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Systematic or Meta-analysis Studies

The local inflammatory response in colorectal cancer – Type, location or density? A systematic review and meta-analysis

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ABSTRACT

Introduction: The host anti-tumour inflammatory response is a strong prognostic indicator, and tumour infiltrating lymphocytes (TILs) are believed to have a complimentary role alongside TNM assessment in dictating future management. However, there is wide disagreement regarding the most efficacious and cost-effective method of assessment.

Methods: A comprehensive literature search was performed of EMBASE, MedLine and PubMed as well as an assessment of references to identify all relevant studies relating to the assessment of the peri-tumoural inflammatory response or TILs and prognosis in colorectal cancer (CRC). A meta-analysis was performed of 67 studies meeting the REMARK criteria using RevMan software.

Results: Intratumoural assessment of both CD3 and CD8 in CRC were significant for disease-free survival (DFS) (combined HRs 0.46; 95%CI: 0.39–0.54 and 0.54; 95%CI: 0.45–0.65), as well as overall survival (OS) and disease-specific survival (DSS). The same was true for assessment of CD3 and CD8 at the invasive margin (DFS: combined HRs 0.45; 95%CI: 0.33–0.61 and 0.51; 95%CI: 0.41–0.62). However, similar fixed effects summaries were also observed for H&E-based methods, like Klintrup-Makinen grade (DFS: HR 0.62; 95%CI: 0.43–0.88). Furthermore, inflammatory assessments were independent of MSI status.

Conclusion: The evidence suggests that it is the density of a co-ordinated local inflammatory infiltrate that confers survival benefit, rather than any individual immune cell subtype. Furthermore, the location of individual cells within the tumour microenvironment does not appear to influence survival. The authors advocate a standardised assessment of the local inflammatory response, but caution against emphasizing the importance of any individual immune cell subtype.

Introduction

Colorectal cancer (CRC) represents a significant health burden with 1.8 million deaths worldwide in 2018 related to the disease [1]. It has increasingly been recognised over recent decades that whilst TNM staging has significant prognostic value in CRC, it does not take account of the interaction between host and tumour [2]. It has been repeatedly demonstrated that the local host inflammatory response to the tumour has significant positive prognostic value, hence the recent addition of immune checkpoint inhibitors to the therapeutic repertoire for CRC [3]. However, an assessment of the local inflammatory response remains only an optional item on the CRC reporting dataset [4].

There remains a drive towards inclusion in the TNM classification system of an assessment of the local immune response [5,6]. However, one of the barriers to implementing routine clinical assessment of the anti-tumour immune response is the heterogeneity in the methodology employed in identifying and quantifying this reaction. It has not, for example, been comprehensively shown whether a haematoxylin and eosin (H&E)-based assessment or immunohistochemical (IHC) assessment of individual inflammatory cells is superior in terms of prognostic benefit, since a recent review of tumour-infiltrating lymphocytes in solid tumours by the International Immuno-Oncology Biomarkers Working Group concluded that, in colorectal cancer, further work was required to define the “most appropriate balance between simplicity and depth of information” [7].

Assessment of TILs has already been established in the pathology reporting dataset for malignant melanoma, although there is ongoing discussion regarding the method of assessment [8]. Indeed, there has been a move to standardise assessment of TILs in many solid tumours and this would be beneficial to enable identification of those that may

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ben-efit from immunotherapy.

Several H&E methods have been employed in addition to many individual inflammatory cells from both the innate and adaptive immune system. A further area of contention relates to which tumour compartment should be assessed for these individual immune cell subtypes. Menon and colleagues described a basement-membrane or "BM-like structure" that appeared to prevent infiltration of immune cells into the tumour, when staining for antilaminin on IHC [9]. There are others who advocate that those tumours that have a strong intratumoural infiltration, so called "immune hot" tumours, are associated with better prognosis [10]. Therefore, it is important to establish whether there is any evidence of superiority or not of assessing intratumoural vs the invasive margin in terms of prognostication.

Furthermore, the use of tissue microarrays (TMAs) has been employed by many to allow rapid throughput of many tumour specimens simultaneously in addition to reducing inter-sample variations in staining. However, TMAs may limit the location-specific assessment of prognosis. Similarly, differences in quantification of immune cell density ranging from semiquantitative assessment and manual counts to digital pathology-based assessments and automated cell counting has led to difficulty in standardising a method for clinical implementation [11–13].

Microsatellite instability (MSI) must also be accounted for when considering tumour infiltrating lymphocytes. Hereditary MSI, in addition to sporadic cases of MSI, account for around 15% of all colorectal cancers [14]. When testing was first introduced for MSI, it was purely based on genetic assessment, which was expensive. High density of tumour infiltrating lymphocytes was one of the markers used in the screening process to select patients for testing [14], since MSI cancers were known to be immunogenic [15–17] – a feature of MSI tumours that has subsequently been attributed to high neo-antigen load [18]. With the advent of more economical immunohistochemical assessment for loss of DNA-mismatch repair proteins and BRAF, it has become more economical to assess for the presence of MSI in all colorectal tumours to guide adjuvant therapy [19]. Immunotherapies are currently only licensed for use in MSI-high colorectal cancer in view of the poor response of microsatellite stable (MSS) tumours during clinical trial [3]. However, the effects of immunotherapies remain untested in selected individuals with a prominent immune response in MSS CRC. It remains to be seen whether these individuals would derive benefit from adjuvant immunotherapy in this context.

This review seeks to present the available evidence for survival outcomes based on assessment of the peritumoural immune response in colorectal cancer and to outline a possible strategy for incorporating assessment of the immune microenvironment in colorectal cancer into clinical practice.

Methods

Search strategy

The aim of the literature search was to identify all primary studies that assessed the anti-tumour inflammatory response in colorectal cancer and its relation to prognosis among patients with resected colorectal cancer. PubMed, Ovid MEDLINE and EMBASE databases were searched on the 1st May 2019 using the following criteria:

1. "Colon cancer" OR "Rectal Cancer" OR "Colorectal cancer" (in Abstract) AND
2. "Survival" OR Prognos$ (in Abstract) AND
   a. "Klintrup-Makinen" OR KM OR "Crohn's-like reaction" OR CLR OR "peritumo$ inflamm$" (in Abstract) OR
   b. "Cytotoxic or CD8 or CD3 or CD4 or T-cell or Tcell or lymphocyte or macrophage or CD68 or CD163 or "natural killer" or CD56 or CD57 or CD45RO or FoxP3 or Treg or T-reg or CD20 or "tumo$ infiltrating lymphocytes" or TILs" (in Abstract) AND
   c. Immunohistochemistry (in any field)

Table 1
Inclusion criteria of prognostic studies of anti-tumour inflammatory response.

1. Either prospective or retrospective design with a well-defined study population
2. Study of rectal, colon or colorectal cancer primary resections
3. Assessment on FFPE slides using a standardised H&E assessment or IHC staining for a specified inflammatory cell population of interest
4. Clear description of the specimen used for assessment, antibodies used, tumour compartment assessed and counting method.
5. Groupings of patients and data cutoffs.
6. Description of statistical analysis methods used.
7. For meta-analysis, only those papers reporting a proportional hazard model used, including details of adjustment variables.

One reviewer (P.G.A) made an inspection of the titles and abstracts of citations to identify relevant studies and obtain full texts. A further search was made of the reference lists to identify studies not identified in the original search. The search was limited to English language, published from 1997 to present and studies in humans.

Methodologic and validity assessment

Study inclusion criteria were derived from published REMARK guidelines [20]. Eligible studies were included if meeting criteria in Table 1.

Data extraction

One author (P.G.A) performed a review of all eligible manuscripts. Any contentious articles were discussed with the other authors (DCM and JHP) and agreement was reached regarding articles for inclusion and exclusion. The extracted study data included: year of publication; stage of disease; time period of sample; specimen used for assessment (whether biopsy, TMA or whole section); for TMA, number and size of cores, if given; whether colon or rectal (if specified) or colorectal; haematoxylin and eosin (H&E) staining or antibody used for IHC; counting method; whether MSI was assessed and how this was handled in statistical analysis; specific survival outcome. On the rare occasion where hazard ratios were given without a 95% CI, the *P*-value was utilised to give an estimation of the standard error. Only studies that performed multivariate analysis were included. However, given the high numbers of studies that combined multiple immune variables in multivariate analysis, univariate hazard ratios were entered into meta-analysis. An assessment of bias was made based on REMARK guidelines criteria and the table is presented in supplementary data.

Statistical analysis

Studies were grouped according to whether they assessed rectal, colon or colorectal cancer for any given variable. In addition, studies were subgrouped by type of survival assessed: whether disease-free survival, which was defined as time to disease recurrence and included the terms "recurrence-free survival" and "progression-free survival"; overall survival, which was defined as time to death from any cause; or dis-ease-specific survival, which was defined as time to cancer-related death and also included the term "cancer-specific survival". The location of inflammatory assessment performed was defined as Intra-Tumoural (IT) or at the Invasive Margin (IM). For IT, studies were included regardless of whether they performed an assessment within cancer cell nests (or intra-epithelial (IE)) or in tumour stroma (ST) or combined (IE + ST). Studies using an "immunoscore-type" method (i.e. combining high scores from both IT and IM areas) for assessing a single inflammatory cell subtype were included in the IT assessment only. Studies with a sample size of less than 100 were excluded from meta-analysis. For single studies of a particular inflammatory assessment, the

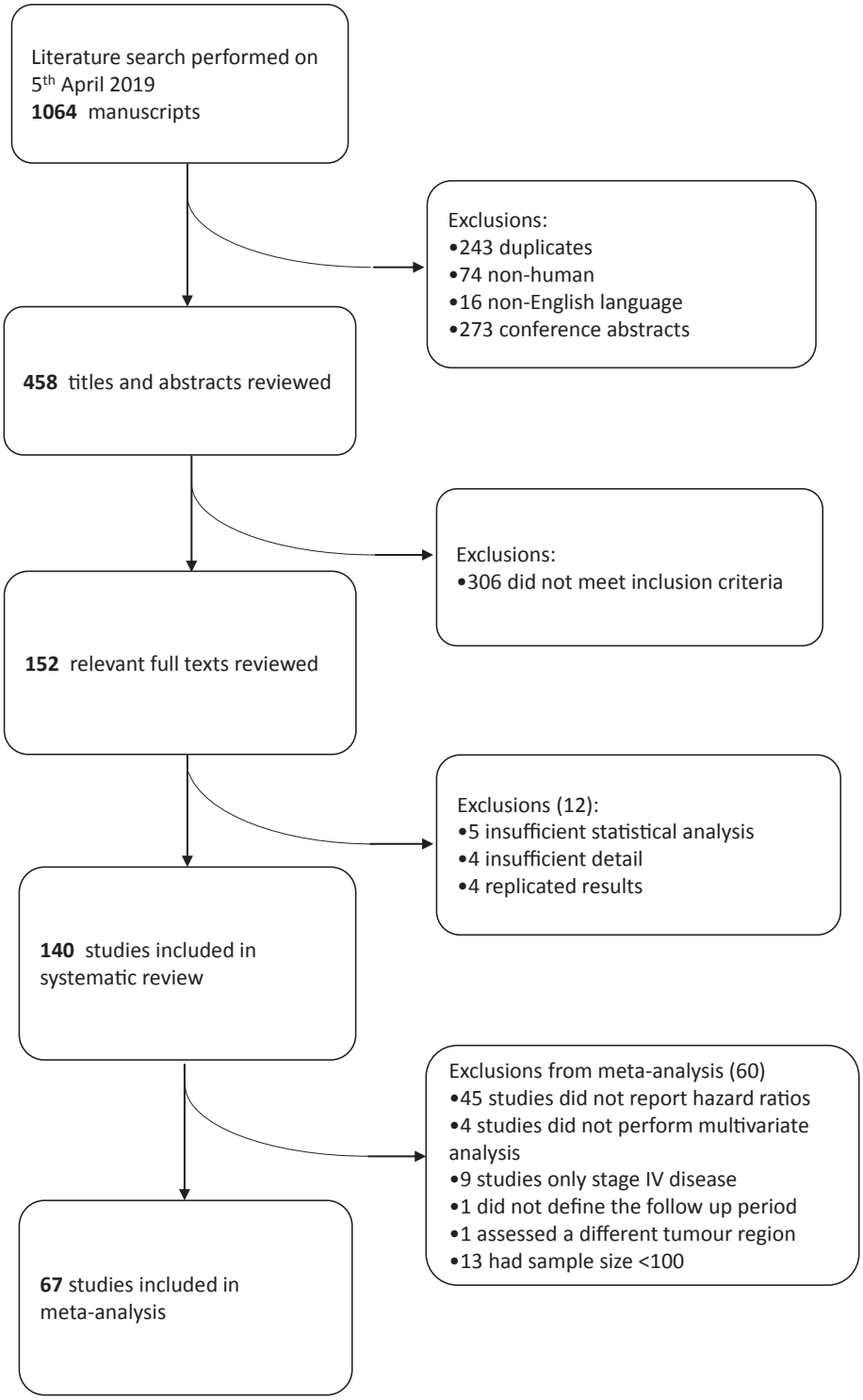

Fig. 1. Flow diagram of literature search and included/excluded studies.

HR and 95% CI for that study are reported. Where multiple studies evaluated the same inflammatory assessment, a fixed effects summary HR is given with 95% CI. Where the Hazard Ratio was greater than 1.0, this indicated a worse survival for higher value of a given inflammatory variable and vice versa. Confidence intervals crossing 1.0 were considered non-significant. Inter-study heterogeneity was also assessed and presented as the I^2 value. Publication bias was assessed by funnel plot. Meta-analyses were performed using REVMAN systematic review and meta-analysis software, version 5.3.

Results

Literature search and exclusion of studies

Literature search yielded a total of 1064 manuscripts for assessment (Fig. 1). After exclusion of duplicates (n = 243 studies), non-human studies (n = 74 studies), non-English language (n = 16 studies) and conference abstracts (n = 273), titles and abstracts were reviewed for 458 studies. Full texts were obtained for the 152 relevant studies. Following careful scrutiny of these, 12 studies were excluded due to

Table 2
Summary table of all papers reporting survival outcomes based on peritumoural inflammatory infiltrate and inflammatory cell subtypes.

Measurement of local inflammatory response	Total number of studies[a]	Studies reporting significant positive association[a] (%)	Studies reporting significant negative association[a]	Studies reporting no survival association[a]
Inflammatory infiltrate on H&E				
Klintrup-Makinen/Jass	18	14 (78)		4
CLR	14	13 (93)		1
TILs (H&E)	9	8 (89)		1
Combined assessment	2	2 (100)		0
Any H&E method	**32**	**30 (94)**		**2**
T-lymphocyte subsets				
CD3 (generic T-cell)	34	24 (71)		10
CD8 (cytotoxic T-cell)	62	46 (74)		16
CD4 (helper T-cell)	15	6 (40)		9
CD45RO (memory T-cell)	15	12 (80)		3
FoxP3 (regulatory T-cell)	34	21[b] (62)	2[b] (6)	12
Combined T-cells	**86**	**67[b] (78)**	**2[b] (2)**	**18**
Immunoscore	14	13 (93)		1
B-lymphocytes (CD20)	6	5 (83)		1
Natural killer cells (CD56, CD57)	6	5 (93)		1
Macrophages (CD68, CD163, CD206)				
CD68	17	10 (59)	1 (6)	6
CD163	7	2 (29)	3 (43)	2
CD206	1	0	1 (100)	0
Combined macrophages	**22**	**12 (55)**	**5 (23)**	**5**
Total	**140**	**119[c] (85)**	**7[c] (5)**	**16**

[a] Numbers in columns will not add up as many studies looked at more than one marker.
[b] One study both positive and negative.
[c] Two studies reported both positive and negative findings.

insufficient statistical analysis, insufficient detail, or replication of results from a previous work. This left 140 studies with sufficiently detailed methodology and patient cohort assessing inflammatory infiltration in colon, rectal and colorectal cancer (Table 2 and supplementary data). When performing meta-analysis, however, a further 60 studies were excluded, for reasons presented in flow diagram (Fig. 1). Finally, the decision was made to exclude 13 studies with small sample size ($n < 100$) due to the low event rate and reliability of results. This left a final total of 67 studies for meta-analysis, summary of included studies in Supplementary Table S1. Assessment of bias is shown in Supplementary Table S2. All studies included in meta-analysis were of good quality with low risk of bias and only one study scoring moderate risk.

Of those studies included in the meta-analysis, 35 performed assessment on whole sections, 29 on TMAs, and the remaining 3 on a combination of TMA and whole sections ($n = 2$) or TMA and biopsy ($n = 1$). TMA sizes varied and included cores ranging from 0.6 mm to 3 mm, with core size not provided for 4 studies. Automated cell counting was utilized in 21 studies, while the remaining 46 used manual assessment methods. Only 27 studies documented that blinding was performed to patient outcome. Sample size varied from 103 up to 2681 patients. The majority ($n = 51$) contained less than 500 patients. The median study size was 285, with an interquartile range of 160–478. Sixty-nine percent of papers assessed the presence of MSI, however 26% of those did not include it in the multivariate analysis. There were over 45 adjustment variables used in multivariate analysis, although the most common were age ($n = 45$), sex ($n = 37$), grade ($n = 35$), N-stage ($n = 29$), TNM ($n = 29$) , T-stage ($n = 27$), MSI ($n = 24$), tumour site ($n = 22$), lympho-vascular/perineural invasion ($n = 21$), other inflammatory assessment ($n = 21$), adjuvant therapy ($n = 12$) and M-stage ($n = 8$).

H&E assessment of inflammatory infiltrate

Since 1997, thirty-two studies were identified that assessed survival in the context of an H&E-based assessment of the peritumoural inflammatory infiltrate in colon, rectal or colorectal cancer: Jass/Klintrup-Makinen, Crohn's like reaction, tumour-infiltrating lymphocyte counts or a combination of these. Four studies had overlapping cohorts [21–24], leaving twenty-eight independent studies with a total of 11423 patients [9,25–51]. Two studies did not find a significant survival difference for any H&E-based method [9,37], whereas the other twenty-six studies found significantly prolonged survival for patients with higher local inflammatory infiltrate with a total number of 10887 patients [25–36,38–51].

Klintrup-Makinen and Jass

The most common method of assessment was the Klintrup-Makinen grade (KM) or Jass scoring systems: two related, but separate systems assessing the quantity of the overall local inflammatory infiltrate. The Jass score was first reported in rectal cancer in 1986 and claimed to be the first to show an independent association of the lymphocytic infiltrate with survival [52]. They described the lymphocytic infiltrate at the invasive margin (IM) or "advancing front" of the tumour as pronounced, moderate, little or none. Prominent inflammation at the IM appeared as a "cap" or continuous layer, whereas moderate inflammation was more broken or interrupted with fewer lymphocytes, while they combined "little" and "none" into one category. They found this 3-point scale was able to stratify rectal cancer survival into 3 distinct bands. Klintrup and colleagues [22] developed a similar phenotypic assessment of the local inflammatory infiltrate at the IM in 2005, with a 4 point scale, scoring a "cup-like" infiltrate as 3, band-like as 2, interrupted band as 1 or minimal inflammation as 0. They further added in the destruction of cancer cells in the top two scores and dichotomised their score with the upper and lower two categories being combined into high vs low, respectively.

Eighteen studies assessed the inflammatory infiltrate using KM or Jass scoring, although one of these had an overlapping cohort [22], leaving seventeen independent studies and a total of 4904 patients: four of these studies found no significant difference [9,21,34,37]; the remaining thirteen found significantly better survival for higher KM or

Table 3
Meta-analysis results for papers meeting inclusion criteria for Colon cancer.

Impact of study methodology on heterogeneity testing (I^2 test) and overall effect							
	Overall effect				Heterogeneity		
Location assessed	Survival type	No. of studies	HR	95% CI	I^2 test (%)	*P*-value	First Author Surname/year
Klintrup-Makinen/Jass							
	OS	1	0.63	0.42–0.95	NA		Hynes
	DSS	1	0.48	0.31–0.75	NA		Hynes
Crohn's-like reaction							
G-A	OS	1	0.64	0.48–0.86	NA		Hynes
	DSS	1	0.60	0.42–0.85	NA		Hynes
Tumour infiltrating lymphocytes (H&E)							
	DFS	1	0.37	0.15–0.90	NA		Turner
	OS	1	0.45	0.23–0.87	NA		Turner
CD3							
IT	DFS	2	0.59	0.38–0.91	3	0.31	**Guidoboni**, Sinicrope
	OS	3	0.49	0.33–0.71	0	0.83	**Guidoboni**, Miller, Sinicrope
	DSS	1	0.35	0.14–0.88	NA		Miller
IM	OS	1	0.48	0.22–1.03	NA		Miller
	DSS	1	0.65	0.50–0.84	NA		Miller
CD8							
IT	DFS	1	0.35	0.16–0.76	NA		**Guidoboni**
	OS	3	0.58	0.41–0.83	34	0.22	**Guidoboni**, Miller, Yoon
	DSS	1	0.77	0.32–1.87	NA		Miller
IM	OS	1	0.84	0.41–1.71	NA		Miller
	DSS	1	0.77	0.32–1.87	NA		Miller
FoxP3							
IT	DFS	1	1.23	0.72–2.13	NA		Sinicrope
	OS	2	0.91	0.59–1.40	86	0.008	Miller, Sinicrope
	DSS	1	0.28	0.12–0.66	NA		Miller
Immunoscore							
	DFS	1	0.63	0.52–0.75	NA		Pages 18
	OS	1	0.70	0.58–0.84	NA		Pages 18

Bold studies: Right sided tumours only

Jass scores, with a total of 4046 patients [25,26,29–33,35,36,38–41]. In terms of interobserver agreement, the original study by Jass quoted interobserver agreement as 0.72 [52]. However, in these 19 studies, interobserver agreement was only reported for KM by two groups with kappa values ranging from 0.05 to 0.48 in one study [26] and 0.50 to 0.79 in another [22].

Nine independent studies assessed the presence of MSI: of which two found peritumoural inflammation to be independent of MSI in survival [26,36]; one only included MSI tumours and did not find peritumoural inflammation to be associated with survival [21]; three found MSI to have no association with survival [38,39,41]; and three did not include MSI in survival analysis [9,33,35].

Only one study met inclusion criteria for meta-analysis for KM in colon cancer finding it significant for OS and DSS (Table 3). Six studies were included for KM in colorectal cancer (Table 4, Fig. 2): three analysing DFS (HR 0.62, 95% CI: 0.43–0.88); two for OS (HR 0.43; 95% CI: 0.26–0.71); and three for DSS (HR 0.40; 95% CI: 0.29–0.55). There was no significant heterogeneity between studies for any survival type. In addition, the funnel plot for KM shows no clear publication bias. There were no studies in rectal cancer that met inclusion criteria for meta-analysis.

Crohn's-like reaction

The Crohn's-like reaction (CLR), described by Graham and Appelman in 1990 [53], which assesses the number of "discrete lymphoid aggregates", with or without germinal centres, at the IM and spread through the muscularis propria. The CLR is considered part of the adaptive immune response against the cancer, with aggregates consisting largely of B-cells, and to a lesser degree T-cells and antigen-presenting cells [41]. The original method of assessing this response scored more than 3 aggregates and at least 1 germinal centre as "intense", vs a "mild" reaction if 2 or fewer aggregates or none if absent [53]. Various groups attempting to validate this method have combined two of these categories into one, whether none and mild vs intense [28,48], or none vs mild or intense [22]. Two adaptations to this have been proposed and validated. The first was proposed by Ueno and colleagues in 2013, scoring CLR on a size-based criteria: any aggregate of > 1 mm in diameter was classed as "active" [50]. A further method of scoring CLR was proposed by Vayrynen and colleagues in 2014, which measures the "density" of reaction by dividing the total number of aggregates by the length of the IM: any density of greater than 0.38 follicles/mm (calculated by ROC curve) is classed as high density [41]. Still others have measured a B-cell response on H&E by performing manual counts of plasma cells at the IM [23] or within the tumour [45].

Fourteen studies reported CLR or plasma cell counts although three of these had overlapping cohorts [21,22,24], leaving eleven independent studies comprising a total of 6595 patients, all of which found a significantly better survival for high CLR [23,26,28,36,41,43,45,46,48–50]. Seven independent studies (3803 patients) used the Graham-Appelman (G-A) method of assessment: four of these (2337 patients) used a cut-off of greater that one (grouping absent and mild together) [28,46,48,54], of which only one found that CLR was not associated with survival and this was in a cohort of solely MSI tumours [54]; while the other three (1466 patients) used a cut-off of greater than zero (grouping mild and intense together) [22,26,36], of which one found that CLR was not associated with survival [22]. Another paper adjusted the criteria for intense staining to greater than 5 lymphoid aggregates but there was only one tumour in this category, therefore effectively this paper also combined mild and intense

Table 4
Meta-analysis results for papers meeting inclusion criteria for Colorectal cancer.

Impact of study methodology on heterogeneity testing (I^2 test) and overall effect

	Overall effect				Heterogeneity		
Location assessed	Survival type	No. of studies	HR	95% CI	I^2 test (%)	*P*-value	First Author Surname/year
Klintrup-Makinen/Jass							
	DFS	3	0.62	0.43–0.88	0	0.39	Climent, Klintrup, Menon
	OS	2	0.43	0.26–0.71	0	0.45	Climent, Ogino
	DSS	3	0.40	0.29–0.55	0	0.91	Ogino, Park 14, Vayrynen 14
Crohn's-like reaction							
G-A	DFS	1	0.87	0.26–2.89	NA		Lee 16
	OS	4	0.68	0.60–0.78	53	0.09	**Bae**, Buckowitz, Ogino, Rozek
	DSS	2	0.64	0.54–0.77	0	0.60	Ogino, Rozek
Ueno criteria	DFS	2	0.49	0.37–0.64	0	0.98	**Kim 15a**, Ueno 15
	DSS	1	0.40	0.20–0.80	NA		Ueno 13
Vayrynen criteria	DFS	1	0.50	0.28–0.89	NA		**Kim 15a**
	DSS	1	0.54	0.37–0.79	NA		Vayrynen 2014
Any method	DFS	3	0.51	0.39–0.66	0	0.84	**Kim 15a**, Ueno 15, Lee 16
	OS	4	0.68	0.60–0.78	53	0.09	**Bae**, Buckowitz, Ogino, Rozek
	DSS	4	0.61	0.52–0.71	0	0.50	Ogino, Rozek, Ueno 13, Vayrynen 14
Tumour infiltrating lymphocytes (H&E)							
	DFS	3	0.65	0.51–0.83	0	0.37	Climent, Lee 16, Ropponen
	OS	4	0.73	0.64–0.84	0	0.73	Climent, Nielsen, Ogino, Rozek
	DSS	3	0.66	0.55–0.78	0	0.95	Ogino, Ropponen, Rozek
Combined H&E inflammatory assessment							
	OS	1	0.50	0.31–0.81	NA		Ogino
	DSS	1	0.31	0.15–0.65	NA		Ogino
CD3							
IT	DFS	6	0.46	0.39–0.54	56	0.04	Chen 16, Deschoolmeester, Erisken, Galon 06, Kim 18, Vayrynen 16
	OS	8	0.57	0.50–0.64	73	< 0.001	Berntsson 17, Chen 16, Deschoolmeester, Eriksen, Galon 06, Kim 18, Nosho, Vayrynen 16
	DSS	6	0.59	0.50–0.70	0	0.82	Dahlin, Nosho, Richards 14, Simpson, Vayrynen 12, Vayrynen 16
IM	DFS	3	0.45	0.33–0.61	0	0.69	Deschoolmeester, Galon 06, Vayrynen 16
	OS	4	0.71	0.59–0.85	67	0.03	Deschoolmeester, Galon 06, Nosho, Vayrynen 16
	DSS	6	0.58	0.48–0.69	10	0.35	Dahlin, Laghi, Nosho, Richards 14, Vayrynen 12, Vayrynen 16
CD8							
IT	DFS	8	0.46	0.39–0.54	48	0.06	Chen 16, Deschoolmeester, Eriksen, Kim 18, Mori, Prall, Tosolini, Vayrynen 16
	OS	15	0.63	0.58–0.67	65	< 0.001	Berntsson 17, Chen 16, Deschoolmeester, Erisken, Kasajima, Kim 18, Naito, Nazemalhosseini-Majorad, Nosho, Oshikiri, Pages 09, Prizment, Salama, Vayrynen 16, Zlobec 2008c
	DSS	9	0.62	0.56–0.69	73	< 0.001	Baker, Chiba, Ling, Nosho, Pages 09, Prall, Prizment, Richards 14, Vayrynen 16
IM	DFS	4	0.50	0.40–0.62	0	0.81	Deschoolmeester, Kim 15b, Tosolini, Vayrynen 16
	OS	4	0.62	0.51–0.75	58	0.05	Deschoolmeester, Kim 15b, Nosho, Vayrynen 16
	DSS	5	0.53	0.45–0.63	53	0.05	Lugli, Matsutani, Nosho, Richards 14, Vayrynen 16
CD4							
IT	DFS	1	0.55	0.32–0.96	NA		Chen 16
	OS	2	0.64	0.42–0.97	0	0.72	Chen 16, Kasajima
	DSS	1	0.64	0.41–0.99	NA		Ling
CD45RO							
IT	DFS	3	0.52	0.40–0.69	83	0.003	Chen 16, Kim 15b, Pages 09
	OS	5	0.68	0.61–0.75	78	0.001	Chen 16, Kim 15b, Nosho, Pages 09, Salama
	DSS	3	0.53	0.44–0.64	83	0.002	Nosho, Pages 09, Richards 14
IM	DFS	1	0.42	0.33–0.54	NA		Kim 15b
	OS	2	0.51	0.42–0.63	77	0.01	Kim 15b, Nosho
	DSS	2	0.57	0.47–0.68	70	0.07	Nosho, Richards 14
FoxP3							
IT	DFS	2	0.52	0.36–0.77	27	0.24	Chen 16, Vayrynen 16
	OS	4	0.72	0.65–0.80	77	0.004	Chen 16, Nosho, Salama, Vayrynen 16
	DSS	3	0.47	0.37–0.61	0	0.40	Nosho, Richards 14, Vayrynen 16
IM	DFS	1	0.42	0.19–0.93	NA		Vayrynen 16
	OS	2	0.47	0.35–0.63	0	0.81	Nosho, Vayrynen 16
	DSS	3	0.57	0.46–0.70	62	0.07	Nosho, Richards 14, Vayrynen 16
Immunoscore							
	DFS	3	0.49	0.41–0.58	91	< 0.001	Mlecnik, Pages 09, Wirta
	OS	3	0.61	0.53–0.70	89	< 0.001	Mlecnik, Pages 09, Wirta
	DSS	5	0.47	0.39–0.55	86	< 0.001	Mlecnik, Nearchou, Pages 09, Park 16, Wirta
CD20							
IT	DFS	1	0.62	0.40–0.96	NA		Chen 16
	OS	2	0.66	0.52–0.89	0	0.50	Berntsson 16, Chen 16

(*continued on next page*)

Table 4 (*continued*)

Impact of study methodology on heterogeneity testing (I^2 test) and overall effect

	Overall effect				Heterogeneity		
Location assessed	Survival type	No. of studies	HR	95% CI	I^2 test (%)	*P*-value	First Author Surname/year
CD56/57							
IT	DFS	2	0.47	0.28–0.78	71	0.06	Chen 16, Tachibana
	OS	2	0.48	0.28–0.84	33	0.22	Chen 16, Tachibana
CD68							
IT	DFS	3	1.21	0.95–1.55	81	0.005	Chen 16, Kim 18, Vayrynen 16
	OS	5	0.91	0.75–1.11	77	0.002	Chen 16, Gulubova, Kim 18, Koelzer 16, Vayrynen 16
	DSS	2	0.58	0.38–0.89	0	0.56	Algars, Vayrynen 16
IM	DFS	1	0.43	0.19–0.96	NA		Vayrynen 16
	OS	3	0.48	0.36–0.64	48	0.15	Gulubova, Li 18a, Vayrynen 16
	DSS	1	0.40	0.20–0.81	NA		Vayrynen 16
CD163/206							
IM	DFS	1	3.68	1.74–7.82	NA		Shibutani 17
	DSS	1	0.66	0.42–1.05	NA		Edin

Bold studies: MSI high only studies

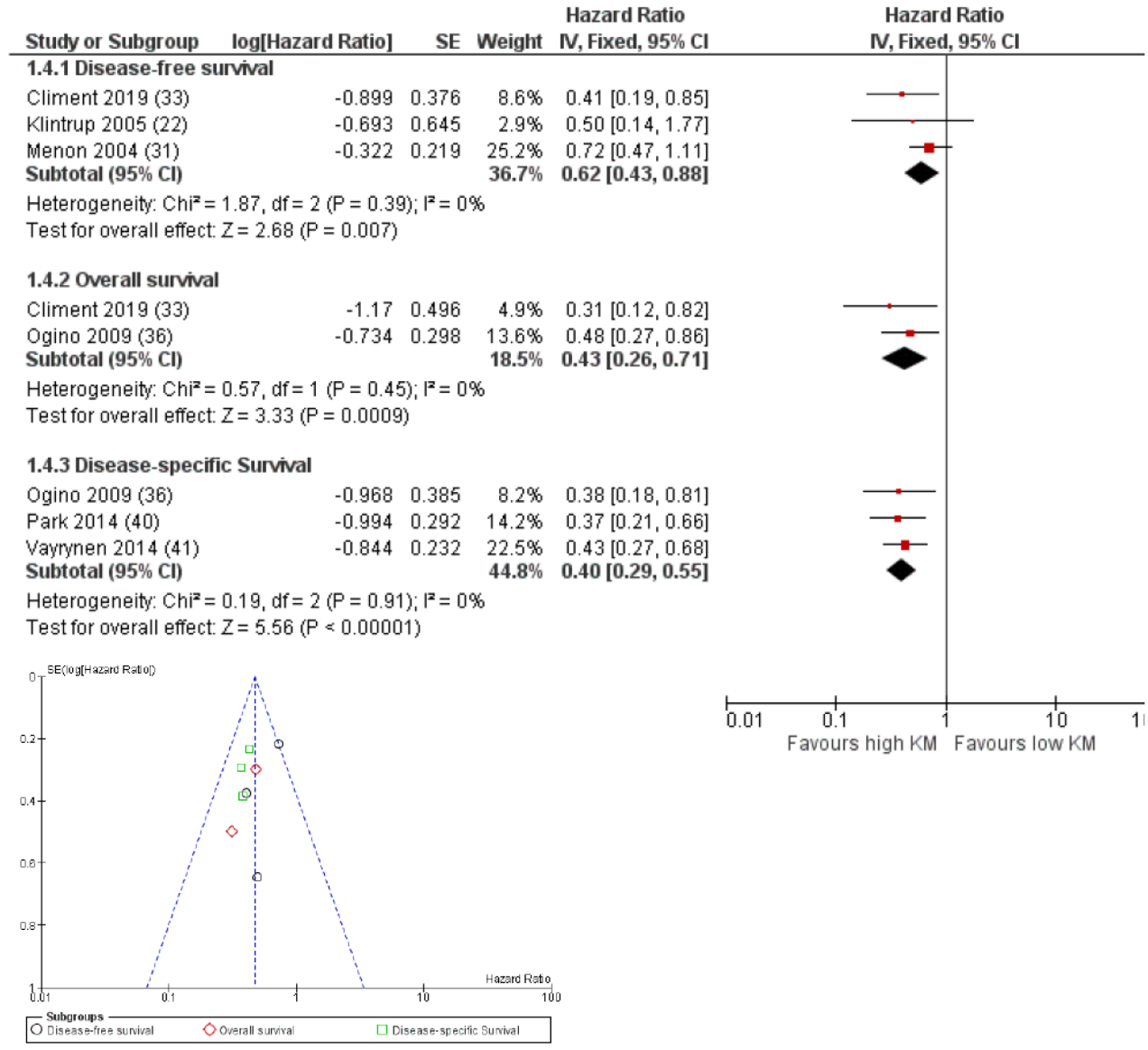

Fig. 2. Forest plot and funnel plot for KM/Jass classification in colorectal cancer according to DFS, OS and DSS.

reactions and found CLR to be significant for survival [43]. The only group to report different cut-offs for the G-A method in the same cohort assessed only patients with MSI and found that a cut-off of greater than 0 (144 high vs 25 low) was significant [21], whereas a cut-off of greater than 1 (48 high vs 164 low) was not [49]. This was probably because the lower cut-off selected out those patients in this cohort who had MSI but a poor local inflammatory response for worse survival. Three independent studies (2073 patients) used a digital pathology method of assessing CLR: one using the size or Ueno method [50]; one using the density or Vayrynen method [41]; one compared the original G-A method with the Ueno and Vayrynen methods in a cohort of MSI-high tumours [54]. All three studies found that Ueno and Vayrynen methods were significant for survival. Interobserver agreement for the G-A method was reported as around 0.50 [49,50] with a range of 0.29 to 0.92 [26,28]. The Ueno method had a reported agreement of 0.56 [49] to 0.67 [50] and the Vayrynen method, 0.71–0.81 [41,49]. The only paper to directly compare all three methods found the Vayrynen density-based method to be the most reproducible [49].

Eight independent groups assessed the presence of MSI: of which four found CLR to be independent of MSI [26,36,46,49]; two found that MSI was not independently significant for survival [41,48]; and two did not include MSI in survival analysis [43,50].

Only one study met inclusion criteria for meta-analysis for CLR in colon cancer using the G-A method and finding it significant for OS and DSS (Table 3). Five studies were included for G-A method in colorectal cancer (Table 4, Fig. 3A): only one assessed DFS finding it to be significant for survival; four assessed OS (HR 0.68; 95% CI: 0.60–0.78); and two DSS (HR 0.64; 95% CI: 0.54–0.77). There was no significant heterogeneity for DSS, although for OS, there was moderate heterogeneity with an I^2 of 53% ($p = 0.09$). Three studies met inclusion criteria for meta-analysis for the Ueno method in colorectal cancer (Table 4, Fig. 3B): two of these assessed DFS (HR 0.49; 95% CI: 0.37–0.64), with no significant heterogeneity and one assessed OS, finding it to be significant for survival. Two studies were included for the Vayrynen method (Table 4, Fig. 3C), both finding it significant for survival: one for DFS and the other for DSS. The funnel plot for the G-A method shows no evidence of publication bias, whereas those for Ueno and Vayrynen criterion, there were too few studies to comment on publication bias. There were no studies assessing CLR in rectal cancer.

TILs on H&E

TILs have been referred to by many groups when considering an H&E based assessment. However, some are referring to an assessment at the invasive margin similar to the KM grade [55]. Papers describing TILs on H&E were considered when an assessment was made of at least one IT compartment, whether IE, ST or combined IE + ST.

There were nine independent studies that assessed TILs in this way in relation to survival in colon or colorectal cancer with a total of 5508 patients [27,33,36,42–47], of which eight studies showed a positive association with survival, comprising 5343 patients [27,36,42–47], while one showed no association [33]. There was no assessment of interobserver variability reported in any of the studies. Six studies assessed the presence of MSI: of which two found that TILs were independent of MSI in survival [36,46]; one found that MSI was not significant for survival [27]; while three did not include MSI in survival analysis [33,43,44]. Four studies assessed the density of TILs semi-quantitatively [27,36,42,43], while five counted cells and assigned a cut-off [33,44–47].

Only one study met inclusion criteria for meta-analysis for TILs on H&E sections in colon cancer, finding it significant for DFS and OS (Table 3, Fig. 4). For colorectal cancer, six studies were included for TILs on H&E sections (Table 4): three assessed DFS (HR 0.65; 95% CI: 0.51–0.83); four assessed OS (HR 0.73; 95% CI: 0.64–0.84); and three assessed DSS (HR 0.66; 95% CI: 0.55–0.78). There was no significant heterogeneity between the studies for any survival outcome. Funnel plot shows no significant publication bias. There were no studies assessing TILs on H&E in rectal cancer.

Combined inflammatory assessment on H&E

Ogino and colleagues developed a combined scoring system in 843 patients assessing KM, CLR, ST TILs and IE TILs. A semiquantitative score was assigned for each element of peritumoural inflammation. Each element of the score was found to be individually significant for survival apart from IE TILs. All four elements were given equal weighting and combined into a total score. An arbitrary cut-off was subsequently set to separate the cohort into 3 categories. The combined inflammatory score was found to be independently significant for OS and CSS and was independent of MSI [36]. Another group performed the same assessment, comparing the score's efficacy in a Caucasian population comprising 159 patients and an Afro-American population comprising 52 patients. The score was not significant in the Caucasian population but was significant in the Afro-American population and independent of MSI [51].

One study had to be excluded from meta-analysis due to lack of follow up data [51]. Hazard ratios for the combined H&E assessment in colorectal cancer (Table 4) were significant for both OS and DSS. There were no studies in rectal or colon cancer utilising the combined H&E assessment.

MSI and H&E assessment of peritumoural inflammation

Sixteen original studies comparing H&E assessment of peritumoural inflammation and survival also took MSI into consideration [9,26,27,33,35,36,38,39,41,43,44,46,48–51]. However, of those sixteen: six studies did not enter MSI into multivariate analysis [9,33,35,43,44,50]; five found that MSI was not significant for survival [27,38,39,41,48]; but the remaining 5 studies found that peritumoural inflammation was independent of MSI for prognostic significance (3820 patients) [26,36,46,49,51]. One of these studies was exclusively in 212 MSI colorectal cancers and found CLR to be significant for survival even in this group [49].

T-lymphocyte subsets

The adaptive immune response includes the actions of a variety of immune cells with varying functions, both pro-inflammatory and immunosuppressive, which includes T-cells with a range of phenotypes: from CD4 helper T-cells, to CD8 cytotoxic T-cells, to FoxP3 regulatory T-cells and CD45RO memory T-cells.

Eighty-six studies were identified that assessed rectal, colon or colorectal cancer survival according to T-cell marker assessment using immunohistochemistry. However, many of these studied overlapping cohorts or assessed more than one marker [21,30,54,56–75]. In terms of independent cohorts studied in relation to different T-cell markers, there was a total of 63 studies, encompassing 14700 patients [9,13,37,39,76–134]. Of the total studies, 67 found a significant positive effect for T-cell infiltration in and around the tumour, but only 47 of these studied independent cohorts, with a total number of 13014 patients [9,13,37,39,76–80,82,83,85,86,89,90,92–95,97–101,103–105,107–109,112,114,116–119,121–125,127–130,132,133]. Two studies found a negative survival impact regarding FoxP3 expression [57,130], of which one found both negative and positive effects [130]. Eighteen studies did not find any significant survival difference according to T-cell infiltration, although only 14 of these assessed independent cohorts [84,87,88,91,96,102,106,110,111,113,115,120,126,134]. Many studies compared more than one T-cell marker and included multiple inflammatory markers or MSI [70,81,83,92,95,132] in multivariate analysis [30,61,78,83,85,90,92,98,104,117–119,127,130], with the result that although each marker was highly significant on univariate analysis, the survival advantage was not independent of other markers of

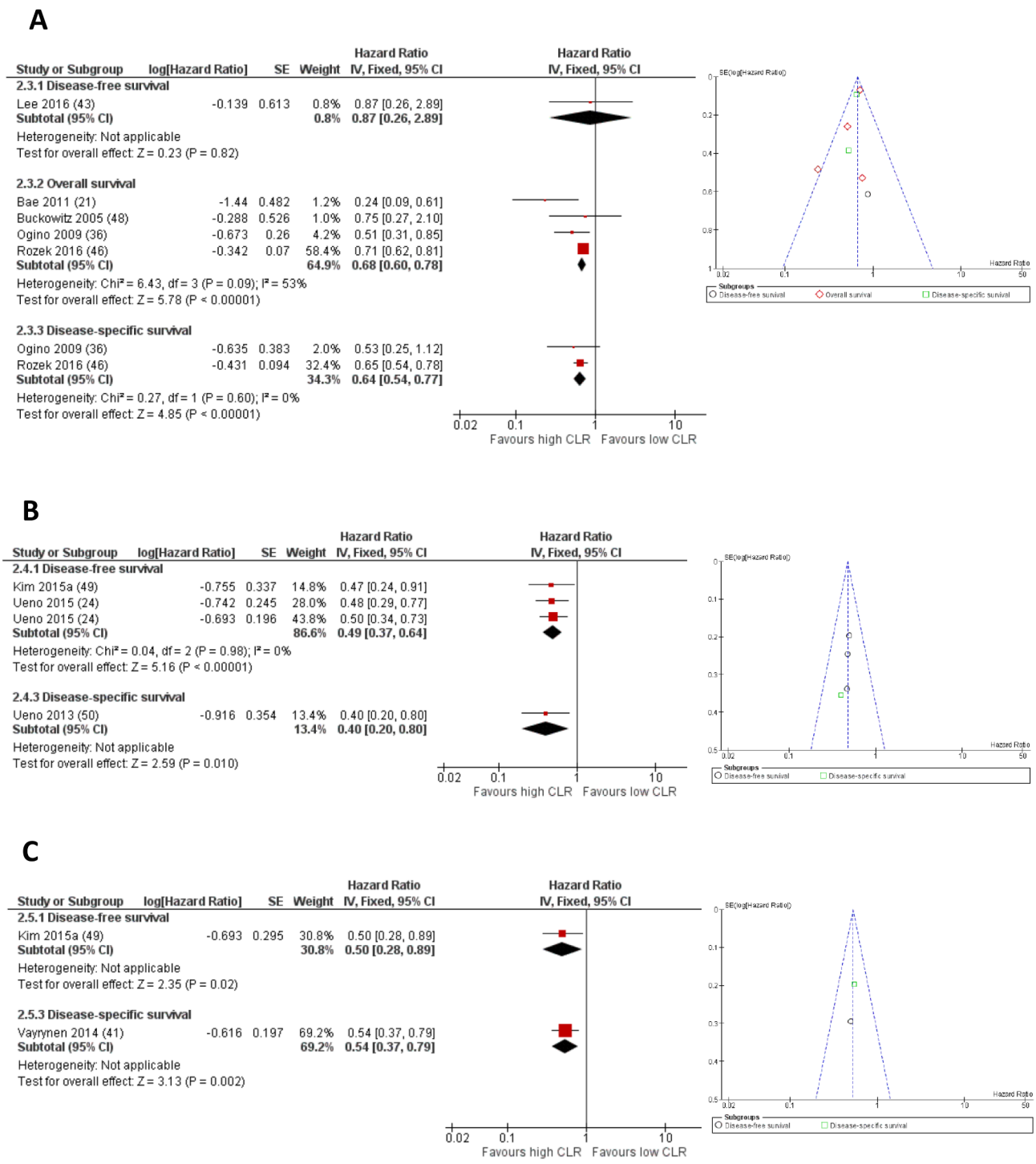

Fig. 3. Forest plots and funnel plots for CLR DFS, OS and DSS in colorectal cancer, as measured by: (A) Graham-Appelman method; (B) Ueno method; and (C) Vayrynen method.

inflammation. This suggests that a functional host immune response results in a better outcome, regardless of which markers are assessed.

CD3 (generic T-cell marker)

CD3, a generic T-cell marker expressed by the majority of T-cells [135], was assessed in thirty-four studies in relation to survival in rectal, colon or colorectal cancer, although two studies had overlapping cohorts [59,61]. There were, therefore, thirty-two independent cohorts comprising a total of 7947 patients [30,58,60,63,68,76–99,102,118,127]. CD3 was found to have a significant positive association with survival in twenty-four of these, of which twenty-three were independent and comprised 5292 patients [30,58,60,63,76–81,83,85,86,89,90,92–95,97–99,127]. Ten studies found no significant association of CD3 with survival [59,68,82,84,87,88,91,96,102,118].

In rectal cancer, there were two papers comprising 229 patients assessing CD3 [76,102], both of which assessed the intratumoural compartments in pre-treatment biopsies, but only one was significant for survival (136 patients) [76]. The latter used a manual method of assessment [76], the other used an electronic counting method [102].

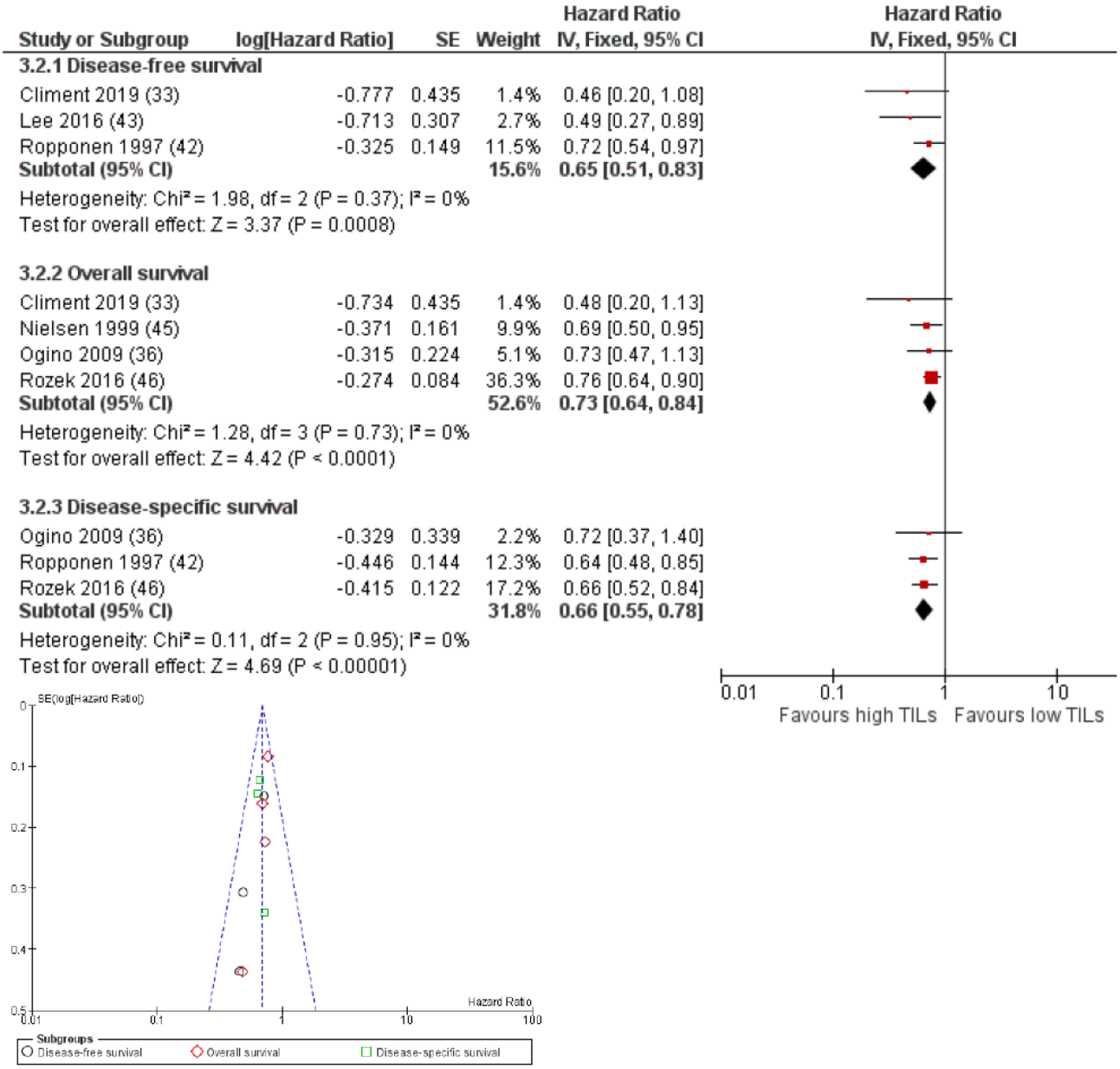

Fig. 4. Forest plot and funnel plot for TILs on H&E in colorectal cancer according to DFS, OS and DSS.

Neither study assessed the presence of MSI. Both studies used a median cut-off with equal group sizes. Neither met criteria for inclusion in meta-analysis.

In colon cancer, there were 6 studies comprising 591 patients that assessed the relation of CD3 infiltration with survival [58,61,77–79,127]. All found a significant association with survival. Five of these (502 patients) assessed the intratumoural compartments [58,61,77,79,127]. Two assessed the invasive margin in addition to other areas of the slide [78,79]. Three studies compared different tumour regions in the same cohort and of these: one found that the IE CD3 was significant for survival, whereas ST CD3 was not [58]; one found both IM CD3 and total slide CD3 were significant for survival [78]; and one found that intratumoural CD3 was significant where IM CD3 was not [79]. Three studies assessed the presence of MSI: one of which found no association of MSI with survival and it was therefore not included in multivariate analysis [79]; whereas 2 were independent of MSI [58,77]. Four papers used an electronic method of assessment [77–79,127], whereas 2 used a manual assessment [58,61]. Five of the studies used various different arbitrary data cut-offs [58,61,77,78,127], whereas one study used a "data-driven" cut-off[79].

Three studies met inclusion criteria for meta-analysis for IT CD3 in colon cancer (Table 3, Fig. 5A): two assessed DFS (HR 0.59; 95% CI: 0.38–0.91), with no significant heterogeneity; three assessed OS (HR 0.49; 95% CI: 0.33–0.71), with no significant heterogeneity; and one study assessed DSS finding a significant survival benefit. There were too few studies to interpret the funnel plot for IT CD3. Only one study met inclusion criteria for IM CD3 in colon cancer (Table 3, Fig. 5B). There was a trend towards a significant survival benefit for OS, although this was not significant and there was no significant survival benefit for DSS.

In colorectal cancer, there were 26 studies comprising 7230 patients assessing the relation of CD3 infiltration with survival [30,59,60,63,68,80–99,118]. Seventeen of these, with 4633 patients, found a positive association of CD3 with survival [30,60,63,80,81,83,85,86,89,90,92–95,97–99], compared with 9 studies (with 2597 patients) finding no significant survival difference [59,68,82,84,87,88,96,118]. Nineteen studies (5414 patients) assessed CD3 in the intratumoural compartments [30,60,68,81–94,96,98] and of these 12

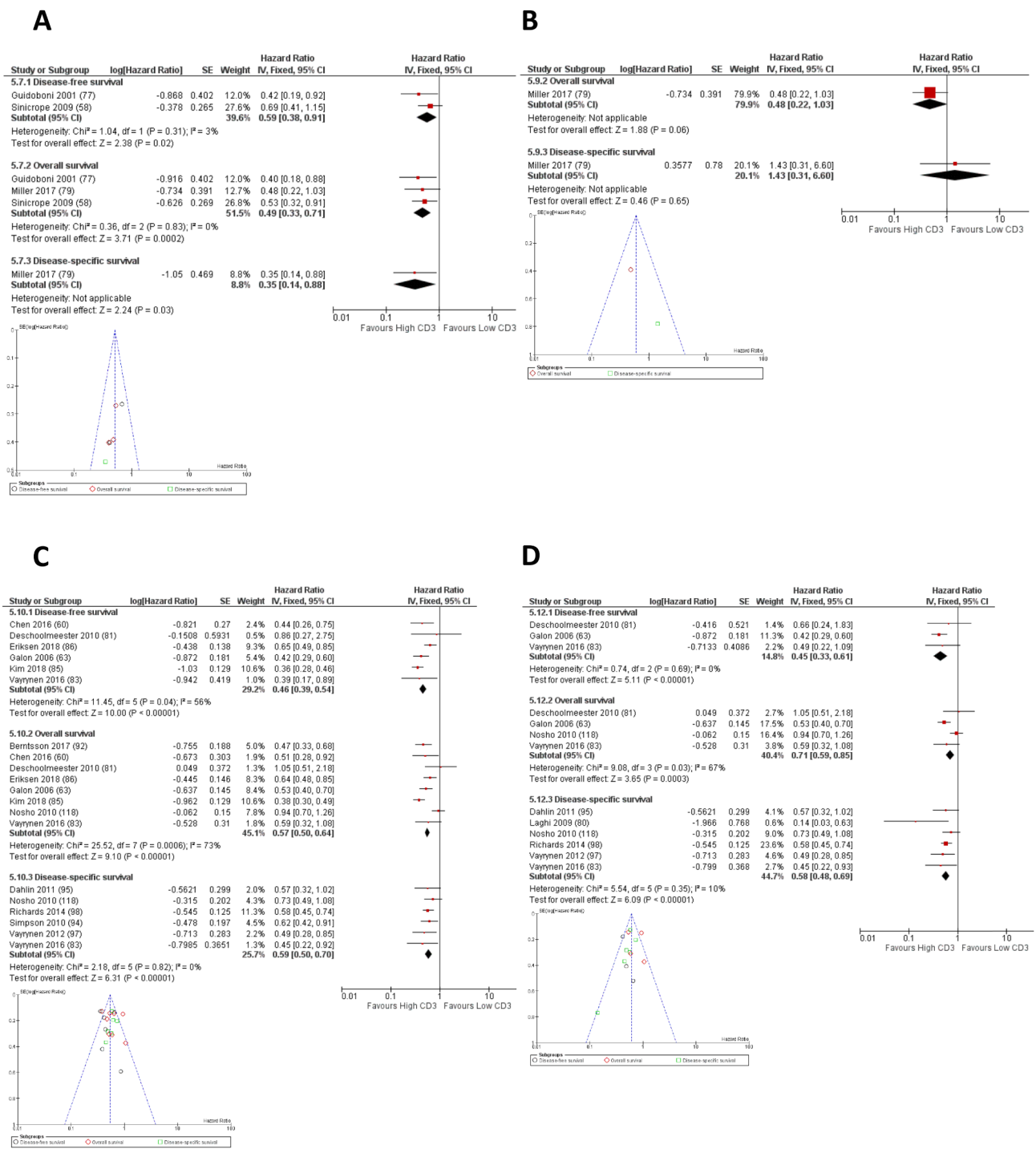

Fig. 5. Forest plots and funnel plots for CD3 according to DFS, OS and DSS: (A) IT in colon cancer; (B) IM in colon cancer; (C) IT in colorectal cancer; (D) IM in colorectal cancer.

studies (3579 patients) found a significant association with survival [30,60,81,83,85,86,89,90,92–94,98]. Nine studies (1641 patients) assessed CD3 and the invasive margin [30,80,81,83,84,87,89,91,98], of which 4 studies (775 patients) were significant for survival [30,80,83,98]. Six studies (1668 patients) assessed CD3 on the whole slide [59,63,95,97,99,118] of which four (906 patients) were significant for survival [63,95,97,99]. Seven studies significant for survival assessed more than one tumour region and of these: three found that IE CD3 and not ST CD3 was significant for survival [81,83,94], whereas three found that both IE CD3 and ST CD3 were significant for survival [85,89,98]; two studies found that intratumoural assessment of CD3 was significant whereas assessment at the invasive margin was not [81,89], while three found that assessment of both intratumoural and invasive margin CD3 was significant for survival [30,83,98]. Nine studies assessed the presence of MSI: of these two studies found CD3 to be independent of MSI [80,83]; two studies found no association of MSI with survival and therefore it was not included in multivariate analysis [86,88]; a further two studies did not include MSI in survival analysis [85,94]; and in 3 studies CD3 was not independent of MSI on multivariate analysis [81,92,95]. Fifteen studies used an electronic method of assessment [59,60,63,80,82,83,85,86,90–93,97,99,118] of which 11 found CD3 to be significant for survival

[60,63,80,83,85,86,90,92,93,97,99]. Twelve studies used a manual assessment method [30,68,81,84,87–89,94–98], of which 7 found CD3 to be associated with survival [30,81,89,94,95,97,98]. The only study to directly compare electronic and manual methods of CD3 as-sessment in colorectal cancer found both to be comparable and significant for survival [97]. Thirteen studies used various different arbitrary data cut-offs [68,81,84–86,88–90,94–96,98,118], whereas 9 studies used data-driven cut-offs [60,63,80,82,83,92,93,97,99]. In four studies, the cut-off method employed was unclear [30,59,87,91].

Twelve studies met inclusion criteria for meta-analysis for IT CD3 in colorectal cancer (Table 4, Fig. 5C): six assessed DFS (HR 0.46; 95% CI: 0.39–0.54), with moderate heterogeneity between these results (I^2 56%; $p = 0.04$); eight assessed OS (HR 0.57; 95% CI: 0.50–0.64), with substantial heterogeneity (I^2 73%; $p < 0.001$); and six assessed DSS (HR 0.59; 95% CI: 0.50–0.70), with no significant heterogeneity. The funnel plot showed no evidence of publication bias. Eight studies were included for IM CD3 (Table 4, Fig. 5D): three assessed DFS (HR 0.45; 95% CI: 0.33–0.61), with no significant heterogeneity; four assessed OS (HR 0.71; 95% CI: 0.59–0.85), with substantial heterogeneity (I^2 67%; $p = 0.03$); and six assessed DSS (HR 0.58; 95% CI: 0.48–0.69), with no significant heterogeneity. Funnel plot did not reveal any publication bias.

CD8 (cytotoxic T-cells)

CD8 is a cytotoxic T-cell marker, whose role is to recognise a foreign antigen presented by an antigen presenting cell and to bind cells expressing that antigen, inducing cell lysis and recruiting other immune cells with the release of cytokines [136]. CD8 was assessed in 62 studies in relation to survival in rectal, colon or colorectal cancer, although there were twelve studies whose cohorts overlapped [21,54,56,59,60,64,65,67–69,75,137], leaving 50 studies assessing CD8 in independent cohorts comprising a total of 12868 patients [9,13,30,37,39,70,71,76–79,81–86,88,89,91–93,98,100–125,134]. CD8 was found to be significant for survival in 46 studies, but only 37 of these were in independent cohorts [9,13,30,37,39,70,71,76–78,81–83,85,86,92,93,98,100,101,103–105,107–109,112,114,116–119,121–125] comprising 11085 patients. CD8 was not significantly associated with survival in 16 studies, of which 13 were independent [79,84,88,89,91,102,106,110,111,113,115,120,134].

In rectal cancer, there were eleven independent studies assessing CD8 and survival comprising 1749 patients [64,69,76,100–106,137]. Nine of these were significant for survival (1463 patients) [64,69,76,100,101,103–105,137]. Seven studies assessed CD8 in intratumoural compartments on pre-treatment biopsies (949 patients) [69,76,100–104]. Of these, three found a significant association of CD8 in pre-treatment biopsies with survival (497 patients) [69,76,103]. While six studies (1269 patients) assessed CD8 in the intratumoural compartment of resected specimens [100,101,103,104,106,137], of which four studies (804 patients) were significantly associated with improved survival [100,101,104,137]. Two studies assessed the combined invasive margin and intratumoural compartments for CD8 infiltration (162 patients), both of which were significant for survival [64,105]. Four studies assessed CD8 infiltration in biopsies taken prior to neo-adjuvant therapy, in addition to post-resection specimens: of these, three studies (346 patients) found that CD8 levels in the resected specimen were significantly associated with survival while those in the biopsies were not [100,101,104]; whereas in 1 study (285 patients) CD8 in the biopsy and not the resected specimen was associated with survival [103]. Only one study in full resection specimens compared more than one tumour compartment, finding ST CD8 to be significantly associated with survival, where IE CD8 was not [101]. Three studies used an electronic method of assessment [64,101,102], of which 2 studies found CD8 to be significant for survival [64,101]. Eight studies used a manual assessment method [69,76,100,103–106,137], of which 7 found CD8 to be significant for survival [69,76,100,103–105,137]. Three studies assessed the presence of MSI: of which 1 did not find MSI to be independently significant for survival [106]; whereas the other 2 did not include MSI in the survival analysis [69,137]. Nine studies used an arbitrary cut-off [69,76,100–106], of which 7 found CD8 to be significant for survival [69,76,100,101,103–105]. Two studies used a data-driven cut-off, both of which were significant for survival [64,137].

Three studies met inclusion criteria for meta-analysis for IT CD8 in rectal cancer (Table 5, Fig. 6A): one assessed DFS and OS, finding only DFS to be significant for survival; and two assessed DSS (HR 0.52; 95% CI: 0.39–0.69), with substantial heterogeneity (I^2 73%; $p = 0.05$). Funnel plot could not be interpreted due since there were too few studies. No studies met inclusion criteria for meta-analysis of IM CD8 in rectal cancer.

In colon cancer, there were six independent studies (811 patients) assessing CD8 and survival: of which four (650 patients) found a significant impact on survival [67,77,78,107], whereas 2 did not [79,134], although one of these was in stage IV disease [134]. All the studies assessing CD8 in colon cancer assessed the intratumoural compartment. The only study assessing both IE CD8 and ST CD8 separately found both to be significant for survival [107]. Two studies (193 patients) assessed CD8 at the invasive margin [78,79], of which only one (89 patients) was significant for survival [78]. Only one study (89 patients) assessed CD8 on the whole slide, finding it to be significant for survival [78]. This study was also the only study with significant results comparing intratumoural and invasive margin CD8, finding both to be significant for survival [78]. Three studies used an electronic method of assessment [77–79], of which two found CD8 to be significant for survival [77,78]. Three studies used a manual method of assessment [67,107,134], of which 2 found CD8 to be significant for survival [67,107]. Four studies

Table 5
Meta-analysis results for papers meeting inclusion criteria for Rectal cancer.

Impact of study methodology on heterogeneity testing (I^2 test) and overall effect

		Overall effect			Heterogeneity		
Location assessed	Survival type	No. of studies	HR	95% CI	I^2 test (%)	*P*-value	First Author Surname/year
CD8							
IT	DFS	1	0.38	0.14–0.99	NA		Chen 19
	OS	1	0.44	0.13–1.53	NA		Chen 19
	DSS	2	0.52	0.39–0.69	73	0.05	Rosenbaum, Zlobec 08a
FoxP3							
IT	DFS	1	0.72	0.56–0.93	NA		Reimers
	OS	1	0.73	0.56–0.95	NA		Reimers
CD56/57							
IT	OS	1	0.23	0.08–0.66	NA		Alderdice

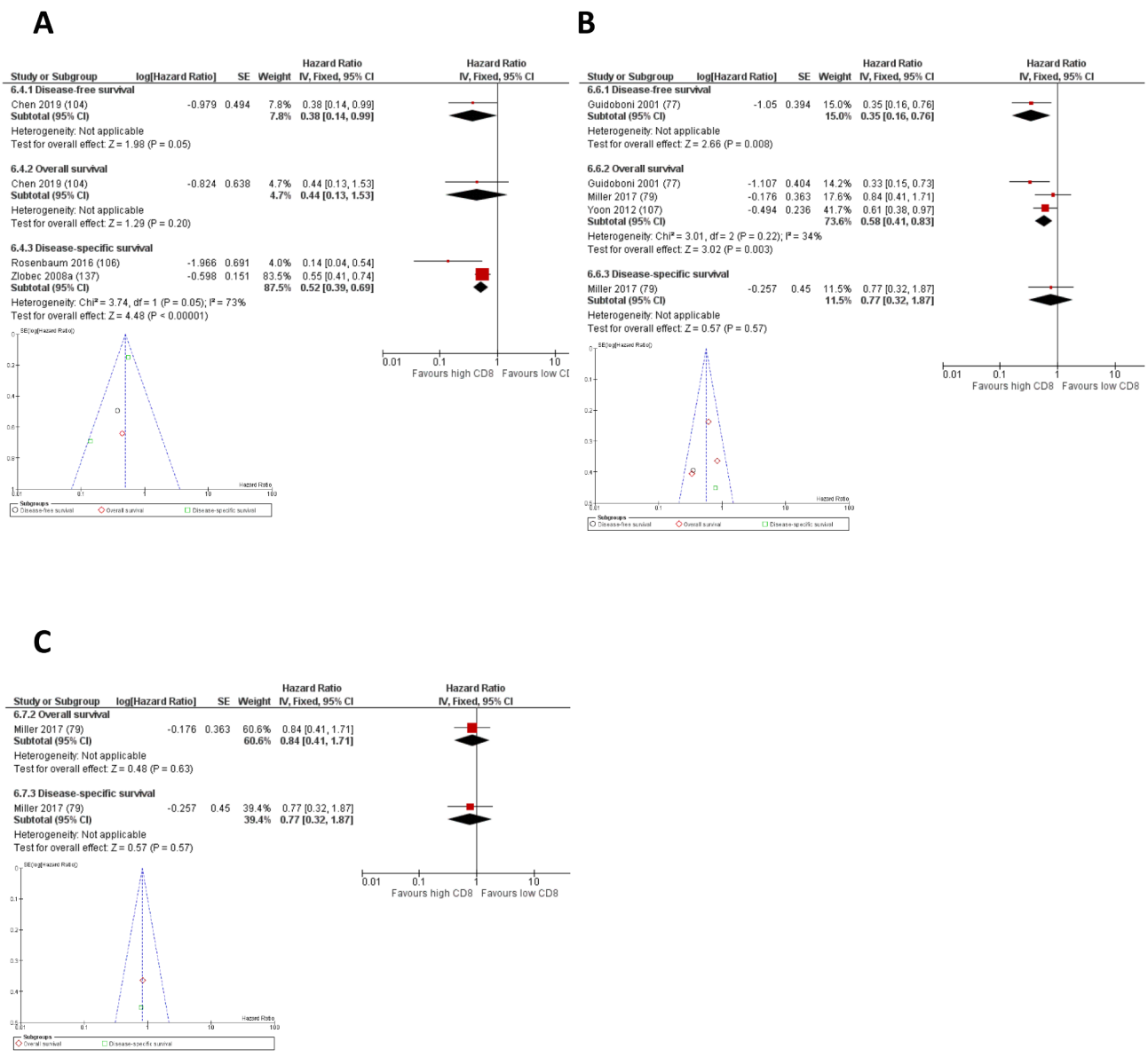

Fig. 6. Forest plots and funnel plots for CD8 according to DFS, OS and DSS: (A) IT in rectal cancer; (B) IT in colon cancer; (C) IM in colon cancer.

assessed the presence of MSI: of which 1 found IE CD8 to be independent of MSI [77]; one found ST CD8, but not IE CD8 to be independent of MSI [107]; one found that MSI was not significant for survival [79]; and one excluded all MSI patients from survival analysis [67]. Four studies used an arbitrary data cut-off for analysis [77,78,107,134], of which 3 were significant for survival [77,78,107]. Two studies used a data-driven cut-off [67,79], of which one was significant [67].

Four studies met inclusion criteria for meta-analysis for IT CD8 in colon cancer (Table 3, Fig. 6C): one assessed and was significant for DFS; three assessed OS (HR 0.58; 95% CI: 0.41–0.83), with moderate heterogeneity (I^2 34%; p = 0.22); and one assessed DSS finding no significant difference in survival. Funnel plot could not be assessed for publication bias. Two studies were included for IM CD8: one assessed DFS finding borderline significance; the other assessed OS and DSS finding no significant survival difference for either.

In colorectal cancer, there were 44 papers assessing CD8, 38 of which (11274 patients) assessed independent cohorts [9,13,30,37,39,59,70,71,81–86,88,89,91–93,98,108–125], of which 26 independent studies (9903 patients) found CD8 to be significant for survival [9,13,30,37,39,70,71,81–83,85,86,92,93,98,108,109,112,114,116–119,121–125]. Thirty-four independent studies (10168 patients) assessed intratumoural CD8 [9,13,30,37,39,70,71,81–86,88,89,91–93,98,108–117,119–122,125], of which 25 (8736 patients) were significant for survival [13,30,37,39,70,71,81–83,85,86,92,93,98,108,109,112,114,116,117,121,122,125]. Of the nine studies that directly compared intratumoural CD8 assessment: three studies (625 patients) found IE significant where ST was not [75,81,121]; two studies (460 patients) found ST significant where IE was not [83,125]; three studies (1294 patients) found both IE and ST to be significant [71,85,98]; and one (291 patients) study compared IE assessment with combined assessment of IE and ST and found IE alone to be significant [37]. There were 17 independent studies (4027 patients) that assessed CD8 at the invasive margin [9,30,54,71,75,81,83,84,89,91,98,109,111,120,123–125], of which eight (2491 patients) found IM CD8 to be significant for survival [9,54,71,98,109,123–125]. Ten studies (2271 patients) compared CD8 at the IM and in the intratumoural compartment: of which only one (96 patients) found IM CD8 to be significant for survival where intratumoural measures were not [9]; four studies (691 patients) found intratumoural CD8 significant where IM CD8 was not [30,75,81,83]; five studies (1484 patients) found both intratumoural and IM CD8 to be significant for survival [54,71,98,109,125]. Four

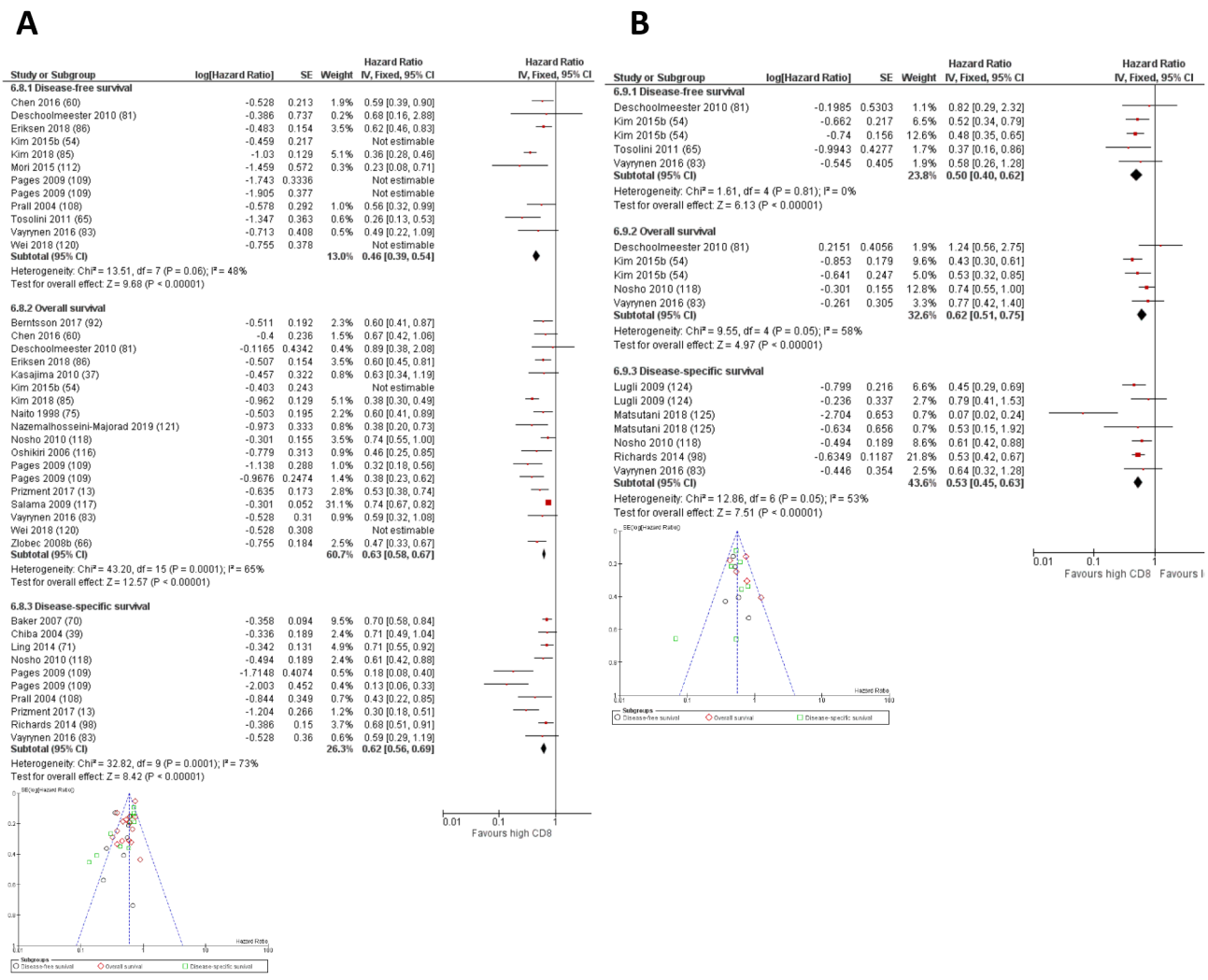

Fig. 7. Forest plots and funnel plots for CD8 according to DFS, OS and DSS: (A) IT in colorectal cancer; (B) IM in colorectal cancer.

independent studies compared CD8 on the whole slide (combined IM and intratumoural): of which three (1335 patients) were significant for survival [71,109,118]; and one was not significant, but this study was small with only 35 patients [59]. Fifteen independent studies (5475 patients) used an electronic method of assessment [13,59,82,83,85,86,91–93,109,111,117–120], of which 11 (4949 patients) found CD8 to be significant for survival [13,82,83,85,86,92,93,109,117–119]. Twenty-two independent studies (5534 patients) used a manual method of assessment [9,30,37,39,70,71,81,84,88,89,98,108,110,112–116,121–123,125], of which 16 (4689 patients) found CD8 to be significant for survival [9,30,37,39,70,71,81,98,108,112,114,116,121–123,125]. Eighteen independent studies assessed the presence of MSI: of which 4 found CD8 to be independent of MSI [71,108,121,125]; five found MSI to be not significant for survival [39,86,88,113,117]; three found that CD8 was not independent of MSI on multivariate analysis [81,83,92]; and six did not include MSI in multivariate analysis [9,13,85,112,114,124]. Twenty-five studies (6853 patients) used an arbitrary data cut-off [9,13,37,39,71,81,84–86,88,89,98,108,110–113,115,117–121,123,125], of which 17 (5936 patients) found CD8 to be significant for survival [9,13,37,39,71,81,85,86,98,108,112,117–119,121,123,125]. Seven studies used a data-driven cut-off, all of which found CD8 to be significant for survival [82,83,92,93,109,114,124]. The cut-off was unclear for 5 studies [30,59,91,116,122].

Twenty-two studies met inclusion criteria for meta-analysis for IT CD8 in colorectal cancer (Table 4, Fig. 7A): eight assessed DFS (HR 0.46; 95% CI: 0.39–0.54), with moderate heterogeneity between these results (I^2 48%; $p = 0.06$); fifteen assessed OS (HR 0.63; 95% CI: 0.58–0.67), with substantial heterogeneity (I^2 65%; $p < 0.001$); and nine assessed DSS (HR 0.62; 95% CI: 0.56–0.69), with substantial heterogeneity (I^2 73%; $p < 0.001$). The funnel plot revealed no significant publication bias, although one study in particular is seen as an outlier for DFS, OS and DSS [109].

Eight studies were included for IM CD8 (Table 4, Fig. 7B): four assessed DFS (HR 0.50; 95% CI: 0.40–0.62), with no significant heterogeneity; four assessed OS (HR 0.62; 95% CI: 0.51–0.75), with substantial heterogeneity (I^2 58%; $p = 0.05$); and five assessed DSS (HR 0.53; 95% CI: 0.45–0.63), with substantial heterogeneity (I^2 53%; $p = 0.05$). The funnel plot shows no significant publication bias. There is one small left-sided outlier skewing results and heterogeneity for DSS, although curiously only for one of the two populations in the same study [125]. Exclusion of this outlying population resulted in no significant heterogeneity for DSS, data not shown.

CD4 (predominantly helper T-cells)

CD4 is a surface marker predominantly expressed by helper T-cells, although it is expressed to a lesser degree by other immune cells and therefore is not entirely helper T-cell specific [138]. CD4 T-cells play a considerable role in anti-cancer immunity with both cytotoxic capabilities and roles in recruitment and priming both cytotoxic T-cells and recruiting B-cells to the tumour microenvironment [139]. There were

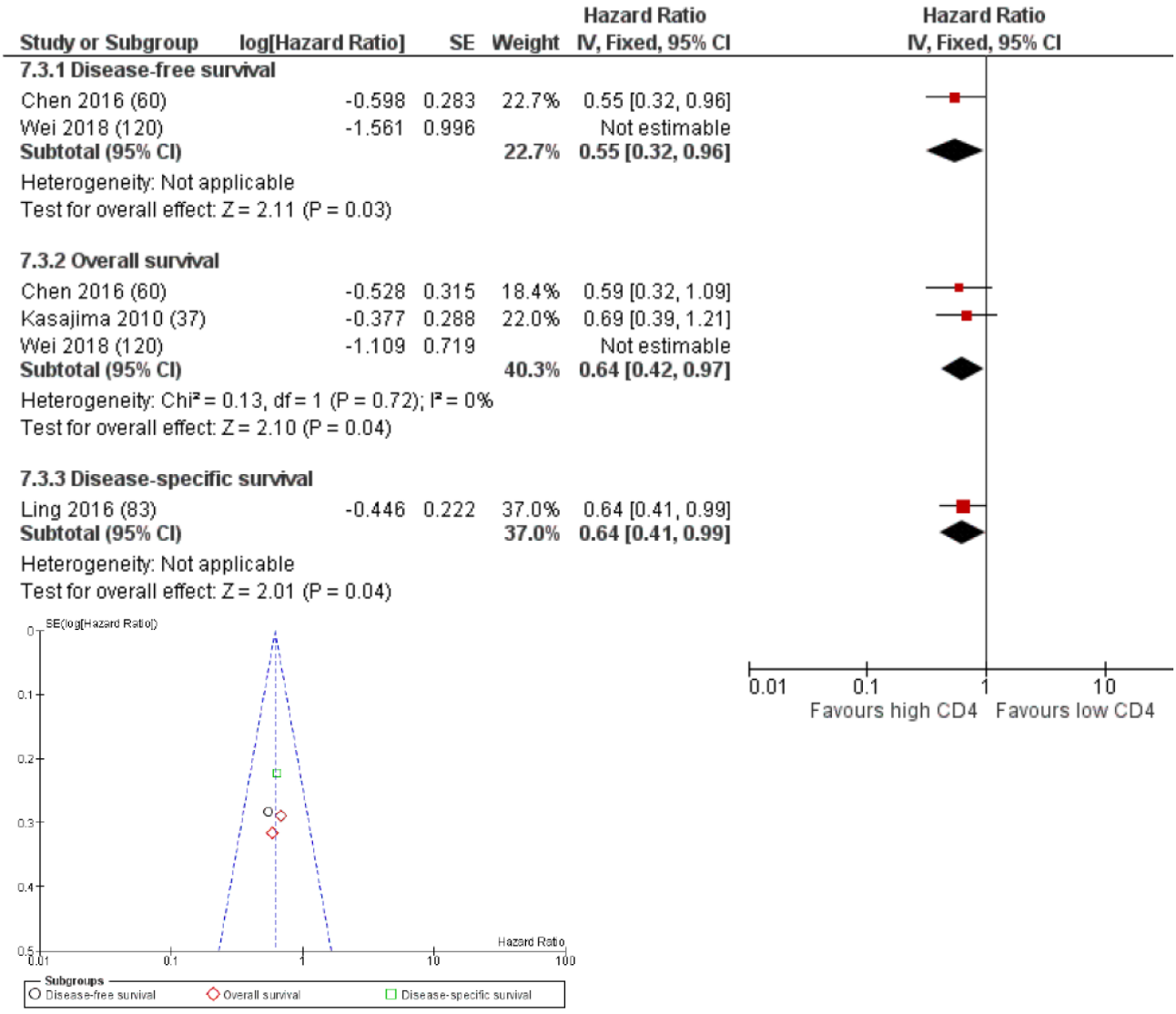

Fig. 8. Forest plot and funnel plot for IT CD4 according to DFS, OS and DSS in colorectal cancer.

fifteen studies on CD4 and rectal or colorectal cancer survival, but the cohort in one overlapped with another [113], leaving fourteen independent studies comprising 2726 patients [9,30,37,56,59,60,71,73,82,91,93,111,125,126]. One of these (62 patients) assessed survival in rectal cancer but did not meet inclusion criteria for meta-analysis. They assessed pre-treatment biopsies but did not find IT CD4 to be significant for survival [56].

The remaining thirteen independent studies (2664 patients) assessed CD4 in colorectal cancer: of which six studies (1471 patients) found CD4 to be significantly associated with better survival [37,60,71,73,82,93]. Whereas the 7 remaining studies (1193 patients) found no association between CD4 and survival [9,30,59,91,111,125,126]. Eleven studies assessed intratumoural CD4 [9,30,37,60,71,73,82,91,93,111,125], of which six found it to be significant for survival [37,60,71,73,82,93]. None of the 5 studies assessing CD4 at the invasive margin, nor the 2 studies assessing CD4 on the whole slide found any significant association with survival [9,30,59,91,111,125,126]. There were no studies with significant findings that compared CD4 in different tumour regions. Six studies used an electronic method of assessment [59,60,82,91,93,111], of which 3 found CD4 to be significant for survival [60,82,93]. Seven studies used a manual method of CD4 assessment [9,30,37,71,73,125,126], of which 3 found it to be significant for survival [37,71,73]. Four studies assessed the presence of MSI: of which 2 found CD4 to be independent of MSI for survival [71,125]; one did not find MSI to be significant for survival [113]; and one did not include MSI in multivariate analysis [9]. Seven studies used an arbitrary data cut-off [9,37,71,73,111,113,125], of which 3 found CD4 to be significant for survival [37,71,73]. Three studies used a data-driven cut-off, all of which were significant for survival [60,82,93]. The data cut-off was unclear for 4 studies [30,59,91,126].

Three studies met inclusion criteria for meta-analysis for IT CD4 in colorectal cancer (Table 4, Fig. 8): one assessed DFS finding a significant survival benefit; two assessed OS (HR 0.64; 95% CI: 0.42–0.97), with no significant heterogeneity; one assessed DSS finding a significant survival benefit. There were too few papers to give meaningful results from funnel plot analysis. No studies reporting IM CD4 were included in meta-analysis. There were no studies identified addressing CD4 in colon cancer.

CD45RO (effector memory T-cells)

CD45RO is a surface marker expressed by effector memory T-cells and has largely been studied in this context. However, it can also be expressed by B-cells and to a lesser degree by other immune cells and therefore is not entirely effector memory T-cell specific [140–142]. The

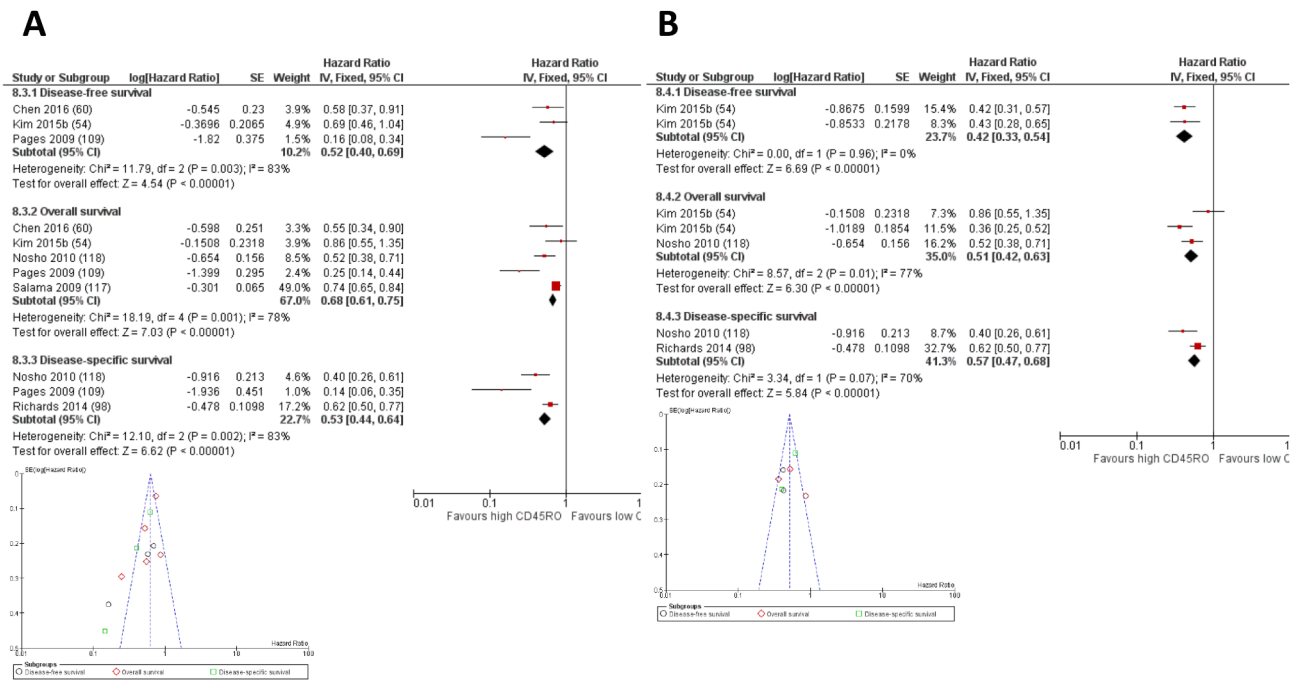

Fig. 9. Forest plots and funnel plots for CD45RO according to DFS, OS and DSS: (A) IT in colorectal cancer; (B) IM in colorectal cancer.

role of effector memory T-cells is to enact a swift response to a recognised foreign antigen [143]. There were fifteen studies assessing CD45RO and survival in rectal, colon or colorectal cancer, one of which had an overlapping cohort [61], leaving 14 independent studies comprising 4235 patients [54,60,69,84,93,98,109,111,117–119,122,127,131]. Twelve studies found CD45RO to be associated with better survival, but only eleven (3992 patients) of these were independent [54,60,69,98,109,117–119,122,127,131].

In rectal cancer, there were two studies (263 patients) assessing CD45RO, both finding it to be significantly associated with survival: one of which performed a manual assessment of CD45RO in pre-treatment biopsies [69], the other an electronic assessment in post-resection specimens [131]. Neither study compared CD45RO in more than one tumour region. One assessed the presence of MSI but did not include this in multivariate analysis [69]. Both studies used an arbitrary data cut-off. Neither study met inclusion for meta-analysis in rectal cancer.

In colon cancer, there were two studies (166 patients) assessing intratumoural CD45RO, both finding it to be significantly associated with survival: one of which used a manual method [61], while the other used an electronic counting method [127]. Neither study assessed the invasive margin, nor the presence of MSI. Both studies used an arbitrary data cut-off. Neither met inclusion criteria for meta-analysis of CD45RO in colon cancer.

In colorectal cancer, there were 11 studies (3885 patients) assessing CD45RO and survival [54,60,84,93,98,109,111,117–119,122], of which 8 studies (3642 patients) found a significant association with survival [54,60,98,109,117–119,122]. In those not primarily assessing stage IV disease, comprising 3590 patients, 7 out of 8 studies reported survival advantage for those with high CD45RO [54,60,98,109,111,117,118,122], with only 1 small study (27 patients) reporting no significance [111]. Ten studies assessed intratumoural CD45RO [54,60,84,93,98,109,111,117,119,122], of which seven found it to be associated with survival [54,60,98,109,117,119,122]. The only study comparing CD45RO in more than one intratumoural compartment found both IE and ST CD45RO to be significantly associated with survival [98]. Five studies assessed CD45RO at the invasive margin [54,84,98,109,119], of which 4 found it to be significant for survival [54,98,109,119]. Of the 4 studies that compared assessment at the invasive margin compared with intratumoural assessment, all found that both areas were associated with better survival [54,98,109,119]. Two studies performed a combined assessment of the full slide (invasive margin and intratumoural) and both found CD45RO to be significantly associated with survival [109,118]. Eight studies used an electronic method of assessment [54,60,93,109,111,117–119], of which 6 found CD45RO to be significant for survival [54,60,109,117–119]. Of the 3 studies using a manual assessment method: two found CD45RO to be significant for survival [98,122], whereas one did not [84]. Two studies assessed the presence of MSI, but neither included it in multivariate analysis [54,117]. Seven studies used an arbitrary data cut-off [54,84,98,111,117–119], of which 5 found CD45RO to be significantly associated with survival [54,98,117–119]. Three studies used a data-driven cut-off [60,93,109] and 2 of these found CD45RO to be associated with survival [60,109]. Data cut-off was unclear in one study [122].

Six studies met inclusion criteria for meta-analysis for IT CD45RO in colorectal cancer (Table 4, Fig. 9B): three assessed DFS (HR 0.52; 95% CI: 0.40–0.69), with substantial heterogeneity between these results (I^2 83%; $p = 0.003$); five assessed OS (HR 0.53; 95% CI: 0.61–0.75), with substantial heterogeneity (I^2 78%; $p = 0.001$); and three assessed DSS (HR 0.53; 95% CI: 0.44–0.64), with substantial heterogeneity (I^2 83%; $p = 0.002$). As with CD8, the funnel plot revealed no significant publication bias, although one study in particular is seen as an outlier for DFS, OS and DSS [109], skewing results to the left and removal of this study from meta-analysis results in a large fall in the heterogeneity to 0%, 51% and 70% for DFS, OS and DSS, respectively (data not shown). Three studies were included for IM CD45RO (Table 4, Fig. 9C): one assessed DFS finding survival benefit; two assessed OS (HR 0.51; 95% CI: 0.42–0.63), with substantial heterogeneity (I^2 77%; $p = 0.01$); and two assessed DSS (HR 0.57; 95% CI: 0.47–0.68), with substantial heterogeneity (I^2 70%; $p = 0.07$). Funnel plot contained too few studies to give meaningful data regarding publication bias, although the plot was narrow indicating larger studies with similar results.

FoxP3 (regulatory T-cells)

FoxP3 is commonly expressed by regulatory T-cells. A large body of research has been performed on regulatory T-cells in a variety of cancers as well as non-neoplastic research. Due to the fact that their function is to regulate the immune system by suppressing T-cell activity, thereby preventing overactivity and autoimmunity [144], their presence in and around colorectal cancer may be expected to be a poor prognostic indicator as some have hypothesised [110]. Some have gone as far as to say that they may be pro-tumour [110,145]. Thirty-four studies were identified that assessed rectal, colon or colorectal cancer prognosis related to FoxP3 expression in TILs, but there were three overlapping cohorts [58,68,102], leaving 31 independent studies (7991 patients) [54,57,59,60,71,74,79,82–84,90,92,98,100,101,107,110,112,115,117–120,126–130,132–134]. Twenty-one studies found FoxP3 to be associated with better survival, but only twenty (6774 patients) of these were independent [54,60,71,79,83,90,92,98,101,107,117–119,127–130,132–134], compared with 2 studies (218 patients) finding FoxP3 to have a detrimental impact on survival [57,130], one of which had both positive and negative findings [130]. Twelve studies found no impact of TILs FoxP3 expression on survival [58,59,74,82,84,100,102,110,112,115,120,126].

In rectal cancer, there were four independent studies (840 patients) assessing FoxP3: of which two (631 patients) found a positive association with survival [101,128]; one (128 patients) found a negative association with survival [57]; and one found no association [100]. Three studies assessed FoxP3 in pre-treatment biopsies: of which only one (153 patients) found it to have a significant positive association with survival [101]; while the other two found no association [100,102]. Four studies assessed the intratumoural compartment of post-resection specimens: of which one study found a significant positive association with survival [128]; one found a significant negative

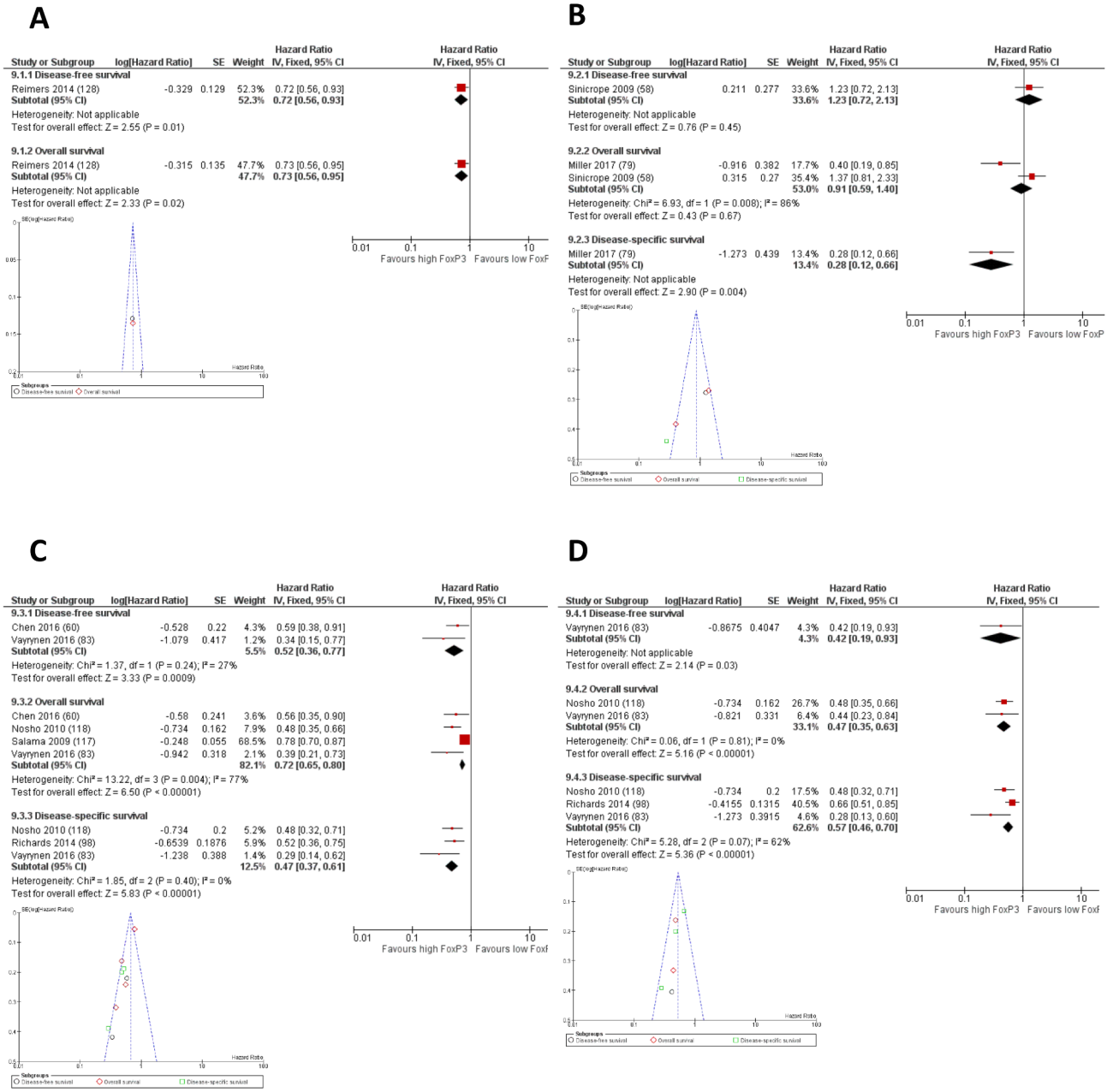

Fig. 10. Forest plots and funnel plots for FoxP3 according to DFS, OS and DSS: (A) IT in rectal cancer; (B) IT in colon cancer; (C) IT in colorectal cancer; (D) IM in colorectal cancer.

association [57]; and two found no significant association [100,101]. One study assessed FoxP3 in both pre-treatment biopsies and post-resection specimens, finding its presence in the biopsy to be significant for better survival, whereas there was no survival significance of FoxP3 in the resected specimen [101]. Of the two studies that assessed the same cohort: one assessed FoxP3 in pre-treatment biopsies, finding no significant difference to survival [102], whereas in the resected specimen, there was a significant negative association of the presence of FoxP3 with survival [57]. No groups assessed the invasive margin or the whole tumour slide for FoxP3 in rectal cancer. Two independent studies used an electronic method of assessment and a data-driven cut-off, of which one (128 patients) found a negative impact of FoxP3 infiltration in the resected tumour on survival [57], whereas one (153 patients) found a positive impact of FoxP3 in the pre-treatment biopsy on survival [101]. Two studies used a manual method of assessment and arbitrary cut-off, of which one (81 patients) found no significant impact on survival in pre-treatment biopsy or resected specimen [100], whereas one (478 patients) found a significant positive impact of FoxP3 on the resected specimen [128]. No studies assessed the presence of MSI.

Only one study met inclusion criteria for meta-analysis of IT FoxP3 in rectal cancer (Table 5, Fig. 10A): assessing DFS and OS, with significant positive survival association for both.

In colon cancer, there were five independent studies (540 patients) assessing FoxP3, all of which found a positive association with survival [79,107,127,129,134]. Four of these assessed the intratumoural compartments [79,107,127,134] and two assessed the invasive margin [79,129]. The only study (104 patients) assessing both intratumoural compartments and the invasive margin found that IT FoxP3 was significant for better survival, whereas IM FoxP3 was not significant [79]. However, the other study of IM FoxP3 (136 patients) found that this was significant for better survival [129]. None of the studies assessed FoxP3 in the whole slide in colon cancer. Three studies used an electronic method of assessment [79,127,134], whereas two used a manual assessment method [107,129]. Three studies assessed the presence of MSI: of which one found the presence of FoxP3 to be independent of MSI [107]; one found that MSI was not significant for survival [79]; and one found that FoxP3 was not independent of MSI [129]. Three studies used an arbitrary data cut-off [107,127,134], while two used a data-driven cut-off [79,129].

Two studies met inclusion criteria for meta-analysis of IT FoxP3 in colon cancer (Table 3, Fig. 10B): one assessed DFS finding no survival association; two assessed OS (HR 0.91; 95% CI: 0.59–1.40), with substantial heterogeneity (I^2 86%; $p = 0.008$); and one assessed DSS, finding a significant positive survival association. The funnel plot was wide and one reason for this is that some of the results tended towards poorer survival and some towards better survival. No comment can be made regarding publication bias since the number of studies was too few.

In colorectal cancer, twenty-two independent studies (6611 patients) assessed the association [54,59,60,71,74,82–84,90,92,98,110,112,115,117–120,126,130,132,133] of FoxP3: of which thirteen (5603 patients) found it to be significant for survival [54,60,71,83,90,92,98,117–119,130,132,133]. Nineteen independent studies (5220 patients) assessed intratumoural FoxP3 [54,60,71,74,82–84,90,92,98,110,112,115,117,119,120,130,132,133], of which eleven studies (4898 patients) were significantly associated with survival [60,71,83,90,92,98,117,119,130,132,133]. One of these papers found both negative and positive influence on survival depending on whether FoxP3 T-cells were found in the intraepithelial compartment or combined intraepithelial and stromal compartments, respectively [130]. There was only one other study with significant results that assessed more than one intratumoural compartment for FoxP3, finding that both IE and ST FoxP3 were significant for better survival [98]. Six independent studies (1597 patients) assessed FoxP3 at the invasive margin [54,71,83,84,98,120], with a positive survival association in four studies comprising 1281 patients [54,71,83,98]. Of the three studies with significant findings that assessed both intratumoural FoxP3 and at the invasive margin, all found the presence of FoxP3 to be associated with better survival, regardless of tumour compartment assessed [71,83,98]. Four studies performed a combined assessment of FoxP3 at the invasive margin and intratumoural [59,71,118,126], of which 2 found a significant positive association with survival [71,118]. Eleven studies (3822 patients) used an electronic method of assessment [54,59,60,82,83,90,92,117–120], of which eight (3336 patients) found a significant association of FoxP3 with better survival [54,60,83,90,92,117–119]. Eleven studies (2789 patients) used a manual method of assessment [71,74,84,98,110,112,115,126,130,132,133], of which five (2267 patients) found a significant effect of FoxP3 on survival [71,98,130,132,133], one of which was both positive and negative [130]. Eight studies assessed the presence of MSI: of which one found FoxP3 to be independent of MSI [71]; two found MSI not to be independently significant for survival [74,117]; one study assessed FoxP3 in MSI and MSS subgroups separately and found FoxP3 to be significant in the MSS subgroup [132]; two groups did not include MSI in multivariate analysis [54,112]; and two groups found that FoxP3 was not independent of MSI for survival [83,92]. Fifteen independent studies (4040 patients) used an arbitrary data cut-off [54,71,74,84,90,98,110,112,115,117–120,130,133], of which nine studies (3357 patients) found a significant association with survival [54,71,90,98,117–119,130,133]. Five studies (2434 patients) used a data-driven cut-off [60,82,83,92,132], four (2246 patients) of which were associated with better survival [60,83,92,132]. For two studies (137 patients), the data cut-off method was unclear [59,126].

Five studies met inclusion criteria for meta-analysis of IT FoxP3 in colorectal cancer (Table 4, Fig. 10C): two assessed DFS (HR 0.52; 95% CI: 0.36–0.77), with no significant heterogeneity; four assessed OS (HR 0.72; 95% CI: 0.65–0.80), with substantial heterogeneity (I^2 77%; $p = 0.004$); and three assessed DSS (HR 0.47; 95% CI: 0.37–0.61), with no significant heterogeneity. Funnel plot appeared to be skewed to the left indicating a bias against smaller studies with no significant difference in survival outcome. Three studies were included for IM FoxP3 (Table 4, Fig. 10D): one assessed DFS finding a significant survival benefit; two assessed OS (HR 0.47; 95% CI: 0.35–0.63), with no significant heterogeneity; and three assessed DSS (HR 0.57; 95% CI: 0.46–0.70), with substantial heterogeneity (I^2 63%; $p = 0.07$). Funnel plot contained too few studies to comment on publication bias.

Immunoscore

The Immunoscore is a patented score and a trademark of Inserm developed by Galon and colleagues over the last two decades [6,63,64,109,146,147]. The score involves an electronic assessment of the presence of two T-cell markers in the tumour centre (IE + ST) and at the IM giving 4 parameters, dichotomised in each region and for each cell type with a ROC curve into high or low. One point is assigned to each parameter scoring “high” and these points are added together to give a maximum score of 4 and a minimum of 0 [109]. The original score used CD3 and CD45RO [63] as the two immune markers. Over time this evolved to use a combination of CD45RO and CD8 [109,147], but was subsequently modified again to use CD3 and CD8 [6,64]. This latter combination has been validated in a large international consortium with 2681 patients [6]. The score can be performed on either TMA [63,64,109,147] or whole sections [6,148]. This work has been replicated by other groups: some using the same software [149], others using alternative electronic cell counting software [12,62,99,150], and others performing a manual assessment using the immunoscore method [98,151]. Still others have adapted the score with additional items in the case of stage IV disease such as the addition of a Granzyme B marker (an additional marker of cytotoxic T-cell activation) [152], assessment of CD3 and CD8 in the tumour metastasis as well as the primary [153,154] or addition of CD163 (an M2 macrophage marker) [153], since stage IV disease is not effectively stratified by the immunoscore

alone [63]. In total, fourteen studies assessed a version of the Immunoscore in rectal, colon or colorectal cancer, although three studies had overlapping populations [64,109,151], leaving eleven independent studies comprising 4624 patients [6,12,62,98,99,147,149,150,152–154], of which only one (60 patients with stage IV disease) found no significant association with survival [154].

In rectal cancer, one study (83 patients) assessed the Immunoscore, which was significant for survival [64]. They used an electronic method, counting CD3 and CD8 stained cells, and a data-driven cut-off. MSI was not assessed and they used the original 5 group split [64]. This study did not meet inclusion criteria for meta-analysis.

There were two studies (2827 patients) in colon cancer [6,12], both of which were significant for better survival. Both studies assessed primary colon cancer, although there were issues in one study with scoring CD8 in the tumour centre, leaving a total score of 3 [12]. Both studies used an electronic method of assessment, although one used the patented software [6] and the other used freeware [12]. Both studies assessed the presence of MSI: one found Immunoscore to be independent of MSI [6]; the other was unclear since they used a forward stepwise model [12]. One study used an arbitrary cut-off [6], whilst one used a data-driven cut-off [12].

One study met inclusion criteria for meta-analysis (Table 3) for immunoscore in colon cancer and this was significant for DFS and OS.

In colorectal cancer, two groups used overlapping cohorts [109,151], leaving nine independent studies comprising 1797 patients [62,98,99,147,149,150,152–154]. Only one of these studies was not significant for survival and this was a small study of 60 patients with stage IV disease [154]. There were four studies assessing the efficacy of

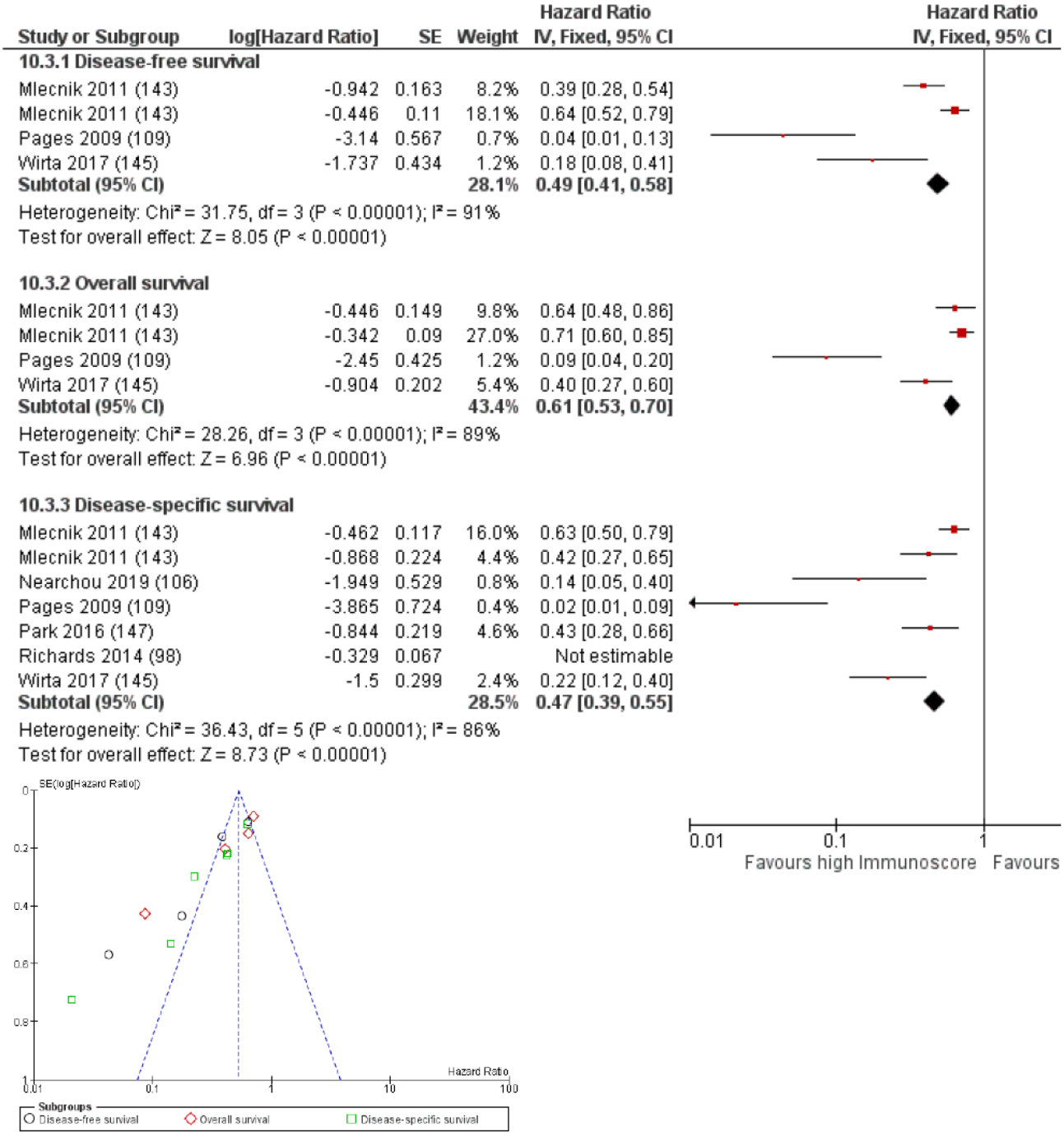

Fig. 11. Forest plot and funnel plot for Immunoscore in colorectal cancer according to DFS, OS and DSS.

the Immunoscore in metastatic disease, all of whom adapted the immunoscore to suit: two groups assessed the immunoscore only on liver metastases [62,152], one of whom added Granzyme B as a third marker in addition to CD3 and CD8 [152]; the other two assessed the immunoscore in the primary tumour centre, invasive margin and in the metastasis [153,154]; one of these also added in assessment of CD163 (as an M2 macrophage marker) in the primary in addition to the immunoscore [153]. Eight used an electronic method of assessment [62,99,147,149,150,152–154], although two of these used freeware [62,150]. One group used a manual method of assessment [98]. Three groups assessed the presence of MSI: of which one found Immunoscore to be independent of MSI [149]; one found that MSI was not significant for survival [151]; and one had only MSS tumours [152]. Three studies employed an arbitrary data cut-off method [62,98,150]. Five studies employed a data-driven cut-off, including the study that did not find a significant difference [99,147,149,153,154]. One study's data cut-off method was unclear [152]. Three groups used the originally proposed 5-category method of splitting cases [98,147,149]; whereas the remaining six dichotomised the data to give a high vs low score [62,99,150,152–154]).

Five studies met inclusion criteria for meta-analysis for Immunoscore in colorectal cancer (Table 4, Fig. 11): three assessed DFS (HR 0.49; 95% CI: 0.41–0.58), with considerable heterogeneity (I^2 91%; $p < 0.001$); three assessed OS (HR 0.61; 95% CI: 0.53–0.70), with considerable heterogeneity (I^2 89%; $p < 0.001$); and five assessed DSS (HR 0.47; 95% CI: 0.39–0.55), with considerable heterogeneity (I^2 86%; $p < 0.001$). Funnel plot assessment was performed which showed that publications were significantly skewed to the left, with no studies showing no significant difference and the plot itself was wide indicating large variations in results. One study in particular was identified as an outlier [109].

CD20 (B-cell marker)

CD20 is a generic B-cell marker, whose role in anti-tumour pathophysiology is to interact with T-cells in co-ordinating the host defence by producing various cytokines and chemokines in order to recruit and activate T-cells, in addition to acting as antigen presenting cells [155,156]. Furthermore, mature B-cells produce antibodies directed against foreign entities [155,156]. They tend to form tertiary lymphoid structures or lymphoid aggregates in colorectal cancer [41], but are also found infiltrating the tumour itself [89,91].

There were six independent studies (1491 patients) staining for CD20 in colorectal cancer [60,89,91,157–159], of which five (1374 patients) found it to be significant for better survival [60,91,157–159]. Four papers assessed CD20 in the intratumoural compartments [60,89,91,159], of which three found a significant association with survival [60,91,159]. Two papers assessed CD20 at the invasive margin [89,158], of which only one was significant for survival [158]. One study assessed CD20 on the whole slide (intratumoural as well as at the invasive margin) and this was significant for better survival [157]. There were no studies with significant findings comparing more than one tumour compartment. Four studies used an electronic method of assessment, all of which were significant for survival [60,91,157,158]. Two studies used a manual method of assessment [89,159], of which one was significant for survival [159]. Only one study assessed the presence of MSI but did not include it in multivariate analysis [159]. Two studies used an arbitrary data cut-off [89,91], of which one was significant for survival [91]. Three used a data-driven cut-off, all of which were significant for survival [60,157,159]. The data cut-off was unclear in one study [158].

Three studies met inclusion criteria for meta-analysis for IT CD20 in colorectal cancer (Table 4, Fig. 12): one assessed DFS finding it to be

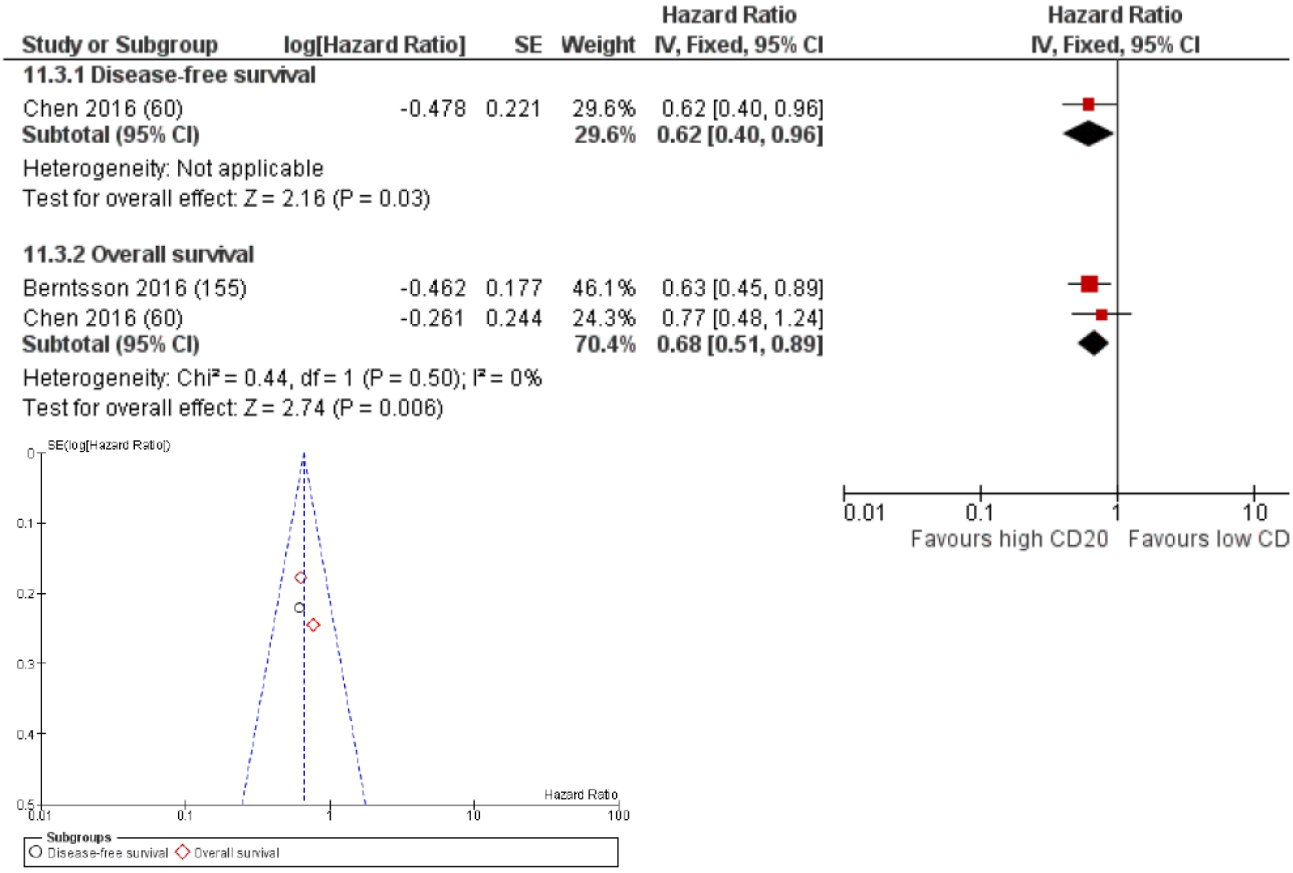

Fig. 12. Forest plot and funnel plot for IT CD20 in colorectal cancer according to DFS and OS.

significant for survival; two assessed OS (HR 0.66; 95% CI: 0.52–0.89), with no significant heterogeneity. Funnel plot provided no meaningful data regarding publication bias.

CD56/CD57 (natural killer cells)

CD56 and CD57 are cell surface markers commonly expressed on the surface of natural killer (NK) cells [160–162], whose role as part of the innate immune system is to induce cell lysis although they may also have an immunoregulatory role [163]. CD56 is a more generic NK cell marker and can potentially also be expressed on other immune cells [160], whereas CD57 is a mature NK cell marker and is suggestive of terminal differentiation [162].

One study (149 patients) assessed natural killer cells in rectal cancer, finding increased density to be associated with survival in the post-resection specimen [164]. They measured intratumoural CD56 using an electronic method and an arbitrary data cut-off. MSI was not assessed. This study met inclusion criteria for meta-analysis (Table 5) and was significant for OS.

There were five studies (634 patients) in colorectal cancer [9,29,30,165,166], of which four (502 patients) were significant for better survival [9,29,165,166]. All five studies assessed the intratumoural compartments, of which three found intratumoural natural killer cells to be significant for survival [29,165,166]. Two studies assessed natural killer cells at the invasive margin [9,30], one of which found a significant effect on survival [9]. Only one paper with significant findings assessed natural killer cells in more than one tumour compartment, finding that their presence at the invasive margin was significant for survival, whereas their presence within the tumour was not [9]. However, three other studies assessing intratumoural natural killer cells found their presence to be significant for better survival [29,165,166]. All the studies in natural killer cells in colorectal cancer used a manual method of assessment. Only one study assessed the presence of MSI but did not include it in multivariate analysis [9]. Two studies used an arbitrary data cut-off, both of which were significant for survival [9,29]. One study used a data-driven cut-off, and this was significant for survival [165]. The cut-off was unclear for the remaining two studies [30,166]. When broken down by antibody markers, neither of the papers assessing CD56 in colorectal cancer found any significant difference in survival [9,30], although the study in rectal cancer found the presence of CD56 positive cells to be associated with better survival [164]. All the papers assessing CD57 were significant for survival [9,29,166]. The only paper comparing CD56 and CD57 staining in colorectal cancer found CD57 to be significant for better survival, where CD56 was not [9]. One group stained for intratumoural Va24, a marker of activated natural killer T-cells, which was also asso-ciated with better survival [165].

Two studies met inclusion criteria for meta-analysis of IT CD56/57 in colorectal cancer (Table 4, Fig. 13): both assessed DFS (HR 0.47; 95% CI: 0.28–0.78), with substantial heterogeneity (I^2 71%; $p = 0.06$); both also assessed OS (HR 0.48; 95% CI: 0.28–0.84), with no significant heterogeneity. Funnel plot did not add meaningful data regarding publication bias since numbers were too few. No studies met inclusion criteria for meta-analysis of IM CD56/57.

Macrophages

Part of the innate immune response, macrophages play a main role in phagocytosis and antigen presentation. They have been implicated in anti-tumour defence and T-cell recruitment [167], but have also been implicated in a pro-tumour role [168] and have been shown to the express immune checkpoint protein PDL1 [169], believed to play a role

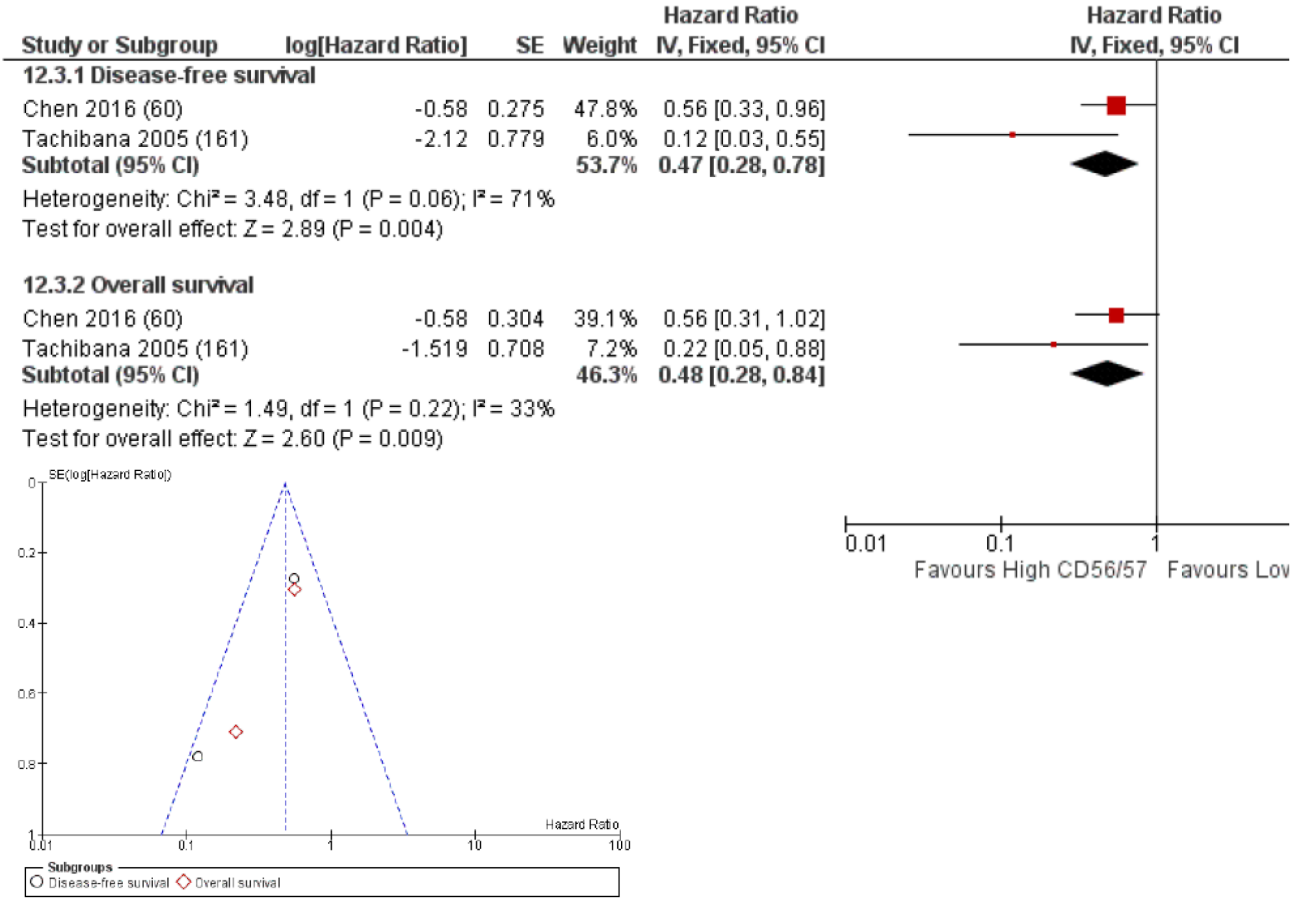

Fig. 13. Forest plot and funnel plot for IT CD56/57 in colorectal cancer according to DFS and OS.

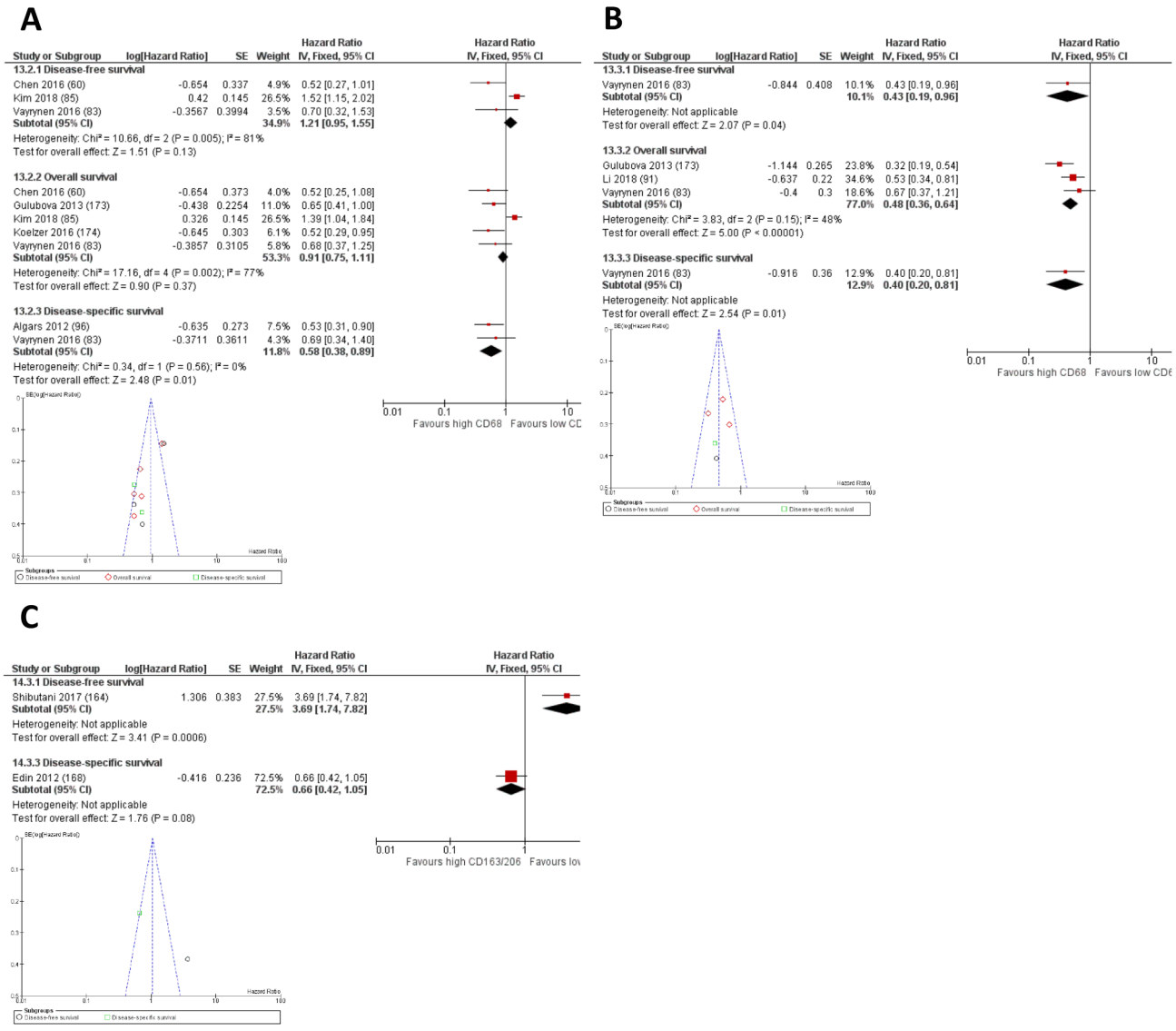

Fig. 14. Forest plots and funnel plots for macrophage markers in colorectal cancer according to DFS, OS and DSS: (A) IT CD68; (B) IM CD68; (C) IM CD163.

in immune resistance [170]. CD68 is a generic macrophage marker, whereas CD163 and CD206 are believed to be markers more specific to M2 tumour associated macrophages that have been associated with a worse outcome [171]. There were twenty-two studies overall in colon or colorectal cancer although one study had an overlapping population [172], leaving twenty-one independent studies comprising 4879 patients [60,83,85,87,89,91,96,111,122,123,168,171,173–181]. Of these, eleven studies (2038 patients) found that higher infiltration of macrophages were associated with a better prognosis [60,83,87,96,122,175–178,180,181], compared with five studies (1965 patients) finding a worse prognosis [85,168,171,173,179].

In colon cancer, two studies (1070 patients) assessed macrophage markers and survival. One study of 835 patients assessed CD68 and CD206 [171]. CD68 assessment in the intratumoural stromal compartment was not associated with survival, whereas CD206 was associated with worse survival [171]. The other paper assessed CD163 in the intratumoural stromal compartment, which was associated with worse survival [173]. One used an electronic assessment method and a data-driven cut-off [171], whereas the other used a manual method and an arbitrary cut-off [173]. Neither paper assessed the invasive margin. Both assessed the presence of MSI: one found that MSI was not significant for survival [171]; the other did not include MSI in survival analysis [173]. Neither study met inclusion criteria for meta-analysis.

Sixteen independent studies (3528 patients) assessed CD68 in colorectal cancer: of which ten (1998 patients) were associated with an improved outcome [60,83,87,96,122,175,177,178,180,181], one (654 patients) was associated with a worse outcome [85] and five (876 patients) found no difference [89,91,111,123,174]. The study that found a negative impact of CD68 on survival measured it in the IE compartment and ST compartment, finding that the presence of CD68 in the IE compartment impacted negatively on survival, whereas CD68 in the ST compartment made no difference to survival [85]. However, another three studies also measured CD68 in the IE compartment: of which two studies (187 patients) found no difference to survival [87,89]; while one (201 patients) found it to be associated with improved survival [178]. Twelve studies in total assessed intratumoural CD68 [60,83,85,87,89,91,96,111,122,174,175,177,178], of which five (796 patients) found it to be associated with better survival [60,96,122,175,178], whereas one found it to be associated with worse survival as already mentioned [85]. Eight studies assessed CD68 at the

invasive margin: of which five found it to be associated with better survival [83,87,177,180,181], compared with 3 that identified no association [89,111,123]. Of the three studies that assessed CD68 both intratumoural and at the invasive margin, all found the invasive margin to be significantly associated with better survival, whereas intratumoural measurement was not significant [83,87,177]. Five studies used an electronic method of assessment [60,83,85,91,111], of which two found CD68 to be associated with better survival [60,83] and one with worse survival [85]. Eleven studies used a manual assessment method [87,89,96,122,123,174,175,177,178,180,181], of which only three found no association with better survival [89,91,123]. Four studies assessed the presence of MSI: of which one found CD68 to be associated with better survival, independent of MSI [181]; one found that CD68 was not independent of MSI [83]; while two did not include MSI in multivariate analysis [85,178]. Eleven studies used an arbitrary data cut-off [85,96,111,122,123,174,175,177,178,180,181], of which only three found no impact on survival [111,123,174]. Two studies used a data-driven cut-off, both of which found a significant association of CD68 with survival [60,83]. The cut-off method was unclear for the other three studies [87,89,91].

Six studies met inclusion criteria for meta-analysis of IT CD68 in colorectal cancer (Table 4, Fig. 14A): three assessed DFS (HR 1.21; 95% CI: 0.95–1.55), with substantial heterogeneity (I^2 81%; $p = 0.005$); five assessed OS (HR 0.91; 95% CI: 0.75–1.11), with substantial heterogeneity (I^2 77%; $p = 0.002$); and two assessed DSS (HR 0.58; 95% CI: 0.38–0.89), with no significant heterogeneity. The funnel plot revealed most results to be grouped towards a positive influence of CD68 on survival, apart from a single large study indicating a negative effect on survival [85], which skews data to the right. Three studies were included for IM CD68 (Table 4, Fig. 14B): one assessed DFS, with a significant positive effect on survival; three assessed OS (HR 0.48; 95% CI: 0.36–0.64), with moderate heterogeneity (I^2 48%; $p = 0.15$); and one assessed DSS with significant positive survival impact.

Six studies (1540 patients) assessed CD163 in colorectal cancer, of which two (508 patients) found it to be associated with an improved survival outcome [172,176], two (241 patients) were associated with a worse outcome [168,179], and two found no association with survival [85,178]. Four studies assess intratumoural CD163 [85,176,178,179], of which one found it to be associated with better survival [176] and one found it to be associated with worse survival [179], while the other two studies found no significant difference in survival outcomes. Two studies assessed IM CD163, of which one was associated with worse survival [168], while the other was associated with better survival [172]. There were no studies that compared CD163 at the invasive margin versus intratumoural. Two studies used an electronic assessment method: one was associated with worse survival [179] and one was not associated with survival [85]. Four studies used a manual assessment [168,172,176,178], of which two were associated with better survival [172,176], while one was associated with worse survival [168]. Three studies assessed the presence of MSI, but none of them included this in survival analysis [85,172,178]. All six studies used an arbitrary cut-off apart from one that used a data driven cut-off [179].

No studies on IT CD163/206 met inclusion criteria for meta-analysis. Two studies for IM CD163 were included in meta-analysis for colorectal cancer (Table 4, Fig. 14C): one assessed DFS finding a significantly worse survival outcome; the other assessed DSS, with no significant survival difference, but a trend towards better survival.

Two studies compared CD68 and CD163 stained cells [85,178]. Both found CD68 to be significant for survival, where CD163 was not. However, one (654 patients) found CD68 was associated with worse survival [85], but the other (201 patients) better [178]. A further group studied the same population (468 patients) in two separate studies, one looking at CD68 [180] and the other at CD163 [172], but they found that both markers were associated with better survival.

Discussion

The complex nature of the interaction between the tumour and host immune system has long been investigated. It is clear from the large body of evidence displayed that a strong local inflammatory response is a positive prognostic indicator. Conversely, lack of a co-ordinated local inflammatory response results in a poor outcome. This issue is somewhat confounded by the presence or absence of MSI and it is remarkable how few studies have taken MSI into account in survival analysis.

The main aim of this review was to present evidence for survival outcomes based on assessment of the local inflammatory response in colorectal cancer. As a result, a secondary aim was to determine whether any particular inflammatory assessment had superior prognostic value, enabling a step towards incorporating assessment of the immune microenvironment in colorectal cancer into clinical practice.

In the past, some have advocated that not only the density of the local inflammatory response, but also the subtype of cells present and the location of these cells within the tumour microenvironment hold equal prognostic information [63,96,182]. However, in terms of cell type, the results of the meta-analysis presented show similar fixed effects summaries regardless of cell type assessed, with the exception of FoxP3 and macrophages. Furthermore, the fixed effects summaries are similar regardless of whether an H&E assessment method or IHC for a specific cell subtype is employed.

In terms of location of inflammatory cells, combined effects models were similar regardless of whether intratumoural assessment or assessment at the invasive margin were performed. In addition, those studies that compared IT assessment with IM did not agree on whether the presence of inflammatory cells in one compartment conveyed superior survival advantage over the other.

When considering FoxP3 regulatory T-cells and macrophages, even these cell subtypes are associated with improved survival on the whole, but there does appear to be more heterogeneity in the literature regarding their positive or negative influence on survival outcomes. The negative impact of FoxP3 on survival has been attributed to the regulatory nature of this T-cell subset in dampening the effects of cytotoxic T-lymphocytes [144]. In the case of macrophages, the negative prognostic effect has been attributed to the protumour effects of the “M2 macrophage” phenotype [168,183]. Both of these immune cell subsets may therefore be implicated in the immunoediting that tumours are believed to perform as they develop and evade or escape the host immunosurveillance [184].

The fact that most, if not all of the inflammatory cells assessed contribute to an improved survival outcome indicates that it is the density of a healthy, functional and co-ordinated immune response of all cell types that drive an effective anti-tumour response [45,98]. Conversely, it might be said that no cell type acting in isolation could effectively oppose tumour growth and metastasis. Hence those tumours with higher densities of infiltrating immune cells are associated with earlier stage, both in terms of depth of invasion and lymphatic and haematogenous spread [98,182].

However, the question remains as to which method of assessing peritumoural inflammatory response is optimal in colorectal cancer. If concordance could be achieved regarding a universal assessment method, then an inflammatory assessment could be incorporated into the colorectal cancer dataset to compliment TNM staging and other clinicopathological variables and guide the most appropriate post-operative management for each patient. This issue is especially important given the increasing use of immunotherapies in identifying which patients would derive greatest benefit.

The most studied immune cell subtypes and therefore those with the greatest combined evidence behind their use are CD8, CD3 and FoxP3. FoxP3, given the heterogeneity in terms of survival outcomes is less ideal for a generalised marker of the anti-tumour immune response. Not

only have the utilities of CD3 and CD8 individually been shown for colorectal cancer, both markers were associated with better prognosis when present in colon cancer and rectal cancer. Furthermore, the presence of CD3 and CD8 in preoperative biopsies was associated with better prognosis [69,76,103].

There is an argument for the use of a simple H&E based method like the Klintrup-Makinen grade: it is a validated method with similar fixed effects summaries to IHC methods; it is cost-effective, using routinely assessed slides in clinical pathology with no requirement for special stains. However, there are fewer studies reporting this method and therefore the evidence is less robust and there is an element of inter-observer variability in the published literature.

Similarly, CLR is able to stratify prognosis, but with even less published literature than for KM and the range of methods that have been used to assess CLR, in addition to the variability in cut-offs used mean that the pooled evidence for CLR is weak. The same could also be said for TILs counted on H&E, CD4, CD45RO, CD20, and CD56/57. All of these assessments have an overall positive survival effect, but the evidence was not strong.

Furthermore, if an IHC method was preferred, the next question would be to agree a standardised assessment approach. There are those who have advocated for digital pathology and the use of patented software to achieve a standardised method with excellent reproducibility [6]. However, the rationale for this approach hinges on the fact that it matters which tumour compartment the inflammatory cells are found in, but the published literature does not support this theory. Furthermore, the incorporation of this method of assessment into routine clinical practice necessitates not only that all colorectal cancer cases are assessed in pathology laboratories with digital pathology facilities, but also that the patented software is purchased on a global scale. In addition, there is evidence of significant publication bias regarding the immunoscore, although the recent large prospective consortium trial provides high quality evidence that it does stratify survival [6].

There are a range of methods of quantifying the extent of infiltration of CD3 and CD8, from semiquantitative scoring and manual counts to digital image analysis and automated counting. Around half of the studies included in meta-analysis for both CD3 and CD8 used a manual assessment and this did not impair their ability to identify those with a higher inflammatory infiltrate, which would suggest that an electronic assessment is not essential to the implementation of an IHC-based method of assessing host anti-tumour immune response in routine clinical practice. The only study to directly compare manual and electronic assessment of IHC for CD3 found a good intraclass correlation coefficient between manual and automated cell counting [97].

MSI is a known confounding factor in colorectal cancer for any inflammatory assessment due to the greater inflammatory response that these tumours stimulate, a feature that has been attributed to greater quantities of neo-antigen generation [18]. Having said this, nearly half of the included studies did not assess for the presence of MSI and even less included this in multivariate analysis. However, in many studies MSI was not found to influence survival and in those where it did, there were several studies that showed that the survival benefit offered by a strong anti-tumour immune response was independent of MSI.

In the same way as patients with MSI and stage II disease are unlikely to require adjuvant therapy [19], those with a dense local inflammatory response would be offered similar protection.

Conclusion

Based on the weight of published evidence available, the authors advocate a standardised approach to assessing the immune response to colorectal cancer in routine clinical practice to further stratify survival and dictate individualised management. However, whether this assessment should be a simple validated H&E method alone, such as the Klintrup-Makinen grade, or whether it should require IHC for immune cell subtypes, or a combination of these methods, requires further assessment. CD3 may act as a surrogate marker of overall T-cell infiltrate, but the authors caution against undue focus on any individual cell type at the expense of the overall inflammatory infiltrate, since it is likely to be a co-ordinated immune response that provides the observed protective effect on survival.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

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