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Phillips Adekoyejo Abiodun

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PROGNOSTIC SIGNIFICANCE OF PHH3, KI-67 AND BCL-2 IN PROSTATE CANCER

BY

DR. ADEKOYEJO ABIODUN PHILLIPS

(STUDENT No: 1688243)

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF MASTER OF SCIENCE IN MEDICINE (ANATOMICAL PATHOLOGY) AT THE UNIVERSITY OF THE WITWATERSRAND

JUNE, 2018

DECLARATION

I declare that this project was carried out by me, Dr. Adekoyejo Abiodun Phillips under appropriate supervision. This dissertation has not been presented to any other examining body, and has not been submitted elsewhere for publication. The findings and opinion expressed in this work are entirely mine, except where due acknowledgment/references have been made, and do not represent the views of the University of the Witwatersrand.

07/06/2018

ADEKOYEJO ABIODUN PHILLIPS

CERTIFICATION

This is to certify that the study in this dissertation "Prognostic significance of PHH3, Ki-67 and Bcl-2 in prostate cancer" was carried out under my supervision

Dr. M. Louw

Consultant Pathologist and Senior Lecturer

Department of Anatomical Pathology,

University of the Witwatersrand

Signature _____ 21/06/2018

DEDICATION

This work is dedicated to my family, trainers, consultants, colleagues and all prostatic adenocarcinoma patients world-wide.

ACKNOWLEDGMENT

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I am very grateful to Dr. Melanie Louw, Prof. M. Hale and Prof. M. Altini for their continuous support and encouragement.

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LIST OF ABBREVATIONS

1. AMACR	Alpha-Methylacyl-CoA Racemase
2. Bak	Bcl-2 homologous antagonist killer
3. Bax	Bcl-2 associated X protein
4. Bcl-2	B-cell lymphoma 2
5. Bcl-xl	B-cell lymphoma-extra large
6. Bim	Bcl-2-like protein 11
7. BRCA gene:	Breast cancer gene
8. CK 5/6	Cytokeratin 5/6
9. DLX-2	Distal-less homeobox 2 gene
10. DPX mountant	Distyrene, Plasticizer and Xylene mountant
11. EAU	European Association of Urology
12. ER	Estrogen receptor
13. ERG	ETS-related gene
14. ETS	Erythroblast-Transformation Specific
15. FASN	Fatty Acid Synthase
16. FFPE	Formalin-fixed paraffin-embedded
17. HER-2	Human epidermal growth factor receptor 2
18. ISUP	International Society of Urological Pathology
19. NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
20. p63	Transformation-related protein 63
21. p53	Transformation-related protein 53
22. PHH3	Phosphohistone H3
23. PMA	prostate carcinoma mucin-like antigen
24. PR	Progesterone receptor
25. PTEN	Phosphatase and tensin homolog
26. PSA:	Prostate specific antigen
27. PSMA	Prostate-Specific Membrane Antigen
28. RB gene	Retinoblastoma gene

ABSTRACT

INTRODUCTION: Prostate cancer is the second most common cancer in men and it is a leading cause of cancer death. In 2012, 1.1 million new cases were diagnosed worldwide, and it accounted for 15% of cancer cases diagnosed. Prostate cancer can be managed either by active treatment or by watchful waiting/ active surveillance. Prognostic factors associated with prostate cancer include Gleason score, age, extracapsular invasion, seminal vesicle invasion, heterogeneity, prostate specific antigen (PSA) assessment and biomarkers. The Gleason score has been described as a quintessential prognostic factor in prostate cancer. Like other prognostic factors, the Gleason score has been modified to improve its reproducibility. The modified Gleason grading system is still subjective in nature although not as much as it was before the 2014 International Society of Urological Pathology (ISUP) modification. Therefore, a need exists to introduce a more objective and reliable method for predicting prognosis in prostate cancer. The use of biomarkers could provide a more reliable approach for the prediction of prognosis in prostate cancer. Some studies have linked the outcome of prostate cancer to genes and proteins involved in cell proliferation and apoptosis.

METHODOLOGY: This is a retrospective study that was done on prostatic cancer specimens received in 2014 in the Department of Anatomical Pathology at the University of the Witwatersrand. The age of patients was retrieved from the Trackcare system. The formalin-fixed paraffin-embedded (FFPE) blocks were retrieved from the departmental archive and the Gleason score were determined by the primary investigator and the supervisor using the 2014 ISUP modification of the Gleason score. Intra-observer and inter-observer reproducibility were then assessed. The proliferative activities of the tumours were assessed using Ki-67 and phosphohistone H3 (PHH3), and the apoptotic activity was assessed using B-cell lymphoma 2

(Bcl-2). Statistically significant association and correlation between ISUP grade groups and the biomarkers were determined using Spearman's correlation, Pearson's correlation and Pearson's chi square. Benign prostatic hyperplasia cases were used as negative control in the study.

RESULT: The 60 – 69 age group was the most affected age group and mean age was 64.7 years. A common morphological observation in the study was the presence of a loose, oedematous, myxoid stroma around malignant glands. ISUP grade group 1 was the most seen ISUP grade group. The intra-observer and inter-observer agreement as measured by Cohen kappa were 87.88% and 93.94% respectively. The proliferative markers, Ki-67 and PHH3, had statistically significant correlation with the ISUP grade group (p-value = <0.05). Similarly, Bcl-2 showed statistically significant correlation with ISUP grade group. Ki-67 and Bcl-2 also showed significant correlation.

CONCLUSION: Ki-67, PHH3 and Bcl-2 biomarkers are of prognostic significance in prostate cancer. However, there needs to be a consensus meeting to determine what value should be used as a cut-off before this may be introduced into clinical practice.

Keywords: Ki-67, PHH3, Bcl-2, prognosis, prostate cancer

CHAPTER ONE

INTRODUCTION

Prostate cancer is the second most common cancer in men [1]. It is a leading cause of cancer related death; accounting for about 307,000 deaths worldwide in 2012. In the same year, 1.1 million new cases were recorded worldwide, and it accounted for 15% of cancer cases diagnosed in men [1].

Prostate cancer can be managed either by active treatment which can be in the form of androgen therapy, radical prostatectomy or brachytherapy or by watchful waiting/ active surveillance. Both forms of management, active treatment and active surveillance, are associated with some degree of decreased quality of life, this is worse in patients undergoing active treatment [2]. Some studies have suggested that untreated, localized low-grade prostate cancer may pose limited risk to patient life. [3,4] It is important to determine patients with innocuous cancer and those with clinically aggressive cancer [5]. Individuals with a germline mutation in the breast cancer (BRCA) gene are at increased risk of developing early-onset prostate cancer which is clinically more aggressive.

Prognostic factors associated with prostate cancer can be divided into clinicopathological factors and biological factors like biomarkers, PSA assessment etc [6]. The clinicopathological factors are those assessed by physical examination, radiological examination, and histological examination. These factors include but are not limited to Gleason score, age, extracapsular invasion, seminal vesicle invasion, heterogeneity [6]. The Gleason score has been described as a quintessential prognostic factor in prostate cancer. It predicts recurrence, biochemical failure and distant metastasis [7]. The Gleason score is derived by summing up the primary (predominant) Gleason grade and secondary (less predominant) Gleason grade observed in a tumour. There are

five Gleason grades; these grades are based on the architectural growth pattern of the tumour, which is assessed on low power (x4 objective).

Like many other grading systems, the Gleason grading system has undergone some modifications notably in 2005 and 2014. The last modification was done during the International Society of Urological Pathology 2014 consensus conference [8]. At the meeting, the architecture of each of the Gleason grade patterns was redefined with emphasis on Gleason grade 3 pattern and Gleason grade 4 pattern. The consensus on the Gleason grade patterns was aimed at reducing intra-observer and inter-observer reproducibility. Also, at the meeting, a new grading system based on the 2014 modification of the Gleason grade was introduced; the new grading system has 5 grades which are designated ISUP grade group 1 to 5. Clinically, the grade group correlates with prognosis better than the previous grading system. Furthermore, it eliminates the false impression that a patient with Gleason score 6 tumour has an intermediate-grade tumour rather than a low-grade tumour. Despite the 2014 modification of the Gleason grades, the problem of inter-observer and intra-observer reproducibility persist although not as much as it was before the modification especially amongst pathologists-in-training and young pathologists. Therefore, a need exists to introduce a more objective and reliable method for predicting prognosis in prostate cancer. The use of biomarkers may provide such a method with higher reliability. Unlike some other cancers grading systems, the prostate cancer grading system does not consider the use of biomarkers although some studies have linked the outcome of prostate cancer management to genes and proteins involved in cell proliferation and apoptosis.

A tumour proliferation index can be determined with immunohistochemical stains like Ki-67 and PHH3. Ki-67 is a protein that is important in cell-cycle regulation; it is expressed in all the phases of cell cycle except in the G_0 resting phase. In prostate cancer, Ki-67 has been described

as the most promising immunohistochemical biomarker [9]. It has been studied extensively and shown to have prognostic significance in prostate cancer [9,10]. PHH3 is a constituent of the human chromatin structure that is phosphorylated during chromatin condensation in mitosis. The phosphorylation occurs exclusively during the late G2 and mitosis phase of the cell cycle, thus it provides a stricter assessment of mitotic activity than Ki-67. Like Ki-67, it has been proven to be of prognostic significance in prostate cancer. [11] However, several studies have concluded that these biomarkers of proliferation are not of prognostic significance in prostate cancer. [12,13,14]

Likewise, immunohistochemistry can be used to determine the apoptotic markers like transformation-related protein 53 (p53), Bcl-2 and Bcl-2 associated X protein (Bax) in prostatic cancer specimen. Bcl-2 is a protein that suppresses apoptosis and it is encoded by the Bcl-2 oncogene. It has been postulated that its over expression in prostate cancer leads to prolonged survival of tumour cells which increase net tumour and facilitate the progression of prostate cancer. Studies done on the prognostic significance of Bcl-2 in prostate cancer have yielded an equivocal result. [15,16,17] Some studies have concluded that it is of prognostic significance while other studies have refuted this claim.

Considering the contrasting results from these studies and the benefit to patient management if these biomarkers could accurately and reliably predict outcome of management, there exist a need to further study the prognostic significance of proliferation and apoptotic markers in prostate cancer.

CHAPTER TWO

LITERATURE REVIEW

2.1 EPIDEMIOLOGY

2.1.1 GLOBAL

According to the 2012 GLOBOCAN, prostate cancer is the fourth most common cancer in both sexes combined and the worldwide 5-year prevalence is 3.85 million cases. [1] The incidence and mortality vary from region to region, North America and Australia have the highest incidence with an age standardized rate (ASR) of 97.2 and 111.6 per 100,000 men respectively. Similarly, the incidence rate is high in other developed parts of the world like Europe. The high incidence rate has been attributed to a well-developed cancer screening programme in these regions. The incidence is relatively low in less developed regions; Northern Africa, Eastern Asia and South-Central Asia have incidence rate of 10.5, 10.5 and 4.5 per 100,000 respectively. [1] On the contrary, some less-developed regions like Caribbean and, Southern Africa have an incidence rate similar to those of developed countries. In terms of mortality, the world-wide mortality rate is about 10 per 100,000. Like the incidence rate, it also varies with region; the mortality rate is least in Asia and highest in Africa and Caribbean region. The mortality rate in America falls between those of Asia and Africa. [1]

2.1.2 AFRICA

Although Africa has been categorized as a low incidence region for prostate cancer, several publications have argued that African men suffer disproportionately from prostatic cancer when compared to men from another region of the world. Within Africa, the disease burden is more in sub-Saharan Africa than North Africa. Genetics, nutrition and level of poverty are some of the

reasons attributed to the disparity in Africa. [18] In 2015, Adeloye and associates carried out a meta-analysis study to determine the incidence of prostate cancer across Africa. The study involved 40 studies in 16 African countries. The incidence ranged between 0.38 to 182.5 per 100,000 men and the continent-wide pooled incidence was 21.95 per 100,000 men. Furthermore, the study showed that the incidence has been increasing; it rose form 2.6 – 16.9 per 100,000 in 1990 to 4.7 – 38.1 per 100,000 in 2007. [18] This increase in incidence across Africa was also confirmed in another study done by Parkin *et al.* [19] In their study, 29,663 cases of prostate cancer were diagnosed in 2002 and the continental age-standardised incidence rate was 16 per 100,000. Southern Africa had the highest incidence rate with 40.5 cases per 100,000 and North Africa had the lowest incidence rate with 5.8 cases per 100,000. East Africa, Central Africa and West Africa had incidence rate of 13.8, 24.5 and 19.3 cases per 100,000. The study also reported increased incidence rate in South Africa, Uganda and Nigeria.

2.1.3 SOUTH AFRICA

The health burden of prostate cancer in South Africa is quite large. According to Babb *et al.* the percentage of cancer among South African men attributed to prostate cancer rose from 7% in 1986 to 17% in 2006. [20] The total number of cases reported during the 20-year period was 63,886. The age-standardised incidence rate rose from 17 cases per 100,000 at the beginning of the study in 1986 to 27 cases per 100,000 at the end of the study in 2006. The mean age of diagnosis was 68 years. Prostate cancer is the second most common cause of cancer death after lung cancer. Like the incidence rate, the mortality rate has been increasing for the last 10 years. In 1999, 1954 deaths were attributed to prostate cancer and by 2009 the number of death had risen to 2332. In 2009, the mean age of death was 74 years.

2.2 MANAGEMENT

Prostate cancer is a heterogeneous disease with variable treatment outcome. The treatment options for prostate cancer depend on specific guidelines e.g. European Association of Urology guidelines. In turns, these guidelines depend on certain parameters like the Gleason score, clinical stage of the tumour, patient's life expectancy, presence of symptoms and patient's opinion. [21] Some of the available treatment options include watchful waiting / active surveillance, radiation therapy, radical prostatectomy, hormonal therapy, cytotoxic therapy and combination of therapies.

Patients with low-risk prostate cancer, which is defined as PSA less than 10ng/mL, Gleason score 6 or clinical stage T1c – T2a, are given the option of watchful waiting, radical prostatectomy and radiation therapy. [22] Intermediate risk prostatic cancer patient (PSA of 10.1 – 20.0 ng/mL, Gleason score 7 or clinical stage T2b-c) are managed in an interdisciplinary setting which comprises urologists and radiation oncologists. The treatment modalities for intermediate risk prostatic cancer include radical prostatectomy and radiation therapy. [22] Patients with high-risk prostatic cancer, which is defined as PSA above 20.0 ng/mL, Gleason score 8-10 or clinical stage T3a and above, are offered neoadjuvant and adjuvant treatment options in a multidisciplinary tumour board. The aforementioned treatment options are associated with significant decrease in the quality of life. [2] Radical prostatectomy may be complicated by erectile dysfunction, post-operative infection and excessive bleeding. Complications associated with radiation therapy include radiation-induced proctitis, recurrent bleeding and erectile dysfunction. [23] Lastly, side-effects of hormonal therapy include hot flushes and erectile dysfunction. [23] Thus, it appears watchful waiting / active surveillance may be the therapeutical option associated with the least risk or complication.

According to data from the Cancer of the Prostate Strategic Urologic Research Endeavor (CaPSURE) Registry, the percentage of patients diagnosed with low-risk prostate cancer increased from 29.8% to 45.3% between 1989 and 1992. [2] The increase in incidence is probably due to the PSA-based prostate cancer screening program which has led to detection of cancer in early stage of progression. Thus, it can be inferred that low-risk prostate cancer with Gleason score 6 and below and clinical stage T1c – T2a will continue to increase and the watchful waiting would be the most common treatment option in the near future. As stated above, the diagnosis of this subset of prostate cancer requires the Gleason score which is subject to inter-observer and intra-observer reproducibility [24] especially among trainee pathologists and young pathologists. Inter-observer and intra-observer reproducibility may lead to under or over grading of prostate cancers which may result in serious consequences. For example, a patient with a Gleason score 6 tumour which comprises only single well-differentiated glands (Gleason grade 3) maybe misinterpreted as a Gleason score 7 with predominantly well-differentiated glands and a small focus of fused-glands by a young pathologist with modest experience in prostate pathology. In this case, the patient would fall into the intermediate-risk prostate cancer group instead of low-risk prostate cancer group and consequently the patient would not be offered active surveillance as a treatment option which is associated with lesser or no complication. Hence, there is a need to introduce an ancillary technique to support Gleason scoring system for pathologist-in-training and young pathologists with modest experience in prostate cancer.

2.3 GLEASON SCORE

The Gleason score is a quintessential prognostic tool, for the pathologists and urologists, in the management of prostate cancer. It predicts biochemical failure, outcome of radical

prostatectomy, local recurrence, lymph node metastasis as well as distant metastasis. The Gleason score was introduced by Donald Gleason in 1966 and refined by Mellinger in 1977. [25] Since 1977, it has undergone two other major modifications in 2005 and 2014 by the International Society of Urological Pathology (ISUP). With respect to prostatectomy specimen, the Gleason score is derived by adding the primary Gleason grade with the secondary Gleason grade. If a tertiary grade exists, it is not added to the score, but it is mentioned as a comment in the report. When a prostatectomy specimen has multiple nodules with different Gleason score, each dominant nodule must be assigned a separate Gleason score. As regards core needle biopsy, the Gleason score is a combination of the highest Gleason grade and the primary Gleason grade which is the predominant Gleason grade.[25] When separate cores from a specimen show different Gleason scores, each core should be assigned a different Gleason score.[25]

At the 2014 ISUP consensus meeting, the five Gleason grades were re-defined, and the various components of each grade are as follow [8]

- The Gleason grade 1 pattern represents a well-circumscribed mass of round glands with uniform shape, size and spacing.
- Gleason grade 2 pattern represents a group of uncircumscribed glands with uniform shape, size and spacing.
- Gleason grade 3 pattern lacks circumscription, it comprises discrete glands with different sizes and shapes. Other features of Gleason grade 3 pattern include; branching glands and back-to-back discrete glands.
- Gleason grade 4 pattern comprises small to large cribriform glands with well-formed lumina or slit-like lumina; glomeruloid structures and fused glands. Other features include poorly-formed glands with peripherally arranged nuclei.

- Gleason grade 5 pattern represents single cells; nest or cords of cells with or without central area of necrosis; nests or cords of cells with vaguely-formed lumina. Other features include sheets of cells with or without rosette formation and cribriform glands with central areas of necrosis.

The new grading system, ISUP grading system, introduced with the 2014 Gleason modification uses the above architectural pattern. The five ISUP grades are defined below. [8]

- ISUP grade group 1: Gleason score 6 (3+3) i.e. tumours with only architectural patterns of Gleason grade 3. [8]
- ISUP grade group 2: Gleason score 7 (3+4) i.e. tumours with predominantly Gleason grade 3 architectural patterns and a minor component which comprises Gleason grade 4 architectural patterns.[8]
- ISUP grade group 3: Gleason score 7(4+3) i.e. tumours with predominantly Gleason grade 4 architectural patterns and a minor component which comprises Gleason grade 3 architectural pattern.[8]
- ISUP grade group 4; Gleason score 8 which can be a combination of the following patterns [8]
    - Gleason score 8 (4+4) i.e. tumour which consists of only Gleason grade 4 architectural pattern
    - Gleason score 8 (3+5) i.e. tumours with predominantly Gleason grade 3 architectural patterns and a minor component which comprises Gleason grade 5 architectural patterns.

- Gleason score 8 (5+3) i.e. tumours with predominantly Gleason grade 5 architectural patterns and a minor component which comprises Gleason grade 3 architectural patterns.

- ISUP grade group 5: Gleason scores 9 and 10 which can be a combination of the following patterns [8]
  - Gleason score 9 (4+5) i.e. tumours with predominantly Gleason grade 4 architectural patterns and a minor component which comprises Gleason grade 5 architectural patterns.
  - Gleason score 9 (5+4) i.e. tumours with predominantly Gleason grade 5 architectural patterns and a minor component which comprises Gleason grade 4 architectural patterns.
  - Gleason score 10 (5+5) i.e. tumour which consists of only Gleason grade 5 architectural pattern.

2.4 BIOMARKERS

Biomarkers are a group of metabolites and chemical agents which are detected in the body fluids, genes and proteins within tissues. The National Cancer Institute of the United States of America defined it as "a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process or of a condition or disease." [26] The functions of biomarkers in oncology maybe categorised into diagnostic [27], pharmacodynamic [26], predictive [26] and prognostic. [26]

- Diagnostic biomarkers: this group of biomarkers helps to establish the diagnosis of cancer and differentiate common mimickers from cancer. A typical example of

biomarkers in this group are basal cell markers (Transformation-related protein p63 [p63] and Cytokeratin 5/6 [CK 5/6]). In prostate cancer, diagnostic biomarkers may be further subclassified into [27]

  - Positive markers of malignancy in primary carcinoma: some of the biomarkers in this sub-group are prostate carcinoma mucin-like antigen (PMA), alpha-methylacyl-CoA (AMACR) racemase and fatty acid synthase (FASN)
  - Markers of prostatic origin: examples in this sub-group include prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA) and prostein.

- Pharmacodynamic biomarkers: these biomarkers are often used in the development of novel anticancer medication. They predict the immediate effect of drugs in cancer patients. [26]
- Predictive biomarkers: this group of biomarkers assesses the benefit of a specific treatment method. [26]
- Prognostic biomarkers: one of the main purposes of this class of biomarkers is to encourage individualised therapy. [27] The application of individualised therapy in prostate cancer will help to determine which patient gets aggressive therapy or watchful waiting. Prognostic biomarkers may serve other functions which include predicting the course of a cancer. Prognostic biomarkers for prostate cancer may be sub-divided into [27]
  - Genomic markers: TMPRSS2-ERG rearrangement
  - Epigenetic alteration: gene methylation, histone modification and microRNA expression

- Protein biomarkers (immunohistochemistry): In histopathology, this is the most popular sub-group of prognostic biomarkers. Proteins that are assessed depend on the malignancy. For prostate cancer, the common ones assessed include p53, Bcl-2, PHH3 and Ki-67. Despite their popularity in prostate cancer, protein biomarkers have not been incorporated into the clinical management of prostatic cancer. Some of the reasons for this are issues pertaining to interference from other factors like tissue handling and fixation as well as scoring of immunohistochemistry stains. [27]

2.5 CELL COUNTING IN IMMUNOHISTOCEMISTRY

Cell counting is an important process in assessing the proliferative and apoptotic index of tumours. Overestimation or underestimation of these indexes greatly affects the research data, and this subsequently affects the overall quality of research work. Different methods have been used to asses proliferative and apoptotic index in research. The methods include eye-balling, automated counting, eye-counting with microscope and manual counting of captured images or printed images. [28]

- Eye-balling: this method is primarily based on estimation. A researcher scans and estimates the number of stained cells on an intermediate-power microscopic field (x 100 magnification). The proliferative or apoptotic index is calculated by dividing the number of stained tumour cells by the total number of tumour cells which is then expressed in percentage. The numbers of cells are not actually counted in this method.

  This is the most common method of cell counting. [28] The advantages of this method include ease of use, low cost as there is no additional cost and no impact on turnaround

time. The demerit of this method is inaccuracy in result since it is based on estimation rather than counting.

- Eye-counting with microscope: this method requires the researcher to count the stained cells in real time. Firstly, the slide is scanned at low-power to identify "hot spots" which are areas with the highest concentration of stained cells. Afterward, the stained tumour cells and total number of tumour cells (both stained and unstained) are counted at high-power (x400 magnification) and expressed in percentage to calculate either the proliferative or apoptotic tumour cells.

  The merits of this method include accuracy, ease of reproducibility, no additional cost and low possibility of counting non-target cells. The demerit of this method is low practicability especially in non-research setting.

- Automated counting method: this method requires the use of an automated cell image cytometer. An entire slide is scanned by an automated scope at x 40 magnification and the digital image is uploaded on a computer. Tumour hot spots are selected by a technician and the proliferative or apoptotic index is calculated.

  The advantage of this method is accuracy. One of the drawbacks of this method is cost; the automated scan cost between $50,000 and $150,000. [28] Other drawbacks include dependence on technician availability and high probability of counting non-target cells. [28]

- Manual counting of camera-captured / printed image: ethod requires scanning of a slide with a microscope at intermediate power to detect hot spots. Afterward, a digital image of the hot spot is captured and printed out on paper. The stained-tumour cells are the marked with a pencil or marker and counted. The proliferation or apoptotic index is calculated.

The advantages of this method are high accuracy, high reproducibility and low probability of counting non-target cells. In terms of costs, it is relatively expensive when compared to the eye-balling and eye-counting with microscope which requires no additional cost. However, when compare to the automated system it is quite affordable. The additional cost for this method is between $500 and $15,000 [28] which is the cost of a digital camera and a printer.

2.6 FALSE POSITIVE AND FALSE NEGATIVE RESULT IN IMMUNOHISTOCHEMISTRY

Accurate identification and characterisation of the molecular signature of tumour cells is very important in modern-day pathology practice because some of these molecular signatures hold important prognostic information. Furthermore, targeted therapies rely on proper identification of these molecular signatures. Immunohistochemistry is the most widely used method to determine the molecular phenotype of tumour cells. [29] Like most other procedures and techniques in laboratory medicine and research, immunohistochemistry is prone to the problem of false positivity and false negativity.

2.6.1 FALSE NEGATIVE RESULT – REDUCED SENSITIVITY

False negativity in immunohistochemistry means that the antigen of interest is present within the tissue however it is not detected during the procedure. In clinical practice, this may imply that a patient will be undertreated or get a wrong form of therapy. For instance, a breast cancer patient with a false negative estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) receptor result will most likely be offered chemotherapy rather than hormonal therapy or Herceptin which are the appropriate lines of management if the tumour

is truly ER positive. Consequently, remission will most likely not occur in this patient due to a false negative result. Common cause of false negative result in immunohistochemistry include [29,30]

- Over-diluted antibodies or improperly optimised antibodies.
- Inappropriate antigen retrieval technique or inadequate antigen retrieval technique for a particular antigen.
- Paucity of antigen for antibodies resulting in lack of detection by light microscopy.
- Conformational changes in epitope leading to lack of binding sites for antibodies.
- Inaccessibility of antibodies to epitope in concealed locations.
- Degradation of molecules during storage.
- Destruction of antigens during tissue processing: tissues are subjected to multiple steps during tissue processing and antigen destruction may occur during the process. Possible causes of antigen destruction include; inappropriate fixation of tissue, the pH of the fixative and the duration of fixation.

2.6.2 FALSE POSITIVE RESULT

In immunohistochemistry, a false positive result implies that antigen that is not present in a tissue is being interpreted or shown as present. Like a false negative result, it may lead to mismanagement of patient. The causes of false positivity in immunohistochemistry may be divided into immunological and non-immunological. [29]

Immunological causes include

- Similarity in antigen of interest with an unintended antigen which may result in antibody binding to the unintended antigen especially if it has a higher affinity for the antibody. The similarity may be due to structural homology and binding affinity. [29]
- Post-translation modification of an unintended antigen.

Non-immunological causes include [29,30]

- Highly-expressed endogenous peroxidise which are unrelated to the antigen-antibody reaction but sufficient enough to produce a positive signal.
- Highly-expressed biotin sufficient enough to produce a positive signal unrelated to an antigen-antibody reaction.
- Formalin pigment which can be mistaken for positive signals.
- Drying artefact.
- Antigen leakage into different tissue compartment.
- Overexposure of tissue to antigen-antibody reaction.
- Inappropriately high concentration of antibody.

2.7 Ki-67

Ki-67 is a protein that is important in cell-cycle regulation; it is expressed in all the phases of cell cycle except in the G_0 resting phase. In prostate cancer, Ki-67 has been described as the most promising immunohistochemical biomarker [9]. Its prognostic significance has been studied in core needle biopsy specimens and radical prostatectomy specimens as well as in conservatively

managed patients and radically managed patients. In a study done by Fisher *et al*, [9] it was observed that the Ki-67 proliferation index correlated significantly with the Gleason score. Although the study did not make use of the ISUP grade group system, because the grade group system had not been introduced before the study, 68.7% of the specimens with Ki-67 proliferation index of >20% had a Gleason score greater than 7. Inversely, 77.2% of specimens with Ki-67 proliferation index of <5% had a Gleason score of 7 and below. In the same study, Ki-67 was also a significant predictor of prostate cancer death. Ki-67 is also known to predict tumour progression, treatment failure and survival. Although there has been little consensus on the cut-off point for Ki-67 proliferation index, this study used a cut-off of 10%. It correlated significantly with Gleason score, nodal metastasis and serum prostatic specific antigen levels.

In another study done by Mitra *et al*, [10] increase in Ki-67 proliferation index had a positive correlation with aggressive prostate cancer and higher Gleason score. In this study, prostate cancer specimens with and without the BRCA mutation were used and both cohorts were divided into tumour with Gleason score greater than 7 and Gleason score 7 and below. Immunohistochemistry staining was scored in this study by calculating the positive cell index (PCI), the PCI ranged from 0-90%. Two cut-offs, 3.5% and 7.1%, were used to assess Ki-67 proliferation index in this study. When 3.5% was used as the cut-off, 91% of specimens from the BRCA mutation cohort with Gleason score greater than 7 had a Ki-67 proliferation index of >3.5% while only 33.4% of the BRCA mutation cohort with Gleason score of 7 and below had a Ki-67 proliferation index above 3.5%. In the cohort of prostate cancer without BRCA mutation, 84% of the specimens with Gleason score greater than 7 had a Ki-67 proliferation index greater than 3.5% while only 55% of the specimens with a Gleason score of 7 and below in this cohort had a Ki-67 proliferation index above 3.5%. Similarly, when a cut-off of 7.1% was used, in the

prostate cancer with BRCA mutation cohort, 73% of the specimens with Gleason score greater than 7 had a Ki-67 proliferation index of above 7.1% while only 33.4% of the specimens with Gleason score 7 and below had a Ki-67 proliferation of above 7.1%. Likewise, in the prostate cancer without BRCA mutation cohort, 80% of the specimens with Gleason score greater than 7 had a Ki-67 proliferation index of above 7.1% while only 27.1% of the specimens with Gleason score 7 and below had a Ki-67 proliferation of above 7.1%. There was no statistically significant difference in the Ki-67 proliferation index between the BRCA mutation cohort and the non-BRCA mutation cohort. Irrespective of the BRCA mutation status, a strong relationship was aggressive prostate cancer and Ki-67 expression.

Green *et al* in 2015 carried out a study to determine the predictive value of Ki-67 and distal-less homeobox 2 gene (DLX-2) which is also proliferative marker. [31] The predictive values of these proliferative markers were compared. Immunohistochemistry was scored using the H-score; a score of ≥ 110 and $10 \geq$ were regarded as positive for Ki-67 and DLX-2 respectively. Ki-67 was positive in 6.8% of the 161 scoreable tissue and DLX-2 was positive in 73% of the 185 scoreable tissue. Ki-67 added predictive value to Gleason score, PSA, metastatic risk and prostate cancer specific survival. Expression of DLX-2 failed to show association with PSA, Gleason score and prostate cancer specific risk. However, it showed positive association with metastasis. Co-expression with elevated Ki-67 was noted in 8.2% of DLX-2 positive tumour. Green and associates concluded that Ki-67 and DLX-2 may be of clinical importance for patients on active surveillance

In another study done at the Zagazig University in Egypt by Abdelbany, high Ki-67 showed statistically significant association with Gleason score >7, elevated PSA, advanced tumour stage, metastasis and disease progression. [32] The study assessed the prognostic significance of Ki-67,

between the basal and secretory cells correlates with the different level of differentiation which is consistent with the theory that basal cells have stem cell functions. [33] In prostatic intraepithelial neoplasia and prostatic adenocarcinoma, Bcl-2 is expressed by the luminal epithelial cells. The expression of the biomarker is increased in androgen-independent tumour. [33]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcriptional regulation of the Bcl-2 p2 promoter is a mechanism by which BCL-2 is upregulated. [33]. Some researchers have explored the potential benefit of inducing apoptosis as a therapeutical option for prostate cancer patient and some of the suggested mechanism include antisense-mediated down-regulation of Bcl-2, blockade of NF-kB, hammered-head ribozyme directed against anti-apoptotic protein like Bcl-2 and activation of pro-apoptotic pathway in vitro. [33]

The prognostic significance of Bcl-2 in prostate cancer is equivocal, some studies have suggested that over-expression of this protein is of prognostic importance while others have refuted its relevance as a prognostic marker.

Park *et al* assessed the prognostic significance of Bcl-2, Ki-67, transformation-related protein 16 (p16), E-cadherin and PTEN in one hundred and twenty-four prostate cancer specimens received at the Yeungnam University Hospital from 2007 to 2008. [15] Reaction to Bcl-2 was described as positive when more than 50% of the tumour cells showed moderate to intense staining and Ki-67 was assessed using the proliferation / labelling index which was expressed in percentage. The Ki-67 proliferation index was stratified into 3 groups <10% (group 1), 10 – 20% (group 2) and >20% (group 3). Bcl-2 was over-expressed in 10% of prostatic adenocarcinoma cases and all benign nodular hyperplasia cases. Its over-expression in prostatic adenocarcinoma showed statistically significant association with metastasis and high Gleason score but it did not correlate with PSA value. Ki-67 proliferation index was lower in benign nodular hyperplasia than prostatic

adenocarcinoma. It also showed statistically significant association with high Gleason score and loss of p16. However, it failed to show statistically significant difference in patients with metastatic and non-metastatic cancer. Similarly, PTEN, p16 and E-cadherin did not predict metastasis. Park and colleagues concluded that a combination of over-expression of Bcl-2, elevated Ki-67 proliferation index and high Gleason score may be a marker of high-risk prostate cancer. Bcl-2 was also suggested as an independent prognostic marker. [15]

In Iran, Anvari and associates assessed the prognostic significance of apoptotic markers, Bcl-2 and Bax, in thirty-seven prostate cancer patients who were referred to the Omid Hospital between 2003 and 2007[16]. In the study, over-expression of Bcl-2 correlated significantly with Gleason score greater than 7. Immunohistochemistry for these markers was scored using the H-score with a range of 0 to 300. A H-score of 50 and above was considered positive and the median H-score was used to distinguish between high and low Bax and Bcl-2 groups. The median H-score for Bcl-2 was 85, and it ranged from 0 to 220. There was over-expression of Bcl-2 in 69.6% of tumour with Gleason score greater than 7 while only 21.4% of tumour with Gleason of 7 and below showed over-expression of Bcl-2. It also correlated with biochemical progression-free survival. On the other hand, Bax did not correlate significantly with Gleason score or biochemical progression-free period. The median H-score was 200 (40 to 300). The authors concluded that high Bcl-2 in hormonally-treated prostatic cancer patients is associated with worse biochemical progression-free rate. [16]

In another study by Bubendorf *et al*, there was no significant correlation between Bcl-2 and Gleason score. [17] The study assessed the prognostic significance of Bcl-2, Ki-67 and p53 on 137 untreated clinically localized prostate cancer patients who had radical prostatectomy. Bcl-2 immunohistochemistry was subjectively estimated as low (scattered staining in tumour cells),

intermediate (10 to 50% of tumour cells) and high (greater than 50% of tumour cells). A cut-off of 10% was set as positive tumour staining. The tumours, based on the Gleason score, were divided into low-grade tumours with Gleason score of 6 and below and high-grade tumours with Gleason score of 7 and above. Bcl-2 was over-expressed in 21% of tumour with Gleason score less than 7 and it was over-expressed in 28% of tumour with Gleason score of 7 and above.

In another study done by Bauer *et al* in United States of America, p53 and Bcl-2 were independent prognostic factors for disease free survival in prostatic cancer patient managed with radical prostatectomy. [34] The study was done on archival specimens from 175 patients diagnosed with prostatic adenocarcinoma between 1986 and 1992 at the Walter Reed Army Medical Centre. The patients were followed up for 1 to 9 years and recurrence was defined as two consecutive increases in PSA level greater than 0.2 ng/mL. Bcl-2 and p53 were scored on the areas with the highest percentage of cytoplasmic and nuclear positivity respectively. The scores ranged from 0 to 4 which were interpreted as score 0 = less than 1% stain, score 1+ = 1 – 25 % stain, score 2+ = 26 to 50% stain, score 3+ = 51 to 75% stain and score 4+ = 76 to 100% stain. 26.9% of the cases showed Bcl-2 over-expression and 65% of the cases showed p53 expression. Bcl-2 was also expressed by basal cells, atrophic glandular cells, seminal vesicles and ejaculatory ducts. Bcl-2 showed increase expression with higher Gleason score, higher tumour stage and disease-free survival. However, it did not show a clear trend with age. Similarly, p53 showed correlation with high Gleason score and tumour stage.

Matsushima *et al* in Japan investigated the predictive value of Bcl-2 and p53 in prostatic adenocarcinoma patients. [35] The study assessed 146 patients who had different form of therapy. The mean age in the study was 71 years and the mean follow-up period was 35.5 months. Specimens with nuclear staining in more than 10% of tumour cells were regarded as p53

positive and those that expressed cytoplasmic staining in more than 10% of tumour cells were regarded as Bcl-2 positive tumours. Bcl-2 positive tumours constituted 19.9% of the 146 tumours and p53 positive tumours constituted 27.3% of the tumours. There was no statistically significant correlation between Bcl-2 and Gleason score however p53 showed statistically significant correlation. Bcl-2 positivity correlated less to shorter progression-free survival than p53 ($p = 0.088$ vs $p = 0.016$ respectively). Bcl-2 showed a statistically significant association with tumour stage. Matsushima conclude that combined p53 and Bcl-2 immunohistochemistry provided additional information on biopsy-diagnosed prostatic adenocarcinoma and Bcl-2 positivity was an independent prognostic factor.

Similarly, Yoshino and colleagues studied the prognostic importance of Bcl-2 in Japan. [36] In their study, they evaluated its importance in hormone-refractory prostate cancer patients on Taxane-based chemotherapy. Three cohorts of prostatic cancer patients were involved in this study. The first cohort comprised 40 patients who had localized prostate cancer and underwent radical prostatectomy. The second cohort consisted of 30 patients with hormone-refractory prostate cancer who were placed on Taxane-based therapy and the third cohort comprised 19 patients with hormone-refractory prostate cancer who were not given Taxane-based therapy. In addition to Bcl-2, other apoptosis related markers like Bcl-2 homologous antagonist killer (Bak) and Bax were also investigated. Specimens in which greater than 10% of cells showed staining were regarded as positive and those in which 10% or less of the cells showed staining were regarded as negative. Among the cohort with localized prostate cancer, Bcl-2 overexpression correlated with higher Gleason score and advanced pathological stage but did not correlate with preoperative PSA. Bax and Bak failed to correlate with Gleason score and tumour stage. None of the apoptotic markers showed significant correlation with biochemical failure after

prostatectomy. In the Taxane-treated hormone refractory prostate cancer cohort, Bcl-2, Bax and Bak were positive in 60%, 43.3% and 33.3% of the 30 cases respectively. In the hormone refractory prostate cancer patients that were not exposed to Taxane-based therapy, Bcl-2, Bax and Bak were positive in 68.4%, 15.8% and 31.6% of the 19 cases respectively. It was noted that Bcl-2 was significantly lower in localized prostate cancer than hormone-refractory cancer, and Bak and Bax were significantly higher in localized cancer than hormone-refractory cancer. Patients who responded partially to Taxane-based therapy had higher Bcl-2 expression than those with stable disease. In hormone refractory cases, there was longer causes-specific survival in patients with Bcl-2 positive tumours than those with Bcl-2 negative tumours. Bcl-2 positivity was an independent predictor of cause-specific survival among hormone-refractory prostate cancer patients on Taxane-based chemotherapy.

Rubio *et al* in Spain also investigated the prognostic importance of Bcl-2, Bax, Cyclooxygenase (Cox) and Ki-67 in 91 patients who were diagnosed by core-needle biopsy and managed with radical prostatectomy. [37] In this study, the mean age was 63.62 years. Biochemical progression was defined as post-operative PSA greater than 0.2 ng/ml at any time during follow up period and the mean follow-up period was 46.5 months. Bcl-2 and Ki-67 were quantified using apoptotic and proliferation index respectively in areas with moderate to high intensity staining. The cut-off for Ki-67 proliferation index was 5% in this study. Cox and Bax were quantified using a combination of intensity and percentage. Intensity was given a score of 0 to 3 which corresponded to no stain, weak stain, moderate stain and strong stain respectively. The percentage was converted into a single digit score of 0 to 4 which corresponded to 0%, 1-25%, 26-50%, 51-75% and 76-100% respectively. The two single digit scores were them multiplied to form the Cox or Bax score. Ki-67 proliferation index showed significant concordance in both

core-needle biopsy specimens and radical prostatectomy specimens. Ki-67 proliferation index showed statistically significant association with the Gleason score and it was an independent predictor of disease free survival. An inverse relationship was observed between Ki-67 proliferative index and Cox-2 expression. Similarly, Cox-2 expression showed significant association with Gleason score and biochemical progression. Bcl-2 expression was too low for statistical analysis and Bax expression showed no statistically significance in the study.

Keshgegian *et al* also investigated the significance of apoptotic and proliferation markers in prostate cancer. [38] Their study involved 206 prostate cancer patients who had radical prostatectomy. These patients were followed up for an average of 4 years. Recurrence in this study was defined as clinical evidence of disease or rising PSA level. Ki-67, retinoblastoma gene product (pRB), p53 and Bcl-2 were assessed in this study. Ki-67 was assessed with the percentage of positively stained cells (proliferation index). Bcl-2, pRB and p53 were assessed semi quantitatively and divided into negative staining, less than 5%, 6-20%, 21-50% and greater than 50%. The Ki-67 proliferation index ranged between 0 and 28.8%. It showed statistically significant association with Gleason score, tumour volume, capsular invasion, seminal vesicle invasion and lymph node metastasis. Bcl-2 was positive in only 13.45% of the 208 cases. It did not show significant correlation with any of the histopathological parameters. Similarly, p53 failed to show significant association with histopathological parameters. pRB showed significant association with only lymph node metastasis. With respect to recurrence-free survival, only Ki-67 and Bcl-2 showed statistically significant association with increased recurrence. Patients with high Ki-67 proliferation index and Bcl-2 positive tumour had worse prognosis than patients with low Ki-67 and Bcl-2 negative tumours. Histopathological parameters that showed significant

association with recurrence were Gleason score, capsular invasion, seminal vesicle invasion and lymph node invasion.

Johnson and colleagues in the United Kingdom also examined the prognostic significance of Bcl-2, p53, Bax and Ki-67 in radical prostatectomy specimens with prostatic cancer, high-grade prostatic intraepithelial neoplasia (HG-PIN) and benign prostatic epithelium. [39] Ki-67 was assessed with proliferation index and Bcl-2 was assessed as + for strong cytoplasmic stain in less than 5% of tumour cells, ++ for 5 -50% and +++ when greater than 50% of tumour cells were positive. The foci with prostate cancer had significantly higher Ki-67 proliferation index when compare to HG-PIN areas. Ki-67 proliferation index was also higher in stage three tumours than stage two tumours. It also increased with the Gleason score, but it failed to reach a statistically significant level. Bcl-2 stained positively in basal cells of benign prostatic glands and seminal vesicle. It was more expressed in areas with HG-PIN than areas with prostate cancer. Only 2.3% of cancer foci showed positive staining for Bcl-2. There was no correlation between Bcl-2 and preoperative PSA, pathological stage and Gleason score.

2.9 PHH3

Phosphohistone H3 (PHH3) is a constituent of the human chromatin structure that is phosphorylated during chromatin condensation in mitosis. The phosphorylation occurs exclusively during the late G2 and mitosis phase of the cell cycle, thus it provides a stricter assessment of mitotic activity than Ki-67. [11] In clinical and in-vitro studies, difference between pre-therapy and post-therapy PHH3 level have been used to assess the effectiveness of mitotic inhibitors. [40] A strong correlation exists between PHH3 and mitotic activity in many tumours, however, when compared to Ki-67 it has not been extensively studied in prostate cancer.

A study done by Goltz *et al,* on a cohort of ERG translocation and androgen expressing prostate cancer, showed correlation between PHH3 and Gleason score. [11] Tissue microarrays were produced by selecting two cores from representative carcinomatous areas of 640 radical prostatectomy specimens. Immunohistochemistry for the biomarkers was assessed with the proliferation rate and index. The proliferation rate was defined as the percentage of tumour cells stained while proliferation index was defined as the number of positive tumour cells per carcinomatous area i.e. combining both epithelial and stroma cells. The proliferation rate ranged between 0% - 0.52% and the mean proliferation index was 0.21/mm^2. Approximately 83% of the cases showed no reactivity for PHH3. The PHH3 proliferation rate and index showed statistically significant association with Gleason score and pathological stage respectively. The Ki-67 proliferation rate ranged between 0.1% - 18% and the proliferation index was 69.22/mm^2. There was a statistically significant correlation between Ki-67 proliferation rate and Gleason score, pathological stage and margin status. It also correlated with the PHH3 rate, PHH3 index and androgen receptor expression. Also, the Ki-67 proliferation rate showed significant association with relapse free survival. However, PHH3 rate or index failed to show an association.

Braun *et al* in Germany investigated the relationship between aneuploidy in prostate cancer and proliferation markers. [41] In their study, the pattern of chromosome number change was studied in 486 cases of prostate cancers which comprised metastatic and non-metastatic diseases. About 60% of the cases had aneuploidy which showed significant increase with tumour stage. There was statistically significant association between rising proliferation index and tumour progression. The mean Ki-67 proliferation index, expressed in percentage, for non-metastatic, nodal metastatic and distant metastatic tumour were 0.62%, 7.11% and 22.10% respectively. The mean PHH3 proliferation index for non-metastatic, nodal metastatic and distant metastatic

tumours were 0.28%, 1.9% and 7.46% respectively. PHH3 and Ki-67 showed statistically significant correlation with extent of aneuploidy. Furthermore, the proliferation index for the markers showed statistically significant difference between aneuploid and diploid tumours.

Nowak *et al* also assessed the prognostic significance of PHH3 and Ki-67, using tissue microarray, on radical prostatectomy specimens from a cohort of Swedish men with prostate cancer. [12] Ki-67 and PHH3 were assessed using proliferation and mitoses indices respectively and both were expressed in percentage. The Ki-67 proliferation index ranged between 0 and 0.45% while the PHH3 mitotic index ranged between 0.01 and 0.26%. The Ki-67 proliferation index correlated significantly with the Gleason score but failed to show significant correlation with biochemical failure, tumour stage and diagnostic PSA. On the other hand, PHH3 correlated significantly with the prostatic specific antigen level at the time of diagnosis and biochemical failure but did not show significant correlation with Gleason scores.

CHAPTER 3

AIM AND OBJECTIVES

AIM

1. The aim of this study was to determine the prognostic significance of proliferation and apoptotic markers in prostate adenocarcinoma.

OBJECTIVES

1. To categorise previously diagnosed prostate cancer into grade groups and to test the reproducibility of the 2014 ISUP modification of the Gleason grading system.
2. To determine the degree of Ki-67, PHH3 and Bcl-2 positivity in prostate adenocarcinoma.
3. To determine if there is a correlation between the Gleason grade groups of prostate adenocarcinoma and markers of proliferation index and apoptosis.
4. To analyse the relationship between Bcl-2 and Ki-67.

CHAPTER FOUR

METHODOLOGY

4.1 STUDY DESIGN

- This study is a retrospective and observational study.

4.2 STUDY SETTING

- This study was done in the Anatomical Pathology Department of the University of Witwatersrand, Johannesburg, South Africa and National Health Laboratory Service (NHLS) Anatomical Pathology Laboratory at the Charlotte Maxeke Academic Hospital, Johannesburg, South Africa.

4.3 STUDY POPULATION

- The study population comprised all prostate specimens in 2014 with histological diagnosis of prostatic adenocarcinoma.

4.4 EXCLUSION CRITERIA

- All prostatectomy cases and these were determined by the histopathological reports.
- All cases reported as insufficient on histopathological report; core needle biopsies which were reported as insufficient to make histological diagnosis were excluded from this study.
- All specimens with insufficient tissue on formalin-fixed paraffin embedded (FFPE) block; the FFPE blocks were examined for adequacy of tissue and blocks without sufficient tissue were subsequently excluded from this study.

- All cases of Gleason grade pattern 5 with extensive tumour necrosis as the areas of tumour necrosis may not be reactive optimally to the immunohistochemical stains. However, tumours with Gleason grade pattern 5 with single cells and solid nest were not excluded the study.

4.5 SAMPLE SIZE

- All the cases of prostatic adenocarcinoma diagnosed in 2014, after applying the exclusion criteria mentioned above, were used in this study.
- The estimated sample size for this study was 50 cases based on the number of cases received in the first quarter of 2012 however 44 were seen after a thorough search of the Trackcare system

4.6 TASK ALLOCATION AND DATA COLLECTION

- A search was done on the National Health Laboratory Service (NHLS) track care system at the Charlotte Maxeke Academic Hospital using the following keywords **"prostate" and "adenocarcinoma"**.
- Another search was done on the NHLS track care system using the keywords **"prostate" and "benign prostatic hyperplasia"** and the search yielded 10 cases of benign prostatic hyperplasia. The benign prostatic hyperplasia cases served as negative controls in the study.
- The histopathological reports of the cases from the search were printed out and the cases reported as insufficient to make diagnosis were excluded from the study.
- The haematoxylin and eosin stained slides and the formalin-fixed paraffin embedded (FFPE) blocks of the remaining cases were retrieved from the departmental archive.

- The exclusion criteria were applied.
- A data collection sheet was created for each case. The data collection sheet contained the following information; serial case number, laboratory number, age of each patient, Gleason score (initial Gleason score, repeat Gleason score and supervisor's Gleason score) and grade group. Other information included were Ki-67 proliferation index, PHH3 proliferation index, Bcl-2 apoptotic index and the single digit score for each of the immunohistochemical stains. Please refer to appendix 1 for details of the data collection sheet.
- The Gleason scores on the histopathological reports were noted and the slides were examined by the primary investigator and the Gleason score was recorded as initial Gleason score
- Two weeks later, the slides were re-examined by the primary investigator and the Gleason score were then recorded on the data collection sheet as primary investigator's Gleason score. This ensured intra-observer reproducibility. The 2 Gleason scores (initial and primary investigator) were then statistically compared and agreement between the two set of scores was done with Cohen kappa calculation. This ensured reliability of the scores.
- Afterward, the cases were passed to the supervisor who also examined the cases. This ensured inter-observer reproducibility. The Gleason scores (primary investigator's vs supervisor's) were statistically compared and agreement between the two set of scores was assessed by kappa calculation. This ensured reliability of the scores.

- Using the 2014 International Society of Urological Pathology Grade group classification each of the cases were assigned to a grade group (please refer to appendix 2 for details of the 2014 ISUP grade group).
- Specific morphological features on each slide were noted and these specific features were
    - Prostatitis
    - Perineural invasion
    - Lymphovascular space invasion
    - High-grade prostatic intraepithelial neoplasia.

4.7 CONTROL CASES

- From the second search result above, 6 FFPE blocks and the corresponding slides of benign prostatic hyperplasia were retrieved from the departmental archive which were examined.

4.8 IMMUNOHISTOCHEMISTRY STAINING PROTOCOL

- From each of the FFPE blocks three, one for each of the immunohistochemical stains, 3μm-thin tissue sections were cut. Each of the sections was placed on a separate glass slide. Antigens were retrieved from the tissue sections and these tissues were stained with PHH3, Ki-67 and Bcl-2 in the presence of adequate positive and negative controls. The antigen retrieval protocol and the immunohistochemical staining protocol used are as follows.
- Antigen retrieval protocol: The 3μm thin tissue sections were dewaxed in xylene for a minute and rehydrated by passing through decreasing concentration of alcohol (100%, 95% and 70%) for a minute. Antigens were retrieved by placing the slides in 100mls of

citrate buffer at pH6 and heating in microwave oven at 100^0c for 15 to 20 minutes. The slide was then washed with distilled water for 2 minutes, flooded twice in phosphate buffer for 3 minutes and rinsed again in distilled water for 2 minutes. The slides were placed in hydrogen peroxide for 10 minutes and washed in running distilled water for 2 minutes. Afterward, the slides were incubated with ultraviolet blocking solution for 10 minutes, the slides were wiped gently with cotton wool to remove excess blocking solution. The slides were then stained with the different antibodies.

- Immunohistochemical stain protocol: the slides were placed in 200µl of the appropriate antibody solution (Dako Ki-67 antibody {clone MIB-1/ code M7240/dilution of 1:100}; Dako Bcl-2 {clone 124/ code M0887/ dilution 1:100} and Cell Marque PHH3 rabbit polyclonal antibodies {dilution of 1:250}) and incubated at room temperature for an hour. The slides were then flooded with phosphate buffer twice. Antibody enhancer were then applied to the slides for 30 minutes and the slides were washed with phosphate buffer for 3 minutes. The slides were dipped in a horseradish peroxidase solution and incubated at room temperature for 45 minutes. The slides were transferred into a phosphate buffer solution for 3 minutes. A solution of 3'3 diaminobenzidine (DAB) tetrahydrochloride was prepared according to manufacturer's protocol and the slides were placed in the solution in a fume hood for 3 minutes. The slides were then washed under running water for 3 minutes. The slides were then counter stained with haematoxylin. The slides were dehydrated in increasing concentration of alcohol and cleared with xylene. Cover-slips were placed on the slides using a distyrene, plasticizer and xylene (DPX) mountant. The slides were allowed to dry and viewed.

- Immunohistochemistry was done by the primary investigator with technical assistance by the laboratory technologist. The procedure was supervised by the supervisor.

4.9 SCORING OF IMMUNOHISTOCHEMISTRY

- After immunohistochemistry had been done, the slides were examined in a semi-quantitative manner. The slides were scanned with a x10 objective lens (magnification of x100) for cytoplasmic staining in Bcl-2 and nuclear staining in PHH3 and Ki-67.
- With a x40 objective lens (magnification of x400), the total number of carcinoma cells in a focus was counted with a grid which was attached to the eyepiece. The use of grid in counting ensured that a cell was not counted twice. The numbers of Ki-67, PHH3 and Bcl-2 positive-stained cells were counted, and the percentage of positive-stained cells was calculated by dividing the total number positive-stained cells by the total number of carcinoma cells and the results were expressed in percentage by multiplying by 100.
- This process was done in 10 random foci and the average percentage of positive stained was used as the proliferative or apoptotic index.
- The average percentage of positive cells was then converted into a single digit score ranging from 0 to 3. Where 0 represent 0% (no staining), 1 represents 1 to 24%, 2 represents 25 to 75% and 3 represents >75%.
- The intensity of the immunohistochemical stains was not assessed in this study.

4.10 STATISTICAL ANALYSIS

- The null hypothesis for this research is that the proliferation index, as measured by Ki-67 and PHH3, and apoptotic index, as measured Bcl-2, does not correlate with the Gleason score. Therefore, they do not indicate prognosis of prostate cancer.

- Statistical analysis: The data from the study was analysed with STATA version 12. The Gleason grade groups were correlated with the individual biomarkers i.e. Gleason grade group vs Ki-67, Gleason grade group vs PHH3 and Gleason grade group vs Bcl-2 using Pearson's correlation co-efficient(r) if the data is parametric or Spearman's rank correlation if the data is non-parametric.

4.11 ETHICAL APPROVAL

- The department has a blanket ethics approval (M1074) to use archived blocks, reports and slides in histological, immunohistochemical and molecular research.
- Furthermore, project specific application (M1606101) was sent to the University of the Witwatersrand ethics review board. This research commenced after ethical approval was given by the ethic review board.

4.12 TIME ALLOCATION ON PROJECT

- The time for completion of this research was 19 months.
- Please refer to accompanying Gantt chart.

CHAPTER FIVE

RESULTS

A total of 44 cases of prostatic adenocarcinoma were identified on the Trackcare system. Out of the 44 cases, 10 cases were excluded from the study after review of the slides with the supervisor. The reasons for exclusion include suboptimal amount of tissue on the slide and a case was excluded due to insufficient tissue according to histopathological report, thus a total of 33 cases were used in this study.

The patients' age ranged between 46 to 79 years and the mean age was 64.7 years. Prostatic adenocarcinoma was most common in the 60-69 age group with 17 cases representing 51.5% of the cases in the study. The 40-49 year age group had the least number of cases with only 2 cases; one of the cases was a ISUP grade group 4 tumour and the other was a ISUP grade group 1 tumour. The 50-59 and 70-79 age groups had 5 and 9 cases respectively. (Figure 1)

In terms of specific morphological features, six of the 33 cases showed perineural invasion, one had neuroendocrine differentiation and one showed basal cell hyperplasia. Perineural invasion was more common in the higher ISUP grade groups; four out of the six cases occurred in ISUP group 3 and 4. Only one of the cases had a Ki-67 proliferation index lesser than the median Ki-67 proliferation index. Similarly, only one case was below the median and mean value for PHH3 and Bcl-2 respectively. The case with neuroendocrine differentiation was seen in a 72-year-old patient and the tumour was an ISUP grade group 2 tumour. (Figure 2) The case with basal hyperplasia was an ISUP grade 1 tumour and it occurred in a 58-year-old patient. In most cases of prostatic adenocarcinoma, an area of loose, oedematous, myxoid stroma was seen around the infiltrating tumour cells. (Figure 3) The stroma was looser or lacked the architectural organisation when compared to the adjacent fibromuscular stroma.

ISUP grade group 1 tumours were the most common ISUP grade group and this group constituted 36.4 % (12/33). There were no cases of ISUP grade group 5 tumour in this study. ISUP grade group 2, 3 and 4 had eight, five and eight cases respectively. (Figure 4) Using the Cohen's kappa coefficient, the Gleason score intra-observer agreement was 87.88% and the kappa was 0.8432; this was interpreted as an excellent agreement. (Table 1a) The inter-observer agreement, between the primary investigator and supervisor, was 21.21% and the kappa was 0.0613 which was interpreted as a poor agreement. (Table 1b) This necessitated the supervisor to re-train the primary investigator on the ISUP 2014 modification of the Gleason score. After a week of re-training, the inter-observer reproducibility was repeated between the primary investigator and supervisor. The Cohen's kappa statistic showed an agreement of 93.94% and the kappa of 0.9148 which was interpreted as an excellent agreement. (Table 1c)

Ten cases of benign prostatic enlargement were seen on the Trackcare system however 6 were available in the departmental archive for assessment. The six cases were selected as negative controls in this study. The patients age ranged between 53 and 78 years, and the mean age was 65.83 years.

Out of the 33 case that were subjected to Ki-67 immunohistochemical stain, 28 cases showed readable staining. The staining could not be assessed in 5 cases due to either the tumour having been washed-off or there were not enough tumour cells to quantify. The Ki-67 proliferation index in the 28 cases ranged between 0-38% (Figure 5) and the median and mean values were 4.65% and 6.88%. The skewness and kurtosis value were 2.6456 and 10.792 respectively and two outliers were noted in the box plot. (Figure 6) Thus the median value of 4.65% was set has the cut-off point. Consequently, tumours with Ki-67 proliferation index of 4.65% and above were regarded as Ki-67 positive tumours and those with Ki-67 proliferation index below 4.65%

were regarded as Ki-67 negative tumours. The association between Ki-67 proliferation index and ISUP grade groups was assessed with Spearman correlation; the Spearman rho was 0.6818 which was statistically significant with a p-value of 0.0001. (Figure 7) The association between ISUP grade groups and Ki-67 negative and positive tumours was also assessed with Pearson's chi-square and there was also a statistically significant association (p-value= 0.012) (Table 2).

For PHH3, twenty-six out of the 33 stained-cases had readable score. The PHH3 proliferation index ranged from 0.05 to 4.4%. (Figure 8) The mean and median values were 0.855% and 0.4% respectively. An outlier was noted in the box plot (Figure 6) and the standard deviation, skewness and kurtosis were 1.026, 1.952 and 6.794 respectively. The median value of 0.4% was used as the cut-off point. Thus, tumours with PHH-3 proliferation index of $\geq$0.4% were regarded as PHH3-positive tumours and vice-versa. Using the Spearman correlation, there was a positive correlation (rho value of 0.714) between PHH3 proliferation index and ISUP grade group (p-value = 0.0000). (Figure 7) Pearson's chi-square also showed statistically significant association (p-value = 0.003) between ISUP grade group and dichotomised PHH3 proliferation index. (Table 2)

Only 2 cases of Bcl-2 were not readable. The apoptotic index, for the remaining 31 cases, ranged between 1.3 to 27.5% (Figure 9). The mean, median, standard deviation, skewness and kurtosis were 10.4%, 9.8%, 6.59%, 0.67 and 2.91 respectively. There was no outlier in the box plot (Figure 6) thus the mean was used as the cut-off value. Consequently, tumours with Bcl-2 apoptotic index less than 10.5% were designated Bcl-2 negative and tumours with Bcl-2 apoptotic index of 10.5% and above were designated Bcl-2 positive tumours. Correlation between ISUP grade groups and Bcl-2 apoptotic index was assessed with Pearson's correlation and their correlation value was 0.6470. (Figure 7) Like the proliferation markers, there was

statistically significant association (p-value = 0.027) between ISUP grade groups and dichotomised Bcl-2 apoptotic index. (Table 2)

The correlation between the different biomarkers was also assessed by Spearman's correlation (Figure 7). Ki-67 proliferation index showed an excellent correlation with PHH3 proliferation index; the correlation value was 0.985. Similarly, Bcl-2 apoptotic index correlated with Ki-67 proliferation index and PHH3 proliferation index with a rho value of 0.686 and 0.707 respectively.

The single-digit score was not used as most of the apoptotic and proliferation index values were 1 and this would create a bias during statistical analysis.

The average age of benign prostatic hyperplasia patients, whose specimens served as control in this study, was 65.83 years (53-78 years). The control cases showed less reactivity with the immunohistochemical stains. Ki-67 proliferation index ranged between 0.6 and 1.9%, and the mean and median Ki-67 proliferation index were 0.93 and 0.75% respectively. The PHH3 proliferation index was between 0 and 0.1%, and the mean PHH3 proliferation index was 0.05%. The mean value for Bcl-2 was 2.57% and the range was 1 – 3.9%.

TABLES AND FIGURES

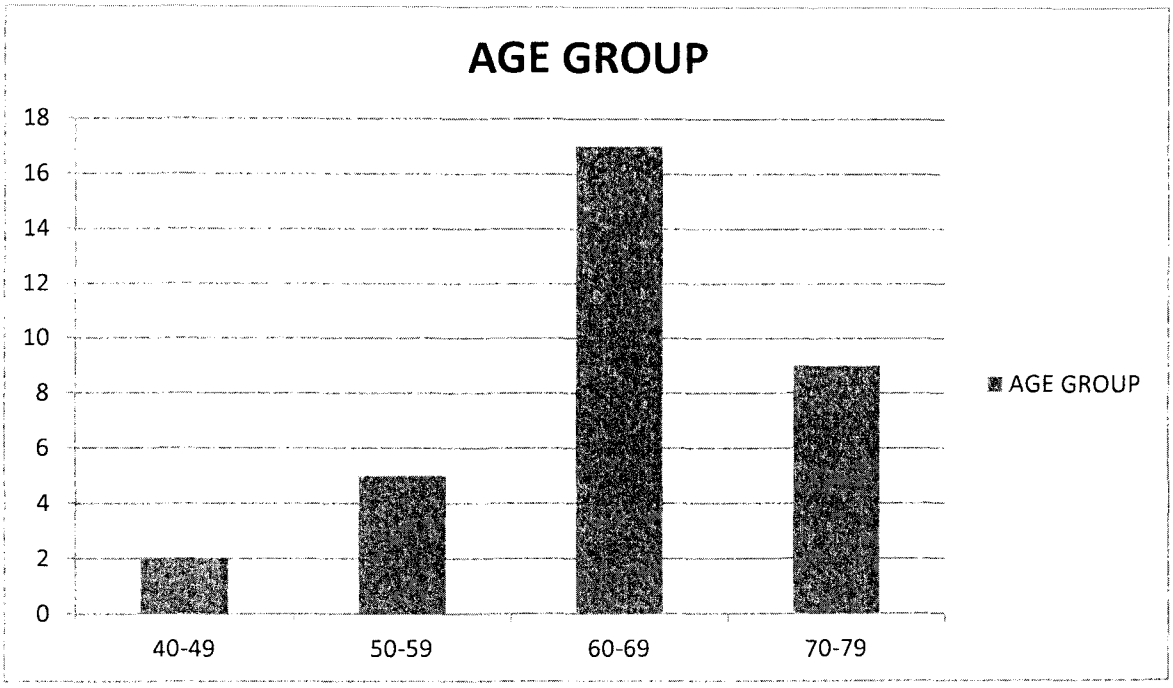


Figure 1: Bar-chart showing the age distribution for prostatic adenocarcinoma.

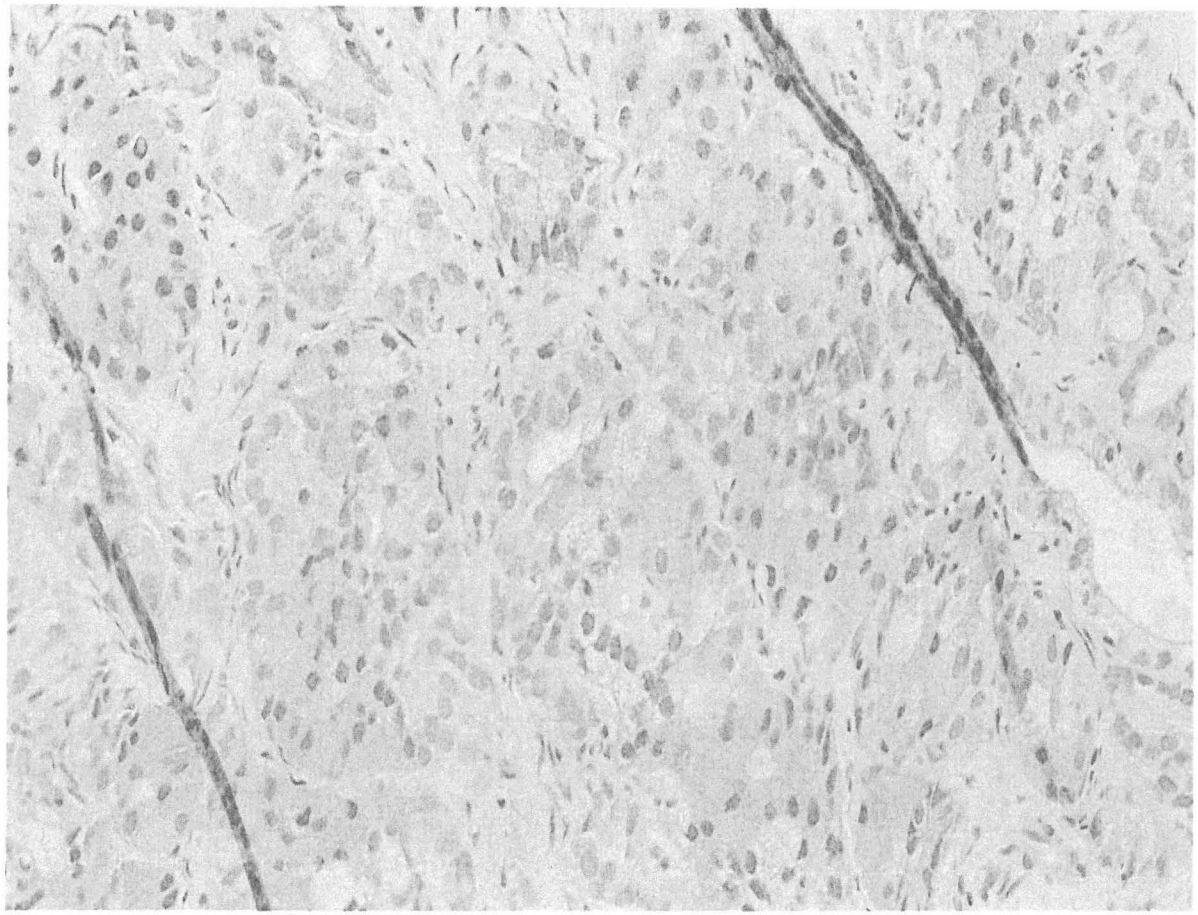


Figure 2: Showing a prostatic adenocarcinoma with neuroendocrine differentiation. The cytoplasm is deeply eosinophilic and the nuclei are monomorphic. (Haematoxylin and Eosin stain at x100 magnification)

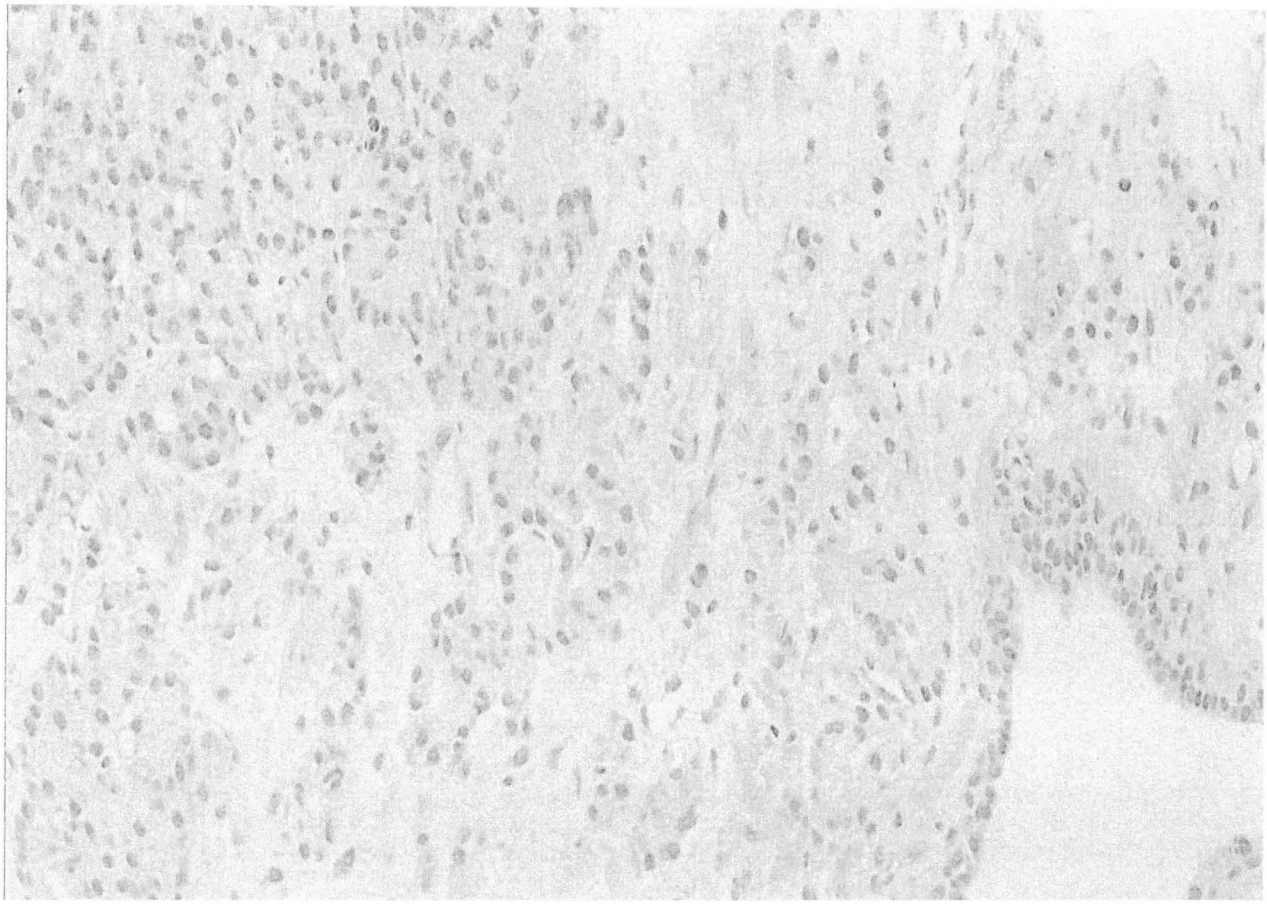


Figure 3: Showing loose, oedematous, myxoid stroma around infiltrating glands / cells (Haematoxylin and Eosin stain at x100 magnification)

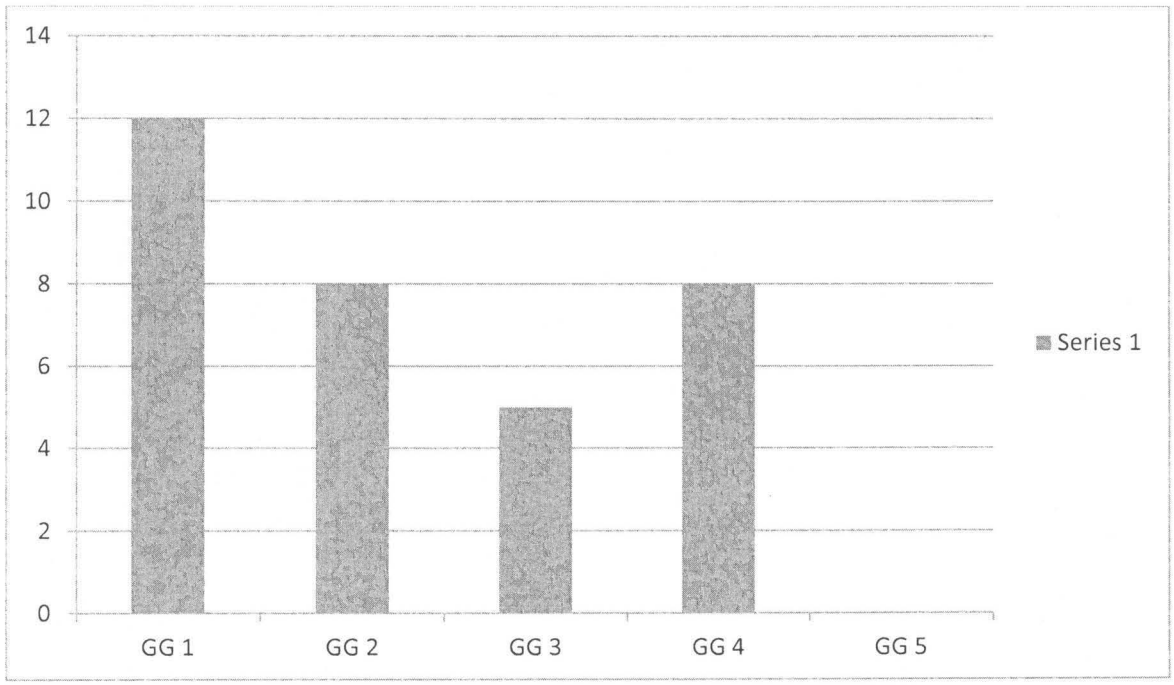


Figure 4: Bar-chart showing the ISUP grade group distribution

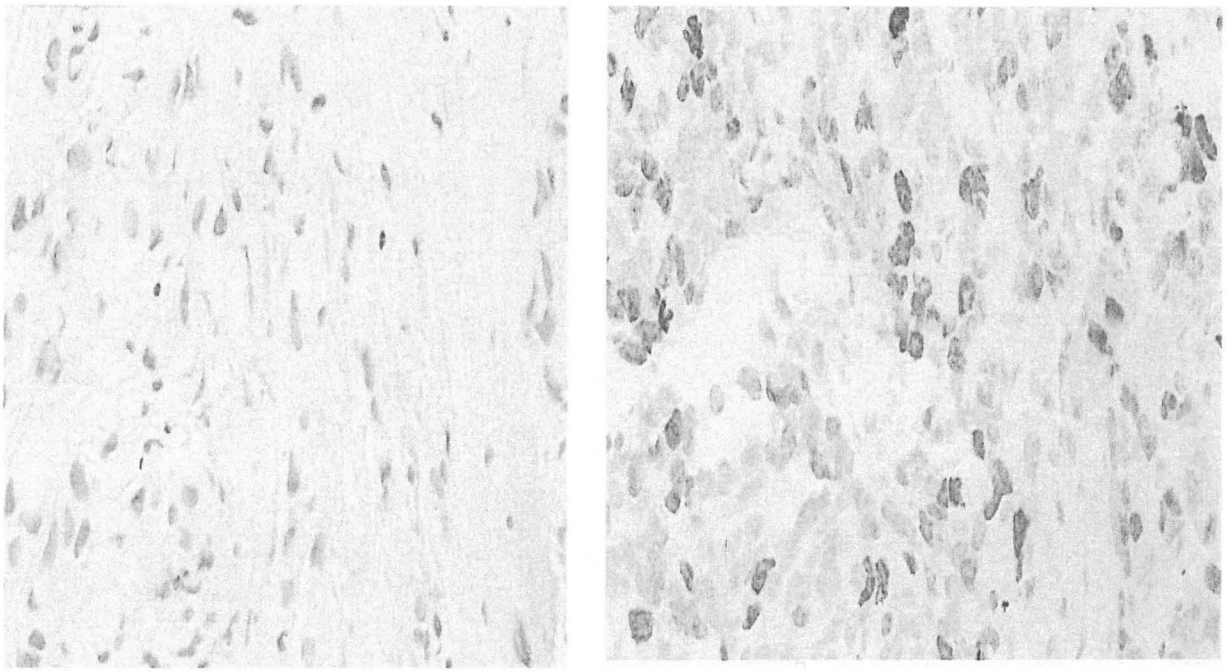


Figure 5: Immunohistochemistry photomicrograph at x100 magnification showing two tumours with a Ki-67 proliferation index of 0% (left) and 38% (right)

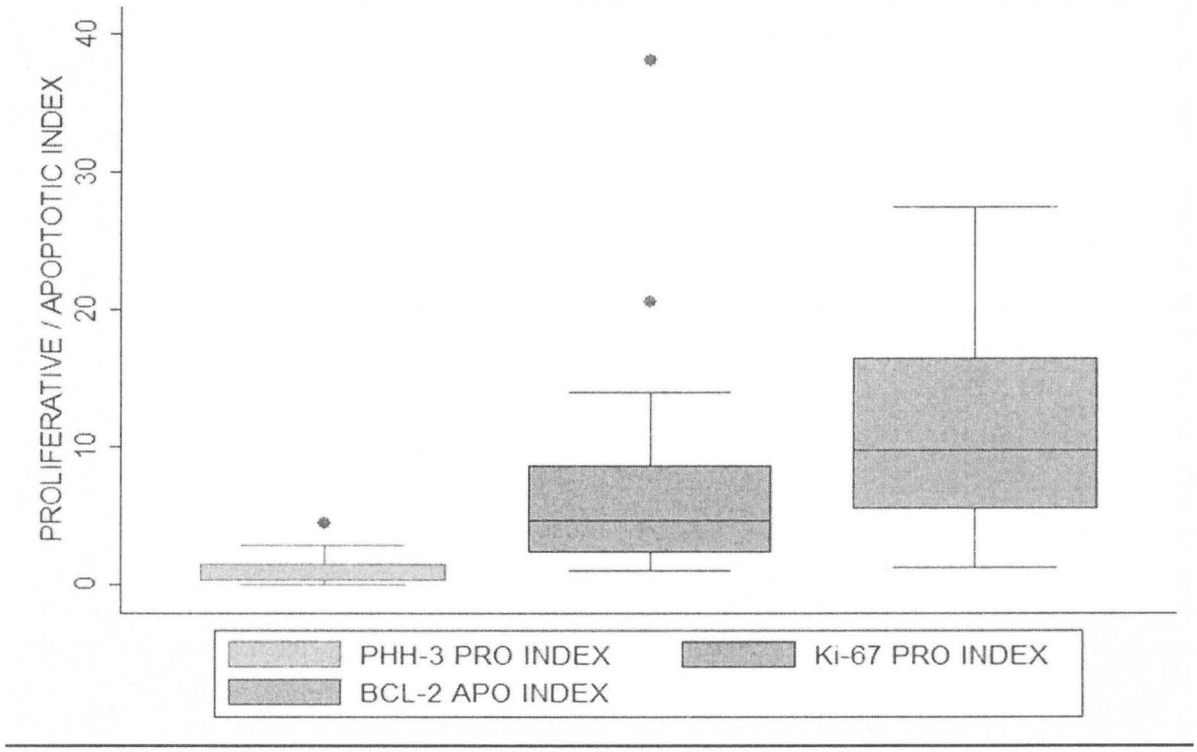


Figure 6: Box plot showing the distribution of proliferation (PRO) and apoptotic (APO) index among the tumours

```
. correlate GRADEGROUP BCL2APOINDEX
(obs=31)

             | GRADEG~P BCL2AP~X
-------------+------------------
  GRADEGROUP |   1.0000
BCL2APOINDEX |   0.6470   1.0000


. spearman GRADEGROUP  PHH3PROINDEX, stats(rho obs p)

 Number of obs =       26
Spearman's rho =   0.7143

Test of Ho: GRADEGROUP and PHH3PROINDEX are independent
    Prob > |t| =   0.0000

. spearman GRADEGROUP   Ki67PROINDEX, stats(rho obs p)

 Number of obs =       28
Spearman's rho =   0.6816

Test of Ho: GRADEGROUP and Ki67PROINDEX are independent
    Prob > |t| =   0.0001
```

```
. spearman Ki67PROINDEX PHH3PROINDEX BCL2APOINDEX
(obs=22)

             | Ki67PR~X PHH3PR~X BCL2AP~X
-------------+---------------------------
Ki67PROINDEX |   1.0000
PHH3PROINDEX |   0.8956   1.0000
BCL2APOINDEX |   0.6859   0.7096   1.0000
```

Figure 7: Showing the Pearson's correlation and Spearman's correlation between ISUP grade groups, apoptotic markers and proliferation markers

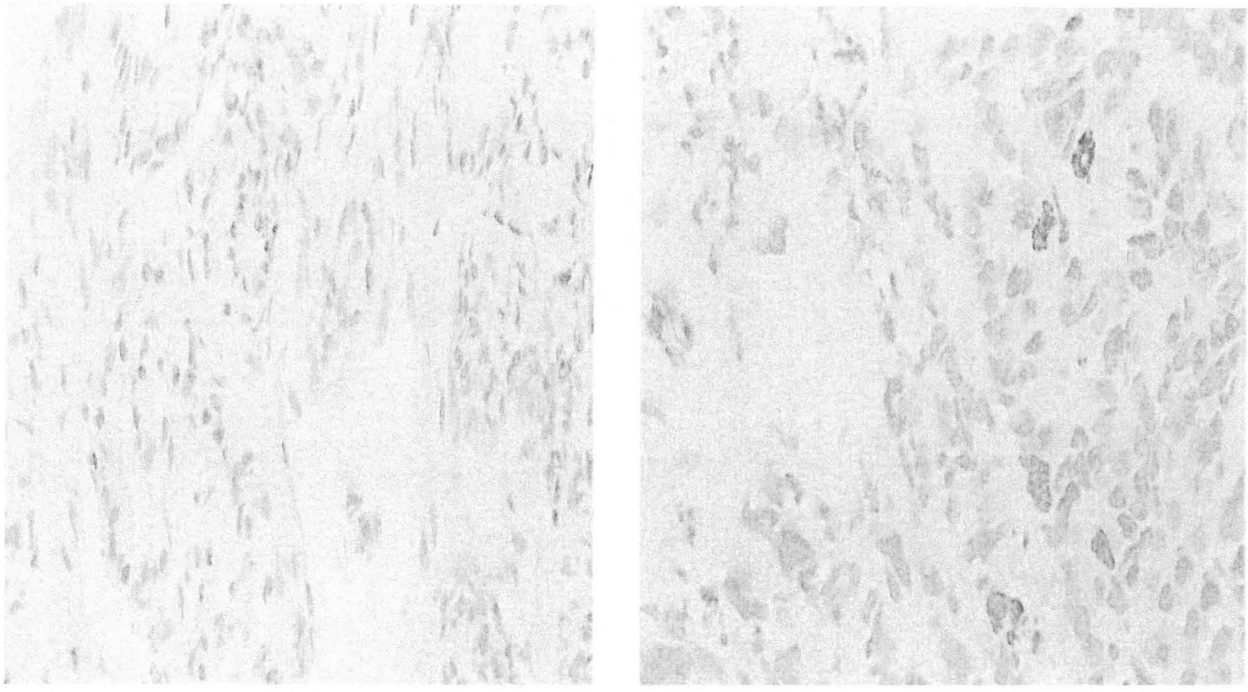


Figure 8: Immunohistochemistry photomicrograph at x100 magnification showing two tumours with PHH3 proliferation index of 0%(left) and 4.4%(right)

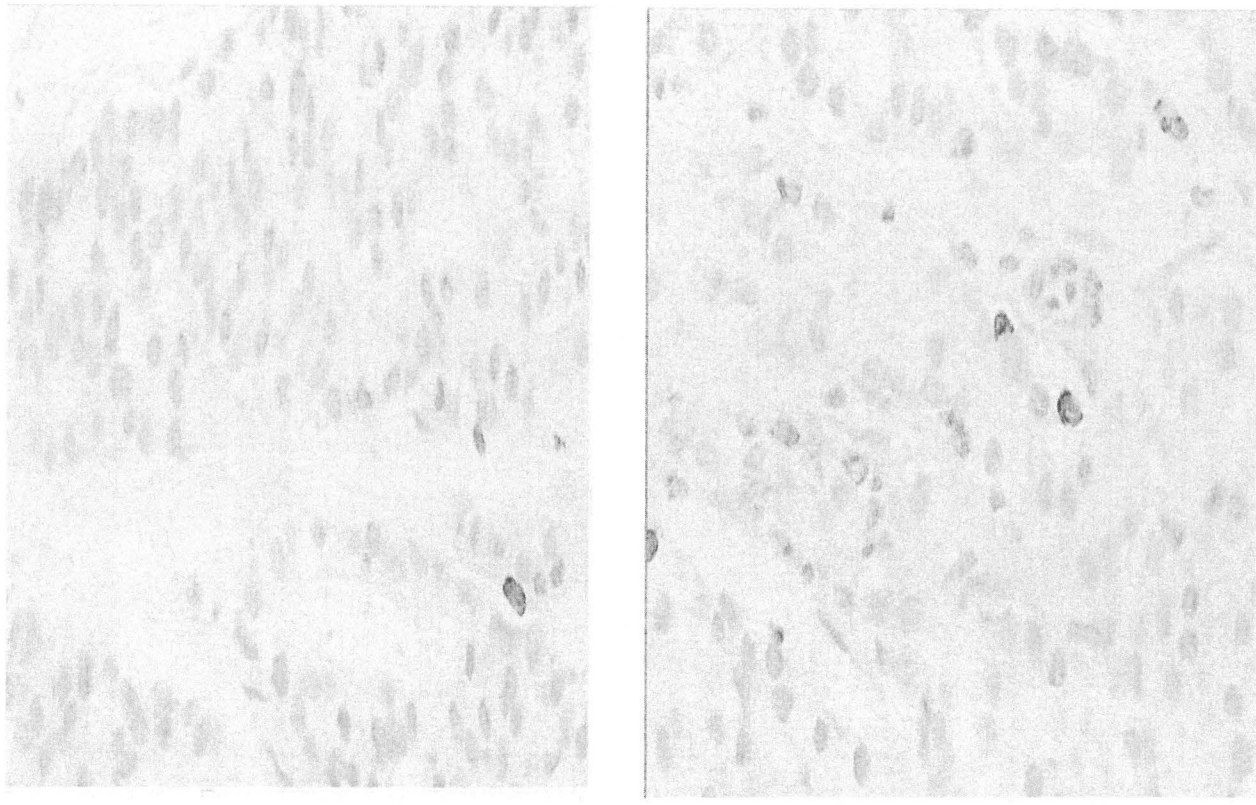


Figure 9: Immunohistochemistry photomicrograph at x100 magnification showing two tumours with Bcl-2 apoptotic index of 1.3% (left) and 27.5% (right)

Table 1a: Table showing the intra-observer reproducibility of the primary investigator as measure by kappa statistic

REPEAT PI's ISUP GRADE	INITIAL PRIMARY INVESTIGATOR's (PI's) ISUP GRADE					
	1	2	3	4	5	Total
1	4	0	1	0	0	5
2	0	6	1	0	0	7
3	0	0	3	0	0	3
4	0	0	0	6	1	7
5	0	0	0	1	10	11
Total	4	6	5	7	11	33

Agreement	Expected Agreement	Kappa	Std. Err.	Z	Prob>Z
87.88%	22.68%	0.8432	0.0905	9.32	0.0000

Table 1b: Table showing the inter-observer reproducibility between primary investigator and supervisor before re-training as measured by kappa statistic

SUPERVISOR 's ISUP GRADE	INITIAL PRIMARY INVESTIGATOR's (PI's) ISUP GRADE 1	2	3	4	5	Total
1	4	4	4	1	1	14
2	0	2	1	4	1	8
3	0	0	0	1	2	3
4	0	0	0	1	7	8
5	0	0	0	0	0	0
Total	4	6	5	7	11	33

Agreement	Expected Agreement	Kappa	Std. Err.	Z	Prob>Z
21.21%	16.07%	0.0613	0.0697	0.88	0.1898

Table 1c: Table showing the inter-observer reproducibility between primary investigator and supervisor after re-training as measured by kappa statistic

SUPERVISOR 's ISUP GRADE	POST-TRAINING PI's ISUP GRADE 1	2	3	4	5	Total
1	13	0	1	0	0	14
2	0	8	0	0	0	8
3	0	0	3	0	0	3
4	0	0	0	7	1	8
5	0	0	0	0	0	0
Total	13	8	4	7	1	33

Agreement	Expected Agreement	Kappa	Std. Err.	Z	Prob>Z
93.94%	28.83%	0.9148	0.1039	8.80	0.0000

Table 2: Showing the Pearson Chi-square statistic between biomarkers and ISUP grade groups.

	ISUP GRADE GROUPS					
BIOMARKERS	1	2	3	4	5	TOTAL
Ki-67 < 4.65%	9	3	1	1	-	14
Ki-67 ≥ 4.65%	1	4	4	5	-	14
TOTAL	10	7	5	6	-	28
	Pearson chi-square (x^2) = 11.00 and p-value = 0.012					
	1	2	3	4	5	
PHH3 < 0.4 %	9	2	0	1	-	12
PHH3 ≥ 0.4%	1	4	4	5	-	14
TOTAL	10	6	4	6	-	26
	Pearson chi-square (x^2) = 13.66 and p-value = 0.0003					
	1	2	3	4	5	
Bcl-2 < 10.5%	10	3	2	2	-	17
Bcl-2 ≥ 10.5%	1	5	3	5	-	14
TOTAL	11	8	5	7	-	31
	Pearson chi-square (x^2) = 9.14 and p-value = 0.027					

CHAPTER SIX

DISCUSSION

Prostate cancer is primarily a disease of the elderly. In this study, the mean age at presentation was 64.7 years and this corroborated the mean age of 68 years reported by Babb *et al* in South Africa. [19] Studies done in other African countries like Egypt and Nigeria have also reported mean age of 64.97 years, 66 years, 67 years and 68 years. [32, 42-44]. These studies show that the 60-69 age group is the most afflicted age group in Africa. Studies from Europe show similar findings; Fisher *et al* and Mitra *et al* in the United Kingdom reported a mean age of 69.6 and 67 years respectively. [9,10] Rubio *et al* in Spain and Burbendorf *et al* in Switzerland reported 63.62 and 63.3 years respectively.[37,17] Based on the above data, it is recommended that prostate cancer screening should be done for African and European men before they attain the age of 60 years. This is in-line with the Cancer Association of South Africa (CANSA) recommendation which states that prostate cancer screening should be performed for men from the age of 50 years [45] and the American Urology Association (AUA) guideline for prostate cancer screening which recommends that screening should be commenced at the age of 55 years in average risk men.[46] However, men with higher risk of developing prostate cancer, family history of prostate cancer of germline mutation in BRCA gene, should commence screening at an earlier age. The screening will enable early detection of prostate cancer before it progresses. Studies from Asian countries appear to report mean age in the 70s. Anvari *et al* in Iran, Yoshino *et al* and Matsushima *et al* in Japan reported 73, 72 and 71 years respectively.[16,36,35] This may suggest that prostate cancer present at a later age in this region, but a more comprehensive study will be required to validate this observation.

In this study, the most common morphological features seen was a loose, oedematous stroma surrounding the infiltrating glands or cells. This morphological feature corresponds to the oedematous / myxoid reactive stroma and the extra-cellular matrix-rich reactive stroma described by De Vivar *et al.* [51] Typically, the stroma was less eosinophilic than the adjacent smooth muscle fibres and lacked the orderly arrangement associated with smooth muscle cells. Cells with cytomorphological features similar to that of myofibroblasts were seen within this area however immunohistochemistry was not done to ascertain the specific histogenesis of the cells as this was beyond the scope of this research. Glands surrounded by oedematous stroma and myxoid stroma should be viewed as suspicious areas especially when they are small-sized glands. It should be pointed out that loose, oedematous and myxoid stroma were also seen around inflammatory cells, thus reactive stroma should not be used alone as a pointer to malignancy rather it should be used in conjunction with other classical architectural and cytomorphological features like glomeruloid glands, increased nucleocytoplasmic ratios and prominent nucleoli. The feature described as Myofibroblastic reactive stroma by De Vivar *et al* was not encountered in our study although this feature was not particularly searched for during the study.[51] De Vivar and colleagues described this reaction as a marked myofibroblastic response which is accompanied by loss of smooth muscle architecture. Unlike the other type of stroma reaction, there is no difference in colour between the area of reaction and adjacent fibromuscular stroma. However, it is hypercellular relative to the adjacent fibromuscular stroma. It is recommended that several extensive studies should be done to determine the prognostic significance of these stroma reactions in prostate cancer. Perineural invasion was seen in 13.6% (6/44) of the cases in this study. This is quite low when compared to 82.4% mentioned in De Vivar's study. The disparity in percentages could be due to the specimen types used in the two

studies; De Vivar *et al* used radical prostatectomy specimen, which provides more tissue for examination, while core biopsy specimens were used in our study. In this study, 66.67% of the cases with perineural invasion were in ISUP grade group 3 and above. This confirms the poor prognosis which is associated with perineural invasion. To buttress this point, 83.33% of cases that had perineural invasion were Ki-67-positive, PHH3-positive and Bcl-2 positive tumours. Thus, prostatic cancer specimens should be thoroughly examined for perineural invasion and it should be documented in patients' reports to help in management decisions. A case of prostatic adenocarcinoma with neuroendocrine differentiation was seen in our study thus this entity represented 2.27% of cases seen. According to Parimi *et al*, neuroendocrine differentiation is seen in 5 – 10% of prostatic adenocarcinoma. [52] The low percentage in this study could be due to short duration of the study and the number of samples used. Furthermore, more cases with neuroendocrine differentiation might have been detected if radical prostatectomy specimens were included in this study because it would provide more tissue for examination. The incidence of prostatic cancer with neuroendocrine differentiation has been on the rise due to increase in the use of androgen ablation therapy in prostate cancer. [52] Some authors have suggested that it is associated with worse prognosis while others believe it is of no prognostic importance. Although only a single case was seen in this study, the high proliferation index (Ki-67 of 14% and PHH3 of 2.9%) and high apoptotic index (Bcl-2 of 10.5%) in this case suggest it might be associated with bad prognosis. A more extensive study should be done to assess the prognostic significance of neuroendocrine differentiation in prostatic adenocarcinoma

During the 2014 ISUP meeting, the ISUP grading system was introduced and the 2005 Gleason score was modified in order to address some certain issues some of which are lack of consensus on some certain grades and grades that were not covered in 2005 Gleason. [8] Furthermore, it is

believed the 2014 modification would improve inter and intra-observer reproducibility. In this study, the pre-training and post-training inter-observer agreement between primary investigator and supervisor showed poor (kappa = 0.06) and excellent (kappa = 0.91) agreement respectively. This emphasizes the importance of training and regular exposure, especially for pathologist-in-training, to cases of prostate cancer. In the study by Kweldam, which assessed inter-observer reproducibility of Gleason grade 4, consensus was reached in most cases with cribriform and glomeruloid pattern [47] which corroborated our findings. The level of agreement was lower in ill-formed and fused glands in the Kweldam study similar observation was noted in our study. Thus, more emphasis should be made on demonstrating the different patterns of Gleason grades for to pathologists-in-training. In the study by Melia, the intra-observer agreement kappa was 0.66 and the overall inter-observer agreement kappa was 0.54. [48] These were quite low when compared to the intra-observer agreement kappa of 0.84 and inter-observer agreement kappa of 0.91 in our study. The difference in the inter-observer agreement may be due to the number of researchers in both studies; 2 researchers were involved in this study while Melia *et al* study had 11 researchers. A bigger study involving more pathologists and trainees is advised to adequately assess inter-observer agreement in our environment. Furthermore, the different grading system used, 2005 ISUP grading system by Melia vs 2014 ISUP Gleason modification in this study may have contributed to the difference in intra and inter-observer agreement. Thus, it may be inferred that the 2014 ISUP Gleason modification may provide a better reproduction of the Gleason patterns. Other studies that used the 2005 ISUP grading system reported inter-observer and intra-observer agreement similar to that of Melia *et al*. Griffiths *et al* reported an intra-observer agreement kappa of 0.07 to 0.82; some of their participants had an intra-observer agreement similar to our study. [49] The inter-observer agreement before and after 40-minute training

session were fair (kappa = 0.33) and moderate (kappa = 0.41) agreement respectively. In our study, the training session lasted for a week and this explains the marked improvement from poor agreement (kappa = 0.06) to excellent agreement (kappa = 0.91). This further buttress the importance of training and teaching session in improving reproducibility of Gleason score. In this study, there was no ISUP grade group 5 tumour. The ISUP grade groups were from 1 to 4 and the most seen was grade group 1 which represented 36.4% of the cases seen. In Leapman *et al* study, ISUP grade group 1 also constituted the majority of the cases and it constituted 64% of cases seen. [50] The wide variation in the percentages (36.4% vs 64%) could be due to the difference in sample size and study design; 33 cases were used in our study whereas Leapman and associates used 10529 cases which came from a combination of 43 centres enrolled in the Cancer of the Prostate Strategic Urologic Research Endeavour (CaPSURE). Furthermore, Leapman study spanned for 20 years (1995 to 2014) while ours was for a year. The high frequency of ISUP grade group 1 tumours in both studies might reflect the PSA-based prostate screening which has led to early detection of prostate cancer.

The Ki-67 proliferation index in this study ranged between 0 – 38% which corroborated with a similar study done in Nigeria by Phillips *et al* (unpublished data) which reported a range of 0 – 35%. Similarly, Burbendorf *et al* and Fisher *et al* reported a range of 0 - 33% and 0 - 30% respectively. [17,9] This shows the diversity in proliferative activity in prostate cancer; some cases have very low Ki-67 proliferation index which is comparable to that seen in benign lesion while other have proliferation activity comparable to aggressive malignancies. Some other authors have reported lower proliferation index range in their studies; 0 – 0.4% by Nowak *et al* and 0.1 – 18% by Goltz *et al*. [12,11] This disparity in figure could be due to method of antigen retrieval, type of wax used during tissue processing and how the tissue blocks were stored prior

to the study. Other factors that can affect immunohistochemical stain include inadequate optimization of antibodies and low concentration of antibodies. The median Ki-67 proliferation index in this study was 4.65% and this corroborated to the median / mean values which have been reported by previous researchers. Keshgegian *et al* and Glotz *et al* reported a median value of 6.4% and 2% respectively. [38,11] Burbendorf *et al* and Fisher *et al* reported a mean value 7.5% and 3.9% respectively. [9,17] Irrespective of the measure of central tendency used the mean / median values were below 8% which may suggest that most prostate cancer is a low proliferating malignancy. The median Ki-67 proliferation index for the benign prostatic hyperplasia (control) cases was 0.75%, this is much lower than the median value (4.65%) for malignant cases which is in-line with Park *et al*'s studies which reported that the proliferative activity is higher in prostatic adenocarcinoma than benign prostatic hyperplasia. [15] Furthermore, the lower median value in the control cases justifies the proliferation index values of the malignant cases recorded in this study. Ki-67 is the most studied biomarker in prostate cancer. In our study, the Ki-67 proliferative index showed statistically significant association with the ISUP grade groups. Although, the ISUP grade group was used in this study, study that were done using the Gleason score showed similar findings. Abdelbary *et al*, Keshgegian *et al*. Fisher *et al*. Mitra *et al* and Park *et al* demonstrated a statistically significant association between Ki-67 proliferation index and Gleason score. [32,38,9,10,15] Thus, irrespective of the grading system used Ki-67 proliferation index remains a powerful prognostic tool in prostate cancer. However, there needs to be a consensus on what should be used as the cut-off. Most studies make use of either the mean or the median value as the cut-off, in this study the median value was used as the cut-off. It is recommended that the Ki-67 proliferation index should be introduced into clinical practice once a consensus has been reached on the cut-off value. Only

one out of the ten cases of ISUP grade group 1 tumours in our study had a Ki-67 proliferation index above the median value. Thus, Ki-67 proliferation index may produce additional information which may be beneficial for patients considering watchful waiting / active surveillance. Furthermore, it may be help guide management decisions for elderly patients with ISUP grade group 2 and 3 tumours whose life expectancy is less than 10 years. In such cases, if the Ki-67 proliferation index is below the cut-off, the patient may decide to opt for watchful waiting which is not the usual method of management for this group of patients. Aside from Gleason score / ISUP grade group, Ki-67 proliferation index has been shown to be predictive of tumour stage, biochemical progression, metastasis and death. [32, 31, 15, 9] The present study only assessed the association of Ki-67 proliferation index with ISUP grade groups. It is recommended that an expanded and prospective study should be done to assess its association with the above mentioned clinicopathological parameter in our environment. Some studies did not show statistically significant association between Ki-67 proliferation index and Gleason score. This may be due to different study designs used, difference in the grading system used in the studies which is based on the year the study was done. Perhaps, if the studies graded the tumours using the 2014 ISUP Gleason modification which was used in our own study the outcome may have been different.

PHH3 is a core histone protein and together with the other histone proteins they form the major protein constituent of chromatin. [40] Only a few studies have assessed the predictive value of PHH3 in prostate cancer. [11,12,41] To the best of our knowledge, no study has assessed its prognostic significant in Africa. In this study, the PHH3 proliferation index ranged between 0.05 to 4.4%. The mean and median values were 0.4 and 0.85% respectively. Different studies have reported different range and median values. Nowak *et al* reported a range of 0.01 to 0.265% and

a median value of 0.04%; Goltz *et al* reported a range of 0 to 0.52% and a mean value of 0.0152%; and Braun *et al* reported a mean value of 0.28%, 1.90% and 7.46% for non-metastatic, nodal metastatic and distant metastatic cases respectively.[12,11,41] This wide disparity could be due to the type of PHH3 antibodies used in the different studies although the rabbit polyclonal PHH3 was used in Nowak *et al* study and Goltz *et al* study.[12,11] It was not specified if it was the anti PHH3 (ser 10) antibody which detects histone 3 when it is phosphorylated at serine 10 or the anti PHH3 (ser 28) antibody that specifically detects histone 3 when it is phosphorylated at serine 28. According to Sun et al study, tissue reactivity for PHH3 (ser 10) is more than that of PHH3 (ser 28). [40] Although their study did not assess the reactivity of these two PHH3 antibodies in prostate cancer, the study examined at least five malignancies which include breast cancer, melanoma, ovarian cancer, gastric cancer and colorectal cancer. In some malignancies like colorectal and breast cancer, PHH3 (ser 28) was not expressed by the malignant cells but PHH3 (ser 10) was expressed. Thus, there is a need to determine the level of expressivity of the two PHH3 antibodies in prostate cancer and a consensus should be made as regards which of the two PHH3 antibodies is better for prostate cancer research. Other factors that can affect expression of protein, which have been mentioned earlier, include lack of optimisation of antibodies and inadequate antigen retrieval method. Furthermore, tissue microarray was used in Goltz *et al* and Nowak *et al* study; the use of tissue microarray would have resulted in limited amount of malignant cells that would express PHH3. Despite the disparity in the level of expressivity, PHH3 shows a statistically significant association with Gleason score and ISUP grading system. In the present study, it showed a positive correlation with the ISUP grade group with a Spearman correlation rho of 0.714. This corroborated the findings in Goltz *et al* study which reported a rho value of 0.174. Although their rho value is much lower than our study, this

could be due to the disparity in the PHH3 proliferation index range and the different material used in the studies i.e. tissue microarray vs core biopsy. In addition, Glotz *et al* did not use the 2014 ISUP Gleason modification which has been said to give a better clinical assessment. Like Ki-67 proliferation marker, the different Gleason systems used in the two study did not affect the predictive outcome of PHH3. The Spearman's rho of 0.714 recorded for the correlation between PHH3 proliferation index and ISUP grade group was slightly higher than the Spearman's rho of 0.6818 observed for the correlation between Ki-67 proliferation index and the ISUP grade group. This may suggest that PHH3 is a better predictor of prognosis in prostate cancer. However, this observation needs to be verified by a more extensive study. There was no statistically significant correlation between PHH3 proliferation index and Gleason score in Nowak *et al* study. The lack of correlation could be due to the use of tissue microarray. Furthermore, the study used the 2005 Gleason grading system. This study also used Pearson's chi square to assess the association of PHH3 proliferation index with ISUP grade groups and a statistically significant association was also demonstrated (p-value = 0.0003). The median value was used as a cut-off value in this study. Cut-off was not used in Nowak *et al* study but Glotz *et al* used the 90^{th} percentile (0.0335%) as the cut-off in their own study. Like the Ki-67 proliferation marker, there is a need to reach a consensus on what should be agreed as the cut-off before the PHH3 proliferation index can be introduced to clinical practice. Due to the paucity of research work on PHH3 on prostate cancer, there is a need for extensive studies to be done in different part of the world before a consensus can be reached on the cut-off point. Aside from its association with the Gleason score and ISUP, PHH3 proliferation index has also been shown to correlate with other clinicopathological parameters like aneuploidy [41], biochemical recurrence [11], adverse outcome for ERG+ or AR+ (androgen receptor positive) prostate cancer patients [11]. The

present study did not assess for other parameters other than Gleason score. Thus, it is recommended that a more elaborate study should be done to assess the association of PHH3 proliferation index with other clinicopathological parameters and management outcomes in Africa.

Apoptosis is a pathway of cell death that involves activation of a well-orchestrated suicide program during which the cell predestined for death activates intrinsic enzymes that destroy its own protein and DNA. [53] The process is tightly regulated by p53 and Bcl-2 family. [16] The Bcl-2 family comprises two subfamilies of proteins; proapoptotic proteins (Bax. Bak and Bcl-2-like protein 11[Bim]) and anti-apoptotic proteins (Bcl-2 and B-cell lymphoma-extra large [Bcl-xl]). [16] Quite a number of studies have assessed the prognostic significant of Bcl-2 in prostate cancer [15 - 17, 33 – 39, 54] with mixed results. In this study, we noticed that Bcl-2 stained basal cells in non-malignant glands, and lymphocytes surrounding some of the prostatic glands. This finding is consistent to what have been documented by previous authors; Krajewska *et al* documented that it is expressed by non-androgen dependent basal cells that line the basement membrane of prostate glands [55], Rubio *et al* reported that it is not expressed in the secretory cell in normal prostate [37] and Matsushima mentioned its present in infiltrating lymphocytes.[35] From the above observation, it can be inferred that Bcl-2 is a marker of basal cells. Thus, like p63 and CK5/6, it could be used to distinguish between malignant prostatic glands which lack a basal layer and non-malignant prostatic glands with preserved basal layer. However, before this can be done there is a need to carry out a study to compare the sensitivity and specificity of p63, CK 5/6 and Bcl-2 in differentiating between malignant and non-malignant prostatic glands. The scoring methods have been used by different researcher to quantify Bcl-2 immunohistochemistry. The present study and some other studies used apoptotic index (number

of stained malignant cells divided by total number of malignant cells in a field expressed in percentage) and some researcher used the H-score which is a combination of intensity and percentage of stained cells. It is difficult to say which of the two scoring methods is more reliable, but it would be better if a single scoring system is adapted as this would make it easier to compare the apoptotic index in different studies. In this study, 94% of the cases showed positivity for Bcl-2. This is quite high when compared to other studies; Yoshino *et al* in Japan reported 68.4% positivity in hormone resistance prostate cancers [36], Anavari *et al* in Iran reported 70.3% positivity [16], Bauer *et al* in Switzerland reported 26.9% positivity [34] and some other authors have reported as low as 2.3% positivity [39]. The disparity in the figures could be due to some of the factors which have been mentioned earlier that affect immunohistochemistry. Furthermore, it could be that apoptosis is more pronounced in prostatic cancer specimens in Africa. More regional studies will be required to refute or confirm this hypothesis. In this study, the Bcl-2 apoptotic index ranged from 1.3% to 27.5% and the mean value was 10.4%. Keshgegian *et al* and Burbendorf *et al* reported 0 - >50%.[38,17] Again, the disparity could be due to factors affecting immunohistochemistry. The present study showed a statistically significant association between the Bcl-2 apoptotic index and ISUP grade groups. This corroborated with the studies done by Bauer *et al* in Switzerland, Anvari *et al* in Iran, Yoshino *et al* in Japan, Park *et al* in South Korea and Rubio *et al* in Spain.[34,16,36,15,37] Despite the difference in scoring methods used by the authors, the prognostic value of Bcl-2 was confirmed. The lack of association reported by Johnson *et al* could be due to the low level of expression in their study; only one out of the 43 cases showed positive cytoplasmic staining. If the expressivity was higher there might have been a positive association between the Gleason score and Bcl-2 apoptotic index. Bubendorf *et al* and Matsushima *et al* also reported no

statistically significant association between Gleason score and Bcl-2.[17,35] The discordance is association could be due to the difference in study design and the Gleason grading system used; both studies used the pre-2005 Gleason grading system while the 2014 ISUP modification of the Gleason grading system was used in this study.

The growth of a neoplasm is dependent on proliferation of tumour cells and programmed cell death within neoplastic lesion. Thus, interaction between proliferative and apoptotic activities is very important. In this study, there was a statistically significant association between proliferation and apoptosis as measured by Ki-67 proliferation index and Bcl-2 apoptotic index respectively (p-value = 0.0001). The Spearman correlation rho was 0.8559. This may imply that the high ISUP grade group tumours with high proliferative activity will also have a significant amount of apoptotic cells which may explain the reason why some high-grade prostatic malignancies do not respond to hormonal therapy and radiation therapy as these forms of therapy are known to stimulate apoptosis and Bcl-2 prevents apoptosis. Further research is required to confirm this hypothesis and if found to be true, therapy that will target Bcl-2 and its related proteins could be developed to treat high ISUP grade group prostate cancer and hormone refractory prostate cancers.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this study demonstrated that prostatic adenocarcinoma is a disease of the elderly and the 60 – 69 year age group is the most afflicted in South Africa, other African countries and most parts of the world. Our study confirms that the 2014 ISUP modification of the Gleason grading system has a high intra and inter-observer reproducibility and this justifies the tremendous amount of research work that was done to during the 2014 ISUP modification of the Gleason. Aside from the architectural and cytomorphology of malignant prostatic glands, a thorough examination of the prostatic stroma is very important in making and supporting the diagnosis of prostate cancer. The presence of a loose, oedematous myxoid stroma or extracellular matrix-rich reactive stroma should raise suspicion of a malignancy in prostatic tissue.

This study demonstrated the prognostic importance of Ki-67, PHH3 and Bcl-2 in prostatic adenocarcinoma. These markers showed a statistically significant association with the Gleason score which is a quintessential prognostic tool in prostate cancer. The correlation between Ki-67 and Bcl-2 which was demonstrated in this study may give an indication to why prostatic malignancy with high Gleason score tend to develop resistance to radiation and hormonal therapy which rely of the stimulation of apoptosis.

Our recommendations are as follows:

1. Commencement of PSA-based prostate cancer screening programme at the age of 50 years in regions and countries that are yet to commence the programme.
2. The programme should be uninterrupted in countries and regions where it is in place.

3. Continuous training and re-training for pathologists-in-training, urologist trainees and oncologists-in-training on the use and importance of the 2014 ISUP Gleason modification and the ISUP grading system.
4. Consensus meeting on what should be used as the cut-off proliferative index value for Ki-67.
5. Multi-centre and multi-national research in different parts of the world to further assess the prognostic value of PHH3 in prostatic cancer because only a few research studies are available on this biomarker with respect to prostatic cancer.
6. Multi-national research in Africa to assess the prognostic significance of Bcl-2 in prostate cancer patients on our continent.
7. Introduction of proliferative marker and apoptotic marker as a prognostic tool in the clinical management of prostate cancer.

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ANNEXURE

APPENDIX 1

DATA COLOLECTION SHEET

- SERIAL CASE number :
- LABORATORY number :
- AGE :
- GLEASON SCORE
    a. Initial (Original slide) :
    b. Primary investigator's :
    c. Supervisor's :
- GRADE GROUP :

	PERCENTAGE OF POSITIVELY-STAINED CELLS IN EACH FOCUS											
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**AVERAGE**	**SINGLE DIGIT SCORE**
% of Ki-67-stained cells												
% of PHH3-stained cells												
% of Bcl-2-stained cells												

- Specific morphological features:

APPENDIX 2

INTERNATIONAL SOCIETY OF UROLOGICAL PATHOLOGY 2014 GLEASON MODIFICATION AND GRADE GROUP.

HISTOLOGICAL DEFINITION OF GLEASON PATTERN:

- Gleason pattern 1: Comprises tumours with closely packed uniform sized and shaped glands.
- Gleason pattern 2: Comprises tumours with variation in size of glands, shape of glands and glands with in-folding.
- Gleason pattern 3: Comprises tumours with branching glands with variation in size and shape, occasional tangentially sectioned glands amongst well-formed small glands with open lumina and back-to-back discrete glands.
- Gleason pattern 4: Comprises tumours with cribriform glands with well-formed lumina or cribriform lumina; fused glands; poorly-formed glands with peripherally arranged nuclei and glomeruloid pattern.
- Gleason pattern 5: Comprises tumours with solid nests with or without rosette-like areas; tumours with unequivocal comedonecrosis even when focal; discrete glands with necrotic debris within the lumen and individual malignant cells.

NB

- A Gleason pattern 4 should only be made at x100 magnification
- A lower pattern should be favoured in cases with borderline morphology between Gleason pattern 3 and 4.
- It should be noted that Gleason pattern 1 and 2 are not assigned in core biopsy specimen.

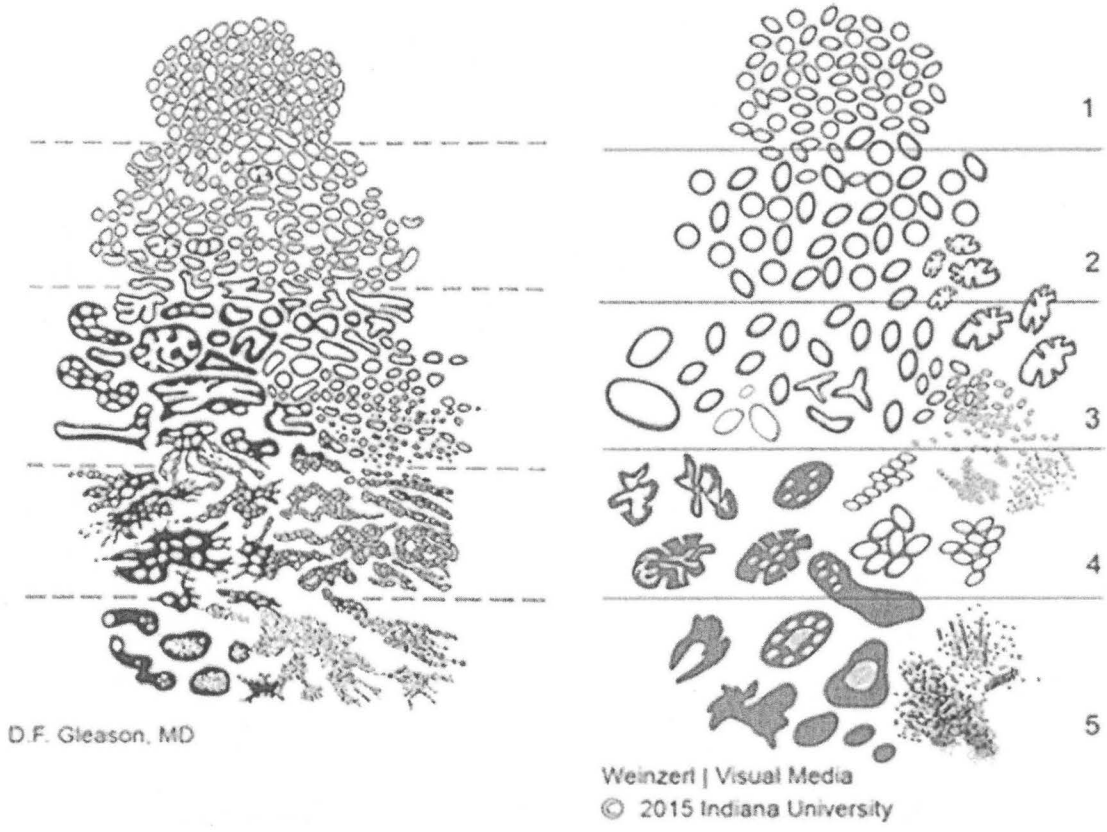


FIGURE 2. Prostatic adenocarcinoma (histologic patterns): original (left) and 2015 Modified ISUP Gleason schematic diagrams.

Epstein, J.I., Egevad, L., Amin, M.B., *et al.* 2016. The 2014 International Society of Urological Pathology (ISUP) consensus conference on Gleason grading of prostatic carcinoma: definition of grading patterns and proposal for a new grading system. *Am J Surg Pathol.* 40(2): 244-252.

DEFINITION OF THE GRADE GROUP SYSTEM.

1. Grade Group 1 (Gleason score < 6): It comprises tumours with only individual discrete well-formed glands.
2. Grade Group 2 (Gleason score 3+4=7): It comprises tumors with predominantly well-formed glands and a lesser component of poorly-formed, fused and/or cribriform glands.
3. Grade Group 3 (Gleason score 4+3=7): It comprise tumors with predominantly poorly-formed, fused and/or cribriform glands and a lesser component of well-formed glands.
4. Grade Group 4 (Gleason score 4+4=8; 3+5=8 and 5+3=8): It comprises tumours with any of the following,
    a. Only poorly-formed, fused and/or cribriform glands (4+4=8)
    b. Predominantly well-formed glands and a lesser component lacking glands (3+5=8).
    c. Predominantly lacking glands and lesser component well-formed glands (5+3=8).
5. Grade Group 5 (Gleason scores 9 and 10); it comprises tumours that lack gland formation or shows areas of necrosis with or without areas with poorly-formed, fused and/or cribriform glands.

NB

- It is not unusual to find poorly-formed, cribriform or fused glands as a minor component of 3+5 or 5+3.

APPENDIX 3: GANTT CHART

	MONTH	JUNE				JULY				AUGUST				SEPTEMBER				OCTOBER				NOVEMBER				DECEMBER				JAN 2017	DEC 2017
	WEEK	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4		
	OBJECTIVE																														
1	Ethical approval from School																														
2	Data retrieval from Track care																														
3	Selection of cases.							x																							
4	Retrieval of blocks and slides																														
5	Examination of blocks & slides/ case selection 2																														
6	Gleason score recording																														
7	Immuno stains																														
8	Immuno review																														
9	Data analysis																														
10	Discussion/Conclusion																													x	x	x	X	X	X	X	X

R14/49 Dr Adekoyejo Abiodun Phillips

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1606101

NAME: (Principal Investigator)	Dr Adekoyejo Abiodun Phillips
DEPARTMENT:	Anatomical Pathology Department of Anatomical Pathology University of the Witwatersrand
PROJECT TITLE:	The Prognostic Significance of PHH3, Ki-67 and Bcl-2 IN Prostate Cancer
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-Study
SUPERVISOR:	Melanie Louw
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	20/07/2016

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 in Room 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised 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 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 review in June and will therefore be due in the month June each year.

M L Louw
Principal Investigator Signature

Date 21/06/2018

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES