Prognostic value of tumor-infiltrating immune cells in primary colorectal cancer and metastases

Steve Zacharias Martin (steve.martin@charite.de)
Charite Universitatsmedizin Berlin https://orcid.org/0000-0003-0653-6458
Katrin E. Tagscherer
Johannes Gutenberg Universitat Mainz
Stefanie Deckert
Johannes Gutenberg Universitat Mainz
David Horst
Charite Universitatsmedizin Berlin
Markus Moehler
Johannes Gutenberg Universitat Mainz
Hauke Lang
Johannes Gutenberg Universitat Mainz
Wilfried Roth
Johannes Gutenberg Universitat Mainz

Research article

Keywords: metastases, metastasized colorectal cancer, prognostic biomarker, colorectal cancer, immune infiltrate, TILs

Posted Date: June 22nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-36627/v1

Abstract

Background

Precise prognostic biomarkers are urgently needed to improve treatment and hence prognosis for patients with metastasized colorectal carcinoma. Tumor-infiltrating CD3 and CD8 positive lymphocytes have been shown to robustly predict survival of colorectal cancer in stages I-III in retro- and prospective studies. Their clinical value in metastasized tumors or the role of other types of immune cells is not well known.

Methods

We performed a detailed characterization of tumor infiltrating immune cells of primary colorectal cancer and metastases of 55 patients. Immune infiltrates were assessed visually and digitally.

Results

High densities of CD3, CD8, CD20, CD4, CD45R0, CD68, FOXP3, PD1 and PD-L1- positive cells in both primary colorectal cancer and metastases were significantly associated with prolonged survival in univariate and multivariate analysis. For CD3-CD8 immunoscore, CD8-CD20 TB cell score, CD45R0, CD68 and PD1 the evaluation of metastatic immune infiltrates showed superior hazard ratios, compared to evaluation of primary tumor-infiltrating immune cells (hazard ratio = 9.2; 5.7; 8.9; 5.7; 6.0, respectively). In case of FOXP3, CD4 and PD-L1 the combined evaluation of primary and metastatic immune infiltrates increased prognostic precision even further (hazard ratio = 5.7; 19.6 and 8.5, respectively). Visual analysis confirmed digital image analysis, but showed inferior hazard ratios.

Conclusions

A broad range of types of immune infiltrates predicts longer survival in metastasized colorectal cancer patients. Of all immune infiltrates, CD4 showed the highest prognostic precision. The combined assessment in primary colorectal cancer and metastases is valuable in most cases. Thus, automated digital analysis of immune infiltrates in colorectal metastases could improve the prognostic stratification of patients.

Background

Colorectal cancer (CRC) is the third most common malignancy in the world and over one-half of these patients will develop liver metastases[1, 2]. Currently, the prognosis and hence stratification to treatment regimens is based solely on anatomical and histopathological criteria of the TNM classification[3] and tumor cell differentiation[4, 5]. Although this approach has proven to be clinically useful in the past, its

predictive accuracy is only moderate[6–8]. In order to improve the prognosis of metastasized colorectal cancer patients, there is a high clinical demand for more accurate prognostic markers to identify patients at high risk. A very promising biomarker is an immunoscore introduced by Galon et al.[6, 9, 10]. Here, the combined density of CD3 and CD8 positive lymphocytes at the invasive margin (IM) and tumor center (CT) of primary colorectal cancer and metastases is measured and a score calculated. Numerous studies have shown that patients with higher immunoscores are characterized by prolonged survival. Furthermore, this immunoscore has successfully been validated in a large prospective multinational and multicenter study[11] and was described to exceed the prognostic power of the TNM classification system[8]. However, the strong evidence of its prognostic value is limited to UICC stages I-III[11–22]. Patients with metastasized colorectal carcinoma have rarely been investigated[23–25], although they present a group of immense clinical importance due to their unfavorable prognosis. Furthermore, only CD3 and CD8 were included in the immunoscore, although tumor-infiltrating immune cells in general are thought to influence tumor progression and metastasis. For example, preliminary data has shown that high densities of CD20 positive B cells and FOXP3 positive regulatory T cells may be associated with better prognosis in colorectal cancer[9, 26–28]. The role of CD4 positive T helper (TH) cells and macrophages, on the other hand, is still controversial, as is the prognostic relevance of key proteins of the checkpoint inhibition system PD1 and PD-L1[9, 29–40].

In the present study, we performed a detailed characterization of a broad range of tumor infiltrating immune cells (CD3, CD4, CD8, CD20, CD45R0, CD68, FOXP3, PD1, and PD-L1) at the primary tumor site and in all available metastasectomy specimens of colorectal cancer patients. It was our aim to determine and confirm the suitability of immune infiltrates as prognostic biomarkers for patients with metastasized tumor stage. Additionally, we present a comparison of digital image analysis and conventional visual analysis.

Methods

Patients

A total of 55 patients with metastasized colorectal cancer were included in this study, which underwent surgical treatment of their primary cancer and metastases at the Department of General Visceral and Transplantation Surgery of the University Medical Center Mainz between 2000 and 2015. Clinical and pathological data were collected using pathology reports and medical charts. To obtain survival data, patients were followed up for a mean of four years [Min = 2 months; Max = 201 months]. Data of patients without the occurrence of death in the follow-up period were censored. 36 of the patients suffered from synchronous colorectal cancer and 19 patients from metachronous disease. The metastasectomy specimens were obtained from the liver (n = 84) or lung (n = 5). Of the 55 patients, 16 received preoperative systemic therapy or radiation treatment and 36 were treated with a postoperative systemic therapy. Table 1 depicts the patient’s clinical characteristics.

Table 1
Clinical characteristics of the patient cohort

Patients		**Primary Cancer**		**Biological Characteristics**	
number of patients	55	**Localization**		KRAS mutation status	
		right-sided	13	mutated	16
Age [years]		left-sided	24	wildtype	24
Median [Min; Max]	66 [27; 84]	rectal	18	unknown	15
< 50 years	6				
≥ 50–60 years	13	**TNM-classification**		NRAS mutation status	
≥ 60–70 years	10	pT1	1	mutated	0
≥ 70–80 years	17	pT2	2	wildtype	14
≥ 80 years	9	pT3	44	unknown	41
		pT4a	7		
Biological gender		pT4b	1	MSS (IHC)	55
male	39	pN0	15		
female	16	pN1a	9	**Metastases**	
		pN1b	8	synchronous	36
Death in follow-up		pN1c	1	metachronous	19
Yes	29	pN2a	13		
Survival Median [Min; Max], months	28 [7; 87]	pN2b	9	number of metastases/patient	
No (censor)	26			1	8
Survival Median [Min; Max], months	42 [2; 201]	**Grading**		2	16
		G2	44	3	8
		G3	11	4	3
				≥ 5	20

Min = minimum; Max = maximum; MSS (IHC) = microsatellite stability evaluated by immunohistochemistry (MLH1, MSH2, MSH6, PMS2)

Patients		Primary Cancer	Biological Characteristics
preoperative treatment		**postoperative treatment**	
yes	16	yes	36
no	36	no	16
unknown	3	unknown	3
Min = minimum; Max = maximum; MSS (IHC) = microsatellite stability evaluated by immunohistochemistry (MLH1, MSH2, MSH6, PMS2)			

Immunohistochemistry

Paraffin embedded diagnostic material of primary colorectal cancer and metastases were selected that included both the tumor center and its invasive margin. The invasive margin was defined as an area of 1 mm width that is centered at the border separating the uninvolved tissue from malignant cells. The tumor center includes all the tissue inside the invasive margin. Immunohistochemical staining was performed automatically using the Dako EnVision FLEX HRP/DAB; K 8010 Kit (Dako, Agilent, Santa Clara, USA) and the Dako Autostainer platform (Dako, Agilent, Santa Clara, USA) according to the manufacturer's specifications. All buffers and chemical agents were included in the kit. While the primary antibodies CD3 (Dako Ref.: IR503, rabbit), CD4 (Dako Ref.: IR649, mouse), CD8 (Dako Ref.: IR623, mouse), CD20 (Dako Ref.: IR604, mouse) and CD68 (Dako Ref.: IR609, mouse) were ready to use, CD45R0 (Cell Marque Ref.: 147M-95, mouse) was diluted 1:300; FOXP3 (Abcam Ref.: ab20034; mouse) and PD1 (Abcam Ref.: ab52587; mouse) were diluted 1:100 and PD-L1 (Abcam Ref.: ab213524, rabbit) was diluted 1:250. For antigen retrieval, sections were heated in a steam cooker for either 20 min at pH 9 in EDTA-buffer (CD3, CD4, CD8, CD20, CD45R0, and CD68) or for 35 min at pH 6 in citrate-buffer (FOXP3, PD1 and PD-L1). The immunostained slides then were digitized at 40x magnification using a NanoZoomer-Series Digital Slide Scanner (Hamamatsu Photonics, Hamamatsu, Japan). Analysis of the immune infiltrate was performed digitally and visually.

Analysis of infiltrates of immune cells and key proteins of the checkpoint inhibition system

The immune infiltrates (CD3, CD4, CD8, CD20, CD45R0, CD68 and FOXP3) and key proteins of checkpoint inhibition (PD1 and PD-L1) were quantified at the invasive margin and tumor center of primary colorectal cancer and at the invasive margin of all metastasectomy specimens. For the purpose of better readability, those will be subsumed as immune infiltrates in the remainder of this article. The tumor center of metastases was not considered due to extensive regressive changes and necrosis.

For visual analysis, immune infiltrates were assessed semiquantitatively based on the IC Score, defined as the proportion of tumor area that is occupied by the positively stained cells [41].

For digital analysis, a ready to use software based on the Halo platform from Indica Labs (Corrales, NM, USA) was used. On the one hand, the total number of stained cells was quantified in relation to the total area of the invasive margin or tumor center (cell-count/mm²). On the other hand, the area of the immune stain was assessed in relation to the total area of the invasive margin or tumor center (stain-area/mm²x10^5). For evaluation of the cell-count/mm², the CytoNuclear module v1.4 was applied and for the evaluation of the stain-area/mm²x10^5 the area quantification module v1.4 was applied. Firstly, the invasive margin and tumor center were manually selected for each whole slide. Then ten representative sections were used to define staining parameters in a training phase (e.g. minimum nuclear optical density, minimum staining optical density, nuclear and cellular size and roundness) for an optimal recognition of positively stained cells. All automated results were visually validated for accuracy and manually corrected for misclassified areas (e.g. artefacts due to air-bubbles, inking or tissue folding). The CD4, FOXP3, PD1 and PD-L1 stained sections showed a strong and non-specific staining of hepatocytes. Therefore, a HALO tissue classifier was trained to recognize and exclude hepatocytes in whole slides of hepatic metastases for those immunostains. Supplement Fig. 1 illustrates the digital image analysis process. Figures 1a and 1b depict digital quantification of all immune infiltrates in box-plots.

Immune infiltrate Scoring System

For survival analysis using the Kaplan-Meier method, patients were dichotomized into two groups showing low grade and high grade immune infiltrates. For visual analysis, the following cut-off values of IC Scores (defined as before) were used: CD3, CD8, CD20, CD4, CD45R0, PDL-1, PD1, FOXP3: 1% and CD68: 50%. Supplement Fig. 2 depicts examples of low grade and high grade immune infiltrates for primary colorectal cancer and Supplement Fig. 3 for the metastases.

For digital analysis, ROC curve analysis was performed related to the patient's overall survival and the Youden`s index and its associated criterion was defined as cut-off value[42]. Additionally, analysis was repeated using the median as cut-off value. Evaluated immune infiltrate values of all stains and cut-off values are depicted in Supplementary Table 1. Immune infiltrates with a value above the cut-off were given a Score 1 (high) and such below a Score 0 (low). CD3 and CD8 positive lymphocytes (immunoscore) as well as CD8 and CD20 positive lymphocytes (TB-cell score) were combined as defined by Galon et al. and Mlecnik et al.[6, 25], to enhance comparability to those studies. Survival analysis was performed for primary carcinomas and metastases individually and combined. Since some patients had more than one metastasis, the least infiltrated, most infiltrated and last resected metastasis was considered in the survival analysis. In case of only one metastasis, the same metastasis was considered as least infiltrated, most infiltrated and last resected.

Final scores of immune infiltrates were calculated using the following rules (symbols: CT = tumor center; IM = invasive margin; P = primary cancer; M = metastasis):

Immunoscore (CD3-CD8):

Primary cancer = $^{P}CD3_{IM} + {}^{P}CD8_{IM} + {}^{P}CD3_{CT} + {}^{P}CD8_{CT}$

Metastasis = $^{M}CD3_{IM} + {}^{M}CD8_{IM}$

Primary and metastasis combined = $^{P}CD3_{IM} + {}^{P}CD8_{IM} + {}^{P}CD3_{CT} + {}^{P}CD8_{CT} + {}^{M}CD3_{IM} + {}^{M}CD8_{IM}$

TB-cell score (CD8-CD20):

Primary cancer = $^{P}CD8_{IM} + {}^{P}CD20_{IM} + {}^{P}CD8_{CT} + {}^{P}CD20_{CT}$

Metastasis = $^{M}CD8_{IM} + {}^{M}CD20_{IM}$

Primary & metastasis combined = $^{P}CD8_{IM} + {}^{P}CD20_{IM} + {}^{P}CD8_{CT} + {}^{P}CD20_{CT} + {}^{M}CD8_{IM} + {}^{M}CD20_{IM}$

CD4, CD45R0, CD68, FOXP3, PD1 and PD-L1 Score (e.g. CD4):

Primary cancer = $^{P}CD4_{IM} + {}^{P}CD4_{CT}$

Metastasis = ^{M}CD4IM

Primary and metastasis combined = $^{P}CD4_{IM} + {}^{P}CD4_{CT} + {}^{M}CD4_{IM}$

Scores had a possible range from 0 to 6. Survival analysis was then performed for each score and again after dichotomization in patients with low grade and high grade immune infiltrates using the Youden`s index or the median as cut-off value.

Statistical Analysis

Quantification of immune infiltrates was presented in box plots indicating the minimum, first quartile, median, third quartile and maximum. The Kaplan-Meier method was used for survival analysis. Survival curves were calculated for overall scores and for dichotomized groups (low grade and high grade). The significance of the differences between low grade and high grade immune infiltrate groups was compared using the logrank test chi². For the overall scores the logrank test chi² was used to determine the likelihood of a survival trend across the scores. Univariate and multivariate regression models based on Cox proportional hazards models were used to determine the hazard ratios (HR), which were visualized in forest plots. The clinicopathological parameters age, postoperative treatment, grading of differentiation, localization, number of metastases, preoperative treatment, pN, pT, biological sex and synchronicity of metastasis were analyzed in univariate analysis and those found to be statistically associated with survival included in the multivariate regression models. Since only 55 patients were included in the study and 29 of them died in follow-up, only 3 covariates should be included in the multivariate regression model. Therefore, not all immune infiltrates could be included at the same time. Multivariate regression analysis was therefore performed for each type of immune infiltrate for primary colorectal cancer, metastases and the combined score individually and the type of metastasis with

highest hazard ratio in univariate analysis was selected. The parameters regression coefficient b, its standard error SE, Wald statistic $(b/SE)^2$, p-value P, Exp(b) and the 95% confidence interval for Exp(b) are provided. A p-value of less than 0.05 was considered as statistically significant. Statistical analysis was performed using MedCalc for Windows, version 18.11 (MedCalc Software, Ostend, Belgium).

Results

Clinicopathological parameters

The prognostic significance of clinicopathological parameters (age, preoperative treatment, grade, localization, number of metastases, pTNM stage, biological sex and synchronicity of metastasis) was analyzed using univariate Cox proportional hazards models. Of all clinicopathological parameters, only high pT-stage (pT4 vs. pT1-3) and preoperative treatment were statistically associated with a poor outcome (see Table 2). Those were included in multivariate regression models.

Table 2
Univariate analysis of clinicopathological parameters

Clinicopathological parameters	Hazard Ratio	Min	Max	p-value
Age (> 65y vs. ≤65y)	0.7762	0.3660	1.6462	0.5089
postoperative treatment (yes vs. no)	0.6353	0.2920	1.3820	0.2526
Grading (G3 vs. G2)	1.3116	0.4953	3.4736	0.5852
localization of primary tumor (right sided vs. left sided)	1.0329	0.4328	2.4648	0.9419
Number of metastases (> 4 vs ≤ 4)	1.1967	0.5685	2.5189	0.6363
preoperative treatment (yes vs. no)	**0.2634**	**0.1012**	**0.6855**	**0.0063**
pN (pN1,2 vs. pN0)	0.839	0.3742	1.8810	0.6699
pT (pT4 vs. pT1-3)	**0.1737**	**0.03906**	**0.7727**	**0.0215**
biological gender (male vs. female)	0.7566	0.3434	1.6670	0.4889
synchronicity of metastasis (metachronous vs. synchronous)	1.0183	0.4789	2.1653	0.9624
Min = Minimum; Max = Maximum				

Primary tumor-infiltrating immune infiltrates as prognostic marker in metastasized CRCs

The prognostic significance of the density of immune infiltrates in the invasive margin and tumor center of primary colorectal cancer was analyzed using univariate Cox proportional hazards models and Kaplan

Meier Curves. Immune infiltrates were assessed digitally and visually and divided into a low grade and a high grade group based on predetermined cut-off values using ROC curve analysis (see Supplementary Table 1). Scores were calculated as specified in the methods section.

Strikingly, survival analysis by the Kaplan Meier method revealed that a low density of all investigated immune infiltrate types (CD3-CD8, CD8-CD20, CD4, CD45R0, CD68, FOXP3, PD1 and PD-L1 positive cells) was statistically associated with a poor outcome (Table 3, Fig. 2) in visual and digital analysis. Only for PD1, visual analysis did not show a statistically significant p-value below 0.05. Hazard ratios were consistently lower for visual analysis compared to digital analysis for all types of immune infiltrates. Digital assessment of stain area and cell-count of immune infiltrates led to comparable hazard ratios, except for CD4 and PD-L1, where quantification of the stain area led to much higher hazard ratios (17.3 vs. 2.8 and 8.2 vs. 3.4 respectively). These two immune infiltrates showed the highest hazard ratios among all other types. Visual analysis confirmed the findings of digital analysis; however hazard ratios were consistently lower. A detailed overview of all Kaplan Meier curves and hazard ratios is presented in Supplementary Figs. 4 and 5.

Table 3
Univariate analysis of primary colorectal cancer tumor-infiltrating immune cells

Immune infiltrate Type	quantification	Hazard Ratio	Min	Max	p-value
CD3-CD8	digital (stain area)	3.6228	1.6692	7.863	0.0011
CD8-CD20		5.5956	2.5895	12.0916	< 0.0001
FOXP3		4.4878	2.0887	9.6427	0.0001
CD4		17.3364	6.3679	47.1978	< 0.0001
CD45R0		2.6225	1.2427	5.5344	0.0114
CD68		3.5983	1.6873	7.6736	0.0009
PD-L1		8.2467	3.7158	18.3027	< 0.0001
PD1		3.543	1.6455	7.6284	0.0012
CD3-CD8	digital (cell-count)	4 2051	1 9865	8 9016	0.0002
CD8-CD20		6.5545	3.0285	14.1897	< 0.0001
FOXP3		3.6889	1.7081	7.9669	0.0009
CD4		2.8025	1.3376	5.8719	0.0063
CD45R0		2.974	1.2682	6.9745	0.0122
CD68		3.1432	1.4944	6.6111	0.0025
PD-L1		3.4242	1.623	7.2242	0.0012
PD1		2.2929	0.986	5.3322	0.054
CD3-CD8	visual	3.1656	1.4854	6.7466	0.0028
CD8-CD20		2.8742	1.3503	6.1179	0.0062
FOXP3		3.0301	1.3191	6.9606	0.009
CD4		2.2813	1.0689	4.8687	0.033
CD45R0		2.4173	1.0954	5.3343	0.0288
CD68		2.4859	1.1675	5.2935	0.0182
PD-L1		2.8483	1.3579	5.9742	0.0056
PD1		1.4597	0.6946	3.0675	0.3182

Min = Minimum; Max = Maximum

Multivariate Cox regression analysis suggests that all types of immune infiltrates contributed to the prediction of survival by digital analysis and all but PD1 and CD4 in visual analysis (see Supplement Table 2). However, only three covariates were included in the regression model (each type of immune infiltrate, pT-stage and preoperative therapy), so comparison of the different types of immune infiltrates is not possible.

Metastatic-infiltrating immune infiltrates as prognostic marker in metastasized CRCs

Beyond evaluating the primary site of colorectal carcinomas, all metastasectomy specimens were investigated as well. For patients with more than one metastasis, the least and most infiltrated metastasis and the last resected metastasis were included in the survival analysis.

A low metastatic density of all investigated immune infiltrate types (CD3-CD8, CD8-CD20, CD4, CD45R0, CD68, FOXP3, PD1 and PD-L1 positive cells) was statistically associated with a poor outcome in digital analysis (Table 4; Fig. 2). Hazard ratios were consistently higher for digitally assessed stain area when compared to cell-count (see Supplementary Table 3). The highest hazard ratios were detected for CD4 immune infiltrates of the most infiltrated metastases (HR = 13.5) and CD3-CD8 immunoscore of the least infiltrated metastases (HR = 9.2). Except for PD-L1 infiltrates, all three types of metastases showed statistically significant hazard ratios.

Table 4
Univariate analysis of metastatic tumor-infiltrating immune infiltrates

Immune infiltrate Type	quantification	type of metastasis	Hazard Ratio	Min	Max	p-value
CD3-CD8	digital (stain area)	least infiltrated	9.2072	3.4681	24.443	< 0.0001
		most infiltrated	3.1557	1.4842	6.7096	0.0028
		last resected	4.1181	1.7225	9.8453	0.0015
CD8-CD20		least infiltrated	6.6241	2.605	16.8442	0.0001
		most infiltrated	2.1278	0.9801	4.6195	0.0562
		last resected	8.5449	3.1329	23.3064	< 0.0001
FOXP3		least infiltrated	3.3253	1.578	7.0075	0.00016
		most infiltrated	3.9237	1.7681	8.7071	0.0008
		last resected	2.6837	1.2275	5.8673	0.0134
CD4		least infiltrated	4.3846	1.9417	9.9009	0.0004
		most infiltrated	13.5044	4.6423	39.2843	< 0.0001
		last resected	3.2375	1.4281	7.3395	0.0049
CD45R0		least infiltrated	2.9541	1.3754	6.3452	0.0055
		most infiltrated	8.9139	3.261	24.3662	< 0.0001
		last resected	3.3246	1.5141	7.3001	0.0028
CD68		least infiltrated	3.9659	1.8896	8.3237	0.0003
		most infiltrated	5.6578	2.5131	12.7372	< 0.0001
		last resected	3.1134	1.4767	6.5643	0.0028
PD-L1		least infiltrated	1.5078	0.7128	3.1897	0.2827
		most infiltrated	2.7459	1.2065	6.2495	0.0161
		last resected	1.9083	0.6916	5.2659	0.2121
PD1		least infiltrated	5.029	2.1525	11.7496	0.0002
		most infiltrated	6.0017	1.9465	18.505	0.0018

Immune infiltrate Type	quantification	type of metastasis	Hazard Ratio	Min	Max	p-value
		last resected	4.0143	1.6404	9.824	0.0023

In visual analysis only CD3-CD8 immunoscore (last resected), CD8-CD20 TB cell score (last resected) and CD45R0 (all types), CD68 (all types), FOXP3 (least and most infiltrated), and PD1 (most infiltrated and last resected) - immune infiltrates were statistically associated with a poor outcome (see Supplementary Table 3). Hazard ratios were consistently lower than those resulting from digital analysis. A detailed overview of all Kaplan Meier curves and hazard ratios is presented in Supplementary Figs. 4 and 5.

Multivariate Cox regression analysis suggests that all investigated types of metastatic immune infiltrates contribute to the prediction of survival in digital analysis, except for PD-L1 (see Supplementary Table 2). In visual analysis, only CD3-CD8 immunoscore and CD45R0, CD68, FOXP3 and PD1 - immune infiltrates contributed to survival prediction. However, only three covariates were included in the regression model (each type of immune infiltrate, pT-stage and preoperative therapy), so comparison of the different types of immune infiltrates is not possible.

Combination of immune infiltrates of primary colorectal cancer and metastases

Cox proportional hazard models revealed that evaluation of metastatic infiltrates of CD3-CD8 immunoscore (least infiltrated: HR = 9.2 vs. 3.6), CD8-CD20 TB cell score (last resected: HR = 8.5 vs. 5.6), CD45R0 (most infiltrated: HR = 8.9 vs.2.6), CD68 (most infiltrated: HR = 5.7 vs. 3.6) and PD1 (most infiltrated: HR = 6.0 vs. 3.5) showed higher hazard ratios than evaluation of infiltrates in primary colorectal cancer in digital analysis (see Tables 3 and 4). For CD4 (most infiltrated: 13.5 vs. 17.3), FOXP3 (most infiltrated: 3.9 vs. 4.5) and PD-L1 (most infiltrated: 2.7 vs. 8.2) evaluation of metastatic infiltrates showed lower hazard ratios than evaluation of infiltrates in primary colorectal cancer in digital analysis. As a next step, we investigated, whether a combined score of immune infiltrates of primary colorectal cancer and metastases shows increased prognostic power compared to the immune infiltrate score of each individually. Only for FOXP3 (most infiltrated: HR = 5.7), CD4 (least infiltrated: HR = 19.6) and PDL1 (least infiltrated: HR = 8.5) did the combined scores show higher hazard ratios (see Table 5).

Table 5
Univariate analysis of combined primary colorectal cancer and metastases tumor-infiltrating immune cells

Immune infiltrate Type	quantification	type of metastasis	Hazard Ratio	Min	Max	p-value
CD3-CD8	digital (stain area)	least infiltrated	4.9777	2.3362	10.6059	< 0.0001
		most infiltrated	5.1221	2.3567	11.1327	< 0.0001
		last resected	4.7393	2.2186	10.1239	0.0001
CD8-CD20		least infiltrated	6.5559	3.0368	14.1531	< 0.0001
		most infiltrated	5.9561	2.7913	12.7091	< 0.0001
		last resected	4.9275	2.2857	10.6226	< 0.0001
FOXP3		least infiltrated	4.4878	2.0887	9.6427	0.0001
		most infiltrated	5.6868	2.6571	12.1712	< 0.0001
		last resected	4.6907	2.1946	10.2334	0.0002
CD4		least infiltrated	19.6348	6.6923	57.6076	< 0.0001
		most infiltrated	17.3364	6.3679	47.1978	< 0.0001
		last resected	17.3364	6.3679	47.1978	< 0.0001
CD45R0		least infiltrated	3.2212	1.5221	6.8167	0.0022
		most infiltrated	3.6182	1.7232	7.5973	0.0007
		last resected	3.8175	1.7431	8.3606	0.0008
CD68		least infiltrated	3.997	1.8625	8.5778	0.0004
		most infiltrated	5.3545	2.5253	11.3535	< 0.0001
		last resected	3.4585	1.6109	7.4251	0.00015
PD-L1		least infiltrated	8.5184	3.757	19.3145	< 0.0001

Immune infiltrate Type	quantification	type of metastasis	Hazard Ratio	Min	Max	p-value
		most infiltrated	7.0818	3.1262	16.0426	< 0.0001
		last resected	5.0107	2.3739	10.5761	< 0.0001
PD1		least infiltrated	3.1683	1.5128	6.6354	0.0022
		most infiltrated	3.0213	1.4316	6.3762	0.0037
		last resected	2.8012	1.338	5.8647	0.0063

In analogy to the non-combined immune infiltrate scores of primary colorectal cancer and metastases, digital quantification of cell-count led consistently to similar or smaller hazard ratios compared to digital quantification of area-stain (see Supplementary Table 4). Similarly, visual analysis led consistently to smaller hazard ratios, except for CD68 and CD45R0 that showed similar hazard ratios (see Supplementary Table 4). A detailed overview of all Kaplan Meier curves and hazard ratios is presented in Supplementary Figs. 4 and 5.

Multivariate Cox regression analysis suggests that all investigated types of combined immune infiltrates of primary colorectal cancer and metastases contributed to the prediction of survival in digital analysis. In visual analysis, all but the CD8-CD20 TB-cell score contributed to the prediction of survival (see Supplementary Table 2). However, only three covariates were included in the regression model (each type of immune infiltrate, pT-stage and preoperative therapy), so comparison of the different types of immune infiltrates is not possible.

Summary of immune infiltrates with highest prognostic precision

The highest hazard ratios were calculated for the combined evaluation of immune infiltrates of primary colorectal cancer and metastases (quantification of stain area) for FOXP3 (HR = 5.7), CD4 (HR = 19.6) and PD-L1 (HR = 8.5) in digital analysis. For CD3-CD8 immunoscore (HR = 9.2; least infiltrated metastases), CD8-CD20 TB cell score (HR = 5.7, last resected metastases), CD45R0 (HR = 8.9, most infiltrated metastases), CD68 (HR = 5.7, most infiltrated metastases) and PD1 (HR = 6.0, most infiltrated metastases) evaluation of metastatic immune infiltrates alone showed highest hazard ratios for digital analysis. The evaluation of immune infiltrates in primary colorectal cancer alone led to consistently lower hazard ratios in digital analysis, as did evaluation using visual analysis.

We repeated all steps of the survival analysis with the median as cut-off value to divide immune infiltrates into low grade and high grade groups. This analysis confirmed findings of ROC curve analysis

driven cut-off values, although hazard ratios were predominantly smaller. Data is presented in Supplementary Table 5.

Discussion

All investigated primary tumor-infiltrating immune infiltrates are prognostic markers in metastasized CRC

In order to improve prognosis of metastasized colorectal cancer patients, there is a high clinical demand for more accurate prognostic markers to identify patients at high risk. In the past years, prognostic relevance of tumor infiltrating lymphocytes was detected in an increasing number of solid tumors[9, 28]. Therefore, in this study, we performed a thorough characterization of a wide panel of tumor immune infiltrates of the primary site and its metastases in a patient collective with microsatellite stable metastasized colorectal carcinoma. Strikingly, a low density of all investigated immune infiltrate types (CD3, CD8, CD20, CD4, CD45R0, CD68, FOXP3, PD1 and PD-L1 positive cells) was statistically associated with a poor outcome in this collective.

The positive prognostic significance of high CD3, CD8 and CD45R0 cell infiltrates in the invasive margin and the tumor center of colorectal carcinomas has been demonstrated several times[11–22, 28, 43]. This led to a proposition of the so-called immunoscore, calculated from both CD3 and CD8 immune infiltrates to predict the clinical course of patients in early and late tumor stages (UICC I-III, but not IV) with high accuracy[6, 10, 11]. In the present study, a prognostic significance of a high CD3-CD8 immunoscore and a high CD45R0 immune infiltrate can now be confirmed in a group of patients with metastatic colorectal carcinoma. Similarly, Bindea et al. and Berntsson et al. showed a significantly prolonged overall survival for a high CD8-CD20 TB cell score for patients of early and late tumor stages [44, 45], as we confirmed in this study.

So far, studies have mainly concentrated on CD3, CD8, CD45 and CD20 lymphocytes. Far less is known about the prognostic significance of other immune cells. The prognostic significance of CD68 positive immune infiltrates has mainly been investigated for patients with non-metastasized colorectal carcinoma and several studies described a prolonged survival of patients with high CD68 intratumoral cell densities [33–35, 37]. Only one study demonstrated this association in patients with advanced tumor stages (UICC stages III-IV)[36], but only based on visual analysis. In this study, we were able to show a link between high CD68 positive cell infiltration of primary colorectal carcinoma and prolonged survival in both visual and digital analysis.

The prognostic significance of T helper cells (TH), however, is still largely unclear. Individual studies detected a positive prognostic significance of high TH1 infiltrates, a negative prognostic significance of high TH17 infiltrates and no significance of intratumoral TH2 cells, when primary colorectal cancer was analyzed[45, 46]. In the present study, we did not subtype the TH cells, but used the well-established pan-TH

cell marker CD4. Strikingly, we detected the highest prognostic power for CD4 positive tumor infiltrates compared to all other types of immune infiltrates with a hazard ratio of 17.3. Noteworthy, digital analysis via area stain performed far better than cell-count and visual analysis. Kuwahara et al. is the only other group to have study intratumoral-infiltrating CD4 positive cells in colorectal carcinoma and found them to be among the strongest prognostic indicators[47]. However, metastasized colorectal cancer has not been included in this study.

Some studies have already detected superior survival of patients that show high FOXP3 positive immune infiltrates in primary colorectal cancer. However, predominantly early and consistently non-metastasized tumor stages were included in those studies. Here, we confirm this prognostic association in a collective of metastasized colorectal carcinomas.

The key proteins of the immune checkpoint system are currently receiving a lot of attention with regard to immunomodulation therapy in numerous malignancies. The prognostic value of immunohistochemical detection in colorectal carcinomas and their metastases, however, is yet to be determined. Only few studies investigated the possible role of PD1 and PD-L1 as prognostic biomarkers in tissue micro array based studies of microsatellite unstable[39] and stable[38, 40] colorectal carcinomas. All of these studies showed a favorable prognosis for the detection of high densities of PD1 and PD-L1 positive cells. Here, we confirmed this ability to predict a favorable prognosis on whole slides and in a metastasized disease stage.

Evaluation of metastatic tumor-infiltrating immune infiltrates increases prognostic power

An important strength of the present work is that not only primary colorectal cancer, but also all resected metastasectomy specimens were examined for immune infiltrates. Only few other groups have performed such a combined immunological characterization and data is only available for few immune infiltrate markers.

In case of the CD3-CD8 immunoscore, Mlecnik et al.[25], Halama et al.[23], Wang et al.[48] and Kwak et al.[24] analyzed the prognostic significance of the metastatic CD3-CD8 immunoscore, of which only the latter group also included the corresponding primary colorectal cancer in their evaluation. All groups reported that the immunoscore predicts prognosis following liver resection. Our data support this hypothesis, and we identified that the immunoscore of the least infiltrated metastases leads to the highest prognostic precision with the highest hazard ratio, compared to analysis of the primary tumor and other types of metastases. This finding is also supported by data of Mlecnik et al.[25], who also described the least infiltrated metastasis as the strongest prognostic marker.

In case of the CD8-CD20 TB cell score, only Meshcheryakova et al.[49] and Halama et al.[23] analyzed infiltrates of metastases and reported prolonged survival for higher scores. Primary colorectal carcinomas were not investigated. Not only can we support their findings, but since we evaluated both primary and metastatic infiltrates, we were able to identify the TB cell score of the last resected

metastasis as the marker with highest prognostic precision, even compared to the evaluation of the primary colorectal cancer alone.

We found a significant association between high metastatic CD4 infiltrates and favorable survival. Only two groups have investigated the prognostic relevance of CD4 in colorectal cancer metastases [50–52]. Katz et al. found a prognostic advantage of high metastatic CD4 positive immune infiltrates in one study, but prognostic disadvantages in another[51, 52]. However, in contrast to our study, they analyzed only small tissue samples on a tissue micro array. Due to the expected heterogeneous spatial distribution of the CD4 positive cells (own unpublished data), an analysis of whole slides seems more preferable and might explain the contradictory findings. Another group to investigate CD4 positive immune infiltrates was Tanis et al. that only performed a visual analysis[50]. In doing so, they found no association between a favorable prognosis and a high density of CD4 positive cells. However, this is not contradicting our data, as we too were not able to detect a prognostic relevance in visual analysis. Only by digital quantification a strong predictive value for survival was apparent. It is noteworthy that a combined evaluation of CD4 positive tumor infiltrates of primary cancer and the most infiltrated metastasis showed highest prognostic power of all other immune markers with a hazard ratio of 19.6.

In case of CD68, only Meshcheryakova et al. evaluated metastatic infiltrates, but did not detect a prognostic relevance[49], that we were able to identify in our study. However, Meshcheryakova et al only evaluated the cell-count/mm² in one metastasis per patient that was selected randomly, ignoring all others. Using this method, only the most infiltrated metastases showed a prognostic significance in our data. Therefore, it seems probable that due to high temporal heterogeneity of distribution of the CD68 positive cells among the metastases (own unpublished data) this group missed the prognostic value. In performing digital quantification by stain area, all types of metastases showed a significant prolonged survival for high CD68 densities. This was also confirmed by visual analysis and emphasizes the advantage of using two separate methods of digital quantification.

High metastatic FOXP3 infiltrates showed a significant positive prognostic value in this study. Furthermore, a combined score of primary and metastatic infiltrates increased prognostic power even further. This is in line with a study performed by Nakagawa et al. that also demonstrated an inferior prognosis for patients with low infiltration of peritumoral regulatory T cells in resected metastases[53]. Katz et al. did not find a prognostic value of FOXP3 positive immune infiltrates, but yet again did only analyze small tissue samples on a tissue micro array that might not sufficiently represent the heterogeneity of spatial distribution (own unpublished data)[51].

To our knowledge, metastatic infiltrates of PD1 and PD-L1 positive cells have not been investigated so far. In this study, we detected a significant prolonged survival in presence of high metastatic infiltrates and showed that evaluation of a combined score increases prognostic power even further in case of PD-L1.

Visual analysis of immune infiltrates carries some prognostic value for metastatic colorectal carcinoma

The evaluation of the prognostic significance of different types of immune infiltrates in colorectal carcinoma is almost solely based on digital image analysis. Most noteworthy is that the immunoscore was recently validated in a prospective multinational and multicenter study without including a visual analysis[11], although latter would be much easier to perform in clinical practice and is already common in breast cancer[54, 55]. In the present study, we therefore additionally assessed the immune infiltrates visually. Remarkably, the visual quantification of the immune infiltrates in the invasive margin of primary colorectal cancer and metastases confirmed the prognostic value of all immunostains, except of PD1 and CD4. However, the hazard ratios were consistently inferior to those of the digital analysis. This is in line with findings of Jakubowska et al. that visually assessed the inflammatory cell infiltrate using light microscopy with hematoxylin and eosin and found a significant shorter survival for patients with a weak infiltrate[56]. Both findings support the prognostic value of visual analysis of immune infiltrates. However, a replacement of digital image analysis by visual assessment cannot be recommended at this time.

Limitations

The here presented study has some limitations. First, only 55 patients were enrolled reducing statistical power. Therefore, multivariate regression analysis could only be performed for each type of immune infiltrate for primary colorectal cancer, metastases and the combined score individually. However, we were able to confirm the well-known prognostic significance of the CD3-CD8 immunoscore in our patient collective, which has been described as robust in numerous studies[11–22, 43]. Therefore, it seems plausible to assume that our case collection is suitable for investigating also other immune markers.

Secondly, the optimal cut-off value for dichotomizing patients in low-grade and high-grade infiltrate groups is not known. However, we analyzed our data using the two widely applied cut-off values Youden's index of ROC curve analysis and median and both showed substantially comparable findings.

Thirdly, only cases with microsatellite-stable colorectal carcinomas were investigated.

Conclusion

In this study, we demonstrate the prognostic relevance of a broad range of tumor immune infiltrates in microsatellite stable primary colorectal carcinomas and their metastases. In addition to confirming the CD3-CD8 immunoscore as a robust prognostic marker in a metastasized disease stage, we identified a broad range of other less well investigated immune infiltrates as prognostically relevant (CD4, CD20, CD45R0, FOXP3, CD68, PD1 and PD-L1) in both primary tumors and metastases. Strikingly, evaluation of CD4 shows by far the highest prognostic power amongst all other; a combined score of primary colorectal cancer and metastases increases accuracy even further. Since visual analysis of immune infiltrates is easier to perform in clinical practice and is already common in breast cancer[54, 55], we

included a visual analysis in this study design. Notably, visual analysis did indeed confirm the prognostic value of most immune markers. However, its accuracy was consistently lower than digital image analysis.

Based on findings in this study, tumor-infiltrating immune cells carry a high prognostic value in microsatellite stable and metastasized primary colorectal cancer and should be evaluated in prospective studies for clinical application. Automated digital analysis of immune infiltrates in colorectal metastases could improve the prognostic stratification of patients even further. In addition to the well-established immunoscore, the marker CD4 seems especially promising.

Abbreviations

CRC colorectal cancer

CT tumor center

HR Hazard Ratio

IM invasive margin

TH T helper cells

Declarations

Ethics approval and consent to participate:

The study was approved by our institution's ethics committee (Joint Ethics Committee of the Faculty of Economics and Business Administration of Goethe University Frankfurt and the Gutenberg School of Management & Economics of the Faculty of Law, Management and Economics of Johannes Gutenberg University Mainz; ethikkommission@wiwi.uni-frankfurt.de). Informed written consent was obtained from all participants of this study.

Consent for publication:

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files, with the exception of data that would compromise the individual privacy of the patients.

Competing interests:

The authors declare that they have no competing interests.

Funding:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions:

Conception and design: WR, SZM. Administrative support: SZM, HL, MM, DH, WR. Study materials and patient data: WR, SZM, MM, KET, SD. Experiments: SZM. Data Analysis: SZM. Manuscript writing: SZM. All authors read and approved the final manuscript.

Acknowledgements:

We want to thank Pimrapat Gebert of the Institute of biometry and clinical epidemiology of the Charité Universitätsmedizin Berlin for the competent statistical consultation of this study. Furthermore, tissue samples were provided by the tissue bank of the University Medical Center Mainz in accordance with the regulations of the tissue biobank and the approval of the ethics committee of University Medical Center Mainz. Special thanks to Stefanie Zimmer, Antonietta Valentino, Silke Mitschke and Bonny Adami.

References

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 2015;136:E359-386.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin.* 2019;69:7–34.
3. Brierley JD, GMK, Wittekind Ch. *TNM classification of malignant tumors, 8th edition*: Wiley- VCH Verlag GmbH & Co. KGaA; 2017.
4. Locker GY, Hamilton S, Harris J, et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol.* 2006;24:5313–5327.
5. Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW. Colorectal cancer. *Lancet.* 2005;365:153–165.
6. Galon J, Mlecnik B, Bindea G, et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol.* 2014;232:199–209.
7. Kirilovsky A, Marliot F, El Sissy C, Haicheur N, Galon J, Pages F. Rational bases for the use of the Immunoscore in routine clinical settings as a prognostic and predictive biomarker in cancer patients. *Int Immunol.* 2016;28:373–382.

8. Lea D, Haland S, Hagland HR, Soreide K. Accuracy of TNM staging in colorectal cancer: a review of current culprits, the modern role of morphology and stepping-stones for improvements in the molecular era. *Scand J Gastroenterol.* 2014;49:1153–1163.
9. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer.* 2012;12:298–306.
10. Galon J, Pages F, Marincola FM, et al. Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med.* 2012;10:205.
11. Pages F, Mlecnik B, Marliot F, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet.* 2018;391:2128–2139.
12. Wirta EV, Seppala T, Friman M, et al. Immunoscore in mismatch repair-proficient and -deficient colon cancer. *J Pathol Clin Res.* 2017;3:203–213.
13. Emile JF, Julie C, Le Malicot K, et al. Prospective validation of a lymphocyte infiltration prognostic test in stage III colon cancer patients treated with adjuvant FOLFOX. *Eur J Cancer.* 2017;82:16–24.
14. Mlecnik B, Bindea G, Angell HK, et al. Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity.* 2016;44:698–711.
15. Mlecnik B, Tosolini M, Kirilovsky A, et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol.* 2011;29:610–618.
16. Pages F, Kirilovsky A, Mlecnik B, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol.* 2009;27:5944–5951.
17. Ogino S, Nosho K, Irahara N, et al. Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype. *Clin Cancer Res.* 2009;15:6412–6420.
18. Laghi L, Bianchi P, Miranda E, et al. CD3 + cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. *Lancet Oncol.* 2009;10:877–884.
19. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* 2006;313:1960–1964.
20. Mei Z, Liu Y, Liu C, et al. Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis. *Br J Cancer.* 2014;110:1595–1605.
21. Nosho K, Baba Y, Tanaka N, et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol.* 2010;222:350–366.
22. Pages F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med.* 2005;353:2654–2666.
23. Halama N, Michel S, Kloor M, et al. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer Res.* 2011;71:5670–5677.

24. Kwak Y, Koh J, Kim DW, Kang SB, Kim WH, Lee HS. Immunoscore encompassing CD3 + and CD8 + T cell densities in distant metastasis is a robust prognostic marker for advanced colorectal cancer. *Oncotarget.* 2016;7:81778–81790.
25. Mlecnik B, Van den Eynde M, Bindea G, et al. Comprehensive Intrametastatic Immune Quantification and Major Impact of Immunoscore on Survival. *J Natl Cancer Inst.* 2018;110.
26. Shang B, Liu Y, Jiang SJ, Liu Y. Prognostic value of tumor-infiltrating FoxP3 + regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep.* 2015;5:15179.
27. Yuen GJ, Demissie E, Pillai S. B lymphocytes and cancer: a love-hate relationship. *Trends Cancer.* 2016;2:747–757.
28. Zhao Y, Ge X, He J, et al. The prognostic value of tumor-infiltrating lymphocytes in colorectal cancer differs by anatomical subsite: a systematic review and meta-analysis. *World J Surg Oncol.* 2019;17:85.
29. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity.* 2013;39:11–26.
30. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* 2010;141:39–51.
31. Wagner DC, Roth W. [Prognostic significance of immune cell infiltrates in tumor pathology]. *Pathologe.* 2018;39:532–538.
32. Edin S, Wikberg ML, Dahlin AM, et al. The distribution of macrophages with a M1 or M2 phenotype in relation to prognosis and the molecular characteristics of colorectal cancer. PLoS One 2012;7:e47045.
33. Chaput N, Svrcek M, Auperin A, et al. Tumour-infiltrating CD68 + and CD57 + cells predict patient outcome in stage II-III colorectal cancer. *Br J Cancer.* 2013;109:1013–1022.
34. Zlobec I, Minoo P, Terracciano L, Baker K, Lugli A. Characterization of the immunological microenvironment of tumour buds and its impact on prognosis in mismatch repair-proficient and -deficient colorectal cancers. *Histopathology.* 2011;59:482–495.
35. Forssell J, Oberg A, Henriksson ML, Stenling R, Jung A, Palmqvist R. High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clin Cancer Res.* 2007;13:1472–1479.
36. Zhou Q, Peng RQ, Wu XJ, et al. The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. *J Transl Med.* 2010;8:13.
37. Koelzer VH, Canonica K, Dawson H, et al. Phenotyping of tumor-associated macrophages in colorectal cancer: Impact on single cell invasion (tumor budding) and clinicopathological outcome. *Oncoimmunology.* 2016;5:e1106677.
38. Droeser RA, Hirt C, Viehl CT, et al. Clinical impact of programmed cell death ligand 1 expression in colorectal cancer. *Eur J Cancer.* 2013;49:2233–2242.

39. Lee LH, Cavalcanti MS, Segal NH, et al. Patterns and prognostic relevance of PD-1 and PD-L1 expression in colorectal carcinoma. *Mod Pathol.* 2016;29:1433–1442.
40. Li Y, Liang L, Dai W, et al. Prognostic impact of programed cell death-1 (PD-1) and PD-ligand 1 (PD-L1) expression in cancer cells and tumor infiltrating lymphocytes in colorectal cancer. *Mol Cancer.* 2016;15:55.
41. Ltd FH-LR. VENTANA PD-L1 (SP142) Assay. Vol 20192019.
42. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics.* 1988;44:837–845.
43. Galon J, Pages F, Marincola FM, et al. The immune score as a new possible approach for the classification of cancer. *J Transl Med.* 2012;10:1.
44. Berntsson J, Nodin B, Eberhard J, Micke P, Jirstrom K. Prognostic impact of tumour-infiltrating B cells and plasma cells in colorectal cancer. *Int J Cancer.* 2016;139:1129–1139.
45. Bindea G, Mlecnik B, Tosolini M, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity.* 2013;39:782–795.
46. Tosolini M, Kirilovsky A, Mlecnik B, et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. *Cancer Res.* 2011;71:1263–1271.
47. Kuwahara T, Hazama S, Suzuki N, et al. Intratumoural-infiltrating CD4 + and FOXP3 + T cells as strong positive predictive markers for the prognosis of resectable colorectal cancer. *Br J Cancer.* 2019;121:659–665.
48. Wang Y, Lin HC, Huang MY, et al. The Immunoscore system predicts prognosis after liver metastasectomy in colorectal cancer liver metastases. *Cancer Immunol Immunother.* 2018;67:435–444.
49. Meshcheryakova A, Tamandl D, Bajna E, et al. B cells and ectopic follicular structures: novel players in anti-tumor programming with prognostic power for patients with metastatic colorectal cancer. *PLoS One.* 2014;9:e99008.
50. Tanis E, Julie C, Emile JF, et al. Prognostic impact of immune response in resectable colorectal liver metastases treated by surgery alone or surgery with perioperative FOLFOX in the randomised EORTC study 40983. *Eur J Cancer.* 2015;51:2708–2717.
51. Katz SC, Bamboat ZM, Maker AV, et al. Regulatory T cell infiltration predicts outcome following resection of colorectal cancer liver metastases. *Ann Surg Oncol.* 2013;20:946–955.
52. Katz SC, Pillarisetty V, Bamboat ZM, et al. T cell infiltrate predicts long-term survival following resection of colorectal cancer liver metastases. *Ann Surg Oncol.* 2009;16:2524–2530.
53. Nakagawa K, Tanaka K, Homma Y, et al. Low infiltration of peritumoral regulatory T cells predicts worse outcome following resection of colorectal liver metastases. *Ann Surg Oncol.* 2015;22:180–186.

54. Denkert C, Loibl S, Budczies J, Wienert S, Klauschen F [Standardized determination of tumor-infiltrating lymphocytes in breast cancer: A prognostic marker for histological diagnosis]. *Pathologe.* 2018;39:520–531.

55. Denkert C, Loibl S, Noske A, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol.* 2010;28:105–113.

56. Jakubowska K, Kisielewski W, Kanczuga-Koda L, Koda M, Famulski W. Diagnostic value of inflammatory cell infiltrates, tumor stroma percentage and disease-free survival in patients with colorectal cancer. *Oncol Lett.* 2017;14:3869–3877.

Figures

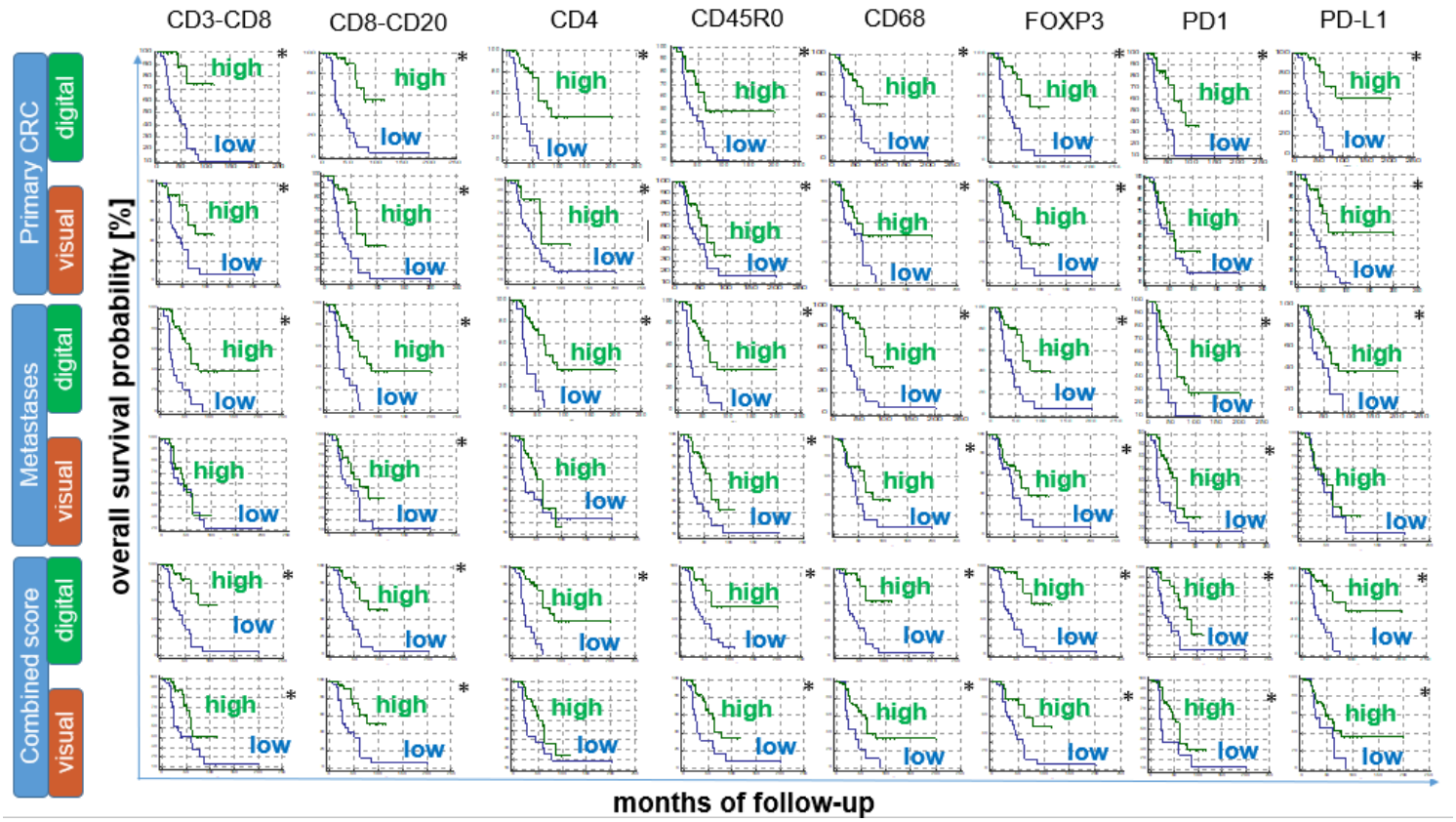

Figure 1

Kaplan Meier survival curves for high and low densities of CD3-CD8, CD8-CD20, CD4, CD68, FOXP3, PD1, PD-L1 and CD45R0 positive cell infiltrates. The upper two rows show the assessment of immune infiltrates of primary colorectal cancer. The middle two rows show the assessment of immune infiltrates of colorectal cancer metastases (CD3-CD8 = least infiltrated metastases, CD8-CD20 = last resected metastases; CD4, CD68, FOXP3, PD1, PD-L1 and CD45R0 = most infiltrated metastases). The lower two rows show the combined assessment of immune infiltrates of primary colorectal cancer and metastases (CD3-CD8= least infiltrated metastases, CD8-CD20 = last resected metastases; CD4, CD68, FOXP3, PD1, PD-L1 and CD45R0 = most infiltrated metastases). Depicted are digital (stain area/mm²x105) and visual

analyses. Censorship is marked. A star (*) in the right upper area of each graph marks a p-value below 0.05 in the logrank test chi².

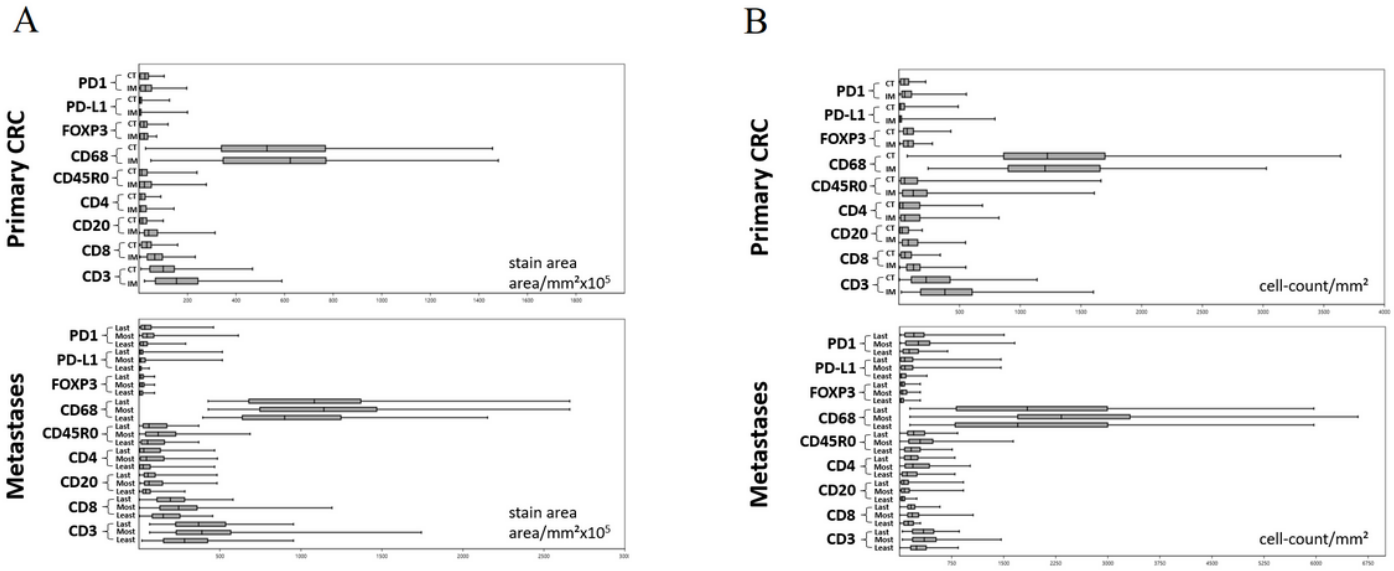

Figure 2

a: Depicted are box plots of the digital analysis of all immune infiltrate types, indicating the minimum, first quartile, median, third quartile and maximum. The upper box plot shows quantification of immune infiltrates of primary colorectal cancer (CRC) in the invasive margin (IM, lower row) and in the tumor center (CT, upper row). The lower box plot shows quantification of immune infiltrates of the metastases. Plots are presented for the last resected metastases (Last, upper), the most infiltrated (Most, middle) and the least infiltrated (Least, lower) metastases. Digital analysis quantified stain area of the immune stain (area/mm²x105). b: Depicted are box plots of the digital analysis of all immune infiltrate types, indicating the minimum, first quartile, median, third quartile and maximum. The upper box plot shows quantification of immune infiltrates of primary colorectal cancer (CRC) in the invasive margin (IM, lower row) and in the tumor center (CT, upper row). The lower box plot shows quantification of immune infiltrates of the metastases. Plots are presented for the last resected metastases (Last, upper), the most infiltrated (Most, middle) and the least infiltrated (Least, lower) metastases. Digital analysis quantified the cell-count of positively immune stained cells (cell-count/mm²).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1.pptx
- AdditionalFile2.pptx
- AdditionalFile3.pptx

- AdditionalFile4.pptx
- AdditionalFile5.pptx
- AdditionalFile6.docx
- AdditionalFile7.docx
- AdditionalFile8.docx
- AdditionalFile9.docx
- AdditionalFile10.docx