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ARTICLE

Molecular Diagnostics

Variant allele frequency in baseline circulating tumour DNA to measure tumour burden and to stratify outcomes in patients with *RAS* wild-type metastatic colorectal cancer: a translational objective of the Valentino study

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INTRODUCTION: In patients with metastatic colorectal cancer (mCRC), baseline circulating tumour DNA (ctDNA) variant allele fraction (VAF) might serve as a surrogate of disease burden and should be evaluated in comparison with CEA and RECIST-defined sum of target lesions.

METHODS: In this pre-planned analysis of the VALENTINO trial, we included patients with *RAS* wild-type mCRC receiving upfront FOLFOX/panitumumab with available baseline liquid biopsy. CtDNA was analysed by means of a 14-gene NGS panel. For each patient, the gene with the highest VAF in ctDNA was selected.

RESULTS: The final cohort included 135 patients. The median VAF was 12.6% (IQR: 2.0–45.2%). Higher VAF was observed in patients with liver metastases and with synchronous metastases presentation. Patients with high VAF had poorer median OS compared to those with low VAF (21.8 vs 36.5 months; HR: 1.82, 95%CI: 1.20–2.76; $p = 0.005$). VAF outperformed baseline CEA and target lesion diameter in the prognostic stratification and remained significantly correlated with OS ($p = 0.003$) in a multivariate model. VAF was not significantly correlated with dimensional response and PFS.

CONCLUSION: CtDNA measured by VAF is prognostic in patients with *RAS* wild-type mCRC. Response and PFS after an anti-EGFR-based first-line strategy are independent from initial tumour burden.

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INTRODUCTION

Beyond several clinical applications of circulating tumour DNA (ctDNA) in early-stage colorectal cancer (CRC), liquid biopsy (LB) has a promising role in the metastatic setting for several purposes, such as prognostic stratification, monitoring of tumour response dynamics and treatment failure and baseline and longitudinal genomic profiling [1, 2]. From a "qualitative" standpoint, tumour-specific alterations detected in the ctDNA could guide the treatment choice according to their association with resistance or sensitivity to specific targeted agents such as anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies [3–7]. On the other hand, the amount of ctDNA is a candidate prognostic factor in patients with cancer [8], including those with metastatic CRC (mCRC) [9].

New surrogate markers of disease extension need to be implemented for the clinical management of patients with advanced cancers. In the setting of mCRC, the use of carcinoembryonic antigen (CEA) is recommended and widespread in the clinical practice to measure the disease burden and the depth of response to available treatment options, since this tumour marker mirrors the amount of active disease. Radiological parameters such as the Response Evaluation Criteria in Solid Tumours

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(RECIST)-defined sum of the target lesions are recorded in clinical trials and tumour diameters are empirically assessed in the clinical practice, with several limitations such as the subjectiveness of evaluations and inter-observer variability. Preliminary data showed that ctDNA response may have greater discriminative ability compared to CEA and RECIST [10], but the prognostic role of baseline ctDNA needs to be investigated in larger data sets and exploratory analyses of randomised clinical trials. Based on these considerations, the variant allele fraction (VAF) of clonal gene alterations detected in ctDNA is a candidate marker of disease burden and may have prognostic utility in patients with mCRC [11].

Here we investigated baseline VAF as a prognostic marker in patients with *RAS* wild-type mCRC treated with an anti-EGFR-based upfront strategy in the frame of the Valentino trial and we compared it with other parameters commonly available in the clinical practice, namely, CEA and RECIST-defined sum of target lesions.

METHODS

Patients' cohort

The Valentino study (NCT02476045) was a multicentre, randomised, non-blinded, open-label phase 2 trial that investigated the progression-free survival (PFS) non-inferiority of maintenance with panitumumab (arm B) vs panitumumab plus 5-FU/LV (arm A) following an induction treatment with panitumumab/FOLFOX-4 in patients with *RAS* wild-type mCRC [12]. In patients signing an optional consent form, LBs were collected at baseline and every 8 weeks during treatment (independently of delays of treatment cycles) until disease progression, consent withdrawal or death.

In this pre-specified exploratory analysis, we included all clinically evaluable randomised patients with evaluable baseline LBs. RECIST 1.1 response, early tumour shrinkage (ETS) and depth of response (DoR) were assessed by blinded independent central review, as previously described [13]. Available tumour profiling data are represented by tissue next-generation sequencing (NGS), as previously reported [14].

Institutional review board approval was obtained from all participating centres and all patients provided written informed consent. The complete list of participating centres is available in the original study publication [12]. The study was conducted in accordance with the Declaration of Helsinki.

NGS of plasma samples

Isolation of cell-free DNA (cfDNA) was performed from 1.5 mL of plasma by means of the RSC cfDNA Plasma Kit (Promega); Qubit® dsDNA HS Assay Kit (ThermoFisher) was used for cfDNA quantification. Targeted libraries were performed using Oncomine™ Colon cfDNA Assay (ThermoFisher), which amplifies 48 amplicons covering 240 key hotspot mutations of 14 genes (*AKT1*, *APC BRAF*, *CTNNB1*, *EGFR*, *ERBB2*, *FBXW7*, *GNAS*, *KRAS*, *MAP2K1*, *NRAS*, *PIK3CA*, *MAD4*, *TP53*) frequently mutated in CRCs. Targeted libraries were prepared with 2–50 ng of cfDNA; briefly, a unique molecular tag was assigned to each cfDNA molecule through a first PCR reaction in a Veriti™ thermal cycler, and subsequently, tagged library fragments were amplified in a second round of PCR to produce independent barcoded libraries. Ion GeneStudio™ S5 Prime system was used to carry on sequencing. For sensitive variant detection down to 0.1% VAF, we targeted a median read coverage >25,000, median molecular coverage >2500 and targets >0.8 median molecular coverage >60%. Sequencing reads that shared the same molecular barcode information were grouped into a single family; to reduce potential false positives, a variant was called when at least two molecular barcode families shared the same mutation, corresponding to two independent mutated alleles. For each patient, the maximum VAF detected among mutated genes was taken as an estimate of the ctDNA amount and investigated as the prognostic marker.

Statistical analysis

Patients were stratified according to candidate prognostic markers (VAF, CEA and sum of RECIST-defined target lesions) using median value as a blind cut-off. PFS was defined as the time from randomisation to documentation of progressive disease/death; overall survival (OS) was defined as the time from randomisation to death from any cause; both parameters were censored at the last follow-up for event-free subjects. Mann–Whitney *U*-test was used for the comparisons of continuous nonparametric data. Correlation between continuous variables was tested with linear regression and measured with R^2. Univariate and multivariate Cox regressions were used to model right-censored variables; prognostic markers were implemented in the multivariate models as continuous variables and corresponding hazard ratios (HRs) were scaled to represent third vs first quartile comparison; variables with a p value < 0.1 were used to build multivariate models. The difference in precision accuracy of different models was tested by means of Likelihood ratio test. Data were imported and handled in R v3.6.1 [15], using ggplot2, dplyr, survminer, survival and finalfit packages.

RESULTS

Patients' population

Patients' selection flow chart is depicted in Supplementary Fig. S1. The final study cohort included 135 patients with measurable disease and available baseline LB. Overall, 125 out of 135 (92.6%) patients had detectable ctDNA. The median VAF was 12.6% (interquartile range (IQR): 2.0–45.2%), whereas the median CEA value was 29.30 ng/ml (IQR: 8.51–139.10 ng/ml) and the median RECIST-defined sum of the target lesions was 80.0 mm (IQR: 50.0–117.5 mm). Median cfDNA concentration was 103.0 ng/µl (IQR: 68.4–176.5 ng/µl). Table 1 depicts the median VAF, CEA and RECIST according to the main baseline characteristics. Of note, VAF was significantly higher in patients with liver metastases and synchronous presentation; a significant imbalance in VAF distribution was observed in the two study arms despite randomisation, with higher VAF in the FU/LV and panitumumab maintenance arm. The values of VAF, CEA and sum of target lesions were linearly correlated (Fig. 1). From a qualitative point of view, *TP53* was the gene with the highest VAF in approximately half of the cohort (73 out of 135 patients, 54.1%), followed by *APC* and *PIK3CA* (31 and 6 patients, respectively; Fig. 2). Consistently, *TP53* was also the gene with the highest median VAF, whereas *RAS* mutations were subclonal, as previously described [3] (Supplementary Fig. S2).

Prognostic impact of VAF

We first evaluated the prognostic impact of VAF using the median value as cut-off. Patients with higher VAF displayed a non-significantly shorter PFS (mPFS: 9.9 vs 12.7 months; HR: 1.27, 95% confidence interval (CI): 0.89–1.82; $p = 0.181$; Fig. 3a) and a markedly and significantly poorer OS (mOS: 21.8 vs 36.5 months; HR: 1.82, 95% CI: 1.20–2.76; $p = 0.005$; Fig. 3b). Supplementary Fig. 3A depicts time-dependent areas under the characteristic curves for CEA, RECIST and VAF, whereas Supplementary Fig. 3B shows their respective OS according to the median cut-offs. The PFS benefit of adding 5-FU/LV to panitumumab in the maintenance phase was independent from VAF (Fig. 3c). Consistently with the trial's results, the maintenance treatment arm did not have a significant impact in terms of OS in both VAF subgroups (Fig. 3d). When modelling VAF as a continuous variable, the risk of death or PFS events was non-linear (Supplementary Fig. S4A), although the use of VAF quartiles did not improve the discriminative ability of this parameter (Supplementary Fig. S4B). Then, all disease burden surrogates were modelled as continuous variables in the univariate and multivariable Cox models for OS and PFS, as shown in Table 2. Notably, only VAF was significantly associated with both OS and PFS in the multivariable analyses, whereas CEA and sum of the target lesions were not.

Supplementary Table 1 shows the association of the three surrogates of disease burden with response dynamics in terms of ETS and DoR. No significant correlation of VAF, CEA and sum of the target lesions with tumour response was observed.

Finally, we investigated the prognostic value of cfDNA quantity. Patients with high and low cfDNA—using the median as a blind cut-off—did not display a significantly different outcome in terms

Table 1. Patient and disease characteristics in the overall population and distribution of the values VAF, CEA and RECIST-defined sum of the target lesions according to such baseline features.

Baseline features		Total	Median VAF (IQR)	p*	Median CEA (IQR)	p*	Median diameters (IQR)	p*
Age, years	<70	105 (77.78)	0.10 (0.02–0.47)	0.649	37.62 (12.00–169.20)	0.239	81.00 (58.00–138.00)	0.007
	≥70	30 (22.22)	0.17 (0.01–0.38)		22.75 (6.90–74.75)		60.50 (32.00–90.50)	
Sex	Male	88 (65.19)	0.14 (0.02–0.46)	0.782	29.30 (8.05–150.68)	0.921	83.50 (57.75–130.25)	0.388
	Female	47 (34.81)	0.10 (0.02–0.44)		32.60 (12.38–128.25)		77.00 (47.00–105.50)	
ECOG PS	0	96 (71.11)	0.11 (0.02–0.43)	0.351	29.30 (8.10–115.95)	0.064	79.50 (49.75–113.75)	0.5
	1	39 (28.89)	0.19 (0.02–0.53)		68.00 (15.23–346.10)		88.00 (51.50–138.00)	
Adjuvant treatment	No	112 (87.50)	0.17 (0.02–0.47)	0.053	38.31 (9.57–171.40)	0.034	81.00 (57.75–130.25)	0.041
	Yes	16 (12.50)	0.03 (0.01–0.11)		14.07 (7.41–28.55)		55.50 (40.75–83.75)	
Resection of primary	No	57 (42.22)	0.25 (0.03–0.49)	0.054	89.49 (25.80–349.90)	<0.001	98.00 (71.00–143.00)	0.001
	Yes	78 (57.78)	0.06 (0.01–0.35)		24.85 (6.46–67.85)		69.50 (46.00–95.75)	
Liver metastases	Yes	105 (77.78)	0.22 (0.03–0.49)	<0.001	67.41 (15.80–271.20)	<0.001	88.00 (65.00–138.00)	<0.001
	No	30 (22.22)	0.01 (0.01–0.04)		9.25 (4.57–29.30)		47.00 (24.25–76.00)	
Lung metastases	Yes	38 (28.15)	0.08 (0.01–0.35)	0.189	32.80 (6.58–241.50)	0.924	83.50 (48.50–136.25)	0.897
	No	97 (71.85)	0.15 (0.02–0.47)		29.30 (10.40–124.20)		79.00 (57.00–108.00)	
Lymph node metastases	Yes	38 (28.15)	0.07 (0.02–0.30)	0.243	16.48 (7.07–75.05)	0.074	76.00 (44.00–103.00)	0.291
	No	97 (71.85)	0.17 (0.02–0.47)		37.46 (12.14–234.00)		81.00 (58.00–121.00)	
Peritoneal metastases	Yes	27 (20.00)	0.05 (0.02–0.41)	0.459	27.90 (6.40–69.50)	0.138	67.00 (46.50–92.50)	0.061
	No	108 (80.00)	0.17 (0.02–0.47)		37.54 (10.28–171.40)		81.50 (55.25–130.25)	
Metastasis timing	Synchronous	110 (81.48)	0.19 (0.03–0.48)	0.001	61.80 (12.83–241.50)	0.001	81.50 (59.25–132.75)	0.024
	Metachronous	25 (18.52)	0.01 (0.01–0.06)		12.65 (6.37–29.30)		58.00 (46.00–95.00)	
No. of metastatic sites	1	74 (54.81)	0.14 (0.02–0.48)	0.455	29.30 (9.03–120.40)	0.524	78.50 (48.25–108.00)	0.426
	>1	61 (45.19)	0.10 (0.02–0.43)		33.00 (8.45–234.00)		88.00 (54.40–134.00)	
Sidedness	Left	116 (85.93)	0.14 (0.02–0.45)	0.367	30.95 (9.94–129.35)	0.699	81.00 (56.35–126.00)	0.025
	Right	19 (14.07)	0.02 (0.01–0.50)		29.30 (6.10–247.55)		67.00 (24.50–89.50)	
Treatment arm	Panitumumab plus FU/LV	69 (51.11)	0.18 (0.03–0.50)	0.028	29.30 (8.10–108.90)	0.442	79.00 (54.40–129.00)	0.716
	Panitumumab	66 (48.89)	0.06 (0.01–0.34)		37.01 (13.28–163.02)		80.50 (49.25–108.75)	

VAF variant allelic fraction, *CEA* carcinoembryonic antigen, *RECIST* Response Evaluation Criteria In Solid Tumours, *IQR* interquartile range, *ECOG PS* Eastern Cooperative Oncology Group performance status, *FU/LV* fluorouracil/leucovorin.
*Mann–Whitney test.

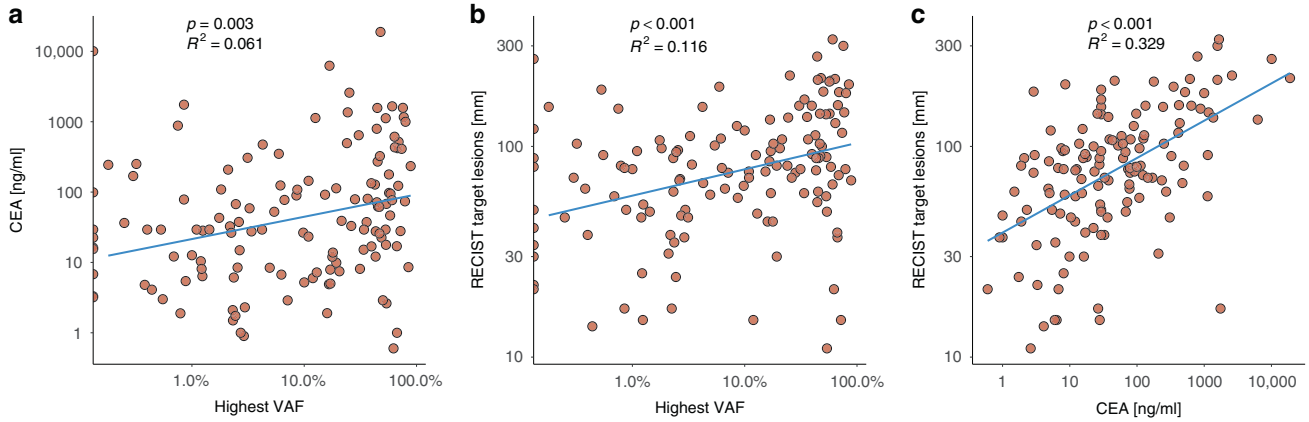

Fig. 1 Correlation of VAF, CEA and RECIST. Scatter plots showing the correlatios between **a** VAF and CEA, **b** VAF and RECIST-defined sum of the target lesions, and **c** CEA and RECIST-defined sum of the target lesions. mm millimeter, ng/ml nanogram per milliliter.

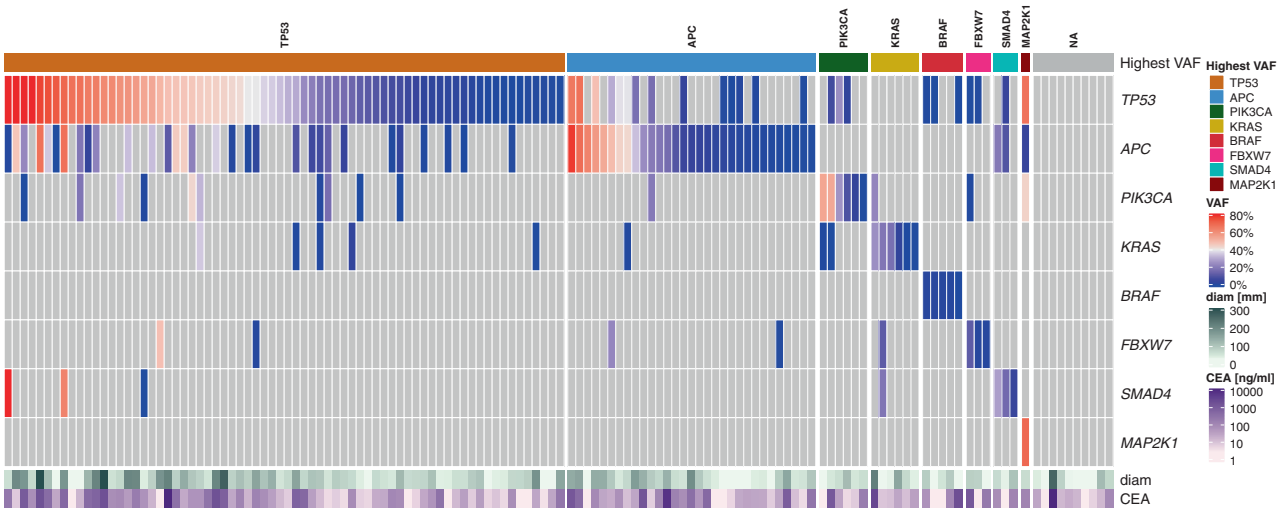

Fig. 2 Heatmap of mutations. Heatmap depicting the VAFs according to the specific gene mutations and their individual association with CEA and RECIST-defined sum of the target lesions. mm millimeter, ng/ml nanogram per milliliter.

of OS (HR: 1.52; 95% CI: 0.86–2.71; $p = 0.153$) or in terms of PFS (HR: 0.95; 95% CI: 0.57–1.58; $p = 0.839$).

DISCUSSION

This report illustrates that the amount of ctDNA as measured by VAF was a prognostic factor in patients with *RAS* wild-type mCRC eligible for initial therapy with panitumumab plus FOLFOX. The discriminative ability of VAF was higher than baseline CEA or RECIST and such comparative evaluations are warranted for this research field. VAF did not have any effect on tumour response parameters and the magnitude of its effect was rather modest in terms of PFS, whereas it was statistically and clinically meaningful in terms of OS. These outcomes may be easily explained by the fact that disease control induced by the upfront use of highly active regimens may be largely independent from tumour burden, whereas tumour burden itself is just prognostic.

In the molecular subgroup of patients included in our study, *TP53*, *APC*, *PIK3CA*, *SMAD4* and *FBXW7* were among the key genes contributing to the VAF value, as biologically supported by the high clonality of mutations in these genes during early steps in CRC development. The very low frequency of *BRAF* mutations in our data set highlights the importance of generating specific data for this molecular subgroup, particularly in the setting of novel targeted strategies [16]. We previously showed that the detection of *RAS* or *PIK3CA* mutations in baseline ctDNA was associated with poorer outcomes of patients with tissue *RAS* wild-type mCRC enrolled in the Valentino study, thus suggesting that such alterations may drive resistance to anti-EGFR therapies [3]. Given the fact that *RAS* mutations were subclonal and *PIK3CA* mutations usually display a higher degree of spatial heterogeneity compared to other mutations, the subset of patients with *RAS* or *PIK3CA* mutations did not overlap with patients with high VAF. In fact, *RAS* or *PIK3CA* determined the highest VAF in a small number ($n = 12$) of individuals. We therefore speculate that the prognostic effect of high VAF is most likely related to disease burden rather than the presence of anti-EGFR resistance mechanisms. Nevertheless, it is fascinating to hypothesise that patients with high disease burden may have an increased risk of developing resistance to EGFR inhibition because of maximal genomic heterogeneity and diversity of clonal evolution in this subgroup.

Most of the previous reports did not aim to quantify the exact amount of ctDNA and used a rather limited subset of genes [17–22]. Also, previous studies stratified the patients according to the presence or absence of mutations in ctDNA [23] or used the total quantity of circulating-free DNA [24]. Our study estimated the quantity of ctDNA by means of VAF as previously proposed by other groups who focussed on mixed cohorts of advanced solid tumours [8] or small data sets of mCRC patients [9, 25, 26].

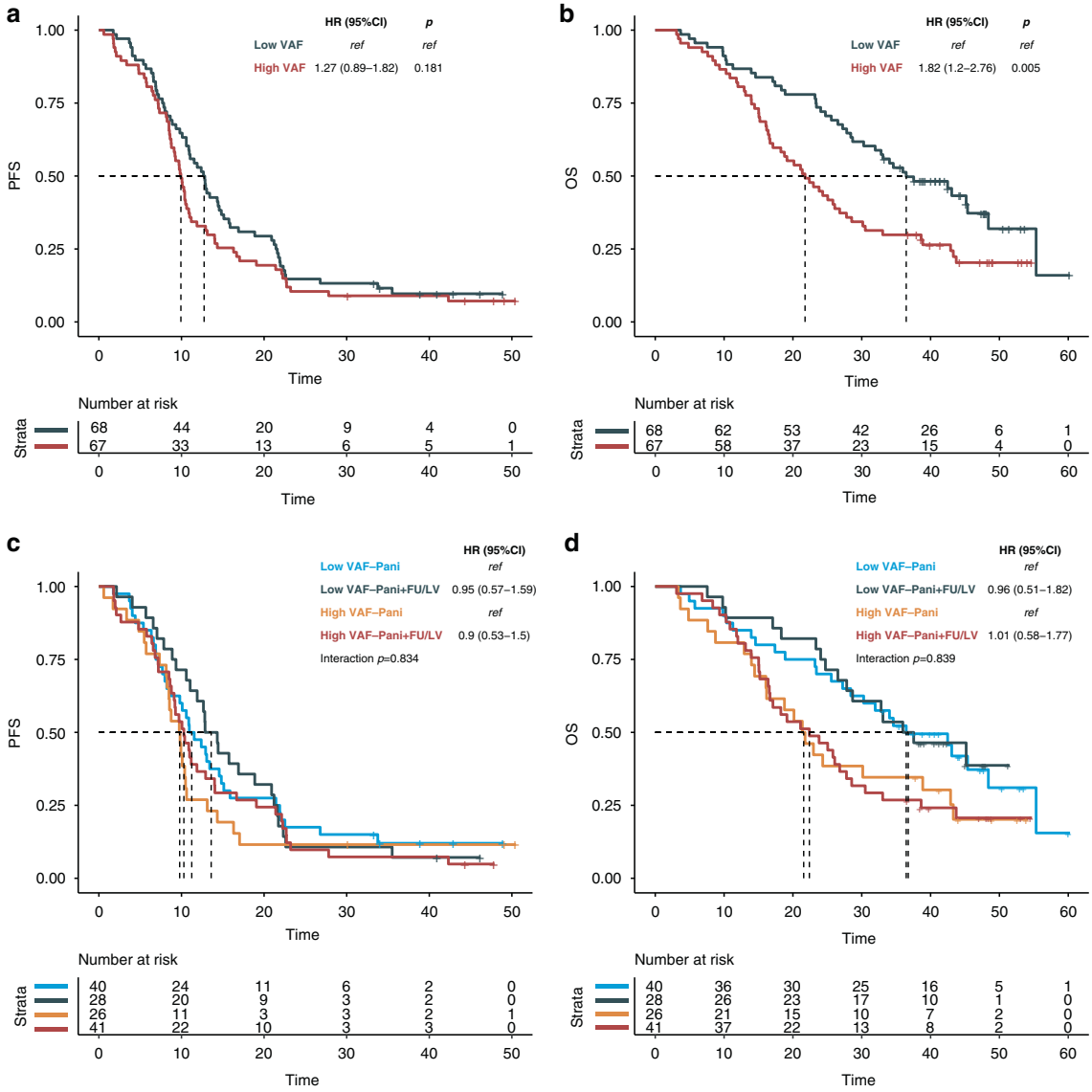

Fig. 3 Impact of VAF on PFS and OS. Kaplan–Meier curves showing OS and PFS according to VAF (**a**, **b**) and interaction between VAF and maintenance treatment arm (**c**, **d**).

Considering that the assessment of baseline VAF is easy and rapid, this marker has translational relevance and several potential clinical applications: first, it might be used as a stratification factor for future randomised trials or as a selection criterion to guide patients' enrolment in trials on intensified upfront regimens, as done in the VISNÙ-1 trial in patients with three or more baseline circulating tumour cells [27]; second, post hoc analyses of randomised studies investigating FOLFOXIRI-based regimens [28, 29] might reveal a predictive role of ctDNA burden and, reasonably, a relatively higher benefit of chemotherapy intensification in the high VAF subgroup. Our study does not allow to draw any conclusions on the cost-effectiveness of VAF in the clinical practice, especially considering the low costs and reproducibility of CEA dosing. Nevertheless, the assessment of VAF may be of relevance for patients with baseline CEA values within the normal ranges. As a matter of fact, ctDNA tests commercialised for non-invasive molecular profiling may allow the concomitant evaluation of VAF. Just a small proportion of patients in our cohort had undetectable ctDNA and this event may not only be related to non-shedding properties of specific histotypes or metastatic sites or to pre-analytical variables but also to low disease burden or to a multiplicity of such factors.

Our study has major limitations. First, the lack of longitudinal assessment of VAF dynamics. Indeed, ctDNA levels decrease earlier than CEA during chemotherapy [23] and may be used as an early marker to monitor the rapidity and depth of tumour response, thus anticipating radiological reassessment [10]. However, ctDNA is undetectable within 4 weeks in almost all patients with RECIST response [26]. Therefore, since the VALENTINO trial scheduled the first on-treatment plasma samples only at 8-week timepoint—concomitantly with radiological re-assessment—we could not assess the prognostic effect of early VAF changes. Second, the widespread use of LB assays for blood monitoring and genotyping may allow physicians to evaluate disease progression and its molecular drivers. Again, the clinical usefulness of repeated LB testing should be established thanks to prospectively validation, especially regarding additional value of such monitoring compared to standard radiology. More intriguingly, rise and falls of ctDNA levels or VAF could inform the design of clinical trials, especially those investigating stop&go chemotherapy and

Table 2. Univariate and multivariate regressions for OS and PFS; features with a $p < 0.1$ in the univariate analyses were used to build the multivariate models.

		OS		PFS	
		HR (univariable)	**HR (multivariable)**	**HR (univariable)**	**HR (multivariable)**
Age	<70	—	—	—	—
	≥70	1.09 (0.68–1.76, $p = 0.711$)	—	1.00 (0.65–1.52, $p = 0.994$)	—
Sex	Male	—	—	—	—
	Female	1.25 (0.82–1.91, $p = 0.304$)	—	1.34 (0.93–1.94, $p = 0.115$)	—
ECOG PS	0	—	—	—	—
	1	1.57 (1.01–2.43, $p = 0.044$)	1.66 (1.05–2.64, $p = 0.030$)	1.34 (0.91–1.97, $p = 0.144$)	—
Adjuvant	No	—	—	—	—
	Yes	0.66 (0.33–1.33, $p = 0.249$)	—	1.11 (0.66–1.89, $p = 0.692$)	—
CEA [ng/ml][a]	8.5	—	—	—	—
	139.1	1.01 (0.99–1.02, $p = 0.334$)	—	1.00 (0.99–1.01, $p = 0.761$)	—
Target lesion size [mm][a]	50	—	—	—	—
	117.5	1.15 (0.94–1.42, $p = 0.174$)	—	1.10 (0.91–1.32, $p = 0.327$)	—
Highest VAF[a]	2%	—	—	—	—
	45.2%	1.46 (1.06–2.01, $p = 0.022$)	1.53 (1.09–2.13, $p = 0.013$)	1.28 (0.95–1.73, $p = 0.092$)	1.38 (1.02–1.87, $p = 0.035$)
No. of metastatic sites	1	—	—	—	—
	>1	2.20 (1.45–3.33, $p < 0.001$)	2.23 (1.45–3.40, $p < 0.001$)	1.74 (1.21–2.48, $p = 0.003$)	1.89 (1.31–2.72, $p = 0.001$)
Sidedness	Left	—	—	—	—
	Right	2.17 (1.28–3.69, $p = 0.004$)	2.41 (1.39–4.17, $p = 0.002$)	1.90 (1.15–3.14, $p = 0.013$)	2.00 (1.20–3.32, $p = 0.008$)
Metastases timing	Synchronous	—	—	—	—
	Metachronous	0.60 (0.34–1.07, $p = 0.085$)	0.60 (0.33–1.09, $p = 0.091$)	0.84 (0.53–1.33, $p = 0.460$)	—

[a]These features were considered as numeric; reported values represent the first and the third quartiles.

pulsatile EGFR inhibition. Third, our data cannot be translated to the population of patients with microsatellite instability-high or *RAS*-mutated mCRC, who are currently treated with other first-line standard regimens: immunotherapy and chemotherapy +/− bevacizumab, respectively.

In conclusion, this report shows that VAF is a promising prognostic marker potentially related to initial disease burden in patients with *RAS* wild-type mCRC eligible for anti-EGFR-based upfront regimens. Efforts on harmonisation of the results of specific assays and implementation in clinical trials should be made to adequately interpret the results of LBs in patients with advanced disease.

CODE AVAILABILITY

Codes can be made available upon request to the corresponding author.

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AUTHOR CONTRIBUTIONS

PM and FP designed the work, interpreted the results and drafted the original version of the manuscript. FP conceived the work. All authors acquired data, revised the manuscript, approved the final version and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Institutional review board approval was obtained from all participating Centres and all patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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