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The clinical significance of current laboratory and other prognostic indicators in the management of South African children with Precursor B cell acute lymphoblastic leukaemia

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THE CLINICAL SIGNIFICANCE OF CURRENT LABORATORY AND OTHER PROGNOSTIC INDICATORS IN THE MANAGEMENT OF SOUTH AFRICAN CHILDREN WITH PRECURSOR B CELL ACUTE LYMPHOBLASTIC LEUKAEMIA

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A Research Report submitted to the Faculty of Health Sciences,

University of the Witwatersrand, Johannesburg

in part fulfilment of the requirements for

the degree of Master of Medicine in the branch of Haematology

Johannesburg, 2008

DECLARATION

I, Elise Schapkaitz declare that this Research Report is my own work. It is being submitted for Master of Medicine (in the branch of Haematology) to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Elise Schapkaitz

é .. day of April 2008

PUBLICATIONS

The following publication was submitted to the International Journal of Pediatric Hematology/Oncology:

E. Schapkaitz, J.E. Poole, R. Schwyzer, B. Goodwin, A.L.H. Mayne, G.G. Sherman.
The utility of measuring early clearance of leukaemic cells by a simplified polymerase chain reaction (PCR) assay in children with Precursor B cell Acute Lymphoblastic Leukaemia

ABSTRACT

Prognostic factors predictive of outcome in childhood Acute Lymphoblastic Leukaemia (ALL) are used to risk assign treatment groups.

This study aimed to identify the relevance of these prognostic features in the modern treatment era in South African children. A retrospective analysis of the presentation clinical and laboratory features and treatment outcomes of all children treated for Precursor B cell ALL at the Johannesburg Hospital was performed.

Between January 1997 and May 2007, 100 children were reviewed. Clinical features (age, race and gender) emerged as significant prognostic variables. Laboratory features (white cell count and genetic features) lacked significance. Early morphologic response on day 15 identified a subgroup associated with a favourable outcome. However the presence of > 5% blasts was not significantly predictive of relapse or death at this time point. Minimal residual disease (MRD) detection by modified immunoglobulin gene rearrangement and flow cytometry techniques did not improve the predictive value of the morphological assessment.

In a low resource setting, the challenge is to design cost effective MRD detection methods to improve the identification of patients at risk for relapse.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following people who assisted me with this research report.

Professor Gayle Sherman for her advice and encouragement. This research report is a product of her dedication to achieving excellence in the field of Paediatric Haematology.

Anthony Mayne for his statistical expertise and assistance.

Dr Janet Poole and the Paediatric Haematology Oncology unit at Johannesburg Hospital for access to patient data. This research report is a tribute to their commitment to the highest level of medical care.

The laboratory staff and department of cytogenetics at the National Health Laboratory Service (NHLS) responsible for performing the testing reported.

Megan Murray and the University of Witwatersrand, GEMP III students of 2005 for assistance with initial data collection and capture.

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NOMENCLATURE

ALL	Acute Lymphoblastic Leukaemia
APC	Allophycocyanin
Ag	Antigen
BFM	Berlin-Frankfurt-Munster
BM	Bone marrow
CCG	Childrenôs Cancer Group
CNS	Central nervous system
COG	Childrenôs oncology group
CI	Confidence interval
DFCI	Dana-Farber cancer Institute
EFS	Event free survival
FC	Flow cytometry
FITC	Fluorescein isothiocyanate
FR1	Framework region one
FR2	Framework region two
FR3	Framework region three
JH	Joining
Ig	Immunoglobulin
IgH	Immunoglobulin Heavy chain
IgK	Immunoglobulin kappa light chain
IgL	Immunoglobulin lambda light chain
MRD	Minimal residual disease
NHLS	National Health Laboratory Service
NPV	Negative predictive value
POG	Paediatric Oncology Group
PB	Peripheral blood
PerCP	Peridinin chlorophyll protein
PE	Phycoerythrin
PCR	Polymerase chain reaction

PPV	Positive predictive Value
RT-PCR	Reverse transcriptase polymerase chain reaction
SJCRM	St Judes Childrenôs Research Hospital
TCRD	T cell receptor delta
TCRB	T cell receptor beta
TCRG	T cell receptor gamma
VH	Variable
WCC	White cell count

1. INTRODUCTION

This is a retrospective record review of the presenting features and clinical outcomes of therapy of children with Precursor B cell Acute Lymphoblastic Leukaemia (ALL). This was conducted at the Johannesburg Hospital Paediatric Oncology Unit over a 125 month period. This is the main referral centre for children with haematological malignancies in Johannesburg.

1.1 Definition

ALL is a clonal proliferation of lymphoblasts, involving the bone marrow (BM) and peripheral blood (PB). ALL is the most common childhood cancer accounting for 30% of childhood malignancies (Pui, *et al* 1995). ALL is sub-typed according to the cell lineage into Precursor B cell ALL and Precursor T cell ALL (Harris, *et al* 1997). Precursor B cell ALL is the most common childhood subtype, which accounts for approximately 84% of cases (Camitta BM 1997). It is further divided into three groups according to the stage of B cell maturation on immunophenotypic analysis.[1]

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[1] Precursor B cell ALL subtypes include pro-B ALL which express early B lineage markers (CD19, CD22, and CD79a but no CD10 and no cytoplasmic or surface immunoglobulin{Ig}), common ALL which in addition to CD19, CD22, and CD79a, also express CD10 and pre-B ALL which express CD10 and cytoplasmic Ig.
B cell ALL is treated differently from Precursor B and T cell ALL with a lymphoma based approach and is no longer included in the World Health Organization classification of ALL (Harris, *et al* 1997).

1.2 Background

Remarkable progress has been achieved in the management of children with ALL such that children diagnosed with Precursor B cell ALL achieve a five year event free survival (EFS) of greater than 80% in large clinical trials (Pui and Evans 1998, Silverman*, et al* 2001).

As a result of improved outcome with treatment intensification, clinical research has focused on identifying clinical and laboratory prognostic features at diagnosis for risk stratification (Kersey 1997). In this way, only high risk cases are treated intensively with less toxic therapy reserved for low risk cases (Friedmann and Weinstein 2000).

More than 50 markers that correlate significantly with prognosis have been identified (Donadieu and Hill 2001). These prognostic features have been incorporated in various combinations into classification systems in order to assign treatment groups into high and standard risk groups (Tables 1.1 and 1.2).

Table 1.1.

Clinical and laboratory prognostic factors in childhood Acute Lymphoblastic Leukaemia (Pui, *et al* 1990, Smith, *et al* 1996)

RISK FACTORS	FAVOURABLE OUTCOME	UNFAVOURABLE OUTCOME
Age	1-10 years	< 1 and > 10 years
Gender	Female	Male
Race	Asian, white	Black
WCC at diagnosis	< 50 x 10^9/l	> 50 x 10^9/l
DNA index	> 1.16	< 1.16
Chromosome number/leukaemic cell	> 50	< 45
Cytogenetics	Trisomies 4,10 and 17	t(4;11) in infants and t(9;22)
Molecular genetics	TEL-AML1	MLL in infants and BCR-ABL
Day 8 prednisone response	< 1 x 10^9/l	> 1 x 10^9/l
Immunophenotype	Precursor B cell	Precursor T cell, B cell
CNS status	CNS 1(no blasts)	CNS 2 (< 5 blasts/ml)-3(Ó5 blasts/ml)
Key: WCC, white cell count; CNS, central nervous system		

Table 1.2.

Current risk classification systems

Group/ Institution	Features
SJCRM	
Low risk	Age 1-9, WCC < 50 x 10^9/l, DNA index 1.16-1.6 or TEL-AML1 fusion, MRD < 0.01% at end of induction, no BCR-ABL fusion or MLL rearrangement or t(1;19), no CNS or testicular disease
High risk	BCR-ABL fusion, induction failure, MRD > 1% at end of induction
BFM	
Standard risk	Prednisone good response, molecular remission on day 33, no BCR-ABL fusion or MLL rearrangement
Medium risk	Prednisone good response, morphologic remission on day 33, no BCR-ABL fusion or MLL rearrangement
High risk	Prednisone poor response, no morphologic remission on day 33, presence of BCR-ABL fusion or MLL rearrangement, Ó0.1% leukaemic cells at end of induction
DFCI	
Standard risk	Age 1-9, WCC<50 x 10^9/l, no BCR-ABL fusion, no T cell immunophenotype, no mediastinal mass, remission day 31
High risk	WCC>50 x 10^9/l, age <1 or >10, CNS 2 or 3, presence of BCR-ABL fusion, T cell, mediastinal mass
COG	
Low	Age 1-9, WCC < 50 x 10^9/l, trisomies 4,10 and 17 or TEL-AML1 fusion, rapid early response, no CNS or testicular disease, no BCR-ABL fusion, E2A-PBX1 fusion or MLL rearrangement
Standard	Age 1-9, WCC < 50 x 10^9/l, no trisomies 4,10 and 17, no TEL-AML1 fusion, or one of these genetic features but slow early response
High	No very high risk features
Very high	BCR-ABL fusion, hypoploidy, DNA index < 0.81, induction failure

Key:

SJCRM, St Judes Childrenôs Research Hospital; DFCI, Dana-Farber cancer Institute;
BFM, Berlin-Frankfurt-Munster; COG, Childrenôs oncology group
CNS, central nervous system; MRD, minimal residual disease

Most study groups use age and white cell count (WCC) at diagnosis for stratification because these features have consistently been shown to be important independent prognostic indictors (Ribeiro and Pui 1993). More recently treatment protocols classify patients on the basis of the genetic features of the leukaemic blasts and an early response to remission-induction treatment (Pui*, et al* 2004c). These features have been shown to predict more accurately the outcome rather than presenting clinical (age, gender, race) and laboratory features (WCC, DNA index, immunophenotype) which have lost prognostic significance with improvements in therapy (Pui*, et al* 2004a).

1.2.1 Acute Lymphoblastic Leukaemia in South African children

Presenting features predictive of outcome have also been described in South African children (Table 1.3).

Table 1.3.

Risk factors evaluated for influence on event free survival in South African children with Acute Lymphoblastic Leukaemia

Study	No. of patients	Centre	Risk factors	5 year EFS	p value
Wessels, et al 1997 (1983-1995)	96	Tygerburg Hospital	Age < 2 and > 8 years	25% and 20%	0.002
			WCC>20 x 10^9/l	21%	0.02
Macdougall 1985 (1974-1982)	130	Johannesburg & Chris Hani Baragwanath Hospitals	Age < 2 and > 10 years	-	0.0006
			Race-black	32%	0.0001
			CNS disease	-	0.006

At Johannesburg Hospital, the Paediatric Oncology Unit have adopted treatment protocols from large co-operative trials namely, the Toronto C (1997-2001) and modified ALL Berlin-Frankfurt-Munster (BFM) 95 (2002 to date) protocols. As a result of the poor survival rates previously reported in South African children with ALL treated according to standard risk protocols (MacDougall 1985), children in this study were not assigned to standard risk treatment. According to the modified BFM 95 protocol, patients were classified into medium and high risk groups on the basis of the genetic features of the leukaemic blasts and an early response to remission-induction treatment (Table 1.2) (Schrappe*, et al* 2000a).

1.2.2 Genetic features

The differences in treatment outcome for distinct age groups and to a lesser extent WCC groups can be attributed to the specific genetic abnormalities that the leukaemic cells harbour (Pui*, et al* 2004a). This is demonstrated by the poor prognosis of infant ALL, which results from the high frequency of MLL-AF4 rearrangement involving chromosome band 11q23 in this age group (Pui*, et al* 2002). Seventy five percent of childhood ALL cases have specific genetic abnormalities with therapeutic and prognostic significance at presentation (Pui*, et al* 1990). The best characterised chromosomal translocation is t (9; 22)/ BCR-ABL rearrangement designated as the Ph chromosome, which is present in three to five percent of childhood ALL cases. This has remained a poor prognostic feature despite treatment intensification (Crist*, et al* 1990).

Favourable genetic features associated with Precursor B cell ALL include hyperdiploidy (> 50 chromosomes or a DNA index > 1.16) and the t(12;21)/ TEL-AML1 rearrangement (Romana, *et al* 1995, Trueworthy, *et al* 1992). However in recent trials, the prognostic influence of hyperdiploidy has been found to closely correlate with other favourable genetic features such as trisomies of chromosome four and ten (Harris, *et al* 1992). The presence of the TEL-AML1 rearrangement has also lost prognostic impact in recent studies (Harbott, *et al* 1997, Satake, *et al* 1997, Seeger, *et al* 1998).

1.2.3 Early response to remission-induction treatment

Because clinical outcome is determined by many factors including the genetic features of the leukaemic cells, measurement of an early response to therapy has consistently shown independent prognostic significance. An early response to therapy is defined as evidence of an initial response to therapy prior to the BM evaluation at the end of induction therapy (Gaynon, *et al* 1997). This is a significant time point in treatment because augmentation of therapy for a slow early response improves survival (Nachman, *et al* 1998). The persistence of blasts in the PB or BM after the first week or two weeks of induction treatment has been found to be highly predictive of disease recurrence. [2]

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[2] Early response has been measured by morphologic evaluation of blasts in the PB after one week of prednisone therapy in BFM protocols since 1986 (Schrappe, *et al* 1996). Investigators from SJCRM have shown that the presence of > 1 x 10^{9}/l PB blasts after one week of conventional induction chemotherapy is also associated with a poor prognosis (Gajjar, *et al* 1995). Other authors have found BM blasts of Ó 5% during induction therapy predict relapse (Sandlund, *et al* 2002, Steinherz, *et al* 1996).

1.2.4 Minimal residual disease

Measurement of an early response to therapy lacks predictive value, as most relapses occur in patients with standard risk features. More recently, the detection of minimal residual disease (MRD) has been demonstrated to provide a more sensitive assessment of treatment response (Cave, *et al* 1998, Coustan-Smith, *et al* 2000, Campana and Coustan-Smith 2004, Dworzak, *et al* 2002,). This is a measure of the lowest level of detectable disease during morphologic remission. Estimates by conventional morphology have limited sensitivity, as even in morphologic remission patients may still have as many as 10^{10} leukaemic cells in the BM (Campana 2003, Szczepanski, *et al* 2001).

Currently several treatment groups (refer to Table 1.2) utilise monitoring of MRD at the end of remission induction therapy and/or sequential time points during treatment in order to identify patients at increased risk for relapse (Pui, *et al* 2004b). The detection of MRD is most frequently performed by Polymerase Chain Reaction (PCR) analysis or multi-parameter flow cytometry (FC). MRD detection by PCR targets clonal Ig gene rearrangements and fusion genes (Beishuizen, *et al* 1994). While only 30-40% of ALL patients have specific chromosomal aberrations with well-defined breakpoint fusion regions, virtually all Precursor B cell ALL patients (> 95%) have rearranged immunoglobulin heavy (IgH) genes (Campana and Pui 1995). The selection of the methods used for assessment of MRD depends on the expertise and facilities present in each centre.

1.2.4.1 PCR

Studies of clonal gene rearrangements have demonstrated a significantly increased risk of relapse in cases with evidence of MRD (Cave, *et al* 1998) (Foroni, *et al* 1999) (van Dongen, *et al* 1998). This is, however, a laborious procedure which requires analysis of multiple genetic targets (to reduce the risk of clonal evolution of the leukaemic clone during the disease course). PCR techniques are currently performed by two methods: these use either patient specific or consensus primers. Most academic centres favour the use of the former more sensitive (one leukaemic cell per 10^{5} -10^{6} cells) and specific method. This detects the DNA sequence of the IgH gene rearrangement of the leukaemic clone by sequencing the rearrangements and synthesizing allele specific primers (Cave, *et al* 1998, van Dongen, *et al* 1998). This is however costly and time consuming and limits application as a routine diagnostic tool. The latter method is simpler and more widely applicable, however it offers a lower level of detection of MRD (one leukaemic cell per 10^{3} -10^{4} cells) (Brisco, *et al* 2001, Nizet, *et al* 1993, Sykes, *et al* 1997). This PCR technique uses consensus primers to amplify junctional sequences for each patient.

1.2.4.2 Flow Cytometry

FC analysis is used to type leukaemic cells by defining their cell surface antigen (Ag) expression. Aberrant Ag expression on ALL blasts can be used to distinguish leukaemic cells from normal lymphoid cells. The use of broad antibody panels is a rapid technique and enables MRD monitoring in more than 90% of patients with a sensitivity of 0.01% (one leukaemic cell per 10^{4} cells) (Kerst, *et al* 2005).

1.2.4.3 Detection of Minimal Residual Disease at Johannesburg Hospital

The presence of MRD at the end of remission induction therapy (day 33) has recently been included in the high risk BFM classification. At the National Health Laboratory Service (NHLS) in Johannesburg, the assessment of MRD is performed by the complementary use of PCR with consensus primers and FC with limited antibody panels. These are performed at sequential time points during therapy. This combination of simplified MRD detection methods was initiated in 1996 on the basis of available technology and cost considerations. In a low resource setting such as South Africa, the monitoring tests employed to detect MRD, have been modified in order to make this testing readily available to all patients. The detection of MRD is currently not used to guide therapy in our unit as the clinical significance of MRD and its predictive value in relation to other prognostic features of ALL has never been formally assessed.

1.3 Research questions

1. Are the clinical and laboratory risk factors (refer to Table 1.1 and 1.3) previously shown to be prognostic in large multi-centre and South African studies of children with Precursor B cell ALL currently predictive of outcome with intensification of therapy in the modern treatment era?

2. Are genetic features and an early treatment response, used to risk assign treatment groups, predictive of outcome in this cohort?

3. Does the detection of MRD by modified techniques at sequential time points during therapy offer an improved sensitivity and specificity when compared to morphologic analysis?

2. PATIENTS AND METHODS

2.1 Patients

This study includes all children with the diagnosis of Precursor B cell ALL who were treated at the Johannesburg Hospital Paediatric Oncology Unit between January 1997 and May 2007. The diagnosis of Precursor B cell ALL was established according to standard morphological (French American British), cytochemical and immunophenotypic criteria (Catovsky and Matutes 1992). Children who were diagnosed and transferred to other centres for management were excluded from this analysis.

Clinical and laboratory data at disease presentation was collected from patient clinic files or the NHLS computer database.

Clinical data included age, gender and race. Laboratory data recorded included white cell count (WCC), FISH analysis for a t(9;22)/BCR-ABL rearrangement; a t(12;21)/TEL-AML1 rearrangement and a t(4,11)/MLL-AF4 rearrangement, the DNA Index/Ploidy as determined by FC and the karyotype on cytogenetic analysis. The presence of central nervous system (CNS) disease was not included as this was not detected in any of the patients studied.

The WCC at diagnosis was categorized as $< 50 \times 10^{9}$/l and $> 50 \times 10^{9}$/l and age as < 2 years, 2-10 years and > 10 years. The age groups applied differed from the National Cancer Institute criteria which has defined a uniform age range of 1- 9.9 (Smith*, et al*

1996). This was because an age range of 2-10 years was previously applied in South African cohorts and was significantly associated with survival.

This study was approved by the University of the Witwatersrandôs Human Research Ethics committee (protocol number MO60632).

2.2 Treatment protocol

All children diagnosed with ALL from January 1997 to December 2001 were treated uniformly according to the Hospital for Sick Childrenôs (SickKids, Toronto, Canada) high risk ALL protocol (Toronto C protocol). This is a chemotherapy regimen adapted from the approach of the BFM study group (Schrappe*, et al* 2000b).

In December 2001, a modified BFM 95 protocol was introduced in order to standardise treatment approaches between the Paediatric Oncology institutions in Gauteng. All children were primarily assigned to a medium risk group. Patients with one of the following features were changed to a high risk protocol: a t(9;22) rearrangement, or a t(4;11) rearrangement or failure to achieve an early response to remission-induction treatment. This was determined by the PB response to prednisone on day eight (a poor response defined as blasts of $> 1 \times 10^{9}$/l) or failure to achieve morphologic remission on day 33 of induction treatment (defined as Ó5% leukaemic blast cells on BM aspirate).

Treatment schedules for patients treated according to the Toronto C and modified ALL BFM 95 protocol are detailed in Appendix A and B. Briefly, the Toronto C protocol

consisted of induction therapy with prednisone, vincristine, daunorubicin and Lôasparaginase; CNS prophylaxis with intrathecal methotrexate (all patients with CNS disease received triple intrathecal chemotherapy with methotrexate, hydrocortisone and Ara-C); consolidation therapy with cyclophosphamide, Ara C and 6-mercaptopurine and intrathecal chemotherapy; re-induction pulses with dexamethasone, vincristine, adriamycin and Lôasparaginase, followed by cyclophosphamide, Ara C and 6-thioguanine and intrathecal chemotherapy; and maintenance therapy with: vincristine, prednisone, methotrexate and intrathecal chemotherapy. Trimethoprim-sulfamethoxole was given to all patients daily for three consecutive days per week post remission induction as prophylaxis against Pneumocystis jiroveci pneumonia. Chemotherapy was continued for 36 months.

The medium risk modified ALL BFM 95 protocol differed from the above regimen in that prednisone monotherapy was administered during induction for seven days; followed by vincristine and daunorubicin weekly. Intrathecal hydrocortisone was administered as part of CNS prophylaxis. The high risk blocks consisted of six cycles of multi-agent chemotherapy on a monthly basis, commencing one week after induction therapy.

2.3 Treatment response

Treatment response was measured by BM examinations performed at times specified by the relevant Toronto C and modified ALL BFM 95 protocols. Namely, an early response to remission induction therapy was measured at day 14 and 15 of treatment respectively (combined and referred to as day 15); remission was determined on day 28 and 33 of

induction therapy respectively (combined and referred to as day 33); and follow-up was performed prior to commencing maintenance therapy, at completion of treatment and if clinically indicated. Evaluation of blasts in the PB after one week of prednisone therapy was included as a measure of an early response from 2001 with adoption of a modified ALL BFM 95 protocol.

With each BM examination, MRD was determined by complementary use of PCR and FC. Follow-up with PCR analysis was not possible in patients who were polyclonal at diagnosis or in whom no baseline PCR was performed. In addition, as PCR was not used to guide management in these patients, owing to socio-economic constraints PCR was not performed at all time points in every patient.

2.4 Morphologic analysis

The diagnosis of Precursor B cell ALL was based on light microscopic appearance of BM smears. Morphologic remission was defined as < 5% leukaemic blasts on a regenerated marrow specimen at the end of remission induction therapy.

The number of leukaemic blasts in the PB on day eight was calculated from the absolute WCC and percentage of blasts. This was determined by morphologic and immunophenotypic analysis of the PB. The presence of $< 1 \times 10^{9}/l$ was defined as an early prednisone response.

2.5 Cytogenetic and FISH analysis

Diagnostic karyotyping of leukaemic cells was performed by the department of cytogenetics, NHLS. Banded chromosomes were prepared from 24 hour cultured preparations of fresh bone marrow.

FISH Analysis of t(12;21) and t(9;22) was initiated in 2000 and 2001 respectively. The LSI BCR-ABL and TEL-AML1 extra signal dual colour DNA probes (VYSIS) were used to detect the presence of t(9;22) and t(12;21) respectively.

2.6 Ploidy analysis

DNA content of the leukaemic blasts was determined by FC. The DNA index was defined as the ratio of DNA content of the leukemic G0/G1 cells versus normal diploid cells. Hyperdiploidy (which correlates with > 50 chromosomes) was defined according to the presence of two peaks and a DNA index > 1.16.

2.7 Minimal residual disease analysis

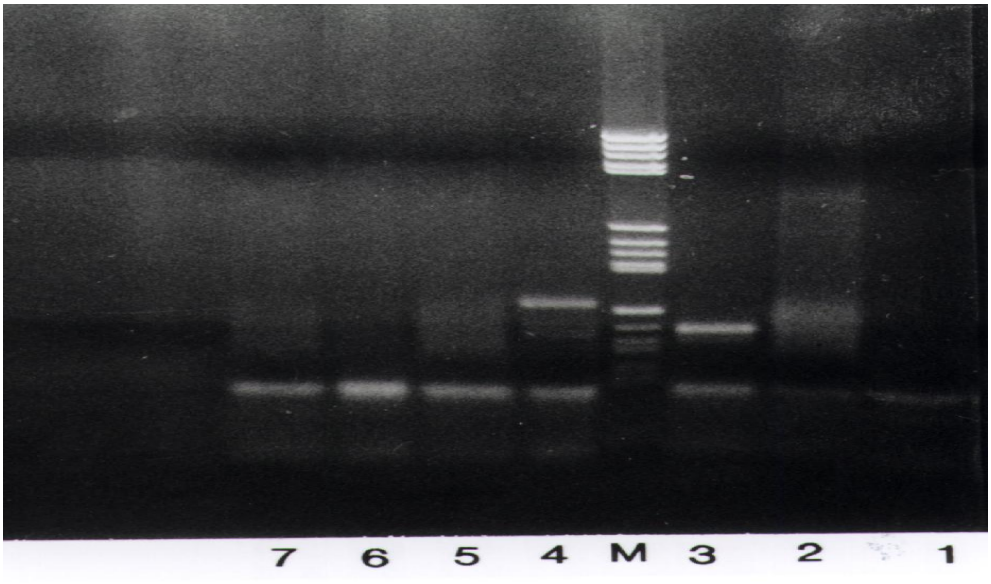
2.7.1 IgH PCR

Genomic DNA was extracted from bone marrow aspirates or EDTA collected PB using High Pure PCR template Preparation Kit (Roche, Mannheim, Germany) as indicated by the manufacturer. For the detection of gene rearrangements within framework region two (FR2) and framework region three (FR3), a nested PCR procedure was performed. The forward primer used for both the first round and nested PCR for the FR2 reaction was: 5ôTGG (G/A) TC CG(C/A) CAG (G/C) C(T/C)(T/C) CN GG 3ôand for the FR3 reaction: 5ôACA CGGC (C/T) (G/C) TGT ATT ACT GT 3ô. The reverse primers used in both the

FR2 and FR3 first round PCR were: 5ô TGA GGA GAC GGT GAC 3ô, and the second round: 5ô GTG ACC AGG GTN CCT TGG CCC CAG 3ô. A final concentration of 10 pmoles/µl of primer was added to each reaction. The annealing temperature was 50°C for the first round and 53°C for the second round.

PCR products were separated in a 2% agarose gel and visualised with Ethidium bromide staining on a UV transiluminator. Accepted size ranges for FR2 and FR3 products ranged from 120 to 300 base pairs and 80 to 120 base pairs respectively.

All samples were amplified with primers specific for the β-globin housekeeping gene to ensure that sufficient and high quality DNA was extracted during the extraction procedure. The primer set used for this amplification included the GF: 5ô AGT GCT GCA AGA AGA ACA ACT ACC 3ô; and the GR: 5ô CTC TGC ATC ATG GGC AGT GAG CTC 3ô. An annealing temperature of 63°C was used to perform a 40-cycle programme. These primers yielded a 330bp sized product to confirm DNA integrity.



Key:
Lane 1: blank
Lane 2: polyclonal control
Lane 3: monoclonal control
Lane M: molecular weight ladder

Figure 2.1.

PCR products in a 2% agarose gel

2.7.2 Flow cytometry

Pre-treatment heparinised bone marrow aspirate samples were submitted for surface marker analysis. Ficol-separated mononuclear cells were stained with monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinin chlorophyll protein (PerCP). The combination of

CD10, CD19, HLA DR and no surface membrane Ig expression was used to define Precursor B cell ALL.

Analysis was performed on a dual-laser FACSCalibur (Becton Dickinson, San Jose, CA, USA) equipped with Cell Quest Pro and PAINT-A-GATE Pro software on a maximum of 10 000 events per sample.

A limited analysis was performed at each follow-up time point during treatment. The monoclonal antibody combinations included: CD19/CD10/CD45 and/or HLADR/CD33/CD34 as well as relevant isotypic controls. These combinations were not standardised and varied according to the presentation phenotype. The combinations used rely on differences in brightness of the expression between leukemic cells and normal lymphoid progenitors. CD19/CD10 co-expressing cells which were large in size and/or cells that accounted for > 5% in number were used to define the presence of MRD.

2.8 Statistical analysis

EFS is defined as survival from the date of diagnosis until the date of relapse, death or last follow-up. EFS rates were estimated by the Kaplan-Meier survival function. The log-rank test was used to test the equality of survival curves. The significance of differences in means was tested using t- and F- tests. A p-value of < 0.05 is considered statistically significant. A Cox regression was used to determine hazard rates. All analyses were conducted using Stata 9.2 software.

3. RESULTS

3.1 Patient characteristics

There were 110 patients diagnosed with Precursor B cell ALL during the study period. There were ten non-disease related deaths from causes including sepsis, haemorrhage, and chemotherapy-related mortality, which were censored at the time of event on EFS analysis.

An average of 11 new cases of ALL were diagnosed and treated annually at Johannesburg Hospital Paediatric Oncology Unit (Figure 3.1).The average length of follow-up for the entire cohort was 55.8 months (range 0.4 ï 122.5). The follow-up was 79.6 months for patients treated according to the Toronto C protocol and 27.8 months for those treated according to a modified ALL BFM 95 protocol.

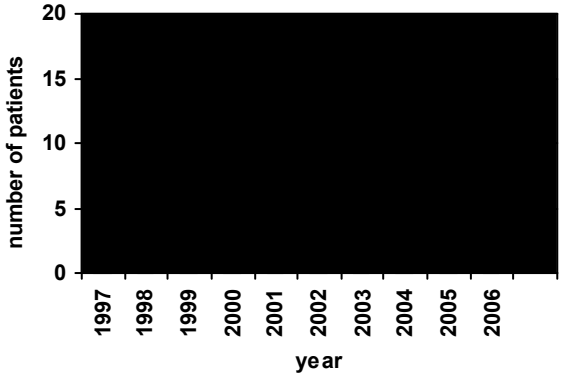


Figure 3.1.

Paediatric cases diagnosed with Precursor B cell Acute Lymphoblastic Leukaemia between 1997 and 2006.

3.1.1 Clinical

The presenting clinical features and treatment outcome of the 100 patients analysed are summarised in Table 3.1 and 3.2.

Table 3.1.

Study population clinical characteristics (n=100)

Features	Number of Patients
Age	
< 2 and > 10 years 2-10 years	9 and 15 76
Gender	
Male Female	57 43
Race	
Black White Asian Mixed	36 46 11 7

Table 3.2:

Treatment outcome according to clinical features

Features	5 year EFS (%) (95% CI)	p value
Age		
< 2 and > 10 years 2-10 years	64.8 [41.4 - 80.7] 86.4 [75.2 - 92.7]	0.018
Gender		
Male Female	68.7 [54.0 - 79.5] 97.6 [83.4 - 98.9]	0.001
Race		
Black Non-black	68.9 [49.7 - 82.1] 87.5 [75.4 ï 93.9]	0.006
Key: CI, confidence interval		

3.1.1.1 Age

As expected the incidence of childhood ALL was highest in the age-group of 2-10 years. Ages of the children in this study ranged from 3 months to 14.6 years. Children aged < 2 years and > 10 years were associated with an increased risk of relapse (Table 3.2).

3.1.1.2 Gender

There were a higher proportion of boys, which is consistent with findings in other large cohorts of children with ALL (Chessells*, et al* 1995). Male gender was predictive of an unfavourable outcome (Table 3.2).

3.1.1.3 Race

In contrast to other published series, black children represented a larger proportion of this study population (36%) as opposed to the 6.5% reported by Pui*, et al* 2003 and 7% by Bhatia*, et al* 2002. Black race was predictive of an unfavourable outcome (Table 3.2). Black children were more likely than non black children to have poor clinical and laboratory prognostic features. Black children were more likely to be male than non black children (74% versus 47%). Black children had a higher frequency of being diagnosed at age > 10 years than non black children (26% black versus 8%) and a slightly higher frequency of t(9;22) (7% of black children versus 4%). These poor risk features did not achieve statistical significance on Cox regression analysis.

3.1.2 Laboratory

The presenting laboratory features and treatment outcome are summarised in Table 3.3 and 3.4.

Table 3.3.

Study population laboratory features (n=100)

Features	Number of patients
Ploidy	
Hyperdiploid Diploid	24 70
t(9;22) BCR-ABL	
Absent Present	47 6
t(12;21) TEL-AML1	
Absent Present	53 17
t(4;11) MLL-AF4	
Absent Present	55 0
WCC (x 10 9/l)	
> 50 < 50	12 88

Table 3.4:

Treatment outcome according to laboratory features

Features	5 year EFS (%)(95% CI)	p value
Ploidy		
Hyperdiploid Diploid	90.8 [68.1-97.6] 76.38 [63.08-85.42]	0.34
t(9;22) BCR-ABL		
Absent Present	69.8 [47.7-83.9] 41.7 [5.6-76.7]	0.1
t(12;21) TEL-AML1		
Absent Present	65.89 [48.7-78.5] 88.2 [60.6-96.9]	0.18
t(4;11) MLL-AF4		
Absent Present	-	-
WCC (x 10 9/l)		
> 50 < 50	80.2 [40.3-94.8 82.1 [71.4-89.0]	0.85

3.1.2.1 WCC

The average WCC at diagnosis was 24.7 x 10^{9}/l (range 0.8-241.6). WCC was not significantly predictive of outcome.

3.1.2.2 Genetic features

In the case of the presence of a t(9;22) or t(12;21) on FISH analysis there were insufficient numbers tested. There were no cases of the MLL-AF4 rearrangement detected in the 55 patients tested.

The most common fusion gene identified was the TEL-AML1 rearrangement. The presence of a TEL-AML1 rearrangement, although not statistically significant, was associated with an improved survival. In this study the TEL-AML1 rearrangement was

associated with a WCC of less than 50 x 10^9/l and an older average age of 6.7 years (range 2.2-13.9).This contrasts to the peak incidence between the ages of one and five years described in other cohorts.

The incidence of the BCR-ABL rearrangement was higher than the three to five percent reported in other large childhood studies (11.3%). The presence of a BCR-ABL rearrangement, although not statistically significant, was associated with a poor prognosis. Children with a Ph chromosome had an average WCC of 84.0 x 10^9/l at diagnosis (range 3.8-241.6) and presented at an older average age of 8.3 years (range 3.4-13.6). Five of these patients were treated according to high risk protocols (there was one induction failure). There were two relapses at 27.3 and 13.4 months respectively after achieving morphologic remission. The former patient transformed to an Acute Myeloid Leukaemia.

Ploidy analysis was performed in 94 patients at diagnosis (Table 3.5). Hyperdiploidy was associated with a favourable prognosis, although this did not reach statistical significance. A Karyotype was performed in 13 of the 24 cases of hyperdiploidy. None of these cases were found to have trisomies of chromosomes four or ten.

Table 3.5

Ploidy groups according to the DNA index on flow cytometric analysis

Group	No of patients
Hyperdiploid >1.16	24
Low Hyperdiploid* (1.05-1.16)	6
Diploid* (0.95-1.05)	42
Pseudodiploid*	22
*Grouped as diploid for Ploidy analysis (n=70). There were no hypodiploid cases	

3.2 Treatment protocol

There were 54 children treated according to the Toronto C protocol and 46 children according to a modified ALL BFM 95 protocol. There was no significant difference between the two treatment protocols in the EFS after 5 years (p=0.8). Eight children treated according to a modified ALL BFM 95 protocol were changed to a high risk protocol (Table 3.6). The use of a more intensive treatment protocol in these patients with high risk features was not significantly associated with a better outcome (p = 0.6).

Table 3.6

Patients treated according to a modified high risk Berlin-Frankfurt-Munster 95 protocol

Patient	High risk criteria	EFS* (months)	Outcome
Patient 1	t(9;22)	64.1	Alive and in remission
Patient 2	t(9;22)	45.0	Alive and in remission
Patient 3	t(9;22)	37.4	Relapse and died
Patient 4	t(9;22)	14.4	Relapse and died
Patient 5	t(9;22)	5.8	Alive and in remission
Patient 6	Day 33 BM > 5%	8.2	Alive and in remission
Patient 7	Day 8 blasts > 1 x 10^9/l	29.9	Alive and in remission
Patient 8	Day 8 blasts > 1 x 10^9/l	6.1	Alive and in remission

*from diagnosis

3.3 Treatment response

In this study the estimated EFS rate after five years was 80.8% (95% CI, 71.1%-87.6%) (including the ten non disease related deaths). There were 19 children who relapsed. Their clinical and laboratory characteristics are tabulated in Appendix C. In the children treated according to the Toronto C protocol, the major cause of treatment failure was early marrow relapse. There were five induction failures on the Toronto C protocol. There were no induction failures on the modified ALL BFM 95 protocol; however there were six early relapses (within the first year after induction therapy). Overall, most relapses occurred during maintenance therapy (n=eight).

3.4 Morphologic response to remission induction therapy

3.4.1 Day eight prednisone response

A PB analysis was performed in 38 patients on day eight. There was evidence of a rapid early response in 36 patients. The numbers were however, too small and the follow-up too short to predict outcome (p=0.4).

3.4.2 Day 15 bone marrow analysis

An early response to remission induction therapy was measured on BM analysis in 95 patients. Results on day 15 were not available in three cases and two patients died prior to reaching day 15 of treatment.

Evaluation of an early response to induction therapy on day 15 by morphologic analysis gave a sensitivity and positive predictive value (PPV) of 85.9% and 87.0% which was significantly predictive of survival. Figure 3.2 illustrates that 77 patients achieved an early response to therapy associated with an EFS after five years of 87.5% (95% CI, 77.2% - 93.3%, $p<0.004$). An early response to therapy predicted survival in 67 of these patients who had not relapsed at closure of the study with an average follow-up of 62.0 months (range 4.9 to 122.3). This contrasts to those with Ó 5% blasts which were associated with an EFS after five years of 51.8% (95% CI, 21.0% - 75.8%).

Relapse was not predicted in ten cases with < 5% blasts on morphologic analysis (refer to Table 3.8). Morphologic analysis on day 15 is often impaired by haemodilution, which makes it difficult to estimate reliably and reproducibly residual disease.

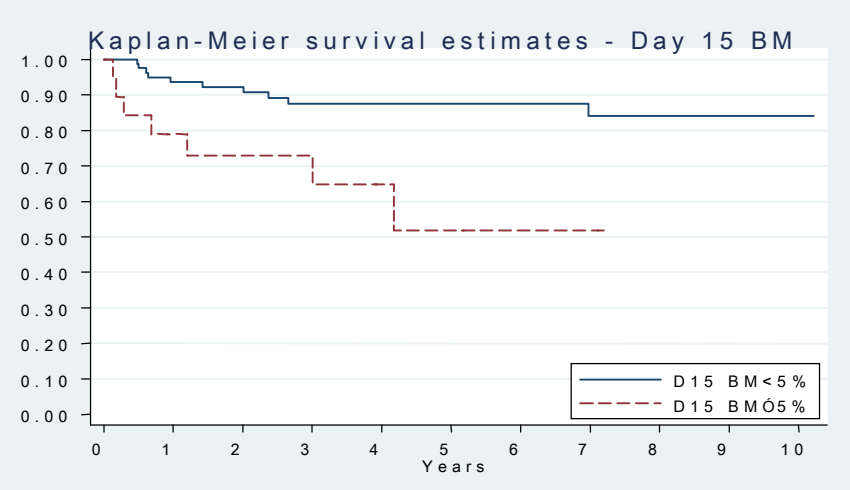


Figure 3.2.

Kaplan-Meier estimates of event free survival for children with Ó5 % and < 5% blasts on morphologic analysis on day 15 of induction therapy

3.4.3 Day 33 bone marrow analysis

A BM analysis was performed in 97 patients on day 33. Results were not available in three patients who died prior to day 33.

The day 33 analysis gave a high sensitivity and PPV (98.8% and 85.1% respectively) which was predictive of survival. Morphologic remission was achieved in 94 patients on day 33 associated with a five year EFS of 84.2% (95% CI, 77.2%-90.6%, p=0.18). Morphologic remission predicted survival in 80 of these patients who had not relapsed at closure of the study (refer to Table 3.9).

Relapse was not predicted in 14 cases with < 5% blasts on morphologic analysis (refer to Table 3.9). These 14 patients relapsed at an average of 24.5 months from diagnosis (range 5.9 to 83.8). The day 33 morphologic analysis yielded a low specificity and negative predictive value (NPV) of 12.5% and 66.7% respectively. Use of morphologic analysis on day 33 as a single investigation risks persistent disease not being detected.

On day 33, Ó5% blasts were detected in three BM assessments. This predicted relapse in two cases (at 2.2 and 3.5 months from diagnosis respectively). The other patient who was treated according to a high risk protocol had not relapsed at closure of the study (8.2 month follow-up).

3.5 Predictive value of minimal residual disease

3.5.1 Minimal residual disease targets identified at diagnosis

An IgH gene rearrangement with FR3 and/or FR2 primers was identified in 68 patients (81.9%) for MRD monitoring (Table 3.7). A leukemic phenotype with CD19, CD10 and/or CD34 expression was identified in all 100 patients for follow -up of MRD.

Table 3.7

Results of PCR analysis performed in 83 patients at diagnosis

PCR result	Number of patients
FR2 monoclonal	48
FR3 monoclonal	60
Polyclonal	11
No result	4
Not done	17
In the present study, the detection frequency of IgH gene rearrangements with FR3 primers was 72.3% versus 57.8% with FR2 primers at diagnosis. Follow-up was possible with at least two targets in 38 (45.8%) patients.	

3.5.2 Minimal residual disease results during treatment

3.5.2.1 Day 15 PCR

PCR results on day 15 were available in only 50 patients thus a direct comparison with the morphology findings (n=100) on day 15 was not possible.

Evaluation of an early response to induction therapy on day 15 molecular analysis was significantly predictive of survival. There were 35 patients with no MRD on day 15 associated with an EFS after five years of 89.3% (95% CI, 70.1% - 96.4%, $p<0.02$). This is compared to those with evidence of MRD associated with an EFS after five years of 66.7% (95% CI, 37.5% - 84.6%) (Figure 3.3). This test predicted the survival of 32 of the patients (refer to Table 3.8). At the closure of the study, these patients had not relapsed within an average follow up of 59.2 months (range 4.9 to 108.3). The use of a simplified PCR assay offered a lower sensitivity (of 76.2%) as compared to conventional morphology on day 15.

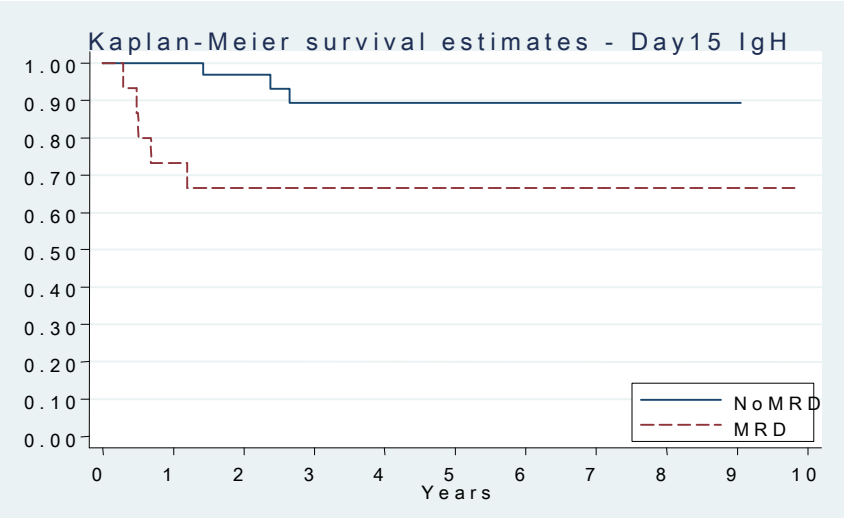


Figure 3.3

Kaplan-Meier estimates of Event Free Survival for children with and without minimal residual disease on PCR analysis on day 15 of induction therapy

MRD was present in 15 patients on day 15. The presence of MRD predicted relapse in five patients, however there were ten patients who had not relapsed at closure of this study. These ten children were more likely to have favourable prognostic features when compared to the five patients who relapsed. The use of a simplified PCR assay was not predictive of relapse.

There were ten patients with < 5% blasts by conventional morphology on day 15 who relapsed. MRD detection was performed in five of these patients and there was evidence of MRD in two cases. The disjoint day 15 morphologic and day 15 molecular tests did not add significant predictive power to the two tests taken singly (refer to Table 3.8).

Table 3.8

Comparison of Day 15 morphologic and minimal residual disease analysis

Tests	Relapsed/died true	Survived true	Total	*Sensitivity (%)	Specificity (%)	*PPV (%)	NPV (%)
BM							
< 5%	10	67	77				
Ó 5%	7	11	18				
	17	78	95	85.9	41.2	87.0	38.9
PCR							
poly	3	32	35				
mono	5	10	15				
	8	42	50	76.2	62.5	91.4	33.3
FC							
< 5%	7	40	47				
Ó 5%	10	24	34				
	17	64	81	62.5	58.8	85.1	29.4

*Sensitivity and PPV is stated in terms of the variable for survival[3]

Key:

PPV, positive predictive value; NPV, negative predictive value;

poly, polyclonal; mono, monoclonal

3.5.2.2 Day 15 flow cytometry

FC analysis performed in 81 patients on day 15 was not significantly predictive of outcome (refer to Table 3.8).

There were 34 patients with Ó 5% CD19/CD10 co-expressing cells. The presence of MRD predicted relapse in ten of these patients. There were 24 children with evidence of MRD, who had not relapsed at closure of the study. Use of a simplified FC assay was an unsatisfactory predictor of relapse on day 15 analysis.

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[3] The Oxford Dictionary of Statistics defines sensitivity as ćthe probability of a test giving a correct resultô In this analysis this relates to actual survival. For example, < 5% BM blasts is an accurate predictor of survival (high sensitivity and PPV). However, the prediction of relapse or death is inaccurate (low specificity and NPV).

3.5.2.3 Day 33 PCR

Molecular analysis performed in 65 patients on day 33 was not significantly predictive of outcome (refer to Table 3.9).

MRD was present in 13 patients at the end of induction. The presence of MRD predicted relapse in one patient. The high percentage of false positive results made this test an unsatisfactory predictor of relapse, which added considerably to the cost of the day 33 analysis (refer to Table 3.9).

Table 3.9

Comparison of Day 33 morphologic and minimal residual disease analysis

Tests	Relapsed/died true	Survived true	Total	*Sensitivity (%)	Specificity (%)	*PPV (%)	NPV (%)
BM							
< 5%	14	80	94				
Ó 5%	2	1	3				
	16	81	97	98.8	12.5	85.1	66.7
PCR							
poly	10	42	52				
mono	1	12	13				
	11	54	65	77.8	9.1	80.8	7.7
FC							
< 5%	12	70	82				
Ó 5%	3	2	5				
	15	72	87	97.2	20.0	85.4	60.0

*Sensitivity and PPV is stated in terms of the variable for survival

Key:

PPV, positive predictive value; NPV, negative predictive value;

poly, polyclonal; mono, monoclonal

3.5.2.4 Day 33 Flow Cytometry

FC analysis was performed in 87 patients on day 33. FC analysis on day 33 was significantly predictive of survival (refer to Table 3.9).

There were < 5% CD19/CD10 co-expressing cells in 82 patients associated with an EFS after 5 years of 84.4% (95% CI,73.4%-91.1%, p<0.01). This predicted survival in 70 of these patients who had not relapsed at closure of the study.

There were Ó5% CD19/CD10 co-expressing cells in five patients. This predicted relapse in three patients. Day 33 represented the most reliable time point for distinguishing leukemic cells from reactive precursor B cells with limited antibody panels.

The detection of MRD by limited FC offered a high sensitivity and PPV (97.2% and 85.4% respectively), however did not significantly improve the morphological assessment of a response to remission induction therapy. Morphology or immunophenotypic analysis or a disjunction of the two were not significantly predictive of relapse or death.

3.5.2.5 PCR prior to commencing maintenance and completion of therapy

A molecular analysis was performed in 66 patients prior to commencing maintenance therapy and in 39 patients at completion of therapy (refers to table 3.10). This was not statistically significantly predictive of outcome. Significance was impaired by the small sample size.

PCR analysis yielded a high number of false positive results on sequential follow-up and was therefore an unreliable predictor of relapse.

Table 3.10

Results of minimal residual disease analysis performed prior to maintenance therapy and at completion of therapy

Tests	Relapsed/died true	Survived true	Total	*Sensitivity (%)	Specificity (%)	*PPV (%)	NPV (%)
PM PCR							
Poly	8	53	61				
mono	1	4	5				
	9	57	66	93.0	11.0	86.9	20.0
PM FC							
< 5%	8	57	65				
Ó 5%	2	12	14				
	10	69	79	82.6	20.0	87.7	14.3
End PCR							
poly	2	27	29				
mono	1	9	10				
	3	36	39	75.0	33.3	93.1	10.0
End FC							
< 5%	3	43	46				
Ó 5%	1	7	8				
	4	50	54	86.0	25.0	93.5	12.5

*Sensitivity and PPV is stated in terms of the variable for survival

Key:

PPV, positive predictive value; NPV, negative predictive value;

poly, polyclonal; mono, monoclonal

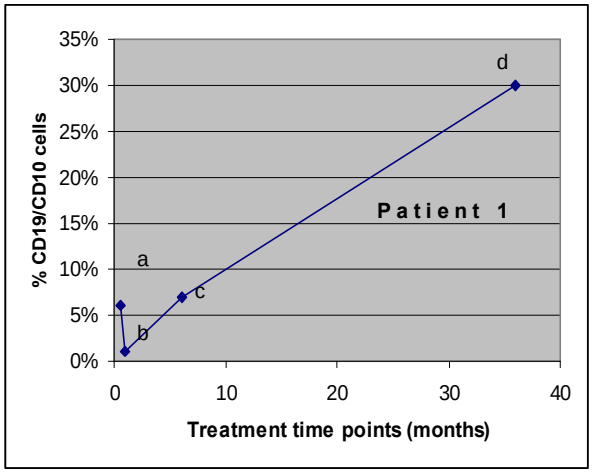
PM, prior to maintenance; End, end of therapy

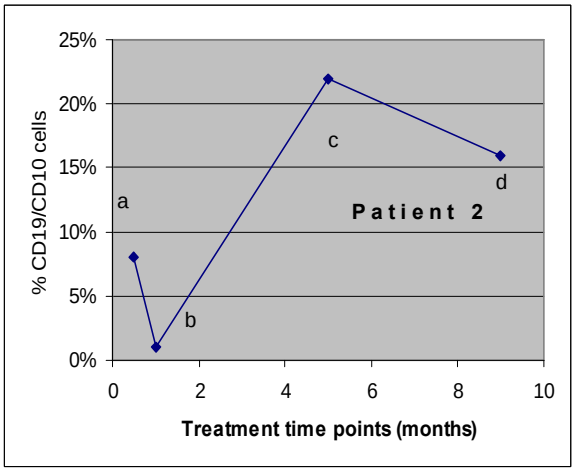
3.5.2.6 Flow cytometry prior to maintenance and completion of therapy

FC analysis was performed in 79 patients prior to commencing maintenance therapy and in 54 patients at completion of therapy (refers to Table 3.10). This was not statistically significantly predictive of outcome.

As expected, the proportion of samples with the presence of MRD on FC was higher on day 15 than on day 33, however did not decrease thereafter at prior to maintenance. At

regenerative time points, such as prior to maintenance therapy, there is an increasing number of immature B cells which represent reactive precursor B cells. Limited immunophenotypic analysis did not accurately distinguish these reactive precursor B cells from leukaemic cells at prior to maintenance (Dworzak*, et al* 2002). As a result, there were two patients (with survivals of 36 and 20.2 months respectively) who demonstrated increasing numbers (Ó 5%) of CD19/CD10 co-expressing cells several months before morphological relapse was detectable (Figure 3.4). These cells, however, resembled reactive precursor B cells on limited analysis and relapse was not predicted. MRD on PCR analysis was not detected in one case and not performed in the other.





b

Key:

a. % CD19/CD10 cells at day 15

b. % CD19/CD10 cells at day 33

c. % CD19/CD10 cells at prior to maintenance therapy

d. % CD19/CD10 cells at the time of morphological relapse

Figure 3.4

Detection of minimal residual disease at sequential time points in therapy by flow cytometry in two relapses

The presence of MRD on more than one occasion was a stronger predictor of relapse. In this study there were three patients that demonstrated MRD by FC at more than two sequential time points in the first six months of therapy. These patients relapsed at 6.6, 13.4 and 48.9 months respectively after achieving morphologic remission.

3.5.3 Stability of minimal residual disease targets at relapse

BM samples at relapse were analysed in six patients with BM involvement. In only one of six of these patients at least one PCR target remained stable i.e., one of 11 targets. The false negative results at relapse are probably due to continuing rearrangements and clonal selection.

In contrast, at the time of relapse, the immunophenotype was stable when compared to primary diagnosis. In all 14 relapses who achieved morphologic remission, MRD was not detected in the BMôs preceding relapse with limited FC. The median interval between these aspirations and relapse was ten months (range 5.9 ï 83.8).

3.6 Prognostic features

Of the risk factors analysed, the prognostic features tabulated in Table 3.11 were predictive of survival. These tests gave prediction rates of EFS which were significantly higher than the five year EFS rate based on treatment alone. The most favourable subgroup was non-black females aged 2-10 years. In this group an EFS rate of 100% was achieved ($p < 0.01$).

Table 3.11

Significant prognostic factors predictive of survival

Tests and factors predictive of survival	EFS at last date*	EFS 5 years	95% CI	p-value
Day 15 BM< 5%	87.0%	87.1%	[76.6%, 93.1%]	<0.004
Day 15 IgH no MRD	91.4%	89.2%	[70.1%, 96.4%]	<0.02
Day 33 %CD19/10 < 5%	85.4%	84.4%	[73.4%, 91.1%]	<0.01
Non-black	87.5%	87.5%	[75.4%, 93.9%]	<0.01
Female	95.3%	97.6%	[84.2%, 99.6%]	<0.01
Age between 2 and 10 years	86.8%	86.3%	[75.1%, 92.7%]	<0.02
* diagnosis to last date may be less than 5 years				

A Cox proportional hazards model was used to compute the ratio of relapse or death in the entire cohort (Table 3.12).

Table 3.12

Multivariate Cox Regression analysis

Tests and factors predictive of relapse or death	Hazard ratio based on a Cox regression	95% CI	p-value
Day 15 BM Ó 5%	3.0	[1.1, 8.4]	<0.004
Day 15 IgH MRD	4.4	[1.0, 18.6]	<0.02
Day 33 %CD19/10 Ó 5%	8.8	[2.4, 32.1]	<0.01
Black	3.4	[1.3, 9.0]	<0.02
Male	14.8	[1.9, 11.3]	<0.01
Age < 2 and >10 years	2.9	[1.1, 7.4]	<0.03

4. DISCUSSIONS AND CONCLUSION

This is a single centre account of the treatment outcome and prognostic factor analysis in a series of 100 children diagnosed with Precursor B cell ALL in the modern treatment era. The following discussion has been formulated according to the research questions outlined in section 1.3.

4.1 Treatment outcome

Treatment was based on modifications of the widely used BFM treatment regimen, namely Toronto C and ALL BFM 95 protocols (Schrappe 2004). Preceding this analysis, studies demonstrated a poor survival rate in South African children (MacDougall 1985, Wessels, *et al* 1997). All children in this study were thus assigned to augmented treatment protocols, which resulted in a significantly improved outcome (a five year EFS of 80.8%). This is consistent with reports from large multi-centre trials (Pui and Evans 1998). Although there was no further increase in EFS after five years with introduction of prednisone monotherapy with the ALL BFM 95 protocol, there was evidence of an improved response to induction therapy.

4.2 Clinical Prognostic Features

The clinical features, race and age emerged as significant prognostic factors in preceding South African studies. In this study, black males aged < 2 and > 10 years were associated with a poor prognosis. In multi-centre studies, in the modern treatment era, the predictive value of clinical features has been lost. The combination of these features in South African children may represent a distinct subgroup at risk for poor survival and relapse.

With treatment intensification and improved access to treatment[4] in this recent study, survival rates across racial groups have increased significantly. Previously an EFS after five years of 32% and 72% respectively were reported in black and white children with ALL in Johannesburg between 1974 and 1982 (Macdougall, *et al* 1986). In this study, black and white children achieved an EFS after five years of 68.9% (95% CI, 49.7%-82.1%) and 86.1% (95% CI, 71.3%-93.6%) respectively.

Even with equal access to treatment, black children continue to fare poorly, whereas outcomes for mixed and Asian children did not differ significantly from those of white children. This is consistent with the findings of the recent Paediatric Oncology Group (POG) and Childrenôs Cancer Group (CCG) reports which showed an EFS after five years of 61% and 69% respectively (Bhatia, *et al* 2002, Pollock, *et al* 2000). In this study, black South African children were more likely to have unfavourable prognostic features than white children. However on multivariate analysis, these features did not explain the poor survival rates in black children. These findings suggest that other risk factors exist in the tumour biology or treatment response of black children, which confers a worse outcome (Kadan-Lottick, *et al* 2003). Recently the presence of genetic polymorphisms affecting drug metabolism of methotrexate and 6-MP have been described in black patients (Pui, *et al* 2004a). Future studies will demonstrate the predictive value of host

[4] Patients with poor socio-economic circumstances were institutionalised for the duration of their therapy to ensure compliance, completion of treatment and access to routine follow-up.

genetic features. In this way pharmocogenetic features at presentation can be used to risk assign treatment groups.

Contrary to these reports, a single institution study indicated that with equal access to treatment black children with ALL can achieve high cure rates (five year EFS of 80.7%) (Pui*, et al* 2003). Black children, however, represented a small proportion of this study.

4.3 Laboratory Prognostic Features

In the case of WCC, the presence of a t(9;22) or t(12;21) and Ploidy there was either insufficient numbers tested or the results were not significantly predictive of outcome.

4.4 Predictive value of genetic features

In order to improve the overall survival rate, therapy is intensified according to the genetic features of the leukaemic blasts. Published series such as the XIIIB Study have demonstrated the independent prognostic significance of the BCR-ABL or MLL-AF4 gene rearrangements (Pui*, et al* 2004c).

In this study, the presence of the BCR-ABL rearrangement was associated with a poor EFS after five years of 41.7% (95% CI, 5.6%-76.7%; p=0.1). This was not statistically significant because of the small sample size. Children with a Ph chromosome were more likely to present at an older age with a higher WCC. This is in contrast to TEL-AML1 positive ALL cases which were more likely to be characterised by favourable presenting features. The presence of the TEL-AML1 rearrangement was associated with an

improved five year EFS, however in keeping with recent reports; this was not predictive of survival (Harbott*, et al* 1997, Satake*, et al* 1997, Seeger*, et al* 1998).

The presence of chromosomal translocations also represents a stable target for the detection of MRD by reverse transcriptase (RT)-PCR amplification in 23 of the children in this study population (de Haas*, et al* 2000, Markus*, et al* 2006, Satake*, et al* 1997, Seeger*, et al* 1998). Although this would only be applicable in a small percentage of ALLôs (BCR-ABL transcripts were detected in six cases and TEL-AML1 transcripts in 17 cases in this study), it is a less complex method than PCR analysis of gene rearrangements which is also limited by clonal evolution.

4.5 Predictive value of Treatment response

4.5.1 Day eight prednisone response

In the case of the day eight PB response to prednisone, there were insufficient numbers tested to determine its predictive value. The leukemic cells in 92.1% of the children in this study demonstrated evidence of a sensitivity to prednisone therapy.

4.5.2 Day 15 analysis

The evaluation of an early response to therapy as determined by morphologic analysis of the day 15 BM identified a subgroup of patients with an ultra fast response to remission induction therapy. This was associated with a favourable outcome. This test gave a prediction rate of EFS which was significantly higher than the five year EFS rate based on treatment alone.

Use of a simplified PCR method on day 15 did not significantly improve the morphological assessment of an early response to remission induction therapy. There was no significant increase in the prediction of EFS by a combination of the two tests. Neither test was significantly predictive of relapse or death. Limited FC was an unsatisfactory predictor of outcome.

4.5.3 Day 33 analysis

Treatment response, according to the modified ALL BFM 95 protocol, is guided by morphologic remission status on day 33. This time point offered the highest sensitivity (98.8%) which was predictive of survival.

Limited FC evaluation of a response to induction therapy did not add to the morphologic assessment. However, day 33 represented the most significant time point for performing limited FC because of the presence of exclusively mature B cells demonstrated early during treatment. Patients with Ó5% CD19/CD10 co-expressing cells on limited FC had a hazards ratio (HR) of 8.8 (95% CI, 2.4-32.1; p=0.01). Molecular analysis did not significantly improve the day 33 analysis.

4.6 Minimal Residual Disease

Routine use of MRD monitoring is limited by the complexity, technical expertise and cost of the assays. In order to extend the benefits of MRD detection to most South African children with Precursor B cell ALL we had instituted relatively simple and inexpensive PCR and FC assays. Both techniques have been employed for the detection

of MRD as reports in the literature have indicated that these two tests should be viewed as complementary to each other in view of their respective limitations (Kerst*, et al* 2005, Pui*, et al* 1999).

The panel of limited antibodies used to study MRD by FC allowed monitoring of 100% of patients, whereas PCR based on IgH primers did not detect 20% of rearrangements at diagnosis for follow-up of MRD. Detection of rearrangements was restricted by the panel of primers used. This is consistent with the findings of previous studies which employed simplified PCR techniques (Potter*, et al* 1993) (Nizet*, et al* 1991).

In this study, the detection of MRD by simplified PCR and FC assays did not provide additional information when compared to conventional morphologic assessment of treatment response. As indicated above, use of a simplified PCR and FC assay did not significantly improve the morphological findings of an early response to therapy but did add to the cost of the assessment of residual disease[5]. Further, assessment of MRD at later time points in treatment did not emerge as a significant predictor of subclinical relapse.

[5] The cost of a routine BM analysis in 2007 was R275.00. Complementary PCR and limited FC was performed at an additional cost of R467.00 per primer and R165.00 per monoclonal antibody. This added an additional cost of at least R1429.00 to the analysis at each time point.

4.6.1 Limitations of a simplified PCR assay

MRD was detected via a qualitative nested PCR using four consensus primers. This is a rapid and simple method compared to other more complex methods which are based on sequencing and the use of patient-specific probes. Although more complex methods are labour intensive, a specificity of 100% can be routinely achieved (Panzer-Grumayer*, et al* 2000). In this study, the specificity never exceeded 62.5%. This can be explained on the basis of the limited panel of primers used and/or improper primer annealing. At least two PCR targets are required per patient for follow-up of MRD because of ongoing secondary rearrangements (Beishuizen*, et al* 1994, Szczepanski*, et al* 2002). In this study, subclinical relapse was not detected in the six available samples. MRD PCR targets present at diagnosis were preserved at relapse in only one patient. In the other five, failure to detect relapse may have been associated with loss of the IgH gene rearrangement.

Use of a simplified PCR assay yielded a lower sensitivity as compared to morphologic analysis. The day 15 analysis gave the lowest sensitivity (76.2%). This is most likely due to the increased presence of non-viable cells during induction therapy as studies using specific PCR techniques also report a low sensitivity (Panzer-Grumayer*, et al* 2000). Another cause to be considered was the possibility of contamination because the same sets of primers were used for different patients. Negative controls are however included with every batch of samples analysed to limit this risk. The inability to differentiate oligo-clonal from monoclonal populations at diagnosis is more likely. Another limitation of this PCR assay was the use of gel electrophoresis to resolve the different sized

amplicon products. This simplification allows for easier routine detection, however polyclonal background levels further limit the sensitivity (one leukaemic cell per 10^3 normal bone marrow cells).

Comparisons of our results on MRD to other clinical trials are difficult as a result of the different techniques employed. Despite this the detection of MRD emerges as a significant predictor of outcome in all studies. This indicates that a uniform method for the detection of MRD is not required, provided the method used can achieve a sensitivity of more than one leukemic cell per 10^4 normal bone marrow cells (Brisco*, et al* 1994, Foroni*, et al* 1999, Wasserman*, et al* 1992).

4.6.2 Limitations of a simplified Flow cytometry assay

Use of extensive panels for follow-up of aberrant expression limits the routine application of FC for MRD follow-up. Most of the reports in the literature are based on aberrant expression (Campana and Coustan-Smith 2004). Other authors have reported the value of a simplified FC assay based on CD19, CD10 and/or CD34 expression using standardised panels (Coustan-Smith*, et al* 1998, Coustan-Smith*, et al* 2006, Farahat*, et al* 1998). This has been shown to be a simple and sensitive technique which can discriminate between leukaemic and reactive precursor B cells at early time points during treatment.

As demonstrated, this FC method reliably discriminated between leukaemic cells and normal cells at day 33 of treatment. Regenerative time points (prior to maintenance) were characterised by a limited sensitivity in a high proportion of cases because of the

presence of immature B cells which resemble leukaemic cells immnophenotypically (Dworzak*, et al* 2002).

The analysis of MRD at a single time point was not sufficient for recognition of patients with poor prognosis as well as patients with good prognosis in this study. In keeping with the findings in the literature, the presence of MRD at more than two sequential time points was a more reliable predictor of relapse (Dworzak*, et al* 2002).

MRD was not detected in the BMôs performed prior to relapse in the 14 relapses studied. This can be explained on the basis of a recent study which showed that MRD could be detected by FC (at a level of >0.1% nucleated cells) in aspirates performed within three months of relapse; however not after six months (Dworzak*, et al* 2002). Most relapses occurred during maintenance therapy and BMôs were performed more than six months prior to relapse. More frequent sampling of patients may be needed, especially in the first two years of treatment, in order to detect relapse. However continuous BM monitoring is a traumatic experience for a child. The findings of this study indicate that measurement of an early treatment response represents a more reliable predictor of survival. In addition, assessment of a response to induction therapy provides the possibility for early treatment intervention (Nachman*, et al* 1998).

4.7 Limitations of the study

There are some limitations in this study which should be considered:

1. Prognostic features used to risk assign therapy namely, the day eight PB response to prednisone therapy and the genetic features of the leukaemic blasts did not reach statistical significance in this study. However, these findings were limited by the small number of patients studied on the ALL BFM 95 protocol (n=46).

2. This study was performed retrospectively and thus PCR and FC results were not available in all patients at all time points for the assessment of MRD. (In most of these instances the tests were not performed because of cost constraints or limited sample material). Direct comparison with the morphology findings was thus not possible. In addition, the panel of FC markers performed at each time point was not standardized. Studies comparing FC and PCR have indicated that the two methods generally yield concordant results (Neale*, et al* 1999). This finding could not be confirmed in this study.

4.8 CONCLUSION AND RECOMMENDATIONS

The results of this study suggest that children of different racial, gender and age groups with Precursor B cell ALL vary in their survival despite treatment intensification. This is important as these factors are not normally considered when risk assigning patients according to the current treatment protocol. WCC, previously predictive of outcome in South African studies, has been replaced by measurement of an early treatment response at day 15. Assessment by morphology offers a limited sensitivity because the presence of < 5% blasts accurately predicted survival in only 85.9% of patients tested on day 15. MRD techniques are required to improve the predictive value of the morphologic assessment. However analysis of MRD as detected by simplified PCR and FC assays did not significantly improve the assessment of conventional morphology. The ALL BFM 95 criteria namely; an early response to prednisone therapy and the genetic features of the leukaemic blasts which are used to risk assign patients, did not emerge as important variables in this study. However it must be emphasised that there were too few cases to assess prognostic significance.

In a low resource setting, the challenge is to design easy and cost effective methods in order to identify all patients at risk for relapse. In view of the results of this analysis, the following measures have been introduced in order to improve the current PCR assay at the NHLS:

In an attempt to standardise the detection of MRD, the primer sets designed by the European BIOMED-2 collaborative study group (van Dongen, *et al* 2003) were

implemented at the NHLS, Johannesburg in 2007. This *InVivo*Scribe Technologiesô Identiclone™ PCR IgH assay combines multiple consensus primers that target FR2, FR3 and framework region one (FR1) within the variable and conserved joining regions, as well as the diversity and joining regions and of the IgH chain locus (Figure 4.1).

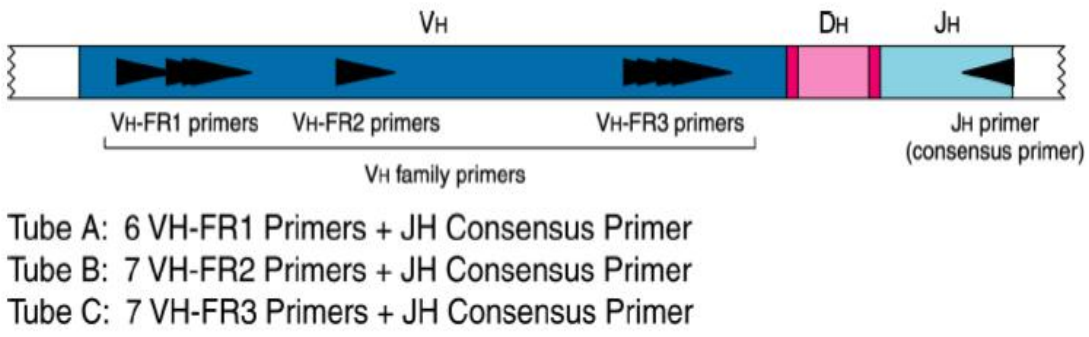


Figure 4.1

Immunoglobulin Heavy chain gene rearrangements

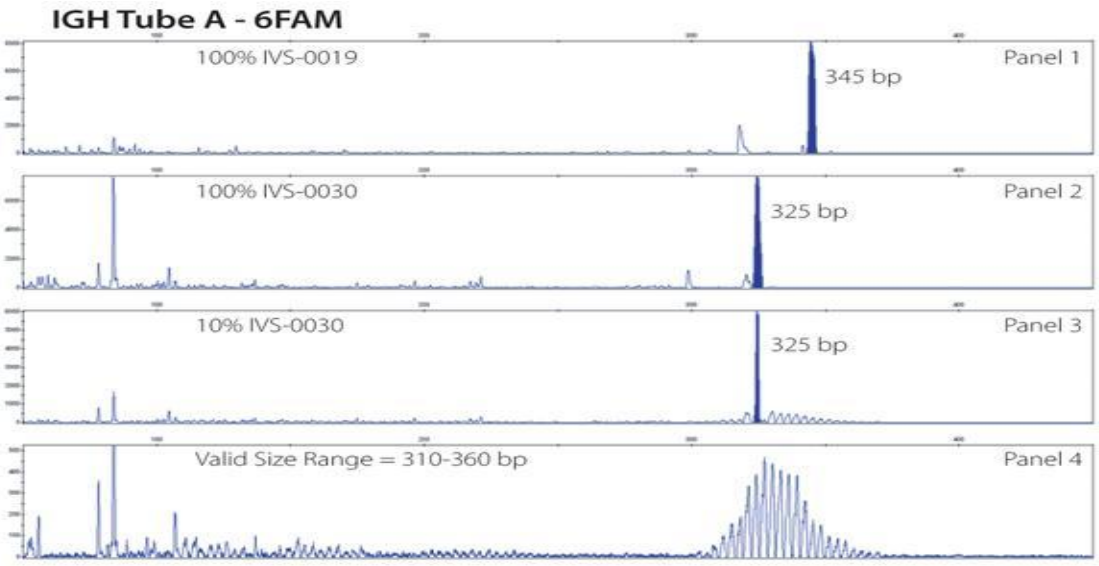
The arrows represent the positions of the primers that target the conserved FR1-3 of the variable (VH) and the conserved joining (JH) region (*InVivo*Scribe Technologiesô Identiclone™ PCR IgH package insert).

If no IgH rearrangement is detected, screening for other clonality markers including Ig kappa light chain (IgK), Ig lambda light chain (IgL), TCR beta (TCRB), TCR gamma (TCRG) and TCR delta (TCRD) genes can be performed. This will increase the percentage of patients covered to > 95%. This will also help to detect all sub clones at

diagnosis and to monitor patients with at least two targets to prevent false negatives during follow-up as a result of continuing secondary rearrangements (Beishuizen, *et al* 1994, Szczepanski, *et al* 2002, van Dongen, *et al* 1998).

The PCR products will be visualised by an alternative method to gel electrophoresis using capillary electrophoresis which detects differential fluorescence. In this way the amplicon products are differentially detected with primers conjugated with fluorescent dyes corresponding to different targeted regions (van Dongen, *et al* 2003).This method eliminates the use of carcinogens such as Ethidium bromide.

Differential detection allows for accurate, reproducible and objective interpretation. In addition, this can be used to provide a semi-quantitative assessment of tumour reduction (Figure 4.2). This is a more affordable alternative to sequencing the initial product and designing allele specific primers (Pongers-Willemse, *et al* 1999). In this way an increased level of MRD during early therapy and/or persistent MRD during the course of treatment will provide a more reliable predictor of relapse.



Key:

Panel 1: 100% alternative clonal control

Panel 2: 100% recommended clonal control

Panel 3: 10% dilution of recommended clonal control

Panel 4: polyclonal control

(*InVivo*Scribe TechnologiesôIdenticlone™ PCR IgH package insert)

Figure 4.2

Differential fluorescent detection of the amplicon products within framework region one

For future MRD detection at the NHLS, the decision to use either one method or the concurrent use of PCR and FC may be determined by cost. The cost of MRD follow-up with the above PCR assay will be increased approximately three fold.

Attempts have also been made to standardise the detection of MRD by FC. Aberrant immunophenotypic profiles have been previously described by the European BIOMED-1 collaborative study group (Lucio*, et al* 2001). This assay uses a combination of five triple

monoclonal antibody combinations, namely TdT/CD10/CD19; CD10/CD20/CD19; CD34/CD38/CD19; CD34/CD22/CD19 and CD19/CD34/CD45. The pattern of antigen expression allows for accurate discrimination of 98% of Precursor B-cell ALL cases at diagnosis which can be used for follow-up. On multi centre analysis aberrant expression was identified with at least two and often three of the triple monoclonal antibody combinations per case.

This profile does not routinely include cross lineage T cell (CD2 and CD7) or myeloid (CD13, CD33, CD15 and CD65) expression. According to the recommendations, these should only be performed in select cases where applicable. Implementation of this standardised FC profile could allow for more accurate assessment of MRD. This will also be of value at regenerative time points to distinguish leukaemic cells from reactive precursor cells without substantially increasing the cost of the assay.

Better predictors of outcome are needed to identify patients requiring aggressive therapy and to avoid over treating those patients likely to do well. Further development in the fields of molecular diagnostics and the detection of MRD are essential in order to refine therapy, in particular for patient subgroups (black race and male gender) with divergent results.

Appendix A

Toronto C high risk Treatment Protocol

Induction therapy (~4 wk)	Prednisone po q day Vincristine^ IVI q week Daunorubicin* IVI q week L-asparaginase IVI x 9 doses
CNS Treatment (~4 wk)	IT Methotrexate x 3 doses IT Ara-C x 1 dose CNS disease-Triple IT q week
Consolidation therapy **Phase II**	 Cyclophosphamide IVI x 2 doses Ara-C IVI x 4 doses q week 6-MP po q day IT Methotrexate q week
Phase III-CNS Intensification	6-MP po x 35 days High dose Methotrexate x 3 doses < 6 years: IT Methotrexate q week > 6 years: 18 Gy cranial irradiation
Phase IV Re-induction/Re-intensification **I** **II**	Dexamethasone po q day Vincristine IVI q week Adriamycin IVI q week L-asparaginase IMI x 4 doses Cyclophosphamide IVI x 1 dose Ara-C IVI x 4 doses q week x 2 6-Thioguanine po 14 days IT Methotrexate q week x 2
Maintenance therapy (3 months, to continue until 3 years from diagnosis)	Prednisone po x 5 days 4 weekly Vincristine IVI x 4weekly Methotrexate po weekly/ IVI 2weekly IT Methotrexate x 1 dose
*commenced on Day 0 of induction therapy Key: IT, intrathecal; IVI, intravenous;	 po, per os; CNS, central nervous system

Appendix B

Modified Berlin-Frankfurt-Munster 95 Medium Risk Treatment Protocol

Induction therapy (~4 wk)	Prednisone po q day Vincristine IVI q week Daunorubicin IVI q week L-asparaginase IVI x 9 doses
CNS Treatment (~4 wk)	IT Methotrexate x 3 doses IT Hydrocortisone x 3 doses IT Ara-C x 1 dose CNS disease-Triple IT q week
Consolidation therapy **Phase II**	Cyclophosphamide IVI x 2 doses Ara-C IVI x 4 doses q week 6-MP po q day IT Methotrexate q week IT Hydrocortisone q week
Phase III-CNS Intensification	6-MP po x 35 days High dose Methotrexate x 3 doses < 6 years: IT Methotrexate q week IT Hydrocortisone q week > 6 years: 18 Gy cranial irradiation
Phase IV Re-induction/Re-intensification **I**	Dexamethasone po q day Vincristine IVI q week Adriamycin IVI q week L-asparaginase IMI x 4 doses Cyclophosphamide IVI x 1 dose
II	Ara-C IVI x 4 doses q week x 2 6-Thioguanine po 14 days IT Methotrexate q week x 2 IT Hydrocortisone q week x 2
Maintenance therapy (3 months, to continue until 3 years from diagnosis)	Prednisone po x 5 days 4 weekly Vincristine IVI x 4weekly Methotrexate po weekly/ IVI 2weekly IT Methotrexate x 1 dose IT Hydrocortisone x 1 dose
Key: IT, intrathecal; IVI, intravenous;	po, per os; CNS, central nervous system

Appendix C: Clinical and laboratory characteristics of the 19 patients who relapsed in this study

Patient	Remission Day 33	Treatment Group	Age	Race	Gender	WCC ($x10^9$/l)	Genetics	Ploidy	EFS (months)
Patient 1	Induction failure	Toronto C	>10	white	male	<50	t(12;21)	diploid	0
Patient 2	Induction failure	Toronto C	<2	black	male	<50	Not done	diploid	0
Patient 3	Induction failure	Toronto C	<2	white	female	<50	Not done	diploid	0
Patient 4	Induction failure	Toronto C	>10	black	male	<50	t(12;21)	diploid	0
Patient 5	Induction failure	Toronto C	2-10	asian	male	>50	t(9;22)	diploid	0
Patient 6	Morphologic remission	Toronto C	2-10	black	male	<50	Not done	diploid	31.8
Patient 7	Morphologic remission	BFM 95-Medium	2-10	white	male	<50	Not detected	hyperdiploid	24.1
Patient 8	Morphologic remission	BFM 95-Medium	>10	black	male	<50	Not detected	diploid	11.5
Patient 9	Morphologic remission	BFM 95-Medium	2-10	white	male	<50	Not detected	diploid	50.1
Patient 10	Morphologic remission	BFM 95-Medium	2-10	black	male	<50	Not detected	diploid	7.7
Patient 11	Morphologic remission	BFM 95-High	2-10	black	male	<50	t(9;22)	diploid	37.4
Patient 12	Morphologic remission	BFM 95-Medium	<2	white	female	<50	Not detected	diploid	7.3
Patient 13	Morphologic remission	BFM 95-High	>10	black	male	>50	t(9;22)	diploid	14.4
Patient 14	Morphologic remission	BFM 95-Medium	2-10	black	male	<50	Not detected	hyperdiploid	5.7
Patient 15	Morphologic remission	BFM 95-Medium	<2	white	male	<50	Not detected	diploid	20.2
Patient 16	Morphologic remission	BFM 95-Medium	2-10	black	male	<50	Not detected	diploid	7.7
Patient 17	Morphologic remission	BFM 95-Medium	<2	mixed	male	<50	Not detected	diploid	36.0
Patient 18	Morphologic remission	Toronto C	2-10	black	male	<50	Not done	hyperdiploid	83.5
Patient 19	Morphologic remission	BFM 95-Medium	2-10	black	male	No result	Not done	diploid	6.0

Appendix D

Protocol

The clinical significance of current laboratory and prognostic indicators in the management of South African children with Precursor B cell Acute Lymphoblastic Leukaemia

Introduction

Approximately 15 new paediatric cases of Acute Lymphoblastic Leukaemia (ALL) are diagnosed and treated annually at Johannesburg General Hospital. ALL is the most common childhood cancer(Pui, *et al* 1995). ALL is sub-typed (immunophenotypically) into B-cell ALL, Precursor B cell ALL and T-cell ALL. Precursor B cell ALL is the most common childhood subtype, which accounts for ~84% of cases (Camitta BM 1997). With advances in diagnosis and management, children diagnosed with Precursor B cell ALL achieve a five year event free survival (EFS) of ~80% in large clinical trials (Pui and Evans 1998, Silverman, *et al* 2001).

Improvements in childhood ALL survival can be attributed to the identification of various risk factors which can predict outcomes (Kersey 1997). Current treatment protocols use clinical and laboratory prognostic features at diagnosis to help identify patients who need additional and more aggressive therapy. In this way only high risk cases are treated intensively, with less toxic therapy reserved for low risk cases (Friedmann and Weinstein 2000).

At Johannesburg Hospital, the Paediatric Oncology Unit have adopted treatment protocols from large co-operative trials. From January 1997 to November 2001, Precursor B cell ALL cases were treated according to the Toronto C protocol. Subsequently in December 2001, the modified ALL Berlin-Frankfurt-Munster (BFM) 95 protocol was introduced and continues to be used to date.

The modified BFM 95 protocol classifies patients into standard, medium and high risk groups on the basis of (Schrappe, *et al* 2000):

1. The genetic features of the leukaemic blast cells (namely the presence of t (9; 22)/ BCR-ABL rearrangement, or a t (4; 11)/MLL-AF4 rearrangement, associated with a poor prognosis).
2. An early response to remission-induction treatment as determined by the day eight prednisone response and the achievement of morphologic remission (defined as < 5% blasts) on day 33 of induction treatment.

Traditionally, studies have identified over 50 clinical and laboratory markers which correlate significantly with prognosis (Donadieu*, et al* 1998). The following have been most consistently associated with a favourable outcome: a low white cell count (WCC) count of < 50 x 10^{9}/l, a presenting age of 1-10 years, female gender, white and Asian race groups and hyperdiploidy (DNA index >1.16) (Pui*, et al* 1990, Smith*, et al* 1996). However the prognostic significance of these features has been abolished with improvements in therapy. More recently, treatment protocols such as BFM 95 are guided by molecular genetic findings (Pui 2000, Rubnitz*, et al* 1997)and remission status (Campana and Pui 1995). These have been shown to predict more accurately the outcome rather than presenting clinical features (Pui*, et al* 2004).

Seventy five percent of childhood ALL cases have specific genetic abnormalities with therapeutic and prognostic significance at presentation (Pui*, et al* 1990).

The effectiveness of therapy and impending relapse in patients is determined by bone marrow morphology at specific time points during treatment. Patients with < 5% lymphoblasts on bone marrow morphologic analysis are said to be in morphologic remission. However estimates by conventional morphology have limited sensitivity and accuracy, as even in morphologic remission patients may still have as many as 10^{10} leukaemic cells in the marrow. This is responsible for relapse in 15-20% of cases (Campana and Pui 1995).

More sensitive and specific methods were developed to assess early treatment response. Such methods measure minimal residual disease (MRD), which is defined

as the lowest level of detectable disease during morphologic remission. MRD detection in ALL patients is most frequently performed by multiparameter flow cytometry of aberrant immunophenotype (Coustan-Smith, *et al* 2000) or polymerase chain reaction (PCR) (Cave, *et al* 1998) since either method is reported to achieve a sensitivity of 0.01%.

Flow cytometry analysis is used to type leukaemic cells by defining their cell surface antigen (Ag) expression. Abnormal Ag expression on ALL blasts and abnormal pattern of expression distinguish leukaemic cells from normal lymphoid cells.

MRD detection by PCR targets clonal immunoglobin (IgH) and T cell receptor (TCR) gene rearrangements and fusion genes. While only 30-40% of ALL patients have specific chromosomal aberrations with well-defined breakpoint fusion regions, virtually all patients (> 95%) have rearranged IgH genes.

The process of IgH and TCR rearrangement gives rise to a unique rearrangement in each B and T cell respectively during normal lymphocyte development. Rearrangement of IgH involves recombination of one of approximately 200 germline variable (V) segments, 30 diversity (D) segments, and 6 joining (J) segments. Further variability is generated at the V-D and D-J junctions by base removal (by exonucleases) and random addition of õNö nucleotides (by TdT). This process generates a hypervariable sequence known as the complementarity-determining region III (CDRIII) of the IgH, which characterizes a given B-cell or B-cell clone.

This sequence can be amplified by PCR using consensus primers to conserved areas of the 5õV region and 3õJ region which flank the CDR-III region. This represents a clonal marker specific for the patientõs disease. In a research setting a patient specific primer is designed from this unique sequence for follow up MRD assessment (Szczepanski, *et al* 2002).

Patients who achieve molecular or immunophenotypic remission (< 10^{-4} leukaemic BM cells) after induction therapy have a significantly more favourable prognosis (Cave*, et al* 1998). MRD detection has thus been incorporated into many current risk classification systems [18].

In our unit, complementary use of PCR (of IgH gene rearrangements) and limited flow cytometry is performed with every bone marrow examination at specific time points during therapy. PCR follow-up is however only performed if an IgH rearrangement can be amplified at diagnosis using FR3/FR2 primers. This was initiated in 1996 on the basis of available technology and cost considerations. The use of multiple approaches is desirable so as to increase the number of patients studied and to minimize the limitations of individual methods (Pui and Campana 2000).

The detection of MRD is currently not used to guide therapy in our unit as the clinical significance of MRD and its predictive value in relation to other prognostic features of ALL has never been formerly assessed.

In a low resource setting such as South Africa, the monitoring tests employed to detect MRD, have been modified as a result of technical and financial constraints. The sensitivity of MRD detection by flow cytometry in our unit is approximately 1%, as only 10 000 events are counted per sample (FACS CALIBUR BD) and limited panels of markers are used for follow up analysis.

The IgH rearrangements are currently being detected via a qualitative nested PCR assay using 4 consensus primers. This allows detection of 65-75% of monoclonal populations (Reed*, et al* 1993). Gel electrophoresis is being used to resolve the different sized amplicon products. However, an alternative method using differential fluorescence detection with primers conjugated with fluorescent dyes corresponding to different targeted regions will be available in early 2007.

It is thus important to assess whether these modified tests are sensitive and specific enough to detect relapse in a disease with such a high survival rate.

Study objectives

1. To characterise and assess the prognostic value of clinical (gender, age, race and treatment protocol) & laboratory (white cell count, ploidy, FISH and cytogenetic findings) risk factors in our patient population diagnosed with Precursor B cell ALL

2. To assess whether treatment response as measured by an early response to therapy and the detection of MRD effectively predicts relapse and/or determines outcome in our patient population diagnosed with Precursor B cell ALL. An early response to remission-induction treatment will be determined by the day eight prednisone response and the achievement of morphologic remission (defined as < 5% blasts) on day 15 and 33 of induction treatment. The presence of MRD will be measured by simplified PCR for IgH gene rearrangements and Flow Cytometry at sequential time points during therapy, namely at presentation, at day 15 of induction therapy, at day 33 of induction therapy, prior to commencing maintenance therapy and at completion of therapy.

Methodology

This is a retrospective record review of the presenting features and clinical outcome of therapy of all (a minimum of 100 cases) of Precursor B cell ALL diagnosed and completing treatment at the Johannesburg Hospital Paediatric Oncology Unit (Ward 294) from 1997 to date.

The following data will be collected from patient clinic files and/ or the NHLS computer database:

1. Patient demographics (Age, gender, race)

2. B-cell ALL subtypes (null, precursor, mature B-cell ALL)
3. Treatment protocol
4. Survival data
5. White Cell Count at presentation
6. FISH for Ph chromosome/ t(9;22), TEL-AML1/ t(12,21), and MLL-AF4/ t(4,11) and Cytogenetic findings at disease presentation
7. Day eight peripheral blood count/prednisone response
8. PCR for IgH gene rearrangement and Flow Cytometry results at diagnosis and specific time points during treatment

This information will be useful in assessing:

1. The clinical use of PCR for IgH gene rearrangement and Flow Cytometry at specific time points during treatment to detect the presence of minimal residual disease.
2. The predictive value of an early response to remission-induction treatment.
3. Clinical and laboratory prognostic factors predictive of outcome.

This is a retrospective study, and the potential biases include lack of randomisation and small sample size. Complete data sets on all patients to be studied are readily available from patient clinic files and/ or the NHLS computer database. However specific tests may not have been performed on a subgroup of patients because of cost constraints or limited sample material. In addition follow-up of MRD at all time points during therapy will not be possible in patients who have not completed therapy at the time of closure of this study.

Data analysis

Data will be recorded in EXCEL. Data will be presented as numbers (frequency).Data will be analysed in Stata 9.2 software. Survival rates will be estimated by the Kaplan-Meier method. The log-rank test will be used to assess differences in univariate analysis. A multiple regression analysis will be performed using the Cox proportional hazards

model to evaluate the independent effect of variables that show a significant influence on survival rates in the univariate analysis.

Ethics

Ethics approval was obtained on 06/07/01 (Protocol number MO60632). Data will be entered from January 1997 to date. Ethical clearance for data collected and analysed between 1997 and 2000 was obtained on 05/05/30 (Protocol number M050523). This research project will incorporate this existing database.

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Appendix E

Data Collection Sheet

Sheet 1 (to be kept confidential)

1. Hospital number
2. Patient name and surname
3. Study number (combination of the hospital number & patient initials)

Sheet 2 (for data collection and analysis)

1. Study number (that can be linked to patient on Sheet 1 if necessary)
2. Demographic data namely:
    a. Date of birth
    b. Gender
    c. Ethnic group
3. Date of presentation / diagnosis
4. T or B-cell ALL and B-cell subtypes (Null, Common, Mature B)
5. Treatment protocol
6. Survival data to determine event free survival
    a. Date of remission according to bone marrow morphology
    b. Date of relapse
    c. Date last examined (assesses cases lost to follow up)
    d. Date and cause of death (namely, disease related or non-disease related e.g. sepsis, haemorrhage, chemotherapy related etc.)
7. PCR (for IgH and TCR gene rearrangements) results at diagnosis & during treatment (including the date of each examination)
8. Flow cytometry (to assess percentage of clonally restricted CD19/CD10 cells, cell size and DNA index) results at diagnosis & during treatment (including the date of each examination)
9. Cytogenetic and FISH findings at presentation if applicable

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