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ORIGINAL ARTICLE

Prognostic assessment of resected colorectal liver metastases integrating pathological features, *RAS* mutation and Immunoscore

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Abstract

Surgical resection of colorectal liver metastases combined with systemic treatment aims to maximize patient survival. However, recurrence rates are very high postsurgery. In order to assess patient prognosis after metastasis resection, we evaluated the main patho-molecular and immune parameters of all surgical specimens. Two hundred twenty-one patients who underwent, after different preoperative treatment, curative resection of 582 metastases were analyzed. Clinicopathological parameters, *RAS* tumor mutation, and the consensus Immunoscore (I) were assessed for all patients. Overall survival (OS) and time to relapse (TTR) were estimated using the Kaplan–Meier method and compared by log-rank tests. Cox proportional hazard models were used for uni- and multivariate analysis. Immunoscore and clinicopathological parameters (number of metastases, surgical margin, histopathological growth pattern, and steatohepatitis) were associated with relapse in multivariate analysis. Overall, pathological score (PS) that combines relevant clinicopathological factors for relapse, and I, were prognostic for TTR (2-year TTR rate PS 0–1: 49.8% (95% CI: 42.2–58.8) versus PS 2–4: 20.9% (95% CI: 13.4–32.8), hazard ratio (HR) = 2.54 (95% CI: 1.82–3.53), $p < 0.0000$; and 2-year TTR rate I 0: 25.7% (95% CI: 16.3–40.5) versus I 3–4: 60% (95% CI: 47.2–76.3), HR = 2.87 (95% CI: 1.73–4.75), $p = 0.0000$). Immunoscore was also prognostic for OS (HR [I 3–4 versus I 0] = 4.25, 95% CI: 1.95–9.23; $p = 0.0001$). Immunoscore (HR [I 3–4 versus I 0] = 0.27, 95% CI: 0.12–0.58; $p = 0.0009$) and *RAS* mutation (HR [mutated versus WT] = 1.66, 95% CI: 1.06–2.58; $p = 0.0265$) were significant for OS. In conclusion, PS including relevant clinicopathological parameters and Immunoscore permit stratification of stage IV colorectal cancer patient prognosis in terms of TTR and identify patients with higher risk of recurrence. Immunoscore remains the major prognostic factor for OS.

Keywords: colorectal cancer; colorectal liver metastases; Immunoscore; pathological score; tumor regression grading; histopathological growth pattern; chemotherapy related liver injury; steatohepatitis; tumor microenvironment

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Conflict of interest statement: *Immunoscore® is a registered trademark from INSERM. JG is co-founder and chairman of the scientific advisory board of HalioDx. JG and BM have patents associated with 'in vitro method for the prognosis of progression of a cancer' (PCT/IB2006/003168, PCT/EP2013/062405).*

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in the world, and worse prognosis is associated with metastasis in liver, lung, and peritoneum [1]. Surgical resection of colorectal liver metastases provides a 5-year survival rate of 50–60% in a subgroup of resectable patients [2]. Advancement of neo-adjuvant therapy, such as anti-VEGF and anti-EGFR treatments combined with chemotherapy, improves tumor response rate and patient eligibility for resection [3]. However, disease recurrence occurs in up to 70% of patients who have undergone curative resection, and approximately 50% of patients have a recurrence in the first 2 years posthepatectomy [4].

In order to identify this high-risk population there is a need to stratify patient using consensus, well-characterized biomarkers. For this purpose, the analysis of patho-molecular findings in resected metastasis specimens could give significant information about the efficacy of the preoperative treatment, the aggressiveness of the tumor and the status of the surrounding liver.

Several studies have reported the prognostic survival relevance after metastasis resection of some clinicopathological parameters such as size and number of lesions [5], status of the surgical margin [6], pathological tumor response assessed by tumor regression grading (TRG) [7,8], histopathological growth pattern (HGP) of metastases in the liver [9], molecular status assessed by the presence of *RAS* and *BRAF* mutation [1], and chemotherapy-associated liver injury (CALI) [10]. Evaluation of CALI often reported sinusoidal obstruction syndrome (SOS), a consequence of endothelial damage related to preoperative treatment, nodular regenerative hyperplasia (NRH), an advanced stage of SOS characterized by a nodular modification of the liver parenchyma, and steatohepatitis [10].

Furthermore, the immune microenvironment, quantified by the consensus Immunoscore (I), assessing the tumor immune infiltration of T and cytotoxic T cells, plays a major role in patient's relapse and survival in primary [11–21] and metastatic settings [22––25]. Multiple metastases occur frequently in CRC patients and are characterized by inter-tumoral genetic [26] and immune heterogeneity [22–25]; hence, one parameter seems not to be enough to characterize them and to stratify patients. To address this problem and improve the predictive accuracy of patho-molecular examination and immune assessment of metastases, we investigated all these recognized prognostic factors to study comprehensively their prognostic impact in stratifying patient prognosis.

Materials and methods

Patient selection and clinical outcome

All synchronous or metachronous metastases from a cohort of unselected patients who underwent curative metastasis resection with or without preoperative treatment at Cliniques Universitaires Saint Luc and Grand Hôpital de Charleroi (Belgium) between 2005 and 2016 were analyzed.

Patient inclusion criteria in the study were: the availability of clinical and survival information, and of formalin fixed-paraffin embedded tissue (FFPE) of all resected metastases from the patient. Patient clinical characteristics and the type of preoperative treatment (chemotherapy alone, chemotherapy + anti-VEGF, chemotherapy + anti-EGFR, no treatment) were monitored.

Chemotherapy regimens were oxaliplatin or irinotecan + fluoropyrimidine; anti-VEGF and anti-EGFR treatments were mainly bevacizumab and cetuximab respectively (monoclonal antibodies). A secure, web-based database was assembled to integrate clinical and pathological datasets. Clinical outcome was assessed in all patients. Overall survival (OS) was defined as the interval between the date of metastasis surgical resection and tumor-related death, or the last or most recent follow-up. The median follow-up period was 44.5 months (range 35.4–46.3). Time to recurrence (TTR) was defined as the interval between metastasis resection and the date of recurrence, which was monitored by clinical and imaging assessments until the patient's death or the end of the follow-up. Approval for this research was obtained from the ethics committees of the Cliniques Universitaires Saint Luc and Grand Hôpital de Charleroi.

Pathology evaluation

FFPE metastases were serially cut into 5 µm thick slices, and examined macroscopically. All sections containing tumor tissue were embedded, as well as surrounding nontumoral tissue. Morphological analysis

was done using H&E, Masson's trichrome blue and reticulin staining. The histological diagnosis was made according to WHO criteria [27]. The pathological response of each metastasis was scored according to the previously reported TRG classification previously reported [7,9]. In brief, TRG is a semi-quantitative classification system comprising five grades (TRG 1–5) based on the proportion of tumoral cells and fibrosis in the tumor (Figure 1A). The TRG grades were further grouped into high TRG (TRG 4–5) reflecting nonpathological response and low TRG (TRG 1–3) meaning pathological response [7,24]. For patients with multiple metastases, the worst TRG (higher score) among all the lesions was selected for the analysis.

HGP was assessed based on the morphology of the tumor–nontumor liver interface as described by Eefsen *et al*. [28]: desmoplastic HGP, pushing HGP, replacement HGP and mixed HGP. Mixed HGP was defined when the tumor comprised more than one pattern in the same lesion (Figure 1B). For patients with several metastases, HGP was assessed as the worst or best

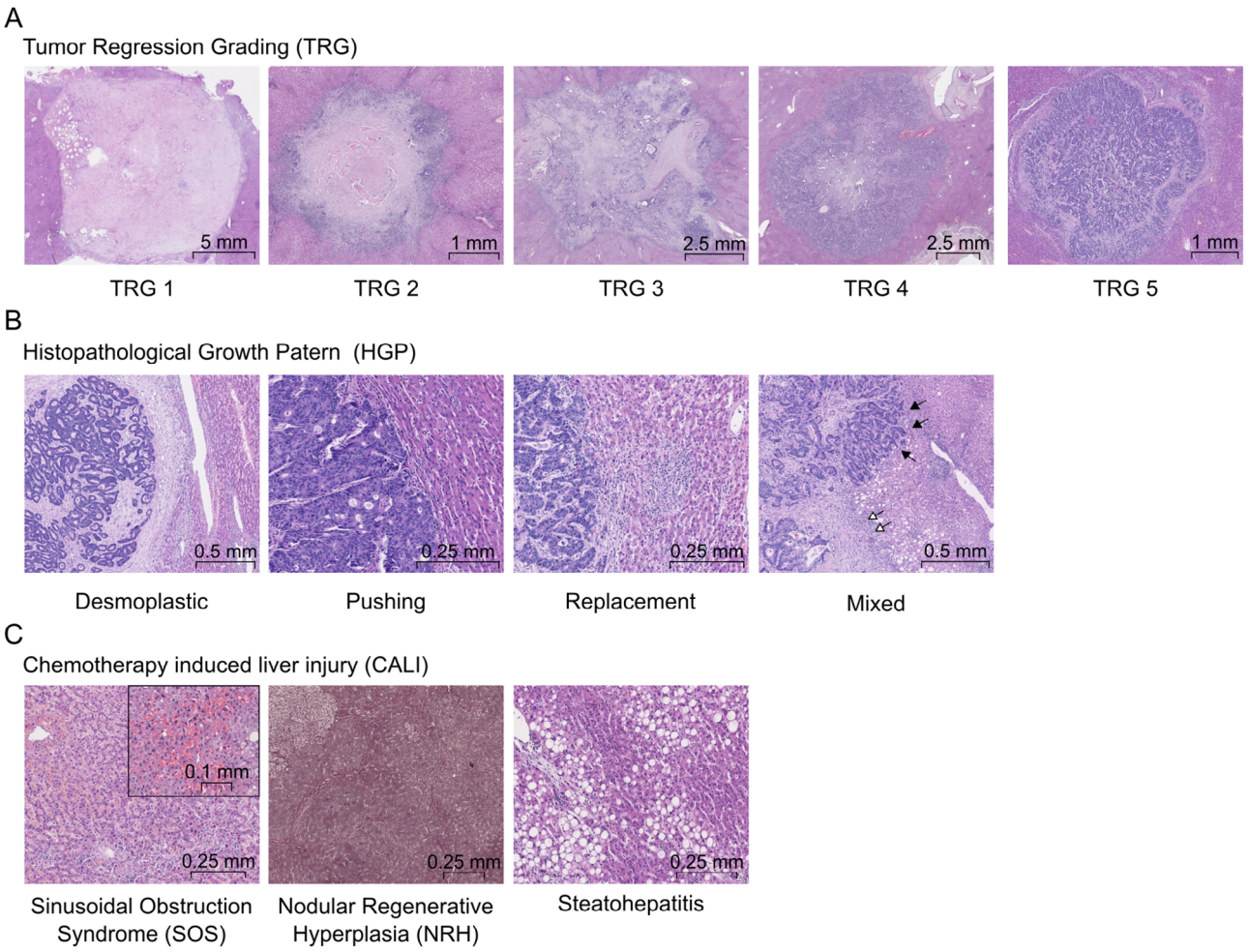

Figure 1. Pathological parameters analyzed. (A) TRG in colorectal liver metastases (H&E). TRG 1 (complete response to the treatment with maximal fibrosis); TRG 2 (major response with only scattered neoplastic cells in a fibrotic context); TRG 3 (minor response with more residual tumor cells but fibrosis predominates); TRG 4 (absence of response with residual cancer cells predominating over fibrosis); and TRG 5 (no signs of regression). (B) Four different HGPs (H&E). Desmoplastic HGP (metastasis is separated from the surrounding liver parenchyma by a desmoplastic rim), pushing HGP (metastasis grows by compressing the liver parenchyma), replacement HGP (metastases growth preserves the architecture of the hepatic tissue), and mixed HGP (a mix of two or more patterns. Desmoplastic HGP (arrow) and replacement HGP (arrowhead) are shown here). (C) Chemotherapy related liver injury (CALI) is classified in: sinusoidal obstructive syndrome (SOS, H&E) (varying degrees of endothelial damage); NRH (reticulmin staining) (nodularity aspect of the liver parenchyma without fibrosis); and steatohepatitis (H&E) (steatosis, ballooning and lobular inflammation).

HGP (the worst being defined by the presence of mixed or replacement HGP recognized to be associated with unfavorable patient prognosis [29]).

In the nontumoral hepatic parenchyma, CALI (including SOS, NRH and steatohepatitis) was assessed (Figure 1C). SOS was graded according to the previously reported histological grading system [30]: 0, absent; 1, mild (centrilobular involvement limited to one-third of the lobular surface); 2, moderate (centrilobular involvement in two-thirds of the lobular surface); and 3, severe (complete centrilobular involvement). SOS was considered as present when the score was grade 2 or 3.

NRH was graded according to the Wanless scoring system [31]: 0, absent; 1, nodules present but indistinct; 2, nodules present but only occasionally distinct; and 3, nodules distinct in most examined areas. NRH was considered as present when the score was grade 2 or 3.

Steatohepatitis was defined as the concomitant presence of steatosis, lobular inflammation, and hepatocellular ballooning [32].

The presence of perineural, vascular and biliary duct invasion was also investigated.

A positive resection margin (R1 status) was defined when the lesion crossed the surgical margin. In multiple cases, the resection margin was assessed as positive if at least one lesion was positive.

Pathoscore

Significant relevant clinicopathological parameters for TTR (multivariate analysis) were combined into a PS, that includes four parameters, namely: more than three lesions, R1 positive margin, replacement or mixed HGP and steatohepatitis. One point was given for each parameter when present and summed-up for pathological scoring.

Immunoscore

As previously reported [21,24], Immunoscore (I) was assessed by the immune densities (cells/mm^2) of CD3 and CD8 positive lymphocytes in the center of the tumor (CT) and at the invasive margin (IM) of the metastasis (see supplementary material, Supplementary materials and methods). Immunohistochemistry for CD3/CD8 was performed on FFPE slides using anti-CD3 (2GV6, Ventana, PA, USA) and anti-CD8 (4B11, Dako, Carpinteria, CA, USA) antibodies. The density of each marker in both region (CT/IM) was digitally quantified using INSERM Definiens Developer XD as described previously [24]. In brief, Immunoscore ranges from 0 (I 0) when low densities of CD3 and CD8 are found in both regions (CT/IM), to 4 (I 4) when high densities of CD3 and CD8 are found in both regions. Immunoscore was analyzed with three (I 0, I 1–2, I 3–4) or two (I 0–2, I 3–4) prognostic groups [21] (Figure 2). For those patients with multiple metastases, the lower Immunoscore among all metastases was selected for further analysis [24].

Molecular analysis: RAS, BRAF, and microsatellite instability tumor status

The presence of *RAS* and *BRAF* mutation and MSI status was assessed for all patients (see supplementary material, Supplementary materials and methods). A screening genotyping for selected mutations in *KRAS* (exon 2–4), *NRAS* (exon 2–4), and *BRAF* (exon 15, codon 600) was performed by PCR followed by pyrosequencing. MSI status was evaluated by immunohistochemistry (MLH1, MSH2, MSH6, and PMS2 antibodies).

Statistical analysis

Kaplan–Meier curves were used to assess the impact of Immunoscore and PS on TTR and OS. Univariate analysis of clinical, pathological, molecular, and immune parameters was performed using log-rank test. The Cox proportional-hazards model was performed to test the simultaneous influence on TTR and OS of all covariates. The assumption of the hazard's proportionality was assessed by the PHA (proportional hazards assumption) test. This test determines if a variable follows the PHA and if the test is positive (P value < 0.05) the variable violates the PHA and should be excluded from the analysis. At the patient level, *t*-test and the Wilcoxon–Mann–Whitney test were applied as well as Fisher's exact test for categorical values. To account for the dependency among metastases from the same patient, all analyses at the metastasis level were done based on a multilevel model approach by using a generalized linear mixed model fit by maximum likelihood (Laplace approximation) with a fixed predictor per individual level. All tests were two-sided and a P value of less than 0.05 was considered statistically significant. Logrank P values obtained for markers were dichotomized using the minimal P value approach. The predictive performance of the models was assessed by Harrell's concordance index (c-index) [33]. The Relative importance of each risk parameter to survival/relapse was estimated using the χ^2 proportion test. This method determines the percentage of relative contribution of

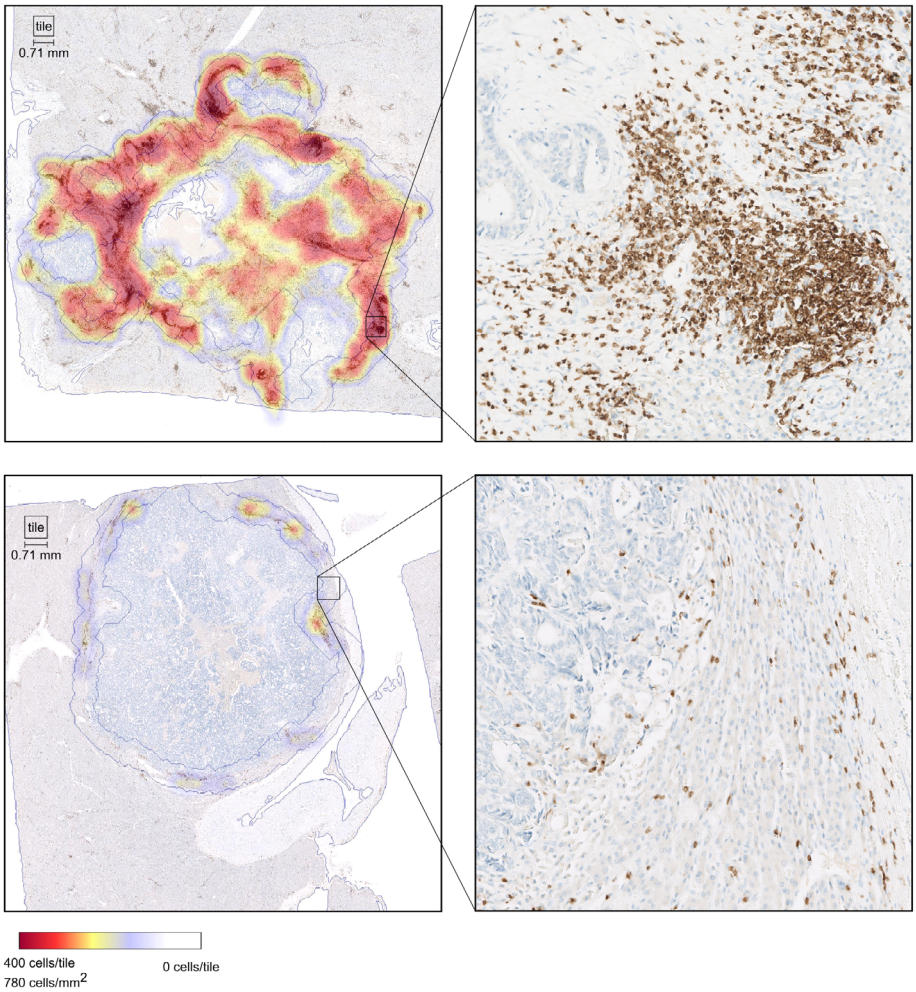

Figure 2. Intrametastatic immune infiltrate. Representative slides of two metastatic lesions with high (up) and low (down) T-cell (CD3+) density. The infiltrate is shown with a high to low density color gradient scaling from red to white, respectively. The immunohistochemical density of CD3 positive lymphocytes (in brown) in the invasive margin (IM) of the lesion is shown for a tile.

each variable explaining the current Cox model. All analyses were performed using R mlmRev, survival, Misc, and survMisc packages.

Results

Patient characteristics, clinicopathological features and Immunoscore

221 patients with their corresponding 582 metastatic lesions were included in the analysis. The patient characteristics are detailed in Table 1. The majority of the patients had a *BRAF* WT and MSS tumor, and had several synchronous metastases resected. Most of the patients received preoperative chemotherapy (84.6%), mainly associated with a targeted therapy (anti-VEGF or anti-EGFR). The clinicopathological features of the included patients were similar in the different preoperative treatment groups with the exception of more *RAS* WT tumors in the group of anti-EGFR treated patients, more metastases with completely resected margin (R0), and fewer metastases per patient in the group of patients without preoperative therapy (see supplementary material, Table S1). Surprisingly, we observed no significant CALI difference among the different preoperative treatment groups. Moreover, steatohepatitis, SOS and NRH were also observed in surrounding liver parenchyma of untreated patients.

For each metastasis, we evaluated the size, the presence of a tumor-positive resection margin (R1), the

Table 1. Patient characteristics

Parameter	Number of patients, n = 221
Age at the diagnosis (years), mean, range	62 (25–88)
Gender	
Male	122 (55.2%)
Female	99 (44.8%)
Primary tumor	
Location	
Right colon	36 (16.3%)
Left colon	32 (14.5%)
Sigmoid colon	94 (42.5%)
Rectum	59 (26.7%)
Pathologic T stage (pT)*	
T0	2 (0.9%)
T1	7 (3.2%)
T2	18 (8.1%)
T3	141 (63.8%)
T4	53 (24%)
Nodal status*	
pN0	73 (33%)
pN+	148 (67%)
RAS status	
WT	121 (54.8%)
Mutated	97 (43.9%)
BRAF status	
WT	213 (96.4%)
Mutated	2 (0.9%)
Microsatellite instability status	
Microsatellite stable (MSS)	215 (97.3%)
Microsatellite instable (MSI)	3 (1.4%)
Metastases	
Number	
Mean	2.67
Min–max	1–24
Size (mm)	
Mean	25.2
Min–max	2–110
Resection margin status**	
R0	555 (95.4%)
R1	27 (4.6%)
Type of metastases	
Synchronous	164 (74.2%)
Metachronous	57 (25.8%)
Preoperative treatment	
Chemotherapy alone‡	47 (21.3%)
Chemotherapy + anti-VEGFR§	79 (35.7%)
Chemotherapy + anti-EGFR¶	61 (27.6%)
No treatment	34 (15.4%)

*TNM seventh edition; pN0, negative lymph nodes; pN+, positive lymph nodes.
†R0, negative surgical margin; R1, positive surgical margin. Tumor cells can be seen microscopically.
‡Chemo: Oxaliplatin or irinotecan + fluoropyrimidine.
§Anti-VEGF: bevacizumab or cediranib.
¶Anti-EGFR: cetuximab or panitumumab.

pathological response assessed by the TRG, the HGP, the presence of bile duct/vascular or perineural tumor involvement and the Immunoscore. The pathological findings are summarized in supplementary material, Tables S1–S3. TRG and HGP were mainly heterogeneous for patients with multiples metastases. Desmoplastic and mixed HGP metastases were the most frequent patterns (see supplementary material, Table S2). Pathological response (TRG 1–3) was significantly associated with desmoplastic and pushing HGP but also with higher Immunoscore (see supplementary material, Table S4). Conversely to Immunoscore, pathological response (low TRG) and Desmoplastic and Pushing HGP were significantly associated with a preoperative treatment. No R1 positive margin status was observed in untreated lesions (see supplementary material, Table S3).

The impact of patho-molecular features and Immunoscore on patient prognosis following metastasis resection

The outcome of patients following metastasis resection was investigated, taking into account clinicopathological features, *RAS* mutation and Immunoscore. In univariate analysis, the following parameters were associated with shorter TTR: the presence of involved lymph nodes (pN+) during primary tumor resection ($p = 0.0172$), the presence of more than three metastases ($p = 0.0001$), R1 positive margin ($p = 0.0017$), the presence of replacement and mixed HGP ($p = 0.0002$), and low Immunoscore ($p = 0.0000$) (Table 2). Recurrence-free rates at 2 years were 60% and 25.7% for I 3–4 and I 0, respectively (hazard ratio [HR; I 3–4 versus I 0] = 2.87; 95% CI, 1.73 to 4.75; $p = 0.0000$). The presence of more than three metastases ($p = 0.0229$), TRG ($p = 0.0471$), Immunoscore ($p = 0.0001$) and the presence of RAS mutation ($p = 0.0109$) were the only parameters significantly prognostic for OS (see supplementary material, Table S6). OS rates at 5 years were 66.3% and 35.2% in Immunoscore High and Low, respectively (HR [I 3–4 versus I 0] = 4.25; 95% CI, 1.95–9.23; $p = 0.0001$), and 47.2% and 59.2% in *RAS* mutant and WT, respectively (HR [mutant versus WT] = 1.76; 95% CI, 1.13–2.74; $p = 0.0109$).

In Cox multivariate analyses (Table 3), replacement and mixed HGP, presence of steatohepatitis, number of resected metastases, resection margin status and Immunoscore were significantly associated with TTR, while only *RAS* mutation and Immunoscore were significant for OS. The relative contribution of each parameter included in multivariate analysis for TTR and OS is reported in supplementary material, Figure S1. Finally, the relative contribution to the risk of each parameter (clinicopathological, *RAS*, Immunoscore) were represented for TTR and OS in

Table 2. Univariate analysis for TTR

	Number of patients (%)	TTR							
		Median months (95% CI)	Rate at 2 year % (95% CI)	Hazard ratio (95% CI)	Logrank *P* value	RMST months (95% CI)	ΔRMST months (95% CI)	RMST *P* value	c-index (95% CI)
Gender									0.51 (0.47–0.56)
Female	99 (44.8)	18.8 (11.2–27.7)	42.9 (34.1–53.9)	1.0 (reference)		42.3 (33.1–51.4)	0.0 (reference)		
Male	122 (55.2)	14.2 (10.6–21.6)	37.9 (30–48)	1.19 (0.86–1.64)	0.3011	34.3 (26.7–41.9)	-8 (−19.9–4)	0.1905	
pT (TNM seventh edition)									0.54 (0.5–0.58)
T0–1–2	27 (12.2)	24.6 (10.7–57.5)	51.3 (34.8–75.6)	1.0 (reference)		37.6 (23.9–51.2)	0.0 (reference)		
T3	141 (63.8)	16.1 (12.6–22.7)	40.4 (32.9–49.6)	1.22 (0.72–2.07)	0.4624	34 (28.1–39.9)	−3.6 (−18.5–11.3)	0.6384	
T4	53 (24)	12.9 (8.2–21.6)	34.7 (23.8–50.5)	1.47 (0.82–2.63)	0.1955	29.8 (20.8–38.8)	−7.8 (−24.2–8.6)	0.3494	
Nodal status									0.55 (0.52–0.59)
pN0	73 (33)	24.6 (17.8–37.6)	54.5 (43.9–67.5)	1.0 (reference)		47 (35.4–58.5)	0.0 (reference)		
pN+	148 (67)	12.9 (10.4–17)	33.2 (26.3–42)	1.54 (1.08–2.19)	0.0172	35.6 (28.2–43)	−11.4 (−25.1–2.4)	0.1054	
Type of metastases									0.51 (0.47–0.55)
Synchronous	164 (74.2)	15.9 (12–19.8)	39.1 (32.1–47.5)	1.0 (reference)		37.5 (30.9–44.1)	0.0 (reference)		
Metachronous	57 (25.8)	21 (10.6–31.4)	43.6 (32.2–59)	0.94 (0.65–1.36)	0.7587	36.6 (25.6–47.6)	−0.9 (−13.8–11.9)	0.8845	
Number of lesions									0.56 (0.53–0.6)
≤3	167 (75.6)	22.5 (15.9–28.1)	46.6 (39.4–55.1)	1.0 (reference)		40.5 (34.1–47)	0.0 (reference)		
≥4	54 (24.4)	9.7 (8.2–11.2)	20.8 (12.3–35.1)	1.97 (1.39–2.8)	0.0001	21.5 (13.7–29.3)	−19 (−29.1–8.9)	0.0002	
Resection margin status									0.54 (0.51–0.57)
R0	197 (89.1)	18.1 (13.2–22.9)	42.6 (36.1–50.3)	1.0 (reference)		23.4 (21.1–25.7)	0.0 (reference)		
R1	24 (10.9)	9.6 (5.8–11.2)	20.8 (9.6–45.4)	2.07 (1.3–3.29)	0.0017	14.1 (8.9–19.3)	−9.3 (−15–3.7)	0.0013	
Mean lesion size (mm)									0.51 (0.46–0.55)
≤10	24 (11)	10.2 (7.8–55.2)	34.8 (19.9–60.9)	1.0 (reference)		31.2 (17.9–44.5)	0.0 (reference)		
>10–30	139 (63.5)	18.1 (13.1–24.1)	42.7 (35–52)	0.9 (0.53–1.54)	0.7079	31.6 (26.2–37)	0.4 (−13.9–14.7)	0.9572	
>30–50	41 (18.7)	12.8 (8.7–31.6)	37 (24.6–55.8)	0.89 (0.48–1.66)	0.7193	32.7 (22.5–42.9)	1.5 (−15.2–18.2)	0.8594	
>50	15 (6.8)	18.8 (5–61.3)	40 (21.5–74.3)	0.92 (0.42–1.98)	0.8230	31.9 (17.1–46.7)	0.7 (−19.1–20.6)	0.9431	
Worst TRG per patient									0.54 (0.5–0.58)
Low TRG (1–2–3)	79 (35.7)	18.5 (13.3–30.6)	45.5 (35.5–58.3)	1.0 (reference)		40.7 (30.2–51.3)	0.0 (reference)		
High TRG (4–5)	142 (64.3)	13.2 (10.2–21)	37.5 (30.2–46.6)	1.23 (0.87–1.72)	0.2397	38.7 (30.9–46.6)	−2 (−15.2–11.1)	0.7640	
Histopathological growth pattern									0.58 (0.54–0.62)
Desmoplastic/pushing	110 (49.8)	22.9 (16.8–35.7)	49.9 (41.1–60.4)	1.0 (reference)		44.9 (36.9–53)	0.0 (reference)		
Replacement/mixed	111 (50.2)	10.7 (9.5–13.8)	30.7 (23.1–40.9)	1.84 (1.33–2.56)	0.0002	26.5 (20–33)	−18.4 (−28.8–8.1)	0.0005	
Sinusoidal obstruction syndrome									0.54 (0.49–0.58)
No	126 (57)	21.6 (13.8–24.8)	46.6 (38.5–56.4)	1.0 (reference)		44.9 (36.3–53.5)	0.0 (reference)		
Yes	95 (43)	12.9 (9.7–18.5)	31.8 (23.5–43)	1.34 (0.97–1.85)	0.0763	33.6 (24.8–42.3)	−11.3 (−23.6–0.9)	0.0696	
Nodular regenerative hyperplasia									0.51 (0.47–0.54)
No	162 (73.3)	15.9 (12.6–22.9)	42.1 (35–50.7)	1.0 (reference)		39.7 (32.2–47.2)	0.0 (reference)		
Yes	59 (26.7)	17.8 (9.7–22.5)	35.3 (24.8–50.3)	1.05 (0.73–1.5)	0.7894	38.6 (26.8–50.4)	−1.1 (−15.1–12.9)	0.8765	
Steatohepatitis									0.53 (0.49–0.56)
No	170 (76.9)	17.8 (12.3–22.5)	41.4 (34.5–49.8)	1.0 (reference)		39.6 (32.7–46.5)	0.0 (reference)		
Yes	51 (23.1)	13.1 (8.7–22.7)	36.1 (24.9–52.3)	1.27 (0.88–1.84)	0.1950	32.6 (21.3–43.8)	−7 (−20.3–6.2)	0.2965	
RAS status									0.51 (0.46–0.55)
WT	122 (55.2)	17.8 (12.6–24.3)	42 (33.8–52.2)	1.0 (reference)		38 (30–45.9)	0.0 (reference)		

(Continues)

Table 2. Continued

	Number of patients (%)	TTR							
		Median months (95% CI)	Rate at 2 year % (95% CI)	Hazard ratio (95% CI)	Logrank *P* value	RMST months (95% CI)	ΔRMST months (95% CI)	RMST *P* value	c-index (95% CI)
Mutated	99 (44.8)	15 (10.7–22.5)	38.2 (29.7–49.1)	1.07 (0.77–1.47)	0.6931	36.2 (27.9–44.4)	−1.8 (−13.3–9.7)	0.7580	
Immunoscore (I)									0.6 (0.56–0.64)
I 0	56 (25.3)	9.5 (8–12)	25.7 (16.3–40.5)	2.87 (1.73–4.75)	0.0000	23.5 (14.9–32.2)	−32.7 (−49.4–16)	0.0001	
I 1–2	117 (52.9)	17.8 (11.1–22.9)	39.1 (31–49.3)	1.85 (1.16–2.94)	0.0088	37.9 (29.8–46.1)	−18.3 (−34.7–1.9)	0.0290	
I 3–4	48 (21.7)	30.6 (15.9-NR)	60 (47.2–76.3)	1.0 (reference)		56.2 (42–70.5)	0.0 (reference)		
Pathological score (PS)									0.61 (0.57–0.65)
PS 0–1	148 (67)	23.2 (17.8–31.4)	49.8 (42.2–58.8)	1.0 (reference)		44.2 (37.3–51)	0.0 (reference)		
PS 2–4	73 (33)	9.5 (8.2–10.4)	20.9 (13.4–32.8)	2.54 (1.82–3.53)	0.0000	18.5 (12.3–24.8)	−25.6 (−34.9–16.3)	<0.0001	

R0, negative surgical margin; R1, positive surgical margin; pN0, negative lymph nodes; pN+, positive lymph nodes; NR, not reached; RMST, restricted mean survival time.

Figure 3A,B. Immunoscore (30%) and HGP (27%) were the most powerful contributive factors for TTR (Figure 3A). Immunoscore (64%) and RAS status (25%) were the parameters with the highest contribution for OS (Figure 3B).

Characteristics of CALI and its clinical impact

Among the 187 treated patients, 129 (68.9%) presented with CALI: SOS in 47 patients (36.4%), NRH in 12 patients (9.3%), steatohepatitis in 32 patients (24.8%), and 30 patients (23.2%) with both SOS and NRH (see supplementary material, Table S1). Steatohepatitis was not associated with the preoperative treatment type or the number of cycles (see supplementary material, Table S5). Moreover, steatohepatitis was not specifically related to CALI but was also present in 7 (20.5%) nontreated patients and all these patients had clinical features of a metabolic condition (such as high BMI [>25], dyslipidemia or diabetes), or history of alcohol abuse. Meanwhile, 11 (34.3%) of the 32 patients with steatohepatitis receiving preoperative treatment, presented with features of a metabolic condition and/or history of alcohol abuse. Similarly to steatohepatitis, NRH and SOS were not associated with preoperative treatment type, while there was a trend to observe more SOS and/or NRH in patients treated with more than six cycles of chemotherapy ($p = 0.0573$, see supplementary material, Table S5).

PS, Immunoscore and *RAS* mutation for patient prognostic assessment

After assessment of the individual impact of clinicopathological parameters for patient outcome (Figure 3A, B and Table 2), their combined power on the patient prognosis was investigated. Relevant clinicopathological parameters from multivariate analysis for TTR (more than three lesions, R1 positive margin, presence of replacement and mixed HGP and steatohepatitis) combined into a PS were evaluated regarding patient outcome. According to this classification, patients were subdivided into 2 (PS 0–1, PS 2–4) or 3 groups (PS 0, PS 1, PS 2–4). PS was significantly associated with TTR in univariate analysis (Table 2). Patients with a worse PS (PS 2–4) have more than 2 times higher risk to relapse compared with patients with a favorable PS (PS 0–1; $p < 0.0000$).

Hence, PS, Immunoscore and *RAS* mutational status were the variables analyzed in the final multivariate model for TTR and OS (Table 3 and Figure 3C,D). Immunoscore (HR [I 3–4 versus I 0] = 0.40 95% CI,

Table 3. Multivariate analysis for TTR and OS

149/221 (events/total)	Time to recurrence			
Variable	Hazard ratio (95% CI)	CoxPH pV	Wald pV	c-index (95%CI)
Initial model				0.65 (0.61–0.7)
Number of lesions (≥4 versus ≤3)	1.47 (1.00–2.16)	0.8107	0.0479	
Steatohepatitis (yes versus no)	1.54 (1.04–2.28)	0.3162	0.0325	
NRH (yes versus no)	0.98 (0.67–1.42)	0.5909	0.9091	
SOS (yes versus no)	1.13 (0.78–1.63)	0.9220	0.5297	
TRG (high [3–5] versus low [1–2])	0.95 (0.67–1.36)	0.0259	0.7811	
Resection margin status (R1 versus R0)	1.81 (1.11–2.94)	0.8735	0.0173	
HGP (replacement/mixed versus desmoplastic/pushing)	1.75 (1.24–2.47)	0.9673	0.0016	
RAS status (mutated versus WT)	1.08 (0.77–1.52)	0.3652	0.6513	
Immunoscore (I 1–2 versus I 0)	0.68 (0.47–0.99)	0.5233	0.0428	
Immunoscore (I 3–4 versus I 0)	0.43 (0.25–0.74)	0.5577	0.0022	
Model after stepwise selection				0.65 (0.61–0.7)
Number of lesions (≥4 versus ≤3)	1.51 (1.03–2.19)	0.8225	0.0324	
Steatohepatitis (yes versus no)	1.51 (1.03–2.21)	0.3232	0.0357	
Resection margin status (R1 versus R0)	1.85 (1.15–2.99)	0.9528	0.0111	
HGP (replacement/mixed versus desmoplastic/pushing)	1.71 (1.22–2.40)	0.9612	0.0017	
Immunoscore (I 1–2 versus I 0)	0.67 (0.46–0.97)	0.5240	0.0329	
Immunoscore (I 3–4 versus I 0)	0.42 (0.25–0.70)	0.9045	0.0010	
Model with PS				0.65 (0.61–0.7)
PS (PS 2–4 versus PS 0–1)	2.32 (1.66–3.24)	0.6605	<0.0001	
RAS status (mutated versus WT)	1.02 (0.74–1.42)	0.3866	0.8880	
Immunoscore (I 1–2 versus I 0)	0.68 (0.47–0.99)	0.5061	0.0423	
Immunoscore (I 3–4 versus I 0)	0.41 (0.24–0.68)	0.5102	0.0005	
81/220 (events/total)	OS			
Variable	Hazard ratio (95% CI)	CoxPH pV	Wald pV	c-index (95%CI)
Initial model				0.68 (0.62–0.75)
Number of lesions (≥4 versus ≤3)	1.31 (0.76–2.28)	0.4747	0.3337	
Steatohepatitis (yes versus no)	1.13 (0.64–2.01)	0.7168	0.6733	
NRH (yes versus no)	0.94 (0.57–1.56)	0.9324	0.8152	
SOS (yes versus no)	1.03 (0.60–1.76)	0.7671	0.9121	
TRG (high [3–5] versus low [1–2])	1.23 (0.73–2.06)	0.4049	0.4360	
Resection margin status (R1 versus R0)	1.05 (0.49–2.26)	0.9599	0.8933	
HGP (replacement/mixed versus desmoplastic/pushing)	1.31 (0.83–2.09)	0.9830	0.2476	
RAS status (mutated versus WT)	1.62 (1.01–2.60)	0.1321	0.0469	
Immunoscore (I 1–2 versus I 0)	0.58 (0.36–0.94)	0.3169	0.0278	
Immunoscore (I 3–4 versus I 0)	0.30 (0.13–0.67)	0.8425	0.0036	
Model after stepwise selection				0.68 (0.62–0.74)
HGP (replacement/mixed versus desmoplastic/pushing)	1.43 (0.92–2.22)	0.9975	0.1140	
RAS status (mutated versus WT)	1.70 (1.09–2.66)	0.1322	0.0195	
Immunoscore (I 1–2 versus I 0)	0.54 (0.34–0.86)	0.3202	0.0099	
Immunoscore (I 3–4 versus I 0)	0.26 (0.12–0.56)	0.3125	0.0006	
Model with PS				0.68 (0.62–0.74)
PS (PS 2–4 versus PS 0–1)	1.34 (0.85–2.12)	0.2651	0.2059	
RAS status (mutated versus WT)	1.65 (1.06–2.57)	0.6694	0.0273	
Immunoscore (I 1–2 versus I 0)	0.56 (0.35–0.89)	0.3001	0.0147	
Immunoscore (I 3–4 versus I 0)	0.27 (0.12–0.59)	0.1507	0.0011	

R0, negative surgical margin; R1, positive surgical margin; CoxPH pV, Cox Proportional Hazard assumption test.

0.24–0.66; $p = 0.0004$) and PS (HR [PS 2–4 versus PS 0–1] =2.09 95% CI, 1.50–2.93; $p < 0.0001$) were significant for TTR. Immunoscore (HR [I 3–4 versus I 0] = 0.27 95% CI, 0.12 to 0.58; $p = 0.0009$), *RAS* (HR [mutated versus WT] = 1.66 95% CI, 1.06–2.58; $p = 0.0265$) were significant for OS. All parameters included in the initial multivariable model for TTR (Table 3) were analyzed for their relative contribution to the risk of TTR and OS (see supplementary material, Figure S1). The most contributive parameters for relapse following metastasis resection were PS (66%) and Immunoscore (34%), while Immunoscore (68%) remained the most important parameter for patient OS (parameters from the final multivariable Cox model, Figure 3C,D).

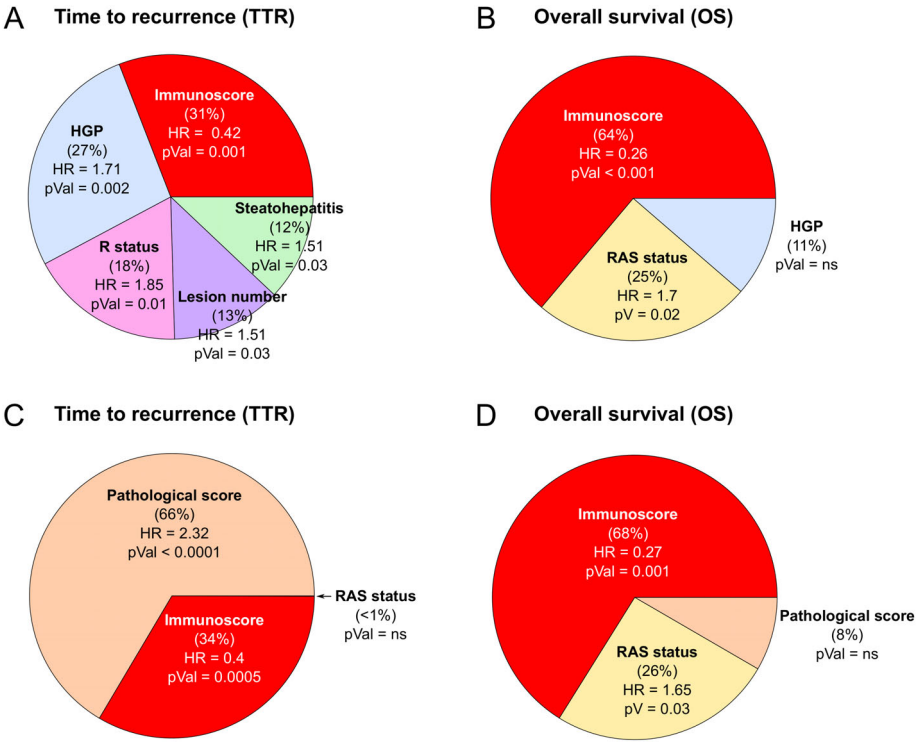

Figure 3. The relative importance of clinicopathological factors, *RAS* mutational status and Immunoscore for patient survival. (A–D) Assessment of the *relative importance* of *each parameter to survival risk using the chi squared proportion* (χ^2) test for clinical parameters + Immunoscore for TTR (left) and OS (left). (A,B) Model with individual parameters: steatohepatitis, HGP, lesion number, R status, RAS status and Immunoscore. (C,D) Model with the PS, RAS status and Immunoscore. The significance of the Cox multivariate regression model was evaluated with the Wald *P* value. *P* value <0.05 was considered significant.

The combination of Immunoscore and PS was evaluated with regard to patient outcome (Figure 4). Patients combining a favorable PS (PS 0–1) and a high Immunoscore (I 3–4) had the lowest risk of relapse (Figure 4E). The worst OS was observed for patients with poor PS (PS 2–4) and low Immunoscore (I 0–2) (Figure 4F). In addition, it was observed that patients combining heterogeneous PS and Immunoscore (favorable, unfavorable, or inversely) were at higher risk of relapse (Figure 4E). Regardless of PS, patients with high Immunoscore had a prolonged survival compared to patients with low Immunoscore (Figure 4F).

Discussion

The aim of our study was to integrate and study all recognized prognostic markers that could help to stratify the prognosis of patients with metastasis. We performed comprehensive analyses of clinicopathological and immune parameters of all resected metastases from patients, thus extending previous knowledge [34]. In the literature, several pathologic parameters have been already described to have prognostic value [1,5–8,28]. However, some of them (e.g. the size of the lesions, R1 positive margin) have lost their relevance in recent years mainly due to advances in chemotherapeutic treatment [35]. In addition, recent studies highlight the prognostic impact of Immunoscore in primary and metastatic settings, and also of molecular factors such as *RAS* mutational status [1,21,24].

Similar to previous reports, we observed that the number of lesions (more than three lesions) [36], a R1 positive margin [37], a replacement or mixed HGP [28], the presence of steatohepatitis in the surrounding liver normal parenchyma [38], and a low Immunoscore [24] were associated with higher risk of relapse. Interestingly, we report the weight and significance of each factor in multivariate analysis. Immunoscore remained a major prognostic factor by itself for TTR and OS confirming that within metastatic CRC the adaptive immune response plays a central role in preventing tumor recurrence

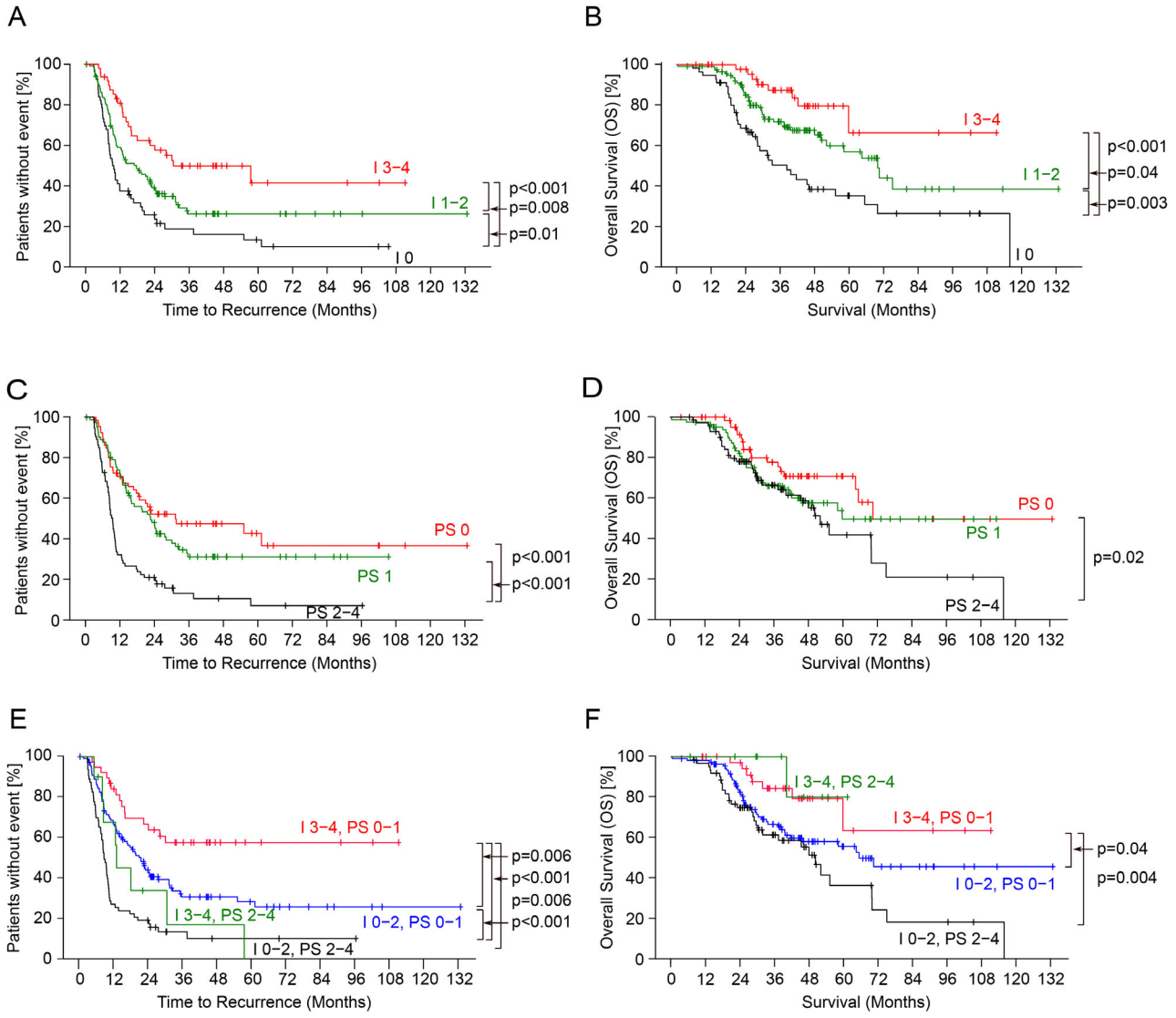

Figure 4. The impact on survival of Immunoscore and PS. Kaplan–Meier curves for TTR and OS according to Immunoscore (A,B), PS (C,D) Immunoscore and PS (E,F). Immunoscore (I) was determined based on the minimum infiltrated metastases. Five groups of patients were defined based on the density of CD3 and CD8 in the center and the invasive margin of the metastasis (minimum *P* value cut-off): I 0 (0Hi), I 1 (1Hi), I 2 (2Hi), I 3 (3Hi), I 4 (4Hi). Immunoscore (I) groups were merged as: I0 (black), I 1–2 (green) and I 3–4 (red). (C,D) PS includes: more than three lesions, R1 positive margin, steatohepatitis, replacement or mixed HGP. One point was given for each parameter when present. PS groups were merged: PS 0–1 (red), PS 1 (green) and PS 2–4 (black). (E,F) Immunoscore and PS groups: I 3–4, PS 0–1 (red); I 3–4, PS 2–4 (green); I 0–2, PS 0–1 (blue); I 0–2, PS 2–4 (black).

[39–43]. Evidence for immunoediting [15,44], and the role of the natural immunity and long-lasting capacity of memory T-cells [45] could play a central role for patients' survival. Interestingly, we observed higher Immunoscore in the largest metastasis. Considering the high specificity of Immunoscore evaluation on a biopsy [25] and the strong prognostication of the worst Immunoscore among all the metastases, the biopsy of the largest metastasis in patient with multiple metastases could be useful to best approach the metastatic immune microenvironment.

In order to improve the assessment of patient prognosis, we defined a PS integrating the most relevant markers associated with shorter TTR after curative resection of metastases. This PS demonstrated high ability to stratify patients for tumor recurrence but was not related to patient survival. In a multivariate analysis, PS and Immunoscore better stratify patients for the

risk of relapse. However, analyzing the contribution of each parameter to survival, we found that Immunoscore remains the major significant contributive parameter for OS, while *RAS* mutation status provided less survival contribution. Interestingly, steatohepatitis and HGP were the pathologic parameters contributing to PS. Steatohepatitis associated with metastasis resection is referred to as chemotherapy-associated steatohepatitis [46]. In our report, we observed steatohepatitis as a bad prognostic factor independently of its origin (CALI, metabolic). Although some articles have described an association between irinotecan and steatohepatitis [47], recent studies involving large cohorts of patients did not find this correlation [48–50]. These studies demonstrated that the only risk factor associated with steatohepatitis seems to be a high patient BMI (>27) [51]. Similar results were observed in our study. We could not find any association of steatohepatitis with irinotecan, but rather with the presence of metabolic syndrome or obesity. Steatohepatitis was observed in resections from patients who did not receive chemotherapy. Obesity and, in particular, adipose tissue expansion and inflammation is a source of a systemic inflammatory state able to trigger oncogenic responses such as cell proliferation, invasiveness, and metastasis [52] and could also cause localized inflammation, which may predispose to the occurrence of liver metastases [53]. Further research is needed to evaluate whether prevention of steatohepatitis (that could be evaluated by pretherapeutic liver biopsy) by nutritional counseling could be helpful in order to ameliorate patient survival and metastasis occurrence [54].

The presence of replacement or mixed pattern HGP resulted in a significantly shorter TTR. Replacement HGP may not stimulate angiogenesis for vascular supply but instead co-opt the sinusoidal vasculature of the liver. This could compromise the response to some types of treatment (bevacizumab-based treatment) and decrease immune infiltration [55]. Mixed HGP correlates with a heterogeneous reaction to treatment, due to the intratumoral heterogeneity [29]. Regarding heterogeneity of pathological features in patients with multiple metastases, our study confirmed that the worst HGP (replacement and mixed) [28] and the lowest Immunoscore [24] were the most relevant parameters for patient outcome. Conversely to another group [56], we did not observe any association between high Immunoscore and desmoplastic HGP in our cohort. Surprisingly, pathological response, a strong prognostic factor for treated patients [2,7], lost its significance in our report. Compared to the other group, we analyzed here all patients with resected liver colorectal metastases unselectively, independently of the preoperative treatment modality. Preoperative chemo- and targeted therapies modify the immune microenvironment [25,57], HGP [56,58], and exclusively determine TRG [2,7,8]. Similarly to steatohepatitis, desmoplastic or pushing HGP and high Immunoscore are also observed in untreated patients. Our results confirm the limit of the treatment impact in our unselected cohort and highlight the strongest prognostic factors, independently of preoperative treatment. In this way, no survival benefit for preoperative treatment has ever been demonstrated for patients with resectable liver colorectal metastases [59]. Since the prognostic value of Immunoscore has been demonstrated in Stage I/II/III, Stage II, Stage III [12,13,15,18–21,39–42,60] and its predictive value of response to chemotherapy demonstrated for Stage III patients [61], this further reinforces the clinical utility of Immunoscore in Stage IV patients [24,25,39–43].

To the best of our knowledge, this is the first study comprehensively analyzing pathological parameters and their association with Immunoscore. Nevertheless, the limit of our study is its retrospective design. Additional studies to validate the importance of PS and the other parameters should be further considered.

In conclusion, a complete pathological and immune evaluation of metastasis and surrounding liver parenchyma permits adequate stratification of Stage IV CRC patient prognosis. Steatohepatitis, which contributes highly to PS, could be further investigated with liver biopsy before metastasis resection in the future. The combination of PS and Immunoscore, both important markers to assess the risk of patient tumor relapse, helps clinicians in the decision-making process, and for the best clinical approach after metastasis resection. Independently of relapse, Immunoscore remains the major determinant of patient OS.

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Author contributions

JG, MVdE, PB and MK conceived and designed the study. PB, MVdE, NHu and MK acquired data. BM, MVdE, GB, PB, JG and MK analyzed and interpreted data. PB, MVdE, GB, JG, BM and MK drafted the manuscript. JG, MVdE, PB, BM, GB, GBe, JC, NH, FM, LL, TF, NL, CH, BN, NHu, FP, AJM and MK critically revised the manuscript. BM and GB carried out statistical analysis. GBe, JC, NH, FM, LL, TF, NHu, CH, BN, NL, FP and AJM provided technical support. JG, MVdE and PB supervised the study.

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Reference 62 is cited only in the supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. The relative importance of clinicopathological factors, *RAS* mutational status and Immunoscore included in the initial model of multivariate analysis for patient survival

Table S1. Clinicopathological features in colorectal liver metastasis per patient

Table S2. Metastasis characteristics

Table S3. Clinicopathological features and Immunoscore in colorectal liver metastasis per lesion

Table S4. Association between histological growth pattern (HGP), tumor regression grading (TRG), and Immunoscore (I) (analysis per lesion)

Table S5. Associations of CALI and treatment

Table S6. Univariate analysis for overall survival