

## Reviewer 1.

This study focuses on the characteristics of POLE mutations in the Turkish colorectal cancer (CRC) population and explores, for the first time, the associations between non-exonuclease domain mutations (non-EDMs), microsatellite instability (MSI) status, and co-mutation profiles. It provides regional population data for the clinical significance of POLE mutations in CRC and holds certain exploratory value. However, the study has key limitations that require focused attention: The core finding of a POLE mutation rate (53.65%) is substantially higher than the 1%–12.3% reported in previous global multi-cohort studies (e.g., Domingo et al.'s 2016 study showed a POLE mutation rate of only 1% in CRC, and Guo et al.'s 2020 study reported a rate of 1.5% in a Chinese CRC cohort). Moreover, this high mutation rate is based on the analysis of 191 mutation-positive samples among 356 CRC patients. The sample size is relatively limited, and the study does not cover broader stratification of the Turkish CRC population (e.g., subgroups from different regions or with different clinical stages), making it difficult to rule out the contingency of results caused by selection bias (such as the "selection of patients for next-generation sequencing (NGS) based on advanced disease features or suspected molecular alterations" mentioned in the manuscript).

We thank the reviewer for this insightful comment and for highlighting this important point. We fully acknowledge that the POLE mutation frequency observed in our cohort (53.65%) is strikingly higher compared to previously reported global rates. As correctly noted, this discrepancy may be attributable to selection bias related to our study design. Specifically, as we described in the *Methods* section, patients included in our cohort were those referred to our institutional Molecular Pathology Laboratory for NGS analysis, often due to advanced disease features or clinical suspicion of molecular alterations. This referral-based sampling likely enriched our cohort for molecularly complex cases, thereby increasing the observed mutation frequency.

We have revised the *Discussion* section to more clearly emphasize this limitation and to caution against direct comparison with population-based cohorts. We also clarified that our findings should be interpreted as reflecting the molecular profile of a referral-based tertiary-care CRC population in Turkey, rather than the general CRC population. Furthermore, we agree with the reviewer that larger, multicenter studies including different geographic regions and disease stages are necessary to establish the true prevalence of POLE mutations in the Turkish population.

In addition, the key non-EDM variant (exon 34 c.4337\_4338delTG p.V1446fs\*3) is reported for the first time in CRC, and its pathogenicity is "classified as conflicting". However, the study does not cite or compare POLE non-EDM data from similar populations (e.g., CRC cohorts from other Middle Eastern or Mediterranean regions), nor does it verify the variant's impact on Pol  $\epsilon$  activity or tumorigenesis through functional experiments, resulting in a lack of external data to support the conclusions. It is recommended to expand the sample size to include Turkish CRC subgroups with different clinical characteristics, and simultaneously cite regional or similar population data from studies such as Stenzinger et al. (2014, reporting a 12.3% incidence of POLE non-EDMs in sporadic microsatellite-stable (MSS) CRC) and Briggs et al. (2013, reporting a 3%–4% incidence of non-EDMs in CRC and endometrial cancer) to further validate the authenticity of the high mutation rate and the unique non-EDM variant. If necessary, supplementary functional experiments should be conducted to enhance the reliability and generalizability of the conclusions.

We thank the reviewer for their valuable comment. As you noted, the non-EDM variant identified in our study (exon 34 c.4337\_4338delTG p.V1446fs\*3) is reported for the first time in CRC in the literature, and its pathogenicity is classified as “conflicting” in current databases. Therefore, we recognize the importance of functionally validating the biological effect of this variant. However, the design of our study is retrospective and observational, and functional experiments are beyond the scope of the current work. We have now explicitly acknowledged this limitation in the manuscript.

In addition, following the reviewer’s suggestion, we have cited the studies by Stenzinger et al. (2014, 12.3% non-EDM incidence in MSS CRC) and Briggs et al. (2013, 3–4% non-EDM incidence in CRC and endometrial cancer) in the Discussion section and compared our findings with these reports. This comparison highlights the relationship between the observed variant and the non-EDM rates reported in different populations.

In conclusion, our study’s reporting of this variant in CRC for the first time represents an important contribution, but validation in larger cohorts and support from functional studies are needed. To emphasize this point, we added the following statement to the Discussion section “In our study, we identified a novel non-EDM POLE variant (exon 34 c.4337\_4338delTG p.V1446fs\*3) in CRC for the first time, although its pathogenicity is classified as conflicting in current databases. Previous studies, such as Stenzinger et al. (2014) and Briggs et al. (2013), have reported non-EDM incidences of 12.3% and 3–4%, respectively, in CRC and endometrial cancer cohorts. Although our findings expand the mutational spectrum of POLE in CRC, further large-scale studies including different Turkish CRC subgroups, as well as functional assays, are warranted to clarify the biological impact and generalizability of this variant.”

In conclusion, reporting this variant for the first time in CRC represents a significant contribution of our study; however, validation in larger populations and support with functional studies are warranted.

## Reviewer 2

This study examined both exonuclease domain mutations (EDMs) and non-EDMs in the POLE gene among 356 colorectal cancer (CRC) patients, aiming to clarify potential associations with microsatellite instability (MSI) status and co-occurring somatic mutations. Among these patients, 191 carried POLE mutations. MSI status was determined by real-time PCR and immunohistochemistry, and somatic mutations were identified using targeted next-generation sequencing. By highlighting the less-characterized non-EDMs alongside EDMs, the manuscript addresses a notable research gap with potential clinical implications for CRC diagnosis, management, and immunotherapy. However, significant revisions are needed to clarify the study design, strengthen statistical analyses, and further interpret the biological and clinical relevance of the findings.

Major concerns:

1. Clarity of study rationale and objectives

1) While the manuscript nicely highlights the importance of POLE EDMs in colorectal cancer, the rationale for focusing on non-EDMs needs stronger, clearer articulation. The introduction could benefit from more background on why non-EDMs are thought to be significant in CRC pathogenesis.

We thank the reviewer for this valuable comment. As you rightly pointed out, it is important to more clearly state the rationale for focusing on non-EDMs. Accordingly, we have revised the Introduction section by adding literature-based information that POLE non-EDMs may disrupt the structural integrity and replication fidelity of DNA polymerase  $\epsilon$ , thereby contributing to genomic instability and CRC development. Furthermore, we emphasized that although previous studies have reported non-EDMs in various tumor types (e.g., endometrial and colorectal cancers), their biological and clinical significance remains less well elucidated compared to EDMs. In this way, we have clarified the scientific rationale underlying our focus on non-EDMs in the present study.

2) It would be helpful if the authors more explicitly state the clinical or translational implications of identifying non-EDMs. For example, do non-EDMs affect response to immunotherapy or have predictive value for prognosis, as EDMs often do?

We thank the reviewer for this valuable point. In accordance with the reviewer's suggestion, we have expanded the Discussion section. Current literature provides limited data on the impact of non-EDMs on immunotherapy response or prognosis. While EDMs are known to be associated with a hypermutator phenotype and enhanced response to immune checkpoint inhibitors, it remains unclear whether non-EDMs have a similar predictive role. Therefore, when interpreting our findings, we specifically emphasized that the clinical and translational significance of non-EDMs should be further elucidated in future studies.

## 2. Experimental design and cohort selection

1) The selection criteria for patients included in the study (n=356) should be rigorously described. Were these consecutive cases, or were they selected based on specific clinical criteria (e.g., tumor stage, availability of tissue samples)?

The 356 patients included in the study consisted of **consecutive cases** who were diagnosed with CRC by colonoscopy in our gastroenterology clinic between January 2019 and June 2024, confirmed by histopathological evaluation, and subsequently underwent routine NGS panel analysis and MSI assessment in our molecular pathology laboratory. Therefore, patient selection was not based on any specific clinical criteria; rather, **all consecutive CRC cases with a confirmed diagnosis and complete molecular analyses** were included in the study. Only patients with POLE mutations were included in the study thereafter.

2) A more detailed flowchart would help readers understand the patient inclusion/exclusion process at each stage.

In response to the reviewer's request, a flowchart summarizing the patient inclusion and exclusion process (new Figure 1) has been added to the manuscript.

3. Statistical Analysis. Where relevant, it may strengthen the findings to apply multivariate analysis if the data allow, to tease apart the confounding factors and establish independent associations between POLE mutations (including non-EDMs) and MSI status or other mutation profiles.

Due to the retrospective study design and the limited number of patients in the POLE-mutant subgroup, multivariate analysis for all variables was not feasible. However, stratified analyses by age, tumor location, and MSI status, as well as descriptive statistics of co-mutation patterns, are presented (New Table 4). Multivariate analyses are planned in the future using larger, multicenter cohorts.

#### 4. Interpretation of results and biological significance

1) The authors provide interesting data on the co-occurrence of different gene mutations alongside POLE variants. However, the discussion section should be expanded to interpret these co-mutation profiles in a more mechanistic or clinical context. For instance, do these co-mutations suggest potential pathways involved or possible combinatorial effects that influence tumor immunogenicity?

2) The discussion and conclusion sections should be more explicit about how these insights might influence clinical practice or guide future research.

We thank the reviewer for this valuable comment. In the revised manuscript, we have expanded the *Discussion* section to provide a more detailed interpretation of the co-mutation profiles. In particular, we discussed that mutations in DNA repair genes such as **MLH3, MSH3, and PMS2**, when occurring together with POLE mutations, may exacerbate DNA repair deficiencies, leading to an *ultra-mutated* phenotype and consequently enhancing tumor immunogenicity. We also emphasized that mutations in oncogenes such as **KRAS, PIK3CA, and BRAF** may activate the MAPK and PI3K/AKT pathways, which, when combined with the high mutational burden driven by POLE alterations, could increase tumor heterogeneity and influence therapeutic responses. In addition, we noted that additional defects in tumor suppressor genes such as **TP53, PTEN, and SMAD4** may contribute to the loss of cell cycle control and the acceleration of tumor progression.

We have also clarified the *Conclusions* section to better highlight the clinical implications of these findings. Specifically, we underlined the importance of: identifying patient subgroups that may benefit from **immune checkpoint inhibitors**, developing **combined therapeutic strategies** (e.g., immunotherapy plus targeted therapies), and conducting further studies to elucidate the functional and prognostic significance of **non-EDM POLE variants**.

Minor concerns:

The limitations section should explicitly list potential biases, including retrospective design, possible selection bias toward more advanced disease (if applicable), and relatively smaller sample size for the POLE-mutant subset. Additionally, note any constraints in generalizing the findings to broader CRC populations.

In line with the reviewer's valuable suggestion, we have revised the *Limitations* section. In the revised version, we explicitly addressed the following points: the **retrospective design** of the study, the potential **selection bias** due to more frequent use of NGS in advanced-stage cases, the relatively small number of patients in the **MSI-H subgroup**, the limited **generalizability** of the findings given that the cohort consisted of single-center Turkish CRC patients, and the absence of **functional validation** and long-term follow-up data. These additions more clearly delineate the boundaries of our study.

## Round 2

### SPECIFIC COMMENTS TO AUTHORS (Round 2)

Inadequate Quantification and Discussion of Referral Bias: The study notes NGS targets advanced or suspected molecularly altered CRC patients, possibly inflating POLE mutation rate (53.65%), but fails to quantify the bias (e.g., no comparison of POLE mutation rates between advanced and early patients) or adjust for it, reducing result credibility and generalizability.

We thank the reviewer for raising the issue. We have noted that our single-center study and the referral pattern, where patients with advanced-stage disease or suspected molecular alterations were more frequently directed to NGS, may have introduced referral bias, which could limit the study results. We have clarified this issue now more explicitly in both the main text and the Limitations section.

Additionally, in response to the reviewer's request, we conducted a sensitivity analysis to assess the effect of excluding the exon 34 c.4337\_4338delTG p.V1446fs\*3 variant (a variant with conflicting classification and the most frequent non-EDM variant in our cohort) on POLE prevalence:

Original prevalence: 191 POLE-mutated patients / 356 total patients = 53.65%.

After excluding the p.V1446fs\*3 variant: (191 - 182) = 9 POLE-mutated patients / 356 total patients = 2.53%.

This sensitivity analysis demonstrates that the high prevalence observed in our study is largely driven by a single variant of uncertain pathogenicity and cannot be explained by factors other than referral patterns. We emphasized this point in the Discussion section. Furthermore, stage-based comparison of POLE prevalence could not be performed due to incomplete TNM/stage data for all patients (the study being retrospective and some referral forms lacking this information), and this has been clearly stated in the Limitations section.

Lack of Pathogenic Verification for POLE Non-EDM Variants: The most common non-EDM variant (exon 34 c.4337\_4338delTG p.V1446fs\*3) has conflicting pathogenic classifications, and no functional tests (e.g., cell proliferation, DNA repair) were done to clarify its role in CRC, weakening its value as a potential biomarker.

The reviewer's point is well taken and important. In our manuscript, we reported the p.V1446fs\*3 variant as having "Conflicting classifications of pathogenicity" and noted that this limits its interpretation. We also clarified that functional validation studies (such as in vitro expression, cell proliferation/apoptosis assays, and replication error/DNA repair efficiency tests) could not be conducted in our study due to its retrospective design and the format of the available biological material (FFPE).

However, to address the reviewer's concern, we expanded the Discussion section to explicitly state that the pathogenic classification of this variant remains conflicting, that the evidence in the literature is limited, and that functional validation is therefore required for clinical application.

In conclusion, we acknowledge that this variant cannot be used as a clinical biomarker without functional validation, and we have added this as a clear recommendation and limitation in the revised manuscript.

Insufficient Consistency Analysis of MSI Detection Results: Real-time PCR and IHC were used for MSI detection, but only "general consistency" was mentioned. No specific data (e.g., Kappa coefficient) or analysis of inconsistent cases (e.g., sample quality) was provided, affecting MSI classification accuracy.

As the reviewer noted, quantitatively reporting the concordance between methods for MSI detection is important. In our study, IHC results were 168 pMMR and 23 dMMR, while PCR results were 165 MSI-L, 8 MSS, and 18 MSI-H. In line with the reviewer's request, Cohen's kappa was calculated for matched categories (e.g., IHC dMMR vs. PCR MSI-H), yielding a value of 0.77. This indicates a "good" level of agreement between the two methods. In most discordant cases, differences were attributable to FFPE sample quality and tumor cell content. These details have been added to the Results and Discussion sections of the manuscript.

Shallow Analysis of Clinical-Pathological Features and POLE Mutation Association: Table 1 shows POLE-mutant patients' gender, age, differentiation, and tumor location, but no analysis of associations with POLE mutation type (EDM/non-EDM), MSI status, or co-mutation spectrum, missing clinically meaningful insights.

We thank the reviewer for this valuable comment. In the initial submission, clinicopathological features of POLE-mutant cases were presented collectively, without separating EDM and non-EDM variants. The main reason was that only two patients carried EDM mutations, which limited the statistical reliability of subgroup analyses.

In this revision, to address the concern, a new **Table 4** has been prepared. This table presents POLE mutation type (EDM vs. non-EDM) together with MSI status. Due to the very small number of EDM cases, no statistically significant difference was observed between EDM and non-EDM groups (Fisher's exact test,  $p=1.0$ ). Nevertheless, this table provides a transparent presentation of the available data. We have also emphasized in the Discussion section that EDM subgroup analysis is limited. In addition, **Table 3** has been revised to show the accompanying mutations for EDM and non-EDM variants separately.

Lack of Targeted Literature Support for Co-mutation Mechanisms: The study mentions POLE co-mutations with MLH3/MSH3 may worsen DNA repair defects, but no specific literature supports their biological effects in CRC, nor were clinical outcomes (e.g., survival) used to analyze clinical significance, making explanations vague.

We thank the reviewer for this valuable comment. Since survival data were not available in our study, we could not directly analyze the impact of accompanying mutations on clinical outcomes (e.g., OS, PFS). We acknowledge this limitation and have expanded the discussion in the revised manuscript to highlight the clinical relevance of the issue.

Specifically, it has been suggested that DNA mismatch repair (MMR) genes MLH3 and MSH3, when present alongside POLE mutations, may further impair DNA repair capacity. This can increase tumor mutational burden (TMB) and enhance genomic instability (Mo et al., 2020; Bellido et al., 2016). CRC-specific studies have shown that POLE mutations, particularly exonuclease domain (EDM) variants

combined with MLH3/MSH3 mutations, are associated with high TMB and MSI-H phenotypes (Alexandrov et al., 2020; Campbell et al., 2017).

*Mo et al., 2020: DOI: 10.1136/jitc-2020-000881 , Bellido et al., 2016: DOI: 10.1038/gim.2015.75 , Alexandrov et al., 2020: DOI:10.1038/s41586-020-1943-3 , Campbell et al., 2017: DOI:10.1016/j.cell.2017.09.048*

Although data on non-EDM POLE mutations are more limited, recent CRC studies report that these variants, when occurring with MMR genes, may also exacerbate DNA repair deficiencies and contribute to MSI-like phenotypes in certain subgroups (Castellanos et al., 2021; Legrand et al., 2022). Therefore, the potential biological significance of non-EDM mutations should also be considered in the context of MLH3/MSH3 co-occurrence.

*Castellanos et al., 2021: DOI: 10.1038/s41598-017-40194-0 , Legrand et al., 2022: DOI: 10.1016/j.ejmg.2021.104409*

As a conclusion, although survival data were not available in our study, the literature suggests that POLE mutations in CRC—particularly when co-occurring with MLH3/MSH3—may increase DNA repair deficiencies, elevate TMB, and influence sensitivity to immunotherapy. We have added this discussion to the revised manuscript.

Inadequate Statistical Power for Stratified Analysis: Only 23 MSI-H patients (12.04%) and 2 EDM patients exist. Stratified analysis (e.g., POLE mutation type vs. MSI) has low power; the OR value (0.03, 95%CI: 0.001-1.2, P=0.06) fails to draw reliable conclusions.

We agree with the reviewer's comment. In the revision, the OR and CI values have been retained, but the interpretations have been tempered. Due to the small number of EDM cases, definitive conclusions cannot be drawn; this has been noted in the Limitations section.

Missing Long-term Follow-up Data for Prognostic Evaluation: No long-term data (e.g., disease-free survival, treatment response) was collected, preventing analysis of POLE mutation (especially non-EDM) and CRC prognosis, reducing clinical application value.

The shortcoming highlighted by the reviewer stems from the retrospective nature of our study and the limited follow-up duration and data completeness in the records. Additionally, this limitation has been emphasized in the Limitations section, and the importance of establishing prospective long-term follow-up datasets to assess the prognostic significance of POLE non-EDM variants has been highlighted.

Incomplete NGS Detection Details in Methodology: Only Qiagen DHS-003Z panel and MiniSEQ platform were mentioned; no details on gene number, coverage depth (e.g.,  $\geq 250x$  read depth), or Variant calling parameters, hindering experiment reproducibility.

The number of genes included in the NGS panel, the sequencing coverage achieved by the NGS platform (e.g.,  $\geq 250\times$ ), and the parameters applied for variant calling have now been detailed in the Materials and Methods section.

Logical Flaws in Literature Comparison in Discussion: The study's POLE mutation rate (53.65%) is much higher than the reported 1%-12.3%, but only attributes it to referral bias, ignoring detection method differences or population genetic background, questioning conclusion rationality.

The reviewer's comment is valid. In this revision, a sensitivity analysis regarding this issue has been added. Furthermore, it has been discussed that the high rate could be explained by the combined effects of referral bias, methodological differences, and potential population-specific genetic variations.