Prevalence and influence on outcome of *HER2/neu*, *HER3* and NRG1 expression in patients with metastatic colorectal cancer

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Our aim was to explore the impact of the *HER2/neu*, *HER3* receptor as well as their ligands' neuregulin (*NRG1)* expression on the outcome of patients with metastatic colorectal cancer (mCRC). *NRG1*, *HER2/neu* and *HER3* expression was evaluated in 208 patients with mCRC receiving 5-FU/LV plus irinotecan or irinotecan plus oxaliplatin as the first-line treatment. Biomarker expression was correlated with the outcome of patients. *NRG1* (low: 192 vs. high: 16), *HER2/neu* (low: 201 vs. high: 7) and *HER3* (low: 69 vs. high: 139) expressions were assessed in 208 patients. High versus low *NRG1* expression significantly affected progression-free survival (PFS) [4.7 vs. 8.2 months, hazard ratio (HR): 2.45; 95% confidence interval (CI): 1.45–4.13; $P = 0.001$], but not overall survival (OS) (15.5 vs. 20.7 months, HR: 1.33; 95% CI: 0.76–2.35; $P = 0.32$). High versus low *HER3* expression (PFS: 7.1 vs. 8.8 months, HR: 1.11; 95% CI: 0.82–1.50; $P = 0.50$; OS: 19.8 vs. 21.1 months, HR: 0.95; 95% CI: 0.70–1.30; $P = 0.75$) and high compared with low *HER2/neu* expression (PFS: 7.7 vs. 8.0 months, HR: 1.07; 95% CI: 0.71–1.60; $P = 0.75$; OS: 16.6 vs. 21.1 months, HR: 1.13; 95% CI: 0.75–1.71; $P = 0.57$) did not influence outcome. High *NRG1* expression was associated with inferior PFS in the FIRE-1 trial. We did not detect a prognostic impact of *HER2/neu* and *HER3* overexpression in mCRC. The frequency of overexpression was comparable with other studies. *Anti-Cancer Drugs* **28:717–722**

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Introduction

Treatment of metastatic colorectal cancer (mCRC) has improved since molecular biomarkers are being evaluated for their predictive and prognostic information, such as *RAS* mutations for epidermal growth factor receptor (EGFR)-targeted treatment [1,2]. Together with the *HER2/neu*, *HER3* and *HER4* receptor, the EGFR belongs to the HER receptor family [3]. These receptors have tyrosine kinase activity. When activated, the receptors engage intracellular signalling pathways leading to proliferation [4–8].

HER2/neu overexpression was identified as valuable target in subpopulations of breast cancer and gastric cancer [9–11]. The HERACLES trial has further identified *HER2/neu* overexpression as a targetable structure in a subset of patients with *KRAS* wild-type mCRC [12]. Besides this potential predictive relevance, the unfavourable outcome of colorectal cancer (CRC) patients has been associated with *HER2/neu* overexpression in previous analyses [13,14].

Potentially treatment-relevant expression of the *HER3* receptor has been reported in several solid cancer types [15]. If neuregulin (*NRG1*) binds to the *HER3* receptor, *HER3* forms heterodimers with the *HER2/neu* receptor [6]. Subsequently, the *PI3K-AKT* and *MAPK* pathways are activated, which stimulate tumour proliferation [6,15]. In the literature, *HER3* expression rates in CRC cells range from 34 to 90% [15]. However, it is not clear if

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HER3 expression is associated with patients outcome in colorectal cancer. *HER3* overexpression was reported to be a negative prognostic marker for patients with CRC without distant metastases [16–18].

In addition, previous investigations focused on the role of *NRG1* in CRC as being the activating ligand of the *HER3* receptor. De Boeck *et al.* [19] found tumour progression to be highly influenced by bone marrow-derived mesenchymal stem cells releasing *NRG1 in vitro* and *in vivo*. Furthermore, two investigations indicated a potential role for predicting lymph node involvement and the occurrence of distant metastases [20–22]. Nevertheless, two cohorts of advanced CRC and one study of a cohort of CRC patients with distant metastases did not confirm effects on outcome [23–25]. Coalteration of *HER2/neu* and *HER3* expression was found rarely, also without impact on the outcome of patients with CRC [23].

This analysis was designed to confirm the prevalence and prognostic impact of *HER2/neu*, *HER3* and *NRG1* expression in a chemotherapy-based study cohort of 208 patients with mCRC (FIRE-1 trial) receiving either 5-FU/LV plus irinotecan or irinotecan plus oxaliplatin as first-line therapy. To our knowledge, the FIRE-1 trial is the first randomized-controlled trial to investigate the impact of *HER2/neu* and *HER3* receptor overexpression in relation to *NRG1* expression [16–18,20–24].

Methods

Study design and treatment schedule

FIRE-1 was a multicentre phase III study. The protocol, primary results and characteristics of patients have been published previously [26]. Also, details on the subpopulation evaluable for translational research have been reported [27]. Information on *RAS* mutation status and EGFR ligand expression was available for the cohort as described previously [27].

Patients

Our analysis included 208 of 479 patients with available tumour material [27] for the analysis of *HER2/neu*, *HER3* and *NRG1* expression.

Ethics

The trial was conducted in accordance with the Declaration of Helsinki (1996). All patients provided written informed consent to be treated within a clinical trial. This investigation was performed as a retrospective evaluation with the approval of the local ethics committee of the University of Munich (registry-number: 545-11).

End points

For this manuscript, overall survival (OS) (time from randomization to death), progression-free survival (PFS) (interval between randomization and death or progression) and response rate (WHO classification: complete remission, partial remission, no change, progressive disease) were used to correlate molecular characteristics with the outcome of patients of the FIRE-1 trial.

Immunohistochemistry

Immunohistochemistry was performed using 5 μm whole standard tissue sections of FFPE tumour samples. For the detection of *HER2/neu*, a prediluted anti-*HER2/neu* rabbit monoclonal antibody (clone 4B5; Ventana Medical Systems, Oro Valley, Arizona, USA) was used as the primary antibody. The staining was performed on a Ventana Benchmark XT autostainer using the XT UltraView diaminobenzidine kit (Ventana Medical Systems) following the manufacturer's protocols. Staining of *HER3* and *NRG1* was performed using the Vectastain ABC-Kit Elite Universal detection system (Vector Laboratories, Peterborough, UK). For *HER3* immunohistochemistry, a monoclonal rabbit antibody was used as the primary antibody (ab93739; Abcam, Cambridge, UK). *NRG1* staining was performed using a polyclonal human antibody (HPA010964; Atlas Antibodies, Stockholm, Sweden).

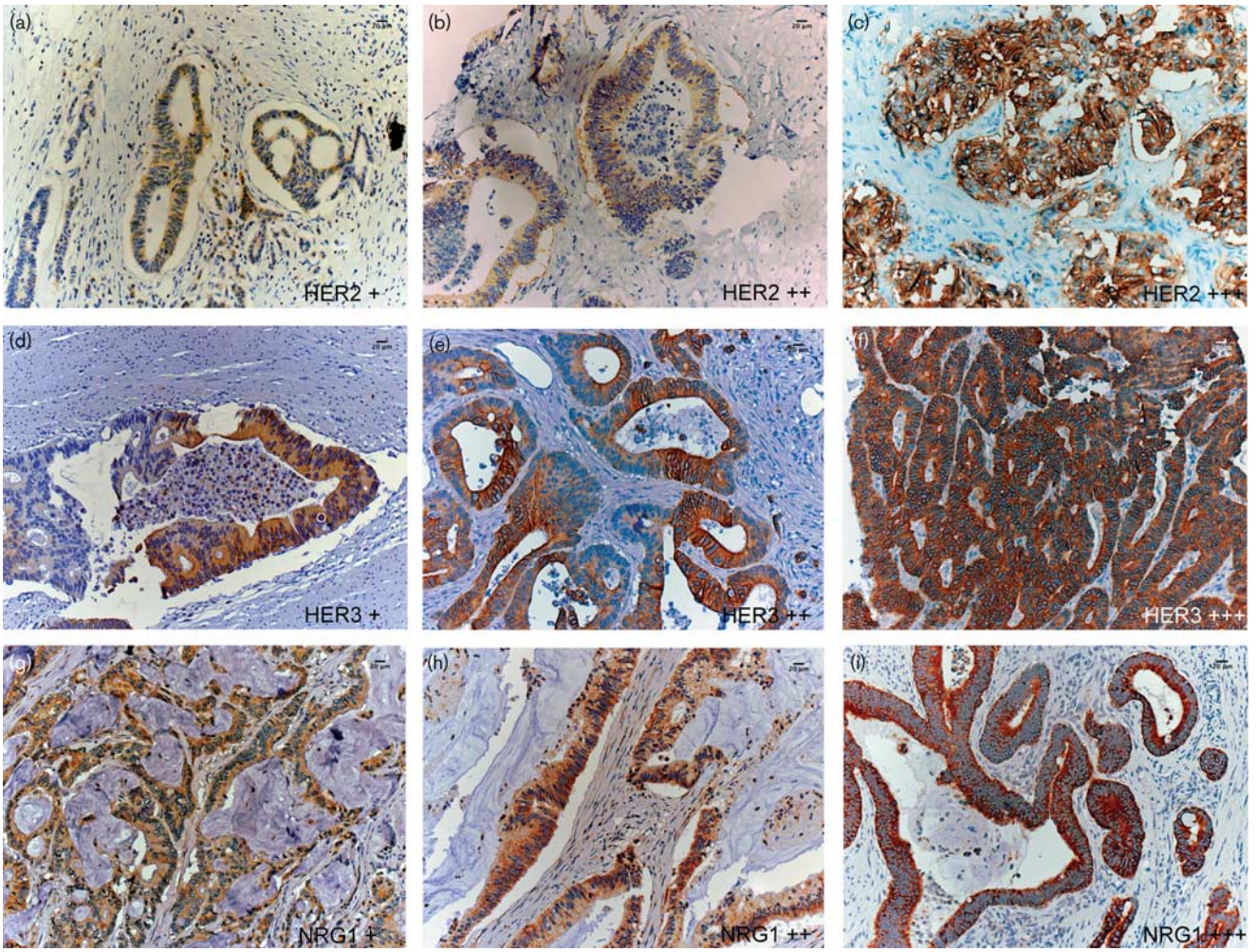
Scoring of high and low expression, FISH

As no evident and standardized method for dividing high and low expression of *HER2/neu*, *HER3* and *NRG1* existed at the time of evaluation, we used the *HER2/neu* score of Rüschoff and colleagues in gastric cancer for scoring complete biomarker expression. Therefore, membrane staining was graduated by intensity (0: none, 1+: weak, 2+: moderate, 3+: strong) and percentage of stained tumour cells (Fig. 1). High expression was defined by a percentage of more than 10% stained tumour cells and at least moderate (2+ or 3+) membrane staining versus no or weak staining (0 or 1+) for low expression. In addition, two-colour fluorescence in-situ hybridization (FISH) was performed in patients showing moderate (2+) *HER2/neu* staining. Chromosome 17 centromere signals (green) as well as *HER2* gene signals (red) were counted in at least 20 nuclei of colorectal tumour cells. Thus, a red to green ratio of at least 2 indicated amplification of *HER2*. Primary tumour slides were evaluated by two independent observers (A.S. and J.N.) using a light microscope. Disagreements (< 5%) were reviewed together, followed by conclusive judgement.

Statistical analysis

OS and PFS stratified by the molecular markers were estimated using Kaplan–Meier analysis. Significant differences were evaluated using the log-rank test and Cox regression analysis. Univariate Cox regression was performed in subgroups. The correlation of clinicopathologic parameters with biomarker expression was assessed using the χ^2-test and the Fisher exact test for nominal variables. All *P*-values of less than 0.05 (two sided) were considered significant. SPSS PASW 18.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis.

Fig. 1



Immunohistochemical staining intensity of *HER2/neu* expression [+: weak (a), ++: intermediate (b), +++: strong (c)], *HER3* expression [+: weak (d), ++: intermediate (e), +++: strong (f)] and *NRG1* expression [+: weak (g), ++: intermediate (h), +++: strong (i)]. NRG, neuregulin.

Results

Study population

HER2/neu, *HER3* and *NRG1* analyses were carried out in 208 tumours. Characteristics of the entire patient population and the evaluable subpopulation have been published before [27]. According to baseline and tumour characteristics as well as PFS and OS, the subpopulation was well comparable with the entire study population [27].

Prevalence of high NRG1, HER3 and HER2/neu expression

Of 208 metastatic colorectal tumours in total, high *NRG1* expression was detected in 16 (7.7%) specimens. 139 of 208 tumours (67%) were diagnosed to have *HER3* overexpression. Twenty-three (11.1%) patients showed moderate *HER2/neu* staining. A subsequent FISH analysis, however, showed a missing gene amplification in all of these 23 patients (*HER2/neu*: chromosome 17 ratio <2.0). Therefore, only strong (3+) stainings in seven (3.3%) patients were accepted as high *HER2/neu* expression.

Correlation of NRG1, HER3 and HER2/neu

A significant correlation of biomarkers with each other could not be detected for *NRG1* and *HER2/neu* ($P=1.00$) or for *NRG1* and *HER3* ($P=0.41$). High *HER2/neu* expression also did not correlate with *HER3* overexpression ($P=0.43$; Table 1).

Association of NRG1, HER3 and HER2/neu expression with RAS mutations and EGFR-ligand expression

HER3 overexpression was significantly correlated with the presence of *RAS* mutations ($P=0.02$; Table 2). *HER3* overexpression showed a trend towards an association

Table 1 Correlation of *HER2/neu* and *HER3* overexpression with *NRG1* expression and coalteration of *HER2/neu* and *HER3* expression

Correlation of *HER2/neu* and *HER3* overexpression with *NRG1* expression				
	Neuregulin 1 [*n* (%)]			
	Low	High	Total [*n* (%)]	*P* (two sided)
HER2/neu				
Low	185 (89.0)	16 (7.7)	201 (96.7)	1.00
High	7 (3.3)	0 (0.0)	7 (3.3)	
Total	192 (92.3)	16 (7.7)	208 (100.0)	
HER3				
Low	62 (29.8)	7 (3.4)	69 (33.2)	0.41
High	130 (62.5)	9 (4.3)	139 (66.8)	
Total	192 (92.3)	16 (7.7)	208 (100.0)	

Coalteration of *HER2/neu* and *HER3* expression				
	HER2/neu [*n* (%)]			
	Low	High	Total [*n* (%)]	*P* (two sided)
HER3				
Low	68 (32.7)	1 (0.5)	69 (33.2)	0.43
High	133 (64.0)	6 (2.8)	139 (66.8)	
Total	201 (96.7)	7 (3.3)	208 (100.0)	

P-values calculated using the χ^2-test.
NRG, neuregulin.

Table 2 Correlation of *HER3* expression and *RAS* status

	HER3 [*n* (%)]			
RAS	Low	High	Total [*n* (%)]	*P* (two sided)
Wild type	42 (20.2)	59 (28.4)	101 (48.6)	0.02
Mutation	27 (12.9)	80 (38.5)	107 (51.4)	
Total	69 (33.1)	139 (66.9)	208 (100.0)	

P-values calculated using the χ^2-test.

with high *EREG* expression. ($P=0.07$). All other combinations did not show associations.

Survival analysis

High versus low *NRG1* expression significantly affected PFS (4.7 vs. 8.2 months, hazard ratio: 2.45; 95% confidence interval: 1.45–4.13; $P=0.001$), but not OS (15.5 vs. 20.7 months, hazard ratio: 1.33; 95% confidence interval: 0.76–2.35; $P=0.32$). *HER3* and *Her2/neu* expression did not influence outcome (Fig 2a–d).

Discussion

To our knowledge, the FIRE-1 trial is the first randomized-controlled trial to investigate the impact of *HER2/neu* and *HER3* receptor overexpression in relation to *NRG1* expression and *RAS* status in mCRC [16–18, 20–24]. Previous investigations focused mostly on analysing *HER2/neu* and *HER3* expression in advanced colorectal or rectal tumours with patients receiving adjuvant radiotherapy or chemotherapy [17,18,20–22]. The FIRE-1 treatment schedule consisted of 5-FU/LV plus irinotecan or oxaliplatin plus irinotecan as first-line therapy, following a recommended second-line therapy with the respective crossover study regimen. With 208 patients enrolled in the analysis, our trial represents a robust investigation [16–18,20–24].

Evaluation of *HER2/neu* expression has been established in gastric cancer by Rüschoff *et al.* [9] using immunohistochemical staining and FISH in intermediate cases. Therefore, we decided to use these validated methods for patients with mCRC in accordance with the literature. *HER3* expression was also evaluated by immunohistochemical stainings using a modified Rüschoff semiquantitative scoring system, as in previous investigations, defining overexpression by cytoplasmatic or membrane staining intensity [16,18,23, 24]. As scoring of *NRG1* expression is not yet standardized for any tumour type [19], we also used an adapted Rüschoff score.

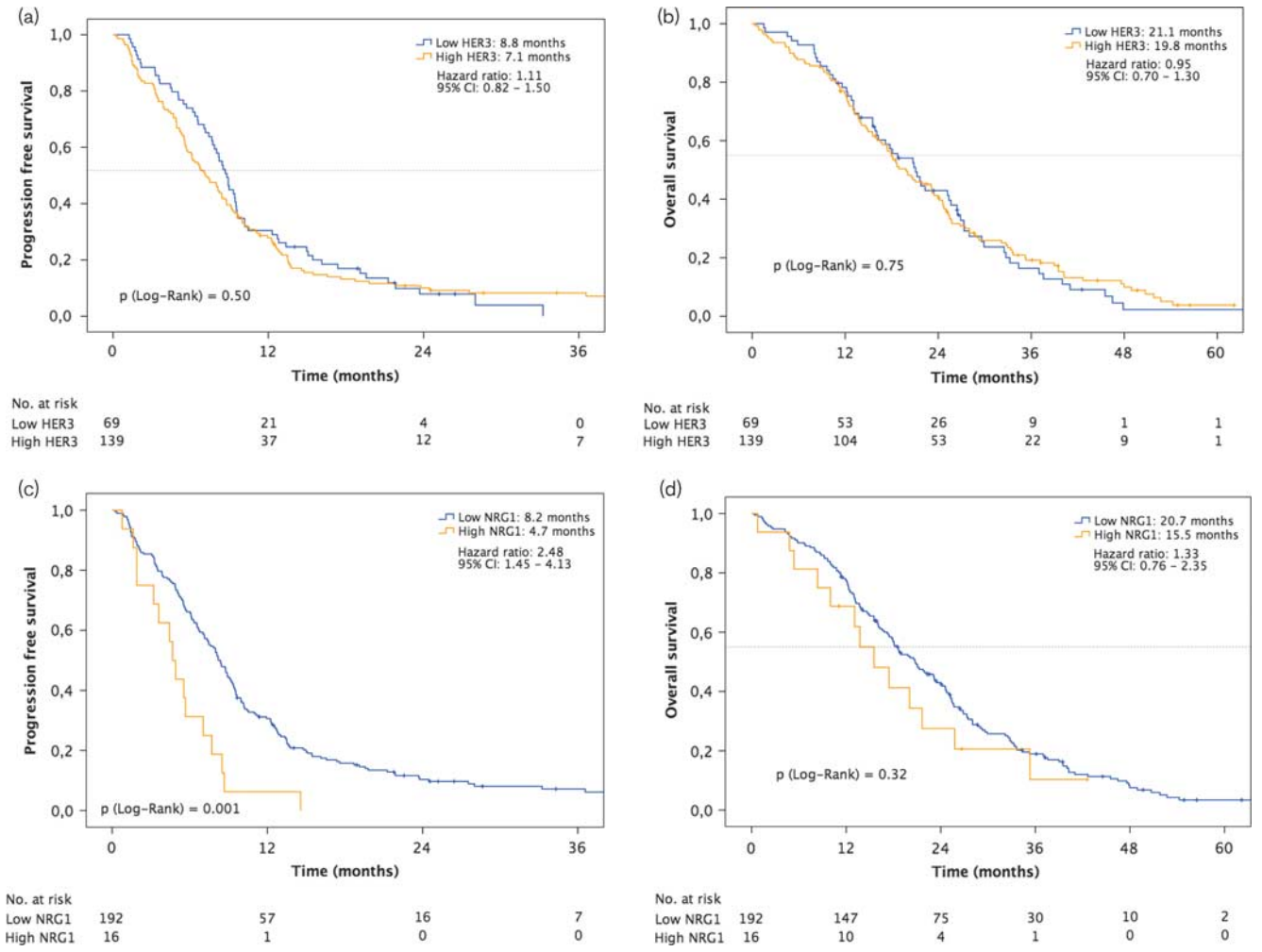
In FIRE-1, 3.3% of tumours showed high *HER2/neu* expression. This compares favourably to the average expression rate of *HER2/neu* in the literature of ~5.0% [12,13,23]. In FIRE-1, 67% of primary tumours showed high *HER3* expression, comparing favourably to recent other cohorts (Seo *et al.* [23]: 69%; Lédel *et al.* [18,22]: 70%). One trial evaluating transmembrane *NRG1* expression in CRC reported a frequency of high expression of 76%, in contrast to 8% in FIRE-1 [19]. However, a standardized scoring system is missing in this case, which may explain the discrepancy.

We attempted to correlate expression rates of *HER* receptors with *HER* ligands (*NRG1*) as well as downstream molecules (*RAS*). Unlike a previous investigation, we could not associate *HER3* expression with *NRG1* expression [19]. In our investigation, regular simultaneous expression of *HER2/neu* and *HER3* was also not detected, although coexpression of *HER2/neu* and *HER3* has been described in cohort of 364 surgically resected CRC patients [28]. The latter discrepancy might be caused in part by the different clinical backgrounds of patients as well as by the different diagnostic methods used.

By contrast, in our cohort, *HER3* expression correlated with *RAS* mutations, although *HER3* expression could not be associated with *KRAS* mutations previously [21]. This observation might have resulted from a higher number of patients enrolled in our investigation in addition to an extended analysis of *RAS* mutations.

In our study, high *NRG1* expression led to a significant decrease in PFS. This finding is supported by a previous investigation that also observed significantly worse 5-year PFS in patients with mCRC and high NRG1 expression [19]. Because of the small numbers of patients showing high NRG1 expression, conclusions are limited. The sample size of patients with *HER2/neu* overexpression in our cohort does not allow for conclusions on the prognostic impact. It is noteworthy that *HER3* overexpression

Fig. 2



Outcomes according to subgroups in FIRE-1; (a): PFS of patients in FIRE-1 comparing low and high *HER3* expression. (b): OS of patients in FIRE-1 comparing low and high *HER3* expression. (c): PFS of patients in FIRE-1 comparing low and high *NRG1* expression. (d): OS of patients in FIRE-1 comparing low and high *NRG1* expression. CI, confidence interval; NRG, neuregulin; OS, overall survival; PFS, progression-free survival.

was not associated with an unfavourable outcome in FIRE-1, although conflicting data may exist [16–18,24, 28]. However, the small number of patients enrolled in some trials limits conclusions.

Our investigation had several strengths. FIRE-1 was a randomized-controlled trial with irinotecan-based treatment, which had a rather small likelihood of bias in terms of the outcome and follow-up information. Unfortunately, although 208 patients were enrolled in this investigation, which represented a rather robust sample size, numbers in subgroups (NRG1, Her2/neu) became small. It might also be argued that treatment in FIRE-1 does not comply with the latest recommendations. In addition, FIRE-1, as well as previous investigations, lacked a validation collective for a proof-of-principle analysis. Therefore, further research is warranted to evaluate prognostic effects.

Conclusion

A significant unfavourable impact on PFS was observed in patients with mCRC with a high *NRG1* expression in the FIRE-1 trial. We did not detect a prognostic impact of *HER2/neu* and *HER3* overexpression in mCRC with respect to PFS and OS.

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Conflicts of interest

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