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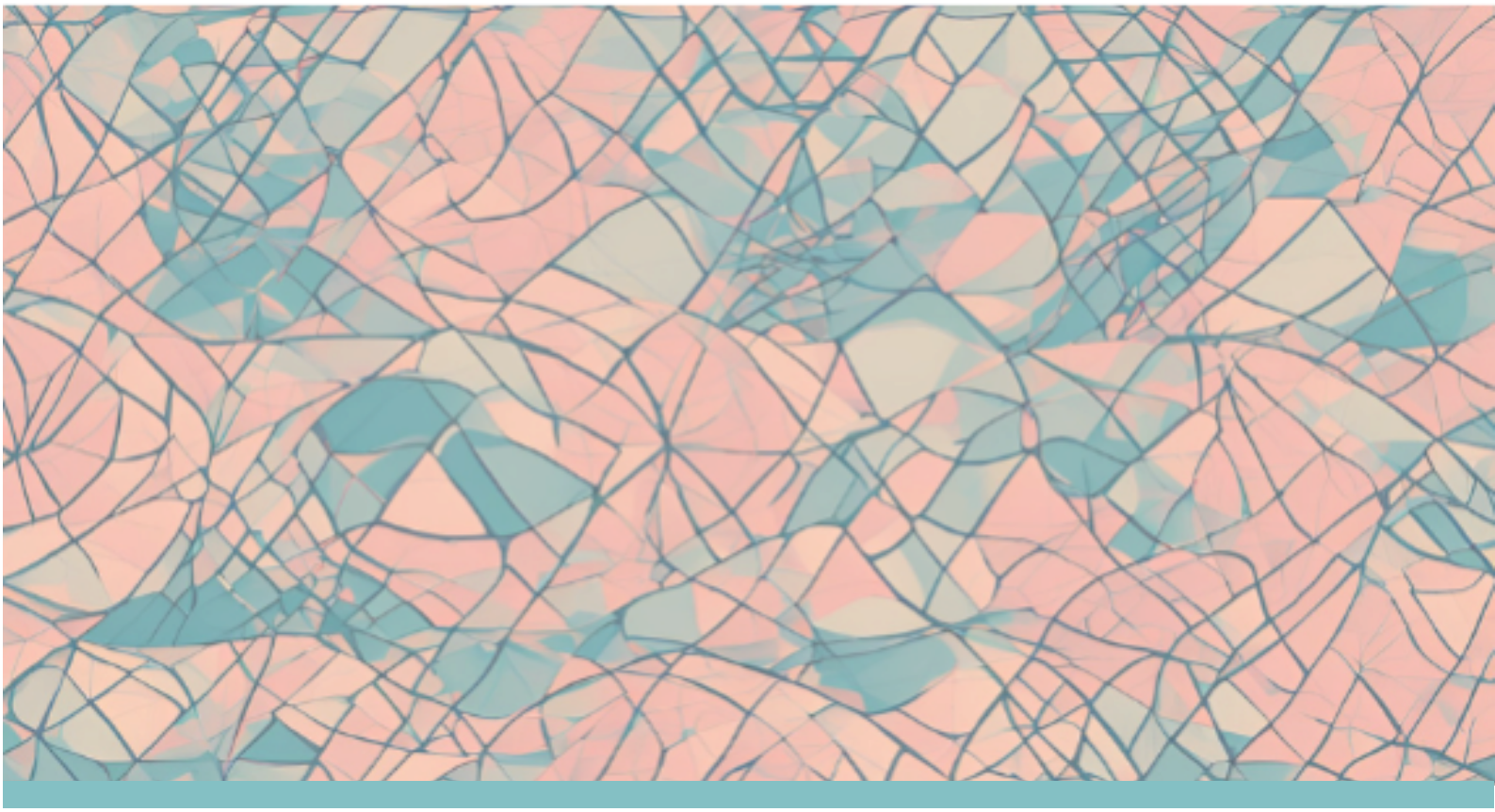
Mutated allele fraction of drive gene in plasma as a biomarker of response to antiangiogenic agents in metastatic colorectal cancer patients

Nadia Saoudi González



Doctoral Thesis

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ABBREVIATIONS

ABBREVIATIONS

A

- APC, Adenomatous polyposis coli

B

- BRAF, v-Raf murine sarcoma viral oncogene homolog B

C

- CEA, Carcinoembryonic antigen
- CEIC, Clinical Research Ethics Committee
- cfDNA, circulant free DNA
- ctDNA, circulant tumor DNA
- CR, complete response
- CRC colorectal cancer
- CT, computed tomography scan
- CTCAE, Common Toxicity Criteria Adverse Events

D

- DFS, disease free survival
- dMMR, deficient mismatch repair
- DNA, deoxyribonucleic acid

E

- ECOG, Eastern Cooperative Oncology Group
- EDTA, Ethylenediaminetetraacetic acid
- EGFR, epidermal growth factor receptor
- EMA, European Medicines Agency
- ESMO, European Society of Medical Oncology

F

- FDA, Food and Drug Administration
- FOLFIRI, 5-flurouracil + leucovorin + irinotecan chemotherapy combination
- FOLFOX, 5-flurouracil + leucovorin + oxaliplatin chemotherapy combination

H

- HER2, human epidermal growth factor receptor 2
- HR, Hazard Ratio

K

- KRAS, Kirsten rat Sarcoma virus.

M

- MAF, mutation allele frequency
- MAPK, mitogen activated protein kinase
- mCRC, metastatic colorectal cancer
- MMR, DNA mismatch repair system
- MRD, minimal residual disease
- MSI, microsatellite instability
- MSS, microsatellite stability
- mut, mutation

N

- NGS, next generation sequencing
- NRAS, neuroblastoma RAS viral oncogene homolog

O

- OS, overall survival

P

- PCR, polymerase chain reaction
- PD, progressive Disease
- PFS, progression free survival
- PIK3CA phosphatidylinositol -4,5-biphospate 3-kinase catalytic subunit-α
- PR, partial response

R

- RNA, acid ribonucleic
- RR, response rate

S

- SD, stable disease

T

- TAS-102, Trifluridine/tipiracil (FTD–TPI)
- TKI, tyrosine kinase inhibitor
- TNM, tumor-node-metastases
- TP53, tumor protein P53

V

- VAF, variant allele frequency
- VEGF, vascular endothelial growth factor
- VHIO, Vall d'Hebron Institute of Oncology

W

- WES, Whole Exome Sequencing
- Wt, wild type, non-mutated gene

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ABSTRACT

ABSTRACT

Antiangiogenic therapies, including bevacizumab, are a cornerstone of metastatic colorectal cancer (mCRC) treatment, but reliable biomarkers to predict patient response remain limited. This study investigates the mutant allele fraction (MAF) of driver genes in circulating tumor DNA (ctDNA) as a potential predictive biomarker. A comprehensive analysis was conducted across three independent cohorts: (1) a first-line cohort of 185 mCRC patients treated with chemotherapy ± bevacizumab, (2) a second-line cohort of 43 patients, and (3) a refractory cohort of 32 patients receiving TAS-102 ± bevacizumab. Circulating tumor DNA was analyzed using BEAMing and NGS techniques, and MAF levels were correlated with clinical outcomes.

In the first-line cohort, high MAF (≥5.8%) was associated with shorter progression-free survival (PFS) and overall survival (OS) (median OS: 17.7 vs. 40.7 months, $p < 0.001$). Multivariable analysis confirmed the value of MAF as an independent predictor of survival. In the subset of 122 patients who did not undergo metastasectomy, high MAF patients who received bevacizumab showed significantly better PFS, while no differences were observed in low MAF patients, with an interaction p-value of 0.026 supporting MAF's predictive role in determining the benefit of antiangiogenic therapy in first-line mCRC. In second-line and refractory settings, similar trends were observed, with high MAF consistently linked to poor clinical outcomes. Mechanistic studies evaluating tumor vascular permeability showed a positive correlation between MAF levels and hemorrhage grades ($p=0.032$), suggesting that heightened vascular leakiness contributes to ctDNA shedding and reflects an aggressive tumor phenotype.

These results underscore the potential of integrating MAF measurements into clinical practice, paving the way for biomarker-driven treatment strategies and personalized management of mCRC. Ongoing research focuses on validating these findings in external cohorts and mechanistic in vivo models, including patient-derived xenografts and tumor microenvironment analysis to explore the mechanistic basis of ctDNA release and the role of angiogenesis.

RESUM

RESUM

Les teràpies antiangiogèniques, incloent-hi el bevacizumab, són una pedra angular en el tractament del càncer colorectal metastàtic (mCRC), però els biomarcadors fiables per predir la resposta dels pacients continuen sent limitats. Aquest estudi investiga la fracció al·lèlica mutada (MAF) dels gens *driver* en l'ADN tumoral circulant (ctDNA) com a potencial biomarcador predictiu. Es va realitzar un anàlisi exhaustiu en tres cohorts independents: (1) una cohort de primera línia de 185 pacients amb mCRC tractats amb quimioteràpia ± bevacizumab, (2) una cohort de segona línia de 43 pacients i (3) una cohort de malaltia refractària de 32 pacients que van rebre TAS-102 ± bevacizumab. El ctDNA es va analitzar mitjançant tècniques BEAMing i NGS, correlacionant els nivells de MAF amb els resultats clínics.

En la cohort de primera línia, un valor de MAF elevat (≥5,8%) es va associar amb una supervivència lliure de progressió (PFS) i una supervivència global (OS) més curtes (mOS: 17,7 vs. 40,7 mesos, $p < 0,001$). L'anàlisi multivariable va confirmar el MAF com a predictor independent de la supervivència. En el subgrup de 122 pacients amb mCRC no sotmesos a cirurgia de les metástasis, els pacients amb MAF elevat que van rebre bevacizumab van mostrar una PFS significativament millor, mentre que no es van observar diferències significatives en els pacients amb MAF baix, amb un valor d'interacció p de 0,026 que recolza el paper predictiu del MAF en determinar el benefici de la teràpia antiangiogènica en la primera línia. En les cohorts de segona línia i refractàries, es van observar tendències similars, amb una associació constant entre MAF elevat i resultats clínics desfavorables. Els estudis mecanístics que van avaluar la permeabilitat vascular tumoral van mostrar una correlació positiva entre els nivells de MAF i els graus d'hemorràgia tumoral ($p=0,032$), suggerint que una major permeabilitat vascular contribueix a l'alliberament de ctDNA i reflecteix un fenotip tumoral més agressiu.

La investigació en curs se centra a validar aquests resultats en cohorts externes i el estudi de xenotransplantaments derivats de pacients amb l'anàlisis del microambient tumoral per explorar la base mecanística de l'alliberament de ctDNA i el paper de l'angiogènesi. Aquests resultats subratllen el potencial d'integrar la mesura del MAF en la pràctica clínica, obrint el camí cap a estratègies de tractament guiades per biomarcadors i la gestió personalitzada del mCRC.

BACKGROUND

1. BACKGROUND

1.1 Epidemiology of colorectal cancer

Colorectal cancer (CRC) is a significant global health issue, ranking in third place in terms of incidence but being the second most common cause of death from oncological diseases worldwide.[1] More than 1.9 million new cases of colorectal cancer and nearly 1 million deaths were estimated to occur in 2022, representing close to one in 10 cancer cases and deaths. CRC incidence is expected to further increase to 3.2 million new cases by 2040.[1] Across different regions, the incidence of CRC varies significantly, with differences of up to eight-fold observed between countries. In nations experiencing significant developmental changes, CRC incidence rates tend to increase consistently with the rising Human Development Index, implying a possible causal connection.[2]

Patients with CRC are diagnosed in stage IV in 25% of cases, but 25-50% of those with early-stage disease will eventually develop metastases during their oncological disease.[3,4] While the overall 5-year survival (OS) across all stages of CRC is around 65%, it drops significantly to just 15% for patients with metastatic disease.[5]

In recent years, CRC incidence in developed countries has declined due to healthier lifestyles and the introduction of screening programs.[1] In contrast to the recent stabilizing or declining trends for all age groups, recent studies on several continents have reported an increase in CRC among younger adults (under 50 years at diagnosis).[6–13] Recognizing this trend, the US Preventive Services Task Force has revised its 2016 guidelines to match those of the American Cancer Society, now recommending that screening begin at age 45.[14] To date, the true magnitude and underlying aetiologies of this increase remain unclear, but they suggest a profound influence of risk factors during early young adulthood such as diet, body weight, lifestyle, and the use of antibiotics. More research is needed to understand the role of the exposome (the environmental exposures that an individual encounters throughout life) and specialized carcinogenesis in early onset CRC with international efforts currently being made to advance in this area.[15] Given its substantial epidemiological burden and the urgent need to improve clinical outcomes, CRC remains a critical focus of research and innovation in healthcare.

1.2. The role of Angiogenesis in Colorectal Cancer Carcinogenesis

In the complex etiology of CRC, the interplay between environmental and genetic factors drives the development of hallmark cancer traits in colon epithelial cells.[16,17] The progression of CRC typically begins with the formation of polyps, which evolve into adenomas and eventually develop into adenocarcinoma. Genomically, at least three major, non-mutually exclusive pathways have been identified as driving this transformation from polyp to adenocarcinoma: chromosomal instability (the most common, characterized by mutations in *APC*, *KRAS*, and *TP53*), microsatellite instability (due to defects in DNA mismatch repair system, secondary to genetic or epigenetic alterations causing loss of DNA mismatch repair (MMR) proteins), and the CpG island methylation pathway (CIMP, involving hypermethylation of gene promoters and often *BRAF* mutations).[18,19] Microsatellites are repeated motifs of short DNA sequences. If the MMR system is deficient (dMMR), these microsatellite replication errors are not corrected, leading to instability in the microsatellite sequence. Mismatch repair deficiency is defined as the loss of at least one of the four MMR proteins: MLH1, PMS2, MSH2, or MSH6, and is typically assessed by confirming the absence of one of these proteins using immunohistochemistry. This deficiency can result from either sporadic genetic mutations or inherited mutations, such as those associated with Lynch syndrome.

The autonomous growth of solid malignant tumors, upon reaching a certain stage, leads to the formation of a microenvironment characterized by severe hypoxia and acidosis, that have been recognized as a hallmark of tumor progression. These hypoxic conditions induce the expression of hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2), which, in turn, upregulate various growth and proangiogenic factors.[20] These proangiogenic factors promote the sprouting of blood vessels from pre-existing ones, a process known as angiogenesis. This process is tightly regulated by a balance between proangiogenic and antiangiogenic factors. When the activity of proangiogenic factors exceeds that of antiangiogenic factors, the angiogenic switch is triggered. The critical role of HIFs in regulating angiogenesis was recognized with the Nobel Prize in Physiology or Medicine in 2019, highlighting the central role of oxygen sensing in tumor vascularization. The vasculature that forms within tumors is highly heterogeneous and poorly perfused, characterized by abnormal and leaky blood vessels.[21] Proangiogenic factors are participants in this equilibrium, such as

vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), transforming growth factor (TGF-α and TGF-β) platelet-derived endothelial cell growth factor (PDGF), and angiopoietins produced from cancer or stromal cells.[22] VEGF and its receptor (VEGFR) constitute a fundamental axis in angiogenesis, which consists of five ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF), and endocrine gland-derived vascular endothelial growth factor (EG-VEGF).[23] VEGF ligands bind to three receptor tyrosine kinases, VEGFR1, VEGFR2 and VEGFR3, predominantly expressed on vascular endothelial cells.[24] VEGFR can also interact with other proteins such as neuropilins, integrins, cadherins or heparin sulphate proteoglycans, which modulates tyrosine kinase receptor activity. [25,26] Upon activation, VEGFR triggers intracellular signaling pathways as PI3K/AKT and p38/MAPK, which drive endothelial cell proliferation, migration, and vascular permeability, promoting angiogenesis crucial for tumor progression.[27] Of the various interactions within the VEGF–VEGFR system, VEGF-A, VEGF-B, and PlGF primarily drive angiogenesis, whereas VEGF-C and VEGF-D are more closely associated with the regulation of lymphangiogenesis.[28] VEGF-A and VEGF-B primarily interact with VEGFR-1 and 2, which are predominantly found on vascular endothelial cells, though also present on certain non-endothelial cell types. In contrast, VEGF-C and D exhibit highest affinity for VEGFR-3, which is mainly expressed on lymphatic cells, playing a central role in lymphangiogenesis.[29]

Anti-angiogenic therapy has been conceptualized over 50 years ago as a promising strategy for cancer treatment by targeting tumor vasculature to limit blood supply.[30] Initially, the goal was to 'starve' tumors by blocking angiogenesis but advances in understanding the tumor microenvironment led to the concept of 'vascular normalization.' This approach involves the judicious use of antiangiogenic drugs to remodel the tumor vasculature, transforming it from a chaotic and dysfunctional state to a more organized and efficient one. This normalization enhances the delivery of therapeutics and oxygen, reduces treatment resistance, and fosters a less hostile microenvironment, thereby improving the overall efficacy of combination therapies.[31]

Despite the discovery of numerous regulators in tumor angiogenesis, anti-angiogenic research predominantly targets the VEGF/VEGFR signaling pathway, with recombinant monoclonal antibodies and small-molecule tyrosine kinase

inhibitors serving as the main therapeutic approaches. Monoclonal antibodies approved in metastatic colorectal cancer include bevacizumab, which binds to VEGF-A; ramucirumab, which inhibits VEGFR-2; and aflibercept, a human recombinant fusion protein that targets VEGF-A, VEGF-B, and PlGF.[31] Tyrosine kinase inhibitors are small-molecule compounds that inhibit a broad range of protein kinases. In the setting of CRC, approved TKIs encompass fruquintinib (high selective action on VEGFR-1,2 and 3) and regorafenib (multi-TKI that targets VEGFR-1,2 and 3, and others such as TIE-2 BRAF, KIT, RET, PDGFR and FGFR). Figure 1 schematizes the main receptors and ligands of the angiogenic pathway, along with the antiangiogenic drugs.

Because VEGF also plays an important role in normal angiogenic physiologic processes – such as vacular homeostasis, coagulation, blood pressure regulation, and wound healing, beyond others – VEGF inhibition carries a unique toxicity. Antiangiogenics reported higher rates of bleeding, arterial thromboembolic events (such as cerebrovascular events, myocardial infarction, transient ischemic attack, and angina), gastrointestinal perforation, impaired wound healing, proteinuria, and hypertension. These adverse events are now broadly recognized as class toxicities associated with anti-VEGF therapy.[32]

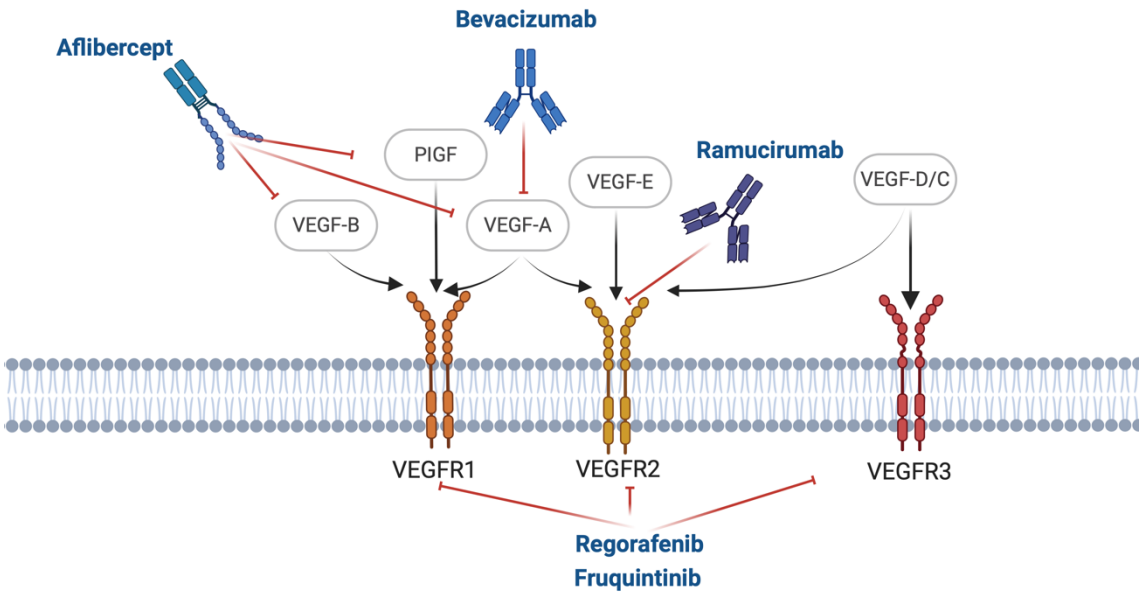


Figure 1: Schematic overview highlights key receptors and ligands in the angiogenic pathway and the main antiangiogenic treatments for mCRC.

1.3. Role of antiangiogenic treatment in colon cancer

1.3.1. Role of antiangiogenic treatment in in locorregional colon cancer

One of the main challenges in the adjuvant setting is the limited advancement in drug development over recent decades, as no therapies have shown improved outcomes beyond the established use of oxaliplatin and fluoropyrimidines. Agents that demonstrate activity in the metastatic setting, such as irinotecan and targeted biologics, have not provided significant benefit over fluoropyrimidines or oxaliplatin in randomized clinical trials.[33–35] The NSABP C-08 trial recruited patients with stage II or III colon cancer, treated with FOLFOX (a combination of the fluoropyrimidine 5FU and oxaliplatin) with or without bevacizumab. No significant effect on disease-free survival (DFS) or OS was shown.[36] The AVANT trial involved patients with resected stage III colon cancer, randomized to receive FOLFOX, FOLFOX-bevacizumab, or CAPOX-bevacizumab (a combination of the oral fluoropyrimidine capecitabine and oxaliplatin). After a median follow-up of more than 6 years, the study found no benefit in adding bevacizumab in terms of DFS and noted a negative effect on OS.[37] The QUASAR2 study recruited patients with stage III or high-risk stage II colorectal cancer, treated with capecitabine with or without bevacizumab, showing initial improvement in DFS within the first 2 years, but increased recurrence after 2 years.[38] Overall, bevacizumab did not show a benefit when added to adjuvant chemotherapy in colorectal cancer, with concerning trends of increased recurrence rates post-treatment and worsened OS.

Several hypotheses may explain why bevacizumab has failed to show clinical benefits in the adjuvant treatment setting. Micrometastasis and macrometastasis have different tumor microenvironment, which may explain why the response to targeted agents differs between adjuvant and metastatic settings. According to Gompertz's principle, tumor growth follows a sigmoidal curve, where early-stage micrometastases proliferate rapidly, while larger tumors grow more slowly due to factors such as limited vascularization and resource competition. Given their cytostatic nature, antiangiogenic therapies may have a limited role in micrometastatic disease, as these rapidly growing lesions are more sensitive to cytotoxic treatments.[39]

Preclinical studies in murine models have shown that short-term anti-VEGF therapy can paradoxically promote more aggressive disease, likely driven by tumor hypoxia and inflammation.[40,41] This mechanism may explain why, clinically, antiangiogenic treatments can initially improve DFS but lead to unfavorable outcomes upon discontinuation. Additionally, adjuvant targeted agents may induce tumor cell dormancy, with regrowth occurring once those agents are discontinued.[42]

1.3.2. Role of antiangiogenic treatment in the metastatic colorectal cancer

1.3.2.1. First-line treatment

To define the optimized strategy treatment for patients with mCRC, is crucial to consider the fitness status of the patient, the sidedness of colon tumor, molecular status of all *RAS* and *BRAF*, MMR status, and resectionability of metastatic disease, besides the patients' preferences. As outlined in the European Society for Medical Oncology (ESMO) guidelines, the combination of biological agents with chemotherapy constitutes the standard first-line treatment for patients with mCRC.[43–50]

Fluoropyrimidines, including intravenous fluorouracil and oral capecitabine, have served as the cornerstone of conventional chemotherapy for mCRC for over five decades. Notably, the choice between oral capecitabine and intravenous fluorouracil has not shown significant differences in patient outcomes.[51] While fluoropyrimidine monotherapy is generally well-tolerated and suitable for frail patients, those who are in reasonably good health often receive therapy with doublet regimens, such as oxaliplatin-based (FOLFOX or CAPOX) or irinotecan-based (FOLFIRI) therapies. Comparisons between FOLFOX and FOLFIRI indicate similar patient outcomes but different toxicity profiles; oxaliplatin causes more sensory neuropathy, whereas irinotecan leads to increased diarrhea and alopecia.[52]

Bevacizumab has been approved for both first and second-line therapy in mCRC patients. Numerous clinical trials conducted over the past two decades have explored the combination of bevacizumab with chemotherapy in this context. In the initial phase III trial published in 2004, the addition of bevacizumab to irinotecan, bolus fluorouracil, and leucovorin (IFL) was compared to IFL alone, demonstrating improvement in OS.[53] Since then, clinical trials have explored and demonstrated the

benefits of adding bevacizumab to chemotherapy regiments containing oxaliplatin, irinotecan, or both, as well as fluoropyrimidine in monotherapy.

In the first-line setting, the phase III NO16966 trial evaluated the addition of bevacizumab to oxaliplatin-based chemotherapy, and showed a significant improvement in PFS. However, the magnitude of benefit was smaller than expected, and no meaningful differences were observed in OS or ORR between patients who received bevacizumab and those who did not.[47] The phase II BECOME trial randomized patients with *RAS* mutant unresectable, liver-limited mCRC to bevacizumab plus FOLFOX versus FOLFOX alone, demostrating higher objective response rate (ORR), median PFS (9.5 versus 5.6 months), median OS (25.7 versus 20.5 months), and complete (R0) resection rates (22.3 % vs 5.8 %).[54]

For patients who are not candidates for doublet chemotherapy, the combination of fluoropyrimidines with bevacizumab has shown greater efficacy compared to fluoropyrimidine monotherapy. The phase III AVEX trial evaluated patients aged 70 or older with mCRC, ineligible for doublet chemotherapy, by randomizing them to bevacizumab plus capecitabine or capecitabine alone. The combination significantly improved median PFS (9.1 months vs. 5.1 months; HR, 0.53; 95% CI, 0.41–0.69) and was well tolerated, though OS was not significantly different between the groups (20.7 months vs. 16.8 months).[55]

In contrast to frail patients, there are fit patients requiring a high response rate of the metastatic disease, thus more intense chemotherapy backbones have been investigated. Different phase II/III have explored the combination of FOLFOXIRI with or without bevacizumab versus doublet combinations with or without bevacizumab. [56–60] A recent individual-patient data meta-analysis of those clinical trials has been done, demonstrating that FOLFOXIRI plus bevacizumab significantly and meaningfully improves OS, PFS, ORR and negative resection margins rates of patients with metastatic colorectal cancer compared with doublets with bevacizumab, but with an increase in toxicity.[61]

Epidermal growth factor receptor (EGFR) inhibitors, cetuximab and panitumumab, demonstrate effectiveness primarily in *KRAS*/*NRAS* wild type (wt) mCRC patients. Cetuximab, a chimeric anti-EGFR monoclonal antibody, and panitumumab, a humanized anti-EGFR monoclonal antibody, have shown effectiveness across various treatment lines.[62] The question of which biologic (antiEGFR or antiVEGF) is

preferable in the first-line treatment of *RAS*wt mCRC has been addressed in different phase III trials. The phase III FIRE-3 trial evaluated previously untreated mCRC patients with *KRAS* wt, initially including all-comers but later amended to exclude those with *KRAS* exon 2 mutations. Patients received FOLFIRI plus cetuximab or FOLFIRI plus bevacizumab, showing no significant difference in the primary endpoint of ORR, but better OS with cetuximab in *KRAS* exon 2 wild type patients. [63] Similarly, the phase III CALGB80405 trial demonstrated that either FOLFIRI or FOLFOX combined with bevacizumab or cetuximab, provided comparable outcomes in patients with *RASwt* tumors, indicating flexibility in treatment options. [64]

The clinical and molecular heterogeneity of mCRC is partly due to the tumor's anatomic location, as left and right-sided tumors originate from different embryonic structures, have distinct physiological functions, varying nutrient exposures, and different exposure to microbiota.[65] Studies have shown that colon cancer sidedness is a predictive biomarker for response to biological agents. From a metanalysis published on 2017 from first line studies, a significant predictive benefit was demonstrated for chemotherapy plus EGFR inhibitors in patients with left-sided tumors (HR=0.75 [0.67-0.84] and 0.78 [0.70-0.87] for OS and PFS, respectively. [66–68] However there was a trend, but no significant benefit for patients treated with chemotherapy with or without bevacizumab with right-sided tumors (HRs=1.12 [0.87-1.45] and 1.12 [0.87-1.44] for OS and PFS, respectively). Recent prospective data from the phase III PARADIGM trial, that randomized patients with *RAS*wt mCRC to be treated with patinumumab in combination with mFOLOFX or bevacizumab combined with mFOLFOX, demonstrated a clear benefit of antiEGFR therapy for patients with left sided colon cancer (OS 37.9 months vs 34.3 months; HR 0.82).[69] Thus, for those patients who have left-sided *RAS*wt disease, cytotoxic doublet plus an anti-EGFR antibody should be the treatment of choice. For the ones with right-sided *RAS*wt disease or *RAS* mutated, cytotoxic combination with bevacizumab is the preferred option. The combination of both VEGF and anti-EGFR treatments is not recommended regarding the results of the phase III PACCE and CAIRO2 trials.[70,71]

Last, maintenance treatment is a therapeutic strategy that envisages a period of high-intensity chemotherapy, after which agents that are mainly responsible for cumulative toxicity are stopped, therefore remaining with a more simple and non-toxic combination of treatments until progression disease. This approach differs

from treatment interruption, where drug withdrawal is permitted with intervals free of treatment. Maintenance is active and must be considered as a part of mCRC treatment strategy, as active maintenance with fluoropyrimidines and bevacizumab has demonstrated improvement of PFS (but not OS). [72,73]

1.3.2.2. Second line treatment

When patients with mCRC relapse, selecting a second-line treatment involves considering factors such as prior chemotherapy exposure, timing of progression, molecular status, tolerance of previous chemotherapy, and patient preferences.

Different antiangiogenic agents have demonstrated efficacy in mCRC in second line. The VELOUR phase III trial randomized 1,226 patients to receive aflibercept or placebo every 2 weeks plus FOLFIRI, demonstrating advantages in OS, PFS, and RR of aflibercept in combination with FOLFIRI versus chemotherapy alone. The results showed an OS benefit in favour of the experimental arm, with an OS of 13.5 vs 12.06 months (HR 0.817; p=0.0032), PFS of 6.9 vs 4.67 months (HR 0.758; p < 0.0001), and ORR of 19.8% vs 11.1 % with aflibercept plus FOLFIRI compared to chemotherapy alone.[46]

The TML18147 trial was a randomized phase III study evaluating the effectiveness of continuing bevacizumab treatment beyond disease progression in patients with mCRC who had previously undergone first-line therapy with bevacizumab.[48] Patients were randomized to receive either bevacizumab with chemotherapy or chemotherapy alone, demonstrating an improvement in OS for patients in the bevacizumab plus chemo-therapy group (11.1 vs 9.8 months).

Ramucirumab, a fully human monoclonal antibody directed against VEGFR-2, was assessed in the phase III RAISE trial for its efficacy and safety when combined with second-line FOLFIRI. The study compared this regimen to FOLFIRI plus placebo in patients with metastatic colorectal cancer who had experienced disease progression following first-line treatment with bevacizumab and FOLFOX, regardless of KRAS mutation status. [43] The findings demonstrated a meaningful increase in both OS and PFS (13.3 versus 11.7 months and 5.7 versus 4.5 months, respectively).

The results of all these phase III trials support the benefit from continuation of VEGF inhibition following prior exposure to bevacizumab. No direct comparison has been

done, but the effects across all studies are of similar magnitude. The selection of bevacizumab, aflibercept or ramucirumab should be individualized by evaluating the toxicity profile, patient preference and reimbursement policy of each country.

1.3.2.3. Refractory setting

Regorafenib is an oral multi-kinase inhibitor that targets multiple protein kinases involved in oncogenesis, the tumor microenvironment, and exhibits anti-angiogenic effects through dual-targeted VEGFR2 tyrosine kinase inhibitio.[74] The phase III CORRECT trial randomized patients 2:1 to receive best supportive care (BSC) plus oral regorafenib or placebo. Median OS was 6.4 months in the regorafenib group versus 5.0 months in the placebo group (HR 0,77; p=0·0052).[75]

Trifluridine/tipiracil (FTD/TPI, TFTD, or TAS-102) is an orally administered combination of a thymidine-based nucleic acid analogue, trifluridine, and a thymidine phosphorylase inhibitor, tipiracil hydrochloride at a molar ratio of 1:0.5. TAS-102 has demonstrated efficacy in terms of OS compared to BSC in patients with refractory mCRC (7.1 vs 5.3 months respectivelly; HR: 0.58 to 0.81; p < 0.001).[76]

A phase II trial assessed TAS-102 with or without bevacizumab in a randomized design.[77] The combination therapy led to a statistically significant improvement in PFS (4.6 vs 2.6 months, HR 0.45 [95% CI 0.29-0.72]) and OS (9.4 vs 6.7 months, HR 0.55 [95% CI 0.32-0.94]). In the SUNLIGHT phase III trial, patients with refractory mCRC who had received no more than two prior chemotherapy regimens showed significant improvements in progression-free survival (HR 0.44 [95% CI 0.36-0.54]; p < 0.001) and OS (HR 0.61 [95% CI 0.49-0.77]; p < 0.001) with the combinatio of TAS-102 and bevacizumab compared to TAS-102 monotherapy.[78]

Fruquintinib is a TKI that selectively targets VEGFR-1, -2, and -3.[79] In the context of the phase III FRESCO study, fruquintinib was evaluated in patients that had received at least two previous lines of chemotherapy with mCRC treated at China. The results showed that fruquintinib resulted in a significant improvement in OS (median of 9.3 months) compared to placebo (median of 6.6 months), as well as progression-free survival. In the international phase III FRESCO-2 study the efficacy and safety of fruquintinib were evaluated in patients with mCRC who had received TAS-102, regorafenib, or both. The study included 691 (who had received an average of four

previous lines of treatment) were randomly assigned (2:1) to receive fruquintinib 5 mg orally daily, 3 weeks on, 1 week off, or placebo, along with the best symptomatic treatment. The results showed a median OS of 7.4 months in the fruquintinib group compared to 4.8 months in the placebo group (HR 0.66 [95% CI 0.55–0.80]; p < 0.0001). [80]

1.4. Biomarkers in the era of precision oncology

1.4.1. Definition of a biomarker in cancer

There are several definitions of the term "biomarker" in the literature. The World Health Organization (WHO) defines biomarkers as any substance, structure, or process that can be measured in the body or its products and that influences or predicts the incidence or outcome of disease.[81] This definition includes functional, physiological, biochemical, and molecular interactions. Similarly, the NIH Biomarkers Definitions Working Group describes biomarkers as objectively measurable characteristics that indicate normal biological processes, disease states, or responses to therapeutic interventions.[82] These definitions highlight the diverse nature of biomarkers, which can range from molecular markers to imaging findings and physiological measurements. Molecular biomarkers can be diverse compounds such as proteins, nucleic acids, antibodies, or peptides, and can reflect changes in diverse biological key processes such as mutatations (genomic), gene expression (transcriptomic), or post-translational modifications (proteomic). Biomarkers have numerous applications in clinical settings for cancer patients, including diagnosis, prognosis, prediction of therapeutic responses, monitoring disease status, and assessing the toxicity of treatments, beyond others.[83]

Prognostic biomarkers predict the disease outcome regardless of treatment, estimating the long-term course of the disease independently of any therapeutic intervention. A biomarker is predictive if the treatment effect (experimental compared with control) is different for biomarker-positive patients compared with biomarker-negative patients. [84] Predictive biomarkers, measured before treatment, are crucial for tailoring the optimal therapeutic strategy for cancer patients to enhance response and minimize treatment-related toxicity.[85]

For a biomarker to be clinically useful and routinely implemented, it must be developed through focused studies on a specific, homogeneous population and correlate with tumor behavior and treatment outcomes. It should be easily accessible with standardized collection and processing protocols, avoiding serial assessments or invasive procedures. The assay must be reproducible with clear cutoffs and validated in an independent cohort. Additionally, the biomarker should guide clinical decisions leading to meaningful outcomes, such as improved survival, better quality of life, or reduced toxicity.[86] Table 1 summarizes the characteristics that an effective, clinically validated biomarker should possess.

Table 1: Characteristics of ideal biomarker

Prediction with High Accuracy
Cost-effective
Rapid turnaround time
Proven useful in each clinical context
Impact confirmed in validation cohort
Reproducible
Easy to interpret
Reliable

1.4.2. Molecular Biomarker classification: the ESCAT

The primary goal of cancer biomarker assays in precision medicine era is to personalize patient care by tailoring treatments to the unique multiomic traits of each individual, moving beyond the "one-size-fits-all" approach to deliver the right therapy to the specific patient at the optimal time, dose, and schedule.[87]

As our understanding of cancer biology improves and access to tumor genomic sequencing technologies expands, using molecular biomarkers as targets for precision cancer medicine is becoming a promising strategy. With an increasing number of patients undergoing multigene sequencing to identify genomic alterations that can be targeted, interpreting these complex sequencing results is becoming increasingly challenging in daily clinical practice. Differentiating between findings of proven clinical value, potential value based on preliminary evidence, and hypothetical gene-drug matches is crucial for managing expectations and ensuring the best care to cancer patients.[88,89] ESMO has unified efforts to create clinical guidelines and recommendations for classifying molecular predictive therapeutical biomarkers, or molecular alterations, based on clinical evidence of utility, aiming to

aid oncologists in prioritizing potential targets for clinical use creating the ESMO Scale of Clinical Actionability for molecular Targets (ESCAT) classification.[90]

Other scientific organizations have developed alternative classification systems for molecular targets. For instance, the AMP/ASCO/CAP guidelines provide a framework for categorizing genetic variants based on their clinical relevance. Similarly, OncoKB classifies molecular targets into different categories according to FDA approvals and the strength of supporting clinical evidence. These classification systems, like ESCAT, aim to guide the interpretation of molecular alterations and their potential role in precision oncology. Figure 2 resumes ESCAT classification. Here we find the definition of each ESCAT category:

- ESCAT I: the match between an alteration and a drug has been validated in clinical trials and should guide treatment decisions in daily practice.
    - I-A: prospective, randomized clinical trials demonstrate that the alteration-drug combination in a specific tumor type produces a clinically significant improvement in a clinical variable related to survival.
    - I-B: prospective, non-randomized clinical trials demonstrate that the alteration-drug match in a specific tumor type produces a clinically significant benefit.
    - I-C: clinical trials in different tumor types or "Basket" clinical trials show a clinical benefit associated with the alteration-drug match, with similar benefit across all tumor types.
- ESCAT II: a drug matching the alteration has been associated with responses in phase I/II or in retrospective analyses of randomized trials.
    - II-A: retrospective studies show that patients with the specific alteration in a particular tumor type experience a clinically significant benefit with the matched drug compared to alteration-negative patients.
    - II-B: prospective clinical trials demonstrates that the alteration-drug match in a specific tumor type leads to an increased response rate when treated with a compatible drug. However, currently there is no data on survival endpoints.
- ESCAT III: alterations validated in another cancer, but not in the disease being treated.

- III-A: demonstrated clinical benefit in patients with the specific alteration (similar to the previous levels I and II) but in a different tumor type. Limited/absence of available clinical evidence for the patient's specific cancer type or overall, for cancer types.
- III-B: an alteration that has a predicted functional impact similar to a previously studied tier I in the same gene or pathway, but lacks associated clinical data support.

- ESCAT IV: hypothetically actionable alterations based on preclinical data.
  - IV-A: evidence that the alteration or a functionally similar alteration influences drug sensitivity in *in vitro* or *in vivo* preclinical models.
  - IV-B: predicted actionability *in silico*.
- ESCAT X: alterations without any scientific evidence regarding actionability.

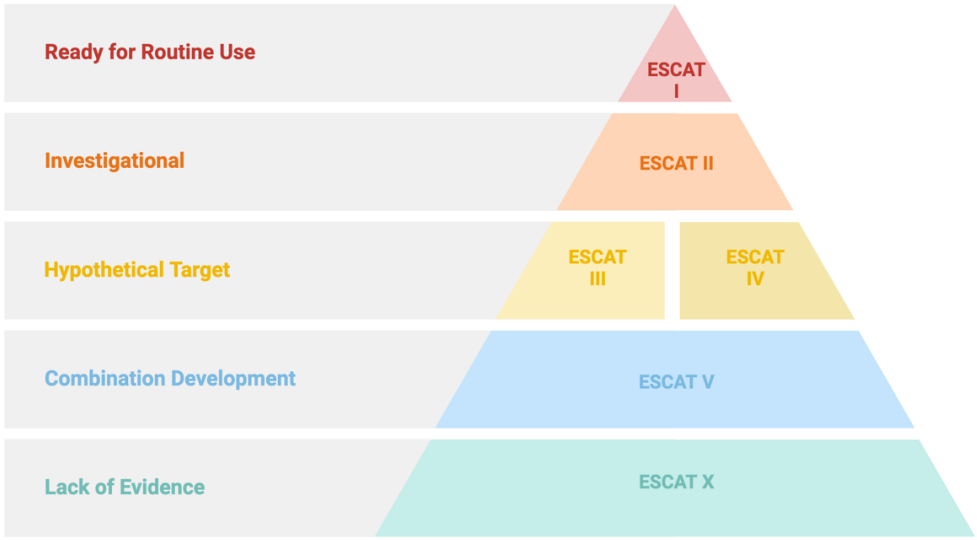


Figure 2: Resume of ESCAT classification (adapted from Mateo et al.[90]).

1.5. Biomarkers in colon cancer

1.5.1. Biomarkers in localized colon cancer

About 75% of newly diagnosed colon cancer patients have early-stage disease, and while surgical resection of the tumor and regional lymph nodes is curative for stages II and III, about 25-50% of these patients may relapse without adjuvant therapy.[91,92] Therefore, adjuvant systemic chemotherapy is crucial for reducing relapse risk by targeting clinically undetectable minimal residual disease (MRD). Currently, adjuvant therapy decisions are guided by clinicopathologic characteristics. Adjuvant

chemotherapy is generally recommended for stage III colon cancer patients, while stage II treatment remains debated and individualized.[93] The current standard of care in stage III is a combination of fluoropyrimidine and oxaliplatin, based on large-scale clinical trials.[94–96] In general, it has been established that adjuvant systemic therapy with fluoropyrimidines alone decreases the risk of death by 10%-15% in stage III disease, with a further 4%-5% improvement for oxaliplatin-containing combinations.[94–96] In order to decrease the risk of long-term peripheral neuropathy from oxaliplatin, the IDEA phase III study was conducted to compare 3-month vs 6-month CAPOX or FOLFOX regimens in more than 12,000 stage III colon cancer patients; however, it did not confirm the noninferiority of the 3-month regimen compared to 6 months.[97]

For stage II CRC with poor mayor prognosis factors (pT4 or <12 lymph nodes assessed during the surgery) the ESMO guidelines recommend adjuvant chemotherapy based on a combination of fluoropyrimidine and oxaliplatin.[98,99] When only one minor prognostic factor is present—such as high-grade histology, vascular, lymphatic or perineural invasion, bowel obstruction or perforation, or elevated preoperative CEA levels—the risk of relapse is relatively low. In this context, fluoropyrimidine monotherapy is recommended for microsatellite stable (MSS) tumors, while patients with stage II MSI tumors typically have an excellent prognosis and are unlikely to benefit from 5-FU alone. [93] Based on expert recommendation, patients with multiple minor prognostic risk factors might benefit from adding oxaliplatin therapy, as indicated by a trend toward increased benefit in the high-risk stage II subgroup of the MOSAIC trial, despite lacking statistical significance.[100–102] Tables 2 and 3 summarize key risk factors and clinical decisions for patients with stage II colon cancer across various guidelines, illustrating the current variability in biomarker use and clinical approaches in this population.

The MSI phenotype occurs in about 15-20% of localized CRC cases, with consistent positive prognostic value in stage II CRC, but results are controversial for patients with stage III CRC.[103,104] Considering the marked sensitivity of MSI mCRC to inmunotherapy, the neoadjuvant treatment landscape is likely to change dramatically in the coming years for this population.[105] Mismatch repair status is the sole approved molecular biomarker for localized CRC, while other genetic markers

like *RAS* and *BRAF* mutations are not recommended for routine assessment of recurrence risk due to their limited impact on adjuvant decision-making.[106]

The Immunoscore assesses the density of CD3+ and CD8+ T cells in tumors and their margins to estimate recurrence risk in localized colon cancer, with high scores correlating with a significantly lower risk of relapse.[107]

Gene signatures like Oncotype DX and GeneFx Colon, validated in stage II colon cancer using tumor samples, offer potential for prognostic stratification but are not routinely recommended for clinical use due to limited predictive value; they may, however, complement clinicopathological information in intermediate-risk cases.[108,109]

Table 2: Risk factors in different guidelines for stage II colon cancer patients

ASCO	Sampling <12 Lymph Nodes in surgery; Obstruction; Perforation; PNI; LI; VI; poorly differentiated; pT4; Budding 3 tumor	
ESMO	**Minor RF**	**Major RF**
	High grade; LI; VI; PNI; Obstruction; Elevated CEA pre -surgery	Sampling <12 Lymph Nodes in surgery; pT4

LV: Lymphatic invasion; VI: Vascular invasion; PNI: Perineural Invasion

Table 3: Summary of treatment decisions in stage II colon cancer

		ASCO (2022)	**ESMO (2020)**
No Risk Factor	**MSS**	Follow Up	Follow Up
	MSI		
1 Risk Factor	**MSS**	6 months fluoropyrimidine	6 months fluoropyrimidine
	MSI	3 or 6 months CAPOX/FOLFOX	Follow Up
>1 Risk Factor	**MSS**	3 or 6 months CAPOX/FOLFOX or 6 months fluoropyrimidine	6 months FOLFOX or 6 months CAPOX or 3 months CAPOX
	MSI	3 or 6 months CAPOX/FOLFOX	
pT4 or <12LN	**MSS**	3 or 6 months CAPOX/FOLFOX or 6 months fluoropyrimidine	6 months FOLFOX or 6 months CAPOX or 3 months CAPOX
	MSI	3 or 6 months CAPOX/FOLFOX	

1.5.2. Biomarkers in metastatic colorectal cancer

After diagnosing mCRC, a multidisciplinary tumor board should discuss to determine the optimal treatment strategy, considering patient comorbidities and preferences, tumor histology, molecular subtype, treatment goals, tumor burden, metastasis locations, primary tumor symptoms, among others.

Molecular biomarkers are crucial in selecting appropriate treatments for mCRC patients. Figure 3 shows the main biomarkers in mCRC. Mutations in *RAS* are found in approximately 40% of mCRC cases and are associated with worse prognosis. [110,111] At the time of mCRC diagnosis, guidelines recommend testing for MMR status as well as *KRAS*, *NRAS* (exons 2, 3, and 4), and *BRAFV600* mutations. This evaluation is crucial for selecting the appropriate first-line therapy, as these mutations serve as prognostic and predictive biomarkers for biological treatment. Moreover, it aids in planning the treatment continuum. Testing for these mutations can be performed on the primary tumor or metastatic sites, as they occur early in CRC and show strong correlation between primary and metastatic tumors. If tissue is unavailable, *RAS* mutations can be tested using ctDNA.[112]

BRAFV600 mutations are found in 8–12% of mCRC patients and are associated with poor prognosis, aggressive tumor behavior, females, right-sided tumors, mucinous histology, and frequent peritoneal metastases. Preclinical and translational studies have revealed that upon BRAF inhibition, there is an immediate signal upregulation via the EGFR pathway, suggesting the addition of an anti-EGFR treatment to the BRAF inhibitor.[113,114] The phase III BEACON trial set the combination of encorafenib/cetuximab as the standard treatment for *BRAF*V600E mCRC after at least one prior systemic therapy.[115,116] Patients were randomly assigned to receive a triplet therapy (encorafenib, cetuximab, binimetinib), doublet therapy (encorafenib, cetuximab), or control treatment. The doublet therapy showed a mOS of 9.3 months compared to 5.9 months in the control group. The phase III BREAKWATER trial is currently evaluating the combination of encorafenib and cetuximab with chemotherapy as a first-line treatment for *BRAFV600* mCRC patients, with preliminary promising results. [117]

MSI testing is essential not only for detecting hered but also to select patients for immune checkpoint inhibition (ICI) as part of the initial molecular work-up.[118,119] In the pivotal KEYNOTE-177 phase III trial, previously untreated dMMR/MSI-H mCRC

patients were randomized to receive pembrolizumab or standard therapy (chemotherapy plus targeted agents, according to investigator). Pembrolizumab exhibited a significant improvement in the primary endpoint of PFS and improving quality of life compared to chemotherapy. Although no significant differences in OS were observed, this may be due to the high percentage of crossover in the chemotherapy arm (60% of patients progressing received an ICB).[119,120] The recent CheckMate 8HW phase III trial showed that untreated MSI-H/dMMR mCR patients presented significantly longer PFS when treated with nivolumab plus ipilimumab than with chemotherapy (72% vs 15% at 24 months of FUP), along with fewer side effects.[121] Moreover, nivolumab plus ipilimumab showed superior compared to nivolumab monotherapy (not reached vs. 39.3 months).[122]

Amplification of the HER2 (human epidermal growth factor receptor 2) gene (*ERBB2*) has been reported in approximately 3% to 4% of all CRC patients, being more common in *RAS/BRAF*wt patients and those with rectal localization. [123,124] *ERBB2* amplification is generally seen as a negative predictor for anti-EGFR therapies based on retrospective data.[125–127] Furthermore, *ERBB2* amplification serves as a predictive factor for the effectiveness of HER2-targeted therapies when used in combination. In the second-line treatment and beyond, HER2-directed therapies, such as the monoclonal antibody trastuzumab, combined with tyrosine kinase inhibitors like tucatinib, lapatinib, or pertuzumab, as well as the antibody-drug conjugate trastuzumab-deruxtecan, have shown clinical effectiveness mCRC patients.[128–134] Building on its efficacy in later treatment lines, the ongoing MOUNTAINEER-03 trial is assessing tucatinib and trastuzumab in combination with FOLFOX compared to FOLFOX plus either bevacizumab or cetuximab in the first-line setting.

In mCRC, the *KRAS* glycine-to-cysteine mutation at codon 12 (*KRAS* G12C) occurs in up to 4% of the patients and is associated with a poor response to standard treatments and shorter OS compared with non-G12C mutations.[135,136] The *KRAS* protein cycles between “on” state, attached to guanosine triphostpahte (GTP), to an “off” state in wich GTP lost one phosphate turning into guanosine diphosphate (GDP). The G12C mutation impairs GTP hydrolysis, which shifts KRAS to the active GTP-binding state promoting tumorigenesis and metastases. [135,137] Despite best efforts for many decades, *RAS* mutations have proved to be challenging to target,

remaining undruggable until recently. Some targeted agents have now demonstrated clinical activity against *KRASG12C* mutations.[138] Initial treatment with KRAS G12C inhibitors in monotherapy revealed that blocking KRASG12C leads to resistance through negative feedback via the EGFR receptor, similar to *BRAFV600* mCRC.[139] Understanding this biology was key to overcoming resistance mechanisms. Subsequent studies incorporated EGFR inhibitors to enhance antitumor effects and improve clinical outcomes by overcoming resistance mechanisms. Adagrasib and sotorasib were the first KRAS inhibitors to enter clinical development. The CodeBreaK 300 trial, which evaluated sotorasib in combination with panitumumab, became the first phase III randomized study to show a clinical benefit of a KRAS inhibitor compared to standard therapy in patients with refractory mCRC.[140] Besides, new KRAS inhibitors such as divarasib or garsorasib have also demonstrated deep clinical activity not only in monotherapy (with disease control rate of 84% and 95% respectively) but also in combination with anti-EGFR agents in the case of divarasib (disease control rate increase to 95.8%). [141,142] Pending full approval by the EMA and FDA, cetuximab-adagrasib and panitumumab-sotorasib have been included in the ESMO live guidelines for mCRC as ESCAT 1A treatments for patients with KRASG12C mutations in third-line therapy or beyond. [140,143]

POLE and *POLD1* mutations play a critical role in DNA replication fidelity, as their exonuclease domains are responsible for proofreading and repairing mismatched bases. Mutations in these domains, present in about 1-2% of mCRC patients, result in loss of proofreading function, leading to the accumulation of genetic mutations. These genes are crucial for maintaining genomic stability through their roles in DNA replication and repair mechanisms.[144] Notably, akin to MSI-h tumors, CRCs with *POLE*/D mutations exhibit heightened infiltration of immune cells and a high tumor mutational burden.[145] Immune checkpoint inhibitors such as nivolumab and durvalumab show promising results in mCRC patients with POLE/D exonuclease domain mutations, suggesting their potential as predictive biomarkers for ICI in MSS mCRC, pending further validation.[146,147] *NTRK* gene fusions, present in 0.2%–1% of mCRC patients and mostly in dMMR *RAS/BRAF*wt patients, lead to overexpression of tropomyosin receptor kinase proteins; many of these patients also have MMR deficiency.[148,149,149,150] Although NTRK fusions are rare in CRC, larotrectinib and entrectinib (pan-tropomyosin-related kinase inhibitors) are potential therapeutic

options for NTRK fusion-positive CRCs after progression on at least two lines of treatment, so testing should be considered whenever a treatment option is available. [151] Gene fusions of *ALK* and *ROS1* occur in 0.2–2.4% of mCRC patients, often in older, female patients with dMMR status.[152]While ALK and ROS1 inhibitors are available for non-small cell lung cancer, none are currently approved for treating mCRC.[153] *RET* fusions have been described in less than 1% of patients with mCRC, and are frequently associated with *BRAF*wt right-sided tumors. The presence of a *RET* fusion confers poor prognosis to patients with mCRC. [154] Some case reports described the efficacy of tyrosine kinase inhibitors in mCRC patients with RET fusions. [154,155]

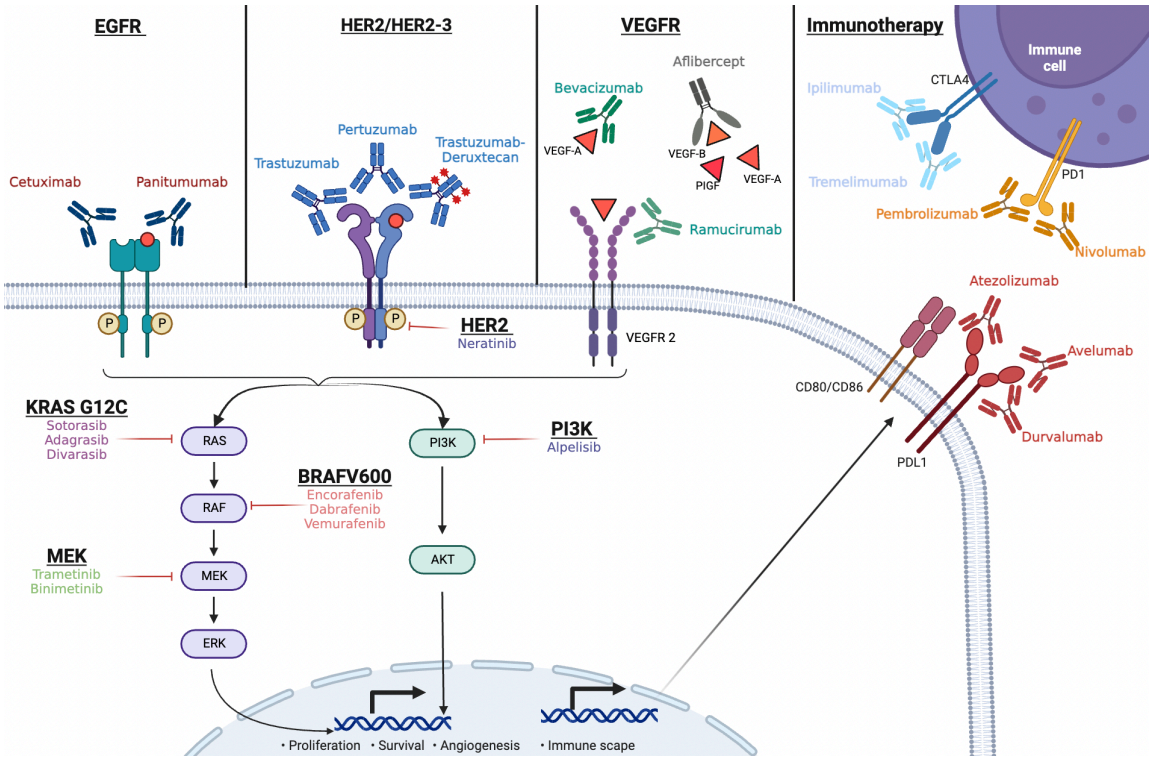


Figure 3: Main Molecular pathways and their main target treatment in mCRC. Adapted from N. Saoudi et al. [156]

1.5.3. Predictive biomarkers to antiangiogenic therapies

Antiangiogenic therapies play a crucial role in mCRC treatment, yet their effectiveness varies among patients, with some experiencing substantial side effects and financial burdens. A major challenge in optimizing these treatments is the absence of reliable biomarkers to predict therapeutic response. While research has explored potential biomarkers, most are based on limited retrospective studies and lack sufficient validation for clinical application. [157,158] This highlights a critical

unmet need for predictive biomarkers to guide patient selection and improve the cost-effectiveness and safety profile of antiangiogenic therapies in mCRC.

Regarding tissue-based biomarkers, single-nucleotide polymorphisms (SNPs) of the VEGF gene have been investigated. The polymorphism of VEGF-Ac.*237C>T was significantly related to time-to-treatment failure in 46 mCRC patients treated with bevacizumab. [159] A retrospective analysis of different VEGF-A, VEGF-C, and VEGFR-1,2,3 SNPs was made in 138 patients with mCRC treated with regorafenib, showing that VEGF-A rs2010963 maintained an independent correlation with PFS and OS. [160] A study of angiogenesis-related proteins in surgical samples suggests that HIF-2α expression levels might help identify patients who would benefit from bevacizumab treatment.[161]

Circulating VEGF is the most studied potential predictive factor for anti-angiogenic treatment in mCRC patients. A post-hoc analysis of the VELOUR trial indicated that higher levels of VEGF-A and PlGF in patients previously treated with bevacizumab may signal resistance. In the placebo group, higher baseline levels of these biomarkers were associated with shorter survival. Conversely, in the aflibercept group, improved OS and PFS were observed regardless of baseline VEGF-A or PlGF levels, confirming aflibercept activity even in patients with bevacizumab-induced resistance.[162] Other retrospective data supports this prognostic role of plasma levels of VEGF-A, but without implications in the prediction of response to antiangiogenic treatment. [163–165]Two phase III studies (HORIZON II and III) evaluating the use of cediranib (a VEGFR TKI) in combination with chemotherapy in first line and compared to placebo or bevacizumab (respective) have evaluated baseline levels of VEGF and soluble VEGFR-2 (sVEGFR-2) as prognostic and predictive biomarkers. High baseline VEGF was associated with worse PFS in both studies and with worse OS in the HORIZON II study. However, these results were not uniformly confirmed.[166] In the phase III prospective ITACa trial, where mCRC patients were randomized to receive FOLFOX/FOLFIRI with or without bevacizumab, the circulating mRNA expression of five biomarkers—VEGF-A, eNOS (Endothelial Nitric Oxide Synthase), EPHB4 (Ephrin type-B receptor 4), COX2 (Cyclooxygenase-2), and HIF-1α—at baseline and during were analyzed. Baseline circulating biomarker levels were not associated with clinical outcomes. However, a >30% reduction in eNOS or VEGF mRNA levels from baseline to the first clinical evaluation was

significantly associated with longer OS in bevacizumab treated patients. [167] In a study of mCRC patients treated with FOLFIRI plus aflibercept in a phase II trial, specific circulating miRNAs, particularly hsa-miR-33b-5p, were identified for distinguishing responders from non-responders and predicting disease progression risk.[168]

Studies on circulating cell-free DNA (cfDNA) and bevacizumab response have identified resistance mechanisms in mCRC. Whole-genome sequencing of plasma DNA in 150 patients revealed a recurrent focal amplification on chromosome 13q12.2 in 8.7% of cases, linked to advanced disease and resistance. This amplification, involving the POLR1D gene, promotes cell proliferation and VEGFA upregulation, a key factor in angiogenesis and resistance. [169] A study quantifying ctDNA before and after treatment in 35 mCRC patients treated with regorafenib found that elevated baseline levels of total ctDNA inversely correlated with PFS, while early reductions in mutant ctDNA fractions predicted longer PFS.[170] Regarding regorafenib, a retrospective analysis from the CORRECT trial (phase III trial comparing regorafenib vs placebo in refractory setting) assessed in 503 mCRC patients using plasma DNA BEAMing technology to identify *KRAS*, *PIK3CA*, and *BRAF* mutations and quantify circulating protein biomarkers. *KRAS* mutations were found in 69% of patients, with 48% of those classified as *KRAS*wt in tissue, showing mutations in plasma DNA, highlighting BEAMing's utility for real-time tumor genotyping. Regorafenib demonstrated consistent clinical benefits across subgroups defined by *KRAS* and *PIK3CA* mutational status or ctDNA concentrations. While most protein biomarkers were not predictive, high TIE-1 (an angiopoietin receptor) concentrations were associated with longer overall survival in the univariable analysis.[171]

Histological metastatic growth patterns in the liver have been correlated with resistance to antiangiogenic treatment. The replacement growth pattern, which relies on microvessel co-option (integrating pre-existing normal blood vessels from the surrounding liver tissue) instead of forming new ones, has been identified as a marker of resistance to antiangiogenic therapy.[172] Some studies are researching the role of image in the assessment of vascularity of mCRC by radiomics of MRI and CT scan, trying to translate medical images into biological information about tumor angiogenic status. [173] Dynamic contrast-enhanced ultrasound imaging reveals

variability in tumor response and could predict treatment outcomes. Pharmacokinetic parameters from dynamic contrast-enhanced MRI offer a promising approach for evaluating response and progression in bevacizumab-treated patients. Perfusion CT, through its integration of anatomical detail and vascular physiology, correlates angiogenesis with microvessel density and serves as a robust tool for therapeutic assessment, though results vary.[174]

1.6. Circulating tumor DNA as a biomarker

1.6.1. Biological bases of ctDNA

Precision oncology uses high-throughput technologies to identify actionable genomic alterations in cancer tissue, improving the efficacy of treatments and reducing toxicity. While traditional methods required tissue samples, liquid biopsies now offer a non-invasive way to obtain genomic data. Liquid biopsy is a minimally invasive method that focuses on blood or body secretions to detect molecular alterations, tumor cells, or metabolites. In medicine, liquid biopsy has been key to avoiding chorionic biopsies by enabling the study of fetal DNA in maternal blood. In other fields, such as infectious diseases, it is changing the way we diagnose microbial infections through the study of microbial cell-free DNA sequencing.[175,176]

Over the past decades, liquid biopsy have gained importance also in the field of oncology, enabling the implementation of MRD detection in solid tumors, advancing research in early diagnosis, and aiding in tracking clonal evolution and drug resistance. [177–181]

The term "circulating cell-free DNA" (cfDNA) refers to fragmented DNA present in the cell-free component of whole blood. This term was introduced by Mandel and Métais in 1948, marking the beginning of a significant area of research in molecular biology. [182] It was not until the 1977 that cfDNA was first observed to increase in cancer patients by Leon et al, highlighting its potential as a tumor biomarker.[183] In 1989, Stroun et al. first reported the appearance of several plasma DNA originated from cancer cells. [184] In 1994, Sorenson et al. achieved a breakthrough by detecting specific cancer mutations, such as *KRAS* mutations, in blood.[185] Subsequent studies, including research on ctDNA mutations of resistance in colon cancer and *EGFR* gene mutations in non-small cell lung cancer, have demonstrated the critical role of

ctDNA in monitoring disease progression and response to therapy. The rise of next-generation sequencing (NGS) in the past decade has improved ctDNA detection, marking a "golden age" for ctDNA research with major impacts on cancer diagnosis and treatment. Beyond the study of DNA, there are multiple biomarkers found in liquid biopsies that provide valuable insights into the molecular insights of cancer, including circulating tumor cells (CTC), extracellular vesicles (EVs), cell-free RNA (cfRNA), microRNA (miRNA), cfDNA, and ctDNA. [181] It has shown that cfDNA has origins from apoptotic and necrotic processes. [186] In healthy individuals, cfDNA is primarily originated from hematopoietic cells and is typically present at low concentrations (around 1–10 ng/mL of plasma).[187] cfDNA exhibits daily fluctuations and often changes in response to various physiological conditions; it typically increases in plasma following exercise, burns, sepsis and trauma, serving as a broad health indicator that spikes during stress or physical activity.[188,189]

Aside from blood, other non-invasive approaches using urine, saliva, and semen, along with invasive methods using cerebrospinal fluid (CSF), and pleural and peritoneal effusions, have been utilized to assess ctDNA.[190] CtDNA in urine includes both short fragments (<100 bp) filtered through the kidneys and longer fragments from tumors in the urinary tract. Saliva may also contain ctDNA from local tumors, but with shorter and less abundant fragments (40–60 bp).[190] The bloodstream is ideal for ctDNA studies because it is fresh, preservable, and can be easily accessed through a simple blood draw. This allows for continuous monitoring of tumor changes over time and across different tumor regions.

Circulating tumor DNA is a subset of cfDNA released specifically from tumors, reflecting the same genomic alterations as the tumors themselves with ctDNA fractions ranging from less than 0.1% to over 50%. [191,192] Cancer patients exhibit higher levels of plasma cfDNA than healthy individuals. [191] Its detection at low fractions has improved with advancements like BEAMING and NGS. [193] The short half-life of ctDNA (minutes to a few hours) allows for real-time monitoring of disease status. [194]

The mechanisms responsible for the release of ctDNA from tumors are not fully understood, and both active and passive release mechanisms lack systematic descriptions. Apoptosis and necrosis as major contributors to ctDNA release, but other mechanisms have been described, such as ferroptosis, pyroptosis, active

secretion in extracellular vesicles and senescence. [195] There is compelling evidence suggesting that the distribution of cfDNA fragment sizes follows a non-random pattern. [196] The size of cfDNA fragments is influenced by the number of nucleosomes wrapping around the DNA.[197] The predominant peak size of cfDNA fragments is 167bp, corresponding to the length of DNA around a single nucleosome (147bp) and a protective linker DNA (20bp) that shields DNA from cleavage. [198,199] Apoptotic cell-derived cfDNA exhibits a ladder-like pattern due to internucleosomal DNA fragmentation, whereas necrotic tumor cells release larger DNA fragments.[200] Other passive release mechanisms involve circulating tumor cells and chromosomal instability. [201] Hypoxia and the tumor's molecular features are implicated in ctDNA release, with hypoxia potentially modulating ctDNA release rates.[202] Additionally, cell death can indirectly induce active ctDNA release through paracrine signaling, potentially affecting treatment resistance.[203] The molecular factors influencing ctDNA release, including tumor genetics and immunity, are not well understood. cfDNA interactions with proteins and extracellular vesicles affect its clearance and degradation, with complexes like monoclonal antibodies and nucleosomes reducing degradation by blocking DNase access.[204] Membrane encapsulation by extracellular vesicles protects cfDNA from degradation. The uptake of cfDNA by cells may also play a role in clearance.[201,205] mCRC patients with metastases limited to the lungs or peritoneum often show lower or undetectable ctDNA levels. [206] In a recent study, patients with localized colon cancer recurrences in the peritoneum, locoregional, or lungs lacked detectable ctDNA, suggesting that the type and location of metastasis affect ctDNA shedding.[207] The biological mechanisms for these patterns remain unclear.

1.6.2. ctDNA detection methods

High sensitivity is essential for accurate ctDNA detection due to its variability influenced by therapy, tumor traits, and individual patient factors.[192,208] While advanced-stage cancer can exhibit ctDNA levels exceeding 10% of peripheral cfDNA, early stage may feature levels as low as 0.1%. [209] Enhanced sensitivity at low VAFs is imperative, as highlighted by the FDA's evaluation of commercial ctDNA assays, emphasizing the critical need for test sensitivity below 0.5% VAF for MRD detection. [210] To address these challenges, solutions have been developed across preanalytical,

analytical, technical, technological, bioinformatics, and biological domains. These advancements greatly reduce the limit of detection (LOD) for ctDNA assays, enabling highly sensitive methods. Detection of ctDNA involves targeted and untargeted approaches. Targeted methods, like digital PCR (dPCR) and targeted NGS, focus on specific known mutations and are useful for tracking known tumor changes. Untargeted methods, such as whole-genome sequencing (WGS) and whole exome sequencing (WES), do not require prior tumor information and can uncover unknown alterations, aiding in the monitoring of clonal evolution and drug resistance.[211] Choosing a ctDNA detection method depends on factors like clinical needs, sensitivity, specificity, target mutations, cost, and availability. NGS offers comprehensive analysis but is costly and complex, while dPCR provides precise quantification and high sensitivity but has a limited target range.

1.6.2.1. Preanalytics

The accurate detection of ctDNA fragments is highly dependent on preanalytical factors, including the type and volume of the specimen, timing of collection, as well as conditions of processing and storage.[212] Current evidence suggests that plasma is preferable to serum for ctDNA detection, and blood should be collected in tubes containing EDTA.[213] Timely plasma separation—ideally within 1 to 2 hours after blood collection—is essential to prevent white blood cell lysis, which can contaminate ctDNA with genomic DNA from leukocytes. When immediate processing is not feasible, specialized cfDNA stabilization tubes offer an alternative, allowing for sample storage over several days without affecting cfDNA integrity.[213] Plasma is typically separated using double centrifugation under slow deceleration and stored at -80 °C until DNA extraction.

Circulating tumor DNA levels may be affected by systemic and local treatments, such as chemotherapy, targeted therapy, immunotherapy, or radiotherapy, but also by other concurrent inflammatory processes such as trauma or surgery. Elevated levels of circulant normal cfDNA is leading to background noise that could effectively dilute the ctDNA and lower sensitivity. Therefore, for the detection of postoperative MRD, blood sampling should ideally be performed at least 2 weeks after surgery. Repeat testing is also suggested to avoid false negatives.[214]

1.6.2.2. Target PCR techniques: Real time PCR and Digital PCR

The initial ctDNA analysis methods were real time target PCR-based, specifically designed to detect single-gene mutations. Real time PCR (rt-PCR) enable the detection of point mutations and short indels at remarkably low frequencies.[211,215] Digital PCR (dPCR) techniques, including BEAMing (beads, emulsions, amplification, and magnetics) and Droplet Digital PCR (ddPCR), represent a substantial advancement over real time PCR in terms of sensitivity for quantifying ctDNA. They are particularly recognized for their capability to identify point mutations and short insertions or deletions (indels) at extremely low frequencies. [211,215] These methods, extensively evaluated across various cancers and clinical scenarios, enhance traditional PCR sensitivity by 10–100 times, consistently detecting VAFs as low as 0.1% to 0.01%. [211,215] To quantify the target DNA, rt-PCR requires standard curves, generated using samples with known concentrations of the target. Unlike RT-PCR, dPCR can detect a single transcript copy, providing absolute quantification without standard curves.[211]

Figure 4 resumes the BEAMing technology. BEAMing uses magnetic beads coated with streptavidin that display primers specific to both mutated and non-mutated alleles on their surface. These beads are emulsified with the DNA to be analyzed, along with all necessary components for amplification. Each compartment contains, on average, no more than one DNA molecule to be analyzed and one bead. Following PCR thermocycling, each bead is coated with thousands of identical copies of the original DNA strand being studied. The emulsion is then broken, and the beads are recovered using a magnet. After DNA denaturation, the beads are incubated with oligonucleotide probes linked to fluorescent substances. The separation of the beads according to the sequences is performed by flow cytometry, allowing for quantification of the different populations.

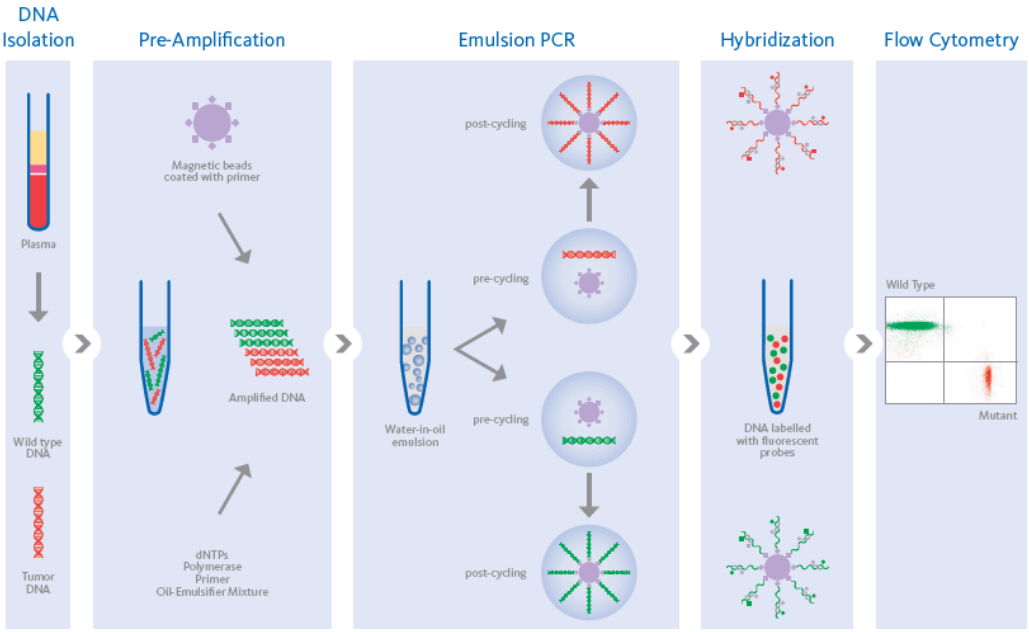


Figure 4: Digital PCR technology based on emulsion PCR with magnetic beads. Adapted from Sysmex®.

1.6.2.3. Targeted Next-Generation Sequencing Tumor-agnostic

Tumor-agnostic NGS approaches address the limitations of traditional methods by not relying on prior genomic information from the patient's tumor. Instead, these tests focus on analyzing specific genomic loci, including clinically relevant single nucleotide variants (SNVs), indels, fusions, and copy number variations (CNVs). By using target enrichment techniques, such as amplicon-based or hybrid capture methods, tumor-agnostic NGS can study selected regions of the genome, often guided by resources like COSMIC to design targeted assays.[211]

These techniques, while highly sensitive (detecting mutant alleles down to the 0.01% allele fraction), face challenges due to increased blood and sequencing artifacts originating from leukocytes and clonal hematopoiesis of indeterminate potential. Mutations associated with clonal hematopoiesis, occurring in common oncogenic drivers (*TP53*, *KRAS*, *PIK3CA*), can contribute to misdiagnosis, especially at low ctDNA VAF.[216–219] Analytical and technical progress in NGS platforms has provided unprecedented throughput, improved read-lengths, and extensive sequencing depth, coupled with minimized sequencing errors. [211,215] These advances extend to library preparation techniques, sequencing chemistries, and innovations in target enrichment strategies, including multiplexing and barcoding

approaches. Bioinformatics pipelines now include advanced variant calling algorithms, robust error correction, strict quality control, and machine learning for enhanced data interpretation.

Amplicon-based ctDNA NGS assays use targeted amplification to detect and analyze specific regions of interest. After ctDNA extraction, a targeted amplification step employs specific primers designed to flank the regions of interest, ensuring amplification of the intended sequences.[220,221] Molecular barcoding techniques are straightforward, involving a three-cycle barcoding PCR, followed by adaptor PCR for library generation, and then bead purification before sequencing. Hybrid capture-based ctDNA NGS assays, utilize biotinylated oligonucleotide baits to selectively capture specific library regions, binding to target cfDNA fragments subsequently isolated using streptavidin. The incorporation of unique molecular identifiers and advanced error correction techniques have demonstrated an initial LOD detection limit of 0.02%. [222]

1.6.2.5. Targeted Tumor-informed NGS

Tumor-informed sequencing, which utilizes genomic analysis of tumor tissue to create a unique "barcode" for tracking mutations in the blood, stands out as a method to enhance sensitivity and specificity. This is because the mutations being tracked are patient-specific, allowing the exclusion of non-tumor clonal hematopoiesis of indeterminate potential mutations. However, tumor-informed ctDNA testing has practical limitations, such as high cost and long turnaround times, which may hinder widespread adoption and timely decision-making. Despite being resource-intensive, tumor-informed sequencing panels typically offer increased sensitivity and have been pivotal in MRD detection. This is particularly significant for patients with early-stage cancer, who generally exhibit lower ctDNA levels compared to metastatic patients, presenting challenges in detection sensitivity that tumor-informed tests are designed to address. [222,223]

1.6.2.6. New strategies: non-targeted techniques

Untargeted methods, such WGS and WES, offer valuable insights into cancer by providing genome-wide copy number profiling and mutation spectrum evaluation, without the need for prior tumor genome information, allowing for the discovery of previously unknown alterations and monitoring of drug-resistant clones. Whole-genmoe sequencing enables the detection of copy number variations, indels, and point mutations across the entire genome. However, it suffers from lower sensitivity and higher costs. Conversely, WES offers a more targeted approach by sequencing only the exome within the genome, which can be more efficient. Despite these advantages, both techniques are currently limited in their applications and require higher concentrations of input material.

Initially, shallow whole-genome sequencing (sWGS) was used for the detection of somatic copy number alterations. Recent studies demonstrate the expanded capabilities of sWGS in uncovering complex tumor characteristics, such as DNA fragmentation patterns and SNVs. [211,215] Advancements in sWGS showcase efficient detection of ctDNA fractions as low as 10^{-5}, offering cost-effective, genome-wide analyses that outperform targeted sequencing in identifying events crucial for cancer detection.

Traditional hotspot mutation analysis using sequencing panels can be limited in early cancer detection due to the absence of detectable mutations in some cancers and the challenge of identifying rare mutations amidst a large amount of normal cfDNA, leading to higher false negatives. To overcome these limitations, researchers are exploring several alternative approaches. One such approach involves analyzing the fragmentation patterns of cfDNA, such as read length, end-motif sequences, and chromosomal distribution, using low-coverage whole genome sequencing analysis.

Fragmentomics studies the fragmentation patterns of cfDNA and ctDNA, revealing that non-random cfDNA fragmentation reflects epigenetic regulation and tissue of origin. [224] The study of specific ctDNA fragmentomes is being implemented in early cancer detection. [225]Table 4 resumes the main ctDNA detection methods and applications.

To further address the limitations of traditional ctDNA liquid biopsy testing, the use of cancer-specific methylation signals has been explored. This approach involves

analysing altered patterns of DNA methylation characteristic of cancer cells and leveraging these features to detect cancer-specific methylation signals in cfDNA. Additionally, it enables differentiation between various types of cancer origins by identifying the cancer-specific DNA methylation pattern in different tissue type. [195] There are several types of methylation analysis in cancer cfDNA using NGS technology including bisulfite sequencing, affinity purification for methylated DNA and methylation sensitive restriction enzyme sequencing. [226]

Table 4: Resume of main ctDNA Detection Methods

Category	Tests/Approaches	Applications
Targeted PCR-based methods	Real-time PCR (Super-ARMS EGFR Mutation Kit)	Targeted therapy selection
	Digital PCR (Bio-Rad QXDX ddPCR, ScodaSafe EGFR T790M assay)	Monitoring specific resistance mutations
Tumor-Agnostic NGS	Amplicon-based (ThermoFisher Oncomine cfDNA, SafeSEQ)	Monitoring MRD, therapy selection, mutation analysis
	Hybrid capture-based (TSO500, FoundationOne Liquid CDX)	Cancer detection, genotyping, MRD, clonal evolution
Tumor-Informed NGS	Personalized panels (Signatera, TARDIS)	MRD detection, recurrence prediction, therapy adjustment
Genome-wide approaches	WGS or WES (MRDetect, INVAR, DREAMS)	Detection of unknown alterations, clonal evolution, rare mutations
Emerging Techniques	Shallow WGS (PlasmaSeq, ichorCNA)	SCNA evaluation, DNA fragmentation analysis, CNV estimation
	Fragmentomics	Tissue of origin analysis, cfDNA fragmentation pattern assessment
	Methylation-based assays (bisulfite sequencing, methylation-sensitive assays)	Differentiating cancer origins, early cancer detection

1.7. ctDNA as a biomarker in colon cancer

1.7.1. ctDNA as a biomarker in locoregional colon cancer

Balancing the benefits of relapse reduction against the risks of toxicity in adjuvant CRC treatment is challenging, making it essential to identify which patients need chemotherapy versus those who can be monitored closely. [227] There is an urgent need for robust predictive biomarkers to better identify relapse risk and assess treatment benefits. Recent efforts are focused on using ctDNA to identify post-surgery MRD in CRC, guiding adjuvant treatment decisions and reducing unnecessary interventions. Circulant tumor DNA offers a noninvasive, highly sensitive, and specific method for assessing MRD and determining the need for adjuvant chemotherapy.

The first evidence of ctDNA as a marker MRD in colorectal cancer was published in 2008 in a study of 18 patients with resected liver metastases. Using the BEAMing assay, the study showed that ctDNA levels decreased after metastasectomy; patients with detectable ctDNA at follow-up had recurrences, while those with undetectable ctDNA did not.[228] This observation inspired different studies to validate ctDNA as a marker of MRD. Tie et al.'s research on stage II colon cancer found that postoperative ctDNA detection independently predicts relapse-free survival, particularly with adjuvant chemotherapy. [229] Another multicenter study links postoperative detectable ctDNA to inferior relapse-free survival, affecting the 3-year recurrence-free interval in those receiving adjuvant chemotherapy, while a prospective cohort demonstrates ctDNA's independent predictive role after surgery in patients with stage I to III CRC.[230–236] The recent GALAXY observational study, encompassing more than 1000 surgically resected CRC patients of all stages, confirmed prospectively ctDNA's prognostic role. Intriguingly, the study revealed that adjuvant treatment improved 6 and 12-month DFS in patients with ctDNA-positive across stages II, III, and IV, with no DFS advantage observed in patients with ctDNA-negative.[236] Table 5 resumes the main observational studies published.

Table 5: Main observational studies published using ctDNA for MRD

Study	n	Stage	ctDNA Assay	ctDNA detection rate	HR for RFS ctDNA+ vs -
Tie J, et al.	230	II	Safe-SeqS	8.7%	13.3, p < 0.001
Tie J, et al.	96	III	Safe-SeqS	21%	3.8, p < 0.001
Taieb J, et al.	805	III	ddPCR	13.5%	1.85, p < 0.001
Reinert T, et al.	130	I-III	Signatera	10.6%	7.2, p < 0.001
Tarazona N, et al.	69	I-III	ddPCR	20.3%	6.96, p < 0.001
Chen G, et al.	240	II-III	NGS (>1 variant; 425 genes)	8.3%	11.0, p < 0.001
Anandappa G, et al.	85	II-III	Signatera	II: 6.5%/III: 26%	10.0, p < 0.001
Parikh AR, et al.	103	I-IV	Guardant Reveal (epigenomic + genomic)	Post-Adjuvant: 24%	11.2, p < 0.001
Kotani D, et al.	1039	II-IV	Signatera	18%	10.0, p < 0.0001

Safe-Seq: 1 variant; 15 genes; ddPCR: 2 methylated markers; Signatera: 16 variants; WES

Observational studies have highlighted that detecting ctDNA after curative treatment indicates a high risk of recurrence, but the benefits of adjuvant therapy for these ctDNA-positive patients remain uncertain. Ongoing prospective clinical trials are exploring how ctDNA can guide treatment localized settings. The Circulating Tumor DNA Analysis Informing Adjuvant Chemotherapy in Stage II Colon Cancer (DYNAMIC) was a randomized phase II trial designed to investigate whether a ctDNA-guided approach as compared with a standard approach in stage II colon cancer could reduce the use of adjuvant treatment without compromising the risk of recurrence. Four hundred and fifty-five patients with stage II colon cancer were randomly assigned to either ctDNA-guided management or standard management, with a lower percentage of ctDNA-guided patients receiving adjuvant chemotherapy (15% vs. 28%). The 2-year recurrence-free survival with ctDNA-guided management was non-inferior to standard management (93.5% vs. 92.4%), demonstrating that a ctDNA-guided approach reduced adjuvant chemotherapy use without compromising recurrence-free survival.[237]

Many ongoing trials with both escalating and de-escalating treatment strategies based on ctDNA assessment are ongoing in early colon cancer, and will further define the utility of ctDNA for adjuvant systemic treatment decisions.

1.7.2. ctDNA as a biomarker in metastatic colorectal cancer

Cancers are characterized by spatial and temporal heterogeneity, which is particularly relevant in mCRC

Tissue samples for genetic studies can be biased due to their invasiveness and the difficulty of sampling multiple metastatic lesions. Circulating tumor DNA provides a more comprehensive view of all tumor clones, offering broader insights than a single biopsy. Metastatic CRC clonal populations can vary due to tumor microenvironment and treatment pressures. The use of ctDNA allows us to track clonal evolution secondary to the treatments in real-time to improve precision medicine.

Selection of first-line treatment in patients with mCRC is largely guided by biomarker profiling[238,239]. Multiple studies have compared driver mutation profiles between solid tissue biopsies and ctDNA, demonstrating a high degree of concordance. However, detection rates can differ depending on metastatic site, with liver metastases typically yielding higher ctDNA detection than peritoneal or lung metastases, likely reflecting variations in ctDNA shedding dynamics. [240–242]

Apart from *RAS* and *BRAF* mutations, various studies have retrospectively expanded the molecular selection strategies to enhance tumor response to EGFR inhibitors. "Negative ultra-selection" efforts have included the retrospective analysis of diverse alterations such as ERBB2, EGFR ECD, FGFR1, PDGFRA, PIK3CA, PTEN, AKT1, MAP2K1, KRAS, ERBB2, MET amplifications, among others. While the clinical validity of those biomarkers in the decision algorithm for anti-EGFR administration remains to be fully established with prospective clinical trials, ctDNA is recognized as a valuable tool for detecting these resistance biomarkers. A post-hoc analysis of the PARADIGM trial further supported negative ultraselection using ctDNA, where ctDNA allowed negative ultra-selection to distinguish those patients experiencing greater benefit to chemotherapy and panitumumab (vs. bevacizumab) regardless of primary tumor sidedness.[243]

Beyond EGFR targeting, ctDNA holds potential for identifying candidates for anti-HER2 regimens in a subset of mCRC patients. ERBB2 copy number assessment in ctDNA has shown concordance with tissue data, and studies like DESTINY-CRC01 with the antibody-drug conjugate trastuzumab-deruxtecan confiming higher ORR and PFS in patients with greater levels of ERBB2 copy number in plasma.[244]

From a prognostic point of view, the quantification of ctDNA through the analysis of the MAF has been demonstrated to be an independent prognostic factor in *RAS*-mutated mCRC patients.[245]This study shows that patients with lower levels of *RAS*-MAF presented better OS and PFS to first line, providing valuable insights into clinical outcomes before initiating treatment. Moreover, in a recent real-world study of 1,725 patients with various metastatic cancers, a MAF of ≥10% was linked to poorer OS, indicating that MAF could serve as a useful, universal prognostic marker across different cancer types.[246] Furthermore, ctDNA has proven valuable as both a prognostic and predictive biomarker for individuals with *BRAF*V600-mutant mCRC.[247]

The rechallenge with anti-EGFR agents has been established as a potential treatment option for chemorefractory *RAS*wt mCRC patients after a period of anti-EGFR-free therapy. Resistant clones that develop during EGFR blockade have been observed to diminish upon discontinuation of these agents, restoring sensitivity to rechallenge strategies. Successive treatments in the second line, not based on EGFR inhibitors, may partially restore sensitive clones, laying the groundwork for the possibility of anti-EGFR rechallenge. [248] The appearance of RAS mutations during disease progression on first-line chemotherapy plus anti-EGFR monoclonal antibodies may be followed by a reduction in the MAF of these mutations. The decay of the MAF of *RAS* and other resistant clones in ctDNA during non-EGFR-based treatment has been estimated to have a half-life ranging between 3.7 and 4.7 months, suggesting clonal evolution during therapy. This time has been used in the past to empirically test EGFR inhibitors' rechallenge, with low ORRs [249]. Circulating tumor DNA has demonstrated significant suitability for identifying patients eligible for rechallenge. This concept was initially proven in 2015, demonstrating that individuals benefiting from multiple anti-EGFR treatments exhibited fluctuating levels of ctDNA RAS mutations, forming the molecular basis for rechallenge efficacy. [250] Subsequent trials, including CRICKET, retrospectively confirmed that having *RAS*wt ctDNA at the time of rechallenge was a mandatory condition for a positive response. [251–253] The multi-center phase II CRICKET evaluated a rechallenge strategy using cetuximab and irinotecan in patients with *RAS* and *BRAF*wt mCRC who had acquired resistance to first-line irinotecan- and cetuximab-based therapy.[253] Among the 28 patients who were enrolled, there was an ORR of 21%, with six patients achieving partial

responses and nine experiencing disease stabilization. A retrospective analysis of baseline ctDNA revealed a correlation between the presence of RAS mutations and shorter PFS, underscoring the necessity of using ctDNA in selecting patients for this rechallenge approach. Subsequent prospective studies, notably the CHRONOS phase II, involved screening patients with tissue *RAS*wt tumors, previously treated with anti-EGFR therapy, through ctDNA. This screening resulted in the exclusion of 31% of patients due to the identification of resistance mutations in ctDNA. [254] Of the patients included in the study, 63% achieved disease control, indicating that utilizing ctDNA-guided anti-EGFR rechallenge could be a safe and effective approach for patients with refractory mCRC.

RATIONAL OF THE DOCTORAL PROJECT

2. RATIONAL OF THE DOCTORAL PROJECT

Despite significant advancements, mCRC remains a major challenge in oncology, with survival rates at five-years around 14%. One way to improve clinical outcomes is by identifying beforehand the main prognostic and predictive factors for treatment response to select more targeted, effective and personalized treatments for mCRC patients.

Prior investigations into baseline MAF of *RAS* in *RAS*-mutated mCRC patients revealed a notable correlation with OS, wherein patients with lower MAF of *RAS* in ctDNA demonstrated significantly prolonged life expectancy. While mutations in RAS pathway have been identified as positive and negative predictive biomarkers for target therapy, there is still a lack of predictive biomarkers for antiangiogenic therapies. Despite the proven survival advantage of antiangiogenics when combined with chemotherapy in across all the lines of treatment, there is currently a lack of biomarkers to effectively identify patients who will most benefit from this approach. Recent findings from the phase III SUNLIGHT trial revealed that refractory mCRC patients receiving TAS-102/bevacizumab combination therapy exhibited superior survival outcomes compared to those not treated with antiangiogenics. This trial marks a significant advancement as the first phase III study in refractory mCRC to demonstrate improved OS compared to an active control, attributed to the incorporation of an antiangiogenic agent. Exploring the benefit of antiangiogenics in the refractory setting and understanding why adding these drugs is important for comprehending the tumor biology of mCRC. The role of ctDNA can shed light in this regard.

We theorize that plasma MAFs may hinge upon the quality and functionality of tumor vasculature. We hypothesized that tumors with greater vascularity, characterized by permeable and leaky vessels, are more likely to shed ctDNA and thereafter shed higher amounts of ctDNA and, subsequently, present higher MAF values of cancer driver genes. Therefore, these tumors are also expected to be more responsive to antiangiogenic treatments, given the reliance of their microenvironment on angiogenic signaling. Considering this, we advocate for a retrospective/prospective study encompassing diverse cohorts throughout all the mCRC patients' oncological history. A cohort in first-line mCRC patients with the aim of confirming the prognostic value of ctDNA and explore the predictive power

of ctDNA for antiangiogenic therapy, while exploring its nexus with cancer tissue vascularization. The biomarker value of ctDNA as a prognostic and predictive biomarker of response will also be assessed in a second cohort of patients treated in the second-line or later line. A third cohort of patients treated with TAS/TAS plus bevacizumab will be explored.

To establish a mechanistic link between tumor vasculature and ctDNA release, we propose conducting vascular permeability studies using formalin-fixed paraffin-embedded (FFPE) tumor tissue from the first-line cohort. These studies aim to determine whether vascular leakiness, as measured by hemorrage in cancer tissue, correlates with plasma MAF levels.

Through this, we aim to elucidate the role of ctDNA as a biomarker for antiangiogenesis treatment and understand the dynamics of ctDNA modulation under antiangiogenic therapy throughout the oncological history of mCRC. The ramifications of this endeavor hold promise in directly augmenting mCRC patients' life expectancy and clinical care while shedding light on broader research avenues across cancer types benefiting from anti-angiogenic interventions.

HYPOTHESIS

3. HYPOTHESIS

3.1. Primary

- The value of MAF in ctDNA of driver genes is predictive of the response to first-line bevacizumab-containing regimens in mCRC patients.

3.2. Secondary

- The value of MAF in ctDNA of driver genes is an independent prognostic factor across all the lines of mCRC patients.

- The value of MAF of driver in ctDNA genes can segregate those mCRC patients that benefit from antiangiogenic therapy in the refractory setting.

- The value of MAF of driver in ctDNA is higher in those patients with more permeable vessels in the primary tumor.

OBJECTIVES

4. OBJECTIVES

4.1. Primary Objective

- Evaluate the predictive role of the allele frequency of driver genes in circulating tumor DNA in mCRC patients receiving first-line bevacizumab containing-regimens.

4.2. Secondary Objectives

- Evaluate the prognostic role of the mutant allele frequency of driver genes in circulating tumor DNA in first, second and subsequent lines of treatment of metastatic colorectal cancer patients.

- Evaluate the correlation between vessel permeability in parafine primary tissue and basal MAF values in ctDNA.

MATERIAL AND METHODS

5. MATERIAL AND METHODS

5.1. Type of study

The present study is observational, as no specific interventions were performed on the included patients. The study is single center, as patients were selected from the Medical Oncology Department of Vall d'Hebron Hospital who met the inclusion criteria specified in section 5.3.

5.2. Ethical considerations

The study was approved by the Ethics Committee of the Vall d'Hebron Hospital with code PR(AG)173-2018**.** Prior to blood extraction, patients were provided with an explanation and a paper copy of an informed consent form, approved by the CEIC (projects: PR(AG)113/2015 or PR(AG)309-2022), detailing the use of plasma samples and clinical data for further analyses in the context of clinical research. After being given time for reflection, patients who chose to participate signed the consent form prior to the extraction of blood and the collection of clinical data.

5.3. Selection of patient cohort and inclusion criteria

Between January 1st, 2017, and May 30th, 2023, adult patients (≥18-years old) with pathological confirmed pMMR metastatic colorectal cancer who met the following inclusion criteria were identified retrospectively and prospectively for the different cohorts as shown in the table 6.

Table 6: Inclusion criteria of the diverse cohorts

Cohort	Inclusion criteria
For all cohorts	- Adult >18 years old - Pathological confirmed pMMR mCRC - Independent of *RAS/BRAF* status - Availability of signed informed consent for the stock and use of plasma samples based on the PR(AG)113/2015 or PR(AG)309-2022 projects for plasma collection for mCRC patients ("Seroteca") - Availability of baseline plasma before the initiation of the intestest line systemic treatment (minimum 8mL of fresh/frozen plasma or 20mL blood in Streck Cell-Free DNA BCT ® (2 tubes of 10mL)
1st Line	- Patients candidates to systemic treatment (at least 1 cycle of treatment) - Informed consent for plasma and subsequent analysis complimented for live patients - Patients receiving chemotherapy alone or in combination with bevacizumab - From 2017 to October 2022, only *RAS* mutated patients were included. After October 2022, all *RAS* patients were included in the analysis
2nd line	- Patients candidates to systemic treatment (at least 1 cycle of treatment) in second line - Patients receiving chemotherapy in combination with antiangiogenic drugs
TAS-102 +/- bevacizumab	- Patients candidates to systemic treatment (at least 1 cycle of treatment) in third or subsequent lines - Patients receiving TAS-102 alone or in combination with bevacizumab

5.4. Clinical, pathological, and molecular variables

After the selection of the patients (from a retrospective way reviewing data patients' records; and prospective in the clinical basis activity), the clinical information was extracted from the medical records of the selected patients and entered a coded database created for this project using the Research Electronic Data Capture (REDCap, Vanderbilt University) software.

The following variables have been collected:

- **Epidemiological data**: date of birth, age, sex.

- **Clinical data**: date of diagnosis of colorectal cancer, staging at diagnosis according to TNM criteria, surgery for primary tumor (yes/no), performance of adjuvant treatment after primary surgery (if applicable), location of primary tumor (right colon from cecum to splenic flexure; left colon including descending colon and sigmoid colon; rectum), sites of metastatic localization (location and number).

- Data related to treatment received: lines of treatment during metastatic disease (dates, number, type of chemotherapy, and biological treatment), surgery performed for metastatic disease, and response to treatment according to RECIST criteria and clinical assessment of the oncologist in charge of the patient.

- Molecular data from solid tissue: the date of tumor sample collection for *RAS*, *BRAF* and other molecular biomarkers (if available) determination, qualitative determination of these in tumor tissue.

- Molecular data from blood ctDNA: date of blood sample collection, the line in which the sample is collected, the status of *RAS* mutation in blood both quantitatively and qualitatively in RAS mutated patients, the status of the driver gene for tissue *RAS*wt mCRC patients.

- Survival data: start and end dates of each treatment in metastatic disease, patient status (alive/deceased), date of death (if applicable), and date of last follow-up.

5.5. Molecular analysis of ctDNA

The patient inclusion period for this study (more than 5 years) has allowed for the observation of advancements in ctDNA analysis technology at our institution. Initially, only *RAS*-mutated patient samples were included and analyzed using digital PCR. In October 2022, our institution transitioned from digital PCR testing to the NGS tissue non-informed ctDNA test (Guardant Health®). With this transition, we expanded the study to include *RAS*wt patients. Since then, all samples, regardless of *RAS* status, have been analyzed using ctDNA NGS from both prospective and retrospective collections.

5.5.1. BEAMing digital PCR ctDNA analisis

RAS status in plasma was analyzed using BEAMing® (Sysmex Corporation, Kobe, Japan) until October 2022. The evaluation was based on the commercially available and previously validated BEAMing RAS plasma mutation panel (see supplementary table 1 for the specific genes tested).[255] Plasma processing followed established protocols. Samples were classified as mutant if the mutation rate exceeded the threshold of 0.02–0.04% determined by the BEAMing RAS panel assay.

This method uses magnetic beads coated with streptavidin that display primers specific to both mutated and non-mutated alleles on their surface. These beads are emulsified with the DNA to be analyzed, along with all necessary components for amplification. Each compartment contains, on average, no more than one DNA molecule to be analyzed and one bead. Following PCR thermocycling, each bead is coated with thousands of identical copies of the original DNA strand being studied. The emulsion is then broken, and the beads are recovered using a magnet. After DNA denaturation, the beads are incubated with oligonucleotide probes linked to fluorescent substances. The separation of the beads according to the sequences is performed by flow cytometry, allowing for quantification of the different populations. The sensitivity of this technique is quantified as capable of detecting one mutated copy among 10,000 non-mutated copies. Figure 4 in the introduction resumes the BEAMing technology.

Blood samples of 10 ml were collected in EDTA tubes. Plasma was isolated within the first hour. A two-step centrifugation was performed: the first for 10 minutes at

1600xg at room temperature. The supernatant was collected and centrifuged again for 10 minutes at 3000xg at room temperature to remove any remaining cells. The supernatant was the plasma, which was then frozen at -80°C until use. To perform the analysis, cfDNA purification was necessary, which was done using the QIAamp Circulating Nucleic Acid Kit according to the manufacturer's instructions. The quantity and quality of DNA were measured using a fluorometer (Qubit). The purified cfDNA samples were then tested using the OncoBEAM RAS Kit (catalog number ZR150048) for colorectal cancer according to the manufacturer's instructions (Sysmex Inostics). The flow cytometry data required for BEAMing result analysis was obtained using the Cube 6i Flow Cytometer with FCS Express™ Software. A mutation detection rate above 0.02% was considered positive. At least 150ng of DNA is required for BEAMing analysis. Multiplex PCR pre-amplification of multiple loci is performed, followed by a second more specific pre-amplification with primers for the desired amplicons. Subsequently, emulsion PCR is performed on the surface of magnetic beads in an oil-in-water emulsion with several thermal cycles. The next step is hybridization with fluorescent probes specific to each mutation, followed by flow cytometry to quantify the PCR product results. This allows calculation of the ratio of mutated alleles to non-mutated alleles. The mutant allele fraction (MAF) or variable allelic fracion (VAF) is defined as the number of beads with mutated DNA divided by the total number of beads analyzed.

5.5.2. NGS tumor agnostic ctDNA analysis

From October 2022 to May 30th, 2023, the plasma samples were analysed using the in-house NGS tumor agnostic panel VHIO360 with outsourced services provided by Guardant Health®. VHIO360 provides information of 74 cancer-associated genes. Table 2 in the supplementary data shows a table of the VHIO360Panel. Blood samples of 10 ml were collected in Streck tubes. A two-step centrifugation was performed: the first for 10 minutes at 1600xg at 4ºC. The supernatant was collected and centrifuged again for 10 minutes at 3000xg at 4ºC to remove any remaining cells. The supernatant was the plasma, which was then frozen at -80°C until use. cfDNA is then extracted from plasma, enriched for targeted regions, and sequenced using the Illumina platform and hg19 as the reference genome. The sequencing data and sample quality assessment and the variant calling step are outsourced services

provided by Guardant Health® through its proprietary Bioinformatics Pipeline (BIP) Analysis software. The downstream steps of curation, classification and reporting of the variants provided in the BIP software output are performed at Cancer Genomics Laboratory (VHIO). Publicly available databases (COSMIC, cBioPortal, ClinVar, VarSome, OncoKB) are used for the classification and interpretation purposes. The types of genomic alterations detected by VHIO360 include single SNVs, gene amplifications, fusions, short indels, longest detected, 70 base pairs, and splice site disrupting events. Microsatellite Instability status is assessed for all cancer types by evaluating somatic changes in the length of repetitive sequences on the VHIO360 panel. A "Not Detected" result in samples where the highest % cfDNA is < 0.2% is an inconclusive result because it does not preclude MSI-High status in tissue. This version of the VHIO360 test is not validated for the detection of other types of genomic alterations, such as complex rearrangements or gene deletions. According to the analytical validation, the LOD of the VHIO360 test is 0.125% of VAF for SNVs and Indels. The test may detect both SNVs and Indels with a VAF below 0.125%. However, sensitivity and specificity of the test at this VAF level could not be determined during the analytical validation phase due to the unavailability of commercial reference material encompassing variants below this threshold. Certain sample or variant characteristics, such as low cfDNA concentration, may result in reduced analytic sensitivity (See table 3 supplementary data for the sensitivity and specificity depending on MAF values). VHIO360 cannot discern the source of circulating cfDNA, and for some variants in the range of 40 to 60% cfDNA, the test cannot easily distinguish germline variants from somatic alterations. VHIO360 is not validated for the detection of germline or de novo variants that are associated with hereditary cancer risk. Tissue genotyping should be considered when plasma genotyping is negative, if clinically appropriate. The Genomic Equivalents (GE) are original genomic copies of the sample that could be analysed with this test.

5.5.3. Considerations for the determinationof MAF value result

Those patients with no ctDNA detected in plasma were treated numerically as patients with MAF value=0 for statistical analysis.

For *RAS* mutated *(RAS*mut) patients analysed with Guardant Health®, the MAF selected for the analysis was the same specifically *RAS* mutation shown in the ctDNA result as the driver mutation of the tumor. For *RAS*wt patients, the MAF value selected for the analysis was the gene that showed higher value of MAF on behalf of the genes in the results.

5.6. Vessel permeability test

To test our hypothesis linking vascular leakiness and MAF, and considering the availability of FFPE samples from primary tumors, we developed a histological approach using surrogate markers of vascular permeability. Given the challenges in directly measuring intravasation and extravasation pressures in FFPE samples (as dynamic techniques like injecting and measuring extravasation of contrast fluxes), we focused on studying haemorrhage in tissue as an indirect indicator of vascular pressure dynamics.[256,257] Specifically, we hypothesized that regions with significant erythrocyte extravasation (detectable as haemorrhages) reflect heightened vascular permeability. The extravasation of fluid from the vessel (into the tumor) is accompanied by intravasation (fluid or cells moving back into the vessel). This re-entry mechanism can be exploited by tumor cells to invade the vasculature, generate metastases, and potentially shed ctDNA. Assuming that intravasation and extravasation pressures are balanced, we hypothesized that tumors presenting hemorrhages are likely to have higher extravasation, so higher intravasation pressures, thus increasing the likelihood of ctDNA shedding and higher plasma MAF levels.

We selected a cohort of first-line mCRC patients with available FFPE samples from primary tumors and known MAF values in plasma. From this cohort, we randomly selected two groups of extreme cases: patients with hepatic disease and high MAF (≥5.8) and patients with low MAF (<5.8). For each patient, 8 to 10 sections (3-5 μm thick) from FFPE primary tumor samples were sent to the IDIBELL laboratory led by Oriol Casanovas. Haematoxylin and eosin (H&E) staining was performed for histopathological evaluation. The nex definition and scoring was performed:

- Non-hemorrhagic areas were defined as regions devoid of extravascular red blood cells.

- Hemorrhagic areas were identified by visible erythrocyte extravasation beyond vessel walls, confirmed through histopathological examination.
- Microhemorrhages were quantified using a scoring system based on the extent and density of erythrocyte extravasation within each tumor section, classified as +, ++, or +++.

A correlation analysis was performed to assess the relationship between the extent of microhemorrhages and plasma MAF levels.

5.7. Statistical analysis

Statistical analyses were performed using R 4.3.1 (Rstudio v.1.2.13) or newer, utilizing data collected in REDCap. The statistical analyses were conducted by the statisticians of the Oncology Data Science Group (OdysSey). Overall survival for first-line treatment is defined as the time from the diagnosis of metastatic colorectal cancer to death from any cause or the last follow-up visit (censored), whichever occurred first. Overall survival for the cohort receiving second-line treatment and TAS/TAS-102 + Bevacizumab was calculated from the date of initiation of the specific treatment line to death from any cause or the last follow-up visit (censored), whichever occurs first. Progression-free survival (PFS) was determined as the duration from the initiation of therapy (of each line of interest) to either disease progression or death, or the date of last follow-up (censoring), whichever event occurred first. Estimations of PFS and OS were conducted using the Kaplan-Meier method, with the log-rank test used for survival comparisons. Cox proportional hazards models were employed to derive hazard ratios (HRs) accompanied by 95% confidence intervals (Cis). All p-values were two-sided, and values of less than 0.05 were considered statistically significant.

For the different variables included in the study, descriptive analyses were performed according to the categorical or continuous nature of the variables. Continuous variables were expressed as median (or mean, as specified), and range (or interquartile range (IQR), as specified). Categorical variables were expressed with absolute values and percentages.

For the univariable analysis, Fisher's or X^2exact test was used for categorical variables, and the Student's t-test or Mann-Whitney test was used for continuous

variables (after checking for the normal distribution of the data with the Shapiro-Wilk test). The Spearman test was used to evaluate associations for continuous variables.

Patients in the first-line cohort were categorized into high (≥ 5.8%) or low (< 5.8%) mutant allele frequency groups, employing the previously established cutoff as a prognostic factor. [245]Other cutoffs were explored during the analysis of the other cohorts.

PRISM GraphPad (v10.2.3) was used for creating some of the graphs for the descriptive part of the analysis.

RESULTS

6. RESULTS

6.1 Cohort first Line

6.1.1. Baseline characteristics of the study population

A total of 185 mCRC patients treated with chemotherapy or chemotherapy in combination with bevacizumab in first line, with available baseline plasma, were included in this analysis. Table 7 presents the main clinical characteristics of the patients overall, according to the treatment received. Figure 5 illustrates main characteristics of the first-line population.

Given the observational nature of the study, without randomized groups, we decided to perform a statistical analysis to compare the distribution of qualitative categories between the two groups (treated or not with bevacizumab). Staging was classified as stage IV or localized. The only significant difference between the two groups was the distribution of *RAS* mutation (p=0.0127), with a higher proportion of patients with *RAS* mutations receiving bevacizumab compared to those without mutation. The other categories showed similar distribution between groups.

One hundred and seventeen patients (63.24%) received chemotherapy in combination with bevacizumab, and 68 patients (36.76%) received chemotherapy alone. The median age of the overall cohort was 66.45 years (range: 33.54 – 86.9), being similar between both groups (65.19 and 67.36, respectively). The age distribution in the two treatment groups did not follow a normal distribution. The age distributions were similar between both groups (p=0.18 for the Mann-Whitney test). Figure 1 in the supplementary data shows the age density distribution for both groups. One hundred and thirteen patients (61.08%) were male and 72 (38.92%) were female. In the bevacizumab cohort, 66 patients (56.41%) were male compared to the 47 patients (69.12%) in the chemotherapy only group.

One hundred forty-six of all the patients (79%) were *RAS*-mutated. In the overall cohort, only 3 were identified as *BRAF*mut. Among these, 2 patients had a *BRAF*V600 mutation, and 1 had the *BRAF*G469V mutation. The two patients with the BRAFV600 mutation were classified as low MAF and did not receive bevacizumab; one had metastatic disease in the peritoneum, and the other in the ovaries. The patient with the *BRAF*G469V mutation, classified as high MAF with metastatic disease in the liver

and lungs, did receive antiangiogenic treatment. Due to the low number of patients in this category, these patients will be categorized as *RAS*mut for the purposes of our analysis.

One hundred thirty-four patients (72.43%) of the global cohort were stage IV at diagnose, with a similar percentage of patients with stage IV CRC at diagnosis in both groups (86 patients in the bevacizumab group (73.5%) vs. 70.59% in the no-bevacizumab population). Regarding tumor site, 74 patients (40%) had a right-sided tumor, while 103 patient (55.68%) had a left-sided or rectal tumor. In the overall cohort, 65 patients underwent metastasectomy surgery, constituting approximately 35% of the total cohort. One hundred and twenty-three patients (66.49%) had surgery for the primary tumor.

The median MAF of the driver gene was 6.49% (IQR: 0.9462 – 18.57). Of the total population, 80 patients (43.2%) were classified as high MAF (≥ 5.8%), and 105 (56.76%) as low. Of those 105 patients, 34 were classified as not ctDNA detectable. Of these 34 patients without detectable ctDNA, 22 (64.7%) received bevacizumab and 12 (35.3%) received chemotherapy alone. Of the 146 *RAS*mut patients, 25 (17.1%) had undetectable ctDNA in plasma. Among these 25 patients, 17 (68%) had no liver disease, and those with liver disease (8 patients) had a low bulky disease. Table 5 in the supplementary data shows the distribution of non-shedder patients between both treatment groups according to their RAS mutation status.

The MAF distribution in the two treatment groups did not follow a normal distribution and was similar between both groups (p=0.34 for the Mann-Whitney test). Figure 6 shows the violin distribution of MAF values in both groups.

In the total population of 185 patients, liver disease was present in 143 individuals (77.3%). This distribution remained consistent among patients treated with bevacizumab (90 patients, 76.92%) and those receiving chemotherapy alone (53 patients, 77.94%). Furthermore, 78 patients (42.16% of the total) experienced metastasis in two or more organs. Among them, 55 patients (47.01% of those receiving bevacizumab) were treated with bevacizumab alongside chemotherapy, while 23 patients (13.69%) received chemotherapy alone. Table 4 in supplementary illustrates the metastatic involvement of the patient population.

Of the total number of patients with a *RAS*mut (n=146), 19 (13%) baseline plasma samples were analyzed using NGS Guardant, while the remaining (127 patients, 87%) samples were analyzed using digital PCR. All 39 patients *RAS*wt were analyzed by NGS Guardant**.** Table 8 describes the genes selected for the *RAS*wt cohort. It is important to note that those 9 patients with *RAS* mutations detected in ctDNA were treated as *RA*Swt patients in the analysis.

Table 7: Main clinical characteristics according to treatment with or without bevacizumab (percentages are compared to group of treatment)

	Overall population (n=185)	Bevacizumab population (n=117)	No bevaciumab population (n-=68)
Sex			
Female	72 (38.92%)	51 (43.59%)	21 (30.88%)
Male	113 (61.08%)	66 (56.41%)	47 (69.12%)
Stage at diagnosis			
I	2 (1.08%)	1 (0.86%)	1 (1.47%)
II	17 (9.19%)	10 (8.55%)	7 (10.29%)
III	32 (17.3%)	20 (17.09%)	12 (17.65%)
IV	134 (72.43%)	86 (73.5%)	48 (70.59%)
Tumor Site			
Right	74 (40%)	45 (38.46%)	29 (42.65%)
Left	68 (36.76%)	46 (39.32%)	22 (32.35%)
Rectum	35 (18.92%)	19 (16.24%)	16 (23.53%)
Unknown	4 (2.16%)	4 (3.42%)	0
Synchronic	4 (2.16%)	3 (2.56%)	1 (1.47%)
Age			
Median (range)	66.45 (33.54 – 86.9)	65.19 (35.09 – 82.94)	67.36 (33.54 – 86.9)
***RAS* status**			
Mut	146 (78.92%)	99 (84.62%)	47 (69.12%)
wt	39 (21.08%)	18 (15.38%)	21 (30.88%)
MAF			
Median (IQR)	6.494 (0.9462 -18.57)	6.309 (1-17)	6.71 (0.79 – 19.5)
MAF classification			
High (>=5.8%)	80 (43.24%)	50 (42.74%)	30 (44.12%)
Low (<5.8%)	105 (56.76%)	67 (57.26%)	38 (55.88%)
Metastatic Surgery			
No	122 (65.95%)	81 (69.23%)	41 (60.29%)
Yes	63 (34.05%)	36 (30.77%)	27 (39.71%)
Primary Tumor Surgery			
No	62 (33.51%)	40 (34.19%)	22 (32.35%)
Yes	123 (66.49%)	77 (65.81%)	46 (67.65%)
Liver disease			
No	42 (22.70%)	27 (23.08%)	15 (22.06%)
Yes	143 (77.30%)	90 (76.92%)	53 (77.94%)
Metastatic sites			
1 site	107 (57.84%)	62 (52.99%)	45 (66.18%)
≥ 2 sites	78 (42.16%)	55 (47.01%)	23 (33.82%)

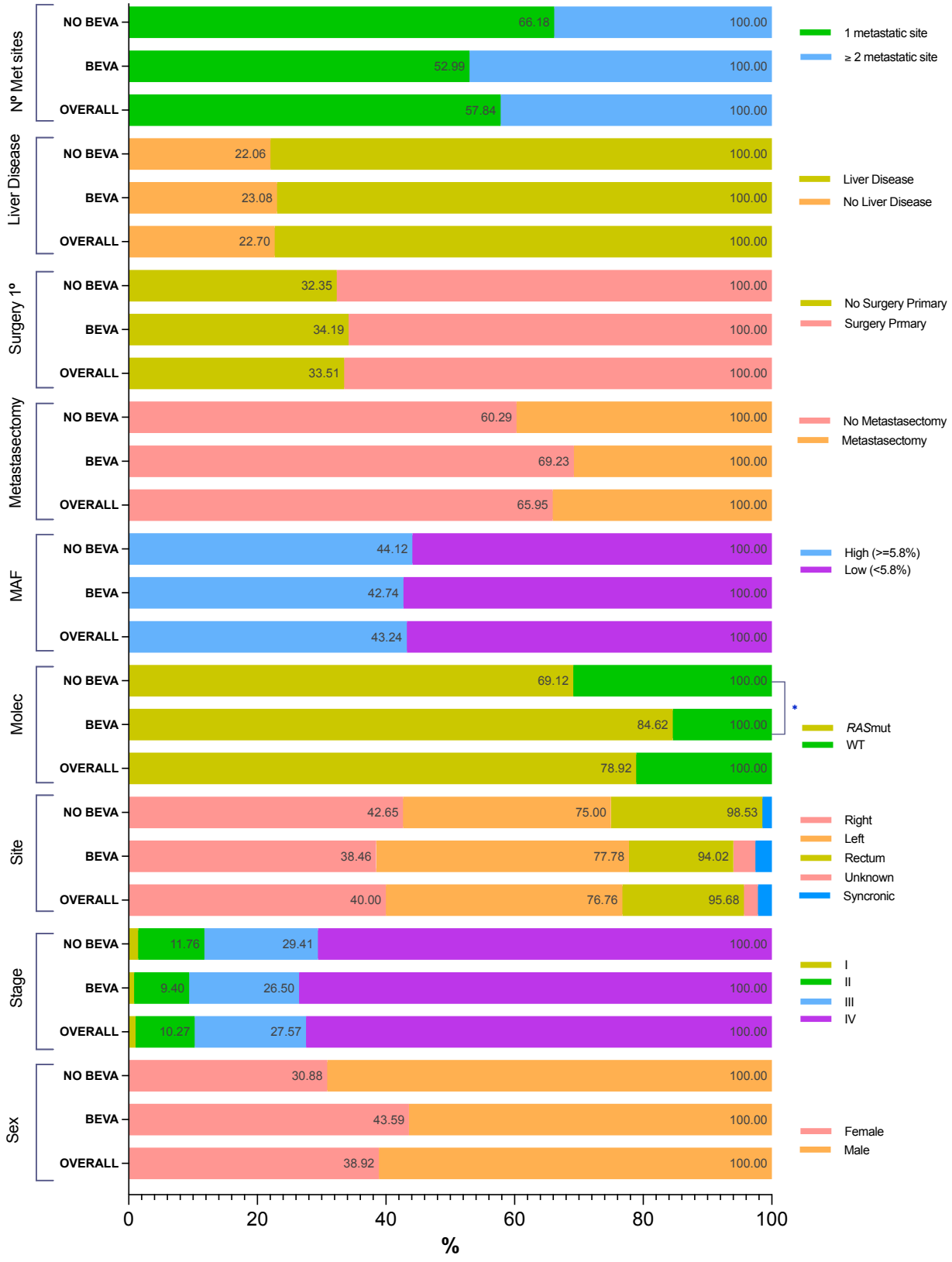


Figure 5: Bar graph with main characteristics of the first-line population.

Table 8: Genes present in the ctDNA selected for the *RAS*wt cohort

GENES	n (n=39)
TP53	10
RAS	9
WT	8
APC	7
ARAF	1
AXIN1	1
GATA3	1
AKAp9	1
AMER1	1

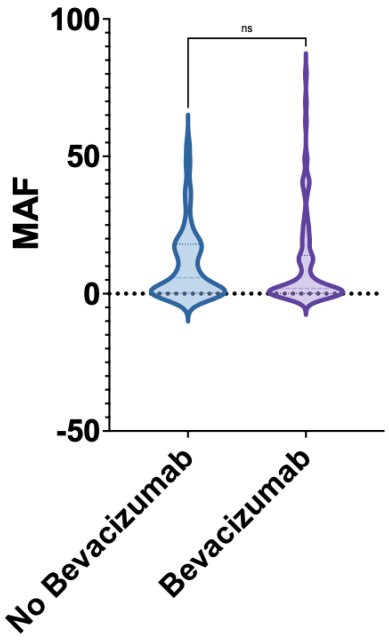


Figure 6: Violin graphics of the distribution of MAF.

6.1.2. Overall survival analysis

We first analyzed the OS of all patients in the first-line cohort. The median OS for these patients was 21.62 months (95% CI: 17.45-26.48). Figure 7 shows the Kaplan-Meier survival curves for the entire population.

When comparing survival between patients based on MAF values, using a previously defined cutoff of 5.8, those with high MAF had a shorter survival compared to those with low MAF (17.74 months vs. 40.67 months, respectively; HR: 0.43 [95%CI: 0.30-0.61], $p < 0.001$). Figure 8 illustrates the Kaplan-Meier survival curves for the entire first-line cohort, stratified by MAF values.

Next, we included treatment with or without bevacizumab in the survival analysis. For patients with high MAF who received bevacizumab, the mOS was 19.22 months (95%CI: 15.93-27.99). In contrast, high MAF patients who did not receive bevacizumab had an mOS of 16.53 months (95%CI: 12.16-22.90), with no statistically significant difference between these two groups (HR: 0.80 [95%CI: 0.49-1.32]; p=0.38).

For patients with low MAF, those who received bevacizumab had a mOS of 43.4 months (95%CI: 35.38-62.36), while those who did not receive bevacizumab had an mOS of 40.61 months (95% CI: 22.97-73.33). There were no statistically significant differences between these two groups (HR: 0.90 [95%CI: 0.55-1.47]; p=0.66).

Figure 9 displays the mOS across different groups based on MAF levels and bevacizumab treatment status. Figures 2 and 3 in the supplementary section present the Kaplan-Meier curves for both high and low MAF populations, comparing those who received bevacizumab to those who did not.

In the multivariable analysis, several clinicopathological factors were evaluated, including treatment with bevacizumab, MAF levels, tumor site, synchronous metastasis, ECOG, resection of metastatic disease, age, and *RAS* mutations. Among these factors, surgery of the metastatic site (HR: 0.26 [95%CI 0.10-0.64]; p=0.004), low MAF (HR: 0.49 [95%CI 0.31-0.77]; p=0.02), and receiving bevacizumab (HR: 0.61 [95%CI 0.38-0.98]; p=0.039) were significantly associated with better OS. Synchronous primary tumors (HR: 6.61 [95%CI: 2.07-21.1]; $p < 0.001$) and synchronous metastatic disease (HR: 6.88 [95%CI: 3.73-12.74]; $p < 0.001$) were

identified as poor prognostic factors in our cohort. Figure 10 illustrates the results of the multivariable analysis for overall survival.

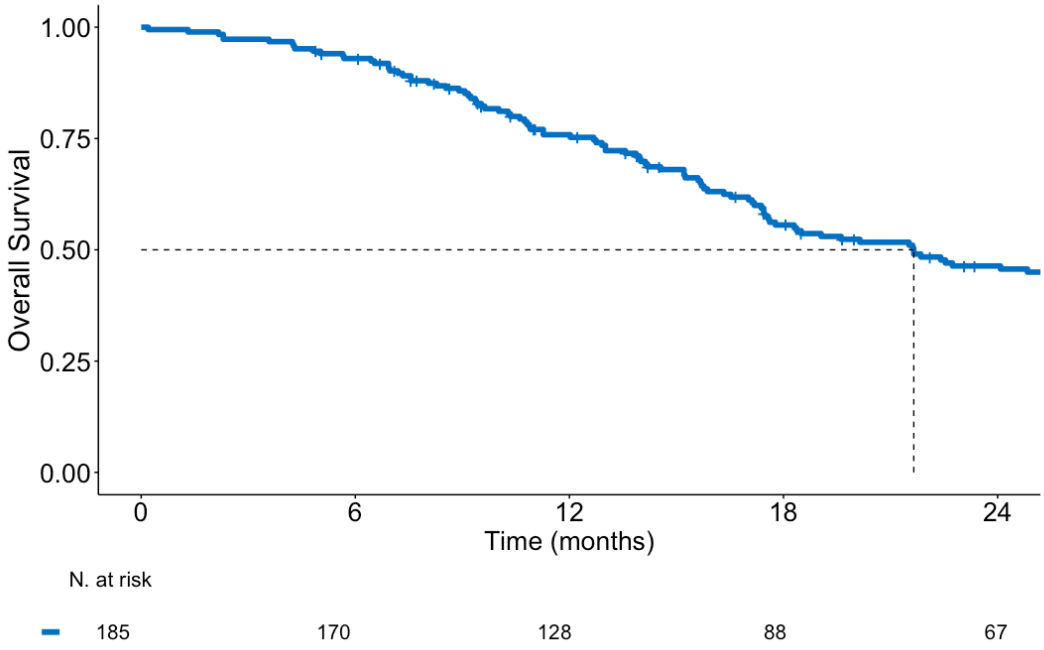


Figure 7: Kaplan-Meier curve for OS in the global cohort of first line.

Factor	n	median IC95%	HR IC95%	p.value
HIGH (>=5.8%)	80	17.74m [15.93 ; 22.9]	Ref. [Ref. ; Ref.]	-
LOW (< 5.8%)	105	40.67m [33.87 ; 54.6]	0.43 [0.3 ; 0.61]	0

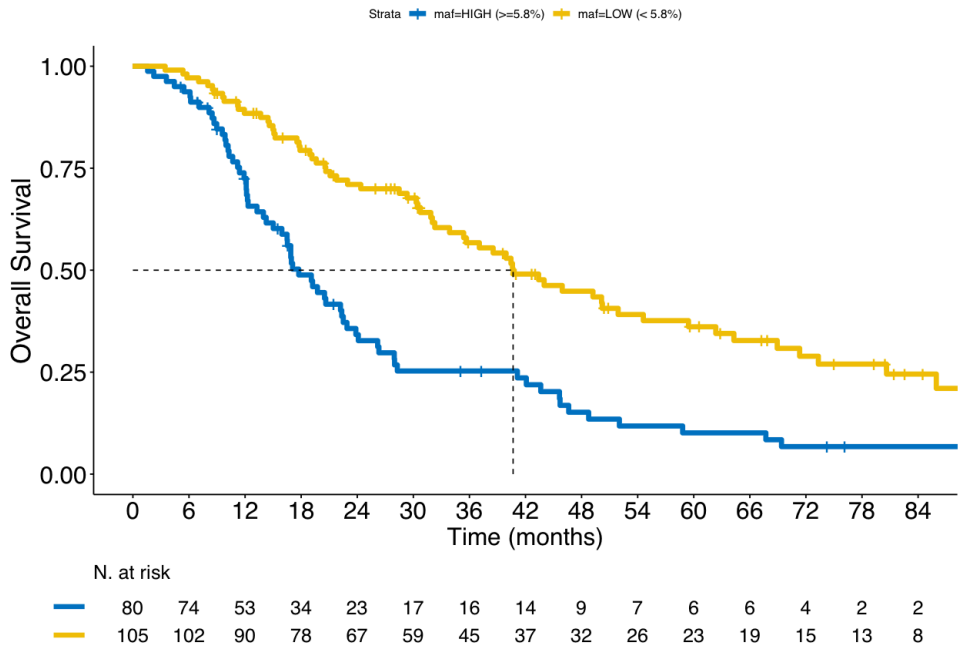


Figure 8: Kaplan-Meier curves for overall survival in the global cohort of first line comparing high (blue) vs low (yellow) MAF population.

Factor	n	median IC95%	HR IC95%	p.value
HIGH (>=5.8%)-Beva	50	19.22m [15.93 ; 27.99]	Ref. [Ref. ; Ref.]	-
HIGH (>=5.8%)-No beva	30	16.53m [12.16 ; 22.9]	1.21 [0.74 ; 1.99]	0.4507
LOW (< 5.8%)-Beva	67	43.4m [35.38 ; 62.36]	0.44 [0.28 ; 0.69]	3e-04
LOW (< 5.8%)-No beva	38	40.61m [22.97 ; 73.33]	0.5 [0.3 ; 0.82]	0.0057

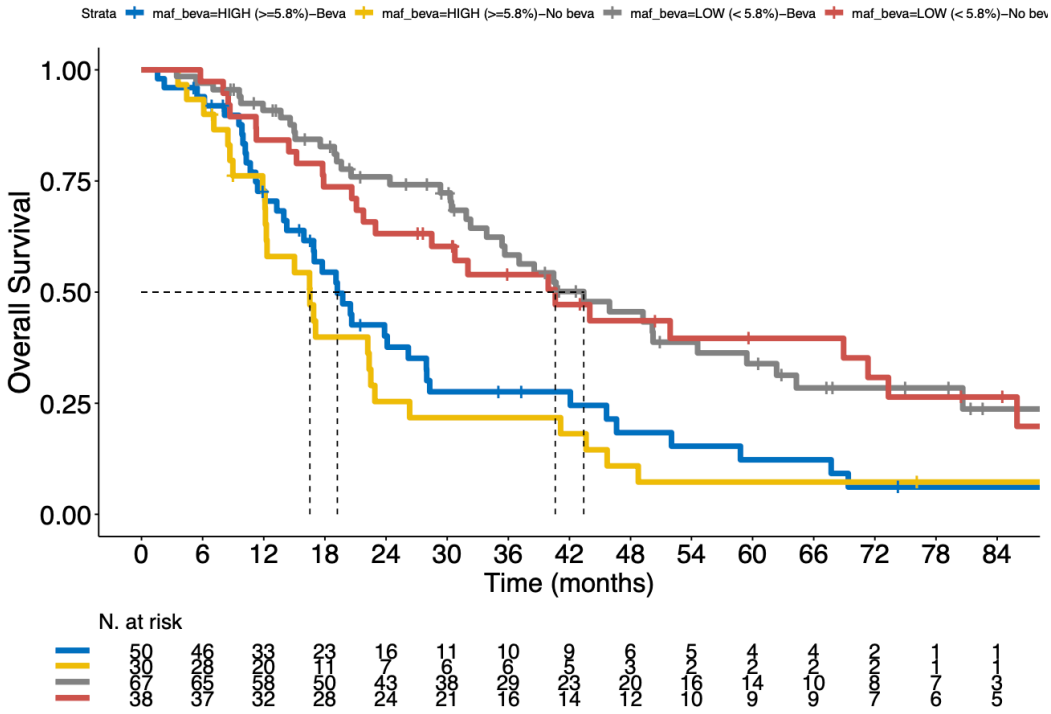


Figure 9: Kaplan-Meier curves for OS in mCRC patients according to MAF levels and treatment with bevacizumab in first-line cohort.

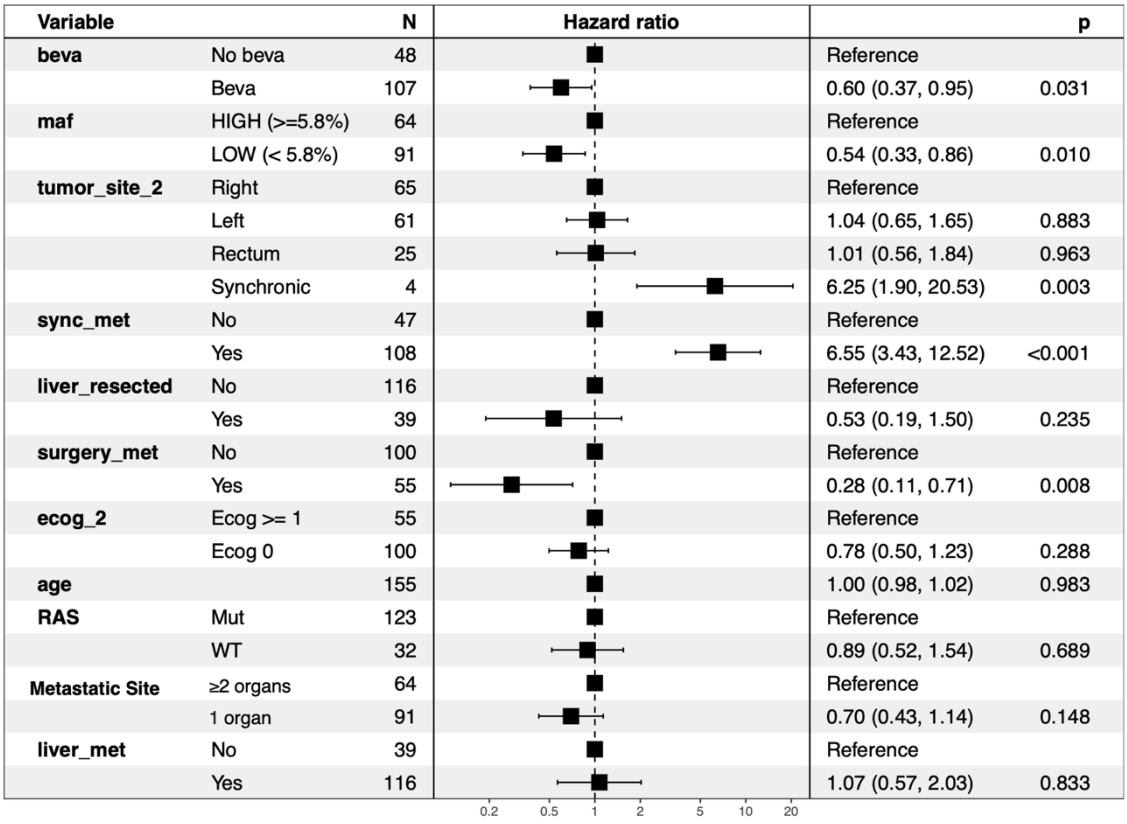


Figure 10: Multivariable Cox model to evaluate the association between clinicopathological factors and OS results in first-line cohort.

6.1.3. Progression free survival analysis

To evaluate the potential role of MAF values as a predictive biomarker for response to bevacizumab in the first line, we conducted several analyses.

Initially, we compared patients with high vs. low MAF to see if PFS Kaplan-Meier analysis diverged based on bevacizumab treatment (Figure 11). In the total population (n = 185), patients with high MAF showed a trend towards improved PFS with bevacizumab, though the difference was not statistically significant (HR: 0.65 [95% CI: 0.40-1.05]; p=0.0802). In contrast, patients with low MAF did not exhibit any significant difference in PFS with or without bevacizumab (HR: 1.34 [95% CI: 0.85-2.09]; p=0.206).

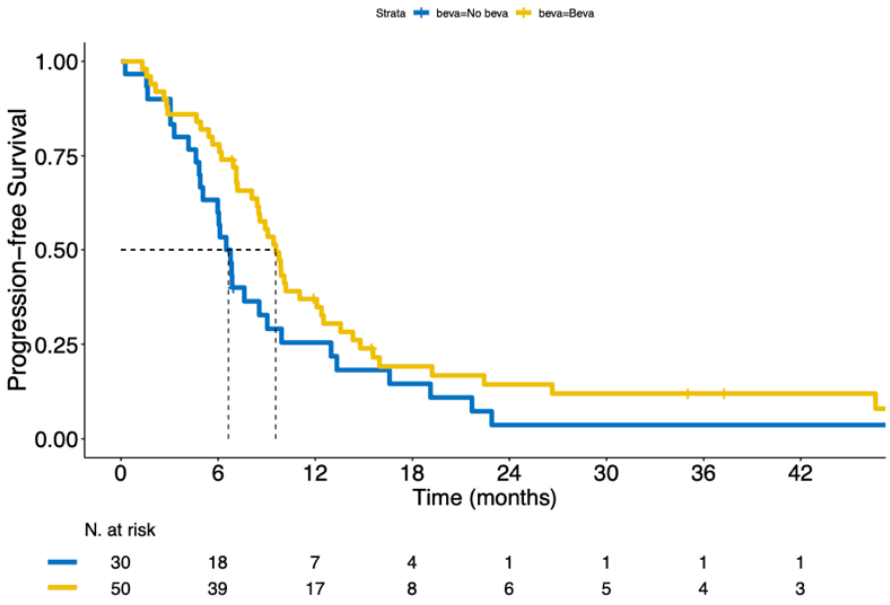
To address potential bias introduced by including patients who had undergone metastasectomy, we performed a separate analysis excluding these patients, resulting in a total sample size of 122. Among the high MAF patients (n = 60), those treated with bevacizumab had significantly better PFS compared to those who did not receive the drug (HR: 1.89 [95% CI: 1.10-3.25]; p=0.0214). However, among patients with low MAF (n = 62), there were no significant differences in PFS between those who received bevacizumab and those who did not (HR: 0.99 [95% CI: 0.54-1.82]; p=0.99) (Figure 12).

In the multivariable analysis for PFS within this cohort, we evaluated several factors, including bevacizumab treatment, MAF levels, tumor site, synchronous metastasis, ECOG performance status, resection of metastatic disease, age, and RAS mutations. We found that low MAF (HR: 0.62 [95% CI: 0.41-0.93]; p=0.021) and ECOG PS 0 (HR: 0.66 [95% CI: 0.44-0.89]; p=0.045) were significantly associated with better PFS. Conversely, synchronous metastatic disease (HR: 2.08 [95% CI: 1.31-3.31]; p=0.002) was identified as an independent poor prognostic factor for PFS in our cohort. Figure 13 displays the results of the multivariable analysis for PFS.

Given the observational nature of this study, we calculated the p of interaction to assess whether the effect of bevacizumab treatment on progression-free survival varies with MAF levels. This analysis aims to determine the predictive role of MAF by indicating if it modifies the treatment effect. The p-value for the interaction test between bevacizumab treatment and MAF levels was 0.026.

A

Factor	n	median IC95%	HR IC95%	p.value
No beva	30	6.64m [5.06 ; 9.92]	Ref. [Ref. ; Ref.]	-
Beva	50	9.56m [8.41 ; 12.39]	0.65 [0.4 ; 1.05]	0.0802



B

Factor	n	median IC95%	HR IC95%	p.value
No beva	38	14.26m [11.6 ; 21.95]	Ref. [Ref. ; Ref.]	-
Beva	67	13.86m [9.72 ; 16.89]	1.34 [0.85 ; 2.09]	0.206

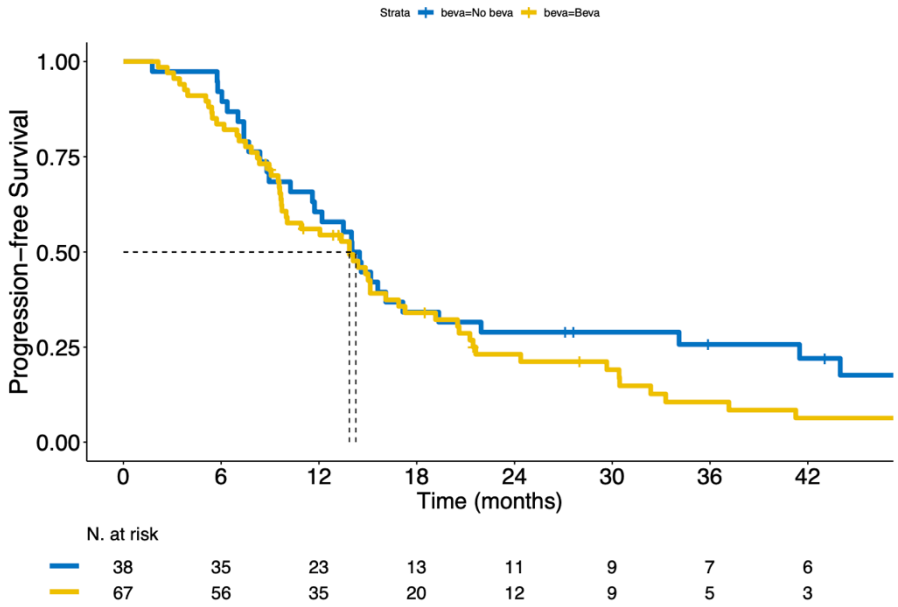
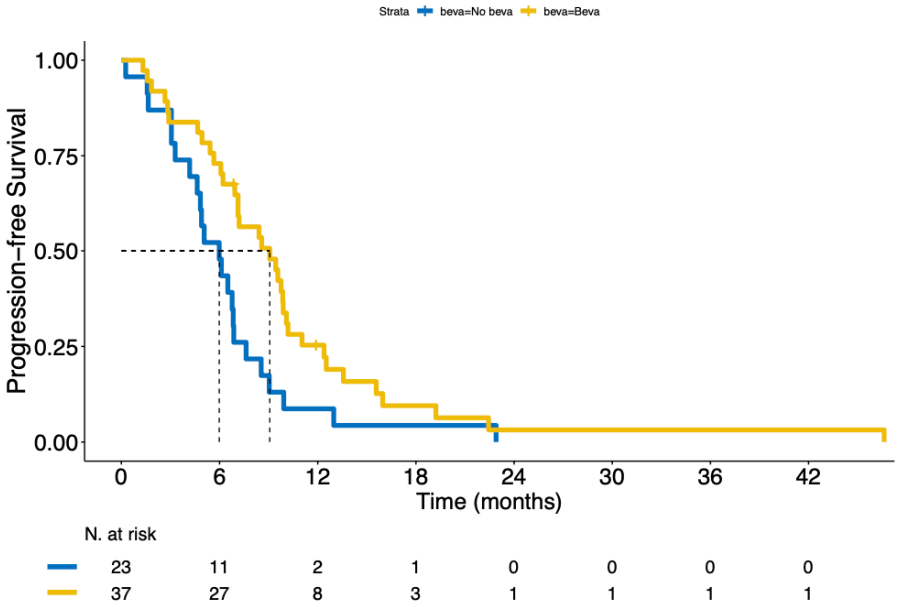


Figure 11: PFS of total population. Fig. A shows patients with high MAF, and figure B patients with low MAF.

A

Factor	n	median IC95%	HR IC95%	p.value
No beva	23	5.98m [4.63 ; 7.62]	Ref. [Ref. ; Ref.]	-
Beva	37	9.07m [7.13 ; 10.18]	0.53 [0.31 ; 0.91]	0.0214



B

Factor	n	median IC95%	HR IC95%	p.value
No beva	18	9.53m [7.39 ; 21.95]	Ref. [Ref. ; Ref.]	-
Beva	44	10.91m [9.56 ; 17.28]	0.99 [0.54 ; 1.82]	0.9861

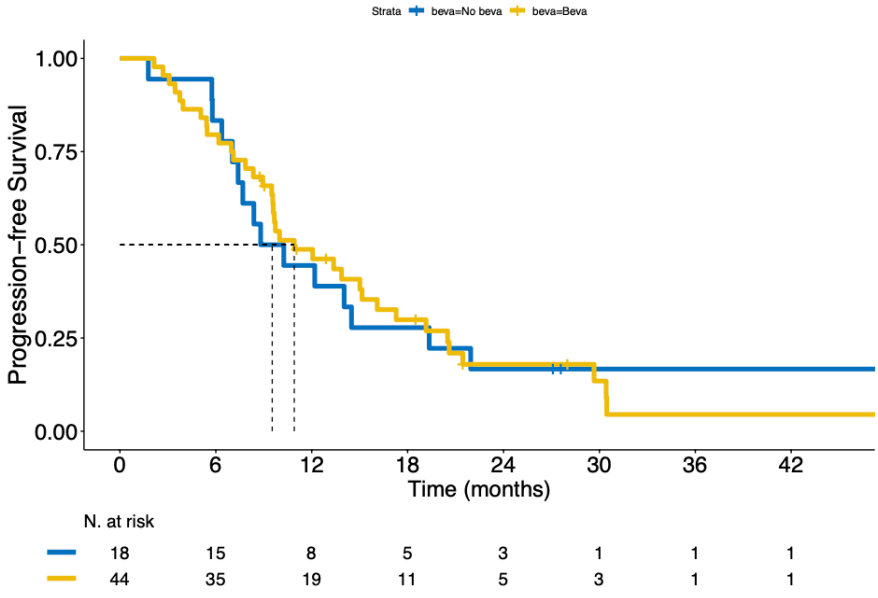


Figure 12: PFS of patients without surgery of metastasis. Fig A shows patients with high MAF, and figure B patients with low MAF.

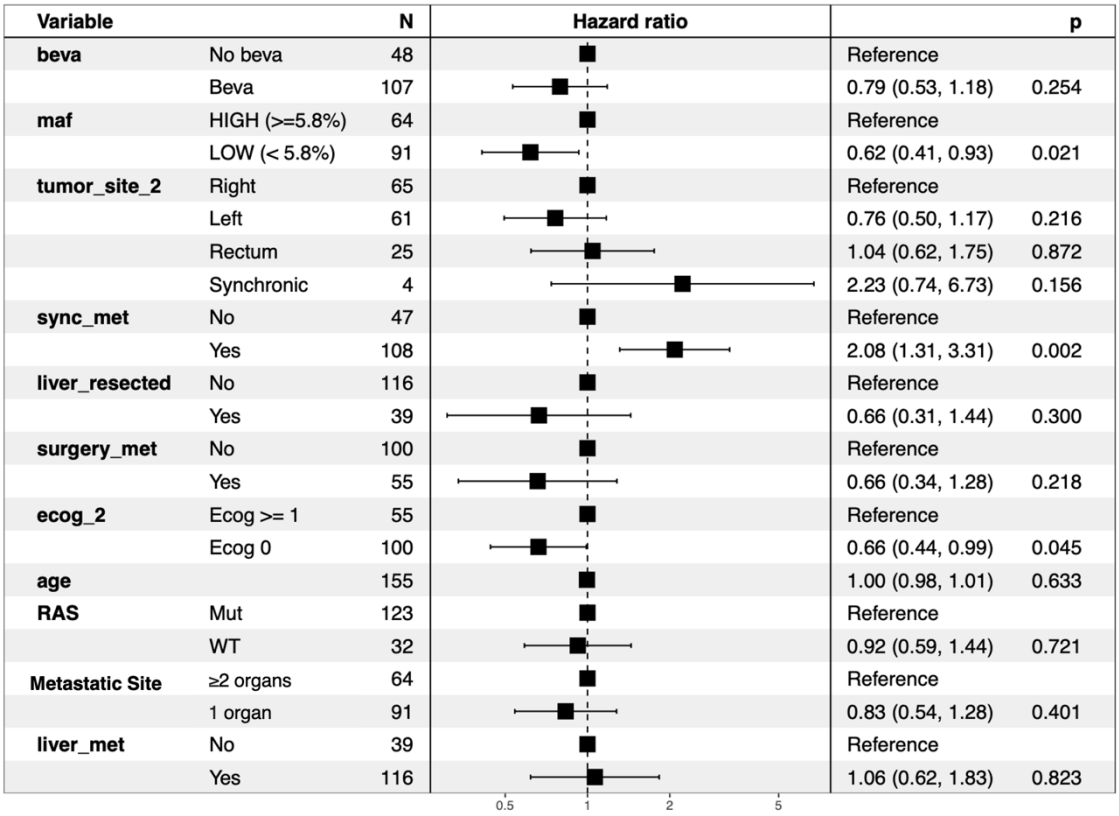


Figure 13: Multivariable Cox model to evaluate the association between clinicopathological factors and PFS in first-line cohort.

6.2. Cohort second line

6.2.1. Baseline characteristics of the study population

A total of 43 patients were included in the second-line cohort, all of whom had baseline plasma available for analysis and were treated with chemotherapy and antiangiogenic therapy. Table 9 and figure 14 describes the main characteristics of this cohort.

We calculated the median MAF in this population, which was 2.09% (IQR: 0.23-14.32%). As the median MAF in this cohort differed from that in the first-line cohort, and since no established cutoff exists for MAF in the second line, we decided to determine a new cutoff of the value of MAF that would better 87egregate this population for OS analysis. The cutoff identified was 1.04%. Therefore, we divided the cohort into two groups: those with high MAF (≥1.04%), comprising 25 patients, and those with low MAF (<1.04%), comprising 18 patients.

Given the observational nature of the study, without randomized groups, we decided to perform a statistical analysis to compare the distribution of qualitative categories between the two groups (high vs low MAF). We found significant differences in the stage at diagnosis between patients with high and low MAF ($p < 0.0001$), with stage IV being more common among those with high MAF. Additionally, there were significant differences in prior antiangiogenic treatment ($p=0.0281$); patients with low MAF were less likely to have received previous antiangiogenic therapy.

Of the 43 patients, 12 were *RAS*wt, with 7 of these in the high MAF cohort. The median MAF of *RAS*wt patients in these patients was 2.82% (IQR 0.2-9.6%). All but two of *RAS*wt patients received anti-EGFR treatment in the first line, with the exception of two elderly female patient who were treated with a combination of fluoropyrimidine monotherapy and bevacizumab in the first line. Table 10 shows the genes selected for the *RAS*wt patients in this cohort.

Eight patients presented *BRAF*V600 tumors, with a median MAF of 12.9% (IQR 0-30.23%), which was higher than the median MAF of the overall population, without finding any significantly different when comparing *BRAF*wt with *BRAF*V600 patients ($p=0.8653$).

Table 9: Main clinical characteristics according to treatment with or without bevacizumab (percentages are compared to group of treatment)

	Overall population (n=43)	High MAF (≥1.04% n=25)	Low MAF (<1.04% n=18)
Sex			
Female	20 (46.51%)	11 (44%)	9 (50%)
Male	23 (53.49%)	14 (56%)	9 (50%)
Stage at diagnosis			
I	0	0	0
II	2 (4.65%)	1 (4%)	1 (5.56%)
III	9 (20.93%)	2 (8%)	7 (38.89%)
IV	32 (74.42%)	22 (88%)	10 (55.56%)
Tumor Site			
Right	18 (41.86%)	10 (40%)	8 (44.44%)
Left	21 (48.84%)	14 (56 %)	7 (38.89 %)
Rectum	4 (9.3 %)	1 (4 %)	3 (16.67 %)
Age			
Median (range)	61.79 (32.25-6.23)	61.79 (32.47-76.23)	61.87 (32.25 – 74.49)
Molecular status			
***RAS*mut**	23 (53.5%)	13 (52%)	10 (55.55%)
***BRAF*mut**	8 (18.6%)	5(20%)	3 (16.67%)
wt	12 (27.9%)	7 (28%)	5 (27.78)
MAF			
Median (IQR)	2.09 (0.23-14.32)		
Chemotherapy received			
FOLFOX	3 (6.98%)	1 (4%)	2 (11.11%)
FOLFIRI	38 (88.37%)	22 (88%)	16 (88.89%)
Other	2 (4.65%)	2 (8%)	0
Previous antiangiogenic			
No	24 (55.81%)	10 (40%)	14 (77.78%)
Yes	19 (44.19%)	15 (60%)	4 (22.22%)
Antiangiogenic treatment received			
Bevacizumab	14 (32.56%)	6 (24%)	8 (44.44%)
Aflibercept	29 (67.44%)	19 (76%)	10 (55.56%)
Liver disease (at the beginning of the treatment)			
No	9 (20.93%)	4 (16%)	5 (27.78%)
Yes	34 (79.07%)	21 (84%)	13 (72.22%)
Surgery of primary tumor			
No	8 (18.6%)	8 (32%)	0
Yes	35 (81.4%)	17 (68%)	18 (100%)
Metastatic sites (at the beginning of the treatment)			
1 site	14 (32.56%)	8 (32%)	6 (33.33%)
≥2 sites	29 (67.44%)	17 (68%)	12 (66.67%)

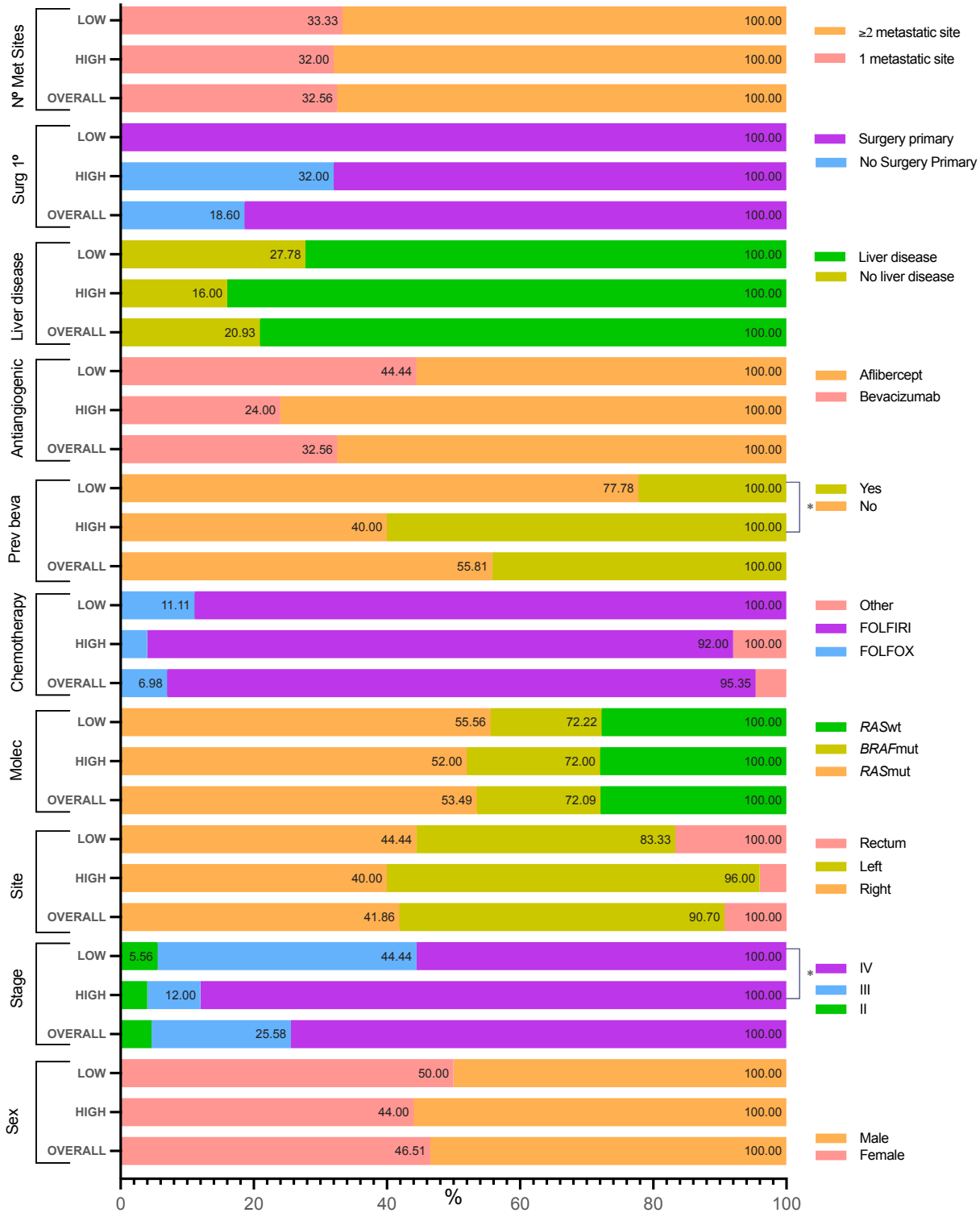


Figure 14: Bar graph with main characteristics of the second line population.

6.2.2. Overall survival analysis

Median OS of the entire population was 11.03 months (95% CI: 8.47-25.1). Figure 15 shows the Kaplan-Meier of all the population in this cohort. When considering the mOS in hight vs low MAF patients, patients with MAF ≥1.4 had a shorter mOS (20.93 months 95% CI: 17.08-35.38) than patients with MAF<1.4 (59.7 95% CI:53.52-NA). The differences in the survival between both groups was statistically significant (HR: 0.26 [95% CI:0.12-0.6]; p=0.0015). Figure 16 shows the Kaplan-Meier differences between high and low MAF patients in this population.

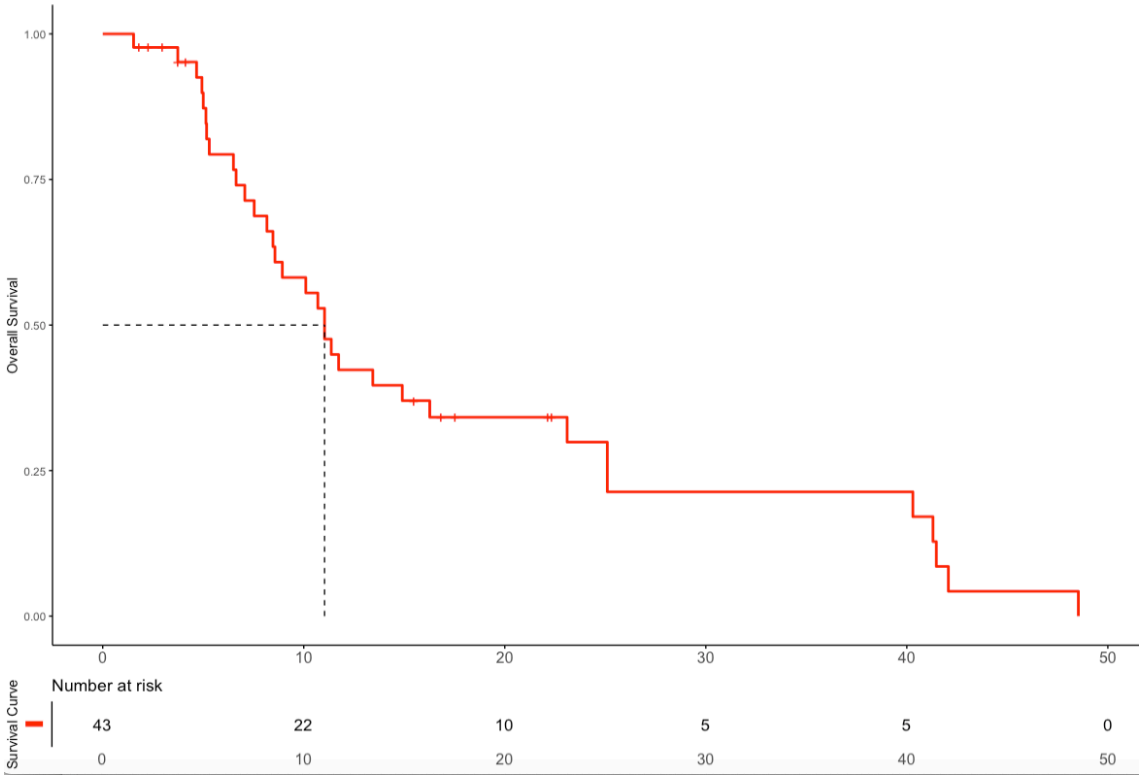


Figure 15: Kaplan Meier graphics of the OS of the cohort of second line patients.

Factor	n	median IC95%	HR IC95%	p.value
HIGH (>=1.04%)	25	20.93m [17.08 ; 35.38]	Ref. [Ref. ; Ref.]	-
LOW (< 1.04%)	18	59.79m [53.52 ; NA]	0.26 [0.12 ; 0.6]	0.0015

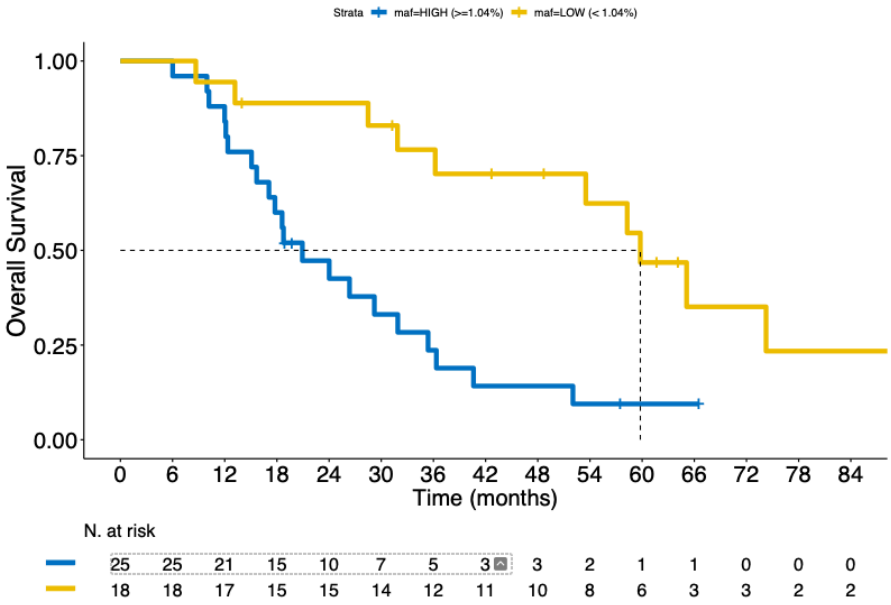


Figure 16: Kaplan Meier graphics and Log rank test of the OS of the cohort of second line patients regarding MAF stratification.

Table 10: Genes selected for the *RAS*wt cohort.

GENES	n (n=12)
APC	4
TP53	2
RAS	1
BRCA1	1
FBXW	1
WT	3

6.2.3. Progression free survival analysis

When considering PFS, median PFS of all cohort was 5.6 months (95% CI: 4.87-6.5). Median PFS of high MAF patients was 5.1 months (95% CI: 2.5-5.63), and median PFS of low MAF patients was 7.5 months (95% CI: 5.9-NR). Figure 17 shows the PFS distribution.

When comparing PFS between two groups, we found statistical differences between PFS of those high MAF patients and those low MAF patients (HR 0.31 [95% CI:1.16-0.62]; p=0.0002, figure 18). Neither an interaction test, multivariable analysis nor separation of the population by MAF level was performed to predict MAF as a predictor of response to antiangiogenic treatment given the low numbers of patients in the general population and the low statistical power.

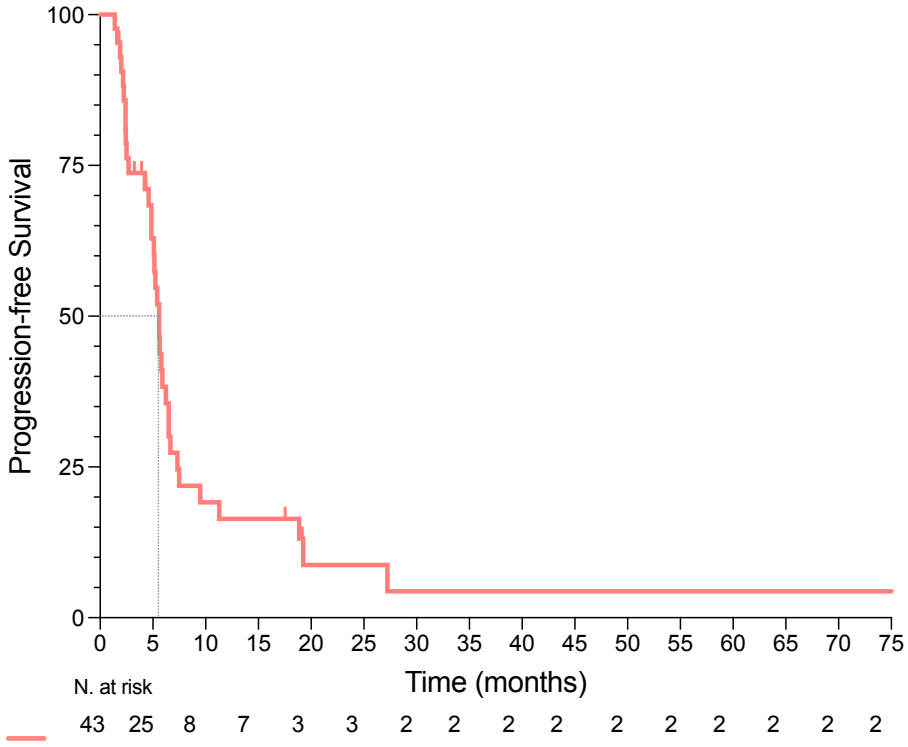


Figure 17: Kaplan-Meier graphics of the PFS for all the cohort of second line.

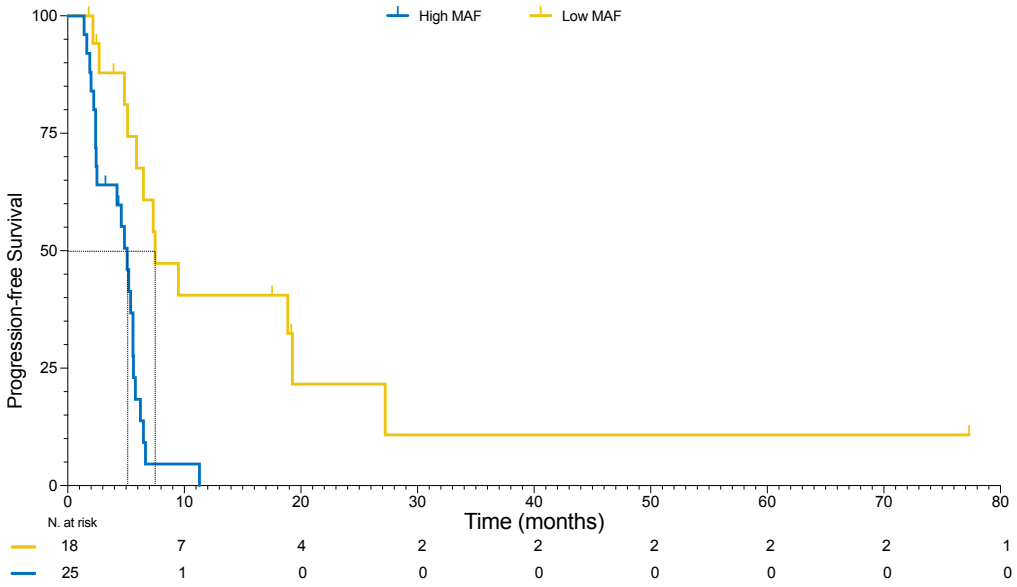


Figure 18: PFS curves between high and low MAF patients in second line cohort.

6.3. Cohort TAS-102/ TAS-102 bevacizumab

6.3.1. Baseline characteristics of the study population

We collected data from patients with mCRC who received TAS-102 or TAS-102 in combination with bevacizumab in the third line or beyond. The basal plasma of all those patients were analysed with NGS test Guardant.

Thirty-two patients with refractory mCRC were included, 14 (43.7%) received TAS-102 in combination with bevacizumab, and 18 (56.3%) received TAS-102 in monotherapy. Median MAF was 9.095 (IQR: 0.53-24.4); being lower in patients treated in monotherapy (3.6 (IQR: 0.2-15.62) in comparison with patients that received TAS-102 in combination with bevacizumab (12.6 IQR:2.098-29.19). Twelve patients were *RAS*wt (37.5%). As the median MAF in this cohort was higher significantly from the two previous first and second line, and since no stablished cutoff exist in this setting, we decided to determine a new cutoff. Patients were stratified as high (≥15,51%) or low (<15,51%) of drive gene. This cutoff was calculated using the cut-off point that best separated the population for survival. Fourteen patients (43.7%) were high MAF, and 18 patients (56.25%) were classified as low MAF. Nineteen patients (59.38%) received the treatment in third line, and the rest (13 patients, 40.62%) in fourth line or more. Table 11 resumes clinical patient characteristics.

Given the observational nature of the study, without randomized groups, we decided to perform a statistical analysis to compare the distribution of qualitative categories between the TAS-102 and TAS-102 + bevacizumab groups. We did not found significant differences in any of the variables included. The distribution of MAF values did not show statistical significant differences between TAS and TAS bevacizumab. Figure 19 illustrates the distribution of the different qualitative variables in the overall population, as well as in the TAS/TAS Bevacizumab cohorts.

Most patients (n=27, 84.4%) had previously received antiangiogenic therapy prior to TAS-102/TAS-102 + Bevacizumab treatment. Five patients did not receive antiangiogenic treatment, three of whom had medical contraindications (haemorrhagic or thrombotic events).

Table 11: Main clinical characteristics according to treatment with or without bevacizumab

	Overall population (n=32)	TAS-102 (n=14)	TAS-102 bevacizumab (n=18)
Sex			
Female	15 (46.88%)	6 (42.86%)	9 (50%)
Male	17 (53.12%)	8 (57.14%)	9 (50%)
Stage at diagnostic			
I	1 (3.12%)	0 (0%)	1 (5.56%)
II	4 (12.5%)	1 (7.14%)	3 (16.67%)
III	10 (31.25%)	6 (42.86%)	4 (22.22%)
IV	17 (53.12%)	7 (50%)	10 (55.56%)
Tumor Site			
Right	8 (25%)	6 (42.86%)	2 (11.11%)
Left	16 (50%)	5 (35.71%)	11 (61.11%)
Rectum	8 (25%)	3 (21.43%))	5 (27.78%)
Age			
Median (range)	59.86 (37.1-81.12)	64.03 (37.1 - 77.22)	53.27 (41.45 - 81.12)
***RAS* status**			
Mut	20 (62.5%)	10 (71.43%)	10 (55.56%)
wt	12 (37.5%)	4 (28.57%)	8 (44.44%)
MAF			
Median (IQR)	9.095 (0.53 - 24.4)	3.62 (0.2 - 15.62)	12.56 (2.098 - 29.19)
High (≥15.51%)	14 (43.75%)	5 (35.71%)	9 (50%)
Low (<15.51%)	18 (56.25%)	9 (64.29%)	9 (50%)
Line treatment			
3rd Line	19 (59.37%)	7 (50%)	12 (66.7%)
>3rd Line	13 (40.63%)	7 (50%)	6 (33.3%)
Liver metastases			
No	18 (56.25%)	6 (53%)	12 (67%)
Yes	14 (43.75%)	8 (57%)	6 (33%)
Previous Antiangiogenic			
Yes	27 (84.37%)	11 (78.57%)	16 (88.9%)
No	5 (15.63%)	3 (21.43%)	2 (11.1%)

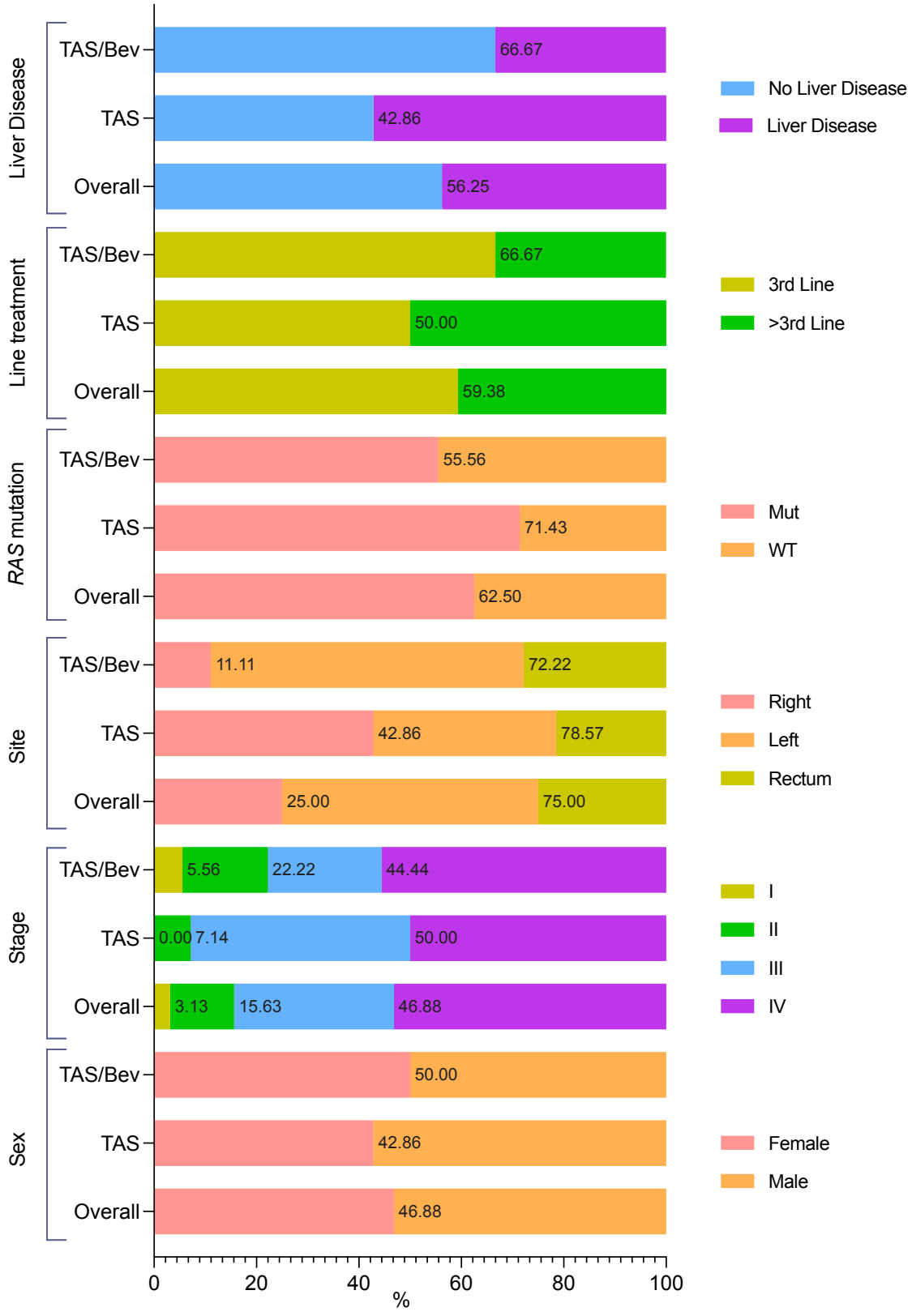


Figure 19: Bar graph with main characteristics of the TAS/TAS Bevacizumab population.

6.3.2. Overall survival results

Median OS of the entire population was 10.10 months (95% CI: 6.64 - 11.90). Figure 20 shows the Kaplan-Meier of all the population in this cohort.

Higher mOS was reached in those patients with low MAF compared to high MAF (11.1 vs 6.64 months, HR 0.31 [95% CI:0.12-0.83]; p=0.019). Figure 21 represents the Kaplan-Meier curves for this population classified with MAF value. This prognostic effect was maintained for low MAF patients in the multivariable analysis (HR 0.18 [95% CI:0.06-0.56]; p=0.03). Patients treated with TAS-102 in monotherapy had a worse prognosis in the multivariable analysis (HR 3.55 (95% CI:1.05-12.02], p= 0.042). Figure 22 shows the multivariable analysis for OS of this cohort.

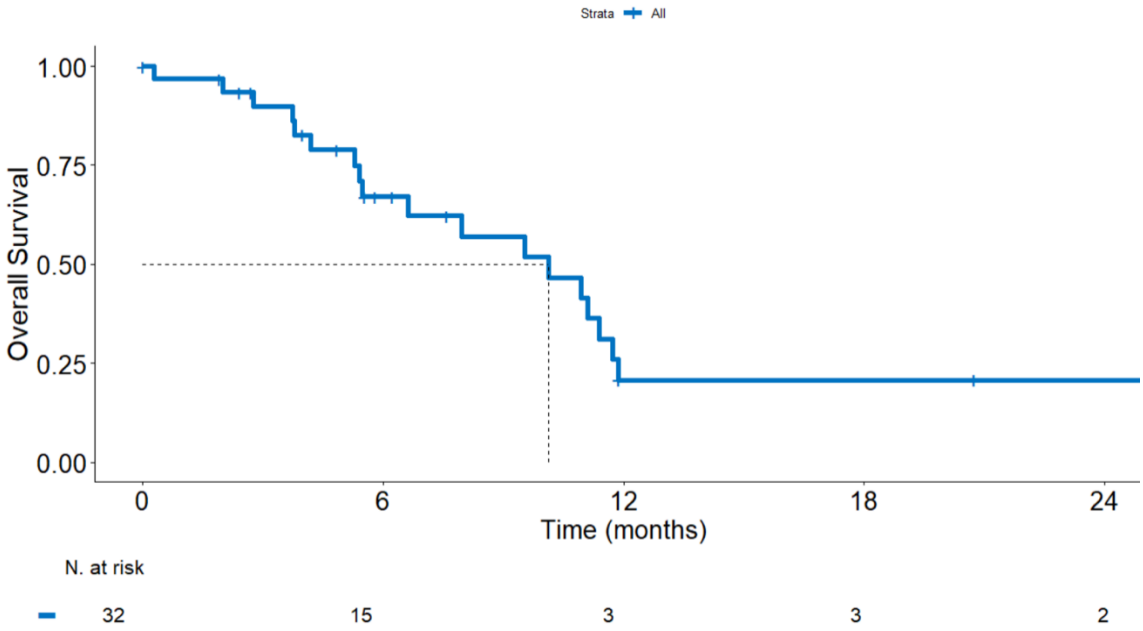


Figure 20: Kaplan-Meier graphics of OS of the cohort of TAS-102/TAS-102+ bevacizumab.

Factor	n	median IC95%	HR IC95%	p.value
HIGH (>=15.51%)	14	6.64m [3.78 ; NA]	Ref. [Ref. ; Ref.]	-
LOW (< 15.51%)	18	11.1m [9.53 ; NA]	0.31 [0.12 ; 0.83]	0.019

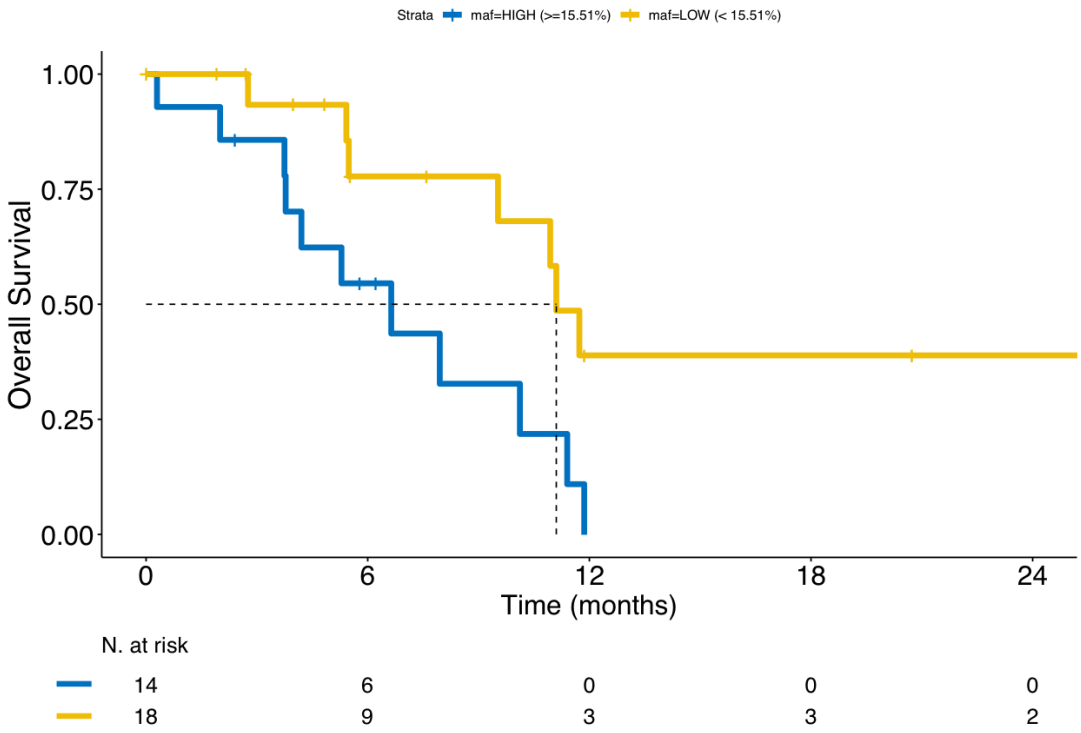


Figure 21: Kaplan-Meier curves of OS of mCRC patients treated with TAS-102/ TAS-102 + bevacizumab according to MAF levels.

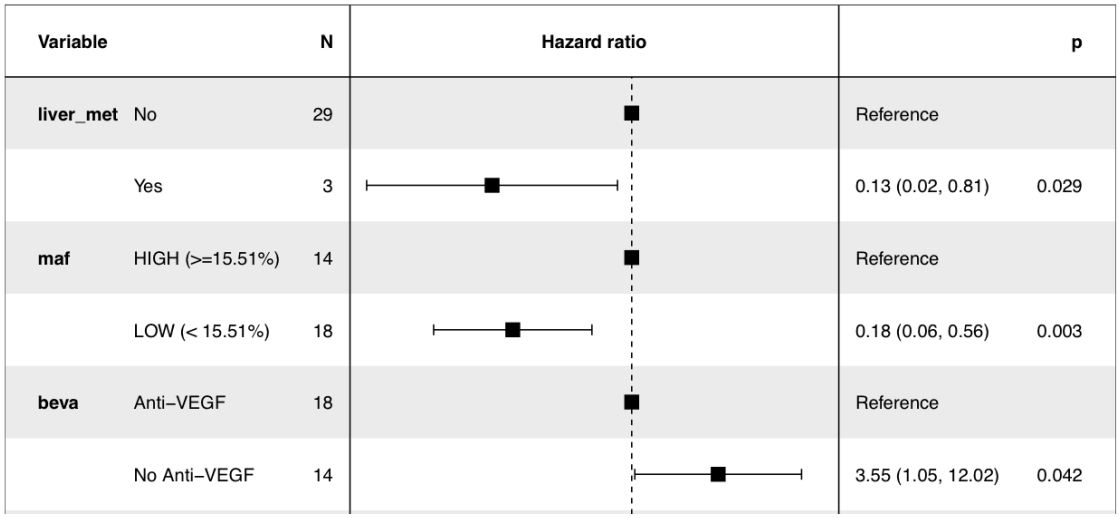


Figure 22: Multivariable Cox model to evaluate the association between clincopathological factors and OS of mCRC patients treated with TAS-102/ TAS-102 + bevacizumab.

6.3.3. Progression free survival analysis

Regarding PFS analysis, there was a trend towards better PFS in those patients with low MAF compared to those with high MAF (HR 0.44 [95% CI:0.19-1.06]; p=0.0667). Figure 23 shows the Kaplan-Meier curves for PFS in this population.

Patients with a low MAF had better PFS in the multivariable study (HR 0.32 [95% CI: 0.12-0.88]; p=0.027). Likewise, being without antiangiogenic therapy was associated with worse PFS in the multivariable analysis (HR 4.4 [95% CI: 1.57-12.32]; p=0.005). Figure 24 shows the multivariable Cox model for PFS. Neither an interaction test nor separation of the population by MAF level was performed to predict MAF as a predictor of response to antiangiogenic treatment given the low numbers of patients in the general population and the low statistical power.

Factor	n	median IC95%	HR IC95%	p.value
HIGH (>=15.51%)	14	3.09m [2.2 ; NA]	Ref. [Ref. ; Ref.]	-
LOW (< 15.51%)	18	3.55m [2.89 ; NA]	0.44 [0.19 ; 1.06]	0.0667

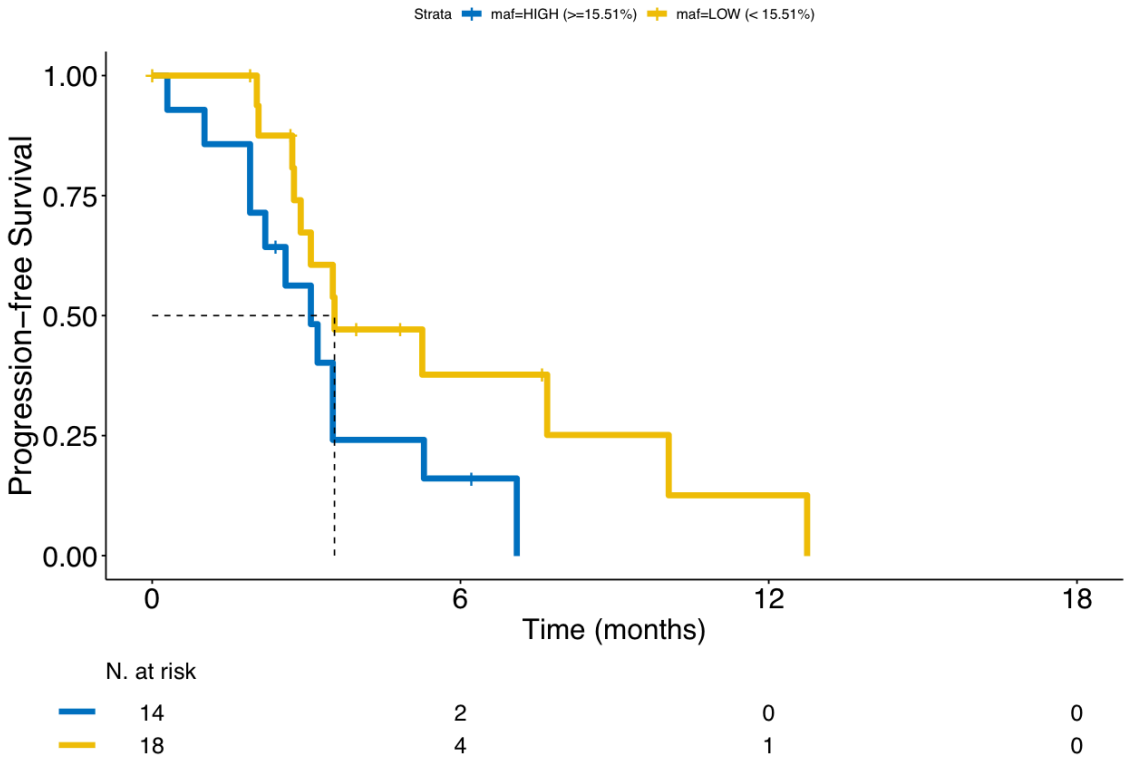


Figure 23: Kaplan-Meier curve for progression free survival of patients treated with TAS-102 or TAS-102 + bevacizumab according to MAF levels.

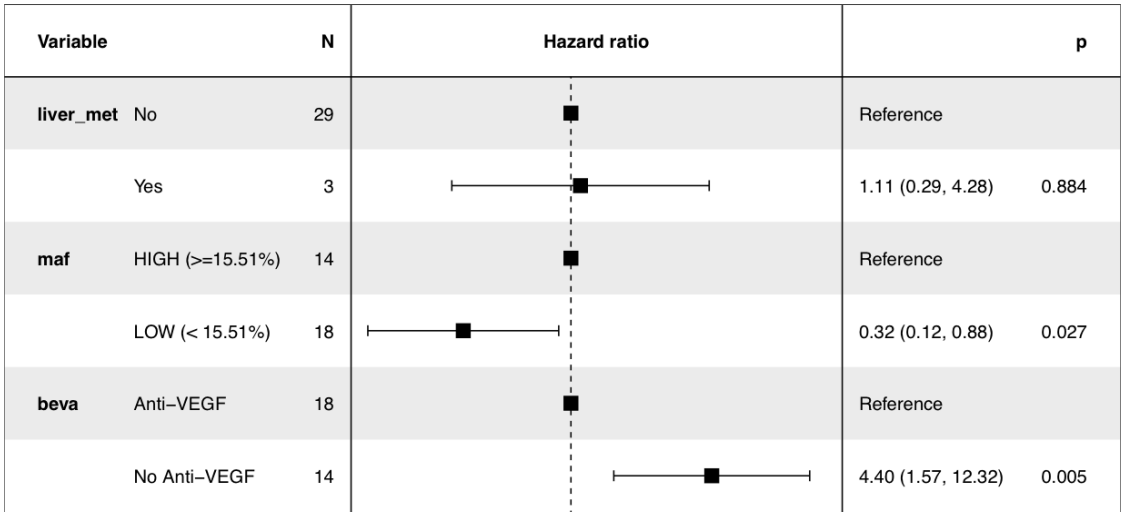


Figure 24: Multivariable Cox model to evaluate the association between clincopathological factors and progression free survival of mCRC patients treated with TAS-102/ TAS-102 + bevacizumab.

6.4. Permeability test of tumor vasculature and correlation with MAF

From the first-line cohort, we randomly selected 56 patients with available MAF values and sufficient primary tumor tissue. We evaluated the primary tumor (biopsy or surgical) for hemorrhaging test as a marker of vascular leakiness and surrogate of intravasation (see Methods). Tissue of patients were classified as ND (not determined), negative for patients whose tissue showed no evidence of macro- or microhemorrhage, and positive for those with detected hemorrhage. Positive cases were further classified into three grades: +, ++, or +++, based on the intensity of the hemorrhage. This analysis was conducted at an external site (IDIBELL), and the pathologist was blind for the MAF value of each. We then collected the MAF values and compared the median MAF across the different classification groups. Table 12 shows the main clinical characteristics of this cohort

The classification results were as follows: 15 patients as ND, 10 patients as negative (-), 10 as +, 10 as ++, and 11 as +++. The distribution of clinical important characteristics such as synchronous disease, the presence of liver disease, and *RAS* mutations was similar across the groups (χ^2 test, $p > 0.05$).

The median MAF values were 19.7% for the ND group, 5.24% for the negative group (-), 11.8% for the + group, 12.5% for the ++ group, and 15.3% for the +++ group. The Kruskal-Wallis test showed statistical significance (p-value = 0.0322). Pairwise comparisons using the Dunn test (excluding the ND group) identified a significant difference between the negative group and the +++ group (adjusted p-value = 0.0209). Figure 25 shows the median MAF values across the different groups. Figure 26 shows examples of FFPE H&E slides for quantification of hemorrhage from negative to +++.

Table 12: Main clinical characteristics of patients tested for hemorrhage in FFPE samples

	+ (n=10)	++ (n=10)	+++ (n=11)	- (n=10)	ND (n=15)
Sex					
Female	6 (40%)	4 (40%)	4 (36.36%)	2 (20%)	7 (46.67%)
Male	4 (40%)	6 (60%)	7 (63.64%)	8 (80%)	9 (60%)
Age					
Median (range)	67 (44 - 78)	72.5 (47 - 81)	62 (40 - 70)	65.5 (42 - 77)	66 (33 - 88)
Bevacizumab					
Yes	5 (50%)	6 (60%)	8 (72.73%)	7 (70%)	9 (60%)
No	5 (50%)	4 (40%)	3 (27.27%)	3 (30%)	6 (40%)
Tumor Site					
Left	3 (30%)	7 (70%)	5 (45.45%)	5 (50%)	8 (53.33%)
Right	7 (70%)	3 (30%)	5 (45.45%)	5 (50%)	7 (46.67%)
MAF					
Median (IQR)	8.27 (1.49-17.37)	10.28 (6.77-14.07)	15 (11.82-18.75)	4.17 (0.62-8.92)	17.04 (7.28-25.62)
Liver Disease					
Yes	8 (80%)	10 (100%)	9 (81.82%)	8 (80%)	14 (93.33%)
No	2 (20%)	0 (0%)	2 (18.18%)	2 (20%)	1 (6.67%)
Type of sample analyzed					
Surgery	8 (80%)	3 (30%)	9 (81.82%)	8 (80%)	4 (26.67%)
Biopsy	2 (20%)	7 (70%)	2 (18.18%)	2 (20%)	11 (73.33%)
***RAS* status**					
Mut	8 (80%)	10 (100%)	11 (100%)	9 (90%)	11 (73.33%)
wt	2 (20%)	0 (0%)	0 (0%)	1 (10%)	4 (26.67%)

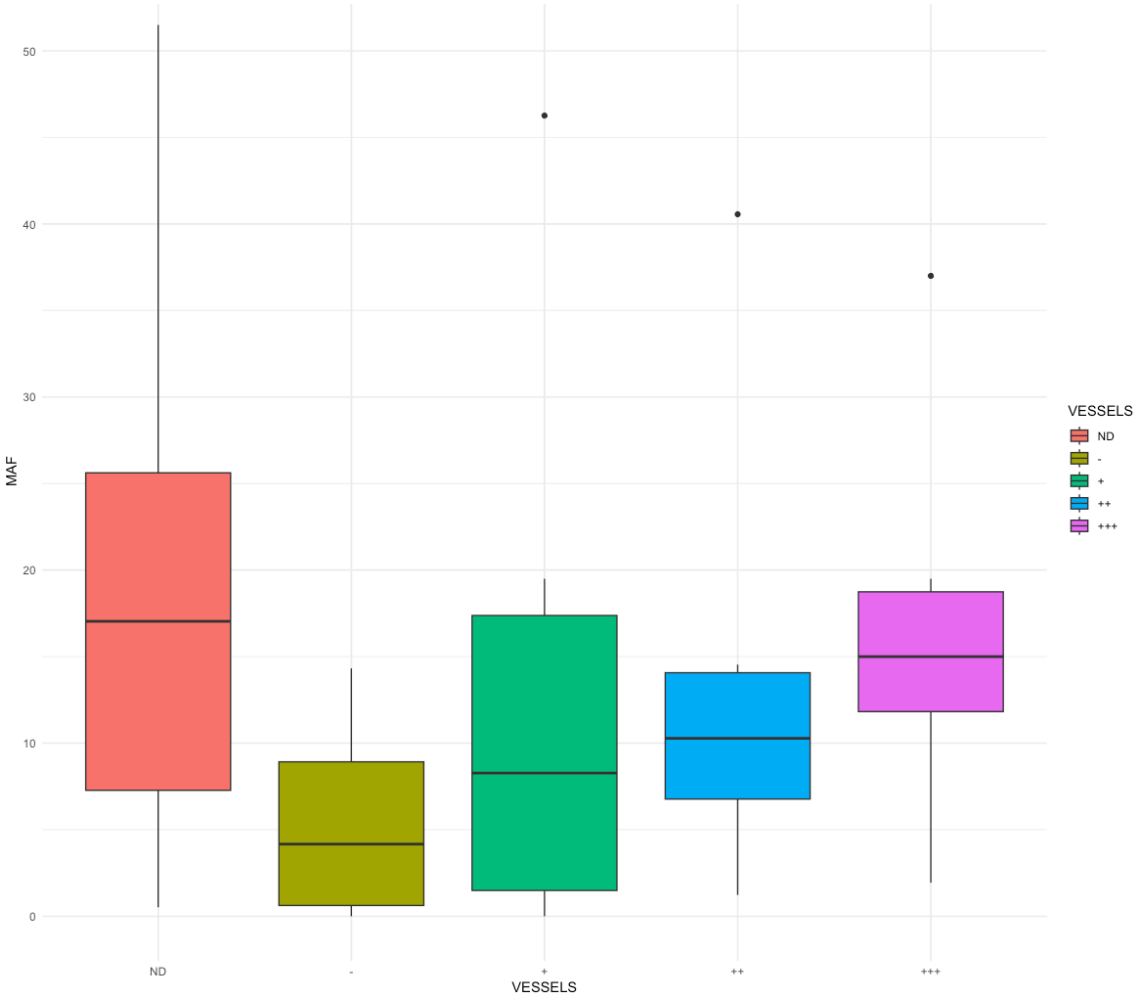


Figure 25: Box Plot showing Median MAF of ND, -, +, ++,+++ groups of patients.

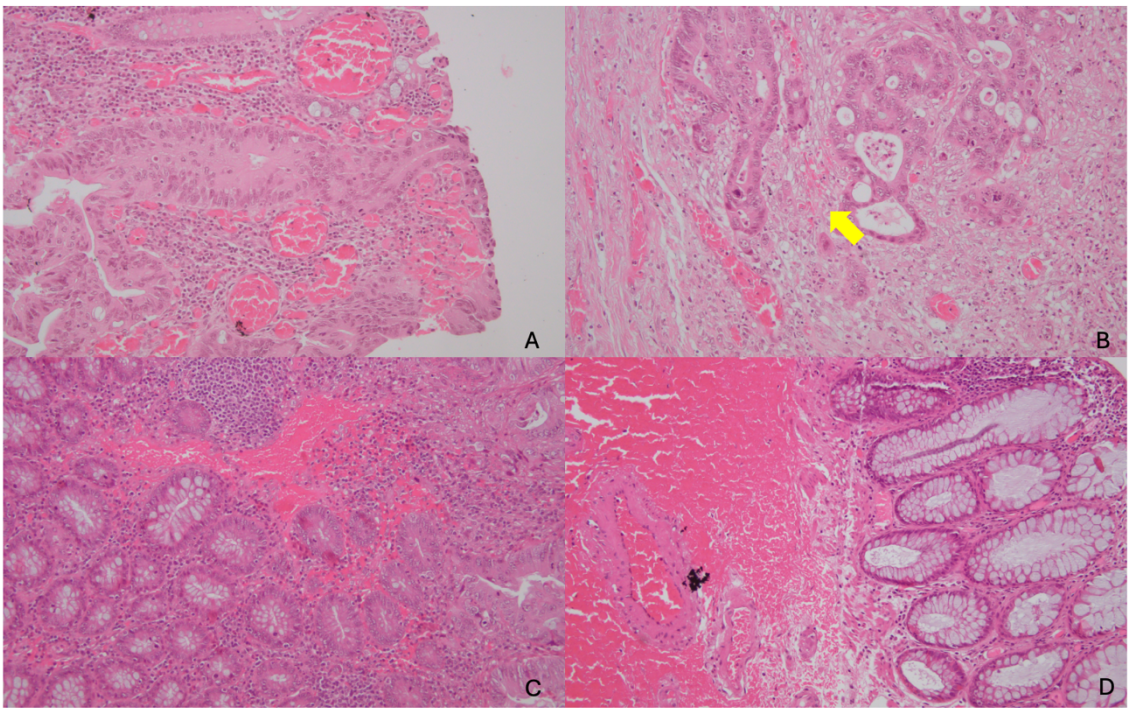


Figure 26: H&E showing used classification: A: negative hemorrhage tumor; B +, C++; D +++. The yellow arrow indicates hemorrhage.

DISCUSSION

7. DISCUSSION

The aim of our study was to elucidate the role of ctDNA as a predictive biomarker for response to antiangiogenic treatment in the first-line setting and to gain a deeper understanding of the dynamics and significance of ctDNA throughout the entire oncological history of mCRC patients. To achieve this, we conducted a comprehensive analysis of mCRC patient cohorts across various treatment lines, evaluating ctDNA's role as both a prognostic and predictive biomarker.

We observed a potential predictive role of MAF in the benefit of bevacizumab in first-line mCRC. This finding, based on a clinical cohort of 185 patients, aligns with the central hypothesis that plasma MAF of driver genes reflects tumor vasculature quality and functionality. Tumors with greater vascularity, characterized by permeable and leaky vessels, are more prone to shedding higher amounts of ctDNA and appear to benefit more from the vascular normalization effect of antiangiogenic therapy. The vascular leaking test, which quantifies hemorrhage as a surrogate for vascular permeability, provides a mechanistic link between our hypothesis and the clinical observations in this study. However, ongoing *in vivo* experiments aim to further explore the mechanistic relationship between ctDNA shedding and angiogenesis. Despite the promising nature of these findings, from a practical clinical standpoint, these results are derived from retrospective, non-randomized data, and cannot yet be used to guide bevacizumab treatment decisions in first-line mCRC. Additionally, validation cohort data is not yet available. Moving forward, validation in an independent cohort and prospective clinical trial designs will be necessary to confirm these observations. The independent prognostic value of MAF in ctDNA across different treatment lines suggests that it could complement other established clinical prognostic markers to refine therapeutic strategies for mCRC patients. Although derived from separate patient cohorts, we observed a dynamic shift in median MAF values across treatment lines: 6.5% in the first line, 2.1% in the second line, and 9.1% in the third line or beyond. This information, which is scarce in the literature, provides a general overview of MAF dynamics throughout the oncological history of mCRC patients. These variations can be attributed to multiple factors, including changes in tumor volume, evolving tumor biology across treatment lines, and the impact of cytostatic treatments in ctDNA shedding.

7.1. First-line cohort

7.1.1. Baseline characteristics of the study population

The main objective of this study was to evaluate the predictive role of allele frequency of driver genes in circulating tumor DNA in patients with mCRC receiving first-line bevacizumab. To achieve this, we selected patients with MSS mCRC who received chemotherapy or a combination of chemotherapy and bevacizumab as first-line treatment. This selection could introduce some bias, which will be further discussed in the limitations section of the study. We collected clinical, molecular, and ctDNA analysis data from 185 patients with mCRC in this cohort. Our cohort is heterogeneous in both clinical presentation and molecular characteristics, but it effectively represents the real-world data of patients undergoing first-line treatment for mCRC.

The median age of the cohort was 66.5 years, consistent with the median age of mCRC patients. Additionally, 61.08% of the patients were male, which aligns with the sex distribution data available in the literature for mCRC patients.[1] One hundred and thirty-four patients (72.4%) were diagnosed at stage IV, which is a higher percentage than what is typically reported in the literature. However, this is consistent with the percentage of stage IV patients we receive as first-visits in VHIO (78%, based on internal statistics from 2023, VHIO).

It is noteworthy that 65 patients (35% of the total cohort) underwent metastasectomy during the first line of treatment. This factor will be considered in the analysis of PFS results. In the literature, it is reported that at the time of diagnosis, 20–25% of mCRC patients have lesions that are resectable or potentially resectable, and additionally, conversion from initially unresectable to resectable disease can occur in 15–50% of patients following chemotherapy, consistent with the percentaje of the first-line cohort patients. [258]

More than half of the patients (63.2%) received antiangiogenic therapy. When comparing the distribution of treatment categories between the two groups (antiangiogenic vs. non-antiangiogenic), the only significant difference observed was the higher frequency of *RAS* mutations in the bevacizumab group. This can be explained by the current treatment guidelines for mCRC, which recommend combining chemotherapy with bevacizumab as the first-line treatment for *RAS*-

mutated patients, regardless of tumor laterality. However, it is worth noting that among the 146 *RAS*-mutant patients, 46 (31.5%) did not receive antiangiogenic treatment. This could be due to several factors, such as early recruitment before the widespread availability of antiangiogenic biosimilars, contraindications to antiangiogenic therapy, or the treating oncologist's preference. This selection might also introduce bias into the results, which will be discussed further.

Due to the extended recruitment period of the study, we initially included patients with *RAS* mutations because of technical limitations for ctDNA analysis, specifically the absence of NGS for ctDNA. After NGS was implemented in our institution, we began to include patients regardless of their *RAS* status. This shift explains the high proportion of *RAS*-mutant patients in the cohort (146, 79%). For the cohort of *RAS*wt patients (39, 21%), we selected the driver gene with the highest MAF value from the NGS ctDNA results. This approach, however, could introduce bias, which we will discuss further. The main driver genes identified included mutations in *p53*, *RAS*, and *APC*, along with alterations in genes related to the MAPK pathway (e.g., *ARAF*), the β-catenin pathway (e.g., *AXIN1* and *AMER1*), and less commonly described genes such as *GATA3* (a transcription factor) and *AKAP9* (a member of the A-kinase anchor proteins family involved in cancer development and metastasis).[259] Interestingly, 9 out 39 patients (23%) were found to be *RAS*-mutant in plasma despite being *RAS*wt in tissue. Of these, 8 were classified as having low MAF, with 7 showing MAF values under 1%. The patient classified as high MAF had a plasma MAF of *KRAS*A146 at 37% by NGS in ctDNA. This patient had right-sided CRC with extensive metastatic disease at diagnosis, including bulky liver involvement. The primary biopsy sample had a very low representation of adenocarcinoma, allowing only digital PCR to be performed on the tissue. The patient was treated with first-line bevacizumab combined with chemotherapy, achieving stable disease but with a PFS of less than 12 months. After switching to second-line treatment with FOLFIRI and Aflibercept, rapid progression to the brain occurred within 4 months.

Several factors could explain the discrepancy between tissue and plasma results in *RAS*wt patients. For the high MAF patient, the low representation of tissue in the biopsy sample likely limited the detection of RAS mutations. For the other patients, with low MAF the discrepancy could be due to spatial heterogeneity of tumor mutations, as biopsies capture the genetic profile of a specific region, while ctDNA

offers a more comprehensive view. Additionally, differences in the timing of tissue and liquid biopsy sampling may have contributed; in some cases, tumor tissue was collected during the nonmetastatic stage, with *RAS* mutations potentially developing later as the disease progressed. Variations in assay sensitivity between the tissue and liquid biopsies could also have played a role.

A prespecified exploratory biomarker analysis of the PARADIGM trial evaluated the association between ctDNA gene alterations and efficacy outcomes, focusing on a broad panel of gene alterations associated with resistance to EGFR inhibition, including *KRAS*, *NRAS*, *PTEN*, *EGFR* extracellular domain mutations, HER2 and MET amplifications, and *ALK*, *RET*, and *NTRK1* fusions.[260] The presence of *RAS* or *BRAF* mutations in this study was 17% in the overall cohort, with *RAS* or *NRAS* mutations present in 7% of cases. A higher incidence was observed in patients with right-sided tumors (40.1%, according to supplementary data, with 10% for *RAS* or *NRAS* mutations). In our cohort, 23% of *RAS*wt patients had mutations in *KRAS* or *NRAS*, which is slightly higher than reported in this and other studies, such as the PERSEIDA trial, where 12.6% of patients initially identified as *RAS*wt in tissue biopsies showed *RAS* mutations in their ctDNA at baseline. [261] *BRAF*-mutant patients are underrepresented (only 3 patients), mainly due to their inclusion in our center of BRAF mutated patients in first-line clinical trials.

The median MAF for the entire cohort was 6.49%. In the literature, basal median MAF values for mCRC patients receiving first-line treatment vary, ranging from 2.3% to 20%, depending on patient characteristics (e.g., presence or absence of liver metastases).[262–266]

An important point to mention is the percentage of patients with undetectable ctDNA in their blood. It is known that some patients do not shed ctDNA into the bloodstream. Currently, the primary factors influencing shedding patterns are attributed to the distribution and bulk of metastatic disease, with shedding being more common in patients with liver metastases compared to those with metastases in other sites such as the lungs or peritoneum. Previous published cohorts indicate from 20-28% of patients do not shed ctDNA, percentage that is similar to our cohort, and maily due to the lack of liver metastatic disease.[267,268]

7.1.2. The role of MAF as a prognostic biomarker in first-line cohort

The role of the MAF of ctDNA as a prognostic factor is well stablished not only in mCRC but also across other cancer types. A recent study in more than 1,500 patients affected from various cancer types showed that ctDNA MAF shows significant independent prognostic impact within a real-world dataset. [246]

The prognostic value of the MAF of *RAS* has been extensively documented across various cohorts of *KRAS*mut mCRC in the literature, with diverse cut-off points established to identify patients with high or low MAF and subsequently determine those with better or worse prognoses. Notably, MAF of *RAS* has been reported as an independent prognostic factor in multivariable analyses. [269–271] Considering other molecular subgroups, recent studies have also highlighted the prognostic value of MAF for *BRAF*-mutated patients, identifying it as an independent prognostic factor.[247] However, the literature on the prognostic role of MAF in *RAS*wt patients is less extensive. Recently, two studies have explored MAF as a prognostic factor in *RAS*wt patients. In a cohort of 412 chemotherapy-naïve patients with mCRC including *RAS*wt tumors (33%), a ctDNA MAF cutoff of 20% served as an independent prognostic marker.[267] The VALENTINO was a phase II clinical trial investigating first-line maintenance therapy strategies in *RAS*wt mCRC patients, demonstrating that continuing panitumumab 5FU after induction therapy was superior in terms of PFS compared to panitumumab alone.[272] In a preplanned analysis of the VALENTINO trial, baseline ctDNA MAF was identified as a robust prognostic marker in 135 patients, outperforming traditional markers such as CEA and target lesion size. [273]

In our cohort, the prognostic value of MAF remains significant, even when including *RAS*wt patients. This underscores the added value of ctDNA analysis in these patients, who can now be categorized based on plasma MAF levels due to advancements in NGS techniques. Importantly, our multivariable analysis shows that the prognostic value of MAF remains independent of other confounding variables such as synchronous disease, metastatic surgery, or the number of affected organs. Moreover, the lack of significance related to the number of affected organs suggests that shedding and MAF values of ctDNA might extend beyond merely serving as surrogate biomarkers for metastatic disease volume. It is likely that the

biological implications of shedding play a fundamental role in tumor aggressiveness in mCRC patients.

7.1.3. The role of MAF as a predictive biomarker for antiangiogenics in the cohort of first line

Currently, there are no predictive biomarkers available to guide bevacizumab prescription in mCRC. Although various VEGF-A gene and VEGFR receptor polymorphisms have been investigated as potential, they have not been demonstrated their applicability in the clinical setting.

A review of the literature reveals that while some studies describe the dynamics of ctDNA MAF values during treatment with antiangiogenic drugs, there is no published data linking the role of ctDNA to response to antiangiogenic therapy.[274]

In order to explore the predictive role of ctDNA for bevacizumab in first-line, we compared patients with high versus low MAF to determine if PFS analysis diverges based on bevacizumab treatment. In the overall population, there was a trend toward statistical differences in PFS for patients with high MAF who received bevacizumab compared to those who did not, although these differences were not statistically significant. Considering the importance bias of metastatsetomy in PFS, we decided to analyse those non-surgical patients. Notably, the differences became statistically significant when we analyzed the subset of 122 patients who did not undergo metastasectomy. In this subset, high MAF patients who received bevacizumab had significantly better PFS compared to those who did not receive the drug. In contrast, no significant differences in PFS were observed for low MAF patients regardless of bevacizumab treatment. Additionally, in the multivariable analysis for PFS within this cohort, low MAF was significantly associated with better PFS. Given the observational nature of this study, we calculated the p-value for the interaction between bevacizumab treatment and MAF levels to assess whether the effect of bevacizumab on progression-free survival varies with MAF levels. The interaction p-value was 0.026. Taking these data toghether, we can say that there is a predictive role of MAF in the benefit of antiangiogenic treatment for first-line mCRC patients.

The journey of a biomarker from discovery to clinical application is long and arduous. The most reliable setting for initial retrospective biomarker discovery is through the analysis of data collected from prospective clinical trials. One of the most significant challenges in biomarker validation is bias, that can be introduced at various stages, including patient selection, specimen collection, specimen analysis, and patient evaluation. Randomization and blinding are critical tools for minimizing bias and ensuring the reliability of study outcomes. It is important to note that the data generated in this study were not derived from a randomized trial, which is a limitation that must be acknowledged.

To identify a predictive biomarker, secondary analyses should ideally be conducted using data from randomized clinical trials, employing an interaction test between the treatment and the biomarker within a statistical model. Reviewing the literature regarding predictive biomarker discovery, we can take as an example the IPASS study.[275] This study enrolled patients with advanced pulmonary adenocarcinoma and randomized them to receive either gefitinib or carboplatin plus paclitaxel (CP). The *EGFR* mutation status of the patients was determined retrospectively after enrollment. The interaction between treatment and *EGFR* mutation status was highly statistically significant ($p < 0.001$). Moreover, patients with *EGFR*-mutated tumors had significantly longer PFS when treated with gefitinib compared to those treated with CP. Conversely, patients with *EGFR*wt tumors experienced significantly shorter PFS when treated with gefitinib compared to CP.

Even if this example from the literature is taking from clinical trial data, we can confirm that our observations from non-randomized data are the same as from other biomarkers discovered in the past.

Validation is "a process to establish that the performance of a test, tool, or instrument is acceptable for its intended purpose. External validation establishes a biomarker's performance in a completely independent dataset not used during development.[276] Several prospective clinical trial designs aim to validate the clinical utility of predictive biomarkers. Although we are not yet at the stage of designing a clinical trial, we plan to validate our results with a cohort of patients from a major tertiary hospital in Madrid.

7.2. Second-line cohort

We selected a cohort of patients who received antiangiogenic treatment as second-line therapy for mCRC. This choice was made due to the limited number of patients treated with chemotherapy alone in the second line and our intention to exclude those treated with other biological therapies (such as anti-EGFR) to align with the study's objectives. For the purpose of this analysis, we aimed to study the differences in OS and PFS between patients with high and low MAF.

A total of 43 patients were included in the second-line cohort. When comparing the median MAF values of the second-line cohort to those of the first-line cohort, the median MAF in the second line (2.82%) is substantially lower than in the first line (6.49%). These differences could be attributed to several factors: first, the possible reduction in metastatic disease volume between first and second-line treatments, which could be related to prior response of disease during first line treatment; second, the selection of a different patient cohort receiving various biological treatments; third, intrapatient variability; and four: the previous treatment with chemotherapy agents in the first-line could explain lower MAF in the second-line cohort. To better understand this, we collected plasma samples from 31 patients in the first-line cohort at progression (included in the first- and second-line cohorts). Of these, 19 (61%) showed no change or a decrease in MAF compared to baseline values, presenting as best response of the disease SD or PR for 16 of them. This could be explained due to reduced metastatic volume in second line or the influence of previous chemotherapy during first-line.

In our analysis of patient distribution across high and low MAF groups, significant differences were observed among patients with synchronous disease, where high MAF was more prevalent. This disparity aligns with expectations, given the more aggressive nature of synchronous disease. Furthermore, this observation may also explain why all patients classified as low MAF had undergone surgery for the primary tumor, as part of the oncological history of metachronous mCRC patients. We identified notable differences in the administration of prior antiangiogenic therapy in the first-line setting. Specifically, 77% of patients with low MAF did not receive antiangiogenic treatment, whereas 40% of patients with high MAF were not treated with such therapy, a difference that reached statistical significance. To further investigate, we examined the cohort of patients who had not previously

received antiangiogenic treatment. Among the 24 patients in this category, 14 harbored either *RAS* or *BRAF* mutations, while 10 were *RAS*wt (all of whom received EGFR inhibitors in the first-line setting). Among the 14 *RAS- or BRAF*-mutant patients, we reviewed the reasons for the absence of first-line bevacizumab treatment. Six patients experienced disease progression within six months following primary tumor resection and were treated with FOLFOX. Three patients received FOLFOX perioperatively as part of metastasectomy treatment, and three *BRAF*-mutant patients participated in clinical trials that did not include antiangiogenic therapy in the first line. The reasons were unspecified for two patients treated in 2014 and 2017, possibly due to the unavailability of antiangiogenic options at that time. The enrichment of low MAF patients among those who did not receive antiangiogenic treatment could be explained by disease progression following surgery, which is associated with a lower tumor burden and may have biased this patient distribution.

Interestingly, in the second-line treatment, MAF levels continued to exhibit prognostic significance, with higher MAF associated with poorer OS and PFS compared to lower MAF patients. We also assessed whether the 12 patients *RAS*wt tumors had received EGFR inhibitors in the first-line setting, as this treatment could have exerted selective pressure on resistant clones, potentially resulting in elevated MAF levels in this population. However, the median MAF for these patients was 2.82%, comparable to the median MAF of 2.09% observed in the overall second-line population.

7.3. TAS-102/TAS-102 + bevacizumab cohort

We collected data from 32 patients treated with TAS-102 or TAS-102 in combination with Bevacizumab. The median MAF for all the cohort was 9.1%. Interestingly, the median MAF in patients treated with TAS-102 monotherapy was lower (3.6%) compared to those receiving TAS-102 with bevacizumab (12.6%). However, these differences were not statistically significant (p=0.1768). Despite patients treated with TAS-102 Bevacizumab displaying poorer prognostic factors, including a higher prevalence of liver disease (57% vs 33%), MAF remained as an independent prognostic indicator in our cohort. The higher prevalence of patients with liver

disease in patients treated with TAS-102 Bevacizumab could be explained for the regulatory approvals in our institution, where TAS-102 in monotherapy is mainly approved for those patients without liver disease based on the analysis of the RECOURSE trial, where patients with more than 18 months since the onset of metastatic disease, no more than two metastatic sites, and no liver involvement experienced a greater magnitude of benefit in overall survival from TAS-102.[277]

We also sought to determine whether the median MAF in our cohort was higher or lower compared to median MAF values reported for refractory mCRC patients. A literature search revealed that, while there are descriptive studies on refractory populations using NGS in ctDNA, particularly regarding clonal evolution dynamics, no studies specifically report the median MAF of ctDNA in this setting. At VHIO, we reviewed MAF data in refractory patients who had ctDNA analysis performed either before or at the time of progression on any treatment line, excluding those involved in the TAS/TAS-bevacizumab analysis. Among 37 patients analyzed with plasma samples from pre-third line to progression on up to an eighth line, the median MAF in this cohort was 9.76%. Despite the heterogeneity of this cohort and the preliminary nature of the result, this data provides context for understanding the median MAF of 9.1 % observed in the TAS/TAS-bevacizumab cohort in the context of refractory setting.

With a MAF of 5.8%, the prognostic value of MAF for OS in later treatment lines was not confirmed, which prompted us to explore a higher cutoff, potentially related to the increased metastatic disease burden in this context. At a cutoff of 15.5%, we observed differences in OS, and MAF retained independent prognostic value in the multivariable analysis. When applying this new cutoff to assess the predictive value in the TAS-102-bevacizumab setting, PFS values in the Kaplan-Meier analysis were not statistically significantly different. In the multivariable analysis, MAF values maintained an independent value for PFS. The discrepancy between the results of the log-rank test and the multivariable analysis can be explained by the confounding factor of receiving bevacizumab and the increased representation of low MAF patients in the TAS-102 monotherapy arm.

7.4. Vascular leaking test

This study aimed to evaluate tumor vasculature permeability through hemorrhage classification and its correlation with the MAF in mCRC. Our approach was grounded on the hypothesis that heightened vascular leakiness, reflected by hemorrhage, is associated with increased tumor intravasation and higher plasma MAF levels. Given the difficulty of directly measuring intravasation and extravasation pressures in FFPE samples, we used hemorrhage as a surrogate marker of vascular pressure dynamics. Hemorrhagic regions were considered indicative of areas where significant erythrocyte extravasation, and to maintain preassures, increased intravasation and by extension fluid, tumor cell and ctDNA intravasation, likely occurred. The median MAF values progressively increased from the negative group (4.17) through the + (11.8), ++ (12.5), and +++ (15.3) groups, highlighting a trend in which higher hemorrhage levels correspond to elevated MAF. The results suggest that hemorrhage, as a marker of vascular leakiness, holds significant biological relevance when stratifying patients based on MAF levels. The distribution of clinical characteristics across the hemorrhage classification groups (ND, -, +, ++, and +++) showed no significant differences in key features such as synchronous disease, liver involvement, and *RAS* mutation status, suggesting a balanced patient distribution and robust study design. Notably, the type of sample analyzed varied considerably, with surgical specimens dominating the + and +++ groups, while biopsies were more prevalent in the ND groups. The higher prevalence of biopsies in the ND groups could be an explanation to not be able to determine the hemorrhage in tissue, as tissue in biopsies normally is underrepresented.

The results are based on a cohort of 56 patients with “extreme cases”, with patients with hepatic disease and high MAF and patients with low MAF. These interesting results must be confirmed in a validation cohort independent of tumor site and MAF value.

7.5. Limitations of the study

While our study provides valuable insights, several limitations must be acknowledged. The observational nature of the study and the absence of randomization between treatment groups may introduce significant biases, such as selection bias, which could impact the interpretation of our findings. Additionally, the cohorts were selected based on real-world clinical practice, which exhibited high heterogeneity among patients. This variability may have further biased our results.

A notable bias in the first-line cohort is the selection of *RAS*-mutated patients who did not receive the combination of chemotherapy and antiangiogenic treatments. Early in the cohort selection process, the limited use of this combination could be attributed to restricted access to biologic-specific treatments and the recent approval of such combinations. The choice of *RAS*-mutated patients who were treated exclusively with chemotherapy, likely due to their fragility or ineligibility for antiangiogenic treatments, introduces a significant bias that may have skewed the results of this study. Given the study's primary objective to explore the predictive role of ctDNA in determining the response to antiangiogenic therapies, and considering its observational nature, this specific population was the only viable option. Additionally, due to the distinct molecular biology of *RAS*wt patients and the potential introduction of another treatment variable, such as EGFR inhibitors, we chose not to include them in the analysis.

For the second-line cohort, our focus was on patients treated with antiangiogenics, which introduced another form of bias. By restricting the cohort to second-line treatments that included antiangiogenics, we achieved homogeneity but excluded patients in third-line or refractory settings, potentially introducing further bias. Moreover, the underrepresentation of *RAS*wt patients, the exclusion of those treated with EGFR inhibitors, and the lack of analysis of ctDNA dynamics in this population may have also contributed to selection bias.

These limitations should be considered when interpreting the results, though the primary biomarker objectives of the study and its observational nature help contextualize these constraints. The relatively small sample size in the second-line and TAS-102/TAS-102+bevacizumab cohorts, particularly in subgroup analyses, may limit the statistical power of our conclusions.

It is also important to note that while we quantified ctDNA using MAF values, other quantitative methods, such as measuring mean tumor molecules per milliliter (MTM/mL), might provide different perspectives and additional quantitative value. Methodologically, the heterogeneity in MAF acquisition between digital PCR and next-generation sequencing (NGS) could have introduced bias due to the differing sensitivity and specificity of these techniques, as the limit of detection (LOD) and the minimum cfDNA required for detection differ between them.

Another significant limitation, particularly concerning the biomarker-focused analysis, is the lack of a validation cohort. However, we are actively working to establish a validation cohort with patients from Hospital General Universitario Gregorio Marañón to confirm the predictive value of MAF for antiangiogenic treatments in first-line patients.

The correlation between MAF and hemorrhage in tissue appears evident. However, this cohort consists of patients with either high MAF and liver disease or low MAF and no liver involvement, representing an “extreme” first cohort. Therefore, these findings require validation in a broader cohort, independent of MAF values and metastatic site, which is currently underway**.** Additionally, the assumption that tissue hemorrhage serves as a surrogate marker for high extravasation pressure—and, consequently, high intravasation pressure leading to ctDNA shedding—requires further confirmation through mechanistic studies in dynamic in vivo models.

CONCLUSIONS

8. CONCLUSIONS

- **The MAF value in ctDNA of driver genes may be a promising predictive biomarker of response to first-line bevacizumab in metastatic colorectal cancer patients. However, these results must be confirmed in an independent cohort.**

- **The MAF value in ctDNA of driver genes is an independent prognostic factor in first-line treatment, retaining significance even after adjusting for other prognostic variables such as liver disease or metastasectomy.**

- **The MAF value in ctDNA of driver genes is an independent prognostic factor across all treatment lines, including later lines with TAS-102 or TAS-102 plus bevacizumab.**

- **Median MAF values vary across treatment lines—6.5% in first line, 2.1% in second, and 9.1% in third or beyond—reflecting changes in tumor burden, treatment effects, and the evolving biology of mCRC.**

FUTURE DIRECTIONS

9. FUTURE DIRECTIONS

Although immediate clinical applications are constrained by the limitations discussed, the findings have generated important hypotheses and highlighted the need for a multimodal approach in biomarker development. The research from this thesis is expected to inspire and inform future studies in the field. Building on the conclusions of this project, we are pursuing several immediate future directions with three main objectives: validating our clinical results from the first-line cohort in an external validation cohort, increasing the number of patients analyzed in subsequent lines, and enhancing our understanding of shedding mechanisms through translational research.

- **Validation Cohort**: We are establishing a validation cohort to confirm the predictive value of driver MAF in ctDNA for antiangiogenic treatment. Samples from this cohort are being sent to our VHIO genomic facility for analysis to verify the findings as a predictive biomarker (Hospital Gregorio Marañon).
- **FFPE Sample Analysis**: We are continuing to analyze formalin-fixed paraffin-embedded (FFPE) samples with the pathology consortium at ICO-Bellvitge to confirm the results observed in this first "extreme" cohort evaluated.
- **Patient-Derived Xenograft (PDX) Models**: We are developing patient-derived xenograft (PDX) animal models to investigate the mechanisms underlying the ctDNA shedding observed clinically. These models are being characterized for shedding levels and treated with or without bevacizumab. We aim to explore transcriptional features using RNA sequencing (RNA-seq) to identify genes related to shedding. Preliminary analyses suggest that RNA-seq data on vascular density and network quality may determine the tumor's ability to shed ctDNA into the bloodstream.
- **CtDNA Baseline Characterization**: We are continuing to collect baseline ctDNA characterization for each patient treated at our center to increase the dataset and improve characterization across diverse cohorts.

From a practical day-to-day clinical perspective, these results, based on retrospective non-randomized data, cannot currently be used to decide on the addition of bevacizumab in the first line of treatment. The OS outcomes observed across different lines of therapy could serve as an indication, alongside other known clinical prognostic markers, to redefine the therapeutic strategy for patients.

The next ideal step for bringing driver-gene MAF in ctDNA into clinical practice as a predictive biomarker would be to validate these findings in a phase II clinical trial. When designing biomarker-guided trials, it is crucial to carefully formulate research questions. A key consideration will be patient selection, potentially including first-line mCRC patients who are not candidates for EGFR inhibitors (e.g., RAS-mutated or right-sided RAS wild-type patients) and who have no contraindications for antiangiogenic treatment. Two main options for trial design include: (1) randomizing patients based on MAF ctDNA levels (e.g., patients with MAF > 5.8% randomized to receive bevacizumab, while those with low MAF are randomized to not receive it) or (2) randomizing patients into ctDNA-guided versus non-ctDNA-guided treatment groups. However, clinical trial design should be deferred until the findings are validated in an external cohort.

The results of this PhD project open promising avenues for further research into predictive biomarkers of antiangiogenic therapies in mCRC and raise new questions, paving the way for future tralsational projects focused on understanding the dynamics of ctDNA shedding, which should be explored and validated through mechanistic in vivo models.

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SUPPLEMENTARY DATA

11. SUPLEMENTARY DATA

Supplementary Table 1: *RAS* panel of mutations for BEAMing analysis

Gene	Exon	Mutation
KRAS	2	G12S/R/C/D/A/V
	2	G13D
	3	A59T
	3	Q61L/H/R
	4	K117N
	4	A146T/V
NRAS	2	G12S/R/C/D/A/V
	2	G13D/R/V
	3	A59T
	3	Q61K/L/R/H
	4	K117N
	4	A164T

Supplementary Table 2: Genes on the VHIO360 Panel. All exons are sequenced in some genes; only clinically significant exons are sequenced in other genes

AKT1	*ALK #*	*APC*	*AR **	*ARAF*	*ARIDIA*	*ATM*	*BRAF**	*BRCA1*
BRCA2	*CCND1 **	*CCND2 **	*CCNE1 **	*CDH1*	*CDK12*	*CDK4 **	*CDK6 **	*CDKNZA*
CTNNB1	*DDR2*	*EGFR**	*ERBB2 **	*ESR1*	*EZH2*	*FBXW7*	*FGFR1 **	*FGFR2 #**
FGFR3 #	*GATA3*	*GNA11*	*GNAQ*	*GNAS*	*HNFIA*	*HRAS*	*IDH1*	*IDH2*
JAK2	*JAK3*	*KIT **	*KRAS **	*MAP2K1*	*MAP2K2*	*MAPK1*	*MAPK3*	*MET **
MLH1	*MPL*	*MTOR*	*MYC **	*NF1*	*NFE2L2*	*NOTCH1*	*NPM1*	*NRAS*
NTRK1 #	*NTRK3*	*PDGFRA **	*PIK3CA **	*PTEN*	*PTPN11*	*RAF1**	*RB1*	*RET #*
RHEB	*RHOA*	*RIT1*	*ROS1 #*	*SMADA*	*SMO*	*STK11*	*TERT §*	*TP53*
TSC1	*VHL*							

§ VHIO360 reports alterations in the promoter region of this gene
# VHIO360 reports fusions events involving this gene
* VHIO360 reports amplifications of this gene

Supplementary Table 3: Sensitivity and specificity for Guardant360 depending on mutant allele fraction (MAF)

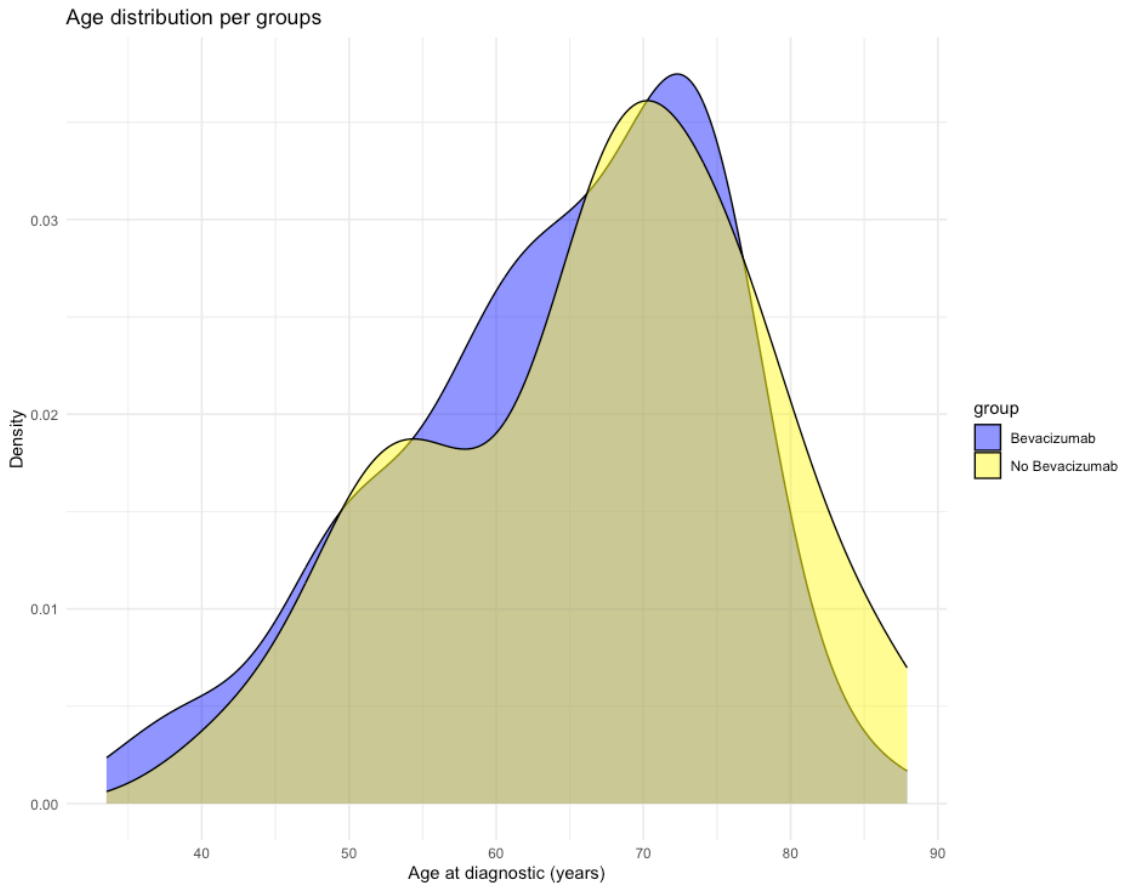
	Sensitivity		**Specificity**	
Mutant allele fraction	**>0,5%**	**0,1-0,5%**	**>0,5%**	**0,1-0,5%**
SNVs	**>0,94**	0.81	1	1
Indels	**>0,95**	0.69	1	1

Supplementary Table 4: Distribution of Non-Shedder patients (n=34)

	Bevacizumab	**No Bevacizumab**
***RAS*mut**	19	6
***RA*swt**	3	6

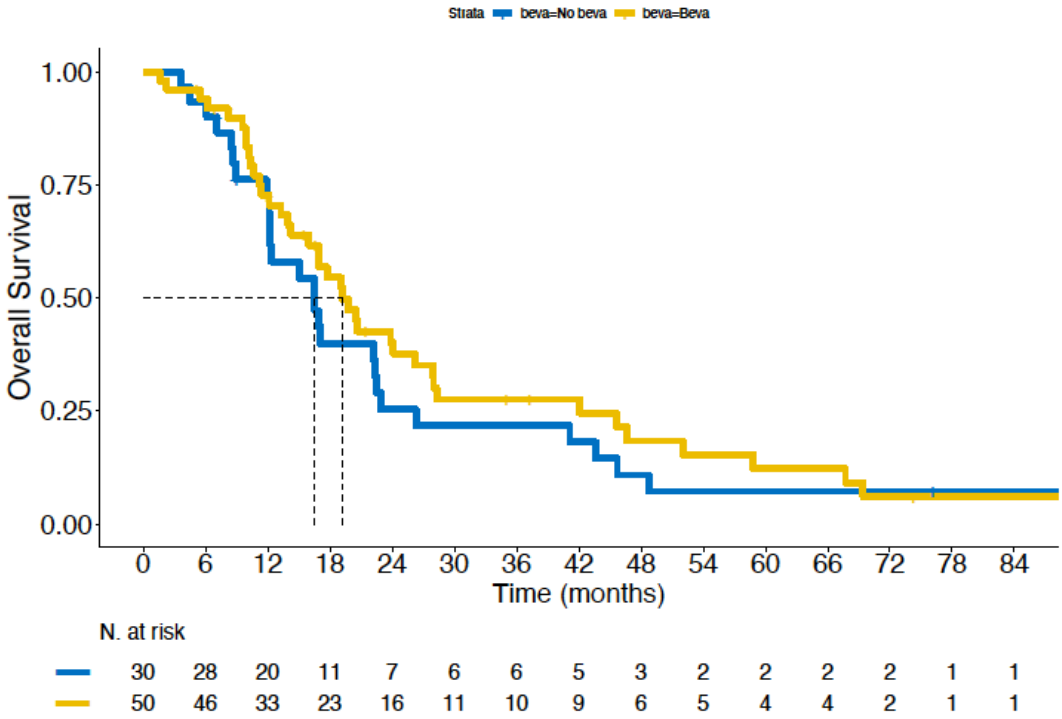
Supplementary Table 5: Localisation metastatic site(s)

	Overall population (n=185)	Bevacizumab population (n=117)	No bevacizumab population (n-=68)
Liver	77 (41.62%)	43 (36.75%)	34 (50%)
Lung	11 (5.95%)	9 (7.69%)	2 (2.94%)
Nodes	6 (3.24%)	3 (2.56%)	3 (4.41%)
Peritoneal	9 (4.86%)	4 (3.42%)	5 (7.35%)
Other	4 (2.16%)	3 (2.56%)	1 (1.47%)
Liver, Lung, Peritoneal	2 (1.08%)	2 (1.71%)	0 (0%)
Liver, Nodes	6 (3.24%)	3 (2.56%)	3 (4.41%)
Liver, Lung	31 (16.76%)	23 (19.66%)	8 (11.76%)
Liver, Nodes, Other	2 (1.08%)	2 (1.71%)	0 (0%)
Liver, Lung, Nodes	7 (3.78%)	6 (5.13%)	1 (1.47%)
Liver, Nodes, Peritoneal	3 (1.62%)	3 (2.56%)	0 (0%)
Lung, Other	1 (0.54%)	1 (0.85%)	0 (0%)
Liver, Peritoneal, Other	2 (1.08%)	0 (0%)	2 (2.94%)
Liver, Bones	1 (0.54%)	0 (0%)	1 (1.47%)
Peritoneal, Other	3 (1.62%)	2 (1.71%)	1 (1.47%)
Liver, Peritoneal	6 (3.24%)	3 (2.56%)	3 (4.41%)
Liver, Other	2 (1.08%)	2 (1.71%)	0 (0%)
Lung, Bones	1 (0.54%)	1 (0.85%)	0 (0%)
Lung, Nodes	5 (2.7%)	4 (3.42%)	1 (1.47%)
Liver, Lung, Nodes, Peritoneal	1 (0.54%)	0 (0%)	1 (1.47%)
Lung, Peritoneal	1 (0.54%)	0 (0%)	1 (1.47%)
Liver, Nodes, Bones, Other	1 (0.54%)	1 (0.85%)	0 (0%)
Liver, Lung, Bones	1 (0.54%)	1 (0.85%)	0 (0%)
Nodes, Other	1 (0.54%)	1 (0.85%)	0 (0%)
Nodes, Peritoneal	1 (0.54%)	0 (0%)	1 (1.47%)



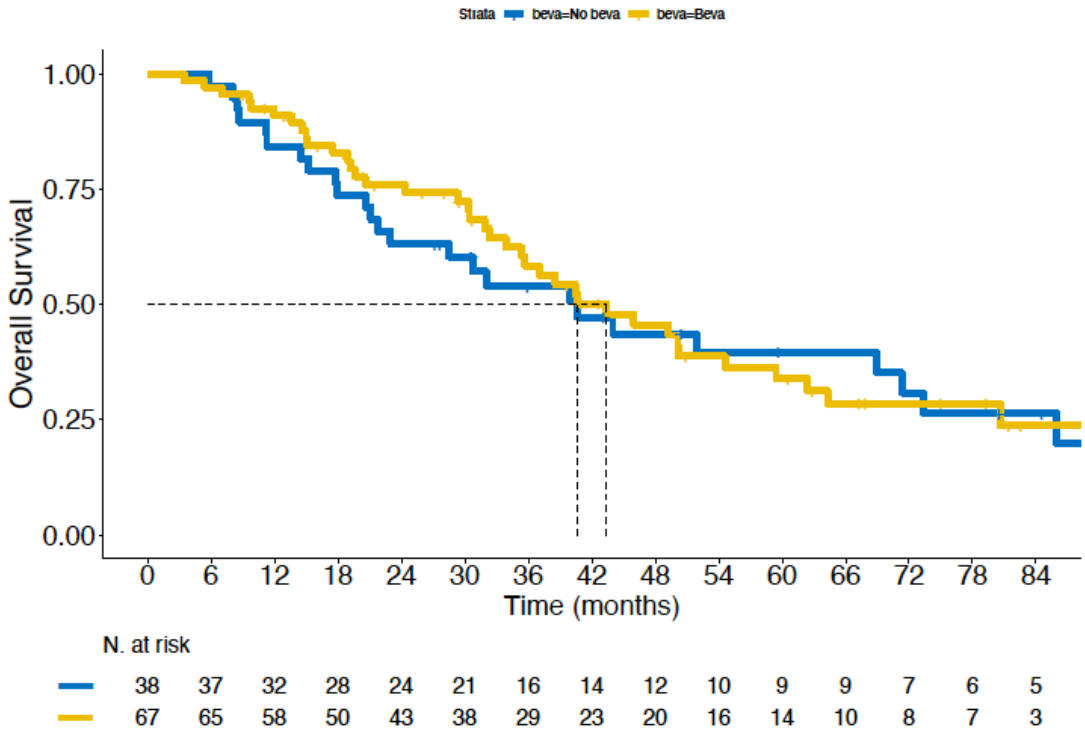
Supplementary Figure 1: Age distribution in the first-line cohort.

Factor	n	median IC95%	HR IC95%	p.value
No beva	30	16.53m [12.16 ; 22.9]	Ref. [Ref. ; Ref.]	-
Beva	50	19.22m [15.93 ; 27.99]	0.8 [0.49 ; 1.32]	0.3843



Supplementary Figure 2: Kaplan-Meier curve for mOS of patients in first-line cohort and high MAF treated with bevacizumab (yellow) or without (blue).

Factor	n	median IC95%	HR IC95%	p.value
No beva	38	40.61m [22.97 ; 73.33]	Ref. [Ref. ; Ref.]	-
Beva	67	43.4m [35.38 ; 62.36]	0.9 [0.55 ; 1.47]	0.661



Supplementary Figure 3: Kaplan-Meier curve for mOS of patients in first-line cohort and low MAF treated with bevacizumab (yellow) or without (blue).