only been partially successful owing to the continued application of morphology as the standard reference for immunophenotypic characterization of leukemia, which according to Paietta should be considered a relic of the past (45).

To overcome these pitfalls, multiparametric analysis has been recommended. This refers to the simultaneous measurements and display of four or five different parameters of scatter

and fluorescence. With appropriate combinations of antibodies, it is frequently possible to distinguish between normal and neoplastic cells and particularly when fluorescent measurements are combined with information from light scatter (46). Multiparametric FCM of minimal residual disease in ALL is a valuable tool for relapse prediction and for the identification of a cohort of patients with very poor prognosis. (47) The technology has illustrated the complexity of differentiation pathways taken by leukemic cells and has highlighted the uniqueness of the phenotype of a particular neoplasm (48, 49). However, these are difficult to use in a routine clinical set up (35).

The lack of a significant difference in TdT percentage expression by cases and controls suggest that morphology and immunological aspects are independent of each other and that morphology is an imperfect marker of biologic potential (26,49,50). This is because it is often difficult to determine accurately the maturation stage of the biologically immature cells by morphological criteria. This observation strengthens the resolve that immunological tests act as ancillary adjuncts to morphological assessment of malignancies-an observation shared by Weir and Borowitz (14, 17).

The usefulness of TdT in differentiating between leukemic marrow samples from those of benign cases has been documented. Indeed, this concept forms the basis for panel markers in acute leukemia and not for benign conditions (1, 2, 12.48). This study has demonstrated a significant difference in the expression of TdT between acute leukemia (ALL & AML when analyzed together) and Non leukemia controls (p=0.025). Implications are that it is unusual for a benign haemopoietic condition to express significantly high levels of TdT and at bright fluorescence. Simultaneous studies of TdT and HLA-DR have implicated the two as markers of cell immaturity in the differentiation sequence with no reference to cell lineage (23, 24, 29) .In addition, ancillary tests using TdT should be judiciously selected on established reactivity patterns rather than on whims.

This study has re-affirmed that bright fluorescence is undoubtedly significant compared to borderline and weakly positive fluorescence. However, in as much as qualitative features of positivity are important, emphasis is shifting to descriptions of fluorescence intensity in addition to simply positive or negative. (16, 32). This has been referred to as fluorescence definition. It allows for detection of aberrant antigen co-expression and the analysis for heterogeneity and clonality of malignant cells in leukemia and lymphomas (32). Fluorescence definition may provide a better descriptor of antigen distribution on leukemic cells than percentage positives. More over it is a more powerful predictor of events free survival than either qualitative dichotomous discrimination of positives and negatives. Studies by Borowitz et al, demonstrated that fluorescence intensity of CD45 and CD 20 was significantly associated with events free survival in B-precursor ALL, while other markers showed no significant correlation (27). This implies that the fluorescence definition for CD45 and CD20 provides prognostic information not available from simple considerations of antigen expressions- as positive or negative- and adds to that obtained from traditional clinical and biologic risk factors (27, 28). Lack of this installation in many flow cytometers has hampered the use of relative fluorescence to compare studies performed over a time.

7.0 CONCLUSION

In summary, this study has indirectly evaluated the practical aspects of setting up an immunophenotypic assessment of leukemia in our clinical setting. Some observations on the clinical utility of a single Mab (Tdt) in ALL were made. It was observed that;

- Morphologic assessment of ALL could be complemented by TdT.
- If it can be considered part of the test panel TdT may have a role in the follow-up of patients with ALL who are undergoing chemotherapy.
- Although there was no significant difference in TdT reactivity between the various categories of ALL on treatment, there was a positive correlation between the blast counts and Tdt activity.
- It was not possible to deduce the proportion of TdT positive ALL in our set-up owing to an insufficient sample size of new cases of ALL. The same case applied to AUL as only one case was recruited in to the study.

8.0 CONSTRAINTS ENCOUNTERED.

In the course of data collection and analysis, the following were some of the limitations encountered.

Sample size

Leukemias being rare outcomes predispose investigators to small sample sizes and especially so in studies of short duration. Our three-month study period exposed the investigation to this probability. The small number of patients recruited made it difficult to observe any significant trends. A wider catchment study area and an extension of the study period would have realized a reasonable number of cases.

On the other hand, there were several instances when potential cases, either previously diagnosed or newly presenting, were excluded from recruitment. These missed opportunities were attributed either:

- To small volumes of BMAs samples obtained and therefore not amenable to flow cytometry.
- Some samples were received in the laboratory clotted in the specimen bottles prior to analysis.
- The investigator was not informed of some cases of ALL due for BMAs examination.

In all instances, the diagnosis (of ALL or other pathology) was made on bone marrow slides. Owing to ethical bonds, we couldn't reach out to re-aspirate such patient purely for study purposes.

Technical aspects.

This being a pioneer study in Kenya there was some but not enough technical expertise locally in the field of Leukemias. However, attempts to seek reference standards and control material abroad were fruitless owing to a paucity of contacts in this field. Only one of the approached authorities responded by sending a methodology used in their laboratory and was not compatible with our resources. The methodology was therefore not considered.

The study had been designed to quantify the fluorescence intensity as well. We were however unsuccessful in setting up to this function in the flow cytometer. This was meant to express fluorescence definition in mean equivalents of soluble fluorochrome (MESF).

Publications

It is difficult to obtain literature locally. Literature citing epidemiological and or clinicopathologic features of patients with ALL against TdT were obtained mainly from older publications. Methods of TdT analysis differed from the one used in this study.Most of the newer publications dealt with panel leukemia markers with TdT only being mentioned. This study however is in line with the recommended protocols for immunological diagnosis of lymphoid malignancies.

9.0 RECOMMENDATIONS

The outcomes of this pioneer study point to a promising utility of flow cytometric studies in patient care. The investigator recommends the following as the way forward

1. That the clinical applications of TdT phenotyping in diagnosis and monitoring of ALL chemotherapy be instituted in our clinical setting.
2. That the use of more immunological markers of disease (MAbs) alongside other ancillary studies like cytochemistry and cytogenetics should be considered in our leukemia patients work-up.
3. That more studies with a wider participation of institutions and encompassing other diseases amenable to FCM evaluation are carried out in our set-up.
4. The significance of fluorescence intensity in TdT phenotype assays ought to be investigated.
5. Training of laboratory personnel in FCM and other novel diagnostic techniques in haematologic oncology needs to be instituted.

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Appendix 1

CONSENT FORM

Serial number...

ACUTE LYMPHOBLASTIC LEUKEMIA: TdT AS AN IMMUNOLOGICAL CELL MARKER.

Patient's Particulars

Name..............................Age...   OP/IP number..................

Ward............   Bed number.

Declaration.

I,.............................(Self/Parent/Guardian) of................................hereby consent to the recruitment and inclusion in this study.

In regards to participation in this study, it is by informed consent that:

1. Recruitment into this study is voluntary and I may opt to withdraw the consent prior to commencement of investigations related to the study.
2. A bone marrow aspirate sample and a blood sample are the bare minimum requirements for the study (and hence the inclusion).
3. Neither financial gains nor any preferential treatment, favors or denial of treatment owed to the subject will be advanced or withdrawn due to my assent or denial to be included in the study.
4. Information and results obtained by virtue of this study shall remain confidential and will only be used to attain the objectives of the study.
5. Results bearing direct relevance to his or her hospitalization or visit to any of the treatment units shall be communicated to the attending clinicians.

Name..

Signature/Thumb print [ ] Date of consenting...d. /...m/...y

Name and signature of witness. Designation.

Appendix 2

ACUTE LYMPHOBLASTIC LEUKEMIA: INTRANUCLEAR TdT AS AN IMMUNOLOGICAL CELL MARKER

DATA COLLECTION SHEET:

SERIAL NO: ____________

DATE OF INTERVIEW: __/__/____

BIOGRAPHIC DATA

Name ____________________________ IP NO.____________ UNIT:

Date of birth Age at recruitment: *Years:* *Months:*

LABORATORY FEATURES

1. **Peripheral blood counts (value)**

   Hb (g/dl.)

   WBC ($x10^9$)

   RIB $x10^{12}$)

   Pelts $x10^9$)

2. **Peripheral blood film**

Red blood cells (tick where appropriate)

1. Normocytic normochromic
2. Microcytic hypochromic
3. Macrocytic
4. Mixed picture

Web Differential counts (value %)

1. Neutrophils (N)
2. Lymphocytes (L)
3. Monocytes (M)
4. Eosinophils (E)
5. Basophils (B)
6. Blasts

Platelets counts $x10^9$

Bone marrow aspirate :(tick where appropriate)

Date of collection: __/__/____

1. Cellularity
   I. Hypercellular
   II. Normocellular
   III. Hypocellular
2. ME ratio
   I. Within normal range (specify value;)
   II. Increased (specify Value;)
   III. Reversed- (specify value;)

	Maintained	Reduced	Increased
Erythropoiesis Leukopoiesis Megakaryopoiesis			

Plasma cells (value %)

Blasts (value %)

Stainable Iron
1. Present
2. Absent

Morphological diagnosis (tick one)
1. ALL
2. AML
3. AUL
4. CLL
5. OTHER (to specify)

ALL subtype
1. L1
2. L2
3. L3

Treatment category
1. Pretreatment
2. Consolidation (>28 days<6 months)
3. Maintenance (>6 months)

Flow cytometry

Date of run: __/__/____

Age of sample (Hrs.)

TdT status
1. Positive
2. Negative

	Peripheral blood	Bone marrow
TdT expression (%)		
Absolute No. Of +ve cells		

Appendix 3

FORMULA FOR CALCULATION OF SAMPLE SIZE

Step 1 (where population is >10,000)

$$Ss = [Z^2 *(p)*(1-p)] / c^2$$

Where

Z= Z value (1.96 for 95% confidence level)

P=percentage picking a choice, expressed as decimal

C=confidence interval, expressed as decimal

Step 2 (where population is < 10,000)

$$Nf = n / [1 + (n/N)]$$

Where

Nf= new sample size for populations < 10,000

n= sample size if population > 10000(ss in step 1 above)

N= current population size (no Less than 10,000)

Appendix 4

PROCEDURE FOR OBTAINING PERIPHERAL BLOOD AND BONE MARROW SPECIMENS

To avoid faults in specimen collection, storage and transport to the laboratory and to ensure reproducibility of results, a standard procedure for collecting and handling blood and bone marrow specimens is be as follows

A) PERIPHERAL BLOOD:

i. Reassure the patient and the guardian prior to beginning the procedure.

ii. Identify a suitable peripheral vein. The antecubital veins are preferable but if not accessible, the dorsal veins of the hand will be a suitable alternative.

iii. Apply a tourniquet around the upper third of the humerus to enhance the prominence of the distally located veins.

iv. Clean the skin overlying the identified vein using 70% alcohol and allow to air dry.

v. Using a gauge 21 needle for the adults and bigger children or gauge 23 for the smaller ones access the lumen of the vein gently and steadily. By means of an evacuated container (vacutainers), draw an amount of blood to the level determined for the anticoagulant in the tube.

vi. Gently rock the vacutainer to mix the collected blood with the anticoagulant.

vii. The blood is then transported to the laboratory for further processing.

viii. Withdraw the vacutainers needle from the vein apply gentle pressure over the puncture site using an alcohol soaked cotton swab until bleeding stops and then fix adhesive dressing over the site.

B) BONE MARROW ASPIRATION:

Biopsy of the bone marrow is an indispensable adjunct to the study of diseases of the blood. A simple and safe procedure seldom fails to provide important information in patients who have a blood disease. In children, the posterior superior iliac spine punctures are preferable to other site owing to their higher marrow yield and relative safety compared to other possible sampling sites.

In this study, reusable sterile bone marrow puncture needles were used for each patient. The procedure, for which asepsis was maintained, was as follows;

i. Reassure the patient as to the nature of the procedure.

ii. Clean the skin overlying the site of biopsy using 70% alcohol.

iii. Infiltrate the skin, subcutaneous tissue and the periosteum with five-ml. of 2% lignocaine for local anesthesia.

iv. By boring movements, penetrate the marrow cavity perpendicularly and once it has been reached, withdraw the stylet of the biopsy needle and attach a ten-ml. syringe to suck up the contents.

v. Prepare marrow films from the aspirated material immediately to avoid clotting of the specimen.

vi. The remainder of the marrow specimen will be transferred in preservative free heparin to the laboratory for further processing.

vii. In cases of re-aspiration owing to specimen inadequacy, a different site from the previous one will be seed to avoid hemodilution.

Appendix 5

PROCEDURE FOR TDT IMMUNOPHENOTYPING

Warning:

All biological specimens and materials being exposed to them are considered biohazards. Handle as if capable of transmitting infections and dispose of with proper precaution in accordance with laid down regulations and procedures. Never pipette by mouth. Wear protective clothing and gloves.

A: Bone marrow aspirate cell separation (to obtain a washed mononuclear cells isolate)

Background and principle;

Histopaque ®- 1077 (Sigma diagnostics Inc) is a solution of polysucrose and sodium diatrizoate adjusted to a density of 1.077 +/- 0.001 g/ml. This medium facilitates rapid recovery of mononuclear cells from small volumes of blood and bone marrow aspirates.

Anticoagulated venous blood and /or bone marrow are layered onto Histopaque®-1077. During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sediment, whereas lymphocytes and other mononuclear cells remain at the plasma-Histopaque interface.

Erythrocyte contamination is negligible. Most extraneous platelets are removed by low speed centrifugation during the washing steps.

Procedure

1) To a 15 ml conical centrifuge tube, add 3.0 ml of Histopaque®-1077™ and bring to room temperature.
2) Carefully layer 3.0 ml whole blood or BMAs onto Histopaque® -1077. Centrifuge at 400g for exactly 30 min at room temperature. Centrifugation at lower temperatures, such as four ^{0}C, may result in cell clumping and poor recovery.
3) After centrifugation, carefully aspirate, with a pasture pipette, the upper layer to within 0.5 cm of the opaque interface containing mononuclear cells. Discard the upper layer.

4) Carefully transfer the opaque interface, with a Pasteur pipette into a clean conical centrifuge tube.
5) Add to this tube (step 4), 10 ml isotonic phosphate buffered saline (PBS) solution and mix by gentle aspiration.
6) Centrifuge at 250g for 10 min.
7) Aspirate the supernatant and discard.
8) Resuspend cell pellet with 5.0 ml isotonic PBS and mix by gentle aspiration with a Pasteur pipette.
9) Centrifuge at 250g for 10 mins.
10) Repeat step 7,8 and 9, discard supernatant and resuspend cell pellet in 0.5 ml isotonic PBS solution.

B: staining with the gating reagent (CD 45) anti-TdT

1) Add 20 µl of fluorochrome-conjugated CD 45 monoclonal antibody to 100 µl of washed mononuclear cells from BMAs or peripheral blood in a 12 X 75 –mm tube.
2) Vortex gently and incubate for 15 to 30 minutes in the dark at room temperature (20 to 30 ^{0}C).
3) Add 2 to 3 ml of fetal bovine serum (FBS-wash buffer) and centrifuge at 500g for five minutes. Remove the supernatant.
4) To fix the mononuclear test cells, add 0.5 ml of 1% formaldehyde and mix thoroughly. Incubate for twenty minutes at room temperature. Centrifuge at 1000 rpm for 10 mins aspirate and discard supernatant.
5) Add 0.5 ml of 0.1% triton X-100 in PBS and incubate for 5 to 10 min. centrifuge at 1000 rpm for ten mins. Aspirate and discard supernatant.
6) Resuspend in 1% FBS in PBS to a final concentration of 1X 10^{6} per 50 µl.
7) Prepare on tube of 50 µl of mononuclear cell suspension and add 20 µl of conjugated anti human Tdt. prepare another tube of 50 µl of cell suspension and add 20 µl of conjugated isotype control Shake gently and incubate in the dark at room temperature for 20 to 30 mins.
8) Wash tubes in 2 ml of wash buffer. Centrifuge for 5 minutes at 1000 rpm, aspirate and discard supernatant.
9) Resuspend in 300 µl of wash buffer and analyze by flow cytometry.

Appendix 6

SUMMARY OF SAMPLES ANALYZED AND THEIR RESULTS

SERIAL NO.	AGE (YRS)	SEX	MORPHOLOGY	RX STATUS	BLASTS%	TDT		
						STATUS	%+Ve	EVENTS /30000
BMA 0602	>15	F	MEGALOBLASTIC ANEMIA	N/A	<3	+	.08	24
0604	3	M	ALL	Y	3	+	.11	33
0605	>15	M	AML-M2	N	92	+	2.88	864
0606	13	F	ALL	N	>90	+	.79	237
0608	10	M	REACTIVE	N/A	<3	+	.65	195
0610	30	M	NORMAL PBF	N/A	0	-	0	0
0614	13	F	AML	N	>90	-	0	0
0616	8	M	ALL	Y	9	+	1.53	459
0622	3.5	M	APLASTIC ANAEMIA	N/A	<3	-	0	0
0623	10	F	ALL	Y	0	-	0	0
0629		M	ALL	N		+	8.57	2572
0801	11	M	IRON DEFICIENCY	N/A	<3	-	0	0
0802		F	REACTIVE	N/A	<3	-	0	0
0803	9 months	M	IRON DEFICIENCY	N/A	<3	+	1.01	303
0804		M	IRON DEFICIENCY	N/A	<3	+	.1	30
0805	6	M	ALL	Y	6	-	.02	6
0806	7	M	NORMAL BMA	Y	<3	+	.51	153
0807			AML			-	.04	12
0808	15	M	REACTIVE BMA	N/A	<3	+	.7	210
0809		M	PLASMA CELL DYSCRASIA	N/A	<3	-	.03	9
0810	12	M	MEGALOBLASTIC ANEMIA	N/A	<3	-	0	0
0811		M	REACTIVE	N/A	<3	-	0	0
0812*	13	M	ALL	N	70	-	0	0
0813	10	M	ALL	Y	13	-	0	0
0814	6	F	REACTIVE	N/A	<3	+	0.08	24
0815	9	M	HYPOPLASIA /DYSPLASIA	N/A	<3	-	0	0
0816	1 & 9 months	F	CDA	N/A	<3	-	0	0
0817	7	F	ALL	Y	6	+	.23	69
0818		M	CLL	N/A	<3	-	.02	6
0819	13	F	ALL	Y	6	+	.23	69
0820		M	ALL	Y	15	-	0	0
2805**		M	ALL	Y	92	+	75.6	22680

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- 0812: Case was signed out as ALL. However, blasts exhibited some myeloid features. Cytochemistry was not done.
- 2805: This was a case of ALL relapse
- In reference to treatment (Rx) status the letters represent

- Y = on chemotherapy
- N = pretreatment cases of acute leukemia
- N/A =chemotherapy not applicable; doesn't preclude appropriate treatment for the diagnosis

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Ref. KNH-ERC/01/1468 Date 21 August 2002

Dr. Albert Giitwa Gachau
Dept. of Human Pathology
Faculty of Medicine
University of Nairobi

Dear Dr. Gachau,

RESEARCH PROPOSAL "ACUTE LYMPHOBLASTIC LEUKEMIA: TERMINAL DEOXYRIBONUCLEOTIDYL TRANSFERASE AS AN IMMUNOLOGICAL CELL MARKER" (P82/7/2002)

This is to acknowledge receipt of your submitted research proposal and to inform you that the KNH-Ethics and Research Committee has reviewed and **approved** your above cited proposal.

On behalf of the Committee, I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely,

DR. L. MUCHIRI
For: SECRETARY, KNH-ERC

cc Prof. K.M. Bhatt, Chairperson, KNH-ERC
The Deputy Director (C/S), KNH
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CMRO