The Added Value of Baseline Circulating Tumor DNA Profiling in Patients with Molecularly Hyperselected, Left-sided Metastatic Colorectal Cancer

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ABSTRACT

Purpose: The routine use of liquid biopsy is not recommended for the choice of initial treatment for patients with metastatic colorectal cancer (mCRC).

Experimental Design: We included patients with left-sided, *RAS/BRAF* wild-type, HER2-negative, and microsatellite stable mCRC, treated with upfront panitumumab/FOLFOX-4 in the Valentino study. We performed amplicon-based genomic profiling of 14 genes in baseline plasma samples and compared these data with tumor tissue ultra-deep sequencing results. Specific gene mutations in circulating tumor DNA (ctDNA) and their clonality were associated with progression-free survival (PFS), overall survival (OS), and radiological dynamics.

Results: Ten and 15 of 120 patients had a mutation of *RAS* and *PIK3CA* in ctDNA, with a positive concordance with tissue deep sequencing of only 31.3% and 47.1%, respectively. Presence of *RAS* or *PIK3CA* mutations in baseline ctDNA was associated with worse median PFS [8 vs. 12.8 months; HR, 2.49; 95% confidence interval (CI), 1.28–4.81; $P = 0.007$ and 8.5 vs. 12.9 months; HR, 2.86; 95% CI, 1.63–5.04; $P < 0.001$] and median OS (17.1 vs. 36.5 months; HR, 2.26; 95% CI, 1.03–4.96; $P = 0.042$ and 21.1 vs. 38.9 months; HR, 2.18; 95% CI, 1.16–4.07; $P = 0.015$). *RAS* mutations in ctDNA were associated with worse RECIST response, early tumor shrinkage, and depth of response, while *PIK3CA* mutations were not. Patients with higher levels of *RAS/PIK3CA* variant allele fraction (VAF) in ctDNA had the worst outcomes (VAF ≥ 5% vs. all wild-type: median PFS, 7.7 vs. 13.1 months; HR, 4.02; 95% CI, 2.03–7.95; $P < 0.001$ and median OS, 18.8 vs. 38.9 months; HR, 4.07; 95% CI, 2.04–8.12; $P < 0.001$).

Conclusions: Baseline ctDNA profiling may add value to tumor tissue testing to refine the molecular hyperselection of patients with mCRC for upfront anti-EGFR–based strategies.

Introduction

Anti-EGFR mAbs, cetuximab and panitumumab, are guideline-recommended treatments for patients with *RAS/BRAF* wild-type metastatic colorectal cancer (mCRC; ref. 1). Both primary tumor sidedness and molecular hyperselection beyond *RAS* and *BRAF* status allow to select patients with the highest likelihood of benefit from EGFR inhibition, that is, those with left-sided tumors and absence of rare genomic alterations associated with primary resistance [*HER2/MET* amplification, gene fusions, *PIK3CA* mutations, and microsatellite instability (MSI); refs. 2, 3]. Tumor tissue profiling still represents the mainstay for the detection of such alterations, but it is limited by spatial and temporal genomic heterogeneity.

In the setting of acquired resistance to EGFR blockade, liquid biopsy overcomes such limitations as it allows to capture the dynamics of clonal evolution with greater precision compared with tumor rebiopsies (4, 5). Specifically, the emergence of resistance alterations (more frequently involving *RAS*) under the selective pressure of therapy may be related to the expansion of preexisting resistant tumor subclones, while their decay during postprogression treatments sustains the use of liquid biopsy to select patients with potential benefit from anti-EGFR retreatment (6, 7).

Nevertheless, limited data are available on the role of liquid biopsy to predict the outcomes of patients clinically eligible for an anti-EGFR–based upfront treatment (ref. 8; Supplementary Table S1). Indeed, some mechanisms of primary resistance to anti-EGFR therapy, such as *HER2* amplification or gene fusions, are usually clonal alterations with the role of oncogenic drivers and may be easily assessed in tumor

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

In this prespecified exploratory analysis of the Valentino trial, next-generation sequencing of circulating tumor DNA (ctDNA) obtained from baseline liquid biopsies allowed the detection of new *RAS* or *PIK3CA* mutations in patients treated with upfront anti-EGFR therapy for metastatic colorectal cancer (mCRC). Patients with left-sided, hyperselected mCRC receiving panitumumab/FOLFOX-4 and with *RAS/PIK3CA* mutations in ctDNA had worse outcomes, particularly those with variant allele fraction ≥5%. Liquid biopsy may refine the molecular selection of patients with highest likelihood of benefit from EGFR blockade as compared with tumor tissue profiling alone and it may guide decisions on upfront therapy in patients with mCRC.

tissue. Other alterations, such as *RAS* and *PIK3CA* mutations, may be subclonal, with a variant allele fraction (VAF) below 5% or even 1%, and therefore may be missed by several standard assays and only detectable by high-sensitivity techniques, such as next-generation sequencing (NGS) with high-depth coverage (3). On top of this, such mutations may also be highly heterogeneous from a spatial point of view, with the chance of being detected only by means of liquid biopsy. Therefore, genomic profiling of baseline circulating tumor DNA (ctDNA) may increase the detection of anti-EGFR resistance alterations and it may allow to quantify their clonality and VAF, with potential association to treatment outcomes (4, 8).

In this prespecified exploratory analysis of the Valentino trial (9), we aimed at assessing the added value of baseline ctDNA NGS profiling in patients with molecularly hyperselected, left-sided mCRC receiving a panitumumab-based upfront treatment strategy.

Materials and Methods

Patients' cohort

The Valentino study (NCT02476045) was a multicenter, randomized, open-label phase II trial that investigated the progression-free survival (PFS) noninferiority of maintenance with panitumumab (arm B) versus panitumumab plus 5-FU/LV (arm A), following an induction treatment with panitumumab/FOLFOX-4 in patients with *RAS* wild-type mCRC (9). In patients signing an optional consent form, liquid biopsies 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 prespecified exploratory analysis, we included all clinically evaluable randomized patients with available baseline liquid biopsies, and molecularly hyperselected (*RAS* and *BRAF* wild-type based on standard assays, HER2-negative, and microsatellite stable status) and left-sided tumors, who were therefore highly enriched for EGFR-dependent cells in their tumors' bulk.

RECIST 1.1 response, early tumor shrinkage (ETS), and depth of response (DoR) were assessed by blinded independent central review, as described previously (10). Available tumor profiling data included: *HER2* status assessed by both IHC and silver ISH, MSI status by multiplex PCR, and presence of *RAS* mutations with low VAF (i.e., <5%, and therefore missed by several standard assays or usually filtered in standard NGS algorithms) and *PIK3CA* mutations by centrally performed ultra-deep NGS (3).

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

NGS of plasma samples

Cell-free DNA (cfDNA) was isolated and quantified from 1.5 mL of plasma using Maxwell RSC cfDNA Plasma Kit (Promega) and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Targeted libraries were performed using Oncomine Colon cfDNA Assay (Thermo Fisher Scientific) that amplifies 48 amplicons covering about 240 key hotspot mutations from 14 genes that are frequently mutated in colorectal cancers (*AKT1, APC BRAF, CTNNB1, EGFR, ERBB2, FBXW7, GNAS, KRAS, MAP2K1, NRAS, PIK3CA, MAD4*, and *TP53*). An amount of 2–50 ng of cfDNA was used to prepare targeted libraries; briefly, each cfDNA molecule was assigned with a unique molecular tag through a first PCR in a VeritiTM thermal cycler and subsequently, tagged library fragments were amplified in a second round of PCR to produce independent barcoded libraries. Sequencing was carried out on the Ion GeneStudioTM S5 prime system. For sensitive variant detection down to 0.1% VAF, we targeted a median read coverage > 25,000, median molecular coverage > 2,500, 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.

Statistical analysis

No formal statistical hypothesis was done due to the exploratory nature of this study. PFS was defined as the time from randomization to documentation of progressive disease/death; overall survival (OS) was defined as the time from randomization to death from any cause. Both parameters were censored at the last follow-up for event-free subjects. χ^2 and Fisher exact tests were used to test the distribution of categorical data. Mann–Whitney U test was used for the comparisons of continuous nonparametric data. Univariate Cox regressions were used to model right-censored variables. Unidimensional k-means algorithm was used to model the VAF cutoff of relevant resistance mutations and their correlation with outcomes. Data were imported and handled in R v3.6.1, using ggplot2, dplyr, survminer, survival, and finalfit packages (11).

Results

Patients population

Supplementary Fig. S1 shows flow chart of the patients included in this study. A total of 120 patients with available and evaluable plasma samples had left-sided, *RAS* and *BRAF* wild-type, HER2-negative, and microsatellite stable (MSS) cancers. **Figure 1A** shows the heatmap of all the alterations detected in ctDNA in the final cohort. Regarding genes with putative role in resistance to anti-EGFR therapy, 10 and 15 patients had *RAS* and *PIK3CA* mutations in liquid biopsy, respectively, with only 4 patients among these showing concomitant *RAS* and *PIK3CA* mutations. **Table 1** shows the specific *RAS* and/or *PIK3CA* mutations, as well as "founder"/clonal mutations, such as *TP53* or *APC*, with the VAF of each mutation in ctDNA, and *RAS* and *PIK3CA* status in matching tumor tissue. Supplementary Fig. S2 reports the VAF distribution for each mutation, reaching the highest median value for *TP53* and the lowest for *RAS*, while Supplementary Fig. S3 shows the VAF distribution of relevant resistance alterations in *RAS* and *PIK3CA*, which was used to build the VAF cutoff for survival analyses. Supplementary Table S2 shows the patients' and disease baseline

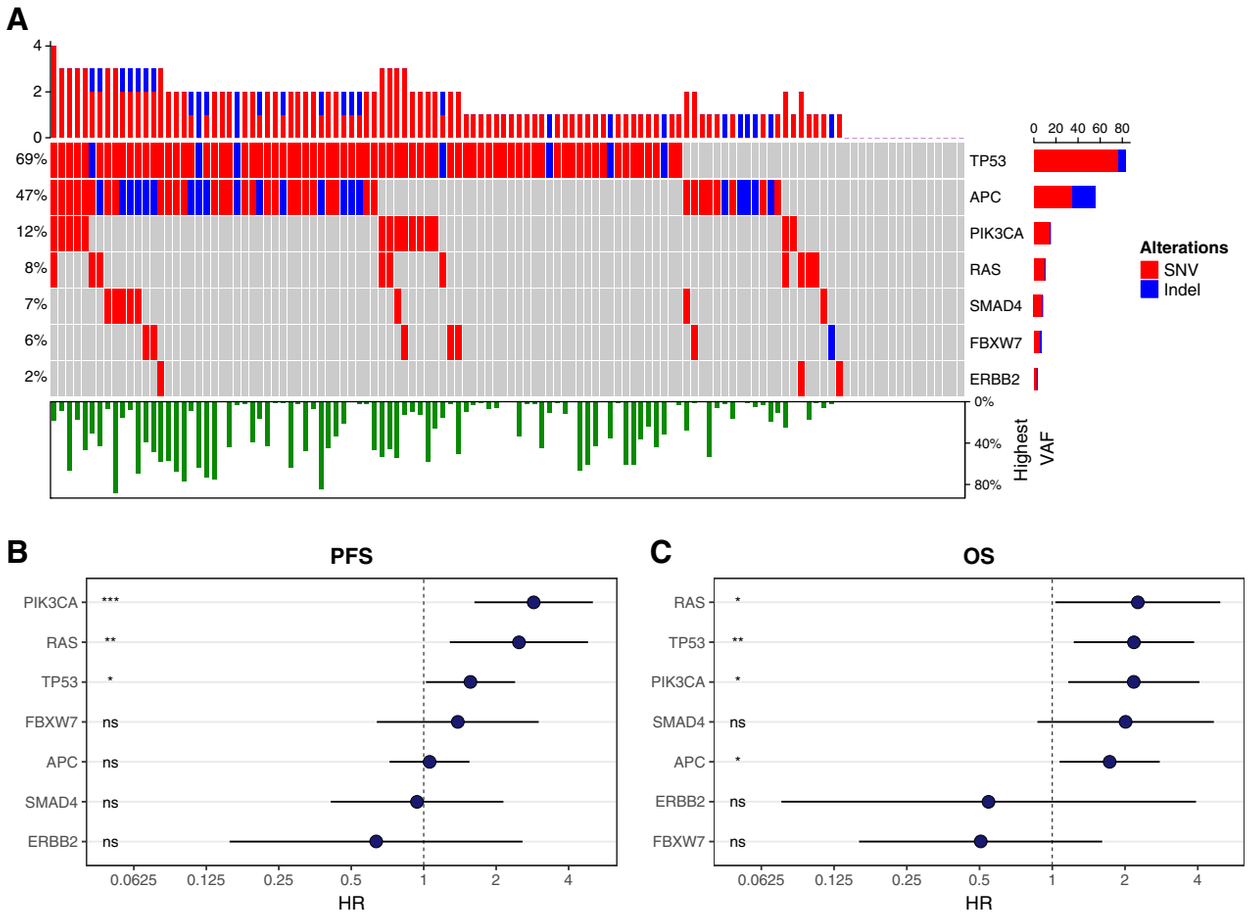

Figure 1.

A, Heatmap representing the gene mutations found in liquid biopsy of patients with hyperselected mCRC included in the study cohort; bottom annotation shows the maximum VAF for each sample. **B** and **C**, The HRs for progression and death with respective CIs for each gene mutation (ns, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

characteristics in the final study cohort, without significant differences according to *RAS* or *PIK3CA* mutational status in liquid biopsy.

Prognostic impact of mutations in ctDNA

Figure 1B and **C** resume the impact of each gene mutation detected in ctDNA in terms of PFS and OS, respectively. Regarding genes with putative role in resistance to anti-EGFR therapy, both PFS and OS were significantly worse in patients bearing a *RAS* mutation in ctDNA versus *RAS* wild-type subgroup [median PFS (mPFS), 8 vs. 12.8 months; HR, 2.49; 95% confidence interval (CI), 1.28–4.81; $P = 0.007$; and median OS (mOS), 17.1 vs. 36.5 months; HR, 2.26; 95% CI, 1.03–4.96; $P = 0.042$; **Fig. 2A** and **B**]. Similar results were observed in patients bearing a *PIK3CA* mutation in ctDNA versus wild-type subgroup (mPFS, 8.5 vs. 12.9 months; HR, 2.86; 95% CI, 1.63–5.04; $P < 0.001$ and mOS, 21.1 vs. 38.9 months; HR, 2.18; 95% CI, 1.16–4.07; $P = 0.015$; **Fig. 2C** and **D**). Despite the low numerosity, a trend for a worse PFS and OS could be observed in patients bearing simultaneously *PIK3CA* and *KRAS* mutations compared with patients carrying only one of them (Supplementary Fig. S4). **Table 2** shows the impact of *RAS* and *PIK3CA* status in ctDNA in terms of tumor response dynamics; notably, the presence of *RAS* mutations in baseline ctDNA was significantly associated with overall response rate according to RECIST v1.1. and ETS, and trended to significance for association with DoR, while *PIK3CA* status was not associated with these parameters.

Comparison of liquid biopsy and tumor tissue NGS

All 120 patients had available tissue NGS deep-sequencing data, as reported previously (3). Supplementary Fig. S5 shows the concordance between liquid biopsy and tissue NGS in terms of *RAS* (Supplementary Fig. S5A) and *PIK3CA* (Supplementary Fig. S5B) mutations. Overall concordance was 109 of 120 (90.8%) for *RAS* status and 111 of 120 (92.5%) for *PIK3CA* status; however, positive concordance in mutated samples (in liquid biopsy and/or tumor tissue) was only 5 of 16 (31.3%) for *RAS* mutations and 8 of 17 (47.1%) for *PIK3CA* mutations. Patients with *RAS* or *PIK3CA* mutations found only in tumor tissue and not in liquid biopsy were very few and their outcomes could not be investigated as a unique subgroup, although they did not seem to show poorer individual outcomes (Supplementary Fig. S6).

Role of *RAS* and *PIK3CA* VAF in liquid biopsy

Median VAFs of *RAS* and *PIK3CA* mutations in liquid biopsy were 1.3% [interquartile range (IQR), 1.1%–14.3%] and 17.1% (IQR, 1.3%–29.3%), respectively. Expectedly, the median VAF of such mutations in liquid biopsy was higher in patients with positive concordance with

Table 1. *RAS* mutations, *PIK3CA* mutations, and founder/clonal mutations with their corresponding VAF for each sample.

Patient ID	Plasma status of KRAS exons 2,3, and 4	Plasma status of NRAS exons 2,3, and 4	Plasma status of PIK3CA (exons 9 and 20)	Type of mutation	Plasma KRAS, NRAS, or PIK3CA mA%	Plasma-associated mutated genes	Type of mutation	mA%	Tumor tissue status of KRAS or NRAS (exons 2, 3, and 4) and PIK3CA (exons 9 and 20) by NGS	Site of tissue sample	Time lapse between tissue and blood collection (months)
001–005	Wild-type	Wild-type	Mutated (exon 9)	p.E545K (c.1633G>A)	0.87%	TP53 (exon 8)	p.R273H (c.818G>A)	8.52%	Wild-type	P	1.3
001–017	Wild-type	Wild-type	Mutated (exon 20)	H1047R (c.3140A>G)	1.16%	FBXW7 (exon 9) TP53 (exon 8)	p.R505C (c.1513C>T) p.C275Y (c.824G>A)	12.58% 0.32%	Wild-type	P	1.5
001–023	Wild-type	Wild-type	Mutated (exon 9)	p.E542K (c.1624G>A)	0.30%	None	NA	NA	Wild-type	P	0.6
001–028	Mutated (exon 2)	Wild-type	Mutated (exon 9)	KRAS: G12V (c.35G>T) PIK3CA: p.E545K (c.1633G>A)	KRAS: 25.24% PIK3CA: 23.23%	None	NA	NA	KRAS: G12V (exon 2) PIK3CA: E545K (exon 9)	P	3.1
001–031	Mutated (exon 2)	Wild-type	Wild-type	G12D (c.35G>A)	17.80%	None	NA	NA	KRAS: G12D (exon 2)	P	0.9
001–041	Wild-type	Mutated (exon 2)	Mutated (exon 9)	NRAS: G13C (c.37G>T) PIK3CA: p.E545K (c.1633G>A)	NRAS: 0.02% PIK3CA: 53.18%	TP53 (exon 7)	p.T230fs (c.688dupA)	8.10%	PIK3CA E545K (exon 9)	M	2.5
001–043	Mutated (exon 4)	Wild-type	Wild-type	p.A146T (c.436G>A)	1.12%	APC (exon 15) TP53 (exon 7)	p.Q1291* (c.3871C>T) p.N235KfsTer12 (c.705_706delCTinsA)	10.40% 30.58%	Wild-type	P	1.8
004–010	Wild-type	Wild-type	Mutated (exon 9)	p.E542K (c.1624G>A)	18.76%	TP53 (exon 8) APC (exon 15)	p.R267SfsTer73 (c.799_815delCGGAA-CAGCTTTGAGGTinsA) p.S932* (c.2795C>G)	66.76% 19.26%	PIK3CA: E542K (exon 9)	P	1.4
004–025	Wild-type	Wild-type	Mutated (exon 20)	H1047L (c.3140A>T)	34.80%	TP53 (exon 5)	p.R175H (c.524G>A)	58.64%	PIK3CA: H1047L (exon 20)	P	1.7
004–033	Wild-type	Mutated (exon 3)	Wild-type	Q61H (c.183A>T)	3.86%	TP53 (exon 7)	p.S241del (c.721_723delTCC)	15.32%	Wild-type	P	6.9
007–002	Wild-type	Wild-type	Mutated (exon 9)	p.E545K (c.1633G>A)	1.50%	TP53 (exon 8) SMAD 4 (exon 9)	p.R282W (c.844C>T) p.D351V (c.1052A>T)	53.95% 0.21%	Wild-type	P	2.8
007–009	Wild-type	Wild-type	Mutated (exon 9)	p.E542K (c.1624G>A)	17.05%	APC (exon 15) TP53 (exon 5)	p.Q1367* (c.4099C>T) p.P152L (c.455C>T)	14.00% 17.11%	PIK3CA: E542K (exon 9)	P	1.9
008–008	Wild-type	Wild-type	Mutated (exon 9)	p.E545K (c.1633G>A)	43.00%	APC (exon 15) TP53 (exon 7)	p.Q1406* (c.4216C>T) p.M246T (c.737T>C)	22% 46.6%	PIK3CA: E454K (exon 9)	P	1.4
008–009	Wild-type	Wild-type	Mutated (exon 20)	H1047R (c.3140A>)	26.23%	BRAF (exon 15) TP53 (exon 5) TP53 (exon 8)	p.V600E (c.1799T>A) p.R175H (c.524G>A) p.R273C (c.817C>T)	0.18% 0.07% 25.11%	PIK3CA: H1047R (exon 20)	P	1.2
008–010	Mutated (exon 4)	Wild-type	Wild-type	A146T (c.436G>A)	0.25%	ERBB2 (exon 8)	p.L313I (c.937C>A)	0.22%	Wild-type	P	2.4

(Continued on the following page)

Table 1. *RAS* mutations, *PIK3CA* mutations, and founder/clonal mutations with their corresponding VAF for each sample. (Cont'd)

Patient ID	Plasma status of KRAS exons 2,3, and 4	Plasma status of NRAS exons 2,3, and 4	Plasma status of PIK3CA (exons 9 and 20)	Type of mutation	Plasma KRAS, NRAS, or PIK3CA mA%	Plasma-associated mutated genes	Type of mutation	mA%	Tumor tissue status of KRAS or NRAS (exons 2, 3, and 4) and PIK3CA (exons 9 and 20) by NGS	Site of tissue sample	Time lapse between tissue and blood collection (months)
015–006	Mutated (exon 2)	Wild-type	Mutated (exon 20)	KRAS: G12D (c.35G>A) PIK3CA: H1047R (c.3140A>G)	KRAS:1% PIK3CA: 2%	APC (exon 15) TP53 (exon 5) TP53 (exon 5)	p.E1408* (c.4222G>T) p.V157F (c.469G>T) p.R175H (c.524G>A)	8% 1.43% 18%	KRAS: G13S (exon 2)	P	20.7
015–008	Wild-type	Wild-type	Mutated (exon 20)	H1047V (c.3134A>T) and H1047R (c.3140A>G)	10% 10%	TP53 (exon 8)	p.R248Q (c.743G>A)	4.41%	PIK3CA: D1045V (exon 20) and PIK3CA: H1047R (exon 20)	P	1.3
020–001	Mutated (exon 2)	Wild-type	Wild-type	G12A (c.35G>C)	1.30%	APC (exon 15) TP53 (exon 7)	p. A1305QfsTer3 (c.3913delG) p.G245S (c.733G>A)	43.20% 38.92%	Wild-type	P	2.5
024–001	Mutated (exon 2)	Wild-type	Mutated (exon 20)	KRAS: G12D (c.35G>A) PIK3CA: H1047R (c.3140A>G)	KRAS: 35.39% PIK3CA: 32.40%	TP53 (exon 5)	p.V157F (c.469G>T)	45.42%	KRAS: G12D (exon 2); PIK3CA: H1047R (exon 20)	M	1.2
025–002	Mutated (exon 3)	Wild-type	Wild-type	p.Q61H (c.183A>C)	1.19%	None	NA	NA	KRAS: Q61H (exon 3)	P	38
025–006	Wild-type	Wild-type	Mutated (exon20)	H1047R (c.3140A>G)	0.84%	TP53 (exon 5) TP53 (exon 5)	p.R158L (c.473G>T) p.V157F (c.469G>T)	0.49% 12.93%	Wild-type	M	1.8

Abbreviations: M, metastasis; mA%, mutation load; NA, not applicable; NGS, next-generation sequencing; P, primary tumor.

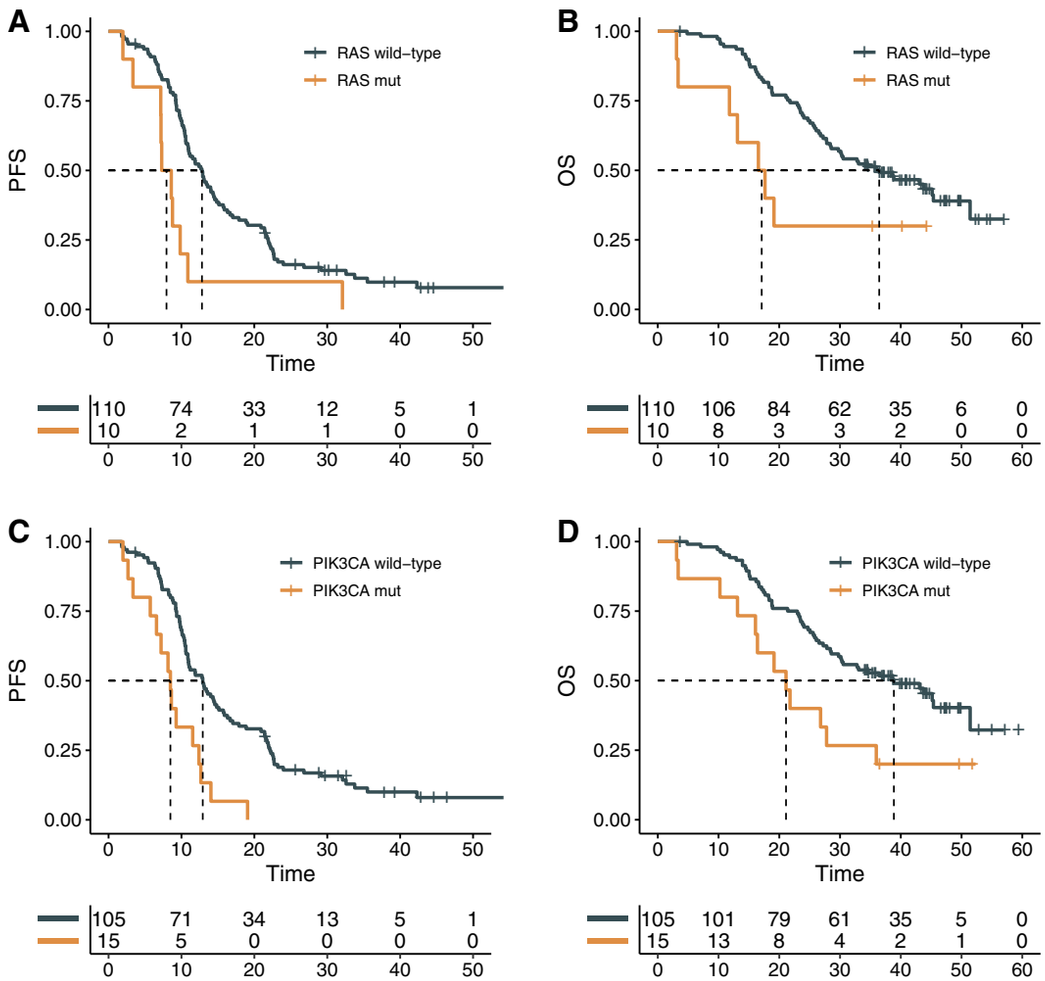

Figure 2.

Kaplan–Meier curves representing PFS and OS for, respectively, *RAS* mutated (mut) versus wild-type status in baseline ctDNA (**A** and **B**) and *PIK3CA* mutated versus wild-type status in baseline ctDNA (**C** and **D**).

tissue NGS versus those with undetectable tissue mutations (median *RAS* VAF, 17.8% vs. 1.1% and median *PIK3CA* VAF, 27.8% vs. 1.2%; Mann–Whitney test $P = 0.056$ and $P = 0.004$; **Fig. 3A** and **D**). *RAS* VAF was also significantly associated with ETS ($P = 0.011$; **Fig. 3B**) and DoR ($P = 0.073$; **Fig. 3C**). Such associations for *PIK3CA* VAF status were not significant (**Fig. 3E** and **F**).

Exploiting the VAFs cutoff derived upon their distribution, we then split patients with *RAS* and/or *PIK3CA* mutations with VAF of at least 5% in liquid biopsy ("clonal" subgroup) versus those with mutations with VAF < 5% ("subclonal" subgroup) and those without mutations in ctDNA. Compared with the all wild-type one, the "clonal" subgroup had the worst PFS (mPFS, 7.7 vs. 13.1;

Table 2. Summary of overall response rate according to RECIST v1.1, ETS, and DoR according to *RAS* and *PIK3CA* mutational status in baseline ctDNA.

		N (%)	RECIST response	No RECIST response	*P*§	ETS	No ETS	*P*§	Median DoR % reduction (IQR)	*P**
RAS	Wild-type	110 (91.7)	92 (86.0)	15 (14.0)	0.039	75 (75.0)	25 (25.0)	0.023	0.47 (0.38–0.60)	0.072
	Mutated	10 (8.3)	5 (55.6)	4 (44.4)		3 (33.3)	6 (66.7)		0.17 (0.16–0.47)	
PIK3CA	Wild-type	105 (87.5)	86 (85.1)	15 (14.9)	0.267	70 (74.5)	24 (25.5)	0.169	0.47 (0.38–0.59)	0.305
	Mutated	15 (12.5)	11 (73.3)	4 (26.7)		8 (53.3)	7 (46.7)		0.43 (0.21–0.57)	

Note: RECIST response was available for 116 of the 120 patients; ETS and DoR were available for 109 of the 120 patients.
§χ^2 test or Fisher test as appropriate.
*Mann–Whitney test.

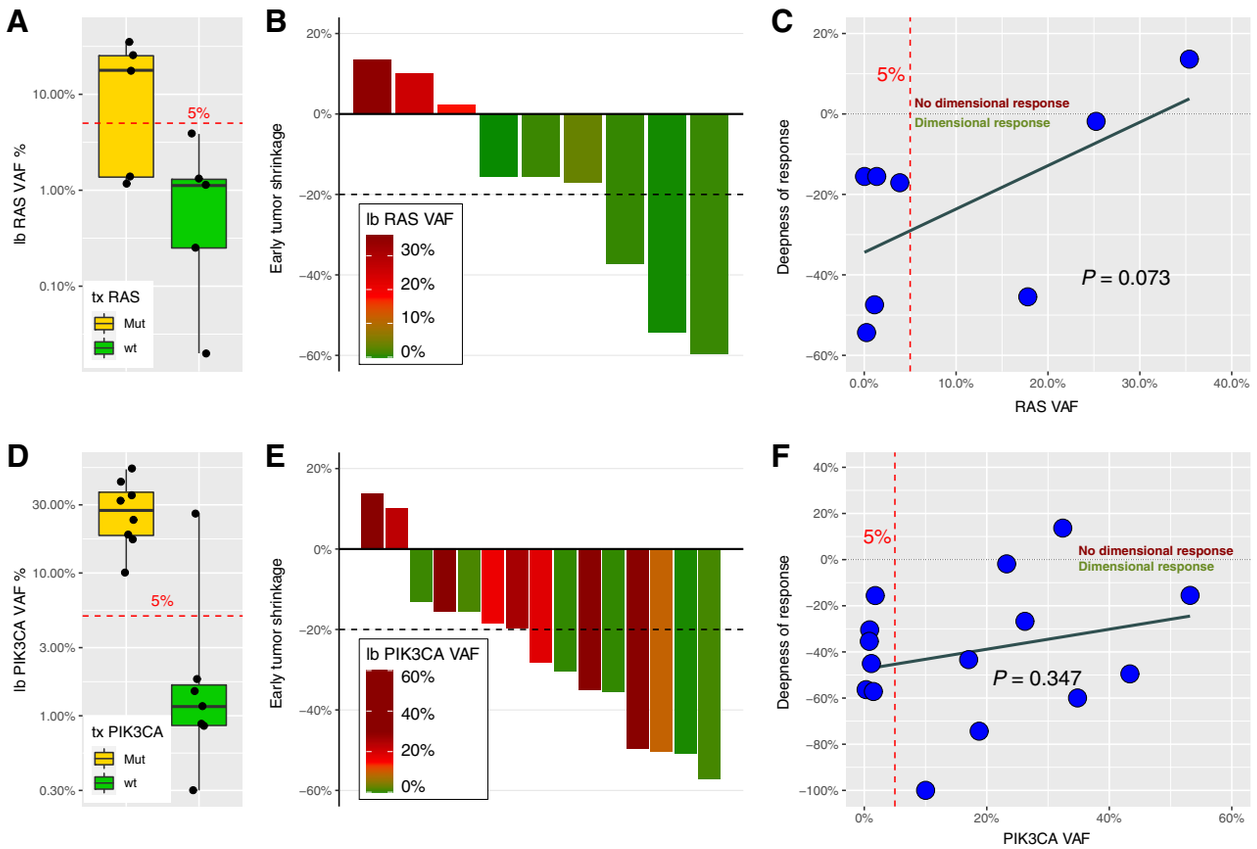

Figure 3.

A, Box plot depicting *RAS* VAF according to the concomitant detection of *RAS* mutation in the matched tumor tissue. **B,** Waterfall plot showing the relationship between ETS and *RAS* VAF. **C,** Scatter plot showing the correlation of *RAS* VAF with DoR. **D,** Box plot depicting *PIK3CA* VAF according to the concomitant detection of *PIK3CA* mutation in the matched tumor tissue. **E,** Waterfall plot showing the relationship between ETS and *PIK3CA* VAF. **F,** Scatter plot showing the correlation of *PIK3CA* VAF with DoR. mut, mutated; wt, wild-type.

HR, 4.02; 95% CI, 2.03–7.95; $P < 0.001$; **Fig. 4A**) and OS (mOS, 18.8 vs. 38.9; HR, 4.07; 95% CI, 2.04–8.12; $P < 0.001$; **Fig. 4B**).

Discussion

Although genomic alterations of primary resistance to EGFR inhibition may be detected in ctDNA of patients with mCRC and potentially missed by genomic profiling of matched tumor tissue, their clinical significance and usefulness remain largely unknown. When focusing on *RAS* testing, pivotal cohort studies showed a good, but not perfect concordance between plasma and tumor genotyping (7, 12–19). In fact, despite the use of tumor profiling assays with high sensitivity for the detection of subclonal alterations, only the use of liquid biopsy may overcome the issue of spatial heterogeneity. On the opposite side, liquid biopsy may have meaningful limitations in patients with low tumor burden and poorly shedding cancers, such as those with peritoneal/lymph nodal lesions and mucinous histotype. Exploratory analyses of the aforementioned studies suggested that *RAS* testing in liquid biopsy results in similar clinical outcomes when compared with tissue testing in patients treated with anti-EGFR–based regimens. However, such evidence was limited by the small sample size, the variety of treatment lines and regimens, and, above all, the lack of survival comparison between subgroups (12, 16–18, 20). Moving forward from this background, Normanno and colleagues showed that, in a subgroup of 92 patients with *KRAS* exon 2 wild-type mCRC receiving upfront FOLFIRI plus cetuximab in the frame of CAPRIGOIM trial, patients with *RAS* mutations detected in plasma or in tumor tissue had significantly worse outcomes versus wild-type counterparts, but without differences in the discriminative ability of plasma versus tissue testing (19).

With the aim of providing new evidence on the potential added value of baseline liquid biopsy genotyping in the first-line setting, we took advantage of the all-*RAS* and *BRAF* wild-type patients' population enrolled in the Valentino study, who received panitumumab/FOLFOX-4 upfront strategy (9) and had been characterized previously by tissue deep sequencing (3). Moreover, with the aim of minimizing the potential confounding effects of the factors associated with primary resistance to EGFR inhibition, we focused only on hyperselected patients with left-sided, HER2-negative, and MSS cancers, who are currently considered as the optimal candidates for an upfront anti-EGFR–based regimen. We showed that patients with *RAS* or *PIK3CA* mutations in baseline ctDNA have worse outcomes in terms of PFS and OS compared with the wild-type subgroups, with *RAS*, but not *PIK3CA*, mutations being also associated with poorer response dynamics in terms of RECIST response, ETS, and DoR. It should be acknowledged that the lack of significant association of *PIK3CA* status

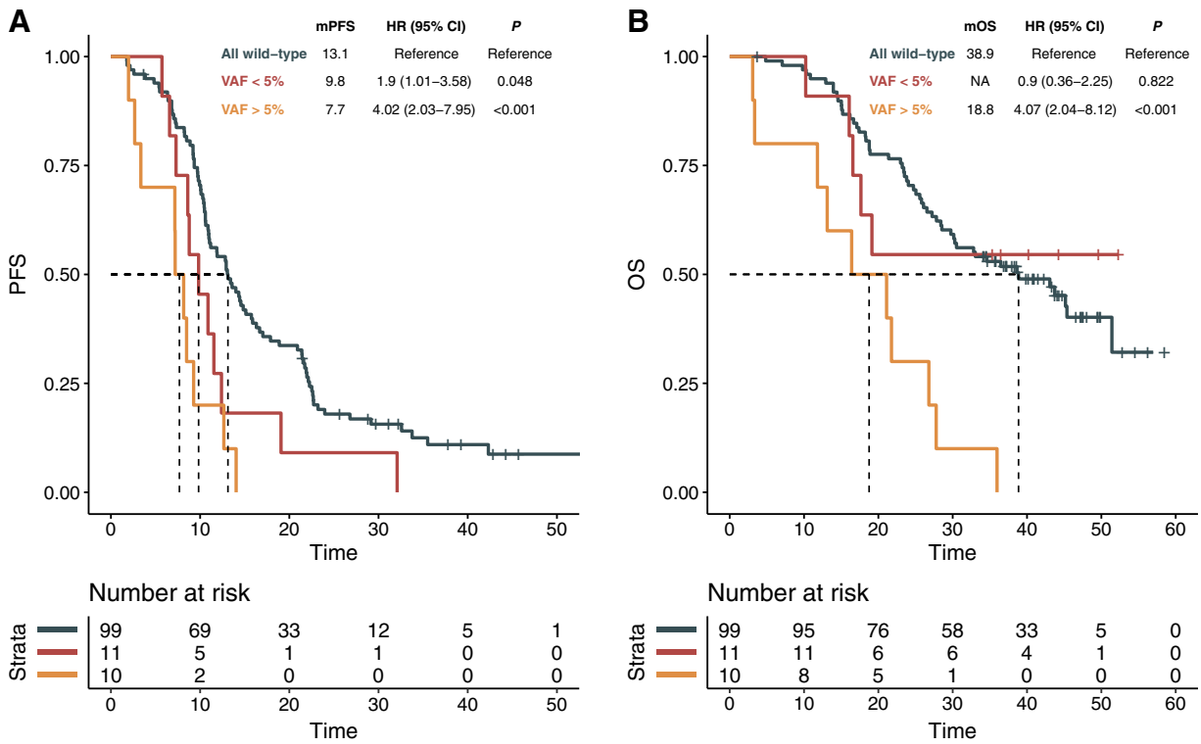

Figure 4.

A and **B,** Kaplan–Meier curves representing PFS and OS for, respectively, patients with at least one *RAS/PIK3CA* mutation VAF > 5% versus patients with *RAS/PIK3CA* mutation with VAF < 5% versus patients with *RAS* and *PIK3CA* all wild-type status in baseline ctDNA.

with response parameters may be related to the low number of patients and to the confounding effects of the associated chemotherapy. Finally, although *TP53* mutations are not an established marker of resistance to anti-EGFR therapy—they had a negative prognostic value in our cohort because these mutations showed the highest median VAF across all samples and the highest prevalence—we speculate that the detection of *TP53* mutations in individual subjects may be at least partly correlated with the higher amount of ctDNA and/or the higher tumor burden.

Regarding the specific negative predictive role of *PIK3CA* mutations in mCRC, their association with other markers of primary resistance to anti-EGFR agents (such as *RAS/BRAF* mutations and right sidedness) has previously limited the clinical transferability of the results of initial investigations (21). Our study showed the potential clinical usefulness of liquid biopsy to assess *PIK3CA* status in mCRC, in light of the high degree of heterogeneity of *PIK3CA* mutations in tumor tissue and the available data in patients with other tumor types, including advanced breast cancer (22). Notably, the use of NGS assays for ctDNA profiling allowed us to concomitantly assess both *PIK3CA* and *RAS* status, as well as other genomic alterations with potential clinical actionability.

RAS or *PIK3CA* mutations with low fractional abundance detected only in tumor tissue by ultra-deep sequencing, but not in ctDNA, may be subclonal and, on top of this, highly heterogeneous from a spatial point of view. The very few patients with this profile did not seem to show poorer individual outcomes, but larger datasets are needed to investigate such patients as a unique subgroup. Consistent with these results, the ULTRA study showed that *RAS* mutations in tumor tissue with a VAF < 5% and only detectable through an ultra-sensitive assay (digital droplet PCR) were not associated with worse outcomes, further corroborating the current clinical use of validated assays with 5% limit of detection for *RAS* testing in tumor tissue (23). On the other side, mutations detected through ctDNA genotyping may mirror the presence of clinically important tumor subclones and could be associated with the rapid emergence of acquired resistance, rather than primary resistance to anti-EGFR drugs. In an attempt to further characterize this phenomenon, we showed that patients with *RAS* and/or *PIK3CA* VAF > 5% in cfDNA have the worst PFS and OS, while patients with *RAS* VAF > 5% also showed worse dynamics of response, including ETS and DoR. It should be pointed out that the VAF may be related to tumor burden and per-se prognostic. Moreover, VAF thresholds of resistance mutations in ctDNA, particularly after adjustment for the VAF of "founder" mutations (such as *TP53* or *APC*) simultaneously identified by NGS, need validation through pooled analyses of clinical trials and larger datasets. Our 5% VAF cutoff, which warrants further validation in similar settings, is based upon the VAF intrinsic distribution and is close to the values reported in previous datasets (16, 24, 25).

Our study has the potential of clinical transferability in several settings. Prospective validation of our data may be achieved by randomized clinical trials investigating alternative upfront regimens or non-anti-EGFR–based maintenance strategies in patients with left-sided and molecularly hyperselected mCRC with baseline *RAS* mutations in ctDNA. Moreover, given the potential actionability of *PIK3CA* mutations when regarded as therapeutic targets rather than contributors to the puzzle of negative hyperselection, liquid biopsy–guided proof-of-concept trials or preclinical studies of anti-EGFR agents combined with alfa-selective PIK3 inhibitors, such as alpelisib, could be planned in the population with "isolated" *PIK3CA* mutations. Clear limitations of our study include its exploratory nature, the lack of data

on longitudinal monitoring of serial liquid biopsy obtained during treatment, and the lack of prospective validation of the mutational VAF in ctDNA. Moreover, the study design did not allow to discriminate between the prognostic value of specific gene mutations in ctDNA and their potential predictive role, because both study arms were panitumumab based. On top of that, the low number of patients in the *RAS/PIK3CA*-mutated subgroups did not allow us to properly assess the interaction with the two treatment arms. Finally, in light of the poor prognostic role of *APC* wild-type status in mCRC (26, 27), we acknowledge that the low frequency of *APC* mutations in our dataset is clearly related to the relatively low coverage of the gene sequence by the hotspots included in this custom panel.

In conclusion, we showed that baseline ctDNA genomic profiling may add value to tumor tissue profiling to refine the molecular hyperselection of patients with the highest likelihood of benefit from upfront anti-EGFR–based strategies. Liquid biopsy data should be incorporated in future clinical trials with targeted agents conducted in patients with mCRC.

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Authors' Contributions

P. Manca: Conceptualization, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **S. Corallo:** Conceptualization, resources, formal analysis, investigation, methodology, writing–original draft, writing–review and editing. **A. Busico:** Data curation, formal analysis, investigation, visualization, methodology, writing–review and editing. **S. Lonardi:** Data curation, validation, visualization, writing–review and editing. **F. Corti:** Data curation, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **C. Antoniotti:** Resources, data curation, visualization, writing–review and editing. **L. Procaccio:** Resources, data curation, visualization, writing–review and editing. **M. Clavarezza:** Resources, data curation, visualization, writing–review and editing. **V. Smiroldo:** Resources, data curation, visualization, writing–review and editing. **G. Tomasello:** Resources, data curation, visualization, writing–review and editing. **R. Murialdo:** Resources, data curation, visualization, writing–review and editing. **A. Sartore-Bianchi:** Resources, data curation, visualization, writing–review and editing. **P. Racca:** Resources, data curation, visualization, writing–review and editing. **F. Pagani:** Resources, data curation, investigation, visualization, writing–review and editing. **G. Randon:** Resources, data curation, investigation, visualization, writing–review and editing. **A. Martinetti:** Resources, formal analysis, investigation, writing–review and editing. **E. Sottotetti:** Resources, investigation, visualization, writing–review and editing. **F. Palermo:** Resources, software, visualization, project administration, writing–review and editing. **F. Perrone:** Resources, data curation, software, formal analysis, supervision, investigation, writing–review and editing. **E. Tamborini:** Resources, data curation, software, formal analysis, supervision, writing–review and editing. **M. Prisciandaro:** Resources, data curation, visualization, writing–review and editing. **A. Raimondi:** Resources, data curation, visualization, writing–review and editing. **M. Di Bartolomeo:** Resources, data curation, validation, visualization, writing–review and editing. **F. Morano:** Resources, data curation, funding acquisition, validation, investigation, visualization, project administration, writing–review and editing. **F. Pietrantonio:** Conceptualization, resources, data curation, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, writing–review and editing.

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