Diffuse large B-cell lymphoma in a South African cohort with a high HIV prevalence: an analysis by cell-of-origin, Epstein-Barr virus infection and survival

by

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DECLARATION

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1. ABSTRACT

Introduction:

Diffuse large B-cell lymphoma, not otherwise specified (DLBCL NOS) is subdivided according to the cell-of-origin (COO) classification into germinal centre B-cell (GCB) and activated B-cell (ABC) subtypes, each with different molecular profiles and clinical behaviour. This study aims to describe the pattern of the COO subtypes, the proportion of Epstein-Barr virus (EBV) co-infection, and their influence on survival outcomes in a setting of high HIV prevalence.

Materials and Methods:

This retrospective cohort study included patients diagnosed with de novo DLBCL NOS at our tertiary academic centre in Cape Town, South Africa over a 14-year period. Immunohistochemical stains were performed for COO classification, according to the Hans algorithm. Tumour EBV co-infection was established by EBV-encoded ribonucleic acid in situ hybridisation (EBER-ISH) staining. The effect of the COO subtypes and EBV co-infection on overall survival were described by means of univariate, bivariate and multivariate analyses.

Results:

A total of 181 patients with DLBCL NOS were included, which comprised 131 HIV-uninfected and 50 HIV-infected patients. There was an equal distribution of GCB and ABC subtypes in the HIV-infected and HIV-uninfected groups. EBV co-infection was detected in 16% of the HIV-infected cases and in 7% of the HIV-uninfected cases (p=0.09). There was no significant difference in the incidence of EBV co-infection between the GCB and ABC subtypes (p=0.67). HIV-infected patients with CD4≥150 cells/mm^3 had similar survival to HIV-uninfected patients (p=0.005). Multivariate regression analysis showed that in the HIV-infected group with marked immunosuppression (CD4<150 cells/mm^3), there was significantly poorer overall survival compared to the HIV-uninfected group (HR 2.4, 95% CI 1.3–4.1). There were no statistically significant differences in overall survival by DLBCL COO subtype.

Conclusions:

There was no difference in the proportion of DLBCL COO subtypes, regardless of HIV status. EBV co-infection was more common in the HIV-infected group, but less than described in the literature. Unexpectedly, there were no significant differences in survival outcomes between the GCB and ABC subtypes. Higher CD4 counts in the HIV-infected

group had good survival outcomes, while lower CD4 counts predicted adverse survival outcomes. Further research is needed to explore the genetic mutational landscape of HIV-associated DLBCL.

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5. ABBREVIATIONS

ABC	Activated B-cell
Ag	Antigen
AID	Activation-induced cytidine deaminase
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
BART	BamHI-A rightward transcripts
BCL	B-cell chronic lymphocytic leukaemia/lymphoma
BCR	B-cell receptor
CARD11	Caspase recruitment domain-containing protein 11
CBM	CARD11/BCL10/MALT1 multi-protein complex
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
cDNA	Complementary deoxyribonucleic acid
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisone
CI	Confidence intervals
COO	Cell-of-origin
CSR	Class switch recombination
DEL	Double-expressor lymphoma
DHL	Double-hit lymphoma
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
EBER	Epstein-Barr virus-encoded small ribonucleic acid
EBER-ISH	Epstein-Barr virus-encoded ribonucleic acid in situ hybridisation
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EPOCH	Etoposide, prednisone, vincristine, doxorubicin and cyclophosphamide
FISH	Fluorescent in situ hybridisation
GC	Germinal centre
GCB	Germinal centre B-cell
GSH	Groote Schuur Hospital
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HR	Hazard ratios
IHC	Immunohistochemical

IL	Interleukin
IQR	Interquartile ranges
ITAM	Immunoreceptor tyrosine-based activation motif
LMP	Latent membrane protein
MUM1	Multiple myeloma oncogene 1
MYD88	Myeloid differentiation primary response 88
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NHLS	National Health Laboratory Service
NIH	National Institutes of Health
NOS	Not otherwise specified
NRF	National Research Foundation
OS	Overall survival
R-CHOP	Rituximab + cyclophosphamide, doxorubicin, vincristine, prednisone
SHM	Somatic hypermutation
THL	Triple-hit lymphoma
TLR	Toll-like receptor
TMA	Tissue microarray
UCT	University of Cape Town
VDJ	Variable, diversity, joining gene segments
WHO	World Health Organisation

6. CHAPTER 1:

Research Protocol

6.1. Title

Diffuse large B-cell lymphoma in a South African population – an analysis by subtype, HIV status, EBV infection and survival.

6.2. Purpose of the study

Primary aim:

- To correctly classify our population of patients with diffuse large B-cell lymphoma (DLBCL) by subtype.

Secondary aims:

- To identify the proportion of human immunodeficiency virus (HIV) positive DLBCL cases.
- To identify the proportion of Epstein-Barr virus (EBV) positive DLBCL cases.
- To compare survival outcomes in DLBCL of the South African population with that of international data.
- To gain insight into the pathobiology of DLBCL in HIV and inform further studies.
- To lay the foundation for further molecular investigation into the driver mutations present in the heterogeneous landscape of DLBCL through subtype classification.

6.3. Background

DLBCL is the most common type of lymphoid malignancy in adults worldwide,[1] accounting for approximately one third of all non-Hodgkin lymphomas in developed countries with an even greater proportion in developing countries.[2]

South Africa is home to the largest HIV epidemic in the world, with 19% of the global population of HIV-infected people.[3] In 2018, the total number of people living with HIV among the South African population was estimated at 7.52 million, with a prevalence of approximately 13.1%.[4]

The South African national antiretroviral treatment guidelines were implemented in 2004 to provide antiretroviral therapy (ART) for HIV-positive patients with a CD4 count of <200 cells/mm^3.[5] These guidelines have since undergone several amendments, becoming more inclusive with each revision.[5] The current 2018 guidelines include all HIV-positive patients, regardless of CD4 count or clinical staging, as being eligible for ART initiation.[5] Despite the changes in ART guidelines, lymphoma has remained a World Health Organisation (WHO) stage IV acquired immunodeficiency syndrome (AIDS)-defining illness and has therefore consistently fulfilled the ART eligibility criteria irrespective of CD4 count.[5]

With the advent of ART for HIV, the risk of developing AIDS-defining cancers has reduced.[6] However, despite the immune reconstitution afforded by ART, the incidence of lymphomas remains elevated.[6]

HIV plays both a direct as well as an indirect role in lymphomagenesis.[6] Direct mechanisms involve HIV-encoded proteins and HIV virions that act as crucial microenvironmental factors in promoting lymphoma development.[6] Indirect mechanisms include chronic B-cell activation, oncogenic viral infection e.g. EBV, cytokine overproduction, and genetic alterations.[6] HIV-associated DLBCL demonstrates a more frequent association with EBV when compared with DLBCL in immunocompetent patients.[6]

EBV infection has been well characterised to play a driving role in lymphomagenesis among immunocompromised hosts; whereas EBV acts rather as a co-factor among immunocompetent hosts.[7] Studies have shown that EBV is associated with 30-60% of HIV-related DLBCL cases, compared with 10% in HIV-unrelated DLBCL cases.[8]

The cell-of-origin classification defines two major subgroups of DLBCL that arise from different stages of normal B-cell differentiation, namely germinal centre B-cell (GCB) and activated B-cell (ABC).[9] This has been incorporated into the 2017 WHO classification of DLBCL, not otherwise specified (NOS) as two molecular subtypes.[2]

Hans *et al*.[10] published the first study that correlated subclassification of DLBCL by gene expression with that of protein expression. By using immunohistochemistry, the combined results of 3 immunostains (i.e. CD10, BCL6 and MUM1) could classify DLBCL into molecular subtypes – GCB and non-GCB.[10] Two recently published studies by Schmitz *et al*.[11] and Chapuy *et al*.[12] have identified genetic subtypes of DLBCL based on shared genomic aberrations. These subtypes differ with respect to genotype, phenotype, epigenetics, clinical characteristics and survival outcomes allowing for better understanding of the pathogenesis of DLBCL.[11-12]

The current standard of therapy for DLBCL comprises of combination immunochemotherapy using cyclophosphamide, doxorubicin, vincristine and prednisone plus the anti-CD20 monoclonal antibody rituximab (R-CHOP).[13] On this protocol, approximately 60% of patients with DLBCL are cured.[14] However, the other 40% of patients will relapse or develop refractory disease with the majority eventually succumbing to the disease.[14]

Patients with GCB DLBCL have better outcomes; with a 5-year overall survival rate of 60% and a relapse rate of around 20%.[13,14] Whereas ABC DLBCL is associated with the poorest prognosis and highest relapse rates of all DLBCLs; with a 5-year overall survival rate of only about 40%.[13]

DLBCL is a heterogeneous malignancy with incredible and continuing advancements being made in increasing our molecular and biological understanding of the disease. This offers a promising future for a change in therapeutic approach by the development of targeted therapeutic options which will ultimately improve overall treatment outcomes in these patients.

A multitude of studies have been conducted on DLBCL cases involving subtyping, various prognostic factors as well as advanced genomic sequencing techniques which have revealed many different somatic driver mutations that contribute to lymphomagenesis. However, most of these studies include patients from Western countries with very limited information available from other population groups, particularly in HIV. Furthermore, there

have been conflicting results regarding the role of molecular subtypes in predicting outcome in HIV-associated DLBCL. [15-18]

This study is a retrospective cohort study that aims to provide a South African perspective on DLBCL by correlating molecular subtype with HIV status, EBV infection as well as survival outcome.

The information gained from this study will enable further insight into the pathobiology of DLBCL, particularly in HIV. This will then lay the foundation and inform further studies into the investigation of known oncogenic driver mutations, as well as to identify possible new mutations in the heterogeneous landscape of DLBCL.

6.4. Methodology

- **Study Design**
    - A retrospective cohort study will be performed. Sequential patients with primary DLBCL, NOS diagnosed by qualified histopathologists, at the National Health Laboratory Service (NHLS) Division of Anatomical Pathology at Groote Schuur Hospital (GSH), from 1 January 2005 to 31 December 2018, will be included for retrospective review. Patients will be categorized by DLBCL subtype, HIV status and EBV infection; with additional testing performed on existing tissue specimens that are found to have missing predictor data (i.e. DLBCL subtype and EBV infection). Patients will be followed up from DLBCL diagnosis until death or last seen alive to determine survival outcomes.

- **Characteristics of the study population**
    - Inclusion criteria:
        - Adult (age ≥18 years) HIV-positive and HIV-negative patients.
        - Patients diagnosed with primary DLBCL, NOS.
        - Patients diagnosed from 1 January 2005 to 31 December 2018.
        - Patients diagnosed by qualified histopathologists, at the NHLS Division of Anatomical Pathology at GSH.
        - Cases with adequate stored tissue specimens available for additional testing.

        - Patients referred from referring hospitals (whose biopsy specimens were tested at the NHLS Division of Anatomical Pathology at GSH) who were subsequently treated at GSH Haematology Lymphoma Clinic, GSH Oncology Lymphoma Clinic, or University of Cape Town (UCT) Private Academic Hospital.
    - Exclusion criteria:
        - Minors (age <18 years).
        - Patients diagnosed with lymphomas other than primary DLBCL, NOS.
        - Patients diagnosed before 1 January 2005.
        - Patients diagnosed by training/unqualified histopathologists.
        - Cases with inadequate/no stored tissue specimens available for additional testing.
        - Patients referred from referring hospitals (whose biopsy specimens were tested at an external laboratory) with no biopsy specimens available at the NHLS Division of Anatomical Pathology at GSH.

- **Recruitment and enrolment**
    - Sequential study patients diagnosed with primary DLBCL, NOS and clinically followed up from 1 January 2005 to 31 December 2018 will be identified by the primary investigator at the NHLS Division of Haematopathology at GSH, using the existing bone marrow database; and at the Department of Medicine, Division of Haematology at GSH using the existing clinical database.
    - Study patients meeting the inclusion criteria (listed above) will be selected.

- **Research procedures and data collection methods**
    - Demographic characteristics and histology results will be obtained by the primary investigator from the NHLS results systems – DISA and TrakCare.
    - Existing/archived tissue specimens that were initially collected for clinical/diagnostic purposes and then stored will be obtained by an archivist, from the NHLS Division of Anatomical Pathology Repository (non-research) at GSH.
    - Tissue slides will be prepared by a qualified medical technologist, at the Division of Anatomical Pathology Research Laboratory at UCT.
    - Additional immunohistochemical (IHC) stains will be performed on the slides of the cases that are found to have missing data, by a qualified medical technologist, at the Division of Anatomical Pathology Research Laboratory at

UCT. These stains will then be interpreted by the co-investigator (qualified histopathologist).

- Currently, the DLBCL cases are not all categorised according to the WHO 2017 classification[2] and will require further IHC staining to classify them (i.e. CD10, BCL6, MUM1). The Hans algorithm (Fig. 1) will be used to correctly classify these patients as completely as possible. Note that 'non-GCB' refers to the ABC subtype.

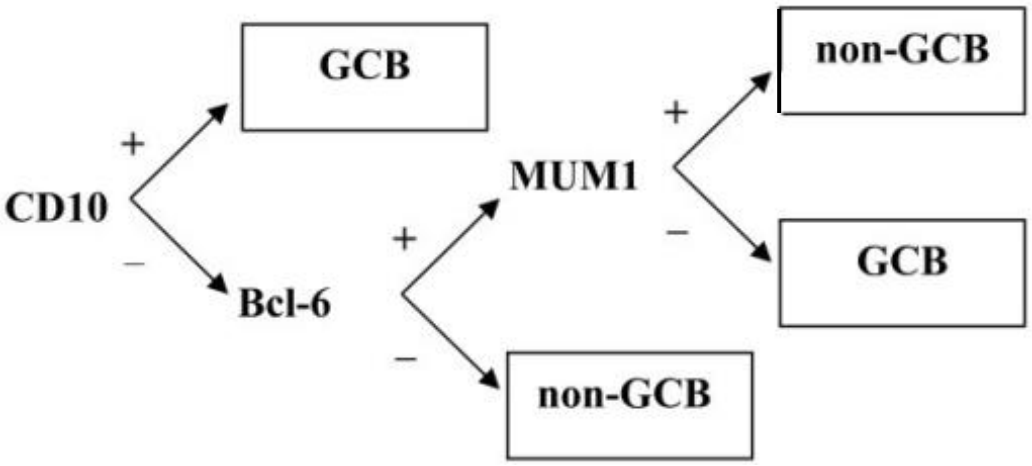

Fig. 1: Hans algorithm. Classifying DLBCL into GCB and non-GCB subtypes according to immunohistochemical (CD10, BCL6, MUM1) expression.[10]

- The EBV status has not been investigated on most of the DLBCL cases. An EBV-encoded ribonucleic acid in situ hybridisation (EBER-ISH) stain will be performed on the specimens by a qualified medical technologist, at the Division of Anatomical Pathology Research Laboratory at UCT. This stain will then be interpreted by the co-investigator (qualified histopathologist). These results will enable identification of the proportion of EBV-positive DLBCL cases.
- MYC and BCL2 co-expression has also not been investigated on the DLBCL cases to determine double-expressor profiles. The c-MYC and BCL2 IHC stains will be performed on the specimens by a qualified medical technologist, at the Division of Anatomical Pathology Research Laboratory at UCT. These stains will then be interpreted by the co-investigator (qualified histopathologist).
- Further information to be collected by the primary investigator on study patients will include:

        - Age and gender
        - HIV status and CD4 count
        - Ki67 proliferation index
        - Site of biopsy specimen
    - The outcome to be measured is survival. Patients will be followed up from DLBCL diagnosis until death or last seen alive to determine survival outcomes. This will be obtained from GSH medical records which will be verified on the GSH administrative database (Clinicom) and confirmed by the South African department of home affairs.

- **Sample size and power**

Table 1: Sample size and power

ABC (percentage)	GCB (percentage)	ABC (no. per group)	GCB (no. per group)	Total sample size
45%	50%	1198	2396	3594
40%	50%	294	587	881
35%	50%	129	258	387
30%	**50%**	**73**	**146**	**219**
25%	50%	46	92	138

    - A study of 219 DLBCL patients with one-third being ABC DLBCL (73 patients) and two-thirds being GCB DLBCL (146 patients) will provide 80% power with a two-tailed significance level of 0.05 (α=0.05, β=0.20) to detect a difference of 20% (i.e. 30% ABC and 50% GCB).

- **Data analysis**
    - Data will be collected and recorded into the REDCap database[19] which is secured behind the UCT firewall and is password protected.
    - Data analysis will be performed in STATA v14.0.[20]
    - Variables for analysis will include: gender, age, HIV status, CD4 count, primary biopsy site, DLBCL subtype, double expressor profile, Ki67 proliferation index and EBV infection. For an association between dichotomous and categorical variables, the Pearson's chi-squared test or Fisher's exact test will be used; and for continuous non-parametric variables, the Wilcoxon rank-sum test will be used.

- Survival analysis will be performed using the Kaplan-Meier method. Overall survival (OS) will be measured as the time from date of diagnosis until date of death regardless of cause or date last seen alive. Patients who are lost to follow-up will be censored at the last follow-up date. Kaplan-Meier survival curves will be restricted to display 5-year OS. The comparison between survival distributions will be determined by means of the log-rank test. A two-sided p-value of <0.05 will be considered statistically.
- A Cox proportional hazards model will be developed to assess the impact of prognostic factors on OS. Covariates selected for this model will be based on results of the univariate analysis.

6.5. Costs

The cost to perform the IHC stains. The number of patients needing each stain is likely to vary depending on what IHC stains were performed as part of routine care.

The total cost is estimated at R100 000.

6.6. Funding

- Fogarty International Center of the National Institutes of Health grants (D43-TW010345, D43-TW010543)
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  - Grant holder – Estelle Verburgh

6.7. Ethics

This protocol has been approved by the UCT Departmental Research Committee and the UCT Human Research Ethics Committee (approval number: 441/2018).

- **Description of risks and benefits**
    - This study involves no more than minimal risk (i.e. anonymous use of specimens).
    - There will not be direct involvement with human patients; there is no risk of discomfort or harm; and there will not be an additional informed consent process.
    - Initial informed consent was obtained at the time of specimen collection for clinical/diagnostic purposes but did not include permission to utilize stored unused specimens for future research. Specimens are stored in a repository created and operated for non-research purposes. However, the stored tissue specimens will be used anonymously after it has been obtained from the repository and the results will not place an individual, family or community at social, psychological or economic risk.
    - This study cannot practically be carried out without the waiver of the additional informed consent due to various factors such as: the number of patients estimated at 200; the 14-year time period of retrospective review; patient mortality; patients lost to follow-up; and patient demographic distribution across the country/continent.
    - The benefits will include improving our understanding of DLBCL which may ultimately lead to better management and therapeutic options, especially in HIV-positive patients.

- **Privacy and confidentiality**
    - The data will be collected and recorded into the UCT secured password protected REDCap database[19] using the personal computer of the primary investigator.
    - Computer-based records will only be available to the investigators involved in the study through the use of access privileges and passwords.
    - Paper-based records will be kept in a secure location (locked office) and will only be accessible to the investigators involved in the study.
    - Investigators will be required to sign statements agreeing to protect the security and confidentiality of identifiable information.
    - Personal identifiers will initially be required to obtain the stored specimens from the repository. However, these identifiers will be promptly deleted/destroyed from the data once the specimens are obtained. These specimens will then be utilized without identifiable details (anonymously) and the results will not be traced back to the individual.

6.8. What happens at the end of the study?

The findings of this study will be written up in the form on an article and will be submitted to a peer-reviewed medical journal for publication.

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7. CHAPTER 2:

Literature Review

7.1. OBJECTIVES

To obtain background information on the following:

- Introduction to diffuse large B-cell lymphoma
    - Epidemiology
    - Etiology
    - Morphology
    - Classification
    - Pathogenesis
- The NF-kB signalling pathway in DLBCL
- The role of EBV in lymphomagenesis
- Subtypes of DLBCL
    - Molecular subtypes – Hans algorithm
    - Genetic subtypes
- Double-/triple-hit and double-expressor lymphomas
- Tumour proliferation index – Ki-67
- DLBCL treatment and outcomes
- HIV and DLBCL
    - Lymphomagenic role of HIV
    - Survival outcomes in HIV-associated DLBCL
    - HIV in South Africa

7.2. SEARCH STRATEGY

This literature search was performed using the PubMed search engine accessing primarily the MEDLINE database maintained by the United States National Library of Medicine at the National Institutes of Health. Search terms used in the PubMed search included *diffuse large B-cell lymphoma* and one of each of the following: *epidemiology, aetiology/etiology, morphology, classification, pathogenesis, NF-kB pathway, EBV, molecular subtypes, Hans algorithm, genetic subtypes, double-hit, triple-hit, double expressor, tumour/tumor proliferation index, HIV, ART, South Africa, treatment, survival, outcomes*. The searches were limited to journal articles, reviews, guidelines and clinical trials; with full text availability; and publication dates between the years 2000 and 2019. Thousands of publications were identified, 307 were selected as relevant research publications, and 61 were ultimately used and referenced in this literature review.

Further information was obtained from the 2017 World Health Organisation classification of tumours of haematopoietic and lymphoid tissues, the UNAIDS South African factsheet, Statistics South Africa mid-year population estimates 2019, and the South African antiretroviral treatment guidelines.

The referencing style used is in accordance with the Pathology journal author guidelines (appendix 9.4).

7.3. SUMMARY OF LITERATURE

7.3.1. Introduction to diffuse large B-cell lymphoma

7.3.1.1. Epidemiology

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoid malignancy in adults worldwide,[1] accounting for approximately one third of all non-Hodgkin lymphomas in developed countries with an even greater proportion in developing countries.[2] DLBCL is an aggressive malignancy that demonstrates marked heterogeneity with regards to histopathology, biology, molecular and clinical features.[3] In the United States, African-American patients have a lower overall incidence of DLBCL, however present at younger ages with more advanced disease stages, when compared to Caucasian patients.[4] DLBCL in patients without an overt immunodeficiency show a varied prevalence of Epstein-Barr virus (EBV) infection ranging from 3% in western populations to 10% in Asian and Latin American populations.[2] Human immunodeficiency virus (HIV)-infected patients with DLBCL have been shown to present at significantly younger ages than their HIV-uninfected counterparts.[5]

7.3.1.2. Aetiology

DLBCLs can either be referred to as primary or secondary depending on their aetiology.[2] The cause of majority of DLBCLs is unknown and are therefore referred to as primary (i.e. tumours arising de novo).[2] The minority of DLBCL cases are then referred to as secondary (i.e. tumours transforming from a less aggressive lymphoma such as chronic lymphocytic leukaemia/small lymphocytic lymphoma, follicular lymphoma, marginal zone lymphoma, and nodular lymphocyte predominant Hodgkin lymphoma).[2]

7.3.1.3. Morphology

The definition of DLBCL according to the 2017 World Health Organisation (WHO) classification is – ‘a neoplasm of medium or large B lymphoid cells whose nuclei are the same size as, or larger than, those of normal macrophages, or more than twice the size of those of normal lymphocytes, with a diffuse growth pattern’.[2]

The morphology of DLBCL include three common variants (centroblastic, immunoblastic, anaplastic) as well as other rare variants.[2] The centroblastic variant is the most common

morphological variant characterised by medium to large lymphoid cells, oval to round nuclear outline, finely dispersed chromatin with 2-4 conspicuous nucleoli, and scanty basophilic/amphophilic cytoplasm (Fig. 1).[2] The immunoblastic variant consists of immunoblasts which are large lymphoid cells with a prominent central nucleolus and a moderate to abundant basophilic cytoplasm, as well as immunoblasts with plasmacytoid differentiation in some cases (Fig. 2).[2] The anaplastic variant is characterised by large to very large lymphoid cells with extensive nuclear pleomorphism or bizarre nuclei (Fig. 3).[2]

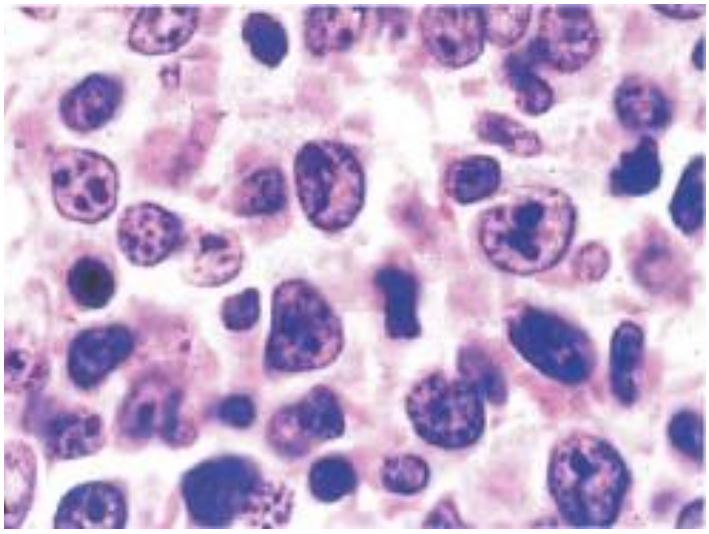

Fig. 1: Photomicrograph of DLBCL, centroblastic variant.[2]

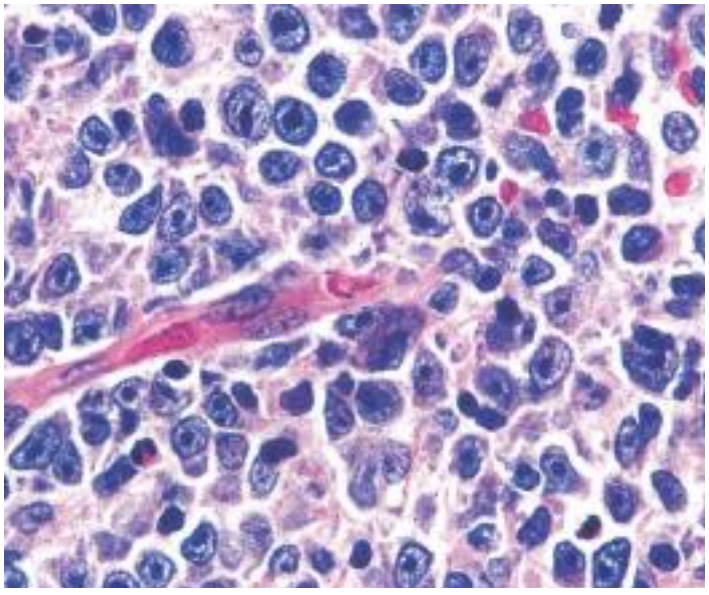

Fig. 2: Photomicrograph of DLBCL, immunoblastic variant.[2]

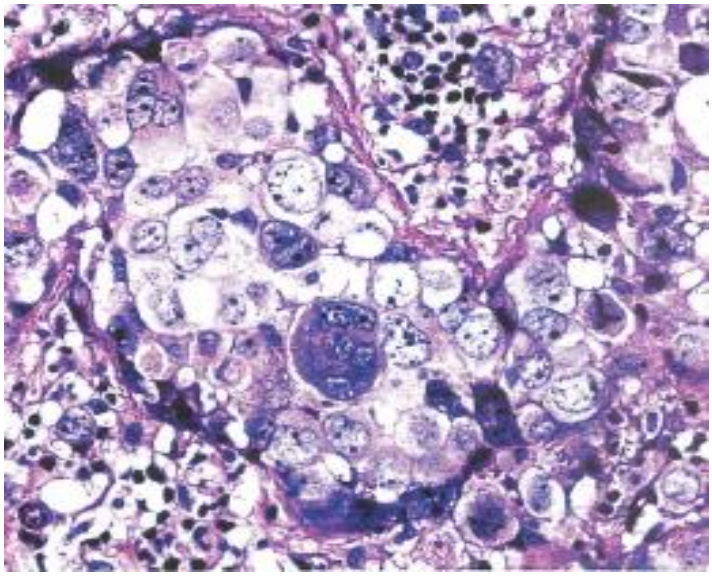

Fig. 3: Photomicrograph of DLBCL, anaplastic variant.[2]

7.3.1.4. Classification

The cell-of-origin (COO) classification, based on gene expression profiling studies, defines two major subgroups of DLBCL that arise from different stages of normal B-cell differentiation, namely germinal centre B-cell (GCB) and activated B-cell (ABC).[6] This has been incorporated into the 2017 WHO classification of DLBCL, not otherwise specified (NOS) as two molecular subtypes – GCB and ABC.[2]

7.3.1.5. Pathogenesis

DLBCL occurs due to the malignant transformation of mature B-cells that have undergone the germinal centre (GC) reaction (Fig. 4).[7] The GC forms when naïve B-cells are exposed to a foreign antigen (Ag); and consists of two distinct areas – the dark zone which is the site for clonal expansion; and the light zone which is the site for antibody affinity maturation.[8-9] The dark zone comprises of proliferating cells that undergo somatic hypermutation (SHM) which mutate the variable region of the immunoglobulin genes.[8-9] The light zone is composed of selected B-cells destined to become memory B-cells or plasma cells due to their high affinity for the Ag, which also experience class switch recombination (CSR).[8-9]

Modern genomic analysis of DLBCL has greatly increased our understanding of the pathogenesis of this disease.[7] A multitude of genomic alterations have been discovered that play a role in both tumour development and maintenance by disrupting normal biological

functions of B-cells.[7] These genomic lesions change the structure and/or the expression of proto-oncogenes and tumour suppressor genes which may then result in the constitutive activation of signal transduction pathways, differentiation block, immune surveillance escape, and epigenetic remodelling.[7]

The mechanisms involved in the pathogenesis of DLBCL include two mechanisms that occur in most tumours (i.e. somatically acquired point mutations and gene copy number changes);[7] as well as a further two mechanisms that occur in B-lymphocytes (i.e. chromosomal translocations and aberrant SHM).[10-11] DLBCL-associated chromosomal translocations occur due to errors in variable, diversity, joining gene segments (VDJ) recombination, SHM and CSR;[11] which leads to constitutive or ectopic activation of proto-oncogenes in the lymphoma cells.[7] Aberrant SHM results in the accumulation of multiple mutations at the normally unmutated 5' end of the deoxyribonucleic acid (DNA) sequence,[10] which then cause structural and functional alterations in various proto-oncogenes and tumour suppressor genes.[7]

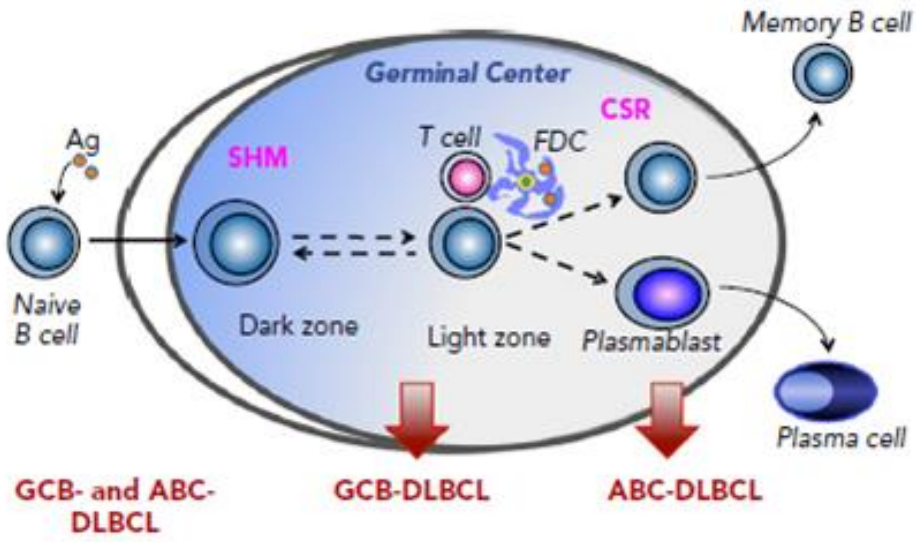

Fig. 4: Cellular origin of DLBCL subtypes. Schematic representation of the GC reaction and its association with GCB DLBCL and ABC DLBCL.[7]

7.3.2. The NF-κB signalling pathway in DLBCL

The nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signalling pathway is transiently activated during the normal B-cell response to antigens resulting in cellular proliferation and suppression of apoptosis.[12] This pathway plays an important role in lymphomagenesis as genetic aberrations result in constitutive activation of NF-κB signalling.[12]

ABC DLBCL preferentially expresses NF-κB target genes when compared with GCB DLBCL.[12] The ABC subgroup thus depends on constitutive NF-κB signalling whereas the GCB subgroup does not.[13] The characteristic constitutive activation of the NF-κB pathway in ABC DLBCL occurs via B-cell receptor (BCR) and/or toll-like receptor (TLR) signalling pathways.[14] This may occur through various somatically acquired mutations such as the well described mutations in *CARD11*, *MYD88*, *CD79A* and *CD79B ITAMs* (Fig. 5).[15]

Caspase recruitment domain-containing protein 11 (*CARD11*) mutations are observed in approximately 10% of ABC DLBCL cases,[16] of unknown HIV/EBV status. *CARD11* is a cytoplasmic scaffolding protein, composed of multiple modular domains, which serves as a signalling adapter.[17-18] This protein forms part of the *CARD11/BCL10/MALT1* (CBM) multi-protein complex which, upon Ag receptor stimulation, activates BCR signalling and ultimately NF-κB activation.[16] Mutations in *CARD11* are gain-of-function mutations that result in chronic active BCR signalling and NF-κB pathway activation.[16-18]

Myeloid differentiation primary response 88 (*MYD88*) mutations are the most frequently encountered genetic alterations, occurring in about 30-40% of patients with ABC DLBCL,[2] of unknown HIV/EBV status. *MYD88* is an adaptor protein that mediates TLR receptor signalling as part of the innate immune response, which then induces NF-κB pathway activation.[16] Somatic gain-of-function *MYD88* mutations cause inappropriate TLR signalling which result in constitutional NF-κB activation.[18]

Cluster of differentiation 79A (*CD79A*) and cluster of differentiation 79B (*CD79B*) immunoreceptor tyrosine-based activation motifs (*ITAM*s) mutations are demonstrated in approximately 20% of ABC DLBCL cases, of unknown HIV/EBV status, with *CD79B* mutations being of higher frequency.[18] *CD79A* and *CD79B* are transmembrane proteins, which are subunits of the B-cell receptor.[2] These two associated proteins serve as a scaffold for the assembly and membrane expression of the BCR, and initiate downstream signalling

to the NF-κB pathway.[13] Crucially involved in this process are two highly conserved tyrosine residues in a conserved stretch of amino acids within the ITAM, which are present in the cytoplasmic tails of both *CD79A* and *CD79B*.[18] Gain-of-function mutations in the *CD79A* and *CD79B ITAMs* thus increase surface BCR expression resulting in chronic active BCR signalling and activation of the NF-κB pathway.[19-20]

Due to the differences in oncogenic driver mutations and subtype specificity, this allows for exciting pharmacological prospects by means of NF-κB pathway inhibitors for ABC DLBCL.

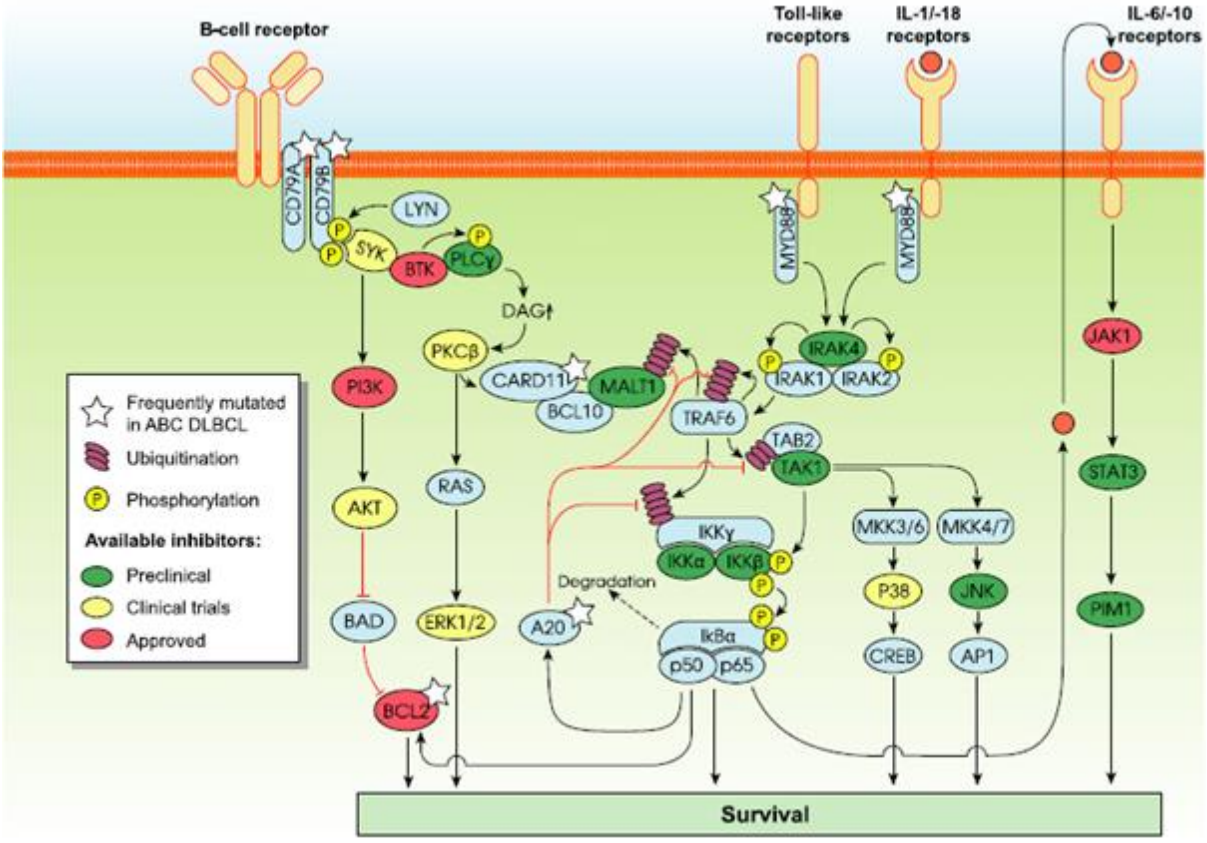

Fig. 5: NF-κB signalling pathways in ABC DLBCL. Schematic representation of the various pathways involved in NF-κB activation leading to the development of ABC DLBCL.[18]

7.3.3. The role of EBV in lymphomagenesis

EBV is a gamma human herpes virus that infects the majority of the world's population.[21]

During primary infection, EBV preferentially infects B-lymphocytes by binding onto the CD21 surface receptor and human leucocyte antigen (HLA) class II co-receptor.[21] Upon entering the host cell, the linear viral genome becomes circular and then persists as an episome.[22] Initially, infected B-cells are controlled by natural killer cells and cytotoxic T-cells; but this response does not destroy all of the infected cells.[22] Thus, leaving a pool of latent EBV-infected memory B-cells that perpetuates within the host (Fig. 6).[22]

EBV normally expresses about 100 viral genes.[21] However, latent EBV-infected B-cells limit the number of genes expressed to nine latent viral proteins in variable patterns thereby evading the host's immune recognition.[21] The nine viral proteins include: three membrane proteins (i.e. latent membrane protein (LMP)-1, -2a and -2b); and six nuclear antigens (i.e. Epstein-Barr nuclear antigen (EBNA)-1, -2, -3a, -3b, -3c and -LP).[21] Epstein-Barr virus-encoded small ribonucleic acid (EBER)-1 and EBER-2, along with BamHI-A rightward transcripts (BART) are also expressed.[21]

LMP-1 is the main transforming protein of EBV as it acts as an oncogene.[22] It induces B-cell activation changes such as cell clumping, cell adhesion and upregulation of CD23, CD39, CD40 and CD44.[22] It also increases the expression of anti-apoptotic proteins BCL-2 and A20, as well as induces cytokine production of interleukin (IL)-6 and IL-8.[22] As a member of the tumour necrosis factor receptor superfamily, LMP-1 is constitutively activated and is able to function as a ligand-independent signalling pathway activator.[22] LMP-1 structurally mimics the CD40 ligand and is therefore able to bind onto the CD40 receptor that is usually located on the cell surface of B-cells, thereby inducing growth and differentiation.[22] It is also able to activate the NF-κB signalling pathway.[22] LMP-2 maintains latency of the infection by preventing reactivation of the virus.[22]

EBNA-1 is a nuclear phosphoprotein that binds viral DNA and is responsible for maintaining the EBV genome as an episome within the host B-cell.[22] EBNA-2 is a viral transcription factor that stimulates the expression of LMP-1, LMP-2 and other cellular proteins involved in B-cell growth and transformation.[22] EBNA-3s also increase the expression of cellular proteins.[22]

EBERs and BART do not encode proteins however may play a role in viral oncogenesis and suppression of apoptosis.[22-23]

The switch from latent to lytic EBV-infection is dependent upon the activation of two genes, *BZLF1* and *BRLF1*, both of which encode for transcription activators that induce transcription of the lytic viral genes.[22] During the lytic cycle of infection, viral genes are expressed that encode for proteins responsible for viral genome replication and particle synthesis.[22]

EBV infection has been well characterised in immunocompromised hosts as it plays a driving role in lymphomagenesis; whereas among immunocompetent hosts, EBV acts as a co-factor rather than the driving influence.[22] Studies have shown that EBV is associated with 30-60% of HIV-related DLBCL cases, compared with 10% in HIV-unrelated DLBCL cases.[24]

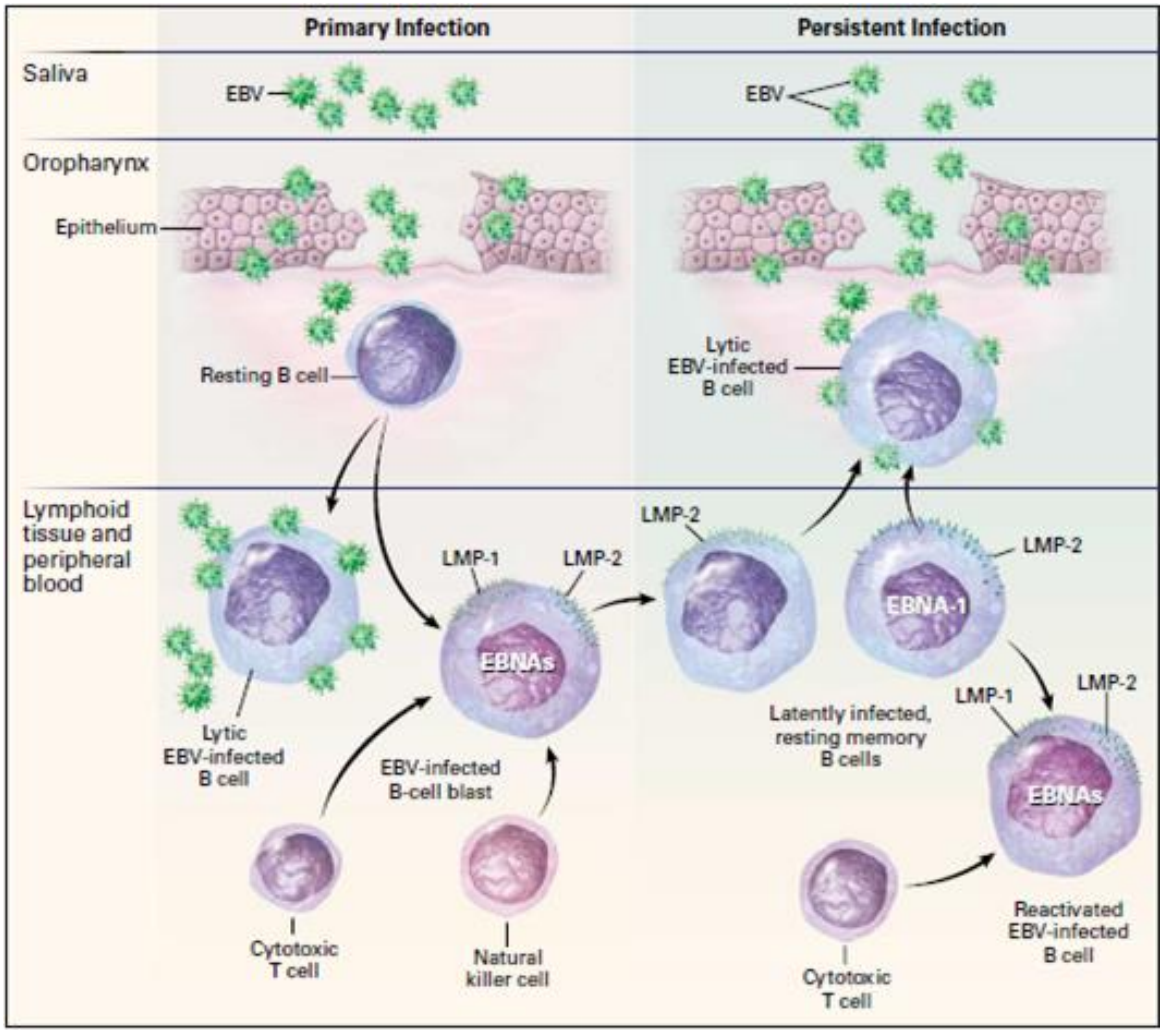

Fig. 6: EBV infection and persistence within the host. Schematic representation of EBV infection in the oropharynx. During primary infection, EBV infects resting B-lymphocytes resulting in lytic infection with viral production or latent viral protein expression. Infected B-cells are controlled by cytotoxic T-cells and natural killer cells. EBV perpetuates in latently infected memory B-cells which express limited latent viral proteins. These cells can be reactivated by EBV with the expression of other latent viral proteins, leading to elimination by cytotoxic T-cells. Latently infected B-cells may also develop into lytic infection with production and shedding of the virus.[25]

7.3.4. Subtypes of DLBCL

7.3.4.1. Molecular subtypes – Hans algorithm

Hans *et al*. published the first study that correlated subclassification of DLBCL by gene expression with that of protein expression.[26] By using immunohistochemistry, the combined results of 3 immunostains, that is, CD10, B-cell chronic lymphocytic leukaemia/lymphoma (BCL) 6 and multiple myeloma oncogene 1 (MUM1) could classify DLBCL into GCB and non-GCB subtypes which were similar to gene expression results of a complementary deoxyribonucleic acid (cDNA) microarray (Fig. 7).[26] This allowed for a widely applicable and more practical method to subclassify DLBCL into molecular subtypes.[26]

The study involved the analysis of 152 de novo DLBCLs using a tissue microarray (TMA) stained with antibodies.[26] Of these, 142 cases had been successfully classified by a cDNA microarray into DLBCL subtypes with GCB, ABC, or type 3 gene expression profiles.[26] Type 3 is heterogenous with outcomes similar to ABC DLBCL.[26] Therefore, the cases evaluated by TMA were classified into GCB and non-GCB subtypes.[26]

GCB DLBCLs were identified if CD10 was positive; or if CD10 was negative with BCL6 positive and MUM1 negative.[26] Cases were assigned as non-GCB DLBCL if both CD10 and BCL6 were negative; or if CD10 was negative with both BCL6 and MUM1 positive (Fig. 8).[26]

CD10 is a membrane-associated neutral endopeptidase with a restricted expression in GC cells of reactive lymphoid tissues.[26] BCL6 is a zinc-finger protein that functions as a transcriptional repressor and is expressed in GC B-cells.[26] MUM1 is a transcription factor of the interferon regulatory factor family and is expressed in plasma cells and some GC cells.[26]

The immunostain panel used with the TMA reproduced the cDNA microarray results in 71% of GCB cases and 88% of non-GCB cases.[26] The DLBCL subgroups determined by the TMA also predicted survival outcomes similar to that reported using the cDNA microarray.[26]

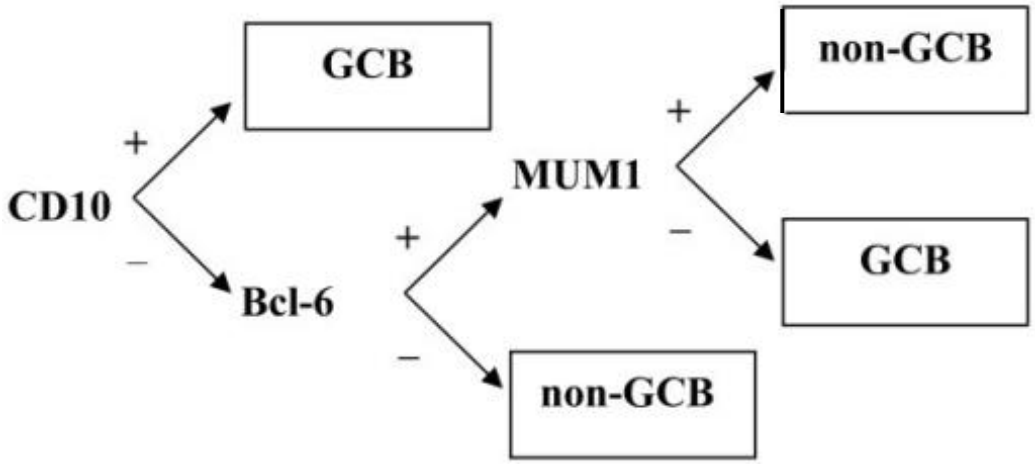

Fig. 7: Hans algorithm. Classifying DLBCL into GCB and non-GCB subtypes according to immunohistochemical (CD10, BCL-6, MUM1) expression.[26]

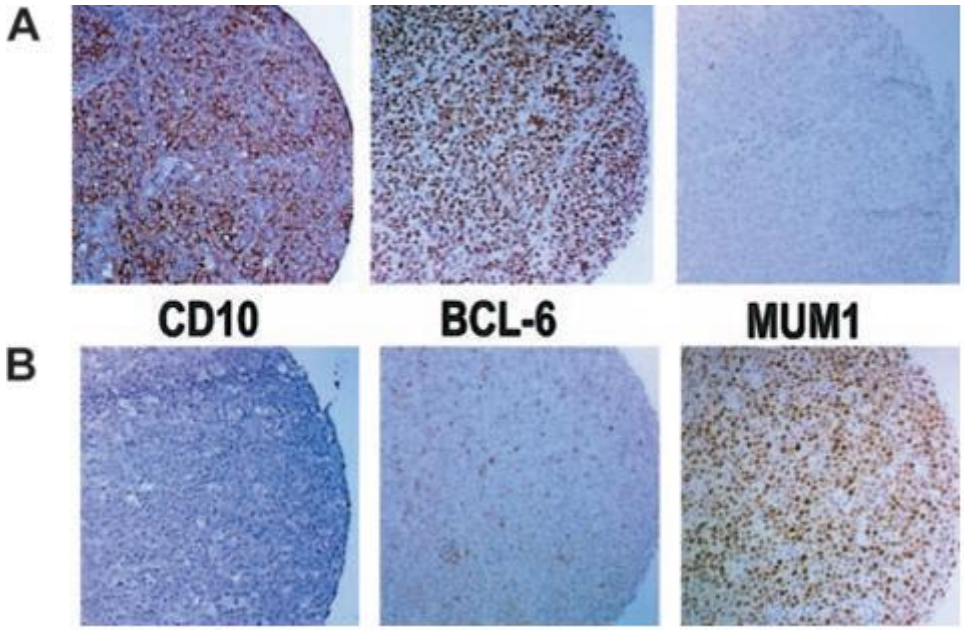

Fig. 8: Immunohistochemical results involved in the subclassification of DLBCL using the Hans algorithm. (A) GCB case that is CD10 positive, BCL6 positive and MUM1 negative. (B) Non-GCB case that is CD10 negative, BCL6 positive and MUM1 positive.[26]

7.3.4.2. Genetic subtypes

Two recently published studies have identified genetic subtypes of DLBCL based on shared genomic aberrations, taking the COO classification to new depth and complexity. These subtypes differ with respect to genotype, phenotype, epigenetics, clinical characteristics and survival outcomes allowing for better understanding of the pathogenesis, prognostication and potentially new treatment targets of DLBCL.

The first study by Schmitz *et al*. investigated 574 DLBCLs using exome and transcriptome sequencing, targeted amplicon resequencing, and array-based DNA copy-number analysis.[27] Four genetic subtypes of DLBCL were identified based on the co-occurrence of genetic abnormalities.[27] These subtypes were termed MCD (*MYD88*^{L265P} and *CD79B* mutations), BN2 (*BCL6* fusions and *NOTCH2* mutations), N1 (*NOTCH1* mutations), and EZB (*EZH2* mutations and *BCL2* translocations).[27] The MCD and N1 subtypes were predominantly ABC DLBCLs; EZB comprised mostly of GCB DLBCLs; and BN2 included ABC, GCB and unclassified DLBCLs.[27] Regarding survival outcomes, the BN2 and EZB subtypes had far more favourable outcomes than the MCD and N1 subtypes.[27]

The second study by Chapuy *et al*. analysed 304 primary DLBCLs using whole-exome sequencing.[28] Five genetic DLBCL subsets, referred to as clusters (C1–C5), were defined using consensus clustering of co-occurring genetic driver alterations.[28] C1 harboured *BCL6* structural variants and *NOTCH2* mutations.[28] C2 was characterised by mutations resulting in biallelic inactivation of *TP53*, as well as *17p* copy loss.[28] C3 demonstrated *BCL2* mutations with structural variants, and mutations in chromatin modifiers such as *EZH2*.[28] C4 exhibited mutations in multiple histone genes, immune evasion molecules, BCR/Pi3K signalling intermediates, RAS/JAK/STAT pathway molecules, and NF-κB modifiers including *CARD11*.[28] C5 harboured *18q* gain with increased BCL2 expression, and concordant *MYD88*^{L265P}/*CD79B* mutations.[28] C0 was a small additional subset without detectable genetic abnormalities.[28] C1 and C5 consisted predominantly of ABC DLBCLs; C3 and C4 comprised primarily of GCB DLBCLs; while C2 included both ABC and GCB DLBCLs.[28] Survival outcome associations revealed that C0, C1 and C4 had better outcomes than C3 and C5; whereas C2 had a progressive decline in outcome over time.[28]

7.3.5. Double-/triple-hit and double-expressor lymphomas

Double-hit lymphoma (DHL) and triple-hit lymphoma (THL) refers to the presence of a *MYC* rearrangement with a *BCL2* and/or *BCL6* rearrangement.[29] This is defined in the WHO 2017 classification as high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements.[2] The frequency of rearrangements in these lymphomas are: DHL with *MYC* and *BCL2* translocations (65%), THL with all three translocations (21%), and DHL with *MYC* and *BCL6* translocations (14%).[29]

Double-expressor lymphoma (DEL) refers to the co-expression of MYC and BCL2 proteins without underlying chromosomal rearrangements.[29] Over-expression of these two proteins occurs due to gene amplification and post-translational processes in the absence of chromosome translocations.[29] The immunohistochemical (IHC) thresholds to define DEL are ≥40% for MYC and >50% for BCL2.[2] This is not a separate diagnostic entity in the current WHO classification and is therefore considered an adverse prognostic indicator in DLBCL, NOS.[29]

DHL and THL constitute 5% to 7% of DLBCLs and correlates significantly with the GCB subtype.[29] DEL comprise 20% to 30% of DLBCLs and correlates highly with the ABC subtype.[29] The majority of DHL/THL will co-express the MYC and BCL2 proteins.[29] Both DHL/THL and DEL are associated with poor prognoses; however, the presence of MYC and BCL2 dual-protein expression results in more inferior survival outcomes.[29-30]

MYC is a proto-oncogene located on chromosome 8q24.[29] It encodes for a transcription factor involved in multiple roles including protein synthesis, cellular differentiation and metabolism.[29] In the setting of lymphoma, MYC expression results in gene instability, genetic amplification, and cellular proliferation.[29] Over-expression of the MYC protein may occur due to various mechanisms such as chromosome translocations, *MYC* gene amplification, aberrant somatic mutations and copy number alterations.[29,31]

BCL2 is an oncogene on chromosome 18q21.[29] It encodes for a protein responsible for maintaining cellular viability by inhibiting apoptosis.[29] In the context of lymphoma, BCL2 over-expression interacts together with *MYC* and other oncogenes leading to the progression of lymphoma.[29] BCL2 over-expression can occur as a result of chromosome translocations and gene amplification.[29]

BCL6 gene is situated on chromosome 3q27.[29] The BCL6 protein functions as a transcriptional repressor and modulator of cellular processes such as cellular activation, differentiation and apoptosis.[29] In a normal B-lymphocyte, BCL6 suppresses the activity of MYC and BCL2.[29] In the setting of lymphoma, there is inhibition of normal BCL6 protein functions.[29] Dysregulation of BCL6 is attributable to chromosomal translocations.[29]

7.3.6. Tumour proliferation index – Ki-67

Ki67 is a nuclear non-histone protein that is synthesized at the beginning of cellular proliferation and is expressed during all active phases of the cell cycle.[32-33] The Ki67 IHC stain is therefore widely used as an index to determine the proliferative activity of lymphoma.[33]

In DLBCL patients treated with standard chemotherapy, a high Ki67 expression indicates a poor prognosis with decreased survival outcomes.[34]

Conversely, in HIV-infected DLBCL patients treated with continuous infusion chemotherapy, a higher Ki-67 expression is associated with a better prognosis and improved survival outcomes.[32] This may be due to the fact that this form of therapy targets dividing cells and is therefore more likely to be effective in tumours with a rapid cellular turnover.[32]

7.3.7. DLBCL treatment and outcomes

The current standard of therapy for DLBCL comprises of combination immunochemotherapy using cyclophosphamide, doxorubicin, vincristine and prednisone plus the anti-CD20 monoclonal antibody rituximab (R-CHOP).[35] On this protocol, approximately 60% of patients with DLBCL are cured.[36] However, the other 40% of patients will relapse or develop refractory disease with the majority eventually succumbing to the disease.[37]

Patients with GCB DLBCL have better outcomes; with a 5-year overall survival rate of 60% and a relapse rate of around 20%.[35,37] Whereas ABC DLBCL is associated with the poorest prognosis and highest relapse rates of all DLBCLs; with a 5-year overall survival rate of only about 40%.[35]

Clinical trials now require that the subtype of DLBCL is known prior to trial enrolment. Early trials using proteasome inhibitors that downregulate NF-κB such as bortezomib have shown improved outcomes in ABC DLBCL.[35] However, this has not yet been incorporated into standard treatment protocols.

7.3.8. HIV and DLBCL

7.3.8.1. Lymphomagenic role of HIV

HIV plays both an indirect as well as a direct role in lymphomagenesis.[38]

Indirect role

Indirect mechanisms of HIV-induced lymphomagenesis include: chronic B-cell activation due to immune dysfunction, oncogenic viral infection (e.g. EBV) due to impaired immune surveillance, cytokine overproduction, and genetic alterations.[38]

Persistent uncontrolled antigenic stimulation may lead to monoclonal B-cell proliferation.[39] This is enhanced by the abnormal production of cytokines such as IL-6 and IL-10, which promote the growth of B-lymphocytes.[39] The dysregulated B-cell clonal expansion is also at an increased risk of acquiring a critical genetic lesion that may eventually result in the development of lymphoma.[38]

HIV-associated DLBCL demonstrates a more frequent association with EBV when compared with DLBCL in immunocompetent patients.[40]

Direct role

Direct mechanisms whereby HIV contributes to lymphomagenesis involve HIV-encoded proteins and HIV virions that act as crucial microenvironmental factors in promoting lymphoma development.[38]

HIV-encoded proteins such as p17 variants and gp120 are secreted and accumulate predominantly within lymph node germinal centres.[41] It persists in HIV-infected individuals, even under antiretroviral therapy (ART) with an undetectable viral load.[41] These proteins have enhanced B-cell activating and growth-promoting properties that act directly on B-lymphocytes, thereby increasing the risk of developing lymphoma.[38]

CD40 ligand (CD40L) is a co-stimulatory molecule that is normally expressed on the surface of activated T-lymphocytes.[42] This molecule can be inserted onto the surface of the HIV particle during budding from the $CD4^+$ T-cell.[42] HIV virions bearing CD40L are then able to mimic physiological stimulation and thus aberrantly activate B-lymphocytes.[42-43] These

hyperactivated B-cells express a DNA editing enzyme called activation-induced cytidine deaminase (AID) which mediates immunoglobulin CSR and SHM.[44] AID is able to induce point mutations and chromosomal translocations that involve oncogenes which may lead to the development of lymphoma.[44-45]

7.3.8.2. Survival outcomes in HIV-associated DLBCL

With the advent of ART for HIV, the risk of developing acquired immunodeficiency syndrome (AIDS)-defining cancers has reduced.[38] However, despite the immune reconstitution afforded by ART, the incidence of lymphomas remains elevated.[46] In the setting of HIV, lymphomas constitute more than half of all AIDS-defining malignancies,[47-48] and is the leading cause of mortality in these patients.[40,49] DLBCL is one of the most common lymphomas that occurs in people infected with HIV.[50-51] These lymphomas are characterised by high-grade features that frequently have extranodal involvement with advanced stage presentation.[38]

Studies reveal conflicting results regarding the role of molecular subtypes in predicting outcome in HIV-associated DLBCL – summarised in table 1.

Table 1: Studies showing conflicting survival outcomes in HIV-associated DLBCL subtypes

Study	No. of patients	Survival outcome between HIV-associated DLBCL subtypes
Chadburn, *et al*. J Clin Oncol. 2009.[33]	81	No significant difference
Dunleavy, *et al*. Blood. 2010.[52]	33	Adverse survival in ABC subtype
Morton, *et al*. Leuk Lymphoma. 2014.[53]	51	Adverse survival in ABC subtype
Chao, *et al*. Clin Cancer Res. 2015.[54]	80	Adverse survival in GCB subtype

In the current ART and rituximab era, HIV-infected individuals with DLBCL achieve significantly improved survival outcomes.[55] The concurrent use of ART with chemotherapy has been shown to be associated with better clinical outcomes due to HIV control, decreased infectious complications and fewer AIDS-defining events.[55] When comparing rituximab use in HIV-positive and HIV-negative DLBCL patients, survival outcomes have been inconsistent with some studies reporting poorer outcomes in the HIV-positive group while other studies report similar outcomes in both groups.[54,56-59]

Amongst HIV-infected patients with DLBCL, survival outcomes differ depending on the type of chemotherapy regimen – intensive vs standard.[55] It has been shown that infusional chemotherapy such as etoposide, prednisone, vincristine, doxorubicin and cyclophosphamide (EPOCH) is associated with significantly improved overall survival when compared with standard cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP) chemotherapy.[55] This association is similar when rituximab is included in the regimen (i.e. R-EPOCH vs R-CHOP).[55]

In the South African population with more advanced HIV presentations, intensive chemotherapy may result in higher treatment-related mortality; therefore, current practice is to use the standard regimen.

7.3.8.3. HIV in South Africa

South Africa is home to the largest HIV epidemic in the world, with 20% of the global population of HIV-infected people.[60] In 2019, the total number of people living with HIV among the South African population was estimated at 7.97 million, with a prevalence of approximately 13.5%.[61]

The South African national antiretroviral treatment guidelines were implemented in 2004 to provide ART for HIV-positive patients with a CD4 count of <200 cells/mm^3.[62] These guidelines have since undergone several amendments, becoming more inclusive with each revision.[62] The current 2018 guidelines include all HIV-positive patients, regardless of CD4 count or clinical staging, as being eligible for ART initiation.[62] Despite the changes in ART guidelines, lymphoma has remained a WHO stage IV AIDS-defining illness and has therefore consistently fulfilled the ART eligibility criteria irrespective of CD4 count.[62] Universal ART coverage for HIV has been implemented in South Africa since 2016, but 20% of people living with HIV still remain untreated.[63]

7.4. LITERARY KNOWLEDGE GAPS & RATIONALE FOR THIS STUDY

As described in the literature, DLBCL is an immensely heterogenous malignancy with incredible and continuing advancements being made in increasing our molecular and biological understanding of the disease. This has resulted in the identification and characterisation of specific oncoproteins and survival pathways that may potentially be amendable to intervention. This offers a promising future for a change in therapeutic approach by the development of targeted therapeutic options which will ultimately improve overall treatment outcomes in these patients.

A multitude of studies have been conducted on DLBCL cases involving subtyping, various prognostic factors including EBV, as well as advanced genomic sequencing techniques which have revealed many different somatic driver mutations that contribute to lymphomagenesis. However, most of these studies include patients from Western countries with very limited information available from other population groups, particularly in HIV. Furthermore, there have been conflicting results regarding the role of molecular subtypes in predicting outcome in HIV-associated DLBCL.

This is a retrospective cohort study that aims to provide a South African perspective on DLBCL by correlating molecular subtype with HIV status, EBV infection as well as survival outcome.

The information gained from this study will enable further insight into the pathobiology of DLBCL, particularly in HIV. This will then lay the foundation and inform further studies into the investigation of known oncogenic driver mutations, as well as to identify possible new mutations in the heterogeneous landscape of DLBCL.

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8. CHAPTER 3:

Publication-ready Manuscript

8.1. Title page

Title

Diffuse large B-cell lymphoma in a South African cohort with a high HIV prevalence: an analysis by cell-of-origin, Epstein-Barr virus infection and survival

Running title

Diffuse large B-cell lymphoma in a high HIV prevalent cohort

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8.2. Abstract

Introduction:

Diffuse large B-cell lymphoma, not otherwise specified (DLBCL NOS) is subdivided according to the cell-of-origin (COO) classification into germinal centre B-cell (GCB) and activated B-cell (ABC) subtypes, each with different molecular profiles and clinical behaviour. This study aims to describe the pattern of the COO subtypes, the proportion of Epstein-Barr virus (EBV) co-infection, and their influence on survival outcomes in a setting of high HIV prevalence.

Materials and Methods:

This retrospective cohort study included patients diagnosed with de novo DLBCL NOS at our tertiary academic centre in Cape Town, South Africa over a 14-year period. Immunohistochemical stains were performed for COO classification, according to the Hans algorithm. Tumour EBV co-infection was established by EBV-encoded ribonucleic acid in situ hybridisation (EBER-ISH) staining. The effect of the COO subtypes and EBV co-infection on overall survival were described by means of univariate, bivariate and multivariate analyses.

Results:

A total of 181 patients with DLBCL NOS were included, which comprised 131 HIV-uninfected and 50 HIV-infected patients. There was an equal distribution of GCB and ABC subtypes in the HIV-infected and HIV-uninfected groups. EBV co-infection was detected in 16% of the HIV-infected cases and in 7% of the HIV-uninfected cases (p=0.09). There was no significant difference in the incidence of EBV co-infection between the GCB and ABC subtypes (p=0.67). HIV-infected patients with CD4≥150 cells/mm^3 had similar survival to HIV-uninfected patients (p=0.005). Multivariate regression analysis showed that in the HIV-infected group with marked immunosuppression (CD4<150 cells/mm^3), there was significantly poorer overall survival compared to the HIV-uninfected group (HR 2.4, 95% CI 1.3–4.1). There were no statistically significant differences in overall survival by DLBCL COO subtype.

Conclusions:

There was no difference in the proportion of DLBCL COO subtypes, regardless of HIV status. EBV co-infection was more common in the HIV-infected group, but less than described in the literature. Unexpectedly, there were no significant differences in survival outcomes between the GCB and ABC subtypes. Higher CD4 counts in the HIV-infected

group had good survival outcomes, while lower CD4 counts predicted adverse survival outcomes. Further research is needed to explore the genetic mutational landscape of HIV-associated DLBCL.

8.3. Keywords

Diffuse large B-cell lymphoma
Cell-of-origin
HIV
Epstein-Barr virus
Survival
South Africa

8.4. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoid malignancy in adults worldwide,[1] accounting for approximately one third of all non-Hodgkin lymphomas in developed countries and a greater proportion in developing countries.[2] The cell-of-origin (COO) classification, based on gene expression profiling studies, delineates two molecular subtypes of DLBCL, not otherwise specified (NOS) namely germinal centre B-cell (GCB) and activated B-cell (ABC).[3] The ABC subtype is reported to be associated with the poorest prognosis and highest relapse rates, in the well-resourced HIV-uninfected setting.[4]

Human immunodeficiency virus (HIV) has been shown to play an important role in lymphomagenesis.[5] South Africa is home to the largest HIV epidemic in the world, with 20% of the global population of HIV-infected people.[6] In 2019, the total number of people living with HIV among the South African population is estimated at 7.97 million, with a prevalence of approximately 19.1% amongst adults aged 15–49 years and an overall prevalence of approximately 13.5%.[7] Universal antiretroviral therapy (ART) coverage for HIV has been implemented in South Africa since 2016, but 20% of people living with HIV still remain untreated.[8] With the advent of ART, the risk of developing acquired immunodeficiency syndrome (AIDS)-defining cancers has reduced.[5] However, in South Africa, late institution of ART and late diagnosis of AIDS-defining cancers remain common. In the setting of HIV, lymphomas constitute more than half of all AIDS-defining malignancies;[9] and are

characterised by high-grade features including extranodal involvement and advanced stage presentation.[5]

Tumour Epstein-Barr virus (EBV) co-infection has been well described in immunocompromised hosts and is more frequently linked with HIV-associated DLBCL than with HIV-unassociated DLBCL.[10] Tumour EBV infection enables the tumour cells to evade the immune response and become immortalised.[11] It is not known whether EBV infection is more likely to drive the development of the GCB or ABC subtype of DLBCL.

DLBCL NOS has been extensively studied for prognostic factors that may inform treatment decisions. These methods include tumour subtyping by immunohistochemistry and genomic sequencing techniques. However, regardless of HIV status, reports of DLBCL patient cohorts are mostly from higher-income countries; and very limited information is available from patients in low- and middle-income countries, particularly those infected with HIV. Immunohistochemistry is an inexpensive and available testing modality in many low- and middle-income countries thus, making this a relatable method of COO determination in many centres around the world. The association of molecular subtypes with outcome in HIV-associated DLBCL is by no means settled.[12-15] It is therefore of interest to determine DLBCL COO subtypes and their outcome in an HIV-endemic setting.

We performed this study among DLBCL NOS patients to describe the pattern of the COO subtypes, hereafter referred to as 'DLBCL subtypes', as well as the proportion of cases with concurrent EBV infection. We also evaluated the effect of DLBCL subtype, HIV status and EBV co-infection on survival outcomes.

8.5. Materials and Methods

8.5.1 Study design and setting

This was a retrospective cohort study set in Groote Schuur Hospital (GSH), Cape Town, South Africa. GSH is one of two tertiary academic centres for public patients in the Western Cape Province. Study patients were identified from the pathology and clinical databases at GSH. Consecutive adults (age≥18 years) diagnosed with de novo DLBCL NOS between 1st January 2005 and 31st December 2018 were selected. This study was approved, including a waiver of consent, by the University of Cape Town Human Research Ethics Committee (reference number: 441/2018).

8.5.2. Patient selection

All cases were diagnosed by qualified histopathologists, at the Division of Anatomical Pathology, GSH. The diagnosis of DLBCL was made based on histologic and immunophenotypic findings, according to the World Health Organisation (WHO) classification in use at the time.[2,16,17] The DLBCL cases were reported as demonstrating large lymphoid cells with a diffuse growth pattern. The morphology displayed was centroblastic, immunoblastic or mixed centroblastic and immunoblastic (Fig. 1). All cases demonstrated diffuse CD20 immunohistochemical (IHC) expression. In the years prior to 2016, many of our cohort cases lacked the specific IHC stains used for COO subtyping according to the Hans algorithm; therefore, only cases with sufficient stored tissue blocks for histological review and additional testing were included for this study.

8.5.3. Research procedures

Patient demographic characteristics and disease variables were recorded including gender, age at diagnosis, HIV status, CD4 count if HIV-infected, primary biopsy site, DLBCL subtype, double expressor profile, Ki67 proliferation index and tumour EBV status. Survival data was obtained from medical records and confirmed by the South African Government Department of Home Affairs. We did not assess individual treatment response; therefore, death by any cause was included.

Archived tissue specimens in the form of formalin-fixed, paraffin wax-embedded tissue blocks were obtained for cases requiring further stains. Stains that were not performed at the initial diagnostic work-up were determined for each case, and the corresponding number of slides prepared. Three-micron sections were cut from the tissue blocks, placed onto silanised slides and heat fixed on a hotplate at 75°C for 30 minutes. Tissue sections were then dewaxed through xylene, cleared in ethanol and rehydrated in water. All IHC stains were performed with the Envision Detection System on a universal staining system, the Dako Autostainer (Dako, Colorado), using routine staining protocols and the antibodies listed in Supplementary Table 1, Appendix. The EBV-encoded ribonucleic acid in situ hybridisation (EBER-ISH) made use of the Ventana ISH iVIEW Blue Plus Detection Kit on an automated slide stainer, the BenchMark ULTRA IHC/ISH System (Roche, California).

IHC stains performed included CD10, BCL6 and MUM1 in order to classify the cases according to the WHO 2017 classification[2] of DLBCL molecular subtypes, using the Hans algorithm.[18] The ‘non-GCB’ group is referred to as the ABC subtype in this study. Cases

were assigned as the GCB subtype if CD10 was positive; or as the ABC subtype if both CD10 and BCL6 were negative. If CD10 was negative and BCL6 was positive, the expression of MUM1 determined the subtype; with MUM1 negativity assigning the GCB subtype, and MUM1 positivity assigning the ABC subtype (Fig. 2). The stains were interpreted as either positive or negative with a threshold of ≥30% tumour cell staining to determine positivity.

The c-MYC and BCL2 IHC stains were performed to determine double-expressor profiles of the DLBCL cases (Fig. 3). The stain thresholds to determine positivity were ≥50% tumour cytoplasmic staining for BCL2, and ≥40% tumour nuclear staining for MYC.

The Ki67 stain was performed to assess the proliferation index with a percentage of tumour cell positivity assigned (Fig. 4).

EBER-ISH, the standard and most sensitive method of EBV detection in tumour cells, was performed to enable identification of the proportion of EBV-positive DLBCL cases and was interpreted as positive with any proportion of tumour cell reactivity up to 80% (Fig. 5).

8.5.4. Statistical analysis

The data was collected and recorded into an online database (REDCap).[19] Variables from all patients were obtained for analysis and included: gender, age at diagnosis, HIV status, CD4 count if HIV-infected, primary biopsy site, DLBCL subtype, double expressor profile, Ki67 proliferation index and tumour EBV status. Statistical data analysis was performed using STATA v14.0 (StataCorp, Texas).[20]

Dichotomous and categorical variables were characterised using frequencies and percentages and compared by HIV status and DLBCL subtype using the Pearson Chi-squared test or Fisher's exact test. Continuous non-parametric variables were summarised using medians and interquartile ranges (IQR) and compared using the Wilcoxon rank-sum test.

Survival analysis was estimated using the Kaplan-Meier method. Overall survival (OS) was measured as the time from date of diagnosis until date of death, regardless of cause, or date last seen alive. Patients who were lost to follow-up were censored at the last follow-up date. Kaplan-Meier survival curves were restricted to display 5-year OS. The comparison between

survival distributions was determined by means of the log-rank test. A two-sided *p*-value of <0.05 was considered statistically significant.

A Cox proportional hazards model was developed to calculate hazard ratios (HR) with 95% confidence intervals (CI) to assess the impact of prognostic factors on OS. Univariate analysis was performed to determine the potential association of prognostic factors with outcome. Factors with a *p*-value of <0.2 or those that were thought to be clinically relevant were ultimately selected as covariates for multivariate analysis. These factors included: HIV status, DLBCL subtype, Ki67 proliferation index and age at diagnosis.

8.6. Results

A total of 362 de novo DLBCL cases were identified. Of this total, 181 cases had inadequate stored tissue specimens available for additional testing and were therefore excluded. As a result, 181 DLBCL NOS cases met eligibility criteria and included 131 HIV-uninfected patients and 50 HIV-infected patients.

Of the 181 patients included, 93 (51%) were men and the median age at diagnosis was 52 years (IQR, 39–63). Fifty patients (28%) were HIV-positive with a median age of 39 years (IQR, 34–49) and a median CD4 count of 148 cells/mm^3 (IQR, 72–337). There was a similar distribution of DLBCL subtypes, even when compared by age and HIV status. Nodal primary biopsy sites (71%) were more common than extranodal sites (29%). Further details of patient baseline characteristics are summarised in Table 1.

Bivariate analysis comparing the various IHC and EBER-ISH results by HIV status and DLBCL subtype are presented in Table 2. DLBCL subtypes were evenly distributed amongst the HIV-infected and HIV-uninfected groups. EBV co-infection was detected in 8 (16%) of the HIV-infected cases and in 9 (7%) of the HIV-uninfected cases, though this did not reach statistical significance (*p*=0.09); with no significant difference found between DLBCL subtypes (*p*=0.67). The proliferation index, Ki67>75%, occurred significantly more frequently in HIV-infected patients (*p*=0.004) and approached significance in the ABC subtype (*p*=0.05). Sixteen patients were double expressors, but there were no statistically significant correlations with HIV status (*p*=0.77) nor DLBCL subtype (*p*=0.87).

Survival analysis of the total DLBCL patient population showed that by the end of the study period, 95 patients (52%) had died and 7 patients had been lost to follow-up. The median

survival time was 30 months (95% CI 16–61 months), based on a total patient follow-up time of 4982 months and median follow-up time of 15 months (range, 3 days–14 years). The 1-year, 2-year and 5-year OS were 65%, 52% and 40% respectively.

HIV-infected patients with a CD4<150 cells/mm^3 had a median survival time of 6 months (95% CI 4–16 months). The Kaplan-Meier curves depicting 5-year OS estimates (Fig. 6) showed that HIV-infected patients with a CD4≥150 cells/mm^3 had similar survival to HIV-uninfected patients; and HIV-infected patients with a CD4<150 cells/mm^3 had a significantly shorter survival than both HIV-infected patients with a CD4≥150 cells/mm^3 and HIV-uninfected patients (p=0.005). No statistically significant differences in survival were found when the ABC subtype was compared with the GCB subtype (p=0.32), nor when both HIV status and DLBCL subtype were combined for analysis (p=0.14).

The Cox regression analysis, shown in Table 3, assessed the prognostic effect of associated variables on OS. HIV infection with a CD4<150 cells/mm^3 was a significantly poorer prognosis in comparison with no HIV infection (HR 2.4, 95% CI 1.3–4.1). DLBCL subtype (HR 1.2, 95% CI 0.8–1.9), Ki67 (HR 1.2, 95% CI 0.8–1.9) and age (HR 1.0, 95% CI 0.6–1.6) were not associated with statistically significant differences in survival.

8.7. Discussion

In this study, performed in a major South African academic referral centre, we found that the DLBCL subtypes (GCB and ABC) were equally distributed among HIV-infected and HIV-uninfected patients. Contrary to expectation, we found that EBV co-infection was detected in only 16% of HIV-associated DLBCLs, although it occurred more frequently in HIV-infected patients as opposed to 7% in HIV-uninfected patients. This is an interesting finding, as previous studies have shown that EBV is positive in 30-60% of HIV-associated DLBCL cases and has been thought to play a significant driving role in lymphomagenesis; compared with 10% EBV positivity in HIV-unassociated DLBCL cases.[10,21]

No significant difference in 5-year OS was demonstrated between the GCB and ABC subtypes. This is in keeping with results of a similar South African study by Pather *et al*.[22] which, albeit a smaller cohort, also established no significant differences in survival between the subtypes. However, international studies from well-resourced countries with low HIV

prevalence report the ABC subtype to have a poorer prognosis with a 5-year OS of approximately 40% compared to the GCB subtype, which has a 60% 5-year OS.[4,23]

HIV has been shown to play both an indirect and direct role in lymphomagenesis.[5] Indirect mechanisms include chronic B-cell activation due to immune dysfunction, oncogenic viral co-infection (e.g. EBV) due to impaired immune surveillance, cytokine overproduction, and genetic alterations.[5] Direct mechanisms involve HIV-encoded proteins and HIV virions that act as crucial microenvironmental factors in promoting lymphoma development.[5] These aetiological mechanisms persist even in those on ART with undetectable viral loads.[24] We hypothesize that these mechanisms are common to the development of either the ABC or GCB subtype, which may explain the equal incidence of the subtypes amongst our HIV-infected cases.

With regards to HIV-infected patients, there was no significant difference in OS by DLBCL subtype. Of four previous published studies, two reported poorer survival outcomes in patients with the ABC subtype,[4,14] one found poorer survival in patients with the GCB subtype,[15] and a fourth study found no significant difference in outcome between the subtypes.[12]

Our study showed that HIV-infected patients with CD4 counts of 150 cells/mm^3 or more had similar survival outcomes to HIV-uninfected patients; which may be explained by similar immune competencies of the two groups. Our study also showed that HIV-infected patients with CD4 counts of less than 150 cells/mm^3 had a significantly shorter survival and a 2.4 times higher risk of mortality in comparison to HIV-uninfected patients. This is likely due to delayed presentations of both HIV infection and lymphoma resulting in severe immunosuppression, as confirmed by the decreased CD4 counts.

A high Ki67 proliferation index has been shown to indicate a poor prognosis with decreased survival outcomes in DLBCL patients treated with standard chemotherapy.[25] We found that Ki67>75% occurred more commonly in HIV-infected patients and the ABC subtype, however its association with adverse survival was not significant.

Double-expressor lymphoma (DEL) refers to the co-expression of MYC and BCL2 proteins without underlying chromosomal rearrangements.[26] DEL is an adverse prognostic indicator which is present in 20% to 30% of DLBCL cases, and has been reported to correlate highly with the ABC subtype.[26] Our cohort of DLBCL patients yielded a small proportion (9%) of double expressors that showed no significant correlation with subtype.

Two recently published studies by Schmitz *et al*.[27] and Chapuy *et al*.[28] have identified genetic subtypes of DLBCL based on shared genomic aberrations involving for example, *MYD88*, *CD79B*, *BCL6*, *BCL2*, *EZH2*, *NOTCH*, *CARD11* and *TP53*. This has taken the COO classification of DLBCL to new depth and complexity. These genetic subtypes have not, to our knowledge, been specifically investigated in HIV.

This study has several limitations. Firstly, due to lack of archived tissue, 50% of our patient cohort could not be subtyped and was excluded. Secondly, in our resource-constrained local setting, ancillary investigations such as fluorescent in situ hybridisation (FISH) and molecular testing are not routinely performed on the majority of cases which may result in double/triple-hit lymphomas being erroneously classified as DLBCL NOS and thus being treated with standard chemotherapy instead of the recommended higher intensity regimens.[29] Thirdly, it is difficult to compare the mortality rates of our study group with international data as rituximab was only implemented into our treatment protocols from 2013; and even then mostly in HIV-uninfected patients due to resource constraints and lack of supportive data. Lastly, we did not collect details on stage of disease, treatment regimens nor specific treatment outcomes other than survival which would have allowed for a more comprehensive analysis of the effect of DLBCL subtype on survival.

The strength of this study is that, for the first time in our HIV-endemic region, we describe a large proportion of our DLBCL population with detailed IHC and EBER-ISH findings as well as survival outcomes.

The information gained from our study will form a basis from which to further explore the pathobiology of DLBCL, particularly in HIV.

8.8. Conclusions

We found that DLBCL subtypes were present in equal proportions, regardless of HIV status. EBV co-infection was also associated equally with both the ABC and GCB subtypes. EBV was present in a much lower proportion of HIV-associated DLBCLs than described in the literature at only 16%. There were no significant differences in survival between the DLBCL subtypes. HIV infection with high CD4 counts had good survival outcomes; and HIV infection with low CD4 counts predict for markedly adverse survival outcomes.

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Acknowledgements: Edward L. Murphy, George Rutherford, Lawrence D. Kaplan and the rest of the UCSF team for assisting in the writing of this manuscript.

8.9. Appendix. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.17632/4m4dz4n2wn.1.

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8.11. Tables

Table 1 Baseline characteristics of DLBCL study patients

Characteristic	Frequency (percentage) or Median (IQR)
Patients	181 (100%)
Gender	
Male	93 (51%)
Female	88 (49%)
Age at diagnosis (years)	
<30	14 (8%)
30-50	65 (36%)
51-70	86 (47%)
>70	16 (9%)
Median age at diagnosis (years)	
Median (IQR)	52 (39–63)
HIV-infected median (IQR)	39 (34–49)
HIV-uninfected median (IQR)	57 (48–65)
DLBCL ABC median (IQR)	55 (39–61)
DLBCL GCB median (IQR)	51 (41–64)
HIV status	
Positive	50 (28%)
Negative	131 (72%)
CD4 count at diagnosis (cells/mm^3)	
Median (IQR)	148 (72–337)
DLBCL subtype	
ABC	94 (52%)
GCB	87 (48%)
HIV status & DLBCL subtype	
HIV-infected + ABC	24 (48%)
HIV-infected + GCB	26 (52%)
Primary biopsy site	
Nodal	128 (71%)
Extranodal	53 (29%)

IQR, interquartile range; HIV, human immunodeficiency virus; DLBCL, diffuse large B-cell lymphoma; ABC, activated B-cell; GCB, germinal centre B-cell.

Table 2 Bivariate analysis comparing immunohistochemical and EBER-ISH results by HIV status & DLBCL subtype

	HIV-uninfected (*n* = 131)	HIV-infected (*n* = 50)	*P*-value	ABC subtype (*n* = 94)	GCB subtype (*n* = 87)	*P*-value
Hans algorithm						
CD10 positive	46 (35%)	23 (46%)	0.18	0 (0%)	69 (79%)	
BCL6 positive	86 (66%)	31 (62%)	0.65	42 (45%)	75 (86%)	<0.001
MUM1 positive	70 (53%)	30 (60%)	0.67	79 (84%)	21 (24%)	
EBV infection						
EBER-ISH positive	9 (7%)	8 (16%)	0.09	8 (9%)	9 (10%)	0.67
EBER-ISH negative	122 (93%)	42 (84%)		86 (91%)	78 (90%)	
Proliferation index						
Ki67>75%	78 (60%)	41 (82%)	0.004	68 (72%)	51 (59%)	0.05
Ki67≤75%	53 (40%)	9 (18%)		26 (28%)	36 (41%)	
Expressor profile						
Double expressor	11 (8%)	5 (10%)	0.77	8 (9%)	8 (9%)	0.87
MYC positive	18 (14%)	7 (14%)	0.96	12 (13%)	13 (15%)	0.67
BCL2 positive	78 (60%)	24 (48%)	0.16	58 (62%)	44 (51%)	0.13

HIV, human immunodeficiency virus; ABC, activated B-cell; GCB, germinal centre B-cell; EBV, Epstein-Barr virus; EBER-ISH, Epstein-Barr virus-encoded ribonucleic acid in situ hybridisation.

Table 3 Multivariate model of prognostic factors associated with overall survival

Selected variables	Hazard ratio	95% Confidence interval	P-value
HIV status & CD4 count (cells/mm^3)			
(HIV-infected + CD4<150 vs HIV-uninfected)	2.4	1.3–4.1	0.003
(HIV-infected + CD4≥150 vs HIV-uninfected)	1.2	0.6–2.4	0.55
DLBCL subtype (ABC vs GCB)	1.2	0.8–1.9	0.35
Ki67 proliferation index (>75% vs ≤75%)	1.2	0.8–1.9	0.44
Age at diagnosis (years) (>45 vs ≤45)	1.0	0.6–1.6	0.97

HIV, human immunodeficiency virus; DLBCL, diffuse large B-cell lymphoma; ABC, activated B-cell; GCB, germinal centre B-cell.

8.12. Figures

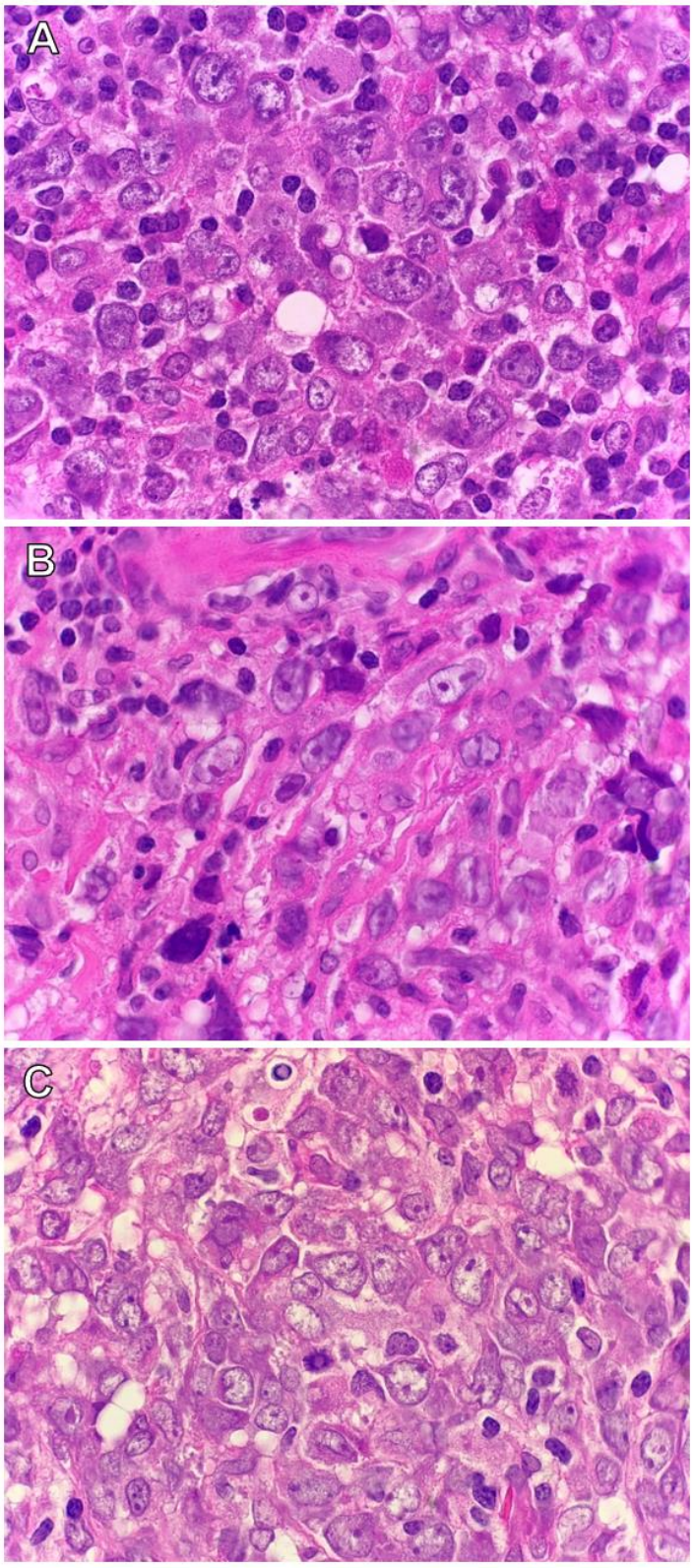

Fig. 1 Morphological variants of DLBCL. Cases of DLBCL demonstrating the (A) centroblastic variant; (B) immunoblastic variant; and (C) mixed centroblastic and immunoblastic variant. (haematoxylin and eosin, original magnification x100)

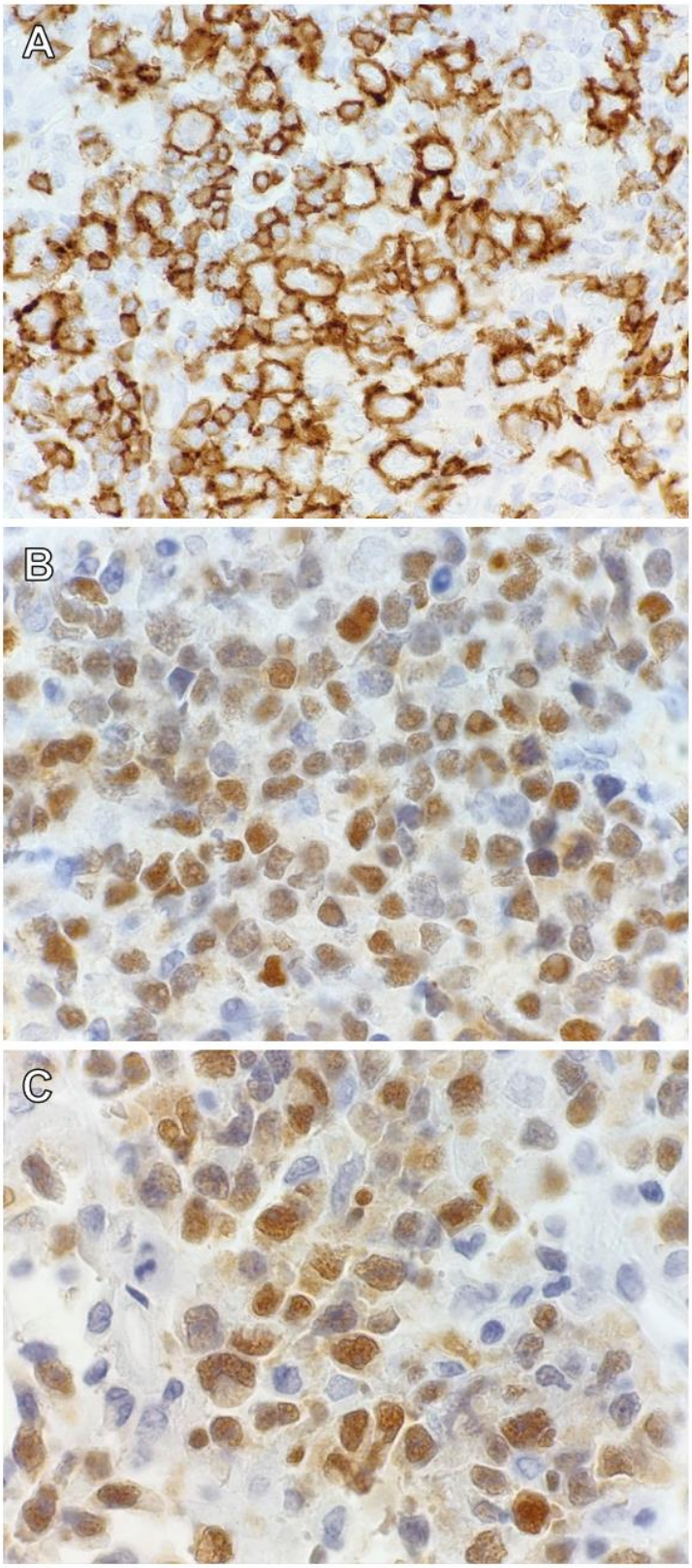

Fig. 2 Immunohistochemical staining patterns used to classify DLBCL NOS according to the Hans algorithm. (A) CD10 membrane staining positivity in a case of the germinal centre B-cell (GCB) subtype. (B) BCL6 nuclear staining and (C) MUM1 nuclear staining in a case of the activated B-cell (ABC) subtype. (original magnification x100)

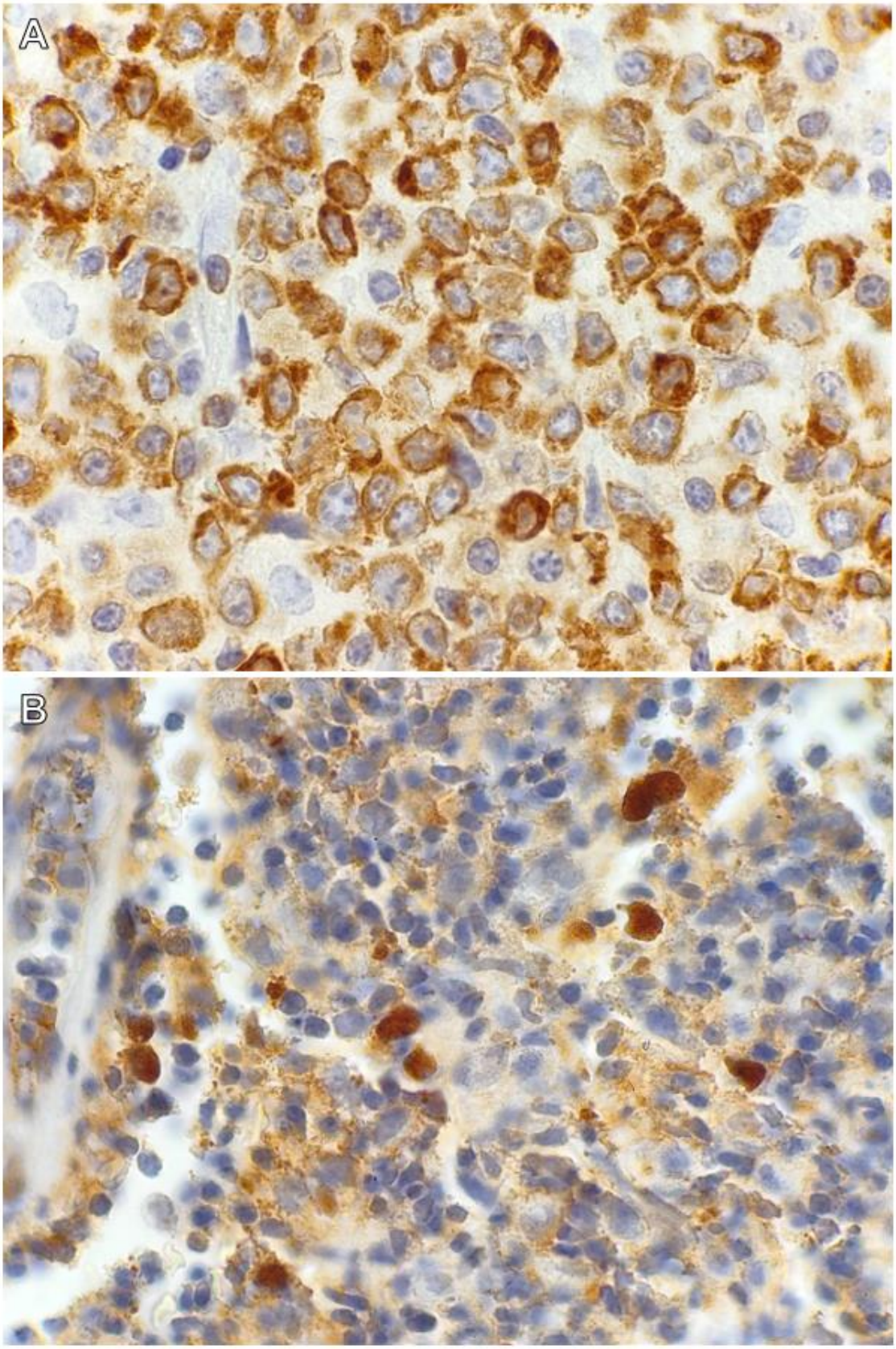

Fig. 3 Immunohistochemical staining patterns used to determine double-expressor profiles of DLBCL. A case of double-expressor DLBCL with (A) BCL2 cytoplasmic staining and (B) c-MYC nuclear staining. (original magnification x100)

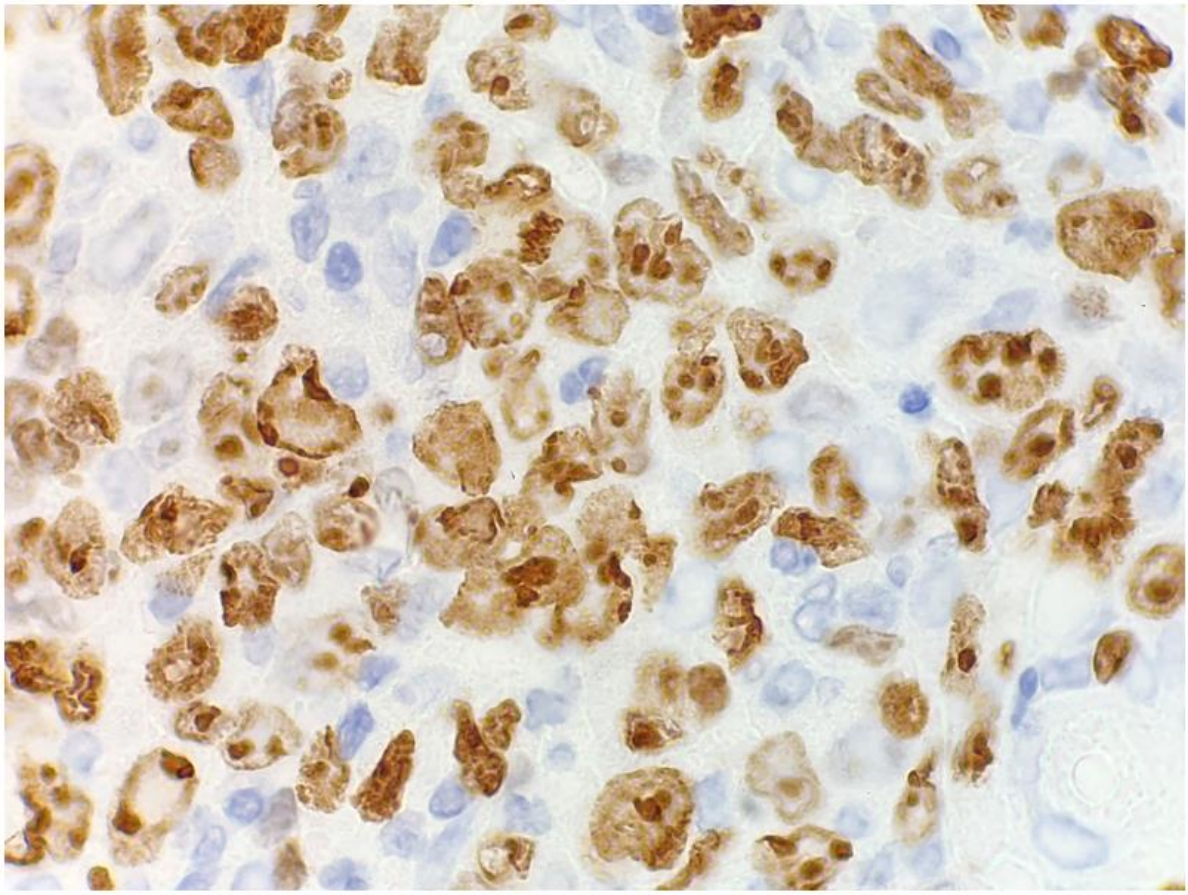

Fig. 4 Ki67 immunohistochemical staining to determine the proliferation index of DLBCL. A case of Ki67 nuclear staining of >75% tumour cell positivity, in an HIV-infected patient. (original magnification x100)

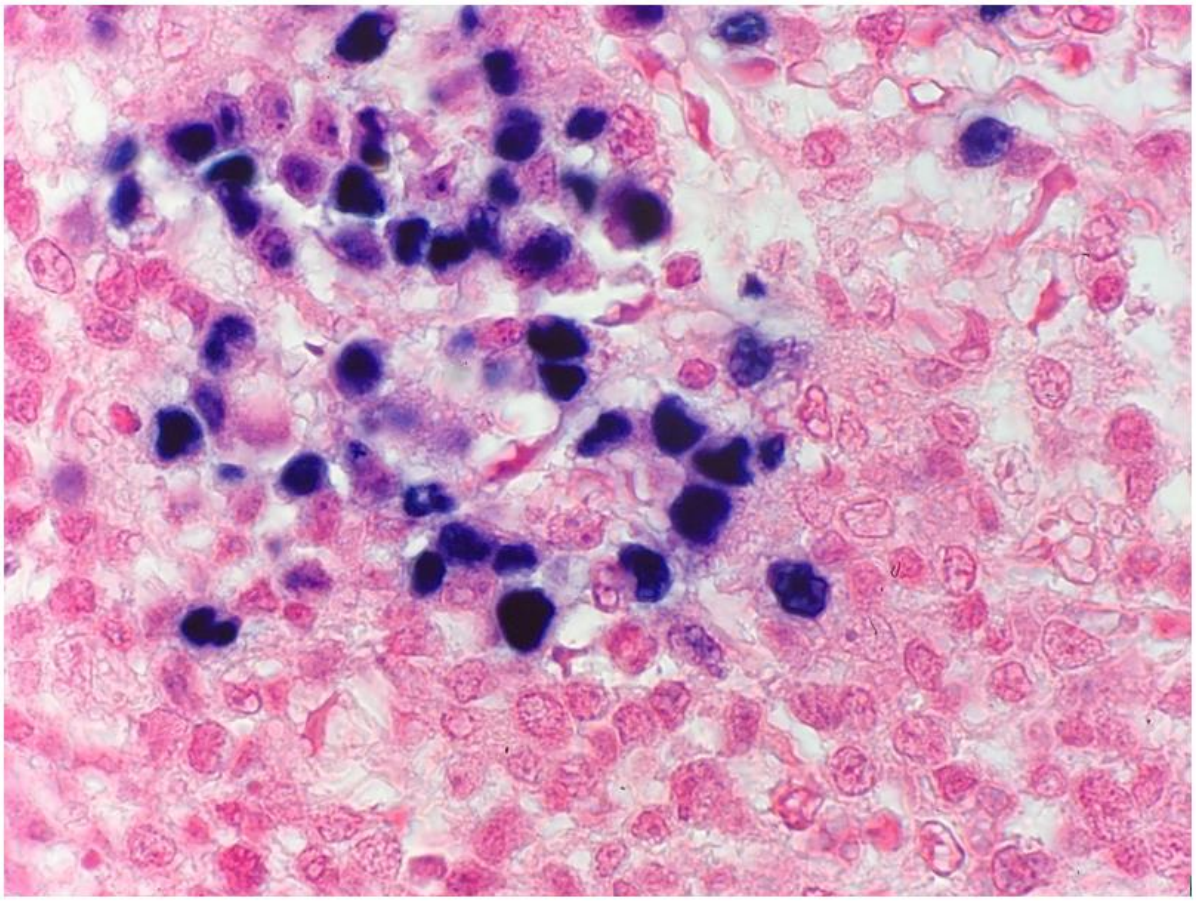

Fig. 5 EBV-encoded ribonucleic acid in situ hybridisation (EBER-ISH) staining of DLBCL to determine the presence of Epstein-Barr virus (EBV). A case of EBER-ISH nuclear stain positivity, in an HIV-infected patient, confirming tumour EBV co-infection. (original magnification x100)

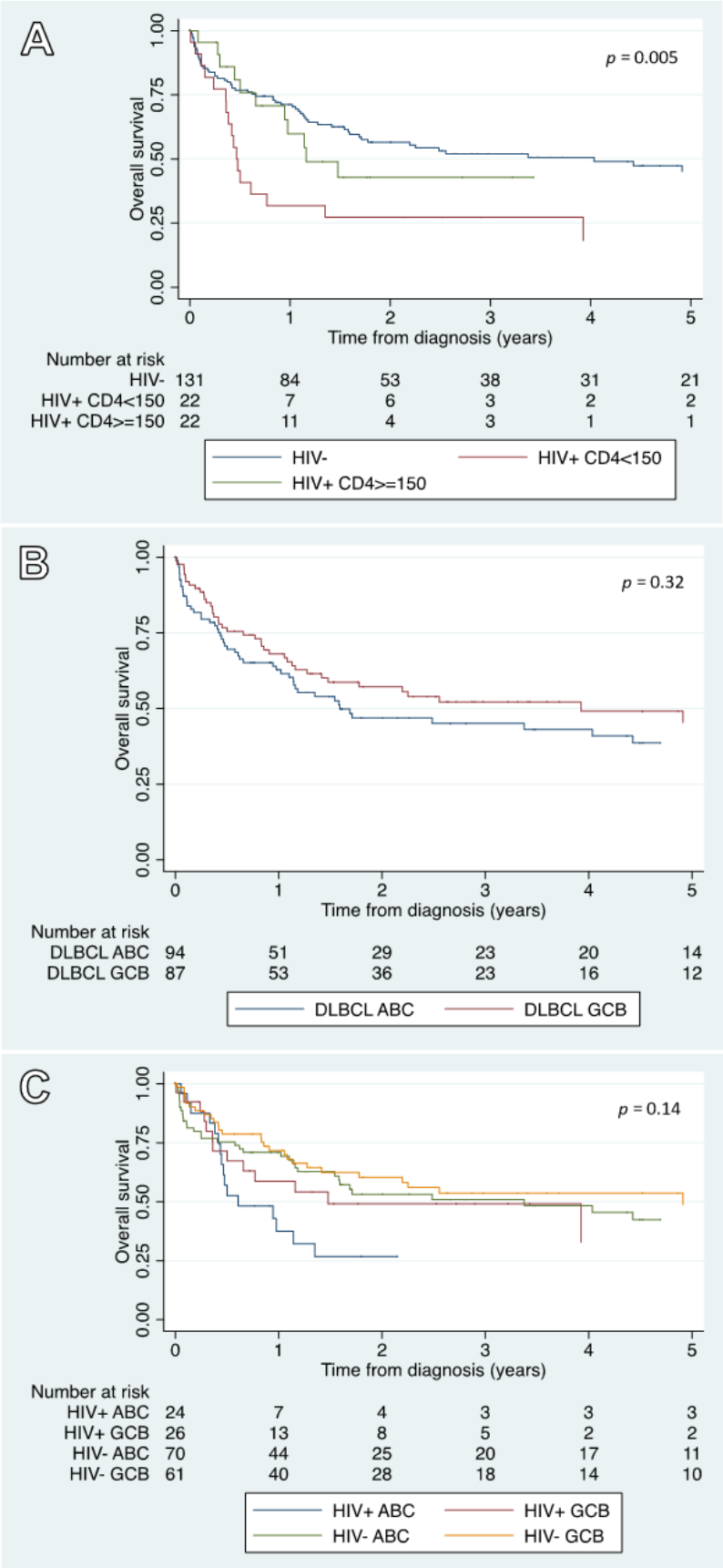

Fig. 6 Kaplan-Meier survival estimates depicting 5-year overall survival of DLBCL study patients by: (A) HIV status, (B) DLBCL subtype, (C) HIV status and DLBCL subtype.

8.13. Appendix

Supplementary Table 1 Antibodies used for all immunohistochemical stains performed in this study

Antibody (monoclonal)	Clone	Supplier	Antigen retrieval	Dilution	Control
CD10	56C6	Dako	1mM EDTA pH9	1:40	Tonsil
BCL6	PG-B6p	Dako	1mM EDTA pH9	1:20	Appendix
BCL2	124	Dako	1mM EDTA pH9	1:20	Appendix
MUM1	MUM1p	Dako	1mM EDTA pH9	1:50	Appendix
c-MYC	9E10	Invitrogen	10mM Citrate pH6	1:75	Tonsil
Ki-67	Mib-1	Dako	1mM EDTA pH9	1:30	Appendix

9. CHAPTER 4:

Appendices

9.1. Ethics research protocol approval

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences

Human Research Ethics Committee

12 July 2018

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

HREC REF: 441/2018

Division of Haematology
E-5, NGSH

Dear Dr Antel

PROJECT TITLE: DIFFUSE LARGE B-CELL LYMPHOMA IN HIV, AN ANALYSIS BY SUBTYPE AND EBV INFECTION- (MMed-Candidate-Dr S Cassim)

Thank you for your response letter dated 11 July 2018, addressing the issues raised by the Human Research Ethics Committee (HREC).

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 July 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Dr Sumaiya Cassim will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical

Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

9.2. Ethics protocol amendment approval

HUMAN RESEARCH ETHICS COMMITTEE
04 OCT 2018
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

UNIVERSITY OF CAPE TOWN
FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

Form FHS006: Protocol Amendment

HREC office use only (FWA00001637; IRB00001938)		
☑ Approved	☑ Type of review: Expedited	☐ Full committee
This serves as notification that all changes and documentation described below are approved.		
Signature Chairperson of the HREC		Date: 5/10/2018

Note: All major amendments must include a local **PI Synopsis** justifying the changes for the amendment. Please note that incomplete amendment submissions will not be reviewed.

Comments from the HREC to the Principal Investigator.
Note: The approval of this protocol amendment does not grant annual approval. Please complete the FHS016 / FHS017 form for annual approval at least one month before study expiration.

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	04 October 2018		
HREC REF Number	441/2018		
Protocol title	Diffuse large B-cell lymphoma in HIV, an analysis by subtype and EBV infection		
Protocol number (if applicable)	N/A		
Principal Investigator	Dr Katherine Antel		
Department / Office Internal Mail Address	C/O Chantal McCarthy E5 Haematology Clinic		
1.1 Is this a major or a minor amendment? (see FHS006hlp) Major (tick box) Minor (tick box)		☐ Major	☑ Minor
1.2 Does this protocol receive US Federal funding?		☐ Yes	☑ No
1.3 If the amendment is a major amendment and receives US Federal Funding, does the amendment require full committee approval? **Note:** Any protocol amendments for **Full Committee** review MUST be submitted on the monthly HREC submission dates (Please email an electronic copy to hrec-enquiries@uct.ac.za)		☐ Yes	☐ No

2. List of Proposed Amendments with Revised Version Numbers and Dates

Please itemise on the page below, all amendments with revised version numbers and dates, which need approval. This page will be detached, signed and returned to the PI as notification of approval. Please add extra pages if necessary.
Research Protocol (FHS015) V6.0 dated 04 October 2018. Amendment 1: (Title) Diffuse large B-cell lymphoma in a South African population – an analysis by subtype, HIV status, EBV infection and survival. Amendment 2: (Secondary aim) To compare survival outcomes in DLBCL of the South African population with that of international data. Amendment 3: (Data collection methods) Survival outcome.

3. Protocol status (tick ✓)

☐	Open to enrolment
☐	No participants have been enrolled
☑	Closed to enrolment (tick ✓)
☑	Research-related activities are ongoing
☐	Research-related activities are complete, long-term follow-up only
☐	Research-related activities are complete, data analysis only

4. Proposed changes will affect: (tick ✓ all the categories that apply)

	Protocol
☑	Study objectives, design (including investigator's brochure, clinical activities, study length)
☐	Study instruments, questionnaires, interview schedules
☐	Sample size
☐	Recruitment methods
☐	Eligibility criteria (inclusion and exclusion criteria)
☐	Drug/device (composition, amount, schedule, route of administration, combination with other drugs/devices, safety information)
☑	Data collection/ analysis
☐	Principal Investigator. (Please attach revised conflict of interest and PI declaration statements. Refer sections 7 and 8.4 in the New Protocol Application Form FHS013)
☐	Consent form and information sheet
☐	Recruitment materials (e.g. advertisements)
☐	Administrative (e.g. change in sponsor's name, change in contact information)

2. List of Proposed Amendments with Revised Version Numbers and Dates

Please itemise on the page below, all amendments with revised version numbers and dates, which need approval. This page will be detached, signed and returned to the PI as notification of approval. Please add extra pages if necessary.
Research Protocol (FHS015) V6.0 dated 04 October 2018. Amendment 1: (Title) Diffuse large B-cell lymphoma in a South African population – an analysis by subtype, HIV status, EBV infection and survival. Amendment 2: (Secondary aim) To compare survival outcomes in DLBCL of the South African population with that of international data. Amendment 3: (Data collection methods) Survival outcome.

3. Protocol status (tick ✓)

☐	Open to enrolment
☐	No participants have been enrolled
☑	Closed to enrolment (tick ✓)
☑	Research-related activities are ongoing
☐	Research-related activities are complete, long-term follow-up only
☐	Research-related activities are complete, data analysis only

4. Proposed changes will affect: (tick ✓ all the categories that apply)

	Protocol
☑	Study objectives, design (including investigator's brochure, clinical activities, study length)
☐	Study instruments, questionnaires, interview schedules
☐	Sample size
☐	Recruitment methods
☐	Eligibility criteria (inclusion and exclusion criteria)
☐	Drug/device (composition, amount, schedule, route of administration, combination with other drugs/devices, safety information)
☑	Data collection/ analysis
☐	Principal Investigator. (Please attach revised conflict of interest and PI declaration statements. Refer sections 7 and 8.4 in the New Protocol Application Form FHS013)
☐	Consent form and information sheet
☐	Recruitment materials (e.g. advertisements)
☐	Administrative (e.g. change in sponsor's name, change in contact information)

9.3. University of Cape Town, Faculty of Health Sciences – MMed Part III (minor dissertation) guidelines for candidates, supervisors and examiners

	UNIVERSITY OF CAPE TOWN **FACULTY OF HEALTH SCIENCES**	
MMed Part III (minor dissertation) Guidelines for candidates, supervisors and examiners		

The MMed minor dissertation is one of three examination components of the MMed degree. This minor dissertation carries one third of the weight of a full master's dissertation in terms of its credit weighting, i.e. 60 credits (nominally 600 hours of work). In order to register as a specialist in South Africa, the Health Professions Council of South Africa (HPCSA) requires all specialist trainees who register for training after 1 January 2011 to have completed a relevant research study. The MMed Part III fulfils HPCSA research requirements as well as research requirements by the specialties who include a research project as part of their examination process by the Colleges of Medicine of South Africa (CMSA).

Educational aims

The research project should demonstrate that the student:

- can work independently and ethically under supervision (contributions/assistance must be acknowledged);
- is sufficiently acquainted with the relevant literature to provide appropriate motivation for the research question;
- can plan research or clinical audit (write a protocol), which is approved by an assessor group (delegated by the head of department) and ethics committee where relevant, that contributes new or additional data to the collective knowledge base (the specific data has not been presented as part of other research), but need not produce a unique contribution to the scientific literature;
- uses an appropriate method/design/technique and analysis;
- can adequately present and discuss the significance of the results of the study;
- can present the study in an academically acceptable manner.

Type and scope of the research

The following types of studies are acceptable:

- A clinical audit with or without a repeat data collection cycle;
- A systematic review of the literature on its own with extraction and extrapolation of data OR a meta-analysis using recognised research methods (eg Cochrane, PRISMA);
- A research study – pro-/retrospective lab or clinical or database review;
- Description and analysis of a case series or cohort, deemed sufficient to supply new knowledge/data, even if only contextual or exploratory;
- Epidemiological research;
- Health service/systems/education research;
- Qualitative research;

Noting:

- *The sample size* can be limited by time (Registrars have limited time allocated/available to collect data and write it up concurrently with their clinical training) - data collection and write up should be possible to complete within two consecutive or cumulative months.

- *Data analysis* may use simple descriptive statistics alone – more advanced analysis can be used, but the student must demonstrate (in the write up) insight into the choice of analysis.

- The above limitations may be associated with the use of descriptive cohort studies based on medical record review; exploratory or pilot studies with small convenience samples; or audits without a repeat data collection cycle to prove quality improvement (QI). Despite limitations, these studies can provide an adequate basis for learning research methodology and can add new data to the collective knowledge base – they may also provide the basis for further publishable work such as a second audit to complete a full QI cycle. As long as these limitations are appropriately acknowledged, these studies should still be acceptable.

- The topic, study design and scope of research may depend on the particular discipline and must be agreed on in consultation with the supervisor(s). The topic must be approved as being suitable for MMed dissertation by the Departmental Research Committee (DRC) and/or a group appointed for this purpose by the head of department.

Submission formats

The dissertation may be presented in one of three formats:

I: Publication-ready format;
II: Published (or accepted for publication) Paper format
III: Monograph format.

As disciplines differ in their requirements, it is important that the format chosen is acceptable to the discipline and appropriate College within the CMSA.

Research protocol

NOTE: All communication from UCT regarding the MMed and the examination process will occur via student UCT e-mail address – [student number]@myuct.ac.za. Students must also make sure they have username and password and are able to access the PeopleSoft Student Administration Self Service.

Candidates intending to register for the MMed Part III are required to submit a research protocol for approval to their respective Departmental Research Committees (DRC). The research protocol should briefly summarise the existing knowledge on the topic and justify the research question; it should clearly describe the objectives and methodology and should be structured according to the guidelines in Form FHS015. Write a synopsis according to Form FHS014. Complete a new protocol application form FHS 013. All FHS forms are available at http://www.health.uct.ac.za/fhs/research/humanethics/forms.

The candidate must then obtain approval from the UCT Faculty of Health Sciences Research Ethics Committee (HREC) prior to conducting their research. Studies that involve the audit of clinical records or services also require formal HREC approval. Any primary research that is taking place in a provincial or local authority health facility, such as public sector hospitals or clinics, must also be

submitted to the provincial government for approval, after the UCT Research Ethics Committee approval has been obtained. Approval to access public sector facilities for research is needed for all provincial and local authority facilities. There are five points where approval for research can be applied for; Groote Schuur Hospital, Red Cross War Memorial Children's Hospital, Tygerberg Hospital), the local authorities and "all other province". Teaching hospitals and the local authorities approve research projects in-house. "All other province" approvals are done via the Directorate: Health Impact Assessment (Sub-directorate: Research) at provincial head office. If research crosses these boundaries, up to five approvals may be needed. Further details can be found at https://www.westerncape.gov.za/general-publication/health-research-approval-process.
The Provincial Health Research Committee does not approve research proposals itself, but oversees this approval process by reviewing difficult applications on referral.

The proposal contents should comply with requirements stipulated in Form D1a. This full research protocol together with FHS 013, a copy of the HREC approval letter and completed Forms D1 (Protocol approval), D3 (supervisor appointment form), and D1a must be submitted to the postgraduate administration office, for approval by the Professional Masters Committee (PMC) Chair and the Board of the Faculty of Health Sciences, prior to commencement of the research. If the title, aims, objectives or any other aspect of the research change following initial submission, an amendment must be submitted to HREC. **All D-forms are available from the post graduate faculty office or on the UCT Vula Mmed/Mphil site (All registrars and supervisors must be added to this site – your departmental programme manager must send names and email addresses to gregory.doyle@uct.ac.za in order to be added to the site).**

Timelines

Submission of the research protocol for approval should generally be made within the first 12 - 24 months of the registrar programme (this varies between disciplines). Heads of Departments or Divisions should meet with their registrars at least biannually to review progress towards their research project. Unless otherwise stipulated by your Division / Department, the research project should generally be completed by the end of Year 3. For a number of specialties, a dissertation must be submitted before writing the Part II examination. Often the research component of specialist training is only initiated after successful completion of the Part I examination.

Supervisors

The supervisor must: have research experience, ideally a Master's degree, equivalent (eg appropriate publications), or higher; be able relate to the candidate's research project; be available for regular discussion and advice; and be someone with whom the candidate can develop a good working relationship. If the primary supervisor does not have adequate experience, then a secondary supervisor who has appropriate experience will need to be appointed in addition. Supervisors who have not had extensive experience supervising are required to attend a supervisor training course. Where specialised equipment and/or laboratory work is required for the study, the supervisor should assist in facilitating access to appropriate facilities.

The primary supervisor may be based outside the candidate's home department, faculty or university. In such a case, a member of UCT staff will also be required as co-supervisor in addition to the primary supervisor, to serve as a guide and link to UCT faculty and discipline-specific procedures. Primary supervisors retain responsibilities to the candidate and the university until the dissertation process is complete. In addition to the forms mentioned above, the supervisor and student must complete D2a which describes the contractual memorandum of agreement (MOU) between supervisor and student regarding the minor dissertation.

The dissertation

Submission of all formats of the dissertation should include the following:

The title page should contain the candidate's name, dissertation title and the name of the university. It must also state the degree, e.g. Master of Medicine (MMed) in, Medicine, Paediatrics, etc.

The table of contents

The declaration page should include a statement to the effect that the research reported is based on independent work performed by the candidate and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. It must also state that this work has not been reported or published *prior to registration* for the abovementioned degree.

The abstract should summarise the study rationale, methods, results, discussion and conclusion in fewer than 500 words.

Acknowledgements and contributions. This section should acknowledge and describe the support or input from supervisors and other co-author(s) if applicable. In a dissertation derived from work started by others, e.g. analysis of data collected for another project, the origin of the data and the candidate's contribution must be clearly stated. The candidate must complete the dissertation after his/her registration for the degree and therefore under supervision. In a published manuscript from a multi-authored project, the candidate must be first author.

List of Tables
List of Figures
Abbreviations

The remainder of the dissertation must be presented in one of three formats:

I: Publication-ready format;
II: Published (or accepted for publication) paper format
II: Monograph format.

I: Publication format

The dissertation must include a manuscript in publication-ready format. The body of the dissertation must be structured as follows:

Chapter 1: Introduction and Literature review

This section must give the background and context of the research question and must include a review of the literature relevant to the subject matter and methods of the study. The review should summarise and interpret the existing knowledge in the field with relevance to the research setting and should identify knowledge gaps and hence the rationale for the dissertation. This chapter should end with a clear statement reflecting the aims and objectives of the research reported in the publication-ready manuscript. References quoted in this chapter should appear at the end of the chapter, not at the end of the thesis. This chapter should be between 2 000 and 5 000 words.

Chapter 2: Publication-ready Manuscript

This chapter must be presented in the form of a manuscript of an article for a named peer reviewed journal, meeting all the requirements of the "Instructions for Authors" of that journal, including the word count and referencing style. Unless specially motivated, the journal chosen should allow for at least 2000 words (not more than 5000 words) excluding abstract, tables, figures and references. The "Instructions to Authors" of the journal must be appended. The co-authors should be listed in the appropriate order, and each of their contributions to the manuscript stated. The journal chosen for publication must be appropriate to the subject matter of the dissertation and listed in the citation index of the Institute for Scientific Information (ISI) or accredited by the Department of Education http://www.lib.uct.ac.za/medical/index.php?html=/libs/accredjnls.htm&libid=24); *other journals with similar review processes, particularly South African journals may be acceptable if permission is obtained from the PMC Chair after appropriate motivation is provided.*

Note 1: In this format, the candidate need not have submitted the article for publication, nor is the acceptance of the article for publication a requirement for passing the degree. However, the norm is to publish the study with the supervisor(s) as co-author(s), and candidates are strongly encouraged to submit their manuscript for publication after examination of the minor dissertation.

NOTE 2: IF THE RESEARCH IS A FULL SYSTEMATIC REVIEW, THERE IS NO NEED FOR A SEPARATE CHAPTER 1 – THE REVIEW SHULD BE SUBMITTED AS ONE CHAPTER.

Appendices

Append all supporting documents including:

- Questionnaire/data capture instrument(s)
- Consent forms and any related participant information sheets
- Technical appendices, including, if considered necessary, any additional tables not included in the main manuscript for the examiner to have available. These should be accompanied by a brief narrative.
- Official Ethics approval letter from the Faculty Research Ethics Committee (except for a full systematic review) and any other approvals required (e.g. Provincial Government).
- Instructions to Authors of the chosen journal

II: Published (or accepted for publication) paper format

A manuscript that has already been published or accepted for publication in a journal that is listed in the citation index of the Institute for Scientific Information (ISI) or accredited by the Department of Education (*other journals with similar review processes, particularly South African journals may be acceptable if permission is obtained from the PMC Chair after appropriate motivation is provided),* may be submitted if the candidate was the first author, the candidate's contribution was completed under supervision during his/her registration for the degree, and the paper is in line with the educational aims and scope of research described in the first part of this document.

The dissertation must be submitted in similar format to the publication-ready format – the only differences being: a separate literature review is not required; the accepted publication is submitted as a single chapter following the same format as described above under "Chapter 2"; and the reviewer comments from the journal should be attached as an appendix. *When this format is used, the contributions of all the authors must be very clearly stated under a sub-heading in the "Acknowledgments and contributions" section in the first part of the thesis.*

III: Standard monograph format

Some disciplines and constituent Colleges of the Colleges of Medicine of South Africa require a standard monograph presented in a comprehensive and scholarly style to be submitted as part of the examination. The length is typically 16 000 to 20 000 words in length, but may vary. If the length is not stipulated, the monograph should be 6000 – 16000 words, excluding references and tables.

A recommended structure for the body of the dissertation is as follows;

Chapter 1: Introduction and Literature review
(see guidelines above)

Chapter 2: Methods
Material and methods of the study must be fully described and factually presented.

Chapter 3: Results

Chapter 4: Discussion and conclusions

Appendices
(see guidelines above - omit the instructions to authors)

Language and writing

Clear, grammatically correct English is essential.

Supervisors may assist candidates in developing scientific communication skills but they are not required to do detailed editing or correction of spelling, grammar, or style. Training in scientific writing is available at the Health sciences Writing Centre. Registrars need to make an appointment via the website: http://www.writingcentre.uct.ac.za/about/healthsciences

Candidates should refer to Form D4, Guidelines on the Layout and Style of the Dissertation or Thesis. As long as the dissertation is readable and internally consistent, any of a number of styles are acceptable. For a publication-ready manuscript, references should be formatted according to the instructions to authors for the journal selected, and candidates should use the same style throughout their dissertation. For a monograph format manuscript, the Harvard style for referencing is recommended, but not compulsory. For reference management, Refworks or Endnote can be downloaded from the ICTS or UCT library website.

Candidates should look at previous examples of Master's dissertations in the library. Master's dissertations are available in the Health Sciences Library. A search will need to be done to obtain a list of titles and authors. This search can be done using search words (e.g. dissertation, health, health sciences, etc.). The librarian can be asked for assistance. Some of these dissertations are available via: http://www.medical.lib.uct.ac.za/hsl/theses-dissertations

Annual approval

After 1 year, apply to HREC for continuing approval Form FHS016 (for intervention study) or FHS017 (for record review) or submit a study closure form, FHS010, if the study is complete. If registration in MMED III is required for more than one year then complete form D2(b) and submit to Post Grad Office when re-registering.

Submission of dissertations

On completion, the dissertation and a Turn-it-in originality report must be submitted to the Faculty Postgraduate Office. The candidate should inform the Faculty Officer one month in advance of the intention to submit, using Form D8 (Intention to submit) online with PeopleSoft system and should subsequently submit their dissertation using the same system **– guidelines for this process and the use of Turn-it-in are on the Mmed/Mphil Vula Website and detailed guidelines are also available in the UCT student help document: " Digital submission of a thesis/dissertation for examination and library access".** This document is available online at http://www.uct.ac.za/usr/current_students/postgrad/digital_upload_dissertations_theses.pdf

Supervisors will be requested by the Faculty Postgraduate Officer to submit a letter supporting submission, and clearly specifying whether the format of submission, so that the appropriate instructions are sent to the examiners. This letter should be supplied by the primary supervisor. If this supervisor is external, the internal supervisor must be kept informed at every stage of the process.

Please note: In the event that any of your external examiners request a hard copy of your dissertation/ thesis, you will be required to supply this. The Faculty office will inform you should this be necessary.

Specific submission requirements may be set by individual disciplines or constituent Colleges of the CMSA, and registrars are obliged to ensure that their research projects and dissertations meet these specific requirements. UCT Dissertation submission deadlines:

1. March 15th for June graduation
2. August 15th for December graduation

Note on fees: To avoid attracting fees, dissertations need to be submitted before the beginning of the first quarter (first day of academic year), and before the start of the second semester (mid July) to qualify for a 50% fee rebate.

Examiners

The full dissertation will be submitted for examination through the Postgraduate Office to two examiners (nominated by the supervisors and HOD) – at least one examiner must be external to UCT. An internal examiner must not be involved in the research.

It is the supervisors' responsibility to submit names of three potential examiners (or two examiners who have already agreed to examine pending approval of the Post Graduate Office) to the Faculty Officer when the candidate is ready to submit. Appointment of examiners from outside South Africa is encouraged. These nominations need to be approved by the Deputy Dean: Postgraduate Affairs on behalf of the Faculty Board and submitted to the Faculty Board for ratification via a Dean's Circular.

Details required for each examiner are: academic qualifications, postal and/or physical address, telephone and fax numbers and e-mail address, and one paragraph description of their standing in the relevant field (drawn from their CV if need be). The examiners will be sent a copy of these guidelines as well as a guideline for marking. *The candidate may not be informed of the identity of the examiners*. After the outcome of the minor dissertation has been finalised, the examiners' identities are made known if the examiners have indicated that they do not object to this.

Publication agreement

The university has a moral responsibility to publish all research undertaken when publication is stated as an anticipated output. A candidate who fails to submit a manuscript to a journal for publication within 1 year of submission of their thesis, must accept that their supervisor(s) are entitled to publish their data on their behalf, with the student as co-author - this should be stated in the memorandum of understanding.

9.4. Pathology journal author guidelines

PATHOLOGY

The Journal of the Royal College of Pathologists of Australasia (RCPA)

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

DESCRIPTION

Pathology is the official journal of the Royal College of Pathologists of Australasia (RCPA). It is committed to publishing peer-reviewed, original articles related to the science of pathology in its broadest sense, including anatomical pathology, chemical pathology and biochemistry, cytopathology, experimental pathology, forensic pathology and morbid anatomy, genetics, haematology, immunology and immunopathology, microbiology and molecular pathology.

In addition to original articles, Reviews, Rapid Communications and Correspondence are published.

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GUIDE FOR AUTHORS

INTRODUCTION

Types of Paper

Contributions falling into the following categories will be considered for publication: original research articles, reviews, rapid communications, and correspondence.
Please ensure that you select the appropriate article type from the list of options when making your submission. Authors contributing to special issues should ensure that they select the special issue article type from this list.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of submitted manuscripts.

Before a decision has been made on a manuscript (ie, while it is still under review): Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Journal Managers will inform the Journal Editors of any such requests and review of the manuscript will be suspended until authorship has been agreed.
After a decision has been made on a manuscript: Any requests to add, delete, or rearrange author names in an article where the review process is complete and a decision has been made will not be considered or granted.

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You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. If the funding source(s) had no such involvement then this should be stated.

Language and language services

Please write your text in good English (British usage is preferred).

Submission

Submission to this journal proceeds totally online. Use the following guidelines to prepare your article. Via the homepage of this journal you will be guided stepwise through the creation and uploading of the various files. The system automatically converts source files to a single Adobe Acrobat PDF version of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e mail and via the author's homepage, removing the need for a hard-copy paper trail. If you are unable to provide an electronic version, please contact the editorial office prior to submission (e-mail: journal@rcpa.edu.au; telephone: +61 2 8356 5809)

Additional Information
Tables and figures may be presented with captions following the main body of the manuscript. Figures should additionally be uploaded as high-resolution files.

PREPARATION

Peer review
This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final.

Use of word processing software
It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts. Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text.
To avoid unnecessary errors, you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure
Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly, for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information
•***Title***. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
•***Author names and affiliations***. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a superscript number immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each author.
•***Corresponding author***. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.
•***Present/permanent address***. If an author has moved since the work described in the article was done, or was visiting at the time, a "Present address" (or "Permanent address") may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Asterisks and related symbols are used for such footnotes.
•***Running title***. The running title should not exceed 50 characters.

Abstract
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should not be included. Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords
Authors are invited to submit keywords associated with their paper.

Abbreviations
Define abbreviations at their first mention in the text. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Nomenclature and units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other quantities are mentioned, give their equivalent in SI.

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp.

Footnotes

Footnotes should be used sparingly and should be indicated with symbols in the following order: * ‖ **, etc.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
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TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g. ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References
Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text in parentheses including the authors names and "Unpublished results" or "Personal communication". Citation of a reference as "in press" implies that the item has been accepted for publication and should be included in the reference list.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references should be included in the reference list.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

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Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript.

Reference style
Consecutive Arabic numbers must be used in superscript form to indicate references in the text, tables and legends. The full references should be listed sequentially in the order in which they are first mentioned, and presented following the text of the manuscript.

Examples of different types of references are given below. Note: the first three authors must be given before *et al*. is used.

***Examples*:**
Journal articles
Goodwin CS, Smith BC. Computer printing and filing of microbiology reports. *J Clin Pathol* 1976; 29: 543-52
Pages from a book
Eisen HN. Immunology: An *Introduction to Molecular and Cellular Principles of the Immune Response*. 5th ed. New York: Harper & Row, 1974; 406-9.
A chapter of a book
Cassidy JT, Petty RE. *Textbook of Pediatric Rheumatology*. 2nd ed. New York: Churchill Livingstone, 1990; Chapter 3, Basic concepts of drug therapy.
A contribution to a book
Anderson RJ, Schrier RW. Acute renal failure. In: Brunswald E, Kurt J, Petersdorf RG, editors. *Harrison's Principles of Internal Medicine*. 11th ed. New York: McGraw-Hill, 1987; 1149-55.

Journal abbreviations source
Journal names should be abbreviated according to Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html.

Data visualization
Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research.

Research data
This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation.

Data linking
If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that gives them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system.

For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

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This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols, and methods) associated with your manuscript in a free-to-use, open access repository. During the submission process, after uploading your manuscript, you will have the opportunity to upload your relevant datasets directly to *MendeleyData*. The datasets will be listed and directly accessible to readers next to your published article online.

Data statement
To foster transparency, we encourage you to state the availability of your data in your submission. This may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you will have the opportunity to indicate why during the submission process, for example by stating that the research data is confidential. The statement will appear with your published article on ScienceDirect.

Submission requirements for specific article styles

Reviews
Submission of reviews on topical issues is strongly encouraged. If accepted for publication, reviews are published in the next available issue, usually within 5 months of acceptance.

Rapid communications
The purpose of the Rapid Communication is timely disclosure of key elements of a study. Submissions should cover sufficient background to put the new information into context and justify urgent publication. Rapid Communications are handled as expeditiously as possible with material being published within 5 months of receipt by the Editor. To facilitate rapid evaluation, it is essential that authors submit written MATERIALS and METHOD information to assist in establishing if the work merits rapid publication.

Case reports
Clinical case reports are mostly published as Correspondence and must be of exceptionally unusual presentation or interest. Please note that *Pathology* no longer accepts single case studies involving DNA mutations.

Correspondence
Correspondence may be refereed by an Editorial Board member or published at the discretion of the Editor. The title must be short and there should be no summary, keywords or subheadings. A brief introduction (a few sentences) should be followed by a succinct report and discussion. A formal, lengthy literature review is not necessary. The article should: (1) have no more than five authors; (2) be no more than 1500 words in total; (3) have a maximum of two figures and one table; (4) have a maximum of 12 references; and (5) report something novel that is not just of regional interest.

Submission checklist

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