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ARTICLE

Translational Therapeutics

Trop-2 and Nectin-4 immunohistochemical expression in metastatic colorectal cancer: searching for the right population for drugs' development

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BACKGROUND: Trop-2 and Nectin-4 are transmembrane proteins overexpressed in many tumours and targets of antibody–drug conjugates (ADC). In metastatic colorectal cancer (mCRC), the role of Trop-2 and Nectin-4 has been poorly investigated.
METHODS: Tumour samples of patients randomised in the phase III TRIBE2 were assessed for Trop-2 and Nectin-4 expression.
RESULTS: Three hundred eighty-six tumours were assessed for Trop-2 expression. 90 (23%), 115 (30%) and 181 (47%) were Trop-2 high, medium and low, respectively. Patients with low Trop-2 tumours achieved longer PFS (12 versus 9.9 months, $p = 0.047$) and OS (27.3 versus 21.3 months, $p = 0.015$) than those with high/medium Trop-2 tumours. These findings were confirmed in multivariate analysis ($p = 0.022$ and $p = 0.023$, respectively). A greater OS benefit from treatment intensification with FOLFOXIRI/bevacizumab was observed in patients with high/medium Trop-2 tumours (p-for-interaction = 0.041).Two hundred fifty-one tumours were assessed for Nectin-4 expression. Fourteen (5%), 67 (27%) and 170 (68%) were high, medium and low, respectively. No prognostic impact was observed based on Nectin-4 expression and no interaction effect was reported between Nectin-4 expression groups and treatment arm.
CONCLUSIONS: In mCRC, expression levels of Trop-2 and Nectin-4 are heterogeneous, suggesting a target-driven development of anti-Trop2 and anti-Nectin-4 ADCs. Medium/high Trop-2 expression is associated with worse prognosis and higher benefit from chemotherapy intensification.

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BACKGROUND

Trophoblast cell-surface antigen-2 (Trop-2) is a 46-kD transmembrane glycoprotein involved in the regulation of cell adhesion that was identified in human placental trophoblasts [1] with minimal or absent levels in cellular membrane of adult somatic tissues [2]. This protein is overexpressed in many epithelial cancers including breast [3, 4], urothelial [5], oral [6], lung [7] and gastrointestinal [8–10] carcinomas, where it stimulates cancer growth by promoting cellular proliferation, survival, and invasion [11–15]. Elevated levels of Trop-2 expression were correlated with aggressive behaviour and poor prognosis in several cancers [3, 15, 16].

Nectin-4 is an immunoglobulin-like cell adhesion transmembrane protein involved in the development and maintenance of adherent junctions in cooperation with cadherins [17]. It is normally expressed in embryonic and placental tissues during foetal development while expression levels decline in adult somatic tissues [18]. Several studies showed that Nectin-4 is overexpressed in various tumours including urothelial [19], breast [20], gastrointestinal [21–23], and lung [24] carcinomas contributing to tumour-cell growth, proliferation, invasion and migration [24, 25]. Moreover, high levels of Nectin-4 were associated with poor prognosis in many cancer types [21, 26–28].

The overexpression of both Trop-2 and Nectin-4 in several cancers together with their limited expression in normal tissues, led to identify these molecules as appealing therapeutic targets [29, 30]. Indeed, two antibody–drug conjugates (ADC),

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sacituzumab govitecan, an anti-Trop-2 antibody coupled to the topoisomerase I inhibitor SN-38 [29], and enfortumab vedotin, an anti-Nectin-4 antibody conjugated to the microtubule-disrupting agent monomethyl auristatin E [30], showed better outcomes compared to standard chemotherapy in two phase III trials enroling patients with advanced triple-negative breast cancer [31] and urothelial carcinoma [32], respectively, thus obtaining FDA and EMA approvals in these settings [33–36].

In metastatic colorectal cancer (mCRC), the role of Trop-2 and Nectin-4 has been poorly investigated [22, 37] and their expression and prognostic value remain to be elucidated.

Drawing from these considerations, we performed an immunohistochemical evaluation of Trop-2 and Nectin-4 on tumour samples of mCRC patients enroled in the TRIBE-2 study, a phase III trial comparing FOLFOXIRI (5-fluorouracil, leucovorin, oxaliplatin and irinotecan)/bevacizumab (bev) with FOLFOX (5-fluorouracil, leucovorin and oxaliplatin)/bev, as first-line treatment [38].

METHODS

Study population

TRIBE2 (NCT02339116) [38] is a phase III randomised, open-label, multicentre trials where 679 initially unresectable mCRC patients (aged 18–70 years with Eastern Cooperative Oncology Group performance status [ECOG-PS] of 2 or less and aged 71–75 years with an ECOG-PS of 0) were randomised in a 1:1 ratio to receive FOLFOX/bev followed by FOLFIRI/bev after disease progression or FOLFOXIRI/bev followed by the reintroduction of the same agents after disease progression. All treatments were administered up to eight cycles, followed by 5-fluorouracil plus bev until disease progression, unacceptable adverse events, or consent withdrawal in both arms. In the present study, only patients with available chemonaïve primary tumour samples for immunohistochemistry analysis were included.

Immunohistochemical assessments

Immunohistochemical assessments were performed by optical microscope and centralised at the Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa. Three micrometres-thick tumour sections were stained with anti-TROP2 Antibody [clone EPR20043 - Abcam, Cambridge, UK] and anti-Nectin-4 Antibody [clone EPR15613-68—Abcam, Cambridge, UK]. Staining was done on an automated IHC/ISH slide staining system (BenchMark ULTRA—Ventana Medical Systems, Inc.) using the ultraView DAB Detection Kit (Ventana Medical Systems, Inc.). Slides were dewaxed, pre-treated by standard cell conditioner at 95 °C for 64 minutes with ULTRA CC1 ready-to-use solution (Ventana Medical Systems, Inc.), and thereafter incubated with anti-TROP2 Antibody and anti-Nectin-4 Antibody, dilution 1:100 at 36 °C for 36 min.

Then slides were counterstained with Hematoxylin for 12 min and Bluing Reagent (Ventana Medical Systems, Inc.) for 12 min. Finally, slides were dehydrated by sequential passages through increasing (from 70% to 100%) concentrations of ethanol, xylene and then were mounted with coverslip. Sections from cervix cancer tissues and human placenta were used as positive control for anti-Trop-2 antibody and anti-Nectin-4 antibody, respectively, as per manufacturer protocol. The positive staining controls were used for each freshly prepared slides or for each run in the case of tissue slides previously prepared from archival tumour samples. Tissue samples were independently evaluated by two pathologists (CU, AP) blinded to clinical information, treatment regimen, and outcome. In discordant cases, a consensus was reached through the evaluation of a third pathologist (G.F.).

The expression of Trop-2 and Nectin-4 were categorised by means of a histochemical score (H-score), calculated as follows: (3 × % cells with strong intensity staining) + (2 × % cells with moderate intensity staining) + (1 × % cells with mild intensity staining). Tissue samples with H-score <100, 100–200 and >200 were defined as low, medium and high, respectively [31, 39, 40] (Supplementary Fig. 1).

Statistics

Strength of concordance between pathologists for the assessment of Trop-2 and Nectin-4 expression was carried out by means of K of Cohen. Descriptive statistics was used to describe the distribution of Trop-2 and Nectin-4 H-scores. Chi-square test, Fisher's exact test, or Mann–Whitney test were used whenever appropriate to compare clinical and molecular baseline characteristics among Trop-2 and Nectin-4 subgroups. Progression-free survival (PFS) and overall survival (OS) were defined as the time from randomisation to the first evidence of disease progression or death, whichever occurred first, and as the time from randomisation to death due to any cause, respectively. Survival curves were estimated by the Kaplan–Meier method and compared with the log-rank test. Hazard ratios (HRs) with 95% confidence intervals (CIs) were estimated with Cox proportional hazards model. The impact of clinical and molecular prognostic variables on PFS and OS was first assessed in univariate analyses. Statistically significantly prognostic covariates ($p < 0.10$) were included in a multivariable Cox proportional hazard model. Subgroup analyses to assess the benefit of FOLFOXIRI/bev versus FOLFOX/bev based on Trop-2 and Nectin-4 subgroups in terms of PFS and OS were carried out using interaction test. Statistical significance was set at a p-value of 0.05 for a bilateral test. The data cut-off for the present analysis was 28 December 2020. All analyses were carried out with MedCalc Statistical Software (https://www.medcalc.org).

RESULTS

Trop-2 expression

Samples from 386 (56.8%) out of 679 patients enroled in the TRIBE2 study, 251 tumour blocks and 135 stored paraffin slides, were available for Trop-2 immunohistochemical assessment (Supplementary Fig. 2).

The strength of agreement between pathologists for the assessment of Trop-2 was very high (K of Cohen = 0.91). The expression of Trop-2 was highly heterogeneous. Indeed, only the 27.7% of samples showed a unique category of immunohistochemical expression (no staining or only 1+ or 2+ or 3+ staining). The distribution of Trop-2 H-scores is summarised in Supplementary Fig. 3, panel B.

Overall, 90 (23%), 115 (30%) and 181 (47%) were classified as high, medium and low, respectively. As shown in Table 1, high Trop-2 tumours were more frequently *BRAF* mutated ($p = 0.0049$) and right-sided ($p = 0.028$) compared to medium and low ones.

Patients with low Trop-2 tumours achieved numerically longer PFS (12.0 versus 10.0 versus 9.6 months, $p = 0.092$) and significantly longer OS (27.3 versus 20.5 versus 23.9 months, $p = 0.044$) than those with medium and high Trop-2 tumours (Fig. 1).

Considering the similar survival, we grouped together patients with medium and high Trop-2 levels. Low Trop-2 tumours were less frequently *BRAF* mutated ($p = 0.0082$) and right-sided ($p = 0.046$) compared to medium/high Trop-2 ones (Supplementary Table 1).

Patients with low Trop-2 tumours achieved longer PFS (12.0 versus 9.9 months, HR: 0.81, 95% CI: 0.66–1.00, $p = 0.047$) and OS (27.3 versus 21.3 months, HR: 0.76, 95% CI: 0.60–0.95, $p = 0.014$) than those with medium/high Trop2 tumours (Supplementary Fig. 4). The prognostic value of Trop-2 expression levels was confirmed in the multivariate analysis in terms of both PFS ($p = 0.022$) and OS ($p = 0.023$) (Table 2).

An Interaction effect was shown between Trop-2 expression and treatment arm with higher benefit from FOLFOXIRI/bev in the medium/high Trop2 cohort in terms of OS (p for interaction = 0.041), but not for PFS (p for interaction = 0.11) (Fig. 2).

Nectin-4 expression

386 tumour samples were assessed for Nectin-4 immunohistochemical expression. However, absence of immunostaining was observed in all the 135 samples with available stored tissue slides, and these cases were considered not evaluable and excluded from the analysis.

The strength of agreement between pathologists for the assessment of Nectin-4 was very high (K of Cohen=0.95). The expression of Nectin-4 was quite heterogeneous. Indeed, the 62.9% of samples showed a unique category of immunohistochemical

Table 1. Patients' characteristics.

	Population assessed for Trop-2 expression $N = 386$				Population assessed for Nectin-4 expression $N = 251$			
	Trop-2 low $N = 181$ (47%) *n* (%)	**Trop-2 medium $N = 115$ (30%) *n* (%)**	**Trop-2 high $N = 90$ (23%) *n* (%)**	***p***	**Nectin-4 low $N = 170$ (68%) *n* (%)**	**Nectin-4 medium $N = 67$ (27%) *n* (%)**	**Nectin-4 high $N = 14$ (5%) *n* (%)**	***p***
Age (years)								
Median	62	61	59	0.44	63	61	55	0.058
Range	(29–75)	(34–74)	(32–75)		(32–75)	(40–75)	(37–68)	
Sex								
Male	106 (58)	62 (54)	47 (52)	0.55	89 (52)	43 (64)	8 (57)	0.25
Female	75 (42)	53 (46)	43 (48)		81 (48)	24 (36)	6 (43)	
ECOG-PS								
0	156 (86)	98 (85)	82 (91)	0.41	152 (89)	60 (89)	9 (64)	**0.019**
1–2	25 (14)	17 (15)	8 (9)		18 (11)	7 (11)	5 (36)	
Site of primary tumour								
Right	64 (35)	46 (40)	47 (52)	**0.028**	77 (45)	25 (37)	1 (7)	**0.016**
Left and rectum	117 (65)	69 (60)	43 (48)		93 (55)	42 (63)	13 (93)	
RAS/BRAF mutational status								
RAS/BRAF wt	48 (26)	19 (17)	14 (15)	**0.0049**	32 (19)	13 (19)	1 (7)	0.78
RAS mut	113 (62)	79 (69)	55 (61)		106 (62)	46 (69)	10 (71)	
BRAF mut	14 (8)	12 (10)	19 (21)		23 (13)	7 (10)	2 (14)	
NA	6 (4)	5 (4)	2 (3)		9 (6)	1 (2)	1 (8)	
Microsatellite status								
pMMR/MSS	162 (89)	111 (96)	85 (95)	0.51	157 (92)	63 (94)	14 (100)	0.37
dMMR/MSI-H	9 (5)	3 (3)	3 (3)		8 (5)	1 (2)	0 (0)	
NA	10 (6)	1 (1)	2 (2)		5 (3)	3 (4)	0 (0)	
Resected primary tumour								
Yes	93 (51)	84 (73)	61 (68)	**<0.001**	118 (69)	29 (43)	2 (14)	**<0.001**
No	88 (49)	31 (27)	29 (32)		52 (31)	38 (57)	12 (86)	
Liver only disease								
Yes	55 (30)	25 (22)	26 (29)	0.24	43 (25)	19 (28)	6 (43)	0.34
No	125 (69)	90 (78)	64 (71)		127 (75)	47 (70)	8 (57)	
NA	1 (1)	0 (0)	0 (0)		0 (0)	1 (2)	0 (0)	
Time to metastases								
Synchronous	162(89)	100 (87)	81 (90)	0.73	152 (89)	63 (94)	12 (86)	0.46
Metachronous	19 (11)	15 (13)	9 (10)		18 (11)	4 (6)	2 (14)	
Treatment arm								
FOLFOX + bev	84 (51)	64 (55)	48 (53)	0.26	86 (51)	36 (54)	8 (57)	0.83
FOLFOXIRI + bev	97 (49)	51 (49)	42 (47)		84 (49)	31 (46)	6 (43)	

bev bevacizumab, *dMMR* deficient mismatch repair, *ECOG-PS* Eastern Cooperative Oncology Group Performance Status, *MSI-H* microsatellite instable high, *MSS* microsatellite stable, *mut* mutated, *N* number, *NA* not available, *pMMR* proficient mismatch repair, *wt* wild-type.
Statistically significant $p < 0.05$ values are in bold.

expression (no staining or only 1+ or 2+ or 3+ staining). The distribution of Nectin-4 H-scores is summarised in Supplementary Fig. 3, panel A.

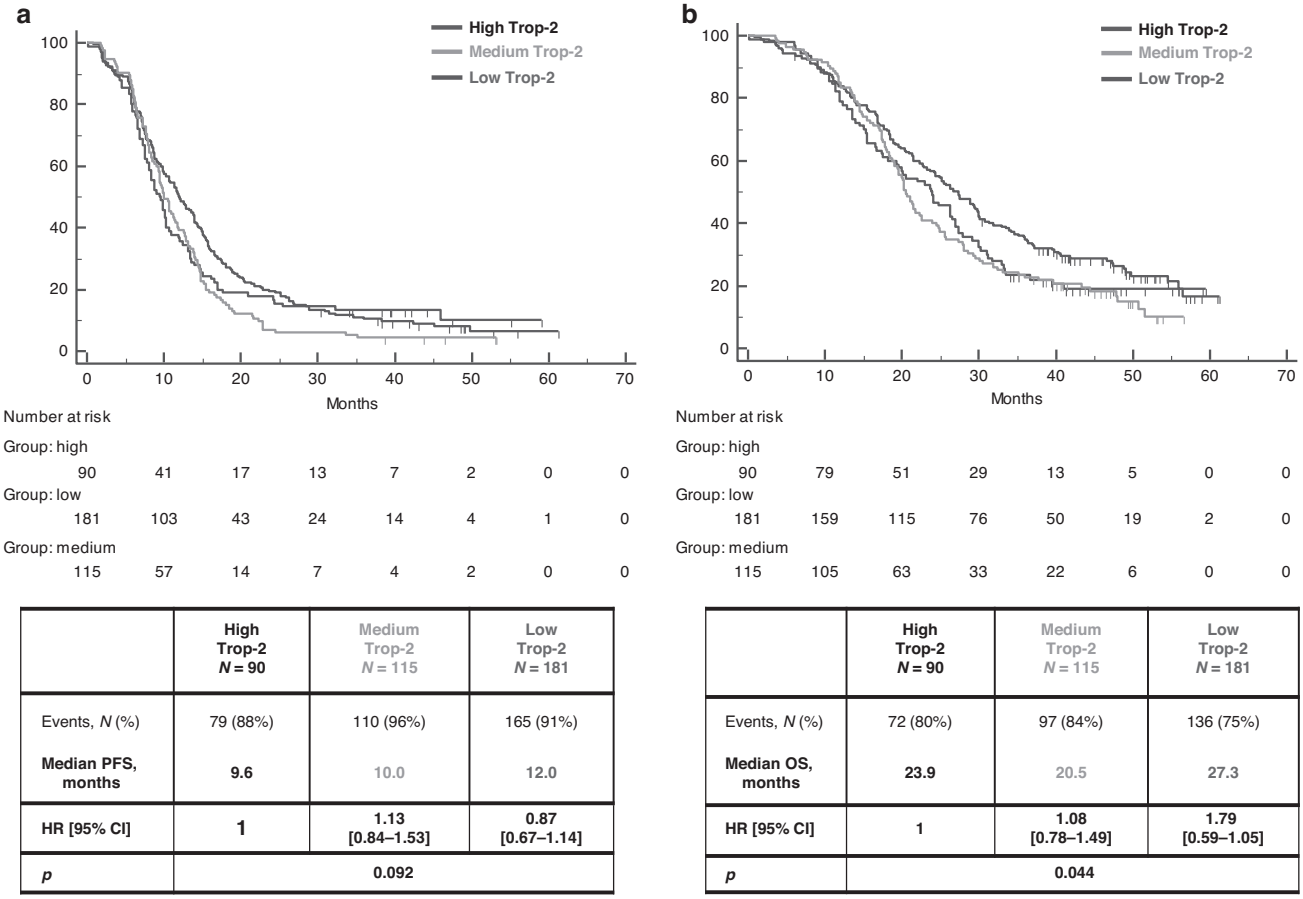
Among 251 tumours blocks with adequate immunostaining, 14 (5%), 67 (27%) and 170 (68%) were classified as high, medium and low, respectively (Supplementary Fig. 2). Patients with high Nectin-4 tumours had more frequently an ECOG-PS of 1–2 ($p = 0.019$) and a left-sided primary tumour ($p = 0.016$) (Table 1).

No differences were observed based on Nectin-4 expression in terms of both PFS ($p = 0.56$) and OS ($p = 0.83$) (Fig. 3).

No interaction effect was evident between Nectin-4 expression groups and treatment arm in terms of both PFS (*p* for interaction = 0.63) and OS (p for interaction = 0.98) (Supplementary Fig. 5).

DISCUSSION

In recent years, ADCs have expanded the oncological therapeutic armamentarium [41–43]. In particular, sacituzumab govitecan, an anti-Trop-2 ADC, and enfortumab vedotin, an anti-Nectin-4 ADC, were recently approved for the treatment of advanced triple-negative breast cancer and urothelial carcinoma, respectively [33–36]. Considering the high expression of Trop-2 and Nectin-4 in the majority of triple-negative breast cancer [31] and urothelial



	High Trop-2 N = 90	Medium Trop-2 N = 115	Low Trop-2 N = 181
Events, N (%)	79 (88%)	110 (96%)	165 (91%)
Median PFS, months	9.6	10.0	12.0
HR [95% CI]	1	1.13 [0.84–1.53]	0.87 [0.67–1.14]
p		0.092	

	High Trop-2 N = 90	Medium Trop-2 N = 115	Low Trop-2 N = 181
Events, N (%)	72 (80%)	97 (84%)	136 (75%)
Median OS, months	23.9	20.5	27.3
HR [95% CI]	1	1.08 [0.78–1.49]	1.79 [0.59–1.05]
p		0.044	

Fig. 1 Kaplan-Meier curves according Trop-2 expression levels. Kaplan–Meier curves of progression-free survival (**a**) and overall survival (**b**) according to Trop-2 expression levels (high versus medium versus low).

carcinoma [40] respectively, testing expression levels of their targets was not required for both drugs [33–36]. Indeed, the median H-score of Nectin-4 in urothelial carcinoma is 290 out of 300 (40) and 80% of triple-negative breast cancer showed a medium/high H-score of Trop-2 [31]. In addition, a subgroup analysis of the ASCENT trial, comparing sacituzumab govitecan versus single-agent chemotherapy at physician's choice in pre-treated triple-negative breast cancer, showed no interaction effect between expression levels of Trop-2 and sacituzumab govitecan efficacy [31]. In mCRC, the expression of Trop-2 and Nectin-4 has been poorly investigated until now and heterogeneous immuno-histochemical scores were adopted, thus hampering the overall interpretation of available results [22, 37, 44, 45].

In the present study, we analysed the expression levels of Trop-2 and Nectin-4 in a population of chemo-naïve mCRC patients using H-score, a numerical value obtained by a weighted sum of the percentage of stained cells as made in more recent studies in solid tumours. H-score offers a dynamic range to quantify biomarker abundance, thus allowing to accurately assess the biomarker expression [31, 39, 40]. Since the expression of Trop-2 and Nectin-4 was heterogeneous in most samples, the lack of repeated assessments on different slides of the same tumour might be a limitation of our study.

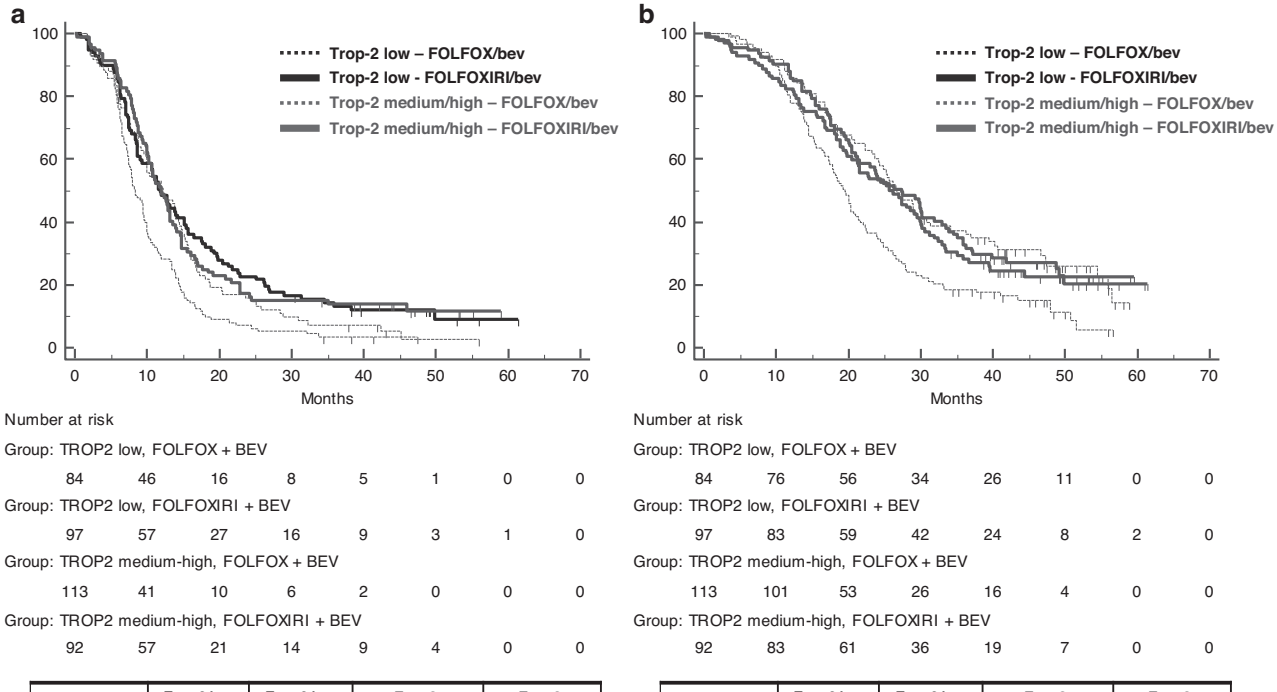
However, expression levels of Trop-2 were low in about half of cases differently than the around 20% of triple-negative breast cancers [31]. The use of H-score as well as the inclusion of chemo-naïve primary tumour samples only from mCRC patients may justify the higher frequency of cases with elevated Trop-2 expression compared with a previous study including early CRCs and adopting a different method for Trop-2 evaluation [46].

Moreover, Nectin-4 expression levels were high only in the 5% of tumour samples compared with more than 90% in urothelial carcinoma [40]. Therefore, the reduced expression levels of these two markers may highly dilute the efficacy of sacituzumab govitecan and enfortumab vedotin in the overall population of mCRC. Indeed, in a phase I/II basket trial assessing the safety and activity of sacituzumab govitecan in solid tumours, an overall response rate (ORR) as low as 3% was reported in the cohort of mCRC, all pre-treated with the topoisomerase I inhibitor irinotecan [46], while patients with small cell lung cancers appeared to respond irrespective of prior treatment with the topoisomerase I inhibitors topotecan or irinotecan (ORR: 13% in patients pre-treated with topoisomerase I inhibitors *versus* 11% in the topoisomerase I inhibitors-naïve group) [47]. Therefore, the low activity of sacituzumab govitecan in mCRC could be due to the lack of patients' selection based on Trop-2 expression levels rather than to the previous exposure to irinotecan. To corroborate this hypothesis, in irinotecan-pre-treated mCRC patients, the ADC trastuzumab-deruxtecan, an anti-HER-2 antibody linked to a topoisomerase I inhibitor, is active only in HER2-positive tumours (IHC 3+ or IHC 2 + /ISH−), but not in HER2-low ones (IHC 2 + /ISH − or IHC 1 +) (ORR: 45% versus 0%). In addition, among HER2-positive patients, the efficacy of trastuzumab-deruxtecan is more pronounced in patients with higher HER2 expression (IHC 3 +) than those who have less (IHC 2 + /ISH positive) (ORR: 57% versus 8%) [48]. Therefore, a more selective approach, including only

Table 2. Uni- and multivariate analyses for progression-free survival and overall survival.

		Progression-free survival					Overall survival				
Characteristics	***N* (%)**	**Median (months)**	**Univariate analysis**		**Multivariate analysis**		**Median (months)**	**Univariate analysis**		**Multivariate analysis**	
			HR (95% CI)	***p***	**HR (95% CI)**	***p***		**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Trop-2 status											
High + medium	205 (53)	9.9	1	—	—	—	21.3	1	—	1	—
Low	181 (47)	12.0	0.81 (0.66–1.00)	**0.047**	0.77 (0.61–0.96)	**0.022**	27.3	0.76 (0.60–0.95)	**0.015**	0.75 (0.69–0.96)	**0.023**
Arm											
FOLFOX + bev	196 (51)	9.6	1	**<0.001**	1	**0.003**	22.2	1	**0.091**	1	0.41
FOLFOXIRI + bev	190 (49)	11.9	0.69 (0.56–0.85)		0.71 (0.57–0.89)		26.2	0.82 (0.66–1.03)		0.90 (0.71–1.15)	
ECOG-PS											
0	336 (87)	11.3	1	**<0.001**	1	**<0.001**	25.3	1	**<0.001**	1	**<0.001**
1–2	50 (13)	7.2	2.14 (1.58–2.89)		1.93 (1.38 –2.70)		15.4	2.48 (1.82–3.38)		2.48 (1.75–3.49)	
Previous adjuvant therapy											
No	381 (99)	10.6	1	0.93	—	—	23.7	1	0.60	—	—
Yes	5 (1)	8.1	1.04 (0.43-2.52)		—		29.5	0.76 (0.28-2.05)		—	
Time to metastases											
Metachronous	43 (12)	10.6	1	0.56	—	—	23.9	1	0.74	—	—
Synchronous	343 (88)	10.6	0.91 (0.65–1.26)		—		25.5	0.94 (0.66–1.34)		—	
Resected primary tumour											
No	148 (38)	9.9	1	**0.10**	1	**0.029**	21.4	1	**0.021**	1	**<0.001**
Yes	238 (62)	11.2	0.84 (0.68–1.04)		0.82 (0.64–1.05)		25.7	0.76 (0.61–0.96)		0.64 (0.50–0.83)	
Liver only disease											
No	279 (71)	10	1	**0.024**	1	0.11	21.5	1	**<0.001**	1	**0.016**
Yes	106 (29)	11.9	0.76 (0.60–0.96)		0.82 (0.64–1.05)		30	0.63 (0.49–0.82)		0.71 (0.54–0.94)	
NA	1 (<1)										
Site of primary tumour											
Right	157 (41)	9.8	1	0.15	—	—	21	1	**0.012**	1	**0.015**
Left and rectum	229 (59)	11.3	0.85 (0.69–1.06)		—		25.2	0.75 (0.59–0.94)		0.73 (0.57–0.94)	
Microsatellite status											
pMMR/MSS	358 (93)	10.2	1	**0.015**	1	0.059	23.9	1	**0.039**	1	0.093
dMMR/MSI-H	15 (4)	17.3	0.46 (0.24–0.86)		0.54 (0.28–1.02)		37.8	0.45 (0.21–0.96)		0.52 (0.24–1.12)	
NA	13 (3)										
RAS/BRAF mutational status											
All wt	81 (21)	11.6	1		1		32.9	1		1	
RAS mut	247 (64)	10.1	1.18 (0.90–1.53)	**0.23**	—	—	23.9	1.71 (1.26–2.32)	**<0.001**	1.59 (1.15–2.19)	**0.005**
BRAF mut	45 (12)	8	1.55 (1.07–2.72)	**0.021**	1.38 (0.92–2.07)	0.12	14.5	2.60 (1.71–3.96)	**<0.001**	2.02 (1.27–3.20)	**0.003**
NA	13 (3)										

bev bevacizumab, *dMMR* deficient mismatch repair, *ECOG-PS* Eastern Cooperative Oncology Group Performance Status, *HR* hazard ration, *MSI-H* microsatellite instable high, *MSS* microsatellite stable, *mut* mutated, *N* number, *NA* not available, *pMMR* proficient mismatch repair, *wt* wild-type.
Statistically significant $p < 0.05$ values are in bold.



	Trop-2 low FOLFOX/ bev *N* = 84	Trop-2 low FOLFOXIRI/ bev *N* = 97	Trop-2 medium/high FOLFOX/bev *N* = 113	Trop-2 medium/high FOLFOXIRI/bev *N* = 92
Events, *N* (%)	79 (94%)	86 (89%)	109 (96%)	80 (87%)
Median PFS, months	**12.2**	**11.8**	8.4	11.9
HR [95% CI]	1	0.83 [0.62 – 1.12]	1	0.59 [0.43 – 0.79]
p	0.19		**<0.001**	
p	**<0.001**			
***p* for interaction**	**0.11**			

	Trop-2 low FOLFOX/ bev *N* = 84	Trop-2 low FOLFOXIRI/ bev *N* = 97	Trop-2 medium/high FOLFOX/bev *N* = 113	Trop-2 medium/high FOLFOXIRI/bev *N* = 92
Events, *N* (%)	63 (75%)	73 (75%)	99 (88%)	70 (76%)
Median PFS, months	**26.7**	**27.3**	19.5	25.7
HR [95% CI]	1	1.07 [0.78 – 1.46]	1	0.66 [0.47 – 0.91]
p	0.68		**0.005**	
p	**0.0020**			
***p* for interaction**	**0.141**			

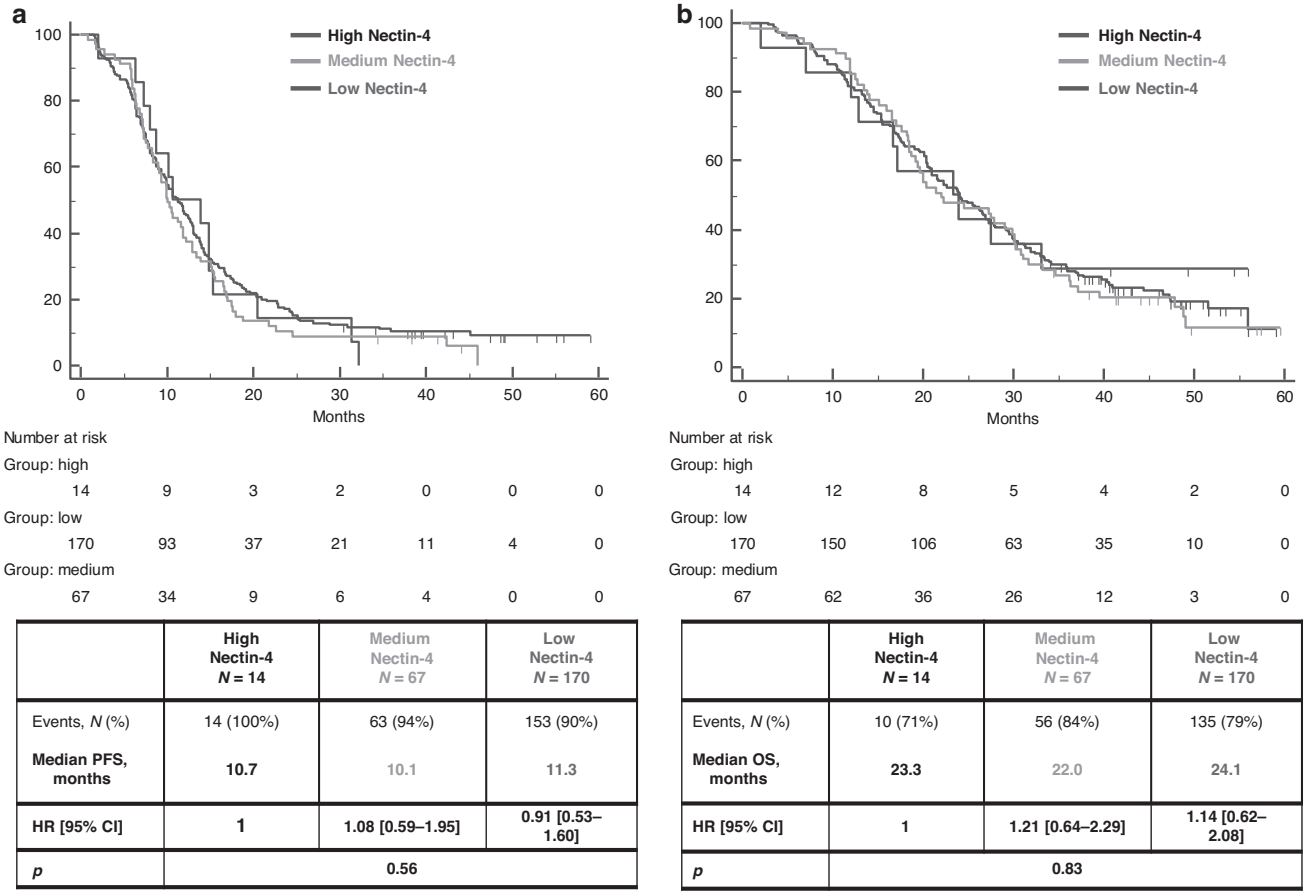
Fig. 2 Kaplan-Meier curves according Trop-2 expression levels and treatment. Kaplan–Meier curves of progression-free survival (**a**) and overall survival (**b**) according to Trop-2 expression levels (medium/high and low) and treatment (FOLFOX/bevacizumab and FOLFOXIRI/bevacizumab).

patients with high or medium-high expression of Trop-2, may be more appropriate for the development of sacituzumab govitecan in mCRC, and the same approach should be likely translated also to the design of basket trials investigating this drug.

Since the expression of Nectin-4 is lower than Trop-2 in the mCRC and since the release of the cytotoxic agent monomethyl auristatin E linked to enfortumab vedotin occurs only after internalisation in tumour cells [49], a clinically relevant efficacy of this drug in unselected mCRC is unlikely, though in the absence of specific data.

In our study, patients with medium/high Trop-2 expression levels showed worse prognosis compared to those with low Trop-2 tumours, as already reported for other cancer types [3, 15, 16]. Notably, the significant association of high/medium Trop-2 expression levels with shorter PFS and OS was confirmed also in the multivariable model, thus suggesting that the prognostic impact of this biomarker is independent of other associated poor prognostic factors, such as right-sidedness and *BRAF* mutational status. In addition, a higher benefit from chemotherapy intensification was observed in the medium/high Trop-2 cohort. On the other hand, Nectin-4 expression did not show any prognostic or predictive impact. However, it should be noted that the assessment of Nectin-4 failed in the 35% of cases, thus reducing the power to detect a possible prognostic value. In particular, Nectin-4 expression was evaluable only in cases with available formalin-fixed paraffin-embedded (FFPE) blocks while the analysis failed in the case of tissue slides previously prepared from archival tumour samples. This is probably due to a time-dependent antigen degradation with consequent loss of immunoreactivity [50]. Considering that not all antigens are equally affected by antigen decay, not all antibodies show reduced detection ability [51]. Indeed, Trop-2 expression was not impaired by this issue. Therefore, the use of slides from FFPE blocks within days or weeks after sectioning would be desirable instead of archival slides for the assessment of Nectin-4 expression levels. Several preclinical data showed an association between Nectin-4 overexpression and 5-fluoruracil resistance in colorectal cancer cells [52–55]. However, we observed no difference in the efficacy of FOLFOXIRI/bev versus FOLFOX/bev and no prognostic impact based on Nectin-4 expression. The administration of 5-fluoruracil in both arms and the technical issue of Nectin-4 assessment in archival slides prevent us from deriving any definitive conclusion regarding the prognostic role and the negative predictive value of Nectin-4 overexpression with regard to the efficacy of 5-fluoruracil.

In conclusion, expression levels of Trop-2 and Nectin-4 are heterogeneous in mCRC, suggesting that only a target-driven development of anti-Trop-2 and anti-Nectin-4 ADCs would be biologically reasonable. Similarly, the appealing combination of these drugs with immune checkpoint inhibitors, whose rationale lies in their potential synergistic effect [52] should be investigated in properly selected populations.



	High Nectin-4 *N* = 14	Medium Nectin-4 *N* = 67	Low Nectin-4 *N* = 170
Events, *N* (%)	14 (100%)	63 (94%)	153 (90%)
Median PFS, months	**10.7**	10.1	11.3
HR [95% CI]	**1**	**1.08 [0.59–1.95]**	**0.91 [0.53–1.60]**
p	**0.56**		

	High Nectin-4 *N* = 14	Medium Nectin-4 *N* = 67	Low Nectin-4 *N* = 170
Events, *N* (%)	10 (71%)	56 (84%)	135 (79%)
Median OS, months	**23.3**	22.0	24.1
HR [95% CI]	**1**	**1.21 [0.64–2.29]**	**1.14 [0.62–2.08]**
p	**0.83**		

Fig. 3 Kaplan-Meier curves according Nectin-4 expression levels. Kaplan–Meier curves of progression-free survival (**a**) and overall survival (**b**) according to Nectin-4 expression levels (high versus medium versus low).

DATA AVAILABILITY

Datasets supporting the results of this work are available to editors, referees and readers promptly upon request.

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AUTHOR CONTRIBUTIONS

Study concepts: RM, CU, MMG, CC. Study design: RM, CU, MMG, MG, CC. Data acquisition: CU, MMG, MG. Quality control of the data and algorithms: CU, MMG, MG. Data analysis and interpretation: RM, CU, MMG, MG, CC. Statistical analysis: RM, MMG. Manuscript preparation: RM, MMG. Manuscript editing: CU, GF, CC. Manuscript review: all authors.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All patients provided written informed consent to study procedures before enrolment. Approvals for TRIBE protocol were obtained from local ethics committees of participating sites.

ADDITIONAL INFORMATION

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