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ORIGINAL ARTICLE

Plasmatic *BRAF*-V600E allele fraction as a prognostic factor in metastatic colorectal cancer treated with BRAF combinatorial treatments

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Background: Combination of a BRAF inhibitor (BRAFi) and an anti-epidermal growth factor receptor (EGFR), with or without a MEK inhibitor (MEKi), improves survival in *BRAF*-V600E-mutant metastatic colorectal cancer (mCRC) over standard chemotherapy. However, responses are heterogeneous and there are no available biomarkers to assess patient prognosis or guide doublet- or triplet-based regimens. In order to better characterize the clinical heterogeneity observed, we assessed the prognostic and predictive role of the plasmatic *BRAF* allele fraction (AF) for these combinations.

Patients and methods: A prospective discovery cohort including 47 *BRAF*-V600E-mutant patients treated with BRAFi −anti-EGFR ± MEKi in clinical trials and real-world practice was evaluated. Results were validated in an independent multicenter cohort ($n = 29$). Plasmatic *BRAF*-V600E AF cut-off at baseline was defined in the discovery cohort with droplet digital PCR (ddPCR). All patients had tissue-confirmed *BRAF*-V600E mutations.

Results: Patients with high AF have major frequency of liver metastases and more metastatic sites. In the discovery cohort, median progression-free survival (PFS) and overall survival (OS) were 4.4 and 10.1 months, respectively. Patients with high *BRAF* AF (≥2%, $n = 23$) showed worse PFS [hazard ratio (HR) 2.97, 95% confidence interval (CI) 1.55-5.69; $P = 0.001$] and worse OS (HR 3.28, 95% CI 1.58-6.81; $P = 0.001$) than low-*BRAF* AF patients (<2%, $n = 24$). In the multivariable analysis, *BRAF* AF levels maintained independent significance. In the validation cohort, high *BRAF* AF was associated with worse PFS (HR 3.83, 95% CI 1.60-9.17; $P = 0.002$) and a trend toward worse OS was observed (HR 1.86, 95% CI 0.80-4.34; $P = 0.15$). An exploratory analysis of predictive value showed that high-BRAF AF patients ($n = 35$) benefited more from triplet therapy than low-BRAF AF patients ($n = 41$; PFS and OS interaction tests, $P < 0.01$).

Conclusions: Plasmatic *BRAF* AF determined by ddPCR is a reliable surrogate of tumor burden and aggressiveness in *BRAF*-V600E-mutant mCRC treated with a BRAFi plus an anti-EGFR with or without a MEKi and identifies patients who may benefit from treatment intensification. Our results warrant further validation of plasmatic BRAF AF to refine clinical stratification and guide treatment strategies.

Key words: colorectal cancer, *BRAF*-V600E mutation, mutant allele fraction, BRAF inhibitor, anti-EGFR, MEK inhibitor

INTRODUCTION

In colorectal cancer (CRC), the *BRAF-600E* mutation occurs in up to 10% of patients.[1-3] It encodes for a serine or threonine-protein kinase associated with mitogen-activated protein kinase (MAPK) pathway activation resulting in cellular proliferation and metastases.[1,4] This mutation is associated with poor prognosis, with median overall survival (OS) of only 11 months, and poor response to standard chemotherapy.[5,6] Unlike in *BRAF*-V600E-mutant melanoma, use of targeted agents in *BRAF*-mutant metastatic CRC (mCRC) did not achieve clinical benefit,[7,8] due to signaling up-regulation via the

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epidermal growth factor receptor (EGFR) with BRAF blockade in monotherapy.[9] This was addressed in subsequent trials by combining an anti-EGFR with the BRAF inhibitor (BRAFi), thereby improving the antitumoral effects. Furthermore, some trials incorporated not only an anti-EGFR but a third drug to enhance clinical activity, including MEK or PI3CA inhibitors.[10,11] The development of targeted therapy for *BRAF*-V600E-mutant mCRC culminated with the BEACON trial.[12] This phase III trial evaluated encorafenib—cetuximab with or without binimetinib versus irinotecan plus cetuximab-based chemotherapy. Confirmed overall response rates (ORRs) were 26.8% for the triplet arm and 19.5% for the doublet arm, versus 1.8% for the control arm, and median OS was 9.3 months for both the triplet and the doublet arms, versus 5.9 months for the control arm.[13] Based on these results and the more favorable toxicity profile of the doublet combination, in May 2020 both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved encorafenib and cetuximab for patients with mCRC whose tumors have the *BRAF*-V600E mutation and who have received at least one prior treatment regimen.

Despite the overall poor prognosis associated with *BRAF* mutations, there is nonetheless a high level of clinical heterogeneity in outcomes, with 10%-20% of patients surviving >2 years whereas 10% of patients survive for <5 months.[12] Various prognostic scores have been developed, based on clinical and pathological data, identifying specific subgroups with relevant differences in life expectancy.[14-16] This clinical heterogeneity has been confirmed in two transcriptomic signatures. Among the four consensus molecular subtypes (CMS) based on gene expression patterns, *BRAF* mutations tend to be more prevalent in the CMS1 immune subtype.[17] Furthermore, two transcriptional subtypes of *BRAF*-V600E-mutant CRC (BM1 and BM2) have been described, with the BM1 subtype associated with a poorer prognosis than the BM2 subtype.[2] Nevertheless, logistically, transcriptomic signatures cannot be carried out easily in routine clinical practice as they require a large amount of tumor tissue, are expensive, and have high turnaround times and turnover requirements. In several tumor types, the allele fraction (AF) of specific mutations such as *BRAF* or *KRAS* has been associated with clinical outcomes; the higher the AF the worse the prognosis.[18-22] The prognostic and predictive value of *BRAF*-mutant AF has been evaluated in previous studies in patients with CRC. Nonetheless, these cohorts included a small number of patients with *BRAF*-V600E-mutant tumors and they were heterogeneously treated.[23-25] However, a prospectively validated AF cut-off has not been established. We therefore aimed to evaluate whether plasmatic *BRAF* AF determined by droplet digital PCR (ddPCR) can be used to improve our understanding of the prognosis of *BRAF*-V600E-mutant mCRC patients treated with BRAFi-based combination therapy.

MATERIALS AND METHODS

Patient population

This retrospective study was approved by the institutional review board or independent ethics committee at each center and was conducted in accordance with the requirements of the regulatory authorities of each country. Consecutive patients with *BRAF*-V600E-mutated mCRC treated with BRAFi—anti-EGFR ± MEK inhibitor (MEKi) at the Vall d'Hebron University Hospital between 2015 and 2020 were evaluated in a discovery set. The Consolidated Standards of Reporting Trials (CONSORT) diagram of patients included in the discovery set is represented in Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2023.02.016. The external validation set included data from consecutive eligible patients treated between 2018 and 2021 in three additional centers from Italy and Spain. Eligible patients had a tissue-confirmed *BRAF*-V600E mutation [by ddPCR or next-generation sequencing (NGS)], available clinicopathological data, and an available plasma sample before initiating treatment with a BRAFi-based combination. Clinicopathological data, treatment type, response, and survival outcomes were collected from patient medical records. Baseline tissue (or plasma if no tissue was available) samples were analyzed by NGS (FoundationOne, Guardant360). Evaluated genomic alterations include: *KRAS*, *NRAS*, *GNAS*, *ARAF*, *PTEN*, *ERBB2*, *EGFR*, *MAP2K1*, and *AKT1* mutations and *MET*, *BRAF*, *EGFR*, and *IGF1R* amplifications.

Droplet digital PCR

Analysis of *BRAF*-V600E mutation in the baseline plasma sample was carried out by ddPCR using a Custom TaqMan SNP genotyping assay. PCR primers and TaqMan probes (FAM or VIC-labeled) were obtained from Thermo Fisher Scientific. Cell-free DNA (cfDNA) extraction was carried out with the QIAamp Circulating Nucleic Acid Kit according to the manufacturer's instructions (QIAGEN Inc.). The final concentration was measured using a Qubit dsDNA HS assay kit and the Qubit 4.0 fluorometer (Life Technologies) following the manufacturer's instructions. Primers (900 nM) and probes (250 nM) [AF for the mutant and VIC for the wild-type (WT) allele] were mixed with 2× Droplet PCR Supermix (Thermo Fisher Scientific) to a final volume of 12 µl. Eight microliters of undiluted cfDNA (equivalent to cfDNA from 1.5 ml of plasma) was used for the ddPCR reaction. Each assay was carried out in triplicate in independent mixes and loaded on to different wells for amplification. The 20-µl reaction mixture was applied to the QX200 Droplet Generator Cartridge (Bio-Rad Laboratories, Hercules, CA) with 70 µl of mineral oil to form droplets in ~35 µl of oil-in-water mixture. The mixture was transferred to a 96-well PCR plate and heat-sealed. The plate was placed in a C1000 Touch thermal cycler (Bio-Rad Laboratories) and amplified to the endpoint PCR. Thermal cycling conditions were 95°C ×10 min (1 cycle), 94°C for 30 s (1 cycle), 57°C ×1 min (40 cycles), and 98°C ×10 min (1 cycle), with a ramp rate of 2°C per second.

After PCR, the 96-well PCR plate was read on a QX-200 Droplet Reader (Bio-Rad Laboratories). The data were analyzed with Quantalife software. Briefly, a threshold line was drawn for channel 1 and channel 2 to separate the two

clusters of negative and positive droplets, respectively. The threshold line for positive droplets was determined by the control samples. Since samples were retrospective and limited plasma was available for analysis, we set a cut-off of a minimum 100 events per replicate in the sample to call a genotype and corresponding AF (*BRAF*-V600E WT or mutant).

Statistical analysis

Progression-free survival (PFS) was defined as the time from anti-BRAF therapy initiation to disease progression or death, whichever occurred first. OS was defined as the time from anti-BRAF therapy initiation to death from any cause. PFS and OS were estimated using the Kaplan−Meier method. Univariable Cox proportional hazards models were used to obtain hazard ratios (HRs) with 95% confidence intervals (CIs). To select variables with the highest prognostic impact for OS, we carried out a least absolute shrinkage and selection operator (LASSO) regression using package 'glmnet' in R software to build the most parsimonious multivariable model. Since there is not an optimal BRAF AF cut-off using ddPCR (previous studies used NGS), all possible cut-offs to dichotomize BRAF AF were explored (in terms of OS HRs and the lower 95% CI) to better understand the behavior of the biomarker and to select a clinically relevant cut-off to stratify patients'. To improve the clinical utility, all possible cut-offs to dichotomize *BRAF* AF were explored. To assess the predictive capacity of *BRAF* AF, interaction tests were used to estimate if the benefit of doublet or triplet therapy differed according to *BRAF* AF group. No data imputation was carried out. The threshold for statistical significance was defined as 0.05 (two-sided). All statistical analyses were carried out using R statistical software.

RESULTS

A total of 76 *BRAF*-V600E mCRC patients were included in this study, 47 patients in the discovery cohort and 29 in the validation cohort. Table 1 presents the patient characteristics overall and according to AF levels ($\geq$2% versus $<$2%). In the discovery and validation cohorts, 49% and 41% of the patients had high baseline *BRAF* AF, respectively, based on median AF in the discovery cohort. In both the discovery and the validation cohorts, patients with high AF presented more frequently with Eastern Cooperative Oncology Group (ECOG) performance status (PS) $\geq$2 (17% versus 0% and 25% versus 0%; $P = 0.004$), $\geq$2 tumor sites (43% versus 29% and 67% versus 24%; $P = 0.05$), liver metastasis (78% versus 17% and 59% versus 41%; $P < 0.001$), and had a higher rate of progressive disease as the best response to BRAFi combinations (30% versus 0% and 50% versus 18%) compared with patients with low AF, respectively. Regarding treatment, 70% and 72% of patients received doublet therapy in the discovery and validation cohorts, respectively. To evaluate whether the presence of subclonal co-mutations is associated with clinical outcomes on targeted therapy, we analyzed baseline NGS in 35/47 (75%) and 29/29 (100%) patients in the discovery and the validation cohorts, respectively. In the discovery cohort the most frequent genomic alterations at baseline were *PTEN* mutation (5.7%), *AKT1* mutation (2.9%), *KRAS* amplification (2.9%), and *EGFR* amplification (2.9%), whereas in the validation cohort, the most frequent alterations were *GNAS* mutation (14%), *MET* amplification (6.9%), *KRAS* mutation (3.4%), *ARAF* mutation (3.4%), *ERBB2* mutation (3.4%), and *EGFR* mutation (3.4%). The complete analysis of subclonal mutations can be found in Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2023.02.016.

Discovery cohort

In the discovery cohort, median OS was 10.1 months (95% CI 6.5-17.5 months) and median PFS was 4.4 months (95% CI 3.6-8.0 months) with a median follow-up of 24.1 months (Figure 1A). Complete or partial response was observed in 30% of the patients, while 80% achieved disease control (complete or partial response or stable disease). As a continuous variable, *BRAF* AF baseline values were associated with OS (HR 10.27, 95% CI 1.79-59.1; $P = 0.009$) and PFS (HR 8.79, 95% CI 1.52-50.7; $P = 0.02$). To better stratify patients in clinical practice, different cut-offs to dichotomize *BRAF* AF were explored. No relevant differences in the lower 95% CI estimation for OS HR were obtained using cut-offs from 0% to 20% (Figure 1B). Based on this, the median value of the *BRAF* AF score in this cohort (2%) was selected as the cut-off for group stratification. Plasma with undetectable *BRAF* mutation was included in the low-*BRAF* AF group. OS outcomes were significantly worse in patients with high *BRAF* AF ($\geq$2%, $n = 23$), with a median OS of 4.6 months, while patients with low *BRAF* AF ($\leq$2%, $n = 24$) had a median OS of 17.5 months (HR 3.28, 95% CI 1.58-6.81; $P = 0.001$). Median PFS was 3.3 months for high-*BRAF* AF patients and 8.3 months for low-*BRAF* AF patients (HR 2.97, 95% CI 1.55-5.69; $P = 0.001$) (Figure 1C and D).

In the univariable analysis, several clinicopathological factors including ECOG PS, number of metastatic sites, liver metastasis, carcinoembryonic antigen (CEA) levels, and albumin levels were associated with OS (Figure 2). Among them, ECOG PS, number of metastatic sites, CEA levels, and *BRAF* AF ($\geq$2% versus $<$2%; HR 4.74, 95% CI 1.52-14.81; $P = 0.008$) maintained statistical significance for OS in the multivariable analysis. Consequently, *BRAF* AF baseline levels maintained their independent statistical significance after adjusting for clinicopathological factors.

Validation cohort

Findings from the discovery cohort were validated in an external independent cohort of 29 *BRAF*-V600E-mutant mCRC patients treated with a BRAFi plus an anti-EGFR with or without a MEKi. Median OS in the validation cohort was 7.3 months (95% CI 6.3-11.3 months) and median PFS was 4.8 months (95% CI 4.0-6.4 months) with a median follow-up of 21.8 months. Complete or partial response was observed in 13% of the patients, and disease control was achieved in 59%. The median *BRAF*-V600E AF value was

Table 1. Baseline characteristics of patients for the discovery and validation cohorts, according to AF levels

Discovery cohort, *n* = 47 (%)	**Overall**	**AF >2%, *n* = 23**	**AF <2%, *n* = 24**
Sex, *n* (%)			
Male	19 (40)	9 (39)	10 (42)
Female	28 (60)	14 (61)	14 (58)
Age (years, range)	61 (33-79)	62 (33-79)	61 (33-79)
ECOG, *n* (%)			
0	23 (49)	8 (35)	15 (63)
1	20 (41)	11 (48)	9 (37)
>2	4 (10)	4 (17)	0
Sidedness, *n* (%)			
Right	30 (64)	15 (65)	15 (63)
Left	17 (36)	8 (35)	9 (37)
CEA (ng/ml, range)	6.3 (0.5-7025)	8.85 (1.2-7025)	4.8 (0.5-1579)
NLR (range)	3.4 (1-16.14)	4.3 (1-16.14)	3.29 (1.26-6.15)
Albumin (g/dl, range)	3.9 (2.8-4.4)	3.7 (2.8-4.4)	4 (2.9-4.4)
LDH (UI/l, range)	331 (144-1501)	380 (202-1501)	320 (144-1126)
MSI, *n* (%)			
MSI	8 (17)	4 (18)	4 (17)
MSS	36 (77)	18 (78)	18 (75)
NA	3 (6)	1 (4)	2 (8)
Previous lines, *n* (%)			
1	22 (47)	13 (57)	9 (38)
2	19 (40)	9 (39)	10 (42)
>2	6 (13)	1 (4)	5 (20)
Tumor sites, *n* (%)			
1	13 (28)	3 (14)	10 (42)
2	17 (36)	10 (43)	7 (29)
>2	17 (36)	10 (43)	7 (29)
Liver metastases, *n* (%)	20 (43)	18 (78)	4 (17)
BOR, *n* (%)			
CR	1 (2)	0	1 (4)
PR	13 (28)	7 (30)	6 (25)
SD	24 (51)	8 (36)	16 (67)
PD	7 (15)	7 (30)	0
NA	2 (4)	1 (4)	1 (4)
BRAF inhibitor combination, *n* (%)			
Doublet	33 (70)	16 (70)	17 (71)
Encorafenib—cetuximab	26 (55)	11 (48)	15 (62)
Vemurafenib—cetuximab	6 (13)	4 (18)	2 (9)
Vemurafenib—irinotecan—cetuximab	1 (2)	1 (4)	0
Triplet	14 (28)	7 (30)	7 (29)
Encorafenib—binimetinib—cetuximab	14 (28)	7 (30)	7 (29)
Validation cohort, n = 29 (%)	**Overall**	**AF >2%, n = 12**	**AF <2%, n = 17**
Sex, *n* (%)			
Male	12 (41)	4 (33)	8 (47)
Female	17 (59)	8 (67)	9 (53)
Age (years, range)	68 (36-82)	64 (36-82)	68 (38-81)
ECOG, *n* (%)			
0	10 (34)	3 (25)	7 (41)
1	16 (55)	6 (50)	10 (59)
>2	3 (11)	3 (25)	0
Sidedness, *n* (%)			
Right	18 (62)	7 (58)	11 (65)
Left	11 (38)	5 (42)	6 (35)
CEA (ng/ml, range)	7.05 (1.7-1489)	6.9 (1.26-1489)	7.2 (1.7-348)
NLR (range)	2.1 (0.28-15)	2.39 (0.8-9.57)	1.82 (0.28-8.1)
Albumin (g/dl, range)	4.4 (3.4-4.8)	4.4 (3.4-4.6)	4.1 (3.8-4.8)
LDH (UI/l, range)	220 (132-796)	290 (190-581)	192 (132-796)
MSI, *n* (%)			
MSI	4 (14)	1 (8)	3 (18)
MSS	25 (86)	11 (92)	14 (82)
NA	0	0	0
Previous lines, *n* (%)			
1	17 (59)	9 (75)	2 (12)
2	9 (31)	1 (8)	6 (35)
>2	3 (10)	2 (17)	9 (53)
Tumor sites, *n* (%)			
1	9 (31)	3 (25)	6 (35)
2	8 (28)	1 (8)	7 (41)

Continued

Table 1. Continued

Validation cohort, n = 29 (%)	Overall	AF >2%, n = 12	AF <2%, n = 17
>2	12 (41)	8 (67)	4 (24)
Liver metastases, *n* (%)	14 (48)	7 (59)	7 (41)
BOR, *n* (%)			
CR	1 (3)	0	1 (5)
PR	3(10)	3 (25)	0
SD	13 (46)	3 (25)	10 (59)
PD	9 (31)	6 (50)	3 (18)
NA	3 (10)	0	3 (18)
BRAF inhibitor combination, *n* (%)			
Doublet	21 (72)	9 (75)	12 (71)
Encorafenib—cetuximab	21 (72)	9 (75)	12 (71)
Vemurafenib—cetuximab	0	0	0
Vemurafenib—irinotecan—cetuximab	0	0	0
Triplet	8 (38)	3 (25)	5 (29)
Encorafenib—binimetinib—cetuximab	8 (38)	3 (25)	5 (29)

AF, allele fraction; BOR, best overall response; CR, complete response; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; MSI, microsatellite instability; MSS, microsatellite stability; NA, not available; PD, progression disease; PR, partial response; PS, performance status; SD, stable disease.

0.9% (interquartile range 0.1%-5.0%), and using the predefined cut-off of 2%, 41% of patients were classified as high AF ($n = 12$) and 59% as low AF ($n = 17$). Consistent with the results obtained in the discovery cohort, high AF was significantly associated with worse PFS (HR 3.83, 95% CI 1.60-9.17; $P = 0.003$) (Figure 3A). The median OS of 6.4 months in high-AF patients was numerically lower than in patients with low AF (8.8 months), showing a trend toward worse OS in the high-AF group, albeit not statistically significant (HR 1.86, 95% CI 0.80-4.34; $P = 0.15$) (Figure 3B). The association between *BRAF*-V600E AF and survival outcomes showed similar results after adjusting for MAPK subclonal co-mutation status (Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2023.02.016). Because of the low number of microsatellite instable (MSI) patients, PFS and OS in this specific population were evaluated mixing both cohorts. Among MSI patients ($n = 12$, 16%), those with low AF ($n = 7$) had better PFS (6.4 versus 2.3 months) and OS (16.7 versus 2.6 months) than MSI patients with high AF ($n = 5$).

BRAF AF as a potential predictive factor for benefit with triplet therapy

We next evaluated the potential role of liquid biopsy to identify a subgroup of patients that could benefit from triplet combination including a MEKi. We used the pooled cohort ($n = 76$) to estimate the benefit of the doublet and triplet combinations in patients with low and high *BRAF* AF levels. In the OS analysis, patients with low *BRAF* AF ($n = 41$) showed similar outcomes with the triplet and the doublet treatment (HR 0.90, 95% CI 0.38-2.14). However, high-*BRAF* AF patients ($n = 35$) obtained better OS outcomes with the triplet combination compared to the doublet (HR 0.17, 95% CI 0.06-0.53; interaction test = 0.002) (Figure 4A). Similarly, no PFS differences were observed regarding the treatment combination in low-*BRAF* AF patients (HR 1.12, 95% CI 0.47-2.68) but a greater benefit from the triplet was observed in high-*BRAF* AF patients (HR 0.27, 95% CI 0.11-0.68; interaction test = 0.005) (Figure 4B). Kaplan—Meier curves are presented in Supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2023.02.016.

DISCUSSION

Unlike the success observed in treating *BRAF*-V600E-mutant melanoma with BRAFi as single agents, the path toward successful targeted blockade in *BRAF*-V600E-mutant mCRC has proved considerably more challenging. It has taken some time since the disappointing results with the BRAFi vemurafenib treatment in BRAF-mutant mCRC[7] to identify the EGFR loop as the main mechanism of resistance,[9,26] along with the subsequent successful development of the doublet and triplet BRAFi combinations. The BEACON trial is the largest phase III trial enrolling patients with *BRAF*-V600E mCRC worldwide. Based on the results of this trial, the encorafenib—cetuximab doublet became the new standard of care for patients with refractory mCRC harboring a *BRAF*-V600E mutation. Nonetheless, the high degree of heterogeneity in clinical responses[12] raised the need to identify prognostic and predictive biomarkers that optimize the clinical management of patients, which included assessing the prognostic role of *BRAF* AF in cfDNA. Although the prognostic significance of *BRAF* AF has been assessed in plasma and tissue samples in two other previous cohorts that included colorectal tumors, the number of patients with *BRAF*-V600E colon cancer in these cohorts was limited, with only five and six patients, respectively.[23,24] A retrospective correlative biomarker study using data from the BEACON trial demonstrated longer OS among patients with low AF compared with those with high AF, by treatment arm (14.8 versus 7.2 months, 14.8 versus 5.4 months, and 9.3 versus 4.2 months for the triplet, doublet, and chemotherapy arms, with low AF and high AF, respectively).[13] However, these results come from a highly selected clinical trial population, which does not accurately reflect the real-world population given that only patients considered fit enough are enrolled in a clinical trial. The correlative analyses from the BEACON trial using circulating

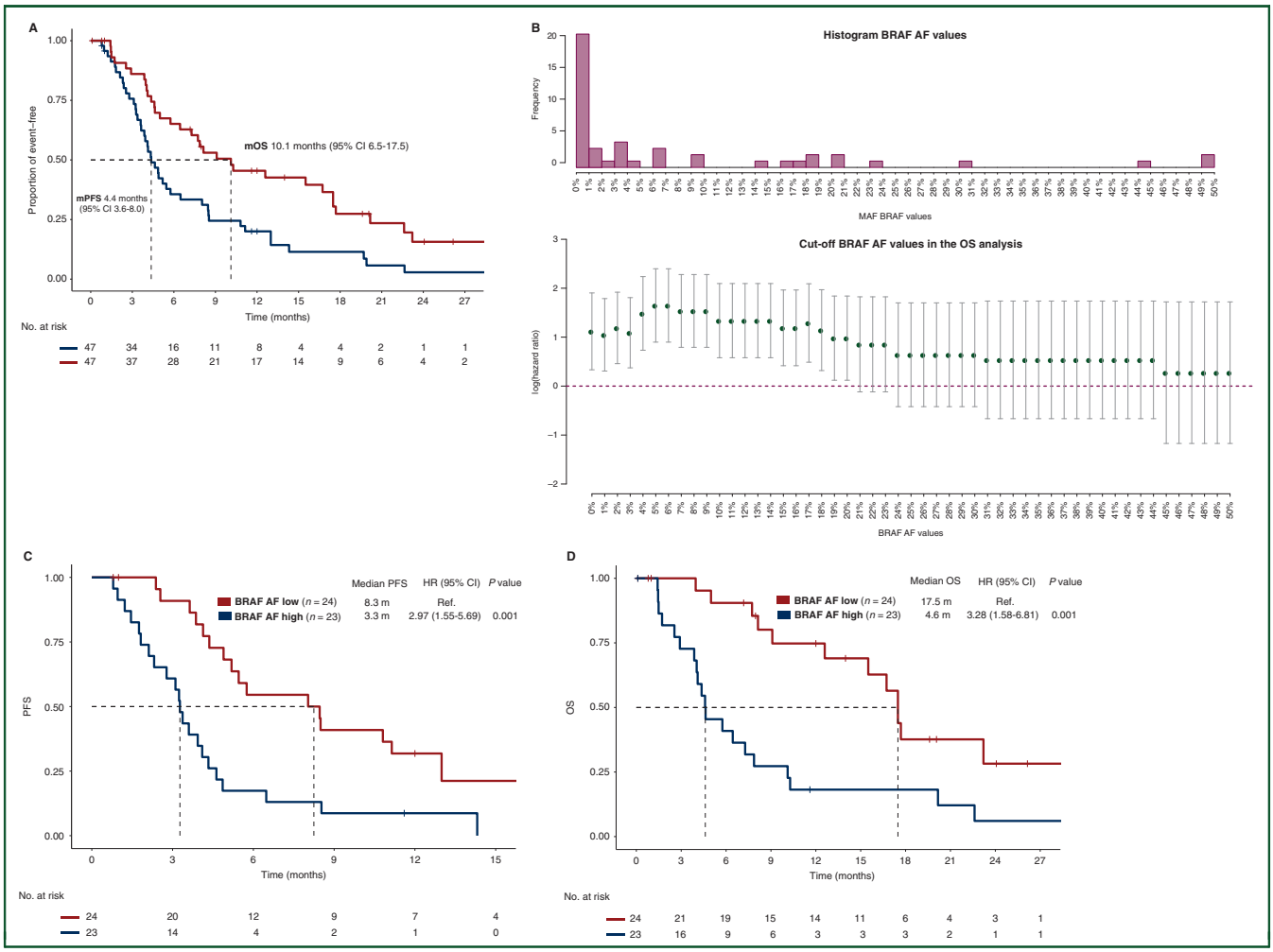

Figure 1. Survival outcomes in the discovery cohort according to *BRAF* AF. (A) Kaplan—Meier estimations of overall survival (OS) and progression-free survival (PFS) in the overall population. (B) Histogram with the distribution of *BRAF* AF values (upper), and dichotomization of BRAF AF values using all potential cut-offs and the impact on the association with OS by means of the HR and 95% CI (lower). (C) PFS according to the *BRAF* AF cut-off. (D) OS according to the *BRAF* AF cut-off. BRAF AF, BRAF allele fraction; CI, confidence interval; HR, hazard ratio; MAF, mutant allele frequency; mOS, median OS; mPFS, median PFS.

tumor DNA genomic profiling (GuardantOMNI) demonstrated the prognostic role of *BRAF* AF.[13] In all treatment arms, OS was significantly longer in patients with low *BRAF* AF (<2%) than in those with high AF ($P < 0.0001$). Patients in the triplet and doublet arms had improved ORR and OS regardless of *BRAF* AF, compared with those in the chemotherapy arm. In our cohort, clinical outcomes were similar to those reported in the BEACON trial, with a median PFS of 4.4 months and a median OS of 10.1 months in the overall cohort. Our study was carried out using ddPCR, which is highly sensitive and easy to implement in routine clinical practice in the real-world setting. Although NGS offers many advantages and will likely be widely adopted in the mid to long term, we consider it important to validate a technique that could easily fill in the gap during the coming years and in settings where NGS will be difficult to implement.

Of note, our cohort includes not only patients from clinical trials (42% of the cohort) but also patients who received BRAFi combinations as compassionate use (48% of the cohort). In both of our cohorts (discovery and validation), patients with high AF tended to have worse PS, were more heavily pretreated, had more metastatic sites including more frequent liver involvement, and poorer response to the BRAFi combination than patients with low AF, suggesting that plasmatic *BRAF* AF might be a surrogate of tumor load with potential prognostic value. Among patients from the discovery cohort, those patients with low AF had significantly longer OS than those with high AF. In the validation cohort, the same tendency was observed; however, it did not reach statistical significance.

These results suggest that plasmatic *BRAF* AF has a survival impact and can be used to identify potentially longer survivors. While our analysis did not validate the results in the validation cohort in terms of OS, there is a clear tendency toward shorter OS among patients with high AF in our population. The added value of our study is the use of the reliable and highly reproducible technique ddPCR. Furthermore, patients were also included outside the context of a clinical trial, suggesting that this approach may be of interest in a real-world population.

Although the doublet combination was approved by both FDA and EMA, data suggest that selected patients can benefit from the addition of a MEKi. In the BEACON trial, to

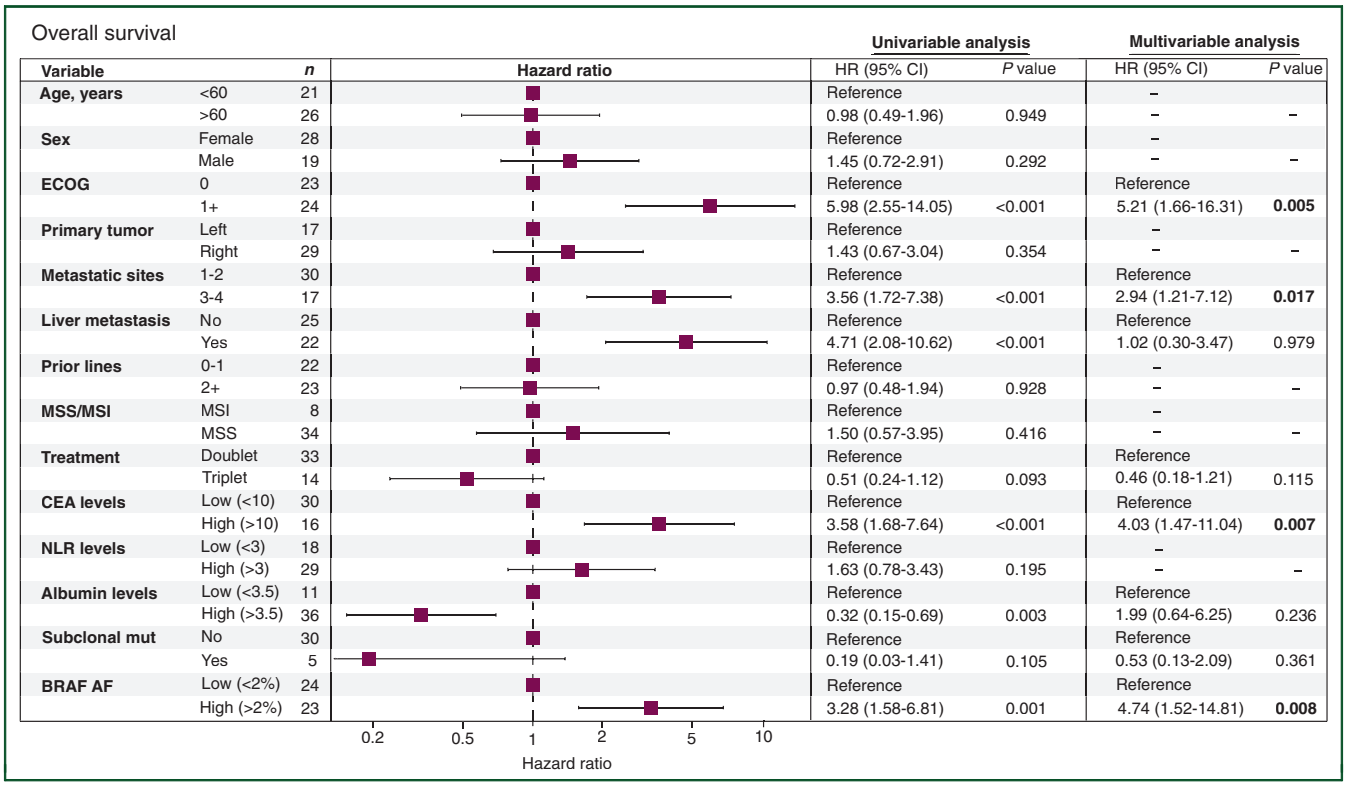

Figure 2. Univariable and multivariable Cox models to evaluate the association between clinicopathological factors and overall survival in the discovery cohort ($n = 47$).
BRAF AF, BRAF allele fraction; CEA, carcinoembryonic antigen; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stability; NLR, neutrophil-to-lymphocyte ratio.

identify molecular correlates of clinical outcome, tissue samples were analyzed using whole-exome sequencing and whole-transcriptome sequencing.[27] In their study, OS according to genetic subtype (CMS or BM classifications)[2,17] was similar between the doublet and the triplet arms. However, a trend favoring treatment with the triplet was observed in CMS4 and BM1 tumors (HR 0.73, 95% CI 0.44-1.21 and HR 0.71, 95% CI 0.43-1.19, respectively). ORR was higher in the triplet arm versus the doublet arm for CMS4 and BM1 tumor groups (33.3% versus 19.2% and 33.3% versus 14.9%, respectively). Regarding liquid biopsy results, ORR was higher among patients from the BEACON trial, independent of *BRAF* AF, in both the triplet and doublet arms compared with the chemotherapy arm. However, in the pooled cohort of our study, among 14 patients treated with the triplet therapy, a longer OS was seen for patients with high AF (HR 0.17, 95% CI 0.06-0.53) compared with patients with low AF (HR 0.90, 95% CI 0.38-2.14) suggesting that there is a subgroup of *BRAF*-mutant tumors that may obtain benefit from the triplet.

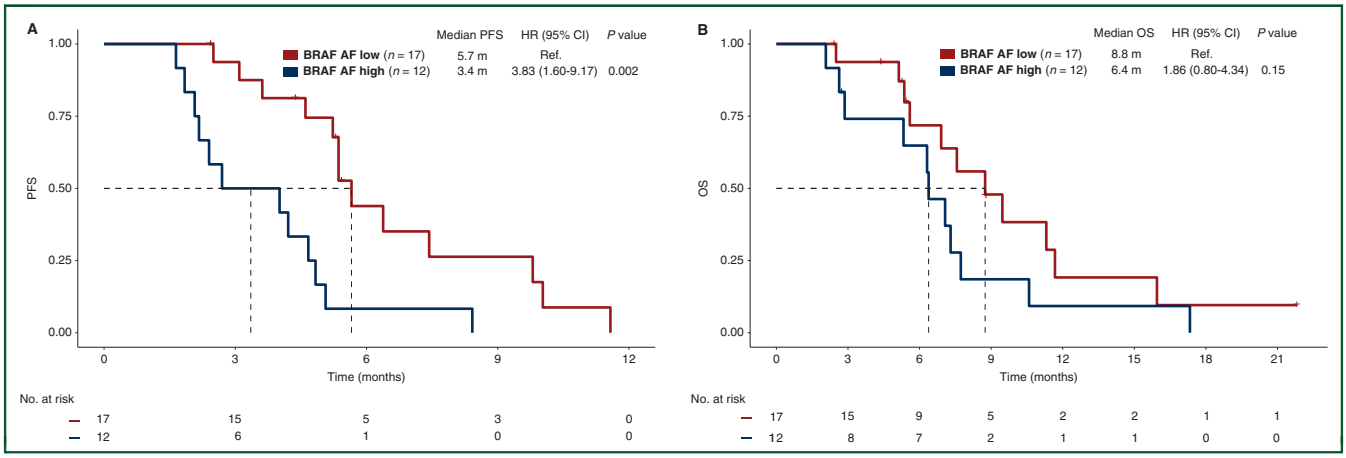

Figure 3. Progression-free survival (PFS) by pre-defined *BRAF* AF groups (A) and overall survival (OS) by pre-defined *BRAF* AF groups (B) in the validation cohort ($n = 29$).
BRAF AF, BRAF allele fraction; CI, confidence interval; HR, hazard ratio.

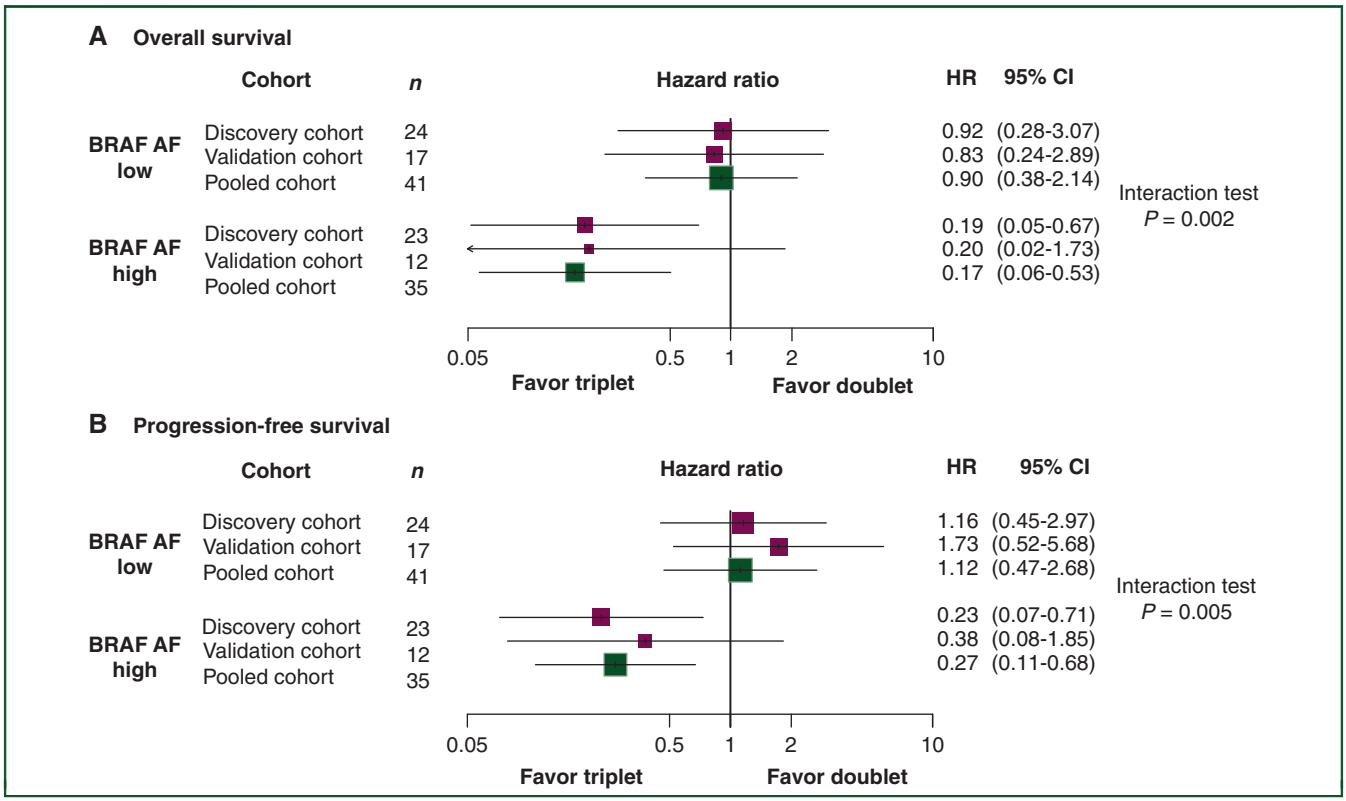

Figure 4. Forest plots evaluating the efficacy of doublet versus triplet combination therapy in patients with low and high *BRAF* AF levels in overall survival (A) and progression-free survival (B). Interaction test was calculated to quantify whether the magnitude of benefit with the triplet combination therapy was different regarding the *BRAF* AF groups.
BRAF AF, BRAF allele fraction; HR, hazard ratio.

Finally, patients with *BRAF*-V600E mCRC receiving BRAFi-based combinations may present many different alterations in the MAPK pathway leading to acquired resistance.[28,29] Previous evidence demonstrated that some *BRAF*-V600E colorectal tumors may harbor subclonal MAPK pathway alterations detectable at trace levels before treatment that likely are responsible for the development of acquired resistance.[30] To confirm the prognostic value of *BRAF* AF regardless of the presence of those co-mutations, we carried out a baseline tissue NGS and found that subclonal mutation status was not associated with survival outcomes, but more importantly, *BRAF*-V600E AF remained statistically significant in the multivariable analysis after adjusting for these subclonal mutations.

The main limitations of our study were the relatively small sample size of the validation cohort and the retrospective nature of the study. Differences between the two cohorts due to the modest sample size should also be taken into consideration, such as a higher number of previous lines and metastatic sites in the validation cohort. Nevertheless, the baseline clinical characteristics did not impact the validation of the prognostic and potentially predictive value of the *BRAF* AF. Furthermore, 17% and 14% of the patients presented with microsatellite instability and 3% and 6% of them received subsequent immunotherapy in the discovery and validation cohorts, respectively. These patients have longer OS compared with their *BRAF*-mutant microsatellite stable counterparts, potentially hampering interpretation of the OS results. Nevertheless, the low frequency of patients with microsatellite instability in these two cohorts would not affect the prognostic concept of the *BRAF* AF.

The identification of robust prognostic factors in *BRAF*-mutant mCRC is of critical importance to guide treatment strategies and modulate patient expectations. Moreover, the current study suggests the existence of a subgroup of patients that can potentially obtain benefit from more intensive treatment including a MEKi. Overall, our results highlight the utility of plasmatic *BRAF* AF using ddPCR, particularly considering the difficulties of obtaining tissue samples in this specific population. Furthermore, the incorporation of plasmatic *BRAF* AF data could support better patient stratification in prospective clinical trials. Prospective validation in a large cohort is needed.

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