Amphiregulin Expression Is a Predictive Biomarker for *EGFR* Inhibition in Metastatic Colorectal Cancer: Combined Analysis of Three Randomized Trials

Arndt Stahler[1], Sebastian Stintzing[2,3], Dominik P. Modest[3,4], Ingrid Ricard[1], Clemens Giessen-Jung[1], Christine Kapaun[5], Boryana Ivanova[5], Florian Kaiser[6], Ludwig Fischer von Weikersthal[7], Nicolas Moosmann[8], Andreas Schalhorn[1], Martina Stauch[9], Alexander Kiani[10], Swantje Held[11], Thomas Decker[12], Markus Moehler[13], Jens Neumann[3,5], Thomas Kirchner[3,5], Andreas Jung[3,5], and Volker Heinemann[1,3]

ABSTRACT

Purpose: Amphiregulin (*AREG*) and epiregulin (*EREG*) are ligands of *EGFR*. Predictive information for anti-*EGFR* treatment in metastatic colorectal cancer (mCRC) was observed, but data for other agents is limited.

Experimental Design: Ligand mRNA expression; *RAS, BRAF, PIK3CA* mutations; and *EGFR* expression were assessed by qRT-PCR, pyrosequencing, and IHC, respectively, in mCRC tumor tissue of patients participating in the randomized controlled trials FIRE-1, CIOX, and FIRE-3. Normalized mRNA expression was dichotomized using median and third quartile. Overall (OS) and progression-free survival (PFS) were estimated by Kaplan–Meier method including univariate and multivariate Cox regression analyses. Penalized spline regression analysis tested interaction of mRNA expression and outcome.

Results: Of 688 patients with available material, high *AREG* expression was detected in 343 (>median) and 172 (>3rd quartile) patients. High *AREG* expression was associated with significantly higher OS [26.2 vs. 21.5 months, HR = 0.80; 95% confidence interval (CI), 0.68–0.94; $P = 0.007$], PFS (10.0 vs. 8.1 months, HR = 0.74; 95% CI, 0.63–0.86; $P = 0.001$), and objective response rate (63.1% vs. 51.6%, $P = 0.004$) compared to low expression at both threshold values. This effect remained significant in multivariate Cox regression analysis (OS: $P = 0.01$, PFS: $P = 0.002$). High *AREG* mRNA expression interacted significantly with the efficacy of cetuximab compared with bevacizumab (OS: $P = 0.02$, PFS: $P = 0.04$) in *RAS* WT mCRC.

Conclusions: High *AREG* mRNA expression is a favorable prognostic biomarker for mCRC which interacted significantly with efficacy of anti-*EGFR* treatment.

[1]Department of Internal Medicine III and Comprehensive Cancer Centre Munich, University Hospital Grosshadern, Ludwig-Maximilian-Universitaet Muenchen, Munich, Germany. [2]Medical Department, Division of Hematology, Oncology and Tumor Immunology (CCM), Charité Universitaetsmedizin Berlin, Berlin, Germany. [3]DKTK, German Cancer Consortium, German Cancer Research Centre (DKFZ), Heidelberg, Germany. [4]Medical Department, Division of Hematology, Oncology and Tumor Immunology (CVK), Charité Universitaetsmedizin Berlin, Berlin, Germany. [5]Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany. [6]VK&K Studien GbR, Landshut, Germany. [7]Klinikum St. Marien, Oncology, Amberg, Germany. [8]Department for Hematology and Oncology, Klinikum Barmherzige Brüder, Regensburg, Germany. [9]Onkologische Praxis, Kronach, Germany. [10]Department of Medicine IV, Klinikum Bayreuth GmbH, Bayreuth, Germany. [11]ClinAssess GmbH, Leverkusen, Germany. [12]Onkologische Praxis, Ravensburg, Germany. [13]I. Department of Internal Medicine, University Medical Center Mainz, Mainz, Germany.

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ClinicalTrial registration: FIRE1 (n/a), CIOX (NCT00254137), FIRE3 (NCT00433927)

Corresponding Author: Arndt Stahler, University Hospital Grosshadern, Ludwig-Maximilan-Universitaet Muenchen, Marchioninistrasse 15, Munich 81377, Germany. Phone: 4989-4400-73126; Fax: 4989-4400-75124; E-mail: arndt.stahler@gmail.com

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Introduction

Antibodies targeting *EGFR* significantly improve outcome of patients with *RAS* wild-type (WT) metastatic colorectal cancer (mCRC; 1, 2). The beneficial effect of anti-*EGFR* antibodies on survival appeared to be limited to left-sided tumors. Present research is focusing on the investigation of additional biomarkers to provide a mechanistic basis for the prediction of anti-*EGFR* treatment efficacy.

Amphiregulin (*AREG*) and epiregulin (*EREG*) are ligands of *EGFR*, which promote activation of the *MAPK* signaling pathway including cell proliferation and invasion (3). It was shown that *EGFR* ligands were regulated by autocrine loops with activation by *integrin α6β4* and *RAS*-independent methylation of intragenic regions (3–6). Moreover, lower ligand expression was observed in right-sided, *BRAF* V600E–mutated (MUT), and microsatellite instable tumors (7, 8). High ligand expression was associated with better prognosis regardless of treatment and higher susceptibility to anti-*EGFR* antibodies in patients with *RAS* WT mCRC, which implicated a potential role of predictive biomarkers (7, 9–13).

Despite their benefit, major problems interpreting these biomarkers were of methodologic nature (e.g., normalization to reference genes and threshold values to dichotomize high and low expression). Moreover, the effect of ligand expression according to primary tumor sidedness was not investigated yet owing to limited sample sizes or missing clinical data. We aimed to address these issues and performed a combined biomarker analysis for *AREG* and *EREG* mRNA expression in the randomized controlled trials FIRE-1, CIOX (FIRE-2), and FIRE-3.

Translational Relevance

Amphiregulin (*AREG*) and epiregulin (*EREG*) are ligands of *EGFR* and were considered as predictive biomarkers for treatment targeting *EGFR* in metastatic colorectal cancer (mCRC). Interaction of *EGFR* ligand expression with anti-*EGFR* treatment was never shown, as control arms without anti-*EGFR* agents were often missing in these investigations. For the first time, we demonstrated significant beneficial treatment interaction of *AREG* mRNA expression with cetuximab-compared with bevacizumab-containing regimen and cytotoxic treatment without biologicals using similarly ascertained data from three randomized controlled trials (FIRE-1, CIOX, and FIRE-3). Moreover, a subgroup of patients with primary tumors of the right colon and high *AREG* expression benefitted from anti-*EGFR* treatment. Therefore, predictive information of *AREG* mRNA expression was confirmed and validated, and *AREG* mRNA expression might be considered as a predictive biomarker for cetuximab treatment independently from primary tumor sidedness.

FIRE-1 was a phase III trial that compared weekly application of 5-fluorouracil, leucovorin, and irinotecan (FOLFIRI) to a modified protocol of irinotecan plus oxaliplatin (IROX) in first-line treatment of mCRC (14). The CIOX phase II trial tested the combination of capecitabine and oxaliplatin (CAPOX) or irinotecan (CAPIRI) plus cetuximab as first-line treatment of mCRC (15). The FIRE-3 phase III trial showed superiority of biweekly FOLFIRI plus cetuximab versus bevacizumab in first-line treatment of *KRAS* WT (and later *RAS* WT) mCRC (16).

Prognostic and predictive effects of *AREG* and *EREG* mRNA expression were assessed in a large collective of patients with mCRC treated with cytotoxic agents (FIRE-1), cetuximab (CIOX, FIRE-3), or bevacizumab (FIRE-3). Two different threshold values were applied for exclusion of threshold specific effects. Subgroup analyses were performed to assess the influence of primary tumor location and IHC *EGFR* expression.

Materials and Methods

Trial designs and patients

Trial designs, treatment protocols, efficacy, and safety of all trials were published elsewhere (14–16). All trials were conducted before *RAS* mutations were identified as relevant biomarker in mCRC. This retrospective analysis included only patients with available qRT-PCR data for *AREG* and *EREG* mRNA expression. All patients provided written informed consent for trial participation and translational research. This analysis was conducted in accordance with the Declaration of Helsinki (1996). Analyses of mRNA expression data in clinical trials were approved by the local ethics committee of the University of Munich (Munich, Germany; registry-nos. 090–04, 545–11, 186–15, respectively).

End points

Primary and secondary endpoints of the original studies were published previously (14–16). This analysis investigated prognostic and predictive effects of high *EGFR* ligand mRNA expression to overall survival (OS), progression-free survival (PFS), and objective response rates (ORR). ORR was defined as percentage of complete and partial remissions (FIRE-1: UICC criteria; CIOX, FIRE-3: RECIST 1.0). PFS and OS were defined as described in their respective protocols and expressed as median values. Patients alive at the end of their study were censored at the last time point of patient contact.

Mutational analysis, extraction of mRNA, and qRT-PCR analysis

DNA mutational analysis was performed by pyrosequencing as described previously (13). mRNA was isolated following the instructions of the RNeasy FFPE Tissue Kit (Qiagen). Transcripted into cDNA were 150 ng (FIRE-1), 20 ng (CIOX), and 150–500 ng (FIRE-3) of mRNA, respectively. mRNA expression was determined in duplicates using the LightCycler 480 and Universal Probe Library system (Roche). *β-Actin* and *GAPDH* (not FIRE-1) were used as reference. A total of 5×10^4 molecules cDNA were used as positive and RNase-free water as negative control. ΔΔCp values were calculated as described previously (13).

Normalization of ΔΔCp gene expression between trials

To correct for methodologic bias, logarithmic $\Delta\Delta Cp$ expression values were considered as real numbers. The difference of each $\Delta\Delta Cp^o$ of a trial and trial-specific minimum $min(\Delta\Delta Cp)$ was divided by the trial-specific expression range of maximum and minimum $\Delta\Delta Cp$.

$$\Delta\Delta Cp_i = \frac{\Delta\Delta Cp^o - min(\Delta\Delta Cp)}{max(\Delta\Delta Cp) - min(\Delta\Delta Cp)}$$

In addition, results were recalculated with unnormalized expression values to gather variance deriving from normalization procedure.

Membranous EGFR expression by IHC

EGFR expression was assessed by IHC in FIRE-1 and CIOX using a prediluted monoclonal mouse antibody clone 3C6 (Ventana Medical Systems) on a Ventana BenchMark XT autostainer. Two independent reviewers (FIRE-1: A. Stahler and J. Neumann; CIOX: C. Kapaun and J. Neumann) assessed the staining intensities from 0 to 3 and the percentage of stained cells. A maximum score of 300 could be achieved by multiplication of these parameters. As published before, a score analogous to Neumann and colleagues dichotomized *EGFR*-positive (score > 22.5) and -negative (score < 22.5) tumors (13, 17).

Statistical analysis

Mean expressions were compared by the *t* test for *AREG* mRNA expression (parametric distribution) in clinical and molecular patient subgroups. Two threshold values (median and third quartile of normalized mRNA expression) dichotomized high and low ligand expression. Kaplan–Meier method estimated OS and PFS; comparisons were made using the log-rank test. Univariate and multivariate Cox regression analyses were performed in subgroups for OS and PFS. Group comparisons including ORR were performed using the χ^2 test. Test on interaction was performed with penalized spline regression analysis of *AREG* expression as a continuous variable. *P* values < 0.05 (two-sided) were considered statistically significant. SPSS PASW 23.0 (SPSS) and R v3.6.1 software were used for statistical analyses.

Results

Trial population

The total trial population of FIRE-1, CIOX, and FIRE-3 consisted of 1,408 patients. Of these, 688 patients (48.9%) provided material for mRNA extraction and gene expression analysis (**Fig. 1**). Data for mutations in *RAS* (no *KRAS* codon 59 and *NRAS* exon 2–4 data in CIOX), *BRAF*, and *PIK3CA* genes by pyrosequencing were available from all trials. IHC expression of *EGFR* was assessed in FIRE-1 and

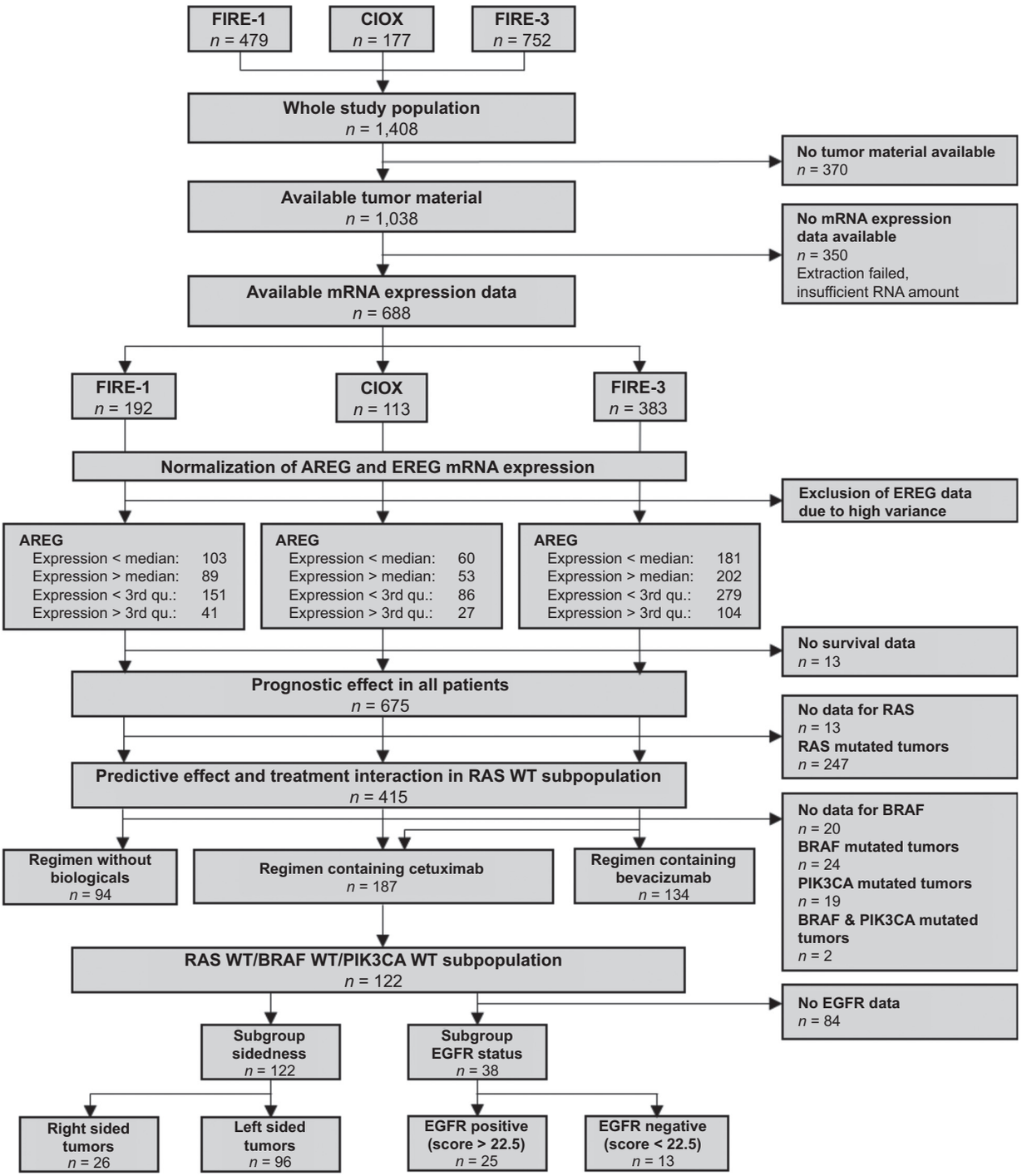

Figure 1.

CONSORT diagram of the investigated population. Available mRNA expression data for *AREG* and *EREG* of the randomized controlled trials FIRE-1, CIOX, and FIRE-3 were pooled and analyzed using two threshold values, which dichotomized the population into high and low expression for *AREG* and *EREG*, respectively. *EREG* data were excluded due to high variance. Prognostic analyses were performed in the complete dataset. Treatment interaction and predictive effects of high *AREG* expression were investigated in the *RAS* WT subpopulation. Impact of *AREG* expression according to sidedness and *EGFR* expression was investigated in patients treated with cetuximab after exclusion of *BRAF*- and *PIK3CA*-mutated tumors.

CIOX. Baseline characteristics of trial subsets were comparable, but differences were observed for primary tumor side ($P = 0.02$), *RAS* [$P < 0.001$, prospective (FIRE-3) versus retrospective (FIRE-1, CIOX) assessment of *KRAS* mutations], *BRAF* ($P = 0.04$), and *EGFR* ($P < 0.001$) status (**Table 1**; Supplementary Fig. S1).

AREG mRNA expression according to baseline characteristics

The distributions of normalized expression were parametric for *AREG* and nonparametric for *EREG* (Supplementary Fig. S2). Normalization failed to harmonize the collective in regard to *EREG* mRNA expression owing to high variance. Thus, *EREG* expression was

Table 1. Baseline characteristics of mRNA expression subsets of FIRE-1, CIOX, and FIRE-3.

	FIRE-1 $n = 192$		CIOX $n = 113$[a]		FIRE-3 $n = 383$[b]		
	FOLFIRI $n = 101$	mIROX $n = 91$	CAPIRI + Cetuximab $n = 56$	CAPOX + Cetuximab $n = 54$	FOLFIRI + Cetuximab $n = 189$	FOLFIRI + Bevacizumab $n = 192$	*P*
Age, median (range)	63 (42–75)	63 (25–76)	63 (32–75)	61 (43–77)	64 (38–76)	66 (31–76)	0.18
<65 years, *n* (%)	59 (58.4)	55 (60.4)	32 (57.1)	31 (57.4)	95 (50.3)	87 (45.3)	
≥65 years, *n* (%)	42 (41.6)	36 (39.6)	24 (42.9)	23 (42.6)	85 (45.0)	102 (53.1)	
Missing, *n* (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (4.8)	3 (1.6)	
Sex							
Female, *n* (%)	37 (36.6)	26 (28.6)	16 (28.6)	15 (27.8)	57 (30.2)	66 (34.4)	0.71
Male, *n* (%)	64 (63.4)	65 (71.4)	40 (71.4)	39 (72.2)	123 (65.1)	123 (64.1)	
Missing, *n* (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (4.8)	3 (1.6)	
ECOG status, *n* (%)							
0–1	98 (97.0)	82 (91.2)	53 (96.4)	50 (92.6)	176 (97.9)	185 (97.9)	0.05
2	3 (3.0)	8 (8.8)	2 (3.6)	4 (7.4)	4 (2.1)	4 (2.1)	
Missing, *n* (%)	0 (0.0)	1 (1.1)	1 (1.8)	0 (0.0)	9 (4.8)	3 (1.6)	
Primary tumor location, *n* (%)							
Right	14 (13.9)	14 (15.4)	16 (28.6)	21 (38.9)	47 (24.9)	54 (28.1)	**0.02**
Left	76 (75.2)	68 (74.7)	40 (71.4)	33 (61.1)	142 (75.1)	136 (70.8)	
Missing, *n* (%)	11 (10.9)	9 (9.9)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.0)	
Sites of metastases, *n* (%)							
1–2	84 (83.2)	73 (80.2)	42 (75.0)	41 (75.9)	143 (75.7)	147 (76.6)	0.67
≥3	16 (15.9)	16 (17.6)	14 (25.0)	13 (24.1)	33 (17.4)	41 (21.3)	
Missing, *n* (%)	1 (0.9)	2 (2.2)	0 (0.0)	0 (0.0)	13 (6.9)	4 (2.1)	
RAS status, *n* (%)							
Wild-type	43 (42.6)	51 (56.0)	29 (51.8)	37 (68.5)	121 (64.0)	134 (69.8)	<**0.001**
Mutated	58 (57.4)	40 (44.0)	27 (48.2)	17 (31.5)	57 (30.2)	51 (26.6)	
Missing, *n* (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	11 (5.8)	7 (3.6)	
BRAF status, *n* (%)							
Wild-type	100 (99.0)	84 (92.3)	49 (87.5)	49 (90.7)	127 (67.2)	139 (72.4)	**0.04**
Mutated	1 (1.0)	7 (7.7)	7 (12.5)	5 (9.3)	18 (9.5)	13 (6.8)	
Missing, *n* (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	44 (23.3)	40 (20.8)	
PIK3CA status, *n* (%)							
Wild-type	95 (94.1)	85 (93.4)	50 (89.3)	44 (81.5)	176 (93.1)	170 (88.5)	0.07
Mutated	6 (5.9)	6 (6.6)	6 (10.7)	10 (18.5)	13 (6.9)	22 (11.5)	
Missing, *n* (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
EGFR IHC score, *n* (%)							
<22.5	63 (62.4)	61 (67.0)	17 (30.4)	15 (27.8)	0 (0.0)	0 (0.0)	<**0.001**
≥22.5	38 (37.6)	30 (33.0)	35 (62.5)	37 (68.5)	0 (0.0)	0 (0.0)	
Missing, *n* (%)	0 (0.0)	0 (0.0)	4 (7.1)	2 (3.7)	189 (100.0)	192 (100.0)	
Normalized *AREG* expression, median (range)	0.50 (0.0–1.0)	0.44 (0.1–0.9)	0.48 (0.1–1.0)	0.49 (0.0–0.7)	0.50 (0.0–1.0)	0.51 (0.0–0.8)	
<Median, *n* (%)	46 (45.5)	57 (62.6)	31 (55.4)	27 (50.0)	89 (47.1)	90 (46.9)	0.13
≥Median, *n* (%)	55 (54.5)	34 (37.4)	25 (44.6)	27 (50.0)	100 (52.9)	102 (53.1)	
<3rd quartile, *n* (%)	74 (73.3)	77 (84.6)	42 (75.0)	41 (75.9)	134 (70.9)	143 (74.5)	0.27
≥3rd quartile, *n* (%)	27 (26.7)	14 (15.4)	14 (25.0)	13 (24.1)	55 (29.1)	49 (25.5)	
Normalized *EREG* expression, median (range)	0.49 (0.0–1.0)	0.42 (0.1–0.8)	0.38 (0.1–1.0)	0.37 (0.0–0.7)	0.27 (0.0–0.8)	0.29 (0.0–1.0)	
<Median, *n* (%)	27 (26.7)	50 (54.9)	17 (30.4)	18 (33.3)	123 (65.1)	130 (67.7)	<**0.001**
≥Median, *n* (%)	74 (73.3)	41 (45.1)	39 (69.6)	36 (66.7)	66 (34.9)	62 (32.3)	
<3rd quartile, *n* (%)	46 (45.5)	55 (60.4)	36 (64.3)	34 (63.0)	170 (89.9)	176 (91.7)	<**0.001**
≥3rd quartile, *n* (%)	55 (54.5)	36 (39.6)	20 (35.7)	20 (37.0)	19 (10.1)	16 (8.3)	

Note: *P* values < 0.05 were considered significant and are displayed bold.
Abbreviation: ECOG, Eastern Cooperative Oncology Group.
[a]Missing treatment information for 3 patients.
[b]Missing treatment information for 2 patients.

excluded from further analyses (**Table 1**). Mean *AREG* expression was comparable for all baseline characteristics including sidedness, *EGFR* expression, and *MSI-H* status. However, median *AREG* and *EGFR* expression were lower in detailed primary tumor locations of the right colon (Supplementary Fig. S3). A trend for lower *AREG* expression was observed in *RAS* WT/*BRAF* MUT tumors (Supplementary Table S1).

Normalized *AREG* mRNA expression is a strong prognostic biomarker

In individual trial data analyses, OS was more pronounced and PFS was significantly longer in the presence of high *AREG* expression (Supplementary Fig. S4).

In pooled data analysis of all trials, high *AREG* expression was significantly associated with longer OS and PFS regardless of threshold value (**Fig. 2**). The favorable prognostic effect of normalized *AREG* expression was confirmed in multivariate Cox regression analyses including baseline and molecular characteristics for OS [HR = 0.46 (95% CI, 0.25–0.85); $P = 0.01$] and PFS [HR = 0.40 (95% CI, 0.22–0.71); $P = 0.002$; Supplementary Table S2).

ORR was significantly higher in the presence of high *AREG* expression [median: 63.1% vs. 51.6%, OR = 1.61 (95% CI, 1.16–2.21), $P = 0.004$; third quartile: 71.5% vs. 52.6%, OR = 2.26 (95% CI, 1.53–3.34); $P < 0.0001$]. Comparable results were observed for unnormalized expression values (Supplementary Table S3).

Impact of normalized *AREG* mRNA expression in relation to molecular biomarkers

Next, the prognostic impact of normalized *AREG* expression was related to biomarkers activating the *MAPK* signaling pathway directly (IHC *EGFR* expression, *RAS, BRAF* MUT) or indirectly (*PIK3CA* MUT).

Among 688 patients, *RAS, BRAF*, or *PIK3CA* MUT and *EGFR* positivity were detected in 253 (36.7%), 51 (7.4%), 62 (9.0%), and 140 (20.3%) patients, respectively. The favorable prognostic effect of high *AREG* expression was observed in *RAS, BRAF, PIK3CA* WT, and *EGFR*-positive tumors for OS and PFS (Supplementary Table S4).

Normalized *AREG* mRNA expression is a predictor of efficacy for cetuximab in RAS WT mCRC

Across all trials, 415 patients had *RAS* WT tumors and of these, 187 (45.1%) were treated with a cetuximab-containing regimen. In this subgroup, OS (36.6 vs. 23.5 months), PFS (10.6 vs. 7.8 months), and ORR (83.3 vs. 63.9%) were significantly higher in the presence of high *AREG* expression (dichotomized by median). Positive effects of high

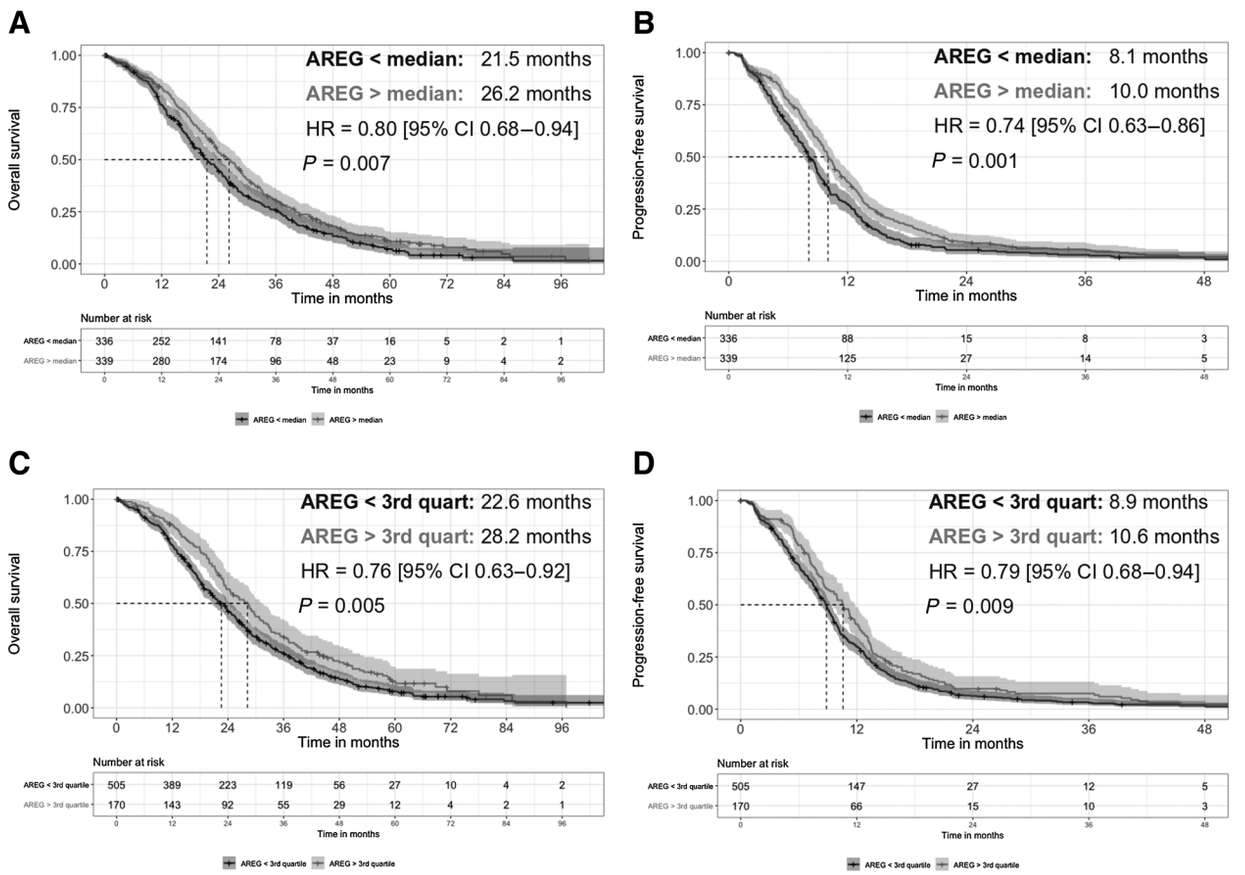

Figure 2.

Kaplan–Meier plots of high versus low *AREG* expression. **A,** OS, when *AREG* expression dichotomized by median, all trials. **B,** PFS, when *AREG* expression dichotomized by median, all trials. **C,** OS, when *AREG* expression dichotomized by third quartile, all trials. **D,** PFS, when *AREG* expression dichotomized by third quartile, all trials.

AREG expression were not observed in *RAS* WT patients treated with bevacizumab or without biologicals (**Table 2**).

AREG expression interacted significantly with cetuximab, not bevacizumab, in patients with *RAS* WT tumors regarding OS ($P = 0.02$) and PFS ($P = 0.04$). However, interaction of *AREG* expression with the respective trial (CIOX or FIRE-3) indicated trial-dependent effect sizes (OS: $P = 0.03$; PFS: $P = 0.05$).

AREG expression in context of cetuximab treatment and EGFR expression

To investigate the impact of high *AREG* expression in the context of isolated *EGFR* expression and cetuximab treatment, we selected 38 patients of CIOX with *RAS, BRAF*, and *PIK3CA* WT tumors and available IHC *EGFR* expression data.

In this specific subgroup, high *AREG* expression was significantly associated with higher ORR and remarkably prolonged OS and PFS in *EGFR*-positive tumors, but also numerically prolonged OS and PFS in *EGFR*-negative tumors (**Table 3**).

Efficacy of cetuximab in tumors with high *AREG* expression according to sidedness

122 patients with *RAS, BRAF*, and *PIK3CA* WT tumors (right-sided: $n = 26$; left-sided: $n = 96$) received cetuximab-containing regimens. In left-sided tumors with high *AREG* expression, OS was more pronounced and PFS was significantly longer. In tumors of the right colon, high *AREG* expression was significantly associated with higher OS [24.5 vs. 16.1 months, HR = 0.32 (95% CI, 0.11–0.87); $P = 0.03$] and numerically higher PFS (**Table 3**).

Discussion

To our knowledge, this combined biomarker analysis represented the largest collective that was analyzed for *AREG* mRNA expression so far. High *AREG* expression was identified as a strong positive prognostic biomarker for OS, PFS, and ORR. Our analysis found a significant interaction of *AREG* expression with the use of cetuximab-containing regimens, whereas no effect was observed for bevacizumab or cytotoxic treatment alone. Finally, patients with right-sided *RAS/BRAF* WT tumors showed longer OS and PFS when treated with cetuximab in the presence of high *AREG* expression.

AREG and *EREG* are ligands of *EGFR*, which promote activation of the *MAPK* signaling pathway by ligand binding, which lead to proliferation and invasion of cancer cells (3, 4). Inhibition of *EGFR* signaling in *RAS* WT patients with high ligand levels resulted in significantly increased OS and PFS (9, 10, 12, 18, 19). Although a strong prognostic effect of high *AREG* expression was observed for treatment strategies with and without anti-*EGFR* antibodies (7, 13), we could confirm significant interaction only with cetuximab-containing regimens in our analysis for *RAS* WT tumors. However, effect sizes depended on the respective trial (CIOX or FIRE-3).

Biologically, autocrine loops were identified which promoted transcription and translation of *EGFR* ligands via *MMP1*, which itself was activated by ligand binding to *EGFR* (3). Tumors with elevated expression of *EGFR* ligands might represent a subgroup of tumors with an *EGFR* overstimulated activation of the *MAPK* pathway, for which *EGFR* signaling is vital for tumor growth. We therefore hypothesized that *EGFR* inhibition would substantially increase survival of patients with tumors showing high *AREG* expression.

In all patients, a major prognostic benefit on OS and PFS was observed irrespectively of treatment in tumors with high simultaneous *AREG* and *EGFR* expression compared to high *AREG*, but low *EGFR*

Table 2. Treatment-dependent outcome of amphiregulin mRNA expression in *RAS* WT patients of FIRE-1, CIOX, and FIRE-3.

	Cetuximab + Chemotherapy				Bevacizumab + Chemotherapy				Chemotherapy			
	AREG ≥median $n = 102$	***AREG*** <median $n = 85$	***AREG*** ≥3rd quartile $n = 64$	***AREG*** <3rd quartile $n = 123$	***AREG*** ≥median $n = 68$	***AREG*** <median $n = 66$	***AREG*** ≥3rd quartile $n = 35$	***AREG*** <3rd quartile $n = 99$	***AREG*** ≥median $n = 40$	***AREG*** <median $n = 54$	***AREG*** ≥3rd quartile $n = 22$	***AREG*** <3rd quartile $n = 72$
Overall survival, months	36.6	23.5	37.1	24.5	27.5	23.8	28.6	23.8	23.2	21.9	23.2	21.8
HR (95% CI)		0.60 (0.43–0.83)		0.61 (0.43–0.87)		0.93 (0.65–1.33)		0.96 (0.65–1.43)		1.50 (0.94–2.40)		0.78 (0.46–1.32)
P, log-rank		**0.002**		**0.006**		0.71		0.85		0.09		0.35
Progression-free survival, months	10.6	7.8	11.2	8.6	11.3	10.3	11.5	10.7	7.5	7.7	7.5	7.7
HR (95% CI)		0.66 (0.49–0.88)		0.77 (0.56–1.05)		0.92 (0.65–1.30)		1.03 (0.70–1.53)		0.82 (0.53–1.27)		0.86 (0.51–1.43)
P, log-rank		**0.006**		0.10		0.63		0.87		0.37		0.55
Objective response rate, %	83.3	63.9	83.6	69.3	68.2	55.9	76.5	57.1	47.5	46.3	59.1	43.1
P, χ^2		**0.006**		0.06		0.20		0.06		1.00		0.23

Note: Survival times are displayed as medians. *P* values < 0.05 are considered significant and displayed bold.

Table 3. Efficacy of cetuximab in *RAS/BRAF/PIK3CA* WT patients with high versus low *AREG* expression according to *EGFR* expression and sidedness.

	***EGFR* status**				**Sidedness**			
	***EGFR* positive** $n = 25$		***EGFR* negative** $n = 13$		**Right-sided tumors** $n = 26$		**Left-sided tumors** $n = 96$	
Endpoints	***AREG*** ≥**median** $n = 13$	***AREG*** <**median** $n = 12$	***AREG*** ≥**median** $n = 7$	***AREG*** <**median** $n = 6$	***AREG*** ≥**median** $n = 10$	***AREG*** <**median** $n = 16$	***AREG*** ≥**median** $n = 53$	***AREG*** <**median** $n = 43$
CR/PR, *n* (%)	8 (61.5)	3 (23.1)	5 (71.4)	4 (66.7)	7 (70.0)	7 (43.8)	40 (75.5)	26 (60.5)
SD/PD, *n* (%)	0 (0.0)	7 (58.3)	2 (28.6)	2 (33.3)	1 (10.0)	7 (43.8)	5 (9.4)	10 (23.3)
Missing ORR, *n* (%)	5 (38.5)	2 (16.7)	0 (0.0)	0 (0.0)	2 (20.0)	2 (12.5)	8 (15.1)	7 (16.3)
P (χ^2)	**0.004**		1.00		0.17		0.08	
OS, months (95% CI)	39.9 (11.4–68.3)	11.3 (0.0–25.9)	40.4 (22.3–58.6)	18.0 (13.8–22.2)	24.5 (n/a)	16.1 (0.9–31.2)	39.9 (35.0–44.7)	26.9 (16.5–37.3)
HR (95% CI)	0.43 (0.16–1.16)		0.35 (0.09–1.26)		0.32 (0.11–0.87)		0.70 (0.44–1.11)	
P (log-rank)	0.10		0.11		**0.03**		0.13	
PFS, months (95% CI)	11.2 (6.9–15.5)	4.3 (3.3–5.3)	11.8 (3.6–20.0)	5.9 (3.6–8.1)	8.5 (2.3–14.7)	4.3 (3.7–5.0)	10.7 (9.2–12.1)	8.0 (6.5–9.5)
HR (95% CI)	0.23 (0.08–0.64)		0.23 (0.05–0.95)		0.47 (0.21–1.09)		0.61 (0.40–0.93)	
P (log-rank)	**0.005**		**0.04**		0.08		**0.02**	

Note: Survival times are displayed as medians. *P* values < 0.05 are considered significant and are displayed bold.
Abbreviations: CR, complete remission; PD, progressive disease; PR, partial remission; SD, stable disease; WT, wild-type.

expression. The subgroup of patients who had all-WT tumors and who were treated with cetuximab derived numerical benefit of high *AREG* expression irrespective of *EGFR* status, though. Thus, a prognostic, but not predictive, impact of *EGFR* expression in combination with *AREG* expression on treatment efficacy of cetuximab was observed in this analysis, analogous to previous results (20, 21). The validity of these findings might be biased by the limited sample size of 38 patients. Confirmation of the potential coherence of *AREG* and *EGFR* expression in a larger collective is therefore warranted and necessary.

Beyond activating mutations within the *MAPK* and *PIK3CA-AKT* signaling pathway, *HER2/neu* overexpression and amplification, respectively, were recognized as additional predictive biomarkers in the subset of patients with *RAS* WT tumors. *HER2/neu*-positive tumors occurred with a prevalence of 5% and were associated with worse response to anti-*EGFR* treatment than negative tumors (22–24). Our analysis did not adjust for this factor, as assessments of *HER2/neu* status across all trials were heterogeneous (FIRE-1: IHC; FIRE-3: next-generation sequencing; CIOX: no assessment). Therefore, *HER2/neu* expression and amplification could have additionally influenced outcome of patients treated with anti-*EGFR* antibodies.

EREG expression was associated with high variance and nonparametric data distribution in our analysis. In contrast to *AREG* expression, combined analysis of all trial collectives with consistent threshold values was not possible. However, we were able to reproduce improvements of OS and PFS in patients of FIRE-1 with high *EREG* expression (data not shown), confirming its prognostic role in patients treated with and without anti-*EGFR* antibodies (10–12, 25, 26). The choice of a consistent threshold value and its translation into clinical practice, however, might be impaired owing to the heterogeneity of mRNA expression in the respective collectives.

In addition to *RAS* status, primary tumor sidedness has been shown to be an important predictor of anti-*EGFR* treatment efficacy (1, 2, 27). Molecular characteristics such as higher prevalence of *BRAF* V600E mutations and high-grade microsatellite instability, higher tumor mutational burden, and enrichment of immune-like gene expression–based phenotypes accumulated in primary tumors of the right colon (28, 29). While *RAS* WT tumors in the left colon were associated with good response to anti-*EGFR* agents, survival of patients with right-sided *RAS* WT tumors was significantly worse (2, 27). An explanation for this phenomenon is still missing. Recommendations for the optimal treatment of *RAS* WT right-sided primary tumors differed across guidelines (ESMO: no stratification by sidedness; NCCN: no anti-*EGFR* antibodies in first-line treatment; ASCO: no anti-*EGFR* antibodies regardless of treatment line; refs. 30–32). Although the impact of primary tumor sidedness on anti-*EGFR* efficacy was retrospectively assessed in larger clinical trials, it should be respected for clinical decision-making in terms of the optimal first-line strategy in metastatic disease. Nevertheless, despite an accumulation of molecular characteristics, these tumors might still represent a heterogeneous subgroup, as some tumors of the right colon derived benefit from treatment with cetuximab (33). Further biomarkers are therefore needed to identify a subpopulation of *RAS* WT primary tumors in the right colon with susceptibility to anti-*EGFR* treatment.

Data on the distribution of *EGFR* ligands according to primary tumor side was limited owing to low sample sizes. However, elevated levels of *EGFR* ligand mRNA expression were observed more frequently in left- than right-sided tumors recently (8). We confirmed this observation by analyzing median expression per detailed primary tumor location. This finding might be associated with reported regulatory mechanisms of *EGFR* ligand expression, as

hypermethylation, which is more frequently found in right-sided tumors (34), correlated with lower and demethylation with higher ligand expression, respectively (4–7). In our analysis, high *AREG* expression was evident in a small subset of ten patients with *RAS* WT right-sided primary tumors and was associated with significantly longer OS and numerically longer PFS, similar to left-sided primary tumors. Nevertheless, this analysis was formally not powered to investigate the hypothesis of different outcome according to the primary tumor side and *AREG* mRNA expression. Therefore, these findings should be interpreted with caution.

Taken together, we hypothesized that the favorable effect of high *AREG* expression on outcomes possibly depended on concomitant positivity of *EGFR* expression, but was independent of primary tumor side. Former inconsistencies in regard to the relevance of *EGFR* expression as a predictive biomarker for anti-*EGFR* treatment efficacy might be explained by differential *EGFR* ligand expression which may represent a missing link in this context (20, 21). Detailed characterization of *MAPK* activation by combined assessment of mutations, *EGFR* and *AREG* expression might therefore support the prediction of cetuximab efficacy regardless of the primary tumor location.

We were able to investigate a large collective of patients with mCRC for mRNA expression at different time points with minor methodologic bias. However, our results are limited by the fact that expression values could have been considered absolute (i.e., global minimal expression in FIRE-3 and high expression in CIOX and FIRE-1; Supplementary Fig. S2) and not trial-specific. This assumption, however, would have implicated major differences in mRNA expression between cohorts within the same entity without adjustment for methodologic bias such as RNA input. Second, we considered logarithmic values as real numbers for rescaling similar data of three trials to one scale. Although this method is vulnerable from a mathematical point of view, we showed comparable results for unnormalized data when trial-specific threshold values were taken into account. Therefore, data normalization produced at best minor variance of these results. Nevertheless, trial-specific data normalization failed for nonparametric data distribution such as *EREG* expression. Finally, baseline characteristics were slightly imbalanced for molecular parameters, as data for *KRAS* codon 59 and *NRAS* testing were missing in CIOX, and IHC *EGFR* expression was not assessed in FIRE-3.

In conclusion, we demonstrated high *AREG* mRNA expression at two threshold values as a strong prognostic biomarker for OS, PFS, and ORR in patients with mCRC. An interaction of *AREG* mRNA expression and anti-*EGFR* treatment was confirmed for OS and PFS in our analyses. Furthermore, positive prognostic effects of high *AREG* expression were observed in patients with IHC *EGFR*-positive tumors. The definition of patient populations with benefit from anti-*EGFR* treatment might be supported by the additional assessment of *AREG* and *EGFR* expression together with *RAS/BRAF/PIK3CA* mutational analyses. This approach may potentially be used to define patients with right-sided *RAS* WT tumors that benefit from *EGFR* antibodies. Prospective analyses are warranted to confirm the predictive role of high *AREG* expression (or surrogate biomarkers) for anti-*EGFR* treatment in mCRC.

Disclosure of Potential Conflicts of Interest

A. Stahler reports grants from German Translational Research Consortium (DKTK; no. n/a), Weigand-Gravenhorst-Bohnewand-Fond (no. n/a, to D.P. Modest), Sanofi-Aventis (support of FIRE-1 trial), Pfizer (support of FIRE-1 and FIRE-3), and Merck KGaA Germany (support of CIOX and FIRE-3) during the conduct of the study; in addition, A. Stahler reports personal fees from Roche Pharma (travel reimbursement, honoraria for talks), AMGEN (travel reimbursement), and Merck (travel reimbursement) outside the submitted work. S. Stintzing reports personal fees from Amgen, Bayer, Pierre-Fabre, Lilly, Merck KGaA, Sanofi, Roche, MSD, Servier, Takeda, and Taiho outside the submitted work. D.P. Modest reports grants from Merck during the conduct of the study; in addition, D.P. Modest reports grants and personal fees from Merck, Amgen, Roche, and personal fees from Servier, Sanofi, BMS, MSD, PierreFabre, and Lilly outside the submitted work. I. Ricard reports personal fees from Roche outside the submitted work. C. Giessen-Jung reports personal fees from Roche (honoraria for talks and travel support) during the conduct of the study. A. Kiani reports personal fees from Merck (advisory board, honoraria) and Roche (advisory board, honoraria) outside the submitted work. T. Decker reports personal fees from Novartis (advisory boards) outside the submitted work. M. Moehler reports personal fees and nonfinancial support from Roche and Servier; grants and personal fees form MSD; grants, personal fees, and nonfinancial support from Merck Serono, BMS, and Bayer during the conduct of the study. A. Jung reports personal fees from Amgen, Pfizer, Archer DX, AstraZeneca, Biocartis, Boehringer Ingelheim, Bristol Myers Squibb (BMS), Merck-Serono, Merck Sharp & Dohme (MSD), Roche Pharma, Takeda, and Thermo Fisher outside the submitted work. V. Heinemann reports grants from Pfizer; grants and personal fees from Boehringer Ingelheim; personal fees from BMS, MSD, Novartis, Pierre Fabre, and Celgene; grants, personal fees and nonfinancial support from Merck, Servier, Bayer, Amgen, and Roche; grants and personal fees from Sanofi; and grants, personal fees, and nonfinancial support from Sirtex outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

A. Stahler: Conceptualization, resources, data curation, formal analysis, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing. **S. Stintzing:** Conceptualization, resources, supervision, funding acquisition, project administration, writing-review and editing. **D.P. Modest:** Conceptualization, resources, supervision, funding acquisition, investigation, project administration, writing-review and editing. **I. Ricard:** Software, formal analysis, validation. **C. Giessen-Jung:** Resources, investigation, writing-review and editing. **C. Kapaun:** Resources, investigation, writing-review and editing. **B. Ivanova:** Resources, investigation, writing-review and editing. **F. Kaiser:** Resources, investigation, writing-review and editing. **L. Fischer von Weikersthal:** Resources, investigation, writing-review and editing. **N. Moosmann:** Resources, investigation, writing-review and editing. **A. Schalhorn:** Resources, investigation, writing-review and editing. **M. Stauch:** Resources, investigation, writing-review and editing. **A. Kiani:** Resources, investigation, writing-review and editing. **S. Held:** Data curation, software, formal analysis, validation, writing-review and editing. **T. Decker:** Resources, investigation, writing-review and editing. **M. Moehler:** Resources, investigation, writing-review and editing. **J. Neumann:** Resources, supervision, validation, investigation, methodology, writing-review and editing. **T. Kirchner:** Resources, supervision, investigation, project administration, writing-review and editing. **A. Jung:** Conceptualization, resources, supervision, investigation, methodology, writing-review and editing. **V. Heinemann:** Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing-original draft, project administration.

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