MEDICAL UNIVERSITY – PLEVEN
FACULTY OF MEDICINE
DEPARTMENT OF PATHOLOGY

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MORPHOLOGICAL CHARACTERISTICS, TUMOR BUDDING, AND IMMUNE STROMAL RESPONSE AND THEIR DEPENDENCE ON THE MUTATIONAL PROFILE OF LEFT- AND RIGHT-SIDED COLORECTAL CARCINOMAS

A B S T R A C T

of a dissertation
for the acquisition of the educational and scientific degree
"DOCTOR"

PLEVEN, 2026

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of a dissertation
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"DOCTOR"
Field of higher education: 7. Healthcare and Sports
Professional field: 7.1 Medicine
Doctoral program: "Pathology and Cytopathology"

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PLEVEN, 2026

The dissertation text comprises 205 standard pages, including 48 tables, 16 figures, and 31 images.

The bibliography contains 294 references: 164 published in the last 10 years and 3 citing Bulgarian authors.

The author is an extramural PhD student at the Department of Pathology, Faculty of Medicine, Medical University of Pleven.

The dissertation was discussed and directed for public defence by an extended Departmental Council of the Department of Pathology, Faculty of Medicine, Medical University – Pleven, held on 17.03.2026.

The public defense of the dissertation will take place on June 23, 2026, at 1:00 p.m. in Ambroise Paré Hall, Medical University – Pleven, 1 Kliment Ohridski St., Pleven, Bulgaria.

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Note: The numbering of the appended figures, tables, and images has been carried out in accordance with the text of the abstract and does not coincide with the numbering in the dissertation.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Abbreviation	Full name
AJCC	American Joint Committee on Cancer
CLR	Crohn-like lymphoid reaction
CK AE1/AE3	Pancytokeratin
dMMR	deficient mismatch repair
ECIS	European Cancer Information System
EMVI	Extramural venous invasion
FFPE	Formalin-fixed paraffin-embedded
H&E	Hematoxylin and Eosin
IMVI	Intramural venous invasion
ITBCC	International Tumour Budding Consensus Conference
CRC	Colorectal carcinoma
LVI	Lymphovascular invasion
MMR	Mismatch repair
NGS	Next-generation sequencing
PNI	Perineural invasion
pMMR	proficient mismatch repair
PTB	Peritumoral budding
ROC	Receiver Operating Characteristic
SPSS	Statistical Package for the Social Sciences
TNM	Tumour, Node, Metastasis classification
WHO	World Health Organization

INTRODUCTION

In “Digestive System Tumours – 5th ed.” of the “WHO classification of tumours series”, adenocarcinomas of the colon and rectum are defined as the second most common malignant disease in women and the third most common malignant disease in men. Emphasis is also placed on the increasing incidence of colorectal carcinomas at a younger age.

The current data of ECIS – European Cancer Information System for Bulgaria for 2022 show expected new cases of patients with colon and rectal carcinomas – 5,086, and deaths from colorectal carcinomas for the same year – 2,759. This makes these neoplasms the most common in incidence and the second leading cause of death from malignant diseases in both sexes.

The data for the European Union show pronounced disproportions in overall care for cancer diseases. They are expressed both within and between countries. Within the EU, only Bulgaria and Romania have not developed, or are in the initial stages of developing, screening programs for carcinomas of the colon and rectum. In countries with active preventive programs for CRC, the coverage ranges from 4% (Cyprus) to 76% (Denmark).

Overcoming these differences in the long term requires improved health education and training, requirements for continuing medical personnel qualifications, and innovations in early detection, diagnosis, and cancer therapy. Public attitudes should also be considered in the development of national programs for the control of malignant diseases and in the construction of databases for the adequate evaluation of cancer patients' care.

It is expected that by 2035, mortality from malignant diseases will exceed mortality from cardiovascular diseases. This requires directing the attention of society as a whole, and in particular health specialists from different levels and scientific fields, to the different aspects of non-medical and medical care related to CRC – prevention, early, precise, and modern diagnosis, personalized treatment using contemporary scientific approaches for prediction and prognostication of the expected potential effects in the individual patients, financial and other support for patients and their families.

The significance and relative shares of colon and rectal cancer incidence and mortality at the global and national levels shaped the direction of our scientific interests and practical efforts within the framework of the present study.

AIM

To investigate MORPHOLOGICAL CHARACTERISTICS, TUMOR BUDDING, AND IMMUNE STROMAL RESPONSE AND THEIR DEPENDENCE ON THE MUTATIONAL PROFILE OF LEFT- AND RIGHT-SIDED COLORECTAL CARCINOMAS.

OBJECTIVES

1. Targeted study, analysis, and evaluation of specific aspects of the epidemiological data of the patients – sex, age, primary tumour localisation - right-sided, left-sided, and rectal; histomorphological evaluation and description of the characteristics of CRC - histological subtype, tumour grading, pathological staging, etc.
2. Histomorphological evaluation and analysis of lymphovascular and perineural invasion; of the status of the lymph nodes; and of specific characteristics of the peritumoral stromal immune reaction.
3. Investigation, morphological evaluation, grading, and analysis of peritumoral budding (PTB):

- Subtask 3A - in endoscopic colorectal biopsies and determination of the factors that influence this process.
- Subtask 3B - in resection material with CRC.

4. Investigation, evaluation, and analysis of tumour MMR status in resection materials from CRC.
5. Investigation and analysis of some of the most frequent mutations of colorectal carcinomas in the specific patient sample.

MATERIALS AND METHODS

PATIENT SAMPLE

This study was conducted retrospectively and includes two groups of patients, selected as follows:

FIRST GROUP - SUBTASK NO. 3A – ENDOSCOPIC MATERIALS FROM COLORECTAL CARCINOMA:

We performed a random retrospective selection of 100 patients with colorectal carcinoma diagnosed by endoscopic examination for the period 2020–2022.

The materials were provided from the archive of the Department of General and Clinical Pathology at UMHAT "Dr Georgi Stranski" – Pleven.

The selection was performed according to the predefined inclusion and exclusion criteria (Table 1).

PATIENT SELECTION CRITERIA - SUBTASK NO. 3A

Inclusion criteria for the study	Exclusion criteria for the study
Patients with primary CRC	Metastases in the colon or rectum from other primary tumours (according to clinical data)
Preoperative endoscopic biopsy	Preoperative therapy performed
	Other primary colorectal tumours are different from CRC.
	Recurrence of CRC
	Biopsy material obtained by a method other than endoscopy
	Biopsy materials at risk of exhaustion

Table 1. Criteria for patient selection and inclusion in the first stage of the study - Subtask No. 3A.

SECOND GROUP - SUBTASK NO. 3B – RESECTION MATERIALS FROM COLORECTAL CARCINOMA:

We performed a random retrospective selection of 100 patients with postoperative histologically proven colorectal adenocarcinoma on resection material in the period 2020–2023.

The materials were provided from the archive of the Department of General and Clinical Pathology at UMHAT "Dr Georgi Stranski" – Pleven.

The selection was performed according to the predefined inclusion and exclusion criteria (Table 2).

PATIENT SELECTION CRITERIA - SUBTASK NO. 3B

Inclusion criteria for the study	Exclusion criteria for the study
Patients with primary CRC	Metastatic tumours of the colon or rectum
Resection biopsy material with carcinoma of the colon and rectum	Neuroendocrine tumors/carcinomas
The study intentionally included a small group of patients who had received preoperative radiotherapy or chemotherapy.	Benign tumors
	Mesenchymal tumors

Table 2. Criteria for patient selection and inclusion in the second stage of the study - Subtask No. 3B.

All patients provided written informed consent in the respective hospital unit.

The study was conducted in accordance with the Declaration of Helsinki. It was approved by the Ethics Committee of Medical University – Pleven, Protocol No. 50/1, May 2020 and OH: 635- KENID/6 November 2020, before its initiation.

Patient data are anonymous and were used without identifying information.

The collected data – sex, age, tumour location, histological type and degree of differentiation, tumour budding, reasons for inability to report, and all additional parameters – were summarised and recorded in a protocol form designed for this purpose.

METHODS

Depending on the type of investigations performed, we used and applied different methods, according to the specifics of each task, namely:

BY TASK NO. 1:

• Analysis of medical documentation to determine the epidemiological data of the patients needed for our study - sex and age.

• Analysis of the patients' medical documentation, as well as macroscopic evaluation of the resection biopsy materials to determine the primary tumour location in the colon and rectum.

• Histological examination of biopsy materials to determine the histological subtype of the tumours and tumour grading.

BY TASK NO. 2:

• Analysis and evaluation of lymphovascular invasion (LVI).

The CAP clinical recommendations indicate the need for separate evaluation and reporting of:

- Invasion of small vessels - corresponding to involvement of lymphatic vessels, capillaries, and postcapillary venules.

- Invasion of large vessels, corresponding to venous invasion - intramural and extramural (IMVI and EMVI). The characteristics of these types of invasions, the method of histological evaluation, and their prognostic value are presented in Table 3:

IMVI and EMVI - characteristics and prognostic value

Parameter	IMVI (Intramural venous invasion)	EMVI (Extramural venous invasion)
Anatomical definition	Venous invasion in the wall: submucosa and/or muscularis propria	Venous invasion beyond the muscularis propria, in pericolic or perirectal adipose tissue
Overall prognostic weight	Adverse factor, but with a more equivocal and weaker prognostic value	Well-established independent adverse prognostic factor

Independent prognostic factor	The evidence is limited and inconsistent between studies	Demonstrated in multiple multivariate analyses
Association with metastases	Present, but weaker and variable	Strong association with hematogenous metastasis, especially liver metastases
Clinical significance	Additional prognostic information, especially in early stages (pT1)	Key marker for risk stratification and therapeutic decisions
Diagnostic features	More difficult to assess; often requires elastin staining. The deepest level of venous invasion is recorded.	Endothelium-lined spaces featuring a distinct smooth muscle layer and/or elastic lamina. Orphan arteries and protruding tongues are marked by increased elastin staining. Well-defined tumor nodules encased by elastic lamina seen in hematoxylin-eosin (H&E) or elastin stains also indicate venous invasion. The most profound level of venous invasion is documented.
Method of assessment used in our study	Examination of H&E-stained slides	Examination of H&E-stained slides

Table 3. Characteristics of IMVI and EMVI, method of histological evaluation, and prognostic value.

• Analysis and evaluation of perineural invasion (PNI).

In 2009, Leibig et al. defined PNI as a tumour near a nerve that either involves at least 33% of its circumference or has tumour cells in any of the three nerve sheath layers.

Miyashita et al. investigated intramural perineural invasion localised to the Plexus nervosus myentericus (Auerbach). They defined this mode of tumour spread as a horizontal intramural myenteric invasion, regardless of the presence of other PNI features.

In our study, we used the following approach in the evaluation of the histological slides:

- staining with H&E.
- tumour located near a nerve, affecting at least 33% of its circumference.
- tumour cells located in each of the three layers of the nerve sheath: epineurium, perineurium, endoneurium.
- for perineural invasion, we accepted invasion in the plexus nervosus myentericus (Auerbach).
- recording in TNM staging - PNI+/-

• Analysis and evaluation of lymph node status (N)

In the TNM Classification of Malignant Tumours, 8th ed., published in 2017, the N categories for metastatic lymph nodes in colorectal carcinomas are defined. An updated version was published in the AJCC 8th ed. and WHO Classification of Tumours Online (BlueBooks).

The algorithm for the evaluation of CRC N status requires:

- Description of the total number of lymph nodes.
- Histomorphological examination of at least 12 lymph nodes.
- Positive lymph nodes outside the regional basin are defined as distant metastases – for example, iliac lymph nodes in carcinoma of the rectosigmoid region are defined as M1A.
- Micrometastases – presence of 10 to 20 tumour cells and a diameter ≥ 0.2 mm, and it is recommended that they be included among the standard positive lymph nodes.
- Presence of micrometastases ≤ 0.2 mm defines them as N0 (i+).

In evaluating lymph nodes in our patient cohort, we adhered to the above algorithm for N status in patients with CRC. The retrospective nature of the study imposed certain limitations related to the available biopsy protocols and, more specifically, to the number of regional lymph nodes described.

• Tumour deposits (TD) - Tumour deposits are defined as discrete tumour nodules in the area of lymphatic drainage of the primary carcinoma, without the presence of recognisable lymphoid tissue, vascular, or neural structure.

Algorithm for determining tumour deposits:

- For the evaluation of tumour deposits, we used standard H&E-stained microscopic slides.

- We carefully excluded the presence of peritumoral lymphoid tissue, capsule, marginal sinus, endothelium, elastic membrane, smooth muscle wall, and peripheral nerve.
- We determined the total number of tumour deposits.
- We staged the existing tumour deposits according to the TNM classification.

• Analysis and evaluation of specific characteristics of the peritumoral stromal immune reaction

The term Crohn-like lymphoid reaction (CLR) was first introduced in 1990 by Graham and Appelman to denote discrete lymphoid aggregates usually localised in the muscularis propria or pericolic adipose tissue, beyond the borders of the invasive tumour front. The morphological spectrum of CLR varies from discrete lymphocytic aggregates to well-formed lymphoid follicles with clearly defined germinal centres (tertiary follicles).

The method of Graham and Appelman classifies CLR into three grades: “intense” - with more than 3 aggregates and at least 1 of them with a germinal centre - Grade 2; “mild” - 2 or fewer aggregates - Grade 1; and “absent” - no lymphocytic aggregates - Grade 0.

In our study, because of the easy applicability and the lack of necessity to apply morphometric parameters, we preferred the use of the original method of Graham and Appelman with the grades described above: “intense or marked”, “mild”, and “absent”, with the assessment performed close to, but not within, the invasive tumour front.

BY TASK NO. 3:

• Analysis and evaluation of peritumoral budding (Peritumoral budding, PTB)

Peritumoral budding was assessed on H&E slides according to the ITBCC algorithm. Ten fields were scanned using a 10× objective to identify the invasive front and the hotspot area. PTB was counted in the hotspot area using a 20× objective, and the result was divided by a normalising factor to calculate the number of buds per 0.785 mm² (in the present study, the normalising factor was 0.810).

Reporting and classification into groups:

Bd1 (low): 0–4 tumour buds.

Bd2 (intermediate): 5–9 tumour buds.

Bd3 (high): ≥10 tumour buds.

IHC for CK was performed on individual biopsy specimens for demonstration purposes.

BY TASK NO. 4:

• Immunohistochemical examination of MMR status and pancytokeratin (CK AE1/AE3)

Tumour resection specimens and endoscopic biopsies fixed in 10% buffered formalin and embedded in paraffin (FFPE) were examined.

The immunohistochemical studies were performed automatically using the Dako Agilent Autostainer Link 48 with the EnVision FLEX visualisation system and High pH (Link) and HRP Rabbit/Mouse (K800021-2). Epitope retrieval (HIER) was performed in DAKO PT Link (PT100/PT101).

- The MMR proteins and pancytokeratin were examined (MLH1, PMS2, MSH2, MSH6, CK AE1/AE3).
- MMR status was determined by immunohistochemical examination of the four major proteins of the mismatch repair system with ready-to-use monoclonal antibodies from Dako/Agilent.

MMR staining was assessed only by nuclear expression in tumour cells.

Controls:

Internal positive controls: normal colorectal mucosa, stromal cells, and lymphocytes on the same section.

External negative controls: according to the manufacturer's kit.

Interpretation criteria:

- pMMR (retained expression): nuclear staining in >10% of tumour cells with positive internal controls.
- dMMR (loss of expression): lack of nuclear staining in tumour cells with retained expression in the control cells.

Subclonal expression was not analysed.

In dMMR cases, a correlation was performed between the missing proteins and the mutational profile (NGS) to assess the probability of Lynch syndrome, without performing additional molecular tests.

We applied the classical four-antibody algorithm (MLH1, PMS2, MSH2, MSH6) to assess MMR deficiency.

- Immunohistochemical examination of CK AE1/AE3 in endoscopic biopsy materials:

In endoscopic biopsies, immunohistochemical examination was performed to confirm epithelial origin using a standardised, ready-to-use pancytokeratin antibody.

The positive reaction was recorded as diffuse cytoplasmic and/or membranous staining in epithelial/tumour cells, used to confirm epithelial differentiation in carcinoma in small biopsy fragments.

BY TASK NO. 5:

• Methods of genetic analysis

The genetic analysis was performed by the team of the Research Laboratory for Precision and Genomic Medicine at the Centre of Competence for Personalised Medicine, 3D and Telemedicine, Robotic and Minimally Invasive Surgery “Leonardo da Vinci” at Medical University – Pleven.

Next-Generation Sequencing (NGS) technology was used.

Representative tumour blocks of formalin-fixed and paraffin-embedded material (FFPE), obtained after surgical resection, were provided. DNA extraction was performed according to the manufacturer's protocols for the NGS panel used.

The TruSight Tumor 15 panel (Illumina Inc., San Diego, USA) was used, which includes analysis of mutations in 15 key genes associated with oncogenesis:

AKT1, BRAF, EGFR, ERBB2 (HER2), FOXL2, GNA11, GNAQ, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA, RET, TP53.

Sequencing was performed on the Illumina NextSeq 550 platform, according to the manufacturer's instructions.

Interpretation of the results was performed by specialists in medical genetics and medical bioinformatics, with clinically significant variants considered.

Additional analysis was performed in dMMR cases.

In cases with a mismatch repair defect (dMMR), a correlation was made between:

- the missing MMR proteins (MLH1, PMS2, MSH2, MSH6),
- the established mutations,
- the probability of the presence of Lynch syndrome.

Within the study, no additional genetic tests were performed to confirm Lynch syndrome, and the research team did not influence the diagnostic and therapeutic process.

• Statistical processing of genetic data

The data from the genetic studies were processed with software and by methods described in the section - Statistical analysis.

STATISTICAL ANALYSIS OF THE RESULTS

The results obtained in the performance and reporting of each of the above-listed tasks were statistically processed using IBM SPSS Statistics v24.0 (Chicago, Illinois, USA).

The raw data obtained by us are presented mainly as categorical variables (tumour location, histological type, degree of differentiation, stage, LVI, PNI, MMR status, degree of tumour budding, immune reaction, presence of mutations) and nonparametric quantitative indicators (age).

To assess the relationships between these categorical variables, the χ^2 (Chi-square) test of independence was used. For tables with small, expected cell counts, the Monte Carlo approximation to the χ^2 test was used. Values of $p < 0.05$ were accepted as statistically significant.

In the analysis of relationships between categorical variables, in addition to the χ^2 test of independence, Cramer's V coefficient was also calculated to determine the strength of association.

For comparison of quantitative indicators across more than two groups, when the distributions were nonnormal, the nonparametric Kruskal–Wallis test was used.

For comparisons of proportions between two groups (e.g., mutation frequency between sexes), a two-proportion Z-test was used.

Data normalisation was performed on distributions by location to standardise scales and avoid disproportionate influence of individual variables. Normalisation was carried out by presenting relative shares (percentages) to ensure comparability across groups of different sizes. Normality of quantitative variable distributions was assessed using the Shapiro–Wilk test.

The analysis included investigation of statistical dependencies between primary tumour location and clinicopathological characteristics; degree of tumour budding and tumour grade/stage, LVI, PNI, lymph node status; peritumoral immune reaction (Crohn-like) and the other prognostic factors; MMR status, histological type, mutational profile.

This statistical approach allows assessment of associative relationships among morphological, immunological, and genetic parameters; however, due to the retrospective nature of the study and the sample size, multivariate regression analysis was not applied.

In 2×2 contingency tables with low expected frequencies, Fisher's exact test was applied, and in larger tables, a Monte Carlo approximation was used. Additionally, logistic regression analysis was used to assess the Aggressive Score, and ROC analysis was performed to evaluate its discriminative ability (described in Appendix 1).

Descriptive statistical analysis was also performed, with categorical variables presented by absolute frequencies and relative shares (%), and quantitative indicators by median and interquartile range.

RESULTS OF THE PERFORMED STUDIES

In this section, we present the results of our study, which have been published in peer-reviewed scientific journals.

Two separate patient cohorts were studied, in accordance with the aims and objectives of our study.

FIRST PATIENT COHORT

In the first patient cohort, we evaluated morphological features, grading, and peritumoral budding (PTB) in endoscopic colorectal biopsies and identified factors influencing this process.

The results that we reported in this group of patients with colorectal carcinoma proven by endoscopic biopsies are as follows:

BY TASK NO. 1:

Distribution by sex in the patient cohort is (n, %): MALE: 63 (63.0%); FEMALE: 37 (37.0%).

Distribution of patients by sex and age - Figure 1:

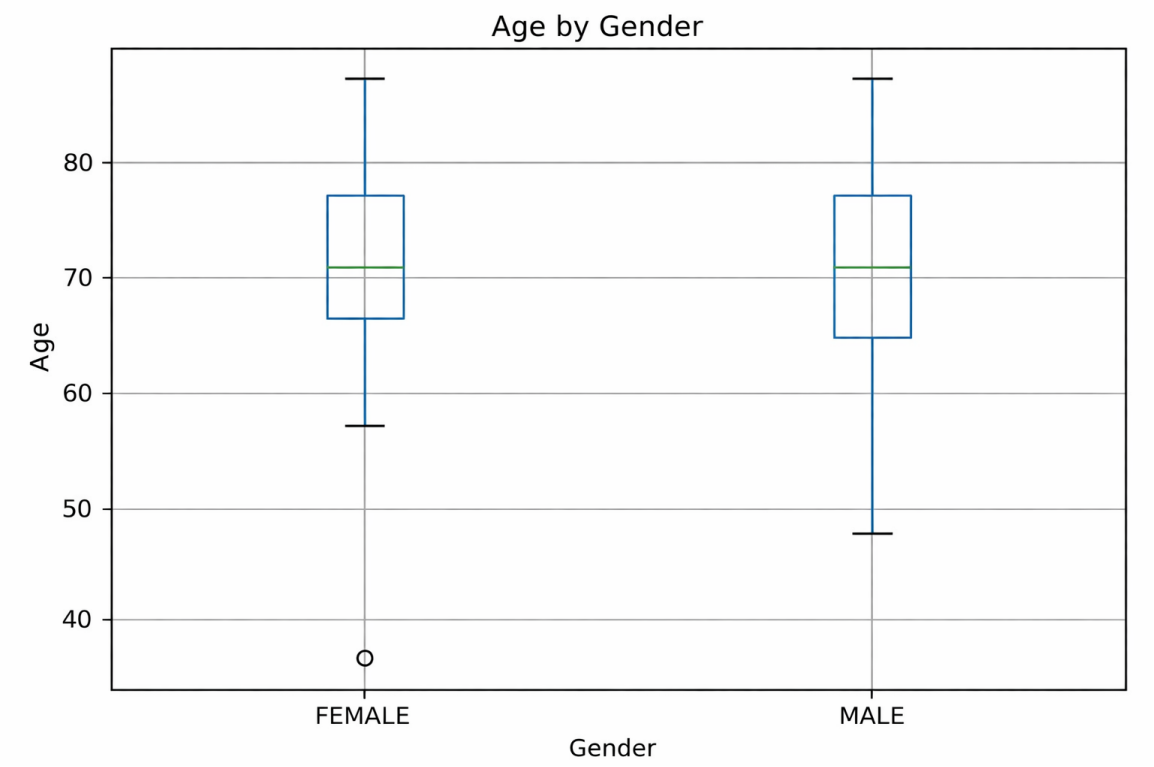

Figure 1. Distribution of patients by sex and age.

Mean age: 70.9 ± 9.1; median 71.0 (IQR 65.0–77.0). In the studied group, the youngest female patient was 35 years old, and the oldest was 87 years old. The presence of isolated patients in the lower age

groups (1 female patient, 30–40 years; 2 patients, 40–50 years - a total of 3 patients under 50 years) does not substantially change the mean age due to the strong concentration of cases in the 60–80 year interval, which is confirmed by the median and interquartile range.

In each age group, the sex distribution of the patients was as follows - Figure 2:

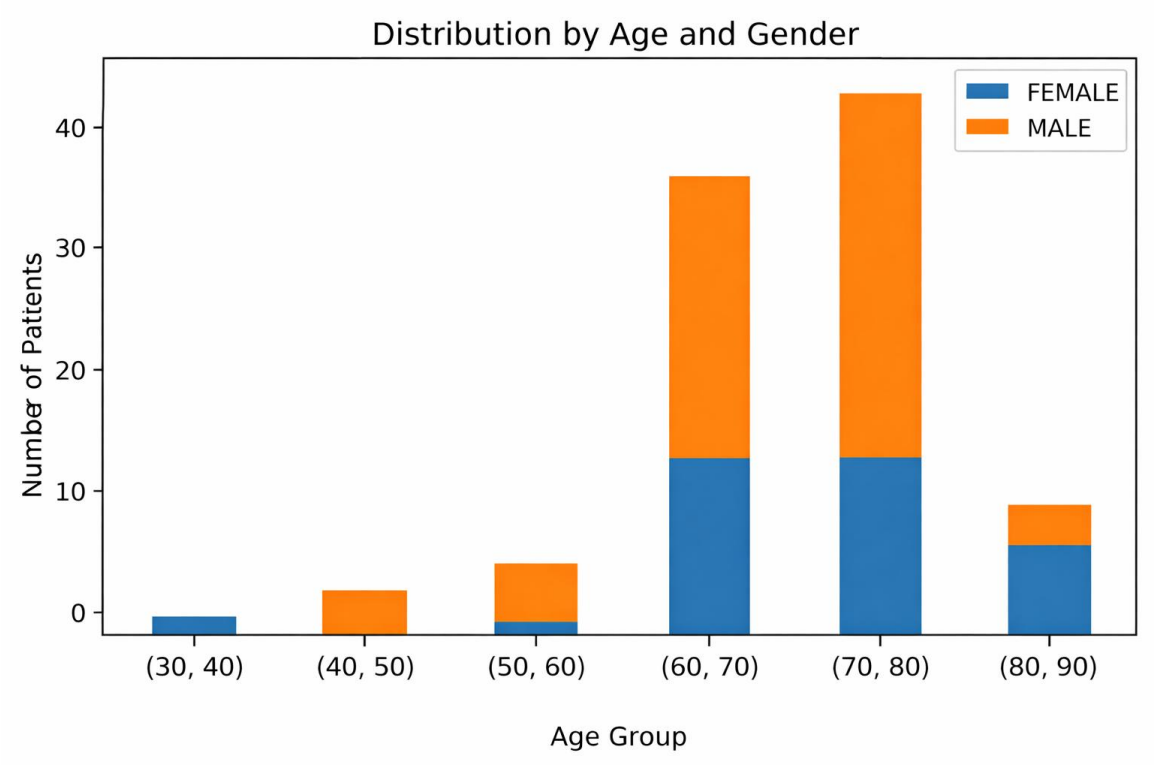

Figure 2. Distribution by sex in the different age groups.

The primary localisation of the tumours in the left colon, right colon, and rectum is respectively: (n=100)

- Left colon: 24 (24.0%)
- Right colon: 29 (29.0%)
- Rectum: 47 (47.0%)

Figure 3 shows the distribution of patients by age and tumour localisation. The boxplot analysis shows the highest median age in left colon tumours and the widest age range in right-sided carcinomas. However, there is no statistically significant difference in the Kruskal–Wallis test. (Kruskal–Wallis H = 4.21, df = 2, p = 0.122)

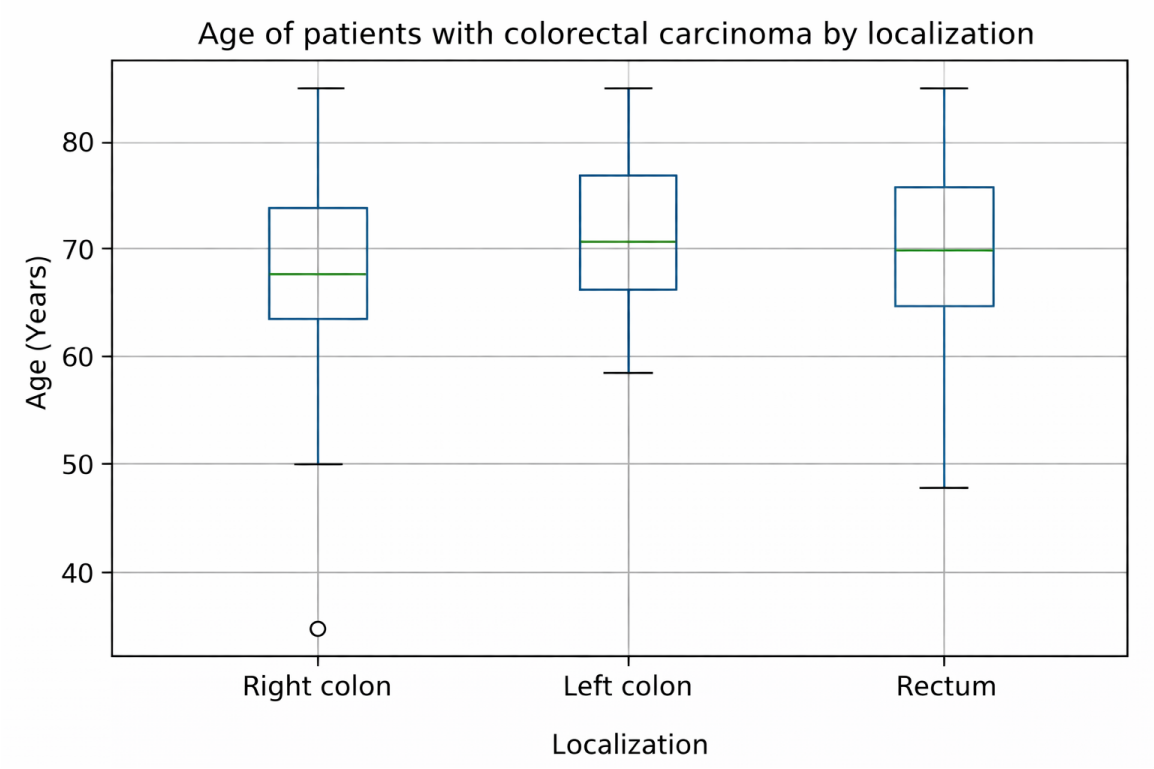

Figure 3. Age distribution of patients according to the primary localisation of adenocarcinomas of the left colon, right colon, and rectum.

The distribution of carcinomas according to localisation - left colon, right colon, and rectum, sex, and age - is as follows: Table 6:

Localization	Sex	n	Min.	Max.	Mean	Median
Right colon	FEMALE	15	35.0	87.0	67.9	68.0
Right colon	MALE	14	50.0	80.0	69.5	71.0
Left colon	FEMALE	8	64.0	85.0	76.9	78.5
Left colon	MALE	16	62.0	87.0	72.8	72.0
Rectum	FEMALE	14	61.0	87.0	73.3	72.0
Rectum	MALE	33	48.0	86.0	69.4	71.0

Table 6. Analysis by age, sex, and localisation.

Right colon carcinomas have a relatively even sex distribution and the widest age range. The youngest patient in the cohort is included here as well. The heterogeneity of right colon tumours is clearly evident from the distribution pattern in this group. Left colon tumours occur in older patients, especially in

women, who form the oldest subgroup in the study. Rectal carcinomas show a clear predominance in men, and men have a lower minimum age. This corresponds to epidemiological data for rectal carcinoma.

The results of the statistical data analysis are as follows:

Kruskal–Wallis test (age × localisation)

The nonparametric Kruskal–Wallis test did not show a statistically significant difference in age among patients with tumours of the right colon, left colon, and rectum (Kruskal–Wallis $H = 4.21$, $df = 2$, $p = 0.122$; $p > 0.05$).

χ^2 test (sex and localisation)

The χ^2 analysis did not establish a statistically significant relationship between sex and the anatomical localisation of the tumour - $\chi^2 = 3.885$; $df = 2$; $p = 0.1434$; $p > 0.05$. The strength of association, measured by Cramer's V, is 0.197, which indicates a weak association between sex and tumour localisation. Despite the greater frequency of rectal involvement in men, this difference does not reach statistical significance.

Mann–Whitney U test (age and sex by localisation)

When comparing age between men and women within each anatomical localization, no statistically significant differences were found: right colon (Mann–Whitney $U = 27.5$, $p = 0.8318$; men $n = 12$, women $n = 5$), left colon, including colon transversum ($U = 137.0$, $p = 0.7483$; men $n = 16$, women $n = 16$), and rectum ($U = 187.5$, $p = 0.3163$; men $n = 33$, women $n = 14$). This allows pooling of the sexes in subsequent analyses without risk of systematic bias.

According to histological grading, there is a predominance of moderately differentiated tumours in the studied group - G2 - 73 patients (73%), followed by high-grade tumours - G3 - 17 patients (17%) and low-grade tumours G1 - 10 patients (10%), without a statistically significant difference between left colon, right colon, and rectum.

The distribution of tumours by grade and localisation is shown in Table 7.

Tumor grade	Right colon	Left colon	Rectum
G1	3	1	6
G2	21	16	36
G3	6	7	4

Table 7. Distribution of tumours according to their grade and localisation.

Analysis of the histological subtype shows a clear predominance of adenocarcinoma with no specific features (92%). The remaining cases include mucinous, medullary, and poorly cohesive variants, each represented by a small number of patients (8%). Statistical analysis of tumour differentiation and patient age did not reveal a statistically significant difference (Kruskal–Wallis H = 0.79, df = 2, p = 0.673).

The histological subtypes of the studied carcinomas are reflected in Figure 4:

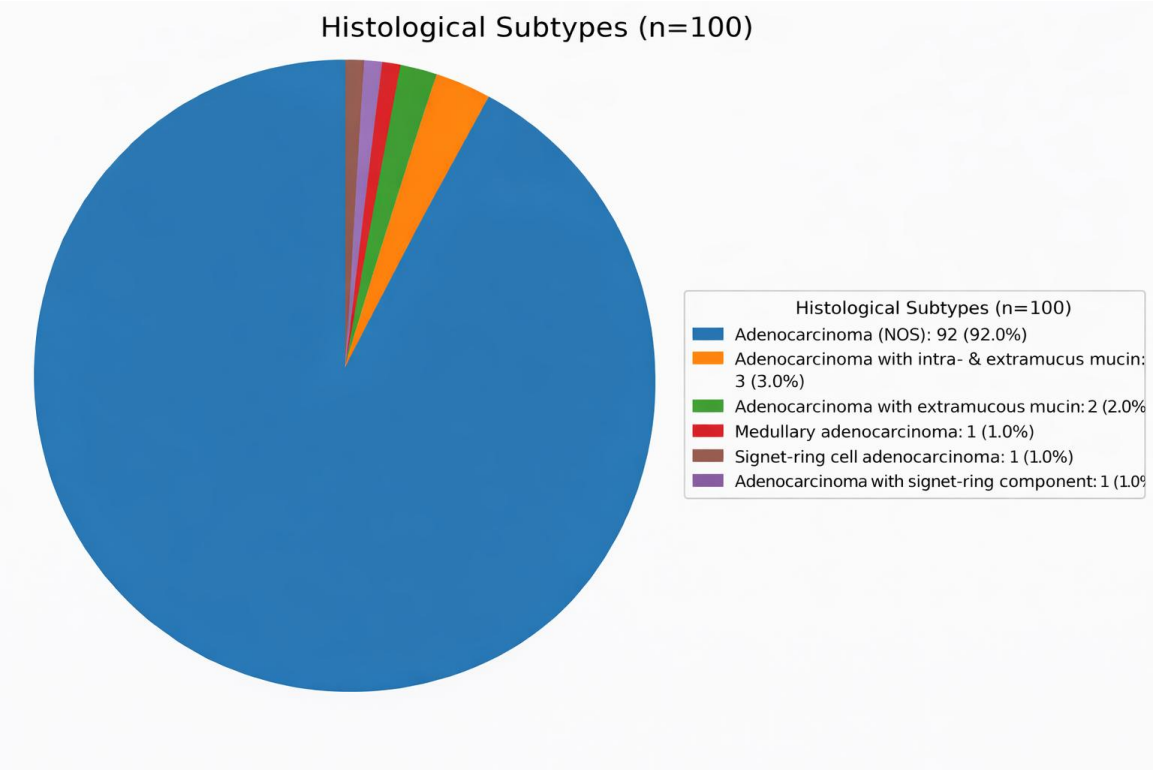

Figure 4. Histological subtypes of the studied carcinomas.

Pathological staging of the tumours in this patient cohort is irrelevant and was not performed.

BY TASK NO. 2:

In this group of patients, the type of biopsy material did not allow it, and we did not aim to investigate lymphovascular, perineural, or intraneural invasion, lymph nodes, or the peritumoral stromal immune reaction; such investigations were not performed.

BY SUBTASK NO. 3A

We investigated and analysed the feasibility of morphological evaluation and grading of peritumoral budding (PTB) in endoscopic colorectal biopsies, as well as the factors influencing this process.

The investigation and grading of peritumoral budding (PTB) were performed according to the ITBCC algorithm described in the “Materials and Methods” section.

Of the total sample of 100 patients, peritumoral budding was reported in only 12 patients (12%).

The distribution of reported/unreported PTB, depending on different factors, is reflected in the following tables:

The distribution of reported/not reported PTB by sex is reflected in Table 8.

Sex	Reported	Unreported	Reported proportion (%)
FEMALE	4	33	10.8
MALE	8	55	12.7

Table 8. PTB reported/not reported distributed by sex.

The distribution by sex does not show a statistically significant difference: ($\chi^2 = 0.27$, df = 1, p = 0.603). Cramer's V = 0.052, which indicates a very weak association between sex and reported tumour budding.

The age distribution is as follows in Table 9.

Budding	n	Mean ± SD	Median	IQR	Min–Max
Not reported	88	70.1 ± 9.1	71.0	65.0–77.0	35–87
Reported	12	76.1 ± 7.9	77.0	70.8–83.2	64–86

Table 9. Age distribution of PTB - reported/not reported.

Age: reported vs unreported

The statistical analysis shows - Mann–Whitney U = 326.0, Z = −2.07, p = 0.038. Patients with reported budding are older (median 77 years versus 71 years; Mann–Whitney U: $p < 0.05$).

Table 10 shows the distribution of reported and unreported tumour budding by localisation.

Localization	Reported	Unreported	Reported proportion (%)
Right colon	2	27	6.9
Left colon	3	21	12.5
Rectum	7	40	14.9

Table 10. Distribution of reported and unreported peritumoral budding according to localisation.

By localisation: The statistical analysis showed that the difference is not statistically significant (χ^2 = 1.11, df = 2, p = 0.574; p > 0.05). Cramer's V = 0.105, which indicates a weak association between localisation and reported peritumoral budding.

Table 11 and Table 12 show, respectively, the distribution of reported/unreported tumour budding according to histological subtype and tumour differentiation:

Subtype	Reported	Unreported	Total
Adenocarcinoma	10	82	92
Adenocarcinoma with intra- and extracellular mucin production	1	2	3
Adenocarcinoma with poorly cohesive component	1	0	1
Adenocarcinoma with extracellular mucin production	0	2	2
Medullary adenocarcinoma	0	1	1
Poorly cohesive adenocarcinoma	0	1	1

Table 11. Distribution of reported/not reported tumour budding according to histological subtype.

Grade	Not reported	Reported
G1	10	0
G2	64	9
G3	14	3

Table 12. Distribution of reported/not reported tumour budding according to tumour grade (G).

Reported budding is most often observed in G2, reflecting G2's dominance in the cohort. There is no statistically significant association between grade and reported budding ($\chi^2 = 1.08$, df = 2, p = 0.58).

From the data and statistical analysis presented so far, we can draw the following conclusion:

Age is an independent factor associated with reported tumour budding.

Tumour grade correlates neither with age nor with budding.

Localisation does not significantly modify this relationship.

There is no statistically significant correlation between histological carcinoma subtypes and grade, or with the reporting of peritumoral budding.

We systematised the factors that determine the likelihood of reporting peritumoral budding in endoscopic biopsies of CRC in the studied patients as follows: Table 13.

Category of biopsy material	Budding not reported	Reported budding	Total
Optimal for evaluation	0 (0%)	10 (10%)	10 (10%)
Technical artefacts hindering evaluation*	55 (55%)	1 (1%)	56 (56%)
Challenges related to tumour characteristics **	33 (33%)	1 (1%)	34 (34%)
Total	88 (88%)	12 (12%)	100 (100%)

Table 13. Factors determining the possibility of reporting peritumoral budding.

Legend:

* Technical artefacts: fragmented material, scant biopsy material.

** Tumour-related characteristics: extensive necrosis, lack of invasive front, low or high degree of differentiation, poorly cohesive morphology, pronounced inflammation.

Upon subsequent subdefinition of the factors determining the reporting of tumour budding in endoscopic biopsies of CRC, the following results were obtained, reflected in Table 14.

Factors influencing	BD0	BD1	BD2	BD3	Total

PTB assessment					
Optimal material	0	3	5	2	10
Lack of invasive front	6	0	0	0	6
Well-differentiated tumor	8	0	0	0	8
Pronounced inflammation	1	0	0	0	1
Necrosis	10	0	0	0	10
Poorly differentiated tumor	7	0	1	0	8
Scant material	12	0	0	0	12
Poorly cohesive tumor	1	0	0	0	1
Fragmented material	43	0	0	1	44
Total	88	3	6	3	100

Table 14. Factors influencing PTB assessment in endoscopic biopsies of CRC.

The possibility of reporting PTB (BD1–BD3) is observed in only 12% of cases (12/100), with 10/12 of these in the presence of optimal material; artefacts or other unfavourable tumour characteristics lead to a predominant BD0. In the following photographs of microscopic slides, we present the main morphological patterns that preclude adequate reporting of peritumoral budding in preoperative endoscopic biopsies of colorectal carcinoma (Photos 1–11).

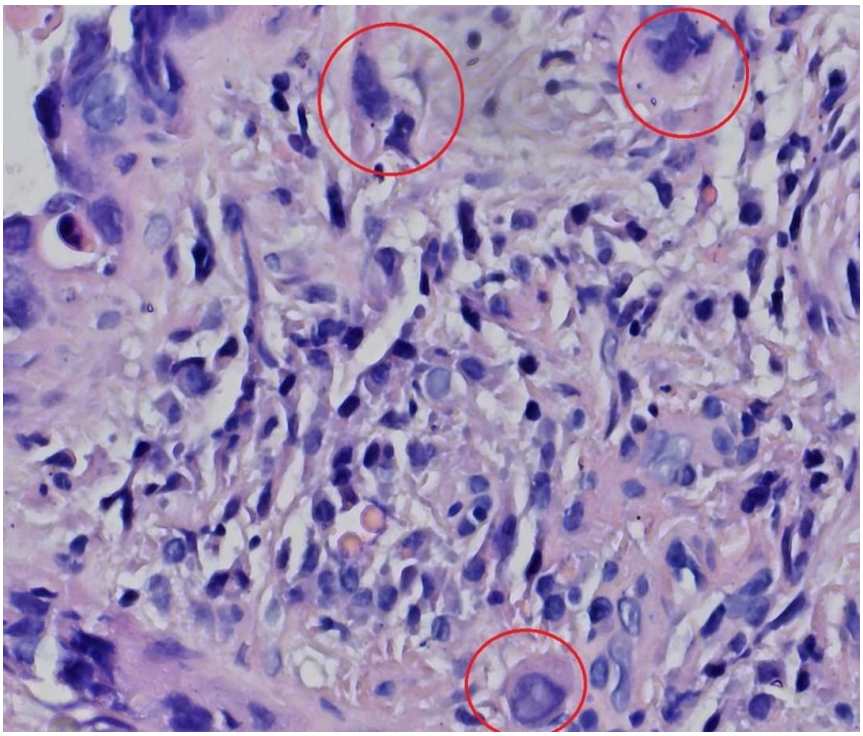

Photo 1. Tumour budding. H&E stain; magnification 40×.

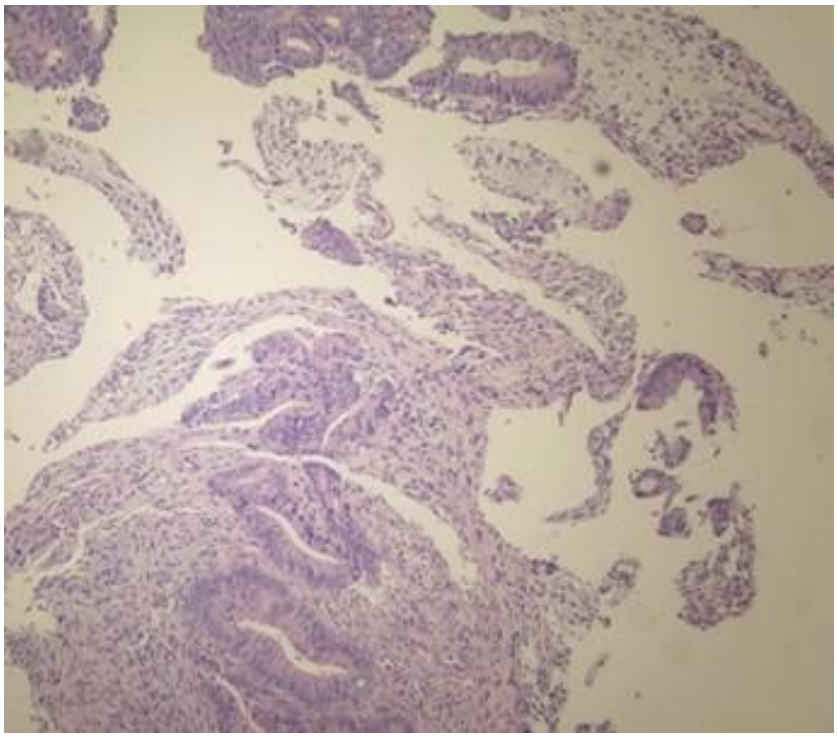

Photo 2. Microscopic appearance of fragmented material from an endoscopic biopsy with CRC. PTB cannot be reported. H&E stain; magnification 20×.

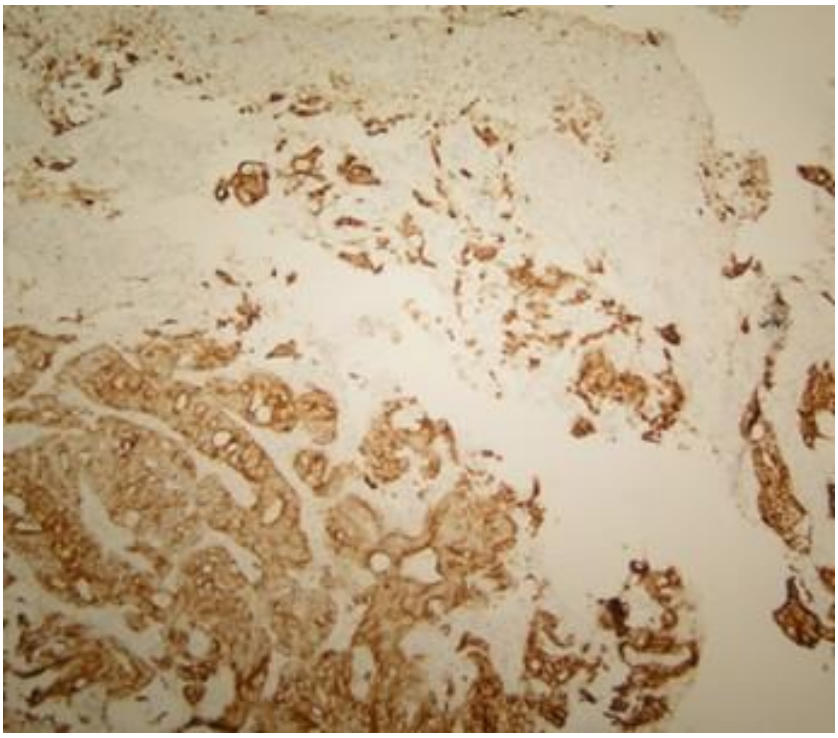

Photo 3. Fragmented material with necrosis. IHC CK AE1/AE3. magnification 20×.

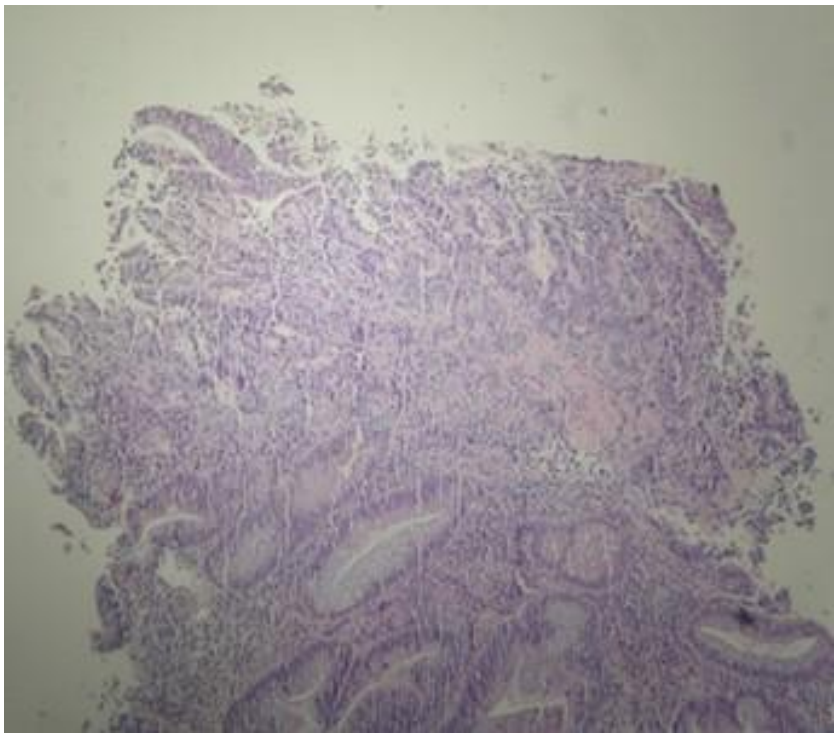

Photo 4. Scant, peripherally located fragmented tumor parenchyma. H&E stain; magnification 20×.

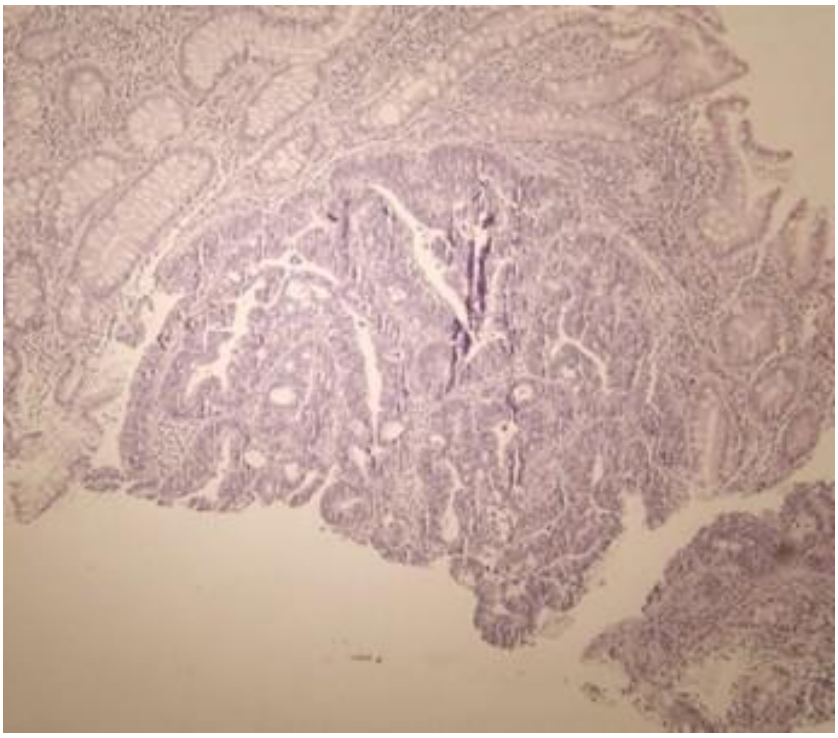

Photo 5. Scant tumour biopsy material without an invasive front. H&E stain; magnification 20×.

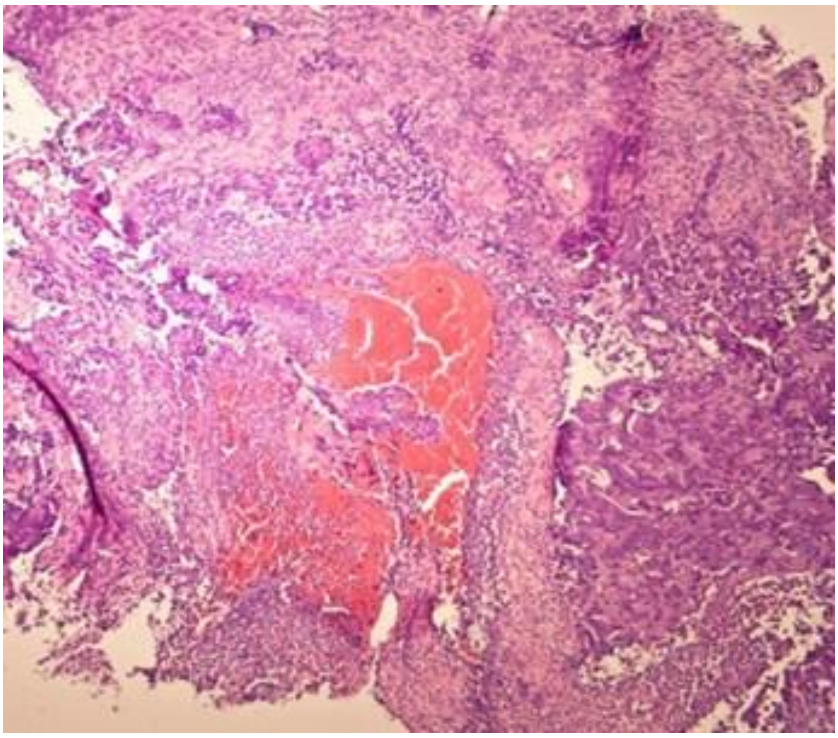

Photo 6. Biopsy material with pronounced necrosis and inflammation. H&E stain; magnification 20×.

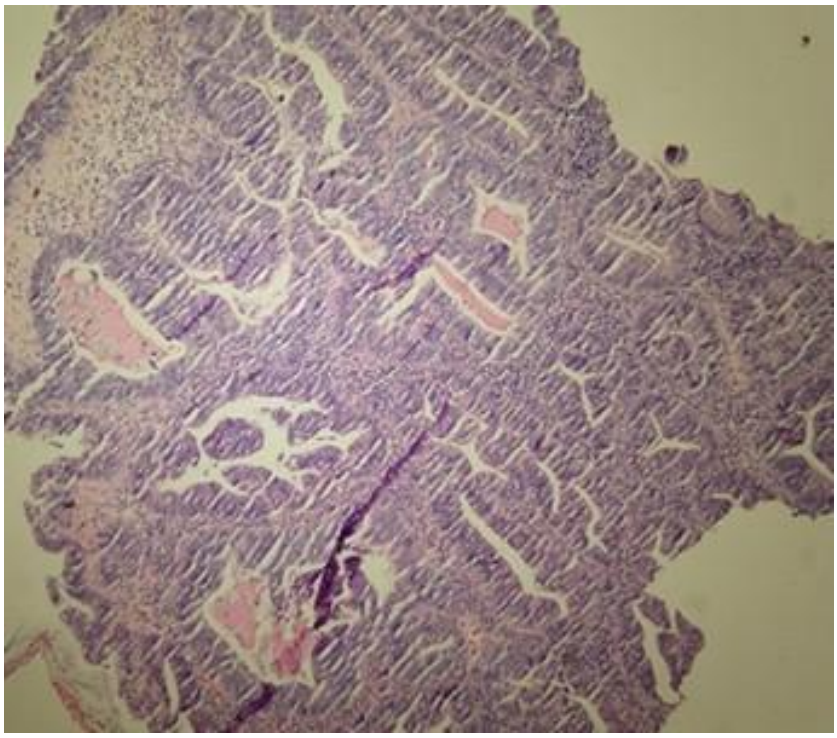

Photo 7. Biopsy material with pronounced artefacts during processing. H&E stain; magnification 20×.

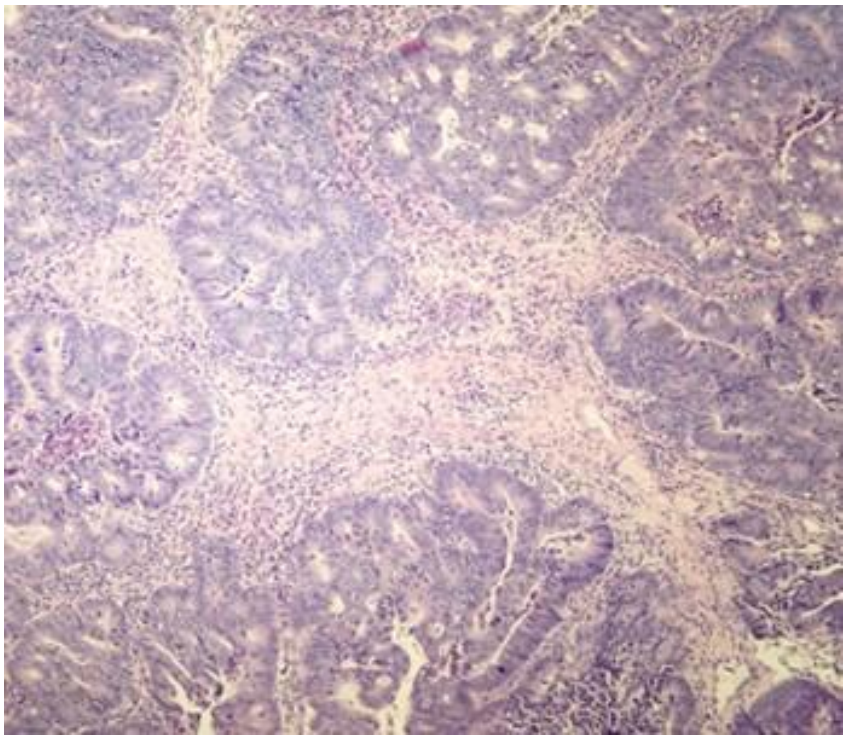

Photo 8. Material from well-differentiated adenocarcinoma of the colon - G1. Tumour budding is absent. H&E stain; magnification 20×.

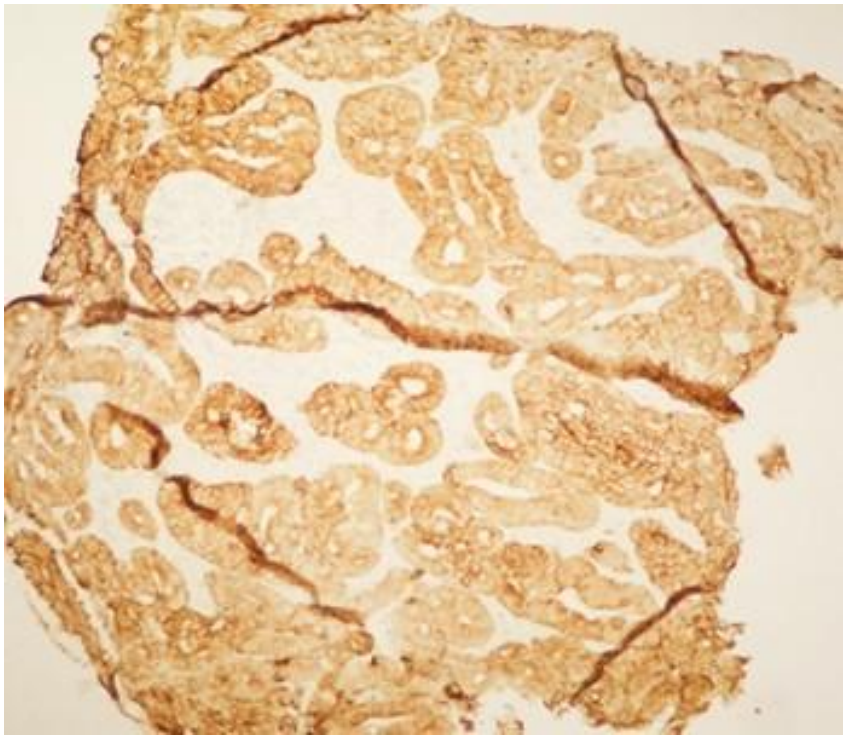

Photo 9. Biopsy material from well-differentiated adenocarcinoma of the colon - G1, without tumour budding. IHC - CK AE1/AE3. magnification 20×.

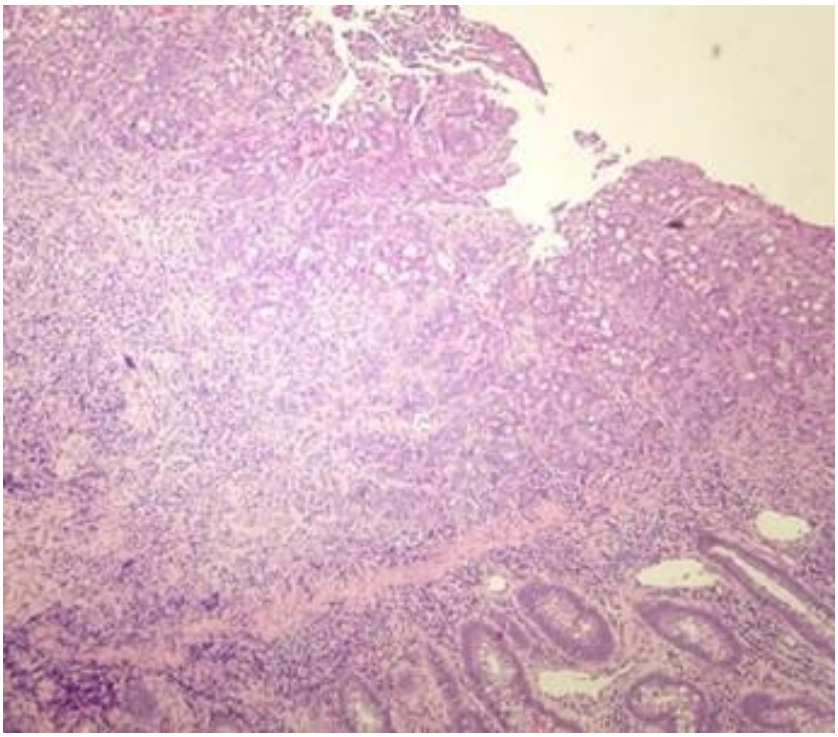

Photo 10. Poorly differentiated colorectal carcinoma with pronounced inflammation. H&E stain; magnification 20×.

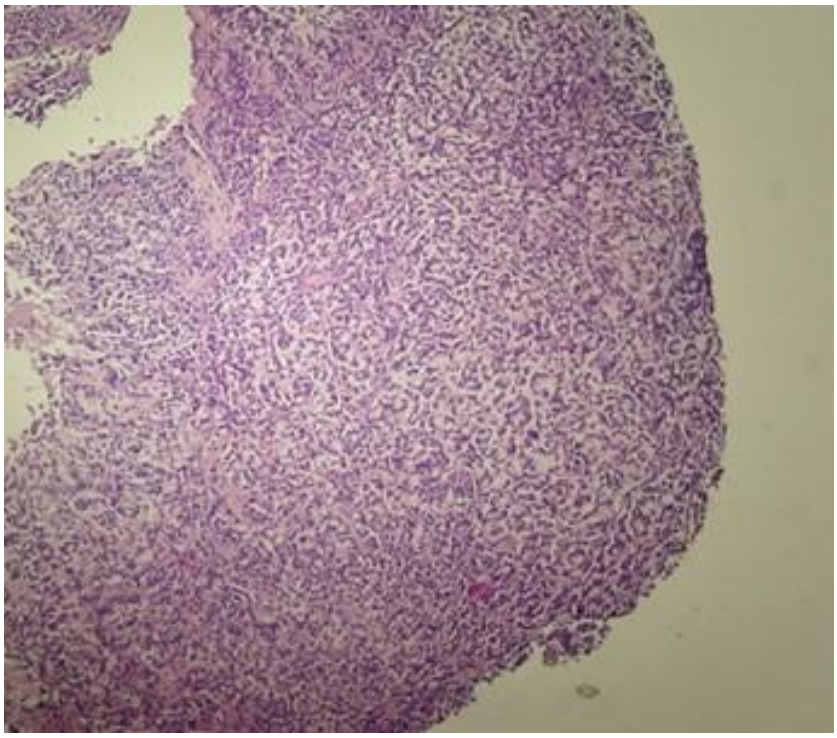

Photo 11. Poorly cohesive colorectal carcinoma. H&E stain; magnification 20×.

The main factor determining the feasibility of evaluating tumour budding is the quality and representativeness of the biopsy material, rather than the tumour's biological characteristics.

With optimal biopsy material, tumour budding can be reliably assessed, whereas in the presence of technical artefacts or unfavourable morphological characteristics, evaluation is impossible in the predominant part of cases.

SECOND PATIENT COHORT

100 patients with postoperative resection-proven colorectal adenocarcinoma were represented in the second cohort during 2020–2023.

BY TASK NO. 1:

• Epidemiological data

Distribution by sex and age

The study included 100 patients, of whom 57 were men (57%), and 43 were women (43%).

The mean age of the patients was 69.23 ± 8.94 years, with a median age of 70.0 years and an age range of 45–88 years.

• Localisation of the primary tumour

After consolidation of the localisations into left colon, right colon, and rectum, the following results were obtained: in women and men, the most frequent localisation was in the right colon (39.5% in women and 43.9% in men), while in the left colon and rectum, the carcinomas showed similar relative shares. The distribution by anatomical localisation was practically similar in both sexes and is reflected in Figure 5.

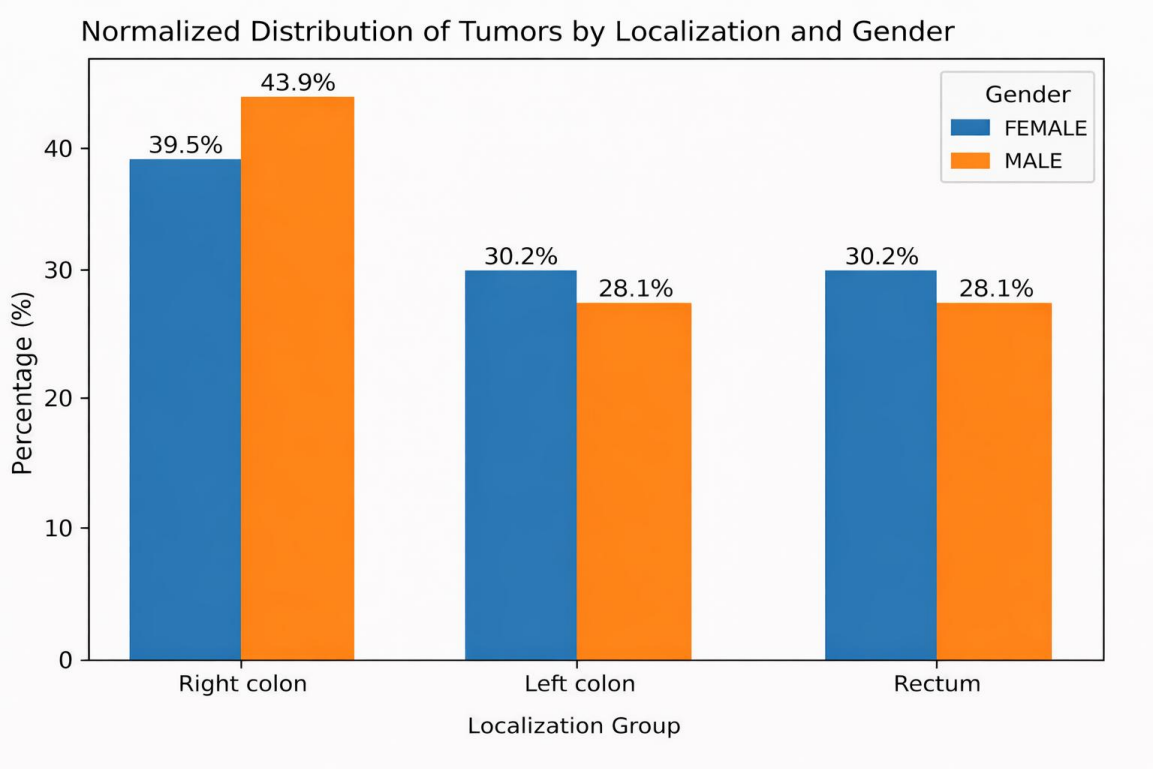

Figure 5. Normalised distribution of tumours in both sexes according to primary localisation.

In the analysis of localisation across the three localisation categories (right colon, left colon, rectum), no statistically significant association was found ($\chi^2 = 0.19$; df = 2; p = 0.91), which confirms the absence of dependence between sex and the anatomical distribution of colorectal carcinoma in the studied cohort. Table 15 presents the median age and age range of patients by tumour anatomical localisation, as well as stratification by sex. The overall median age of the studied cohort was 70.0 years (range 45–88 years), with no difference observed between men and women (median age 70.0 years in both sexes).

When comparing age across the individual anatomical segments, no statistically significant difference was observed (Kruskal–Wallis H = 1.53; df = 2; p = 0.465, when grouped into right colon, left colon, and rectum). Stratification by sex within the individual localisations also did not reveal a substantial difference in age distributions, with the age ranges for men and women overlapping considerably. Due to the single case in fl. hepatica, a test excluding this segment from the age analysis was also performed to avoid statistical instability. After exclusion, the Kruskal–Wallis test yielded H = 3.25 and p = 0.777.

Localization	Median age (patients)	Range (patients)	Total patients	Median (men)	Range (men)	Total men	Median (women)	Range (women)	Total women
Ascendens	70.5	55–88	14	71.0	67–88	7	61.0	55–75	7
Cecum	71.5	56–86	16	71.5	57–86	10	71.5	56–83	6
Descendens	75.0	48–81	6	76.0	57–81	4	61.5	48–75	2
Fl. hepatica	68.0	68–68	1	68.0	68–68	1	—	—	0
Fl. lienalis	69.5	61–78	4	71.0	68–78	3	61.0	61–61	1
Rectum	71.0	45–86	29	72.5	45–86	16	68.0	57–77	13
Sigmoideum	68.0	48–77	19	68.0	57–77	9	68.5	48–76	10
Transversum	65.0	57–84	11	65.0	57–84	7	73.0	61–75	4
Total	70.0	45–88	100	70.0	45–88	57	70.0	48–83	43

Table 15. Median age and age range according to tumour localisation and sex.

From the above, it follows that no statistically significant dependencies are observed among sex, age, and the anatomical localisation of colorectal carcinomas.

• Histological tumour subtype

In the analysis of grouped histological subtypes versus anatomical localisation (right colon, left colon, and rectum), no statistically significant relationship was established between the two variables (χ^2 = 19.937; df = 18; p = 0.336).

The additional stratified analysis by sex also did not demonstrate significant differences: men: χ^2 = 12.826; p = 0.685; women: χ^2 = 8.573; p = 0.380.

The obtained results show that the distribution of histological subtypes is similar across anatomical localisations and is not influenced by patients' sex in the studied cohort.

• Tumour grade (G) and tumour stage (T)

In the analysis of the relationship between anatomical localisation (right colon, left colon, and rectum) and tumour grade, no statistically significant dependence was established (χ^2 = 9.53; df = 6; p = 0.146). The stratified analysis by sex also did not demonstrate significant differences: in men: χ^2 = 4.38; df = 6; p = 0.626, and in women: χ^2 = 7.88; df = 4; p = 0.096. After combining the rare histological categories in men, the result remained statistically nonsignificant (χ^2 = 3.60; df = 4; p = 0.463), confirming the lack of dependence between tumour localisation and degree of differentiation.

About tumour stage (pT), no statistically significant relationship with localisation was established (χ^2 = 15.46; df = 10; p = 0.116). The analysis by sex confirmed the lack of statistically significant dependence (men: χ^2 = 10.70; df = 8; p = 0.219, and women: χ^2 = 14.44; df = 10; p = 0.154).

In a direct comparison between degree of differentiation (G) and tumour stage (pT), a statistically significant relationship was established (χ^2 = 35.27; df = 15; p = 0.0023), which indicates the expected biological dependence between lower differentiation and more advanced stage, which is also schematically reflected in Figure 6.

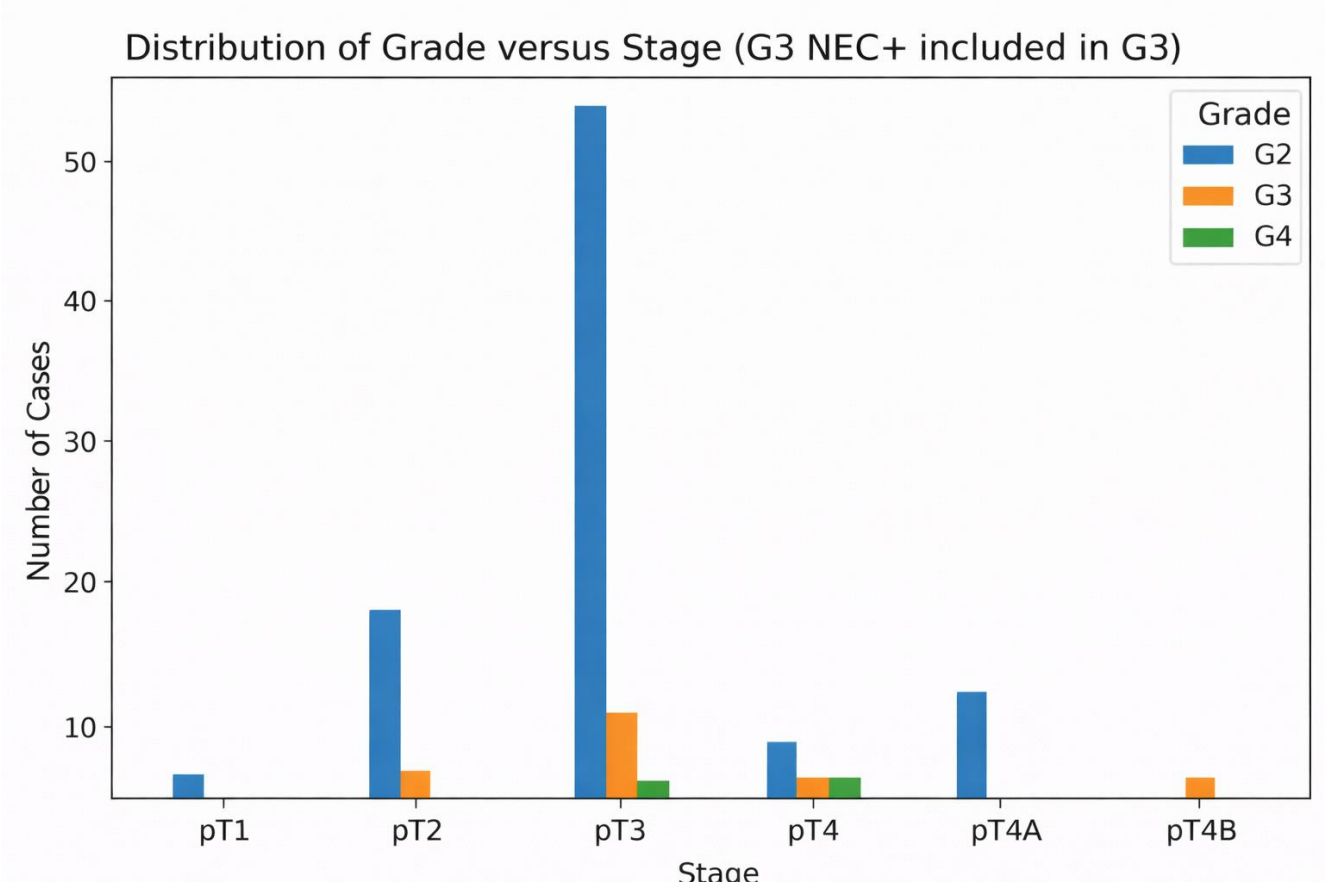

Figure 6. Distribution of tumour Grade (G) versus tumour Stage (T).

• Lymph node status

In the patient cohort, no statistically significant differences in nodal status (N) according to the localization of the primary tumor (right colon, left colon, and rectum) were established in the entire sample (χ^2: 11.811; p-value: 0.621; df = 14), and this remains valid also in the separately conducted analysis by sex: men (χ^2: 12.821; p-value: 0.382; df = 12; women (χ^2: 8.376; p-value: 0.869; df = 14).

When comparing lymph node status and degree of tumour differentiation (Grade), after combining category G3 NEC+ into G3, the χ^2 analysis again did not demonstrate a statistically significant relationship (χ^2: 19.163; p-value: 0.159; df = 14). Additionally, in the analysis of the relationship between lymph node status and tumour stage (pT), no statistically significant differences were established (χ^2: 35.678; p-value: 0.436; df = 35).

In summary, the results show that, in the present cohort, lymph node involvement is not statistically significantly associated with tumour localisation, degree of differentiation, or depth of tumour invasion.

BY TASK NO. 2: IMPORTANT TUMOUR CHARACTERISTICS WITH PROGNOSTIC VALUE

• Lymphatic and vascular invasion (LVI)

In our study, due to the small sample of patients in whom lymphovascular invasion was established (26 of 100 patients (26%)), we did not set as our aim the separate evaluation of these patients. Also, the small sample and the low percentage of patients with established LVI did not allow us to consider intramural (IMVI) and extramural (EMVI) vascular invasion separately. For this reason, we considered them together.

In the individual tumour localisations – right colon, left colon, and rectum, the following picture of lymphovascular invasion was established:

- right colon: negative - 28; positive - 14.
- left colon: negative - 22; positive - 7.
- rectum: negative - 24, positive - 5.

The statistical analysis of the relationship between lymphovascular invasion (LVI) and localization of the primary tumor (right colon, left colon, and rectum) does not establish a statistically significant dependence ($\chi^2 = 2.382$; df = 2; p = 0.304), the strength of association measured by Cramer's V is 0.154, which indicates a weak association between LVI and tumor localization. Despite the relatively higher absolute number of positive cases in right-sided tumours, this difference does not reach statistical significance. Although statistical significance was not established, the observed results are as follows: 33% (14/42) of right colon tumours are LVI+; 24% (7/29) in the left colon and 17% (5/29) in the rectum. A tendency toward a higher frequency of LVI in tumours localised in the right colon is observed, but the sample is not large enough to prove a statistical difference.

In investigating the dependencies between lymphovascular invasion (LVI) and tumour differentiation (G), tumour stage (T), and nodal status (N), we obtained the results reflected in Table 16, Table 17, and Table 18:

Stage	LVI negative	LVI positive
pT1	2	0
pT2	17	1
pT3	49	15
pT4	3	5
pT4A	2	4

pT4B	1	1

Table 16. LVI and tumour stage (pT).

Grade	LVI negative	LVI positive
G2	69	16
G3	5	7
G4	0	3

Table 17. LVI and tumour differentiation (G).

N status	LVI negative	LVI positive
N0	52	7
N1	4	1
N1a	3	1
N1b	5	7
N1c	4	3
N2	4	0
N2a	1	3
N2b	1	4

Table 18. LVI and N status.

The statistical analysis of these data shows a statistically significant relationship between LVI and tumour stage (pT) ($\chi^2 = 16.13$; df = 5; p = 0.0065; Cramer's V = 0.40), with the frequency of LVI increasing with increasing depth of invasion. This shows that more advanced tumours are more likely to invade the vasculature.

A highly statistically significant relationship is established between LVI and the degree of tumour differentiation (Grade) ($\chi^2 = 17.33$; df = 2; p = 0.00017; Cramer's V = 0.42), with poorly differentiated tumours more often demonstrating lymphovascular invasion. This confirms LVI's role as a marker of biological aggressiveness.

The strongest association is observed between LVI and lymph node status (N) ($\chi^2 = 27.75$; df = 7; p = 0.00024; Cramer's V = 0.53), indicating a strong dependence between the presence of lymphovascular invasion and metastases in lymph nodes.

The lack of a statistically significant direct relationship between tumour stage and lymph node status in the present sample is probably due to heterogeneity in the categories and possible limitations in the data. In contrast, LVI appears as a more direct morphological indicator of metastatic potential.

Lymphovascular invasion and histological tumour subtype

In the comparative investigation of these two parameters, the following results were obtained: $\chi^2 = 15.91$; df = 9; p = 0.0687. The statistical analysis of the relationship between lymphovascular invasion and histological subtype does not demonstrate a statistically significant dependence. Due to the presence of rare histological categories with low frequency, the applicability of the classical χ^2 test is limited, and therefore, the result should be interpreted cautiously. A borderline tendency toward association is observed, which could reach statistical significance in a larger sample.

• Perineural invasion (PNI)

The data on the statistical dependencies that we observed about PNI and primary tumour localisation, histological subtype, grade (G), stage (pT), lymphovascular invasion, and nodal status are reflected in the following Tables 19, 20, 21, 22, 23, 24:

Table 19 shows perineural invasion in relation to the primary tumour localisation: right colon, left colon, and rectum.

Category	PNI–	PNI+
Right colon	26	16
Left colon	20	9
Rectum	19	10

Table 19. Perineural invasion in relation to primary tumour localisation.

No statistically significant relationship is established between PNI and anatomical localisation ($\chi^2 = 0.381$; df = 2; p = 0.8267; p > 0.05), with Cramer's V = 0.062, indicating a very weak association.

Table 20 shows PNI and histological subtypes of CRC.

Category	PNI–	PNI+
Adenocarcinoma NOS + mucinous component	0	1
Adenocarcinoma NOS + intra and extracellular mucin production	0	1

Adenocarcinoma NOS	54	23
Adenocarcinoma NOS with mucinous and poorly cohesive component	0	1
Medullary	0	2
MiNEN	1	0
Mucinous	6	5
Serrated adenocarcinoma	3	1
Synchronous- Cecum-mucinous, Rectum-Adenocarcinoma NOS	1	0
Undifferentiated	0	1

Table 20. PNI and histological subtypes.

No statistically significant association is established between PNI and histological subtype. Because of the rarity of the categories, the result should be interpreted cautiously. ($\chi^2 = 13.815$; df = 9; p = 0.1291), Cramer's V = 0.372, which indicates a moderate association.

Table 21 – PNI and tumour differentiation (G).

Grade	PNI (-)	PNI (+)	Total
G1	9	1	10
G2	49	24	73
G3	9	8	17
Total	67	33	100

Table 21. PNI and tumour differentiation (G).

Analysis of the relationship between perineural invasion (PNI) and degree of tumour differentiation (G) shows a tendency toward a more frequent presence of PNI in more poorly differentiated tumours. The highest relative share of PNI is observed in G3 carcinomas, whereas in well-differentiated tumours (G1), perineural invasion is encountered rarely. In the statistical analysis, no statistically significant

association is established between tumour grade and the presence of PNI ($\chi^2 = 4.11$; df = 2; $p > 0.05$). The strength of the relationship, assessed by Cramer's V coefficient, is 0.20, which indicates a weak association between the two indicators.

Table 22 – results of perineural invasion and tumour stage (pT).

Category	PNI–	PNI+
pT1	2	0
pT2	15	3
pT3	41	23
pT4	4	4
pT4A	1	5
pT4B	2	0

Table 22. PNI and tumour stage (pT).

A statistically significant relationship is established between PNI and pT stage ($p < 0.05$) ($\chi^2 = 11.790$; df = 5; p = 0.03778), which supports PNI as a marker of locally advanced disease.

Table 23. PNI and lymphovascular invasion (LVI).

Category	LVI–	LVI+
PNI–	58	7
PNI+	16	19

Table 23. PNI and lymphovascular invasion (LVI).

There is a strong association between PNI and LVI ($p < 0.05$). The combination of PNI+ and LVI+ outlines a more aggressive morphological phenotype. ($\chi^2 = 20.187$; df = 1; $p = 7.0 \times 10^{-6}$; $p < 0.001$), Cramer's V = 0.449, which indicates a moderate to strong association.

Table 24. PNI and nodal status (N).

Category	PNI–	PNI+
N0	45	14
N1	3	2
N1a	3	1
N1b	3	9

N1c	6	1
N2	2	2
N2a	2	2
N2b	1	4

Table 24. PNI and nodal status (N).

PNI is statistically significantly associated with N status ($\chi^2 = 18.527$; df = 7; p = 0.009806; $p < 0.05$), with a higher frequency of PNI+ in some N+ categories. Cramer's V = 0.430, indicating a moderate to strong association.

The obtained results show that perineural invasion (PNI) is more strongly associated with indicators of tumour aggressiveness and potential for dissemination than with anatomical localisation or histological subtype. The established statistically significant relationship between PNI and pT stage supports PNI as a marker of locally advanced disease, since deeper invasion increases the probability of perineural space involvement. The borderline association between PNI and Grade ($p \approx 0.057$) is biologically plausible. It suggests that lower differentiation may favour invasive growth patterns, but the sample size and imbalance between categories limit the power to provide formal proof.

When investigating the interaction between PNI and LVI and PNI and N status, we observed an association between PNI and LVI ($p < 0.001$), which points to the presence of invasive phenotypes – vascular/lymphatic invasion and perineural spread, probably sharing common mechanisms related to impaired cell adhesion, stromal remodelling, and migration. The significant relationship between PNI and N status ($p < 0.05$) further supports PNI as an indicator of metastatic risk.

• Aggressive Score

In view of the established biological relationship among perineural invasion, lymphovascular invasion, and low degree of differentiation, we attempted to construct a composite morphological index (Aggressive Score) to provide an integrated evaluation of invasive potential. The combined Aggressive Score (PNI+LVI+High Grade) demonstrated a statistically significant association with N positivity (p = 0.003), which has potential practical value: rather than relying on isolated findings, the integrated morphological profile may provide more stable risk stratification. The data on the methodology and calculations of the Aggressive Score in our sample are reflected in Appendix 1. Aggressive Score is a research index, not an official clinical prognostic model, and its applicability needs to be confirmed in an independent study of a larger patient cohort.

• Peritumoral immune response

In our study, we evaluated the “Crohn-like” immune reaction observed in colorectal carcinomas.

Photo 12 shows an example of a “Crohn-like” inflammatory peritumoral reaction.

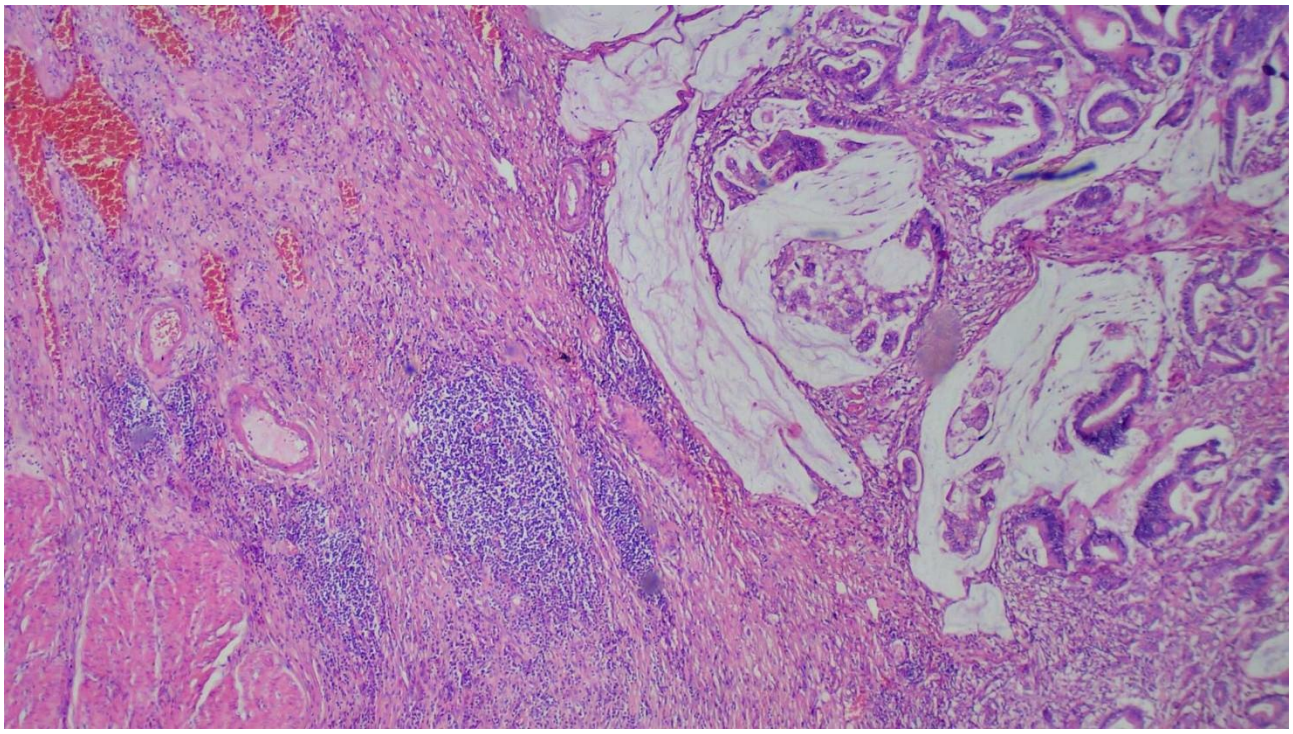

Photo 12. Crohn-like inflammatory peritumoral reaction. H&E stain, magnification 10×.

Peritumoral immune response and primary tumour localisation

Figure 7 presents a graphical image of the distribution of the different degrees of peritumoral immune response according to the localisation of carcinomas in the right colon, left colon, and rectum.

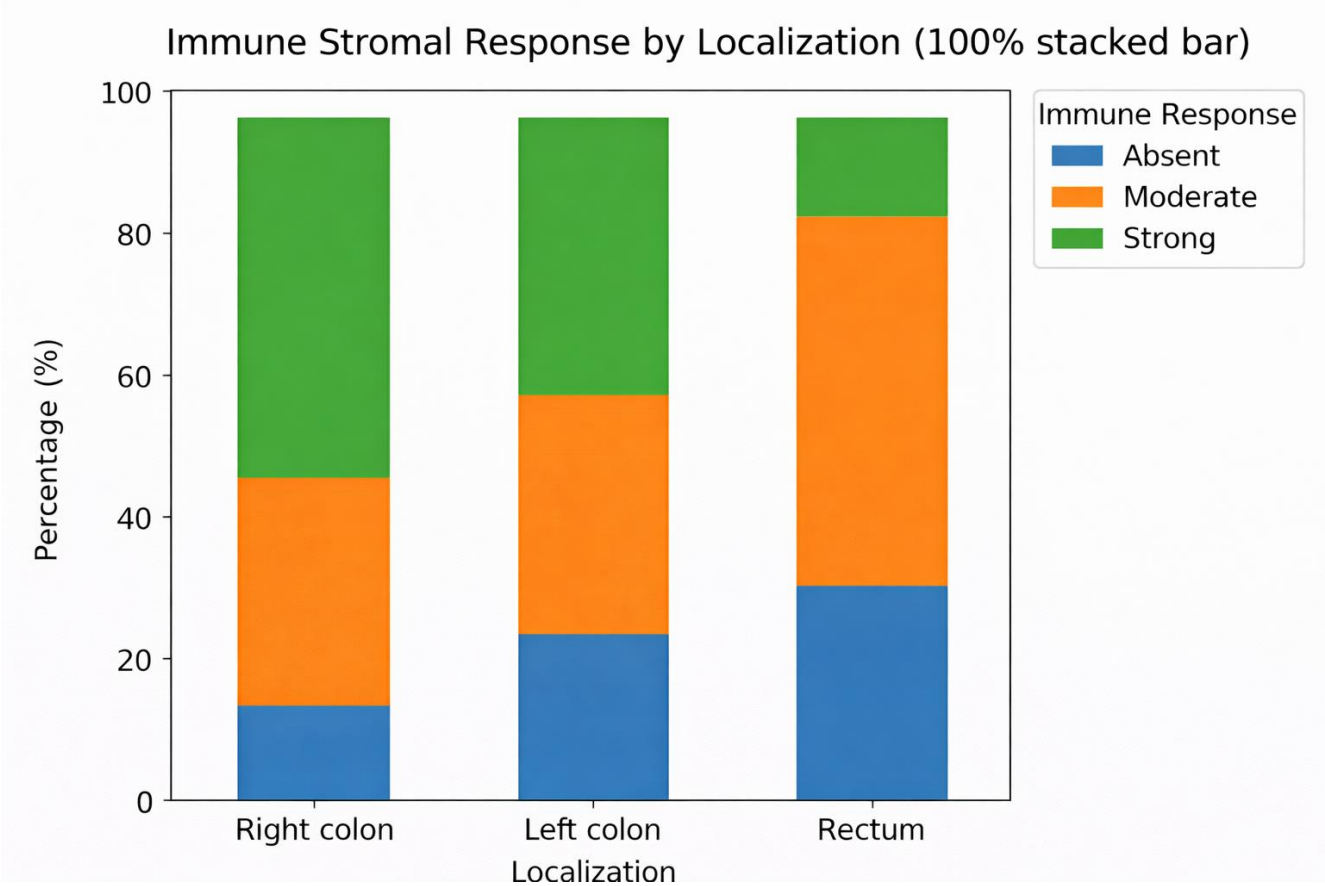

Figure 7. Crohn-like peritumoral immune response and primary tumour localisation - right colon, left colon, rectum.

In absolute values, the reported peritumoral stromal inflammatory reaction is as follows from Table 25:

Localization	Absent	Moderate	Marked
Right colon	5	13	24
Left colon	7	10	12
Rectum	9	16	4

Table 25. Absolute values of the distribution of the peritumoral stromal inflammatory reaction (determined semiquantitatively) according to primary tumour localisation.

The performed statistical analysis showed ($\chi^2 = 14.05$; df = 4; $p = 0.0071$), that there is a statistically significant association between the intensity of the immune stromal (Crohn-like) response and the anatomical localization of the tumor ($p < 0.05$), Cramer's V = 0.265, which indicates a moderate association between tumor localization and the intensity of the immune reaction. Right-sided carcinomas

show the highest share of marked immune reaction, whereas rectal tumours are characterised predominantly by a moderate reaction and a significantly lower percentage of marked response.

Peritumoral immune response and histological subtype of colorectal carcinomas.

The statistical analysis of the dependence between the peritumoral stromal immune response of the "Crohn-like" type and the histological subtype of the tumour, after clinically justified combining of the rare histological variants into a common category, showed a lack of statistically significant association ($\chi^2 = 9.84$; df = 6; p = 0.132). The probable reason is the dominance of adenocarcinoma NOS and the small number of cases in the rare histological variants, which limits the stability of the multicategorical χ^2 analysis.

In the comparative investigation and statistical assessment of the dependencies between the peritumoral "Crohn-like" stromal inflammatory reaction and the other tumour characteristics shown in the table, the following results were obtained: Table 26:

Analysis	χ^2	df	p-value	Statistical significance
Immune reaction vs Tumour Grade (G1–2 vs G3)	$\chi^2 = 1.742$	df = 2	p = 0.418	Lack of significance
Immune reaction vs Stage (pT)	$\chi^2 = 5.411$	df = 15	p = 0.988	Lack of significance
Immune reaction vs N status (N0 vs N+)	$\chi^2 = 0.923$	df = 2	p = 0.630	Lack of significance
Immune reaction vs LVI	$\chi^2 = 2.713$	df = 3	p = 0.438	Lack of significance
Immune reaction vs PNI	$\chi^2 = 3.715$	df = 3	p = 0.294	Lack of significance

Table 26. Dependencies between the peritumoral "Crohn-like" stromal reaction and G, pT, N, LVI, PNI.

In the present cohort, the peritumoral stromal immune reaction does not show a statistically significant relationship with any of the classical prognostic factors: Grade, pT, N status, LVI, or PNI. This suggests that, in this sample, the immune response probably represents an independent morphological phenomenon that is not directly related to traditional parameters of tumour aggressiveness.

BY SUBTASK NO. 3B: PERITUMORAL BUDDING DETERMINED IN RESECTION MATERIALS FROM COLORECTAL CARCINOMA

In our study, the aim was to determine the presence of peritumoral budding (PTB) at the invasive tumour front. The results obtained for PTB and the correlations with different tumour characteristics, such as tumour differentiation (G), stage (pT), lymphovascular invasion (LVI), nodal status (N), and perineural invasion (PNI), are reflected in Table 27:

Tumour grade (G)	Bd1 (Number of patients)	Bd2 (Number of patients)	Bd3 (Number of patients)
G2	35	3	1
G3 + G3 NEC	29	1	0
G4	21	8	2
Tumour stage (pT)	Bd1 (Number of patients)	Bd2 (Number of patients)	Bd3 (Number of patients)
T1	1	0	1
T2	7	5	6
T3	24	22	18
T4	4	1	3
T4A	1	2	3
T4B	2	0	0

Table 27. Peritumoral budding is distributed by grade according to the degrees of tumour differentiation (G) and staging (T).

Examples of peritumoral budding are presented in Photos 13 and 14:

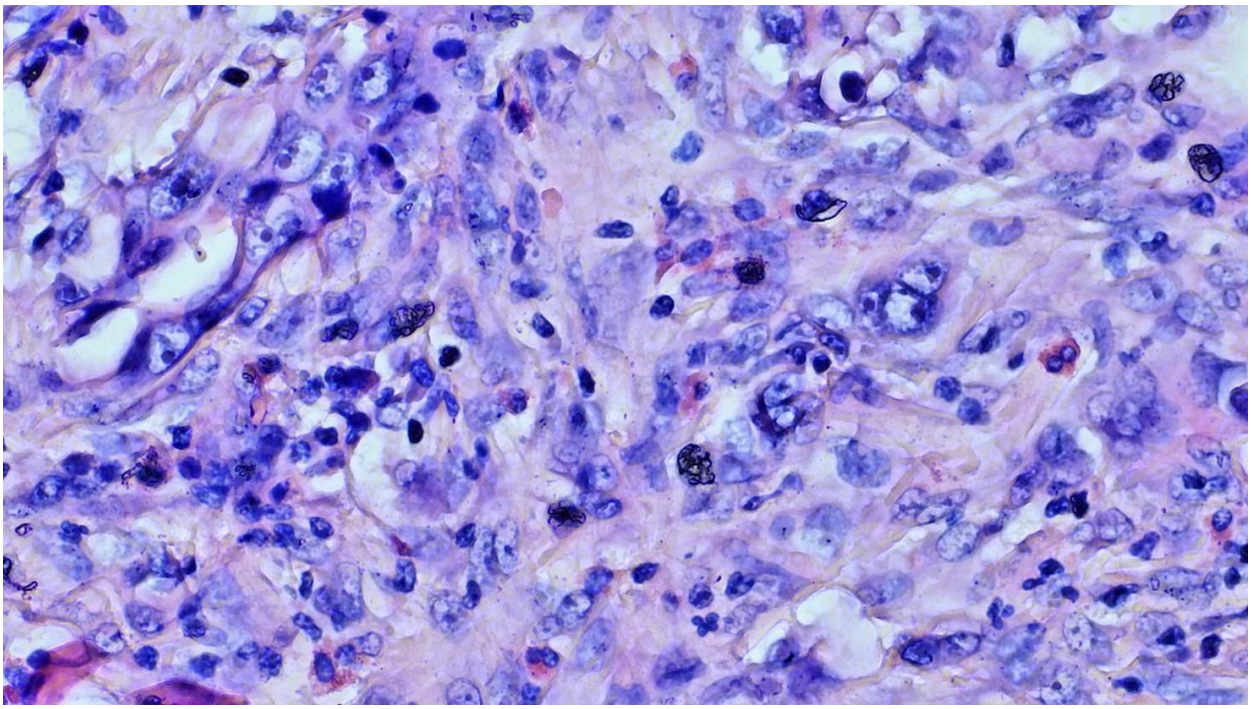

Photo 13. Peritumoral budding - single tumour cells at the invasive front of the tumour. H&E stain, magnification 40×.

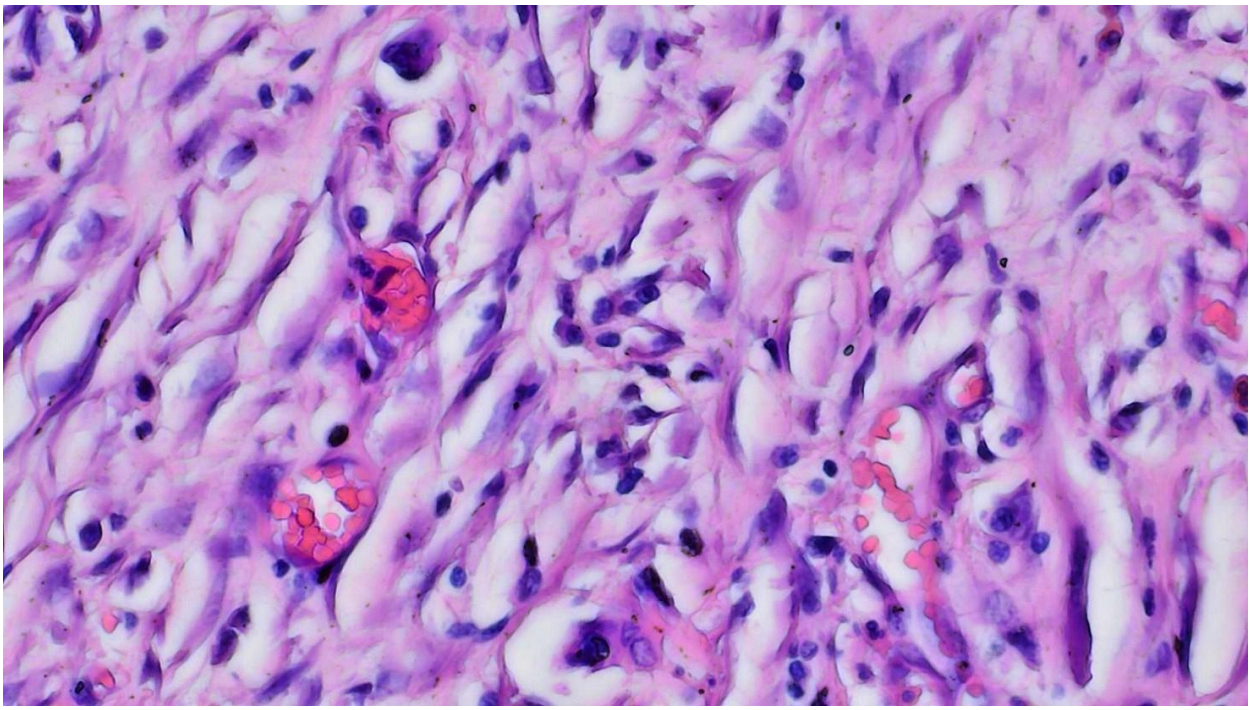

Photo 14. Peritumoral budding. H&E stain, magnification 40×.

The results of statistical processing with respect to these indicators are as follows:

Tumor differentiation (G) and PTB: $\chi^2 = 14.39$; df = 6; p = 0.0255

Tumor stage (pT) and PTB: $\chi^2 = 7.36$; df = 10; p = 0.6908

LVI and PTB: $\chi^2 = 8.59$; df = 2; p = 0.0137

PNI and PTB: $\chi^2 = 5.59$; df = 2; p = 0.0611

N status and PTB: $\chi^2 = 19.93$; df = 14; p = 0.1325

The statistical analysis confirms relationships between tumour differentiation (G) and peritumoral budding, and between lymphovascular invasion and PTB. No statistically significant relationship was observed between PTB and tumour stage (pT), lymph node status (N), or perineural invasion (PNI). The relationship between higher grades of peritumoral budding and lymphovascular invasion supports the concept that tumour budding reflects an aggressive invasive phenotype in colorectal carcinoma. The lack of association with pT stage and lymph node status (N) suggests that peritumoral budding results from specific biological tumour behaviour that is not fully explained by conventional TNM parameters. The borderline p-value for PNI suggests a possible biological interaction that may require larger cohorts for validation. Overall, peritumoral budding remains a reliable morphological marker associated mainly with tumour differentiation and lymphovascular dissemination, but, unlike other studies, we are unable to confirm a direct relationship with nodal status.

BY TASK NO. 4: IMPORTANT PREDICTIVE AND PROGNOSTIC BIOMARKERS IN COLORECTAL CARCINOMAS - MMR

Among predictive and prognostic biomarkers for colorectal carcinomas, we investigated MMR status in CRC and the most frequent mutations (as defined by the TruSight Tumor 15 panel).

• Assessment of MMR status

The following Table 28 summarises the results obtained in the statistical processing of the data concerning the MMR status of colorectal carcinomas and the different epidemiological and tumour characteristics:

Analysis	χ^2	df	p (Pearson)	p (Monte Carlo)	p (used)
MMR and Sex	0.160	1	0.689	not applicable	0.689
MMR and Localisation	20.279	2	0.000039	not applicable	0.000039

MMR and Histological subtype	27.872	9	0.001	0.002	0.002
MMR and Grade	10.841	2	0.004	not applicable	0.004
MMR and pT stage	2.702	5	0.746	0.803	0.803
MMR and Nodal status	0.990	1	0.320	not applicable	0.320
MMR and LVI	0.237	1	0.627	0.556	0.556
MMR and PNI	0.191	1	0.662	not applicable	0.662
MMR and Peritumoral Immune Reaction	4.177	2	0.124	0.151	0.151
MMR and Peritumoral budding	0.648	2	0.723	not applicable	0.723

Table 28. Statistical dependencies between MMR status and various epidemiological and tumour characteristics in CRC.

As is evident from the table, statistical processing shows several statistically significant results. In the following presentation, we will consider and comment on them separately:

MMR and sex - Table 29:

Distribution: n (%)	dMMR	pMMR
Female	9 (20.9%)	34 (79.1%)
Male	9 (15.8%)	48 (84.2%)

Table 29. MMR and sex. Result: $\chi^2 = 0.160$; df = 1; p = 0.6895

The association between MMR status and the considered variable is not statistically significant (χ^2 = 0.160; df = 1; p = 0.689); Pearson χ^2 test with Yates continuity correction. Cramer's V = 0.040, which indicates a very weak association.

MMR and localisation - Table 30:

Distribution: n (%)	dMMR	pMMR
Right colon	16 (38.1%)	26 (61.9%)
Left colon	2 (6.9%)	27 (93.1%)
Rectum	0 (0.0%)	29 (100.0%)

Table 30. MMR and Localisation. Result: χ^2 = 20.279; df = 2; p = 0.000039

The association between MMR status and primary tumour localisation is highly statistically significant (χ^2 = 20.279; df = 2; p < 0.0001). Standard Pearson χ^2 test, Cramer's V = 0.450, which indicates a moderate to strong association.

Analysis of tumour distribution by MMR status shows a clear dependence between molecular profile and anatomical localisation.

• dMMR (deficient mismatch repair)

The greater part of dMMR tumours – 88.9% (n=16) are localised in the right colon. Only 11.1% (n=2) are located in the left colon, and rectal tumours are 0% (n=0). This emphasises the characteristic right-sided localisation of dMMR carcinomas and confirms the well-known biological association between microsatellite instability and the right colon.

• pMMR (proficient mismatch repair)

The tumours in the pMMR group are relatively evenly distributed across the three localisations: right colon (31.7%), left colon (32.9%), and rectum (35.4%). Rectal carcinomas are almost exclusively represented in the pMMR group, which underscores their distinct biological profile.

The statistical analysis confirms the presence of a strong and statistically significant association between MMR status and tumour localisation: $\chi 2$ = 20.279; df = 2; p < 0.0001

These results show that tumour distribution is not random and that MMR status is closely related to the embryologically and biologically different segments of the colon.

MMR and histological subtype - Table 31:

Distribution: n (%)	dMMR	pMMR
Adenocarcinoma NOS with mucinous component	0 (0.0%)	1 (100.0%)
Adenocarcinoma NOS + with intra- and extracellular mucin production	0 (0.0%)	1 (100.0%)
Adenocarcinoma NOS	8 (10.4%)	69 (89.6%)
Adenocarcinoma NOS with mucinous and poorly cohesive component	0 (0.0%)	1 (100.0%)
Medullary carcinoma	2 (100.0%)	0 (0.0%)
MiNEN	1 (100.0%)	0 (0.0%)
Mucinous carcinoma	6 (54.5%)	5 (45.5%)
Serrated adenocarcinoma	1 (25.0%)	3 (75.0%)
Synchronous tumours – Cecum- mucinous, Rectum- Adenocarcinoma NOS	0 (0.0%)	1 (100.0%)
Undifferentiated carcinoma	0 (0.0%)	1 (100.0%)

Table 31. MMR and Histological subtype. Result: $\chi^2 = 27.872$; df = 9; p (Pearson) = 0.0010; p (Monte Carlo) = 0.0016

The association between MMR status and histological subtypes of CRC is statistically significant. (Result: $\chi^2 = 27.872$; df = 9; p (Pearson) = 0.0010; p (Monte Carlo) = 0.0016). Monte Carlo permutation was used due to small expected values (E < 5). Cramer's V = 0.528, which indicates a strong association.

MMR and grade (G) - Table 32:

Grade	dMMR n (%)	pMMR n (%)
G2	11 (61.1%)	74 (90.2%)
G3	5 (27.8%)	7 (8.5%)

G4	2 (11.1%)	1 (1.2%)

Table 32. MMR and tumour grade (G). Result: $\chi^2 = 10.841$; df = 2; p = 0.004424.

The association between MMR status and tumour grade (G) is statistically significant ($\chi^2 = 10.841$; df = 2; p = 0.004424), with Cramer's V = 0.329, indicating a moderate association.

MMR and pT stage - Table 33:

Distribution: n (%)	dMMR	pMMR
pT1	0 (0.0%)	2 (100.0%)
pT2	2 (11.1%)	16 (88.9%)
pT3	12 (18.8%)	52 (81.2%)
pT4	2 (25.0%)	6 (75.0%)
pT4A	1 (16.7%)	5 (83.3%)
pT4B	1 (50.0%)	1 (50.0%)

Table 33. MMR and pT. Result: $\chi^2 = 2.702$; df = 5; p (Pearson) = 0.7457; p (Monte Carlo) = 0.8026.

The association between MMR status and pT is not statistically significant ($\chi^2 = 2.702$; df = 5; p (Pearson) = 0.7457; p (Monte Carlo) = 0.8026), Cramer's V = 0.164, which indicates a weak association. Monte Carlo permutation was used due to small expected values (E<5).

MMR and nodal status - Table 34:

Distribution: n (%)	dMMR	pMMR
N+	5 (12.2%)	36 (87.8%)
N0	13 (22.0%)	46 (78.0%)

Table 34. MMR and nodal status.

The association between MMR and N status is not statistically significant ($\chi^2 = 0.990$; df = 1; p = 0.3198). Pearson χ^2 test with Yates continuity correction), Cramer's V = 0.099, which indicates a weak association.

MMR and LVI - Table 35:

Distribution: n (%)	dMMR	pMMR
LVI+	6 (23.1%)	20 (76.9%)
LVI-	12 (16.2%)	62 (83.8%)

Table 35. MMR and LVI.

The association between MMR status and LVI is not statistically significant ($\chi^2 = 0.237$; df = 1; p (Pearson) = 0.6265; p (Monte Carlo) = 0.5557), Cramer's V = 0.049, which indicates a very weak association. Monte Carlo permutation was used due to small expected values (E<5).

MMR and PNI are reflected in Table 36:

Distribution: n (%)	dMMR	pMMR
PNI+	5 (14.3%)	30 (85.7%)
PNI-	13 (20.0%)	52 (80.0%)

Table 36. MMR and PNI.

The association between MMR status and PNI is not statistically significant ($\chi^2 = 0.191$; df = 1; p = 0.6624), with Cramer's V = 0.044, indicating a very weak association—Standard Pearson χ^2 test.

MMR and peritumoral immune reaction - Table 37:

Distribution: n (%)	dMMR	pMMR
Absent	2 (9.5%)	19 (90.5%)
Marked	11 (27.5%)	29 (72.5%)
Moderate	5 (12.8%)	34 (87.2%)

Table 37. MMR and Peritumoral immune reaction. Result: $\chi^2 = 4.177$; df = 2; p (Pearson) = 0.1239; p (Monte Carlo) = 0.1512.

The association between MMR status and the Crohn-like peritumoral stromal inflammatory reaction is not statistically significant ($\chi^2 = 4.177$; df = 2; p (Pearson) = 0.1239; p (Monte Carlo) = 0.1512), Cramer's V = 0.204, which indicates a weak to moderate association. Monte Carlo permutation was used due to small expected values (E<5). Visually, these data are also presented in Figure 8:

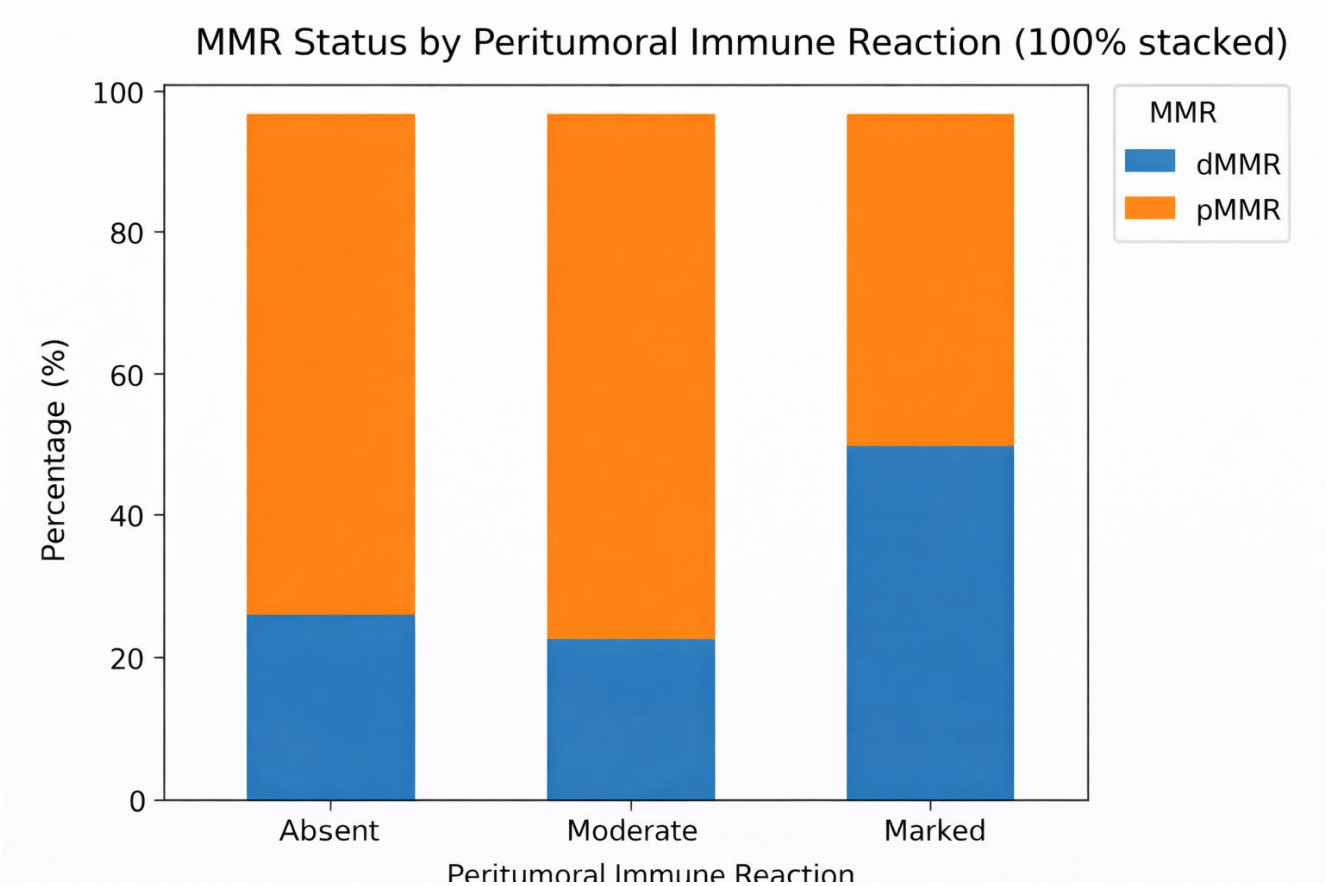

Figure 8. MMR and peritumoral immune reaction.

MMR and peritumoral budding (PTB) - Table 38:

Distribution: n (%)	dMMR	pMMR
Bd1 (0+1)	8 (20.5%)	31 (79.5%)
Bd2	4 (13.3%)	26 (86.7%)
Bd3	6 (19.4%)	25 (80.6%)

Table 38. MMR and Peritumoral budding. Result: $\chi^2 = 0.648$; df = 2; p (Pearson) = 0.7232.

The association between MMR status and PTB is not statistically significant ($\chi^2 = 0.648$; df = 2; p = 0.7232). Cramer's V = 0.080, which indicates a weak association. Standard Pearson χ^2 test.

In Table 39 in this section, we attempted to summarise patients with dMMR status and BRAF V600E gene mutations, alone or in combination with other mutations, in the context of Lynch Syndrome. In the study, we did not set as our aim, and we did not have the full possibilities for definitive clarification of cases suspected of hereditary-type colorectal carcinoma:

Patient	MMR	MLH1	PMS2	MSH6	MSH2	BRAF V600E mutation	Mutations and combinations	Lynch syndrome
10	dMMR	d	d	p	p	BRAF V600E	BRAF + TP53	Not suspected
16	dMMR	d	d	p	p	BRAF V600E	BRAF + TP53	Not suspected
19	dMMR	d	d	d	p	BRAF V600E	BRAF + TP53	Not suspected
20	dMMR	d	d	p	p	BRAF V600E	BRAF + TP53	Not suspected
33	dMMR	d	d	p	p	BRAF V600E	BRAF	Not suspected
52	dMMR	d	d	p	p	BRAF V600E	BRAF + PIK3CA	Not suspected
56	dMMR	d	d	p	p	BRAF V600E	BRAF	Not suspected
58	dMMR	d	d	p	p	BRAF V600E	BRAF	Not suspected
60	dMMR	d	d	p	p	No data	TP53	Suspected
65	dMMR	p	p	d	d	No data	No data	Suspected
67	dMMR	p	p	d	p	No data	PIK3CA + TP53 + KRAS	Suspected
75	dMMR	d	p	p	d	No data	No data	Suspected

84	dMMR	d	d	p	p	BRAF V600E	BRAF	Not suspected
88	dMMR	d	d	p	p	BRAF V600E	BRAF + AKT1	Not suspected
92	dMMR	d	d	p	p	No data	NRAS + TP53	Suspected
94	dMMR	d	d	p	p	BRAF V600E	BRAF + PIK3CA	Not suspected
95	dMMR	d	d	p	p	BRAF V600E	BRAF + TP53	Not suspected
96	dMMR	d	d	p	p	No data	PIK3CA	Suspected

Table 39. dMMR status, corresponding MLH1, PMS2, MSH6, and MSH2 results; major gene mutations and combinations; relationship with Lynch syndrome. “d” – deficient expression; “p” – retained (proficient) expression.

The table shows that in most cases, MLH1 and PMS2 are downregulated, which correlates with the presence of the BRAF V600E mutation and the absence of clinical suspicion for Lynch syndrome. This pattern is typical of sporadic dMMR and is related to MLH1 promoter hypermethylation. In a small subset of cases, retained expression of MLH1/PMS2 and isolated loss of MSH2/MSH6, or other combinations without BRAF mutation, are observed, raising suspicion for Lynch syndrome. This profile is more consistent with a hereditary aetiology. The remaining available mutations (e.g., TP53, PIK3CA, KRAS, NRAS) reflect molecular heterogeneity within the dMMR group but do not change the main conclusion: dMMR tumours in the studied cohort are predominantly sporadic, and a limited number of cases have molecular criteria suggestive of Lynch syndrome. There is a clear distinction between sporadic and potentially hereditary dMMR profiles.

In the present cohort, MMR status was analysed in relation to the main clinicomorphological characteristics of colorectal carcinoma, including anatomical localisation, tumour grade (G), pT stage, nodal status (N), lymphovascular invasion (LVI), perineural invasion (PNI), and peritumoral budding. The most substantial and statistically significant relationship of MMR status is with primary tumour

localisation. dMMR tumours show a clearly expressed dominance in the right colon, whereas pMMR tumours are significantly more evenly distributed between the right colon, left colon, and rectum. This finding fully aligns with established molecular-pathogenetic concepts of differences between right-sided and left-sided colorectal carcinomas, as well as with the described relationship between microsatellite instability and proximal localisation. About the remaining prognostic indicators, no statistically significant associations with MMR status were established.

Examples of different histological subtypes of colorectal carcinomas with different MMR status are presented in the following microscopic Photos 15 – 24:

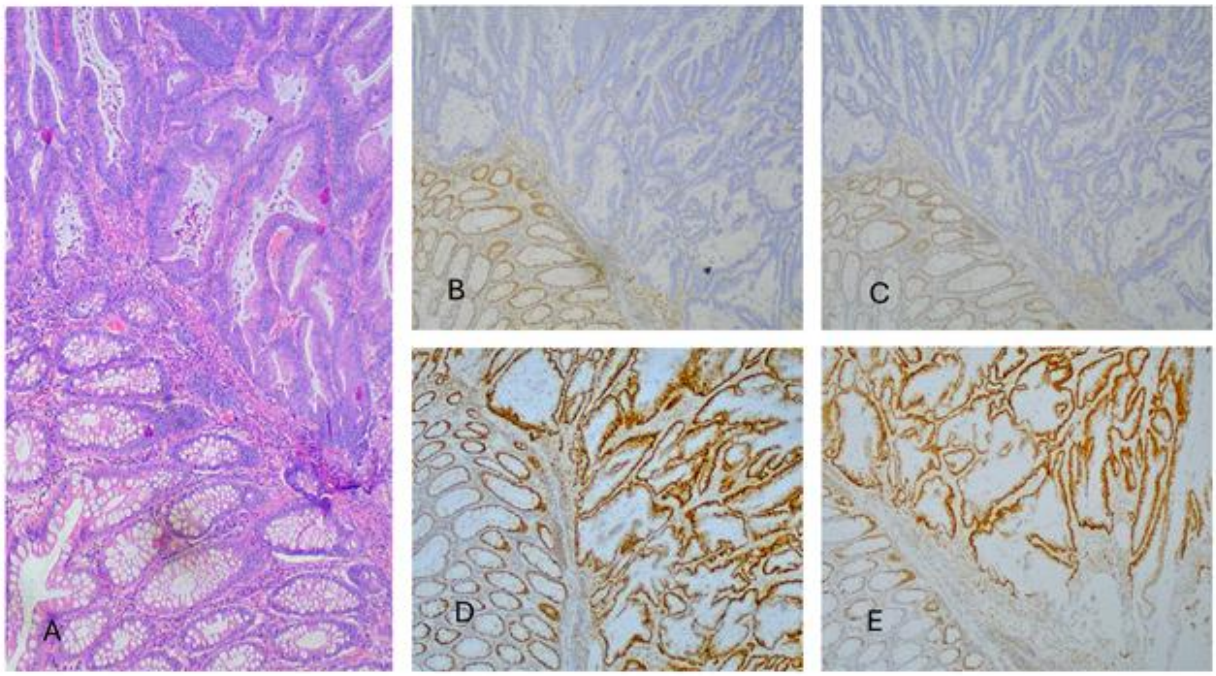

Photo 15. Adenocarcinoma G2- NOS. A- Histological slide - H&E stain; B-dMLH1; C-dPMS2; D-pMSH2; E- pMSH6. magnification 10×.

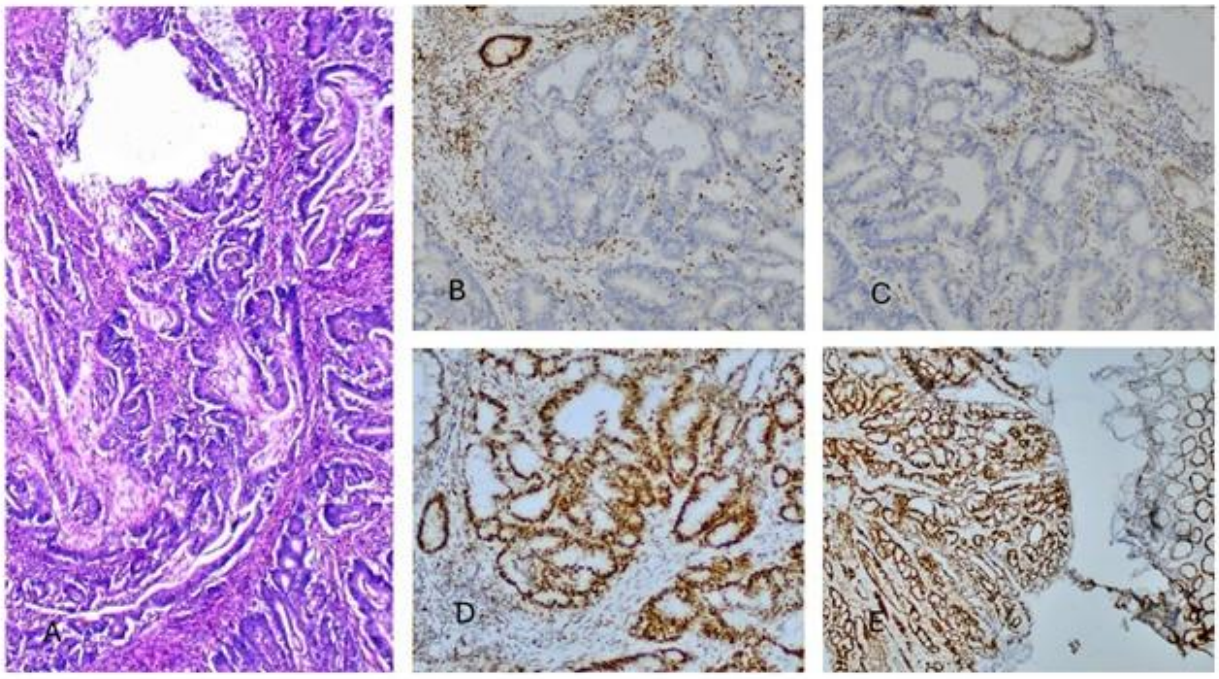

Photo 16. Adenocarcinoma G2- NOS. A- Histological slide - H&E stain; B-dMLH1; C-dPMS2; D-pMSH2; E- pMSH6. magnification 10×.

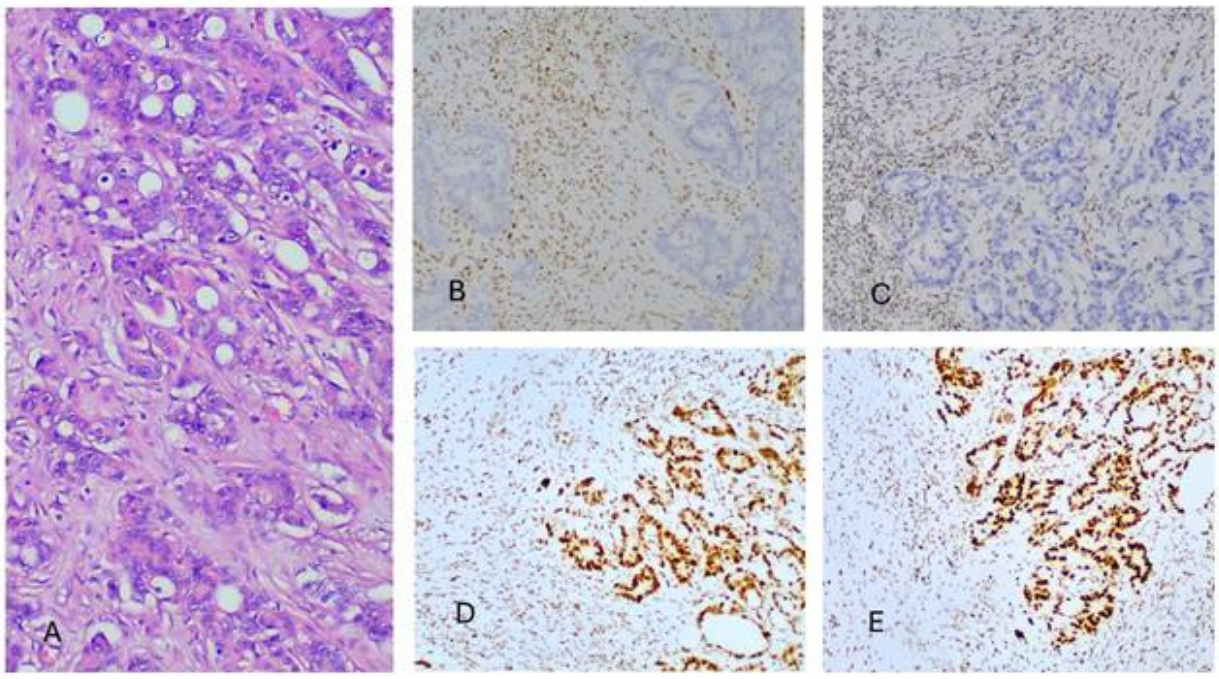

Photo 17. Adenocarcinoma G3- NOS. A- Histological slide - H&E stain; B-dMLH1; C-dPMS2; D-pMSH2; E- pMSH6. magnification 10×.

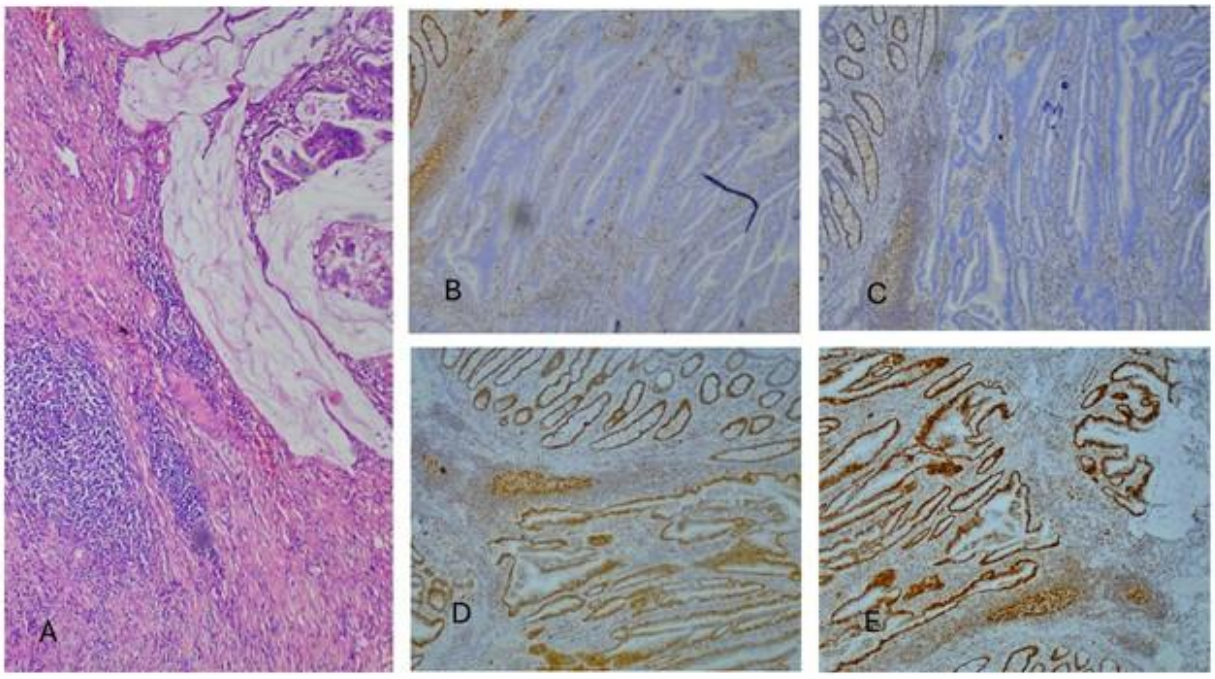

Photo 18. Adenocarcinoma G2- NOS with mucinous component and marked Crohn-like inflammatory reaction. A- Histological slide - H&E stain; B-dMLH1; C-dPMS2; D- pMSH2; E- pMSH6. magnification 10×.

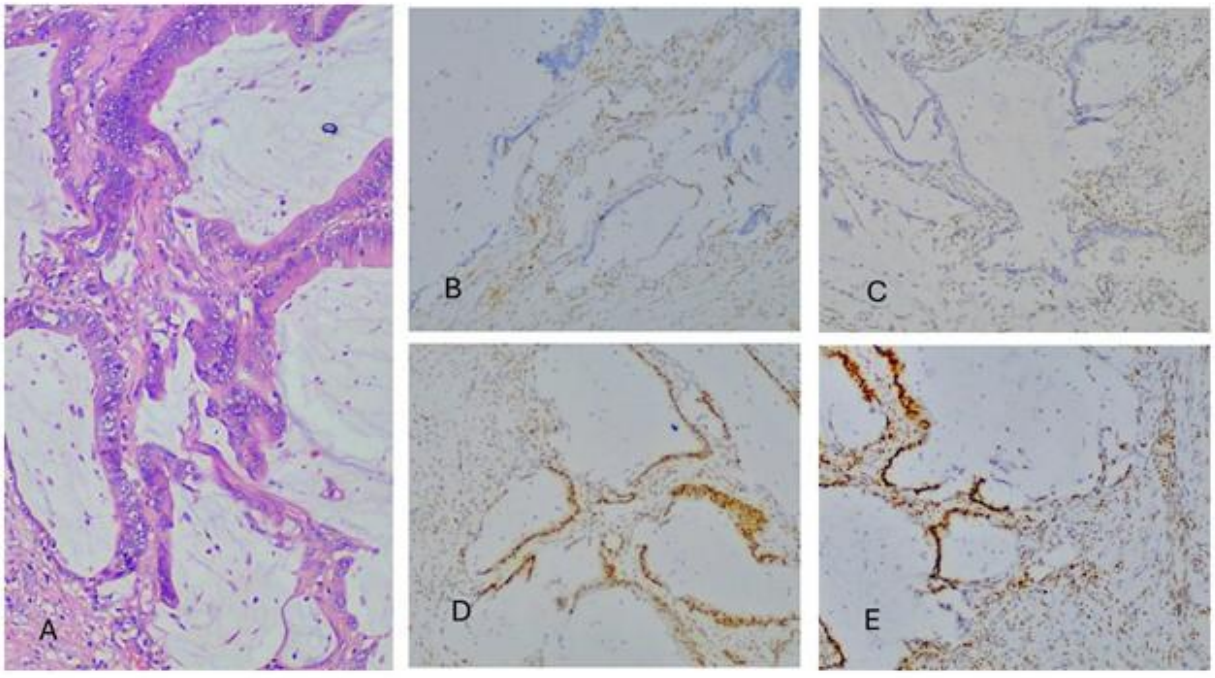

Photo 19. Mucinous carcinoma G2- extracellular mucin production. A- Histological slide - H&E stain; B-dMLH1; C-dPMS2; D- pMSH2; E- pMSH6. magnification 10×.

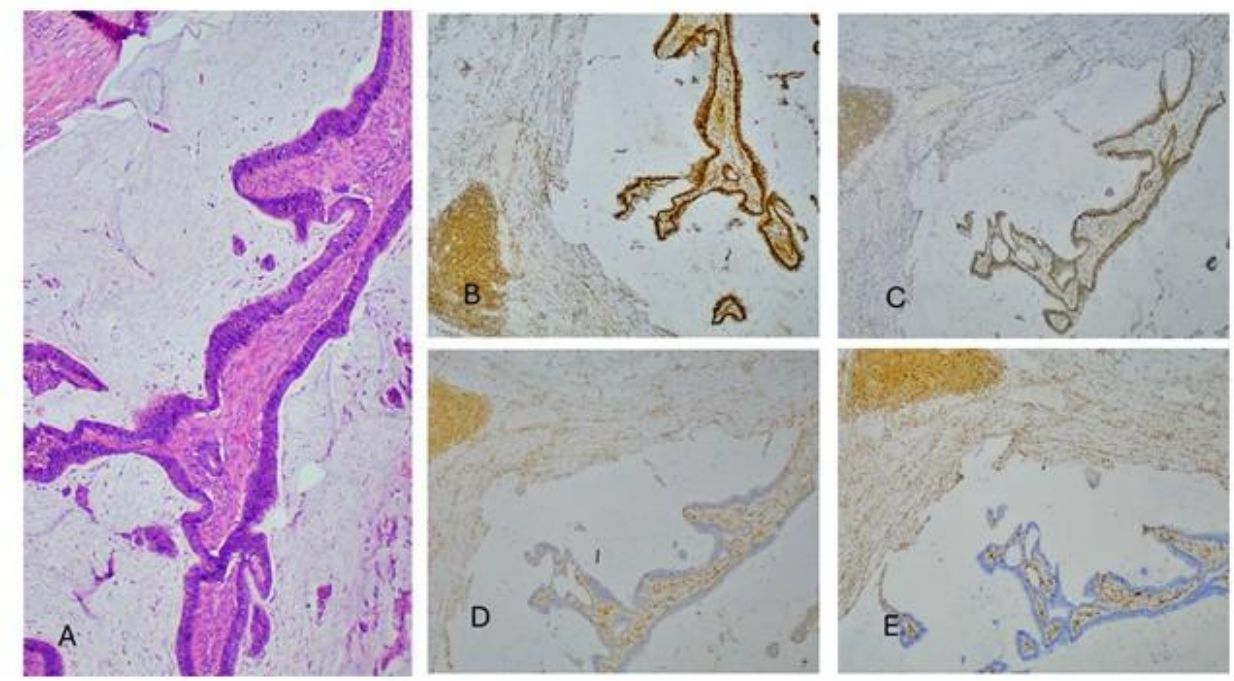

Photo 20. Mucinous carcinoma G2- extracellular mucin production. A- Histological slide - H&E stain; B-pMLH1; C-pPMS2; D- dMSH2; E- dMSH6. magnification 10×.

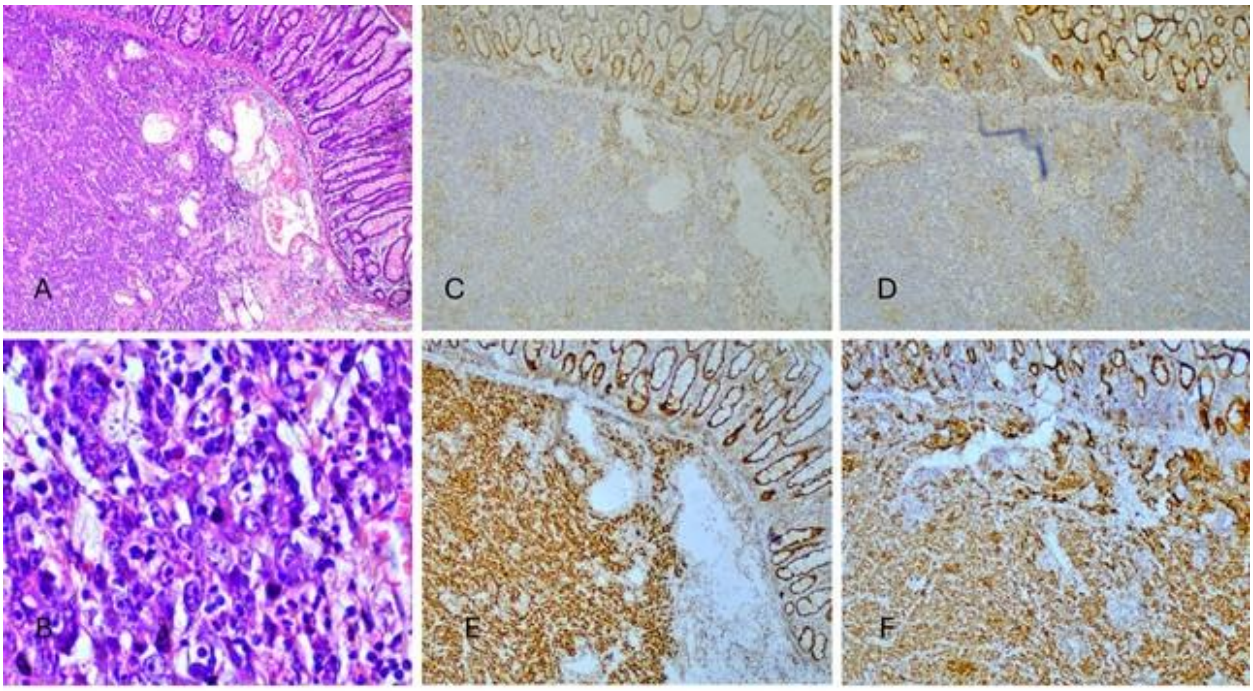

Photo 21. Medullary carcinoma. A and B- Histological slide - H&E stain, magnification 10×, 40×; C-dMLH1; D-dPMS2; E- pMSH2; F- pMSH6. magnification 10×.

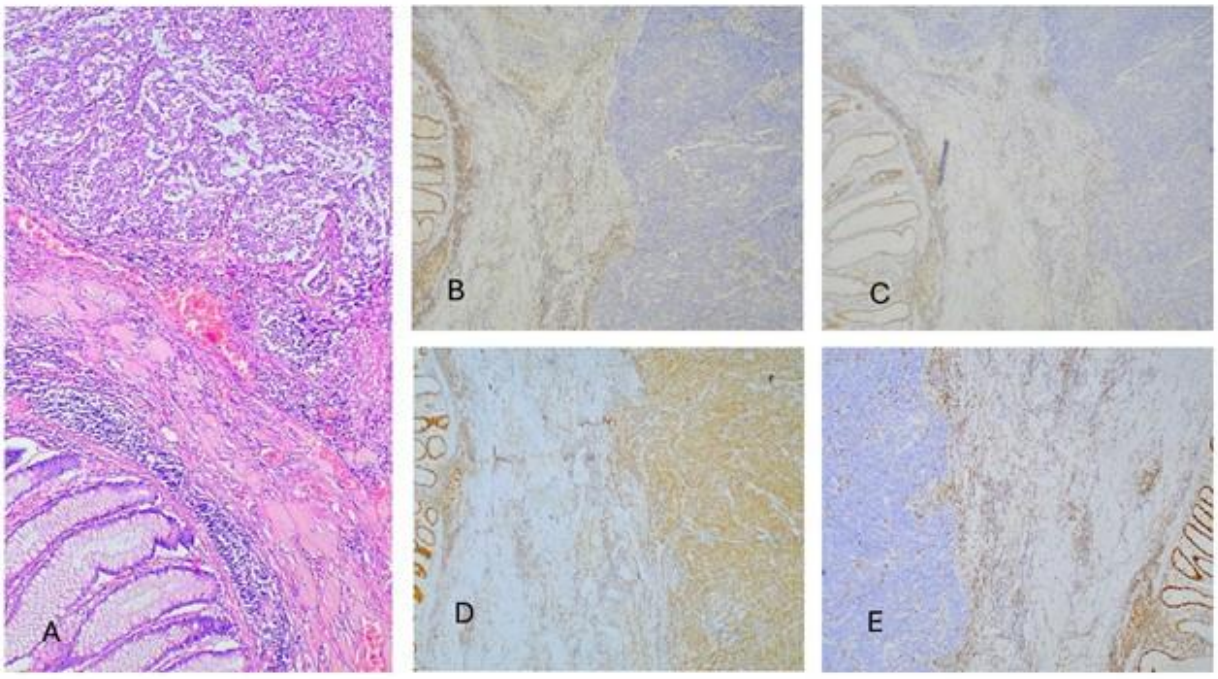

Photo 22. MiNEN. A- Histological slide - H&E stain; B-dMLH1; C-dPMS2; D- pMSH2; E- dMSH6. magnification 10×.

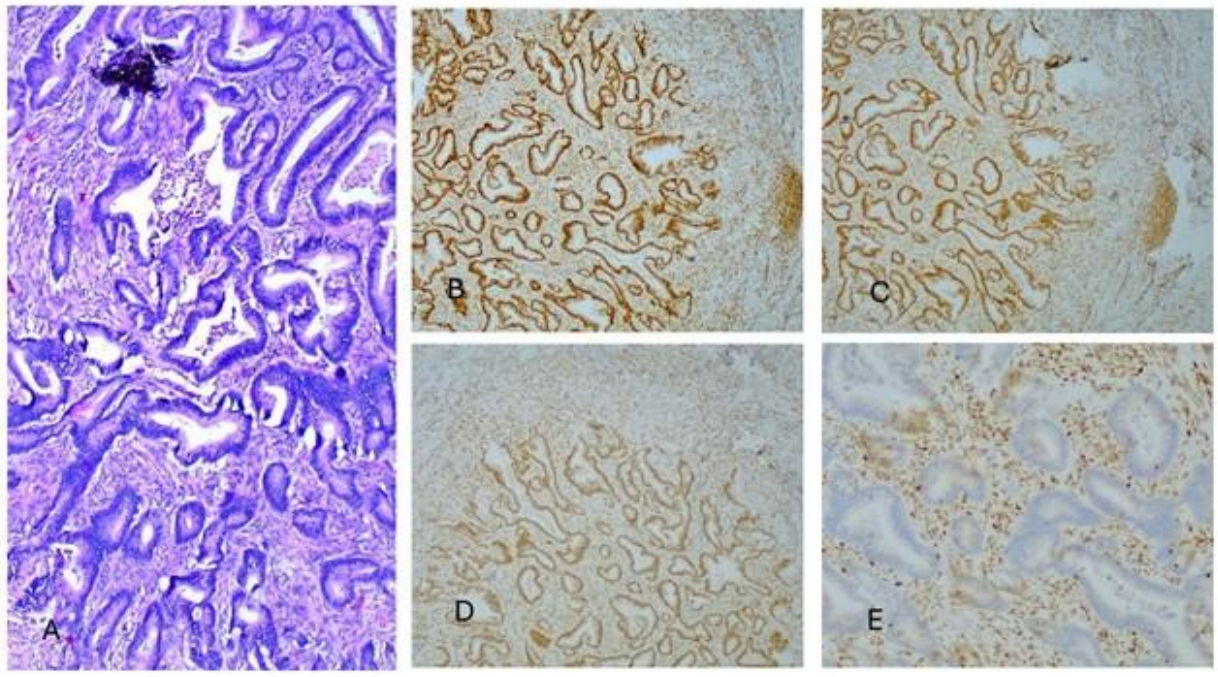

Photo 23. Adenocarcinoma G2- NOS. A- Histological slide - H&E stain; B-pMLH1; C-pPMS2; D-pMSH2; E- dMSH6- isolated deficiency. magnification 10×.

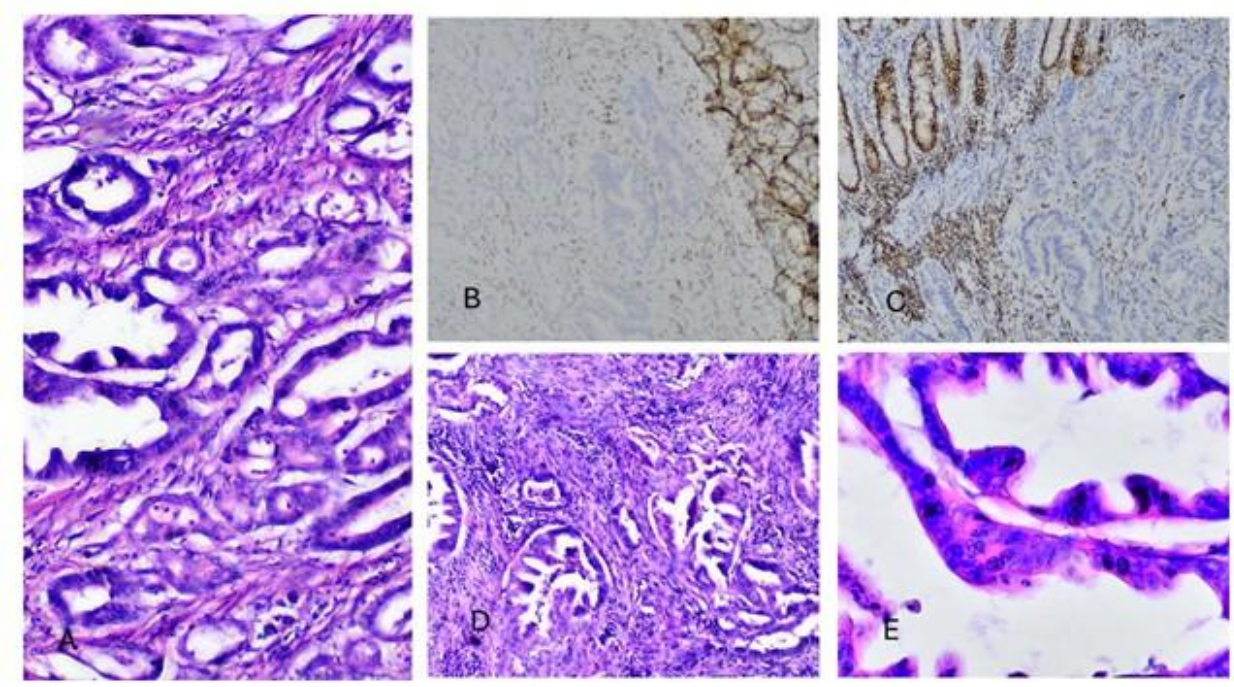

Photo 24. Serrated adenocarcinoma G2. A, D, E- Histological slide - H&E stain, magnification 10×, 40×; B-dMLH1; C-dPMS2. magnification 10×.

BY TASK NO. 5: MUTATIONS OF SOME GENES IN CRC, INVESTIGATED BY NGS

In our study, mutations in colorectal carcinomas were investigated using Next-Generation Sequencing (NGS). We used the Illumina panel - "TruSight Tumor 15". The genes included in this panel are: AKT1, BRAF, EGFR, ERBB2(HER2), FOXL2, GNA11, GNAQ, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA, RET, TP53.

In the sample of 100 patients, NGS data are available for 88, of whom 38 are women, and 50 are men. For the remaining 12 patients, specific data from the TruSight Tumor 15 panel are missing due to technical reasons, such as insufficient tumour material in the paraffin block.

The results obtained from the 88 patients show the following:

• Mutational profile and sex - Table 40:

Parameter	Women n= 38	%	Men n= 50	%
TP53	29	76.3%	33	66.0%
KRAS	15	39.5%	22	44.0%
BRAF	8	21.1%	9	18.0%
PIK3CA	4	10.5%	7	14.0%

NRAS	2	5.3%	2	4.0%

Table 40. Distribution of mutations determined through NGS, by sex.

• In the present sample, TP53 is the most frequently encountered mutation in both sexes. In women, TP53 is established in 76.3% of cases, followed by KRAS (39.5%), BRAF (21.1%), PIK3CA (10.5%), and NRAS (5.3%). In men, TP53 is the most frequent mutation (66.0%), followed by KRAS (44.0%), BRAF (18.0%), PIK3CA (14.0%), and NRAS (4.0%). A similar mutational profile is observed between the sexes, with no substantial differences in the hierarchy of individual gene frequencies.

• Mutational profile and primary tumour localisation - right colon, left colon, rectum.

Table 41 reflects the distribution of mutations by primary tumour localisation: right colon, left colon, and rectum. NGS data are available in n=88 patients (right colon n=36, left colon n=26, rectum n=26).

Localisation (NGS n)	TP53	KRAS	BRAF	PIK3CA	NRAS	AKT1
Right colon (n=36)	20	14	14	7	1	2
Left colon (n=26)	21	9	3	3	1	0
Rectum (n=26)	21	14	0	1	2	0

Table 41. Absolute number of patients with mutation by gene and localisation in the right colon, left colon, and rectum. For 12 of the patients, information on mutations is lacking. Note: It should be noted that in most tumours, mutations in 2 or more genes are detected.

Table 42 shows the results of the statistical tests for the distribution of mutations according to primary tumour localisation:

Gene	χ^2	df	p (Pearson)	minimum expected	p (Monte Carlo)
TP53	6.497	2	0.038837	7.682	
KRAS	2.222	2	0.329208	10.932	
BRAF	16.082	2	0.000322	5.023	

PIK3CA	3.389	2	0.183645	3.250	0.636039
NRAS	0.882	2	0.643402	1.182	0.973167
AKT1	2.956	2	0.228085	0.591	0.657356

Table 42. Results of the statistical tests for the distribution of mutations according to primary tumour localisation in the right colon, left colon, and rectum.

Visually, the distribution by frequency is reflected in Figure 9:

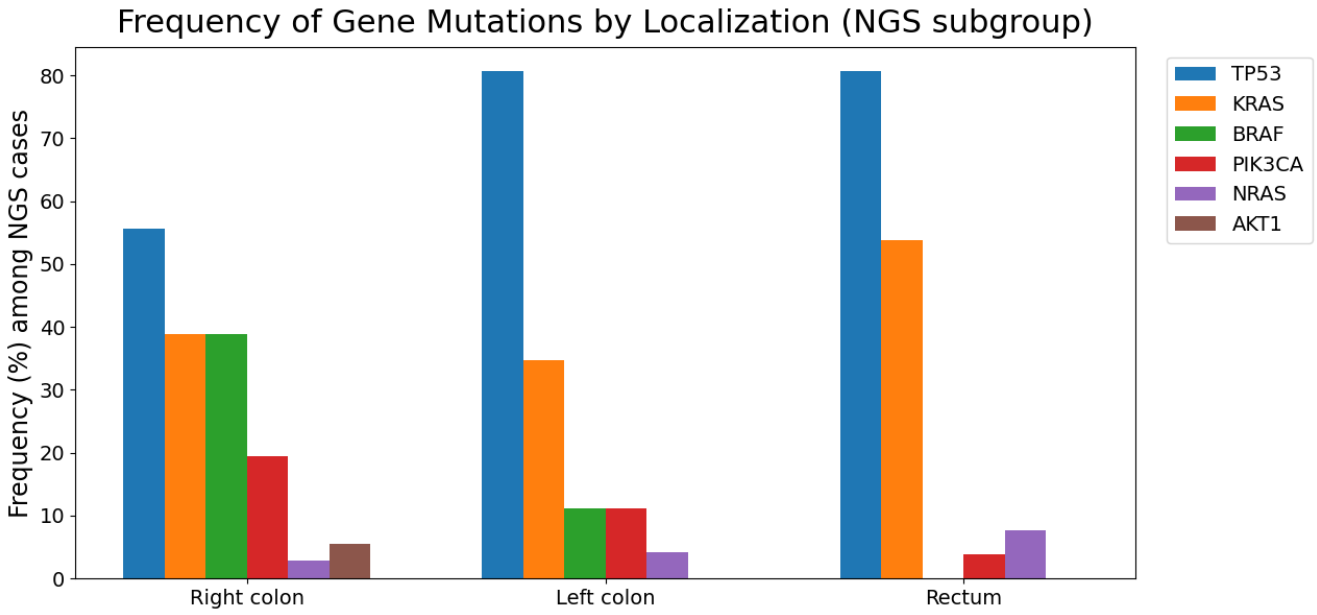

Figure 9. Frequency (%) of mutations by localisation.

The most pronounced association with localisation is observed for BRAF: the frequency is highest in right colon tumours (38.9%), significantly lower in left colon tumours (11.5%), and absent in rectal tumours (0%). This is confirmed by the Pearson χ^2 test ($p = 0.000322$). For the remaining genes, no convincing statistically significant dependence on localisation is established in this NGS subgroup.

Right colon: Right colon tumours have the highest total number of mutations (48) - TP53, KRAS, BRAF, PIK3CA, and AKT1. BRAF mutations are more frequent in this localisation compared with the others. KRAS mutations are less frequent and are similar to those in rectal carcinomas. The high frequency of BRAF mutations in the right colon is consistent with the well-known molecular characteristics of colorectal carcinoma. It points toward a sporadic pathway of carcinogenesis rather than Lynch syndrome.

Left colon: In the left colon, tumours have fewer total mutations (37) than in the right colon. The dominant mutations are in TP53 and KRAS, while PIK3CA is also encountered, but with lower frequency.

Rectum: Rectal carcinomas have a total of 38 mutations, with TP53 having the highest frequency and no BRAF mutations being established, which constitutes a substantial distinction. The profile of rectal carcinomas differs significantly from that of right-sided tumours, which more often demonstrate BRAF mutations, and the presence of KRAS and NRAS mutations in rectal tumours outlines a mutational spectrum more characteristic of sporadic forms of colorectal carcinoma than of hereditary syndromes such as Lynch.

• Mutations and histological subtype of colorectal carcinomas

In the analyses performed, it was established that:

BRAF and histological subtype: Pearson χ^2 test: $\chi^2 = 18.019$; df = 8; p = 0.0211. In this analysis, note that many mutations across histological subtypes are low-frequency. Therefore, we also performed a Monte Carlo permutation test, which showed no statistically significant results (p = 0.3366).

Additionally, we performed grouping of the histological subtypes into three groups: adenocarcinoma NOS, mucinous variants (all mucinous variants), and rare variants (e.g., medullary carcinoma and poorly cohesive), which led to the results in Table 43:

Histological group	BRAF+	Total (n)	BRAF (%)
Adenocarcinoma NOS	8	70	11.4%
Mucinous variants	5	9	55.6%
Rare variants	0	9	0.0%

Table 43. BRAF mutations according to combined histological subgroups - adenocarcinoma NOS, mucinous histological variants, and rare histological variants

The obtained results showed the following: Pearson χ^2 test: $\chi^2 = 11.820$; df = 2; p = 0.0027; Monte Carlo approximation (in rare cells): p = 0.0031.

The highest frequency of BRAF mutation is observed in the mucinous variants (55.6%), followed by Adenocarcinoma NOS (11.4%). In the group of rare variants, BRAF mutations are not established. The obtained results show that BRAF mutation is statistically significantly related to mucinous morphology in the present NGS subsample.

The remaining mutations investigated - TP53, KRAS, PIK3CA, NRAS, AKT1 - did not show statistically significant dependencies with the histological subtypes.

This indicates that the distribution of BRAF mutations across the combined histological groups is statistically significant. These data support the relationship between BRAF mutation and mucinous morphology in colorectal carcinoma.

When investigating dependencies of other tumour characteristics with respect to mutational status in the NGS subsample (n=88), only BRAF mutations showed statistically significant dependence ($\chi^2 = 9.640$; df = 2; p = 0.0081) with tumour grade (G). When comparing the mutational status of the different genes in the patient sample, no statistically significant associations are established between most of the investigated mutations and tumour stage (pT). Only NRAS shows a borderline result in this category, which, however, does not reach statistical significance ($\chi^2 = 10.626$; df = 5; p = 0.0593). With respect to nodal metastases (N status), only TP53 shows borderline, but statistically nonsignificant results ($\chi^2 = 5.988$; df = 2; p = 0.0501). Similar borderline results for BRAF mutations are also obtained when compared with LVI ($\chi^2 = 3.603$; df = 1; p = 0.0577).

The result for PIK3CA and PNI is also borderline and close to statistically significant ($\chi^2 = 3.664$; df = 1; p = 0.0556).

• Peritumoral immune “Crohn-like” reaction in relation to mutations.

The analysis of the relationship between stromal peritumoral immune reaction and genetic mutations showed a statistically significant association only for NRAS ($\chi^2 = 7.21$; df = 2; p = 0.027). This suggests a possible relationship between NRAS mutational status and tumour immune microenvironmental characteristics. For the remaining genes (TP53, KRAS, BRAF, PIK3CA, AKT1), no statistically significant dependence was established ($p > 0.05$).

• Peritumoral budding versus gene mutations:

Bd1 (Bd0 + Bd1): Mutations in TP53 and KRAS are the most frequent, and BRAF and PIK3CA are of moderate frequency. The frequency is similar to that of Bd2.

Bd3 has the highest frequency of TP53, followed by KRAS, BRAF, and some PIK3CA mutations.

• Gene mutations determined through NGS and MMR status of colorectal carcinomas.

In the statistical processing, the following results were obtained:

TP53: $\chi^2 = 3.931$; df = 1; p = 0.0474; Cramer's V = 0.198 - statistically significant

KRAS: $\chi^2 = 10.283$; df = 1; p = 0.00134; Cramer's V = 0.321 - statistically significant

BRAF: $\chi^2 = 38.900$; df = 1; $p < 0.0001$; Cramer's V = 0.624 - statistically significant

PIK3CA: $\chi^2 = 2.794$; df = 1; p = 0.0946; Cramer's V = 0.167 - without statistical dependence

NRAS: $\chi^2 = 0.131$; df = 1; p = 0.7174; Cramer's V = 0.036 - without statistical dependence

AKT1: $\chi^2 = 1.393$; df = 1; p = 0.2379; Cramer's V = 0.118 - without statistical dependence

These results show a strong association between these specific mutations and the MMR status of colorectal tumours, suggesting that KRAS and BRAF mutations are associated with specific MMR statuses. The latter may be of importance in the choice of treatment methods.

DISCUSSION – FIRST PATIENT COHORT

Tumour budding is a standardised morphological manifestation of tumour aggressiveness, reflecting the complex interactions among parenchymal tumour cells, tumour stroma, and the host immune response. The concept linking tumour growth activity, cellular dissociation, and patient prognosis dates to the observations of Broders et al. as early as 1920. Later, Imai introduced the term “tumour sprouting” in the context of gastric carcinoma, emphasising the biological significance of single tumour cells and small cell groups at the invasive front. These early observations laid the foundation for the modern understanding of tumour budding. In contemporary studies, tumour budding is regarded as a histological correlate of epithelial-mesenchymal transition (EMT), characterised by reduced cell adhesion, increased migratory ability, and invasive potential of tumour cells. This phenomenon explains the relationship between tumour budding and an unfavourable clinical outcome, as established in several studies.

Tumour budding has been established as an unfavourable prognostic factor not only in CRC, but also in several other malignant diseases, including carcinomas of the oesophagus, stomach, biliary tract, oral cavity, lung, urinary bladder, uterine cervix, and breast.

Despite its established prognostic significance, the evaluation of tumour budding in preoperative endoscopic biopsies remains highly limited. Numerous authors emphasise problems related to the small volume of the material, fragmentation, necrosis, inflammation, lack of an invasive front, and the extreme degrees of differentiation. Our results fully correspond to these data. In the present sample, tumour budding was assessable in only 12% of cases, whereas in 88% of the biopsies this was not possible. The most frequent limiting factors are fragmentation of the material (43%), scant biopsy material (12%), tumour necrosis (10%), well-differentiated tumours (8%), poorly differentiated tumours (7%), lack of an invasive front (6%), and pronounced inflammation (1%). This clearly shows that the inability to report peritumoral budding in endoscopic biopsies is, above all, a methodological rather than a biological problem. It should be emphasised that these factors do not represent independent biological variables, but structural and technical limitations of the biopsy material. For this reason, their statistical modelling as predictors for the inability to report PTB would be methodologically incorrect and of limited interpretative value.

Reported tumour budding is observed almost exclusively in biopsies of optimal quality, emphasising the importance of adequate biopsy sampling. The presence of an invasive tumour front is a mandatory

condition for reliable assessment; therefore, statistical modelling of the reasons for inability to report is methodologically incorrect, since these factors represent deterministic limitations.

No significant relationship is observed between peritumoral budding and sex or localisation of the primary tumour. Interestingly, patients with reported tumour budding are statistically older. Nevertheless, due to the limited sample size, this result should be interpreted cautiously and requires confirmation in larger series. The observed age difference may reflect a more advanced stage of tumour development, a better morphological representation of the invasive front in older patients, or changes in the tumour microenvironment. Still, such a hypothesis remains speculative and cannot be confirmed in the present sample.

Additional studies show that the reproducibility of tumour budding assessment is significantly lower in biopsy specimens than in resection specimens. Rogers et al. demonstrated limited interobserver agreement in assessing tumour budding in biopsies, whereas reproducibility improved significantly in resection material. Both Rogers et al. and Giger et al. report that, respectively, in 20% and 17% of endoscopic preoperative biopsies from colorectal carcinoma, tumour budding can be reported. (In both publications, intratumoral budding is assessed). Our results regarding the possibility of assessing peritumoral budding in CRC in endoscopic biopsies are significantly lower, only 12%. The logical explanation for this is that, due to its small volume, endoscopic biopsy material does not fully represent the invasive tumour front, which limits the ability to report PTB and makes it difficult to differentiate ITB from PTB. This methodological discrepancy between biopsy and resection material further limits direct comparability of the results and emphasises that tumour budding is a parameter initially developed and validated for assessment on resection specimens.

Zlobec et al. propose a new system for assessing tumour budding (TB) that includes an additional category designated “zero” budding. In their study, it was established that this new “0” budding score is observed in more than 10% of colorectal carcinomas. In the present study, cases with BD=0 were also observed; nevertheless, according to the current recommendations of the International Tumour Budding Consensus Conference (ITBCC), all cases with “zero” budding were classified as BD1. This new grading system for tumour budding is not widely accepted and implemented in clinical practice. It implies a more comprehensive approach to assessing tumour budding, encompassing a broader spectrum of tumour dissociation patterns, including cases without budding activity. As with any new concept, additional studies, validation, and achievement of expert consensus are necessary before such modifications can be routinely integrated into clinical practice.

In conclusion, the present study confirms that technical and morphological factors highly limit the evaluation of tumour budding in endoscopic colorectal biopsies. Although tumour budding remains an extremely important prognostic marker in resection material, its routine evaluation in endoscopic biopsies should be performed with increased caution and clear awareness of the methodological and technical limitations.

DISCUSSION – SECOND PATIENT COHORT

In our study, a total of 100 patients were investigated, and the demographic data show a slight predominance of men, 57 patients (57%) compared with 43 women (43%). The ratio of men to women studied is approximately 1.33:1, with a mean patient age of around 70 years. These results are consistent with global demographic data on colorectal carcinoma, which show that its incidence increases with age. Our data coincide with the data published by Siegel et al., who indicate that the probability of occurrence of invasive carcinoma (in the colon and rectum section) is respectively: 2.7% for men and 2.2% for women in the age of 65–84 years and 1.8% for men and 1.7% for women over 85 years of age. In one of their studies, White et al. established age-sex differences in the frequency of colorectal carcinomas in a cohort of English patients and note that the incidence frequency above 45 years of age is significantly higher in men than in women, with this difference being most pronounced in the 70–74 years age group, with a male-to-female ratio of 1.7:1. Joo et al. report no sex-age differences regarding age at initial diagnosis of colorectal carcinoma in men versus women; at the same time, however, diagnosis of CRC in asymptomatic patients is almost 49% in men versus 42% in women.

Many authors investigate differences related to sex, age, and localisation of the primary carcinomas of the colon and rectum. These studies are based on data published by Bufill et al., who analyse the characteristics of colorectal carcinoma according to the embryonic origin of the anatomical segments of the colon and rectum. Subsequently, multiple authors report similar observations regarding the localisation characteristics of colorectal carcinomas. In our study, we also categorized tumor localizations into three groups: right colon, left colon, and rectum, and the results obtained by us showed a lack of statistically significant dependencies between sex and tumor localization ($\chi^2 = 2.95$; df = 7; p = 0.89) in each separate anatomical localization, as well as a lack of such dependencies when combining right-sided, left-sided, and rectal tumor localizations ($\chi^2 = 0.19$; df = 2; p = 0.91). Both approaches confirm the lack of dependence between sex and the anatomical distribution of colorectal carcinoma in

our studied patient cohort. From the above, it follows that we do not observe statistically significant associations among sex, age, and the anatomical localisation of colorectal carcinomas.

About the histological subtypes of colorectal carcinoma, our study established the following distribution: Adenocarcinoma NOS - n= 77 (77%); Mucinous adenocarcinoma – n= 11 (11%); Serrated adenocarcinoma – n= 4 (4%); Medullary carcinoma – n= 2 (2%); Others – n= 6 (6%) (The category "Others" includes: undifferentiated carcinoma, MiNEN, synchronous tumors, adenocarcinoma with mucinous component, adenocarcinoma with mucinous and poorly cohesive component, and adenocarcinoma with intra- and extracellular mucin production).

These data are consistent with published literature, which reports that conventional adenocarcinoma is the dominant histological type in colorectal carcinoma, accounting for 70–80% in many cohorts. In contrast, the remaining CRC types are relatively rarer.

Mucinous adenocarcinoma in our series accounts for about 11% of cases and falls within the 10–15% range described in the literature. This relative stability among different populations probably reflects the well-defined diagnostic criteria (>50% extracellular mucin).

Serrated adenocarcinoma in our study accounts for 4%, which is lower than the 7–12% reported in many publications. Several factors can explain this discrepancy:

- variability in histological criteria and differences in subjective evaluation.
- different distribution of proximal versus distal tumours;
- particular features of the patient's cohort.

Medullary carcinoma was observed in approximately 2% of patients in our cohort, which is at the upper end of published rates. This is a rare tumour variant, and the observed frequency in our study may be due to a specific molecular profile of CRC in the patients selected by us (MSI/dMMR tumours) or to an unintended selection effect.

The rare variants - MiNEN, undifferentiated carcinoma, mixed histological subtypes - mucinous/poorly cohesive and synchronous forms of carcinomas - were observed overall in about 6% of the patients, a frequency described in different publications.

The analysis of grouped histological subtypes versus anatomical localisation (right colon, left colon, and rectum) did not establish a statistically significant relationship between the two variables ($\chi^2 = 19.937$; df = 18; p = 0.336). The additional analysis by sex also did not demonstrate statistically significant dependencies (men: $\chi^2 = 12.826$; p = 0.685; women: $\chi^2 = 8.573$; p = 0.380). The obtained results show

that the distribution of histological subtypes is similar across anatomical localisations and is not substantially influenced by patient sex.

Tumour grade (Grade) and pT stage

The degree of differentiation (G) is a morphological marker of biological tumour behaviour, an instrument for prognostication and clinical stratification, and an integral part of algorithms for personalised oncotherapeutic decisions.

In the present cohort, no statistically significant relationship is established between anatomical localization (right colon/left colon/rectum) and tumor grade ($\chi^2 = 9.53$; df = 6; p = 0.146), including upon stratification by sex (men: $\chi^2 = 4.38$; df = 6; p = 0.626; women: $\chi^2 = 7.88$; df = 4; p = 0.096).

In a direct comparison between Grade and pT, a statistically significant association is observed ($\chi^2 = 35.27$; df = 15; p = 0.0023), consistent with the expected relationship between lower differentiation, more invasive tumour growth, and greater depth of infiltration. The possible explanation for this dependence is that changes in cell adhesion, gland architecture, the degree and type of interaction between the tumour and stroma, and certain invasion patterns accompany a lower degree of differentiation. The literature data show that aggressive invasive phenotypes (including tumour budding/EMT) are associated with more unfavourable characteristics and prognosis.

Lymph node status (N)

Nodal status is a major component of the TNM system and a surrogate marker of regional tumour spread. In the patient cohort studied by us, no statistically significant differences in N status are established according to localization ($\chi^2 = 11.811$; df = 14; p = 0.621), including in the analysis by sex (men: $\chi^2 = 12.821$; df = 12; p = 0.382; women: $\chi^2 = 8.376$; df = 14; p = 0.869). The lack of association between localisation and N status may reflect a combination of factors: heterogeneity in the N categories, varying volumes, possibly insufficient lymphatic dissection, and a limited predictive role in the rare tumour subcategories. In our case, this means that localisation alone is insufficient to predict regional lymphatic dissemination.

Lymphovascular invasion (LVI)

Lymphovascular invasion is a key prognostic morphological parameter, reflecting both local invasiveness and metastatic tumour potential. The multiple diagnostic challenges and variability in pathological assessment have led to the development of more objective criteria for LVI in CRC, some of which are based on Delphi consensus. In the present cohort, LVI is positive in 26/100 patients (26%). By localisation groups: right colon - 14; left colon - 7; rectum - 5. A tendency toward a higher frequency

of LVI in right-sided tumours is observed, but without statistical significance ($\chi^2 = 2.382$; df = 2; p = 0.304). At the same time, LVI shows significant associations with key markers of aggressiveness and dissemination potential: pT stage ($\chi^2 = 16.13$; df = 5; p = 0.0065; Cramer's V = 0.40), Grade ($\chi^2 = 17.33$; df = 2; p = 0.00017; Cramer's V = 0.42), and N status ($\chi^2 = 27.75$; df = 7; p = 0.00024; Cramer's V = 0.53), with the latter, most strongly expressed, association between LVI and N status supporting the concept of LVI as a direct morphological substrate of lymphogenous dissemination and practically justifying the systematic and careful search for and reporting of LVI in all resection specimens.

Perineural invasion (PNI)

Perineural invasion is a marker of invasive phenotype and, in several tumours, is associated with a more unfavourable course. In our investigation, PNI does not show dependence on the localisation of the primary tumour ($\chi^2 = 0.381$; df = 2; p = 0.8267), and there is also no statistically significant association with the histological subtypes of colorectal carcinomas ($\chi^2 = 13.815$; df = 9; p = 0.1291), with interpretation limited by rare categories.

Unlike the above, PNI shows significant relationships with tumour pT stage ($\chi^2 = 11.790$; df = 5; p = 0.03778), LVI ($\chi^2 = 20.187$; df = 1; $p < 0.001$), and N status ($\chi^2 = 18.527$; df = 7; p = 0.009806). These dependencies support PNI as a marker of locally advanced disease and increased risk of metastasis. The pronounced association of PNI with LVI suggests a concentration of invasive characteristics in parts of the tumours, probably due to common mechanisms of migration and stromal remodelling. From a practical point of view, the combination of PNI+ and LVI+ is an especially alarming morphological feature in tumours.

Peritumoral immune response (Crohn-like lymphoid reaction)

Crohn-like lymphoid reaction is regarded as a morphological indicator of active immune response and the presence of tertiary immune lymphoid structures. Literature data show influence on staging in right-sided colorectal carcinomas, whereas more contemporary publications emphasise the immunological and potentially therapeutic significance of the existing immune response. Relationships with TIL and survival have also been observed, and the criteria for evaluating this type of immune response are under validation. In the present cohort, the Crohn-like peritumoral stromal immune response was evaluated semiquantitatively (absent/moderate/marked). There is a statistically significant association between the intensity of the immune response and anatomical localisation ($\chi^2 = 14.05$; df = 4; p = 0.0071). The right colon shows the highest proportion of marked reaction (absent 5; moderate 13; marked 24), and the rectum – predominantly moderate reaction and a low proportion of marked response (absent 9; moderate

16; marked 4). At the same time, no statistically significant relationships are demonstrated between the Crohn-like reaction and the classical prognostic parameters: Grade ($\chi^2 = 1.742$; df = 2; p = 0.418), pT ($\chi^2 = 5.411$; df = 15; p = 0.988), N status ($\chi^2 = 0.923$; df = 2; p = 0.630), LVI ($\chi^2 = 2.713$; df = 3; p = 0.438), and PNI ($\chi^2 = 3.715$; df = 3; p = 0.294).

The results in our study support the thesis that the peritumoral immune response is more closely related to the biology of the anatomical segment than to the morphological markers of aggressiveness. This is compatible with the concept of differences in the molecular and immune microenvironments of the proximal and distal colon and with the idea that these lymphoid structures reflect the local immune antitumor response.

In conclusion, we may note that our patient cohort demonstrates clear internal consistency of the morphological prognostic factors: tumor differentiation (G) is associated with pT; LVI is strongly related to pT, G, and N; PNI is associated with pT, LVI, and N; and the Crohn-like peritumoral immune response depends on localization, but not on G/pT/N/LVI/PNI. These results emphasise the role of LVI and PNI as objective markers of invasiveness and metastatic risk, and support the need for standardised, detailed reporting of these parameters.

Peritumoral budding (PTB)

Peritumoral budding is a validated morphological marker in colorectal carcinomas that indicates invasiveness and metastatic potential. In the present study, TB was considered an integral component of the invasive phenotype and was evaluated alongside pT, lymph node status, LVI, PNI, tumour grade, and peritumoral immune response. Due to its high clinical value and proven independent prognostic power, TB is the subject of international standardisation and is recommended for routine reporting.

Our results showed significant associations between LVI/PNI and pT/N/grade. PTB should be considered an additional “invasive indicator” that could shed light on differences in the clinical behaviour of parts of tumours with the same pT or grade, as PTB may capture the subpopulation of cells with the highest migratory potential. This corresponds to the clinical observations that TB is associated with more aggressive pathological characteristics and worse prognosis. For example, in pT3/pT4 tumors, the presence of high PTB strengthens the risk profile of the tumors, especially if combined with LVI+ and/or PNI+; in stage II (N0), high PTB may be grounds for more aggressive follow-up and discussion of adjuvant therapy; in biopsy materials, ITB may point to more in-depth preoperative staging and adequate clinical decision-making. ITBCC recommends that PTB be assessed on H&E. Still, in certain situations with limited visibility of budding cells – dense inflammatory infiltrate, significant stromal

reaction, or artefacts, pancytokeratin immunohistochemistry may increase the sensitivity for detection of budding cells and facilitate counting. Some authors demonstrate the possibility of more complex and accurate assessment of PTB through CK staining, but also emphasise the risk of "overcalling" due to differences between H&E-based and IHC-based methods. Our analysis showed that high tumour budding does not represent an isolated histological phenomenon but is part of invasive tumour characteristics, including depth of tumour infiltration, lymphovascular invasion (LVI), perineural invasion (PNI), and regional lymphatic dissemination. No dependence on anatomical localisation (right colon, left colon, rectum) is established.

The association between tumour budding and pT stage is statistically significant. Tumours with pT3–pT4 show a higher frequency of marked budding. Also significant is the association between high tumour budding and the presence of LVI. Budding cells are dissociated tumour units with increased migratory ability, whereas LVI proves entry into vascular structures. The combination TB+ and LVI+ outlines a subgroup of patients with pronounced tumour-invasive potential. We also established a statistically significant relationship between high tumour budding and positive nodal status. Tumour budding may be regarded as a morphological predictor of regional lymphatic dissemination and an indicator of micrometastatic activity. Significant is also the association between high budding and perineural invasion. The combination TB+ and PNI+ suggests an active invasive front using different anatomical structures for progression.

There is a tendency toward higher budding at higher grades, but budding is not equivalent to the degree of differentiation. It adds independent prognostic information beyond classical grading.

In the present cohort, we did not establish a statistically significant dependence between tumour budding and the intensity of the peritumoral immune response. This suggests that the quantitatively assessed stromal immune infiltrate does not directly modulate the invasive phenotype within the investigated cases.

Within an integrated tumour model, the most unfavourable morphological profile includes high budding, pT3/pT4, LVI+, PNI+, and N+. These parameters form a consistent complex of aggressiveness, in which tumour budding occupies a central role as an indicator of the active invasive front.

In our patient cohort, tumour budding confirms its role as an independent marker of aggressiveness and complements pT, N, and LVI in risk stratification. The observed statistical dependencies justify their routine reporting.

In conclusion, tumour budding is a standardizable and clinically significant parameter which reflects the active invasive front of CRC. According to ITBCC and subsequent consensus reports, TB should be routinely reported, as it provides independent prognostic information beyond pT, grade, and nodal status.

Mismatch repair (MMR)

In the present cohort, the frequency of dMMR (18%) falls within the 10–15% range, consistent with international population data. Our analysis demonstrates a strong statistically significant association between dMMR status and right-sided localisation ($p < 0.001$), which is in complete agreement with the published series showing that 70–80% of MSI-H carcinomas are localised proximally to the fl. lienalis. This pattern reflects the different embryological, microbiome, and molecular environment of the proximal colon. In the literature, it is accepted that right-sided tumours more often follow the serrated pathway with MLH1 hypermethylation and a CIMP-high profile.

Regarding MMR and histological subtype, in our cohort, dMMR tumours are more frequently mucinous or medullary, consistent with the WHO classification (2019), which classifies medullary carcinoma as closely associated with the MSI phenotype. In their studies, Yamauchi et al. report a statistically significant relationship between MSI and mucinous morphology. Therefore, the observed dependence in our series is also supported by international data.

Our study does not establish a statistically significant, stable dependence between MMR status and tumour grade. In the literature, MSI-H tumours are often described as poorly differentiated yet paradoxically have a better prognosis in the early stages. This emphasises the biological uniqueness of dMMR carcinomas – morphologically aggressive, but immunologically "visible" to the organism.

We did not establish a statistically significant relationship between MMR status and pT category. Similar results have also been reported in the TCGA analysis, in which MSI does not show a direct dependence on depth of invasion. MMR status reflects the molecular pathway of carcinogenesis, rather than the degree of local infiltration.

MMR and nodal status - in the present study, no categorical dependence is observed between MMR status and N stage. The literature data are contradictory – some series report a lower frequency of nodal dissemination in MSI-H. In contrast, others do not establish an independent effect after multivariate analysis, with a possible explanation being increased immune activity in dMMR tumours, which limits early lymphogenous dissemination.

The analysis of MMR status and the mutations of colorectal carcinomas established by NGS in patients in our cohort shows a strong association between dMMR and the BRAF V600E mutation ($p < 0.001$),

confirming the sporadic MSI pathway through MLH1 promoter hypermethylation. The combination of dMMR + BRAF practically excludes Lynch syndrome and has direct significance for genetic counselling. Regarding the remaining mutations we established, KRAS mutations are more frequent in pMMR tumours, which corresponds to the classical CIN pathway; likewise, TP53 mutations dominate in this group, supporting the concept of chromosomal instability as an alternative mechanism.

The tumour immune microenvironment did not exhibit significant peritumoral lymphoid infiltration in the current series of dMMR tumours, in contrast to the literature, which indicates a high tumour mutational burden and increased neo-antigen load in MSI-H tumours, thereby promoting an activated CD8+ T-cell response. This also explains the high therapeutic response to PD-1 inhibitors (ORR ~40–50%) in metastatic MSI-H colorectal carcinoma.

The data regarding the MMR status of colorectal carcinomas presented so far are closely related to the clinical significance of this tumour characteristic - MMR status has a dual prognostic and predictive value. In stage II, dMMR tumours are associated with better survival and limited benefit from 5-FU monotherapy. In the metastatic context, dMMR is one of the strongest predictive biomarkers for immunotherapy.

In conclusion, MMR status represents a central molecular axis in colorectal carcinoma. The present cohort demonstrates the expected associations with localisation, mutational profile, and immune reaction, thereby confirming the validity of molecular stratification. Integration of MMR testing into routine diagnostics is mandatory from both prognostic and therapeutic perspectives.

Mutational status of colorectal carcinomas

KRAS mutations show the expected association with the pMMR phenotype and the classical chromosomal instability (CIN) pathway, and lead to activation of the MAPK signalling pathway and stimulate cell proliferation. They do not show a stable dependence on localisation or stage, which supports their role as an early initiating event in the adenoma-carcinoma sequence. From a therapeutic point of view, KRAS is an established predictor of lack of response to anti-EGFR therapy.

NRAS mutations are rare and do not show statistically significant associations, consistent with the meta-analyses.

BRAF V600E shows a significant association with dMMR status and right-sided localisation, as well as with mucinous histological subtypes. This pattern fully corresponds to the serrated pathway concept. BRAF-positive tumours are associated with a more unfavourable prognosis in metastatic disease, while targeted therapy improves survival.

TP53 mutations dominate in the pMMR group, reflecting the CIN pathway of carcinogenesis and are the most frequent genetic alteration in CRC. Their effects are reflected in disturbed apoptosis and increased genomic instability.

A tendency toward association with lymph node metastases is observed, but our results are borderline, and interpretation requires caution.

PIK3CA and AKT1 represent components of the PI3K/AKT signalling axis. Their frequency in the present series is consistent with international data, with no significant dependence on tumour stage or primary tumour localisation. The literature data emphasise potential sensitivity to aspirin adjuvant therapy.

Comparison with the literature confirms that the mutational profile in the cohort investigated by us aligns with the two main biological models: MSI/dMMR/BRAF and CIN/KRAS/TP53, thereby validating the results and underscoring the significance of molecular stratification in clinical practice.

CONCLUSIONS

• The colorectal carcinomas in the patient cohort studied by us show clearly expressed histopathological and molecular heterogeneity, reflecting the contemporary concepts of stepwise and multistep carcinogenesis.

• We observed a substantial dependence between tumour differentiation and depth of invasion (pT), with the higher-grade correlating with a statistically significantly advanced tumour stage and a higher frequency of lymphogenous dissemination.

• Lymph node metastases are associated with unfavourable morphological tumour characteristics – high degree of tumour dedifferentiation, presence of lymphovascular invasion (LVI), perineural invasion (PNI), and marked tumour budding, which confirms its role as one of the key prognostic factors in colorectal carcinomas.

• Lymphovascular invasion (LVI) is associated with a higher tumour stage (pT), positive lymph nodal status, and increased tumour aggressiveness, and is established as an independent marker of unfavourable prognosis.

• Perineural invasion (PNI) is also associated with a more advanced tumour stage (pT) and a higher frequency of regional tumour dissemination, which supports the concept of its role as an additional indicator of biological aggressiveness.

• Deficiency in the mismatch repair system (dMMR) in the group of patients studied by us is within the limits of the published international data and demonstrates characteristic morphological and clinicopathological associations corresponding to the known immunohistochemical and molecular profile of these tumours.

• Mutational status (KRAS, BRAF, TP53, etc.) correlates with localisation, histological subtype, and stage of the disease, which emphasises the significance of molecular tumour characteristics in determining therapeutic behaviour.

• The peritumoral immune response (Crohn-like stromal inflammatory reaction) and the degree of peritumoral budding (PTB) show substantial interrelationships with prognostic indicators, which confirms the role of the tumour microenvironment in the progression of colorectal carcinoma.

CONTRIBUTIONS OF THE DISSERTATION

THEORETICAL CONTRIBUTIONS

The dissertation presents a comprehensive morphological and molecular analysis of a cohort of patients with colorectal carcinoma, which systematises the interactions between classical histopathological factors and contemporary molecular markers.

The relationships between tumour differentiation (G), pT stage, lymph nodal status, LVI, PNI, and peritumoral budding (PTB) are analysed in detail, and their complex prognostic interrelationships are shown.

The role of dMMR tumour status as a biologically distinct subtype with characteristic clinicopathological features within the studied population has been confirmed.

An in-depth analysis of the mutational profile of colorectal carcinomas in the studied patient cohort has been performed, and its interaction with morphological parameters has been shown, contributing to a more precise understanding of the molecular classification of the disease.

For our study, we developed and attempted to apply an integral morphological index – Aggressive Score, which combines several established prognostic markers into a unified evaluation system for biological tumour aggressiveness: tumour grade (G), lymphovascular invasion (LVI), and perineural invasion (PNI), which shows correlations with high pT stages and unfavourable tumour morphological characteristics. This integrative indicator would provide additional prognostic tools but requires investigation in larger patient cohorts.

METHODOLOGICAL CONTRIBUTIONS

An integrative approach combining morphological, immunohistochemical, and molecular-genetic analyses was applied, enabling a comprehensive evaluation of tumour biology.

Contemporary statistical methods were used to evaluate associations and dependencies between prognostic factors, ensuring objectivity and reliability of the results.

We attempted to introduce an additional quantitative system for evaluating combined prognostic risk by integrating morphological tumour parameters.

PRACTICAL CONTRIBUTIONS

The results have direct clinical value for refining prognostic assessment and therapeutic stratification of patients with colorectal carcinoma.

The results obtained by us support the routine inclusion of the evaluation of peritumoral budding (PTB), LVI, PNI, and MMR status in the standardised pathological protocol.

We proposed a scientifically and practically justified method for dissecting biopsy resection materials from CRC, justified by the need for a more adequate evaluation of prognostic factors in this group of tumours, and, more specifically, for the complete evaluation of LVI, IMVI, and EMVI.

Aggressive Score could be used as an additional prognostic tool for clinical stratification of patients with colorectal carcinoma.

APPENDICES

APPENDIX 1

Methodology for constructing and statistically evaluating the Aggressive Morphological Score in colorectal carcinoma

1. Conceptual rationale

The Aggressive Morphological Score is a research composite index designed to integrate the evaluation of morphological markers of tumour aggressiveness in colorectal carcinoma (CRC). The index combines three established unfavourable prognostic factors: perineural invasion (PNI), lymphovascular invasion (LVI), and low degree of differentiation (High Grade – G3/G4).

2. Definitions of the included parameters

PNI (Perineural invasion): 0 = absent; 1 = present.

LVI (Lymphovascular invasion): 0 = absent; 1 = present.

Grade: 0 = G1/G2; 1 = G3/G4.

3. Calculation formula

Aggressive Score = PNI (0/1) + LVI (0/1) + Grade (0/1).

Minimum value: 0.

Maximum value: 3.

4. Interpretation

Score 0 – absence of aggressive morphological characteristics.

Score 1 – presence of one unfavourable factor.

Score 2 – presence of two unfavourable factors.

Score 3 – presence of all three unfavourable factors: high-risk profile.

5. Distribution in the studied cohort (n = 100)

Score 0 – 53 cases; Score 1 – 25 cases; Score 2 – 15 cases; Score 3 – 7 cases.

6. Statistical analysis

The χ^2 test of independence was used to assess associations between the Aggressive Score and clinicopathological parameters. In the presence of cells with a small expected number of observations,

a Monte Carlo approximation was used. Values of $p < 0.05$ were accepted as statistically significant. To evaluate the strength of association, Cramer's V coefficient was calculated.

7. Aggressive Score and N status

A statistically significant association was established between higher Aggressive Score and lymph nodal positivity ($\chi^2 = 13.916$; df = 3; $p = 0.003$), Cramer's V = 0.373. The results show a progressive increase in the frequency of N+ with increasing Score.

8. Aggressive Score and pT stage

No statistically significant dependence was established between Aggressive Score and binarised pT stage (pT1–2 versus pT3–4) ($\chi^2 = 5.950$; df = 3; $p = 0.114$), Cramer's V = 0.244, which indicates a weak to moderate association. In this cohort, the index is more closely associated with lymph status than with local depth of invasion.

9. Logistic model and discriminative ability

A logistic regression analysis was performed using N positivity as the outcome. The comparison between a model that included the individual morphological factors and a model that included the Aggressive Score showed comparable discriminative ability (AUC: 0.749 versus 0.737). Due to the limited sample size, no internal validation of the results was performed using bootstrapping. This represents a potential limitation of the analysis, since such an approach would allow a more precise evaluation of the stability and reproducibility of the obtained statistical results.

10. Limitations

The Aggressive Score is a research index and is not part of the TNM system or international consensus. An external validation cohort is necessary to confirm clinical applicability (>200 patients).

11. Conclusion

The integration of PNI, LVI, and degree of differentiation into a unified morphological index provides a preliminary tool for risk stratification in patients with colorectal carcinoma. The methodology is easily reproducible and based on routine histopathological parameters.

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COURSES AND SPECIALIZATIONS IN THE SCIENTIFIC SPECIALTY

1. 13th International Pathology Course – "Victor Babes" National Institute of Pathology; Romanian division of the IAP; Digestive Pathology Association – 6–7.11.2020, Bucharest, Romania.
2. 14th Course of Digestive Pathology – "Carol Davila" University of Medicine and Pharmacy / ENGIP – European Society of Pathology – 05–06.11.2021, Bucharest, Romania.
3. ÖGPath-Pannonian Online Course: Pathology of the Large Bowel – 07–08.05.2021 – Austrian Society of Pathology.
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15th Course of Digestive Pathology – "Carol Davila" University / ENGIP – 04–05.11.2022, Bucharest, Romania.

5. Continuing medical education: Standards, rules, and challenges in clinical pathology – 1–4 June 2023, Golden Sands resort.
6. 16th Course on Digestive Pathology – "Victor Babes" National Institute of Pathology / Romanian division of IAP – 03–04.11.2023, Bucharest, Romania.
7. Pannonian Online Course: Pathology of the Stomach – 12–13 May 2023, Czech Medical Chamber.
8. London GI Pathology Update – 1–2.06.2023, London, Great Britain.
9. London GI Pathology Update – 10–12.04.2024, London, Great Britain.
10. 17th Digestive Pathology Course – «Victor Babes» National Institute of Pathology; Romanian division of the IAP, Bucharest, 1–2 November 2024.
11. 18th Digestive Pathology Course – Bucharest, 7–8 November 2025.

Weekend of Gastrointestinal Pathology 2025 – The University of Miami, 26–27 April 2025.

12. Biannual Meeting of the Pannonian Working Group of GI Pathology – Zagreb, 16–17 May 2025.
13. London GI Pathology Update Course – London, 29 April – 2 May 2025.
14. The 37th European Congress of Pathology – Vienna, 6–10 September 2025.
15. Biannual Meeting of the Pannonian Working Group of GI Pathology – Zagreb, 16–17 May 2025.
16. London GI Pathology Update Course – London, 29 April – 2 May 2025.

PARTICIPATION IN RESEARCH PROJECTS

1. No.18/2020 – Study of the IHC expression of MMR proteins, aspects of the antitumor immune response in colorectal carcinoma with left- and right-sided localisation.
2. No.20/2023 – Clinicopathological correlations in endometrial carcinoma with respect to the immunohistochemical expression of the proteins of the MMR system and the tumour suppressor genes p53 and PTEN.
3. "Centre of Competence in Personalised Medicine, 3D and Telemedicine, Robotic and Minimally Invasive Surgery", reg. No. BG05M2OP001-1.002-0010, financed by the "Operational Program Science and Education for Smart Growth".

Note: The English translation and language editing of this abstract were supported by OpenAI language models and Grammarly, with all final revisions and scientific responsibility assumed by the author.