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[Krasimir Todorov Petrov](#)*, Ivan Nedkov Ivanov, Savelina Lubenova Popovska, [Tatjana Marinova Betova](#), Zornica Bogomilova Kamburova

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Article

Colorectal Cancer: A Brief and Simplified Analysis of a Complex Disease

Krasimir Petrov ^{1,2,*}, Ivan Ivanov ^{1,2,†}, Savelina Popovska ^{1,2}, Tatyana Betova ^{1,2} and Zornica Kamburova ³

¹ Department of Pathology, Faculty of Medicine, Medical University- Pleven, Pleven, 5800, Bulgaria

² Department of General and Clinical Pathology, UMHAT Georgi Stranski- Pleven, Pleven, 5800, Bulgaria

³ Department of Medical Genetics, Faculty of Pharmacy, Medical University- Pleven, Pleven, 5800, Bulgaria

* Correspondence: krasimir.petrov@mu-pleven.bg

 UMBAL Georgi Stranski, Georgi Kochev 8A Blvd., Pleven 5800, Bulgaria.

† These authors contributed equally to this work.

Abstract: In this study we summarized our work on various aspects of colorectal adenocarcinomas, focusing on tumor distribution (left- sided, right- sided, and rectal cancers), histological type, patient age, gender and several factors affecting prognosis. These predictive and prognostic factors include peritumoral budding, lymphovascular and perineural invasion, lymph node metastasis, mismatch repair status, and correlations with some frequently known mutations in colorectal adenocarcinomas, detected using Next- Generation Sequencing (NGS) platform. This comprehensive approach allowed us to explore how these factors interplay and influence the clinical outcome of patients with colorectal adenocarcinoma. Additionally, our findings contribute to the understanding of how genetic mutations may correlate with histological features and prognostic markers. In our study, we do not claim to have exhausted the research on the topic, and we fully recognized the need for more extensive and in-depth studies in this area of tumor pathology.

Keywords: colorectal adenocarcinoma; left and right- sided; histologic type; peritumoral budding; lymphovascular and perineural invasion; lymph node metastasis; MMR status; mutations; NGS

1. Introduction

Cancers of colon and rectum are among the leading causes of morbidity and mortality worldwide. These cancers significantly contribute to the global burden of disease, with increasing incidence rates in many regions. [1].

In the United States, it is estimated that there will be 152,810 new cases of colon and rectal cancers combined for both sexes. Additionally, the estimated number of deaths from these cancers in both sexes will be 53 010 in 2024. Colorectal cancer has now become a leading cause of death in men and the second leading cause in women under the age of 50.[2]

In Bulgaria, the estimated number of new cases of colorectal cancer was reported to be 5,096 with an estimated 2,079 deaths, making it the second most common cause of cancer mortality for both sexes in 2022.[3]

Based on data from the Global Cancer Observatory (International Agency for Research on Cancer), the estimated number of new cases of colorectal cancer is projected to increase by approximately 61 % between 2022 and 2045. Similarly, the number of deaths from colorectal cancer is expected to rise by approximately 77 % during the same period.[4] This alarming trend highlights the growing impact of colorectal cancer and underscores the need for enhanced prevention, early detection, and treatment strategies grounded in the principles of precision and personalized medicine. It further calls for the exploration of new widely- used predictive and prognostic biomarkers in this area of oncology, which could play a pivotal role in tailoring treatments and improving patient outcomes.

Recent understanding of colorectal carcinoma reveals that its etiology and development are influenced by a multitude of factors, such as diet [5] [6] [7] [8] [9] [10], lifestyle habits [11] [12] [13]

[14] [15] [16] [17], social and economic status [18] [19][20] and environmental exposures, and some of which can be modified and can be addressed through prevention strategies. However, other factors, like driver gene mutations and certain genetic predispositions, are not modifiable, highlighting the complexity of the disease.[21] [22] [23] [24] [25] [26]

Many studies emphasize several macroscopic, morphological, and histological characteristics of tumors that are closely related to patient prognosis and survival. These include primary tumor location(left- sided, right- sided or rectal tumors), tumor morphology, lymphatic and vascular invasion, neural invasion, host immune response, tumor budding(peri or intratumoral), poorly differentiated clusters and others. [27] [28] [29] [30] [31] [32] [33] [34] [26] [35] [36] [37][38][39] [40] [41][42] [43][44] [45][46][47][48][49][50][51] [52]

In our study, we focus on certain aspects of the aforementioned tumor characteristics, including some patients' epidemiological data, primary tumor location, tumor histomorphology, perineural or intraneural invasion, lymph node status and tumor deposits, peritumoral budding, MMR status, and some of frequently observed mutations, assessing their prognostic and predictive role, as well as the possible interrelationships between them. This involves investigating the significance of these factors for disease progression and their influence on the selection of appropriate therapeutic regimens.

2. Materials and Methods

The study was conducted retrospectively, with 100 patients diagnosed between 2020 and 2023 with colorectal adenocarcinoma. These patients were selected from the archives of the Department of Clinical Pathology at "Georgi Stranski" University Hospital in Pleven- Bulgaria.

Patient selection:

Inclusion criteria:

In selecting patients for the study, we adhered to the following inclusion criteria:

- patients who provided informed consent to participate in the study;
- patients who had undergone resection of colon or rectum for primary adenocarcinoma.

The selection was carried out randomly. A small group of patients who had received preoperative radiation or chemotherapy was intentionally not excluded.

Exclusion criteria:

- presence of other histological tumor types or metastatic tumors of the colon or rectum;
- patient who declined to provide informed consent for participation.

The study was approved from the Ethics Committee of Medical University – Pleven prior to its commencement.

Methods

Assessment of peritumoral budding:

We used biopsy samples fixed in 10% buffered formalin, embedded in paraffin, cut at 4 μm , and stained with H&E.

Tumor buds were counted on H&E-stained slides according to the algorithm described by the International Tumor Budding Consensus Conference[47]:

Scanning 10 fields at a 10 $\times$ objective to identify the invasive front of the tumor and the TB hotspot area.

Counting PTB in the hotspot area at a 20 $\times$ objective, and dividing the result by the normalization factor to calculate the TB count at 0.785 mm² (in our study, the normalization factor was 0.810).

Tumor budding was graded as follows:

Bd1: Low – 0–4 tumor buds;

Bd2: Intermediate – 5–9 tumor buds;

Bd3: High – 10 or more tumor buds

Assessment of tumor MMR status:

IHC procedure

Tumor resection specimens from patients with colorectal adenocarcinoma were fixed in 10% buffered formalin for 24 to 36 hours at room temperature, dissected, and embedded in paraffin. A pathologist selected 5 µm thick parallel sections of representative invasive tumor tissue and normal mucosa, confirmed by H&E-stained slides.

Epitope retrieval was performed for 20 minutes at 97°C using DAKO PT Link (Code PT100/PT101).

The tumor sections were stained with the following, ready to use antibodies (DAKO Denmark©):

MLH1: ES05 (Monoclonal mouse Anti- Human MutL Protein Homolog 1, (Ready to use; Ref.№ IR079)

MSH2: FE11 (Monoclonal mouse Anti- Human MutS Protein Homolog 2 (Ready to use; Ref.№ IR085)

MSH6: EP49 (Monoclonal rabbit Anti- Human MutS Protein Homolog 6 (Ready to use; Ref.№ IR086)

PMS2: EP51 (Monoclonal rabbit Anti- Human Postmeiotic Segregation Increased 2 (Ready to use; Ref.№ IR087)

EnVision FLEX (K800021-2), High pH (Link), HRP. Rabbit/Mouse. High pH, Immunohistochemistry Visualization system kit was used.

Incubation time for all antibodies was 20 min. at room temperature.

A **Dako Agilent Autostainer Link 48** slide stainer was used to perform the staining. External reagent negative controls were prepared as part of the manufacturer's reagent kit. For internal positive controls, normal colonic mucosa, stromal cells, and stromal lymphocytes from the same slides containing the tumor were used.

The results were analyzed by a pathologist. The expression was categorized as follows:

Normal (Retained): Nuclear expression present in >10% of tumor cells, along with the retained expression in internal controls.

Negative (Loss): Absence of nuclear expression in tumor cells, with the retained expression of internal controls.

In our study, we did not aim to evaluate the subclonal expression of MMR proteins or use immunohistochemistry for the confirmation of peritumoral budding.

Investigation of lymphovascular invasion- LVI:

Due to the relatively small sample size and the limited number of colorectal cancer patients with observed lymphatic and/or vascular invasion, we did not aim to separately analyze these two morphological characteristics. Additionally, we did not focus on distinguishing between intramural and extramural vascular invasion, despite existing evidence indicating their negative prognostic significance[53][54][55].

Assessment of perineural invasion- PNI:

The assessment of perineural invasion (PNI) is based on the identification of tumor cells within any of the three layers of the nerve sheath, following the definition by Liebig C. et al.[56]

NGS testing:

Genetic testing of tumor tissues was performed by NGS, with the TruSight Tumor 15 panel (Illumina. Inc.). Representative tumor tissue blocks, fixed in 10% neutral buffered formalin and embedded in paraffin (FFPE), were selected and cut into 5 µm sections, ensuring sufficient tumor content for analysis. DNA Extraction, quality and quantity assessment, and library preparation were carried out according manufacturer's protocols. The sequencing platform used was the Illumina NextSeq 550 (Illumina. Inc.), following the manufacturer's guidelines. Data analysis and result interpretation were conducted by qualified medical bioinformatics and medical genetics personnel.

Statistical analysis

The results and other data, were recorded, and analyzed using IBM SPSS Statistics. The Chi-Square test was used to compare categorical data with values of $p < 0.05$ considered statistically significant.

For parametric data, if the data did not follow a normal distribution, the Kruskal-Wallis test was employed. We used normalization to adjust the data within a specific range, ensuring that features or variables with different scales do not disproportionately influence the results of the analyses.

3. Results

- Epidemiological data

As mentioned above, in our study, a group of 100 patients was examined, with the following epidemiological characteristics- number and age distribution by sex - presented in Table 1 and Figure 1:

Table 1. Distribution of patients by sex

Sex	Number	%	Total
Female	43	43	43
Male	57	57	57
Total	100	100	100

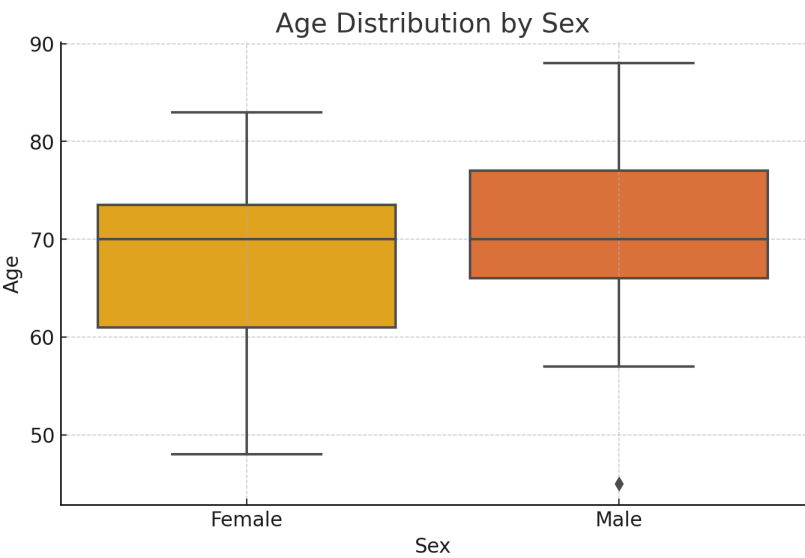


Figure 1. Age distribution by sex: t- test: t: 2.18; p: 0.0318; Df: 90.15

The age ranges were as follows: females ranged from 48 to 83 years old, and males from 45 to 88 years old, with a median age 70 years for both sexes.

Tumor localization across different anatomical sites for the entire patient group is presented in Figure 2, along with the results normalized for sample sizes in Figure 3. There is no statistically significant association between tumor localization and sex. (χ^2 : 2.95; p-value: 0.89; Df : 7)

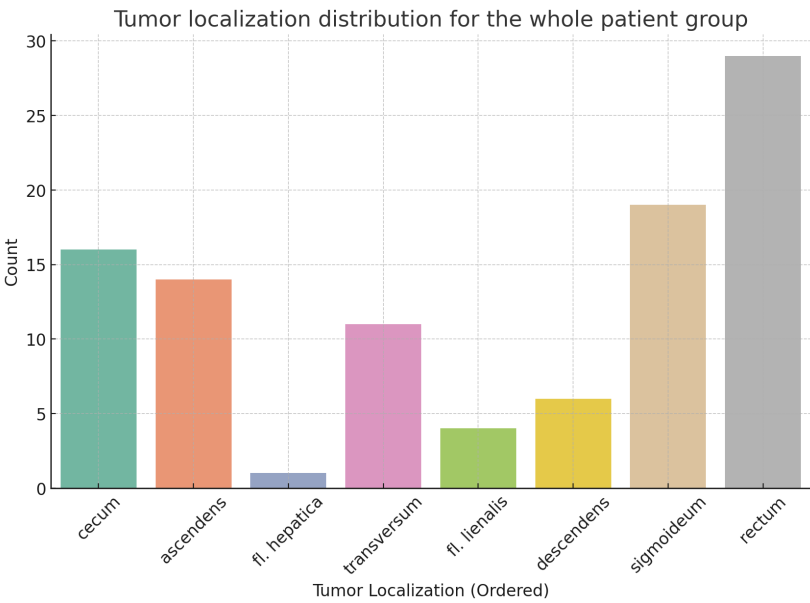


Figure 2. Tumor localization distribution for the whole patient group(count)

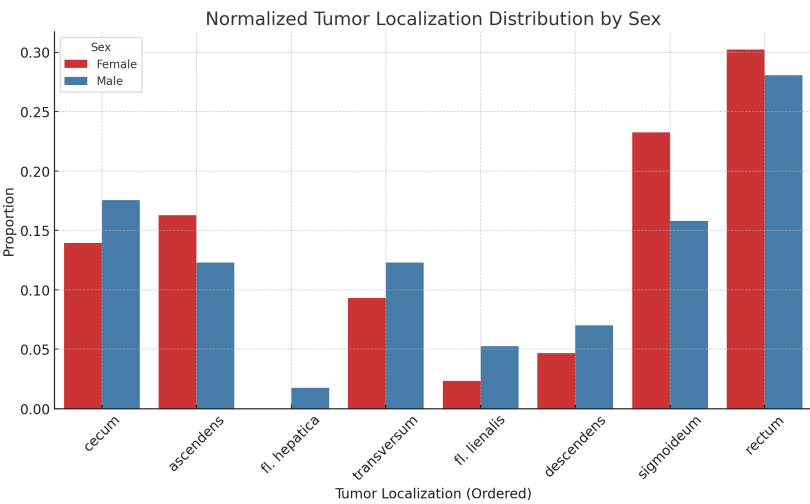


Figure 3. Normalized tumor localization distribution by sex

After consolidating the tumor localizations, we formed groups of patients with left-sided, right-sided, and rectal localization of the primary tumor. After normalization, we obtained the following results- Figure 4.

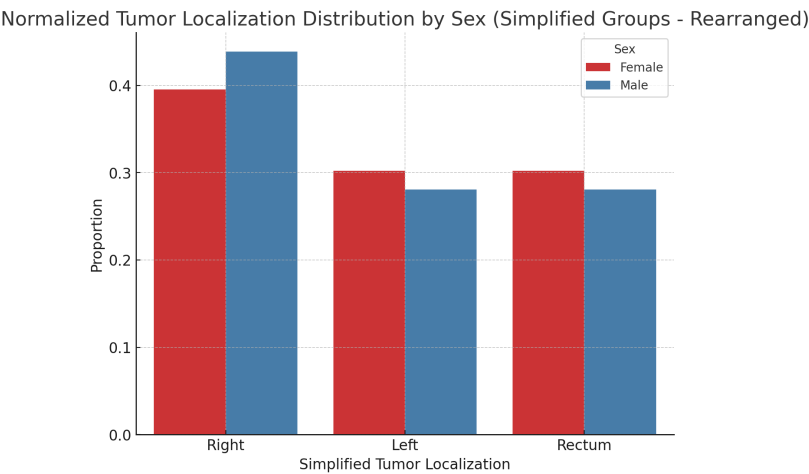


Figure 4. Normalized simplified tumor localization groups: **Right colon, Left colon, and Rectum**

• **Tumor characteristics data**

In the studied patient group, the results regarding some characteristics of the primary tumor are as follows: 1. Primary tumor localization and:

• **Age**

Figure 5. shows a box plot illustrating the distribution of the sample of studied patients based on the location of the primary tumor. Table 2. provides the median age of patients relative to tumor localization in the overall patient group, and differentiated by sex, as well as age ranges. Statistical analysis indicates no statistically significant differences in the distribution by sex(χ^2 : 2.95; p-value: 0.938) and age(Kruskal-Wallis test statistic is approximately 3.25, p-value: 0.777) in relation to different tumor localizations.

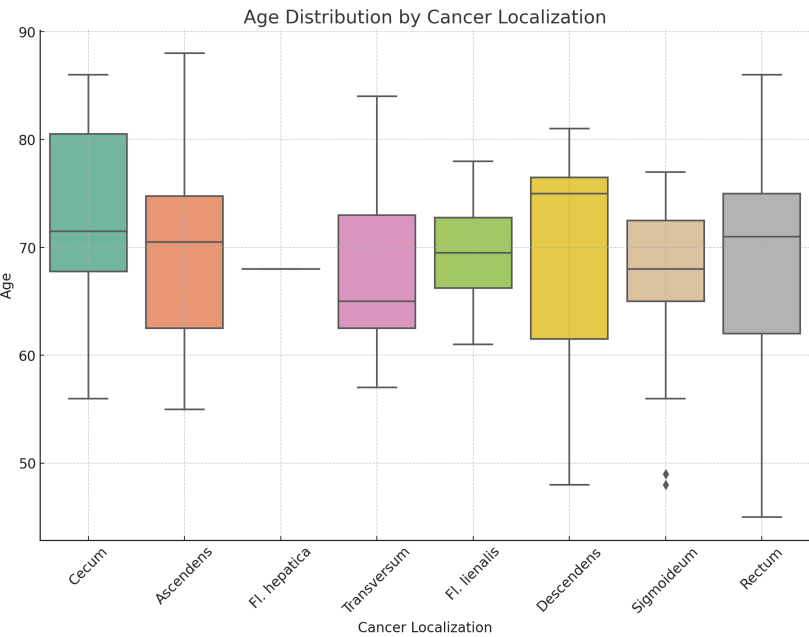


Figure 5. Age distribution by cancer localization

Table 2. Distribution of patients by age and sex based on primary tumor localization.

	Median Age	Range	Total Patients	Median Males	Range Males	Total Males	Median Females	Range Females	Total Females
Ascendens	70.5	55 - 88	14	71.0	67 - 88	7	61.0	55 - 75	7.0
Cecum	71.5	56 - 86	16	71.5	57 - 86	10	71.5	56 - 83	6.0
Descendens	75.0	48 - 81	6	76.0	57 - 81	4	61.5	48 - 75	2.0
Fl. hepatica	68.0	68 - 68	1	68.0	68 - 68	1			
Fl. lienalis	69.5	61 - 78	4	71.0	68 - 78	3	61.0	61 - 61	1.0
Rectum	71.0	45 - 86	29	72.5	45 - 86	16	68.0	57 - 77	13.0
Sigmoideum	68.0	48 - 77	19	68.0	57 - 77	9	68.5	48 - 76	10.0
Transversum	65.0	57 - 84	11	65.0	57 - 84	7	73.0	61 - 75	4.0
Total	70.0	45 - 88	100	70.0	45 - 88	57	70.0	48 - 83	43.0

Based on the different embryological origins of the right side of the colon (cecum, ascending colon, hepatic flexure, and the proximal two-thirds of the transverse colon) and its left side (splenic flexure, descending colon, and sigmoid colon), as well as extensive experimental and clinical data on differences in morphology, genetics, and clinical characteristics, we consolidated the primary tumor localizations into three groups: right colon, left colon, and rectum. This approach also facilitated the subsequent statistical analysis to some extent. Figure 6. and Figure 7. present the results concerning these three localization groups and their relationship with the age and sex of patients with colorectal carcinoma. The subsequent statistical analysis showed no statistically significant differences regarding the primary tumor localization, age, and sex of the patients.

The results of the Kruskal-Wallis tests are as follows: Females: The test statistic is 0.805 with a p-value of 0.669; Males: The test statistic is 0.418 with a p-value of 0.811.

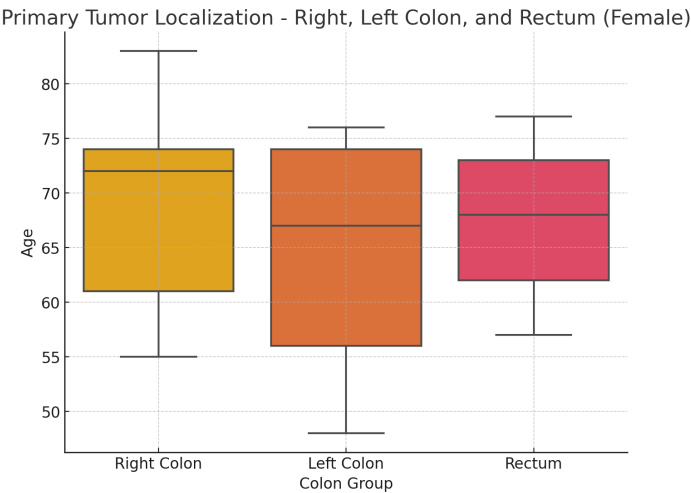


Figure 6. Age distributions across the right colon, left colon, and rectum-females

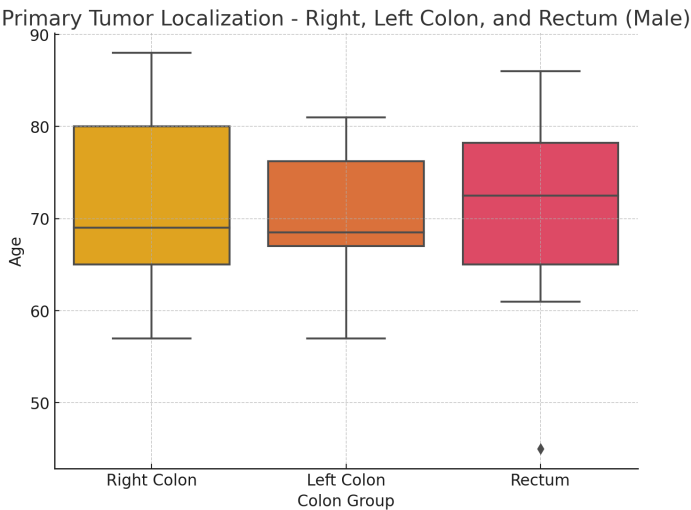


Figure 7. Age distributions across the right colon, left colon, and rectum-males

- Histological subtype

The following bar chart displays the normalized proportions of histological subtypes as a grouped bar chart, with each localization group (Right Colon, Left Colon, and Rectum) represented in Figure 8. In summary, even with normalized proportions, there is no statistically significant relationship between localization and histological type (χ^2 : 19.937; p-value: 0.336; df=18). The statistical results

of the chi-square test for the above indicators, separated by sex (not shown), also do not reveal any statistically significant differences.(Males: p-value: 0.685; Females: p-value: 0.380)

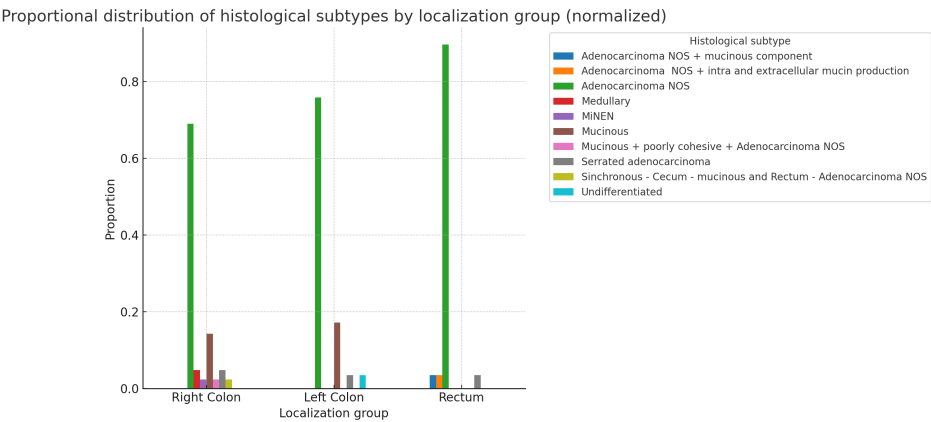


Figure 8. Proportional distribution of histological subtypes by localization group (normalized)

• Tumor Grade and Stage

The following two charts in Figure 9. and Figure 10*. illustrate the possible relationships between primary tumor localization in the Right colon, Left colon, and Rectum, and the corresponding tumor grade and stage. As seen from the charts and the results of the chi-square test, no statistically significant differences are found. Additionally, when analyzing these parameters separately by sex, no statistically significant differences are detected for either tumor grade(Males: p-value: 0.429, Df=6; Females: p-value: 0.096, Df=4) or stage(Males: p-value: 0.230, Df=8; Females: p-value: 0.154, Df=10).(*The ypT categories were added to their respective T groups).

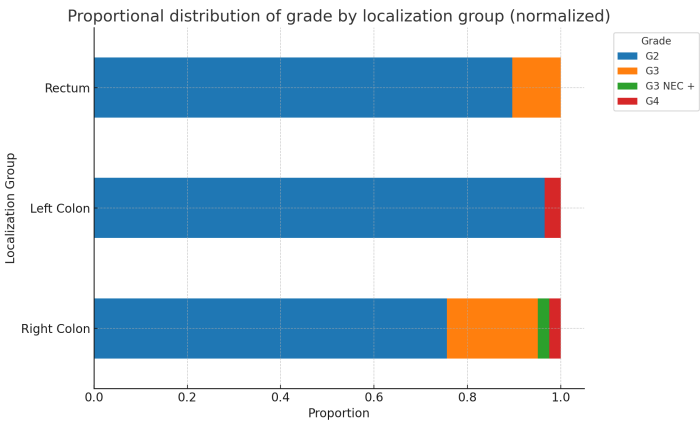


Figure 9. Proportional distribution of grade by localization groups(χ^2 : 9.107; p-value: 0.168; Df=6)

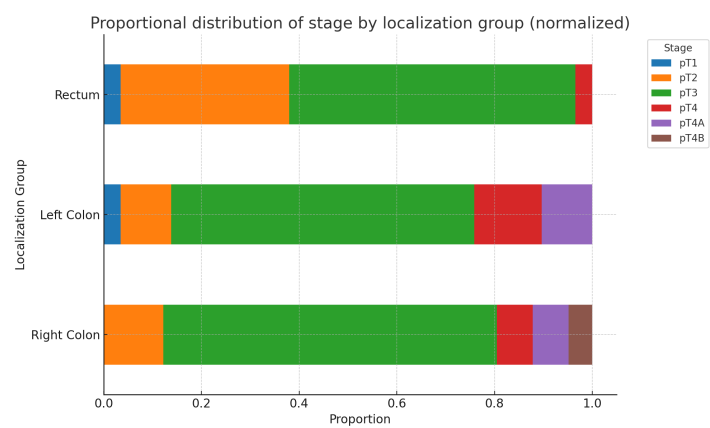


Figure 10. Proportional distribution of stage by localization groups(χ^2 : 15.248; p-value: 0.123; Df=10)

However, when we compare tumor differentiation(G) and tumor stage(pT), the following expected results are obtained- Figure 11:

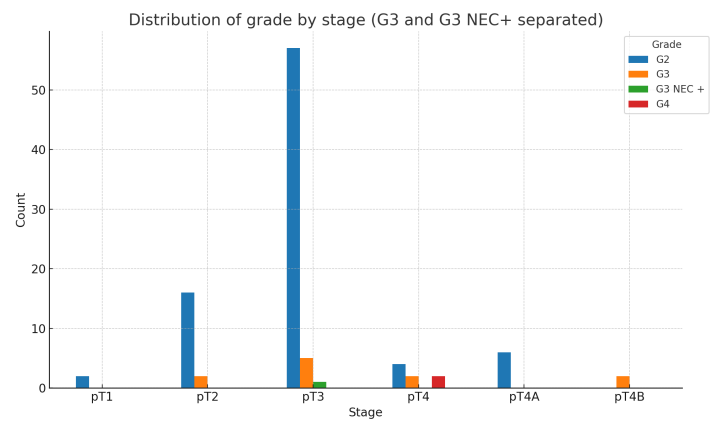


Figure 11. Proportional distribution of G2, G3, and G4 within each stage

As expected, the chi-square test shows that the relationship between grade and stage is highly statistically significant.(χ^2 : 42.69; p-value:0.00000565; Df=10)

• Lymph node status

The statistical analysis of the patient sample showed no statistically significant differences in the N status of patients with colorectal adenocarcinoma based on the localization of the primary tumor—Right colon, Left colon, or Rectum—for the entire patient group(χ^2 : 11.811; p-value: 0.621; df=14). The same conclusions remain valid when analyzed separately by sex: Males: χ^2 : 12.82; p-value: 0.382; df=12; Females: χ^2 : 8.38; p-value: 0.869; df=14 Figure 12 represents the whole patient group.

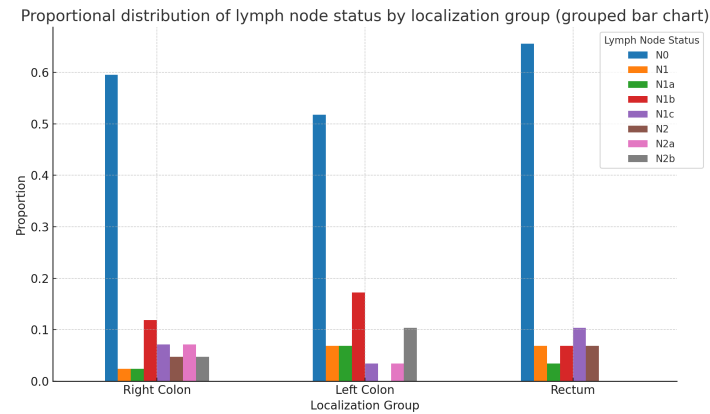


Figure 12. N status and tumor localization- Right colon, Left colon, and Rectum(whole group count)

When we compared lymph node status with the degree of tumor differentiation and tumor stage, the results indicated a lack of statistically significant differences once again, as is most clearly seen from the statistical analysis- Table 3.

Table 3. Chi-Square test results for lymph node status vs. tumor grade and tumor stage

Chi- square test	Grade	Stage
Chi-square value	16.945	36.590
Dof	14	35
p- value	0.259	0.395

- **Important histological features of the tumor:**
- Lymphatic and vascular invasion

In our study, due to the relatively small sample size and the limited number of colorectal cancer patients with observed lymphatic and/or vascular invasion, we did not aim to separately analyze these two morphological characteristics. We also did not focus on distinguishing between so-called intramural and extramural vascular invasion, despite evidence of their negative prognostic significance. For this reason, they will be considered together in the following brief section.

LVI and primary tumor localization: the following Figure 13 and Table 4 visually present the relationships between the localization of the primary tumor and lymphovascular invasion (LVI). The results of the chi-square test for the association between LVI status and localization are as follows: Chi-Square value: 2.38; df: 2; p-value: 0.304. The p-value is greater than 0.05, indicating that there is no statistically significant association between LVI status and localization.

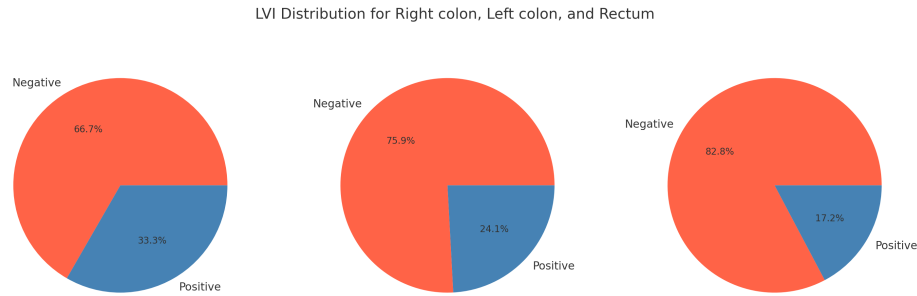


Figure 13. LVI distribution for Right colon, Left colon, and Rectum

Table 4. LVI count by localization group

LVI count by localization	Negative	Positive
Right colon	28	14
Left colon	22	7
Rectum	24	5
Total	74	26

LVI and histopatological subtype- Figure 14 shows the proportion of negative and positive statuses across different tumor histological subtypes. chi-square test The subsequent statistical analysis shows no statistically significant association between lymphovascular invasion (LVI) and histological subtype. The Monte Carlo approximation of the chi-square test for the association between LVI status and histological subtype yields the following results: Chi-Square value: 15.93; df: 9; Monte Carlo p-value: 0.068 However, the p-value is close to significance, suggesting a potential trend that may warrant further investigation.

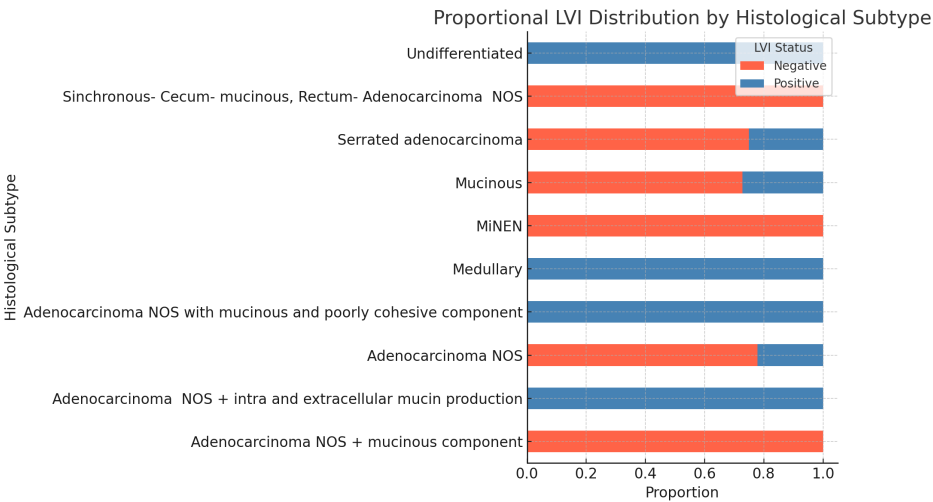


Figure 14. Proportional LVI Distribution by Histological Subtype

LVI and tumor Grade and Stage- As we expected, there is a statistically significant association between lymphovascular invasion and tumor grade and stage. The results of the chi-square tests are shown in the following Table 5 and Figure 15.

Table 5. Chi-square test results for LVI and tumor Grade and Stage

Chi- square value	Grade	Stage
χ^2 value	17.33	16.13
df	2	5
p- value	0.00017	0.006

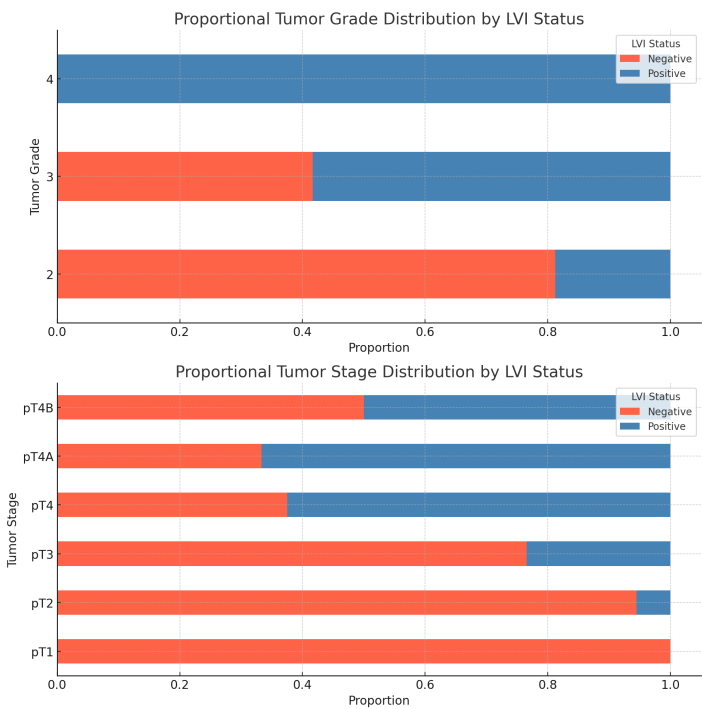


Figure 15. Proportional distribution of LVI status across both tumor grade and tumor stage

• Perineural invasion

The assessment of perineural tumor invasion is based on the presence of tumor cells in any of the three layers of the nerves or tumor cells involving at least one-third of the nerve’s circumference.

In our study, regarding this tumor characteristic, we observed the following results as shown in Figure 16 and Table 6. There are no statistically significant associations between PNI and any of the variables: primary tumor localization, tumor histological subtype, tumor grade, or tumor stage (all p-values > 0.05).

Table 6. Results of the chi-square tests for the association between Perineural Invasion (PNI) and Localization, Histological subtype, Grade, and Stage.

	Localization	Histological subtype	Grade	Stage
χ^2 value	1.46	7.31	2.15	2.46
p- value	0.984	0.605	0.542	0.783
Df	7	9	3	5

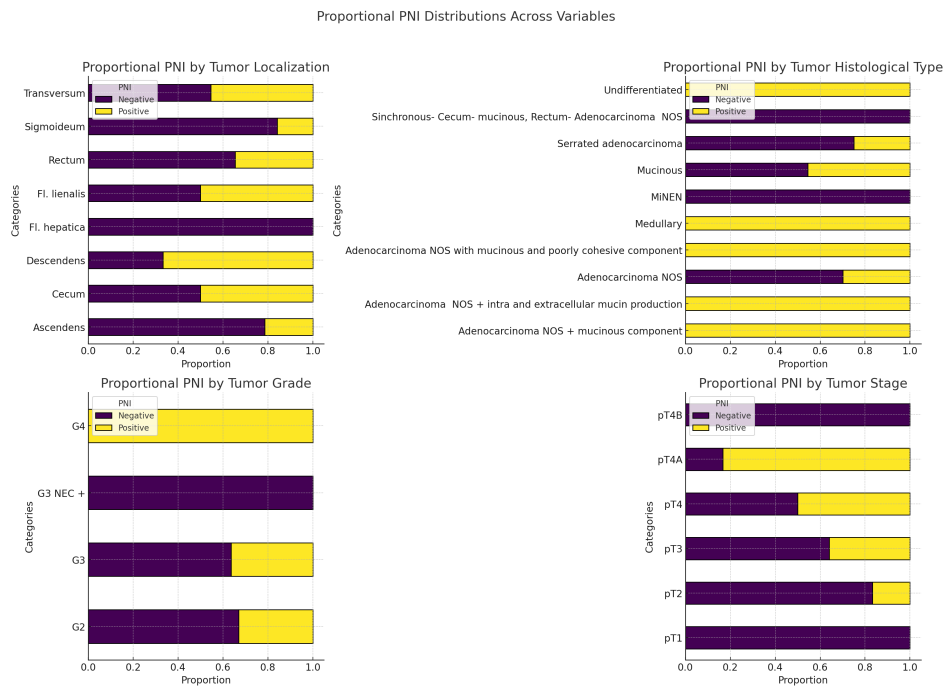


Figure 16. Proportional distribution of Perineural Invasion (PNI) across the following variables: Primary tumor localization, Histological subtype, Tumor grade, Tumor stage.

• Immune response

In our study, we focused on evaluating the peritumoral stromal inflammatory reaction at the invasive tumor front, specifically assessing the immune response of the so-called "Crohn-like" type. Our interest lay in investigating the presence or absence of interactions between this immune reaction and other prognostic factors such as tumor budding, MMR status, mutational status, and primary tumor localization, among others. For practical purposes, we conducted a semi-quantitative assessment of the peritumoral stromal inflammatory reaction, categorizing it into three levels: Absent, Moderate, and Marked inflammatory response. After data processing, we obtained the following results:

-Immune response and primary tumor localization- Figure 17:

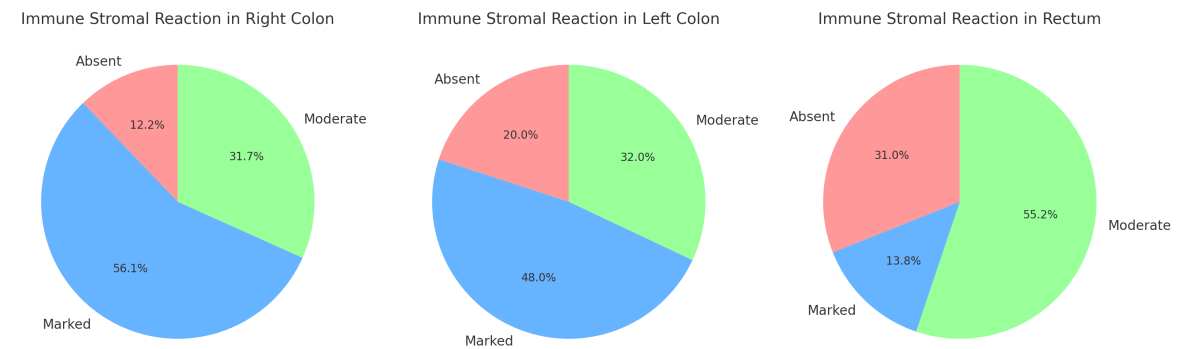


Figure 17. Immune stromal reaction and primary tumor localization

The Right Colon has the highest percentage of marked reactions (56.1%), while absent reactions are relatively low.

The Left Colon shows a more balanced distribution, with the majority of cases presenting marked reactions (48%).

The Rectum is unique in having the highest percentage of moderate reactions (55.2%) and the lowest percentage of marked reactions (13.8%).

Results from the Chi-Square test: χ^2 value: 14.05; (dof): 4; p-value: 0.007. The p-value is statistically significant (below the typical threshold of 0.05). This suggests that there is a strong indication that the distribution of peritumoral stromal immune reactions is not uniform across the three tumor localizations (Right Colon, Left Colon, and Rectum). In other words, the type or intensity of the immune response differs significantly depending on the tumor’s primary location.

-Immune response and histological subtype

The chart compares the average peritumoral stromal reaction for each histological subtype. Each point represents the average immune response (from absent to marked) within each histological type- Figure 18:

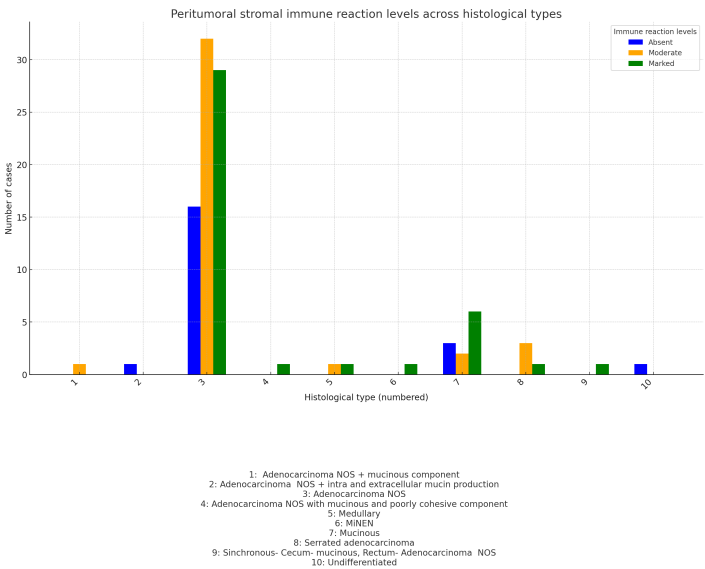


Figure 18. Average peritumoral stromal reaction by histological subtype

As shown in the chart, adenocarcinomas NOS with an additional tumor component (such as Synchronous- Cecum- mucinous + Rectum- Adenocarcinoma NOS, Adenocarcinoma NOS + intra- and extracellular mucin production, or MiNEN with an exception) exhibit a marked immune response. Speculatively, this could be due to the tumor heterogeneity and the higher immunogenicity of these tumors.

The results from the Chi-square test are: χ^2 value: 18.76; p-value: 0.4067; Degrees of freedom (dof): 18 These results show no statistically significant association between histological type and the peritumoral stromal reaction. This suggests that while some histological subtypes may show a pronounced immune response, there isn’t a consistent or significant pattern across the different histological subtypes studied.

-Immune response and tumor grade and stage

In the following Table 7—the relationships between tumor grade and peritumoral stromal immune reaction, as well as tumor stage and peritumoral stromal immune reaction, are presented. The Chi-square tests conducted for these two characteristics show no statistically significant results for either variable, indicating no strong association between tumor grade or tumor stage and the intensity of the peritumoral stromal immune reaction:

1. Tumor Grade (G1, G2, G3) vs. Peritumoral stromal immune reaction (Normalized): Chi-square value (χ^2): 0.185; p-value: 0.996; Degrees of freedom (dof): 4.

2. Tumor Stage (T1, T2, T3, T4) vs. Peritumoral stromal immune reaction (Normalized): Chi-square value (χ^2): 0.685; p-value: 0.995; Degrees of freedom (dof): 6.

This suggests that the immune response in the tumor’s surrounding stroma does not significantly correlate with the differentiation (grade) or progression (stage) of the tumor in this particular cohort.

Table 7. Results for tumor grade and tumor stage and peritumoral stromal immune reaction(count)

Tumor grade	Absent	Moderate	Marked
G2	18	35	32
G3	2	3	7
G4	1	1	1
Tumor stage			
T1	1	1	0
T2	3	9	6
T3	13	23	28
T4	4	6	6

-Immune response and lymph node status

The chi-square test results for the relationship between peritumoral stromal immune reaction and lymph node status are as follows:

Chi-square value: 14.47; p-value: 0.416; Degrees of Freedom: 14.

These results indicate that there is no significant association between peritumoral stromal immune reaction and lymph node status, as the p-value is greater than 0.05. This suggests that variations in the immune reaction in the tumor stroma do not correlate significantly with whether lymph node involvement is present or not.

-Immune response and lymphovascular (LVI) and perineural invasion (PNI)

The following Figure 20 shows the correlation between peritumoral stromal immune reaction and lymphovascular and perineural invasion. As seen from the results of the chi-square tests, there is no statistically significant association between these three examined parameters.

1. Peritumoral stromal immune reaction vs. lymphovascular invasion (LVI):

Chi-square value: 2.23; p-value: 0.327; Degrees of freedom: 2.

2. Peritumoral stromal immune reaction vs. perineural invasion (PNI):

Chi-square value: 3.08; p-value: 0.214; Degrees of freedom: 2.

The lack of a significant correlation suggests that the peritumoral stromal immune reaction does not directly influence lymphovascular or perineural invasion in the context of the cases studied. These results can be interpreted to mean that the immune reaction in the tumor stroma is not a primary predictor of tumor invasiveness in these aspects.

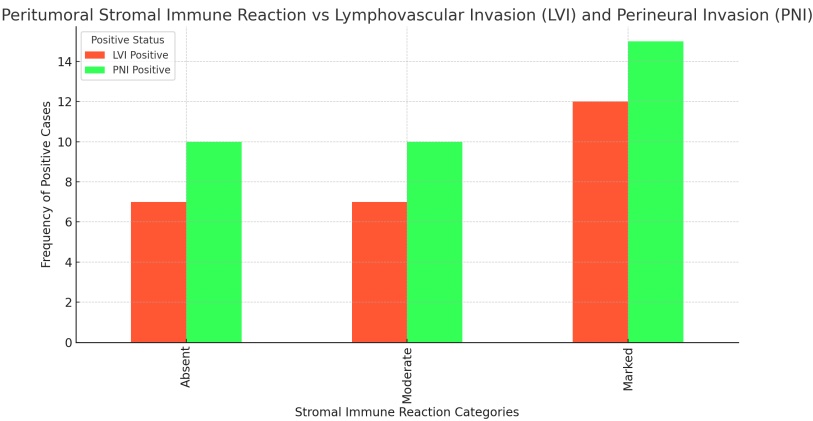


Figure 19. Peritumoral stromal immune reaction vs lymphovascular invasion (LVI) and perineural invasion (PNI)

- Tumor budding

In our study, when assessing tumor budding, we followed the classical scheme described by the International Tumor Budding Consensus Conference (ITBCC) . During the evaluation, we also encountered cases with zero budding, which were included in Bd1 group.

After evaluating the tumor budding, we obtained the following results:

- Peritumoral budding and primary tumor localization- Right colon, Left colon, Rectum:

In the following Figure 20, the relationship between peritumoral budding Bd1 = (Bd0 + Bd1), Bd2, and Bd3 and the primary tumor localization (Right colon, Left colon, Rectum) is illustrated. As seen in the graph, all grades of peritumoral budding are more prevalent in the right colon, followed by the left colon and rectum. Notably, the Bd1 group in the rectum shows a higher value compared to the left colon.

The subsequent statistical analysis did not reveal statistically significant differences among these groups. The results of the Kruskal-Wallis test indicate: Test statistic: 3.37; p-value: 0.185.

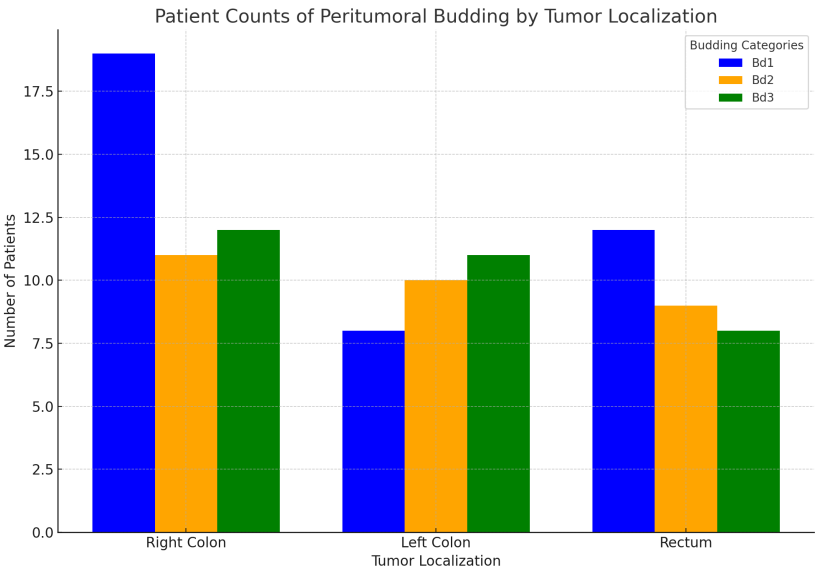


Figure 20. Peritumoral budding distribution across tumor localizations (Bd0+Bd1 Combined)

- Peritumoral budding and histological subtype:

In Figure 21, we can observe the distribution of peritumoral budding across different histological tumor subtypes. The results of the Kruskal-Wallis test for the peritumoral budding groups (Bd1, Bd2, Bd3) across these histological types are as follows: Test statistic: 7.39, p-value: 0.597. These results indicate that there is no statistically significant difference in peritumoral budding across the different histological subtypes, suggesting that the subtype does not have a meaningful impact on the distribution of tumor budding grades for the studied cohort of patients.

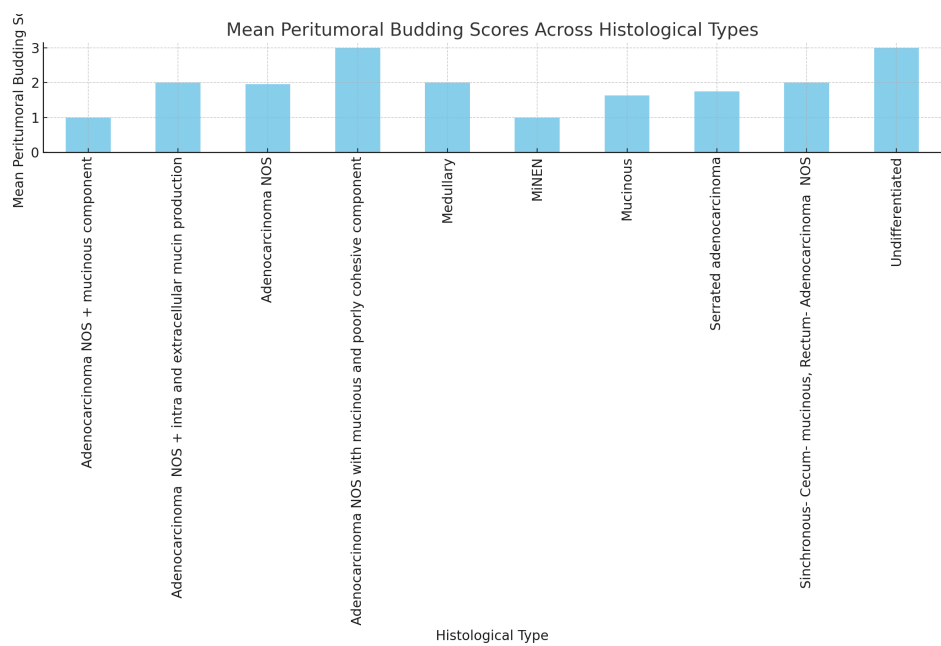


Figure 21. Peritumoral budding distribution across tumor histological subtypes

The undifferentiated tumors, along with adenocarcinoma NOS that include other tumor components such as mucinous and poorly cohesive types, exhibit the highest average peritumoral budding grade (Bd3). This suggests the correlation between these tumor types and more aggressive behavior.

- Peritumoral budding and tumor grade and stage:

The results for peritumoral budding grades and tumor Grade and Stage are shown in the next Table 8

Table 8. Results for tumor grade and tumor stage and peritumoral budding- Bd1(Bd0+ Bd1), Bd2, Bd3

Tumor grade	Bd1	Bd2	Bd3
G2	35	3	1
G3+G3 NEC	29	1	0
G4	21	8	2
Tumor stage			
T1	1	0	1
T2	7	5	6
T3	24	22	18
T4	4	1	3
T4A	1	2	3
T4B	2	0	0

The results of the chi-square tests for the association between peritumoral budding and both tumor grade and tumor stage are as follows: and both tumor grade and tumor stage:

1. Tumor grade vs. Peritumoral budding: Chi-square value: 14.39; p-value: 0.0255; Degrees of freedom: 6. This result indicates that there is a statistically significant association between peritumoral budding and tumor grade, as the p-value is below 0.05.
2. Tumor stage vs. Peritumoral budding: Chi-square value: 7.36; p-value: 0.691; Degrees of freedom: 10.

This result suggests that there is no statistically significant association between peritumoral budding and tumor stage, as the p-value is much higher than 0.05.

-Peritumoral budding and lymphovascular invasion(LVI) and lymph node status:

The analysis of the association between peritumoral budding and lymphovascular invasion indicates a statistically significant correlation between the two, as illustrated in Figure 22. The results from the chi-square test are as follows:

Chi-square value: 8.59; p-value: 0.0137; Degrees of freedom: 2

These results suggest that there is a meaningful relationship between peritumoral budding and lymphovascular invasion, implying that higher levels of budding may be associated with an increased likelihood of lymphovascular spread in colorectal cancer. This finding highlights the importance of assessing both peritumoral budding and lymphovascular invasion in the prognostic evaluation of tumors.

On the other hand, the analysis of the association between peritumoral budding and lymph node status shows a lack of statistical significance in this group:

Chi-square value: 19.93; p-value: 0.132; Degrees of freedom: 14.

These results indicate that there is no significant relationship between peritumoral budding and lymph node status in this cohort.

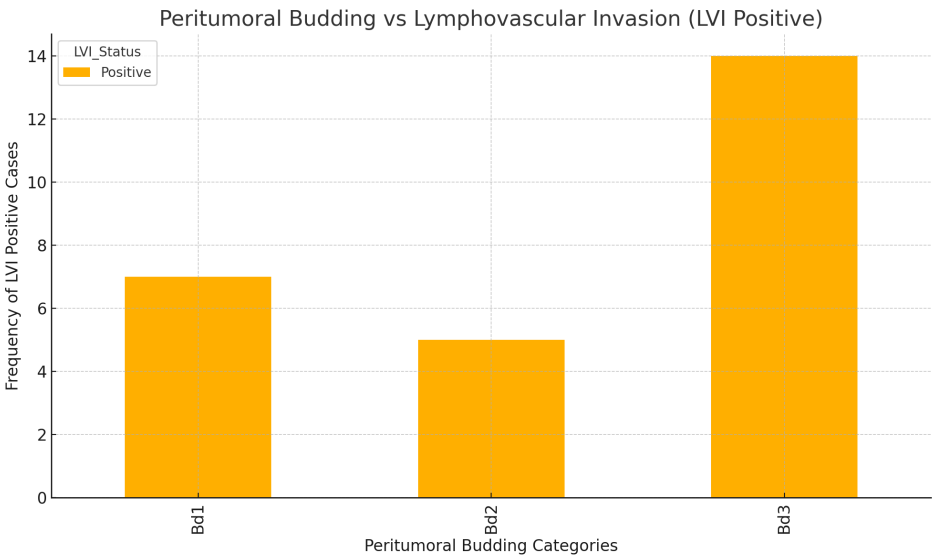


Figure 22. Peritumoral budding and LVI

-Peritumoral budding and perineural invasion(PNI)

The chi-square test results for the association between peritumoral budding and perineural invasion (PNI) are as follows:

Chi-square value: 5.59; p-value: 0.061; Degrees of freedom: 2

These results suggest a marginally non-significant association, as the p-value is slightly above the common threshold of 0.05. This indicates that while there may be some correlation between peritumoral budding and PNI, it does not achieve statistical significance.

3. Important prognostic and predictive biomarkers of Colorectal carcinoma (CRC)

-MMR status and primary tumor localization:

The following Table 9 presents the results of MMR status in colorectal carcinomas within the studied cohort. As illustrated in the corresponding Figure 23, the findings can be summarized as follows:

1. dMMR (Deficient mismatch repair): Right-sided tumors dominate: A significant majority of tumors in the dMMR group are localized in the right colon, approximately 89%. This strong association suggests that dMMR status is more prevalent in right-sided colon cancer. Left-sided and rectal tumors

are rare: There are very few left-sided tumors and no rectal tumors associated with dMMR, indicating a distinct localization pattern.

2. pMMR (Proficient mismatch repair): Even distribution: Tumors in the pMMR group are more evenly distributed across all localization groups (right, left, rectum), suggesting that pMMR tumors do not have a strong preference for a specific side. Common rectal tumors: Rectal tumors are notably common in the pMMR group, accounting for nearly 35% of cases. This highlights a significant presence of rectal cancer in patients with proficient MMR. Relatively frequent left-sided tumors: Left-sided tumors are also observed more frequently in the pMMR group compared to dMMR, reinforcing the variability in tumor location based on MMR status.

-Statistical analysis:
The chi-square test results confirm a statistically significant association between MMR status and tumor localization (right, left, rectum):
Chi-square value: 20.28; p-value: 0.000039; Degrees of freedom: 2. These results indicate a strong correlation between MMR status and the localization of colorectal tumors.

Table 9. MMR status and primary tumor localization

MMR status/ Localization	Right	Left	Rectum
dMMR	16	2	0
pMMR	26	27	29

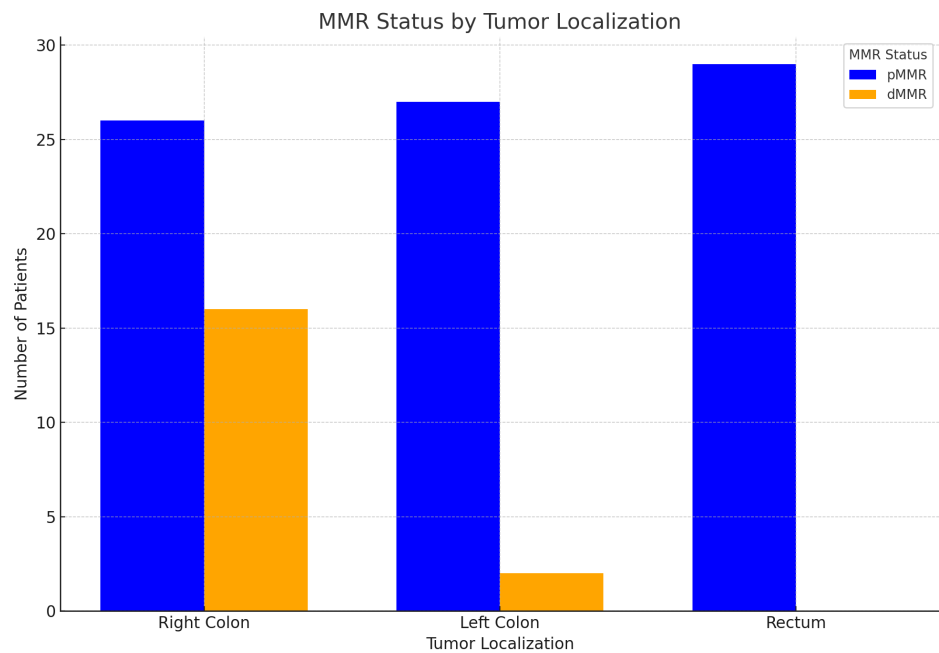


Figure 23. MMR status and primary tumor localization

-MMR status and sex:
For both sexes, the pattern for dMMR is as follows:
-Right Colon: dMMR is most prevalent in the right colon for both males and females. There are 8 cases for males and 8 cases for females localized in the right colon.
-Left Colon: There is only 1 case of dMMR for both males and females in the left colon, showing a much lower prevalence compared to the right colon.
-Rectum: dMMR is absent in the rectum for both males and females. In summary, dMMR is predominantly found in the right colon in both males and females, with very few cases in the left colon and none in the rectum.

-Chi-square value: 0.16; p-value: 0.689; Degrees of Freedom: 1

These results indicate that there is no statistically significant association between MMR status and sex.

-MMR status and histological subtype:

The results for MMR status, histological subtype, and tumor localization are presented in the next crosstab- Table 10. The key findings for dMMR are as follows:

Adenocarcinoma NOS: All 8 cases of dMMR-associated Adenocarcinoma NOS are located in the right colon.

Medullary: Both cases of Medullary tumors are located in the right colon.

MiNEN: The single case of MiNEN is also found in the right colon.

Mucinous: Most dMMR-associated Mucinous cases (4 out of 6) are found in the right colon, with 2 in the left colon.

Serrated adenocarcinoma: The single case is located in the right colon.

No dMMR-associated tumors were found in the rectum.

The results of the chi-square test for statistical significance for MMR status vs histological subtype and tumor localization are as follows:

Chi-square value: 36.72; p-value: 0.0467; Degrees of freedom: 24.

There is a statistically significant association between MMR status, histological subtype, and tumor localization, as the p-value is slightly below 0.05.

When we examined the relationship between MMR status and histological subtype separately, the following results from the chi-square test were obtained:

Chi-square value: 27.87; p-value: 0.001; Degrees of Freedom: 9.

This indicates that there is a statistically significant association between MMR status and histological subtype, as the p-value is well below the 0.05 threshold.

Table 10. MMR status, histological subtype, and primary tumor localization

MMR Status	Histological Type	Right	Left	Rectum
dMMR	Adenocarcinoma NOS	8	0	0
	Medullary	2	0	0
	MiNEN	1	0	0
	Mucinous	4	2	0
	Serrated adenocarcinoma	1	0	0
pMMR	Adenocarcinoma NOS + mucinous component	0	0	1
	Adenocarcinoma NOS + intra and extracellular mucin production	0	0	1
	Adenocarcinoma NOS	21	22	26
	Adenocarcinoma NOS with mucinous and poorly cohesive component	1	0	0
	Mucinous	2	3	0
	Serrated adenocarcinoma	1	1	1
	Synchronous- Cecum- mucinous + Rectum- Adenocarcinoma NOS	1	0	0
	Undifferentiated	0	1	0

-MMR status and peritumoral stromal immune reaction

Our results indicated a lack of statistically significant association between MMR status and peritumoral stromal immune reaction. This suggests that the immune response in the peritumoral stroma does not appear to be influenced by the MMR status of the tumors in the studied cohort.

Chi-square value: 4.18; p-value: 0.1239; Degrees of freedom: 2.

-MMR status and lymph node status:

The next chart visualizing the relationship between MMR status (dMMR and pMMR) and lymph node status- Figure 24.

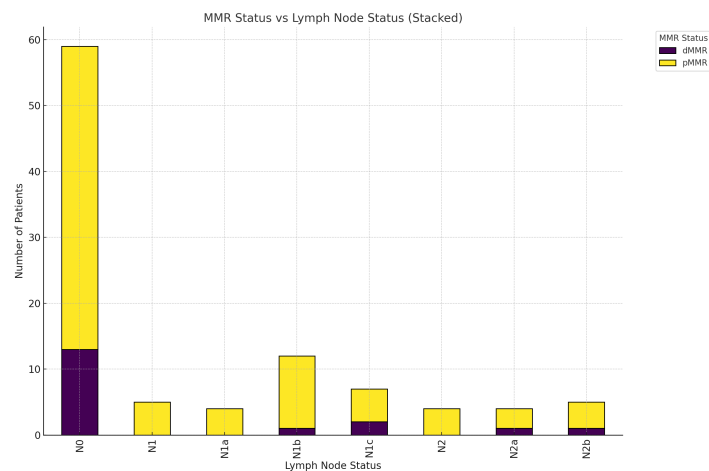


Figure 24. MMR status and lymph node status: Chi-square value: 4.94; p-value: 0.667; Degrees of freedom: 7- there is no statistically significant association

-MMR status and lymphovascular invasion (LVI) and perineural invasion (PNI) The statistical analysis indicates a lack of statistical significance for the association between MMR status and both lymphovascular invasion (LVI) and perineural invasion (PNI). This suggests that MMR status may not play a significant role in predicting these specific invasive behaviors in the tumors analyzed.

1. MMR Status vs LVI: Chi-square value: 0.24 p-value: 0.63; Degrees of freedom: 1.
2. MMR Status vs PNI: Chi-square value: 0.19; p-value: 0.66; Degrees of Freedom: 1.

In a Table 11, patients with dMMR and the corresponding MLH1, PMS2, MSH6, and MSH2 abnormalities identified through immunohistochemistry (IHC) are shown, along with the NGS-analyzed mutations in key genes and their combinations. In the last col- umn, we have indicated cases where further investigation for Lynch syndrome is war- ranted. In cases with a suspicion of Lynch syndrome, no additional tests were conducted in our research to confirm the diagnosis. The research team declared a refusal to influence specific diagnostic or therapeutic decisions.

Table 11. dMMR status with corresponding MLH1, PMS2, MSH6, and MSH2 status, main gene mutations, and mutated gene combinations and their relation to Lynch syndrome.

Patient	MMR	MLH1	PMS2	MSH6	MSH2	BRAF gene mutations	Mutated genes and combinations	Suspicion for Lynch Syndrome
10	dMMR	neg	neg	pos	pos	BRAF	BRAF+ TP53	Not Suspected
16	dMMR	neg	neg	pos	pos	BRAF	BRAF+ TP53	Not Suspected
19	dMMR	neg	neg	neg	pos	BRAF	BRAF+ TP53	Not Suspected
20	dMMR	neg	neg	pos	pos	BRAF	BRAF+ TP53	Not Suspected
33	dMMR	neg	neg	pos	pos	BRAF	BRAF	Not Suspected
52	dMMR	neg	neg	pos	pos	BRAF	BRAF+PIK3CA	Not Suspected
56	dMMR	neg	neg	pos	pos	BRAF	BRAF	Not Suspected
58	dMMR	neg	neg	pos	pos	BRAF	BRAF	Not Suspected
60	dMMR	neg	neg	pos	pos		TP53	Suspected
65	dMMR	pos	pos	neg	neg		No Result	Suspected
67	dMMR	pos	pos	neg	pos		PIK3CA + TP53+ KRAS	Not Suspected
75	dMMR	neg	pos	pos	neg		No Result	Suspected
84	dMMR	neg	neg	pos	pos	BRAF	BRAF	Not Suspected
88	dMMR	neg	neg	pos	pos	BRAF	BRAF + AKT1	Not Suspected
92	dMMR	neg	neg	pos	pos		NRAS + TP53	Suspected
94	dMMR	neg	neg	pos	pos	BRAF	BRAF+PIK3CA	Not Suspected
95	dMMR	neg	neg	pos	pos	BRAF	BRAF+ TP53	Not Suspected
96	dMMR	neg	neg	pos	pos		PIK3CA	Suspected

- Gene mutations identified by NGS

In our study, we utilized the "TruSight Tumor 15" panel from Illumina for Next-Generation Sequencing (NGS). This panel is designed to detect mutations across 15 key genes associated with various cancers, which include: AKT1, BRAF, EGFR, ERBB2(HER2), FOXL2, GNA11, GNAQ, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA, RET, TP53.

The results obtained from NGS are as follows:

-Observed mutations by sex:

-Proportions of gene mutations by sex:

Females: TP53 is the most frequent mutation, appearing in about 67.4% of cases. KRAS follows with a proportion of 34.9%. Other gene mutations include BRAF (18.6%), PIK3CA (9.3%), and NRAS (4.7%).

Males: TP53 is also the most common, but slightly less frequent than in females, at 57.9%. KRAS appears in 38.6% of cases. Other gene mutations include PIK3CA (12.3%), BRAF (15.8%), and NRAS (3.5%).

Both sexes show a higher prevalence of TP53 mutations, but females have a slightly higher proportion. PIK3CA mutations appear more often in males compared to females.

The two-proportion Z-test for each gene mutation between males and females are as follows:

AKT1: Z-Statistic: 0.202; p-value: 0.840;

TP53: Z-Statistic: 0.974; p-value: 0.330;

KRAS: Z-Statistic: -0.381; p-value: 0.703;

BRAF: Z-Statistic: 0.371; p-value: 0.711;

PIK3CA: Z-Statistic: -0.471; p-value: 0.637;

NRAS: Z-Statistic: 0.289; p-value: 0.773;

All p-values are greater than the common significance level of 0.05, indicating no statistically significant differences in the proportions of any gene mutation between males and females.

-Observed mutations vs primary tumor localization:

The distributions of gene mutations by count and frequency in relation to tumor localization are presented in the Table 12 and Figure 25 below. These visualizations illustrate the variations in mutation prevalence across different tumor sites.

Table 12. Mutations and primary tumor localization (*For 12 of the studied patients, there is a lack of data regarding the mutational profile due to insufficient material, technical reasons, and other factors.)

Localization	Total Mutations	AKT1	TP53	KRAS	BRAF	PIK3CA	NRAS
Right colon	42	2	20	14	14	7	1
Left colon	25	0	18	7	2	3	1
Rectum	29	0	21	14	0	1	2

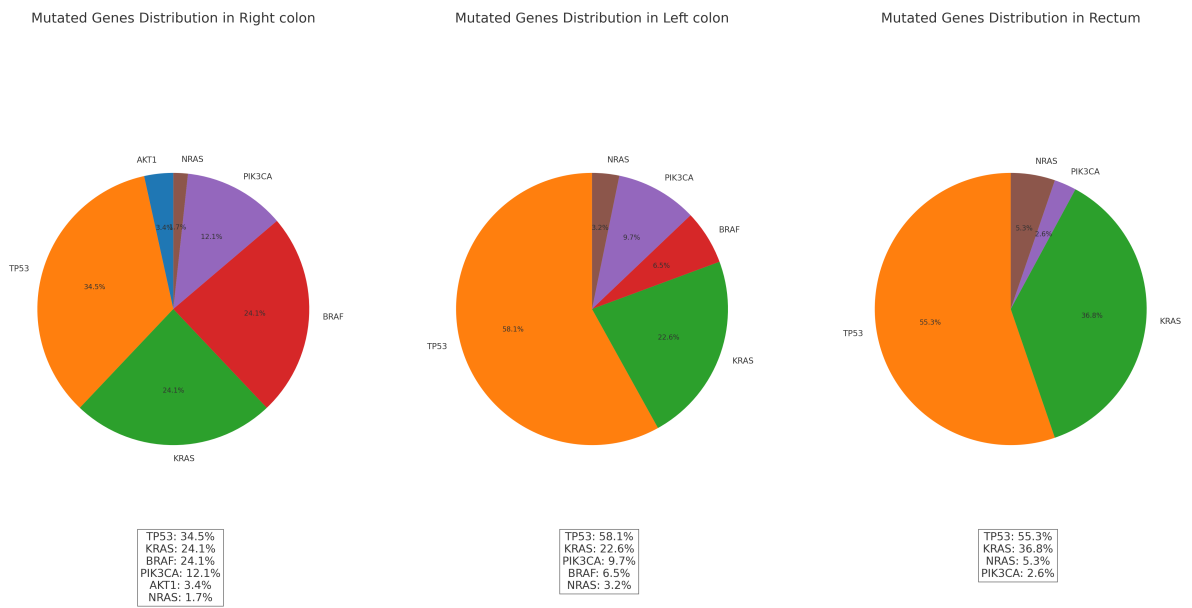


Figure 25. Mutated genes distribution by primary tumor localization- Right colon, Left colon, and Rectum

The chi-square test results for the association between gene mutations and localizations are as follows: Chi-square value: 22.59; p-value: 0.012; Degrees of freedom: 10. The p-value (0.012) is less than 0.05, indicating that there is a statistically significant association between gene mutations and localization. This suggests that the distribution of gene mutations differs significantly across the Right colon, Left colon, and Rectum.

Based on the statistical analysis, there is a significant association between gene mutations and localization:

1. Right colon:

Tumors located in the Right colon tend to exhibit the highest number of mutations overall (42), with a variety of gene mutations such as TP53, KRAS, BRAF, PIK3CA, and AKT1. BRAF and KRAS mutations are more frequent here compared to other locations. The high presence of mutations like BRAF in the right colon aligns with known patterns in colorectal cancer, and they indicates a sporadic pathway rather than Lynch syndrome.

2. Left colon:

The left colon tumors have fewer total mutations (25) compared to the right colon. The dominant mutations here include TP53 and KRAS, with PIK3CA also present but at a lower frequency. The mutations in the left colon tend to align more with classic genetic alterations seen in colorectal cancer. The lower count of BRAF mutations in this area differentiates it from the right colon.

3. Rectum:

The rectal carcinomas has 29 total mutations, with a higher frequency of TP53 mutations. Unlike the right colon, the rectum does not show any BRAF mutations, which is a key distinction. This suggests that the genetic profile of rectal cancers in this dataset differs significantly from right-sided colorectal cancers, which often exhibit BRAF mutations. The presence of KRAS and NRAS mutations indicates a different mutation spectrum in the rectum, which might be more associated with sporadic colorectal cancers rather than hereditary syndromes like Lynch.

-Mutations across tumor histological subtype:

The only gene mutation showing a significant association with histological type is BRAF. This suggests that the presence of BRAF mutations may vary depending on the histological subtype. The Table 13 displays the results of the Chi-square test comparing mutated genes with histological subtypes.

Table 13. Chi- square test results for mutations vs histological subtype

Gene	Chi-square value	p-value	Dof
AKT1	0.61	1.0	9
TP53	7.49	0.586	9
KRAS	10.982	0.277	9
BRAF	20.292	0.016	9
PIK3CA	10.988	0.277	9
NRAS	1.245	0.999	9

-Mutations across tumor grade and stage:

The performed chi-square test for gene mutations in relation to tumor grade and stage yielded the following results:

Tumor grade vs gene mutations:

AKT1 Chi-Square: 0.360; DOF: 2; p-value: 0.835.
TP53 Chi-Square: 5.194; DOF: 2; p-value: 0.074.
KRAS Chi-Square: 0.102; DOF: 2; p-value: 0.950.
BRAF Chi-Square: 12.070; DOF: 2; p-value: 0.002.
PIK3CA Chi-Square: 0.509; DOF: 2; p-value: 0.775.
NRAS Chi-Square: 0.761; DOF: 2; p-value: 0.684.

BRAF shows a statistically significant association with tumor grade.

G2 is the grade with the highest frequency of mutations, particularly TP53 and KRAS. G3 shows TP53 as the most frequent mutation, with a slight increase in BRAF mutations. G4 has the fewest mutations overall.

Tumor stage vs gene mutations:

AKT1 Chi-Square: 1.591; DOF: 5; p-value: 0.902.
TP53 Chi-Square: 4.116; DOF: 5; p-value: 0.533.
KRAS Chi-Square: 1.814; DOF: 5; p-value: 0.874.
BRAF Chi-Square: 6.304; DOF: 5; p-value: 0.278.
PIK3CA Chi-Square: 3.742; DOF: 5; p-value: 0.587.
NRAS Chi-Square: 11.929; DOF: 5; p-value: 0.036.

NRAS shows a statistically significant association with tumor stage.

Stages pT1 and pT2: These early stages demonstrate limited mutations, with TP53 and KRAS mutations appearing occasionally.

Stages pT3 and pT4: In these advanced stages, TP53 and KRAS mutations become more frequent. Additionally, mutations in BRAF and PIK3CA are observed in some cases, indicating a shift toward a more aggressive tumor profile.

Lymph node status and lymphovascular invasion vs gene mutations:

Lymph node status vs gene mutations:

Negative lymph node status: TP53 (27 cases) and KRAS (18 cases) are the most frequent mutations. BRAF appears in 10 cases.

Positive lymph node status: TP53 is the most common (34 cases), followed by KRAS (19 cases) and PIK3CA (8 cases).

Lymphovascular invasion vs gene mutations:

Negative Lymphovascular Invasion: TP53 (38 cases) and KRAS (28 cases) are prominent. BRAF and PIK3CA mutations are also present but less frequent.

Positive Lymphovascular Invasion: TP53 remains frequent (24 cases), but KRAS drops to 9 cases. BRAF is seen in 9 cases, and PIK3CA appears in 5 cases.

The results of the performed chi-square test indicate significant associations between specific gene mutations and both lymph node status and lymphovascular invasion in colorectal cancer:

TP53 Mutations: There is a notable association with both lymph node status and lymphovascular invasion.

Chi-square: 7.423; Dof: 2; p-value: 0.024 (significant association).

BRAF Mutations: A significant association with lymphovascular invasion was also observed for BRAF mutations.

Chi-square: 4.853; Dof: 1; p-value: 0.028 (significant association).

Analyses for gene mutations versus peritumoral budding, stromal immune reaction, and perineural invasion:

1. Peritumoral budding vs gene mutations:

Bd1(Bd0 + Bd1): TP53 and KRAS mutations are most frequent, with moderate occurrences of BRAF and PIK3CA.

Bd2: Similar frequency for TP53, with KRAS and BRAF appearing less frequently.

Bd3: Highest occurrence of TP53, followed by KRAS, BRAF, and some PIK3CA mutations.

2. Stromal immune reaction vs gene mutations:

Absent: TP53 and KRAS mutations are common, with minimal BRAF and PIK3CA.

Moderate: TP53 and KRAS are frequent, with a presence of BRAF but fewer PIK3CA mutations.

Marked: TP53 remains frequent, with higher occurrences of BRAF and PIK3CA.

3. Perineural invasion vs gene mutations:

Negative: TP53 and KRAS mutations are most frequent; BRAF and PIK3CA appear less frequently.

Positive: TP53 remains common, and there is an increase in PIK3CA mutations compared to negative cases.

The summary of the performed chi-square test for the associations reveals significant findings:

TP53 and peritumoral budding: Chi-Square = 6.071; p-value = 0.048; DOF = 2. This indicates a statistically significant association between TP53 mutations and peritumoral budding, suggesting that the presence of TP53 mutations may influence the tumor’s invasive characteristics, potentially impacting prognosis and treatment options.

NRAS and stromal immune reaction: Chi-square = 7.645; p-value = 0.022; DOF = 2. The significant association between NRAS mutations and the stromal immune reaction indicates that NRAS may play a role in the immune landscape surrounding tumors, which could have implications for therapeutic strategies.

These results highlight the importance of genetic profiling in colorectal cancer, as understanding the relationships between specific mutations and clinical features can help inform prognosis and treatment decisions.

MMR status (pMMR and dMMR) versus gene mutations:

Lastly, in this section, we will examine the association between gene mutations and MMR status. In Table 14, the results of the MMR status in relation to the mutations of individual genes are presented. This table highlights the correlation between the MMR (mismatch repair) status and specific gene mutations, which can provide insights into the genetic landscape of colorectal cancer.

Table 14. MMR status in relation to the mutations

MMR status	AKT1	TP53	KRAS	BRAF	PIK3CA	NRAS
dMMR	1	8	1	12	4	1
pMMR	1	54	36	5	7	3

Only KRAS and BRAF mutations show significant associations with MMR status:

KRAS: Chi-square: 7.739; p-value: 0.005; Dof: 1.

BRAF: Chi-square: 34.204; p-value: <0.001; Dof: 1.

These results indicate a strong relationship between these specific mutations and the MMR status of colorectal tumors. The significant p-values suggest that the presence of KRAS and BRAF mutations is associated with specific MMR status groups, which may have implications for treatment decisions and prognostic outcomes.

4. Discussion

1. Patient demographics:

Age and Sex Distribution The cohort consists of 100 patients, with a slight male predominance (57%). The median age of diagnosis is 70 years, consistent with global CRC demographics where incidence increases with age. Studies like those by Siegel et al. confirm this age range, reporting similar median ages in CRC populations globally[2]. Notably, the analysis indicated no statistically significant association between sex and tumor localization (χ^2 : 2.95, p-value: 0.938), aligning with the findings of Kishiki et al., who also found no gender-specific localization preference[57].

2. Tumor localization and its clinical implications. In this cohort, right-sided colon cancers are predominant, consistent with observations that right-sided tumors often feature distinct molecular profiles, such as dMMR status. The study reports that 89% of dMMR cases occur in right-sided tumors, supporting findings from Yahagi et al., which highlight the prevalence of genetic anomalies like BRAF mutations in this localization[50]. The statistically significant correlation between MMR status and tumor localization (χ^2 : 20.28, p-value: 0.000039) confirms the distinct biological behavior observed in right-sided CRCs.

3. Tumor grade and stage

Most tumors in the cohort are of moderate grade (G2), with advanced stages (T3/T4) being common. There is a significant correlation between tumor grade and lymphovascular invasion (LVI) (χ^2 : 17.33; p-value: 0.00017), supporting the hypothesis that higher grades are associated with more aggressive behavior. These results align with Saito et al., who also identified higher rates of LVI in advanced CRC grades[48]. The study found no significant relationship between tumor budding and overall stage ($p > 0.05$), a result that mirrors findings by Benedix et al., who noted that tumor budding's impact varies based on additional factors like tumor localization and genetic mutations[21].

4. Lymphovascular invasion (LVI) and perineural invasion (PNI)

Lymphovascular invasion (LVI) and perineural invasion (PNI) LVI was detected in 26% of cases, showing significant association with higher tumor grades and stages ($p < 0.05$). This aligns with the work of Des Guetz et al., who identified LVI as a reliable marker for predicting poor prognosis and increased metastasis risk in CRC patients [58]. However, PNI did not exhibit statistically significant correlations with tumor grade, stage, or localization (all p-values > 0.05). This reflects findings from studies like Lee et al., who reported the inconsistent prognostic value of PNI across CRC cases[59].

5. Lymph node status and tumor stage

Correlation lymph node involvement increased with tumor stage, notably in T3 and T4 stages, a pattern consistent with established CRC literature that underscores the role of lymphatic spread in tumor progression. Lugli et al. highlight the importance of lymph node evaluation, stating that node positivity is one of the strongest indicators of disease progression. The lack of a statistically significant association between tumor budding and lymph node status (p-value: 0.132) suggests that lymph node involvement is influenced by multiple histological and molecular factors beyond peritumoral budding.

6. Mutations

Mutations and MMR status: Genetic analysis identified several key mutations, including KRAS, BRAF, and TP53. The majority of BRAF mutations were in right-sided tumors, in line with the literature on sporadic CRC cases. dMMR were prevalent in 89% of right-sided cases, reinforcing their role as biomarkers for this subtype. This matches findings by Boland and Goel, who confirm the diagnostic and therapeutic significance of MSI and MMR status in CRC management[60].

7. Comparison with other studies

Tumor budding and immune microenvironment Tumor budding was significantly correlated with higher tumor grades in this cohort, validating its role as a marker for aggressive tumor behavior. Lugli et al. noted similar patterns, emphasizing the association between tumor budding and adverse clinical outcomes[47]. Additionally, the immune response, correlate with better prognosis, consistent with Saito et al., who demonstrated the prognostic value of immune engagement in MSI-H cases[48].

8. Pathological implications of mutations in CRC

The study highlights the prognostic significance of mutations like KRAS, BRAF, and TP53. KRAS mutations, found in various tumor stages and locations, are known to confer resistance to anti-EGFR therapies, necessitating alternative treatment strategies. BRAF mutations, predominantly in right-sided cancers, are associated with poor prognosis and necessitate targeted approaches, echoing findings from Kopetz et al[61]. BRAF mutations in colorectal cancer (CRC) have been associated with a higher likelihood of metastatic disease, highlighting the aggressive nature of this subtype. Research by Domingo et al. emphasizes that BRAF-mutated CRC is characterized by distinct biological behaviors and poorer prognostic outcomes. These mutations are often linked to a more advanced stage at diagnosis and a tendency for early metastasis, particularly to the liver and peritoneum[62]. TP53 mutations are frequently observed across various tumor stages and localizations in colorectal cancer (CRC), highlighting their significant role in tumor progression. These mutations contribute to the molecular complexity and heterogeneity of CRC, influencing both the biological behavior of tumors and their responses to therapies[63].

5. Conclusions

Our team leaves the opportunity for the esteemed audience to draw their own conclusions.

Author Contributions: The contributions of the individual authors in this study are as follows: Krasimir Petrov: conceptualization, methodology, software, resources, validation, investigation, data curation, writing, project administration. Ivan Ivanov: conceptualization, methodology, software, resources, validation, investigation, data curation, supervision. Savelina Popovska: conceptualization, methodology, supervision, project administration, funding acquisition. Tatyana Betova: investigation. Zornica Kamburova: NGS testing -methodology, validation, formal analysis, investigation, data curation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data from this study is available on the Science Data Bank website: <https://www.scidb.cn>. Interested parties may access the data by submitting a request to the authors of the study.

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Abbreviations

The following abbreviations are used in this manuscript:

CRC	Colorectal cancer
PNI	Perineural invasion
LVI	Lymphovascular invasion
MMR	Mismatch repair
NGS	Next generation sequencing

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