Medical University - Pleven

Faculty of Medicine

Department of Pathoanatomy

Elitsa Petrova Kraevska, MD

Muscle-invasive carcinoma of the urinary bladder – morphological and immunohistochemical characteristics with a focus on prognostic and predictive biomarkers

ABSTRACT

of a dissertation

for the acquisition of an educational and scientific degree

"Doctor"

Pleven, 2026

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With gratitude to my scientific supervisor, from whom I learned that science is not just a collection of facts, but a way of thinking – a dialogue between curiosity and responsibility!

The dissertation is presented on 202 standard pages and is illustrated with 14 tables, 14 figures, 17 microphotographs, and 1 appendix. The literature review comprises 221 titles, of which 1 is in Cyrillic, and 220 are in Latin, with 178 of them published in the last 10 years.

The author is a full-time PhD student at the Department of Pathoanatomy, Faculty of Medicine, Medical University of Pleven.

Note: The numbering of figures and tables does not correspond to that in the dissertation.

The dissertation has been discussed and approved for public defense by the extended Department Council of the Department of Pathoanatomy, Faculty of Medicine, Medical University of Pleven, held on November 26, 2025.

The public defense of the dissertation will take place on February 5, 2026, at 1:00 p.m. in Ambroise Pare Hall at MU-Pleven, 1 Kliment Ohridski Street.

Scientific jury composed of:

Prof. Vladislav Rosenov Dunev, MD, PhD

Prof. Veselin Todorov Belovejdov, MD, PhD

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Prof. Sergey Dimitrov Iliev, MD, PhD

The materials related to the defense are available on the website of MU-Pleven:

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CONTENTS

List of abbreviations used:

- BC – Bladder carcinoma
- MIBC – Muscle-invasive bladder cancer
- UC – Urothelial carcinoma
- IHC – Immunohistochemistry
- RC – Radical cystectomy
- TURB – Transurethral resection of the bladder
- CT – Chemotherapy
- NAC – Neoadjuvant chemotherapy
- ICIs – Immune checkpoint inhibitors
- HER2 - Human epidermal growth factor receptor 2
- FISH/*C*ISH - Fluorescence/Chromogenic *in situ* hybridization
- ADC – Antibody–Drug Conjugate
- FGFR3 – Fibroblast Growth Factor Receptor 3
- BCG – Bacillus Calmette-Guérin
- ASCO/CAP – American Society of Clinical Oncology/College of American Pathologists
- EAU – European Association of Urology
- ESMO – European Society for Medical Oncology
- NCCN – National Comprehensive Cancer Network, USA
- ISUP – International Society of Urological Pathology
- OS – Overall Survival
- DFS – Disease-Free Survival

"I belong to the number of those who see great beauty in science."

Marie Skłodowska-Curie

INTRODUCTION

Bladder cancer (BC) is a significant health problem. According to the latest GLOBOCAN data (2022), the disease ranks ninth in terms of frequency, with 614,298 newly diagnosed cases and 220,596 deaths worldwide. The development of BC is associated with various risk factors, including smoking, occupational and chemical exposures, chronic inflammation (e.g., Schistosoma infection), and genetic predisposition. Approximately 25% of patients diagnosed with BC present with a muscle-invasive form of the disease at diagnosis (stage ≥ pT2). Muscle-invasive bladder cancer (MIBC) is characterized by aggressive progression and high mortality, which necessitates early diagnosis and optimization of therapeutic approaches.

Bladder cancer is morphologically diverse. Urothelial carcinoma (UC) occurs in over 90% of diagnosed cases. The WHO classification (2022) stratifies UC into morphologically distinct groups: papillary UC, micropapillary UC, microcystic UC, sarcomatoid UC, plasmacytoid UC, clear cell UC, lipid-rich UC, nested UC, lymphoepithelioma-like UC, and giant cell UC. Morphological subtypes represent urothelial carcinoma with a morphology different from the conventional one. They occur in 20-30% of resected tumors. The different histology is a mandatory element that must be described as a percentage in the pathology report. Often, the identification of such histology in otherwise conventional urothelial carcinoma is associated with aggressive tumor behavior and plays a key role in both diagnosis, prognostic assessment, and treatment.

Over the past decade, accelerated scientific research has led to a deeper understanding of the molecular biology of urothelial tumors. Studies, based on high-tech gene expression analyses, have led to the discovery of molecular variants of urothelial carcinoma similar to those found in breast carcinoma. In 2020, Kamoun et al. created a consensus molecular classification based on a meta-analysis of 1,750 MIBC transcriptomic profiles from a total of 18 published databases. They identified six different molecular variants: basal-squamous (Ba/Sq); luminal papillary (LumP); luminal unstable (LumU); luminal nonspecific (LumNS); neuroendocrine-like (NE-like), and stroma-rich. Molecular typing is an independent predictor of bladder

carcinoma. It allows tumors to be divided into clinically significant groups that demonstrate specific molecular profiles, different prognostic characteristics, and response to therapy.

The high cost associated with molecular analysis and the limited availability of the technological platforms required to perform it are reasons for its low applicability. Clearly defined and easy-to-use surrogate molecular categories based on immunohistochemistry (IHC) are needed to make typing cost-effective and useful in routine daily practice. To better understand the disease, it is important to investigate the relationship between the histological subtype and the molecular profile of the tumor.

The role of prognostic and predictive biomarkers in bladder cancer is essential for assessing the clinical course of the disease and determining appropriate therapeutic strategies. Prognostic biomarkers provide information about the likely outcome of the disease, including the risk of recurrence, progression, and overall survival. Predictive biomarkers play a key role in personalized medicine, allowing treatment to be tailored to the individual characteristics of the patient.

Human epidermal growth factor receptor 2 (HER2) is a well-studied biomarker in breast and stomach cancer. HER2, also known as ERBB2, is a receptor from the tyrosine kinase family that plays a role in cell proliferation, differentiation, and tumorigenesis. The role of HER2 in urothelial carcinoma is of considerable scientific interest, with research focusing on expression frequency, testing methodology, and the relationship to clinical outcomes and prognosis. The frequency of HER2 expression and detection methods (immunohistochemistry and in situ hybridization) in UC reveals significant heterogeneity, requiring a standardized and personalized approach to assessment. Differences in HER2 positivity between different histological subtypes and disease stages highlight the need for individualized diagnostic and therapeutic strategies that meet the specific needs of patients. The clinical significance of HER2 status is related to its prognostic and predictive value. Patients with HER2-positive tumors often demonstrate more aggressive disease and a higher risk of metastasis. Therapeutic approaches involving anti-HER2 therapies offer new treatment options, especially in advanced or refractory cases. Preliminary data from clinical trials show promising activity of new therapies, which is encouraging for the future treatment of patients with HER2-positive urothelial carcinoma.

The tumor suppressor protein p16INK4A is routinely used as an indirect indicator of abnormalities in the RB1 (retinoblastoma protein) pathway. In urothelial carcinoma, loss of function along the p16–RB axis can occur either through deletion/methylation of the *CDKN2A* gene (leading to a lack of p16) or through mutation of *RB1* (leading to persistently high levels

of p16). Accordingly, p16 expression varies among different molecular variants. Published data show almost universal expression of p16 in neuroendocrine-like (NE-like) tumors and frequent expression in basal tumors, while luminal papillary tumors are most often p16-negative. Studying these relationships could aid diagnosis (by using p16 as a surrogate marker for molecular variant) and provide new prognostic indicators.

This dissertation is devoted to the study of the morphological and immunohistochemical characteristics of muscle-invasive bladder cancer, with a focus on *prognostic and predictive biomarkers*. Through systematic analysis of 100 consecutive cases of muscle-invasive bladder cancer over a period of 4 years (2021-2024), the aim is to define, through immunohistochemical methods, the molecular variants according to the consensus molecular classification, as well as to evaluate the expression of HER2 in the different tumor categories. The working hypothesis is that the combination of classical morphological classification and immunohistochemical profile can stratify MIBC into clinically significant groups that have the potential to serve as predictive and prognostic biomarkers.

GOAL AND OBJECTIVES

Objective

To study the morphological characteristics of muscle-invasive bladder cancer and to define molecular variants according to the consensus molecular classification of MIBC using surrogate immunohistochemical markers. Also, to study the expression of the HER2 proto-oncogene and to look for a correlation with the different morphological subtypes and molecular variants.

To achieve this goal, we set the following tasks:

Tasks

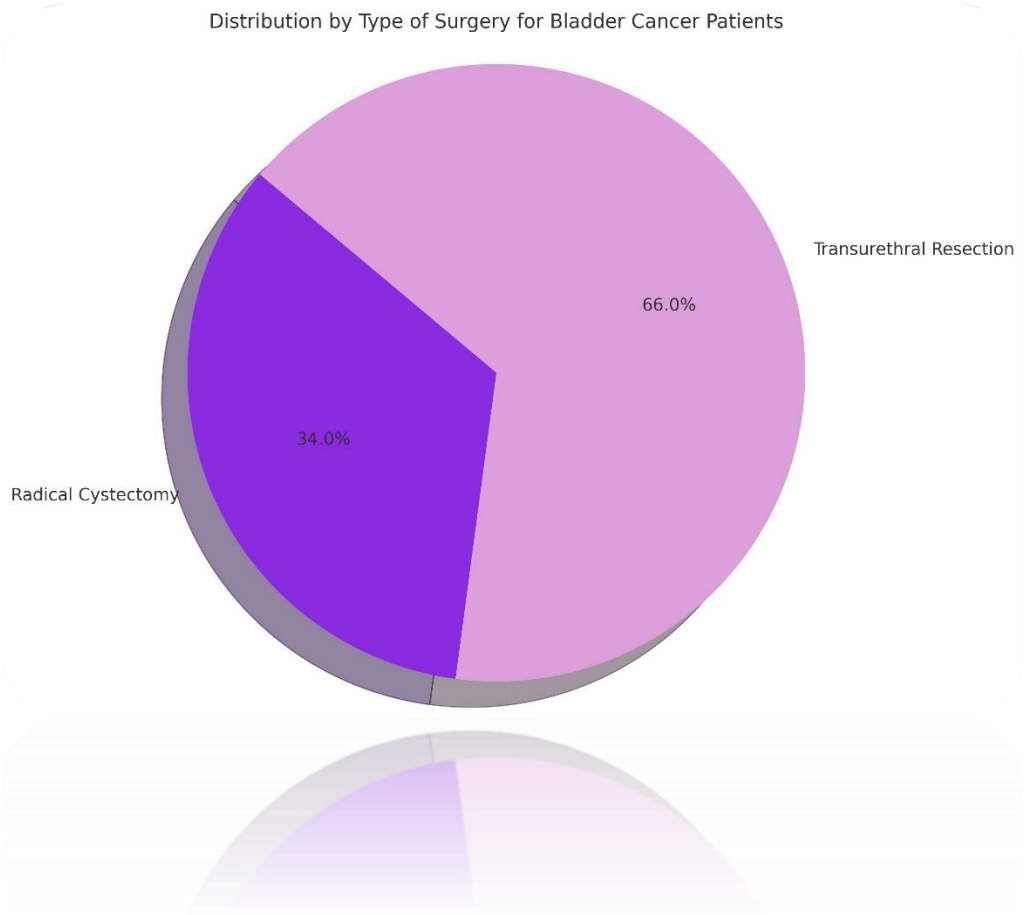
1. To study the frequency of different histological subtypes of MIBC in the cohort studied (n = 100).
2. To determine the molecular variant of the different histological subtypes of MIBC using IHC methods and to study their frequency.
3. To look for a correlation between the classic (histological) and molecular classifications.
4. To determine HER2 expression in different histological subtypes and molecular variants and to study their frequency.
5. To investigate p16 expression in different molecular variants.

MATERIALS AND METHODS

Clinical contingent

One hundred consecutive patients with histologically proven muscle-invasive bladder cancer were studied. The patients were treated at the Urology Clinic of the St. Marina University Hospital in Pleven, Bulgaria, over 4-years (January 2021 – December 2024). Sixty-six patients (66%) underwent transurethral resection (TURB), and the remaining 34 (34%) underwent radical cystectomy (RC). Figure 1.

Figure 1. Frequency distribution of patients according to the surgical intervention performed.



The study group included 86 men (86%) and 14 women (14%), which largely corresponds to the known gender ratio in favor of men (Fig. 2). The average age of the patients was 69.5 years (standard deviation ±8.7 years), ranging from 47 to 87 years (Fig. 3). This suggests that the disease mainly affects older people, typically over 55-60 years of age, which is consistent with the epidemiological data in the literature.

Figure 2. Frequency distribution of patients by gender (n=100).

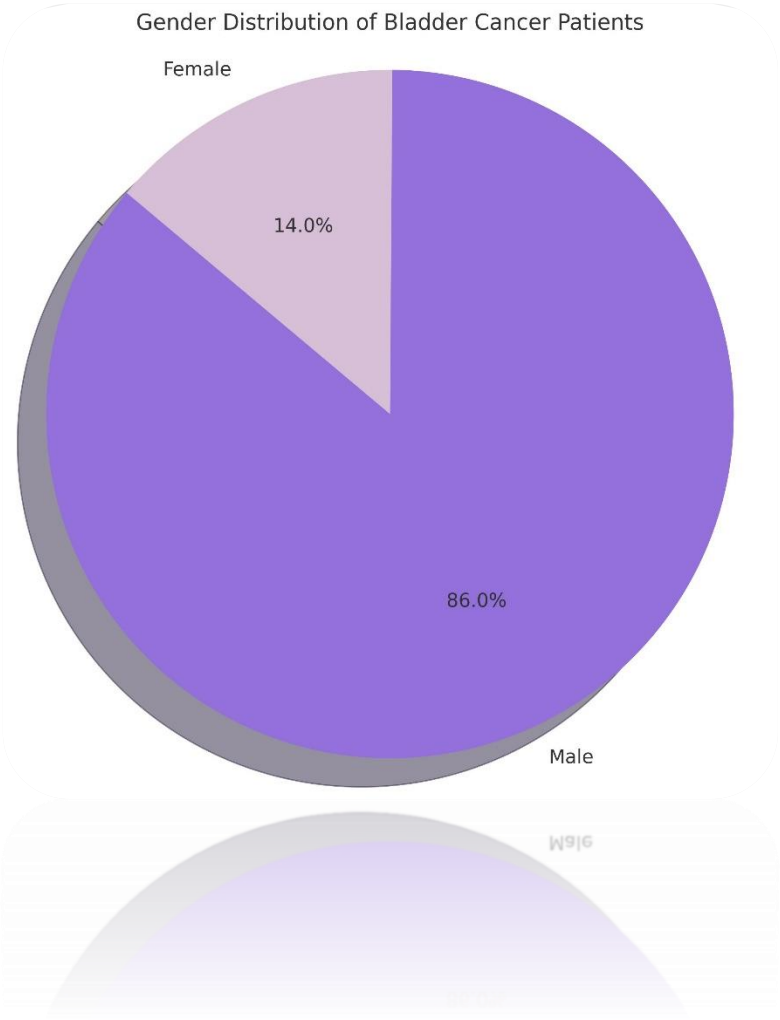
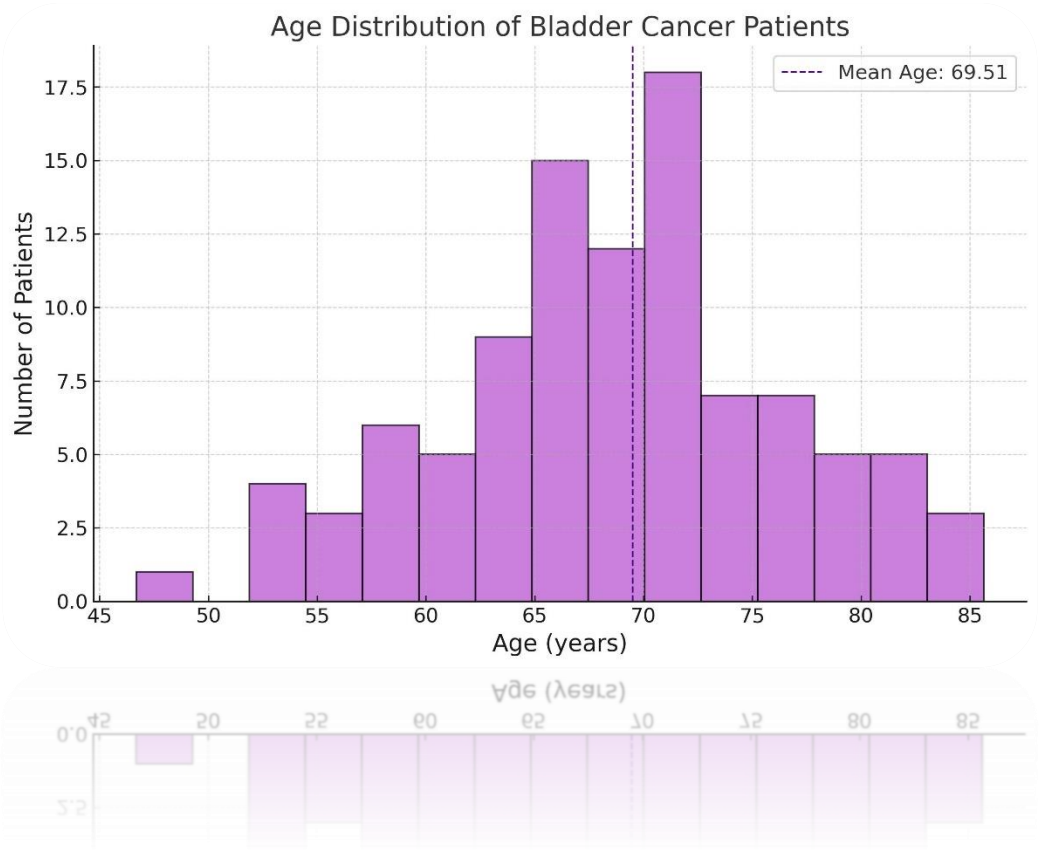


Figure 3. Age distribution of patients.



Diagnostic methods for assessment:

All tumor samples were fixed in neutral buffered formalin and embedded in paraffin according to standard protocol. Histological sections 4 μm thick were prepared and stained with hematoxylin-eosin (H&E) for routine morphological evaluation. Based on H&E staining of the specimen, a histological diagnosis was made for each case according to the recommendations of the WHO (5th edition, 2022) and UICC TNM (8th edition), with special attention paid to the histological subtype. Combined tumors containing more than one histological component were classified as a mixed subtype (≥ 2 morphologically different tumors).

Note: All micrographs in this paper are from the author's personal archive and include cases from the studied cohort.

Immunohistochemistry (IHC):

Paraffin blocks were used to prepare additional sections for immunohistochemical (IHC) staining. The molecular profile of each tumor was determined using a selected panel of surrogate immunohistochemical markers. Specifically, the following primary antibodies were used:

CK5/6 (cytokeratin 5/6, mouse monoclonal antibody, *clone D5/16* B4, ready-to-use, *Dako/Agilent*) and **CK20** (cytokeratin 20, mouse monoclonal antibody, *clone Ks20.8*, ready-to-use, *Dako/Agilent*) – to distinguish between basal and luminal immunophenotypes. CK5/6 is normally expressed in basal cells, and CK20 is in the differentiated surface layer of the urothelium (umbrella cells). Appropriate controls were used for each of the two markers: benign prostatic hyperplasia for CK5/6 and appendix for CK20.

P16 - ready-to-use monoclonal antibody, clone JC2 (Diagnostic BioSystems, *California, USA*). Cervical squamous cell carcinoma associated with HPV and overexpressing p16 was used as a positive control for p16. Criteria for interpreting p16 staining: we considered the result positive if strong or moderate nuclear staining (regardless of cytoplasmic staining) was observed in ≥50% of tumor cells. Absent, weak, or focal (<50%) nuclear signal, as well as only cytoplasmic staining, was reported as a negative result.

Immunohistochemical staining for HER2 was also performed. A standardized **HercepTest™** diagnostic system (Dako/Agilent) and polyclonal rabbit antibody (code A0485) were used, following a protocol similar to that used for breast cancer. Internal control samples were included in each staining cycle – a provided multi-control slide (four cell lines with expression

levels 0, 1+, 2+, 3+) and additional negative controls from the tumor tissue for each patient. This ensures the specificity and reproducibility of the reaction.

A standard immunohistochemistry (IHC) laboratory, equipped with instrumentation provided by the Medical University of Pleven through the Center of Competence for Personalized Medicine, 3D and Telemedicine, Robotic and Minimally Invasive Surgery, was used. IHC staining was performed on an automated *Dako Autostainer Link 48* platform. Visualization was performed using DAB chromogenic and enzyme marker polymer (EnVision™ FLEX) after appropriate epitope retrieval (PT Link, low pH buffer).

Determination of the molecular variant**:** A combination of the above-mentioned IHC markers was used to classify tumors into a specific molecular variant, according to the following IHC algorithm:

- ☑ Basal variant**:** CK5/6 (positive in >10% of tumor cells), CK20 (negative).
- ☑ Luminal papillary variant**:** CK5/6 (negative), CK20 (positive in >10% of tumor cells), p16 (negative).
- ☑ Luminal unstable variant: similar to luminal papillary in CK5/6/CK20, but p16 is strongly and diffusely positive in over 50% of tumor cell nuclei.
- ☑ Luminal nonspecified variant: CK5/6 (negative), CK20 (focal in <10% of tumor cells) with urothelial morphology. This is an intermediate profile that does not fall into the above luminal categories.
- ☑ Stroma-rich variant: characterized mainly by abundant stroma and inflammatory infiltrate; the immunophenotype is double negative for CK5/6 and CK20. In our study, this variant is difficult to recognize with IHC alone. Therefore, cases without a clearly defined basal or luminal profile but with abundant stroma were assigned to this category.
- ☑ Neuroendocrine-like variant: in the presence of neuroendocrine morphology +/- immunohistochemical expression of synaptophysin, chromogranin, and CD56. These tumors are CK5/6 and CK20-negative and have distinctive, frequent double mutations *TP53+RB1* and diffuse p16 expression.
- ☑ Basal-luminal variant: co-expression of both markers (CK5/6 and CK20) in different areas of the tumor (double positive).

Each case in the study was classified into one of the categories listed above based on the results of a combination of immunohistochemical markers supported by the morphological picture. In mixed tumors (with more than one histological component), a separate IHC profile was assessed for each component, as far as technically feasible, and classified according to the described algorithm. In the presence of co-expression of both markers (CK5/6 and CK20), the tumor is placed in a separate category of basoluminous molecular variant.

Statistical methods

The data were processed using the Statistical Package for the Social Sciences v.25 (SPSS Inc., Chicago, IL, USA) software package. Qualitative variables (e.g., gender, type of surgery, frequencies of histological subtypes and molecular variants, HER2 expression) are described by their absolute and relative frequencies (number and percentage). For quantitative variables (e.g., age), the median, minimum, and maximum values (Mdn, Min-Max) were calculated due to the asymmetric distribution of cases. The correlation between histological classification and molecular classification was assessed using cross-tabulation. Differences between groups were tested using Pearson's chi-square ($\chi 2$) test. A significance level of $p < 0.05$ was considered statistically significant.

Results

Analysis of the frequency of different histological subtypes of muscle-invasive carcinoma of the urinary bladder

Of the 100 cases of MIBC studied, a significant proportion (66%, including mixed tumors) demonstrated at least one different histological component. The most common in the cohort studied were papillary urothelial carcinoma, 27 cases (27%), and combined/mixed tumors, also 27%. This was followed by urothelial carcinoma with squamous cell differentiation – 17% of cases. Less common but significant subtypes in our series were: sarcomatoid urothelial carcinoma (5%), small cell carcinoma (5%), micropapillary subtype (4%), urothelial carcinoma with glandular differentiation (3%), plasmacytoid subtype (3%), nested (2%), and poorly differentiated (anaplastic) urothelial carcinoma (3%). In 4% of cases, the tumor does not show specific morphology and is classified as high-grade urothelial carcinoma (designated as NOS – not otherwise specified).

The data on the relative share of the main histological categories are summarized in Table 1:

Table 1: Frequency distribution of histological types in the study cohort.

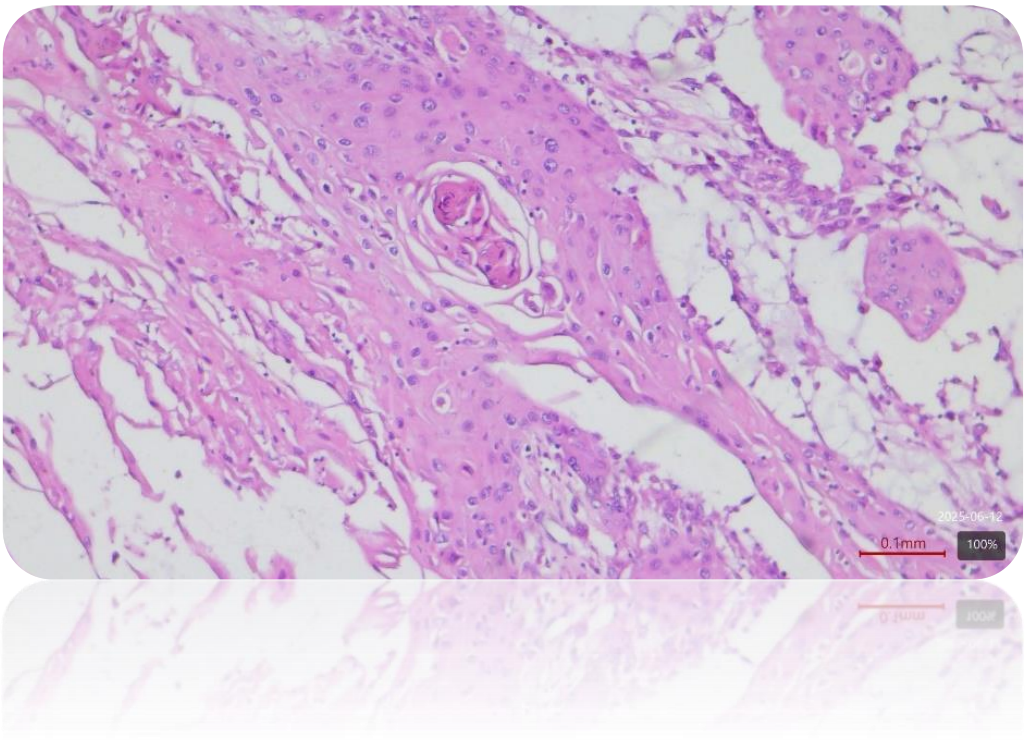
Histological type	Number of cases (n)	Share (%)
Papillary urothelial carcinoma	27	27.0
Mixed (≥2 components)	27	27
UK with flat-cell differentiation	17	17
Sarcomatoid urothelial carcinoma	5	5
Small cell carcinoma	5	5
Micropapillary UK	4	4
UK (NOS)	4	4
UK with glandular differentiation	3	3
Plasmacytoid subtype	3	3
Poorly differentiated UK	3	3
Nested	2	2
Total	10	100

Table 1 shows that papillary and mixed carcinomas form the largest subgroups (27% each). It is assumed that, upon detailed histological examination, most UCs demonstrate some form of divergent differentiation or different morphology. Our results also reflect the fact that all cases are ≥ pT2 (muscle-invasive). It is known that morphologically different subtypes are more common in advanced stages of the disease.

Microscopic findings:

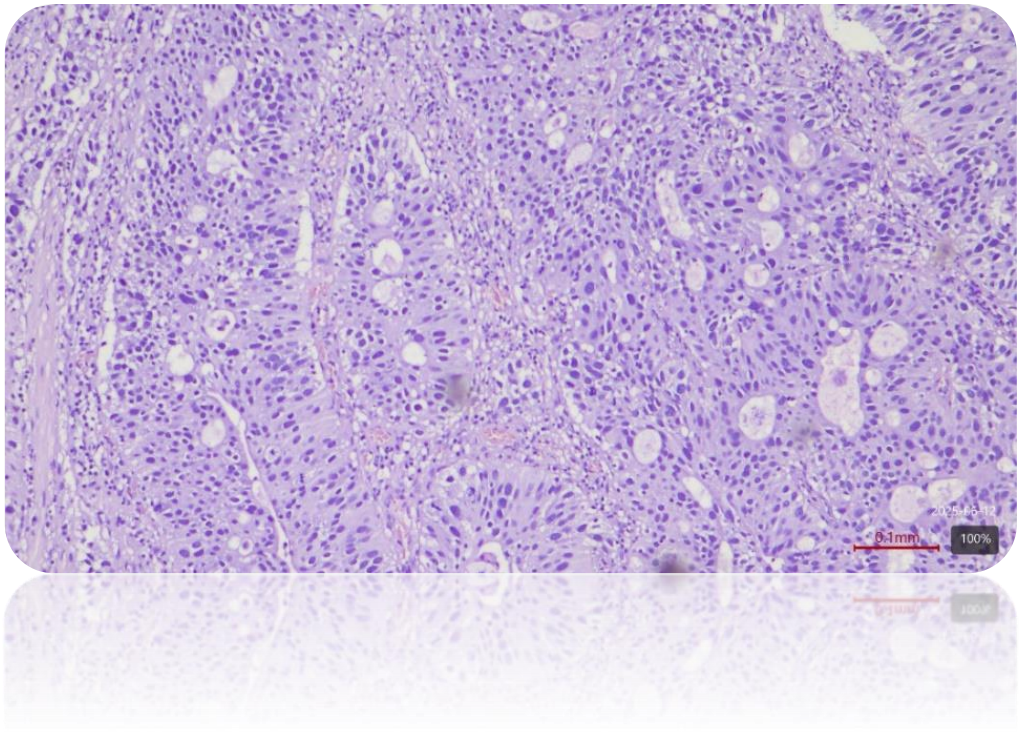
Each histological subtype in the series was validated by its characteristic morphological features. For example, squamous cell differentiation manifests itself as areas of keratinization or intercellular bridges in the tumor. These were found in 17% of cases, which corresponds to the reported frequency of 10–25%.

Microphotographs 1: UC with squamous cell differentiation, magnification x100



- **Glandular differentiation** (glandular structures in a urothelial tumor) is less common – in 3% of our cases, according to the literature (~5-6%).

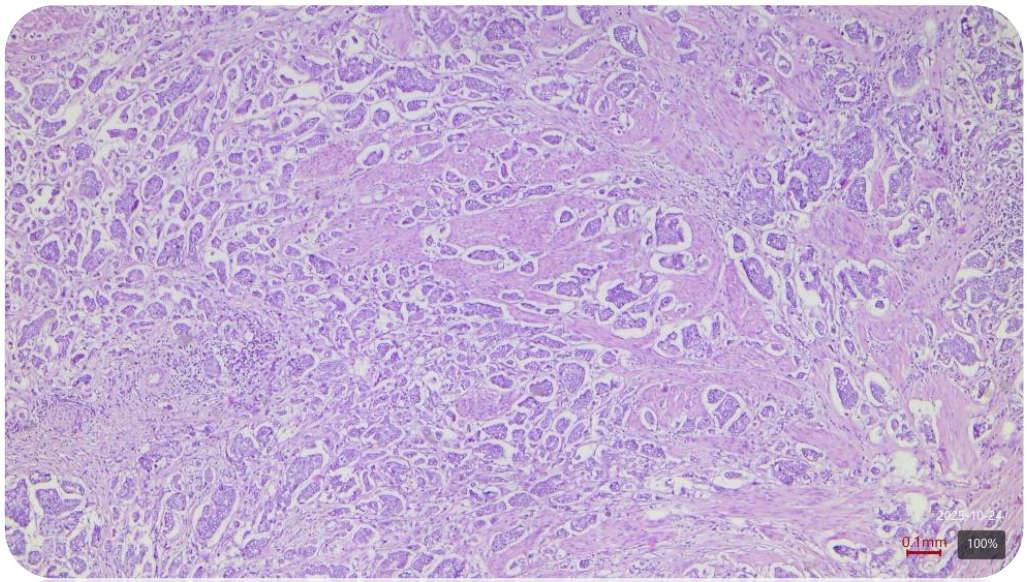
Microphotographs 2: UC with glandular differentiation, magnification x100



- The micropapillary subtype (characterized by delicate papillary structures without a

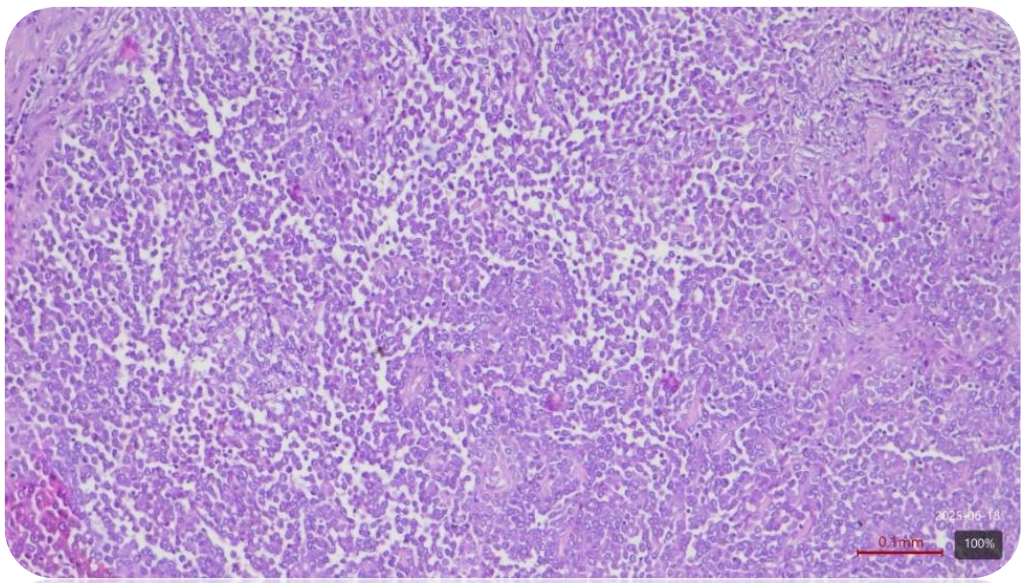
fibrovascular axis) was identified in 4% of cases. This value corresponds to the published range (usually 2–6%).

Microphotographs 3: Micropapillary SC, magnification x40.



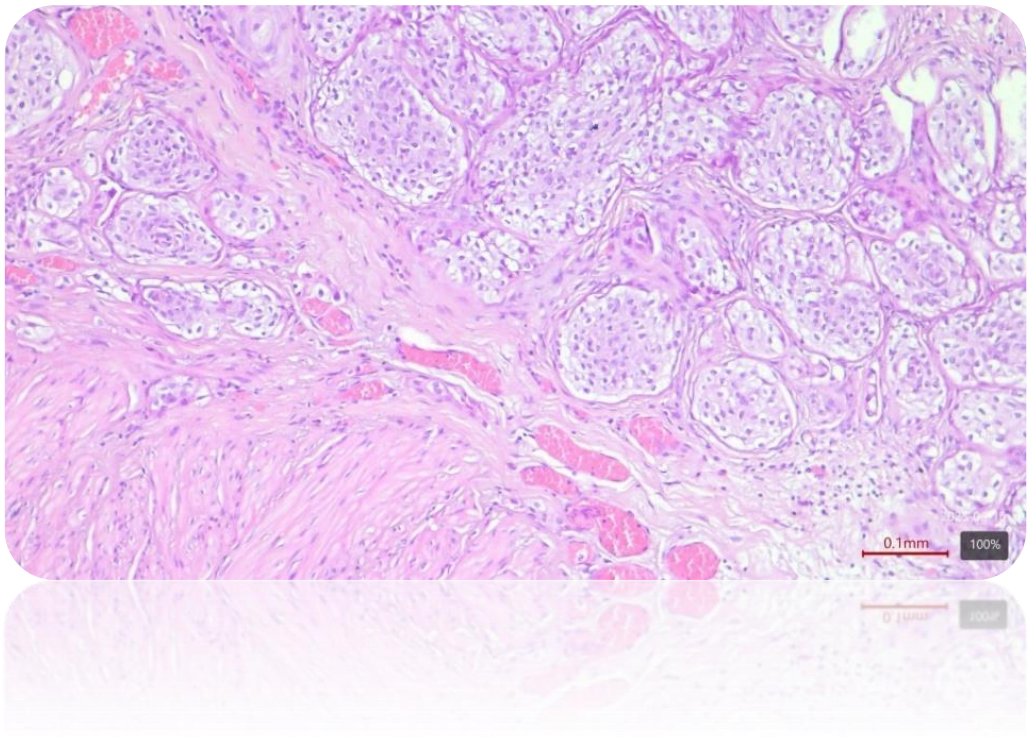
- The plasmacytoid subtype (diffuse, with plasma-like cells) was found in 3% of cases, which falls within the known range of 1–3%.

Microphotographs 4: Plasmacytoid UC, magnification x100.



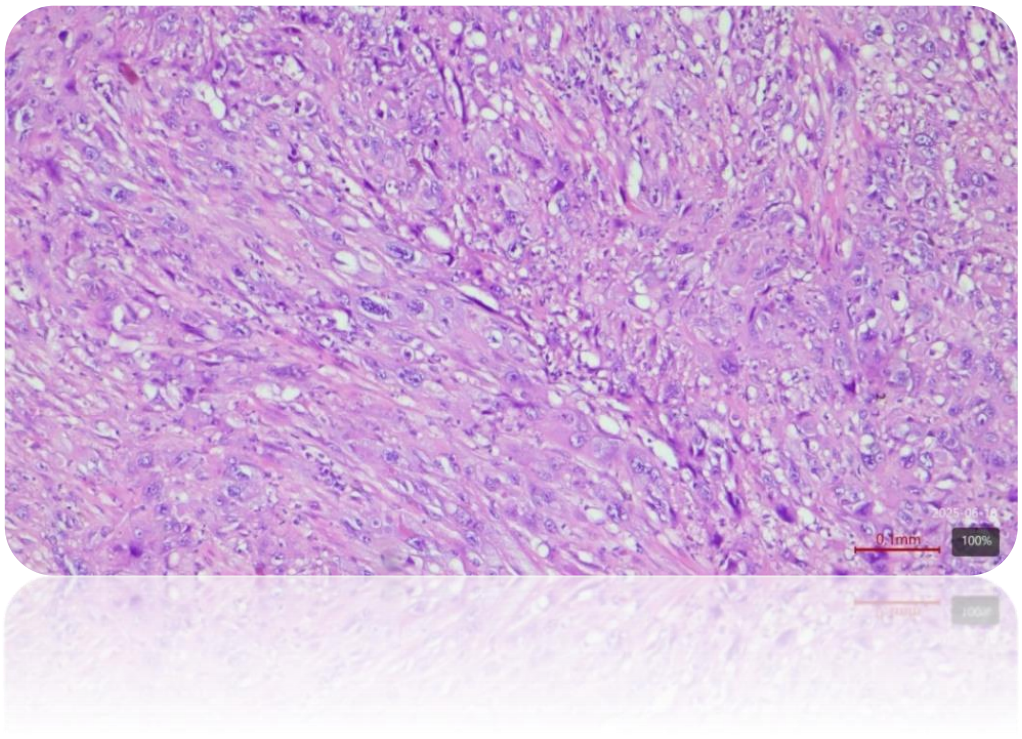
- We found the nested subtype in 2% of tumors. This is slightly above the expected rate for this extremely rare variant (about 1%).

Microphotographs 5: Nested subtype UC, magnification x100.



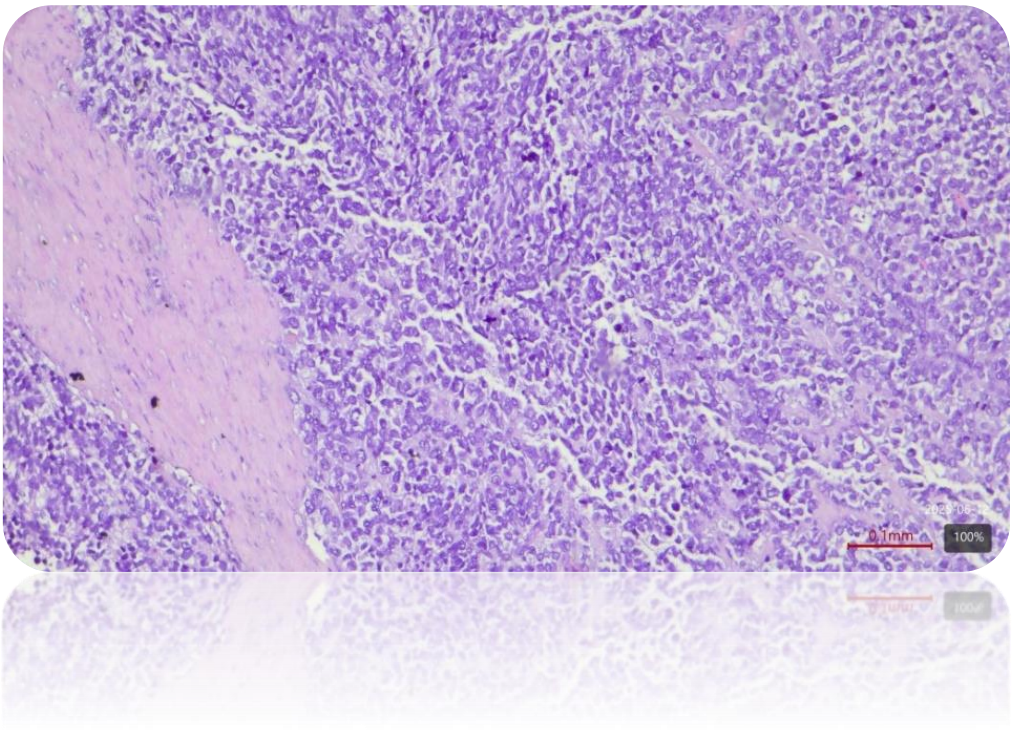
- Sarcomatoid (verrucous cell) carcinoma is observed in 5% of cases, which is significantly higher than the typical <1% reported in the literature.

Microphotographs 6: Sarcomatoid SC, magnification x100



- **Small cell** carcinoma also accounts for 5% in our series, with an expected frequency of less than 1%.

Microphotographs 7: Small cell carcinoma, magnification x100.



This increased representation of certain rare subtypes (sarcomatoid, small cell, and nested) may also be due to the referral of complex cases to university clinics, as well as the small sample size, which allows for statistical fluctuations. Regardless, the presence of these subtypes highlights the aggressiveness of the cohort studied and the need for their accurate identification due to their impact on prognosis and therapeutic behavior.

ANALYSIS OF THE MAIN MOLECULAR VARIANTS OF MIBC AND STUDY OF THEIR FREQUENCY

Currently, the defined molecular categories refer to scientific research based on DNA or RNA studies. For clinical purposes, it is cost-effective to use the IHC method. It should be noted that the two methods do not completely overlap, and it is important to specify which technique is used. In this paper, the method used is immunohistochemistry, but the names of the variants will be used regardless of the method of definition.

For each of the 100 MIBCs, a molecular variant was determined according to the consensus

classification (Ba/Sq, LumP, LumU, LumNS, Stroma-rich, NE-like). The distribution of cases by variant is shown in Table 2:

Table 2*: *Frequency distribution of molecular variants in our cohort according to the consensus molecular classification of MIBC (2020).

Molecular variant	Number of cases (n)	Proportion (%)
Basal/Squamous	3	33
Luminal Papillary	24	24
Luminal Unstable	16	16
Basal/Luminal	9	9
Neuroendocrine-like (NE-like)	6	6
Stroma-rich	2	2
Luminal NS	10	10
Total	10	10

✎ *Note: Frequency of molecular variants in MIBC in the studied series (N = 100). Seven categories were reported: basal, luminal papillary, luminal unstable, luminal nonspecified, basoluminal, neuroendocrine-like, and stromal-enriched. The basoluminal (double-positive) variant was defined as a separate category in the present study.*

As shown in Table 2, the basal molecular variant was identified in 33% of cases (33/100), while luminal variants (papillary, unstable, and unspecified) accounted for a total of approximately 50% (24% + 16% + 10%) of all tumors. We additionally identified a group of **"basal-luminal"** tumors (9%) that co-express basal and luminal markers in different components of the tumor. These cases reflect tumors most often classified as "mixed histological subtype" and, as expected, show differences in their molecular immunoprofile. The remaining tumors fall into the double-negative categories: neuroendocrine-like (NE-like) **–** 6%**,** and stroma-rich – 2% of the series. The distribution of variants obtained in our study is very close to the published data in large series.

Table 3 presents a comparison between the frequencies of variants in our cohort and two key reference studies – the consensus classification by Kamoun et al. (2020) and The Cancer Genome Atlas (TCGA, 2017).

Table 3. Comparison of the percentage distribution of molecular variants between the current study (n = 100) and published sources:

Molecular variant	Our study (N=100)	Kamoun et al. 2020 (N≈1750)	TCGA 2017 (N=408)
Basal (Basal/Squamous)	3	35	35% (Basal/Sq)
Luminal papillary	24	24	35% (Luminal-papillary)
Luminal unstable	16	15	– (included in Lum. Inf.)
Luminal unspecified	10	8	6% ("Luminal, umbrella")
Stroma-enriched (infiltrate)	2	15	19% ("Luminal-infiltrated")
Neuroendocrine-like	6	3	5% ("Neuronal")
Basal luminal (double positive)	9	No separate class	No separate class

📖 *Sources:* Kamoun et al., Eur Urol 2020; Robertson et al., Cell 2017 (TCGA)

Our results show a high degree of agreement with the consensus classification. For example, basal tumors account for about 33%, which is practically identical to the approximately 35% found in large series. Similarly, the luminal papillary variant accounts for 24%, which is identical to the proportion found by Kamoun et al. (2020). The luminal unstable variant is 16% in our study, which is almost identical to about 15% in the consensus. The luminal nonspecified variant is 10% compared to about 8% in the published data. The presentation of the stroma-rich variant is slightly different: only 2% in our study, compared to about 15–19% in other sources. This may be due to the limitations of the IHC methodology used – we may have classified some stromal-rich tumors by their epithelial component (basal or luminal) rather than as a separate group. Some sarcomatoid or conventional UCs that have undergone EMT and have been designated as "Mes-like" in previous studies are less common in our study compared to the reported RNA class Stroma-rich. In short, stroma-rich is not only a biological subtype, but also depends on other factors that are likely to be circumvented by in situ methods. It is worth noting that we identified a basoluminous (double-positive) variant in 9% of cases – a separate class that is not distinguished in transcriptomic classifications, but the presence of such hybrid tumors has also been noted by other authors. Our basal-luminal cases (9%) have both basal and luminal immunoexpression and emphasize that some tumors do not actually fit unambiguously into only one of the two main groups. It is appropriate to continue the study of this category with other methods, such as RNA sequencing.

In summary, the luminal and basal categories among our 100 cases are in a ratio of approximately 60:40, which supports the dual-core ("luminal vs. basal") concept in MIBC. The total proportion of luminal variants (LumPap, LumU, LumNS) in our series is approximately 50%, comparable to approximately 47% in the consensus. Basal tumors account for 33% (close to 35% in large series). The main differences are the lower proportion of stroma-enriched tumors in our series (2% vs. about 15%) and the presence of a separate basoluminal category.

ANALYSIS OF CORRELATION BETWEEN HISTOLOGICAL AND MOLECULAR CLASSIFICATION

The study of the relationship between tumor morphology and molecular variant reveals several significant trends. We observe that certain histological subtypes prefer specific molecular categories. To assess this correlation, cross-tabulations between histological and molecular classification were constructed, and a $\chi 2$ test was applied. The results show a statistically significant dependence between the two classification approaches ($\chi 2$, $p < 0.001$).

Table 4: Cross-tabulation examining the correlation between histological subtype and molecular variant.

			Molecular variant							Total
			Basal	Luminal papillary	Luminal unstable	Basal-luminal	Neuroendocrine-like	Stroma-rich	Luminal nonspecific	
Histological subtype	Urothelial carcinoma (NOS)	Count	1	0	0	0	0	0	3	4
		% within Histological subtype	25	0	0	0	0	0	75.0	100.0
		% within Molecular variant	3.0	0	0	0	0	0	30.0	4.0
		% of Total	1	0	0	0	0	0	3.0	4.0
	Papillary UK	Count	0	17	6	2	0	0	2	27
		% within Histological subtype	0	63	22	7.4	0	0	7.4	100.0
		% within Molecular variant	0	70.8	37.5	22.2	0	0	20.0	27.0
		% of Total	0	17	6.0	2.0	0	0	2.0	27.0
	UK with squamous cell differentiation	Count	1	0	0	0	0	0	0	17
		% within Histological subtype	100	0	0	0	0	0	0	100.0
		% within Molecular variant	51.5	0.0	0	0	0	0	0	17.0
		% of Total	17	0	0	0	0	0	0	17
	Glandular differentiation	Count	1	0	2	0	0	0	0	3
		% within Histological subtype	33	0	66	0	0	0	0	100.0
		% within Molecular variant	3.0	0	12.5	0	0	0	0	3.0
		% of Total	1.0	0	2.0	0	0	0	0	3.0
	Sarcomatoid UK	Count	3	0	0	0	0	2	0	5
		% within Histological subtype	60	0	0	0	0	40.0	0	100.0
		% within Molecular variant	9.1	0	0	0	0	100.0	0	5.0
		% of Total	3.0	0	0	0	0	2.0	0	5
	Plasmacytoid UK	Count	0	1	0	1	0	0	1	3
		% within Histological	0.0	33.3	0	33	0	0	33.3	100.0

		subtype								
		% within Molecular variant	0	4.2	0	11.1	0	0	10.0	3.0
		% of Total	0	1	0	1.0	0	0	1.0	3.0
	Nested UK	Count	0	2	0	0	0	0	0	2
		% within Histological subtype	0	100	0	0	0	0	0	100.0
		% within Molecular variant	0	8.3	0	0	0	0	0	2.0
		% of Total	0	2	0	0	0	0	0	2.0
	Micropapillary UK	Count	0	1	3	0	0	0	0	4
		% within Histological subtype	0	25	75	0	0	0	0	100.0
		% within Molecular variant	0	4.2	18.8	0	0	0	0	4.0
		% of Total	0	1.0	3	0	0	0	0	4.0
	Small cell carcinoma	Count	0	0	0	0	5	0	0	5
		% within Histological subtype	0	0	0	0	100	0	0	100.0
		% within Molecular variant	0	0	0	0	83.3	0	0	5.0
		% of Total	0	0	0	0	5	0	0	5
	Poorly differentiated UK	Count	2	0	0	0	1	0	0	3
		% within Histological subtype	66.7	0	0	0	33.3	0	0	100.0
		% within Molecular variant	6.1	0	0	0	16.7	0	0	3.0
		% of Total	2.0	0	0	0	1.0	0	0	3
	Mixed subtype	Count	9	3	5	6	0	0	4	27
		% within Histological subtype	3	11	18	22	0	0	14.8	100.0
		% within Molecular variant	27.3	12.5	31.3	66.7	0	0	40.0	27.0
		% of Total	9.0 %	3.0	5.0	6.0	0	0	4.0	27.0
Total		Count	3	24	16	9	6	2	10	100

	% within Histological subtype	33	24	16	9	6	2	10	100.0
	% within Molecular variant	100	100	100.0	100.0	100.0	100.0	100.0	100.0
	% of Total	33.0	24.0	16.0	9	6	2	10	100.0

The table shows, for example, that all small cell carcinomas fall entirely within the neuroendocrine-like molecular variant. Squamous cell differentiation corresponds to the basal molecular variant (100%). All sarcomatoid cases fall within the basal or double-negative spectrum. Micropapillary tumors are mainly distributed in the luminal categories, while nests are also predominantly luminal. The plasmacytoid subtype showed a more heterogeneous distribution, but predominantly in the luminal spectrum. Papillary urothelial carcinomas, as expected, fall mainly into the luminal papillary variant. Mixed cases were distributed among different variants depending on the dominant component. Interestingly, 9 of the 27 mixed tumors showed a "basal-luminal" profile, i.e., expression of markers for both types, which confirms the need for these cases to be further investigated using other methods (e.g., RNA sequencing).

Statistical analysis showed that the relationship between histological subtype and molecular variant was not random (χ^2 test with 60 degrees of freedom, $p < 0.001$).

- In summary, luminal molecular variants mainly comprise tumors with papillary, micropapillary, plasmacytoid, and nest morphology, while the basal variant mainly comprises tumors with squamous differentiation and sarcomatoid histological subtype. Small cell carcinoma falls into the neuroendocrine-like (NE-like) variant category. These relationships suggest that knowledge of the histological subtype can guide the probable molecular profile and, conversely, the immunohistochemical profile can predict the presence of hidden morphological components.

ANALYSIS OF HER2 EXPRESSION IN DIFFERENT MOLECULAR VARIANTS AND HISTOLOGICAL SUBTYPES

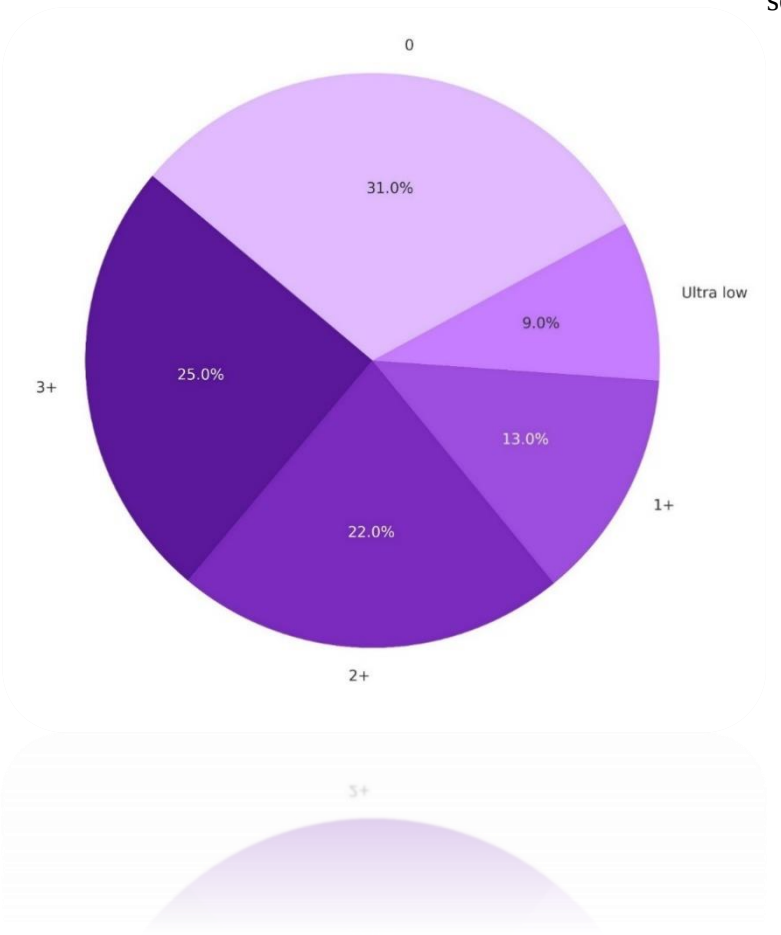
The expression of the HER2 (ERBB2) oncogene was examined in all 100 cases of MIBC by immunohistochemistry, following the ASCO/CAP (2025) criteria used in breast cancer. Each case was categorized with a HER2 result: 0 (complete absence of membrane staining), 0+ (minimal membrane staining, which is incomplete and weak in ≤ 10% of tumor cells) 1+ (incomplete and weak/difficult to detect membrane staining in >10% of tumor cells), 2+ (complete and weak to moderate membrane staining in >10% of tumor cells or intense complete membrane staining, but in < 10% of tumor cells – borderline result) or 3+ (complete, strong membrane staining in >10% of tumor cells). If the result falls into the borderline category (2+), it is appropriate to continue the examination with in situ hybridization (ISH) methods. No additional in situ hybridization was performed in the present study, and this will be the subject of ongoing research. HER2-low (low expression) corresponds to HER2 1+ or HER2 2+ with a negative result from the in situ hybridization method. In a narrow sense, HER2-positive is considered to be 3+ or 2+ with proven amplification. In recent years, the term "HER2-ultra low" has gained popularity, referring to minimal incomplete membrane expression in ≤10% of tumor cells. Such cases would traditionally be classified as 0, but we have placed them in a separate category (0+) in view of the possible benefit of new targeted therapies that show results even with low HER2 expression.

Distribution of HER2 status:

Of the 100 cases, a quarter (25%) had strong HER2 expression (3+), and an additional 22 cases were 2+ (moderate, unconfirmed expression). This means that a total of 47% of tumors have at least moderate HER2 expression, with 25% of them showing HER2 overexpression (3+). The remaining tumors are distributed as follows: HER2-0 (negative) – 31%, HER2-1+ – 13%, and HER2-ultra low – 9%. This is graphically represented in *Fig. 4.*

Figure 4: Overall distribution of HER2 expression in 100 cases of MIBC.

The pie chart shows the percentages of tumors in each category: 0 (31%), ultralow/0+ (9%), 1+ (13%), 2+ (22%), and 3+ (25%). It is evident that more than half of the cases (69%) show some HER2 positivity, although only 25% are strongly positive (3+).



These results show a higher proportion of HER2-positive tumors compared to some older studies. A possible explanation for the difference is that our series includes specific histological subtypes and molecular variants in which HER2 is more common, or due to advanced stage (pT2+). This "enrichment" of the cohort may lead to a higher overall frequency of HER2.

HER2 and molecular variants:

A detailed analysis of HER2 status by molecular variants was performed, the results of which are summarized in *Table 5:*

Table 5: The cross-tabulation shows the distribution of HER2 expression by molecular variants. *For each molecular class, the percentage of cases with a specific HER2 status (0, 0+, 1+, 2+, 3+) is presented. The contrast between the basal and luminal groups is clearly visible: HER2-negative tumors predominate in the basal group, while in the luminal group there are large proportions with 2+ and 3+.*

			HER-2 status					Total
			0	1	2	3	0	
Molecular variant	Basal	Count	1	6	3	0	6	33
		% within Molecular variant	54.5	18.2	9	0	18	100.0
		% within HER-2 status	58.1	46.2	13.6	0	66.7	33.0
		% of Total	18.0	6	3	0	6	33.0
	Luminal papillary	Count	3	2	9	8	2	24
		% within Molecular variant	12.5	8.3	37	33	8	100.0
		% within HER-2 status	9.7	15.4	40.9	32.0	22	24.0
		% of Total	3.0	2.0	9.0	8.0	2	24.0
	Luminal unstable	Count	1	1	5	9	0	16
		% within Molecular variant	6.3	6	31	56.3	0	100.0
		% within HER-2 status	3.2	7.7	22.7	36.0	0	16.0
		% of Total	1.0	1.0	5.0	9.0	0	16.0
	Basoluminous	Count	0	4	2	3	0	9
		% within Molecular variant	0	44.4	22	33	0	100
		% within HER-2 status	0	30.8	9.1	12.0	0	9.0
		% of Total	0	4.0	2	3.0	0	9
	NE-like	Count	6	0	0	0	0	6
		% within Molecular variant	100	0	0	0	0	100.0
		% within HER-2 status	19.4	0	0	0	0	6.0
		% of Total	6	0	0	0	0	6.0
	Stroma-rich	Count	1	0	0	0	1	2
		% within Molecular variant	50	0	0	0	50.0	100.0
		% within HER-2 status	3.2	0	0	0	11.1	2.0
		% of Total	1.0	0	0	0	1.0	2.0
	Luminal nonspecified	Count	2	0	3	5	0	10
		% within Molecular variant	20	0	30	50	0	100.0
		% within HER-2 status	6.5	0	13.6	20.0	0	10.0
		% of Total	2.0	0	3.0	5.0	0	10
Total		Count	31	13	22	25	9	10
		% within Molecular variant	31	13.0	22	25	9	100.0
		% within HER-2 status	100	100.0	100.0	100.0	100.0	100.0
		% of Total	31.0	13.0	22.0	25.0	9	100.0

Specifically, in the basal variant (33 cases), no tumors with strong 3+ expression were found. In more than 50% of basal tumors, HER2 was completely negative (0), while the rest showed a weak signal (1+ or rarely 2+), with about 18% classified as "ultra low." Conversely, luminal variants (LumP, LumU, LumNS) showed significantly higher HER2 expression. For example, of the 24 luminal papillary tumors, 33% were 3+ and 38% were 2+, i.e., a total of 71% had moderate to strong HER2 expression. In the luminal unstable variant (16 cases), this proportion is even higher: 56% are 3+ and 31% are 2+, or a total of 87% HER2-positive, including more than half with 3+. The luminal nonspecified variant (10 cases) also shows a high frequency of HER2: 50% are 3+ and 30% are 2+, making a total of 80%. Interestingly, the basalo-luminal hybrid subtype (9 cases) occupies an intermediate position – 33% are 3+, 22% are 2+, and the remaining 44% are 1+; no basalo-luminal tumor was HER2-null. As expected, the double-negative categories (stroma-enriched and NE-like) had no cases of HER2 overexpression (0% 3+). All NE-like tumors (6/6) were HER2-0, while of the two Stroma-rich tumors, one showed 0 and the other showed ultra low expression. These data convincingly demonstrate that HER2 status is closely related to the molecular profile of the tumor: luminal carcinomas express HER2 much more frequently than basal ones (the difference is statistically significant, $p<0.001$). Therefore, strong membrane HER2 immunoreactivity may also serve as a marker for the luminal tumor phenotype.

HER2 and histological subtypes

The relationship between HER2 expression and specific morphological subtypes was analyzed. Of the 27 papillary carcinomas in our study, 12 (44%) demonstrated 3+ HER2 expression, while another 7 (26%) were 2+. This means that approximately 70% have at least some expression, including a high percentage of strongly positive cases. It was found that micropapillary carcinomas are almost always HER2-positive – all four cases showed ≥2+ expression, with three of them being 3+ and one being 2+. This is an important finding, as the micropapillary subtype is known for its aggressiveness. Furthermore, the literature data show frequent HER2 expression, which is confirmed by our data. Urothelial carcinomas with glandular differentiation (3 cases) showed conflicting data for HER2: 2 cases were 0, and one case was 2+, but the small number of these cases does not allow for statistically significant conclusions. Plasmacytoid tumors (3 cases) showed results of 1+, 2+, and 3+, respectively, which also makes assessment difficult, but places them more in the group of HER2-expressing tumors, as is consistent with the literature. This corresponds to the fact that plasmacytoid tumors usually belong to the luminal category. They also have other molecular features, such

as *CDH1* loss. Nodular carcinomas (2 cases) were at both extremes: one case HER2-0 and 3+, respectively. We can expect HER2 overexpression in them, but research on larger cohorts is needed. Sarcomatoid (5 cases) are 0 (4 cases) or 0+ (1 case), which is somewhat expected given their frequent basal profile. Small cell (5 cases) are 0 (corresponding to NE-like). Poorly differentiated (3 cases) are negative (0). Squamous cell differentiations (17 tumors) are predominantly HER2-negative: 8 cases are 0.5–1+, 4 are 0+; there are no 3+ or 2+ cases.

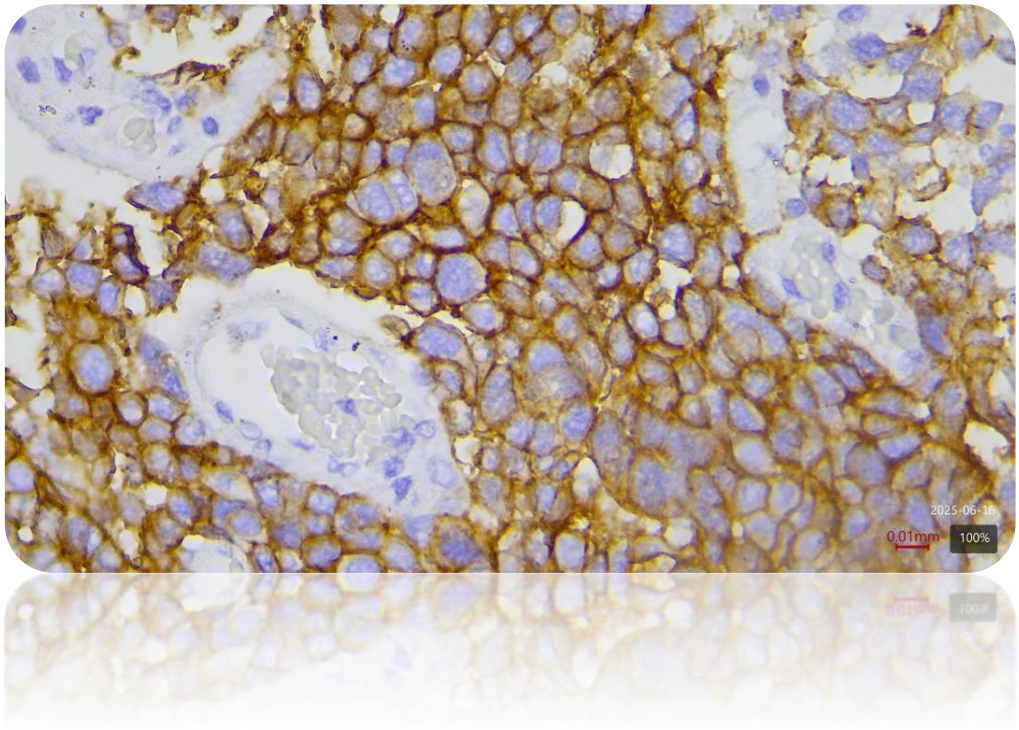
Table 6: The cross-tabulation shows the distribution of HER2 expression by histological subtype of urothelial carcinoma. The percentages of HER2 statuses (0, 0+, 1+, 2+, 3+) for each subtype are shown.

			HER-2 status					Total
			0	1	2	3	0	
Histological type	Urothelial carcinoma NOS	Count	3	0	1	0	0	4
		% within Histological type	75.0	0	25	0	0	100.0
		% within HER-2 status	9.7	0	4.5	0	0	4.0
		% of Total	3.0	0	1.0	0	0	4.0
	Papillary UK	Count	2	4	7	12	2	27
		% within Histological type	7.4	14.8	25.9	44	7	100.0
		% within HER-2 status	6.5	30.8	31.8	48.0	22.2	27.0
		% of Total	2.0	4.0	7.0	12.0	2	27
	UK with squamous cell differentiation	Count	8	5	0	0	4	17
		% within Histological type	47.1	29.4	0	0	23.5	100.0
		% within HER-2 status	25.8	38.5	0	0	44.4	17.0
		% of Total	8	5	0	0	4	17.0
	Glandular differentiation	Count	2	0	1	0	0	3
		% within Histological type	66.7	0	3	0	0	100.0
		% within HER-2 status	6.5	0	4.5	0	0	3.0
		% of Total	2.0	0	1.0	0	0	3.0
	Sarcomatoid UK	Count	4	0	0	0	1	5
		% within Histological type	80	0	0	0	20.0	100.0
		% within HER-2 status	12.9	0	0	0	11.1	5.0
		% of Total	4.0	0	0	0	1.0	5.0
	Plasmacytoid UK	Count	0	1	1	1	0	3
		% within Histological type	0	33	3	3	0	100
		% within HER-2 status	0	7.7	4.5	4	0	3.0
		% of Total	0	1.0	1	1.0	0	3.0
	Nested UK	Count	1	0	0	1	0	2
		% within Histological type	50	0	0	50	0	100.0
		% within HER-2 status	3.2	0	0	4.0	0	2.0
		% of Total	1.0	0	0	1.0	0	2.0
	Micropapillary UK	Count	0	0	1	3	0	4
		% within Histological type	0	0	25	75	0	100.0
		% within HER-2 status	0	0	4.5	12	0	4.0
		% of Total	0	0	1.0	3.0	0	4
	Small cell carcinoma	Count	5	0	0	0	0	5
		% within Histological	100	0	0	0	0	100.0

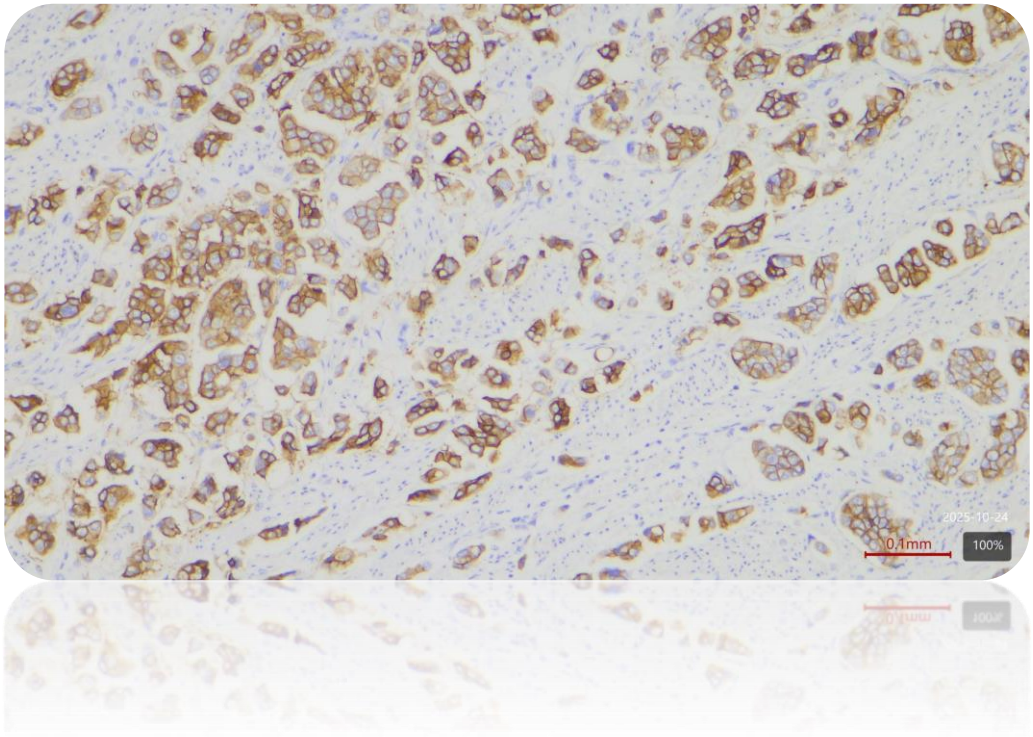
		type						
		% within HER-2 status	16.1	0	0	0	0	5.0
		% of Total	5	0	0	0	0	5.0
	Weakly differentiated UK	Count	3	0	0	0	0	3
		% within Histological type	100	0	0	0	0	100.0
		% within HER-2 status	9.7	0	0	0	0	3.0
		% of Total	3	0	0	0	0	3.0
	Mixed option	Count	3	3	11	8	2	27
		% within Histological type	11	11	40.7	29.6	7.4	100.0
		% within HER-2 status	9.7	23.1	50.0	32.0	22.2	27.0
		% of Total	3	3	11.0	8.0	2	27.0
Total		Count	31	13	22	25	9	100
		% within Histological type	31	13	22	25	9	100.0
		% within HER-2 status	100	100.0	100	100.0	100	100.0
		% of Total	31.0	13.0	22.0	25.0	9	100.0

- In summary, HER2 overexpression is most common in papillary and luminal molecular pathway-related (including micropapillary) tumors. Squamous cell and sarcomatoid UCs rarely show HER2 3+. These results have important therapeutic implications: they suggest that patients with luminal tumors (which are more likely to express HER2) may be candidates for anti-HER2 targeted therapy. Conversely, targeting HER2 is unlikely to be effective in basal tumors, as they do not express the target protein; other approaches (e.g., immunotherapy, platinum-based chemotherapy) are more appropriate for them.

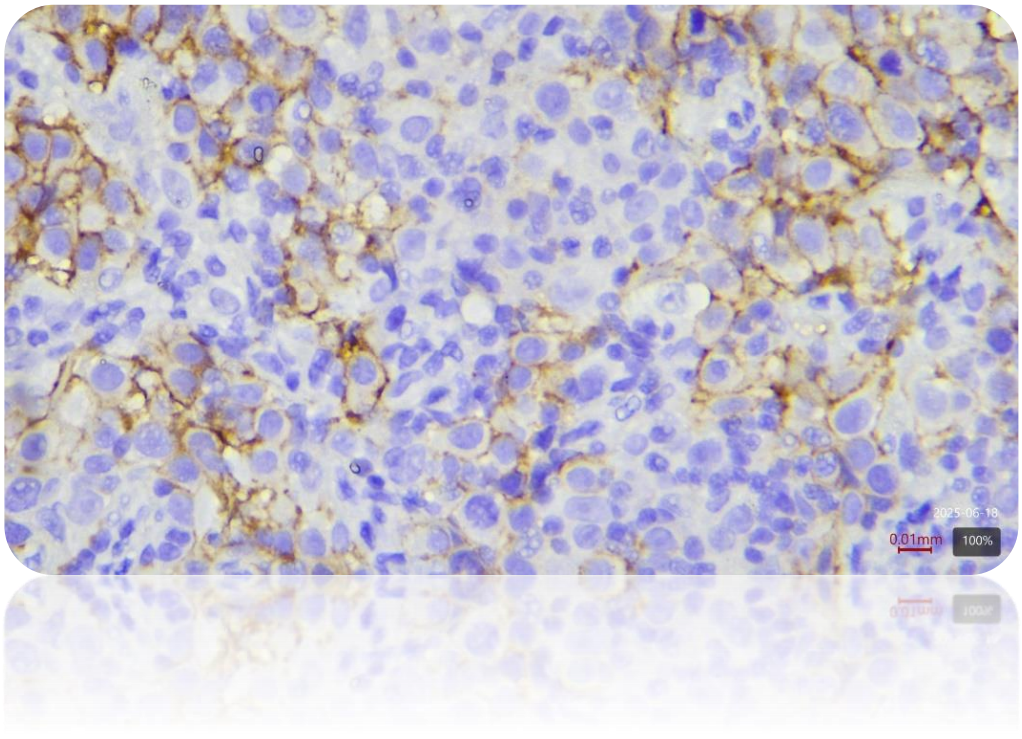
Microphotographs 8: Urotelial papillary carcinoma, luminal papillary molecular variant with strong full membrane expression in over 10% of tumor cells / HER2 3+.



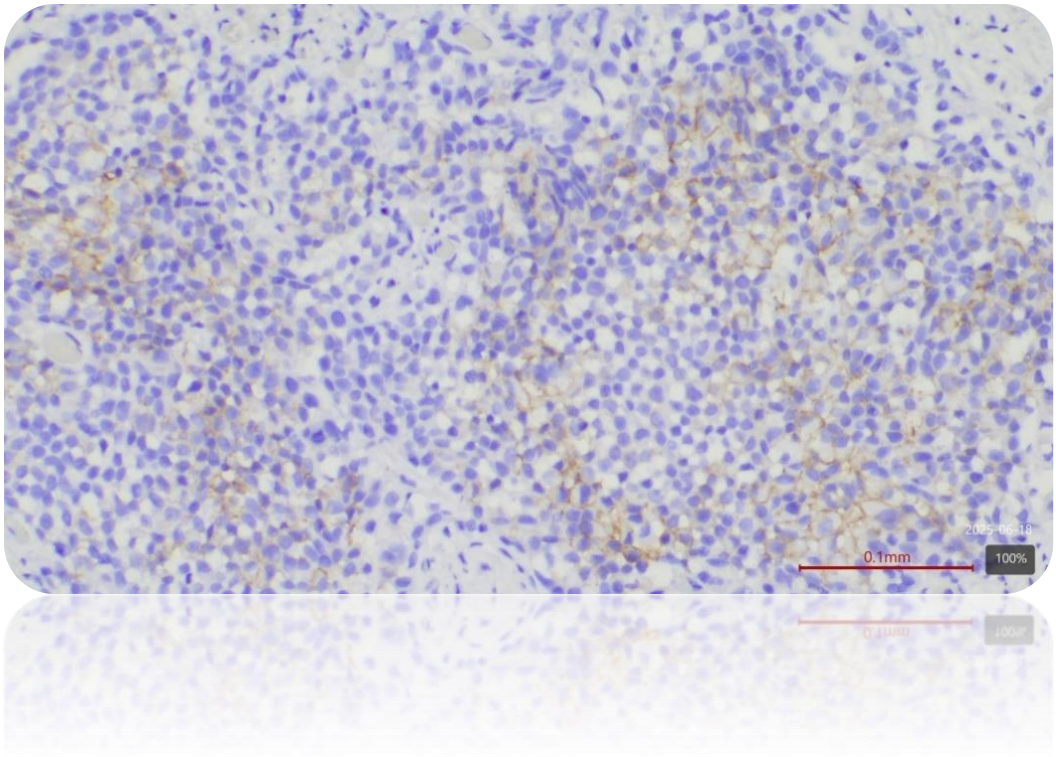
Microphotographs 9: HER2 3+ expression (strong and complete membrane staining in >10% of tumor cells in micropapillary structures), magnification x100.



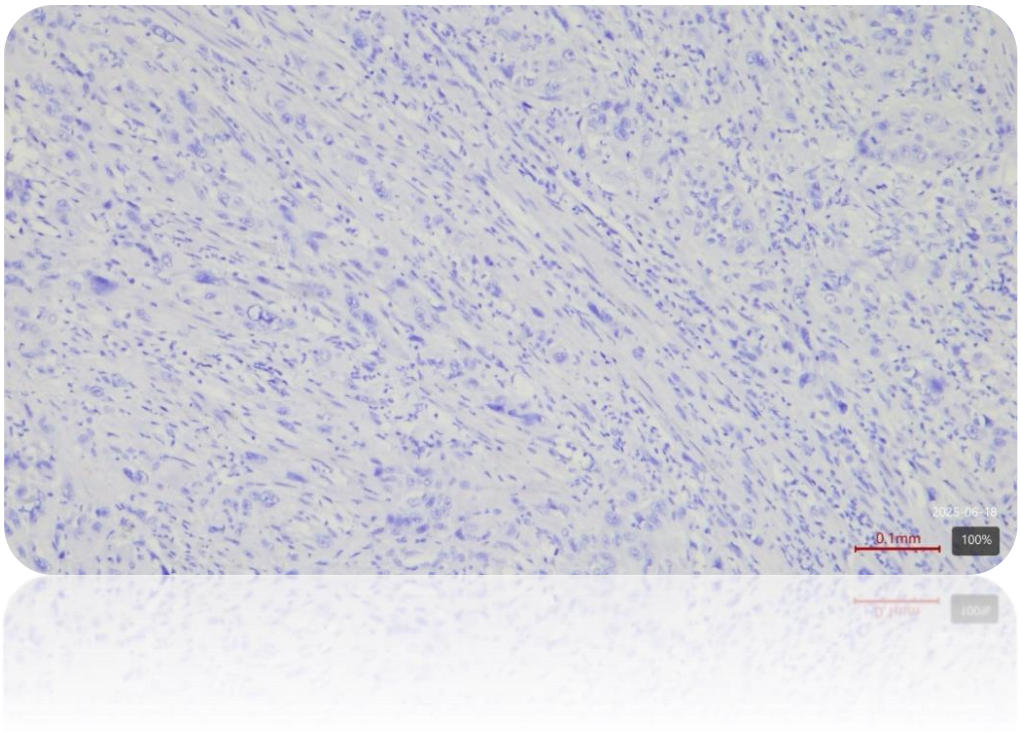
Microphotographs 10: Lymphoepithelioma-like carcinoma with HER2 2+ expression, magnification x400.



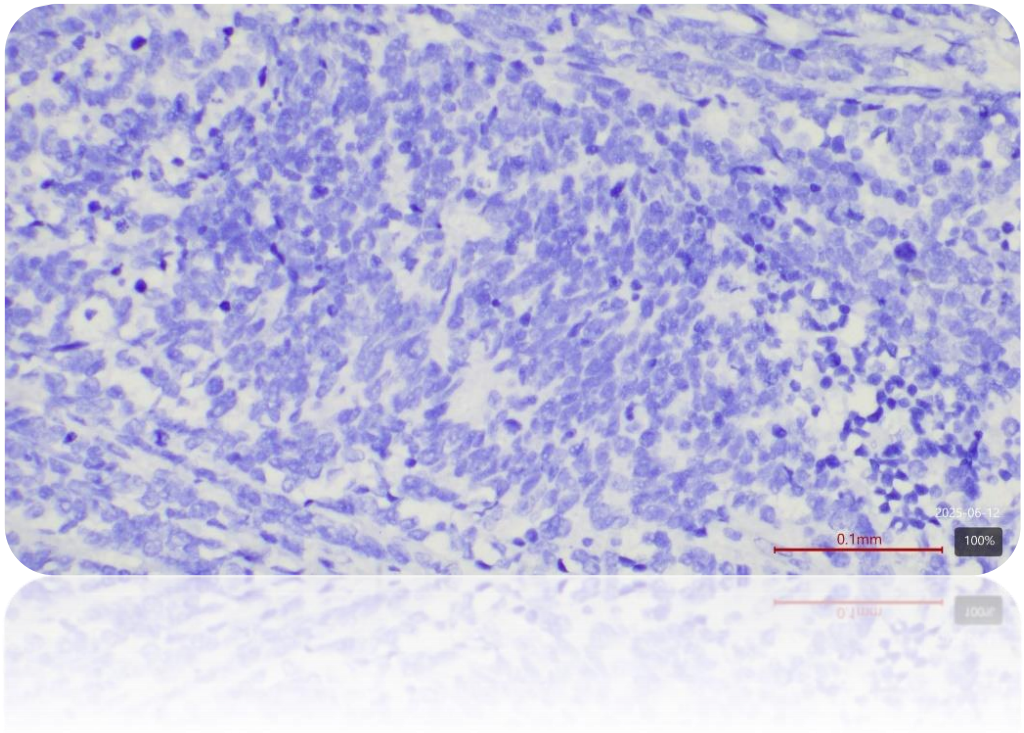
Microphotographs 11: Incomplete and weak staining in more than 10% of tumor cells/HER2 1+ expression in the plasmacytoid subtype, magnification x200.



Microphotographs 12: Sarcomatoid subtype UK, basal molecular variant HER2 (0) complete absence of membrane staining magnification x100.



Microphotographs 13: Small cell carcinoma with complete absence of membrane staining/HER2 (0), magnification x200.



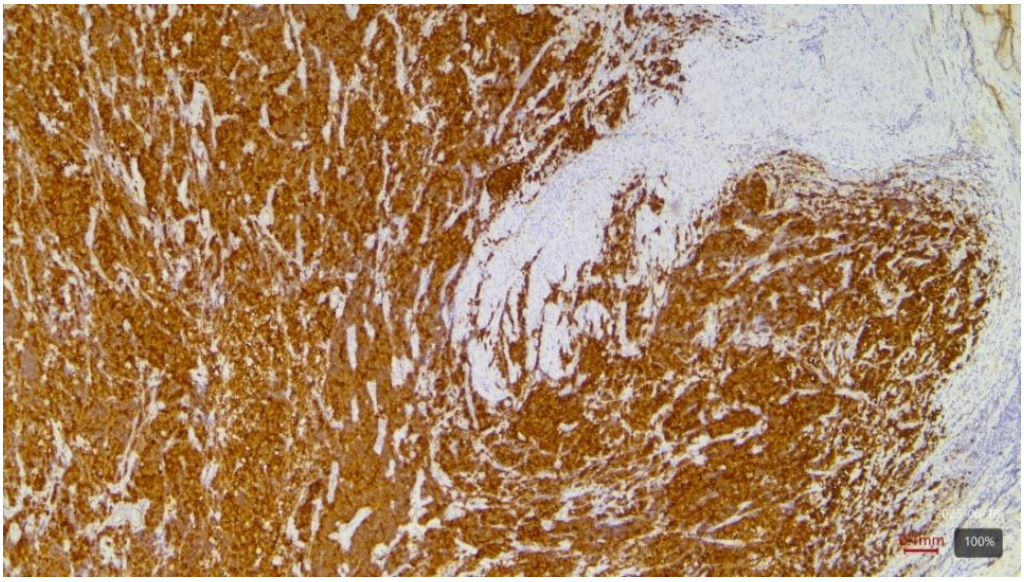
ANALYSIS OF P16 EXPRESSION

The p16INK4A (CDKN2A) protein is a tumor suppressor whose expression serves as a marker for (non)functionality of the RB pathway. There are *two main mechanisms* for inactivation of cell cycle control in urothelial carcinoma: (1) loss or inactivation of *CDKN2A*, leading to a lack of p16; or (2) mutation/loss of *RB1*, leading to non-functional pRb and compensatory overexpression of p16. Different molecular variants favor one mechanism or the other. We used p16 as an additional immunohistochemical classifier of the luminal category, as well as in double-negative and double-positive cases. All luminal unstable (LumU) tumors (16/16, 100%) were p16-positive, which is expected given the frequent *RB1* mutations in this variant. Thus, high p16 levels in LumU are an indicator of Rb function loss (non-functional pRb) – a phenomenon also known as "paradoxical p16 overexpression". In contrast, luminal papillary (LumP) tumors are p16-negative – in our series, all p16-negative luminal tumors were classified in this category. This corresponds to the indirect inactivation of the Rb pathway in LumP through the loss of *CDKN2A* (9p21 deletion), leading to a lack of p16 protein. The luminal nonspecified (LumNS) variant showed mixed results – out of 10

cases, 2 were p16-negative, and 8 were positive, suggesting some heterogeneity in the mechanisms of these undefined luminal tumors (probably some are similar to LumP, and others to LumU in terms of the RB pathway). Basal-luminal tumors (9 cases) showed only 22% p16 positivity (2/9), i.e., a p16-negative profile predominates in them. This confirms that our basoluminal group tends more towards the luminal (loss of CDKN2A) than towards the basal axis.

The characteristic molecular feature of NE-like variants is the simultaneous inactivation of TP53 and RB1. It has been reported that most NE-like tumors have mutations in both genes simultaneously**,** a pattern similar to that seen in small cell lung carcinoma. This double loss leads to complete release of the cell cycle from tumor suppressor control. From the point of view of p16, this means that CDKN2A is rarely lost by deletion, as there is no selective need for this additional alteration – the Rb pathway is already compromised at the RB1 level. On the contrary, due to the loss of functional Rb protein, p16INK4A usually accumulates in NE-like tumor cells (negative feedback is disrupted). Therefore, NE-like carcinomas have strong p16-positive immunostaining in the majority of cases. Our data also reflect this: of the 6 tumors with NE-like morphology in our cohort, 5 are p16-positive (approximately 83%), and only 1 is p16-negative. The single negative case could be explained by additional loss or promoter methylation of CDKN2A in this tumor, but overall, our data confirm that the NE-like variant is predominantly p16 positive.

***Microphotographs 14: Strong nuclear and cytoplasmic expression of p16 in small cell carcinoma*, magnification x40.**



The two tumors classified as "stroma-rich" in our series are p16-positive (100%). This small number does not allow conclusions to be drawn, but it shows that it is possible for stroma-rich tumors to express p16 under certain circumstances – probably when there is a loss of RB1 in tumor cells.

Microphotographs 15: Stroma-enriched molecular variant with strong nuclear expression of p16, magnification x400.

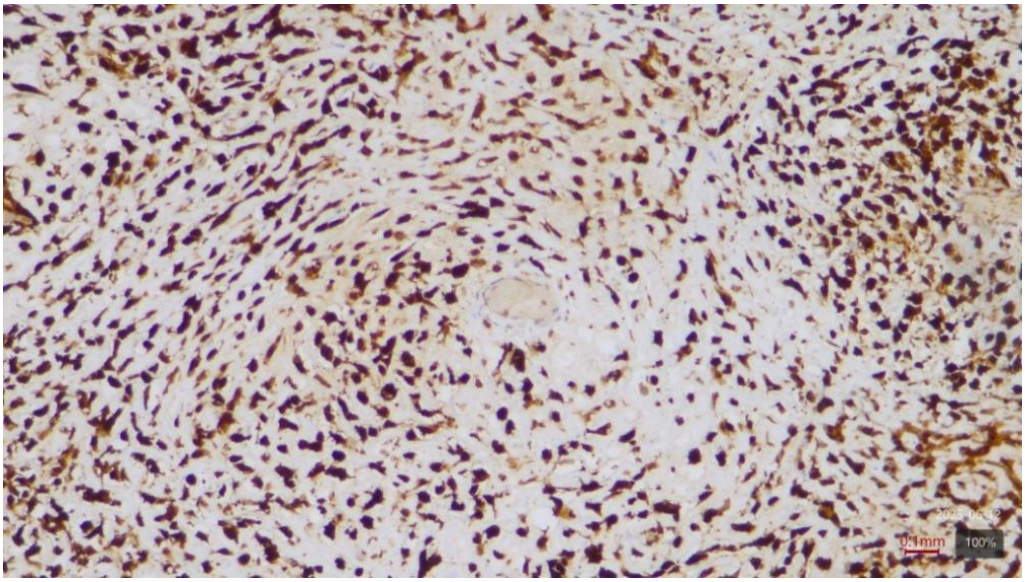


Table 7. Cross-tabulation showing p16 expression in different molecular variants.

			p16		Total
			p16 positive	p16 negative	
Molecular variant	Luminal papillary	Count	0	24	24
		% within Molecular variant	0.0	100.0	100.0
		% within p16	0	70.6	35.8
		% of Total	0	35.8	35.8
	Luminal unstable	Count	16	0	16
		% within Molecular variant	100	0	100.0
		% within p16	48.5	0	23.9
		% of Total	23.9	0	23.9
	Basoluminous	Count	2	7	9
		% within Molecular variant	22	77.8	100.0
		% within p16	6.1	20.6	13.4
		% of Total	3.0	10.4	13.4
	NE-like	Count	5	1	6
		% within Molecular variant	83	16.7	100.0
		% within p16	15.2	2.9	9.0
		% of Total	7.5	1.5	9.0
	Stroma rich	Count	2	0	2
		% within Molecular variant	100	0	100.0
		% within p16	6.1	0	3.0
		% of Total	3	0	3.0
	Luminal unspecified	Count	8	2	10
		% within Molecular variant	80	20	100.0
		% within p16	24.2	5.9	14.9
		% of Total	11.9	3.0	14.9
Total		Count	33	34	67
		% within Molecular variant	49.3	50.7	100
		% within p16	100	100	100.0
		% of Total	49.3	50.7	100.0

P16 and morphological subtypes:

Overall, p16 status correlates more with molecular profile than directly with morphology. For example, small cell (NE-like) carcinomas showed diffuse p16 in 5 cases (≈83% positive), which is expected in the NE-like variant with double TP53/RB1 mutation.

- In summary, luminal papillary tumors do not have p16, while luminal unstable and NE-like tumors almost always express p16. Intermediate variants (LumNS, basoluminal) show mixed behavior. Our results confirm the role of p16 as a distinguishing feature between different biological pathways: p16 is lowly expressed in tumors with CDKN2A deletion (most commonly LumP) and highly expressed in tumors with RB1 mutation (LumU, NE-like).

From a diagnostic point of view, p16 can be used as an additional immunohistochemical marker for characterizing MIBC. Strong p16 (+) staining in a large percentage of cells indicates an unfavorable molecular profile (Luminal Unstable or NE-like), which is associated with aggressiveness but also potentially with sensitivity to chemotherapy (e.g., NE-like tumors respond to platinum-based CT). On the other hand, a p16 (−) result in MIBC suggests a luminal papillary variant, which, although with a better prognosis, may not respond to certain therapies (LumP are more resistant to neoadjuvant chemotherapy according to some data).

Overall, our data confirm that the p16 expression profile is a valuable molecular marker for each category. It reflects fundamental differences in tumorigenesis: luminal tumors most often evade the cell cycle pathway through loss of p16, while non-luminal tumors do so through loss of RB1 and, accordingly, have high levels of p16. This complements the other immunohistochemical markers and helps to more accurately determine the molecular variant.

Discussion

This study provides insight into the morphological subtypes and molecular characteristics of muscle-invasive urothelial carcinoma of the bladder, thereby filling some gaps in knowledge and providing a valuable local perspective. The results confirm the high heterogeneity of MIBC at both the tissue (morphological) and molecular levels. The discussion below focuses on the key findings, their context in light of published data, and their clinical significance.

1. Histological subtypes and their frequency: We found that 66% of MIBCs have a histological component different from the conventional one. This is higher than the classically cited frequencies of histological subtypes (around 25%) in bladder cancer. One reason may be the thorough search for and reporting of even minimal foci with different morphology. It is known that in routine practice, up to 50% of histological subtypes may initially remain un, especially if they are small foci. In our series, the targeted review of all resections has probably led to a more complete identification of these components. Another factor is selection: all cases are muscle-invasive (pT2+), whereas statistics from the literature often include superficial tumors, and invasive tumors have a higher tendency toward divergent differentiation. Our data may also reflect the fact that more complex, "atypical" cases are referred to university clinics, enriching the percentage of rare subtypes.

The specific frequencies of histological subtypes in our study largely fall within the known ranges. Squamous cell differentiation (17%) is as expected and is the most common divergent form in urothelial carcinoma, with a reported frequency of 10–25%. Some authors even note that if minimal squamous cell foci are specifically sought, the proportion may reach 60%, which highlights how widespread but often underestimated squamous cell metaplasia is in urothelial tumors. Glandular differentiation (3% in our study) is slightly below the typical 5–6%; we categorized some of the mixed cases with small glandular areas simply as "mixed subtype" without separately reporting the glandular component. The micropapillary subtype (4%) falls within the commonly cited range of 3–5%. The plasmacytoid subtype (3%) coincides with the literature range of 1–3%. It is rare but very aggressive and is often diagnosed at an advanced stage (pT3/T4). The nested subtype (2%) is above the expected (~0.3–1%), which probably reflects increased vigilance towards this otherwise difficult-to-recognize subtype, often confused with benign lesions. The sarcomatoid subtype (5%) and small cell carcinoma (5%) are significantly above the literature data (<1% each), but these

deviations are probably an artifact of the small sample size and "luck" – 5 cases out of 100. However, the presence of so many sarcomatoid and small cell carcinomas highlights the aggressiveness of the population under consideration. These two variants are among the worst prognostic forms of bladder cancer, associated with early metastasis and poor prognosis.

In conclusion of the morphological part, our series supports the thesis that tumors with variations in histology are not an exception, but rather a rule in muscle-invasive bladder carcinoma - two-thirds of cases have some other deviating feature (squamous cell, glandular, sarcomatoid, etc.). This has a direct impact on clinical practice. It is recommended that pathologists actively look for such variations, as their identification may change the grading. For example, the presence of a sarcomatoid component automatically makes the tumor high-grade/grade 3, which is associated with more aggressive behavior. This improves therapeutic planning: for example, micropapillary tumors, even with limited invasion, require early cystectomy due to their tendency to disseminate.

2. Molecular classification – comparison with consensus. Our immunohistochemical profiling successfully stratified almost all (91%) of the cases to the corresponding molecular variant according to the consensus classification. The distribution achieved (33% basal, 50% luminal, 9% basoluminal, 8% double-negative) coincides with large reference studies. This demonstrates that the IHC method reproduces transcriptomic classification through a selected panel of markers. The difference in the proportion of stroma-rich (2% vs. 15%) is the most significant deviation. One possible explanation is methodological: the consensus definition of a stroma-enriched variant is based primarily on genes related to the microenvironment, such as the expression of fibroblast and immune markers (collagens, PD-L1, etc.). Purely quantitatively, we cannot directly measure the "amount of stroma" or the "level of immune infiltrate"; we assess these characteristics morphologically. It is entirely possible that some of the tumors that would fall into the stroma-enriched class transcriptomically have been classified according to their epithelial profile- for example, a tumor with abundant stroma but also with pronounced basal markers may have been classified as basal instead of being listed separately. This would reduce the number of separately identified "stroma-rich" cases.

Secondly, the inclusion of a basoluminal variant in our classification is an interesting feature. In the consensus, these tumors are not separated but are "distributed" according to a dominant feature (i.e., if a tumor expresses both basal and luminal markers, the algorithm usually places it in either the basal or luminal category, depending on which prevails). However, the presence of double-positive (DP**)** tumors is well documented in some studies. In our study,

these account for about 9% of cases. From a clinical perspective, these DP tumors also deserve attention, as they may represent a true hybrid variant combining characteristics (and potentially inheriting therapeutic resistance) of both main classes. Another hypothesis is that these DP profiles actually reflect intratumor heterogeneity, i.e., the tumor contains a mixture of separate clones, some with a basal signature and others with a luminal signature. To clarify this, additional studies are needed, such as RNA analysis of individual cells from DP tumors, to see whether they are genetically a mixture or a hybrid branch. In practical terms, the basalo-luminal tumors in our study behaved intermediately in terms of biomarkers: their HER2 expression was between that of purely luminal and purely basal tumors, and their p16 profile tended toward luminal (only 22% were p16 positive).

Ultimately, the molecular profiling we applied almost completely overlaps with the frequency of consensus classes even in a limited sample. This means that consensus classification is largely reproducible and can be implemented in diagnostic practice using an IHC panel. This would allow pathology departments that do not have high-tech sequencing capabilities to classify their cases in a biologically meaningful way. For clinicians, knowing the variant is beneficial. For example, there are already clinical trials demonstrating different therapeutic approaches for different molecular variants – for example, neoadjuvant chemotherapy works better in basal tumors, while targeted therapy, immunotherapy in relapse, etc. are being considered for luminal tumors. Our data give us confidence that immunohistochemical typing would be reliable enough to inform such decisions.

3. Morphology-molecular correlation: The correlation obtained between the classical histological diagnosis and the molecular profile is largely expected, but nevertheless valuable for confirmation. It shows that the morphological phenotype reflects the underlying genotype/transcriptome. For example, the observation that squamous differentiation almost always indicates a basal variant is consistent with previous studies. This is logical – basal tumors have gene expression similar to basal urothelial cells, and the latter have an affinity for squamous metaplasia, so tumors inherit this morphological feature. Papillary architecture, on the other hand, is an almost certain indicator of luminality. All papillary tumors in our series were either LumP or LumU. However, there is no absolute 1:1 correspondence; sometimes a tumor combines characteristics of more than one variant. For example, mixed urothelial carcinoma with glandular differentiation and a small cell component (we have such a case in our cohort) has a molecular profile that contains elements of both Luminal and NE-like. It was

difficult to classify such cases unambiguously. As mentioned, their profile often reflects the proportions of their components, and some appear as DP (basal-luminal).

The correlation between morphology and molecule has *a* direct *diagnostic effect*. If the pathologist sees a certain rare subtype, he can predict certain molecular features: for example, the micropapillary subtype is likely to be luminal unstable (with high Ki-67, possible *ERBB2* alterations); the plasmacytoid subtype will be luminal (with impaired *E-cadherin* adhesion); the small cell subtype will be NE-like (with mutations in *TP53+RB1*, high expression of p16, will respond to chemotherapy). Conversely, if immunohistochemistry reveals a basal profile, and the morphology is, for example, "clear urothelial carcinoma," the reserve material can be searched for a missed small squamous cell area that would justify the profile – i.e., molecular information can help to highlight a hidden histological component.

4. HER2 as a prognostic/predictive biomarker: One of the significant findings in our study is the high frequency of HER2 overexpression in muscle-invasive bladder cancer, especially in certain groups. A total of 25% of all cases are 3+ on IHC, making them potential candidates for anti-HER2 therapy, and another 22% are 2+ (borderline; some of them would likely show gene amplification on additional testing). In other words, approximately one-quarter to one-third of MIBC carry HER2 as a therapeutic target. A wide range of HER2 expression frequencies has been observed in the literature. These differences stem from the lack of uniform criteria and the heterogeneity of the cohorts. Our study uses the latest ASCO/CAP guidelines (2025), which are stricter and better adapted. For example, we also reported the "ultra low" category, which did not appear in previous publications. In our study, 9% of tumors were "ultra low," and if not separated into a separate group, they would have gone into the 0 category and would have artificially increased the number of "negatives" to 40%. We believe that reporting the "ultra low" category is important because even these minimally HER2-expressing tumors may respond to some new ADC therapies.

The most important conclusion regarding HER2 is its strong association with the luminal differentiation pathway**.** Our data clearly show that luminal tumors (LumP, LumU, LumNS) often express HER2, while basal tumors hardly express it. This confirms the observations of Damrauer et al. (2014), who classify bladder carcinomas as "Luminal (plus their variants)" – HER2 (+), and "Basal" – HER2 (−). In our series, more than half of LumU are 3+, most LumP are ≥2+, while none of the basal tumors are 3+. This correlation has both therapeutic and prognostic value. Basal-squamous tumors generally respond better to chemotherapy and do not have HER2, so the standard treatment for them remains chemotherapy and

immunotherapy in case of progression. Luminal unstable tumors are unfavorable in nature (high grade, frequent metastases), but interestingly, the presence of HER2 as a target in them opens up a new possibility–targeted therapy. Clinical trials are already underway (e.g., THOR – Trial of Herceptin in Bladder Cancer) to evaluate the effect of adding anti-HER2 drugs in HER2-positive MIBC. Our results suggest that if a patient is strongly HER2(+), their tumor is likely to be lumina–land there is evidence that luminal tumors are less sensitive to conventional chemotherapy. Therefore, for such a patient, a targeted approach would be a more reasonable course of action. Conversely, if the tumor is HER2(-) and we know it is basal, we would prefer classic chemotherapy, because they benefit from neoadjuvant chemotherapy (it has been shown that basal cases gain 10% more survival with the addition of neoadjuvant treatment, while luminal cases show no statistical benefit). Of course, these strategies require further clinical validation, but they outline a personalized approach: molecular profiling and HER2 status can guide optimal therapy.

In addition, HER2 is also a prognostic marker. In our study, HER2(+) tumors generally correlate with luminal variants, which have a different prognosis from basal variants. Some studies associate HER2 overexpression with more aggressive behavior (shorter progression-free survival), but this is not unanimous and may depend on concomitant therapy. There is a hypothesis that HER2(+) tumors are more aggressive if left untreated, but if treated with targeted agents, the results improve dramatically – an analogy with breast cancer. Currently, HER2 is not routinely tested for bladder cancer in clinical practice, but based on our data, we recommend the introduction of HER2 testing at least in patients with muscle-invasive carcinoma, especially if the tumor demonstrates luminal characteristics (papillary growth, micropapillary component, etc.). This could open the door to the use of drugs approved for other indications, especially in metastatic or platinum-refractory cases where treatment options are limited.

5. Role of p16 and RB pathway: Our findings regarding p16 complement the molecular profile of variants with functional information about the cell cycle status. The LumU variant is characterized by RB1 inactivation – our group of LumU tumors is p16(+), which is a direct sign of pathway loss (due to RB mutation, p16 is released and accumulated). Interestingly, the consensus classification of GU (Genomic Subtype) defines LumU precisely as a p16(+) and RB1(−) IHC profile, combined with the absence of FGFR3 and high Ki-67. On the other hand, LumP tumors were p16 (−). It is known that about 50–60% of superficial papillary carcinomas have a deletion of 9p21 (where *CDKN2A* is located), which leads to a lack of p16 protein. Our data suggest that this is also the case with invasive papillary tumors. Here, we can also make a

connection with clinical observation: papillary (LumP) tumors are often multiple, recurrent superficial lesions associated with the so-called field effect (the entire mucosa is susceptible to papillary formations). The 9p21 deletion is an early event that may explain this diffuse predisposition. The loss of p16 gives a clonal advantage to urothelial cells throughout the surface, leading to multifocal papillary growths. However, the absence of p16 makes them less sensitive to cytotoxic chemotherapy (as they have a complete G1 arrest), which may contribute to the fact that the LumP variant – although more indolent – does not respond well to neoadjuvant chemotherapy. This observation is discussed in the literature: basal tumors (usually p16-positive) are affected by neoadjuvant treatment, while luminal tumors are not affected, or at least not to the same extent. One possible biological explanation is that p16-negative cells have alternative cell cycle control and may be less sensitive to DNA-damaging agents, damaging DNA, unlike p16-positive cells, which have disrupted Rb and a hyperactive cycle, making them vulnerable to chemotherapeutic agents.

The NE-like variant, on the other hand, almost always combines *TP53+RB1* mutations and, accordingly, is almost always p16-positive (83% in our study). This is consistent with what has been described in small-cell lung cancer: there, almost all cases have RB1 loss and are p16-positive.

In general, it can be said that p16 expression serves as a functional marker reflecting the status of two key tumor suppressors – RB and p53. When RB and p53 are lost (as in NE-like), p16 is strongly positive, but this does not stop anything – the cells are in an uncontrolled cycle, which makes these tumors very aggressive. When RB is lost, but p53 is preserved (as is often the case in LumU), p16 is high, but without RB, this only marks the problem; it does not solve it; these tumors have high proliferation. And when RB is preserved, and p16 is lost (LumP), the cells enter the cycle slowly, but with a functioning RB, they control the pace to some extent, so these tumors are often more indolent. These nuances are important and suggest that, for example, combination therapy, targeting the RB pathway, may have different effects depending on the variant. Currently, there are no specific therapies to restore RB function or increase p16, but the idea is that by knowing which pathway is disrupted, we could adapt our approach in the future. For example, CDK4/6 inhibitors would only work if RB is intact, i.e., in LumP, but not in LumU.

The study has direct implications for the future diagnosis and treatment of bladder carcinoma. First, we showed that immunohistochemical classification is possible, i.e., any pathology center with a basic set of antibodies can categorize MIBC tumors into molecular variants in a large percentage of cases (91% in our study). This can be incorporated into standard protocols,

similar to breast cancer, where each case is mandatorily classified as Luminal A/B, HER2-enriched, or Basal-like based on IHC (ER, PR, HER2, Ki-67). Our data show that antibodies against CK5/6, SK20, and p16 can be used to achieve a classification that provides prognostic information. Second, we identified the group of HER2-positive MIBC as a potential target for personalized treatment. In the era of precision medicine, the presence of a targetable mutation or overexpression is a valuable opportunity. Until recently, there were no approved targeted therapies for BC, but now there are FGFR3 inhibitors for FGFR3-mutated tumors, PD-1/PD-L1 inhibitors for appropriate CPS, and ADC against Nectin-4. It is possible that anti-HER2 agents will soon be added to the treatment arsenal. Our study is the first in Bulgaria to systematically investigate HER2 in urothelial carcinoma and demonstrate its significance.

Furthermore, combining HER2 and molecular data, we propose a risk stratification scheme: NE-like variant (high risk, sensitive to chemotherapy), Basal variant (high risk, but sensitive to chemotherapy and immunotherapy), LumU (high risk, potentially targetable with HER2 inhibitors), LumP (lower risk, insensitive to chemotherapy but potentially targetable with FGFR3 inhibitors in the presence of FGFR3 mutations), Basal-like (medium risk, requiring further investigation, e.g., with RNA-seq) and Stroma-rich (unclear risk, responding to immunotherapy). Of course, these are hypothetical considerations that require clinical validation. Presenting the data in this way is one of the contributions of the dissertation.

- **Study limitations:**

It should be noted that the study covers 100 patients from a single center, which is a relatively small sample size and may not fully reflect the population picture. Some rare subtypes (lipid-enriched, lymphoepithelial-like, etc.) were found in mixed carcinomas and in negligible numbers, to conclude. Also, there is no long-term follow-up of patients; our focus was on pathological correlations rather than survival or therapeutic response. Monitoring of this cohort is ongoing, and in the future, we may be able to analyze the prognostic value of the variants, e.g., whether basal variants have worse overall survival than luminal variants, etc. Another potential drawback is that immunohistochemical assessment has a subjective element; for example, the determination of luminal unstable versus luminal nonspecific is somewhat dependent on judgment. We tried to adhere strictly to the criteria, but small inaccuracies are still possible. The use of digital scores (image analysis) would reduce subjectivity and is recommended for future studies. A significant drawback is the lack of further clarification of patients with HER2 2+. Our study of this group will continue, and they will be further clarified using in situ hybridization methods (SISH). Another target group and

field for our future research will be the group of mixed tumors. At present, mixed tumors represent a serious diagnostic and therapeutic challenge.

- **Comparison with other studies:**

Our results are consistent with global trends. For example, the TCGA project (2017) and the consensus classification by Kamoun et al. (2020) largely overlap with our results. These coincidences give confidence in the correctness of our approach.

- **New hypotheses and suggestions:**

Based on the accumulated data, we propose the following:

- Routine immunohistochemical profiling for HER2 and basal/luminal markers should be included in all cases of MIBC, especially in younger patients or when planning systemic therapy, as this may influence the choice of treatment.

- Further investigation of the basoluminal variant is needed – for example, through RNA-sequencing – to determine whether it represents a separate class with a unique transcriptional signature or is simply a mixture.

- Conduct prospective clinical studies to validate the prognostic significance of the identified variants and biomarkers in the Bulgarian population – for example, whether basal patients really benefit from neoadjuvant chemotherapy in our conditions, whether HER2(+)/LumU patients benefit from the inclusion of anti-HER2 therapy, etc.

- To implement a multidisciplinary approach to the treatment of bladder cancer – for example, preliminary molecular typing, to be discussed at a tumor board meeting together with all data, similar to the practice for breast cancer. We have already presented the results at our center and received feedback – in aggressive phenotypes (basal, NE-like), earlier adjuvant treatment is being considered, and in HER2(+) metastatic cases, referral to appropriate clinical trials.

Conclusions:

1. **Frequency of histological variants:**

Muscle-invasive bladder cancer demonstrates considerable morphological heterogeneity. This proportion of morphological variation in our study is higher (66%) than traditionally reported due to the focused search and advanced stage of the tumors included.

2. **Molecular classification:**

The application of an immunohistochemical panel allowed the stratification of 91% of our cohort into one of the consensus molecular variants. The distribution of variants in the studied cohort largely coincides with the published data from large-scale studies. The main differences from the consensus are the lower relative proportion of stroma-enriched tumors and the separation of a basal-luminal category in our study.

3. **Correlation between morphology and molecular variant:**

There is a correlation between the histological subtype of the tumor and its molecular variant (χ^2, $p < 0.001$). These relationships confirm that the morphological phenotype reflects the underlying molecular mechanisms in the tumor. This knowledge can be used for better diagnosis and prognosis.

4. **HER2 expression:**

HER2 overexpression (IHC 3+) was found in a significant proportion of MIBCs – 25% of cases. Overall, more than half of the tumors (69%) show some HER2 positivity (including low and ultra-low expression). HER2 status correlates with molecular and histological profiles. These data identify HER2 as a potential prognostic and predictive biomarker.

5. p16 status:

The expression of p16 shows a biphasic distribution among MIBCs, reflecting the different mechanisms of Rb pathway disruption. P16 serves as a surrogate marker for further subtyping within the luminal category. This makes p16 a valuable additional tool in the immunohistochemical classification of MIBCs. Furthermore, p16 has prognostic and predictive value; for example, p16-positive tumors have higher proliferative activity and are more likely to respond to therapies targeting the cell cycle.

6. Clinical significance:

The combination of morphological and immunohistochemical data allows for better prognostic stratification of patients with MIBC. The proposed immunohistochemical algorithm for MIBC typing is applicable in routine diagnostic practice and provides information that can optimize the therapeutic decision-making for each patient through a personalized approach.

The dissertation enriches the knowledge about MIBC, as for the first time in our country: (a) it provides a detailed quantitative description of the frequency of different histological subtypes in MIBC at the local level; (b) demonstrates a practical approach to the molecular classification of MIBC through a selected immunohistochemical panel and confirms the reproducibility of this classification; (c) reveals the existence of a hybrid basaloid category of tumors with a specific immunophenotype; (d) establishes the frequency of HER2 overexpression in MIBC and its relationship to the luminal differentiation pathway, which has direct clinical significance; (e) analyzes p16 expression as a marker for RB1 status and demonstrates its value in distinguishing between variants; (f) integrates morphological and molecular data into clear algorithms and recommendations for improving the diagnosis and treatment of patients with bladder cancer.

Contributions of the scientific work

The dissertation contains the following original scientific and applied scientific contributions:

1. Clarification of the morphological spectrum of MIBC in a Bulgarian cohort**.** For the first time, the frequency of the different histological subtypes of muscle-invasive urothelial carcinoma of the bladder in Bulgaria has been systematically documented. A higher than expected prevalence of variant morphology (66% of cases) has been established, including rare and aggressive subtypes (sarcomatoid, micropapillary, plasmacytoid, etc.), which emphasizes the need for increased attention in the histopathological diagnosis of these tumors.

2. Introduction of immunohistochemical-based classification of MIBC: An immunohistochemical algorithm for molecular stratification of MIBC has been developed and applied in accordance with the consensus classification (Basal/Sq, Luminal Pap, Luminal Unst, Luminal NS, NE-like, Stroma-rich). The reproducibility of this approach has been demonstrated; the distribution of variants obtained is consistent with international data. This is a scientific and applied contribution showing that complex molecular classification can be implemented in routine practice using a relatively accessible IHC panel.

3. Identification of a basoluminous (double-positive) variant: A distinct group of 9% of cases with a hybrid immunophenotype (simultaneous expression of basal and luminal markers) has been identified. It has been proposed that these tumors be treated as a separate "basal-luminal" variant, as they demonstrate intermediate characteristics (e.g., moderate HER2 expression, predominantly p16-negativity) and may have specific biological behavior. This observation contributes to the understanding of the heterogeneity of urothelial carcinomas and raises new questions about the genomic origin of these neoplasms.

4. Study of HER2 status in MIBC**:** For the first time in a Bulgarian series, the expression status of HER2 in bladder cancer has been systematically studied. It was found that a significant proportion of MIBC (25%) demonstrated HER2 overexpression (3+), with

positivity strongly correlating with luminal molecular variants and certain morphological subtypes (micropapillary). This contribution has direct clinical significance: it identifies the subgroup of patients with MIBC who could potentially benefit from anti-HER2 targeted therapy and provides arguments for the introduction of HER2 testing in advanced bladder cancer.

5. Analysis of p16 as a marker for RB1 status: It has been shown that p16 expression has a dichotomous distribution reflecting the mechanism of cell cycle inactivation – luminal papillary tumors (with CDKN2A deletion) are p16(−), while luminal unstable and NE-like tumors (with RB1 mutation) are p16(+). This is a contribution that confirms the practical benefit of including p16 in the immunohistochemical panel for classification.

6. Morphology-molecule correlation and data integration**:** A comprehensive correlation between the histological and molecular characteristics of each tumor has been established. This has led to the formulation of specific dependencies (e.g., squamous cell component → basal variant; papillary growth → luminal variant; HER2(+)/p16(+) profile → luminal unstable variant, etc.), which represent scientific and applied guidelines for diagnostic practice. Based on these relationships, recommendations for optimal treatment were derived (e.g., indications for targeted therapy in HER2-positive luminal tumors, aggressive behavior in the NE-like variant, LumP resistance to chemotherapy, etc.), which is a significant contribution to the introduction of personalized therapeutic approaches.

7. Local population database: The information collected from 100 consecutive cases forms the first local database of its kind for MIBC in Bulgaria, including detailed morphological and immunohistochemical parameters. This database can be used for future studies – for example, tracking survival and response to treatment according to subtype or variant, comparison with other populations, as well as for educational purposes (a set of characteristic microphotographs has been created for each subtype and variant).

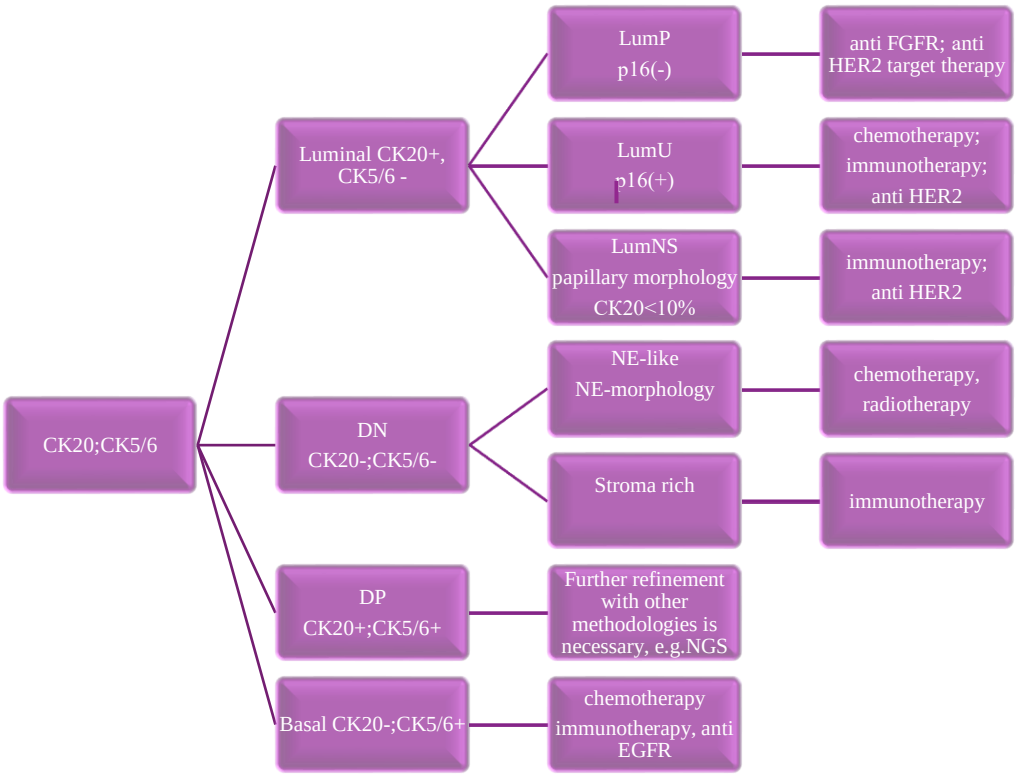
📖 *In conclusion, our study demonstrates the close relationship between morphology, molecular profile, and expression of key biomarkers (HER2, p16) in muscle-invasive bladder cancer.*

These results contribute to the understanding of the pathogenesis of the disease and provide a basis for personalized diagnostic and therapeutic strategies.

While classical pathology remains the backbone of diagnosis, the addition of targeted immunohistochemical markers allows for finer stratification, which is essential in the era of precision medicine.

Applications

Application 1:



***Note:**
Therapeutic models are hypothetical considerations that require clinical validation.

PUBLICATIONS, PARTICIPATION IN SCIENTIFIC FORUMS, COURSES, AND PROJECTS RELATED TO THE DISSERTATION:

PUBLICATIONS OF SCIENTIFIC RESULTS IN FULL-TEXT ARTICLES:

1. **Kraevska, E.**, Popovska, S. *Immunohistochemistry-Based Molecular Profiling of Muscle-Invasive Bladder Cancer: Analysis of 100 Consecutive Cases with Morphological Correlation*. *Medical Sciences*, 2025, *13*(3): Article number 202; e-ISSN: 2076-3271; Web of Science, Scopus

2. **Kraevska, E.**, Popovska, S. *Analysis of HER2 Expression in Different Histological Subtypes and IHC-Based Molecular Variants of Muscle-Invasive Bladder Carcinoma*. *Medicina*, 2025, *61*(10): Article number 1759; ISSN: 1010-660X; Web of Science, Scopus

3. Vasilev, P., Popovska, S., **Kraevska, E**., Karamanliev, M., Dimitrov, D., Yordanova, I. *Relationship Between PD-L1, PD-1, CD8 and Clinicopathological Factors in Primary SCCs*. Dermatology Practical and Conceptual, 2024, 14(3): Article number e2024176; ISSN: 2160-9381; Web of Science, Scopus

PARTICIPATION IN SCIENTIFIC FORUMS RELATED TO THE DISSERTATION

1. 1-3 June 2023 National Conference on Pathology, Golden Sands Resort

   "Histological and immunohistochemical changes occurring during clinical progression in muscle-invasive bladder cancer - a case study"

   E. Kraevska, S. Popovska

2. April 24-27, 2025 XIV National Congress on Pathology "Pathology - a compass for accurate diagnosis and treatment" Sandanski

   "Combined small cell carcinoma of the bladder: morphological, immunohistochemical, imaging, and clinical characteristics-a clinical case"

   E. Kraevska, S. Popovska, A. Vanov, R. Trifonov

3. 29-31 August 2025 Regional scientific meeting "Hippocratic School - Pass it on"

   Plovdiv "Histological subtypes of bladder cancer and their diagnostic and therapeutic significance" **Dr. Elitsa Petrova Kraevska**

PARTICIPATION IN SPECIALIZED COURSES IN THE FIELD OF THE THESIS TOPIC:

- 1st Course on Urothelial Cancers 2023: Tumor heterogeneity and therapeutic resistance, Institut Curie, Paris, France, December 7-9, 2023.
- Vincent Academy of Pathology - Diagnostic Pathology of the Urinary System and Male Genital Organs October 16–18, 2024, Linz, Austria
- Advanced Bladder Pathology Course January 20–21, 2025, BAUP, UK, London
- Neuroendocrine GU tumors, March 5, 2025, BAUP, UK, London
- Staging of Bladder Cancer, May 7, 2025, BAUP, UK, London
- Mimics of bladder cancer, June 5, 2025, BAUP, UK, London
- Grading of urothelial carcinoma April 2, 2025, BAUP, UK, London
- Challenges and new opportunities in the treatment of renal cell and urothelial carcinoma 30.11.- 01.12.24 Borovets, Bulgaria
- Evolution in *HER2* diagnostics – new concepts and practical guidelines

  May 30-31, 2025, Sofia, Bulgaria
- National Academy of Pathology: HER2 expression in breast cancer – the spectrum unfolds, Guest lecturer: Prof. Giuseppe Viale, University of Milan, Italy, 20.04.2024, Sofia
- *Breast HER2 Dual ISH Proficiency Test 509, June 2023*
- Breast HER2 Dual ISH Proficiency Test 509, June 2023
- HER2 Testing Summit: Re-evaluating approaches to HER2 scoring in breast cancer
- Breast HER2 (4B5) Proficiency Test 418, August/September 2024
- Breast HER2 IHC Feedback_18 September 2024, 18th September 2024
- HER2 Diagnostics Summit - EU.-12.oct.2024

- Breast HER2 (4B5) Proficiency Test 414, April 2024
- Breast HER2 IHC Feedback_18 April 2024
- HER2 (4B5) Proficiency Test 418, August/September 2024
- HER2 IHC Feedback_18 September 2024, 18th September 2024
- Breast HER2 (4B5) Proficiency Test 414, April 2024
- Breast HER2 IHC & HER2 Dual ISH Case Review Session 422/522, February 12, 2025
- Breast HER2 Dual ISH Proficiency Test 522, January/February 2025
- Breast HER2 Dual ISH Education session 522, January 29, 2025
- Breast HER2 (4B5) Proficiency Test 422, January/February 2025

PARTICIPATION IN RESEARCH PROJECTS:

1. Project No. D7/2024, funded by the Medical University of Pleven, "Muscle-invasive bladder cancer - morphological and immunohistochemical characteristics with a focus on certain predictive biomarkers."
2. "Center of Excellence in Personalized Medicine, Health Insurance and Telemedicine, Robotic and Minimally Invasive Surgery," reg. No. BG05M2OP001-1.002-0010, funded by the Operational Program "Science and Education for Smart Growth."
3. "Competence Center for Personalized Medicine, 3D and Telemedicine, Robotic and Minimally Invasive Surgery", funded by the Operational Program "Scientific Research, Innovation and Digitization for Smart Transformation," co-funded by the European Union, project No. BG16RFPR002-1.014-0002
4. Project No. BG-RRP-2.004-0003, funded by the European Union - NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria