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ORIGINAL ARTICLE

Negative hyper-selection of metastatic colorectal cancer patients for anti-EGFR monoclonal antibodies: the PRESSING case–control study

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Background: Refining the selection of metastatic colorectal cancer patients candidates for anti-epidermal growth factor receptor (EGFR) monoclonal antibodies beyond *RAS* and *BRAF* testing is a challenge of precision oncology. Several uncommon genomic mechanisms of primary resistance, leading to activation of tyrosine kinase receptors other than EGFR or downstream signalling pathways, have been suggested by preclinical and retrospective studies.

Patients and methods: We conducted this multicentre, prospective, case–control study to demonstrate the negative predictive impact of a panel of rare genomic alterations [PRESSING (PRimary rESiStance IN *RAS* and *BRAF* wild-type metastatic colorectal cancer patients treated with anti-eGfr monoclonal antibodies) panel], including *HER2/MET* amplifications, *ALK/ROS1/NTRK1-3/RET* fusions and *PIK3CA* mutations. Hypothesizing a prevalence of candidate alterations of 15% and 0% in resistant and sensitive *RAS* and *BRAF* wild-type patients, respectively, with two-sided α and β errors of 0.05 and 0.20, 47 patients per group were needed.

Results: Forty-seven patients per group were included. PRESSING panel alterations were significantly more frequent in resistant (24 out of 47, 51.1%) than in sensitive (1 out of 47, 2.1%) patients ($P < 0.001$) and in right- (12 out of 29, 41.4%) than left-sided (13 out of 65, 20.0%) tumours ($P = 0.03$). The predictive accuracy of PRESSING panel and sidedness was 75.3% and 70.2%, respectively. Among hyper-selected patients, right-sidedness was still associated with resistance ($P = 0.002$). The predictive accuracy of the combined evaluation of PRESSING panel and sidedness was 80.4%. As a secondary analysis, 8 (17.0%) resistant and 0 sensitive patients showed microsatellite instability ($P < 0.001$).

Conclusion: The investigated panel of genomic alterations allows refining the selection of *RAS* and *BRAF* wild-type metastatic colorectal cancer patients candidates for anti-EGFRs, partially explaining and further corroborating the predictive ability of primary tumour sidedness.

Key words: anti-EGFR monoclonal antibodies, predictive factors, case–control study, genomic alterations

Introduction

RAS and *BRAF* testing provides the only available biomarkers to molecularly select metastatic colorectal cancer (mCRC) patients to receive the anti-EGFRs cetuximab and panitumumab in clinical practice [1, 2]. Also excluding patients bearing *RAS* or BRAF V600E mutations, primary resistance still represents a relevant issue [3, 4].

To elucidate the molecular bases underlying this clinical scenario, genomic and non-genomic mechanisms have been investigated. Constitutive activation of tyrosine kinase receptors other than EGFR

through uncommon genomic events (*HER2* amplification and mutations, *MET* amplification, *NTRK/ROS/ALK/RET* rearrangements) negatively affects the susceptibility to EGFR inhibition in preclinical models [5–9]. Recent studies confirm these findings [6, 10–13] and support the clinical value of such resistance mechanisms as actionable drivers and predictors of benefit from alternative targeted strategies [11, 14–16]. Noteworthy, these events are 'flags' of oncogene addiction, and even if their frequency is low in all comers (4% for *HER2* alterations; around 1% for *MET* amplification or gene fusions), they are enriched in *RAS* and *BRAF* wild-type tumours.

Up today the translation of these biomarkers into clinical practice has been halted by the lack of a formal demonstration of their predictive impact. At the same time, their low prevalence makes unrealistic prospective validation studies or *post-hoc* analyses of randomized trials. However, refining the negative selection of candidates for anti-EGFRs would be important not only to spare a costly and potentially toxic therapy to resistant patients but also to identify promising therapeutic targets, whose blockade might be a more effective strategy.

Oncogenic events inducing deregulation of the PI3K/PTEN/AKT axis were associated with resistance to anti-EGFRs in retrospective series, and even if their rationale seems biologically sounded, such analyses were biased by the inappropriate molecular selection. Among *PIK3CA* mutations, stronger evidence was provided with regard to those affecting exon 20, but their role has never been fully assessed [17–20].

Drawing from these considerations, we designed the present case–control study [PRESSING (PRimary rESiStance IN *RAS* and *BRAF* wild-type metastatic colorectal cancer patients treated with anti-eGfr monoclonal antibodies)], with the primary objective to demonstrate the negative predictive role of a panel of candidate genomic alterations (PRESSING panel) in *RAS* and *BRAF* wild-type mCRC patients treated with anti-EGFRs and to estimate the role of negative hyper-selection beyond *RAS* and *BRAF* in maximizing the efficacy of anti-EGFRs.

As secondary objective, since primary tumour location was identified as a clinical surrogate of a complex landscape of molecular predictors of resistance or sensitivity to EGFR blockade [21], we investigated whether this effect was still evident following genomic hyper-selection. Finally, we explored whether microsatellite instability was associated with resistance to anti-EGFRs, since MSI-high tumours are typically hypermutated and rely on multiple mechanisms for their growth [22].

Patients and methods

Study population

Consecutive patients with *RAS* and *BRAF* wild-type mCRC treated with anti-EGFR-containing regimens at four Italian centres were included in two cohorts of resistant *versus* sensitive patients. Resistant patients were those experiencing disease progression at the first computed tomography scan reassessment during any anti-EGFR-containing regimen, independently of the line of treatment and the association with chemotherapy. Sensitive patients were those achieving Response Evaluation Criteria in Solid Tumors (RECIST) response or a disease stabilization lasting at least 6 months when receiving anti-EGFRs as single agents. The combination with irinotecan was allowed only in irinotecan-refractory disease (i.e. with previous disease progression during or within 3 months from the last dose of irinotecan-containing therapies).

Other inclusion criteria were as follows: *RAS* and *BRAF* wild-type status assessed by means of CE-IVD methods; at least one measurable lesion according to RECIST 1.1; at least two consecutive radiological reassessments by computed tomography scan; written informed consent to study participation.

Study design

PRESSING was a multicentre, case–control study, based on prospective translational hypothesis. Independent cases (resistant group) and controls (sensitive group) with one control per case were planned. Hypothesizing a prevalence of candidate alterations included in the PRESSING panel equal to 0% and 15% among controls and cases, respectively, 47 cases and 47 controls were needed to be able to reject the null hypothesis of equally prevalent alterations, with α and β errors of 0.05 and 0.20. The study was approved by the local Ethics Committees of participating institutions.

Molecular analyses

PRESSING panel included the following genomic alterations: *HER2* amplification/activating mutations; *MET* amplification; *NTRK/ROS1/ALK/RET* rearrangements; *PIK3CA* exon 20 mutations, *PTEN* inactivating mutations, *AKT1* mutations.

Briefly, immunohistochemistry for HER2/MET and dual-colour silver in situ hybridization for both genes were carried out and scored as described previously [23, 24]. Immunohistochemistry for ALK/ROS1/panTRK/RET was carried out as screening method using standard protocols for pan-Trk (including TrkA, TrkB, TrkC; Cell Signaling Danvers, Massachussets, USA, clone C17F1, 1 : 25 dilution), ROS1 (Cell Signaling, clone D4D6, 1 : 500 dilution), ALK (Cell Signaling, clone D5F3, 1 : 500 dilution) and RET (Abcam Cambridge, UK, clone EPR2871). All samples with any immunohistochemistry (IHC) staining underwent RNA-seq for confirmation of the gene fusions and identification of its partner [13].

Oncogenic mutations in the hotspot regions of 50 cancer-related genes (Hotspot Cancer Panel v2), including *HER2* and *PIK3CA*/*PTEN*/*AKT1*, were assessed by means of Targeted Next-Generation Sequencing (T-NGS) through the Ion Torrent Personal Genome platform (Life Technologies® Waltham, Massachussets, USA), as described previously [25]. At the same time, this technique allowed to centrally re-assess *RAS* status with deeper coverage. The fractional abundance of *RAS* mutations was reported after correction for tumour cellularity [25].

Statistical analysis

An uncorrected chi square statistic was used to compare the prevalence of alterations in the PRESSING panel and in other alterations between resistant and sensitive patients.

The impact of alterations included in the PRESSING panel and of primary tumour location on progression-free survival (PFS) and overall survival (OS) was investigated. PFS was defined as the time from the beginning of the anti-EGFR-containing treatment to the radiological evidence of disease progression or last follow-up. OS was defined as the time from the beginning of an anti-EGFR-containing treatment to death or last follow-up. PFS and OS analyses were determined according to the Kaplan–Meier method and survival curves were compared using the log-rank test.

The predictive accuracy of proposed assessments was calculated as the sum of true positive and true negative observations relative to the total number of observations.

Results

Study population

As shown in supplementary Figure S1, available at *Annals of Oncology* online, the study population included 47 resistant

Table 1. Prevalence of candidate genomic alterations of the PRESSING panel in samples from resistant (cases) versus sensitive (controls) patients

Molecular alterations	Resistant patients *N = 47*	Sensitive patients *N = 47*
HER2 amplification	7	0
HER2 mutations	1 (G776V, exon 20)	0
MET amplification	4	0
NTRK rearrangements	2 (*SCYL3-NTRK1* and *TPM3-NTRK1*)	0
ALK rearrangements	0	0
ROS1 rearrangements	0	0
RET rearrangements	1 (*CCDC6-RET*)	0
PIK3CA exon 20 mutations	1 (A1035V, exon 20)	1 (H1047R, exon 20)
AKT1 mut	1 (R25C)	0
PTEN mutations	3 (L247S, R233stop and del P248, exon 7)	0
Patients with candidate alterations	20	1

(cases) and 47 sensitive patients (controls). Patients' characteristics are summarized in supplementary Table S1, available at *Annals of Oncology* online. No relevant differences between resistant and sensitive patients were evident, except for a significantly higher prevalence of right-sided primary tumours among resistant (51.1%) versus sensitive (10.6%) patients ($P < 0.001$). At a median follow-up of 36.3 [95% confidence interval (CI), 22.2–78.3] months, median PFS with anti-EGFR treatment was 2.0 (95% CI, 1.5 to 2.8) among resistant and 8.1 (95% CI, 7.3–10.7) months among sensitive patients. Median OS was 10.6 (95% CI, 7.2–13.3) and 20.3 (95% CI, 16.5–26.2) months, respectively.

Candidate alterations are significantly more frequent among resistant patients

As summarized in Table 1 and depicted in Figure 1, one candidate alteration included in the PRESSING panel was found in samples from 20 (42.6%) out of 47 resistant versus only 1 (2.1%) out of 47 sensitive patients, respectively ($P < 0.001$). Noteworthy, all alterations were mutually exclusive. *HER2* amplification was the most frequent alteration in resistant patients (seven cases, 14.9%), followed by *MET* amplification (four cases, 8.5%).

In samples from resistant patients, T-NGS analysis allowed identifying *RAS* mutations at low fractional abundance in four (8.5%) tumours deemed *RAS* and *BRAF* wild-type by means of the CE-IVD techniques previously used (Figure 1A). In particular, KRAS G12V, G12D and Q61H mutations (fractional abundance: 6%, 8% and 10%, respectively), and NRAS Q61R mutation (fractional abundance: 10%) were observed in samples not bearing other candidate alterations. Therefore, predictors of resistance to anti-EGFRs were reported in samples from 24 (51.1%) versus 1 (2.1%) resistant and sensitive patient, respectively ($P < 0.001$). Mutations reported by means of T-NGS in all analysed samples are detailed in supplementary Table S2, available at *Annals of Oncology* online. Interestingly, variants of uncertain significant potentially affecting anti-EGFRs' efficacy (i.e. MET E168D, ALK L1198P or KRAS T50I) were found in resistant cases not bearing any other candidate alteration.

Overall, based on our findings, the 51.1% of resistant cases was associated to one of these candidate molecular alterations, and excluding these patients from the treatment with anti-EGFRs could allow increasing the clinical benefit rate from 50.0% to 67.0% (supplementary Figure S2, available at *Annals of Oncology* online). A clear separation of PFS curves in favour of the molecularly hyper-selected subgroup was evident (Figure 2A). The median PFS of patients not bearing any alteration in the PRESSING panel was 6.3 versus 2.7 months among patients bearing any alteration [HR (hazard ratio): 0.18 (95% CI, 0.09–0.35), $P < 0.001$). No significant difference was observed in terms of OS [median OS: 15.2 versus 17.3 months, HR: 1.05 (95% CI, 0.60–1.83), $P = 0.876$] (Figure 2B).

Primary tumour location affects outcome also following molecular hyper-selection

Candidate alterations of the PRESSING panel were found in 41.4% (12 out of 29) of right-sided tumours versus 20.0% (13 out of 65) of left-sided ($P = 0.03$) (Figure 1). When focusing only on hyper-selected patients (i.e. after excluding those bearing any candidate mechanism of primary resistance), primary tumour location was still associated with clinical outcome. In fact, primary tumours were right-sided in the 43.5% (10 out of 23) of resistant patients versus the 10.9% (5 out of 46) of sensitive patients ($P = 0.002$). The negative prognostic impact of the right-sidedness was evident also among hyper-selected patients in terms of both PFS [3.3 versus 7.3 months, HR: 4.77 (95% CI, 2.06–11.1), $P < 0.001$, Figure 2C] and OS [median OS: 8.4 versus 16.6 months, HR: 2.04 (95% CI, 1.02–4.07), $P = 0.04$, Figure 2D].

The accuracy of primary tumour location and PRESSING panel assessment in predicting the treatment outcome was 70.2% and 74.5%, respectively, while it increased up to 79.8% with the combined evaluation of both sidedness and PRESSING panel.

Microsatellite instability is often associated with candidate alterations of primary resistance

Tumours from 8 (17.0%) resistant and 0 (0%) sensitive patients were MSI-high ($P < 0.001$). MSI-high status was associated with candidate molecular alterations included in the PRESSING panel in six (75.0%) out of eight cases. Six (75.0%) out of eight MSI-high tumours were right sided.

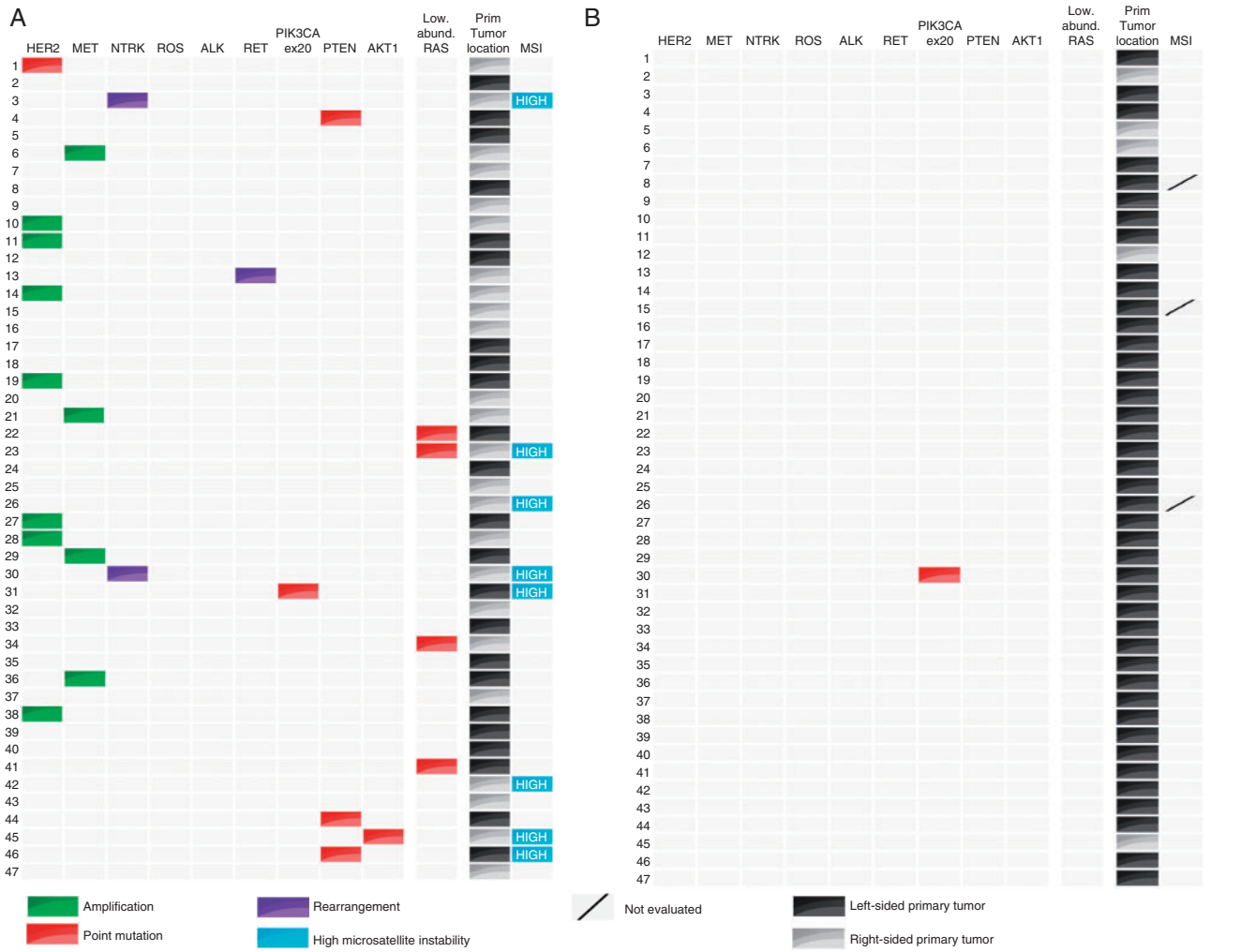

Figure 1. Comparison of PRESSING panel alterations, primary tumour location and microsatellite instability among resistant (A) and sensitive (B) patients.

Discussion

Tailoring treatments based on patients' and tumours' characteristics is a challenge of modern oncology. Nowadays, biomarkers routinely used to guide therapeutic choices in mCRC are negative predictors of benefit from anti-EGFRs, so that the selection of candidate patients is based on the exclusion of *RAS* and *BRAF* mutated, rather than the positive selection of sensitive ones. The role of several rare genomic alterations in refining negative selection has been investigated by preclinical and retrospective studies on each single biomarker [3–6, 8–10, 12, 13, 17–20].

In order to validate these findings, conducting new randomized trials stratified according to candidate markers would be methodologically appropriate, but rather unfeasible. Similarly, assessing the impact of each candidate alteration would be difficult because of their low prevalence. Drawing from these considerations and searching for a pragmatic approach to tackle these limitations, we designed the present case–control study based on a formal a priori statistical hypothesis.

With the aim of embedding the results of this study in the current 'best clinical practice', our analysis was restricted to molecularly and clinically selected patients. In particular, we only allowed the registration of *RAS* and *BRAF* wild-type patients—the optimal candidates for anti-EGFRs in the daily practice—assessable for the actual benefit from anti-EGFRs, avoiding the confounding effect of combined chemotherapy regimens. Here, we show that the evaluation of the PRESSING panel may allow excluding from anti-EGFR therapies a substantial (around 50%) percentage of resistant patients, thus representing a step forward in the way towards negative molecular hyper-selection.

The mutual exclusivity of alterations of the PRESSING panel indirectly suggests their role as oncogenic drivers. Moreover, the lack of benefit from anti-EGFRs further strengthens the interest towards these alterations as actionable targets for molecularly-defined patients' subgroups.

Finally, though in the absence of a randomized control group not receiving the anti-EGFR that clearly represents a limitation of this study, the evidence of significant differences in treatment sensitivity and PFS, but not in OS, seems to corroborate the role of alterations included in the PRESSING panel as predictive rather than prognostic markers.

Our results also emphasize the emerging role of deep-sequencing for the detection of *RAS* mutations, given their

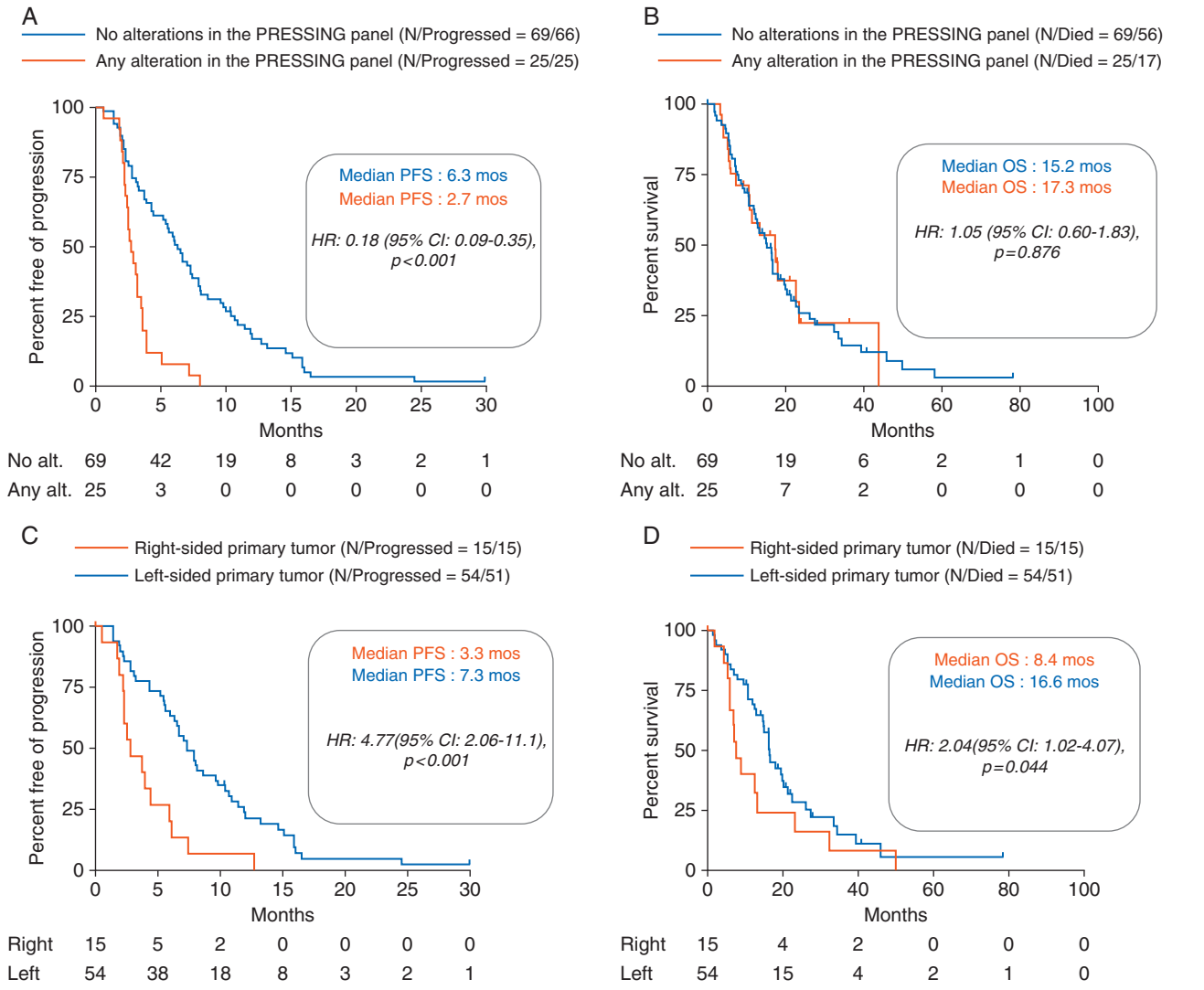

Figure 2. Kaplan–Meier estimates of progression-free survival (PFS) and overall survival (OS) according to the presence of PRESSING panel alterations (A, B) in the study population and according to primary tumour location in hyper-selected patients (C, D).

negative predictive impact, even when at low fractional abundance [26, 27]. The large-scale diffusion of T-NGS technology in the daily practice clearly allows a massive parallel multigene sequencing. At the same time, the potential clinical interest of other variants of unknown significance identified at T-NGS, such as MET E168D, ALK L1198P or KRAS T50I should be functionally validated by preclinical experiments. In fact, although NGS enables the simultaneous evaluation of multiple genomic alterations with potential predictive interest, they also require cautious interpretation in the daily practice.

A growing amount of clinical evidence, mainly deriving from subgroup analyses of randomized trials, underlines that primary location affects the sensitivity to anti-EGFRs [21]. In particular, while the magnitude of benefit from these drugs is significant in left-sided tumours, right-sided ones seem to derive modest or no benefit and clearly show poor prognosis. These findings are supported by a biologic rationale, given the higher prevalence of several molecular mechanisms potentially associated with resistance to anti-EGFRs in right-sided tumours [13, 28]. In our study, a higher prevalence of alterations in the PRESSING panel was reported in right-sided tumours, thus supporting the role of primary tumour location as a clinical surrogate marker underpinning the complex molecular landscape of primary resistance. Of note, among hyper-selected patients (i.e. following the exclusion of those bearing alterations of the PRESSING panel) right-sidedness was still more frequent among resistant then sensitive patients, and retained its negative prognostic impact. The combined evaluation of primary tumour location and PRESSING panel alterations provides the best predictive accuracy with regard to the efficacy of anti-EGFRs. Our preclinical and translational knowledge about resistance to EGFR blockade in most right-sided tumours should be deepened including not only targeted genomics but also gene expression profiling studies. On the other hand, a small subset of patients with right-sided cancers may benefit from anti-EGFRs.

In the last months, immune checkpoint inhibitors emerged as a practice-changing treatment in patients with MSI-high mCRC [29, 30], characterized by high mutational/neoantigen load [22, 31] and abundant lymphocytic infiltration. We wondered whether the high mutational burden of these tumours,

determining the activation of multiple oncogenic signals, could hamper the efficacy of strategies that rely on the blockade of a single pathway. Although a significantly higher percentage of MSI-high samples were found among resistant patients, microsatellite instability was associated in most cases with other predictors of primary resistance and with right-sidedness. For this reason, the role of MSI-high as determinant of resistance to anti-EGFRs should be further investigated.

In conclusion, while present results introduce the new concept of negative hyper-selection of patients to be excluded from anti-EGFRs, their further validation in post hoc analyses of randomized trials would be warranted. Moreover, a substantial step forward would be the refinement of negative hyper-selection by investigating non-genomic mechanisms. As a further step, the positive identification of highly EGFR-addicted tumours, including those bearing *IRS2* mutations/amplification preclinically related with sensitivity to anti-EGFRs [5] and not detected by the T-NGS platform that we adopted, would mark a fundamental paradigm shift in the molecular characterization of mCRC.

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Disclosure

The authors have declared no conflicts of interest.

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