COMPARISON AND CORRELATION OF KI67 EXPRESSION AND GLEASON SCORE IN PREDICTION OF BIOLOGICAL BEHAVIOUR OF PROSTATIC ADENOCARCINOMA IN TANZANIAN PATIENTS

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By

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Medicine (Anatomical Pathology) of Muhimbili University of Health and Allied Sciences

Muhimbili University of Health and Allied Sciences

September 2013

CERTIFICATION FOR THE DEGREE AWARD

The undersigned certify that they have read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled ***Comparison and correlation of Ki67 expression and Gleason score in the prediction of biological behaviour of prostatic adenocarcinoma in Tanzanian Patients,*** in partial fulfilment of the requirements for the degree of Master of Medicine (Anatomical Pathology) of Muhimbili University of Health and Allied Sciences.

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(Supervisor)

Date: _____

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Prof. J.N. Kitinya

(Supervisor)

Date: _____

DECLARATION AND COPYRIGHT

I, **Belson Rugwizangoga**, declare that this **dissertation** is my own original work and that it has not been presented and will not be presented to any other university for a similar or any other degree award.

Signature……. Date…….....……..

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"May each one of you find through this work, the fruit of the unforgettable rendered service".

DEDICATION

To Almighty my God

To my beloved wife Vénantie Uwimana

To my precious son Cierto Rey Ngoga

To my dear parents

To all my family

To God's Flock Choir Family

To all my teachers

To all my friends and colleagues,

This work is dedicated.

ABBREVIATIONS

%:	Percentage
AMACR:	α-methylacyl-CoA-racemase
BSO:	Bilateral subcapsular orchiectomy
CAB:	Combined (or complete) androgen blockade
CD:	Cluster of differentiation
CEA:	Carcinoembryonic antigen
CI:	Confidence interval
CK:	Cytokeratin
CPL:	Central Pathology Laboratories
CT:	Computerized Tomography
DAB:	Diaminobenzidine
DHT:	Dihydrotestosterone
DHT–AR :	dihydrotestosterone–androgen receptor complex
DNA:	Deoxyribonucleic acid
DPX:	distyrene plasticiser xylene
EMA:	Epithelial membrane antigen
EMT:	Epithelial-to-mesenchymal transition
ERG:	ETS (E twenty-six) related gene
ETS:	E twenty-six
ETV1:	ETS (E twenty-six) variant 1
H_2O:	Water
H_2O_2:	Hydrogen peroxide
H&E:	Haematoxylin and eosin
HIV:	Human immunodeficiency virus
HPIN:	High-grade prostatic intraepithelial neoplasia
HRP:	Horse radish peroxidase
IHC:	Immunohistochemistry
ISUP:	International Society of Urological Pathology
kDa:	Kilodalton
LHRHa:	Luteinising hormone-releasing hormone analogue

M:	molar (concentration)
MAB:	Maximal androgen blockade
mL:	Millilitre
mm^2	Squared millimetre
MNH:	Muhimbili National Hospital
MRI:	Magnetic resonance imagery
mRNA:	messenger ribonucleic acid
MUHAS:	Muhimbili University of Health and Allied Sciences
ng:	nanogram
NSE:	Neuron-specific enolase
ORCI:	Ocean Road Cancer Institute
PAP:	Prostatic acid phosphatase
PBS:	Phosphate buffered saline
PCa3:	Prostate cancer antigen 3
PDEF:	Prostate-derived ETS factor
pH:	*Potenz* (German: Power) + *H*: the reciprocal of hydrogen-ion concentration
PNI:	Perineural invasion
PSA:	Prostate-specific antigen
RNA:	Ribonucleic acid
SPSS:	Statistical package for social sciences
TA:	Transactivating
TNM:	Tumour, node (lymph node), metastasis
TMPRSS2	Transmembrane protease, serine 2
TURP:	Transurethral resection of prostate
US, (USA):	United States (of America)
VACURG:	Veterans Administration Cooperative Urological Research Group
WHO:	World Health Organization
$\chi 2$:	Chi-square

DEFINITIONS OF TERMS

1. **Staging:**

Staging is an act of assigning a stage to a process. A stage is a definite or distinct phase as of development of a disease.

In Oncology, a stage in the extent of a cancer, especially whether it has spread or metastasized, the determination of which is used to guide future therapy and determine prognosis (McGraw-Hill Concise Dictionary of Modern Medicine, © 2002 by The McGraw-Hill Companies, Inc.).

In this study, the statistical analysis was based on the standard prognostic grouping system as presented in Background, and this comprises the clinical staging (tumour, node involvement, distant organ metastasis) combined with Gleason score and serological testing of prostate-specific antigen.

2. **Progression**

Progression is the process of spreading or becoming more severe (Dorland's Medical Dictionary for Health Consumers. © 2007 by Saunders, an Imprint of Elsevier Inc.).

In this study, progression refers to a development of documented sign of the prostate cancer becoming more severe, that is, to a more advanced stage, or the same stage but with new element, for example development of a new metastasis, any other disease-related complication.

3. **Survival**

Survival is defined as struggle to remain living. Survival analysis is a statistical technique to analyse longevity. In this study, survival duration/time is the period of time from the date of diagnosis to event (death or end of the follow-up period).

4. **Outcome**

An outcome is an end result. It is, in the context of this study, defined as the condition of a patient at the last follow up (death, lost to follow-up or alive at the expiration of study period). (Mosby's Medical Dictionary, 8th Edition, © 2009, Elsevier Inc.).

5. **Biological behaviour:**

Biological behaviour, in this study, refers to parameters which characterise the degree of advancement of the prostate cancer disease. They include stage, serum PSA levels, extent of tumour in the prostate, progression and survival duration since the time of diagnosis.

ABSTRACT

Background

Prostate cancer is the second most commonly diagnosed and sixth most lethal cancer in men worldwide, affecting Blacks more than Whites and Asians, with geographical variations. Not all men diagnosed with prostate cancer will develop a progressive disease; some cancers remain indolent and usually need active surveillance, while others are aggressive thus needing treatment. Gleason score is the most recognized of progression-associated prognosticators, but it misses some cases. Development of prostate cancer progression marker is anxiously awaited, to detect prostate cancer progressive cases missed by Gleason score system. Proliferation markers have been shown to be involved in progression of some human tumours. Ki67 expression contribution in predicting prostate cancer biological behaviour needs to be determined, for use in a combination to Gleason score to offer better prognosis information.

Aim

To compare and correlate Gleason score with Ki67 expression in predicting the biological behaviour of prostatic adenocarcinoma in Tanzanian patients, in regards to prostatic-specific antigen (PSA), stage, progression of the disease and survival duration.

Methods

256 cases of prostatic adenocarcinoma diagnosed at Muhimbili National Hospital (MNH) from January 2005 to December 2006 were retrieved; 214 were retained in the study. The types of specimens were 188 (87.9%) core biopsies, 24 (11.2%) transurethral resection of prostate (TURP) and 2 cases (0.9%) of bone biopsies. A retrospective follow-up of 48 patients was done over 60 months using patients' files; 42 excluded cases consisted of 29 cases missing tissue blocks and 13 cases without clinical data. Sections of archival tissue blocks were stained with Haematoxylin and Eosin (H&E) and MIB-1 to respectively determine Gleason score and Ki67 index by determining percent of positive cells among at least 500 cells in hotspots. Gleason score was correlated and compared to Ki67 index in terms of PSA, stage, disease

progression and patient's survival among others. Data entry and analysis were done through computer software; P value and 95% confidence interval (CI) were used as measure of variable association.

Results

Prostatic cancer was diagnosed in 34.73% (256/737) of prostate specimens; all cases were acinar adenocarcinoma. Mean age was 70.5 years, with the range of 42 to 96 years. Clinical data suffered poor documentation. Pre-treatment PSA levels were high, mean was 79.578 (2.32 to 500.00) ng/mL. 68.0% had advanced clinical disease, 26.1% had distant metastases. High grades predominated (Gleason score 8-10 in 34.6%, Gleason score 7 in 38.3% of cases). Median Ki67 index in prostatic adenocarcinoma was 10.39% (range 0.0% to 50.94%). 48 cases were followed up along 0.56 to 60 months (mean was 31.2 months); 2 of 15 patients were alive at last follow-up. The mean survival of fully followed-up cases was 24.50 months (95% CI=13.09 to 35.91 months). Gleason score correlated with age (P= 0.000, 95% CI=70.705 to 71.676 years), pre-treatment serum PSA level (P=0.001; 95% CI=75.45 to 84 ng/mL), average tumour extent in tissue (P= 0.000; 95% CI=66.26 to 69.49%), tumour stage (P=0.009), survival duration (P=0.029) and patient's outcome (P=0.011). Ki67 index decreased with age (P=0.006), increased with Gleason score (P=0.001) and average tumour extent (P=0.032), correlated with disease progression (P=0.020; 95% CI= 1.163 to 6.885) and survival duration (P=0.21). Pre-treatment PSA level significantly correlated with Gleason score but not with Ki67 index; Gleason score rather than Ki67 index predicted tumour stage; both Gleason score and Ki67 index similarly predicted survival duration, but Ki67 index depicted progressive cancer disease cases missed by Gleason score system.

Conclusion

A combination of Gleason score and Ki67 index offers better prognostic information than either alone. The challenge to Ki67 index use is its cost and time consumption. A multicentre prospective study is recommended to confirm these findings. Standard documentation and preventive education on prostate cancer should be implemented and monitored in Tanzania.

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CHAPTER 1. BACKGROUND

1.0 Introduction

Urological diseases are a major problem to the general population worldwide [1]. Among these diseases, prostate cancer is nowadays the second most commonly diagnosed and the sixth most lethal cancer in males worldwide [2]. The prostate is an organ which plays important roles in semen production in men [3]. With aging, this gland has the potential to acquire atrophic, hyperplastic, metaplastic and dysplastic changes and eventually prostatic cancer [3]. It is essential to outline the developmental anatomy, physiology, histology and other biological behaviour of the normal prostate, in order understand the biological behaviour and pathological changes leading to the life-threatening cancer of the prostate.

1.1 Prostate

1.1.1 Overview

1.1.1.1 Introduction

The prostate is a bulky gland with internal and external secretion pertaining to the male genital apparatus; it is located under the urinary bladder, in the confluence of the genital and urinary tracts, crossed by the urethra and incompletely surrounded by the striated sphincters [4]. The prostate is the seat of 3 main affections: prostate cancer, prostatic hyperplasia/hypertrophy and prostatitis. Prostate adenocarcinoma is influenced by the hormones [1, 3, 5].

1.1.1.2 Developmental anatomy

The prostate gland develops during the twelfth week of gestation under the influence of androgenic hormones produced by the foetal testis [3]. The epithelial outgrowths of urogenital sinus endoderm develop into earliest prostatic ducts [3, 5]. The primitive mesenchyme associated with these epithelial outgrowths differentiates into fibromuscular elements. The glands distal to the verumontanum arch into the peripheral posterolateral region of the gland. The glands of the peripheral and transition zone develop from endoderm of the urogenital sinus. The glands of the central zone are thought to be of Wolffian duct origin [3].

1.1.1.3 Prostate in adult

1.1.1.3.1 Anatomy of the prostate

The prostate of an adult man is funnel shaped and weighs 20 grams; it measures 4x3x2 centimetres [3]. It is located within the true pelvis between bladder neck considered as base of prostate and urogenital diaphragm made up of levator ani muscle, considered as apex of prostate. The seminal vesicles extend from the posterior aspect of the prostate to posterior surface of the bladder. The prostate and seminal vesicles are separated from the rectum by Denonvillier's fascia [3, 5].

There exist different anatomical models of the prostate gland [1]. The current model is called McNeal model (Figure 1); it defines transitional, central, peripheral and periurethral zones; the outer or cortical zones are termed "peripheral"; "central" is towards base and, the inner or periurethral zone is termed "transitional" [6].

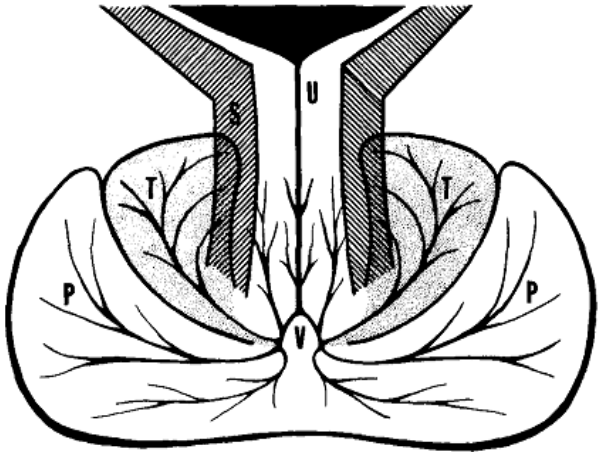


Figure 1. Diagram of the prostate in its original McNeal anatomical model

The proximal ducts of the peripheral zone (P) radiate laterally from the verumontanum (V). Ducts of the transition zone (T) exist from urethra just proximal to the base of the verumontanum, lying between the peripheral zone and the sphincter (S). Periurethral ducts arising along the proximal urethra are confined to the periurethral zone (U) and run proximally toward the bladder [6].

Blood supply is from the inferior vesical arteries which are branches of the internal iliac arteries [3]. Prostatic veins drain into the prostatic plexus and eventually into the internal iliac veins [3]. The lymphatics drain mainly into the internal iliac lymph nodes, while others drain to the external iliac and sacral lymph nodes [3]. A rich neural plexus supplies the prostate, and multiple autonomic ganglia are located near the capsule. The perineural spaces are of least resistance, thus the adenocarcinoma spreads through them [3, 5].

1.1.1.3.2 Physiology

The prostatic exocrine secretion is a major component of seminal fluid [3, 4]. The main components of the prostatic secretion are acid phosphatase and citric acid, and the proteolytic enzymes involved in the liquefaction of coagulated human semen which include fibrinolysin, fibrinogenase, and aminopeptidase [3]. The sympathetic and parasympathetic stimulations partly control prostatic secretion through stromal smooth muscle fibre contraction [3]. Human prostate gland contains high concentrations of zinc which are under hormonal regulation [1, 3]. The prostate carcinoma cells show a marked and persistent decrease in zinc and citrate levels [3].

The main circulating androgen which is testosterone is metabolized into the active intracellular hormone dihydrotestosterone (DHT) by the action of the enzyme 5-α-reductase [7]. DHT binds to a cytosolic androgen receptor and is transported to the nucleus where the DHT receptor complex induces ribonucleic acid (RNA) and protein synthesis as well as cell replication [7]. Alterations in hormonal production which occur with advancing age may result in the development of hyperplasia and carcinoma [8, 9, 10].

1.1.1.3.3 Histology of the prostate

The transition zone represents 5% of the prostatic volume [1, 3, 4, 5]. It is the site of nodular prostatic hyperplasia. The large duct carcinomas arise from the transitional zone and comprise 10% of prostate cancers [1, 3, 5].

The central zone comprises 25% of prostatic volume and is the site of 5% of prostate cancers [3, 5]. The ducts of central zone are large and irregular; glands are complex with tall columnar, pseudostratified epithelium with papillary infoldings. The stroma is denser in central zone than in transition zone and than in peripheral zone respectively [7].

The peripheral zone makes up 70% of prostatic volume [1, 3, 4, 5]. It is a site of 80% of prostate cancers [1, 3, 5]. It has moderate gland complexity and loose fibromuscular stroma with spaced smooth muscle bundles [3, 5].

The “capsule” consists of fibromuscular layer which is most prominent along base and posterior portion of lateral borders, and is an inseparable component of prostatic non-glandular stroma [3]. The stroma contains abundant smooth muscle, which squeezes out secretions [3, 4].

The aging prostate gland is commonly affected by both atrophy and hyperplasia [3]. Atrophy usually occurs after the sixth decade of life but can be seen from the third decade. The aging atrophy is diffuse and involves the peripheral zone [3]. The atrophic glands are usually organized, and though crowded, the glands are regularly distributed in the stroma; the basal cell layer is maintained and there is no atypia [3, 6].

1.1.1.3.4 Types of cells and immunohistochemical features

The cell types in prostate consist of secretory cells, basal cells, scattered neuroendocrine cells, urothelium and ejaculatory duct/seminal vesicle cells [4]. The secretory cells are located along glandular lumens [4, 5]. They stain positively with prostatic acid phosphatase (PAP), prostate-specific antigen (PSA), vimentin, keratin, Leu7/CD57, epithelial membrane antigen (EMA) in 80% of the cases, and carcinoembryonic antigen (CEA) in 25% of the cases [3, 11]. The secretory cells don’t stain with a high molecular weight keratin CK903 (antibody also called 34βE12) [3].

The basal cells consist of low cuboidal epithelium and may have prominent nuclear grooves and prominent nucleoli [4, 5]. The basal cells are present in normal and benign conditions, while they are absent in adenocarcinoma. The absence of basal cells is determined on routine microscopy in many cases, but a high molecular weight cytokeratin stain may be required in difficult interpretations [3]. They stain positively with CK903, p63 and androgen receptors; they don't stain with PSA, PAP, S100 and actin [3].

The neuroendocrine cells are haphazardly distributed in prostate glands [12]. They stain positively with chromogranin A, B, secretogranin II, peptide hormones and PSA. They don't stain with androgen receptors [4].

The urothelium is found in the proximal 2 millimetres of the prostatic ducts [1, 3, 5]. The ejaculatory ducts and seminal vesicles are lined by double cell layer of pseudostratified epithelium and contain a golden-brown lipofuscin pigment; their nuclei are large, hyperchromatic (monster) nuclei; and they may have intranuclear inclusions [3, 4, 8]. Normal mucins are neutral, while most adenocarcinomas secrete acidic and neutral mucins [3, 4].

The prostatic stromal cells may be highlighted with antibodies against vimentin, desmin, pan-muscle actin, and smooth muscle actin; however, normal prostatic epithelium may also show focal staining for vimentin [3].

1.1.1.3.5 Prostate-specific antigen

Prostate-specific antigen (PSA) was discovered in 1979 as a glycoprotein specific to the prostate [13]. PSA appears at the time of puberty [3]. The antibodies directed against PSA show diffuse and strong positivity in secretory cells of normal and hyperplastic glands from any prostatic zones. PSA staining is also observed in periurethral glands in both males and females, anal glands in males, urachal remnants, cystitis cystica and cystitis glandularis of the urinary bladder [3], as well as in the extraprostatic tumours including urethral and bladder adenocarcinoma, extramammary Paget's disease of the penis, some gastrointestinal

adenocarcinomas, mature teratomas, salivary gland pleomorphic adenoma and salivary gland carcinomas in males [14, 15].

1.1.1.3.6 Prostatic acid phosphatase

The role of prostatic acid phosphatase (PAP) in the diagnosis and follow-up of patients with metastatic prostate adenocarcinoma was established in 1938 [16] but has been superseded by serum PSA as a prostatic tumour marker. PAP is found in normal and hyperplastic prostate glands and also in most prostatic adenocarcinomas [17]. PAP is also identified in some normal tissues and neoplasms of nonprostatic origin [1, 14, 18] including pancreatic islet cells, parietal cells of the stomach, adenocarcinoma of the bladder, cloacogenic carcinoma of the anus and mature teratoma among others [14, 15].

1.1.2 Prostatic carcinoma

1.1.2.1 Introduction

Prostatic carcinoma arises from the prostate glandular secretory cells. If not treated prostate cancer may follow a natural course, starting as a tiny group of cancer cells that can grow into a large aggressive tumour [8]. A large number of screening detected cases will remain indolent; some reports showed that case-fatality rate is about 16%, while other affected men will die with but not from prostatic carcinoma [24]. There is no known means to prevent prostate cancer. Currently, the key to early diagnosis is regular physical examination and serum PSA test [25].

1.1.2.2 Epidemiology

Prostate cancer is the second most commonly diagnosed and the sixth most lethal cancer in men worldwide; it remains the most prevalent, and the sixth lethal cancer in men in developed countries, while it is considered sixth both most commonly diagnosed and lethal cancer in men in developing countries [2]. The annual incidence of the prostate cancer increases with the age [1, 3, 5]. Survival is significantly greater in high-incidence countries (80% in the USA vs. 40%

in developing countries). This favourable prognosis may be due to more latent cancer being detected by PSA screening procedures in developed countries. Incidence rates, per 100,000, vary by nearly 60-fold worldwide, ranging from 3.9 in India to 228.7 in United States of America (US) blacks. The highest rates are recorded in North America, Oceania, and Northern and Western Europe, whereas the lowest rates are found in Asia and North Africa [26]. Increasing numbers of prostate cancer patients is attributable to increasing longevity of population and therefore higher proportion of older men in population, along with the more widespread use of PSA screening.

In the USA, a general lifetime risk of developing the prostate cancer for American men is about 1 in 6 [27]; while a specific lifetime risk of developing prostate cancer for African American men is 1 in 5 [28]. The age adjusted incidence is increasing, 99% with clinical disease are above 50 years of age. The latent cancers are found in 20% in men in 50's, 70% in men in 70's. It is usually necessary to examine entire gland to find the latent cancer [29]. In the USA, the prostate cancer disease incidence rates per 100,000 are 228.7 for Blacks, 141.0 for Whites and 77.2 for Asian Americans [30]; but the rates are higher in Scandinavians [3]. However, all the groups have similar incidence of latent cancers, suggesting the role of environmental or other genetic factors for the development of the clinical disease [3].

In Denmark, 57% of the prostate cancer patients die of the prostate disease while 41% die of other causes; the median survival time from the time of diagnosis is estimated to 3.5 years, with a range of 0.01 to 15.6 years, while the median age at diagnosis is 75 years, with a range of 49 to 95 years [31].

Sub-Saharan Africa shows regional and ethnic variations in prostate cancer epidemiology [32]. The relatively high incidence (and mortality) recorded in African populations is reflected in populations of African descent elsewhere. Thus, within the United States, the black population has the highest incidence (and mortality) rates [3, 30]. Southern Africa [32] appears to have the highest rates (40.5 per 100,000). Rates of histologically diagnosed prostate cancer in South Africa are 40.1 per 100,000 in whites versus 14 per 100,000 in blacks, although for blacks,

access to diagnostic facilities has been limited [32]. In Zimbabwe, rates for whites and blacks were 70 versus 25 per 100,000 respectively [32]. Central Africa follows with rates of 24.5 per 100,000 [32]. Surprisingly, in West Africa, where the majority of African-American men originated, the incidence rate of prostate cancer was estimated as 19.3 per 100,000 in 2002, compared with about 228.7 per 100,000 in the United States [30, 32]. High rates are observed in other places with populations that are descended from West Africa, for example, the Bahamas, Barbados and Trinidad [32].

In South Africa, [33] the lifetime risk to develop prostate cancer is 1 for 6 men. Prostate cancer is the leading cancer in males and more than 4,000 men are diagnosed yearly with prostate cancer in South Africa. In Cape Town in the 1950s prostate cancer was more common in Blacks than in Whites [34] but for the present, the situation is the opposite [32]. Increases over time have also been noted in Kampala and in Ibadan, but it is unclear how much of these increases can be attributed to increased awareness or a greater readiness to perform prostatectomy for urinary symptoms in elderly men [32]. In Nigeria prostate cancer accounts for 16.5 % of all cancers reported in 1,001 men based on a 10-year analysis of data from the Kano Cancer Registry, from 1995 to 2004 [35].

In the East-African Community, Burundi had an estimated age-standardized incidence of 31/100,000 in 2008, while the estimated age-standardized death rate was 21/100,000 [36]. In Kenya, [36] the estimated age-standardized incidence in 2008 was 34/100,000, while the age-standardized death rate was estimated at 23/100,000. A study in Nairobi in 2000 showed that 25% of prostatic carcinoma patients had poorly differentiated cancers and that these had a very poor prognosis [37]. The estimated age-standardized incidence of prostate cancer in Rwanda in 2008 was 28/100,000, while the estimated age-standardized death rate was 21/100,000 [36]. In Uganda, the incidence of and mortality from cancer in general and specifically the prostate cancer has been profoundly impacted by the HIV epidemic [38]. For the period 1982-2000, prostate cancer was the fourth most common malignancy identified in Kampala, representing 5.8 percent of all cases [38].

In the United Republic of Tanzania, the estimated number of new cases per year is 1,248 and many more may have prostate cancer but not know about it [36]; but the extrapolated figures of incidence using global figures are 25,063 cases per year [39]. The estimated age-standardized rate of new cases per year is 20.1/100,000, while the estimated age-standardized number of deaths per year is 17.4/100,000 [36]. The predominant histological type is adenocarcinoma which represents 99.1% of prostate cancer [40]. Moderately differentiated adenocarcinoma represents 45.1% of the cases; 61% have a Gleason score of 5-7, and 81.5% of the cases present with PSA levels of more than 20.0 ng/mL [40].

Polymorphisms on the androgen receptor gene may vary by ethnic group and may provide some explanation for the geographic variation observed [32]. Recommendations for studies on interethnic variations in androgen receptor polymorphisms throughout Africa have been made [32].

1.1.2.3 Genetics in prostate cancer

Though the screening of prostatic carcinoma with serum PSA is widespread, PSA levels are not cancer-specific because serum levels are commonly elevated in benign conditions; on the other hand, 15% of men with normal PSA levels, explicitly between 0 and 4.0 ng ⁄ ml, have prostate carcinomas diagnosed on prostate biopsy, and 15% of these men have high Gleason grade disease [14, 15, 41]. Therefore, the strategies in prostate cancer early detection rely primarily on other biomarkers that are specific and sensitive to prostate carcinoma [41].

The alternative approach is to develop clinically strong assays for genomic and transcriptomic biomarkers which are at the same time cancer-specific and predictive for prostate cancer [41]. Several genomic rearrangements exist in prostatic carcinomas and may point to a complex mechanism for tumour cells to gain growth advantage [41]. Also, inherited genetic variants can predispose to disease progression, potentially favouring specific somatic events [41].

A majority of prostate carcinomas exhibit common gene fusions involving androgen-regulated 5 prime partners, namely TMPRSS2, SLC45A3 and NDRG1, and E twenty-six (ETS)

transcription factors, specifically ERG and ETV1, the latter being less involved in gene fusion [41]. The fusion of TMPRSS2 and ERG loci at the chromosomal level and consequent overexpression of the TMPRSS2:ERG transcript and truncated ERG protein product have been found to be 100% specific for the presence of prostate cancer; the TMPRSS2:ERG mRNA is detectable in urine [41]. Urine TMPRSS2:ERG level is associated with tumour size, high prostatectomy Gleason score [41]. A non-coding transcript PCa3 is a prostate-specific gene overexpressed in more than 95% of prostate cancers and is detectable in urine [41]. The urine TMPRSS2:ERG, in combination with urine PCa3, enhances the utility of serum PSA for predicting prostatic carcinoma and clinically relevant cancer on biopsy [41]. All these findings have immediate and future implications in diagnosis, prognosis, prediction and therapy of prostatic carcinoma [41].

1.1.2.4 Clinical aspects

1.1.2.4.1 Natural history of prostate cancer

Cancer of the prostate develops from secretory cells of the prostate glands. It often develops very slowly, and remains localised at the beginning [1, 3, 5]. When cancer advances, it may extend to the nearby organs and can metastasize to distant structures like lymph nodes, bones etc. If cancerous cells penetrate into the lymphatic vessels of the prostate, they are transported towards the pelvic lymph nodes [1, 3, 5].

The symptoms are urinary frequency and urgency, dysuria, mictalgia and bone pain; they settle quickly, become permanent and worsen in a few months [1, 3, 5]. Prostate carcinoma can be detected [3] with digital rectal examination, transurethral ultrasound and elevated PSA levels above 4ng/ml or increasing over time. Prostate carcinomas secrete ten times more PSA than normal prostate tissue [3]. The other differential diagnoses of firm prostate are granulomatous prostatitis, nodular hyperplasia, tuberculosis, infarct and lithiasis [3]. The other differential diagnoses of elevated PSA are nodular hyperplasia where there is mild increase in PSA, prostatitis, infarct, and trauma and rarely other tumours like salivary duct carcinoma [42]. For benign disease, the increase in PSA is usually transient [3].

Other tests for prostate cancer detection include transrectal ultrasound with biopsies, local lymph node evaluation, alkaline phosphatases, creatinine to check renal function; computerized tomography (CT) scan to detect lymph node or distant metastasis; magnetic resonance imagery (MRI) to detect extraprostatic extension of the cancer, and osseous scintiscanning to detect prostate cancer extension to the bones [3]. The tables of Partin are used to calculate the risk of extracapsular extension of the prostate cancer [43]. Majority of cancers develop in prostate peripheral zone, 15% in transitional zone and 5% in central zone. Clinical progression seems related to stage and degree of the tumour differentiation [3, 12].

There is however an inherent difficulty in knowing which prostate cancer bearers will have progressive or aggressive disease, as against those who will have slowly progressive or indolent disease; markers to distinguish these two scenarios would be very valuable.

1.1.2.4.2 Staging

The stage of the disease is defined by "the TNM system" [3] which describes the extension of the primary tumour (T), lymph node metastases (N), and absence or presence of distant metastases (M). The clinical staging of the primary tumour is summarized in table 1.

Table 1. Clinical staging of prostate primary tumour (T)

Clinical staging of the prostate primary tumour
TX: Primary tumour cannot be assessed
T0: No evidence of primary tumour
T1: Clinically inapparent tumour neither palpable nor visible by imaging
T1a: Tumour incidental histological finding in 5% or less of tissue resected
T1b: Tumour incidental histological finding in more than 5% of tissue resected
T1c: Tumour identified by needle biopsy (e.g. because of elevated PSA)
T2: Tumour confined within prostate
T2a: Tumour involves one half of one lobe or less
T2b: Tumour involves more than one half of one lobe, but not both lobes
T2c: Tumour involves both lobes
T3: Tumour extends through the prostatic capsule
T3a: Extracapsular extension (unilateral or bilateral)
T3b: Tumour invades seminal vesicle(s)
T4: Tumour is fixed or invades adjacent structures other than seminal vesicles

Source: [3].

The histological evaluation of the prostate cancer enables the pathological staging of the tumour, as indicated in table 2.

Table 2. Pathological staging of prostatic primary tumour (pT)

Pathological staging of the prostatic primary tumour
pT2: Organ confined
pT2a: Unilateral, one half of one side or less
pT2b: Unilateral, involving more than one half of one side, but not both sides
pT2c: Bilateral disease
pT3: Extraprostatic extension
pT3a: Extraprostatic extension or microscopic invasion of bladder neck
pT3b: Seminal vesicle invasion
pT4: Invasion of rectum, levator muscles or pelvic wall

Source: [3].

There is no pathologic T1 classification. The margins are important, and margin status is independent of T classification. The positive margins are classified as either focal or extensive based on the length of involvement of the inked line of resection.

The regional lymph nodes (N) involvement staging is the next step of tumour extension evaluation [3].

NX: Regional lymph nodes were not assessed

N0: No regional lymph node metastasis

N1: Metastasis in regional lymph node(s)

The pathological lymph node stages pNX, pN0 and pN1 are the same as the clinical lymph node stages cNX, cN0 and cN1 respectively. Metastases to periprostatic or periseminal vesicle lymph nodes suggests poor prognosis [44].

Distant metastases (M) of the prostate tumour are the important features of prognosis. This staging is summarized in table 3.

Table 3. Staging of distant metastases (M) of the prostate tumour

Distant metastases of the prostate tumour
M0: No distant metastasis
M1: Distant metastasis
M1a: Distant metastasis to non-regional lymph node(s)
M1b: Distant metastasis to bone(s)
M1c: Distant metastasis to other site(s) with or without bone disease

Source: [3].

When more than one site of metastasis is present, the most advanced category is used, that is, pM1c.

The TNM staging findings are grouped with the PSA levels and tumour grades, as summarized in table 4.

Table 4. Prognostic groups of prostate tumour

Stage	***TNM, PSA levels and grade features***				
Stage I	T1a-2a	N0	M0	PSA<10	Gleason 6 or less OR
	T1-2a	N0	M0	PSA unknown	Gleason unknown
Stage IIA	T1a-c	N0	M0	PSA < 20	Gleason 7 OR
	T1a-c	N0	M0	PSA 10-19.9	Gleason 6 or less OR
	T2a-b	N0	M0	PSA < 20	Gleason 7 or less OR
	T2b	N0	M0	PSA unknown	Gleason unknown
Stage IIB	T2c	N0	M0	Any PSA	Any Gleason OR
	T1-2	N0	M0	PSA 20 or higher	Any Gleason OR
	T1-2	N0	M0	Any PSA	Gleason 8 or higher
Stage III	T3a-b	N0	M0	Any PSA	Any Gleason score
Stage IV	T4 or N1 or M1				

Source: [3].

When either PSA or Gleason is not available, grouping should be determined by T stage or either PSA or Gleason as available [3]. Gleason score correlates with the clinical stage of the tumour [12].

1.1.2.4.3 Biological characteristics of prostatic carcinoma

a. Tumour distribution and extension

About 70% of prostatic carcinomas arise from the peripheral zone (posterior, lateral, anterior), usually spares periurethral zone except in late stages; more than 90% of radical prostatectomy specimens usually have tumour posteriorly and 65% anteriorly; the anterior tumour is associated with higher tumour volume and extraprostatic extension [45]. Tumour extension is by local invasion via seminal vesicles and bladder base, rarely into prostatic urethra; rectal invasion is rare due to tough Denonvillier's fascia. Seminal vesicle invasion occurs via either direct spread along ejaculatory duct complex, spread outside prostate, through capsule, then into seminal vesicle, or patients with better prognosis who had isolated deposits in seminal vesicle with no contiguous prostatic primary [3]. Genetic and environmental metabolic disorders may be implicated in prostatic carcinoma initiation and progression [24].

Perineural invasion (PNI) is found in up to 85% of all tumours; the diameter of perineural invasion may be a prognostic factor [46]. It may mediate local tumour spread via tumour expression of nerve cell adhesion molecule [47].

b. Metastases

About 35% of prostatic adenocarcinomas have metastases, most common sites are bone (90%), lung (46%), liver (25%), pleura (21%), adrenals (13%); spine involvement is common even in small tumours and tends to be upward spread from lumbar to cervical level [48]. The angiolymphatic invasion is not commonly seen. The increased vessel cellularity has been shown to be associated with higher grade tumours [49].

1.1.2.5 Histological diagnostic criteria for prostate carcinoma

The establishment of the diagnosis of malignancy in minimal amount of glands in needle biopsy tissue is a big challenge [50]. In these cases, the differential diagnoses of carcinoma should be considered, and they include the atypical adenomatous hyperplasia (adenosis), atrophy, high-grade prostatic intraepithelial neoplasia (HPIN) and focal glandular atypia or

atypical small acinar proliferation [50]. Most cases of minimal prostatic adenocarcinoma can be diagnosed based on H&E-stained sections, few need adjunctive study using antibody 34ßE12 [12, 50]. The minimal carcinomas in prostate needle biopsy specimens are mostly of intermediate grade and most of the corresponding prostatic carcinomas in the whole prostate gland are pathologically significant [12, 50].

The major criteria for diagnosis of prostatic adenocarcinoma are the architectural patterns which include among others, the infiltrative small glands or cribriform glands too large or irregular to represent HPIN, single cell layer because of absence of basal cells; other major criteria for diagnosis of prostatic adenocarcinoma concerns the nuclear atypia defined by nuclear and nucleolar enlargement [50]. The minor criteria for the diagnosis of prostatic adenocarcinoma include the intraluminal wispy blue mucin, pink amorphous secretions, mitotic figures, intraluminal crystalloids, adjacent high-grade PIN, amphophilic cytoplasm and nuclear hyperchromasia [50].

The Stout criteria for diagnosis of prostatic adenocarcinoma include the glandular pattern, with small irregular glands with no particular relation to the adjacent stroma or normal glands; the glandular epithelium arrangement shows lack of basal cells in cancer, while the cellular details concern large deeply staining nucleoli [50]. The minimal prostatic adenocarcinoma commonly exhibits the presence of small glands between benign glands [50].

1.1.2.6 Histological classification of prostate tumours

The histopathological diagnosis and grading have an important role in the management of prostate cancer [51]. Table 5 summarises the World Health Organization (WHO) histological classification of the prostate tumours.

Table 5. WHO (2004) histological classification of tumours of the prostate

Type of tumours	Subtype	Histological diagnosis	Variants
Epithelial tumours	Glandular neoplasms	Adenocarcinoma (acinar)	Atrophic; pseudohyperplastic; foamy; colloid; signet ring; oncocytic; lymphoepithelioma-like
		Carcinoma with spindle cell differentiation	Carcinosarcoma, sarcomatoid carcinoma
		Ductal adenocarcinoma	Cribriform; papillary; solid
		Prostatic intraepithelial neoplasia (PIN)	
	Urothelial	Urothelial carcinoma	
	Squamous tumours	Adenosquamous carcinoma	
		Squamous cell carcinoma	
	Basal cell tumours	Basal cell adenoma	
		Basal cell carcinoma	
Neuroendocrine tumours		Endocrine differentiation within adenocarcinoma; carcinoid tumour; small cell carcinoma; paraganglioma; neuroblastoma	
Prostatic stromal tumours		Stromal tumour of uncertain malignant potential; stromal sarcoma	
Mesenchymal tumours		Leiomyosarcoma, Rhabdomyosarcoma, Chondrosarcoma, Angiosarcoma, Malignant fibrous histiocytoma, Malignant peripheral nerve sheath tumour, Haemangioma, Chondroma, Leiomyoma, Granular cell tumour, Haemangiopericytoma, Solitary fibrous tumour.	
Hematolymphoid tumours		Lymphoma; Leukaemia	
Miscellaneous tumours		Cystadenoma, Nephroblastoma (Wilms tumour), Rhabdoid tumour, Germ cell tumours (Yolk sac tumour, Seminoma, Embryonal carcinoma & teratoma, Choriocarcinoma), Clear cell adenocarcinoma, Melanoma	
Metastatic tumours			

Source: [51].

1.1.2.7 **Grading of the prostatic adenocarcinoma**

Different systems have been used in grading the prostate cancer, but the most universal is the Gleason grading system [1, 3, 5, 12]. The Gleason grading system is only used for the

adenocarcinomas and is an established prognostic indicator [12]. The Gleason grading method was devised in the 1960s and 1970s by Dr Donald F Gleason and members of the Veterans Administration Cooperative Urological Research Group (VACURG) [12, 52].

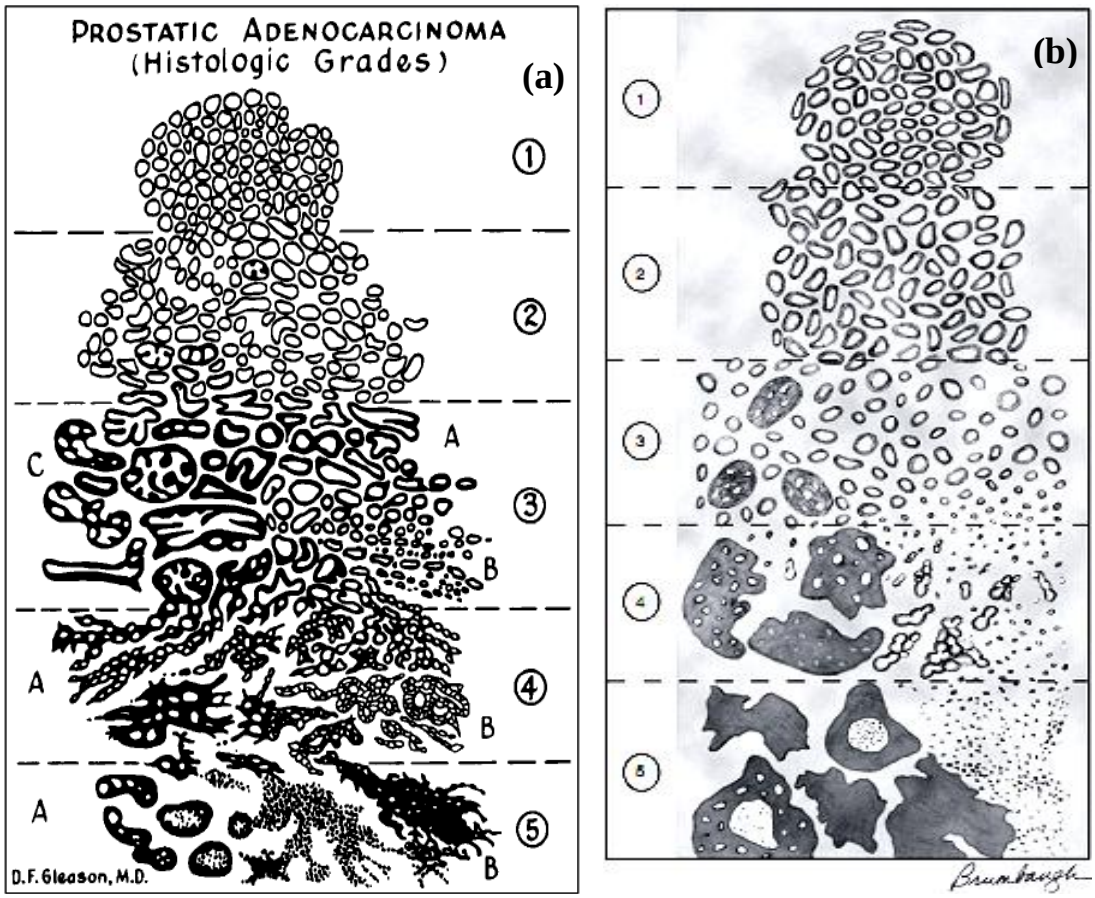


Figure 2. Gleason grades: standard drawing (a) and modified Gleason grading (b). Courtesy of Humphrey (Figure 2a) [12], and Delahunt *et al.* (Figure 2b) [27].

This grading system is based on the histological pattern of malignant epithelial cells on H&E-stained sections. Five basic grade patterns are used to generate a histological score, which can range from 2 to 10 [12, 52]. These patterns are illustrated in a standard drawing which serves as a guide for recognition of the specific Gleason grades (Figure 2a), the system which was modified in 2005 [27] by the International Society of Urological Pathology (ISUP) (Figure 2b). Increasing Gleason grade is directly related to a number of histopathological end points,

including tumour size, margin status, and pathologic stage [12]. Indeed, models have been developed that allow for pre-treatment prediction of pathologic stage based upon needle biopsy Gleason grade, total serum PSA level, and clinical stage. Gleason grade has been linked to a number of clinical end points, including clinical stage, progression to metastatic disease, and survival [12].

Nine growth patterns were consolidated into five grades and these were illustrated in a drawing by Dr Gleason (Figure 2a). The five basic grade patterns are used to generate a histological score, which can range from 2 to 10, by adding the primary grade pattern and the secondary grade pattern [12, 52]. The primary pattern is the one that is predominant in area, by simple visual inspection. The secondary pattern is the second most common pattern. If only one grade is in the tissue sample, that grade is multiplied by two to give the score [12, 52]. As examples, if the prostate sections show adenocarcinoma with pattern 3 predominance, and the second predominant pattern is 4, then the Gleason score will be 3+4=7; if the pattern is purely 3, then the Gleason score will be 3+3 (or 3x2)=6. Moreover, the modified Gleason grading suggests that for the radical prostatectomy specimens the tertiary pattern should be considered when grading the tumour, if the tertiary pattern has a higher grade than the secondary or primary pattern, or represents more than 5% of the whole tumour [27]. However, in the 2005 ISUP consensus conference, it is agreed that the Gleason score should be based upon the primary and secondary pattern, with only a comment regarding any tertiary pattern [27].

Gleason grades

Grade 1 is characterized by very well-differentiated growth of closely packed but separate, uniform, rounded to oval, medium-sized acini. The back-to-back glands should be separated by thin stromal rims [12, 27, 52].

Grade 2 is like grade 1 but with more variability in gland shape and more stroma separating glands; most glands are separated by less than one average gland diameter; less circumscribed at periphery, although no infiltration into stroma or between benign glands [12, 27, 52].

Grade 3 has single, separate, much more variable glands, may be closely packed but usually irregularly separated, ragged, poorly defined edge, but still in circumscribed structure, looser than nodule, slightly infiltrative [12, 52]. There are three patterns of Gleason grade 3 prostatic adenocarcinoma [12, 52]. Grade 3A is the most common pattern, characterized by well-formed, relatively uniform glands infiltrating between benign glands; glands may be angulated or compressed, separated by > 1 gland diameter, tend to have basophilic cytoplasm and larger nucleoli than grades 1 or 2. Grade 3B exhibits small glands with inconspicuous or absent lumina, glands are still separate. Grade 3C has papillary or cribriform patterns, with smooth, rounded, pushing type edges without stromal infiltration; cribriform unit is small (size of normal prostate gland) and well circumscribed without a ragged or infiltrative border [12, 52].

Two patterns of Gleason grade 4 prostatic adenocarcinoma are recognized [12, 52]. Pattern 4A is the most common and consists of small acinar structures, some with well-formed lumina, fusing into cords or chains. Pattern 4B is recognized by papillary-cribriform tumours with irregular or invasive edges; it includes many but not all ductal carcinomas; nodule of cribriform gland should be larger than normal prostate gland; large nodules of cribriform Gleason 4 lack supporting stroma and tend to fragment. The hypernephroid pattern presents with nests of clear cells, small, hyperchromatic nuclei; there is fusion of acini into more solid sheets with the appearance of back to back glands without intervening stroma [12, 52].

Grade 5 has two patterns [12, 52]. Pattern 5A is characterized by comedocarcinoma, papillary or cribriform carcinomas with central necrosis. The pattern 5B consists of carcinomas with minimal glandular differentiation, ranging from infiltrating single cells (including signet ring cells) to solid sheets of tumour cells. The presence of Gleason grade 5 and high percent carcinoma at prostatectomy predicts early death [12].

1.1.2.8 Diagnostic markers for prostate cancer

The basal cells of prostate glands stain with both the broad spectrum and low molecular weight keratins as well as with high molecular keratins [3], while the secretory cells are negative for

high molecular weight cytokeratin, specifically 34βE12 [3, 8, 20, 21]. Other markers of basal cells include p63 and cytokeratin 5/6. Basal cell-specific staining is important diagnostically as a complete absence of basal cells in an acinar proliferation is an important criterion of malignancy [3, 8, 20, 21, 53]. The absence of basal cells is determined on routine microscopy in many cases, but a high molecular weight cytokeratin stain may be required in difficult interpretations [3].

CK5/6 stains dark the cytoplasm of basal cells in the benign glands and HPIN glands [53]. CK5/6 is an excellent prostate basal cell marker and it can be used in a double immunoassay or individually [53].

p63 is a homologue of the p53 tumour suppressor gene, and encodes for at least six major isotypes [54]. Three of these 6 isotypes (TAp63α, TAp63β, and TAp63γ) contain the transactivating (TA) domain and are able to transactivate p53 reporter genes and induce apoptosis; the other three isotypes (ΔNp63α, ΔNp63 β, and ΔNp63γ) are transcribed from an internal promoter, they lack the TA domain, and act as dominant-negatives to suppress transactivation by both p53 and TAp63 isotypes [54]. p63 is selectively expressed in the basal cell compartment of a variety of epithelial tissues including among others, the prostate [54]. p63 is therefore essential for the maintenance of a stem cell population in various epithelial tissues [54].

The complete lack of 34βE12, CK5/6 or p63 staining indicates absence of basal cells and leads to the diagnosis of prostatic carcinoma [3, 8, 20, 21, 53, 54].

The product of α-methylacyl-CoA-racemase (AMACR) gene is an enzyme involved in the β-oxidation of branched-chain fatty acids; the AMACR is overexpressed in prostate cancer, and is potentially an important biomarker for prostate cancer [55]. AMACR shows circumferential and subluminal or cytoplasmic finely granular staining in the secretory epithelial cells in prostate cancer and HPIN glands [53]. AMACR stains positively all variants and unusual morphological patterns of prostatic carcinoma, including ductal, mucinous, pseudohyperplastic, and atrophic patterns. AMACR has been incorporated in the

immunohistochemical panel used to distinguish benign prostatic atrophy from prostate cancer associated with atrophic features on needle biopsy; but when AMACR is used alone is not sufficiently discriminatory in the differential diagnosis of atrophic prostate cancer versus benign atrophy. Positive AMACR immunostaining in combination with negative basal cell markers is recommended in the differentiation of atrophic prostate cancer and benign atrophy. The triple (p63 + 34βE12 + AMACR) commercial antibody cocktails exist [53].

1.1.2.9 Role of markers of malignancy and progression

The E twenty-six (ETS) family of transcription factors acts in most biological pathways involved in normal cell as well as tumour cell growth and development [56]. One of the members of ETS family is the epithelial-specific, Prostate-derived ETS factor (PDEF) [56]. PDEF mRNA overexpression is identified in breast and prostate tumour. PDEF has tumour-suppressive functions substantiated by a correlation between PDEF protein loss and increased aggressiveness in breast, prostate, ovarian and colon cancer [56]. Tumour cells activate specific pathways associated with epithelial-to-mesenchymal transition (EMT) in order to promote decreased cell-to-cell contacts, increased cell motility and invasiveness [56].

SLUG, or SNAI-2, is one of the transcription factors which induces a partial or sometimes full EMT during cancer progression [56]. PDEF directly alters the expression resulting in the loss of the cell adhesion molecule E-cadherin, causing the expression of mesenchymal proteins including among others, vimentin and N-cadherin, which are identified as critical features of EMT in tumour progression. PDEF also alters the expression of EMT-associated proteins independent of SLUG expression [56]. On the other hand, exogenous SLUG expression restores the migratory phenotype inhibited by PDEF occupancy of the SLUG promoter [56].

ETS factors act as positive or negative regulators of the expression of the genes involved in diverse biological processes, including those that control cellular proliferation, differentiation, haematopoiesis, apoptosis, metastasis, tissue remodelling, angiogenesis and transformation

[57]. A number of ETS factors can function as either activators or repressors, depending upon the type of promoter and cellular environment [57].

Others metabolic markers are also thought to be associated with prostatic carcinoma progression; they include adiponectin and leptin, both of which can act either through autocrine, paracrine or endocrine pathways [24]. The decrease in adiponectin and /or the increase in leptin are thought to be associated with prostate cancer progression [24].

1.1.2.10 Treatment overview

Prostate cancer continuum is a concept that describes the natural progression of prostate cancer; it encompasses all stages of the disease, namely, localized prostate cancer (organ-confined with no regional lymph node involvement, no distant metastases), locally advanced prostate cancer (not organ-confined, has spread beyond the capsule, in some cases to regional lymph nodes, but involves no distant metastases), advanced prostate cancer (distant metastases are present), and hormone-refractory prostate cancer (insensitive to hormone treatment) [58]. A large number however remain indolent, and the case-fatality rate is estimated at 16% in some studies [24], but one study which randomised men with localised prostate cancer between radical prostatectomy and watchful waiting found that the risk of metastatic disease was 13% in men randomised to surgery versus 27% in men randomised to watchful waiting after 8 years of follow-up, thus confirming the overall survival benefit in treated men [59].

Prostate cancer growth depends upon the presence of androgens, namely the dihydrotestosterone–androgen receptor complex (DHT–AR) which regulates gene expression within the cancer cells; without androgens the proliferation of prostate cancer cells is not possible [10]. Therapeutic options include surgery, hormonotherapy, chemotherapy and radiotherapy, with the aim of androgen blockade (castration).

Surgery consists essentially of bilateral subcapsular orchidectomy (BSO); this aggressive castration started in 1940’s and was found to be associated with reduction in androgen levels, slow progression of the disease and increased survival rates; androgen blockade temporally

halted cancer growth in 60 to 80% of prostate cancer patients [10]. A disadvantage of surgical castration is the psychological trauma with impairment of the quality of life.

Luteinising hormone-releasing hormone analogues (LHRHa) were developed in 1980's; these agents block testicular production of androgens without the need for surgery, and LHRHa therapy was shown to be equivalent to both surgical castration and oestrogen therapy in terms of reduced disease progression and survival rates [10]. LHRHa monotherapy reduces plasma androgen levels by about 90–95%, because the adrenal glands continue to produce androgens [10]. Addition of an antiandrogen improves the efficacy of hormonal therapy by preventing the remaining androgens from activating the intraprostate androgen receptors [10].

Complete androgen blockade is done using combination hormonal therapy by an antiandrogen and a luteinising hormone-releasing hormone analogue (LHRHa) to treat patients with advanced prostate cancer [10]. Combination hormonal therapy is also called maximal androgen blockade (MAB), or combined or complete androgen blockade (CAB) [10].

The increasing screening efficacy leads to an increasing proportion of cases presenting with early-stage disease; combination hormonal therapy is therefore increasingly used in the neoadjuvant or adjuvant setting with radiotherapy, as well as after localised therapy if there is PSA recurrence [10].

Radiotherapy may be indicated for localized disease, locally advanced disease or metastatic disease, and consists of either external beam radiotherapy or brachytherapy.

Cytotoxic chemotherapy is indicated in hormone refractory and advanced disease, and includes Docetaxel, Cyclophosphamide, Prednisone and Mitoxantrone. Provenge is a vaccine developed to halt progression of prostate cancer into hormone refractory disease.

Prevention of prostate cancer disease is done by digital rectal examination and serum PSA test. PSA test may be used to establish a baseline from an initial determination and then follow the

patient with sequential PSA tests, every 3 or 6 months to evaluate the trend; if PSA is increasing, it is a sign of prostate abnormality, commendable of more investigations [60, 61].

There is still, however, a difficulty in determining which prostate cancer bearers will need aggressive treatment (for progressive or aggressive disease), as against those who merely need an active surveillance (for slowly progressive or indolent disease); markers to distinguish these two scenarios would be very valuable.

1.2 Ki67 antigen

The Ki67 antigen is a non-histone nucleoprotein, which is selectively expressed by actively cycling cells, that is, G1, S, G2 and M-phases, but it is absent in resting cells (G0-phase), and is demonstrable in paraffin-embedded histological sections using the MIB-1 antibody [62, 63]. Two isoforms of 345 and 395 kDa have been identified [64]. During mitosis most of the protein is relocated to the surface of the chromosomes. The antigen is rapidly degraded as the cell enters the non-proliferative state [65], and there appears to be no expression of Ki67 during DNA repair processes. Ki67 expression is known to correlate with the clinical stage, tumour differentiation and prognosis in Western populations [31].

Even though the human prostate adenocarcinoma is considered as a slow growing tumour, some tumours show aggressiveness that is highlighted by MIB-1 antibody [31]. The tumour proliferation rate is determined by scoring the percentage of MIB-1-stained cells among the tumoural cells [31, 62, 64]. High and low risk groups are differently defined by respective authors depending on the type of investigated cancer [31, 66]. The series investigating the lymphoma proliferation used MIB-1 expression score of more than 40% to define a high risk group [66], while the series which investigated the prostate carcinoma proliferation determined the high and low risk groups by dividing into 2 equal groups using the median MIB-1 score, and this grouping showed a direct correlation with Gleason score and patient's outcome [31].

CHAPTER 2. AIMS OF THE STUDY

2.1 Rationale of the study

Urological diseases are a major problem to the general population worldwide [1]. Prostate cancer ranks the second most commonly diagnosed cancer in males worldwide, while it is the first in developed countries and the sixth in developing countries. It the sixth leading cause of cancer death in men likewise worldwide, in developed countries as well as in developing countries [2]. Racial and genetic factors are among the risk factors for prostate cancer [67]. Globally, Black men are more affected, followed by Whites and Asians, and geographical and epidemiological variations exist within the same respective races [1, 3, 5, 12]. In Tanzania, the estimated number of new cases of prostate cancer per year is 1,248 [36], but the extrapolated figures of incidence, using global figures, are 25,063 cases per year [39]. A related study done in Tanzania confirmed the direct correlation between Gleason score and pre-treatment serum PSA levels; it also showed that adenocarcinoma represents 99.1% of all prostate cancers [40].

A large number of screening detected cases will remain indolent; some reports showed that case-fatality rate is about 16%, while other affected men will die with but not from prostatic carcinoma [24]. However, an overall survival benefit was seen in a randomised treated group [59], but a stress brought on by an aggressive, sometimes unnecessary, treatment cannot be ignored [10, 58]. The main challenge in prostatic carcinoma management is to predict which case will be indolent thus needing merely active surveillance versus aggressive cases needing aggressive treatment. A number of prognostic factors have been established to assist in the prediction of disease progression. These factors include generally Gleason score and, in some settings, tumour proliferation markers.

2.2 Problem statement

Ki67 expression is known to correlate with the clinical stage, Gleason score and prognosis in Western populations [31]. Gleason score may not be representative of the true histological grading of the tumour in some cases of small prostate core biopsies [12, 52]. A combination of these two factors have not yet been performed to determine if it may be the best predictor of

the prostatic adenocarcinoma biological behaviour than either alone. Moreover, the correlation between Gleason score and biological behaviour of prostatic adenocarcinoma has not been investigated in Tanzania. This study aims to compare and correlate Ki67 expression with Gleason score in predicting the biological behaviour of prostatic adenocarcinoma in Tanzanian patients, in order to improve patients' management.

The study question is, "Would a combination of Ki67 expression and Gleason score offer better prognostic information than either alone?"

2.3 Objectives

2.3.1 Broad objective

To compare and correlate Ki67 expression and Gleason score in predicting the biological behaviour of prostate adenocarcinoma in Tanzanian patients, in order to improve patients' management.

2.3.2 Specific objectives

1. To determine the Gleason score of prostatic adenocarcinoma in Tanzanian patients who attended the MNH Histopathology Unit from January 1st, 2005 to December 31st, 2006;
2. To determine the Ki67 index of prostatic adenocarcinoma in Tanzanian patients who attended the MNH Histopathology Unit from January 1st, 2005 to December 31st, 2006;
3. To establish the clinical stage (TNM) at initial presentation and the survival rate of MNH patients with prostatic adenocarcinoma during the study period;
4. To compare and correlate the Gleason score versus Ki67 index, in regard to biological behaviour of prostatic adenocarcinoma in Tanzanian patients who attended the MNH Histopathology Unit from January 1st, 2005 to December 31st, 2006.

2.4 Hypothesis

A combination of Ki67 expression and Gleason score offers better prognostic information than either alone.

CHAPTER 3. MATERIALS AND METHODS

3.1 Study population

3.1.1 Study area

The study was conducted at MNH, which receives referred patients from all over the country, serves as a national consultant Hospital and serves as the Teaching Hospital for the Muhimbili University of Health and Allied Sciences (MUHAS). The mainstay of the study was the Histopathology Unit of the Department of Diagnostic Laboratory Services and the Department of Medical Records of MNH. The Histopathology laboratory of MUHAS has the capacity to do routine surgical pathology, cytology, histochemical special stains and immunohistochemistry. Record keeping in the Histopathology Unit at MNH is done through filing investigation forms, physical and electronic pathology reports, slides, paraffin wax embedded tissue blocks and temporarily the gross specimens. Data and study material were therefore retrieved and used in our study. Retrieved, formalin-fixed paraffin wax-embedded tissues were processed for H&E stains and MIB-1 staining, at MUHAS Pathology Laboratory. Clinical data and patient's outcome information were retrieved in patient's files in the Departments of Medical Records of MNH.

3.1.2 Sampling

Study population (Figure 3) included all cases of prostate adenocarcinoma diagnosed on partial prostatectomy, on transurethral resections and prostate needle biopsies from January 1st 2005 to December 31st, 2006 at MNH. The recruitment of the cases was retrospective in nature, collecting all the archival specimens. Patient's identification, biodata and subsequent experimental data were entered in data collection sheets.

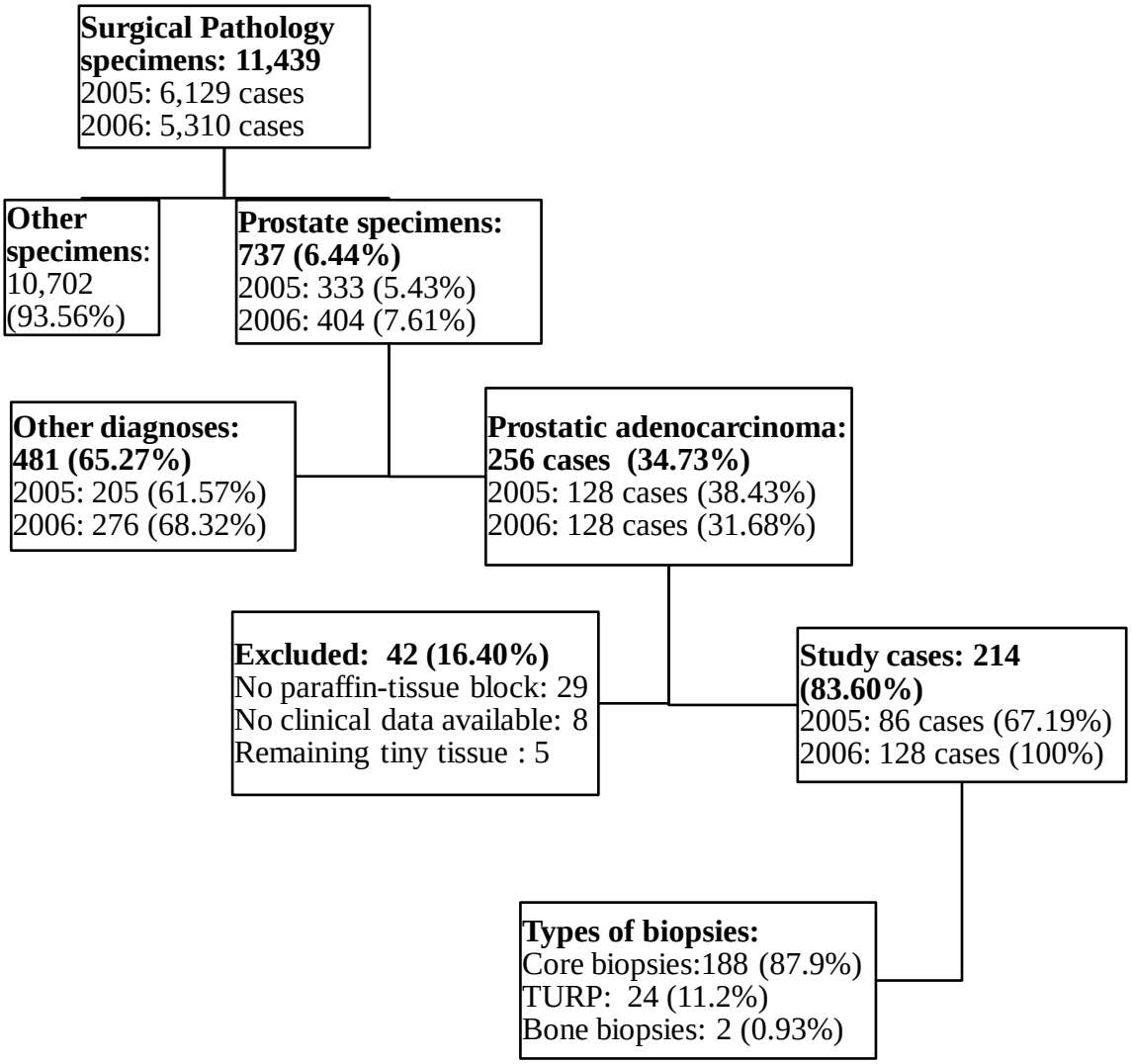


Figure 3. Methodology algorithm

Figure 3 shows that 34.73% of prostate tissues consist of prostatic adenocarcinoma. About 83.60% of the recruited cases were retained in the study.

3.1.3 Pilot study

A pilot study had been done prior to this proposal; 20 case files were randomly selected, staging was proven possible in 12 cases using provided clinical information and this was considered adequate.

3.1.4 Inclusion criteria

The confirmed cases of prostate adenocarcinoma diagnosed between the 1st January 2005 and the 31st December 2006 at MNH Histopathology unit, for which the paraffin wax-embedded tissue blocks and patients' clinical data were available.

3.1.5 Exclusion criteria

The exclusion criteria from this study were unconfirmed diagnosis of prostatic adenocarcinoma, unavailability of paraffin wax-embedded blocks, and/or clinical data.

3.2 Laboratory methods

3.2.1 Preparation of histological sections

Paraffin wax-embedded tissue blocks were cut to obtain four 4 μm-thick sections [68]. The sections were de-waxed by placing the slides on hot plate and microwaved in the oven at 60°C for 50 minutes, and then placed in xylene. Hydration was done by incubating the sections in absolute ethanol, 95%, 80% and 70% ethanol to water. Then the sections were stained by routine H&E stains, dehydrated and cleared in xylene and coverslipped using distyrene plasticiser xylene (DPX) [68]. Gleason score was assigned to each case using the standard Gleason grading system [12]. For the specimens in which more than 2 patterns were present, and the worst grade is neither the primary nor the secondary grade, the primary and highest grade among the latter two was chosen to arrive at a score, as recommended [69]. Cancer percentage on each core/section was estimated as percent of the core/section length involved by cancer; subsequently, the percentage of tumour involvement was estimated by averaging the results from all cores/sections analyzed for each case [70]; it is the average percent of tumour involvement which was reported (Appendix IV for references).

Control development, assessment and documentation were done under light microscope. The standard operating procedure for prostate surgical pathology specimens is presented in appendices of this report (Appendix IV). The investigator and two pathologists individually gave Gleason score and average tumour extent on tissue for each case. In case of discrepancy, a consensus was obtained.

3.2.2 Immunohistochemistry (IHC)

The specimens for the immunostaining procedures were retrieved from the formalin-fixed, paraffin wax-embedded tissue used for the original histopathological grading. One representative 4 μm thick section per tissue block was chosen. The sections were de-waxed by placing the slides on hot plate and microwaved in the oven at 60°C for 50 minutes, and then placed in xylene. Hydration was done by incubating the sections in absolute ethanol, 95% and 70% ethanol to water. A ring was made around the section using a hydrophobic Pap pen, to limit the spread of antibody solutions. 2 drops of a 3% hydrogen peroxide solution were added to each section for 15 minutes to block endogenous peroxidase, and then the slides were put into water. The slides were incubated in citrate buffer at pH 6.0 at the concentration of 0.01 M within a pressure cooker from which the slides were removed 2 minutes after full pressure. The slides were washed using washing buffer, tris phosphate buffered saline (PBS) for 3 minutes. PBS was then drained from slides to add the primary antibody MIB-1 and allow incubation overnight at 2-8 °C. The slides were then washed with PBS for 5 minutes and then 1 drop of streptavidin- Horseradish Peroxidase (HRP) – conjugate was added to each section for 20 minutes. Thereafter, 1 drop of HRP was added to each section for 30 minutes. The slides were again washed with PBS for 5 minutes. PBS was drained from the sections, the chromogen diaminobenzidine (DAB) added to the sections for 5 minutes. The sections were washed with water for 1 minute, counterstained in haematoxylin for 10 seconds, differentiated by 2 dips into 1% acid alcohol. The sections were blued in warm water for 2 minutes, dehydrated through 70%, 95% and 100% ethanol, and then cleared in 2 changes of xylene for 10 minutes. The sections were finally coverslipped using DPX, ready for assessment and interpretation.

Negative controls for the immunostain were achieved by omitting the primary antibody. Positive controls for MIB-1 were known cases of diffuse large B cell lymphoma, and some cases of benign prostatic hyperplasia included to be compared to carcinoma cases. The standard operating procedure for immunostaining method used in this study is presented in appendices of this report (Appendix V).

3.2.3 Quantitation of immunoreactivity

The areas of highest proliferative activity (hot spots**)** were found by scanning the tumour sections at low magnification of 40X (10X ocular and 4X objective). Inside these areas the tumour cell counts were performed by a random sampling technique at 400X (10X ocular and 40X objective) using a 10 x 10 grid (0.0625 mrn^2) in the eyepiece. Criteria for MIB-1 positivity were strong and complete brown-yellowish cell nuclear coloration. Ki67 positive cells were counted in a field of at least 500 cells by an observer blinded from respective patient's clinical data and Gleason score. The MIB-1 score represented the number of stained tumour cell nuclei expressed as a percentage of the total number of tumour cell nuclei counted.

3.3 Statistical methods

Data were collected using pre-established data collection sheets, which were tested before use. The data collection sheets were coded for easy computer use. The data were entered and analyzed using Statistical Package of Social Sciences (SPSS) 16.0 for Windows and Win Pepi version 11.1 (2010). Statistical test to assess the validity of association were Chi-square test (χ^2) for subgroups; Log-rank test (or Kaplan-Meier method) was used to assess the validity of association for the survival analysis.

The minimal number of cases needed for statistical analysis was determined through the Survey Systems Software of the Creative Research Systems (15 Lone Oak, Suite 2 Petalum CA94952, USA). With the study population of 216 subjects, fixing the Confidence Level at 95% and the Confidence Interval (CI) at 50 (acceptable: 0.1-50; where 50 is recommended to be used for determination of a general level of accuracy of a sample), therefore the minimal number of cases needed for analysis was determined to be four (4) cases.

The analysis of comparative survival data using both Gleason score and Ki67 expression using stratification of clinical stages was don't done because of the paucity of the cases in the strata. The significance of association was determined by calculations of *P value* and 95% CI. The two-sided p value <0.05 was considered confirming statistically significant association. Gleason scores were compressed for evaluation and correlations [71]. Concerning Ki67

expression, low and high risk groups were determined by dividing the patients into two equal groups using the median MIB-1 score.

The progression of prostatic adenocarcinoma disease was determined by comparing the tumour stage at diagnosis with tumour stage at the end of follow-up for stages IIA, IIB, and III; for cases with stage IV at diagnosis, progression was determined by occurrence of new metastasis or complication or death by prostate cancer. Incidence rate times were calculated from the date of diagnosis to the occurrence of signs of disease progression. The correlation of incidence rates to other variables was performed using Poisson distribution; the analysis was performed through the software WinPepi 11.1 for Windows.

3.4 Limitations of the study

Clinical data generally suffered poor documentation and archiving system. All 256 cases had their investigation request forms and histology reports in the archives of MNH Histopathology Unit. Although 142 cases had MNH registration numbers, only 75 cases (52.81%) were provided with in-wards patients' files. Among the 75 cases with patients' files, only 48 (64.00%) cases presented with documented follow-up after diagnosis of prostatic adenocarcinoma. To cope with this shortcoming, clinical information provided through laboratory investigation forms was used for analyses other than follow-up related ones. After compilation of clinical data from files and from investigation request forms, only 8 cases were excluded from the study on the poor clinical data background (Figure 3). 29 cases were excluded from the study because they lacked archival paraffin-embedded tissue blocks; this is attributed to the migration of the laboratory during renovation of the Central Pathology Laboratories (CPL) building between 2003 and 2006.

The available patients' files showed poor documentation. Though available investigations were done and documented for most of the in-ward case files, the clinical staging however was never done in a single case (0%). This limitation was handled by staging 153 cases using all the information provided in files, investigation forms and laboratory test findings including histology in case evidence of locoregional of distant organ involvement by the prostate cancer

was confirmed. Moreover, a prior pilot study had shown the study to be possible. The investigation request forms had in some cases adequate clinical information, that is, PSA level or clinical and/or radiological findings. PSA levels were provided on histology investigation request forms in only 18 cases of benign prostatic lesions, therefore preventing further correlation to cancer cases; the mean was 19.11ng/mL, ranging from 1.6 to 67.7 ng/mL. Six cases were provided with serum PSA levels mentioned > 10ng/mL; as these seem to have been obtained through a testing (assay type not mentioned in the files) in which the upper limit of results is 10 ng/mL, that variable was considered inadequate for analysis for these cases. Concerning the adequacy of prostate cancer reporting, only 28.9% of cases (21.8% in 2005 and 35.9% in 2006) had Gleason score in the histology reports; 42.2% of the reports mentioned the grade (for example, Prostatic adenocarcinoma Grade IV) or differentiation (for example, Prostatic adenocarcinoma Poorly differentiated), the remaining 28.9% of reports had no mention about Gleason score or grade. The average extent of tumour on tissue sections was never mentioned in reports (0%). These two shortcomings in histology reporting did not affect this study, since all the study cases had their paraffin-tissue blocks sectioned, stained and reported anew, according to the prostate cancer reporting standards. Financial and time constraints prevented a multicentre study, and recommendations shall be formulated to conduct a multicentre study on prostate cancer biological behaviour prediction factors.

3.5 Ethical considerations

Ethical clearance and approval was obtained from MUHAS Ethical Clearance Committee to conduct the study (Appendix II). Permission was also sought from the management of the MNH to use the archival data in this study (Appendix III). All patients' information in this study was treated as being strictly confidential and their identities were and will not be revealed.

CHAPTER 4. RESULTS

4.1 Demographic data

The mean age of the study population was 70.46 years, and was close to the median (70.00 years) and the mode (70 years). The age ranged from 42 years to 96 years. Approximately 80.6% of the patients had the age between 65 and 85 years; only 3 patients were less than 50 years of age. Figure 4 is a histogram of the age distribution of the study population.

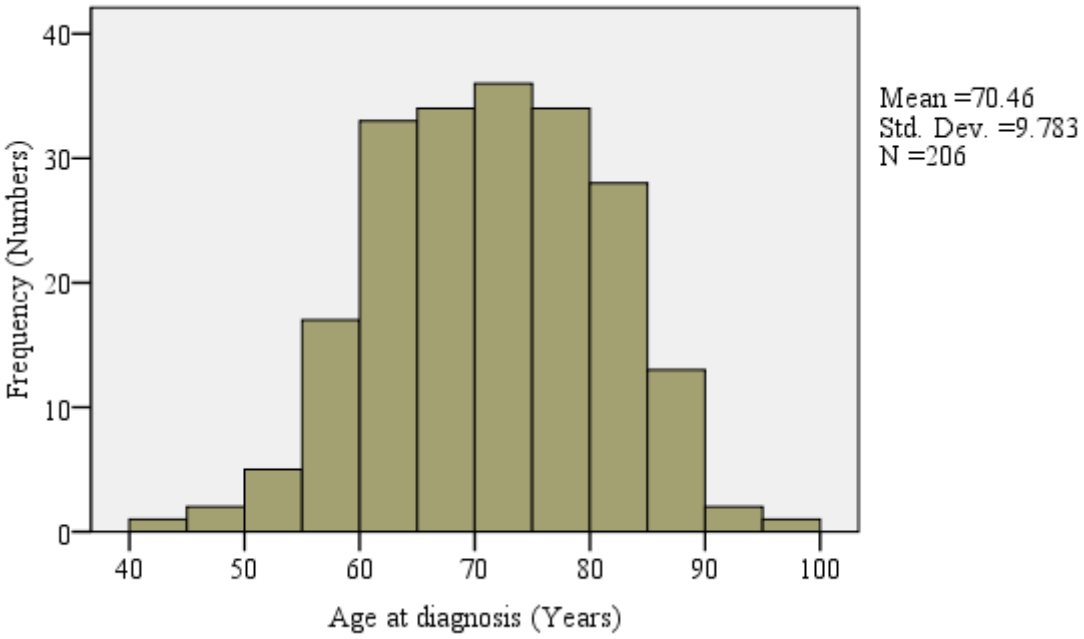


Figure 4. Age distribution of the study population

Among the 214 patients included in this study, 206 had their age documented, while it was not mentioned in 8 cases.

In this study population, Africans represented 94.4%, while the remainder comprised the cases of unspecified race in files. The residence of patients was specified in 92 cases. Half of the study population (46) came from Dar es Salaam City; many of other patients came from Zanzibar (9), Coastal Region (8), Morogoro (4) and Dodoma (4) Regions.

4.2 Distribution of the variables

The pre-treatment serum PSA levels were provided in 140 cases; the distribution of pre-treatment PSA levels showed that 61.82% of the patients had pre-treatment PSA level of more than 100 ng/mL (Figure 5). The mean was 79.578 ng/mL, ranging from 2.320 to 500.000 ng/mL. The mode was equal to the median (100 ng/mL). PSA levels were provided on histology investigation request forms in only 18 cases of benign prostatic lesions, and the mean was 19.11ng/mL (range 1.6 to 67.7 ng/mL, only 2 cases had normal PSA ranges). This limited the ability to compare PSA levels in malignant and benign lesions.

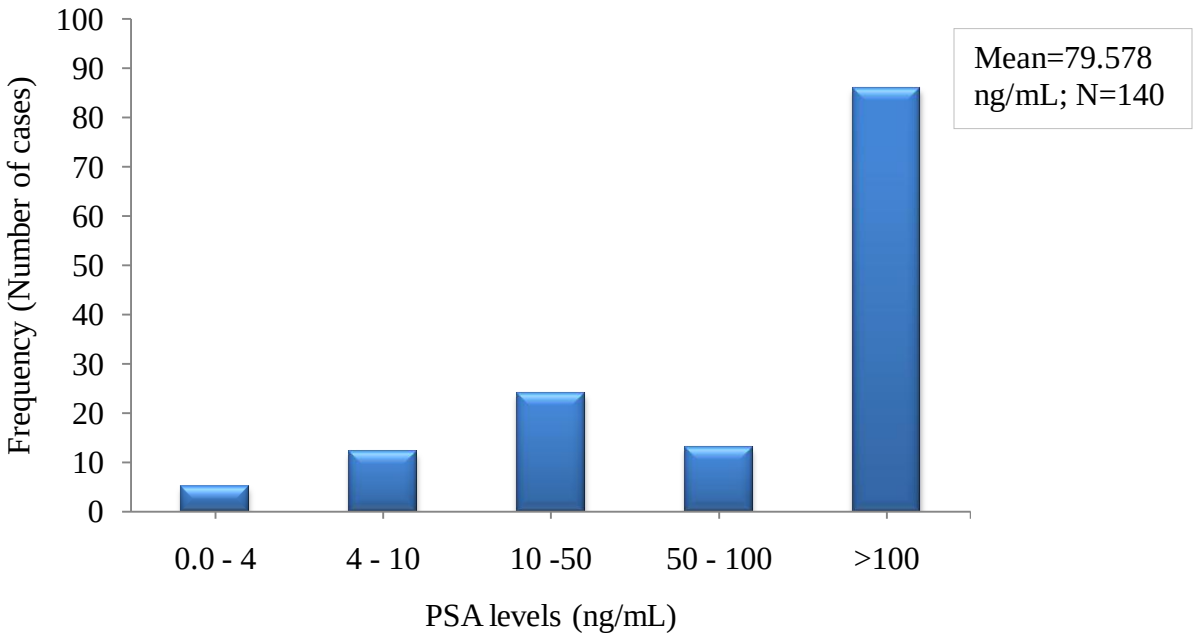


Figure 5. Distribution of pre-treatment PSA levels

Concerning the type of prostate biopsy specimens in this study, 188 cases (87.9%) were prostate needle core biopsies, 24 cases (12.1%) TURP specimens, while 2 cases (0.9%) were bone biopsies from metastatic lesions. No case of radical prostatectomy was found.

Gleason score 7 (moderately poorly differentiated adenocarcinoma) was the most encountered, seen in 82 (38.3%) cases (Figure 6, n=214). Gleason scores 2-4 (well differentiated adenocarcinoma) was seen in 5 (2.4%) cases; Gleason scores 5-6 (moderately differentiated adenocarcinoma) were encountered in 53 (24.7%) cases while Gleason scores 8-10 (poorly differentiated adenocarcinoma) were seen in 74 (34.6%) cases.

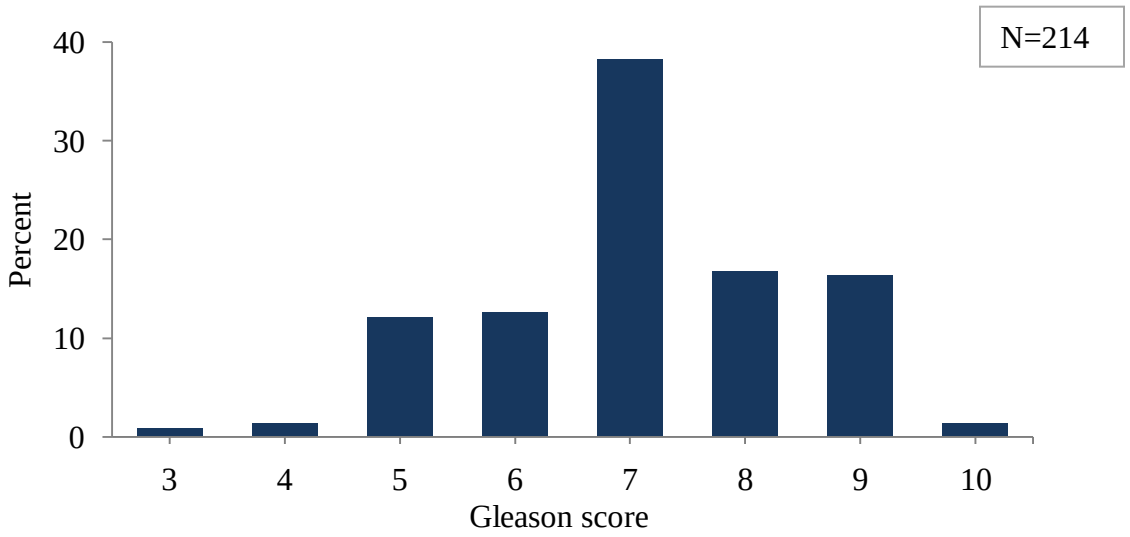


Figure 6. Gleason score distribution

Figure 6 shows illustrations of Gleason scores and other histological findings.

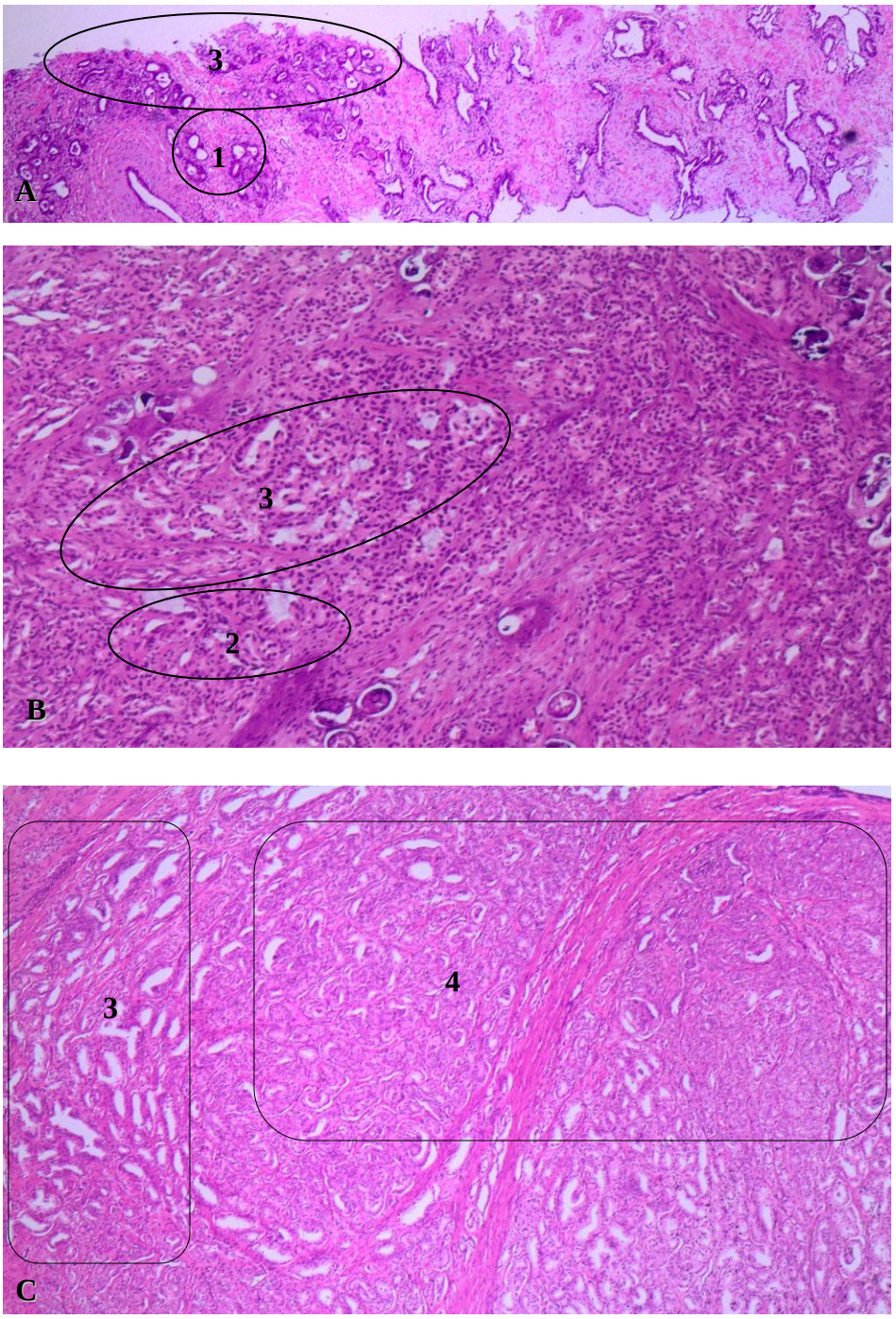


Figure 7. Photomicrographs illustrating the Gleason patterns

Routine H&E stained sections. **A**, Male 83 years had carcinoma with a Gleason score 3+1=4 (x40). **B**, Male 77 years had a carcinoma showing a Gleason score 3+2=5 with schistosomiasis (x100). **C**, Male 73 years presented with a carcinoma showing a Gleason score 4+3=7 (x40).

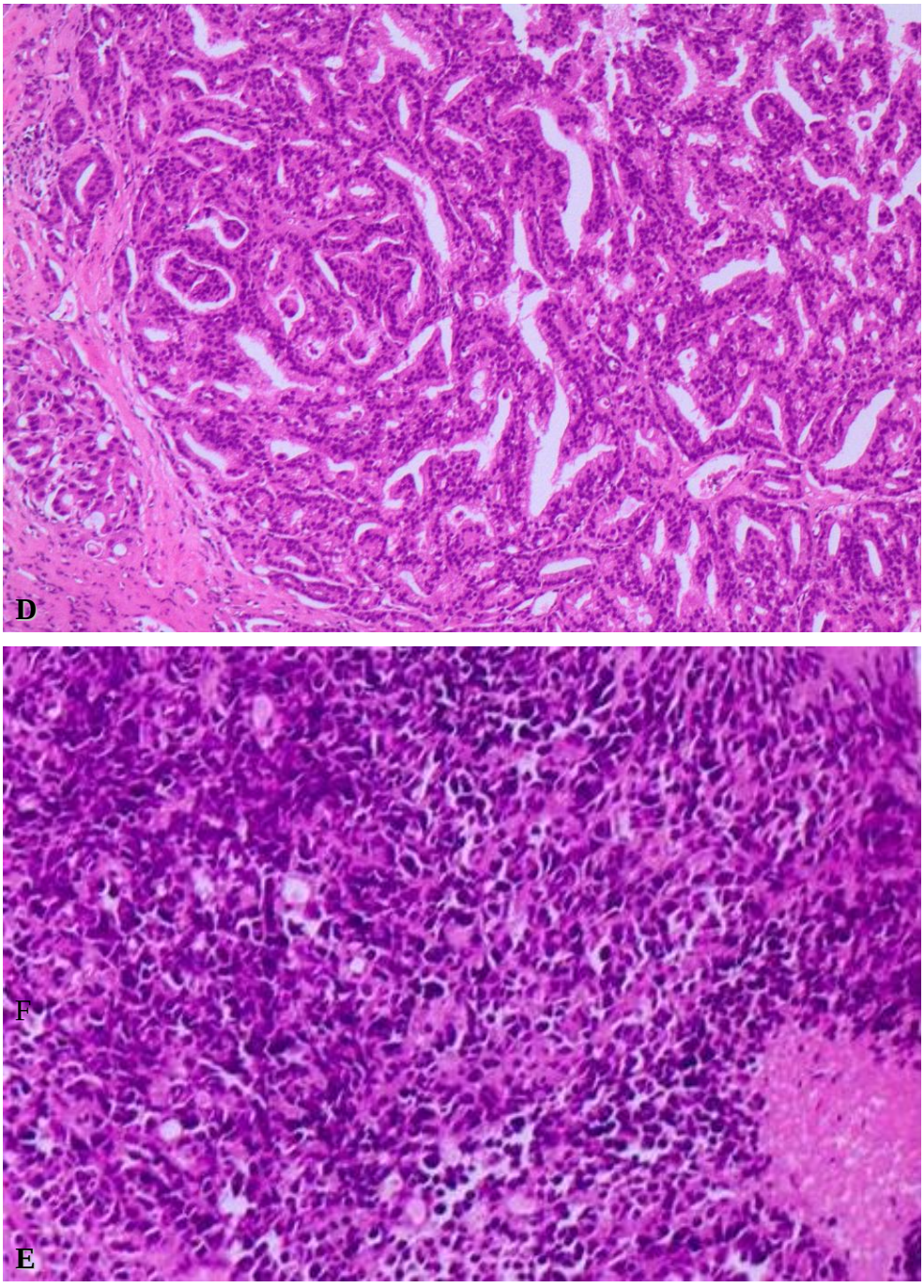


Figure 7 (continued). Photomicrographs illustrating the Gleason patterns

Routine H&E stained sections. **D**, Male 60 years of age, had carcinoma showing a Gleason score 4+4=8 (x100). **E**, Male 84 years presented with a prostatic adenocarcinoma showing a Gleason score 5+5=10, with necrosis at right lower side (x100).

Figure 8 shows that more than 42% of the cases presented with the average tumour extent of at least 80% of the section area (Figure 9A for illustration). The mean tumour extent of tissue sections was 67.88%, ranging from 20 to 99.5%; the median was 70%.

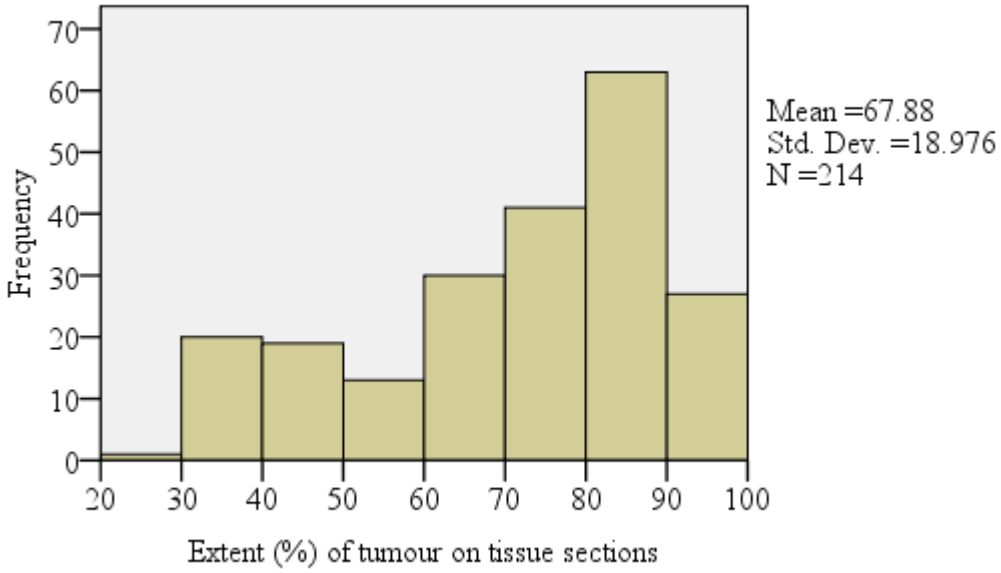


Figure 8. Distribution of the average extent of tumour on histology sections

Perineural invasion (PNI) and vascular invasion by the tumour were seen in 50.92% of the cases (Table 6). About 33.17% of the cases presented only with PNI by the tumour, and 4.47% presented with vascular invasion by the tumour, while 13.08 % of the study population had both PNI and vascular invasion. Figure 9B is an illustration of PNI, while Figure 9C is an illustration of vascular invasion.

Table 6. Perineural and vascular invasion

Findings	Frequency	Percent
Perineural invasion and vascular invasion	28	13.08
Perineural invasion alone	71	33.17
Vascular invasion alone	10	4.67
Subtotal (either perineural and/or vascular invasion)	*109*	*50.93*
No PNI or vascular invasion	105	49.07
Total	214	100

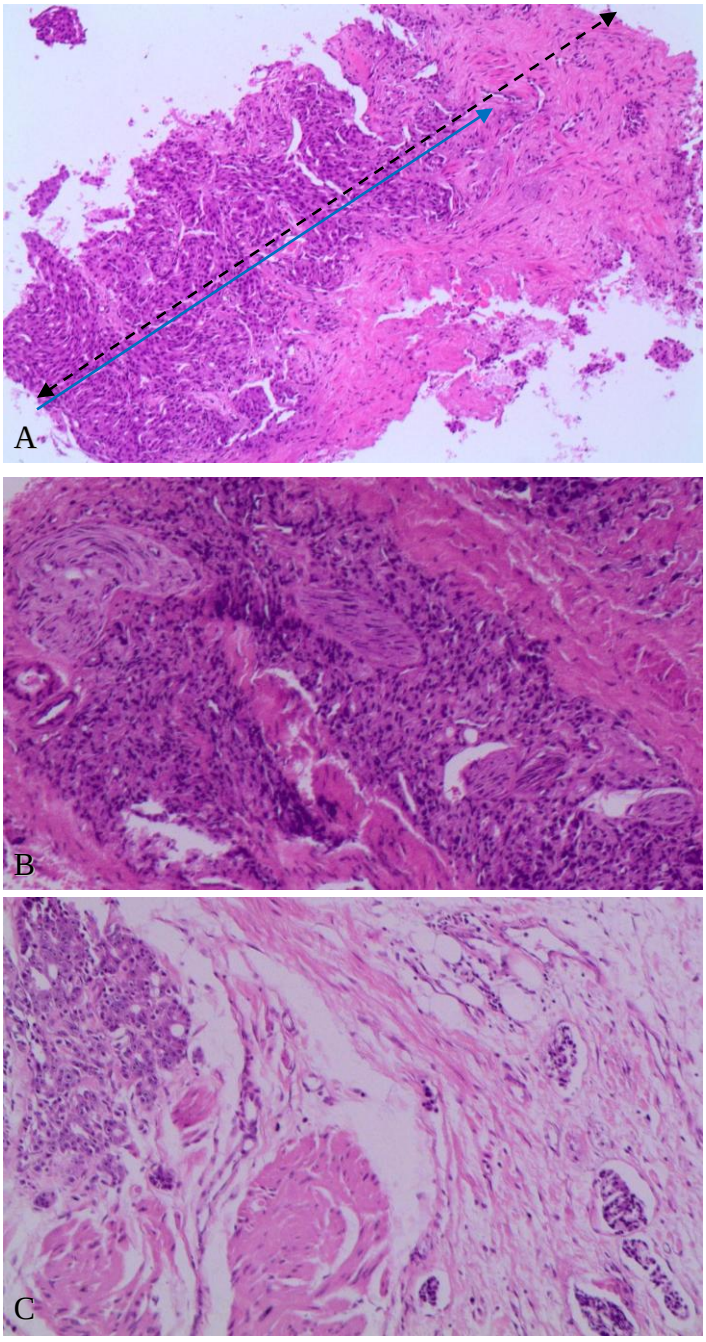


Figure 9. Illustrations of average tumour extent, PNI and vascular invasion

A, Male 66 years had carcinoma Gleason score 4+5=9; tumour (blue arrow) extent to 70% of this core (dash-line arrow) length (H&E, x40). **B**, same patient as in A, this section shows PNI (H&E, x40). **C**, Male 63 years had a carcinoma with Gleason score 4+5=9 with vascular invasion (H&E, x100).

All (100%) study cases were of acinar prostatic adenocarcinoma type (Table 7). Other associated diagnoses were prostatic adenomatous hyperplasia in 39 cases (18.2%) and schistosomiasis in 2 cases (0.9%). Rectal invasion by the tumour, ganglion cells within the tumour and skeletal muscle invasion by the tumour contributed to revise the stage of tumour, and were found in 18 (8.4%), 6 (2.8%) and 2 (0.9%) respectively.

Table 7. Diagnosis and other histology findings

Histology findings		**Frequency**	**Percent**
Diagnosis	Acinar adenocarcinoma	214	100
Other associated findings	Prostatic Adenomatous Hyperplasia	39	18.2
	Tumour invading the rectum	18	8.4
	Ganglion cells in invaded tissue	6	2.8
	Schistosomiasis	2	0.9
	Spine bone involvement	2	0.9
	Skeletal muscle involvement	2	0.9

Many of the patients were diagnosed with prostatic adenocarcinoma at advanced disease stage; 39.9% were at stage IV, 28.1% at stage III, 28.1 at stage IIB and 3.9% at stage IIA (Table 8). No case of stage I was found. Only 153 cases (70.09%) could be staged based on the provided clinical, radiological PSA level and histological information.

Table 8. Stage of the tumour at diagnosis

Stage at diagnosis	**Frequency**	**Percent among staged cases**	**Overall percent**
IIA	6	3.9	2.8
IIB	43	28.1	20.1
III	43	28.1	20.1
IV	61	39.9	28.5
Subtotal (staged cases)	*153*	*100.0*	*71.5*
Non-staged cases	61	-	28.5
Total	214	-	100

Ki67 index in prostatic adenocarcinoma ranged from 0.0% to 50.94%, as shown in Figure 10. The mean was 12.00%, while the median was 10.39%. Ki67 staining was done in 103 cases.

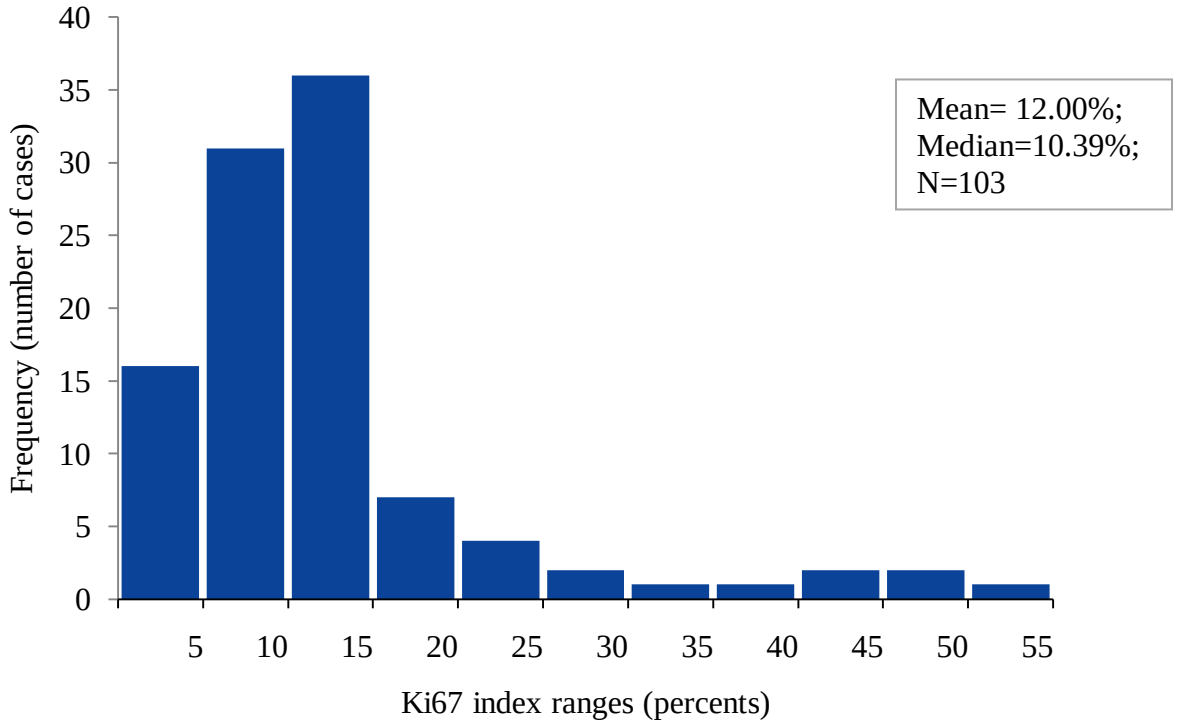


Figure 10. Distribution of Ki67 index

Ki67 index in sections of benign prostatic hyperplasia (BPH) used as either external or internal positive controls ranged from 0.0% to 1.5%. The illustrations of immunoperoxidase MIB-1 stained sections are presented in Figure 11.

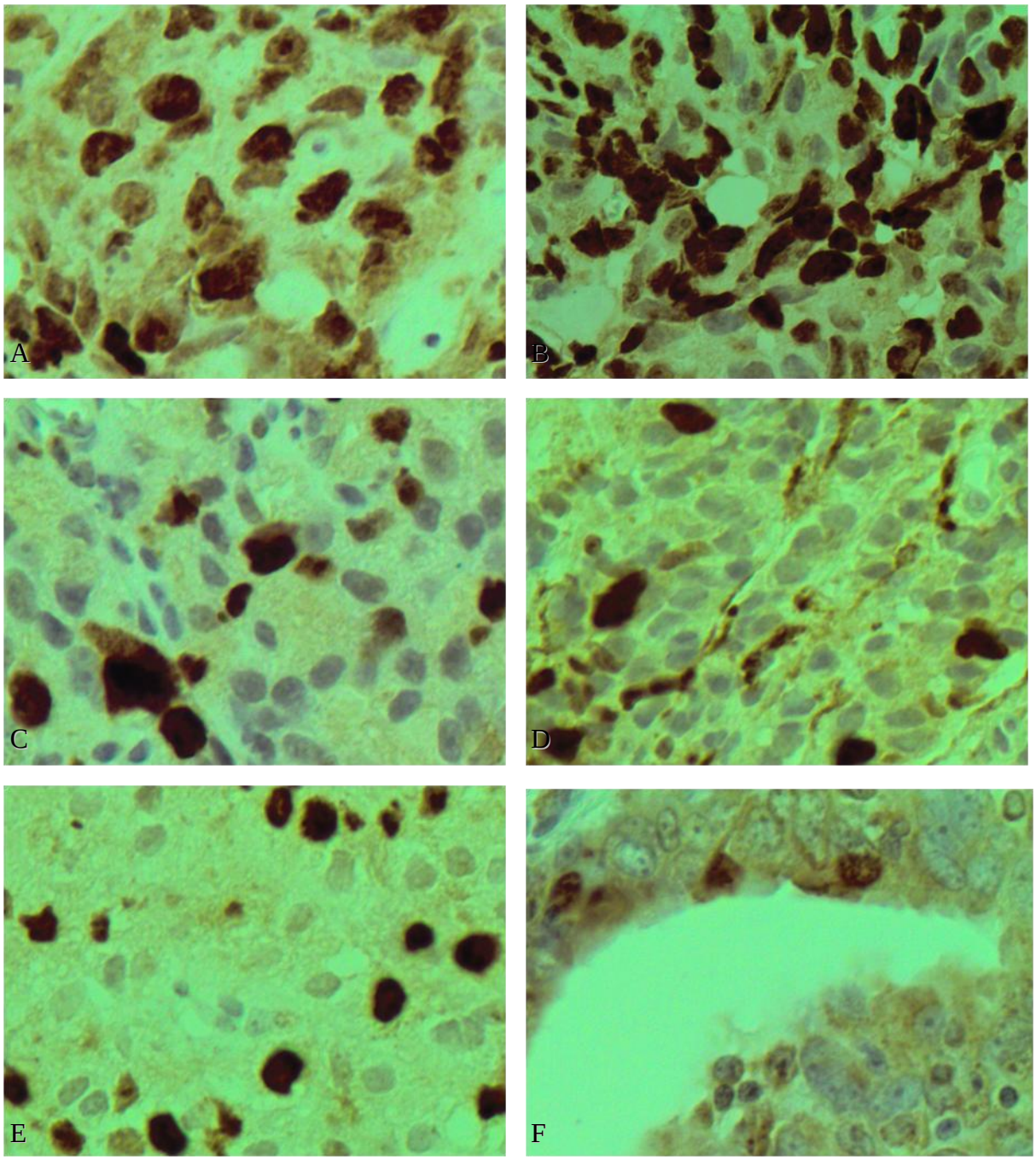


Figure 11. Photomicrographs of immunoperoxidase MIB-1 stained sections

A-E: Prostatic adenocarcinoma, with respective Ki67 indexes, **A**, 50.9% (Gleason 5+4=9). **B**, 45% (Gleason 4+4=8). **C**, 20.1% (Gleason 4+5=9). **D**, 11.8% (Gleason 4+5=9). **E**, 10.9% (Gleason 5+4=9). **F**, 0.9%, benign prostatic hyperplasia. All are at high power (x400).

Only 48 patients had documented follow-up after diagnosis. Among them 32 (87.5%) of them had an advanced cancer disease (stages III and IV) at the last follow-up, as shown in Table 9.

Table 9. Stage of the disease at last follow up

Stage	Frequency	Percent
IIA	2	4.17
IIB	4	8.33
III	10	20.83
IV	32	66.67
Total	48	100.0

The mean duration of follow-up was 31.2 months (0.56 to 60 months). Two patients out of 15 with complete follow-up were living with the disease at 60 months; one was 68 years of age, tumour extent of 40%, Gleason score 3+4=7, Ki67 index 11.45% and stage IIA which progressed to stage III at last follow-up; another patient was 70 years of age, tumour extent of 60%, Gleason score 3+3=6 and stage IIA, which progressed to stage IIB at last follow-up. One patient died at 60 months of follow-up. Survival analysis of the 15 patients for whom the outcome was known is presented in Table 10 and Figure 12. The mean survival was 24.50 months, ranging from 0.56 to 60 months.

Table 10. Survival Table

Patient	Survival time (Months)	Cumulative Proportion Surviving at the Time		Outcome at the end of follow-up
		Estimate	Std. Error	
1	.560	.933	.064	Dead
2	1.000	.867	.088	Dead
3	1.250	.800	.103	Dead
4	1.750	.733	.114	Dead
5	4.000	.667	.122	Dead
6	15.250	.600	.126	Dead
7	19.000	.533	.129	Dead
8	21.750	.467	.129	Dead
9	23.000	.400	.126	Dead
10	25.000	.333	.122	Dead
11	26.000	.267	.114	Dead
12	49.000	.200	.103	Dead
13	60.000	.000	.000	Dead
14	60.000	-	-	Alive
15	60.000	-	-	alive

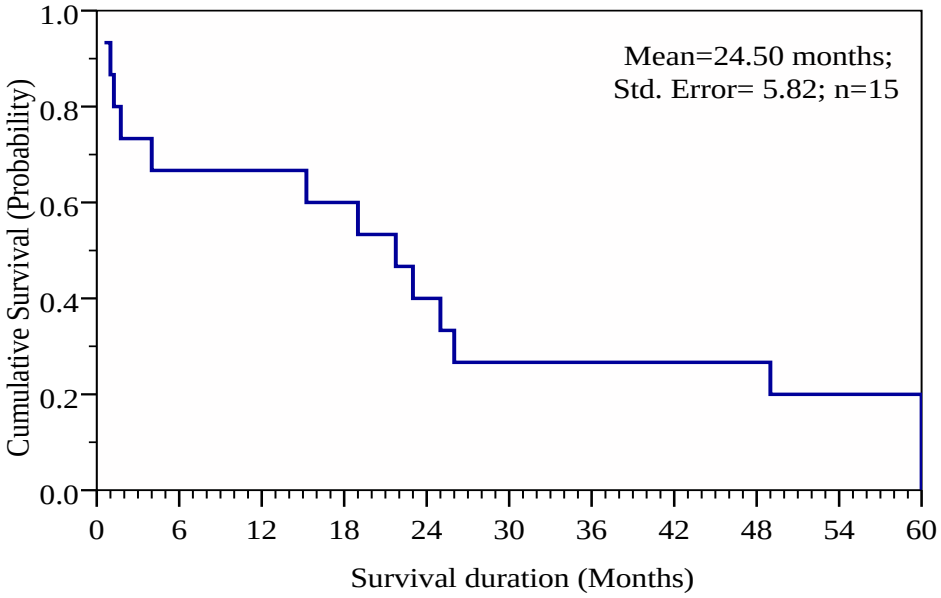


Figure 12. Kaplan-Meier curve for the survival duration of the study population

Thirty-eight out of 40 (95%) metastatic prostatic adenocarcinoma involved bones (Table 11). This was mainly confirmed by clinico-radiological findings and for 2 cases by also histology.

Table 11. Distribution of the metastatic sites by prostatic adenocarcinoma

Site of metastasis	***Frequency***	***Percent (over 153 cases)***	***Overall percent***
Humerus	1	0.65	0.47
Iliac bone	5	3.27	2.34
Liver	1	0.65	0.47
Lymph node	1	0.65	0.47
Spine	32	20.91	14.95
Subtotal (all metastases)	*40*	*26.14*	*18.70*
No metastases	*113*	*73.86*	*52.80*
Non-documented cases	61	-	28.50
Total	214	-	100

Concerning the outcome of the patients, 33 (68.7%) of the 48 followed up patients were incompletely followed up, 13 (27.1%) died within 60 months after diagnosis and only 2 (4.2%) were alive with the disease at the end of 60 months after diagnosis (Table 12). 12 out of 13 (92.3%) death cases died of prostate cancer disease. The remainder case of death was a male 63 years of age with stage IV (rectal involvement) prostatic adenocarcinoma Gleason score 3+4=7, Ki67 index of 1.0% and average tumour extent on 70% of the tissue, and died of diabetes mellitus 49 months after diagnosis of prostatic adenocarcinoma.

Table 12. Outcome of the prostatic adenocarcinoma patients

Outcome	Frequency	Percent
Alive	2	4.2
Lost-to-follow up	33	68.7
Dead	13*	27.1
Total	48	100.0

*One died of diabetes mellitus

4.3 Correlation of variables

Gleason score tends to decrease with an increase in the age of the patient, as shown in Figure 13; this correlation is statistically significant (ANOVA *P*= 0.000) and precise (95% CI for mean is 70.705 to 71.676 years).

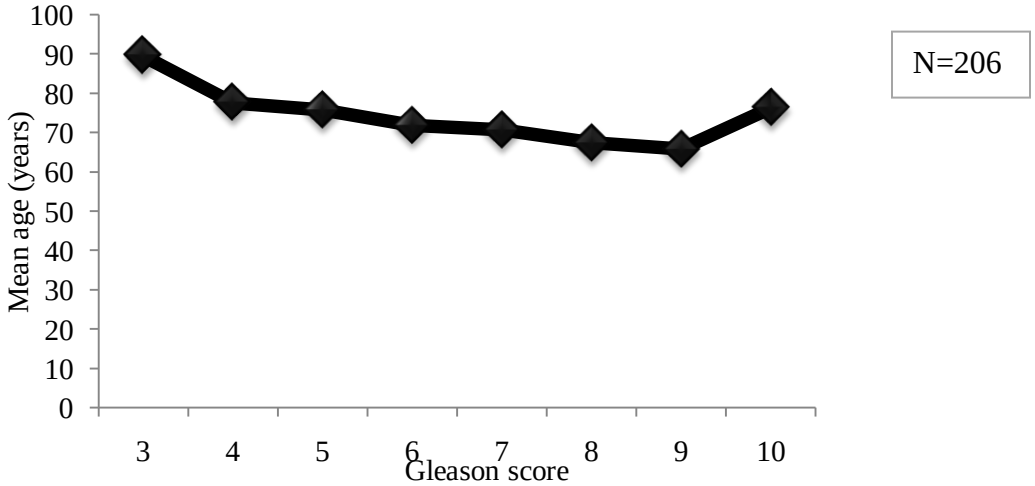


Figure 13. Correlation of Gleason score and age of the patient

Figure 14 shows that pre-treatment serum PSA level was associated with Gleason score (ANOVA *P*= 0.001; 95% CI for mean is 75.45 to 84 ng/mL, n=140). Both patients with Gleason score 4 had outlier high mean PSA values, of respectively 44 and 100 ng/mL.

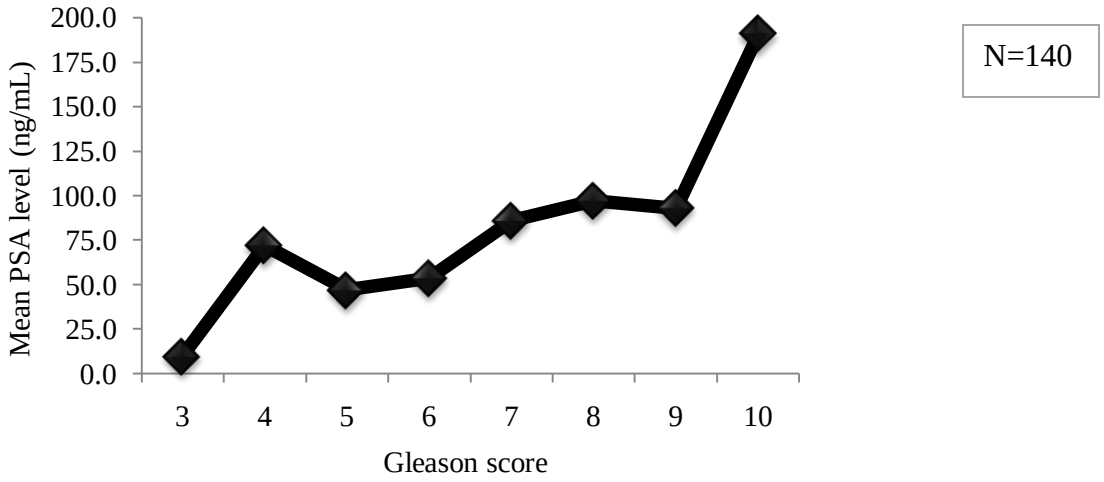


Figure 14. Correlation of Gleason score to pre-treatment PSA level

The average extent of tumour on tissue generally increased with the Gleason score (*P*= 0.000; 95% C.I. for mean is 66.26 to 69.49%), as shown in Figure 15. One of 2 patients with Gleason score 3 had an outlier high average tumour extent of 80%; 2 of 3 patients with Gleason score 4 had average tumour extent of 75% each.

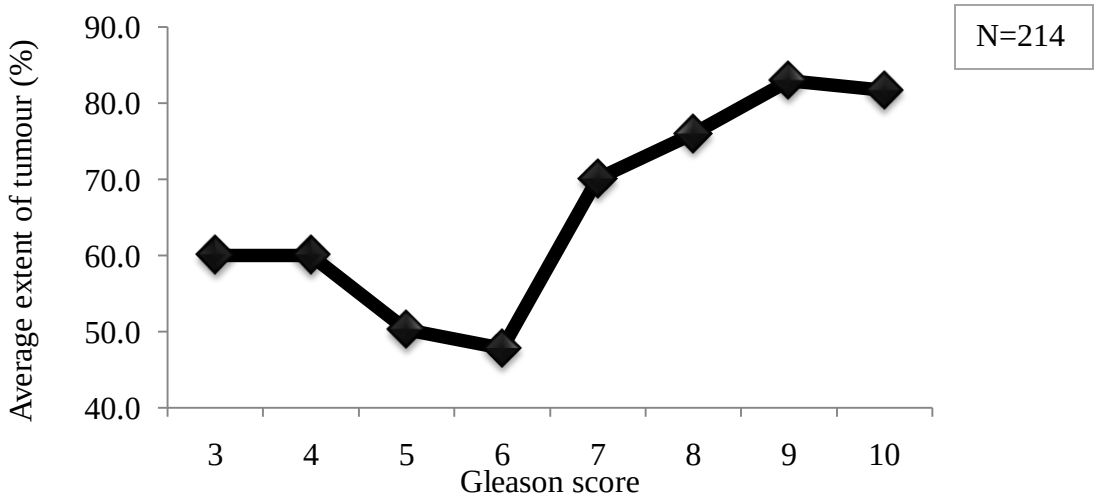


Figure 15. Correlation of Gleason score to the extent of tumour on tissue sections

Gleason score in this study was directly associated with tumour stage at diagnosis, as shown in Table 13 (Fisher's exact P value = 0.009). Higher grades were associated with higher stages.

Table 13. Correlation of Gleason score and tumour stage at diagnosis

Stage at diagnosis	Gleason score								Total
	3	***4***	***5***	***6***	***7***	***8***	***9***	***10***	
IIA	0	0	0	5	1	0	0	0	6
IIB	0	1	6	3	18	7	7	1	43
III	0	0	6	4	17	10	5	1	43
IV	0	0	3	5	28	9	16	0	61
Total	0	1	15	17	64	26	28	2	153
Fisher's exact *P*= 0.009									

Serum PSA level at last follow-up (not shown) was not directly associated with the Gleason score of prostatic adenocarcinoma (*P*= 0.816; 95% C.I. for mean is -21.75 to 266.52 ng/mL). Only 10 cases were provided with PSA level at the end of the follow-up.

The pre-treatment serum PSA level did not correlate with serum PSA level (not shown) at last follow-up (P = 0.279; 95% CI for mean is -21.7511 to 266.5271 ng/mL). The post-operative increase of PSA levels may reflect progressive and/or refractory disease. The patient who had an increase in PSA from 14.5 to 500 ng/mL consisted of a 76-year-old patient who presented with a stage IV prostatic adenocarcinoma (involving rectum) at diagnosis; average tumour extent of tissue was 75%, there was PNI. The Ki67 index was 7.6%. He developed no distant metastasis, and was lost-to-follow up at 21.5 months.

Average tumour extent on tissue was directly associated with the tumour stage at diagnosis, as shown in Figure 16. The association was statistically significant (Cochrane-Armitage tests for linear trend *P*=0.011) and precise (95% CI for mean is 69.70 to 71.29 %; n=153).

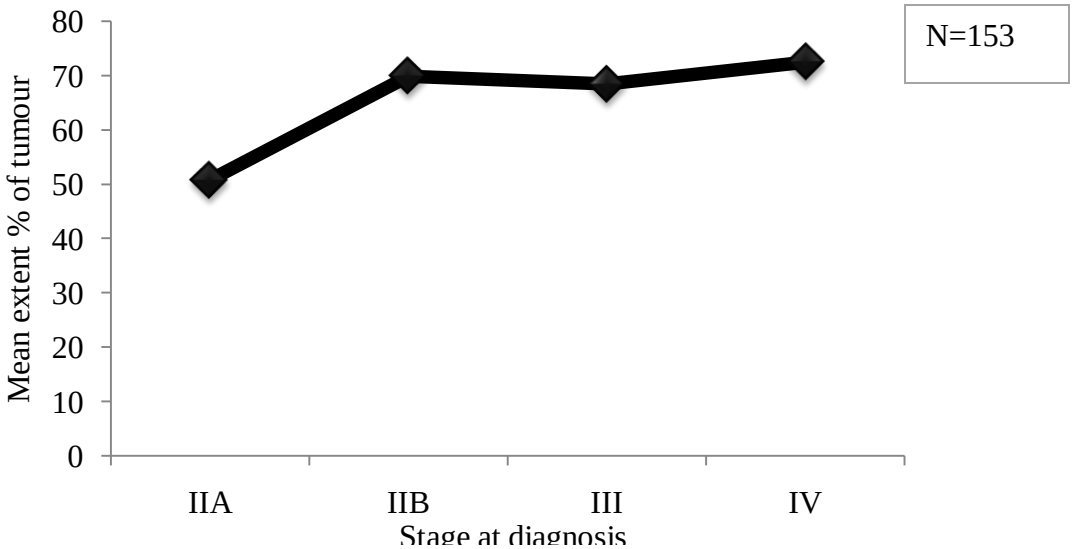


Figure 16. Correlation of tumour stage and average extent of tumour on tissue sections

Table 15 showed that neither PNI nor vascular invasion was associated with the tumour stage at diagnosis (Cochrane-Armitage tests for linear trend, $P = 0.428$ and $P = 0.447$, respectively). However, no stage IIA case had PNI or vascular invasion.

Table 14. Correlation of PNI and vascular invasion with tumour stage at diagnosis

Stage	PNI		Total	Vascular Invasion		Total
	Yes	No		Yes	No	
IIA	0	6	6	0	6	6
IIB	27	16	43	7	36	43
III	19	24	43	12	31	43
IV	27	34	61	11	50	61
Total	73	80	153	30	123	153
Cochrane-Armitage test for linear trend: $P = 0.428$				Cochrane-Armitage test for linear trend: $P = 0.447$		

Despite the high rate of incomplete follow-up, the outcome of the patient was directly associated with the tumour stage at diagnosis, as shown in Table 15 (P=0.011). Both patients who were alive at the end of follow-up were diagnosed with stage IIA cancer. Death risk increased with advanced stage at diagnosis.

Table 15. Correlation of tumour stage at diagnosis and outcome of the patient

Stage at diagnosis	Outcome			Total
	Alive	Incomplete follow-up	Dead	
IIA	2	2	1	5
IIB	0	8	3	11
III	0	12	2	14
IV	0	11	7	18
Total	2	33	13	48
Cochrane-Armitage tests for linear trend P=0.011				

The outcome of the patient was not associated with the Gleason score. This finding was the same if the Gleason scores were considered separately or Gleason score 7 grouped in low risk group (Cochrane-Armitage tests for linear trend P = 0.280), or in intermediate risk group, or in high risk group. Both patients who were alive at the end of follow-up were in low risk group (they had Gleason scores of 6 and 7 respectively).

The survival duration of the 15 patients with complete follow-up inversely correlated with the tumour stage at diagnosis (Figure 17), the association was statistically significant (Log Rank P=0.018; 95% CI for mean is 13.09 to 35.91 months). The mean survival duration was 48.67 months for stage IIA, 21.08 months for stage IIB, 40.87 months for stage III, and 10.93 months for stage IV.

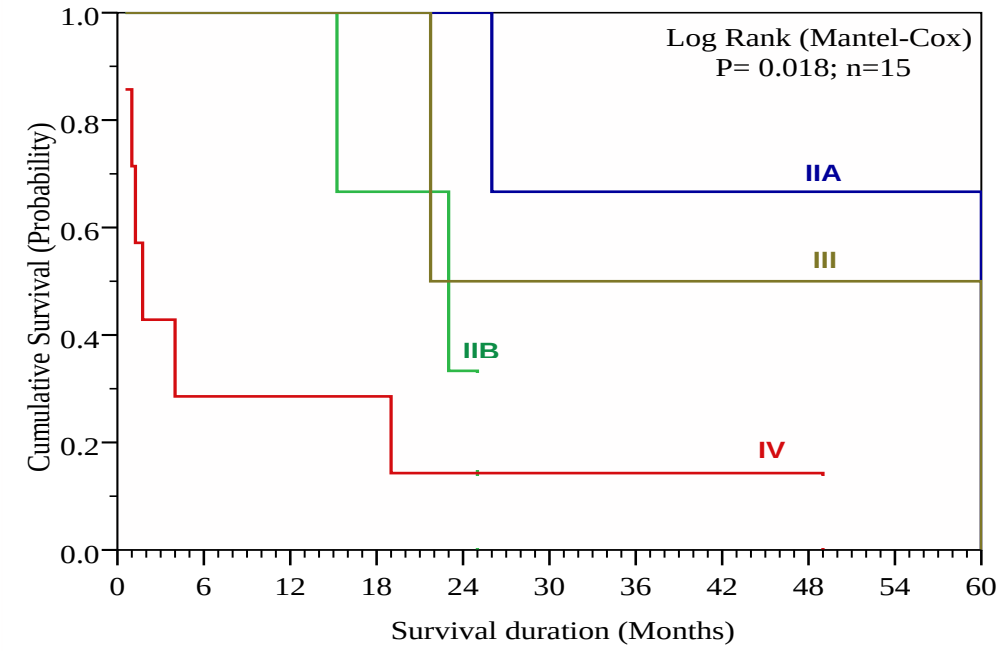


Figure 17 Correlation of tumour stage at diagnosis and survival of the patients

In correlating survival duration to the average tumour extent on tissue sections, three alternatives were used to define the best cut-point. Considering the slope of the curve of distribution of average tumour extent (Figure 8), the cut-points of 50%, 60% and the median (as used in other studies) were used respectively (Table 16); however, none of these cut-points

showed a statistically significant association between the survival and the average tumour extent. The use of median average tumour extent seemed to be more rational.

Table 16. Correlation of survival to the average tumour extent on tissue sections.

Average tumour extent grouping method	Prognostic groups	Mean survival (months)	95% CI (months)	P value (Log Rank)
50% as cut-point	<50% (n=2)	32.00	0.00 to 86.88	0.484
	≥50% (n= 13)	23.351	11.80 to 34.89	
60% as cut-point	<60% (n=3)	29.66	0.00 to 61.68	0.539
	≥60% (n=12)	23.21	10.66 to 35.76	
Median (70%) as	<70% (n=4)	37.25	10.17to 64.33	0.146
cut-point	≥70% (n=11)	19.87	8.14to 31.59	
Overall		24.50	13.09 to 35.91	
	n=15			

Gleason score was correlated to survival utilizing Gleason score grouping in order to have enough samples in respective subgroups. Three alternatives of Gleason score grouping were done, namely considering Gleason score 7 as low, intermediate or high grade (Table 17).

Table 17. Correlation of survival duration to the Gleason score

Gleason score grouping method	Prognostic groups	Mean survival (months)	95% CI (months)	Log Rank (Mantel-Cox) P value
Gleason score 7	Gleason score 2-6	28.75 (n=4)	6.04 to 51.45	0.483
considered high grade	Gleason score 7-10	22.96 (n=11)	9.22 to 36.69	
Gleason score 7	Gleason score 2-6	28.75 (n=4)	6.04 to 51.45	
considered	Gleason score 7	34.35 (n=5)	7.67 to 61.03	0.101
intermediate grade	Gleason score 8-10	13.46 (n=6)	5.39 to 21.54	
Gleason score 7	Gleason score 2-7	31.86 (n=9)	14.91 to 48.81	0.029
considered low grade	Gleason score 8-10	13.46 (n=6)	5.39 to 21.54	
Overall	n=15	24.50	13.09 to 35.91	-

In this study, there was a statistically significant association between survival duration and Gleason score if Gleason score 7 is considered as low grade (Table 17 and Figure 18, P=0.029). Higher Gleason score implied short survival duration.

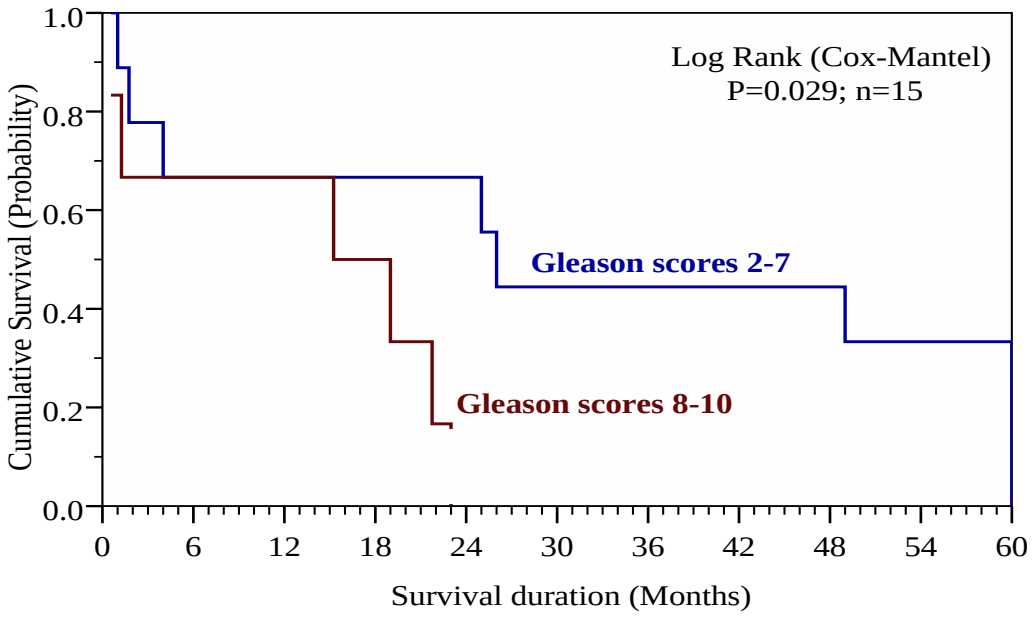


Figure 18. Correlation of the survival of patients to the Gleason score

The numbers of cases in the respective Gleason score groups were small, thus hindering further stratified survival analysis.

Ki67 index directly correlated with the survival duration of prostatic adenocarcinoma patients, as shown in Figure 19 [Log Rank (Mantel-Cox) P=0.021; n=15; 95% CI for mean is 13.09 to 35.91 months].

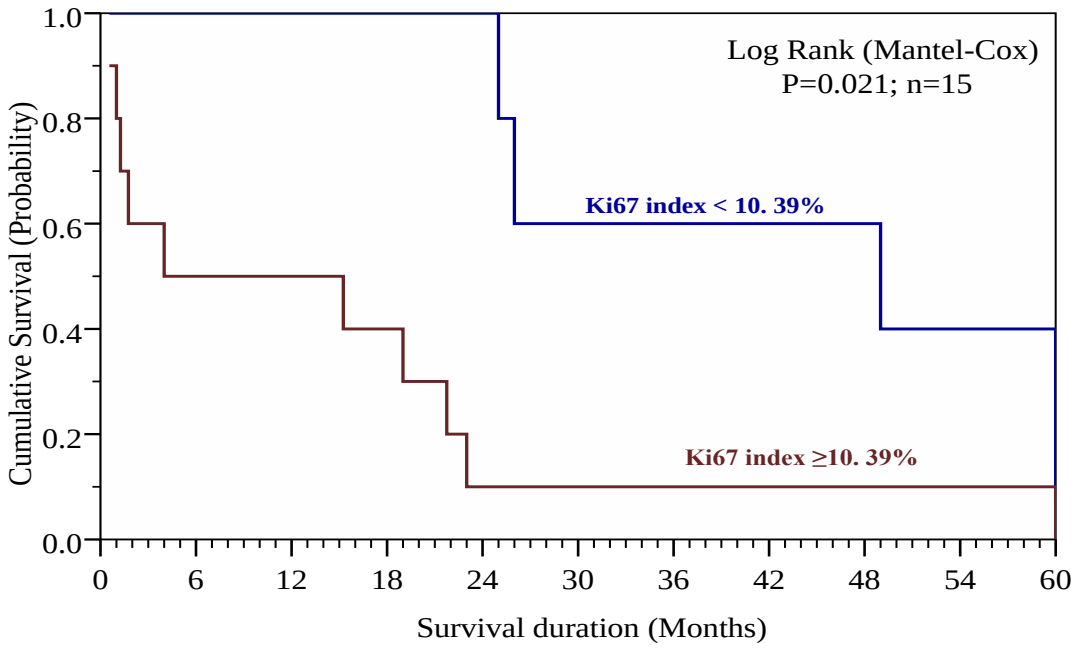


Figure 19. Kaplan-Meier survival correlated to Ki67 index

The mean survival duration for the cases in which Ki67 index was below the median Ki67 value (10.39%) was 44.00 months, while for those in which Ki67 index was above the median Ki67 value (10.39%) the mean survival was 14.75 months.

Gleason score directly correlated with the Ki67 index (Table 18); Ki67 index increased with Gleason score (P=0.000). The mean Ki67 index in Gleason score 7 cases is close to that of the Gleason score 2-6 group; Gleason 7 was therefore classified in low risk group..

Table 18. Correlation of Gleason score and Ki67 index

Gleason score	Ki67 index (%)				
	Mean	N	Standard deviation	Minimum	Maximum
Gleason score 2-7	9.0949	63	7.6023	0.00	45.61
Gleason score 8-10	16.5887	40	12.43429	0.00	50.94
Total	12.0051	103	10.37869	0.00	50.94
			P=0.000		

A patient with Gleason score 4 and Ki67 index 28.32% had a stage IIB disease at diagnosis, tumour extent at 75% of the sections, but the patient was not followed-up. The two patients with Gleason score 10 had Ki67 indexes of 4.47% and 0.0% respectively; the one who had Ki67 index of 0.0% was 77 years of age, with stage III cancer at diagnosis, pre-operative serum PSA level of 467 ng/mL, PNI and average tumour extent at 80% of the tissue, he was not followed-up. Another patient with Gleason score 10 and Ki67 index of 4.47% was 68 years- of age, pre-treatment PSA level of 100 ng/mL, PNI and tumour extent at 70% of the tissue, he was not followed-up and had no useful clinical data for disease staging.

Concerning correlation of Ki67 expression to other variables, Ki67 index significantly decreased with age (Chi-square P=0.006) and increased with average tumour extent on sections (Chi-square P=0.032). Ki67 index did not correlate with PNI (Chi-square P=0.106), tumour stage at the end of follow-up (P = 0.135) and patient's outcome (P = 0.226).

4.4 Comparison of Gleason score and Ki67 index to predict biological behaviours

Figure 20 shows that Gleason score was directly associated with pre-treatment serum PSA level (P=0.000, n=140), a higher PSA level predicting a higher Gleason score. Ki67 index did not correlate with the pre-treatment serum PSA level (P=0.825, n=58). Gleason score, rather than Ki67 index, significantly correlated with pre-treatment serum PSA levels.

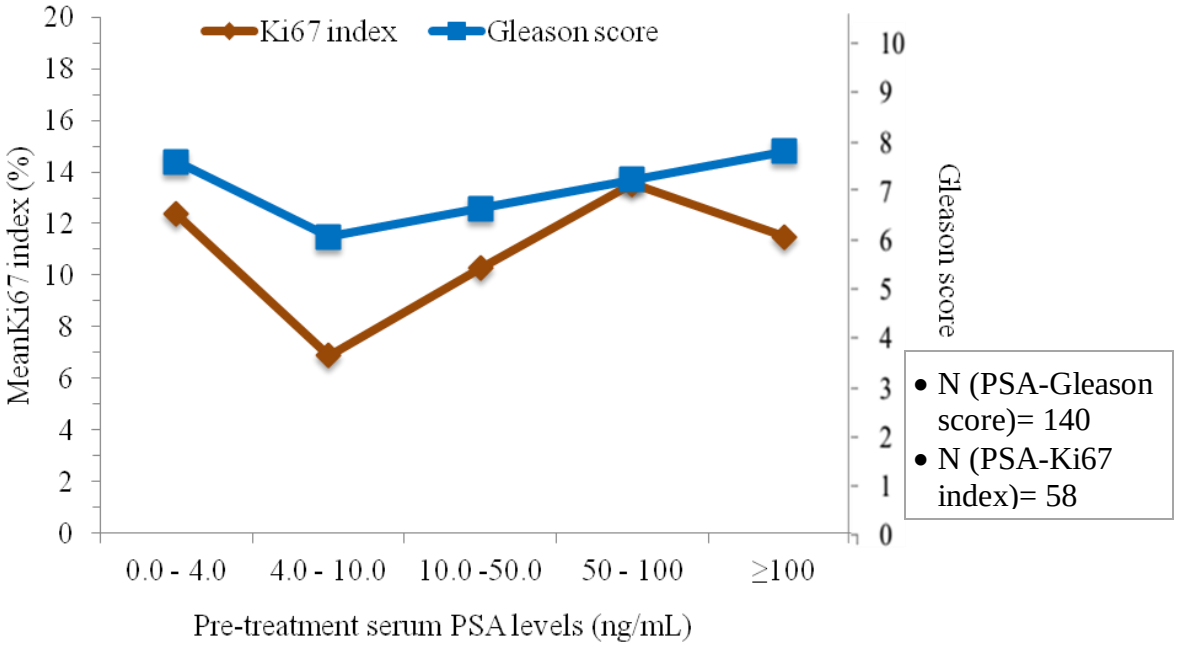


Figure 20. Gleason score and Ki67 index compared on basis of pre-treatment PSA levels

As shown in Figure 20, the five patients who had normal ranges of pre-treatment serum PSA levels had however high grade disease (one had Gleason score 9, another had 8, two had Gleason score 7 and one had a Gleason score of 5), high Ki67 index (range 6.9% to 18.7%), high average extent of tumour on tissue (respectively 80% for 2 cases, 60% for 2 cases and 30% for one case), progressive disease (one case was stage IV, two had stage III and other 2 had stage IIB). Two of the 5 patients with normal pre-treatment serum PSA levels died within 1 month and 22 months of diagnosis respectively; the other 3 cases were incompletely followed up (2 to 47 months). This shows that Gleason score and Ki67 index depict aggressive prostate cancer cases missed by PSA test.

Figure 21 shows that Gleason score directly correlated with the tumour stage at diagnosis (P=0.007, n=153), a higher Gleason score being associated with higher stage. Ki67 index did not correlate with the tumour stage at diagnosis (P=0.573, n=93). Gleason score, rather than Ki67 index, may significantly predict the tumour stage.

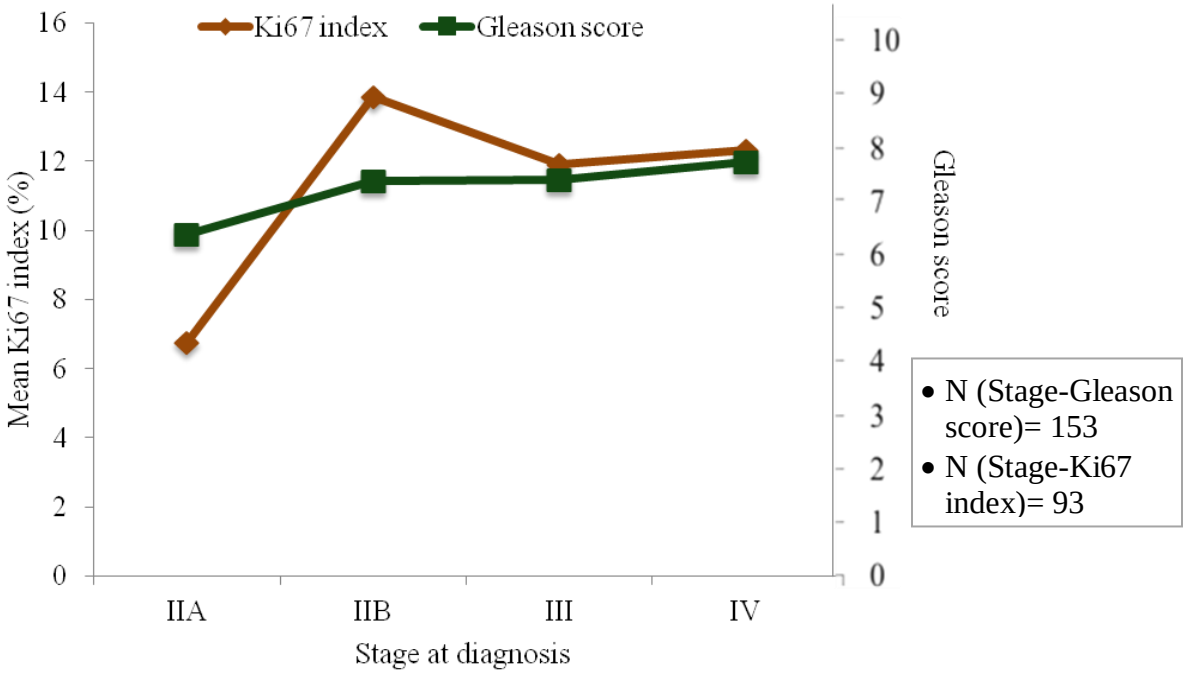


Figure 21. Gleason score and Ki67 index compared by tumour stage at diagnosis

Poisson distribution of disease progression incidence rates is presented in Table 19. In most categories, progression to higher disease stage was demonstrated: 3 out of 5 (60%) of stage IIA, 8 out of 11 (72.7%) of stage IIB, 8 out of 14 (57.1 %) of stage III, and 6 out of 18 cases (33.3%) of stage IV acquired additional metastases, developed complications or died of the disease. The remaining 23 cases had no evidence of progression.

Table 19. Gleason score and Ki67 index compared on basis of disease progression

Risk group	**Gleason score**		**Ki67 index**		**Comparison of Gleason score and Ki67**		
	Rate per 1,000 person months	Attribu-table fraction	Rate per 1,000 person months	Attribu-table fraction	Ratio	SE	Fraction of Ki67-detected (Gleason score-missed) cases
Low	24.976	19.0%	21.944	38.3%	0.8786	0.399	6.6%
High	61.605	59.5%	60.702	63.8%	0.9853	0.438	13.3%
Overall	30.845	-	35.571	-	1.153	0.283	-
Fisher's Exact test	P value = 0.073 95% CI is 0.921 to 6.030 months		P value = 0.020 95% CI is 1.163 to 6.885 months		P value = 0.715 95% CI is 0.635 to 2.093 months		

Note: SE = standard error for ratio. N=48

Gleason score did not significantly predict the progression of prostate cancer disease (Fisher's exact test P value = 0.073; 95% CI is 0.921 to 6.030); Ki67 index predicted prostate cancer progression (Fisher's exact test P value = 0.020; 95% CI is 1.163 to 6.885). Ki67 index was able to detect progressive disease cases missed by Gleason score system, respectively 6.6% of the low risk group and 13.3% of high risk group cases. Therefore, when compared to Gleason score, Ki67 index would help to detect an additional prospective progressive prostatic adenocarcinoma case for 18 cases followed up 1 year (or 212 cases followed up for 1 month).

Concerning the prediction of survival duration, Figure 19 shows that the median Ki67 index significantly determines two distinct risk groups; on other hand, when Gleason score 7 is considered in low group risk, two distinct risk groups are significantly defined (Table 17 and Figure 18). The next step is to determine among Gleason score and Ki67 index, the best predictor of the patient's survival duration, as presented in Table 20.

Table 20. Comparison of Gleason score and Ki67 index in predicting the survival

Survival groups	**Gleason score (Table 17, Figure 18)**			**Ki67 index (Figure 19)**			**Difference**		
	Mean (months)	SE	n	Mean (months)	SE	n	Mean (months)	SE	Chi square P value
Low risk group	31.86	8.649	9	44.00	7.817	5	12.140	13.089	0.372
High risk group	13.46	4.120	6	14.75	5.812	10	1.290	8.222	0.878
Total	24.50	5.820	15	24.50	5.820	15	10.850	15.042	
P value	Log Rank P=0.029			Log Rank P=0.021			Chi square P = 0.483		
95% CI	13.09 to 35.91			13.09 to 35.91			-21.646 to 43.346		

Note: SE = standard error for mean; n= number

Data in Table 21 show that there was no statistically significant difference between Gleason score and Ki67 index in predicting the survival of prostatic adenocarcinoma patient (P=0.483, 95% CI for mean difference is -21.646 to 43.346 months), both tests were equivalent in predicting the patient's survival duration.

CHAPTER 5. DISCUSSION

5.1 Introduction

Prostate cancer constitutes a life-threat in men worldwide without geographical, racial or economical class sparing though variations exist [1, 2, 3, 26, 28, 29, 30, 32, 33, 34]. Prostate cancer usually is a slow-progressing disease, a number of patients die with but not from prostate cancer [24]; once diagnosed, prostate cancer may remain indolent or progress to localized prostate cancer, to locally advanced cancer, to advanced metastatic disease and finally hormone-refractory prostate cancer, and death may occur at any stage [10, 58].

One of the challenges in management of prostate cancer is to determine, at the time of diagnosis, which case will be indolent or slowly progressive thus needing an active surveillance, or which case will be aggressive and therefore needing aggressive treatment. Gleason score is one of the established prognosticators of prostatic adenocarcinoma, but Gleason score on a needle core biopsy sometimes may not reflect the dominant grade on prostatectomy, and therefore mislead management decision making.

The use of tumour progression markers to distinguish these two scenarios would be very valuable; some related investigations are being done [24, 56, 57]. Proliferative markers may add inputs to the present central role played by Gleason score to predetermine patient's risk to progressive disease [31]. The expression of Ki67, as a nuclear antigen expressed during the cell cycle and highlighted by MIB-1 immunoperoxidase stain, may detect progressive disease in cases missed by Gleason score risk grouping system.

This study aimed to compare Ki67 index with Gleason score in prediction of biological behaviour of prostatic adenocarcinoma, as to provide an additional input in evidence-based management of prostate cancer. It was conducted in a national referral hospital of an East African country, Tanzania.

5.2 Data overview

In 2005 and 2006, a number of 737 prostate specimens out of 11,439 (6.44%) surgical pathology specimens were received at MNH. This rate seems to be slowly increasing because in 2003, prostate specimens were 311 out of 5,037 surgical specimens (6.17%), while 593 prostate specimens out of a total number of 8,823 (6.72%) surgical specimens were received in 2011. This may reflect the increased awareness in population and/or improved screening and diagnostic facilities. However, the diagnosed cases are still few, because prostate cancer estimate only in 60-69 years-old men is 65% [60]; more general population sensitization is therefore needed regarding awareness and screening. Prostatic adenocarcinoma was found in 34.73% of all prostate specimens: the male population must be aware that at least one third of all those having prostate symptoms have a cancer; early medical care is recommended to them.

Available investigations were done and documented for most of the in-ward case files, but the clinical staging was done in none (0%). It is recommended to always clearly determine the stage of the prostate cancer [69]. The number of prostate biopsy cores determines the adequacy of Gleason score at radical prostatectomy; at least 10 cores should be taken in patients with prostate volumes of less than 50 cm^3, while for patients with prostate volumes of 50 cm^3 or more, a number of cores does not affect the prediction of radical prostatectomy Gleason score [72]. As prostate transrectal ultrasound (TRUS) guided needle core biopsies represented 87.9 % of the study cases, it is imperative for surgeons to abide by the good clinical practices of prostate biopsies; Appendix IV of this report provides useful guidelines on this matter.

The adequacy of prostatic adenocarcinoma histological reporting was low, only 28.9% of cases (21.8% in 2005 and 35.9% in 2006) had Gleason score in the histology reports; 42.2% of the reports mentioned the grade or differentiation, the remaining 28.9% of reports had no mention about Gleason score or grade. It should be made compulsory to report the Gleason score in all cases of prostatic adenocarcinoma which had no previous treatment [12, 52]. Average extent of tumour on tissue sections was mentioned in none (0%). Prostatic adenocarcinoma diagnosis should contain the extent of tumour in cores because it is a significant predictor of total tumour volume at radical prostatectomy [73].

The use of checklists/datasets may solve this quality problem; it is known that Pathology departments in which checklists are not routinely used have a lower rate of reports that include all the required elements [69, 74, 75]. A checklist of minimum elements to include in histology report of prostatic adenocarcinoma is presented in Appendix IV of this report.

Until the period of this study, majority of the cases of prostatic adenocarcinoma in Tanzania and East-Africa in general were treated by hormonal manipulation using the surgical technique of bilateral subcapsular orchidectomy (BSO) [37]. The literature seems to advise medical castration rather than surgical one; complete androgen blockade is done using combination hormonal therapy with an antiandrogen and a luteinising hormone-releasing hormone analogue (LHRHa) to treat patients with advanced prostate cancer [10, 58]. The cost of the hormonal therapy remains a challenge to Tanzanians.

As far as prevention of prostatic adenocarcinoma is concerned, it is recommended that PSA test may be used to establish a baseline from an initial determination and then follow up with sequential PSA tests, every three or six months, in order to evaluate the trend where, if it is increasing, is a sign of abnormality of the prostate, indicating the need for more investigations [60, 61].

5.3 Demographic data and variable correlations

Demographics

The mean age in this study population is 70.46 years, and ranged from 42 years to 96 years. Only 3 patients (1.40%) were less than 50 years of age. This mean age was similar to that found in Uganda (70 years) [76]; it was also similar to the mean age of 68.12 years found in a study done from November 2006 to May 2008 at MNH [40]. The androgen deficiency, or andropause, is one of the recognized risk factors of prostatic adenocarcinoma; andropause affects 1 in 200 aging men [77], explaining higher incidence of prostatic adenocarcinoma in elderly men. A number of studies confirm the increase of prostate carcinoma with age [48, 77]. In South Africa, the mean age at diagnosis is significantly higher in Whites (69.7 years)

than Coloured (67.9 years) and Blacks (68.9 years) [78]. Whether the age of the patient is a significant independent prognostic factor or not remains controversial [42, 71].

Race was specified in 94.4% of the cases, all were Africans. Although Tanzania has got significant number of Asians especially Indians, no specified Asian case was found in this study. The explanation may be the fact that Asian Tanzanians consult private hospitals more than the public ones including MNH. It is also known that Asian population has lower incidence of prostatic adenocarcinoma than Blacks, respectively 3.9 in India and 228.7 per 100,000 in Africans Americans [30]. A recent study done in USA showed that prostatic adenocarcinoma tends to be of higher grade and extent in Blacks than Whites when stratified by stages [79]; while another recent large study in USA showed that among prostatic adenocarcinoma patients, Blacks were younger, had higher Gleason scores and PSA levels, and had higher average extent tissue cancer involvement [70]. Apart from race, a strong familial clustering is confirmed in first-degree family history of prostate cancer [80]. This was not investigated in this retrospective study done in a setting with poor clinical data documentation.

Half of the study population came from Dar es Salaam City; most of the remaining came from regions neighbouring Dar es Salaam. Prostate cancer awareness and medical care-seeking behaviour are usually higher in cities. The problem of accessibility to competent health facilities as MNH may explain the high proportion of cases from within and around Dar es Salaam, and advocates for improved health services in all regions of Tanzania.

PSA level

Pre-treatment serum PSA levels were provided in patient's files and/or investigation request forms in only 140 cases (65.42%); the mean was 79.578 ng/mL. Though the mean pre-treatment PSA levels in prostatic benign lesions was relatively lower (19.11 ng/mL, ranges 1.6 to 67.7 ng/mL), only 2 cases of benign lesions had normal ranges. On other hand, 5 high grade adenocarcinoma cases had normal ranges of pre-treatment serum PSA levels; this emphasizes the role of PSA as a screening and follow-up tool, but not a diagnostic test. In a study done in

Detroit, USA, the mean preoperative PSA level in prostate cancer cases was 26.8 ng/mL [81]. The high PSA levels in this study population reflect the lack of prior screening and a significant delay in medical care, and presumably the black race [70]. Pre-treatment serum PSA level in this study directly correlated to the Gleason score. A number of studies have confirmed the association between pre-treatment serum PSA level and Gleason score [12, 37, 40, 82], tumour stage [61], as well as the importance of post-surgery biochemical follow-up by periodical measurement of serum PSA levels [83]. Serum PSA level in normal prostates increases with each decade of age and with the volume of the prostate which itself also increases with the age [13]. A diligent interpretation of PSA levels must consider the age of patient and the volume of prostate. Since considerable disparities in PSA results from different assays have been observed, it is better to interpret the obtained values accordingly [84].

Only 8 cases in this study were provided with PSA levels in post-BSO. This is an indicator of poor follow-up of the prostatic carcinoma patients in Tanzania.

Histological findings

All (100%) prostate cancer cases in this study were acinar adenocarcinoma. A previous study done at MNH had found that 99.1% of the cases were adenocarcinoma while one case was an embryonal rhabdomyosarcoma [40].

Gleason score 7 (moderately poorly differentiated adenocarcinoma) was encountered in 38.3% of the cases. Well differentiated adenocarcinoma (Gleason scores 2-4) cases represented 2.4%, moderately differentiated adenocarcinoma (Gleason scores 5-6) cases represented 24.7% while poorly differentiated adenocarcinoma (Gleason scores 8-10) cases represented 34.6%. The preponderance of Gleason score 7 was confirmed by other studies [12, 79, 85], and was around 43% in some studies [79, 85]. Some reports consider Gleason 7 (3+4) and Gleason 7 (4+3) separately because of difference in prognosis [86]. In the original Gleason score system, Gleason score 6 was the most predominant, encountered in 39.05% of the cases [52]. The overtime increase in case proportions of Gleason score 7 and above has been well documented [27, 87]. In one study which recruited cases of organ-confined prostatic adenocarcinoma,

Gleason scores 8-10 were encountered in 42.9% of Blacks and only 18.2% of Whites [79]. In this Tanzanian study, Gleason score correlated with the age of patients, pre-treatment serum PSA levels, tumour extent on tissue sections, tumour stage, Ki67 index and with survival of patients. A number of studies confirmed the correlation between Gleason score and pre-treatment serum PSA levels [12, 27, 37, 40, 82], tumour stage [27, 52], Ki67 index, [31] and prognosis [12, 27, 52, 71]. A recent report showed that patients with high grade, and not stage, especially African-Americans, are more prone to readmission within 30 days after prostatectomy than others, but indications for readmission were not specified [88].

The mean of average tumour extent of tissue sections in this study was 67.88%, ranging from 20 to 99.5%, the median was 70%. The mean extent of tumour on tissue sections in one study was 16.3 % ranging from 0.1% to 86% [89]. The higher extent of tumour at diagnosis is our series is an indicator of late medical care and/or high grade malignancy, leading to a large, infiltrating tumour. The extent of tumour on tissue sections in this study was directly associated with Gleason score, Ki67 index and the tumour stage at diagnosis but not to the survival of patients. In the American series, average extent of tumour was significantly related to the tumour stage, Gleason score and overall survival [89]. The lack of correlation of average tumour extent and patient's survival is attributed to the high rates of incomplete follow-up in this study resulting in a small number of cases available for analysis. Tumour extent on tissue sections is a recognized prognostic factor classified in category II [71], and must appear in histology report [69, 74].

Perineural invasion (PNI) by the tumour was seen in 46.25% of cases, while vascular invasion was encountered in 17.55%. In 13.08 % of cases, both PNI and vascular invasion were seen. PNI rates in previous reports varied from 20.5% [81], to 27.27% [90], to 75% [46]. In one study, the density of nerve in benign biopsy cores was found to be higher than that in malignant biopsy cores [90]. Neural cell adhesion molecule (N-CAM) expression was reported to be significantly upregulated in nerves with PNI than nerves without PNI, suggesting the possible involvement of N-CAM in PNI [47]. PNI is reported to have a statistically significant association with extraprostatic extension and seminal vesicle invasion by the prostate

carcinoma [69, 81]. In this study, however, neither PNI nor vascular invasion was associated with the tumour stage at diagnosis, but also no PNI was seen in stage IIA disease. Some authors recommend the measurement of the PNI diameter in radical prostatectomy, since it is an independent prognostic factor [46, 69]. PNI is classified in category III of the prognostic factors of prostate cancer [71], and must be mentioned in histology report [69].

Schistosomiasis in prostate was found in 2 (0.9%) cases of prostate carcinoma in this study. The literature shows that schistosomiasis in prostate is more frequently associated with benign prostatic hyperplasia than cancer [91]. Prostatic schistosomiasis in association to prostatic adenocarcinoma is an incidental finding; no causative relationship is yet documented..

Stage of prostatic adenocarcinoma

In this study, 67.3% of patients were diagnosed with advanced prostatic adenocarcinoma, namely 38.6% at stage IV and 28.7% at stage III; 28.7% of cases were at stage IIB and 4.0% at stage IIA. In 1974, Gleason had found that stage IV was seen in 36.72% and stage III in 48.83% [52]. In a recent study in USA, stage IV was found in 0.3%, stage III in 27.9% of prostatectomy specimens [92]. A more recent study in USA showed that about 91% of all prostate cancers among African Americans are diagnosed at a local or regional stage, compared with 93% in Whites [28]. There is a clear overtime improvement in early medical care-seeking behaviour in USA (advanced cancer in 85.55% of cases in 1974 to 28.2% in 2012) [28, 52, 92], thanks to PSA screening [87]. In Tanzania in 2006, advanced prostate cancer (stages III and IV) was found in 67.3% of cases, making a cry for general population sensitization for early medical care-seeking behaviour in Tanzania. Tumour stage at diagnosis in this study correlated with Gleason score, average tumour extent on tissue sections, tumour stage at the end of follow-up, outcome and survival duration, and with Ki67 index. Similarly in literature, tumour stage is known to correlate with pre-treatment serum PSA level [61], Gleason score [12, 52], PNI [46, 90], Ki67 expression [31] and is an established prognostic factor in category I [37, 42, 52, 71, 92].

Forty out of 153 (26.14%) patients with adequate clinical information presented with distant metastasis of prostatic adenocarcinoma; 95% cases of metastatic lesions involved the bones, essentially the spine; 12 (7.84%) of the cases developed metastases during the 60 months-follow-up. Only 4 cases (2.61%) had clinical lymph node involvement by prostatic adenocarcinoma, 3 of them had other distant organ metastasis. One large study reported pathological lymph node involvement in 4.4% of prostate carcinoma cases [44], suggesting that many cases of lymph node involvement are clinically missed in our settings. The rate of metastatic disease in prostatic adenocarcinoma has been reported in literature to be around 35% [48]. The predilection of prostatic carcinoma for bone metastases is well established and confirmed in this study [48, 93].

Survival duration of the patients

The mean survival of the patients who had a complete follow-up was 24.50 (ranges 0.56 to 60) months; and only 2 were arrive at 60 months, while one died at 60 months of follow-up. In a prospective study on survival of 56 patients having advanced prostate cancer in Nairobi, 37.29% of patients were still alive at 48 months [37]. In this Tanzanian study, there was a statistically significant association between patient survival and variables such as tumour stage at diagnosis, Gleason score and Ki67index; but survival was not significantly associated with average extent of tumour, probably reflecting a challenge of incomplete follow-up. Various studies have confirmed the association between patient survival and Ki67 expression, [31] tumour stage, Gleason score and average tumour extent on sections [12, 27, 37, 52, 71, 76, 87, 92]. The prognostic value of different modes of treatment of prostatic adenocarcinoma needs to be investigated in Tanzania.

Concerning the mortality, 13 out of our 15 (86.666%) completely followed-up patients had died within 60 months, 12 of them due to prostate cancer disease itself. Though many cases were incompletely followed up, this mortality rate is alarming. In Kenyan series in which only advanced prostate cancer patients were followed-up, 63.71% died within 48 months [37]. Prostate cancer mortality trends have decreased in younger patients in developed countries since the introduction of PSA screening, currently reaching a stable plateau [94]. A number of

environmental and genetic risk factors for prostate cancer have been identified, but none of these is strongly associated with disease [95]. Serum PSA screening remains until now the method of secondary prevention and follow-up; the test must be made available and accessible, and awareness about its importance taught to all men.

Ki67 index in prostatic adenocarcinoma

The median percent Ki67 positive cells (index) in prostatic adenocarcinoma was 10.39% (range 0.0% to 50.94%); the mean was 12.00%. These findings are similar to those of a Danish study done on tumour cell proliferation and survival in 221 patients with prostate cancer followed expectantly, in which the median Ki67 index was 10.30% (ranged 0 to 46.1%) [31].

In this study, Ki67 index significantly decreased with the age of patients, and significantly increased with Gleason score. These findings support the fact that a low grade and therefore slowly progressive malignancy will be symptomatic after long time at advanced age, as compared to a high grade, aggressive malignancy which will be rapidly symptomatic and therefore diagnosed at relatively younger age. Similarly, the Danish series showed that Ki67 index significantly correlated with the tumour grade [31]. Age still remains a controversial independent prognostic factor is prostate cancer [42, 71].

Moreover in this study, Ki67 index correlated with the survival but not the outcome. Likewise, various studies showed Ki67 index to be one of the survival-associated prognosticators in all stages of prostatic cancer [31, 42]. Ki67 index was also reported to be one of the progression-associated prognosticators in organ-confined disease but not with the advanced disease [42, 48]; there was therefore no association between Ki67 index and the outcome of the patients in this study because the majority of patients were diagnosed with already advanced prostate cancer. Though Ki67 index is well-established category III prognostic marker in prostate cancer, there is however until now, no definitive cut-off point of Ki67 index value determining a low/high risk groups of patients [71]. In this study, Ki67 index was associated with tumour stage at diagnosis. Similarly in most of other studies, Ki67 index was significantly associated with tumour stage [31, 42, 48].

5.4 Comparison of Gleason score and Ki67 expression

Gleason score rather than Ki67 index significantly correlated with pre-treatment PSA levels (respectively P=0.000 and P=0.432), that is, serum PSA level decreases with tumour differentiation, but may not correlate with tumour proliferation rate. On the other hand, Gleason score rather than Ki67 index significantly predicted the tumour stage at diagnosis (respectively P=0.007 and P=0.352). Moreover, Ki67 index rather than Gleason score significantly predicted the progression of prostatic adenocarcinoma; when compared to Gleason score, Ki67 index would help to predict prostatic adenocarcinoma progression in 1 additional case for 18 cases followed up for 1 year. This finding is contrary to that reported by Buhmeida *et al*. who found Gleason score to be an independent progression-associated prognosticator in all regardless of prostate disease stage, while Ki67 index was progression-associated prognosticator only in organ-confined prostate disease [42]. The incomplete follow-up and loss-to follow-up in this Tanzanian series may be the cause of discrepancy, but related larger multicentre and prospective study in our settings is recommended for more accurate conclusion.

Last but not least, Gleason score and Ki67 index are equivalent tests in predicting the patient's survival duration; both of them were associated with the patient's survival, and there was no significant difference in survival durations of respective low risk and high risk groups as defined by either test Gleason score and Ki67 index. This conclusion is also contrary to that of Buhmeida *et al.* who reported Gleason score to be an independent survival-associated prognosticator in all regardless of prostate disease stage, while Ki67 index was survival-associated prognosticator only in organ-confined prostate disease [42]. The fact that Gleason score is classified in category I while Ki67 index is in category III of prostate cancer prognostic factors does not undermine the role of Ki67 in predicting prognosis, but it is solely because it is among factors not sufficiently studied to demonstrate their prognostic value [71].

Globally, this study showed that Gleason score and Ki67 index synergistically offer better prognostic information than either alone, in management of prostatic adenocarcinoma.

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

1. Conclusion

Prostatic adenocarcinoma remains a threatening disease in men worldwide, with racial and geographical variations. In Tanzania, fewer cases than expected are diagnosed and, unfortunately at advanced stages, mainly because screening programme using digital rectal examination and serial PSA tests is not yet integrated in national health system. Adequate clinical and histological documentations are still a challenge in management of prostatic adenocarcinoma.

Gleason score is a known prognostic factor, yet in some cases, it does not predict the disease progression; other factors suggested to be potentially survival- and progression-associated prognosticators in prostatic adenocarcinoma include Ki67 expression among others.

This study compared Gleason score and Ki67 index in predicting the biological behaviours of prostatic adenocarcinoma in Tanzanian patients, the hypothesis supposed that a combination of Ki67 expression and Gleason score offer better prognostic information than either alone. This hypothesis was verified. To improve the management of prostate cancer, we therefore suggest key recommendations.

2. Recommendations

The Government and stakeholders in health system should seriously consider addressing scientifically the issues related to prevalence, incidence and distribution of prostate cancer.

Primary, secondary and tertiary preventive programmes should be planned, implemented and monitored; these include among others, availability and accessibility of the preventive and curative services including PSA tests, and training of health professionals.

Health professionals' associations should take the first step to alert the population's awareness regarding prostate cancer, as well as consider abiding by standards of good practices.

The elements of awareness about prostate cancer include:

- Removal of the taboo ideology culturally given to sexual organ diseases and/or consequences of any intervention on these organs;
- Knowledge concerning the prevalence and age distribution of prostate cancer, symptoms as well as the available methods of intervention;
- The ethical and financial issues pertaining to the awareness about prostate cancer should be addressed through the elaboration and implementation of national guidelines which are to be discussed and adopted by a multidisciplinary committee.

The good practice in health care facilities should include among others:

- The medical internal audits in Histopathology consisting of internal assessment of quality of the Department services.
- The assessment of the requesting consultant (clerical and clinical details), technical and secretarial performance and Pathologist' s performance which include turnaround time, macroscopic description together with block selection and orientation and clerical details in report among others.
- The regular meetings and discussion which serve educational and feedback opportunities.
- The datasets of specimen collection, processing and reporting should be disseminated by the Department of Pathology to other partner services.
- Clinical Departments should adequately document all the cases using international standards.

The contribution of Ki67 index in predicting prostate cancer behaviour is promising, and needs confirmation through multiregression analysis in larger multicentre studies on prostatic cancer prognosticators.

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APPENDICES

Appendix I. Data Collection Sheet

I. Patient's Particulars

1. Data Collection Sheet Number: [ ][ ][ ]
2. Hospital Registration Number: [A][ ][ ][ ][ ][ ][ ]
3. Age at Diagnosis (Years): [ ][ ]
4. Race African [ ] Asian [ ] Caucasian [ ] Mixed [ ]
5. Date of Diagnosis (yyyy/mm/dd): [2][0][0][ ] [ ][ ] [ ][ ]
6. Region of Residence at Diagnosis: [ ][ ][ ][ ][ ][ ][ ][ ]

II. Clinical information

7. Serum Pretreatment PSA Level (ng/mL): [ ][ ][ ] . [ ][ ][ ]
8. Type of Biopsy
   a. Needle Core Biopsy [ ]
   b. TURP or partial prostatectomy [ ]
   c. Radical Prostatectomy [ ]
9. TNM staging: T [ ] N [ ] M [ ]

III. Initial Prognostic grouping

10. Prognostic stage: I [ ] IIA [ ] IIB [ ] III [ ] IV [ ]

IV. Routine Histopathology Findings

11. Predominant Grade ☐

12. Second Predominant Grade ☐

13. Gleason Score ☐

14. Perineural invasion Yes ☐ No ☐

15. Vascular invasion Yes ☐ No ☐

16. Extent of tumour (%) on tissue sections ☐☐☐

V. Ki67 Immunoreactivity

17. Percentage (%) of positive cells: ☐☐☐.☐☐

VI. Clinical staging at the end of follow up

18. TNM staging: T☐ N☐ M☐

19. Date of disease progression documentation ☐☐☐☐ ☐☐ ☐☐

VII. Prognostic grouping at the end of follow up

20. Prognostic stage: I ☐ IIA ☐ IIB ☐ III ☐ IV ☐

VIII. Outcome at the end of follow up

21. Outcome: 1. Alive ☐ 2. Lost-to-follow up ☐ 3. Dead ☐

4. Referred to nearby Hospital ☐ ; If the answer is not (1), proceed:

22. Date of last visit / death (yyyy/mm/dd): ☐☐☐☐ ☐☐ ☐☐

23. Cause of death: 1. Prostate cancer 2. Other

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Ref. No. MU/PGS/SAEC/Vol. VI/

31st July, 2012

Dr.Belson R ugwizangoga
M.Med. Anatomical Pathology,
MUHAS.

RE: APPROVAL OF ETHICAL CLEARANCE FOR A STUDY TITLED "COMPARISON AND CORRELATION OF Ki67 EXPRESSION AND GLEASON SCORE IN THE PREDICTION OF BIOLOGICAL BEHAVIOR OF PROSTATIC ADENOCARCINOMA IN TANZANIA PATIENTS"

Reference is made to the above heading.

I am pleased to inform you that, the Chairman has on behalf of the Senate approved ethical clearance for the above-mentioned study.

Thus ethical clearance is granted and you may proceed with the planned study.

Please liaise with bursar's office to get your research fund.

Prof. Z. Premji
DIRECTOR, POSTGRADUATE STUDIES

/emm

c.c. Vice Chancellor, MUHAS
c.c. Deputy Vice Chancellor – ARC, MUHAS
c.c. Dean, School of Medicine - MUHAS

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In reply please quote:
Ref:

7TH July 2012

TO WHOM IT MAY CONCERN
MUHIMBILI NATIONAL HOSPITAL

RE: RESEARCH CLEARANCE NO 238 2012/2013

Name of Researcher	**DR BELSON RUGWIZANGOKA**
Research Title	COMPARISON AND CORRELATION OF Ki67 EXPRESSION AND GLEASON SCORE IN THE PREDICTION OF BIOLOGICAL BEHAVIOUR OF PROSTATIC ADENOCARCINOMA IN TANZANIAN PATIENTS.
Type of Research	RETROSPECTIVE STUDY WHICH WILL BE CONDUCTED AT THE HISTOPATHOLOGY LABORATORY AND MEDICAL RECORD
Valid Between	**AUGUST TO DEC 2012**

The above named has been allowed to conduct the stated research.

Please accord him/her and his/her assistants the necessary assistance/cooperation.

Sincerely,

Dr. H.F. Swai
DIRECTOR OF MEDICAL SERVICES

Appendix IV. Standard operating procedures (SOP) for prostate surgical pathology specimens

I. Biopsy specimens

1. Transrectal ultrasound (TRUS) needle biopsy:

- Wide-bore needle (18 gauge) is used to take biopsy: at least 10 cores (5 from each lobe)
- Cores are counted and measured (in mm), submitted separately if labelled
- Process for initial histological examination through 3 levels.
- Careful handling is necessary to avoid crush artefact.
- Cores may be painted with alcian blue (so are easily visible on facing the paraffin block).

2. Transurethral resection of prostate (TURP) chippings:

❖ If aggressive treatment (radical prostatectomy) might be considered, for example in a younger patient, all tissue is embedded and examined histologically, as is also the case if there is any clinical suspicion of malignancy.

❖ Otherwise, guidelines of initial sampling of TURP specimens are:

➲ For ≤ 12g of tissue: all is processed;
➲ For >12g: 12 g + an extra 2 g for every 5 g of tissue in excess of 12 g is processed.
➲ One level is examined from each block.
➲ If carcinoma (or high-grade PIN) is found, all tissue should be processed to give an accurate stage (pT1a tumour in ≤ 5% of tissue resected; pT1b tumour in > 5% of tissue).
➲ If there is a prior diagnosis of carcinoma of the prostate: 6 g or 4 cassettes are embedded.

II. Resection specimens

1. Simple (retropubic/suprapubic)

- Occasionally done, prostatectomy is performed for BPH.
- Orientation is not usually possible, although two distinct lobes should be identifiable.
- Weighed and measure (three dimensions, mm) the specimen
- Then serially section at 3–4 mm intervals.
- Carefully examine for areas suspicious of carcinoma (yellow, firm)
- 6-8 cassettes of tissue processed, labelling the two lobes separately.

2. Radical prostatectomy

Initial procedure

- Orientate the specimen using seminal vesicles and by placing a probe into prostatic urethra.
- This will allow identification of the flat base superiorly (proximal, bladder, base margin) and the more conical apex antero-inferiorly (distal, urethral, apical margin).
- Weigh the entire specimen, measure in 3 dimensions (mm) and give the lengths (cm) of the attached seminal vesicles and vasa deferentia.
- Paint the right, left, anterior, posterior, superior and inferior surfaces of the prostate using 6 different-coloured inks, including the soft tissue around the base of the seminal vesicles.
- Note of any areas where the prostatic tissue has been disrupted by the surgical knife.
- Fix the specimen by immersion in 10% formalin for at least 24–36 hours.

Sections:

- Dissect off the seminal vesicles and vasa deferentia and serially section these.
- Amputate proximal and distal 5 mm of prostate (bladder and urethral margins respectively) and serially section these at 3 mm intervals perpendicular to the amputating cut (Figure).
- Serially section at 3–4 mm intervals in the coronal plane from anterior to posterior.
- Carefully examine the slices, maintaining orientation with the help of the coloured inks.
- Asymmetry between lobes may be another clue.

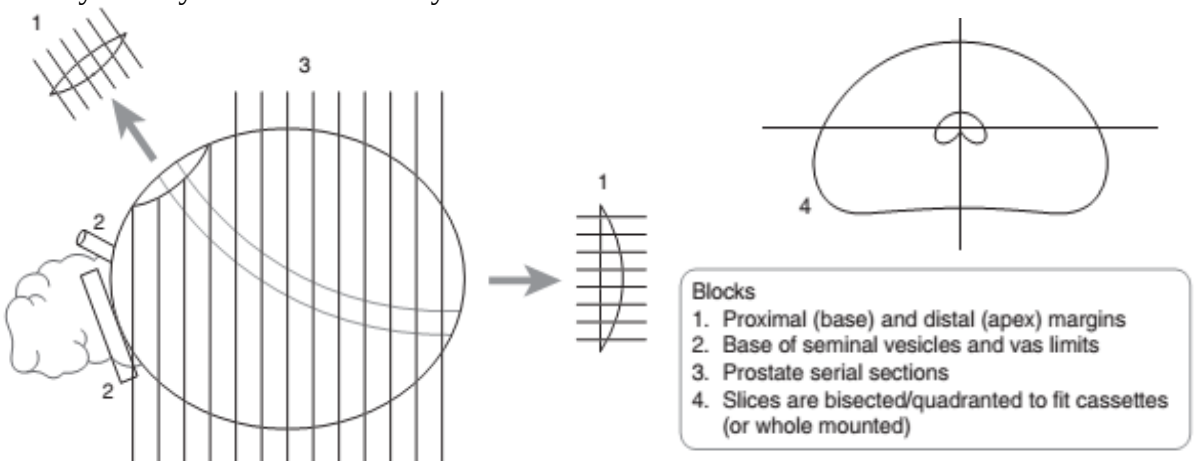


Figure: Blocking a radical prostatectomy specimen

Description

- Tumour: Site (right/left, peripheral/central, anterior/posterior).
  Size (mm).
  Multifocality.
  Appearance (soft/firm, pale/yellow/granular).
  Edge (circumscribed/irregular).
  Extension beyond capsule/into seminal vesicles.
- Non-neoplastic tissue: appearance (colour, consistency, nodularity).

Blocks for histology (see figure)

- Sample proximal (base) and distal (apical) margins as described.
- Sample seminal vesicles at their bases (junction with prostate) and vasa deferentia
- Each serial section is bisected into right and left halves (and if necessary into superior and
- Inferior quadrants) to fit into routine cassettes and the entire gland processed for histological examination, labelling each block carefully to aid microscopic interpretation.
- All pelvic lymph nodes (usually submitted separately) should be counted and sampled.

III. Histopathology report

- Tumour type: acinar (proximal duct) adenocarcinoma/other.
- Tumour differentiation: Gleason grading system. Don't grade if there has been prior treatment
- Tumour volume/average extent of tumour on sections:

  The tumour is outlined microscopically on each glass slide and the area involved measured (mm^2). The areas for all sections are summed and the overall tumour volume (mm^3) is derived from multiplying by the average slice thickness (3–4 mm).

  This may be expressed as a proportion of the total volume of the prostate, to give the percentage gland involvement.

- Tumour edge: pushing/infiltrative.
- Extent of local tumour spread (stage)
- Lymphovascular invasion:
  - perineural and lymphovascular space.
  - Present/not present.
  - Inside/outside capsule.
- Regional lymph nodes.
  - Pelvic nodes below the bifurcation of the common iliac arteries.
  - pN0 no regional lymph node metastasis.
  - pN1 metastasis in regional lymph node(s).
- Excision margins
  - Proximal (base), distal (apical), circumferential margins involved/uninvolved.
  - Distances (in mm) to nearest margins.
- Other pathology
  - High-grade PIN, effects of radiotherapy or androgen-deprivation therapy (glandular atrophy, apoptosis, vacuolation, stromal fibrosis)

SOP References

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Appendix V. Standard operating procedure for immunoperoxidase stains

Make tissue block sections 4 μm thick, put on immunohistochemistry (IHC) slides

1. De-wax by placing the slides on hot plate at 60°C for 50 minutes
2. Immerse the slides in xylene in 2 batches for 5 minutes each.
3. Tissue must be rehydrated before commencing staining protocol.
    a. Immerse the slides in 100% alcohol in 2 batches for 10 dips each.
    b. Immerse the slides in 95% alcohol in 2 batches for 10 dips each.
    c. Immerse the slides in 70% alcohol in 1 batch for 10 dips.
    d. Rinse the slides with deionized water.
4. Surround the tissue with a hydrophobic barrier using a barrier pen.
5. To quench endogenous peroxidase activity, incubate the sample with 2 drops peroxidase blocking reagent (3% H_2O_2 in water or methanol) for 15 minutes.
6. Bring the slides into water for 5 minutes.
7. Prepare antigen retrieval solution to unmask the antigens
    - e.g., 100 ml of Citrate Buffer at pH 6.00 into 900 ml of distilled water
    - e.g., 150 ml of Citrate Buffer at pH 6.00 into 1350 ml of distilled water
8. Heat antigen retrieval solution in pressure cooker (not closed) until it starts boiling
9. Place slides in antigen retrieval solution in pressure cooker and close the lid
10. Time for 2 minutes at full pressure
11. Remove the slides from pressure cooker and place in water (never allow slides to dry)
12. Add phosphate buffered saline (PBS) wash buffer for 3 minutes
13. Drain slides of PBS, and wipe away any excess wash buffer, add the primary antibody
14. Incubate overnight at 2-8 °C the sample with primary antibody.

    Note: Appropriate controls are critical for the accurate interpretation of IHC results. All IHC experiments should include negative and positive controls.

15. Wash 3 times with wash buffer for 5 minutes each, and drain the slides.
16. Incubate the sample with 1-3 drops of High Sensitivity Streptavidin-HRP (horse rabbit peroxidase) conjugate (HSS-HRP) for 30 minutes.
17. Rinse with wash buffer 3 times for 15 minutes each and drain the slides.
18. Incubate the sample with 1-3 drops of biotinylated secondary antibodies (HRP) for 30-60 minutes (time depends on thickness of the section).

Note: High Sensitivity Streptavidin is a chemical analog of Streptavidin that has little net positive charge at neutral or slightly alkaline pH and will interact only with biotin attached to secondary antibodies.

19. Rinse and wash 3 times in wash buffer (PBS) for 2 minutes each.

20. Calculate the required working volume of DAB Chromogen Solution given that 100-200 µL is required to cover the entire tissue section on a single slide. Make up DAB: 1 drop of DAB for each 1 mL of substrate buffer.

21. Add 1-5 drops of DAB Chromogen Solution to cover the entire tissue section and incubate for 3-20 minutes. Colored precipitate will localize to the sites of antigen expression as the chromogenic substrate is converted by HRP enzyme into insoluble end product.

    Note: DAB and AEC are hazardous materials. Gloves and safety glasses should be worn and all steps performed inside a fume hood.

22. Rinse the sample with wash buffer (PBS) 3 times for 10 minutes each.

23. Rinse in deionized H_2O and drain the slides.

24. Counterstain in hematoxylin for 10 seconds.

25. Quick dip in 1% acid alcohol, 2 dips

26. Blue in warm water for 2 minutes

27. Dehydration through ascending concentrations of alcohol

    a. Immerse the slides in 70% alcohol in1 batch for 10 dips.
    b. Immerse the slides in 95% alcohol in 2 batches for 10 dips each.
    c. Immerse the slides in 100% alcohol in 2 batches for 10 dips each.

28. Clear in xylene in 2 batches for 3 minutes each

29. Coverslip

30. Visualize staining of tissue under a microscope using a bright-field illumination.

Immunohistochemistry SOP reference

1. Leste SC. *Manual of Surgical Pathology.* 3rd Edition. Boston: Elsevier Saunders, 2010.