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Systematic review and meta-analysis of immunohistochemical prognostic biomarkers in resected oesophageal adenocarcinoma

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Background: Oesophageal adenocarcinoma (OAC) is one of the fastest rising malignancies with continued poor prognosis. Many studies have proposed novel biomarkers but, to date, no immunohistochemical markers of survival after oesophageal resection have entered clinical practice. Here, we systematically review and meta-analyse the published literature, to identify potential biomarkers.

Methods: Relevant articles were identified via Ovid medline 1946–2013. For inclusion, studies had to conform to REporting recommendations for tumor MARKer (REMARK) prognostic study criteria. The primary end-point was a pooled hazard ratio (HR) and variance, summarising the effect of marker expression on prognosis.

Results: A total of 3059 articles were identified. After exclusion of irrelevant titles and abstracts, 214 articles were reviewed in full. Nine molecules had been examined in more than one study (CD3, CD8, COX-2, EGFR, HER2, Ki67, LgR5, p53 and VEGF) and were meta-analysed. Markers with largest survival effects were COX-2 (HR = 2.47, confidence interval (CI) = 1.15–3.79), CD3 (HR = 0.51, 95% CI = 0.32–0.70), CD8 (HR = 0.55, CI = 0.31–0.80) and EGFR (HR = 1.65, 95% CI = 1.14–2.16).

Discussion: Current methods have not delivered clinically useful molecular prognostic biomarkers in OAC. We have highlighted the paucity of good-quality robust studies in this field. A genome-to-protein approach would be better suited for the development and subsequent validation of biomarkers. Large collaborative projects with standardised methodology will be required to generate clinically useful biomarkers.

Oesophageal adenocarcinoma (OAC) is one of the fastest rising cancers in men in the UK and now accounts for more than 5700 new cases per year (Rouvelas *et al*, 2005; National Oesophago-Gastric Cancer audit, 2013; Peng *et al*, 2013). There is an urgent need to identify prognostic subtypes of OAC as despite potentially curative treatment, 5-year survival is only 35–40% and current pathological prognostic markers are unreliable. The systematic identification of molecular prognostic markers would allow for improved prognostic information for the patient and a better understanding of the underlying tumour biology. This will help in the logical development of novel targeted therapies for these patients.

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OAC is an area of unmet research need and has been highlighted as a research priority by governments and in the strategies of large funding bodies (Chen *et al*, 2012). Predicting prognosis after potentially curative surgery for OAC is difficult and inaccurate. It is currently based on internationally accepted tumour staging (Tumour Node Metastasis), with the addition of other important pathological criteria including resection margin status, the presence of vascular or neural invasion and of signet ring histology (Sobin *et al*, 2010; O'Neill *et al*, 2013; Schoppmann *et al*, 2013b; Yendamuri *et al*, 2013). It is now well recognised that in addition to pathological scoring systems other features of tumours, for example, constituents of the microenvironment, immune infiltration and response to neoadjuvant chemotherapy (NAC), are critical to tumour progression (Courrech Staal *et al*, 2011). A good response to NAC has been consistently shown to predict for better outcome (Mandard *et al*, 1994; Fareed *et al*, 2009; Noble *et al*, 2013). This is being used by some clinicians to determine adjuvant treatment protocols, but this can only be accurately determined after oesophageal resection, and even in those patients where a poor local tumour response to NAC is observed, a proportion may benefit from systemic treatment by virtue of nodal downstaging (Noble *et al*, 2013).

Recent advances in OAC have focused on early diagnosis and understanding the genetic landscape of the disease (Kadri *et al*, 2010; Liu *et al*, 2014). Next-generation sequencing studies may ultimately lead to molecular phenotype therapeutics in OAC but the widespread application of near-patient whole-genome sequencing at the time of diagnosis is likely to be many years away (Dulak *et al*, 2013; Weaver *et al*, 2014). Immunohistochemical (IHC) analysis of differentially expressed proteins is currently superior to DNA-based biomarkers in terms of availability, labour requirements, determining cellular localisation of a marker and takes into consideration post-transcriptional processing. IHC is routinely used in pathology laboratories to differentiate between subtypes of oesophageal cancer and guides targeted therapy with biological agents in a range of cancer types (DiMaio *et al*, 2012; Ward *et al*, 2013). A number of IHC-based prognostic biomarkers have been reported in OAC, but none have entered clinical practice (Waterman *et al*, 2004; Ong *et al*, 2013).

In this systematic review and meta-analysis, we sought to carefully assess the available published literature on prognostic IHC biomarkers of survival in the resected tumour from patients with OAC. The objective of the study was to identify prognostic markers to provide improved risk stratification in addition to highlighting molecular targets that could offer strategies for the development of novel therapies for patients with OAC.

MATERIALS AND METHODS

Identification of literature. The aim of the search was to identify all primary literature examining IHC markers of prognosis in OAC. A search strategy combining Plain Text and Medical Subject Heading terms was developed:

(1) (esoph* OR oeoph*) AND (carcinoma OR adenocarcinoma OR cance* OR neoplas* OR tumo*) AND (Prognos* OR Surviv* OR Mortal*) AND (protei* OR marke* OR biomark*)

OR

(2) Oesophageal Neoplasms/AND (prognosis/or disease-free survival (DFS)/OR Survival/OR mortality/or 'cause of death'/or fatal outcome/or survival rate/) AND (biological markers/or exp antigens, differentiation/or genetic markers/or exp tumour markers, biological/OR genes/or exp genes, neoplasm/) AND Adenocarcinoma/.

The search term was entered into Ovid MEDLINE (1946 to November 2013) without limits and 3059 articles were returned (Figure 1). Existing systematic reviews and reference lists were crosschecked for studies missed by the search term. In cases where studies were derived from the same data set, the more recent or most complete article was retained. Only published results are included in this review. The Preferred Reporting Items for Systematic Review and Meta-Analysis were utilised (Moher *et al*, 2009).

Screening. Two independent reviewers (FN and LMM) examined 3059 study titles. From these study titles, 695 abstracts were brought forward as relevant to this study. On review of the abstracts, potentially eligible full-text articles were retrieved with relevant appendices and Supplementary Information.

Eligibility and data extraction. Full-text articles were reviewed against quality criteria (Table 1) derived from the REporting recommendations for tumour MARKer prognostic studies criteria (REMARK – published guidelines for quality reporting in IHC-based tumour biomarker studies; McShane *et al*, 2005).

For relevant articles, variables were extracted. These included the following: first name author, IHC target, year of publication, number of cases, primary antibody used, dilution of primary antibody, reference group for statistical analysis, number of positive stained cases, univariate or multivariate analysis, hazard ratio (HR), 95% confidence interval (CI), *P*-value, location of stain and type of survival (overall survival (OS), cancer-specific survival (CSS) or DFS. Only CSS and OS were pooled in the meta-analysis).

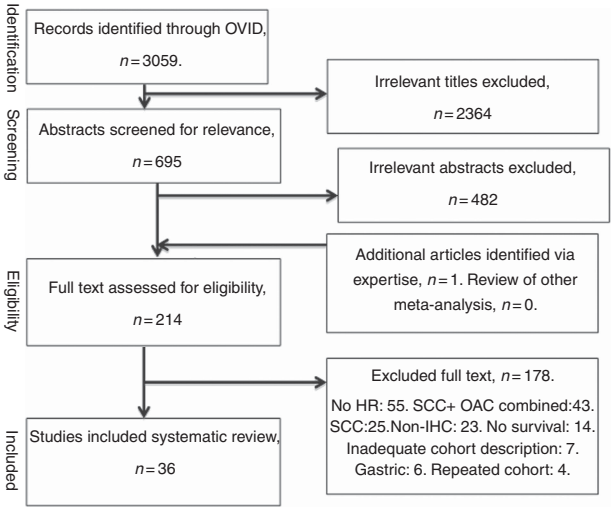

Figure 1. PRISMA flow chart illustrating stages of selection of final articles for meta-analysis.

Table 1. Inclusion criteria adapted from REMARK criteria, utilised at eligibility stage of selection

1	Prospective or retrospective cohort design with a well-defined study population with justification for excluded cases
2	Assay of primary/neoadjuvant resected OAC tumour specimens
3	Clear description of methods for tissue handling and IHC, including antigen retrieval, selection, and preparation of both primary and secondary antibodies, as well as visualisation techniques
4	A clear statement on the choice of positive and negative controls and on the outcome of the assay to ensure that the primary antibody used was a well-validated reagent
5	Statistical analysis using univariate or multivariate hazards modelling
6	Reporting of the resulting HRs including 95% CIs and *P*-values

Abbreviations: CI = confidence interval; IHC = immunohistochemical; HR = hazard ratio; OAC = oesophageal adenocarcinoma; REMARK = REporting recommendations for tumor MARKer.

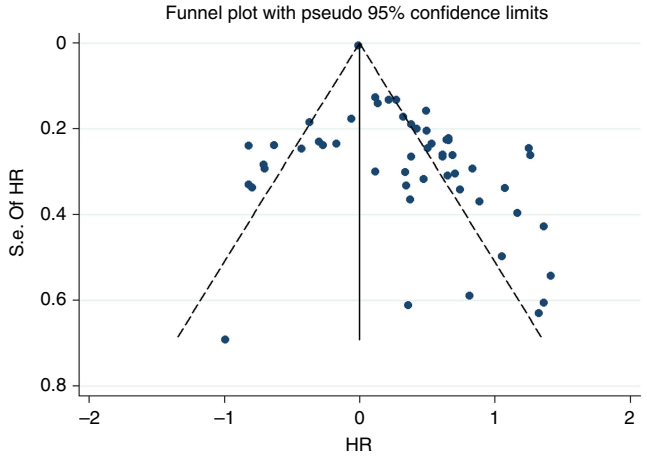

Figure 2. Funnel plot showing publication bias for the 58 included studies providing HR and CI. Plotted points are frequently seen away from the '0'.

Synthesis and statistical analysis. Both univariate and multivariate results were considered for the meta-analysis, with univariate analysis used preferentially when both were available. Univariate analysis was preferred due to the variability of analysis used (univariate $n=3$; multivariate $n=5$; and both $n=27$). In addition, there was variability in the method and variables used to derive the final multivariate model making comparative analysis across studies biased (Supplementary Figure 1). Of 36 studies included in the review, 27 (75%) stated HR and CI derived by multivariate analysis. Of these, the method used to make the model was described in only nine (33%). The method used to make the model varied as follows: entering all variables on univariate analysis into the model in 6 (22%); using backward stepwise regression in 2 (7%) and it was impossible to accurately assess the method used in 19 (70%). The number of variables used to create the multivariate model varied and was anywhere between 3 and 13. Where studies considered opposite degrees of expression, the inverse HR and CI was calculated to give results for high expression. For biomarkers analysed in more than one study, HR and CIs were entered into a random-effects model on Stata Statistical Software, SE 12 (StataCorp LP, College Station, TX, USA). The synthesised HR is reported as increase of risk of death from OAC within the individual study's reference group with $HR>1$ indicating increased risk of death, and $HR<1$ indicating decreased risk of death.

The heterogeneity of results between studies was assessed using I2 statistics (a measure of consistency of results between studies) with increasing heterogeneity implying less utility in generalising across studies (Higgins *et al*, 2003). Sensitivity analysis was carried out by removing individual studies from the meta-analysis and assessing the effect on the pooled result. Presence of publication bias was formally evaluated using funnel plots (Figure 2) (Sterne *et al*, 2001).

RESULTS

Excluded studies. Of the 3059 articles returned, 2364 were excluded on review of title and 482 on examination of abstract, leaving 214 articles considered relevant. Crosschecking of existing systematic review reference lists revealed no further relevant articles (Vallbohmer and Lenz, 2006; Ong *et al*, 2010; Chan *et al*, 2012; Chen *et al*, 2012, 2013; Peng *et al*, 2013; Gowryshankar *et al*, 2014).

Upon careful review of the 214 articles against the REMARK inclusion criteria, 56 did not provide a HR, 43 combined OAC and SCC subtypes for statistical analysis, 25 examined only SCC, 23 used non-IHC methodology, 14 did not examine survival, 7 had inadequate cohort description, 6 examined gastric cancer and 4 repeated use of a cohort. This left only 36 articles that conformed to REMARK inclusion criteria.

Included studies. 26 individual research centres contributed to the 36 articles. 20 studies (56%) reported cohorts of patients who underwent surgery only; in 6 studies (17%) the authors reported that some patients had undergone neoadjuvant therapy and in 10 studies (28%) no information were given regarding preoperative treatment. Of the six studies, where some kind of neoadjuvant treatment was reported, this consisted of chemoradiotherapy in five (14%) and chemotherapy in three (8%). The percentage of patients who had undergone neoadjuvant therapy varied from 8 to 100%. The specific neoadjuvant treatment regimes that were used were only reported in three out of six (50%) studies. Little overlap in methodology was seen with every centre using different antibodies at different dilutions or different scoring systems. Variable cohort sizes were used, ranging from 24 to 259 cases. 50 HRs, CIs and relevant variables were extracted from these studies. Extracted data is reported in Table 2 with biomarkers grouped according to the hallmark of cancer with which a functional role for that molecule has been most closely attributed (Figure 3; Hanahan and Weinberg, 2011).

19 of the 36 articles examined one or more of the same nine molecules, making them suitable for meta-analysis. Upon pooling studies, six of the nine molecules showed prognostic significance: COX-2, CD3, CD8, p53, EGFR and HER2 in order of HR, with LgR5, VEGF and Ki67 not reaching significance. Forrest plots are shown in Figure 4.

In three of the studies included in the meta-analyses, only multivariate analysis was stated. Where sensitivity analysis was not possible due to lack of appropriate, robust literature, agreement on prognostic value was considered with other studies on OAC.

COX-2. COX-2 is a rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins and has multiple functions in immune evasion, angiogenesis and proliferation. COX-2 is consistently detected with varying expression in OAC (Lagorce *et al*, 2003). Subsequently, inhibitors of COX-2 have been shown to be protective of progression from Barrett's oesophagus to OAC, and have shown some promise in improving prognosis when used alongside NAC (Corley *et al*, 2003; Tuynman *et al*, 2005).

Three studies, consisting of a total of 382 patients, contributed to quantify the effect of COX-2, which was found to correlate negatively with prognosis (Buskens *et al*, 2002; Bhandari *et al*, 2006; Prins *et al*, 2012). Although consistent overexpression is noted in OAC, differences in cutoff values for staining positivity, and variability in numbers of positive staining cases between studies are seen here (27% and 79% positive) (Buskens *et al*, 2002; Prins *et al*, 2012). Within other prognostic studies on OAC, not providing HR, both significant (three studies, $n=194$) and non-significant (three studies, $n=139$) results have been reported (Lagorce *et al*, 2003; France *et al*, 2004; Kulke *et al*, 2004; Heeren *et al*, 2005; Mobius *et al*, 2005; Tuynman *et al*, 2008).

CD3. CD3+ cells are mature T lymphocytes and quantification of CD3+ has been commonly used to evaluate immunological response against solid tumours (Dahlin *et al*, 2011). Two studies identified CD3 as an independent predictor of improved survival in OAC (Rauser *et al*, 2010; Zingg *et al*, 2010). Methods of exploration varied; with one study using an automated scoring system across 10 random high-power fields *vs* central CD3+ lymphocyte count (Rauser *et al*, 2010; Zingg *et al*, 2010). However, the studies show good agreement ($I^2=0.00\%$) and similar weighting on meta-analysis.

Table 2. Extracted data from biomarker articles

	IHC target	*N*	Positive cases	Primary antibody (dilution)	Reference group	Uni or Multivariate	Survival	HR	CI	*P*-value
Evasion of growth supressors										
Evangelou *et al*, 2008	Caspase-3	35	NA	Cell signalling Bioline (1 : 100)	Labelling index	Univariate	DFS	<0.01[a]	0.298–3.302[a]	0.990
Evangelou *et al*, 2008	E2f-1	35	23 (66%)	Santa Cruz (1 : 100)	>35% cells labelling index	Univariate	DFS	3.908	0.153–0.992[a]	0.048
Cavazzola *et al*, 2009	p53	38	24 (52%)	Sigma Biosciences (1 : 100)	>10%	Multivariate	CSS	1.429	0.429–4.725	0.514
Moskaluk *et al*, 1996	p53	88	40 (45%)	Novocastra (NA)	>50%	Univariate	OS	1.46	0.87–2.46	0.155
Madani *et al*, 2010	p53	142	48 (34%)	Dako (1 : 50)	+2 to +8	Univariate	OS	1.64	1.1–2.45	0.014
Sustained Proliferative signalling										
Langer *et al*, 2006	EGFR	137	72 (53%)	Cytomed (1 : 60)	>10% cells +ve.	Univariate	OS	0.99	0.98–1.00[a]	0.039
Ong *et al*, 2013	EGFR	359	36 (10%)	Novocastra (1 : 10)	+2, +3	Univariate	OS	1.520	1.03–2.26	0.040
Ong *et al*, 2013[b]	EGFR	663	NA	Novocastra (1 : 10)	+1	Univariate	OS	0.83	0.66–1.04	.10
			100 (15%)		+2			1.41	.05–1.91	0.02
					+3			0.94	0.58–1.52	0.80
Wang *et al*, 2007	EGFR	103	33 (32%)	Dako (NA)	>5%	Univariate	OS	1.93	1.24–3.02	0.004
Nakamura *et al*, 1994	HER2	62	15 (19%)	Boehringer Mannheim Biochemica (NA)	+2	Univariate	OS	4.100	1.4–11.8	0.015
Yoon *et al*, 2012	HER2	708	119 (17%)	Herceptest (NA)	+2, +3	Univariate	OS	0.760	0.59–0.96	0.024
Phillips *et al*, 2013	HER2	135	31 (23%)	Ventana (NA)	+2, +3	Multivariate	OS	0.840	0.53–1.33	0.470
Dutta *et al*, 2012	Ki67	98	NA	Dako (1 : 50)	Slidepath scoring algorithm. Tertiles.	Univariate	CSS	1.460	1.01–2.12	0.048
Evangelou *et al*, 2008	Ki67	35	NA	Dako (1 : 100)	<35% cells +ve, labelling index	Univariate	DFS	3.757	0.986–11.68	0.050
Falkenback *et al*, 2008	Ki67	59	50 (85%)	Dako (1 : 1000)	0–10%	Univariate	CSS	3.900	1.7–9.1	<0.001
Tuynman *et al*, 2008	MET	145	78 (54%)	Zymed (1 : 100)	+2, +3	Univariate	DFS	2.300	1.3–4.1	0.004
Prins *et al*, 2013	p-mTOR	147	29 (19.7%)	Cell Signalling Technology (1 : 50)	2+, 3+	Univariate	CSS	1.648	1.019–2.664	0.042
Ong *et al*, 2013	PAPSS2	337	216 (64%)	Abcam (1 : 600)	+2, +3	Univariate	OS	1.240	0.96–1.61	0.100
Schoppmann *et al*, 2012	pSTAT3	179	72 (40%)	Cell Signalling Technology (1 : 100)	>10 (>median)	Univariate	OS	1.982	1.186–3.311	0.050
Bettstetter *et al*, 2013	PTEN	117	101 (86%)	Cell Signalling Technology (1 : 50)	>75%	Multivariate	OS	0.451	0.233–0.873	0.018
Escape from immune surveillance										
Loos *et al*, 2011	B7-H1	101	74 (73%)	Abcam (NA)	> +4 (Intensity + proportion of cells)	Univariate	OS	2.92	1.50–5.66	<0.001
Rauser *et al*, 2010	CD3	99	57 (58%)	NeoMarkers (1 : 100)	>2.0 Labelling indices	Univariate	OS	0.49	0.28–0.85	0.012
Zingg *et al*, 2010	CD3 central	105	NA	Dako (1 : 50)	>563 (>median count)	Univariate	OS	0.53	0.33–0.84	0.008
Zingg *et al*, 2010	CD4 central	105	58 (55%)	NeoMarkers (1 : 40)	>30 (>median count)	Univariate	OS	0.74	0.47–1.16	0.187
Zingg *et al*, 2010	CD25 central	105	NA	NeoMarkers (1 : 10)	>33 (>median count)	Univariate	OS	0.76	0.48–1.22	0.262
Zingg *et al*, 2010	CD8 central	105	51 (49%)	Dako (1 : 50)	>225 (>median count)	Univariate	OS	0.44	0.27–0.69	<0.001
Dutta *et al*, 2012	CD8 tertiles	98	NA	Dako (1 : 100)	Slidepath scoring algorithm	Univariate	CSS	0.69	0.48–0.99	0.048
Rauser *et al*, 2010	CD45RO	110	93 (85%)	Dako (1 : 1200)	>0.9 Labelling indices	Univariate	DFS	0.44	0.23–0.84	0.013
Dutta *et al*, 2012	CD68	98	NA	Dako (1 : 200)	Slidepath scoring algorithm. Tertiles.	Univariate	CSS	1.38	0.99–1.94	0.061
Zingg *et al*, 2010	FoxP3 central	105	46 (43%)	eBioscience (1 : 50)	>117 (>median count)	Univariate	OS	0.65	0.40–1.05	0.079
Deregulation of cellular energetics										
Birner *et al*, 2011	CAIX	182	85 (47%)	Abcam (1 : 1000)	>median score (20 out of score 0–300)	Univariate	OS	1.844	1.11–3.08	0.007
Tumour promoting inflammation										
Wang *et al*, 2006	ANXA1	104	41 (39%)	BD Biosciences (1 : 100)	>25%	Univariate	OS	1.930	1.25–2.99	0.003
Bhandari *et al*, 2006	COX-2	90	NA	Cayman Chemical (1 : 100)	>200	Univariate	CSS	3.530	2.11–5.89	<0.001
Buskens *et al*, 2002	COX-2	145	115 (79%)	Cayman Chemical (1 : 200)	+2, +3	Univariate	OS	3.200	1.5–7.1	0.002
Prins *et al*, 2012	COX-2	147	39 (27%)	Cayman Chemical (1 : 100)	+3	Univariate	OS	1.700	1.07–2.69	0.023
Tuynman *et al*, 2008	COX-2	145	78 (54%)	Cayman Chemical (1 : 200)	+2, +3	Univariate	DFS	1.400	0.8–2.6	0.234
Evasion of apoptosis										
Ong *et al*, 2013	DCK	355	126 (36%)	Lifespan Biosciences (1 : 10)	+2, +3	Univariate	OS	0.980	0.75–1.28	0.860
Chandra *et al*, 2002	GSTπ	15	6 (40%)	Vector laboratories	3+	Univariate	DFS	2.250	0.71–7.17	0.350
Ong *et al*, 2013	MTMR9	356	88 (25%)	Novus (1 : 350)	+2, +3	Univariate	OS	1.140	0.87–1.51	0.340
Ong *et al*, 2013	NEIL2	357	198 (55%)	Sigma-Aldrich (1 : 50)	+2, +3	Univariate	OS	1.120	0.87–1.43	0.390
Ong *et al*, 2013	SIRT2	359	156 (44%)	Atlas Antibodies (1 : 100)	+2, +3	Univariate	OS	1.310	1.03–1.67	0.030
Ong *et al*, 2013[b]	SIRT2	663	NA	Atlas Antibodies (1 : 100)	2	Univariate	OS	1.69	1.10–2.60	0.02
			290 (44)		1			1.81	1.24–2.64	<0.01
					0			1.37	0.96–1.97	0.08
Ong *et al*, 2013	WT1	358	19 (5%)	Dako (1 : 800)	+2, +3	Univariate	OS	0.710	0.39–1.30	0.270

Table 2. (Continued)

	IHC target	*N*	Positive cases	Primary antibody (dilution)	Reference group	Uni or Multivariate	Survival	HR	CI	*P*-value
Inducing angiogenesis										
Dutta *et al*, 2012	CD34	98	NA	Dako (1 : 150)	Slidepath scoring algorithm. Tertiles.	Univariate	CSS	0.94	0.67–1.34	0.736
Cavazzola *et al*, 2009	VEGF	38	22 (48%)	Santa Cruz (1 : 400)	>30% cells stained	Multivariate	CSS	0.369	0.095–1.436	0.115
Prins *et al*, 2012	VEGF	143	90 (63%)	R&D systems (1 : 50)	> +1	Univariate	CSS	1.900	1.22–2.96	0.005
Xie *et al*, 2013	VEGF-C	128	96 (75%)	Santa Cruz (1 : 50)	>0.18 Mean optical density	Multivariate	DFS	3.491	2.156–5.652	<0.0001
Tissue invasion and metastasis										
Hector *et al*, 2010	AXL	92	56 (61%)	R&D systems (1 : 100)	+3	Multivariate	OS	1.91	1.04–3.49	0.036
Falkenback *et al*, 2008	E-cadherin	59	44 (75%)	Dako (1 : 100)	Absent/reduced	Univariate	CSS	3.900	1.2–12.9	0.017
Becker *et al*, 2010	LgR5	24	NA	MBL Internation Co (1 : 50)	>5 (Intensity + proportion)	Univariate	OS	2.860	1.08–7.61	0.040
von Rahden *et al*, 2011	LgR5	60	51 (85%)	Abcam (NA)	>15%	Univariate	OS	2.418	1.17–4.99	0.033
Grimm *et al*, 2010	MMP-1	60	33 (55%)	Hiddenhausen (NA)	>46%	Univariate	OS	1.453	0.7101–2.9718	0.307
Streppel *et al*, 2012	Mucin 16	95	66 (70%)	Abcam (1 : 200)	Moderate/Diffuse	Univariate	NA	1.410	0.734–2.709	0.303
Wijnhoven *et al*, 2005	p120	96	65 (67%)	Transduction laboratories (1 : 1000)	<90%	Multiivariate	OS	2.100	1.1–4.2	0.006
Schoppmann *et al*, 2013b	Podoplanin (lymphovascular invasion)	194	81 (42%)	Ventana (NA)	Tumour cluster in podoplanin decorated space	Univariate	OS	1.863	1.086–0.195[a]	<0.01
Schoppmann *et al*, 2013a	Podoplanin (CAFs)	200	118 (59%)	Venatana (1 : 300)	>10% CAFs	Univariate	OS	1.843	1.097–3.096	0.001
Birner *et al*, 2012	RKIP	179	NA	Upstage/Millipore (1 : 1000)	>80 out of score 0–300 (>median score)	Multivariate	DFS	0.494	0.278–0.878	0.016
Ong *et al*, 2013	TRIMM44	349	197 (56%)	Protein Tech group (1 : 50)	+2, +3	Univariate	OS	1.310	1.01–1.70	0.040
Ong *et al*, 2013[b]	TRIMM44	655	NA	Protein Tech group (1 : 50)	+1	Univariate	OS	1.46	0.89–2.44	0.4
			442 (67%)		+2			1.59	0.96–2.63	0.07
					+3			1.94	1.09–3.44	0.02
Laerum *et al*, 2012	uPAR (Cancer cells)	60	37 (62%)	Raised in-house	+2, +3, +4	Univariate	OS	2.020	1.11–3.66	0.021
Laerum *et al*, 2012	uPAR (Macrophages)	60	57 (95%)	Raised in-house	+2, +3, +4	Univariate	OS	1.120	0.62–2.01	0.710
Laerum *et al*, 2012	uPAR (myofibroblasts)	60	39 (65%)	Raised in-house	+2, +3, +4	Univariate	OS	1.600	0.86–2.99	0.140

Abbreviations: CI = confidence interval; CSS = cancer-specific survival; DFS = disease-free survival; IHC = immunohistochemical; HR = hazard ratio; OS = overall survival.
[a]Values as documented in original articles. Incorrect values excluded from meta-analysis.
[b]Validation cohorts from same study not used in meta-analysis due to differences in cut-offs.

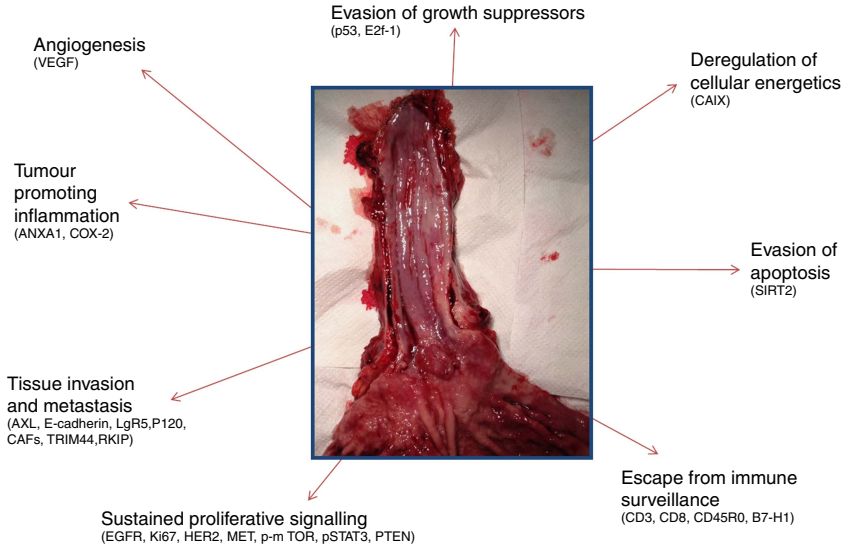

Figure 3. Statistically significant prognostic biomarkers from at least one study in resected oesophageal adenocarcinoma covering all hallmarks of cancer.

CD8. CD8 is a marker of cytotoxic T cells. CD8 + cells kill cancer cells via release of granzyme and perforin or via Fas ligand presentation (Owen *et al*, 2013). This is an area of considerable interest with trials in a number of solid organ cancers examining strategies to enhance tumour-cell killing. The discovery of the role of PD-L1 on tumour cells and its interaction with the PD-1 receptor on cyctotoxic T cells leading to immune cell exhaustion have led to the development of antibodies targeting both the

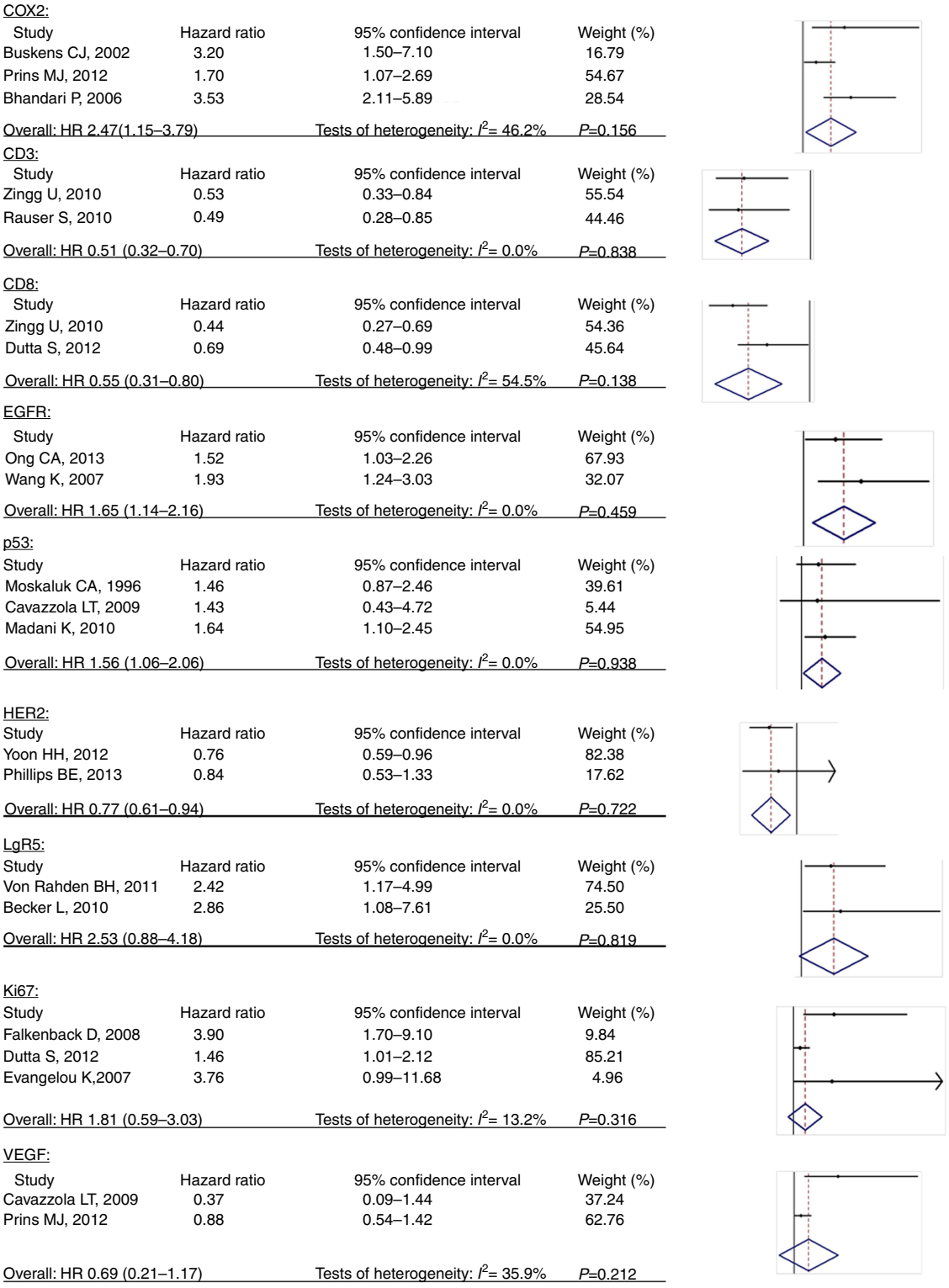

Figure 4. Forest plots with associated hazard ratio (HR) and 95% confidence interval. Weights calculated using a random effects model. HR > 1 implies worse survival with overexpression, HR < 1; improved survival (vertical black line indicates HR of 1; red vertical dotted line indicates overall HR). A full colour version of this figure is available at the *British Journal of Cancer* journal online.

receptor and its ligand (McDermott and Atkins, 2013). Two studies were pooled examining CD8 (cytotoxic, T-cell effector), comprising a total of 203 cases (Zingg *et al*, 2010; Dutta *et al*, 2012). Moderate heterogeneity is observed ($I^2 = 54.5\%$). Methodological differences may be the cause of the heterogeneity, with observation of increasing CD8+ count across three tertiles, *vs* CD8 with a cutoff set for high *vs* low expression (Zingg *et al*, 2010; Dutta *et al*, 2012).

EGFR. EGFR is a receptor tyrosine kinase, shown to have effects on cancer differentiation, proliferation, invasion and metastasis (Grandis and Sok, 2004). EGFR targeting is used in the treatment

of colorectal cancer and non-small cell lung cancer (Mahipal *et al*, 2014). A total of 642 patients were pooled from two studies (Wang *et al*, 2007; Ong *et al*, 2013). Combined, an overall, slight-negative, prognostic effect of EGFR overexpression was found. This is in agreement with other studies in OAC that were unsuitable for meta-analysis (Mukaida *et al*, 1991; Yacoub *et al*, 1997; Lennerz *et al*, 2011). This effect on prognosis with overexpressed EGFR has been noted in both colorectal cancer and gastric adenocarcinoma (Rego *et al*, 2010; Hong *et al*, 2013).

p53. p53 acts as a hub for multiple intra-cellular surveillance systems, constantly reporting on cellular integrity. When stress is detected, a damaged cell can initiate DNA repair, senescence and/or apoptosis. TP53 mutation can increase protein stability, meaning IHC detection correlates with mutation (Bellini *et al*, 2012). However IHC does not account for all mutations, with between 52% and 80% agreement between IHC and PCR with truncating and missense mutations (Bian *et al*, 2001). *TP53* is the most commonly mutated gene in OAC and has recently been found to have a mutational frequency that would distinguish between disease stages and thus identify progression towards malignancy (Weaver *et al*, 2014).

Eleven studies examining p53 were reviewed in full (Flejou *et al*, 1994; Casson *et al*, 1995; Sauter *et al*, 1995; Moskaluk *et al*, 1996; Hardwick *et al*, 1997; Casson *et al*, 1998, 2003; Jiao *et al*, 2003; Heeren *et al*, 2004; Cavazzola *et al*, 2009; Madani *et al*, 2010). Only three of these were suitable for inclusion in the systematic review and subsequent meta-analysis, containing a total of 268 patients and showing a pooled effect of worse prognosis with increased expression (Moskaluk *et al*, 1996; Cavazzola *et al*, 2009; Madani *et al*, 2010). Good agreement is seen between the three included studies. However, five other studies not included in the review failed to reach significance, suggesting that the prognostic value of p53 may not be as obvious as the meta-analysed results suggest (Duhaylongsod *et al*, 1995; Coggi *et al*, 1997; Hardwick *et al*, 1997; Langer *et al*, 2006; Falkenback *et al*, 2008).

HER2. HER2 exhibits extensive homology with EGFR, frequently dimerising with it or another member of the EGFR family HER3 (Wolf-Yadlin *et al*, 2006). HER2 is overexpressed in a number of cancers and undergoes a ligand-independent activation, with consequent downstream signals involved in proliferation and migration (Wolf-Yadlin *et al*, 2006). HER2 provides a target for the monoclonal antibody trastuzumab, which has proven efficacy in breast and gastric cancer treatment (Hynes and Lane, 2005; Bang *et al*, 2010). HER2 targeting in OAC is being assessed in the feasibility arm of the MRC STO3 clinical trial (Okines *et al*, 2013).

Two studies were suitable for pooling, showing an OS benefit with overexpression of HER2 (Nakamura *et al*, 1994; Yoon *et al*, 2012; Phillips *et al*, 2013). Both studies used the ToGA trial protocol to assess HER2 overexpression, including FISH analysis of *ErbB2* gene amplification. This effect has failed to be reproduced by smaller studies, potentially as a result of under powering (Polkowski *et al*, 1999; Reichelt *et al*, 2007; Hu *et al*, 2011; Thompson *et al*, 2011). The overall protective effect seen here is in contrast to studies investigating OAC using techniques other than IHC, where a negative prognostic effect is noted, as well as in breast cancer, where a dramatically worse prognosis is seen with overexpression (Andrulis *et al*, 1998; Chan *et al*, 2012).

LgR5. The R-spondin receptor Lgr5 is a stem cell marker in multiple organs in mice and humans. Single Lgr5 stem cells derived from the intestine can be cultured to build epithelial structures that retain hallmarks of the *in vivo* epithelium (Sato and Clevers, 2013). In tumours, Lgr5 expression is believed to define cancer stem cells and may have prognostic effects by promoting invasion and metastasis as well as initiating self-renewal pathways (Reya and Clevers, 2005). Despite the vast majority of cancer deaths being attributable to invasion and metastasis, Lgr5 was the only suitable biomarker for meta-analysis with its main function associated with this hallmark of cancer (Becker *et al*, 2010; von Rahden *et al*, 2011).

Lgr5 failed to reach statistical significance as a prognostic marker. This was due to the wide, asymmetric CIs, resulting from under powering with only 84 cases in total across the two studies (Becker *et al*, 2010; von Rahden *et al*, 2011).

VEGF. VEGF is upregulated in response to hypoxia, acting as a key mediator of angiogenesis and affecting vessel permeability, potentially enhancing haematogenous dissemination (Hicklin and Ellis, 2005). Two studies contributed to VEGF meta-analysis, with a total of 181 patients, producing a non-significant effect on survival (Cavazzola *et al*, 2009; Prins *et al*, 2012). Again, few studies have examined prognosis and angiogenesis, with contradictory results seen in small cohorts (Couvelard *et al*, 2000; Saad *et al*, 2005). With emerging targeted therapies, further work will be required to confirm whether VEGF is a true driver of cancer aggressiveness (Shah *et al*, 2011).

Ki67. Despite the common use of Ki67 to index cellular proliferation, its biological function in the tumour remains elusive. It seems to co-localise with ribosomal RNA during mitosis suggesting a role in protein synthesis and, more recently, chromatin remodelling (Bullwinkel *et al*, 2006).

Here, three studies were pooled, comprising a total of 192 patients (Evangelou *et al*, 2008; Falkenback *et al*, 2008; Dutta *et al*, 2012). A non-significant result was observed. Again, this could be due to a combination of asymmetrical wide CIs in two studies, combined with marginal prognostic value in the other. In breast cancer, increased cellular proliferation index has been studied as a negative prognostic marker and in directing use of chemotherapy against rapidly dividing tumours (Martin *et al*, 2004; de Azambuja *et al*, 2007; Yerushalmi *et al*, 2010). However, Ki67 expression is understudied in OAC, and prognostic significance remains inconclusive.

Publication bias. Within the 214 relevant articles, 92 of these provided HRs and statistical significance, 52 (57%) of these provided non-significant results. This is in contrast to the final 36 articles that met REMARK inclusion criteria, where only six (18%) centred on non-significant results. Asymmetry was noted when all data was viewed on a funnel plot (Figure 2) suggesting positive publication bias.

DISCUSSION

Previous meta-analysis of oesophageal cancer examining individual molecules of prognosis have combined OAC and SCC in addition to using different investigational techniques for analysis (Vallbohmer and Lenz, 2006; Ong *et al*, 2010; Chan *et al*, 2012; Chen *et al*, 2012, 2013; Peng *et al*, 2013; Gowryshankar *et al*, 2014). There is consensus that OAC and SCC should be considered as separate biological entities and current clinical trials in oesophageal cancer reflect this approach. To date, this is the first meta-analysis that has synthesised the literature associated with all IHC markers solely in resected OAC. Using a validated prognostic marker-reporting tool to inform our strict inclusion and exclusion criteria, we identified 36 high-quality articles providing reliable HRs and CIs (McShane *et al*, 2005). From these articles, nine markers were suitable for meta-analysis and of these six markers showed significant correlation with survival. These markers were COX-2, CD3, CD8, HER2, EGFR and p53. Several other molecules have been assessed in good quality studies that met the REMARK inclusion criteria, but do not have a second study available for pooling. Of particular interest, MET, B7-H1, CAIX, ANXA1 and

VEGF-C all showed significant, highly prognostic effects in cohorts containing over 100 cases but still require validation and/or elucidation of the underlying biology.

A number of the molecules identified in this review are related to emerging therapies. Four of the nine meta-analysed markers (COX-2 – celecoxib, EGFR – gefitinib, HER2 – trastuzumab and VEGF – bevacizumab) focussed on molecules with targeted therapeutics either already in use or in development, and two lymphocyte markers representing the presence of effectors of anti-tumour immunity, which can be induced by new therapies (Zhang *et al*, 2003; Galon *et al*, 2006; Ekman *et al*, 2008; Mei *et al*, 2014; Ward *et al*, 2014). As well as new therapies, there is an increasing interest in the role the cancer microenvironment has in OAC progression (Courrech Staal *et al*, 2011). Here, CD3 and CD8 demonstrate the greatest protective prognostic impact, illustrating the importance of the immune response to OAC. However, IHC analysis of other components of the microenvironment have been largely neglected, for example, only two papers comment on the impact of cancer-associated fibroblasts on prognosis (Laerum *et al*, 2012; Schoppmann *et al*, 2013a).

The most striking observation of this meta-analysis is the scarcity of high-quality articles, with 66% (69 out of 104) of potentially suitable studies not conforming to REMARK criteria. In similar meta-analyses published on the two cancers with worse prognoses than OAC, 83 suitable articles were pooled for pancreatic cancer prognosis, and in lung cancer, enough data were found to analyse 17 markers studied in four or more papers (Zhu *et al*, 2006; Jamieson *et al*, 2011; Peng *et al*, 2013). This suggests that prognostic marker research in OAC is lacking. In addition, despite the majority of patients now receiving some form of neoadjuvant therapy before resection for OAC (Noble *et al*, 2013), we found this to be poorly reported in these studies. It was therefore impossible to make any attempt to discriminate between markers prognostic after primary resection or after neoadjuvant therapy. Future reports should include a detailed description of the types of multimodal treatment given to patients and preferably include an analysis based on these treatment types.

A trend was noticed towards more robust methodology when authors used larger data sets. The largest study identified used independent generation and validation data sets to confirm the prognostic significance of the novel markers SIRT2 and TRIMM44. The analysis was performed in two different patient cohorts from separate centres and was of high quality (Ong *et al*, 2013). However, we were unable to include this study in the meta-analysis because the cutoffs used to assess the HRs in the two cohorts were different. SIRT2 and TRIMM44 require validation using the same methodology and cutoffs in another cohort. Despite this, the study by Ong *et al* (2013) describes a sophisticated approach to the development of a biomarker based on genetic analysis carried through to the protein level. Genome sequencing studies such as the UK ICGC project in OAC (Weaver *et al*, 2014) will deliver more potential markers of prognosis in selected sub-groups and methods such as those described by Ong *et al* (2013) will be required to translate these findings into meaningful clinical outcomes.

Authors who appeared more than once in the 214 initial articles, often adhered to REMARK criteria, and provided log-rank or cox regression hazard calculations. This suggests a gradual uptake of REMARK criteria, since it's inception in 2005. Another potential reason for this poorer reporting in smaller studies may be due to more frequent negative results due to inadequate powering. With an overall reluctance towards negative reporting, it is quite possible that these results are left out as redundant data, with the larger data sets having more positive results, and a greater likelihood of publication.(Kyzas *et al*, 2007)

Limitations. Meta-analysis is able to enhance power that leads to more robust generalisations within a field. However, there are notorious confounding factors (Altman, 2001).

Here, only one study was prospective in design (Madani *et al*, 2010). Retrospective analysis allows potential issues in reporting and selection bias. With differences in multivariate or univariate analysis, CSS or OS, size of cohorts, cutoffs, primary antibody at different dilutions and occasionally radically different numbers of positive staining cases lead to less validity when combining results. Future work will require multi-centre efforts to gather large enough, prospective cohorts to provide robust clarification of truly prognostic markers.

With this meta-analysis we have only included IHC detectable markers of survival. Both IHC and RT-PCR have their own limitations; however, IHC is seen to be the most practical way to assess protein expression in solid cancers, with IHC survival biomarkers well described in other malignancies (Zhu *et al*, 2006; Jamieson *et al*, 2011).

In future work, multivariate modelling will give an insight into interaction between different variables in OAC. In this study, univariate analysis was used preferentially, to limit heterogeneity between methods of producing HRs, as a multivariate HR can be altered by use of different prognostic factors or model types in individual models. In fact, it is likely that a combination of markers will be required to give meaningful prognostic information to an individual patient, perhaps covering multiple Hallmarks of Cancer, rather than considering individual biomarkers in isolation. There are existing data from oesophageal cancer biology to support this strategy (Kadri *et al*, 2010; Peters *et al*, 2010; Liu *et al*, 2014).

CONCLUSION

Current methods have not delivered clinically useful molecular prognostic biomarkers in OAC. We have highlighted the paucity of good-quality robust studies in this field. This may be because little attention has been focused on OAC research compared with other cancers, or perhaps it is an indication of the molecular complexity of the disease that is only just beginning to be appreciated. The development of new and novel biomarkers in OAC will require understanding of this complexity and in this context IHC alone seems inappropriate. A genome to protein approach would be better suited for the development and subsequent validation of biomarkers. Large collaborative projects with standardised methodology will be required to generate clinically useful biomarkers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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