The Association between Epstein-Barr virus and nasopharyngeal carcinoma

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DECLARATION

I, Mbuyelo Abbygale Mabaso (student number 1826114), hereby declare that this research report is my own work. It is submitted for the degree of Master of Medicine in the Division of Anatomical Pathology, School of Pathology, Faculty of Health Sciences at the University of the Witwatersrand.

19/11/2021

DEDICATION

I dedicate this research report to the All-Mighty God. To my parents who always encourage me. To my dear husband who always supports me and to my children who believe so much in me.

ACKNOWLEDGEMENTS

I would like to thank the following people who played a role in this research project:

- My supervisor Dr Dawn-Lee Van Der Byl for dedicating her time and guidance.
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ABSTRACT

Background: Nasopharyngeal carcinoma (NPC) is a rare malignant neoplasm which has a wide age range but affects predominantly adult males. NPC is highly associated with Epstein-Barr virus. There are 3 histological subtypes described by the World Health Organisation (WHO), the commonest being the non-keratinizing subtype.

Methods: In this retrospective study, confirmed cases of nasopharyngeal carcinoma were identified over a six and half year period in a South African population. The patient age, gender, HIV status and CD4 cell count were recorded where available. The site and type of biopsy, histological subtypes and EBER-ISH status of the tumour were also noted.

Results: There were 120 cases of nasopharyngeal carcinoma during the study period. The age range was 10-75 years. The male to female ratio was 1.8:1. The most frequently biopsied site was the nasopharynx which constituted 74 (60.12%) cases.

Of the 120 cases analysed, 110 cases (81.6 %) were classified as non-keratinizing nasopharyngeal carcinoma and there were two cases (1.68%) which were classified as keratinizing nasopharyngeal carcinoma. Eight cases (6.72%) could not be classified into any category. There were no cases of basaloid nasopharyngeal carcinoma in this cohort.

EBER-ISH had been performed in 102 cases and 96 (94.1%) were positive. In these 96 cases, 87 (90.6%) cases were of the non-keratinizing subtype of NPC.

HIV results were only available in 19 cases with 9 of these cases occurring in HIV positive patients. Of these 19 cases, CD4 cell counts were only available in 3 cases with a range of 300-900 cells/mm^3.

Conclusion: The study confirms that South African population shows nasopharyngeal carcinoma in a wide age range similar to other parts of the world. The most common histological subtype is non-keratinizing nasopharyngeal carcinoma. Nasopharyngeal carcinoma is strongly associated with Epstein-Barr virus in this population.

TABLE OF CONTENTS

LIST OF FIGURES

Figures **Page numbers**

LIST OF TABLES:

Tables **Page numbers**

LIST OF ABBREVIATIONS IN ALPHABETIC ORDER

AIDS:	Acquired immunodeficiency syndrome
BARF:	BamH1-A Reading Frame-1
BCL:	B-cell lymphoma
BL:	Burkitt lymphoma
CHBAH:	Chris Hani Baragwanath Academic Hospital
CDK:	Cyclin dependent kinase
CMJAH:	Charlotte Maxeke Johannesburg Academic Hospital
CT:	Computer tomography
DNA:	Deoxyribonucleic acid
EBER:	Epstein-Barr encoded RNA (ribonucleic acid)
EBER-ISH:	Epstein-Barr virus encoded RNA (ribonucleic acid) In Situ Hybridization
EBNA:	Epstein–Barr nuclear antigen
EBV:	Epstein-Barr virus
HAART:	Highly active antiretroviral therapy
HIV:	Human immunodeficiency virus

HL:	Hodgkin lymphoma
HLA:	Human leucocyte antigen
IQR:	Interquartile range
LMP:	Latent membrane protein
MAPK:	Mitogen-activated protein kinase
MLL:	Mixed-lineage leukemia
MRI:	Magnetic resonance imaging
NHLS:	National Health Laboratory Service
NPC:	Nasopharyngeal carcinoma
PI3K:	Phosphoinositide 3-kinase
PCR:	Polymerase chain reaction
RNA:	Ribonucleic acid
SA:	South Africa
SCC:	Squamous cell carcinoma
USA:	United State of America
WHO:	World Health Organisation

Chapter 1 Introduction

1.1 Background:

Nasopharyngeal carcinoma (NPC) is a rare malignant neoplasm arising from epithelial cells of the nasopharynx which has a high prevalence rate in Asia where it is considered endemic (1,2,3,4). It has been reported to be the third most common cancer among men with an incidence of 50 per 100 000 (1). Incidence rates for Southern Africa are not known although parts of North Africa are also classified as endemic with an intermediate incidence (4). NPC has a wide age distribution which shows a bimodal peak, in children and adults with males being more affected compared to females (1,2). The aetiology of NPC is multifactorial, including genetic make-up and environmental influences. Epstein-Barr virus has also been associated (3,4).

Patients present in different ways depending on the site involved and the presence or absence of metastasis to other organs (3). The most common presentation relates to nasal symptoms including discharge, bleeding and obstruction accounting for 46% of cases (3, 5).

According to the World Health Organisation (WHO) classification of tumours, there are three subtypes of nasopharyngeal carcinoma, keratinizing squamous cell carcinoma, non-keratinizing squamous cell carcinoma and basaloid squamous cell carcinoma (3, 5).

Definitive diagnosis and subtyping of NPC is made by histological evaluation of an endoscopically guided biopsy of the primary nasopharyngeal tumour (6).

South Africa (SA) is home to the largest Human immunodeficiency virus (HIV) seropositive population in the world, with 7·2 million individuals (7). Epstein-Barr virus (EBV) is associated with the development of both lymphoid and epithelial tumours with nasopharyngeal carcinoma being one of those epithelial tumours. Ninety-six percent of patients who have a CD4 cell count of 100-400 cells/mm3 also have EBV infection. The incidence of EBV associated tumours in HIV infected patients are found to be 100 times higher than in the general population (8, 9). At present, few research studies conducted indicate that patients with HIV/ Acquired immunodeficiency syndrome (AIDS) are at an increased risk of developing mucosal squamous cell carcinoma, nasopharyngeal carcinoma, lymphoepithelial carcinoma of the salivary gland, and Merkel cell carcinoma (10).

Definitive correlation of HIV and NPC has not been established. In view of the high HIV burden in South Africa and the prevalence of Epstein-Barr virus in HIV infected individuals, additional research on the association of EBV and NPC in a South African context will be of value.

The research findings will serve to document the epidemiological characteristics of nasopharyngeal carcinoma in a South African population. Epidemiological data such as the age, gender ratio, site, histological subtypes, association with Epstein-Barr virus and HIV

with CD4 cell count will contribute to the academic knowledge and give an overview of nasopharyngeal carcinomas in SA.

Chapter 2 Aims and objectives

2.1 Aims of the study:

The aim of this study was to describe nasopharyngeal carcinoma in a South African population and the association between Epstein-Barr virus and nasopharyngeal carcinoma in this population.

2.2 Objectives:

2.2.1. To describe the epidemiology of nasopharyngeal carcinoma including age and gender.

2.2.2. To determine the HIV status and CD4 cell counts in patients diagnosed with nasopharyngeal carcinoma.

2.2.3. To determine the frequency of histopathological subtypes of nasopharyngeal carcinoma.

2.2.4. To determine the type of specimen and site of specimen including frequency of metastasis.

2.2.5. To determine the frequency of Epstein-Barr Virus Encoded RNA (Ribonucleic Acid) In Situ Hybridization (EBER –ISH) in nasopharyngeal carcinoma.

2.2.6. To determine the correlation between subtypes, EBER-ISH status and HIV status.

Chapter 3 Literature review

Nasopharyngeal carcinoma is a rare malignant neoplasm arising from epithelial cells of the nasopharynx (1, 2, 3). NPC has a unique and specific ethnic and geographic distribution (4) with Southern China known to have the highest incidence rates of NPC globally (3, 5, 11). NPC usually involves the pharyngeal recess (Rosernmuller's fossa) and posteromedial crura of the eustachian tube opening into the nasopharynx (2, 3, 12).

Nasopharyngeal carcinoma is rare in most parts of the world; however, it is considered endemic in Southern China (5) where its incidence is as high as 25 per 100 000 (1) and has been reported to be the third most common cancer among men with an incidence of 50 per 100 000 (5). In non-endemic countries such as United State of America (USA), the incidence is much lower with an incidence rate of 0.5 – 2 per 100 000 (5). Incidence rates for Southern Africa are not known although parts of North Africa are also classified as endemic (5) with an intermediate incidence (4). It accounts for 7-10% of all cancers in North Africa (4) where there is a higher incidence of the disease in men.

The age distribution of nasopharyngeal carcinoma is wide (12) and is more common in the adult population (3,4) than in children. NPC can account for up to 20% of cases in the 10 to 25-year age group in North Africa (4).

NPC is highly associated with Epstein-Barr virus (2, 3, 11, 13) with most NPC's expressing EBV proteins (11). The aetiology is multifactorial including environmental factors such as smoking (4), occupational exposures (12) and certain dietary intakes (2, 3, 4, 5, 12, 13) which have been shown to increase the risk of NPC. In Asia in particular, a high intake of salt-cured fish and meat contribute to the high NPC incidence (2, 3, 5, 12, 13). The increased risk of NPC is also associated with the consumption of rancid butter products in North Africa. These are considered as a potential EBV activator and therefore a possible cause of nasopharyngeal carcinoma (4). Genetic factors principally human leucocyte antigen (HLA) class 1 alleles (4, 5) contribute to the disease. In particular, HLA A0207 (5), has been singled out.

Patients present in different ways depending on the site involved and the presence or absence of metastasis to other organs. Up to 10% of tumours are asymptomatic with 5% of cases presenting as a metastasis (3). In early disease, the symptoms are non-specific; therefore, most patients present with late disease (5). The most common presentation relates to nasal symptoms including discharge, bleeding, and obstruction accounting for 46% of cases. Aural symptoms and a neck mass are also frequently encountered accounting for 42% each. The other symptoms which have been reported include headaches, visual abnormalities, facial numbness, speech or swallowing disorders and weight loss (3).

There are three subtypes of nasopharyngeal carcinoma classified by the World Health Organisation; keratinizing squamous cell carcinoma, non-keratinizing squamous cell carcinoma and basaloid squamous cell carcinoma (3, 12). The non-keratinizing subtype is further divided into differentiated and undifferentiated subtypes. Non-keratinizing squamous cell carcinoma and basaloid squamous cell carcinoma are associated with elevated Epstein-Barr virus titres; however, keratinizing squamous cell carcinoma is rarely infected by the virus (2). Undifferentiated nasopharyngeal carcinoma is distinguished from other tumours by its prominent infiltration of lymphocytes (12, 14). The prognostic relevance of this characteristic has been a controversy in the past years. The infiltration by lymphocytes is proven to be beneficial in this particular tumour. The presence of the infiltration may prolong the time to regional metastasis of the tumour to the cervical lymph nodes (14). In endemic areas, more than 95% of NPC are classified as the undifferentiated non-keratinizing squamous cell carcinoma and is universally associated with EBV. In non-endemic areas, 25% are classified as keratinizing squamous cell carcinoma (SCC) and 12% as non-keratinizing differentiated SCC, which are associated with classic head and neck aetiological factors including alcohol and tobacco (1). The remaining 63% of nasopharyngeal carcinomas are classified as non-keratinizing undifferentiated nasopharyngeal carcinoma (3).

Definitive diagnosis of NPC is made by histological evaluation of an endoscopically guided biopsy of the primary nasopharyngeal tumour (6). The non-invasive diagnostic tools include basic history and clinical examination including examination of the cranial nerves and lymph nodes (2, 6). Multiple radiological modalities have been employed in the work-up of NPC including chest X-rays, bone scans, magnetic resonance imaging (MRI), computer tomography (CT) and positron emission tomography. Magnetic resonance imaging is

considered the preferred modality; however, positron emission tomography is being investigated and already is showing high sensitivity (6).

In most nasopharyngeal carcinomas, there is constant expression of Epstein-Barr virus genes encoding Epstein-Barr encoded ribonucleic acid (EBER). Latent membrane protein (LMP) 1 is present in 30% to 50% cases of nasopharyngeal carcinomas and is required for EBV mediated transformation of B-lymphocytes as well as inhibition of epithelial differentiation (12).

Epstein-Barr virus is a Herpes virus that falls into γ-subgroup. There are eight types of human herpesviruses which are divided into three subgroups according to the type of cells they infect and the site of latency. The other subgroups include α-group which infect predominantly epithelial cells and establish their latency in the neurons. Examples of this group includes human herpes simplex type 1 and 2 and varicella-zoster virus. The viruses in the β-group include cytomegalovirus and human herpes virus type-6 and 7 infect various types of cells and establish their latency in various types of cells. The last group, as mentioned above, Epstein-Barr virus and human herpes type-8 infects predominantly lymphoid cells and establish latency in lymphoid organs. Epstein-Barr virus is transmitted by saliva and infects lymphocytes and epithelial cells. The virus usually become latent while expressing eight virus proteins including Epstein-Barr nuclear antigen (EBNA) 1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1 and LMP2 (LMP2A and LMP2B) which promote proliferation and transformation of nasopharyngeal epithelial cells into malignant cells (4, 11).

In most, if not all nasopharyngeal carcinoma, there is constant expression of Epstein-Barr virus genes encoding Epstein-Barr encoded RNA (EBER), Epstein-Barr nuclear antigen 1, latent membrane protein 1, latent membrane protein 2, BamH1-A Reading Frame-1 (BARF) 0, and BARF1. Latent membrane protein 1 and BARF1 are the only ones which are known to be capable of inducing malignant transformation. With this mentioned, these two are considered as viral oncogenes in nasopharyngeal carcinoma. In BARF1 gene, the 21 to 56 sequences of amino acid can induce transformation into malignancy and also activation of B-cell lymphoma (BCL) 2. BCL2 is an anti-apoptotic nuclear factor which provides neoplastic cells with indefinite survival. BARF1 is constantly expressed in approximately 98% of nasopharyngeal carcinomas, therefore, it indicates that this protein plays an important role in the carcinogenesis of the disease. LMP1 is present in 30% to 50% cases of nasopharyngeal carcinomas, and it is known for its B cell immortalization and oncogenic properties. Traits of genetics also play a role that is important in the pathogenesis of nasopharyngeal carcinoma, for example, human leukocyte antigen (HLA) haplotypes namely HLA-B13, -A3, -B5, 15 and allele in HLA-B18 (6, 15).

Other studies performed indicated recurrent genomic alteration including deletion of P16-locus on cytoband 9p21, amplification of cyclin dependent kinase (CDK) 1, mutation of TP53, inactivation of mutation in negative regulators of the pathway of NF-$_K$B, mutation in mitogen-activated protein kinase (MAPK) including Phosphoinositide 3-kinase (PI3K) signaling pathway and mixed-lineage leukemia (MLL) genes 318, 19, 21,an epigenetic regulator, also contributes to the carcinogenesis of the disease (2, 6). Also reported is inactivation of the genes that suppresses the tumour; SPLUNC1, UBAP1, BRD7, Nor1,

NGX6 and LTF (3). Epigenetic alteration, for example microRNA was also found to contribute to the progression of NPC.

Analysis of plasma of quantitative EBV deoxyribonucleic acid (DNA) in high-risk population can be used for screening, prognosis, and treatment response. It can elevate the efficiency of screening in high-risk populations. Besides screening purposes, EBV DNA together with EBV antibodies can be used for early diagnosis, prognosis, and monitoring (16). Definitive diagnosis is confirmed by performing a biopsy of the nasopharyngeal mass followed by histological examination. CT scan and MRI are valuable imaging tool in patients for staging diagnosis of NPC, however, NPC is commonly diagnosed late due to its deep location and vague symptoms. Current sensitive in situ hybridization methods allow for the detection of EBV-encoded small RNAs, in almost 100% of cases of NPC, irrespective of their histologic subtypes (10, 17, 18).

Detecting EBV genomic latent membrane protein and Epstein-Barr virus nuclear antigen in nasopharyngeal swabs can be used to verify nasopharyngeal carcinoma with a sensitivity of 91.4% and specificity of 98.3%. The nasopharyngeal swab coupled with polymerase chain reaction (PCR) based Epstein-Barr virus latent membrane protein and Epstein-Barr nuclear antigen detection could serve as a good supplement to pathological diagnosis of nasopharyngeal carcinoma (9)**.** PCR targeting a repeat sequence within the EBV genome confirmed a significant association between NPC pathology and an elevated level of EBV

DNA in plasma (19). Detection of EBV by quantitative PCR is inexpensive, reliable, and accurate in diagnosing NPC. This can also be used in clinical management (15, 20).

Nasopharyngeal carcinoma is highly sensitive to radiotherapy (12, 14, 21, 22) more especially in the early stages (stage I and II) (6). Radiation is usually given to the primary tumour and to the draining cervical lymph nodes even if metastasis to these nodes is not established (6). In cases of metastatic disease, some consider chemotherapy a preferable modality (21).

Tumours or lesions involving the nasopharynx can be extremely difficult to classify. Infectious lesions, for example mucormycosis can mimic a tumour. Amongst the neoplastic lesions, differentiation between benign and malignant can also be difficult with clinical examination only. All the lesions in the nasopharynx may present with similar or same symptoms. With clinical examination, a little bit more can be elicited to differentiate between benign and malignant neoplasms. Radiological examination, more especially CT and MRI may assist in categorizing infectious, benign, and malignant lesions. These radiologic imaging may show extension to soft tissue which favours a malignant neoplasm (23). In children, the most common neoplasms are benign, however the differential diagnosis of malignant neoplasms includes rhabdomyosarcoma, carcinoma, and lymphoma (24). In adults, the malignant tumour includes adenocarcinoma, neuroendocrine carcinoma, and lymphoma (24).

South Africa is home to the largest Human immunodeficiency virus (HIV) seropositive population in the world, with 7·2 million individuals. Since the country's first case of HIV in 1982, South Africa has come a long way to reduce HIV/ AIDS-related rates of infection, morbidity, and mortality with the use of highly active anti-retroviral therapy (HAART) (7). EBV is associated with the development of both lymphoid and epithelial tumours. The virus contributes to the oncogenesis as evidenced by its frequent detection in certain tumours, namely Burkitt lymphoma (BL), post-transplant B cell lymphomas, Hodgkin lymphoma (HL) and NPC. Polymerase chain reaction was used to determine the relationship between EBV viral load in peripheral blood in HIV infected patients. Ninety-six (96) % of patients who had a CD4 cell count of 100-400 cells/mm^3 were found to have EBV infection (8, 9). The incidence of EBV associated tumours in HIV infected patients was found to be 100 times higher than in the general population (18). In one of the earlier studies performed by Frisch et al., it was revealed that there is no increased risk of NPC seen in patients with HIV/AIDS in the USA (25).

Chapter 4 Materials and Methods

4.1 Study design:

A retrospective study design was used to describe the epidemiology, histological spectrum and the association between Epstein-Barr virus and nasopharyngeal carcinoma.

4.2 Target population:

The target population included all the cases of NPC diagnosed in patients of all ages during the period from 1 July 2013 to 31 December 2019 at the National Health Laboratory Service (NHLS), Histopathology Division of Anatomical Pathology at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and Chris Hani Baragwanath Academic Hospital (CHBAH), Gauteng, South Africa. Laboratories at these two hospitals cover mostly the Southern, Western and Eastern parts of Gauteng.

4.3 Sampling:

The cohort of patients was obtained using Corporate Data Warehouse- based search of the NHLS laboratory information system. Stained slides which had been archived were retrieved and reviewed where needed.

4.4 Ethical considerations:

Ethical approval was obtained from the University of the Witwatersrand Human Research Ethics Committee (clearance certificate number M200433 on 3 June 2020) for this retrospective study (Appendix 2).

4.5 Inclusion and exclusion criteria:

All cases of nasopharyngeal carcinoma which were diagnosed between 1 July 2013 and 31 December 2019 were included in the study. All the cases with an uncertain diagnosis where NPC may have formed part of the final differential diagnosis were excluded.

4.6 Data collection:

Patient data was retrieved from the histopathological reports only and patients were anonymised. This data was recorded on the data collection sheets and then stored in a Microsoft Excel spread sheet. A total of 120 cases were obtained. The following variables were obtained and analysed in each case: age, gender, topographic region of involvement at presentation, type of presentation, type of specimen, HIV status, CD4 cell count, histopathological subtypes and EBER-ISH status where available on the report. The histopathological diagnosis was obtained from histopathology report.

In the cases where the histologic subtype and EBER-ISH results were not obtained from the report, stained slides were retrieved from the archives of the Division of Anatomical Pathology at CMJAH and CHBAH.

4.7 Data Analysis:

Descriptive statistics were performed. Age was presented as mean with interquartile range (IQR) and median.

Categorical data was summarized as frequencies and percentages.

Chapter 5 Results

5.1 Sample size:

There were 121 cases of nasopharyngeal carcinoma diagnosed at the National Health Laboratory Services between the period of 1 July 2013 and 31 December 2019. Two of these cases were for the same patient and were taken two weeks apart. These two cases were therefore combined and described together. A final sample size of 120 cases of nasopharyngeal carcinoma was reached.

5.2 Demographic data:

5.2.1 Age at presentation:

Of the 120 cases analysed, there were 2 cases in which the age was not specified. With the remaining 118 cases, 15 (12.71%) patients were between the age 0-18 years, the youngest being 10 years old. There were 58 (49.15%) patients who presented between 19-50 (young adults) years of age and the remaining 45 (38.14%) patients were above 50 years of age. The oldest age at presentation was 75 years of age. The IQR is 52% and the median age is 43 years.

5.2.1: Age at presentation

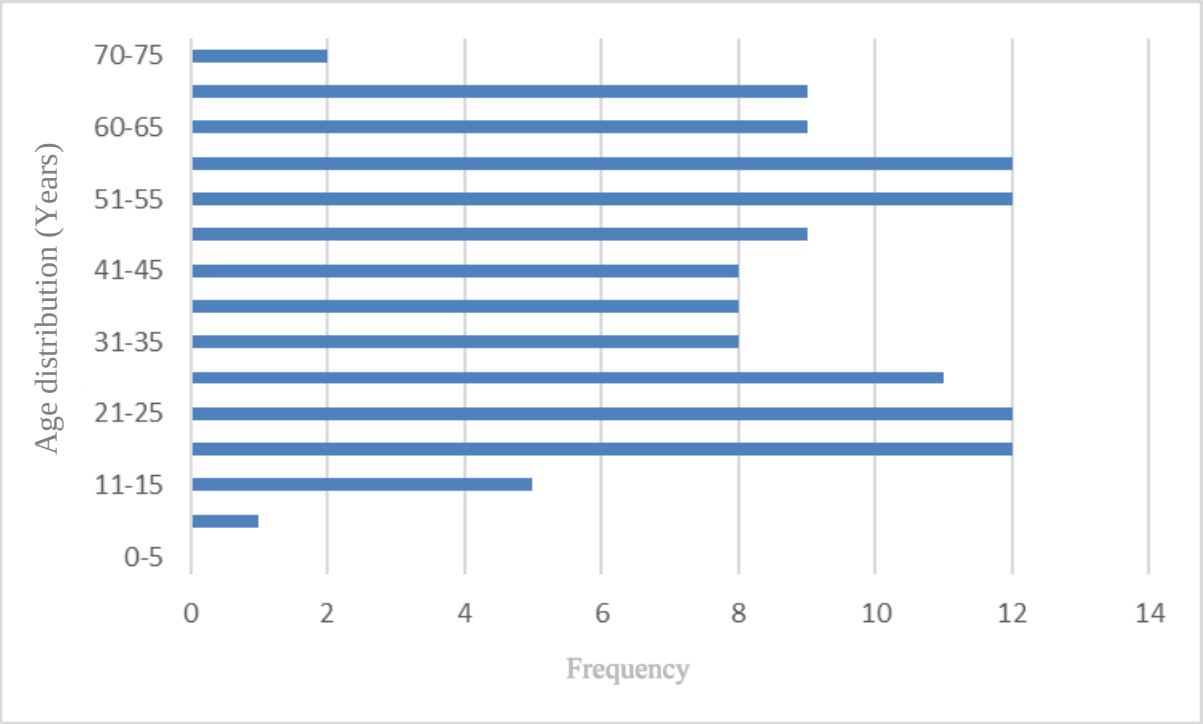

Figure 1: Distribution of age at presentation.

5.2.2 Gender Ratio:

A total number of 77 males (64.17%) and 43 (35.83%) females were diagnosed with nasopharyngeal carcinoma during this study period. The male to female ratio is 1.8:1 for all cases of nasopharyngeal carcinoma.

The cases of males ranged between 10 years and 67 years with a median of 39 years and the cases in females ranged between 12 years and 75 years with a median of 43 years.

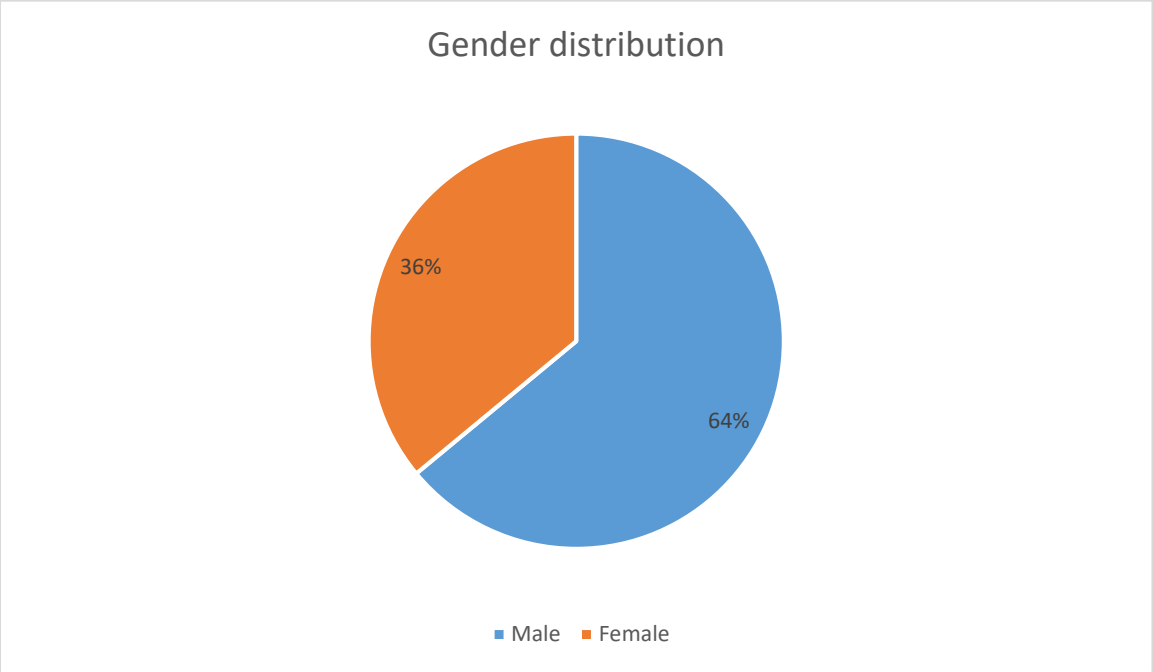

Figure 2: Pie chart illustrating the gender frequency of patients diagnosed with nasopharyngeal carcinoma.

5.3 HIV status and CD4 cell count:

Of the 120 cases, HIV status was only provided in 19 cases on the histological reports obtained. Of these cases, nine (47.37%) patients were HIV positive and the remaining 10 (52.63%) cases were negative for HIV. Amongst the paediatric group, HIV status was provided in two cases, both of whom were HIV negative.

There were three cases in which the CD4 cell count of patients was submitted. The CD4 cell count ranged from 300 to 900 cells/mm^3. The median is 600 cells/mm^3 and the IQR is 45%.

5.4 Site and type of biopsy:

Biopsies were submitted from various sites. In 3 cases, 2 biopsies were submitted simultaneously, bringing the total to 123 different biopsy sites. The most frequently biopsied site was the nasopharynx which constituted 74 (60.12%) cases. In 3 of these cases, a second site was also biopsied. The second site was a lymph node in 2 cases and a parotid gland in 1 case. These 3 cases were seen in the young adult (19-50-year) age group.

A lymph node was the second most common biopsy site with 37 lymph node biopsies submitted. In 35 of these cases, the lymph node biopsy was submitted alone.

The less common sites of involvement included the parotid gland in 5 cases (one with a simultaneous nasopharynx biopsy) and 4 cases in the bone marrow. A skin, subauricular and unspecified soft tissue biopsy site each accounted for 1 case.

Table 1: Frequency of biopsy site:

Site of biopsy	Number of cases	Percentage of cases
Nasopharynx	74	60.12%
Lymph node	37	30.1%
Bone marrow	4	3.3%
Parotid gland	5	4.1%
Others	3	2.4%

In the paediatric group, eight (53.33%) cases presented as primary nasopharyngeal disease. The remaining 7 (46.66%) cases had locoregional involvement or distant metastasis.

Of the 123 biopsies submitted, 101 (82.11%) patients underwent an incisional biopsy, and 22 (17.88%) patients underwent an excisional biopsy. Of the 22 excision biopsies, 17 (80.9 %) were of a lymph node.

In this cohort, most cases presented with a primary nasopharyngeal mass accounting for 74 (60.2%) cases. In 3 of these 74 cases, the presentation was that of a primary nasopharyngeal mass with locoregional involvement or metastasis to a lymph node in 2 cases. There was locoregional (parotid gland) involvement in 1 case. Thirty-seven (37) cases (30.1%) showed

involvement of the lymph nodes, this is uncertain whether it was distant lymph node metastasis or regional lymph node (locoregional) involvement. The remaining 4 (3.3%) cases presented with a metastasis to the bone marrow. In the cases derived from sites other than the nasopharynx, 32 (69.56%) were in males and 14 (30.43%) were in females.

5.5: Histological subtypes (WHO classification):

Of the 120 cases analysed, 110 cases (81.6 %) were classified as non-keratinizing nasopharyngeal carcinoma and there were 2 cases (1.68%) which were classified as keratinizing nasopharyngeal carcinoma. Thirteen cases were reviewed by the researcher and the supervisor to subtype the tumour and state the EBER-ISH results. No cases were reclassified after review. In eight cases (6.72%), slides could not be retrieved for subtyping due to filing errors and these cases were left as "unclassified". There were no cases of basaloid nasopharyngeal carcinoma in the cohort.

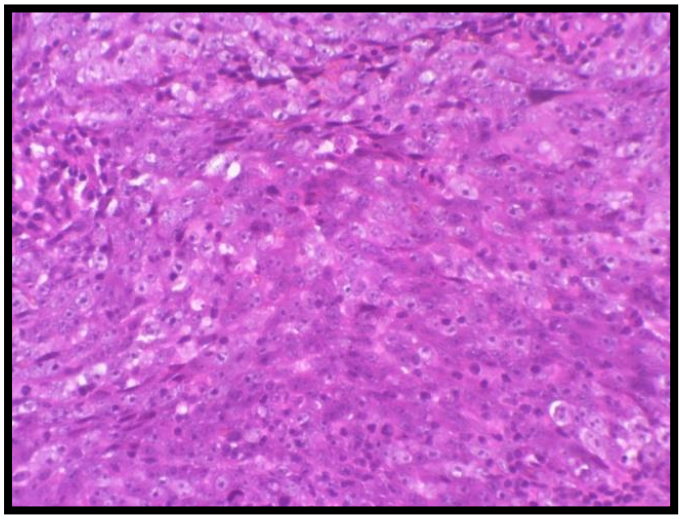

Figure 3: Non-keratinizing nasopharyngeal carcinoma characterised by sheets of epithelial cells intermingled with lymphocytes. (X200 magnification).

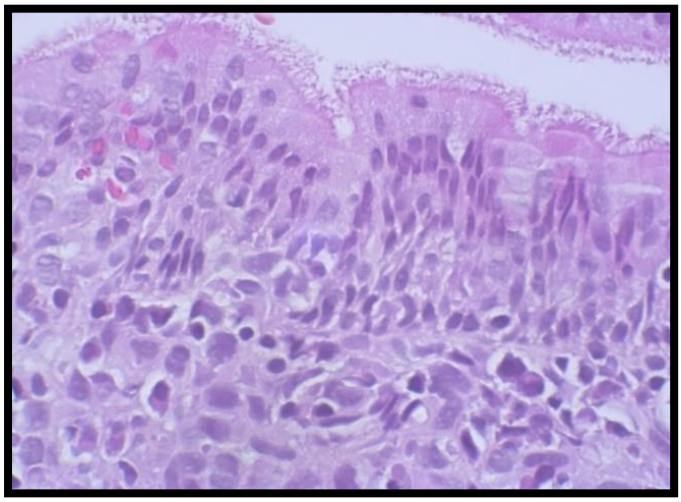

Figure 4: Non-keratinizing (undifferentiated) nasopharyngeal carcinoma arranged in a syncytial growth pattern with hyperchromatic nuclei and overlying respiratory epithelium (X400 magnification).

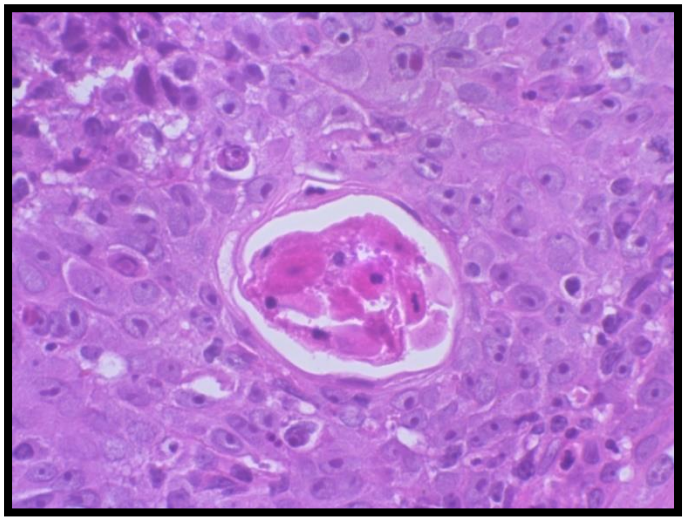

Figure 5: Keratinizing nasopharyngeal carcinoma showing malignant cells with central keratinization (X400 magnification).

Both cases of keratinizing nasopharyngeal carcinomas were seen in adults above 60 years of age (61 and 75 years old respectively).

Of the 15 cases seen in the paediatric group, 14 (93.33%) were non-keratinizing nasopharyngeal carcinoma and 1 (6.66%) could not be classified.

Of the nine HIV positive cases, eight (88.9%) showed a non-keratinizing subtype and 1 (11.1%) case could not be classified. Of the 10 HIV negative cases, 9 (90%) were non-keratinizing and 1 (10%) could not be classified.

5.6 EBER-ISH status:

An in-situ hybridization stain for EBER protein, EBER-ISH, had been performed in 102 cases. Of these 102 cases, 96 (94.1%) were positive for EBER-ISH. The EBER-ISH positive cases occurred in non-keratinizing NPC in 87 (90.6%) of cases, in both (2,1%) the keratinizing NPC and in 7 (7,3%) of the EBER-ISH positive cases the histology was unclassified.

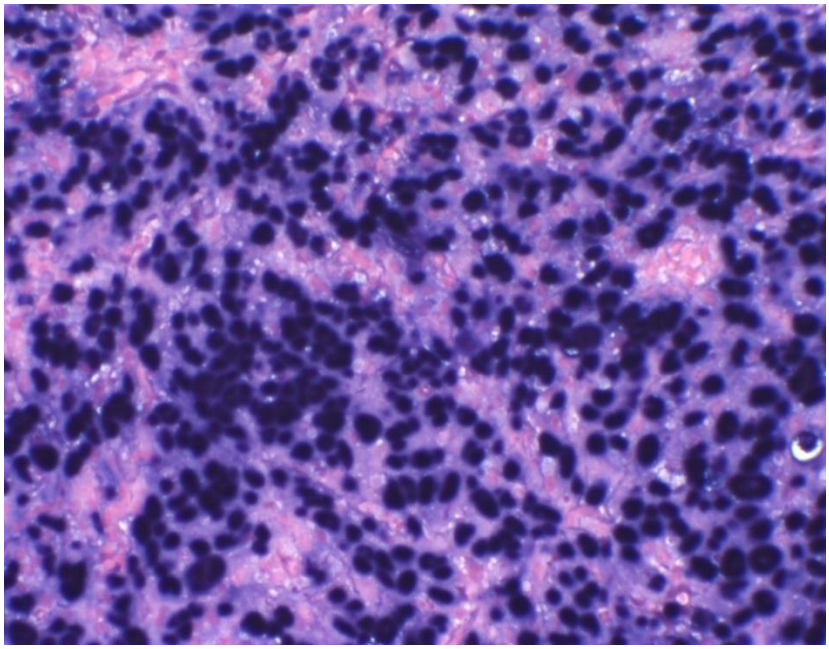

Figure 6: Positive nuclear staining of EBER-ISH in non-keratinizing nasopharyngeal carcinoma.

There were 6 (5.9%) cases of NPC that were negative for EBER-ISH. All these cases (100%) were non-keratinizing NPC.

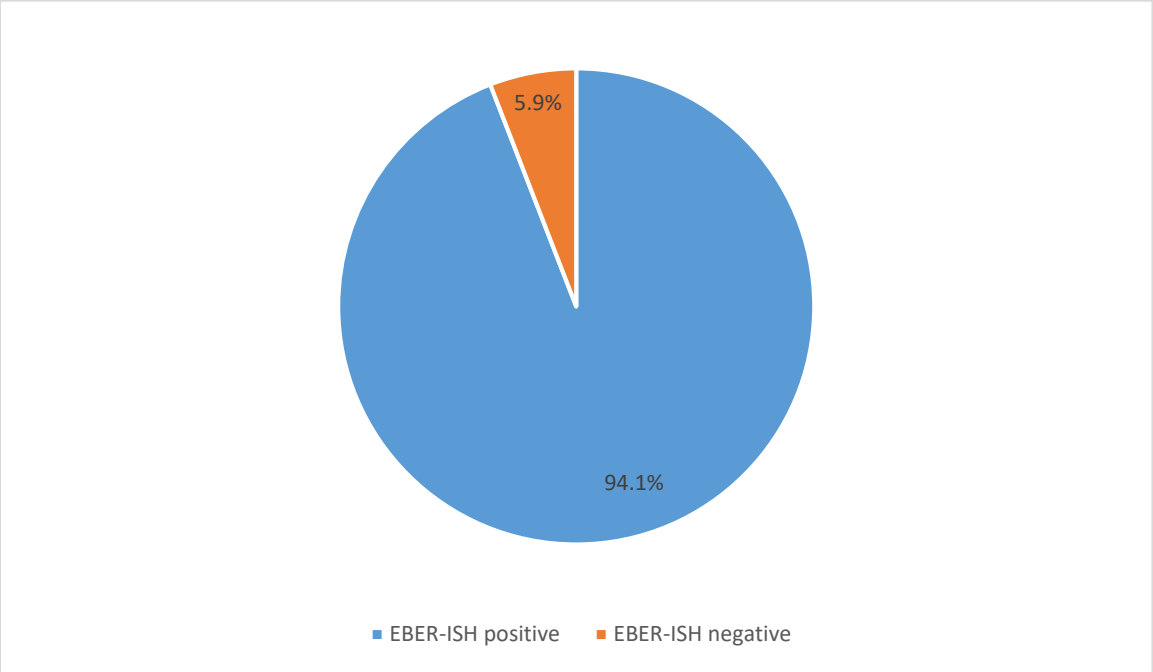

Figure 7: Pie chart demonstrating the number of EBER-ISH positive and negative cases.

Table 2: EBER-ISH results in histological subtypes:

	EBER-ISH results			
	Positive	(%)	Negative	(%)
Non-keratinizing NPC	87	90,6	6	100
Keratinizing NPC	2	2,1	0	0
Unclassified	7	7,3	0	0
Total	96	94.1%	6	5.9%

Of the 15 cases seen in the paediatric group, 14 (93.33%) were EBER-ISH positive. In the remaining 1 case, EBER-ISH was not performed.

Of the 9 HIV positive cases, six (66.6%) were positive for EBER-ISH and in the remaining 3 cases, EBER-ISH was not performed. In all 10 HIV negative cases, EBER-ISH was positive (100%).

Chapter 6 Discussion

6.1 Major findings

The major findings in this study show that South African population has a bimodal pattern of presentation of nasopharyngeal carcinoma which is similar to other parts of the world. The most common histological subtype is non-keratinizing nasopharyngeal carcinoma, and it is highly associated with Epstein-Barr virus.

6.2 Incidence, age, and gender distribution:

In this cohort study, the incidence of nasopharyngeal carcinoma was 120 cases in the six and half-year period (average of 24 cases per year). This is similar to the study performed in India, an endemic area, where 303 cases were identified in the period of 10 years (average of 30.3 cases per year) (26).

Nasopharyngeal carcinoma was seen in patients between 10-75 years of age and there were no cases in children under 10 years old in this study. Vasef et al. and the WHO confirm that the age distribution is wide and is more common in the adult population than in children (3,12).

The most common age of presentation of nasopharyngeal carcinoma (NPC) is between reported 30-50 years of age (3, 4), however, a bimodal distribution of NPC has been reported by Petersson et al. (3). In this study, a bimodal distribution was noted, the first in the 16-25-

year age group and the second in the 51–59-year age group with 24 cases in each of these groups.

In the study done by Laantri et al. (4), their cases revealed NPC involving children from 10 years of age which is the same as in this study where the youngest age of presentation was 10 years.

In this study, nasopharyngeal carcinoma shows a slight predilection for males (64.1%) over females (35.8%). This is in keeping with the data reviewed by Tabuchi et al. (5) which stated that there is a higher incidence in males compared to female. The World Health Organisation also show a male predominance with a male to female ratio of 3:1 (3).

The reason for the higher incidence in males could be due to aetiological factors related to lifestyle. Laantri et al. (4) noted that more males are found to be smokers and consume alcohol compared to females. Smoking and alcohol are known risk factors for NPC (4). The male predominance could change as the number of female smokers increase.

Overall, the demographic data in this cohort from South Africa is similar to that reported in the literature with a wide age range, inclusive of children and a male predominance.

6.3 Site and type of biopsy:

The type of presentation was predominately primary, in which patients presented with a nasopharyngeal mass. There were also a number of cases which presented involving regional spread to sites including the lymph nodes and parotid gland. In several studies, the cervical lymph nodes were most commonly involved (2, 3, 12). In this study, however, the site of the lymph node biopsy was not stated in these cases.

Metastasis to the bone marrow was noted in 3.3%, and this is similar to the data collected by Petersson et al. who reported 5% of cases with metastasis to the bone (3).

Most patients underwent an incisional biopsy compared to excisional biopsy. Chan et al. stated that the definitive diagnosis of NPC is made by histological evaluation of an endoscopically guided biopsy of the primary nasopharyngeal tumour (6). In this study, most of the excision biopsies were performed in those patients with locoregional metastasis to the lymph node (80.9 % of excisions) and all the incisional biopsies were performed in those patients with primary tumour.

6.4 Histological subtypes:

In this study, it was revealed that the most predominant WHO subtype of NPC is the non-keratinizing subtype of NPC. The cases of keratinizing subtype were less than 2% with no cases of basaloid NPC seen at all. This is similar to the data reviewed by Chan et al. who reported a predominance of non-keratinizing nasopharyngeal

carcinoma in an endemic area like Southern China (1). They stated that the non-keratinizing NPC accounts for almost 95% on the NPC cases. The WHO also stated that non-keratinizing NPC is predominant in the non-endemic areas (26). The reason for the low numbers of keratinizing NPC could not be established in this study, although there were cases which were not classified into any group.

This cohort from the Gauteng region of South Africa shows the same distribution of the histological subtypes of NPC to the other parts of the world which are endemic regions.

The basaloid subtype of NPC has a morphological appearance analogous to many other tumours occurring in the head and neck region and has been infrequently reported to occur as a primary nasopharyngeal tumour (26). There were no cases classified as basaloid NPC in this research study and this is in keeping with the other studies from both endemic and non-endemic countries (26). This raises the question as to whether this subtype has been misdiagnosed as other basaloid malignancies in the head and neck region. Of interest, in several other studies, it is stated that it could simply be ignored (26), raising its validity in being included in the WHO classification.

Furthermore, the prognostic value of histological subtypes is not well established. Some studies suggest that the non-keratinizing subtype is less locally advanced compared to the keratinizing subtype and the non-keratinizing subtype has a better prognosis than the keratinizing subtype (26). In this study, there are too few cases of keratinizing NPC to allow for prognostic comparison with the non-keratinizing subtype, should survival data be collected in a subsequent study.

6.5 EBER-ISH status:

Epstein-Barr virus (EBV) was seen in 94.1% of the cases of nasopharyngeal carcinoma in this study. This is in keeping with several other studies (2, 3, 11, 13) that showed that EBV is found in almost all the NPCs, including studies from endemic and non-endemic areas. The study by Chan et al. (1) contradicted these studies and stated that EBV is mostly found in the non-keratinizing and basaloid subtype. As most cases in this study was of the non-keratinizing subtype with no cases from the basaloid subtype, the finding by Chan et al. that EBV is less common in keratinizing NPC cannot be confirmed (1).

The prevalence of EBV confirms that Epstein-Barr virus plays a major role in the carcinogenesis of NPC. However, it is not known whether EBV is the initiating or the driving factor in carcinogenesis. In this study, the other risk factors such as smoking and alcohol use, were not assessed. In those cases, that were negative for EBER-ISH, other risk factors could have played a more significant role.

EBV has been proven to be the present in the majority of cases of NPC. Considering the lack of resources in Africa, this raises the question on the necessity of performing EBER-ISH in all cases of NPC. Based on the findings in this study, I would recommend that if the clinical, radiological, and histological findings are consistent with a nasopharyngeal carcinoma, then there is no need to test for EBER-ISH, however; EBER-ISH is specific and sensitive for NPC when comparing tumours included in the differential diagnosis. The value of EBER-ISH in the context of metastatic diseases or locoregional involvement where an undifferentiated or poorly differentiated squamous cell carcinoma is seen still remains.

6.6 Association of HIV with nasopharyngeal carcinoma and EBER-ISH:

HIV status was not provided in the report in most cases (84.16%) in the study. South African is one of the countries with the highest prevalence rate of HIV (9) and there is established literature that HIV is highly associated with EBV. Other EBV associated tumours such as lymphoma (Hodgkin and non-Hodgkin), and EBV associated smooth muscle tumour are quite common in South Africa (18).

Unfortunately, as HIV status was not stated in many of the reports, the correlation of HIV with EBV and NPC could not be established. This may be due to the lack of awareness of the association of NPC with EBV and therefore possibly with HIV. Similar to HIV, the CD4 cell count was available in only 3 cases and no inferences could be made.

Considering that there is no literature review regarding the association of HIV and NPC directly, this would be of a value in a South African population. Further research into the association of HIV and nasopharyngeal carcinoma should be pursued.

The limitation of this study is that HIV status including the viral load was not stated in most cases. The association of HIV and nasopharyngeal carcinoma was one of the objectives, but this question was not answered in this study. The study design and ethical considerations did not allow for HIV and CD4 cell counts to be obtained in ways other than from the report. This limitation should have been considered and alternative ways of retrieving the HIV status and CD4 results of patients should have been considered and applied for. Further research on the association of HIV and nasopharyngeal carcinoma could be performed by obtaining such data from Corporate Data Warehouse once ethical clearance is obtained.

A take home message of this study is that NPC occurs in South Africa with a bimodal age distribution. Since nasopharyngeal carcinoma has many mimickers, one needs to have a high index of suspicion and where necessary, test for EBER-ISH.

Chapter 7 Conclusion

The overall findings in this study confirm that this cohort of NPC is comparable to NPC from endemic countries in terms of the epidemiology, histological subtypes, and the association with EBV. The finding of 90.6% of EBV positivity in this cohort confirms the association of EBV with NPC in a South African setting.

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Appendix 1: Data collection sheet

Study number:

Age (years):

Gender:

Male	Female	Unknown

HIV status:

Positive	Negative	Unknown

CD4 cell count: _____cells/mm^3

Type of presentation:

Primary	Metastatic	Recurrence

Type of specimen:

Incisional biopsy	Excision

Site of biopsy:

Nasopharynx	Lymph node	Other: _____

Histological subtype:

Keratinizing	Non-keratinizing	Basaloid	Cannot be determined

EBER-ISH:

Positive	Negative	Unknown

If recurrence or metastasis, time interval since first diagnosis (months): _____

Appendix 2: Ethics clearance letter

WITWATERSRAND
JOHANNESBURG

R14/49 Dr Mbuyelo Mabaso

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M191022

NAME: (Principal Investigator)	Dr Mbuyelo Mabaso
DEPARTMENT:	Anatomical pathology National Health Laboratory Services
PROJECT TITLE:	The Association between Epstein-Barr Virus and nasopharyngeal carcinoma
DATE CONSIDERED:	25/10/2019
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Dawn-Lee van der Byl
APPROVED BY:	Dr. C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	01/11/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **October** and will therefore be due in the month of **October** each year. Unreported changes to the application may invalidate the clearance given by the

Appendix 3: Turnitin report

Turnitin Originality Report

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