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Multi-colour FISH in oesophageal adenocarcinoma—predictors of prognosis independent of stage and grade

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Background: Oesophageal adenocarcinoma or Barrett's adenocarcinoma (EAC) is increasing in incidence and stratification of prognosis might improve disease management. Multi-colour Fluorescence *in situ* hybridisation (FISH) investigating *ERBB2*, *MYC*, *CDKN2A* and *ZNF217* has recently shown promising results for the diagnosis of dysplasia and cancer using cytological samples.

Methods: To identify markers of prognosis we targeted four selected gene loci using multi-colour FISH applied to a tissue microarray containing 130 EAC samples. Prognostic predictors (P1, P2, P3) based on genomic copy numbers of the four loci were statistically assessed to stratify patients according to overall survival in combination with clinical data.

Results: The best stratification into favourable and unfavourable prognoses was shown by P1, percentage of cells with less than two *ZNF217* signals; P2, percentage of cells with fewer *ERBB2*- than *ZNF217* signals; and P3, overall ratio of *ERBB2*-/*ZNF217* signals. Median survival times for P1 were 32 vs 73 months, 28 vs 73 months for P2; and 27 vs 65 months for P3. Regarding each tumour grade P2 subdivided patients into distinct prognostic groups independently within each grade, with different median survival times of at least 35 months.

Conclusions: Cell signal number of the *ERBB2* and *ZNF217* loci showed independence from tumour stage and differentiation grade. The prognostic value of multi-colour FISH-assays is applicable to EAC and is superior to single markers.

Within the last three decades the incidence of oesophageal adenocarcinoma (EAC) has increased in Western countries and exceeded the levels of other cancers (Blot, 1994; Heitmiller and Sharma, 1994; Devesa *et al*, 1998; Vizcaino *et al*, 2002; Pohl and Welch, 2005; Brown *et al*, 2008; Umar and Fleischer, 2008). Otherwise, the 5-year survival of EAC has remained at less than 10% in the past two decades (Blot *et al*, 1991; Devesa *et al*, 1998). EAC is believed to develop *via* a stepwise process from Barrett's metaplasia as a predisposing condition (Hamilton and Smith, 1987;

Hameeteman *et al*, 1989; Miros *et al*, 1991; Reid, 1991; Haggitt, 1994; Spechler, 1994, 2002). Within a reflux-metaplasia-dysplasia-carcinoma sequence, chronic damage by gastro oesophageal reflux leads to development of metaplastic Barrett's epithelium (BE). Further mutagenic events result in a low- to high-grade intraepithelial neoplasia-dysplasia-sequence and finally in invasive cancer (Spechler, 2002).

To detect EAC at an early, potentially curable stage, guidelines for gastro oesophageal reflux disease (GERD) outline that patients

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with GERD might benefit from an early endoscopic evaluation for Barrett's oesophagus (BE), considering its conversion rate to EAC of 0.5–1% per year (Peitz and Malfertheiner, 2007; Thomas *et al*, 2007). The gold standard in estimating the prognosis of EAC remains to be clinical classifications like TNM and grading of EAC. However, these classifications are not suitable for every situation, for example, biopsy collection, and could be complemented by new biomarkers (Cestari *et al*, 2007; Huang, 2010). In EAC the detection of *MDM2*, *VEGF* and *p53* have been suggested as markers of poor prognosis, but showed contradictory results across studies (Cavazzola *et al*, 2009; Prins *et al*, 2012; Renouf *et al*, 2013). A recent multi-centric study (OCCAM group) identified *DCK*, *PAPSS2*, *SIRT2* and *TRIM44* as prognostic biomarkers in EAC by extensive gene expression profiling and immunohistochemistry (Peters *et al*, 2010). New molecular methods like multiplex FISH analysis could be utilised for predictive stratification of patients with EAC to select individualised treatment.

Gain and loss of certain gene loci can contribute to the progression from BE to EAC, but less is known about their prognostic significance as biomarkers. As one example, *CDKN2A* (previously known as p16; 9p21) is involved in the clonal expansion of EAC progenitor cells (Barrett *et al*, 1999). Other gene loci such as *ERBB2* (also known as Her2) (17q12), *MYC* (8q24) and *ZNF217* (20q13.2) are also implicated in EAC development. All these genomic loci have shown promising results in detecting progression from BE to dysplasia and EAC in oesophageal brush cytology specimens (Brankley *et al*, 2006; Rygiel *et al*, 2007; Fritcher *et al*, 2008). Although it could be assumed that functionally important markers for progression might have an impact on prognosis in EAC, the Fluorescence *In Situ* Hybridisation (FISH) assay using multi-colour Locus-Specific Identifier (LSI) probes has never been applied to advanced EAC.

In brief, the single markers in this multi-colour FISH assay are characterised as follows:

ERBB2 is part of the human epidermal growth factor receptor family. It stimulates cell proliferation using the RAS-MAP-kinase pathway and blocks apoptosis using the mTOR pathway. *ERBB2* was first evaluated as a target for trastuzumab therapy in invasive breast cancer, and later in gastric cancer and EAC (Huoher *et al*, 2006).

ZNF217, also known as Zinc finger protein 217, encoded by *ZNF217*, blocks signals resulting from dysfunction and may promote neoplastic transformation and progression of malignancy (Huang *et al*, 2005).

ZNF217 has recently been implicated in ovarian cancer. There it could be linked to activated PIK3CA signalling, which puts it in a possible context to influence *ERBB2* and MAPK signalling (Hou *et al*, 2014; Yoda *et al*, 2014).

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*, p16^{Ink4A}), also known as multiple tumour suppressor 1 (MTS-1), is a tumour suppressor encoded by *CDKN2A* (Nobori *et al*, 1994). Alterations such as loss of *CDKN2A*, usually by deletion, mutation or promoter hypermethylation, cause increased risk of developing malignant tumours such as adenocarcinoma, including EAC, because of its function in cell cycle regulation (Igaki *et al*, 1994).

MYC is a regulator gene that encodes a transcription factor and is located on chromosome 8. It is believed to regulate the expression of 15% of all genes through binding to enhancer box sequences. Mutations in *MYC* that cause its persistent expression have been found in many cancers (Cotterman *et al*, 2008). This leads to deregulated expression of genes involved in cell proliferation and results in cancers like Burkitt's lymphoma (Hummel *et al*, 2006).

As a proof-of-principle, multiplex FISH analysis can be applied to formalin-fixed paraffin-embedded (FFPE) tissue, for example, for *MYC* and *ZNF217* (Wiech *et al*, 2009).

In this study we have applied four locus-specific probes (*ERBB2* (17q12), *MYC* (8q24), *CDKN2A* (9p21), *ZNF217* (20q13.2)) to FFPE EAC tissue specimens and assessed the prognostic value of these markers. A large, clinically well-defined EAC patient group with follow-up data was investigated on tissue microarrays (TMA).

MATERIALS AND METHODS

Patient samples, tumour classification and clinical data. One hundred and thirty EAC patient tissue samples were collected at the Institute of Pathology, Technical University of Munich (TUM) from 1991 to 2004 as previously described (Langer *et al*, 2006; Rauser *et al*, 2007). The presence of Barrett's adenocarcinoma was confirmed histologically according to the tumour site and histological categories. None of the patients had received neoadjuvant chemotherapy or radiation. The average patients' age was 63.4 years, ranging from 33 to 83 years. The median follow-up time was 43 months (see Table 1). All histological samples were FFPE. The tumours were classified according to the 2002 WHO classification of tumours from 2002 and the 6th edition of the TNM-classification for oesophageal tumours (Hamilton and Aaltonen, 2000). Invasion into blood vessels, lymphatic vessels or extension of tumour beyond submucosal gland tissue was documented separately. All cases were re-evaluated by an experienced pathologist (MS, TTR), including exact location of the tumour area on haematoxylin/eosin-stained (HE) slides before extracting tissue for the TMA. The clinical follow-up data were assembled separately from patient records. Twenty tissue samples of cardiac mucosa without metaplasia or dysplasia were used as representative control tissues. National and local ethical guidelines were followed.

TMA procedure. TMAs were constructed for each tumour as described previously (Bubendorf *et al*, 2001; Klopocki *et al*, 2004; Baumann *et al*, 2006). A representative tumour section was selected from a HE section of the paraffin block with the centre and representative region of tumour tissue identified (detailed in Supplementary Technical Notes).

Four-colour FISH. Analysis of multi-colour FISH on TMA slides is a suitable tool to identify specific genomic changes in tumours (Schwarz *et al*, 2008). FISH was performed on FFPE slides as previously described (Sassen *et al*, 2008). We used directly labelled LSI probes for *ERBB2* (17q12), *MYC* (8q24), *CDKN2A* (9p21) and

Table 1. Major characteristics of the EAC study group (N = 130)

Median age (range)	Gender, n (%)		Stage, n (%)					Grade, n (%)		
	Male	Female	I	IIA	IIB	III	IV	1	2	3
63.5 years (33–83)	118 (90.8)	12 (9.2)	55 (42.3)	22 (16.9)	16 (12.3)	30 (23.1)	7 (5.4)	12 (9.2)	56 (43.0)	62 (47.7)

Abbreviation: EAC = oesophageal adenocarcinoma.

ZNF217 (20q13.2). The four-colour LSI FISH probe set was provided by Abbott Molecular (Des Plaines, IL, USA) and applied on TMA sections following the manufacturer's protocols (detailed information in Supplementary Technical Notes).

Signal evaluation by epifluorescence microscopy. Slides were evaluated using a fluorescence microscope equipped with five bandpass filters specific for DAPI and each probe signal (*ERBB2* (green), *MYC* (blue), *CDKN2A* (red) and *ZNF217* (gold, Aristoplan from Leitz, Wetzlar, more details in Supplementary Technical Notes): Images were obtained with a Plan-Apochromatic lens (63 \times , numerical aperture 1.4) and an Axio-Cam MRm camera (both from Zeiss, Jena, Germany). HE pictures were taken with a Leica DM 4000 B and DFC 320 camera (Leica AG, Wetzlar, Germany).

Enumeration and evaluation of FISH signals. To determine copy numbers at *ERBB2* (17q12), *MYC* (8q24), *CDKN2A* (9p21) and *ZNF217* (20q13.2) chromosomal loci, FISH signals for each of the four probes were enumerated in 130 EAC specimens with acceptable hybridisation quality. A conventional HE stain was performed to identify tumour areas for FISH analysis, as well as guarantee the representativeness of the TMA spots, and for reference histology.

For each patient specimen, a representative tumour area was selected for evaluation on one of the three replicate punch samples on the TMA, and 50 consecutive tumour cells were counted in the selected area. To assure the sampling method was representative, a 50-cell enumeration was conducted on all three punch samples of 10 individual, randomly selected EAC cases. The representation and homogeneity of the selected tumour areas during TMA generation was statistically confirmed for each of the four probes (*ERBB2* (17q12), *MYC* (8q24), *CDKN2A* (9p21) and *ZNF217* (20q13.2)) in the three resulting data sets by student's *t*-test and interclass correlation (Cohen's Kappa).

In the control group, 19 tissue specimens with acceptable hybridisation quality were enumerated. Nuclei in the cardiac glands were examined in the same way as the nuclei of tumour cells in the carcinoma group. For quality control, two reviewers experienced in FISH analysis evaluated at least 15% of all punches and comparison of their results showed high agreement.

Statistical analysis. In total, 130 EAC patient specimens were evaluated in this study. To select the final FISH parameter(s) that have significant impact on patients' overall survival, the enumerated data for the 130 patients were split into two parts. A randomly selected subset of 64 patients was used as the training dataset, and the remaining subset of 66 patients was treated as the validation dataset.

Multiple (>30) FISH parameters derived from the copy numbers of the four FISH-LSI probes were first evaluated as possible prognostic indicators of overall survival within a training subset; the statistically significant parameters were further validated within the full 130-patient dataset. The FISH parameters were created from all possible combinations of the four probes used in the assay, and were derived either from percentages of cells with an abnormal copy number of one or more probes, or from the copy number ratios.

Correlations between FISH parameters and important clinical variables (age, gender, tumour grade and stage), were evaluated using the χ^2 -test. Frequency tables were generated to show the patient distribution within each level.

To select optimal cutoff values for each of the FISH parameters, patients were categorised into FISH positive (+) and FISH negative (-) groups based on each of the possible cutoff values. The log-rank test was used to compare overall survival between the FISH+ and FISH- groups. The hypothesis was that FISH+ patients have worse overall survival than FISH- patients. For each

FISH parameter, the optimal cutoff value separating patients into favourable and unfavourable survival outcome groups was selected as the value that yields the lowest log-rank *P*-value. The parameters with log-rank *P*-values < 0.05 at the optimal cutoff value were chosen for further analysis.

With the most promising FISH parameters selected and their optimal cutoffs derived from the log-rank test, a multivariate Cox proportional hazard model was used to select the final FISH parameters after adjusting for key clinical factors (age, gender, tumour grade and stage). This exercise was done using the training dataset. Once the final model was selected, it was confirmed with the validation dataset and applied to the full dataset. Furthermore, Kaplan-Meier method was used to obtain the overall survival curves, stratified by important FISH and clinical predictors, together with the corresponding median overall survival time. All analyses were performed using SAS (Version 8.2, SAS Institute Inc., Cary, NC, USA).

RESULTS

Morphological homogeneity in different TMA punches from the tumour centre correlates with FISH results.

Initially a series of 10 tumour specimens were analysed. In HE staining, the punch biopsies from tumour centres showed the same morphology, cytology and tumour grading. To exclude molecular heterogeneity in the tumour punches taken during TMA generation, three separate sets of 50 consecutive cells were counted for each of our individual probes. Student's *t*-tests showed no significant differences between means and each counted subset; further on, interclass correlation coefficients were calculated and showed significant homogeneity between the chosen tumour punch biopsies in the TMA (details in Supplementary Statistical Notes).

The effect of nuclear truncation is comparable in EAC tissue and normal tissue.

In this study, FISH analysis was performed on histological specimens. In comparison to cytological specimens, FISH on histologic tissues showed a loss of up to 30% owing to nuclear truncation, which depends on slide thickness (Nielsen *et al*, 2012). The effect was equal in tumour and normal tissues. There was no effect of the greater diameter of tumour cell nuclei vs those of normal tissue (data not shown). Therefore, these FISH-stained 3 μ m-thick TMA sections were deemed appropriate for FISH analysis. In normal tissue the mean values of signals/cell for *CDKN2A*, *ERBB2*, *ZNF217* and *MYC* were determined to be 1.68, 1.78, 1.77 and 1.74, respectively.

Fluorescence *in situ* hybridisation parameters distinguish between normal and malignant tissue.

The multi-colour probe set utilised in this study (*ERBB2*, *MYC*, *CDKN2A* and *ZNF217*, Figure 1) was originally selected to aid in diagnosing dysplasia and EAC in patients with Barrett's metaplasia (Brankley *et al*, 2006). We observed that EAC was associated with gains in the *ZNF217* and *ERBB2* signals. As expected, *MYC* also showed a slight gain in signal, while *CDKN2A* showed a signal loss in EAC. *MYC* and *CDKN2A* indicated malignancy, but revealed no further prognostic potential when analysed in multivariate analysis. *ERBB2* as a single marker correlated with tumour grade.

Table 2 shows the performance of selected FISH parameters with cutoffs (percentage of positive cells or ratio of two probe signals) for normal tissue and EAC. As is evident from the table, we confirmed that the probes used in this study were highly sensitive and specific for EAC when compared to normal tissue, with the highest sensitivity and specificity achieved with *ERBB2* and *ZNF217*, or composite parameters based on these loci.

Selected FISH parameters predict overall survival in EAC patients. Multiple parameters derived from the copy numbers of

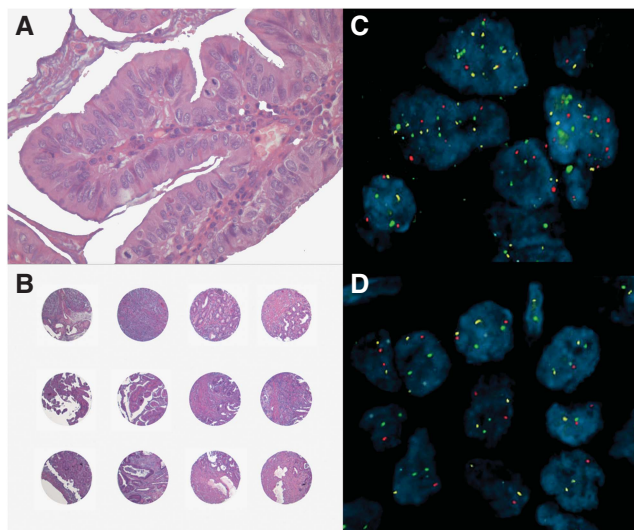


Figure 1. (A) Oesophageal adenocarcinoma with HE staining (TMA): magnification $400\times$, for verifying carcinoma cells against epithelium, low- or high-grade dysplasia and normal soft tissue in between tumour cells. (B) Oesophageal adenocarcinoma with HE staining (TMA): magnification $500\times$, for counting and verifying carcinoma tissue and epithelium, low- or high-grade dysplasia and normal soft tissue. (C) Representative sample of EAC (TMA): FISH showing gain in *ERBB2* and *ZNF217*, loss of *CDKN2A* and unspecific signals for *MYC*. Fluorescence *in situ* hybridisation–LSI probes for *ERBB2* (green), *MYC* (blue), *CDKN2A* (red) and *ZNF217* (gold). Note the gain in strong *ERBB2* spots in comparison to the low number of *ZNF217*, indicating an unfavourable EAC with regard to our predictors P1–P3. (D) Tissue from normal cardiac mucosa with the FISH–LSI probes for *ERBB2* (green), *MYC* (blue), *CDKN2A* (red) and *ZNF217* (gold) as the control group and standard values.

Table 2. Performance of selected parameters based on FISH–LSI probes and probe combinations in identifying EAC (130 EAC and 19 cardia mucosa (normal control)), in order of descending sensitivity and specificity

Parameter	Cutoff	Sensitivity	Specificity
% Cells with <i>ZNF217/CDKN2A</i> > 1	> 35%	98%	95%
<i>ZNF217</i> /cell	> 2.0	96%	100%
<i>ERBB2</i> /cell	> 2.0	96%	95%
% Cells with gains in any two loci	> 20%	95%	100%
<i>ERBB2/CDKN2A</i>	> 1.2	94%	100%
% Cells with gains in any three loci	> 2.0%	93%	100%

Abbreviations: EAC = oesophageal adenocarcinoma; FISH–LSI = fluorescence *in situ* hybridisation–locus-specific identifier. Varied cutoff values were used for each FISH parameter to calculate the sensitivity and specificity in discriminating EAC from normal controls.

ERBB2, *MYC*, *CDKN2A*, and *ZNF217* were screened by univariate log-rank analysis of overall survival at multiple cutoff values within a randomly selected training subset, as described in the Materials and Methods section.

For each of the parameters, the optimal cutoffs were selected that minimised log-rank *P*-values. Those parameters with optimal cutoffs that were statistically significant at the 0.05 level were selected as potential prognostic indicators of overall survival. The selected parameters were further evaluated for significance in univariate analysis with a validation data subset, as well as with the complete 130-specimen dataset. With the selected cutoffs, parameters P1, P2 and P3 listed below were shown to have statistically

significant effects on survival in randomly selected training and validation subsets of the data, as well as in the full dataset, discriminating patients with longer median survival from those with shorter survival times. Therefore, parameters P1, P2 and P3 were shown to dichotomise between favourable and unfavourable outcomes with respect to overall survival:

- P1: percentage of cells with less than two signals for the *ZNF217* locus, with favourable outcome below a cutoff of 16%.
- P2: percentage of cells with fewer *ERBB2* than *ZNF217* signals (*ERBB2/ZNF217* < 1), with favourable outcome observed at or above a cutoff of 28%.
- P3: the overall ratio of *ERBB2* to *ZNF217* signals, with favourable outcome observed below a cutoff of 1.5.

The results of the log-rank test for each of the selected predictors in the combined full dataset of 130 patients are shown in Table 3.

Analysis of covariates and proportional hazards model to predict overall survival.

As shown above, FISH parameters were found to be potential predictors of survival. These parameters were further evaluated in a covariate analysis with clinico-pathological variables to understand the relative importance of molecular and clinical parameters that might be associated with disease outcome. Correlation between several clinico-pathological variables, as well as correlation of these variables with FISH, was assessed in univariate and multivariate (Cox proportional hazards) analyses, testing the effect of these covariates on patient survival. The clinico-pathological variables included anatomical stage (I, IIA, IIB, III and IV), blood vessel invasion, lymphangiosis carcinomatosa, depth of tissue infiltration (T-stage), lymph node status (N-stage) and tumour grade. In a univariate analysis these were the best predictors of survival. However, when entered in a multivariate model, it was observed that the clinical parameters, including lymph node status, were correlated with the anatomical stage. Therefore, out of the clinical parameters tested, two were selected for further analysis: tumour grade and anatomical stage.

Tumour grade and anatomical stage were also found to be correlated with FISH parameters P1, P2 and P3. Therefore, a stratified analysis was performed to assess the prognostic value of FISH in the presence of these important clinico-pathological covariates.

Multivariate model analysis of stratified data. The greatest predictive power in stratifying the patient population in this study was observed when FISH was combined with grade or stage in the multivariate analysis (Figures 2–5 and Table 4). In the multivariate model analysis carried out using SAS, the PHRED procedure, statistical significance was achieved with both the training and validation sets for all three FISH parameters (P1–P3, log-rank $P < 0.0001$). The model with the highest statistical scores of all models possible for all given covariates is shown in Table 4. This model is based on the entire patient group (complete dataset) and reports survival times and hazard ratios for each FISH parameter.

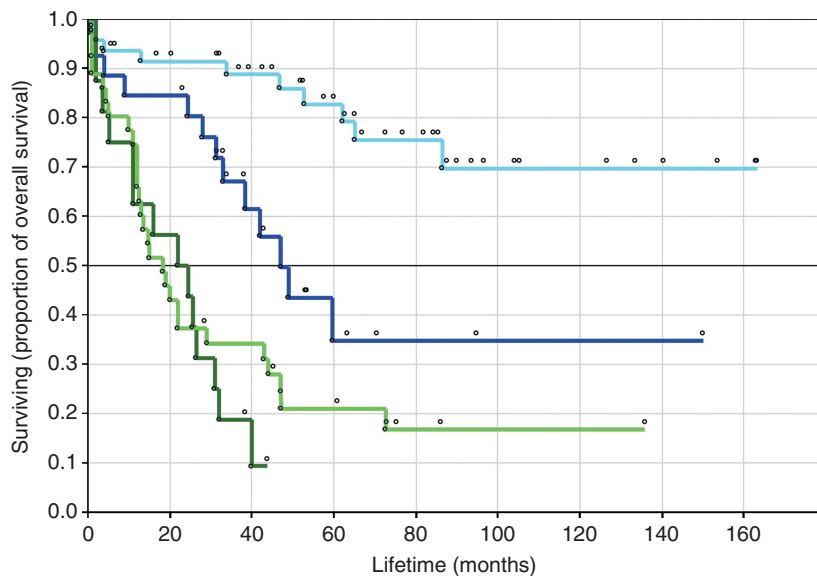
Each of the predictors P1, P2 and P3 adds additional prognostic information to histological grade.

As is evident from Table 4, each of the three FISH predictors, P1, P2 and P3 added prognostic value to that of each histological grade in the common tripartite grading system. Furthermore, defining the FISH+ group of patients as those with poorer prognosis for each of the selected FISH parameters, the same trend for a combination of all three parameters combined (P positive defined as either P1, P2, or P3 positive) was shown in the survival analysis (Figure 5A). For each grade, FISH (P) stratified patients into FISH positive and negative groups based on the difference in the overall survival. For example, in well differentiated tumours patients' survival could be separated

Table 3. Univariate survival analysis of the selected FISH predictors P1, P2 and P3 (different combinations of *ERBB2* and *ZNF217*, analysed by SAS, favourable and unfavourable groups in months, hazard ratio (HR) and *P*-value in log-rank test)

Predictor and definition	Median Survival		Median values		Log-rank test	
	Favourable	Unfavourable	Favourable	Unfavourable	HR	<i>P</i> -value
P1: % of cells with <2 <i>ZNF217</i> signals, cutoff 16%	72.7	33	6.00	20.00	1.764	0.0267
P2: % of cells with <i>ERBB2</i> < <i>ZNF217</i> signals, cutoff 28%	72.7	31.3	26.53	52.00	1.996	0.0066
P3: Overall ratio of <i>ERBB2</i> / <i>ZNF217</i> signals, cutoff 1.5	65.2	27.3	0.94	2.15	2.112	0.0086

Abbreviation: FISH = fluorescence *in situ* hybridisation.



Stage	Log-rank <i>P</i> -value (FISH within stage)	FISH status	Number of patients	Number surviving	Median survival (months)	Plot line
IA and IIA	0.0015	Favourable	47	37	> Study duration	Light blue line
		Unfavourable	27	14	47.00	Dark blue line
IIB, III and IV	0.4363	Favourable	36	8	18.30	Green line
		Unfavourable	16	2	23.25	Dark green line

Figure 2. Predictor 1 (P1): P1 discriminates patient survival the best in low-stage groups. Kaplan–Meier survival analysis of P1: percentage of cells with less than two signals for *ZNF217*. Best dichotomization of data for favourable outcome below a cutoff of 16%. Results grouped by clinical stage. Median survival in months for each category is shown in parentheses (log-rank test, *P*<0.0001).

in longer than study duration in comparison to 59.67 months as well as in poor differentiated tumours with a distinction of 47.0 vs 11.00 months, as is evident from Figure 5.

Fluorescence *in situ* hybridisation parameters and tumour stages. In contrast, the contributions of P1, P2 and P3, as well as the three parameters in combination, were more significant at low stages (I and IIA), where FISH discriminated between patients with favourable prognosis and those with survival similar to that of higher-stage patients (IIB, III and IV) (Figures 2–4 and 5B). Specifically, the effect was most significant for Stage IIA patients (data not shown). As stage IIA and IIB differ by the status of lymph node metastasis, it is possible that FISH allows detection of a subset of patients with worse outcome, possibly owing to occult metastasis, who may need to be re-evaluated for treatment.

***ZNF217*/cell ratio and *ERBB2*/cell ratio influence.** Fluorescence *in situ* hybridisation parameters P1, P2 and P3, derived from the copy number of *ZNF217* loci per cell, have high-prognostic value. Thus, we examined this FISH parameter in more detail.

ZNF217/cell did not show statistical significance in the initial analysis; however, it was significant when combined with stage or grade (Figure 6). Interestingly, we found evidence that a relatively low level of *ZNF217*/cell (<2.8) was associated with a poorer prognosis, similar to the trend observed with P1 and P2 (Figure 6), while higher ratios were associated with a more favourable outcome. This trend was consistent with the effect of *ZNF217* copy number on P1, P2 and P3.

In contrast, the *ERBB2*/cell number was directly correlated with poorer prognosis. We found a significant correlation between *ERBB2* gain and poorer survival. Kaplan–Meier survival analysis indicated a median survival time greater than the study duration for patients with low *ERBB2* copy number (<43% cells with *ERBB2* >2 signals/cell) and 43 months for patients with higher copy number (Supplementary Figure 1A). This trend is consistent with the effect of *ERBB2* copy number on P2 and P3. So besides the three predictors P1–P3, the percentage of cells with *ERBB2* >2 signals/cell may serve as a predictor.

Combining *ERBB2* and *ZNF217* into a ratio to yield a single predictor was fulfilled with our predictor P3, which could be used

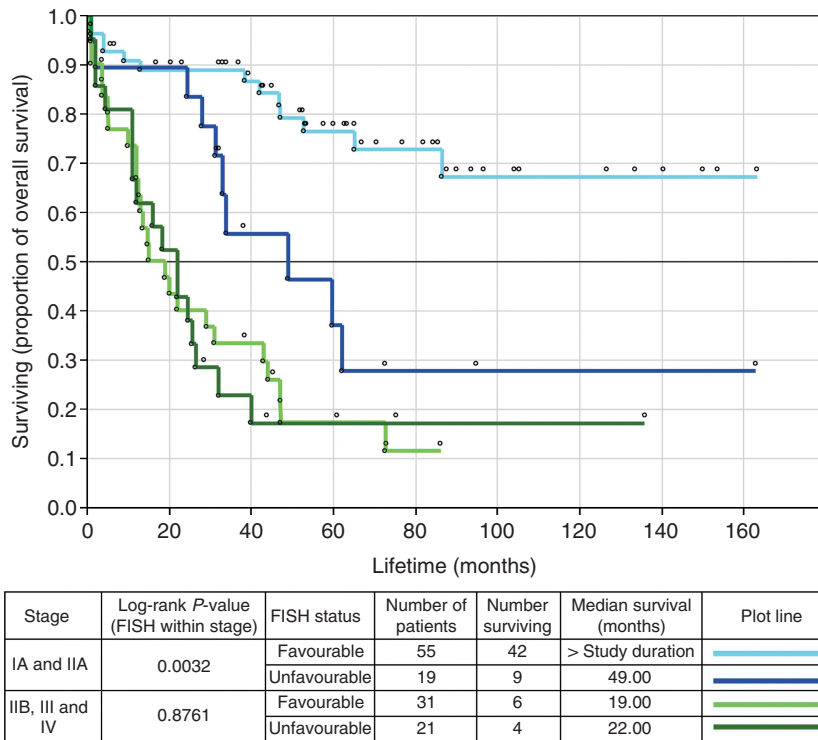


Figure 3. Predictor 2 (P2): P2 is able to separate patients with low-stage tumours into groups with good and poor prognosis. Kaplan–Meier survival analysis of P2: percentage of cells with fewer *ERBB2* than *ZNF217* signals ($ERBB2/ZNF217 < 1$), with favourable outcome observed at or above a cutoff of 28%, grouped by clinical stage. Median survival in months for each category is shown in parentheses (log-rank test, $P < 0.0001$).

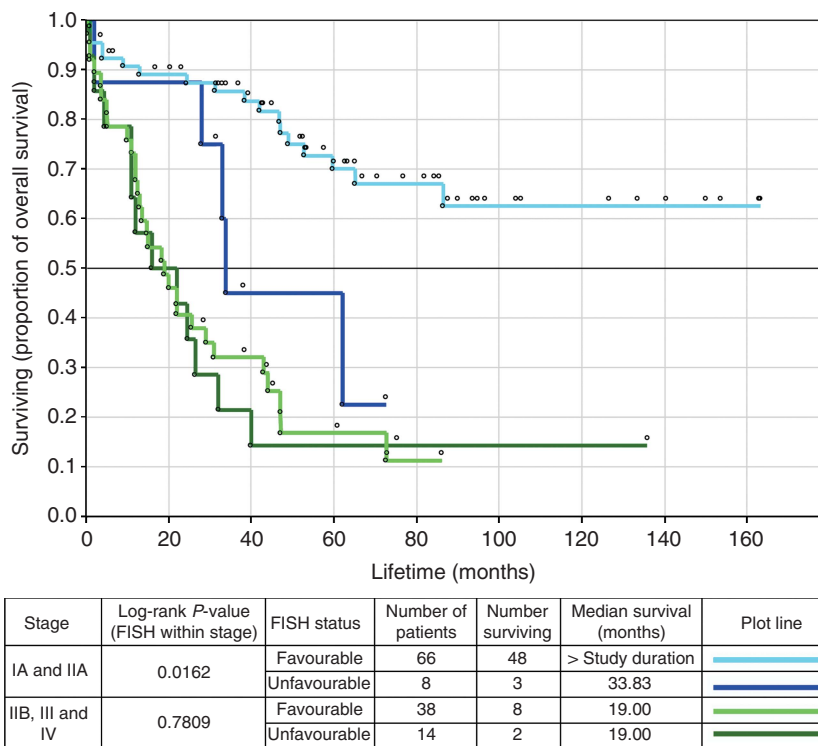


Figure 4. Predictor 3 (P3): P3 is able to separate patients with low-stage tumours into groups with good and poor prognosis. Kaplan–Meier survival analysis of P3: the overall ratio of *ERBB2* to *ZNF217* signals, with favourable outcome observed below a cutoff ratio of 1.5, grouped by clinical stage. The late crossing of the curves in high-stage groups is owing to the small number of patients with these tumour stages. Median survival in months for each category is shown in parentheses (log-rank test, $P < 0.0001$).

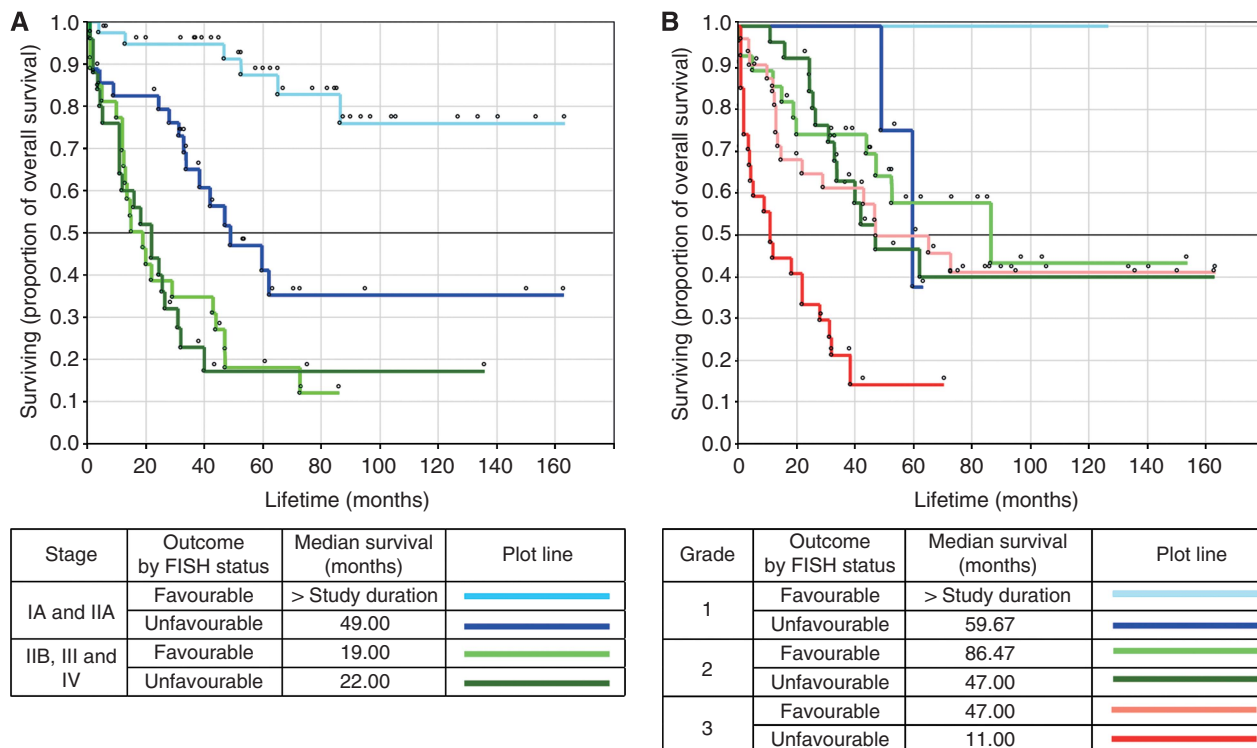


Figure 5. A combination of predictors is able to identify individuals with a poorer prognosis in low- and high-tumour stages (A), and by grade in the standard tripartite grading system (B). Prognosis grouped by histological grade or stage and FISH results, favourable if one of the three FISH predictors P1–P3 = P is favourable in Kaplan–Meier survival analysis. Median survival in months for P for each category is shown in tables under the plots. Regarding stage (A), FISH results separate low stages IA and IIA significantly (log-rank test $P < 0.0001$) but not higher stages (log-rank test, $P = 0.8060$). Regarding grade (B), FISH results tend to separate G1 EAC (log-rank test, $P = 0.0586$), G2 EAC not significantly (log-rank test, $P = 0.5596$), but especially G3 EAC (log-rank test $P = 0.0007$).

Table 4. Multivariate survival analysis of the selected FISH predictors stratified by grade

Predictor	Grade 1		Grade 2		Grade 3		HR
	Median survival		Median survival		Median survival		
	Favourable	Unfavourable	Favourable	Unfavourable	Favourable	Unfavourable	
P1	> study duration	59.7	86.5	42.0	43.0	11.0	2.193
P2	> study duration	54.3	86.5	40.0	46.8	12.0	2.529
P3	> study duration	No patient	86.5	40.0	38.4	11.0	2.513
FISH	> study duration	59.7	86.5	47.0	47.0	11.0	2.463

Abbreviation: FISH = fluorescence *in situ* hybridisation. Also shown is the overall FISH status, defined as: if any one, or any combination of the three predictors is positive, the overall FISH status is positive; otherwise, the status is negative (hazard ratio (HR), analysed by SAS, PHRED Procedure, median survival in months).

as a separate prognostic marker for the whole study collective (Supplementary Figure 1B).

DISCUSSION

The clinical management of EAC is still limited in stratifying patients into prognostic groups (Esofago, 1991) and a search for comprehensive markers to improve disease management is warranted (Ong *et al*, 2010; Moyes and Going, 2011).

Biomarkers in EAC are being investigated with a plethora of molecular techniques. In addition to classical histology, immunohistochemistry, methylation patterns or genetic aberrations like

LOH of a certain chromosomal region (Albrecht *et al*, 2004; Ong *et al*, 2010), as well as single nucleotide polymorphism microarray (SNP-chip), are being utilised as research tools, but still lack clinical significance (Akagi *et al*, 2009; Wiech *et al*, 2009).

Regarding prognosis, *MDM2*, *VEGF* and *p53* evolved as additional single markers in EAC but don't seem to be robust across studies (Cavazzola *et al*, 2009; Prins *et al*, 2012; Renouf *et al*, 2013). Hence, extending the analysis to multiple targets and evaluating its feasibility for routine protocols is required. Fluorescence *in situ* hybridisation meets these criteria.

Multi-colour FISH used on cytological oesophageal brushing specimens has shown potential to serve as a diagnostic tool to determine the progression from BE to high-grade dysplasia and EAC with high sensitivity and specificity (Fritcher *et al*, 2008).

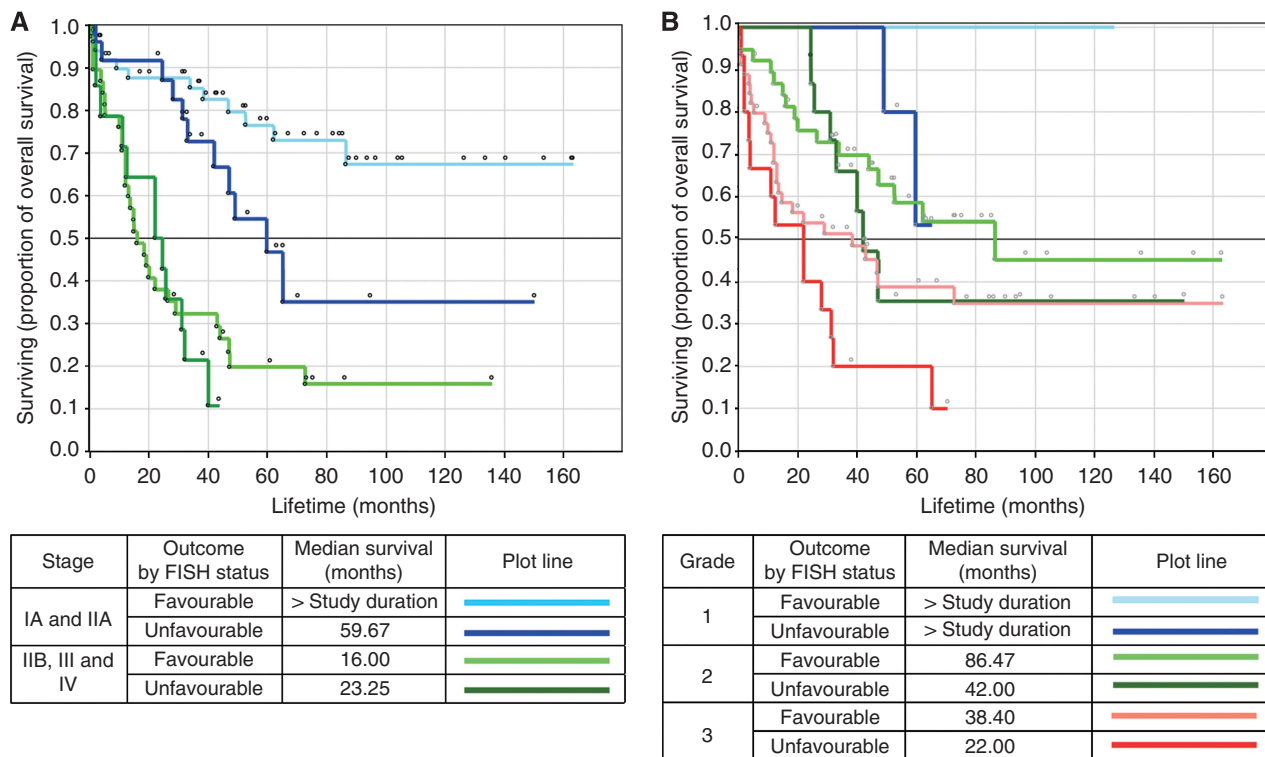


Figure 6. *ZNF217/cell* serves as a predictor and subdivides individuals into stage groups and within each grade according to prognosis. Kaplan–Meier survival analysis of the FISH parameter *ZNF217/cell*; definition and cutoff: *ZNF217/cell* with a cutoff of 2.8 and a favourable outcome above the cutoff, grouped by clinical stage (A), and histological grade (B). *ZNF217/cell* and stages I and IIA, III and IV (log-rank test, $P < 0.0001$); *ZNF217/cell* and grades 1–3 (log-rank test, $P = 0.0009$). Median survival in months for each category is shown in tables under the plots.

This probe set consisting of *ERBB2*, *CDKN2A*, *ZNF217* and *MYC* was transferred to manifested EAC to address a possible prognostic impact. To our knowledge this is the first study in EAC combining FISH biomarkers with outstanding clinical follow-up data on 130 individuals over 160 months.

We defined three essential predictors of overall survival: P1, P2 and P3, and compared them to the clinico-pathological parameters in univariate and multivariate analysis (Cox proportional hazards) to analyse the effect of these covariates on patient survival. We determined that each of the FISH parameters added additional predictive information independent of histological grade. Thus, FISH may be able to detect individuals with high-risk EAC, adding information to the classical histopathological staging (Figures 2–4).

In patient follow-up of over 160 months, all three FISH parameters P1, P2 and P3 predicted significant survival differences of about 40 months. This allowed the identification of patients with at least 25% prolonged lifetime. We further developed a subgroup analysis in low- and high-grade stages (I and IIA vs IIB, III and IV). Fluorescence *in situ* hybridisation predictors were able to better distinguish favourable and unfavourable outcomes in low tumour stages. Hence, the useful multi-colour FISH approach for the analysis of progression from BE to EAC in brush cytology sends further light on EAC's prognosis (Brankley *et al*, 2012; Yao *et al*, 2008). In detail, the biomarkers *ZNF217* and *ERBB2* added more prognostic significance than *CDKN2A* or *MYC*. The latter still provided diagnostic power for detection of EAC. As seen from Table 2, all four gene loci are increased in EAC at high sensitivity and specificity, but our data support a particular role of *ZNF217* and *ERBB2* in EAC.

Taking a closer look at the effect of the *ZNF217* locus on survival, *ZNF217* showed a relatively low-level gain that was associated with a poorer prognosis, while higher copy number

was linked to better outcome. Low-level gain in *ZNF217*, in contrast to high-copy number, appears to be unfavourable for survival in this analysis. This interesting finding may be related to the notion that initial genomic instability is followed by the selection of more stable tumour cells with low-level to moderately amplified *ZNF217* in aggressive cancer cells, similar to ovarian clear cell carcinoma (Rahman *et al*, 2012). Our finding that EAC patients possessing higher copy number *ZNF217* have better outcomes contrasts to literature reports, where Huang *et al* (2005) showed that a higher copy number of *ZNF217* was associated with a poorer prognosis in breast cancer. Littlepage *et al* (2012) showed that *ZNF217* as a candidate oncogene could be a target of treatment in breast cancer and is associated with poor prognosis. Those findings regarding *ZNF217* seem not to be a universal phenomenon and actually are still under debate. Our data on *ZNF217* are so far the first correlated to prognosis in EAC. It seems that *ZNF217* has a different role in EAC prognosis as compared with breast cancer. In our study the clinical associations of the three predictors (P1–P3) have in common the finding that higher levels of *ZNF217* are associated with better outcome.

ERBB2 represents the second essential part of our predictors (P1–P3). Knowledge about *Her2/ERBB2* has grown substantially within the last years since the introduction of trastuzumab as a targeted therapy for gastric cancer and EAC as well (Ross and McKenna, 2001; Bookman, 2003; Gravalos and Jimeno, 2008; Aitken *et al*, 2010; Bang *et al*, 2010; Rüschoff *et al*, 2010). It is known that up to 25% of EACs overexpress *Her2/ERBB2* in immunohistochemistry or FISH analysis. These patients might therefore be eligible for targeted therapy and benefit from a therapy-associated better outcome. Assessment of *ERBB2* by means of a theranostic marker evoked the problem of tumour heterogeneity in EAC. It is strongly recommended to use whole tumour

sections for diagnostic purposes. Additionally, immunohistochemistry was judged superior to FISH analysis as it maintains an overview of a possibly heterogeneous cancer sample (Rüschoff *et al*, 2010, 2012). In a study setting, TMA techniques are generally very useful for the simultaneous analysis of large sample numbers, as in our case. TMA generation itself is targeted on homogeneity in collected core biopsies. Possibly this is the reason behind our more homogenous *ERBB2* results. The perspectives of whole slide imaging and analysis by automated digital pathology will be applicable for Multi-colour FISH analysis in the near future (Dobson *et al*, 2010; Braun *et al*, 2013; Furrer *et al*, 2013). This will enable a concrete mapping of tumour heterogeneity with FISH comparable to immunohistochemistry. Beyond theranostic aspects, the role of *ERBB2* as a prognostic marker is still under debate (Bang *et al*, 2010). We found a significant correlation between low levels of *ERBB2* and better outcome and an association with tumour grade and stage. Specifically, low copy number of *ERBB2*, which in this context is statistically defined as less than 43% of cells with *ERBB2* > 2/cell, was significantly associated with improved survival (Supplementary Figure 1A). This is in line with a recent meta-analysis showing 7 months of gain in survival in patients without *Her2/ERBB2* expression. However, Gowryshankar *et al* (2014) also referred to the heterogeneous results of the prognostic impact of *ERBB2* in EAC among different studies (Tanner *et al*, 2005; Gravalos and Jimeno, 2008; Yoon *et al*, 2012a, b).

To obtain more objective detection methods and classifications of *Her2/ERBB2*, positivity should be standardized for study purposes, for example, with the envisioned partially automated sample scoring system (Dobson *et al*, 2010).

This is particularly mandatory as multiplex approaches complicate the number of possible combinations—we calculated $n > 30$ mathematical terms—and might imply confusing thresholds. This accounts for our markers P1 and P2, which achieved the best discriminative *P*-values, but might be regarded as counter-intuitive with respect to their definitions and cutoffs.

Fluorescence *in situ* hybridisation parameters defined as ratios are more convenient. Thus, P3 is described as the overall ratio of the *ERBB2* to *ZNF217* signals. Its cutoff is set at 1.5 and maintains high significance. That makes P3 the most valuable for current routine diagnostics. Additionally, ratio-based FISH predictors are advantaged as they are less dependent on the thickness of slides or nuclear truncation (see additional Supplementary Technical Notes). However, the combination of all three predictors (P1–P3) added the utmost prognostic value in our study.

In conclusion, we see great advantage in analysing several biomarkers in a single assay. This can be demonstrated by multi-colour FISH in comparison to single marker approaches, for example, investigation of *p53*, *MDM2* and *VEGF* in EAC (Reid *et al*, 2001; Cavazzola *et al*, 2009; Prins *et al*, 2012; Renouf *et al*, 2013). An extensive whole genome analysis from the OCCAM group detected *DCK*, *PAPSS2*, *SIRT2* and *TRIM44* as potential prognostic biomarkers on the transcriptional and immunohistochemical level (Peters *et al*, 2010). Taking into account that tumour genetics and gene expressions interfere in a complex manner, FISH is an ancillary approach investigating prognosis in EAC. Our multi-colour-FISH assay is easy to use, ready for clinical application on FFPE-material, and combines the sensitivity, specificity and diagnostic value of *CDKN2A*, *ZNF217*, *ERBB2* and *MYC* and the prognostic value of *ZNF217* and *ERBB2* into predictors like P1, P2 and P3. In particular the predictor P3 seems to be appropriate for diagnostic feasibility (Supplementary Figure 1B). In addition, it is important to note that our predictors P1–P3 were not planned *a priori* and, therefore, the definitions and their results might require validation in an independent study. All predictors might provide an additive opportunity for early intervention and improved outcome in patients with EAC (Reid *et al*, 2001; Quaroni *et al*, 2009).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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