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THE ASSOCIATION OF EPSTEIN-BARR VIRUS AND ACUTE LEUKAEMIAS IN NAMIBIA

By

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I, Marien Naudé, hereby declare that the work contained in the thesis entitled: "The association of Epstein-Barr virus and Acute Leukaemias in Namibia", is my own original work and that I have not previously in its entirety or in part submitted it at any university or other higher education institution for the award of a degree.

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"If I do my best, the Lord shall do the rest"

Thielle Schiele

DEDICATIONS

To my cousin's daughter, Jessica Kotze at the age of only five years old lost her battle with Acute Leukaemia.

To my grandfather, parents, husband and children, who have motivated and supported me throughout my academic journey.

Finally to all the suffering diagnosed Acute Leukaemia patients.

ABSTRACT

There are two viruses found to be direct aetiological agents of leukaemia, namely Epstein-Barr virus (EBV) and Human T-cell leukaemia virus. Viral infections were estimated to cause 15 – 20 % of all human cancers, by its capability to release virus-encoded proteins into its host. These proteins reprogram host cell signalling pathways within deoxyribonucleic acid (DNA), which are responsible for differentiation and proliferation. Furthermore, 90% of the world's population is infected with EBV as a life-long and dormant infection of B lymphocytes. EBV is a considerably known an etiological factor in various tumours, but very little is known of the relationship between EBV and Acute Leukaemia (AL), taking into consideration any type of AL, gender or age. AL is a serious disease and the actual cause of cancer remains unknown. This study, therefore, aimed to establish the presence of Epstein-Barr virus in patients with Acute Leukaemias.

The patients who were previously diagnosed with AL and who visited the Oncology ward (Windhoek Central Hospital) within the specified time frame (01 March 2017 – 31 July 2018), were screened for the presence of EBV IgG and IgM antibodies, by the use of the Enzyme-linked Immunosorbent Assay (ELISA) method. The same Ethylene Diamine Tetra Acetic Acid (EDTA) samples, which were sent to Namibian Institute of Pathology (NIP) for routine AL check-up screening, were used for this study. Controls were randomly selected among non-AL patients of different ages. The AL patients which relapsed within the period of interest were screened for the presence of EBV DNA. The same amount of patients were picked as a control and also screened for EBV DNA by Real-Time Polymerase Chain Reaction (PCR). All variables were compared between cases and controls to determine any significant association of EBV with AL. Statistical differences between the AL patients and controls were determined by the use of Chi-square testing.

The AL patient group presented with the following results: 66 out of 95 (69%) were Acute Lymphoblastic Leukaemia (ALL) patients and 29 out of the 95 (31%) were Acute Myeloid Leukaemia (AML) patients. Additionally, 8 out of 95 (8%) relapsed within the period of interest. EBV IgG was the most common marker among the AL patients of which 95 out of the 95 (100%) patients were positive for EBV IgG following, EBV IgM of which 0 out of the 95 were positive for EBV IgM (0%). In the control group, 54 out of the 95 patients were positive for EBV IgG (57%) and 41 out of the 95 were negative for EBV IgG (43%). Furthermore, 17 out of the 95 were positive for EBV IgM (18%) and 78 patients out of the 95 were negative for EBV IgM (82%). Positive results were considered for titer concentrations of more than and equal to 1.2 for EBV IgG, and, more than and equal to 1.24 for EBV IgM. EBV DNA was based on qualitative measures. Chi-square results: EBV IgG p=0.0002*** for AL. EBV IgG

p=0.0002*** for ALL. EBV IgG p=0.0002*** for AML. EBV DNA p=0.03* for the relapsed AL cases. A P-value less than or equal to 0.05 was considered statistically significant.

Overall it can be concluded that EBV is common amongst AL patients of Namibia, as a lifelong latent infection, which could have contributed to the development of AL regardless of the type of AL (ALL or AML), neither the age affected nor the gender. Furthermore, it's concluded that there is also an association between EBV and relapsing AL. These conclusions broaden the knowledge that EBV can initially infect anyone and the consequences in the long term may be fatal. Prevention interventions considering EBV should already be taken at newborn infancy period. For those previously infected with EBV should maintain a strong immunity to prevent the EBV from going viral.

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

GLOSSARY

AIDS:	Acquired Immunodeficiency Syndrome
AICR:	American Institute of Cancer Research
AML:	Acute Myeloid Leukaemia
AL:	Acute Leukaemia
ALL:	Acute Lymphoblastic Leukaemia
CD 21:	Cluster of Differentiation 21
DNA:	Deoxyribonucleic acid
EBV:	Epstein-Barr virus
EDTA:	Ethylene Diamine Tetra Acetic Acid
ELISA:	Enzyme Linked Immunosorbent Assay
FAB:	French American British
HIV:	Human Immunodeficiency Virus
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
ISO:	International Organisation for Standardization
ISR:	Immune Sensitivity Ratio
NIP:	Namibia Institute of Pathology
NUST:	Namibia University of Science and Technology
OD:	Optical Density
ONC:	Oncology ward
PCR:	Polymerase Chain Reaction
POW:	Paediatric Oncology ward
RNA:	Ribonucleic
SANAS:	South African National Accreditation System
WHO:	World Health Organization
WPP:	World Press Photo
UN:	United Nations

DEFINITION OF CONCEPTS

1. **Epstein Barr Virus (EBV):** A virus which was found to have oncogenic effects on its host (Elawad *et al*., 2014).

2. **Acute Leukaemia (AL):** According to the World Health Organization (WHO), classification of Acute Leukaemia is a blood cancer of which the bone marrow or peripheral smear consists of 20% blasts or more (WHO, 2016).

3. **Acute Myeloid Leukaemia (AML):** Acute Myeloid Leukaemia is a form of blood cancer which is characterized by the infiltration of the bone marrow, blood and other tissues by proliferated, abnormally differentiated and clonal cells of the hematopoietic system (Döhner *et al.,* 2015).

4. **Acute Lymphoblastic Leukaemia (ALL):** Acute Lymphoblastic Leukaemia is a malignant disease that arises from several genetic mutations in a single B- or T-lymphoid progenitor, which leads to altered blast cell proliferation (Pui, 2011).

5. **Relapse Acute Leukaemia:** When the reappearance of cancerous blasts occurs after remission was reached (American Cancer Society, 2017).

6. **Association:** A link between two variables (Guan *et al*., 2017).

7. **Namibia:** South-Western Country of Africa consisting of a population of +/- 2.5 million (WPP & UN, 2017).

8. **Adult classification for AL patients:** AL patients with the age of 16 years or more (WHO, 2009).

9. **Children classification for AL patients:** AL patients with the age of 15 years or less (WHO, 2009).

10. **ISO15189:2012:** A global initiative that aims at creating a standard measure of quality in medical laboratories, version 2012 (Plebani *et al.,* 2015).

CHAPTER 1: INTRODUCTION

1.1 Introduction and Background

Acute Leukaemia (AL) is a haematological malignancy originating from the bone marrow and it's categorized by the overproduction or proliferation of cancerous stem cells (Angle, 2013). The cancerous stem cells will then overproduce cancerous committed stem cells or blasts, which are either of leucocyte, erythrocyte or platelet origin (Tewari *et al*., 2017). The cancerous blasts will accumulate in the bone marrow and overspill into the blood. Furthermore, the cancerous blasts will spread to the extravascular organs like the kidneys, lungs, liver or spleen (Hoffbrand & Moss, 2011). There are two types of AL, namely Acute Lymphoblastic Leukaemia (ALL) or Acute Myeloid Leukaemia (AML) (Bennett *et al*., 1976). AL is a serious disease with a worldwide incidence rate of 46.7 per million per year for children under fifteen years of age, for the year 2009 (WHO, 2009). South Africa has a high AL incidence rate of 45 per million per year, affecting many families (Stefan *et al.,* 2015). According to a study done in Namibia, ALs are the most common malignancy in Namibian children, with an incidence rate of 22.5 per million per year (Stefan *et al*., 2014).

AL has a high mortality rate of which an estimated 50% of the affected patients will be deceased and therefore it claims millions of lives worldwide (Orioal, 2010). The life expectancy of AML if detected early is about 20 – 40% of patients surviving for 60 months minimal (Kottaridis *et al*., 2001). ALL shows similar statistics for adults approximately 40%; however, in children, about 80% of affected could be completely cured with early diagnosis, with the correct treatment regime and depending on the treatment response (Legrand *et al*., 1998). Cost of treatment for AL is extremely high; and in the year 2016, United States total mean rate per patient for AL treatment was +/- 38,245 – 163,350 US Dollars (Hayati H, 2016). The causes of ALs vary and efforts are being made to reduce their occurrence, by experimenting on various predisposing risk factors and gaining knowledge of these particular risk factors. This knowledge could be eventually used to develop measures to reduce potentially harmful exposures and decrease the risk of AL (Belson *et al*., 2010).

There are two viruses found to be a direct aetiological agent or risk factor of AL, namely Epstein-Barr virus (EBV) and Human T-cell leukaemia virus (Jarrett, 2006). Viral infections were estimated to cause 15 – 20 % of all human cancers, by its capability to release virus-encoded proteins into its host (Margaret & Munger, 2008). The proteins reprogram host cell signalling pathways within the

deoxyribonucleic acid (DNA) of the host, which are responsible for differentiation, proliferation, cell death, genomic integrity and the recognition process by the immune system (Margaret & Munger, 2008).

A previous study has shown that approximately 90% of the world's population is infected with EBV as a life-long, latent or dormant infection of B lymphocytes (Stannard, 2013). Malignancies associated with EBV include B cell lymphomas, Hodgkin's disease and nasopharyngeal carcinoma (Cohen, 2011), and an estimated 200 000 cases of cancers are caused by EBV across the world (Smith, 2014). It was found that the Epstein-Barr virus (EBV) is a common herpes virus linked to infectious mononucleosis, multiple cancers and was the first virus to be linked to cancer (Cohen, 2011).

In developing countries, EBV occurs at a much earlier age of life and approximately 90% of the children above two years are seropositive with EBV (Stannard, 2013). Infected people with EBV produce EBV in their saliva and therefore transmission occurs through close contact between the infected and uninfected individuals. Primary infection with seroconversion is usually asymptomatic (Epstein *et al*., 1998). EBV has the tendency to maintain latency infection with the virus genome retained in the host cells, without the active production of infectious virions. EBV attacks the B lymphocytes by the use of a receptor cluster differentiation 21 (CD21) and achieves a latent infection *in vivo* or *in vitro* (Hopwood *et al.,* 2002). The actual causes of AL were hypothesized that the role of physical, chemical and biological factors could be responsible for the development of AL, provided that EBV involvement may be a contributing factor towards the formation (Elawad *et al*., 2014).

EBV was concluded not to be the cause of Acute Lymphoblastic Leukaemia (ALL) among Mexican children (Sanchez, 2014), but in Sweden specifically, EBV was suggested to be the cause towards ALL (Altier *et al*., 2006). In another study, it was noted that EBV was the etiological factor of AL among Sudanese ALL children (Elawad *et al*., 2014). Currently, there is no data available concerning an association between EBV and AL patients of Namibia. Clearly the situation differs from country to country; therefore it's important to determine the situation specifically for Namibia, concerning EBV and AL because the incidence rate for AL continues to rise within Namibia.

The actual cause of AL remains under investigation (Abdul-Hay & Terwilliger, 2017). EBV has been associated with various tumours, but very little is known about the relationship between EBV and AL, taking into consideration any type of AL (Guan *et al.,* 2017). This study was the first study within Namibia to be conducted showing the link between EBV and AL. The aim of this study was to determine

the association between EBV and AL and to show possible involvement of EBV towards relapsing AL in Namibia. The information gained from this study perhaps can be used for future purposes as a preventative tool to save many lives.

1.2 Literature review

1.2.1 Epstein Barr Virus

According to Glaser, *et al.* (1997) EBV has been suspected to be the etiological factor of Hodgkin's disease. Furthermore, EBV is a type of virus that usually infects people in early infancy or childhood, which presents asymptomatically (Gershburg, 2005). If EBV infection subsides to infection in adolescent or young adults, then EBV often results in Infectious Mononucleosis with lymphadenopathy, fever, splenomegaly and a sore throat (Cohen *et al.,* 2009).

A study conducted in Northern Africa highlighted the possibility of an association of EBV by assessing the presence of EBV in Egyptian ALL children, with the use of specific immunoglobulins assay for capsid protein antigens and molecular methods (Zaki *et al.,* 2014). From their study, Zaki *et al*. (2014) concluded that EBV IgG was the most common marker among children with ALL (29%), followed by EBV DNA (25.8%) and EBV IgM (12.9%) respectively. In the control group, only 15 children were positive for EBV IgG (15%) and 5 children were positive for EBV IgM (5%) and none were positive for EBV DNA (Zaki *et al.,* 2014). This data shows that there was a statistically significant relationship in presence of EBV IgG (P=0.0001) and EBV DNA (P=0.04) between patients and controls, which led to the authors' conclusion that EBV is common in children with ALL (Zaki *et al.,* 2014).

According to Seghal *et al*. (2010), there was a significant increase in EBV in ALL patients ($p < 0.05$) by the use of chi-square analysis. PCR for BamH1-W EBV nuclear antigen (EBNA) promoter was used to screen 25 ALL children. Furthermore, they found 8 out of the 25 ALL (32%) patients were positive for EBV DNA and therefore came to the conclusion that there is an association between EBV and ALL in Indian children (Sehgal, 2010). This is similar findings to Sakajiri (2002), which reported increased EBV infection in a patient with T ALL but in this case, used Southern blotting and in situ hybridization (ISH) and was for Sweden AL patients in general.

1.2.1.1 EBV morphology and biology

Some studies emphasized that the EBV particles appear smaller than the morphology of the normal sized Herpes virus particles (Arikawa, 1997). Although there is a significant size difference between EBV and the other members of the Herpes virus, morphologically EBV appears as all other Herpes viruses (Epstein & Achong, 2012). An immature particle of EBV will measure about 80 *u*m in diameter and contain a central dense ring-shaped nucleoid or core (Epstein *et al*., 1964) (Figure 1). EBV particles appear as hexagonal forms with triangular surfaces covered in hallow, tubular capsomeres (Yamaguchi, 1967) (Figure 1). A mature EBV particle measures 120 *u*m in diameter (Epstein *et al*., 1964).

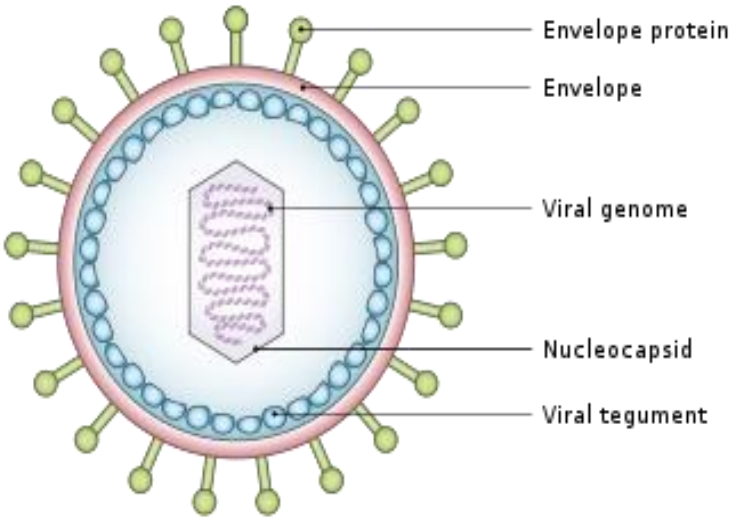

Figure 1: The general structure of the EBV particle

The immature particle of EBV consisting of a viral genome contained within the nucleocapsid, surrounded by viral tegument. The envelope of the particle protects the content inside. The envelope protein is contained on the outer surface of the particle and covers the entire surface (Epstein & Achong, 2012).

1.2.1.2 Lifecycle and transmission

EBV has two phases within its lifecycle, an active replication phase, also known as the release of the virus (Germi *et al*., 2016), and the latent viral infectious phase, which cannot reveal its latency from the host's immune system (Baer, 1984). The active replication phase involves the production of new virions and causes the shedding of viral particles into its host (Germi *et al*., 2016). While throughout the latency period, the viral genome will be present and the virulent forms are able to persist over a long period of time or even indefinitely in the host, without necessarily causing damage (Eligio *et al*., 2010).

The double-stranded EBV DNA genome is of 186-kb in size and the genome codes for a number of structural and non-structural genes (Epstein & Achong, 2012). The same port of entry is the same port of exit such as the oropharynx. The EBV replicates in the epithelial cells and in the B cells of the oropharynx, after entry (Jeffrey & Cohen, 2000). The EBV spreads through the body via infected B cells while the latent genes perform other activities (Germi *et al*., 2016). The activities are either to drive the B cells to the EBV lytic cycle or acquire the status of latency (Eligio *et al.,* 2010). EBV follows a productive lytic infection like other herpes viruses and establishes latent infection in its host (Rickinson & Kieff, 2001). The latent infection is reached by self-replicating extra-chromosomal nucleic acid, known as episomes (Eligio *et al.,* 2010). There are three different latencies i.e.: latencies I, II, and III, due to the differential expressions of certain subsets of EBV-specific genes (Germi *et al*., 2016). Under circumstances such as B-cell homing by an antigen-driven trigger and other so far unknown triggers, EBV sporadically reactivates from the latency period (Elawad *et al.,* 2014). The virus is intermittently shed from saliva, therefore, the main route of transmission is then directly from person to person; however, transmission via transplantation, blood products, and sexual transmission can take place (Epstein *et al*., 1998). EBV transforms B cells *in vivo* and *in vitro*, thus immortalizing B cells (Rickinson & Kieff, 2001).

The knowledge of the EBV life cycle is of the essence to better understand the EBV diagnosis. EBV antibodies are frequently used to describe the infection status of the disease. Using IgM and IgG antibodies, present and past infection can be determined respectively (Germi *et al*., 2016). The presence of EBV IgM and IgG together indicates acute or present infection, whereas the presence of EBV IgG without IgM indicates typical past infection (De Paschale, 2012).

When viral infections strike, the body responds by commanding B cells within the immune system to push out antibodies to battle the invading pathogen. However, when EBV infections occur, an unusual immune response happens (Tangye *et al*., 2017). The EBV virus invades the B cells themselves, reprograms them within the DNA, and takes over control of their functions. The immune system is therefore faulty and unable to stop the EBV infection (Tangye *et al*., 2017). High titer antibodies against EBV structural and early lytic antigens is the indication of either sporadic reactivation from latency in malignant cells, however, antibodies may not be able to block EBV infection because EBV also has the capability of spreading through the cell to cell contact (Young *et al.,* 2015).

Viruses such as EBV my promote leukemogenesis indirectly by initiating an abnormal immune response, resulting in autonomous B-cell precursor proliferation. EBV DNA persists even during remission and can be detectable by PCR (Bartenhagen, et al., 2017). EBV is known as a virus which is integrated into the genome (DNA) of the precursor B cells, therefore may promote leukemogenesis by acting on proliferation and differentiation. EBV DNA, in this case, shall be persistent and can be

detectable in leukemic cells (Bartenhagen, et al., 2017). In this particular study of Bartenhagen *et al.,* (2017), 11 of the 14 AL cases contained EBV DNA. However, the same response was seen towards other viruses like (*Anelloviridae, Herpesviridae*, and *Parvoviridae*). Additionally, to this study, they found that an integrated virus such as EBV is truly essential for leukemic cell characteristics which can be expected to be persistent at relapse. According to the findings of Bartenhagen *et al*., (2017) persistence of viruses was analysed in 6 patients for whom both diagnosis and relapse bone marrow was available and found in 5 patients with viral DNA.

According to Wood *et al*., (2017) EBV uses enhancers to activate *MYC*, a gene that is a key driver of lymphoma development and switch off *BCL2L11*, a gene that normally triggers cell death and prevents lymphoma. EBV encodes transcription factors (EBNAs) that activate *M*YC and silence BCL2L11 (Wood, *et al*., 2016). Furthermore, EBNA2 transactivator activates multiple MYC enhancers and reconfigures the MYC locus to increase upstream and decrease downstream enhancer-promoter interactions (Wood, *et al.*, 2016). In Burkitt's lymphoma, the EBV virus has been detected in the genome of the malignant cells (integrated virus) leading to the formation of an oncogene (cMyc). This oncogene (cMyc) disrupts the proliferation control of the cell, causing the over-proliferation or production of cancerous precursor stem cells of no functional use (malignant clone) (Wood, *et al*., 2016).

1.2.1.3 Pathology

EBV was discovered in 1964, by the examinations of cultured Burkitt's lymphoma tissue under the electron microscope (Epstein *et al.,* 1964). EBV was the first herpes virus found to be oncogenic in humans (Smets & Sokal, 2014). Those who do contract EBV at a younger age are suspected to be at a higher risk of developing B-cell lymphoid tumours. B-cell lymphoid tumours have an accelerated proliferation rate, increasing the chances of genetic errors to occur during DNA replication (Elawad *et al.,* 2014). Specific chromosomal transformations occur, like chromosomal translocation rearrangement of elements in immunoglobulins, contained in affected lymphoid cells tend to give rise to leukemic clones (Henrik *et al.,* 2007). EBV has been known to show a variety of signs and symptoms such as rashes, fatigue, sore throat, swollen glands in neck, fever, weakness, sore muscles, lack of appetite, swollen liver, headache, enlarged spleen and in more serious cases, it may lead to upper airway obstruction and spleen rupture (Baer, 1984).

1.2.1.4 Diagnosis of EBV

Several techniques have been used in the diagnosis of EBV. ELISA is one of the techniques used. This is a plate-based method which is used for detecting and quantifying substances such as antibodies and hormones (Weber-Nordt *et al.,* 2017). An enzyme conjugated with an antibody reacts with a colourless substrate to produce a colour product known as a chromogenic substrate (Gan & Patel, 2013). The principle of the ELISA method is based on the intensity of the colour produced as an indication of the amount of the specific antigen or antibody present (Edwina, 2011).

The confirmatory test of EBV diagnosis is known as Real-Time Polymerase Chain Reaction (PCR), of which DNA is isolated from plasma samples. PCR is a molecular technique which monitors the amplification of extracted and targeted DNA molecules (Van Aelst *et al.,* 2012).

1.2.1.5 Treatment for EBV

According to Cohen, (2011), EBV vaccine was undergoing clinical trials. Overall EBV possesses a variety of proteins when it is in the active and latent phases, therefore vaccine candidates are designated to involve proteins from both phases to develop a successful vaccine (Drouet, 2016). There is hope with the long term usage of Valacyclovir drug, which might allow the eradication of EBV from the body, but only if re-infection doesn't occur (Hoshino, 2009). Both inhibitors of EBV replication in cell culture and clinical trials of acyclovir for the treatment of patients with Infectious Mononucleosis have failed to be of benefit for the study patients (Gershburg, 2005). Another study concluded that the age-specific prevalence of EBV infection is required to determine when a prophylactic vaccine is needed to be administrated (Balfour, 2013).

1.2.2 Acute Leukaemia

1.2.2.1 Diagnosis and Incidence of Acute Leukaemia

AL is defined as the presence of over 20% blasts in the peripheral blood or the bone marrow at clinical presentation (Hunger & Mullighan, 2015). Confirmatory testing will follow after a bone marrow aspiration and trephine biopsy morphology analysis. These methods include Flow cytometry and Cytogenetic studies of which blasts of myeloid or lymphoid lineage can be determined, and can localise the stage of cellular differentiation, as well as, determine the stage of the disease (Hoffbrand, 2011).

According to Robak *et al.* (2016), Leukaemia is a malignancy of the blood cells or known as blood cancer because leucocytes undergo genetic changes to become leukaemic cells. The leukaemic cells proliferate and survive better than normal healthy leucocytes (Abdul-Hay & Terwilliger, 2017). Over a period of time, leukaemic cells suppress the development of other normal cells; therefore patients with leukaemia are more prone to contract infection (Robak *et al.*, 2016). Symptoms of AL patients include fatigue, weakness, dizziness, easy bruising, nose bleeds, bleeding gums, persistent infections, pale skin and etc. (Seiter, 2017) (American Cancer Association, 2017).

ALL is the most common malignancy among children worldwide (Sanchez, 2014). AML is the most common AL in adults. There are particular age groups which are affected by various leukaemias of which most childhood leukaemias presents as AL (Abdul-Hay & Terwilliger, 2017). ALL mostly affects children of the ages 2 -10 years. However, AML accounts for only 15 -20% of ALs within children (Angle, 2013). AML is more common in older individuals, as the incidence of AML increases with age and is approximately 1.3% in persons younger than 65 years, but approximately 12.2% in persons older than 65 years (Döhner, 2015) (Leukaemias, 2014).

Approximately 1 300 Namibians are newly diagnosed with cancer annually, excluding non-melanoma skin cancers (Namibia Cancer Resources, 1996). The incidence rate of AL among Namibian children in 1995 was at 55.5 per million per year (Wessels & Hesseling, 1996). However, another study showed that AL is the most common malignancy in Namibian children, with an incidence rate of 22.5 per million per year (Stefan *et al*., 2014).

Of all cancers, childhood cancers only compromise of 1%, however, more than 200 000 new cases of childhood cancers are reported globally per annum and 70% comes from developing countries including Namibia (Ribeiro & Pui, 2005). Based on the findings of Stefan *et al*. (2014) up to 80% of children with malignancies can be cured among those of developed countries. Unfortunately, for developing countries, 70-80% of children with malignancies become long term survivors and 95% only have 5 years survival rates (Belson, *et al*., 2010). Stefan *et al*. (2014) focused on malignancies recorded from January 2003 up to December 2010. AL and retinoblastomas occurred mostly among the 191 patients admitted to the Windhoek Central Hospital (Paediatric Oncology 8 West) selected for the study. AL had a frequency rate of 22.5% of which most of the patients suffering from AL suffered from ALL with a frequency rate of 88.4% (Stefan *et al*., 2014).

1.2.2.2 Acute Lymphoblastic Leukaemia

Acute Lymphoblastic Leukaemia may also be referred to as Acute Lymphocytic Leukaemia or Acute Lymphoid Leukaemia (ALL). This type of leukaemia originates from the immature lymphoid precursor cells in the bone marrow. This leukaemia rapidly progresses and is fatal after a few months if not treated (Abdul-Hay & Terwilliger, 2017). The overproduction of leukaemic cells replaces the normal haematopoietic cells of the bone marrow and multiplies continuously, causing damage and death by inhibiting the production of normal erythrocytes, leucocytes and platelets (Seiter, 2017).

According to Hoffbrand & Moss (2011), ALL is sub-classified according to the underlying genetic effect (e.g. B lymphoblastic leukaemia/lymphoma with t(9;22)(q34;q11.2); (BCR-ABL1) by the World Health Organisation (2016). B cell ALL consists of several specific genetic subtypes such as those with the t(9;22) or t(12;21) translocations, rearrangements of the MLL gene or alteration in chromosome number, such as diploid (Arber, *et al*., 2016). Identifying the subtype acts as an essential guide to the optimal treatment protocol and prognosis (Abdul-Hay & Terwilliger, 2017). An abnormal karyotype is found in 50 – 70% of T cell ALL cases and the Notch signalling pathway is activated in most cases (Hoffbrand & Moss, 2011). When classifying ALL, the type of lymphocyte (whether B cell or T cell) and the maturity of the cell are taken into consideration (Belson *et al*., 2010).

ALL WHO Classification:

B-Cell ALL

- Early pre-B ALL, which constitutes approximately 10% of cases.
- Common ALL, which constitutes approximately 50% of cases.
- Pre-B ALL, which constitutes approximately 10% of cases.
- Mature B-Cell ALL, or Burkitt Leukaemia, which constitutes approximately 4% of cases (Wang, 2014) (Table 1).

T-Cell ALL

- Pre-T ALL, which constitutes approximately 5% to 10% of cases.
- Mature T-Cell ALL, which constitutes about 15 to 20% of cases (Table 1).

Mixed Lineage Acute Leukaemias: acute leukaemias consisting of both myeloid and lymphoid cells, usually the AL which formed after transformation from either chronic leukaemias or myelodysplastic syndromes (Pre-Leukaemic phase) (Wang, 2014) (Table 1).

Table 1: WHO classification of precursor B-cell and T-cell neoplasms

Lymphoid Leukaemia	Subtype
Precursor B lymphoblastic leukaemia / lymphoblastic lymphoma	(precursor B-cell acute lymphoblastic leukaemia)
Precursor T lymphoblastic leukaemia / lymphoblastic lymphoma	(precursor T-cell acute lymphoblastic leukaemia)
Mature B-cell neoplasms	Chronic Lymphocytic leukaemia / small lymphocytic lymphoma
	B-cell prolymphocytic leukaemia
	Lymphoplasmacytic lymphoma / Waldenström macroglobulinaemia
	Splenic marginal zone lymphoma (±villous lymphocytes)
	Hairy cell leukaemia
	Plasma cell myeloma
	Mantle cell lymphoma
	Follicular lymphoma
	Diffuse large B cell lymphoma
	Burkitt's Lymphoma
Mature T-cell and NK-cell neoplasms	(amongst others):
	T-cell prolymphocytic leukaemia
	T-cell large granular lymphocytic leukaemia
	Aggressive NK cell leukaemia
	Adult T-cell leukaemia/lymphoma (HTLV-1)
	Mycosis fungoides / Sézary syndrome

Note: The above table represents the WHO classifications of precursor B-cell and T-cell neoplasms with their related sub-types, which are of essence for a correct diagnosis for treatment purposes (Wang, 2014).

1.2.2.3 Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML), also known as Acute Myelocytic Leukaemia, Acute Myelogenous Leukaemia or Acute Myeloblastic Leukaemia, originates from the immature myeloid precursor cells in the bone marrow (Döhner *et al.,* 2015). AML usually do not form tumours, as it generally occurs in the bone marrow (Dombret & Gardin, 2016). In most cases, AML moves into the blood and can spread to other parts of the body, such as the lymph nodes, spleen, liver and central nervous system. It occurs in both adults and children (Hoffbrand & Moss, 2011). AML is staged differently from other leukaemias (Dombret & Gardin, 2016).

The subtype of AML is essential for treatment administration, as Acute Promyelocytic Leukaemia (M3) is treated using different drugs instead of the drugs used to treat other subtypes of AML (Döhner *et al*., 2015). AML is mostly sub-classified according to the French-American-British (FAB) classification, which was established by a group of French, American and British leukaemia experts. It is sub-classified from M0 to M7, based on the type of myelogenous cell and the maturity of the cells, which can be identified microscopically through routine staining (Döhner *et al*., 2015) (Table 2).

In Namibia, the FAB classification of Acute Myeloid Leukaemia is commonly used, although the WHO Classification is the recommended Classification to be used worldwide and complicated cases require the WHO Classification referencing (Döhner *et al*., 2015) (Table 2). In the WHO classifications of AML, the leukaemias with consistent cytogenetic abnormalities and those that are MDS related were taken into separate groups; the rest of the old FAB classification was put under the "AML not otherwise categorized" entry (Dombret & Gardin, 2016) (Table 2).

Table 2: WHO Classification versus FAB Classification of Acute Myeloid Leukaemia

WHO Classification (AML)	FAB Classification (AML)
Acute myeloid leukaemia with recurrent genetic abnormalities Acute myeloid leukaemia with t(8;21)(q22;q22); (AML1(CBFa)/ETO) Acute myeloid leukaemia with abnormal bone marrow eosinophils inv(16)(p13q22) or t(16;16)(p13;q22); (CBFb/MYH11) Acute Promyelocytic Leukaemia (AML with t(15;17)(q22;q12) (PML/RARa) and variants. Acute myeloid leukaemia with 11q23 (MLL) abnormalities	MO Undifferentiated AML M1 Myeloblastic, without maturation M2 Myeloblastic, with granulocytic maturation - with a translocation between chromosomes 8 and 21 M3 Promyelocytic, or Acute Promyelocytic Leukaemia (APL) - with a translocation between chromosomes 15 and 17 M4 Myelomonocytic M4eo Myelomonocytic together with bone marrow eosinophilia M5a Monoblastic leukaemia M5b Monocytic Leukaemia M6 Erythrocytic, or Erythroleukaemia M7 Megakaryoblastic - with a translocation between chromosomes 1 and 22
Acute myeloid leukaemia with multilineage dysplasia With prior MDS Without prior MDS	
Acute myeloid leukaemia and myelodysplastic syndromes, therapy related. Alkylating agent-related Epipodophyllotoxin related Other types	
Acute myeloid leukaemia not otherwise categorized Acute myeloid leukaemia minimally differentiated Acute myeloid leukaemia without maturation Acute myeloid leukaemia with maturation Acute myelomonocytic leukaemia Acute monoblastic and monocytic leukaemia Acute erythroid leukaemias Acute megakaryoblastic leukaemia Acute basophilic leukaemia Acute panmyelosis with myelofibrosis Myeloid sarcoma	

Note: The above table represents the comparison between the WHO and the FAB classification of AML, which as well are of essence for the correct diagnosis for treatment purposes (Dombret & Gardin, 2016) (Döhner *et al*., 2015).

1.2.2.4 Predisposing risk factors contributing to the development of Acute Leukaemia

There are a number of environmental risk factors that influence the development of leukaemia.

1. Genetic factors increase the risk of AL in children with Down's syndrome and other rare genetic changes and play a role in CLL (Belson *et al*., 2010).
2. Radiation, especially in survivors of atomic bomb explosions, mine workers and people who received radiation therapy for certain medical conditions (Abdul-Hay & Terwilliger, 2017).
3. Chemicals, such as benzene and cigarette smoking increase the risk of developing AML (Belson *et al*., 2010).
4. Country of origin, those coming from Japan, particular regions in Africa and the Caribbean, induces the development of leukaemia via a virus known as HTLV (human T-cell leukaemia/lymphoma virus) (Belson *et al*., 2010).

The predisposing risk factors only explain a few cases of AL, however, being exposed to these risk factors does not mean that the development of AL will definitely occur, but it might contribute towards its development (Angle, 2013).

1.2.2.5 Treatment protocols for Acute Leukaemia

Tyrosine kinase inhibitors (TKIs) are the main targeted drugs used to treat leukaemia, a drug like Imatinib mesylate (Glivec) (Joshua & Dziba, 2004). Treatment for ALL depends on the specific subtype of the Leukaemia. It is treated with the use of Chemotherapy, and a combination of drugs including steroids given in several cycles (Mason, 2017). The combinations of drugs which are used for ALL treatment are cyclophosphamide, vincristine sulphate, adriamycin and dexamethasone which stands for "CVAD" (Maserejian *et al.,* 2015).

AML is treated with a combination of chemotherapy with or without radiation therapy, as well as, stem cell transplant depending on the specific subtype of leukaemia. Unsuccessful AML treatment is most probably due to bone marrow failure caused by the acquisition of primary infection (Barrett & Battiwalla, 2012). The immune-compromised state is caused by chemotherapy, therefore, more susceptible to primary infection (Barrett & Battiwalla, 2012). The importance of a correct diagnosis of leukaemia is crucial for the treatment regime (Dombret & Gardin, 2016).

AML is treated completely different from ALL (Dombret & Gardin, 2016). Namibia, therefore, uses flow cytometry and cytogenetic analysis studies as confirmatory testing, after an AL was diagnosed on bone marrow and trephine analysis. Confirmatory testing is performed to ensure correct diagnosis and respective correct treatment regime, therefore, to follow (Hoffbrand & Moss, 2011).

Relapsing leukaemia is treated with cytoreductive chemotherapy followed by donor leucocyte infusion (Dombret & Gardin, 2016). Relapsing leukaemia after allogeneic stem cell transplantation remains a problem with an unknown cause for the action to occur (Kurnaz, 2016). Therefore, one of the goals of this study is to determine if EBV might be a possible contributing factor towards relapsing ALs.

1.2.2.6 Cost of treatment and epidemiology

According to the American Institute of Cancer Research (AICR), cancer costs the world each year more money than any other known disease. Cancer stands at US$895 billion annually (American Cancer Society, 2017). In 2017, the American Cancer Society estimated that an estimated 62 130 new cases of leukaemia would be diagnosed and approximately 24500 deaths would occur (American Cancer Society, 2017). In the year 2014, there was research done in the United Kingdom to determine how many lives were taken by AL, and an estimated 4 584 deaths occurred, concluding 8 deaths for every 100 000 leukaemia cases in males and 6 deaths in every 100 000 leukaemia cases in females (Mason, 2017). According to Health Profile Namibia (2014), 21 Namibian citizens died of a particular type of leukaemia in 2014, which accounted for 0.16% of deaths in Namibia in 2014. This particular statistics is expected to increase due to the growing population of Namibia. Larger numbers of Namibian population will be exposed to aetiological factors causing leukaemia as EBV found at a higher incident rate in developing countries such as Namibia (Pui, 2011).

1.3 Research Problem Statements

Currently, there is hardly any data available concerning the association between EBV and AL patients, particularly amongst the Namibian population. The same problem applies to the relapse of AL and its association with EBV. The incident rate of AL in Namibia might be increasing, as well as, it's related mortality rates, because this is happening in other countries and most probably the same in Namibia. EBV has been shown to be associated with AL in other countries. Large numbers of AL patients' who are in remission relapse back to AL, but the cause of this relapse is still unknown. A question remains whether or not EBV is also the possible cause of other ALs such as Acute Myeloid Leukaemia (AML). Virtually no information is available concerning any association between EBV and AML in Namibia. EBV

targets the B-lymphocytes thus there is this perception that EBV is only found in lymph proliferating diseases and not in myeloid lineage diseases.

It was concluded that there was a high number of childhood patients with ALL, which showed active replication of EBV (Sehgal, 2010). Furthermore, an oncology patient in Namibia who has AL should be taken into consideration whether that patient is diagnosed with ALL or AML, concerning EBV as the possible cause of AL. As the majority of the known EBV studies involve ALL diagnosed children, there is a gap for similar studies involving adults and gender. Therefore, in this study adults of various age groups and genders, diagnosed with AL, regardless of its type, were investigated in connection with EBV infection, in order to rule out any bias strategies and draw more conclusions about EBV cases.

EBV is an asymptomatic virus that can persist for a lifetime. If symptoms appear later in adolescent life then it's known as Infectious Mononucleosis. EBV only causes cancer in immune suppressed patients (Fujiwara, 2014).

Before any treatment may be given to AL patients, the stage of the disease must be determined in order, to evaluate treatment options. Leukaemia is staged differently from other cancers as it originates in the bone marrow cells, thus staging are based on the spread of the cancerous cells to other organs and the cancerous cell counts. Ultimately the disease type is also very important (McCay, 2016).

When the patients with AL receive chemotherapy, their immune system is highly suppressed due to treatment, which stops DNA and RNA replication. Furthermore, combined with radiation, chemotherapy also destroys the bone marrow stem cells. Bone marrow stem cells are the producing cells of the white blood cells, which is the defence system or immune system of the body. If the immune system is suppressed then EBV leaves its latent phase and becomes an active viral infection. Cancer is caused because the immune system is too weak to fight off the EBV, causing an overproduction of B-Lymphocytes. The patients receiving chemotherapy have a decreased immune system, thus are at high risk of the EBV to cause cancer. EBV is likely to be the cause of the relapse of AL patients, which will be partially investigated in this study as well.

1.4 Objectives

1.4.1 Broad Objective:

- Explore the association between Epstein-Barr virus and Acute Leukaemias in Namibia.

1.4.2 Specific Objectives:

- Establish the presence of Epstein-Barr virus in patients with Acute Leukaemias.
- Confirm the association between relapsing Acute Leukaemia and Epstein-Barr virus infection.
- Investigate the relationship between Epstein-Barr virus and Acute Myeloid Leukaemia.

1.5 Research Questions

- Is there an association between Epstein-Barr virus and Acute Leukaemias in Namibia?
- Do patients with Acute Leukaemia exhibit specific immunoglobulins for the Epstein- Barr virus?
- Does the Epstein-Barr virus contribute to the relapse of Acute Leukaemia patients within Namibia?
- Is Epstein-Barr virus also a contributing factor towards Acute Myeloid Leukaemia?

1.6 Research Hypothesizes

It is possible that there is a significant relationship between EBV and AL patients in Namibia. The majority of Namibian AL patients will likely be previously infected with EBV (IgG Immunoglobulin positive); therefore, it may be that EBV is the cause of AL among Namibian Oncology patients. An association between EBV and relapse AL patients is estimated, which EBV could be the attributing cause of the relapsing AL. If EBV Immunoglobulins are found in both ALL and AML, compared to a control group, then the type of Leukaemia probably does not play a role and it could be partially concluded that EBV causes any AL type.

CHAPTER 2: RESEARCH METHODOLOGY

2.1 Study Design

This was a prospective study by use of experimental quantitative study design and a partial qualitative design. The patients who were previously diagnosed with Acute Leukaemia and who visited the Oncology ward (Windhoek Central Hospital) within the specified collection time frame (01 March 2017 – 31 July 2018) were screened for the presence of EBV IgG and IgM antibodies. The same Ethylene Diamine Tetra Acetic Acid (EDTA) samples sent to Namibian Institute of Pathology (NIP) for routine check-up screening were used for this study.

Frequencies were then analyzed to determine an association between the variables concerning EBV immunoglobulins and AL. The screening process included the ELISA method for IgG and IgM immunoglobulins. Controls were randomly selected among non Acute Leukaemia patients of different ages. All variables were compared between cases and controls to determine any significant association of EBV with AL.

A retrospective descriptive study design was used for the deceased patients who were previously diagnosed with AL and tested for the presence of EBV immunoglobulins IgG and IgM.

2.2 Study Population

2.2.1 Inclusion criteria

The AL patient samples were collected and batched from the AL patients who visited the oncology unit from the period 01 March 2017 – 31 July 2018 (Annexure 2). Each AL patient had two EDTA samples collected on different days to ensure that the outcomes of the results were precise. Those AL patient cases that were appearing on the AL registry of adult and paediatric oncology Windhoek Central Hospital were included in the study. Patients who were in the remission phase of AL were also tested. All the AL relapse patients who relapsed within the period of sample collection were used as well within this research. This study was not aged specific and all cases encountered were taken into consideration.

For the control group, EDTA samples which were also sent in for routine Haematology were batched. The same sample size was used as per above group, except they were from non-leukemic patients. These patients didn't require being healthy, they had to be non-leukaemic, of which any age or gender was included in the study to allow the broadening of the findings and respective conclusions.

As part of the inclusion criteria, were also the relapse patients that relapsed back to AL, during the specified collection time frame. The control group used for the relapse study were of the same sample size as non-relapsed AL cases.

2.2.2 Exclusion criteria

For the AL patient samples, all non-AL patients and all other cancer types were excluded from the study. The deceased AL patients, of which no previous EBV testing was performed concerning IgG and IgM, were also excluded from the study.

The control group patients, all leukaemia cases were excluded. The control group patients were randomly selected from patients of Windhoek Central Hospital to have the same amount of patients as the AL patients.

The relapse patients, the cases, which did not relapse back to AL within, the specified time frame of the collection, were excluded for this phase of the research. The control group pertaining to the relapse study which was non-AL cases were also excluded.

All the EDTA samples which were not centrifuged and aliquot within two hours after sample collection were excluded in this study as a general Good Laboratory Practice for prevention of haemolysis and for further preservation.

2.3 Sample method

Non-probability sampling technique of convenience was used to collect the samples. EDTA samples were collected, centrifuged, red cells and plasma was separated (aliquot) and stored at -70 ◦C. These samples were transported to the lab within 30 minutes because they belong to the critical wards which are provided with prompt delivery services (porters). The separation occurred within two hours after

sample collection to minimize the degeneration of red cells, which could interfere with the testing system (haemolysis). These samples were batched until the testing phase was reached and initiated.

A sample method of convenience was used; therefore EDTA plasma samples were used instead of serum samples. This was because it was difficult to get hold of all the AL patients across Namibia. It was, therefore, more convenient for picking the same EDTA samples which were sent in for a routine check-up or for routine screening of blasts within Haematology (NIP). By this method, the sample size grew much quicker than waiting for a serum sample coming in. In some cases by the time the researcher reached the oncology ward, the patient has already left and some already started travelling back home outside Windhoek. EDTA plasma samples are in some countries, like Europe the preferred sample type for ELISA testing rather than serum (Nilson *et al.,* 2007). According to Robinson *et al*., (2014) they concluded that EDTA doesn't affect the conjugate activity of the ELISA method. Furthermore, Robinson *et al*. (2014) debated that if EDTA presence interfered with the conjugate activity then no yellow colour production would have occurred in their experiments. These findings are similar to Doucet *et al*., (2013) which came to the conclusion that EDTA plasma samples are reliable to use for screening of EBV IgG and IgM.

The package inserts for EBV IgG and EBV IgM ELISA was extensively reviewed. One important note was highlighted that the High positive, Low positive control, Calibrator 1, Calibrator 2 and Calibrator 3 of which the kit provides to ensure quality assurance monitoring are made from plasma or serum. With this information, it can be concluded that plasma can be used for this testing system as well (Captia EBV VCA IgM, Capita EBV VCA P-18 IgG; Trinity Biotech).

The same EDTA samples which were from the relapse category were sent for further testing (RT-PCR). This allowed for, not the need to recollect EDTA samples for molecular testing; however, serum is not allowed in this case of testing. Relapse patients sample size were much smaller and if the option of the serum used initially for ELISA testing the sample size would have been even less and then possible couldn't rely on the conclusions drawn. It was very hard to get hold of the relapse samples; therefore, the sampling method of convenience played an advantage to this stage of the study.

2.4 Sample size

A sample size of convenience was used. The sample size was based on all the AL cases collected in the period of interest. The sampling collection took place as the cases presented themselves at the adult oncology or 8 west paediatric oncology for routine treatment and general checkups. The sample size

was 95 AL cases of which 8 relapse cases. The same sample size was used for the control groups, comprising of 95 non-leukemic and 8 non-relapsed AL patients.

2.5 Data collection methods

Blood samples from both adult and paediatric oncology ward (Windhoek Central Hospital), which were collected for routine screening (check-up), were used for this study. Samples were screened for EBV IgG and IgM antibodies by the use of the ELISA method and tabulated on the latest version of Excel. Refer to Annexure 4.2: Acute Leukaemia Patient Group Data

The control group samples were randomly picked from patients of Windhoek Central Hospital and selected according to the inclusion and exclusion criteria. These samples were screened for EBV IgG and IgM antibodies by the use of the ELISA method and tabulated on the latest version of Excel. Refer to Annexure 4.1: Control Group Data

For the relapsed AL patients, blood samples were screened for EBV PCR and the results were respectively computed on the latest version of Excel. Refer to Annexure 4.4: Relapse AL Data

The non relapse AL or control group, the same amount as the relapse cases were picked, but these cases were non relapse AL cases. These samples were screened with EBV PCR and the results were also computed on the latest version of Excel. Refer to Annexure 4.5: Non-Relapse Control Group Data

2.6 Ethical consideration of research

Ethical approval for this study was obtained from the Health Research Ethics Committee of the Namibia University of Science and Technology (NUST). Furthermore, ethical approval and consent were granted to allow patient records to be used from the Ministry of Health and Social Services (MoHSS) of Namibia. Refer to Annexure 3.1: Ethical approval letter MoHSS (17/3/3 MN)

No patient's name will be made public in this study. Ethical approval was granted from the Namibia Institute of Pathology as the only centre to collect samples and as the testing facility for the patient samples. Refer to Annexure 3.2: NIP Ethical approval letter

2.7 Experimental Methods

2.7.1 ELISA method

ELISA was the main test used in this study and was used to screen for the presence of EBV IgG and IgM immunoglobulins. ELISA is a technique designed for detecting and quantifying peptides, proteins, antibodies and hormones by use of a plate-based assay. An antigen must be immobilized to a solid surface and then respectively complexed with an antibody which is linked to an enzyme (Gan & Patel, 2013). The detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The detection strategy is based on specific antibody-antigen interactions (Gan & Patel, 2013). The ELISA plates are analysed by spectrophotometry, at a 450nm wavelength to obtain Optical Density units (OD units). Furthermore, calculations were performed using the calibration factor obtained from the specific package insert of the kit. Immune Status Ratio (ISR) values are then respectively computed for each patient to get a titer concentration. This was a semi-quantitative method used.

2.7.2 Real-Time PCR

Real-Time PCR was used to screen the cases which relapsed back to AL. The PCR was used to determine the presence of EBV DNA, from extracted blood of the relapsed AL patients and non relapsed patients (control group). Primers directed to conserved regions of the EBV genome encoding capsid protein gp220 and Epstein-Barr nuclear antigen 1 were used to determine the presence of EBV, at an ideal condition and temperature of 25◦C (Telenti, 1990). These samples were referred to Johannesburg, South Africa Lancet Referral Laboratory. Lancet is an accredited and a selected long term referral laboratory of NIP as per contract.

2.8 Measures to ensure validity and reliability

A control group was screened through the same process of the ELISA method mentioned above, except the control group contained non-leukemic patients. The EBV ELISA method is an accredited method with NIP and complies with the ISO 15189:2012 (SANAS accreditation). Each plate had a Blank; Calibration 1; Calibration 2; Calibration 3; High Positive and Low Positive controls, which were used to ensure the plate and respective results, were reliable. All the AL patients were performed in duplicates to ensure the true positivity and true negativity of the patient results. Refer to Annexure 4.3: Acute Leukaemia Patients Additional Data after Re-testing

Summarized percentages (prevalence) of IgG and IgM immunoglobulins were computed into respective variable groups including AL, ALL and AML and analysed for significant relationships pertaining to frequencies. All variable groups were compared to a control group (non-leukemic) to determine significant statistical differences in the results.

All cases found to have relapsed back to AL were screened with Real-Time PCR to determine for the presence of EBV DNA. With each batch of PCR run, there were controls in place to ensure the reliability of the batch results. The same amounts of relapse cases were screened with EBV PCR as a control group consisting of non-relapse AL patients. The Real-Time PCR is an accredited test of Lancet (SANAS accreditation). At this phase of the research aimed to determine if EBV infection was present and possibly contributing to the relapse of AL.

2.9 Data management and storage

Generated data was stored as laboratory printouts raw data and stored under lock and key. Data was also stored in a soft copy form for back-up purposes. Soft copy form was on a hard drive disk, CD-ROM and flash drives. Only authorized personnel had access to data generated and stored.

2.10 Data analysis

Data was entered and statistically analyzed using the latest version Excel analysis tools. The statistical analysis was mainly carried out considering the three categories of serological response to EBV as follows: EBV negative, EBV acute infection and EBV latent infection. Frequencies concerning positivity and negativity rates were compared between patients and controls.

The use of the Chi-square test was used to determine statistically significant differences between AL patients and controls. A P-value was derived from the Chi-square test, of which a P- value less than or equal to 0.05 was considered statistically significant.

The Microsoft Office Excel spreadsheet was designed in a tabulated format, whereby the requisition number, stage of AL, specific type of AL (ALL or AML), IgG titration concentration, IgM titration concentration, age, gender, IgG result, IgM result, IgG optical density, IgM optical density and PCR were used as headings. Refer to Annexure 1: Format of the Excel Spreadsheet used to capture the data.

According to the Namibian National Cancer Registry for distinguishing between children and adults, are defined by the following age category: Acute Leukaemia children ≤15 years and Acute Leukaemia adults 15 > years respectively (Carrara *et al*., 2017).

CHAPTER 3: RESULTS

The AL patients consisted of ALL and AML patients. It was noted that 66 out of 95 (69%) were ALL and 29 out of the 95 (31%) were AML. The ALL were double as many compared to the AML patients. Furthermore, a small portion relapsed within the period of interest and consisted of 8 (8%) out of the 95 AL patients (Table 3).

The AL patients consisted of 51 which were females (54%) and 44 out of the 95 which were males (46%) (Table 3). The AL patients compared to the control group regarding age divisions were different in each age category, which made it possible to look at most possible scenarios in both groups concerning EBV evaluation (Table 3).

The AL patient group compared to the control group were more or less the same proportions regarding gender. However, in the AL patient group, there were more males compared to the control group. The adult or child category showed different proportions, in which the AL patient group there were children, but in the control group, there were more adults (Table 3).

EBV IgG was the most common marker among the AL candidates of which 95 were positive for EBV IgG(100%), following EBV IgM of which none were positive (0%). However different results showed in the control group of which 54 out of the 95 were positive for EBV IgG (57%) and 41 out of the 95 were negative for EBV IgG (43%). Furthermore, 17 out of the 95 were positive for EBV IgM (18%) and 78 patients out of the 95 were negative for EBV IgM (82%). Positive results were considered for concentrations of more than and equal to 1.2 for EBV IgG, and more than and equal to 1.24 for EBV IgM (Table 3).

Table 3: Overall participant general characteristics

Characteristics	Control (n=95)	AL Patients (n=95)	P value
Males	37	44	
Females	58	51	
Adults	84	44	
Children	11	51	
ALL	Non leukaemic	66	
AML	Non leukaemic	29	
Relapse	Non leukaemic	8	
Remission	Non leukaemic	82	
Initial	Non leukaemic	2	
Deceased	Non leukaemic	3	
Age (median)	34	14	
EBV IgG (% pos)	57	100	
EBV IgG titre (mean) (ISI value)	4.40	10.87	0.0002 ***
EBV IgM (% pos)	18	0	
EBV IgM (mean) (ISI value)	0.75	0.07	0.2345

Note: The above table represents the general and overall characteristics found in the AL patients and the control group. These samples were screen by ELISA testing for EBV immunoglobulins.

The AL patients had a total of 46 children (56%) and 36 adults (44%) in remission, with a total of 82 (85%), which was in remission. The remission category had the highest prevalence of which the children were more in the remission stage (Table 4 & Figure 2).

The relapse stage consisted of 7 adults (88%) and 1 child (12%) of which the adults were the majority which relapsed (Table 4 & Figure 2).

The initial stage of the disease consisted only of 1 adult and 1 child of which was part of the newly diagnosed patients which didn't initiate chemotherapy yet. The deceased cases consisted of 3 children (38%) of which none of the adults had representation in this stage (Figure 2).

In the Non-relapse group or Control group, 4 out of the 8 were males (50%) and 4 out of the 8 were females (50%). The adults were 8 (100%) and no children were in this control group. Only 1 out of the 8 was tested positive for EBV PCR which was a female (12.5%). The rest of the control patients were 3 females and 4 males which were negative for EBV DNA (Table 4).

Table 4: EBV DNA in Relapsed AL

Characteristics	Non Relapse AL Control (n=8)	Relapse AL Patients (n=8)	P value
Males	4	1	
Females	4	7	
Adults	8	7	
Children	0	1	
Age (median) (years)	47	31	
EBV DNA Positive	1	3	0.0325 *
EBV DNA Negative	7	5	

Note: The above table represents the results found from the relapsed AL patients compared to the Non-Relapsed AL control group. These samples were screened by the used of RT PCR.

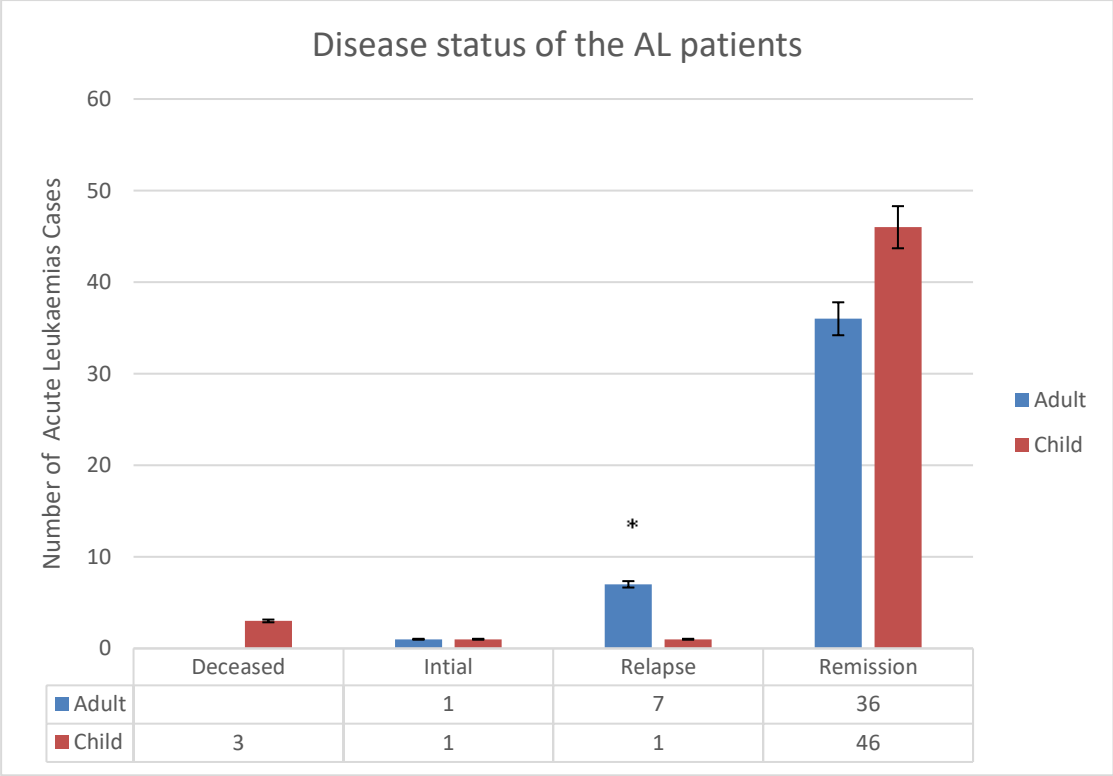

Figure 2: Bar chart showing the different disease status of the AL patients

Among the AL patients; there was a peak in the remission category and the least among the initial diagnosis stage. More adults relapsed than children, but more children deceased than adults (*p<0.05, **p<0.01, ***p< 0.001). The method of "Error bar of the Mean" was used to determine the error bar significance figures.

Furthermore now looking at the age for the whole AL patients, 44 out of the 95 (46%) were adults (15 > years), and 51 out of the 95 (54%) were children (≤15 years) (Table 5). However, in the control group, 84 out of the 95 (88%) were adults and 11 out of the 95 which were children (12%) (Table 5).

For the AL patients, it was noted that 51 out of the 51 children were EBV IgG positive (100%) and 0 out of the 51 children were EBV IgG negative (0%). Furthermore, 0 out of the 51 children were EBV IgM positive (0%) and 51 out of the 51 children were EBV IgM negative (100%) (Table 5).

Among the AL adults, 44 out of the 44 were EBV IgG positive (100%) and 0 out of the 44 (0%) were EBV IgG negative. Additionally, for the adults, 0 out of the 44 were EBV IgM positive (0%) and 44 out of the 44 were EBV IgM negative (100%) (Table 5).

Table 5: EBV in AL children versus adults

Characteristics	**AL Child (≤15 years)**	**AL Adult (>15 years)**	**P value**
AL patients	51	44	
Age (median) (years)	7	34	
EBV IgG (% pos)	100	100	
EBV IgG titre (mean) (ISI value)	10.83	10.93	0.0002 ***
EBV IgM (% pos)	0	0	
EBV IgM titre (mean) (ISI value)	0.08	0.06	0.2345

Note: The above table represents the EBV results found in the AL patients comparing the children and adults results against each other.

For the 66 ALL cases, all were positive for EBV IgG (100%) and all 66 were negative for EBV IgM (100%) (Table 6).

Table 6: EBV in ALL

Characteristics	Control (n=66)	ALL Patients (n=66)	P value
Males	32	32	
Females	34	34	
Age (median) (years)	38	13	
EBV IgG (% pos)	56.06	100	
EBV IgG titre (mean) (ISI value)	4.8	10.88	0.0002 ***
EBV IgM (% pos)	15.15	0	
EBV IgM titre (mean) (ISI value)	0.79	0.08	0.248

Note: The above table represents the EBV results found in the ALL patients compared to the control group results with the same number of males and females.

Furthermore, 28 out of the 66 (42%) were adult ALL patients and 38 out of the 66 (58%) were children ALL patients (Table 7).

In the ALL group, there were more children than adults, whereas in the AML group there were more adults than children (Table 7 & 9).

Table 7: EBV in ALL children versus adults

Characteristics	ALL Child (≤15 years)	ALL Adult (>15 years)	P value
ALL patients	38	28	
Age (median) (years)	8	30	
EBV IgG (% pos)	100	100	
EBV IgG titre (mean) (ISI value)	10.92	10.82	0.0002 ***
EBV IgM (% pos)	0	0	
EBV IgM titre (mean) (ISI value)	0.09	0.07	0.248

Note: The above table represents the EBV results found in the ALL patients comparing the children and adults results against each other.

Of the AML patients, 29 cases were EBV IgG positive (100%) and 29 cases were EBV IgM negative (100%) (Table 8).

Table 8: EBV in AML

Characteristics	Control (n=29)	AML Patients (n=29)	P value
Males	12	12	
Females	17	17	
Age (median) (years)	32	16	
EBV IgG (% pos)	45	100	
EBV IgG titre (mean) (ISI value)	3.75	10.85	0.0002 ***
EBV IgM (% pos)	10.34	0	
EBV IgM titre (mean) (ISI value)	0.48	0.06	0.238

Note: The above table represents the EBV results found in the AML patients compared to the control group results with the same number of males and females.

Additionally, 16 out of the 29 (55%) were adult AML patients, and 13 out of the 29 (45%) were children AML patients (Table 9).

Table 9: EBV in AML children versus adults

Characteristics	AML Child (≤15 years)	AML Adult (>15 years)	P value
AML patients	13	16	
Age (median) (years)	7	25	
EBV IgG (% pos)	100	100	
EBV IgG titre (mean) (ISI value)	10.54	11.11	0.0002 ***
EBV IgM (% pos)	0	0	
EBV IgM titre (mean) (ISI value)	0.07	0.06	0.238

Note: The above table represents the EBV results found in the AML patients comparing the children and adults results against each other.

From the above data sets, there was a statistically significant difference in presence of EBV IgG (P=0.0002***) between the AL patients and the control group of which $P \leq 0.05$ was considered

statistically significant. For EBV IgM (P=0.224) it didn't show statistical significance between the AL patients and the control group (Table 10 Part 1) (*p<0.05, **p<0.01, ***p< 0.001).

Furthermore, there was a statistically significant difference in presence of EBV IgG (P=0.0002***) between the ALL patient group and the control group, P ≤ 0.05 was considered statistically significant in ALL patients. EBV IgM (P=0.278) didn't show statistical significance in ALL patients (Table 10 Part 1).

There was a statistically significant difference in presence of EBV IgG (P=0.0002***) between the AML patient group and the control group, of which P ≤ 0.05 was considered statistically significant in AML patients. EBV IgM (P=0.238) didn't show statistical significance in AML patients (Table 10 Part 1).

It was found that there was as well a statistical significant difference between the AL relapse patients and the non-relapse control group for EBV PCR (P=0.03) of which P ≤ 0.05 was considered statistically significant (Table 10 Part 2).

Among the comparisons between gender of AL patients and the control group, it was found that there was a statistical significant association in the presence of EBV IgG, however no association in the presence of EBV IgM (Table 10 Part 3).

Table 10: Table association between EBV and Acute Leukaemias

Part 1	**EBV IgG Concentration (IU/ml)**					**EBV IgM Concentration (IU/ml)**				
	Control Group		**Patient Group**		**P value**	**Control Group**		**Patient Group**		**P value**
	Children	**Adults**	**Children**	**Adults**		**Children**	**Adults**	**Children**	**Adults**	
AL	6.54	4.12	10.83	10.93	0.0002 ***	0.92	0.72	0.08	0.06	0.244
ALL	6.54	4.12	10.92	10.82	0.0002 ***	0.92	0.72	0.09	0.07	0.248
AML	6.54	4.12	10.54	11.11	0.0002 ***	0.92	0.72	0.07	0.06	0.238
Part 2	**Real Time PCR (EBV DNA)**									
	Non Relapse AL (Control group)		**Relapse AL (Patient Group)**		**P value**					
Positive	1		3		0.0325 *					
Negative	7		5							
Part 3	**EBV IgG Concentration (IU/ml)**					**EBV IgM Concentration (IU/ml)**				
	Control Group		**Patient Group**		**P value**	**Control Group**		**Patient Group**		**P value**
	Males	**Females**	**Males**	**Females**		**Males**	**Females**	**Males**	**Females**	
AL	4.12	6.54	11.11	10.54	0.0002***	0.70	0.77	0.07	0.08	0.276
ALL	3.36	5.06	11.43	10.37	0.0002 ***	0.70	0.77	0.08	0.08	0.278
AML	4.12	6.54	11.29	10.55	0.0001 ***	0.70	0.77	0.05	0.07	0.265

Note: The first part of the above table represents the Chi-square testing between AL patients and the control group concerning adult versus children. The second part of the table represents the Chi-square testing between the Relapse AL group and the Non-Relapse AL group. The third part of the table represents the Chi-square testing between AL patients and the control group concerning males versus females. P ≤ 0.05 was considered a statistically significant relationship (*p<0.05, **p<0.01, ***p< 0.001).

CHAPTER 4: DISCUSSION

A previous study targeting EBV infection has shown that approximately 90% of the world's population is infected with EBV as a life-long latent or dormant infection of B lymphocytes (Tangye *et al*., 2017). In our research study, the EBV past infection positivity rates (EBV IgG) shows different results compared to Tangye *et al.* (2017), which found in total 75% of the AL and non-leukemic patients together were previously exposed to EBV infection. This, in turn, shows that different results are presentable for different countries concerning EBV infection positivity rates.

A study conducted among Egyptian ALL children showed that only 29% of their patients were positive for EBV IgG, but still concluded that their results are statistically significant (p=0.0001) and concluded that EBV is involved in the development of ALL Zaki *et al*., (2014). Furthermore, Zaki *et al.* (2014) showed that EBV DNA had a significant difference between patients and controls (p=0.04). In this research study, the results are very similar concerning EBV IgG (p=0.0002) and EBV DNA (p=0.04) but different concerning the contribution towards both ALL and AML and not just ALL. Furthermore despite neither the age nor gender; and overall shows that previous exposure (lifetime latent infection) contributes to the development of AL and not recent EBV infection.

According to Hoffbrand & Moss (2011), EBV is associated with many cases of haematological malignancies of a lymphogenous nature, especially concerning B Cell ALL, and is most likely responsible for genetic mutations occurring in lymphogenous cells. Furthermore, according to Hoffbrand & Moss (2011), ALL is a form of leukaemia which mostly occurs in children and accounts for approximately 20% of ALs. In our research study, the results were similar of which the children were more infected with ALL (58%); compared to the adults (42%), but our adult percentages were higher compared to Hoffbrand & Moss (2011). Approximately half of the patients were adults and half were children, this is because the patients were picked as they presented themselves at the oncology and paediatric oncology ward. It can be drawn from the results that the most common AL type among the patients was ALL which was double as many as the AML, but this finding was patientive to the prevalence of the disease and sampling technique used. ALL were the most common among the children patients which are similar to the findings of Hunger & Mullighan (2015), which they found that ALL is the most common cancer among children in the United States.

In our research study the adults (55%) were more common among the AML group which is similar to a study of Juliusson *et al.* (2017); which concluded that AML is a disease of adults (95%) among Swedish AML patients, but their frequencies were much higher compared to our research study. It can be debated that countries differ with age prevalence rates of AML (Juliusson *et al.* 2017). Out of the AML patients, 59% were females which were as well similar to Juliusson *et al.* (2017), which their conclusions were at 51% were females in AML. Additionally, it can be debated that most probably females have higher prevalence towards AML, but further studies are required to explore this conclusion drawn.

According to Sehgal (2010), it was discussed that there was no evidence that EBV contributes to AML development in children, which these findings are different from our research study which shows EBV does contribute to AML, regardless of neither the age nor gender affected.

It was interesting to find that some of the control patients tested positive for EBV IgM. This could be because non randomisation sampling techniques were used to obtain 95 control patients. The majority of these control patients were patients from Windhoek Central Hospital that were hospitalised and were of non-leukaemic diagnosis. This suggests that the control patients were recently infected with EBV thus can't contribute to the development of AL yet because EBV contributes to the development of AL as a lifetime latent infection and not a recent infection.

Another observation was noted from the control group was that the children were showing higher EBV infection rates, average optical densities and titer concentrations for both EBV IgG and EBV IgM compared to the adults. These findings are similar to the world's findings that EBV infects usually in the early age of life (Tangye *et al*., 2017). EBV is mostly acquired early in life and carried as an asymptomatic infection of the B lymphoid system, but when the host's immunity is compromised then the EBV virus generates its pathogenic potential (Taylor *et al.,* 2015). Further studies are required to explore why the immunity of the AL patients was initially compromised before the development of AL post infection of EBV.

A small portion of patients relapsed within the specified time frame of sample collection. Out of this small portion of patients, the majority were adult females of ALL origin. All the patients which relapsed with AL were screened by use of Real-Time PCR for the presence of EBV DNA. This, in turn, suggests that EBV relapse from the latent phase might contribute to the relapse of the AL because the results showed statistical significance in the relationship. Only one candidate of the control group was tested

positive for EBV DNA compared to the three AL relapsed patients who tested positive. In this study, 7 adults (majority) relapsed and only 1 child (minority) relapsed. The deceased compared patterns show different frequencies of which 3 children (majority) were deceased of which none of the adults (minority) was deceased. This shows an indication that when the children relapse with AL they pass away and don't recover from the relapse. The adults, on the other hand, recover from the relapse. The fatality of relapsing is worse for children compared to adults. There was a previous study based on a case-control of a male AML patient, which relapsed 11 years after a transplant and post-chemotherapy, of which at the stage of AL relapse the patient had an acute EBV infection. In that particular study, they concluded that EBV might have caused the relapse of the AL (Kikushige *et al*., 2006). With regard to our study and these study findings of Kikushige *et al*., it is clear that there is a need for further studies regarding EBV as a contributing factor towards relapse AL.

Additionally the exploring of maternal transmission of EBV infection should be investigated; as it was established that there is an association between maternal EBV and activation of ALL in the respective offspring (Tedeschi, 2007), the establishment should be made in AML as well as a crucial aspect could have been blindsided that EBV only contributes towards ALL.

CHAPTER 5: CONCLUSION

The current study shows that it does not matter the type of AL, EBV might contribute to the development of both ALL and AML following previous exposure to EBV. On completion of the study, and the statistics have been drawn up, it was also concluded that EBV contributes to the relapse of AL.

The association of AL with EBV played a significant role in the study, as the virus may have been a predisposing factor among the paediatric and adult AL patients. It should, therefore, be implemented as a testing algorithm, that every individual diagnosed with AL be screened for the presence of EBV IgG and IgM antibodies in their serum. Furthermore, which will in return, prevent the development of AL when interventions are taken. It's more important to vaccinate all newborn infants against EBV, to prevent initial EBV infection from development which contributes to the development of AL. This may reduce the incidence of AL in Namibia. The AL patients should maintain a strong immunity to prevent EBV from contributing to relapse of AL.

It can be strongly suggested that all AL patients tested within this research study were previously infected with EBV because all the patients tested positive for EBV IgG. None of the AL patients was positive for EBV IgM, thus no active EBV infection or recent EBV infection took place.

The overall conclusion drawn was that EBV is common amongst AL patients of Namibia, as a lifelong latent infection, which could have contributed to the development of AL regardless of the type of AL (ALL or AML), neither the age affected nor the gender. This conclusion broadens the knowledge that EBV can initially infect anyone and the consequences in the long term may be fatal. AL associated with EBV should, therefore, remain being considered a serious matter which requires the absolute, complete attention of medical personnel to ensure the best possible treatment and patient management of the disease. Prevention interventions considering EBV should already be taken at newborn infancy period. For those previously infected with EBV should maintain a strong immunity to prevent the EBV from going viral.

Further studies and research can be conducted to determine the EBV viral load level upon relapse of AL patients of Namibia, in order to determine more information on immunity versus EBV number of

viral copies. This also creates an opportunity so that further studies can be carried out also on viral infections, not only EBV but all other types of viruses linked to AL.

LIMITATIONS AND SCOPE OF RESEARCH

Some patients after being diagnosed seek treatment in more developed countries, thus their names don't appear on the AL registry causing the sample size to be decreased. In order to obtain a high sample size, the deceased AL patients who were previously screened for EBV immunoglobulins were included in this study.

There are a lot of causes to cancer proven previously with other research studies, with a variety of environmental circumstances of the patients taken into account, however, in this study, all these causes were excluded and not taken into account. The main aim of this study was to determine a crude association between EBV and ALs in Namibia, therefore the focus was not on the other causes towards cancer.

Another limitation to this study was, not knowing that a proper AL registry is always kept and updated in a regular manner, this will cause some of the patients to be left out of the study. In order to minimize the effect of this limitation, NIP has a regularly updated registry within Haematology department, Windhoek Reference Laboratory, which was used in this study as well, trying to pick up on the cases which were not updated on the AL registry.

Patients who appear on the AL registry were only those patients who had a confirmatory Flow cytometry or Cytogenetics testing was done on either their bone marrow or peripheral blood. Due to the fact that some patients are diagnosed with AL-only in a late critical state on the peripheral smear, some patients are too poor for regular check-ups with their doctors, the majority of these patients die before their names are entered on the AL registry.

Some cases were hard to find concerning relapsing patients into AL, therefore proper communication between the oncology wards and the researcher was maintained.

EBV IgM might only appear later in infection, or might be produced transiently, or might appear at extremely low concentrations that laboratory testing is unable to pick it up (Ralf, 2004). This was another limitation towards this study as this research depended mostly on ELISA testing, but PCR testing is much more sensitive thus for the relapse cases confirmatory testing took place using EBV PCR method.

Seems that PCR remains the gold standard for diagnosis of EBV infections of which both Seghal *et al.* (2010) and Sakajiri *et al*. (2002) used the methods to screen their patients, to come to a conclusion that there is an association between EBV and AL. However in our research study the cost constraints was a challenge and therefore the ELISA method was a much cheaper option for screening purposes, however, the relapse cases were screened by RT-PCR.

A possible major limitation to the study was the inclusion of unhealthy patients as the control group, which could have had a higher EBV infection level than a normal population. This could have caused bias in the results and could have caused conclusions drawn which might have been different if the control group was chosen differently. In this case, the results, however, did still show statistical differences between patients and controls, even though the EBV level was assumed to be higher in the control group and therefore still considered valid control group.

Finally, a possible major limitation to the study could have been the use of EDTA plasma samples, instead of serum samples. EDTA was suspected to prevent the conjugate activity; however, in our research study, the colours of the ELISA tests were clear and prominent for both the AL patients and controls. This is similar to the findings of Robinson *et al*., (2014), which concluded that EDTA doesn't affect the conjugate activity in ELISA testing. The test package inserts of our study's, rather emphasize that the degeneration of red cells could possibly cause haemolysis and this could interfere with the testing process. To minimize this interfering factor, all EDTA samples were centrifuged, aliquot and frozen at -70 ◦C within two hours after sample collection. Both Robinson *et al.,* (2014) and Nilson *et al*., (2002) used EDTA plasma for ELISA testing of EBV IgG and IgM screening. They came to reliable results and conclusions that EDTA plasma is worthy using if samples are separated and aliquot within 8 hours after sample collection (Robinson *et al*., 2014) (Nilson *et al*., 2002). These findings are similar to Doucet *et al*., (2013) which came to the conclusion that EDTA plasma samples are reliable to use for screening of EBV IgG and IgM.

SIGNIFICANCE OF RESEARCH STUDY

The results and the conclusions made from this study can be used as a prevention tool and will tremendously decrease the prevalence of AL in Namibia in the near future and save many lives. The association of EBV as the possible cause of relapsing AL will likely contribute to thousands of lives if used as prevention.

This was the first study which was done in Namibia on the association between EBV and AL patients of Namibia. The cause of relapsing AL and the reason for it is still unknown. This study will contribute to broadening the knowledge of EBV as the possible cause for the relapse of the AL.

Currently, all newly diagnosed children with ALL are being screened for EBV infection as a guideline for paediatric oncology. From this study's conclusions drawn the guideline should be changed to all newly diagnosed patients with AL despite the age or type of AL, should as well be screened for EBV infection.

RECOMMENDATIONS

The need for further studies to be done on a successful vaccine against AL patients, who were previously infected with EBV, so that it can be used as a preventative tool against relapsing AL. This could possibly contribute to being a follow-up study on this research study.

Additionally, further studies are required to determine the time frames pertaining to the development of AL post-exposure to EBV infection. This could then contribute as a prevention tool for patients who were already infected by EBV and to maintain strong immunity at the peak periods of EBV fatal implication stages.

FUTURE WORK

After the conclusions were drawn it is noted that this research study requires additional work to be done for future purposes. Some of the work would be linked to screening all the AL patients for the presence of EBV DNA by the use of Real-Time PCR, to pick up the presence of EBV DNA before the

development of EBV IgM immunoglobulins. Furthermore, this shall allow early detection of recent EBV contraction and therefore interventions can be taken early enough to have an impact on the disease progression.

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ANNEXURE 01: DATA COLLECTION SHEET

NAMIBIA UNIVERSITY OF SCIENCE AND TECHNOLOGY

Data Collection Sheet: EBV versus Acute Leukaemia **Sheet Number: 01**

Date	Identification Number	Patient Name	Status of Disease: (Initial diagnosis, remission, relapse phase or deceased)	Specific Diagnosis (Acute Lymphoblastic Leukaemia OR Acute Myeloblastic Leukaemia)	EBV IgG Concentration OD=OD units; Titer Con= ISR value	EBV IgM Concentration OD=OD units; Titer Con= ISR value	PCR Positive Or Negative (Only Relapse Cases)
	01						
	02						
	03						
	04						
	05						
	06						
	07						
	08						
	09						
	10						
	11						
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	22						
	23						
	24						
	25						
	26						
	27						
	28						
	29						
	30						
	TOTALS						
	Percentages out of 30						

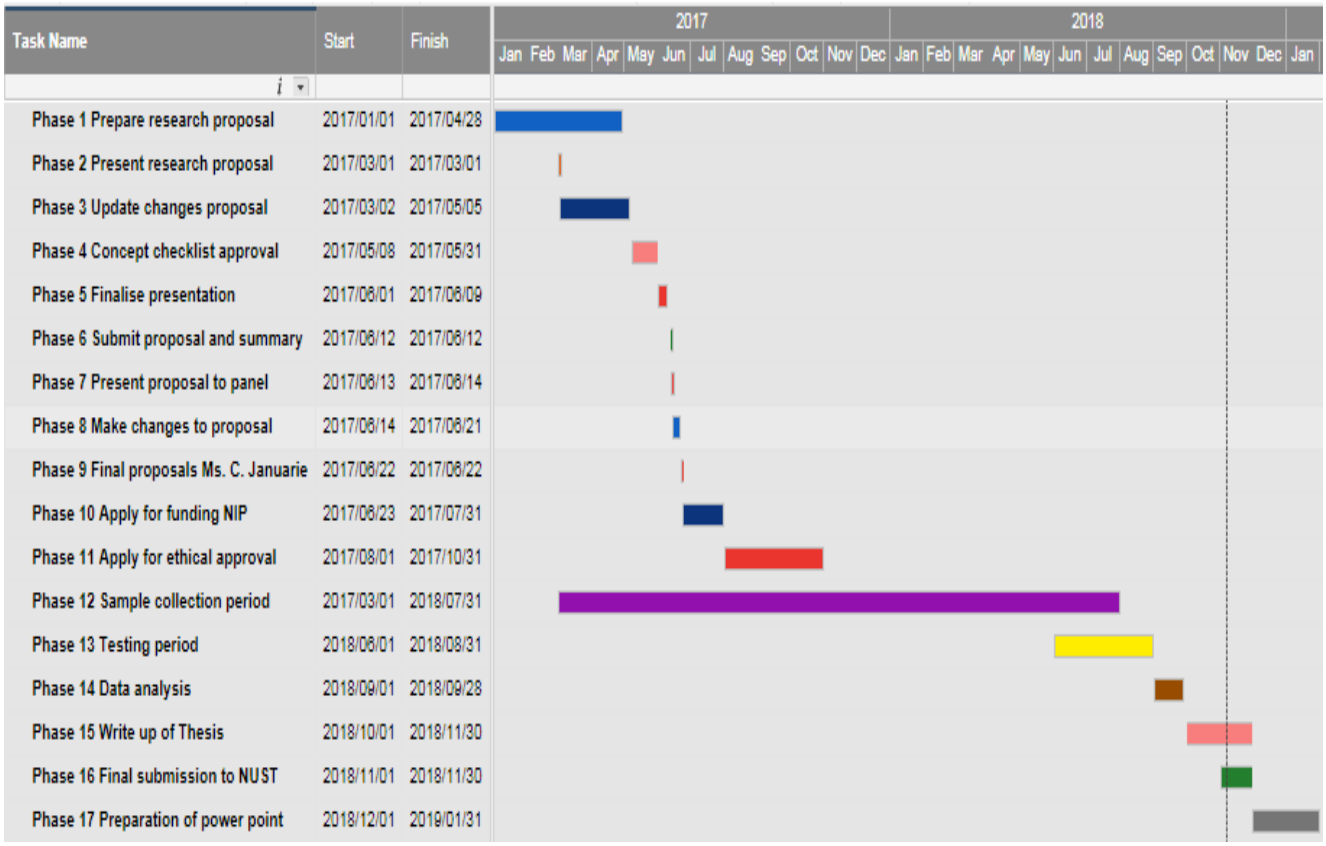
OD = OD units

Titer Concentration = ISR value

Reviewed by:

Date reviewed:

ANNEXURE 02: WORK PLAN



Task Name	Start	Finish
Phase 1 Prepare research proposal	2017/01/01	2017/04/28
Phase 2 Present research proposal	2017/03/01	2017/03/01
Phase 3 Update changes proposal	2017/03/02	2017/05/05
Phase 4 Concept checklist approval	2017/05/08	2017/05/31
Phase 5 Finalise presentation	2017/06/01	2017/06/09
Phase 6 Submit proposal and summary	2017/06/12	2017/06/12
Phase 7 Present proposal to panel	2017/06/13	2017/06/14
Phase 8 Make changes to proposal	2017/06/14	2017/06/21
Phase 9 Final proposals Ms. C. Januarie	2017/06/22	2017/06/22
Phase 10 Apply for funding NIP	2017/06/23	2017/07/31
Phase 11 Apply for ethical approval	2017/08/01	2017/10/31
Phase 12 Sample collection period	2017/03/01	2018/07/31
Phase 13 Testing period	2018/06/01	2018/08/31
Phase 14 Data analysis	2018/09/01	2018/09/28
Phase 15 Write up of Thesis	2018/10/01	2018/11/30
Phase 16 Final submission to NUST	2018/11/01	2018/11/30
Phase 17 Preparation of power point	2018/12/01	2019/01/31

ANNEXURE 03: ETHICAL APPROVAL LETTERS

3.1 Ethical approval of MoHSS

REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198 | Ministerial Building | Tel: 061 – 2032150
Windhoek | Harvey Street | Fax: 061 – 222558
Namibia | Windhoek | Email: shimenghipangelwa71@gmail.com

OFFICE OF THE PERMANENT SECRETARY

Ref: 17/3/3 **MN**
Enquiries: Mr. J. Nghipangelwa

Date: 04 October 2017

Ms. Marien Naude
Namibia University of Science and Technology
Windhoek
Namibia

Dear Ms. Naude

Re: Association of Epstein-Barr virus and Acute Leukemias in Namibia.

1. Reference is made to your application to conduct the above-mentioned study.

2. The proposal has been evaluated and found to have merit.

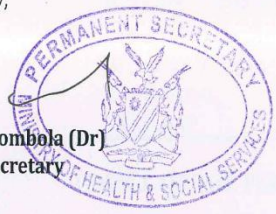
3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**

3.1 The data to be collected must only be used for academic purposes;
3.2 No other data should be collected other than the data stated in the proposal;
3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects' should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;
3.4 A quarterly report to be submitted to the Ministry's Research Unit;
3.5 Preliminary findings to be submitted upon completion of the study;

3.6 Final report to be submitted upon completion of the study;

3.7 Separate permission should be sought from the Ministry of Health and Social Services for the publication of the findings.

Yours sincerely,



Andreas Mwoombola (Dr)
Permanent Secretary

3.2 NIP Ethical approval letter

OFFICE OF THE CHIEF OPERATIONS OFFICER

Enquiries: Mr Boniface Makumbi; Tel.: 061-295 4210

Date: 01 November 2017

Ms Marien Naude
Namibia University of Science and Technology (NUST)
Windhoek
Namibia

Dear Ms Naude

RE: The Association of Epstein-Barr Virus and Acute Leukaemias in Namibia

1. The above mentioned research proposal was referred to the Research/Ethics Committee of the Namibia Institute of Pathology Limited for review.
2. It is a pleasure to inform you approval was granted for you to proceed with the research on condition that the following be complied with:

2.1 Make the necessary corrections and clarifications as discussed, under the following sections:

- In the Abstract, there should be no citation of references.
- Include section 2.2.1 in the second paragraph of under section 1.2
- Clarify the following sentence under section 1.6 " The association of EBV as the possible cause of relapsing AL will likely contribute to thousands of lives if used as prevention".
- In section 2.2.4, remove "etc" and start the sentence with The major/Common symptoms of AL….."
- Clarify the PCR test methodology that you are going to use, e.g your target genes, probes, primers, etc Verify your cost estimates and make sure you have not understated the costs

2.2 Observe and adhere to all ethical considerations and confidentiality to protect patient information.

2.3 Ensure that you have gotten the approval of the Ministry of Health and Social servicesto conduct this research, and adhere to all terms and conditions as stipulated by the Ministry of Health and Social services

2.4 Final report to be shared with the Namibia Institute of Pathology Limited.

Yours Sincerely

pp

Harold T Kaura
Chief Operations Officer

3.3 NIP Financial assistance for Master of Health Sciences

NAMIBIA INSTITUTE OF PATHOLOGY LIMITED
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DIVISION OF TRAINING AND DEVELOPMENT

Enquiries: R.L Matiti
Ext: 4235

10 July 2018

Namibia University of Science and Technology
Private Bag 13388
Windhoek
NAMIBIA

Dear Sir/Madam

FINANCIAL ASSISTANCE: I.R.O MARIEN NAUDÉ, STUDENT NUMBER: 200801139

I can confirm that Namibia Institute of Pathology (NIP) Ltd is willing to accept liability for all tuition fees due in respect of Marien Naudé.

Please arrange for an invoice to be issued to Namibia institute of Pathology.

Yours sincerely

.....
MATHEW NGHIPUMBWA
NIP TRAINING COODINATOR

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