Retrospective analysis of the Hans algorithm and BCL2 expression in HIV positive and negative patients with diffuse large B-cell lymphoma.

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg; in partial fulfilment for the degree of Master of Medicine (Anatomical Pathology).

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DECLARATION

I, Sarah Kate Hooper, declare that this research report is my own work. It is being submitted in partial fulfilment for the degree of Master of Medicine in Anatomical Pathology. It has not been submitted before for any degree or examination at this or any other university.

Sarah Kate Hooper

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DEDICATION

In memory of my mother and grandmother, Jennifer Ann Taryn Watkins and Jean Alison Watkins, who nurtured my learning with wisdom and love.

ABSTRACT

Introduction:

Diffuse large B-cell lymphoma (DLCBL) is the most commonly occurring non-Hodgkin lymphoma. Several DLBCL variants are described, with varying morphological, genetic and molecular characteristics, as well as clinical features. With the use of a wide range of diagnostic tools,these tumours have been usefully classified and prognosticated.

The cell of origin (COO) theory and its immunohistochemical interpretation by the Hans algorithm is currently regarded as the most useful immunohistochemical tool for classification and prognostication. It recapitulates whole genome sequencing by approximately 80%, separating DLBCL into a germinal centre (GCB) and an activated B cell (ABC) phenotypes. Tumours of germinal centre phenotype have a significantly better prognosis than those of activated B cell phenotype.

The B-cell lymphoma 2 (*BCL2*) family of genes encode for a group of proteins that regulate cellular apoptosis. Twenty-five proteins are included in this group; one of these is known as BCL2. The role of *BCL2* upregulation as a predictor of disease prognosis in DLBCL is controversial. Studies yield inconclusive and sometimes conflicting results, with some authors demonstrating adverse outcomes in *BCL2* expressing neoplasms of the ABC DLBCL subgroup.Other studies have shown BCL2 protein to be a marker of poor prognosis in germinal centre phenotype DLBCL but not in activated status phenotype DLBCL.

In HIV positive individuals, DLBCL is recognised to be a more aggressive disease than in HIVnegative individuals.

Methods and Aims:

The interrelationship of these various factors forms the basis of this study, which is to establish the immunohistochemical classification of DLBCL according to the Hans algorithm, whether thetumours express BCL2 immunophenotypically and whether these two prognostic factors have anyrelationship to the HIV status.

Results:

The majority of patients included in the study were HIV positive (63/72, 87,5%) and the minority were negative (9/72, 12,5%). Consensus microscopy of the cases determined the majority to be of the centroblastic morphological subtype (62/72, 86,1%) with others classified as anaplastic (10/72, 13.9%). No immunoblastic or "other" morphological subtypes were identified. Of the cases evaluated, the majority were of GCB by the Hans immunohistochemical panel (49/72, 68%), and the minority were ABC subtype (23/72 32%). Of the HIV positive cases, 37.9% (25/66) were BCL2 negative and 62.1% (41/66) were BCL2 positive. Of the HIV negative cases, 0.00 % (0/9) were BCL2 negative and 100% (9/9) were BCL2 positive. A statistically significant difference in BCL2 positivity rates in HIV positive and HIV negative patients was seen (100% versus 37.9%) (two-tailed Fisher's Exact test, p-value = 0.02).

Conclusion:

Our findings demonstrate a higher prevalence of DLBCL in HIV infected patients compared to uninfected patients. We were not able to find a reliable conclusion as to the relationship between COO by Hans algorithm and HIV status. We showed a higher correlation of BCL2 positivity with ABC COO subtype, over GCB subtype. We were able to demonstrate a significant association between BCL2 positivity and HIV uninfected DLBCL patients, compared to a markedly lower association in HIV infected patients.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABC: Activated B Cell

ART: Antiretroviral therapy

ATP: Adenosine triphosphate

BCLU: B cell lymphoma unclassifiable

BCL2: B cell lymphoma 2

BCL6: B cell lymphoma 6

BAK: BCL2 antagonist killer 1

BAX: BCL2-associated X protein

BOK: B CL2-related ovarian killer

CDKN1A Cyclin kinase inhibitor 1A

CDKN1B Cyclin kinase inhibitor 1B

COO: Cell of origin

DISC: Death-inducing signalling complex

DLBCL: Diffuse large B cell lymphoma

DNA: Deoxyribose nucleic acid

EBV: Epstein Barr Virus

GCB: Germinal centre B

GEP: Gene expression profiling

H&E: Haematoxylin and Eosin

HGBCL-DH: High grade B cell lymphoma-double hit

HGBCL-TH: High grade B cell lymphoma-triple hit

IHC: Immunohistochemistry

IPI: International prognostic index

LDH: Lactate Dehydrogenase

MOMP: Mitochondrial outer membrane permeabilization complex

MUM1: Multiple myeloma oncogene 1

NHLS: National Health Laboratory Service

NOS: Not otherwise specified

PCR: Polymerase chain reaction

R- CHOP: Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone

SMAC: Second mitochondria-derived activator of caspases

SMI: Small molecule inhibitor

UV: Ultraviolet

WHO: World Health Organisation

LIST OF FIGURES

Chapter 3:

Chapter 4:

Chapter 5:

LIST OF TABLES

Chapter 5:

CHAPTER 1- INTRODUCTION

Diffuse large B cell lymphoma (DLCBL) is the most commonly occurring non-Hodgkin lymphoma and makes up around 40 percent of lymphoma cases (Chao et al., 2015). It is the fifth most common cancer worldwide (Gouveia et al., 2012), and in South Africa is the ninth most common in men, and the tenth in women (National Cancer Registry, South Africa). The DLBCL group of neoplasms is characterised by a diffuse proliferation of large and mature B-cells. They arise in lymph nodes in 60 percent of cases, with 40 percent occurring in solid organs, primarily the gastrointestinal tract but also the central nervous system, the skin, bone marrow, salivary gland, liver, lung and kidney. Secondary bone marrow involvement is found in 11% to 27% of cases, but peripheral blood involvement is rare. (Gouveia et al., 2012).

Several DLBCL variants are described, with varying clinical, morphological, immunophenotypic, genetic and molecular characteristics (Swerdlow et al., 2017). The advent of next generation whole genome sequencing has allowed for major advances in the categorisation and prognostication of these cancers based on distinct genetic nuances. This has been the basis of the cell of origin (COO) classification of DLBCL. These genetic nuances have been found to be recapitulated with 80% accuracy by the application of an immunohistochemical panel known as the Hans algorithm (Hans et al., 2004).

The Hans algorithm is currently regarded as the most useful tool interpreting the COO classification of DLBCL. It is important for prognostication, separating DLBCL into a germinal centre phenotype (GCB) and an activated B-cell phenotype (ABC) based on an immunohistochemical panel. The panel includes immunohistochemical markers CD10, BCL6, and MUM1. GCB DLBCL has been shown to have a better overall survival than ABC DLBCL (Hans et al., 2004).

The variants of DLCBL arise from mature B lymphocytes at different stages in their development and differentiation. Mutations most frequently occur in the germinal centres of the secondary lymphoid follicle after antigen presentation to the B-lymphocyte. The common mutations in DLBCL include translocations and amplifications of *BCL2* and *MYC*, point mutations of *P53,* promotor region changes in *BCL6,* and somatic hypermutation in *BCL6, MYC, PAX5* and *PIM1* (Gouveia et al., 2012).

In DLBCL, BCL2 protein expression has been well established as a predictor of chemotherapy resistance and poor treatment response in routinely used chemotherapeutic regimens. The role of *BCL2* as a predictor of overall disease prognosis in this group is still controversial. Studies yield inconclusive and sometimes conflicting results, with some authors only demonstrating adverse outcomes in the BCL2 expressing neoplasms of the ABC DLBCL subgroup (Amen et al., 2007; Wu and Keating, 2006). Two studies showed BCL2 protein to be a marker of poor prognosis in germinal centre B-cell DLBCL (GCB) but not in activated B-cell DLBCL (ABC) (J. et al., 2011; Visco et al., 2013).

Human immunodeficiency virus (HIV) infection status imparts a further dimension to the prognostic prediction in cases of DLBCL. DLBCL is recognised to be a more aggressive disease with an inferior clinical outcome in HIV infected compared with HIV uninfected individuals. The pathogenesis of DLBCL in the context of HIV infection more frequently involves over-expression of transcriptional regulators *cMYC*, *BCL6* and *MUM1*. Elevated *cMYC* expression in HIV-related DLBCL has been shown to predict inferior survival and may explain the more aggressive clinical course of these lymphomas (Chao et al., 2010).

The interrelationship between the GCB and ABC immunophenotype as determined by the Hans algorithm, BCL2 expression by immunohistochemistry and HIV status in the pathogenesis of

DLBCL is complex. This study aimed to provide an epidemiological appreciation of the relationship between these three factors in a cohort of DLBCL patients in Charlotte Maxeke Academic Hospital, Johannesburg.

CHAPTER 2- AIMS AND OBJECTIVES

2.1 Aims

To determine the association between the COO classification of DLBCL (germinal centre B-cell phenotype / activated B-cell phenotype) by Hans algorithm, and BCL2 immunohistochemical expression in HIV positive and HIV negative individuals.

2.2 Objectives

1. To source a cohort of diffuse large B-cell non-Hodgkin lymphoma from HIV positive and HIV negative individuals.

2. To classify the tumours according to cell of origin by the Hans algorithm into germinal centre type or activated B-cell type by retrospective review of CD10, BCL6 and MUM1 immunohistochemistry.

3. To establish BCL2 immunohistochemistry findings for the cases.

4. To assess the relationships among Hans algorithm subtype, BCL2 expression and HIV status.

CHAPTER 3- LITERATURE REVIEW

3.1 Hans algorithm and DLBCL

DLCBL is the most commonly occurring non -Hodgkin lymphoma and makes up 40 percent of lymphoma cases (Chao et al., 2015). It is the fifth most common cancer worldwide (Gouveia et al., 2012), and in South Africa is the ninth most common in men, and the tenth in women (National Cancer Registry, South Africa).

The DLBCL group of neoplasms is characterised by a diffuse proliferation of large and mature B-cells. DLBCL usually arises as a primary malignancy but can also arise from an indolent lymphoma, such as follicular lymphoma, chronic lymphocytic leukaemia, marginal zone lymphoma or nodular lymphocytic predominant Hodgkin lymphoma.

The DLBCL group of neoplasms are characterised by a diffuse proliferation of large and mature B-cells. The nucleus of these cells is usually larger than the size of a macrophage or endothelial cell nucleus. These neoplasms arise in the lymph node in 60 percent of cases. The remaining 40 percent are extra-nodal, occurring in solid organs, primarily the gastrointestinal tract, but also the central nervous system, the skin, bone marrow, salivary, liver, lung, and kidney. Bone marrow involvement is found in 11% to 27% of cases, but DLBCL rarely infiltrates the peripheral blood (Gouveia et al, 2010).

Several DLBCL variants are described, with varying clinical, morphological, immunophenotypic, genetic and molecular characteristics (Swerdlow et al., 2017). The advent of next generation whole genome sequencing has allowed for major advances in the categorisation and prognostication of these cancers based on distinct genetic nuances. In combination with morphology, immunohistochemistry, flow cytometry, polymerase chain reaction, and tissue microarray, these tumours have been usefully classified and prognosticated. These advances have enhanced the widely used International Prognostic Index (IPI) model in determining disease outcomes and prognosis (Schneider et al, 2011).

Gene expression profiling comparing normal and DLBCL lymphocytes identified three unique genetic signatures, denoting three unique subtypes of disease based on cell of origin (COO); the germinal centre B-cell (GCB)-like subtype, which resembles the gene expression profile (GEP) of normal GCBs, the activated B-cell (ABC)-like subtype, which resemble normal ABCs, and unclassifiable disease in the remaining 10%-15% of samples (Alizadeh et al., 2000). ABC subtype shows NF-kB activation and recurrent mutation of *MYD88* and *CD79B* (Knittel et al, 2016),

while GCB subtype reveals more frequent rearrangement and recurrent mutation of *BCL2*, *TNFRSF14* (Dubois et al., 2016), *EZH2* (Morin et al., 2010), and *GNA13* (Morin et al, 2013).

While COO was first identified by GEP, this assay is expensive with limited availability and hindered by the need for fresh frozen tissue. (Lui et al, 2019).

These genetic nuances have been observed to be recapitulated with 80% accuracy by the application of an easily accessible immunohistochemical panel known as the Hans algorithm (Hans et al., 2004). The Hans algorithm is currently regarded as the most useful tool for prognostication, separating DLBCL into a GCB and an ABC subtype based on an immunohistochemical panel. The panel includes immunohistochemical markers CD10, BCL6, and MUM1. DLBCL cases which express CD10 or cases that express BCL6 and are negative for MUM1 are classified as GCB phenotype. Cases that express MUM1 with or without expression of BCL6 are assigned to the ABC subtype. (Hans et al, 2004).

Tumours of germinal centre phenotype have a significantly better prognosis than those of activated B cell phenotype (Hans et al, 2004).

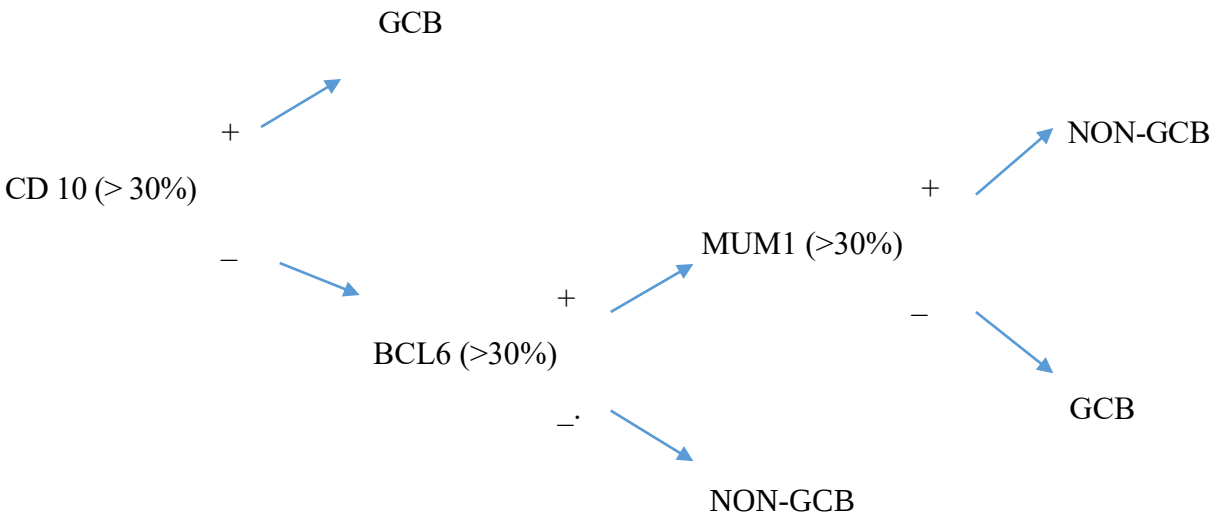


Figure 3.1. Schematic diagram of the Hans algorithm

3.2 BCL2 and DLBCL

The variants of DLCBL arise from mature B lymphocytes at various stages in their development and differentiation. These mutations most frequently occur in the germinal centres of the secondary lymphoid follicle after antigen presentation to the B lymphocyte. The common mutations occurring to promote a malignant cell proliferation include translocations and amplifications at *BCl2* and *MYC*, point mutations at *p53*, promotor region changes in *BCL6*, and somatic hypermutation in *BCL6, MYC, Pax5* and *Pim1* (Rodrigues et al, 2010).

The homeostasis of normal cell populations relies on the delicate balance between cell proliferation and cell death. This is true for encapsulated organs, as well as the circulating cells that comprise the haematopoietic system (Stanley et al, 2018). Oncogenes are genes which, when mutated, will lead to either increased cell proliferation or decreased cell death. Through mutation these genes become either gain-of-function oncogenes or loss-of-function oncogenes.

Oncogenes are divided into three broad functional categories. The first category consists of the growth and proliferation genes (for example *MYC, Ras*) which gain function when mutated and lead to increased downstream signalling of transcription factors. The second category consists of the tumour suppressor genes (for example *p53, Rb*) which lose function and therefore no longer perform their role as gatekeepers of abnormal transcription and DNA replication. And the third category is composed of the programmed cell death genes (*BCL2, p53*) which, in most cases, lose their function as pro-apoptotic genes (Stanley et al, 2018).

It is important to note that in the entity of high-grade lymphoma, the mechanism of genetic insult plays a key role in prognosis and outcome. Depending on whether the genetic insult is a translocation, amplification, copy number variation, point mutation, or somatic hypermutation, varying amounts of protein products will be expressed (Sesques et al, 2017). Translocations and amplifications have the most significant effect on the expression of the oncogenic proteins *BCL2, MYC and BCL6*. This is the reason that fluorescent in situ hybridisation is the gold standard in prognosticating and detecting these neoplasms. Immunohistochemistry gives a good indication of protein expression but does not indicate the mechanism under which this has occurred, which is important in the diagnosis of high grade B cell lymphoma-double hit (HGBCL-DH).

The B-cell lymphoma 2 (*BCL2*) family of genes encode for a group of proteins that regulate cellular apoptosis. Twenty-five proteins are included in this group; one of these is known as BCL2. Apoptosis is a form of genetically mediated programmed cell death. Well-regulated apoptosis is fundamental to normal tissue homeostasis, and cellular and tissue response to injury.

Dysfunctional apoptosis is implicated in many disease processes including oncogenic processes, degenerative disorders, and tissue ischaemia (Siddiqui et al, 2015). BCL-2 proteins are either pro-apoptotic, or anti- apoptotic in function. While the term *BCL2* denotes the entire family of proteins, one of these proteins is called BCL2 in its own right and will be the main focus of the discussion in its role as an inhibitor of apoptosis and as an oncogene in DLBCL.

On a biochemical cellular level, two main pathways of apoptosis have been described in detail. These are the extrinsic, or cell death pathway and the intrinsic, or mitochondrial pathway (Igney et al, 2002). A third, less understood, pathway has been identified which is T-cell mediated and is signalled by granzyme A or B (Martinvalet et al, 2005).

The extrinsic pathway, the intrinsic pathway and the granzyme B pathway all converge and activate the common execution pathway. This pathway prepares the cellular contents for apoptosis by the activation of caspase 3. Caspase 3 activates intracellular endonuclease and protease and cleaves the cytoskeleton into portions ready to be packed into smaller cell membrane bound vesicles known as apoptotic bodies. Apoptotic bodies are then taken up by phagocytes of the immune system (Siddiqui et al, 2015). Granzyme A acts independently of caspases to degrade single stranded DNA (Martinvalet et al. 2005).

The extrinsic and intrinsic pathways are so named owed to the mechanisms by which they are activated, and do not have different outcomes as they converge on the common execution pathway. The intrinsic pathway is dependent on intracellular distress signals issued primarily to the mitochondria, from where pro-apoptotic signalling chemicals are released, including the BCL2 family of pro-apoptotic proteins. The extrinsic pathway is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of a death-inducing signalling complex (DISC).

The BCL2 family of proteins are components of the intrinsic pathway. The pathway is activated by intracellular sensors responding to stressors. The stressors include DNA damage, loss of growth hormone signalling, the unfolded protein response, and increased numbers of intracellular reactive oxygen species (Hodgkiss, et al 2013). These injurious mechanisms cause the mitochondrial membrane to become more permeable leading to the release of pro-apoptotic proteins into the cytosol from the intermitochondrial membrane space (Hodgkiss, et al 2013). These proteins include second mitochondria-derived activator of caspases (SMAC) and cytochrome C. SMAC binds to and deactivates the proteins that normally inhibit apoptosis by

deactivating caspases. Through this process the intrinsic pathway switches off the inhibitors of apoptosis. This affects mitochondrial membrane permeability.

Cytochrome C is released from mitochondria through the mitochondrial outer membrane permeabilization (MOMP) complex (Green et al, 2004), transiently formed in the outer mitochondrial membrane during the intrinsic pathway activation process.

Cytochrome C binds with Apoptotic protease activating factor – 1 (*Apaf-1*) and ATP. These in turn, bind with pro-caspase-9 to form an apoptosome which cleaves the pro-caspase into its active form of caspase-9, which in turn activates the caspase-3 and the execution pathway.

The interactions between anti-apoptotic and pro-apoptotic proteins of the BCL2 family of proteins either inhibit or activate MOMP, determining the outcome of apoptosis or cell preservation.

The mammalian B cell lymphoma-2 (BCL2) family of proteins consists of both pro- apoptotic and anti- apoptotic proteins which interact with one another in complex molecular dynamics to determine homeostasis in apoptosis pathways.

BCL2 antagonist killer 1 (BAK), BCL2-associated X protein (BAX), and possibly BCL2- related ovarian killer (BOK) are termed the effectors. They mediate MOMP and are pro-apoptotic. BCL2, BCL-xL, BCL-w, Myeloid cell leukaemia 1 (MCL-1), and BCL2-related gene A1 (A1) inhibit MOMP and are anti-apoptotic (Moldoveanu et al, 2015). A subfamily of BCL2 are the BH3-only proteins which regulate the other two classes. When there is genetic dysregulation of the homeostasis of expression of these proteins, oncogenesis can occur.

The *BCL2* gene is located at chromosome 18q21.33. It consists of 3 exons (Javadaker et al, 2017). Gene rearrangements, translocations and point mutations can all occur at this locus and cause over or under expression of this gene and its protein products in B lymphocytes and other cells in which it is expressed.

The loss of homeostasis in the *BCL2* gene favours oncogenesis through the inhibition of the timeous apoptosis of tumour cells, allowing for prolonged cell survival, replication and mutagenesis. BCL2 inhibits apoptosis by binding to and neutralizing activated proapoptotic BCL2 family members, including the mitochondrial outer membrane permeabilizers, BAX and BAK, as well as the intracellular stress sensors BIM and Puma that activate BOX and BAK (Courier et al, 2015).

BCL2 is a novel proto-oncogene in that in that it localises to mitochondria rather than the nucleus, as well as only affecting apoptosis with no effect on cell proliferation (Stanley et al, 2018). Because of this, often, such as in diffuse large B cell lymphoma (DLBCL), these effects need to occur in combination with the effects of other oncogenes which promote proliferation (such a *MYC* and *BCL6* in DLBCL) for a malignancy to arise.

The most common mutation affecting *BCL2*, is a translocation between chromosome 14 and 18 (q32;q21). This translocation juxtaposes the BCL2 and immunoglobulin transcription factor locus. Chromosome 18 gains material which leads to increased *BCL2* transcription and BCL2 protein expression (Galteland et al, 2005). This is by far the most frequently occurring translocation in lymphoid tumours. It occurs in 80 percent of follicular lymphomas and 20 percent of diffuse large B cell lymphomas (Fukuhara et al, 1979). In the WHO category of DLBCL, a separate entity of high grade B cell lymphoma- double hit (HGBCL-DH) was described in 2008. These lymphomas have *MYC* and *BCL2* and/or *BCL6* rearrangements (Swerdlow et al, 2016).

BCL2 is over-expressed in the above-mentioned lymphomas. It is also expressed in chronic lymphocytic leukaemia, plasma cell dyscrasia and non-lymphoid tumours such as breast, prostate, lung and liver neoplasms. BCL2 is also a normal finding in healthy T lymphocytes, as well as the B lymphocytes of the mantle zone of lymphoid follicles. The above entities all share the feature of being immunohistochemically positive for BCL2 protein. It is important to note that this over-expression does not arise from the t (14, 18) translocation in every case.

In DLBCL, BCL2 protein expression has been established as a predictor of chemotherapy resistance and poor treatment response in routinely used chemotherapeutic regimens. Increased expression of BCL2 leads to resistance to chemotherapeutic drugs and radiation therapy. BCL2 protects cells from a wide range of cytotoxic insults, including cytokine deprivation, ultraviolet (UV) - and γ-irradiation, and chemotherapeutic drugs.

However, the role of *BCL2* as a predictor of overall disease prognosis in this group is still controversial. Studies yield inconclusive and sometimes conflicting results, with some authors only demonstrating adverse outcomes in the BCL2 expressing neoplasms of the ABC DLBCL subgroup (Amen et al, 2007; Wu et al, 2006). Two subsequent studies showed BCL2 protein to be a marker of poor prognosis in GCB DLBCL but not in a-ABC DLBCL (Iqbal et al, 2011; Visco et al, 2013). Another showed BCL2 expression as a determinant of a poorer prognosis and more aggressive course of disease in DLBCL (Sesques et al, 2017).

3.3 BCL2 and Double and Triple hit lymphoma

High-grade B-cell lymphomas-double hit (HGBCL-DH) are a relatively recently and separately described entity of aggressive non-Hodgkin lymphoma characterised by *MYC* and *BCL2* and/or *BCL6* re- arrangements. Because of their double, and sometimes triple mutation genetic makeup they are often referred to as double-hit lymphomas, and sometimes, triple-hit lymphomas. Ninety-three percent of these lesions arise from the germinal centres of lymphoid follicles (Ghandi et al, 2013).

The 2016 revised World Health Organisation (WHO) Classification of Lymphoid Tumours, described this group as a separate entity, marked out for its aggressive course and as having a notably poor prognosis and chemotherapy treatment response. It includes tumours in the diffuse large B cell lymphoma group, high grade B cell lymphomas and certain Burkitt lymphomas. The new WHO classification also assisted in better classification of the 2008 version of the B-cell lymphoma unclassifiable (BCLU) group with features intermediate between DLBCL and Burkitt lymphoma.

There are no distinguishing histological features to these tumours. Most are highly aggressive, with cells that are medium to large in size and associated with a high proliferative index on Ki67 staining and have a mature B-cell immunophenotype (Sesques et al, 2017). Other general and more variable morphological features include monomorphism or pleomorphism, a classic 'starry sky' pattern, diffuse growth, cytoplasmic eosinophilia, necrosis, mitotic activity, apoptosis, vesicular nuclei, lobed nuclei, a prominent nucleolus, endothelial proliferation, stromal sclerosis, and infiltration of the adjacent soft tissues (Oliviera et al, 2017).

More than 80 percent of patients with HGBCL-DH have translocations in both *MYC* and *BCL2* (Aukema et al, 2011). The remainder have *MYC* and *BCL6* translocations and usually express BCL2 protein despite not having necessarily having a *BCL2* translocation (Li et al, 2016).

Most HGBCL-DH are initiated by a *BCL2* translocation that leads to the over expression of the BCL2 protein and inhibition of apoptosis as already discussed.

The *MYC* gene is located at chromosome 8 q24. It encodes a transcription factor, which under the right genetic, epigenetic and microenvironmental setting can become over- expressed and induce the oncogenesis of an aggressive lymphoma. *MYC* holds a pivotal role in cellular metabolism, the synthesis of proteins, cellular differentiation, the renewal of stem cells, mRNA and micro-RNA regulation and the cellular stress response (Meyer et al, 2008). Downstream of *MYC*, are the

cyclin-dependent kinases which control cell cycle progression. In addition, *MYC* increases the expression of tumour suppressor *TP53* and the proapoptotic protein BIM. While one would expect these changes to be protective against cell proliferation, in the case of HGBL-DH, this leads to genomic instability, more somatic mutations and the counter-intuitive outcome of a malignancy (Sesques et al, 2017). The overall effect of the translocation at *MYC* is vastly increased transcription, cell proliferation and genomic instability (Sesques et al, 2017).

In addition to the primary role of BCL2 as an inhibitor of apoptosis and promotor of cell survival, BCL2 induces tumour resistance to chemotherapy and enhances and supports the effects of other oncogenes. BCL2 and MYC have a particularly synergistic working relationship in oncogenesis. One of the transcription products of *MYC* is a protein called BIM which is one of the pro-apoptotic proteins of the broader BCL2 family of proteins. In the absence of BCL2, BIM would bind to effector proteins BAX or BAK and cause mitochondrial depolarisation and apoptosis (Sesques et al, 2017). The overall consequence of this relationship is an enhanced and relentlessly aggressive promotion of tumour growth.

BCL6 is a transcription factor which, under normal circumstances, plays a significant role in the germinal centre reaction (Basso et al, 2012). When a translocation or other mutation occurs at BCL6, its homeostasis becomes dysregulated, and this interferes with B cell differentiation in the germinal B cells and confers further genomic fragility and oncogenesis. (Sesques et al, 2017).

It is important to note that in the entity of high-grade lymphoma, the mechanism of genetic insult plays an important role in prognosis and outcome. Depending on whether the genetic insult is a translocation, amplification, copy number variation, point mutation, or somatic hypermutation, varying amounts of protein products will ultimately be expressed (Sesques et al, 2017). Translocations have by far the most significant effect on the expression of the oncogenic proteins *BCL2*, *MYC* and *BCL6*.This is the reason that fluorescent in situ hybridisation is the gold standard in prognosticating and diagnosing these neoplasms. Immunohistochemistry gives a good indication of protein expression but does not indicate the mechanism under which this has occurred, which is important in the diagnosis of HGBCL-DH.

The clinical significance of the entity of HGBCL-DH is its dismal prognosis, poor response to therapy and propensity for relapse. Patients with HGBCL-DH frequently present with extra-nodal involvement, particularly of the central nervous system.

There is no standardized approach to the chemotherapy of these cancers. The role of first line stem cell transplantation in these patients is poorly defined but shows some potential. Patients with HGBCL-DH have a median overall survival that ranges from 4.5 months to 34 months, compared with a median 2-year overall survival of 70% for patients with DLBCL treated with R-CHOP (Ashley et al, 2016).

3.4 BCL2 and Targeted Chemotherapy

BCL2 has proven of interest and potential in novel molecular cancer therapy development. The ideal aim of effective therapy is to eliminate the anti-apoptotic function of BCL2. Many trials have been conducted and are currently underway at various phases to develop these drugs. The most promising field of research lies in the development of small molecule inhibitors (SMIs). SMIs are organic molecules of low molecular mass (usually < 750 Dalton) (Azmi et al, 2010). A very simplified explanation of their function is that they are able to target and bind to small molecular binding sites within the BCL2 protein. These sites would otherwise bind to pro-apoptotic proteins from the BCL2 family – such as BAX – in the process of blocking their function and hindering apoptosis. By blocking these sites, BCL2 is essentially "switched off" and rendered indolent. The overall concept is that rather than attempting to eliminate BCL2, the chemotherapy inactivates it. This contrasts with preceding trends in the development of BCL2 targeted chemotherapy which included silencing strategies using antisense technology to diminish BCL2 expression levels, as well as antibody directed therapy against BCL2 (Azmi et al, 2010). Both experimental therapies faced extensive challenges and disappointing results. SMIs show far more promise.

3.5 HIV and DLBCL

DLBCL can preferentially occur in patients with immunodeficiency, such as in solid organ transplantation recipients or in patients infected with HIV who have low CD4 counts. (Testoni et al, 2015).

Twenty percent of the world's HIV infected individuals reside in South Africa; this is the highest prevalence of infection worldwide (Joint UNAIDS, 2019). An estimate of 7.97 million people were said to be infected with HIV in the South African population in a 2019 study. This study

demonstrated a prevalence of approximately 19.1% amongst adults aged 15–49 years and an overall prevalence of approximately 13.5% (Statistics South Africa).

Further to this, 20% of people living with HIV in South Africa are not yet on antiretroviral therapy (ART) despite a national treatment programme being initiated in 2016 (Larson et al, 2019).

The likelihood of developing AIDS-defining cancers has reduced with the successful treatment of HIV, however the prevalence of lymphoma in HIV positive patients remains high in South Africa, comprising 50% of AIDS-defining malignancies (Patel et al., 2015).

In HIV infected individuals, DLBCL is recognised to be a more aggressive disease than in HIV negative individuals, with more frequent high stage and younger age at presentation, and an inferior clinical outcome in HIV infected compared with HIV uninfected individuals (Nandikolla et al, 2016).

The study of HIV and its effects on DLBCL course, prognosis and treatment response comprises many complex independent and interrelated variables. These include lower $CD4^+$ T-cell counts, uncontrolled HIV-1 viral load, age over 50 years and high International Prognostic Index (IPI) score (Little et al, 2003; Kaplan et al., 2005; Mounier et al, 2006; COHERE Study Group et al, 2009). In addition, extra-nodal versus intranodal site, as well as in patients on antiretroviral therapy versus those not on antiretroviral therapy have a role to play (Besson et al., 2017). Lastly Epstein Barr virus (EBV) co-infection is also implicated in the course, prognosis and treatment response of DLBCL (Morton et al., 2014).

The role of molecular subgroups, including COO and oncogenes such as *BCL2* in prognosticating HIV-associated DLBCL remains uncertain. A recent study has shown that, in cases with similar histological features, the molecular pathogenesis of immunodeficiency-associated lymphomas differs from lymphomas of the immunocompetent host (Capello et al., 2011). It is postulated that in the setting of immunocompromise, immune deregulation, viral infections, and chronic antigenic stimulation may provide alternative cell survival signals that render neoplastic B cells less dependent on genetic lesions otherwise important for lymphomagenesis in immunocompetent host (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012).

3.6 The relationship between HIV and the Hans algorithm in DLBCL

In a recent South African study DLBCL subtypes (GCB and ABC) were equally distributed among HIV infected and HIV uninfected patients (Cassim et al., 2020). One international study found ABC DLBCL to be substantially more common in HIV infected (83%) than in HIV uninfected (54%) cases ($p < 0.001$) (Morton et al., 2014).

Minimal research has been performed to elucidate the prognostic implications of Hans algorithm subtype in HIV infection versus non infection. A study showed interesting findings whereby the Hans algorithm was not predictive of prognosis in HIV infected patients, in contrast to non- HIV-associated DLBCL, where the ABC subtype has an inferior prognosis. Higher expression of c-MYC present in HIV- associated DLBCL may negate the better prognosis of GCB subtype (Chao et al., 2015). This study concluded that this needs to be explored further (Nandikolla et al., 2016).

3.7 The relationship between HIV and BCL2 expression in DLBCL

The pathogenesis of DLBCL in the context of HIV infection more frequently involves over-expression of transcriptional regulators *cMYC*, *BCL6* and *MUM1*. Elevated cMYC expression in HIV-associated DLBCL has been shown to predict inferior survival and may explain the more aggressive clinical course of these lymphomas (Chao et al., 2010).

These relationships, particularly regarding BCL-2 expression, have not been extensively explored or correlated in the current literature. One study showed significant differences in common oncogene translocations in HIV infected versus HIV uninfected patients. Within the group of HIV uninfected cases, *MYC* and *BCL2* translocations were significantly more common and *BCL6* significantly less common in GCB-DLBCL than in ABC-DLBCL (Morton et al., 2014). Within the group of HIV infected cases, translocations other than *MYC* were rare ($\leq 6\%$) and unrelated to COO (Morton et al., 2014). Another study showed a strong independent poor prognosis associated with BCL2 expression in HIV infected cases of DLBCL (Phillipe et al, 2019). A study by Chen et al., showed significantly reduced BCL2 protein expression in HIV infected populations. Interestingly, they showed that HIV infected individuals had a significantly higher proliferation index by Ki67 staining than HIV uninfected individuals. An inverse relationship was illustrated between Ki67 and BCL2 protein expression by IHC in HIV infected patients. This study also showed reduced gene expression of cell cycle inhibitors such as *CDKN1A, CDNK1B* as well as other *BCL2* family pro-apoptotic genes, such a *BIM, BMF, BAX* and *PUMA*. It was

concluded that, in the setting of HIV-infection, DLBCL has less dependence on the traditional anti-apoptotic mechanism of *BCL2* in tumorigenesis. It was suggested that in HIV infection, mechanisms preventing cell cycle progression and the induction of apoptosis may involve alternative pathways (Chen et al., 2019).

A subsequent study was conducted in Malawi, also investigating the molecular characterisation of HIV-associated DLBCL. This study showed that, in the setting of HIV-infected patients with DLBCL, the HIV infected cases show a unique and relatively heterogenous gene expression profile. Compared with HIV negative cases, HIV-associated DLBCL cases were enriched for hypoxia- induced genes and expression modules related to oxidative stress. In addition, genes related to angiogenesis were favoured over the anti-apoptotic genes were favoured in HIV uninfected cases (Fedoriw et al, 2020). These findings are in keeping with previously published histologic and phenotypic observations, demonstrating unique stromal reactions, and morphological features and pathways of angiogenesis in HIV-associated lymphoma (Liapis et al, 2013).

CHAPTER 4 – MATERIALS AND METHODS

4.1 Study Design and Sample

This study was a retrospective case review of diffuse large B-cell non-Hodgkin lymphoma cases in HIV positive and HIV negative individuals, assessing the immunohistochemical findings that inform the Hans algorithm, and BCL2 protein expression. It was conducted in the Division of Anatomical Pathology, University of the Witwatersrand, under a broader departmental study. Ethics clearance has been obtained for the broader study (Appendix A).

Individual ethics from the University of the Witwatersrand Human Research Ethics Committee (HREC) has been obtained for this study (Appendix B), and written permission was obtained from the holder of the database to be used in this study (Appendix C). The database was compiled in the following way: A search was performed on TrakCare with the following SNOMED codes (M-96873, M-96803, M-95953, M-95903) for cases of high grade B-cell lymphomas in the period 2013-2017. A total of 707 cases were retrieved consisting of DLBCL cases, BL and BCLU cases.

All cases of DLBCL with known HIV status and subtype were retrieved off the existing database and assigned study numbers. A total study number of 72 cases were selected, 63 from HIV positive individuals and 9 from HIV negative individuals. The time of reporting for the cases ranged from 2007 to 2017.

4.2 Slide, Immunohistochemistry and patient demographic Review

Slides and reports of the cases were retrieved and reviewed by the candidate and two consultant histopathologists, to achieve a consensus diagnosis of DLBCL. DLBCL morphological variant subtyping was done according to the WHO Classification of Haematopoietic and Lymphoid Tissues (Swerdlow et al., 2017).

The following patient demographic information was captured on a data sheet: Patient age (>16 years), gender, race, biopsy site, and HIV infection status (HIV status was regarded as positive if stated as such by the referring clinician on the request form or if a positive HIV serology or (polymerase chain reaction) PCR result was available on the NHLS database. Similarly, HIV status was regarded as negative if stated as such by the referring clinician on the request form or if a negative HIV serology or PCR result was available on the NHLS database).

The demographic and DLBCL characteristics were compared between patients who were HIV infected and uninfected. All cases and slides were reviewed by two lymphoma-specialist pathologists and the candidate with a consensus opinion at a multi-headed microscope.

The cases were classified into centroblastic, anaplastic, immunoblastic or 'other' variants, based on morphological features at 100-400 x magnification. DLBCL centroblastic variant was assigned to cases with large lymphoid cells with vesicular nuclei containing vesicular chromatin and 2-4 nucleoli, often in apposition to the nuclear membrane, and scant basophilic cytoplasm. In order for cases to be considered immunoblastic variant, they needed to show over 90% of the cells as immunoblastic, cells with a large round oval nucleus, a single prominent central nucleolus and considerable basophilic cytoplasm. (Xie et al, 2015). The anaplastic morphology was assigned to cases where the neoplastic lymphocytes had one to several, large, pleomorphic nuclei, mimicking Hodgkin/Reed-Sternberg cells, or the tumour cells of anaplastic large cell lymphoma (ALCL) (Chen et al, 2019).

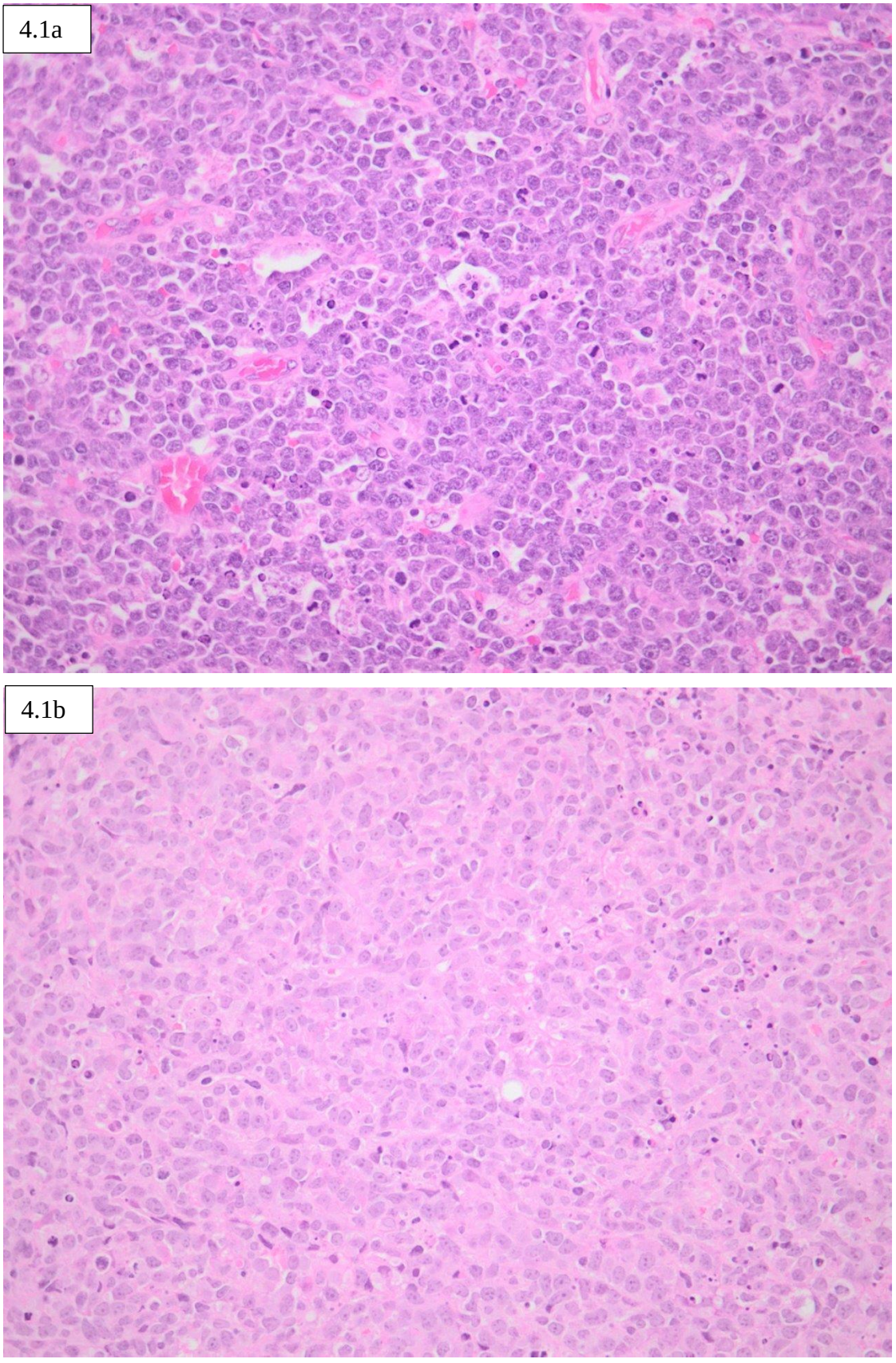
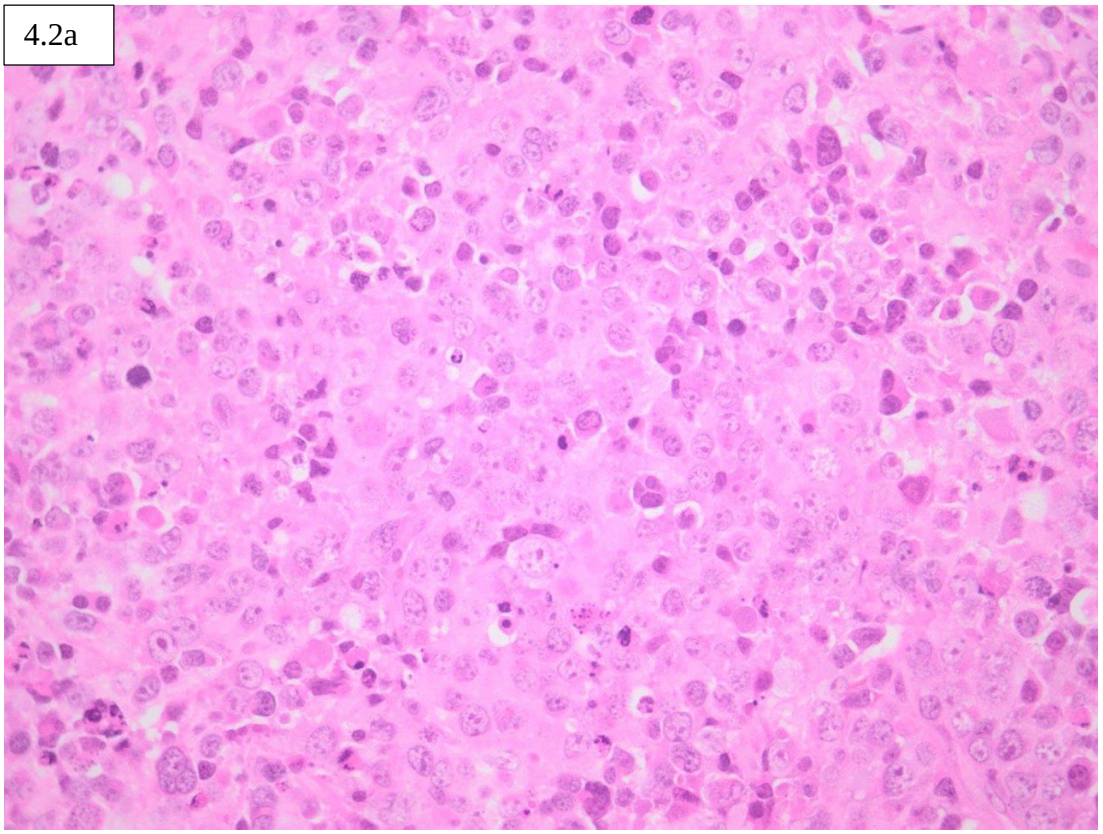


Figure 4.1: Photomicrographs of examples of DLBCL, NOS, with centroblastic cytomorphology (H&E stain, x400). Image 4.1a is that of a small bowel DLBCL in a HIV infected patient. Image 4.1b is a liver DLBCL in an HIV reactive patient.

4.2a



4.2b

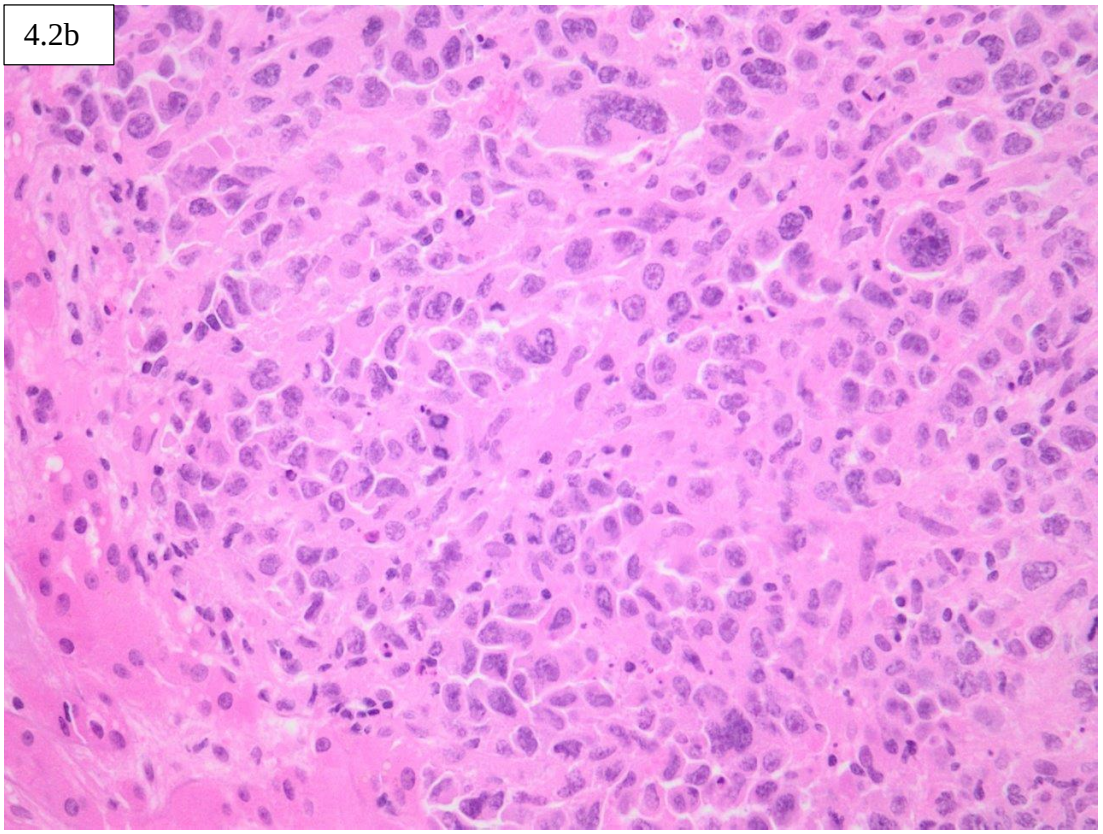


Figure 4.2: Photomicrographs of examples of DLBCL, NOS, with anaplastic cytomorphology (H&E stain, x400). Image 4.2a is that of a neck node DLBCL in a HIV infected patient while 4.2b is a liver DLBCL in an HIV uninfected patient.

The Hans algorithm was applied to all cases, according to CD10, MUM1 and BCL6 status, and BCL2 protein expression assessed immunophenotypically. BCL2 was considered positive where the reactivity of lymphoma cells with antibody was greater than 20% membranous or cytoplasmic staining (Mahmoud et al, 2011). CD10, MUM1 and BCL6 were interpreted as per the Hans algorithm with a cut off of 30% or more positive staining.

4.3 Data Analysis

Microsoft Excel ® was used to store data as a spreadsheet and the statistical analysis software, the R program, performed statistical calculations.

Tumour marker expression was considered positive or negative, based on previously published cut-off values.

Numerical data was described using the mean and standard deviation if normally distributed and median and interquartile range if not normally distributed. Categorical data was described by way of frequencies and percentages.

The relationships between categorical variables (e.g. HIV status and Hans algorithm subtype and HIV status and BCL2 expression) was assessed using a Fisher Exact test (individual categories are less than 10).

The differences in age by HIV status, gender, tumour site, Hans algorithm subtype and BCL2 status were assessed using a T-test as age was normally distributed.

A p-value of < 0.05 was used to determine statistical significance in all inferential statistics.

4.4 Inclusion and Exclusion criteria

Inclusion:

1. Patients 16 years old or older
2. BCL2 and Hans algorithm immunohistochemistry complete
3. HIV test result available at time of diagnosis

4. Case confirmed as diffuse large B-cell lymphoma by review

Exclusion:

1. Patients < 16years old

2. Incomplete BCL2 and/or Hans algorithm immunohistochemistry

3. HIV status unknown

4. Cases showing discordance in histological and immunohistochemical features by review.

4.5 Ethical considerations

All cases were anonymised and assigned a unique study number. The spreadsheet containing the results was stored on the work desktop computers of the investigators. These computers are password protected. No information other than that specified on the data collection sheet was collected. The raw data remained password protected, and unshared. The summarised results of this study will be shared by way of peer reviewed publication.

CHAPTER 5- RESULTS

5.1. Sample size and description

A total of 72 cases were included in the study. The samples included biopsies of DLBCL cases from both extra-nodal biopsy sites (44/72, 61%) and nodal sites (28/72, 39%). The sites of biopsy and distribution of the study samples per site are summarised in Table 5.1.

Table 5.1. Sites biopsied and number of samples taken from each

Site	Number of cases	%
Nodal	28	39%
Axillary node	4	
Cervical node	7	
Inguinal node	3	
Lymph node NOS	14	
Extra-nodal	44	61%
Supraclavicular mass	1	
Neck mass	2	
Oropharynx	5	
Nasopharynx	1	
Breast	3	
Lung	1	
Cervix	1	
Bone	3	
Soft tissue	3	
Stomach	5	
Small bowel	5	
Oesophagus	1	
Liver	4	
Spleen	1	
Kidney	2	
Anus	1	
Omentum	1	
Abdominal mass	4	

5.2 Age

The ages of cases in the cohort were normally distributed, as illustrated in Figure 2, and ranged from 16 to 66 years of age (range of 50 years) with a mean of 41.7 and a standard deviation of 11.0 years. There was no significant difference between the ages of females and males in the cohort (T-test, p=0.41).

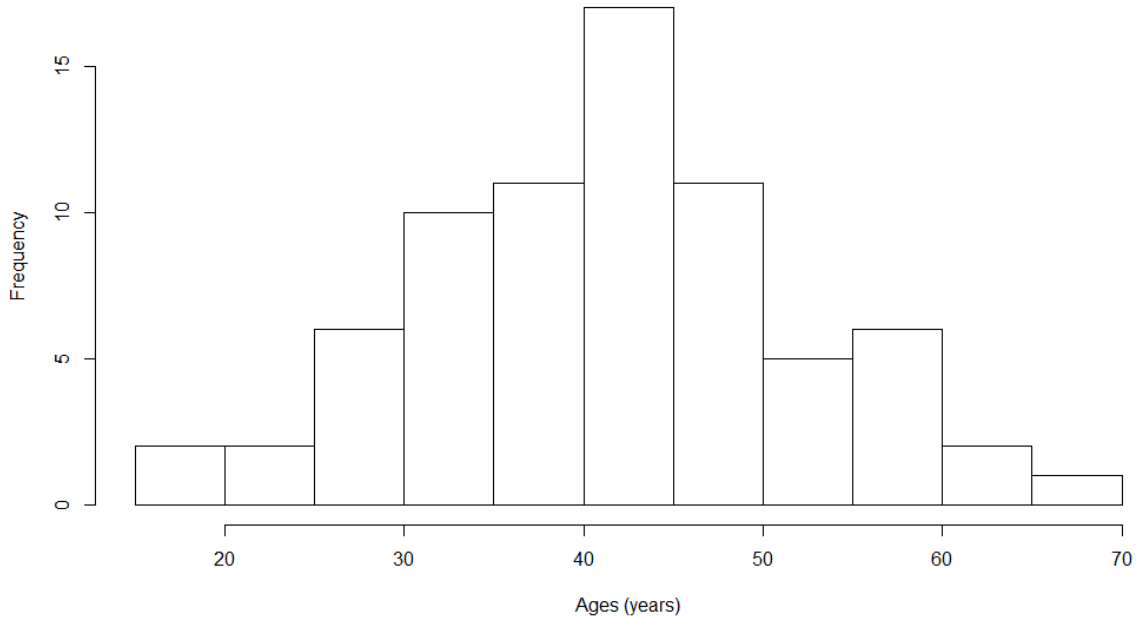


Figure 5.1. Histogram showing Distribution of age in the study population

Males comprised the majority of cases in the cohort (42/ 72, 58%), and females comprised a slight minority of cases (30/72, 42%)

5.3. HIV Status

The majority of patients included in the study were HIV positive (63/72, 87,5%) and the minority were negative (9/72, 12,5%).

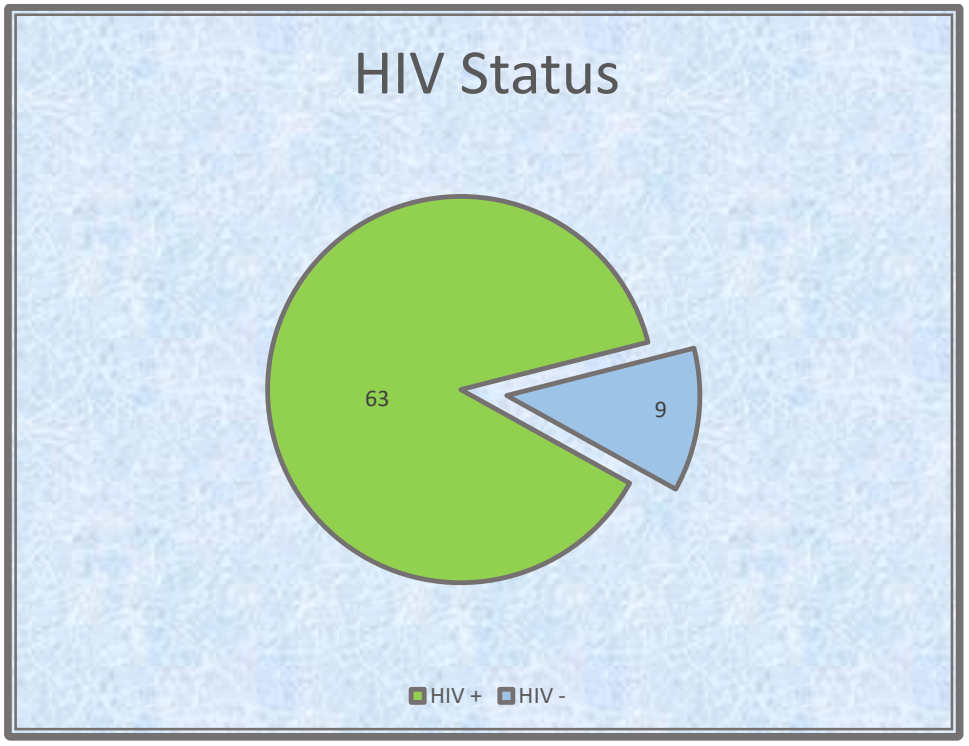


Figure 5.2. Pie chart showing HIV infection versus non-infection in the study population

5.4. Morphology

Consensus microscopy of the cases determined the majority to be centroblastic (62/72, 86%) with others classified as anaplastic (10/72, 14%). No immunoblastic or "other" cases were identified.

5.5. Hans Algorithm Subtypes

Of the cases evaluated, the majority were of GCB subtype by the Hans immunohistochemical panel (49/72, 68%), and the minority were ABC subtype (23/72 32%). The immunohistochemical findings determining this outcome are illustrated in Table 5.2.

Table 5.2. Immunohistochemical findings contributing to Hans algorithm interpretation.

	CD10	MUM1	BCL6
Positive	48	40	66
Negative	24	32	6

5.6. Association between the Hans algorithm classification of DLBCL (germinal centre B-cell phenotype/ activated B-cell phenotype) and BCL2 immunohistochemical expression

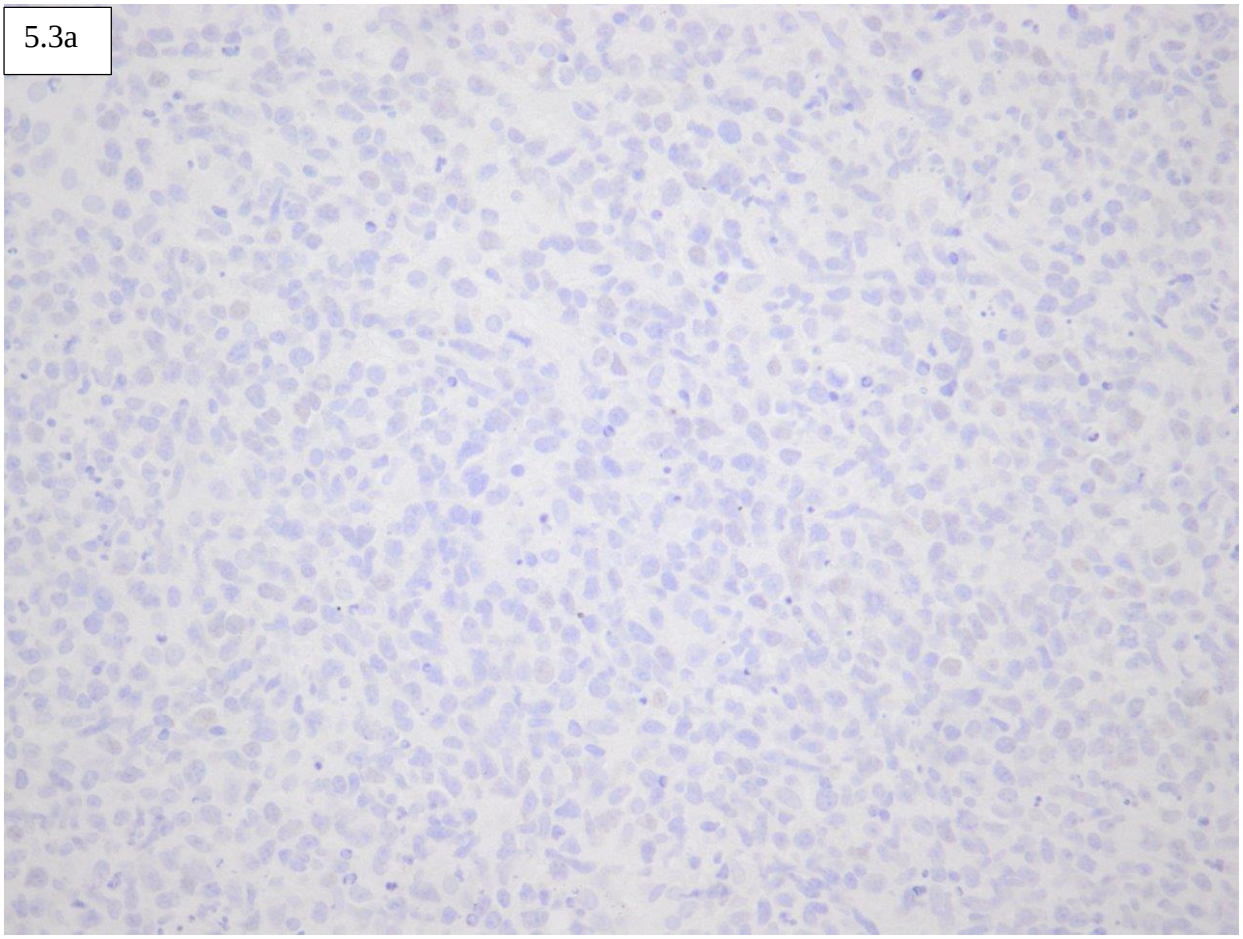
BCL2 immunohistochemical staining was assessed for all 72 cases with 49 positive and 23 negative as demonstrated in Figure 5.4, with a cut off value of 20 % or more membranous or cytoplasmic staining to call a positive result. Regarding the relationship between BCL2 status and Hans algorithm subtype, 8,6% (2/23) of ABC cases were BCL2 negative and 91,4 % (21/23) were BCL2 positive. Of the GCB cases 44,8% (22/49) were BCL2 negative and 55,2% (27/49) were BCL2 Positive. This data is summarised in Table 5.3 and illustrated in Figure 5.4.

Table 5.3. Summary of Data for BCL2 immunohistochemistry and Hans algorithm subtype

	ABC	%	GCB	%
BCL2 Positive	21/23	91,4 %	27/49	55,2 %
BCL2 negative	2/23	8,6%	22/49	44,8%

A statistically significant difference in BCL2 positivity rates in ABC and GCB patients was seen (91,4% versus 55,2%) (two-tailed Fisher's Exact test, p-value = 0.003).

5.3a



5.3b

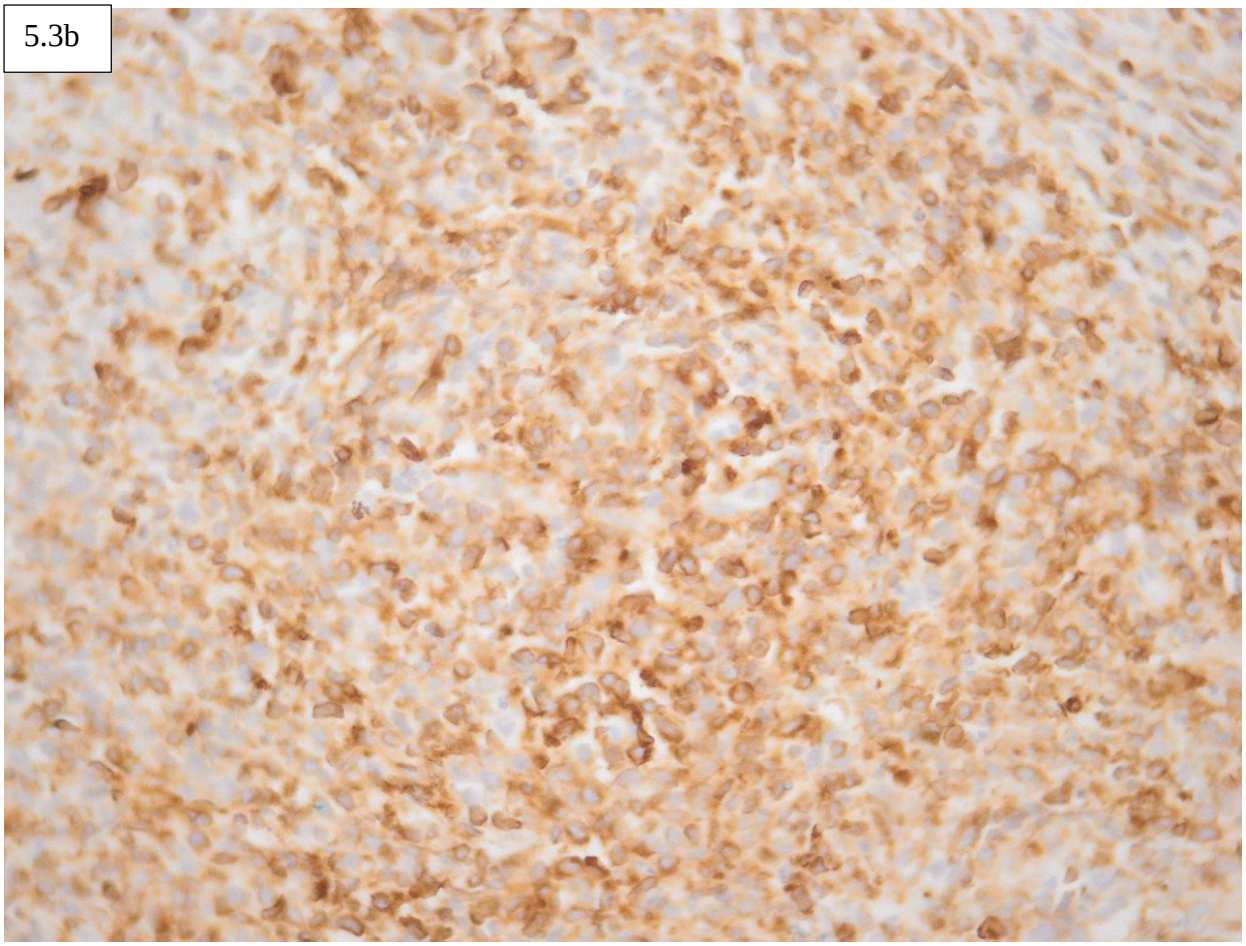


Figure 5.3: Photomicrographs of examples of DLBCL, NOS, staining for BCL2 antibody (400x). Image 5.3a is that of a BCL2 negative DLBCL in a HIV infected patient. Image 5.3b is a BCL2 positive DLBCL in an HIV uninfected patient.

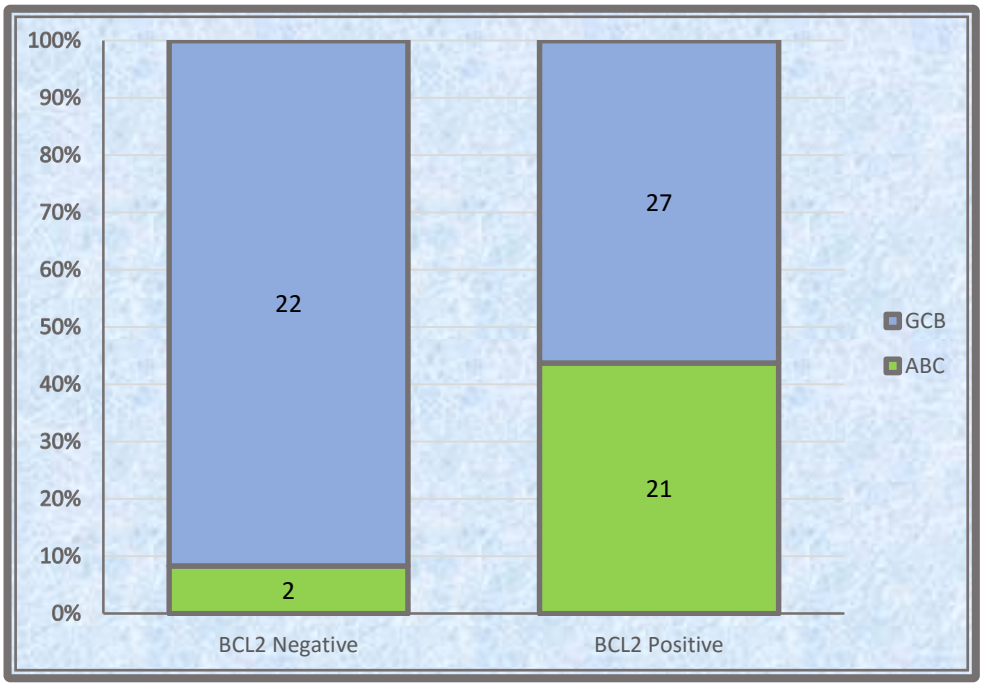


Figure 5.4. Correlation of BCL2 immunohistochemistry with Hans Algorithm

5.7 Association between HIV status and BCL2 immunohistochemical expression

Regarding the relationship between BCL2-status and HIV status, 38% (24/63) of HIV positive cases were BCL2 negative and 62% (39/63) were BCL2 positive. Of the HIV negative cases, 0.00 % (0/9) were BCL2 negative and 100% (9/9) were BCL2 positive. This data is summarised in Table 5.4 and illustrated in Figure 5.5.

A statistically significant difference in BCL2 positivity rates in HIV positive and HIV negative patients was seen (100% versus 38%) (two-tailed Fisher's Exact test, p-value = 0.02)

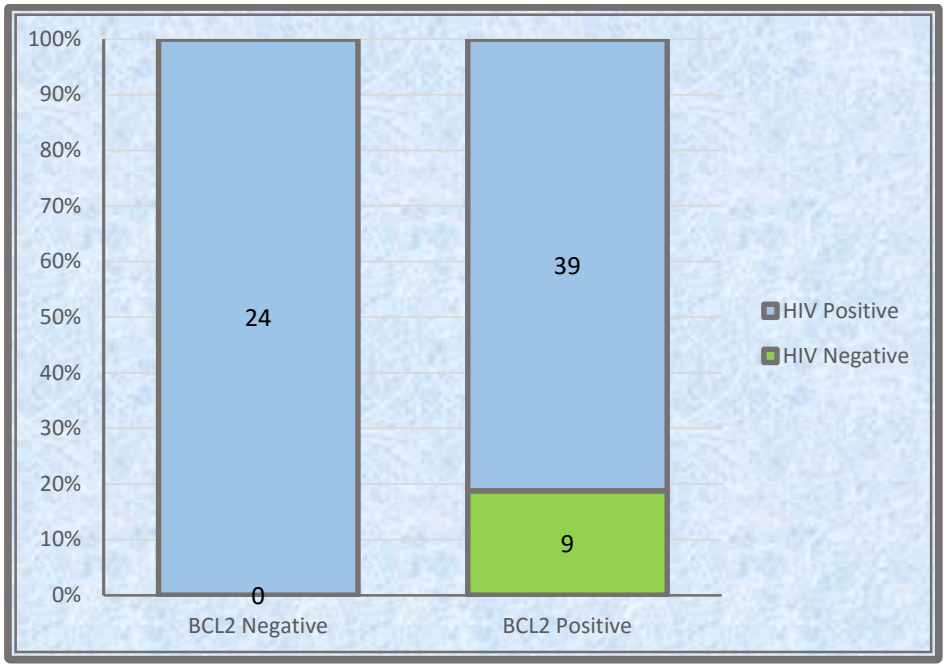


Figure 5.5. Correlation of BCL2 immunohistochemistry with HIV status

Table 5.4. Summary of Data for BCL2 immunohistochemistry and HIV status

	HIV Positive	%	HIV Negative	%
BCL2 Positive	39/63	62%	9/9	100%
BCL2 negative	24/63	38%	0/9	0%

5.8 Association between HIV status and Hans Algorithm subtype

Regarding the relationship between HIV status and Hans algorithm subtype, 29% (18/63) of HIV positive cases were ABC and 71% (45/63) were GCB. Of the HIV negative cases 56% (5/9) were ABC and 44% (4/9) were GCB. This data is summarised in Table 5.5 and illustrated in figure 5.6.

A difference in HIV positivity rates in ABC and GCB negative subtypes was seen (100% versus 38%) (two-tailed Fisher's Exact test, p-value = 0.1334). This difference was not statistically significant.

Table 5.5: Summary of Data for HIV status and Hans algorithm subtype

	HIV Positive	%	HIV Negative	%
ABC	18/63	29%	5/9	56%
GCB	45/63	71%	4/9	44%

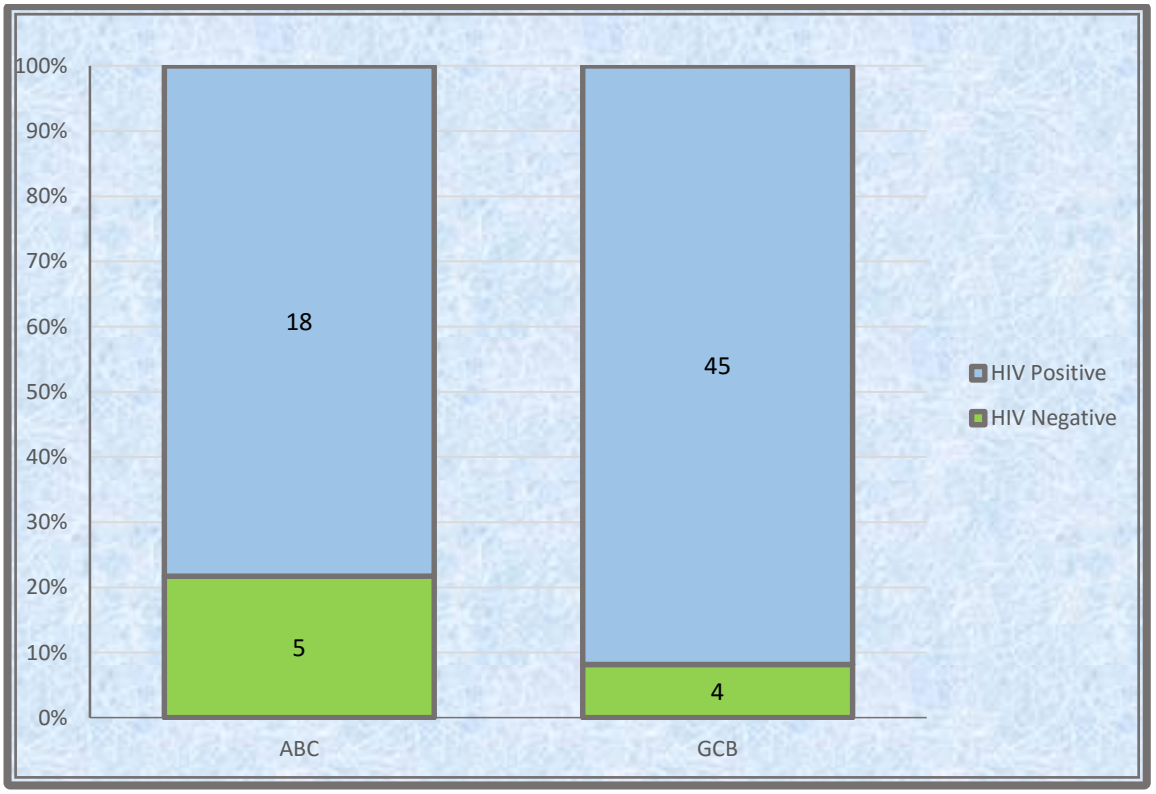


Figure 5.6 Correlation of HIV Status with Hans algorithm subtype

CHAPTER 6 – DISCUSSION

The pathogenesis, cell of origin and molecular profile of DLBCL comprises complex independent and interrelated factors. These include patient immune status, age, co-infection with EBV, tumour cell of origin and the genetic profile of the lymphoma. Each variable has either confirmed, or potential, implications for clinical presentation, prognosis and treatment response for patients. Minimal research has been conducted to elucidate the inter-relationships between these variables.

This study was a small pilot investigation assessing the prevalence, and potential significance of relationships between HIV infection, BCL2 protein expression and COO by Hans algorithm subtype in DLBCL within the unique patient demographic of South Africa. Research in this field is limited, despite the possibility of impactful outcomes regarding findings showing correlations. This is the first study to compare these three variables within a single patient cohort. It is the first South African study aiming to determine BCL2 protein expression within the HIV infected and HIV uninfected patient populations and stands among one other sub-Saharan study (Fedoriw et al., 2020) and one international study (Maguire et al., 2019) in this regard.

In the recent past, classification of DLBCL variants that did not conform to specific subtypes, were described as centroblastic, immunoblastic, or anaplastic based on morphology (Swerdlow et al, 2008). Studies of the clinical relevance of these morphological variants show poor reproducibility and have conflicting results, some studies showing that centroblastic tumours have a better clinical outcome compared to immunoblastic tumours (Ott et al, 2010). This morphological assessment and classification has become relatively redundant in the age of more accurate and reproducible molecular classifications such as GEP, COO and Hans algorithm, and is no longer recommended in the WHO 2017 guidelines (Swerdlow et al, 2017). Our study, for completeness, did include this morphological classification and consensus microscopy of the cases determined the majority to be centroblastic (62/72, 86%) with others classified as anaplastic (9/72, 14%). None showed immunoblastic, or other morphology.

DLBCL can preferentially occur in patients with immunodeficiency, including in HIV infection particularly in those patients with low CD4 counts (Testoni et al, 2015). South Africa has a unique HIV infection profile in that it has the highest worldwide prevalence of approximately 19.1% amongst adults aged 15–49 years and an overall prevalence of approximately 13.5% (Statistics South Africa) and continues to have a relatively high group of patients who are HIV infected but

not on antiretroviral therapy (Larson et al, 2019). South Africa's social and economic landscape also contribute exacerbating factors to the incidence and severity of HIV related morbidity and mortality. These include co-infection with other virus and infectious diseases (EBV, Human papilloma virus and Tuberculosis), poor access to healthcare impeding early diagnosis, treatment, and follow-up, as well as potential nutritional deficiencies and other chronic illnesses.

The prevalence of lymphoma in HIV infected patients is high in South Africa comprising 50% of AIDS defining malignancies (Patel et al., 2015). Within this cohort, our study noted more study subjects with HIV than not. Sixty three out of 72 of patients included in the study were HIV infected (87,5%) and 9 out of 72 were uninfected (12,5%). This matches similar international studies claiming HIV infection as the strongest risk factor for lymphoma with a 77-fold associated risk for non-Hodgkin lymphoma (NHL), and an 11-fold risk for Hodgkin lymphoma (Patel et al., 2015; Engels et al 2008; Grulit et al 2007). A 2015 study at Chris Hani Baragwanath Academic Hospital (CHBAH), Soweto, Johannesburg showed 70% of the patients with non-Hodgkin lymphoma were HIV infected, with tumours tending to me more aggressive and atypical, with a worse prognosis than HIV uninfected patients (Patel et al, 2015). Our study shows consensus with the previously reported local and international prevalence of DLBCL in HIV infected patients.

Our study did not aim to elucidate the prognostic implications of HIV infection in DLBCL in our cohort, or the effects of ARV therapy, CD4 count, age, viral load, or EBV co-infection on the prevalence and prognosis of DLBCL in the HIV infected South African population. This is an opportunity for further research. In multiple other studies, HIV infection predicts a more aggressive DLBCL disease profile than in HIV uninfected individuals, with more frequent high stage and younger age at presentation and poor clinical outcome. (Nandikolla et al, 2016; Patel et al, 2015)

In overseas studies, the incidence of AIDS-related DLBCL decreased in HIV infected patients on antiretroviral therapy, with changes in the HIV-associated relative risks affecting the decrease (Polesel et al, 2008; Robbins et al, 2014; Shiels et al, 2013).

Due to the prevalence of HIV in DLBCL in South Africa, and our relatively small study number, it was not possible to match the sample sizes of the HIV infected and HIV uninfected cohorts. This is a shortcoming of the study with regards to the objectives of correlating HIV status to Hans algorithm. A difference in HIV infection rates in ABC and GCB subtypes which was not statistically significant was seen in our study for this reason (100% versus 38%). Because of this, it was not possible to corroborate with conflicting international and South African results

in this question, although our study suggests that HIV infection correlates more frequently with ABC subtype. One international study found ABC-DLBCL to be substantially more common in HIV infected (83%) than in HIV uninfected (54%) cases ($p < 0.001$) (Morton et al., 2014), whereas a local study showed GCB and ABC subtypes were equally distributed among HIV-infected and HIV-uninfected patients. (Cassim et al., 2020). The extremely high number of HIV infected cases of DBCL in South Africa make it a challenge to collect equitable data numbers of HIV uninfected cases. However, it would be of interest to do further study in this regard, particularly given the implications on prognosis and treatment in both HIV and COO. Questions include the prevalence of ABC versus GCB subtypes in HIV infected populations and the presence, if any, of a cumulative poor prognosis in those cases which are ABC subtype, and occur in HIV infected individuals, and which, if any, is the more important prognostic indicator.

The International Prognostic Index (IPI) (INHLPI, 1993) was developed in 1993 to extrapolate prognosis in aggressive NHL based on five clinical factors: age, stage, the number of extra-nodal sites, performance status, and Lactate dehydrogenase (LDH). With the subsequent advent of molecular techniques, particularly next generation whole genome sequencing, distinctive DLBCL subtypes have been described by COO and molecular features. These tools can be independently utilized outside of the IPI to identify high-risk disease and predict failure of chemotherapy.

The Hans algorithm is widely regarded as the most useful tool for prognostication of DLBCL based on the COO classification, separating it into a germinal centre phenotype (GCB) and an activated B-cell phenotype (ABC) on the basis of an immunohistochemical panel. The panel includes immunohistochemical markers CD10, BCL6, and MUM1. Tumours of germinal centre phenotype have a significantly better prognosis than those of activated B-cell phenotype (Hans et al., 2004).

Of the cases evaluated in this study, the majority were of GCB subtype by the Hans immunohistochemical panel (49/72, 68%), and the minority were ABC (23/72, 32%).

In previous studies, BCL2 protein was overexpressed in approximately half of patients with GCB-DLBCL and three quarters of patients with ABC-DLBCL (Visco et al., 2013). Our study partially replicates this. Of the ABC cases 8,6% (2/23) was BCL-2 negative and 91,4 % (21/23) were BCL2 positive. Of the GCB cases, 44,8% (22/49) were BCL2 negative and 55,2% (27/49) were BCL2 positive. However, our methods differed from this study in that we used a cut off value of 20% positive membranous or cytoplasmic staining for a positive BCL2 score, whereas the Visco study

used a 50% staining cut off value to call BCL2 positive. The literature shows significant variation in staining cut-off values for BCL2 positivity, which is not yet validated or standardised.

A limitation of our study in this regard was that we did not perform FISH cytogenetics on the cases. We used BCL2 immunohistochemistry rather than FISH to establish BCL2 expression. This meant that we were not able to ascertain the nature of the molecular insult that resulted in over-expression. This is relevant in that the nature of the genetic alteration plays a significant role in prognosis, and that specific genetic insults occur more frequently in each COO subtype. Studies utilising FISH demonstrate that *BCL2* translocations are more frequently found in the GCB subtype, whereas *BCL2* 18q21 locus amplification is more common in the ABC subtype of DLBCL (Huang et al, 2002; Rosenwald et al, 2002).

While our study does not explore prognostic implications of these findings, this is explored in other series. In these, BCL2 overexpression had prognostic value only in the GCB subtype in the context of *BCL2* translocation. It was not predictive of outcome otherwise. The GCB patients with isolated *BCL2* translocations had outcomes correlating with those of patients with the ABC subtype, but less dire prognosis than patients with double hit lymphoma (Visco et al, 2013). This would be an opportunity for a future study with clinical and cytogenetic additions to our data set. It also has not been investigated in the context of concurrent HIV infection.

The molecular characterisation of HIV-associated lymphomas has only recently been explored, and in a limited number of studies. Our study investigation into the prevalence of BCL2 positive IHC in HIV infected versus uninfected populations likely holds the most scientific relevance of the entire study, as it contributes to the growth of a very small pool of global investigation on the subject.

In our study series, 62.1% (39/63) of HIV-reactive cases were BCL2 negative and 37,9 %(24/63) were BCL2 positive on IHC. Of the HIV uninfected cases, 0 % (0/9) were BCL2 negative and 100 % (9/9) were BCL2 positive on IHC. A statistically significant difference in BCL2 positivity rates in HIV infected and HIV uninfected patients was seen (100% versus 38%). From this, it can be surmised that BCL2 expression occurs less frequently in HIV infected patients compared to HIV uninfected patients.

This finding correlates with a recent seminal study investigating gene expression profiling in the HIV infected DLBCL population versus the HIV uninfected DLBCL population. It was the first

study investigating these relationships. The researchers showed significantly reduced BCL2 protein expression on IHC, as well as transcriptionally on FISH. Interestingly, they showed that HIV infected individuals had a significantly higher proliferation index by Ki67 staining than HIV uninfected individuals. An inverse relationship was shown to exist between Ki67 and BCL2 protein expression by IHC in HIV infected patients. This study also showed reduced gene expression of cell cycle inhibitors such as *CDKN1B, CDNK1A*, as well as other BCL2 family pro-apoptotic genes, such a *BIM, BMF, BAX and PUMA*. It was concluded that, in the setting of HIV infection, DLBCL has less dependence on the traditional anti-apoptotic mechanism of BCL2 in tumorigenesis generally found in HIV uninfected individuals. The authors suggested that, in a setting of HIV, mechanisms preventing cell cycle progression and the induction of apoptosis may be alternative pathways (Maguire et al, 2019). This is in keeping with previous postulations that in immunocompromised individuals, immune deregulation, viral infections, and chronic antigenic stimulation may provide alternative cell survival signals that render neoplastic B cells less dependent on genetic lesions otherwise important for lymphomagenesis in immunocompetent host (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012). Further study needs to be conducted into these mechanisms and their prognostic implications.

A subsequent sub-Saharan study was conducted in Malawi, also investigating the molecular characterisation of HIV-associated DLBCL. This was the second published study in the world. It is of special interest to our study, as Malawi is a sub-Saharan country that mirrors South Africa's high HIV burden. This article confirms that, in the setting of HIV-infected patients with DLBCL, the HIV infected cases show a unique and relatively heterogenous gene expression profile. Compared with HIV negative cases, HIV-associated DLBCL cases were enriched for hypoxia-induced genes and expression modules related to oxidative stress. In addition, genes related to angiogenesis were favoured over the anti-apoptotic genes were favoured in HIV uninfected cases. (Fedoriw et al, 2020). These findings are in keeping with previously published histologic and phenotypic observations, demonstrating unique stromal reactions, and morphological features and pathways of angiogenesis in HIV-associated lymphoma (Liapis et al, 2013).

These genomic studies are pioneering in that they are beginning to demonstrate a unique genetic profile for HIV-associated DLBCL, that differs in molecular phenotype and the prognostic implications to the well-studied HIV-unassociated DLBCL. It is promising that our study contributes to this body of knowledge, albeit in a small cohort, and only examining BCL2 expression by IHC in HIV infected individuals (rather than whole genome profiling). Our results

open the opportunity for further investigation into, not only BCL2, but other molecular profiling in this context.

A shortcoming of our study in this aspect, is that we did not include the patients' ART treatment status and CD4 cell counts in our data series. The Malawian group showed that patients on long standing ART had molecular profiles more in line with those in the HIV uninfected population. Our study, once again, did not incorporate prognosis into the data set, which the other studies do. This relationship seems complex and is an opportunity for further discussion and investigation.

Within South Africa, where the HIV burden is so high, biomarker validation in this population group would have an important impact on tumour prognostication, and the acquisition and allocation of limited treatment resources in an underfunded setting. There is significance in expanding this pilot trial into a full study of gene expression profiles in HIV infected patients with DLBCL. This could lead to follow up investigations into prognostic and treatment implications of these gene expression differences.

CHAPTER 7- CONCLUSION

This study aimed to establish the relationships between DLBCL cases that are of either GCB or ABC phenotype (as determined by the Hans algorithm), BCL2 protein expression in these two groups and whether HIV infection status bears any influence on the expression of these two prognostic parameters.

Our findings confirm those of previous studies showing a higher prevalence of DLBCL in HIV infected patients compared to uninfected patients. We are not able to find a reliable conclusion as to the relationship between COO by Hans algorithm and HIV status. Our findings demonstrate a higher correlation of BCL2 positivity with ABC COO subtype, over GCB subtype. They are also able to demonstrate a significant association between BCL2 positivity and HIV uninfected DLBCL patients, compared to a markedly lower association in HIV infected patients, adding to a growing body of work on the emerging unique genetic mechanisms involved in DLBCL tumour development in the setting of HIV infection.

This was a pilot study, comparing broad variables with small study numbers. There is scope to elaborate the study number, as well as the detail of the variables in each group of comparisons. Within the molecular category, there is enough evidence to justify further investigation and molecular classification of HIV associated DLBCL, particularly given the high disease burden of both

conditions within South Africa.

The final purpose of these investigations would be to collate a set of reliable prognostic indicators, including IHC, COO, and molecular factors, in the setting of HIV infection and DLBCL. This would contribute to patient care and information, treatment guidelines and the development of effective targeted drug therapies for these aggressive malignancies, and a possible increased opportunity of hope for these patients, their families and the clinicians managing them.

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APPENDIX A: Ethics certificate for broader study

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Dr P Magangane et al
School: Pathology
Department: Anatomical Pathology
NHLS

E-mail: Pumza.Magangane@wits.ac.za

CC: Supervisor: Dr Y Perner <Yvonne.Perner@nhls.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 21/02/2018

REF: *R14/49*

PROTOCOL NO: W-CBP-180221-02 *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Characterization of high grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 translocations*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

APPENDIX B: Ethics certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

21/02/2018

Ref: W-CBP-180221-02

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Dr P Magangane et al
Student No. (if appropriate): N/A

Supervisor: Dr Y Perner

School: Pathology
Department: Anatomical Pathology
NHLS

Project title: *Characterization of high grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 translocations*

Reason: Record review.
No human participants will be involved in the study.

Professor CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu and Rhulani Mkansi.

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
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Website:http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Postgraduate Affairs

TO: Dr S Hooper
School of Pathology
Department of Anatomical Pathology
Medical School
University

E-mail: sarah.kate.hooper@gmail.com

CC: Supervisor: Drs Y Perner, E McAlpine and P Magangane
Yvonne.Perner@nhls.ac.za
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 7 December 2020

REF: R14/49

PROTOCOL NO: **M200817** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *Retrospective analysis of the Hans algorithm and BCL2 expression in HIV positive and negative patients with diffuse large B-cell lymphoma*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Dr S Hooper

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200817

NAME: (Principal Investigator)	Dr S Hooper
DEPARTMENT:	School of Pathology Department of Anatomical Pathology Medical School University
PROJECT TITLE:	Retrospective analysis of the Hans algorithm and BCL2 expression in HIV positive and negative patients with diffuse large B-cell lymphoma
DATE CONSIDERED:	28 August 2020
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Drs Y Perner, E McAlpine and P Magangane
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	7 December 2020

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

DECLARATION OF INVESTIGATORS

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

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

Signature of Principal Investigator Date

APPENDIX C: Consent for access to NHLS database

NATIONAL HEALTH LABORATORY SERVICE
UNIVERSITY OF THE WITWATERSRAND – JOHANNESBURG
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Division of Anatomical Pathology
Faculty of Health Sciences
7 York Road
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Practice Number: 5200296

16 April 2020

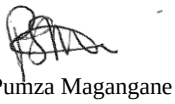
Re: Access to database

To Whom It May Concern

I, Pumza Samantha Magangane, do hereby give consent to the MMed student, Dr Sarah Hooper (0404330P) to have access to the database of retrieved pathological data from the project entitled: "Characterization of high grade B cell lymphoma, with MYC and BCL2 and/or BCL6 translocations". Their study entitled: "A retrospective review of the relationship between HIV-status, Hans algorithm subtype and BCL2 immunohistochemical expression in diffuse large B-cell lymphoma" forms part of this larger study.

Kindly find attached the ethics approval certificate for the larger study.

Yours sincerely



Dr Pumza Magangane

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Dr Y Perner MBBCh (Witwatersrand) FCPath (SA); MMed (Witwatersrand)

April 20, 2020

Human Research Ethics Committee (Medical)
University of the Witwatersrand
Johannesburg
2000

Re: Consent for access to NHLS database

This letter serves to confirm that the Department of Anatomical Pathology at the University of the Witwatersrand and NHLS is happy to grant Dr Sarah Hooper access to patient reports on the TrakCare system for her study entitled, "Retrospective analysis of the Hans algorithm and BCL2 expression in HIV positive and negative patients with diffuse large B-cell lymphoma. "

The Department of Anatomical Pathology is happy for the above research to be carried out on archived material and records in the Division of Anatomical Pathology.

Dr Hooper will ensure that she registers on the NHLS AARMS system.

With best wishes.

Yours sincerely,

Dr Yvonne Perner
Head: Department of Anatomical Pathology