*Imaging, Diagnosis, Prognosis*

Clinical Cancer Research

# Prognostic and Predictive Values of the Immunoscore in Patients with Rectal Cancer

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## Abstract

**Purpose:** To determine whether the tumor immune infiltrate, as recently evaluated with the Immunoscore methodology, could be a useful prognostic marker in patients with rectal cancers.

**Experimental design:** The influence of the immune infiltrate on patient's outcome was investigated in patients with or without preoperative chemoradiation therapy (pCRT). The density of total ($CD3^+$) and cytotoxic ($CD8^+$) T lymphocytes was evaluated by immunohistochemistry and quantified by a dedicated image analysis software in surgical specimens of patients with rectal cancer ($n = 111$) who did not receive pCRT and in tumor biopsies performed before pCRT from additional 55 patients. The results were correlated with tumor recurrence, patient's survival, and response to pCRT.

**Results:** The densities of $CD3^+$ and $CD8^+$ lymphocytes and the associated Immunoscore (from I0 to I4) were significantly correlated with differences in disease-free and overall survival (HR, 1.81 and 1.72, respectively; all $P < 0.005$). Cox multivariate analysis supports the advantage of the Immunoscore compared with the tumor–node–metastasis (TNM) staging in predicting recurrence and survival (all $P < 0.001$). Lymph node ratio added information in a prognostic model (all $P < 0.05$). In addition, high infiltration of $CD3^+$ and $CD8^+$ lymphocytes in tumor biopsies was associated with downstaging of the tumor after pCRT ($CD3^+$ cells; Fisher exact test $P = 0.01$).

**Conclusions:** The Immunoscore could be a useful prognostic marker in patients with rectal cancer treated by primary surgery. The determination of the immune infiltrate in biopsies before treatment could be a valuable information for the prediction of response to pCRT. *Clin Cancer Res; 20(7); 1891–9. ©2014 AACR.*

## Introduction

In rectal cancers, the worldwide used American Joint Committee on Cancer/Unio Internationale Contra Cancrum tumor–node-metastasis (AJCC/UICC-TNM) system (TNM; ref. 1) is of major importance but gives limited prognostic information and no prediction on the benefit of a chosen therapy (2, 3). Additional prognostic and predictive markers are needed (3). With the exception of microsatellite instability, which only concerns a minority of rectal cancers, recent genetic and molecular biology analyses of rectal cancers did not provide novel prognostic markers (4). One possible reason for this limited prognostic accuracy was the assumption, until recently, that tumor progression would be essentially a cell-autonomous process not influenced by the microenvironment (5). The paradigm could be now different as a large body of data from retrospective cohorts of human solid tumors demonstrated that the *in situ* immune infiltrate deeply influences the outcome of the patients (6).

We provided evidence that the type, the density, and the location of immune cells within tumor samples strongly influence the evolution of human colorectal cancers (stage I–IV; ref. 7). Thus, the adaptive immune reaction composed of T lymphocytes ($CD3^+$) with cytotoxic ($CD8^+$) and memory ($CD45RO^+$) phenotype within the core of the tumor (CT) and the invasive margin (IM) is a highly significant parameter to predict recurrence and survival (7). To promote the use of this immune investigation as a routine

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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## Translational Relevance

In previous publications, we demonstrated that the immune infiltrate within colorectal tumors strongly influenced the outcome of patients with colorectal cancer. We then created an "Immunoscore" to quantify the immune infiltrate. This method is currently tested worldwide on retrospective cohorts of patients with colonic cancers (22 centers; >9,000 patients) to promote the Immunoscore in routine clinical settings. Because of distinct clinicopathologic features, tumor markers, and treatment regimens when compared with colonic cancers, it has been decided not to consider rectal tumors until a dedicated study evaluating the impact of the immune infiltrate on the tumor progression, prognosis, and response to preoperative chemoradiation therapy (pCRT) in rectal cancers was performed. This is the scope of this study. On the basis of the positive results of this study, the evaluation of the immune marker on an international multicenter study should now be initiated.

testing for cancer classification, we established a methodology named "Immunoscore" that provides a score based on the numeration of $CD3^+$ and $CD8^+$ lymphocytes in the CT and in the IM regions of tumors (c.f., Material and Methods for details). This classification seems to be more accurate to anticipate the outcome events than the TNM classification (8). Importantly, an independent international panel of 22 expert laboratories has started to work on large retrospective cohorts of colon cancers to promote the Immunoscore in routine clinical settings (9, 10). In a first step, it has been decided not to consider rectal tumors due to distinct clinicopathologic features, tumor markers, and treatment regimens when compared with colonic adenocarcinomas (11–13). A dedicated study evaluating the impact of the immune infiltrate on the tumor progression, prognosis, and response to preoperative chemoradiation therapy (pCRT) in rectal cancers is now required.

The aims of this study were (i) to evaluate the prognostic performance of the Immunoscore on surgical specimens of patients with clinically localized rectal cancer treated by primary surgery; (ii) to compare prognostic accuracies of the Immunoscore and of the TNM; and (iii) to evaluate the performance of the immune infiltrate to predict the response to pCRT in patients with advanced rectal cancers.

## Material and Methods

### Patients and database

*Surgery cohort.* In the surgery cohort were included 111 prospectively registered patients with a rectal cancer who underwent radical surgery with mesorectum excision as a primary treatment (14) at the Laennec/HEGP hospitals, Paris, France between 1987 and 2004. Clinical findings and conventional histopathologic parameters were scored according to TNM (Supplementary Table S1; ref. 1). Tumor locations and staging were as follows: stage I–IV, high rectum ($n = 79$); stage I (T1–T2, N0, and M0), middle and low rectum ($n = 32$). Postoperative cares were in accord with the general practice for patients with rectal cancer. The mean duration of follow-up was 74 months. The extreme values until progression/death or last follow-up were 0 and 244 months, respectively. A secured database, the Tumoral MicroEnvironment DB (TME.db), was constructed for the management of patient's data (15). Ethical, legal, and social implications were approved by an ethical review board.

*Neoadjuvant therapy cohort.* In the neoadjuvant therapy cohort were included 55 patients with TNM stage II and III middle or low rectal cancer registered between 2007 and 2012 (Supplementary Table S2) from the St. Spiridon Hospital/Regional Oncologic Institute (Iasi, Romania), an international expert center where the Immunoscore is prospectively evaluated and where preoperative biopsies are available. The neoadjuvant therapy was applied following the guidelines of the European Society for Medical Oncology. The Research Ethics Committee of the University of Medicine and Pharmacy "Gr. T. Popa," Iasi approved the study.

### Histopathologic analysis

All the hematoxylin and eosin (H&E) sections of the rectal cancers were examined by pathologists for evaluation of TNM stage, tumor differentiation, lymph node ratio (LNR) defined as the number of positive lymph nodes divided by the total number of lymph nodes examined (16), presence of tumor emboli in vascular, lymphatic, or perineural structures (VELIPI status; ref. 17), and the quality of resection (R status; ref. 4). "Downstaging" was defined as any pathologic stage (ypTNM) less than pretreatment imagistic stage. Tumor regression grade (TRG) based on tumor-fibrosis ratio was determined as recommended (18).

### Tissue microarray construction, staining, and analysis

For tissue samples harvested on surgical specimens, two cores were taken from CT and two cores from IM (Fig. 1A) for tissue microarray (TMA) construction as previously described (7). Slides immunostained for CD3 and CD8 (SP7 and 4B11, respectively; Neomarkers) were quantified using an image analysis workstation (Spot Browser; ALPHELYS). The "minimum *P* value" approach was applied to obtain the cutoff providing the best separation between the groups of patients (high vs. low) related to their disease-free survival (DFS) outcome. Accordingly, the cutoff values determined for $CD3^+$ and $CD8^+$ cell densities were 256 (Fig. 1B) and 202 cells/$mm^2$ in the CT and 144 and 50 cells/$mm^2$ in the IM, respectively.

### Determination of the Immunoscore

Patients were stratified according to the "Immunoscore" ("I") ranging from I0 to I4, depending on the total number of high densities observed ($CD3^+$ cells and $CD8^+$ cells in the tumor regions; refs. 8-10). For example, I4 refers to a tumor with high densities of $CD3^+$ and $CD8^+$ cells in CT and IM regions of the tumor (4-Hi); I0 refers to tumors with

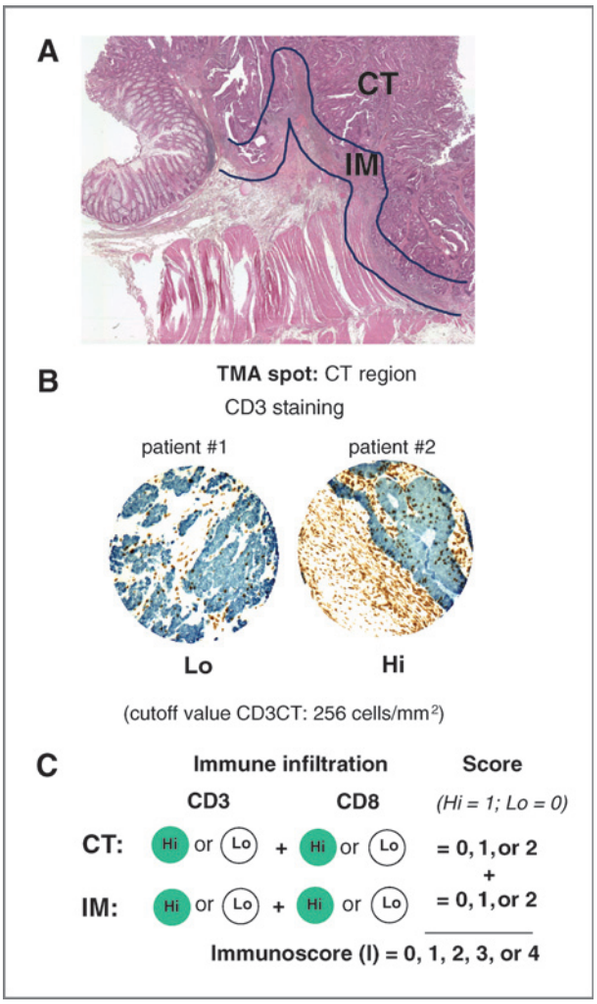



Figure 1. A, H&E section of rectal cancer (original magnification, ×200) showing the tumor regions of interest; CT and IM. B, two spots immunostained for CD3 showing the heterogeneity of $CD3^+$ cell infiltration within tumors among patients. C, establishment of an Immunoscore, based on the numeration of two lymphocyte populations (CD3 and CD8) in CT and in IM. The density of $CD3^+$ cells and $CD8^+$ cells is determined using an image analysis workstation. Each tumor is categorized into Hi or Lo density for each marker in each tumor region, according to a predetermined cutoff value (CD3 CT, 256 cells/$mm^2$; CD3 IM, 144 cells/$mm^2$; CD8 CT, 202 cells/$mm^2$; CD8 IM, 50 cells/$mm^2$) determined using the minimum *P* value approach. Patients are stratified according to a score ranging from I0 to I4 (8-10), depending on the total number of high densities observed (two markers assessed in CT, two markers assessed in IM). For example, I4 refers to a tumor with high densities of $CD3^+$ and $CD8^+$ cells in CT and IM regions of the tumor (4-Hi). I3 refers to tumors with three high densities. Patients with low densities of CD3 and CD8 in both tumor regions (0 Hi density) is classified I0. CT, core of the tumor; IM, invasive margin.

low densities of CD3 and CD8 in both tumor regions (0-Hi; Fig. 1C).

### Biopsy samples and staining

Biopsy samples were incubated for 32 minutes at 37°C with mouse monoclonal antibodies against CD8 (C8/144B; Dako; 1:50 dilution) and 20 minutes at 37°C with rabbit monoclonal antibodies against CD3 (2GV6; Ventana). The ultraView Universal DAB IHC Detection Kit (Ventana) was applied for detecting primary antibodies. High-resolution digital slides were obtained with a NanoZoomer scanner (Hamamatsu). The density of stained cells in the tumor areas was determined using the computerized image analysis system Developer XD (Definiens). Each tumor area was divided into tiles of 0.8 mm sides. The density of the immune cells stained in each biopsy was expressed as the mean density of the three most infiltrated tiles.

### Statistical analysis

Parametric (Student *t* test) and nonparametric (Wilcoxon–Mann–Whitney test) tests were used to identify markers with a significantly different expression among patient groups. Kaplan–Meier curves were used to visualize differences between DFS and overall survival (OS). Significance among patient groups was calculated by using the log-rank test. DFS log-rank *P* values were corrected using the formula proposed by Altman and colleagues (19). We used a multivariate Cox proportional hazards model to determine HRs. HRs were corrected as suggested by Holländer and colleagues (20). All tests were two-sided, and a $P < 0.05$ was considered statistically significant. All analyses were done using the statistical software R (survival package) implemented as a statistical module in TME.db (15).

## Results

### Prognostic factors in patients with rectal cancer treated by primary surgery

*Clinicopathologic data.* The prospectively registered cohort of 111 patients with rectal cancer who underwent a primary resection of the tumor with mesorectum excision was investigated. Univariate analysis showed that TNM staging, T stage, and LNR significantly influenced DFS and OS ($P \leq 0.05$ for all comparisons). In addition, the OS was also influenced by the age of the patients, N stage, and the presence of tumor emboli (Table 1).

*Impact of the immune infiltration.* The densities of $CD3^+$ and $CD8^+$ immune infiltrates were assessed in CT and IM regions (Fig. 1A) by immunohistochemical-based TMA analyses (Fig. 1B) with image analysis software. Positive significant associations were observed between densities of $CD3^+$ and $CD8^+$ cells in tumor regions and clinical outcomes for DFS and OS (Supplementary Table S3). A combined analysis of tumor regions was performed. Patients with high density of a marker in both CT and IM regions were classified "HiHi"; patients with low density of such marker in both tumor regions were classified "LoLo"; patients with a high density of such marker in a single tumor region (CT or IM) were classified "Het." HRs were 4.57 and 5.18 for CD3 and 5.88 and 6 for CD8 between patient groups (HiHi vs. LoLo) for DFS and OS, respectively (all $P \leq 0.004$ by the log-rank tests; Table 1). This combined analysis of CT plus IM regions was more efficient to discriminate patient's outcome when compared with single region analysis (Table 1 and Supplementary Table S3). Kaplan–Meier curves illustrate the significant longer DFS (Fig. 2) and OS

**Table 1.** Univariate analyses for DFS and OS among patients with rectal cancer eligible for primary surgery

| | | DFS | | | OS | | |
|---|---|---|---|---|---|---|---|
| Characteristic | No. of pts (%) | 5-year % (95% CI) | HR (95% CI)[a] | *P*[b] | 5-year % (95% CI) | HR (95% CI) | *P* |
| Age (y) | | | 1.07 (0.72–1.58) | 0.6502 | | 1.55 (1.18–2.04) | 0.0152 |
| <65 | 46 (41.4) | 70.3 (57.5–85.9) | 1.0 (reference) | | 77.3 (65.8–90.7) | 1.0 (reference) | |
| 65–75 | 32 (28.8) | 73 (57.7–92.4) | 0.79 (0.32–1.99) | 0.7729 | 57.2 (41.9–78) | 1.69 (0.84–3.41) | 0.1359 |
| 75–85 | 25 (22.5) | 59.7 (39.9–89.4) | 1.47 (0.61–3.56) | 0.6793 | 42.5 (26.6–68) | 2.73 (1.34–5.55) | 0.0038 |
| 85 | 8 (7.2) | 83.3 (58.3–100) | 0.68 (0.09–5.22) | 0.4901 | 37.5 (15.3–91.7) | 3.04 (1.09–8.46) | 0.0249 |
| UICC (TNM) stage | | | 1.62 (1.11–2.38) | 0.0590 | | 1.69 (1.3–2.2) | 0.0002 |
| 0–I | 58 (52.3) | 79 (68.7–90.9) | 1.0 (reference) | | 74.9 (64.3–87.3) | 1.0 (reference) | |
| II | 27 (24.3) | 65.9 (48.1–90.3) | 1.34 (0.53–3.37) | 0.5294 | 60.8 (44.4–83.1) | 1.52 (0.76–3.04) | 0.2339 |
| III | 16 (14.4) | 52.5 (32.2–85.6) | 2.54 (1.01–6.39) | 0.0397 | 37.5 (19.9–70.6) | 2.27 (1.09–4.74) | 0.0244 |
| IV | 10 (9) | 0 (NA-NA) | 4.6 (1.02–20.81) | 0.0294 | 20 (5.8–69.1) | 5.7 (2.32–13.99) | <0.0001 |
| T stage | | | 1.72 (1.12–2.64) | 0.0244 | | 1.62 (1.19–2.21) | 0.0159 |
| pTis-1 | 24 (21.6) | 95.5 (87.1–100) | 1.0 (reference) | | 87.5 (75.2–100) | 1.0 (reference) | |
| pT2 | 41 (36.9) | 67.2 (53.5–84.3) | 9.71 (1.28–73.9) | 0.0070 | 62.4 (49.1–79.5) | 3.32 (1.14–9.7) | 0.0198 |
| pT3 | 39 (35.1) | 53.7 (37.8–76.3) | 12.34 (1.61–94.45) | 0.0019 | 46.1 (32.5–65.5) | 4.66 (1.6–13.52) | 0.0019 |
| pT4 | 7 (6.3) | 75 (42.6–100) | 5.37 (0.34–85.93) | 0.1822 | 42.9 (18.2–100) | 5.22 (1.39–19.53) | 0.0062 |
| N stage[c] | | | 1.71 (0.98–2.98) | 0.0714 | | 2.07 (1.44–2.98) | 0.0002 |
| N0 | 88 (79.3) | 74.2 (64.8–85) | 1.0 (reference) | | 70.1 (60.9–80.7) | 1.0 (reference) | |
| N1 | 13 (11.7) | 42.4 (20.6–87.2) | 2.7 (1.09–6.72) | 0.0257 | 30.8 (13.6–69.5) | 2.35 (1.16–4.77) | 0.0150 |
| N2 | 10 (9) | 60 (29.3–100) | 2.06 (0.48–8.8) | 0.3183 | 20 (5.8–69.1) | 3.91 (1.77–8.6) | 0.0003 |
| LNR[d] | | | 2.13 (1.28–3.54) | <0.0001 | | 2.04 (1.51–2.74) | <0.0001 |
| 0 | 88 (81.5) | 72.8 (63.3–83.8) | 1.0 (reference) | | 68.8 (59.5–79.6) | 1.0 (reference) | |
| <0.33 | 10 (9.3) | 60 (33.1–100) | 1.54 (0.46–5.15) | 0.4803 | 40 (18.7–85.5) | 1.95 (0.82–4.66) | 0.1248 |
| 0.33–0.66 | 4 (3.7) | 66.7 (30–100) | NA (NA-NA) | NA | 25 (4.6–100) | NA (NA-NA) | NR |
| >0.66 | 6 (5.6) | 0 (NA-NA) | 56.73 (7.78–413.75) | <0.0001 | 0 (NA-NA) | 9.71 (3.57–26.36) | <0.0001 |
| VELIPI | | | 1.55 (0.54–4.48) | 0.4117 | | 2.05 (1.05–4) | 0.0323 |
| No | 92 (84.4) | 71.7 (62.3–82.5) | 1.0 (reference) | | 64.7 (55.4–75.6) | 1.0 (reference) | |
| Yes | 17 (15.6) | 58.9 (34.6–100) | 1.55 (0.54–4.48) | 0.4117 | 35.3 (18.5–67.2) | 2.05 (1.05–4) | 0.0323 |
| CD3 (CT/IM)[e] | | | 2.0 (1.23–3.22) | 0.0983 | | 2.04 (1.33–3.12) | 0.0019 |
| LoLo | 7 (7.7) | 21.4 (3.8–100) | 4.57 (1.94–10.75) | 0.0030 | 28.6 (8.9–92.2) | 5.18 (2.01–13.37) | 0.0002 |
| Het | 25 (27.5) | 65.5 (47.8–89.9) | 1.15 (0.92–1.44) | NA | 50.3 (33.8–74.8) | 1.88 (0.99–3.58) | 0.0502 |
| HiHi | 59 (64.8) | 71.9 (60.4–85.7) | 1.0 (reference) | | 66.2 (54.8–79.9) | 1.0 (reference) | |
| CD8 (CT/IM)[e] | | | 2.27 (1.43–3.70) | 0.0817 | | 2.27 (1.47–3.45) | 0.0004 |
| LoLo | 13 (15.3) | 38.9 (16.9–89.7) | 5.88 (2.16–15.97) | 0.0040 | 30.8 (13.6–69.5) | 6 (2.23–16.11) | <0.0001 |
| Het | 40 (47.1) | 56.3 (41.2–76.8) | 2.98 (1.26–7.06) | 0.2611 | 47.3 (33.8–66.3) | 3.43 (1.57–7.47) | 0.00101 |
| HiHi | 32 (37.6) | 85.7 (73.6–99.7) | 1.0 (reference) | | 83.3 (70.9–97.9) | 1.0 (reference) | |

NOTE: VELIPI denotes the presence of vascular emboli (VE), lymphatic invasion (LI), and perineural invasion (PI), alone or in combination (information not available for 2 patients).

Abbreviations: LNR, lymph node ratio; NA, not applicable.

[a]HR corrected (20).

[b]log-rank *P* value corrected (19).

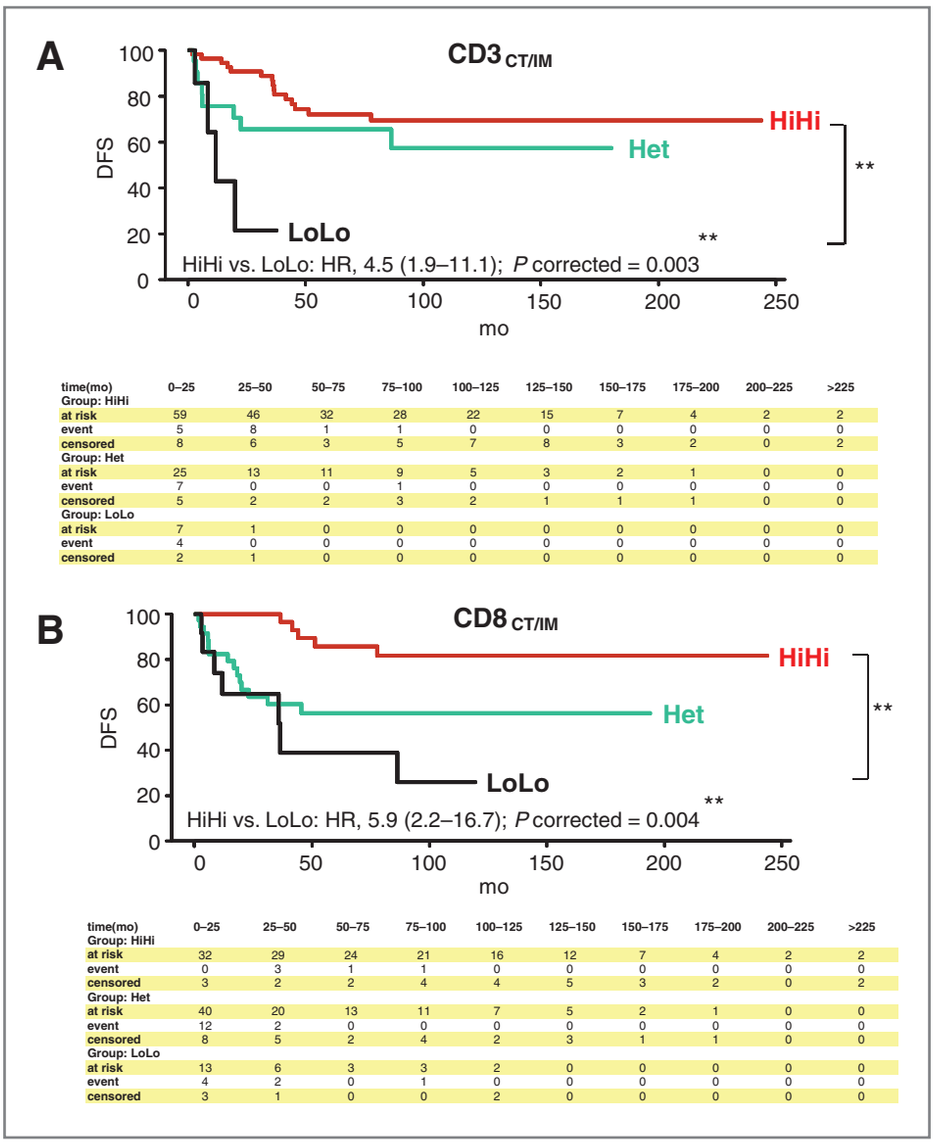
[c]TNM 6th edition.

[d]Information not available for 3 patients.

[e]For patients with data available on TMA analyses.

times (Supplementary Fig. S1) for patients with tumors highly infiltrated in combined tumor regions for CD3 and CD8. Thus, the assessment of the natural immune infiltration of $CD3^+$ and $CD8^+$ T lymphocytes in tumor regions (CT/IM) classified patients treated by primary surgery in subgroups with statistically different clinical outcomes.

***Impact of the Immunoscore.*** The "Immunoscore" uses the numeration of $CD3^+$ and $CD8^+$ cells in the CT and the IM regions to provide a score (from I0 to I4) depending on the total number of high densities observed (two markers assessed in CT, two markers assessed in IM; Fig. 1C). According to the Immunoscore, repartitions of the cohort




| time(mo) | 0–25 | 25–50 | 50–75 | 75–100 | 100–125 | 125–150 | 150–175 | 175–200 | 200–225 | >225 |
|---|---|---|---|---|---|---|---|---|---|---|
| Group: HiHi | | | | | | | | | | |
| at risk | 59 | 46 | 32 | 28 | 22 | 15 | 7 | 4 | 2 | 2 |
| event | 5 | 8 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| censored | 8 | 6 | 3 | 5 | 7 | 8 | 3 | 2 | 0 | 2 |
| Group: Het | | | | | | | | | | |
| at risk | 25 | 13 | 11 | 9 | 5 | 3 | 2 | 1 | 0 | 0 |
| event | 7 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| censored | 5 | 2 | 2 | 3 | 2 | 1 | 1 | 1 | 0 | 0 |
| Group: LoLo | | | | | | | | | | |
| at risk | 7 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| event | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| censored | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| time(mo) | 0–25 | 25–50 | 50–75 | 75–100 | 100–125 | 125–150 | 150–175 | 175–200 | 200–225 | >225 |
|---|---|---|---|---|---|---|---|---|---|---|
| Group: HiHi | | | | | | | | | | |
| at risk | 32 | 29 | 24 | 21 | 16 | 12 | 7 | 4 | 2 | 2 |
| event | 0 | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| censored | 3 | 2 | 2 | 4 | 4 | 5 | 3 | 2 | 0 | 2 |
| Group: Het | | | | | | | | | | |
| at risk | 40 | 20 | 13 | 11 | 7 | 5 | 2 | 1 | 0 | 0 |
| event | 12 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| censored | 8 | 5 | 2 | 4 | 2 | 3 | 1 | 1 | 0 | 0 |
| Group: LoLo | | | | | | | | | | |
| at risk | 13 | 6 | 3 | 3 | 2 | 0 | 0 | 0 | 0 | 0 |
| event | 4 | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| censored | 3 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |

**Figure 2.** A, Kaplan–Meier curves for the duration in months of DFS according to T-cell ($CD3^+$) density evaluated in combined tumor regions (CT and IM). B, Kaplan–Meier curves for the duration of DFS according to cytotoxic T-cell ($CD8^+$) density evaluated in combined tumor regions (CT and IM). For each marker (CD3 and CD8), significant difference ($P < 0.005$) was observed for DFS times between patients with high densities in the CT and in the IM (HiHi; red line), and low densities in the CT and in the IM (LoLo; black line). Patients at risk at each interval in the Kaplan–Meier survival curves for the duration of DFS are shown.

were as follows: 35% I4, 28% I3, 25% I2, 7% I1, and 5% I0, with an increasing risk of relapse from I4 to I0, with the associated HRs: 1, 1.69, 2.69, 3.1, and ∞ respectively, for the DFS (log-rank test corrected $P = 0.0038$) and HRs of 1, 2.63, 4.45, 4 and ∞ respectively, for the OS (log-rank test $P = 0.0003$; Supplementary Table S3). Kaplan–Meier curves illustrating the DFS and OS times according to the Immunoscore are shown in Fig. 3. Significant differences between patient groups for survival times were also observed after grouping the patients I0 and I1, which experienced the poorest postoperative outcome (Supplementary Fig. S2).

When combining the Immunoscore with the clinicopathologic markers, only the Immunoscore and the lymph node ratio (LNR) remained significant for DFS and OS in the model after stepwise-based Cox multivariate analysis (Immunoscore: $P = 0.007$ and $P = 0.002$; LNR: $P = 0.04$ and $P = 0.0007$, for DFS and OS respectively; Table 2). We then performed a Cox multivariate regression analysis by adding TNM staging to the Immunoscore into the model. Strikingly, the Immunoscore remained highly significantly associated with DFS, whereas the TNM staging did not reach significance. A strong impact of the Immunoscore on the OS was also evidenced (HR of 0.62 $P = 0.0004$; Table 2). As a result, the Immunoscore seems to be a highly significant prognostic factor in the group of patients treated by primary surgery.

### Is the natural immune infiltration, in patients treated by pCRT before surgery, a prognostic factor?

To question this issue, we first investigated a historic series of 33 patients that would be nowadays eligible for pCRT (21), to evaluate whether the natural immune infiltration could influence the clinical outcome. Significant

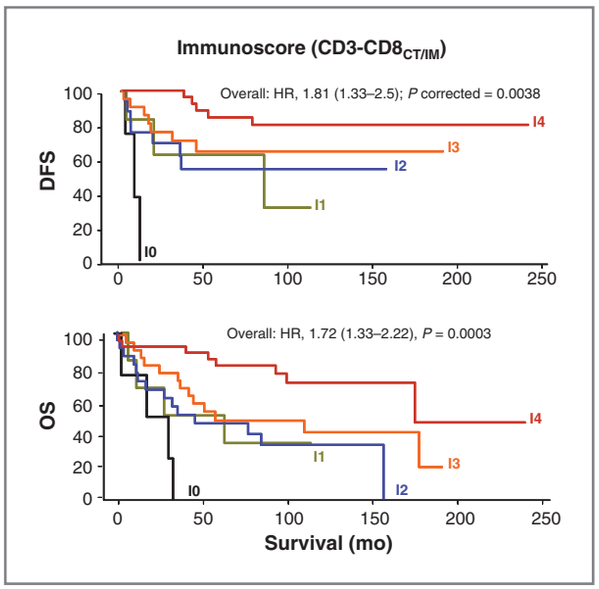



Figure 3. Kaplan–Meier curves for the duration of DFS and OS according to the Immunoscore in patients with rectal cancer eligible for a primary surgery (log-rank statistical test $P < 0.005$ for all comparisons). Patients at risk at each interval in the Kaplan–Meier survival curves for the duration of DFS and OS according to the Immunoscore are shown in Supplementary Table S4. Patients with an Immunoscore of 0 or 1 experienced a very poor postoperative outcome and thus could be grouped together. Kaplan–Meier curves and associated statistics after grouping are shown in the Supplementary Fig. 2.

higher densities of $CD3^+$ (Fig. 4A) and $CD8^+$ cells (data not shown) were observed in tumor regions (CT and IM) of patients who did not experience recurrence (all $P < 0.05$).

pCRT induces histologic reactions precluding the realization of an Immunoscore as the architecture of a treated tumor is deeply modified and the delineation of the analyzed tumor regions (CT and IM) is often no longer practicable.

To circumvent this issue, biopsies performed before pCRT for diagnosis were investigated for the immune infiltrate in a recent cohort of 55 patients (Fig. 4B). We asked whether the immune infiltration could predict the response to pCRT, as pCRT induces cell death forms with immunogenic potential in rectal tumors (22). The ypTNM downstaging and TRG were used as endpoints to evaluate response to pCRT (4, 18). High infiltration of $CD3^+$ cells in tumor biopsies predominated (72% of the cases) in the subgroup of responders (complete or partial response) to pCRT (Fig. 4B), whereas 63% of the biopsies with a low infiltration of $CD3^+$ cells belong to the group of nonresponders to pCRT (for CD3, Fisher exact test $P = 0.015$). The same pattern was observed for $CD8^+$ cells (data not shown). The TRG4, 3, 2, 1, 0 evaluated on surgical specimens from patients treated by pCRT were found in 7.3%, 45.4%, 32.7%, 7.3%, and 7.3% of the cohort, respectively. The lowest infiltration of CD3 and CD8 was observed in patients TRG0, without any sign of tumor regression (data not shown). Thus, the assessment of the immune infiltrate in biopsies could help to anticipate the patient's response to pCRT.

## Discussion

Rectal cancer is a major public health issue with 80,000 new cases per year in Europe (23). Current therapeutic strategies for rectal cancers, which may strongly impact patient's quality of life (24), are based on clinicopathologic

**Table 2.** Multivariate Cox proportional hazard analysis for DFS and OS among patients with rectal cancer eligible for primary surgery

| | DFS | | OS | |
|---|---|---|---|---|
| | HR (95% CI) | *P* | HR (95% CI) | *P* |
| Model before stepwise (stepAIC) selection | | | | |
| Age | 1.14 (0.74–1.75) | 0.5646 | 1.77 (1.3–2.42) | 0.0003 |
| Tumor (T) stage | 1.63 (0.95–2.8) | 0.0750 | 1.23 (0.8–1.88) | 0.3535 |
| N stage | 0.42 (0.08–2.28) | 0.3174 | 1.24 (0.43–3.54) | 0.6885 |
| LNR | 4.03 (0.88–18.46) | 0.0727 | 1.69 (0.7–4.1) | 0.2426 |
| VELIPI + (tumor emboli) | 0.79 (0.18–3.45) | 0.7564 | 0.57 (0.19–1.74) | 0.3244 |
| Immunoscore[a] (I0 to I4) | 0.62 (0.44–0.87) | 0.0061 | 0.67 (0.5–0.89) | 0.0053 |
| Model after stepwise (stepAIC) selection | | | | |
| Age | | | 1.66 (1.25–2.22) | 0.0005 |
| Tumor (T) stage | 1.59 (0.94–2.7) | 0.0836 | | |
| LNR | 1.88 (1.02–3.46) | 0.0414 | 1.89 (1.31–2.72) | .0007 |
| Immunoscore[a] (I0 to I4) | 0.62 (0.44–0.88) | 0.0069 | 0.65 (0.49–0.85) | 0.0019 |
| UICC TNM Staging | 1.43 (0.94–2.19) | 0.0977 | 1.41 (1.01–1.98) | 0.0437 |
| Immunoscore[a] (I0 to I4) | 0.55 (0.39–0.79) | 0.0009 | 0.62 (0.47–0.81) | 0.0004 |

NOTE: All categorical covariates were transformed into numeric codes before they entered into the Cox model.
Abbreviation: AIC, Akaike information criterion.
[a]Leave-one-out method. Correction using $C = 1 - (SE[coef]/coef)^2$; heuristic shrinkage factor corrected with Holländer et al. (20).

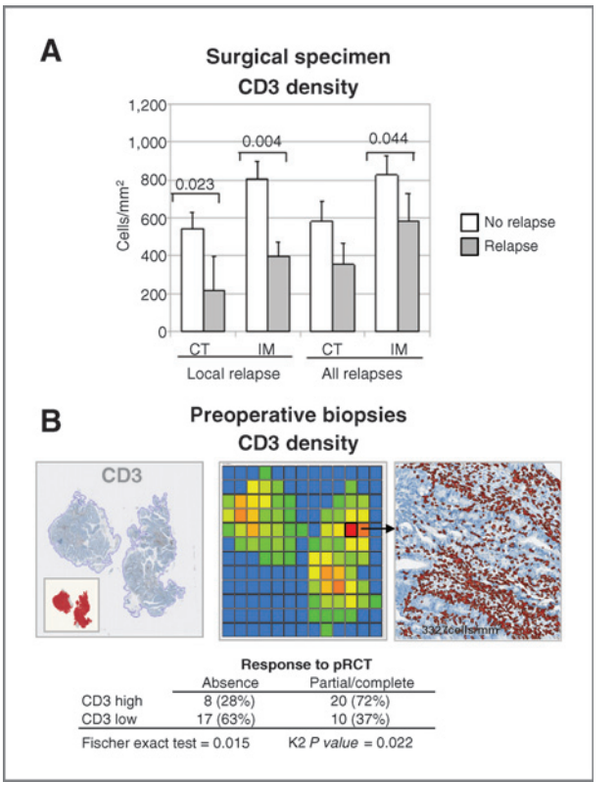



Figure 4. A, comparison of the mean (±SEM) densities of $CD3^+$ cells in the CT and IM regions of tumor from patients eligible for a pCRT, without (white bars) or with (gray bars) tumor recurrence. B, biopsies immunostained were analyzed for the densities of $CD3^+$ cells in tumor areas (in red in the small square) using a computerized image analysis system. The tumor area is divided into tiles for the analysis. A heat map view shows the densities of the stained cells in all tiles, from the minimum (green) to the maximum density (red). Right, illustration of the detection of $CD3^+$ cells (in red) in a tile (magnification ×20). Bottom, distribution of high and low densities of $CD3^+$ cells in biopsies of patients that will experience a complete/partial response or an absence of response to pCRT according to the ypTNM downstaging of the tumor. The categorization of the patient's densities for $CD3^+$ cells (high vs. low) was performed at the median of the cohort. The Fisher exact test comparing the distribution of Hi and Lo infiltration in each group, $P < 0.05$ was considered significant.

staging systems that do not take into account biologic features of the tumor (4). There is a need for new prognostic and predictive markers to avoid under- or overtreatment in the neoadjuvant and adjuvant settings (3).

Our study was designed to address these questions. Period of inclusion is stated between 1987 and 2003 as in our institution mesorectal excision was routinely performed since 1987, and as in rectal cancer a 10-year follow-up is required for clinical studies (25). With respect to these limitations, surgical results and outcomes of our series were comparable with published data (26, 27).

We herein show that the densities of $CD3^+$ and $CD8^+$ lymphocytes and the associated Immunoscore (from I0 to I4) significantly correlated with DFS and OS times. These results are in line with publications showing a beneficial impact of cytotoxic T lymphocytes and the associated Th1 immune orientation in tumors of diverse origins: melanoma, head and neck, breast, bladder, urothelial, ovarian, renal, prostatic, lung, colorectal (6–8, 28–31), and few series of rectal cancer (32–34). This corpus of data strongly suggests that tumor behavior should now be considered as the result of a balance between the invasive tumor process and the response of the host of which the local immune reaction is a major component (5, 6).

We further illustrate the beneficial impact of a coordinated immune reaction in specific tumor regions (i.e., the core of the tumor and the invasive margin) to prevent recurrence and increase survival, as we observed in colon cancers (7). As a result, we demonstrate the prognostic power of the Immunoscore, which summarizes the information of the immune-cell densities in these tumor regions (8). The Immunoscore classified nearly 50% of the patients with very distinct behaviors: 35% with very a good outcome (I4) as opposed to 12% with a poor outcome (for I0 and I1). This study confirms that there is an inverse relationship between tumor invasion and the extent of immune cell infiltration (8); 90% of the patients with the highest Immunoscore I4 presented with a localized cancer (stage I–II). But importantly, 34% of the patients with a localized cancer (stage I–II) presented with an Immunoscore associated with a very poor outcome (I0–I2); conversely, 16% of the patients with an advanced rectal cancer (stage III–IV) presented with an Immunoscore associated with a very good outcome (I4; Supplementary Table S5). These data illustrate how the Immunoscore overcomes the TNM scoring system in multivariate analyses, as we observed in colon cancer (8). To reinforce the confidence on the statistical association observed, patients with poor postoperative outcome (I0 and I1) were pooled (Supplementary Fig. S2); again, the multivariate analysis showed the prognostic power of the Immunoscore.

We also show that LNR is the only parameter adding information to the Immunoscore to better predict the DFS and the OS. LNR, which evaluates the dynamic balance between the number of positive lymph nodes and the total number of lymph nodes analyzed, has been shown to be a more accurate prognostic marker than the absolute number of positive lymph nodes that is currently used in the TNM staging system (16). The information carried by the LNR rather reflects complementary aspects of the antitumor immunity not depicted by the Immunoscore than mechanistic filtering activities attributed to lymph nodes (35).

For patients treated by pCRT, assessment of antitumor immunity by the Immunoscore is inappropriate as pCRT induces deep changes (18) with tumor regression, fibrosis, or mucous secretion that preclude a precise delimitation of the tumor and the invasive margin. In this context, biopsies performed for diagnosis purpose are the sole material free of radiation or chemotherapy effects. We evidenced a significant correlation between densities of $CD3^+$ cells (and of $CD8^+$ cells, data not shown) and the response to pCRT, as recently reported in a series of 48 patients (36). One hypothesis explaining this correlation could be that pCRT is an immune adjuvant acting through both the innate and

adaptive immune responses (37). Future accurate tools predicting response to pCRT should probably take into account both the immune components and the genetic features of the tumor (38). We are currently evaluating, on a large cohort with a 10-year follow-up (24), whether the immune infiltrate in biopsies could predict DFS and OS times, as suggested by the immune investigation performed on surgical specimens of our historic cohort of 33 patients. A positive result could provide a rationale to assess the immune infiltrate in biopsies to predict responders to pCRT and to select patients achieving complete clinical tumor regression for their inclusion in prospective studies evaluating new strategies with minimal or even no surgery (24).

In conclusion, our work highlights the performance of the immune infiltration and the Immunoscore to predict the clinical behavior of the patients. Evaluation of the immune marker on an international multicenter study should now be initiated.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

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