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Dissecting tumor lymphocyte infiltration to predict benefit from immune-checkpoint inhibitors in metastatic colorectal cancer: lessons from the AtezoT RIBE study

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ABSTRACT

Background Tumor immune cells influence the efficacy of immune-checkpoint inhibitors (ICIs) and many efforts aim at identifying features of tumor immune microenvironment able to predict benefit from ICIs in proficient mismatch repair (pMMR)/microsatellite stable (MSS) metastatic colorectal cancer (mCRC).

Methods We characterized tumor immune cell infiltrate, by assessing tumor-infiltrating lymphocytes (TILs), Immunoscore, Immunoscore-IC, and programmed death ligand-1 (PD-L1) expression in tumor samples of patients with mCRC enrolled in the AtezoTRIBE study, a phase II randomized trial comparing FOLFOXIRI/bevacizumab/atezolizumab to FOLFOXIRI/bevacizumab, with the aim of evaluating the prognostic and predictive value of these features.

Results Out of 218 patients enrolled, 181 (83%), 77 (35%), 157 (72%) and 162 (74%) specimens were successfully tested for TILs, Immunoscore, Immunoscore-IC and PD-L1 expression, respectively, and 69 (38%), 45 (58%), 50 (32%) and 21 (13%) tumors were classified as TILs-high, Immunoscore-high, Immunoscore-IC-high and PD-L1-high, respectively. A poor agreement was observed between TILs and Immunoscore or Immunoscore-IC (K of Cohen <0.20). In the pMMR population, longer progression-free survival (PFS) was reported for Immunoscore-high and Immunoscore-IC-high groups compared with Immunoscore-low (16.4 vs 12.2 months; HR: 0.55, 95% CI: 0.30 to 0.99; p=0.049) and Immunoscore-IC-low (14.8 vs 11.5 months; HR: 0.55, 95% CI: 0.35 to 0.85; p=0.007), respectively, with a significant interaction effect between treatment arms and Immunoscore-IC (p for interaction: 0.006) and a trend for Immunoscore (p for interaction: 0.13). No PFS difference was shown according to TILs and PD-L1 expression. Consistent results were reported in the overall population.

Conclusions The digital evaluation of tumor immune cell infiltrate by means of Immunoscore-IC or Immunoscore identifies the subset of patients with pMMR mCRC achieving more benefit from the addition of the anti-PD-L1 to the upfront treatment. Immunoscore-IC stands as the most promising predictor of benefit from ICIs.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ In proficient mismatch repair (pMMR) metastatic colorectal cancer (mCRC) Immunoscore-IC predicts the benefit from adding atezolizumab to FOLFOXIRI and bevacizumab, as recently showed in the subgroup analysis of the phase II randomized AtezoTRIBE trial. However, no data are available about how this information overlaps with other potential biomarkers of microenvironment immunogenicity including tumor infiltrating lymphocytes (TILs), Immunoscore and programmed death ligand-1 (PD-L1), and the relative weight of these features.

WHAT THIS STUDY ADDS

⇒ We observed a poor agreement between TILs and Immunoscore or Immunoscore-IC on tumor samples of patients with mCRC enrolled in the AtezoTRIBE study. Both Immunoscore and Immunoscore-IC predict the efficacy of atezolizumab addition to FOLFOXIRI and bevacizumab in the pMMR population, while TILs and PD-L1 expression do not.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Digital pathology-based markers show the highest level of accuracy in identifying patients with pMMR mCRC that might benefit by the treatment with immune checkpoint inhibitors. Considering the lower performance of Immunoscore compared with Immunoscore-IC, and the technical issues mainly related to the need to evaluate also the invasive margin, the latter stands as the most promising predictor.

BACKGROUND

The host immune response in the tumor microenvironment has recently caught growing interest for its prognostic impact in several solid tumors.[1] With the introduction

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of new immunotherapeutic agents in the therapeutic armamentarium, immune tumor microenvironment has been extensively investigated, with the aim of identifying features of tumor immunogenicity able to predict benefit from immune-checkpoint inhibitors (ICIs) in different clinical scenarios.[2]

In early-stage colon cancer, both tumor-infiltrating lymphocytes (TILs) assessed in H&E-stained sections and Immunoscore, a digital immunohistochemistry-based scoring system quantifying cytotoxic T cells in the tumor core and at the invasive margin, showed a strong prognostic effect irrespective of stage, lymph node count and molecular features including mismatch repair system (MMR) status /microsatellite instability and *RAS/BRAF* mutational status.[3–6]

On the other hand, in metastatic colorectal cancer (mCRC), the prognostic value of the tumor immune infiltrate has been investigated[7–13] but the predictive impact for patients treated with cytotoxic and targeted agents with or without ICIs still needs to be elucidated.

To this regard, in a retrospective study, among 85 patients with deficient mismatch repair (dMMR)/microsatellite instability-high (MSI-H) mCRC treated with ICIs, those with a higher number of TILs achieved higher response rates and longer survival, thus supporting the potential role of TILs count as a predictive biomarker of ICIs efficacy in the dMMR/MSI-H population.[14] These results are in line with those obtained in the setting of advanced non-small cell lung cancer (NSCLC). Indeed, in a retrospective study of patients with NSCLC treated with nivolumab or chemotherapy, high TILs were associated with favorable outcomes in the cohort receiving the ICI, but not in patients treated with chemotherapy.[15]

Although the majority of proficient mismatch repair (pMMR)/microsatellite stable (MSS) tumors has an absent or inactive infiltrate of CD8+ T cell, recent evidence postulated a relevant heterogeneity also among these tumors.[6] Indeed, according to the post hoc subgroup analysis of the AtezoTRIBE study, a phase II trial comparing upfront FOLFOXIRI (5-fluorouracil, leucovorin, oxaliplatin and irinotecan)/bevacizumab (bev) alone or in combination with the anti-programmed death ligand-1 (PD-L1) atezolizumab, Immunoscore-IC, a novel assay measuring the densities of PD-L1+ and CD8+ cells as well as the proximity among these cells, stands as a potential predictor of benefit from the addition of the ICI. Notably, the predictive impact of Immunoscore-IC, being Immunoscore-IC high tumors more likely to derive benefit from the use of atezolizumab in terms of progression-free survival (PFS), seems independent of MMR status and tumor mutational burden, and clearly evident also among patients with pMMR tumors.[16] Consistently, an exploratory analysis of the CheckMate 9×8 study, a phase II trial comparing upfront FOLFOX (5-fluorouracil, leucovorin, and oxaliplatin)/bev alone or in combination with the anti-programmed cell death protein-1 (PD-1) nivolumab, suggested that among patients with pMMR/MSS tumors, CD8+ T cell levels ≥2% in the tumor microenvironment could identify patients deriving benefit from the addition of the anti-PD-1 to the standard upfront therapy.[17]

PD-L1 expression is regarded as a predictive marker of ICI efficacy[18 19] and is adopted for selecting patients to be treated with ICIs in several solid tumors.[20–22] However, the benefit from ICIs in dMMR/MSI-H mCRC is observed irrespectively of PD-L1 expression.[23–25] At the same line, an exploratory subgroup analysis of the above mentioned CheckMate 9×8 study showed that the PD-L1 expression does not affect the efficacy of ICIs in combination with chemotherapy in mCRC population composed by pMMR/MSS tumors for the 95% of cases.[17]

Based on these considerations, we performed a comprehensive characterization of the tumor immune cell infiltrate and PD-L1 expression on tumor samples of patients with mCRC enrolled in the AtezoTRIBE study,[16] in order to assess the concordance among TILs, Immunoscore and Immunoscore-IC, and to explore their clinical relevance as prognostic and predictive factors with regard to the use of ICIs.

METHODS

Study population

AtezoTRIBE[16] (NCT03721653) was a phase II randomized, open-label, multicenter trial where 218 patients with initially unresectable mCRC (18–70 years old with Eastern Cooperative Oncology Group performance status (ECOG-PS) of 2 or less or 71–75 years old with an ECOG-PS of 0) were randomized in a 1:2 ratio to receive FOLFOXIRI/bev or the same regimen plus the anti-PD-L1 atezolizumab. All treatments were administered up to eight cycles, followed by 5-fluorouracil plus bev with or without atezolizumab, according to the randomization group, until disease progression, unacceptable adverse events, or consent withdrawal in both arms.

Patients with availability of an archival tumor sample collected before starting the study treatment and adequate for tumor immune cell infiltrate assessment were included in the present analysis.

Immune-markers associated with clinical outcome in the control arm of the AtezoTRIBE study, would have been validated in sample from patients previously enrolled in the TRIBE2 trial[26] (NCT02339116), a phase III randomized, open-label, multicenter trials where 679 patients with initially unresectable mCRC (aged 18–70 years old with ECOG-PS of 2 or less or 71–75 years old with an ECOG-PS of 0) were randomized 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.

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TILs, Immunoscore, Immunoscore-IC and PD-L1 and tumor mutational burden assessment

TILs assessment was performed by optical microscope and centralized at the Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa. Tissue samples were independently evaluated by two pathologists (CU and AP) blinded to clinical information, treatment regimen, and outcome. The density of TILs was defined as the mean value of five random observations and counts at high-power fields (40×) of tumor-enriched areas made of >60% of neoplastic cells on H&E-stained sections. In paucicellular tumors, such as mucinous adenocarcinomas, the analysis was performed within fields with the highest tumor cell density. Only tumor epithelium infiltrating lymphocytes were retained for scoring, while stromal lymphocytes were not scored. Tumors showing an average number of TILs <2.0 were defined as TILs-low, whereas those with ≥2.0 TILs were defined as TILs-high.[3] Immunoscore, Immunoscore-IC and PD-L1 were assessed at the laboratories of Veracyte (Marseille, France), blinded to patients' clinical data, treatment received, and outcome, as previously described.[5 16]

For Immunoscore evaluation, only surgically resected specimens from primary tumor or liver metastases were considered eligible, while biopsy samples were excluded. One formalin-fixed paraffin-embedded tumor sample containing the tumor core and invasive margin was independently selected by two pathologists (CU and AP) at the Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa. Two tumor sections were processed by immunohistochemistry for CD3 and CD8 staining. Counterstained slides were digitalized at ×10 magnification and 0.45 µm/pixel resolution (NanoZoomer-XR, Hamamatsu, Japan) and CD3+ and cytotoxic CD8+T-stained cells in the tumor core and invasive margin were quantified by digital pathology (Immunoscore Analyzer, Veracyte SAS). The Immunoscore test for each patient was calculated from the mean of four density percentiles (two markers and two regions) and then, according to predefined cut-offs, dichotomized as low or high if a 0–25% or 25–100% density was scored, respectively.

For Immunoscore-IC test, surgically resected specimens or biopsies from either primary tumor or metastatic sites were deemed eligible. A single tumor tissue section was processed by immunohistochemistry for PD-L1 and CD8 staining. Stained slides were scanned with a high-resolution scanner (NanoZoomer XR, Hamamatsu Photonics, Hamamatsu, Japan) to obtain 20×digital images. The densities of PD-L1+ cells and cytotoxic CD8+T cells in the tumor core were quantified by digital pathology on the HALO platform (Indica Labs, Corrales, New Mexico, USA), as well as their proximity and clustering. In particular, five parameters, measured as linear values, were selected for inclusion into the Immunoscore-IC score (density of total CD8, density of CD8-free (without PD-L1+ cell in proximity), density of CD8-cluster (CD8 cells in proximity of less than 20 micrometers of another CD8), density of PD-L1 cells, and distance between CD8-positive and PD-L1-positive cells). The risk-score was computed incorporating the five parameters and dichotomized into two categories: the low-risk group, characterized by high values of Immunoscore-IC markers, was defined as Immunoscore-IC-high, whereas the high-risk group, with low markers density values, was defined as an Immunoscore-IC-low.

PD-L1 immunohistochemical expression was evaluated on surgically resected specimens or biopsies from either primary tumor or metastatic sites. Stained slides were scanned with a high-resolution scanner (NanoZoomer XR, Hamamatsu Photonics, Hamamatsu, Japan) to obtain 20× digital images. The density of PD-L1+ tumor cells was quantified by digital pathology using the HALO platform (Indica Labs, Corrales, New Mexico, USA). A cut-off of 1% was adopted to differentiate tumors with high and low PD-L1 expression (online supplemental table 1).

Tumor mutational burden (TMB) was assessed by means of FoundationOne CDx assay (Foundation Medicine, Cambridge, Massachusetts, USA). A cut-off of 10 mutations/megabase was adopted to differentiate tumors with high and low TMB.[27]

Statistics

X^2 test, Fisher's exact test, or Mann-Whitney test were used when appropriate to compare clinical and molecular baseline characteristics among subgroups defined according to TILs, Immunoscore, Immunoscore-IC and PD-L1 expression. Strength of concordance between TILs and Immunoscore or Immunoscore-IC was carried out by means of K of Cohen. PFS was defined as the time from randomization to the first evidence of disease progression or death, whichever occurred first, while and overall survival (OS) was defined as the time from randomization to death. Objective response rate (ORR) was defined as the percentage of patients who achieved partial or complete response according to RECIST (Response Evaluation Criteria in Solid Tumors) V.1.1 criteria.[28] Survival curves were estimated by the Kaplan-Meier method and compared with the log-rank test. HRs with 95% CIs were estimated with Cox proportional hazards model. ORs with 95% CIs were estimated with a logistic regression model. Subgroup analyses to assess the benefit of FOLFOXIRI/bev plus atezolizumab versus FOLFOXIRI/bev based on TILs, Immunoscore, Immunoscore-IC and PD-L1 expression subgroups in terms of PFS were carried out using interaction tests. The abovementioned analyses were separately performed in the pMMR subgroup. Statistical significance was set at a p value of 0.05 for a bilateral test. Considering the exploratory nature of the present study, no adjustment for multiple testing was performed. The data cut-off for the present analysis was August 1, 2021. All analyses were carried out with SAS V.9.4.

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RESULTS

Out of 218 patients enrolled in the AtezoTRIBE study, 181 (83%), 77 (35%), 157 (72%) and 162 (74%) tumor specimens were successfully tested for TILs, Immunoscore, Immunoscore-IC and PD-L1 expression, respectively (online supplemental figure 1).

Patients' characteristics are listed in table 1.

Overall, 69 (38%), 45 (58%), 50 (32%) and 21 (13%) tumors were classified as TILs-high, Immunoscore-high, Immunoscore-IC-high and PD-L1-high, respectively. TILs-high and PD-L1-high tumors were more frequently dMMR compared with TILs-low (12% vs 3%, p=0.021) and PD-L1-low (19% vs 5%, p=0.040), while Immunoscore-IC-high tumors had more often a high TMB compared with Immunoscore-IC-low (24% vs 8%, p=0.018). In addition, TILs-high had less frequent liver metastases with respect to TIL-low (64% vs 79%, p=0.020). No other significant differences were reported among TILs, Immunoscore, Immunoscore-IC and PD-L1 expression subgroups (table 1).

Similar results were observed in the pMMR population (online supplemental table 2).

Among TILs-high tumors (n=69) with paired data available for Immunoscore (n=21, 30%) and Immunoscore-IC (n=54, 78%), 13 (62%) and 21 (39%) cases were Immunoscore-high and Immunoscore-IC-high, respectively. Similarly, among TILs-low samples (n=112) with paired data available for Immunoscore (n=53, 47%) and Immunoscore-IC (n=97, 87%), 24 (45%) and 73 (75%) were Immunoscore-low and Immunoscore-IC-low, respectively. Therefore, the strength of agreement between TILs and Immunoscore or Immunoscore-IC was poor (K of Cohen=0.055 and 0.15, respectively) (figure 1, panel A). Consistent results were reported in the pMMR population (figure 1, panel B). In pMMR population, no differences were observed between TILs-high and TILs-low tumors in terms of PFS (p=0.36) (online supplemental figure 2, panel A), as well as between PD-L1-high and PD-L1-low tumors (p=1.0) (online supplemental figure 2, panel D). On the other hand, patients with Immunoscore-high or Immunoscore-IC-high tumors achieved longer PFS than those with Immunoscore-low (–16.4 vs 12.2 months; HR: 0.55, 95% CI: 0.30 to 0.99 p=0.049) (online supplemental figure 2, panel B) Immunoscore-IC-low (–14.8 vs 11.5 months; HR: 0.55, 95% CI: 0.35 to 0.85; p=0.007) (online supplemental figure 2, panel C). Interaction effects between TILs, Immunoscore and Immunoscore-IC subgroups and treatment arm were observed in terms of PFS, while the benefit of the addition of ICIs to FOLFOXIRI/bev was independent of PD-L1 expression (figures 2 and 3). Unexpectedly, a higher benefit in favor of the addition of atezolizumab was reported in patients with TILs-low tumors (p for interaction=0.039). On the other hand, patients with Immunoscore-IC-high tumors showed a higher PFS advantage from the addition of atezolizumab (p for interaction=0.006). The same trend for higher benefit in favor of FOLFOXIRI/bev plus atezolizumab was observed in patients with Immunoscore-high tumors (p for interaction=0.13). No interaction effect was shown between the four immune-biomarkers and treatment arm in terms of ORR (online supplemental figure 3).

The prognostic effect of TILs, Immunoscore, Immunoscore-IC and PD-L1 expression was assessed in the group of patients with pMMR not treated with atezolizumab. In this cohort, patients with TILs-high tumors achieved longer PFS than those with TILs-low (15.0 vs 10.8 months; HR: 0.46, 95% CI: 0.22 to 0.96; p=0.034) (figure 4, panel A) populations, while no difference was observed based on Immunoscore, Immunoscore-IC and PD-L1 expression (figure 4, panels B–D).

Consistent results were observed in the overall population (online supplemental figure 4; figure 3; online supplemental figure 5). In order to validate the prognostic impact of TILs, we evaluated samples from patients enrolled in the previous TRIBE2 study of FOLFOXIRI/bev versus FOLFOX/bev as upfront therapy in unresectable mCRC. However, among 434 pMMR/MSS tumors, no differences were observed between TILs-high (N=263, 61%) and TILs-low (N=171, 39%) groups in terms of both PFS (p=0.39) (online supplemental figure 6, panel B) and OS (p=0.86) (online supplemental figure 6, panel D). Consistent results were shown in the overall population (online supplemental figure 6, panels A and C).

DISCUSSION

The introduction of ICIs has deeply improved the prognosis of several solid and hematological malignancies, including melanoma, lymphoma, lung, kidney, and urothelial cancers.[29] However, ICIs remain largely ineffective in mCRC, where the benefit of immunotherapy is currently limited to a small subset of patients with tumors harboring dMMR/MSI-H (4–5%)[23 30–32] or pathogenetic mutations of *POLE* gene (0.5–1%).[33]

Recently, the phase II randomized AtezoTRIBE study suggested that among pMMR/MSS tumors, a subgroup of tumors has an immunogenic tumor microenvironment and, consequently, may benefit from the anti-PD-L1 addition to upfront chemotherapy and bev.[16] In particular, Immunoscore-IC test is able to identify about 30% of pMMR tumors with an 'activated' immune microenvironment, accountable for preliminary evidence of benefit from adding atezolizumab to FOLFOXIRI/bev in the AtezoTRIBE trial.[16] Recently, the post hoc analysis of another phase II randomized trial, CheckMate 9×8, reported a higher benefit from the addition of nivolumab to FOLFOX/bev among patients with CD8+ T cells levels higher than 2%.[17]

In the present study, we compared the predictive impact of two other candidate biomarkers depicting the immune tumor microenvironment, TILs and Immunoscore, in patients enrolled in the AtezoTRIBE trial. Although TILs and Immunoscore showed a clear prognostic effect in early-stage colorectal cancer (CRC)[3–5] and their predictive value with regard to response to immunotherapy has

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Table 1 Patients' characteristics according to TILs, Immunoscore, Immunoscore-IC and PD-L1-TPS in the overall population

Characteristics	Population assessed for TILs N=181			Population assessed for Immunoscore N=77			Population assessed for Immunoscore-IC N=157			Population assessed for PD-L1-TPS N=162		
	TILs high N=69 n (%)	TILs low N=112 n (%)	P value	Immunoscore high N=45 n (%)	Immunoscore low N=32 n (%)	P value	Immunoscore-IC high N=50 n (%)	Immunoscore-IC low N=107 n (%)	P value	PD-L1 high N=21 n (%)	PD-L1 low N=141 n (%)	P value
Age (years)												
Median	59	62	0.092*	62	58	0.22*	61	61	0.56*	56	62	**0.0075***
Range	(20–75)	(36–75)		(40–75)	(38–74)		(41–75)	(20–75)		(41–71)	(20–75)	
Sex												
Male	44 (64)	65 (58)	0.44†	29 (64)	20 (63)	0.86†	28 (56)	70 (65)	0.26†	11 (52)	89 (63)	0.35†
Female	25 (36)	47 (42)		16 (36)	12 (37)		22 (44)	37 (35)		10 (48)	52 (37)	
ECOG-PS												
0	59 (86)	97 (87)	0.83†	43 (96)	27 (84)	0.12‡	45 (90)	87 (81)	0.17†	19 (90)	117 (83)	0.53‡
1–2	10 (14)	15 (13)		2 (4.4)	5 (16)		5 (10)	20 (19)		2 (10)	24 (17)	
Site of primary tumor												
Right	33 (48)	45 (40)	0.31†	25 (56)	15 (47)	0.45†	23 (46)	46 (43)	0.72†	7 (33)	64 (45)	0.30†
Left and rectum	36 (52)	67 (60)		20 (44)	17 (53)		27 (54)	61 (57)		14 (67)	77 (55)	
RAS/BRAF mutational status												
RAS/BRAF wt	10 (14)	19 (17)	0.26†	6 (13.3)	5 (16)	0.91†	8 (16)	15 (14)	0.16†	3 (14)	22 (16)	0.47†
RAS mut	48 (70)	83 (75)		32 (71)	23 (72)		32 (65)	83 (78)		14 (67)	104 (74)	
BRAF mut	11 (16)	9 (8)		7 (16)	4 (12)		9 (18)	9 (8)		4 (19)	14 (10)	
NA	–	1		–	–		1	–		–	1	
Microsatellite status												
pMMR	59 (88)	108 (97)	**0.021**‡	40 (89)	29 (94)	0.69‡	47 (94)	100 (94)	1.0‡	17 (81)	131 (95)	**0.040**‡
dMMR	8 (12)	3 (3)		5 (11)	2 (6)		3 (6)	7 (6)		4 (19)	7 (5)	
NA	2	1		–	1		–	–		–	3	
Resected primary tumor												
Yes	29 (42)	58 (52)	0.20†	41 (91)	31 (97)	0.40‡	20 (40)	60 (56)	0.061†	12 (57)	70 (50)	0.52†
No	40 (58)	54 (48)		4 (9)	1 (3)		30 (60)	47 (44)		9 (43)	71 (50)	
Prior adjuvant chemotherapy												
Yes	4 (6)	5 (4)	0.73‡	3 (7)	3 (9)	0.69‡	1 (2)	7 (6)	0.43‡	3 (14)	5 (3)	0.07‡
No	65 (94)	107 (96)		42 (93)	29 (91)		49 (98)	100 (94)		18 (86)	136 (97)	
Number of metastatic sites												
1	34 (49)	42 (37)	0.12†	18 (40)	13 (41)	0.96†	22 (44)	36 (34)	0.21†	8 (38)	52 (37)	0.91†
>1	35 (51)	70 (63)		27 (60)	19 (59)		28 (56)	71 (66)		13 (62)	89 (63)	

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Table 1 Continued

Characteristics	Population assessed for TILs N=181			Population assessed for Immunoscore N=77			Population assessed for Immunoscore-IC N=157			Population assessed for PD-L1-TPS N=162		
	TILs high N=69 n (%)	TILs low N=112 n (%)	P value	Immunoscore high N=45 n (%)	Immunoscore low N=32 n (%)	P value	Immunoscore-IC high N=50 n (%)	Immunoscore-IC low N=107 n (%)	P value	PD-L1 high N=21 n (%)	PD-L1 low N=141 n (%)	P value
Liver only disease												
Yes	20 (29)	28 (25)	0.56†	9 (20)	11 (34)	0.16†	15 (30)	25 (23)	0.37†	6 (29)	35 (25)	0.71†
No	49 (71)	84 (75)		36 (80)	21 (66)		35 (70)	82 (77)		15 (71)	106 (75)	
Liver metastases												
Yes	44 (64)	89 (79)	**0.020**†	28 (62)	26 (81)	0.074†	34 (68)	85 (79)	0.12†	17 (81)	104 (74)	0.60‡
No	25 (36)	23 (21)		17 (38)	6 (19)		16 (32)	22 (21)		4 (19)	37 (26)	
Time to metastases												
Synchronous	61 (88)	94 (84)	0.40†	30 (67)	26 (81)	0.16†	43 (86)	89 (83)	0.65†	18 (86)	119 (84)	1.0‡
Metachronous	8 (12)	18 (16)		15 (33)	6 (19)		7 (14)	18 (17)		3 (14)	22 (16)	
Treatment arm												
FOLFOXIRI/bev	21 (30)	45 (40)	0.19†	12 (27)	11 (34)	0.47†	18 (36)	39 (36)	0.96†	7 (33)	51 (36)	0.80†
FOLFOXIRI/bev/atezo	48 (70)	67 (60)		33 (73)	21 (66)		32 (64)	68 (64)		14 (67)	90 (64)	
Tumor mutational burden												
High (≥10 Mut/Mb)	8 (17)	7 (8)	0.12†	8 (21)	2 (7)	0.17‡	8 (24)	7 (8)	**0.018**†	3 (21)	13 (12)	0.39‡
Low (<10 Mut/Mb)	38 (83)	78 (92)		30 (79)	25 (93)		25 (76)	79 (92)		11 (79)	95 (88)	
NA	23	27		7	5		17	21		7	33	

Bold values indicate statistically significant data ($p < 0.05$).
*Kruskal-Wallis p value;
†Fisher's p value;
‡χ^2 p value.
atezo, atezolizumab; bev, bevacizumab; dMMR, deficient mismatch repair; ECOG-PS, Eastern Cooperative Oncology Group performance status; mut, mutated; Mut/Mb, mutations/megabase ; N, number; NA, not available; PD-L1, programmed death-ligand 1; pMMR, proficient mismatch repair; TPS, Tumor Proportion Score; wt, wild-type.

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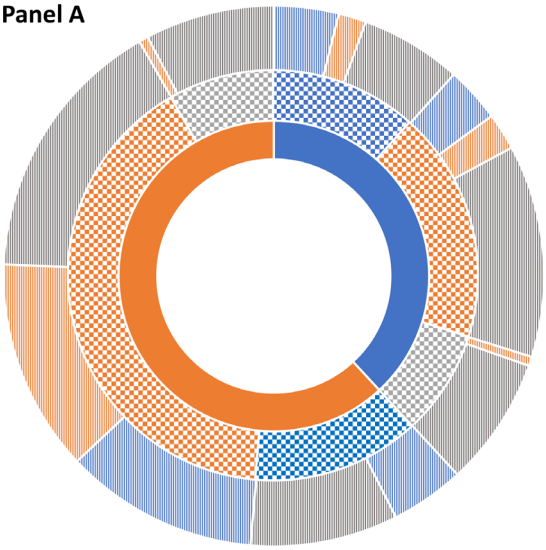
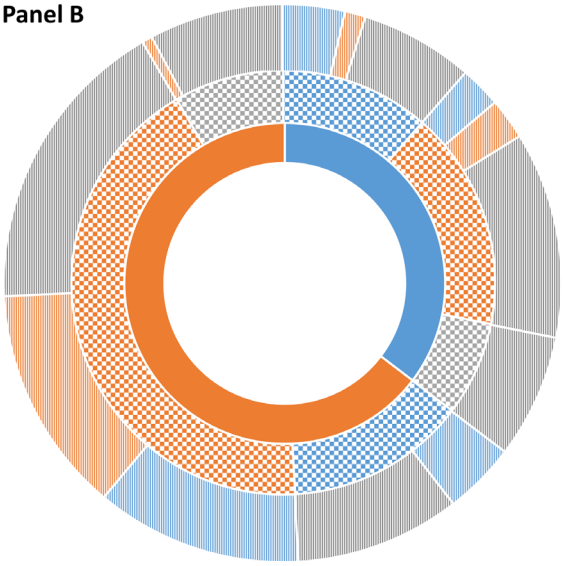
Panel A



	TILs-high N=69	TILs-low N=112	*K of Cohen*
Immunoscore			
High	13 (62%)	29 (55%)	0.055
Low	8 (38%)	24 (45%)	
NA	48	59	
Immunoscore-IC			
High	21 (39%)	24 (25%)	0.15
Low	33 (61%)	73 (75%)	
NA	15	15	

Panel B



	TILs-high N=59	TILs-low N=108	*K of Cohen*
Immunoscore			
High	10 (63%)	27 (54%)	0.059
Low	6 (37%)	23 (46%)	
NA	43	58	
Immunoscore-IC			
High	19 (40%)	23 (24%)	0.16
Low	28 (60%)	71 (76%)	
NA	12	14	

Figure 1 Donut plot showing concordance among TILs, Immunoscore and Immunoscore-IC in overall population (panel A) and in proficient mismatch repair subgroup (panel B) of the AtezoTRIBE study. TILs, tumor-infiltrating lymphocytes.

strongly been suggested,[2] whether these two biomarkers might predict the efficacy of ICIs in pMMR/MSS mCRC is currently poorly investigated. In addition, we also assessed the role of PD-L1 expression on tumor cells for its predictive value of ICI efficacy observed in other solid tumors.[18 19]

Surprisingly, higher levels of TILs were associated with lower, instead of higher, benefit from the addition of atezolizumab to upfront therapy. Conversely, as expected, a larger PFS benefit from the ICI-based strategy was detected in patients with Immunoscore-high or Immunoscore-IC-high tumors. No interaction effect was observed between treatment arms and PD-L1 expression.

It is well-known that tumor immune infiltrate includes multiple cell types with possible opposite immune-stimulating or immune-suppressive effects.[34] To this regard, a recent study reported that TILs composition in mCRC was rather heterogeneous with some tumors showing CD8+ prevalence, others having CD4+ preponderance, others being characterized by a predominance of regulatory T cells.[35] Therefore, TILs assessment, providing a rough evaluation of lymphocytes in the tumor specimen, while not distinguishing T cells according to their functions, does not adequately depict the immunogenicity of tumor microenvironment, at least in pMMR/MSS mCRC. Indeed, there is a poor agreement between TILs and Immunoscore or Immunoscore-IC (K of Cohen <0.20) in our study and similar results were showed by Pagès *et al*, where a 48% discordance was found between Immunoscore and TILs density.[5] This result might be explained by the fact that while both Immunoscore and Immunoscore-IC are measures that objectively quantify predefined T-cell subsets and describe their spatial distribution in specific tumor regions by means of digital pathology, TILs testing provides a semi-quantitative evaluation of undefined cell populations in randomly selected tumor areas, as determined by a visual operator-dependent assessment. Indeed, a weak concordance was

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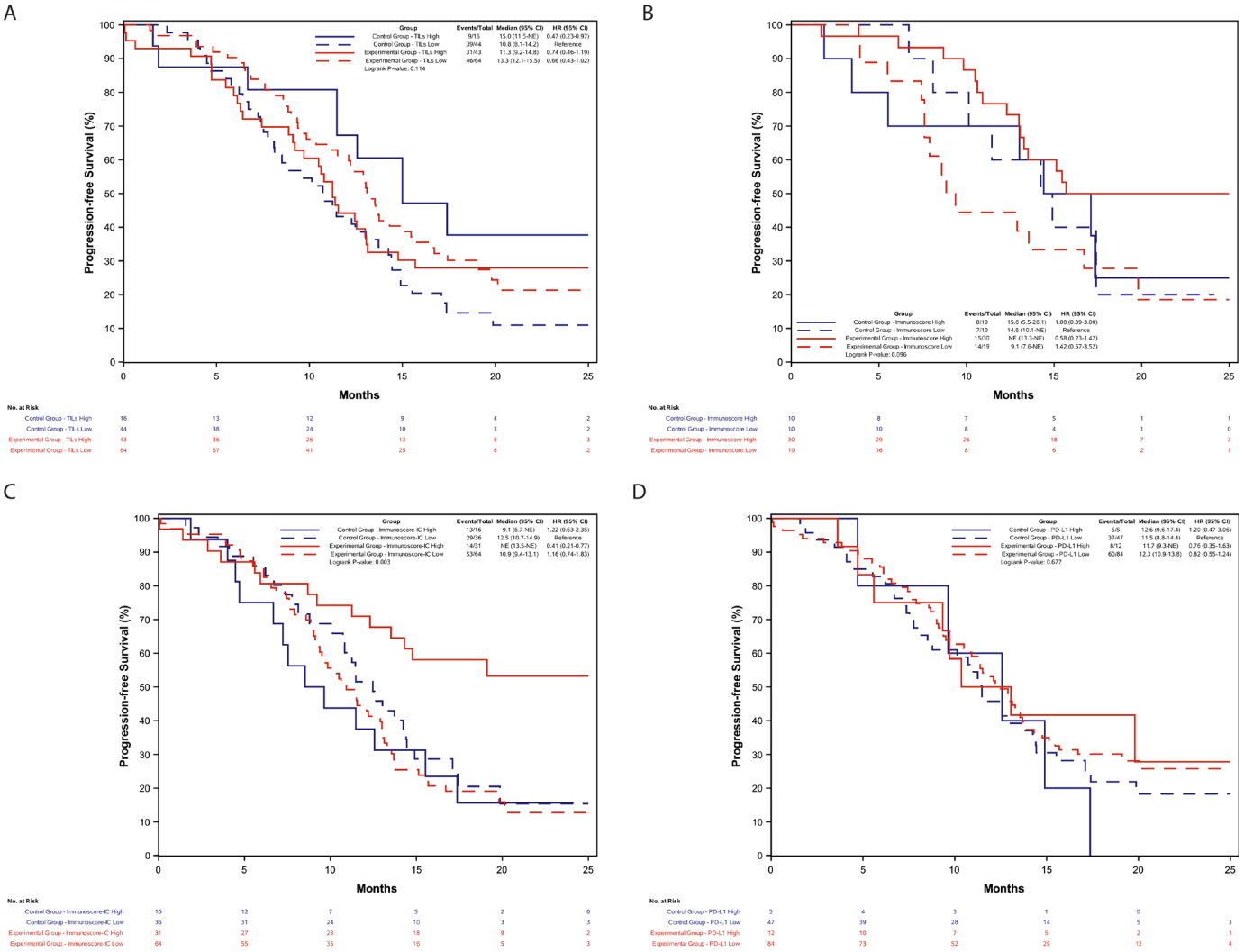

Figure 2 Kaplan-Meier curves of progression-free survival based on treatment and according to TILs (panel A), Immunoscore (panel B), Immunoscore-IC (panel C) and PD-L1 expression (panel D) in the proficient mismatch repair population of the AtezoTRIBE study. PD-L1, programmed death ligand-1; TILs, tumor-infiltrating lymphocytes.

previously reported between pathologist visual scoring of the CD3+ and CD8+ T-cell densities at the tumor site and Immunoscore for early-stage colon cancer and minimal agreement among pathologists was observed.[36] However, whether a digital assessment might better quantify TILs leading to a more suitable evaluation of their predictive value for ICIs efficacy in mCRC remains to be established. To this regard, a recent retrospective study showed that a machine learning-based analysis of H&E digital images of patients with metastatic NSCLC treated with single-agent ICIs found a higher response rates and longer PFS and OS in tumors with higher levels of TILs.[37] Moreover, a digital quantification of a subset of TILs, like PD-1+TILs and PD-1+CD8+T cells, could even more accurately catch the subgroup of patients that benefit of ICIs, as recently showed in metastatic NSCLC[38] and in early pMMR CRC.[39]

Although OS data are not yet available in our study for immature follow-up, Immunoscore and Immunoscore-IC showed no prognostic impact in terms of PFS in patients with mCRC treated without ICIs, consistently with a previous study reporting a less prognostic relevance of Immunoscore in the metastatic setting compared with early stages CRC.[10] Similarly, although patients with TILs-high tumors had better PFS when treated with FOLFOXIRI/bev in the AtezoTRIBE study, the prognostic value of TILs was not confirmed in the larger cohort of the TRIBE2 study. Indeed, immunogenic cells density decreases across stages in CRC[40 41] and therefore we can speculate that the whole lymphocyte assessment by means of TILs test appropriately catches the immunogenicity and prognosis of CRCs only in early stages when cytotoxic T cells are prevalent.

In our series both Immunoscore and Immunoscore-IC predict efficacy of ICIs addition to FOLFOXIRI/bev, but data about Immunoscore are less robust, due to the small number of evaluable cases. To this regard, a major limitation for assessing the performance of the Immunoscore in the metastatic setting is that a representative invasive margin is required for this analysis, which is lacking in the case of metastatic patients with unresected primary tumors. Indeed, in the present study, Immunoscore data was available only for 77 patients, as compared with 157

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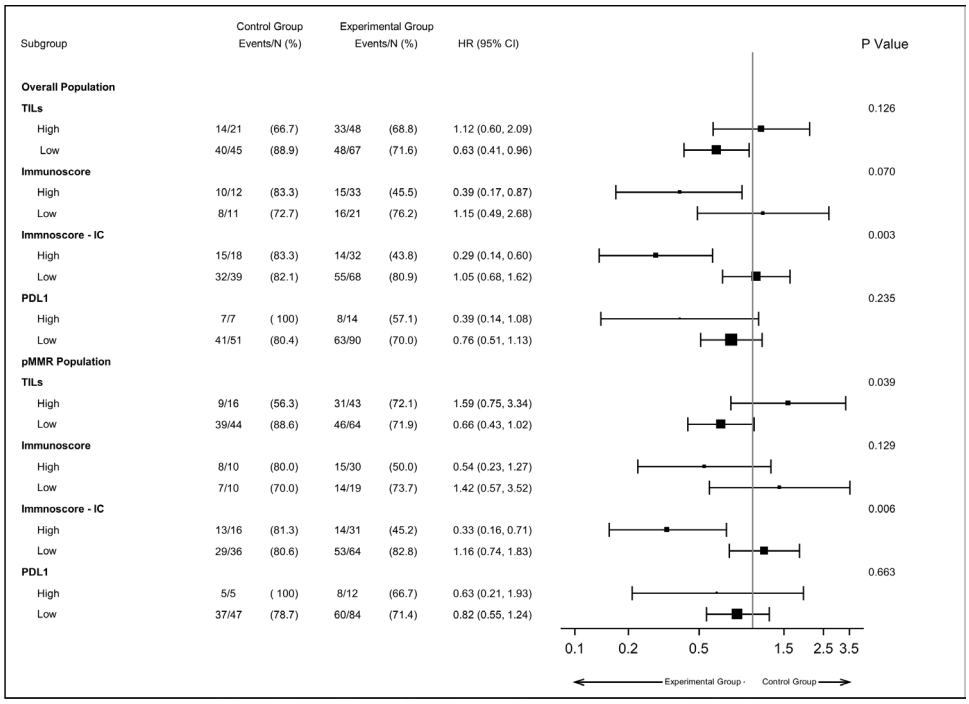

Figure 3 Forest-plot according to TILs, Immunoscore, Immunoscore-IC and PD-L1 expression of progression-free survival in the overall population and in the pMMR subgroup of the AtezoTRIBE study. PD-L1, programmed death ligand-1; TILs, pMMR, proficient mismatch repair; TILs, tumor-infiltrating lymphocytes.

cases in which Immunoscore-IC was successfully assessed. Moreover, the Immunoscore results could be potentially biased by the enrichment in the assessable population of patients with resected primary tumors that could have better prognosis than unresected patients[42] and not be suitably representative of the overall study population. More recently, a biopsy-adapted Immunoscore, a digital immunohistochemistry quantification of CD3+ and CD8+

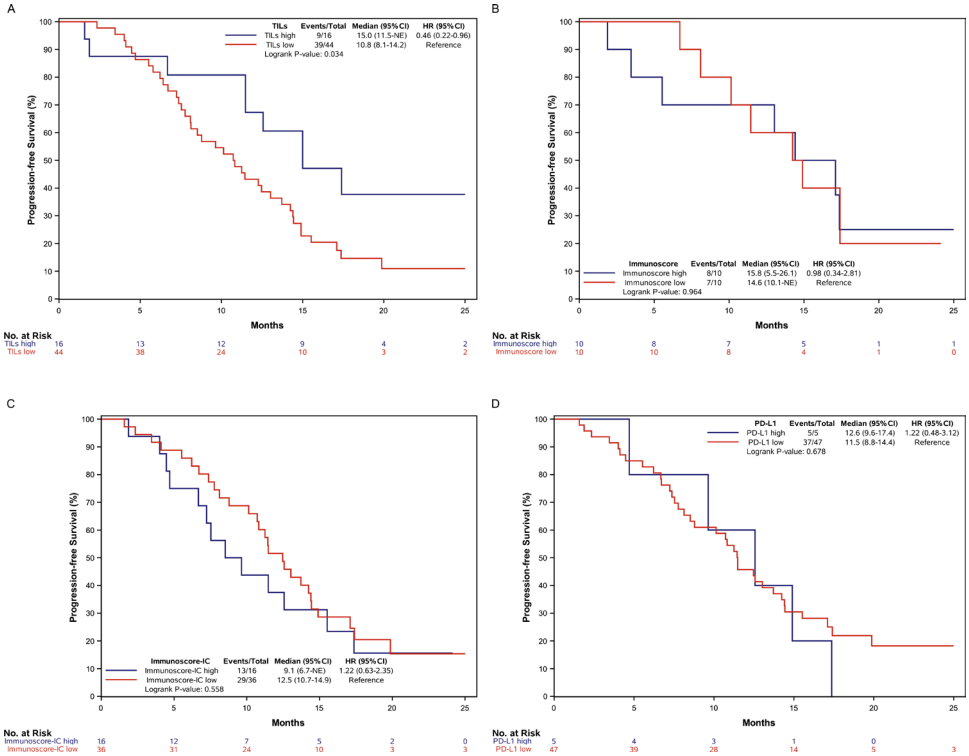

Figure 4 Kaplan-Meier curves of progression-free survival according to TILs (panel A), Immunoscore (panel B), Immunoscore-IC (panel C) and PD-L1 expression (panel D) in the proficient mismatch repair treated with FOLFOXIRI/bevacizumab of the AtezoTRIBE study. PD-L1, programmed death ligand-1; TILs, tumor-infiltrating lymphocytes.

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T cells in the tumor region of biopsy samples, was developed for locally advanced rectal cancer. In this setting, biopsy-adapted Immunoscore positively correlates with histopathological response to neoadjuvant chemoradiotherapy and disease relapse thus supporting this biomarker, in addition to post-neoadjuvant treatment images, as a promising tool for selecting patients candidate to watch-and-wait strategy.[43 44] However, the prognostic impact of biopsy-adapted Immunoscore is not validated in early-stage colon cancer and in mCRC.

Differently from other solid tumors,[18 19] but in accordance with other studies both in dMMR/MSI-H[23–25] and pMMR/MSS[17] mCRC, PD-L1 expression showed no predictive role for ICIs efficacy in our analysis. However, whether PD-L1 expression assessed in both tumor and immune cells (CPS: Combined Positive Score) or in tumor infiltrating immune cells (IC score) might be a better predictor of benefit from the addition of ICIs to chemotherapy in mCRC, as reported for other solid tumors,[45] is still to be established.

In conclusion, our study shows that a subset of patients with pMMR mCRC with immunogenic tumor microenvironment benefit from the addition of anti-PD-L1 to upfront treatment and that Immunoscore-IC stands as the most promising marker to identify this subgroup. Considering that the analysis of investigated immune-related biomarkers was not preplanned and no correction for multiple testing was applied, our data should be regarded as hypothesis-generating. Moreover, with specific regard to Immunoscore-IC, the reliability of this assessment should be validated in samples from other trials evaluating the use of ICIs in pMMR mCRC and in a prospectively conceived randomized trial.

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Competing interests AC, AK, JF: are Veracyte employees. JG: has patents associated with the immune prognostic and predictive biomarkers, is co-founder of HalioDx, a Veracyte company. SL: has a consulting or an advisory role for Amgen, Merck Serono, Lilly, AstraZeneca, Incyte, Daiichi-Sankyo, BMS, Servier, and MSD; has received research funding from Amgen, Merck Serono, Bayer, Roche, Lilly, AstraZeneca, and BMS; and has received speakers' fees from Roche, Lilly, BMS, Servier, Merck Serono, Pierre-Fabre, GlaxoSmithKline, and Amgen. FP: honoraria from Amgen, Bayer, Servier, Merck-Serono, Lilly, MSD, Organon, BMS, AstraZeneca, Pierre-Fabre; research grants from Bristol-Myers Squibb, AstraZeneca, Agenus and Incyte. LS: speakers' and consultant's fee from MSD, AstraZeneca, Servier, Bayer, Merck, Amgen, Pierre-Fabre. GM: received speakers' fees—Merck, Amgen. CC: honoraria—Amgen, Bayer, Merck, Roche and Servier. Consulting or advisory role—Amgen, Bayer, MSD, Roche. Speakers' Bureau—Servier. Research funding—Bayer, Merck, Servier. Travel, accommodations and expenses—Roche and Servier. All other authors have declared no conflicts of interest.

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