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# High tumor mutational burden assessed through next-generation sequencing predicts favorable survival in microsatellite stable metastatic colon cancer patients

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## Abstract

**Background** Microsatellite instability (MSI) is a well-established predictive biomarker for immune checkpoint inhibitor (ICI) response in metastatic colon cancer. Both high MSI and tumor mutational burden (TMB) are markers of genomic instability. However, the prognostic and predictive value of TMB in patients with microsatellite stable (MSS) tumors remains unclear.

**Methods** We evaluated the prognostic significance of TMB levels in MSS metastatic colon cancer patients undergoing standard treatments. Tumor responses were assessed using RECIST v1.1 criteria. Comprehensive clinical and molecular profiling was conducted, including next-generation sequencing (NGS) for TMB evaluation with the TruSight Oncology<sup>®</sup> kit. Overall survival (OS) was the primary endpoint. Multivariate Cox regression analysis was utilized to assess the relationship among potential prognostic factors.

**Results** Among 102 MSS metastatic colon cancer patients, high TMB (> 10 mut/mb) was associated with a significantly longer median OS compared to low TMB (70.0 vs 45.0 months, respectively; HR: 0.45; 95% CIs 0.21 to 0.96; P = 0.0396). Multivariate analysis, adjusting for age, gender, number of metastatic sites, response to first-line chemotherapy, RAS mutational status, and liver involvement, identified TMB as an independent prognostic factor, along with response to first-line chemotherapy.

**Conclusions** Our results highlight the prognostic significance of TMB in MSS metastatic colon cancer patients, suggesting its potential role in patient stratification and treatment decision-making.

**Keywords** Microsatellite instability, Tumor mutational burden, Colon cancer, Prognosis, NGS

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## Background

Colorectal cancer (CRC) is one of the most common malignancies worldwide, with over 1 million new cases and 600,000 deaths annually. Despite significant advances in early detection and treatment, about 40% of patients present with or will eventually develop metastatic disease [1]. In recent years, the treatment of metastatic colorectal cancer (mCRC) has been enriched with new biologic drugs, including small molecules and monoclonal antibodies, which can be administered as monotherapy or in combination with chemotherapy [2]. However, despite the improvement in median survival to over 24 months through a continuum-of-care approach using all available treatments, mCRC remains a significant cause of morbidity and mortality, with a five-year survival rate of only 10–15% [1, 2]. One of the latest advancements in treatment is immunotherapy. The Food and Drug Administration (FDA) has recently approved pembrolizumab, an anti-PD-1 immune checkpoint inhibitor (ICI), for treating microsatellite instability (MSI) or high tumor mutational burden (TMB) solid tumors, including mCRC. Pembrolizumab works by restoring the immune system ability to attack tumor cells [3, 4].

MSI tumors are characterized by numerous errors in microsatellite DNA sequences due to a deficient mismatch repair system (dMMR), leading to mutation accumulation [5]. Microsatellite status can be characterized using different techniques (from immunohistochemistry to next-generation sequencing, NGS), all of which show good concordance [6]. Only about 5% of mCRC patients exhibit MSI status, limiting the patient population that can benefit from ICIs.

Another biomarker that directly measures the mutational propensity of neoplastic cells is the TMB. TMB is typically defined as the total number of somatic non-synonymous mutations per coding region of a tumor genome (mutations per megabase, mut/mb) [7]. Similar to MSI, the focus on TMB as a biomarker in solid tumors is primarily due to its potential to predict response to immunotherapy. A higher TMB likely indicates a greater presence of neoantigens capable of eliciting an antitumor immune response. In melanoma and non-small cell lung cancer (NSCLC), the predictive role of TMB is well-established, showing that high TMB correlates with excellent responses to ICIs [8, 9]. Prognostically, the results are mixed. In lung cancer, high TMB (>8 mut/Mb vs ≤8 mut/Mb) is associated with better survival, especially in stage I-III patients who underwent lung resection and standard post-operative treatments. High TMB is an independent variable in multivariate analysis adjusted for treatment type, age, sex, performance status, histology, and T/N stage [10]. Assessing the prognostic role of TMB in melanoma outside the context of ICIs is

challenging, as ICIs are the standard treatment. However, a significant study evaluated the mortality risk of patients with high versus low TMB across various solid tumors, both before and after the introduction of ICIs treatment. In NSCLC and melanoma, high TMB shifted from a negative to a positive prognostic factor with the introduction of ICIs. In contrast, high TMB consistently favored prognosis in colorectal, endometrial, and bladder cancers, independently from ICI treatment [11].

However, in CRC, the results are contradictory. A study analyzing two public datasets (MSKCC, Memorial Sloan Kettering Cancer Center, and TCGA, The Cancer Genome Atlas) did not find a significant difference in survival between patients with high vs low TMB, except in those with *KRAS* mutations, where high TMB was associated with better prognosis. However, these patients were highly heterogeneous, especially regarding staging, which included all stages from I to IV [12]. Another study examining TMB in mCRC patients enrolled in the FIRE-3 trial found no prognostic role for TMB (high >8 Mut/Mb vs low ≤8 Mut/Mb) [13]. Another study, which categorized TMB as low, intermediate, or high (<7, 7–16, or ≥17 Mut/Mb, respectively), suggested a trend indicating that high TMB is associated with improved prognosis in metastatic colorectal cancer [14].

The present study was undertaken to measure the prognostic effect of TMB in a well-characterized cohort of MSS metastatic colon cancer patients outside the context of ICIs treatment.

## Methods

### Study design and primary objective

This single-center, observational study aimed to explore the prognostic power of TMB in metastatic colon cancer (mCC) patients with MSS tumors and who received standard treatments.

### Patients' selection and clinical management

Between January 2016 and January 2024, patients were enrolled and managed according to European Society of Medical Oncology (ESMO) guidelines [15]. To control prognostic variables, exclusions were made for patients with Performance Status (PS) Eastern Cooperative Oncology Group (ECOG) ≥2, cachexia risk >1 [16], peritoneal carcinomatosis, and life expectancy <3 months (as assessed by the oncologists involved in the study). Inclusion criteria included age >18 years, MSS tumors confirmed by immunohistochemical analysis of MMR proteins [17] and verified through NGS. Only patients with colon cancer were included to ensure homogeneous and interpretable results, recognizing the distinct clinical, biological, and molecular characteristics of colon versus rectal

cancers. Patients were monitored regularly with total body Computed Tomography (tbCT) scans and/or Magnetic Resonance Imaging (MRI) every six months, and treatment responses were evaluated according to Response Evaluation Criteria In Solid Tumors (RECIST v1.1) [18]. The disease control (DC) rate was defined as the proportion of patients achieving complete or partial responses or stable disease, while progressive disease indicated a lack of disease control.

As the study was non-therapeutic, formal ethics approval was not required per institutional guidelines. The study adhered to the Declaration of Helsinki principles, and all participants provided written informed consent before treatment or genetic testing.

### Tumor specimens and sequencing

Primary colon cancer samples were collected as formalin-fixed and paraffin-embedded (FFPE) tissue specimens, and tumor cell microdissection was performed under morphological guidance. DNA was extracted using the MGF03-Genomic DNA FFPE One-Step Kit following the manufacturer's instructions (MagCore Diatech). The DNA quality was evaluated in triplicate with the FFPE QC Kit, following the manufacturer's guidelines (Illumina, San Diego, USA). Libraries were created using the TruSight Oncology<sup>®</sup> 500 kit, which targets 523 cancer-related genes (full list in Supplementary File 1). This assay detects a range of genomic alterations, including small nucleotide variants (SNVs), indels, splice variants, copy number variants, fusions, and immunotherapy biomarkers like TMB and microsatellite status. Sequencing was performed on an Illumina NovaSeq 6000 platform (San Diego, USA). TMB quantification was performed as described by Chalmers et al. [19]. This process included all coding somatic base substitutions and insertions/deletions (indels) within the targeted regions, encompassing synonymous mutations. Variant calling and TMB calculation algorithms were kept independently to ensure accurate coding variant counts (see Manufacturer Instructions at <https://emea.support.illumina.com/>). The targeted coding genomic region spanned 1.9 Mb. An accurate exome-based predictive model for MSI classification was used, employing a statistical classifier derived from somatic mutation profiles to differentiate MSI from MSS tumors. This classifier was trained on 999 exome-sequenced TCGA tumor samples with known MSI status (based on mononucleotide markers) and demonstrated a positive predictive value of 98.9% and a negative predictive value of 98.8% in an independent test set of 427 samples (see Manufacturer Instructions at <https://emea.support.illumina.com/>).

### Bioinformatics analysis and data presentation

The bioinformatics pipeline of Illumina TruSight Oncology 500 was used for the analysis of sequencing data. A median of 117 million reads was generated per sample, with coverage in the target region exceeding the manufacturer's suggested threshold of 150X. Sequence data were aligned to the human reference genome GRCh37 using the Burrows–Wheeler Aligner with default parameters [20]. Population- and cancer-specific variants were cross-referenced with several databases, including GENCODE, dbNSFP, ICGC-PCAWG, COSMIC, 1000Genomes, ClinVar, CancerMine, OncoScore, CIViC, and CBMDB, to evaluate clinical significance. Variants were filtered using unmatched normal datasets and excluded if the global minor allele frequency was <1%. Prioritization of variants followed a four-tiered structure (Tier 1–4), in line with AMP/ACMG joint consensus recommendations [21]. Variants with strong clinical significance in cancer were identified based on evidence levels from databases such as CIViC and Cancer Biomarkers.

The prognostic impact of clinic-pathological variables on overall survival (OS) was investigated. OS was calculated from metastatic disease diagnosis until death from colon cancer (cancer-specific survival). Progression-free survival (PFS) was not included due to heterogeneous treatments and radiologic evaluations, making vital status a more reliable outcome. Data were obtained from an electronic database (analyzed in April 2024) containing clinical and pathological information of mCC patients treated at the unit of Innovative Therapies for Abdominal Metastases of the Istituto Nazionale Tumori di Napoli “G. Pascale”. Covariates were dichotomized for univariate and multivariate analyses: age ( $\geq 70$  vs <70 years), gender (male vs female), metastatic involvement (more than one site vs one site), response to first-line therapy (disease control vs no disease control), *RAS* mutational status variants (wild-type vs mutated), liver involvement (yes vs no), and TMB high (high  $\geq 10$  mut/mg vs low <10 mut/mg). Regarding the definition and dichotomization of TMB, given the FDA approval of pembrolizumab as an agnostic treatment for all metastatic solid tumors with  $\geq 10$  mut/Mb, and considering the effectiveness of this cut-off in real-world populations as a predictive biomarker for immunotherapy [22, 23], a consensus among the authors led to the adoption of this 10 mut/Mb cut-off. OS was assessed using the Kaplan–Meier method, with statistical significance determined by the two-tailed log-rank test for univariate analysis. Multivariate analysis, based on the Cox proportional-hazards regression model, examined prognostic interactions between OS and covariates, with Hazard Ratios (HRs) reported alongside 95% confidence intervals (CIs). Covariates were carefully selected based on clinical relevance. The “enter” method

was used for the Cox regression model, incorporating all selected covariates simultaneously to assess their independent prognostic significance. No stepwise selection method was applied; all variables were retained in the model to ensure consistency and minimize potential biases associated with variable exclusion. The t-test was used to compare the mean values of continuous variables (ages) between high TMB and low TMB groups. Associations between clinicopathological variables and classification groups were studied using contingency tables and the chi-square test. A p-value less than the conventional level of 0.05 was considered significant. Statistical analyses were conducted using Excel software and MedCalc® version 20.112.

## Results

### Clinicopathological characteristics of patients and tumors

The flowchart describing the selection of the cohort is shown in Fig. 1, and the clinical and pathological characteristics of the study population are summarized in Table 1.

The median age of the entire cohort was 64 years, ranging from 31 to 82 years. When stratified by TMB, the median age was 63 years (range 31–82) in the TMB < 10 mut/mb group and 68 years (range 48–81) in the TMB ≥ 10 mutations/mb group. The difference in median age between the two TMB groups was statistically significant ( $P=0.0389$ ). Among the 102 patients, 36 (35.3%) were female and 66 (64.7%) were male. The primary tumor was predominantly located on the left side (68.6%), and most tumors were graded as G2/3 (93.1%). At initial diagnosis, 13 patients (12.7%) had pT1/2 stage tumors, 52 patients (50.9%) had pT3 stage tumors, 15 patients (14.7%) had pT4 stage tumors, and the pT stage was unknown for 22 patients (21.6%) since these patients did not undergo resection of the primary tumor. The majority of patients had pN1 pathological stage tumors (33.3%). Seventy-eight patients (76.5%) developed metastasis at one organ, while 24 patients (23.5%) had more than two metastatic sites at the time of diagnosis. Liver involvement was observed in 70.6% of cases. The distribution of gender, tumor side, pT stage, pN stage, number of metastatic sites, and liver as a metastatic site did not differ significantly between the two TMB groups ( $P>0.05$ ).

### Associations between TMB, gene mutations, and treatment responses

With an exploratory and descriptive aim, we conducted analyses of TMB levels within genetic profiles of patients (*BRAF* p.V600E, *PIK3CA*, *RAS*, and *TP53*) (Table 2) and their response to treatments (Table 3).

The analysis revealed a significant association only between *BRAF* p.V600E mutation status and TMB levels ( $P=0.0221$ ), indicating that patients with this mutation are more likely to have higher TMB. No significant correlations were found between TMB levels and mutations in *PIK3CA* ( $P=0.6386$ ), *RAS* ( $P=0.1001$ ), and *TP53* ( $P=0.8818$ ).

The correlation between TMB levels and the propensity for mutations may be linked to variations in responses to first-line chemotherapies. Upon further examination, we found that, although there were no significant associations between TMB levels and response rates in the overall cohort or among patients treated with anti-EGFR therapies, our analysis suggests a potential effect of TMB levels on the efficacy of treatment regimens containing bevacizumab. Specifically, patients with low TMB showed a slightly better response compared to those with high TMB ( $P=0.0497$ ).

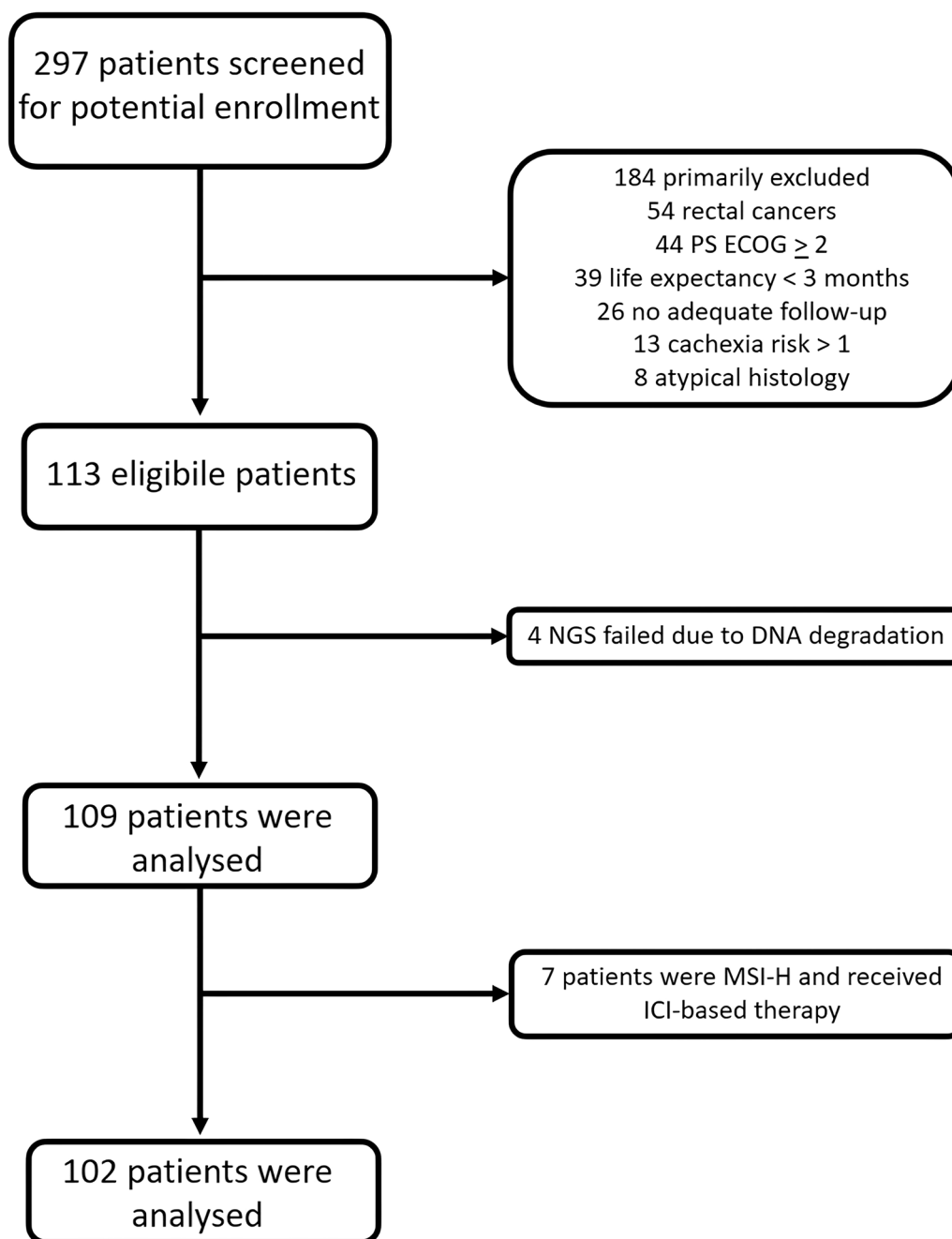
### Prognostic significance of TMB

The primary objective of this study was to investigate the prognostic significance of TMB in patients with MSS metastatic colon cancer (mCC). All participants had an ECOG performance status of 0–1, a life expectancy of more than three months (as assessed by the oncologists involved in the study), and a cachexia risk score below 1. After a median follow-up of 46 months, there were 53 deaths attributed to colon cancer. Kaplan–Meier survival curves for overall survival (OS) revealed a distinct separation based on TMB levels (low vs. high), with the difference becoming particularly evident after one year of follow-up (Fig. 2).

The median OS (mOS) was 70.0 months for patients with high TMB compared to 45.0 months for those with low TMB ( $P=0.0372$ , log-rank test; HR: 0.47; CI 0.22–0.99). In multivariate analysis, after adjusting for age (≥ 70 years vs < 70 years), gender (male vs female), extent of metastatic disease (multiple sites vs single site), response to first-line chemotherapy (disease control vs no disease control), *RAS* mutational status (mutated vs wild-type), and liver involvement (involved vs not involved), TMB levels remained an independent prognostic factor ( $P=0.0396$ ), alongside the response to first-line chemotherapy ( $P=0.0134$ ). HRs with CIs for each covariate are presented in Table 4.

## Discussion

The present study aimed to explore the prognostic value of TMB in a well-characterized cohort of metastatic colon cancer patients with microsatellite stable tumors who received standard treatments. None of the enrolled patients received immune checkpoint inhibitor (ICI)-based therapy, as per European pharmacy



**Fig. 1** Flowchart describing the inclusion and exclusion process of screened and analyzed patients, with reasons for exclusion listed on the right

regulatory guidelines. Our findings suggest that high TMB (> 10 mut/Mb), observed in 26.5% of the patients, is associated with improved overall survival (OS). Specifically, patients with high TMB had a median OS of 70 months, compared to 45 months for those with low TMB (P=0.0372). Importantly, TMB remained an independent prognostic factor in multivariate analysis, highlighting its potential significance as a biomarker in

colon cancer. Previous studies by Valero et al. [11] and Wang et al. [12] identified high TMB as a positive prognostic factor in colon cancer. In contrast, the analysis of patients in the FIRE-3 trial found no prognostic role for TMB [13], while another study demonstrated only a trend suggesting high TMB as a potential positive prognostic factor [14]. Our study contributes to clarify these discrepancies by focusing on a single-center, consecutive,

**Table 1** Clinico-pathological characteristics of analysed patients

Variable	No. (%)	TMB		P
		< 10	≥ 10	
Age				
Median (range)	64 (31–82)	63 (31–82)	68 (48–81)	0.0389
Gender				
Female	36 (35.3)	28	8	0.4748
Male	66 (64.7)	47	19	
Side				
Left	70 (68.6)	51	19	0.8208
Right	32 (31.4)	24	8	
Grading				
G1	7 (6.9)	4	3	0.3109
G2/3	95 (93.1)	71	24	
pT				
1/2	13 (12.7)	9	4	0.9591
3	52 (50.9)	38	14	
4	15 (14.7)	11	4	
Unknown	22 (21.6)	17	5	
pN				
0	29 (28.4)	20	9	0.7859
1	34 (33.3)	26	8	
2	17 (16.7)	12	5	
Unknown	22 (21.6)	17	5	
No. of metastatic sites				
1	78 (76.5)	56	22	0.4763
≥ 2	24 (23.5)	19	5	
Liver involvement				
Yes	72 (70.6)	53	19	0.9770
No	30 (29.4)	22	8	

**Table 2** Associations between tumor mutational burden (TMB) and mutations in specific key driver genes

Gene status	Total no. (%)	TMB		P
		Low	High	
<i>BRAF</i> p.V600E				
Wild-type	96 (94.1)	73	23	0.0221
Mutated	6 (5.9)	2	4	
<i>PIK3CA</i>				
Wild-type	86 (84.3)	64	22	0.6386
Mutated	16 (15.7)	11	5	
<i>RAS</i>				
Wild-type	58 (56.9)	39	19	0.1001
Mutated	44 (43.1)	36	8	
<i>TP53</i>				
Wild-type	39 (38.2)	29	10	0.8818
Mutated	63 (61.8)	46	17	

**Table 3** Associations between tumor mutational burden (TMB) and Response to First-Line Chemotherapy

Variable	Total no. (%)	TMB		P
		Low	High	
Response to first-line CT				
DC	80 (78.4)	59	21	0.9237
No DC	22 (21.6)	16	6	
Response to first-line anti-EGFR CT				
DC	49 (87.5)	31	18	0.2449
No DC	7 (12.5)	6	1	
Response to first-line anti-VEGF* CT				
DC	31 (67.4)	28	3	0.0497
No DC	15 (32.6)	10	5	

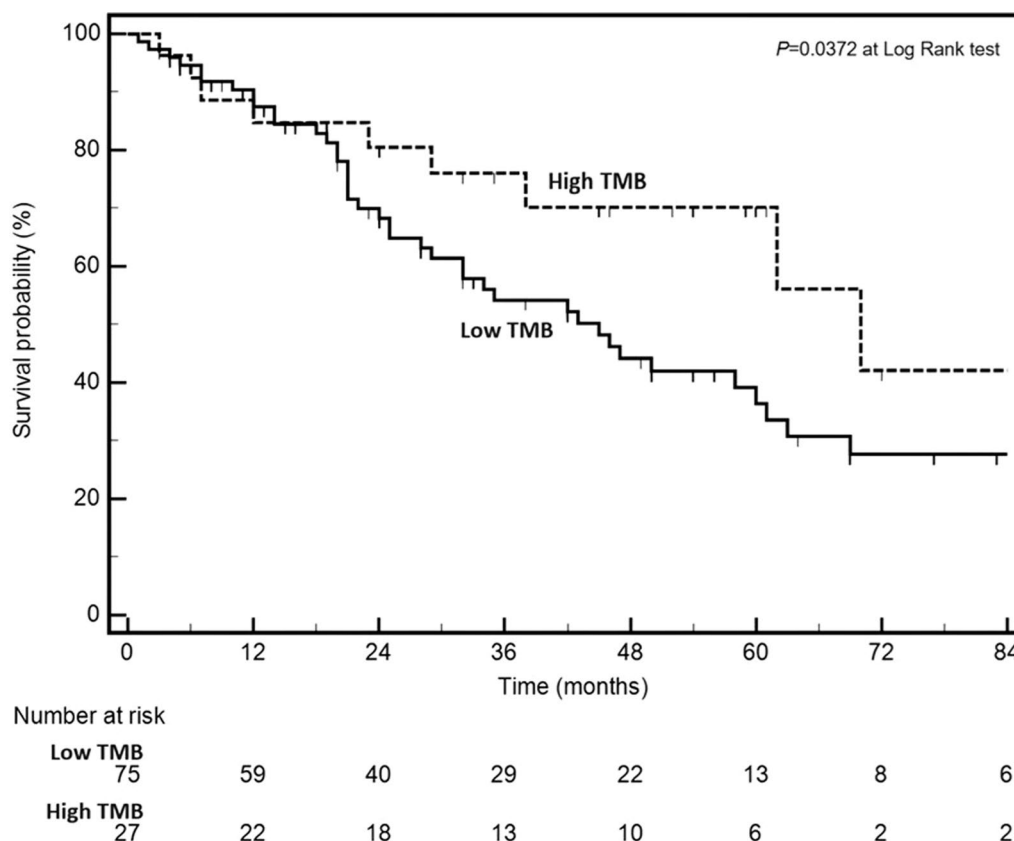
CT: chemotherapy; DC: Disease control; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor

\* bevacizumab

and homogeneous cohort of MSS mCC patients characterized using the same NGS test. Factors such as variability in sequencing platforms, panel design (e.g., the size of the targeted genomic region used to define TMB), patient characteristics, analyzed tissue types (colon vs. rectum, primary vs. metastatic), tumor location (left vs. right-sided primary), and differing thresholds for classifying low versus high TMB may all contribute to the inconsistent and non-uniform results observed across studies.

It is interesting to note that the first association emerging from our analysis is with age. The median age tends to be higher in patients with high TMB. This phenomenon has been extensively described elsewhere [24] and partially explains the improved response to ICIs in some tumor types with advanced age. However, in younger patients, hereditary conditions such as Lynch syndrome, which are more prevalent in this population, are often associated with microsatellite instability (MSI) and elevated TMB [25, 26]. In contrast, in sporadic cases involving older patients, aging cells exhibit epigenetic and genetic changes associated with increased replication errors, telomere shortening, and reduced DNA repair efficiency [27, 28]. These factors collectively contribute to the accumulation of somatic mutations and an age-related increase in mutational burden.

Additionally, although not a primary objective of our study, an exploratory analysis suggested that high TMB levels might influence the efficacy of first-line anti-VEGF therapy (i.e. bevacizumab). Specifically, patients with low TMB demonstrated a better response compared to those with high TMB. Given the limited number of cases and the heterogeneity of chemotherapy regimens combined with bevacizumab, these results should be interpreted with caution. However, this finding warrants further



**Fig. 2** Kaplan–Meier survival curves for patients with high tumor mutational burden (TMB) versus low TMB show a significantly divergent course ( $P=0.0372$ , log-rank test), favoring patients with high TMB. Hazard ratios and confidence intervals are reported in Table 4

**Table 4** Uni- and multi-variate analyses of the prognostic impact of TMB with selected covariates

Co-variate	Dicothomization	Median survivals (months)	No. of events/patients	P at univariate	HR	95% CIs	P at multivariate
Age	≥70y vs <70y	47.0 vs 87.0	39/73 vs 14/29	0.4929	0.94	0.48 to 1.84	0.8689
Gender	M vs F	61.0 vs 42.0	36/66 vs 17/36	0.5199	1.14	0.61 to 2.10	0.6704
No. of metastatic sites	> 1 vs 1 site	32.0 vs 61.0	16/24 vs 37/78	0.0038	0.61	0.31 to 1.19	0.1514
Response to first-line CT	DC vs no DC	29.0 vs 63.0	36/80 vs 17/22	0.0002	0.44	0.22 to 0.84	0.0134
RAS mutational status	Mutated vs wild-type	34.0 vs 62.0	26/44 vs 27/58	0.0983	1.29	0.73 to 2.27	0.3798
Liver involvement	Yes vs No	45.0 vs 70.0	41/72 vs 12/30	0.1303	1.35	0.66 to 2.75	0.4022
TMB high vs low	Yes vs No	70.0 vs 45.0	9/27 vs 44/75	0.0372	0.45	0.21 to 0.96	0.0396

CI: Confidence Interval; CT: chemotherapy; DC: Disease Control; F: Female; HR: Hazard Ratio; M: Male; y: years; TMB: Tumor mutational burden

discussion. In fact, it is known that anti-VEGF drugs and immune checkpoint inhibitors (ICIs) demonstrate synergistic clinical efficacy in terms of response and survival improvements in hepatocellular carcinoma, renal cell carcinoma, and soft tissue sarcomas [29–31]. The mechanistic and molecular bases of this synergy remain unclear. One possible hypothesis is that high TMB may lead to the activation of alternative angiogenic pathways,

thereby influencing the response to these therapies. Recent studies have shown that PD-L1 overexpressing non-small cell lung cancers (NSCLCs) secrete angiogenic factors such as angiopoietin-1, platelet-derived growth factor-beta, and fibroblast growth factor-alpha, promoting neo-angiogenesis independent of the canonical VEGF-dependent pathway [32]. It is conceivable that tumors with higher TMB may evade VEGF inhibition

by upregulating immunological checkpoints and secreting pro-angiogenic factors, thereby enhancing their survival. These hypotheses are scientifically intriguing and warrant further investigation to elucidate the underlying mechanisms.

Moreover, our findings are consistent with previous reports linking the *BRAF* p.V600E variant with TMB levels in larger cohorts [33]. *BRAF* p.V600E mutations are more common in microsatellite instability (MSI) tumors, and similar increased mutational propensity and genomic instability observed in high TMB tumors may account for a higher incidence of *BRAF* mutations [33–35]. However, the molecular basis of these associations remains unclear beyond this hypothesis. Additionally, while *BRAF* mutations are generally associated with a poor prognosis in colon cancer [36], our study found that high TMB was linked to better survival outcomes. This apparent paradox suggests a complex interplay between TMB and specific genetic alterations, which influences tumor biology and patient prognosis. The impact of different treatments on clinical outcomes should also be considered in this context.

The mechanism by which high TMB, independently of MMR protein deficiency, is associated with a good prognosis in MSS patients could be linked to the greater accumulation of somatic mutations, resulting in the generation of neoantigens [37]. A similar phenomenon is believed to be responsible for the better prognosis of metastatic patients with MSI [38]. These neoantigens arise from non-synonymous alterations in the coding regions of the tumor genome and are recognized as foreign by the immune system. Consequently, high TMB tumors possess a broader repertoire of neoantigens, increasing the likelihood of eliciting an immune response. The presence of these neoantigens can lead to tumor cell recognition and subsequent destruction by cytotoxic T lymphocytes (CTLs) and other immune effectors [39] conferring a better prognosis. This subgroup, which represents a larger proportion than the mere 5% of MSI patients, likely has additional prognostic impact and warrants specific biological and immunological characterization.

As previously explained, TMB is considered a marker of tumor neoantigen load. High TMB increases the likelihood of generating neoantigens, which can trigger an immune response. However, the immunoeediting process plays a critical role in determining whether these neoantigens are successfully targeted or if the tumor evolves mechanisms to evade immune detection [40, 41]. Immunoeediting reflects the dynamic interplay between the immune system and tumor evolution. Initially, immune surveillance suppresses tumorigenesis by recognizing and eliminating abnormal cells. However, as tumors accumulate mutations (especially those driving immune

evasion) they enter a state of equilibrium, where immune responses are unable to fully eradicate the tumor. Eventually, tumor cells that adapt to immune pressure escape detection, leading to cancer progression. Thus, TMB is intrinsically linked to the immunoeediting process. Without intending to delve into exhaustive technical details, it is important to note that the quantification of immunoeediting is an emerging biomarker [42, 43] that, in the near future, could integrate and enhance the prognostic and predictive significance of TMB.

Some limitations deserve to be highlighted. Firstly, the retrospective nature of the study, although partially mitigated by the single-center design and the homogeneous NGS technique, may introduce potential selection biases related to clinical characteristics such as disease severity, access to care, and treatment eligibility, all of which can influence outcomes. The single-center design may not fully capture the diversity of clinical and demographic characteristics seen in broader, multi-institutional cohorts, which could limit the generalizability of our findings to other clinical settings or geographic regions. However, it is important to emphasize that the single-center design also offers certain strengths, such as the homogeneity of treatments and the completeness of follow-up data, which enhance the robustness of our study. Secondly, although we adopted the FDA-approved cut-off of 10 mut/mb, there is inherent arbitrariness in this threshold, and different studies may use varying cut-offs. Finally, rather than a limitation, a distinctive feature of this cohort is its higher median survival of 50 months compared to the approximately 30 months typically reported for metastatic colon cancer. This is attributable to the patient selection criteria: patients with excellent performance status (85% with ECOG PS 0 and the remainder with PS 1), an estimated life expectancy of at least 3 months, absence of peritoneal disease, and a cachexia risk < 1. These criteria identify patients with uniformly excellent overall clinical conditions. This methodological approach is intended to highlight the prognostic impact of biological factors that may operate independently of the multiple factors related to overall clinical status.

## Conclusions

Our study reinforces the prognostic value of high tumor mutational burden (assessed through next generation sequencing) in microsatellite stable metastatic colon cancer patients. This finding adds to the growing evidence supporting tumor mutational burden as a crucial biomarker in oncology.

## Abbreviations

CRC	Colorectal cancer
mCRC	Metastatic colorectal cancer

FDA	Food and Drug Administration
PD-1	Programmed cell death 1
ICI	Immune checkpoint inhibitor
MSI-H	Microsatellite instability-high
TMB-H	Tumor mutational burden-high
dMMR	Deficient mismatch repair
NGS	Next-generation sequencing
NSCLC	Non-small cell lung cancer
MSKCC	Memorial Sloan Kettering Cancer Center
TCGA	The Cancer Genome Atlas
KRAS	Kirsten RAT Sarcoma viral oncogene homolog
ESMO	European Society of Medical Oncology
PS	Performance status
ECOG	Eastern Cooperative Oncology Group
MSS	Microsatellite stable
tbCT	Total body Computed Tomography
MRI	Magnetic Resonance Imaging
RECIST	Response Evaluation Criteria In Solid Tumors
DC	Disease control
FFPE	Formalin-fixed and paraffin-embedded
SNVs	Small nucleotide variants
AMP	Association for Molecular Pathology
ACMG	American College of Medical Genetics
OS	Overall survival
PFS	Progression-free survival
HR	Hazard ratios
CI	Confidence interval
BRAF	V-raf murine sarcoma viral oncogene homolog B
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
TP53	Tumor Protein p53
EGFR	Epidermal Growth Factor Receptor
PD-L1	Programmed Death-Ligand 1
VEGF	Vascular Endothelial Growth Factor
mOS	Median overall survival
CT	Chemotherapy
F	Female
M	Male
CTLs	Cytotoxic T lymphocytes

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-024-05927-9>.

Supplementary Material 1

### Acknowledgements

We thank Daniela Capobianco for technical editing and writing assistance. We would like to express our gratitude to the TRIAL scientific association (CF: 92088670622) for their invaluable and unwavering collaboration on this work.

### Author contributions

Conceptualization, A.D.M., M.S., A.O., M.C., G.N.; methodology, A.D.M., M.S., A.O., G.S., M. C.; software, A.O., A.C.C., R.S.; validation, R.S., G.S., F.S., A.C., A.O.; formal analysis, R.S., G.S., A.O.; investigations, A.D.M., M.S., R.S., U.P., R.P., A.M.C., M.B., G.S., G.F., M.C.; resources, A.O.; data curation, A.D.M., M.S.; writing-original draft preparation, A.D.M., M.S., A.O.; writing-review and editing, G.N., M.C.; supervision, M.C., A.O. All authors have read and agreed to the published version of the manuscript.

### Funding

This work was supported by grants from the Italian Government, Ministry of Health (Ricerca Corrente L4/8) and by the “Ministero delle Imprese e del Made in Italy” project number F/310034/01-03/X56, “Epi-MET—Funzionalizzazione delle aberrazioni (epi)genomiche nei tumori metastatici.” We also extend our deepest gratitude to the late Mrs. Antonietta Nacca, a private citizen, for her generous and unconditional financial support.

### Availability of data and materials

The genetic data supporting the findings of this study are available upon reasonable request, which can be sent to [giovanni.savarese@centroames.it](mailto:giovanni.savarese@centroames.it).

### Declarations

#### Ethics approval and consent to participate

The Ethical Committee of the University of Campania “Luigi Vanvitelli” approved this study with the numbers 790 and 68 for project i-Cure. All patients provided written informed consent before undergoing treatment administration and molecular assessments.

#### Consent for publication

Not applicable.

#### Competing interests

Roberto Sirica, Giovanni Savarese, and Monica Ianniello are employed by AMES, Centro Polidiagnostico Strumentale srl, 80013 Naples, Italy. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Received: 9 October 2024 Accepted: 27 November 2024

Published: 5 December 2024

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