Expression levels of miRNA-127 in a cohort of HIV-positive and HIV-negative Diffuse Large B-Cell Lymphoma.

By

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Declaration

I, Chera Olivier…, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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DEDICATION

I dedicate this to my rock, my mother, who always pushes me to be my best, and to her mothers (Dora and Susan) who taught her to be the best.

I also dedicate this to the person who supports me day and night and who cheers me on even when I wanted to give up, Howard.

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ABSTRACT

Diffuse Large B Cell Lymphoma is one of the most common Non-Hodgkin's Lymphomas. It is prevalent in older age patients but as of late there has been a rise in the younger population in South Africa due to the rise of HIV. DLBCL is quite an aggressive cancer but can be treated, however the relapse rate is high. There are prognostic indicators which can be seen as factors which can be indicative of a poor outcome for patients.

Micro-RNA(miRNA) are small non-coding RNA which can remain stable to be tested. There are several miRNAs which may be linked to prognosis, including miRNA-21 whose upregulated expression has been associated with bad prognosis. However, this is not specific to DLBCL and some studies done have indicated that there may be other miRNAs which are better suited to be biomarkers for DLBCL. Studies have pointed to the direction of miRNA-127 as a more reliable microRNA in its association with prognosis in breast, cervical and gastric cancer as well as DLBCL.

Objective: The primary aim of this study was to determine the association between miRNA-127 and prognostic markers including immunohistochemical stains, survival status and the IPI factor to determine its significance as a prognostic indicator. An additional aim was to determine the correlation between HIV status and expression level of miRNA-127.

Design: A total of 42 DLBCL cases were collected from the archive of Division of Anatomical Pathology, University of Cape Town/NHLS Groote Schuur. The H&E slides were assessed before RNA was extracted from FFPE tissue and converted to cDNA. Real time quantitative RT-qPCR was used to assess the expression of the microRNA. Normal tissue as well as reactive lymph node tissue were used as controls. The expression patterns were also correlated to the clinical information to determine if there was any relationship.

Results: Out of the 42 cases used, 10 cases were silenced, and 31 cases had high miRNA-127 expression. The expression levels were correlated with the IPI factors and the other clinicopathologic features however no significant conclusion were determined.

Conclusion: We found high expression of miRNA-127 cases in the majority of the DLBCL cases. There was no correlation between HIV status and the expression of miRNA-127, nor between the expression and any of the clinicopathological feature. For future studies it is advised that more equally distribution of samples (both HIV status and gender) are obtained, this will allow for a better comparative study.

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List of Abbreviations

Δ - Delta

°C - Degrees Celsius

γ – Gamma

≤ - less than or equal to

μm – Micro meter

μl – Micro litre

μM – Micro molar

% - Percent

3′ UTR – Three prime Untranslated Region

ABC- Activated B-Cell

cDNA - Complementary DNA

Ct – Threshold Cycle

DLBCL- Diffuse Large B-Cell Lymphoma

DNA - Deoxyribonucleic Acid

DNase - Deoxyribonuclease

dNTP - Deoxynucleoside triphosphate

FFPE - Formalin Fixed Paraffin Embedded

FGF - Fibroblast Growth Factor

GCB- Germinal Centre B-cell

HREC - Human Research Ethics Committee

IHC – Immunohistochemistry

miRNA /miR- Micro RNA

mRNA - Messenger RNA

PCR – Polymerase Chain Reaction

Pre-miRNA - Precursor miRNA

RISC- RNA induced silencing complex

RNA- Ribonucleic Acid

RNase - Ribonuclease

RT-PCR - Reverse Transcriptase-Polymerase Chain Reaction

ZFs - Zinc Fingers

CHAPTER 1: INTRODUCTION

MicroRNA are small non-coding RNA that post transcriptionally regulate gene expression and are derived from genome encoded stem loop precursors. They function through TNA induced silencing complex (RISC) mediated binding to target mRNA by pairing to the 3' untranslated region. MiRNA have been associated with many cancers and because of the stability in some bodily fluid may help diagnostic testing.

One of the most common lymphomas is Diffuse Large B-Cell lymphoma (DLBCL). This cancer is curable but if left untreated can lead to death in a short time span and although curable DLBCL has a high relapse rate. With the rise of HIV in South Africa there has been a rise of DLBCL cases, not only in adults but there has also been in a rise in younger patients.

The purpose of this study was to determine the expression levels of miRNA-127 and investigate the prognostic value it may hold in Diffuse Large B-cell Lymphoma (DLBCL). A quantitative reverse transcription PCR will be performed to detect miRNA-127 expression in the cohort and correlated it with the clinicopathological factors. The study will also investigate whether the miRNA-127 expression levels between a cohort of HIV positive and HIV negative patients differ.

In many cancers miRNA-127 has been reported to be significantly downregulated, where low levels are also associated with advanced clinical stage and metastases. Lower miRNA-127 was therefore indicative of a poor prognosis. Therefore miRNA-127 may be a potential biomarker for predicting survival of patients with DLBCL. No literature has investigated the relationship between miRNA-127 expression and HIV status, and it is hypothesised that with a higher CD4 count miRNA-127 expression may be lower.

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

It has been estimated that in 2018, 18.1 million new cases and 9.6 million cancer deaths will be reported worldwide.[121] This estimate shows a big increase in the figures estimated in 2012, where new cancer cases were an estimated 14.2 million and 8.2 million cancer-related deaths, were reported worldwide.[1] According to the 2002 Revised Burden of disease estimates in South Africa, lymphoma was the 12th most common cancer, with lymphoma related deaths ranking at 2.5% with 1032 deaths, of which 601 were males. According to the National Health Laboratory Services (NHLS) Cancer Registry[2] in 2011, 804 new Non-Hodgkin's Lymphomas cases were reported in males and 712 in new female patients. The highest prevalence for males was in the 40-44-year age group and the 35-39-year age group for women.

Cancer is a genetic disease characterised by the uncontrollable proliferation and evasion of cell death of damaged cells.[3] Cancer cells can invade the entire body by moving through the basement membrane into the blood and lymphatics vessles. A study by Stephens and Aigner showed that a single cell with 6-12 mutations is believed to cause cancer.There are a number of factors that cause damage to the genetic blueprint, thus resulting in mutations.[4] Mutations in the genes that encode for proto-oncogenes and tumour suppressors can lead to the gene becoming activate or inactivate.[16] Many factors may cause epigenetic or genetic changes which can lead to the development of cancer.[76]

Cancer is distinguished by the type of cell and where it originates from, with the cells being classified according to their appearance. Cancers are named according to the cells of which they are composed, e.g.; epithelial malignancies are carcinomas, connective tissue malignancies are sarcomas and lymphocyte malignancies are lymphomas.

2.1.1 Carcinogenesis

Carcinogenesis is the development of cancer from normal cells to a cancerous cell. There are three steps in carcinogenesis: tumour initiation, promotion and progression illustrated in figure 1, below. Tumour initiation occurs when there is an interaction between the carcinogen and the cell DNA. This may cause a break or damage to occur in the DNA strand. If the damage is repaired, no initiation step will take place. In the promotion step, the damaged cell multiplies, causing the damaged genome to be replicated. Progression, the irreversible step, is where there is further growth of mutated cells followed by angiogenesis, allowing the cells to grow. Once cancerous cells cross the basement membrane they are able to metastasize to other parts of the body.[16]

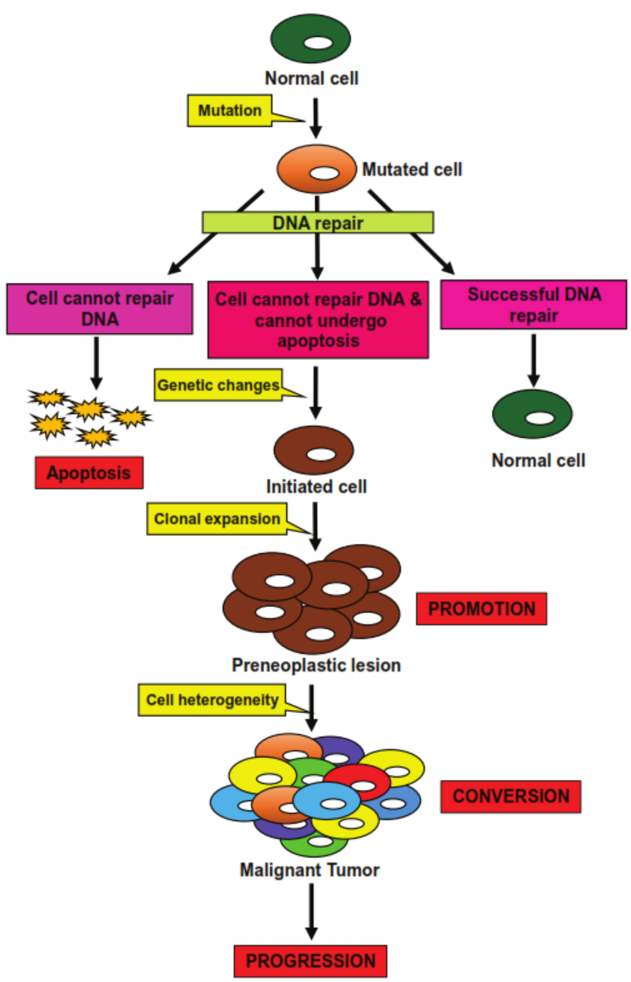

Figure 1: Carcinogenesis stages. When a cell is damaged it will either be repaired or if it cannot be repaired the cell will undergo apoptosis. However, a cell may undergo a mutation where it is unable to undergo apoptosis and neither repair itself, and this is the start of the development of cancer. The cell with the mutated gene will then divide and duplicate forming more cells with the mutation leading to promotion of the cancer cell and later progression. [74]

2.1.2 Aetiology/Epidemiology

There are a number of factors that may increase the risk of developing cancer. They include tobacco, alcohol use, certain infectious agents, radiation, poor nutrition, certain gynaecological and environmental factors[6]. The International Agency for Research on Cancer (IARC)[7] has developed a classification system based on the carcinogenic relationship in humans - Group 1 comprises agents that are carcinogenic to humans; Group 2 is divided into two subtypes: 2A which is probably carcinogenic to humans (limited human carcinogenic evidence but sufficient animal carcinogenic relationship) and subtype 2B comprising possibly carcinogenic to humans (limited human evidence, and not sufficient animal evidence); Group 3 comprise not classifiable agents, and Group 4 which is probably not carcinogenic to humans.[8]

2.1.2.1 Tobacco and alcohol use

According to WHO, tobacco in any form (snuff, cigarettes, pipe smoking) is the cause of 20% of cancer deaths worldwide, as well as 70% of lung cancer. Whilst tobacco can cause lung, oesophageal or mouth cancer, alcohol consumption can increase the chances of development of cancers, especially cancers affecting the liver, colon and even the female breast. According to IARC both are Group 1 carcinogens.[9]

2.1.2.2 Diet and poor nutrition

Poor diet plays a role in the development of cancer. The lack of essential vitamins needed for cell development or even the absence of proper proteins may result in the body not being properly protected or having the required amino acids, therefore, causing mutations which may lead to cancer development. This may not have a direct effect but may have a ripple effect. With mass production of food, more hormone additives that may lead to hormonal imbalance are being consumed, and may lead to cancer.

A high obesity rate has been correlated with a higher risk of cancer development in adults, seeing an increase of development of endometrial cancer in women, but also cancer of the kidneys, gallbladder or the colon in men.[6]. Obesity may also affect prognosis and survival. Certain foods have a carcinogenic effect, for example, salty fish (Chinese-style) and processed meat like bacon and salami are Group 1 carcinogens according to IARC and are linked to causing nasopharynx and stomach cancer.[9]

2.1.2.3 Infection

There are several infectious organisms that play a role in the development of cancer. They are responsible for 15% of cancers deaths. These infectious organisms include viruses, parasites and bacteria and are all Group 1 carcinogens according to IARC.[9]. Viral infection including

Human Immunodeficiency Virus (HIV), Human Papilloma Virus (HPV), Hepatitis B and C, Human T-cell lymphotropic virus type 1 (HTLV-1), Epstein-Barr virus and Human Herpes virus (HHV-8). *Helicobacter pylori* and *Chlamydia trachomatous* are bacterial infections whereas *Schistosoma haematobium, Opisthorchis viverrine* and *Clonorchis Sinensis* are parasitic agents. HPV, which is commonly linked to cervical cancer and Epstein–Barr virus, is commonly linked to lymphomas.[6]

2.1.2.4 Radiation

Radiation causes 2% of cancer deaths. Forms of radiation may include sun rays (UV radiation), X-rays, cellphones (radiofrequency waves) or appliances like microwaves. Naturally occurring radon gas may also be hazardous.[6]

2.1.2.5 Environmental factors and occupational hazard

With the growth of cities and urbanisation, pollution and deforestation greatly affect the environment and people. Pollution and water contamination are some of the main side effects that may contribute to the development of cancer. Water contaminants may lead to a variety of cancers, for example, pesticide contamination has been suspected to lead to Non-Hodgkin's lymphoma. Water disinfectant by-products have also been found to lead to certain cancers, including bladder and colorectal cancer, which may be due to the chlorination.

There are other hazards in the working environment that may have a small contribution to the development of cancer. For example, hairdressers who work with hair dye tend to get bladder cancer. There are other factors which are more commonly noted to cause damage, like working with asbestos that may lead to mesothelioma or even lung cancer, similar to railway workers who inhale diesel fumes.[6]

2.1.2.6 Gynaecologic Factors

Hormones may play an important part in the development of cancers. Menstrual cycles, the age of initial pregnancy or onset of menopause may all be an indication of the risk of development of cancer.

According to Trichopoulos et al., an increase in the number of pregnancies in women may contribute to a lower risk of development of cancer of the endometrium, ovary or breast.[6]

Table 1: Age of onset of certain gynaecological factors governed mainly by hormones may be an indication of which cancer is inclined to develop.[6]

Gynaecological factor	Period more inclined to cause cancer	Cancer that tends to develop
Menstrual cycle	Under 13	Breast cancer
First pregnancy	Over 35	Breast cancer
Menopause	Over 60	Breast cancer

2.1.2.7 Hereditary factors and age

Genetic defects play a significant role in the development of cancer. If a family has a mutated gene or is prone to mutation of a gene, these genes may be inherited.[6] Inherited mutations are present in the first cells formed, the mutation may be present in either the egg or the sperm. Therefore, inherited cancers may start developing from an early age, but some may require certain mutations to occur for cancer to be present.[10]. With age, the incidence of cancer tends to increase, which may be attributed to accumulations of mutations over the years.[5]

2.1.3 Epigenetic Regulation

Environmental stresses increase genomic instability which can lead to genes undergoing epigenetic regulation. Epigenetic refers to all heritable changes not coded in the DNA sequence but in gene expression and chromatin structure. Epigenetic mechanisms may include either the addition of the methyl group to the promotor region of the gene or histone modification which allow for stable propagation of gene activity but when these processes are disrupted it may lead to tumorigenesis .[73] Figure 2. illustrates the initiation phase of cancer. Here, DNA is damaged due to a variety of factors that may cause different types of mutations to occur. It is seen that the damage caused may lead to either somatic or epigenetic alterations if DNA damage is not repaired. Germ line mutations can also occur which can affect the DNA repair genes. The DNA damage that leads to epigenetic alterations can then be classified into histone modification or CpG island methylation. [11]

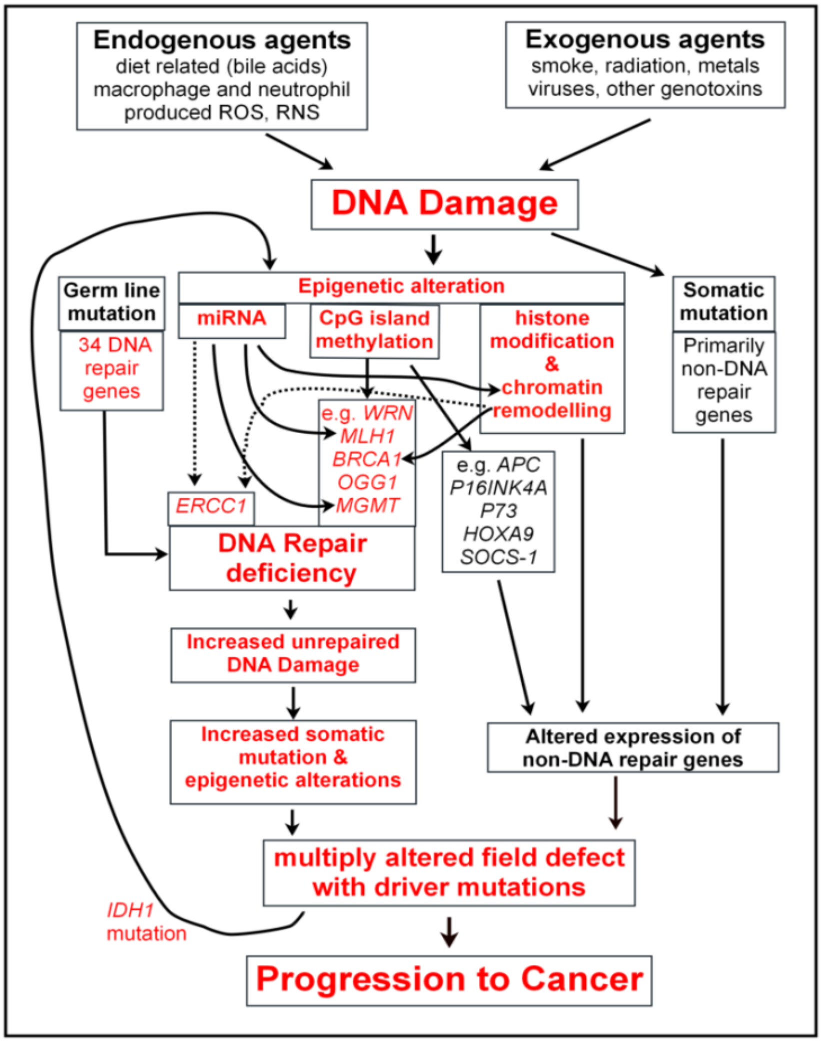

Figure 2: Initiation of cancer from the initial influence of the risk factors.[11]

In the case of histone modification, histones may become more tightly wound, making certain DNA inaccessible, or may have a non-histone protein binding to the histone tail, thus decoding the message.

With DNA methylation, the methyl groups may join to CpG island which result in transcriptional silencing (seen in tumour suppresor genes) or a methyl group may be removed (normally seen in oncogenes) [75]. Figure 3. illustrates DNA methylation, where on the left side, it represents hypomethylation, a methyl group is removed, and on the right side, hypermethylation is shown where a methyl group is added to the CpG island. The CpG island is a short stretch of DNA where the frequency of C-G sequence is higher than others. In the case of miRNA genes, they too can undergo epigenetic alterations. This will then result in the miRNA expression being deregulated, causing overexpression or underexpression of certain genes. For instance, RET may be overexpressed due to increased level of the miRNA, causing increased growth.[11,12,13] CpG island methylation is emerging as a common hallmark of a variety of tumours according to Guo et al.[14]

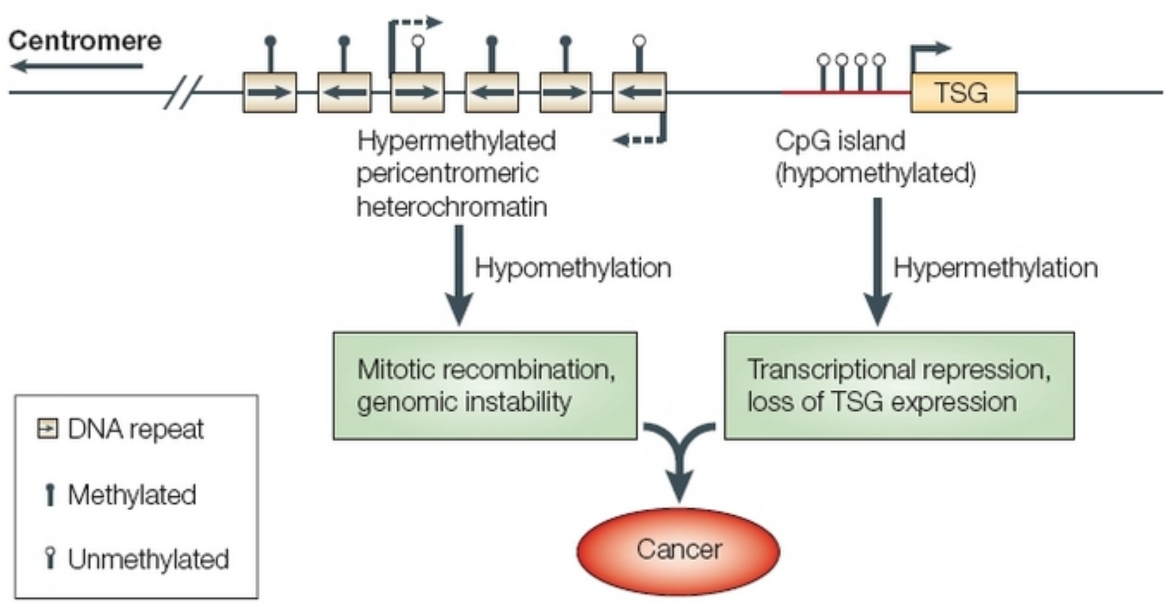

Figure 3: DNA Methylation[15]

Phenotypic changes occur due to alternations in the genes. The abnormalities can affect two classes of genes, the tumour suppressor genes and the oncogenes. There are a variety of both classes of genes that target different areas and functions.[16]

2.1.3.1 Oncogenes

Proto-oncogenes play an important role in cellular growth and proliferation.[71] When proto-oncogenes mutate to become permanently activated, they are called oncogenes. Oncogenes are classified into six main groups: transcription factors, tyrosine kinase, growth factors, receptor tyrosine kinase, regulatory GTPase, and serine or threonine kinases.[17]

They are involved in organism development, including activation of the receptors for cell proliferation, inducing cell proliferation, transducing signals for cell growth, regulating transcription and apoptosis.

2.1.3.2 Tumour suppressors genes

When functioning normally, these are genes that help slow cell division, repair DNA damage or initiate apoptosis. Kinzler and Vogelstein[18] have classified these genes according to the function, grouping them as caretakers, gatekeepers and landscapers. Gatekeepers refer to tumour suppressors which control growth by regulating cell cycle and inducing apoptosis; caretakers are DNA repair proteins which prevent genetic instability; and landscapers, which are the least well characterised, normally regulate tissue morphogenesis and intercellular signalling.[16] Therefore, when a mutation occurs that cause the tumour suppressor gene to be inactivated, it may lead to the formation and progression of cancer. For tumour suppressor genes to be inactivated both gene copies are required to be inactivated, this mechanism is referred to as the Knudson “two” hit hypothesis.[71]

Figure 4, by Weinberg[19], displays a number of oncogenes and tumour suppressors that are commonly involved in cancer. Here Weinberg illustrates that whether a tumour suppressor or an oncogene, in different cancers they work on different areas of the cells or pathways.

ONCOGENES	
Genes for growth factors or their receptors	
PDGF	Codes for platelet-derived growth factor. Involved in glioma (a brain cancer)
erb-B	Codes for the receptor for epidermal growth factor. Involved in glioblastoma (a brain cancer) and breast cancer
erb-B2	Also called *HER-2* or *neu*. Codes for a growth factor receptor. Involved in breast, salivary gland and ovarian cancers
RET	Codes for a growth factor receptor. Involved in thyroid cancer
Genes for cytoplasmic relays in stimulatory signaling pathways	
Ki-ras	Involved in lung, ovarian, colon and pancreatic cancers
N-ras	Involved in leukemias
Genes for transcription factors that activate growth-promoting genes	
c-myc	Involved in leukemias and breast, stomach and lung cancers
N-myc	Involved in neuroblastoma (a nerve cell cancer) and glioblastoma
L-myc	Involved in lung cancer
Genes for other kinds of molecules	
Bcl-2	Codes for a protein that normally blocks cell suicide. Involved in follicular *B* cell lymphoma
Bcl-1	Also called *PRAD1*. Codes for cyclin D1, a stimulatory component of the cell cycle clock. Involved in breast, head and neck cancers
MDM2	Codes for an antagonist of the p53 tumor suppressor protein. Involved in sarcomas (connective tissue cancers) and other cancers
TUMOR SUPPRESSOR GENES	
Genes for proteins in the cytoplasm	
APC	Involved in colon and stomach cancers
DPC4	Codes for a relay molecule in a signaling pathway that inhibits cell division. Involved in pancreatic cancer
NF-1	Codes for a protein that inhibits a stimulatory (Ras) protein. Involved in neurofibroma and pheochromocytoma (cancers of the peripheral nervous system) and myeloid leukemia
NF-2	Involved in meningioma and ependymoma (brain cancers) and schwannoma (affecting the wrapping around peripheral nerves)
Genes for proteins in the nucleus	
MTS1	Codes for the p16 protein, a braking component of the cell cycle clock. Involved in a wide range of cancers
RB	Codes for the pRB protein, a master brake of the cell cycle. Involved in retinoblastoma and bone, bladder, small cell lung and breast cancer
p53	Codes for the p53 protein, which can halt cell division and induce abnormal cells to kill themselves. Involved in a wide range of cancers
WT1	Involved in Wilms' tumor of the kidney
Genes for proteins whose cellular location is not yet clear	
BRCA1	Involved in breast and ovarian cancers
BRCA2	Involved in breast cancer
VHL	Involved in renal cell cancer

Figure 4: Key genes involved in the development of cancer. Both oncogenes and tumour suppressor play different roles .[19]

Depending on the gene that has mutated, the cancer may progress faster or slower. The reason for this is each gene plays a different part in the normal pathways, and once one is either silenced or overexpressed it will have a direct effect on all systems in the pathway.

A mutation initially occurs in one cell, but if that cell does not undergo apoptosis or DNA repair the mutated cell will divide. When mutations occur in genes that regulate the cell cycle, this may lead to increased cell division. This rapid growth may lead to a mutation of the initial mutation which is referred to as a variant of the mutation. This may then lead to a different gene being affect hence leading to different people having the same cancer, have may have different gene involvement and hence different prognosis. [16]

2.1.4 Hallmarks of cancer

Cancer cells have certain acquired characteristics that are a result of their changes. These six hallmarks are referred to as the hallmarks of cancer[25]

1. Evading apoptosis

   Apoptosis is the process of programmed cell death. It is needed to ensure there is proper division and development of cells. Cancer cells are able to evade this process; therefore, cancer cells are not broken up.

2. Inducing angiogenesis

   All cells require nutrients and oxygen to survive which is required from blood vessels. Cancers cells can induce new blood vessels to be formed which is referred to as angiogenesis.

3. Evading growth suppressors

   Cancer cells are able to evade signals that suppress cell proliferation.

4. Activate tissue invasion

Cancer cells are able to dissociate from primary tumor site and migrate through the tissue. They can move through the basement membrane into the blood vessels and travel through the blood stream, until they adhere and invade a new area.

5. Limitless replicative potential

Senescence is referred to the point where after a normal cell enters a stage of permanent growth arrest and they can no longer replicate. Cancer cells are able to disrupt the telomerase mechanism allowing them to have limitless replicative potential.

6. Self-sufficient growth

The cancer cell produces growth factor so that they are able to be self-reliant and sustain constant proliferation.

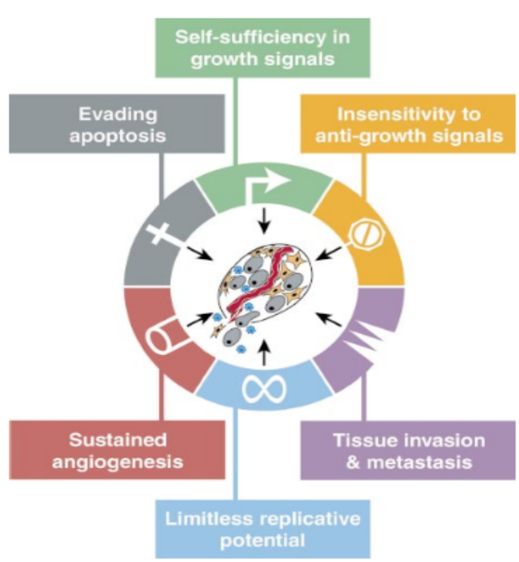

Figure 5: The hallmarks of cancer.[25]

2.2 MiRNA

2.2.1 MicroRNA biogenesis and function

2.2.1.1 Function

MiRNAs are short single-stranded ribonucleic acid (RNA), which regulate gene expression. They can anneal to messenger RNA(mRNA) and, therefore, affect translation of proteins.[20]. MiRNA regulate genes by binding to 3' untranslated regions of the targeted gene.[21] MiRNA can regulate expression of 30-60% of protein-coding sequences and, therefore, plays a role in the development, differentiation, cell proliferation, cell stress response and apoptosis.[22].

MiRNAs are stable in not only body fluids but also in fresh and archived tissue. It can be extracted and therefore measured non-invasively.[64 65] They been found to have prognostic and diagnostic value, where profiling panels have been established to assist in profiling cancers.[21]

2.2.1.2 Biogenesis

Figure 6 illustrates the biogenesis of miRNA. This process starts from the intron of the DNA and move out into the cytoplasm to silence proteins.[23] Each gene is transcribed by RNA polymerase II, thus producing a regulatory RNA. This transcript is a primary microRNA and can fold into a hairpin structure. DGCR8 (DiGeorge syndrome chromosomal region 8) recognises the structure and its associated enzyme, Drosha, then interacts to form a processing complex, thus helping to cut it into a smaller miRNA that is able to be transported into the cytoplasm. Exportin 5 is then able to transport the miRNA into the cytoplasm where the enzyme DICER, is able to recognise and cut the loop. The remaining double strand RNA molecule and DICER then interact with AGO 2, causing the double strand to unwind leaving only one strand. The strand with AGO 2 is able to form the RNA Induced Silencing Complex (RISC). RISC binds to messenger RNA (mRNA), where it either cuts the mRNA strand or binds so that the strand cannot be read, thus leading to silencing of protein encoded by the mRNA.[24]

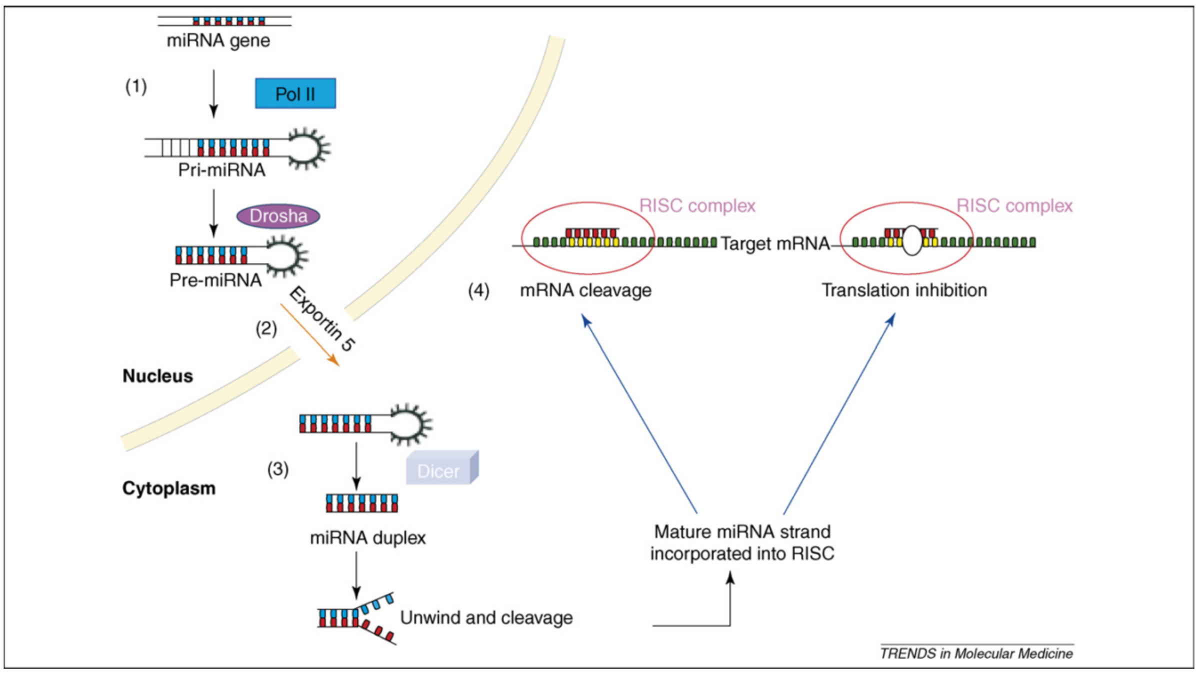

Figure 6: Biogenesis of miRNA.[23]

2.2.2 MicroRNA in cancer

Meola[22] investigated the role of miRNA in a variety of diseases and suggested that miRNA may contain the mutations that lead to the genetic conditions. miRNA target mRNA which lead to either the expression or suppression of proteins, but in cancer the miRNAs expression is affected hence leading to protein expression being affected. In some cancers, miRNAs that would normally target oncogenic transcripts and therefore supress oncogenic transformation, are downregulated, these are known as tumour suppressor miRNAs. Oncogenic miRNAs promoting development of cancer are upregulated.[26]

MiRNAs that have high expressions levels are known as oncomiRs. These oncogenic miRNAs are upregulated in certain cancers; miRNA-21 and miRNA-155 are examples of oncomiRs leading to high expression of target genes. miRNA-127 is an example of a tumour suppressor. These miRNAs have an inverse relationship with their target genes; low miRNA expression is associated with high gene expression.[13, 39]

The location of the miRNAs dictates whether the miRNA may be transcribed singularly directly from its promoter or in clusters from a shared promoter. These miRNAs may be exonic (found in exons of a gene), intronic (located in the intron) or intergenic (found in between genes). [37]

2.2.2.1 MicroRNA-127

According to Malumbres[19] miRNA-127 was the first microRNA found to be epigenetically regulated, where miRNA-127 is hypermethylated in tumour cells.[19] MiRNA-127 is located on chromosome 14q32.2[31], where it overlaps with miRNA-433 on the 3' end. It targets BCL6, which is involved in apoptosis and DNA damage response.[31,32] The primary miRNA(pri-miRNA) that is transcribed explained in the biogenesis of miRNA (figure 6), can fold into a hairpin and they're then processed into pre-miRNA. These structures are transported into the cytoplasm to further development into the secondary structures. The structure illustrated below is the secondary structure of miRNA-127. [23,33]

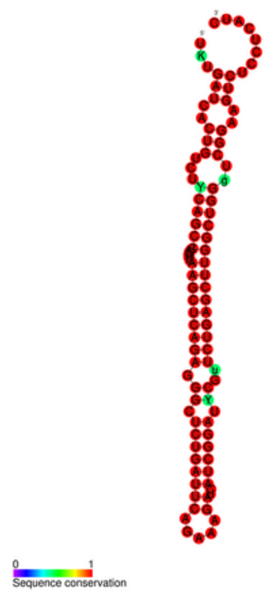

Figure 7: Secondary Structure of miRNA-127. [33]

2.2.2.1.1 MicroRNA-127 in cancer

In a study by Wang et al.,[30] decreased expression levels of miRNA-127 play a very important part in breast cancer progression. Low expressions levels have been associated with a decreased overall survival and increased lymph node metastasizes. In this study, miRNA-127 was administered and this upregulation saw the reduction of colony formation, enhanced apoptosis and suppression of the growth of cells.[30]

The study by Lee et al.,[35] found that miRNAs were associated with prognostic factors and disease progression. They found that miRNA deregulation is detectable in the early stages of cervical cancer and that miRNA-127 was significantly associated in cases with lymph node involvement.[35]

In gastric cancer, lower expression of miR-127 has been associated with higher grade tumours.[14]

Roehle et al.,[27] carried out a study which looked at the expression signatures of 58 miRNAs in Diffuse Large B-Cell Lymphoma (DLBCL). A correlation between miRNA expression and survival (event-free and overall) was done. From the 58 miRNAs tested, a panel of 16 miRNAs had a p-value of <0.1. Eight of the 16 miRNAs had a significant correlation with overall survival (p-value <0.05). Of the eight miRNAs, miRNA-127 was the only miRNA that had both overall survival and event-free survival ($p<0.05$).[27] Although miRNA-127 is not highly expressed, the expression of this miRNA still has prognostic value as low expression levels are indicative of a poor prognosis in DLBCL.[27]

Low levels of miRNA-127 and high levels of BCL6 give the DLBCL cells proliferative advantage as B-cells keep proliferating in the GC centres.[36] In DLBCL, miRNA-127 has a low expression and is linked to poor prognosis.[27,37]

2.3 Lymph Nodes and Lymphomas

2.3.1 Lymph Node Anatomy and Histology

The lymphatic system is part of the immune system. Its function is to filter and remove excess fluid and waste particles including excess fats, and to defend the body against disease. The lymphatic system is divided into two parts, primary lymphoid organs where B and T cells are formed and mature, and the secondary lymphoid organs where B and T cell further differentiate and are activated.[38] The primary lymphoid organs are the bone marrow and thymus, whereas the secondary group consists of the lymph, lymph nodes, tonsils, spleen, Peyer's patch and mucosa-associated lymphoid tissue.[39]

The lymph nodes are small, encapsulated nodes situated throughout the body along the lymphatic vessels.[40] Lymph nodes can be found in a variety of areas in the body as shown in Figure 8. The cervical lymph nodes drain the head and neck, whereas the axillary nodes drain the upper limbs down to the umbilicus and the inguinal lymph nodes drain the abdomen below the umbilicus and the lower limbs.[41]

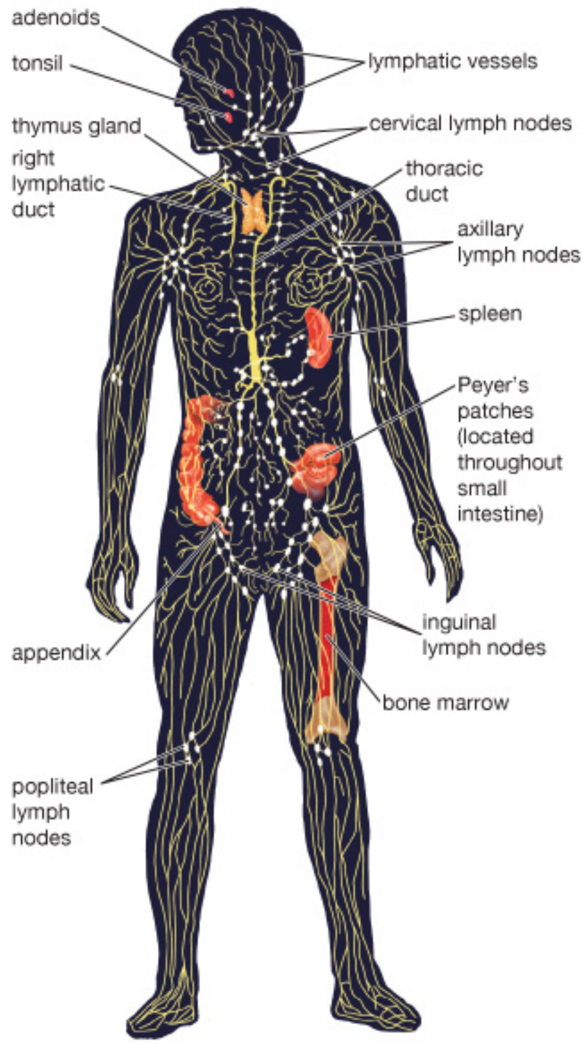

Figure 8: There are a number of lymph nodes across the body[29]

The unfiltered lymph enters throughout the afferent vessels and once filtered with the help of lymphocytes it is expelled back into the circulation via efferent vessels.[39] The lymph node is made up of four layers: the capsule and the inner and outer cortex and the medulla. The capsule is composed of connective tissue and ensures that the lymph node is protected. The outer cortex contains germinal centres which are sites where mature B lymphocytes not only proliferate and differentiate, but here antibodies also switch class as part of an immune response.[38]

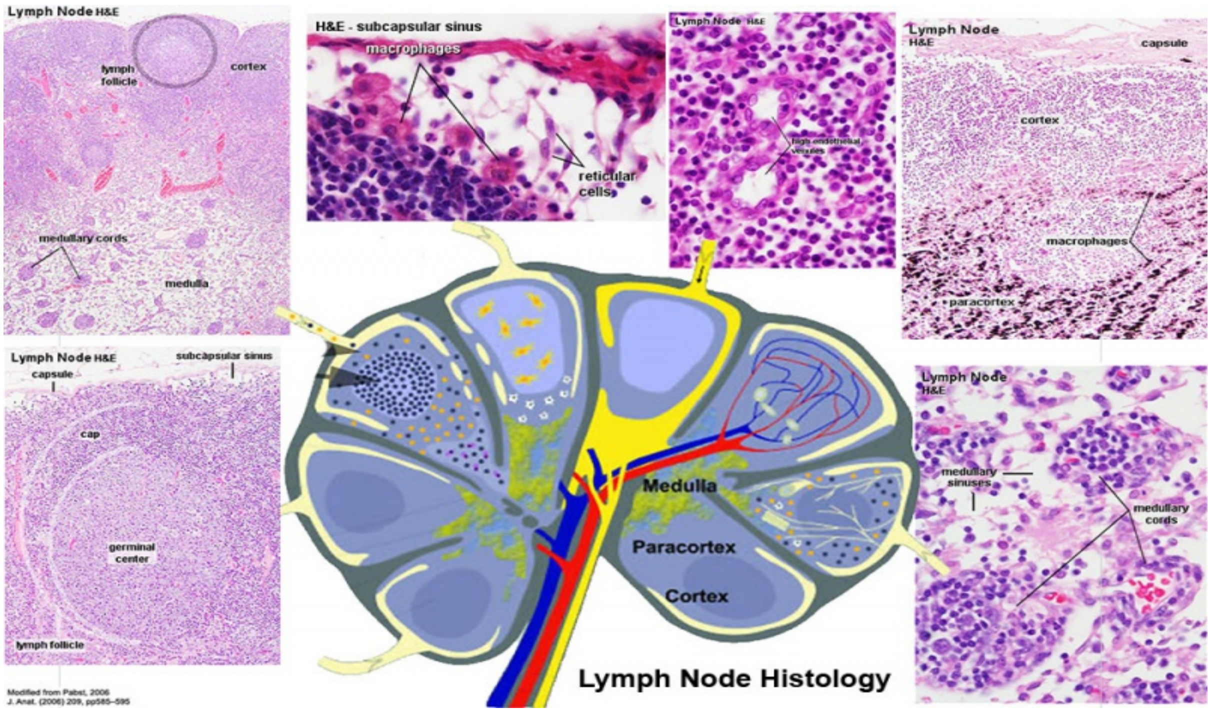

Figure 9: A schematic view of the lymph node along with the microscopic picture (H&E stained) demonstrating the different regions in the lymph node. The lymph enters the lymph node through the afferent lymphatic vessel and the filtered lymph leave via the efferent lymphatic vessels. The cortex which on presentation of an antigen can develop into a germinal centre consists mainly of B cells, where plasma cells and B cells are the central region and the medulla. The blood enters through the paracortex which is also the region which is high in T-cells [101,102]

2.3.2 B-cell abnormalities

Abnormalities in B-cell development can lead to autoimmune diseases, leukaemia or lymphomas. B cell malignancies can arise at different stages of the b-cell development. This gives rise to a range of malignancies with a variety of morphological and clinical presentations. [77]

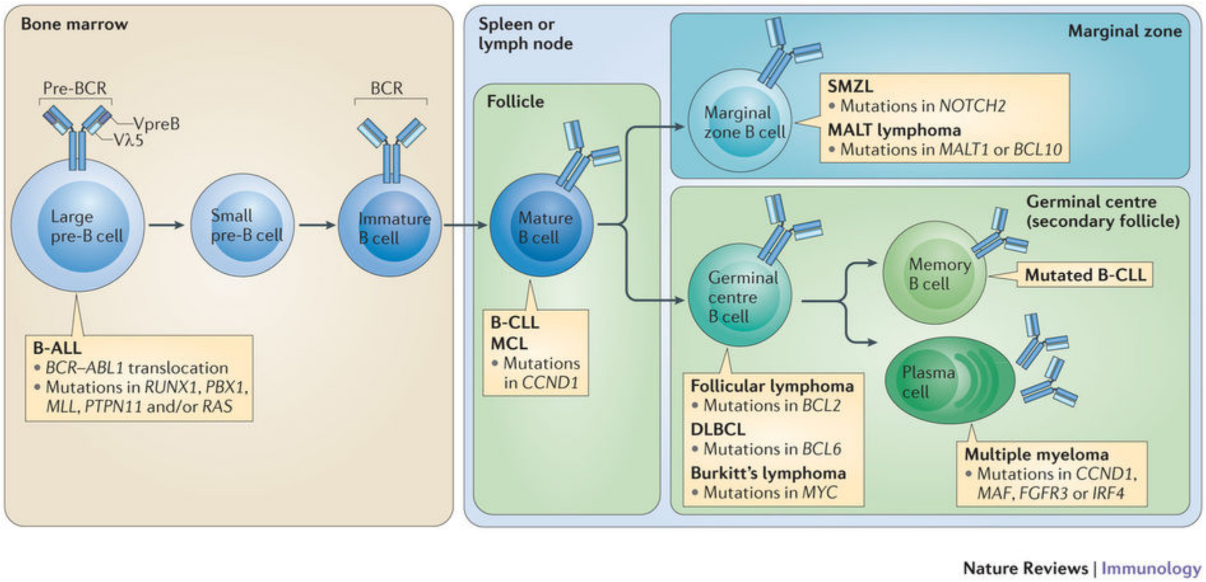

Figure 10: There are a variety of malignancies which can arise from the different B-cell developmental stages. Cancers can arise in the bone marrow, spleen or lymph node. The type of cancer and the cells that dominate in each type is based on the area in which the cancer formed.[78]

2.3.3 Lymphoma

Lymphoma is the most common blood malignancy which was discovered in 1832 by Thomas Hodgkin's, [42] This tumour is characterised by an uncontrollable growth of lymphocytes.[66] The development of lymphomas is largely unknown, however, the interaction between the environment factors and the host is said to play a role in its development.[43] In 2012 lymphoma developed in 450,000 worldwide with 44% resulting in death ranking it the 7th most common cancer in the world.[67,79]

2.3.3.1 Symptoms

The symptoms associated with lymphoma are the same for both Hodgkin's and non-Hodgkin's lymphomas. The symptoms may include sudden weight loss, night sweats, fatigue, enlarged painless nodes, loss of appetite and fever.[44] In DLBCL systemic B symtoms (fever, night sweats, weight-loss) is seen in 30% of cases and has also been assoiciated with a poor prognosis.[68]

2.3.3.2 Types

Lymphomas can be divided into 2 subtypes; Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL).[37,44,45]

In the United States of America, there are an estimated 761,600 people in remission or living with lymphoma, where NHL cases are estimated at 584,100.[46] The reported survival rate is 71.4% in NHL and 87.7% in HL. In the United States, approximately 20,000 people die from lymphoma every year; 6% from HL and 94% from NHL.[46]

TABLE 4: WHO classification of the mature B-cell, T-cell, and NK-cell neoplasms (2008)

Mature B-cell neoplasms

Chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic lymphoma/leukemia, unclassifiable
- *Splenic diffuse red pulp small B-cell lymphoma**
- Hairy cell leukemia-variant*

Lymphoplasmacytic lymphoma
Waldenström macroglobulinemia
Heavy chain diseases
- Alpha heavy chain disease
- Gamma heavy chain disease
- Mu heavy chain disease

Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone lymphoma
Pediatric nodal marginal zone lymphoma
Follicular lymphoma
Pediatric follicular lymphoma
Primary cutaneous follicular center lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma (DLBCL), NOS
- T-cell/histiocyte-rich large B-cell lymphoma
- *EBV+ DLBCL of the elderly*
- *DLBCL associated with chronic inflammation*
- Lymphomatoid granulomatosis

Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary cutaneous DLBCL, leg type
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma arising in HHV-8–associated multicentric Castleman disease
Primary effusion lymphoma
Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classic Hodgkin lymphoma

Mature T-cell and NK-cell neoplasms

T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK cells*
Aggressive NK cell leukemia
Systemic EBV+ T-cell lymphoproliferative disease of childhood
Hydroa vacciniforme-like lymphoma
Adult T-cell leukemia/lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Enteropathy-associated T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous CD30+ T-cell lymphoproliferative disorders
- Lymphomatoid papulosis
- Primary cutaneous anaplastic large cell lymphoma

Primary cutaneous gamma-delta T-cell lymphoma
*Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma**
*Primary cutaneous CD4+ small/medium T-cell lymphoma**
Peripheral T-cell lymphoma, NOS
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma, ALK+
*Anaplastic large cell lymphoma, ALK−**

Hodgkin lymphoma

Nodular lymphocyte-predominant Hodgkin lymphoma
Classic Hodgkin lymphoma
- Nodular sclerosis classic Hodgkin lymphoma
- Lymphocyte-rich classic Hodgkin lymphoma
- Mixed cellularity classic Hodgkin lymphoma
- Lymphocyte-depleted classic Hodgkin lymphoma

Posttransplantation lymphoproliferative disorders (PTLDs)

Early lesions
Plasmacytic hyperplasia
Infectious mononucleosis-like PTLD
Polymorphic PTLD
Monomorphic PTLD (B and T/NK-cell types)†
Classic Hodgkin lymphoma type PTLD†

*Provisional entities for which the WHO Working Group thought there was insufficient evidence to recognize as distinct diseases at this time.
†These lesions are classified according to the leukemia or lymphoma to which they correspond. Diseases shown in italics were newly included in the 2008 WHO classification.

Figure 11: WHO classification of the mature B-cell, T-cell, and NK-cell neoplasms.[47]

2.3.3.2.1 Hodgkin's

HL is the rarer of the two, where the malignant cells arise from the B lymphocytes.[48] Reed-Sternberg cells are seen in HL, and this is a diagnostic factor that helps distinguish the lymphomas. Both lymphomas that have spread and are *in situ* can be cured.[48]

2.3.3.2.2 Non-Hodgkin's lymphoma

NHL is a group of heterogeneous diseases, which originates in the B-, T-, or natural killer (NK) lymphocytes. Between 85% and 90% of these lymphoproliferative disorders originate from B lymphocytes where the remaining disorders arise from T lymphocytes or NK lymphocytes.[45] There are two clinical categories: indolent, which shows fewer symptoms and slower growth, and aggressive, which has fast growth.[46]

2.3.3.2.2.1 Diffuse large B cell lymphoma

DLBCL is one of the most frequent lymphoma seen. Studies by Perry et al. collected data from 1985 to 1999 and from the 487 samples collected 38.2% was DLBCL[86,87]. Anderson et al., collected a total of 1378 samples and looked at Non-Hodgkin's lymphoma in a variety of locations including Hong Kong, Cape Town, Vancouver, London, Lyon, Gottingen, Locarno and Omaha. In London, Vancouver, Omaha and Cape Town there were slightly more follicular lymphoma cases than DLBCL but in all other locations DLBCL had the highest numbers. In all locations, the percentage of DLBCL was more than 25%.[89]

Diffuse large B cell lymphoma (DLBCL) is one of the non-Hodgkin's lymphomas which tends to be very aggressive.[66] It is a clinically heterogeneous disease that presents itself at different anatomical sites. There are two subtypes for DLBCL: germinal B-cell-like (GCB) and activated B-cell-like (ABC).[50,36, 3] GCB DLBCL has a better prognosis than ABC DLBCL.[80]

The WHO's classification system defines 14 subtypes, each of which can be differentiated based on the location of the tumour, the presence of other cells within the tumour (such as T cells), and whether the patient has certain other illnesses related to DLBCL. One of these well-defined groupings of particular note is primary mediastinal (thymic) large B cell lymphoma, which arises within the thymus or mediastinal lymph nodes.[51]

In some cases, a tumour may share many features with both DLBCL and Burkitt's lymphoma. In these situations, the tumour is classified as simply "B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt's lymphoma". A similar situation can arise between DLBCL and Hodgkin's lymphoma; the tumour is then classified as "B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Hodgkin's lymphoma".[47]

When a case of DLBCL does not conform to any of these subtypes, and is also not considered unclassifiable, then it is classified as "diffuse large B-cell lymphoma, not otherwise specified" (DLBCL, NOS). The majority of DLBCL cases fall into this category.[47]

GCB DLBCL and non-GCB DLBCL

The subtypes are believed to arise due to the different stages of the lymphoid differentiation. In the initial stage of differentiation, the precursor B-cell develops into a naïve B-cell in the peripheral lymphoid tissue. Once the naïve B-cell enters the primary follicle, a germinal centre (GC) is formed, where it matures to a centroblast expressing CD10- and BCL6-. The centroblast will then later mature further into a plasma-cell or memory b-cell. For further maturation to occur a slight downregulation of BCL6- by the MUM1 protein may occur. [76 77]

2.3.3.2.2.1.1 Risk Factors

The risk factors associated with the development of lymphoma include several immune deficiency disorders. Some of these include HIV infection, ataxia telangiectasia and Wicott-Aldrich syndrome.[51,76] Some viral, as well as bacterial infections, have been linked to increased incidences. Organ transplants have also shown to increase the risk of developing DLBCL by 10-100-fold.[51]

There are a number of risk factors that are directly linked to the prognosis. These include the age group above 60 years, and having more than one extranodal involvement. The International Prognostic Index of risk factors illustrates the profound effect on mortality and survival rates (see Figure 12).[53]

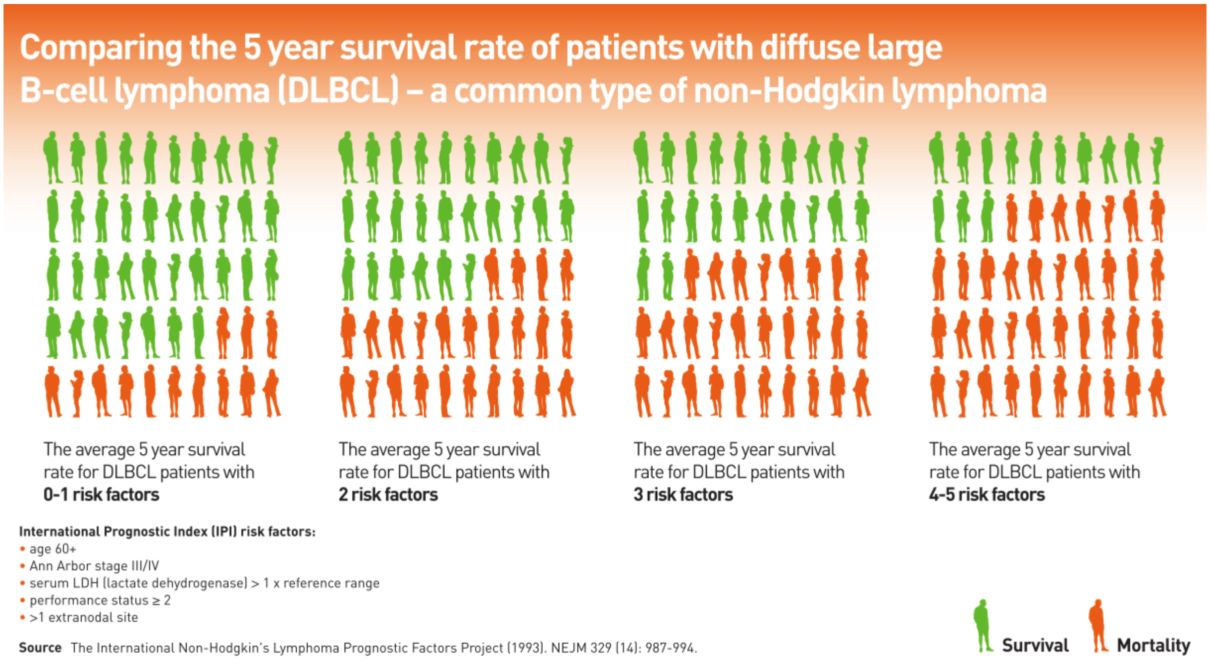

Figure 12: Relationship between the survival and mortality based on the International Prognostic Index risk factors.[49]

HIV/AIDS increases the chance of the development of lymphoma by 100 times. It is believed that the drop in CD4 count due to HIV infection increases the risk of developing lymphoma, especially DLBCL.[54] HIV- related lymphomas (HRL) are more aggressive than Non-HIV-related lymphomas. In addition, they have a shorter life expectancy. However, since the HIV treatment regimen (ARVs) has become available to more people, life expectancy has increased in these individuals.[55,54]

2.3.3.2.2.1.2 Diagnosis

The diagnostic process includes a physical examination to check for enlarged lymph nodes and blood tests (including full blood count, LDH). A fine needle aspiration (FNA) can be done on enlarged lymph nodes to check for malignant cells if the lymph node is not easily assessable.

A biopsy of the lymph node or tissue is done followed by confirmatory immunohistochemistry and flow cytometry testing.[56] The panel used for immunohistochemistry consists of CD20, CD3, CD5, CD10, Bcl-2/Bcl-6, Ki-67, MUM-1 and MYC. The flow cytometry panel used is Kappa/lambda, CD45, CD3, CD5, CD19, CD10 and CD20.[69]

DLBCL is characterized by large abnormal lymphocytes (larger than the normal cells) and the fact that the abnormal cells are spread diffusely throughout the tumour. Large lymphoid cells that are irregularly shaped with dense chromatin and indistinct nucleoli as illustrated in Figure 13 is the picture that is seen when DLBCL is present.[57]

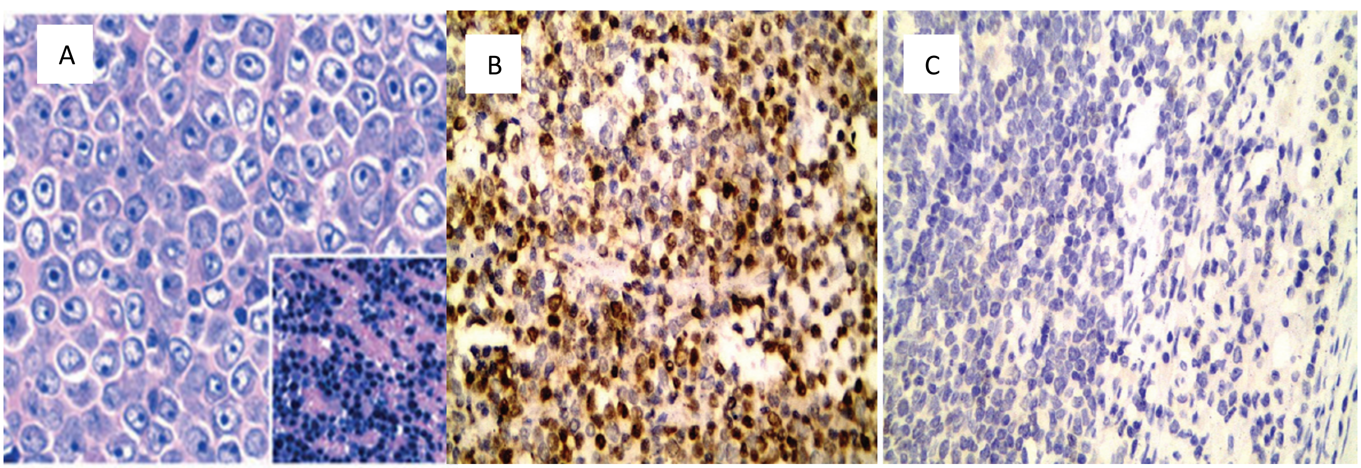

Figure 13: Microscopic investigation of DLBC demonstrate large atypical cells, with abundant cytoplasm and prominent nucleoli (A), Immunochemistry staining is done to help confirm a diagnosis or for subtyping DLBCL cases, B illustrates BCL6+ and C illustrates a BCL6- case[57,100]

2.3.3.2.2.1.3 Immunohistochemistry and prognosis

Subtyping between GCB and ABC-DLBCL can be done by using different immunochemical algorithms. The Hans Algorithm uses three markers to classify diffuse large B cell lymphoma from germinal (CD10+, BCL6 +, MUM1-) and non-germinal subtypes (CD10-, MUM1+. BCL6-); the Choi algorithm adds two stains, FOXP1, GCET1[58] and is thought to be more accurate at classifying, as well as Muris algorithm which uses BCL2 instead of BCL6. It has been shown that the Hans Algorithm is sufficient when investigating prognosis.[59]

The study by Amen et al suggested that the expression of Cyclin D2 and BCL2 are the best indicators of a bad prognosis .[78]In 5% of DLBCL there is chromosomal rearrangements of both *MYC* and *BCL2*, this is known as a double hit lymphoma (DHL) the prognosis for these patients is very poor[70] Ki-67 antigen is present in all cycling cells including cancer therefore reflects the proliferation rate of the tumour. A study by Broyde et al (2007) suggested a cut-off of 70% with a higher expression being indicative of a poor survival.[79]

2.3.3.2.2.1.4 Cancer staging

After cancers has been positively diagnosed then cancer is put into different stage based on its growth and whether it if has metastasised. Cancer staging is the process of determining how far the cancer has developed. There are 4 main stages of DLBCL: stage I and II where a group or two or more groups have been affected on only one side of the diaphragm. Stage III and IV are regarded as advance stage, where stage III is where lymph nodes are affected on both sides of the diaphragm and stage IV is when lymphoma is outside of the lymphatic system.

A letter may accompany the number, which is an indication of the presence of B symptoms and in the cause of "E" this indicates extranodular involvement. [17,93,122]

2.3.3.2.2.1.5 Treatments

DLBCL is a curable disease, and common anthracycline-based chemotherapy is used to treat it.[60]. Rituximab was later discovered and is thought to cause apoptosis and cell death. The current treatment regimen of three cycles of CHOP and rituximab is used in conjunction with radiation.[50,60] Not all patients with advanced stage DLBCL have been cured by the R-CHOP therapy. Therefore, the future therapies may include Bortezomib, Enzastaurin, Lenalidomide, and Bevacizumab.[50]

After treatment, the 5-year survival rate of the subtypes are 60% for GCB and 35% for ABC pathway.[61]

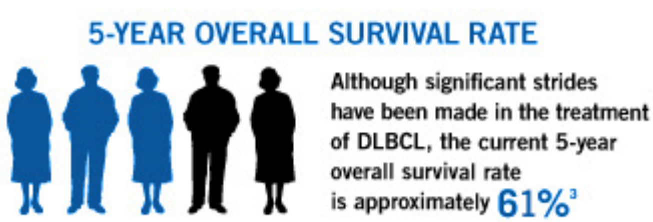

Figure 14: Overall survival rate of DLBCL even with treatment is only 61%.[67]

2.3.3.2.2.1.6 MicroRNA in DLBCL

In DLBCL, the level of expression of certain miRNAs can play a major role due to their prognostic and diagnostic value.[21] Eight miRNAs were found to correlate with overall and event-free survival in DLBCL. These are miR-21, miR-127, miR-34a, miR-195, miR-19A, miR-23A, miR-27A and miR-LET7G.[31 62]

2.4 Biomarkers

Detection, diagnosis, and prognosis of cancer are just some of the ways in which biomarkers have provided to be important in oncology. Biomarkers have also been found to be useful in determining therapeutic intervention in a variety of cancers. Biomarker are simply indicator of biological status, originating from within the cell their three main sources are proteins, metabolites and nucleic acids. [90] Although biomarkers may not be very helpful when only one is used as they lack specificity that links them to one cancer, it is therefore necessary that the biomarkers are used as a panel. A panel will then consist of a number of biomarkers that will be specific to each cancer, with further testing helping to type cancers and even determine prognosis.

Although there are a number of biomarkers there are three biomarkers that stand out in literature when investigating prognosis, BCL2, Ki-67 and BCL6. [78,79,91]

2.4.1 BCL6

The zinc finger transcription factor BCL6 proto-oncogene is necessary for germinal centre (GC) formation and is found to be frequently translocated in DLBCL.[91,93]

2.4.2 BCL2

Part of the family of apoptotic regulatory proteins BCL2 is an anti-apoptotic protein important in normal B-cell development and differentiation. BCL2 is frequently overexpressed, GCB the t(14;18) translocation is usually the cause of the upregulation whereas in the non-germinal subtype, ABC is overexpressed due to the consecutive activation of the NF-ϰB pathway. [92]

2.4.3 Ki67

The nuclear protein Ki67 is used to determine the proliferative activity of the lymphoma by determining the number of cell positive for Ki67 and dividing it by the total number of cells. Ki67 is expressed by dividing cells throughout the active part of the cell cycle. Ki67 staining of about 70% is regarded as having a poor prognosis. [79,91]

2.5 HIV and Lymphoma

HIV is classified as a virus called a ‘retrovirus’. It weakens your immune system by destroying CD4+ T cell leading to polyclonal proliferation of B cells. The constant proliferation then increases the chance of accumulation of mutations.[75]

36.9 million people are currently living with Human Immunodeficiency Virus (HIV) worldwide with 67.8% in Sub-Saharan Africa.[73,50] According to Shisana et al. (2012) the estimated HIV incidence in South Africa increased to 6.4 million people in 2014, with an estimated 321 000 patients diagnosed in the Western Cape.[74] HIV infection is an important risk factor that has been linked to an increased development of diffuse large B cell lymphoma (DLBCL).

2.6 The gap in the research

A higher number of DLBCL cases have been seen within the HIV positive cohort. With the high HIV statistics, this has led to an increased risk of developing DLBCL. Although treatable HIV-related DLBCL is very aggressive and if not treated may lead to death within a year. Research has not yet established whether miRNA-127 expression is lower in a HIV positive cohort or whether miRNA-127 remains unaffected, hence not affecting the prognosis.

Our study will investigate the expression of miR-127 in a cohort of HIV-positive and HIV-negative DLBCL cases. In addition, an investigation of its prognostic potential in HIV positive DLBCL will be analysed since no study has determined the relationship in such a cohort.

In 2013, a retrospective study done at Red Cross Children's Hospital in Cape Town, South Africa, over a period of 10 years, showed that 6.5% of HIV-negative children were diagnosed with DLBCL whereas children who were HIV-positive, 34.6% DLBCL cases were reported.[63]

There is, however, no information that shows how the HIV status affects the expression levels of miR-127 and how this affects the prognosis.

CHAPTER 3: MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Ethics Approval

Ethics approval for the project was obtained from the Human Ethics Research Committee, Faculty of Health Sciences (HREC REF: 755/2015), at the University of Cape Town.

3.2 Sample size determination

The sample size was determined using the sample size calculation for a qualitative variable.[99] The number of cases selected was based on the prevalence of DLBCL in the population in the study by Ferlay[75] Other literature was also examined, and the similar sample sizes were used in other studies.

3.3 Sample Collection

Cases for this study were selected by initially doing a search on the DISALab system of the NHLS to find DLBCL cases submitted for histopathological analysis at the NHLS. The Formalin-Fixed Paraffin-embedded (FFPE) tissue was used in our study. The original Haematoxylin and Eosin (H&E) stained slides corresponding to the samples were examined by a pathologist, from the Division of Anatomical Pathology, NHLS/UCT. Inclusion criteria was DLBCL with a population of more than 50% abnormal lymphocytes. The tissue blocks of the corresponding cases were then obtained from the archives of the Division of

Anatomical Pathology, National Health Services(NHLS), University of Cape Town, Groote Schuur Hospital.

3.4 Pathology patient report

Patient reports for the approved cases were accessed from the Department of Radiation Oncology at the Groote Schuur Hospital, Cape Town under the supervision of the Head of oncology department (Collaborators). The age, stage, serum lactate dehydrogenase levels, performance status and involvement of extranodal sites were noted. The date of death (if relevant), sex and HIV status were also noted.

3.5 H&E staining

The DLBCL samples were cut into 3μm sections and dewaxed in xylene. The sections were dehydrated through a descending series of alcohol followed by being washed under running water. The slides were then placed in Meyers Haematoxylin for 5 minutes to stain the nucleus before being rinsed and then blued with Ammoniated water for 3 minutes. The slides were washed again and put into the counterstain, phloxine Eosin for 15 minutes. Finally, slides were washed, dehydrated through an ascending series of ethanol, cleared in xylene and mounted with Entellan .

After staining, slides were then examined and demarcated by a pathologist, thus indicating tumour regions. This is done to ensure that no necrotic material or other tissue was scraped, to prevent false readings or contamination. After slides were demarcated, more sections were cut at 8μm to be used for the RNA extraction.

3.6 RNA Extraction

3.6.1 Tissue Preparation

RNA was extracted from FFPE tissue. 8μm tissue section were cut and placed on glass slides and baked on a 60˚C hot plate for an hour to melt the wax. The slides then passed through a series of xylene and alcohol washes, to enable dewaxing and clearing respectively. Finally, the slides were rinsed well before being left overnight at room temperature to dry.

When the slides were sufficiently dry they were superimposed onto the demarcated H&E slides previously marked by pathologist, to ensure that the correct area is scraped. The tissue was then scraped using scalpel blades before being transferred to autoclaved Eppendorf tubes. For each case three 8μm sections of tissue was used.

3.6.2 RNA extraction

The High Pure FFPE RNA Micro Kit Version 7 by Roche was used for RNA extraction. The protocol provided by Roche was utilised. This started with 60μl tissue lysis buffer and 10μl 10% Sodium Dodecyl (lauryl) Sulfate (SDS) being added to the scraped tissue. The SDS solubilizes the lipids and proteins in the membrane thus exposing the chromosome.

30μl Proteinase K was then added followed by spinning and incubating in a water bath at 55℃ for 3 hours. The incubation ensures the proteinase is able to digest the protein and remove contamination. After incubation 200μl binding buffer and ethanol were added to the eppendorf tube, mixed well with the vortex and spun down. The lysate was then pipetted into the top reservoir of the micro filter tubes provided. These tubes ensure that the RNA is captured on the filters allowing all debris to pass through and be discarded. 30μl DNase solution was added to the dry micro filter tube and was then incubated at room temperature for 15 minutes. The filter was then washed 3 times, once using wash buffer I and twice with wash buffer II, centrifuging after each addition to ensure clean RNA remains on the filter. The High pure micro filter tube was added to a new micro-centrifuge tube before the 20μl elution butter was added to help release the RNA from the filter. The flow through was pure total RNA.

3.7 RNA quantification and Polyadenylation

The RNA samples were quantified using a Nanodrop (Thermo Scientific, Wellington, DE 19810 USA) instrument. Initially 1μl RNAse-free water was used to blank the Nanodrop before using a volume of 2μl RNA from the extraction sample for the procedure.

The concentration of the samples were diluted to 100ng to be used for the addition of the Poly A tail. The Poly A reaction was carried out in a final volume of 20μl consisting of template RNA (100ng); 5 x PAP buffer; 25mM MnCl2; 100mM ATP; nuclease free water and 2U/μl of poly (A) polymerase. A negative control (nuclease free water instead of RNA) was included in the reaction. The product was then loaded on to the PCR (GeneAmp PCR System 9700, Applied Bioscience). The PCR conditions used were 37°C for 75 minutes and 65°C for 25 minutes.

3.8 cDNA synthesis

Subsequent to the poly A reaction, cDNA was synthesized in a final volume of 20μl using 100ng Poly A product, dH2O, 10mM dNTPs, 5x transcription reverse transcriptase reaction buffer, 25mM $MgCl_2$, 40U/μl protector RNase inhibitor, 20U/μl transcriptor reverse transcriptase and 50mM adaptors. The PCR conditions were as follows:

- 42°C for 60 minutes
- 42°C for 75 minutes
- 85°C for 10 minutes

The cDNA products were then diluted to 1000ng and stored at -20°C.

3.9 miRNA- 127 primer design

Primers for miRNA-127 was performed using the method outlined in the study by Balcells, Ciera and Busk (2011) where 18S and U6 were used as a housekeeping gene for each sample. The primers sequences in table 1 were synthesised in the Department of Molecular cell Biology at the University of Cape Town (UCT).

Table 2: Primer set sequences for miR-127 and two references genes used, 18S and U6

	Forward primer	**Reverse primer**
miRNA-127	CGCAGCTGAAGCTCAGA	CAGGTCCAGTTTTTTTTTTTTTTTATCA
18S	TTTCGCTCTGGTCCGTCTTG	TTCGGAACTGAGGCCATGAT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

3.10 Primer Optimization

In order to optimise RT-PCR conditions and to verify the specificity of the primer pair, the optimal primer concentration and volume were tested using the KicqStart SYBR Green (Sigma Aldrich). The primer titration was preformed using concentrations of the primer ranging from .05μm to 300μm for the total reaction volume of 20μl. In all wells 10ul SYBR green as well as 3μl cDNA was used, the optimal primer volume was also tested from ranging from 0.5μl to 2μl.

3.11 miRNA-127 quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

For each primer, U6, 18s and miRNA-127 a master mix was prepared using 2x Sybrgreen (KapaBiosystems), dH2O and 10μM of the primers (Table 2.4) in separate eppendorf tubes. The eppendorf tubes were then briefly vortexed and a volume of 19.3μl from each master mix was added to 96 well plates, alternating rows between the two primers. 0.7μl cDNA (1000ng) from each of the samples was added into the 19.3μl master mix in triplicates. A blank (B), in which sterile water was added instead of cDNA was included to normalise the reaction. The 96 well plates were then covered with adhesive sealing films to prevent evaporation, leakage and contamination between the wells. The plates were then centrifuged at high speed and placed in a Light Cycler480® instrument (Roche) to perform the qRT-PCR using the conditions below. The qRT-PCR was performed twice to verify the quantitative data generated by the instrument.

Table 3: The qRT-PCR process

Stage	**Number of cycles**	**Temperature (°C)**	**Time**	**Ramp (°C/s)**
Pre-incubation	1 cycle	95	5 m	4.4
Amplification	45 cycles	95 (Denaturation)	10s	4.4
		60 (Annealing)	15s	2.2
Melting curve	1 cycle	95	5s	4.4
		65	1s	2.2
		97	Continuous	0.11
Cooling	1 cycle	40	30s	2.2

3.12 Statistical analysis

The expression patterns of miR-127 were compared to the clinicopathological features using Chi-square test. A p value of ≤ 0.05 indicated statistical significance.

3.13 Relative Quantification

Expression levels of miR-127 relative to 18s and U6, the reference genes, in each sample were determined by dividing the absolute concentration of the target gene by the absolute concentration of the housekeeping gene. The resulting ratio expressed the amount of target relative to the reference gene in each sample.

The fold change can then be determined by using an experimental sample with lower normalization value as the calibrator. Each normalized value is divided by the calibrator to determine the fold change.

The calculation used in the study by Robertus et al.,[34] was also used to compare the results obtain against their findings. In their study they used the U6 as a reference gene. They normalized the target miRNA relative to the reference gene using the following formula:

$\Delta C_t = (\Delta C_{t\ miRNA\text{-}127} - \Delta C_{t\ U6})$. Relative expression levels were expressed as $2^{-\Delta Ct}$.

3.14 Immunohistochemistry method and evaluation

All staining is done using thin FFPE tissue sections of 3µm. The sections first undergo antigen unmasking to help the antibody binding site to be revealed. The stains have labelled antibodies that will bind to their respective antigen and therefore stain positive.

For the evaluation of the three biomarkers that were used, BCL6, BCL2 and Ki67, immunohistochemical staining results from the patient file was used, where available. BCL6 and BCL2 are done in the lab to help classify DLBCL into GCB and ABC, which may help with treatment. In the case of Ki67, the cell proliferation marker, a highly statin tissue is said to be more aggressive. Ki67 is also used routinely to determine treatment efficacy where high percentage reflect worse outcome.

When making the diagnosis, the percentage of positively stained cells are scored.

CHAPTER 4: RESULTS

Results

4.1. The clinicopathologic/demographic features of all the DLBCL cases

A total of 42 DLBCL cases were enrolled in the study (appendix A). There were more females (57%) than males. The ages ranged from 15 years to 90 years, with the majority of cases being over 60 years. For the 42 cases the mean age of the patients was 50, although HIV positive DLBCL cases had a lower age average (37 years) and HIV negative cases had an age average of 60 years old. There were 10 HIV positive cases and 30 HIV-negative cases, and 2 cases had no HIV status recorded.

Furthermore, 22% cases presented with stage I, 19% with stage II, 16% with stage III and only 44% presented with stage IV of the tumour, but 10 cases were undefined. There were 11 cases which presented with extranodal involvement, but 17 cases had no record, whether there was involvement or not, 33% of cases were cervical lymph nodes, with the sites ranging from inguinal node to axillary node.

Only 14 patients out of 42 had been recorded as deceased, with the majority occurring as a result of cancer related problems (Table 4).

Table 4: The clinicopathologic features of the 42 cases

Clinicopathologic features	***Number of cases with valid entry***
Age	<60 = 23
	>60 =19
Sex Ratio (M: F)	18:24
Ann Arbour Stage	I = 7
	II =6
	III =5
	IV =14
	Not recorded = 10
Extranodal involvement	Involved = 11
	Not Involved =14
	Not recorded = 17
HIV cases	HIV positive = 10
	HIV negative = 30
Deceased patients	14
GCB/ABC	GCB = 14
	ABC =16
	NR=10
Cervical node	14

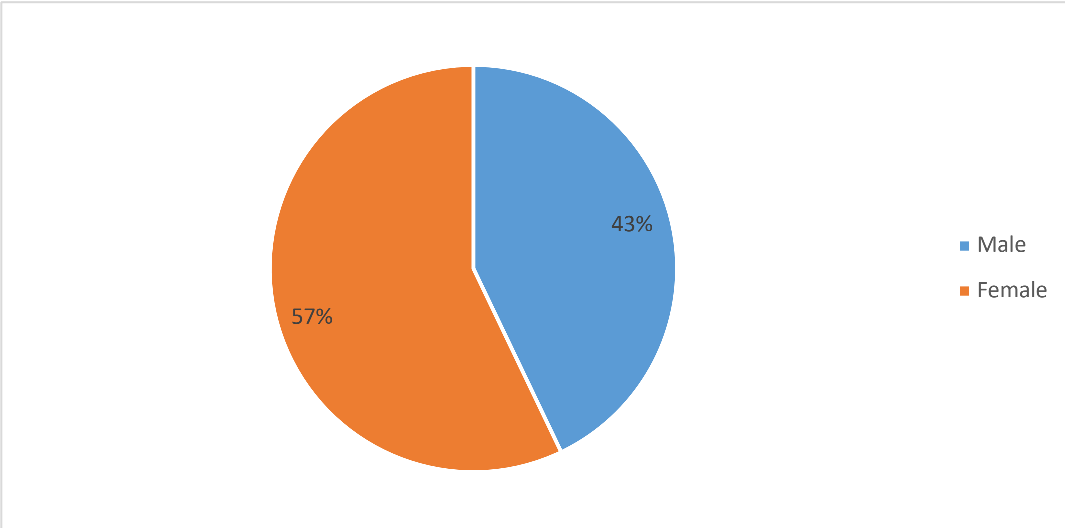

Figure 15*:* The distribution of gender in the DLBCL cohort

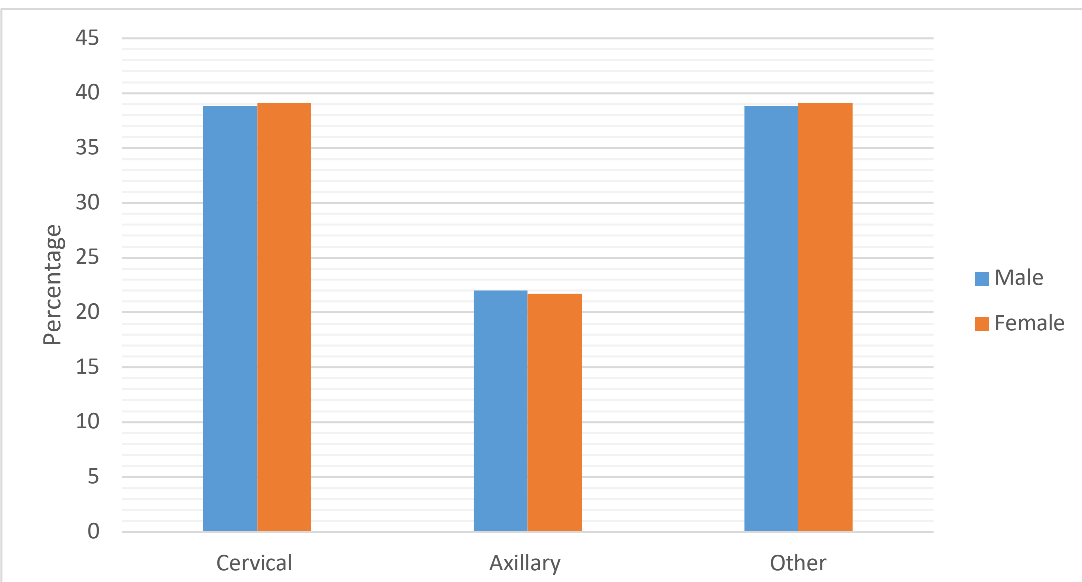

Figure 16*:* The relationship between gender in the distribution of site of cancer in the DLBCL cohort

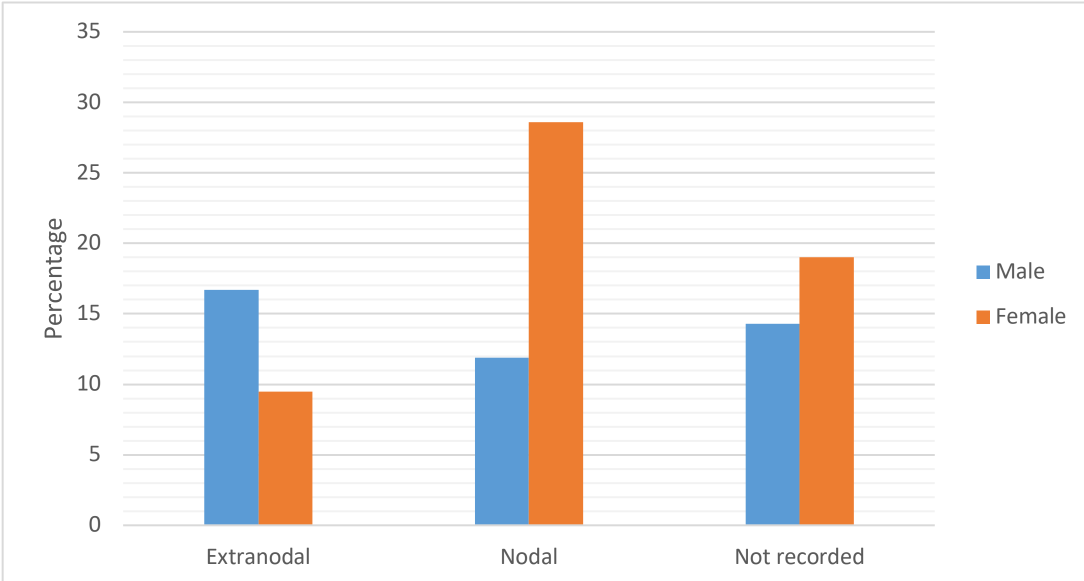

Figure 17*:* The relationship between gender and extranodal involvement

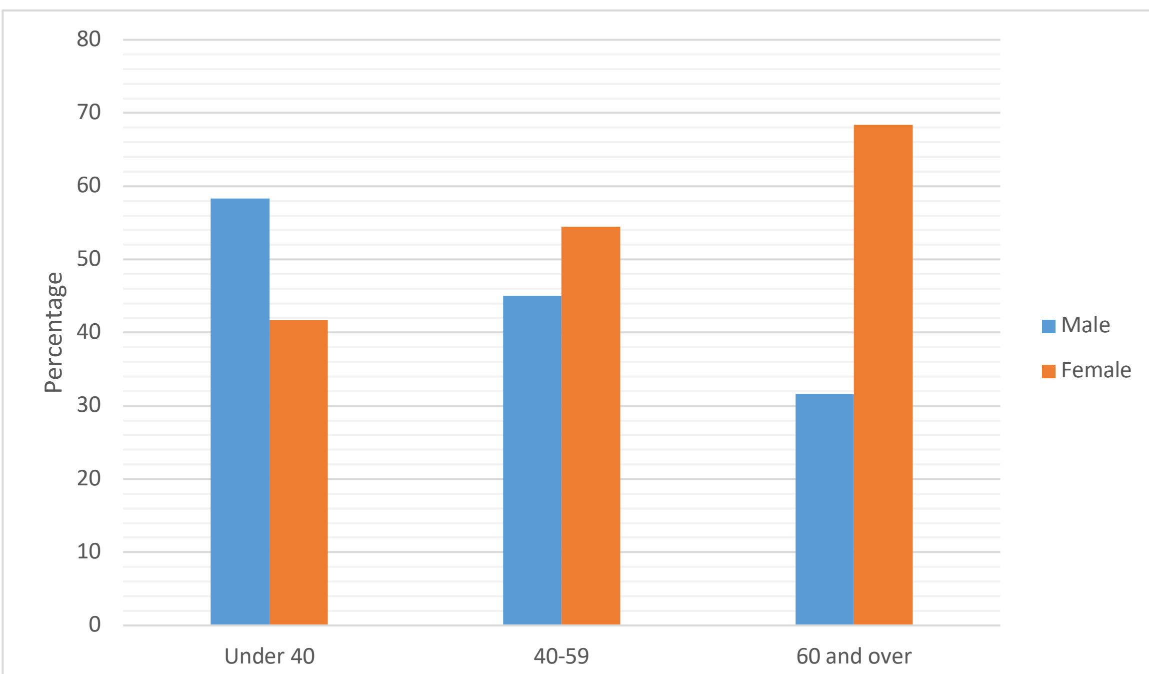

Figure 18*:* The relation between gender in the distribution of age in the DLBCL cohort

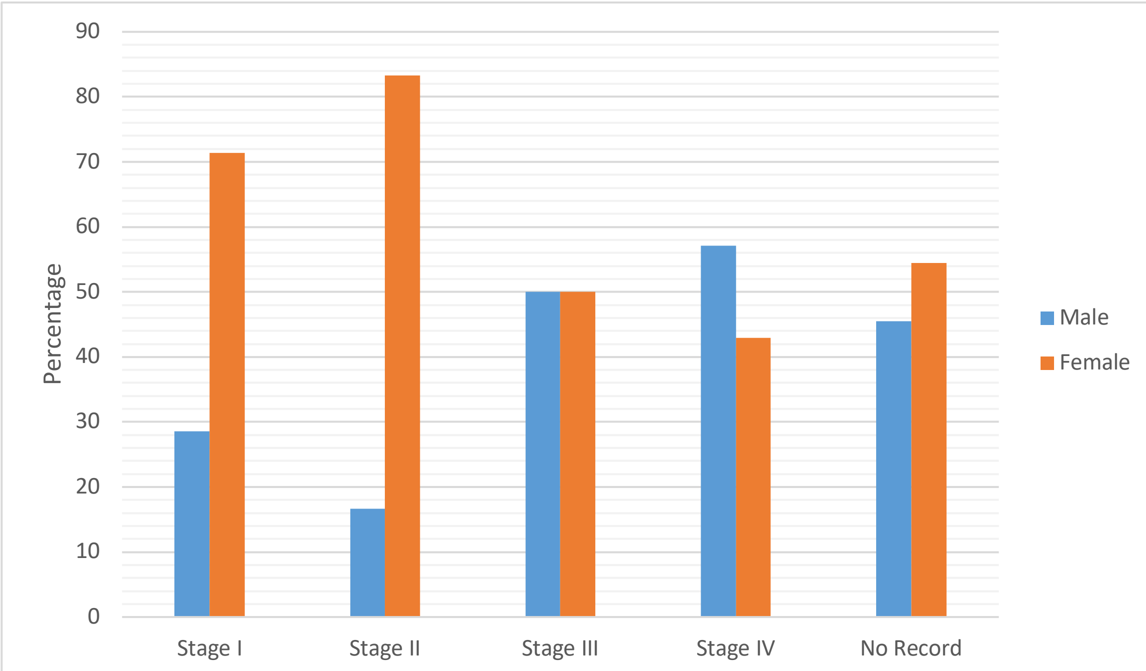

Figure 19*:* The relation between gender in the distribution of stage of cancer in the DLBCL cohort

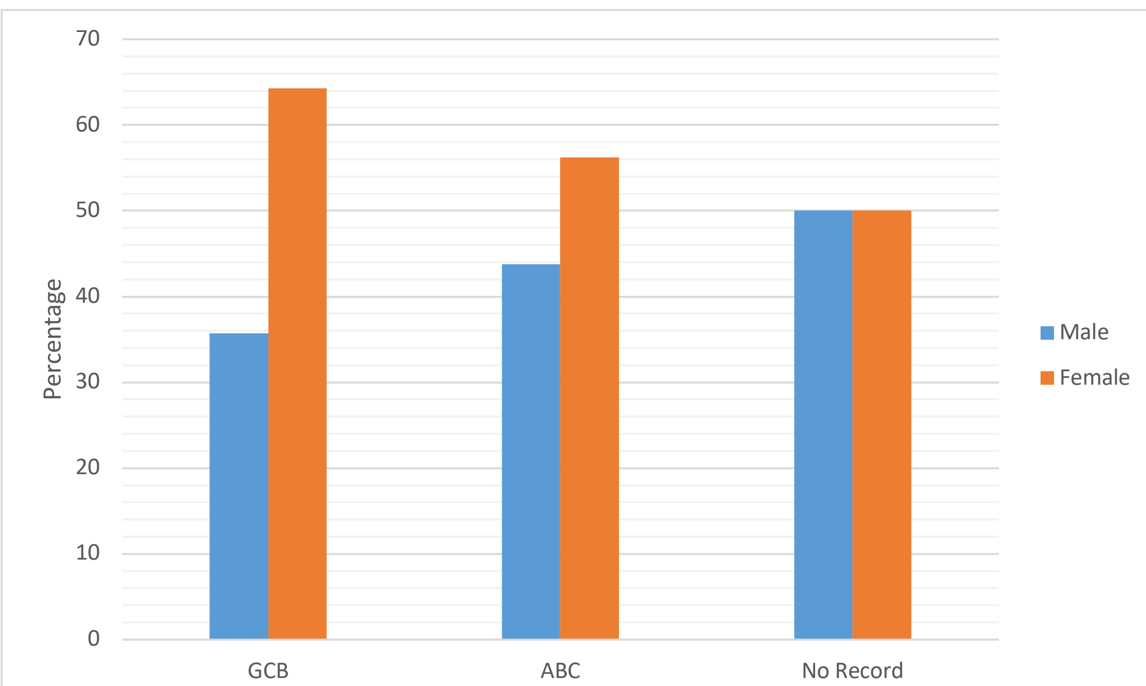

Figure 20: The relation between gender in the distribution of subtype in the DLBCL cohort.

*(GCB – Germinal Centre B-cell. ABC – Activated B-cell)

4.2 Comparison of HIV positive vs HIV negative cases

Table 5: Comparison of the clinicopathologic features and HIV status

	HIV- DLBCL	*HIV+ DLBCL*	*No record*
Number of cases	**30**	**10**	**2**
Age Range	**28-90**	**15-63**	
Age average	**60**	**37**	
Ann Arbor Stage			**11 (2)**
I	**6**	**1**	
II	**3**	**3**	
III	**3**	**1**	
IV	**11**	**3**	
Elevated LDH (>480=U/L)	**14**	**6**	**11 (3)**
ECOG Performance status			**18 (5)**
0	**2**	**0**	
1	**7**	**2**	
2	**3**	**1**	
3	**6**	**2**	
4	**1**	**0**	
Site			**18 (4)**

Nodal	**8**	**5**	
Extranodal	**10**	**1**	
Subtype			**10(2)**
GCB	**10**	**4**	
ABC	**11**	**5**	
IPI (≥3) *	**14**	**2**	

***IPI was determined only for cases that had record of all five factors**

There were 31 and 24 cases with Ann Arbor stage and performance status (PS) recorded, respectively (Table 5). The majority of the HIV negative cases were classified as stage IV (47.8%). In contrast, the majority of cases in the HIV positive group were classified as stage II and stage IV (37.5%).

There were 16 cases with IPI more or equal to 3 recorded, of which only 2 were HIV positive.

Just 57% of all DLBCL cases had record of nodal involvement, where 54% were nodal lymphomas. 55% of HIV negative cases were recorded as having extranodal involvement, in contrast to the HIV positive cases being only16.6%.

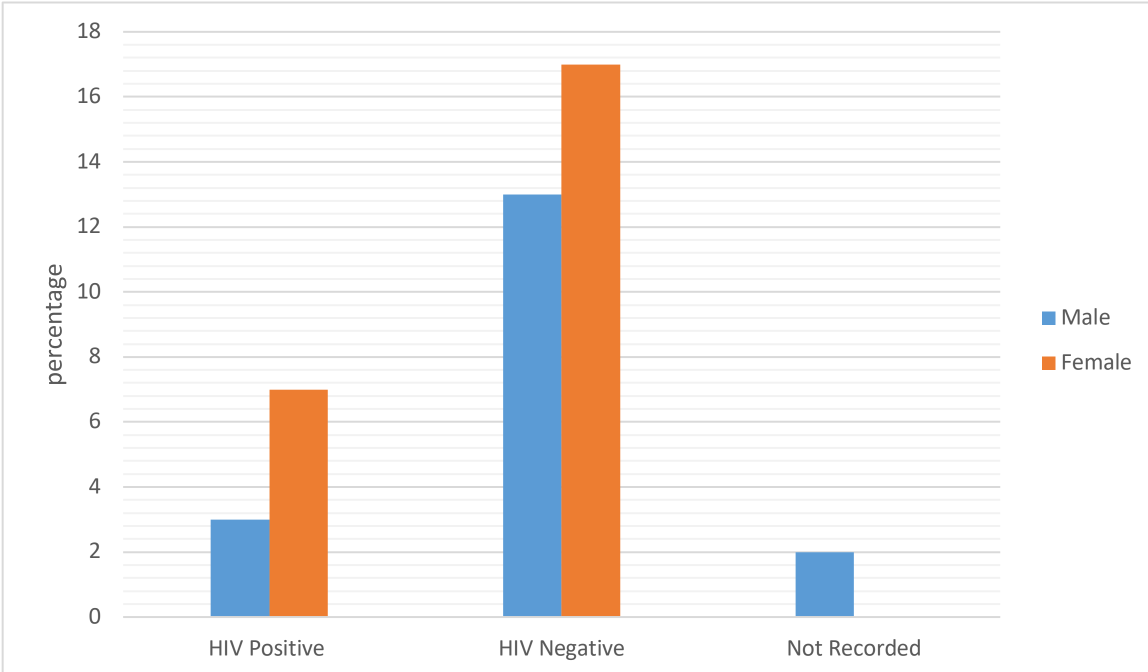

Figure 21: The relation between gender and HIV status in the DLBCL cohort

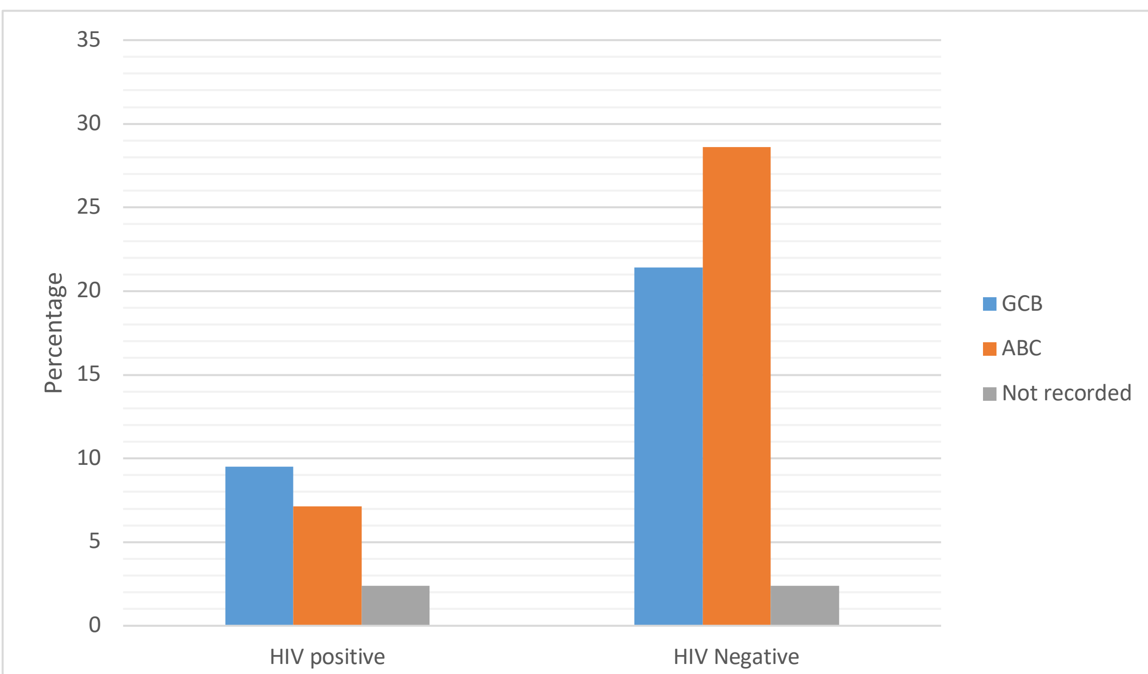

Figure 22: The relation between HIV status and DLBCL subtype in the DLBCL cohort

*(GCB – Germinal Centre B-cell, ABC – Activated B-cell)

4.3 Comparisons of HIV status and immunohistochemical stains

Table 6: Comparison of the immunohistochemistry stains and HIV status

IMMUNOHISTOCHEMICAL STAIN	***HIV- DLBCL***	***HIV+ DLBCL***	***Cases with no data***
BCL2 positive (Total)	**18 (25)**	**5 (6)**	**11**
BCL6 positive (Total)	**22 (29)**	**7(10)**	**3**
KI67 ≥70% (Total)	**16 (22)**	**7 (7)**	**13**

Only 74% and 69% of DLBCL cases had BCL2 Ki67 recorded respectively, whereas 93% of cases had BCL6 results. All HIV positive cases had a Ki67 results higher have 70%, where HIV negative had only72.7% cases that were higher than 70%. 83% of HIV positive cases were possible for BCL2 in contrast to the 72% HIV negative cases. The BCL6 results were closers, with 76% positive cases for HIV negative cases and 70%postive cases for HIV positive cases.

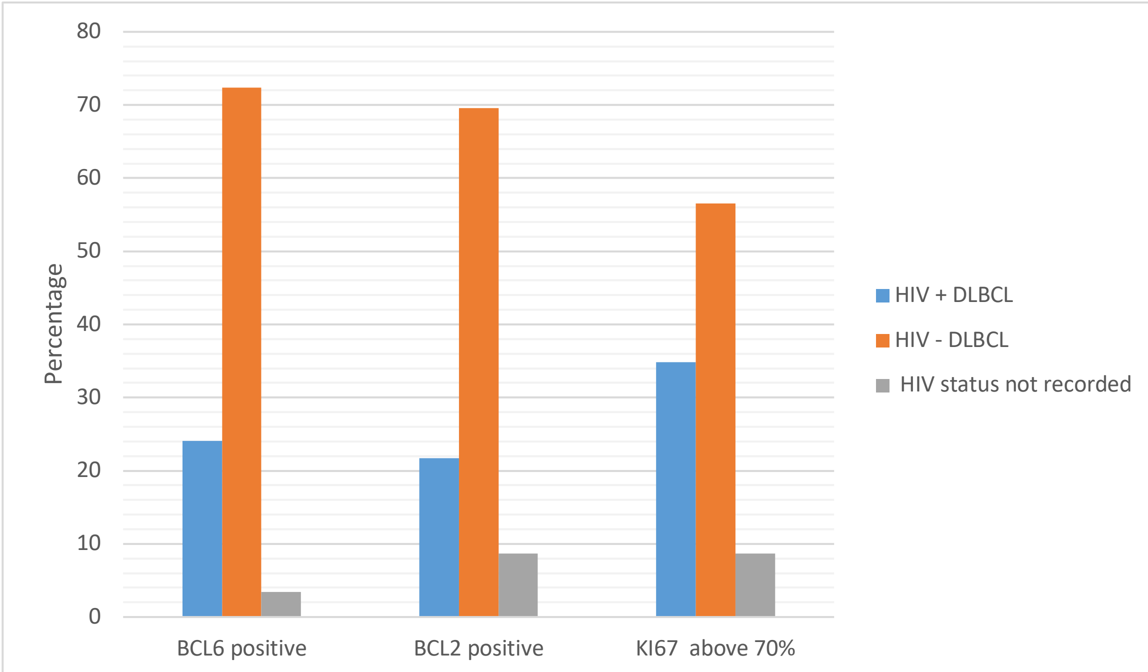

Figure 23: The relation between HIV status and the positive immunohistochemistry staining in the DLBCL cohort

4.4 MiRNA-127 expression levels

73.8% of the DLBCL cases had higher miR-127 expression levels than the normal and reference genes. 41.9% of cases are over 60 years old and 57.8% HIV-negative. Disregarding the cases without staining data, 75% of cases were BCL6 positive, 76.2% BCL2 positive and 73.7% had a Ki67 above 70%. 35.5% of cases were patients whom had died within a year of diagnosis.

26.2% of DLBCL cases recorded no miR-127 expression level and was regarded as being silenced. 63.6 of these cases are female and 36.4% are HIV-positive. All these cases expect 1 had Ki67 percentages greater than 70% and all but 3 had positive BCL6. It should be noted that the 3 cases which were BCL6 negative were all BCL2 positive. Although there was one case that had no BCL2, out of the remaining cases only 30% of cases were BCL2 negative and all these cases were BCL6 positive. Only 27.2% of cases were cases where patients had died within a year of diagnosis.

Table 7: Comparison of the clinicopathologic features and miR-127 expression levels

VARIABLE	***MiRNA-127 EXPRESSION***		
	High Expression	**Silenced**	**Not recorded**
Sex (F:M)	17:14	7:4	**0**
Age			**0**
Above 60	13	5	
Ann Arbor Stage			**11**
I	6	2	
II	4	2	
III	3	1	
IV	9	4	
Elevated LDH (>480=U/L)	13	7	**11**
ECOG Performance status			**18**
0	**1**	**1**	
1	**7**	**2**	
2	**4**	**0**	

3	**6**	**2**	
4	**1**	**0**	
Nodular Involvement			**18**
Nodal	**11**	**2**	
Extranodal	**8**	**3**	
Subtype			11
GCB	**9**	**5**	
ABC	**12**	**4**	
IPI (≥3)	**12**	**4**	**3**
Positive Immuno stains			
BCL6	**21**	**8**	3
BCL2	**16**	**7**	11
*Ki67 (**≥70%**)*	**14**	**8**	13
HIV Status			2
Positive	**6**	**4**	
Negative	**23**	**7**	

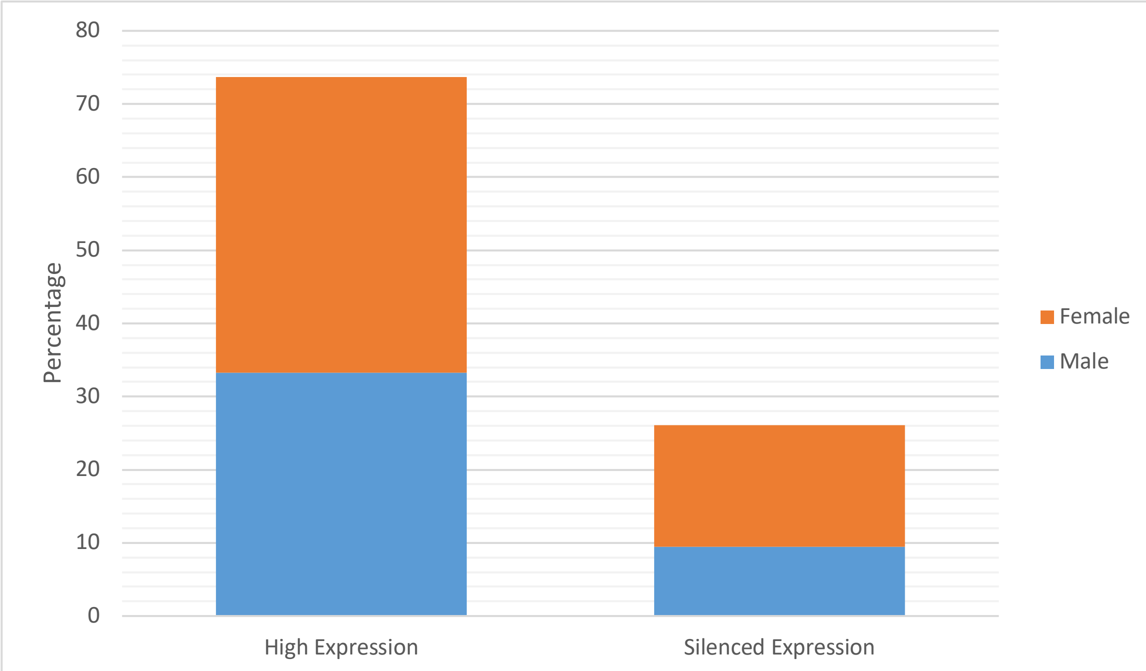

Figure 24: The relation between gender and expression levels of miR-127 the DLBCL cohort

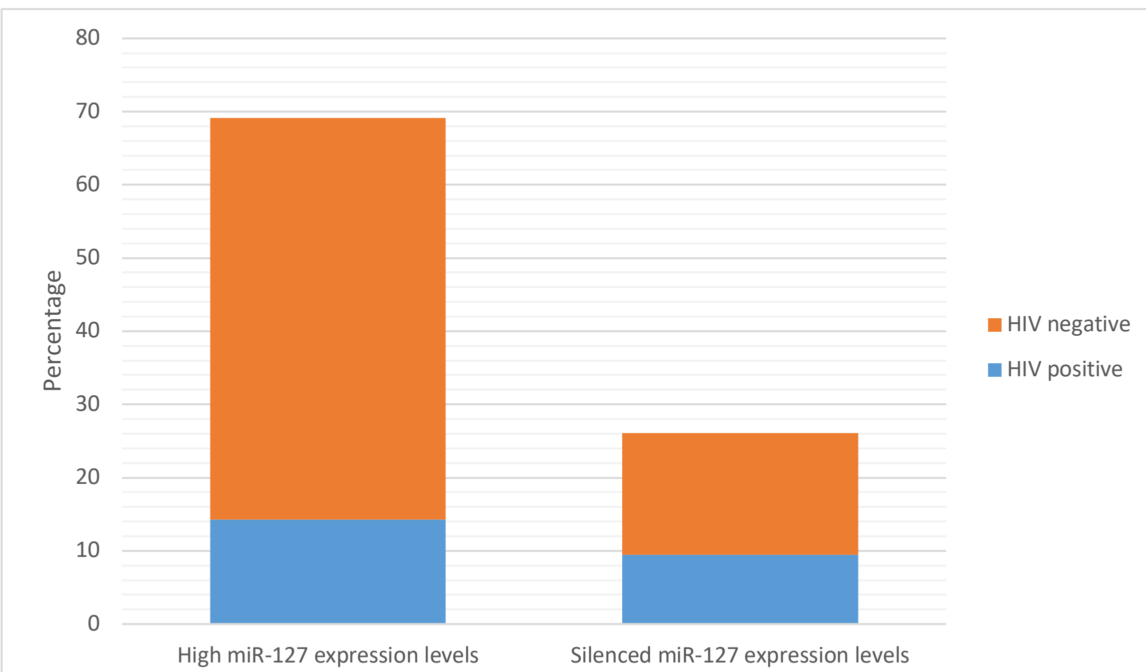

Figure 25: The relation between HIV status and expression levels of miR-127 the DLBCL cohort

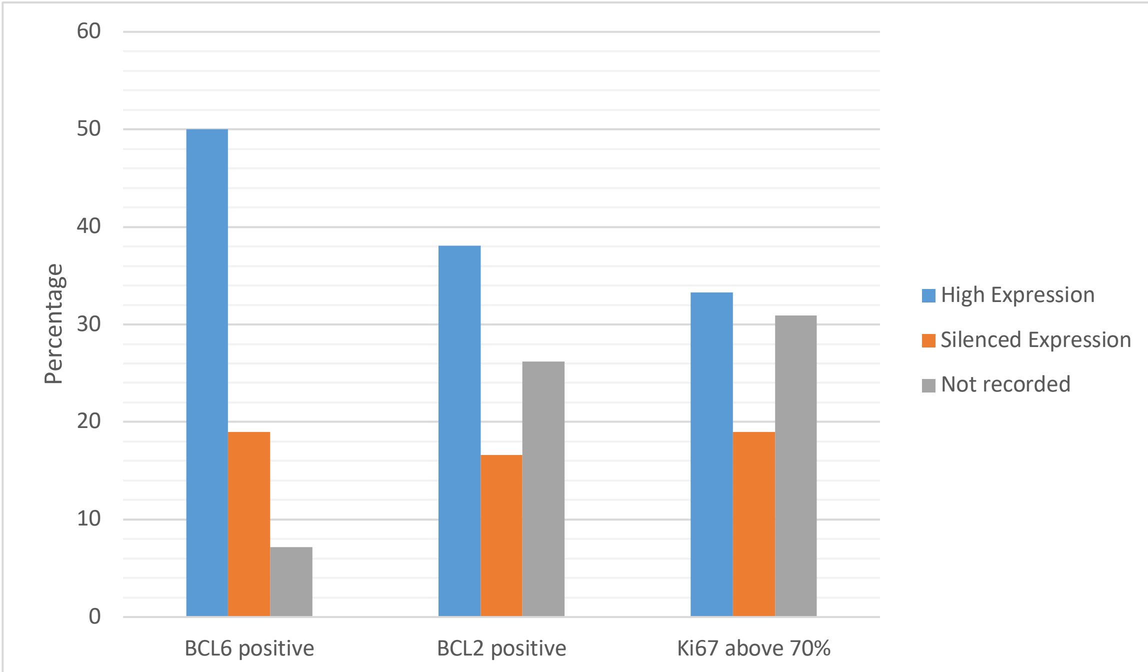

Figure 26: The relation between positive immunohistochemical stains and expression levels of miR-127 the DLBCL cohort

4.5 Significance of clinicopathological feature in association with miRNA-127 expression

Out of the cases used 73.8% had higher expression levels than that of the normal tissue and that of the reference genes, and 26.2% were silenced. However, there is no statistical significance of any of the clinical feature in respect to the expression levels of miRNA-127.

Table 8: Significance of the clinicopathologic features and expression of miRNA-127

Clinical Feature	***Significance in respect to miRNA-127***
Sex	**Not significant (p=0.796)**
Age (Above 60)	**Not significant(p=1)**
Subtype	**Not significant(p=0.311)**
HIV status	**Not significant(p=0.417)**
BCL6 positive	**Not significant(p=0.322)**
BCL2 positive	**Not significant(p=0.498)**
Ki67 above 70%	**Not significant (p=0.546)**

CHAPTER 5: DISCUSSION AND CONCLUSION

Discussion

The expression levels of miR-127 were correlated to clinicopathological features thought to be important for prognosis, as well as factors on the International Prognostic Index (IPI). These factors included age, Lactate Dehydrogenase levels, Performance status, stage of cancer and extranodal involvement. We also correlated the expression levels with HIV status, sex, survival status and three immunohistochemical stains thought to have prognostic value.

DLCBL is a curable disease but if left untreated leads to death within a year of diagnosis.[46] Lower expression levels of miR-127 has also been associated with a bad prognosis and it was therefore important to determine which factor are significant to the expression levels of miR-127, if any.

Regarding gender distribution, we found that the majority of the cohort were female (57%) whilst the males were the minority (18/42). Studies by Sarkozy et al.,[104] Hasselblom et al.,[105] and Robertus et al.,[32] all demonstrated a different relationship with the majority of case being male in these studies.These studies correlate with the Ferlay global statistics where there are a higher number of males diagnosed with non-Hodgkin's lymphomas. [75] This may well be due to the smaller sample size but could also be due to the difference in the epidemiology in the studies. However, there was a study done by Pinnark et al.,[113] that did have a cohort similar to our study with the female patients being 59.1%.

Regarding the age range, the majority of female cases ages were concentrated above the age of 60. In contrast, the male DLBCL were had more than 50% of their cases under the age of 40 which is similar to other published data. A study by Hedström et al., [106] found that the male cohort was more at risk of developing DLBCL when under 50 as opposed to the female cohort who only were at higher risk after the age of 60. Even in a much younger cohort as seen in the study by Burkhardt et al., the older the female patients were still at higher risk than the younger patinets.[107] In the study by Beheshti et al., the older female group were found to have less DLBCL progression when compared to younger female group and to males. They found that the *JUN* signalling was linked to the age, and *CYCS* signalling pathway was linked to the differences in gender.[109,110]

In our study there was no significance association found between gender, overall survival and progression free survival.

In our study, gender and age was compared to the site of the DLBCL, there was no difference in cervical lymph nodes (33%) based on gender. When the extranodal involvement was investigated it was established that 26.2% of cases had extranodal involvement of which16.7% was male. In our study there was no significance association with extranodal involvement. In a study by Yao et al.,[111] however, it was shown that the extranodal involvement was associated with a worse prognosis of which 58% of the extranodal cases were males.

In the study by Robertus et al.,[31] they were able to establish that the expression of miR-127 was linked with the site of presentation, as the testicular DLBCL had much higher expression than the CNS DLBCL presentation.

The study by Cote et al.,[117] demonstrated that HIV increased the risk of developing DLBCL by more than 150-fold, making HIV status an important factor to investigate when looking at prognosis. Regarding the HIV status, our study had a higher number of HIV negative cases

(71.4%) to that of Pinnark et al.,[113] who had 93.7% HIV negative cases. In a comparison between the HIV-positive and HIV-negative DLBCL cohorts there were higher number of females in the HIV-positive cohort in comparison with males. However, in the HIV-negative cohort there were also more females. So, there was no correlation made between HIV status and sex nor was a relationship establish on how they relate to the miR-127 expression. The fact that the groups (sex and HIV status) were not equally represented made it difficult to determine if there was any correlation between them. In addition, there is a higher number of females in the HIV positive cohort, this is in line with literature which showed the higher proportion of females infected with HIV in South Africa.[73,74]

In addition, we were also unable to observe any relationship between IPI, stage, age and performance status between the HIV negative and positive DLBCL groups. This is in contrast to the study by Baptista et al.,[102] which indicated that HIV-positive cases were more inclined to have higher performance status, later staging and a worst overall survival rate. Coutinho et al.,[115] showed that HIV positive patients had significantly longer disease-free state and overall survival than the HIV negative cohort.

Overall the expected outcome was a lower expression of miR-127 in all the DLBCL cases with a marked decreased in the HIV positive cases. Although no other literature showed the relationship between HIV-positive cases and low miR-127 expression in DLBCL, it was expected that HIV positive cases may have a lower expression or may even be silenced. In addition, it was expected that factors on the IPI may also be associated with lower expression. This was however not the case as only 40% of HIV positive cases were silenced. Overall only 26.2% of cases were silenced and the other 73.8% had higher expression levels than the normal sample ranges.

Another factor investigated for prognostic value was the subtyping found in DLBCL, germinal centre B-cell (GCB) and activated B-cell (ABC). In our study 12 cases could not be subtyped but of the rest, 46.7% were GCB and 53.3% were ABC DLBCL. Although the studies by Nowakowski and Czuczman [119] and by Frick et al., [120] were able to establish a relationship between subtype and prognosis, concluding that ABC DLBCL had a worse prognosis than GCB DLBCL. However, we were unable to prove such a relationship. Chen et al.,[112] were also able to support the finding by Frick et al.,[120] and were able to further demonstrate that there was a relationship between extranodal involvement and subtype; as well as stage and LDH. In their study, they had majority GCB DLBCL and majority males. 84.6% of the GCB DLBCL cases had extranodal involvement and 84.2% of ABC DLBCL cases had extranodal involvement. With more than half (61.5%) of GCB cases being stage 3 and 4 DLBCL, and 81.3% of ABC cases were stage 3 and 4.

The prognostic value BCL6, BCL2 and Ki67. When the stain profile was correlated with miR-127 expression in our cohort of 42 DLBCL cases all but 10 of our cases had a higher expression levels of miR-127. These 10 cases were silenced but had no significant clinicopathological feature. In the 10 cases all but 3 cases had positive BCL2 immunostaining and were above 70% for Ki67. This was also seen to be the case in the study by Iqbal et al., where BCL2 had no significance in prognosis of DLBCL and although other studies noticed a relationship between deceased overall survival and BCL2 there was no true significance.[94, 108] However the study by Pather et al., [114] did determine that in the case of GCB DLBCL BCL2 positivity was indicative of an improved survival.

Xie et al.,[116] determined that KI67 should be regarded as an important indicator of prognosis stating that its presence was associated with a poor prognosis as they showed there was a relationship between LDH, stage and age.

In our study we determined that BCL6 had not significant prognostic value, as only 25% had been silenced which was similar to the study by Kawamoto et al.,[118] who also showed that BCL6 had no significant relationship with overall survival. However, the study by Lossos et al[95] demonstrated a relationship between BCL6 and overall survival, deeming the presence of BCL6 as an indicator of a good prognosis.

Our study included reactive nodes to compare the expression level of miRNA-127, all these cases had an elevated miRNA-127 expression level. Although less than half of the group were HIV-positive, all miRNA-127 expression levels were high with a higher average expression than the HIV negative DLBCL cases. In some of these cases the expression levels were two-fold higher than that of the normal patient sample. The chip by Qiagen had also shown an upregulated miRNA-127 expression in our DLBCL cohort when the samples were pooled, which then supported our results using a different method.

The correlation between clinicopathological features and the miRNA-127 expression levels showed that there was no significant association with age, staging, sex and serum LDH which was in keeping with other published literature.[29,96] We were also unable to establish any statistical significance relationship between miRNA-127 expression and HIV status.

CONCLUSION

To our knowledge this was the first study that investigated the correlation between miRNA-127 and HIV status in a cohort of DLBCL using FFPE tissue in the South African cohort. There were seven factors that were investigated for prognostic value, age, gender, HIV status, Ki67, BCL6, BCL2 and miRNA-127 expression levels.

For age 45% of cases were above the age of 60, where it was expected to be more than 50%. Regarding gender there was a female to male ratio (female:male) was 4:3. There was no marked difference so we were unable to establish a clear prognostic value. The majority of the cases were HIV negative with the ratio to positive cases at 3:1(negative: positive). This was expected to be the other way around, as there has been in increased number of DLBCL cases as HIV cases has increased. [54] The immunostaining results had a bigger difference margin than the above mentioned factors, with Ki67 86% of cases had a high Ki67 percentage. The BCL2 had 74% positive cases and BCL6 had 70% positive cases. Regarding the miRNA-127 expression there were only 26% of cases that were silenced where 74% had a higher expression than the normal range. When comparing the cases with silenced miRNA-127 expression and immunostaining results, Ki67 and BCL6 stained positive for all the cases expect 3 cases for BCL6 and 1 case in Ki67.

In conclusion we found no correlation between miRNA-127 expression and the possible prognostic biomarkers but Ki67 used in conjuction with BCL6 could be good biomarkers to use as almost all cases that were silenced were positive for BCL6 and Ki67.

Regarding future approaches the biggest hurdle encountered in our study was the distribution of not only sex but also HIV status. It would therefore be good to open up the time period from which samples can be taken to ensure that an equal number of men and women that are HIV positive and HIV negative with DLBCL are obtained. It would also be beneficial to look at the studies that have all the information (biomarkers) recorded to help determine if there is a relationship.

It would also be interesting to investigate reactive nodes, as all the reactive nodes had a higher expression level of miRNA-127 than the normal range

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APPENDIX

APPENDIX

Appendix A: Staging of Cancer

There are 4 main stages of DLBCL:

- **Stage I**: 1 group of lymph nodes affected
- **stage II**: 2 or more groups of lymph nodes affected, above or below the diaphragm
- **stage III**: lymph nodes affected on both sides of the diaphragm
- **stage IV**: lymphoma is found in organs outside the lymphatic system or in the bone marrow.

A letter may accompany the number.

- **A:** no B symptoms
- **B**: B symptoms present (fever, night sweats, weight loss)
- **E**: extranodal lymphoma (outside of the lymph nodes).

Stage 1A and stage 2A referred to as ‘early-stage’ DLBCL. ‘Advanced-stage’ DLBCL is stage 3 and stage 4.

Appendix B: The clinicopathologic data of 42 cases of DLBCL

Case #	Age (Y)	Gender	Grade	Lymph nodes	Stage	HIV status	GC/non-GG
1	65	Female	1	Yes	IV	Left	Distal
2	35	Female	3	No	II	Left	Distal
3	57	Female	1	Yes	III	Left	Distal
4	32	Female	1	Yes	III	Left	Distal
5	60	Female	3	Yes	III	Right	Proximal
6	56	Male	2	No	I	Right	Proximal
7	42	Male	1	No	I	Right	Proximal
8	62	Female	1	No	III	Left	Distal
9	51	Female	2	No	I	Left	Distal
10	83	Male	1	No	III	Left	Distal
11	65	Male	1	Yes	IV	Left	Distal
12	59	Male	2	Yes	III	Right	Proximal
13	65	Male	3	Yes	III	Right	Proximal
14	70	Female	2	Yes	I	Left	Distal

15	68	Male	2	No	II	Right	Proximal
16	51	Male	2	Yes	III	Left	Distal
17	52	Female	2	Yes	III	Left	Distal
18	50	Male	2	No	I	Left	Distal
19	72	Female	3	Yes	III	Right	Proximal
20	58	Male	2	No	I	Left	Distal
21	75	Female	1	No	II	Left	Distal
22	74	Male	2	No	III	Right	Proximal
23	50	Male	2	No	III	Left	Distal
24	59	Female	2	No	I	Right	Proximal
25	65	Female	2	No	I	Left	Distal
26	61	Female	2	No	I	Right	Proximal
27	51	Female	2	No	IV	Left	Distal
28	63	Female	2	No	III	Right	Proximal
29	60	Female	2	No	I	Left	Distal
30	70	Male	2	No	III	Right	Proximal
31	64	Male	2	No	III	Left	Distal
32	65	Male	2	No	III	Left	Distal

33	80	Male	2	No	I	Left	Distal
34	77	Male	2	No	I	Right	Proximal
35	55	Male	2	Yes	III	Left	Distal
36	60	Female	2	Yes	IV	Right	Proximal
37	73	Male	2	No	II	Right	Proximal
38	84	Female	2	No	III	Left	Distal
39	40	Male	2	No	I	Right	Proximal
40	30	Female	2	No	I	Right	Proximal
41	76	Female	2	No	III	Right	Proximal

Appendix C: DLBCL classification types using immunohistochemistry stains

There are a number of algorithms that can be used to determine the subtype of DLBCL. For the purposes of my study the Hans' Algorithm is sufficient and was used to subtype DLBCL cases.[95]