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ORIGINAL ARTICLE

BRAFV600 variant allele frequency predicts outcome in metastatic melanoma patients treated with BRAF and MEK inhibitors

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Abstract

Background: The prognostic impact of variant allele frequency (VAF) on clinical outcome in *BRAFV600* mutated metastatic melanoma patients (MMPs) receiving BRAF (BRAFi) and MEK inhibitors (MEKi) is unclear.

Materials and Methods: A cohort of MMPs receiving first line BRAFi and MEKi was identified by inspecting dedicated databases of three Italian Melanoma Intergroup centres. VAF was determined by next generation sequencing in pre-treatment baseline tissue samples. Correlation between VAF and *BRAF* copy number variation was analysed in an ancillary study by using a training and a validation cohort of melanoma tissue samples and cell lines.

Results: Overall, 107 MMPs were included in the study. The VAF cut-off determined by ROC curve was 41.3%. At multivariate analysis, progression-free survival (PFS) was significantly shorter in patients with M1c/M1d [HR 2.25 (95% CI 1.41–3.6, $p < 0.01$)], in those with VAF >41.3% [HR 1.62 (95% CI 1.04–2.54, $p < 0.05$)] and in those with ECOG PS ≥1 [HR 1.82 (95% CI 1.15–2.88, $p < 0.05$)]. Overall survival (OS) was significantly shorter in patients with M1c/M1d [HR 2.01 (95% CI 1.25–3.25, $p < 0.01$)]. Furthermore, OS was shorter in patients with VAF >41.3% [HR 1.46 (95% CI 0.93–2.29, $p = 0.06$)] and in patients with ECOG PS ≥1 [HR 1.52 (95% CI 0.94–2.87, $p = 0.14$)]. *BRAF* gene amplification was found in 11% and 7% of samples in the training and validation cohort, respectively.

Conclusions: High VAF is an independent poor prognostic factor in MMP receiving BRAFi and MEKi. High VAF and *BRAF* amplification coexist in 7%–11% of patients.

Mario Mandalà and Giuseppe Palmieri: Equally contributed first authorship.

Daniela Massi and Pietro Quaglino: Equal senior contribution.

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INTRODUCTION

The last decade represents a renaissance era in the systemic treatment landscape of metastatic melanoma, with the development of two main strategies, targeted therapy and immunotherapy. In particular, the discovery that 40%–50% of melanoma patients harbour *BRAF* mutations led to identify, for the first time, a truly actionable molecular target.[1,2] This genetic alteration is an important driver in melanoma pathogenesis, leading to the constitutive activation of the downstream MAPK pathway (RAS/RAF/MEK/ERK proteins), which sustains tumour cell proliferation. This discovery paved the way to the development of BRAF (BRAFi) and MEK (MEKi) inhibitors that are one of the standard treatments in advanced and adjuvant setting.[2–7] While clinical responses to MAPKi may be dramatic and some patients treated with BRAFi/MEKi may stay in remission for years, the median duration of response is 11–13 months for patients treated with BRAFi/MEKi.[5–7]

For this reason, there is intense investigation into alternative or complementary therapeutic strategies, and to identify biomarkers of resistance. Precise selection of patients is crucial for optimal use of BRAFi/MEKi. *BRAF* mutations may be detected in archived formalin-fixed, paraffin-embedded (FFPE) melanoma tissue; however, it is currently unclear whether primary tumours or consecutive metastases should be preferentially analysed because of the possibility of inter-tumour heterogeneity. It is also unclear whether the *BRAFV600* variant allele frequency (VAF) of *BRAFV600* mutations correlates with response to MAPKi, and most importantly with patient outcome.

The potential correlation between *BRAF* VAF and clinical outcome of BRAFi/MEKi treated patients has been reported in previous studies with controversial results.[8–12] Several factors could influence these conflicting results including: small patients' cohort, assessment of *BRAF* VAF in primary and metastatic disease and treatment with both BRAFi alone or BRAFi in combination with MEKi.

Based on this evidence, we planned a retrospective study in three Italian Melanoma Intergroup (IMI) centres, including melanoma patients prospectively annotated in specific dataset, with the main objective to correlate the *BRAFV600* VAF with overall response and outcome of metastatic melanoma patients (MMPs) receiving MAPKi.

MATERIALS AND METHODS

Patient characteristics

The cohort of 107 MMPs ('discovery cohort') was identified by inspecting the electronic databases of all MMPs treated at three IMI centres (Perugia, Florence, Turin) from 15 July 2013 to 22 September 2021.

All the patients included in the discovery cohort had stage IV metastatic melanoma (26 stage IVM1a, 17 stage IVM1b, 38 stage IVM1c and 26 stage IVM1d) (Table S1). We retrieved data concerning clinical outcome and MAPKi treatment from consecutive MMPs. The local Ethics Committees approved the study protocol. The study was conducted in compliance with the World Medical Association Declaration of Helsinki. Information on age, gender, histopathology, surgical and medical treatments were retrieved for each patient, as well as data on tumour objective response rate (ORR), progression-free survival (PFS) and overall survival (OS). Data on treatment and survival were collected prospectively. Medical records and/or tissue quality control and review of pathology material confirmed accuracy in histopathological classification.

Tumour stage was assessed according to the American Joint Committee on Cancer (AJCC) TNM (Tumour, Node, Metastasis) staging system classification (VIII edition). Tumour assessment was carried out by total body computed tomography (CT) scan at baseline and every 12 weeks until disease progression or death. Clinical response to BRAFi/MEKi was assessed by RECIST v1.1 criteria. Timing of follow-up was similar according to IMI rules. Four categories were identified based on the response to treatment: disease progression (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.

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

Tissue samples with a content of tumour-vs-non-tumour cells ≥50% (evaluated at the observation of haematoxylin & eosin-stained slides) were included in the analysis. For the discovery cohort, among 107 MMPs, BRAF analysis was performed in 37 cases on the primary tumour samples and in 70 on metastatic samples from different sites. The tumour area was macroscopically dissected to concentrate tumour tissue. DNA was extracted using QIAamp DNA FFPE Tissue kit (Qiagen GmbH) according to the manufacturer's instructions on QiaCube instrument (Qiagen GmbH). More details on NGS analysis are reported in Appendix S1.

Evaluation of copy number variation (CNV) by ddPCR and VAF by NGS

BRAF copy number variation (CNV) was evaluated by digital droplets PCR (ddPCR) Copy Number Determination Assays (#dHsaCP2500366; Bio-Rad). More details on ddPCR are reported in Appendix S1.

The ddPCR assays were performed using the QX200 Droplet Digital PCR System (Bio-Rad), following manufacturer instruction. Briefly, 11 μL of 2X ddPCR Supermix for probes (no dUTP), 1 μL of 20X *BRAF* (dHsaCP2500366) target primer/probe mix and 1 μL of 20X AP3B1 (dHsaCP2500348) REF target primer/probe mix

were premixed. Final reaction was prepared combining the ddPCR reagents with 15 ng of DNA for a final volume of 22 μL. Each 20 μL reaction was dispensed into a separate well of a disposable eight channel droplet generator cartridge, and a volume of 70 μL of droplet generation oil was loaded into each of the corresponding oil wells. Emulsions obtained with the droplet generator were transferred in a 96-well plates then PCR amplified on a T100 Touch thermal cycler (Bio-Rad) with the following program: 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 1 min, 98°C for 10 min, with a ramp rate of 2.5°C/s. After PCR, readout of droplet fluorescence was performed by the droplet reader QX200 (Bio-Rad) and analysed with the QuantaSoft Analysis Pro Software Version 1.7.4 (Bio-Rad). The ratio of absolute quantities of the target locus (*BRAF*) to the absolute quantities of the reference locus (AP3B1) was used for CNV quantification. *BRAF* gene amplification was classified with CNV value ≥4 copies.

To validate the relationship between *BRAF* CNV and VAF, a validation cohort including cases with known *BRAF* mutational status was used: 90 DNA samples from advanced melanoma patients carrying a *BRAFV600* mutation ($N=27$; 10 primary tumours and 17 metastases) or a *BRAF* wild-type gene ($N=63$; 15 primary tumours and 48 metastases) (Table S2). Since the validation cohort was not intended to replicate the correlation between the *BRAF* allele frequency and the clinical outcome but to only focus on the relationship between the two molecular features affecting the gene (CNV and VAF), the patients here included were not investigated for their disease outcome.

The mutation analysis was carried out on the Ion Oncomine Comprehensive Assay (OCA) Plus panel, which provides a highly multiplexed target selection of over 500 genes implicated in cancer research and provides a comprehensive genomic profiling solution appropriate for FFPE tissues (at https://f.hubspotusercontent10.net/hubfs/4584212/PG2682-PJT8457-COL117107-Update-OCA-PlusOnGS-Flyer-Global-FHR.pdf). In the NGS-based analysis of the validation cohort, the use of the OCA Plus panel allowed to simultaneously infer both CNV and VAF values for *BRAF* gene (this was not possible for the 22-gene Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2), with no need for further investigations using the ddPCR assay.

While mutation analysis for the discovery cohort was based on a 22-gene targeted panel routinely used into the clinical practice, the validation cohort was indeed screened using a 500-gene panel, since this latter series is a part of a much larger patients' collection being investigated for different purposes within a parallelly ongoing study.

Libraries were generated from 10 ng of input DNA using the Ion AmpliSeq Library Kit Plus, barcoded with Ion Xpress Barcode Adapters (Life Technologies) and purified with Agencourt Ampure XP Beads (Beckman Coulter Life Sciences). The obtained PCR amplicons were diluted to a final concentration of 70 pM and pooled together; emulsion PCR and Chip (Ion 540) loading steps were performed by the Ion Chef Instrument. Again, sequencing of libraries was done with the Ion S5™ System (Thermo Fisher Scientific), and sequencing data were processed with the Ion Torrent Suite (V5.2.1). Using the OCA Plus panel, CNVs are identified along with all other classes of variants (SNVs, indels and splice variants), whose description is, however, out of the purposes of the present work. *BRAF* gene amplification was classified with CNV value ≥4 copies.

Melanoma cell lines

Melanoma cell lines were kindly provided by *Istituto Dermopatico dell'Immacolata* in Rome, *Istituto Nazionale dei Tumori* in Milan and University of Genoa. They were established in vitro from primary and metastatic melanomas of donor patients with documented histological diagnosis, after obtaining their informed consent, as previously described by our group.[13,14] Further information and culturing protocols about the used cell lines could be provided on request.

Statistical analysis

Descriptive statistics were calculated including frequencies and percentages for categorical variables, median and mean ± standard deviation (SD) for quantitative variables. Categorical variables were evaluated by chi-square or Fisher's exact test when appropriate.

The time-dependent endpoints were analysed according to the Kaplan–Meier method. Survival curves were compared using the log-rank test. Cox regression model was applied to estimate Hazard ratio and 95% CI to identify prognostic factors independently associated with survival. Stepwise backward-selection approach was used for evaluate variables to be included in the Cox's multivariate analysis to find a reduced model that best explains the data. The assumption of proportional risks was verified by the Therneau and Grambsch test.

Optimal cut-off of allelic frequency was obtained by Youden approach (area under ROC curve at cut-off 0.62) and confirmed by Liu approach.[15,16] The overall accuracy level of the model was measured via the Harrell c-index.

Statistical analyses were performed with STATA v.16.1 (StataCorp LP). Further details on Statistical analysis are reported in Appendix S1.

RESULTS

The study cohort included 107 MMPs (42 females and 65 males), with a median age of 63 years (range 27–87 years). DNA samples were obtained from metastatic specimens in 70 patients (65.4%) and from primary tumour specimens in 37 patients (34.6%). Table S1 reports demographic features, the anatomical sites in which VAF was analysed, VAF for each patient.

Table S3 reports characteristics of treated patients according to *BRAF* variant allele frequencies.

Overall, 43 patients experienced PD (40.2%) and 17 SD (15.9%). Of the 47 responding patients (44%), 35 achieved PR and 12 a CR (32.7% and 11.2%, respectively) (Table S3). The median follow-up was 15.8 months (range 6.7–39 months). The median PFS was 10.2 months (95% CI 8.1–13.8 months). Overall, the median OS was 15.8 months (95% CI 11.1–26.7 months). The *BRAFV600E* mutation represented the most prevalent variant (76.6%). In addition, 25 cases carried the *BRAFV600K* substitution. The mean and the median VAF were 43% and 41.3%, respectively. VAF values did not correlate with the M score (M1a/M1b vs M1c/M1d: $p = 0.21$) (Table S3). All patients received the combination of BRAFi and anti-MEKi. For all patients, the baseline mutational *BRAF* profile was assessed before starting treatment. The Kernel density estimate of *BRAFV600* variant allele frequencies is reported in Figure S1.

To estimate the influence of *BRAFV600* VAF on patient outcome, according to the ROC curve and Liu approach, we used the cut-off of 41.3% VAF to dichotomize cases with low and high VAF. ORR (RP + RC) was 43.4% and 44.4% ($p = 0.3$), respectively in patients with VAF levels ≤41.3% and >41.3% (Table S3). At multivariate analysis, patients with M1C + M1D disease had a significant lower response than patients with M1a + M1b disease (Table S4). At univariate analysis, PFS was significantly shorter in patients with M1c/M1d vs M1a/M1b (HR 2.23, $p < 0.001$), ECOG PS ≥1 (HR 1.64 $p < 0.03$) and in those with VAF > 41.3% (HR 1.67, $p < 0.02$) (Table 1). At multivariate analysis, PFS was significantly shorter in patients with M1c/M1d [HR 2.25 (95% CI 1.41–3.6, $p < 0.001$)], in those with VAF >41.3% [HR 1.62 (95% CI 1.04–2.54, $p = 0.03$)] and in those with ECOG PS ≥1 [HR 1.82 (95% CI 1.15–2.88, $p < 0.01$)] (Table 1, Figure 1a). OS was significantly shorter in patients with M1c/M1d [HR 2.01 (95% CI 1.25–3.25, $p < 0.01$)]. Furthermore, OS was shorter in patients with VAF >41.3% [HR 1.46 (95% CI 0.93–2.29, $p = 0.1$)] and in patients with ECOG PS ≥1 [HR 1.52 (95% CI 0.94–2.87, $p = 0.09$)] (Table 1, Figure 1b). A statistically significant OS reduction in patients with high VAF was found at univariate analysis [HR 1.6 (95% CI 1.01–2.47, $p = 0.04$)], but only with borderline significance at multivariate analysis [HR 1.46 (0.93–2.29, $p = 0.1$)].

The impact of VAF on PFS and OS was also evaluated separately by analysing primary and metastatic tumour samples (Table S5). A significant correlation was found between VAF and PFS for metastatic sites samples [HR 1.99 (95% CI 1.09–3.67, $p < 0.05$)] and a trend for primary melanoma (HR 1.86 95% CI 0.79–4.4). Furthermore, a trend of significant association with OS was found both for VAF in metastatic sites (HR 1.69, 95% CI 0.9–3.15) and primary melanoma (HR 1.88, 95% CI 0.69–5.1).

Table S6 summarizes the results of the additional genes mutated in the discovery and validation cohort. The presence of one or more mutations in one of the additional 22 genes considered did not show any significant impact on disease outcome; indeed, patients with or without mutations in one of these genes did not show statistically significant differences in PFS ($p = 0.635$) or OS ($p = 0.722$). The validation cohort showed similar findings.

Subsequent lines of therapy in the overall cohort of 107 MMPs upon MAPKi progression are reported on Table S7.

TABLE 1 Univariate and multivariate analysis: prognostic factors for PFS and OS.

	Progression-free survival (95% CI)		Overall survival (95% CI)	
Characteristic	**HR univariate**	**HR multivariate**	**HR univariate**	**HR multivariate**
Gender				
Male (ref.)	–	–	–	–
Female	1.09 (0.71–1.69) $p = 0.69$	1.54 (0.98–2.44) $p = 0.06$	1.03 (0.66–1.63) $p = 0.88$	
ECOG performance status				
0 (ref.)	–	–	–	–
≥1	1.64 (1.04–2.57) $p = 0.03$	1.82 (1.15–2.88) $p = 0.01$	1.67 (1.05–2.68) $p = 0.03$	1.52 (0.94–2.87) $p = 0.09$
Metastasis				
M1a/M1b (ref.)	–	–	–	–
M1c/M1d	2.23 (1.42–3.49) $p < 0.001$	2.25 (1.41–3.6) $p < 0.001$	2.11 (1.32–3.39) $p < 0.01$	2.01 (1.25–3.25) $p < 0.01$
Variant allelic frequency				
≤41.3% (ref.)	–	–	–	–
>41.3%	1.67 (1.09–2.56) $p = 0.02$	1.62 (1.04–2.54) $p = 0.03$	1.6 (1.01–2.47) $p = 0.04$	1.46 (0.93–2.29) $p = 0.1$
Histology type				
Superficial spreading (ref.)	–	–	–	–
Nodular melanoma	1.31 (0.83–2.08) $p = 0.45$		1.21 (0.74–1.98) $p = 0.68$	

Note: Multivariate models were adjusted for age and estimated by stepwise backward-selection method ($p < 0.3$).

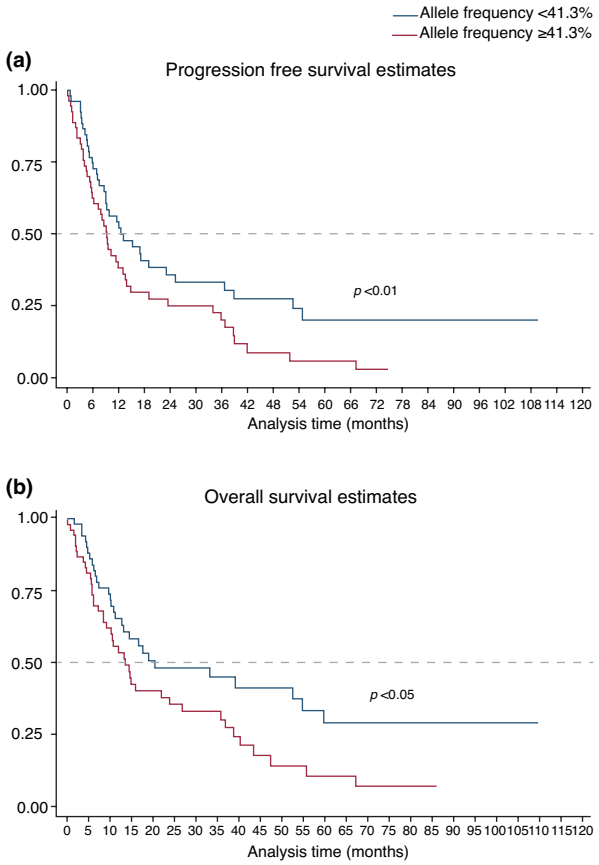

FIGURE 1 Progression-free and overall survival according to *BRAFV600* allele frequencies in melanoma patients treated with MAPKi.

Correlation between *BRAFV600* VAF and CNV in melanoma samples and cell lines

We then evaluated the potential correlation between CNV and VAF of mutated *BRAF* gene. Among available DNA samples ($N = 54$) of the training cohort (107 patients), the incidence of *BRAF* amplification was 11.1% (6/54) (Figure 2a). Although an amplified *BRAF* gene was exclusively observed in melanoma samples with a *BRAFV600* VAF value ≥41.3% (6/33; 18.2%), the vast majority of *BRAF*-mutated cases did not show the co-occurrence of the *BRAF* gene amplification at baseline (before starting targeted therapy) (Figure 2a). In an exploratory analysis, we looked at the outcome according to the presence or not of *BRAFV600* amplification. Among 6 patients with *BRAF* amplification, the median PFS was 9.4 months and OS 10.6 months vs 9.1 months and OS 16.6 months in those without *BRAF* amplification.

To further investigate the relationship between the increased *BRAF* gene copy numbers and the *BRAF* mutational status, we evaluated the *BRAF* CNV values in a validation cohort of melanoma tissues classified for *BRAF* mutational status through an NGS-based approach. In such validation cohort, consisting of 90 DNA samples from advanced melanoma patients (Table S2) carrying a *BRAFV600* mutation ($N = 27$; 10 primary tumours and 17 metastases) or a *BRAF* wild-type gene ($N = 63$; 15 primary tumours and 48 metastases), 3 out of 27 (11.1%) *BRAFV600* mutated cases showed an amplified *BRAF* gene; interestingly, coexistence of *BRAF* gene amplification—in terms of a significantly increased CNV value—was also found in 2/63 (3.2%) *BRAF* wild-type cases (Figure 2b).

The analysis of the distribution of CNV according to the VAF percentage assuming as cut-off 40% is summarized in Table S8. Although an amplified BRAF gene was exclusively observed in melanoma samples with a BRAFV600 VAF value ≥40% (6/33; 18.2%), the vast majority of BRAF-mutated cases did not show the co-occurrence of the BRAF gene amplification at baseline (before starting targeted therapy) (Figure 2a). Overall, an increased CNV value was observed in a limited fraction of *BRAFV600* mutated samples (<15%).

Finally, we evaluated the co-occurrence of both phenomena in melanoma cell lines that represent the melanomas with the most malignant phenotype due to their highly uncontrolled cell proliferation and growth. In our series, 9/43 (21%) *BRAFV600* mutated cell lines revealed a *BRAF* gene amplification (Figure 2c), which was also found in 3 out of 27 (13%) *BRAF* wild-type cell lines. Thus, our data show that *BRAF* gene amplification is an independent event from the occurrence of driving mutations in the *BRAF* gene.

DISCUSSION

One of the major issues in exploiting BRAFi/MEKi in MMPs lies in the interpatient clinical variability, duration of response and outcome: some patients derive no benefit, others have long-term dramatic response with symptom relief, and the remainder is somewhere in between. Hence, one of the unmet medical needs is to identify biomarkers allowing accurate establishment of the best treatment approach in the individual patient with *BRAF*-mutated melanoma. In this context of uncertainty, our results may be relevant and timely in the routine activity and for the design of future clinical trials.

In *BRAFV600* mutated MMPs treated with first line BRAFi/MEKi, our study provides four striking results: (1) High VAF is associated with a significantly shorter PFS; (2) High VAF is associated with a shorter OS; (3) ORR is not associated with VAF; (4) *BRAFV600* gene amplification, determined by CNV, is independent from VAF in the vast majority of patients.

A similar correlation was found in a subgroup analysis of the COLUMBUS study[17] on cell-free circulating tumour DNA and not tissue derived as in our study. Indeed, in an exploratory analysis of part 1 of the COLUMBUS study, Dummer et al. suggested that baseline *BRAFV600E/K* VAF is prognostic for PFS and OS, and that a more significant reduction in *BRAFV600E/K* VAF in response to encorafenib + binimetinib treatment occurred in responders compared with non-responders.[17] In this study, cfDNA was prognostic as well; hence, in the future longitudinal ctDNA

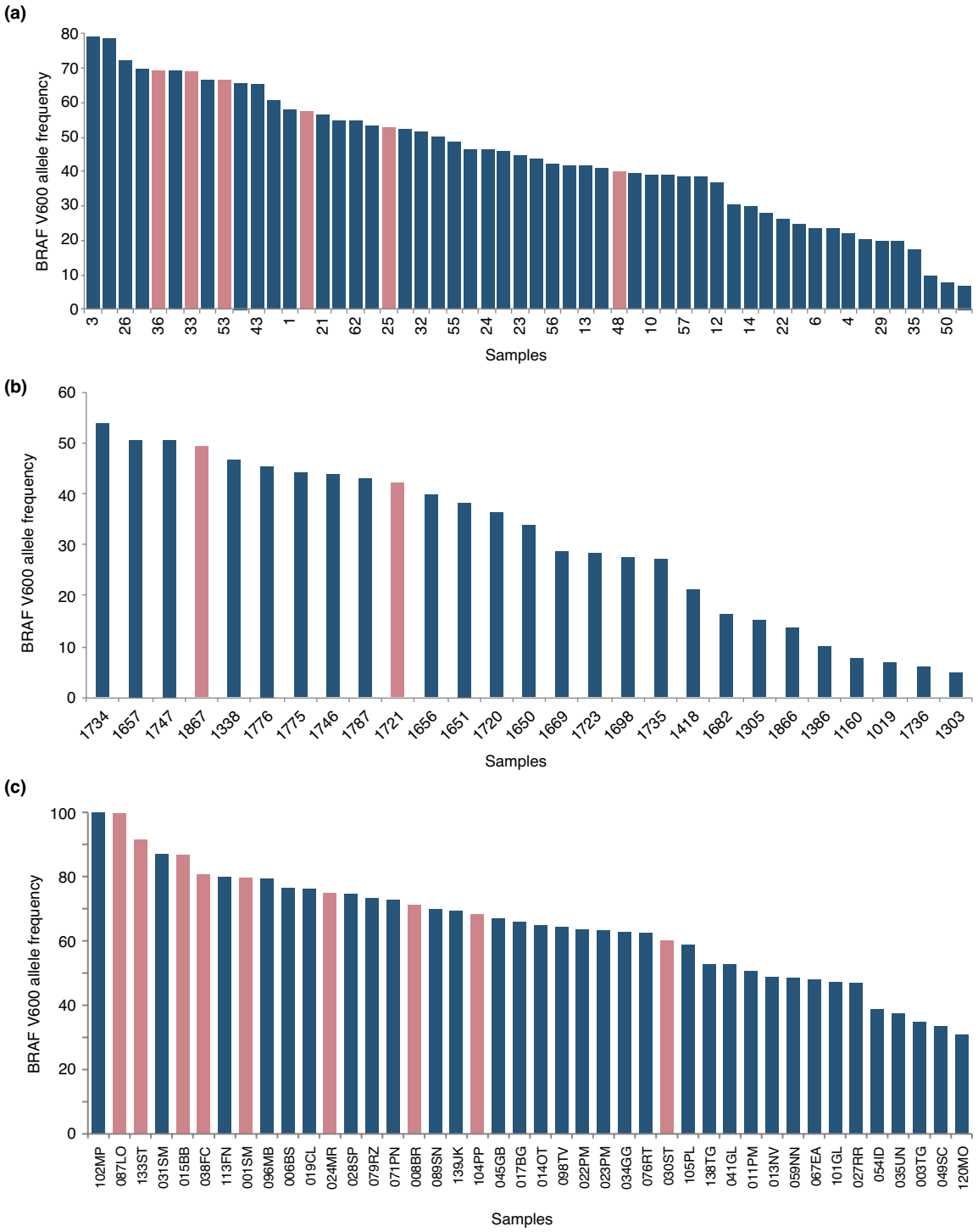

FIGURE 2 Distribution of melanoma samples according to the *BRAFV600* variant allele frequency. In red, samples carrying the *BRAF* gene amplification (>4 copies; see Methods). Results on melanoma tissues from the discovery (a) and validation (b) cohorts as well as on melanoma cell lines (c).

is a promising tool that may impact treatment decisions for patients with *BRAFV600* mutations.[17]

Our results are in disagreement with two recent reports.[11,12] Berrino et al.[12] analysed VAF in 327 patients at different clinical stages, showing a better outcome in patients with higher VAF. In their series, however, only a minority of patients was treated with targeted therapy and correlated with outcome; moreover, patients treated either with BRAFi alone or BRAF plus MEKi were included and VAF was determined by pyrosequencing and not by NGS as in our study. Furthermore, correlation between VAF and CNV was not analysed. Similarly, in the study

by Stagni et al. study, only 47 patients have been included, treated with BRAFi alone or BRAFi plus MEKi, in different lines of treatment and VAF was determined by qPCR and not by NGS.[11] Our results are partially in line with a previous report by Lebbe et al. showing that high VAF is associated with lower response rate and a worse outcome.[8] In this study, all patients received BRAFi alone and VAF was determined by qPCR.[8] NGS beyond the high diagnostic sensitivity (detection limit of 1%–2%) and specificity (100% comprehensive test)[18] is feasible and cost-affordable and provides additional potentially actionable information for patients with *BRAFV600E/K* negative metastatic melanoma.

Our study found a worse outcome, in terms of PFS and OS, in patients with high VAF. In IMI centres, melanoma patients who do not enter clinical trials are assessed according to standard guidelines at baseline and upon treatment. Specifically, before starting treatment, all patients undergo total body computed tomography (CT), ECG and blood laboratory analysis. Physical examination, including dermatological evaluation, is carried out every 4 weeks. The first disease assessment is planned after 8 weeks and thereafter every 12 weeks. The CT scan is anticipated if there is a suspicion of disease progression and/or in the presence of worsening symptoms. Overall, the time schedule for disease assessment is similar for all patients. Hence, our data were not influenced by the way enrolled patients were managed, which was homogeneous in different centres.

Several biological reasons can justify our findings: (i) melanomas with high VAF harbour more target to be inhibited by BRAFi and MEKi; (ii) *BRAF* mutation is associated with an immunosuppressive microenvironment, and this seems to be related to the amount of mutation found in melanoma cells[19,20]; (iii) since one of the main mechanisms of resistance relies on the reactivation of the MAPK, a higher number of cells harbouring the *BRAF* mutation may drive the reactivation of MAPK pathway in a number of melanoma cells exposed to BRAFi and MEKi.

Interestingly, the response rate was not different in MMP with low VAF compared to those with high VAF. Thus, our data suggest that VAF influences the emergence of secondary resistance without an impact on primary resistance. The first response to BRAFi/MEKi is driven by the presence of *BRAFV600* mutation but emergence of clones resistant to BRAFi and MEKi may be partially influenced by VAF.

To determine the relationship between gene amplification and VAF, we made additional experiments in a training series, validated in an independent cohort and in cell lines. Our data support the notion that only a minority of patients with high VAF harbours a *BRAF* gene amplification, in the order of 7%–11%. It is to be underlined that such a scenario is present at baseline, being modified during BRAFi treatment. Indeed, the gene amplification at the *BRAF* locus or *BRAF* copy number alteration involving the chromosome 7 (whole chromosome or chromosome arm) may strongly contribute to the variations of *BRAF* mutant allele frequency in melanoma and therefore interfere with the clinical efficacy of mutant–protein targeted therapy during the entire course of the BRAFi treatment.[21,22]

This study has several strengths: (i) we provide a molecular biomarker associated with outcome in patients treated with BRAFi/MEKi; (ii) all patients have been uniformly treated with BRAFi/MEKi; and (iii) all patients were managed homogeneously in the context of IMI centres.

The limitations of our report are as follows: (i) this is retrospective study and a validation dataset is needed; (ii) we did not evaluate VAF in melanoma samples at progression and did not explore its relationship with MAPK reactivation; (iii) patients with stage III disease receiving adjuvant therapy were not included in this study and further study are needed to test the hypothesis that VAF can impact outcome in high-risk melanoma patients, radically resected; (iv) most of them did not receive any further treatment because of rapid progression of disease (Table S7); (v) our study did not find any prognostic role of additional mutated genes ($p = 0.24$) (Table S6), but it is underpowered due to relative limited cohort included.

Another limit of our study is that not all the analysed samples derived from a metastatic site, specifically, among 107 patients, 39 have been studied by analysing the primary tumour. When splitting the analysis between primary and metastatic sites, the results are therefore more robust, in terms of PFS, for the metastatic sites, the limited number of primary tumour samples do not allow to draw conclusions, although a positive trend is observed in primary melanoma as well (Table S5).

In conclusion, our study supports VAF, determined by NGS, as a prognostic biomarker of *BRAFV600* mutated receiving first line BRAFi/MEKi. Whether MMP with high VAF should receive alternative treatments, this should be addressed by ad hoc designed clinical studies.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The patients in this manuscript have given written informed consent to publication of their case details.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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