The Clinical Role of Ancillary Investigations in Staging Diffuse Large B-cell Lymphoma

Dipti Talaulikar

A thesis submitted for the degree of
Doctor of Philosophy
of the
Australian National University

January 2009

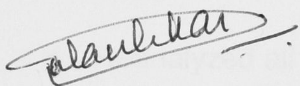
Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

Albert Einstein

Declaration:

I declare that:

- This thesis represents my original work and contains no material written or published by another person except where due reference is made.
- The thesis is a compilation of peer-reviewed papers which have been published, are in press, or have been submitted for publication during the tenure of my PhD.
- The material presented in this thesis has not been submitted for any other degree.



Dipti Talaulikar

January 2009

Contribution of author, Dipti Talaulikar:

Each study presented in this thesis was designed and managed by Dipti Talaulikar. In particular, Dipti undertook the following work during her candidature:

- Designed each study, prepared all ethics and grants applications, and set up each database
- Retrieved all cases from medical and laboratory records, and all archived slides and paraffin-embedded trephine blocks
- Performed all the data extraction and data entry, and analyzed and computed all clinical data
- Reanalyzed all histology slides
- Re-interpreted all flow cytometry data
- Cut sections, labeled and stained slides for immunohistochemistry with help
- Reported and computed immunohistochemistry slides
- Cut sections from paraffin-embedded trephine biopsy blocks for molecular analysis with help
- Performed all DNA extractions
- Developed all molecular methods under supervision
- Performed all molecular analysis with technical guidance as required
- Performed descriptive statistical analysis and attended analytical analyses performed by Dr. Bruce Shadbolt
- Wrote first draft of all papers

Contribution of other authors:

Jane Dahlstrom provided overall supervision of the project. She helped in formulating the reporting system for immunohistochemistry and trained me in performing all immunohistochemical analysis. She was one of the two blinded immunohistochemistry reviewers and also reviewed all papers and presentations.

Bruce Shadbolt helped in setting up the database and trained me in statistical analysis. All statistical analyses were performed under his supervision, and he reviewed relevant papers and presentations.

Anne McDonald was the second blinded reviewer for routine histology for most cases whereas Michael Pidcock was the second reviewer for some of the cases used in the pilot study. Anne McDonald helped review relevant papers and presentations.

Michelle McNiven trained me in molecular techniques, and helped me in developing the molecular methods. She was technical advisor on all molecular studies and reviewed relevant papers and presentations.

Amy Broomfield helped train me in immunohistochemistry processing such as cutting blocks. She helped with the cutting of sections and staining immunohistochemistry slides.

Jill Bell and Kowsar Khan helped train me in flow cytometry techniques and also helped in reanalyzing the flow cytometry data for interpretation.

James Gray was not involved directly in any research but owing to his background in molecular biology, presented some of the findings at a local conference on behalf of the other authors and reviewed one paper.

Intellectual planning: DT (90%), JED: (5%) BS: (5%)

Experimental work: DT (95%) JED: (2%) AMcD: (1%) JB and KK: (1%), AB: (1%)

Analyses: DT (70%) BS (30%)

Writing: DT (97%) JED (2%) BS (0.5%) MM (0.5%)

The contribution of all authors and of acknowledged persons have been individually listed after each published paper under the title "author contributions".

Acknowledgements:

I would like to thank my supervisor, Professor Jane Dahlstrom for her unwavering support and encouragement, her scientific guidance and her patience during the tenure of my PhD. I could not have asked for a better mentor.

I have received enormous support from Dr. Bruce Shadbolt who has always being there at a moment's notice. I am particularly grateful for his honest feedback at all times and his patience during the numerous statistical analysis sessions.

I am deeply grateful to Dr. Anne McDonald, for doing the hard yards and being the second blinded reviewer for all histology slides. Her common sense approach while reviewing some of my papers has helped me maintain my focus. Her timely help in all matters relating to my research is deeply appreciated.

My sincere thanks to Michelle McNiven, chief scientist at the Molecular Pathology laboratory, ACT Pathology for her scientific and technical help with all the molecular pathology work. She as well as Jennifer Ridgway, Anita Campbell and Tracey Cowey, trained me in molecular pathology laboratory techniques and were always there to guide me in laboratory work. I am grateful for Amy Broomfield's help with cutting sections and staining for immunohistochemical analysis. The histopathology laboratory has been unfailingly supportive of my

research. I thank Jill Bell and Kowsar Khan for the reanalysis of raw flow cytometry data for the project and also for training me to perform the analysis.

I would also like to thank Professor Frank Bowden for encouraging me to take up the PhD and for his support during this period. The support of the former Dean of the Australian National University Medical School, Professor Paul Gatenby has been invaluable. The advice from Professor Helen O'Neill at various stages during my research has helped me tremendously.

There are a number of other people and departments I would like to thank. Gloria Spyropoulos from the medical records department was always there to help me create and re-create the patient lists. Phillip Baron from the Haematology department, ACT Pathology taught me how to perform data extraction from laboratory records. Dr. Elango Subromania, advanced trainee in haematology, determined the trephine length in a large proportion of cases and presented the findings at a local conference.

The research presented in this thesis would not have been possible without the support of funding bodies. I would like to thank the Private Practice Trust Fund at the Canberra Hospital for providing me the initial funding for the project. I also received a small equipment grant from the Leukaemia Foundation.

All the laboratory work for this research was performed at ACT Pathology and I would like to thank the Director of ACT Pathology, Professor Julia Potter for supporting my research.

I would like to thank the Haematology laboratory, not only for their moral support but also for their friendship, for being there to listen and care during some of the difficult times. In particular, I would like to thank Kerrie Andriolo, Jill Bell, Jovanco Naumovski and Jackie Pratt.

None of this would be possible without my family. The quiet support and encouragement I have received from my husband during all my highs and lows has been my anchor. My little daughter, Sakshi has been amazingly understanding of “mamma’s research” including the stress levels, the late nights and odd hours, and missed fun times.

Lastly, I would like to dedicate this research to patients with Diffuse Large B-cell Lymphoma and other sub-types of Non-Hodgkin Lymphoma.

Abstract:

Advanced stage disease in Diffuse Large B-cell Lymphoma (DLBCL) confers a poor prognosis. Staging investigations performed at diagnosis include histological examination of bone marrow biopsy.

This study addresses the impact of the use of flow cytometry, immunohistochemistry and molecular studies using immunoglobulin heavy chain (IgH) and light chain (IgL) gene rearrangements, on overall survival and on the International Prognostic Index (IPI).

Bone marrow trephines from 156 DLBCL patients were assessed on routine histology, then by immunohistochemistry using T-cell, B-cell and light chain markers. Raw flow cytometry data were reanalysed and reinterpreted blindly. DNA was extracted from formalin-fixed trephine biopsy specimens and IgH and IgL gene rearrangement analysis performed on all cases using the InVivo Scribe kit based on the BIOMED2 protocols. Clinical data was obtained from medical and laboratory records. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 14.0.

The median age of the cohort was 62 years (range 20-86 years). Nine patients treated with palliative intent were excluded from all survival analyses. Others were treated with anthracycline based chemotherapy; 36 patients received concomitant Rituximab. Median overall survival was 6.1 years (95% CI: 3.8, 8.4).

Histological involvement of the bone marrow by lymphoma was noted in 30 cases (19.2 %). Flow cytometry and immunohistochemistry detected an additional 17 cases (10.9%) each with 4 cases detected by both methods. Thirty (19.2 %) patients were upstaged to stage IV (stage I: 6, stage II: 12, stage III: 12). Kaplan Meier curves demonstrated that positivity on flow cytometry and immunohistochemistry resulted in a significantly worse overall survival and progression free survival compared to negative cases. Cox proportional hazards models showed that flow cytometry added significant independent prognostic value over histology in predicting survival [Histology: 2.1, 95% CI 1.0, 4.3, p=0.05; Flow cytometry: 2.0, 95% CI 1.0, 3.8, p=0.04] while immunohistochemistry showed co linearity with histology in prediction of survival but added significantly more than histology alone [Histology: 1.3, 95% CI 0.5, 3.0, p=0.6; immunohistochemistry: 2.3, 95% CI 1.1, 5.0, p = 0.03]. Flow cytometry and immunohistochemistry together were a stronger predictor of survival over and above histological diagnosis [Histology: 1.8, 95% CI 0.8, 3.7, p =0.1; Flow cytometry/immunohistochemistry: 2.7, 95% CI 1.2, 6.2, p=0.02].

Feasibility studies prior to molecular testing showed lower quality of DNA was obtained from formalin-fixed decalcified paraffin-embedded trephines compared to fresh-frozen samples. DNA amplification was obtained from 133/155 (84.7%) formalin-fixed trephine blocks with progressively reduced amplification at higher base pair length. Forty one cases were positive on IgH (n=19) and IgL (n=34) gene analysis with 12 cases positive on both. To establish tumour origin, molecular tests were performed on 17 available primary formalin-fixed tissue blocks and showed comparable clonal bands in 10 cases. Two of these were positive on routine histology. Thus, using stringent criteria to account for false

positivity, routine molecular staging on formalin-fixed trephine biopsy tissue upstaged 8 (5.1%) cases. Molecular staging did not affect overall survival in our study.

A change in the IPI was noted in 18 cases (11.5%) on immunophenotyping and 22 (14.1%) cases on immunophenotyping + molecular testing. Revised IPI models using immunophenotyping alone (rIPI1), and immunophenotyping with molecular studies (rIPI2) were compared to baseline IPI in a Cox regression model. Results showed that rIPI1 provides the best differentiation between the IPI categories.

In conclusion, this research demonstrates that the routine use of ancillary investigations improves detection of BM involvement in DLBCL and upstages ~20% of patients. Immunophenotyping (flow cytometry + immunohistochemistry) has additional benefit over routine histology in predicting survival and improves the predictive value of the IPI. These results suggest that current guidelines should incorporate immunophenotyping in routine staging and recommend the use of a new and more inclusive definition of bone marrow involvement within the IPI.

Research outcomes:

This thesis was planned prospectively, with permission, as a series of publications. These are listed below, with the experiments they relate to and the chapters they are included in (ref: section 1.5).

Publication	Experiment	Chapter
Talaulikar D, Dahlstrom JE, Broomfield A, McNiven M, Shadbolt B. **"Occult marrow involvement in Diffuse Large B-cell Lymphoma: results of a pilot study."** Pathology. 2007 Dec; 39(6):580-5.	I	3.1
Talaulikar D, Shadbolt B, Bell J, Khan K, and Dahlstrom JE: **"Clinical role of flow cytometry in redefining bone marrow involvement in Diffuse Large B-cell Lymphoma – a new perspective."** Histopathology 2008; 52: 340-347.	II	4.1
Talaulikar D, Dahlstrom JE, Shadbolt B, Broomfield A, McDonald A. **"Role of Immunohistochemistry in staging diffuse large B-cell lymphoma"**. Journal of Histochemistry and Cytochemistry, 2008 Oct;56(10):893-900. Epub 2008 Jun 23.	III	5.1
Talaulikar D, Dahlstrom JE, Shadbolt B, McDonald A. **"Clinical Implications of Immunophenotyping in Staging Diffuse Large B-Cell Lymphoma"**. Blood, (ASH annual meeting abstracts) 2008; 112: Abstract 5279.	III	5.2
Talaulikar D, Gray JX, Shadbolt B, McNiven M, Dahlstrom JE: **"A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsies using two different methods."** J Clin Pathol. 2008 Jan; 61(1):119-23. Epub 2007 Jun 1.	IV	6.1
Talaulikar D, Shadbolt B, McNiven M, Dahlstrom JE: **"DNA amplification from formalin-fixed decalcified paraffin-embedded bone marrow trephine specimens – does the duration of storage matter"?** Pathology, 2008 Dec; 40(7): 702-706.	IV	6.2
Talaulikar D, Shadbolt B, Dahlstrom JE, McDonald A. "**Routine use of ancillary investigations in staging Diffuse Large B-cell Lymphoma improves the International Prognostic Index (IPI)"** Submitted for publication.	V	7.1
Talaulikar D, and Dahlstrom JE. "**Staging bone marrow in Diffuse Large B-cell Lymphoma: the role of ancillary investigations**". Pathology 2009; in press.	-	8.1

Presentations:

Talaulikar D, Dahlstrom JE, Broomfield A, McNiven M, Prosser IW, Shadbolt B. Occult marrow involvement in Diffuse Large B-cell Lymphoma: incidence and prognostic value. Oral presentation at the RCPA Pathology Update, Sydney, Australia, 2006. *Won David S Nelson prize for best oral presentation*

Talaulikar D, McNiven M, Prosser IW, Dahlstrom JE. Efficiency of extraction and amplification of DNA from formalin-fixed paraffin-embedded archival bone marrow trephine specimens. Poster presentation at the 11th Congress of Human Genetics, Brisbane, Australia, 2006

Talaulikar D, Dahlstrom JE, Brun M, Broomfield A. Comparison of flow cytometry and immunohistochemistry in detection of occult marrow disease in Diffuse large B-cell Lymphoma. Poster presentation at the Australian division, International academy of Pathologists (IAP) Conference, Sydney, Australia, June 2006

Talaulikar D, Gray J, McNiven M, Shadbolt B, Dahlstrom J; Molecular Staging of Diffuse Large B-cell Lymphoma (DLBCL) is Best Performed with Freshly Frozen Marrow Specimens. Oral presentation at the annual meeting of the Haematology Society of Australia and New Zealand, Hobart, Australia, 2006

Talaulikar D, Shadbolt B, Bell J, Khan K, and Dahlstrom JE: Clinical role of flow cytometry in redefining bone marrow involvement in Diffuse Large B-cell Lymphoma – a new perspective. Poster at European Congress of Pathology, Turkey, 2007 and at the RCPA Pathology Update, Sydney, Australia, 2008

Talaulikar D, Dahlstrom JE, Shadbolt B, Broomfield A, McDonald A. "Role of Immunohistochemistry in staging diffuse large B-cell lymphoma" poster presentation at annual meeting of International Society of Laboratory Haematology, 2008 and Canberra Region Annual Scientific Meeting (CRASM), Canberra, Australia, 2008. *Won best clinical poster prize at CRASM, 2008*

Talaulikar D, Choudhury A, Shadbolt B, Brown M: "Lymphocytopenia as a Prognostic Marker for Diffuse Large B Cell Lymphoma". Oral presentation at the Canberra Region Annual Scientific Meeting, Canberra, Australia, 2008

Invited talks:

Immunohistochemistry and Flow cytometry in Lymphomas. Histotechnology group of New South Wales (NSW) Annual Meeting, Canberra, Australia, 2007

Staging in Diffuse Large B-cell Lymphoma. Cancer Biology Forum, Australian National University, Canberra, Australia, November 2007

Staging in Diffuse Large B-cell Lymphoma. 13th World Congress on Advances in Oncology and 11th International Symposium on Molecular Medicine, Greece, October 2008

Additional Peer-reviewed Publications:

Talaulikar D, Choudhury A, Shadbolt B, Brown M: "Lymphocytopenia as a Prognostic Marker for Diffuse Large B Cell Lymphoma". Leukaemia and Lymphoma, May 2008; 49(5): 959-964

TABLE OF CONTENTS:

DECLARATION..........i
PREFACE..........ii
ACKNOWLEDGEMENTS..........v
ABSTRACT..........viii
RESEARCH OUTCOMES..........xi

TABLE OF CONTENTS..........1

LIST OF TABLES AND FIGURES:..........4

ABBREVIATIONS:..........9

CHAPTER I: CONTEXT AND PROGRAM OF RESEARCH..........12

1.1 INTRODUCTION:..........13
1.2. HYPOTHESIS:..........16
1.3. AIMS:..........16
1.4. EXPERIMENTS:..........17
1.4.1. Experiment I: Pilot study..........17
1.4.2. Experiment II: Clinical role of flow cytometry in staging DLBCL..........17
1.4.3. Experiment III: Clinical role of immunohistochemistry in staging DLBCL..........18
1.4.4. Experiment IV: Clinical role of molecular staging in DLBCL..........19
1.4.5. Experiment V: Impact of ancillary staging investigations on the IPI..........19
1.5. PROGRAM OF RESEARCH:..........20

CHAPTER II:LITERATURE REVIEW..........22

2.1. INTRODUCTION:..........23
2.2. PATHOLOGIC CLASSIFICATIONS:..........24
2.3. HISTOLOGY:..........26
2.3.1 Macroscopy:..........26
2.3.2. Microscopy:..........26
2.3.3. Morphologic variants:..........27
2.3.4. Other variants:..........29
2.3.4.1. Mediastinal large B-cell lymphoma:..........29
2.3.4.2. Plasmablastic:..........29
2.3.4.3. DLBCL expression of full-length ALK:..........31
2.4. IMMUNOPHENOTYPE:..........32
2.5. GENETICS:..........33
2.5.1. BCL6:..........34
2.5.2. BCL2:..........35
2.5.3. cMYC:..........35
2.5.4. Cytogenetic abnormalities:..........35
2.5.5. Somatic hypermutations:..........36
2.6. CELLULAR DERIVATION:..........37
2.7. MOLECULAR PATHOGENESIS OF DLBCL:..........38
2.8. EPIDEMIOLOGY OF DLBCL:..........39

2.9. CLINICAL FEATURES: 41
2.9.1. Initial presentation: 41
2.9.2. Sites of involvement: 41
2.10. INVESTIGATIONS: 42
2.10.1. Diagnosis: 42
2.10.2. Staging: 43
2.10.2.1. Histological examination of the bone marrow: 45
2.10.2.2. Flow cytometric analysis of BM samples: 49
2.10.2.3. Immunohistochemical analysis of the BM: 54
2.10.2.4. Molecular staging of BM using IgL and IgL analysis: 56
2.11. PROGNOSTIC SYSTEMS: 61
2.11.1. International Prognostic Index (IPI): 61
2.11.2. Cell regulatory proteins: 63
2.11.2.1. p53: 63
2.11.2.2. Ki67: 63
2.11.3. Apoptotic proteins: 64
2.11.3.1. BCL2: 64
2.11.4. B-cell ontogenic proteins: 64
2.11.4.1. BCL6: 64
2.11.4.2. Others: 64
2.11.5. Morphologic variants: 64
2.11.6. Gene expression profile: 65
2.12. TREATMENT: 70

CHAPTER III:PILOT STUDY 93

3.1 OCCULT BONE MARROW INVOLVEMENT IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA: RESULTS OF A PILOT STUDY. PATHOLOGY 2007, 39(6):580-585 94

CHAPTER IV:CLINICAL ROLE OF FLOW CYTOMETRY 104

4.1 CLINICAL ROLE OF FLOW CYTOMETRY IN REDEFINING BONE MARROW INVOLVEMENT IN DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) – A NEW PERSPECTIVE. HISTOPATHOLOGY 2008, 52; 340-347 105

CHAPTER V:CLINICAL ROLE OF IMMUNOHISTOCHEMISTRY 116

5.1 ROLE OF IMMUNOHISTOCHEMISTRY IN STAGING DIFFUSE LARGE B-CELL LYMPHOMA. J HISTOCHEM CYTOCHEM 2008, 56(10):893-900 117

5.2. CLINICAL IMPLICATIONS OF IMMUNOPHENOTYPING IN STAGING DIFFUSE LARGE B-CELL LYMPHOMA. BLOOD, (ASH ANNUAL MEETING ABSTRACTS) 2008; 112: ABSTRACT 5279. 128

CHAPTER VI:PRELIMINARY MOLECULAR STUDIES 129

6.1: A COMPARATIVE STUDY OF THE QUALITY OF DNA OBTAINED FROM FRESH FROZEN AND FORMALIN-FIXED DECALCIFIED PARAFFIN-EMBEDDED BONE MARROW TREPHINE BIOPSIES USING TWO DIFFERENT METHODS. J CLIN PATHOL. 2008 JAN; 61(1):119-23. EPUB 2007 JUN 1. 131

6.2. TALAULIKAR D, SHADBOLT B, MCNIVEN M, DAHLSTROM JE: DNA AMPLIFICATION FROM FORMALIN-FIXED DECALCIFIED PARAFFIN-EMBEDDED BONE MARROW TREPHINE SPECIMENS – DOES THE DURATION OF STORAGE MATTER? PATHOLOGY, 2008 DEC; 40(7): 702-706. 138

CHAPTER VII:SUMMATION OF RESULTS & IMPACT ON INTERNATIONAL PROGNOSTIC INDEX (IPI) **146**

7.1 ROUTINE USE OF ANCILLARY INVESTIGATIONS IN STAGING DIFFUSE LARGE B-CELL LYMPHOMA MAY IMPROVE THE INTERNATIONAL PROGNOSTIC INDEX (IPI). SUBMITTED FOR PUBLICATION 147

CHAPTER VIII:RESEARCH PLACED IN CONTEXT **178**

8.1 STAGING BONE MARROW IN DIFFUSE LARGE B-CELL LYMPHOMA: THE ROLE OF ANCILLARY INVESTIGATIONS. PATHOLOGY 2009; IN PRESS. 179

CHAPTER IX:CONCLUSIONS AND FUTURE DIRECTIONS **220**

9.1 CONCLUSIONS: 221
9.2. STAGING INVESTIGATIONS: 223
9.2.1. Histology: 223
9.2.2. Flow cytometry: 224
9.2.3. Immunohistochemistry: 225
9.2.4. Gene rearrangement studies: 226
9.3. FUTURE DIRECTIONS: 228

APPENDIX: **230**

APPENDIX I: CLINICAL INFORMATION 231
APPENDIX II: CHESON'S STANDARDISED CRITERIA FOR HISTOLOGY 234
APPENDIX III: PROFORMA FOR REPORTING HISTOLOGY ON BONE MARROW BIOPSY 235
APPENDIX IV: PROFORMA FOR REPORTING IMMUNOHISTOCHEMISTRY 236
APPENDIX V: PICTURES OF CONTROL SLIDES FOR IMMUNOHISTOCHEMISTRY 237
APPENDIX VI: EXAMPLE OF KAPPA GENE REARRANGEMENT ANALYSIS: 238
APPENDIX VII: ADDITIONAL RESEARCH OUTCOMES: 239
APPENDIX VIII: TABULATION OF RESULTS 246

ADDENDUM: 250

LIST OF TABLES AND FIGURES:

TABLES:

Table 1.1: Publications arising from period of research included in the thesis 21
Table 2.1: World health organization classification of lymphoid neoplasms 25
Table 2.2: Major recurring genetic events in diffuse large B-cell lymphoma 33
Table 2.3: Occupational, medical and lifestyle risk factors associated with prognosis in lymphoma 40
Table 2.4: Summary of studies on bone marrow histology in diffuse aggressive B-cell lymphoma 48
Table 2.5: Summary of comparative studies on occult marrow involvement using flow cytometry 51
Table 2.6: Summary of comparative studies on discordance between bone marrow histology and immunohistochemistry in lymphoma 55
Table 2.7: Summary of comparative studies on PCR positivity on bone marrow in NHL 60
Table 2.8: Comparison of gene expression platforms used in diffuse large B-cell lymphoma studies 66
Table 4.1: Aspirate, trephine and flow cytometry results 110
Table 5.1.1: IHC results on individual T-cell and B-cell antibodies 121
Table 5.1.2: Cox regression model showing the effect of different definitions of bone marrow involvement on survival after adjusting for age and performance status 122
Table 6.1.1: Target genes and primers used to assess the quality of DNA in the biomed-2 protocol 133
Table 6.1.2: Standard biomed-2 PCR protocol and gel electrophoresis 134
Table 6.2.1: Target genes and primers used to assess the quality of DNA in the biomed-2 .protocol 141
Table 6.2.2: Standardized biomed-2 PCR protocol and gel electrophoresis 141
Table 7.1: Results on immunoglobulin heavy chain (IgH) and light chain (IgL) gene rearrangement studies 162
Table 7.2: Summary table showing the hazard ratios from cox regression analyses for the three IPI models, baseline IPI, rIPI1 and rIPI2 164
Table 8.1: Summary of studies on bone marrow histology in diffuse large B-cell lymphoma 187

Table 8.2: Summary of comparative studies on occult marrow involvement using flow cytometry and impact on outcome measures **193**

Table 8.3: Summary of comparative studies on discordance between bone marrow histology and immunohistochemistry in NHL **197**

Table 8.4: Summary of comparative studies on PCR positivity on bone marrow samples in NHL **203**

FIGURES:

Figure 1.1:	Overall survival based on IPI prognostic categories	**14**
Figure 2.1A:	Macroscopic appearances in diffuse large B-cell lymphoma	**26**
Figure 2.1B:	Microscopic appearances in diffuse large B-cell lymphoma	**26**
Figure 2.2:	Morphologic variants of DLBCL	**28**
Figure 2.3:	T-cell/ histiocyte rich variant of diffuse large B-cell lymphoma	**28**
Figure 2.4:	Mediastinal large B-cell lymphoma	**30**
Figure 2.5:	Plasmablastic variant of diffuse large B-cell lymphoma	**30**
Figure 2.6:	Physiologic and aberrant somatic hypermutation	**36**
Figure 2.7:	Normal B-cell development	**37**
Figure 2.8:	Model for pathogenesis of DLBCL	**38**
Figure 2.9:	Examples of bone marrow involvement in DLBCL	**46**
Figure 2.10:	Flow cytometric involvement of the bone marrow in DLBCL	**52**
Figure 2.11:	Examples of gene rearrangement studies in lymphoma	**57**
Figure 2.12:	Gene expression profiles in diffuse large B-cell lymphoma	**67**
Figure 2.13:	Predictive value of germinal centre and activated B-cell signatures	**68**
Figure 3.1:	Kaplan Meier curve showing the median survival of the patient cohort	**96**
Figure 3.2:	Survival of patients with and without bone marrow involvement using morphology alone (M)	**97**
Figure 3.3:	Survival of patients with and without bone marrow involvement as defined by a new definition including morphology (M), and occult cases detected by flow cytometry, immunohistochemistry and IgH gene rearrangement (M+O)	**98**
Figure 4.1:	Overall survival of the entire patient cohort was 55 months	**107**
Figure 4.2:	Flow cytometric involvement of the bone marrow in DLBCL	**108**
Figure 4.3a:	Sections from an indeterminate case show several small interstitial lymphoid aggregates	**109**
Figure 4.3b:	Immunohistochemical analysis using CD20 shows a greater number of lymphoid aggregates	**109**
Figure 4.4:	Cases with and without morphologically apparent bone marrow involvement (M) show no difference in overall survival	**110**

Figure 4.5:	When cases positive on flow cytometry are included with morphologically apparent cases to define bone marrow involvement (M+F), cases with and without involvement show a significant difference in overall survival	**110**
Figure 4.6:	Kaplan Meier curves show that cases positive on morphology and/or flow cytometry (M+F) have a significantly shorter time to relapse than patients without involvement	**111**
Figure 5.1.1:	When routine histology was used to look for bone marrow involvement, the difference in survival between patients with and without involvement was 40 months vs. 81 months	**121**
Figure 5.1.2:	When immunostaining was used as a routine in all cases, the difference in survival between patients with and without marrow involvement was 28 months vs. 81 months	**121**
Figure 5.1.3:	Examples in which routine histology showed no evidence of involvement	**123**
Figure 6.1.1:	Differences in mean DNA concentration between fresh and formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsy specimens	**133**
Figure 6.1.2A:	Gel picture showing amplification of DNA up to 600 bp in all fresh frozen trephine biopsy specimens	**134**
Figure 6.1.2B:	Gel picture showing loss of larger DNA fragments in formalin-fixed decalcified paraffin-embedded trephine biopsy samples	**134**
Figure 6.1.3:	DNA amplification from formalin-fixed decalcified paraffin-embedded trephine biopsy specimens	**134**
Figure 6.1.4:	Box plots showing the difference in mean maximum length of amplicons generated by fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsy specimens	**135**
Figure 6.1.5:	Box plot showing a significant difference in the mean crossing thresholds of fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine samples on real-time amplification of the β-globin gene using the LightCycler	**135**
Figure 6.1.6:	Agarose gel pictures at 1 hour and 1.5 hours showing the difference between DNA from fresh and formalin-fixed decalcified paraffin-embedded trephine samples	**135**
Figure 6.2.1:	Distribution of cases across different durations of storage	**141**
Figure 6.2.2:	Gel picture showing preferential amplification of smaller DNA fragments	**142**
Figure 6.2.3:	Graphical representation of DNA amplification at different fragment lengths showing progressive reduction in amplification at higher fragment lengths	**142**

Figures 7.1 (A, B and C): The three Kaplan Meier curves show differences in cumulative survival between low-risk, low-intermediate, high-intermediate and high-risk categories using a baseline IPI model (A) and two revised IPI models (B) and (C) **164**

Figure 8.1: Flow chart showing the recommended staging investigations in diffuse large B-cell lymphoma **218**

ABBREVIATIONS:

ALCL: Anaplastic large cell lymphoma

ANOVA: Analysis of variance

B-ALL: B-cell acute lymphoblastic lymphoma

BCL: B-cell CLL/lymphoma

BCSH: British Committee for standards in Haematology

BL: Burkitt lymphoma

BM: Bone marrow

CD: Cluster of differentiation

CHOP: Cyclophosphamide, Doxorubicin, Oncovin, Prednisolone

CI: Confidence interval

CLL/SLL: Chronic lymphocytic leukaemia/ small lymphocytic lymphoma

CNS: Central nervous system

CR: Complete remission

CT: Computerized tomography

df: Degrees of freedom

DFS: Disease free survival

DLBCL: Diffuse large B-cell lymphoma

DNA: Deoxyribose nucleic acid

EBV: Epstein Barr virus

ECOG: Eastern Cooperative Oncology group

EPOCH: Etoposide, Vincristine, Doxorubicin, Cyclophosphamide, Prednisolone

EUC: Electrolytes, urea, creatinine

FBC: Full blood count

FC: Flow cytometry

FFDPE: Formalin-fixed decalcified paraffin-embedded

FISH: Fluorescent in-situ hybridization

FL: Follicular lymphoma

FNA: Fine needle aspiration

FR: Framework

GELA: Groupe d'Etude des Lymphomes de l'adulte

HCL: Hairy cell leukaemia

H&E: Haematoxylin and eosin

HIV: Human immunodeficiency virus

HL: Hodgkin lymphoma

HR: Hazards ratio

ICE: Ifosfamide, Carboplatin, Etoposide

ICSH: International Council for Standardization in Haematology

IgH: Immunoglobulin heavy chain

IgL: Immunoglobulin light chain

IHC: Immunohistochemistry

IPI: International prognostic index

LDH: Lactate dehydrogenase

LLMPP: Lymphoma and Leukaemia Molecular Profiling Project

LR: Logistic regression

MCL: Mantle cell lymphoma

mRNA: messenger Ribonucleic acid

MZL: Marginal zone lymphoma

NF-κ: Nuclear factor-kappa

NHL: Non-Hodgkin lymphoma

NHMRC: National Health and Medical Research Council

OD: Optical density

OS: Overall survival

PBS: Phosphate buffered saline

PEN: Prednisolone, Etoposide, Novantrone

PET: Positron emission tomography

PFS: Progression free survival

PLL: Prolymphocytic leukaemia

PR: Partial remission

R-CHOP: Rituximab, Cyclophosphamide, Doxorubicin, Oncovin, Prednisolone

REAL: Revised European-American classification of lymphoid neoplasms

rIPI: Revised International Prognostic Index

RNA: Ribonucleic acid

RR: Relative risk

RT-PCR: Reverse transcriptase – polymerase chain reaction

SD: Standard deviation

SHM: Somatic hypermutation

SPSS: Statistical package for social sciences

TCR: T-cell receptor

TMA: Tissue microarray

tRNA: transfer Ribonucleic acid

WHO: World Health Organization

WM: Waldenstrom's macroglobulinaemia

CHAPTER I:

Context and program of research

1.1 Introduction:

Diffuse Large B-cell Lymphoma (DLBCL) is the commonest sub-type of Non-Hodgkin Lymphoma (NHL) accounting for 25-30% of all cases. [1, 2] It is defined in the World Health Organization classification of tumours as a diffuse proliferation of large malignant B-cells. [1] This category has been recognized to include more than one disease entity, with heterogeneity in morphology, clinical presentation and response to treatment. [3] The median five-year overall survival in DLBCL is only ~40-50% [4] with variable outcomes depending on pre-treatment clinical and laboratory characteristics. [5]

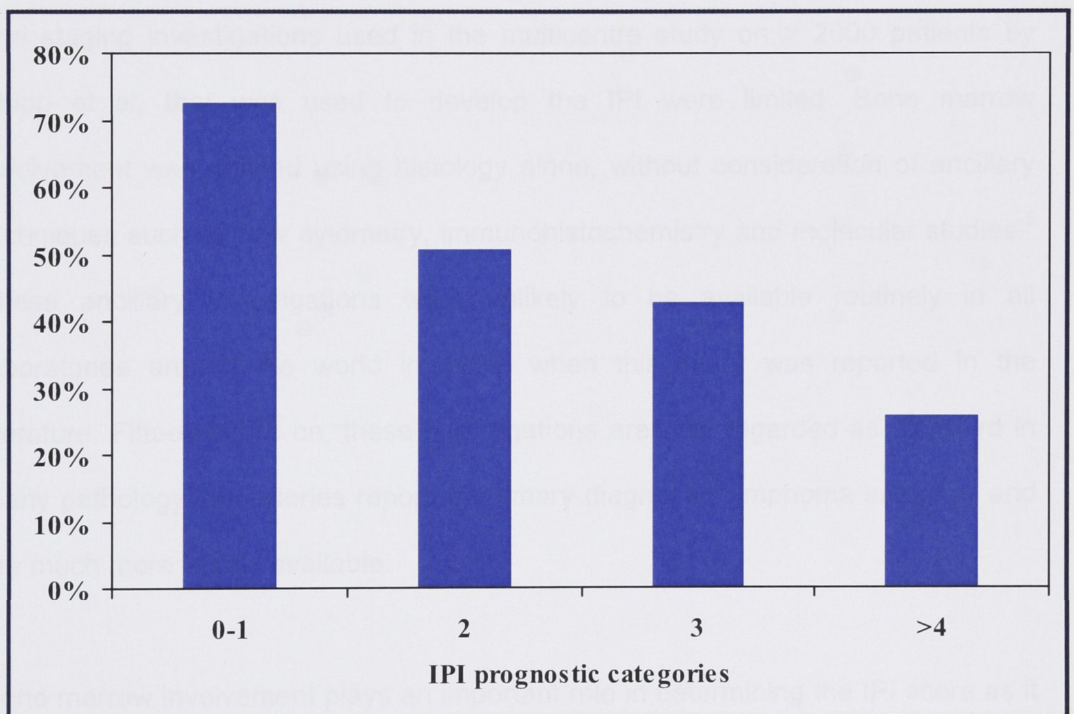
Staging investigations are performed after diagnosis is established and include: chest X-ray; computerized tomography (CT) scans; functional scans such as positron emission tomography (PET) scans; pathology investigations including lactate dehydrogenase (LDH); and examination of the bone marrow (BM).[6] These investigations contribute to the International Prognostic Index (IPI),[5] and may influence treatment decisions.

The IPI is a standard clinical tool that is widely used to predict outcome for patients with aggressive NHL, including DLBCL. It uses a number of clinical and laboratory markers present at the time of diagnosis to predict survival. Age > 60 years, widespread or stage III/IV disease defined by results of radiological investigations and bone marrow biopsy, elevated LDH level, Eastern Cooperative Oncology Group (ECOG) performance status ≥ 2 and more than one extra nodal site of disease, are scored 1 each, and depending on the final score ranging from 0-5,

four prognostic categories are created. These are: low risk correlating with IPI of 0-1, low-intermediate risk with IPI of 2, high-intermediate risk with IPI of 3, and high risk with IPI of 4-5. Five year overall survival ranges from 73% to 26% [5] **(Figure 1.1)**.

Figure 1.1:

Overall survival based on IPI prognostic categories



However, limitations of the IPI are well recognized owing to the heterogeneity in clinical outcomes within IPI groups. Although gene expression profiling has been used to determine subtypes of DLBCL based on stages of B-cell differentiation [7], such studies are a largely limited to the research setting. Efforts to improve clinical outcomes in DLBCL using reliable prognostic markers are ongoing.[8, 9] In this study, we assessed the impact of improved staging investigations using easily available ancillary investigations on overall survival and on the IPI.

The staging investigations used in the multicentre study on > 2000 patients by Shipp et al, that was used to develop the IPI were limited. Bone marrow involvement was defined using histology alone, without consideration of ancillary techniques such as flow cytometry, immunohistochemistry and molecular studies.[5] These ancillary investigations were unlikely to be available routinely in all laboratories around the world in 1993, when this study was reported in the literature. Fifteen years on, these investigations are now regarded as standard in many pathology laboratories reporting primary diagnostic lymphoma samples, and are much more widely available.

Bone marrow involvement plays an important role in determining the IPI score as it has the potential to affect two pre-treatment characteristics. The first of these is the stage of the disease, as bone marrow involvement upstages the disease to stage IV. Additionally, bone marrow is also classified as an extranodal site, and can therefore increase the IPI score in the presence of one additional extranodal site of involvement. Another limitation of the IPI is that it applies the same importance to all extra nodal sites of involvement, and fails to recognize that some extranodal

sites such as bone marrow may have increased significance compared to other sites. Besides implying increased tumour burden,[10] it may also affect tolerability of chemotherapy if the degree of infiltration is significant, as marrow reserve may be compromised.

1.2. Hypothesis:

The working hypothesis behind this research is that in the absence of routine use of ancillary investigations for staging DLBCL, a proportion of patients are being under staged with a potential impact on prognosis.

This thesis lists the results of a series of experiments designed and performed prospectively to test this hypothesis.

1.3. Aims:

1. To determine the rates of occult or histologically inapparent marrow involvement in DLBCL using ancillary investigations i.e. flow cytometry, immunohistochemistry and molecular studies.
2. To re-define bone marrow involvement using a summative model.
3. To determine the impact of routine use of these investigations on clinical outcomes such as overall survival.
4. To determine if these investigations could improve the predictive value of the IPI.

1.4. Experiments:

1.4.1. Experiment I: Pilot study

- A pilot study was planned as the first step to determine the rate of occult bone marrow involvement using ancillary investigations on a small cohort of bone marrow trephine samples.
- Blinded review of routine histology [preferably four sections stained with haematoxylin and eosin, one cut at 2 microns and another cut at 4 microns, one reticulin stained section and one Giemsa stained section] and flow cytometry results of 36 patients diagnosed with DLBCL was performed.
- Immunohistochemical analysis was performed using T-cell, B-cell and light chain markers.
- Molecular studies for Immunoglobulin heavy chain gene rearrangement were performed on paraffin-embedded bone marrow trephines.
- Rates of occult or histologically inapparent bone marrow involvement were determined.
- The predictive value of a summative model of bone marrow involvement (encompassing ancillary staging investigations) was compared with the conventional definition (using histology alone) in determining prognosis in DLBCL.

1.4.2. Experiment II: Clinical role of flow cytometry in staging DLBCL

- Flow cytometry is known to detect clonal populations in histologically inapparent cases. I sought to study the rate of detection of flow cytometric marrow involvement in histologically negative cases and to correlate that with overall survival.

- Blinded reanalysis and re-interpretation of flow cytometry data was performed in 113 DLBCL cases.
- Blinded histology review was performed.
- Review of literature suggested that only a small number of cases were expected to be detected on flow cytometry alone. As these numbers were not adequate to allow meaningful determination of clinical outcome in flow cytometry positive, histology negative cases, I tested a summative model to determine if addition of flow cytometry to histology would affect overall survival.

1.4.3. Experiment III: Clinical role of immunohistochemistry in staging DLBCL

- Immunohistochemistry is used more widely in the context of diagnosis rather than staging, and there is very limited data on the role of immunohistochemistry in staging.
- I aimed to study the role of immunohistochemistry in detecting occult bone marrow involvement in DLBCL and to correlate that with overall survival.
- Immunohistochemical analysis was performed in all cases using T-cell, B-cell and light chain markers.
- The clinical role of immunohistochemistry relative to histology was determined. As there were adequate numbers to allow for meaningful statistical analysis, the summative model used in the pilot study and flow cytometry experiment was not required.

1.4.4. Experiment IV: Clinical role of molecular staging in DLBCL

- I planned to study the impact of molecular staging on outcome in DLBCL using immunoglobulin heavy chain (IgH) and light chain (IgL) analysis (InVivo Scribe kits based on BIOMED-2 protocols).
- Fresh frozen bone marrow aspirate samples were not available and collecting samples prospectively was unlikely to yield adequate numbers for the study. I therefore determined to perform the molecular analyses on archived formalin-fixed paraffin-embedded trephine biopsy samples.
- A sub-study was planned to compare the yield of DNA amplification from fresh frozen and paraffin-embedded trephine samples, and to determine the feasibility of obtaining DNA for IgH and IgL analysis using our trephine processing protocols.
- DNA amplification from formalin-fixed decalcified paraffin-embedded trephine biopsy tissue was performed and as the samples varied in age from < 2 years to >20 years, I studied the effect of age on DNA yield.
- Similar clonal bands were sought in primary tissue to establish tumour origin .

1.4.5. Experiment V: Impact of ancillary staging investigations on the IPI

- This last experiment aimed to study the impact of these ancillary investigations on the International Prognostic Index.
- Overall results from the study for the three ancillary investigations were computed.
- The impact of improved staging using these investigations on the IPI was studied.

1.5. Program of research:

The research was planned prospectively, with permission, as a series of publications with the intention of submitting the thesis as a compilation of published papers. The structure of the thesis is intended such that each paper functions as an independent chapter or sub-chapter. Instead of having separate chapters on methods and results, and a final discussion chapter in which limitations and future directions are discussed, each of the 6 original papers has its own introduction, methods, results and discussion. Limitations of each paper and future directions of research are listed within each paper. **Table 1.1** lists the papers prospectively published during the period of this research.

The two unpublished sections of the thesis are the introduction/ literature review (Chapter II) and a final conclusions chapter (Chapter IX) which besides listing the conclusions of the research, offers reflections on the project, limitations and future directions.

Table 1.1:

Publications arising during the period of research included in the thesis

Publication	Experiment	Chapter
Talaulikar D, Dahlstrom JE, Broomfield A, McNiven M, Shadbolt B. **"Occult marrow involvement in Diffuse Large B-cell Lymphoma: results of a pilot study."** Pathology. 2007 Dec; 39(6):580-5.	I	3.1
Talaulikar D, Shadbolt B, Bell J, Khan K, and Dahlstrom JE: **"Clinical role of flow cytometry in redefining bone marrow involvement in Diffuse Large B-cell Lymphoma – a new perspective."** Histopathology 2008; 52: 340-347.	II	4.1
Talaulikar D, Dahlstrom JE, Shadbolt B, Broomfield A, McDonald A. **"Role of Immunohistochemistry in staging diffuse large B-cell lymphoma"**. Journal of Histochemistry and Cytochemistry, 2008 Oct;56(10):893-900. Epub 2008 Jun 23.	III	5.1
Talaulikar D, Dahlstrom JE, Shadbolt B, McDonald A. **"Clinical Implications of Immunophenotyping in Staging Diffuse Large B-Cell Lymphoma"**. Blood, (ASH annual meeting abstracts) 2008; 112: Abstract 5279.	III	5.2
Talaulikar D, Gray JX, Shadbolt B, McNiven M, Dahlstrom JE: **"A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsies using two different methods."** J Clin Pathol. 2008 Jan; 61(1):119-23. Epub 2007 Jun 1.	IV	6.1
Talaulikar D, Shadbolt B, McNiven M, Dahlstrom JE: **"DNA amplification from formalin-fixed decalcified paraffin-embedded bone marrow trephine specimens – does the duration of storage matter"?** Pathology, 2008 Dec; 40(7): 702-706.	IV	6.2
Talaulikar D, Shadbolt B, Dahlstrom JE, McDonald A. "**Routine use of ancillary investigations in staging Diffuse Large B-cell Lymphoma improves the International Prognostic Index (IPI)"** Submitted for publication.	V	7
Talaulikar D, and Dahlstrom JE. "**Staging bone marrow in Diffuse Large B-cell Lymphoma: the role of ancillary investigations**". Pathology 2009; in press.	-	8

CHAPTER II:

Literature review

2.1. Introduction:

Diffuse Large B-cell Lymphoma (DLBCL) is the commonest sub-type of Non-Hodgkin Lymphoma (NHL) accounting for 25-30% of all cases. [1, 2] It is defined in the World Health Organization (WHO) classification of tumours as a diffuse proliferation of large malignant B-cells. [1] This category has been recognized to include more than one disease entity, with heterogeneity in morphology, clinical presentation and response to treatment. [3]

The pathologic classifications of NHL, histology and pathogenesis, epidemiology, clinical features, evaluation, prognosis and treatment of DLBCL have been reviewed in this chapter. Special emphasis has been placed on the literature surrounding staging as background to the project.

2.2. *Pathologic classifications:*

The WHO classification of tumours of the haemopoietic and lymphoid tissues was set up as a collaborative project of the European Association for Haematopathology and the Society for Haematopathology in 1995. It is based on the principles defined in the "Revised European-American Classification of Lymphoid Neoplasms" (REAL) classification. [3] The REAL classification, which was published in 1994, defined a consensus list of lymphoid neoplasms using all available information to define a disease entity, including morphology, immunophenotyping, genetic features and clinical features. [3]

The REAL classification differed from previous lymphoma classifications in two respects: [3]

i) It focused on defining real and relevant entities instead of subtleties of pathology

ii) It used a consensus approach to lymphoma diagnosis recognising that broad agreement was required across multiple fields of pathology.

The WHO classification (2001) recognizes three major categories of lymphoid neoplasms: Hodgkin Lymphoma (HL), B-cell neoplasms, and T/ NK cell neoplasms. Two sub-categories are recognized within the B and T/NK cell categories, the precursor neoplasms, and peripheral or mature neoplasms. [1] **(Table 2.1)** DLBCL is a mature B-cell neoplasm as defined in the WHO classification.

Table 2.I:

World Health Organization classification of lymphoid neoplasms. [1]

B-cell neoplasms	**T and NK-cell neoplasms**	**Hodgkin Lymphoma (HL)**
* **Precursor B-cell neoplasm** Precursor B- acute lymphoblastic leukaemic/ lymphoma	* **Precursor T-cell neoplasm** Precursor T- acute lymphoblastic leukaemia/ lymphoma	* **Nodular Lymphocyte predominant HL**
* **Mature (peripheral) B-cell neoplasms** - B-cell Chronic lymphocytic leukaemia/ small lymphocytic lymphoma (CLL/ SLL) - B-cell Prolymphocytic Leukaemia (PLL) - Lymphoplasmacytic lymphoma - Splenic marginal zone B-cell lymphoma - Hairy cell leukaemia - Plasma cell Myeloma/ plasmacytoma - Extranodal marginal zone B-cell lymphoma - Nodal marginal zone B-cell lymphoma -Diffuse Large B-cell Lymphoma -Follicular Lymphoma - Mantle cell Lymphoma -Burkitt Lymphoma/Burkitt leukaemia	* **Mature T-cell neoplasms** - T-cell prolymphocytic leukaemia -T-cell granular lymphocytic leukaemia - Aggressive Nk-cell leukaemia - Adult T-cell leukaemia/ lymphoma - Extranodal NK/ T-cell lymphoma, nasal type - Enteropathy type T-cell lymphoma - Hepatosplenic T-cell lymphoma - Subcutaneous panniculitis T-cell lymphoma - Mycosis fungoides/ Sezary syndrome - Anaplastic large cell lymphoma, T/null cell, primary cutaneous type - Anaplastic large cell lymphoma, T/null cell, primary systemic type - Angioimmunoblastic T-cell lymphoma - Peripheral T-cell lymphoma, not otherwise characterized	* **Classical HL** - Nodular sclerosis HL - Lymphocyte predominant HL - Mixed cellularity HL - Lymphocyte depleted HL

From: The World Health Organization Classification of Tumours book, IARC press, 2001

2.3. *Histology:*

2.3.1 Macroscopy:

In lymph nodes, DLBCL causes replacement of the structure of the tissue with a homogeneous fish-flesh appearance. In extra nodal tissues, DLBCL forms a tumour mass with or without fibrosis. These appearances can be modified by haemorrhage into or necrosis of the tissue. [1]**(Figure 2.1A)**

2.3.2. Microscopy:

DLBCL causes diffuse infiltration and replacement of lymphoid or extra nodal tissue by large transformed malignant cells. Histologically, the nuclear size of the malignant lymphoid cells is equal to or exceeds normal macrophage nuclei, or is twice the size of normal lymphocytes. [1]

Cytological or morphologic variants are known although these have high intra-observer and inter-observer variability and are not generally associated with distinct immunophenotypic or genotypic variants. [1, 3] **(Figure 2.1B)**

Figure 2.1A:
Macroscopic appearances in diffuse large B-cell lymphoma. [1]

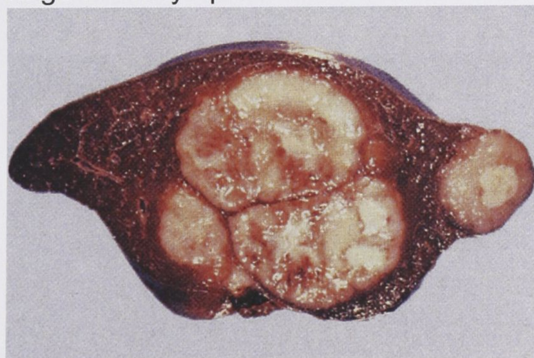
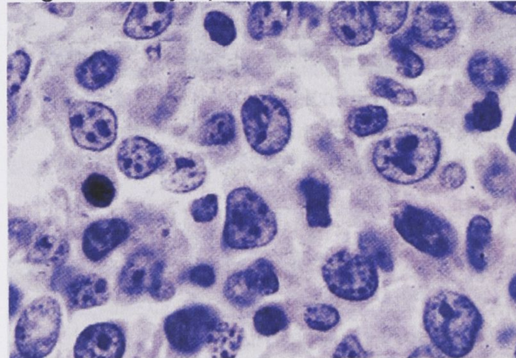


Figure 2.1B:
Microscopic appearances in diffuse large B-cell lymphoma. [1]



These figures are from the World Health Organization Classification of Tumours book, IARC press, 2001

2.3.3. Morphologic variants:

Four morphologic variants are recognized.

1. Centroblastic: This variant has medium to large-sized lymphoid cells with scanty cytoplasm, round nuclei and 2-4 nucleoli. Both monomorphic and polymorphic forms are known. [11] **(Figure 2.2)**

2. Immunoblastic: This variant is characterized by the presence of > 90% immunoblasts, and < 10% centroblasts. The immunoblasts are large cells with basophilic cytoplasm and a single central nucleolus. [11] **(Figure 2.2)**

3. T-cell/histiocyte rich: In this variant, a background infiltrate of non-malignant T-cells is seen around scattered malignant B-cells. These are large cells comprising <10% of the infiltrate. This subtype of DLBCL can be confused with Hodgkin's Lymphoma. [12] **(Figure 2.3)**

4. Anaplastic: Anaplastic Large B-cell Lymphoma is characterized by large cells with bizarre nuclei, which may sometimes grow in a cohesive fashion. [12, 13] **(Figure 2.2)**

Figure 2.2:

The morphologic variants of DLBCL. Panel A shows the centroblastic variant, panel B, the immunoblastic variant, panel C shows anaplastic lymphoma and panel D, plasmablastic lymphoma.[14]

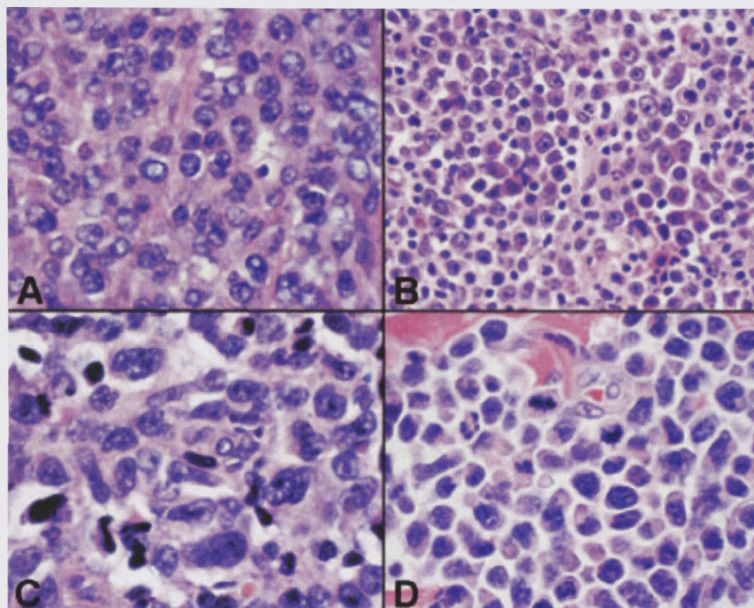


Figure from: Hunt KE, Reichard KK. Diffuse large B-cell lymphoma. Arch Pathol Lab Med. 2008 Jan;132(1):118-24.

Figure 2.3:

H&E staining and immunostaining with CD20 in a case with T-cell/ histiocyte rich B-cell lymphoma.[15]

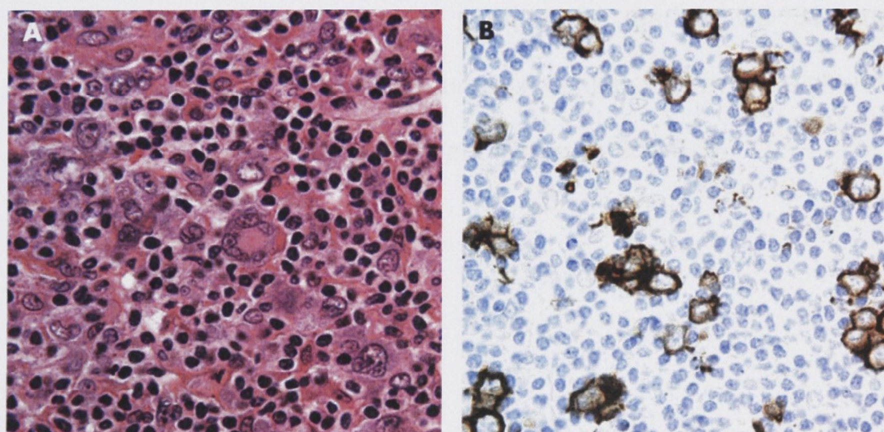


Figure from: Prakash S, Swerdlow SH. Nodal aggressive B-cell lymphomas: a diagnostic approach. J Clin Pathol. 2007 Oct;60(10):1076-85.

2.3.4. Other variants:

2.3.4.1. Mediastinal large B-cell lymphoma:

This is a distinct clinicopathologic entity which classically presents in young women with a mediastinal mass and distinctive clear cell morphology and sclerosis **(Figure 2.4)**. [1] Immunophenotypic profile is similar to classical DLBCL. Recent reports suggest that immunohistochemical markers can be used to distinguish these cases from classical DLBCL. Nuclear c-Rel containing NF-κB transcriptional complexes, and cytoplasmic tumour necrosis factor receptor associated factor-1, TRAF1, which is a NF-κB target, are noted in 53% of mediastinal lymphoma cases as compared to 2% of DLBCL cases. [16]

2.3.4.2. Plasmablastic:

This subtype of DLBCL usually presents on a background of HIV infection, and occurs most commonly in the oral cavity.[17, 18] A large proportion of cases are EBV positive.[17] Morphologically, this subtype can be difficult to distinguish from immunoblastic DLBCL and at times from plasma cell myeloma. However, the cells are CD20 negative, express the plasma cell marker, CD138, and have a high proliferative fraction.[19] These features, in conjunction with the characteristic clinical profile, help to distinguish this variant **(Figures 2.2, 2.5)**.

Figure 2.4:

Histology of mediastinal large B-cell lymphoma showing cells with pale cytoplasm and the associated fine sclerosis. [15]

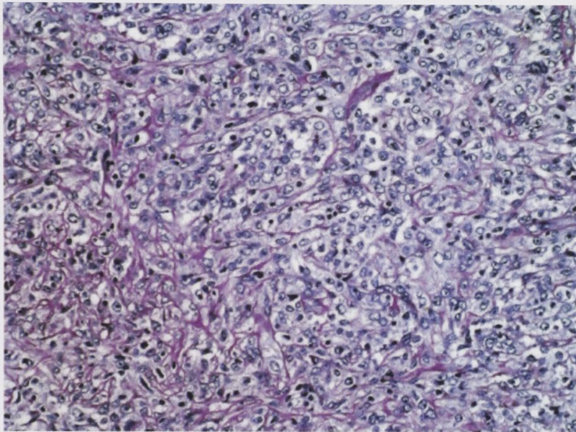


Figure from: Prakash S, Swerdlow SH. Nodal aggressive B-cell lymphomas: a diagnostic approach. J Clin Pathol. 2007 Oct;60(10):1076-85.

Figure 2.5:

Morphology in the plasmablastic variant of diffuse large B-cell lymphoma. Panels A-D show tumours with typical morphology. These include moderate cytoplasm, Golgi zone clearing and prominent central nucleoli. Panel E shows reputed "Centroblastic" variant of plasmablastic lymphoma and panel F shows a morphologic variant with small cells.[20]

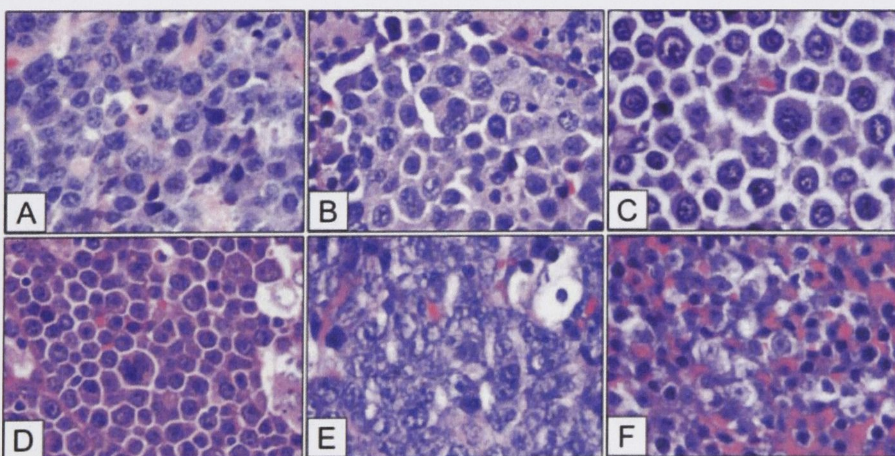


Figure from: Teruya-Feldstein J, Chiao E, Filippa DA, Lin O, Comenzo R, Coleman M, et al. CD20-negative large-cell lymphoma with plasmablastic features: a clinically heterogenous spectrum in both HIV-positive and -negative patients. Ann Oncol. 2004 Nov;15(11):1673-9.

2.3.4.3. DLBCL expression of full-length ALK:

This aggressive subtype of DLBCL usually occurs in adults with a male preponderance. Lymph nodes are massively infiltrated with monomorphic large malignant cells with abundant basophilic cytoplasm, large round nuclei, and prominent central nucleoli. Immunophenotypically, the cells express CD45, but lack other T-cell and B-cell markers except for CD4 and CD57. CD30 is negative but the cells express an endoplasmic reticulum-associated marker detected by antibody VS38. Intracytoplasmic IgA is noted with light chain restriction. Antibodies against ALK detect granular positivity, although t(2;5) and the NPM:ALK fusion gene cannot be detected.[21]

2.4. Immunophenotype:

DLBCL cases express pan-B markers such as CD19, CD20, CD22 and CD79a.[1, 3] However, CD20 expression may be lacking in certain circumstances including morphologic variants with plasmablastic features or those with expression of full length ALK. [20, 22] Cases transformed from follicular lymphoma that have previously been treated with Rituximab have also been shown to be CD20 negative. [23] Surface expression of immunoglobulins M (IgM), G (IgG) or A (IgA) may be seen in 50-75% of cases.[24, 25] Approximately 10% of cases are positive for CD5 and 25-50% are CD10 positive.[26, 27] BCL2 is positive in 30-50% of cases.[28, 29] A high degree of staining (>40%) with the proliferative marker Ki67 is seen, although occasionally it may be higher than 90%.[28] CD30 expression is seen in most of the anaplastic variants and in some non-anaplastic cases.[29, 30]

2.5. Genetics:

Several genetic abnormalities have been identified in subsets of DLBCL although none are typical or diagnostic of the disease. [31] Most tumours demonstrate rearrangement of the immunoglobulin heavy chain (IgH) and light-chain (IgL) genes and somatic hypermutation of the variable regions. Somatic hypermutation occurs during germinal centre B-cell development and can be used as a marker for this stage of maturation. It is beneficial in that it generates antibody diversity and increases antigen affinity. However, it also increases the risk of chromosomal translocations and mutagenesis. [32] BCL2, BCL6 and MYC are the three most frequently deregulated genes, all of which result from translocations that bring the genes under the control of an Ig regulatory element. [31]

Table 2.2:

Major recurring genetic events in DLBCL. [31]

Genetic defect	Frequency	Location	Mechanism of deregulation
BCL6	35-40%	3q27	t(3;..) and SHM
BCL2	t(14;18) 13%, amplification 24%	18q21	t(14; 18) and gene amplification
cMYC	15%	8q24	t(8;..) and SHM
FAS(CD95)	20%	10q24	Death domain mutations, ? SHM
#SHM	45%	Physiologic: Ig(v), FAS, BCL6 Aberrant: BCL6, PIM1, cMYC, PAX5, RhoH/TTF, ?FAS	SHM
P53	16%	17p	Mutation, deletion

Table from: Abramson JS, Shipp MA. Advances in the biology and therapy of diffuse large B-cell lymphoma: moving toward a molecularly targeted approach. Blood. 2005 Aug 15;106(4):1164-74.

2.5.1. BCL6:

The BCL6 is a marker of germinal centre origin.[33, 34] It is a transcriptional repressor that is involved in the regulation of lymphoid development and function.[35] It represses p53 and a large number of other genes involved in apoptosis, cell cycle regulation, and lymphocyte development.[7, 36]

Approximately 30% of DLBCL cases demonstrate chromosomal translocations affecting the 3q27 region involving the candidate proto-oncogene BCL6.[37] BCL6 translocation can involve one of three immunoglobulin loci i.e. 14q32 (IgH), 2p11 (Ig κ) and 22q11 (Ig λ). It can also involve at least 10 other chromosomal sites that are unrelated to non-Ig partner genes.[35] These translocations lead to juxtaposition of heterologous promoters to the BCL6 coding domain, resulting in increased BCL6 expression due to promoter substitution.[38] In the remaining ~ 70% of DLBCL, the BCL6 gene contains mutations in the 5' non-coding region. These occur in the absence of chromosomal abnormalities affecting rearrangement of the BCL6 locus.[39, 40] These mutations most commonly involve genomic sequences adjacent to the BCL6 promoter region suggesting that both mutations and rearrangements may be selected for their ability to alter the BCL6 promoter.[40] Together, rearrangements and mutations occur in ~100% cases, suggesting that the 5' non-coding region is essential to the lymphomagenesis in DLBCL.[40]

2.5.2. BCL2:

BCL2 is an anti-apoptotic protein involved in the t(14;18)(q32;q21) translocation. [41] This juxtaposes the BCL2 gene from chromosome 18 to the IgH locus resulting in over-expression of BCL2.[42, 43] Translocation of the BCL2 gene is a hallmark of Follicular Lymphoma and is seen in 20-60% of DLBCL cases.[37, 42, 44, 45] Other mechanisms such as amplification of BCL2 can also cause over-expression of BCL2. [46, 47]

2.5.3. cMYC:

cMYC is a transcription factor associated with Burkitt's Lymphoma and is deregulated in 15% of DLBCL. [37] This usually results from t(8;14) which juxtaposes the cMYC gene on chromosome 8 to the IgH locus.[48]

2.5.4. Cytogenetic abnormalities:

Recurring chromosomal translocations are reported to occur in ~50% of cases. [49] The t(14;18) is present in ~30% of DLBCL. [50]Chromosomal imbalances of 1q,[50] 1p, 3p, 3q, 5, 6q, 7q, 8q, 14, 18q and 22q are known to occur in DLBCL.[51] Moreover, chromosomal and gene amplification is well-recognized in DLBCL .[47]

2.5.5. Somatic hypermutations:

Physiologically, somatic hypermutation causes Ig gene editing of normal germinal centre B-cells by targeting regulatory sequences downstream of the Ig variable region (IgV) gene promoter. [32] Aberrant somatic hypermutation is postulated to be an additional pathogenetic mechanism in DLBCL [52] and includes genes like BCL6, cMYC, PAX5 and FAS (CD95). This is demonstrated in **Figure 2.6**.

Figure 2.6:

Physiologic and aberrant somatic hypermutation. [31]

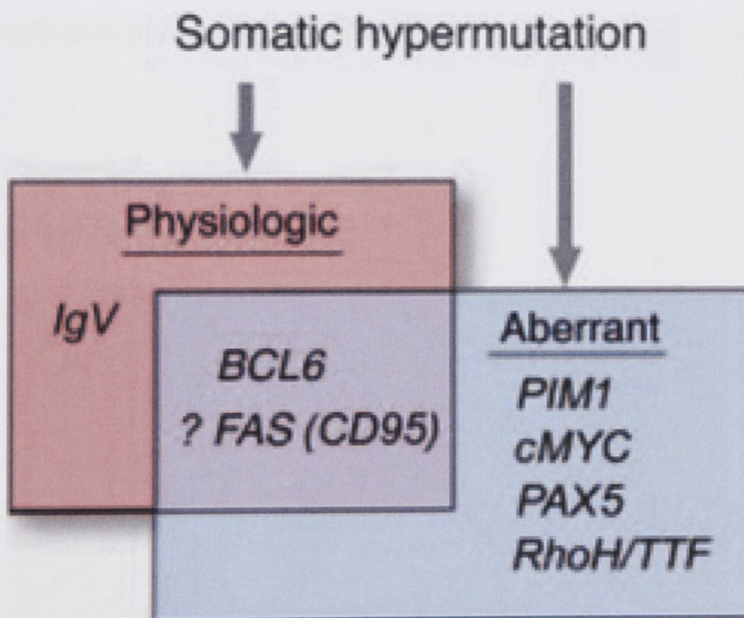


Figure from: Abramson JS, Shipp MA. Advances in the biology and therapy of diffuse large B-cell lymphoma: moving toward a molecularly targeted approach. Blood. 2005 Aug 15;106(4):1164-74.

2.6. Cellular derivation:

B-cell lymphomas arise from normal B lymphocytes at different stages of ontogeny. The stage of maturation at which malignant transformation occurs can be determined on the basis of a number of factors. Normal naïve lymphocytes prior to transit through the germinal centre shown in **Figure 2.7**, have unmutated immunoglobulin chain genes, whereas those that have transited the germinal centre, have somatic mutations in the variable part of the immunoglobulin genes. DLBCL is believed to arise from peripheral B-cells that have migrated through the germinal centres of lymph nodes or secondary lymphoid organs. The presence of ongoing somatic mutations indicates germinal centre origin whereas absence indicates post germinal centre origin.

Figure 2.7:

Normal B-cell development [15]

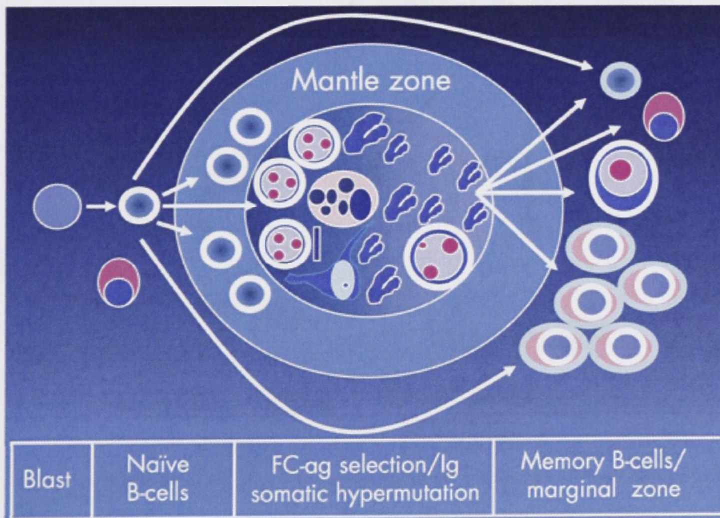


Figure from: Prakash S, Swerdlow SH. Nodal aggressive B-cell lymphomas: a diagnostic approach. J Clin Pathol. 2007 Oct;60(10):1076-85.

2.7. Molecular pathogenesis of DLBCL:

The pathogenesis of DLBCL is complex and represents a multi-step process **(Figure 2.8)**. It involves the accumulation of a number of genetic lesions resulting in generation of the malignant clone. The complexity in the molecular pathogenesis of DLBCL results from a number of factors. These include the cell of origin (ref: section 2.7), the presence of chromosomal translocations (ref: section 2.5.4), aberrant somatic hypermutation (ref: section 2.5.5), tumour surveillance and host responses, and other aberrations such as gene amplification/ deletion. [53]

Figure 2.8:

Model for pathogenesis of DLBCL with common pathways shown.[9]

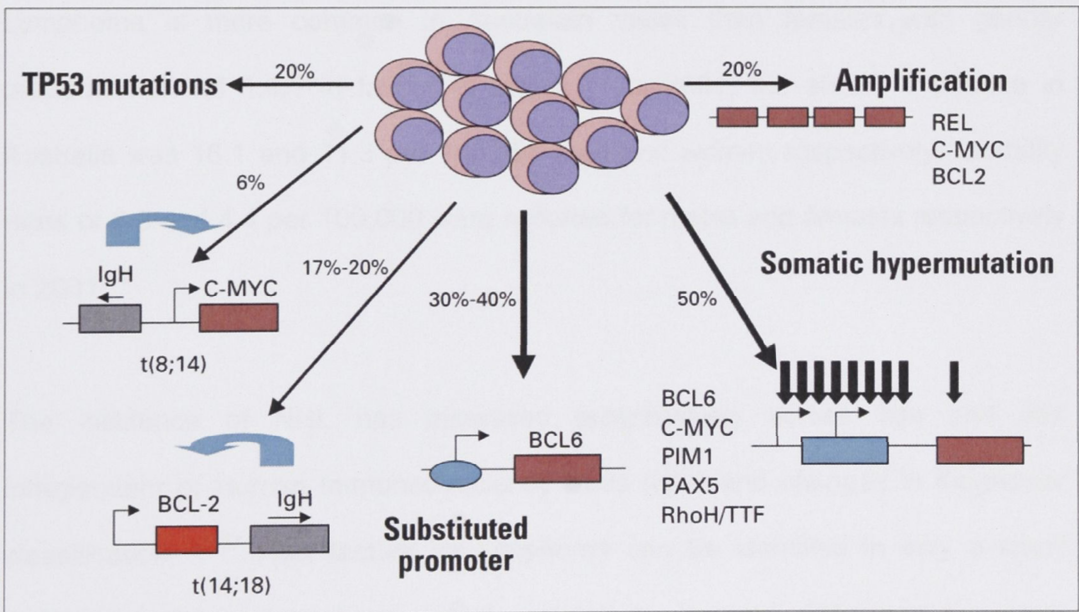


Figure from: Lossos IS, Morgensztern D. Prognostic biomarkers in diffuse large B-cell lymphoma. J Clin Oncol. 2006 Feb 20;24(6):995-1007.

2.8. Epidemiology of DLBCL:

Lymphoma occurs primarily in adults, and most commonly in the 7th decade. It affects 3500 people in Australia every year and constitutes 4% of all newly diagnosed cancers.[6] Data from the Australian Institute of Health and Welfare shows lymphoma occurs as the 6th most common cancer in men, after prostate, colorectal, lung, melanoma and bladder, while in women, it is the 5th most common, after breast, colorectal, melanoma and lung. In children, only lymphoid leukaemia and brain/CNS malignancies occur more commonly. [54] American figures demonstrate the incidence varies by ethnicity; Caucasian Americans have been reported to have higher incidence rates than Blacks, Asians and native Americans or Alaskan Inuit in order of decreasing incidence in an American study. [2]

Lymphoma is more common in Australian males than females with gender predominance of 1.4:1 in favour of males. [54] In 2001, the annual incidence in Australia was 16.1 and 11.3 per 100,000 men and women respectively. Mortality rates of 6.3 and 4.4 per 100,000 were reported for males and females respectively in 2001.[54]

The incidence of NHL has increased progressively across age and sex independent of Human Immunodeficiency Virus (HIV) and changes in diagnosis/ classification. [55-57] Risk factors for lymphoma can be identified in only a small proportion of cases and are largely related to immune deficiency i.e. post-transplant immunosuppression[58], HIV[59-61] and congenital immune deficiency[62], and specific infections such as EBV [63, 64] and Helicobacter pylori[65]. The Australian

Cancer Network Diagnosis and Management of Lymphoma Guidelines Working Party, in the 2005 guidelines for the diagnosis and management of lymphoma, have identified a number of other conditions associated with NHL risk on varying strength of evidence.[6] These are listed in **Table 2.3**.

Table 2.3:

Occupational, medical and lifestyle risk factors associated with prognosis in lymphoma. [6]

Occupational risk:
- Exposure to pesticides or herbicides (NHL)
- Farming (NHL, HL)
- Work in wood-related industry (HL)

Medical and co morbidity risk:
- Childhood appendectomy (NHL)
- Skin cancer (NHL)
- Diabetes (NHL)
- Tuberculosis (NHL)
- Infectious mononucleosis (HL)

Lifestyle risk:
- Cigarette smoking (NHL especially Follicular lymphoma, HL)

Table from: Australian Cancer Network Diagnosis and Management of Lymphoma Guidelines Working Party: Guidelines for the Diagnosis and Mangement of Lymphoma. Sydney: The Cancer Council Australia and Australian Cancer Network; 2005.

B-cell NHL represents >85% of all NHL globally and two entities, DLBCL and Follicular Lymphoma account for > 50% of all NHL. [66] Aggressive lymphomas comprise approximately 50% of all lymphomas and DLBCL ~30-40% of all adult lymphomas.[67]

2.9. *Clinical Features:*

2.9.1. Initial presentation:

Patients present with rapidly enlarging nodal or extranodal disease. Disseminated disease is known to occur in a proportion of cases. [66] B symptoms include night sweats, fevers > 38°C, and weight loss of > 10% over a period of 6 months and occur in ~30% of cases.[68] Serum lactate dehydrogenase is elevated in ~50% of cases. [68]

2.9.2. Sites of involvement:

The common sites of extranodal involvement are gastrointestinal, skin, CNS, testes, bone, soft tissue, lung, kidney, liver, spleen, female genital tract, Waldeyer's ring and salivary gland. Extranodal involvement is known to occur at initial diagnosis in ~40% of cases and the clinical profile may differ from that of nodal disease.[69] The gastrointestinal tract is the commonest extra-nodal site of involvement. Bone marrow involvement has been reported to occur in 12-30% of cases. [70-73]

2.10. Investigations:

These include those performed at initial presentation for diagnostic purposes and thereafter, for staging.

2.10.1. Diagnosis:

Lymphoma diagnosis is best established on surgical biopsies[74] with removal of intact lymph nodes where possible. However, initial testing can be usefully performed through a less invasive procedure i.e. fine needle aspiration (FNA). This also helps by obtaining material for flow cytometry and fluorescent in-situ hybridization (FISH). Although FNA may help to differentiate malignant infiltration from benign lymphoid infiltrates and in detecting high-grade transformation of low grade disease[75-77], it cannot be used reliably in the definitive diagnosis of lymphoma or lymphoma sub-classification.[78-80]

Surgical excision biopsy should be performed in cases where lymph nodes or extranodal sites are superficial and surgically accessible. In deeply situated masses not accessible through routine surgery, needle core biopsies may be used under radiological guidance to obtain tissue to allow the study of some degree of architectural detail[81-83], particularly where surgical excision is not feasible. Surgical endoscopic biopsies are also acceptable alternatives to surgical excision.[84-86]

2.10.2. Staging:

Established staging investigations performed after diagnosis include: history and examination; chest X-ray; CT scans; functional scans such as PET scans; pathology investigations including LDH; and examination of the bone marrow (BM).[6] These investigations help in determining the prognosis by contributing to the International Prognostic Index (IPI),[5] and may influence treatment decisions. The clinical practice guidelines for the diagnosis and management of lymphoma published by the National Health and Medical Research Council (NHMRC) recommend staging BMs be scored as positive, negative or indeterminate based on histology alone. The use of ancillary techniques including immunophenotyping (flow cytometry and immunohistochemistry) and molecular studies are discussed in the context of detection of minimal residual disease following treatment, but not in the context of staging at initial diagnosis.[6] The Alberta Cancer Board guidelines recommend the use of immunophenotyping on BM aspirates at the time of diagnosis by flow cytometry, if facilities are available; however the guidelines do not address the role of other ancillary investigations.[87]

Although previous guidelines produced by the British Committee for Standards in Haematology (BCSH) recommended that ancillary investigations such as flow cytometry, immunohistochemistry (IHC) and molecular studies be available in laboratories involved in lymphoma diagnosis, they have not specifically provided guidelines on their usage.[88] The current 2008 BCSH guidelines on lymphoma diagnosis and staging however do recommend that immunophenotyping by flow cytometry be used to aid in lymphoma diagnosis.[89] An appendix to these guidelines, The Specific Disease Index, recommends that flow cytometry be

performed routinely on all diagnostic and staging bone marrows in DLBCL.[90] There is more limited evidence in the guidelines to recommend performing immunohistochemistry as it may supplement the information provided by flow cytometry.[90]

2.10.2.1. Histological examination of the bone marrow:

Examination of both BM aspirate and trephine biopsy are recommended in the staging of NHL[6], although it is recognized that trephine biopsies are more likely to show involvement as compared to aspirates biopsies. [70, 91] Generally, primary diagnosis and sub-typing of NHL on BM findings is not recommended unless no other tissue is accessible. [1, 6, 90]

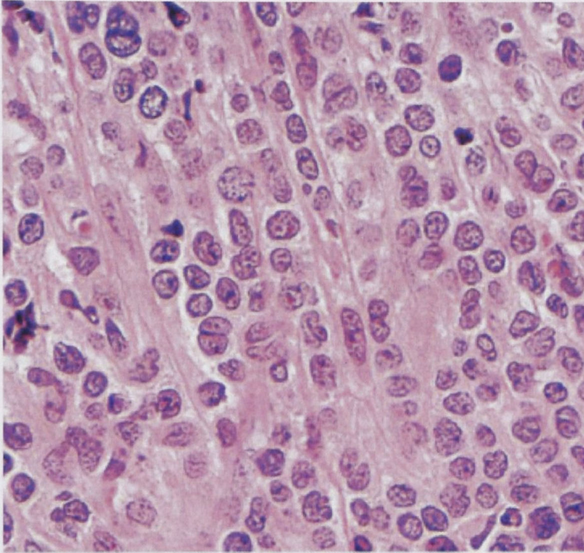
For a long time, conventional wisdom advocated the use of bilateral BM sampling for lymphoma staging with demonstrated improved rates of 10–20% on bilateral sampling.[70, 92, 93] However, more recent data suggests that bilateral sampling is not required. Optimal demonstration of histological BM involvement may be obtained with a trephine biopsy from a single site if it is ≥ 20 mm in length, and multiple levels (at least 4) of the biopsy are examined.[94] From a practical viewpoint, it is not always possible to obtain optimal samples and this may account for lower rates of positivity on BM samples using routine histology alone.

For staging, BM biopsies are reported as positive, negative or equivocal. Use of standardized criteria such as Cheson's criteria for classifying aggregates as benign, malignant or indeterminate can help to minimize inter-observer and intra-observer variability..[92] Cases with paratrabecular lymphoid aggregates, large-sized aggregates, large numbers of aggregates, presence of large cells within aggregates and increased reticulin response are classified as positive for involvement. Benign lymphoid aggregates are classified as those that are intertrabecular in location, small in size, well-circumscribed and containing

predominantly small cells admixed with reactive cells.[92] Examples of BM involvement with DLBCL are shown in **Figure 2.9.**

Figure 2.9:

Examples of bone marrow involvement in DLBCL. [95]



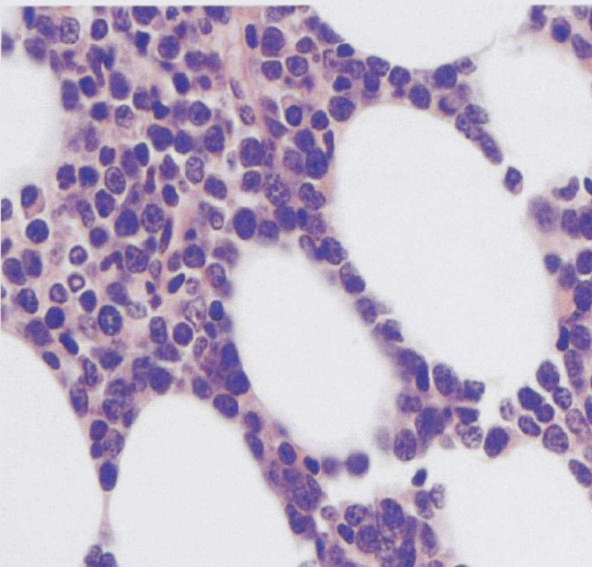


Figure from: Arber DA, George TI. Bone marrow biopsy involvement by non-Hodgkin lymphoma: frequency of lymphoma types, patterns, blood involvement, and discordance with other sites in 450 specimens. Am J Surg Pathol. 2005 Dec;29(12):1549-57.

It is recommended that the extent and pattern of infiltration be reported along with the cell morphology as this has clinical implications. **Table 2.4** summarizes the published studies on BM histology in DLBCL. Yan *et al.* studied 60 adult patients with aggressive B-cell NHL and BM involvement at initial presentation. They demonstrated that the presence of concordant BM involvement with >50% large cells, diffuse infiltration and increased amount of BM infiltration (>70%) correlated with worse overall survival. On multivariate analysis, they found only the pattern of BM involvement to have an impact on survival.[96] More recently, Campbell *et al.* reviewed BMs in their series of newly diagnosed DLBCL patients (*n*=172) and noted BM involvement in 27% of patients. They found that within this group, a greater degree of BM infiltration correlated with a greater incidence of concordant involvement and that these parameters correlated inversely with survival outcomes.[71] Chung *et al.* reported their findings in an even larger cohort of patients (*n*=489) and found ~11% BM positivity rate. They found that although patients with BM involvement had a poorer outcome compared to those who did not have BM involvement, those with concordant BM disease had a particularly bad outcome. They found the presence of large cells in the BM to have prognostic significance independent of the IPI.[72]

TABLE 2.4:

Summary of studies on bone marrow histology in diffuse aggressive B-cell NHL

Author, Year of publication	*N*	**Restaging BM included**	**% Positivity on histology**	**Histological characteristics associated with outcome**	**Outcome measures**
Yan *et al.*, 1995*	60	No	100	Pattern of infiltration	OS
Campbell *et al.*, 2006	172	No	27	Degree of infiltration, concordant involvement	OS, PFS
Chung *et al.*, 2007	489	No	11	Concordant involvement	OS

OS, Overall survival; PFS, progression-free survival; BM, bone marrow
*Included cases with BM involvement only; NA, information not available.

Overall, rates of BM involvement at initial diagnosis in DLBCL vary from ~11%[72] to >25%.[71, 97]

2.10.2.2. Flow cytometric analysis of BM samples:

BM aspirate samples can be submitted for flow cytometric analysis which is an important tool in the diagnosis and characterization of lymphoid malignancies. Owing to the ability to rapidly and sensitively analyse multiple properties of cells in small samples and obtain quantitative results, it can be a useful complement to histological diagnosis. There is limited data on the rates of BM involvement in DLBCL and on its impact on clinical outcomes. [98]

Reported rates of histologically inapparent lymphoma detected by flow cytometry vary depending on the study design and histological subtype. Most studies have addressed the role of flow cytometry in B-cell NHL as a single disease entity rather than studying specific subtypes and have often included restaging marrows in addition to the initial diagnostic ones. Palacio *et al.* found only three histology negative, flow cytometry positive cases in his series of 79 patients (4% discordance), of which the first was a diagnostic DLBCL case, the second a diagnostic follicular lymphoma case, and the third a follow-up follicular lymphoma case.[97] Perea *et al.* found, in their series of 388 B-cell NHL samples including 58 (15%) DLBCL cases, that there was discordance in 82 (21%) cases with 46 morphology positive, flow cytometry negative (BM+/ FC–) cases and 36 morphology negative, flow cytometry positive (BM–/FC+) cases.[99] The 58 cases of DLBCL included 3 (5.2 %) BM–/FC+ cases and 12 (20.7%) BM+/FC– cases. Thus the ability of flow cytometry to detect histologically inapparent disease in DLBCL was low compared to those in follicular lymphoma (24/188, 13%). Duggan *et al.* included staging and restaging BM in their series, but excluded cases with chronic lymphocytic leukaemia (CLL).[100] The total number included 227; however specific

subtypes are not listed in the paper.[100] Of the 27 cases (12%) detected by flow cytometry with negative histology, more cases (*n*=12/27, 44.4%) were of the follicular subtype as compared to DLBCL cases (*n*=4/27, 14.8%). These two studies are in contrast to the findings of Naughton *et al.*, who although reporting very low rates of BM–/FC+ cases among his series of B-cell NHL (3/273, 1.09%) made the interesting observation that two of the three cases were those of large cell lymphoma.[101] Hanson *et al.*, similarly, reported that all five of the 175 B-cell NHL cases (2.9%) that were BM–/FC+ were those of large cell lymphoma. All of these patients were clinically characterised by bulky, stage I disease.[102] In their series of 110 patients, Sah *et al.* addressed the issue of flow cytometry in chronic lymphoid disorders (chronic lymphocytic leukaemia=65, B-cell NHL=39 and hairy cell leukaemia=6), and found 3/39 (8%) cases of B-cell NHL had flow cytometric involvement in the presence of negative histology.[103] Similarly, Dunphy found that 5/188 (2.6%) cases (including 20 cases of DLBCL) had histologically inapparent flow cytometric positivity.[104] Although there are no specific studies on rates of flow cytometric positivity in DLBCL, overall, most studies have shown flow cytometry to make a small but useful addition to histological analysis of BM in NHL[101, 102] in terms of increasing the yield of BM detection. **Table 2.5** summarizes published data in NHL, and particularly, DLBCL. **Figure 2.10** demonstrates a flow cytometric histogram with light chain restriction in a case with B-cell NHL.

TABLE 2.5:

Summary of comparative studies on occult marrow involvement using flow cytometry and impact on outcome measures

Author, year of publica tion	*n*	Lymphoma subtypes	Restaging BM included	% Discor- dance	BM^- $/FC^+$	BM^+/FC^-	Outco me
Palacio *et al.*, 2001	79	DLBCL (31), FL (48)	No	4%	3, 3.8% (1 DLBCL, 2 FL)	0	NA
Perea *et al.*, 2004	380	FL (188), DLBCL (58), MCL (57), MZL (31), WM (25), ^BL (7), others (14)	Yes	21%	36, 9.5% (DLBCL : 3/58, 5.2%)	46, 12% (DLBCL: 12, 20%)	NA
Duggan *et al.*, 2000	227	Not listed	Yes	22%	27, 12% (DLBCL : 4/27, 14.8%#)	16, 7%	NA
Naught on *et al.*, 1998	273	DLBCL (141), FL (76), MCL (8), SLL (9), BL (8), B-ALL (5), T-NHL (6) others (20)	Yes	10%	3, 1.1% (2 DLBCL)	25, 9.1%	NA
Hanson *et al.*, 1999	175	DLBCL (74), FL (58), MZL (12), MCL (10), SLL (9), WM (9), BL (1), others (2)	No	9%	5, 3% (5 DLBCL)	10, 5.7%	NA
Sah *et al.*, 2003	110	CLL (65), low grade NHL (39), HCL (6)	Yes	CLL: 12%, NHL: 31%	CLL: 4/65, 6.2% NHL: 3/39, 8%	CLL: 2/65, 3.1% NHL: 2/39, 5.1%	NA

FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; WM, Waldenstrom's macroglobulinaemia; BL, Burkitt lymphoma; SLL, small lymphocytic lymphoma; CLL, chronic lymphocytic leukaemia; B-ALL, B-cell acute lymphoblastic lymphoma; HCL, hairy cell leukaemia; BM, bone marrow; FL, flow cytometry; OS, overall survival; NA, information not available. #: 14.8% refers to the % of DLBCL amongst the BM-/FC+ cases, and not the toal number of DLBCL cases.

Figure 2.10:

This example of flow cytometric involvement of the bone marrow in B-cell NHL demonstrates kappa light chain restriction.[102]

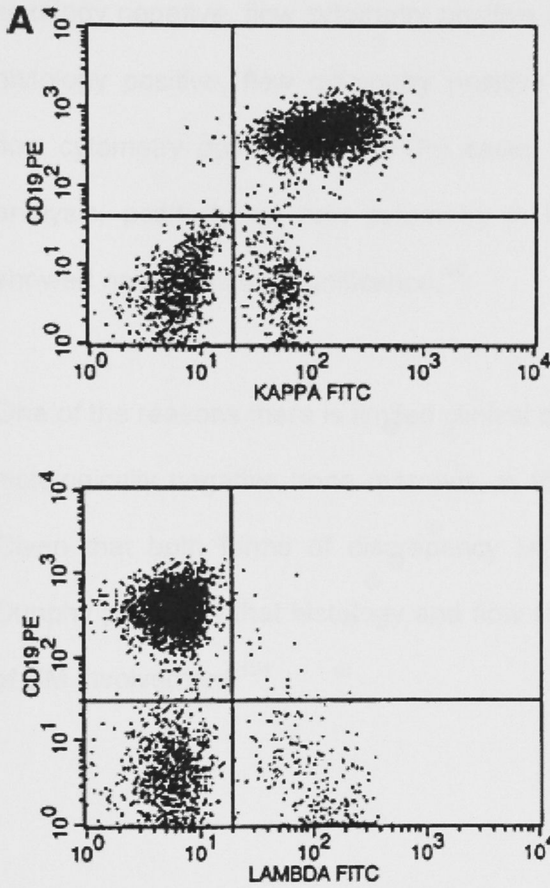


Figure from: Hanson CA, Kurtin PJ, Katzmann JA, Hoyer JD, Li CY, Hodnefield JM, et al. Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. Blood. 1999 Dec 1;94(11):3889-96.

Only a few studies have looked at the clinical impact of flow cytometric involvement on BM in NHL. Perea *et al.* studied outcome measures in a subgroup of follicular lymphoma patients and found no significant differences between the groups of patients that were histology negative, flow cytometry negative (BM–/FC–) and histology negative, flow cytometry positive (BM–/FC+).[30] However, as compared to histology positive, flow cytometry positive cases (BM+/FC+), histology negative, flow cytometry positive (BM–/FC+) cases did significantly better. On multivariate analysis, positivity on flow cytometry in histologically negative or occult cases showed no prognostic significance.[99]

One of the reasons there is limited clinical data on positive flow cytometry results in histologically negative bone marrows, is that the number of such cases is small. Given that both forms of discrepancy i.e. BM+/FC– and BM–/FC+ are known, Dunphy proposed that histology and flow cytometry be combined in the evaluation of BM involvement.[104]

2.10.2.3. Immunohistochemical analysis of the BM:

IHC is currently seen more as a tool for characterizing tumours in primary diagnostic tissue specimens rather than staging BM, and many centres around the world continue to use IHC in a limited manner on BM trephines. Currently its role is limited to histologically ambiguous cases, primarily in differentiating malignant from benign lymphoid aggregates. This seems to be related to the lack of published evidence regarding its value in initial staging. Haematologists appear to prefer immunophenotypic analysis using flow cytometry on BM aspirates. Perhaps this is because the results obtained with flow cytometry are quantitative and are considered to be less prone to errors or because traditionally immunohistochemistry has been reported by histopathologists rather than haematologists. In addition, it is known that routine histology and IHC may be subjective, particularly in inexperienced hands.[105-107]

Most published studies **(Table 2.6)** on the use of IHC in detection of lymphoma in the bone marrow have confined the study group to indeterminate cases on histology or have not reported results for routine histology and immunostaining separately so that comparisons cannot be easily made.[97, 99, 102, 108, 109] Chetty *et al.* looked at 27 cases with follicular lymphoma and found 5 cases that were negative histologically but had minimal involvement on IHC.[110] In an analysis of 42 anaplastic large cell lymphoma cases, Fraga *et al.* reported involvement on routine histology in 17% cases. The group performed routine immunohistochemical analysis using CD30 in all cases, and detected occult disease in a further 23% of cases.[111] Hanson *et al.* have previously reported no benefit from flow cytometry or immunohistochemistry to routine histological diagnosis in all subtypes of NHL.[102]

TABLE 2.6:

Summary of comparative studies on discordance between bone marrow histology and immunohistochemistry

Author, year of publication	*N*	NHL subtypes	BM$^-$/IHC$^+$	BM$^+$/ IHC$^-$	Outcome measures
Chetty *et al.*, 1995	27	FL	5, 18.5%	NA	NA
Fraga *et al., 1995*	42	ALCL	NA, 23%	NA	OS
Hanson *et al., 1999*	175	DLBCL (74) FL (58) MZL (12) MCL (10) SLL (9), WM (9), BL (1) others (2)	1, 0.5%	3, 1.7%	NA

FL, follicular lymphoma; ALCL, anaplastic large cell lymphoma; DLBCL, diffuse large B-cell lymphoma; OS, overall survival; NA, information not available.

2.10.2.4. Molecular staging of BM using IgH and IgL analysis:

Analysis of clonality of B-cell lymphoid populations using polymerase chain reactions (PCR) of immunoglobulin (Ig) genes can be a useful supplementary tool in the diagnosis of B-cell lymphoproliferative disorders.

PCR is simple and easy to use[112, 113] and can be applied to small fresh samples as well as archived paraffin-embedded samples.[114, 115] The immunoglobulin heavy chain (IgH) is usually targeted as most B-cell lymphoproliferative disorders have rearranged IgH genes.[116]

However, somatic mutations involving the IgH genes are known to occur in up to 30% of DLBCL and can render the genes unamenable to amplification.[114, 116] False negative results can also result from improper annealing of PCR primers to gene segments.[117] Lack of standardization of PCR protocols resulted initially in different groups developing individual primer sets with differing sensitivity and applicability with most studies showing a sensitivity of ~60 %.[118, 119] However, the development and standardization of the BIOMED-2 multiplex PCR protocols by the BIOMED-2 European Consortium increased the rates of molecular positivity in histologically proven B-cell neoplasms to unprecedented high levels of 99% (98% in DLBCL).[117] Confirming the results of previous studies, they reported the additional value of immunoglobulin light chain (IgL) analysis and recommended a combination of the two strategies to maximize clonality detection **(Figure 2.11)**.

Figure 2.11:

Examples of clonal bands in FR2 IgH clonality assay (panel A) and kappa light chain clonality assay (panel B). [117]

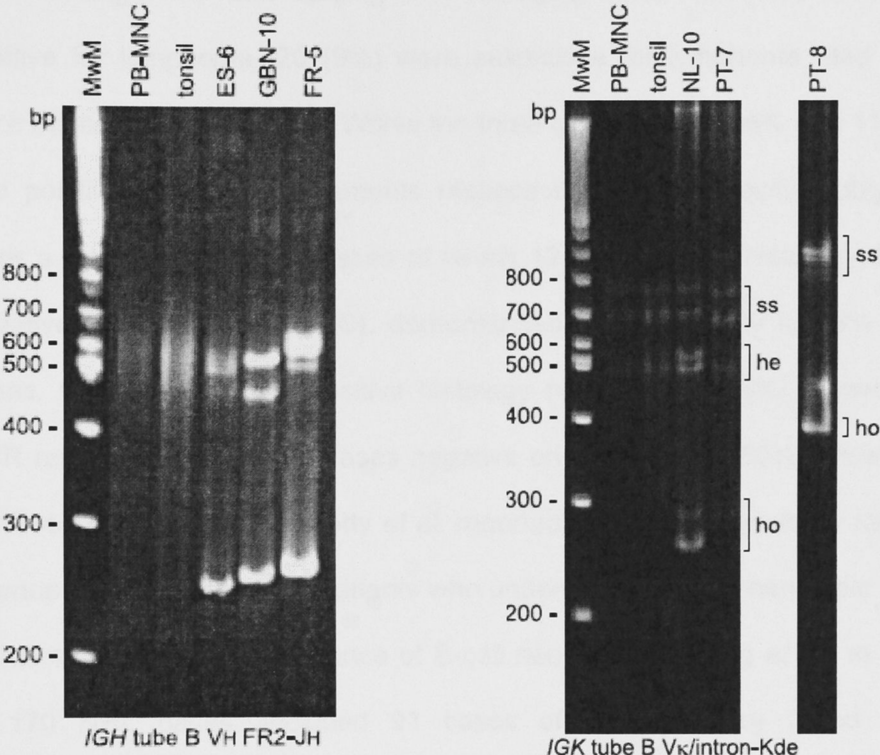


Figure from: van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003 Dec;17(12):2257-317.

Results from a number of studies show a degree of overlap with histology and with other diagnostic modalities[118-120]. One of the earliest studies to correlate the findings of BM histology and PCR for IgH gene rearrangements was by Coad *et al.*[118] Amongst the 225 staging and restaging bone marrows, 60 (27%) were positive for lymphoma, 20 (9%) were suspicious for lymphoma, and 123 (54%) were negative for lymphoma. Within the three groups, 57%, 25% and 11% of cases had positive gene rearrangements respectively. Within specific subtypes, there were a total of 85 DLBCL cases of which 12 had positive histology and 62 had negative BM histology. DLBCL demonstrated PCR positivity in 58% of positive cases. Of the cases with positive histology (*n* =12), 5 (41.6%) showed negative PCR results, and of the 62 cases negative on histology, 6 (10%) showed positive PCR reactions. Similarly, Crotty *et al.* reported a 67% PCR positivity rate amongst a group of 100 consecutive patients who underwent immunophenotypic analysis, of which 40 had objective evidence of B-cell neoplasia.[119] Kang *et al.*, in their series of 170 NHL cases, included 91 cases of DLBCL. They found histological involvement in 21 (23%) cases and observed PCR positivity in only 12 (57%) of these cases.[121] More interestingly, they found 42/70 (60%) cases with negative histology had positive PCR using IgH alone, which is much higher than rates reported in other studies. In a landmark study, Mitterbauer-Hohendanner *et al.* demonstrated the prognostic value of IgH and IgL gene rearrangements in 155 patients with DLBCL. They reported clonal IgH +/– IgL gene rearrangements in 14/19 (74%) cases with histological involvement. Occult molecular positivity was noted in 21/130 (16%) cases. They found a significant difference in overall survival at 5 years in patients with negative histology and negative PCR results (66%), negative histology but positive PCR results (37%), and positive histology as well as

positive PCR results (12%).[73] However, they used fresh peripheral blood and bone marrow aspirate samples, and as discussed in previous sections, BM involvement in DLBCL is seen more often on trephine rather than aspirate samples. Previous reports have demonstrated that PCR results correlate better with trephine samples as compared to aspirate samples. In one study, 10 out of 11 cases with established NHL had negative PCR results on BM aspirates but positive PCR results on trephine biopsy specimens.[118] Thus the yield from molecular staging is likely to be improved with the use of trephine samples rather than aspirates. **Table 2.7** lists relevant studies in NHL and DLBCL.

TABLE 2.7:

Summary of comparative studies on PCR positivity on bone marrow samples in NHL

Author, year of publication	*N*	NHL subtype	Restaging BM included	BM^+/PCR^-	BM^-/PCR^+
Coad *et al.*, 1997	225	FL (86), DLBCL (85), misc* (54)	Yes	26/60, 43.3% (DLBCL: 5/12, 41.6%)	13/123, 11% (DLBCL: 6/62, 10%)
Crotty *et al.*, 1998	60	Not specified	No	13/40#, 32.5%	NA
Kang *et al.*, 2002	170	FL (7), MCL (8) MZL (9) DLBCL (91) SLL (5) BL (4) B-ALL (1) others (5)	Yes	9/21, 42.9%	42/70, 60%
Mitterbauer *et al.*, 2004	155	DLBCL	No	5/19, 26.3%	21/130, 16%

DLBCL, diffuse Large B-cell Lymphoma; NHL, non-Hodgkin lymphoma; BM, bone marrow; PCR, polymerase chain reaction; NA, information not available.

*Including small lymphocytic lymphoma, mantle cell lymphoma, Burkitt lymphoma, marginal zone lymphoma, myeloma, cutaneous B-cell lymphoma, splenic lymphoma with villous lymphocytes.

40 refers to the total number of cases on which clonality was detected, and not histologically positive cases.

2.11. Prognostic systems:

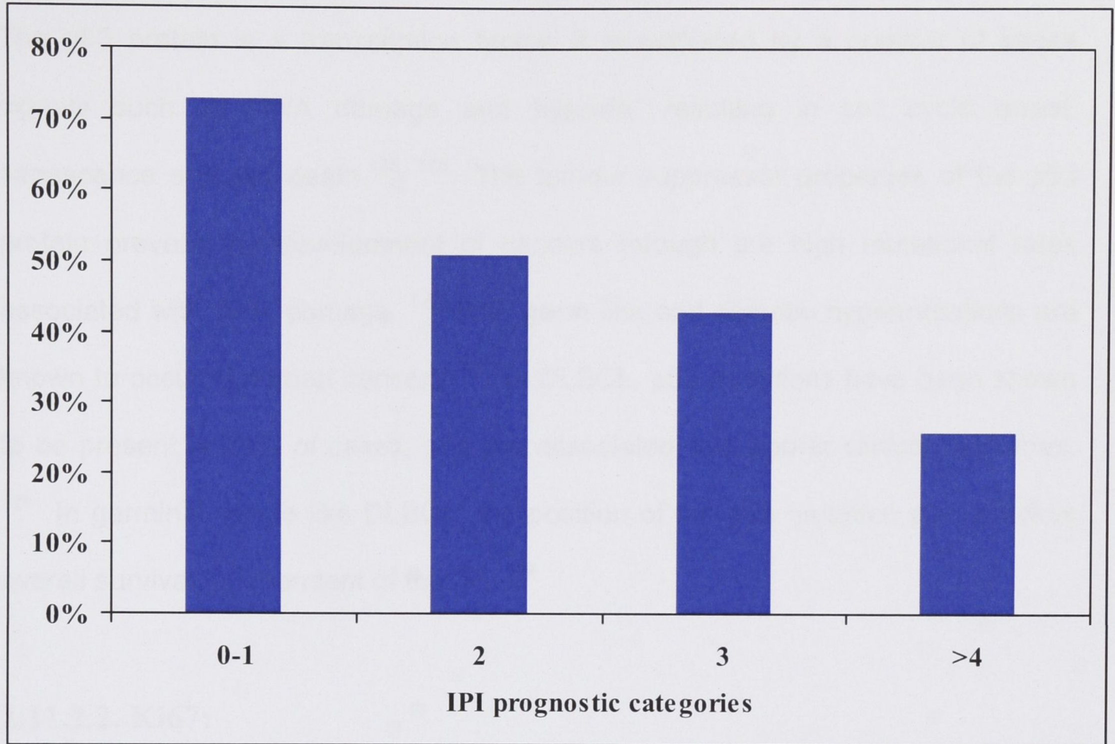
The variable outcomes noted in patients with DLBCL reflect the heterogeneous nature of DLBCL, with varied clinical features and genetic abnormalities. Ann Arbor staging has been used to determine prognosis in NHL.[122] This staging system was developed for patients with Hodgkin Lymphoma where the spread of lymphoma occurs in a contiguous manner to adjacent lymph nodes.[122] As the spread of disease in NHL occurs in a more unpredictable manner[123], the Ann Arbor classification is not as accurate in NHL.[124]

2.11.1. International Prognostic Index (IPI):

The landmark study performed under the Non-Hodgkin's Lymphoma Prognostic Factors Project attempted to classify prognosis for intermediate grade lymphoma using a number of clinical and laboratory pre-treatment characteristics [5]. The proposed International Prognostic Index (IPI) is widely used as a prognostic tool in DLBCL. The Index uses a number of negative pre-treatment clinical and laboratory characteristics to risk-stratify cases. These include: age > 60 years; stage III/IV disease; number of extranodal sites (>1); high lactate dehydrogenase (LDH) levels; and poor performance status measured using the Eastern Cooperative Oncology Group (ECOG) score (≥ 2).[5] Each negative characteristic is assigned a score of 1, and depending on the total score, cases are risk-stratified as low, low-intermediate and high-intermediate and high-risk groups with approximate 5-year survivals of 73%, 51%, 43%, and 26% respectively. This information is currently used to determine treatment and to predict outcome. **(Ref: Figure 1.1)**

Figure 1.1:

Overall survival based on IPI prognostic categories.



However, heterogeneity in clinical outcomes within prognostic scores is well-recognized. This is generally attributed to genetic and molecular prognostic biomarkers which control tumour progression, aggressiveness of disease and patient survival. These include genetic changes within cell cycle regulatory proteins such as TP53, Cyclin D1 and Ki67, aberrations within apoptotic proteins such as BCL2 and caspases and abnormalities in B-cell ontogenic markers such as BCL6, CD5 and CD10. [9]

2.11.2. Cell regulatory proteins:

2.11.2.1. p53:

The p53 protein is a transcription factor. It is activated by a number of stress signals such as DNA damage and hypoxia, resulting in cell cycle arrest, senescence and cell death.[125, 126] The tumour suppressor properties of the p53 protein prevent the development of cancers through the high mutational rates associated with DNA damage. [125] Both germ line and somatic hypermutations are known to occur in human cancers. [127] In DLBCL, p53 mutations have been shown to be present in 24% of cases, and are associated with poorer clinical outcomes. [128] In germinal centre like DLBCL, the position of the p53 mutation also predicts overall survival independent of the IPI. [128]

2.11.2.2. Ki67:

This is a nuclear antigen found in dividing cells and is a marker of proliferative activity as measured by immunohistochemistry. [129] Variable results have been noted in studies assessing the impact of Ki67 on overall survival in DLBCL. [19, 29, 44, 129, 130] The variation in results relate to variable definitions of positivity, and to the fact that Ki67 expression may affect resistance to chemotherapy rather than having a direct effect on survival. [131]

2.11.3. Apoptotic proteins:

2.11.3.1. BCL2:

Translocations involving BCL2 by themselves do not correlate with outcome measures in DLBCL. [45, 132, 133] High BCL2 expression however, has been shown to correlate with poor disease free survival (DFS) in DLBCL. [132] This effect appears to be offset in patients treated with Rituximab. [134]

2.11.4. B-cell ontogenic proteins:

2.11.4.1. BCL6:

High expression of BCL6 has been shown to be associated with a better prognosis in some [35, 44] but not all studies. [37] This may be related to whether the BCL6 translocation involves one of the three immunoglobulin loci or a non-immunoglobulin partner. [135] The BCL6/non-Ig translocation confers a worse prognosis than the BCL6/Ig translocation.[135]

2.11.2.2. Others:

Several other individual B-cell ontogenic proteins including CD10, CD5, FOXP1, HGAL, PKC-β, CD21, CD44, Vascular endothelial growth factor, Endostatin, and Matrix metalloproteinase 9 have been shown to affect prognosis is some studies.

2.11.5. Morphologic variants:

Although several morphologic variants of DLBCL are recognised, these are not definitely associated with outcome. [136]

2.11.6. Gene expression profile:

Owing to the inability of individual prognostic biomarkers to accurately refect the complexity of biologic processes causing the heterogeneity of malignant cells in DLBCL, large scale gene expression profiling using DNA microarrays has been adopted in recent years. This technique allows the analysis of thousands of genes or expressed sequences simultaneously in tumour specimens and therefore, is better able to explore the relationships between prognosis and the molecular features of tumours. DNA microarrays are created in 2 basic forms:

1) Spotted arrays: These are made by deposition of cDNA (PCR amplified, double-stranded) which are approximately 500 base pair (bp) long or long oligonucleotides (50-70 bp) on a glass slide. The Lymphochip is an example of a spotted array and was designed with a selection of genes known/ suspected to have a role in tumour biology and/ or preferentially expressed in lymphoid cells.

2) Oligonucleotide arrays: are generated by in-situ synthesis of short oligonucleotide fragments 20-24 bp long. The Affymetrix array (Affymetrix Inc, Santa Clara, CA) is a commercially available example of an oligonucleotide array.

Both techniques analyse thousands of genes simultaneously and can be applied to tRNA obtained from fresh (frozen) or paraffin-embedded tissue. Different detection methods with specially designed computer software are used to analyse the levels of gene expression of tumour tissue, generating gene expression profiles. Both supervised (structured depending on prior knowledge about tumour samples) and

unsupervised (structured without regard for prior knowledge) methods of analysis can be used. [137] **(Table 2.8)**

Table 2.8:

Comparison of the gene expression platforms used in DLBCL studies. [31]

Characteristic	**Affymetrix**	**Lymphochip**	**Real-time Taqman PCR**
Platform	Commercially available in-situ synthesized oligonucleotide array	Custom-spotted PCR amplified cDNA array	Commercially available primer/ probe preloaded low density arrays
No. of genes tested	Thousands	Thousands	Tens
Target specificity	High	Good	Very high
Relative dynamic range of expression	Intermediate	Lowest	Highest
RNA source	Frozen/ fresh tissue	Frozen/ fresh tissue	Frozen/ fresh tissue/? FFPE
RNA quality	5-10 µg tRNA	5-10 µg tRNA	20-30 ng/gene

Table from: Abramson JS, Shipp MA. Advances in the biology and therapy of diffuse large B-cell lymphoma: moving toward a molecularly targeted approach. Blood. 2005 Aug 15;106(4):1164-74.

The pivotal study using micro arrays in DLBCL used an unsupervised approach on the Lymphochip cDNA array. [7] Tumour samples from 42 patients treated with anthracycline based chemotherapy were analysed using a hierarchical clustering learning method, resulting in the identification of two distinct subgroups of DLBCL based on GC signature genes and activated B-cell signature genes[7] **(Figure 2.12).** Some of the genes in these signatures were those that previously identified as individual prognostic biomarkers. These sub-groups had different clinical profiles with 5 year survival for GC subtypes being significantly better than activated B-cell subtypes in a proportion of low-risk DLBCL patients (76% vs. 16%, p<0.01).

Figure 2.12:

Gene profiling in DLBCL. [7]

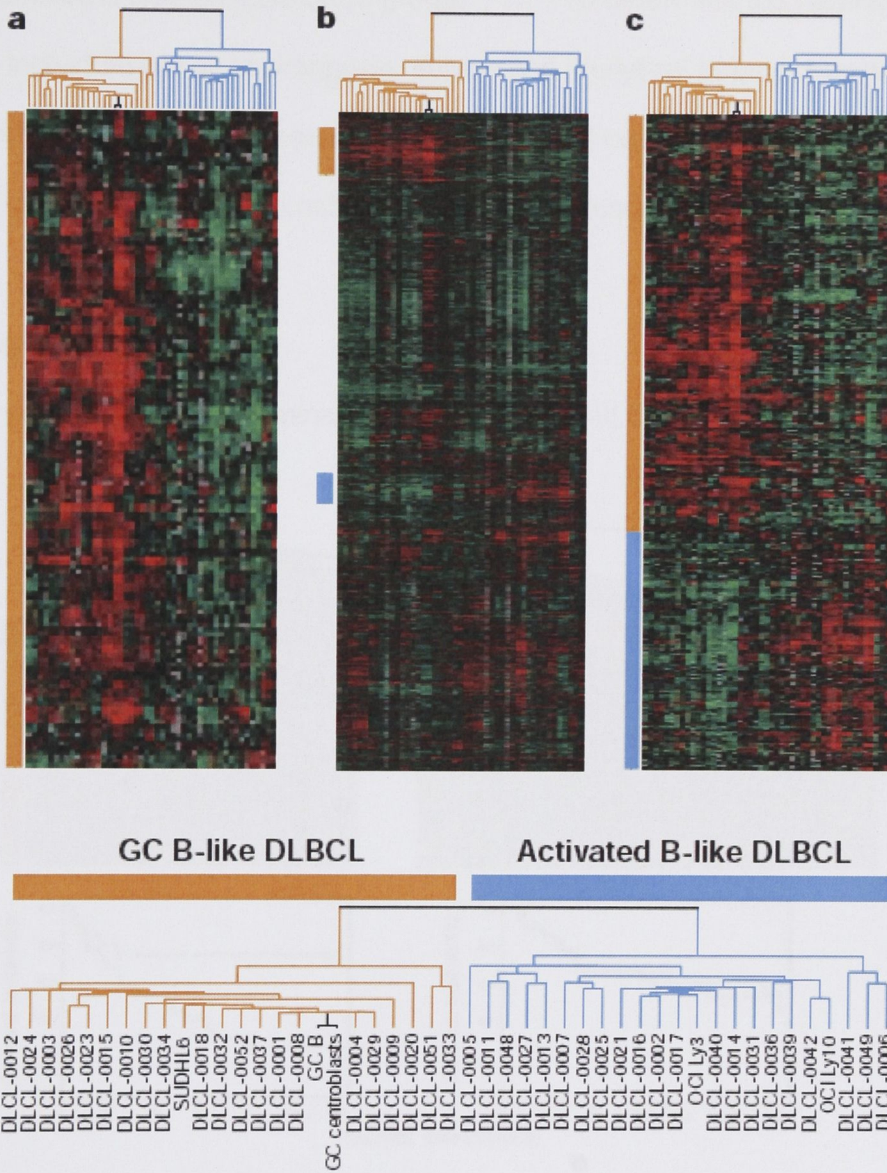


Figure from: Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000 Feb 3;403(6769):503-11.

This was later substantiated in a larger study on 240 patients performed by the Lymphoma and Leukaemia Molecular Profiling Project (LLMPP), which also identified a type 3 DLBCL sub-group. [138, 139] To determine the relative contribution of individual genes to prognosis, supervised analytical approaches were then adopted by several groups. [7, 138, 140] Shipp et al have used oligonucleotide arrays **(Figure 2.13)** [140] while Lossos and colleagues used RT-PCR. [44]

Figure 2.13:

Predictive value of germinal centre and activated B-cell signatures.

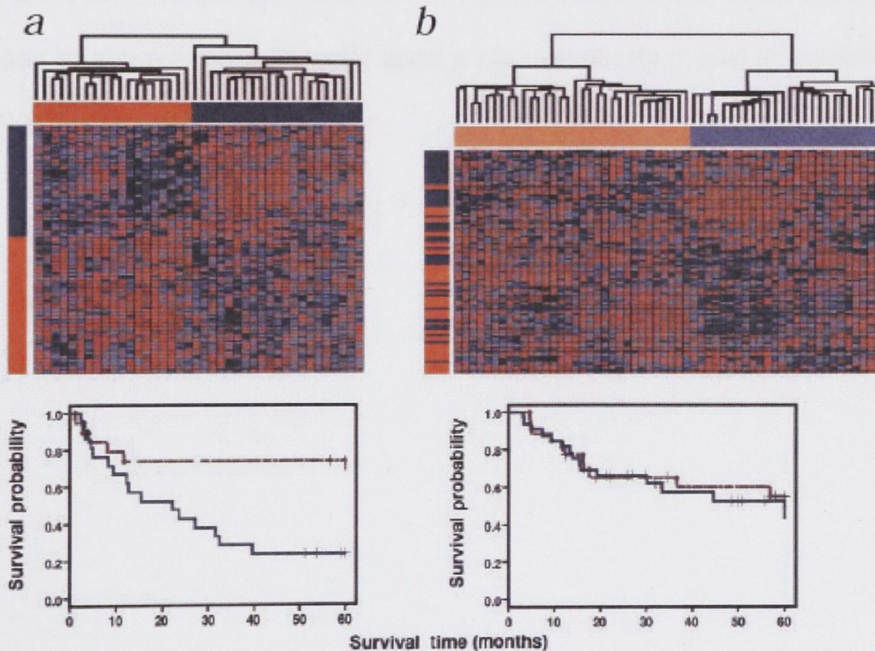


Figure from: Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC, et al. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med. 2002 Jan;8(1):68-74.

Examination of mRNA expression targeting hundreds of genes is difficult to implement in clinical practice owing to the expense involved as well as the complexity of analysis. A set of 6 genes (LMO2, BCL6, FN1, CCND2, SCYA3, and BCL2) that were the strongest predictors was identified by Lossos et al and used to construct a survival prediction model. This has identified three prognostic subgroups of DLBCL patients with low-risk, intermediate-risk and high-risk of dying. [44] Tissue micro arrays (TMA) which allow high-throughput protein expression studies have been used to develop IHC prognostic models using 3 antibodies against CD10, MUM1, and BCL6. [141] Barrans showed that IHC using CD10, BCL2 and BCL6 in conjunction with the IPI, could improve risk stratification in DLBCL[142]. Saez et al have more recently used a eight-antibody model in combination with the IPI to predict outcome. [143]

2.12. Treatment:

The first report of successful treatment of DLBCL was in 1972 [144]. Cyclophosphamide, Vincristine, Doxorubicin and Prednisolone (CHOP) became standard therapy based on phase II studies by Elias et al who reported OS rates of 39%. [145] In 1993, the US Intergroup study reported that the CHOP regimen was associated with similar rates of complete remission (CR), progression-free survival (PFS) and overall survival (OS) as that of more complicated and more toxic regimens. [146] The CHOP regimen resulted in CR rates of 30-40% OS at 5 years, and failure of response or progression of disease in ~30% cases. [146] Attempts to improve outcomes have been made by increasing dose frequency, and by dose-intensification. [147, 148] These studies by Pfreundschuh showed that increasing the frequency of treatment to every 14 days instead of 21 days improved outcomes both in the elderly and in young patients with aggressive DLBCL. In the young, addition of etoposide was noted to be more toxic but well tolerated and improved PFS but not OS, although this was not the case in the elderly population. [147, 148] The role of upfront autologous transplantation after completion of induction chemotherapy has been difficult to establish with studies reporting varied outcomes. [149-151]

Rituximab is a recombinant humanized monoclonal antibody against CD20. [152] It causes killing of CD20 positive tumour cells via a number of immune-mediated and direct mechanisms. [153] Rituximab was noted to be effective in phase II studies in aggressive B-cell lymphomas in the late 1990s. [154, 155] The first randomized control trial to report a benefit for elderly patients treated with R-CHOP came from the

Groupe d'Etude des Lymphomes de l'adulte (GELA) group in 2000 [156] and other reports with similar results followed. [68, 157] The role of rituximab as an addition to CHOP chemotherapy in young, good-risk DLBCL has also been established. [157, 158]

References:

1. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol. 1999 Dec;17(12):3835-49.

2. Morton LM, Wang SS, Devesa SS, Hartge P, Weisenburger DD, Linet MS. Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001. Blood. 2006 Jan 1;107(1):265-76.

3. Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood. 1994 Sep 1;84(5):1361-92.

4. Coiffier B. State-of-the-art therapeutics: diffuse large B-cell lymphoma. J Clin Oncol. 2005 Sep 10;23(26):6387-93.

5. Shipp MA, Harrington, D.P., Anderson, J.R., Armitage, J.O., Bonadonna, G., Brittinger, G., Cabanillas, F., Canellos, G.P., Coiffier, B., Connors, J.M., Cowan R.A., Crowther, D., Dahlberg, S., Engelhard, M., Fisher, R.I., Gisselbrecht, C., Horning, S.J., Lepage, E., Lister, T.A., Meerwald, J.H., Montserrat, E., Nissen, N.I., Oken, M.M., Peterson, B.A., Tondini, C., Velasquez, W.A., & Yeap, B.Y. . A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med. 1993 Sep 30;329(14):987-94.

6. Australian Cancer Network Diagnosis and Management of Lymphoma Guidelines Working Party: Guidelines for the Diagnosis and Mangement of

Lymphoma. Sydney: The Cancer Council Australia and Australian Cancer Network; 2005.

7. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000 Feb 3;403(6769):503-11.

8. Gascoyne RD. Emerging prognostic factors in diffuse large B cell lymphoma. Curr Opin Oncol. 2004 Sep;16(5):436-41.

9. Lossos IS, Morgensztern D. Prognostic biomarkers in diffuse large B-cell lymphoma. J Clin Oncol. 2006 Feb 20;24(6):995-1007.

10. Smithers DW. Summary of papers delivered at the Conference on Staging in Hodgkin's Disease (Ann Arbor). Cancer Res. 1971 Nov;31(11):1869-70.

11. Engelhard M, Brittinger G, Huhn D, Gerhartz HH, Meusers P, Siegert W, et al. Subclassification of diffuse large B-cell lymphomas according to the Kiel classification: distinction of centroblastic and immunoblastic lymphomas is a significant prognostic risk factor. Blood. 1997 Apr 1;89(7):2291-7.

12. Maes B, Anastasopoulou A, Kluin-Nelemans JC, Teodorovic I, Achten R, Carbone A, et al. Among diffuse large B-cell lymphomas, T-cell-rich/histiocyte-rich BCL and CD30+ anaplastic B-cell subtypes exhibit distinct clinical features. Ann Oncol. 2001 Jun;12(6):853-8.

13. Weisenburger DD, Anderson JR, Diebold J, Gascoyne RD, MacLennan KA, Muller-Hermelink HK, et al. Systemic anaplastic large-cell lymphoma: results from the non-Hodgkin's lymphoma classification project. Am J Hematol. 2001 Jul;67(3):172-8.

14. Hunt KE, Reichard KK. Diffuse large B-cell lymphoma. Arch Pathol Lab Med. 2008 Jan;132(1):118-24.

15. Prakash S, Swerdlow SH. Nodal aggressive B-cell lymphomas: a diagnostic approach. J Clin Pathol. 2007 Oct;60(10):1076-85.

16. Rodig SJ, Savage KJ, LaCasce AS, Weng AP, Harris NL, Shipp MA, et al. Expression of TRAF1 and nuclear c-Rel distinguishes primary mediastinal large cell lymphoma from other types of diffuse large B-cell lymphoma. Am J Surg Pathol. 2007 Jan;31(1):106-12.

17. Brown RS, Campbell C, Lishman SC, Spittle MF, Miller RF. Plasmablastic lymphoma: a new subcategory of human immunodeficiency virus-related non-Hodgkin's lymphoma. Clin Oncol (R Coll Radiol). 1998;10(5):327-9.

18. Teruya-Feldstein J. Diffuse large B-cell lymphomas with plasmablastic differentiation. Curr Oncol Rep. 2005 Sep;7(5):357-63.

19. Colomo L, Lopez-Guillermo A, Perales M, Rives S, Martinez A, Bosch F, et al. Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. Blood. 2003 Jan 1;101(1):78-84.

20. Teruya-Feldstein J, Chiao E, Filippa DA, Lin O, Comenzo R, Coleman M, et al. CD20-negative large-cell lymphoma with plasmablastic features: a clinically heterogenous spectrum in both HIV-positive and -negative patients. Ann Oncol. 2004 Nov;15(11):1673-9.

21. Delsol G, Lamant L, Mariame B, Pulford K, Dastugue N, Brousset P, et al. A new subtype of large B-cell lymphoma expressing the ALK kinase and lacking the 2; 5 translocation. Blood. 1997 Mar 1;89(5):1483-90.

22. Chikatsu N, Kojima H, Suzukawa K, Shinagawa A, Nagasawa T, Ozawa H, et al. ALK+, CD30-, CD20- large B-cell lymphoma containing anaplastic lymphoma kinase (ALK) fused to clathrin heavy chain gene (CLTC). Mod Pathol. 2003 Aug;16(8):828-32.

23. Alvaro-Naranjo T, Jaen-Martinez J, Guma-Padro J, Bosch-Princep R, Salvado-Usach MT. CD20-negative DLBCL transformation after rituximab treatment in follicular lymphoma: a new case report and review of the literature. Ann Hematol. 2003 Sep;82(9):585-8.

24. Doggett RS, Wood GS, Horning S, Levy R, Dorfman RF, Bindl J, et al. The immunologic characterization of 95 nodal and extranodal diffuse large cell lymphomas in 89 patients. Am J Pathol. 1984 May;115(2):245-52.

25. Stein H, Lennert K, Feller AC, Mason DY. Immunohistological analysis of human lymphoma: correlation of histological and immunological categories. Adv Cancer Res. 1984;42:67-147.

26. Harada S, Suzuki R, Uehira K, Yatabe Y, Kagami Y, Ogura M, et al. Molecular and immunological dissection of diffuse large B cell lymphoma: CD5+, and CD5- with CD10+ groups may constitute clinically relevant subtypes. Leukemia. 1999 Sep;13(9):1441-7.

27. Matolcsy A, Chadburn A, Knowles DM. De novo CD5-positive and Richter's syndrome-associated diffuse large B cell lymphomas are genotypically distinct. Am J Pathol. 1995 Jul;147(1):207-16.

28. Wilson WH, Teruya-Feldstein J, Fest T, Harris C, Steinberg SM, Jaffe ES, et al. Relationship of p53, bcl-2, and tumor proliferation to clinical drug resistance in non-Hodgkin's lymphomas. Blood. 1997 Jan 15;89(2):601-9.

29. Zhang A, Ohshima K, Sato K, Kanda M, Suzumiya J, Shimazaki K, et al. Prognostic clinicopathologic factors, including immunologic expression in diffuse large B-cell lymphomas. Pathol Int. 1999 Dec;49(12):1043-52.

30. Piris M, Brown DC, Gatter KC, Mason DY. CD30 expression in non-Hodgkin's lymphoma. Histopathology. 1990 Sep;17(3):211-8.

31. Abramson JS, Shipp MA. Advances in the biology and therapy of diffuse large B-cell lymphoma: moving toward a molecularly targeted approach. Blood. 2005 Aug 15;106(4):1164-74.

32. Klein U, Goossens T, Fischer M, Kanzler H, Braeuninger A, Rajewsky K, et al. Somatic hypermutation in normal and transformed human B cells. Immunol Rev. 1998 Apr;162:261-80.

33. Winter JN, Weller EA, Horning SJ, Krajewska M, Variakojis D, Habermann TM, et al. Prognostic significance of Bcl-6 protein expression in DLBCL treated with CHOP or R-CHOP: a prospective correlative study. Blood. 2006 Jan 31.

34. Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, de Waard R, et al. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. Nat Genet. 1997 Jun;16(2):161-70.

35. Ohno H. Pathogenetic role of BCL6 translocation in B-cell non-Hodgkin's lymphoma. Histol Histopathol. 2004 Apr;19(2):637-50.

36. Morgensztern D, Martin MG, Lossos IS. Gene expression profiling in diffuse large B-cell lymphoma. Leuk Lymphoma. 2007 Apr;48(4):669-82.

37. Kramer MH, Hermans J, Wijburg E, Philippo K, Geelen E, van Krieken JH, et al. Clinical relevance of BCL2, BCL6, and MYC rearrangements in diffuse large B-cell lymphoma. Blood. 1998 Nov 1;92(9):3152-62.

38. Shaffer AL, Yu X, He Y, Boldrick J, Chan EP, Staudt LM. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity. 2000 Aug;13(2):199-212.

39. Pasqualucci L, Migliazza A, Basso K, Houldsworth J, Chaganti RS, Dalla-Favera R. Mutations of the BCL6 proto-oncogene disrupt its negative

autoregulation in diffuse large B-cell lymphoma. Blood. 2003 Apr 15;101(8):2914-23.

40. Cattoretti G, Chang CC, Cechova K, Zhang J, Ye BH, Falini B, et al. BCL-6 protein is expressed in germinal-center B cells. Blood. 1995 Jul 1;86(1):45-53.

41. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 1990 Nov 22;348(6299):334-6.

42. Weiss LM, Warnke RA, Sklar J, Cleary ML. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. N Engl J Med. 1987 Nov 5;317(19):1185-9.

43. Lipford E, Wright JJ, Urba W, Whang-Peng J, Kirsch IR, Raffeld M, et al. Refinement of lymphoma cytogenetics by the chromosome 18q21 major breakpoint region. Blood. 1987 Dec;70(6):1816-23.

44. Lossos IS, Czerwinski DK, Alizadeh AA, Wechser MA, Tibshirani R, Botstein D, et al. Prediction of survival in diffuse large-B-cell lymphoma based on the expression of six genes. N Engl J Med. 2004 Apr 29;350(18):1828-37.

45. Hill ME, MacLennan KA, Cunningham DC, Vaughan Hudson B, Burke M, Clarke P, et al. Prognostic significance of BCL-2 expression and bcl-2 major breakpoint region rearrangement in diffuse large cell non-Hodgkin's lymphoma: a British National Lymphoma Investigation Study. Blood. 1996 Aug 1;88(3):1046-51.

46. Monni O, Joensuu H, Franssila K, Klefstrom J, Alitalo K, Knuutila S. BCL2 overexpression associated with chromosomal amplification in diffuse large B-cell lymphoma. Blood. 1997 Aug 1;90(3):1168-74.

47. Rao PH, Houldsworth J, Dyomina K, Parsa NZ, Cigudosa JC, Louie DC, et al. Chromosomal and gene amplification in diffuse large B-cell lymphoma. Blood. 1998 Jul 1;92(1):234-40.

48. Ladanyi M, Offit K, Jhanwar SC, Filippa DA, Chaganti RS. MYC rearrangement and translocations involving band 8q24 in diffuse large cell lymphomas. Blood. 1991 Mar 1;77(5):1057-63.

49. Chaganti RS, Nanjangud G, Schmidt H, Teruya-Feldstein J. Recurring chromosomal abnormalities in non-Hodgkin's lymphoma: biologic and clinical significance. Semin Hematol. 2000 Oct;37(4):396-411.

50. Offit K, Lo Coco F, Louie DC, Parsa NZ, Leung D, Portlock C, et al. Rearrangement of the bcl-6 gene as a prognostic marker in diffuse large-cell lymphoma. N Engl J Med. 1994 Jul 14;331(2):74-80.

51. Cigudosa JC, Parsa NZ, Louie DC, Filippa DA, Jhanwar SC, Johansson B, et al. Cytogenetic analysis of 363 consecutively ascertained diffuse large B-cell lymphomas. Genes Chromosomes Cancer. 1999 Jun;25(2):123-33.

52. Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R, et al. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature. 2001 Jul 19;412(6844):341-6.

53. Lossos IS. Molecular pathogenesis of diffuse large B-cell lymphoma. J Clin Oncol. 2005 Sep 10;23(26):6351-7.

54. Australian Institute of Health and Welfare, Australasian association of Cancer Registries. Cancer in Australia 2001. Canberra: Australian Institute of Health and Welfare, 2004.

55. Hartge P DS, Fraumeni JF, Jr. Hodgkin's and non-Hodgkin's lymphomas. Cancer Surv. 1994;19-20:423-53.

56. Devesa SS FT. Non-Hodgkin's lymphoma time trends: United States and international data. Cancer Res. 1992;52:5432s–40s.

57. Carli PM BM, Maynadie M, Bailly F, Caillot D, Petrella T. Increase in the incidence of non-Hodgkin's lymphomas: evidence for a recent sharp increase in France independent of AIDS. Br J Cancer. 1994;70:713–5.

58. Opelz G HR. Incidence of non-Hodgkin lymphoma in kidney and heart transplant recipients. Lancet. 1993;342:1514–6.

59. Grulich AE WX, Law MG, Coates M, Kaldor JM. Risk of cancer in people with AIDS. AIDS. 1999;13:839–43.

60. Franceschi S DML, La Vecchia C. Advances in the epidemiology of HIV-associated non-Hodgkin's lymphoma and other lymphoid neoplasms. Int J Cancer. 1999;83:481-85.

61. Grulich AE WX, Law MG, et al. B-cell stimulation and prolonged immune deficiency are risk factors for non-Hodgkin's lymphoma in people with AIDS. AIDS. 2000;14:133-40.

62. Filipovich AH MA, Kamat D, Shapiro RS. Primary immunodeficiencies: genetic risk factors for lymphoma. Cancer Res. 1992;52:5465s–7s.

63. N. M. Overview of the epidemiology of malignancy in immune deficiency. J Acquir Immune Defic Syndr. 1999;21 Suppl 1:S5–10.

64. International Agency for Research on Cancer , Working Group on the Evaluation of Carcinogenic Risks to Humans. Epstein-Barr virus and Kaposi's sarcoma herpes virus/human herpes virus 8. 1997;70 edn. Lyon: International Agency for Research on Cancer.

65. Helicobacter and Cancer Cooperative Group. Gastric cancer and Helicobacter pylori: a combined analysis of 12 case control studies nested within prospective cohorts. Gut. 2001;49:347–53.

66. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. Blood. 1997 Jun 1;89(11):3909-18.

67. Armitage JO, Weisenburger DD. New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. J Clin Oncol. 1998 Aug;16(8):2780-95.

68. Feugier P, Van Hoof A, Sebban C, Solal-Celigny P, Bouabdallah R, Ferme C, et al. Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. J Clin Oncol. 2005 Jun 20;23(18):4117-26.

69. Moller MB, Pedersen NT, Christensen BE. Diffuse large B-cell lymphoma: clinical implications of extranodal versus nodal presentation--a population-based study of 1575 cases. Br J Haematol. 2004 Jan;124(2):151-9.

70. Juneja SK, Wolf MM, Cooper IA. Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. J Clin Pathol. 1990 Aug;43(8):630-2.

71. Campbell J, Seymour JF, Matthews J, Wolf M, Stone J, Juneja S. The prognostic impact of bone marrow involvement in patients with diffuse large cell lymphoma varies according to the degree of infiltration and presence of discordant marrow involvement. Eur J Haematol. 2006 Jun;76(6):473-80.

72. Chung R, Lai R, Wei P, Lee J, Hanson J, Belch AR, et al. Concordant but not discordant bone marrow involvement in diffuse large B-cell lymphoma predicts

a poor clinical outcome independent of the International Prognostic Index. Blood. 2007 Aug 15;110(4):1278-82.

73. Mitterbauer-Hohendanner G, Mannhalter C, Winkler K, Mitterbauer M, Skrabs C, Chott A, et al. Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). Leukemia. 2004 Jun;18(6):1102-7.

74. Gascoyne RD. Establishing the diagnosis of lymphoma: from initial biopsy to clinical staging. Oncology (Williston Park). 1998 Oct;12(10 Suppl 8):11-6.

75. Hanson CA. Fine-needle aspiration and immunophenotyping. A role in diagnostic hematopathology? Am J Clin Pathol. 1994 May;101(5):555-6.

76. Levitt S, Cheng L, DuPuis MH, Layfield LJ. Fine needle aspiration diagnosis of malignant lymphoma with confirmation by immunoperoxidase staining. Acta Cytol. 1985 Sep-Oct;29(5):895-902.

77. Steel BL, Schwartz MR, Ramzy I. Fine needle aspiration biopsy in the diagnosis of lymphadenopathy in 1,103 patients. Role, limitations and analysis of diagnostic pitfalls. Acta Cytol. 1995 Jan-Feb;39(1):76-81.

78. Dong HY, Harris NL, Preffer FI, Pitman MB. Fine-needle aspiration biopsy in the diagnosis and classification of primary and recurrent lymphoma: a retrospective analysis of the utility of cytomorphology and flow cytometry. Mod Pathol. 2001 May;14(5):472-81.

79. Young NA, Al-Saleem TI, Ehya H, Smith MR. Utilization of fine-needle aspiration cytology and flow cytometry in the diagnosis and subclassification of primary and recurrent lymphoma. Cancer. 1998 Aug 25;84(4):252-61.

80. Meda BA, Buss DH, Woodruff RD, Cappellari JO, Rainer RO, Powell BL, et al. Diagnosis and subclassification of primary and recurrent lymphoma. The

usefulness and limitations of combined fine-needle aspiration cytomorphology and flow cytometry. Am J Clin Pathol. 2000 May;113(5):688-99.

81. Ben-Yehuda D, Polliack A, Okon E, Sherman Y, Fields S, Lebenshart P, et al. Image-guided core-needle biopsy in malignant lymphoma: experience with 100 patients that suggests the technique is reliable. J Clin Oncol. 1996 Sep;14(9):2431-4.

82. Silverman SG, Lee BY, Mueller PR, Cibas ES, Seltzer SE. Impact of positive findings at image-guided biopsy of lymphoma on patient care: evaluation of clinical history, needle size, and pathologic findings on biopsy performance. Radiology. 1994 Mar;190(3):759-64.

83. de Kerviler E, Guermazi A, Zagdanski AM, Meignin V, Gossot D, Oksenhendler E, et al. Image-guided core-needle biopsy in patients with suspected or recurrent lymphomas. Cancer. 2000 Aug 1;89(3):647-52.

84. Gossot D, de Kerviler E, Brice P, Mariette X, Meignin V, Cazals-Hatem D, et al. Surgical endoscopic techniques in the diagnosis and follow-up of patients with lymphoma. Br J Surg. 1998 Aug;85(8):1107-10.

85. Mann GB, Conlon KC, LaQuaglia M, Dougherty E, Moskowitz CH, Zelenetz AD. Emerging role of laparoscopy in the diagnosis of lymphoma. J Clin Oncol. 1998 May;16(5):1909-15.

86. Lefor AT. Laparoscopic interventions in lymphoma management. Semin Laparosc Surg. 2000 Jun;7(2):129-39.

87. Alberta Cancer Board. Clinical practice guidelines - Lymphoma. 2006.

88. The Haemato-oncology Task Force of British Committee for Standards in Haematology. BCSH guidelines on nodal non-Hodgkin's lymphoma. 2002.

89. Parker A BB, Devereux S, Gatter K, Jack A, Matutes E, Rooney N, Ross F, Wilkins B, Wotherspoon A, Ramsay A. . Best practice in lymphoma diagnosis and reporting. British Committee for Standards in Haematology. Royal College of Pathologists. 2008:1-52.

90. Parker A BB, Devereux S, Gatter K, Jack A, Matutes E, Rooney N, Ross F, Wilkins B, Wotherspoon A, Ramsay A. . Best Practice in Lymphoma Diagnosis and Reporting - Specific Disease Appendix. British Committee for Standards in Haematology. Royal College of Pathologists. 2008:52-60.

91. Bain BJ. Bone marrow aspiration. J Clin Pathol. 2001 Sep;54(9):657-63.

92. Cheson BD, Horning SJ, Coiffier B, Shipp MA, Fisher RI, Connors JM, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol. 1999 Apr;17(4):1244.

93. Wang J, Weiss LM, Chang KL, Slovak ML, Gaal K, Forman SJ, et al. Diagnostic utility of bilateral bone marrow examination: significance of morphologic and ancillary technique study in malignancy. Cancer. 2002 Mar 1;94(5):1522-31.

94. Campbell JK, Matthews JP, Seymour JF, Wolf MM, Juneja SK. Optimum trephine length in the assessment of bone marrow involvement in patients with diffuse large cell lymphoma. Ann Oncol. 2003 Feb;14(2):273-6.

95. Arber DA, George TI. Bone marrow biopsy involvement by non-Hodgkin's lymphoma: frequency of lymphoma types, patterns, blood involvement, and discordance with other sites in 450 specimens. Am J Surg Pathol. 2005 Dec;29(12):1549-57.

96. Yan Y, Chan WC, Weisenburger DD, Anderson JR, Bast MA, Vose JM, et al. Clinical and prognostic significance of bone marrow involvement in patients with diffuse aggressive B-cell lymphoma. J Clin Oncol. 1995 Jun;13(6):1336-42.

97. Palacio C, Acebedo G, Navarrete M, Ruiz-Marcellan C, Sanchez C, Blanco A, et al. Flow cytometry in the bone marrow evaluation of follicular and diffuse large B-cell lymphomas. Haematologica. 2001 Sep;86(9):934-40.

98. Stetler-Stevenson M, Braylan RC. Flow cytometric analysis of lymphomas and lymphoproliferative disorders. Semin Hematol. 2001 Apr;38(2):111-23.

99. Perea G, Altes A, Bellido M, Aventin A, Bordes R, Ayats R, et al. Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL). Histopathology. 2004 Sep;45(3):268-74.

100. Duggan PR, Easton D, Luider J, Auer IA. Bone marrow staging of patients with non-Hodgkin lymphoma by flow cytometry: correlation with morphology. Cancer. 2000 Feb 15;88(4):894-9.

101. Naughton MJ, Hess JL, Zutter MM, Bartlett NL. Bone marrow staging in patients with non-Hodgkin's lymphoma: is flow cytometry a useful test? Cancer. 1998 Mar 15;82(6):1154-9.

102. Hanson CA, Kurtin PJ, Katzmann JA, Hoyer JD, Li CY, Hodnefield JM, et al. Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. Blood. 1999 Dec 1;94(11):3889-96.

103. Sah SP, Matutes E, Wotherspoon AC, Morilla R, Catovsky D. A comparison of flow cytometry, bone marrow biopsy, and bone marrow aspirates in the detection of lymphoid infiltration in B cell disorders. J Clin Pathol. 2003 Feb;56(2):129-32.

104. Dunphy CH. Combining morphology and flow cytometric immunophenotyping to evaluate bone marrow specimens for B-cell malignant neoplasms. Am J Clin Pathol. 1998 May;109(5):625-30.

105. Chudgar U, Khanduri U. Intraobserver and interobserver reproducibility of the FAB classification in acute leukaemia. Indian J Pathol Microbiol. 1992 Jul;35(3):229-36.

106. Diebold J, Weisenburger D, MacLennan KA, Muller-Hermelink HK, Nathwani BN, Harris NL, et al. [Reproducibility and prognostic value of histopathological classifications of malignant lymphomas. Prolegomena for the 1st international classification proposed by WHO. Group of the non-Hodgkin's Malignant Lymphoma Classification Project]. Bull Acad Natl Med. 1998;182(7):1537-48; discussion 48-9.

107. Hanby AM, Hall PA, Rooney N, Dennis P, James P, Richman P, et al. An inter-observer and intra-observer variability study on the diagnosis of lymph node biopsy specimens. Eur J Cancer. 1992;28A(11):1858-62.

108. Mazur G, Halon A, Wrobel T, Jelen M, Kuliczkowski K. Contribution of flow cytometric immunophenotyping and bone marrow trephine biopsy in the detection of lymphoid bone marrow infiltration in non-Hodgkin's lymphomas. Neoplasma. 2004;51(3):159-63.

109. Stacchini A, Demurtas A, Godio L, Martini G, Antinoro V, Palestro G. Flow cytometry in the bone marrow staging of mature B-cell neoplasms. Cytometry B Clin Cytom. 2003 Jul;54(1):10-8.

110. Chetty R, Echezarreta G, Comley M, Gatter K. Immunohistochemistry in apparently normal bone marrow trephine specimens from patients with nodal follicular lymphoma. J Clin Pathol. 1995 Nov;48(11):1035-8.

111. Fraga M, Brousset P, Schlaifer D, Payen C, Robert A, Rubie H, et al. Bone marrow involvement in anaplastic large cell lymphoma. Immunohistochemical detection of minimal disease and its prognostic significance. Am J Clin Pathol. 1995 Jan;103(1):82-9.

112. Trainor KJ, Brisco MJ, Story CJ, Morley AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. Blood. 1990 Jun 1;75(11):2220-2.

113. Trainor KJ, Brisco MJ, Wan JH, Neoh S, Grist S, Morley AA. Gene rearrangement in B- and T-lymphoproliferative disease detected by the polymerase chain reaction. Blood. 1991 Jul 1;78(1):192-6.

114. Wan JH, Trainor KJ, Brisco MJ, Morley AA. Monoclonality in B cell lymphoma detected in paraffin wax embedded sections using the polymerase chain reaction. J Clin Pathol. 1990 Nov;43(11):888-90.

115. Achille A, Scarpa A, Montresor M, Scardoni M, Zamboni G, Chilosi M, et al. Routine application of polymerase chain reaction in the diagnosis of monoclonality of B-cell lymphoid proliferations. Diagn Mol Pathol. 1995 Mar;4(1):14-24.

116. Diss TC, Pan L. Polymerase chain reaction in the assessment of lymphomas. Cancer Surv. 1997;30:21-44.

117. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003 Dec;17(12):2257-317.

118. Coad JE, Olson DJ, Christensen DR, Lander TA, Chibbar R, McGlennen RC, et al. Correlation of PCR-detected clonal gene rearrangements with bone

marrow morphology in patients with B-lineage lymphomas. Am J Surg Pathol. 1997 Sep;21(9):1047-56.

119. Crotty PL, Smith BR, Tallini G. Morphologic, immunophenotypic, and molecular evaluation of bone marrow involvement in non-Hodgkin's lymphoma. Diagn Mol Pathol. 1998 Apr;7(2):90-5.

120. Maes B, Achten R, Demunter A, Peeters B, Verhoef G, De Wolf-Peeters C. Evaluation of B cell lymphoid infiltrates in bone marrow biopsies by morphology, immunohistochemistry, and molecular analysis. J Clin Pathol. 2000 Nov;53(11):835-40.

121. Kang YH, Park CJ, Seo EJ, Huh J, Kim SB, Kang YK, et al. Polymerase chain reaction-based diagnosis of bone marrow involvement in 170 cases of non-Hodgkin lymphoma. Cancer. 2002 Jun 15;94(12):3073-82.

122. Carbone PP, Kaplan HS, Musshoff K, Smithers DW, Tubiana M. Report of the Committee on Hodgkin's Disease Staging Classification. Cancer Res. 1971 Nov;31(11):1860-1.

123. Freeman C, Berg JW, Cutler SJ. Occurrence and prognosis of extranodal lymphomas. Cancer. 1972 Jan;29(1):252-60.

124. Rosenberg SA. Validity of the Ann Arbor staging classification for the non-Hodgkin's lymphomas. Cancer Treat Rep. 1977 Sep;61(6):1023-7.

125. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell. 1997 Feb 7;88(3):323-31.

126. Levine AJ, Vosburgh E. P53 mutations in lymphomas: position matters. Blood. 2008 Oct 15;112(8):2997-8.

127. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science. 1991 Jul 5;253(5015):49-53.

128. Young KH, Leroy K, Moller MB, Colleoni GW, Sanchez-Beato M, Kerbauy FR, et al. Structural profiles of TP53 gene mutations predict clinical outcome in diffuse large B-cell lymphoma: an international collaborative study. Blood. 2008 Oct 15;112(8):3088-98.

129. Grogan TM, Lippman SM, Spier CM, Slymen DJ, Rybski JA, Rangel CS, et al. Independent prognostic significance of a nuclear proliferation antigen in diffuse large cell lymphomas as determined by the monoclonal antibody Ki-67. Blood. 1988 Apr;71(4):1157-60.

130. Hasselblom S, Ridell B, Sigurdardottir M, Hansson U, Nilsson-Ehle H, Andersson PO. Low rather than high Ki-67 protein expression is an adverse prognostic factor in diffuse large B-cell lymphoma. Leuk Lymphoma. 2008 Aug;49(8):1501-9.

131. Jerkeman M, Anderson H, Dictor M, Kvaloy S, Akerman M, Cavallin-Stahl E. Assessment of biological prognostic factors provides clinically relevant information in patients with diffuse large B-cell lymphoma--a Nordic Lymphoma Group study. Ann Hematol. 2004 Jul;83(7):414-9.

132. Hermine O, Haioun C, Lepage E, d'Agay MF, Briere J, Lavignac C, et al. Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma. Groupe d'Etude des Lymphomes de l'Adulte (GELA). Blood. 1996 Jan 1;87(1):265-72.

133. Gascoyne RD, Adomat SA, Krajewski S, Krajewska M, Horsman DE, Tolcher AW, et al. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. Blood. 1997 Jul 1;90(1):244-51.

134. Mounier N, Briere J, Gisselbrecht C, Emile JF, Lederlin P, Sebban C, et al. Rituximab plus CHOP (R-CHOP) overcomes bcl-2--associated resistance to chemotherapy in elderly patients with diffuse large B-cell lymphoma (DLBCL). Blood. 2003 Jun 1;101(11):4279-84.

135. Ueda C, Akasaka T, Ohno H. Non-immunoglobulin/BCL6 gene fusion in diffuse large B-cell lymphoma: prognostic implications. Leuk Lymphoma. 2002 Jul;43(7):1375-81.

136. Salar A, Fernandez de Sevilla A, Romagosa V, Domingo-Claros A, Gonzalez-Barca E, Pera J, et al. Diffuse large B-cell lymphoma: is morphologic subdivision useful in clinical management? Eur J Haematol. 1998 Mar;60(3):202-8.

137. Johnston A, Salles G. Prognostic systems for lymphomas. Hematol Oncol Clin North Am. 2008 Oct;22(5):839-61.

138. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med. 2002 Jun 20;346(25):1937-47.

139. Rosenwald A, Staudt LM. Gene expression profiling of diffuse large B-cell lymphoma. Leuk Lymphoma. 2003;44 Suppl 3:S41-7.

140. Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC, et al. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med. 2002 Jan;8(1):68-74.

141. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004 Jan 1;103(1):275-82.

142. Barrans SL, O'Connor SJ, Evans PA, Davies FE, Owen RG, Haynes AP, et al. Rearrangement of the BCL6 locus at 3q27 is an independent poor prognostic factor in nodal diffuse large B-cell lymphoma. Br J Haematol. 2002 May;117(2):322-32.

143. Saez AI, Saez AJ, Artiga MJ, Perez-Rosado A, Camacho FI, Diez A, et al. Building an outcome predictor model for diffuse large B-cell lymphoma. Am J Pathol. 2004 Feb;164(2):613-22.

144. Levitt M, Marsh JC, DeConti RC, Mitchell MS, Skeel RT, Farber LR, et al. Combination sequential chemotherapy in advanced reticulum cell sarcoma. Cancer. 1972 Mar;29(3):630-6.

145. Elias L, Portlock CS, Rosenberg SA. Combination chemotherapy of diffuse histiocytic lymphoma with cyclophosphamide, adriamycin, vincristine and prednisone (CHOP). Cancer. 1978 Oct;42(4):1705-10.

146. Fisher RI, Gaynor ER, Dahlberg S, Oken MM, Grogan TM, Mize EM, et al. Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med. 1993 Apr 8;328(14):1002-6.

147. Pfreundschuh M, Trumper L, Kloess M, Schmits R, Feller AC, Rube C, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of elderly patients with aggressive lymphomas: results of the NHL-B2 trial of the DSHNHL. Blood. 2004 Aug 1;104(3):634-41.

148. Pfreundschuh M, Trumper L, Kloess M, Schmits R, Feller AC, Rudolph C, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of young patients with good-prognosis (normal LDH) aggressive

lymphomas: results of the NHL-B1 trial of the DSHNHL. Blood. 2004 Aug 1;104(3):626-33.

149. Haioun C, Lepage E, Gisselbrecht C, Salles G, Coiffier B, Brice P, et al. Survival benefit of high-dose therapy in poor-risk aggressive non-Hodgkin's lymphoma: final analysis of the prospective LNH87-2 protocol--a groupe d'Etude des lymphomes de l'Adulte study. J Clin Oncol. 2000 Aug;18(16):3025-30.

150. Gisselbrecht C, Lepage E, Molina T, Quesnel B, Fillet G, Lederlin P, et al. Shortened first-line high-dose chemotherapy for patients with poor-prognosis aggressive lymphoma. J Clin Oncol. 2002 May 15;20(10):2472-9.

151. Milpied N, Deconinck E, Gaillard F, Delwail V, Foussard C, Berthou C, et al. Initial treatment of aggressive lymphoma with high-dose chemotherapy and autologous stem-cell support. N Engl J Med. 2004 Mar 25;350(13):1287-95.

152. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood. 1994 Jan 15;83(2):435-45.

153. Maloney DG, Smith B, Rose A. Rituximab: mechanism of action and resistance. Semin Oncol. 2002 Feb;29(1 Suppl 2):2-9.

154. Coiffier B, Haioun C, Ketterer N, Engert A, Tilly H, Ma D, et al. Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study. Blood. 1998 Sep 15;92(6):1927-32.

155. Vose JM, Link BK, Grossbard ML, Czuczman M, Grillo-Lopez A, Gilman P, et al. Phase II study of rituximab in combination with chop chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma. J Clin Oncol. 2001 Jan 15;19(2):389-97.

156. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002 Jan 24;346(4):235-42.

157. Sehn LH, Donaldson J, Chhanabhai M, Fitzgerald C, Gill K, Klasa R, et al. Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. J Clin Oncol. 2005 Aug 1;23(22):5027-33.

158. Pfreundschuh M, Trumper L, Osterborg A, Pettengell R, Trneny M, Imrie K, et al. CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol. 2006 May;7(5):379-91.

CHAPTER III:

Pilot study

3.1 Occult bone marrow involvement in patients with diffuse large B-cell lymphoma: results of a pilot study. Pathology 2007, 39(6):580-585

Pathology (December 2007) 39(6), pp. 580–585

HAEMATOLOGY

Occult bone marrow involvement in patients with diffuse large B-cell lymphoma: results of a pilot study

DIPTI TALAULIKAR*, JANE E. DAHLSTROM†, BRUCE SHADBOLT‡, MICHELLE MCNIVEN§, AMY BROOMFIELD‡ AND MICHAEL PIDCOCK*

*Departments of *Haematology, †Anatomical Pathology, ‡Epidemiology, and §Molecular Pathology, The Canberra Hospital, Australian Capital Territory, Australia*

Summary

Aims: It is known that advanced stage disease in diffuse large B-cell lymphoma (DLBCL) confers a poor prognosis, and staging investigations are routinely performed at diagnosis, including a bone marrow (BM) biopsy. However, examination of the BM is usually limited to routine light microscopy, with the role of ancillary investigations remaining unestablished. The aim of this pilot study was to estimate the incidence of occult marrow involvement using flow cytometry, immunohistochemistry and immunoglobulin heavy chain (IgH) gene rearrangements, and to determine the impact on survival.

Methods: Clinical and pathological data were obtained on 36 patients diagnosed with DLBCL. Immunohistochemistry using CD3, CD45RO, CD20 and CD79a, and polymerase chain reaction (PCR) to look for IgH gene rearrangements were performed on formalin fixed BM trephines.

Results: Nine patients had morphologically apparent BM involvement. Occult marrow involvement was found in seven of 36 (19.4%) patients using the additional diagnostic modalities. When these cases were included with morphologically apparent cases in a proposed new definition of marrow involvement, the median survival of patients with BM involvement was statistically worse ($p=0.02$) than those without involvement.

Conclusions: The results indicate that use of additional tests on BM at diagnosis can upstage disease for a proportion of patients, which appears to correlate adversely with survival.

Key words: Diffuse large B-cell lymphoma, staging, bone marrow, flow cytometry, immunohistochemistry, immunoglobulin heavy chain gene rearrangement.

Received 18 September 2006, revised 8 March, accepted 26 March 2007

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoid tumour worldwide, accounting for approximately 40% of all cases of B-cell non-Hodgkin's lymphoma (NHL) in the Western world.[1] This category was defined both in the revised European-American lymphoma (REAL) and World Health Organization (WHO) classifications, aiming to include all malignant lymphomas characterised by the large size of the neoplastic cells, B-cell derivation, aggressive clinical presentation and the need for highly effective chemotherapy regimens.[2]

A standard approach is adopted in the investigation of patients with lymphadenopathy and possible NHL in Australia. Full blood count with a blood film, and when indicated, immunophenotypical analysis on peripheral blood is carried out initially to exclude chronic lymphocytic leukaemia (CLL). In patients <30 years old, a monospot is also often performed. If these investigations fail to provide a diagnosis, it is usual to proceed to surgical lymph node biopsy. The diagnosis on biopsy is established presently by morphology and immunohistochemistry with or without flow cytometry. Molecular studies with immunoglobulin heavy chain gene (IgH) and T-cell gamma chain gene are carried out in ambiguous cases to establish the diagnosis. If NHL is diagnosed on biopsy, further staging investigations including radiology and bone marrow (BM) aspirate and trephine biopsies are performed. Flow cytometric analysis is performed on the aspirate in many centres, but is not universally recommended by the current practice guidelines published by the National Health and Medical Research Council (NHMRC).[1] The use of immunohistochemistry and molecular studies is restricted to cases that are equivocal on routine morphology.

Although the British Committee for Standards in Haematology (BCSH) recommends that laboratories involved in the diagnosis of nodal NHL have the facilities to perform immunophenotypical and molecular studies, there are no clear guidelines on the application of these modalities in DLBCL.[3]

It is our hypothesis that, in the absence of established guidelines for the use of ancillary investigations in staging bone marrow, a proportion of cases is being under-staged, which may have an impact on prognosis. Therefore, the aim of the proposed study was to estimate the rate of occult (i.e., histologically inapparent) marrow involvement by more sensitive flow cytometric, immunohistochemical and molecular studies, and to determine whether this impacts on prognosis. This may have therapeutic implications in the future for selection of poor prognosis patients for more intensive treatment modalities.

There has been a great deal of interest in the determination of prognostic factors in DLBCL, as it is recognised that there is significant clinical heterogeneity within the histologically defined entity. Using gene expression profiling, two molecularly distinct forms of DLBCL have been identified corresponding to different stages of B-cell differentiation. One type expresses genes characteristic of

ISSN 0031-3025 printed/ISSN 1465-3931 © 2007 Royal College of Pathologists of Australasia
DOI: 10.1080/00313020701684417

germinal centre B cells and the second type expresses genes normally induced during *in vitro* activation of peripheral blood B cells.[4] Several prognostic markers linked to these two subtypes have been determined for clinical use, e.g., CD10, bcl-2 and bcl-6,[5] but none have been universally accepted.

There is a paucity of data on the impact of occult or low-level, morphologically inapparent bone marrow involvement on prognosis. Therefore, a retrospective cohort study was planned to estimate the rate of occult (i.e., histologically inapparent) marrow involvement by using flow cytometry, immunohistochemical and molecular studies, and the results of a pilot study are presented. For survival analysis, cases with occult marrow involvement (O) were included with those apparent on histology (M) in a new proposed definition of BM involvement (M+O).

PATIENTS AND METHODS

Patients

All patients diagnosed with DLBCL at The Canberra Hospital with a staging BM biopsy performed at diagnosis were included in the study. In our institution, a staging BM biopsy is performed for all patients for whom there is intention to treat, irrespective of the age or clinical stage of disease. A preliminary case identification was carried out from histopathology records using Snomed codes from the Laboratory Information System and from a review of medical records. Approximately 150 patients diagnosed from 1985 to 2004 were identified for recruitment in this retrospective cohort study. After approval was obtained from the Australian Capital Territory (ACT) Human Research Ethics Committee, clinical information on patients was collected from the Medical Records department at The Canberra Hospital.

For the purpose of this pilot study, 36 patients on whom clinical and laboratory data were available and all the requisite investigations had been performed were randomly selected. The mean age of the patient cohort was 65 years (range 36–85 years) with a preponderance of males (male:female 2.6:1.0). Performance status at diagnosis was well distributed between stages 0 and 3.

The mean laboratory values at diagnosis were essentially within normal reference range, although lactate dehydrogenase (LDH) was found to be slightly higher than the upper limit of normal.

Of the 36 patients, 12 (33.3%) were recorded as having stage I disease at first diagnosis, six (16.6%) with stage II disease, and nine (25%) each with stage III and IV disease, according to the Ann Arbor Staging system.[6] All patients with stage IV disease had obvious bone marrow involvement on routine light microscopy using H&E staining.

The overall median survival of the entire patient group was 47.7 months (Fig. 1).

Treatment

All patients were treated with standard anthracycline-containing chemotherapy regimens, except for two who were treated with palliative intent because of age and poor performance status. Twenty-nine of 36 patients received six cycles of CHOP[7] or variations of CHOP chemotherapy,[8,9] two received the PEN protocol[10] and one the Hyper CVAD regimen.[11] One patient each received EPOCH[12] and ICE chemotherapy protocols.[13] Twenty-eight of 36 (77.7%) patients achieved a complete remission (CR) as assessed after completion of the initial chemotherapy regimen. A further four of 36 (11.1%) patients achieved a partial remission (PR) and four (11.1%) either had no response to the administered chemotherapy or demonstrated progression of disease while on chemotherapy. Seventeen of the 32 patients that responded had a subsequent relapse.

Review of morphology and flow cytometry results

At The Canberra Hospital, examination of the staging bone marrow biopsy at initial diagnosis is performed on H&E stained preparations of the aspirate and trephine biopsy. At least two squash and smear preparations of the aspirate were retrieved.

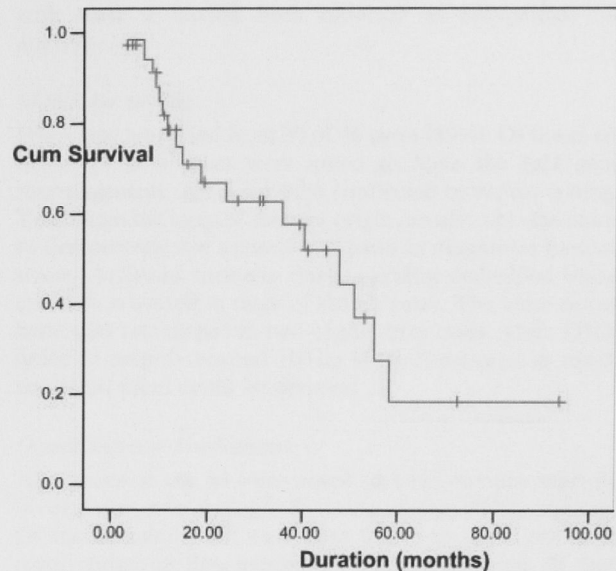


Fig. 1 This Kaplan–Meier survival curve shows that the median survival of the entire patient cohort was 47.7 months.

All trephines performed at our centre are fixed in buffered formalin and acetic acid, decalcified with 5% nitric acid, embedded in paraffin and stained with H&E and Giemsa. Besides this, silver impregnation is performed for reticulin. H&E, Giemsa and reticulin preparations on the trephine biopsy were retrieved for review.

Flow cytometry is also performed on all bone marrow biopsies and results were easily retrieved from laboratory records. An experienced haematologist reviewed all bone marrow slides and flow cytometry results in a blinded fashion. For flow cytometry, monoclonality was defined as a $\kappa:\lambda$ ratio of $>3:1$ or $<0.3:1$.[14]

Immunohistochemistry

Archived formalin fixed, paraffin embedded trephine biopsies were easily accessible and were used to perform immunohistochemistry stains. The mean trephine length for the patient cohort was 19.63 mm, with six cases having a trephine length of less than 15 mm and only one less than 10 mm.

Paraffin sections from each sample were immunostained with the following monoclonal antibodies using the streptavidin-biotin method: two T-cell markers (CD3 and CD45RO) and two B-cell markers (CD20 and CD79a). Tonsillar tissue was used as positive control. A formal system of reporting each antibody was devised, whereby for each antibody staining was reported by two independent observers as present or absent and normal or abnormal. For each antibody, it was also recorded whether staining was present in clusters or individually, and what percentage of involvement was noted on the section.

Molecular testing

Samples for molecular studies were obtained from paraffin embedded trephine sections. DNA extraction was performed manually using the Roche High Pure PCR Template Preparation Kit (Roche, Switzerland) from two 10 μm, formalin fixed, paraffin embedded sections according to the manufacturer's instructions.

Immunoglobulin heavy chain gene rearrangement analysis was performed targeting the conserved framework regions (FR) FR1 and FR3 using the InVivo Scribe kit (InVivo Scribe, USA) based on the BIOMED2 protocols.[15] This consists of five multiplex master mixes, three of which target the conserved framework regions (FR1, FR2 and FR3) and joining regions (J), and two of which target the diversity (D) and joining regions. For the purposes of this pilot study only FR1 and FR3 were analysed. FR1 and FR3 gene rearrangements are reported as clonal,

polyclonal or not detected. These together are estimated to account for approximately 70% of all rearrangements and the target genes are expected to be 310–360 bp and 100–170 bp in length.

The PCR reactions consisted of 45 μL of either FR1 or FR3 master mix solution, 2.5 U of Amplitaq Gold (Applied Biosystems, USA) and 5 μL of template DNA. Thermocycling was performed according the kit protocol on a Perkin Elmer 9600 thermocycler (Perkin Elmer, USA). The controls consisted of a positive clonal control, a cell line positive control, a polyclonal control, negative extraction control and negative PCR control (water).

Non-denaturing polyacrylamide gel electrophoresis was used to resolve the different sized amplicons. PCR product (25 μL) was loaded onto a 6% polyacrylamide gel and 250 V applied for 1.25 h for FR1 and 1.5 h for FR3. After electrophoresis, the gels were stained with ethidium bromide and visualised under UV light. The expected sizes of the PCR products were 310–360 bp for FR1 and 100–170 bp for FR3.

To verify the integrity of the DNA extracted from the paraffin sections, and to validate results, all samples were amplified with the control master mix provided in the kit. This is a multiplex PCR that targets multiple genes and generates a series of amplicons 100, 200, 300, 400 and 600 bp in length.

Statistical analysis

Response and survival data were obtained for each patient and recorded. Data analysis was performed using the software programme SPSS version 14.0 (SPSS, USA). In addition to descriptive analyses, Kaplan–Meier curves and log rank tests were used to test the benefits of bone marrow involvement predicting survival. A stepwise multivariate Cox regression analysis was used to adjust for age, performance status and treatment intent and to determine the most important predictor of survival.

RESULTS

Morphology and flow cytometry

There were nine cases with obvious marrow involvement on routine H&E staining, of which three showed involvement on aspirate but not on trephine and three showed involvement on trephine biopsy but not on aspirate. Only three of the nine cases showed involvement on both aspirate and trephine.

In total, there were nine cases that were detected to be involved with lymphoma on flow cytometry. The three cases that were found to be involved morphologically on both aspirate and trephine biopsy were also found to be involved immunophenotypically. One of the three cases that were involved on aspirate but not on trephine biopsy, and all of the three cases that were involved on trephine biopsy but not on aspirate, showed involvement on flow cytometry.

Immunohistochemistry

Again, there was a total of nine cases that were noted to be positive on immunohistochemistry. Two of these were also noted to be involved morphologically on aspirate and trephine biopsy, one was positive on aspirate but not on trephine, and two were involved on trephine biopsy but not on aspirate. Four cases were morphologically and immunophenotypically inapparent. All four cases had low level involvement on immunohistochemistry with small cells. Case 1 had small clusters in an intertrabecular location, which were noted on retrospective review of H&E morphology. These had been reported as benign. The remaining three cases also had low level involvement with one to two small clusters of cells in an intertrabecular location. Review of H&E morphology revealed no clusters, with Case 2 having been reported as suboptimal for analysis.

Molecular testing

DNA was amplified from 30 of 36 cases (83%). Of these, six of 36 (16.6%) cases were noted to have the IgH gene rearrangement. All cases were confirmed by repeat testing. To address the issue of tumour origin, an attempt was made to demonstrate the same clonal band in diagnostic tumour tissue. Archived formalin fixed, paraffin embedded tissue could be retrieved in three of the six cases. The same clonal band was established in two of the three cases, while DNA failed to amplify beyond 100 bp in the third case, in which no clonal band could be detected.

Occult marrow involvement

Nine cases of the 36 were noted to have obvious marrow involvement morphologically using routine H&E staining. Of the remaining 27, two were found to have low level involvement on flow cytometry. Of the remaining 25 that were negative for involvement on H&E staining and flow cytometry, a further four were noted to be positive on immunohistochemistry. Of the 21 cases negative using these staging investigations, a single case was found to have IgH gene rearrangements using the FR1 and FR3 reactions.

In other words, seven cases (19.4%) with morphologically inapparent bone marrow involvement were detected using flow cytometry, immunohistochemistry and IgH gene rearrangements studies.

Effect on survival

When examination of the aspirate and biopsy specimens was performed using H&E staining, and this was used to define marrow involvement at staging (9/36 cases), no obvious difference in survival was noted between patients with and without involvement ($p=0.13$) (Fig. 2)

However, when besides routine H&E staining the additional three diagnostic modalities were also used to define marrow involvement (16/36), a significant difference in survival was noted between patients with and without involvement ($p=0.02$) (Fig. 3).

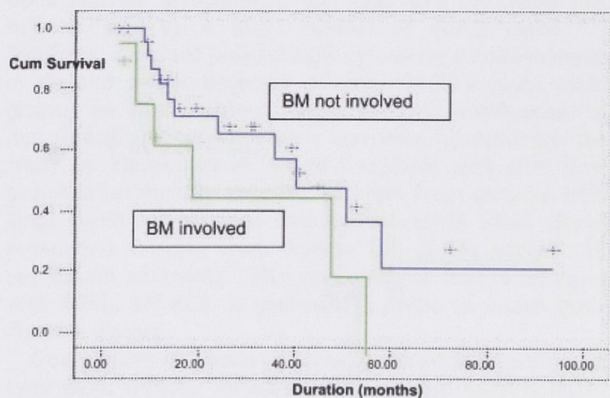


Fig. 2 Survival of patients with and without bone marrow involvement using the conventional definition using morphology alone (M). These Kaplan–Meier curves demonstrate that although there was some difference in survival of patients with and without histologically apparent bone marrow involvement at diagnosis, this was not statistically significant ($p=0.13$)

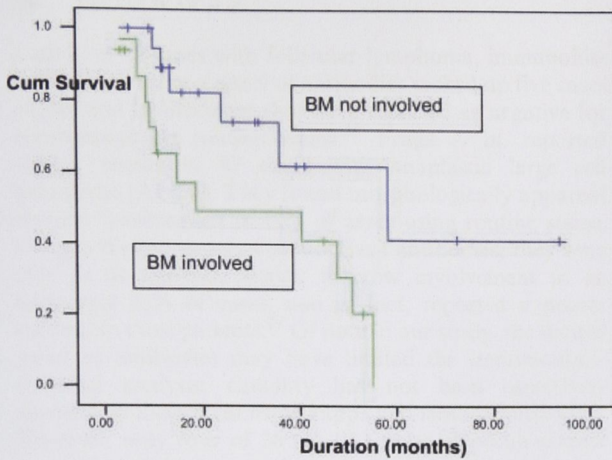


Fig. 3 Survival of patients with and without bone marrow involvement as defined by the proposed definition including morphology, and occult cases detected by flow cytometry, immunohistochemistry and IgH gene rearrangements (M+O). These Kaplan–Meier curves demonstrate that when the proposed definition is used, including cases of occult involvement with cases detected on morphology, there is a significant difference in survival between patients with and without involvement ($p=0.02$).

To establish that inferior outcome was related to disease related issues rather than unrelated causes, time to relapse was determined for patients with marrow involvement as defined by the addition of other diagnostic modalities to routine morphological analyses. The median time to relapse of patients with and without marrow involvement was 12 months versus 64 months, $p=0.003$.

A stepwise Cox regression analysis found that, after adjusting for age, ECOG score and treatment intent, the most significant predictor of survival was the new proposed definition of bone marrow involvement [relative risk (RR)=5.40, 95% confidence interval (CI)=1.37, 21.22; $p<0.02$]. Based on a stepwise (LR) multivariate Cox regression, morphologically apparent marrow involvement used in the conventional definition of BM involvement (M) was competitively considered with the new proposed definition of BM involvement that included cases of occult involvement (M+O) to determine their contribution to predicting survival. In the analysis it was found that the proposed definition contributed significantly more to explaining survival than the conventional one. After the new proposed definition incorporating multiple diagnostic modalities was included in the regression model, the conventional definition of involvement using morphological assessment on H&E alone did not significantly contribute to predicting survival ($p=0.12$).

DISCUSSION

Although there have been several previous studies on the use of ancillary tests in staging bone marrow in lymphoma, most have focused on detection of occult disease with only occasional studies attempting to correlate laboratory results with outcome data. Moreover, there is marked heterogeneity in the investigations used and in the study population. To our knowledge, this is the first study proposing that several ancillary investigations be used in a summative model along with morphology to better define bone marrow involvement in a single subtype of B-cell lymphoma. We also present a comparison of the conventional definition of BM involvement (M) with the proposed new definition, including occult and morphologically apparent cases (M+O) as a predictor of survival.

The international prognostic index (IPI) is the most widely utilised prognostic marker for aggressive lymphoma and uses a number of clinical features, including number of extra nodal sites.[16] One potential limitation of the IPI is related to several reports that specific extra nodal sites like the bone marrow may have prognostic significance.[17–19] This may be because it is thought to reflect a high tumour burden, increased invasive potential and decreased ability to tolerate chemotherapy. Also, the IPI limits the definition of bone marrow involvement to morphologically apparent cases and does not take into account occult or morphologically inapparent marrow involvement. Certainly there are reports as far back as a couple of decades ago that suggest that the rate of occult marrow involvement in aggressive lymphomas using culture techniques could be as high as 15–17%.[20] Although the clinical applicability of culture techniques is obviously limited, increased sensitivity of detection can potentially also be obtained by the use of routinely available additional ancillary techniques like flow cytometry, immunohistochemistry and IgH gene rearrangement, resulting in detection of cases with occult marrow involvement.

Previous reports attempting to study the role of these in detecting occult disease in the bone marrow have also looked at the issue of concordance between these tests. The discordance between results on aspirate and trephine biopsies using routine staining noted in our study has been reported previously, and postulated to be so because of differences in sampling.[21] As with most other studies, we have reported cases that were noted to be positive on either aspirate or trephine biopsy as involved morphologically.

Reports of lack of concordance between positivity on flow cytometry and morphology in broad based studies looking at cases with B-cell NHL with or without a marrow based lymphoproliferative disorder such as chronic lymphocytic leukaemia have indicated that neither morphological examination (of BM aspirate and trephine biopsy) nor flow cytometry alone are adequate to pick up all cases of bone marrow involvement, and that flow cytometry can help to pick up a small number of occult cases.[22–24] Similarly, we noted positive flow cytometry results in seven of the nine morphologically involved DLBCL cases, while picking up two additional cases of occult involvement in morphologically negative bone marrows. Interestingly, the study by Hanson *et al.* found five cases that were flow positive/morphology negative; all were from patients with large B-cell lymphomas, with an extremely small clonal population ranging from 0.09 to 3% of the overall cell population analysed.[24] This may indicate that of all cases with NHL, DLBCL is particularly prone to occult bone marrow disease.

Comparison of studies utilising immunohistochemistry is even more difficult as most studies utilise immunohistochemical stains in selected cases to assist in morphological diagnosis without the results being reported separately.[22,24,25] In general there is a paucity of data regarding the role of immunohistochemical analysis in picking up histologically inapparent infiltrates in the bone marrow. In

a study of 27 cases with follicular lymphoma, immunohistochemistry using a panel of antibodies picked up five cases of minimal involvement that were reported as negative for involvement on routine stains.[26] Fraga *et al.* reported similar results in 47 cases with anaplastic large cell lymphoma (ALCL). They found morphologically apparent marrow involvement in 17% of cases using routine stains. Using a limited panel of monoclonal antibodies, they were able to demonstrate occult marrow involvement in an additional 23% of cases, and in fact, reported a poorer survival in these patients.[27] Of note in our study, the limited panel of antibodies may have limited the immunohistochemical analysis; clonality has not been objectively assessed on these cases using kappa and lambda antibodies. However, only four of 36 (11.1%) cases of occult disease were detected by immunohistochemistry alone, which is a conservative figure compared with the published literature. This indicates that a conservative rather than liberal approach in assigning positivity was adopted. Also, the negative correlation with survival suggests that involvement defined by staining using B-cell markers may be adequate as a poor prognostic marker.

PCR-detected clonal gene rearrangements have been correlated in various studies, with morphology, immunophenotype and immunohistochemistry and a degree of overlap between positive cases on different modalities of investigation being reported.[28–30] Again, heterogeneity is apparent in these studies with variability in both molecular methods and the samples to which they are applied. A recent study by Mitterbauer-Hohendanner *et al.* using a standardised protocol for IgH and TCR gene rearrangements on fresh bone marrow aspirates, assessed the prognostic significance of molecular staging and reported a negative correlation of molecular positivity with survival.[31] Our study looks at molecular positivity for FR1 and FR3 using the BIOMED2 protocol on bone marrow trephines. This may be particularly important in DLBCL, where involvement on trephine biopsy is reported more commonly than on bone marrow aspirates.[32] The BIOMED2 protocol has been reported previously in a modified form on bone marrow trephines.[33] Our overall rate of molecular positivity (~16%) is lower than that described in the literature.[32] This may be because molecular testing was performed from small amounts of archived formalin fixed, paraffin embedded trephine tissue, which is known to give false negative results. Even though a large number of specimens were amplifiable with the control PCR master mix, not all specimens were consistent in amplifying the larger products and this may have produced false negative results. Also, false negativity on heavy chain gene analysis is known to be associated with false negative molecular results in DLBCL due to somatic hypermutation.[15] PCR analysis for kappa and lambda light chains may further increase the rate of molecular positivity.

Overall, when we looked at positivity on either of the ancillary staging tests, we found occult marrow involvement in almost 20% cases, which compares well with rates reported using sensitive culture techniques.[28] Our argument is that as each test has high specificity but inherent limitations in sensitivity, they must be used in a summative rather than concordance model to define bone marrow involvement. The hierarchy used in defining involvement is arbitrary at present, and our results so far do not seem to indicate that altering the order would affect the positivity rate or survival.

Our patient population has been randomly selected based on the availability of clinical and laboratory data, and the performance of all requisite additional investigations, and that may explain the unusual male preponderance compared with that reported in literature.[2] However, as gender is not considered to significantly affect either the rate of bone marrow involvement or survival, we do not expect that to have significantly affected our results. Other demographic and basic clinical and laboratory characteristics were in keeping with the literature. The practice in our institution to perform staging bone marrow biopsies in all cases of DLBCL, irrespective of clinical stage of the disease, reduces the risk of a selection bias. The poor overall survival of the patient cohort remains unexplained at this point. We believe it is unlikely to be related to stage of the disease as the distribution of stages in our study is similar to that in others.

Although our study is a retrospective one and our study population small, our results are significant, as we have proposed a more broad-based definition of bone marrow involvement that includes occult cases with those that are morphologically apparent. More significantly, we have shown that this definition is a better predictor of survival than the conventional model using morphology alone. We hope to report the results of our larger retrospective study, including the possible impact on IPI, in the near future.

Acknowledgements The authors would like to acknowledge the financial support provided for the study by the Private Practice Trust Fund, The Canberra Hospital, and the equipment grant provided by The Leukaemia Foundation, Australia. The principal investigator/author has received a supplementary scholarship from the Arrow Bone Marrow Transplant Foundation, New South Wales, for the project. Initial help with the project plan provided by Dr Ian Prosser is gratefully acknowledged.

Address for correspondence: Dr D. Talaulikar, Department of Haematology, The Canberra Hospital, PO Box 11, Woden, ACT 2606, Australia. E-mail: dipti.talaulikar@act.gov.au

References

1. Australian Cancer Network Diagnosis and Management of Lymphoma Guidelines Working Party, *Guidelines for the Diagnosis and Management of Lymphoma*. Sydney: The Cancer Council Australia and Australian Cancer Network, 2005.
2. Jaffe ES, Harris NE, Stein H, *et al. The World Health Organization Classification of Tumours. Pathology and Genetics: Tumours of the Hematopoietic and Lymphoid Tissues*. Lyon: IARC Press, 2001.
3. The Haemato-Oncology Task Force of British Committee for Standards in Haematology, *BCSH Guidelines on Nodal Non-Hodgkin's Lymphoma: Draft 2, August 2002*. Oxford: Blackwell Publishing, 2002.
4. Alizadeh AA, Eisen MB, Davis RE, *et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; 403: 503–11.
5. de Leval L, Harris NL. Variability in immunophenotype in diffuse large B-cell lymphoma and its clinical relevance. *Histopathology* 2003; 43: 509–28.
6. Carbone PP, Kaplan HS, Musshoff K, *et al.* Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res* 1971; 31: 1860–1.
7. Fisher RI, Gaynor ER, Dahlberg S, *et al.* Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. *N Engl J Med* 1993; 328: 1002–6.

8. Sonneveld P, de Ridder M, Van der Lelie H, *et al.* Comparison of doxorubicin and mitoxantrone in the treatment of elderly patients with advanced non-Hodgkin's lymphoma using CHOP versus CNOP chemotherapy. *J Clin Oncol* 1995; 13: 2530–9.
9. Lambertenghi DG, Butti C, Baldini L, *et al.* A cooperative study of epirubicin with cyclophosphamide, vincristine and prednisolone (CEOP) in non-Hodgkin's lymphoma. *Haematologica* 1995; 80: 318–24.
10. Goss P, Burkes R, Rudinskas L, *et al.* A phase II trial of prednisolone, oral Etoposide, and novantrone (PEN) as initial treatment of non-Hodgkin's lymphoma in elderly patients. *Leuk Lymphoma* 1995; 18: 145–52.
11. Lone SW, Crawford J, Kenealy M, *et al.* Safety and efficacy of pegfilgrastim compared to granulocyte colony stimulating factor (G-CSF) supporting a dose-intensive rapidly cycling anti-metabolite containing chemotherapy regimen (hyper-CVAD) for lymphoid malignancy. *Leuk Lymphoma* 2006; 47: 1813–7.
12. Khaled HM, Zekri ZK, Mokhtar N, *et al.* A randomised EPOCH vs. CHOP front-line therapy for aggressive non-Hodgkin's lymphoma patients: long-term results. *Ann Oncol* 1999; 10: 1489–92.
13. Kingreen D, Beyer J, Kleiner S, *et al.* ICE – an efficient drug combination for stem cell mobilization and high-dose treatment of malignant lymphoma. *Eur J Haematol* 2001; 64: 46–50.
14. Marti GE, Rawstron AC, Ghia P, *et al.* Diagnostic criteria for monoclonal B-cell lymphocytosis. *Br J Haematol* 2005; 130: 325–32.
15. van Dongen JJ, Langerak AW, Bruggemann M, *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003; 17: 2257–317.
16. No authors listed. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. *N Engl J Med* 1993; 329: 987–94.
17. Campbell J, Seymour JF, Matthews J, Wolf M, Stone J, Juneja S. The prognostic impact of bone marrow involvement in patients with diffuse large cell lymphoma varies according to the degree of infiltration and presence of discordant marrow involvement. *Eur J Haematol* 2006; 76: 473–80.
18. Nicolaides C, Fountzilas G, Zoumbos N, *et al.* Diffuse large cell lymphomas: identification of prognostic factors and validation of the International Non-Hodgkin's Lymphoma Prognostic Index. A Hellenic Cooperative Oncology Group Study. *Oncology* 1998; 55: 405–15.
19. Yan Y, Chan WC, Weisenburger DD, *et al.* Clinical and prognostic significance of bone marrow involvement in patients with diffuse aggressive B-cell lymphoma. *J Clin Oncol* 1995; 13: 1336–42.
20. Benjamin D, Magrath IT, Douglass EC, *et al.* Derivation of lymphoma cell lines from microscopically normal bone marrow in patients with undifferentiated lymphomas: evidence of occult bone marrow involvement. *Blood* 1983; 61: 1017–9.
21. Sah SP, Matutes E, Wotherspoon AC. A comparison of flow cytometry, bone marrow biopsy, and bone marrow aspirates in the detection of lymphoid infiltration in B cell disorders. *J Clin Pathol* 2003; 56: 129–32.
22. Stacchini A, Demurtas A, Godio L, *et al.* Flow cytometry in the bone marrow staging of mature B-cell neoplasms. *Cytometry B Clin Cytom* 2003; 54: 10–8.
23. Duggan PR, Easton D, Luider J, *et al.* Bone marrow staging of patients with non-Hodgkin lymphoma by flow cytometry: correlation with morphology. *Cancer* 2000; 88: 894–9.
24. Hanson CA, Kurtin PJ, Katzmann JA, *et al.* Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. *Blood* 1999; 94: 3889–96.
25. Perea G, Altes A, Bellido M, *et al.* Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL). *Histopathology* 2004; 45: 268–74.
26. Chetty R, Echezarreta G, Comley M, *et al.* Immunohistochemistry in apparently normal bone marrow trephine specimens from patients with nodal follicular lymphoma. *J Clin Pathol* 1995; 48: 1035–8.
27. Fraga M, Brousset P, Schlaifer D, *et al.* Bone marrow involvement in anaplastic large cell lymphoma. Immunohistochemical detection of minimal disease and its prognostic significance. *Am J Clin Pathol* 1995; 103: 82–9.
28. Coad JE, Olson DJ, Christensen DR, *et al.* Correlation of PCR-detected clonal gene rearrangements with bone marrow morphology in patients with B-lineage lymphomas. *Am J Surg Pathol* 1997; 21: 1047–56.
29. Crotty PL, Smith BR, Tallini G. Morphologic, immunophenotypic, and molecular evaluation of bone marrow involvement in non-Hodgkin's lymphoma. *Diagn Mol Pathol* 1998; 7: 90–5.
30. Maes B, Achten R, Demunter A, *et al.* Evaluation of B cell lymphoid infiltrates in bone marrow biopsies by morphology, immunohistochemistry, and molecular analysis. *J Clin Pathol* 2000; 53: 835–40.
31. Mitterbauer-Hohendanner G, Mannhalter C, Winkler K, *et al.* Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). *Leukemia* 2004; 18: 1102–7.
32. Juneja SK, Wolf MM, Cooper IA. Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. *J Clin Pathol* 1990; 43: 630–2.
33. Lassmann S, Gerlach UV, Technau-Ihling K, *et al.* Application of BIOMED-2 primers in fixed and decalcified bone marrow biopsies: analysis of immunoglobulin H receptor rearrangements in B-cell non-Hodgkin's lymphomas. *J Mol Diagn* 2005; 7: 582–91.

Author contributions:

Talaulikar D, Dahlstrom JE, Broomfield A, McNiven M, Shadbolt B. **"Occult marrow involvement in Diffuse Large B-cell Lymphoma: results of a pilot study."** Pathology. 2007 Dec; 39(6):580-5.

Dipti Talaulikar:

1. Designed study, prepared ethics and grants applications, and set up database
2. Retrieved all cases from medical and laboratory records, and all archived slides and paraffin-embedded trephine blocks
3. Performed all the data extraction, analysed and computed all clinical data, and performed all data entry
4. Reported all histology slides as the first blinded reviewer
5. Interpreted all flow cytometry analysis
6. Cut sections, labeled and stained slides for immunohistochemistry with help
7. Reported and computed immunohistochemistry slides
8. Cut sections from paraffin-embedded trephine biopsy blocks for molecular analysis
9. Performed all DNA extractions
10. Developed all molecular methods under supervision
11. Performed all molecular analysis
12. Performed descriptive analysis. Other under supervision of and help from Dr. Bruce Shadbolt
13. Wrote the first draft of the paper

Jane Dahlstrom:

1. Development of formal system of reporting IHC
2. Review of IHC slides
3. Review of final paper

Bruce Shadbolt:

1. Setting up database
2. Analytical data analysis
3. Review of final paper

Michelle McNiven:

1. Technical advice on molecular analysis
2. Review of final paper

Amy Broomfield:

1. Technical advice with preparing all immunohistochemistry slides
2. Approval of final paper

Michael Pidcock:

1. Blinded 2nd reviewer of H & E slides
2. Approval of final paper

Ian Prosser:

1. Initial help with project plan

Intellectual planning: DT (95%), JED: (2%) BS: (2%) IWP (1%)

Experimental work: DT (95%) JED: (2%) AMcD: (1%) JB and KK: (1%), AB: (1%)

Analyses: DT (70%) BS (30%)

Writing: DT (97%) JED (2%) BS (0.5%) MM (0.5%)

CHAPTER IV:

Clinical role of flow cytometry

4.1 Clinical role of flow cytometry in redefining bone marrow involvement in diffuse large B-cell lymphoma (DLBCL) – a new perspective. Histopathology 2008, 52; 340-347

Histopathology 2008, **52**, 340–347. DOI: 10.1111/j.1365-2559.2007.02961.x

Clinical role of flow cytometry in redefining bone marrow involvement in diffuse large B-cell lymphoma (DLBCL) – a new perspective

D Talaulikar,[1,2] B Shadbolt,[2,3] J Bell,[1] K Khan,[1] J E Dahlstrom,[2,4] A McDonald,[5] M Pidcock[1] & A Broomfield[4]

[1]*Department of Haematology,* [2]*Australian National University Medical School,* [3]*Department of Epidemiology,* [4]*Department of Anatomical Pathology, The Canberra Hospital, and* [5]*National Capital Private Hospital, Canberra, ACT, Australia*

Date of submission 12 April 2007
Accepted for publication 21 August 2007

Talaulikar D, Shadbolt B, Bell J, Khan K, Dahlstrom J E, McDonald A, Pidcock M & Broomfield A
(2008) *Histopathology* **52**, 340–347

Clinical role of flow cytometry in redefining bone marrow involvement in diffuse large B-cell lymphoma (DLBCL) – a new perspective

Aims: The clinical role of flow cytometry in staging bone marrow in diffuse large B-cell lymphoma (DLBCL), especially its impact on outcome, remains uncertain. The aim was to determine the contribution of flow cytometry to conventional staging, and to study the impact of this revised staging on survival.

Methods and results: One hundred and thirteen cases of DLBCL diagnosed at The Canberra Hospital from 1996 to 2005 were identified. Blinded analysis of bone marrow (BM) morphology and flow cytometric data showed involvement on morphology (M) in 25 (22.1%) cases, on flow cytometry (F) in 21 (18.6%) cases and overall (M + F) in 32 cases (28.3%); discordance was noted in 16 cases (16.1%). Cases with and without marrow involvement on conventional staging alone (M) had no significant difference in survival (P = NS). However, when BM involvement was defined as positivity on morphology and/or flow cytometry (M + F), the median survival of patients with involvement was significantly worse than patients without involvement ($P = 0.026$).

Conclusions: Flow cytometry-positive cases should be included with those positive on morphology in a summative model to define BM involvement in DLBCL, as it may have a potential impact on predicting outcome.

Keywords: bone marrow, cytology, diffuse large B-cell lymphoma, flow cytometry, staging

Abbreviations: BM, bone marrow; CI, confidence interval; DLBCL, diffuse large B-cell lymphoma; F, flow cytometry; H&E, haematoxylin and eosin; IPI, international prognostic index; M, morphology; NHL, non-Hodgkin's lymphoma; PBS, phosphate-buffered saline; RR, relative risk

Introduction

The clinical work-up routinely performed on patients with diffuse large B-cell lymphoma (DLBCL) includes staging investigations to determine the extent of involvement. The British Committee of Standards in Haematology recommends performing a bone marrow (BM), with routine morphological examination of the aspirate and trephine biopsy specimens.[1] Using the Ann Arbour staging system, involvement of BM upgrades a patient to stage IV, with potential implications for both prognosis and treatment.[2]

Ancillary laboratory techniques are now routinely available in most laboratories that could potentially aid in the assessment of BM disease in non-Hodgkin's lymphoma (NHL) by detecting clonal B-cell populations. These include immunophenotyping, immunohistochemistry and molecular testing to detect immunoglobulin gene rearrangements. However, their clinical role remains uncertain, with most centres

Address for correspondence: Dr D Talaulikar, Department of Haematology, The Canberra Hospital, PO Box 11, Woden, ACT 2606, Australia. e-mail: dipti.talaulikar@act.gov.au

© 2008 The Authors. Journal compilation © 2008 Blackwell Publishing Limited.

using the tests merely to aid in the diagnosis of suspicious cases. All of these techniques are believed to be more sensitive than morphology and have been reported to detect low-level morphologically inapparent disease in a proportion of patients.[3]

Of these techniques, flow cytometry is one that is readily available and many centres routinely perform flow cytometric analysis to aid in the morphological assessment of BM specimens, despite the lack of concrete scientific evidence of its benefits. A number of studies have attempted to determine the role of flow cytometry in NHL,[4–9] but patient selection criteria and methodology have been variable which makes some of them difficult to interpret. Most studies have looked at B-cell lymphomas as a whole, including both low-grade and high-grade NHL. This may limit the clinical relevance of these results, as the significance of low-level BM involvement in low-grade NHL is likely to be less than that in the more aggressive lymphomas. In high-grade lymphomas, almost all cases require some form of treatment at diagnosis in contrast to low-grade lymphoma, where often a watch and wait approach is adopted. Furthermore, staging affects treatment options more often in high-grade lymphomas than in low-grade disease. The inclusion of both staging and restaging BM introduces further confounders that may affect the clinical relevance of the results. Another major limitation is that most studies have not attempted to determine the clinical relevance of flow cytometry by correlating positive results with response rates or survival.

This study has attempted to address the impact of incorporating flow cytometry routinely as a staging investigation on the outcome of patients. The morphological and flow cytometric results obtained from staging BMs at first diagnosis of DLBCL in 113 cases were retrospectively reanalysed, and an attempt was made to determine the clinical utility of these investigations by correlating positivity with overall survival.

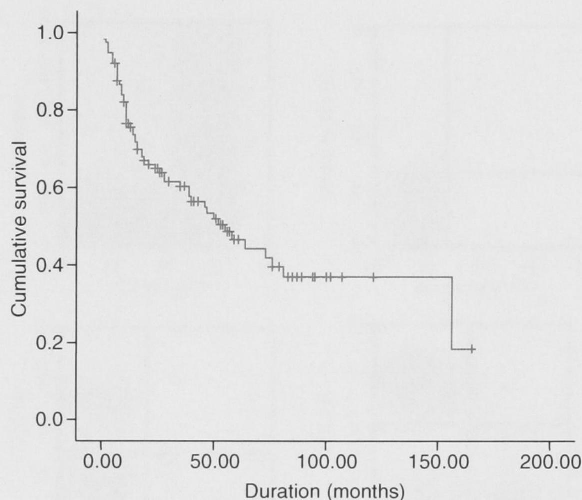


Figure 1. The overall survival of the entire patient cohort was 55 months.

Patients and methods

PATIENTS

We identified 113 retrospective cases diagnosed with histologically proven DLBCL at The Canberra Hospital from 1996 to 2005, on whom staging BM biopsies had been performed. After approval was obtained from the Australian Capital Territory Human Research Ethics Committee; clinical information on patients was collected from the Medical Records department at The Canberra Hospital.

The average age of the patient cohort was 59 years (range 22–86 years), and the male:female ratio was 1.6:1. Using routine staging procedures, 29/113 (25.6%) on whom data were available were found to have stage I disease, 26 (23%) to have stage II and 31 (27.4%) and 27 (23.9%) stage III and IV disease. Treatment data were available on 107 patients with almost all patients having been treated with anthracycline-based regimens. Six patients were treated with palliative intent with steroids alone or in combination with non-anthracycline based drugs. The median overall survival of the entire patient group was 55 months (Figure 1).

BONE MARROW MORPHOLOGY

BM aspirate and trephine biopsy slides at first diagnosis were retrieved and reviewed blindly. At least two squash and smear preparations on the haematoxylin and eosin (H&E)-stained aspirate slides were retrieved. All trephines performed at the centre are routinely fixed in buffered formalin and acetic acid, decalcified with 5% nitric acid, embedded in paraffin and stained with H&E and Giemsa. Besides this, silver impregnation is performed for reticulin. H&E, Giemsa and reticulin preparations on the trephine biopsy were retrieved for review.

All slides were reviewed in a blinded fashion by two experienced haematologists, with discrepant cases being reviewed blindly by a third. Morphological evaluation was carried out on H&E sections and reticulin stains.

© 2008 The Authors. Journal compilation © 2008 Blackwell Publishing Ltd,

Only unambiguous cases (i.e. those where atypical lymphoid cells make up >5% of the total nucleated cells) were classified as positive on aspirates. Trephine biopsy specimens were reported as positive, negative or indeterminate based on Cheson criteria.[10] Positive cases were decided on the basis of paratrabecular infiltrates, large size of aggregates, presence of large cells and increased reticulin response. Discordant marrow involvement was defined as cases with <50% large cells. Benign lymphoid aggregates were classified as those that were intertrabecular in location, small in size, well circumscribed and contained small cells admixed with other reactive cells. Immunohistochemical analysis using two B-cell markers (CD20 and CD79a), two T-cell markers (CD3 and CD45RO), κ and λ stains was performed and reported in conjunction with H&E to resolve indeterminate cases.

FLOW CYTOMETRY

Raw immunophenotypic data on all BM biopsy specimens were retrieved from laboratory records. Four-colour multiparametric flow cytometric analysis was routinely performed, with marrow cells immunophenotypically labelled by direct four-colour immunofluorescence using a panel of antibodies. The panel of antibodies used included: CD45, B-cell markers, including CD19, CD20, and CD22, CD10, HLA-DR, κ and λ, T-cell markers, including CD2, CD3, CD5 and CD7, and natural killer cell markers such as CD16 and CD56. All antibodies were obtained from Becton Dickinson (North Ryde, Australia), with CD45, CD19, CD20, CD3, CD23, κ and λ antibodies of the IgG1 isotype; CD2, CD5, CD7, CD10 and HLA-DR antibodies of the IgG2a isotype; and CD22 antibody of the isotype IgG2b.

Two millilitres of marrow aspirate were incubated with 10 ml of ammonium chloride for 5 min to lyse red cells. The cells were washed in phosphate-buffered saline (PBS) after centrifugation. A cell suspension of 1×10^6 cells per tube was then incubated with the monoclonal antibody for 30 min at room temperature. Each cell suspension was then washed and resuspended in a solution of PBS and fetal calf serum. Isotypic controls IgG1 and IgG2 were used.

Data acquisition was performed in a Becton Dickinson flow cytometer starting with control samples. At least 2000 lymphocytes were counted in each sample. Bright CD45 fluorescent staining and intermediate side scatter were the primary gating strategies used to identify the lymphocyte population. Further gating was performed as necessary on lymphoid populations based on cell size or using back gating on CD19 positive events. Figure 2 demonstrates an example of the gating strategies used in a case with involvement.

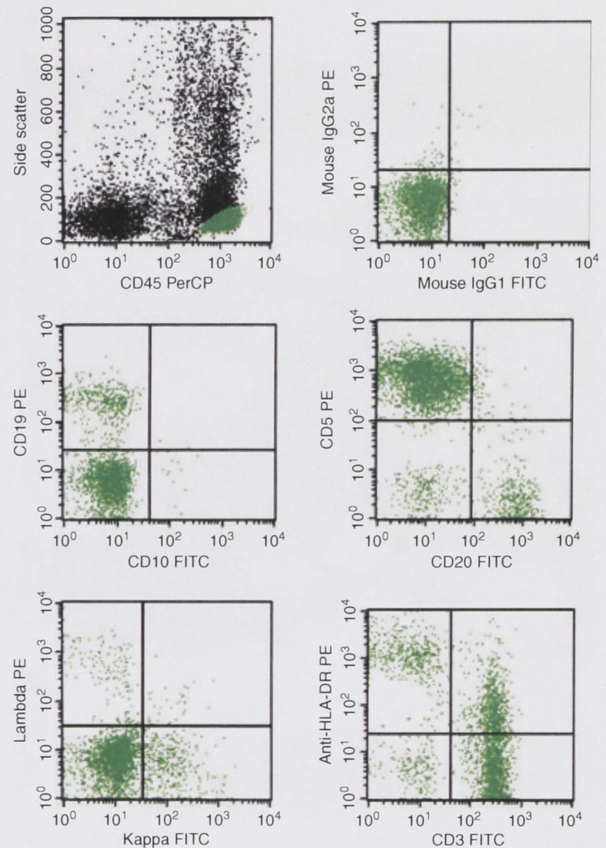


Figure 2. An example of a positive result on flow cytometry. The top row from the left shows the primary gating strategy used to identify the abnormal population and the controls used. The boxes in the middle row show positivity with pan B-cell markers, and clonal light chain restriction (κ) is demonstrated in the last row.

For the purposes of this study, samples were not run again, but data were re-analysed by senior scientists, who also determined the population of lymphocytes to be gated blindly. A haematologist then reviewed and reported the flow cytometric results in a blinded fashion. Positive results on flow cytometry were defined as light chain clonal restriction with a κ:λ ratio of >3:1 or <0.3:1.[11] Predominance of B cells in the gated population alone was not considered as a positive result. Results were compared with those previously reported and discrepant results (n = 5) were re-analysed by a third blinded person and re-interpreted.

STATISTICAL ANALYSIS

Response and survival data were obtained for each patient and recorded. In addition to descriptive

© 2008 The Authors. Journal compilation © 2008 Blackwell Publishing Ltd,

analysis, Kaplan–Meier curves were created for death and relapse as outcomes. Stepwise (logistic regression) multivariate Cox regression analysis was used to establish whether morphology alone or a combination of morphology and flow cytometry was a better predictor of survival. All analyses were performed using the software program Statistical Package for Social Sciences (SPSS) version 14.0 (SPSS Inc., Chicago, IL, USA). Similar analysis was also performed to assess whether a revised international prognostic index (IPI) computed by addition of flow cytometry-positive cases was better at predicting survival compared with the baseline IPI.

Results

MORPHOLOGY

Twenty-five out of 102 cases on which morphological data were available were found to be positive on routine morphological and immunohistochemical analysis of BM. Aspirates were unavailable in three cases because of inadequate samples or dry tap; all three were negative on trephine biopsy. Of the remaining 99 cases, six were positive on aspirate alone, 11 on trephine biopsy and seven on both. There was lack of concordance between aspirate and trephine biopsy samples in 17 out of 102 (16.6%) cases. Overall, there were four cases with an indeterminate report on trephine biopsy using H&E alone. Two were classified as positive and two as negative on immunohistochemical analysis. Figure 3 shows an example where immunohistochemical analysis was used to confirm presence of BM involvement in a case that was classified as indeterminate on routine H&E immunohistochemistry.

FLOW CYTOMETRY

Overall, 21 cases of 99 on whom morphological and flow cytometric data were available were reported as having flow cytometric involvement (Table 1). Of these, seven cases were positive on trephine biopsy alone, five were positive on both aspirate and trephine biopsy and one was positive on aspirate alone. Flow cytometry detected occult BM involvement in eight cases with normal morphology. Of all the cases negative on flow cytometry, 11 were found to have involvement on aspirate and/or trephine biopsy. Five cases were positive on aspirate alone, four on trephine biopsy alone and two on BM aspirate and trephine biopsy. Lack of concor dance between morphology (defined as cases positive on aspirate or trephine biopsy) and flow cytometry was noted in 16 cases out of 99 (16.1%).

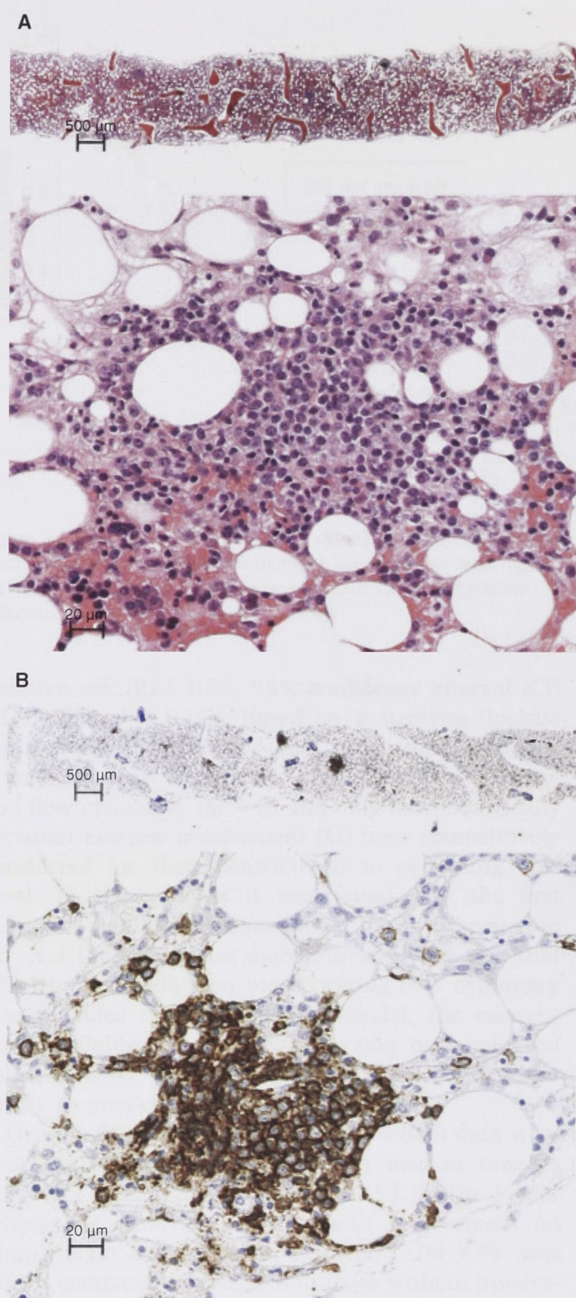


Figure 3. **A**, Histological sections from a case classified as indeterminate on H&E staining (the same case as in Figure 2) shows small interstitial lymphoid aggregates composed predominantly of small cells. **B**, Immunohistochemical analysis using CD20 shows a greater number of lymphoid aggregates than detected on H&E staining. On higher power, these were noted to be intermediate to large in size. κ light chain staining was noted establishing clonality (not shown).

© 2008 The Authors. Journal compilation © 2008 Blackwell Publishing Ltd,

Table 1. Aspirate, trephine and flow cytometry results

Flow cytometry	Trephine involved	Trephine not involved	Trephine not available
Positive			
Aspirate involved	5	1	
Aspirate not involved	7	7	
Aspirate not available		1	
Negative			
Aspirate involved	2	5	
Aspirate not involved	4	65	
Aspirate not available		2	14

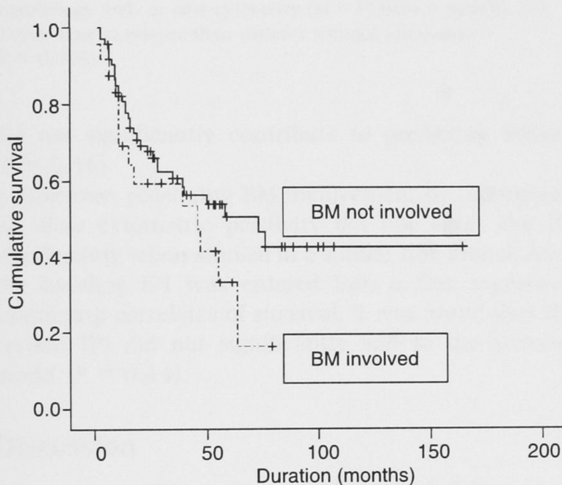


Figure 4. Cases with and without morphologically apparent bone marrow involvement (M) show no difference in overall survival ($P = 0.173$).

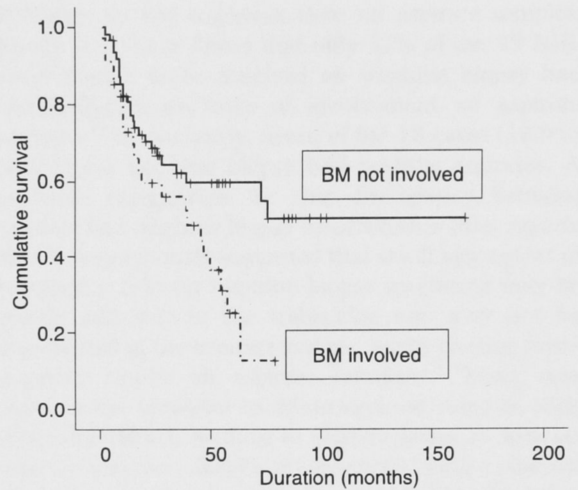


Figure 5. When cases positive on flow cytometry are included with morphologically apparent cases to define bone marrow involvement (M + F), cases with and without involvement show a significant difference in overall survival ($P = 0.026$).

EFFECT ON OUTCOME

The difference in survival between patients with and without morphologically apparent BM involvement was not statistically significant ($P = 0.173$) (Figure 4). However, when BM involvement was defined as positivity on either morphology or flow cytometry, the difference in survival between patients with and without involvement became significant ($P = 0.026$) (Figure 5).

Stepwise Cox regression analysis found that, after adjusting for age, Eastern Cooperative Oncology Group (ECOG) status- and palliative chemotherapy, the most significant predictor of survival was BM involvement on morphology and/or flow cytometry [relative risk (RR) 1.89, 95% confidence interval (CI) 1.00, 3.57; $P = 0.05$]. Based on a stepwise (logistic regression) multivariate Cox regression, marrow involvement as defined by the use of morphology and flow cytometry (M + F) and only morphologically apparent marrow involvement (M) were competitively considered for their contribution to predicting survival. In the analysis it was found that the first definition significantly contributed more to predicting survival than the latter more restricted one and that after the new definition incorporating flow cytometry was included in the regression model, the conventional definition of involvement using morphological assessment on H&E alone did not contribute significantly to predicting survival ($P = 0.73$).

Overall, 60 patients out of 106 on whom data were available relapsed (56.6%), with a median time to relapse of 37 months (95% CI 20.3, 53.7). The 5-year relapse-free survival for cases with involvement on morphology and/or flow cytometry (M + F) was 10.6% compared with 52.0% in those without involvement ($P = 0.005$) (Figure 6). This further establishes that the inferior outcome in terms of poor survival in cases with BM involvement is related to disease rather than other causes. A further Cox regression analysis was carried out to determine the most important predictor of relapse. It showed similar results to those for survival (RR 2.43, 95% CI 1.30, 4.54; $P = 0.006$). After the new definition incorporating cases positive on flow cytometry was included in the regression model, the conventional definition using morphology alone

© 2008 The Authors. Journal compilation © 2008 Blackwell Publishing Ltd,

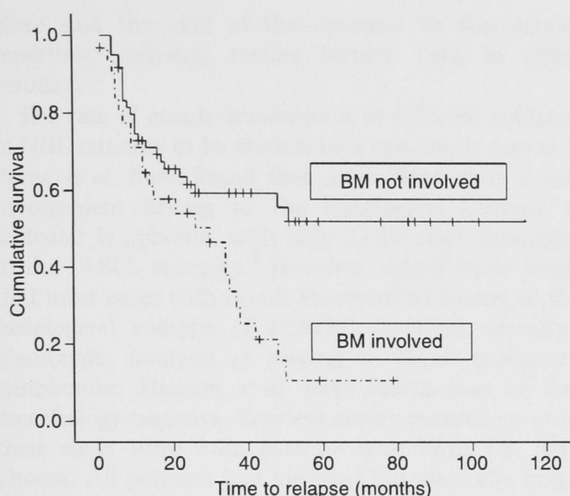


Figure 6. The Kaplan–Meier curves show that cases positive on morphology and/or flow cytometry (M + F) have a significantly shorter time to relapse than patients without involvement ($P = 0.005$).

did not significantly contribute to predicting relapse ($P = 0.36$).

However, redefining BM involvement by incorporating flow cytometric positivity did not affect the IPI significantly when studied in a similar Cox model. After the baseline IPI was entered into a Cox regression examining correlates of survival, it was found that the revised IPI did not significantly add to the survival model ($P = 0.44$).

Discussion

Our results indicate that morphology and flow cytometry should be used together to define BM involvement in NHL. The definition of marrow involvement, i.e. positivity on morphology and/or flow cytometry, appears to be a better prognostic indicator than morphology alone.

Several previous studies have demonstrated a concordance of ~80–90% between morphology, including immunohistochemistry, and flow cytometry.[4,12,13] In our study, we found similar rates of concordance (~84%). Although this degree of concordance has been considered satisfactory by previous authors, it is obvious that there are cases positive on morphology that are likely to be negative on flow cytometry, and similarly, cases positive on flow cytometry that are negative morphologically. A number of causes may be responsible for this discordance.

Discordance between aspirate and trephine biopsy samples is well recognized. The incidence of BM disease is higher on BM trephines than on aspirate samples. Juneja *et al.* have found that only 57% of the 99 NHL cases known to be involved on trephine biopsy had morphological evidence of involvement on aspirate samples.[14] In our study, seven of the 18 cases (38.9%) positive on trephine biopsy had positive aspirates. A probable explanation for this discrepancy between aspirate and trephine biopsy results comes from reports that have previously suggested that small aggregates of lymphoma cells on trephine biopsy specimens may be tightly adherent to the trabeculae and may not be represented in the aspirate sample, again causing false-negative results on aspirate samples.[15] There also remains the potential to dilute aspirate samples with peripheral blood, leading to false-negative results on aspirate samples, despite involvement being noted on the biopsy specimens. Overall, we noted concordance of ~82% between aspirate and trephine biopsy specimens. It is interesting that there were six cases that gave negative results on trephine biopsy but were reported as positive on aspirates. The overall median trephine length of our patient cohort was close to the recommended optimum trephine length of 20 mm, which makes that an unlikely reason for the negative biopsy results.[16] Possible explanations may be related to reports that bilateral trephine biopsies or examination of a single site biopsy at multiple levels can improve the yield on morphological analysis in NHL.[16,17] We did not perform bilateral biopsies and the levels examined in our retrospective study ranged from 1 to 8. This may have yielded false-negative results on our trephine biopsy specimens. It may therefore be reasonable to conclude that in many ways, aspirate and trephine samples, even when taken from the same site, have the potential to behave as different specimens.

As flow cytometry is usually performed on BM aspirates, it follows that discrepancies are likely to be inevitable between flow cytometry, aspirate and trephine biopsy results. Furthermore, it is well recognized that flow cytometry is a more sensitive technique, and can detect low-level involvement in cases where there is <5% infiltration.[5] In other words, flow cytometry can detect cases with occult or morphologically inapparent disease. The definition of monoclonality used, and the gating strategies employed by different groups, vary in different studies. This may be responsible for the varying rates of occult involvement reported. κ:λ ratios used to define monoclonality vary from >3:1 to >6:1, with λ to κ ratios of 0.3:1[4] and <0.5:1.[5] It is recommended that each laboratory sets up its own normal ratios, as has been done in our laboratory. The stringency of control measures in

place and the skill of the operator or the person reporting/analysing results further tend to affect results.

The rate of occult involvement in different subtypes of NHL remains to be studied in a systematic manner. Perea *et al.* have found that most cases with occult involvement belong to the histological subtype of follicular lymphoma, with only 3/36 cases belonging to the DLBCL category.[3] However, others have found that most cases with occult involvement belong to the histological subtype of DLBCL.[4] In their immunophenotypic analysis of staging in B-cell malignant lymphomas, Hanson *et al.* have found that all five morphology-negative, flow cytometry-positive cases in their study were from patients with large cell lymphoma. All patients had localized but clinically bulky disease and were morphologically negative on bilateral biopsies. Whether this indicates that large cell lymphoma cases are more prone to developing occult marrow involvement remains to be clarified.

The clinical relevance in terms of patient outcome, of occult disease in NHL overall, and specifically in DLBCL remains to be established. Positivity on flow cytometry or on any of the other ancillary tests such as molecular studies using *Ig* gene rearrangement upgrades the stage on the Ann Arbour system to grade IV. Although it is recognized that this may have implications in terms of prognosis, there is limited literature addressing the issue. Perea *et al.* have studied the impact of occult disease in patients with follicular lymphoma on the duration of complete remission, and found it had no significant impact on outcome. Patients with negative morphology and positive flow cytometry did no worse than those negative on morphology and flow cytometry. However, they had significantly better outcomes than patients with obvious marrow involvement on morphology.[3]

Part of the difficulty in determining the clinical relevance of occult disease is related to the small number of such cases in any series. A previous report has suggested another way to address the issue, recommending that morphology and flow cytometry be combined in the evaluation of BM specimens for involvement with NHL.[18] If positivity on the two testing strategies were used together in a summative 'either/or' model to define BM involvement, it would take into account the limitations of each test and account for the discordance inherent in staging. A pilot study by our group has also demonstrated that such a summative model, which includes cases detected on ancillary investigations such as flow cytometry, immunohistochemistry and molecular studies to morphologically apparent ones, works as a better predictor of survival than the conventional definition using morphology alone.[19] Morphological staging already utilises this model, with positivity on either aspirate or biopsy being classified as involvement.

Our study is the first to demonstrate the clinical relevance of using both morphology and flow cytometry in a summative model to define BM involvement in patients with DLBCL by correlating it with overall survival. Although our study population was small, we were able to demonstrate that incorporating occult cases into the definition of BM involvement makes a small but significant difference in both overall survival and time to relapse. This allows the discussion regarding this population perhaps to move out of the laboratory into clinical practice. It is not surprising that the revised definition failed to affect significantly the IPI in our study – only cases that upgrade the stage of disease from I/II to IV change the IPI; upstaging from III to IV fails to effect a change. It is possible that our numbers were too small to capture a significant change and larger studies are required to address this issue further, particularly in intermediate and high-grade lymphomas. In future, if these results are corroborated by larger studies, this more comprehensive approach to staging may help to include cases that may have been previously classified as low risk. A prospective, randomized study may help to clarify the role of more aggressive upfront management, including autologous transplantation in these patients.

Acknowledgements

The authors would like to acknowledge the financial support provided for the study by the Private Practice Trust Fund, The Canberra Hospital, and the equipment grant provided by The Leukaemia Foundation, Australia. The principal investigator/author has received a supplementary scholarship from the Arrow Bone Marrow Transplant Foundation, New South Wales for the project.

References

1. The Haemato-oncology Task Force of British Committee for Standards in Haematology. *BCSH guidelines on nodal non-Hodgkin's lymphoma.* Available at: http://www.bcshguidelines.com/pdf/NHL_200504.pdf, 2002. Last accessed 21 January 2008.
2. Miller TP, Dahlberg S, Cassady JR *et al.* Chemotherapy alone compared with chemotherapy plus radiotherapy for localized intermediate- and high-grade non-Hodgkin's lymphoma. *N. Engl. J. Med.* 1998; **339**; 21–26.
3. Perea G, Altes A, Bellido M *et al.* Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL). *Histopathology* 2004; **45**; 268–274.

© 2008 The Authors. Journal compilation © 2008 Blackwell Publishing Ltd,

4. Hanson CA, Kurtin PJ, Katzmann JA *et al.* Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. *Blood* 1999; **94**; 3889–3896.
5. Stacchini A, Demurtas A, Godio L, Martini G, Antinoro V, Palestro G. Flow cytometry in the bone marrow staging of mature B-cell neoplasms. *Cytometry B Clin. Cytom.* 2003; **54**; 10–18.
6. Gomyo H, Shimoyama M, Minagawa K *et al.* Morphologic, flow cytometric and cytogenetic evaluation of bone marrow involvement in B-cell lymphoma. *Haematologica* 2003; **88**; 1358–1365.
7. Duggan PR, Easton D, Luider J, Auer IA. Bone marrow staging of patients with non-Hodgkin lymphoma by flow cytometry: correlation with morphology. *Cancer* 2000; **88**; 894–899.
8. Chetty R, Echezarreta G, Comley M, Gatter K. Immunohistochemistry in apparently normal bone marrow trephine specimens from patients with nodal follicular lymphoma. *J. Clin. Pathol.* 1995; **48**; 1035–1038.
9. Mitterbauer-Hohendanner G, Mannhalter C, Winkler K *et al.* Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). *Leukemia* 2004; **18**; 1102–1107.
10. Cheson BD, Horning SJ, Coiffier B *et al.* Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. *J. Clin. Oncol.* 1999; **17**; 1244.
11. Marti GE, Rawstron AC, Ghia P *et al.* Diagnostic criteria for monoclonal B-cell lymphocytosis. *Br. J. Haematol.* 2005; **130**; 325–332.
12. Naughton MJ, Hess JL, Zutter MM, Bartlett NL. Bone marrow staging in patients with non-Hodgkin's lymphoma: is flow cytometry a useful test? *Cancer* 1998; **82**; 1154–1159.
13. Palacio C, Acebedo G, Navarrete M *et al.* Flow cytometry in the bone marrow evaluation of follicular and diffuse large B-cell lymphomas. *Haematologica* 2001; **86**; 934–940.
14. Juneja SK, Wolf MM, Cooper IA. Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. *J. Clin. Pathol.* 1990; **43**; 630–632.
15. Pittaluga S, Tierens A, Dodoo YL, Delabie J, De Wolf-Peeters C. How reliable is histologic examination of bone marrow trephine biopsy specimens for the staging of non-Hodgkin lymphoma? A study of hairy cell leukemia and mantle cell lymphoma involvement of the bone marrow trephine specimen by histologic, immunohistochemical, and polymerase chain reaction techniques. *Am. J. Clin. Pathol.* 1999; **111**; 179–184.
16. Campbell JK, Matthews JP, Seymour JF, Wolf MM, Juneja SK. Optimum trephine length in the assessment of bone marrow involvement in patients with diffuse large cell lymphoma. *Ann. Oncol.* 2003; **14**; 273–276.
17. Wang J, Weiss LM, Chang KL *et al.* Diagnostic utility of bilateral bone marrow examination: significance of morphologic and ancillary technique study in malignancy. *Cancer* 2002; **94**; 1522–1531.
18. Dunphy C. Combining morphology and flow cytometric immunophenotyping to evaluate bone marrow specimens for B-cell malignant neoplasms. *Am. J. Clin. Pathol.* 1998; **109**; 625–630.
19. Talaulikar D, Dahlstrom JE, Broomfield A, McNiven M, Shadbolt B. Occult marrow involvement in diffuse large B-cell lymphoma: results of a pilot study. *Pathology* 2007; **39**; 580–585.

© 2008 The Authors. Journal compilation © 2008 Blackwell Publishing Ltd, *Histopathology*, **52**, 340–347.

Author contributions:

Talaulikar D, Shadbolt B, Bell J, Khan K, and Dahlstrom JE: **"Clinical role of flow cytometry in redefining bone marrow involvement in Diffuse Large B-cell Lymphoma – a new perspective."** Histopathology 2008; 52: 340-347.

Dipti Talaulikar:

1. Designed study, prepared ethics and grants applications, and set up database
2. Retrieved all cases from medical and laboratory records, and all archived slides and paraffin-embedded trephine blocks
3. Performed all the data extraction, analysed and computed all clinical data, and performed all data entry
4. Reported all histology slides as the first blinded reviewer
5. Interpreted all flow cytometry analysis
6. Performed all statistical analysis under supervision of and help from Dr. Bruce Shadbolt
7. Wrote the first draft of the paper

Bruce Shadbolt:

1 Setting up database

2 All data analysis

3 Review of final paper

Jill Bell & Ms. Kowsar Khan:

1. Reanalysis of dot plots for flow cytometry
2. Approval of final paper

Jane E Dahlstrom:

1. Photographs of slides
2. Approval of final paper

Anne McDonald:

1. Blinded 2nd reviewer of H & E slides on remaining ~75 cases
2. Approved final paper

Michael Pidcock:

1. Blinded 2nd reviewer of H & E slides on pilot study
2. Approved final paper

Amy Broomfield:

1. Technical advice with preparing all immunohistochemistry slides
2. Approved final paper

Intellectual planning: DT (97%), JED: (1%) BS: (2%)

Experimental work: DT (95%) JED: (1%) AMcD: (1%) JB and KK: (2%), AB: (1%)

Analyses: DT (70%) BS (30%)

Writing: DT (97%) JED (2%) BS (1%)

CHAPTER V:

Clinical role of immunohistochemistry

5.1 Role of immunohistochemistry in staging diffuse large B-cell lymphoma. J Histochem Cytochem 2008, 56(10):893-900

Volume 56(10): 893–900, 2008
Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

Role of Immunohistochemistry in Staging Diffuse Large B-cell Lymphoma (DLBCL)

Dipti Talaulikar, Jane Esther Dahlstrom, Bruce Shadbolt, Amy Broomfield, and Anne McDonald

Department of Haematology (DT), Australian National University Medical School (DT,JED,BS), Department of Anatomical Pathology (JED,AB), and Department of Epidemiology (BS), The Canberra Hospital, Canberra, Australia, and National Capital Private Hospital, Canberra, Australia (AM)

SUMMARY The use of immunohistochemistry (IHC) in staging bone marrow in non-Hodgkin's lymphoma (NHL) is largely limited to ambiguous cases, particularly those with lymphoid aggregates. Its role in routine clinical practice remains unestablished. This study aimed to determine whether the routine use of IHC in diffuse large B-cell lymphoma (DLBCL) would improve the detection of lymphomatous involvement in the bone marrow. It also sought to determine the impact of IHC on predicting survival compared with routine histological diagnosis using hematoxylin and eosin (H&E), Giemsa, and reticulin staining. The bone marrow trephines of 156 histologically proven DLBCL cases were assessed on routine histology, and IHC using two T-cell markers (CD45RO and CD3), two B-cell markers (CD20 and CD79a), and κ and λ light chains. IHC detected lymphomatous involvement on an additional 11% cases compared with histology alone. Although both routine histology and IHC were good predictors of survival, IHC was better at predicting survival on stepwise multivariate Cox regression analysis. IHC performed routinely on bone marrow trephines has the ability to improve detection of occult lymphoma in experienced hands. Furthermore, it is a better predictor of survival compared with routine histological examination alone.
(J Histochem Cytochem 56:893–900, 2008)

KEY WORDS
immunohistochemistry
histology
staging
bone marrow trephine biopsy
diffuse large B-cell lymphoma
International Prognostic Index

IMMUNOHISTOCHEMISTRY (IHC) is a very valuable tool that, in the last three decades, has revolutionalized the practice of diagnostic histopathology. It is widely used to improve characterization of a number of tumors and to detect occult metastases, especially in lymph nodes. Applications in lymphoma mainly relate to providing information on the classification and subclassification of lymphomas in diagnostic primary tissue. Other applications include determining biological factors of prognostic significance (Lossos and Morgensztern 2006) in some subtypes of non-Hodgkin's lymphoma (NHL) and a role in staging bone marrow (BM) biopsies once diagnosis is established on primary tissue. Staging BM is generally examined using routine histology, with ancillary studies such as immunophenotyping being performed in some cases to confirm lymphomatous involvement. This may involve flow cytometric assessment of BM aspirate samples or IHC on trephine biopsy samples.

Reported rates of BM involvement in NHL differ based on the subtype of NHL, with low-grade lymphomas having much higher rates of involvement (Harris et al. 1999). The reported rates of BM involvement in diffuse large B-cell lymphoma (DLBCL) vary from 12% to 35% (Conlan et al. 1990; Campbell et al. 2003). Patterns of infiltration vary from subtle focal infiltrates to almost complete effacement by lymphoma cells (Harris et al. 1999). Histological discordance with the presence of large cells in primary tissue and predominantly small cells in the BM is known in DLBCL, with reported rates of ~50-70% (Conlan et al. 1990; Campbell et al. 2006; Chung et al. 2007).

The implications of BM involvement also vary depending on the grade of lymphoma. Marrow involvement in DLBCL has implications for prognosis; lymphomatous infiltration in the marrow has been reported to correlate with poor survival (Yan et al. 1995;

Correspondence to: D. Talaulikar, MBBS, FRACP, FRCPA, GradCertHE, Department of Haematology, The Canberra Hospital, PO Box 11, Woden, ACT 2606, Australia. E-mail: dipti.talaulikar@act.gov.au
Received for publication February 11, 2008; accepted June 11, 2008 [DOI: 10.1369/jhc.2008.951087].

© The Histochemical Society, Inc. 0022-1554/08/$3.30

The Journal of Histochemistry & Cytochemistry

Campbell et al. 2006). Furthermore, marrow involvement in DLBCL affects the International Prognostic Index (IPI) both by increasing the stage of the disease to stage IV and by being assessed as extranodal involve ment (Shipp et al. 1993). Treatment of high-grade lymphomas, in many centers, may vary depending on the stage of the disease or the IPI.

The role of IHC in staging BM has been upstaged, to some extent, by immunophenotyping by flow cytometry of BM aspirates. This is perhaps because the results obtained with flow cytometry are quantitative and perceived to be less prone to the errors that may be associated with a more subjective diagnostic tool such as IHC, particularly in inexperienced hands. The ready availability of flow cytometric analysis in most laboratories has also led to its widespread use. Indeed, there is recent evidence that flow cytometry plays an important role in staging of DLBCL and has an impact on outcome factors (Talaulikar et al. 2008).

It is well known that BM involvement in NHL is more often apparent on trephine biopsy specimens rather than BM aspirates (Juneja et al. 1990; Horlyck and Thorling 1991; Bain 2001). This is likely to relate to the presence of BM fibrosis causing false-negative results on aspirate samples. It follows that flow cytometric assessment of aspirates can also yield false-negative results (Hanson et al. 1999; Perea et al. 2004; Talaulikar et al. 2008), whereas if flow cytometric assessment of trephine biopsy samples was performed, a greater positivity rate may be obtained. There is no published literature addressing this issue; this may be because using some of the biopsy specimen for flow cytometric analysis could compromise availability of tissue for histological evaluation. In most cases of NHL, it is recognized that biopsy specimens need to be at least 16 mm in length and that several levels require examination for optimal results (Bain 2001; Campbell et al. 2003). Obtaining additional biopsy tissue for flow cytometric analysis may inconvenience the patient significantly. Immunophenotyping on BM trephines using IHC is therefore a logical ancillary study in staging BM.

Most studies have focused on the use of IHC on BM trephines in ambiguous cases to differentiate between benign lymphoid aggregates and malignant infiltration. We are aware that many centers within and outside Australia do not use IHC routinely in staging bone marrows, partly because of the cost involved and partly because of the lack of evidence regarding its use. Other studies have looked at the use of IHC alone but have not reported results for routine morphology and immunostaining separately (Palacio et al. 2001; Mazur et al. 2004).

The role of IHC in routine clinical practice to detect occult lymphoma in the BM and its effect on patient survival is largely unknown. This study aimed to assess the clinical use of routine IHC analysis in staging bone marrows in DLBCL, including its effect on outcome factors such as survival.

Materials and Methods

Patients

One hundred fifty-six retrospective cases diagnosed with histologically proven DLBCL at The Canberra Hospital from 1986 to 2005, on whom staging BM biopsies had been performed, were identified for the purpose of the study. After approval was obtained from the Australian Capital Territory (ACT) Human Research Ethics Committee, clinical information on patients was collected from the Medical Records department at The Canberra Hospital.

The average age of the patient cohort (known in 154 cases) was 61 years (range, 20–87 years), and the male to female ratio was 1.5:1. Staging data were available in 148 patients. Using routine staging procedures, 37/148 (25%) had Stage I disease; 35/148 (23.6%) had Stage II disease; 45/148 (30.4%) had Stage III disease; and 31/148 (20.9%) had Stage IV disease. Baseline assessment of IPI was possible in 148 patients. Fourteen patients had an IPI of 0 (9.5%), 23 patients had an IPI of 1 (15.5%), and 36 had an IPI of 2 (24.3%). IPIs of 3, 4, and 5 were noted in 46 (31.1%), 22 (14.9%), and 7 patients (4.7%), respectively. The mean baseline IPI of the patient cohort was 2.41 with an SD of 1.3.

Treatment data were available on 142 patients, with almost all patients having been treated with anthracycline-based regimens. Nine patients were treated with palliative intent with steroids alone or in combination with non–anthracycline-based drugs. Only 36 patients (22.2%) received rituximab. The median overall survival of the entire patient group was 58 months [95% confidence interval (CI), 33; 82 months].

BM Histology

BM biopsies are performed as a routine assessment for all cases with NHL at first diagnosis in our institution. Routinely, all trephines are fixed in buffered formalin and acetic acid. Decalcification is performed using 5% nitric acid. Samples are embedded in paraffin, and an attempt is made to stain four sections with hematoxylin and eosin (H&E). Additionally, for one to two sections, a Giemsa stain is performed. Silver impregnation is performed for reticulin. H&E, Giemsa, and reticulin preparations on the trephine biopsy were retrieved for review. The mean trephine length for the patient cohort was 17.6 mm, with a range of 8–36 mm, and the mean number of levels on H&E sections was 3.7 (range, 1–8).

All slides were reviewed blindly by two hematologists, with discrepant cases (n=20) being reviewed by

a third. Cheson criteria were used to classify trephine biopsy samples as positive, negative, or indeterminate (Cheson et al. 1999). Cases with paratrabecular lymphoid aggregates, large-sized aggregates, presence of large cells within aggregates, and increased reticulin response were classified as positive for involvement. Benign lymphoid aggregates were classified as those that were intertrabecular in location, small in size, well circumscribed, and contained predominantly small cells admixed with reactive cells.

IHC

IHC analysis was performed on a Ventana Benchmark NexES machine (Ventana Medical Systems Inc; Tucson, AZ) for all cases on archived formalin-fixed, paraffin-embedded trephine biopsies. The streptavidin-biotin method was used to immunostain sections using the following monoclonal antibodies: CD3 (Dako clone CD3, DakoCytomation Inc, Carpenteria, CA; 1:200 dilution), CD45RO (Novacastra clone UCLH-1, Novacastra Laboratories Ltd, Newcastle, UK; 1:1000 dilution), CD20 (Zymed clone L26, Zymed Laboratories, Invitrogen Immunodetection Corp, Carlsbad, CA; 1:50 dilution), CD79a (Dako clone JCB117, 1:500 dilution), κ (Novacastra clone kp-53, 1:750 dilution), and λ (Novacastra clone Hp-6054, 1:750 dilution). CD20 and CD3 are known to be most sensitive at assigning lineage in diffuse aggressive NHL (Chadburn and Knowles 1994) and are the most commonly used antibodies in our laboratory. The two additional antibodies, CD79a and CD45RO, were selected to maintain consistency because these are the antibodies used for diagnostic tissue sections in our laboratory. Heat retrieval was used for all antibodies, and tonsillar tissue was used as a positive control. A formal system of reporting was developed and was followed for all stains. Staining for each antibody was reported as positive/negative, normal/abnormal, individual scattered cells/clusters, small/large cells, and percent of biopsy involved (<1%, 1–5%, or >5%). All slides were reported by two pathologists blinded to the previous assessment on histology.

The features described to define abnormality on IHC reflected the Cheson criteria for routine histology, although a fairly conservative approach was adopted by the pathologists to avoid false positives. Overall, location of infiltrates, size of cells, number of cells compared with controls, and nuclear morphology were used.

The presence of clusters of B cells was classified as abnormal or malignant when there were large number of clusters, the clusters were large sized, or they contained disproportionate numbers of larger cells. Scattered malignant cells may be missed quite easily on routine histology (Fraga et al. 1995) but be more easily discernible on IHC analysis. Such cases were classified positive when numbers of B cells were increased compared with controls. These were cases with morphologically normal marrows ($n=6$) that were used to create a visual impression of normal amounts of background T and B cells. Overall, a fairly conservative approach was adopted to avoid false positives.

Statistical Analysis

Response and survival data were recorded for each patient. Besides descriptive analysis, Kaplan Meier curves were created with death as outcome. Stepwise (LR) multivariate Cox regression analysis was used to establish how IHC compared with routine histology as a predictor of survival. Similar analysis was also performed to assess whether a revised IPI computed by addition of immunostaining-positive cases was better at predicting survival compared with the baseline IPI. Patients treated with palliative intent were excluded from all survival analyses. All analyses were performed using the software program Statistical Package for Social Sciences (SPSS) version 14.0 (SPSS Inc; Chicago, IL).

Results

Histology

Of the 156 cases on which BMs could be evaluated, 24 were noted to be positive on routine histology; 6 cases were reported as indeterminate using Cheson criteria. Rather than using immunostaining to aid diagnosis, an attempt was made to resolve these on consensual review of routine H&E slides; all cases were agreed on as being positive for involvement on consensual review. H&E stains showed no evidence of involvement in 126 cases.

IHC

Paraffin-embedded blocks were available in 154 cases. IHC using T- and B-cell markers showed involvement in 43 cases. Of these, routine H&E slides were unavailable for comparison in one case. Of the remaining, routine histology showed involvement in 25 cases, whereas 17 cases were reported as not involved. In other words, use of IHC routinely on all cases detected 17 additional cases (11.0%) with BM involvement. Of the remaining 112 cases, 10 were reported initially as possible for involvement. Using a fairly conservative approach, all cases were classified as negative on consensual review. Of the remaining 102, 5 were reported as involved on H&E, including 2 cases that were previously reported as indeterminate. IHC results were reviewed again after being unblinded to routine histology results and showed that there were small involved areas on routine histology slides that were not apparent on deeper sections taken for IHC. Together, routine histology and IHC detected involvement in 48 cases. Variable expression was noted on the two B-cell markers CD20 and CD79a, with CD20 showing more consistent and strong expression.

The Journal of Histochemistry & Cytochemistry

Table 1 IHC results on individual T-cell and B-cell antibodies

Variables	Normal	Abnormal	Clusters	Individual
CD3 (n=43)	22	21	36	7
CD45RO (n=43)	22	21	31	12
CD20 (n=43)	0	43	39	4
CD79a (n=38*)	7	31	28	10

*Five cases were reported negative on CD79a.

The IHC findings for individual antibodies are listed in Table 1. Eleven (25.6%) of the 43 involved cases had small cells, whereas the remaining 32 (74.4%) had large cells. Fourteen (32.6%), 10 (23.3%), and 19 cases (44.2%) had <1%, 1–5%, and >5% of B cells infiltrating the marrow, whereas T-cell infiltration was as follows: 32 (74.4%) cases with <1%, 4 (9.3%) cases with 1–5%, and 7 (16.3%) cases with >5% infiltration. Of all cases reported as positive on B-cell markers, only 15 showed clear evidence of light chain restriction. Twenty-three cases showed no light chain restriction.

Effect on Outcome

IPI. The results of IHC were added to those of routine histology to redefine BM involvement. A new revised IPI (rIPI) was computed for all cases based on the IHC results. Changes to the IPI essentially occurred when stage of disease was upgraded from Stage 1 or 2 to Stage 4 disease. Marrow involvement was also calculated as an additional extranodal site of involvement, further changing the IPI in some cases. Of 144 cases where rIPI was assessable, 9 cases had an rIPI of 0, 18 had an rIPI of 1, and 33 had an rIPI of 2. Forty, 31, and 13 cases had an rIPI of 3, 4, and 5, respectively.

Survival. Kaplan-Meier curves were created to assess the impact of BM involvement on overall survival. When routine histology alone was used to define BM involvement on H&E staining, the difference in median survival between patients with and without involvement was 40 vs 81 months (p=0.02; Figure 1). Using positivity on IHC to define BM involvement, the median survival of patients with lymphoma in their BM was 28 vs 82 months (p<0.0001; Figure 2).

Kaplan-Meier curves were created to assess the impact of small cell vs large cell infiltration, and the degree of B-cell infiltration. The median survival times for cases with discordant (small cell) involvement were better than those for concordant or large cell involvement (33 and 22 months), although the difference was not significantly significant (p=0.7). The median survival of cases with <1%, 1–5%, and >5% B-cell infiltration was 40, 33, and 15 months. In other words, prognosis seemed to worsen with increased B-cell infiltration, although this again was not statistically significant (p=0.7).

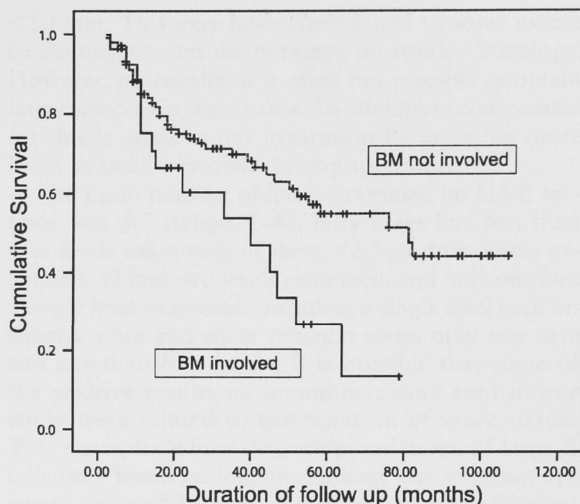


Figure 1 When routine histology on trephine biopsies was used to look for bone marrow involvement, the difference in survival between patients with and without involvement was 40 vs 81 months (p=0.02).

Multivariate Analysis. Using a multivariate forward stepwise (likelihood ratio method) Cox regression, marrow involvement as defined by three different methods was competitively considered for the contribution to predicting survival. The three definitions used were positivity on histology alone (H), positivity on immunostaining alone (I), and positivity on histology or immunostaining (H + I). In the analysis, it was found that the definition using immunostaining alone (I) contributed significantly more to explaining survival than the

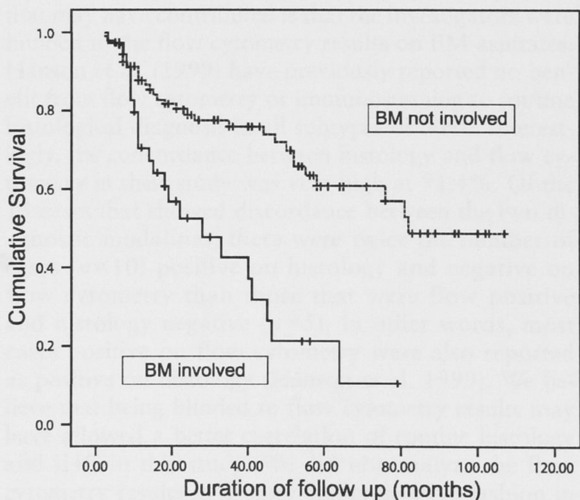


Figure 2 When immunostaining was used as a routine in all cases, the difference in survival between patients with and without marrow involvement was 28 vs 81 months (p<0.0001).

The Journal of Histochemistry & Cytochemistry

other two and that, after the immunostaining model was included in the regression model, the definitions using histology alone and that using both modalities together did not significantly contribute to predicting survival ($p=0.1$, 0.66; Table 2). Degree of malignant infiltration on the biopsy and concordant vs discordant involvement was also included in the model but did not significantly contribute over and above the listed variables ($p=0.95$ and $p=0.56$).

To study the effect of treatment with rituximab on survival, this too was considered, but not found to contribute significantly to the Cox regression model ($p=0.8$).

However, when a similar Cox regression analysis was performed to compare revised and baseline IPI, rIPI did not contribute significantly to survival over and above baseline IPI ($p=0.4$). Although the mean rIPI among patients who did not survive was higher than the mean baseline IPI [3.22 (95% CI, 2.87, 3.57) vs 2.92 (95% CI, 2.59, 3.25)], this was also the case in patients who were alive at the time of analysis [2.38 (95% CI, 2.01, 2.63) vs 2.03 (95% CI, 1.73, 2.32)].

Discussion

This study looked at the role of routine IHC in staging DLBCL at initial diagnosis. Our results indicate that, in experienced hands, use of immunostaining surpasses routine histology by detecting occult disease in ~10% of cases. Moreover, immunostaining alone is a stronger predictor of survival than routine histology.

Detection of occult BM involvement by lymphoma hinges on the adequacy of both routine histological diagnosis and IHC analysis. There are several variables that affect adequacy of routine histological diagnosis on BMs. It was previously believed that bilateral trephine biopsies increased the yield from staging BMs in NHL (Juneja et al. 1990). However, it is now established that bilateral sampling is not required if adequate tissue (including adequate trephine length and number of levels examined) is available for diagnosis (Campbell et al. 2003). Our mean trephine length was reasonable at 17.6 mm, with a range of 8–36 mm. Fifty-five cases had a less than recommended trephine length of <16 mm, although only 7 had a length <10 mm. This may have contributed to some extent to the negative results obtained on routine histology. However, practically, it is often not possible to obtain larger samples in some cases. An attempt to compensate for this is made in our institution by assessing more levels in smaller trephine biopsy specimens.

The mean number of levels examined on H&E sections was 3.7 (range, 1–8). Fifty cases had less than four levels examined; of these, 12 had three levels examined, 37 had two levels examined, and only one had a single level examined. Including a single level each on Giemsa stain and silver reticulin stain, only one case had less than four levels. It is possible that some of the positive results on immunostaining seen in our study were related to examination of more tissue. Two examples where discrete populations of large B cells were noted on immunostaining that were not apparent on careful review of routine histology slides are shown in Figure 3. A previous study by Campbell et al. (2003) established that four levels is the optimal number that need to be examined for routine morphological assessment in staging DLBCL. A similar study is required to assess how much of the benefit obtained on immunostaining is caused by examination of multiple levels.

It is acknowledged that the detection of lymphoma in the marrow is subjective and depends on the experience and skill of the observers, particularly in interpretation of lymphoid aggregates as being benign or malignant. The use of standardized criteria is now therefore recommended for classifying cases as positive, negative, or indeterminate for involvement with lymphoma (Cheson et al. 1999). This was followed in our study and may have been responsible for more cases being classified as negative. Another likely factor that may have contributed is that the investigators were blinded to the flow cytometry results on BM aspirates. Hanson et al. (1999) have previously reported no benefit from flow cytometry or immunostaining to routine histological diagnosis in all subtypes of NHL. Interestingly, the concordance between histology and flow cytometry in their study was very high at 91.4%. Of the 15 cases that showed discordance between the two diagnostic modalities, there were twice the number of cases ($n=10$) positive on histology and negative on flow cytometry than those that were flow positive and histology negative ($n=5$). In other words, most cases positive on flow cytometry were also reported as positive on histology (Hanson et al. 1999). We believe that being blinded to flow cytometry results may have allowed a better correlation of routine histology and IHC in this study. We did not analyze the flow cytometry results in a more comprehensive fashion in this study. However, previous results comparing flow cytometry and morphology showed slightly lower concordance rates of 83% (Talaulikar et al. 2008).

Table 2 Cox regression model showing the effect of different definitions of bone marrow involvement on survival after adjusting for age and performance status*

Significant variables	Hazards ratio (95% CI)	*p* value
Age (years)	1.04 (1.01, 1.06)	0.004
ECOG	1.42 (1.09, 1.84)	0.01
IHC (I)	1.84 (1.0, 3.44)	0.05

*Histology alone (H), and histology + immunostaining (H + I) were considered in the model but did not significantly contribute after immunostaining alone was included. Additional variables (i.e. concordant vs discordant involvement and degree of malignant infiltration) did not contribute to the model either. ECOG, Eastern Cooperative Oncology Group; CI, confidence interval.

The Journal of Histochemistry & Cytochemistry

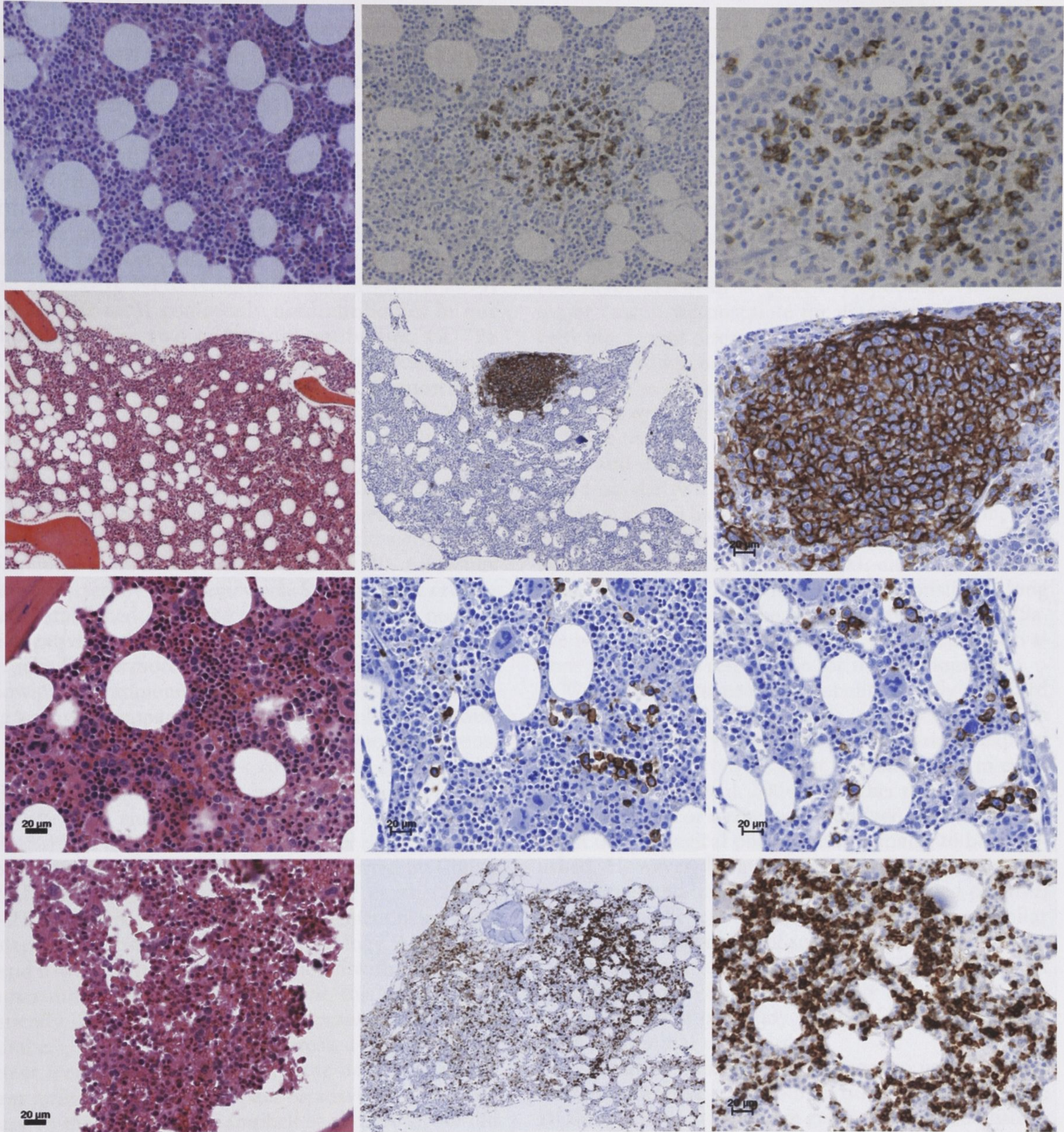


Figure 3 Examples in which routine histology showed no evidence of involvement. Horizontally, top and second panels show cases where, when further sections were cut for IHC analysis, discrete populations of large B cells were noted. Third panel shows a case where routine histology failed to detect scattered large abnormal lymphoid cells that were picked up on IHC. Bottom panel shows a case with scattered small cell disease on IHC with B-cell clusters.

There are other differences between our study and that of Hanson et al. (1999). They included cases of all subtypes of NHL of which only 74 cases were DLBCL (42.2%). They reported 6% BM involvement. This is much lower than published rates of involvement in DLBCL (Conlan et al. 1990; Campbell et al. 2003) and those found in our patient population (30/156, 19.2%). Moreover, in the study by Hanson et al. (1999), only 107/175 cases related to initial presentation; the rest were restaging marrows. This may have had an impact on the final outcome. The mean trephine length of biopsies is not indicated in the study of Hanson et al.

(1999). However, bilateral biopsies were performed in all cases, and at least three levels per biopsy were examined. This indicates that routine histological analysis was more comprehensive; however, this, in our experience, is often not practically possible.

Adequacy of IHC analysis is a contentious issue prone to the same subjective errors as routine histology. We used two B-cell and two T-cell markers to corroborate our findings. CD20 and CD3 have been shown to be most sensitive at assigning lineage in diffuse aggressive NHL (Chadburn and Knowles 1994) and are the most commonly used antibodies in our laboratory. The two additional antibodies, CD79a and CD45RO, were selected to maintain consistency because these are the antibodies used in diagnostic tissue sections. CD79a has an advantage over PAX5 in that it helps to differentiate T cell–rich B-cell lymphomas from Hodgkin Reed Sternberg cells (Isaacson 2007). CD45RO was preferred over CD5 as a T-cell marker because of reports that CD5 expression can occur in DLBCL and may have prognostic significance (Taniguchi et al. 1998; Harada et al. 1999). All results were assessed by two reviewers. Standardized criteria were established and used to classify cases as positive or negative as described in the Materials and Methods section. Furthermore, we believe that our survival data showing that immunostaining alone is better at predicting survival (compared with routine histology) indicates that our immunostaining results are likely to be rigorous.

We found in our series of patients that, of the 43 cases detected to have involvement on IHC, 11 (25.6%) had small cells, whereas the majority (32, 74.4%) had large cells. This is a higher degree of concordant involvement than has been reported by Chung et al. (2007) (~50%) and Campbell et al. (2006) (~28%) and may be caused by involvement being measured on IHC rather than routine histology. Although a trend toward better survival was noted with discordant rather than concordant involvement, this was not statistically significant and may be because of the small numbers, especially when cases treated with palliative intent were excluded. Similarly, the degree of malignant infiltration did not impact on survival. Also, contrary to the findings of Campbell et al. (2006), we did not find a significant association between the degree of infiltration and concordant involvement (χ^2: 3.8, df: 2, $p=0.15$). This is likely to be because we defined positive cases on IHC rather than routine histology.

A few studies have addressed the issue of detection of histologically inapparent disease using immunostaining. Fraga et al. (1995) reported that in a case series ($n=42$) of anaplastic large cell lymphoma, routine histology detected marrow involvement in 17% cases. Routine IHC analysis using CD30 detected occult disease in a further 23% cases (Fraga et al. 1995). We found BM involvement on routine histology in 19.2%, with a more modest 11.1% rate of occult involvement. The authors of the previous study postulated that the inability of routine histology to detect low level disease was related to the scarcity of malignant cells among normal hemopoietic cells and the difficulty in distinguishing them from immature hemopoietic cells. They recommended the systematic use of immunostaining to detect BM involvement in anaplastic lymphoma, which they reported as a poor prognostic factor. We believe a similar phenomenon occurs with low level marrow involvement in large cell lymphoma. The other major reason we postulate for detection of histologically inapparent disease in the BM by IHC is the examination of additional sections. This was reflected in our series by the cases that showed clusters of B cells on IHC that were not seen on routine histology.

Some technical issues were also highlighted by the study and deserve comment. Variable expression was noted on the two B-cell markers CD20 and CD79a. Previous studies have reported that decalcification, particularly with 5% nitric acid, has negative effects of certain clones of CD20, CD79a, CD5, and CD43 (Miller et al. 2000). This may have been responsible, along with other factors, for the weak expression of CD79a. We were unable to find any other published observations that may be responsible for this phenomenon.

We were able to establish clonality in only one third of cases (15/42, 35.7%). There is some literature to suggest that flow cytometry may show variable expression of antigens from different anatomic sites in cases of NHL (Gervasi et al. 2004). Whether this is related to technical issues or a true loss of antigen expression as a result of a biological phenomenon remains to be established. However, because strong positivity was noted on external and internal (plasma cells stained positive) controls, we believe the latter is more likely. Similar variability of expression of some antigens has also been reported to occur over time in B-cell NHL (Echeverri et al. 2002). However, that is unlikely to be responsible for the results in our study because all biopsy samples were from initial diagnosis.

There are a number of advantages to use of routine immunostaining of trephine biopsies for staging DLBCL. IHC techniques are generally well established, available in most centers, and relatively cost effective. They enable the examination of a greater number of levels of the biopsy in a more comprehensive and sensitive manner and are useful in the detection of additional clusters of malignant cells and that of scattered malignant cells among normal hemopoietic cells.

Despite the use of more realistic measures of routine histological diagnosis such as unilateral biopsies and assessment of less than optimal trephine lengths, we have been able to show that immunostaining in experienced hands is more effective at predicting survival than routine histology. There are several other limita-

tions of this study besides those inherent to retrospective data. We acknowledge that results of IHC analysis are subjective and can be very variable in less experienced hands. Also, there may be a degree of variability caused by the use of different antigen retrieval methods and processing of specimens (Miller et al. 2000).

In summary, we showed that IHC analysis using B- and T-cell markers detects morphologically inapparent marrow involvement in ~11% of cases of DLBCL at initial diagnosis. Moreover, immunostaining alone is a stronger predictor of survival than routine histology alone. Although the findings are interesting and suggest that routine immunostaining should be incorporated into staging of DLBCL, further large series of prospective data are required to corroborate the findings, especially in the rituximab era. Further studies are also required to establish whether the lack of light chain expression on BM trephine samples is related to a biological phenomenon.

Acknowledgments

The authors acknowledge the financial support provided for the study by the Private Practice Trust Fund, The Canberra Hospital, and the equipment grant provided by The Leukaemia Foundation, Australia. The principal investigator/author received a supplementary scholarship from the Arrow Bone Marrow Transplant Foundation, New South Wales, for the project.

Literature Cited

Bain BJ (2001) Bone marrow trephine biopsy. J Clin Pathol 54: 737–742

Campbell J, Seymour JF, Matthews J, Wolf M, Stone J, Juneja S (2006) The prognostic impact of bone marrow involvement in patients with diffuse large cell lymphoma varies according to the degree of infiltration and presence of discordant marrow involvement. Eur J Haematol 76:473–480

Campbell JK, Matthews JP, Seymour JF, Wolf MM, Juneja SK (2003) Optimum trephine length in the assessment of bone marrow involvement in patients with diffuse large cell lymphoma. Ann Oncol 14:273–276

Chadburn A, Knowles DM (1994) Paraffin-resistant antigens detectable by antibodies L26 and polyclonal CD3 predict the B- or T-cell lineage of 95% of diffuse aggressive non-Hodgkin's lymphomas. Am J Clin Pathol 102:284–291

Cheson BD, Horning SJ, Coiffier B, Shipp MA, Fisher RI, Connors JM, Lister TA, et al. (1999) Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol 17:1244

Chung R, Lai R, Wei P, Lee J, Hanson J, Belch AR, Turner AR, et al. (2007) Concordant but not discordant bone marrow involvement in diffuse large B-cell lymphoma predicts a poor clinical outcome independent of the International Prognostic Index. Blood 110: 1278–1282

Conlan MG, Bast M, Armitage JO, Weisenburger DD (1990) Bone marrow involvement by non-Hodgkin's lymphoma: the clinical significance of morphologic discordance between the lymph node and bone marrow. Nebraska Lymphoma Study Group. J Clin Oncol 8:1163–1172

Echeverri C, Fisher S, King D, Craig FE (2002) Immunophenotypic variability of B-cell non-Hodgkin lymphoma: a retrospective study of cases analyzed by flow cytometry. Am J Clin Pathol 117:615–620

Fraga M, Brousset P, Schlaifer D, Payen C, Robert A, Rubie H, Huguet-Rigal F, et al. (1995) Bone marrow involvement in anaplastic large cell lymphoma. Immunohistochemical detection of minimal disease and its prognostic significance. Am J Clin Pathol 103:82–89

Gervasi F, Lo Verso R, Giambanco C, Cardinale G, Tomaselli C, Pagnucco G (2004) Flow cytometric immunophenotyping analysis of patterns of antigen expression in non-Hodgkin's B cell lymphoma in samples obtained from different anatomic sites. Ann NY Acad Sci 1028:457–462

Hanson CA, Kurtin PJ, Katzmann JA, Hoyer JD, Li CY, Hodnefield JM, Meyers CH, et al. (1999) Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. Blood 94:3889–3896

Harada S, Suzuki R, Uehira K, Yatabe Y, Kagami Y, Ogura M, Suzuki H, et al. (1999) Molecular and immunological dissection of diffuse large B cell lymphoma: CD5+, and CD5- with CD10+ groups may constitute clinically relevant subtypes. Leukemia 13: 1441–1447

Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, et al. (1999) World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol 17:3835–3849

Horlyck A, Thorling K (1991) Bone marrow examination in non-Hodgkin's lymphoma: comparison of the diagnostic value of marrow aspirations and trephine biopsy. Eur J Haematol 46:54–56

Isaacson PG (2007) Haematopathology practice: the commonest problems encountered in a consultation practice. Histopathology 50:821–834

Juneja SK, Wolf MM, Cooper IA (1990) Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. J Clin Pathol 43:630–632

Lossos IS, Morgensztern D (2006) Prognostic biomarkers in diffuse large B-cell lymphoma. J Clin Oncol 24:995–1007

Mazur G, Halon A, Wrobel T, Jelen M, Kuliczkowski K (2004) Contribution of flow cytometric immunophenotyping and bone marrow trephine biopsy in the detection of lymphoid bone marrow infiltration in non-Hodgkin's lymphomas. Neoplasma 51:159–163

Miller RT, Swanson PE, Wick MR (2000) Fixation and epitope retrieval in diagnostic immunohistochemistry: a concise review with practical considerations. Appl Immunohistochem Mol Morphol 8:228–235

Palacio C, Acebedo G, Navarrete M, Ruiz-Marcellan C, Sanchez C, Blanco A, Lopez A (2001) Flow cytometry in the bone marrow evaluation of follicular and diffuse large B-cell lymphomas. Haematologica 86:934–940

Perea G, Altes A, Bellido M, Aventin A, Bordes R, Ayats R, Remacha AF, et al. (2004) Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL). Histopathology 45:268–274

Shipp MA, Harrington DP, Anderson JR, Armitage JO, Bonadonna G, Brittinger G, Cabanillas F, et al. (1993) A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med 329:987–994

Talaulikar D, Shadbolt B, Bell J, Khan K, Dahlstrom JE, McDonald A, Pidcock ME, et al. (2008) Clinical role of flow cytometry in redefining bone marrow involvement in diffuse large B-cell lymphoma (DLBCL): a new perspective. Histopathology 52:340–347

Taniguchi M, Oka K, Hiasa A, Yamaguchi M, Ohno T, Kita K, Shiku H (1998) De novo CD5+ diffuse large B-cell lymphomas express VH genes with somatic mutation. Blood 91:1145–1151

Yan Y, Chan WC, Weisenburger DD, Anderson JR, Bast MA, Vose JM, Bierman PJ, et al. (1995) Clinical and prognostic significance of bone marrow involvement in patients with diffuse aggressive B-cell lymphoma. J Clin Oncol 13:1336–1342

The Journal of Histochemistry & Cytochemistry

Author contributions:

Talaulikar D, Dahlstrom JE, Shadbolt B, Broomfield A, McDonald A. **"Role of Immunohistochemistry in staging diffuse large B-cell lymphoma"**. Journal of Histochemistry and Cytochemistry, 2008 Oct;56(10):893-900. Epub 2008 Jun 23.

Dipti Talaulikar:

1. Designed study, prepared ethics and grants applications, and set up database
2. Retrieved all cases from medical and laboratory records, and all archived slides and paraffin-embedded trephine blocks
3. Performed all the data extraction, analysed and computed all clinical data, and performed all data entry
4. Reported all histology slides as the first blinded reviewer
5. Interpreted all flow cytometry analysis
6. Cut sections, labeled and stained slides for immunohistochemistry with help
7. Reported and computed immunohistochemistry slides
8. Performed all statistical analysis under supervision of and help from Dr. Bruce Shadbolt
9. Wrote the first draft of the paper

Jane Dahlstrom:

1. Development of formal system of reporting IHC
2. Review of IHC slides
3. Review of final paper
4. Photographs of slides

Bruce Shadbolt:

1. Setting up database
2. All data analysis
3. Review of final paper

Amy Broomfield:

1. Technical advice with preparing all immunohistochemistry slides
2. Approved final paper

Anne McDonald:

1 Blinded 2nd reviewer of H & E slides on remaining ~75 cases

2 Approved final paper

Intellectual planning: DT (95%), JED: (3%) BS: (2%)

Experimental work: DT (95%) JED: (3%) AMcD: (1%), AB: (1%)

Analyses: DT (70%) BS (30%)

Writing: DT (97%) JED (2%) BS (1%)

5.2. Clinical Implications of Immunophenotyping in Staging Diffuse Large B-Cell Lymphoma. Blood, (ASH annual meeting abstracts) 2008; 112: Abstract 5279.

Blood (ASH Annual Meeting Abstracts) 2008 112: Abstract 5279
© 2008 American Society of Hematology

Publication Only

Non-Hodgkin's Lymphoma - Biology, Excluding Therapy

Clinical Implications of Immunophenotyping in Staging Diffuse Large B-Cell Lymphoma

Dipti Talaulikar, MBBS, FRACP, FRCPA, GradCertHE[1,*], Jane E Dahlstrom, MBBS, FRCPA, PhD, GradCertHE[2,*], Bruce Shadbolt, BSc, Grad. Dip(Sc), PhD(Epi)[3,*] and Anne McDonald, MBBS, FRACP, FRCPA[4,*]

[1] Department of Haematology, The Canberra Hospital, Canberra, Australia, [2] Department of Anatomical Pathology, The Canberra Hospital, Canberra, Australia, [3] Department of Epidemiology, The Canberra Hospital, Canberra, Australia, [4] National Capital Private Hospital, Canberra, Australia

Abstract

Diffuse Large B-cell Lymphoma (DLBCL) is the commonest subtype of Non-Hodgkin Lymphoma (NHL). Staging bone marrow (BM) biopsies in NHL are conventionally examined using histology alone, although the use of immunophenotyping to aid diagnosis is increasing. This study addresses the clinical impact of routine use of flow cytometry (FL) and immunohistochemistry (IHC) in 156 histologically proven DLBCL cases at initial diagnosis. The median age of the patient cohort was 62 years (range 20–86 years) and the gender ratio was 1.53 in favor of males. Nine patients were treated with palliative intent and were excluded from all survival analyses. The rest were treated with anthracycline based chemotherapy regimens; 36 patients received concomitant Rituximab. Median overall survival (OS) was 6.1 years (95% CI: 3.8, 8.4). Histological involvement was noted in 30 cases (19.2 %) using standardized Cheson criteria [Cheson BD, Horning SJ, Coiffier B et al. J Clin Oncol 1999; 17:1244]. FL and IHC (using T-cell, B-cell, and light chain markers) detected an additional 17 cases (10.9%) each with 4 cases detected by both methods. Thirty (19.2 %) patients were upstaged to stage IV with the use of these investigations (stage I: 6, stage II: 12, stage III: 12). Kaplan Meier curves demonstrated that positivity on FL, IHC and both (FL + IHC) resulted in a significantly worse OS and progression free survival (PFS) compared to negative cases. Cox proportional hazards models were used to determine the additional benefit of FL and IHC in predicting OS over histological involvement, after adjusting for age, performance status and use of Rituximab. The first analysis compared FL with histology and showed that FL added significant independent prognostic value [Histology: HR = 2.1, 95% CI 1.0, 4.3, p=0.05; FL: HR = 2.0, 95% CI 1.0, 3.8, p=0.04]. The second analysis compared histology with IHC and showed that the two had co linearity in their prediction of survival with IHC adding significantly more than histology alone [Histology: HR = 1.3, 95% CI 0.5, 3.0, p=0.6; IHC: HR = 2.3, 95% CI 1.1, 5.0, p = 0.03]. In the final model, we examined the interactive effect of FL and IHC over and above histological diagnosis and found the interaction to be the stronger predictor of OS [Histology: HR = 1.8, 95% CI 0.8, 3.7, p =0.1; FL/IHC: HR = 2.7, 95% CI 1.2, 6.2, p=0.02]. In conclusion, our results show that FL and IHC performed routinely can improve detection of BM involvement in DLBCL and upstage ~20 % patients to stage IV disease. We found that FL added significant independent prognostic value while IHC showed greater co linearity with routine histological diagnosis. The interactive effect of FL/IHC had additional benefit over routine histology in predicting survival. These results suggest that staging BM in DLBCL should have FL and IHC performed routinely at initial diagnosis.

CHAPTER VI:

Preliminary molecular studies

6.1: A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsies using two different methods. J Clin Pathol. 2008 Jan; 61(1):119-23. Epub 2007 Jun 1.

Downloaded from jcp.bmj.com on 3 January 2008

A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsy specimens using two different methods

D Talaulikar,[1,2] J X Gray,[1] B Shadbolt,[2,3] M McNiven,[4] J E Dahlstrom[2,5]

[1] Department of Haematology, The Canberra Hospital, Garran, Australia; [2] Australian National University Medical School, The Canberra Hospital, Garran, Australia; [3] Department of Epidemiology, The Canberra Hospital, Garran, Australia; [4] Department of Molecular Medicine, The Canberra Hospital, Garran, Australia; [5] Department of Anatomical Pathology, The Canberra Hospital, Garran, Australia

Correspondence to: Dr Dipti Talaulikar, Department of Haematology, The Canberra Hospital, Yamba Drive, Garran, Canberra, ACT 2606, Australia; dipti.talaulikar@act.gov.au

Accepted 7 April 2007
Published Online First 1 June 2007

ABSTRACT

Background: Given its prognostic value, there is renewed interest in molecular staging in non-Hodgkin's lymphoma (NHL) using immunoglobulin heavy and light chain (IgH, IgL) gene rearrangements.

Aims: To compare the efficiency of DNA amplification from fresh frozen and formalin-fixed decalcified paraffin-embedded (FFDPE) bone marrow trephines for use in molecular staging using two methods.

Methods: After manually extracting DNA from 13 FFDPE and 14 fresh frozen trephine biopsy specimens, two methods were used to test for amplifiability: use of the amplification control master mix supplied in the In Vivo Scribe immunoglobulin heavy chain (IgH) clonality kit, which creates 5 amplicons between 96–600 base pairs (bp); and real-time amplification of the β-globin gene.

Results: Using the first method, the mean maximum length of amplicons generated from FFDPE trephines was statistically lower at 300 bp compared to fresh frozen samples, all of which generated amplicons up to 600 bp in size ($p<0.001$). Real-time amplification of the β-globin gene showed that the mean crossing threshold of fresh frozen samples was statistically lower than that of FFDPE samples (23.48 (95% CI 22.47 to 24.48) vs 33.64 (95% CI 32.15 to 35.12); $p<0.001$).

Conclusions: Although amplifiable DNA can be extracted from both fresh-frozen and FFDPE trephine samples for IgH/IgL analysis, freshly frozen specimens are superior as a source of template DNA, especially for higher base pair PCR products.

Staging with bone marrow (BM) biopsy is important in non-Hodgkin lymphoma (NHL), as BM involvement correlates with poor overall survival.[1 2] Earlier reports on molecular staging in NHL using PCR to detect immunoglobulin heavy chain (IgH) gene rearrangements have not found molecular staging to be particularly useful for detection of bone marrow involvement.[3] This may be related partly to failure of the IgH gene to amplify in 25–30% of NHL cases. The primers corresponding to the framework (FR) regions have until recently been designed based on a limited number of FR sequences, resulting in a lack of PCR amplification.[4] Cases of follicular lymphoma and diffuse large B-cell lymphoma are particularly prone to this phenomenon as translocations involving the IgH gene may render one of the IgH loci inaccessible to PCR.[5] Furthermore, somatic hypermutations of the IgH gene, acquired while traversing the germinal centre, may cause false negative results on IgH PCR.[6] This was demonstrated by Kang *et al*, who showed that compared to the 100% concordance noted between morphology and PCR based clonality in small lymphocytic lymphoma, follicular lymphoma showed a concordance of only 66%, whereas diffuse large B-cell lymphoma (DLBCL) showed an even lower concordance of 57%.[7] Recently however, newer methods based on the standardised BIOMED-2 protocol have used multiplex assays employing several upstream primers that have increased the sensitivity of IgH PCR.[8] Methods based on this protocol, when applied to fresh peripheral blood and bone marrow aspirates, have demonstrated the prognostic significance of molecular staging in DLBCL.[9]

However, it is well known that marrow involvement in NHL is more commonly observed on bone marrow trephines than on aspirates.[7 10] Coad *et al* have established the superiority of trephines over aspirates for performing IgH analysis by demonstrating that 10 of the 11 established cases of NHL, which gave negative PCR results using aspirate samples, were found to be positive when trephine samples were used.[11] Our group is currently studying the utility of the BIOMED-2 based protocol using the In Vivo Scribe IgH clonality kit in determining the rate of molecular involvement in bone marrow trephine biopsy specimens. Although clinical laboratories in many parts of the world provide clonality studies on bone marrow trephines, there is little published information on the use of the BIOMED-2 based protocol in formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsy specimens.[8] This is important as archived FFDPE trephines provide a valuable resource for research purposes. To our knowledge, there is only one study that has attempted to use a modified BIOMED-2 protocol on bone marrow trephines.[12]

This feasibility study was performed to directly compare the efficiency of DNA amplification from fresh frozen and FFDPE trephines using the amplification control provided with the In Vivo Scribe kit. Efficiency of amplification was also assessed by real-time amplification of the β-globin gene using the Roche LightCycler Control DNA kit.

METHODS

Samples

Thirteen FFDPE bone marrow trephines were retrieved from archives. All samples were less than

Downloaded from jcp.bmj.com on 3 January 2008

Table 1 Target genes and primers used to assess the quality of DNA in the BIOMED-2 protocol

1.	Human thromboxane synthase gene (TBXAS1, exon 9; GenBank accession no. D34621)	
	5'GCCCGACATTCTGCAAGTCC3'	3'TTGGGAAGGGCCGTTGTGG5'
2.	Human recombination activating gene (RAG1, exon 2; GenBank accession no. M29474)	
	5'TGTTGACTCGATCCACCCCA3'	3'AAGTCGGTTTGAACGTCGAGT5'
3.	Human promyelocytic leukaemia zinc-finger gene (PLZF, exon 1; GenBank accession no. AF060568)	
	5'TGCGATGTGGTCATCATGGTG3'	3'CGGAGTCTGCTGTTACTGTGC5'
4.	Human AF4 gene (exon 11; GenBank accession no. Z83687)	
	5'CCGCAGCAAGCAACGAACC3'	3'CCTCGGCGGTCTCCTTTCG5'
5.	Human AF4 gene (exon 3; GenBank accession no. Z83679)	
	5'GGAGCAGCATTCCATCCAGC3'	3'AATACAGGCCGGGTACCTAC5'

12 months old and had a trephine length of at least 20 mm. Fourteen fresh frozen bone marrow trephines were collected from patients after obtaining informed consent as required by the Australian Capital Territory Tissue Bank. The haematological diagnoses from patients in both groups were similar. FFDPE samples were retrieved from archives from six cases with lymphoproliferative disorder; two cases had diagnoses of immune thrombocytopenic purpura (ITP), two of a myeloproliferative disorder and one of reactive thrombocytosis. Two bone marrows, one performed to evaluate longstanding fevers, and the second to look for a non-haematological malignancy were also included. Fresh trephine samples were collected from five patients with acute leukaemia, five with lymphoproliferative disorder, and four with myeloproliferative disorder. All bone marrows were morphologically normal and had normal counts. All malignant cases were in haematological remission.

All FFDPE samples had been fixed in 10% neutral buffered formalin with acetic acid for at least 2 hours. Decalcification was performed using 5% nitric acid for a period of 2 hours.

DNA extraction

DNA extraction was performed manually using the Roche High Pure PCR Template Preparation Kit from two 10 μm FFDPE trephine samples and from 25–50 mg of thawed fresh bone marrow trephine specimens. Paraffin slices from bone marrow trephine blocks were deparaffinised using xylene and rehydrated using graded ethanol concentrations. Samples were digested overnight with proteinase K and then processed according to the manufacturer's instructions with no modifications. DNA was eluted in a final volume of 50 μl.

DNA quantification using spectrophotometry

DNA quantification using spectrophotometry was performed with 1:100 dilutions of DNA with distilled water. Optical density was read at 260 nm on a BioSpec-mini-spectrophotometer (Schimadzu) using quartz cuvettes. The DNA concentration was then calculated based on the standard formula that for double stranded DNA, an OD_{260} of 1 = 50 μg/ml.

Mean DNA concentration for the fresh and FFDPE specimens was estimated.

Amplification of DNA using the specimen control size ladder

Amplification of a control size ladder from the IgH gene clonality kit from In Vivo Scribe Technologies based on the BIOMED-2 protocol was performed on all fresh frozen bone marrow trephines and 12 FFDPE samples. PCR reaction could not be performed in one FFDPE specimen because of insufficient sample. This master mix creates five amplicons of 96, 200, 300, 400 and 600 base pairs targeting the genes listed in table 1.

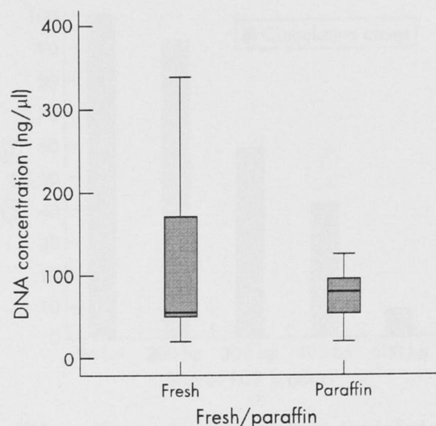


Figure 1 Difference in mean DNA concentrations between fresh and formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsy specimens. There was no statistical difference in the mean DNA concentrations obtained from fresh and FFDPE trephine biopsies (p = 0.5). The horizontal bars depict median values; the boxes depict 95% CI, and the vertical lines depict the range.

The PCR reaction consisted of 45 μl of the specimen control size ladder master mix solution, 2.5 units of Amplitaq Gold and 5 μl of template DNA (with an average template DNA concentration of 300–400 ng/μl). Thermocycling was performed according to the kit protocol (box 1) with no modifications on a Perkin Elmer 9600 thermocycler. Controls consisted of a positive DNA control, negative extraction control and negative PCR control. Water was used as a negative control in both cases.

Non-denaturing polyacrylamide gel electrophoresis was used to resolve the different sized amplicons. PCR product (25 μl) was loaded onto a 6% polyacrylamide gel and 250 V applied for 1.5 hours. After electrophoresis, the gels were stained with ethidium bromide for 5 minutes and visualised under ultraviolet light.

Real-time amplification of the β-globin gene

Real-time amplification of the β-globin gene was performed on all fresh frozen and FFDPE samples using the Roche LightCycler DNA Control kit with no modifications. This amplifies a 110 bp fragment of the human β-globin gene. Amplification of the target DNA was monitored using probes that hybridise to an internal sequence of the amplified fragment. Template DNA (5 μl) was amplified in a total PCR volume of 20 μl on the Roche LightCycler. A positive control along with negative extraction and negative PCR controls (water) were included in the analysis.

Statistical analysis

Statistical analysis for comparing means using ANOVA was performed using the Statistical Package for Social Sciences (SPSS) V.14.0 software. Differences in mean values between the two groups were depicted by box plots, which also included 95% CI and range.

RESULTS

DNA quantification using spectrophotometry

The difference in the mean DNA concentration (in ng/μl) obtained from fresh and FFDPE trephine biopsy tissue was not

Downloaded from jcp.bmj.com on 3 January 2008

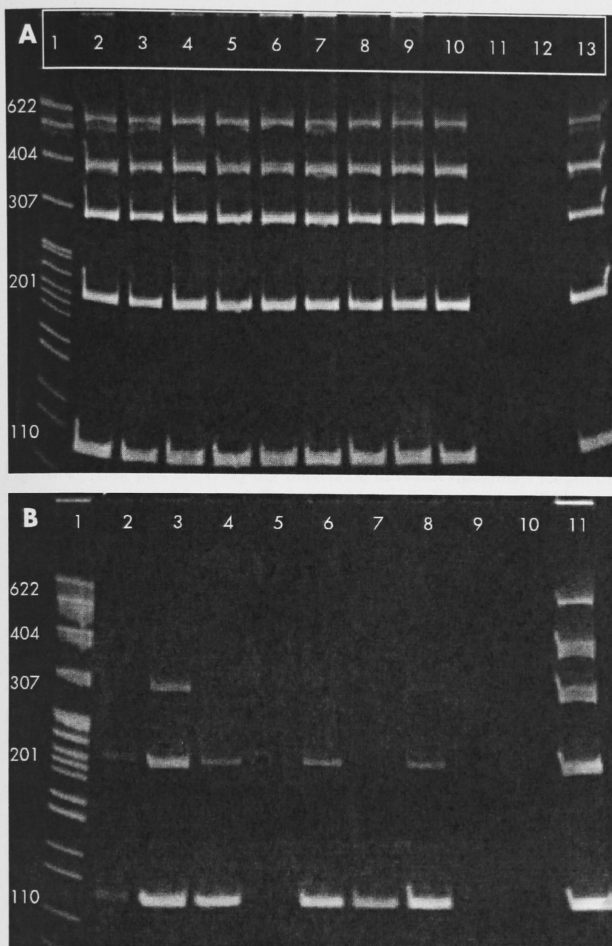


Figure 2 (A) Gel picture showing amplification of DNA up to 600 bp in all fresh frozen trephine biopsy specimens. Lane 1, pBR322 MspI control size ladder; lanes 2–10, fresh trephine samples; lanes 11–12, negative controls; lane 14, positive DNA control. (B) Gel picture showing loss of larger DNA fragments in formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsy samples. Lane 1, pBR322 MspI control size ladder; lanes 2–4, 6–8*, FFDPE trephine samples; lanes 9–10, negative controls; lane 11, positive DNA control. *Lane 5 blank.

statistically significant (108.9, 95% CI 45.9 to 171.9; vs 84.6, 95% CI 54.8 to 114.4; p = 0.5; fig 1). The difference in log-normalised concentrations also did not achieve statistical significance (4.29, 95% CI 3.8 to 4.8; vs 4.26, 95% CI 3.8 to 4.6; p = 0.9).

Amplification of DNA using the specimen control size ladder

All 14 fresh frozen bone marrow trephine specimens yielded amplicons up to and including 600 bp in length (fig 2A). In contrast, there was a progressive dropout of larger PCR products in FFDPE specimens, as shown by the gel picture (fig 2B). Numbers of FFDPE samples that generated amplicons of 96, 200, 300, 400 and 600 bp are shown graphically in fig 3, with all cases generating 96 bp PCR products and only 1 case (8%) generating 600 bp products. The difference in the mean maximum length of amplicons generated by fresh frozen and FFDPE bone marrow trephines was statistically significant (p<0.001) (fig 4).

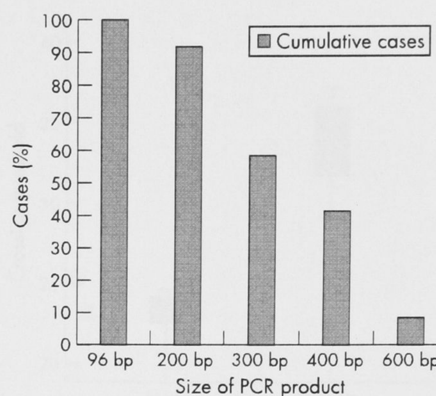


Figure 3 DNA amplification from formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsy specimens. This bar diagram shows that there is a progressive drop out in DNA amplification from FFDPE samples at larger fragment lengths.

Real-time amplification of the β-globin gene

On the LightCycler, the mean crossing threshold of fresh frozen samples was statistically lower than FFDPE samples (23.48, 95% CI 22.47 to 24.48; vs 33.64, 95% CI 32.15 to 35.12; p<0.001) as shown in fig 5. DNA from fresh samples amplified to beyond the threshold of detection approximately 10 cycles earlier than that from FFDPE samples.

DISCUSSION

This feasibility study has shown that amplifiable DNA can be extracted from both fresh-frozen and FFDPE bone marrow trephines for IgH and IgL analysis using the BIOMED-2 protocol. Freshly frozen specimens were however superior as a source of template DNA, especially for higher base pair PCR products. Spectrophotometry was used to assess DNA quantity; two PCR methods were then used to assess the efficiency of

Box 1 Standardised BIOMED-2 PCR protocol and gel electrophoresis

PCR reaction
45 µl of the master mix solution
2.5 units of Amplitaq Gold
5 µl of template DNA

Thermocycling
On Perkin Elmer 9600 thermocycler
Denaturing: 95° for 45 s
Annealing: 60° for 45 s
Amplification: 72° for 90 s
35 cycles

Controls
Positive DNA control
Negative extraction control
Negative PCR control

Gel electrophoresis
Non-denaturing 6% polyacrylamide gel electrophoresis
25 µl of PCR product
250 V applied for 1.5 h
Stained with ethidium bromide and visualised under UV light

Downloaded from jcp.bmj.com on 3 January 2008

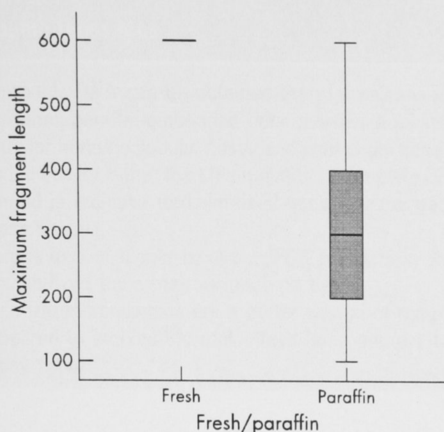


Figure 4 Box plot showing the difference in mean maximum length of amplicons generated by fresh frozen and formalin-fixed decalcified paraffin-embedded (FFDPE) bone marrow trephine samples. There is a statistical difference in the mean maximum length of PCR products amplified from fresh and FFDPE trephine biopsy specimens ($p<0.001$).

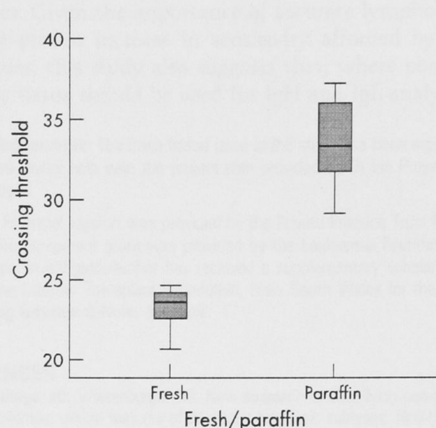


Figure 5 Box plot showing a significant difference in the mean crossing thresholds of fresh frozen and formalin-fixed decalcified paraffin-embedded (FFDPE) bone marrow trephine samples on real-time amplification of the β-globin gene using the LightCycler ($p<0.001$).

amplification of DNA. The first of these used the amplification control master mix provided in the In Vivo Scribe IgH gene clonality kit. The second PCR method was the housekeeping β-globin gene amplification method. Some technical issues highlighted by the study are also discussed.

Results on the amplification control master mix showed that DNA extracted from fresh tissue was consistently able to generate larger products that are up to 600 bp in size. This was in contrast to FFDPE material, which yielded the 96 bp sized fragment in 100% of cases, but was able to generate the 600 bp fragment in only 8% of cases. This has been corroborated by Santella *et al*, who reported that the size of the product extracted from paraffin-embedded sections tended to be smaller than that from fresh tissue.[13]

Comparison of the "real-time" amplification of the β-globin gene showed a statistically significant difference in the mean crossing thresholds between fresh frozen trephine material and FFDPE material (23.48 cycles, 95% CI 22.47 to 24.48; vs 33.64 cycles, 95% CI 32.15 to 35.12; $p<0.001$). This further confirms that fresh frozen samples are a better source of high quality DNA.

Gel electrophoresis provided further confirmation that it is the quality and not the quantity of DNA obtained from FFDPE material that is affected. DNA (1 μg) extracted from three fresh and three FFDPE samples was run on a representative 0.8% agarose gel, 60 V for 1.5 hours (fig 6A,B). Lambda Hind III (1.5 μg) was used as the molecular weight marker. DNA extracted from the fresh samples demonstrated the presence of high molecular weight DNA greater than 23 kb. DNA from the fixed samples produced a faint smear consistent with degraded or poor quality DNA.

Although this appears obvious, previous reports on skin biopsy specimens and on aspirate clots have shown that there is no statistically significant difference in amplifiable DNA extracted from fresh frozen and paraffin-embedded tissue.[14 15] This suggests that it is the process of decalcification that most likely degrades the genomic DNA. In a direct comparison between fixed trephine biopsy specimens and fixed lymph-node sections, Provan *et al* noted that only 1/13 trephine biopsies were able to generate high molecular weight DNA compared to 3/4 nodal specimens, and concluded that the poorer result obtained from trephine biopsy specimens was related to the decalcification process.[16] Also, the type of decalcification agent used can have an impact on the nucleic acid yield, with EDTA being known to provide a higher yield than nitric acid.[17]

The findings from our study suggest that it is not the quantity, but the quality of DNA that is affected by the processing of FFDPE specimens. All of the FFDPE specimens demonstrated amplification at a lower amplicon size of 96 bp, indicating that PCR products of low molecular weight can be obtained from nearly all cases. This is a very gratifying observation, as it implies that lower length DNA fragments can be amplified from all FFDPE cases. However, it must be remembered that our study population was small, and that these results need to be confirmed on larger studies.

Also, we found no significant difference in the median quantity of DNA obtained from fresh frozen and FFDPE trephine biopsy specimens as measured by spectrophotometry (55 ng/μl vs 80 ng/μl, $p = 0.5$). The two fresh frozen samples

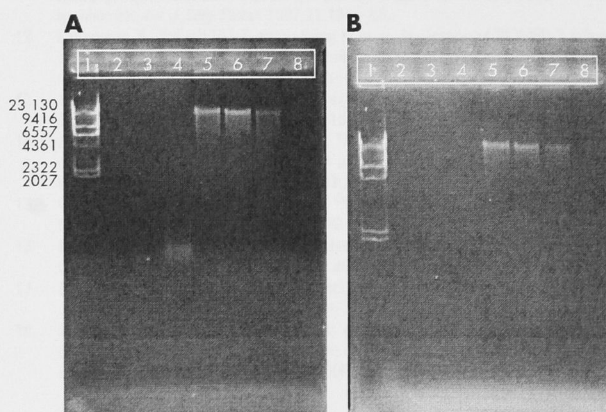


Figure 6 Agarose gel pictures at 1 hour (A) and 1.5 hours (B), showing the difference between DNA from fresh and formalin-fixed decalcified paraffin-embedded (FFDPE) trephine samples. The fresh samples show defined high molecular weight bands while only ill-defined smears are noted with FFDPE samples. Lane 1, λ HindIII molecular weight marker; lanes 2–4, FFDPE trephines; lanes 5–7, fresh trephines; lane 8, negative control.

J Clin Pathol 2008;**61**:119–123. doi:10.1136/jcp.2006.045294

Downloaded from jcp.bmj.com on 3 January 2008

Take-home messages

- Amplifiable DNA can be obtained from formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsy tissue for immunoglobulin heavy and light chain gene analysis.
- It is the quality rather than the quantity of template DNA that is affected in archived formalin-fixed bone marrow trephine biopsy tissue.
- There is loss of higher base pair PCR products in archived formalin-fixed bone marrow trephine tissue.
- Fresh frozen specimens are a better source of template DNA compared to archived formalin-fixed bone marrow trephine biopsy tissue.

that showed concentrations >250 ng/μl could not be accounted for by differences in the haematological diagnoses or the length of the trephine biopsies.

For this study, we used the Roche High Pure PCR Template Preparation kit for DNA extraction. Although the kit has not been widely used in published literature, it is validated in our laboratory for both fresh frozen and FFDPE samples. Our observation is that it is possible to obtain adequate quantities of DNA from two 10 μm slices of FFDPE samples where traditionally as many as 10–15 slices of similar thickness have been used.[16 18] Researchers developing the multiplex assays for IgH/IgL analysis using the BIOMED-2 protocol have found that the best results were obtained at DNA concentrations of 50–100 ng/μl.[6] In this study, we did not adjust the DNA concentration, but opted to use a standard volume of 5 μl, as we found no statistically significant difference in the quantity of DNA obtained from fresh frozen and FFDPE tissue. As limited tissue is often available for DNA extraction from FFDPE trephine biopsy blocks, such an approach potentially prevents the waste of valuable extracted DNA on spectrophotometric analysis.

The practical implications of our findings are that there are limitations of DNA amplification from FFDPE trephine tissue, especially for molecular staging. Target fragment lengths of 110–360 bp are required for IgH analysis and of 120–400 bp for IgL analysis in kits based on the BIOMED-2 protocol. In this current feasibility study, 60% of FFDPE samples were able to generate amplicons of up to 300 bp in size; this suggests that it should be possible to reliably perform IgH/IgL analysis on FFDPE tissue in approximately 60% of cases. Provan *et al* similarly were able to amplify a 294 bp fragment from 6/10 FFDPE trephine biopsy specimens.[16] A major advantage of using the amplification control provided in the gene clonality kit over other methods like the β-globin method is the confirmation of amplifiable DNA of different fragment lengths.

Further, this study suggests that molecular staging performed on FFDPE trephine samples using IgH/IgL analysis based on the BIOMED-2 protocol has the potential for false negative results and the erroneous downstaging of patients. Therefore, negative results should be interpreted with caution to account for false negatives. Given the importance of accurate lymphoma staging and the proven increase in sensitivity afforded by molecular techniques, this study also suggests that, where possible, fresh trephine tissue should be used for IgH and IgL analysis.

Acknowledgements: The fresh tissue used in the study has been stored by the ACT Tissue Bank. Initial help with the project plan provided by Dr Ian Prosser is gratefully acknowledged.

Funding: Financial support was provided by the Private Practice Trust Fund, Canberra Hospital. An equipment grant was provided by the Leukaemia Foundation, Australia. The principal investigator/author has received a supplementary scholarship from the Arrow Bone Marrow Transplant Foundation, New South Wales for the project.
Competing interests: None declared.

REFERENCES

1. **Armitage JO,** Weisenburger DD. New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol* 1998;**16**:2780–95.
2. **Yan Y,** Chan WC, Weisenburger DD, *et al.* Clinical and prognostic significance of bone marrow involvement in patients with diffuse aggressive B-cell lymphoma. *J Clin Oncol* 1995;**13**:1336–42.
3. **Campbell J,** Seymour JF, Matthews J, *et al.* The prognostic impact of bone marrow involvement in patients with diffuse large cell lymphoma varies according to the degree of infiltration and presence of discordant marrow involvement. *Eur J Haematol* 2006;**76**:473–80.
4. **Inghirami G,** Szabolcs MJ, Yee HT, *et al.* Detection of immunoglobulin gene rearrangement of B cell non-Hodgkin's lymphomas and leukemias in fresh, unfixed and formalin-fixed, paraffin-embedded tissue by polymerase chain reaction. *Lab Invest* 1993;**68**:746–57.
5. **Bagg A,** Braziel RM, Arber DA, *et al.* Immunoglobulin heavy chain gene analysis in lymphomas: a multi-center study demonstrating the heterogeneity of performance of polymerase chain reaction assays. *J Mol Diagn* 2002;**4**:81–9.
6. **Kuppers R,** Zhao M, Hansmann ML, *et al.* Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. *Embo J* 1993;**12**:4955–67.
7. **Kang YH,** Park CJ, Seo EJ, *et al.* Polymerase chain reaction-based diagnosis of bone marrow involvement in 170 cases of non-Hodgkin lymphoma. *Cancer* 2002;**94**:3073–82.
8. **van Dongen JJ,** Langerak AW, Bruggemann M, *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003;**17**:2257–317.
9. **Mitterbauer-Hohendanner G,** Mannhalter C, Winkler K, *et al.* Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). *Leukemia* 2004;**18**:1102–7.
10. **Juneja SK,** Wolf MM, Cooper IA. Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. *J Clin Pathol* 1990;**43**:630–2.
11. **Coad JE,** Olson DJ, Christensen DR, *et al.* Correlation of PCR-detected clonal gene rearrangements with bone marrow morphology in patients with B-lineage lymphomas. *Am J Surg Pathol* 1997;**21**:1047–56.
12. **Lassmann S,** Gerlach UV, Technau-Ihling K, *et al.* Application of BIOMED-2 primers in fixed and decalcified bone marrow biopsies: analysis of immunoglobulin H receptor rearrangements in B-cell non-Hodgkin's lymphomas. *J Mol Diagn* 2005;**7**:582–91.
13. **Santella RM.** Approaches to DNA/RNA extraction and whole genome amplification. *Cancer Epidemiol Biomarkers Prev* 2006;**15**:1585–7.
14. **Hughes J,** Weston S, Bennetts B, *et al.* The application of a PCR technique for the detection of immunoglobulin heavy chain gene rearrangements in fresh or paraffin-embedded skin tissue. *Pathology* 2001;**33**:222–5.
15. **Crisan D,** Cadoff EM, Mattson JC, *et al.* Polymerase chain reaction: amplification of DNA from fixed tissue. *Clin Biochem* 1990;**23**:489–95.
16. **Provan AB,** Hodges E, Smith AG, *et al.* Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA. *J Clin Pathol* 1992;**45**:763–5.
17. **Srinivasan M,** Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol* 2002;**161**:1961–71.
18. **Wickham CL,** Boyce M, Joyner MV, *et al.* Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies. *Mol Pathol* 2000;**53**:19–23.

Author contributions:

Talaulikar D, Gray JX, Shadbolt B, McNiven M, Dahlstrom JE: **"A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsies using two different methods."** J Clin Pathol. 2008 Jan; 61(1):119-23. Epub 2007 Jun 1.

Dipti Talaulikar:

1. Designed study, prepared ethics and grants applications, and set up database
2. Retrieved all cases from medical and laboratory records, and all archived paraffin-embedded trephine blocks
3. Performed all the data entry
4. Performed all DNA extractions
5. Developed all molecular methods under supervision
6. Performed all molecular analysis
7. Performed all statistical analysis under supervision of and help from Dr. Bruce Shadbolt
8. Wrote the first draft of the paper

James X. Gray:

1. Presented the findings at local conference
2. Reviewed final paper

Bruce Shadbolt:

1. Setting up database
2. All data analysis
3. Review of final paper

Michelle McNiven:

1. Technical advice on molecular analysis
2. Input into and reviewing final paper

Jane E. Dahlstrom:

1. Reviewed and approved final paper

Intellectual planning: DT (95%), BS: (2%), MM (2%)

Experimental work: DT (98%) AB: (0.5%) MM (1.5%)

Analyses: DT (70%) BS (30%)

Writing: DT (97%) JED (0.5%) BS (0.5%) MM (2%)

6.2. Talaulikar D, Shadbolt B, McNiven M, Dahlstrom JE: "DNA amplification from formalin-fixed decalcified paraffin-embedded bone marrow trephine specimens – does the duration of storage matter"? Pathology, 2008 Dec; 40(7): 702-706.

Pathology (December 2008) 40(7), pp. 702–706

EXPERIMENTAL PATHOLOGY

DNA amplification from formalin-fixed decalcified paraffin-embedded bone marrow trephine specimens: does the duration of storage matter?

DIPTI TALAULIKAR*†, BRUCE SHADBOLT†‡, MICHELLE MCNIVEN§ AND JANE E. DAHLSTROM†||

*Department of Haematology, †Australian National University Medical School, and Departments of ‡Epidemiology, §Molecular Medicine and ||Anatomical Pathology, The Canberra Hospital, Garran, Australian Capital Territory, Australia

Summary

Introduction: Examination of bone marrow (BM) trephines has been traditionally limited to histological analysis. With renewed interest in their use in molecular diagnostics, studies assessing the impact of duration of storage on DNA amplification from archived BM trephines are required.
Methods: DNA extraction was performed on 169 formalin-fixed decalcified paraffin-embedded (FFDPE) trephine blocks with a mean duration of storage of 59.4 months (range 4–200 months). An amplification control master mix from the In Vivo Scribe Immunoglobulin Heavy Chain Clonality kit was used to determine DNA amplification at different amplicon sizes [96–600 base pairs (bp)].
Results: Amplification at 96 bp was noted in 145 of 169 specimens (85.8%). Progressive reduction in amplification was noted at higher base pairs with 99 of 169 (58.6%) amplifying at 200 bp, 56 cases (33.1%) at 300 bp, 35 (20.7%) at 400 bp and 19 (11.2%) at 600 bp. The impact of duration of storage on DNA amplification was not statistically significant.
Conclusions: DNA was obtained from archival BM trephine specimens in approximately 86% of cases, indicating good potential for utilisation in polymerase chain reaction (PCR) techniques. The duration of storage of FFDPE tissue had no impact on the ability to amplify specimens.

Key words: DNA, polymerase chain reaction (PCR), gene amplification, bone marrow trephines, formalin-fixed paraffin-embedded.

Received 24 September, revised 12 December 2007, revised 7 February, accepted 11 February 2008

INTRODUCTION

Bone marrow (BM) biopsy is considered an integral investigation in the diagnosis and staging of haematological disorders. In cases such as lymphoproliferative disorders, it is generally believed that trephine biopsies provide more diagnostic yield than bone marrow aspirates.[1] Traditionally, however, examination of BM trephine biopsy specimens has been limited to histological and immunohistochemical stains.[2] The availability of molecular techniques in the last few decades has increased the scope of diagnostics possible on BM trephine biopsies.

It has been known for some time that archival formalin-fixed decalcified paraffin-embedded (FFDPE) bone marrow trephine tissues are an invaluable source of nucleic acids for retrospective molecular analysis in cases on which fresh material cannot be, or has not been, stored.[3] Over and above their use in conditions such as myeloproliferative disorders, they can also be used for polymerase chain reaction (PCR) amplification of immunoglobulin heavy chain (IgH) and immunoglobulin light chain (IgL) gene rearrangements in the differential diagnosis of lymphoid aggregates.[4] Furthermore, in cases such as diffuse large B-cell lymphoma (DLBCL), the prognostic significance of molecular staging using IgH gene rearrangements on fresh BM aspirates is now well-established.[5] This may have implications for similar testing on trephines as there is literature to suggest that PCR for IgH gene rearrangements in non-Hodgkin's lymphoma cases from trephines rather than aspirates correlates better with morphology.[6]

The major difficulty with the use of archival FFDPE trephine tissue for molecular testing is degradation of the DNA with significantly reduced amplification at higher fragment lengths compared with fresh frozen tissue.[7] This may be related to processing of samples at collection, which is common to all fixed tissues (i.e., soft tissues and bony tissues) and includes fixation variables such as the type, condition and duration of fixation used, and/or post-fixation factors such as the duration and temperature of storage.[8] Another point to consider is that although there is extensive literature on the use of formalin-fixed paraffin-embedded tissue for molecular diagnostics, this is largely focussed on soft tissue specimens such as lymph nodes, tonsils and skin.[9–11] Compared with soft tissue, bone marrow trephines are believed to pose additional problems with DNA yield, with some evidence to suggest this may be related to the additional processing, particularly decalcification, required for BM trephines.[3] Control amplification is considered mandatory for molecular testing on paraffin-embedded material and it is recommended that single copy gene fragments of adequate size, usually 250–400 base pairs (bp) be used.[2] However, with the development of multiplex formats such as those designed by BIOMED-2, it is now possible to obtain information on control PCR for amplicons of different sizes.[12]

Print ISSN 0031-3025/Online ISSN 1465-3931 © 2008 Royal College of Pathologists of Australasia
DOI: 10.1080/00313020802436410

The aim of this study was to determine the feasibility of DNA amplification at different fragment lengths from FFDPE archival bone marrow trephine samples using the BIOMED-2 designed multiplex control PCR. As the specimens in the study had been stored for periods as long as 200 months, we also attempted to determine the correlation of duration of storage of tissue blocks with amplification of DNA using statistical methods.

METHODS

Samples

A total of 169 archival FFDPE bone marrow trephine blocks from 165 patients were retrieved. All samples were from cases of DLBCL diagnosed at The Canberra Hospital between 1985 and 2006.

All FFDPE samples had been fixed in 10% neutral buffered formalin with acetic acid for at least 2 hours prior to decalcification. Decalcification was performed using 5% nitric acid for a period of 2 h. The biopsies were processed overnight and embedding into paraffin was performed using standard laboratory protocols.

Age of specimens

The mean age of the FFDPE blocks could be determined in 164 cases and was 59.4 months, with a range of 4–200 months. Twenty-eight blocks (17%) were less than 12 months old, 58 (35.4%) between 1 and 5 years, 56 (34.2%) between 5 and 10 years, 19 (11.6%) between 10 and 15 years and three (1.8%) more than 15 years old (Fig. 1).

DNA extraction

DNA extraction was performed manually using the Roche High Pure PCR Template Preparation Kit (Roche, Switzerland) from two 10-μm sections of FFDPE trephine samples. Paraffin slices were deparaffinised using xylene and rehydrated using graded ethanol concentrations. Samples were digested overnight with proteinase K and then processed according to the manufacturer's instructions with no modifications. DNA was eluted in a final volume of 50 μL.

DNA quantification using spectrophotometry

DNA quantification using spectrophotometry could not be performed in all cases due to inadequate amounts of DNA. However, 40 cases were randomly selected to represent the cohort, and spectrophotometry was performed with 1:100 dilutions of DNA with distilled water. Quartz cuvettes were used and the optical density read at 260 nm on BioSpec-mini Spectrophotometer (Shimadzu, Japan). DNA concentration was calculated based on the standard formula for double stranded DNA, an OD_{260} of 1 = 50 μg/mL.

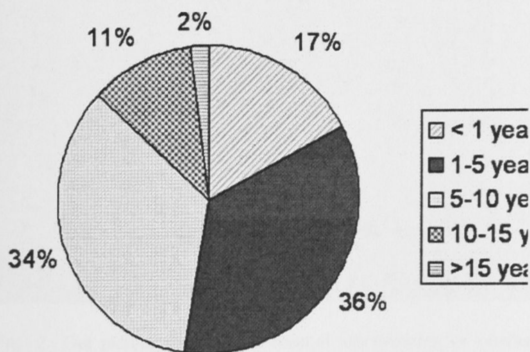


Fig. 1 Distribution of cases across different durations of storage.

Amplification of DNA using the specimen control size ladder

Amplification of a control size ladder from the IgH gene clonality kit from In Vivo Scribe Technologies based on the BIOMED-2 protocol (IgH Gene Clonality Assay - Gel Detection; InVivoScribe Technologies, USA) was performed for all FFDPE samples. This master mix creates five amplicons of 96, 200, 300, 400 and 600 bp targeting the genes listed in Table 1.

The PCR reaction consisted of 45 μL of the specimen control size ladder master mix solution, 2.5 U Amplitaq Gold (Applied Biosystems, USA) and 5 μL of template DNA with an average template DNA concentration of 300–400 ng/μL. Thermo-cycling was performed according to the kit protocol (Table 2) with no modifications on a Perkin Elmer 9600 thermocycler. Controls consisted of a positive cell-line control (Hibbitt R85/005054, which is a B-lymphoblastoid cell line, a gift from Dr Frank Christiansen, Department of Clinical Immunology and Immunogenetics, PathWest, Royal Perth Hospital, Perth, Western Australia), a normal DNA control (polyclonal peripheral blood lymphocytes) and two negative water controls for extraction and PCR.

Non-denaturing polyacrylamide gel electrophoresis was used to resolve the different sized amplicons. Twenty-five μL of PCR product was loaded onto a 6% polyacrylamide gel and 250 V applied for 1.5 h. After electrophoresis, the gels were stained with ethidium bromide for 5 min and visualised under ultraviolet light.

Table 1 Target genes and primers used to assess the quality of DNA in the BIOMED-2 protocol

1. Human thromboxane synthase gene
   (TBXAS1, exon 9; GenBank accession no. D34621)
   $^{5'}$GCCCGACATTCTGCAAGTCC$^{3'}$
   $^{3'}$TTGGGAAGGGCCGTTGTGG$^{5'}$
2. Human recombination activating gene
   (RAG1, exon 2; GenBank accession no. M29474)
   $^{5'}$TGTTGACTCGATCCACCCCA$^{3'}$
   $^{3'}$AAGTCGGTTTGAACGTCGAGT$^{5'}$
3. Human promyelocytic leukaemia zinc-finger gene
   (PLZF, exon 1; GenBank accession no. AF060568)
   $^{5'}$TGCGATGTGGTCATCATGGTG$^{3'}$
   $^{3'}$CGGAGTCTGCTGTTACTGTGC$^{5'}$
4. Human AF4 gene
   (exon 11; GenBank accession no. Z83687)
   $^{5'}$CCGCAGCAAGCAACGAACC$^{3'}$
   $^{3'}$CCTCGGCGGTCTCCTTTCG$^{5'}$
5. Human AF4 gene
   (exon 3; GenBank accession no. Z83679)
   $^{5'}$GGAGCAGCATTCCATCCAGC$^{3'}$
   $^{3'}$AATACAGGCCGGGTACCTAC$^{5'}$

Table 2 Standardised BIOMED-2 PCR protocol and gel electrophoresis

PCR reaction
- 45 μL master mix solution
- 2.5 U Amplitaq Gold
- 5 μL template DNA

Thermocycling
- On Perkin Elmer 9600 thermocycler
- Denaturing: 95° for 45 s
- Annealing: 60° for 45 s
- Amplification: 72° for 90 s
- 35 cycles

Controls
- Positive DNA control
- Negative extraction control
- Negative PCR control

Gel electrophoresis
- Non-denaturing 6% polyacrylamide gel electrophoresis
- 25 μL PCR product
- 250 V applied for 1.5 h
- Stained with ethidium bromide and visualised under UV light

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences version 14.0 software (SPSS, USA). Descriptive analysis was used to determine distributions. A Friedman test was used to determine if there was a significant difference in the number of cases showing amplification at different DNA fragment lengths.

Univariate logistic regression was used to examine the relationship between amplification of DNA (present/absent) and the age of blocks in months. Further, the correlation between the largest size of DNA fragments amplified (96–600 bp) and the age of the blocks in months was determined using Spearman's rho correlation. All tests were two-sided with a probability of less than 0.05 considered significant.

RESULTS

DNA quantification using spectrophotometry

On a random sample of 40 cases, the mean DNA concentration was 53.62 ng/μL with a range of 10–350 ng/μL.

Amplification of DNA using the specimen control size ladder

Amplification was obtained in 145 of 169 (85.8%) of cases. However, lower fragment lengths were preferentially amplified as shown in the gel picture in Fig. 2. Numbers of FFDPE samples that generated amplicons of 96, 200, 300, 400 and 600 bp are demonstrated graphically in Fig. 3, with 99/169 (58.6%) amplifying at 200 bp, 56 cases (33.1%) at 300 bp, 35 (20.7%) at 400 bp and 19 (11.2%) at 600 bp. The progressive dropout of larger PCR products in FFDPE specimens was statistically significant ($\chi^2 = 274.5$, df = 4, $p < 0.0001$).

The largest size of DNA fragments amplified was also assessed. Forty-six cases (31.7%) showed maximum amplification up to 96 bp, 43 (29.7%) up to 200 bp and 21 (14.5%) up to 300 bp. Only 16 (11.0%) showed maximum amplification to 400 bp and 19 (13.1%) to 600 bp. Surprisingly, 39 cases (23.1%) showed loss of amplification of smaller DNA fragments despite evidence of amplification at larger amplicon sizes.

Correlation of DNA amplification with age of paraffin-embedded tissue

The results of the univariate logistic regression analysis suggested that there was no significant correlation between amplification of DNA and age of tissue blocks in months ($\chi^2 = 2.7$, df = 1, $p = 0.1$).

Further, Spearman's rho correlation indicated that there was no significant correlation between the largest size of DNA fragments amplified and the age of the blocks in months (rho = 0.1, $p = 0.1$).

DISCUSSION

This study reports the results of DNA amplification at different fragment lengths in a large sample size of archival bone marrow trephines using a multiplex PCR amplification control. Standardised fixation methods across all samples and a wide storage range of 4 months to >15 years has also allowed for statistical analysis of the impact of the age of tissue blocks on the ability to amplify DNA of different fragment lengths.

Methods used for DNA extraction can affect the final product obtained. Reports on a small number of samples have demonstrated success in producing large DNA fragments in bone marrow trephines,[13] and several groups have attempted to optimise procedures to improve the yield of DNA from archival tissue.[14–17] However, these methods can be labour-intensive and need to be validated in individual laboratories to produce consistent results. For practical purposes, many laboratories continue to use standard methods of DNA extraction for paraffin-embedded tissues. We used the Roche High Pure PCR Template Preparation kit as this is validated for FFDPE tissue in our laboratory.

Our results indicate that some degree of amplification, particularly of smaller DNA fragments is possible from a large proportion of archived bone marrow trephine tissue; we were able to amplify the 96 bp fragment in approximately 86% of cases. These results are an improvement on those reported for tissues fixed in mercury based fixatives such as B5[18] and are comparable with others using formalin-based fixatives.[3] The poorer results found in our study compared with others that have used formalin-fixed trephine tissue may be related to differences in the decalcification agent used; most studies showing higher rates of DNA amplification from bone marrow trephines

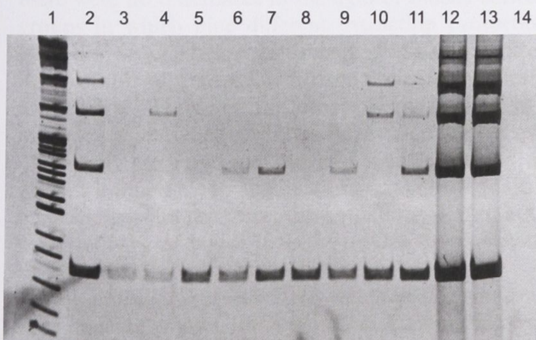


FIG. 2 Gel picture showing preferential amplification of smaller DNA fragments. Lane 1, pBR-322-MspI control size ladder; Lanes 2–11, FFDPE trephine samples; Lane 12–13, positive controls; Lane 14, negative DNA control.

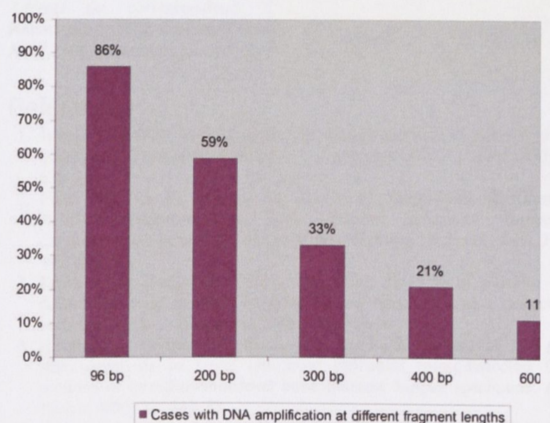


FIG. 3 Graphical representation of DNA amplification at different fragment lengths showing progressive reduction in amplification at higher fragment lengths ($p < 0.0001$).

use EDTA as a decalcification agent.[4,13] Our centre routinely uses acid decalcification rather than EDTA due to the longer processing time required for EDTA decalcification.

We have previously published a comparative study looking at DNA amplification from fresh and FFDPE BM trephine tissue and were able to demonstrate amplification at ~100 bp in 100% of FFDPE cases.[7] One reason for the inferior results in this study (~86% amplification) may be related to smaller amounts of DNA in some samples. Whereas the mean DNA concentration using spectrophotometry in the previous study was 84.6 ng/μL, (95% confidence interval 54.8, 114.4), the mean concentration in a random sample of 40 cases in this study was lower at 53.62 ng/μL with a range of 10–350 ng/μL. This indicates that loss of DNA during the extraction process may have been responsible for lower rates of amplification noted in this study. The length of the tissue is another factor known to affect efficiency of DNA amplification; a trephine biopsy length of 16 mm is considered to be adequate for most diagnostic purposes.[19] The size of tissue is also considered to affect DNA yield.[20] The samples in our previous study were selected such that they were >20 mm in length. In this study, although the mean trephine length was 17 mm, the size range was wide (7–36 mm) and 59 cases (42.4%) had a trephine length lower than the recommended minimum length. Although the mean trephine length in cases that showed amplification was larger than in those cases that did not, the difference was not statistically significant (17.5 mm versus 15.9 mm, $p = 0.219$) using the Yates continuity correlation factor. To further address the issue, amplification in cases with optimal and suboptimal trephine lengths was assessed; no detectable difference was noted between the two groups ($p = 0.2$, Mann–Whitney U test). However, loss of DNA during the extraction process for the smaller samples cannot be conclusively excluded.

Duration of storage is a well recognised post-fixation parameter known to affect tissue preservation and therefore potentially impact on the content and integrity of nucleic acids. A number of studies have standardised their sample groups for age to ensure that the duration of storage does not act as a confounder by affecting the yield of DNA. Coombs *et al.*, in their research into optimising DNA and RNA extraction from archival formalin-fixed tissue, used samples ranging from 1 to 30 years old, and ensured that there were no differences in the ages of blocks between the groups in which nine different extraction protocols were trialled.[17] Several other researchers have demonstrated that it is possible to obtain DNA from archival samples as old as 25 years.[21] However, the impact of age on yield of DNA has not been studied statistically. As our sample size was reasonably large and our range of storage was <1 to >15 years, we were able to demonstrate statistically that the age of specimens did not affect the amplification of DNA. This is likely to be of major interest to researchers working on FFDPE tissues.

The limitations of our study are that our samples were fixed in acid fixatives, instead of EDTA which is considered superior for preservation of nucleic acids. Additionally, standard rather than specialised DNA extraction protocols were used. However, despite these limitations, and the limitation of the additional decalcification required for bone marrow trephines compared with soft tissues, we were able to extract DNA from a comparable number of samples to that described in literature. We also acknowledge that given that most of our samples were between 1 and 15 years old, statistical validity of our results beyond this duration of storage remains to be established. This research addresses the issue of feasibility of DNA amplification at different fragment lengths rather than the yield of DNA. We have not quantified the amount of DNA using spectrophotometric analysis or other methods on all cases, owing to limited quantities of DNA available for analysis. A random sample of 40 cases representative of the cohort was subjected to spectrophotometry. As the OD260/280 ratio of DNA obtained from FFDPE tissue using this extraction process in our laboratory is consistently 1.8 or above, we did not perform it on all samples for this study. We believe the failure of some specimens to amplify is unlikely to have been caused by impure or unclean DNA.

This study has established the impact of duration of storage on the feasibility of DNA amplification at different fragment lengths from FFDPE trephine tissue. As more research using molecular diagnostics on FFDPE tissue occurs, the focus on methods of tissue processing needs to be broadened to preserve more than histological detail. A standardised protocol has recently been proposed to allow maximal preservation of trephine biopsy specimens for histology while still maintaining the integrity of nucleic acids.[22] There is a need for further rigorous research to address issues such as the most effective type and duration of fixation and decalcification, using statistical methods where possible. The best methods for DNA extraction and the impact of other variables such as size/thickness of tissue and temperature of storage also need to be studied.

ACKNOWLEDGEMENTS The authors would like to acknowledge the financial support provided for the study by the Private Practice Trust Fund, The Canberra Hospital, and the equipment grant provided by The Leukaemia Foundation, Australia. The principal investigator/author has received a supplementary scholarship from the Arrow Bone Marrow Transplant Foundation, New South Wales for the project. Ms Amy Broomfield's help with sectioning of archival BM tissue blocks, and Mr Mark Gobbin's help with spectrophotometry is gratefully acknowledged.

Address for correspondence: Dr D. Talaulikar, Department of Haematology, The Canberra Hospital, Yamba Drive, Garran, Canberra, ACT 2606, Australia. E-mail: dipti.talaulikar@act.gov.au

References

1. Juneja SK, Wolf MM, Cooper IA. Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. *J Clin Pathol* 1990; 43: 630–2.
2. Fend F, Bock O, Kremer M, Specht K, Quintanilla-Martinez L. Ancillary techniques in bone marrow pathology: molecular diagnostics on bone marrow trephine biopsies. *Virchows Arch* 2005; 447: 909–19.
3. Provan AB, Hodges E, Smith AG, Smith JL. Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA. *J Clin Pathol* 1992; 45: 763–5.
4. Kremer M, Cabras AD, Fend F, *et al.* PCR analysis of IgH-gene rearrangements in small lymphoid infiltrates microdissected from sections of paraffin-embedded bone marrow biopsy specimens. *Hum Pathol* 2000; 31: 847–53.
5. Mitterbauer-Hohendanner G, Mannhalter C, Winkler K, *et al.* Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). *Leukemia* 2004; 18: 1102–7.

6. Coad JE, Olson DJ, Christensen DR, *et al.* Correlation of PCR-detected clonal gene rearrangements with bone marrow morphology in patients with B-lineage lymphomas. *Am J Surg Pathol* 1997; 21: 1047–56.
7. Talaulikar D, Gray JX, Shadbolt B, McNiven M, Dahlstrom JE. A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsies using two different methods. *J Clin Pathol* 2008; 61: 119–23.
8. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol* 2002; 161: 1961–71.
9. Hoeve MA, Krol AD, Philippo K, *et al.* Limitations of clonality analysis of B cell proliferations using CDR3 polymerase chain reaction. *Mol Pathol* 2000; 53: 194–200.
10. Hughes J, Weston S, Bennetts B, *et al.* The application of a PCR technique for the detection of immunoglobulin heavy chain gene rearrangements in fresh or paraffin-embedded skin tissue. *Pathology* 2001; 33: 222–5.
11. Amara K, Trimeche M, Ziadi S, Sriha B, Mokni M, Korbi S. PCR-based clonality analysis of B-cell lymphomas in paraffin-embedded tissues: diagnostic value of immunoglobulin kappa and lambda light chain gene rearrangement investigation. *Pathol Res Pract* 2006; 202: 425–31.
12. van Dongen JJ, Langerak AW, Bruggemann M, *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003; 17: 2257–317.
13. Wickham CL, Boyce M, Joyner MV, *et al.* Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies. *Mol Pathol* 2000; 53: 19–23.
14. Sepp R, Szabo I, Uda H, Sakamoto H. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *J Clin Pathol* 1994; 47: 318–23.
15. Sato Y, Sugie R, Tsuchiya B, Kameya T, Natori M, Mukai K. Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues. *Diagn Mol Pathol* 2001; 10: 265–71.
16. Wu L, Patten N, Yamashiro CT, Chui B. Extraction and amplification of DNA from formalin-fixed, paraffin-embedded tissues. *Appl Immunohistochem Mol Morphol* 2002; 10: 269–74.
17. Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Res* 1999; 27: e12.
18. Nagasaka T, Lai R, Chen YY, *et al.* The use of archival bone marrow specimens in detecting B-cell non-Hodgkin's lymphomas using polymerase chain reaction methods. *Leuk Lymphoma* 2000; 36: 347–52.
19. Bain BJ. Bone marrow trephine biopsy. *J Clin Pathol* 2001; 54: 737–42.
20. Alizadeh AA, Eisen MB, Davis RE, *et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; 403: 503–11.
21. Gillio-Tos A, De Marco L, Fiano V, *et al.* Efficient DNA extraction from 25-year-old paraffin-embedded tissues: study of 365 samples. *Pathology* 2007; 39: 345–8.
22. Naresh KN, Lampert I, Hasserjian R, *et al.* Optimal processing of bone marrow trephine biopsy: the Hammersmith Protocol. *J Clin Pathol* 2006; 59: 903–11.

Downloaded By: [RCPA] At: 11:58 7 January 2009

Author contributions:

Talaulikar D, Shadbolt B, McNiven M, Dahlstrom JE: **"DNA amplification from formalin-fixed decalcified paraffin-embedded bone marrow trephine specimens – does the duration of storage matter"?** Pathology, 2008 Dec; 40(7): 702-706.

Dipti Talaulikar:

1. Designed study, prepared ethics and grants applications, and set up database
2. Retrieved all cases from medical and laboratory records, and all archived paraffin-embedded trephine blocks
3. Performed all the data entry
4. Performed all DNA extractions
5. Developed all molecular methods under supervision
6. Performed all molecular analysis
7. Performed all statistical analysis under supervision of and help from Dr. Bruce Shadbolt
8. Wrote the first draft of the paper

Bruce Shadbolt:

1. Setting up database
2. All data analysis
3. Review of final paper

Michelle McNiven:

1. Technical advice on molecular analysis
2. Input into and reviewing final paper

Jane E. Dahlstrom:

1. Reviewed and approved final paper

Intellectual planning: DT (96%), BS: (2%) MM (2%)

Experimental work: DT (97%) MM (2.5%) MG (0.5%)

Analyses: DT (70%) BS (30%)

Writing: DT (97%) JED (0.5%) BS (0.5%) MM (2%)

CHAPTER VII:

Summation of results

& impact on

International Prognostic Index (IPI)

7.1 *Routine use of ancillary investigations in staging Diffuse Large B-cell Lymphoma may improve the International Prognostic Index (IPI)" Submitted for publication*

Abstract:

Aims: To determine the effect of the routine use of flow cytometry, immunohistochemistry and molecular studies in staging Diffuse Large B-cell lymphoma (DLBCL) upon the International Prognostic Index (IPI).

Methods and results: Bone marrow trephines of 156 histologically proven DLBCL cases at initial diagnosis were assessed on routine histology, and immunohistochemistry (IHC) using two T-cell markers (CD45RO and CD3), two B-cell markers (CD20 and CD79a) and kappa and lambda light chains. Raw flow cytometry data on all samples were reanalysed and reinterpreted blindly. DNA extracted from archived paraffin-embedded trephine biopsy samples was used for immunoglobulin heavy chain (IgH) and light chain (IgL) gene rearrangement analysis. Using immunophenotyping (flow cytometry and immunohistochemistry), 30 (19.2%) cases were upstaged to stage IV. A further 8 (5.1%) cases were upstaged using molecular studies. A change in IPI was noted in 18 cases (11.5%) on immunophenotyping alone, and 22 (14.1%) cases on immunophenotyping and molecular testing. Comparison of revised IPI models using immunophenotyping alone (rIPI1), and immunophenotyping with molecular studies (rIPI2), with baseline IPI was performed using a Cox regression model and showed that rIPI1 provides the best differentiation between the IPI categories.

Conclusions: Improved bone marrow staging improves the predictive value of the IPI.

Keywords: Staging, Bone Marrow Biopsy, Diffuse Large B-cell Lymphoma, International Prognostic Index, flow cytometry, immunohistochemistry, immunoglobulin gene rearrangement

Introduction:

Diffuse Large B-cell Lymphoma (DLBCL) is defined by the World Health Organization (WHO) as a heterogeneous entity, encompassing morphologic and genetic variants, and variable clinical presentations and outcomes. [1] It accounts for 80% of all aggressive lymphomas. [1] The median overall long-term overall survival (OS) in DLBCL is only ~40-50% [2] with variable outcomes depending on pre-treatment clinical and laboratory characteristics. [3]

The International Prognostic Index (IPI) is a standard clinical tool that is widely used to predict outcome for patients with aggressive Non-Hodgkin Lymphoma (NHL), including DLBCL. It uses a number of clinical and laboratory markers present at the time of diagnosis to predict survival. Age > 60 years, stage III/IV disease defined by results of radiological investigations and bone marrow (BM) biopsy, elevated lactate dehydrogenase (LDH) level, Eastern Cooperative Oncology Group (ECOG) performance status ≥ 2 and more than one extranodal site of disease, are scored 1 each, and depending on the final score ranging from 0-5, 4 prognostic categories are created. These are: low risk correlating with IPI of 0-1, low-intermediate risk with IPI of 2, high-intermediate risk with IPI of 3, and high risk with IPI of 4-5. Five year overall survivals (OS) range from 73% to 26%. [3] However, limitations of the IPI are well recognised owing to the heterogeneity in clinical outcomes within IPI groups. Although gene expression profiling has been used to determine subtypes of DLBCL based on stages of B-cell differentiation, [4] such studies are a largely limited to the research setting. Efforts to improve clinical outcomes in DLBCL using reliable prognostic markers are ongoing. [5, 6] In this study, we assessed the impact of improved staging investigations using easily available ancillary investigations on the IPI.

BM involvement was defined using histology alone in the large multicentre study from which the IPI was developed. [3] Ancillary tests such as flow cytometry, immunohistochemistry and molecular studies were not considered as part of staging towards the IPI. As these investigations have become more routinely available in laboratories around the world and their usage has increased, attempts have been made to define their clinical role.

However, the impact of the routine use of these tests on the IPI has not been formally studied. Only when patients with stage I/ II disease with histologically inapparent bone marrow involvement have positive results on ancillary tests, is there likely to be change in the IPI. Patients with stage III disease, even when upstaged because of BM involvement, will not result in a change in the IPI.

This study demonstrates that a significant change in the predictive value of the IPI can be brought about by incorporating ancillary investigations over and above routine histological diagnosis.

Materials and methods:

Patients:

One hundred and fifty six retrospective cases diagnosed with histologically proven DLBCL at the Canberra Hospital from 1986-2005, on whom staging BM biopsies had been performed, were identified for the purpose of the study. After approval was obtained from the Australian Capital Territory (ACT) Human Research Ethics Committee, clinical information on patients was collected from the Medical Records department at the Canberra Hospital.

The average age of the patient cohort was 61 years (range 20-87 years), and the male to female ratio was 1.5:1. Baseline staging data using routine staging procedures [computerised tomography (CT) scan, gallium/ positron emission tomography (PET) scan and histological examination of BM] was available in 150 patients. Thirty nine (26%), 35 (23%), 45 (30%), and 31 (21%) were found to have stage I, stage II, stage III and stage IV disease respectively. Baseline assessment of IPI was possible in 148 patients as reported in our previous study. [7] Thirty seven (25%) had an IPI of ≤1 of which 14 (9.5%) had an IPI of 0, and 23 (15.5%) an IPI of 1. IPIs of 2 and 3 were noted in 36 (24.3%) and 46 (31.1%) respectively. Twenty nine (19.6%) cases had an IPI of ≥ 4 of which 22 (14.9%) and 7 (4.7%) and an IPI of 4 and 5 respectively. The mean baseline IPI of the patient cohort was 2.41 with a standard deviation of 1.3. [7]

Treatment data showed that of 152 patients on whom data was available, most were treated with anthracycline based regimens. One hundred and twenty nine patients (82.7%) were treated with CHOP [8] or variations of CHOP chemotherapy protocols. [9, 10] Two patients were treated with ICE,[11] 5 patients

with PEN,[12] 1 with EPOCH,[13] 2 with Hyper-CVAD,[14] 3 with TROG [15] and 1 with MACOP-B[16] Nine patients were treated with palliative intent with steroids alone or in combination with non-anthracycline based drugs. Treatment details were not available in 2 patients and 2 were lost to follow-up. Only 36 patients (22.2%) received Mabthera. The median overall survival of the entire patient group was 6 years (95% confidence interval [CI]: 3.8, 8.4 years).

BM histology:

BM biopsies are performed as a routine assessment for all cases with Non-Hodgkin's Lymphoma at first diagnosis in our institution. All trephines are fixed in buffered formalin and acetic acid for 24 hours and then decalcified using 5% nitric acid. Samples are then embedded in paraffin and sections stained with Haematoxylin and Eosin (H&E), Giemsa stain and silver impregnation for reticulin. Archived H&E, Giemsa and reticulin preparations on the trephine biopsy were retrieved for review. The mean trephine length for the patient cohort was similar to our previous reports, 17.6 mm with a range of 8-36 mm and the mean number of levels on H&E sections were 3.7 (range 1-8). [7]

Two haematologists reviewed all slides blindly, and discrepant cases (n=20) were resolved by consensus. Standardised criteria were used to classify trephine biopsy samples as positive, negative or indeterminate. [17]

Flow cytometry:

Raw immunophenotypic data on all bone marrow biopsies was retrieved from laboratory records and analysed as previously described.[18] Multiparametric flow cytometric analysis was performed, with marrow cells immunophenotypically labeled by direct four-colour immunofluorescence using a panel of antibodies. The panel of antibodies used included: CD45, CD19, CD20, CD22, CD10, HLA-DR, Kappa, Lambda, CD2, CD3, CD5, CD7, CD16 and CD56. All antibodies were obtained from Becton-Dickinson. CD45, CD19, CD20, CD3, CD23, Kappa and Lambda antibodies were of the IgG1 isotype; CD2, CD5, CD7, CD10 and HLA-DR antibodies of the IgG2a isotype; and CD22 antibody was of the isotype IgG2b.

Red cells were lysed by incubating 2 ml of marrow aspirate with 10 ml of ammonium chloride for 5 minutes. Cells were washed in phosphate buffered saline (PBS) after centrifugation and a cell-suspension of 1 $X10^6$ cells per tube was then incubated with the monoclonal antibody for 30 minutes at room temperature. Each cell-suspension was then washed and resuspended in a solution of PBS and foetal calf serum. Isotypic controls used were IgG1 and IgG2.

A Becton-Dickinson flow cytometer was used for data acquisition starting with control samples. A minimum of 2000 lymphocytes were counted in each sample. Bright CD45 fluorescent staining and intermediate side scatter were employed as the primary gating strategies to identify the lymphocyte population. Further gating was performed as required based on cell size or using back gating on CD19 positive events.

Previously archived raw data were reanalysed by senior scientists, including blinded re-determination of the population of lymphocytes to be gated. A Haematologist then reviewed and reported the flow cytometry results in a blinded fashion. Positive results on flow cytometry were defined as light chain clonal restriction with a kappa: lambda ratio of >3:1 or <0.3:1. [18, 19] Predominance of B-cells in the gated population alone without light chain restriction was not considered as a positive result. After the investigator was unblinded to previous results, discrepant results were identified (n=5) and re-analysed and re-interpreted by a third blinded person.

Immunohistochemistry:

Immunohistochemical analysis was performed as described in previous reports [7] on a Ventana Benchmark NexES machine. Sections from archived formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsies were immunostained using the streptovidin-biotin method. The following monoclonal antibodies were used: CD3 [Dako clone CD3, 1:200 dilution], CD45RO [Novacastra clone UCLH-1, 1:1000 dilution], CD20 [Zymed clone L26, 1:50 dilution], CD79a [Dako clone JCB117, 1:500 dilution], Kappa [Novacastra clone kp-53, 1:750 dilution], and Lambda [Novacastra clone Hp-6054, 1:750 dilution]. All antibodies are validated and routinely used in our laboratory. CD20 and CD3 are reported to be sensitive at assigning lineage in diffuse aggressive NHL [20] and CD79a and CD45RO were selected over others owing to familiarity and to maintain consistency. These are the antibodies used for diagnostic tissue sections in our laboratory. Heat retrieval was used for all antibodies and tonsillar tissue was used as a positive control. A standardised system of reporting was

adopted and was followed for all stains by two pathologists blinded to previous assessment on histology.

Features used to define involvement on immunohistochemistry reflected standardised histology criteria. The presence of B-cell aggregates was classified as abnormal or malignant when there were large numbers of aggregates, the aggregates were large-sized, or contained disproportionate numbers of larger cells. Controls (six morphologically normal marrows) were used to create a visual impression of normal amounts of background T and B-cells. Scattered small or large B-cells were classified positive only when the numbers were substantially increased as compared to controls. A conservative approach adopted to avoid false positives. Discrepancies between the two pathologists were resolved consensually.

Molecular studies:

Samples for molecular studies were obtained from FFDPE trephine sections. DNA extraction was performed manually using the Roche High Pure PCR Template Preparation Kit from two 10-micron FFDPE trephine sections according to the manufacturer's instructions. To verify the integrity of the DNA extracted from the paraffin sections, and to validate results, all samples were amplified with the control master mix provided in the Immunoglobulin heavy chain (IgH) gene clonality kit from Invivo Scribe Technologies based on the BiOMED2 protocols (IgH Gene Clonality Assay – Gel Detection; InVivo Scribe Teachnologies, USA). This is a multiplex PCR that targets multiple genes and generates a series of amplicons 100, 200, 300, 400 and 600 base pairs (bp) in length. Amplification was obtained in 133/155 cases (84.7%) with amplification

at 96 base pairs (BP), 200 bp, 300 bp, 400 bp and 600 bp noted in 125 (79.6%), 74 (47.1%), 32 (20.4%), 25 (15.9%) and 18 (11.5%) cases. These results are similar to those reported previously. [21]

IgH gene rearrangement analysis was performed on all cases, targeting the conserved framework regions (FR) FR1 [IGH_A: V_HFR1-J_H] and FR3 [IGH_C: V_HFR3-J_H] using the InVivo Scribe kit based on the BiOMED2 protocols. [22] Only FR1 and FR3 were analysed owing to limited amounts of DNA and based on reports from other groups. [23] This was combined with light chain gene rearrangement analysis and included two reactions targeting Ig Kappa (IgK) variable and joining regions [$IGHK_A$: V_k-J_k;] and IgK variable and intragenic regions [IGK_B: V_k-K_{de}]. The PCR reactions consisted of 45μL of the FR1, FR3 or IGK master mix solution, 2.5 units of Amplitaq Gold (Applied Biosystems, USA) and 5μl of template DNA (with an average template DNA concentration of 300-400 ng/μl). Thermo cycling was performed according to the kit protocol with no modifications on a Perkin Elmer 9600 thermocycler. Controls consisted of a positive DNA control, negative extraction control and negative PCR control. Water was used as a negative control in both cases.

Non-denaturing polyacrylamide gel electrophoresis was used to resolve the FR1 and FR3 PCR products. 25uL of PCR product was loaded onto a 6% polyacrylamide gel and 250V applied for 1.25 hours for FR1 and 1.5 hours for FR3 reactions. After electrophoresis, the gels were stained with ethidium bromide and visualised under UV light.

For the IGK reactions, PCR products were denatured at 94°C for five minutes and subsequently cooled at 4°C for 60 minutes to induce duplex formation. Non-denaturing polyacrylamide gel electrophoresis was used to resolve the PCR products. 25uL of PCR product was loaded onto a 6% polyacrylamide gel and 250V applied for 1.5 hours each for both reactions.

FR1, FR3 and IgK gene rearrangements were reported as clonal, polyclonal or not detected. The expected sizes of the PCR products were 310-360 bp for FR1 and 100-170 bp for FR3 which together are estimated to account for approximately 70% of all rearrangements.[23] IGK PCR products were expected to be in the following ranges: 120-160 bp, 190-210 bp, 260-300 bp for IgKA and 210-250, 270-300, 350-390 for IgKB.

Statistical analysis:

Survival data were recorded for each patient. Besides descriptive analysis, Kaplan Meier curves were created with cumulative survival as the outcome. Forward stepwise multivariate Cox regression analysis using the likelihood ratio method was used to establish a comparison between baseline IPI and two revised IPI models. The first (rIPI1) was based on routine use of immunophenotyping alone (flow cytometry and immunohistochemistry) and the second (rIPI2) was based on routine use of immunophenotyping and molecular results. A probability of 0.05 was used as the entry criterion and 0.1 was considered for removal. Patients treated with palliative intent were excluded from all survival analyses. All analyses were performed using the software programme Statistical Package for Social Sciences (SPSS) version 14.0.

Results:

Histology:

As reported previously, [7] of the 156 cases on which bone marrow histology slides were available, 24 were positive on routine histology. Six cases were reported as indeterminate using Cheson criteria, and agreed upon as being positive for involvement after consensual review. H&E stains showed no evidence of involvement in 126 cases.

Flow cytometry:

Flow cytometry data was evaluable in 152 cases, of which 27 (17.3%) cases were noted to be positive for involvement using standardised light chain ratios. Ten of these 27 cases were also positive on routine histology. Flow cytometry detected histologically inapparent involvement in 17 cases (11%).

Immunohistochemistry:

These results have been published previously. [7] IHC using T and B-cell markers showed involvement in 43 cases of 154 available cases; one was not comparable with routine histology due to unavailability of routine H&E slides. Routine histology showed involvement in 25/ 42 cases. An additional 17 cases (11%) with BM involvement were detected on routine use of IHC.

Molecular studies:

Forty one cases of 155 evaluable ones were positive on immunoglobulin heavy and light chain gene analysis. Three of these showed no amplification on amplification controls. Of these, 34 were positive on light chain analysis with all cases showing a clonal band with kappa A; three cases also showed clonal

reactions with kappa B. Overall, 19 cases were positive on heavy chain analysis (FRIII: 18 cases, and FRI: 4 cases) Of these, three cases were positive on both reactions. Overlap with light chain analysis is shown in **table I**. Overall, 12/ 41 cases were positive and 29 negative on routine histology.

Table I:

Results on immunoglobulin heavy chain (IgH) and light chain (IgL) gene rearrangement studies

	#IGK +ve	IgK –ve	**Total**
*FR3 +ve	13	5	**18**
**FR3 –ve	21	116	**137**
Total	**34**	**121**	**155**
FR1 +ve	2	2	**4**
FR1 –ve	32	119	**151**
Total	**34**	**121**	**155**

* FR1: framework I
** FR3: framework III
\# IGK: immunoglobulin kappa

To establish tumour origin, DNA was extracted from 17 available primary FFDPE tissue blocks and gene rearrangement analysis performed. Comparable clonal bands could be identified in only 10 cases. Of these, 2 were positive on routine histology.

Thus, using stringent criteria to account for false positivity, routine molecular staging on FFDPE trephine biopsy tissue yielded positive results in eight (5.1%) histologically negative cases. Clonal populations on flow cytometry were noted in eight cases and abnormal populations were noted on IHC in two cases.

Effect on stage and IPI:

Thirty cases were upstaged using immunophenotyping alone with 6 cases upstaged from stage 1 to 4, 12 from stage 2 to 4, and 12 from stage 3 to 4. When molecular results were added, two additional cases were upstaged from stage 1, 2 from stage 2 and 4 from stage 3.

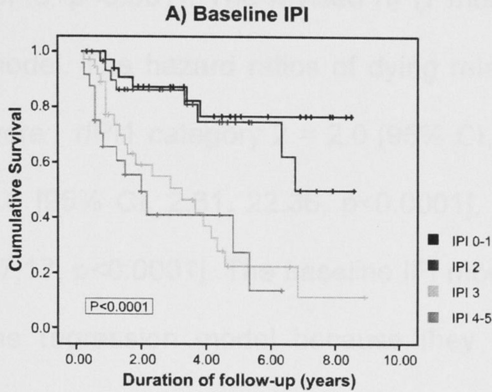
Two new revised IPI (rIPI) models were computed for all cases. The first (rIPI1) was based on immunophenotyping results alone i.e. flow cytometry and immunohistochemistry and the second (rIPI2) on immunophenotyping and molecular results. Changes to the IPI essentially occurred when stage of disease was upgraded from 1 or 2 to stage 4 diseases. Of 148 cases where IPI and rIPI were assessable, three cases were upgraded from IPI 0 to a rIPI1 of 1, 4 cases of IPI 1 changed to a rIPI of 2, 5 cases of IPI 2 were upgraded and 6 cases of IPI 3. No changes were noted in IPI 4-5 group. Overall, 18 patients had a change in their IPI. Of these, three changed their IPI from 0 to 1, which was not apparent when only four prognostic categories were considered. Incorporating molecular results, rIPI2 was found to be upgraded in 6, 7 and 6 cases of IPI 0-1, 2 and 3 respectively.

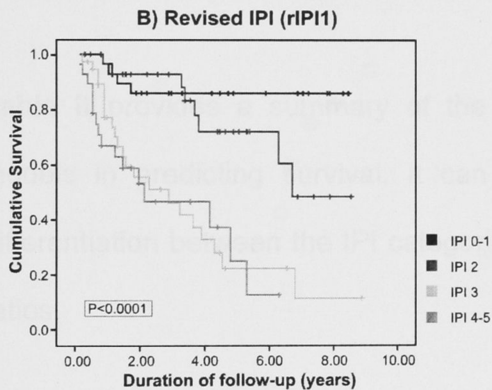
Survival:

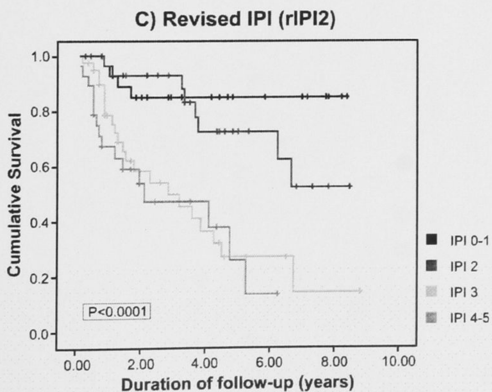
Kaplan Meier curves were created to assess the impact of baseline IPI and the two new revised IPI models rIPI1 and rIPI2 on overall survival. **Figure I** shows the cumulative survival of the four IPI categories using a baseline IPI model, a revised model using immunophenotyping (rIPI1) and a revised model incorporating molecular studies and immunophenotyping (rIPI2) respectively. All three models were statistically significant with p values of <0.0001.

Figure I:

The three Kaplan Meier curves show differences in cumulative survival between with low-risk, low-intermediate, high-intermediate and high-risk categories using a baseline IPI model **(A)** and two revised IPI models. The first (rIPI1) incorporates flow cytometry and immunohistochemistry as routine staging **(B)** and the second IPI model (rIPI2) additionally incorporates molecular testing using IgH/ IgL analysis **(C)**.







Multivariate analysis:

Using a multivariate forward stepwise (likelihood ratio method) Cox regression, the three IPI models were competitively considered for their contribution to predicting survival. The score tests before inclusion into the model were: Baseline IPI: 28.5 (df=3, P<0.001), rIPI1: 31.2 (df=3, p<0.001) and rIPI2: 27.9 (df=3, p<0.001). The revised rIPI1 model was then entered into the regression model. The hazard ratios of dying relative to the 0/1 IPI prognostic categories were: rIPI1 category 2 = 2.0 [95% CI, 0.61, 6.76, p=0.248], rIPI1 category 3 = 7.6 [95% CI, 2.61, 22.36, p<0.0001], rIPI1 category 4/5 = 8.9 [95% CI, 2.94, 27.17, p<0.0001]. The baseline IPI model and rIPI2 models were excluded from the regression model because they did not further contribute to explaining survival [p=0.5, p=0.6 respectively].

Table II provides a summary of the relative performances of the three IPI models in predicting survival. It can be seen that rIPI1 provides the best differentiation between the IPI categories and the largest point estimate hazard ratios.

Table II:

Summary table showing the hazard ratios from Cox regression analyses for the three IPI models, baseline IPI, rIPI1 and rIPI2

	rIPI1[#] Hazard ratios (95% CI)	Baseline IPI[†] Hazard ratios (95% CI)	rIPI2[‡] Hazard ratios (95% CI)
Low-risk IPI (score 0/1)	Reference	Reference	Reference
Low-intermediate IPI (score 2)	2.0 (0.61, 6.76) p=0.248	1.56 (0.54, 4.51) P=0.4	1.8 (0.5, 6.0) P=0.3
High-intermediate IPI (score 3)	7.6 (2.61, 22.36) p<0.0001	5.32 (2.14, 13.22) P<0.0001	6.4 (2.2, 18.6) P=0.001
High-risk IPI (score 4/5)	8.9 (2.94, 27.17) p<0.0001	6.9 (2.61, 18.32) P<0.0001	8.1 (2.7, 24.6) P<0.001

[#]Cox regression using forward likelihood ratio method (X^2: 31.5, df=3, p<0.0001)
[†]Cox regression entering baseline IPI into the model first (X^2: 27.4, df=3, p<0.0001)
[‡]Cox regression entering rIPI2 in to the model first (X^2: 28.2, df=3, p<0.0001)

To study the effect of treatment with Mabthera on survival, this too was considered, but not found to contribute significantly to the Cox regression model (p:0.96).

Discussion:

In this study, we have shown a significant improvement in the predictive value of the IPI using ancillary staging investigations, particularly immunophenotyping, on the BM. By upstaging a proportion of cases, routine use of immunophenotyping provides better differentiation across the IPI prognostic categories. These results suggest that current guidelines should consider incorporating immunophenotyping in routine staging and recommend the use of a new and more inclusive definition of BM involvement within the IPI.

There have been several previous studies on the clinical role of ancillary investigations such as flow cytometry, [18, 19, 24-28] and IHC [7, 19, 29, 30] in NHL, although variable results are noted depending on the histological subtypes of NHL.

Overall, flow cytometry has been reported to be more sensitive than histology alone, and detection of flow cytometry positive cases have been reported in 3-11% of histologically negative cases, with rates in DLBCL varying from negligible to ~15%. [18, 19, 24-26, 28, 31] The converse is also true and 5-20% histologically positive DLBCL cases have been reported to be negative on flow cytometry. [18, 19, 24, 25, 28] This may relate to a number of factors such as sampling and adequacy of histological diagnosis. The clinical utility of using flow cytometry with histology has been demonstrated by our group previously. [18]

Similarly, IHC is reported to detect marrow involvement in histologically negative cases in ~10-23% of cases depending on the histological diagnosis and the antibodies used. [7, 29, 30] This is considered to be due to examination of a

greater number of levels and also to easier detection of scattered malignant cells within normal haemopoietic tissue.

Overall, we found that use of immunophenotyping i.e. flow cytometry and immunohistochemistry in staging bone marrow biopsies upstages ~20-22% of patients with DLBCL. [31] We also previously reported that flow cytometry adds significant independent prognostic value over and above histology. IHC has greater co-linearity with histology, although it adds significantly more to predicting survival than histology alone. [31] Flow cytometry is generally performed on aspirate samples and can be expected to add independent prognostic value. IHC, on the other hand, is performed on the trephine. Although it does not add independent prognostic value, it is a more sensitive technique than histology alone.

The interactive effect of flow cytometry and immunohistochemistry has been shown to have additional greater predictive value over routine histology in terms of predicting survival. [31] This study shows the value of incorporating these tests as a routine rather than using them in histologically ambiguous cases only. Better differentiation into low-risk, low-intermediate, high-intermediate and high-risk IPI categories is obtained by using these tests on all staging bone marrows.

There are several previous studies addressing the role of gene rearrangement (IgH/ IgL) studies [32-36] in NHL. Of particular interest is the study by Mitterbauer-Hollander et al which showed 16% of histologically negative cases had clonal IgH and/ or IgL genes within the bone marrow. [35] The authors demonstrated a significant difference in OS at 5 years amongst patients with positive histology

and molecular studies, negative histology but positive molecular studies, and negative histology and molecular studies. In our study, only ~5% of histologically negative cases were found to have rearranged immunoglobulin genes. We were unable to demonstrate a difference in OS or a change in the predictive value of the IPI by inclusion of molecular staging. This is likely to be related to the unavailability of archived fresh frozen trephine tissue or DNA for our study resulting in all molecular analyses being performed on FFDPE trephine tissue. This is in contrast to the previous study, in which all molecular analyses were performed on fresh bone marrow aspirates. [35] It is well known that fresh tissue yields better quality DNA compared to FFDPE tissue. [37] It should also be noted that the BIOMED2 based protocols are not as well established on FFDPE tissue, [22] although occasional groups have modified the protocols with improved results. [38] It may be of interest to determine if the use of such modified protocols would improve the prognostic significance of molecular staging on FFDPE tissue. Other alternatives to PCR staging may be staging using Fluorescent in-situ hybridisation (FISH) probes with some recent literature demonstrating that FISH using IgH/ BCL2 may give improved results as compared to PCR on paraffin-embedded sections. [39]

Besides the obvious advantage of availability of archived trephine biopsy tissue, the other reason for choosing to perform molecular staging on trephine biopsy rather than aspirate is that histological bone marrow involvement is noted more commonly on trephine biopsies. This has been demonstrated in previous studies [18, 36, 40] and largely attributed to sampling and the tendency of lymphoma cells to adhere to bony trabeculae. [41] As such, there would be greater likelihood of detecting clonal gene rearrangements on trephine biopsy rather than aspirate

samples. Collecting additional trephine biopsy samples for such testing may be logistically difficult. Improved DNA extraction methods and modification of the BIOMED2 protocols may be the best realistic option.

We acknowledge the limitations of our study. This is a small retrospective study in DLBCL cases at initial diagnosis. The proportion of patients treated with Rituximab in our study was small. Although we demonstrated that use of Rituximab was not a significant confounding factor in our multivariate analysis, a prospective study may be required to confirm that the results are valid in Rituximab treated patients. The role of molecular staging in improving the prognostic significance of the IPI may have been demonstrated if fresh tissue had been analysed.

In conclusion, we were able to demonstrate an improvement in the prognostic significance of the IPI by use of simple, relatively inexpensive and readily available staging investigations such as flow cytometry and IHC. Our results suggest a prospective study to assess the impact of molecular staging on the IPI is warranted.

References:

1. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol. 1999 Dec;17(12):3835-49.

2. Coiffier B. State-of-the-art therapeutics: diffuse large B-cell lymphoma. J Clin Oncol. 2005 Sep 10;23(26):6387-93.

3. Shipp MA, Harrington, D.P., Anderson, J.R., Armitage, J.O., Bonadonna, G., Brittinger, G., Cabanillas, F., Canellos, G.P., Coiffier, B., Connors, J.M., Cowan R.A., Crowther, D., Dahlberg, S., Engelhard, M., Fisher, R.I., Gisselbrecht, C., Horning, S.J., Lepage, E., Lister, T.A., Meerwald, J.H., Montserrat, E., Nissen, N.I., Oken, M.M., Peterson, B.A., Tondini, C., Velasquez, W.A., & Yeap, B.Y. . A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med. 1993 Sep 30;329(14):987-94.

4. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000 Feb 3;403(6769):503-11.

5. Gascoyne RD. Emerging prognostic factors in diffuse large B cell lymphoma. Curr Opin Oncol. 2004 Sep;16(5):436-41.

6. Lossos IS, Morgensztern D. Prognostic biomarkers in diffuse large B-cell lymphoma. J Clin Oncol. 2006 Feb 20;24(6):995-1007.

7. Talaulikar D, Dahlstrom JE, Shadbolt B, Broomfield A, McDonald A. Role of Immunohistochemistry in Staging Diffuse Large B-cell Lymphoma (DLBCL). J Histochem Cytochem. 2008 Oct;56(10):893-900.

8. Fisher RI, Gaynor ER, Dahlberg S, Oken MM, Grogan TM, Mize EM, et al. Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med. 1993 Apr 8;328(14):1002-6.

9. Sonneveld P, de Ridder M, van der Lelie H, Nieuwenhuis K, Schouten H, Mulder A, et al. Comparison of doxorubicin and mitoxantrone in the treatment of elderly patients with advanced diffuse non-Hodgkin's lymphoma using CHOP versus CNOP chemotherapy. J Clin Oncol. 1995 Oct;13(10):2530-9.

10. Lambertenghi Deliliers G, Butti C, Baldini L, Ceriani A, Lombardi F, Luoni M, et al. A cooperative study of epirubicin with cyclophosphamide, vincristine and prednisone (CEOP) in non-Hodgkin's lymphoma. Haematologica. 1995 Jul-Aug;80(4):318-24.

11. Kingreen D, Beyer J, Kleiner S, Reif S, Huhn D, Siegert W. ICE--an efficient drug combination for stem cell mobilization and high-dose treatment of malignant lymphoma. Eur J Haematol Suppl. 2001 Jul;64:46-50.

12. Goss P, Burkes R, Rudinskas L, King M, Chow W, Myers R, et al. A phase II trial of prednisone, oral etoposide, and novantrone (PEN) as initial treatment of non-Hodgkin's lymphoma in elderly patients. Leuk Lymphoma. 1995 Jun;18(1-2):145-52.

13. Khaled HM, Zekri ZK, Mokhtar N, Ali NM, Darwish T, Elattar I, et al. A randomized EPOCH vs. CHOP front-line therapy for aggressive non-Hodgkin's lymphoma patients: long-term results. Ann Oncol. 1999 Dec;10(12):1489-92.

14. Lane SW, Crawford J, Kenealy M, Cull G, Seymour JF, Prince HM, et al. Safety and efficacy of pegfilgrastim compared to granulocyte colony stimulating factor (G-CSF) supporting a dose-intensive, rapidly cycling anti-metabolite containing chemotherapy regimen (Hyper-CVAD) for lymphoid malignancy. Leuk Lymphoma. 2006 Sep;47(9):1813-7.

15. O'Brien PC, Roos DE, Liew KH, Trotter GE, Barton MB, Walker QJ, et al. Preliminary results of combined chemotherapy and radiotherapy for non-AIDS primary central nervous system lymphoma. Trans-Tasman Radiation Oncology Group (TROG). Med J Aust. 1996 Oct 21;165(8):424-7.

16. Zinzani PL, Martelli M, Magagnoli M, Pescarmona E, Scaramucci L, Palombi F, et al. Treatment and clinical management of primary mediastinal large B-cell lymphoma with sclerosis: MACOP-B regimen and mediastinal radiotherapy monitored by (67)Gallium scan in 50 patients. Blood. 1999 Nov 15;94(10):3289-93.

17. Cheson BD, Horning SJ, Coiffier B, Shipp MA, Fisher RI, Connors JM, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol. 1999 Apr;17(4):1244.

18. Talaulikar D, Shadbolt B, Bell J, Khan K, Dahlstrom JE, McDonald A, et al. Clinical role of flow cytometry in redefining bone marrow involvement in diffuse large B-cell lymphoma (DLBCL) - a new perspective. Histopathology. 2008 Feb;52(3):340-7.

19. Hanson CA, Kurtin PJ, Katzmann JA, Hoyer JD, Li CY, Hodnefield JM, et al. Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. Blood. 1999 Dec 1;94(11):3889-96.

20. Chadburn A, Knowles DM. Paraffin-resistant antigens detectable by antibodies L26 and polyclonal CD3 predict the B- or T-cell lineage of 95% of diffuse aggressive non-Hodgkin's lymphomas. Am J Clin Pathol. 1994 Sep;102(3):284-91.

21. Talaulikar D, Shadbolt B, McNiven M, Dahlstrom JE. DNA amplification from formalin-fixed decalcified paraffin-embedded bone marrow trephine specimens: does the duration of storage matter? Pathology. 2008 Dec;40(7):702-6.

22. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003 Dec;17(12):2257-317.

23. Liu H, Bench AJ, Bacon CM, Payne K, Huang Y, Scott MA, et al. A practical strategy for the routine use of BIOMED-2 PCR assays for detection of B- and T-cell clonality in diagnostic haematopathology. Br J Haematol. 2007 Jul;138(1):31-43.

24. Naughton MJ, Hess JL, Zutter MM, Bartlett NL. Bone marrow staging in patients with non-Hodgkin's lymphoma: is flow cytometry a useful test? Cancer. 1998 Mar 15;82(6):1154-9.

25. Duggan PR, Easton D, Luider J, Auer IA. Bone marrow staging of patients with non-Hodgkin lymphoma by flow cytometry: correlation with morphology. Cancer. 2000 Feb 15;88(4):894-9.

26. Palacio C, Acebedo G, Navarrete M, Ruiz-Marcellan C, Sanchez C, Blanco A, et al. Flow cytometry in the bone marrow evaluation of follicular and diffuse large B-cell lymphomas. Haematologica. 2001 Sep;86(9):934-40.

27. Sah SP, Matutes E, Wotherspoon AC, Morilla R, Catovsky D. A comparison of flow cytometry, bone marrow biopsy, and bone marrow aspirates in the detection of lymphoid infiltration in B cell disorders. J Clin Pathol. 2003 Feb;56(2):129-32.

28. Perea G, Altes A, Bellido M, Aventin A, Bordes R, Ayats R, et al. Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL). Histopathology. 2004 Sep;45(3):268-74.

29. Chetty R, Echezarreta G, Comley M, Gatter K. Immunohistochemistry in apparently normal bone marrow trephine specimens from patients with nodal follicular lymphoma. J Clin Pathol. 1995 Nov;48(11):1035-8.

30. Fraga M, Brousset P, Schlaifer D, Payen C, Robert A, Rubie H, et al. Bone marrow involvement in anaplastic large cell lymphoma. Immunohistochemical detection of minimal disease and its prognostic significance. Am J Clin Pathol. 1995 Jan;103(1):82-9.

31. Talaulikar D, Dahlstrom, Jane E, Shadbolt, Bruce, McDonald, Anne. Clinical Implications of Immunophenotyping in Staging Diffuse Large B-Cell Lymphoma. Blood (ASH Annual Meeting Abstracts) 2008;112: Abstract 5279.

32. Coad JE, Olson DJ, Christensen DR, Lander TA, Chibbar R, McGlennen RC, et al. Correlation of PCR-detected clonal gene rearrangements with bone marrow morphology in patients with B-lineage lymphomas. Am J Surg Pathol. 1997 Sep;21(9):1047-56.

33. Crotty PL, Smith BR, Tallini G. Morphologic, immunophenotypic, and molecular evaluation of bone marrow involvement in non-Hodgkin's lymphoma. Diagn Mol Pathol. 1998 Apr;7(2):90-5.

34. Kang YH, Park CJ, Seo EJ, Huh J, Kim SB, Kang YK, et al. Polymerase chain reaction-based diagnosis of bone marrow involvement in 170 cases of non-Hodgkin lymphoma. Cancer. 2002 Jun 15;94(12):3073-82.

35. Mitterbauer-Hohendanner G, Mannhalter C, Winkler K, Mitterbauer M, Skrabs C, Chott A, et al. Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). Leukemia. 2004 Jun;18(6):1102-7.

36. Talaulikar D, Dahlstrom JE, Shadbolt B, McNiven M, Broomfield A, Pidcock M. Occult bone marrow involvement in patients with diffuse large B-cell lymphoma: results of a pilot study. Pathology. 2007 Dec;39(6):580-5.

37. Talaulikar D, Gray JX, Shadbolt B, McNiven M, Dahlstrom JE. A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsy specimens using two different methods. J Clin Pathol. 2008 Jan;61(1):119-23.

38. Lassmann S, Gerlach UV, Technau-Ihling K, Werner M, Fisch P. Application of BIOMED-2 primers in fixed and decalcified bone marrow biopsies: analysis of immunoglobulin H receptor rearrangements in B-cell non-Hodgkin's lymphomas. J Mol Diagn. 2005 Nov;7(5):582-91.

39. Espinet B, Bellosillo B, Melero C, Vela MC, Pedro C, Salido M, et al. FISH is better than BIOMED-2 PCR to detect IgH/BCL2 translocation in follicular lymphoma at diagnosis using paraffin-embedded tissue sections. Leuk Res. 2008 May;32(5):737-42.

40. Juneja SK, Wolf MM, Cooper IA. Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. J Clin Pathol. 1990 Aug;43(8):630-2.

41. Pittaluga S, Tierens A, Dodoo YL, Delabie J, De Wolf-Peeters C. How reliable is histologic examination of bone marrow trephine biopsy specimens for

the staging of non-Hodgkin lymphoma? A study of hairy cell leukemia and mantle cell lymphoma involvement of the bone marrow trephine specimen by histologic, immunohistochemical, and polymerase chain reaction techniques. Am J Clin Pathol. 1999 Feb;111(2):179-84.

Author contributions:

Talaulikar D, Shadbolt B, Dahlstrom JE, McDonald A. **"Routine use of ancillary investigations in staging Diffuse Large B-cell Lymphoma improves the International Prognostic Index (IPI)"** Submitted for publication.

Dipti Talaulikar:

1. Designed study, prepared ethics and grants applications, and set up database
2. Retrieved all cases from medical and laboratory records, and all archived slides and paraffin-embedded trephine blocks
3. Performed all the data extraction, analysed and computed all clinical data, and performed all the data entry
4. Reported all histology slides as the first blinded reviewer
5. Interpreted all flow cytometry analysis
6. Cut sections, labeled and stained slides for immunohistochemistry with help
7. Reported and computed immunohistochemistry slides
8. Performed all DNA extractions
9. Developed all molecular methods under supervision
10. Performed all molecular analysis
11. Performed all the data entry
12. Performed all statistical analysis under supervision of and help from Dr. Bruce Shadbolt
13. Wrote the first draft of the paper

Bruce Shadbolt:

1. Setting up database
2. All data analysis
3. Review of final paper

Jane Dahlstrom:

1. Development of formal system of reporting IHC
2. Review of IHC slides
3. Review of final paper
4. Photographs of slides for paper

Anne McDonald

1. Blinded 2nd reviewer of H & E slides on remaining ~75 cases
2. Approved final paper

Intellectual planning: DT (95%), JED: (3%) BS: (2%)

Experimental work: DT (95%) JED: (3%) AMcD: (1%), AB: (1%)

Analyses: DT (70%) BS (30%)

Writing: DT (97%) JED (2%) BS (1%)

CHAPTER VIII:

Research placed in context

8.1 Staging bone marrow in Diffuse Large B-cell Lymphoma: the role of ancillary investigations". Pathology 2009; in press.

Staging bone marrow in diffuse large B-cell lymphoma: the role of ancillary investigations

DIPTI TALAULIKAR*† AND JANE DAHLSTROM†‡

**Department of Haematology, The Canberra Hospital, Canberra, ACT, Australia, †Australian National University Medical School, ACT Australia and ‡Department of Anatomical Pathology, The Canberra Hospital, Canberra, ACT, Australia*

Summary

Diffuse large B-cell lymphoma (DLBCL) is an aggressive non-Hodgkin lymphoma (NHL) that requires treatment at initial diagnosis. Treatment decisions may be guided by staging investigations, which include assessment of bone marrow (BM). Traditionally this assessment has included light microscopic examination of the bone marrow aspirate and trephine biopsy. Ancillary testing—including immunophenotyping using flow cytometry on BM aspirates, immunohistochemistry (IHC) on BM trephines and molecular studies using immunoglobulin heavy (IgH) or light (IgL) chain gene rearrangements—are performed as required to aid histological diagnosis. This review examines the evidence for the routine use of ancillary tests in the initial staging of DLBCL, including the limitations, potential pitfalls and possible clinical implications of their use in this setting.

Key words: B-cell lymphoma, non-Hodgkin's lymphoma, bone marrow, aspirates, trephine biopsy, flow cytometry, immunohistochemistry, immunophenotyping, molecular, prognosis, staging.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) accounts for ~ 30–40% of all cases of adult Non-Hodgkin lymphoma (NHL). [1]The initial diagnosis is established on tissue biopsy and the World Health Organisation (WHO) criteria characterises DLBCL as a diffuse proliferation of large malignant lymphoid cells with an aggressive clinical course. [2]

Established staging investigations performed after diagnosis include: history and examination; chest X-ray; computerised tomography scans; functional scans such as positron emission tomography scans; pathology investigations including lactate dehydrogenase; and examination of the bone marrow (BM).[1] These investigations help in determining the prognosis by contributing to the International Prognostic Index (IPI),[3] and may influence treatment decisions. The clinical practice guidelines for the diagnosis and management of lymphoma published by the National Health and Medical Research Council (NHMRC) recommend staging BMs be scored as positive, negative or indeterminate based on histology alone. The use of ancillary techniques including immunophenotyping (flow cytometry and immunohistochemistry) and molecular studies are discussed in the context of detection of minimal residual disease following treatment, but not in the context of staging at initial diagnosis.[1] The Alberta Cancer Board guidelines recommend the use of immunophenotyping on BM aspirates at the time of diagnosis by flow cytometry, if facilities are available; however the guidelines do not address the role of other ancillary investigations.[4]

Although previous guidelines produced by the British Committee for Standards in Haematology (BCSH) recommended that ancillary investigations such as flow

cytometry, immunohistochemistry (IHC) and molecular studies be available in laboratories involved in lymphoma diagnosis, they did not specifically provided guidelines on their usage.[5] The current 2008 BCSH guidelines on lymphoma diagnosis and staging however do recommend that immunophenotyping by flow cytometry be used to aid in lymphoma diagnosis.[6] An appendix to these guidelines, The Specific Disease Index, recommends that flow cytometry be performed routinely on all diagnostic and staging bone marrows in DLBCL.[6] There is more limited evidence in the guidelines to recommend performing immunohistochemistry as it may supplement the information provided by flow cytometry.[7]

This review will examine the available literature for the routine use of ancillary tests in the initial staging of DLBCL. It also examines the limitations and potential pitfalls of these tests and the possible clinical implications of their routine use.

BONE MARROW INVOLVEMENT AND THE INTERNATIONAL PROGNOSTIC INDEX (IPI)

The International Prognostic Index (IPI) is a widely used prognostic tool in DLBCL. The Index uses a number of negative pre-treatment clinical and laboratory characteristics to risk-stratify cases. These include: age > 60 years; stage III/IV disease; number of extranodal sites (>1); high lactate dehydrogenase (LDH) levels; and poor performance status measured using the Eastern Cooperative Oncology Group (ECOG) score ($\geq$ 2).[3] Each negative characteristic is assigned a score of 1, and depending on the total score, cases are risk-stratified as low, low-intermediate, high-intermediate and high-risk groups with approximate 5-year survival ranging from ~25% to ~75%. This information is currently used to determine treatment and to predict outcome.

The IPI was developed from a multicentre study by Shipp *et al.* that included > 2000 patients. Bone marrow involvement in this study was defined using histology alone, without consideration of ancillary techniques such as flow cytometry, IHC and molecular studies.[3] These ancillary investigations were unlikely to be available routinely in all laboratories around the world in 1993, when this study was reported in the literature. Fifteen years on, these investigations are now regarded as standard in many pathology laboratories reporting primary diagnostic lymphoma samples, and are much more widely available.

Bone marrow involvement plays an important role in determining the IPI score as it has the potential to affect two pre-treatment characteristics. The first of these is the stage of the disease, as BM involvement upstages the disease to

stage IV. Additionally, BM is also classified as an extranodal site, and can therefore increase the IPI score in the presence of one additional extra nodal site of involvement. For example, a young patient presenting with a breast lump as a single extra nodal site of disease combined with a normal performance status and LDH level, scores 1 on the IPI score. However, if the patient is found to have BM involvement, the IPI increases to 3, as the disease is classified as stage IV, with now two extranodal sites of involvement. Thus BM involvement at initial diagnosis often plays a crucial role in determining treatment and predicting outcome. In the above example, it would change the predicted 5-year survival from ~75% to ~45%. A higher IPI score may also influence the choice of initial therapy.

A limitation of the IPI is that it applies the same importance to all extra nodal sites of involvement, and fails to recognise that some extranodal sites such as BM, may have increased significance compared to other sites. Besides implying increased tumour burden,[8] it may also affect tolerability of chemotherapy if the degree of infiltration is significant, as BM reserve may be compromised.

HISTOLOGICAL EXAMINATION OF THE BONE MARROW

The likelihood of BM involvement in NHL varies depending on the subtype of NHL with low-grade lymphomas having higher rates of involvement than aggressive lymphomas.[2, 9] However, marrow involvement in low-grade versus aggressive lymphomas has differing clinical implications in terms of treatment decisions.[10]

There is conflicting data on the rate and prognostic significance of BM involvement in DLBCL using routine histology. Rates of involvement vary from ~11%[11] to >25%.[12, 13] This variation may be related to two main factors: adequacy of sampled tissue; and accuracy of reporting.

For a long time, conventional wisdom advocated the use of bilateral BM sampling for lymphoma staging with demonstrated improved rates of 10–20% on bilateral sampling.[14-16] However, more recent data suggests that bilateral sampling is not required. Optimal demonstration of histological BM involvement may be obtained with a trephine biopsy from a single site if it is ≥ 20 mm in length, and multiple levels (at least four) of the biopsy are examined.[17] From a practical viewpoint, it is not always possible to obtain optimal samples and this may account for lower rates of positivity on BM samples using routine histology alone.

The other reason for differing rates of BM involvement is inter-observer variation in reporting. There is no literature on the rates of inter and intra-observer variability in reporting staging BM in lymphoma. Significant discrepancies in reporting both bone marrows for unrelated conditions,[18] and the primary tissue

for malignant lymphomas, is however known to occur.[19, 20] Lymphoid aggregates may quite easily be classified as benign by one pathologist and malignant by another. Often, this is compounded by the pathologist's knowledge of clinical or other laboratory events. One way of addressing this is by the use of standardised criteria such as Cheson's criteria for classifying aggregates as benign, malignant or indeterminate.[14] Cases with paratrabecular lymphoid aggregates, large-sized aggregates, large numbers of aggregates, presence of large cells within aggregates and increased reticulin response are classified as positive for involvement. Benign lymphoid aggregates are classified as those that are intertrabecular in location, small in size, well-circumscribed and containing predominantly small cells admixed with reactive cells.[14]

The studies that have addressed the impact of histological BM involvement on outcome measures such as survival and progression-free survival in aggressive B-cell NHL have varied in their results depending on the study design and population.[11, 13, 21-23] The most important predictors appear to be the degree of infiltration, pattern of infiltration and the presence of large (concordant involvement) or small cells (discordant involvement) in the BM [11, 13] **(Table 1).** Yan *et al.* studied 60 adult patients with aggressive B-cell NHL and BM involvement at initial presentation. They demonstrated that the presence of concordant BM involvement with >50% large cells, diffuse infiltration and increased amount of BM infiltration (>70%) correlated with worse overall survival. On multivariate analysis, they found only the pattern of BM involvement to have an impact on survival.[24] More recently, Campbell *et al.* reviewed BMs in their series of newly diagnosed DLBCL patients (*n*=172) and noted BM involvement in 27% of patients. They found that within this group, a

greater degree of BM infiltration correlated with a greater incidence of concordant involvement and that these parameters correlated inversely with survival outcomes.[13] Chung *et al.* reported their findings in an even larger cohort of patients (*n*=489) and found ~11% BM positivity rate. They found that although patients with BM involvement had a poorer outcome compared to those who did not have BM involvement, those with concordant BM disease had a particularly bad outcome. They found the presence of large cells in the BM to have prognostic significance independent of the IPI.[11]

TABLE 1:
Summary of studies on bone marrow histology, including histological characteristics and impact on outcome measures in diffuse large B-cell NHL

Author, Year of publication	*N*	**% Positivity on histology**	**Histological characteristics associated with outcome**	**Outcome measures**
Yan *et al.*, 1995[*]	60	100	Pattern of infiltration	OS
Campbell *et al.*, 2006	172	27	Degree of infiltration, concordant involvement	OS, PFS
Chung *et al.*, 2007	489	11	Concordant involvement	OS
Talaulikar *et al.*, 2007	156	19	NA	OS

OS, Overall survival; PFS, progression-free survival; BM, bone marrow
[*] Included cases with BM involvement only; NA, information not available.

Our group's systematic review of the clinical role of individual diagnostic modalities in DLBCL has shown varying results. Previous studies showed that histological involvement did not significantly affect overall survival.[21] However, a recent larger study (n = 156) detected BM involvement histologically in 30 cases (19%) and demonstrated, in keeping with the findings of other groups, that BM involvement had a negative impact on overall survival.[23] We believe the discrepancy between these studies is related to histological characteristics, with a greater representation of discordant involvement in the earlier series.

.

To summarise, BM involvement is reported to occur in 10 to >25% of cases of DLBCL at initial diagnosis and impact on clinical outcomes may vary depending on the degree and pattern of infiltration as well the presence of concordant or discordant involvement.

FLOW CYTOMETRIC ANALYSIS

Flow cytometry is a useful ancillary investigation that can be used to establish clonality and aid in histological diagnosis in patients with B-cell NHL. It is attractive as a means of confirming the more subjective histological diagnoses. It is important to remember that almost all studies report a degree of discordance (of ~10–20%) between histology and flow cytometry.[12, 22, 25, 26] Thus, cases positive on routine histology may be negative on flow cytometric analysis and vice versa.

Generally in NHL, and more specifically in DLBCL, lymphoma involvement is better noted on trephine biopsy specimens than BM aspirates.[15, 22, 26] A false negative result on flow cytometry is therefore understandable, as flow cytometric analysis is usually performed on BM aspirates and not on trephine biopsy samples. This is largely because BM aspirate samples are more easily available than trephine biopsy samples. Performing flow cytometry on trephine samples may compromise histological diagnosis and/or inconvenience the patient to a greater extent.

There are several reasons for the higher rates of lymphomatous involvement noted on trephine biopsies. For example, dilution of aspirate samples with peripheral blood can result in false negative results on aspirates, and consequently, on flow cytometric analysis. Additionally, small aggregates of lymphoma cells may become adherent to bony trabeculae on trephine biopsies,[27] thus being missed in aspirate and flow cytometric analysis. Other causes of discrepant results include technical reasons such as the definition of monoclonality, control measures used in the laboratory and the skill and

experience of the operator. Definition of monoclonality can vary from >3:1 to >6:1 for κ:λ ratios, and <0.3:1 to <0.5:1 for λ:κ ratios.[12, 25, 26, 28] Reference ranges are thus best established by individual laboratories. Additionally, some NHL cases, including DLBCL, may not be light chain restricted raising the potential for false negative results on flow cytometry.[29] The choice of B-cell antibodies may also be important and care should be taken to account for CD20 negative DLBCL cases if CD20 is used as one of the pan B-cell markers. CD19 may be preferable in such cases. [30-32]

Positive results on flow cytometry in the absence of involvement on routine histology carry greater significance as flow cytometry is known to be a more sensitive technique with the ability to detect clonal populations when there is <5% lymphomatous infiltration.[28, 33] Additionally, optimal sampling for histological diagnosis (i.e. trephines of >20 mm with at least four levels examined) is often difficult to obtain and may result in false negative results on histology. Again, as with flow cytometry, the skill and experience of the observer can change histological results; although this can be minimised to some extent by the use of standardised histological criteria to define involvement.[14]

Reported rates of histologically inapparent lymphoma detected by flow cytometry vary depending on the study design and histological subtype **(Table 2)**. Most studies have addressed the role of flow cytometry in B-cell NHL as a single disease entity rather than studying specific subtypes and have often included restaging marrows in addition to the initial diagnostic ones. Palacio *et al.* found only three histology negative, flow cytometry positive cases in his series of 79 patients (4% discordance), of which the first was a diagnostic

DLBCL case, the second a diagnostic follicular lymphoma case, and the third a follow-up follicular lymphoma case.[12] Perea *et al.* found, in their series of 388 B-cell NHL samples including 58 (15%) DLBCL cases, that there was discordance in 82 (21%) cases with 46 morphology positive, flow cytometry negative (BM+/ FC–) cases and 36 morphology negative, flow cytometry positive (BM–/FC+) cases.[33] The 58 cases of DLBCL included three (5.2 %) BM–/FC+ cases and 12 (20.7%) BM+/FC– cases. Thus the ability of flow cytometry to detect histologically inapparent disease in DLBCL was low compared to those in follicular lymphoma (24/188, 13%). Duggan *et al.* included staging and restaging BM in their series, but excluded cases with chronic lymphocytic leukaemia (CLL).[34] The total number included 227; however specific subtypes are not listed in the paper.[34] Of the 27 cases (12%) detected by flow cytometry with negative histology, more cases (*n*=12/27, 44.4%) were of the follicular subtype as compared to DLBCL cases (*n*=4/27, 14.8%). These two studies are in contrast to the findings of Naughton *et al.*, who although reporting very low rates of BM–/FC+ cases among his series of B-cell NHL (3/273, 1.09%) made the interesting observation that two of the three cases were those of large cell lymphoma.[26] Hanson *et al.*, similarly, reported that all five of the 175 B-cell NHL cases (2.9%) that were BM–/FC+ were those of large cell lymphoma. All of these patients were clinically characterised by bulky, stage I disease.[25] In their series of 110 patients, Sah *et al.* addressed the issue of flow cytometry in chronic lymphoid disorders (chronic lymphocytic leukaemia=65, B-cell NHL=39 and hairy cell leukaemia=6), and found 3/39 (8%) cases of B-cell NHL had flow cytometric involvement in the presence of negative histology.[35] Similarly, Dunphy found that 5/188 (2.6%) cases (including 20 cases of DLBCL) had

histologically inapparent flow cytometric positivity.[36] In our study, 8/113 (7%) DLBCL cases were detected on flow cytometry alone.[22]

Overall, most studies have shown flow cytometry to make a small but useful addition to histological analysis of BM in NHL[25, 26] in terms of increasing the yield of BM detection.

Only a few studies have looked at the clinical impact of flow cytometric involvement on BM in NHL. Perea *et al.* studied outcome measures in a subgroup of follicular lymphoma patients and found no significant differences between the groups of patients that were histology negative, flow cytometry negative (BM–/FC–) and histology negative, flow cytometry positive (BM–/FC+).[30] However, as compared to histology positive, flow cytometry positive cases (BM+/FC+), histology negative, flow cytometry positive (BM–/FC+) cases did significantly better. On multivariate analysis, positivity on flow cytometry in histologically negative or occult cases showed no prognostic significance.[33]

TABLE 2:
Summary of comparative studies on occult marrow involvement using flow cytometry and impact on outcome measures

Author, year of publication	n	Lymphoma subtypes	Staging (S) Restaging (RS) BM	% Discordance	BM^-/FC^+	BM^+/FC^-	Outcome
Palacio et al., 2001	79	DLBCL (31), FL (48)	S	4%	3, 3.8% (1 DLBCL, 2 FL)	0	NA
Perea et al., 2004	380	FL (188), DLBCL (58), MCL (57), MZL (31), WM (25), BL (7), others (14)	S, RS	21%	36, 9.5% (DLBCL: 3/58, 5.2%)	46, 12% (DLBCL: 12, 20%)	NA
Duggan et al., 2000	227	Not listed	S, RS	22%	27, 12% (DLBCL: 4/27, 14.8%#)	16, 7%	NA
Naughton et al., 1998	273	DLBCL (141), FL (76), MCL (8), SLL (9), BL (8), B-ALL (5), T-NHL (6) others (20)	S, RS	10%	3, 1.1% (2 DLBCL)	25, 9.1%	NA
Hanson et al., 1999	175	DLBCL (74), FL (58), MZL (12), MCL (10), SLL (9), WM (9), BL (1), others (2)	S, RS	9%	5, 3% (5 DLBCL)	10, 5.7%	NA
Sah et al., 2003	110	CLL (65), low grade NHL (39), HCL (6)	S, RS	CLL: 12%, NHL: 31%	CLL: 4/65, 6.2% NHL: 3/39, 8%	CLL: 2/65, 3.1% NHL: 2/39, 5.1%	NA
Talaulikar et al., 2007	113	DLBCL	S	16%	8, 7%	11, 9.7%	OS

FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; WM, Waldenstrom's macroglobulinaemia; BL, Burkitt lymphoma; SLL, small lymphocytic lymphoma; CLL, chronic lymphocytic leukaemia; B-ALL, B-cell acute lymphoblastic lymphoma; HCL, hairy cell leukaemia; BM, bone marrow; FL, flow cytometry; OS, overall survival; NA, information not available. [#]: 14.8% refers to the % of DLBCL amongst the BM-/FC+ cases, and not the total number of DLBCL cases.

One of the reasons there is limited clinical data on positive flow cytometry results in histologically negative bone marrows, is that the number of such cases is small. Given that both forms of discrepancy i.e. BM+/FC– and BM–/FC+ are known, Dunphy proposed that histology and flow cytometry be combined in the evaluation of BM involvement.[36] We devised a revised definition of BM involvement in DLBCL using a summative model with positivity on flow cytometry and/or histology classified as 'involved'. We then used a multivariate regression model to compare the conventional definition of BM involvement (p=0.173) with the revised definition and found that the revised definition had greater prognostic significance (p=0.005).[22] Additionally, we have demonstrated that flow cytometry adds independent prognostic value in predicting OS over and above histological diagnosis in a larger study [Histology: 2.1, CI 1.0, 4.3, p=0.05; FL: 2.0, CI 1.0, 3.8, p=0.04].[37]

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemistry (IHC) is another valuable tool for immunophenotypic analysis of staging BM in lymphoma cases. However, there is limited data on the role of IHC in routine clinical practice in terms of detection of histologically inapparent disease and on clinical outcome measures.

IHC is currently seen more as a tool for characterising tumours in primary diagnostic tissue specimens rather than staging BM, and many centres around the world continue to use IHC in a limited manner on BM trephines. Currently its role is limited to histologically ambiguous cases, primarily in differentiating malignant from benign lymphoid aggregates. This seems to be related to the lack of published evidence regarding its value in initial staging. Haematologists appear to prefer immunophenotypic analysis using flow cytometry on BM aspirates. Perhaps this is because the results obtained with flow cytometry are quantitative and are considered to be less prone to errors or because traditionally immunohistochemistry has been reported by histopathologists rather than haematologists. In addition, it is known that routine histology and IHC may be subjective, particularly in inexperienced hands.[18-20]

The limitation of flow cytometry detailed in the previous section can largely be overcome by the addition of immunohistochemical analysis on bone marrow trephines. As BM involvement in NHL is known to be more easily apparent on trephine biopsy specimens than BM aspirates, it follows that immunophenotypic analysis performed on bone marrow trephines using IHC is likely to be more productive than flow cytometric analysis on aspirate samples.

The ability of IHC to detect histologically inapparent disease is related to two factors. Fraga *et al.* postulated that routine histology often failed to detect low level disease in aggressive lymphomas, due to the paucity and scattered distribution of malignant cells amongst normal haemopoietic cells, and the difficulty in recognising them as different from immature haemopoietic cells.[38] This has been corroborated in subsequent studies.[23] The other reason may be that the examination of additional sections for IHC allows the detection of foci of lymphomatous involvement missed on routine histology.[23]

Most published studies on the use of IHC in detection of lymphoma in the bone marrow have confined the study group to indeterminate cases on histology or have not reported results for routine histology and immunostaining separately so that comparisons cannot be easily made.[12, 25, 28, 33, 39] The studies that have addressed the issue of detection of histologically inapparent disease using immunohistochemistry are summarised in **Table 3**. Chetty *et al.* looked at 27 cases with follicular lymphoma and found 5 cases that were negative histologically but had minimal involvement on IHC.[40] In an analysis of 42 anaplastic large cell lymphoma cases, Fraga *et al.* reported involvement on routine histology in 17% cases. The group performed routine immunohistochemical analysis using CD30 in all cases, and detected occult disease in a further 23% of cases.[38] In a pilot study on 36 patients with DLBCL, we found 4 BM–/IHC+ cases (11.1%).[21] Similar results were noted on a larger study of 156 patients, with BM involvement on routine histology in 30 (19.2%) cases and a 11.1% (17 cases) rate of occult involvement using IHC.[23] Hanson *et al.* have previously reported no benefit from flow cytometry or immunohistochemistry to routine histological diagnosis in all subtypes of NHL.[25]

TABLE 3:
Summary of comparative studies on discordance between bone marrow histology and immunohistochemistry

Author, year of publication	*n*	NHL subtypes	BM–/IHC+	BM+/ IHC–	Outcome measures
Chetty *et al.*, 1995	27	FL	5, 18.5%	NA	NA
Fraga *et al., 1995*	42	ALCL	NA, 23%	NA	OS
Hanson *et al., 1999*	175	DLBCL (74) FL (58) MZL (12) MCL (10) SLL (9), WM (9), BL (1) others (2)	1, 0.5%	3, 1.7%	NA
Talaulikar *et al., 2007*	156	DLBCL	17, 11%	5, 3%	OS

FL, follicular lymphoma; ALCL, anaplastic large cell lymphoma; DLBCL, diffuse large B-cell lymphoma; OS, overall survival; NA, information not available.

IHC has its limitations and may fail to detect bone marrow involvement by lymphoma even in histologically obvious cases.[41] [23] The reported reasons include the appropriateness of the antibody panel e.g. CD20 would be of little use in staging if the primary tumour was CD20 negative [30-32], the quality of immunohistochemical analysis, the experience of the pathologist [42], and the processing techniques of bone marrows with pre-fixation, fixation and post-fixation factors affecting the quality of staining.[43, 44] IHC is prone to the same subjective errors as routine histology, and requires the use of standardised criteria.[14] Standardised criteria that reflect the Cheson criteria, and the use of morphologically normal marrows as controls to get a visual impression of the number of B-cells and T-cells in the BM, have been used in some studies.[23] Currently there are no standardised criteria for reporting bone marrow involvement on IHC.

The use of appropriate T-cell and B-cells markers is essential. It is recommended that at least two B-cell and T-cell markers be used to prevent false results relating to the antibody, with CD20 and CD3 being reported as the most sensitive in assigning lineage in diffuse aggressive NHL.[45] The other options in B-cell markers include CD79a, PAX5, 4KB5, and KiB3, and often the antibody of choice is the one the laboratory has validated and is most comfortable with using. These antibodies may be particular relevance in cases where the primary tumour is CD20 negative. [30-32] CD79a has an advantage over PAX5 in that it helps to differentiate T-cell rich B-cell lymphomas from Hodgkin Reed-Sternberg cells; although this is more relevant to primary diagnostic tissue than staging BM.[46] T-cell options are more varied and include CD45RO, CD5, Leu-22, MT-1 and OPD4. CD5 is not preferred as a T-cell marker as CD5

expression can occur in B-cell neoplasms including DLBCL.[47,48] Notably, variable expression can be noted with certain antibodies; decalcification with 5% nitric acid has been reported to negatively affect certain clones of CD20, CD79a, CD5 and CD43.[42] In our study, we found CD20 to be much more strongly and consistently expressed than CD79a with our processing protocol.[23]

It should be remembered that clonality may be difficult to establish in B-cell NHL using IHC for kappa and lambda light chains. We were able to establish clonality in only one third of cases (15/42, 35.7%).[23] There are reports that suggest flow cytometry may show variable expression of antigens from different anatomic sites in cases of NHL.[49] This may be caused by technical factors or by true loss of antigen expression as a result of a biological phenomenon. The use of external, as well as internal (plasma cells stained positive), controls may help to resolve this. Variable antigen expression is also reported to occur over time in B-cell NHL cases and this needs to be considered in relation to follow-up BM samples.[50]

Studies looking at the impact of IHC on clinical outcomes are hard to find. We have reported that IHC is a stronger prognostic marker than routine histology in a Cox regression analysis model.[23] Histology and IHC show co linearity in their prediction of survival with IHC adding significantly more than histology alone [Histology: 1.3, CI 0.5, 3.0, p=0.6; IHC: 2.3, CI 1.1, 5.0, p = 0.03].[37]

To summarise, there are advantages in the routine use of IHC on trephine biopsies for staging DLBCL. Usually, IHC is technically well established in most centres, and relatively cost effective as only a small number of markers (T-cell

and B-cell markers, light chain markers) are required. IHC also allows a larger number of levels of the biopsy to be examined using a more sensitive technique. The two ways IHC may be useful is in detection of additional foci of lymphomatous involvement, and of scattered large malignant cells amongst normal haemopoietic cells. However, IHC can also be subjective and results may vary depending on observer skill and experience, the use of different antigen retrieval methods and differing methods of processing specimens.[42] There is a need to develop and use standardised methods to process BM samples and to report IHC.

MOLECULAR STUDIES IN DLBCL

Analysis of clonality of B-cell lymphoid populations using polymerase chain reactions (PCR) of immunoglobulin (Ig) genes can be a useful supplementary tool in the diagnosis of B-cell lymphoproliferative disorders.

PCR is simple and easy to use[51, 52] and can be applied to small fresh samples as well as archived paraffin-embedded samples.[53, 54] The immunoglobulin heavy chain (IgH) is usually targeted as most B-cell lymphoproliferative disorders have rearranged IgH genes.[55] Consensus primers directed against framework 3 (FRIII), and less frequently against framework 2 (FRII) and framework 1 (FRI) can be used to establish B-cell clonality.[54]

However, somatic mutations involving the IgH genes are known to occur in up to 30% of DLBCL and can render the genes unamenable to amplification.[53, 55] False negative results can also result from improper annealing of PCR primers to gene segments.[56] Lack of standardisation of PCR protocols resulted initially in different groups developing individual primer sets with differing sensitivity and applicability with most studies showing a sensitivity of ~60 %.[57, 58] However, the development and standardisation of the BIOMED-2 multiplex PCR protocols by the BIOMED-2 European Consortium increased the rates of molecular positivity in histologically proven B-cell neoplasms to unprecedented high levels of 99% (98% in DLBCL).[56] Confirming the results of previous studies, they reported the additional value of immunoglobulin light chain (IgL) analysis and recommended a combination of the two strategies to maximise clonality detection.

Results from a number of studies show a degree of overlap with histology and with other diagnostic modalities[57-59] **(Table 4)**. One of the earliest studies to correlate the findings of BM histology and PCR for IgH gene rearrangements was by Coad *et al.*[57] Amongst the 225 staging and restaging bone marrows, 60 (27%) were positive for lymphoma, 20 (9%) were suspicious for lymphoma, and 123 (54%) were negative for lymphoma. Within the three groups, 57%, 25% and 11% of cases had positive gene rearrangements respectively. Within specific subtypes, there were a total of 85 DLBCL cases of which 12 had positive histology and 62 had negative BM histology. DLBCL demonstrated PCR positivity in 58% of positive cases. Of the cases with positive histology (*n* =12), 5 (41.6%) showed negative PCR results, and of the 62 cases negative on histology, 6 (10%) showed positive PCR reactions. Similarly, Crotty *et al.* reported a 67% PCR positivity rate amongst a group of 100 consecutive patients who underwent immunophenotypic analysis, of which 40 had objective evidence of B-cell neoplasia.[58] Kang *et al.*, in their series of 170 NHL cases, included 91 cases of DLBCL. They found histological involvement in 21 (23%) cases and observed PCR positivity in only 12 (57%) of these cases.[60] More interestingly, they found 42/70 (60%) cases with negative histology had positive PCR using IgH alone, which is much higher than rates reported in other studies. In a landmark study, Mitterbauer-Hohendanner *et al.* demonstrated the prognostic value of IgH and IgL gene rearrangements in 155 patients with DLBCL. They reported clonal IgH +/– IgL gene rearrangements in 14/19 (74%) cases with histological involvement. Occult molecular positivity was noted in 21/130 (16%) cases. They found a significant difference in overall survival at 5 years in patients with negative histology and negative PCR results (66%), negative histology but positive PCR results (37%), and positive histology as well

as positive PCR results (12%).[61] However, they used fresh peripheral blood and bone marrow aspirate samples, and as discussed in previous sections, BM involvement in DLBCL is seen more often on trephine rather than aspirate samples. Previous reports have demonstrated that PCR results correlate better with trephine samples as compared to aspirate samples. In one study, 10 out of 11 cases with established NHL had negative PCR results on BM aspirates but positive PCR results on trephine biopsy specimens.[57] Thus the yield from molecular staging is likely to be improved with the use of trephine samples rather than aspirates.

TABLE 4:
Summary of comparative studies on PCR positivity on bone marrow samples in NHL

Author, year of publication	*n*	NHL subtype	Staging (S) Restaging (RS) BM	BM+/ PCR–	BM– /PCR+	Outcome measures
Coad *et al.*, 1997	225	FL (86), DLBCL (85), misc* (54)	S, RS	26/60, 43.3% (DLBCL: 5/12, 41.6%)	13/123, 11% (DLBCL: 6/62, 10%)	N/A
Crotty *et al.*, 1998	60	Not specified	S	13/40#, 32.5%	NA	N/A
Kang *et al.*, 2002	170	FL (7), MCL (8) MZL (9) DLBCL (91) SLL (5) BL (4) B-ALL (1) others (5)	S, RS	9/21, 42.9%	42/70, 60%	N/A
Mitterbauer *et al.*, 2004	155	DLBCL	S	5/19, 26.3%	21/130, 16%	OS
Talaulikar *et al.* , 2007	36	DLBCL	S	4/9, 44.4%	1/9, 11.1%	OS

DLBCL, diffuse Large B-cell Lymphoma; NHL, non-Hodgkin lymphoma; BM, bone marrow; PCR, polymerase chain reaction; NA, information not available.
*Including small lymphocytic lymphoma, mantle cell lymphoma, Burkitt lymphoma, marginal zone lymphoma, myeloma, cutaneous B-cell lymphoma, splenic lymphoma with villous lymphocytes.
#40 refers to the total number of cases on which clonality was detected, and not histologically positive cases.

Trephine biopsy samples can either be used fresh or sections can be taken from archived paraffin-embedded blocks. Although amplifiable DNA for IgH and IgL analysis can be extracted from both fresh frozen and paraffin-embedded trephine samples, it has been reported that fresh frozen specimens are a better source of DNA (mean amplicon size 600 bp, 300 bp, p <0.001).[62] A number of studies have demonstrated that routine DNA extraction methods are efficient enough to amplify DNA from approximately 60–85% of archived formalin-fixed paraffin-embedded trephine specimens.[63-65] This rate may be lower in trephines fixed in mercuric acid based fixatives such as B5 instead of formalin.[65]. Inghirami *et al* have shown similar positivity rates (64%, 63%) with fresh unfixed and paraffin-embedded tissue using FRIII IgH gene rearrangement.[66] The applicability of the BIOMED-2 primers on fixed tissues remains doubtful,[56] although there are occasional reports of modified protocols being more effective in formalin fixed trephine samples.[67] In a pilot study, we have reported rates similar to the study by Mitterbauer-Hohendanner *et al.*[55] (performed on aspirate samples), with 6/36 cases (16.6%) showing clonality on IgH FRI and FRIII analysis on formalin-fixed decalcified paraffin embedded (FFDPE) trephine tissue.[21] We were able to use a summative model to define bone marrow involvement (positive histology, flow cytometry, IHC and/or IgH studies) and to better predict prognosis in these patients. However, a larger study performed on 156 FFDPE trephine biopsies has failed to demonstrate that molecular staging with IgH/ IgL affects prognosis. We believe this is likely to be related to the limitations of the use of FFDPE tissue.

To summarise, molecular studies using a combination of IgH and IgL analysis can be used to establish clonality; although a degree of overlap may be seen

owing to sampling, technical and biological issues. The strategic use of standardised IgH and IgL primers can detect histologically inapparent disease which appears to correlate adversely with clinical outcome. Further in cases where primary tissue is not available, BM trephine tissue can be used for gene expression profiling and this may have particular relevance in the future. [68].

CONCLUSIONS

Histological BM involvement occurs in 10–25% of DLBCL cases. Ancillary investigations can improve the rate of detection of BM involvement and may influence clinical outcome. Figure I shows a flow diagram with the recommended staging investigations after diagnosis is established in DLBCL.

Although a high degree of discordance is noted between histology and flow cytometry (5–20%), the routine use of this flow cytometry can improve the rate of detection of lymphoma in the BM by up to 10%, and in conjunction with histology, potentially influence clinical outcome. The routine use of immunohistochemistry (IHC) can improve detection by up to 20 %. Recent data suggests that IHC is a stronger predictor of survival than routine histology. Molecular studies using a combination of IgH and IgL analysis in a standardised multiplex PCR format improve rates of detection of lymphoma in BM by a further 10–15% and there is clear evidence that this better predicts survival of these patients.

Overall, there is enough evidence to warrant studies to assess the impact of the routine use of these investigations on the IPI in aggressive B-cell NHL. Studies by our group show a significant improvement In the predictive value of the IPI with the addition of immunophenotyping i.e. flow cytometry and IHC in addition to routine histology (unpublished observation, submitted for publication). We found that immunophenotyping upstaged 30/156 (19.2%) cases to stage IV. A further 8 (5.1%) cases were upstaged using molecular studies. A change in IPI was noted in 18 cases (11.5%) on immunophenotyping alone, and 22 (14.1%) cases on immunophenotyping and molecular testing. Revised IPI models using

immunophenotyping alone (rIPI1), and immunophenotyping with molecular studies (rIPI2) were compared to baseline IPI in a Cox regression model. Results showed that rIPI1 provides the best differentiation between the IPI categories indicating that improved bone marrow staging improves the predictive value of the IPI. We were unable to show a benefit to the IPI from the addition of molecular studies to BM staging due to the lack of availability of fresh tissue for analysis as all molecular studies in our study were performed on paraffin-embedded tissue. Larger multicentre studies are required to validate our findings and to establish whether a new definition of the IPI is required.

Acknowledgements:

The authors would like to thank Dr. Anne McDonald of the National Capital Private Hospital, Canberra, Australia, for comments and suggestions on the final manuscript.

Address for correspondence: Dr D Talaulikar, Department of Haematology, The Canberra Hospital, PO Box 11, Woden, ACT 2606, AUSTRALIA. E-mail: dipti.talaulikar@act.gov.au.

References:

1. Australian Cancer Network Diagnosis and Management of Lymphoma Guidelines Working Party: Guidelines for the Diagnosis and Mangement of Lymphoma. Sydney: The Cancer Council Australia and Australian Cancer Network; 2005.
2. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol. 1999 Dec;17(12):3835-49.
3. Shipp MA, Harrington, D.P., Anderson, J.R., Armitage, J.O., Bonadonna, G., Brittinger, G., Cabanillas, F., Canellos, G.P., Coiffier, B., Connors, J.M., Cowan R.A., Crowther, D., Dahlberg, S., Engelhard, M., Fisher, R.I., Gisselbrecht, C., Horning, S.J., Lepage, E., Lister, T.A., Meerwald, J.H., Montserrat, E., Nissen, N.I., Oken, M.M., Peterson, B.A., Tondini, C., Velasquez, W.A., & Yeap, B.Y. . A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med. 1993 Sep 30;329(14):987-94.
4. Alberta Cancer Board. Clinical practice guidelines - Lymphoma. 2006.
5. The Haemato-oncology Task Force of British Committee for Standards in Haematology. BCSH guidelines on nodal non-Hodgkin's lymphoma. 2002.
6. Parker A BB, Devereux S, Gatter K, Jack A, Matutes E, Rooney N, Ross F, Wilkins B, Wotherspoon A, Ramsay A. . Best Practice in Lymphoma Diagnosis and Reporting - Specific Disease Appendix. British Committee for Standards in Haematology, Royal College of Pathologists. 2008:52-60.

7. Parker A BB, Devereux S, Gatter K, Jack A, Matutes E, Rooney N, Ross F, Wilkins B, Wotherspoon A, Ramsay A. . Best practice in lymphoma diagnosis and reporting. British Committee for Standards in Haematology, Royal College of Pathologists. 2008:1-52.

8. Smithers DW. Summary of papers delivered at the Conference on Staging in Hodgkin's Disease (Ann Arbor). Cancer Res. 1971 Nov;31(11):1869-70.

9. Arber DA, George TI. Bone marrow biopsy involvement by non-Hodgkin's lymphoma: frequency of lymphoma types, patterns, blood involvement, and discordance with other sites in 450 specimens. Am J Surg Pathol. 2005 Dec;29(12):1549-57.

10. Horning SJ, Rosenberg SA. The natural history of initially untreated low-grade non-Hodgkin's lymphomas. N Engl J Med. 1984 Dec 6;311(23):1471-5.

11. Chung R, Lai R, Wei P, Lee J, Hanson J, Belch AR, et al. Concordant but not discordant bone marrow involvement in diffuse large B-cell lymphoma predicts a poor clinical outcome independent of the International Prognostic Index. Blood. 2007 Aug 15;110(4):1278-82.

12. Palacio C, Acebedo G, Navarrete M, Ruiz-Marcellan C, Sanchez C, Blanco A, et al. Flow cytometry in the bone marrow evaluation of follicular and diffuse large B-cell lymphomas. Haematologica. 2001 Sep;86(9):934-40.

13. Campbell J, Seymour JF, Matthews J, Wolf M, Stone J, Juneja S. The prognostic impact of bone marrow involvement in patients with diffuse large cell lymphoma varies according to the degree of infiltration and presence of discordant marrow involvement. Eur J Haematol. 2006 Jun;76(6):473-80.

14. Cheson BD, Horning SJ, Coiffier B, Shipp MA, Fisher RI, Connors JM, et al. Report of an international workshop to standardize response criteria for non-

Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol. 1999 Apr;17(4):1244.

15. Juneja SK, Wolf MM, Cooper IA. Value of bilateral bone marrow biopsy specimens in non-Hodgkin's lymphoma. J Clin Pathol. 1990 Aug;43(8):630-2.

16. Wang J, Weiss LM, Chang KL, Slovak ML, Gaal K, Forman SJ, et al. Diagnostic utility of bilateral bone marrow examination: significance of morphologic and ancillary technique study in malignancy. Cancer. 2002 Mar 1;94(5):1522-31.

17. Campbell JK, Matthews JP, Seymour JF, Wolf MM, Juneja SK. Optimum trephine length in the assessment of bone marrow involvement in patients with diffuse large cell lymphoma. Ann Oncol. 2003 Feb;14(2):273-6.

18. Chudgar U, Khanduri U. Intraobserver and interobserver reproducibility of the FAB classification in acute leukaemia. Indian J Pathol Microbiol. 1992 Jul;35(3):229-36.

19. Diebold J, Weisenburger D, MacLennan KA, Muller-Hermelink HK, Nathwani BN, Harris NL, et al. [Reproducibility and prognostic value of histopathological classifications of malignant lymphomas. Prolegomena for the 1st international classification proposed by WHO. Group of the non-Hodgkin's Malignant Lymphoma Classification Project]. Bull Acad Natl Med. 1998;182(7):1537-48; discussion 48-9.

20. Hanby AM, Hall PA, Rooney N, Dennis P, James P, Richman P, et al. An inter-observer and intra-observer variability study on the diagnosis of lymph node biopsy specimens. Eur J Cancer. 1992;28A(11):1858-62.

21. Talaulikar D, Dahlstrom JE, Shadbolt B, McNiven M, Broomfield A, Pidcock M. Occult bone marrow involvement in patients with diffuse large B-cell lymphoma: results of a pilot study. Pathology. 2007 Dec;39(6):580-5.

22. Talaulikar D, Shadbolt B, Bell J, Khan K, Dahlstrom JE, McDonald A, Pidcock ME, Broomfield A. Clinical role of flow cytometry in redefining bone marrow involvement in Diffuse Large B-cell Lymphoma (DLBCL) – a new perspective. Histopathology. 2008;52:340-7.

23. Talaulikar D, Dahlstrom JE, Shadbolt B, Broomfield A, McDonald A. Role of Immunohistochemistry in Staging Diffuse Large B-cell Lymphoma (DLBCL). J Histochem Cytochem. 2008 Oct;56(10):893-900.

24. Yan Y, Chan WC, Weisenburger DD, Anderson JR, Bast MA, Vose JM, et al. Clinical and prognostic significance of bone marrow involvement in patients with diffuse aggressive B-cell lymphoma. J Clin Oncol. 1995 Jun;13(6):1336-42.

25. Hanson CA, Kurtin PJ, Katzmann JA, Hoyer JD, Li CY, Hodnefield JM, et al. Immunophenotypic analysis of peripheral blood and bone marrow in the staging of B-cell malignant lymphoma. Blood. 1999 Dec 1;94(11):3889-96.

26. Naughton MJ, Hess JL, Zutter MM, Bartlett NL. Bone marrow staging in patients with non-Hodgkin's lymphoma: is flow cytometry a useful test? Cancer. 1998 Mar 15;82(6):1154-9.

27. Pittaluga S, Tierens A, Dodoo YL, Delabie J, De Wolf-Peeters C. How reliable is histologic examination of bone marrow trephine biopsy specimens for the staging of non-Hodgkin lymphoma? A study of hairy cell leukemia and mantle cell lymphoma involvement of the bone marrow trephine specimen by histologic, immunohistochemical, and polymerase chain reaction techniques. Am J Clin Pathol. 1999 Feb;111(2):179-84.

28. Stacchini A, Demurtas A, Godio L, Martini G, Antinoro V, Palestro G. Flow cytometry in the bone marrow staging of mature B-cell neoplasms. Cytometry B Clin Cytom. 2003 Jul;54(1):10-8.

29. Picker LJ, Weiss LM, Medeiros LJ, Wood GS, Warnke RA. Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol. 1987 Jul;128(1):181-201.

30. Teruya-Feldstein J, Chiao E, Filippa DA, Lin O, Comenzo R, Coleman M, et al. CD20-negative large-cell lymphoma with plasmablastic features: a clinically heterogenous spectrum in both HIV-positive and -negative patients. Ann Oncol. 2004 Nov;15(11):1673-9.

31. Chikatsu N, Kojima H, Suzukawa K, Shinagawa A, Nagasawa T, Ozawa H, et al. ALK+, CD30-, CD20- large B-cell lymphoma containing anaplastic lymphoma kinase (ALK) fused to clathrin heavy chain gene (CLTC). Mod Pathol. 2003 Aug;16(8):828-32.

32. Alvaro-Naranjo T, Jaen-Martinez J, Guma-Padro J, Bosch-Princep R, Salvado-Usach MT. CD20-negative DLBCL transformation after rituximab treatment in follicular lymphoma: a new case report and review of the literature. Ann Hematol. 2003 Sep;82(9):585-8.

33. Perea G, Altes A, Bellido M, Aventin A, Bordes R, Ayats R, et al. Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL). Histopathology. 2004 Sep;45(3):268-74.

34. Duggan PR, Easton D, Luider J, Auer IA. Bone marrow staging of patients with non-Hodgkin lymphoma by flow cytometry: correlation with morphology. Cancer. 2000 Feb 15;88(4):894-9.

35. Sah SP, Matutes E, Wotherspoon AC, Morilla R, Catovsky D. A comparison of flow cytometry, bone marrow biopsy, and bone marrow aspirates in the detection of lymphoid infiltration in B cell disorders. J Clin Pathol. 2003 Feb;56(2):129-32.

36. Dunphy CH. Combining morphology and flow cytometric immunophenotyping to evaluate bone marrow specimens for B-cell malignant neoplasms. Am J Clin Pathol. 1998 May;109(5):625-30.

37. Talaulikar D, Dahlstrom JE, Shadbolt B, McDonald A. Clinical Implications of Immunophenotyping in Staging Diffuse Large B-Cell Lymphoma. Blood (ASH Annual Meeting Abstracts) 2008;112: Abstract 5279.

38. Fraga M, Brousset P, Schlaifer D, Payen C, Robert A, Rubie H, et al. Bone marrow involvement in anaplastic large cell lymphoma. Immunohistochemical detection of minimal disease and its prognostic significance. Am J Clin Pathol. 1995 Jan;103(1):82-9.

39. Mazur G, Halon A, Wrobel T, Jelen M, Kuliczkowski K. Contribution of flow cytometric immunophenotyping and bone marrow trephine biopsy in the detection of lymphoid bone marrow infiltration in non-Hodgkin's lymphomas. Neoplasma. 2004;51(3):159-63.

40. Chetty R, Echezarreta G, Comley M, Gatter K. Immunohistochemistry in apparently normal bone marrow trephine specimens from patients with nodal follicular lymphoma. J Clin Pathol. 1995 Nov;48(11):1035-8.

41. Schwonzen M, Pohl C, Steinmetz T, Wickramanayake PD, Thiele J, Diehl V. Bone marrow involvement in non-Hodgkin's lymphoma: increased diagnostic sensitivity by combination of immunocytology, cytomorphology and trephine histology. Br J Haematol. 1992 Jul;81(3):362-9.

42. Miller RT, Swanson PE, Wick MR. Fixation and epitope retrieval in diagnostic immunohistochemistry: a concise review with practical considerations. Appl Immunohistochem Mol Morphol. 2000 Sep;8(3):228-35.

43. Leong AS. Pitfalls in diagnostic immunohistology. Adv Anat Pathol. 2004 Mar;11(2):86-93.

44. Naresh KN, Lampert I, Hasserjian R, Lykidis D, Elderfield K, Horncastle D, et al. Optimal processing of bone marrow trephine biopsy: the Hammersmith Protocol. J Clin Pathol. 2006 Sep;59(9):903-11.

45. Chadburn A, Knowles DM. Paraffin-resistant antigens detectable by antibodies L26 and polyclonal CD3 predict the B- or T-cell lineage of 95% of diffuse aggressive non-Hodgkin's lymphomas. Am J Clin Pathol. 1994 Sep;102(3):284-91.

46. Isaacson PG. Haematopathology practice: the commonest problems encountered in a consultation practice. Histopathology. 2007 Jun;50(7):821-34.

47. Taniguchi M, Oka K, Hiasa A, Yamaguchi M, Ohno T, Kita K, et al. De novo CD5+ diffuse large B-cell lymphomas express VH genes with somatic mutation. Blood. 1998 Feb 15;91(4):1145-51.

48. Harada S, Suzuki R, Uehira K, Yatabe Y, Kagami Y, Ogura M, et al. Molecular and immunological dissection of diffuse large B cell lymphoma: CD5+, and CD5- with CD10+ groups may constitute clinically relevant subtypes. Leukemia. 1999 Sep;13(9):1441-7.

49. Gervasi F, Lo Verso R, Giambanco C, Cardinale G, Tomaselli C, Pagnucco G. Flow cytometric immunophenotyping analysis of patterns of antigen expression in non-Hodgkin's B cell lymphoma in samples obtained from different anatomic sites. Ann N Y Acad Sci. 2004 Dec;1028:457-62.

50. Echeverri C, Fisher S, King D, Craig FE. Immunophenotypic variability of B-cell non-Hodgkin lymphoma: a retrospective study of cases analysed by flow cytometry. Am J Clin Pathol. 2002 Apr;117(4):615-20.

51. Trainor KJ, Brisco MJ, Story CJ, Morley AA. Monoclonality in B-lymphoproliferative disorders detected at the DNA level. Blood. 1990 Jun 1;75(11):2220-2.

52. Trainor KJ, Brisco MJ, Wan JH, Neoh S, Grist S, Morley AA. Gene rearrangement in B- and T-lymphoproliferative disease detected by the polymerase chain reaction. Blood. 1991 Jul 1;78(1):192-6.

53. Wan JH, Trainor KJ, Brisco MJ, Morley AA. Monoclonality in B cell lymphoma detected in paraffin wax embedded sections using the polymerase chain reaction. J Clin Pathol. 1990 Nov;43(11):888-90.

54. Achille A, Scarpa A, Montresor M, Scardoni M, Zamboni G, Chilosi M, et al. Routine application of polymerase chain reaction in the diagnosis of monoclonality of B-cell lymphoid proliferations. Diagn Mol Pathol. 1995 Mar;4(1):14-24.

55. Diss TC, Pan L. Polymerase chain reaction in the assessment of lymphomas. Cancer Surv. 1997;30:21-44.

56. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003 Dec;17(12):2257-317.

57. Coad JE, Olson DJ, Christensen DR, Lander TA, Chibbar R, McGlennen RC, et al. Correlation of PCR-detected clonal gene rearrangements with bone marrow morphology in patients with B-lineage lymphomas. Am J Surg Pathol. 1997 Sep;21(9):1047-56.

58. Crotty PL, Smith BR, Tallini G. Morphologic, immunophenotypic, and molecular evaluation of bone marrow involvement in non-Hodgkin's lymphoma. Diagn Mol Pathol. 1998 Apr;7(2):90-5.

59. Maes B, Achten R, Demunter A, Peeters B, Verhoef G, De Wolf-Peeters C. Evaluation of B cell lymphoid infiltrates in bone marrow biopsies by

morphology, immunohistochemistry, and molecular analysis. J Clin Pathol. 2000 Nov;53(11):835-40.

60. Kang YH, Park CJ, Seo EJ, Huh J, Kim SB, Kang YK, et al. Polymerase chain reaction-based diagnosis of bone marrow involvement in 170 cases of non-Hodgkin lymphoma. Cancer. 2002 Jun 15;94(12):3073-82.

61. Mitterbauer-Hohendanner G, Mannhalter C, Winkler K, Mitterbauer M, Skrabs C, Chott A, et al. Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL). Leukemia. 2004 Jun;18(6):1102-7.

62. Talaulikar D, Gray JX, Shadbolt B, McNiven M, Dahlstrom JE. A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsy specimens using two different methods. J Clin Pathol. 2008 Jan;61(1):119-23.

63. Provan AB, Hodges E, Smith AG, Smith JL. Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA. J Clin Pathol. 1992 Sep;45(9):763-5.

64. Talaulikar D, Shadbolt B, McNiven M, Dahlstrom JE. DNA amplification from formalin-fixed decalcified paraffin-embedded bone marrow trephine specimens – does the duration of storage matter? . Pathology. 2008;in press.

65. Nagasaka T, Lai R, Chen YY, Chen W, Arber DA, Chang KL, et al. The use of archival bone marrow specimens in detecting B-cell non-Hodgkin's lymphomas using polymerase chain reaction methods. Leuk Lymphoma. 2000 Jan;36(3-4):347-52.

66. Inghirami G, Szabolcs MJ, Yee HT, Corradini P, Cesarman E, Knowles DM. Detection of immunoglobulin gene rearrangement of B cell non-Hodgkin's lymphomas and leukemias in fresh, unfixed and formalin-fixed, paraffin-

embedded tissue by polymerase chain reaction. Lab Invest. 1993 Jun;68(6):746-57.

67. Lassmann S, Gerlach UV, Technau-Ihling K, Werner M, Fisch P. Application of BIOMED-2 primers in fixed and decalcified bone marrow biopsies: analysis of immunoglobulin H receptor rearrangements in B-cell non-Hodgkin's lymphomas. J Mol Diagn. 2005 Nov;7(5):582-91.

68. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000 Feb 3;403(6769):503-11.

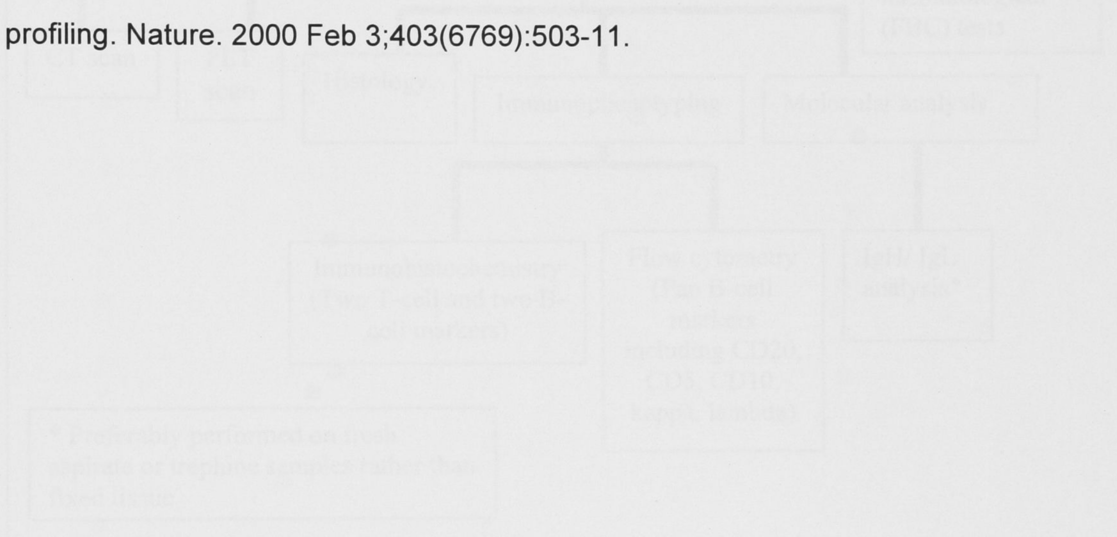
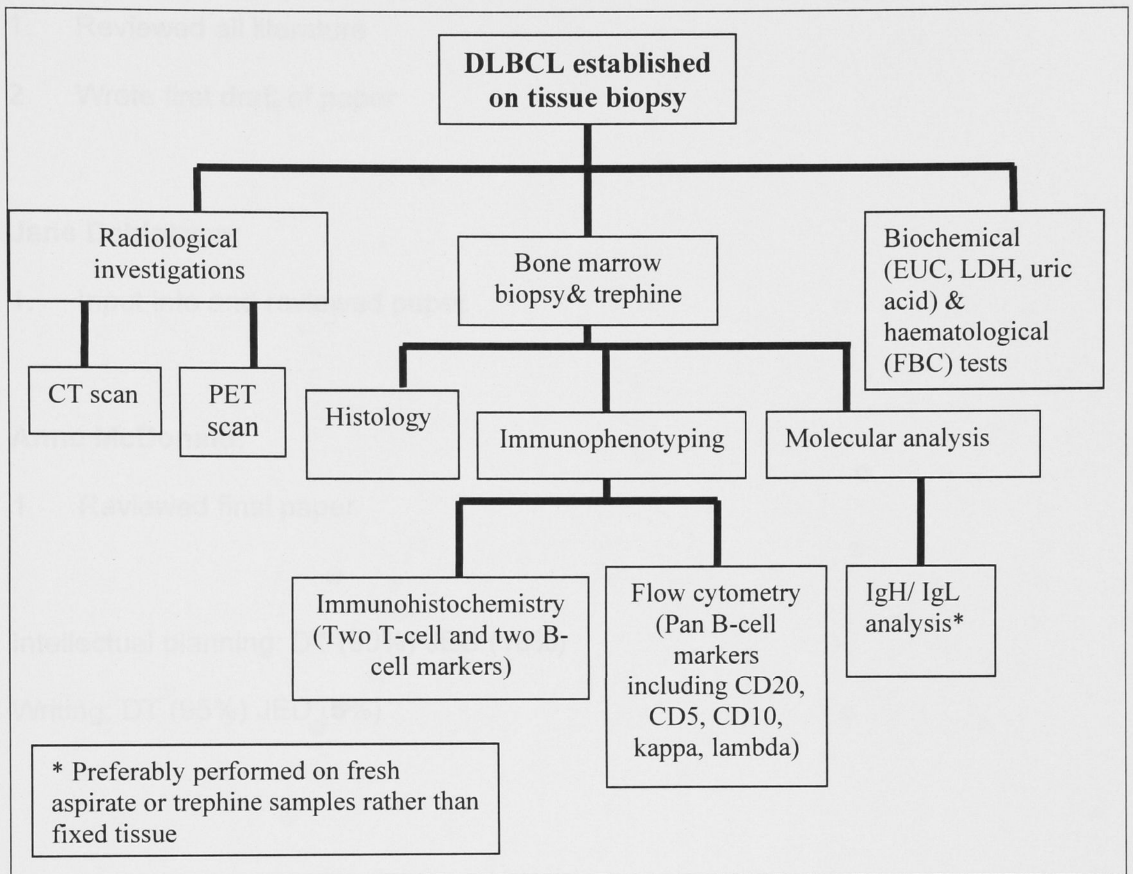


Figure I:

Flow diagram showing recommended investigations to be performed for staging in DLBCL



CT: Computerized Tomography; PET: Positron Emission Tomography; EUC: electrolyes, urea and creatinine; LDH: lactate dehydrogenase; FBC: full blood count; IgH: Immunoglobulin heavy chain; IgL: Immunoglobulin light chain

Author contributions:

Dipti Talaulikar:

1. Reviewed all literature
2. Wrote first draft of paper

Jane Dahlstrom:

1. Input into and reviewed paper

Anne McDonald:

1. Reviewed final paper

Intellectual planning: DT (90%) JED (10%)

Writing: DT (95%) JED (5%)

CHAPTER IX:

Conclusions and future directions

9.1 Conclusions:

Staging investigations in DLBCL contribute to the IPI and affect prognosis. This research aimed to determine the rates of histologically inapparent bone marrow involvement using flow cytometry, immunohistochemistry and gene rearrangement studies, and to determine the impact of using these tests on clinical outcomes. The important conclusions from the research are summarized below:

- Routine histology using Haematoxylin and Eosin staining detected lymphomatous involvement in 30/156 cases (19.2%). Rates of 11% to 27% are reported in literature.
- Trephine biopsies showed involvement more often than aspirate samples as previously reported in literature.
- Flow cytometry detected lymphomatous involvement ~10% of histologically negative cases. It also failed to detect lymphoma in 18 (11.5%) of histologically obvious cases suggesting that there are limitations with both histology and flow cytometry techniques related to sampling and/or technical issues.
- Flow cytometry was an independent prognostic marker of survival in DLBCL [Histology: 2.1, CI 1.0, 4.3, p=0.05; FL: 2.0, CI 1.0, 3.8, p=0.04].
- Immunohistochemistry was more sensitive than routine histology and detected bone marrow involvement in an additional ~10% cases. This was related to:

1) Examination of additional levels resulting in detection of lymphoid aggregates not apparent on routine histology preparations, and

2) Detection of scattered malignant cells which were difficult to visualize amongst normal haemopoietic tissue.

- As a prognostic marker, immunohistochemistry showed significant co-linearity with histology although it contributed more to predicting survival than routine histology [Histology: 1.3, CI 0.5, 3.0, p=0.6; IHC: 2.3, CI 1.1, 5.0, p = 0.03].
- Overall, immunophenotyping using flow cytometry and immunohistochemistry detected marrow involvement in ~20% of cases negative on routine histology. Use of these immunophenotyping techniques over and above routine histology improved prediction of overall survival [Histology: 1.8, CI 0.8, 3.7, p =0.1; FL/IHC: 2.7, CI 1.2, 6.2, p=0.02], suggesting that flow cytometry and immunohistochemistry should be performed as a routine in staging bone marrow at initial presentation in DLBCL.
- There are major limitations independent of sample age, to using archived formalin-fixed decalcified paraffin-embedded trephine biopsy tissue for molecular testing as the quality of DNA is poor especially at larger amplicons sizes.
- DNA amplification at lower fragment lengths was obtained in ~85% of formalin-fixed decalcified paraffin-embedded trephine samples in our study.
- In this retrospective study, gene rearrangement studies were performed on formalin-fixed decalcified paraffin-embedded trephine biopsy tissue due to lack of availability of archived fresh bone marrow aspirates. Clonal populations were detected in in 19% cases. However, concrete evidence of tumour origin was established only in 6.4% cases. Molecular testing using these stringent criteria detected bone marrow involvement in only ~5% histologically negative cases.
- This study was unable to demonstrate that molecular staging affects overall survival. This is likely to be technical limitations causing false negative results.

Previous studies have demonstrated the clinical role of molecular staging performed on bone marrow aspirates.

- Based on this research and previous studies, ancillary investigations i.e. flow cytometry, immunohistochemistry and molecular studies should be performed as a routine on all bone marrows at initial diagnosis, as the detection of occult disease in morphologically normal marrow has been shown to affect clinical outcome in DLBCL.
- The use of immunophenotyping with routine histology as staging investigations within the IPI resulted in a significant improvement in the predictive value of the IPI. In this study, molecular analysis did not further contribute in improving the prognostic significance of the IPI.

9.2. Staging investigations:

Laboratory investigations i.e. routine histology, flow cytometry, immunohistochemistry and gene rearrangement studies, used on staging bone marrow, have limitations related to technical issues and/ or sampling.

9.2.1. Histology:

Using routine histology, positivity on aspirate samples is particularly hard to define, especially when only a small number of atypical cells are present. In the absence of clear guidelines, we used a cut-off of at least 5% atypical cells to define marrow involvement on the aspirate. It may be debated that marrow involvement in lymphoma is seen more commonly on trephine samples than on aspirate samples, and that involvement on marrow aspirate in the absence of obvious involvement on the trephine should be ignored. For our first two experiments, we defined bone marrow involvement as positivity on aspirate

and/ or trephine biopsy. This was related partly to concerns regarding adequacy of sampling of trephine biopsies. Recommended trephine lengths of 16-20 mm with examination of at least 4 sections of the biopsy can be difficult to achieve in practice resulting in false negative results. The other concern with using trephine results alone was that flow cytometry is performed on aspirate samples and was expected to correlate better with aspirate results. However, we found only a small number of cases were positive on aspirate alone, and from experiment III onwards, we defined histological bone marrow involvement on trephine biopsy results alone.

The classification of lymphoid aggregates within the bone marrow as benign or malignant can be particularly difficult and may result in variability in reporting. The use of standardized reporting criteria in our study may have helped reduce the error rate. A literature search revealed that there is no data on rates on intra and inter-observer variability in reporting staging bone marrow in DLBCL or other sub-types of NHL. A project is currently underway in our institution to establish these rates in the two main sub-types of NHL, Follicular Lymphoma and Diffuse Large B-cell Lymphoma.

9.2.2. Flow cytometry:

Flow cytometry is a tidy, easily quantifiable test which appears to be less fallible. However, sampling issues may relate to dilution of aspirate samples with peripheral blood, resulting in false negative cases. False negative results may also result from the adherence of lymphoma cells to bony trabeculae, resulting in failure of the malignant cells to be aspirated. A major technical issue

that needs to be considered is the setting up of appropriate cut-offs for light chain ratios within individual laboratories.

9.2.3. Immunohistochemistry:

Immunohistochemical analysis is a relatively inexpensive, sensitive and readily available technique for detection of lymphomatous involvement in the bone marrow. Detection of small populations can be difficult, particularly as clonality can be difficult to demonstrate in some types of NHL. Additionally, there can be technical limitations with light chain analysis. Usually, B-cell or T-cell markers are relied upon to detect malignant populations. We chose to use CD20 in our study as all primary tumours expressed CD20. In CD20 negative tumours, alternative antibodies may be required. It must be remembered that normal bone marrow contains scattered B-cells and T-cells and also that lymphoid aggregates may be benign or malignant. Quantitation using morphometry can help determine if the number of lymphocytes are increased and cut-offs need to be established. Use of dual staining such as a B-cell marker with a light chain marker may be useful in some cases although these techniques may not be available at all centres. We used controls to overcome this problem for this study. We selected morphologically normal bone marrows from patients who had no known lymphoma and used B-cell and T-cell markers to obtain a visual impression of background normal lymphocyte populations.

We also attempted to use standardized criteria for differentiating benign from malignant lymphoid aggregates. There is a need for developing standardized and widely acceptable criteria for reporting immunohistochemical stains in lymphoma staging.

Issues such as validation of individual antibodies within laboratories, and the choice of the antibodies used can also affect results with immunohistochemistry. The processing of bone marrow during formalin-fixation, decalcification and paraffin-embedding can affect immunohistochemical stains; we found CD20 to be a much a stronger B-cell marker than CD79a, and believe this may be related to nitric acid decalcification as the application of these antibodies to lymph nodes produced similar intensity of staining.

9.2.4. Gene rearrangement studies:

Molecular testing for gene rearrangement analysis has been performed to detect lymphoma within the bone marrow since the 1980s. There is controversy as to whether molecular analysis should be performed on bone marrow aspirates or trephine biopsy samples. As lymphomatous detection in trephine biopsies is higher than in aspirates, it follows that molecular analysis on trephine biopsies is likely to yield more positive results than that performed on aspirate samples. However, aspirates are easy to obtain and additional samples for such testing do not pose greater inconvenience to the patient. Another option is to use formalin-fixed trephine biopsies for molecular analysis as we did. However, DNA amplified from such samples may be reduced or of poor quality, making such analysis difficult.

The sensitivity of gene rearrangement assays has been a major issue until recently. The development of standardized multiplex PCR reactions by the BIOMED2 European Consortium has increased the sensitivity of these assays markedly. However, these assays are not as well established on formalin-fixed

and decalcified tissues. We have been unable to demonstrate the utility of molecular staging on the IPI and believe this is primarily because of the limitations of using formalin-fixed rather than fresh tissue. Although the impact of molecular staging on survival in DLBCL has been demonstrated by other authors, further research is required to study the impact on the IPI.

The optimal method for processing trephine biopsies for molecular analysis remains unknown. So far, processing of such samples has been aimed at obtaining the best histological results rather than improved DNA yield from processed samples. Standardized protocols for processing samples for archival purposes such that best results are obtained on histology and molecular studies should be developed.

Larger multicentre prospective trials are now required to corroborate the findings of this research. This will allow some possible confounders such as chemotherapy regimen +/-Rituximab to be examined. It will also be possible to perform sub-group analysis to determine if there are differences in rates of occult marrow involvement in different histological subtypes of DLBCL. More importantly, it will allow gene expression profiling to be taken into account in the survival analyses. This was not considered feasible in our study due to lack of availability of fresh tissue. Fresh samples are now being tissue banked with patient consent for such studies in the future.

9.3. Future directions:

This thesis has identified that improvements in the predictive value of the IPI are possible using simple, easy to use investigations that are available in routine practice and can be performed in a timely manner i.e. usually within 48-72 hours to assist in clinical management. Some ancillary investigations have crept into clinical practice based on their availability rather than on evidence. The experiments performed during the course of the research highlight the importance of generating evidence for and reviewing the use of these tests in different situations including clinical scenarios. Several areas of future research that are likely to be highly relevant to clinical practice have been identified.

In the setting of a single diagnosis, it has identified that individual tests have technical limitations, and has raised questions on how best to define involvement on routine histology and immunohistochemistry. This includes the need for standardized reporting of histology and immunohistochemistry reducing rates of intra-observer and inter-observer variability. Guidelines such as those recently released by the **International Council for Standardization in Haematology (ICSH),** for standardization of bone marrow specimens and reports (Int J Lab Hematol, 2008 Oct; 30 (5):349-64) are the first step in this direction. There is also a need to develop a standardized method for quantifying stained cells on immunohistochemical analysis.

This study raises valid questions on whether molecular staging should be performed on bone marrow aspirates or trephine biopsy samples. Without compromising histological diagnosis, adequate fresh trephine biopsy samples may not be available for molecular analysis. This indicates that such analysis

may require to be performed on formalin-fixed samples. It highlights the need to develop standardized methods for processing samples to allow preservation of more than just histological detail. Optimal preservation of nucleic acid integrity will ensure archived samples remain a valuable resource or future generations. Improved and standardized methods of DNA extraction from formalin-fixed tissues are also required.

This thesis has identified the need for larger multi-centre prospective studies to study the impact of ancillary investigations on the IPI. This will allow the study of variables in diagnosis i.e. histological sub-type and gene expression profile, and treatment i.e. use of Rituximab that were beyond the scope of this study. In the future, this may translate into more robust prognostic systems which allow tailored therapies and improved outcomes in this heterogeneous disease.

Appendix:

Appendix I: Clinical information

A) PROFORMA FOR DATA RETRIEVAL FROM MEDICAL & LABORATORY RECORDS

DEMOGRAPHICS:

NAME:
AGE:
SEX:
UR NUMBER:
DATE DIAGNOSED:

CLINICAL FEATURES:

B SYMPTOMS:
HEPATOSPLENOMEGALY:
LYMPHADENOPATHY:
PERFORMANCE STATUS:

PERIPHERAL BLOOD:

FBC, DIFFERENTIAL, PLATELETS:
CREATININE:
ELECTROLYTES:
CALCIUM:
LDH:
LFT:
URIC ACID:
HIV TEST:
MONOSPOT:

BIOPSY:

TISSUE:
HISTOLOGY:
IMMUNOHISTOCHEMISTRY:
FLOW CYTOMETRY:
MOLECULAR STUDIES:
DATE:

BONE MARROW BIOPSY:

DATE:
ASPIRATE:
FLOW CYTOMETRY:
TREPHINE:
IMMUNOHISTOCHEMISTRY:
MOLECULAR STUDIES:

RADIOLOGICAL DIAGNOSIS:

CHEST X-RAY:
CT SCAN:
GALLIUM SCAN:

LUMBAR PUNCTURE:

INTERNATIONAL PROGNOSTIC INDEX (IPI):

AGE:
TUMOUR STAGE:
NUMBER OF EXTRANODAL SITES:
PERFORMANCE STATUS:
LDH:

OUTCOME FACTORS:

RESPONSE TO INITIAL THERAPY:
DISEASE FREE SURVIVAL:
RELAPSE:
DEATH:

B) ANN ARBOR STAGING IN LYMPHOMA:

STAGE I:	Limited to one lymph node group or extranodal site on one side of the diaphragm
STAGE II:	Spread to two lymph node groups, or one or more lymph node groups with/ without extranodal involvement, on the same side of the diaphragm
STAGE III:	Spread to two lymph node groups, with/without partial involvement of an extranodal organ or site above and below the diaphragm
STAGE IV:	Extensive or diffuse involvement in one organ or site, with/without involvement in distant lymph nodes
A: absent systemic symptoms **B:** presence of any of the following B symptoms: fever (greater than 101.5°), drenching night sweats, unexplained weight loss of 10% or more within the last 6 months	

Ref: Smithers DW. Summary of papers delivered at the conference on staging in Hodgkin's disease (Ann Arbor). Cancer Res. 1971 Nov; 32 (11): 1869-70

C) EASTERN COOPERATIVE ONCOLOGY GROUP (ECOG) PERFORMANCE SCORE:

0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited self care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self care. Totally confined to bed or chair
5	Dead

Ref: Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.

Appendix II: Cheson's standardised criteria for histology

Scoring	Histological characteristics
Positive	Unequivocal cytological or architectural evidence of involvement
Negative	No lymphoid aggregates or only few well-circumscribed aggregates
Indeterminate	Increased number or size of lymphoid aggregates without cytological or architectural atypia

Tabulated from: Cheson et al, Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group, J Clin Oncol, 1999

It is recommended that the percentage of involvement and lymphoma sub-type should also be reported to account for discordant involvement.

Appendix III: Proforma for reporting histology on bone marrow biopsy

X number	
Trephine Length	
Cellularity - Hypocellular Normocellular Hypercellular	
Aspirate- Involved (>5% atypical cells) Not involved	
Trephine- Involved Not involved Indeterminate	
Lymph aggregates- Number Size (small/medium/large) Location (Intertrabecular, paratrabecular)	
Reticulin- Normal Increased	
Comments-	

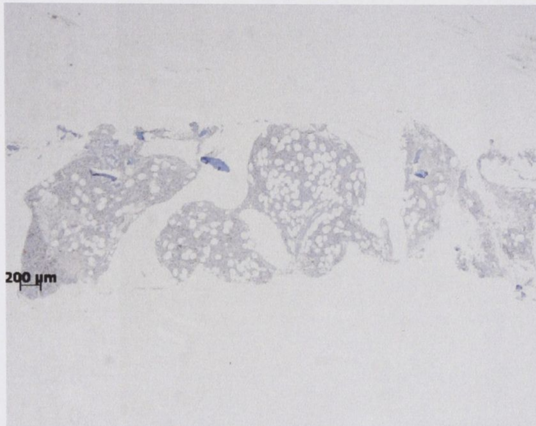
Appendix IV: Proforma for reporting immunohistochemistry

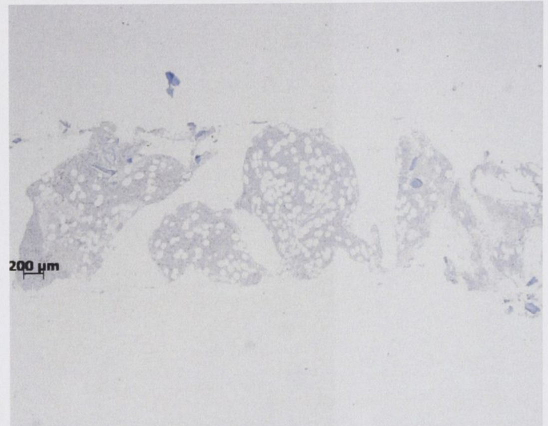
Antibody	CD3	CD45RO	CD20	CD79A	kappa	Lambda
Staining- Present Absent						
Cellular morphology- Normal Abnormal						
Staining (compared to controls)- Normal Increased						
Present as- Individual cells clusters						
% involvement						

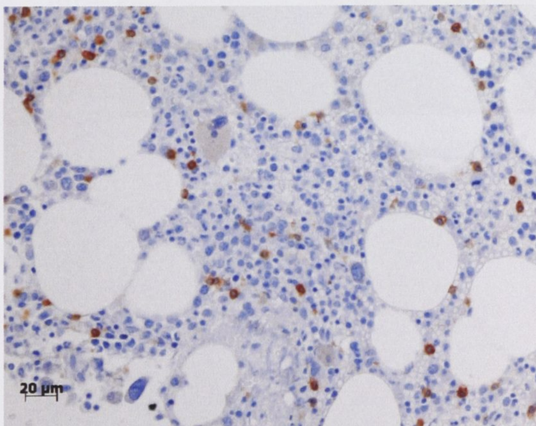
Appendix V: Pictures of control slides for immunohistochemistry

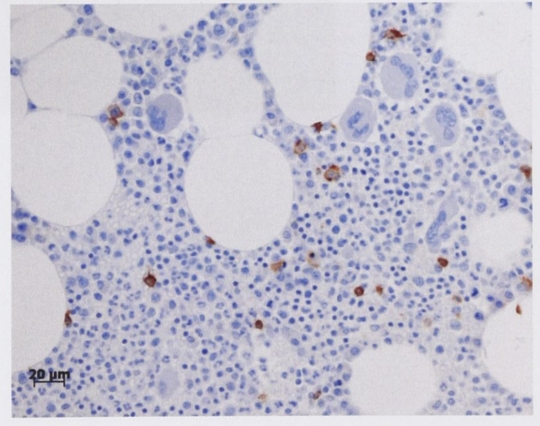
Immunohistochemical staining with CD3 (Panel A) and CD20 (Panel B) shows normal scattered T-cells and B-cells with normal cytological features

PANEL A: CD3 | **PANEL B: CD20**



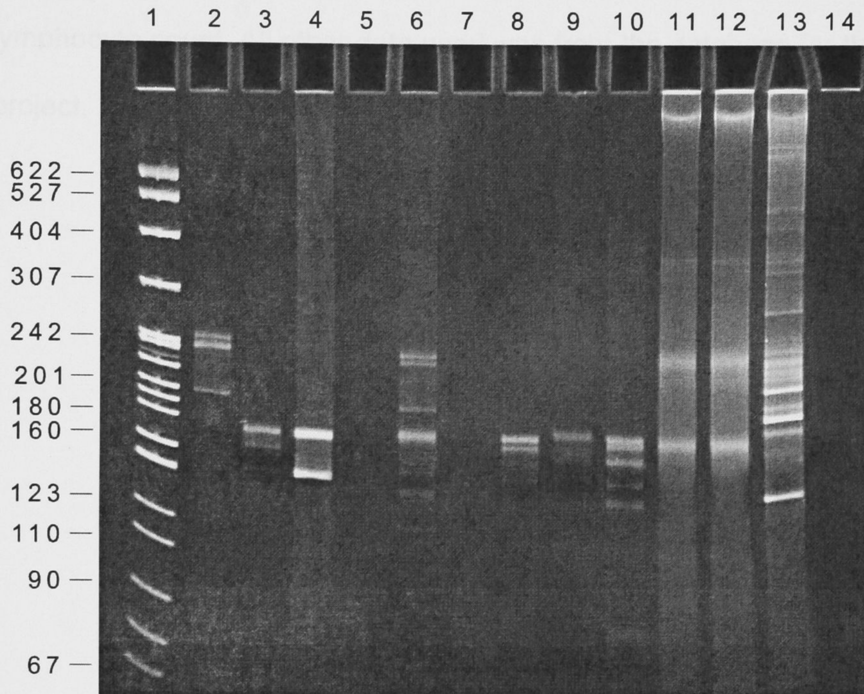






Appendix VI: Example of Kappa gene rearrangement:

This gel picture shows kappa gene rearrangement using heteroduplex analysis from the study. Lane 1: pBR-322-MspI control ladder; lanes 2-10: patient samples; Lane 11-12: polyclonal controls; lane 13: clonal control; lane 14: negative control.



Appendix VII: Additional research outcomes:

The following paper was an outcome of a research project performed by a medical student under the supervision of Dipti Talaulikar. The student, Ayesha Choudhury, performed data extraction from laboratory records for the absolute lymphocyte count. All other data used was from the database for the main project.

Leukemia & Lymphoma, May 2008; 49(5): 959–964

informa
healthcare

ORIGINAL ARTICLE: CLINICAL

Lymphocytopenia as a prognostic marker for diffuse large B cell lymphomas

DIPTI TALAULIKAR[1], AYESHA CHOUDHURY[2], BRUCE SHADBOLT[3], & MICHAEL BROWN[4]

[1]*Department of Haematology, ACT Pathology, The Canberra Hospital, Garran, Canberra 2605, Australia,* [2]*ANU Medical School, Australian National University, Canberra, Australia,* [3]*Department of Epidemiology, The Canberra Hospital, Canberra, Australia, and* [4]*Department of Anatomical Pathology, ACT Pathology The Canberra Hospital, Canberra, Australia*

(Received 19 December 2007; revised 31 January 2008; accepted 2 February 2008)

Downloaded By: [The Canberra Hospital] At: 21:10 15 August 2008

Abstract

Lymphocytopenia has been reported to confer adverse outcomes in a number of hematological malignancies. Recently, it has been reported to be associated with poor outcome in diffuse large B-cell lymphoma (DLBCL). The aim of this study was to determine the incidence of lymphocytopenia at diagnosis in patients with DLBCL, and to confirm its significance as a prognostic factor, particularly in relation to the international prognostic index (IPI). Medical and laboratory records of 165 patients diagnosed with DLBCL were retrieved and analysed. Lymphocyte counts were correlated with overall survival and role as a prognostic marker independent of IPI was determined. Lymphocytopenia (lymphocyte count $\leq 1 \times 10^9$/L) was noted in 35.8%; it correlated adversely with overall survival (3.4 years vs. 10.3 years, $p = 0.002$). A Cox regression model established that the prognostic significance was independent of the IPI.

Keywords: *Diffuse large B cell lymphoma, lymphocytopenia, international prognostic index, non-Hodgkin lymphoma, survival*

Introduction

Diffuse large B-cell lymphoma (DLBCL) accounts for ~30% of all newly diagnosed cases of Non-Hodgkin lymphoma (NHL) and for 80% of all aggressive lymphomas. The World Health Organization (WHO) classifies DLBCL as a heterogeneous entity, encompassing a number of morphologic variants, various molecular and genetic abnormalities, and variable clinical presentations and outcomes [1]. Overall, long-term clinical remissions are achieved in only 50–60% of patients, although improved survival rates are known in selected populations [2].

Despite an improved understanding of the diversity of DLBCL, the mainstay of therapy remains the CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) chemotherapy regimen [3]. The addition of rituximab, a CD20 monoclonal antibody, to the chemotherapy regimen [4], and dose-dense treatments [5], are recent modifications with demonstrated benefit.

The international prognostic index (IPI) is a standard clinical tool used to predict outcome for patients with aggressive NHL. The IPI estimates survival time from a number of negative prognostic markers present at the time of diagnosis. These markers include age greater than 60 years, stage III/IV disease, elevated lactate dehydrogenase (LDH) level, eastern cooperative oncology group (ECOG) performance status $\geq$2 and more than one extra nodal site of disease. This model creates four prognostic categories, low-risk, low-intermediate risk, high-intermediate risk and high risk, with 5-year overall survival (OS) ranging from 26 to 73% [6].

However, it is well recognized that there is considerable heterogeneity within the IPI groups, and gene expression analyses have been used to

Correspondence: Dr. D. Talaulikar, MBBS, FRACP, FRCPA, GradCertHE, Department of Haematology, The Canberra Hospital, P.O. Box 11, WODEN ACT 2606 Australia. Tel: +61-2-62442487. Fax: +61-2-62442892. E-mail: dipti.talaulikar@act.gov.au

ISSN 1042-8194 print/ISSN 1029-2403 online © 2008 Informa UK Ltd.
DOI: 10.1080/10428190801959026

determine subtypes of DLBCL based on cell of origin and outcome after therapy [2]. This technology, unfortunately, is not routinely available for clinical use, and an ongoing search continues for identifying reliable prognostic markers, with the hope of developing tailored treatment regimens to provide best care to individual patients. Indeed, a limited number of factors were considered in the large multi centre study that led to the development of IPI, and it is possible that readily accessible clinical and/or laboratory markers subsequently demonstrated to have prognostic significance could be potentially added to the IPI model to improve it further.

The presence of lymphocytopenia, or low absolute lymphocyte counts (ALCs), has been linked to adverse outcomes in a number of non-malignant conditions. Hawkins et al. have identified it as a factor that increases morbidity and mortality in sepsis [7]. Other studies have found the lymphocyte count to be effective as a prognostic marker in conditions such as Crohn's disease and acute heart failure [8,9]. More relevantly, studies have correlated lymphocytopenia persisting after conventional, or high dose chemotherapy (in the setting of peripheral blood stem cell transplantation) with poor outcome in several hematological malignancies [10,11]. Low lymphocyte counts at diagnosis have also been found to be an important prognostic marker in Follicular Lymphoma in terms of predicting overall survival and response to immunotherapy [12,13], and in Hodgkin lymphoma [14]. Kim et al. have recently reported that lymphocytopenia is predictive of response to chemotherapy and survival in DLBCL patients treated with CHOP or R-CHOP chemotherapy regimens. [15]

The aim of this study was to determine the incidence of lymphocytopenia in DLBCL, and to confirm its prognostic significance, particularly in relation to the IPI.

Materials and methods

Patients

One hundred and sixty-five patients diagnosed with DLBCL from 1985 to 2004 at The Canberra Hospital were recruited for the study. After approval was obtained from the Australian National University Human Research Ethics Committee, full blood count (FBC) data was retrospectively retrieved for each patient from laboratory records. Relevant clinical information, including performance status of patients, stage of disease, treatment and response was collected from Medical Records. The mean age of the patient cohort was found to be 61 years (range, 20–87 years) with a higher proportion of males (male:female = 1.62:1).

Of the 154 patients for whom staging data was available, 40 (25.9%) were recorded to have stage I disease at first diagnosis. Thirty six (23.4%) had stage II disease, 46 (29.9%) had stage III disease and 32 (20.8%) had stage IV disease, according to the Ann Arbor Staging system [16].

Of the 155 patients for whom the IPI could be calculated, 14 had an IPI of 0. Out of the remaining patients, 22 had an IPI score of 1, and 39 a score of 2. Fifty patients had a score of 3, 23 a score of 4 and 7 patients had an IPI score of 5.

Laboratory parameters

FBC results used in this study were the earliest ones available up to one month prior to performance of staging bone marrow (BM) biopsy at first diagnosis. Of the 165 patients recruited for the study, six patients had incomplete or absent FBC records; laboratory results of 159 patients were used in this study. The mean laboratory values of hemoglobin (Hb), absolute neutrophil count (ANC) and ALC were 128.2 g/L, 5.7×10^9/L and 1.4×10^9/L, respectively.

Treatment

Treatment data was available on 151 patients of which 10 patients were treated with palliative intent. The remaining 141 were treated with anthracycline-based chemotherapy regimens. Thirty-six patients received Mabthera whereas 115 did not.

Patient outcomes

Response data was available for all 151 patients. Overall, 117 patients (77.5%) achieved a complete remission (CR) after completion of the initial chemotherapy regimen. A further 19 patients (12.6%) achieved partial remission (PR) and the remaining 15 patients (9.9%) either had no response to the administered chemotherapy or demonstrated progression of disease while on therapy.

Seventy-three (48.3%) of those who had responded to therapy had a subsequent relapse. The overall median survival of the entire patient group was 6.3 years with a 95% confidence interval of 4.3 to 8.6 years (Figure 1).

Statistical analysis

Survival data were obtained for each patient and recorded. Data analysis was done using Statistical Package for Social Science version 14.0. Baseline comparisons of patients with lymphocyte counts $> 1.0 \times 10^9$/L, and those $\leq 1.0 \times 10^9$/L were done

Downloaded By: [The Canberra Hospital] At: 21:10 15 August 2008

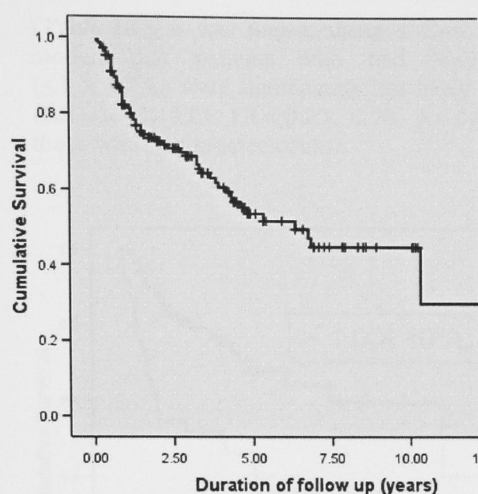


Figure 1. Median survival of the entire patient cohort was found to be 6.3 years (95% CI, 4.3–8.6 years).

for age, gender, Hb, LDH, ECOG performance status, tumour stage, curative chemotherapy, response to treatment, relapse, and death. Chi square and Mann–Whitney U tests were performed to determine significance depending on the measurement type. Kaplan–Meier curves were used to test the association between lymphocyte count at first diagnosis and survival rate. A stepwise multivariate Cox regression analysis was used to adjust for age, performance status and treatment, and to assess prognostic significance independent of the IPI. Patients treated with palliative intent were excluded from all survival analyses.

Results

Lymphocyte count

The mean lymphocyte count in the entire patient cohort (n =159) was determined to be 1.41 × 10^9 /L (range, 0.2 × 10^9 /L–5.4 × 10^9/L). The number of patients with a lymphocyte count less than or equal to 1.0 × 10^9/L was 57 (35.8%). The remaining patients (n = 102, 64%) had a lymphocyte count greater than 1.0 × 10^9/L. Table I shows the baseline patient characteristics of patients with ALCs equal to or < 1.0 × 10^9/L, and those > 1.0 × 10^9/L. There were no significant differences in age, gender, performance status, clinical stage or treatment intent between the two groups.

Lymphocyte counts and survival

Mean lymphocyte count in surviving patients was significantly higher than in those patients that did not survive (1.54 × 10^9/L vs. 1.24 × 10^9/L, $p = 0.01$). At a lymphocyte cut-off of 1 × 10^9/L, an obvious difference in survival rates was noted. Patients with levels above the designated cut-off had a better median survival time, in comparison with those who had lower counts (10.3 years vs. 3.4 years, $p = 0.002$) (Figure 2).

Table I. Baseline patient characteristics between low and normal lymphocyte counts.

Patient characteristics	Lymphocyte count ≤1.0 × 10^9/L (n = 57)	Lymphocyte count >1.0 × 10^9/L (n = 102)	p-Value
Age [median, (range), years]	61.9 (27.6–86.2)	62.6 (20.6–85.4)	0.6
Gender			
Male	28 (54.9%)	66 (67.3%)	0.2
Female	23 (45.1%)	32 (32.7%)	
Hemoglobin [median, (range), g/L]	118.5 (51–167)	137 (75–183)	<0.0001
Lactate dehydrogenase [median, (range), U/L]	712 (377–4195)	519 (258–3331)	<0.0001
ECOG			
0–1	12 (25%)	39 (41.9%)	0.1
2–5	36 (75%)	54 (58.1%)	
Tumour stage			
1–2	23 (46%)	50 (51.5%)	0.7
3–4	27 (54%)	47 (48.5%)	
Treatment			
Curative	43 (93.5%)	88 (92.6%)	0.9
Palliative	3 (6.5%)	7 (7.4%)	
Response			
Yes	29 (64.4%)	79 (82.3%)	0.06
No	16 (35.6%)	17 (17.7%)	
Relapse			
Yes	28 (65.1%)	40 (42.6%)	0.01
No	15 (34.9%)	54 (57.4%)	
Death			
Yes	31 (60.8%)	32 (33.7%)	0.002
No	20 (39.2%)	63 (66.3%)	

ECOG, eastern cooperative oncology group.

Multivariate analysis

Using a multivariate forward stepwise (likelihood ratio method) Cox regression, patients who had lymphocytes ≤1 × 10^9/L were significantly less likely to survive (Relative risk = 0.42, 95% CI = 0.23, 0.77, $p = 0.005$) than patients who had lymphocyte count >1 × 10^9/L. This result was noted after adjusting for the significant effects of age, ECOG, and clinical stage (Table II).

On examining the additional effect of lymphocyte count in predicting survival independent of the IPI

Downloaded By: [The Canberra Hospital] At: 21:10 15 August 2008

(Table III), it was found, using a Cox regression model, that patients who had lower counts ($\leq 1 \times 10^9$/L) were significantly less likely to survive (RR = 0.43, 95% CI = 0.23, 0.78, $p = 0.006$) than those who had greater counts.

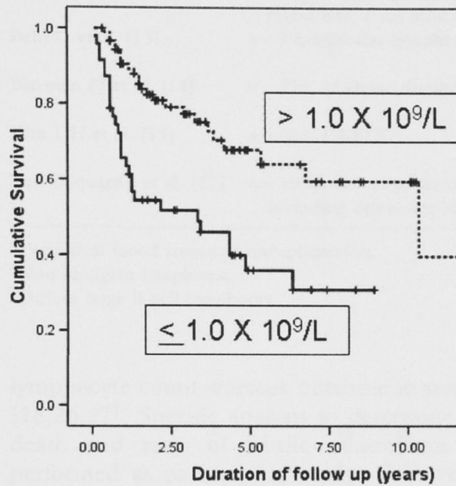


Figure 2. Patients with a lymphocyte count above 1.0×10^9/L had significantly prolonged overall survival than those with counts equal to or below 1.0×10^9/L (median survival of 10.3 years vs. 3.4 years, $p = 0.002$).

Table II. Cox regression model showing the effect of lymphocyte count on survival after adjusting for demographic, clinical and pathological indicators* ($n = 4$).

Significant variables	Hazards ratio (95% CI)	p-Value
Age (years)	1.03 (1.01, 1.06)	0.008
ECOG status (0–4)	1.32 (1.01, 1.73)	0.04
Clinical stage (1–4)	1.40 (1.05, 1.88)	0.02
Lymphocyte count ($\leq 1 \times 10^9$/L)	0.42 (0.23, 0.77)	0.005

ECOG, eastern cooperative oncology group.
*Mabthera was considered in the model, but did not significantly contribute after other parameters were included.

Table III. Cox regression model showing the effect of lymphocyte count on survival after adjusting for IPI.*

Significant variables	Hazards ratio (95% CI)	p-Value
IPI (0–5)	1.66 (1.29, 2.13)	< 0.0001
Lymphocyte count (< 1 × 10^9/L)	0.43 (0.23, 0.78)	0.006

IPI, international prognostic index.
*Age, performance status, and tumour stage were considered in the model, but did not significantly contribute after IPI was included.

Discussion

Conlan et al. established more than 15 years ago that abnormal hematological parameters are noted in more than 60% of patients with NHL. They found anaemia to be the most common abnormality and noted leucopenia in only 6% of their study population [18]. Several years later, early lymphocytopenia (at day 5) was reported in 41% of a retrospective patient population with high-grade NHL on chemotherapy [19]. The same French group, more recently, reported low leukocyte counts in 3% of their patient population of lymphoma and solid tumours, but found a higher incidence of low lymphocyte counts (22%). Another group has reported low lymphocyte counts in more than 50% of cases in a more specific patient population with Angioimmunoblastic T-cell Lymphoma [20]. In our study population, 35.8% of patients had low lymphocyte counts.

One reason for this reported variation between different studies may be related to differing definitions of lymphocytopenia, ranging from below normal reference range to 0.5×10^9/L (Table IV). Confounding factors such as surgical intervention and infections may also be expected to affect hematological parameters [21]. In our study, we used the earliest lymphocyte counts available, within a month before diagnosis, to account for patients having undergone major surgery to obtain tissue for histological diagnosis. Other confounders such as steroid therapy [22] and concurrent infections such as HIV [23,24] are known to influence lymphocyte counts. A further exploration of all known parameters may be warranted to standardize the best definition of lymphocytopenia at diagnosis in malignancies, so as to allow comparison of different studies.

Although a definitive link between lymphocytopenia and poor outcome has been established in several studies (Table IV), the causes for the poor outcomes remain speculative. Porrata et al. reported that early lymphocyte recovery after autologous stem cell transplantation predicted superior survival for cases with NHL and Multiple Myeloma, and postulated that early immune reconstitution may protect against disease progression from residual tumor cells [11]. Indeed, it is recognized, using both animal and clinical models, that cancer cells can cause immunosuppressive networks to develop during malignant progression via a number of mechanisms, including suppression of dendritic cells and T-cells by tumor-derived growth factors [25]. Additionally, the correlation of lymphocytopenia with chemotherapy-induced febrile neutropenia means that this could potentially be another mechanism through which low

Downloaded By: [The Canberra Hospital] At: 21:10 15 August 2008

Table IV. Published studies looking at the outcome of low-lymphocyte counts in lymphoma.

Study	Number of patients (n), diagnosis	Lymphocyte threshold used	Effect on outcome
Porrata LF et al. [11]	$n = 126$, lymphocyte recovery post PBSCT* for multiple myeloma/NHL†	0.5×10^9/L	Improved overall survival and progression free survival ($p < 0.0001$)
Siddiqui M et al. [12]	$n = 228$, lymphocyte recovery post-PBSCT for follicular lymphoma	1.0×10^9/L	Improved overall survival ($p < 0.0001$)
Behl D et al. [13]	$n = 79$, follicular lymphoma	0.89×10^9/L	Poor response to Rituximab and longer time to progression ($p < 0.0001$, < 0.009)
Bierman PJ et al. [14]	$n = 379$, Hodgkin disease	0.6×10^9/L	Poor event-free survival and overall survival post-PBSCT* ($p = 0.003$, 0.01)
Kim DH et al. [15]	$n = 223$, DLBCL‡	1.0×10^9/L	Poor overall survival and event-free survival ($p < 0.001$)
Ray-Coquard I et al. [17]	$n = 1051$, cancer patients including aggressive NHL	0.7×10^9/L, day 5 post-chemotherapy	Increased risk of early death post-chemotherapy ($p < 0.01$)

*Peripheral blood stem-cell transplantation.
†Non-Hodgkin lymphoma.
‡Diffuse large B-cell lymphoma.

lymphocyte count worsens outcome in malignancies [18,26,27]. Specific analysis to determine causes of death and rates of febrile neutropenia was not performed as part of this study. However, relapse data was available and showed that patients with lymphocytopenia were likely to relapse earlier than those without low lymphocyte counts (median relapse time of 2 years vs. 6.3 years, $p = 0.005$), suggesting that early relapses were one likely cause of poor survival in our patients. To what degree the incidence of febrile neutropenia would contribute to outcome in this specific group of patients remains unestablished.

Another potential mechanism for affecting overall survival may be response to Rituximab therapy. Rituximab is a chimaeric monoclonal antibody against CD20 [28], which is now considered standard of care in DLBCL [4]. The exact mechanisms of action of Rituximab are not established in in-vivo models, but in-vitro models have shown that the drug induces apoptosis, complement-mediated lysis, and antibody-mediated cellular cytotoxicity [29]. Behl et al. from the Mayo clinic have recently demonstrated the correlation between low lymphocyte counts at initiation of Rituximab therapy and therapeutic efficacy, based on the hypothesis that a competent host immune system would improve response rates to Rituximab [13]. Our study was a retrospective study with patients from as far back as 1985; only 36 patients in the cohort had received Rituximab. Hence, analysis to determine correlation between low lymphocyte counts and therapeutic efficacy to Rituximab therapy in DLBCL was not attempted.

In summary, this study shows that low lymphocyte counts can occur in up to one-third of patients with DLBCL at presentation. Furthermore, despite the patient population being small and the treatment received heterogeneous, we have confirmed lymphocytopenia at initial diagnosis as a new, independent prognostic factor in DLBCL. The results are of particular importance because it has been clearly shown that lymphocyte count has an additional effect in predicting survival over and above the IPI, and thus, may have the potential to improve the risk stratification model. Further studies are also required to determine correlation between low lymphocyte counts and efficacy to Rituximab in DLBCL, and to determine if specific lymphocyte subpopulations are associated with poor outcome.

References

1. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol 1999;17:3835–3849.
2. Coiffier B. State-of-the-art therapeutics: diffuse large B-cell lymphoma. J Clin Oncol 2005;23:6387–6393.
3. Fisher RI, Gaynor ER, Dahlberg S, Oken MM, Grogan TM, Mize EM, et al. Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med 1993;328:1002–1006.
4. Sehn LH, Donaldson J, Chhanabhai M, Fitzgerald C, Gill K, Klasa R, et al. Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. J Clin Oncol 2005;23:5027–5033.
5. Rueda A, Sabin P, Rifa J, Llanos M, Gomez-Codina J, Lobo F, et al. R-CHOP-14 in patients with diffuse large B-cell lymphoma younger than 70 years: a multicentre, prospective study. Hematol Oncol 2007, Sept 17, Epub ahead of print.

6. Shipp MA, Harrington, DP, Anderson, JR, Armitage JO, Bonadonna G, Brittinger G, et al. A predictive model for aggressive non-Hodgkin's lymphoma. The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med 1993;329:987–994.
7. Hawkins CA, Collignon P, Adams DN, Bowden FJ, Cook MC. Profound lymphopenia and bacteraemia. Intern Med J 2006;36:385–388.
8. Heimann TM, Bolnick K, Aufses AH Jr.Prognostic significance of severe preoperative lymphopenia in patients with Crohn's disease. Ann Surg 1986;203:132–135.
9. Rudiger A, Burckhardt OA, Harpes P, Muller SA, Follath F. The relative lymphocyte count on hospital admission is a risk factor for long-term mortality in patients with acute heart failure. Am J Emerg Med 2006;24:451–454.
10. Behl D, Porrata LF, Markovic SN, Letendre L, Pruthi RK, Hook CC, et al. Absolute lymphocyte count recovery after induction chemotherapy predicts superior survival in acute myelogenous leukemia. Leukemia 2006;20:29–34.
11. Porrata LF, Gertz MA, Inwards DJ, Litzow MR, Lacy MQ, Tefferi A, et al. Early lymphocyte recovery predicts superior survival after autologous hematopoietic stem cell transplantation in multiple myeloma or non-Hodgkin lymphoma. Blood 2001;98:579–585.
12. Siddiqui M, Ristow K, Markovic SN, Witzig TE, Habermann TM, Colgan JP, et al. Absolute lymphocyte count predicts overall survival in follicular lymphomas. Br J Haematol 2006;134:596–601.
13. Behl D, Ristow K, Markovic SN, Witzig TE, Habermann TM, Colgan JP, et al. Absolute lymphocyte count predicts therapeutic efficacy of rituximab therapy in follicular lymphomas. Br J Haematol 2007;137:409–415.
14. Bierman PJ, Lynch JC, Bociek RG, Whalen VL, Kessinger A, Vose JM, et al. The International Prognostic Factors Project score for advanced Hodgkin's disease is useful for predicting outcome of autologous hematopoietic stem cell transplantation. Ann Oncol 2002;13:1370–1377.
15. Kim DH, Baek JH, Chae YS, Kim YK, Kim HJ, Park YH, et al. Absolute lymphocyte counts predicts response to chemotherapy and survival in diffuse large B-cell lymphoma. Leukemia 2007;21:2227–2230.
16. Smithers DW. Summary of papers delivered at the Conference on Staging in Hodgkin's Disease (Ann Arbor). Cancer Res 1971;31:1869–1870.
17. Ray-Coquard I, Ghesquiere H, Bachelot T, Borg C, Biron P, Sebban C, et al. Identification of patients at risk for early death after conventional chemotherapy in solid tumours and lymphomas. Br J Cancer 2001;85:816–822.
18. Conlan MG, Bast M, Armitage JO, Weisenburger DD. Bone marrow involvement by non-Hodgkin's lymphoma: the clinical significance of morphologic discordance between the lymph node and bone marrow. Nebraska Lymphoma Study Group. J Clin Oncol 1990;8:1163–1172.
19. Blay JY, Chauvin F, Le Cesne A, Anglaret B, Bouhour D, Lasset C, et al. Early lymphopenia after cytotoxic chemotherapy as a risk factor for febrile neutropenia. J Clin Oncol 1996;14:636–643.
20. Lachenal F, Berger F, Ghesquieres H, Biron P, Hot A, Callet-Bauchu E, et al. Angioimmunoblastic T-cell lymphoma: clinical and laboratory features at diagnosis in 77 patients. Medicine (Baltimore) 2007;86:282–292.
21. Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. Crit Care Med 1996;24:1125–1128.
22. Fauci AS. Mechanisms of corticosteroid action on lymphocyte subpopulations. I. Redistribution of circulating T and b lymphocytes to the bone marrow. Immunology 1975;28:669–680.
23. Treacy M, Lai L, Costello C, Clark A. Peripheral blood and bone marrow abnormalities in patients with HIV related disease. Br J Haematol 1987;65:289–294.
24. Stebbing J, Sawleshwarkar S, Michailidis C, Jones R, Bower M, Mandalia S, et al. Assessment of the efficacy of total lymphocyte counts as predictors of AIDS defining infections in HIV-1 infected people. Postgrad Med J 2005;81:586–588.
25. Kim R, Emi M, Tanabe K, Arihiro K. Tumor-driven evolution of immunosuppressive networks during malignant progression. Cancer Res 2006;66:5527–5536.
26. Choi CW, Sung HJ, Park KH, Yoon SY, Kim SJ, Oh SC, et al. Early lymphopenia as a risk factor for chemotherapy-induced febrile neutropenia. Am J Hematol 2003;73:263–266.
27. Ray-Coquard I, Borg C, Bachelot T, Sebban C, Philip I, Clapisson G, et al. Baseline and early lymphopenia predict for the risk of febrile neutropenia after chemotherapy. Br J Cancer 2003;88:181–186.
28. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, et al. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 1994; 83:435–445.
29. Cartron G, Watier H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. Blood 2004;104:2635–2642.

Appendix VIII: Tabulation of results

Case Number	Histology (Trephine)	Flow Cytometry	[$]IHC	Molecular	Baseline [&]IPI	Revised IPI
1	[#]I	*NI	I	I	2	2
2	NI	NI	NI	NI	1	1
3	NI	I	NI	NI	3	4
4	NI	NI	NI	NI	2	2
5	NI	NI	NI	NI	3	3
6	NI	NI	I	NI	3	3
7	NI	NI	NI	NI	4	4
8	NI	NI	NI	NI	3	3
9	NI	NI	NI	NI	2	2
10	NI	NI	NI	NI	3	3
11	NI	NI	I	NI	2	3
12	NI	NI	I	NI	4	4
13	NI	I	NI	NI	4	4
14	I	NI	I	I	2	2
15	NI	NI	NI	NI	3	3
16	NI	NI	I	NI	3	3
17	NI	NI	NI	NI	1	1
18	NI	NI	NI	I	4	4
19	NI	NI	NI	NI	4	4
20	NI	NI	NI	I	2	2
21	I	NI	I	I	3	3
22	NI	NI	NI	NI	1	1
23	NI	NI	NI	NI	2	2
24	NI	NI	I	NI	3	3
25	NI	NI	NI	NI	1	1
26	NI	NI	NI	NI	1	1
27	I	I	I	I	4	4
28	NI	NI		I	1	1
29	NI	NI	NI	NI	4	4
30	I	NI	NI	NI	3	3
31	NI	NI	NI	I	1	1
32	I	NI	I	NI	2	2
33	NI	NI	NI	NI	3	3
34	NI	NI	NI	NI	1	1
35	NI	NI	NI	NI	2	2
36	I	NI	I	NI	3	3
37	I	I	I	NI	4	4
38	NI	NI	NI	NI	3	3
39	NI	NI	I	NI	3	4
40	I	I	I	NI	3	3
41	NI	NI	NI	NI	1	1
42	I	I	I	NI	3	3
43	NI	NI	NI	I	1	1
44	I	NI	I	I	3	3
45	NI	NI	NI	NI	4	4

Case Number	Histology (Trephine)	Flow Cytometry	[$]IHC	Molecular	Baseline [&]IPI	Revised IPI
46	NI	NI	NI	NI	1	1
47	NI	NI	NI	NI	3	3
48	NI	NI	NI	NI	2	2
49	NI	NI	I	I	2	3
50	NI	NI	NI	I	2	2
51	NI	NI	NI	NI	1	1
52	I	NI	I	NI	4	4
53	I	I	I	NI	3	3
54	NI	NI	NI	NI	2	2
55	NI	NI	NI	NI	3	3
56	NI	I	NI	NI	1	1
57	NI	NI	NI	NI	2	2
58	NI	NI	I	NI	3	4
59	I	NI	I	NI		
60	NI	NI	NI	NI	4	4
61	NI	NI	NI	I	2	2
62	NI	NI	NI	I	2	2
63	NI	NI	NI	NI	3	3
64	NI	I	NI	NI	3	4
65	NI	NI	I	I	3	3
66	NI	NI	NI	NI	1	1
67	NI	I	NI	NI	3	3
68	NI	I	NI	NI	3	4
69	NI	NI	NI	NI	4	4
70	NI	I	I	I	4	4
71	NI	I	I	NI	3	3
72	I	I	I	I	4	4
73	NI	NI	NI	I	3	3
74	NI	NI	NI	NI	2	2
75	NI	NI	NI	NI	1	1
76	NI	NI	NI	NI	1	1
77	NI	NI	NI	NI	2	2
78	I	I	I	I	4	4
79	NI	NI	NI	NI	1	1
80	NI	NI	NI	NI	2	2
81	I	I	I	I	3	3
82	I	NI	I	NI	3	3
83	NI	I	NI	I	2	3
84	NI	NI	NI	NI	1	1
85	NI	NI	NI	I	1	1
86	NI	NI	NI	I	1	2
87	NI	NI	NI	I	2	2
88	NI	NI	I	I	1	2
89	NI	NI	NI	NI	2	2
90	NI	NI	NI	NI	1	1
91	I	I	I	I	3	3
92	NI	NI	NI	NI	4	4
93	NI	NI	NI	NI	4	4
94	NI	NI	NI	NI	4	4

Case Number	Histology (Trephine)	Flow Cytometry	$IHC	Molecular	Baseline &IPI	Revised IPI
95	I	NI	I	NI	3	3
96	NI	NI	NI	I	3	3
97	I	NI	NI	NI		
98	NI	I	NI	NI	3	3
99	NI	NI	NI	I	1	2
100	NI	NI	NI	NI	3	3
101	NI	NI	NI	NI	4	4
102	I	NI	NI	I	2	3
103	I	I	I	I	4	4
104	NI	NI	NI	NI	2	2
105	NI	NI	NI	I	2	3
106	NI	NI	NI	NI	4	4
107	NI	NI		NI	3	3
108	NI	I	I		1	2
109	NI		NI	I	3	3
110	NI	I	NI	NI	1	1
111	NI	I	NI	NI	1	2
112	NI	NI	NI	NI		
113	NI	NI	NI	NI		
114	NI	NI	NI	NI	3	3
115	NI	NI	NI	NI	1	1
116	I	NI	NI	NI	2	2
117	I		I	NI	3	3
118	NI	NI	NI	NI	1	1
119	NI	NI	NI	NI	1	1
120	NI		I	NI	3	4
121	NI	NI	NI	NI		
122	NI	I	NI	I	2	3
123	NI	NI	NI	NI	2	3
124	NI	NI	I	NI	1	2
125	NI	NI	NI	NI	1	1
126	NI	NI	NI	NI	1	1
127	NI	I	NI	NI	3	3
128	NI	NI	NI	NI		
129	NI	NI	NI	I		
130	NI	NI	NI	NI		
131	NI	NI	NI	NI	2	2
132	NI	NI	NI	NI	3	3
133	NI	NI	NI	NI	2	2
134	I	NI	I	NI	4	4
135	NI	NI	NI	NI	1	1
136	NI	I	NI	NI	1	1
137	I	NI	I	NI	2	2
138	I		NI	I	2	2
139	NI	NI	NI	NI	4	4
140	NI	NI	NI	NI	3	3
141	NI	NI	NI	I	3	3
142	NI	NI	NI	I	4	4
143	I	NI	I	NI	2	2

Case Number	Histology (Trephine)	Flow Cytometry	[$]IHC	Molecular	Baseline [&]IPI	Revised IPI
144	NI	NI	NI	NI	3	3
145	NI	NI	NI	NI	2	2
146	NI	NI	NI	NI	4	4
147	NI	NI	NI	NI	1	1
148	NI	NI	I	NI	4	4
149	NI	NI	NI	NI	1	1
150	NI	NI	NI	I	3	3
151	NI	NI	NI	NI	1	1
152	NI	I	I	NI	4	4
153	NI	NI	NI	I	2	2
154	NI	NI	NI	I	4	4
155	NI	NI	NI	I	2	2
156	I	NI	I	NI	4	4
157						

*NI: not involved, [#]I: involved, [$]IHC: immunohistochemistry, [&]IPI: international prognostic index

Addendum

The new WHO classification of Tumours of haemopoietic and Lymphoid Tissues was released in late 2008 and is not included in the text of the thesis. This classification is more comprehensive than the one quoted.

In particular, the category of Diffuse Large B-cell Lymphoma has been expanded to include several other entities and sub-entities. An updated classification is listed and the individual entities described in this addendum.

World Health Organization classification of lymphoid neoplasms (2008).

A) B-cell neoplasms

*** PRECURSOR B-CELL NEOPLASM**

- Precursor B- acute lymphoblastic leukaemic/ lymphoma

*** MATURE (PERIPHERAL) B-CELL NEOPLASMS**

- Chronic lymphocytic leukaemia/ small lymphocytic lymphoma (CLL/ SLL)
- B-cell Prolymphocytic Leukaemia (PLL)
- Splenic marginal zone B-cell lymphoma
- Hairy cell leukaemia
- *Splenic B-cell lymphoma/ leukaemia, unclassifiable*
- *Splenic diffuse red pulp small B-cell lymphoma*
- *Hairy cell leukaemia-variant*
- Lymphoplasmacytic lymphoma
- Waldenstrom's macroglobulinaemia
- Heavy chain diseases
- Alpha heavy chain disease
- gamma heavy chain disease
- Mu heavy chain disease
- Plasma cell Myeloma
- Solitary plasmacytoma of bone
- Extraosseous plasmacytoma
- Extranodal marginal zone lymphoma of mucosa-associated lymphoma type (MALT lymphoma)
- Nodal marginal zone lymphoma
- *Paediatric nodal marginal zone lymphoma*
- Follicular lymphoma
- *Paediatric follicular lymphoma*
- Primary cutaneous follicle centre lymphoma
- Mantle cell lymphoma
-Diffuse large B-cell lymphoma (DLBCL), NOS
- T-cell/ histiocyte rich large B-cell lymphoma
- Primary DLBCL of the CNS
- Primary cutaneous DLBCL, leg type
- *EBV positive DLBCL of the elderly*
- DLBCL associated with chronic inflammation
- Lymphomatoid granulomatosis
- Primary mediastinal (thymic) large B-cell lymphoma
- Intravascular large B-cell lymphoma

- ALK positive large B-cell lymphoma
- Plasmablastic lymphoma
- Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
- Primary effusion lymphoma
- Burkitt lymphoma
- B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
- B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma

B) T and NK-cell neoplasms

*** PRECURSOR T-CELL NEOPLASM**

- Precursor T- acute lymphoblastic leukaemia/ lymphoma

*** MATURE T-CELL NEOPLASMS**

- T-cell prolymphocytic leukaemia
-T-cell granular lymphocytic leukaemia
- *Chronic lymphoproliferative disorder of NK-cells*
- Aggressive Nk-cell leukaemia
- Systemic EBV positive T-cell lymphoproliferative disorder of childhood
- Hydroa vacciniforme-like lymphoma
- Adult T-cell leukaemia/ lymphoma
- Extranodal NK/ T-cell lymphoma, nasal type
- Enteropathy type T-cell lymphoma
- Hepatosplenic T-cell lymphoma
- Subcutaneous panniculitis T-cell lymphoma
- Mycosis fungoides
- Sezary syndrome
- Primary cutaneous CD30 positive T-cell lymphoproliferative disorders
- Lymphomatoid papulosis
- Primary cutaneous anaplastic large cell lymphoma
- Primary cutaneous gamma-delta T-cell lymphoma
-*Primary cutaneous CD8 positive aggressive epidermotropic cytotoxic T-cell lymphoma*
- *Primary cutaneous CD4 positive small/medium T-cell lymphoma*
- Peripheral T-cell lymphoma, NOS
- Angioimmunoblastic T-cell lymphoma
-Anaplastic large cell lymphoma, ALK positive
- *Anaplastic large cell lymphoma, ALK negative*

C) Hodgkin Lymphoma (HL)

- Nodular Lymphocyte predominant HL
- Classical HL
- Nodular sclerosis classical Hodgkin lymphoma
- Lymphocyte-rich classical Hodgkin lymphoma
- Mixed cellularity classical Hodgkin lymphoma
- Lymphocyte-depleted classical Hodgkin lymphoma

Diffuse large B-cell lymphoma: variants, subgroups and subtypes/entities

1) Diffuse large B-cell lymphoma, NOS

This category includes those DLBCL cases that do not fit into any of the specific subtypes or disease entities mentioned in the classification and constitute ~23-30% of adult NHL cases in the western world. This category includes:

Common morphologic variants

Centroblastic –described in main text on page 27.
Immunoblastic – described in main text on page 27.
Anaplastic – described in main text on page 27.

Rare morphologic variants

Variations in morphology rarely noted within the category of DLBCL, NOS include presence of myxoid stroma, pseudo-rosette formation, spindle shaped malignant cells, presence of cytoplasmic granules, microvillous projections and intercellular junctions.

Molecular subgroups:

Germinal centre B-cell like – described in main text on page 66.
Activated B-cell like – described in main text on page 66.

Immunohistochemical sub-groups

CD5 positive DLBCL- CD5 +vity is seen in ~10% of DLBCL cases and although it may rarely arise from CLL/SLL, is more often seen in de-novo DLBCL.
Germinal centre B-cell like (GCB) – Include cases in which >30% of cells express CD10, as well as cases that are CD10-, Bcl6+ and IRF/MUM1- .
Non-germinal centre B-cell like (non-GCB) – includes those cases that fulfil the above criteria on immunophenotyping.

2) Diffuse large B-cell lymphoma subtypes

T-cell/ histiocyte rich large B-cell lymphoma – described in main text on page 27. Where previously it was included as a morphologic variant, in the new classification, it is listed as a separate sub-type.
Primary DLBCL of the CNS – includes all primary intracerebral and intraocular lymphomas and represents <1% of all NHL.
Primary cutaneous DLBCL, leg type – is a primary cutaneous DLBCL composed only of large transformed B-cells that arises most commonly in the leg. It occurs primarily in elderly women and constitutes ~20% of all primary cutaneous B-cell lymphomas.
EBV positive DLBCL of the elderly – occurs as an EBV positive clonal B-cell proliferation in patients older than 50 years of age in the absence of previous immunodeficiency or lymphoma.

3) Other lymphomas of large B-cells

Primary mediastinal (thymic) large B-cell lymphoma – described in main text on page 29.
Intravascular large B-cell lymphoma – is a rare subtype of DLBCL characterised by selective growth of malignant cells within the lumina of the smaller blood vessels. Lymph nodes are usually spared in this subtype.
DLBCL associated with chronic inflammation – is associated with EBV virus and occurs in the context of long-standing inflammation, usually in body cavities or narrow spaces. It generally occurs >10 years after onset of chronic inflammation.
Lymphomatoid granulomatosis – is an EBV-driven lymphoproliferative disease that occurs with greater frequency in patients with underlying immunodeficiency. It is an angiodestructive disorder that involves the lungs in >90% of cases. The tumour can be graded depending on the proportion of EBV positive cells compared to that of the background reactive lymphocytes; clinical aggressiveness depends on the grade of the tumour.
ALK positive large B-cell lymphoma – described in main text on page 31.
Plasmablastic lymphoma – described in main text on page 29.
Large B-cell lymphoma arising in human herpes virus (HHV)8-associated multicentric Castleman disease – occurs in HIV positive patients with HHV8-associated multicentric Castleman disease but can also occur in HIV negative cases where HHV8 is endemic. Although the HHV8 malignant cells resemble plasmablasts morphologically and carry Intracytoplasmic Ig, they differ from those in plasmablastic lymphoma in that they correspond to naïve B-cells that have not undergone somatic hypermutation.
Primary effusion lymphoma – is associated with kaposi sarcoma herpes virus (KSHV) and occurs in patients with underlying HIV and severe immunodeficiency. It presents as serous effusions without tumour masses and carries a poor prognosis.

4) Borderline cases

B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma – is not a distinct clinical entity, but a heterogeneous group of disorders that have features that are intermediate between DLBCL and BL.
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma – this category is used to describe cases that carry morphologic, immunophenotypic or clinical features that are intermediate between DLBCL and classical HL. They occur most commonly in the mediastinum but can also be seen in other areas.