Journal of Translational Medicine

RESEARCH

Open Access

High BRAF variant allele frequency predicts poor outcomes in metastatic melanoma patients treated with BRAF/MEK inhibitors

Michele Guida[1], Benedetta Apollonio[1,24*], Luca Romano[2], Francesco Spagnolo[3,4], Pietro Quaglino[5], Roberta Depenni[6], Rosamaria Pinto[7], Teresa Squicciarini[1], Livia Fucci[8], Piergiorgio Di Tullio[9], Maria Chiara Scaini[10], Maria Teresa Maccallini[11], Alice Indini[12], Teresa Troiani[13], Iole Natalicchio[9], Sonia Brugnara[14], Maurizio Lombardo[15], Cristina Pellegrini[16], Paola Queirolo[17], Fabiana Perrone[18], Alessandro Minisini[19], Marco Tucci[20], Raffaele Conca[21], Silvia Costabile[2], Miriam Macrì[2], Enrica Tanda[3,4], Elena Croce[3,4], Rebecca Senetta[5], Paolo Fava[5], Giuseppe Pugliese[6], Stefania Pellegrini[10], Elisa Melucci[22], Michele Del Vecchio[12], Francesco Caraglia[13], Salvatore Girlando[23], Simona De Summa[7†], Sabino Strippoli[1†] and on behalf of the Italian Melanoma Intergroup (IMI)

Abstract

Background BRAF/MEK inhibitors have improved the outcome in metastatic melanoma (MM) patients harboring a BRAF mutation, but no biomarker predictive of response has been identified.

Methods We conducted a retrospective analysis on 264 MM patients that had received first-line targeted therapy with BRAF/MEK inhibitors. Next-generation sequencing (NGS) was performed on tissue biopsies, and samples with > 30% tumor cellularity were included in the study. The impact of BRAF variant allele frequency (BRAF-VAF) on clinical treatment outcomes was analyzed.

Results BRAF-VAF was dichotomized using two approaches. (1) The "surv_cutpoint" function identified two different cut-off for progression-free survival (PFS: 44.05%) and overall survival (OS:45.1%). Patients with BRAF-VAF > 44.05% showed a significantly lower PFS (median PFS: 10 months, 95% CI: 7–13 months), compared to patients with BRAF-VAF < 44.05% (median PFS: 13 months, 95% CI: 12–21 months). Moreover, patients with higher VAF (> 45.1%) experienced a lower OS (median OS: 26 months, 95% CI: 19–38 months), compared with patients with VAF < 45.1% (median OS: 29 months, 95% CI: 29–51 months). (2) The ROC analysis significantly predicted PFS but not OS. BRAF-VAF normalized with neoplastic cellularity (nVAF) showed a strong association with both PFS, and OS compared to BRAF-VAF alone. nVAF also emerged as an independent predictor for PFS in the multivariate analysis (HR: 3.88, 95% CI: 1.84–8.20), with a higher nVAF score associated with a 3.88-fold increased risk of progression.

†Simona De Summaa and Sabino Strippoli contributed equally to this work.

* These authors equally contributed to this work.

*Correspondence:
Benedetta Apollonio
b.apollonio@oncologico.bari.it

Full list of author information is available at the end of the article

Conclusions Our study demonstrated the role of the BRAF-VAF as predictor of response in MM patients treated with BRAF/MEK inhibitors. Moreover, VAF normalization predicts PFS better than BRAF-VAF alone.

Highlights

- The role of BRAF-VAF in predicting response to BRAF/MEK inhibitors therapy in melanoma has not been elucidated yet.
- In 264 metastatic melanoma patients treated with first-line targeted therapy, high BRAF-VAF values correlated with worse clinical outcomes.
- This evidence is further strengthened when BRAF-VAF was normalized using neoplastic cellularity (nVAF).
- BRAF-VAF can be used as predictor of clinical outcomes in metastatic melanoma patients treated with first-line targeted therapy.

Keywords Melanoma, BRAF mutation, Variant allele frequency (VAF), BRAF/MEK target therapy

Background

About 50% of melanomas carry a somatic mutation of the BRAF gene, encoding for a serine-threonine kinase involved in the MAPK pathway (BRAF/MEK/ERK). The BRAF V600E pathogenic variant is detected in up to 90% of the BRAF mutated melanomas, along with other rarer variants in the same codon (V600K, V600D, V600R, and V600M), or in the surrounding amino acid residues. Notably, BRAF mutation represents a negative independent prognostic factor in early and advanced treatment-naive melanomas [1, 2], and predicts the activity and efficacy of BRAF inhibitors (BRAFi) combined with MEK inhibitors (MEKi).

Approval of BRAFi/MEKi has dramatically increased the response rate and survival of BRAF-mutated metastatic melanoma (MM) patients. However, although response is observed in about 80% of MM patients, the median response duration is about 11–13 months, with approximately 30% of patients achieving a full response and long-term benefits [3, 4]. Moreover, the majority of patients experience disease relapse when treatment is stopped [5].

Also, modern immunotherapy with checkpoint inhibitors as a single agent or in combination has greatly improved the survival of many patients with MM regardless of the BRAF mutational status [6–11].

The major advantage of immunotherapy lies in its durable, long-term benefits that can persist even after treatment discontinuation. In contrast, targeted therapy is characterized rapid clinical responses with symptoms improvement and high response rates. Given these complementary strengths, combining or sequencing the two approaches was expected to enhance outcomes in patients with BRAF-mutated melanoma. Preclinical studies also supported the potential for synergistic effects, demonstrating that activation of the MAPK pathway promotes an immunosuppressive tumor microenvironment that can be reversed by BRAF inhibition [12]. However, clinical trials evaluating combined targeted and immune therapy in the first-line setting have not demonstrated a significant survival advantage. For this reason, other studies have been addressing whether different therapy sequence was essential to improve outcomes. Two randomized phase II studies, SECOMBIT and DREAMseq, explored this question. Both trials demonstrated superior efficacy with first-line ICI therapy, while the SECOMBIT study further showed that the "sandwich" approach—initial targeted therapy followed by immunotherapy and subsequent targeted therapy—yields comparable results [13]. Collectively, these findings support initiating combination treatment with immunotherapy as the most effective first-line strategy for BRAF mutant melanoma patients.

Accurately predicting patient response, especially for BRAF-mutated patients, remains a critical and unmet need. New tools that forecast long-term response are needed to guide optimal treatment selection (targeted vs. immunotherapy) and potentially improve patient outcomes. For example, different clinical responses to targeted therapy could be partially explained by inter-patient heterogeneity and tumor sub-clonality [14, 15]. Variant allele frequency (VAF), which measures the proportion of mutated alleles to total alleles in the examined cells, has emerged as a potential factor influencing clinical and pathological features, as well as predicting the response to targeted therapies [16]. Nevertheless, currently available data are still controversial and come from small and heterogeneous melanoma cohorts. Moreover, these studies are often affected by the presence of numerous biases, including the assessment of BRAF status on primary tumor or metastatic disease, different BRAF pathogenic variants (V600E vs. non-V600E), multiple techniques used to identify BRAF mutations (pyrosequencing, Real Time-PCR, NGS), and treatment used (BRAFi alone or combined with MEKi) [17–21]. Recent data demonstrated that high BRAF-VAF is an independent poor prognostic factor in MM receiving BRAFi and MEKi [22].

To validate this hypothesis, we evaluated BRAF-VAF in a large retrospective cohort of MM patients treated

with first-line BRAFi/MEKi. To obtain more robust and reliable data, we selected only cases in which BRAF mutations were assessed by Next-Generation Sequencing (NGS). We correlated BRAF-VAF with melanoma features, patient characteristics, and clinical outcomes to targeted therapy. Normalizing allele frequency to tumor cellularity is essential for accurately interpreting somatic mutations in cancer genomics. Tumor samples often consist of a mixture of tumor and normal cells, and this heterogeneity directly impacts the observed variant allele frequencies. Without normalization, clonal mutations can appear subclonal, and zygosity or copy number alterations may be misinterpreted. Several studies have emphasized this point. Failure to normalize can lead to misinterpretations of clonality and copy number alterations. Techniques such as All-FIT [23], CLONETv2 [24], and PUREE [25] show that accounting for tumor purity improves the accuracy of downstream analyses, including ploidy, mutation burden, and gene expression. Overall, integrating tumor purity into VAF normalization enhances the reliability of downstream analyses such as clonal architecture inference, mutation timing, and therapeutic response prediction [26]. Thus, we further developed a normalized BRAF-VAF score (nVAF), incorporating both BRAF-VAF and the percentage of neoplastic cells analyzed, to address the potential influence of the number of tumor cells on BRAF-VAF data reliability.

Materials and methods

Patient characteristics

264 MM patients were identified by inspecting the databases of 18 referral melanoma Centers belonging to the Italian Melanoma Intergroup (IMI) from 15 July 2013 to 22 September 2021. All patients had stage IV MM and were treated with first-line MAPKi. Information on age, gender, histopathology, surgical and medical treatments was retrieved for each patient, as well as data on tumor objective response rate (ORR), progression-free survival (PFS), and overall survival (OS). Data on treatment and survival were collected prospectively. Patients without sufficient pathological and clinical data, life expectancy < 3 months, no measurable lesions according to RECIST v1.1 criteria, and with no evaluation of response were excluded from the analysis. Tissue quality was reviewed by pathologists to confirm histopathological classification and tumor cellularity.

The tumor stage was assessed according to the American Joint Committee on Cancer (AJCC) TNM (Tumor, Node, Metastasis) staging system classification (VIII edition) [27]. Tumor assessment was carried out by whole body computed tomography (CT) scan at baseline and approximately every 12 weeks until disease progression or death. Clinical response to BRAFi/MEKi was assessed by RECIST v1.1 criteria [28]. The timing of follow-up was similar across all participating centers, according to Italian association of medical oncology (AIOM) guidelines. Four categories were identified based on the response to treatment: progressive disease (PD), stable disease (SD), partial response (PR), or complete response (CR). OS was defined as the time from starting the treatment to death or to the last follow-up, PFS as the interval between the beginning of the therapy to disease progression or death. The local Ethics Committees of all centers approved the study protocol. The study was conducted in accordance with the World Medical Association Declaration of Helsinki.

DNA extraction and *BRAFV600* VAF analysis by next-generation sequencing (NGS)

Archived formalin-fixed, paraffin-embedded (FFPE) melanoma tissue samples (primary tumors or metastasis) with a tumor content ≥ 30% (evaluated by pathologists on hematoxylin-eosin-stained slides) were included in the analysis. BRAF analysis was performed in 92 cases on the primary tumor samples and in 163 on metastatic samples from different sites. All samples underwent the standard diagnostic procedures. Briefly, the tumor area was macroscopically dissected to concentrate tumor tissue. DNA was extracted either manually or automatically. Following the pathologist's assessment of neoplastic cell percentage, all samples underwent macrodissection of the designated area. Samples were sequenced using both liquid capture (Illumina Miseq or NextSeq550) and amplicon-based (Thermofisher S5 or Genexus) platforms, according to the manufacturer's instructions. Somatic mutations were called and annotated using the proprietary software provided by the platform company. A coverage of > 500X was considered, and only variants with an allele frequency > 5% were included in the analysis.

Statistical analysis

Statistical analyses were performed in R v3.1.1, considering results significant when p-value < 0.05. In detail, "survival" and "survminer" packages were used for survival analyses. To identify the best cut-off for BRAF-VAF, the "pROC" R package [29] was used. The optimal threshold was identified with Youden's statistics that identifies the cut-off that maximizes the distance to the identity line. "maxstat" R package was used to run *surv_cutpoint*" function. Such a function was implemented to identify the optimal cutpoint in survival analyses for two groups through Maximally selected rank statistics. Univariate and multivariate analysis were performed through the *coxph* function implemented in "survival" package. Graphs were created using the "ggplot2" package in R [30]. Normalized BRAF-VAF (nVAF) was calculated through the formula proposed by Kanchi et al. [31]

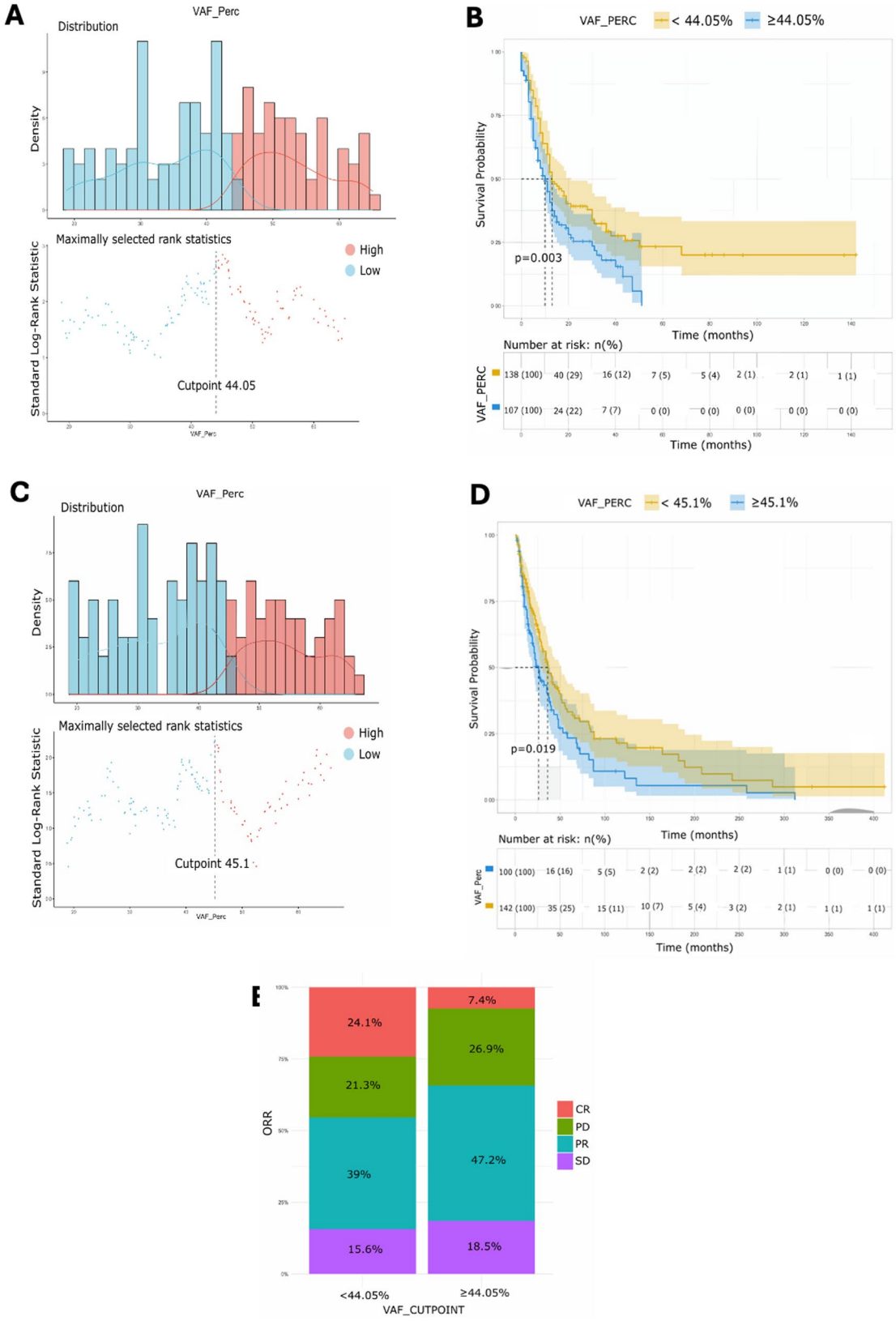

Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 **(A)** Graph showing the "surv_cutpoint" function used to determine an optimal cut-off point for BRAF-VAF that best stratifies patients based on PFS. **(B)** PFS Kaplan-Meier curve comparison with the identified BRAF-VAF cut-point of 44.05% ($p = 0.0033$). **(C)** Graph showing the "surv_cutpoint" function used to determine an optimal cut-off point for BRAF-VAF that best stratifies patients based on OS. **(D)** OS Kaplan-Meier curve comparison with the identified BRAF-VAF cut-point of 45.1% ($p = 0.019$). **(E)** ORR of patients dichotomized into high vs. low BRAF-VAF according to the 44.05% cut-off point identified in (**A**). CR: complete response, PD: progressive disease, PR: partial response, SD: stable disease

$$nVAF = \frac{tVAF - (1 - Pt) * normVAF}{Pt}$$

Where:

- tVAF is the tumoral BRAF-VAF;
- normVAF is BRAF-VAF in the normal tissue.
- Pt represents the neoplastic cellularity.

Considering that BRAF alterations are not present in the normal tissue, nVAF= $\frac{tVAF}{Pt}$.

Results

Cohort description and univariate analysis

Samples were selected according to the workflow described in Supplementary Fig. 1. All the 264 patients had MM (23.5% stage IVM1a, 12% stage IVM1b, 36% stage IVM1c, and 20.5% stage IVM1d), and were treated with first-line MEK + BRAF inhibitors, with a median follow-up of 24 months (range: 1–41 months). BRAFV600E was detected in 81.4% of cases, while other rarer variants were detected in the remaining 18.6%. Among the analyzed patients, 19.3% experienced PD, 18.6% SD, 43.9% PR, and 17.4% CR. The mean PFS was 16.1 months, and the mean OS was 39.4 months. Additional features are reported in Table S1.

First, we performed univariate Cox hazard regression analyses for both PFS and OS. Normal LDH level, metastatic stage M1a-b, and a better ECOG performance status (0–1 vs. 2–3) were associated with significantly longer PFS, while the occurrence of toxicities during targeted therapy was associated with a longer PFS. Regarding OS, increased age at diagnosis, normal LDH level, histological source for BRAF-VAF evaluation (metastasis vs. primary), and M1a-b stage were instead associated with a significantly longer OS. All the statistically significant clinical features were considered in the multivariate analyses (Table S2).

BRAF variant allelic frequency and clinical outcomes

We first investigated whether BRAF-VAF could predict clinical outcomes to target therapy. BRAF-VAF as a continuous variable exhibited limited predictive power for patient outcomes (Supplementary Fig. 2A, B). Therefore, we explored its potential as a categorical variable. We employed two approaches to identify BRAF-VAF cut-offs that could stratify patients and predict clinical outcomes.

First, we utilized the "surv_cutpoint" function to determine an optimal cut-off point. This analysis revealed a BRAF-VAF cutoff of 44.05% that best stratified patients based on PFS. In detail, patients with BRAF-VAF < 44.05% had a longer median PFS (13 months, 95% CI: 12–21) than patients with BRAF-VAF > 44.05% (median PFS: 10 months, 95% CI: 7–13) (Fig. 1a, b). Similarly, a cut-point was identified for OS. Patients with BRAF-VAF ≥ 45.1% had a shorter median OS of 26 months (95% CI: 19–38 months) compared to a median OS of 29 months (95% CI: 29–51 months) for patients with BRAF-VAF < 45.1% (p-value = 0.019) (Fig. 1c, d). Analysis of the overall response rate to targeted therapy revealed a significantly higher percentage of complete responses (CR) in patients with BRAF-VAF below the threshold of 44.05% (CR rate: 24.1%) compared to those with higher BRAF-VAF (CR rate: 7.4%, p-value = 0.006). (Fig. 1e).

Our second approach employed ROC analysis. In this analytical design, the aim was the identification of the best BRAF-VAF cut-off to stratify patients in terms of response (responder (CR + PR) versus non-responder (PD + SD)). The estimated cut-off for BRAF-VAF according to ROC analysis was 41.35%. Univariate and multivariate analysis indicated VAF ≥ 41.35% as independent risk factor for progression (univariate: HR: 1.47, 95% CI: 1.08–2.01, multivariate: HR: 1.65, 95% CI: 1.14–2.40) (Fig. 2a). In contrast, OS did not exhibit a statistically significant association with BRAF-VAF in both univariate (HR: 1.32; 95% CI: 0.97–1.79) and multivariate analysis (HR:1.08, 95%CI:0.72–1.61) (Fig. 2b).

When the ROC-identified cut-off was used to generate Kaplan-Meier curves, patients with VAF < 41.35% displayed a significantly improved median PFS compared to those with higher BRAF-VAF (13 months vs. 11 months, respectively; 95%CI:12–28 vs. 95%CI: 8–13, p-value = 0.013) (Fig. 3a).

Patients with lower BRAF-VAF showed only a trend towards improved OS (median OS: 40 months (95%CI: 29–52) vs. 27 months (95%CI: 22–37), $p = 0.08$) (Fig. 3b). ORR analysis showed that patients with VAF < 41.35% displayed higher complete response rates (26.5% vs. 7.8%, Fig. 3c).

Interestingly, the same trends for PFS were observed for the BRAF mutation subtype V600K alone (Supplementary Fig. 3a, b).

In our cohort, BRAF-VAF was assessed on both primary tumor and metastatic tissue. Interestingly, neither surv_cutpoint nor ROC analysis identified a correlation

A

Variable		N	Hazard ratio	p
LDH	<ULN	88	Reference	
	>2xULN	28	2.26 (1.36, 3.75)	0.002
	>ULN	63	1.59 (1.06, 2.38)	0.025
ECOG	0-1	159	Reference	
	2_3	20	2.34 (1.32, 4.17)	0.004
VAFroc	<41.35%	88	Reference	
	>=41.35%	91	1.65 (1.14, 2.40)	0.008
Toxicities	no	118	Reference	
	yes	61	0.47 (0.31, 0.72)	<0.001
Metastatic_Stage	M1a/M1b	53	Reference	
	M1c/M1d	126	1.79 (1.15, 2.80)	0.010

B

Variable		N	Hazard ratio	p
Age		158	1.01 (1.00, 1.02)	0.16
Site	head	25	Reference	
	limbs	47	1.10 (0.57, 2.09)	0.78
	torso	79	0.78 (0.43, 1.41)	0.41
	unknown primary	7	2.37 (0.89, 6.31)	0.08
LDH	<ULN	79	Reference	
	>2xULN	23	1.76 (1.02, 3.03)	0.04
	>ULN	56	1.26 (0.80, 1.99)	0.33
Material	metastasis	93	Reference	
	primary	65	1.76 (1.14, 2.73)	0.01
Metastatic_Stage	M1a/M1b	48	Reference	
	M1c/M1d	110	1.52 (0.98, 2.35)	0.06
VAFroc	<41.35%	78	Reference	
	>=41.35%	80	1.08 (0.72, 1.61)	0.71

Fig. 2 Multivariate Cox hazard regression analysis for **(A)** PFS and **(B)** OS considering BRAF-VAF dichotomization using ROC curve (cut-off = 41.35%)

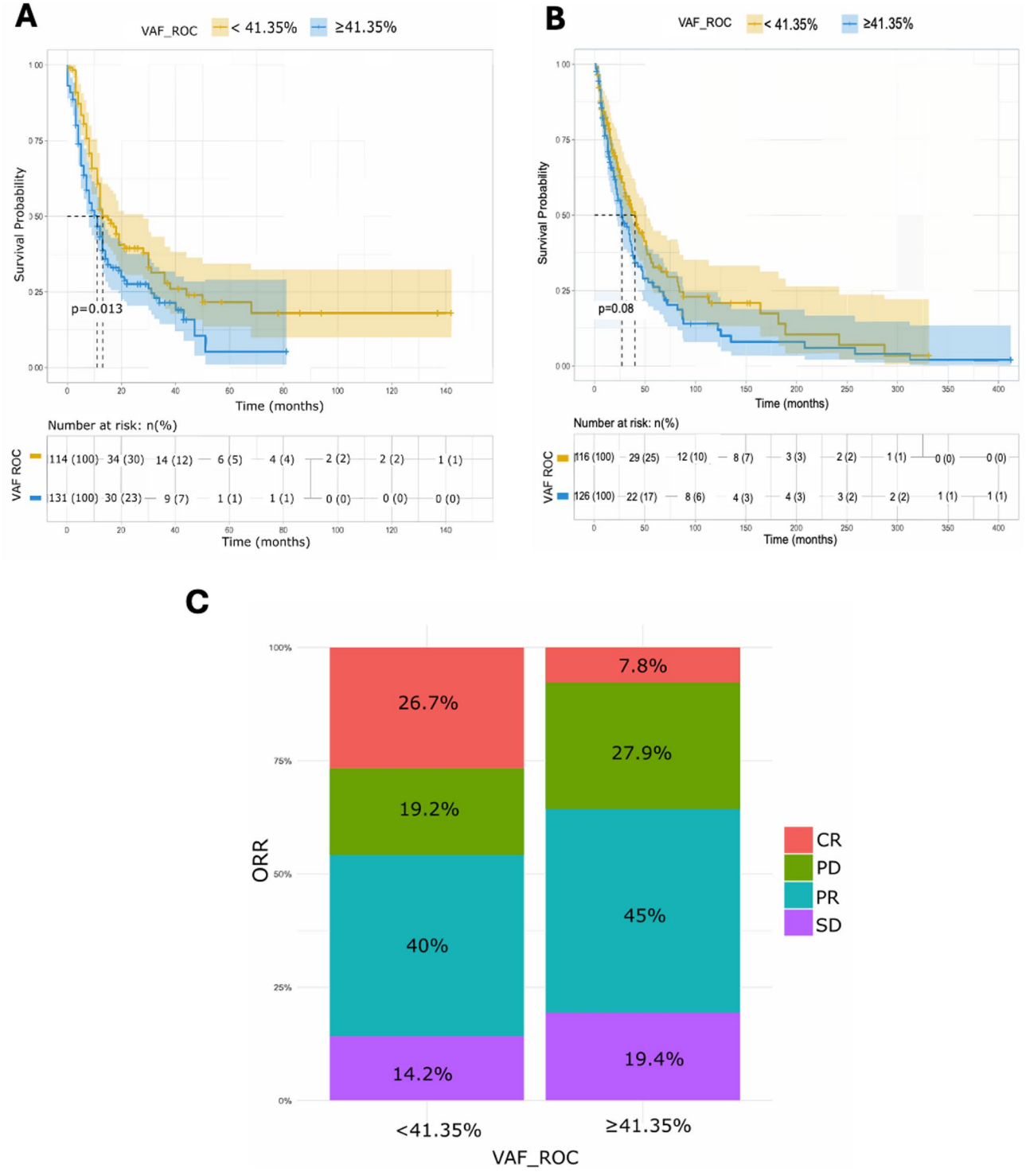

Fig. 3 **A**) Kaplan-Meier survival curves for PFS considering BRAF-VAF dichotomization using ROC curve (cut-off = 41.35%, $p = 0.013$). **(B)** Kaplan-Meier curve comparison for OS considering BRAF-VAF dichotomization using ROC curve (cut-off = 45.1%, $p = 0.08$) **(C)** ORR of patients dichotomized into high vs. low VAF according to the 45.1% cut-off point identified in (**A**). CR: complete response, PD: progressive disease, PR: partial response, SD: stable disease

between tissue source (primary vs. metastasis) and PFS. However, both analyses revealed a significant association between BRAF-VAF assessed in primary tumor and poorer OS (surv_cutpoint: HR 1.76, $p = 0.01$, Fig. 2b; ROC: HR 1.77, $p = 0.02$, Fig. 4b). This finding is particularly noteworthy because, despite similar response rates to initial treatment (Supplementary Fig. 4a), patients with BRAF-VAF assessed in metastatic tissue received

A

Variable		N	Hazard ratio	p
LDH	<ULN	79	Reference	
	>2xULN	27	2.19 (1.30, 3.70)	0.003
	>ULN	52	1.91 (1.24, 2.95)	0.004
ECOG	0-1	141	Reference	
	2_3	17	1.98 (1.07, 3.67)	0.029
nVAF		158	3.88 (1.84, 8.20)	<0.001
Toxicities	no	107	Reference	
	yes	51	0.42 (0.26, 0.68)	<0.001
Metastatic_Stage	M1a/M1b	49	Reference	
	M1c/M1d	109	1.92 (1.19, 3.09)	0.008

B

Variable		N	Hazard ratio	p
Age		145	1.01 (0.99, 1.02)	0.25
Site	head	24	Reference	
	limbs	44	1.03 (0.55, 1.94)	0.93
	torso	70	0.81 (0.45, 1.46)	0.48
	unknown primary	7	2.42 (0.90, 6.49)	0.08
LDH	<ULN	74	Reference	
	>2xULN	23	1.71 (0.99, 2.98)	0.06
	>ULN	48	1.31 (0.81, 2.12)	0.27
Material	metastasis	85	Reference	
	primary	60	1.77 (1.11, 2.83)	0.02
Metastatic_Stage	M1a/M1b	47	Reference	
	M1c/M1d	98	1.52 (0.98, 2.36)	0.06
nVAF		145	1.19 (0.62, 2.27)	0.60

Fig. 4 Multivariate Cox hazard regression analysis for **(A)** PFS and **(B)** OS considering nVAF

second-line therapy more frequently than those with BRAF-VAF evaluated in primary tumors (Supplementary Fig. 4b). This disparity in access to subsequent therapies suggests a potential explanation for the observed difference in OS.

BRAF-VAF and tumor cellularity relationship

Since low tumor cellularity could lead to BRAF-VAF underestimation [9], we evaluated the correlation between the percentage of cancer cells and BRAF-VAF. To this end, we calculated a normalized BRAF-VAF (nVAF) score as described in the Methods section. Univariate analysis showed a strong association between nVAF and both PFS (HR: 3.29, 95% CI: 1.73–6.26) and OS (HR: 1.61, 95% CI: 1.01–2.59). Interestingly, nVAF had a predictive value also in the multivariate analysis for PFS (HR: 3.88, 95% CI: 1.84–8.20) (Fig. 4a), indicating that a higher nVAF score is associated with an approximately 3.88-fold increased risk of progression. Regarding OS, nVAF showed only a trend in univariate and multivariate analysis (Fig. 4b).

To better understand how nVAF influences patient outcomes, we explored its potential as a continuous predictor for both PFS and OS. This analysis went beyond nVAF alone and considered how other clinical factors like LDH levels, metastatic stage, ECOG performance status, and treatment side effects might affect survival probability within different nVAF ranges.

Specifically, the area under the survival probability curve was split into ten ranges. We then investigated which of these ranges were associated with worse outcomes when combined with the other clinical factors. For PFS, patients with nVAF between 1.71 and 2.79 had a lower chance of survival (Supplementary Fig. 5). This association between nVAF and poorer PFS became even stronger when patients also had elevated LDH levels (Supplementary Fig. 5a), advanced metastatic cancer (M1c/M1d) (Supplementary Fig. 5b), a lower ECOG performance score (2–3) (indicating more limitations in daily activities), or no treatment-related side effects (Supplementary Fig. 5d). Similar trends were observed for OS, with lower survival probability in patients with elevated LDH (Supplementary Fig. 6a), advanced metastatic cancer (M1c/M1d) (Supplementary Fig. 6b), and unknown primary tumors (Supplementary Fig. 6c).

To identify the optimal thresholds for Kaplan-Meier survival curves, we employed the "maxstat" statistical package to analyze nVAF values for both PFS and OS. The analysis identified a cut-point of 0.301 for PFS and 0.643 for OS. Patients with nVAF < 0.301 exhibited a significantly longer median PFS of 30 months (95% CI: 12-NA) compared to patients with nVAF above this cut-off (median PFS: 11 months, 95% CI: 8–12 months, p-value = 0.0044) (Fig. 5a). When the analysis was focused solely on the V600K BRAF mutation, we only observed a trend between nVAF and PFS, though it did not reach statistical significance (Supplementary Fig. 7a).

In addition, patients with nVAF < 0.643 had a significantly higher median OS (p-value = 0.019) of 38 months (95%CI: 34–49 months), while those with nVAF exceeding 0.643 had a median OS of 22 months (95% CI: 16–28 months) (Fig. 5b). Significant associations (p-value = 0.024) were observed between nVAF and therapy response, and patients with lower nVAF experienced higher rates of complete response (Fig. 5c).

Discussion

BRAF-mutant melanomas include heterogeneous tumors that are difficult to treat by targeting a single pathway [32]. While targeted therapies with BRAF and MEK inhibitors initially induce impressive tumor regression in most patients, resistance emerges in over half of the cases, ultimately limiting long-term survival benefits [3, 4].

Many factors may contribute to the great variability in patient outcomes, and the impact of BRAF-VAF on response to targeted therapy remains unclear. While some studies suggest a link between BRAF-VAF and treatment response, the currently available data are scarce, often conflicting, and deriving from small patient cohorts with significant heterogeneity [17–21]. In an exploratory analysis of part 1 COLUMBUS study, baseline *BRAFV600E/K* VAF, performed on cell-free circulating tumor DNA, resulted prognostic for PFS and OS, and correlated with reduced response to encorafenib + binimetinib treatment [4]. Recently, *Mandalà* and colleagues reported a correlation between BRAF-VAF and outcomes to BRAFi/MEKi as front-line therapy in a cohort of 107 BRAFV600 mutated MM patients. They demonstrated that a BRAF-VAF value above the cut-point of 41.3% had a poor prognostic significance for PFS and OS, a higher disease load, and poorer ECOG performance status [22].

We have reported data from the largest population published so far. Our cohort included 264 BRAF-mutated patients treated with BRAF + MEK inhibitors as first-line therapy. To achieve a more homogenous study population, we restricted our analysis to patients whose BRAF mutation was analyzed by NGS and whose tumor samples had at least 30% tumor cellularity. We conducted a comprehensive analysis to investigate the relationship between BRAF-VAF, tumor cellularity, and response to target therapy. While analyzing BRAF-VAF as a continuous variable yielded unpromising results, dichotomizing BRAF-VAF using two distinct methods (surv_cutpoint function and ROC analysis) demonstrated that patients with BRAF-VAF below the identified cut-offs had better PFS, OS, and response to therapy. Our data confirmed that BRAF-VAF is a strong predictor

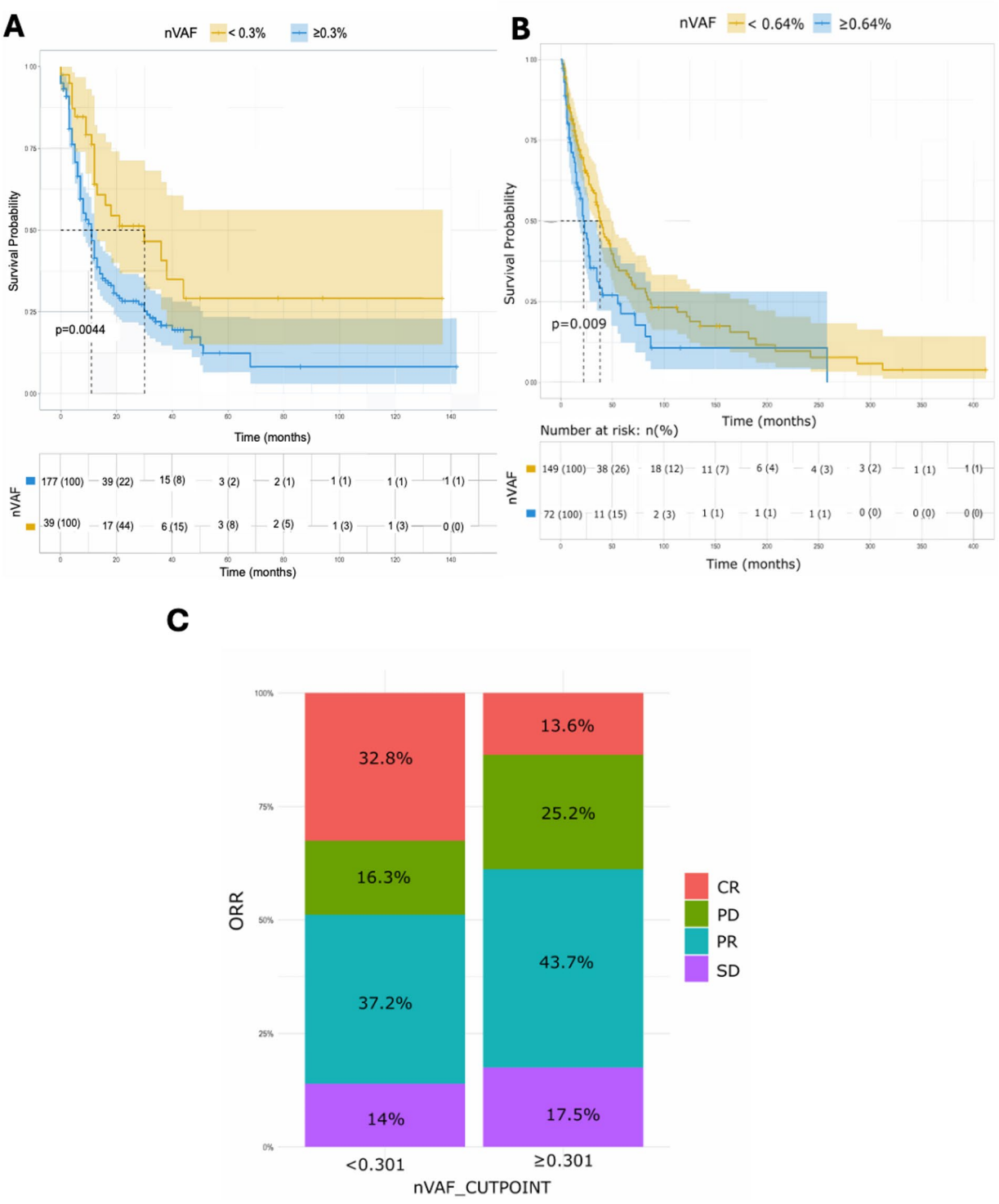

Fig. 5 **A**) Kaplan-Meier survival curves for PFS considering BRAF-VAF dichotomization using "maxstat" statistical package (cut-off = 0.301, $p = 0.0044$). **(B)** Kaplan-Meier survival curves for OS considering nVAF dichotomization using "maxstat" statistical package (cut-off = 0.643, $p = 0.009$). **(C)** ORR of patients dichotomized into high vs. low nVAF according to the 0.301 cut-off point identified in (**A**)

of progression in MM patients receiving first-line MAPK inhibitor therapy, in line with previously published data [22].

Previous studies have shown that low tumor cellularity could lead to underestimation of VAF in various cancers [21, 33, 34], and a consistent correlation between tumor cellularity and VAF has not yet been definitively established. The lack of a linear relationship between VAF and neoplastic cellularity has been reported in both

non-small cell lung cancer (NSCLC) and colorectal carcinoma (CRC) [35]. When we normalized BRAF-VAF according to the tumor cellularity (nVAF), we observed a stronger association between nVAF and PFS. This finding highlights the potential of combining VAF and tumor cellularity to improve the prediction of response to targeted therapy.

Accurately identifying which BRAF-mutated patients are likely to respond to target therapy remains a critical unmet clinical need. In clinical practice, targeted therapy is generally preferred for symptomatic patients, especially those with neurological involvement, rapidly progressive disease, and/or elevated LDH levels [12]. In our study elevated BRAF-VAF values have been associated with poorer clinical outcomes under targeted therapy, suggesting that immunotherapy may be a more suitable option for patients with high BRAF-VAF. However, this hypothesis requires validation in prospective clinical trials designed to confirm the predictive value of BRAF-VAF. Such studies should clarify whether patients with high BRAF-VAF/nVAF derive greater benefit from immune checkpoint inhibition or from emerging alternative therapeutic strategies compared with standard BRAF/MEK inhibition.

Regarding OS, nVAF showed only a trend in univariate and multivariate analysis without achieving statistical significance. This result could reflect the influence of successive lines of therapy, such as immunotherapy with checkpoint inhibitors offered to 34% of our patients. In conclusion, we demonstrated that the robustness of BRAF VAF to predict disease progression is improved when BRAF-VAF is normalized with tumoral cellularity and preserved even when sample cellularity is low (30%-50%).

Regarding the optimal tissue source for BRAF-VAF assessment, it is currently unclear whether the primary tumor or consecutive metastases should be preferentially analyzed for BRAF mutation status because of the possibility of inter-tumor heterogeneity [36]. Indeed, although most patients have a homogeneous BRAF mutation status, BRAF mutational heterogeneity among different tumor sites of a single individual has been reported in about 10% of patients, and about 15% of BRAF-mutated melanomas may have intratumor BRAF heterogeneity [32]. Moreover, dynamic and unpredictable changes in mutant allele fractions of BRAF and NRAS during visceral progression of cutaneous melanoma have also been reported [37].

In our MM population, BRAF status was detected in 98 (40.8%) primary tumors and in 165 (62.5%) metastatic samples. Unexpectedly, while there was no correlation between tissue source and PFS, we found a significantly poorer OS in patients whose BRAF-VAF was determined in primary tumors compared with those in whom it was determined in metastases (HR 1.58, CI 1.16–2.17, p = 0.004). Although paired primary–metastasis samples were not analyzed, this finding may reflect differences in access to second-line therapies between the two groups (33% in the primary tumor group vs. 64% in the metastatic group), rather than intrinsic biological differences related to tissue origin. Notably, since BRAF-VAF was correlated with PFS but not with OS in both groups, this parameter appears to be a predictive factor for response to targeted therapy rather than a purely prognostic one. As supported by current literature and by the progressive improvement in melanoma survival outcomes over recent years [38, 39] unequal access to subsequent treatment lines likely accounts for the observed OS difference between patients whose BRAF status was assessed in primary versus metastatic samples.

Moreover, our findings demonstrate that both primary and metastatic tissues are reliable sources for BRAF mutation analysis for therapeutic purposes. Therefore, primary tumor tissue can be considered an acceptable alternative for guiding systemic treatment decisions when a metastatic sample is unavailable.

Our study provided evidence for the strong prognostic and predictive role of BRAF-VAF in a large patient population homogeneously treated with first-line BRAFi/MEKi. Our study presents several key strengths: (1) High-Performance NGS for VAF Assessment: we employed a highly reliable NGS approach to evaluate BRAF-VAF in all patients, ensuring accurate and consistent measurement. (2) Unveiling the BRAF-VAF and Cellularity Link: we demonstrated for the first time a significant relationship between BRAF-VAF and tumor cellularity in melanoma. (3) Robust VAF for Predicting Disease Progression: our findings reveal that BRAF-VAF is a robust predictor of disease progression even when tumor cellularity falls below 50%. (4) PFS Independence from tumor source: by analyzing a large patient cohort with data from both primary tumors and metastases, we demonstrate that PFS is not significantly influenced by the source of the tumor tissue.

Our study also has some limitations, which include: (1) Multicenter Retrospective Design: this study employed a retrospective design across multiple centers. While valuable, data validation using a separate prospective cohort would strengthen the findings. (2) Limited analysis at progression: we did not evaluate melanoma samples obtained at disease progression. This could provide valuable insights into mechanisms of resistance to targeted therapy, including the emergence of additional mutations. (3) Decentralized NGS and cellularity assessments: both NGS analysis and neoplastic cellularity evaluation were performed at individual participating centers without a centralized approach. Standardization across centers could potentially improve data consistency. (4) Heterogeneous Second-Line treatments: treatment regimens employed after initial BRAF/MEK inhibitor therapy varied among patients. This heterogeneity limits our ability to draw definitive conclusions about the impact

of BRAF-VAF on response to subsequent therapies. (5) Pathologist-scored cellularity: neoplastic cellularity was determined by pathologists according to established guidelines. While this approach is a standard procedure, utilizing digital pathology scoring could potentially provide more precise and objective cellularity measurements. (6) Exclusion of low cellularity samples: our study excluded analysis of samples with neoplastic cellularity below 30%. Further investigation of VAF in these low-cellularity samples is warranted.

Conclusions

In conclusion, our study confirmed the role of the BRAF-VAF as a negative predictive and prognostic biomarker in MM patients treated with BRAF/MEK inhibitors. Moreover, our data demonstrated that the robustness of BRAF-VAF to predict disease progression is improved when BRAF-VAF is normalized to neoplastic cellularity, and it is preserved even when the cellularity of samples is low (30%-50%). Prospective clinical trials are warranted to validate nVAF as a biomarker for stratifying patients with BRAF mutated melanomas receiving first-line MAPKi therapy. Specifically, these studies should determine if patients with high nVAF derive greater benefit from checkpoint inhibitors or alternative treatment strategies compared to BRAF/MEKi.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-07434-x.

Supplementary Material 1

Author contributions

Conception and design: MG, SS. Development of methodology: MG, SDS, SS. Acquisition of data (managed patients, provided samples, technical analysis of tumor samples): MG, BA, LR, FS, PQ, RD, ST, TS, LF, PDT, MCS, MMT, AI, TT, IN, SB, ML, CP, PQ, FP, AM, MT, RC, SC, MM, ET, EC, RS, PF, GP, SP, EM, MDV, FC, SG, SDS, SS. Analysis and interpretation of data (e.g., statistical analysis, biostatistics): MG, BA, SDS, SS. Writing the manuscript: MG, BA, SDS, SS. Review, and/or revision of the manuscript: MG, BA, SDS, SS. All authors read and approved the final manuscript.

Funding

This work was supported by Ricerca Corrente 2025 funding from the Italian Ministry of Health.

Data availability

The data presented in this study are available upon reasonable request from the corresponding author. The data are not publicly available due to privacy and ethical reasons.

Declarations

Ethics approval and consent to participate

This study was approved by the local Ethics Committee of Istituto Tumori "Giovanni Paolo II" of Bari (prot. no 1085/2022 CE) and the Declaration of Helsinki guidelines were followed.

Competing interests

The authors declare that they have no competing interests. The authors affiliated to the IRCCS Istituto Tumori "Giovanni Paolo II", Bari are responsible for the views expressed in this article, which do not necessarily represent the Institute.

This study was approved by the local Ethics Committee of Istituto Tumori "Giovanni Paolo II" of Bari (prot. no 1085/2022 CE) and the Declaration of Helsinki guidelines were followed.

Author details

[1]Rare Tumors and Melanoma Unit, IRCCS Istituto Tumori Giovanni Paolo II, Bari, Italy
[2]Unit of Medical Oncology, University of Perugia, Perugia, Italy
[3]Department of Internal Medicine and Medical Specialties (DiMI), University of Genoa, Genoa, Italy
[4]Genetics of Rare Cancers, IRCCS Ospedale Policlinico San Martino, Genoa, Italy
[5]Department of Medical Sciences, Section of Dermatology, University of Turin, Turin, Italy
[6]Oncology Unit, AOU Policlinico Modena, Modena, Italy
[7]Molecular Diagnostics and Pharmacogenetics Unit, IRCCS Istituto Tumori "Giovanni Paolo II", Bari, Italy
[8]Pathology Unit, IRCCS Istituto Tumori Giovanni Paolo II, Bari, Italy
[9]Unit of Medical Oncology and Biomolecular Therapy, Department of Medical and Surgical Sciences, University of Foggia, Policlinico Riuniti, Foggia, Italy
[10]Immunology and Molecular Oncology Unit, Veneto Institute of Oncology - IOV IRCCS, Padua, Italy
[11]UOSD Sarcomas and Rare Tumors-IRCCS Regina Elena National Cancer Institute, Rome, Italy
[12]Unit of Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
[13]Department of Precision Medicine, University of Campania Luigi Vanvitelli, 80131 Naples, Italy
[14]Department of Medical Oncology, Santa Chiara Hospital, Trento, Italy
[15]Department of Dermatology, ASST Sette Laghi, Varese, Italy
[16]Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy
[17]Division of Medical Oncology for Melanoma, Sarcoma, and Rare Tumors, European Institute of Oncology IRCCS, Milan, Italy
[18]Medical Oncology Unit, University Hospital of Parma, Parma, Italy
[19]Department of Oncology, Azienda Sanitaria Universitaria Integrata di Udine, Udine, Italy
[20]Department of Interdisciplinary Medicine, Aldo Moro University of Bari, Bari, Italy
[21]Division of Medical Oncology, Department of Onco-Hematology, IRCCS-CROB, Referral Cancer Center of Basilicata, Rionero in Vulture, Italy
[22]Pathology Department-IRCCS Regina Elena National Cancer Institute, Rome, Italy
[23]Pathological Anatomy, Santa Chiara Hospital, Trento, Italy
[24]Viale Orazio Flacco 65, 70124 Bari, Italy

Received: 2 October 2025 / Accepted: 1 November 2025
Published online: 17 December 2025

References

1. Barbour AP, Tang YH, Armour N, Dutton-Regester K, Krause L, Loffler KA, Lambie D, Burmeister B, Thomas J, Smithers BM, et al. BRAF mutation status is an independent prognostic factor for resected stage IIIB and IIIC melanoma: implications for melanoma staging and adjuvant therapy. Eur J Cancer. 2014;50(15):2668–76.
2. Claeson M, Tan SX, Lambie D, Brown S, Walsh MD, Baade PD, Pandeya N, Whitehead KJ, Soyer HP, Smithers BM, et al. The association between BRAF-V600E mutations and death from thin (=1.00 mm) melanomas: A nested case-case study from Queensland, Australia</at. J Eur Acad Dermatol Venereol. 2023;37(9):e1168–72.
3. Robert C, Grob JJ, Stroyakovskiy D, Karaszewska B, Hauschild A, Levchenko E, Chiarion Sileni V, Schachter J, Garbe C, Bondarenko I, et al. Five-Year outcomes with Dabrafenib plus Trametinib in metastatic melanoma. N Engl J Med. 2019;381(7):626–36.

4. Dummer R, Flaherty KT, Robert C, Arance A, JW BdG, Garbe C, Gogas HJ, Gutzmer R, Krajsova I, Liszkay G, et al. COLUMBUS 5-year update: a randomized, open-label, phase III trial of Encorafenib plus binimetinib versus Vemurafenib or Encorafenib in patients with BRAF. Future Oncol. 2023;19(16):1091–8.
5. Lee J, Ahmed T, Maurichi A, Di Guardo L, Stagno AM, Warburton L, Taylor AM, Livingstone E, Rehman S, Khattak A, et al. BRAF inhibitor cessation prior to disease progression in metastatic melanoma: Long-term outcomes. Eur J Cancer. 2023;179:87–97.
6. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Lao CD, Cowey CL, Schadendorf D, Wagstaff J, Dummer R, et al. Five-Year survival with combined nivolumab and ipilimumab in advanced melanoma. N Engl J Med. 2019;381(16):1535–46.
7. Nathan P, Ascierto PA, Haanen J, Espinosa E, Demidov L, Garbe C, Guida M, Lorigan P, Chiarion-Sileni V, Gogas H, et al. Safety and efficacy of nivolumab in patients with rare melanoma subtypes who progressed on or after ipilimumab treatment: a single-arm, open-label, phase II study (CheckMate 172). Eur J Cancer. 2019;119:168–78.
8. Dummer R, Corrie P, Gutzmer R, Meniawy TM, Del Vecchio M, Lebbe C, Guida M, Dutriaux C, Dreno B, Meyer N, et al. First-Line, Fixed-Duration nivolumab plus ipilimumab followed by nivolumab in clinically diverse patient populations with unresectable stage III or IV melanoma: checkmate 401. J Clin Oncol. 2023;41(23):3917–29.
9. Ascierto PA, Mandala M, Ferrucci PF, Guidoboni M, Rutkowski P, Ferraresi V, Arance A, Guida M, Maiello E, Gogas H, et al. Sequencing of ipilimumab plus nivolumab and Encorafenib plus binimetinib for untreated BRAF-Mutated metastatic melanoma (SECOMBIT): A Randomized, Three-Arm, Open-Label phase II trial. J Clin Oncol. 2023;41(2):212–21.
10. Wang DY, Johnson DB, Davis EJ. Toxicities associated with PD-1/PD-L1 Blockade. Cancer J. 2018;24(1):36–40.
11. Atkins MB, Lee SJ, Chmielowski B, Tarhini AA, Cohen GI, Truong TG, Moon HH, Davar D, O'Rourke M, Stephenson JJ, et al. Combination Dabrafenib and Trametinib versus combination nivolumab and ipilimumab for patients with advanced BRAF-Mutant melanoma: the DREAMseq Trial-ECOG-ACRIN EA6134. J Clin Oncol. 2023;41(2):186–97.
12. Dummer R, Welti M, Ramelyte E. The role of triple therapy and therapy sequence in treatment of BRAF-mutant metastatic melanoma. Response to overall survival with first-line Atezolizumab in combination with Vemurafenib and Cobimetinib in BRAFV600 mutation-positive advanced melanoma (IMspire150): second interim analysis of a multicentre, randomised, phase 3 study. J Transl Med. 2023;21(1):529.
13. Ascierto PA, Mandalà M, Ferrucci PF, Guidoboni M, Rutkowski P, Ferraresi V, Arance A, Guida M, Maiello E, Gogas H, et al. Sequencing of ipilimumab plus nivolumab and Encorafenib plus binimetinib for untreated. J Clin Oncol. 2023;41(2):212–21.
14. Long GV, Grob JJ, Nathan P, Ribas A, Robert C, Schadendorf D, Lane SR, Mak C, Legenne P, Flaherty KT, et al. Factors predictive of response, disease progression, and overall survival after Dabrafenib and Trametinib combination treatment: a pooled analysis of individual patient data from randomised trials. Lancet Oncol. 2016;17(12):1743–54.
15. Hauschild A, Larkin J, Ribas A, Dreno B, Flaherty KT, Ascierto PA, Lewis KD, McKenna E, Zhu Q, Mun Y, et al. Modeled prognostic subgroups for survival and treatment outcomes in BRAF V600-Mutated metastatic melanoma: pooled analysis of 4 randomized clinical trials. JAMA Oncol. 2018;4(10):1382–8.
16. Soria X, Vilardell F, Maiques O, Barcelo C, Siso P, de la Rosa I, Velasco A, Cuevas D, Santacana M, Gatius S, et al. BRAF(V600E) Mutant Allele Frequency (MAF) influences melanoma clinicopathologic characteristics. Cancers (Basel) : 2021;13(20).
17. Lebbe C, How-Kit A, Battistella M, Sadoux A, Podgorniak MP, Sidina I, Pages C, Roux J, Porcher R, Tost J, et al. BRAF(V600) mutation levels predict response to Vemurafenib in metastatic melanoma. Melanoma Res. 2014;24(4):415–8.
18. Satzger I, Marks L, Kerick M, Klages S, Berking C, Herbst R, Volker B, Schacht V, Timmermann B, Gutzmer R. Allele frequencies of BRAFV600 mutations in primary melanomas and matched metastases and their relevance for BRAF inhibitor therapy in metastatic melanoma. Oncotarget. 2015;6(35):37895–905.
19. Mesbah Ardakani N, Leslie C, Grieu-Iacopetta F, Lam WS, Budgeon C, Millward M, Amanuel B. Clinical and therapeutic implications of BRAF mutation heterogeneity in metastatic melanoma. Pigment Cell Melanoma Res. 2017;30(2):233–42.
20. Stagni C, Zamuner C, Elefanti L, Zanin T, Bianco PD, Sommariva A, Fabozzi A, Pigozzo J, Mocellin S, Montesco MC, et al. BRAF gene copy number and mutant allele frequency correlate with time to progression in metastatic melanoma patients treated with MAPK inhibitors. Mol Cancer Ther. 2018;17(6):1332–40.
21. Berrino E, Balsamo A, Pisacane A, Gallo S, Becco P, Miglio U, Caravelli D, Poletto S, Paruzzo L, Debernardi C, et al. High BRAF variant allele frequencies are associated with distinct pathological features and responsiveness to target therapy in melanoma patients. ESMO Open. 2021;6(3):100133.
22. Mandala M, Palmieri G, Ludovini V, Baglivo S, Marasciulo F, Castiglione F, Gili A, Osella Abate S, Rubatto M, Senetta R, et al. BRAFV600 variant allele frequency predicts outcome in metastatic melanoma patients treated with BRAF and MEK inhibitors. J Eur Acad Dermatol Venereol. 2023;37(10):1991–8.
23. Loh JW, Guccione C, Di Clemente F, Riedlinger G, Ganesan S, Khiabanian H. All-FIT: allele-frequency-based imputation of tumor purity from high-depth sequencing data. Bioinformatics. 2020;36(7):2173–80.
24. Prandi D, Demichelis F. Ploidy- and Purity-Adjusted Allele-Specific DNA analysis using CLONETv2. Curr Protoc Bioinf. 2019;67(1):e81.
25. Revkov E, Kulshrestha T, Sung KW, Skanderup AJ. PUREE: accurate pan-cancer tumor purity Estimation from gene expression data. Commun Biol. 2023;6(1):394.
26. Haider S, Tyekucheva S, Prandi D, Fox NS, Ahn J, Xu AW, Pantazi A, Park PJ, Laird PW, Sander C, et al. Systematic assessment of tumor purity and its clinical implications. JCO Precis Oncol. 2020;4.
27. Keung EZ, Gershenwald JE. The eighth edition American joint committee on cancer (AJCC) melanoma staging system: implications for melanoma treatment and care. Expert Rev Anticancer Ther. 2018;18(8):775–84.
28. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45(2):228–47.
29. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Muller M. pROC: an open-source package for R and S + to analyze and compare ROC curves. BMC Bioinformatics. 2011;12:77.
30. Villanueva RAM, Chen ZJ. ggplot2: elegant graphics for data analysis, 2nd ed. Meas-Interdiscip Res. 2019;17(3):160–167.
31. Kanchi KL, Johnson KJ, Lu C, McLellan MD, Leiserson MD, Wendl MC, Zhang Q, Koboldt DC, Xie M, Kandoth C, et al. Integrated analysis of germline and somatic variants in ovarian cancer. Nat Commun. 2014;5:3156.
32. Ito T, Tanaka Y, Murata M, Kaku-Ito Y, Furue K, Furue M. BRAF heterogeneity in melanoma. Curr Treat Options Oncol. 2021;22(3):20.
33. McCord M, Jamshidi P, Thirunavu V, Santana-Santos L, Vormittag-Nocito E, Dittman D, Parker S, Baczkowski J, Jennings L, Walshon J, et al. Variant allelic frequencies of driver mutations can identify gliomas with potentially false-negative MGMT promoter methylation results. Acta Neuropathol Commun. 2023;11(1):175.
34. Yang SR, Lin CY, Stehr H, Long SR, Kong CS, Berry GJ, Zehnder JL, Kunder CA. Comprehensive genomic profiling of malignant effusions in patients with metastatic lung adenocarcinoma. J Mol Diagn. 2018;20(2):184–94.
35. Zarabi SK, Zhai L, Cheng YW. A challenging correlation between tumor cellularity and somatic variant allele fraction in lung and colorectal cancers-specimens of low tumor percentage should be analyzed with caution. Biomolecules 2024;14(2).
36. Boscolo Bielo L, Trapani D, Repetto M, Crimini E, Valenza C, Belli C, Criscitiello C, Marra A, Subbiah V, Curigliano G. Variant allele frequency: a decision-making tool in precision oncology? Trends Cancer. 2023;9(12):1058–68.
37. Doma V, Karpati S, Raso E, Barbai T, Timar J. Dynamic and unpredictable changes in mutant allele fractions of BRAF and NRAS during visceral progression of cutaneous malignant melanoma. BMC Cancer. 2019;19(1):786.
38. Namikawa K, Nakano E, Ogata D, Yamazaki N. Long-term survival with systemic therapy in the last decade: can melanoma be cured? J Dermatol. 2024;51(3):343–52.
39. Eisemann N, Schumann L, Baltus H, Labohm L, Kraywinkel K, Katalinic A. Longer survival from melanoma in Germany. Dtsch Arztebl Int. 2024;121(2):45–51.

Publisher's note