Artificial Intelligence–Assisted Amphiregulin and Epiregulin IHC Predicts Panitumumab Benefit in *RAS* Wild-Type Metastatic Colorectal Cancer AC

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ABSTRACT

Purpose: High tumor mRNA levels of the EGFR ligands amphiregulin (AREG) and epiregulin (EREG) are associated with anti-EGFR agent response in metastatic colorectal cancer (mCRC). However, ligand RNA assays have not been adopted into routine practice due to issues with analytic precision and practicality. We investigated whether AREG/EREG IHC could predict benefit from the anti-EGFR agent panitumumab.

Experimental Design: Artificial intelligence algorithms were developed to assess AREG/EREG IHC in 274 patients from the PICCOLO trial of irinotecan with or without panitumumab (Ir vs. IrPan) in *RAS* wild-type mCRC. The primary endpoint was progression-free survival (PFS). Secondary endpoints were RECIST response rate (RR) and overall survival (OS). Models were repeated adjusting separately for *BRAF* mutation status and primary tumor location (PTL).

Results: High ligand expression was associated with significant PFS benefit from IrPan compared with Ir [8.0 vs. 3.2 months; HR, 0.54; 95% confidence interval (CI), 0.37–0.79; $P = 0.001$]; whereas low ligand expression was not (3.4 vs. 4.4 months; HR, 1.05; 95% CI, 0.74–1.49; $P = 0.78$). The ligand-treatment interaction was significant ($P_{interaction} = 0.02$) and remained significant after adjustment for *BRAF*-mutation status and PTL. Likewise, RECIST RR was significantly improved in patients with high ligand expression (IrPan vs. Ir: 48% vs. 6%; $P < 0.0001$) but not those with low ligand expression (25% vs. 14%; $P = 0.10$; $P_{interaction} = 0.01$). The effect on OS was similar but not statistically significant.

Conclusions: AREG/EREG IHC identified patients who benefitted from the addition of panitumumab to irinotecan chemotherapy. IHC is a practicable assay that may be of use in routine practice.

Introduction

The anti-EGFR mAbs, panitumumab and cetuximab are used in the treatment of metastatic colorectal cancer (mCRC; refs. 1, 2). Somatic activating mutations in the *RAS* oncogene have been shown to preclude a response to these agents (3–6). *Post hoc* analyses of the seminal trials of anti-EGFR agents have further identified right-sided primary tumor location (PTL; ref. 7) and activating mutations in *BRAF* (8) as possible negative predictive markers for clinical benefit among *RAS* wild-type (*RAS*-wt) patients. However, despite these advances in patient selection, approximately 40% of those receiving these drugs do not achieve a radiological response, while being exposed to their financial costs and potential toxicities, necessitating the development of further predictive biomarkers.

Autocrine and paracrine stimulation of EGFR by its ligands, amphiregulin (AREG) and epiregulin (EREG), are mechanisms of colorectal cancer EGFR pathway dependence (9). The two molecules are commonly overexpressed in colorectal cancer (10, 11) and are highly coexpressed at the transcriptional (9, 11) and protein levels (10). An association between high *AREG/EREG* mRNA expression levels and a positive response to anti-EGFR therapy among *RAS*-wt patients has been demonstrated in a number of studies (11–17), including in patients with right-sided PTL (18). However, *AREG/EREG* pretreatment analysis has not been adopted into routine clinical practice in part due to a lack of broad access to mRNA quantification techniques, and the potential for analytical imprecision resulting from variability in tissue processing and fixation times in different health care environments.

IHC analysis of formalin-fixed, paraffin-embedded (FFPE) tumor tissue represents an attractive alternative method for quantifying AREG and EREG expression. IHC is routinely performed in histopathology laboratories responsible for the diagnosis of colorectal cancer and shorter turnaround times may increase the opportunity to influence treatment decisions. Moreover, IHC is capable of accurately delineating ligand expression within the tumor area specifically, which cannot be achieved with mRNA quantification. That said, to date there is only preliminary evidence that ligand protein expression correlates with the effectiveness of anti-EGFR therapy (19).

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Translational Relevance

The EGFR ligands amphiregulin (AREG) and epiregulin (EREG) are commonly overexpressed in colorectal cancer. High ligand mRNA levels are associated with favorable outcomes from anti-EGFR therapy. However, this biomarker has not yet been adopted into routine practice, in part due to a lack of broad access to mRNA quantification techniques. IHC is routinely performed in histopathology laboratories responsible for colorectal cancer diagnosis and may represent a preferable technique.

In this prospectively planned retrospective analysis of the PICCOLO trial (irinotecan with or without panitumumab in advanced colorectal cancer), we are the first to demonstrate a predictive effect of AREG/EREG IHC for an anti-EGFR therapy. The effect was independent of primary tumor location and *BRAF* mutation status, suggesting it is possible to identify subgroups of these patients who benefit from panitumumab. While AREG/EREG IHC can be interpreted by eye, we also developed artificial intelligence algorithms to improve the efficiency and reproducibility of ligand quantification.

In this prospectively planned, retrospective analysis of the phase III PICCOLO trial of irinotecan with or without panitumumab in fluoropyrimidine-resistant *RAS*-wt mCRC (20), we assessed the relationship between AREG and EREG IHC and outcomes with panitumumab. We chose *a priori* to use a single combined model, where high expression of either AREG or EREG was regarded as indicative of high ligand expression. We also investigated AREG and EREG as individual predictive biomarkers, and independence from confounders such as *BRAF* mutation status, PTL, and peritoneal metastases in secondary analyses. To further aid the timely delivery of AREG/EREG interpretation in future practice, we have developed artificial intelligence (AI) algorithms to semiautomate the process of IHC ligand quantification.

Materials and Methods

The PICCOLO trial has been reported previously (20, 21). Following UK national ethical approval, and with the written informed consent of all participants, 460 patients with *KRAS* c.12,13,59-61 wt mCRC progressing during or after fluoropyrimidine treatment (with or without oxaliplatin) were randomized 1:1 to irinotecan (Ir) versus irinotecan with panitumumab (IrPan), continued until disease progression or unacceptable toxicity. The University of Leeds Clinical Trials Research Unit managed the trial, overseen by an independent trial steering committee. Both the PICCOLO trial and the translational work presented here were conducted in accordance with Good Clinical Practice guidelines and the provisions of the Declaration of Helsinki.

Ethical approval for the current translational study was given by the North East York Research Ethics Committee. The study includes all patients who, at the point of consent for the PICCOLO trial, had agreed to the use of their archival tissue in future research and for whom adequate stored FFPE tumor tissue remained. Additional mutational analysis for *KRAS* c.146, *NRAS* c.12,13,59-61, and *BRAF* c.1799T>A has been performed previously (20). The primary analysis in this study was conducted in patients who were *KRAS* c.12,13,59-61,146 and *NRAS* c.12,13,59-61 wt (hereafter "*RAS*-wt").

Endpoints

The primary endpoint was PFS; secondary endpoints were overall survival (OS) and RECIST response rate (RR). PFS and RR data were unchanged from the primary PICCOLO trial analysis but updated 2-year OS data were used in this analysis.

IHC

Three 4-μm-thick tissue sections from each FFPE block were cut onto separate Superfrost Plus slides (VWR). One section was stained with anti-AREG (SP272) [Roche Tissue Diagnostics (RTD)] and another with anti-EREG (SP326; RTD) rabbit mAbs using a BenchMark ULTRA instrument (RTD) and preprogrammed protocols. The third section was stained with hematoxylin and eosin using Mayer hematoxylin and Scott tap water substitute as the bluing reagent. Digital images of whole slides were generated using a VENTANA DP 200 scanner at 200 times magnification.

AI algorithm development and verification

Pathologists blinded to patients' treatment allocations, mutation profiles, and clinical outcomes annotated tumor areas on the digital whole slide images and excluded nontumor areas, including tissue artefact and areas of necrosis. Whole slide analysis algorithms (RTD) were developed using machine learning (ML) and computer vision (CV) techniques to compute the percentage of positively stained tumor cells within the tumor areas for AREG and EREG (**Fig. 1A** and **B**). Separate algorithms were developed for each ligand, each consisting of the following sequential steps: cell detection, tumor versus nontumor cell classification, and positive versus negative IHC classification of tumor cells.

To verify the performance of CV and ML algorithms, 30 fields of view (FOV) for each marker were selected by RTD pathologists from 30 patients in PICCOLO as most representative of the staining patterns and morphology found in colorectal cancer. Three RTD pathologists manually labeled positive and negative tumor cells in each FOV. A total of 15,000 cells on AREG stained slides and 17,000 cells on EREG stained slides were manually counted. Manual counts were compared with algorithm-derived results (**Fig. 1C–H**). Quantification of tumor *AREG* and *EREG* mRNA expression within PICCOLO has been reported previously (16). In a second internal validation, algorithm-derived IHC results and ligand mRNA expression were compared in the subset of PICCOLO patients where both were available.

Statistical analysis

Stata was used for all statistical analyses [Stata Statistical Software, Release 16 (2019); StataCorp, RRID:SCR_012763]. Baseline patient characteristics were compared between treatment arms (IrPan vs. Ir) using two-tailed *t* tests, Wilcoxon rank-sum tests (for variables with nonnormally distributed frequency distributions), and Pearson χ^2 tests (for categorical variables). Patient characteristics were compared with the whole trial population using the same tests. Box plots and Wilcoxon rank-sum tests were used to compare the distributions of continuous AREG and EREG IHC percentage positivity between *BRAF*-wt and mutant cases, left (splenic flexure to rectum) versus right PTL, and finally presence versus absence of peritoneal metastases.

In our assessment of *AREG* and *EREG* mRNA within PICCOLO, we described the rationale for dichotomizing the sample into patients with high (*AREG* and/or *EREG* mRNA expression in the top tertile) and low ligand expression (neither *AREG* nor *EREG* expression in the top tertile; ref. 16). To produce a measure with clinical utility, we again dichotomized the sample in this study by IHC percentage positivity (either AREG or EREG high vs. both AREG and EREG low). A ROC

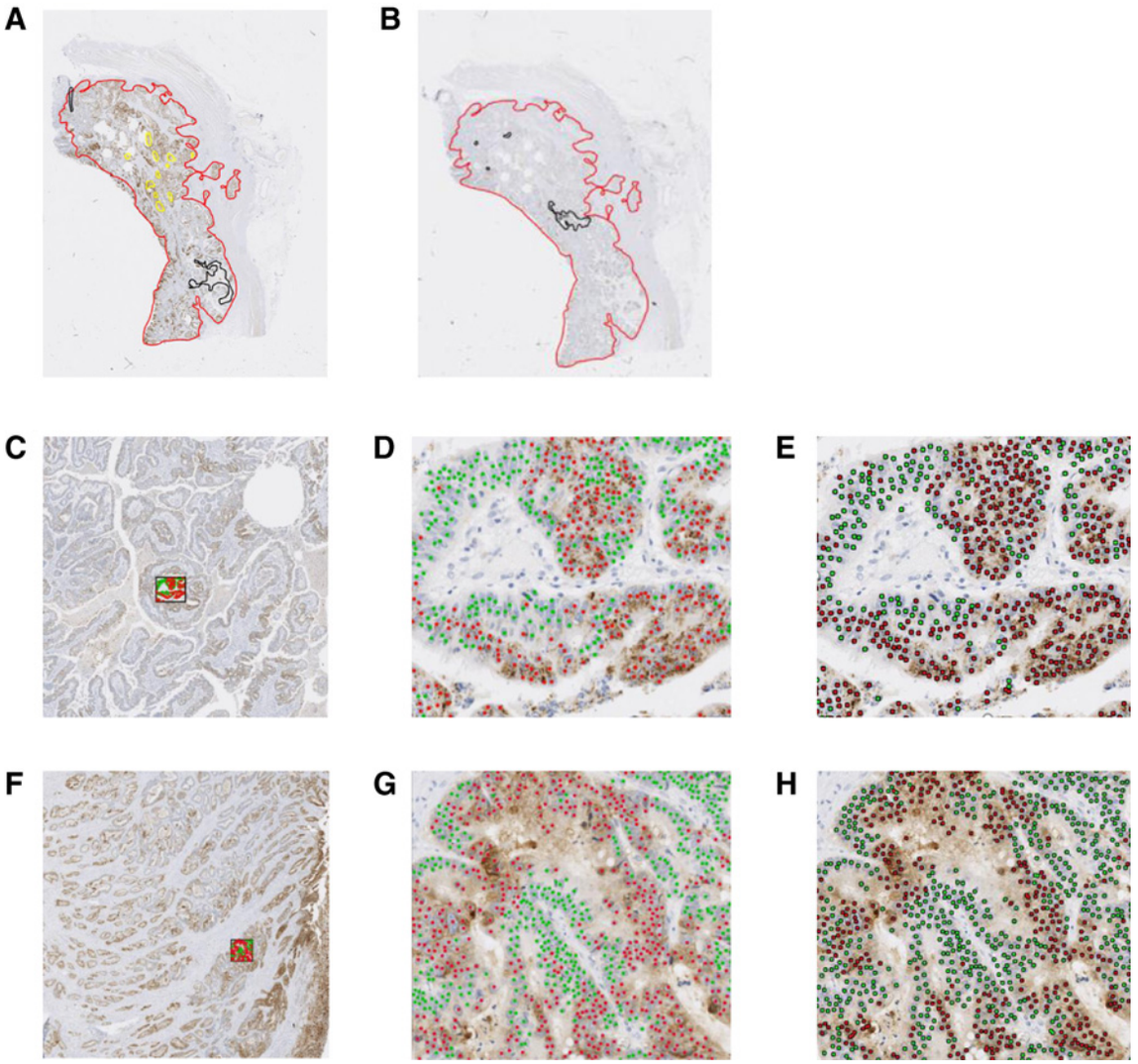

Figure 1.

AI algorithm development for interpretation of AREG and EREG IHC in colorectal cancer. AREG (**A**) and EREG (**B**) stained whole-tissue sections were manually annotated to outline the tumor regions (red contours) and exclude blank regions or artifacts (black contours) and areas of necrosis (yellow contours). To verify the algorithms, 30 FOVs for each marker, representative of typical colorectal cancer morphology and staining patterns, were selected on AREG (**C**) and EREG (**F**) stained slides. Tumor cells within each FOV were annotated by pathologists as positive (red) or negative (green) for AREG (**D**) and EREG (**G**), and compared with algorithm-derived results for the respective ligand [AREG (**E**), EREG (**H**)]. The AREG algorithm showed a Lin concordance correlation coefficient (CCC) of 0.96, 0.91, 0.84, and 0.98 for positive tumor cells, negative tumor cells, all tumor cells, and percentage of positive tumor cells, respectively, while the EREG algorithm showed a CCC of 0.97, 0.93, 0.88, and 0.97 for these measures.

curve was generated, referring to the previous mRNA dichotomization as the basis for sensitivity and specificity calculations. The maximal Youden index was calculated (22) to identify the IHC cut-off point that best aligned with that of the mRNA assay for use in the primary analysis.

Ligand IHC percentage positivity was assessed as a prognostic marker in all patients treated with irinotecan alone—using both the dichotomous classifier (high vs. low) and each ligand separately as continuous variables—in Cox proportional hazards models. Where AREG and EREG were assessed as continuous variables, ligand percentage positivity was scaled down by a factor of 10 to enhance the interpretability of HRs. Analyses were first performed unadjusted and then adjusted for World Health Organization performance status, response to previous therapy, and previous chemotherapy (yes vs. no).

Ligand expression was then assessed as a predictive marker for panitumumab therapy benefit on PFS and OS in unadjusted Cox proportional hazards models, stratifying by ligand IHC status (high vs. low), assessing treatment effects (IrPan vs. Ir), and testing for ligand-treatment interactions using likelihood ratio tests. These models were then repeated adjusting separately for *BRAF* mutation, PTL, and presence of peritoneal metastases to determine whether ligand-treatment interactions persisted after adjustment for these potential confounding factors. The unadjusted models were also repeated in the *RAS*-wt and *BRAF*-wt group and also considering dichotomous AREG

and dichotomous EREG separately. Kaplan–Meier curves were plotted. The concordance probability was assessed using Harrell C index.

Unadjusted RRs for the effect of IrPan versus Ir on RR were estimated from generalized linear models stratified by dichotomous ligand IHC. The likelihood ratio test for ligand-treatment interaction was then performed.

Results

AREG/EREG IHC was completed in 313 (68%) of 460 patients randomized in PICCOLO. A total of 39 patients with *KRAS* c.146 and/or *NRAS* c.12,13,59-61 mutations were excluded, leaving 274 (60%) "*RAS*-wt" patients for inclusion in the primary analysis. 49 (18%) of 274 patients had a *BRAF* c.1799T>A mutation (**Fig. 2**). A total of 242 (88%) *RAS*-wt patients had a disease progression event and 248 (91%) had died. Only the availability of mRNA ligand data and the proportion of resection versus biopsy specimens examined differed significantly between the patient cohort in this biomarker study and the main trial population (Supplementary Table S1).

Ligand expression

AREG and EREG IHC percentage positivity were strongly correlated (Spearman correlation coefficient 0.77, $P < 0.0005$). Among the subset of patients for whom both IHC and mRNA data were available [186 (59%) of 313 patients], the two measures were positively correlated for each of the ligands (Spearman correlation coefficient: AREG 0.64, $P < 0.0001$; EREG 0.80, $P < 0.0001$). The maximal Youden index was 0.581, which determined high versus low cut-off points of 47% IHC positivity for AREG and 52% for EREG. Sensitivity and specificity with these cut-off points were 0.716 and 0.865, respectively. To facilitate manual AREG/EREG interpretation in future clinical practice, the optimal cut-off points were rounded to 50% for both markers. Here, sensitivity and specificity were 0.707 and 0.827, respectively (Supplementary Table S2). Use of the 50% cut-off points divided the

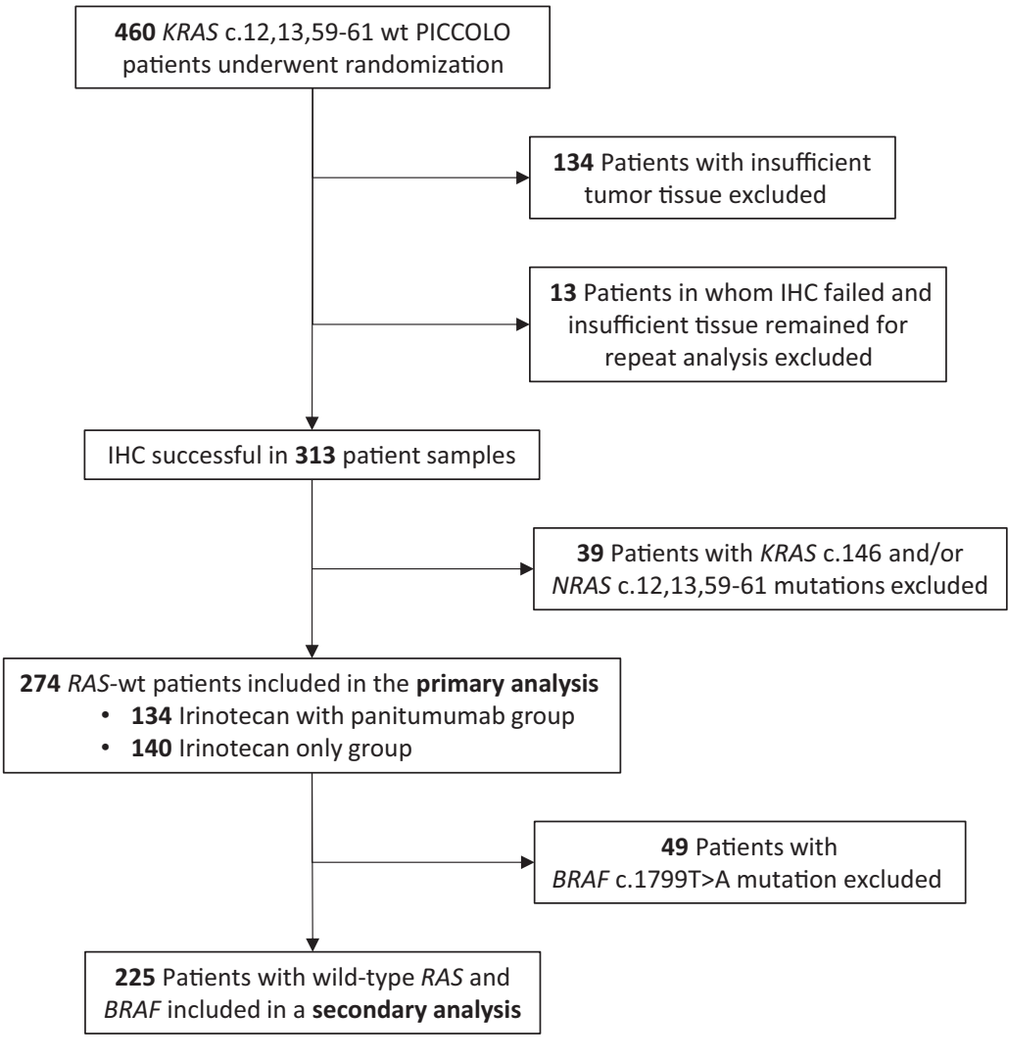

Figure 2.
Flow diagram demonstrating the breakdown of the study sample. wt, wild-type; IHC, immunohistochemistry.

primary analysis sample ($n = 274$) into 132 (48%) high and 142 (52%) low cases (Supplementary Fig. S1).

The high and low ligand groups had an even distribution of patients in each treatment group. There were significantly more patients with right-sided PTL, *BRAF*-mutant status, and peritoneal metastases in the low than the high ligand group (**Table 1**).

AREG/EREG performance as a combined dichotomous biomarker

There was no evidence for a prognostic effect of IHC ligand status (high vs. low) on PFS [unadjusted HR, 1.22 (95% confidence interval, CI, 0.86–1.75); $P = 0.27$] or OS [unadjusted HR, 1.01 (95% CI, 0.71–1.43); $P = 0.98$] in patients treated with irinotecan alone, or in adjusted analyses (Supplementary Table S3).

The primary hypothesis was that high ligand IHC positivity would be predictive of PFS benefit from panitumumab in *RAS*-wt patients. This hypothesis was supported by the data, as shown in **Table 2** and **Fig. 3**.

Among *RAS*-wt patients with high ligand IHC positivity, median PFS with IrPan was 8.0 months, compared with 3.2 months with irinotecan alone [HR, 0.54 (95% CI, 0.37–0.79); $P = 0.001$]. Conversely, there was no benefit from panitumumab in patients with low ligand IHC positivity [median PFS, IrPan vs. Ir: 3.4 vs. 4.4 months; HR, 1.05 (95% CI, 0.74–1.49); $P = 0.78$] (**Table 2**; **Fig. 3**). The ligand-treatment interaction was significant whether unadjusted ($P = 0.02$) or adjusted ($P = 0.02$). The results for OS were less marked ($P_{\text{interaction}} = 0.19$), most likely due to the lack of OS effect of panitumumab overall in the trial. Within the *RAS*-wt and *BRAF*-wt subpopulation the effect sizes were similar, although ligand-treatment interactions did not reach significance, likely due to the smaller sample size (PFS $P_{\text{interaction}} = 0.21$; **Table 2**).

Table 1. Descriptive statistics of characteristics of *RAS*-wt patients in low and high ligand expression groups (as determined by IHC) and *P* values for association.

Patient characteristic	Category	Low ligand expression (≤50% AREG and ≤50% EREG) ($n = 142$)	High ligand expression (>50% AREG or >50% EREG) ($n = 132$)	*P* value for differences between low and high ligand expression[a]
Treatment arm	Ir	74 (52.9)	66 (47.1)	0.73
	IrPan	68 (50.8)	66 (49.2)	
Age at randomization (years)		Mean 61.8 (sd 10.3)	Mean 61.9 (sd 10.3)	0.86
Sex	Male	93 (48.7)	98 (51.3)	0.14
	Female	48 (58.5)	34 (41.5)	
	Unknown	1	0	
Primary tumor location	Left (including rectal)	76 (40.4)	112 (59.6)	<0.0005
	Right	64 (78.1)	18 (21.9)	
	Unknown	2	2	
Peritoneal metastases	No	97 (46.9)	110 (53.1)	0.001
	Yes	42 (71.2)	17 (28.8)	
	Unknown	3	5	
$BRAF_{c.1799T>A}$	Wild-type	98 (43.6)	127 (56.4)	<0.0005
	Mutant	44 (89.8)	5 (10.2)	
Performance status	0–1	134 (51.7)	125 (48.3)	0.90
	2	8 (53.3)	7 (46.7)	
mRNA ligand data available	No	41 (38.7)	65 (61.3)	0.001
	Yes	101 (60.1)	67 (39.9)	
Resection or biopsy	Resection	104 (60.8)	67 (39.2)	<0.0005
	Biopsy	38 (36.9)	65 (63.1)	
Overall survival time (months)		Median 9.3 (IQR, 4.3–19.2)	Median 11.5 (IQR, 7.9–20.0)	0.24[b]
Death event	No	12 (46.2)	14 (53.8)	0.54
	Yes	130 (52.4)	118 (47.6)	
Progression-free survival time (months)		Median 3.8 (IQR, 2.7–8.1)	Median 5.5 (IQR, 2.8–9.2)	0.56[b]
Progression event	No	15 (46.9)	17 (53.1)	0.55
	Yes	127 (52.5)	115 (47.5)	
Best response	Complete or partial response	27 (43.6)	35 (56.4)	0.12
	Stable disease or progression of disease	113 (54.9)	93 (45.1)	
	Unknown	2 (33.3)	4 (66.7)	

Note: Data are *n* (%) unless stated otherwise.
Abbreviations: IQR, interquartile range; Ir, irinotecan; IrPan, irinotecan with panitumumab; sd, standard deviation.
[a]Unknown category excluded from tests. Wilcoxon rank-sum test used for age, and Pearson χ^2 test or Fisher exact test used for categorical variables.
[b]*P*-value from log-rank test for equality of survivor functions.

Table 2. Analysis of the predictive effect of the combined AREG/EREG dichotomous classifier on survival outcomes.

Outcome	Mutation subgroup	All patients		Low ligand expression (≤50% AREG and ≤50% EREG)		High ligand expression (>50% AREG or >50% EREG)		*P* value for ligand-treatment interaction
		Events/ patients	Unadjusted HR (95% CI)	Events/ patients	Unadjusted HR (95% CI)	Events/ patients	Unadjusted HR (95% CI)	
PFS	*RAS*-wt	242/269	0.77 (0.60–1.00) $P = 0.05$	127/140	1.05 (0.74–1.49) $P = 0.78$	115/129	0.54 (0.37–0.79) $P = 0.001$	0.02
	RAS-wt and *BRAF*-wt	197/221	0.65 (0.49–0.86) $P = 0.003$	87/97	0.80 (0.52–1.23) $P = 0.31$	110/124	0.53 (0.36–0.78) $P = 0.001$	0.21
OS	*RAS*-wt	248/274	1.03 (0.80–1.32) $P = 0.81$	130/142	1.21 (0.86–1.71) $P = 0.28$	118/132	0.87 (0.60–1.25) $P = 0.44$	0.19
	RAS- and *BRAF*-wt	202/225	0.90 (0.68–1.18) $P = 0.44$	89/98	0.95 (0.62–1.45) $P = 0.81$	113/127	0.84 (0.58–1.21) $P = 0.34$	0.65

Note: Estimated crude HRs and 95% confidence intervals for the effect of treatment (irinotecan with panitumumab vs. irinotecan) on progression-free survival and overall survival in *RAS* wild-type patients, then *RAS* wild-type and *BRAF* wild-type patients, stratified by the dichotomous classifier and including likelihood ratio tests for ligand-treatment interaction.

Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; -wt, wild-type.

The RECIST RR was significantly improved by the addition of panitumumab to irinotecan among *RAS*-wt patients with high ligand IHC positivity [IrPan vs. Ir: 48% vs. 6%; RR, 7.8 (95% CI, 2.90–20.69); $P < 0.0001$] but not those with low ligand IHC positivity [IrPan vs. Ir: 25% vs. 14%; RR, 1.8 (95% CI, 0.89–3.65); $P = 0.10$]. The ligand-treatment interaction was significant ($P_{interaction} = 0.01$; Supplementary Table S4).

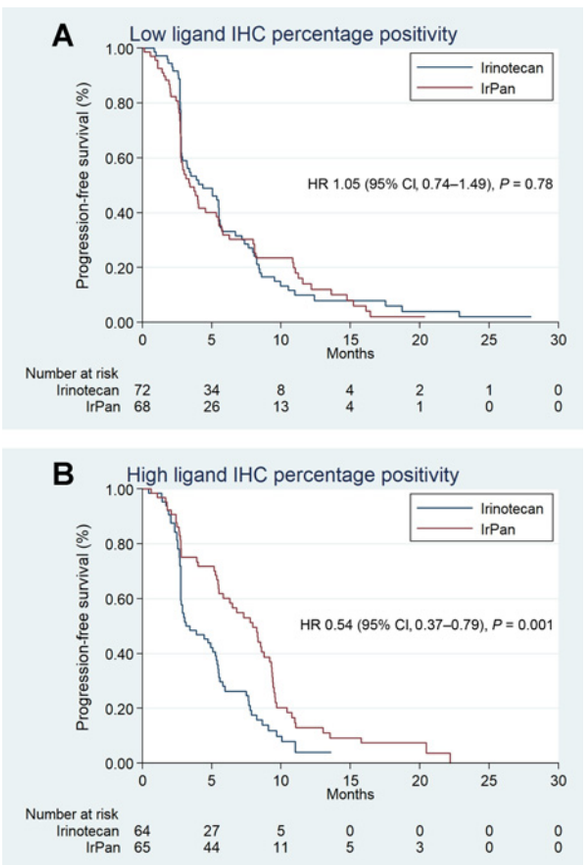

Figure 3.

PFS Kaplan–Meier curves for RAS wild-type patients with low (**A**) and high (**B**) ligand expression. CI, confidence interval; HR, hazard ratio; IHC, immunohistochemistry; IrPan, irinotecan with panitumumab.

AREG and EREG as separate biomarkers

As a secondary analysis, AREG and EREG IHC percentage positivity were examined separately, both as continuous variables and dichotomized at a 50% cut-off point. Neither AREG nor EREG were prognostic for PFS or OS among those treated with irinotecan only (data not shown). EREG was predictive of PFS benefit from panitumumab, both when dichotomized ($P_{interaction} = 0.02$) and when examined as a continuous variable ($P_{interaction} = 0.01$). Although a similar effect was seen among the smaller number of patients who exhibited high AREG expression, the ligand-treatment interactions did not reach significance, and neither AREG nor EREG were predictive of panitumumab therapy OS benefit (**Table 3**; Supplementary Table S5).

Effect of possible confounding factors

Given that *BRAF* mutation, right-sided PTL, and the presence of peritoneal metastases were associated with low ligand IHC positivity, we explored whether the differential treatment effects by ligand level seen for PFS in the *RAS*-wt patients were instead driven by these factors. The dichotomous classifier remained a significant predictor of panitumumab therapy benefit after adjustment for *BRAF* ($P_{interaction} = 0.02$), PTL ($P_{interaction} = 0.01$), and peritoneal metastases ($P_{interaction} = 0.01$). Similar results were found when each ligand was examined separately as a continuous variable (Supplementary Table S6). Thus, the ligand-treatment effect appears to be independent of these potential confounders.

Interrogation of combined AREG/EREG model—alternative cut-off points

For exploratory purposes, panitumumab benefit on PFS using different cut-off points was examined (Supplementary Table S7). Lowering the cut-off point to 20% identified patients with low ligand expression who in fact had significantly inferior PFS with the addition of panitumumab [HR, 1.73 (95% CI, 1.02–2.95); $P = 0.04$] and were perhaps harmed by this treatment. In the high ligand group, the HRs for IrPan versus Ir on PFS showed marked benefit with HRs around 0.55 for the 20% up to the 50% cut-off point. The ligand-treatment interaction remained significant throughout this range, indicating

Table 3. Analysis of the predictive effect of AREG and EREG as individual dichotomized biomarkers on survival outcomes.

		All patients		Low ligand expression (≤50%)		High ligand expression (>50%)		*P* value for ligand-treatment interaction
Outcome	Ligand	Events/ patients	Unadjusted HR (95% CI)	Events/ patients	Unadjusted HR (95% CI)	Events/ patients	Unadjusted HR (95% CI)	
PFS	AREG	242/269	0.77 (0.60–1.00), $P = 0.05$	167/186	0.88 (0.65–1.19), $P = 0.41$	75/83	0.58 (0.36–0.92), $P = 0.02$	0.20
	EREG	242/269	0.79 (0.61–1.02), $P = 0.07$	140/155	1.05 (0.75–1.46), $P = 0.79$	102/114	0.53 (0.35–0.79), $P = 0.002$	0.02
OS	AREG	248/274	1.03 (0.80–1.32), $P = 0.81$	170/188	1.08 (0.79–1.46), $P = 0.64$	78/86	0.95 (0.60–1.49), $P = 0.82$	0.69
	EREG	248/274	1.05 (0.82–1.34), $P = 0.72$	144/157	1.31 (0.94–1.82), $P = 0.11$	104/117	0.78 (0.53–1.15), $P = 0.21$	0.04

Note: Estimated crude hazard ratios and 95% confidence intervals for the effect of treatment (irinotecan with panitumumab vs. irinotecan) on progression-free survival and overall survival in *RAS* wild-type patients stratified by AREG (high vs. low) and EREG (high vs. low) IHC percentage positivity (50% cut-off point), including likelihood ratio tests for ligand-treatment interaction.
Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; -wt, wild-type.

potential for the use of different cut-off points in different clinical scenarios.

Comparison of biopsies and resections

A total of 171 (62%) of 274 tumor specimens were taken from resections and 103 (38%) from biopsies. To ensure our dichotomous model remained reliable irrespective of the tumor specimen type examined, we also analyzed these subgroups separately. Regardless of ligand expression, patients who had undergone resections gained PFS benefit from panitumumab [HR, 0.62 (95% CI, 0.44–0.86); $P = 0.005$] while those with biopsy specimens did not [HR, 1.06 (95% CI, 0.70–1.62); $P = 0.78$], perhaps because these patients were more likely to have presented with advanced metastatic disease rather than recurrence of disease detected during routine postoperative surveillance. For both specimen types, there was a trend to suggest the addition of panitumumab to irinotecan might benefit patients with high ligand expression and not those with low ligand expression (Supplementary Table S8). The ligand-treatment interactions did not reach significance, likely due to the smaller sample sizes in these subgroup analyses.

Discussion

In this analysis, patients with low AREG/EREG expression obtained no PFS benefit from the addition of panitumumab to irinotecan chemotherapy, while those with high expression of either ligand gained significant benefit. We are the first to demonstrate the utility of IHC in this context, and the first to have developed AI algorithms to aid AREG/EREG quantification.

Mechanisms for growth and survival through EGFR in colorectal cancer are complex: on ligation, EGFR can form either homodimers or heterodimers with other members of the ErbB receptor family, expression of which can be amplified; there are multiple endogenous ligands, which are often overexpressed by tumor cells; and downstream intracellular signaling pathways overlap, with constituent molecules that are frequently affected by activating mutations (23). Consequently, *RAS*- (24), *BRAF*- (25), and *PIK3CA*-mutation status (26), and PTEN protein expression (27) have all been examined as potential predictive biomarkers for anti-EGFR therapy benefit, as have *EGFR* (28), *HER2* (29), and *HER3* (30) amplification and mutation, and *EGFR* gene copy number (31). However, with the clear exception of *RAS*-mutation status, none of these biomarkers have proven as consistent as *AREG* and *EREG* in the prediction of outcomes from anti-EGFR therapy.

Despite the wealth of evidence supporting *AREG/EREG* mRNA quantification to inform anti-EGFR treatment decisions (11–18), this knowledge is not yet utilized in routine practice. Reasons for this include a lack of broad access to mRNA quantification assays and issues with analytic precision in real-world settings. IHC is a preferable technique as it is routinely performed in histopathology laboratories responsible for the diagnosis of colorectal cancer and as such can normally be provided at the point of care, resulting in shorter turnaround times. When partnered with AI, IHC may therefore increase the feasibility of bringing AREG/EREG quantification into routine clinical use.

Adenocarcinomas that originate from the right side of the colon (caecum to splenic flexure) are more frequently associated with *BRAF, PTEN*, and *PIK3CA* mutations, as well as mismatch repair enzyme deficiencies (32). The relationship between tumor sidedness—as a proxy for such molecular characteristics—and anti-EGFR therapy has been extensively examined, with retrospective analyses of the CRYSTAL (FOLFIRI ± cetuximab; ref. 7) and PRIME (FOLFOX ± panitumumab; ref. 33) trials demonstrating OS benefit from anti-EGFR agents in *RAS*-wt patients with left-sided but not right-sided PTL. These data have been used to inform U.S. national treatment guidelines, which counsel against anti-EGFR therapy in the first-line setting for those with right PTL (2). Here, AREG and EREG protein expression were higher with left-sided than right-sided PTL. However, our dichotomous classifier remained a significant predictor of panitumumab benefit after adjustment for this factor, indicating its potential clinical utility in identifying right PTL patients who could be appropriately offered anti-EGFR therapy early in their treatment course.

BRAF-mutant status is known to be associated with decreased panitumumab efficacy (25). In this study, interaction testing in the dual *RAS*- and *BRAF*-wt subgroup did not reach statistical significance. However, effect sizes were strongly trended in a similar manner to that of the total study population. The lack of statistical significance in the PFS analysis is therefore likely a consequence of the approximately 30% reduction in power to detect a ligand-treatment interaction in this smaller subgroup. Adjustment for *BRAF* mutation status provided a more powerful analysis than the subgroup analysis, and our dichotomous AREG/EREG classifier remained a significant predictor of

panitumumab therapy benefit in that model. We can therefore be confident that the ligand-treatment interaction observed was independent of *BRAF*-mutation status.

We chose to combine AREG and EREG into a single dichotomous outcome measure, where a positive biomarker outcome was recorded if either AREG or EREG was high and a negative outcome only if both were low. This model allows information from both ligands to be incorporated into a clinically useable measure. It is known that both AREG and EREG can activate EGFR (34–36), but they are highly coexpressed so any multivariable model attempting to include both would be affected by multicoIlinearity. In prior studies using multivariable models, AREG proved more useful in some (17, 37, 38), whereas EREG outperformed AREG in others (11, 12, 15). Here, EREG appeared to represent a more useful individual biomarker. However, given the fact that a number of tumors demonstrate overexpression of one ligand—not both—it is preferable to combine information from both ligands to avoid denying anti-EGFR treatment to the subgroup of patients with high AREG expression alone.

A significant ligand-treatment interaction has previously been demonstrated in this dataset with *AREG/EREG* mRNA quantification. To provide a rationale for our IHC cut-off point selection, we chose that which best aligned with the mRNA cut-off point. The 50% IHC cut-off point this method generated divided the sample into roughly even high and low groups and gave a strong ligand-treatment interaction ($P_{\text{interaction}} = 0.02$). The sensitivity of the IHC cut-off point when using the mRNA results as the ground truth was 71%. While the IHC assay therefore generates fewer positive results at this cut-off point, the two measures are assessing different aspects of AREG/EREG expression and so are not likely to provide identical results. To provide further reassurance, we examined other potential IHC cut-off points in an exploratory analysis. When the cut-off point was lowered to 20%, the high ligand group continued to obtain significant benefit. The 20% cut-off point also identified patients with very low ligand expression who appeared to be harmed by the addition of panitumumab to chemotherapy. This echoes previous studies where treatment with anti-EGFR agents resulted in inferior PFS for *KRAS*-mutant patients (39–41). AREG/EREG testing may therefore identify a subgroup of *RAS*-wt patients similarly at risk of harm from anti-EGFR therapy. A potential mechanism could be that EGFR inhibition induces rapid feedback activation in patients with very low AREG/EREG expression, as seen with *BRAF* inhibitor monotherapy in patients with *BRAF*-mutant cancers. However, further characterization of tumors of this type is required to determine the biological mechanisms underlying this observation.

Ultimately, it may be useful to have two cut-off points: a lower level to ensure patients are not offered anti-EGFR therapy where they would likely be harmed by it; and a higher marker to identify patients with moderate AREG/EREG expression where the risks and benefits of anti-EGFR therapy should be weighed against additional factors such as availability of immunotherapy for mismatch repair deficient tumors, angiogenesis inhibitors for patients with right-sided primaries, and BRAF and MEK inhibitors for *BRAF*-mutant tumors. Here, the predictive effect of AREG/EREG was independent of the potential confounders of PTL, *BRAF*-mutation status, and peritoneal carcinomatosis. We suggest that AREG/EREG assessment could have an important role in aiding the personalization of care in an era of increasing therapeutic options for mCRC.

Interestingly, while it could be hypothesized that raising the AREG/EREG cut-off point might identify a group with very high ligand expression who markedly benefit from anti-EGFR therapy, this was not borne out in our data. In fact, above 50%, the HR for progression among those with high ligand expression remained fairly static regardless of the cut-off point level. Instead, it may rather be that a threshold level is reached after which further increases in the availability of AREG or EREG do not impact upon EGFR activation or panitumumab efficacy.

The mean drug acquisition cost of panitumumab for a 60 kg patient is $8,000 per month and so a validated IHC predictive biomarker offers the potential for significant savings for health care systems having to manage escalating cancer treatment costs. Introducing a new biomarker test to the routine mCRC diagnostic work-up does, however, place greater strain on pathologists, who would be required to deliver a result in a clinically meaningful timeframe. Here we have tried to mitigate that burden by developing AI algorithms to automate as far as possible the quantification of AREG and EREG IHC percentage positivity. AI algorithms are likely to become integral to health care delivery in coming years but themselves necessitate investment in digital pathology technologies and computing power. While we have envisaged AI algorithms assisting with quantification of this biomarker in future clinical practice, the simple cut-off point of 50% IHC positivity for both AREG and EREG means it remains possible for health care providers to implement ligand testing by eye in settings where infrastructural support for AI is not yet in place.

There is clearly potential for overfitting of data in a retrospective analysis. We assessed a single combined biomarker and prespecified a primary endpoint. The endpoints were correlated, as were the various regression models, and therefore no formal correction for multiple testing was applied. However, we would urge caution in the interpretation of borderline significant results in this paper. A larger prospective study should be performed to confirm the findings presented here. Other limitations of the study include the fact that tissue was only available for 313 (68%) of 460 *KRAS* c.12,13,59-61 wt patients in the IrPan versus Ir randomization in PICCOLO. Reassuringly, however, the baseline characteristics and outcomes of the study sample were not dissimilar from those of the main trial population.

Although there was a marked PFS benefit seen with panitumumab therapy in *RAS*-wt patients with high ligand expression, this did not translate into longer OS. For the reason that a PFS benefit but no OS benefit was seen among *RAS*-wt patients in PICCOLO and other second-line mCRC studies (42, 43), we chose PFS as the primary endpoint in this study. There was little post-trial cross-over in the original PICCOLO trial and the discrepancy between PFS and OS findings may rather be explained by shorter survival after progression in patients who had received panitumumab. Establishing a mechanism for this observation would be a useful aim of future work.

This study demonstrates the utility of AREG/EREG IHC as a combined biomarker predictive of panitumumab benefit in mCRC. IHC is a feasible method for quantifying AREG and EREG in routine clinical practice and we have further improved its practicality by developing AI algorithms to semiautomate histopathologic interpretation. Such a biomarker allows identification of patients likely to gain benefit from anti-EGFR agents and, equally importantly, identifies those for whom alternative treatment options should be pursued at a timepoint in their disease course when there is most to be gained from systemic therapies. Prospective verification of these findings is required to ensure they are replicated in the first-line setting and with cetuximab as well as panitumumab. Examination of the predictive power of AREG/EREG in the adjuvant and neoadjuvant settings would also be an interesting aim of future work.

Authors' Disclosures

C.J.M. Williams reports grants from Innovate UK, Yorkshire Cancer Research, and CRUK and grants and nonfinancial support from Roche Tissue Diagnostics and Amgen Inc during the conduct of the study; in addition, C.J.M. Williams has a patent for Prediction of response to EGFR-directed therapies using epiregulin and amphiregulin pending to University of Leeds. J.F. Seligmann reports personal fees from Merck Serono, Pierre Fabre, Roche Diagnostics, and Servier outside the submitted work. M. Shires reports grants from Roche Tissue Diagnostics during the conduct of the study. S. Brown reports grants from Cancer Research UK during the conduct of the study. S. Singh reports a patent for US 10,852,304 issued and a patent for US 16/949,252 pending. A. Muranyi reports personal fees from F. Hoffman-La Roche LTD during the conduct of the study, as well as nonfinancial support from F. Hoffman-La Roche Ltd outside the submitted work; in addition, A. Muranyi has a patent for Materials and methods for performing histochemical assays for human pro-epiregulin and amphiregulin pending to F. Hoffman-La Roche Ltd and a patent for Histochemical systems and methods for evaluating EGFR and EGFR ligand expression in tumor samples pending to F. Hoffman-La Roche Ltd. C. Guetter reports other from Roche outside the submitted work; in addition, C. Guetter has a patent for US 62/706,988 pending. A. Lorsakul reports a patent for Materials and methods for performing histochemical assays for human pro-epiregulin and amphiregulin issued to US 10,852,304, patent US 16/949,252 pending, patent US 63/021,627 pending, and patent US 62/706,988 pending. Z. Zhao reports a patent for Digital pathology analysis of EGFR ligands in colorectal cancer pending. J. Martin reports other from Roche Tissue Diagnostics during the conduct of the study, as well as other from Roche Tissue Diagnostics outside the submitted work; in addition, J. Martin has a patent for US 63/021,627 pending to Roche and a patent for US 62/706,988 pending to Roche. X. Wang reports a patent for Prediction of response to EGFR-directed therapies using epiregulin and amphiregulin pending. W.-W. Liu reports personal fees from Roche Tissue Diagnostics outside the submitted work; in addition, W. Liu has a patent for Prediction of response to EGFR-directed therapies using epiregulin and amphiregulin pending. D. Yan reports personal fees from Roche Tissue Diagnostics and Roche Tissue Diagnostics outside the submitted work. N.P. West reports grants from Innovate UK during the conduct of the study, as well as grants from Yorkshire Cancer Research outside the submitted work. J.H. Barrett reports grants from Roche during the conduct of the study. M. Barnes reports personal fees from Roche during the conduct of the study, as well as personal fees and nonfinancial support from Roche outside the submitted work; in addition, M. Barnes has a patent for Epi/amphiregulin digital pathology predictive algorithm pending to Roche. P. Quirke reports grants, personal fees, and nonfinancial support from Roche/Ventana and grants and personal fees from Amgen during the conduct of the study; in addition, P. Quirke has a patent applied for on this work pending and with royalties paid from Roche, University of Leeds. K. Shanmugam reports other from Roche Diagnostics Solutions outside the submitted work; in addition, K. Shanmugam has a patent for Prediction of response to EGFR-directed therapies using epiregulin and amphiregulin pending and a patent for Materials and methods for performing histochemical assays for human pro-epiregulin and amphiregulin pending. No disclosures were reported by the other authors.

Authors' Contributions

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