Validation of Biomarkers in patients with Urothelial Bladder Carcinoma

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For the award of the degree of

DOCTOR OF PHILOSOPHY

IN THE FACULTY OF MEDICINE

By

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(Reg No: KLEU/Ph.D./2019-2020/DO1219005)

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SEPTEMBER - 2023

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May God shower the above cited personalities with success and honour in their life.

Shadab Rangrez

LIST OF ABBREVIATION

µl	microlitre
5-ALA	5-Aminolevulinic Acid
AUA	American Urological Association
BCa	Bladder Cancer
BCG	bacille Calmette-Guerin
BLC	Blue Light Cystoscopy
BMI	Body mass index
BPH	Benign Prostatic Hyperplasia
BTA	Bladder Tumour Antigen
CFHR	Complement factor H-related protein
CIA	Colorimetric Immunoassay
CIS	Carcinoma In-situ
COPD	Chronic Obstructive Pulmonary Disease
CT	Computer Tomography
CVD	Cardio Vascular Disease
CYFRA	Cytokeratine fragment
DWI	Diffusion weighted imaging
EAU	European Association of Urology
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose
FISH	Fluorescence In-situ Hybridization
GFR	Glomerular Filtration Rate
GLOBOCON	Global Cancer Observatory
H & E	Hematoxylin and Eosin
HAL	Hexaminolevulinic Acid
HPR	Horseradish Peroxidase

HR	Hazard risk
IHC	Immno Histochemistry
LUTS	Lower Urinary Tract Symptoms
MIBC	Muscle Invasive Bladder Cancer
ml	milliliter
MRI	Magnetic Resonance Imaging
N/C	Nucleocytoplasmic ratio
NAC	Neoadjuvant Chemotheraphy
NBI	Narrow Band Imaging
ng	nanogram
NGS	Next Generation Sequencing
NIS	Nikon software
nm	nanometer
NMIBC	Non-Muscle Invasive Bladder Cancer
NMP	Nuclear Matrix Protein
NPV	Negative Predictive Value
OD	Optical Density
OS	Overall Survival
PAH	Polycylic aromatic hydrocarbon
PAP	Papanicolaou
PBGS	Propholinogen Synthase
PDD	Photodynamic Diagnostic
PET	Positron Emission Tomography
PPIX	Protoporphyrin IX
PPV	Positve Predictive Value
PUNLMP	Papillary Low grade urothelial neoplasm
ROC	Receiver operating characteristic curve

rpm	Rotation per minute
RR	Relative risk
RT-qPCR	Reverse transcriptase- quantitative polymerase chain reaction
SCoA	Succinyl Coenzyme
SEER	Surveillance, Epidemiology and end result
SIA	Sandwich Immunoassay
T2DM	Type 2 Diabetes Mellitus
TCA	Tricarboxylic Acid
TCC	Transitional Cell Carcinoma
TERTp	Telomerase reverse transcriptase gene
TUR	Transurethral Resection
UBC	Urothelial Bladder Carcinoma
WLC	White Light Cystoscopy

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ABSTRACT

INTRODUCTION:

Bladder cancer is one of the most common urological cancers. Bladder cancer is generally diagnosed by urethra-cystoscopy, which permits direct imagining of tumours and confirmation by biopsy and pathological analysis. Nevertheless, Cystoscopy and voided urine cytology are effective diagnostic methods for investigation of superficial bladder cancer. Flexible cystoscopy being a minimally invasive procedure and has made flexible cystoscopy more acceptable to patients. Voided urine cytology remains the method of choice for the non-invasive detection of bladder cancer, yet whilst it has a specificity of 93%, its sensitivity is only 25-40%, especially for low-grade and T-stage tumours.

The clinical spectrum at present can be divided into those with (i) non-muscle invasive bladder cancer, (ii) muscle-invasive bladder cancer, and (iii) metastatic disease. The muscle invasive bladder cancer has a high recurrence rate of 50- 70% as it reoccurs despite conservative measures such as transurethral resection of bladder tumor (TURBT) and intravesical therapy. A wide range of alternative procedures and markers have been proposed and studied for the detection of recurrent bladder tumours. These include 5-Aminolevulinic Acid (5-ALA) cytology, nuclear matrix protein 22 (NMP22), BTA test etc. The principle behind 5-ALA fluorescence cytology is based on the metabolism of heme biosynthesis. 5-ALA being the precursor of heme metabolism will selectively get accumulated as protoporphyrin IX (PPIX) in tumor cells.

For quantitative analysis, FDA approved commercial test were used which follows the principle of enzyme linked immunoassay. First test includes NMP-22, it is a nuclear mitotic apparatus protein present in the nuclear matrix of all cell type, located in the mitotic spindle during mitosis and is involved in the proper supply of chromatin to daughter. Similarly, BTA has been identified as a human complement factor H related protein (hCFHrp), which is produced by bladder tumour cells in cell cultures and not by any other epithelial cell lines.

The present study aimed to detect urothelial bladder carcinoma using 5- ALA and its comparison with conventional cytology, NMP-22 and BTA TRAK quantitative test to estimate activity in the urine of the patients with bladder cancer in a trial to assess their

value in the detection of the tumours and to find a reliable non-invasive technique for the diagnosis of cancer bladder. Sensitivity and specificity of these tumour markers was compared to conventional cytology in bladder cancer.

MATERIAL AND METHODS:

The study was conducted between September 2019 and February 2022 at the Urology clinic in a tertiary care centre of South India. The study was reviewed and approved by the institutional ethics committee (KAHER/EC/20-21/001/05). A total of 422 patients with 150 Bladder carcinoma (Cases) and 272 non-cancerous (Controls and Lower Urinary Tract Symptoms Patients) were included in the study.

Collection of voided urine sample Pre-operative urine sample of 150 cc was collected and samples were divided into four different groups, and the following procedures were performed within 1 h for pathological examination. We separated these samples for the conventional cytology, ALA-induced fluorescent cytology, NMP-22 and BTA-TRAK.

RESULTS:

Bladder cancer occurred 3.4% of the time in our institution. The mean age of controls (65.25 ± 18.15 years) was similar to patients with cases (65.28 ± 17.63 years). Significant differences in occupation were found, particularly for those with agricultural and industrial backgrounds. Additionally, a higher relative risk of bladder cancer between 1.5 to 4 times was seen for lifestyle choices such drinking alcohol, smoking, and chewing tobacco.

There were 71 cases of bladder cancer with low-grade tumors, 79 cases with high-grade tumors, and 79 cases with tumor aggression. There were 105 cases of bladder cancer without muscle invasion and 45 cases with muscle invasion. According to our findings, the NMP-22 test had 76% sensitivity and 94.85% specificity. Like other markers, sensitivity showed a strong correlation with an increasing BC tumor grade. Sensitivity and specificity both greatly increased, with respective values of 73.33% and 95.58%.

With AUCs of 0.867 and 0.851, respectively, vs cytology's AUC of 0.625, the NMP-22 test and BTA TRAK surpassed traditional cytology using a ROC curve, and the difference was statistically significant (P = 0.015). With a ROC curve threshold value of

10.7 units/mL, the sensitivity and specificity in the current experiment are equal. Another non-FDA authorized biomarker that has been used is 5-Aminolevulinic Acid (5-ALA). According to our research, 5-ALA-induced fluorescence cytology was substantially more sensitive than commonly used biomarkers like conventional cytology (63.63% and 57.74%) for pTa stage malignancies (88.63% [39/44]) and low-grade tumors [65/71)]. ($p=0.0045$, $p=0.001$, $p<0.01$).

Low and High Grades, Ta, T1 stages, and the sample we provided show the biggest differences in sensitivity between 5-ALA cytology and the other two tests (82% in LG and 97% in HG using 5-ALA cytology; sensitivity 95% in Ta and 95% in T1). Despite the advantages and disadvantages of each biomarker, 5-ALA-induced fluorescent urine cytology has consistently demonstrated good diagnostic efficacy in terms of sensitivities, PPVs, NPVs. and accuracy for the diagnosis of bladder cancer.

CONCLUSION:

In conclusion, 5-ALA based fluorescent cytology in the detection of bladder cancer in voided urine sample represents a set of novel diagnostic assays that can be employed for an early and accurate detection of BC, with highest sensitivity as compared to other routine methods. These can also efficiently detect low-grade Urothelial tumors by showing positivity in different filters. Our study was a single-centered assessment and needs to be validated in other cohorts & multiple centers. We suggest validation and inscription of these markers in urine as independent diagnostic tests or in a panel to be recommended as these may play a vital role in early detection and diagnosis of bladder cancer.

KEYWORDS: Bladder cancer, Non-invasive diagnosis, 5-Aminolevulinic Acid, Nuclear Matrix Protein, Bladder Tumor Antigen.

INTRODUCTION

Cancer is a cluster of rampantly growing cells caused by dysregulation of molecular mechanisms due to series of genetic changes. Almost any type of tissue or organ in the body can develop cancer which can easily spread to other organs. Cancer has been the second growing cause of mortality worldwide which has surpassed cardio-vascular diseases.[1] According to the GLOBOCAN (Global Cancer Observatory) the estimated 24.1 million is the estimated incidence rate and 13 million death rate worldwide by 2030.[2]

The principal factor that catalyses cancer is the mutation in genetic code caused by radiations or mutagenic compounds.[1] Urothelial Bladder Carcinoma (UBC) has become the second leading cause of genito-urinary cancer, with an estimated prevalence of 41.2% in men and 37.9% in females by 2030.[2] Transitional cell carcinoma is a mixed neoplasm that includes both muscle and non-muscle invasive bladder cancer.

Risk factors associated with bladder cancer are age,[3] gender,[4] occupations,[5] smoking,[6] dietary patterns,[7] physical activities[8] and ethnicity.[9] the re-occurring pattern of UBC makes it one of most outrageous human malignancy and difficult to cope with.

1.1 THE URINARY TRACT:

The urinary tract comprises of renal pelvis and ureters (upper tract) and the bladder and urethra (lower tract). Its function is to collect and excrete urine. Urine is produced by the kidneys and stored within the pyelum. Urine passes down the ureters towards the urinary bladder, where it is held until it is time to urinate. An

adult excretes 1.4 - 1.5 litres of urine every day on average. In clinical practise, an acceptable production of 0.5 ml/kg body weight every hour is regarded as typical.

Because water is the predominant component of urine (91% - 96%), the output is determined by fluid consumption. The other components include nitrogen (in the form of urea), phosphorous, creatinine, potassium and calcium.[10] Organic components such as urothelial cells, bacteria, and inflammatory cells are also present.

Histologically, the urinary tract is composed of many cell layers and inner lining is the urothelium made up of specialised urothelial cells which is 3-6 layers of thick cells approximately.[11] The urothelium is separated from lamina propria by a basement membrane (Figure 1). This lamina propria consists of connective tissue made of small blood vessel, stromal cells and smooth muscle fibers known as muscularis mucosa. The thickness of muscularis propria also referred as muscular detrusor varies at different locations. During expansion of bladder the muscle is relaxed to allow its expansion whereas during urine excretion the detrusor muscle contracts and bladder is emptied. The urinary bladder is hollow and able to store urine for number of hours. It can be inspected using a cystoscope via the urethra and operations of bladder can be done transurethral. The bladder can be flushed with the fluid to avoid systemic treatment. The urine can be used as non-invasive tool for urinary biomarker research.[12]

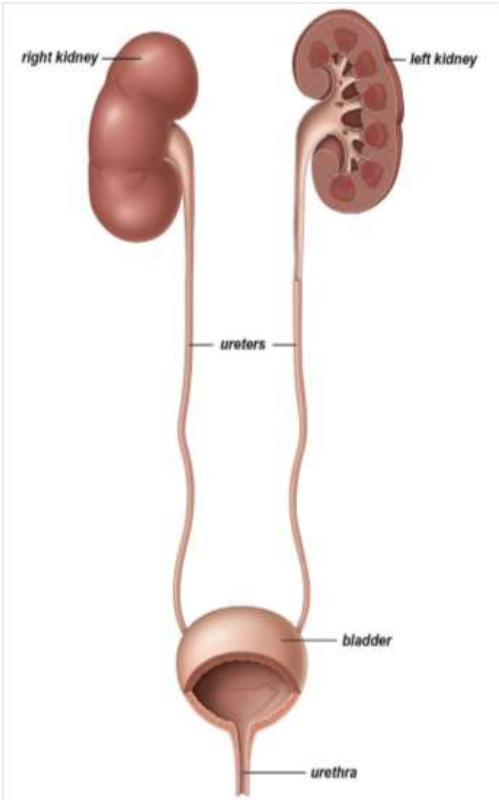

Figure 1: Schematic representation showing Urinary tract with kidneys connected to urinary bladder via ureters and the urethral opening.[12]

1.2 BLADDER CANCER:

Urinary bladder cancer contributes to overall tumour burden worldwide and is 2nd among genitourinary tract malignancies and 6th rmost collective malignancy in men and 17th most common cause of deaths due to cancer in women. Factors like age, gender, hormonal, exposure to aromatic amines and tobacco influence the outcome of bladder cancer. [13]

Bladder cancer has an erratic history. Among bladder cancer, Urothelial carcinoma accounts 90% cases. Tumours arise from the lining epithelium which is transitional cell type. Therefore, it is also called transitional cell carcinoma (TCC). Depending on morphology, the tumours are grouped as low grade or high grade. (Figure 2) Each grade further classified into stages based on depth of invasion.

Muscle invasive bladder cancers (MIBC) are 20-30% whereas Non Muscle Invasive Bladder cancer (NMIBC) is 70-80%. NMIBC originate from flat metaplastic or hyperplastic changes in urothelium lining the bladder, ureter and urethra. These are histologically low grade that grow as superficial non-invasive papillary protrusions. The choice of treatment for NMIBC is transurethral resection followed by adjuvant chemo/immunotherapy depending on stage and grade. Adjuvant chemotherapy and immunotherapy has been used as a prophylactic measure to reduce the frequency of recurrences with variable success rate. Inspite of the adjuvant intravesical therapy, 30% cases show recurrence of tumour, sometimes within two months or may even progress to muscle invasive tumours in 10-20% cases.[14,15,16]

Muscle Invasive bladder cancer develops as a result of carcinoma in-situ or severe dysplasia. It is associated with early metastasis in contrast with NMIBC. The dissection of bilateral pelvic lymph nodes and cysto-prostatectomy in addition to urethro-rectomy for men and anterior exenteration in females are the treatment options for this type of cancer. The standard treatment includes neoadjuvant chemotherapy, radiotherapy in conjunction with radical cystectomy. However, such patients experience disease relapse in 50% of cases. In case of metastasis, the treatment outcome is poor with 5-years survival rate of 5%.[17,18]

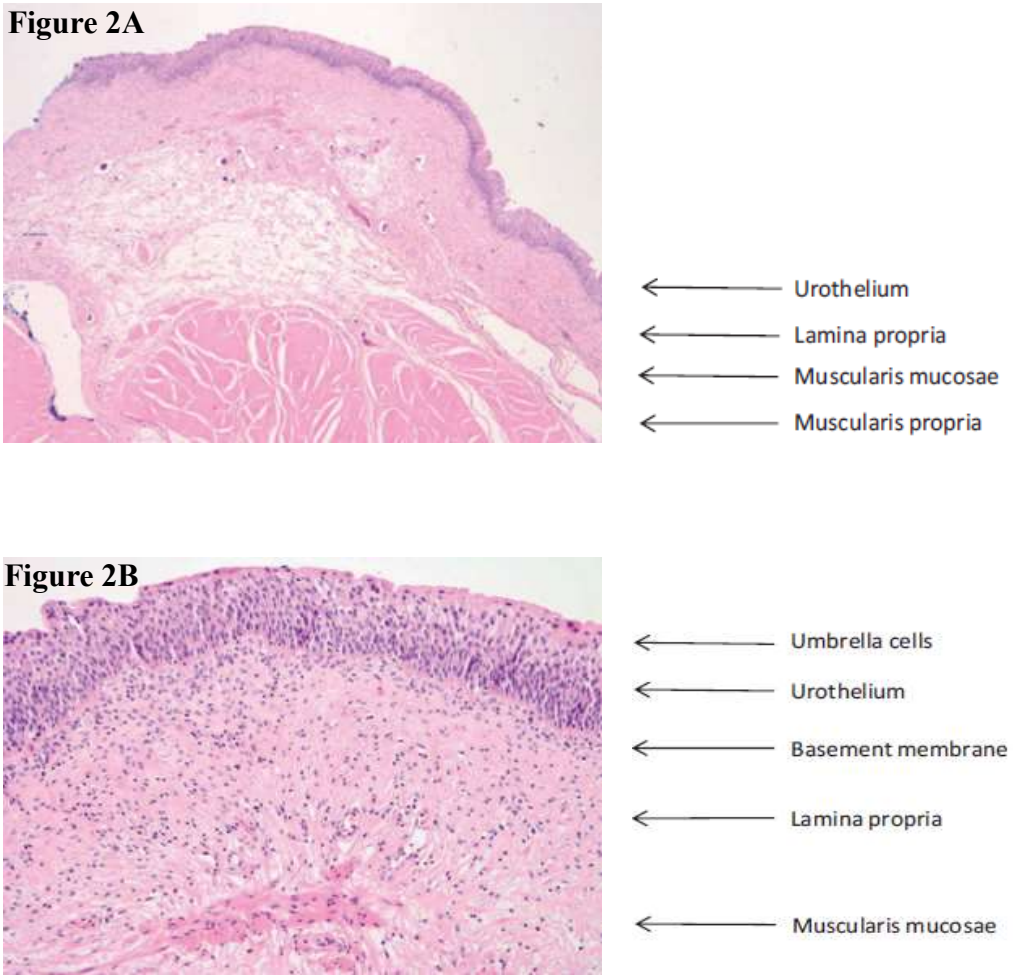

Figure 2: Cross-sectional histology slides with hematoxylin and eosin (H&E) staining showing the different cell layers of the urinary bladder wall at 20x magnification (A) and at 100x magnification (B).[16]

1.3 SIGNS AND SYMPTOMS:

Symptoms of bladder cancer can range from dysuria to frequency and urgency. The vast majority of patients, however, develop hematuria: red blood cells within the urine. Hematuria can be microscopic (not visible with the naked human eye) or macroscopic (which can be seen with the naked eye). Individuals having microscopic hematuria are often detected at random during regular urinalysis. Hematuria can be caused by either malignant or benign conditions. Renal cancer, prostate cancer, and bladder cancer are examples of carcinogenic reasons for

hematuria. Exercise-induced hematuria, Trauma, vascular malformations, urinary tract infections, benign prostate hyperplasia, or renal calculi are examples of benign causes. Only around 3-28% of hematuria patients are going to be identified as having bladder cancer. In a consequence, over seventy percent of patients develop hematuria from various reasons, most of them being benign.

Cystoscopy is the gold standard in bladder cancer diagnosis, which includes histologically proven tumour cells in a sample. Urine cytology is performed in high-risk individuals to confirm the existence of a bladder tumour. In cytology, a pathologist examines cells obtained from urine under a microscope. Upper urinary tract imaging, such as a computed tomography (CT) scan or ultrasound, can also help in the diagnosis of upper urinary tract problems.[19,20]

1.4 TREATMENT FOR BLADDER CANCER PATIENT:

Patients who have a suspicious lesion in their bladder will be first detected with a transurethral resection (TUR) where a resectoscope is placed via the urethra during this surgery to see, examine, and remove the bladder tumour from the bladder wall with a scraper. A pathologist will evaluate the removed cells to verify their diagnosis of underlying bladder cancer and also to determine the histological subtype of the bladder tumour.

The histological diagnosis of a bladder carcinoma influences treatment and follow-up (Table 1, Figure 3). Non-muscle-invasive bladder cancer (NMIBC) arises when a bladder cancer is localised within the urothelium (stage Tis or Ta) or if the tumour exhibits invasive growth within the bladder's lamina propria (T1). A muscle-invasive bladder cancer (MIBC) develops when a bladder tumour enters the musculus detrusor (T2), perivesical tissue (T3), or nearby organs (T4).[20,21]

Table 1: TNM classification for malignant bladder tumours.[16]

	T - Primary Tumor
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma *in situ*: "flat tumor"
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscle
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue:
T3a	microscopically
T3b	macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumor invades prostate stroma, seminal vesicles, uterus, or vagina
T4b	Tumor invades pelvic wall or abdominal wall
	N - Regional Lymph Nodes
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph-node metastasis
N1	Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N2	Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N3	Metastasis in common iliac lymph node(s)
	M - Distant Metastasis
M0	No distant metastasis
M1	Distant metastasis

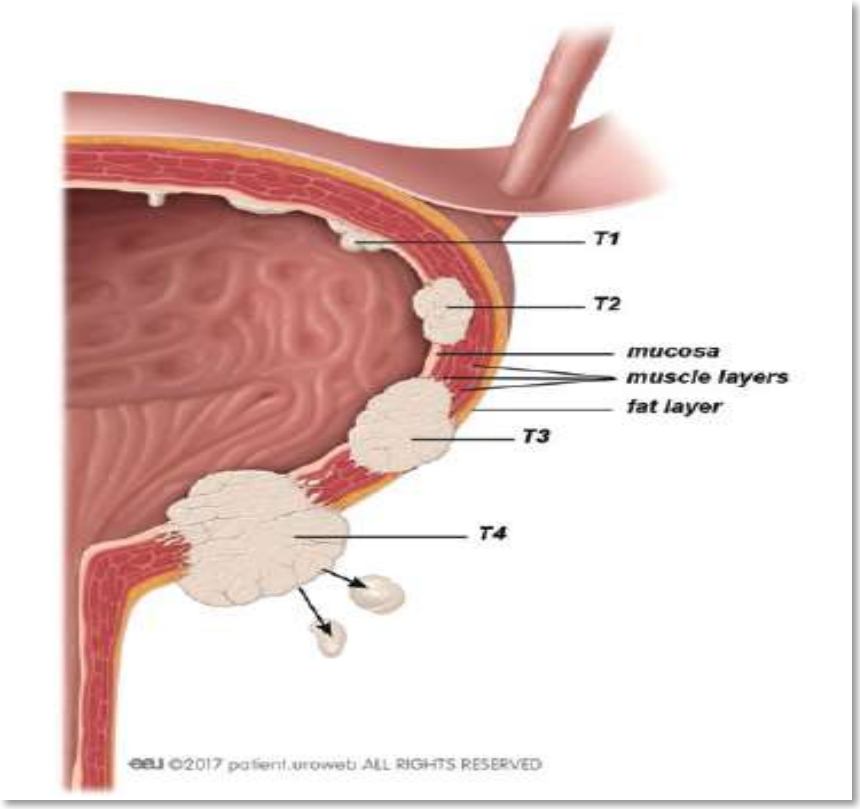

Figure 3: A schematic representation of the extent of invasion in the various stages of carcinoma of the bladder.[16]

Positive node disease can occur in a single lymph node (N1), several loco-regional lymph nodes (N2), or lymph nodes farther distant from the bladder (N3). Distant metastases (for example, metastases to the bone, brain, lung, or liver) are classed as either No metastases (M0) or metastases (M1) (Table 1). NMIBC patients are treated with TUR and, in certain cases, further intravesical instillation of immunotherapeutic or chemotherapeutic medicines. More invasive therapy is necessary if the bladder tumour has migrated further into the detrusor muscle. The primary curative treatment for MIBC is radiation cystectomy with pelvic lymph node dissection, which is occasionally accompanied by four doses of systematic cisplatin-based neoadjuvant chemotherapy (NAC). During a radical cystectomy, the whole bladder is removed, then urine diversion is built from the small bowel. Because treatment response varies so greatly between individuals, NAC only results in 6% improvement in 10-year survival.[22,23]

1.5 SURVEILLANCE AND PROGNOSIS OF BLADDER CANCER:

NMIBC has an overall as well as cancer-specific mortality rate that is more than ninety percent, however there is a considerable recurrence rate (more than 50%), and the recurrences could lead towards MIBC (in approximately 5 to 10%).[24,25] MIBC survival is mostly determined by extensive surgery including the condition of lymph nodes adjacent to the tumour. The rate of survival after five years for pT2N0 is estimated to be 74%, while it drops to 45% with pT4N0. Patients with node-positive disease at the point of radical cystectomy had a poor prognosis, having a 5-year mortality rate of only 22%.[26]

A large study on recurrence after cystectomy showed that more than eighty percent of patients having node-positive cancer (pN1-3) returned, compared to just 20% of individuals having node-negative organ-confined sickness.[27] MIBC patients are monitored on a modified schedule. These patients will have regular CT scans as well as blood tests to assess their physical health and illness status.[28] The psychological and social challenges associated with having to have a radical cystectomy, as well as the challenges of living with a urinary diversion, have received increased attention. Uro-oncology nurses provide both practical and emotional assistance. It is also explored how bladder cancer therapy affects sexuality. Overall, monitoring both the NMIBC and MIBC will require a minimum of 5 years, if not significantly longer.[28]

1.6 AWARENESS AND COST FOR BLADDER CANCER DISEASE:

The prolonged period of surveillance, along with (occasionally) invasive surgical therapies, repeated imaging, and cystoscopies, adds to the cancer of the bladder being among the costliest cancers in the West. Urothelial cancer makes up 3.5% of all tumour-related costs in the countries of the Europe.[29] Bladder cancer is a severe public health concern because of its substantial prevalence and recurring rates, in addition to its short survival rate and high expenditures. Nonetheless, bladder cancer is severely understudied, and public awareness is lacking. The primary cause of this problem is a lack of funding for developments and advancements in bladder cancer research. This again entails a significant duty and challenges for all those active in the area of uro-oncology studies in the future.[30]

1.7 URINARY BIOMARKERS:

The ideal biomarker would be easy to comprehend with high sensitivity and specificity, unbiased, inexpensive and have easy, quick results. Urothelial cancer biomarkers have four goals: (i) prevent recurrence; (ii) decrease the need for recurrent invasive procedures; (iii) identify optimal treatment response; and (iv) Identify progression to invasive disease.[31,32] Because of the contact between urine and urothelium, it can be used as intriguing tool for identifying cancer cells in a less intrusive manner. Importantly, this liquid biopsy approach would enable several longitudinal tumour samples to be taken to determine the existing carcinoma, grade, and genetic background for improved clinical follow-up.[33,34]

Previous study on BCa biomarkers has supported this idea of liquid biopsies predominantly included proteins, nucleic acids, inflammatory, and metabolite indicators. Because these biopsies involve the identification of any molecular or

cellular biomarker in patient bodily fluids, a separate biomarkers array has arisen. The majority of indicators exist as free molecules generated by cancer cells or other tumour microenvironment components (Figure 4).[35,36]

The liquid biopsy concept is growing in acceptance because (i) The biomarkers that have been observed had significant applications over diagnosis and monitoring of stage as well as recurrence; (ii) using minimally invasive techniques for predicting therapeutic response and disease prognosis; as well as (iii) aiding therapeutic clinical decision-making based on identified molecular changes. [35-37]

In recent years, a number of intriguing biomarkers have been studied in clinical trials, only those that have been recognised by laboratory-based diagnostic regulatory agencies to be diagnostics markers for use and supplements for cystoscopy for the initial evaluation and follow-up of BCa have been commercialised. Taking all studies into account, innovative urine biomarkers led to better sensitivity but poorer specificity than cytology, preventing them from being included in international standards guidelines.[33,34,38-41]

Table 2: Urine-based assays to enhance clinical reasoning in bladder cancer.[42-55]

Test	Sample	Biomarker	Assay	Purpose	Sensitivity	Specificity
BTA TRAK®	Protein	Complement factor H-related	CIA	Follow-Up	0.64 (0.58–0.69)	0.77 (0.73–0.81)
BTA Stat®	Protein	Complement factor H-related	SIA	Follow-Up	0.65 (0.54–0.75)	0.74 (0.64–0.82)
NMP22 BC test®	Protein	NMP-22	SIA	Follow-Up	0.69 (0.62–0.75)	0.77 (0.70–0.83)
NMP22 BladderChek test®	Protein	NMP-22	SIA	Diagnosis	0.47 (0.33–0.61)	0.93 (0.81–0.97)
				Follow-Up	0.70 (0.40–0.89)	0.83 (0.75–0.89)
ImmunoCyt/uCyt+™	Sediment	Tumor associated cellular antigens (M344; LDQ10; 19A11)	IF cytology	Diagnosis	0.85 (0.78–0.90)	0.83 (0.77–0.87)
				Follow-Up	0.75 (0.64–0.83)	0.76 (0.70–0.81)
UroVysion™	Sediment	Aneuploidy for chromosomes 3; 7; 17; and loss of 9p21 locus	FISH	Diagnosis	0.73 (0.50–0.88)	0.95 (0.87–0.98)
				Follow-Up	0.55 (0.36–0.72)	0.80 (0.66–0.89)
CxBladder Detect®	mRNA	IGFBP5; HOXA13; MDK; CDK1; CXCR2	RT-qPCR	Diagnosis	0.74 (0.65–0.81)	0.82 (0.79–0.84)
CxBladder Monitor®	mRNA	IGFBP5; HOXA13; MDK; CDK1; CXCR2	RT-qPCR	Follow-Up	0.91 (0.88–0.99)	0.96 (NPV)
AssureMDx™	DNA	FGFR3; TERT; HRAS; OTX1; ONECUT2; TWIST1	DNA methylat	Diagnosis	0.93	0.86
Xpert® Bladder Cancer	mRNA	UPK1B; IGF2; CRH; ANXA10; ABL1	RT-qPCR	Follow-Up	0.84 (0.69–0.93)	0.91 (0.83–0.96)
UBC®	Protein	Cytokeratin 8 and 18 fragments	SIA	Diagnosis	0.61–0.65	0.77–0.82

Abbreviations: BTA; bladder tumor antigen; CIA; colorimetric immunoassay; IF; immunofluorescence; NMP; nuclear matrix protein; UBC; urinary bladder cancer a fluorescence in situ hybridization; RT-qPCR; reverse transcription-quantitative polymerase chain reaction; SIA; sandwich immunoassay.

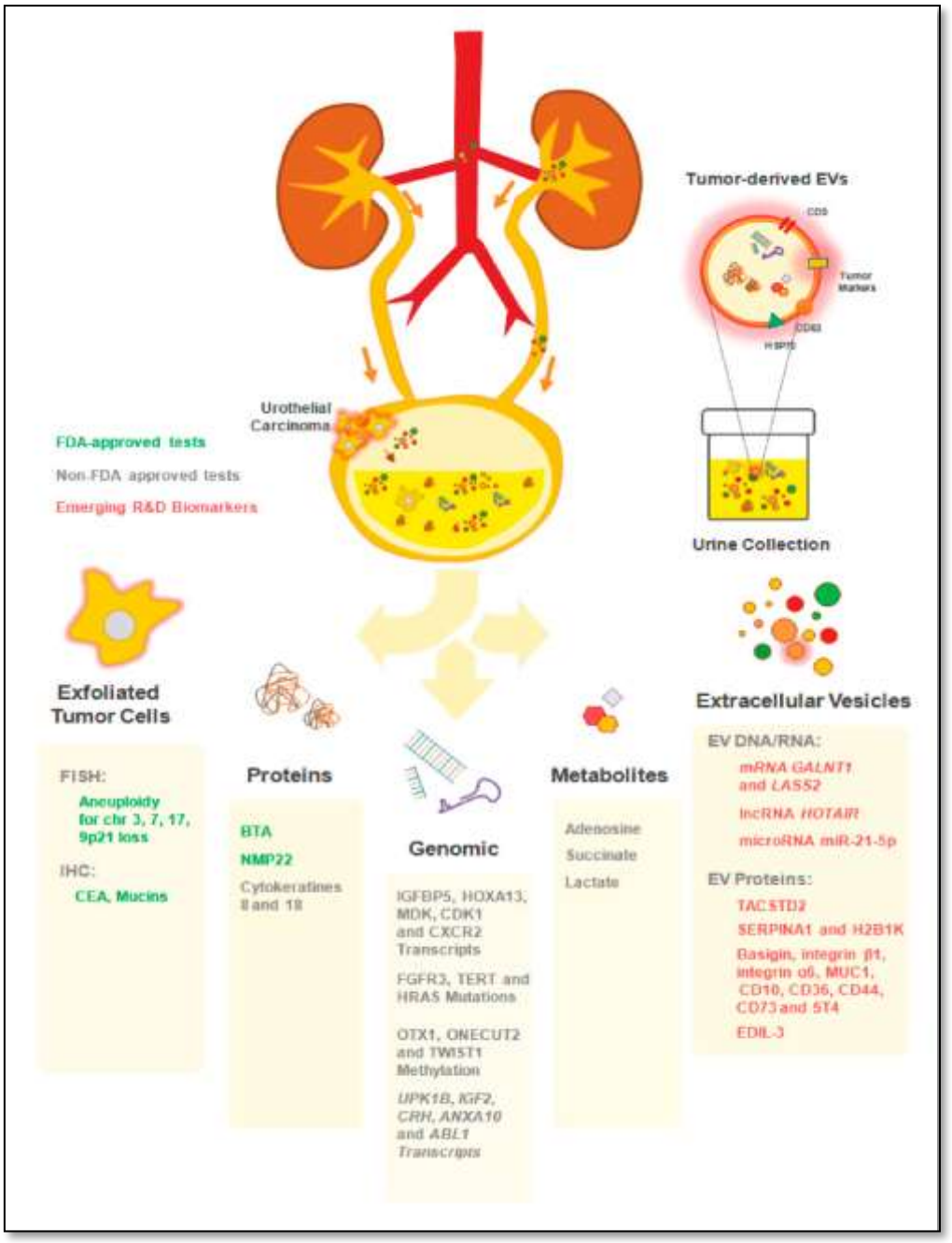

Figure 4: Urine biomarkers for the detection and monitoring of bladder cancer (BCa).[35]

HYPOTHESIS OR RESEARCH QUESTIONS:

1 Is there a non-invasive urinary biomarker which can detect Urothelial bladder cancer or its reoccurrence?

2 What is the biomarker's sensitivity and specificity in comparison to the standard approaches used in everyday practice to identify bladder carcinoma?

3 What are the risk factors related to bladder carcinoma in this part of region?

AIM

To validate the biomarkers in patients for the detection of urothelial bladder carcinoma.

OBJECTIVES

PRIMARY OBJECTIVES:

- Validation of 5-ALA staining using Fluorescence microscopy with Gold standard technique.

SECONDARY OBJECTIVE:

- Validation of Kit based method for the detection of Urothelial Bladder carcinoma.
- To study the various risk factors involved in Urothelial Bladder carcinoma.

4.1 INCIDENCE:

GLOBOCAN 2020 revealed an anticipated 19.3 million cancer occurrences worldwide. Cancer of the breast (2.28 million cases, 11%), lung cancer (2.24 million, 11.4%), colorectal cancer (1.9 million, 10%), and prostate cancer (1.4 million, 7.3%) were the most prevalent malignancies diagnosed globally. There were nearly half of overall cancer cases incidence rates registered in Asia (49.47%), for Europe (22.39%), Northern America (13.25%), Latin America (7.29%), Africa (5.7%) and for Oceanic (1.32%). For Bladder cancer, there were 573000 new cases according GLOBOCAN 2020. Asia reported highest number of incidence rates (36.3%), Europe reported (35.6%), North America (15.7%) and Latin America and Africa with 5% of incidence rate.[56] (Figure 5 A, B & C)

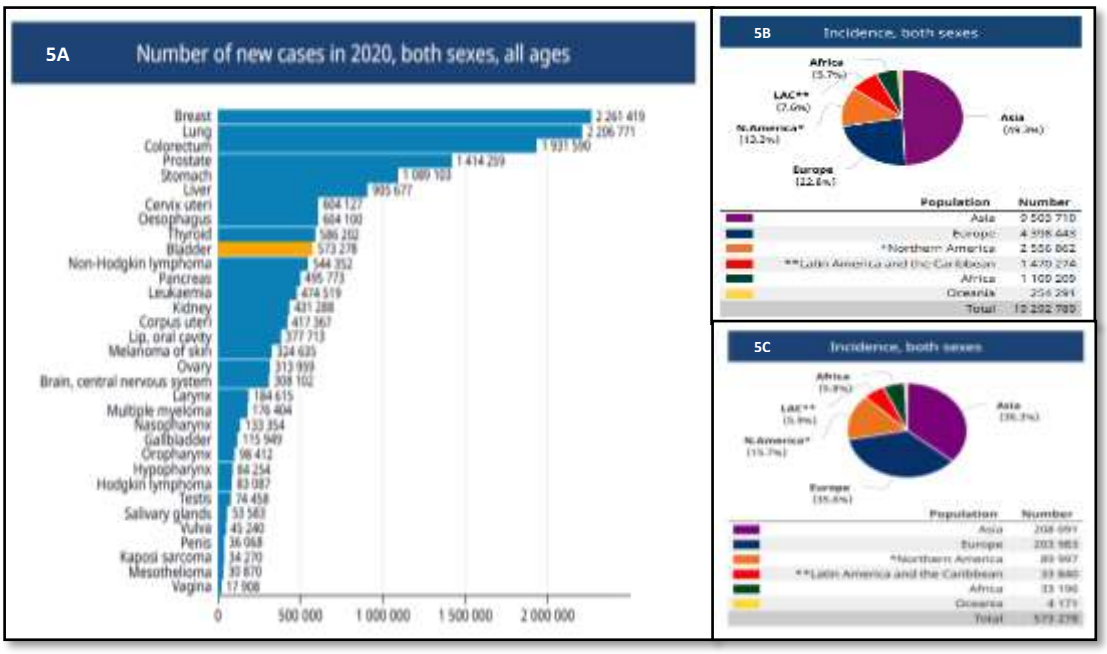

Figure 5: A. Number of new cases in all cancer cases worldwide, B. Incidence of overall cancer cases in both the sexes and C. Incidence of Bladder cancer cases in both the sexes worldwide.[56]

4.2 MORTALITY:

Coming to the mortality rates the cumulative mortality from cancer (Figure 5), In gender-disaggregated cancer prevalence and mortality data, among the most common types of cancer found in men consisted of lung (14.34%), prostate (14.12%), non-melanoma skin (7.20%), and stomach (7.12%) cancers, while the most common cancers identified in females were breast (24.52%), lung (8.42%), and cervix (6.54%) cancers. Males died from lung (21.5%), liver (10.44%), and stomach (9.12%) cancers, while females died from the breast (15.52%), lung (13.77%), and cervix (7.75%) cancers were the most common causes of death in women (Figures 5). For Bladder cancer the number of deaths registered were 2,12,536 cases (2.13%). The gender distribution ratio was 3:1 for males and females respectively. Asia showed highest mortality rates (42.6%), Europe (31.7%), North America (9.9%), Africa and Latin America with 8.8% and 6.2% respectively.[57] (Figure 6 A, B & C)

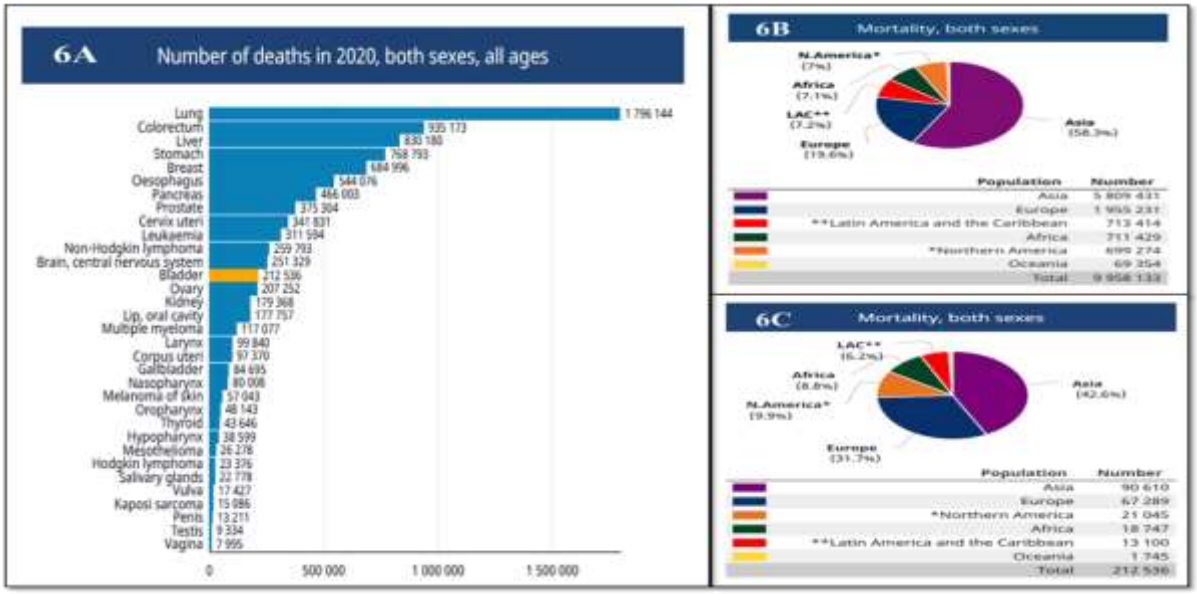

Figure 6: A. Number of Deaths cases in all cancer cases worldwide, B. Mortality of overall cancer cases in both the sexes and C. Mortality of Bladder cancer cases in both the sexes worldwide.[57]

In 2020, the global 5-year prevalence for Bladder cancer was 1721000 cases. With Europe (38.1%), Asia (33.8%), North America (17.5%) showing highest Death rates based on age (per 100,000 persons per year) for both sexes. On a global scale projected the ASRs for bladder cancer occurrence have not changed considerably between 1990 and 2019, showing varying incidence rates in different parts of the world. According to estimates, eastern Asia (the percentage change: 56%), the region of North Africa including the Arabian Peninsula (53%), and the centre of Europe (50%), have witnessed significant rises. In terms of estimated age-standardised death rates, there were general declines worldwide, although a large rise (18%) was seen in Central Asian population. GLOBOCAN predictions of future BC disease burden by continent indicate Africa (101%), the continent of Latin America and the Caribbean (85%), Asia (79%), and Pacific (70%; Fig. 7 A and B) will see the greatest percentage rises in incident BC cases from 2020 to 2040.[58]

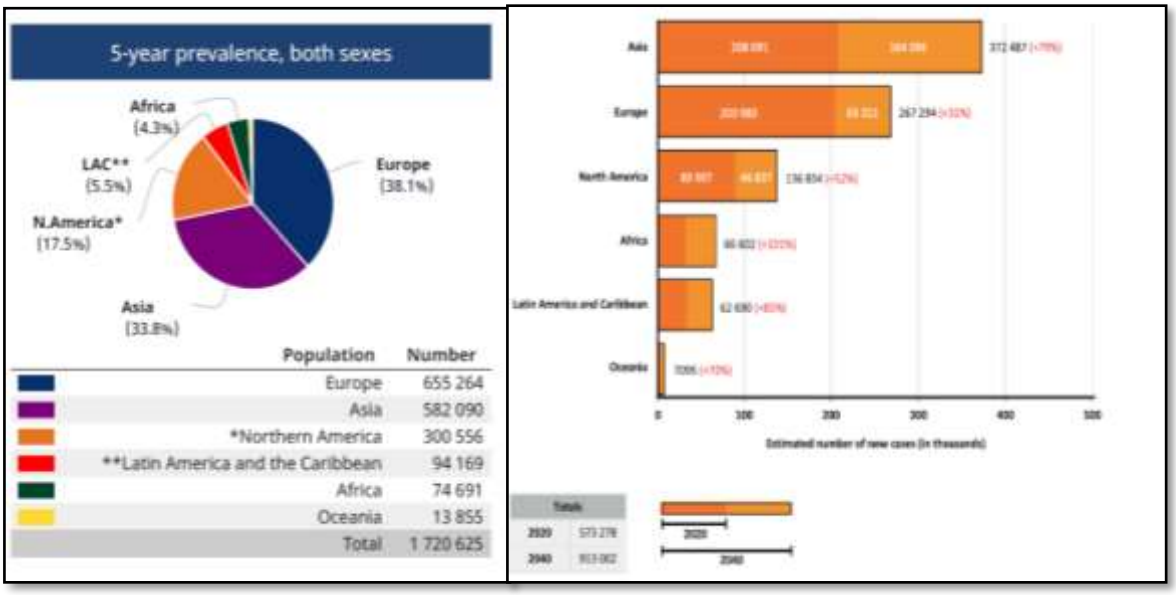

Figure 7: A. Prevalence of number of Bladder cancer cases in 5-years and B. Estimated number of cases projected for next 20 years.[58]

4.3 SURVIVAL:

In the US, there is a 77.1% 5-year survival rate for bladder cancer. While cases diagnosed in situ, which account for 51 percent of all diagnoses, have a 5-year survival rate of 95.8%, that rate drops to 69.5% for localised disease (which accounts for 34 percent of all cases), 36.3% for regional disease (7 percent of all cases), and only 4.6% for metastatic disease (5 percent of all cases) (Figure 8).[59] These figures illustrate both the benefits of early detection and the dismal outlook for bladder cancer with metastatic spread. Over the previous 40 years, the US 5-year survival rate has increased, going from 71.9% for diagnoses in 1975 to 79.3% for diagnoses in 2011.[60] In the US, the 10-year survival rate is 70% and the 15-year survival rate is 65%.[61]

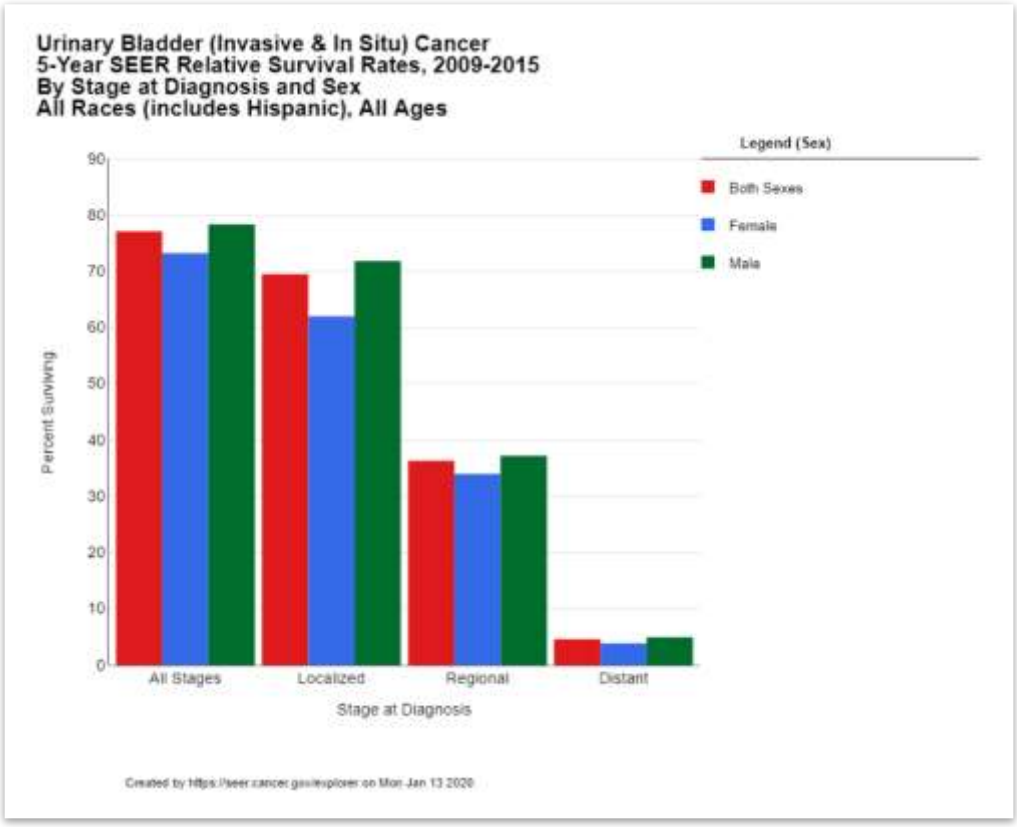

Figure 8: 5-Year SEER Relative Survival Rates for Urinary Bladder (Invasive & In Situ) Cancer, 2009-2015.[59]

4.4 ETIOLOGY:

The 90% urothelial cancer worldwide are prevalent in industrialized countries and are due to direct urothelial exposure to chemical, tobacco smoke or aromatic amines.[59]

Beyond the urothelium the tumours can spread to sub-mucosa, lamina propria, serous layers and muscles. These tumours can also spread nearby organs like uterus and prostate/ vagina. Another 5% of global bladder cancers are squamous cell type, commonly found in Africa as a result of a protozoal infection known as schistosomiasis. The other subtypes like adenocarcinoma, bladder metastases and sarcoma constitute the remaining 5%.[62]

Anything that might change a person's vulnerability to developing a disease is a risk factor. Therefore, it's critical to recognise these risk factors so that an individual may take preventative action and adjust his or her lifestyle. To decrease its prevalence and cure it at an early stage, UBC screening and surveillance are also crucial.[63]

4.5 AWARENESS AND COST FOR BLADDER CANCER DISEASE:

4.5.1 AGE RELATED RISK:

The risk for UBC is known to increase with Age and the median age of diagnosis was 65 to 75 years, 6465 UBC instances were found in the South Australian Cancer Registry (1977–2013),[64] with a mean age of 65.7 years and around 50% of patients between the ages of 50 and 75. Age of the patient at the time of diagnosis was associated with a 1.06-fold increased risk of death in multivariate analysis.[65] Indian patients at UBC had a median age of 59 years, according to a retrospective investigations that looked at 419 patients from the year 2013 as a whole. For the purpose of examining the importance of age and survival, Feng and colleagues

retrieved UBC cases recorded between the years of 1998 and 2005 from the Surveillance, Epidemiology and End Results database. Individuals under 65 years old had a greater rate of OS than individuals over 65.[66]

4.5.2 GENDER RELATED RISK:

Men are 3–4 times more likely than women to develop UBC, however women are typically diagnosed with severe stages of the disease.[67] According to a research, 36% of men and 28% of women had primary bladder tumours that were more aggressive. Numerous research has emphasized that hormonal differences are the cause of the gender variation in UBC incidence. Premenopausal women are at lower risk of UBC than postmenopausal women.[68]

4.5.3 ETHNICITY RELATED RISK:

According to histological stratification, the five-year survival rate for papillary TCC is 89.0% for non-Hispanic whites and Hispanic whites and 86.2% for Asian/Pacific Islanders. Asian/Pacific Islanders had the highest survival rate for non-papillary TCC, at 61.3%, followed by Non-Hispanic Whites at 58.1%, Hispanic Whites at 56.1%, and Blacks at 42.8%. Blacks have been found to have lower disease-specific survival rates than whites, Hispanics, Asian/Pacific Islanders, and whites.[69]

4.6 LIFESTYLE ASSOCIATED RISK FACTORS:

4.6.1 SMOKING:

The main risk factor for UBC in both sexes is tobacco use, and smokers have four times the risk than non-smokers. The risk estimations are 4.06 for current smokers and 2.22 for ex-smokers for both sexes. Aldehydes, aromatic amines, heterocyclic amines, PAHs, and N-nitrosamines are some of the carcinogens present in tobacco smoke. These carcinogens increase DNA adduct formation, methylation of metabolites, and DNA damage in bladder cells. Tobacco smoke mostly disables the

DNA repair machinery, which results in UBC. Recently, it was shown that aldehydes frequently because DNA adducts in the bladder. According to a meta-analysis research, the risk of developing UBC for current smokers is 2.57 (95% Confidence Interval; CI= 2.20-3.00) times higher than it is for non-smokers, even after accounting for the quantity and duration of cigarettes smoked.[70] Additionally, a research revealed that among current and previous smokers, the number of cigarettes smoked influences their likelihood of developing high risk NMIBC as opposed to low risk NMIBC (Odd ratio; OR per year smoked=1.01, 95% CI=1.00-1.03, OR=1.02 per cigarette smoked, 95% CI=1.00-1.04).[71] Bostrom and colleagues examined the illness outcomes following radical cystectomy (RC) in 564 UBC patients. Smokers (52%) and non-smokers (66%), as demonstrated in Fig significant disease-specific survival curves, were both affected. A greater disparity between smokers (37%) and non-smokers (62%) with OS is seen in Figure 9.[72,73,74]

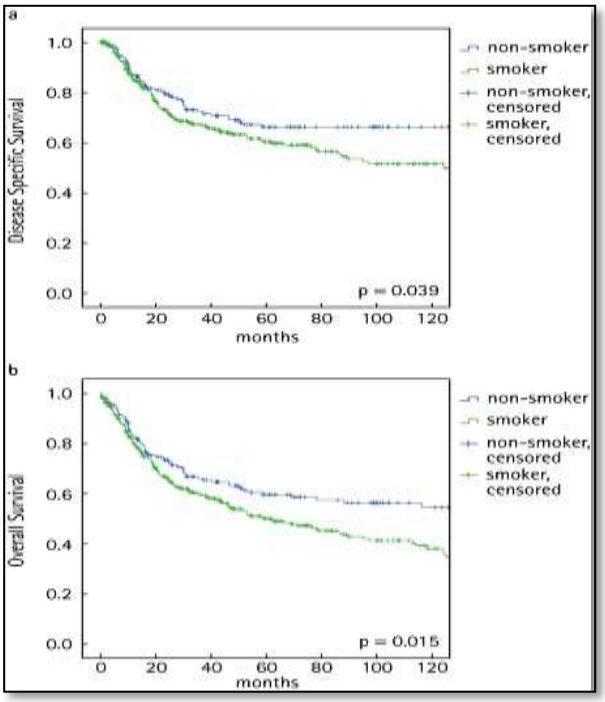

Figure 9: Kaplan-Meier survival analysis (a) Disease specific survival analysis of UBC smokers and non-smokers, (b) OS analysis of UBC smokers and non-smokers.[73]

4.6.2 DIETARY PATTERN:

Dietary routines or behaviours affect the survival, progression, and recurrence of UBC patients. Seafood, pork, vegetable oils, tea, and other food products include benzo[e]pyrene and benzo[b]fluoranthene both compounds are recognised as risk factors.[75] Additionally, a European prospective study on cancer and nutrition found that 3% increase in energy intake from animal protein is related with a 15% rise in the risk of UBC (95% CI=3-30%), whereas a 2% increase in energy from plant protein reduces that risk to 23% (95% CI=36-7%).[58] High intake of red meat was linked to an increased risk of UBC, according to a meta-analysis of 25 trials involving 1,558,848 individuals.[76]

Despite this, multiple epidemiological studies revealed no link between eating red meat and the incidence of UBC.[77] A common food among East Asians and Native Americans is Pteridium aquilinum (bracken fern), which contains the carcinogenic active ingredient ptaquiloside. It is clear from several animal studies that ptaquiloside causes bladder tumours to develop.[78]

4.6.3 PHYSICAL ACTIVITY:

Sedentary behaviour and physical inactivity have been identified as independent risk factors for UBC. The chance of acquiring UBC is reported to be 73% greater throughout the course of lifetime physical inactivity. In those who engage in a lot of physical activity, the lowered summary relative risk (RR) for UBC was 0.85 (95% CI=0.4-0.98),[79] according to a meta-analysis of 15 studies. According to figure 10, the risk of UBC decreases from the 25th, 50th, and 75th percentile levels of physical activity to 10%, 14%, and 17%, respectively. Exercise not only lowers the risk of developing UBC but also enhances the quality of life for those who have

already had the disease. According to a study, patients who engage in high levels of physical exercise see an increase in health-related quality of life of 2.2 times.[80]

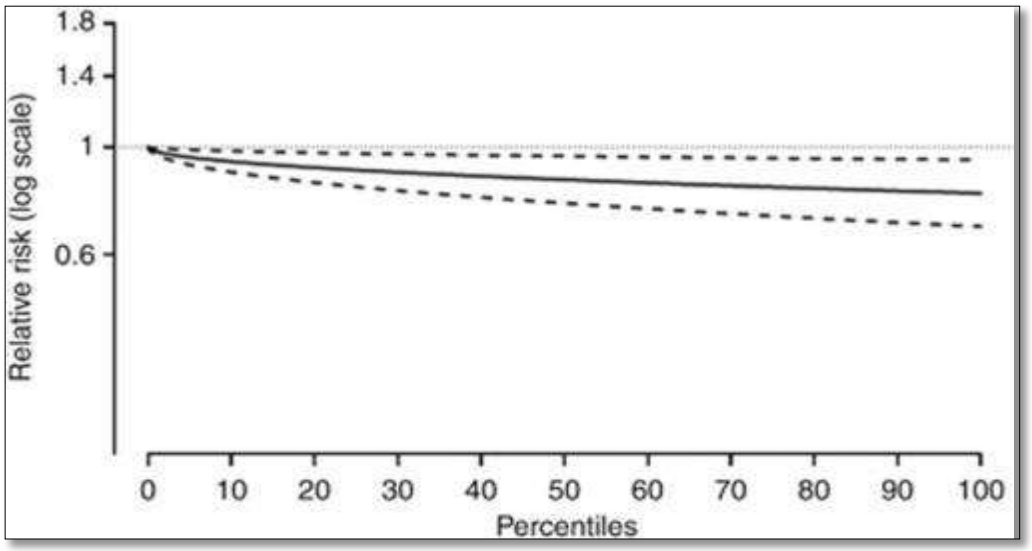

Figure 10: Relationship between growing physical activity percentile and relative risks for UBC.[80]

4.7 RISK FACTORS ASSOCIATED WITH ENVIRONMENTAL:

4.7.1 OCCUPATIONAL RISK:

Exposure to PAHs and aromatic amines (benzidine, 2-naphthylamine, and 4-aminobiphenyl) are the two most significant risk factors for UBC. More employees are exposed to these chemicals/carcinogens in the rubber, dye, and textile production industries.[81] A case-control study's findings revealed that males who handle metalworking fluid had a higher chance of developing UBC. (OR=1.74, 95% CI 1.14-2.54).[82] Another case-control research found that farmers had lower UBC risk but males working as machine operators in the printing sector had higher risk (HR=5.41, 95% CI 1.61-17.72).[83] The risk is around two times higher for weavers with more than ten years of experience in sizing, winding, and warping (95% CI 0.97-5.34). The kind of material handled has been shown to be a

significant risk factor in the weaving sector. The risk is somewhat lower for weavers exposed to cotton (OR=2.00, 95% CI=1.04-3.87) compared to those in contact with artificial substances (OR=2.62, 95% CI 1.1-6.04).[84] A population-based case-control research, on the other hand, found that personal use of hair colour is not directly linked to this illness (HR=0.87, 95% CI=0.65-1.18).[85]

4.7.2 ARSENIC TOXICITY:

Arsenic in drinking water has been noted as a known risk factor for UBC. Low levels of arsenic in water have been strongly linked to UBC in the United States.[86] The role of arsenic in drinking water in the development of UBC in smokers and non-smokers was examined. For UBC, smokers were at higher risk than nonsmokers. Similar to this, Tsuji et al.[74] found that there was a considerably greater risk of arsenic exposure in drinking water among smokers (RR=1.21, 95% CI=0.80-1.84).[87]

4.8 OTHER RISK FACTORS:

4.8.1 MEDICATIONS:

Pioglitazone was approved by the US Food and Drug Administration in 1999 for the treatment of type 2 diabetes mellitus. It is a member of the thiazolidinedione family. Neumann et al.[88] discovered that pioglitazone had a cumulative effect on UBC incidence, with a rate of 49.4 per 100,000 person-years for diabetic individuals using the medication. However, a different research found no evidence of a connection between pioglitazone prescription and the risk of UBC in the Indian population.[89] In a similar vein, there is no connection between long-term pioglitazone usage and the risk of developing UBC (HR=1.07, 95% CI=0.96-1.18), according to a meta-analysis of 12 studies employing HR conducted by Filipova et al. Utilisation of pioglitazone elevates UBC risk globally by 15%.[90]

4.9 ANATOMY:

The bladder is one of the genito-urinary organ which temporarily stores urine due to the rugae, a folded internal lining, it may contain up to 400–600 ml of urine in healthy people. Micturition causes the bladder's muscle to contract, which results in the discharge of urine. The bladder is a hollow, inflatable pelvic viscus that is ovoid when filled and tetrahedral when empty. It mostly consists of collagen and smooth muscle, with very little elastin. A piece of allantois fibre known as the urachus defines its superior part. The urachus connects the bladder apex to the front abdominal wall.[91]

Bladder's exterior characteristics include:

Apex: Positioned superiorly and pointed in the direction of the pubic symphysis. The median umbilical ligament, which is still a part of the urachus, connects it to the umbilicus.

Body: Between the apex and the fundus (or base), the bladder's main portion is called the body. It is triangular in form and has a triangle's tip that faces backward.

Neck: The fundus and the two inferolateral surfaces comprise the neck. It drains continually into the urethra. Urine enters the bladder via the left and right ureters and escapes via the urethra. The trigone, a triangular region within the fundus, serves as a marker for these orifices internally. The trigone, which is created at the base of the bladder by the union of two mesonephric ducts, has smooth walls in contrast to the rest of the internal bladder, which can be understood by its unique embryological origin.[91,92]

In men, the bladder is located between the rectum and pubic symphysis; in females, it is located between the rectum and uterus/vagina. Connective tissue, retropubic fat, and perivesical fat surround the bladder anteriorly, inferiorly, and laterally. The trigone of the bladder is a triangular region of smooth muscle located between the internal

urethral meatus and the two ureteral orifices. The Bell's muscle, which is thicker and runs between each ureteral orifice and the internal-urethral meatus, helps the eye identify the trigone from the remainder of the bladder. The trigone forms from the Wolffian ducts of the mesoderm, according to the conventional hypothesis of the formation of the bladder and trigone, while The remainder of the bladder develops from the endoderm's urogenital sinus.[92-95] (Figure 11)

In men, the bladder base and bladder neck are supported by the endopelvic fascia and the pelvic floor muscles, respectively. The endopelvic fasciae and the prostate hold the bladder neck in place, which is situated 3 to 4 cm behind the symphysis pubis. A layer of smooth muscle covers the bladder neck in this region, forming the involuntary internal-urethral sphincter. The bottom of the bladder and urethra maintains the anterior vaginal wall of the female body. Females' internal urethral sphincters are less developed.[96]

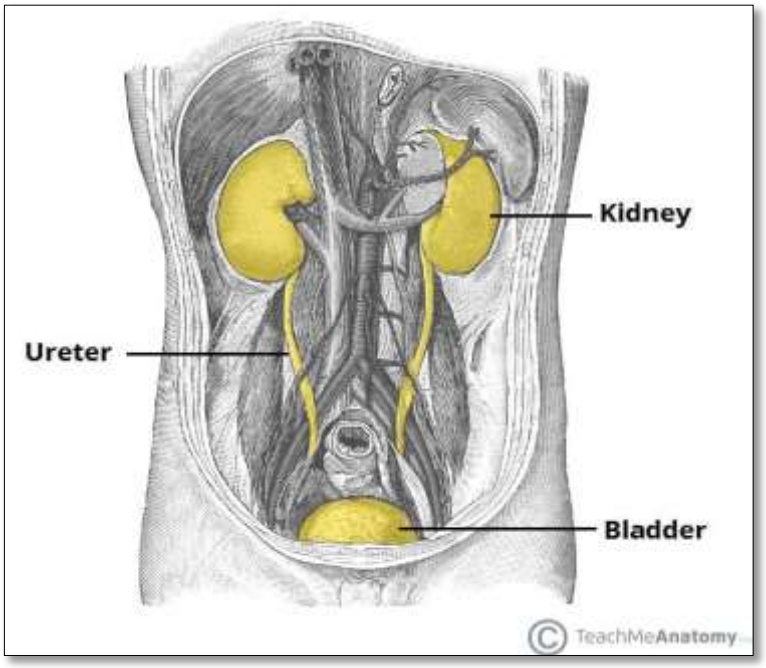

Figure 11: Overview of the urinary tract.[92]

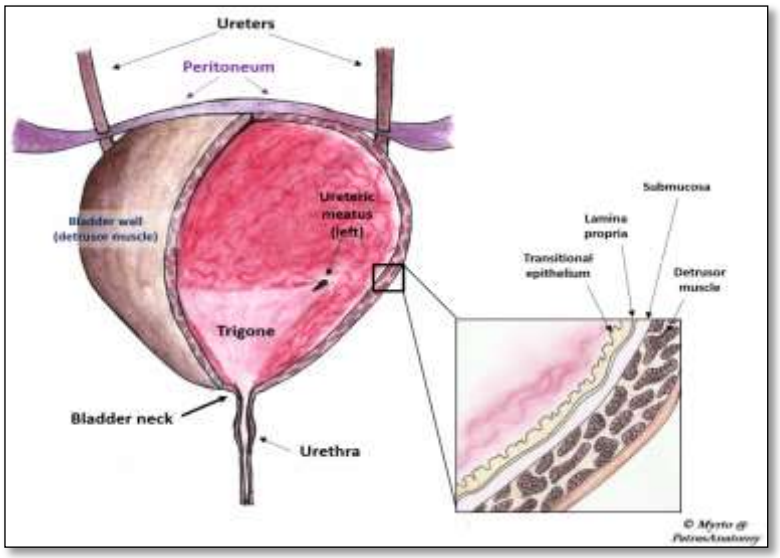

Figure 12: Anatomical features of the bladder.[93]

4.9.1 Musculature:

Urine storage and elimination rely heavily on the bladder's musculature.

Detrusor muscle, a kind of specialised smooth muscle found in the bladder wall, contracts during micturition. It preserves structural integrity when stretched due to the different ways in which its fibres are arranged. Both the parasympathetic and sympathetic nerve systems innervate it.[97]

The detrusor muscle's fibres commonly enlarge (appearing as big trabeculae) to compensate for the increased stress of bladder emptying. This is extremely usual for diseases that restrict urine from leaving the body, such as benign prostatic hyperplasia. Additionally, the urethra has two muscular sphincters:

4.9.2 Internal urethral sphincter:

It is composed of fibres that are smooth and circular and is governed by the autonomic nervous system in men. It's thought to prevent seminal reflux when you're ejaculating. Moreover, in women it is believed to have a functioning sphincter It is created by the proximal urethra and bladder neck architecture.

The external urethral sphincter is structurally identical in both sexes. It is skeletal muscle that is controlled voluntarily. The external sphincteric mechanism is more complicated in men since it also connects with fibres in the rectourethralis and levator ani muscles.[97]

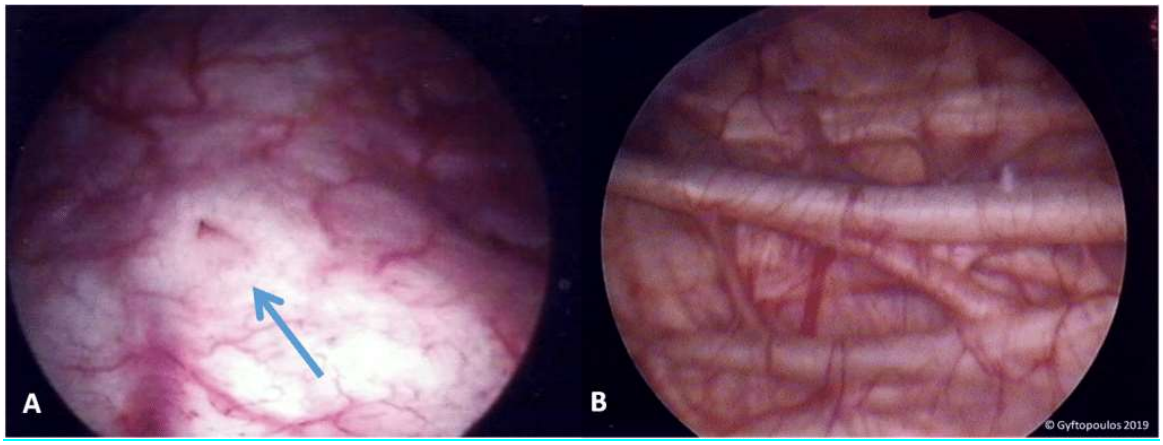

Figure 13: The bladder as seen through an endoscope. (A) The ureteric orifice and trigone. (B) Trabeculae seen in the bladder wall.[97]

Internal iliac arteries serve as the main source of the bladder's vasculature. Arterial flow is provided by the superior vesical branch of the internal iliac artery. The vaginal arteries help to enhance this. In women and the inferior vesical artery in males. In both sexes, the obturator and inferior gluteal arteries may also produce small branches. Venous drainage is made easier by the vesical venous plexus, which empties into the internal iliac veins. At the retro-pubic area, the male vesical plexus and prostate venous plexus, also referred to as the "plexus of Santorini," and this also draws blood through the penis's dorsal vein, are connected.[97]

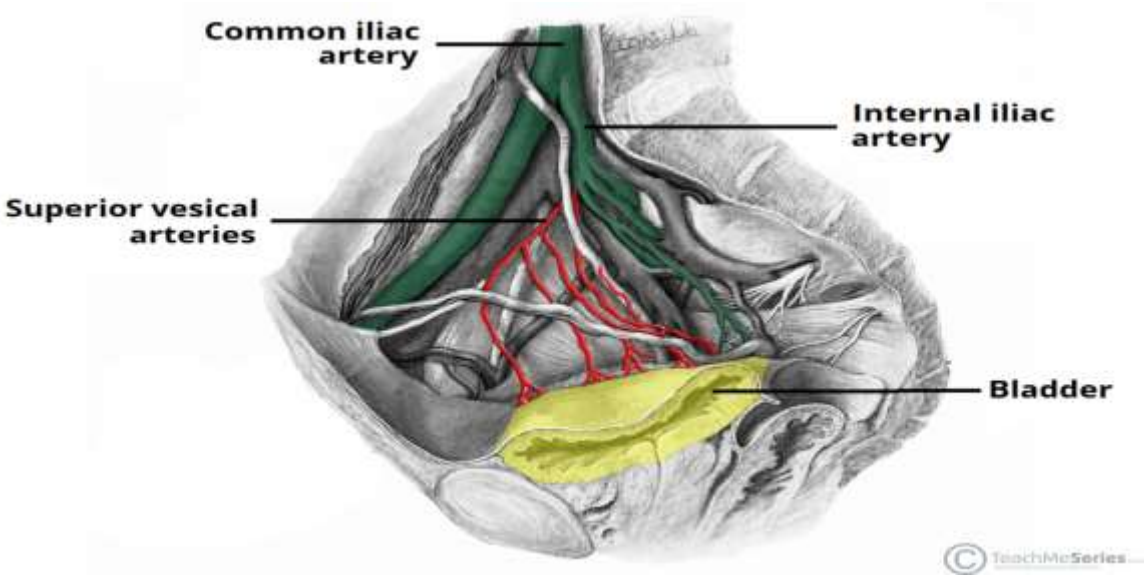

Figure 14: Superior vesical arteries provide the bladder with blood flow.

4.9.3 Lymphatics:

The supero-lateral side of the bladder drains into the external iliac lymph nodes. Drainage begins in the neck and proceeds to the internal iliac, sacral, and external iliac nodes.[98]

4.9.4 Nervous Supply:

The bladder's neurological regulation is complex, involving input from the nervous system's somatic and autonomic: Sympathetic hypogastric nerve. As a result, the detrusor muscle relaxes, which aids in urine retention. S2-S4 pelvic nerve parasympathetic. This nerve's increased impulses induce the detrusor muscle to contract, which assists in micturition. S2-4 is the somatic pudendal nerve. It gives the user voluntary authority over urination by supplying the external urethral sphincter. Aside from the efferent neurons that supply the bladder, there are additionally sensory nerves that communicate with the brain. They are positioned in the bladder wall and notify the person using them when the bladder is about to empty.[99]

4.9.5 Normal Urinary Bladder Morphology:

The basal, intermediate, and umbrella cell types make up the majority of the urothelial mucosa, which typically contains 5-7 cell layers. "Umbrella cells" are the rounded, protruding surface epithelial cells. In the constricted bladder, the intermediate cell layer is roughly six cells thick and is positioned with the long axis perpendicular to the basement membrane. The nuclei are oblong and have nuclear grooves, and the chromatin is coarsely granular. The cytoplasm is potentially vacuolated, somewhat abundant, and amphophilic. [100]

Desmosomes link the individual cell membranes, which make them different. The cuboidal cells that make up the basal layer are one cell thick. The muscularis mucosae, a layer of compact fibrovascular tissue with varying numbers of smooth muscle fibres, make up the lamina propria. The muscularis proper (detrusor muscle) and the epithelium are often located on each side of this layer. Three distribution types of smooth muscle fibres include continuous layer, discontinuous or interrupted layer, and dispersed thin bundles. Thick bundles of smooth muscle that are randomly distributed make up the detrusor muscle. Both the muscularis propria and the lamina propria may include adipose tissue. The adventitia, a layer of fibroelastic tissue and subcutaneous fat, surrounds the muscularis.[101]

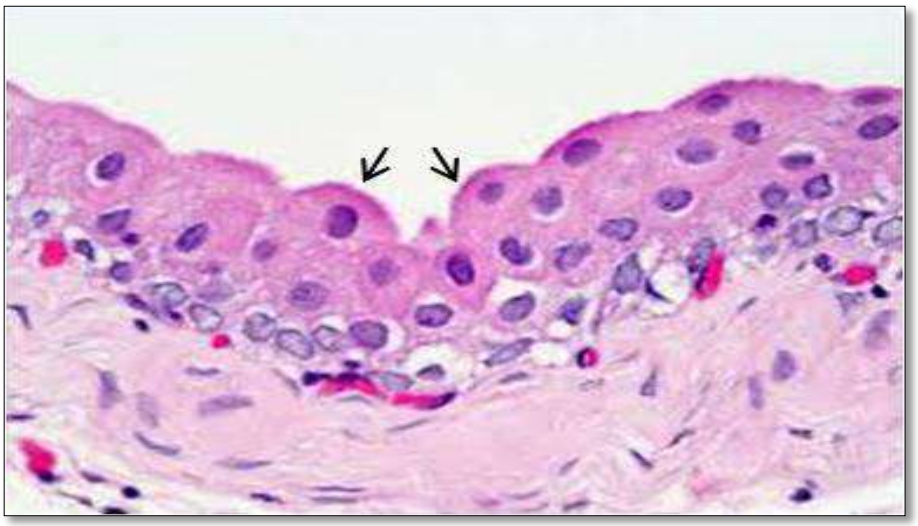

Figure 15: Normal mucosa of urinary bladder showing 3-4 cell layer of epithelial cells with umbrella cells (arrows) in the luminal aspect.

4.10 Classification of Bladder Cancer:

On the basis of morphological characteristics, growth patterns, and depth of invasion, bladder cancer is categorised. For the first time in 1960, UICC under the leadership of Mostofi made an effort to develop a morphologic categorization that would be acceptable to a global organisation. The categorization included the depth

of invasion, or stage, to demonstrate conduct and to direct the therapy because it has been shown that the microscopic appearance, or grade, does not always correlate to the clinical behaviour. Mucosal (T1), muscular (T2), perivesical (T3), and pelvi-fixation (T4) stages were suggested by Wallace [100] The pTa stage, which is limited to the urothelium, was not identified until 1956.

Table 3: The staging system

STAGING SYSTEM	TUMOUR	NODE	METASTASES
Stage 0a	Ta	N0	M0
Stage 0is	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2a	N0	M0
	T2b	N0	M0
Stage III	T3a	N0	M0
	T3b	N0	M0
Stage IV	T4a	N1	M1
	T4b	N1-3	M1

Table 4: Clinical staging of Bladder Cancer.[101]

Tumour	Ta	Papillary tumour that is non-invasive.
	Tis	Flat Urothelium Tumour which is non-invasive
	T1	The layer of tissue and blood vessels above the urothelium, the lamina propria, contains a tumour.
	T2	The muscularis propria, the layers of muscle that surround the bladder.
	T3	Tumor in the layer of perivesical tissue.
	T4	Adjacent tissues, such as the prostate, uterus, or pelvic wall, have been invaded by the tumour.
Node	N0	Lymph nodes have not been affected by cancer.
	N1	One lymph node in the pelvic has cancer.
	N2	Multiple lymph nodes in the pelvic have cancer.
	N3	An abdominal lymph node has been affected by cancer.
Metastases	M0	The spread of cancer has not reached remote areas of the body.
	M1	The liver is one of the distal bodily areas where cancer has spread.

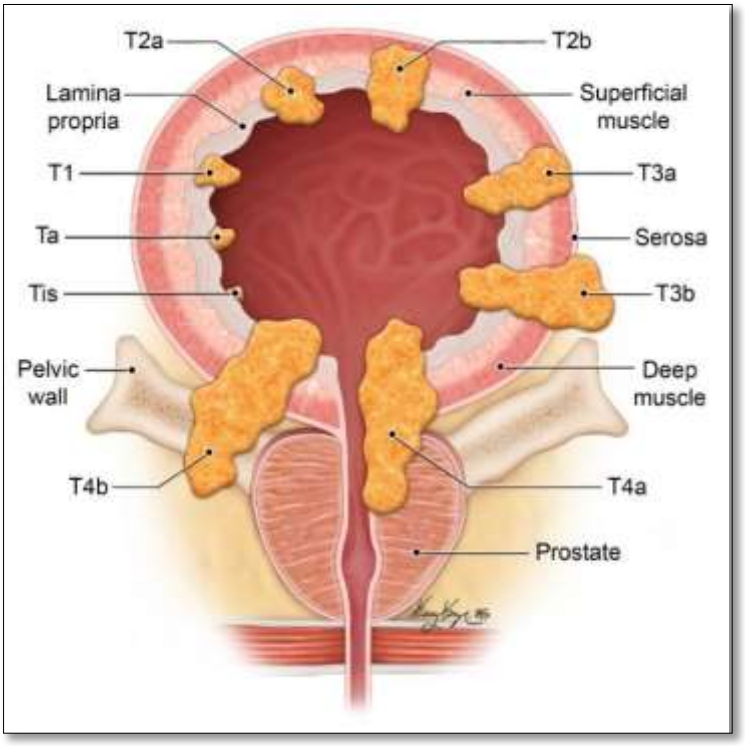

Figure 16: Diagram depicting the various T-stages of bladder cancer.

As previously stated, 90% of urinary bladder tumours are urothelial cancers (transitional cell cancer). Squamous cell carcinoma (5%), adenocarcinoma (2%), small cell, and sarcomatoid cancer make up the remainder. Cancer of the urinary bladder, the epithelium changes, and the severity of the changes determines whether the cancer is classed as Papillary Urothelial Neoplasia of Low Malignant Potential, Low Grade, or High Grade.[101]

4.10.1 Papillary Low-Grade Urothelial Neoplasm:

Papillary low-grade urothelial neoplasm (PUNLMP) has no ability to penetrate or spread. The histological architecture is similar to that of normal urothelium, and the nuclear characteristics are just marginally aberrant. The cells vary from typical epithelial cells in that they lack cytoplasmic clearance and are bigger in size. They

contain several cell layers, with the surface cell layer and fragile fibrovascular stalks preserved. The border of the cell is somewhat defined, and the cytoplasm is homogenous amphophilic to acidophilic. The cytoplasmic clearance is diminished. Nuclei might be circular or elongated, but they always remain perpendicular to the surface and the basal lamina. Chromatin is coarsely granular and equally distributed, while nuclei are tiny or nonexistent (Figure 17). Mitosis occurs seldom.[101]

4.10.2 Low Grade Urothelial Carcinoma:

Cells are organised to cover papillary stalks and architecturally and cytologically resemble PUNLMP. These tumours can infiltrate and (in rare cases) metastasize. The top layer has been maintained in part. There are papillary projections with 10-12 cell thick epithelium, slight polarity loss, and occasional mitoses. Cells are homogeneous in size and distribution, with no discernible boundaries and little to no cytoplasmic clearing. Nuclear boundaries are uneven, rounded, and somewhat pleomorphic. Chromatin is coarsely granular and equally distributed (Figure 18). Mitosis can be detected all across the epithelium, not just at the basal layer.[102]

Figure 17: Papillary urothelial neoplasm (PUNLMP) exhibiting low malignant potential.[102]

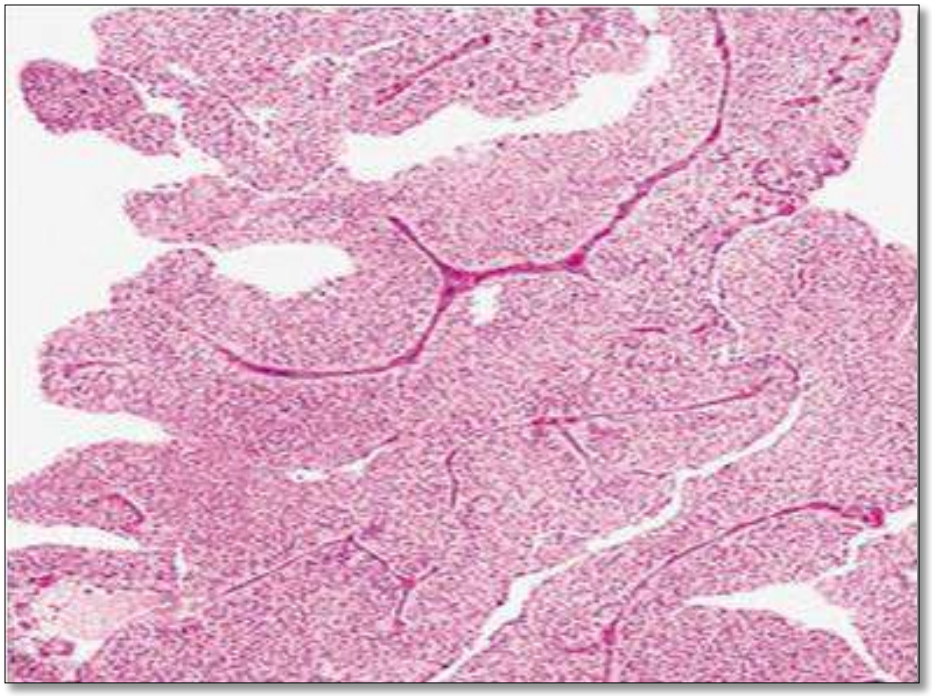

Figure 18: Urothelial cancer of low grade.

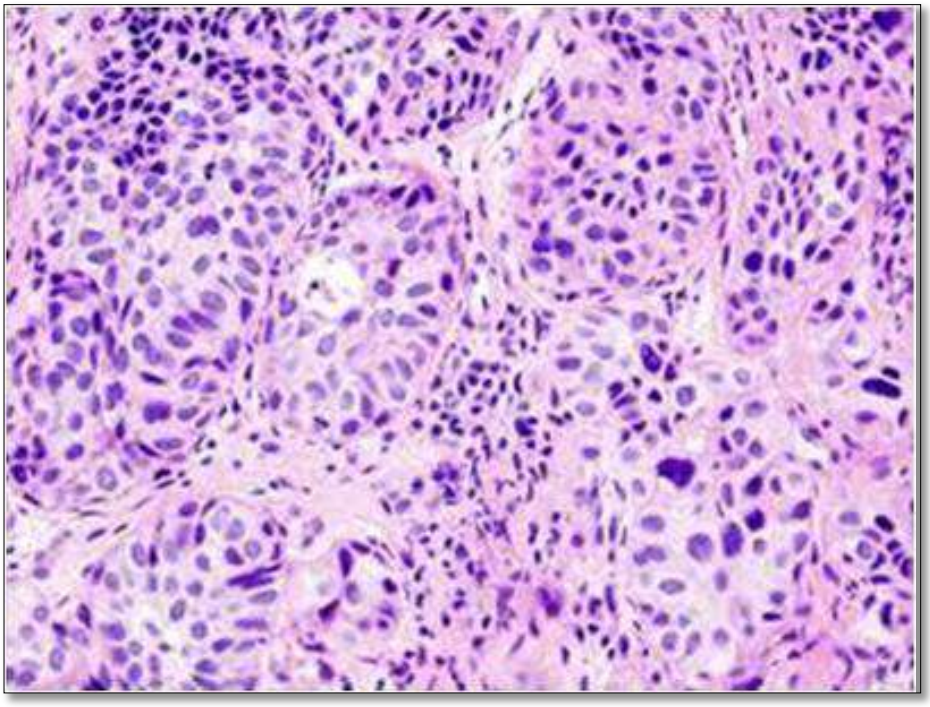

Figure 19: High grade urothelial carcinoma.

4.10.3 High Grade Urothelial Carcinoma:

High-grade tumours are frequently invasive and may be papillary or nodular in nature. High-grade neoplasms account for at least 50% of all urothelial neoplasms and 60-80% of all carcinomas. They are often invading, with cells organised in sheets, nests, and cords. If the papillary component is present, the cells covering the stalk lose their polarity. Cells have fuzzy boundaries. The cytoplasm is homogenous and has the potential to be vacuolated. Nuclei tend to cluster and vary in form significantly (Figure 19). The nuclear chromatin is scattered unevenly and coarsely. Mitoses are prevalent and can be dangerous. With foci of malignant glandular and squamous development, intracellular and extracellular mucins may be present.[102]

4.10.4 Adenocarcinoma:

Urinary bladder cancer may be the site of either primary or secondary adenocarcinoma. The pattern of the tumour cells could be a well-differentiated adenocarcinoma with gland arranged in back-to-back appearance with or without mucin secretion (Figure 20A). Signet ring cell cancer has also been found, with signet cells resting in lakes of mucin. (Figure 20B)[103]

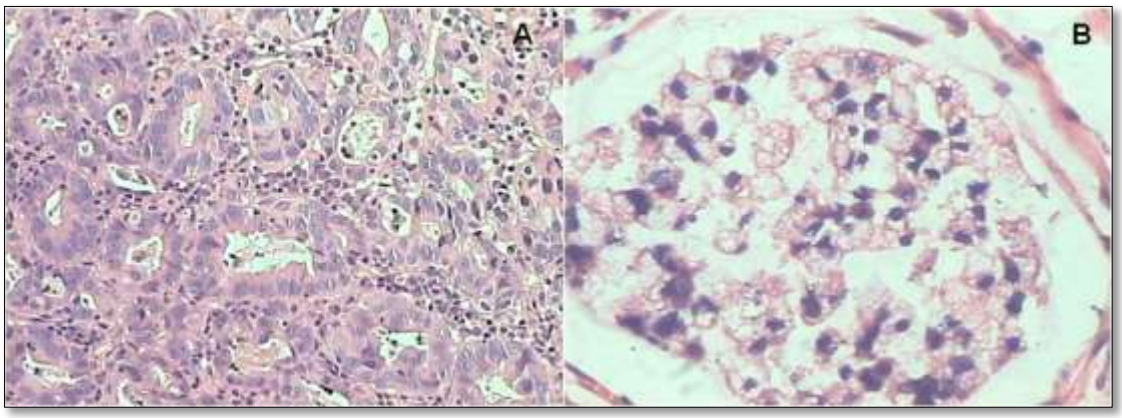

Figure 20: A) Primary adenocarcinoma of bladder with glandular pattern, B) Signet ring cells carcinoma.[103]

4.10.5 Squamous cell carcinoma:

It is more likely in locations where Schistosomiasis is prevalent. Well-differentiated squamous cells are seen along with the presence of keratin pearls and individual cell keratinisation. Eggs of Schistosoma hematobium are identified in the histopathologic section (Figure 21).

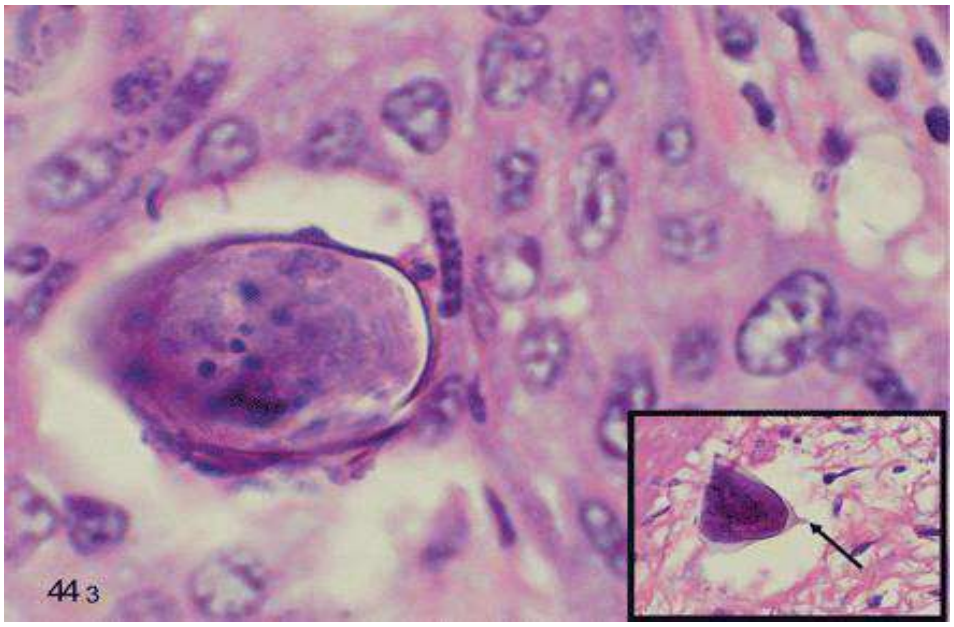

Figure 21: Squamous cell carcinoma of bladder.

Recent molecular classifications of non-muscle invasive bladder cancer based on tumour suppressor gene methylation found three subgroups: Low-Grade pTa, Low-Grade pT1, and High-Grade pT1. In non-muscle invasive subgroups, TSG methylation was similarly found to be predictive of relapse. Furthermore, few studies on molecular classification of bladder cancer found that it was equivalent to breast carcinoma. There are three distinct subtypes.[104]

4.11 Stages of bladder cancer treatment:

4.11.1 Stage 0 (TaN0M0, TisN0M0):

Non-invasive papillary tumours (Ta) and flat non-invasive tumour (Tis) are two types of non-invasive cancer of stage 0 bladder cancer since the tumour has not spread beyond the muscularis mucosae. Transurethral resection (TUR) is performed at this stage, followed by surveillance or intravesical therapy to avoid recurrences. The most frequent intravesical therapy is Bacille-Calmette Guerin (BCG), which is augmented with either chemotherapeutic drugs (Cisplatin, Doxorubicin, Mitomycin C) or Interferon alpha2b. Because high-grade non-invasive papillary (Ta) tumours are more likely to return, 6 weekly intravesical BCG treatments are indicated, beginning a few weeks after TUR and continuing every 3 to 6 months. Close follow-up after treatment is advised, including cystoscopy and urine cytology every 3 to 6 months for at least 2 years to screen for cancer recurrence.[105,106]

4.11.2 Stage I (T1N0M0):

Stage I bladder tumours have infiltrated the bladder's subepithelial connective tissue (the lamina propria) but have not penetrated the muscular layer. Transurethral resection (TUR) is used to treat patients. Half of these tumours (50%) recur. A follow-up cystoscopy/TUR is indicated several weeks later if the cancer is of low grade. Intravesical BCG or mitomycin is administered if the tumour is entirely excised. To avoid invasion, a radical cystectomy may be indicated in high-grade tumours, or a repeat transurethral resection (TUR) followed by intravesical BCG may be required.[105,106]

4.11.3 Stage II (T2aN0M0/ T2bN0M0):

Stage II cancers invade the muscular layer of the bladder wall. The procedure of transurethral resection (TUR) is used to identify the extent of the cancer. Radical cystectomy is the recommended course of treatment for muscle-invasive diseases. Perivesical lymph node resection is also performed. Neoadjuvant or adjuvant chemotherapy is often given to prevent metastases. TUR, radiation, or chemotherapy may be the sole therapeutic option for people with other significant medical or surgical issues.[107]

4.11.4 Stage III (T3aN0M0/ T3bN0M0/ T4aN0M0):

In stage III the tumor extends perivesical and into adjacent organs. Radical cystectomy and perivesical lymph node excision are the primary treatments. To decrease the tumour, neoadjuvant chemotherapy is frequently administered before surgery. This is especially beneficial for T4a tumours that have spread outside of the bladder. [107]

4.11.5 Stage IV (T4bN0M0/Any TN1-3M0/AnyTAnyNM1):

T4b tumours expand to the abdomen or pelvic wall and may have regional lymph nodes or distant metastases. In such circumstances, radical cystectomy is ineffective and should be avoided. Palliative care is used in these instances. Chemotherapy (with or without radiation) is the initial treatment in instances without metastasis, and if metastasis is present, chemotherapy is occasionally followed by radical cystectomy or radiation therapy. [108]

4.11.6 Recurrent bladder cancer:

Treatment and prognosis for recurring bladder cancer are affected by the location, size, and kind of prior treatment as well as the prognosis and course of action. Localized non-invasive bladder cancers frequently come back. Recurrences of bladder cancer can occur in the same area as the original disease or in different areas of the bladder. A repeat TURBT is typically used to treat these tumors. Radical cystectomy is used to treat patients who experience frequent recurrences. Other chemotherapy courses of therapy, such as gemcitabine with doxorubicin, methotrexate or cisplatin, cisplatin and vinblastine, are used to treat recurrent instances.[106]

4.12 UROTHELIAL CARCINOMA IDENTIFICATION:

Painless gross hematuria was the major symptom in 85% of people who have newly diagnosed bladder cancer, although microscopic hematuria affects almost everyone. Because hematuria is frequently intermittent and can be caused by Valsalva manoeuvres, each instance of gross hematuria has to be investigated, even if a subsequent urinalysis is negative. According to Khadra,[109] twenty percent of individuals having gross hematuria are going to be diagnosed with a urologic malignancy, and 12% will be diagnosed with a bladder tumour., and 50% may be the result of an unknown cause. During the first six years, the risk of cancer is virtually non-existent in people with recurrent gross or microscopic hematuria that has had a thorough, negative examination. This should be considered when proposing further tests for individuals with recurrent hematuria.[110]

The AUA recommends cystoscopy, upper tract imaging, and urine cytology for assessment.[111,112] According to the recommendations, low-risk individuals with

microscopic hematuria should be considered for re-examination, and high-risk patients should have a repeat evaluation every six months with urine, cytology, and blood pressure readings. The two basic diagnostic methods for bladder cancer are cystoscopy and biopsy. The gold standard is white light cystoscopy (WLC); flexible office cystoscopy is as reliable as rigid endoscopy and has great sensitivity and specificity for papillary tumours but is very poor for CIS. Cystoscopy with porphyrin dye may be more sensitive in detecting CIS. [113]

Fluorescence caused by Photoactive porphyrins such as hexaminolevulinate, which preferentially concentrate in malignant tissue and emit red fluorescence under blue-wavelength light, are used in cystoscopy. The following could render CIS and tiny papillary lesions easier to identify. A phase 3 study using WLC with blue light cystoscopy in individuals with known or suspected tumours has been completed. Blue light cystoscopy revealed 58% of CIS versus 15% with WLC. Nevertheless, blue light sensitivity was 85-88% and white light sensitivity was 80-85% at the patient level. The false-positive rate for blue light cystoscopy is 39%. More study is needed to understand the particular clinical role of blue light cystoscopy, as its true influence on bladder cancer detection is unknown.[114]

Narrow-band imaging (NBI) is a method for improving the differentiation among mucosal surfaces or microvascular systems that employs no dyes. More light can enter the bladder wall deeper as the wavelength increases. NBI produces light with a narrow bandwidth onto the mucosal surface in the blue (415 nm) and green (540 nm) light spectrums, they are widely absorbed by haemoglobin. As a result, when compared to the pink or white mucosa, the vascular structures look dark brown or green. NBI and WLC are integrated into commercially available equipment, allowing the NBI wavelengths to be activated by just pressing a button. White light

and NBI cystoscopy were performed on 427 individuals with a history of NMIBC.[115]

It is recommended that random bladder biopsies be performed to search for tiny papillary tumours or CIS in endoscopically healthy urothelium. Overall, CIS or small papillary tumours are detected in 2.5% of random biopsies from individuals with known or suspected bladder tumours.[114] Random biopsies are feasible in high-risk people, such as those undergoing post-intravesical treatment or individuals who have favourable cytology results yet a negative endoscopic bladder screening. [116]

Urine cytology, pioneered by Papanicolaou in 1945, assesses the morphologic alterations associated with bladder cancer and serves as the gold standard urine marker against which other indicators are measured. Cytology has sensitivity and specificity of 40% to 62% and 94% to 100% in identifying bladder cancer, respectively.[117] Positive urine cytology is almost always indicative of a bladder tumour, albeit there are few situations when the tumour is not apparent endoscopically. The sensitivity and specificity of urine cytology are determined by the cytopathologist, the quantity of samples examined, and the tumor's stage and grade. Despite the fact that an invasive technique is required, instrumented urine during cystoscopy has increased sensitivity and specificity.[118] An underlying malignancy is present in 15% of individuals with abnormal cytology that is not indicative of cancer. Patients with abnormal cytology should be evaluated more frequently or have random bladder biopsies repeated.[119]

4.13 IMAGING'S IMPACT IN STAGING:

4.13.1 COMPUTED TOMOGRAPHY:

Patients with an elevated risk of bladder cancer ought to undergo a CT abdomen and pelvis with as well as using contrast during the bladder emptying phase before doing TURBT, according to NCCN recommendations. (Figure 22). If imaging of the upper tract has not previously been performed, it is recommended during the initial examination and surgical treatment. Around the stage of diagnosis, two percent of people having bladder urothelial carcinoma will have a synchronous upper tract tumour, and 6% will have a metachronous lesion. The single-bolus approach and the split-bolus technique are two commonly utilised CT urography procedures.[120]

A single full-dose contrast injection is administered, and the patient is scanned throughout the optimum period of renal parenchymal enhancement, additionally during the excretory phase. Because collectively phase of contrast needs a discrete scan, the emission dosage is increased, but the result is a greater single injection volume with enhanced picture quality. By injecting a lower dosage of contrast early and the majority of contrast after a delay, the split bolus approach integrates the excretory phase and parenchymal enhancement into a single scan. Although smaller volume injections may result in less collecting system distension and parenchymal enhancement, fewer scans suggest a lower radiation dosage.[121]

Other treatments used to treat upper tract distension include oral or IV hydration. CT urography has a sensitivity and specificity of 79-93% and 83-99% for detecting bladder cancer, respectively.[122-123] CT is better for T3b and T4 sickness for bladder cancer local staging.[124] Despite the fact that inflammation and a desmoplastic

response might appear identical, especially after biopsy, nodularity and stranding across the bladder surface indicate invasion beyond the serosa.[125]

A study that looked at the effectiveness of CT urography in detecting bladder cancer in those who had hematuria or were under surveillance discovered 13/710 false negatives and 47/710 instances of false positives.[123] The lesions could not be detected in hindsight, therefore 11 of the 13 false negatives were owing to technological constraints; nonetheless, cystoscopy revealed carcinoma in situ. Two false negatives were caused by practical issues: single case had a significant post-void residual and inadequate bladder opacification, and the other involved bilateral hip arthroplasties with bladder artefacts. All false-positive instances required interpretation mistakes, with 12 cases involving BPH and 9 cases involving the bladder.[123]

Urothelial carcinoma may show up on a CT scan as a localised thickening, papillary or nodular tumour, or both. Because urothelial carcinoma demonstrates initial augmentation, obtaining numerous pictures of the bladder during the urothelial phase may aid in tumour detection. With a sensitivity of 89.3% vs. 70.5%, a prospective analysis of 61 lower tract tumours demonstrated better bladder tumour identification when comparing the urothelial phase to the delayed excretory phase.[124] In 5% of instances, calcification is visible (Figure 22). Small flat lesions might be difficult to identify and go undetected. Size is the most often used CT parameter to evaluate nodal involvement, yet this approach is inconsistent since tiny nodes might be reactive while big nodes can be metastatic. [125]

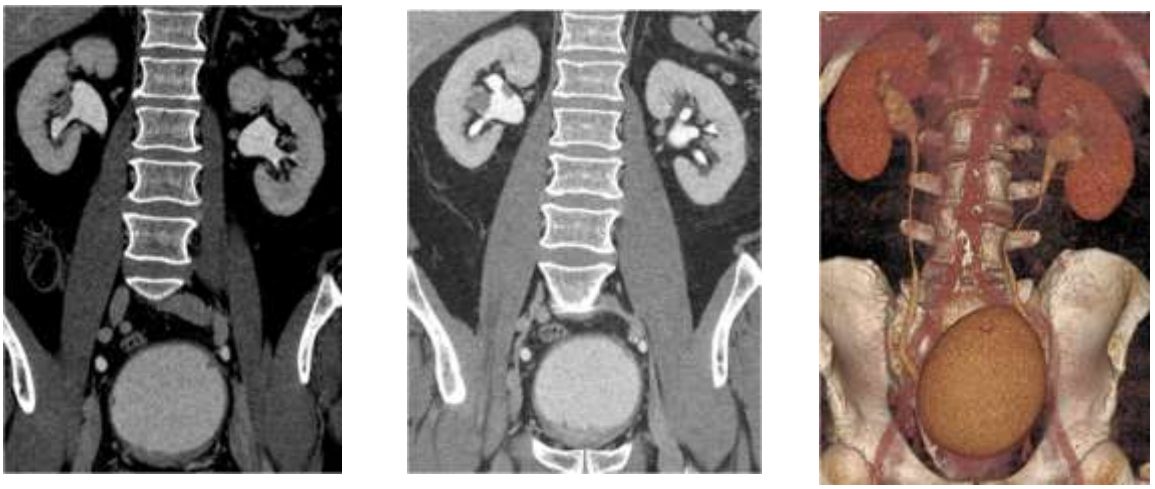

Figure 22: (a) Coronal CT picture in the excretory phase indicates several minor bladder lesions as well as synchronous upper tract illness.

(b) Coronal CT picture from the back, indicating several upper tract lesions.

(c) Coronal 3D volume depiction of another bladder lesion confirms the findings.

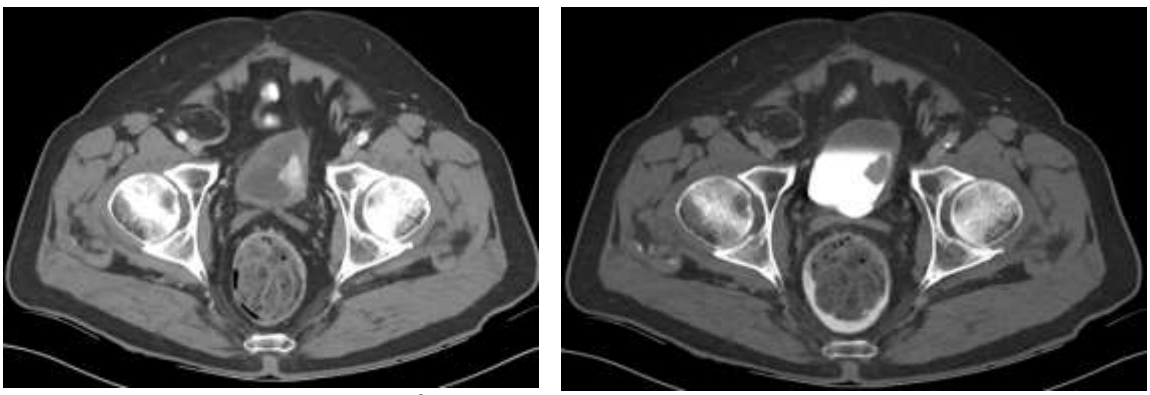

Figure 23: CT scan of a bladder tumour in the excretory phases

(a) An CT picture from the urothelial phase reveals a growing bladder mass. Bladder tumours are hypervascular, and screening the pelvis on CT during the urothelial phase may help in tumour assessment.
(b) An excretory phase axial CT picture reveals the mass as an opening defect covered by urine in the bladder.

4.13.2 MAGNETIC RESONANCE IMAGING (MRI):

Because of MRI's better soft tissue contrast, there is a lot of attention in using multi-parametric MRI for bladder cancer local staging. Diffusion-weighted imaging and dynamic post-contrast imaging are two functional sequences used in multi-parametric MRI alongside anatomic T1 and T2 weighted imaging. On an MRI, the muscularis layer shows a low signal on T2WI and a medium signal on DWI and ADC. On T2WI or DWI, the inner layer, which comprises the urothelium and lamina propria, fails to be evident and shows signs of early amplification, unlike the muscularis, which shows a late and increasing rise. A worrisome lesion had early amplification and limited diffusion with a T2-intermediate signal as compared to muscle and urine. (Figure 24) [126]

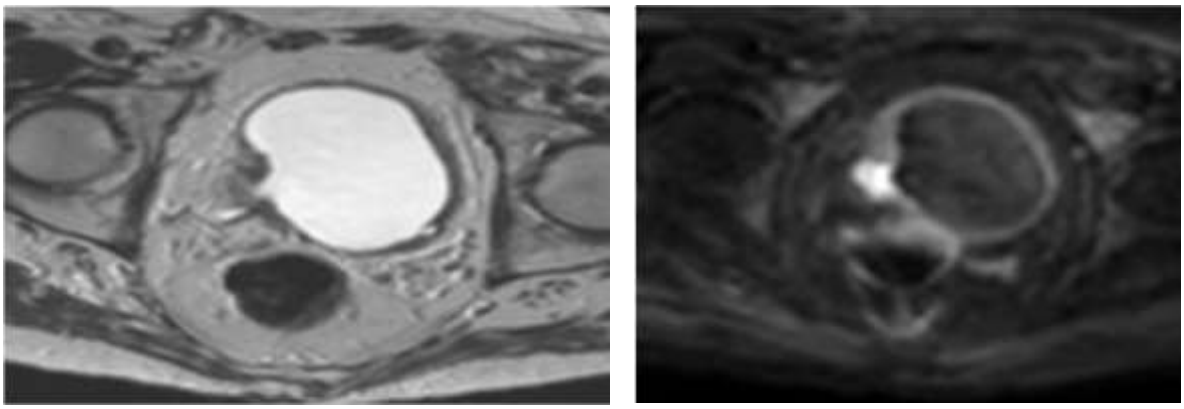

Figure 24: MRI of muscle-invasive bladder cancer (a) Axial T2WI demonstrates a muscle invasive mass in the right bladder wall, seen as a T2 intermediate signal lesion (solid white arrow) invasive into the muscularis propria and T2 hypointense. Because the tumour is near the ureterovesicular junction, it causes hydroureter (dashed white arrow). (b) Axial DWI picture demonstrating a hyperintense mass invading the muscularis propria, indicating a T2 intermediate signal on DWI.

Recently, a comprehensive review of the predictive reliability of multi-parametric MRI (mpMRI) for localised staging was reported..[127] The study discovered that mpMRI has sensitivity of 92% and specificity of 88% for distinguishing between T1 and T2 tumours. The combined sensitivity (0.71) and specificity (0.77) for

distinguishing T2 and T3 cancers were less than optimal. The pooled sensitivity and specificity for distinguishing T1 and T2, T2 and T3, and T4b and pT4b were found to be 0.85 and 0.98, 0.83 and 0.87, and 0.83 and 0.87, respectively, in another meta-analysis.[128] When CT urography is necessary, various guidelines, including those from the AUA, the EAU, and the NCCN, recommend employing MR urography to scan the upper tracts.

According to visualisation ratings, MR urography had slightly better ureter visualisation and slightly worse distal ureter visualisation, whereas CT urography had marginally better intrarenal cavity visualisation. CT urography revealed increased visibility and diagnostic confidence in a retrospective comparison of split-bolus CT urography with 1.5T MR urography. Longer scan periods, poorer special resolution, and a more restricted ability to discern calcifications are some of the disadvantages of MR urography as compared to CT urography.[129]

4.13.3 POSITRON EMISSION TOMOGRAPHY (PET):

Due to FDG excretion in the urine, PET/CT has limited utility in assessing the urinary collecting system, but it is effective in monitoring for distant metastases. (Figure 25).[131] A thorough evaluation of metastatic lesions revealed a pooled sensitivity of 0.82 and a pooled specificity of 0.89.[132] FDG PET/CT affected treatment in 16-65% of patients as compared to conventional CT.[125] Furthermore, the NCCN suggests that FDG PET/CT may be beneficial in a minority of T2 patients and may change management in cT3 illness.[133] For staging lymph nodes, FDG PET/CT has little use. For initial pelvic lymph node staging, one meta-analysis reported a pooled sensitivity of 57% and specificity of 92%. When contrasted with conventional CT, FDG PET/CT did not enhance diagnostic accuracy for identifying lymph node metastases in a

prospective analysis of 61 patients undergoing radical cystectomy and delayed pelvic lymph node dissection.

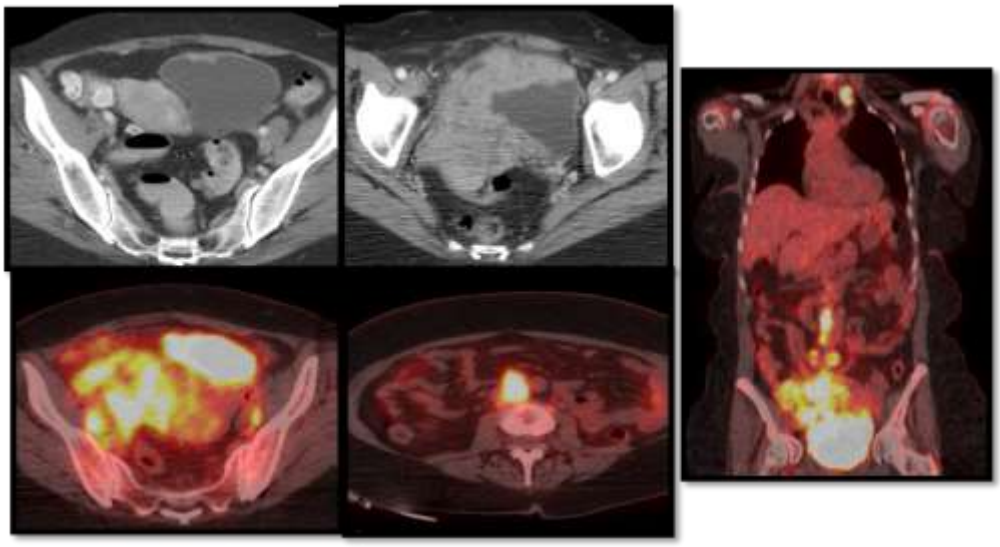

Figure 25: Locally advanced bladder cancer has progressed to metastatic illness. (a) An axial CT picture shows a minor lesion in the anterior bladder that was left untreated for 16 months. (b) Axial CT scan shows considerable growth of the mass, which now involves the uterus, right adnexa, and right pelvic side wall in addition to the bladder. (c) An axial PET/CT picture of the locally progressed tumour with bilateral pelvic lymphadenopathy. (d) An axial PET/CT scan demonstrating metastatic retroperitoneal lymphadenopathy. (e) A Coronal PET/CT scan with metastatic left supraclavicular lymphadenopathy is also seen.

Experimental tracers are being studied, but have not demonstrated enough improvement to warrant their use in clinical practise.[125] A small prospective trial demonstrated no significant advantage of 11C-choline PET/CT versus 18F-FDG PET/CT for urothelial carcinoma staging.[134] FDG PET/MRI is a potentially novel approach to bladder cancer imaging that capitalises on the characteristics of both technologies: MRI's greater contrast resolution including multi-parametric evaluation, and PET's metabolic assessment.

PET/MRI was shown to be more accurate than MRI alone in diagnosing bladder cancer (88% vs. 76%), metastatic pelvic lymph nodes (94% vs. 75%), and non-nodal pelvic malignancy (100% vs. 92%) in a prospective pilot study of 22

PET/MRI scans. PET lowered suspicion in 36% of the cases, 52% (36% increased suspicion, 64% decreased suspicion), and 9% (100% increased suspicion) for bladder tumour.[135] The lack of individuals with true pathologic lymph nodes makes determining nodal status challenging.[133]

4.13.4 ULTRASOUND:

In 45-50% of instances, transabdominal grayscale US may overstaging superficial lesions and understaging advanced malignancies.[134] Ultrasonography showed a sensitivity of 65% and a specificity of 98% in diagnosing bladder urothelial cancer in one study of 1007 people with severe hematuria.[135] A papillary hypoechoic tumour or a discrete area of wall thickening is a classic clinical indication of bladder cancer.[136] (Figure 26).

Bladder cancer eagerly enhances on contrast-enhanced ultrasonography, but the muscularis propria hypoenhancingly enhances. Contrast enhanced ultrasonography, when compared to grayscale ultrasound, may distinguish between illnesses that affect the muscles and those that do not. In one research of 34 patients who had both grayscale and contrast-enhanced ultrasonography before TURBT, contrast-enhanced ultrasound performance reached that of the TURBT reference standard (area under receiver operating characteristic curve, 0.996). [133]

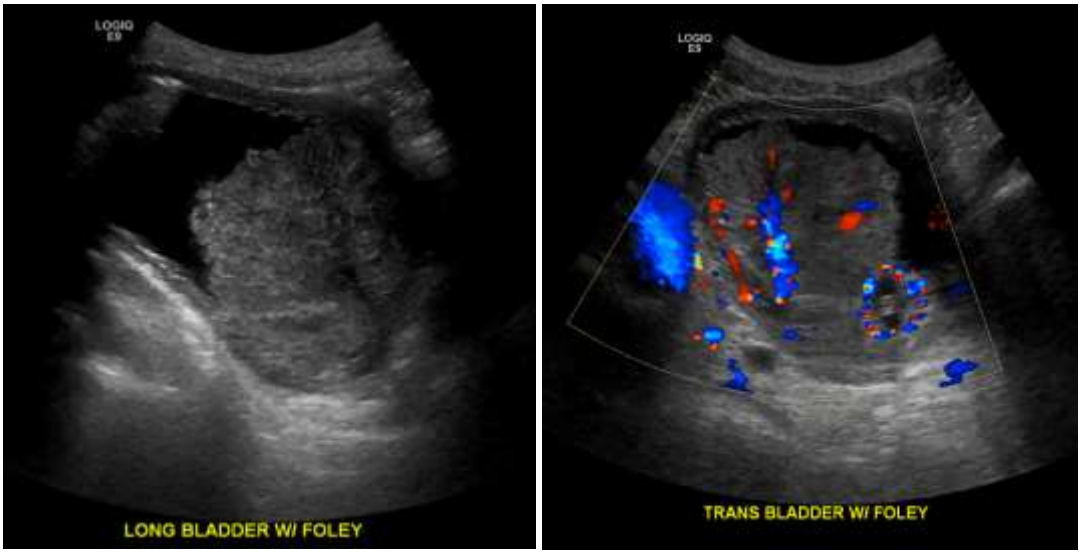

Figure 26, 27: Non-muscle invasive bladder cancer ultrasound and CT pictures. (a) A big mass within the bladder is visible in a longitudinal grayscale ultrasonography picture of the bladder. (b) A transverse colour Doppler picture of the bulk reveals vascularity.

4.13.5 MANAGEMENT:

Bladder cancer is classified into two types: non-muscle invasive and muscle invasive. These two types of bladder cancer have very different management strategies, therapeutic objectives, and survival and recurrence rates. The objective of non-muscle invasive illness is to stop progression and reduce recurrence. A good prognosis with a 20-30% rate of growth is typical for the 75% of bladder tumours that are non-invasive, however, recurrence rates of 60-70% are common.[137] The major goal in muscle-invasive illness is to decide whether to maintain or remove the bladder, and if local bladder treatment alone or systemic therapy is required. Muscle invasive bladder tumours make for about 25% of cases.[138] The initial examination for suspected bladder cancer includes abdominal imaging prior to surgery. A CT urography is suggested. Patients without acute renal failure who have a low GFR or an allergy to iodinated contrast may benefit from MR urography. If contrast is not

permitted, non-contrast MRI using T2 sequences can be utilised to evaluate the urinary system. Further alternatives for assessing the upper tracts include retrograde ureter pyelography with renal ultrasonography, non-contrast CT, and/or ureteroscopy. In cases of sessile or high-grade malignancies, MRI pelvis without and with IV contrast might be used for local staging.[139]

Cystoscopy is also a part of the first assessment of bladder cancer suspicion.[139] Modern improved cystoscopy techniques such as blue light cystoscopy (BLC) and narrow-band imaging (NBI) improve lesion identification when compared to classic white-light cystoscopy (WLC). BLC is a photodynamic diagnostic (PDD) approach that employs substances injected into the bladder and absorbed by urine epithelial cells, including hexaminolevulinate (HAL) or 5-aminolevulinic acid (5-ALA). These are utilised to synthesise photoactive precursor porphyrins, which preferentially aggregate in malignant cells and light after activation, appearing bright pink or red under blue light cystoscopy. BLC enhances the identification of non-muscle invasive bladder cancer, according to many multicenter prospective studies.[139]

When compared to typical white light cystoscopy, one research indicated that using HAL boosted the identification of any cancer by 23%, papillary lesions by at least 12%, and CIS by 43%. In the same research, 25% of patients had lesions that could only be detected by BLC. Narrow band imaging (NBI) produces two bands of blue and green light, which are significantly absorbed by haemoglobin and vascular tissues.[140,141] Bladder tumours are simpler to detect due to their increased vascularity since they contrast with the typical mucosal background of white or pink to look dark brown or green.[142]

NBI identifies 9 to 56% more malignancies as compared to WLC.[143] According to a meta-analysis of randomised controlled studies, There was no distinction in recurrence rates between NBI and HAL-based BLC, a lower repetition with 5-ALA-based BLC compared to NBI and HAL-based BLC, and a lower recurrence with NBI compared to WLC. Additional meta-analysis [144] revealed similar sensitivity and specificity for HAL and 5-ALA-based BLC. The goal of a TURBT is to remove all visible cancer while also confirming pathology and evaluating the depth of invasion. The sample is considered adequate if there is enough muscle in it to assess whether or not there has been muscular invasion.[139] The next course of treatment will be determined by whether the illness is muscle-invasive or non-invasive. Repeat (restaging) TURBT is often suggested for non-muscle invasive bladder cancer due to the possibility of disease progression with a change in therapy in 24-49% of high-grade T1 tumours.[137]

Recurrence rates are lower in patients who have had repeat TURBT, 16% compared to 58% in individuals who have not had repeat TURBT. [145] and persistent tumour after initial TURBT is frequent. In the presence of risk factors for recurrence and progression, such as high-grade disease, higher T-stage (Ta vs. Tis or T1), large size, history of frequent recurrences, multifocality, histologic variants, lymphovascular invasion, and greater depth of invasion (deep T1 tumour), induction with maintenance intravesical chemotherapy may be used instead of a single dose post-TURBT intravesical chemotherapy.[146,147]

4.14 IMAGING SURVEILLANCE:

CT monitoring is not recommended for non-muscle invasive bladder cancer with no risk factors. Because the frequency is less than 1%, regular monitoring for upper tract sickness in asymptomatic low-risk persons is not indicated.[148] In one study, 51 upper tract urothelial carcinomas were discovered in 934 individuals; 15 were discovered during routine imaging, and the remaining 24 were discovered after the patients began having symptoms. 15 CT tests out of 3074 showed a 0.49% imaging effectiveness. Follow-up abdominal-pelvic imaging for muscle-invasive bladder cancer should be done over the first two years, at Six month intervals, then yearly for up to five years, and as needed after that, according to the NCCN.[137] Patients who return after a radical cystectomy usually have a distant recurrence rather than a local recurrence. In the research of 311 patients, 75% of instances of recurrence were distant, with a 12-month median time to recurrence, and 25% were local, with an 18-month median time to recurrence.[149]

The risk of local recurrence following radical cystectomy has been reported to be over 40% in earlier literature and between 6 and 13% in more current study. Improved local control may be linked to neoadjuvant treatment and improved surgical procedures. A distant recurrence occurs in up to 50% of cystectomy patients; the most common non-nodal locales are the bone, lung, and liver, whereas the brain, skin, vagina, and peritoneum are less commonly affected.[150]

4.15 URINE MARKERS FOR UROTHELIAL CANCER:

Cystoscopy of bladder tumours is the gold standard for identifying BC, with a sensitivity range of 62-84% and a specificity range of 43-98%. Because of the time- and temperature-dependent degradation of cells, DNA, and RNA in urine samples, which as a result, the number and quality of cell and DNA molecules vary, as do the sensitivities and specificities, urine biomarker test performance characteristics are overestimated.[151] Urinary biomarkers, on the contrary hand, are appealing because the testing is non-intrusive and inexpensive, and collecting samples is straightforward. This test involves checking for these cells in discarded urine. Urine biomarker tests have a wide range of uses nowadays.[151]

Urine biomarkers, for example, are examined both before to and following transurethral resection operations or intravesical instillation to identify recurrent or progressive BC. The features of a tumour during surgery can also give information about how aggressive and invasive it is. This information is especially valuable for determining the optimal surgical depth, resection margin, and if the detrusor muscle should be included in high-grade malignancies and CIS.[152,153] Urine biomarker monitoring following transurethral resection surgery may be a less intrusive alternative to cystoscopy. Urinary biomarkers can be utilised to assess treatment progress following neoadjuvant chemotherapy, adjuvant chemotherapy, or intravesical instillation. Because of the low frequency of BC, they are not suggested either as a screening method. Urine cytology and cystoscopy both improve the accuracy of BC diagnosis, but more non-invasive, cost-effective, sensitive, and specific diagnostic procedures are still needed. Here, we talk about and evaluate a number of potential urine biomarkers.[151]

4.16 URINARY BIOMARKERS APPROVED BY THE FDA:

Considering a specificity and sensitivity ranging from 48% to 86%, urine cytology is the most reliable urinary biomarker test for detecting exfoliated cancer cells in urine. With 38-90% sensitivity and 98-100% specificity, It aids in the original diagnosis as well as the recurrence tracking of high-grade or CIS BC that receives intravesical therapy. [154,155] However, its sensitivity for low-grade breast cancer is only 4-31%, and the 12% false-positive rate indicates a limited ability to screen out persons with inflammation, atypical urothelial cells, or a history of past cancer therapy. Conventional cytology has significant limits, particularly in patients with recurrent inflammation or who have already received treatment. Furthermore, whenever abnormal urothelial cells are discovered using urine cytology, the results should not be utilised as a diagnostic alternative for cystoscopy because cystoscopy data can be more repeatable than urine cytology data.[158]

4.16.1 Bladder Tumor Antigen STAT and TRAK:

The BTA stat is a simple protein biomarker test that uses an immunochromatographic technique to identify and track BTA in past diagnosed BC patients. Human complement factor H and complement factor H-related proteins, the two of whom have been associated to cancer cell proliferation and immune evasion, are detected by the BTA stat and BTA TRAK assays; their levels increase during BC cell invasion.[159,160] The sensitivity and specificity of BTA stat are 55-80% and 62-90%, correspondingly, while the sensitivity and specificity of BTA TRAK are 70-75% and 46-80%, respectively.[168-170] For monitoring reasons, BTA stat has a better overall sensitivity than urine cytology, but a poorer specificity. BTA TRAK outperforms urine cytology in terms of sensitivity with regard to high-grade and low-grade BC. However, due to their poor specificity and high rate of false positives caused by the

discovery of benign illnesses that include hematuria, urinary tract infections, stones, and in situ ureteral stenting, BTA tests cannot replace urine cytology.[163,164]

4.16.2 NMP22 kit and NMP22 BladderChek:

The NMP22 test and NMP22 BladderChek comprise two ELISA tests aimed at targeting the NMP22 protein, which is found more often in BC cells compared with normal urothelium and is secreted in urine when urothelium cells die.[165,66] The NMP22 surveillance kit has a sensitivity and specificity of 50-60% and 85-90%, respectively. The NMP22 BladderChek's sensitivity and specificity for diagnosis are 60-75% and 70-85%, respectively.[167,168] The high probability of false positives generated by detecting benign diseases limits the usefulness of these diagnostics.[169] The NMP22 test offers greater diagnosing sensitivity for microscopic hematuria as well as increased detection power when compared to urine cytology because of the greater incidence of cellular death reported in low-grade, low-stage, and high-grade BC. However, because of its poor specificity, NMP22 test cannot completely replace urine cytology. [170-173]

4.16.3 IMMUNOCYT ASSAY:

The ImmunoCyt assay, which blends urine cytology and immunohistochemistry staining using fluorescent-labeled monoclonal antibodies, is the sole test available for therapeutic follow-upThe experiment employs carcinoembryonic antigen and 2 BC-associated mucins to identify exfoliated BC cells when employed for diagnostic and monitoring purposes, the assay's overall specificity varied from 60- 80%, while its sensitivity ranged from 65- 100%.[174] Cystoscopy is not required due to this assay's great sensitivity for detecting higher pathological grades in addition to low-grade, low-risk BC.[175] There are various drawbacks, including the need for specialised laboratory equipment and test interpreters with expertise and experience, and it has

having less specificity in urine cytology. Furthermore, due to the fact that it identifies benign illnesses, it possesses an elevated rate of false positives and a low level of specificity (65%) for hematuria.[176]

4.16.4 UROVYSION:

The UroVysion employs a multi-target fluorescence in situ hybridization assay for detecting aneuploidy in chromosomal copy numbers 3, 7, 17, and 9p21, as well as deletion of the P16 tumour suppressor gene. The test has a greater sensitivity (55-85%) and specificity (75-90%) for low-grade BC than urine cytology.[177] This test is useful for finding BC in high-risk individuals with ambiguous cystoscopic results and atypical cells, confirming BCG adaptation in those who did not react, and detecting BC recurrence in NMIBC patients with negative cystoscopy but suspicious cytology. Costs are increased by the requirement for testing equipment and test-reading skills, and also by labor-intensive specimen processing. There is a significant chance of false positive results, particularly in people examined within one year of having a bladder biopsy. The presence of additional primary tumours with chromosomal abnormalities increases the likelihood of false positive results.[178]

4.17 Non-FDA approved urinary Biomarkers:

4.17.1 SURVIVIN:

The inhibitor of apoptosis protein family is a new protein family that adversely affects cell death. urvivin is unique among the members of this family because it is created throughout embryonic and foetal growth, declines in typical adult tissue, and can be detected in altered cellular and a wide range of human cancers. Survivin has been discovered as the 4th largest transcriptome in human malignancies, although it is not found in normal human tissue. Survivin has been shown to be over-expressed

in multiple cancer types in relation to normal tissue, and preferential survivin amplification has shown connected to physiologically severe illness, therapy resistance, and poor clinical outcomes in individuals with various malignancies.[179]

4.17.2 ADXBLADDER:

ADXBLADDER uses ELISA to identify mini-chromosome management protein 5 that is present during DNA replication and has been found to be higher in hematuric BC patients. While the sensitivity for high-risk and muscle-invasive BC is expected to grow to 90-95%, The predicted total sensitivity as well as specificity are 76% and 69%, respectively.[180] Average negative predictive value (NPV) is 97.5%, with a high-risk BC (58) NPV of 99.5%. As a consequence, as a BC diagnosis tool, this type of biomarker might replace or supplement urine cytology. This test has the benefit of being unaffected by benign disorders in terms of diagnostic accuracy.[181,182]

4.17.3 UBC® RAPID TEST:

The UBC® Rapid test is a point-of-care ELISA that identifies solubility fragments of cytokeratins 8 and 18 from urine BC antigen, which are associated with tumour infiltration. With a sensitivity of 50-75% and a specificity of 82-93.8%, this test is more sensitive than low-grade tumours in identifying high-grade NMIBC during diagnosis and follow-up. It is expected to become a urine protein biomarker testing that is sensitive and specific for diagnosing patients with high-grade malignancies that are challenging to detect with cystoscopy, regardless of user, group, or study site.[183]

4.17.4 UROMONITOR:

Real-time PCR is used in the genetic biomarker test Uromonitor to identify TERTp and FGFR3 alterations within exfoliated cancer cells. For NMIBC monitoring, the Uromonitor-V2 test offers 100% sensitivity, 83.5% specificity, 66.5% PPV, and

100% NPV. Uromonitor performs similarly to cystoscopy in all phases and grades of post-transurethral bladder resection recurrence surveillance, serving as a substitute test for those who are unwilling to have a cystoscopy, having a sensitivity of 73% and a specificity of 75%. When used in conjunction with cystoscopy, the uromonitor offers 100% sensitivity and 88% specificity. The detection rate rises to 62.5% in the case of repeated low-grade BC and 75% in the case of recurrent high-grade BC. Inflammation has no effect on the Uromonitor test results.[186]

4.17.5 UroSEEK:

DNA biomarker detection assay in both monitoring and diagnostic, UroSEEK is more accurate to cytology. Individuals at high risk for BC, as well as individuals with abnormal urine cytology, are suitable candidates for initial diagnosis. Monitoring had a sensitivity of 75-95%, a specificity of 70-85%, and an NPV of 50-97%. The drawbacks of the test include The low sensitivity of the Next-generation sequence (NGS) technique used to detect alterations as well as poor performance after follow-up testing of BC patients who have past diagnoses and those with upper bladder urothelial carcinoma.[187,188,189]

4.17.6 CXBLADDER ASSAY:

The CxBladder transcriptome panels test was developed at a lab that looked into five mRNAs connected to BC and one mRNA linked to nonmalignant conditions. Because of the acquisition of clinical factor data, the CxBladderTriage has the advantage of characterising people at risk and possessing a 100% rate of detection for high-grade BC without gross hematuria. It also has a better sensitivity (68% for pTa) and specificity (85%) for low-grade BC than urine cytology. All other evaluative tests have a 93% sensitivity across all levels and grades.[190,191]

4.17.7 XPERT BLADDER CANCER MONITOR:

The XPERT BC Monitor, a transcriptomic panel test targeting five mRNAs shows a similar overall specificity to urine cytology of 90-91% and a higher total sensitivity of 75% (better than urine cytology). In terms of sensitivity (85% vs. 30%, p 0.01) and negative predictive value (93% vs. 76%, p 0.01), this test surpasses urine cytology for lowgrade (75%) and pTa illness (80%).[192]

4.17.8 EPICHECK:

To detect the recurrence of NMIBC, the EpiCheck epigenetic DNA methylation biomarker test is designed to detect 15 altered DNA methylation biomarkers. The probability technique, EpiScore, possesses an NPV of 95.2%, a sensitivity of 68%, and an accuracy of 88%. Urine cytology is more sensitive but has a lower specificity. The high sensitivity for high-grade BC and 83.0% NPV of this test are advantages, as is the reality that urinary tract inflammation has no influence on the results. Costly and technically complicated operations are drawbacks.[193,194]

4.17.9 TaqMan® Arrays:

Based on a qRT-PCR test for BC detection, this 12 + 2 gene-set panel offers a sensitivity of 95% and a specificity of 99%.[200,201] This test has a sensitivity of (75% vs. 85% in the control) and a specificity of (90% vs. 82% in the control) for predicting BC aggressiveness.[195,196,197]

4.17.10 Cytokeratin-19 Proteolytic region:

In terms of evaluation, prognosis, follow-up, and early identification of recurrence, CYFRA 21-1 has been identified as a general tumour biomarker for numerous neoplastic disorders, including BC. As a soluble molecule, it may be detected in serum and other bodily fluids. The ELISA technique is used in the CYFRA 21-1 test, which has sensitivity and specificity of 72-88% and 75-85%, respectively.[198]

4.17.11 BLCA-4:

In the early stages of BC, it's a transcription factor from the NMP family. Despite its high sensitivity and specificity (90-95% and 95-100%, correspondingly), additional study is needed to prove its use as a breast tumour diagnostic biomarker. The fact that BLCA-4 levels are low in persons with a variety of benign diseases adds further to its potential. However, in order to use the BLCA-4 test in hospitals, other procedures that require no urine precipitate analyses are necessary.[199]

4.17.12 Hyaluronic acid:

It is a cell adherence and reproduction promoting chemical. The hyaluronidase enzyme (HAase) catalyzes HA interactions during cancer metastasis to promote cellular proliferation and motility.[204] For NMIBC, the HA testing has accuracy and specificity of 85-100% and 85-95%, respectively.[201]

4.17.13 Telomeres:

Urinary telomerase is linked to the recurrence of NMIBC and possesses an antioxidant function in cancer cell genomes. When telomerase activity is combined with cytology, the sensitivity and specificity increase by 60-85% and 64-88%, respectively. Since the PPV for superficial stages is 84.3%, Telomerase activity correlates with lower grades and stages of BC in 42.3% of all invasive stages, 83.5% of grade 1 tumours, 66.8% of grade 2 tumours, and 40.2% of grade 3 tumours.[202]

4.18 ANALYSIS OF BIOMARKERS:

There are numerous methods to assess novel biomarkers. The approach to be employed is determined by the study question. During the early phases of developing a novel test, the primary concern was whether diagnostics possess sufficient potential as well as how affordable they were. We concentrate on biomarker risk prediction using validation of risk scores in the later phases of evaluation, as well as cost-effectiveness in a clinical setting.

Health Technology Assessment (HTA) is a systematic method for evaluating new medical technology. HTA is a multidisciplinary policy analysis discipline that investigates not only the medical but also the socioeconomic ramifications of health technology development and usage. The goal of an HTA study is to analyse the consequences of novel biomarker testing, which includes innovative health technologies. It helps answer issues about effectiveness, comparative effectiveness, and cost-effectiveness. Figure 28 depicts the steps in the HTA process. A novel biomarker should not be studied in isolation, but rather within the context of existing information. These models usually consider binary events that either already exist in a patient (disease, i.e. diagnosis) or will take place in the future (events, that is, prediction). These danger prediction algorithms can assist physicians in making more customised decisions for those they treat. [203]

The evaluation of biomarkers with applications in urological cancers is the topic of this thesis. It is a common procedure to utilize extended models to assess the additive predicted or diagnosis accuracy of a new biomarker, followed by a comparison of predictive values obtained by the baseline model and the model enlarged with the new biomarker. There are several approaches for doing this evaluation. [204,20]

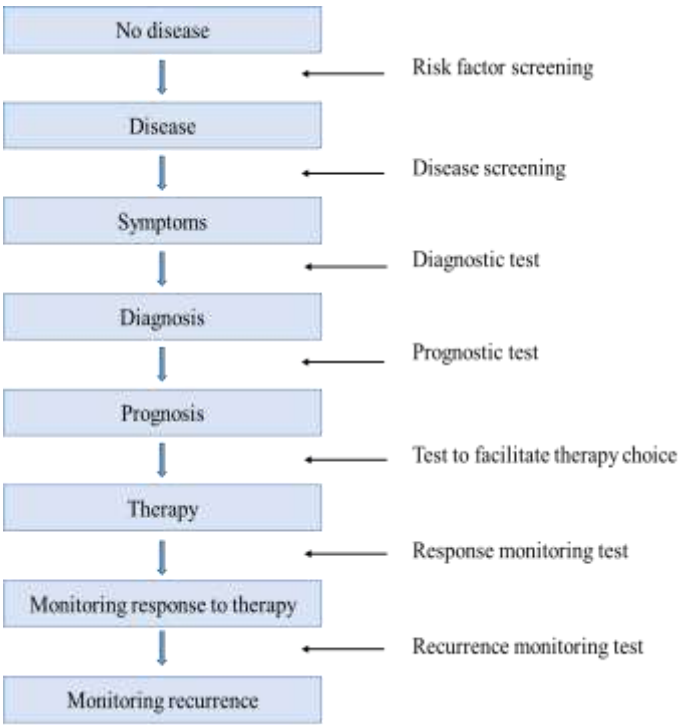

Figure 28: Biomarkers used in the spectrum of disease.

Figure 29: Steps in the Health Technology Assessment Process.

4.19 Methods for assessing the efficacy of Biomarkers:

Sensitivity and specificity are the traditional performance parameters for a binary classification test. Sensitivity is defined as the fraction of positives that are correctly recognised in this manner, i.e. a positive test result when a disease is present. Specificity is the percentage of correctly identified negatives, or results that are not favourable when a disease is not present. Several statistical techniques rely on sensitivity and specificity to characterised the degree of increased prediction capacity. Area under the curve of a receiver operating characteristic curve with all viable prediction cut-offs, Net Reclassification Improvement (NRI), and decision curve analysis (DCA) over a suitable range of prediction cut-offs are examples. The cost-effectiveness analysis is the final step in the evaluation of biomarkers.[206]

4.20 Cost-effectiveness of Biomarkers:

The relative costs and results of multiple solutions are compared in a cost-effectiveness study. The cost-effectiveness ratio is the ratio of outputs to additional expenditures, as a ratio between the outputs and the incremental expenses, which are often monetary values. The Quality Adjusted Life Year (QALY) is a common health outcome examined in medicine. The goal of QALYs is to assess the quality and quantity of life using utilities as effectiveness metric. The summary figure is the cost/QALY ratio. It is commonly analysed in connection to a willingness-to-pay threshold using sensitivity studies that take uncertainties and assumptions into account.[207]

METHODLOGY

5.1 Clinical samples:

Patients enrolled in the Department of Urology at Dr. Prabhakar Kore Hospital and Medical Research Centre in Belagavi, India, between 2019 and 2022 were included in our study. These individuals were evaluated clinically for painless gross / microscopic haematuria, as well as bladder irritation, such as urgency and increase urine frequency (in CIS patients). Individuals with non-muscle invasive tumours experienced very little discomfort, dysuria, urgency, or pain. Patients with muscle invasive tumours, on the other hand, had flank pain due to ureteral obstruction or abdominal/ pelvic/ bone discomfort from distant metastases.

To examine tumour size, number, site, mucosal abnormalities and appearance modalities like Urine cytology, ultrasonography, cystoscopy, bimanual examination, Intravenous Pyelography or CT scan, magnetic resonance imaging, chest X-ray and bone scan were important gold standard methods used to diagnose patients with bladder cancer.

5.2 Sample Collection:

The study trial recruited 422 participants in total. Urine samples have been collected and aliquoted into three different research groups. First group was individuals with Lower Urinary Tract symptoms, second group were individuals attending the hospital for regular health checkups and the third group were patients with confirmed histologically proven bladder cancer cases. After obtaining the informed consent, all individuals were personally interviewed to obtain the information on demographic details. The Institutional Review Board of J.N Medical College in Belagavi, India, granted ethical approval (KAHER/EC/20-21/001/05). The analysis made use of anonymised data gathered after each patient volunteered to participate in the study.

One hundred fifty mid-stream urine samples of patients, positive for carcinoma histologically were divided into non-muscle invasive tumours [stage Ta-T1; 67.61% (71/105) were low grade cases; 32.38% (34/105) were high grade cases] and 45 muscle invasive tumours [stage T2-T4; 100% (45/45) high grade].

5.2.1 Criteria for Inclusion:

Patients with Lower Urinary Tract symptoms , regular health checkup and Bladder cancer proven cases presented for Urology OPD.

5.2.2 Criteria for Exclusion:

Metastatic Cancers.

Coexisting tumours.

Severe recurrent bacterial cystitis.

Bladder cancer patients already on treatment

Imaging of the chest, abdominal ultrasonography, and CT of the abdomen (as previously described) were performed to detect common metastatic locations. Histopathology results from radical cystectomy patients were reviewed for lymph node positivity/metastasis. Regardless, patients who had TURBT were evaluated for clinical staging (based on radiological tests) before being evaluated for pathological staging.

5.3 Summary of clinical and histological findings in patients with non-muscle invasive bladder cancer [stage: Ta-T1]:

Patients suffering from non-muscle invasive disease were 92. Out of which, 41.30% (38/92) patients were of age less than 60 years and 58.69% (54/92) were of age more than 60 years. Out of the total 92 cases, 68.48% (63/92) patients were identified to have positive history of smoking or tobacco chewing and 31.52% (29/92) patients had negative smoking/ tobacco chewing history. Histo-pathologically, 56.52% (52/92)

were classified as low grade and (40/92) were identified as high grade tumors. Thirty-eight out of the 92 NMIBC patients, 41.30% patients were presented with recurrent tumor. Tumors resected from 77.17 % (71/92) patients were examined for its size greater than 3cm while 22.82 % (21/92) patients were observed to have tumor of less than 3 cm. As per radiological records, none of the non-muscle-invasive bladder cancer patients were observed with metastasis or lymph node involvement.

5.4 Clinical and histopathological summary of patients with muscle invasive bladder cancer [stage: T2-T4]:

Among the 58 patients diagnosed with muscle invasive disease, twenty-six (44.82%) patients were of age less than 60 years and 55.18 % (32/58) were of age more than 60 years. Tumors resected during surgical procedure were examined and its size was found to be greater than 3 cm in 75.86% (44/58) cases and less than 3 cm in size in 24.14% (14/58) cases. Thirty-Nine out of fifty-eight patients (67.24 %) had a history of smoking or tobacco whereas 32.76 % (19/58) presented to have negative history of smoking or tobacco chewing. Histo-pathologically, all 58 high stage tumors were classified as high grade tumors. Thirty MIBC patients (51.72%) were presented with recurrent tumor.

5.5 MORPHOLOGY AND URINE CYTOLOGY:

For Cytological assessment, the catheterised or voided urine sample is used commonly to assess the urinary tract. As long as the samples are collected and processed appropriately, it is a patient-friendly approach for examining both the upper and lower urinary tracts. While men can get by with voided specimens, catheterization is frequently recommended for women to prevent contaminating by menstrual blood and

vaginal cells. Furthermore, when bladder tumours are detected, symptomatic persons should have washings of the urine bladder obtained during cystoscopy.

The procedure provides excellent cytological preparations. The following diagnosis of urothelial tumours and aspects were assessed when evaluating the efficacy of urine cytology:

1. Low-grade papillary tumours surrounded by urothelium and exhibiting only mild morphological.

2. High-grade papillary tumours, sessile tumours, and carcinoma in situ with cytomorphologically aberrant urothelial cells.

3. Urothelial tumours are frequently synchronous or metachronous, and they might affect various system of the urinary tract.

Urinary cytology therefore proves particularly valuable for monitoring those who have previously been diagnosed with urothelial cancer. After voiding early morning on three consecutive days, samples of 50 to 100 ml were collected. If a delay in transportation to the laboratory was anticipated, the samples were collected in 50% ethanol. The first morning samples were ignored because cells degenerate The morphology of cells maintained overnight in bladder urine appeared severely degraded when exposed to acidic urine.[208]

5.5.1 Fixation, Fixatives and Smear preparation:

To guarantee the preservation of cytomorphological characteristics, every material for cytological testing was appropriately fixed. The samples were placed in fifteen millilitre centrifuge tubes and centrifuged for 10 minutes at 1500 g. After centrifugation, the supernatant was decanted, and smears were made from the remains with a glass pipette. Smears was wet-fixed in 95% ethanol (or air-dried in some cases). After that, the smears were stained with either the Papanicolaou (Pap) or the

haematoxylin and eosin stains. Exfoliative cytology smears can sometimes be air-dried and correctly coloured..

We solely used 95% ethanol for fixing. Smears made in the laboratory using urine samples instantly submerged in 95% ethanol and fixed before being allowed to dry. Aeration produced cell deformation and cytoplasmic staining abnormalities. At room temperature, the fixation time was 10 to 15 minutes. [209]

FDA APPROVED BIOMARKERS [210]

5.6 Nuclear Matrix Protein (NMP-22) ELISA Kit:

5.6.1 PRINCIPLE:

This assay uses the competitive inhibition enzyme immunoassay technique. This package comprises an antigen-coated microtiter plate. The necessary microtiter plate wells are filled with antibodies specific for NMP-22 and antibody conjugated with Horseradish Peroxidase (HRP). The colour development is observed after adding a substrate solution into the mixture. The colour development halted using a stop solution, and the colour intensity was measured.

Detection range of the assay: 0.15 - 40 ng/ml.

Sensitivity of the assay:

Human NMP-22 has a minimum detectable dosage of less than 0.15ng/ml. The assay's sensitivity, or Lower Limit of Detection (LLD), was estimated as the smallest human NMP-22 concentration which might be distinguished from zero.

Specificity of the assay:

The test is very sensitive and specific for detecting human NMP-22. Human NMP-22 and its analogues exhibited no significant cross-reactivity or interference.

5.6.2 COLLECTING AND STORING THE SAMPLES:

Urine samples were collected in a sterile container and centrifuged for 15 minutes at 1000xg, 2 - 8°C, before being analysed or aliquoted and kept at -20°C or -80°C. A number of freeze-thaw cycles were avoided.

5.6.3 PREPARATION OF THE REAGENTS:

Each of the reagents were permitted to cool down to room temperature (18-25°C) for 30 minutes preceding use, and each experiment began with a new standard. The test samples were to be used within 4 hours, and then discarded. Direct serial dilution in wells was not permitted. Antibody (1x) vial was centrifuged before opening. A 100-fold dilution of the antibody was required were 10 µl of Antibody + 990 µl of Antibody was used. Centrifuge before opening the vial of HRP-conjugate (1x). HRP-conjugate must be diluted 100 times. 20 ml of wash buffer concentration (25x) was dissolved in deionized or distilled water to generate 500 ml of Wash Buffer (1x). Before opening, the standard solution was centrifuged for 30 seconds at 6000-10000rpm. Prior to dilutions, the stock standard solution (40 ng/ml) was maintained at room temperature for 30 minutes to warm up. 50 ml of sample diluent was pipetted into each S0-S4 tube, and a 4-fold dilution series with stock solution was performed. The undiluted Standard acted as high standard and the zero standards were sample diluents.

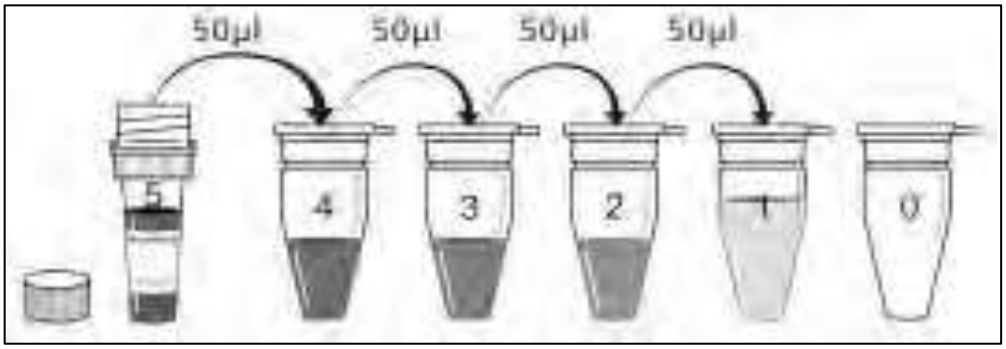

Figure 30: Different Standards Concentration for NMP-22.

Table 5: Different Standards Concentration for NMP-22.

Standards	S5	S4	S3	S2	S1	S0
Concentrations	40	10	2.5	0.6	0.1	0

5.6.4 PROCEDURE FOR ASSAY:

Reagents or samples were brought to room temperature. Once the material was thawed, centrifuge it again before the test. It was strongly recommended that all samples and standards be evaluated twice.

1. All reagents, standards, and tests were produced as indicated in the following sections.

2. The number of wells that would be utilised was decided.

3. Each well received 50 µl of standard and sample, as well as 50 µl of Antibody (1x). The pipette was used to thoroughly mix it for 1 minute.

4. Incubated for 30 minutes at 37°C.

5. After aspirating each well, the washing process was done two more times for a total of four washing. Each well was filled with the wash buffer and left for 2 minutes; thorough liquid drainage at each phase was necessary for excellent performance.

6. 100 µl HRP-conjugate (1x) to each well was added immediately (but not to the blank well). Adhesive strip was used to cover and incubated for 30 minutes at 37°C.

7. Step 6's aspiration/washing procedure was repeated for five times more.

8. Microplate reader was set to 450 nm and was determined by optical density of each well in 5 minutes.

5.6.5 CALCULATION OF RESULTS:

It is suggested that you use the professional soft "Curve Expert" to design a standard curve, which may be obtained from the maker's website. For each standard and sample, the average optical density of Blank was subtracted from the average optical density of duplicate readings. The data was reduced using computer algorithms capable of constructing a four-parameter logistic curve-fit to establish a standard curve. Alternatively, graph the average absorbance for each standard on the x-axis versus the concentration on the y-axis to build the appropriate curve covering the points on the graph. Using regression analysis, the best fit line was determined by plotting the log of the NMP-22 concentrations vs the log of the O.D.

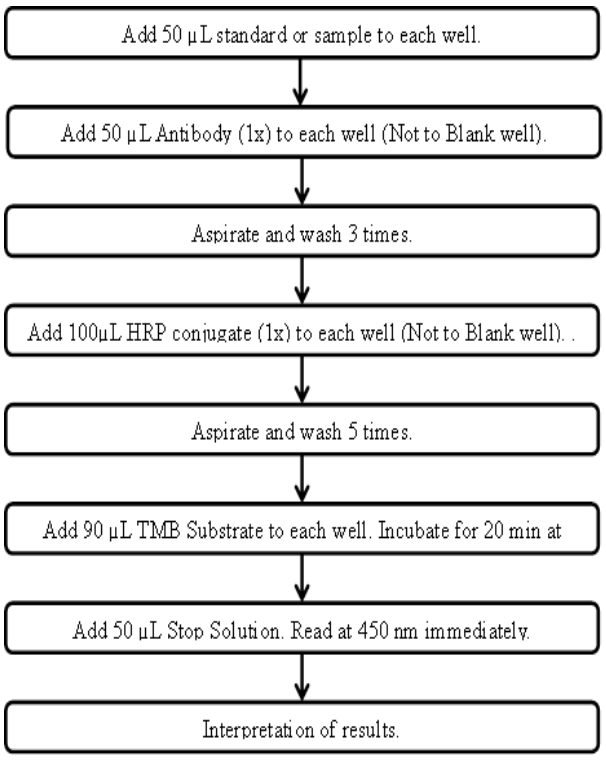

Figure 31 : NMP-22 PROTOCOL SUMMARY.

5.7 Human BTA (Bladder Tumor Antigen) ELISA Kit [211]

5.7.1 INTENDED APPLICATION:

This ELISA kit is used to determine the quantities of Human BTA in serum, plasma, and other biological fluids in vitro.

● The **Sensitivity-** 0.19 ng/ml, Detection Range is in between 0.3 -20ng/mL

● The **Specificity** of Human BTA in samples is identified by this kit. There was no substantial cross-reactivity or interference between Human BTA and analogues.

5.7.2 PRINCIPLE OF THE ASSAY:

This ELISA kit employs the Sandwich-ELISA approach. This kit contains a micro ELISA plate already applied using an antibody designed for Human BTA. In the micro ELISA plate wells, standards or samples are combined with the appropriate antibody. The microplate wells are then incubated using a biotinylated detection antibody specific for Human BTA comprising an Avidin-Horseradish Peroxidase (HRP) combination. Unwanted substances are rinsed away. Each well receives the substrate solution. The only blue wells contain human BTA, biotinylated detection antibody, as well as Avidin-HRP conjugate. The hue changes to yellow when a stop solution is added to the enzyme-substrate process. Optical density (OD) is determined spectrophotometrically at 450 nm and 2 nm wavelengths.

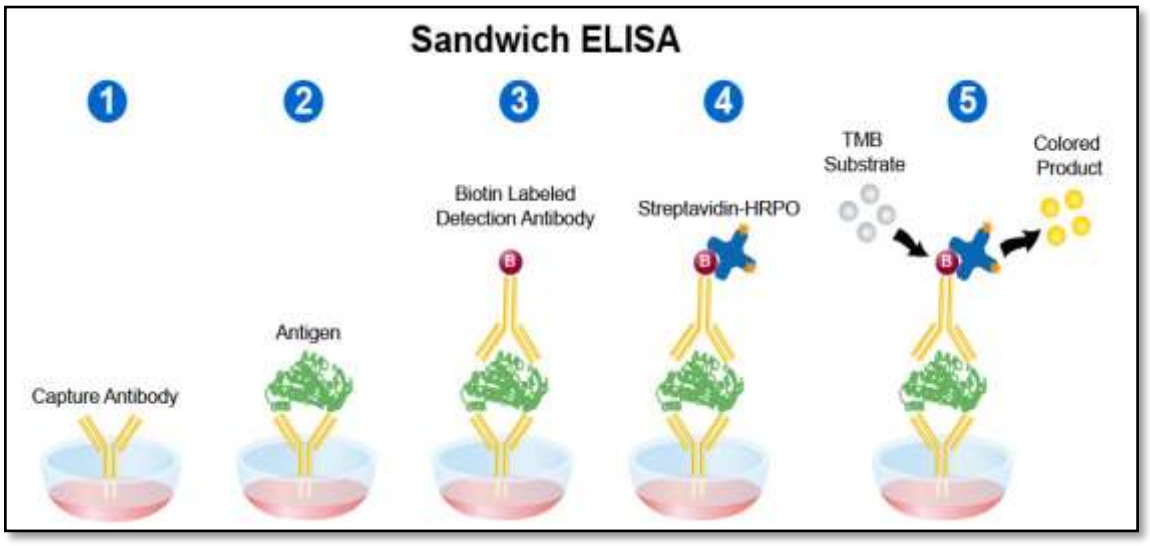

Figure 32: Principle behind BTA TRAK-Sandwich ELISA Method.

5.7.3 Storage & Kit:

Unopened kits can be stored at 4°C approximately one month. If the kit isn't used within a month, store the parts individually in accordance with the instructions below.

5.7.4 SAMPLE COLLECTION:

Urine:

Urine samples were gathered in a sterile container and centrifuged for 15 minutes at 1000xg, 2 - 8°C, before being analysed right away or alliquoted and kept at -20°C or -80°C. A number of freeze-thaw cycles were avoided. Finally, before assaying, one more cycle of centrifugation was done to remove any further forms precipitates that may have accumulated during storage.

5.7.5 REAGENT PREPARATION:

Before applying any reagents, they were warmed to room temperature (18-25°C). 30 mL of Concentrated Wash Buffer was combined mixed 720 mL of deionized or distilled water to generate 750 mL of Wash Buffer.

5.7.6 STANDARD OPERATING PROCEDURE:

The standard was centrifuged at 10,000g for 1 minute before allowing 1.0 mL of Reference Standard and Sample Diluent to rest for 10 minutes before gently turning it several times. A pipette was used to thoroughly mix and dissolve the solution. At a concentration of 20ng/mL, this reconstitution generated a useable solution. Make more dilutions as needed. The dilution gradient was 20, 10, 2.5, 1.25, 0.63, 0.31, and 0 ng/mL. Dilution method: 500 µL of Reference Standard and Sample Diluent were poured into 7 EP tubes. A 500 µL pipette of the 20ng/mL working solution was pipetted into the first tube and combined to make a 10ng/mL working solution. Using this method, 500 µL of solution was pipetted from the initial tube into the second one.

Working solution for Biotinylated Detection Ab:

The needed amount was calculated (100 µL/well) before beginning the experiment. It was preferable to prepare somewhat more than is calculated. Before using, the stock tube was centrifuged, and the 100 Concentrated Biotinylated Detection Ab was diluted with Biotinylated Detection Ab Diluent to make a practical solution.

HRP Conjugate Concentrated Working Solution: To guarantee precise results for the experiment, the kit suggested calculating the necessary amount beforehand at 100 µL/well and prepare some extra solution as a precautionary measure. Concentrated HRP Conjugate Diluent to dilute 100 Concentrated HRP Conjugate into one working solution was used. It was important to note that the last tube acted as your blank and should not have any solutions pipetted from previous tubes.

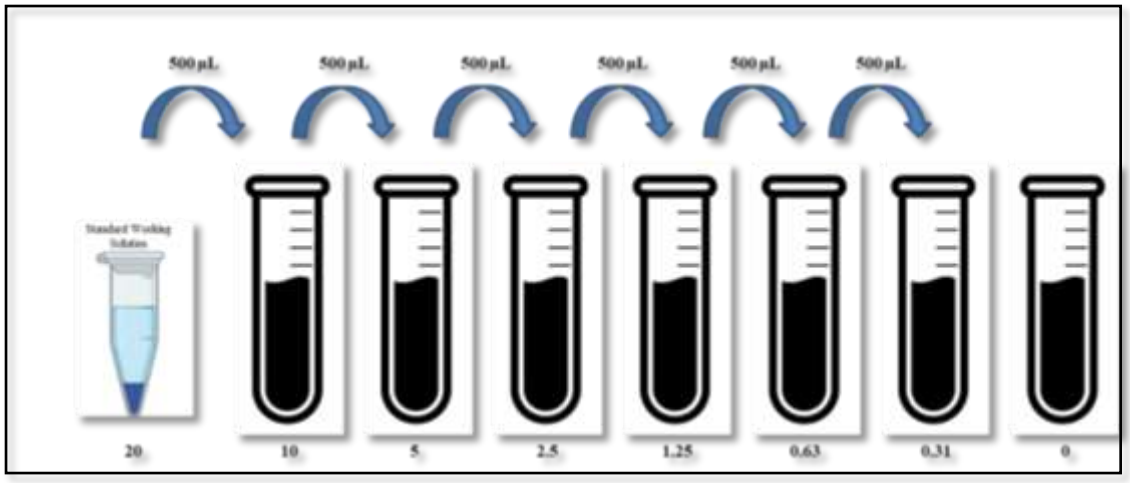

Figure 33: Different Standards Concentration for BTA TRAK.

Table 6: Different Standards Concentration for BTA TRAK.

Concentration (ng/mL)	**20**	**10**	**5**	**2.5**	**1.25**	**0.63**	**0.31**	**0**
Optical Density (OD)	2. 264	1. 44	0. 861	0. 479	0. 238	0. 182	0. 138	0. 092

5.7.7 PROCEDURE:

To guarantee our research results are conclusive and accurate in this instance- We recommended adding duplicate solution concentrations side by side in separate wells; with no more than an allowance of exactly lOO µL per cell. Followed by allocation of respective samples (amounting each also exactly lOO µL), into neighbouring vacant ones. The covering sealer contained within your received package must also subsequently be applied without exception over the entirety of micro ELISA plate wells before carefully placing it within an incubator set at precisely established temperature settings - (37°C) securely for no less than ninety minutes duration. Be cautious when introducing initial solutions and ensuring they are deposited calmly without making contact or creating bubbles on the micro ELISA well wall.

To guarantee exact results in the experiment, all leftover liquid present in all wells was removed without first cleaning them. Following that, proceeded by carefully pouring exactly 100 µL of Biotinylated Detection Ab working solution into each individual well at once and securely covering with sealing material provided with plate after gently but thoroughly mixing towards the centre region to avoid any edge effects observed during processing as per standard regulations guided by protocol guidelines. It was allowed for incubation at a steady and regulated temperature of 37°C for duration of 60 either collecting or separating the mixture from each well.

Then, simply approximately 350 µL was added of an appropriate wash buffer solution to every well and waited patiently for around 12 minutes before draining off any residue from individual wells using proper pipetting technique before patting them dry thoroughly with absorbent paper.

Each well received 100 L of HRP Conjugate working solution before being sealed with the Plate sealer. Finally, at 37°C for 30 minutes.

Each well received 90 µL of Substrate Reagent and was sealed with a new plate sealer. At 37°C, incubated for around 15 minutes. The plate was shielded from the light.

Each well received 50 µL of Stop Solution.

The optical density (OD value) of each well was assessed simultaneously using a microplate reader set to 450 nm.

5.7.8 CALCULATION:

The average optical density of the zero standards was calculated by averaging the repeated measurements for each standard and sample. A four-parameter logistic curve was built on log-log graph paper, using standard concentration on the x-axis and OD values on the y-axis. If the samples were diluted, multiply the typical curve concentration by the dilution factor. The samples were retested with an appropriate dilution if its OD exceeds the top limit of the standard curve. The real concentration was obtained by multiplying the calculated concentration by the dilution factor.

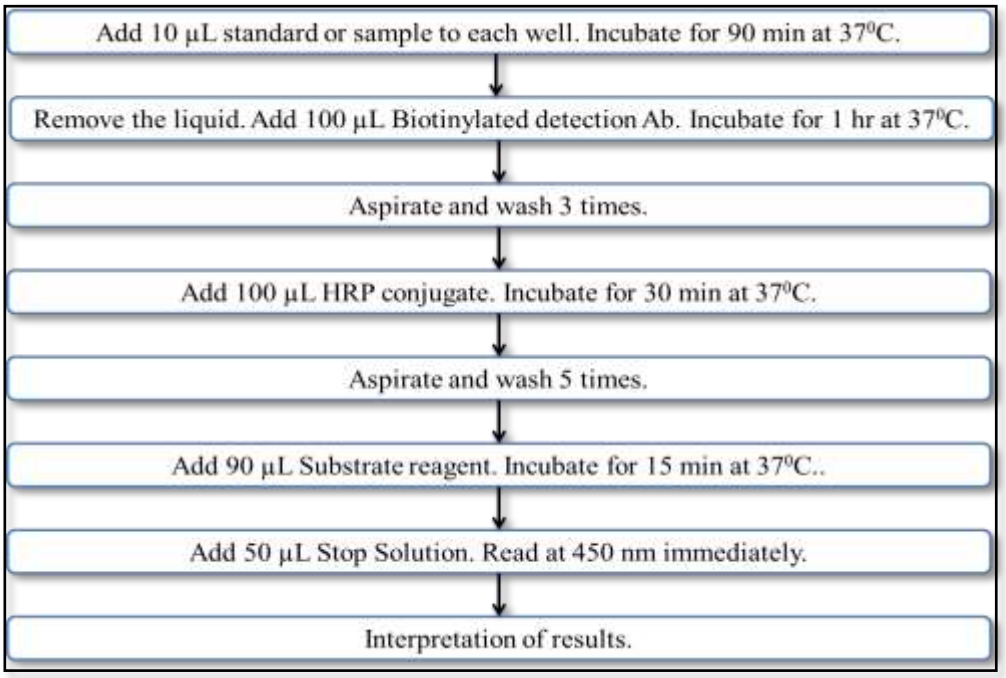

Figure 34: BTA TRAK PROTOCOL SUMMARY.

5.8 5-AMINOLEVULINIC ACID FLUORESCENT CYTOLOGY:

5-ALA (Figure 35) is a whole separate element of Photodynamic Technique. PpIX is not photosensitive in and of itself, but it is produced in situ when there is an excess of 5-ALA. [212]

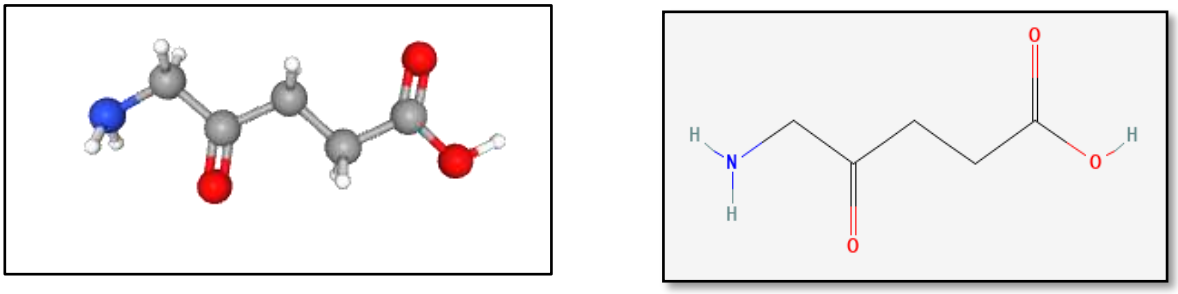

Figure 35: Zwitterionic form of 5-Aminolevulinic acid, with the carbon atoms numbered.

5.8.1 METABOLISM OF 5-AMINOLEVULINIC ACID:

5-ALA (amino-4-oxopentanoic acid) constitutes a delta amino acid with a carbonyl group on carbon four. 5-ALA can be present in a wide range of species. 5-ALA is derived from glutamate or succinyl-Coenzyme A (sCoA) and glycine. Plants, algae, and cyanobacteria, as well as the vast majority of bacteria and archaea, use the multistep C5 pathway to create 5-ALA, whereas humans, mammals, yeasts, and a few bacteria use the one-step C4 pathway.[213] The C4 pathway is linked to the tricarboxylic acid cycle (TCA cycle) in eukaryotes via sCoA.The mitochondrial enzyme aminolevulinic acid synthase uses -sCoA and glycine as substrates. ALAS is a homodimer with a subunit interface active site which is symmetrical linked by dual pyridoxal 5'-phosphate cofactors. ALAS catalyses the decarboxylative synthesis of glycine and sCoA, with the rate-determining step being the release of 5- ALA. The first enzyme responsible for heme synthesis is ALAS. [214,215]

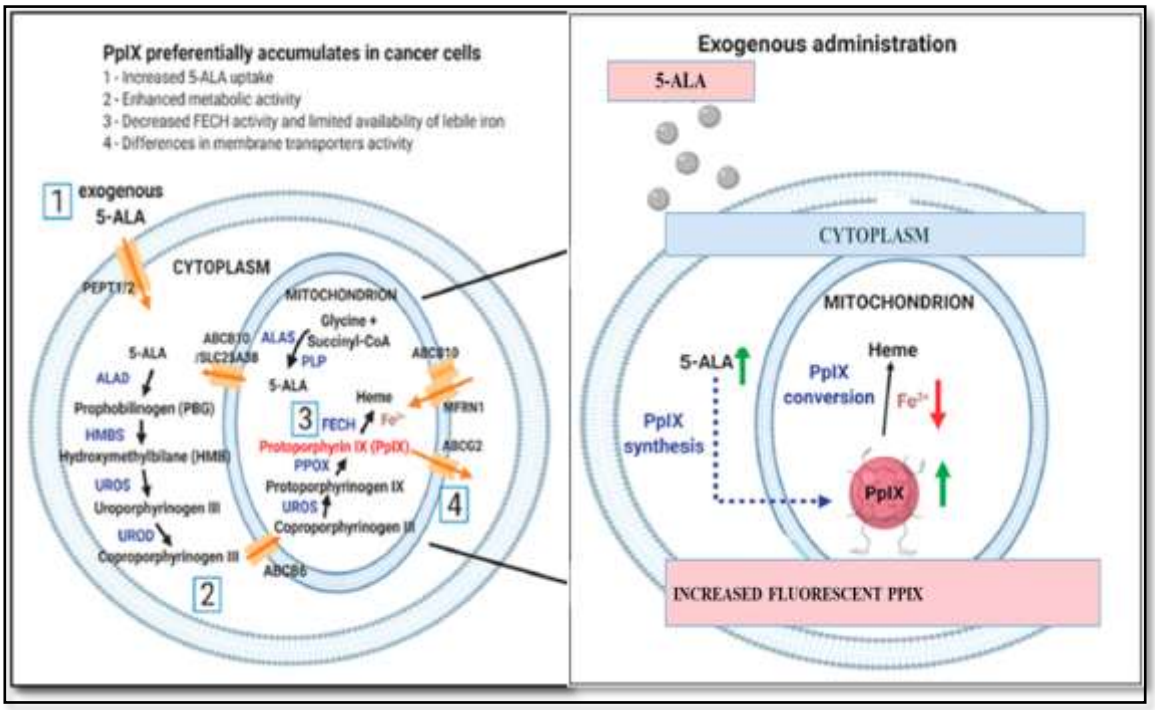

Figure 36: A simplified diagram showing heme production, which occurs in both the cytoplasm and the mitochondria.

Porphobilinogen synthase, porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen III decarboxylase, coproporphyrinogen III oxidase, protoporphyrinogen IX oxidase, and ferrochelatase are other enzymes involved in heme synthesis. The latter three are found in mitochondria, whereas the others are found in the cytoplasm. (Figure 36).[216]

5.8.2 Porphobilinogen synthase (PBGS):

The subsequent enzyme in the heme pathway is phosphobilinogen synthase, commonly known as 5-ALA dehydratase. The pyrrole porphobilinogen (PBG) is formed by the union of two 5-ALA molecules. Unlike ALAS, which is present in the mitochondria, PBGS can be discovered in the cytoplasm. PBGS is a metalloenzyme that is most active when it is homooctameric. A hexamer structure can be produced in human PBGS by a spontaneous single mutation of phenylalanine to leucine (F12L),

albeit with substantially lesser activity.[217-225]

The active region of PBGS is currently revealed to be highly conserved across several species. All PBGS residues are now identified by yeast numbering. The A- and P-sites of the active site, in particular, include two lysine residues, Lys210 and Lys263. After their respective locations, the acid groups (acetyl- and propionyl-) of the product PBG generated from the carboxylate moiety of the 5-ALA substrates are named. According to mutagenesis tests, the latter lysine is essential for enzyme catalysis, while the first must be present for initial substrate binding.[226-234]

Furthermore, the active site has a high number of polar groups that establish hydrogen bonds with the carboxylate moiety of the 5-ALAs via the P- (Ser292 and Tyr328) and A-site (Gln238). Several residues (Ser178, Asp132, and Tyr 208) form a polar area with or hydrogen link to the 5-ALA substrates' terminal amino group. Substrate analogues missing the terminal amino group, on the other hand, have been shown to be effective competitive inhibitors, at least for the P-site. As a result, it is claimed that interactions between the 5-ALA amino group and the enzyme, at least for binding, are not necessary. When the 5-ALAs bind, a flexible part of PBGS is thought to seal the active site33. [235-247]

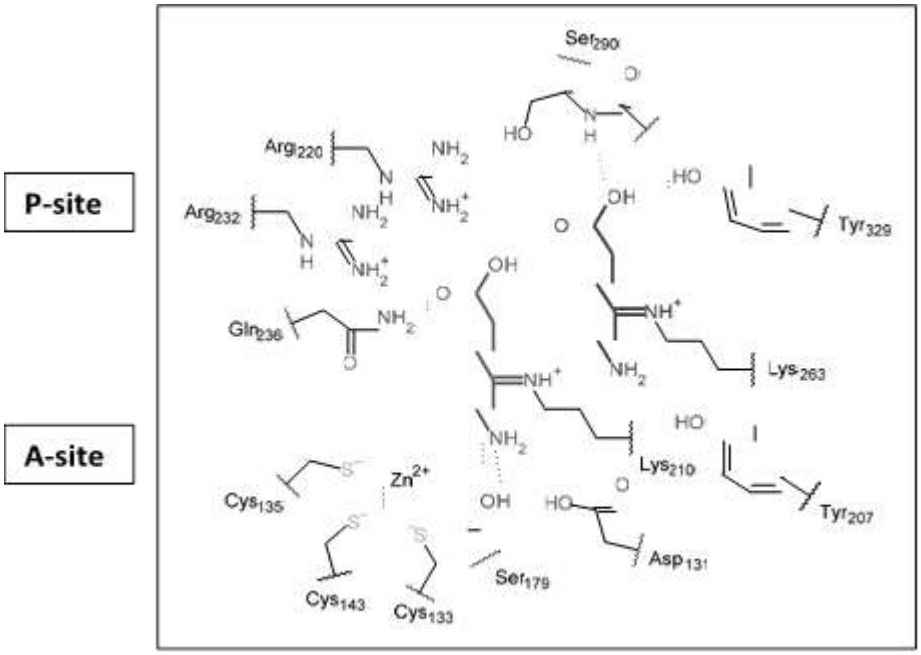

Figure 37: Based on yeast PBGS crystal structures, a schematic representation of the active area of PBGS is presented, with the two 5-ALA substrate molecules chemically connected at the A- and P-sites through Schiff-base linkages. 1H7O34 and 1OHL35 are PDB IDs.

5.8.3 Uroporphyrinogen III decarboxylase:

Uroporphyrinogen III (URO-III) is the first cyclic molecule formed during the heme production process. Uroporphyrinogen III decarboxylase catalyses the decarboxylation of URO-III's acetyl chains to yield coproporphyrinogen III (CP-III), where P symbolises the propionate side chains. The enzyme begins decarboxylation in the acetyl chain of ring D at physiological substrate concentrations, followed by the acetyl chains that make up the A, B, and C rings; however, at greater concentrations, the sequence is random.[248,249]

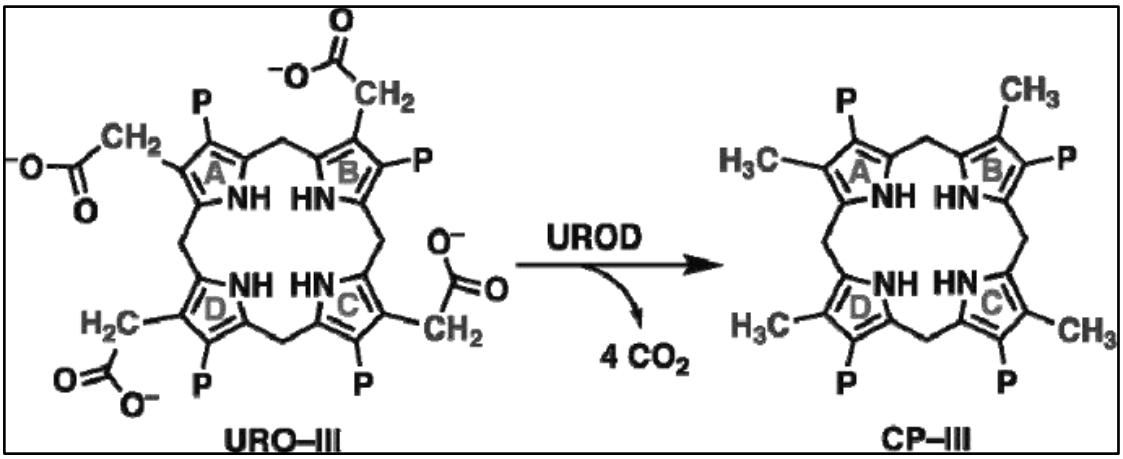

Figure 38: Schematic illustration of the active site of PBGS with the two 5-ALA substrate molecules covalently bound at the A- and P-site via Schiff- base linkages based on the yeast PBGS crystal structures PDB ID: 1H7O34 and 1OHL35

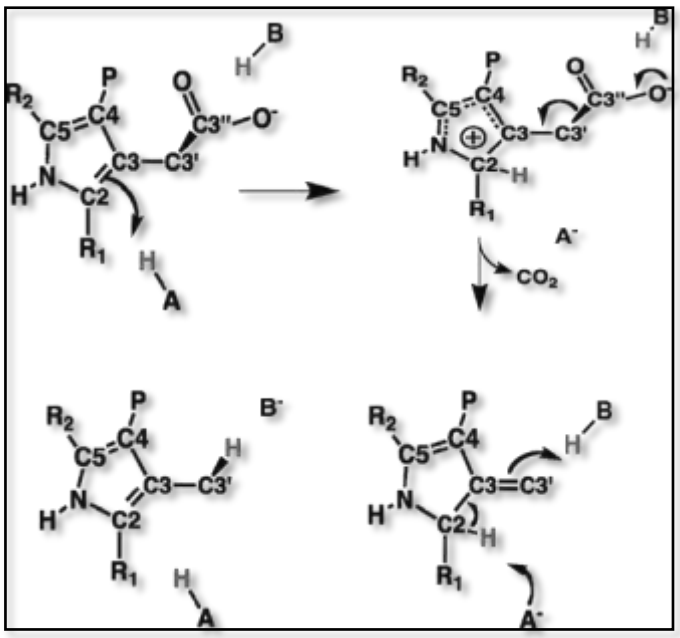

Figure 39: The acid/base mechanism for pyrrole acetate decarboxylation in URO-III is postulated. The general acids are represented by HA and HB.56-58

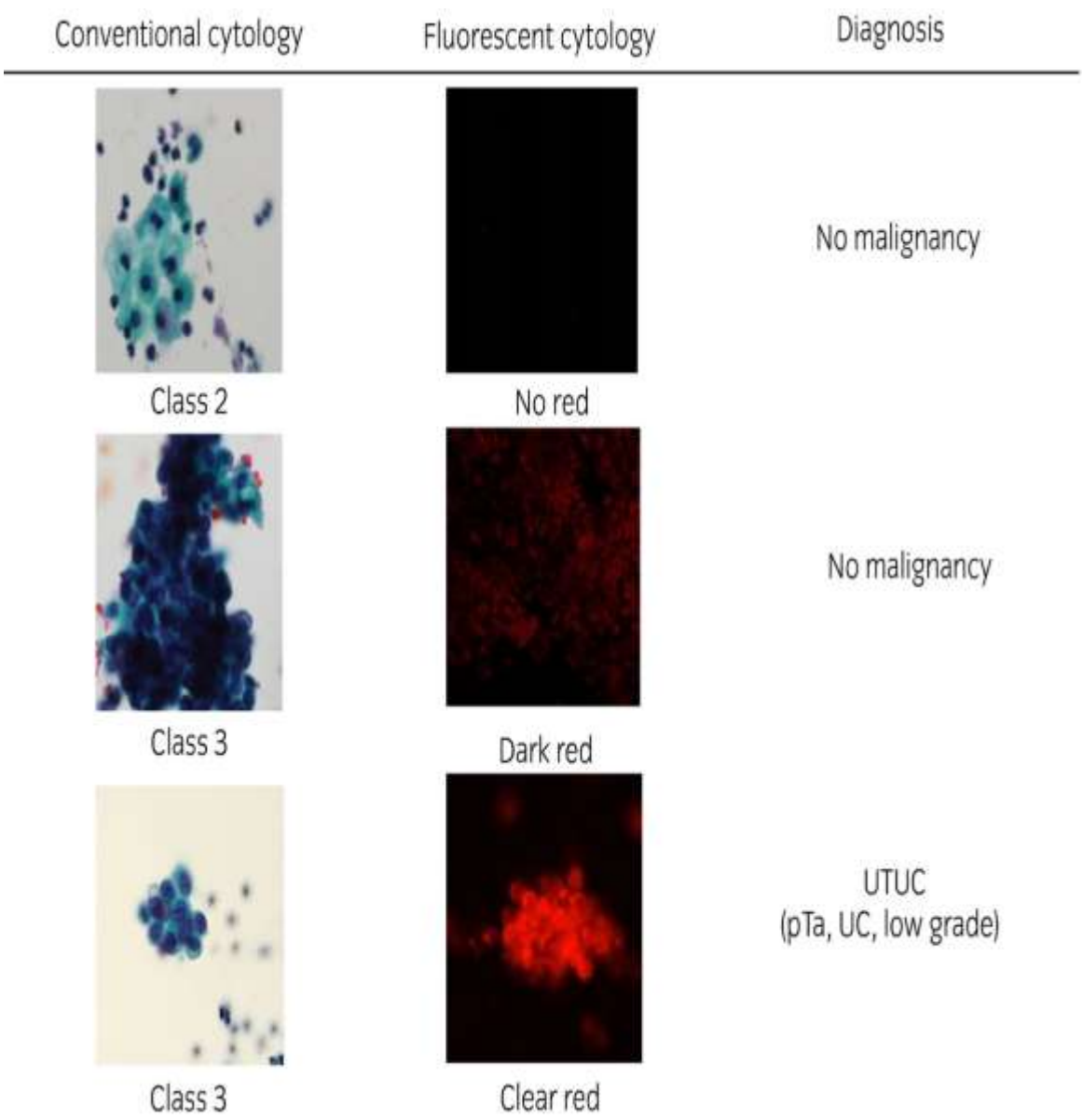

Figure 40: Images for conventional cytology and fluorescence cytology produced by 5-ALA.

The same examiners assessed upper tract urine cytology. In conventional urine cytology, we determined that classes 1, 2, and 3 urine cytology were negative while classes 4 and 5 urine cytology were positive. Similarly, in 5-ALA-induced fluorescence selective upper tract urinary cytology, a urine sample with no red or dark red was declared negative, while a urine sample with clear red was considered positive. (Fig. 40).

Finally, based on "The Paris system for reporting urinary cytology," two expert pathologists reached the final judgement after analysing the same urine sample for both conventional cytology and 5-ALA-induced fluorescence cytology. For malignant cells, conventional urine cytology was either positive or negative. The presence of little or dark red light in 5-ALA-induced fluorescence cytology was classed as negative, while clear red light was defined as positive. We conducted this study in accordance with the Helsinki Declaration's Ethics Principles of 2013, and the ethics committee of J.N. Medical College, Belagavi approved it.

RESULTS

6.1 THE OVERALL CHARACTERISTICS OF THE STUDY SUBJECTS:

In all, 422 total samples were analysed in the current investigation. The Department of Urology provided 122 urine samples for controls, 150 for lower urinary tract symptoms (LUTS), and 150 for histological Urothelial bladder tumours (UBC). The average age of UBC patients was 65.28 ± 17.63 (mean SE), 64.12 ± 18.54 for LUTS participants, and 66.39 ± 17.85 for controls. The UBC patients ranged in age from 31 to 89 years, whereas the control participants ranged from 24-83 years. Men made up 72% of the 150 UBC patients, while women made up 28%. The male to female ratio was 2.5:1 with 108 men and 42 women. Males had a mean age of 66.47 ±15.74, while females had a mean age of 61.77 ± 18.9.

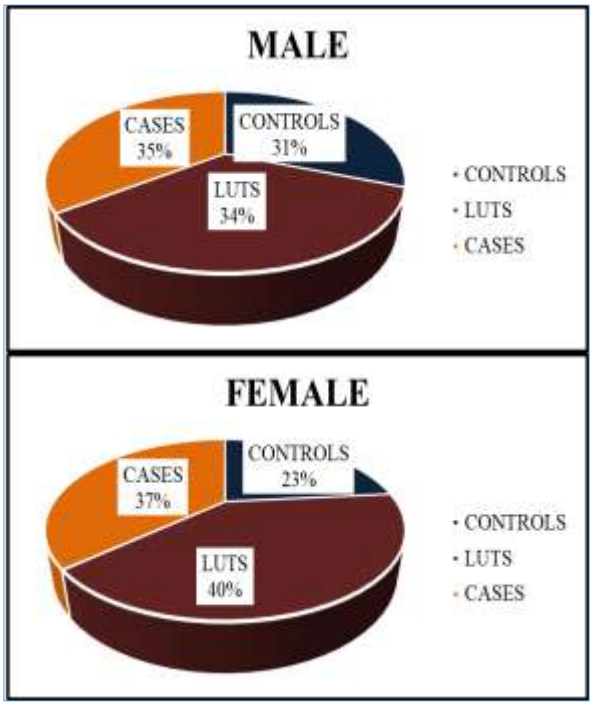

Figure 41: The gender of the enrolled UBC patients, Controls, and LUTS cases in the study (n=422) is shown via a pie diagram.

Farmers made up the majority of UBC patients, accounting for 25.34% (38/150). Businessmen made up the smallest proportion of those present (8%) (12/150) and white coloured jobs 8% (12/150) like teachers, healthcare workers, computer operators and lawyers. Approximately 85.71% (36/42) of the females were housemakers. The trends for control and LUTS groups were rather different and constituted more numbers in business, white-coloured jobs and Homemaking categories. (Table 7)

Table 7: Occupational representation of all the groups of subjects.

Occupation	CONTROLS (n=122)	%	LUTS (n=150)	%	CASES (n=150)	%
	n	%	n	%	n	%
Farming	21	17.213	23	15.333	38	25.333
Menial worker	12	9.836	14	9.333	18	12.000
Industry worker	10	8.197	12	8.000	34	22.667
White-collared job	31	25.410	29	19.333	12	8.000
Business	21	17.213	31	20.667	12	8.000
Homemaker	27	22.131	41	27.333	36	24.000

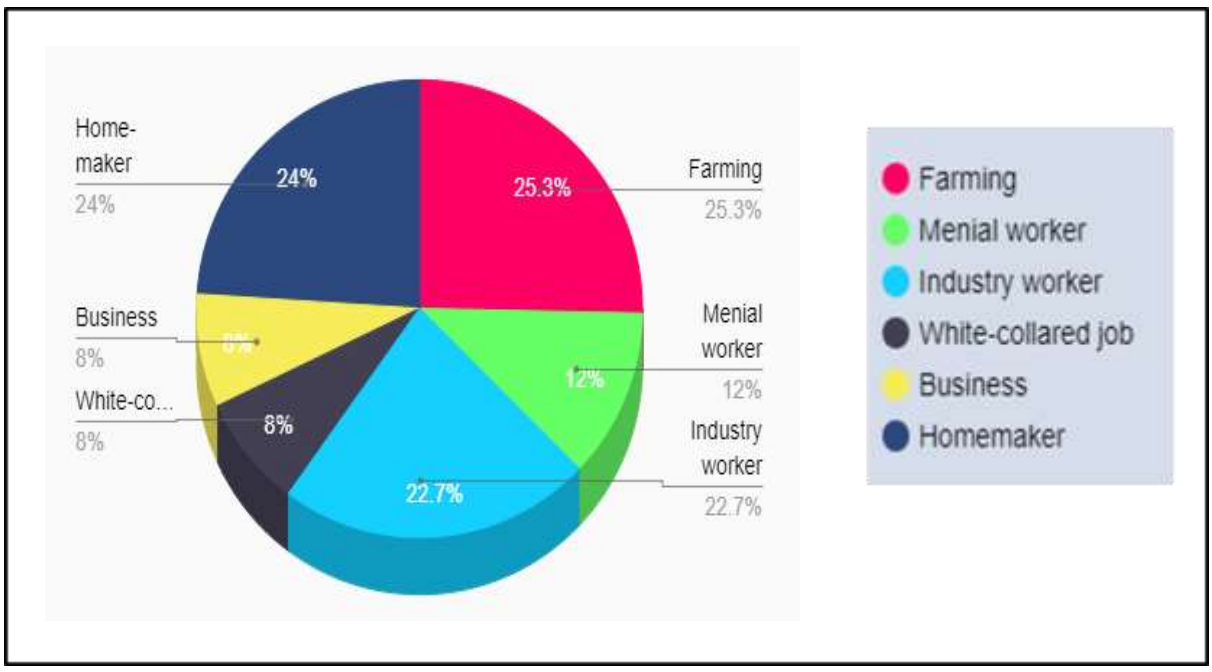

Figure 42: The occupations of the enrolled UBC patients in the study are shown by a pie diagram. (n=150).

Comorbidities were observed in 96.7% of the patients (145/150). Approximately 3.34% (5/150) of the patients had no other illness except UBC. Patients with hypertension made up 21.34% (32/150), whereas those with type 2 diabetes made up 34% (51/150). As shown in table 2, some patients had both type 2 diabetes mellitus and hypertension. The proportion was 11.34% (17/150). Arrhythmia, congestive heart failure, stroke, non-alcoholic fatty liver disease, and cholelithiasis were the least common comorbidities. The data patterns for the other two categories were mostly identical.

Table 8: Comorbidities prevalent in the enrolled study.

VARIABLES	CONTROLS (n=122)	%	LUTS (n=150)	%	CASES (n=150)	%
Hypertension	41	33.607	41	27.333	32	21.333
CVD	22	18.033	32	21.333	31	20.667
COPD	7	5.738	8	5.333	11	7.333
Diabetes	38	31.148	45	30.000	51	34.000
Dyslipidemia	2	1.639	3	2.000	8	5.333
Mixed	12	9.836	21	14.000	17	11.333

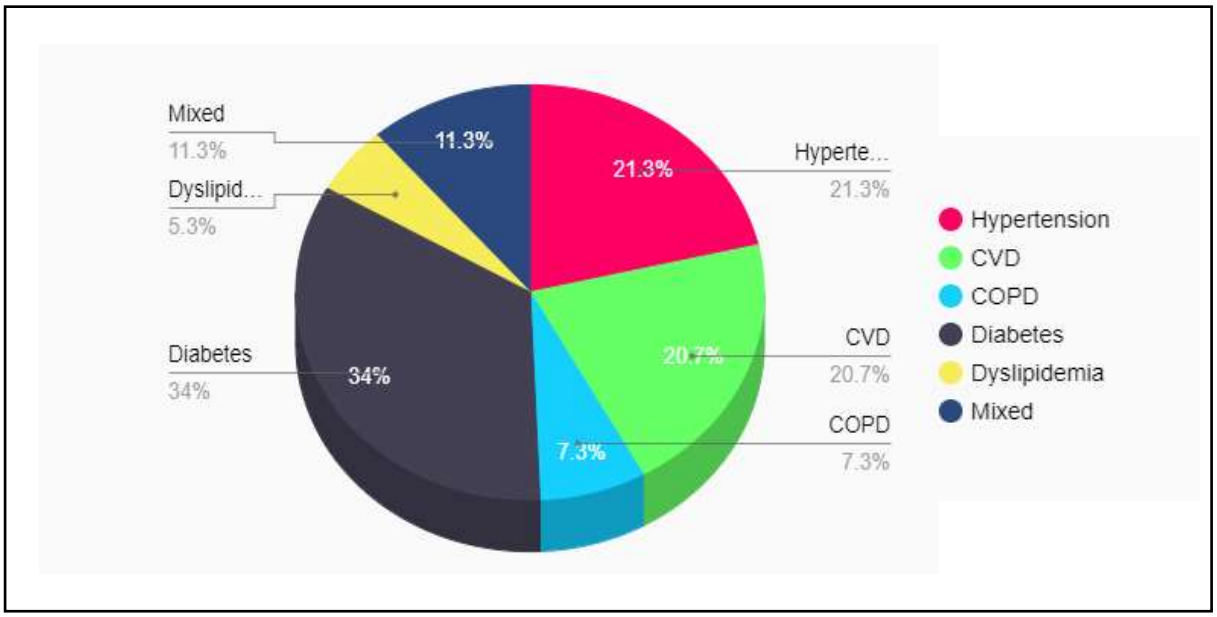

Figure 43: Comorbidities common among enrolled urinary bladder cancer patients are depicted in a pie diagram.

According to the urological history, 28.69 (35/150) of the patients had hematuria and blood clots, as shown in Fig. 4.4. Hematuria, frequency, and urgency were prevalent symptoms in 54.10% (66/150) of patients with mixed symptoms. Urolithiasis, renal calculi and BPH cases had fewer percentage of patient's presentation (2.6%; 4/150) (Table 8). All 35 individuals who had clots in their urine also had lower urinary tract symptoms as well as hematuria. Hematuria was also present in the individuals who had a urinary tract infection. For LUTS group, the highest cases were for hematuria and pain while urination (21.31%, 26/150) followed by BPH and pain during urination and frequent urination respectively (18.03%, 17.21 & 11.48%). For controls groups no significant numbers were observed and 71.31% (87/122) subject showed no symptoms.

Table 9: Signs and Symptoms in various groups.

VARIABLES	**CONTROLS (n=122)**	**%**	**LUTS (n=150)**	**%**	**CASES (n=150)**	**%**
	n	%	n	%	n	%
Blood clots / hematuria	0	0.00	26	21.31	35	28.69
Pain/ Burning sensation during urine	2	1.64	21	17.21	16	13.11
Frequent urination	1	0.82	14	11.48	14	11.48
Nocturia	1	0.82	12	9.84	2	1.64
lower back pain	2	1.64	15	12.30	11	9.02
BPH	2	1.64	22	18.03	1	0.82
Urolithiasis	2	1.64	15	12.30	2	1.64
Renal Calculi	10	8.20	4	3.28	1	0.82
Mixed	15	12.30	21	17.21	66	54.10
NONE	87	71.31	0	0.00	2	1.64

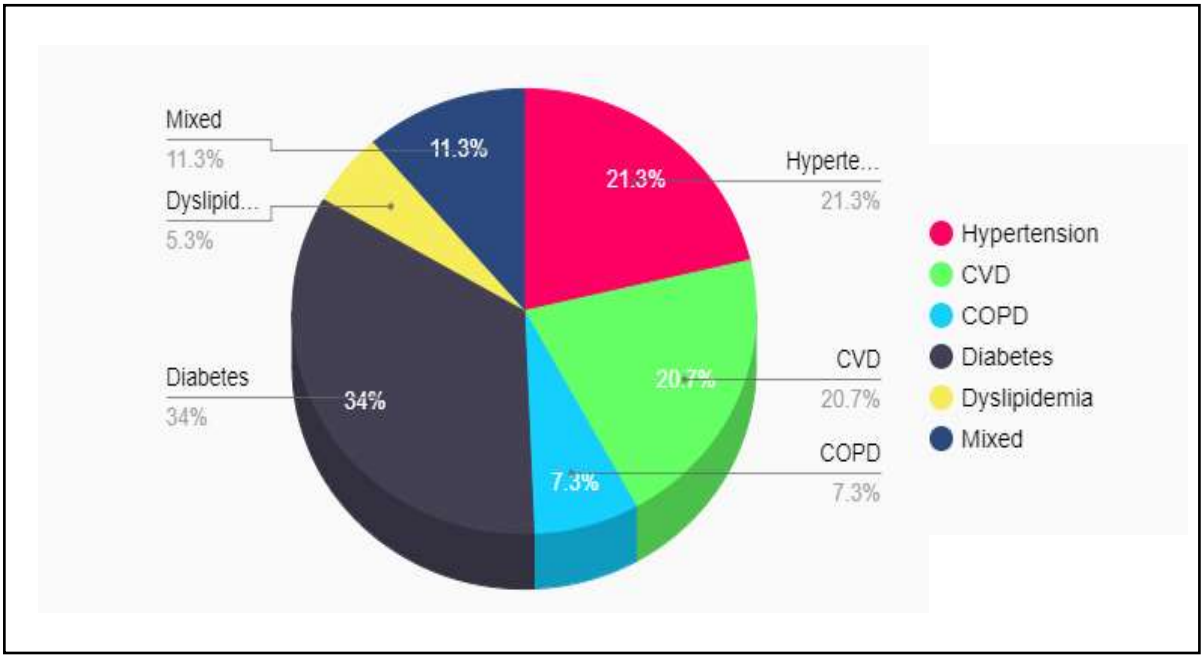

Figure 44: Signs and symptoms in urinary bladder cancer patients were shown as a pie chart.

Continuous data is shown in mean SE in Table 4, whereas categorical/discrete data is depicted in counts with their respective percentage (%). The biochemical parameters serum urea and serum creatinine were significantly associated with the enrolled UBC cancer patients, LUTS cases, and controls ($p < 0.0001^*$; $\chi 2$ test). Similarly, demographic characteristics such as BMI differed significantly between patients and controls ($p < 0.0001^*$; $\chi 2$ test). Clinical criteria such as hematuria showed no statistically significant correlation. ($p < 0.3$). (Table 10)

Table 10: General characteristics of UBC cases, LUTS and Controls.

Factors		CONTROLS (n=122)	LUTS (n=150)	CASES (n=150)	p-value
Age groups					
15-40		9 (7.37%)	12 (8%)	11 (7.33%)	0.4229
41-59		25 (20.49%)	33 (22%)	34 (22.67%)	
60+		88 (72.13%)	105 (70%)	105 (70%)	
Age (Mean ± Std.Dev)		66.39 ± 17.85	64.12 ± 18.54	65.28 ± 17.63	0.9856
Hematuria	**No**	122 (100%)	95 (63.4%)	65 (43.34%)	0.3
	Microscopic	0 (0%)	21 (14%)	40 (26.67%)	
	Gross	0 (0%)	34 (22.67%)	45 (30%)	
Occupation related activity	**Sedentary**	54 (44.26%)	71 (47.34%)	60 (40%)	0.2
	Physical activity	68 (55.73%)	79 (52.67%)	90 (60%)	
Dietary pattern	**Vegetarian**	78 (63.93%)	98 (65.34%)	78 (52%)	**0.019***
	Non-vegetarian	44 (36.06%)	52 (34.66%)	72 (48%)	
Drinking water	**Tap water**	67 (54.91%)	78 (52%)	85 (56.67%)	0.417
	Ground water	55 (45.08%	72 (48%)	65 (43.33%)	
BMI (kg/m2)		26.45 ± 0.5	27.4 ± 0.2	26.6 ± 0.6	**0.0001***
Serum urea (mg/dL)		24.8 ± 1.2	25.6 ± 1.5	34.7 ± 1.4	**0.0001***
Serum Creatinine (mg/dL)		0.8 ± 0.05	0.44± 0.05	1.24 ± 0.07	**0.001***

As noted in many studies related to cancer epidemiology, diet plays a major role. In our study, highest cases had a regime of non-vegetarian food (48%). Cigarette smoking was more prevalent among UBC patients (64% vs. 38.96%). Current smokers' risk of UBC was elevated by one and half times (95% CI, 0.889-2.078) as compared to never smokers. There was more than twofold increase in intensity of cigarette intensity in subjects who smoked for more than 25 cigarettes per day. Furthermore, There was a significant increase in Odds ratio in association to duration in subjects smoking for more than 35 years, (OR= 2.727, 95% CI 0.816-9.116). Similarly results were observed for tobacco exposed cases which showed approximate 3.8 folds increase of risk (95% CI 2.060-6.603) in current cases compared to never exposed subjects. Tobacco duration, but not intensity,

was shown to be substantially linked with UBC risk in former participants. There was more than threefold increase of risk in patients which consumed tobacco for more than 35 years (95% CI 1.126-11.830). Furthermore, analyses for alcohol status showed three folds increase in current subjects (95% CI 1.165-6.394) and also a significant association with cases to controls (p=0.0330), and similar association was also observed for intensity and duration of subjects with history of daily and often consumption of alcohol also had a great risk for cancer. (p=0.034 and p=0.0115) respectively. (Table 11)

Table 11: Lifestyle routine habits of controls and cases.

Lifestyle parameters		**CONTROLS**	**CASES**	**ODDs RATIO**
SMOKING STATUS	**Never**	166 (61.02%)	45 (30%)	1
	Former	64 (23.52%)	48 (32%)	0.889
	Current	42 (15.44%)	57 (32%)	**1.576**
TOBACCO STATUS	**Never**	184 (67.64%)	41 (38%)	1
	Former	57 (20.95%)	44 (27.34)	1.39
	Current	31 (11.39%)	65 (29.33%)	**3.802**
ALCOHOL STATUS	**Never**	184 (67.64%)	45 (30%)	1
	Former	47 (17.27%)	35 (23.34%)	1.35
	Current	41 (15.074%)	70 (46.66%)	**3.095**

6.2 DIAGNOSTIC PERFORMANCE FOR FDA APPROVED BIOMARKERS:

Table 12: Diagnostic Performance of FDA-Approved Biomarkers.

DIAGNOSTIC PERFORMANCE OF FDA APPROVED BIOMARKERS	NMP-22	BTA-TRAK
MALIGNANT on histopathology (True positive)	**114**	**110**
BENIGN on histopathology (True Negative)	**258**	**260**

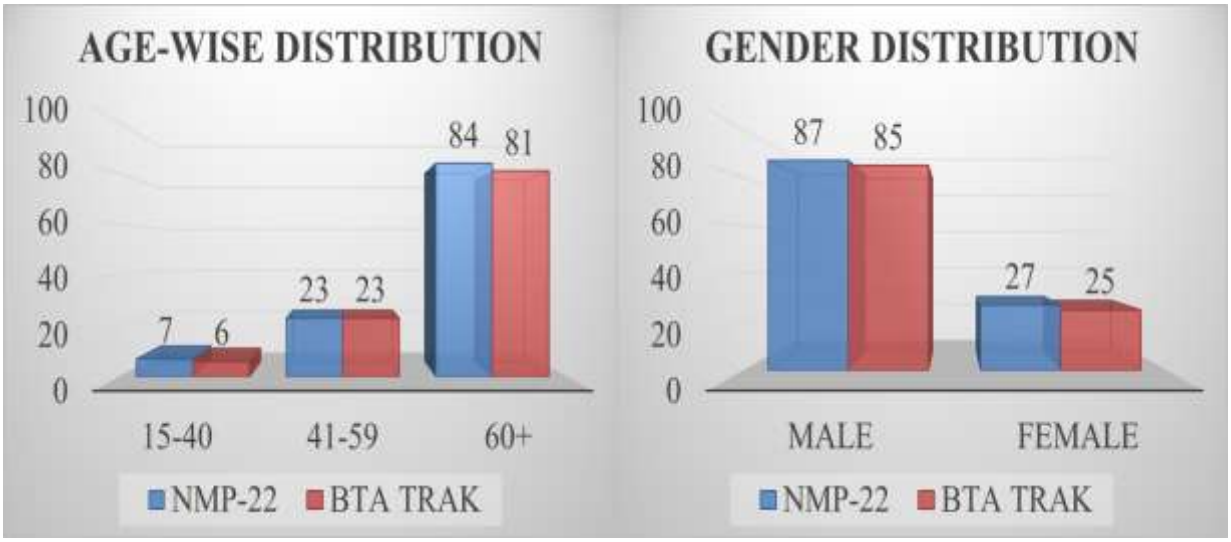

Figure 45: Age wise and Gender distribution for FDA Approved Biomarkers.

For age wise distribution, FDA Approved Biomarkers i.e. NMP-22 test and BTA TRAK both Showed higher amount for all the age groups. NMP-22 test showed positive results 63.63% (7/11) which as compared to BTA TRAK 54.54% (6/11) was comparatively more. In the age group of 60> years the NMP-22 test showed 80% (84/105) when compared with BTA TRAK 77.14% (81/105). Similar results were observed for gender distribution as it did not show any significant co-relation for male population for NMP-22 test with 80.55% (87/108) of positive cases with comparison to BTA TRAK 77.15% (85/108) ($p=0.121$, $p<0.001$).

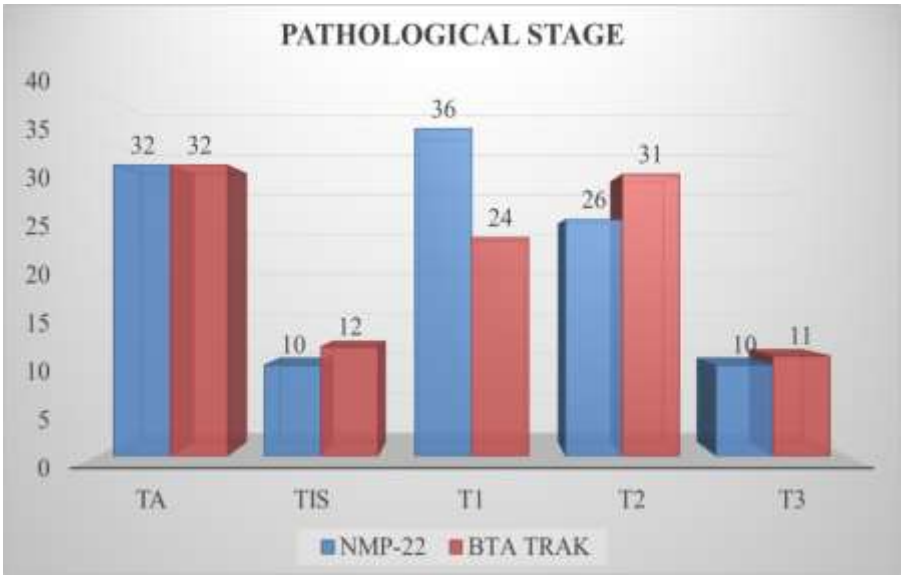

Figure 46: Pathological Stage distribution for FDA Approved Biomarkers.

In Pathological staging for Ta, NMP-22 test and BTA TRAK both showed similar result 72.72% (32/44). Conversely for T1, NMP-22 test showed 85.71% (36/42) when compared with BTA TRAK 57.1% (24/42) ($p=0.0094$, $p<0.01$. Almost all the staging were similar in numbers when compared with each tests.

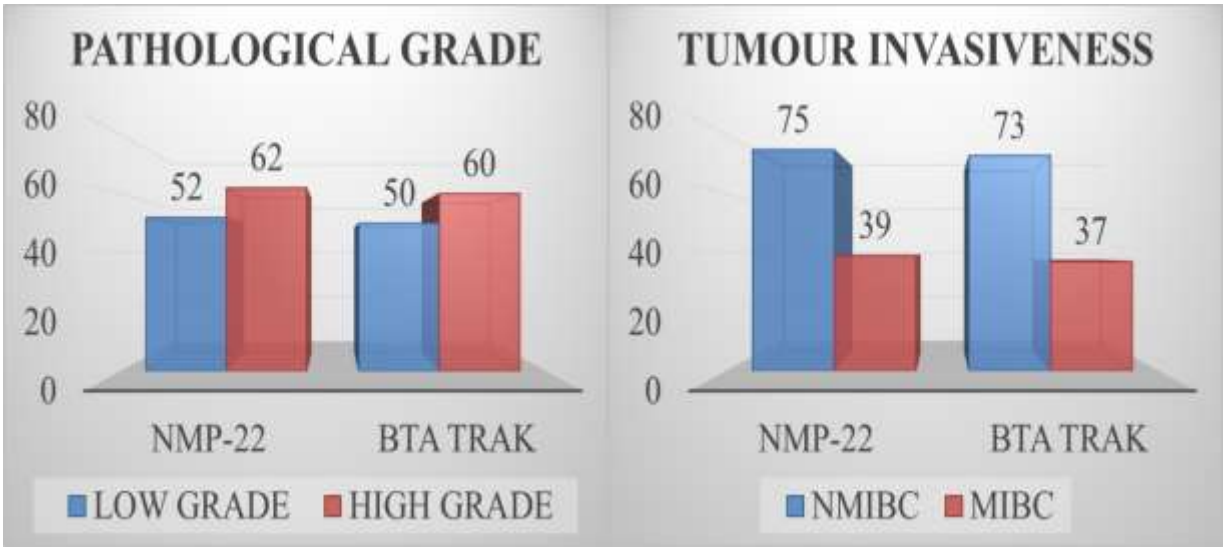

Figure 47: Pathological Grade and Tumour Aggressiveness distribution for FDA Approved Biomarkers.

The test resulted in similar findings for both low and high grade tumours. The NMP-22 test was positive in 52 of 71 (73.23%) instances, whereas the BTA TRAK test was positive in 50 of 71 (70.4%). Similar results were observed for High Grade it was 78.4% (62/79) for NMP-22 test and for BTA TRAK test it was 75.9% (60/79). For tumour invasiveness, BTA TRAK showed no significant association for non-muscle invasive tumour where it was seen, NMP-22 test with values 71.42% (75/105). For BTA TRAK test it was seen that it had similar positive rate for NMIBC 69.52% (73/105) ($p=0.0114$, $p<0.001$). Similarly, for MIBC, both the test showed equal prediction rates.

Table 13: Area under the Curve for NMP-22 and BTA test.

Variables	Area	Std Error	95% Confidence Interval	
			Low limit	High limit
NMP22	0.867	0.054	0.762	0.972
BTA	0.851	0.052	0.750	0.952

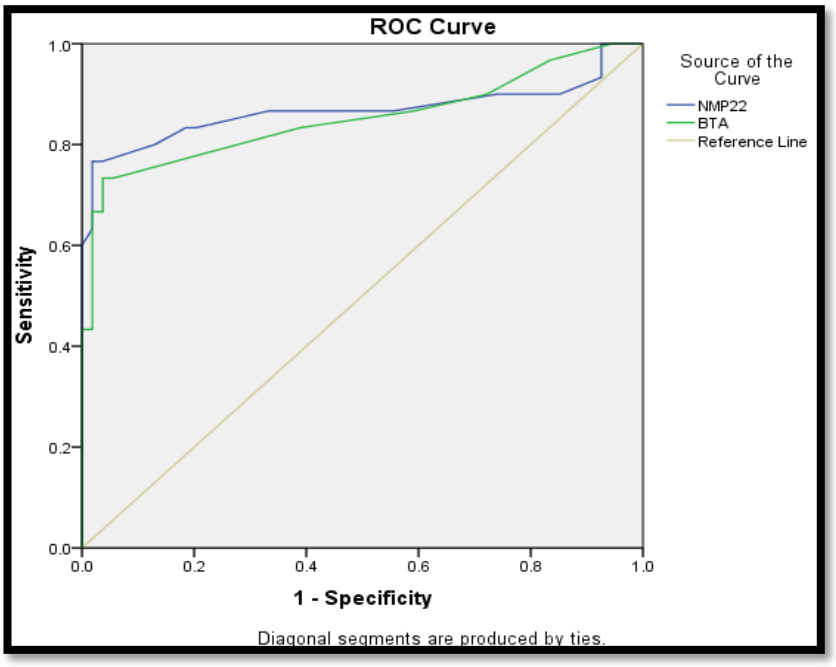

Figure 48: ROC curves for NMP-22 test and BTA TRAK test.

The area under the curve for the NMP-22 test was 0.867, which was quite comparable to the area under the curve for the BTA TRAK test, which was 0.851. The result suggested both the FDA approved test were similar in predicting sensitivity and specificity (Table 13, Figure 48)

6.3 CLINICAL DETAILS ON URINARY BLADDER CANCER:

The Department of Urology, Urinary Biomarkers Research Centre, Dr. Prabhakar Kore Hospital and MRC, Belagavi, examined the histopathology of 422 urothelial urine samples (n=150 UBC cases and n=272 control and LUTS individuals). The UBC instances were gathered from patients who had either TURBT (69.5%) or RC (30.4%). Histopathological investigation comprised tumour differentiation and muscle invasion detection (Figure 49). Two competent pathologists examined the Formalin-Fixed Paraffin-Embedded (FFPE) slices using a penta-head microscope.

In the current study, 47.34% (71/150) of the participants were classified as having low grade NMIBC, 22.67% (34/150) as having high grade NMIBC, and 30% (45/150) as having high grade MIBC. Pathological staging was pTa-1 in 70% (105/150) of the UBC patients and pT2-4 in 30% (45/150). The images below depict the presence of low grade and high grade tumours in NMIBC and MIBC instances, as well as pathological staging (Figure 50).

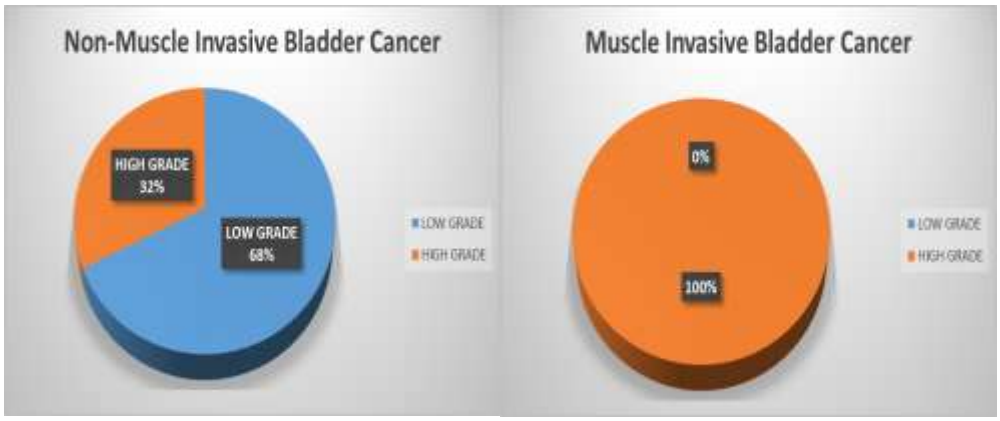

Figure 49: A pie chart illustrating low and high grade tumours in non-muscle invasive and muscle invasive bladder cancer.

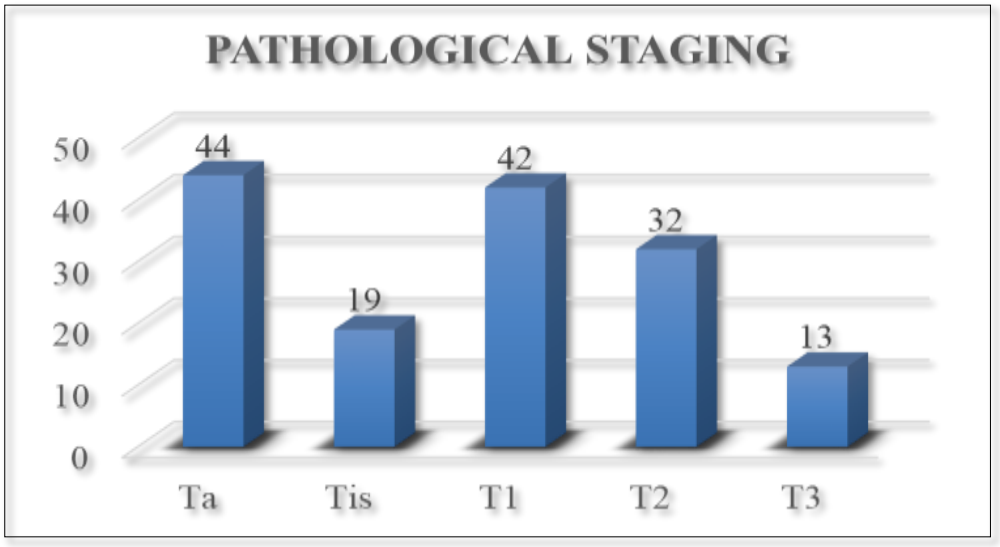

Figure 50: Graphical representation of Pathological staging.

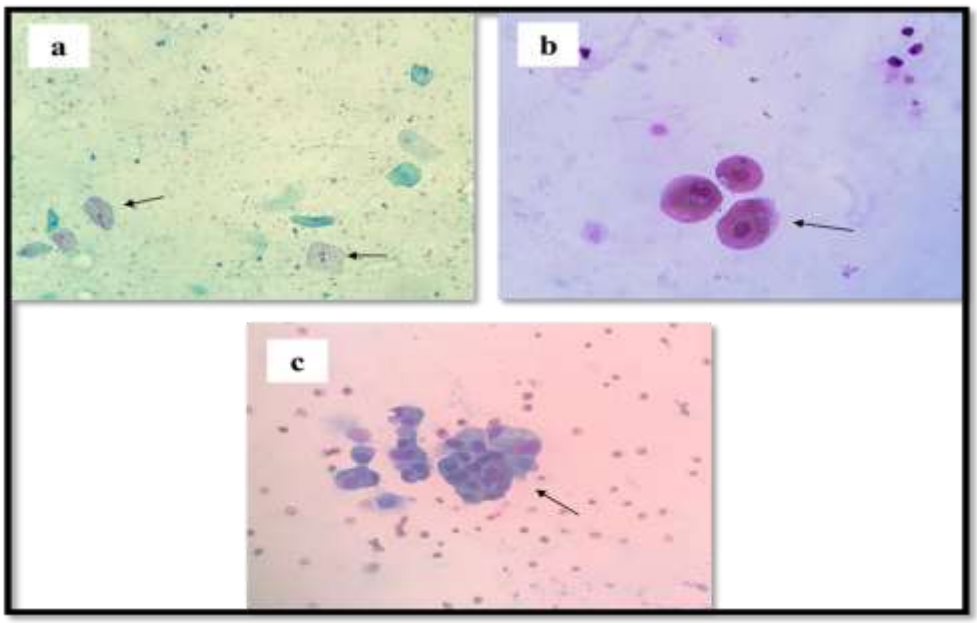

Figure 51: Urine cytology showing (a) Squamous epithelial cells, H&E X 100; (b) Benign looking transitional cells, H&E X 100; (c) H&E X 400 cytopathology smear of malignant cells on a neutrophil background with a high nucleocytoplasmic (N/C) ratio, hyperchromasia, and an uneven nuclear membrane.

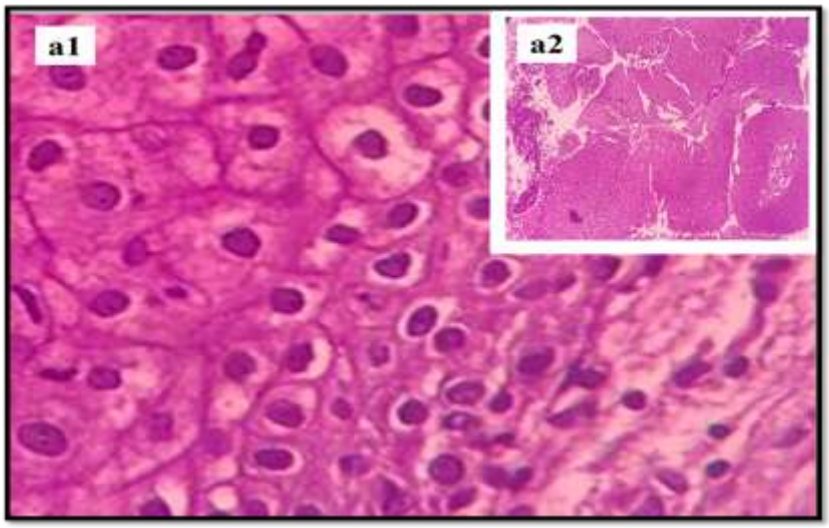

Figure 52: A low grade TCC histopathological segment. (a) H&E X 400 section analysis of a neoplastic growth comprised of cancer cells grouped in a papillary pattern with numerous layers surrounding papillae and a fibrovascular core; (b) H&E X 100 scan picture of the same section.

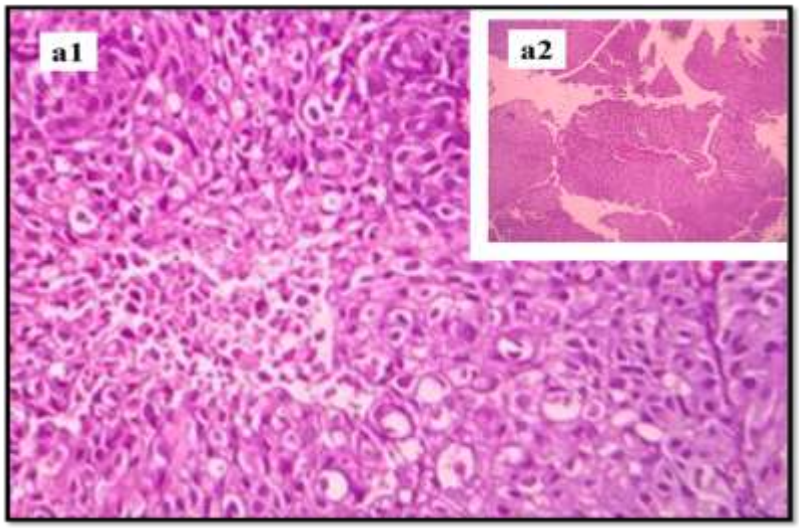

Figure 53: High-grade TCC histopathological segment. (a) Tumour with a solid pattern. Tumour cells are extremely dysplastic, with nuclear and cytoplasmic pleomorphism, a high N/C ratio, mitosis, hyperchromasia, and conspicuous nucleoli, H&E X 400; (b) H&E X 100 scan image of the same region.

6.4 OBJECTIVE 1: TO VALIDATE 5-ALA STAINING USING FLUORESCENCE MICROSCOPY OF UROTHELIAL BLADDER CARCINOMA PATIENTS:

5-ALA Cytology used Fluorescence Microscopy to examine urine samples from 422 patients for the identification of Urothelial Bladder cancer. Table 5 shows the representative positive and negative instances for 5-ALA Cytology and Conventional Cytology. To emphasize the clinical significance of this biomarker in UBC it's staining intensity with the tumour histological variables: tumour grade and stage were analyzed. Furthermore, 5-ALA Cytology assorted staining patterns were studied with the demographic, clinicopathological characteristics and prognostic results.

6.4.1 DIAGNOSTIC PERFORMANCE FOR 5-ALA CYTOLOGY AND CONVENTIONAL CYTOLOGY:

Table 14: Diagnostic Performance for 5-ALA Cytology and Conventional Cytology.

DIAGNOSTIC PERFORMANCE	**5-ALA Fluorescent Cytology**	**Conventional Cytology**
MALIGNANT on histopathology (True positive)	136 / 150	93 / 150
BENIGN on histopathology (True Negative)	262 / 272	268 / 272

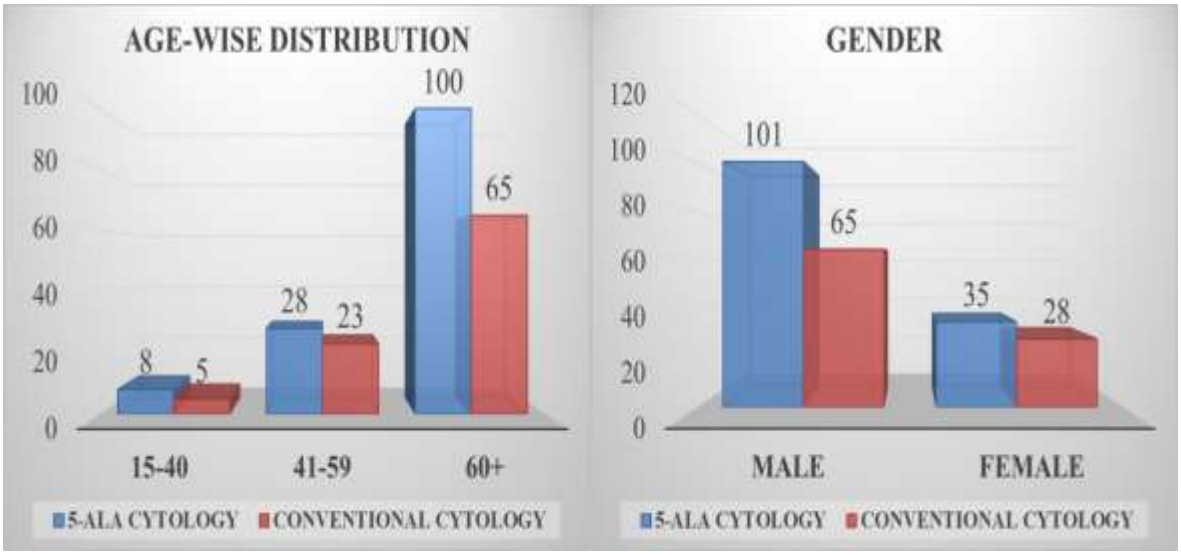

Figure 54: Age wise and Gender distribution for 5-ALA Cytology and Conventional Cytology.

For age wise distribution, 5-ALA Cytology showed higher amount for all the age groups For 15-45 years group 5-ALA Cytology showed positive results 72.27% (8/11) which as compared to Cytology 45.4% (5/11) was more. In the 60+ age group, 5-ALA was significant at 95.23% (100/105) when compared to cytology at 61.9% (65/105) (p =0.001*, p <0.001). Similar findings were seen for gender distribution, with males having a strong co-relation for 5-ALA cytology with 93.52% (101/108) all positive cases compared to cytology 60.18% (65/108) (p=0.001*, p <0.001). (Table 54)

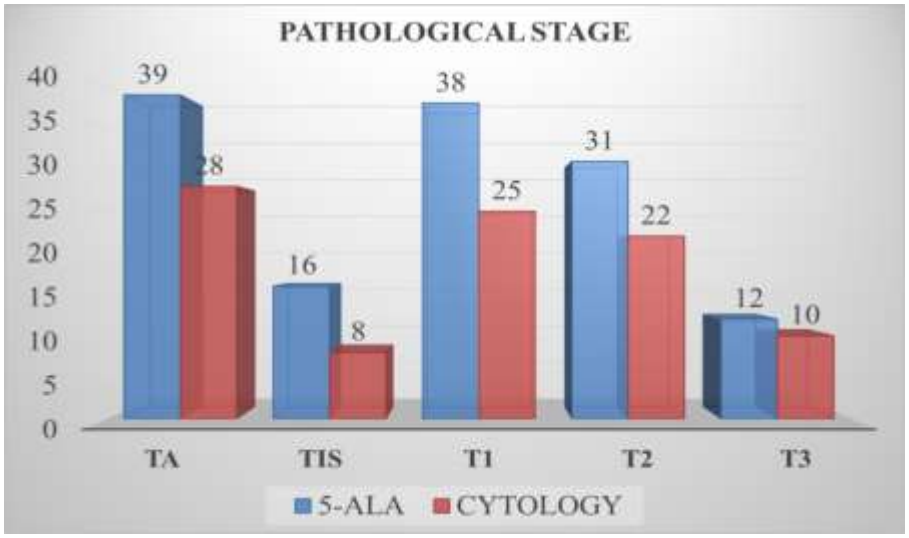

Figure 55: Pathological Stage distribution for 5-ALA Cytology and Conventional Cytology.

In Pathological staging for Ta, 5-ALA cytology showed 88.63% (39/44) which was significantly correlated with cytology 63.63% (28/44) (p=0.0045, p<0.01). Similarly for T1, it showed 90.47% (38/42) when compared with cytology 59.23% (25/42) (p=0.0074, p<0.01). (Table 55) Moreover, it was interesting to observe the positive prediction rate for Tis stage which was very remarkable when we compared both the test as it showed a much higher result for 5-ALA 84.21% (16/19).

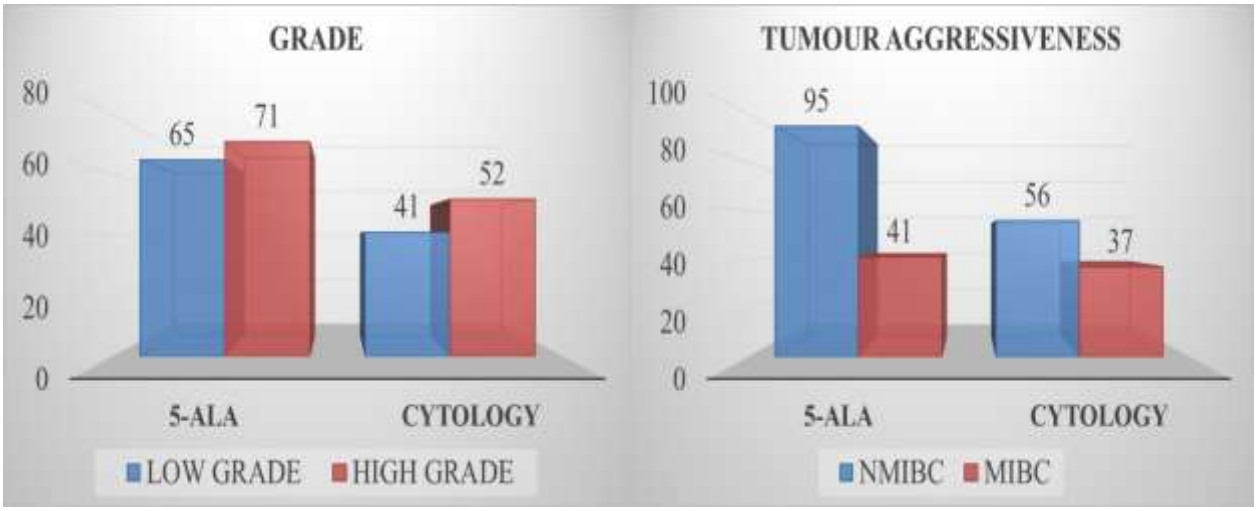

Figure 56: Pathological Grade and Tumour Aggressiveness distribution for 5-ALA Cytology and Conventional Cytology.

For Low Grade Tumours, 65 out of 71 (91.45%) cases were positive for 5-ALA and for cytology it was 41 out of 71 (57.74%). For High Grade it was 89.87 (71/79) for 5-ALA and for cytology it was 65.82% (52/79). For tumour invasiveness, 5-ALA showed a significant association for non-muscle invasive tumour where it was seen, 5-ALA with higher values 90.47 (95/105) (p=0.0001*, p<0.001). (Table 56) For cytology it was seen that it had very low positive rate for NMIBC 39.04% (41/105). Similarly for MIBC, Cytology results showed low predictive rates 82.23% (37/45) when compared with 5-ALA 91.12% (41/45).

6.4.2 5-ALA CYTOLOGY ACCORDING TO THE INTENSITY OF FLUORESCENCE AND WAVELENGTH (NM) ON DISTINCT PATHOLOGICAL STAGES, GRADES, AND CANCER INVASIVENESS:

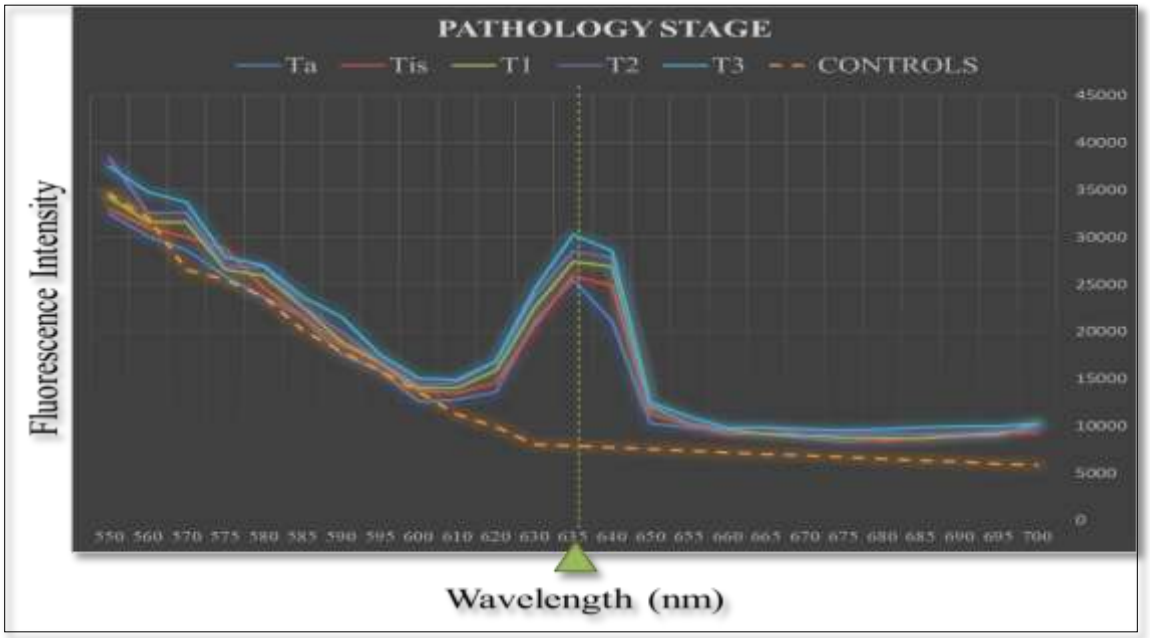

Figure 57: 5-ALA staining on Pathological stage according to the intensity of fluorescence and wavelength (nm).

Protoporphyrin IX fluorescence was measured in each compartment using a fluorescent microscope set to appropriate parameters. A graph was constructed with Fluorescence Intensity (Y-axis) and Wavelength (nm) (X-axis) to assess the Fluorescence at different pathogenic factors. When the sample intensity was out of range, the gain of the microscope was adjusted. The spectra of bladder cancer patients treated with ALA displayed a peak at 635 nm, while the spectrum of ALA-treated controls had no peak. In control patient samples, the peak was undetectable. From the plot it was observed that the peak for T3 stage was higher when compared with the other lower stages i.e Ta, Tis, T1 and T2. (Table 57)

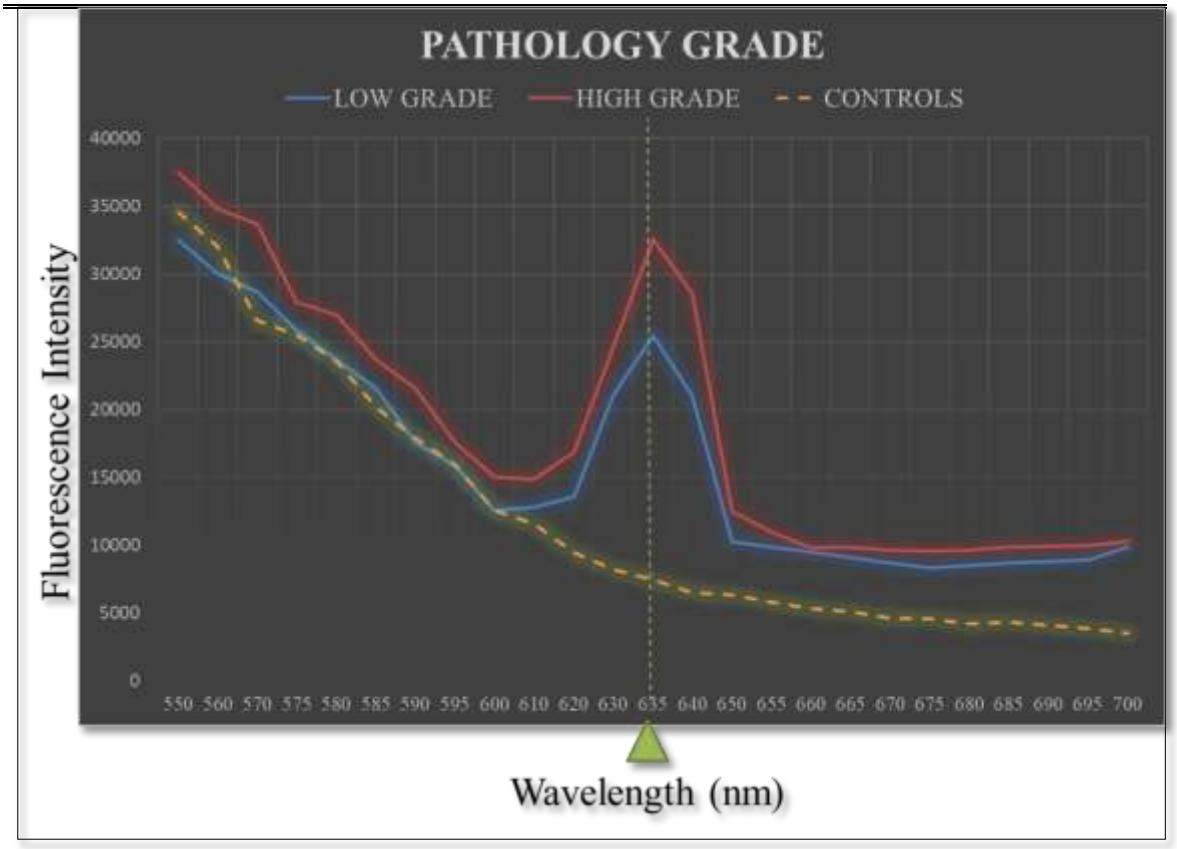

Figure 58: 5-ALA staining on Pathological grade based on Fluorescence intensity and wavelength (nm).

The spectra of samples administered ALA from bladder cancer patients exhibited a peak at 635 nm for both High and Low grade urine samples for pathological grade of the tumour. It was also shown that high grade tumours showed the largest intensity peak as contrasted with low grade tumours, but ALA-treated with controls did not. The peak remained undetectable in control patient samples, and the line was flat.

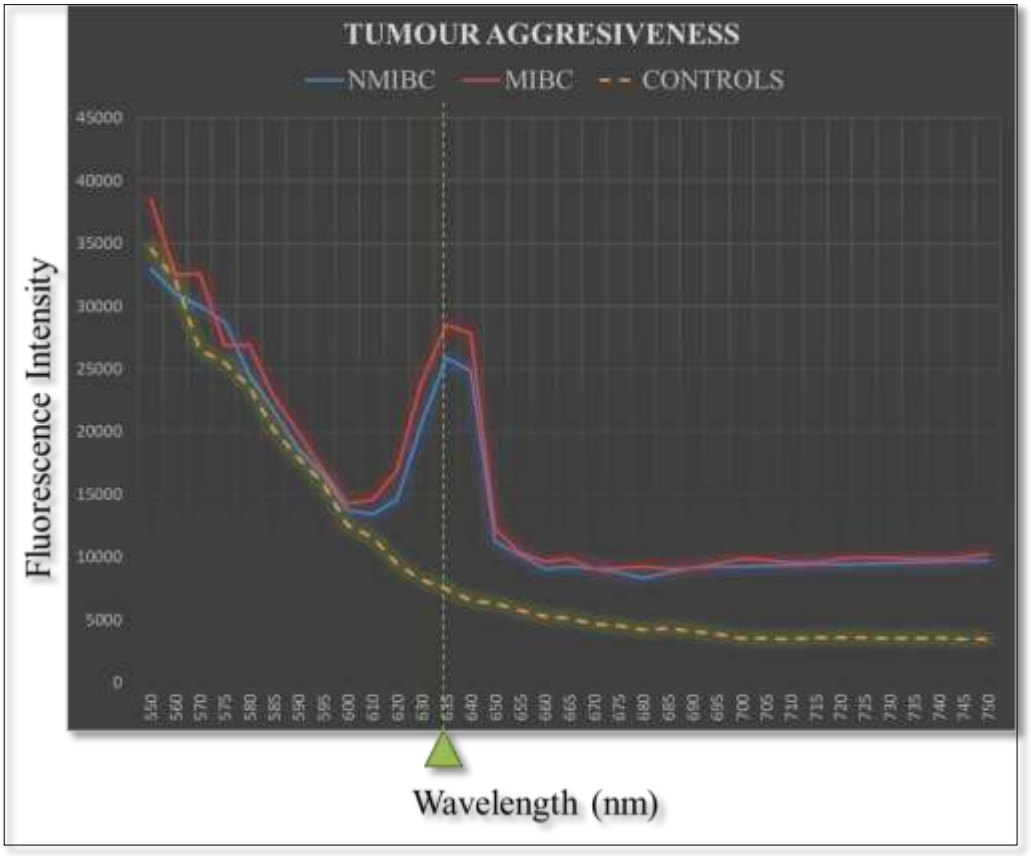

Figure 59: 5-ALA staining on Pathological grade based on Fluorescence intensity and wavelength (nm).

The spectra of samples administered 5-ALA luminescence from carcinoma cases exhibiting an emission peak for both NMIBC and MIBC urine samples for tumour aggressiveness. It was clearly observed that the MIBC had a highest intensity peak when compared to NMIBC, whereas that of ALA-treated with controls did not show peak (Figure 59). The peak was flat with no raised peak or intensity.

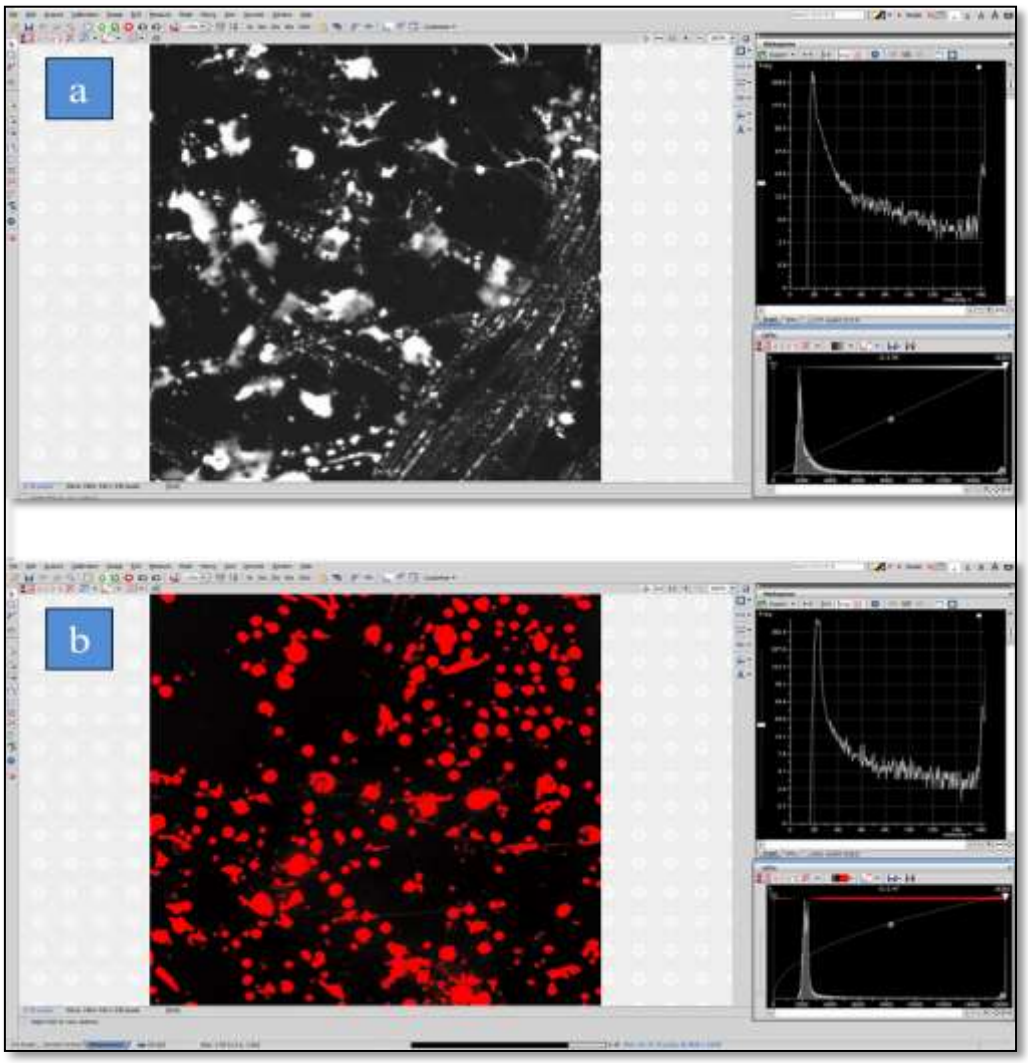

Figure 60: NIS-Elements Viewer: Image (a) show the visual representation in the software used showing morphology of the bladder cancer cells with Histogram in the right upper corner. Image (b) show the visual representation in the software used showing red fluorescence against black background of the bladder cancer cells with Histogram in the right upper corner.

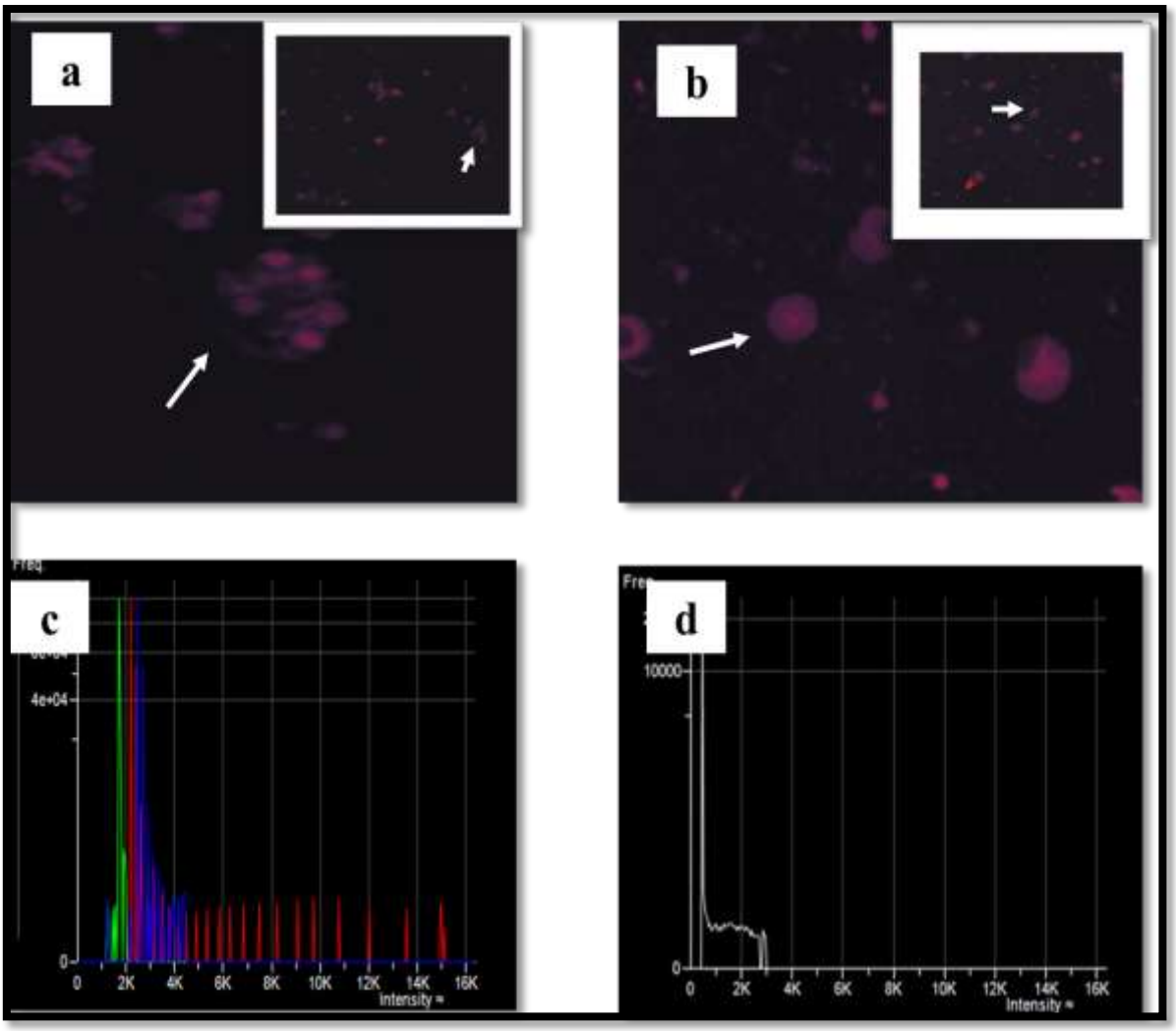

Figure 61: This graphic depicts the effect of 5-ALA on urine. Figures (a and b) depict cells that are light red or pink on a black background, indicating that they are benign urothelial cells. (200X). The histogram in figures (b & d) reveals that the intensity (x-axis) versus frequency (y-axis) is minimal or normal.

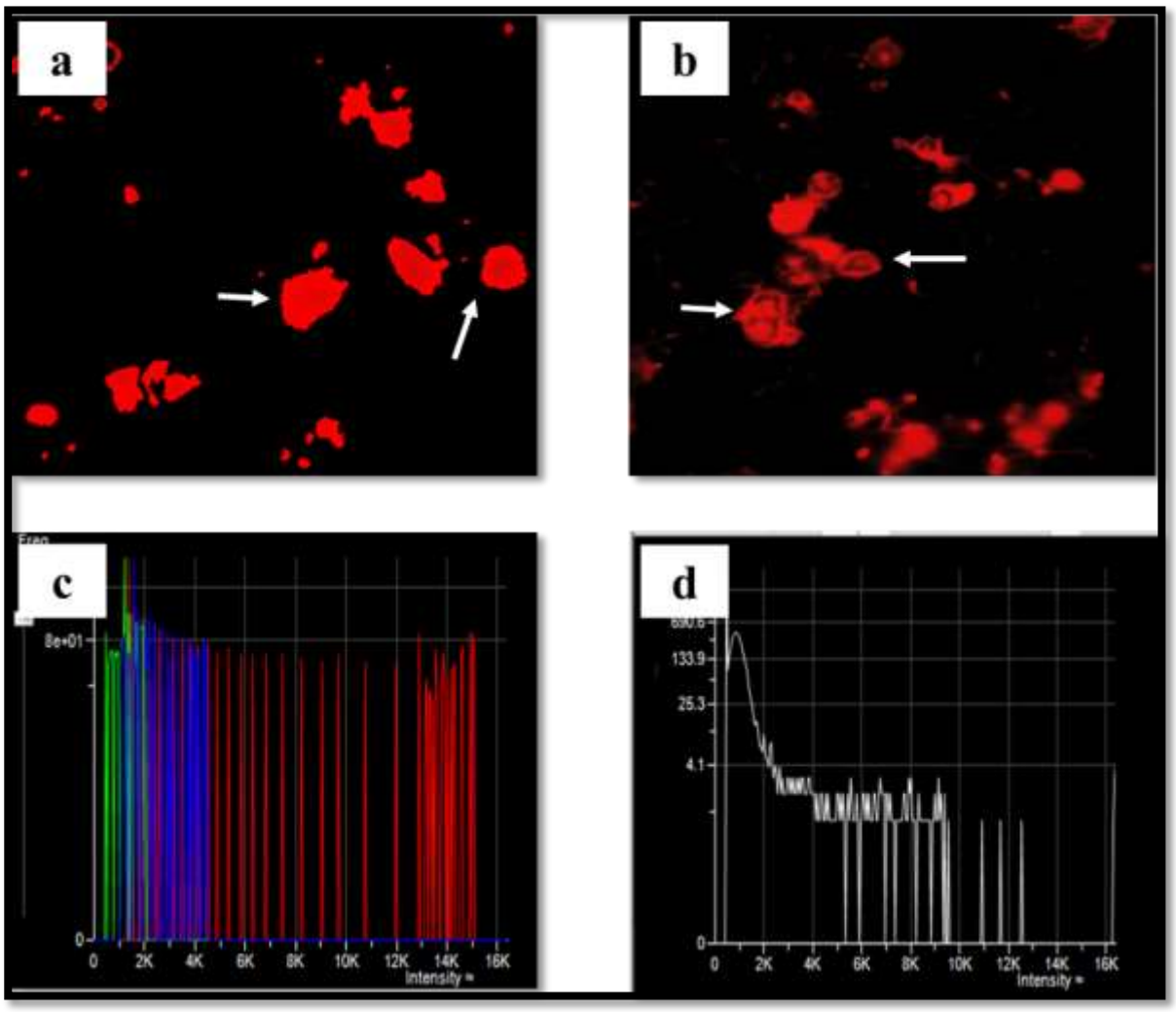

Figure 62: The cells in the picture are bright red or dark red on a black backdrop, indicating cancer urothelial cells. (a & b), (200X). The histogram in figures (c & d) illustrates the intensity (x-axis) versus frequency (y-axis) to be raised.

Table 15: Area under the Curve For 5-ALA cytology and Conventional Cytology.

Variables	Area	Std. Error	95% Confidence Interval	
			Low Limit	Higher Limit
5-ALA CYTOLOGY	0.906	0.009	0.889	0.943
CONVENTIONAL CYTOLOGY	0.625	0.036	0.595	0.736

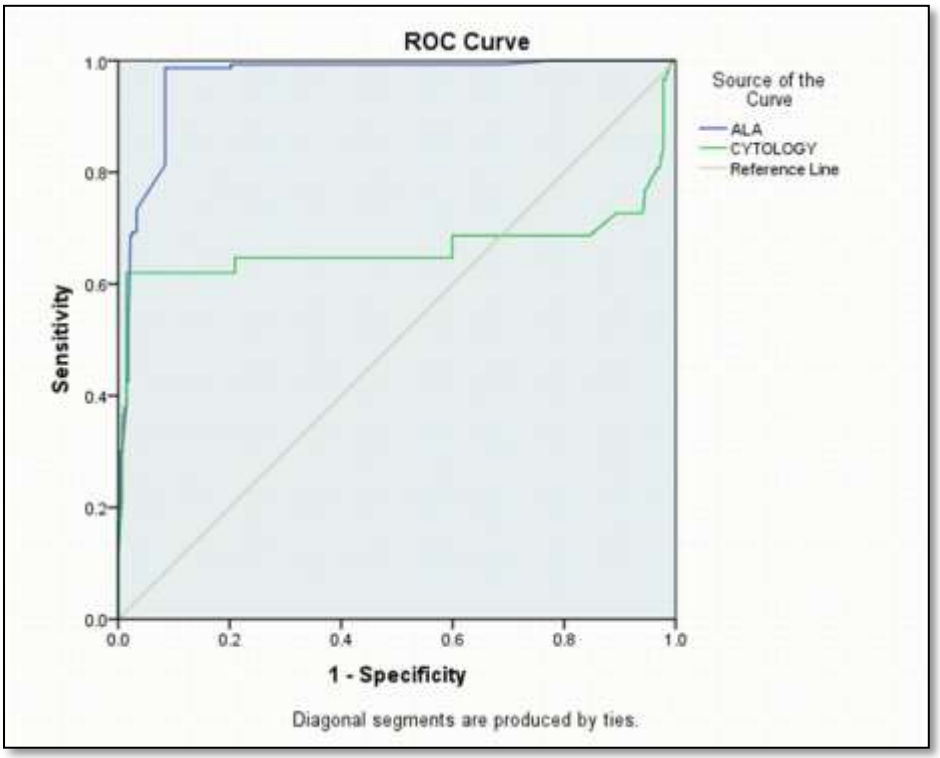

Figure 63: ROC curve for 5-ALA and Conventional Cytology.

The area under the curve for 5-ALA Cytology was 0.906, which was much higher than the area under the curve for Cytology, which was 0.625. The result suggested 5-ALA Cytology higher sensitivity and specificity compared to Conventional Cytology (Table 15, Figure 63)

6.5 DIAGNOSTIC PERFORMANCE OF BIOMARKERS IN DETECTING UROTHELIAL BLADDER CARCINOMA:

Table 16: Comparison of specificity and sensitivity of Biomarkers in detection of Urothelial Bladder carcinoma.

DIAGNOSTIC PERFORMANCE		5-ALA Fluorescent Cytology	Conventional Cytology	NMP-22	BTA-TRAK
n=150	**MALIGNANT on histopathology(True positive)**	136	93	114	110
n= 272	**BENIGN on histopathology (True Negative)**	262	268	258	260
False positive		10	4	14	12
False negative		14	57	36	40
Sensitivity (%)		**90.66***	62	76	73.33
Specificity (%)		**96.32***	98.53	94.85	95.58
PPV (%)		93.15	95.88	87.76	86.67
NPV (%)		94.93	82.46	88.15	87.68
Diagnostic accuracy (%)		**94.31***	85.55	86.72	85.14
Cohen's Kappa (K)		0.875	0.657	0.728	0.719
Time for Inspection		180 min	1-2 days	300 min	240 min

The table 16 above demonstrates the diagnostic efficacy of several biomarkers with traditional cytology. The study comprised 150 Histo-Pathologically Confirmed Malignant Cases and 272 LUTS and Controls. The specificity (96.32% vs. 98.53% vs 94.85 vs 95.58, p = 0.024) and positive predictive value (93.15% vs. 95.88%, vs 87.76 vs 86.67, p = 0.27) of 5- ALA-induced fluorescence cytology and other biomarkers were comparable.

For 5-ALA Cytology a total of 136 cases were positive with 90.66% specificity. For Conventional Cytology 93 cases were positive with sensitivity of 62%. The other FDA Biomarkers showed similar result with sensitivity (73% - 76). The diagnostic accuracy was highest for 5-ALA Cytology with 94.31% and for BTA TRAK test is was marginally lowest 85.14% when compared to Conventional Cytology (85.55%) and NMP-22 Test (86.72%). When compared to all other tests, The greatest Cohen's Kappa (K) value was 0.8745 for 5-ALA induced fluorescence cytology, showing near perfect agreement.

False positives in conventional cytology were identified among four patients: two of the men had bladder prostatic hyperplasia (BPH), while the other two had a urinary tract infection (UTI). Five patients had BPH, three had UTI, and two had urinary stones, and five had false positives in 5-ALA-induced fluorescence cytology. In fourteen individuals, false positives in the NMP-22 test were discovered: 7 with BPH, 3 with UTI, two had urinary stones, and two had radiographic cystitis. Likewise, twelve BTA TRAK test cases had false positive findings.

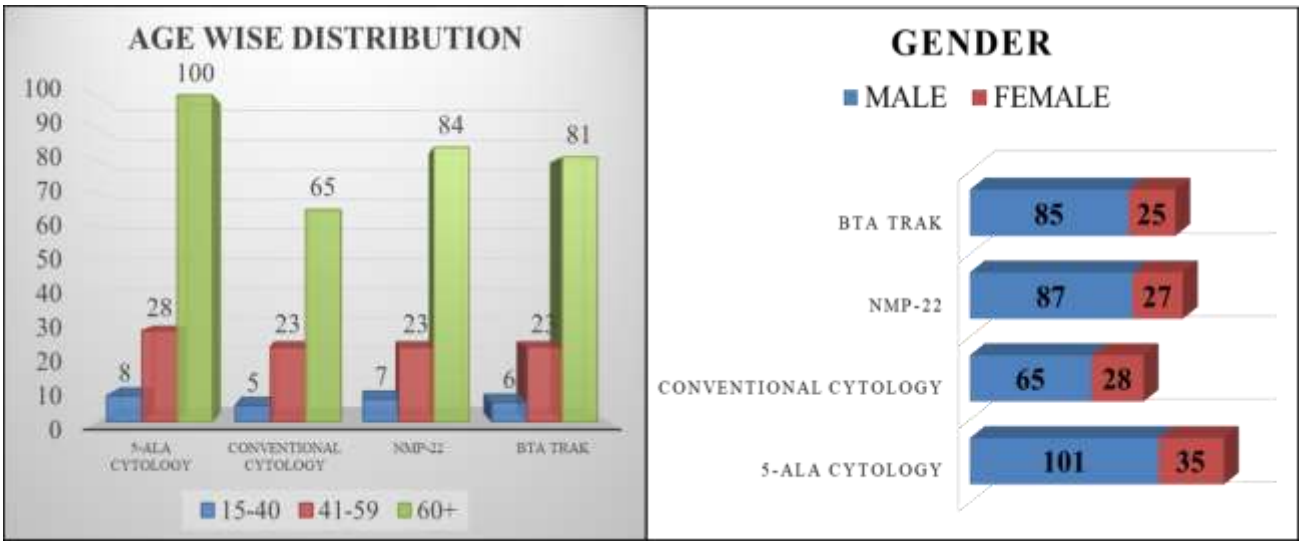

Figure 64: Age wise and Gender distribution for 5-ALA Cytology, Conventional Cytology and FDA approved Biomarkers.

For age wise distribution, 5-ALA Cytology showed higher significant predictive value for all the age groups. Predominantly For 60 years and above group, the predictive values (95.23% vs 61.90% vs 80% vs 77.14%, p= 0.0081*) was high for 5-ALA Cytology. Similar results were observed for gender distribution as it showed a significant co-relation (93.51% vs 60.18% vs 81.55 vs 78.70%, p= 0.0004*) for 5-ALA cytology.

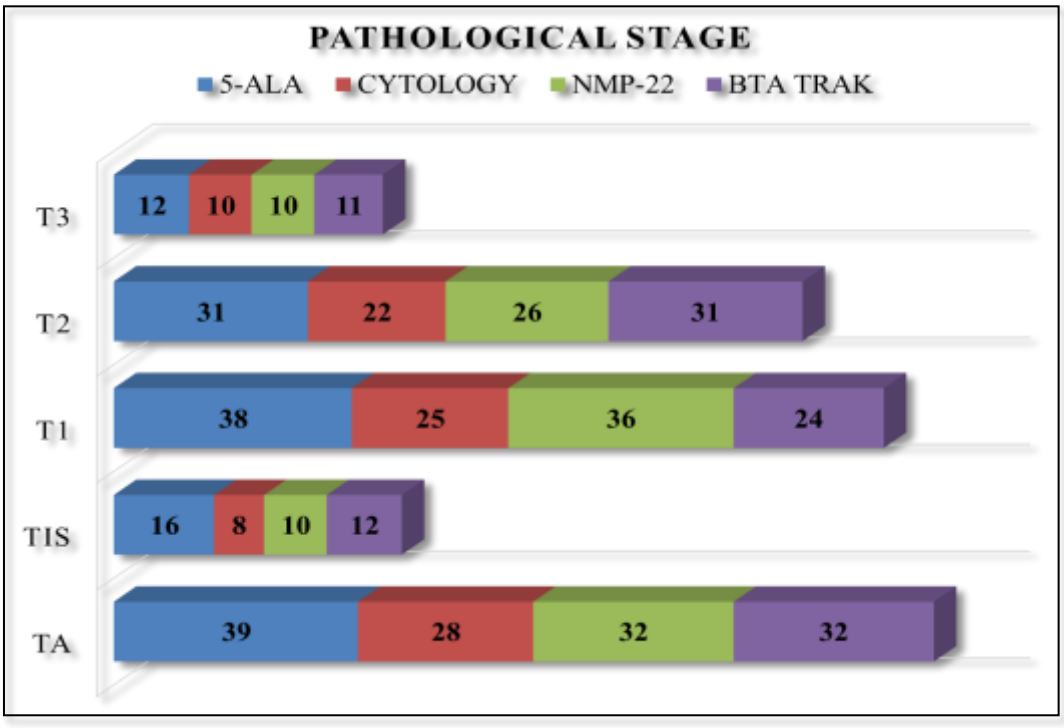

Figure 65: Pathological Stage distribution for 5-ALA Cytology, Conventional Cytology and FDA approved Biomarkers.

In Pathological staging for Ta, 5-ALA cytology showed 88.63% (39/44) which was significantly with conventional cytology 63.63% (28/44) (p=0.0045, p<0.01). For 5-ALA and NMP-22 test it was 88343% vs 72.72% (p=0.0055, p<0.01) and similarly result was observed for 5-ALA Cytology and BTA TRAK test. For T1, 5-ALA showed again a significant result (90.47% vs 59.52% vs 85.71% vs 57.14 %) for Cytology, NMP-22 test and BTA TRAK respectively. Moreover, it was interesting to observe the positive prediction rate for Tis stage which was very remarkable high for 5-ALA cytology when

we compared the other biomarkers test as it showed a much higher (84.21% vs 42.10% vs 59.63% vs 53.15%).

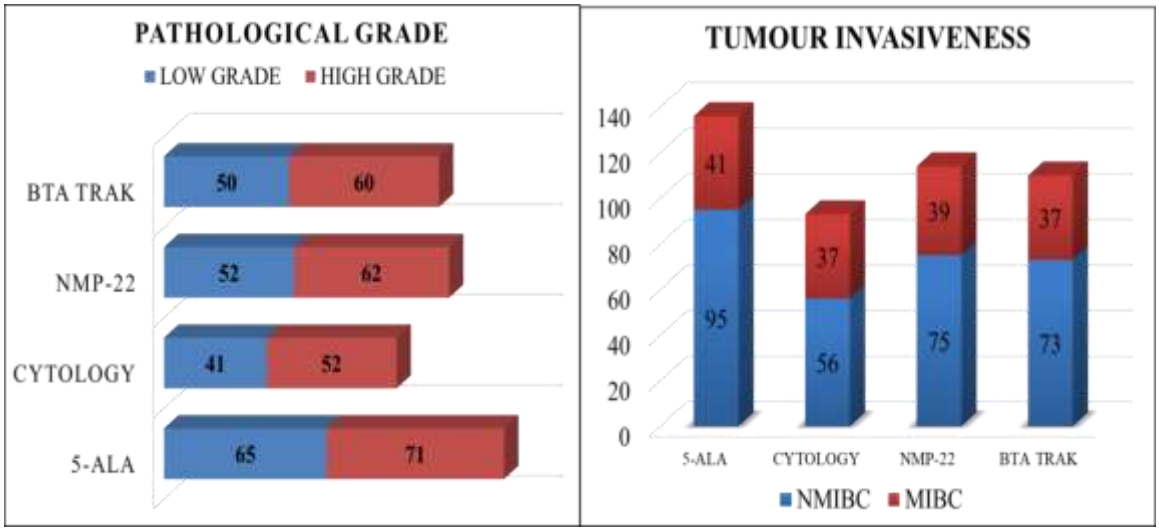

Figure 66: Pathological Grade and Tumour Aggressiveness distribution for 5-ALA Cytology and Conventional Cytology.

For Low Grade Tumours, the specificity for all the biomarkers were 91.45% vs 57.74% vs 73.23% vs 70.64% (p=0.0001*, 0.0191, 0.0057*). The p-value for 5-ALA cytology was assessed against Conventional Cytology, NMP-22 Kit test, and BTA TRAK test. Similarly, High Grade it was 89.87 (71/79) for 5-ALA cytology. For cytology it was 65.82% (52/79) and for FDA approved biomarkers it was 78.48% and 75.94%. For tumour invasiveness, 5-ALA showed a significant association for non-muscle invasive tumour where it was seen, 5-ALA with higher values 90.47 (95/105) (p=0.0001*, p<0.001). For cytology it was seen that it had very low positive rate for NMIBC 39.04% (41/105). Likewise, for BTA TRAK test it was 90.47% vs 69.52% (p=0.0054*, p<0.01) Similarly for MIBC, Cytology and BTA TRAK test both showed results with low predictive rates 82.23% (37/45) when compared with 5-ALA 91.12% (41/45).

6.6 5-ALA Cytology, Conventional Cytology and FDA approved Biomarkers on different Pathological stage, grade and tumour invasiveness based on Fluorescence intensity and wavelength (nm).

Table 17: Area under the Curve for overall Biomarkers.

Variables	Area	Std. Error	95% Confidence Interval	
			Low Limit	Higher Limit
ALA CYTOLOGY	0.906	0.009	0.889	0.943
CONVENTIONAL CYTOLOGY	0.625	0.036	0.595	0.736
NMP-22	0.891	0.035	0.822	0.959
BTA TRAK	0.911	0.031	0.850	0.971

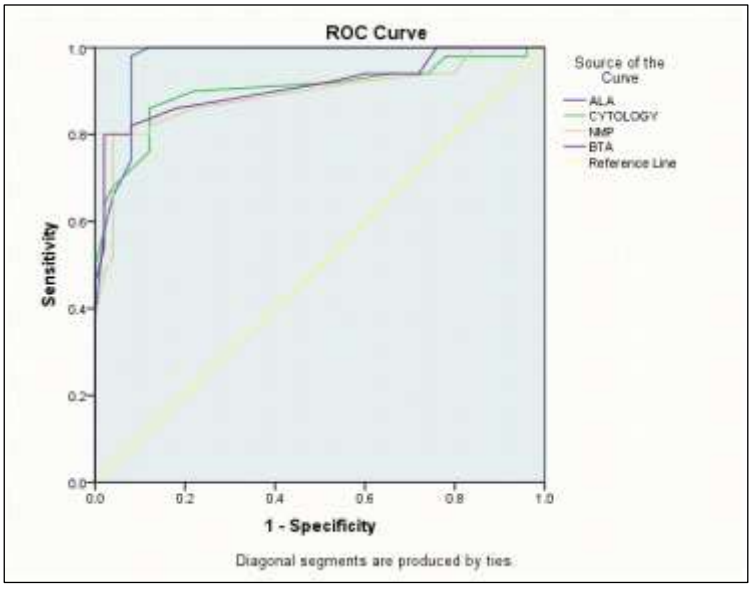

Figure 67: ROC curve for 5-ALA Cytology, Conventional Cytology and FDA approved Biomarkers.

The ROC curve for 5-ALA Cytology Conventional Cytology and FDA approved Biomarkers presented the area under the curve for 5-ALA to be 0.906 which was much more compared to Cytology (0.625), NMP-22 test to be 0.867 which was much similar compared to BTA TRAK test which was 0.851. The result suggested 5-ALA Cytology higher sensitivity and specificity compared to Conventional Cytology and FDA approved biomarkers (Table, Fig)

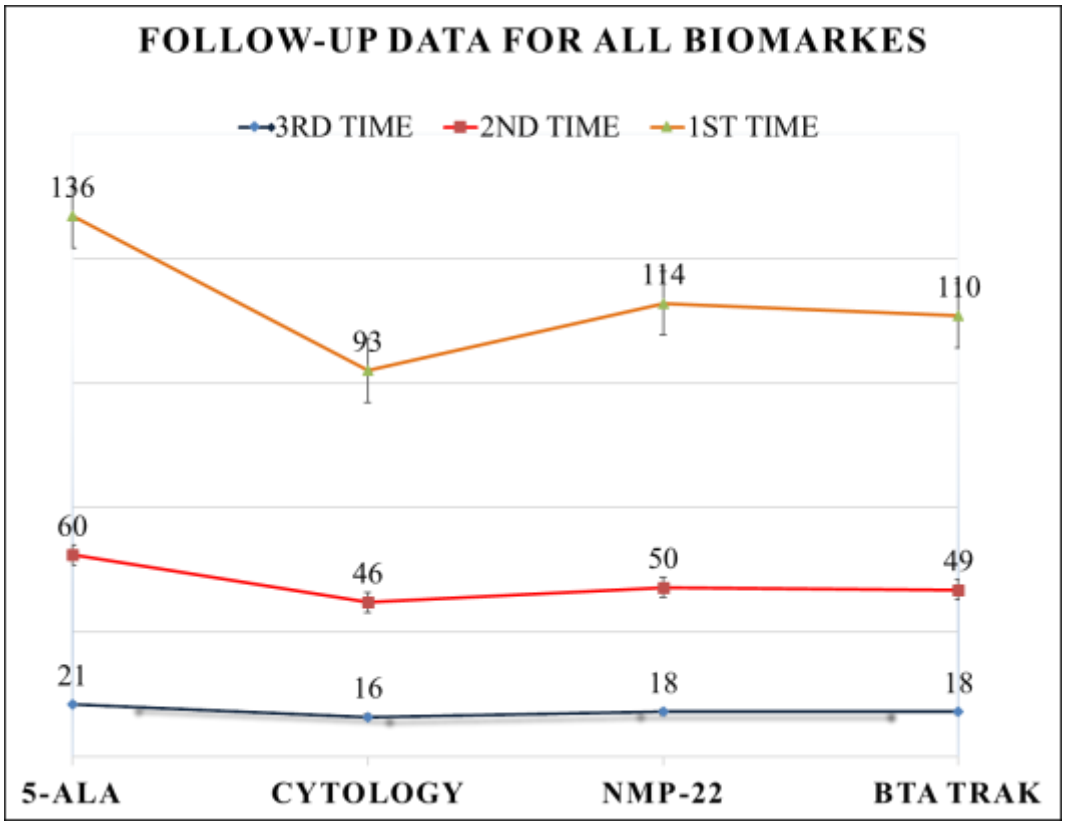

Figure 68: Figure shows the follow-up data for all biomarkers.

In the above figure it is observed the follow-up of cases at 6-12 months of period. In the 1st visit the highest number of positive prediction value was for 5-ALA Cytology 90.7% (136/150) and the lowest was for Conventional Cytology 61.8% (93/150). For 1st follow-up again the graph showed highest rate for 5-ALA Cytology 92% (60/65) and again lowest for Conventional Cytology 69% (46/65). For NMP-22 test and BTA TRAK test it was 75.9% and 74.7% respectively.

For 2nd follow-up checkup a total of 23 cases visited the OPD. The specificity was highest for 5-ALA Cytology with 90.4% (21/23) against 67.9% for Conventional Cytology, 77.5% for NMP-22 test and 74.6% for BTA TRAK test.

Table 18: Table shows the follow-up data for all biomarkers for pathological Grades.

Note: For 1st visit Low Grade =71, High Grade =79, 2nd time Low Grade =22, High Grade =43, and For 3rd time Low Grade =8, High Grade =15.

BIOMARKERS	GRADE	1ST TIME	MEAN ± STDV	p-vlaue	2ND TIME	MEAN ± STDV	p-vlaue	3RD TIME	MEAN ± STDV	p-vlaue
5-ALA	LOW	65	68 ± 4.242	1	20	30 ± 14.142	1	7	10.5 ± 4.949	1
	HIGH	71			40			14		
CYTOLOGY	LOW	41	46.5 ± 7.778	0.0001*	14	23 ± 12.727	0.0097*	5	8 ± 4.242	0.1149
	HIGH	52			32			11		
NMP-22	LOW	51	56.5 ± 7.778	0.0001*	16	25 ± 12.727	0.056	6	9 ± 4.242	0.3205
	HIGH	62			34			12		
BTA TRAK	LOW	50	55 ± 7.071	0.0001*	16	24.5 ± 12.02	0.0331*	5	9 ± 5.65	0.3824
	HIGH	60			33			13		

The following table 18 provided visit-by-visit follow-up results for Low and High Grade tumours. For the first visit for Bladder cancer cases, the 5-ALA Cytology had 65/71 for low grade and 71/79 for high grade vs 41/71 for low grade and 52/79 for high grade (68± 4.242, 46.5 ±7.78, p=0.0001, p0.01). 5-ALA was favourably significant p=0.0001 (p<0.01) for both FDA-approved biomarkers. For the first follow-up for Bladder cancer patients, 5-ALA Cytology had 20/22 for low grade and 40/43 for high grade vs 14/22 for low grade and 32/43 for high grade (30 ±14.142, 23± 12.727 ; p=0.0097, p<0.01). The low grade for the BTA TRAK test was 16/22, while the high grade was 33/43. (30 ± 14.142, 24.5 ± 12.02; p=0.031, p<0.01).

DISCUSSION

Bladder cancer is a particularly prevalent kind of urological cancer and rank sixth among cancers in men. In regard to incidence, it ranks top among malignant urinary tract tumours and second behind prostate cancer in the Developed world. Transitional cell carcinomas account for more than 90% of bladder malignancies, squamous cell carcinomas account for 5%, and adenocarcinomas account for less than 2%. When first diagnosed, Non-muscle invasive bladder cancer (NMIBC) accounts for 70-85% of cases, whereas muscle-invasive bladder cancer (MIBC) accounts for 15%-30%.[14] Ta, T1, and carcinoma in-situ are the pathological stages of NMIBC, often known as superficial cancer. Ta accounts for 70% of cases, T1 accounts for 20%, and carcinoma in situ accounts for 10%. MIBC, commonly known as invasive bladder cancer, has three pathological stages: T2, T3, and T4. Up to 80% of NMIBC patients relapse after 5 years; 30% of Ta patients acquire MIBC; and T1 and carcinoma in situ patients are more inclined to be diagnosed with MIBC. [15]

BCa strikes males three to four times more often than women. Several ideas have been suggested for clarifying the gender disparity in incidence, including differences associated with bladder cancer risks and the possibility of sex steroid hormone control. When women are diagnosed with bladder cancer, they are more likely to acquire locally advanced tumours. [13] Furthermore, females have been linked to higher risks of cancer recurrence, development, and death following therapy, albeit this is not uniform. Dobruch and colleagues[47] discovered a gender gap in BCa incidence that was unrelated to disparities in exposure risk, such as smoking status. Gender differences in carcinogen processing by hepatic enzymes, resulting in unequal urothelium exposure to carcinogens, were possible biological

causes. Furthermore, the operation of the sex steroid hormone system was linked to the development of bladder cancer, suggesting that androgens and oestrogens might have biological effects on bladder cancer in vitro as well as in vivo. The speed and depth of haematuria inquiry varied by gender, with women incurring significantly greater delays in urologic referrals and receiving guideline-concordant imaging less frequently. When females received diagnosed with bladder cancer, their tumours were more advanced. Even after controlling for tumour stage and treatment approach, females had greater cancer-specific mortality.[49] Our research also found that males are three times as likely as females to get bladder cancer.

Toxin exposure at work has been connected to bladder cancer.[72] Since the late 1800s, uncommon cases of bladder cancer have been reported in the industry. In the British rubber industry,[250] Case and Hosker discovered an extraordinarily high rate of bladder cancer. A number of other substances and occupations were eventually linked to an elevated likelihood of bladder cancer.[251] Polycyclic aromatic hydrocarbon exposure has also been linked to greater hazards for the aluminium, coal tar, and coal gasification industries.[252] Low-skilled employees, such as painters, have bladder cancer has been associated to an increased probability. Several epidemiological studies have discovered that painters had RRs of bladder cancer that ranged from 1.2 to 1.5. According to certain research, a period of time subjected to paint components raises the likelihood of bladder cancer.[253] Numerous epidemiological investigations have also revealed that truck, taxi, and bus drivers had a greater prevalence of bladder cancer. In these occupational studies, the RRs for bladder cancer diverse from 1.5-2.3. The majority of research revealed positive trends with duration of exposure. An

element of diesel exhaust fumes is the most likely causative agent. These correlations, however, are not always confirmed and are now being challenged.[254]

The study on the influence of certain agricultural crop activities or vocations on bladder cancer risk is limited, rarely adjusted for smoking, based on a small number of exposed cases, and does not investigate exposure response with exposure duration and intensity. According to four exposed patients and controlled for smoking status, Settimi and colleagues[255] observed a non-significantly greater risk of bladder cancer among female vegetable producers [odds ratio (OR) 2.9, 95% CI (0.81-12.20)]. As a result, we haven't been able to create more thorough exposure scenarios. We discovered, however, that women were at a greater risk than males, hinting that certain exposure situations may escalate risk in women. Other non-significantly elevated risks of bladder cancer associated with pea seeding and greenhouse use were discovered, which had not previously been addressed in the literature.[256]

After controlling for possible confounders (gender and smoking), we discovered a statistically significant higher risk of cancer of the bladder among farmers (25.34% (38/150), with an exposure-response connection for duration. Due to the small number of exposed patients, the considerable impact caused by duration should be viewed with caution. Our finding might be attributable to the limited number of exposed individuals; another explanation is that smoking is a significant risk factor for bladder cancer, so beneficial correlations with other exposures may be disguised among smokers. Some articles indicated a similar tendency, with pesticide linkages found only in never smokers to a larger extent. Being exposed to particular pesticides, if by initial use, returning tasks, or secondary exposure. Pelaez and colleagues evaluated the influence of exposure to

pesticides duration using a case-control technique. Participants who had used pesticides for the longest amount of time (15 years) had a significantly higher risk of bladder cancer [OR 3.4, 95% CI (1.4-6.5)]. [257]

Comorbidities comprise cardiovascular disease, type 2 diabetes (T2DM), high blood pressure, and other conditions. These conditions are frequent in cancer patients and can have an impact on treatment choices, prognosis, and survival outcomes. Regardless of disease stage, the degree of comorbidities has a significant dose-dependent impact on survival. Patients with bladder cancer have several competing risks due to their age and/or the presence of other diseases that enhance morbidity and mortality. [258] We studied the predictive value of co-morbidities among individuals with BC in this study. Comorbidities comprise cardiovascular disease, type 2 diabetes (T2DM), while neither hypertension nor T2DM increased the incidence of BC, a subgroup of T2DM patients with features like race, age, and kidney failure did. T2DM causes and renin-angiotensin system abnormalities that could result in carcinogenesis have been investigated, and possible correlations with high blood sugar levels, autoimmune diseases, resistance to insulin, and endocrine destabilization have been established. T2DM or any other Co-morbidities, on the other hand, did not increase the incidence of BC in our research (Figure 2). The probability of BC rose over time, although not statistically significantly. (Table2) [259]

Without any confounding factors, overall BMI was a risk factor for bladder cancer. BMI increase was a risk associated with bladder cancer that wasn't influenced by confounding factors.[260] The positive connection between bladder cancer and BMI increased beyond the reference BMI, with the mean and standard deviation for UBC (27.6±0.4) and Controls (26.1±0.5) being significant

($p=0.0001$, $p<0.001$) (Table 4). Obesity may be carcinogenic, according to mounting data.

Comorbidities comprise cardiovascular disease, type 2 diabetes (T2DM), Obesity boosts insulin production, which may lead to cancer formation, according to one research on how obesity may promote carcinogenesis.[75] Adipose tissue expansion enhances the production of pro-inflammatory proteins and cytokines (such as tumour necrosis factor- and interleukin-6) while lowering adiponectin synthesis.

Tobacco usage and alcohol use are the most often co-abused medications worldwide. A vast number of research investigations that describe the relationships among tobacco smoking, alcohol intake, and the risk of bladder cancer have been published. Tobacco usage has long been connected to the invasiveness and grade of bladder cancer, [53] These tumour features are closely associated to the papillary feature, and are critical in determining whether non-invasive bladder tumours are Ta or Tis in the TNM classification. Polesei and colleagues [261] examined the effects of smoking on several histological subtypes of transitional cell carcinoma of the bladder (TCC). When compared to never smokers, TCC risk was three times greater in former smokers (95% CI 2.1-4.3) and more than six times higher in current smokers (95% CI 4.8-9.2). Tobacco use is an important risk indicator for bladder cancer, accounting for around half of all occurrences in both men and women. People who smoke extensively are between three and five times more probable than nonsmokers to die, with an evident dose-response relationship for intensity. An aggregated evaluation of 11 case-control studies also found a risk plateau at roughly 20 smokes per day. Furthermore, the duration of smoking has a significant relative risk. Furthermore, the duration of

smoking has a significant RR. Long-term smokers are five times more likely to die than never smokers.[262] Our analysis also revealed that current smokers had a higher relative risk of bladder cancer (3.802 (95% CI, 1.489-5.514) than former smokers (0.889 (95% CI, 0.247-1.965).

Some studies have argued that persistent cigarette smoking confounding might explain an increased risk from alcohol use.[263] We aimed to characterise cigarette smoking behaviours using smoking status, quantity, and duration to best describe bladder cancer. Smoking correction, on the contrary hand, had no influence on the prevalence rate ratios. As a consequence, while some effect cannot be ruled out, the identified link between alcohol use and bladder cancer risk hadn't been anticipated to be entirely attributable to lingering smoking ambiguity. According to a recent meta-analysis based mostly on case-control studies, male bladder cancer risk is modestly enhanced by alcohol use (Odds Ratio = 1.3, 95 percent CI: 0.5- 2.4), a figure that may not be realistic. The purpose of this meta-analysis was to see if the summary odds ratio for male alcohol consumers against people who don't remain steady after incorporating the current study. 3.195 (95 percent CI: 1.76-5.51) was the corrected age-related alcohol-adjusted summary odds ratio. In nine investigations, certain alcoholic drinks were connected to an increased risk of bladder cancer. [264,265]

Bladder cancer possesses a high incidence, progression, and recurrence rate, according to data. It is critical to discover and screen individuals with preclinical bladder cancer, as well as monitor on a regular basis, postoperative bladder patients with cancer (Table 3). Bladder cancer is detected by cystoscopy examination and biopsy, imaging modalities, urinary cytology, fluorescence in situ

hybridization, and urine protein detection. Urinary cytology and cystoscopy/biopsy are currently considered the gold-standard diagnostic procedures for bladder cancer. The cystoscopy/biopsy procedure is invasive and may cause pain, bleeding, urinary tract infections, and other problems. Furthermore, cystoscopy may have difficulty detecting malignancies in isolated areas of the bladder, limiting its clinical use.[114] Cytology, on the other hand, is non-invasive, simple to use, inexpensive, and effective. This approach, however, has significant limitations, including limited sensitivity, low cost efficiency, an absence of interobserver variability, and technological instability. Furthermore, in various investigations, its sensitivity ranges from 11 to 76%. Several variables influence cytology sensitivity, including specimen quality, the amount of scrubbed cells, and pathologist skill. Non-invasive urine indicators are sometimes used to augment or replace cystoscopy as a way of detecting bladder cancer.[155] Furthermore, the pathologist's experience influences cytological interpretation. Because of these approaches' limitations, various urine-bound tests for early identification of cancer of the bladder have been established.

Nuclear Matrix Protein -22 and Bladder Tumour Antigen test have just been approved for clinical usage as bladder cancer markers. NMP-22 is found in the nuclear matrices of all cell types as well as the mitotic spindle throughout mitosis, and its presence is crucial in ensuring the proper flow of chromatin to the daughter. In detecting preclinical T-stage bladder cancer, NMP-22 is twice as effective as cytology, and it can be as sensitive as ninety percent and specific as ninety percent. It was shown that individuals with bladder cancer might have 25-fold higher urine levels of NMP22 than healthy persons.[165] Chou and colleagues [33] reported a meta-analysis in 2015 that discovered qualitative NMP22 with 69%

sensitivity and 77% specificity, as well as qualitative NMP22 with 83% specificity and 70% sensitivity. In a 2017 meta-analysis, Wang and colleagues[266] discovered a pooled sensitivity of 56% and specificity of 88% for bladder cancer detection across 19 studies. Our results were comparable, with 76% sensitivity and 94.85% specificity. NMP22 tracks the cell turnover that occurs when a bladder tumor's surface detaches. In benign situations like inflammation, infection, bladder stones, and hematuria, this process occurs, resulting in false-positive findings.

In a comparable manner BTA is a human complement factor H-associated protein (hCFHrp) that is produced in the culture medium by BCa cells but not by other epithelial cell lines. As the tumour invades the stroma, BTA is released into the urine of bladder cancer patients. According to early studies, the BTA test showed better sensitivity but poorer specificity than cytology.[160]

Four trials were analysed, and the results were comparable, having a sensitivity of 65% and a specificity of 74%. Sensitivity, like other indicators, demonstrated a positive connection with the total tumor grade of the BC. Sensitivity and specificity improved significantly, 73.33% and 95.58%, respectively. [163]

Using a ROC curve, the NMP-22 test and BTA TRAK outperformed conventional cytology, with AUCs of 0.867 and 0.851, respectively, compared to cytology's AUC of 0.625, and the distinction was of statistical significance (P = 0.015). When compared to the specificity and sensitivity of 100.0% 92.0% published by Abd El Gawad et al [267] in Egypt at a greater cutoff value of 78 units/mL, the current study has comparable sensitivity and specificity at a ROC curve cutoff value of 10.7 units/mL. 5-Aminolevulinic acid (5-ALA) is another

unapproved biomarker that has been employed. Around the world, it has been authorised as a photosensitizer of photodynamic diagnostic (PDD) for cancer. For example, ALA has been approved for use as an optical imaging medication to aid in the intraoperative detection of malignant gliomas and bladder cancer.[268] A recent investigation on 5-ALA staining of samples of urine during extracorporeal interaction discovered that PDD sensitivity is effective in BT (81% vs. 44%, respectively), especially in low-grade and low-stage tumours, and that specificity is equal (81% vs. 98%, respectively). However, they only evaluated 61 persons with BT, so there were significant drawbacks, including conventional cytology having extremely low sensitivity (18%) for low-grade BT. and not providing non-cancer group characteristics. In the same authors' experience, urine cytology accuracy is substantially lower, never topping 44%.[269] The current study covered a larger number of patients (n = 422). Our data revealed that the sensitivity of 5-ALA-induced fluorescence cytology was significantly greater for pTa stage cancers 88.63% (39/44) and low-grade tumours 91.45% (65/71) than that of commonly used biomarkers such as conventional cytology 63.63% and 57.74% (p=0.0045, p=0.001, p0.01). The difference in sensitivity between 5-ALA cytology and the other two tests is noticeable in Low and High Grades and Ta, T1 stages, not only in our series, but also in Yamamichi and colleagues' [270] in earlier comparison research, sensitivity increased steadily as you advanced in grade and stage in each one of the assessments.

False-positive 5-ALA cytology findings can be produced by a variety of factors, including inflammation, infection, hyperplasia, and inexperience, according to various sources. There were only six incidences of 5-ALA cytology false positives in the current study that were probably due to inflammation

associated with infection and calculi. There are two plausible explanations for this: first, cells were found in the urine samples that had been emptied, including second, urinary cellular components as well as cancer cells were deceased as time passed between samples pooling to pathological testing. In this circumstance, most cancer cells may decrease mitochondrial metabolic activity, and so 5-ALA cannot be metabolised. In the current work, we administered after a single hour of obtaining urine samples, the extracorporeal ALA was cultivated to avoid the death of these cancer cells.[271]

There are various limitations to this study. Because our study is based on a single institution's patients, the current findings must be reproduced in several cohorts to establish the high diagnostic effectiveness of 5-ALA-induced fluorescent urine cytology for UC. To develop a practical, non-invasive approach for detecting bladder cancer, all patients' voided urine samples were tested for NMP-22, BTA-TRAK, conventional cytology, and 5-ALA cytology. If the medical history and physical examination are suspicious/suggestive of bladder cancer, a non-invasive screening approach may provide an instant diagnosis while avoiding cystoscopy and biopsy. A urine test's ability to detect tumour relapse A helpful technique for selecting instances for control cystoscopies in the surveillance of superficial individuals who have bladder cancer.

There is an understanding and study desire to develop a simple efficient test that can screen a patient, operate as a kind of diagnostic, minimise the number of unnecessary biopsies, resulting in less morbidity and lower medical costs, and play a crucial part in assessing the success of treatment regimens. A successful biomarker for bladder cancer should (i) be inexpensive, tangible, swift to analyse, and easy to comprehend along with excellent sensitivity and specificity; (ii) reduce

the need for prevalent invasive procedures; (iii) detect recurrence; (iv) identify development to invasive disease; and (v) estimate a favourable response. Our findings reveal that 5-ALA-induced cytology has greater sensitivity and negative predictive values (NPVs) than established techniques like urine cytology, BTA-TRAK detection, and NMP-22 detection. Other research' findings corroborate ours. Our discovery using 5-ALA cytology provides a straightforward, reliable, viable, and efficient way to detecting BC that can be performed in the majority of cancer centres.

CONCLUSION

In conclusion, our study provides a highly accurate and noninvasive means of diagnosing Urothelial bladder cancer by using voided urine sample. The 5-ALA fluorescent Cytology is associated with high sensitivity (90.66%) irrespective of the grade of the tumour when compared with conventional cytology (62%) as well as the two FDA approved diagnostic tests namely NMP-22 test (76%) and BTA TRAK test (73.33%). 5-ALA fluorescent Cytology is not only highly sensitive (96.32%), but is also highly specific similar to conventional cytology (98.53%) but however, more specific than NMP-22 test (94.85%) and BTA TRAK test (95.58%).

It has been long desired to have a biomarker test which is reliable, cost-effective and non-invasive. 5-ALA fluorescent Cytology fulfills all these criteria. It is highly accurate, reproducible, easy to perform, non-invasive and inexpensive when compared to the presently available FDA approved tests. Our study is a single institutional study and these results need to be reconfirmed by multi-centric studies so that this test could be used in the diagnosis of Bladder cancer in patients with superficial bladder cancer, which are on follow-up thus avoiding expensive preoperative tests such as Ultra Sonography, CT scan and Invasive Cystoscopy.

As per our study bladder cancer has been linked to a number of risk variables. One such variable is the gender. The link between gender and cancer of the bladder is complicated, and it is probably affected by a number of biological or epidemiological variables. Numerous factors, including the importance of the steroid hormone structure, gender disparities in chemical exposure, metabolic enzyme activity, dietary differences, and India's use of nicotine and alcoholic drinks, might be driving these demographic shifts.

Our study has showed the use of tobacco chewing, cigarette smoking and tobacco contacting product to be a key risk factors in cause of bladder cancer. Our study, confirms the use of biomarkers in the diagnosis of Bladder cancer in clinical decision making and enhancing clinical outcomes.

FUTURE GOALS

Despite recent breakthroughs in molecular diagnostic and prognostic significance in UBC, advanced bladder cancer stages have higher morbidity and fatality rates. As a result, more precise and sensitive Urinary biomarkers are necessary for cancer treatment. The evidence presented shows that 5-ALA Cytology be used as a diagnostic technique for detecting low grade particularly flat UBC.

This investigation could potentially be carried out as follows using the already available data.:

- Validation of these biomarkers in urine samples is necessary to build a non-invasive diagnostic approach capable of replacing the present gold standard cystoscopy.
- Manipulation of 5-ALA Cytology using fluorescence intensity and frequency to determine bladder tumour stage, grade, and aggressiveness.
- Establish a technique for detecting UBC risk prediction models and, as a result, stratifying individuals.
- Evaluating 5-ALA fluorophore molecule with other instruments like flow-cytometry and spectrophotometry and compare the result with florescence microscopy cytology.
- The use of the 5-ALA molecule in clinical decision-making, i.e., predicting who will benefit from conventional treatment/therapy.

SUMMARY

Bladder cancer is a frequent type of cancer that is distinguished by the uncontrolled proliferation of cells that are abnormal in the bladder lining. Early identification of bladder cancer is critical to enhancing patient outcomes since the illness is largely curable when caught early. Urinary biomarkers are increasingly recognised as a promising technique in non-intrusive BCa detection and monitoring. Urine diagnostics markers are substances discovered in urine that signal the existence or progression of a disease. In the circumstance of bladder cancer, several urinary biomarkers have been investigated for their potential diagnostic and prognostic value. These biomarkers can be broadly categorized into genetic, epigenetic, protein, and metabolite markers.

Genetic biomarkers include alterations in specific genes or chromosomal regions associated with bladder cancer. Mutations in the TP53 gene, deletions or mutations in the FGFR3 gene, and ERBB2 gene amplifications are examples of genetic biomarkers. Several laboratory techniques, including polymerase chain reaction (PCR) and fluorescence in situ hybridization, are used, can identify these genetic alterations. Epigenetic biomarkers are DNA structural alterations that can impact gene expression without changing the basic sequence of DNA. DNA methylation is a common epigenetic alteration associated with bladder cancer. Methylation-specific PCR or other methylation detection methods can identify aberrant DNA methylation patterns in specific genes, such as RASSF1A, CDKN2A, or TWIST1, which may indicate the presence of bladder cancer.

Protein biomarkers are proteins that are either overexpressed or under expressed in bladder cancer compared to healthy individuals. Some commonly studied protein biomarkers for bladder cancer include BTA stat, NMP22, and UroVysion. These proteins can be measured using immunoassays, such as enzyme-linked immunosorbent assays

(ELISA) or immunohistochemistry (IHC), in urine samples. Metabolite biomarkers represent the metabolic products or by-products of cellular processes that can be altered in bladder cancer. Metabolomics studies have identified potential metabolite biomarkers such as sarcosine, lactate, and N-acetylglucosamine. Mass spectrometry and nuclear magnetic resonance spectroscopy are commonly used techniques to detect and quantify these metabolites in urine samples. While urine biomarkers show significant potential for bladder cancer diagnosis and surveillance, further research is still needed to validate their clinical utility.

Numerous investigations indicate that particular biomarkers have good sensitivity and specificity; however, the overall performance and consistency of these biomarkers across different patient populations and stages of bladder cancer remain to be established. The detection of urothelial bladder carcinoma, commonly known as bladder cancer, is crucial for early diagnosis and treatment. Several urinary biomarkers, in addition to standard diagnostic approaches such as urine cytology, have been investigated to increase the precision and effectiveness of bladder cancer diagnosis. This summary provides an overview of the role of urine-based biomarkers, including 5-aminolevulinic acid (5-ALA) cytology, Conventional cytology, nuclear matrix protein 22, and bladder tumor-associated antigen TRAK, in the detection of urothelial bladder carcinoma.

4 Urine Cytology: It is a frequently used method which involves examining urine samples under a microscope to detect cancer cells discharged from the bladder lining. While it is non-invasive, its sensitivity is modest, particularly for low-grade and early-stage bladder tumours. False-negative findings are widespread, which limits its use as a single diagnostic tool.

5 5-Aminolevulinic Acid (5-ALA): It is an initial form of the photosensitive agent protoporphyrin IX seen in cancer cells. It is utilised in photodynamic diagnostics

(PDD) to improve bladder cancer detection during cystoscopy. 5-ALA is metabolised in the body and preferentially accumulates in cancer cells, making them luminous under blue light illumination when taken orally or intravenously. This approach enhances the visibility of tumour lesions during cystoscopy, which aids in the identification of bladder cancer.

6 Nuclear Matrix Protein 22 (NMP-22): In bladder cancer cells, it constitutes a nuclear matrix protein which is overexpressed. Immunoassays can be used to detect it in urine samples. NMP-22 testing is a non-invasive approach for detecting bladder cancer that has demonstrated encouraging results for tumours of both the highest and lowest grade. However, the level of sensitivity and specificity varies depending on the investigation, and results that are false-positive can arise owing to various benign urological problems or infections of the urinary tract.

7 Bladder Tumor-Associated Antigen (BTA) TRAK: It is a class of urinary biomarkers which detects complement factor H-related protein (CFHR)-1 and bladder tumour antigen (BTA) in urine. These biomarkers have been linked to the existence of bladder cancer. BTA TRAK tests use immunoassay methods to diagnose bladder cancer in a non-invasive manner.

They had low sensitivity and specificity., and combining them with urine cytology can improve diagnostic accuracy. Total number of 422 subjects were enrolled during the study period of 36 months, in that 150 subjects were Bladder cancer confirmed cases, 150 lower urinary tract symptoms cases and 122 controls. The incidence of Bladder cancer in our hospital was 3.4%. Age was similar in cancer cases (65.28 ± 17.63 years) when compared with controls (65.25 ± 18.15 years). There was significant difference in Occupation mainly for subjects with Farming and Industrial background. For Co-morbidities prevalence subjects and patients with Signs and Symptoms there was no

significant association for cases and controls. For Dietary Habits, Meat eating and dairy consumers were significantly associated for bladder cancer ($p=0.019$, $p<0.05$). Lifestyle habits like smoking, tobacco chewing and alcohol also showed higher relative risk between 1.5-4 folds among bladder cancer cases.

Bladder cancer cases for low grade tumours were 71 and High grade tumours were 79; and for tumour aggression Non-muscle invasion bladder cancer cases were 105 and Muscle invasion bladder cancer were 45. Our results showed result of 76% sensitivity and 94.85% specificity respectively for NMP-22 test. BTA has also been identified as a human complement factor H related protein (hCFHrp), which is produced in cell culture by bladder cancer cells but not in other epithelial cell lines. According to early studies, the BTA test showed better sensitivity but poorer specificity than cytology. Sensitivity, like other indicators, demonstrated a significant connection to rising tumour grade of the BC. Sensitivity and specificity improved significantly, 73.33% and 95.58%, respectively.

Using a ROC curve, the NMP-22 test and BTA TRAK outperformed conventional cytology, with AUCs of 0.867 and 0.851, respectively, compared to cytology's AUC of 0.625, and the distinction was of statistical significance ($P = 0.015$). In the current investigation, the sensitivity and specificity are equivalent, with a ROC curve the threshold value of 10.7 units/mL. 5-Aminolevulinic Acid (5-ALA) is another non-FDA authorised biomarker that has been employed. Our findings revealed that the sensitivity of 5-ALA-induced fluorescence cytology was significantly higher for pTa stage malignancies 88.63% (39/44) and low-grade tumours 91.45% (65/71) than for commonly used biomarkers such as conventional cytology (63.63% and 57.74%, respectively). ($p=0.0045$, $p=0.001$, $p<0.01$).

The difference in sensitivity between 5-ALA cytology and the other two tests is most noticeable in Low and High Grades, Ta, T1 stages, and in the sample we provided (sensitivity 95% in Ta and 95% in T1; 82% in LG and 97% in HG using 5-ALA cytology). There were only six instances of 5-ALA cytology false positives in the current study, that were most likely due to inflammation associated with infection and calculi. There are two possible causes for this: cellular elements in the voided urine samples, and urinary cellular components, in addition to cancer cells, expired during the time before sample pooling and pathological evaluation. In this case, most cancer cells may reduce mitochondrial metabolic activity, preventing 5-ALA from being metabolised. Finally, with goal of developing a reliable, non-invasive approach for diagnosing bladder cancer, all patients' voided urine samples were tested for 5-aminolevulinic acid (5-ALA), cytology, nuclear matrix protein 22 (NMP-22), and bladder tumor-associated antigen (BTA) TRAK). While each biomarker has benefits and disadvantages, 5-ALA-induced fluorescent urine cytology consistently shown good diagnostic effectiveness and accuracy for bladder cancer diagnosis. Furthermore, it may be employed in the future as an upgraded tool in earlier treatment and enhanced outcomes for patients. According to our findings, 5-ALA-induced cytology has higher sensitivities and NPVs than established approaches such as urine cytology, BTA-TRAK detection, and NMP-22 detection. Our findings agree with those of other studies. Our research employing 5-ALA cytology revealed a simple, reliable, feasible, and efficient approach for diagnosing BC that could be performed in most cancer centres.

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ANNEXURE - I

12.1 ETHICAL CLEARANCE LETTER

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Formerly known as KLE University)
(Deemed-to-be-University established u/s 3 of the UGC Act, 1956)
Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)
JNMC Campus, Nehru Nagar, Belagavi-590 010, Karnataka State, India
☎: 0831-2444444 FAX: 0831-2493777 Web: http://www.kledeemeduniversity.edu.in E-mail: info@kledeemeduniversity.edu.in

Ref.No.KAHER/EC/20-21/ 001/05 4th September 2020

To,
Mr. Shadab Rangrez
Full-Time Ph.D. Research Scholar,
2019-20 Batch, Faculty of Medicine,
KAHER, Belagavi.

Dear Research Scholar,

The KAHER Ethics Committee on Human Subjects for Ph.D. Research Project met on **Thursday, the 20th February, 2020** to consider your application for approval of the research project **"Validation Of Biomarkers In Patients With Urothelial Bladder Carcinoma."**

As there are no ethical issues involved in your proposed research project, the committee has provided approval for this research project.

You are requested to report to Ethical Committee of the following:

1. Any deviation from or change of the protocol.
2. Any changes in study documents.

(Dr. Anita Dalal)
Member-Secretary
Ethical Committee (Human) for Ph. D. Research
KAHER, Belagavi.

(Dr. B.C. Kotintot)
Chairman
Ethical Committee (Human) for Ph. D. Research
KAHER, Belagavi.

CC to: - The Director Research Foundation, KAHER, Belagavi.
- The Director Academic Affairs, KAHER, Belagavi.
- The Registrar, KAHER, Belagavi.
- Special Officer to Hon. Vice Chancellor, KAHER, Belagavi.

ANNEXURE - II

12.2 PATIENT INFROMATION SHEET

"Validation of Biomarkers in patients with Urothelial Bladder Carcinoma"

PRINCIPAL INVESTIGATOR: Mr. Shadab Rangrez
Ph.D. Research Scholar (Full Time)
Faculty of Medicine
J.N. Medical College, KAHER, Belgaum

GUIDE: Dr. R.B. Nerli
Professor and HOD
Department of Urology
J.N. Medical College, KAHER, Belgaum

INTRODUCTION AND PURPOSE:

This study is being carried out to validate biomarkers for diagnosis of urinary bladder cancer. The present method for used for diagnosis is cystoscopy which is an invasive procedure and painful to the patients. Therefore, we are working on non-invasive techniques to validate biomarkers using urine samples.

PROCEDURE:

First voided urine sample will be collected prior to cystoscopy. This sample will be used for various tests viz. routine cytology, fluorescence microscopy, kit method and metabolite profiling.

RISKS AND BENEFITS:

It is highly unlikely that the patient will be injured from their participation in this study. This procedure will be carried out at the time when the patient is undergoing routine urine tests. Hence, the patient will not need to get an additional risk for the urine sample.

PRIVACY AND CONFIDENTIALITY:

No names will be associated with the study data, which will be coded. Access to coded data in computers is by access codes and will be limited to the investigators.

INSTITUTION / SPONSOR'S POLICY:

Not applicable for this study.

FINANCIAL INCENTIVES FOR PARTICIPATION:

No incentive or gifts will be offered to the patients for their participation.

AUTHORIZATION TO PUBLISH THE RESULTS:

The results of the study will be published in the form of collective data, without an disclosure of the patient information.

QUERIES AND CONTACT:

In case of the queries during study or in future you may contact following persons,

Dr. R.B. Nerli

Professor

Department of Urology

JNMC, KAHER, Belgaum

Mr. Shadab Rangrez

Investigator,

Ph.D. Research Scholar,

JNMC, KAHER, Belgaum

Mob: 9008553021

CONSENT FORM:

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any of my legal rights by signing this form. My signature below indicates that I have read this consent form, or it has been read to me, this consent form and have had all the questions answered.

Signature / Left Thumb print of the Participant or legally authorized Representative

Participant's name:	
Signature / Left Thumb impression of the participant:	
Name of the legally authorized representative / guardian:	
Signature / Left thumb impression:	
Witness' Name:	
Signature / Left impression:	
Investigator's name and signature:	

Date:

Place:

ANNEXURE - III

12.3 PROFORMA

Detailed Proforma of the Patient					
OPD/ IPD Number:					
Name:					
Date of Sampling:			**Place of Sampling:**		
Address (Rural/Urban):					
Gender:	Female □		Male □		
Stage:			**Grade:**		
Education:	illiterate □	10* □	>10*□	Diploma □	Higher Education □

Occupation:				
Job of patient				
If he/she is working in a chemical industry/mine, what industry/mine?				
Name of the compounds/chemicals to which the patient supposedly is exposed:				
If he/she is working in farms (agricultural activities)?				
Do they generally/mostly use or are exposed to pesticides?				
Name of the pesticides/compounds:				
Is there any plant or factory near the residential area of the patient?				
What kind of factory?				
The distance is about:	< 1 km □	1-5 km □	6-10 km □	>10 km □

Smoking habits:	YES □		NO □	
Type of smoking:	Cigarette □	Beedi □	Cigar/Pipe □	Any other
The status of inhalation:	Filtered □		Non-filtered □	
At what age was smoking started by the patient?	How many cigarettes/beedi in a day are smoked by the patient?			
How long has the patient been smoking?	<5 years □	5-10 years □	11-20 years □	>20 years □
Does the patient chew tobacco/hukka/betel nut/pan parag?	YES □		NO □	
Does the patient use any kind of addicted drugs?	YES □		NO □	
Is there a member in the family, who is a smoker?	YES □		NO □	

Dietary Habits:				
Patient is:	Pure veg. □	Most frequent ve	Most frequent non-veg. □	
If the patient is non-vegetarian, what kind of meat does he/she eat:	Chicken □	Lamb/Mutton □	Buff □	Fish (other sea foods) □
How many cups of milk does the patient consume per day?	Never □	1 cup/day □	2 cups/day □	More than twice □
Does the patient use a lot of milk/dairy products daily like cheese, curd, lassi etc. which are calcium				
What kind of oil/fat is usually used for cooking food?	Butter □	Olive Oil □	Refined Oil □	Mustard Oil □
What kind of utensil/container is generally used for cooking?	Steel □	Copper □	Non-Stick □	Other □
How many glasses of water does the patient consume per day? (1 litre ~ 4 glasses)	1 litre or lesser □	2 litres □	3 litres of more □	
What is the source of water	Natural Water □	Boiled/Filtered	Mineral water from the r	quaguard/other refining

Medication and Health History:		
Does the patient have blood pressure?	YES □	NO □
Does the patient have diabetes?	YES □	NO □
Did/does the patient have any kind of serious disease/lesion in his/her uro-	YES □	NO □
Is there anybody in his/her family or close relatives who has/had cancer of	YES □	NO □
Has the patient been on any medication or any vitamin for a long time	YES □	NO □
Has the patient ever been exposed to UV, X-ray or any other radiation due	YES □	NO □
Is he/she exposed to sun-light for more than 3 hours a day?	YES □	NO □
Has the patient used hair dyes or any other related cosmetic compound?	YES □	NO □
Is/was the patient under a lot of stress/tension during his/her job or due to	YES □	NO □
Important Remarks:		

Patient's Signature

ANNEXURE - IV

PAPER PUBLICATIONS

5-ALA-Induced Fluorescent Cytology in the Diagnosis of Bladder Cancer—a Preliminary Report

Rangrez Shadab, Rajendra B. Nerli, Bidi

R. Saziya, S. C. Ghagane,Chandra Shreya

Indian Journal of Surgical Oncology

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Indian Journal of Surgical Oncology
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ORIGINAL ARTICLE

5-ALA-Induced Fluorescent Cytology in the Diagnosis of Bladder Cancer—a Preliminary Report

Rangrez Shadab[1] · Rajendra B. Nerli[1] · Bidi R. Saziya[2] · S. C. Ghagane[2] · Chandra Shreya[2]

Received: 6 October 2020 / Accepted: 29 April 2021

Abstract
5-Aminolevulinic acid (ALA)-induced fluorescence cystoscopy has established itself in the detection of flat and/or small lesions. This is explained by the simple fact that there is increased uptake of ALA, altered activity of certain enzymes, and altered intracellular redistribution and storage of protoporphyrin IX (PPIX) in the malignant cells. Intracellular PPIX allows red fluorescence detection. In this preliminary study, the efficacy of 5-ALA-induced fluorescent urine cytology was compared with conventional cytology in the diagnosis of bladder tumours. In this prospective study, patients ≥18 years of age admitted to the department of urology with non-malignant conditions formed the controls and patients ≥18 years of age with imaging confirmed bladder tumours formed the study group. Freshly voided urine sample was collected from these patients and divided into two samples of 50 cc each. One of these samples was sent in for conventional cytology examination, whereas the other sample was sent in for 5-ALA fluorescent photo dynamic diagnosis. Conventional cytology and 5-ALA-induced fluorescent cytology were evaluated by the same pathologist. A total of 100 patients were included in the study of which 75 patients were controls and the remaining 25 were patients with bladder tumours. The sensitivity of conventional cytology and 5-ALA-induced fluorescent cytology was 64% and 100% respectively, whereas the specificity was 96% and 98.67% respectively. The sensitivity of conventional cytology was 61.19% in low-grade cancers as compared to 75% in high-grade cancers, whereas the sensitivity was 100% with 5-ALA-induced fluorescent cytology both in low- as well as high-grade cancers. Our study shows that 5-ALA-induced fluorescent cytology is highly sensitive test to diagnose bladder cancer and shows a significant difference especially in low-grade bladder cancer when compared to conventional cytology.

Keywords 5-Aminolevulinic acid · Urothelial carcinoma · Cytology · Sensitivity · Specificity

Introduction

There has been an increased attention focused recently on the use of photodynamic technology using 5-ALA (5-aminolevulinic acid) as a photosensitizer to solve the clinical problem of bladder cancer.[1, 2] ALA-PDD (photo dynamic diagnosis) is indeed useful to detect and identify CIS (carcinoma in situ) in the bladder during TURBT (Transurethral resection of bladder tumour). 5-ALA is a natural amino acid found in animals and plants that is a common precursor of haemoglobin and chlorophyll. Endogenous 5-ALA, synthesized from succinyl coenzyme A and glycine in mitochondria, and exogenously administered 5-ALA follow the same metabolic synthetic pathway.[3] Protoporphyrin IX (PpIX) is a metabolic product of 5-ALA and accumulates in the mitochondria following its administration[4] PpIX is then catalysed by ferrochelatase and binds with ferrous ion resulting in the production of heme.[3]

Cancer cells utilize the glycolysis pathway for production of adenosine triphosphate (ATP), but do not run the tricarboxylic acid cycle or electron transport chain in mitochondria even in normoxia, which is called the Warburg effect and is typical for hyperplasia.[3] The ferrochelatase is inactive in these conditions, because of the lack of electron supply from the tricarboxylic acid cycle, which is essential for reduction of ferric ion to ferrous ion to complete the production of heme by

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ferrochelatase.[3] Biological features that are common to cancer cells, such as abnormal activity of transporters including ATP-binding cassette transporter (ABCG2) and porphyrin synthetic enzymes, can promote PpIX production and inhibit PpIX catabolism. This results in excess accumulation of PpIX in cancer cells. In particular, PpIX is accumulated 9–16-fold higher in urothelium and is highly tumour selective.[3, 5] PpIX is photoactive and gets excited at certain wavelengths of light, particularly visible blue light (375–445 nm), and emits red fluorescence (600–740 nm).[3] This has been the underlying principle of ALA-PDD.[6, 7] Similarly, this principle has been used to make the diagnosis of bladder cancer.[8–10]

The feasibility of diagnosing bladder cancer using 5-ALA was first reported in 1994.[11] Kriegmair et al.[11] instilled 5-ALA intravesically in 68 patients, followed by fluorescence cystoscopy with violet light from a krypton ion laser that produced fluorescence excitation. Tumour lesions were sharply marked with brightly shining red fluorescence. Correlation of fluorescence and microscopic findings gave a sensitivity of 100% and a specificity of 68.5%. Currently, 5-ALA is approved as a photosensitizer of PDD for carcinoma around the world.[12] Recently, Nakai et al.[13] reported on the 5-ALA staining of urine specimens and showed that PDD sensitivity to be effective, compared with conventional cytology in bladder tumours (82% vs. 49%, respectively), particularly in low-grade and low-stage tumours, and to have comparable specificity (80% vs. 100%, respectively). In this preliminary study, we have evaluated the efficacy of 5-ALA-induced fluorescent urine cytology in comparison with conventional cytology in the diagnosis of bladder tumours.

Materials and Methods

This prospective study was conducted after obtaining clearance from the University/Institutional ethical committee. Patients ≥18 years of age admitted to the department of urology with non-malignant conditions such as benign prostatic hyperplasia (BPH), urinary stone, urinary tract infection (UTI), uretero-pelvic junction (UPJ) obstruction, and radiological cystitis formed the controls. Patients ≥18 years of age with imaging (ultrasonography/computed tomography) confirmed bladder tumours formed the study group. Freshly voided urine sample was collected from these patients and divided into two samples of 50 cc each. One of these samples was sent in for conventional cytology examination, whereas the other sample was sent in for 5-ALA fluorescent cytology diagnosis.

Urine Samples and Treatment with 5-ALA The urine sample was centrifuged at 1500 rpm for 5 min and the supernatant was decanted. The pellet was suspended in minimum essential medium (MEM) with 5-aminolevulinic acid hydrochloride (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, 2020), and the concentration was adjusted to 200 µg/mL. Then, the suspension was stored in the dark at 37 °C for 2 h. After that, the sample was centrifuged again at 1500 rpm for 5 min, and the pellet was resuspended in MEM. Finally, the urine sample was tested for protoporphyrin IX fluorescence using a fluorescent microscope (Nikon ECLIPSE Ni; Nikon Corporation, Tokyo, Japan) at appropriate settings (excitation wavelength of 405 nm and emissions wavelength of 600–650 nm).

Evaluation Conventional cytology and 5-ALA-induced fluorescent cytology were evaluated by the same pathologist using the same urine sample. The conventional urine cytology was considered either negative or positive for malignant cells based on the "The Paris system for reporting urinary cytology". The 5-ALA-induced fluorescent cytology showing no red light or dark red was classified as negative and that showing clear red as positive. The final reading was confirmed by two pathologists (Fig. 1).

Comparison with Histopathology All patients with imaging confirmed bladder tumour underwent cystoscopy/biopsy/transurethral resection of bladder tumour. The surgical specimens were sent in for histopathological examination and reported by the same pathologist. The reports of histopathology were compared to results of conventional cytology and 5-ALA induced fluorescent cytology.

Statistical Analysis Data was analysed using the Wilcoxon test or chi-square test. Differences were considered statistically significant when $p < 0.05$. Statistical analyses were performed using (SPSS version 22.0. Armonk, Chicago, USA).

Results

During the study period Dec 2019–Aug 2020, a total of 100 patients were included in the study of which 75 patients were controls and the remaining 25 were patients admitted to the urology wards with imaging confirmed bladder tumours. The demographics of the patients was as shown in Table 1. Of the 75 patients who were controls, 20 (26.67%) had urinary stones, 30 (40%) had benign prostatic hyperplasia, 20 (26.67%) were admitted for reconstructive surgery, and 5 (6.67%) had chronic renal failure awaiting surgery.

The conventional cytology was negative for malignant cells in 72 patients in the control group (Table 2) whereas it was negative in 74 patients with 5-ALA-induced fluorescent cytology. Conventional cytology was positive for malignant cells in 16/25 (64%) cases, whereas ALA-induced fluorescent cytology was positive in all 25 (100%) cases of bladder tumours. The specificity of conventional cytology in

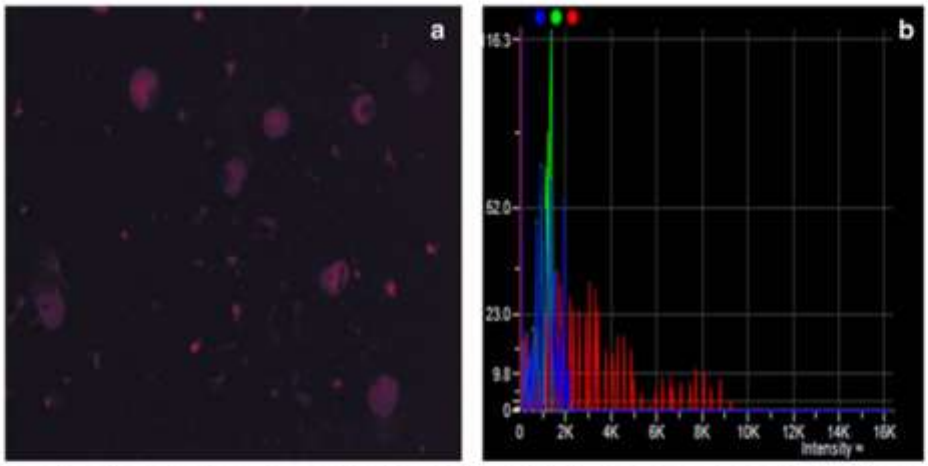

Fig. 1 **a** shows cells emitting faint red fluorescence suggestive of a non-malignant cell. **b** Histogram of frequency (x-axis) versus intensity (y-axis) of a non-malignant cell

comparison with 5-ALA-induced fluorescent cytology was 96%versus 98.67% (Fig. 2).

When histopathological results of the patients with bladder cancer were compared with conventional cytology and 5-ALA-induced fluorescent cytology, it was found out that latter was more sensitive and specific, more so in low-grade cancers. The sensitivity of conventional cytology was 61.19% in low grade cancers as compared to 75% in high-grade cancers, whereas the sensitivity was 100% with 5-ALA-induced fluorescent cytology both in low as well as high-grade cancers (Fig. 3).

Discussion

Bladder cancer is a common disease affecting both men and women. Its incidence ranks first among malignant cancers of the urinary system and second only to prostate cancer in the Western world. More than 90% of bladder cancers are of the transitional cell variety, 5% are squamous cell carcinomas, and less than 2% are adenocarcinomas.[14] At initial diagnosis of bladder cancer, 70 to 85% are non–muscle-invasive bladder cancers (NMIBC) and 15 to 30% are muscle-invasive bladder cancers (MIBC).[15] NMIBC is also known as superficial bladder cancer and includes pathological stages Ta (papillary), T1 (infiltration of lamina propria), and carcinoma in situ. Ta patients make up 70% of cases, T1 roughly 20% and carcinoma in situ about 10%. MIBC is commonly known as invasive bladder cancer and includes pathological stages T2, T3, and T4.[15, 16] Up to 80% of NMIBC patients relapse within 5 years; 30% of Ta patients progress to MIBC, while those with T1 and carcinoma in situ are more likely to develop MIBC.[1, 17] Statistically bladder cancer is associated with high incidence, progression, and recurrence rates.[18]

It is extremely important to accurately diagnose and assess patients with early bladder cancer and also to monitor high-risk postoperative bladder cancer patients on a regular basis (Table 3). Bladder cancer is commonly diagnosed using cystoscopy and biopsy, imaging methods, urinary cytology, fluorescence in situ hybridization, and urine protein detection.[1, 19] As of today, urinary cytology and cystoscopy/biopsy remain the gold standard examination tools to make a diagnosis of bladder cancer. Cystoscopy/biopsy is invasive and is associated with pain, bleeding, urinary tract infections and other complications. Moreover, it may be difficult for cystoscopy to detect tumours in secluded corners of the bladder, which restricts its clinical application. In comparison cytology is non-invasive, simple to use, inexpensive, and performs well, although it has low sensitivity and low diagnostic efficiency, especially with low-grade bladder cancer.[18]

Table 1 Demographics of patients

No	Variables	Controls (*n* 75)	Bladder tumour (*n* 25)
1	Age (years)<40	20 (26.67%)	–
	41–50	5 (6.67%)	5 (20%)
	51–60	30 (40%)	–
	61–70	15 (20%)	10 (40%)
	71–80	5 (6.67%)	10 (40%)
	Median Age	56 ± 15.75	67 ± 12.54
2	Gender Male	45(60%)	25(100%)
	Female	30(40%)	–
3	Co-morbidities hypertension	20 (26.67%)	10 (40%)
	Type II diabetes M	10 (13.33%)	5 (20%)

Table 2 Results of conventional cytology in comparison with 5-ALA fluorescent cytology

	Conventional cytology		5-ALA fluorescent cytology	
	negative	positive	negative	Positive
Controls (n =75)	72	3	74	1
Bladder tumour (n =25)	9	16	–	25
Sensitivity	64%		100%	
Specificity	96%		98.67%	

5-Aminolevulinic acid (ALA)-induced fluorescence cystoscopy has established itself in the detection of flat and/or small lesions that are barely visible under white-light cystoscopy.[20, 21] Fu et al.[22] investigated ex vivo urine fluorescence cytology as a biopsy-free means for detecting bladder cancers. Sediment of urine samples was extracted and incubated with a novel photosensitizer, hypericin. Laser confocal microscopy was used to capture the fluorescence images at an excitation wavelength of 488 nm. Images were subsequently processed to single out the exfoliated bladder cancer cells from the other cells based on the cellular size. The study suggested that the fluorescence intensity profiles of hypericin in bladder cells could potentially provide an automated quantitative means of early bladder cancer diagnosis.

Pytel and Schmeller[23] investigated urinary cytology for induced fluorescence of urothelial cells and detected by fluorescence microscopy. The results were compared with the conventional cytologic and histologic findings. They detected ALA-induced fluorescence in 34 of 38 cases. One of the four histologically negative cases had a false-positive finding and 1 case of urothelial carcinoma did not show fluorescence. They concluded that fluorescence cytology was more sensitive than other non-invasive tests. Tauber et al.[24] evaluated as to whether tumour cells could be detected in bladder lavage fluid samples. Lavage sediments of all patients with histologically confirmed TCC bladder caused red fluorescence peaking at 635 nm, indicating protoporphyrin IX. The authors concluded that lavage sample sediments could be used to detect tumour associated red fluorescence in TCC of bladder.

Miyake et al.[25] used three different modalities of 5-aminolevulinic acid (ALA)-based photodynamic diagnostic tests to diagnose bladder cancer. They developed a compact-size, desktop-type device quantifying red fluorescence in cell suspensions, named "Cellular Fluorescence Analysis Unit" (CFAU). Urine samples from 58 patients with bladder cancer were centrifuged, and urine sediments were then treated with ALA. ALA-treated sediments were subjected to three fluorescence detection assays, including the CFAU assay. The overall sensitivities of conventional cytology, fluorescence cytology, fluorescent spectrophotometric assay, and CFAU assay were 48%, 86%, 86%, and 87%, respectively. The three different ALA-based assays showed high sensitivity and specificity. The ALA-based assay detected low-grade and low-stage bladder urothelial cells at higher rate (68–80% sensitivity) than conventional urine cytology.

Yamamichi et al.[26] evaluated the diagnostic efficacy of 5-ALA-induced fluorescent urine cytology for urothelial carcinoma. They included 318 patients comprising 158 non-cancer patients, 84 bladder tumour patients, and 76 upper urinary tract urothelial carcinoma patients. The sensitivity of 5-ALA-induced fluorescent urine cytology was significantly higher than that of conventional urine cytology (86.9% vs.

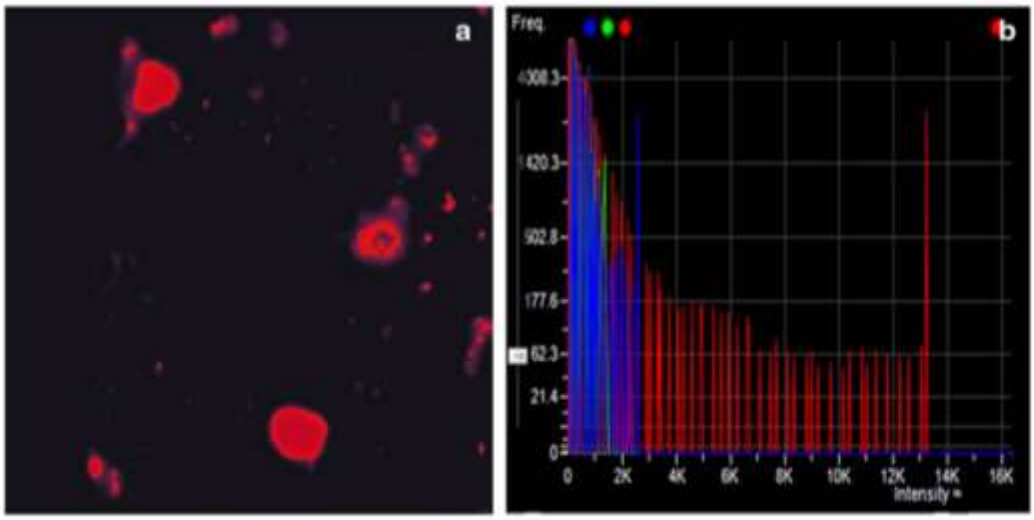

Fig. 2 **a** shows cells emitting intense red fluorescence suggestive of malignant tumour cells at ×200 magnification. **b** Histogram of frequency (x-axis) versus intensity (y-axis) of a malignant tumour cell

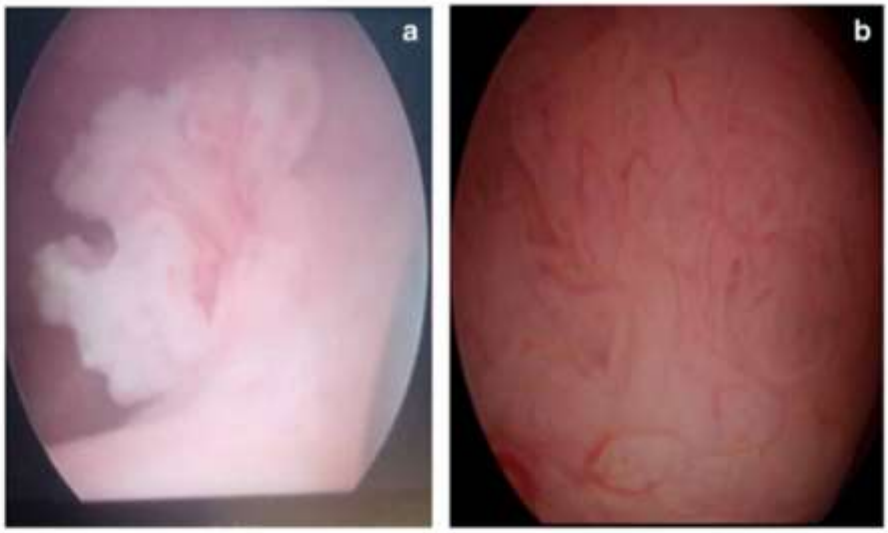

Fig. 3 Cystoscopic images of bladder tumours. **a** shows a papillary lesion. **b** shows a sessile lesion

69.4%; $p = 0.0002$), and the specificity was equivalently high (96.2% vs. 95.6%; $p = 1.0$). In subgroup analysis, the high sensitivity of 5-ALA-induced fluorescent urine cytology was also detected regardless of age, sex, and tumour type. However, in terms of stage and grade, differences were only detected in patients with less than pTa stage (89.2% vs. 52.1%; $p = 0.0001$) and low-grade tumour (91.5% vs. 51.1%; $p < 0.0001$). The authors concluded that 5-ALA-induced fluorescent urine cytology was significantly more effective for urothelial carcinoma diagnosis when compared with the conventional cytology, especially in patients with low-stage and low-grade tumours.

All these abovementioned studies show that 5-ALA-induced fluorescent cytology is highly sensitive even in low-grade and low-stage tumours. These findings suggest that it might be possible to follow up using only 5-ALA-induced fluorescent cytology without cystoscopy in low- and intermediate-risk bladder tumours. Occasionally, false-positive findings of 5-ALA can be reported in conditions such as infection, inflammation, hyperplasia, and inexperience with the use of PDD.[27–29] 5-ALA-induced fluorescent cytology is associated with false negative reports whenever there is lack of cellular components in the voided urine specimen, or when cellular components including the cancer cells die out over the passage of time.

Table 3 Bladder cancer stage and grade

No	Bladder cancer (n =25)	Conventional cytology n (%)	5-ALA-induced fluorescent cytology n (%)	Total
1	Stage pTa	8(72.72)	11(100)	11
2	pTis	2(40)	5(100)	5
3	pT1	3(60)	5(100)	5
4	≥pT2	3(75)	4(100)	4
5	Low grade	13(61.19)	21(100)	21
6	High grade	3(75)	4(100)	4

Conclusion

Our study shows that 5-ALA-induced fluorescent cytology is highly sensitive test to diagnose bladder cancer and shows a significant difference especially in low-grade bladder cancer when compared to conventional cytology. Our study has several limitations that include small sample size and single-center study. Our results need to be further validated in other cohorts so as to identify the high diagnostic efficacy of 5-ALA-induced fluorescent urine cytology for bladder cancer.

Declarations

Conflict of Interest The authors declare no competing interests.

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Original Article

Risk Factors for Bladder Cancer: Results of a Survey of Hospital Patients

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Abstract

Introduction: Several risk factors have been identified in the occurrence of bladder cancer. These include genetic and hereditary factors, smoking and tobacco use, increased body mass index, occupational exposure to certain chemicals and dyes, medical conditions such as chronic cystitis and infectious diseases such as schistosomiasis. This study aimed to evaluate risk factors in patients with bladder cancer. **Materials and Methods:** All patients presenting to the uro-oncology department of the hospital with imaging and histology confirmed bladder cancer were included in the study. Age- and gender-matched patients presenting to the department of urology with benign disorders were prospectively included as controls. All the study subjects and the controls completed a self-administered structured questionnaire. **Results:** Seventy-two (67.3%) of the participants with bladder cancer were males. The mean age of participants with bladder cancer was 59.24 ± 16.28 years. Most participants with bladder cancer worked as farmers (35.5%) or industrial workers (24.3%). Recent history of recurrent urinary tract infections was seen in 85 (79.4%) of the participants with bladder cancer and 32 (30.8%) of controls. Diabetes mellitus was more common among participants with bladder cancer. A significant number of participants with bladder cancer used tobacco and smoked compared to controls. **Conclusions:** This study highlights numerous potential biological and epidemiological factors that may act as a risk factors for bladder cancer. These factors could explain the gender differences observed in the incidence of bladder cancer. In addition, the study indicates the intense risk of tobacco products and smoking on the incidence of bladder cancer.

Key words: Alcohol, bladder cancer, epidemiology, gender, genetics, occupation, risk factors, tobacco smoking

Introduction

The bladder is an organ that remains in constant contact with the environment and is, therefore, sensitive to the effects of environmental carcinogens and inflammation.[1] Tobacco smoking and occupational hazards account for the two most frequent routes of environmental exposure. Tobacco smoke is full of aromatic amines. These amines, when hydroxylated, lead to DNA adduction and damage.[1]

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Original Article

Several risk factors have been identified for the occurrence of bladder cancer. These include genetic risk factors, hereditary factors, smoking and tobacco use, increased body mass index, occupational exposure to certain chemicals and dyes, medical conditions such as chronic cystitis and infectious diseases such as schistosomiasis. Indirect medical causes of bladder cancer are typically unintended side effects of treatment with certain drugs such as anti-diabetic drug pioglitazone, radiation, chemotherapy agents such as cyclophosphamide and environmental pollution through exposure to arsenic in drinking water.[1]

Bladder cancer is a global disease, with a worldwide incidence of 540,000 new cases and 188,000 deaths in 2015.[1] Internationally, the incidence of bladder cancer varies about 10-fold. The disease is reported most often in Europe and North America and least often in several areas of Asia.[2] Epidemiologic data clearly show that bladder cancer is much more common among men, Caucasians, and the elderly.[3] In most high-income countries, men have at least a 3 times greater risk than women.[4]

This study aimed to review the current state of knowledge regarding Bladder cancer risk factors, including factors related to occupation, diet, fluid intake, tobacco and smoking habits and concurrent medical conditions.

Materials and Methods

The research was carried out at the Department of Urology of Doctor Prabhakar Kore Hospital and Medical Research Centre in Belagavi between September 2019 and May 2022. This prospective study was taken up after obtaining ethical clearance from the Institutional Review Board (KAHER/EC/20-21/001/05). All patients presenting to the uro-oncology outpatient department, the hospital admitted patients with signs or symptoms suggestive of bladder cancer and patients followed up for a history of treated bladder cancer were included in the study as cases. Age- and gender-matched patients presenting to the department of urology with symptoms and diagnosis of benign prostatic hyperplasia, urinary stones, urinary tract infection and voiding dysfunction were prospectively included as controls.

All the study subjects and the controls completed a self-administered structured questionnaire (*available on request from the corresponding author*). Trained interviewers monitored the process. The questionnaire collected information on sociodemographic factors, lifestyle habits and diet 2 years before diagnosis, anthropometric measurements, problem-oriented medical history and family history of cancer. Two-specific sections investigated lifetime occupational exposure and exposure to chemicals known (or suspected) to be related to bladder cancer, including the use of hair dyes. Information on smoking included lifetime status (i.e., never, former, or current smoker), the daily number of cigarettes/beedis and grams of tobacco chewed, age at starting, duration of the habit and age at stopping for former smokers.

Odds ratios (ORs) and the corresponding 95% confidence intervals (CIs) were calculated using unconditional logistic regression models. ORs for status, intensity and duration of years were adjusted [Figure 1].

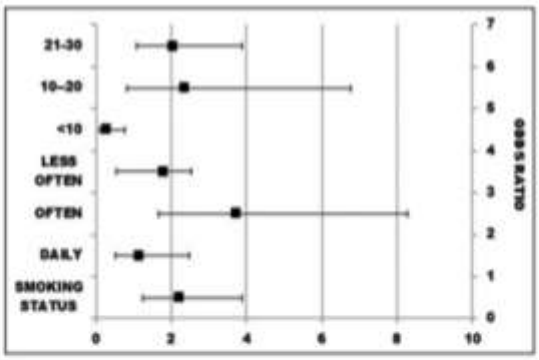

Figure 1: Shows Odds ratios (ORs) and corresponding 95% confidence interval (CI) for smoking status, daily exposure, and duration of years.

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Table 1: Age and gender distribution of patients with bladder cancer

Variables	Bladder cancer (%)			Controls n (%)			*P*-value
Males	72			68			0.001
Females	35			36			
Age (years)	Males	Females	Total	Males	Females	Total	
<30	3	2	5 (4.7%)	4	2	6 (5.8%)	0.001
31–60	32	17	49 (45.8%)	29	17	46 (44.2%)	
>70	37	16	53 (49.5%)	35	17	52 (50%)	

Results

The study included 107 participants with imaging and histopathology confirmed bladder cancer and 104 participants were age and gender-matched controls. Seventy-two (67.3%) participants with bladder cancer were males, and the remaining 35(32.7%)were females. The cancer was significantly more common among males ($P < 0.001$) when compared to females [Table 1]. The mean age of participants with bladder cancer was 59.24 ± 16.28 years (males 59.53 ± 16.16 and females 58.66 ± 16.75). About 50% of the participants with bladder cancer were >70 years old. Most of the participants with bladder cancer worked as farmers (35.5%) or industrial workers (24.3%) [Table 2]. Most of the industry workers worked in chemical industry plants dealing with oils, rubber, paints and heavy metal chemicals.

Most participants in the bladder cancer group (89.7%) and controls (91.3%) were married. Recent history of recurrent urinary tract infections was noted in 85 (79.4%) of participants with bladder cancer and in 32 (30.8%) participants from the control group. Diabetes Mellitus was more common among participants with bladder cancer and a significantly high number of participants with bladder cancer gave a history of cancer (including bladder cancer) in the family. About 45% of the participants with bladder cancer gave an account of ingesting red meat [Table 3]. A significant number of participants with bladder cancer used tobacco and smoked compared to controls. Moreover, the number of participants continuing to use tobacco products, including cigarettes, even after diagnosis was significantly more [Table 4].

Table 2: Occupation of the patients with bladder cancer

Occupation	Bladder Cancer		Controls		*P*-value
	n	%	*n*	%	
Farming	38	35.5	32	30.8	0.8347
Menial worker	8	7.5	13	12.5	0.7484
Industry worker	26	24.3	15	14.4	0.0364*
White-collared job	9	8.4	14	13.5	0.3571
Business	13	12.1	5	4.8	0.01422*
Homemaker	13	12.1	25	24.0	0.0897
Total	107	100.00	104	100.00	

Table 3: Association of various factors with bladder cancer

Variable	Bladder cancer		Controls		Odds ratio
	n	%	*n*	%	
Married	96	89.7	95	91.3	0.826
Recent h/o recurrent UTI	85	79.4	72	69.2	1.7172
Family h/o cancer	11	10.3	2	1.9	5.843
Diabetes mellitus	31	29.0	12	11.5	3.127
Fluid intake (1Lt/day)	25	23.4	21	20.2	0.666
Non-vegetarian diet	86	84.1	75	72.1	2.0471
Pure vegetarian diet	21	15.9	29	27.8	1.458
Red meat consumption	41	45.6	25	29.1	2.265
Alcohol consumption	35	32.7	18	17.3	1.622

Table 4: Use of tobacco products and smoking in patients with bladder cancer

???	Bladder cancer		Controls		*P*-value
Tobacco users	60	56.1	40	38.4	0.010*
Smoking	51	47.7	32	30.8	0.0058*

Discussion

Bladder cancer is a common urological cancer and ranks as the seventh most common cancer among men.[5] Bladder cancer is considerably more common in men than in women (the worldwide ratio is about 3.5:1), which has been regarded as a possible indication of an occupational origin.[6] Bladder cancer has been associated with occupational exposure to industrial carcinogens.[7] As early as the late 19th century, it has been reported that unusual incidences of bladder cancer exist in the industry.[8] Case and Hosker[9] reported an exceptionally high incidence of bladder cancer in the British rubber industry. Several other agents or occupations were later shown to be associated with an increased risk of bladder cancer. Exposure to polycyclic aromatic hydrocarbons in the aluminium, coal tar and coal gasification industries was also reported to be an increased risk.[7]

Painters were reported to be at high risk for developing bladder cancer.[10-13] Several epidemiological studies reported relative risks (RRs) between 1.2 and 1.5 among painters for bladder cancer.[7] Some studies have also reported on the increased risks of bladder cancer with increasing duration of exposure to paint components.[14,15] Several epidemiological studies have also reported an increased incidence of bladder cancer among lorry drivers, taxi drivers and bus drivers.[16-18] The RRs for bladder cancer in studies of these occupational groups varied from 1.3 to 2.2. Most studies found positive trends with the duration of exposure.[7] The most likely causal agent is the constituents of the diesel exhaust emissions. However, these associations have not always been confirmed and are still debated.

A bladder cancer diagnosis is 3–4 times more common in men than women. Numerous explanations for this gender discrepancy in incidence have been offered, including disparate exposures to bladder cancer risk factors and the potential for sex steroid hormone regulation.[19] Women diagnosed with bladder cancer are more likely to have locally advanced tumours at the time of diagnosis.[20] Moreover, the female gender has been reported – albeit not uniformly – to be associated with higher risks of disease recurrence, progression and mortality after the treatment.[21-25] Dobruch *et al*.[19] reported that the gender difference in bladder cancer incidence was independent of differences in exposure risk, including smoking status. Potential molecular mechanisms included the disparate metabolism of carcinogens by hepatic enzymes between men and women, resulting in differential exposure of the urothelium to carcinogens. In addition, the sex steroid hormone pathway activity also played a role in bladder cancer development, demonstrating that both androgens and oestrogens have biological effects on bladder cancer *in vitro* and *in vivo*. Importantly, gender differences do exist in the timeliness and completeness of haematuria evaluation, with women experiencing a significantly more delay in urologic referral and less frequently undergoing guideline-concordant imaging. Correspondingly, women had more advanced tumours at the time of bladder cancer diagnosis. Interestingly, higher cancer-specific mortality was noted among women even after adjusting for tumour stage and treatment modality. Our study also shows that the incidence of bladder cancer is 2–3 times more common in men than in women.

Contemporary studies have underscored the importance of loss of heterozygosity on chromosomal arms 9p and 9q and inactivation of the p16 tumour suppressor gene as potential initiating events in the development of bladder cancer in adults.[26-28] The mutation of important cell cycle regulators, such as the p53 tumour suppressor gene on chromosomal arm 17p, has been tentatively associated with disease

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progression and a poor prognosis after surgical therapy for invasive tumours in adults.[29,30]

No association between alcohol and bladder cancer was found in a multicentric U.S. study of 2982 cases and a comparable number of controls.[31] On the contrary, a meta-analysis gave an overall RR for current drinkers versus non-drinkers of 1.3, a borderline significant.[32] Similarly, Pelucchi *et al*.[33] reported in a large case–control study on the issue for a population with frequent and heavy alcohol consumption revealed no consistent association between alcohol consumption and bladder cancer risk, the RRs being below unity also for six or more drinks of alcoholic beverages per day.

Tobacco smoking is a significant risk factor for bladder cancer,[34] responsible for about half the cases in both men and women.[34] The risk is generally threefold to fivefold higher in heavy smokers compared with never smokers, with a clear dose-response relationship for intensity.[35] A plateau in risk at approximately 20 cigarettes/day has also been reported in a pooled analysis of 11 case-control studies.[36] Furthermore, smoking duration has a high RR The risk is fivefold higher in long-lasting smokers than in never smokers.[35,37,38] Although most studies focused on smoking intensity rather than duration,[35] the pooled analysis results suggested that smoking duration is the overriding factor in determining the risk of bladder cancer.[36]

Tobacco smoking has been consistently associated with bladder cancer invasiveness and grading.[39-42] These tumour characteristics strongly correlate with the papillary feature, which is crucial in the TNM classification to classify non-invasive bladder cancers into Ta or Tis. Polesei *et al*.[43] evaluated the impact of tobacco smoking on specific histological subtypes of transitional cell carcinoma of the bladder (TCC). Compared to never smokers, TCC risk was 3-fold higher in former smokers (95% CI 2.07–4.18) and more than 6-fold higher in current smokers (95% CI 4.54–9.85). TCC risk steadily increased with increasing intensity (OR for C25 cigarettes/day 8.75; 95% CI 3.40–22.55) and duration of smoking (OR for C50 years 5.46; 95% CI 2.60–11.49). No heterogeneity emerged between papillary and non-papillary TCCs for smoking intensity and duration. Still, the risk for those who had smoked for C50 years was twice for non-papillary TCC (OR 10.88) compared with papillary one (OR 4.76). The risk for a 10-year increase in duration among current smokers grew across intensity strata (P = 0.046). Conversely, the risk for a 5-cigarette/day increase in smoking intensity was relatively steady across duration strata (P = 0.18). The authors concluded that the duration of smoking outweighed the intensity in determining TCC risk, with little differences across histological subtypes. Elimination of tobacco smoking could prevent about 65% of bladder TCC.

Conclusion

In conclusion, according to the findings of this study, bladder cancer is associated with several risk factors, one of which is gender. The link between gender and bladder cancer is complicated and it is most likely influenced by various biological and epidemiological characteristics. Numerous factors, including the importance of the steroid hormone pathway, gender differences in chemical exposure, metabolic enzyme activity, disparities in dietary factors and India's consumption of alcohol and tobacco products, may explain these demographic trends. Tobacco use, cigarette smoking and industrial chemicals are the leading risk factors for bladder cancer.

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Author Contributions

Conceived and designed the analysis: SR, RBN and SCG, Collected the data: SR and SRB, Contributed data or analysis tools: SR, SRB and SCG, Performed the analysis: SR and SRB and Wrote the paper: SR, RBN and SCG.

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EMERGING ROLE OF URINARY BIOMARKERS IN DETECTION OF UROTHELIAL BLADDER CARCINOMA IN SOUTH INDIAN POPULATION

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ABSTRACT

Urine cytology is used for screening of exfoliated bladder cells from voided urine but lacks sensitivity. This study aims to check the efficacy of 5-aminolevulinic acid (5- ALA) fluorescence cytology and establish a high sensitivity approach in detecting flat, *in-situ* and/or small lesions that are hardly visible under conventional cystoscopy. Intracellular PPIX allows red fluorescence detection. In this study, 5-ALA fluorescent cytology using urine was compared with conventional cytology in the diagnosis of bladder tumors. In this prospective study, we compared the sensitivity and specificity between conventional cytology, 5-ALA fluorescent cytology and FDA approved commercially available kits (NMP-22 and BTA). The percentage of Protoporphyrin IX facilitated by 5-ALA was amplified in cancer urothelial cells compared to normal urothelial cells. The sensitivity of conventional cytology and 5-ALA fluorescent cytology was 64% and 96% respectively, whereas the specificity was 92% and 98.67% respectively. In conclusion, 5-ALA induced fluorescent urine cytology demonstrated promising outcomes in the detection of bladder carcinoma cells. Furthermore, low grade and low stage tumor cells as well as flat lesions were also positively and accurately interpreted using 5-ALA fluorescent cytology.

Keywords: Bladder cancer, Non-invasive diagnosis, 5-Aminolevulinic Acid, Nuclear Matrix Protein, Bladder Tumor Antigen.

1. INTRODUCTION

Bladder cancer is one of the most common urological cancers. Bladder cancer is generally diagnosed by urethra-cystoscopy, which permits direct imagining of tumours and confirmation by biopsy and pathological analysis [1]. Nevertheless, Cystoscopy and voided urine cytology are effective diagnostic methods for investigation of superficial bladder cancer. Flexible cystoscopy being an invasive procedure has made cystoscopy more acceptable to patients [2]. Voided urine cytology remains the method of choice for the non-invasive detection of bladder cancer, yet whilst it has a specificity of 93%, its sensitivity is only 25-40%, especially for low-grade and T-stage tumours. Bladder cancer in males is 7th most common carcinoma as of 2020, and it has affected around 8.5 males and 3.2 females in every 100000 cases, around the globe. The bladder invariably comes in contact with the environment making it sensitive to environmental carcinogens and inflammation. Tobacco smoke containing aromatic amines, which when hydroxylated, lead to DNA damage [3].

Environmental carcinogens have been implicated in BC with a rising trend in the past decade due to increase in smoking habits. About 70-80% of the BC is diagnosed as non-muscle invasive (NMIBC) and 20-30% as muscle invasive (MIBC). An early diagnosis and detection of recurrence is of utmost importance as 10-30% of NMIBC progress to MIBC [4]. Cystoscopy and biopsy remain the gold-standard tests for the diagnosis of bladder cancer and enable the urologist to identify and resect all visible tumors. Cystoscopy has an overall sensitivity of 62-84%

and specificity of 43-98%, is cost-intensive, invasive and operator-dependent and has limited ability to pick small papillary tumors, low-grade urothelial neoplasms (LGUN) and *in-situ* tumours (CIS) [5]. The clinical spectrum at present can be divided into those with (i) non-muscle invasive bladder cancer, (ii) muscle-invasive bladder cancer, and (iii) metastatic disease. The muscle-invasive bladder cancer has a high recurrence rate of 50-70% as it reoccurs despite conservative measures such as transurethral of bladder tumor (TURBT) and intravesical therapy. However, screening individuals who are at high risk for bladder cancer, with a history of tobacco, occupational exposure, cyclophosphamide exposure, or pelvic radiation, may be helpful for the early detection of bladder cancer [6].

A wide range of alternative procedures and markers have been proposed and studied for the detection of recurrent bladder tumours. These include 5-Aminolevulinic Acid (5-ALA) cytology [7], nuclear matrix protein 22 (NMP-22) [8], BTA test [9] etc. The European Association of Urology in 2006 approved in using 5-Aminolevulinic Acid (5-ALA) for photodynamic diagnosis (PDD) of bladder tumor by cystoscopy as an effective procedure in detection and treatment for various cancer such as skin tumor, brain tumor, and oesophageal tumors. The principle behind 5-ALA fluorescence cytology is based on the metabolism of heme biosynthesis. 5-ALA being the precursor of heme metabolism will selectively get accumulated as protoporphyrin IX (PPIX) in tumor cells. The accumulation of ALA mediated PpIX was earlier reported to be 17 times higher than in normal mucosa. Moreover, ALA is impermeable through lipid bilayers and the metabolism of heme occurs both in cytosol and mitochondria, hence the efficacy of ALA dependent PDD is limited by cellular uptake of ALA and additional accumulation of photosensitizer PpIX [10, 11]. This occurrence is due to the numerous facts such as increased uptake of ALA in tumor cells, mitochondrial properties, and modification of enzyme activity in enzymes such as porphobilinogen deaminase and ferrochelatase activity and storage of PPIX in malignant cells. The sensitivity of PDD with 5-ALA for Transurethral resection of bladder tumor (TURBT) is higher compared to routine white light cystoscopy [12].

However, cystoscopy with 5-ALA has a higher false-positive rate than white light cystoscopy. In contrast, Use of Aminolevulinic Acid provides great benefits compared to conventional cytological procedures. Previous reports have investigated the feasibility and usefulness of urine base tests taking advantage of ALA-fluorescence [13]. Furthermore, the detection modalities are based on fluorescence cytology, fluorescence spectrophotometry, and flow cytometry. However, detection modalities such as spectrophotometry and flow cytometry require cumbersome procedures and costly equipment. In the search for a highly specific, sensitive, and objective method for detection for urothelial tumor cells, we have utilized the ability of fluorescence using 5-ALA for the diagnosis of urothelial tumor cells [14].

For quantitative analysis, FDA approved commercial test were used which follows the principle of enzyme linked immunoassay. First test include NMP-22, it is a nuclear mitotic apparatus protein is present in the nuclear matrix of all cell types and located in the mitotic spindle during mitosis is involved in the proper supply of chromatin to daughter. In bladder cancer, NMP-22 is twice as sensitive as cytology in detecting early T-stage cancers, and up to 90% sensitive and 99% specific [15]. Similarly, BTA has been identified as a human complement factor H related protein (hCFHrp), which is produced by bladder tumour cells in cell cultures and not by any other epithelial cell lines. BTA is released into the urine of patients with bladder cancer as the tumour invades the stroma. Initial reports indicated that BTA test had higher sensitivity and lower specificity than cytology [16]. The present study aimed to detect urothelial bladder carcinoma using 5-ALA and its comparison with conventional cytology, NMP-22 and BTA TRAK quantitative test to estimate activity in the urine of the patients with bladder cancer in a trial to assess their value in the detection of the tumours and to find a reliable non-invasive technique for the diagnosis of cancer bladder. Sensitivity and specificity of these tumour markers was compared to conventional cytology in bladder cancer.

2. MATERIAL AND METHODS

2.1. Patients

The study was conducted between September 2019 and February 2022 at the Urology clinic in a tertiary care centre of South India. The study was reviewed and approved by the institutional ethics committee (KAHER/EC/20-21/001/05). A total of 250 patients with 128 Bladder carcinoma (Cases) and 122 non-cancerous (Controls) were studies. Patients with signs or symptoms suggestive of bladder cancer and patients followed up for a past history of treated bladder cancer were included in the study. Furthermore, patients with a history of renal involvement (e.g. calculi, nephritis or

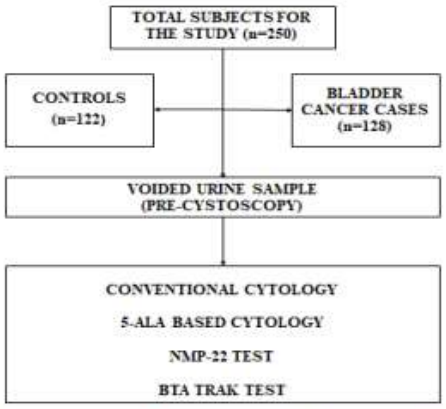
renal cancer); as these conditions may affect bladder cancer antigen detection; recent trauma of the bladder (e.g. cystoscopy, vesical washing, biopsy, or surgery); radiotherapy in the last 3 months; systemic chemotherapy in the last month; gross haematuria in the sample and urine infection formed the control group.

2.2. Collection of voided urine sample

Pre-operative urine sample of 150 cc was collected and samples were divided into four different groups, and the following procedures were performed within 1 h for pathological examination. We separated these samples for the conventional cytology [18], ALA-induced fluorescent cytology [10], NMP-22 and BTA-TRAK (Fig. 1).

2.3. Evaluation of ALA based cytology

Two ml of Minimum Essential Medium (MEM 11095080, GibcoTM, Thermo Fisher Scientific, Waltham, MA, USA) was added to the urine sediment. 5-ALA (5-Aminolevulinic Acid Hydrochloride, Sigma-Aldrich, ©Merck KGaA, Darmstadt, Germany, 2020) was added to the cell suspension to 200µg/ml and incubated for 2 hours at 37°C in a light-shielded incubator. Then, centrifuged the mixture again (1500 rpm for 5 min). The cell suspension was loaded on a slide and was observed using a fluorescence microscope (Nikon ECLIPSE Ni; Nikon Corporation, Tokyo, Japan) loaded with a 400-440 nm bandpass filter for excitation and a 610 nm long-pass filter for absorption, and the presence of fluorescent and reddish urothelial epithelial cells was diagnosed. The brightness and contrast of the fluorescence microscope were set to the recommended default settings for fluorescent urine cytology (Fig. 2).



Total subjects enrolled in the study were divided into two sub-groups i.e. cases and controls. Voided urine samples were subjected to urinary test such as conventional cytology, 5-ALA based cytology, NMP-22 test and BTA TRAK test. ALA- Aminolevulinic acid; NMP22-Nuclear matrix protein; BTA-Bladder tumour antigen.

Fig. 1: Samples for different tests

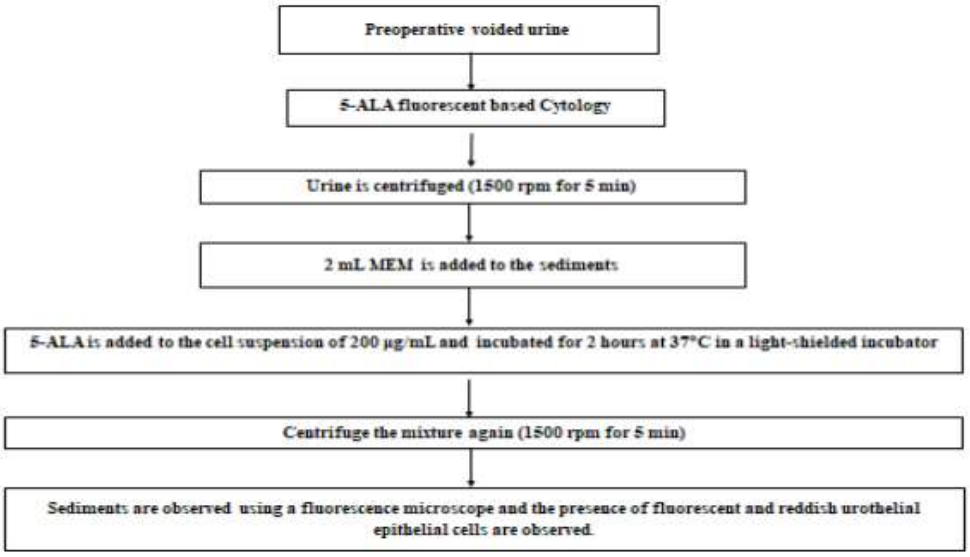


Fig. 2: The protocol of the 5-Aminolevulinic acid fluorescence detection assays. MEM- Minimum essential media

The cells with faint red or pink colour and histogram depicting peaks with low intensity versus frequency were considered as negative for malignancy. Cells showing bright red fluorescence against black background and its histogram depicting peaks (>200, 10K) of high intensity versus frequency were considered malignant (Fig. 3 & 4).

Cells were incubated with 5-ALA for 1 hr. The sediments were observed under fluorescence microscope. For Fig. (a & c), cells showed light red or pink colour against black background suggesting benign urothelial cells. (200X). For Fig. (b & d) the histogram showed intensity (x-axis) vs frequency (y-axis) to be minimum or normal.

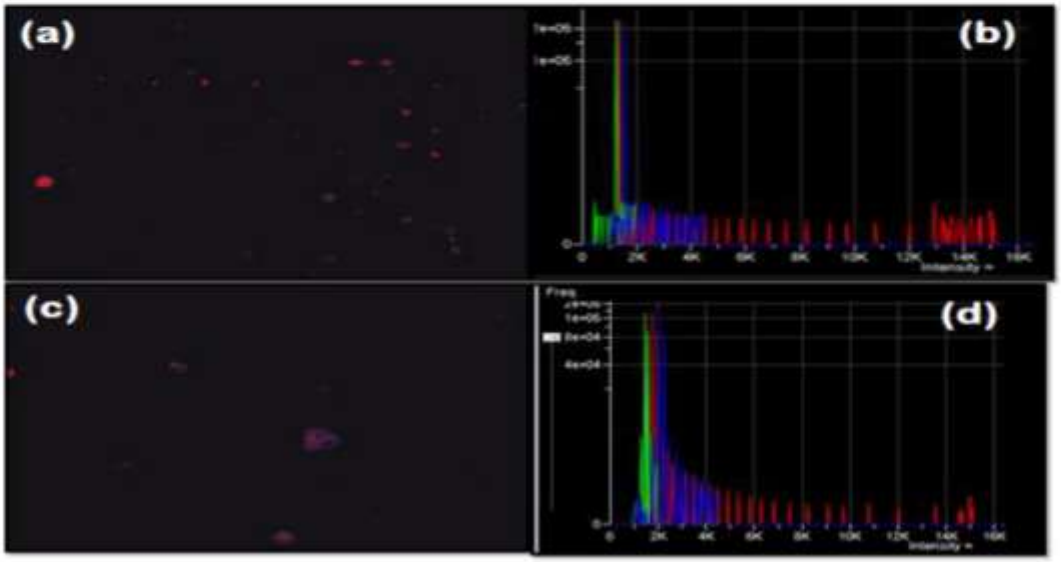


Fig. 3: Effect of 5-ALA on voided urine

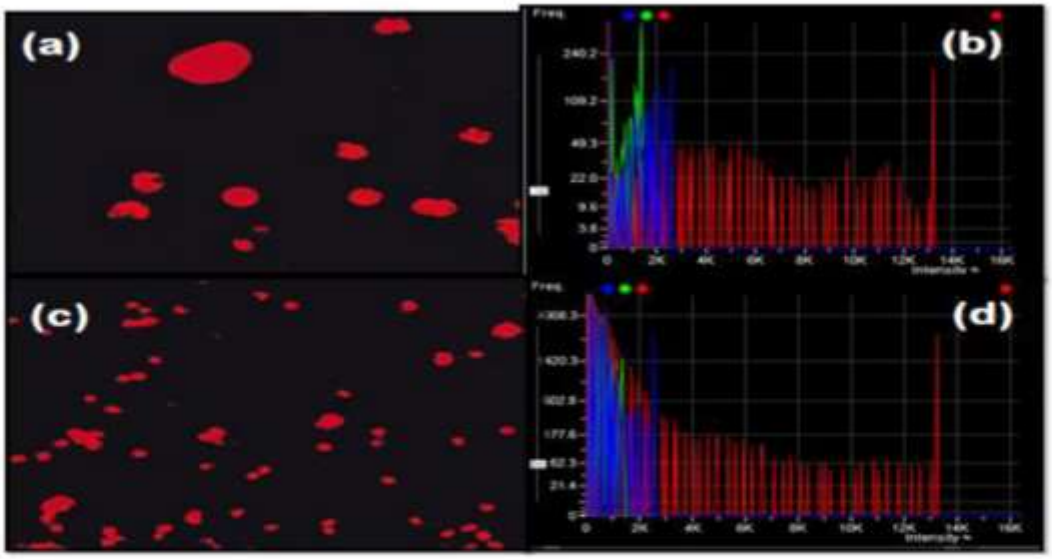


Fig. 4: Effect of 5-ALA on voided urine

Cells were incubated with 5-ALA for 1 hr. The sediments were observed under fluorescence microscope. For Fig. (a & c), cells showed bright red or dark red colour against black background suggesting

malignant urothelial cells. (200X). For Fig. (b & d) the histogram showed intensity (x-axis) vs frequency (y-axis) to be increased.

2.4. Evaluation with commercially available KIT methods

The cell free urine partwas used to evaluate NMP-22 and BTA TRAKby an enzyme immunoassay (EIA) employing two monoclonal antibodies that were specific for the NMP-22 antigen moiety of nuclear mitotic apparatus protein, were used to determine NMP-22 levels in stabilized voided urine (MyBiosource NMP22® test kit, South California, USA). Assay sensitivity, i.e. the lowest concentration of NMP22 antigen that can be measured reliably, is 0.156ng/ml.; within- and between-run coefficients of variation were 4.9 and 9.5%, (Detection Range: 0.156 ng/ml-40 ng/ml.) according to manufacturer. The BTA TRAK assay (MyBiosource NMP22® test kit, South California, USA) is an immunoenzymatic assay (IEMA) utilizing monoclonal antibodies to bind specifically to bladder tumor antigen in urine. The lowest concentration of BTA detectable was estimated to be 19ng/mL. Assay reproducibility as indicated by kit purchaser yielded 5 and 10% variation in intra- and extra-series tests, respectively (Detection Range: 0.31-20ng/mL).

2.5. Analysis of results

Average o fthe duplicate readings for each standard and samples was calculated, then subtracted the average zero standard optical density. a four-parameter logistic curve on log-log graph paper was plotted, with standard concentration on the x-axis and OD values on the y-axis. If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, a re-test must be done with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

2.6. Statistical Analysis

Data were compared using the Wilcoxon test or Chi-square test. Differences were considered statistically significant when $p<0.05$. Statistical analyses were performed using SPSS 22.0.

3. RESULTS

Table 1 shows the patients' clinico-pathological characteristics. The group with UC was older (median age 54 years old) than the non-cancer group (median age 53 years old)($p < 0.01$). The UC group included 128 patients with BT. The non-cancer group included 58 patients with BPH, 45 with urinary stones, 13 with UTI, and 6 with other with urological diagnosis. In the cancer group, 115 patients had cancer of pT1 or less (40, 18, and 57 patients with pTa, pTis, and pT1, respectively), 13 had cancer of pT2 or more, 57 had a low-grade tumor, and 70 had a high-grade tumour (Table 2).

The comparison of conventional cytology, 5-ALA cytology, NMP-22 test and BTA TRAK testwith respect to sensitivity in all specimens are presented in (Table 2). In subgroup analysiswe found that 5-ALA-induced fluorescent cytology tended to be more sensitive than conventional cytology and other kit based biomarkers regardless of patient age (classified by the median age of 54 years old) and sex. The sensitivity was significantly higher for 5-ALA-induced fluorescent cytology (91%) versus that for conventional cytology (55%), NMP-22 test (60%) and BTA TRAK test (64%). Similar trends were seen in both low grade (82%) and high grade (97%) tumours for 5-ALA cytology. Out of 115 BT cases pT1 104 cases were positive for 5-ALA cytology (90%) and for pT2 or more the sensitivity was significant higher compared to other biomarkers (92%, <0.0001).

However, in (Table 3), the specificity (98.2% vs. 95.6% vs 89.21 vs 92.10, $p = 1.0$) and positive predictive value (97.22% vs. 95.08%, vs 85.56 vs 89.13$p = 0.57$) were equivalently high between 5-ALA-induced fluorescent cytology and other biomarkers. In conventional cytology, false positives were found in two patients: one with Bladder prostate hyperplasia (BPH), and one with urinary tract infection (UTI). In 5-ALA-induced fluorescent cytology, false positives were found in six patients: two with BPH, two with UTI, and two with urinary stones. In NMP-22 test, false positives were found in thirteen patients: six with BPH, three with UTI, two with urinary stones and two in radiological cystitis. Similarly, for BTA TRAK test ten cases shown false positive results.

In cases judged as false positive by the conventional cytology or 5-ALA-induced fluorescent cytology, transurethral bladder random biopsy, selective upper tract urine cytology, and contrast-enhanced CT were performed to examine the urinary tract tumor. There were no findings to indicate UC either by additional imaging or pathological examinations, or in the

subsequent follow-up of all patients, urine cytology was re-examined every 3 months for over 1 year and confirmed to be negative. These results suggested that ALA-induced fluorescent cytology may be a superior tool to conventional cytology in clinical practice. When compared among all test, 5-ALA induced fluorescence cytology showed highest Cohen's Kappa (K) of 0.8745 complying to near perfect strength of agreement.For this study, therespective sensitivity and specificity of rangedfrom 40-70% for the conventional cytology, 80-95% for the 5-ALA based cytology, 40-60% for the nuclear matrix protein (NMP) 22 test, and 50-65% for the bladder tumor-associated antigen (BTA) test. The time taken for the examination of one urine specimen is about 1-2 days for conventional cytology, 180 min for 5-ALA based cytology, 300 min for NMP 22 and 240 min for BTA. In addition, the cost of one test is INR 350-400 for conventional cytology, INR 250 for ALA-induced fluorescent cytology andINR 1000-1500 for both NMP 22 and BTA TRAKas described above, 5-ALA-induced fluorescent cytology showed high sensitivities within a short period of time and at much lower cost compared with these other urinary biomarkers (Table 3).

Table 1: Characteristics of Study Subjects

Parameters	Bladder Cancer (%) (n=128)	Controls (%) (n=122)
Mean Age (Range, Years)	54.14 (29-85)	53.34 (24-90)
Male: Female	(114:14)	(105:17)
Comorbidity		
Hypertension	32 (25)	16 (13)
Type- I DM	2 (2)	1 (1)
Type- II DM	24 (19)	15 (12)
Chronic Kidney Disease	11 (9)	3 (2)
Ischemic Heart Disease	8 (6)	4 (3)
Thyroid Disorder	2 (2)	0
Multiple comorbidity	11 (9)	7 (6)
None	38 (30)	76 (62)
Diagnosis		
Bladder Carcinoma	128 (100)	0
BPH	-	58 (48)
Urolithiasis	-	45 (37)
Infection	-	13 (11)
Others	-	6 (5)
Occupational Exposure		
YES	79 (62)	47 (39)
NO	49 (38)	75 (61)
Family History of Any Cancer		
YES	21 (16)	8 (7)
NO	107 (84)	114 (93)
History of Urological Infection		
YES	60 (47)	36 (30)
NO	68 (53)	86 (70)

Table 2: Diagnostic Sensitivity Comparison Between Urinary Biomarkers

Diagnostic Test	Bladder Cancer							Total (%)	Control (%)
	Tumour Grade (%)		Pathological T-Stage (%)						
	Low	High	Ta	Tis	T1	T2	T2>		
	57	71	40	18	57	8	5	128	122
Conventional Cytology	18 (32)	52 (73)	18(45)	8(44)	37(65)	4(50)	3(60)	70(55)	2(2)
5-ALA Cytology	47 (82)	69 (97)	38 (95)	12 (67)	54 (95)	7 (88)	5 (100)	116 (91)	6 (5)
NMP-22 TEST	26 (46)	53 (75)	19 (48)	12 (67)	35 (68)	6 (75)	5 (100)	77 (60)	13 (11)
BTA TRAK TEST	27 (47)	55 (77)	25 (63)	13 (72)	35 (61)	5 (63)	4 (80)	82 (64)	10 (8)

Table 3: Sensitivity, Specificity, Inspection Time, and Cost of Urinary Biomarkers

Variables	Conventional Cytology	5-ALA Cytology	NMP-22 TEST	BTA TRAK TEST
Sensitivity	55%	91%	60%	64%
Specificity	98%	95%	89%	92%
Positive Predictive Value (PPV)	97%	95%	86%	89%
Negative Predictive Value (NPV)	67%	91%	68%	71%
Accuracy	76%	93%	75%	78%
Cohen's KAPPA (K)	0.4561	0.8745	0.4214	0.4917
Inspection Time	1-2 days	180 min	300 min	240 min
Cost (INR)	350-400	250	1000-1500	1000-1500

4. DISCUSSION

Although numerous new urine based test for the detection of bladder cancer are available. Invasive approach like cystoscopy is the number one diagnostic modality for the diagnosis new or recurrent bladder carcinoma.Non-invasive methods likevoided urinary cytology remains the most established non-invasive method for detecting bladder cancer. However, this method has several drawbacks including low sensitivity, low-cost effectiveness, a lack of inter observer variability, and technical instability [17]. Moreover, its sensitivity is low: between 11% and 76% in various studies [18]. Several factors affect the sensitivity of cytology, including specimen quality, number of exfoliated cells and pathologist expertise. The overall low sensitivity of cytology is due to its low sensitivity in detecting low-grade bladder tumours. Non-invasive urine markers can offer an alternative to the standard mode of detecting bladder cancer or they can be used as an adjunct to cystoscopy [19]. In addition, cytological evaluation is subject to the pathologist's experience. The limitations of both techniques led to the development of several urine-bound tests for the early diagnosis of bladder cancer [20]. For this purpose, new bladder cancer markers have recently been introduced into clinical practice.

Currently, 5-ALA is approved as a photosensitizer of photo dynamic diagnosis (PDD) for carcinoma around the world. For example, ALA as an optical imaging medicine was approved to enhance intraoperative detection of malignant glioma and also to detect bladder cancer [21]. A recent report on 5-ALA staining of urine specimens in an extracorporeal exposure showed PDD sensitivity to be effective compared with conventional cytology in BT (82% vs. 49%, respectively), particularly in low-grade and low-stage tumors, and to have comparable specificity (80% vs. 100%, respectively) [14]. However, they studied a small number of patients (61 with BT only), and there were some limitations in that conventional cytology for low-grade BT had very low sensitivity (18%), and the details of the non-cancer group were not described. For NMP-22 test, Research has found that patients with bladder cancer may have urinary levels of NMP22 that are 25-fold greater than levels in healthy subjects [22]. Similarly, BTA TRAK assay have so far been promising, with the finding of an overall sensitivity of about 70%. Conversely, urine cytology sensitivity is much lower in the same authors' experience, never exceeding 44% [23].

The present study included more patients (n = 250). Our results indicated that the sensitivity of 5-ALA-induced fluorescent cytology was significantly higher than that of conventional cytology, NMP-22 test and BTA TRAK test, especially for tumours of pTa stage and low-grade tumors. In Low and High Grades and Ta, T1 stages, the sensitivity between 5-ALA cytology and the other two tests is more evident, not only in our series (sensitivity 95% in Ta and 95% in T1; 82% in LG and 97% in HG with 5-ALA cytology) but also in the studies by Yamamichi et al.[7]In all the three tests, a gradual improvement in the sensitivity with increasing grade and stage was noted, as similar to other comparison studies [24, 25].

Some reports indicated that the false-positive findings of 5-ALA cytology can be induced by several factors such as infection, inflammation, hyperplasia, and inexperience [26]. There were only six cases of false positives by 5-ALA cytology in the present study, probably because of inflammation associated with infection and calculi. There are couple of possible reasons for this, the first cellular components in the voided urine specimen, and the second being that urinary cellular components together with the cancer cells expired out over the passage of time from sample pooling to pathological examination. In this case, those

most cancers cells can notably lose their mitochondrial metabolic action, and therefore, 5-ALA cannot be metabolized. To prevent the death of these cancer cells in the present study, we treated the extracorporeal ALA culture within 1 h of collecting the urine samples.

There are some limitations in this study. Our study is a prospective study of patients from a single institution, and thus, the present results need to be validated in other cohorts to definitively identify the high diagnostic efficacy of 5-ALA-induced fluorescent urine cytology for UC. With an objective to find reliable, non-invasive technique for the diagnosis of bladder cancer, all the voided urine samples of patients were evaluated for NMP-22, BTA-TRAK, conventional cytology, and 5-ALA cytology. When the clinical history and examination is suspicious/suggestive of bladder cancer, a non-invasive diagnostic procedure may lead to an immediate diagnosis surpassing the need of cystoscopy and biopsy. In the follow-up of superficial bladder cancer patients, the ability of a urine test to detect a tumor recurrence could be a useful tool for the selection of cases to be submitted for control cystoscopies [25].

There is an unmet need for a simple reliable test that can screen a patient, serve as a diagnostic test, reduce the number of unnecessary biopsies creating less morbidity, decrease the healthcare costs and play a vital role in the determination of the effectiveness of therapeutic interventions. An ideal biomarker to detect bladder carcinoma should be (i) cost-effective, tangible, fast to process, and easy to interpret, with high sensitivity and specificity (ii) reduce the need for frequent invasive procedures; (iii) exclude recurrence; (iv) detect progression towards invasive disease; (v) predict effective treatment response [28, 29]. From our study we have clearly seen that the sensitivities and NPVs of 5-ALA induced cytology is higher than the conventional methods such as urine cytology, detection of BTA-TRAK and NMP-22. Our results are comparable with those obtained by other investigators. Our study with 5-ALA cytology is simple, reliable, feasible and efficient way to diagnose BC and can easily be done in most of the centres dealing with patients of BC.

5. CONCLUSION

In conclusion, 5-ALA based fluorescent cytology in the detection of bladder cancer in voided urine sample represents a set of novel diagnostic assays that can be employed for an early and accurate detection of BC, with highest sensitivity as compared to other routine methods. These can also efficiently detect low-grade urothelial tumors by showing positivity in different filters. Our study was a single-centred assessment and needs to be validated in other cohorts & multiple centres. We suggest validation and inscription of these markers in urine as independent diagnostic tests or in a panel to be recommended as these may play a vital role in early detection and diagnosis of bladder cancer.

Conflicts of Interest

The authors declared conflict of interest as None.

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ANNEXURE - 5

TRAINING UNDERGONE

The following training programs, webinars and conferences in the last six months:

1. As a part of curriculum, NPTEL online course on "HEALTH RESEARCH FUNDAMENTALS" had enrolled and passed the exam held in December 2020.
2. Workshop on Study Designs and Statistical Methods using SPSS/EXCEL held from 16th September to 20th September 2019 organized by Department of Epidemiology and Biostatistics in collaboration at KAHER, Belagavi 590010.
3. Webinar attended on "Teaching and Learning in Digital Age" organized by KLES B.K Arts, Science and Commerce College, Chikodi, on 08th July 2020.
4. Webinar attended on 'Effective Scientific Writing Skills' organized by KLES B.K Arts, Science and Commerce College, Chikodi, on 30th July 2020.
5. Webinar attended on 'Role of PCR in Diagnosis' organized by KLES B.K Arts, Science and Commerce College, Chikodi, on 12th August 2020
6. Participated in Training Programme on 'Laboratory Procedures' organized by KAHER'S Dr. Prabhakar Kore BSRC Belagavi during 17th to 21st December 2020.
7. Webinar attended on "Basics of Research Methodology and use of statistics in Biology" on 1st April 2021.
8. Webinar attended on "On the eve of World Environment Day" organized by KLES B.K Arts, Science and Commerce College and Karnataka State Pollution Control Board, Chikodi, on 6th June 2021.
9. Has participated in the workshop entitled "Clinical and Research application of flow cytometry on 15th April 2022 Organized by KAHER'S Dr.P.K BSRC, Belagavi as a delegate.

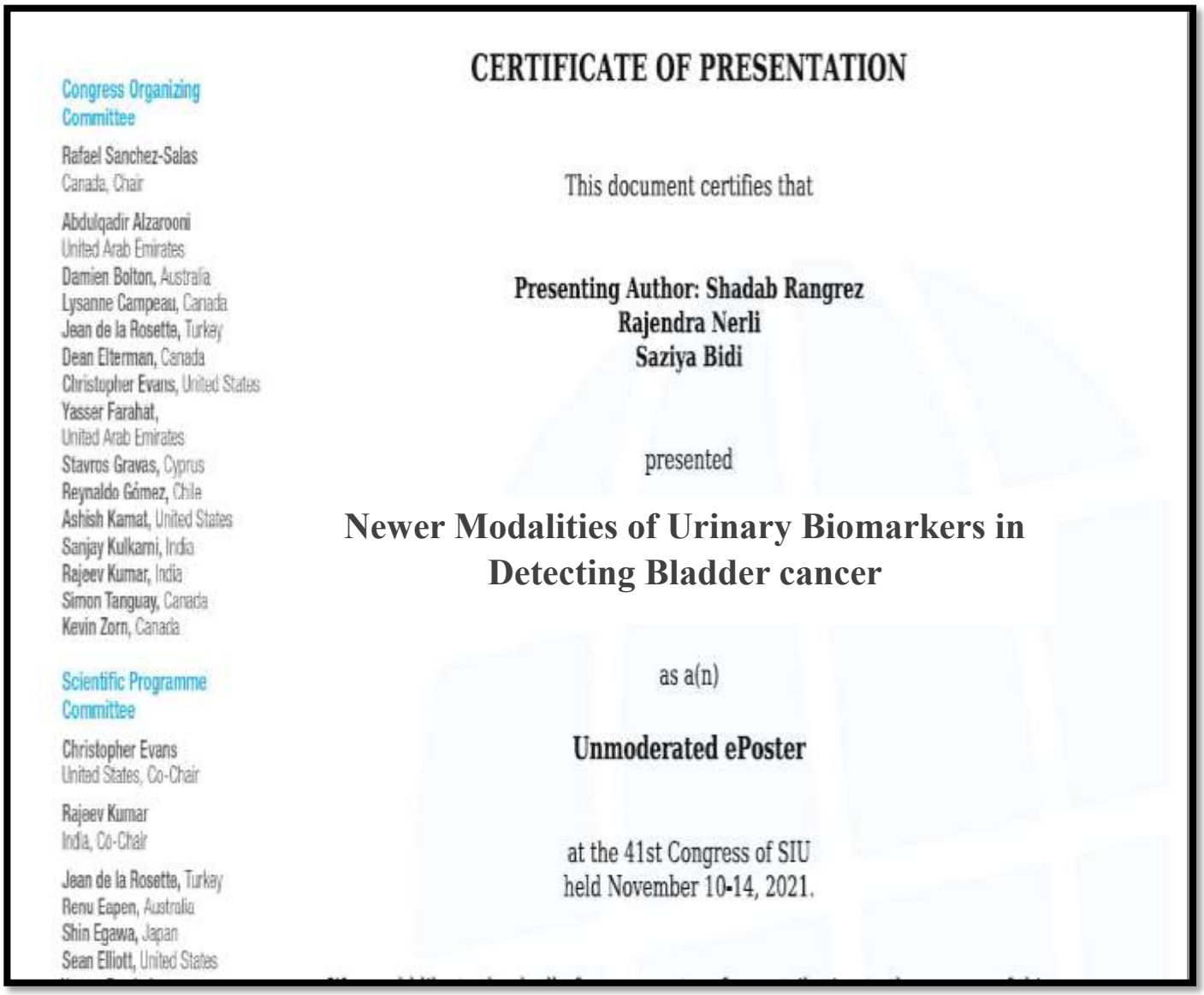
10. Has participated in the seminar on "BIG 21 - Workshop on proposal writing" on 7th July 2022 conducted by Centre for Cellular and Molecular Platforms.
11. Has participated in the seminar on "Molecular Biology of Infectious Diseases" on 26th July 2022 Organized by KAHER'S Dr.P.K BSRC, Belagavi.
12. Had completed a course "COURSERA- CHEMICAL BIOLOGY" which was associated to my research methodology and successfully completed it.

ANNEXURE - 6

POSTER PRESENTATIONS

Presented at 2nd Oman Urological Society Conference Hosted by Oman Urology Society (OUS)

Presented at 41st Congress of the Société Internationale d'Urologie (SIU) held in conjunction with the 10th Emirates International Urological Conference of the Emirates Urological Society (EUS) November 10-14 in Dubai.



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Sean Elliott, United States

CERTIFICATE OF PRESENTATION

This document certifies that

Presenting Author: Shadab Rangrez
Rajendra Nerli
Saziya Bidi

presented

Newer Modalities of Urinary Biomarkers in Detecting Bladder cancer

as a(n)

Unmoderated ePoster

at the 41st Congress of SIU
held November 10-14, 2021.

Podium Presentation at 1st Annual Conference of Andrology Section, USI New-Delhi from 14th – 16th July 2023


THIS CERTIFICATE PROUDLY STATES THAT

Dr Shadab Rangrez

DELIVERED A PODIUM PRESENTATION AT THE 1ST ANNUAL CONFERENCE OF ANDROLOGY SECTION, USI HELD AT HOTEL SHERATON ITC, NEW DELHI FROM 14-16 JULY 2023.

DATTESWAR HOTA
CHAIRMAN, ANDROLOGY SECTION - USI

VINEET MALHOTRA
SECRETARY, ANDROLOGY SECTION - USI
ORGANIZING CHAIRMAN - ANDROCON 23

RAMAN TANWAR
ORGANIZING SECRETARY - ANDROCON 23

Androcon
14-16 July, 2023 | New Delhi
at Hotel Sheraton ITC, Saket

CERTIFICATE OF PARTICIPATION