Associations between AI-Assisted Tumor Amphiregulin and Epiregulin IHC and Outcomes from Anti-EGFR Therapy in the Routine Management of Metastatic Colorectal Cancer

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ABSTRACT

Purpose: High tumor production of the EGFR ligands, amphiregulin (AREG) and epiregulin (EREG), predicted benefit from anti-EGFR therapy for metastatic colorectal cancer (mCRC) in a retrospective analysis of clinical trial data. Here, AREG/EREG IHC was analyzed in a cohort of patients who received anti-EGFR therapy as part of routine care, including key clinical contexts not investigated in the previous analysis.

Experimental Design: Patients who received panitumumab or cetuximab ± chemotherapy for treatment of *RAS* wild-type mCRC at eight UK cancer centers were eligible. Archival formalin-fixed paraffin-embedded tumor tissue was analyzed for AREG and EREG IHC in six regional laboratories using previously developed artificial intelligence technologies. Primary endpoints were progression-free survival (PFS) and overall survival (OS).

Results: A total of 494 of 541 patients (91.3%) had adequate tissue for analysis. A total of 45 were excluded after central extended *RAS* testing, leaving 449 patients in the primary analysis population. After adjustment for additional prognostic factors, high AREG/EREG expression ($n = 360$; 80.2%) was associated with significantly prolonged PFS [median: 8.5 vs. 4.4 months; HR, 0.73; 95% confidence interval (CI), 0.56–0.95; $P = 0.02$] and OS [median: 16.4 vs. 8.9 months; HR, 0.66 95% CI, 0.50–0.86; $P = 0.002$]. The significant OS benefit was maintained among patients with right primary tumor location (PTL), those receiving cetuximab or panitumumab, those with an oxaliplatin- or irinotecan-based chemotherapy backbone, and those with tumor tissue obtained by biopsy or surgical resection.

Conclusions: High tumor AREG/EREG expression was associated with superior survival outcomes from anti-EGFR therapy in mCRC, including in right PTL disease. AREG/EREG IHC assessment could aid therapeutic decisions in routine practice.

See related commentary by Randon and Pietrantonio, p. 4021

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Translational Relevance

The anti-EGFR agents, cetuximab and panitumumab, are used in the palliative management of metastatic colorectal cancer with variable efficacy. High tumor amphiregulin (AREG) and/or epiregulin (EREG) protein production predicted benefit from panitumumab in a retrospective analysis of clinical trial data. This study of patients who received anti-EGFR therapy ± chemotherapy during routine care at eight UK cancer centers provides real-world validation of those findings. Crucially, a prognostic effect was observed among patients receiving either of the approved anti-EGFR agents, and those receiving oxaliplatin- or irinotecan-based concurrent chemotherapy. The effect was similar for patients with right or left primary tumor location (PTL), meaning AREG/EREG may have particular clinical utility in identifying a subgroup of patients with right PTL who benefit from anti-EGFR therapy. IHC was successfully performed at six regional laboratories with the use of formalin-fixed paraffin-embedded tumor tissue acquired either by biopsy or through surgical resection, with quantification assisted by artificial intelligence algorithms.

Introduction

Amphiregulin (AREG) and epiregulin (EREG) are ligands of the EGFR. Excess production by a subset of colorectal adenocarcinomas is thought to denote EGFR pathway dependence, and hence sensitivity to EGFR blockade (1). Indeed, in a retrospective analysis of the PICCOLO trial of second-line irinotecan chemotherapy with or without panitumumab (2), high AREG and/or EREG expression was able to discriminate between patients who did and did not benefit from anti-EGFR therapy (3).

A number of issues need to be addressed before AREG/EREG assessment can be recommended for use to inform treatment decisions in routine clinical practice. First, it must be ensured that AREG/EREG IHC can be reliably and reproducibly performed in different laboratory and clinical settings, and using routinely collected formalin-fixed paraffin-embedded (FFPE) tumor tissue derived from either surgical resections or biopsy procedures. Second, it must be demonstrated that AREG/EREG IHC is associated with benefit from either of the approved anti-EGFR agents—that is, cetuximab as well as panitumumab. Third, the utility of AREG/EREG IHC must also be explored in patients treated under the current standard of care, which involves the use of anti-EGFR therapy in the first-line management of metastatic colorectal cancer (mCRC) in combination with doublet chemotherapy [5-fluorouracil and irinotecan (FOLFIRI), or 5-fluorouracil and oxaliplatin (FOLFOX)] (4, 5)—rather than second-line single-agent irinotecan chemotherapy as in PICCOLO.

Finally, patients with right-sided primary tumors do not appear to benefit from anti-EGFR therapy as an overall group (6, 7)—an observation that was confirmed in recently presented results from the phase III PARADIGM trial of FOLFOX with cetuximab versus FOLFOX with bevacizumab (8). However, tumor sidedness is a proxy indicator for the higher frequency of molecular subtypes with primary resistance to anti-EGFR therapy on the right than the left of the colon (9), and improved selection may identify patients with right primary tumor location (PTL) who stand to benefit from anti-EGFR treatment. Indeed, within PARADIGM, exclusion of patients with alterations in genes associated with anti-EGFR resistance in pretreatment circulating tumor DNA (ctDNA) pointed toward the possible existence of a subset of patients with right PTL with superior median overall survival (OS) with panitumumab rather than bevacizumab [38.9 vs. 30.9 months; HR, 0.82; 95% confidence interval (CI), 0.50–1.35; $P = 0.431$; $P_{\text{interaction}} = 0.145$] (10). In PICCOLO, AREG/EREG IHC predicted progression-free survival (PFS) benefit independently of PTL (3). Demonstration in an external cohort of a significant effect of AREG/EREG IHC on survival outcomes in patients with right PTL would therefore strongly support the use of this biomarker to guide treatment decisions in an area where there is currently significant unmet need.

To address these issues, the results of an observational cohort study recruiting patients who received or were receiving anti-EGFR therapy with cetuximab or panitumumab as part of standard care for mCRC at eight UK cancer centers are presented here. The study was conducted across eight recruiting sites, with AREG and EREG IHC performed in six regional study laboratories. We hypothesized that patients with more than 20% of tumor cells staining positive for AREG and/or EREG would have superior PFS and OS, both across the whole cohort and within the key clinical subsets described.

Materials and Methods

Study eligibility

Patients aged 18 years or older with *RAS* wild-type (-wt), advanced, histologically proven colorectal adenocarcinoma (either inoperable metastatic disease at diagnosis or inoperable recurrent disease) who had received or were receiving palliative cetuximab or panitumumab as part of routine care were eligible for recruitment.

Initial registration for the study was permitted where *RAS* status was unknown at the time of treatment. As AREG and EREG do not identify a subgroup of patients with *RAS*-mutant tumors who benefit from anti-EGFR therapy (11), central *RAS* testing by next-generation sequencing (NGS) was performed for patients where extended *RAS* testing (c.12,13,59,61,117,146 for both *KRAS* and *NRAS*)—as per the American Society of Clinical Oncology definition (12)—had not been performed locally at the time of treatment, and where sufficient tissue remained for analysis. Patients with *RAS*-mutant disease on central *RAS* testing, or with unknown *RAS* status due to insufficient tissue for central analysis, were excluded from the primary analysis population. Patients with *BRAF*-mutant disease were included in the primary analysis population. Results of the FIRE-4.5 trial were published during the course of the current study, showing a negative predictive effect of *BRAF* mutation on benefit from anti-EGFR therapy (13). A secondary analysis population of patients with dual *RAS*- and *BRAF*-wt disease was therefore included.

Sufficient pretreatment archival FFPE tumor tissue must have been available for biomarker analysis within the study. Patients may have received anti-EGFR therapy as part of any line of palliative treatment, with or without combination or single-agent chemotherapy. Where patients received anti-EGFR therapy as part of first-line treatment, two cycles of chemotherapy alone were permitted prior to commencement of anti-EGFR therapy if *RAS* testing results were pending, with the time of treatment commencement for the purposes of the study remaining cycle 1, day 1 of palliative chemotherapy. Patients were ineligible where they received anti-EGFR therapy as part of neoadjuvant systemic treatment prior to surgery with curative intent, or as part of BRAF-targeted therapy.

Ethical approval

Ethical approval was granted by the South Yorkshire Research Ethics Committee (19/YH/0235). All study activities were conducted

in accordance with Good Clinical Practice guidelines and the provisions of the Declaration of Helsinki. Written informed consent was required from all living participants prior to study entry. There was no consent process for deceased participants. Section 251 support to access the records of deceased participants for the purposes of data collection within the study was granted by the UK Health Research Authority's Confidentiality Advisory Group (19/CAG/0221).

Treatment, response assessment, and measurements

In this observational study, cetuximab, panitumumab, and chemotherapy had been prescribed and administered in accordance with local protocols.

Baseline imaging must have been performed a maximum of 8 weeks prior to commencing treatment, or up to 1 week after. Follow-up and response assessments were conducted according to local protocols, with the local radiologist's interpretation of response recorded. Patients were followed to the completion of their first continuous period of anti-EGFR therapy. Treatment breaks of up to 6 weeks were permitted.

Where available, results of routine blood tests [white cell count ($\times 10^9$/L), lymphocyte count ($\times 10^9$/L), neutrophil count ($\times 10^9$/L), platelet count ($\times 10^9$/L), albumin (g/L), alkaline phosphatase (ALP; iu/L), carcinoembryonic antigen (CEA; μg/L)] performed a maximum of 1 week prior to commencing treatment (a maximum of 4 weeks for CEA) were recorded.

Endpoints

The joint primary outcomes were PFS (time from commencement of anti-EGFR therapy until radiological or clinical evidence of disease progression, or death from any cause, whichever was sooner) and OS (time from commencement of treatment to death). Secondary outcome measures were locally assessed response rate (RR; complete or partial response on first follow-up imaging), and disease control rate (DCR; stable disease, complete or partial response on first follow-up imaging). For the purposes of RR and DCR, those without follow-up imaging were assumed to have progressed.

IHC

AREG and EREG IHC were performed using archival FFPE tumor tissue and interpreted with the assistance of artificial intelligence technologies (calculating the percentage of tumor cells stained positively for each ligand) as described previously (ref. 3; Supplementary Fig. S1). Samples without definitive evidence of invasive adenocarcinoma were excluded.

Prior to commencement of study activities, a cross-site quality assurance check was performed. A single anonymized FFPE colorectal adenocarcinoma resection block, surplus to diagnostic requirements, was sectioned in the study central laboratory in Leeds, United Kingdom. Three slide-mounted sections were sent to each participating laboratory (Liverpool, Manchester, Newcastle, Nottingham, Sheffield) for hematoxylin and eosin (H&E), AREG, and EREG staining. Slides were digitized using locally-installed VENTANA DP 200 scanners. Variations in hematoxylin intensity (percentage) and hue (degrees) were assessed by way of calculating medians and interquartile ranges, as were differences in AREG and EREG outcomes. The first three and last three sections from the block were stained in Leeds to ensure differences in AREG/EREG outcomes were not due to changes in tumor characteristics with repeated sectioning.

A prestudy assessment of the impact of variations in fixation time was also performed. Surplus anonymized colorectal adenocarcinoma tissue from 4 patients was fixed by immersion in 10% neutral buffered formalin for 48 hours (the standard fixation time in routine clinical practice). Tissue samples from different regions of the same tumors were similarly fixed for a total of 96 hours. Following fixation, tumor tissue was embedded in paraffin (one standard fixation time block, and two long fixation time blocks prepared per patient) prior to sectioning and staining for H&E, AREG, and EREG. AREG/EREG outcomes from long and standard fixation time blocks were compared.

Central extended *RAS* and *BRAF* mutation testing

Mutation calling was performed using methods and a subset of primers developed previously (14, 15). A detailed description of methods for DNA extraction and amplification, library preparation, and sequencing is provided in the Supplementary Materials and Methods. Briefly, a targeted panel of PCR primers covering somatic mutations in *KRAS* and *NRAS* c.12,13,59,61,117,146 and *BRAF* c.1799 had previously been designed using Primer3 (RRID: SCR_003139; refs. 14, 15). Each somatic mutation was covered by two separate PCR primer pairs to allow for redundancy and internal validation. Primer details are given in Supplementary Table S1.

Each DNA sample was amplified with PCR primers chosen to capture either a putative somatic mutation or a putative wt gene. PCR was performed using Amplitaq Gold Fast PCR master mix (Applied Biosystems). The 12 PCR products from each sample (two for each region tested) were pooled in equimolar amounts and prepared for NGS using NEBNext Ultra library preparation kits (New England Biolabs) with custom-designed index tags to identify each sample. The samples were sequenced on a single run of an MiSeq instrument (Illumina) using paired 150 bp reads.

Following sequencing and demultiplexing, adapters were removed using Cutadapt 3.4 (RRID:SCR_011841). Sequences were aligned to the human genome version hg38 using BWA 0.7.17 (RRID: SCR_010910), and processed using GATK 4.2.0 (RRID:SCR_001876). Using the Pysam wrapper for Samtools (RRID:SCR_002105), aligned reads were split into separate files according to whether their coordinates matched those of the expected PCR products. Variants were called using VarScan 2.4.4 (RRID:SCR_006849). Mutated DNA was noted as present where the variant allele frequency was ≥5% and the read depth exceeded 100.

Cut-off point

In our previous retrospective analysis of the PICCOLO trial, a pragmatically selected cut-off point of 50% AREG and/or EREG tumor cell positivity was chosen *a priori*, with a significant predictive effect on PFS demonstrated (3). A tumor cell was regarded as positively stained if there was evidence of membranous, cytoplasmic, cytomembranous, or punctate staining. In an exploratory analysis, a cut-off point of 20% had a stronger predictive effect. The 20% cut-off point has therefore been taken forward for use in the primary analysis of the data presented here. A further decision was taken *a priori* to also analyze the data using the original 50% cut-off point should no significant prognostic effect be seen at 20%.

Sample size and power

The sample size calculation was performed under the assumption that, using a 20% cut-off point, the AREG/EREG IHC combined dichotomous measure would classify approximately three quarters of participants as having high ligand expression and approximately one quarter of participants as having low ligand expression (3). In the panitumumab arm of PICCOLO (IrPan), patients with high

AREG/EREG IHC percentage positivity (20% cut-off point) had superior PFS compared with those with low ligand expression, with an unadjusted HR of 0.50 (95% CI, 0.32–0.78; $P = 0.002$). On the basis of a sample size of 480 participants and using a 5% significance level, if 60% of participants in the "high" expression group experienced events then the power to detect a HR of 0.5 would be approaching 100%. If only 40% of the participants in the "high" expression group experienced events, there would be 98% power to demonstrate a HR of 0.50.

Statistical analysis

Stata was used for all statistical analyses [Stata Statistical Software, Release 16 (2019); StataCorp; RRID:SCR_012763]. Descriptive statistics of all measures were derived and tabulated. Pairwise correlation between continuous AREG and EREG tumor cell percentage positivity was assessed using the Spearman correlation coefficient, rho (ρ), and a scatterplot of AREG versus EREG, including a line of best fit, plotted. Pairwise associations between the combined dichotomous classifier (20% cut-off point) and age at commencement of treatment, sex, World Health Organization (WHO) performance status (PS; 0, 1, 2–3), PTL (left or rectum, right), primary surgery (excised, *in situ*), number of metastatic sites (<2, ≥2), peritoneal metastases (no, yes) and grade (poor, moderate or well) were assessed using mean and sd, frequencies, and percentages, Two-tailed *t* test for age, and Pearson χ^2 tests for the categorical measures.

In addition to the measures listed above, other predictors used in modeling were: pretreatment white cell count, lymphocyte count, neutrophil count, neutrophil lymphocyte ratio, platelet count, albumin, ALP, and CEA. The extent of missingness was assessed for all predictor variables and, where missing values were missing at random, multiple imputation was implemented prior to modeling and the imputation was repeated 20 times. First, both categorical and continuous predictor variables were evaluated in univariable Cox proportional hazards regression models and HRs and 95% CIs estimated. Prior to modeling, continuous variables were tested for skewness and graphed, with appropriate transformations or scaling applied. In the primary analysis, AREG/EREG expression was assessed using the combined dichotomous classifier at the 20% cut-off point (both ligands "low" vs. either ligand "high") and Kaplan–Meier curves plotted. In secondary analyses, AREG and EREG were assessed separately as individual continuous variables, and separately dichotomized at the 20% cut-off point. If the combined dichotomous ligand measure at the 20% cut-off point was found to be prognostic, multivariable Cox proportional hazards models were then performed, adjusted for the measures found to be prognostic in univariable analyses. If the combined dichotomous ligand measure at the 20% cut-off point was not found to be prognostic in the univariable model, then multivariable models were performed using the most strongly prognostic ligand measure. The proportionality assumption was tested by using the Schoenfeld and scaled Schoenfeld residuals.

Unadjusted ORs and 95% CIs were estimated from logistic regression for the RR and DCR outcomes and then adjusted models were performed similar to the Cox proportional hazards analyses described above.

Prespecified subgroup analyses were conducted for all outcomes stratifying by: PTL, chemotherapy backbone (FOLFOX vs. FOLFIRI), anti-EGFR agent (first-line cetuximab vs. first-line panitumumab), and tumor tissue type examined (biopsy vs. resection).

Data availability

The data generated in this study are available upon request from the corresponding author. Raw DNA sequences are available from the European Nucleotide Archive, accession number PRJEB63103 (http://www.ebi.ac.uk/ena/data/view/PRJEB63103).

Results

Cross-site quality assurance

During prestudy quality assurance, staining of serial sections from the same tumor at each of the participating laboratories (total of seven sections for each ligand) revealed little variation in hematoxylin intensity [AREG: median, 41%; interquartile range (IQR), 39%–43%; EREG: median, 41%; IQR, 37.5%–45%] and hue (AREG: median, 63%; IQR, 62%–63.5%; EREG: median, 63%; IQR, 62.5%–63.5%). AREG percentage tumor positivity ranged from 51% to 70% (median, 60%; IQR, 59.5%–65.5%), and EREG from 79% to 92% (median, 85%; IQR, 84%–85.5%).

Effect of variation in tissue fixation time

Prestudy assessment of the impact of variations in tissue fixation time revealed little effect on AREG/EREG staining outcomes, except in one case where AREG percentage positivity was 41.3% following standard fixation for 48 hours but 81.3% and 90.6% following 96 hours fixation, possibly a result of intratumoral variability (Supplementary Table S2).

Patient characteristics

A total of 541 patients treated with anti-EGFR therapy were registered for the study. A total of 528 (97.6%) patients were known to be *KRAS*-wt by local testing and 13 (2.4%) had unknown *RAS* status. A total of 47 (8.7%) patients were excluded following histopathologic analysis of their archival FFPE tumor tissue: 19 had no evidence of invasive tumor, 27 had no visible tumor cells, and a block from a non-colorectal cancer procedure was received for 1 patient (Supplementary Fig. S2).

Extended *RAS* testing had not been performed locally for 393 of 494 (79.6%) patients with evaluable tumor tissue. There was sufficient available residual tumor tissue for DNA extraction for 281 (71.5%) of these patients. Central *KRAS* testing was successful in 255 patients, and *NRAS* testing was also successful in 255 patients (both successful in 254 common individuals), revealing 34 *KRAS* and nine *NRAS* mutations in 41 individuals. NGS failed in 1 individual with an unknown *RAS* status and 3 patients with *RAS*-unknown status were included among those with insufficient residual tissue for analysis. Hence, 45 of 494 patients were excluded after central *RAS* testing, leaving 449 patients in the primary analysis population. Exclusion of 26 patients with *BRAF*-mutant tumors and 34 patients with unknown *BRAF* status left 389 patients in the *RAS*-wt and *BRAF*-wt secondary analysis population (Supplementary Fig. S2).

At the time of the analysis, 13 of 449 patients (2.9%) remained on treatment, 405 (90.2%) had died, and 422 (94.0%) had experienced a progression event (**Table 1**).

Biomarker expression

As in the PICCOLO dataset, AREG and EREG IHC percentage positivities were strongly correlated (Spearman correlation coefficient 0.77, $P < 0.00005$; Supplementary Fig. S3). Mean AREG tumor cell percentage positivity was 52.6% (sd 32.7%) while mean EREG percentage positivity was 49.8% (sd 35.8%). At a cut-off point of 20%, 339 (75.5%) patients were AREG high and 313 (69.7%) were EREG high. Using the combined AREG/EREG model (both AREG and EREG low

Table 1. Baseline patient demographics and disease characteristics.

Variable	Category	*n* (%) *Unless otherwise stated*
Age at starting treatment (years)		Mean 60.7 (sd 10.9)
Sex	Male	299 (66.6)
	Female	150 (33.4)
Performance status	0	171 (38.1)
	1	149 (33.2)
	2–3	31 (6.9)
	Unknown	98 (21.8)
Chemotherapy	FOLFIRI	286 (63.7)
	FOLFOX	98 (21.8)
	Other	21 (4.7)
	None	44 (9.8)
Anti-EGFR agent	Cetuximab	304 (67.7)
	Panitumumab	145 (32.3)
Dose reduction	No	255 (56.8)
	Yes	192 (42.8)
	No, but treatment ongoing	2 (0.4)
Primary tumor location	Left/Rectum	342 (76.2)
	Right	107 (23.8)
Primary surgery	Excised	250 (55.7)
	In situ	199 (44.3)
Previous neoadjuvant radiotherapy (rectal cancer only)	No	95 (21.2)
	Yes	51 (11.4)
	Not applicable	303 (67.5)
Previous adjuvant chemotherapy	No	296 (65.9)
	Yes	152 (33.9)
	Unknown	1 (0.2)
Line of treatment	First line	371 (82.6)
	Second line or later	78 (17.4)
Tumor grade	Poor	64 (14.2)
	Moderate/Well	307 (68.4)
	Unknown	78 (17.4)
MMR/MSI	dMMR or MSI-H	9 (2.0)
	pMMR, MSS or MSI-L	111 (24.7)
	Unknown	329 (73.3)
Local recurrence	No	198 (44.1)
	Yes	48 (10.7)
	Not applicable	201 (44.8)
	Unknown	2 (0.5)
Peritoneal metastases	No	331 (73.7)
	Yes	109 (24.3)
	Unknown	9 (2.0)
Number of metastatic sites grouped	0 or 1	163 (36.3)
	2 or more	277 (61.7)
	Unknown	9 (2.0)
FFPE tumor tissue examined	Biopsy	239 (53.2)
	Resection	210 (46.8)
Reason for stopping anti-EGFR therapy	Radiological disease progression	276 (61.5)
	Unacceptable toxicity	70 (15.6)
	Clinical disease progression	20 (4.5)
	Death	30 (6.7)
	Patient choice	38 (8.5)
	Loss of funding	1 (0.2)
	Not yet stopped anti-EGFR treatment	13 (2.9)
	Compassionate access to nivolumab	1 (0.2)
KRAS mutation status	Wild-type	449 (100)
NRAS mutation status	Wild-type	440 (98.0)
	Unknown	9 (2.0)
BRAF mutation status	Wild-type	389 (86.6)
	Mutant	26 (5.8)
	Unknown	34 (7.6)
Overall survival event	Censored	44 (9.8)
	Died	405 (90.2)
Progression-free survival event	Censored	27 (6.0)
	Progressed/died	422 (94.0)
Response rate	SD/PD	185 (41.2)
	CR/PR	205 (45.7)
	No scan	59 (13.1)
Disease control rate	PD	85 (18.9)
	SD/PR/CR	305 (67.9)
	No scan	59 (13.1)
Continuous AREG		Mean 52.6 (sd 32.7)
AREG 20%	≤20%	110 (24.5)
	>20%	339 (75.5)
Continuous EREG		Mean 49.8 (sd 35.8)
EREG 20%	≤20%	136 (30.3)
	>20%	313 (69.7)
Ligand dichotomous measure	AREG and EREG ≤20%	89 (19.8)
	AREG or EREG >20%	360 (80.2)

Abbreviations: AREG, amphiregulin; CR, complete response; dMMR, deficient mismatch repair; EGFR, epithelial growth factor receptor; EREG, epiregulin; FFPE, formalin-fixed paraffin-embedded; FOLFIRI, 5-fluorouracil, irinotecan, and folinic acid chemotherapy; FOLFOX, 5-fluorouracil, oxaliplatin, and folinic acid chemotherapy; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; PD, progression of disease; pMMR, proficient mismatch repair; PR, partial response; sd, standard deviation; SD, stable disease.

vs. either AREG or EREG high), 360 (80.2%) patients were AREG/EREG high.

The proportion of patients with high versus low AREG/EREG levels did not differ by age, sex, or WHO PS. As in PICCOLO, there were significantly more patients with right PTL, peritoneal metastases, poorly differentiated tumors, and *BRAF* mutant tumors in the low than the high ligand group (**Table 2**).

AREG/EREG in the primary analysis population

The primary hypothesis was that the combined AREG/EREG model, dichotomized at a 20% cut-off point, would have a prognostic effect on PFS and OS among *RAS*-wt patients treated with anti-EGFR therapy. The hypothesis was supported by the data, both when unadjusted (PFS: HR, 0.62; 95% CI, 0.49–0.79; $P = 0.0002$; OS: HR, 0.57; 95% CI, 0.45–0.72; $P < 0.00005$) and adjusted for the additional prognostic variables of performance status, PTL, primary surgery, tumor grade, neutrophil-to-lymphocyte ratio, platelet count, albumin, ALP, CEA, and peritoneal metastases (PFS: adjusted HR, 0.73; 95% CI, 0.56–0.95; $P = 0.02$; OS: adjusted HR, 0.66; 95% CI, 0.50–0.86; $P = 0.002$; **Fig. 1A** and **B**; **Table 3**). Findings were similar in the subgroup of patients who were also *BRAF*-wt (**Fig 1C** and **D**; **Table 3**). Median PFS in the high AREG/EREG group was 8.5 months, compared with 4.4 months in the low AREG/EREG group; median OS was 16.4 versus 8.9 months. Findings were similar when AREG and EREG were

Table 2. Descriptive statistics of characteristics of patients in low and high ligand expression groups with *P* values for association.

Variable	Category	Low ligand expression (≤20% AREG and ≤20% EREG) ($n = 89$) *n* (%) unless otherwise stated	High ligand expression (>20% AREG or >20% EREG) ($n = 360$) *n* (%) unless otherwise stated	*P* value for differences between low and high ligand expression
Age at starting anti-EGFR therapy (years)		Mean 61.2 (sd 10.6)	Mean 60.6 (sd 10.9)	0.64
Sex	Male	56 (62.9)	243 (67.5)	
	Female	33 (37.1)	117 (32.5)	0.41
Performance status[a]	0	31 (41.3)	140 (50.7)	
	1	36 (48.0)	113 (40.9)	
	2–3	8 (10.7)	23 (8.3)	0.35
Primary tumor location	Left colon	25 (28.1)	171 (47.5)	
	Rectum	23 (25.8)	123 (34.2)	
	Right colon	41 (46.1)	66 (18.3)	**<0.0005**
Primary surgery	Excised	46 (51.7)	204 (56.7)	
	In situ	43 (48.3)	156 (43.3)	0.40
Number of metastatic sites	0 or 1	31 (36.1)	132 (37.3)	
	2 or more	55 (63.9)	222 (62.7)	0.83
Peritoneal metastases	No	45 (52.3)	286 (80.8)	
	Yes	41 (47.7)	68 (19.2)	**<0.0005**
Tumor grade[a]	Poor	27 (34.6)	37 (12.6)	
	Moderate/Well	51 (65.4)	256 (87.4)	**<0.0005**
BRAF mutation status[a]	Wild-type	65 (82.3)	324 (96.4)	
	Mutant	14 (17.7)	12 (3.6)	**<0.0005**

Abbreviation: sd, standard deviation.
[a]Test excludes unknowns.

examined separately, both as continuous variables and dichotomized at a 20% cut-off point (**Table 3**).

In both the primary analysis population and the *RAS*- and *BRAF*-wt subgroup, a significant prognostic effect on locally assessed RR was seen for AREG and EREG as continuous variables, and when EREG—but not AREG—was dichotomized at the 20% cut-off point, in both unadjusted and adjusted models. Consequently, the combined ligand model was not associated with radiological response at the 20% cut-off point. However, both AREG (unadjusted OR, 1.79; 95% CI, 1.20–2.69; $P = 0.005$) and the combined model (adjusted OR, 1.60; 95% CI, 1.01–2.54; $P = 0.04$) were associated with response when dichotomized at the 50% cut-off point. This contrasted with DCR, where all measures were prognostic at the 20% cut-off point in unadjusted and adjusted analyses (**Table 4A** and **B**).

Analysis of key subgroups and clinical contexts

AREG/EREG had a significant prognostic effect on OS for all key subgroups and clinical contexts. Findings were similar for PFS, although in the subgroup of patients with right PTL, the trend toward superior PFS in those with high ligand percentage positivity did not reach statistical significance (**Fig. 2**). There was however a significant prognostic effect on both endpoints when the higher cut-off point of 50% was used (PFS: unadjusted HR, 0.62; 95% CI, 0.41–0.93; $P = 0.02$; OS: unadjusted HR, 0.42; 95% CI, 0.27–0.65; $P = 0.00009$).

A significant prognostic effect on PFS at a 20% cut-off point was similarly seen for patients who received single agent, later-line cetuximab under earlier clinical guidelines (i.e., without simultaneous chemotherapy, $n = 41$; unadjusted HR, 0.35; 95% CI, 0.16–0.74; $P = 0.007$) but not for OS (unadjusted HR, 0.84; 95% CI, 0.42–1.66; $P = 0.61$). Where patients received first-line anti-EGFR therapy in combination with either FOLFOX or FOLFIRI ($n = 359$)—the most common current treatment strategy—prognostic effects on both survival outcomes were observed (PFS: unadjusted HR, 0.64; 95% CI, 0.49–0.84; $P = 0.001$; OS: unadjusted HR, 0.54; 95% CI, 0.42–0.71; $P = 0.00001$).

Discussion

The primary hypothesis, that high AREG/EREG protein production (using a combined model dichotomized at a 20% cut-off point) would be associated with superior survival outcomes among *RAS*-wt patients treated with anti-EGFR therapy, was supported by the data in this cohort study. Critically, the prognostic effect remained significant when analyses were adjusted for other key prognostic variables: performance status, primary tumor location, primary surgery, tumor grade, neutrophil-to-lymphocyte ratio, platelet count, albumin, alkaline phosphatase, carcinoembryonic antigen, and peritoneal metastases.

AREG/EREG IHC has previously only been examined in patients treated with panitumumab, and not those who received the other approved anti-EGFR agent, cetuximab (3). In agreement with previous studies reporting a relationship between *AREG/EREG* mRNA and outcomes from cetuximab (16–22), here AREG/EREG IHC had a prognostic effect among patients receiving either agent.

There has been debate regarding the relative efficacy of anti-EGFR therapy in combination with different chemotherapy backbones. A meta-analysis showed both a PFS and OS advantage with anti-EGFR therapy in trials that used an irinotecan-based chemotherapy backbone (FOLFIRI or irinotecan alone), but not those where an oxaliplatin backbone was used (23). However, this included data from patients treated with CAPOX and cetuximab in the COIN trial, a subgroup shown to have a particular lack of benefit from anti-EGFR therapy (24). When these patients were excluded, a significant PFS benefit from anti-EGFR therapy in the oxaliplatin group was observed. Consequently, current European and American guidelines support the first-line use of an anti-EGFR agent in combination with either FOLFOX or

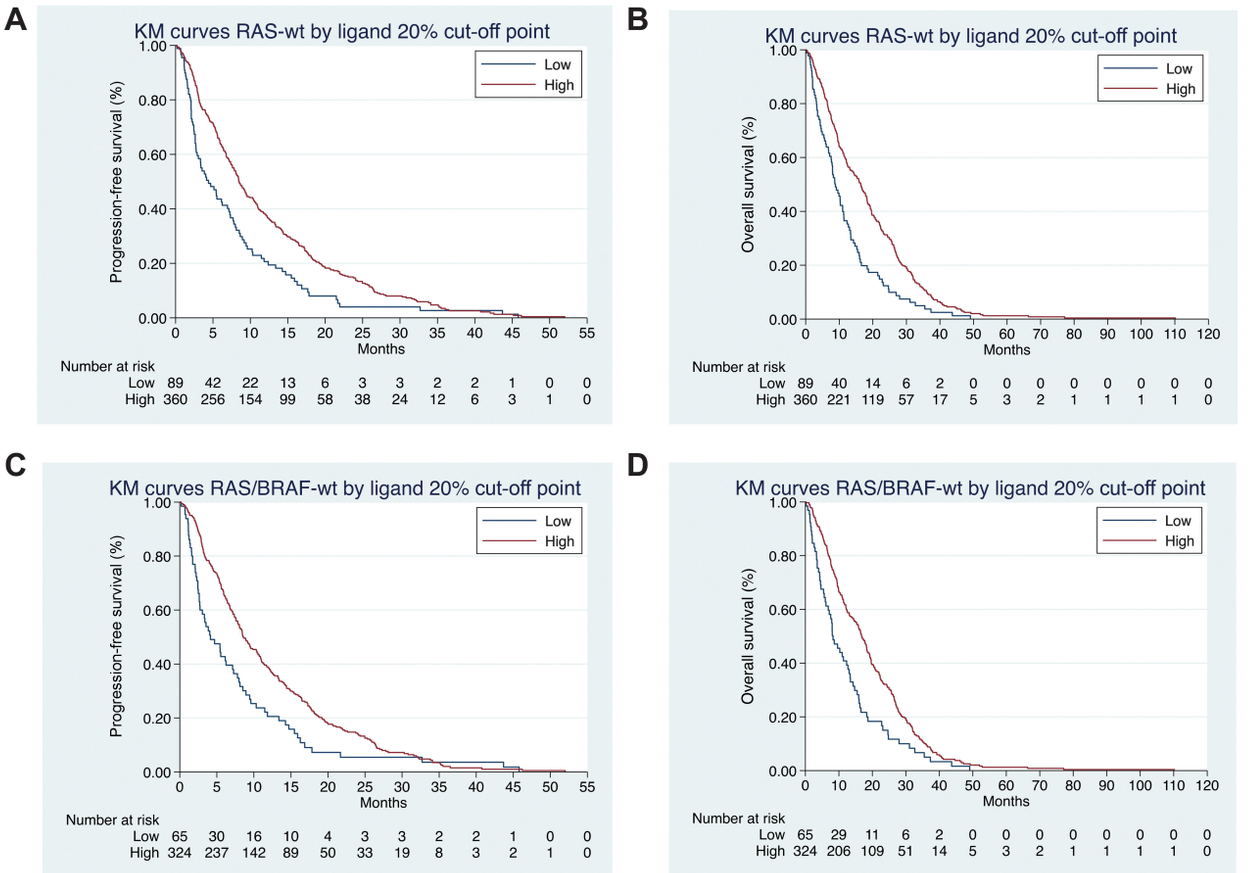

Figure 1.

PFS (**A**) and OS (**B**) Kaplan–Meier (KM) curves for *RAS*-wt patients and PFS (**C**) and OS (**D**) for *RAS*- and *BRAF*-wt patients with low (blue line) and high (red line) AREG/EREG expression (AREG and EREG ≤20% vs. AREG or EREG >20%).

FOLFIRI (5, 25). Crucially for the applicability of AREG/EREG IHC as a biomarker in an unselected patient population, a prognostic effect was demonstrated here among patients receiving either chemotherapy combination. Interestingly, the effect size was numerically greater with FOLFOX. Reasons for this observation might include a stronger contribution to survival outcomes from chemotherapy than anti-EGFR therapy in patients treated with FOLFIRI: without the problematic toxicity of peripheral neuropathy, it may be that patients were able to tolerate a higher dose intensity/duration of FOLFIRI than FOLFOX. However, confidence intervals substantially overlapped and caution is urged regarding overinterpretation of the data.

The design of targeted therapies is based on the concept that different molecular subtypes of cancer exist in different patients—intertumor (interpatient) heterogeneity. However, it is also recognized that heterogeneity exists within individual tumors—intratumoral heterogeneity (26). Through the outgrowth of treatment-resistant clones, this is a mechanism by which secondary resistance to therapies can develop (27, 28). Colorectal cancer biopsies taken at endoscopy are small, superficial fragments of the primary tumor. Concerns that AREG/EREG IHC performed on biopsy specimens might underrepresent the heterogeneity of ligand production within the tumor therefore needed addressing.

Studies examining *AREG/EREG* mRNA have variously analyzed tumor tissue from biopsies (16, 17), a mix of biopsies and resections (18–22), and resections only (11)—with positive findings. In PICCOLO, AREG/EREG IHC appeared to be predictive of panitumumab benefit in patients with tissue of either specimen type—although statistical significance was not reached, likely due to the small patient numbers in the two subgroups (3). Reassuringly, in this study, AREG/EREG IHC had a strong prognostic effect on OS and PFS in both patients with biopsy material and those with resection specimens. Further reassurance can also be obtained from the results of the prestudy quality assurance process, which not only showed good consistency of AREG/EREG IHC results between sites and following alterations to tissue handling, but also showed that results were broadly equivalent when tissue was taken from different regions of the same tumor.

It was again observed here that AREG and EREG protein production is significantly higher in left-sided primary tumors than in those originating on the right of the colon. This correlates with theories that fewer right PTL tumors are dependent on the EGFR pathway for growth and survival (6, 7). Significantly, the AREG/EREG model was prognostic for OS among patients with both right and left PTL tumors—providing further evidence that AREG/EREG might be useful in identifying a subgroup of patients with right PTL who benefit from anti-EGFR therapy. Interestingly, the trend toward superior PFS with high AREG/EREG production in the right PTL subgroup did not reach significance at the 20% cut-off

Table 3. Analysis of the prognostic effect of AREG and EREG on PFS and OS in *RAS*-wt patients, and *RAS*-wt and *BRAF*-wt patients.

Variable	Mutation status	Outcome	Category	Unadjusted HR (95% CI)	*P*	Adjusted HR (95% CI)	*P*
AREG/EREG: combined model	*RAS*-wt	PFS	AREG and EREG ≤20%	1.0		1.0	
			AREG or EREG >20%	0.62 (0.49–0.79)	0.0002	0.73 (0.56–0.95)	**0.02**
		OS	AREG and EREG ≤20%	1.0		1.0	
			AREG or EREG >20%	0.57 (0.45–0.72)	<0.00005	0.66 (0.50–0.86)	**0.002**
	RAS-wt *BRAF*-wt	PFS	AREG and EREG ≤20%	1.0		1.0	
			AREG or EREG >20%	0.63 (0.48–0.83)	0.001	0.73 (0.54–1.00)	**0.05**
		OS	AREG and EREG ≤20%	1.0		1.0	
			AREG or EREG >20%	0.59 (0.45–0.78)	0.0002	0.63 (0.47–0.85)	**0.003**
AREG: continuous (per 10 percentage points)	*RAS*-wt	PFS		0.96 (0.93–0.99)	0.007		
		OS		0.95 (0.92–0.98)	0.001		
	RAS-wt *BRAF*-wt	PFS		0.97 (0.94–1.00)	0.06		
		OS		0.96 (0.93–0.99)	0.03		
AREG: high vs. low	*RAS*-wt	PFS	≤20%	1.0			
			>20%	0.70 (0.56–0.87)	0.002		
		OS	≤20%	1.0			
			>20%	0.63 (0.51–0.79)	0.0001		
	RAS-wt *BRAF*-wt	PFS	≤20%	1.0			
			>20%	0.68 (0.53–0.88)	0.003		
		OS	≤20%	1.0			
			>20%	0.65 (0.50–0.83)	0.001		
EREG: continuous (per 10 percentage points)	*RAS*-wt	PFS		0.95 (0.92–0.98)	0.0002		
		OS		0.94 (0.92–0.97)	<0.00005		
	RAS-wt *BRAF*-wt	PFS		0.96 (0.93–0.99)	0.01		
		OS		0.96 (0.93–0.99)	0.006		
EREG: high vs. low	*RAS*-wt	PFS	≤20%	1.0			
			>20%	0.62 (0.51–0.77)	<0.00005		
		OS	≤20%	1.0			
			>20%	0.63 (0.51–0.78)	<0.00005		
	RAS-wt *BRAF*-wt	PFS	≤20%	1.0			
			>20%	0.68 (0.53–0.88)	0.003		
		OS	≤20%	1.0			
			>20%	0.70 (0.56–0.89)	0.003		

Note: Estimated crude HRs and 95% CIs for the effect of: (i) the combined AREG/EREG model, (ii) continuous AREG and EREG (scaled by a factor of 10 to enhance HR interpretability), and (iii) each biomarker dichotomized at a 20% cut-off point on PFS and OS in *RAS*-wt patients, and *RAS*-wt and *BRAF*-wt patients, treated with anti-EGFR therapy. In adjusted analyses, the combined model was adjusted for performance status, primary tumor location, primary surgery, tumor grade, neutrophil-to-lymphocyte ratio, platelet count, albumin, alkaline phosphatase, carcinoembryonic antigen, and peritoneal metastases.
Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; -wt, wild-type.

point, but did when the cut-off point was raised to 50%. This finding may simply have been the result of smaller patient numbers with right PTL and AREG/EREG IHC positivity above 20% [66/107 (61.7%) patients with right PTL vs. 294 or 342 (86.0%) with left PTL], limiting the power to detect a significant prognostic effect on PFS. It should also be considered that PFS was a less reliable endpoint in this dataset, where the maximum time limit for pretreatment imaging was generous (8 weeks) to allow for real-world variations in practice. There was only a small difference in the point estimate of the HR (0.71 compared with 0.62), and no trend when the cut-off point was varied between 20% and 80% (data not shown), so there is insufficient evidence to suggest that a higher cut-off point is required for right than left PTL tumors. That said, it is conceivable that a higher cut-off point might be required for this subgroup to ensure molecular subtypes resistant to or antagonized by anti-EGFR agents are effectively excluded.

Historically, it has been controversial as to whether *BRAF* mutation status is a definitive negative predictive biomarker for benefit from anti-EGFR therapy. During the course of this study, positive results from the BEACON trial introduced a new targeted therapeutic option for patients with *BRAF*-mutant tumors (29), lessening the requirement for a positive predictive biomarker for anti-EGFR therapy in this subgroup. Subsequent results from the FIRE-4.5 trial provided prospective evidence for a lack of benefit from anti-EGFR therapy in *BRAF*-mutant disease (13). We therefore repeated our analyses in a secondary population of patients with dual *RAS*- and *BRAF*-wt tumors. Despite the reduction in statistical power from the exclusion of the *BRAF*-mutant subgroup, results were concordant with the primary analysis population.

High ligand production using the combined AREG/EREG model, dichotomized at a 20% cut-off point, was associated with superior DCR but not RR. However, RR was significantly greater among patients with AREG/EREG tumor cell positivity of greater than 50%. This is an interesting finding as it suggests that, in situations where disease control is the goal of treatment—such as the palliative setting where anti-EGFR agents are most commonly used—a 20% cut-off point is satisfactory. However, in situations where a reduction in tumor volume is important—such as when attempting to convert unresectable metastatic disease to resectable—a higher cut-off point might be beneficial. Alternatively, it is possible that locally reported radiological response was overestimated, explaining the discrepancy between RR and OS results. Caution is therefore required regarding interpretation

Table 4A. Association between AREG and EREG and locally assessed response rate.

Variable	Mutation status	Category	Statistic	SD/PD	CR/PR	Unadjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
AREG/EREG: combined model	*RAS*-wt	AREG and EREG ≤20%	*n* (%)	39 (54.2)	33 (45.8)	1.0			
		AREG or EREG >20%		146 (45.9)	172 (54.1)	1.39 (0.83–2.33)	0.21		
	RAS-wt *BRAF*-wt	AREG and EREG ≤20%	*n* (%)	25 (51.0)	24 (49.0)	1.0			
		AREG or EREG >20%		132 (45.1)	161 (54.9)	1.27 (0.69–2.33)	0.44		
AREG: continuous (per 10% points)	*RAS*-wt		Mean (sd)	4.82 (3.23)	5.84 (3.18)	1.10 (1.04–1.17)	**0.002**		
	RAS-wt *BRAF*-wt		Mean (sd)	5.19 (3.15)	6.05 (3.06)	1.09 (1.02–1.17)	**0.01**		
AREG: high vs. low	*RAS*-wt	≤20%	*n* (%)	48 (53.3)	42 (46.7)	1.0			
		>20%		137 (45.7)	163 (54.3)	1.36 (0.85–2.18)	0.20		
	RAS-wt *BRAF*-wt	≤20%	*n* (%)	32 (50.0)	32 (50.0)	1.0			
		>20%		125 (45.0)	153 (55.0)	1.22 (0.71–2.11)	0.47		
EREG: continuous (per 10% points)	*RAS*-wt	≤20%	Mean (sd)	4.10 (3.55)	6.02 (3.35)	1.17 (1.10–1.24)	<**0.00005**		
	RAS-wt *BRAF*-wt	>20%	Mean (sd)	4.49 (3.54)	6.24 (3.24)	1.16 (1.09–1.24)	<**0.00005**		
EREG: high vs. low	*RAS*-wt	≤20%	*n* (%)	73 (65.2)	39 (34.8)	1.0		1.0	
		>20%		112 (40.3)	166 (59.7)	2.77 (1.76–4.38)	<**0.00005**	2.27 (1.34–3.82)	**0.002**
	RAS-wt *BRAF*-wt	≤20%	*n* (%)	55 (64.7)	30 (35.3)	1.0		1.0	
		>20%		102 (39.7)	155 (60.3)	2.79 (1.67–4.64)	**0.00008**	2.20 (1.24–3.92)	**0.007**

of these findings and prospective validation is necessary in settings where RR is important.

The recently presented results of the Japanese phase III PARADIGM trial, comparing first-line FOLFOX and panitumumab vs. FOLFOX and bevacizumab in patients with *RAS*-wt mCRC, demonstrated improved OS (HR, 0.84; 95% CI, 0.72–0.98; $P = 0.030$), RR and R0 resection rate with panitumumab rather than bevacizumab (8). However, no significant difference in OS was detected among the subgroup with right PTL (HR, 1.09). ctDNA had been collected prior to treatment in an optional biomarker substudy. Building on the work of prior studies demonstrating a subgroup of patients with gene alterations related to primary resistance to anti-EGFR therapy and inferior outcomes with anti-EGFR therapy (30, 31), Shitara and colleagues performed a "hyperselection" analysis, excluding those with ctDNA alterations in *KRAS*, *NRAS*, *PTEN*, and extracellular domain *EGFR* mutations, *HER2* and *MET* amplifications, and *ALK*, *RET*, and *NTRK1* fusions. This identified a subgroup of patients with right PTL with longer median OS with panitumumab than bevacizumab (38.9 vs. 30.9 months; HR, 0.82; 95% CI, 0.50–1.35), but the difference was not statistically significant (10). While a ctDNA-guided approach has attractions, it would be interesting to examine AREG/EREG IHC in this dataset

Table 4B. Association between AREG and EREG and locally assessed DCR.

Variable	Mutation status	Category	Statistic	PD	SD/CR/PR	Unadjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
AREG/EREG: combined model	*RAS*-wt	AREG and EREG ≤20%	*n* (%)	26 (36.1)	46 (63.9)	1.0		1.0	
		AREG or EREG >20%		59 (18.6)	259 (81.4)	2.48 (1.42–4.33)	**0.001**	1.92 (1.05–3.54)	**0.04**
	RAS-wt *BRAF*-wt	AREG and EREG ≤20%	*n* (%)	18 (36.7)	31 (63.3)	1.0		1.0	
		AREG or EREG >20%		49 (16.7)	244 (83.3)	2.89 (1.50–5.58)	**0.002**	2.63 (1.31–5.30)	**0.007**
AREG: continuous (per 10% points)	*RAS*-wt		Mean (sd)	4.53 (3.40)	5.58 (3.16)	1.11 (1.03–1.19)	**0.009**		
	RAS-wt *BRAF*-wt		Mean (sd)	4.99 (3.43)	5.82 (3.04)	1.09 (1.00–1.18)	0.05		
AREG: high vs. low	*RAS*-wt	≤20%	*n* (%)	28 (31.1)	62 (68.9)	1.0			
		>20%		57 (19.0)	243 (81.0)	1.93 (1.13–3.28)	**0.02**		
	RAS-wt *BRAF*-wt	≤20%	*n* (%)	19 (29.7)	45 (70.3)	1.0			
		>20%		48 (17.3)	230 (82.7)	2.02 (1.09–3.76)	**0.03**		
EREG: continuous (per 10% points)	*RAS*-wt		Mean (sd)	3.77 (3.69)	5.48 (3.46)	1.15 (1.07–1.23)	**0.0001**		
	RAS-wt *BRAF*-wt		Mean (sd)	4.34 (3.74)	5.70 (3.37)	1.12 (1.04–1.21)	**0.004**		
EREG: high vs. low	*RAS*-wt	≤20%	*n* (%)	40 (35.7)	72 (64.3)	1.0			
		>20%		45 (16.2)	233 (83.8)	2.88 (1.74–4.75)	<**0.00005**		
	RAS-wt *BRAF*-wt	≤20%	*n* (%)	28 (32.9)	57 (67.1)	1.0			
		>20%		39 (15.2)	218 (84.8)	2.75 (1.56–4.84)	**0.0005**		

Note: Estimated crude ORs and 95% CIs for the effect of: (i) the combined AREG/EREG model, (ii) continuous AREG and EREG (scaled by a factor of 10 to enhance odds ratio interpretability), and (iii) each biomarker dichotomized at a 20% cutoff point on the odds of complete or partial response (**Table 4A**) and the odds of stable disease, complete or partial response (**Table 4B**) in *RAS*-wt patients, and *RAS*- and *BRAF*-wt patients, treated with anti-EGFR therapy. In adjusted analyses, response rate was adjusted for performance status, primary tumor location, tumor grade, lymphocyte count, and carcinoembryonic antigen; and DCR was adjusted for sex, performance status, primary tumor location, tumor grade, lymphocyte count, neutrophil-to-lymphocyte ratio, and platelet count.
Abbreviations: CI, confidence interval; CR, complete response; PD, progression of disease; PR, partial response; SD, stable disease; sd, standard deviation.

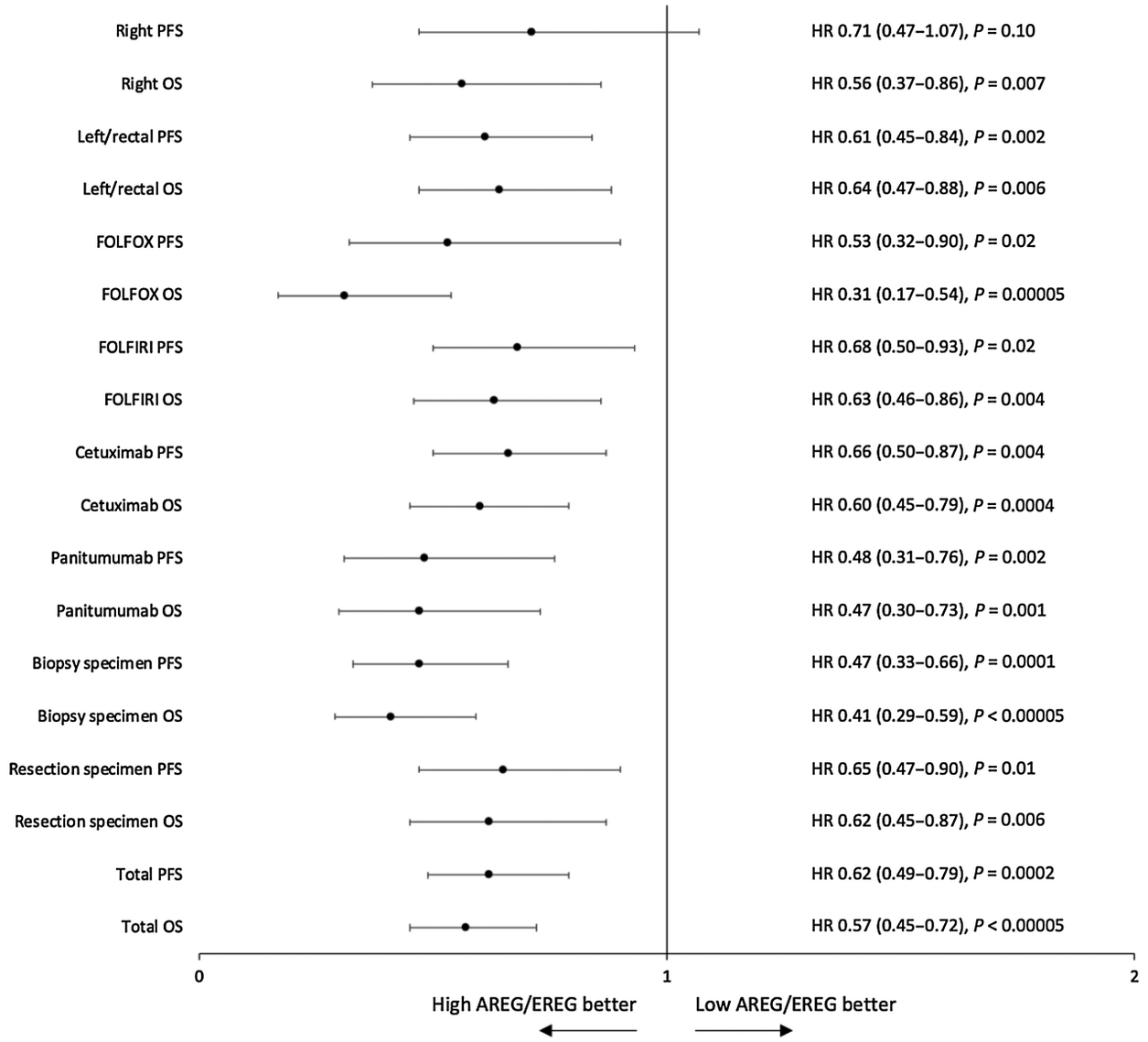

Figure 2.

Forest plot displaying the prognostic effect of AREG/EREG on PFS and OS, stratified by key variables. Estimated crude HRs and 95% CIs for the effect of the combined AREG/EREG model (20% cut-off point) on PFS and OS in *RAS*-wt patients treated with anti-EGFR therapy, stratified by primary tumor location, chemotherapy backbone, anti-EGFR agent (restricted to patients who received anti-EGFR therapy as part of first-line treatment), and tumor specimen type. FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin; FOLFIRI, 5-fluorouracil, leucovorin, and irinotecan; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; PTL, primary tumor location.

to determine whether greater precision could be achieved through combination of these biomarkers.

The design of this study—a largely retrospective cohort study—was efficient and sufficiently addressed queries about the performance of AREG/EREG IHC among clinical subsets not represented in the PICCOLO trial. It was also possible to observe in an external dataset that AREG and EREG tumor cell percentage positivities were similarly distributed and correlated with each other, with similar mean values to those seen in PICCOLO. Central extended *RAS* and *BRAF* mutation testing was successfully performed, improving the reliability of results —and highlighting the necessity to improve access to such testing in routine care environments. Previously observed associations between key clinicopathologic variables and ligand production (PTL, presence/absence of peritoneal metastases, tumor grade) were also confirmed. The main limitation of the study design was that the lack of a control group meant biomarker–treatment interactions could not be tested. That said, AREG/EREG IHC had no prognostic effect among patients receiving irinotecan chemotherapy alone in the PICCOLO trial—only those receiving panitumumab—and so the prognostic effect seen in patients receiving anti-EGFR therapy here goes some way to validating the earlier findings. Full prospective validation of the PICCOLO findings is being explored in the ongoing ARIEL trial of chemotherapy plus anti-EGFR therapy versus chemotherapy alone in patients with right PTL with high *AREG/EREG* mRNA expression

(ISRCTN11061442; ref. 32), with plans to subsequently assess the biomarker using IHC.

Conclusion

In this cohort study, a significant prognostic effect of AREG/EREG IHC was seen among patients receiving anti-EGFR therapy as part of routine care for mCRC. High AREG/EREG protein production was associated with improved OS in all prespecified clinical subsets studied, including patients with right PTL, those receiving either of the approved anti-EGFR agents, those whose backbone chemotherapy was FOLFOX or FOLFIRI, and those with FFPE tissue obtained by biopsy or surgical resection. It was further demonstrated that IHC could be effectively performed across six different laboratories. Prospective validation of AREG/EREG IHC in a randomized controlled trial is now planned to enable the introduction of this biomarker into routine clinical practice.

Authors' Disclosures

C.J.M. Williams reports grants and nonfinancial support from Roche Diagnostics and grants from UKRI: Innovate UK during the conduct of the study as well as personal fees from Roche Diagnostics, Merck Serono, and Servier outside the submitted work; in addition, C.J.M. Williams has a patent for PCT/US2021/050777 pending to University of Leeds. F. Aghaei reports grants from UKRI and Roche Diagnostics during the conduct of the study. A. Muranyi reports personal fees from, employment/salary from, stock in, nonfinancial support from, and travel/accommodation expenses from Roche outside the submitted work; in addition, A. Muranyi has a patent for AU2016288461 issued to Roche, a patent for AU2021273603 pending to Roche, a patent for AU2021273604 pending to Roche, a patent for CA2990214 pending to Roche, a patent for CA3162586 pending to Roche, a patent for CA3162816 pending to Roche, a patent for EP3313878 issued to Roche, a patent for EP3514174 issued to Roche, a patent for EP3978525 pending to Roche, a patent for US 10,852,304 issued to Roche, a patent for US 16/949252 pending to Roche, a patent for CN202180033615.2 pending to Roche, a patent for EP21724604.0 pending to Roche, a patent for JP2022-567539 pending to Roche, and a patent for US 18/050425 pending to Roche. D. Yan reports personal fees from Roche Diagnostics Solutions during the conduct of the study as well as personal fees from Roche Diagnostics Solutions outside the submitted work. M. Shires reports grants from Roche Diagnostics during the conduct of the study as well as grants from Roche Diagnostics outside the submitted work. H.M. Wood reports grants from Yorkshire Cancer Research during the conduct of the study. S.D. Richman reports a patent for PCT/US2021/050777 pending. L. Galvin reports grants from Roche Diagnostics and UKRI during the conduct of the study. A. Sudan reports personal fees from Roche outside the submitted work. K. Lambert reports grants from UKRI and Roche Diagnostics during the conduct of the study. I. Bibby reports grants from UKRI and Roche Diagnostics during the conduct of the study. A. Montazeri reports nonfinancial support from Astella outside the submitted work. T. Gochera reports grants from UK Research and Innovation (UKRI) and Roche Diagnostics during the conduct of the study. M. Saunders reports personal fees from Servier, MSD, and Bayer outside the submitted work. H. Stott reports grants from UKRI and Roche Diagnostics during the conduct of the study. A. Mukherjee reports grants from UKRI and nonfinancial support from Roche Diagnostics during the conduct of the study as well as grants from Bowel Research UK, Liverpool Centre for Mathematics in Healthcare, and MRC-Innovate UK outside the submitted work. M. Ilyas reports grants from Roche Diagnostics and UK Research and Innovation (UKRI) during the conduct of the study as well as other support from Roche Diagnostics outside the submitted work. D. Sculthorpe reports grants from UKRI and nonfinancial support from Roche during the conduct of the study as well as grants from Bowel Research UK outside the submitted work. N. Brown reports personal fees from Amgen, AstraZeneca, Merck, Pfizer, and Bristol Myers Squibb and other support from Roche outside the submitted work. N.P. West reports grants from Innovate UK and Roche Tissue Diagnostics during the conduct of the study as well as personal fees from Astellas, GSK, Bristol Myers Squibb, and Amgen outside the submitted work. J.F. Seligmann reports grants and personal fees from Merck Serono, Pierre Fabre, and Seagen and personal fees from Servier and Zentalis outside the submitted work. K. Shanmugam reports other support from Roche during the conduct of the study as well as other support from Roche outside the submitted work; in addition, K. Shanmugam has a patent for Roche Diagnostics pending and collaboration between Roche and Leeds University. P. Quirke reports grants and personal fees from Roche during the conduct of the study as well as grants and personal fees from Amgen and personal fees from Bayer outside the submitted work; in addition, P. Quirke has a patent for Amphiregulin and epiregulin and prediction of response to anti-EGFr agents by IHC pending. No disclosures were reported by the other authors.

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