A diagnostic biopsy-adapted immunoscore predicts response to neoadjuvant treatment and selects patients with rectal cancer eligible for a watch-and-wait strategy

The biopsy-adapted Immunoscore in rectal cancer

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120–150-word statement of translational relevance

Biopsy-adapted Immunoscore (IS_B) performed on rectal cancer biopsy samples is an adaptation of the standardized IS performed on the surgical specimen in colon cancer. First, IS_B provides a strong and independent prognostic factor for disease free survival of rectal cancer patients. Secondly, IS_B predicts the response to neoadjuvant chemoradiotherapy (nCRT). Lastly, IS_B combined with imaging post-nCRT discriminates the group of patients with a complete histological response to nCRT (no residual tumor) that should benefit from less invasive therapeutic strategies (ie. Watch-and-Wait or minimally invasive surgery), avoiding a disabling and useless rectal amputation surgery.

Abstract

Purpose:

No biomarker to personalize treatment in locally advanced rectal cancer (LARC) is currently available. We assessed in LARC whether a diagnostic biopsy-adapted Immunoscore (IS_B) could predict response to neoadjuvant treatment (nT) and better define patients eligible to an organ preservation strategy ("Watch-and-Wait").

Experimental Design:

Biopsies from two independent cohorts (n_1=131, n_2=118) of patients with LARC treated with nT followed by radical surgery, were immunostained for CD3+ and CD8+ T cells and quantified by digital pathology to determine IS_B. The expression of immune-related genes post-nT was investigated (*n*=64 patients). Results were correlated with response to nT and disease-free survival (DFS). The IS_B prognostic performance was further assessed in a multicentric cohort (n=73 patients) treated by Watch-and-Wait.

Results:

IS_B positively correlated with the degree of histologic response (*P*<0.001) and gene expression levels for Th1 orientation and cytotoxic immune response, post-nT (*P*=0.006). IS_B High identified patients at lower risk of relapse or death compared to IS_B Low (HR=0.21, 95% CI=0.06 to 0.78; *P*=0.009). Prognostic performance of IS_B for DFS was confirmed in a validation cohort. IS_B was an independent parameter, more informative than pre- (P<0.001) and post-nT (P<0.05) imaging to predict DFS. IS_B combined with imaging post-nT discriminated very good responders that could benefit from organ preservation strategy. In the "Watch-and-Wait" cohort (n=73), no relapse was observed in patients with IS_B High (23.3%).

Conclusions: IS_B predicts response to nT and survival in LARC patients' treated by surgery. Its usefulness in the selection of patients eligible for a Watch-and-Wait strategy is strongly suggested.

Key words:

Immunoscore, rectal cancer, neoadjuvant treatment, TNM classification, Watch-and-Wait, Prognosis

Introduction

Colorectal cancer is the third most common cancer in the world with an increasing incidence especially in younger adults (1). In locally advanced rectal cancer (LARC) neoadjuvant chemoradiotherapy (nCRT) followed by radical surgery is recommended by international guidelines (2,3). Tumor recurrence and patient's survival are strongly influenced by the quality of response to neoadjuvant treatment (nT) (4–6). Recent advances in the management of LARC patients have shown that it could be conceivable to avoid amputation of the rectum (preservative strategy; eg. Watch and Wait) in patients with clinical and imaging features compatible with a complete response to nT (7,8). These patients experience acceptable outcomes, however, about 25% of them develop early tumor regrowth (9). There are currently no molecular markers to predict responses to nCRT, and guide treatment decision (3), such as optimization or modification of nT in non-responding patients, and better selection of patients eligible to preservative strategy.

Ionizing radiation has the capacity to prime/reinforce an adaptive T-cell mediated immune response, which acts in the mechanisms of local tumor regression and of distant tumor inhibition and rejection (i.e. the abscopal effect) (10–12). This suggests that the quality and intensity of the natural immune reaction at tumor site before nT could influence the magnitude of response to nT and provide a predictive marker of response. Natural immune reaction at tumor site has been further associated with a favorable prognosis in various cancers (13), including colorectal cancer, treated by surgery alone (14,15). Recent advances in digital pathology and image analysis have allowed a translation of immune assessment into a clinically relevant application (16). Using these technologies, the first standardized immune-based assay for colon cancer called "Immunoscore" (IS; i.e. the combination of CD3+ and CD8+ T cell densities in the tumor and its invasive margin) has been developed. Its robustness and prognostic performance in stage I-III colon cancer has been consolidated through an international validation study (17). Thus, IS provides a reliable evaluation of the natural immune reaction at tumor site.

Preliminary studies in rectal cancer have suggested that the natural immune reaction of tumors could be evaluated on biopsies (18–20), the only sample material available before treatment. A derivation of the Immunoscore performed in initial biopsies (IS_B) before nT has the benefit of evaluating the quality of the initial immune response in the tumor and its potential influence on both the degree of response to nT and clinical outcome.

The aim of this study was to determine whether the IS_B correlates with response to nT, the *in situ* immune status after nT, and clinical outcome. We finally tested whether IS could help to better select the subgroup of patients eligible to a preservative strategy, with acceptable outcome.

Patients and Methods

Patient population

Two retrospective consecutive cohorts of LARC patients (n_1 = 131, n_2 = 118) with available biopsies, treated by nT and radical surgery by total mesorectal excision (TME) were analyzed. Cohort 1 was a monocentric cohort and cohort 2 was multicentric (**Supplementary Table S1 and Supplementary Figure S1**). Inclusion period ranged from 1999 to 2016. Neoadjuvant treatment and surgery criteria were defined by each institution. Overall, 64.2% of patients were male and the median age at diagnosis was 65 years (interquartile range [IQR] = 53.3-74.1). Patients were treated by nT (short [3.7%] or long [96.3%] course of radiation; 5-fluorouracil-based chemotherapy [CT; 82%]; 18% did not receive CT). Rectal tumors were classified as cTNM (UICC TNM 8th edition) I (1.2%), II (27.3%), III (71.5%) according to baseline staging information provided by pelvic magnetic resonance and chest/abdominal computed tomography imaging. An additional cohort of patients (n = 73) with a complete/nearly complete response to nT (ycTNM 0-1), followed by a Watch-and-Wait strategy, was analyzed (**Supplementary Table S2**). A flow chart illustrating the studied population in each part of the investigations is provided in **Supplementary Figure S2**. The median

duration of follow-up for DFS of the cohort 1+2 was 45,4 months (IQR = 25,7-65,6). Duration of follow-up of each cohort for DFS, TTR and OS with the number of events is provided in **Supplementary Table S3**. The study was performed in accordance with the Declaration of Helsinki. Approval of all the centers Institutional Review Board committee was obtained for the study. Signed informed consents patients were obtained in each center.

Clinical outcomes

Patients were compared according to the degree of tumoral response to nT, using different tumor regression grade (TRG) scoring systems: **i/** the Dworak classification (21) defined as complete (Dworak 4), near complete (Dworak 3), moderate (Dworak 2), minimal (Dworak 1) and no regression (Dworak 0), **ii/** the neoadjuvant rectal (NAR) score classification (5), calculated using the equation [5pN-3(cT-pT) + 12]^2/9.61, and classified as low (<8), intermediate (8-16), and high (>16), **iii/** the ypTNM stage, ie. the postsurgical pathological T and N evaluation, and **iv/** downstaging of the tumor (4), defined as complete (ypT0N0), intermediate (ypT1-2N0), or weak/absent (ypT3-4 or N+). For patients who underwent surgery, the events were local, systemic recurrences and death from the date of surgery for disease-free survival (DFS), recurrences for time to recurrence (TTR), and death from any cause for overall survival (OS). All patients who were managed with the Watch-and-Wait strategy were considered to have clinical complete response (ycTNM0) and were offered a strict surveillance protocol.

Immunohistochemistry

Initial biopsies of all patients performed for diagnosis purpose were retrieved from all centers. Two formalin-fixed paraffin-embedded (FFPE) tumor tissue sections of 4 µm were processed for immunochemistry with antibodies against CD3+ (2GV6, 0.4 µg/mL; Ventana, Tuscon, AZ, USA) and

CD8+ (C8/144B, 3 µg/mL; Dako, Glostrup, Denmark) according to the previously described protocol (17) revealed with the Ultraview Universal DAB IHC Detection Kit (Ventana, Tuscon, AZ, USA), and counterstained with Mayer's hematoxylin.

Biopsy-adapted Immunoscore (IS_B) determination

Digital images of stained tissue sections were obtained with a 20X magnification and a resolution of 0.45 µm/pixel (Nanozoomer HT, Hamamatsu, Japan). Delimitation of the tumoral component excluding normal tissue and low/high grade dysplasia-associated lesions was performed by an experienced pathologist (CL). The mean densities of CD3+ and CD8+ T cells in the tumor region were determined with a dedicated IS module of the Developer XD image analysis software (Definiens, Munich, Germany; **Figure 1A**). The mean and distribution of the staining intensities were monitored providing an internal staining quality control. A final quality check was performed to remove nonspecific staining detected by the software. Determination of IS_B was directly derived from the methodology used to determine the Immunoscore® (IS) in the international validation cohort of IS in colon cancers which have shown a strong inter-observer reproducibility (17). CD3+ and CD8+ T cells densities in the tumoral region of each patient were compared to that obtained for the whole cohort of patient and converted accordingly into percentile. Then, the mean of the two percentiles (CD3 and CD8) was translated into one of the three IS_B categories (**Figure 1B)**: IS_B Low (0-25%), IS_B Intermediate (>25-70%), and IS_B High (>70-100%). The IS_B determination was performed blinded to the study endpoint.

RNA extraction and transcriptomic analysis by NanoString technology

Total RNA from 20 µm FFPE tumor tissue sections from all patients for which both biopsies and the corresponding surgical specimen post-nT was available (cohort 1 and 2; n=62) and from colorectal cancer patients not treated with nT (n= 13) was isolated using the RecoverAll™ Total Nucleic Acid

Isolation Kit (Ambion ThermoFisher, Monza, Italy). Distribution of tumor extension T and N stages among patients with or without nT did not display any statistical difference. The quality and quantity of the isolated RNA was measured using Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA) and NanoDrop 2000 (ThermoFisher Scientific, Waltham, USA) and 100-400 ng RNA of each sample was processed using an in-house panel of 44 immune-related genes (Nanostring Technologies, Seattle, WA, USA; **Supplementary Table S4**). Reporter-capture probe pairs were hybridized and the probe/target complexes were immobilized and counted on the nCounter analyzer. Background subtraction was applied to raw data and normalization based on the geometric mean of positive control and internal housekeeping genes (GUSB, SP2) was performed using the nSolver Analysis software, version 2.5.

Statistical analysis and data visualization

Statistical analyses and data visualizations were performed using the R software version 3.5.1 with the add-on survival, survminer, ggpubr, ggplot2, rms and coin packages. The associations between IS_B and clinical characteristics were assessed through the chi-square or Fisher tests of independence. Association level between CD3+ and CD8+ cell densities was measured by Pearson's correlation coefficient r and related *P* value. Survival univariate analyses were performed using the log-rank test and the Cox proportional hazards model. Survival curves were estimated by the Kaplan-Meier method. The log-rank test for trend from the survminer package was performed to detect ordered differences in survival curves. Multivariate survival analyses were performed with Cox proportional hazards model to test the simultaneous influence of all covariates. The proportional hazards assumption (PHA) for each covariate was tested using the cox.zph function. The relative importance of each parameter to survival risk was assessed by the chi-square from Harrell's rms R package. The association between IS_B and nT ordinal response level was assessed using a unilateral linear-by-linear association test. The associations between nT response levels

and CD3+, CD8+ T cells densities and gene intensities were assessed by Kendall's correlation test, T test, and Mann-Whitney U test. Wilcoxon test adjusted to control false discovery rate by using the Benjamini and Hochberg procedure was used to test treatment response level in transcriptional analysis. The ycTNM staging and IS_B were included in the proportional odds ordinal logistic regression model to predict good histopathologic response to nT. P values <0.05 were considered statistically significant. Principal component analysis (PCA) was performed with PCA and fviz_pca_ind functions from packages FactoMineR and factoextra. Linear weighted kappa was used to measure the agreement between resected tumors and biopsy samples in IS calculation.

Results

Biopsy-adapted Immunoscore (IS_B) determination on the rectal cancer diagnostic tissues

CD3+ lymphocytes and cytotoxic CD8+ cells were assessed on initial tumor biopsies performed for diagnosis purpose of LARC (n=322) treated by nT. The immunostaining intensity was monitored to ensure a valid detection and counting of stained cells with the image analysis software (**Figure 1A**). Seven patients were excluded after biomarker quality control (2.8%), and 4 patients were excluded after clinical data quality control (1.2%). The median density of CD3+ and CD8+ T cells in the tumor were 1363 cells/mm^2 and 274 cells/mm^2, respectively (**Supplementary Figure S3A**). The CD3+/CD8+ T cells ratio was highly variable among patients, with a coefficient of determination (r^2) between both markers of 0.58 (**Supplementary Figure S3B**). IS_B was derived from the CD3+ and CD8+ T cells densities (**Figure 1B**). CD3 and CD8 densities in the tumor were converted into percentiles referring to the densities observed in all patients. IS_B mean percentile of CD3 and CD8 was calculated for each biopsy (IS_B mean score). No statistical difference for the mean score was observed between the two cohorts (Cohort 1: 51.9 ± 29.1, Cohort 2: 48.8 ± 23.4 (means ± standard deviation); p=0.36). After converting the mean score into IS_B scoring system, overall 22.7%, 52.5%, and 24.8% of patients had IS_B Low, Intermediate and High, respectively. Of note, IS_B Intermediate

category was more represented in the cohort 2 (61.9%), as compared to cohort 1 (43.5%). Representative tumor biopsies from patients with IS_B Low and IS_B High are shown in **Figure 1C**.

Biopsy-based Immunoscore (IS_B) associated prognostic value

Distribution analysis of IS_B did not display any association with age, sex or tumor location (**Supplementary Table S1**). The magnitude and reproducibility of the IS_B prognostic performance were tested in two independent cohorts. In cohort 1 (n_1 = 131), a significant difference in DFS between patients stratified by IS_B was observed (*P* test for trend [P_{tft}] = 0.012; $HR_{[High\ versus\ Low]}$ = 0.21 (95% CI 0.06-0.78) and illustrated by Kaplan-Meier curves; **Figure 1D**). Patients with IS_B High were at low-risk of relapse, with the 5-year DFS of 91.1% (95% CI 82.0-100) versus 65.8% (95% CI 49.8-86.9) in patients with IS_B Low. These results were confirmed in second independent cohort (n_2 = 118; P_{tft} = 0.021; $HR_{[High\ versus\ Low]}$ = 0.25, 95% CI 0.07-0.86; **Figure 1E**). Identical results were obtained when removing the 3 patients with UICC-TNM stage I tumors (**Supplementary Figure S4**). In pooled analysis (n = 249), a significant difference between patient's groups stratified by IS_B was evidenced by univariate analysis (**Supplementary table S5**) and illustrated by Kaplan-Meier curves for TTR (P < 0.001), DFS (P < 0.005), and OS (P = 0.04; **Supplementary Figure S5**).

Biopsy-based Immunoscore (IS_B) and response to neoadjuvant treatment

We investigated whether the prognostic value associated to IS_B was at least partly a consequence of a relationship between IS_B and the quality of the nT response. The quality of response to nT was assessed 6 to 8 weeks after nT by imaging (ycTNM) and microscopic examination of the resected tumor, by the Dworak classification, a tumor regression grading system, ypTNM, downstaging and the neoadjuvant rectal (NAR) score. In our cohorts (n=249 patients), high CD3+ and CD8+ T cells densities were significantly associated with a good response to nT evaluated by both Dworak classification and ypTNM staging (all *P* < 0.005; **Figure 2A**). The mean of CD3+ and CD8+

percentiles (IS_B mean score) was correlated with the NAR score, Dworak classification, and ypTNM staging (**Figure 2B**). The IS_B level and distribution was positively correlated with tumor response to nT (**Figure 2C**). IS_B High patients were not found in the non-responder Dworak 0 group, and 52.9% of patients with undetectable tumor cells (ie. the Dworak 4 group) were IS_B High (P = 0.0006). The same correlation was observed with the ypTNM, tumor downstaging, and NAR (**Figure 2C**). Good responders to nT were six times more frequent in the IS_B High group than in the IS_B Low group according to the NAR scoring system (**Supplementary Figure S6**). Immune consequences of nT were then investigated on post-nT tumor samples (Dworak 0-4; n=62) by analyzing 44 immune-related genes (**Supplementary Table S4**). Gene expression levels were highly variable among patients (**Supplementary Figure S7**). Unsupervised hierarchical clustering showed that 30.6% (n=19) of patients presented with signs of local immune activation after nT **(Figure 3A**). The immune activation status after nT was positively correlated with the densities of CD3+ and CD8+ T cells (i.e. IS_B) before treatment (**Figure 3B**; P = 0.006). Non-responder tumors (Dworak 0-1) presented similarly low level of immune-related genes expression compared to tumors not treated by nT (**Figure 3A**). Patients with a partial/complete response to neoadjuvant treatment had a significantly higher expression of genes associated with adaptive immunity (*CD3D, CD3E, CD3Z, CD8A*), Th1 orientation (*TBX21/Tbet, STAT4*), activation (*CD69*), cytotoxicity (*GZMA, GZMH, GZMK, PRF1*), immune checkpoints (*CTLA-4, LAG3*), and chemokines (*CCL2, CCL5, CX3CL1*), as compared to patients non-responders to nT (**Figure 3C**). This suggests a link between the quality of the natural adaptive cytotoxic immune response (IS_B), the presence of a post-nT immune activation and the degree of response to nT. Gene expression data analysis through a Principal Component Analysis (PCA) visualization further reinforced the putative link existing between the response to nT and the immune environment by showing distinct patterns of gene expression depending on the degree of response to nT (**Figure 3D**). "The combination of the second and third dimension was the most accurate to discriminate responders/non-responders patients.

Biopsy-adapted Immunoscore (IS_B) - a biomarker to optimize patient care

We investigated whether the IS_B could provide valuable prognostic information when combined with clinic and pathologic criteria available (i) before nT (i.e. initial imaging, cTNM (UICC TNM 8th edition)), (ii) after nT (ie. imaging post-nT, ycTNM) and (iii) after surgery (pathologic examination, ypTNM). In Cox multivariate analysis, IS_B was a stronger predictive marker of DFS than other clinicopathological parameters including cTNM (IS_B High versus IS_B Low: HR = 0.2, *P< 0.001*) and ycTNM (IS_B High versus IS_B Low: HR = 0.25, *P* = 0.039). IS_B further remained a significant independent parameter associated with DFS when combined to ypTNM (**Table 1**). The important contribution of IS_B for the prediction of the relapse and/or death compared to other clinical parameters is illustrated in **Figure 4A**. It is known that the accuracy of the complete response post-nT defined by imaging is imperfect. Only 25 to 50 % of clinical complete responders have no residual tumor (i.e. complete histologic response) (22–24). IS_B combined to imaging post-nT (ycTNM) increased the accuracy of prediction of histological good responders (ypTNM 0-I) as compared to ycTNM alone (**Figure 4B**). Three out of 32 patients with good response to nT (ycTNM = 0-I, n=32) experienced distant relapses, and no local relapse were observed. Importantly, no relapse was observed in IS_B High patients (**Figure 4C**). Thus, IS_B could help to select patients who could achieve a very favorable outcome and be eligible to a Watch and Wait strategy.

IS_B in patients managed with Watch-and-Wait strategy

In a series of patient treated by Watch-and-Wait strategy (n=73), we retrieved the initial diagnostic biopsies to evaluate the IS_B and the associated clinical outcome. Overall, 23%, 51%, and 26% were classified as IS_B High, IS_B Intermediate, and IS_B Low, respectively. Time to relapse was significantly different among patients stratified for IS_B ($P_{[\text{High versus Low}]}$ = 0.025; **Figure 5A**). No evidence of relapse was noticed during the follow-up period in IS_B High patients. Under the Cox proportional hazards

regression model, the 5-year probability of survival without recurrence ranged from 46% to 89% according to IS_B Mean Score (**Figure 5B**). In Cox multivariable analysis, IS_B was related to the patient's TTR, independent of age, tumor location, and the cTNM classification (UICC TNM 8th edition) (*P*= 0.04; **Supplementary Table S6**). The relative contribution of IS_B to the prediction of occurrence of disease relapse is illustrated in **Figure 5C**.

Discussion

This work highlights the links between (i) the quality of natural intra-tumor immunity evaluated by the IS_B, (ii) the intensity of *in situ* immune reaction post-nT, (iii) the extend of the tumor regression post-nT and (iv) the clinical impact in terms of prevention of tumor recurrence and survival. From a clinical point of view, IS_B provides a reliable estimate of both the quality of response after nT and of the risk of recurrence and death in LARC patients. IS_B combined with imaging, could further identify patients with a complete clinical response whom can benefit from a close surveillance strategy post-nT, thus avoiding a disabling and useless rectal amputation surgery.

IS_B can be performed on routine diagnosis biopsies without any additional medical procedure. The rigorous and standardized quantification of immune cell infiltrates was achieved as for the IS colon study (17).

In the current study, IS_B was positively and significantly correlated with tumor response to nT. This observation is consistent with our previous preliminary result (18) and with studies using an optical semi-quantitative evaluation of immune cell infiltrates (19,20,25). In the IS_B Low group (22.7% of the cohort), only 5% of patients experienced complete response (Low NAR score), suggesting that an optimization or modification of nT such as adjunctive therapies (26), immunotherapy (27), or drug repositioning may provide greater benefits for these patients in order to achieve a better response. We evidenced an association between signs of *in situ* cytotoxic adaptive immune response and inflammatory interferon type I-associated molecules production post-nT and the

response to treatment. Type I IFNs play a key role in antitumoral immunity by promoting the maturation and presentation capacity of dendritic cells and their migration to lymph nodes (28). This immune state was influenced by the quality and the intensity of the natural immune response preexisting before nT. IS_B High could not only favor nT-dependent tumor cell death, but also promote the presence of resident immune components that could be essential to avoid local recurrences in organ preservative strategies such as Watch-and-Wait. Of note, few IS_B high patients did not achieve a good response, highlighting that treatment resistance is also guided by independent tumor intrinsic factors (29) or the presence of a suppressive microenvironment (30). Neoadjuvant treatment with development of clinical complete response post-nT has raised the possibility of organ-preserving strategies, as radical resection of the rectum results in functional outcomes, immediate morbidity, and even mortality rates (31). However, imaging after nCRT (ycTNM) has low accuracy in predicting pathologic complete response due to over or under-staging (32). Importantly, no relapse was observed in good responders with IS_B High patients. In addition, IS_B increased the accuracy prediction for very good responders (ypTNM 0-I) evaluated by imaging and identified a subgroup of patients treated with an organ-preserving strategy (Watch-and-Wait) with a very favorable outcome. No biomarker is currently available to help selection of good responders eligible to Watch-and-Wait strategy (9). These results may have significant implication in selecting potential candidates for organ-preservation including patients with IS_B High and complete clinical response to nT, but also those with a delayed complete clinical response (i.e. "nearly-complete responders") that are presently classified as incomplete responders (33).

This study has some limitations. The immune densities associated with predefined cut points (i.e. 25^{th} and 70^{th} percentile) are closely linked to the clinical characteristics of the studied cohort. The densities used as cut point are relevant to LARC patients treated by nT before surgery. In addition, assessment of IS_B was performed on initial biopsies; this implies analysis of only a small fraction of the tumor (10-15% of the surface of cut from a tumor block available after TME) and no analysis of

the invasive margin absent on biopsies. In order to evaluate the correspondence of IS_B and IS in resected tumor, we analyzed 33 colon cancer biopsies and their associated resected tumor, we found a partial correlation between these two specimens (**Supplementary Table S7**, kappa=0.45, p=0.0004). All discrepancies were only observed between 2 consecutive categories of IS. Despite this limited surface analysis and the absence of invasive margin, the prognostic value of the IS_B was retained suggesting the accuracy of the immune evaluation on initial diagnostic biopsy when the surgical piece is unavailable or is impossible to analyze due to architectural changes secondary to the neoadjuvant treatment. In addition, performing IS on post-operative specimen would not allow an assessment of its predictive value of the response to nT. Furthermore, due to the deep histological modifications after nT (no clear delimitation of the tumor and its invasive margin) an IS on post nT specimen is not feasible. The study was performed on patients who came from different countries and received standard-of-care treatment in real-life clinical practice. Despite the size of the specimen and the multiple types of patient care, the strong and constant prognosis value associated with IS_B, highlight the robustness of the test, and its generalizability. Prognostic parameters such as mismatch repair, KRAS, and BRAF status not available in our study, were not included in multivariate analysis with IS scoring system. However, MSI+ cases are rare in rectal cancer (<5%) (34), and we recently evidenced that IS was an independent prognostic parameter for survival when associated with MSI, KRAS and BRAF status in colonic cancers (35). Most of the rectal cancer included in this study was adenocarcinomas. A sub-analysis by histologic subtypes could not be performed due to the large multicentric character of the cohorts studied, with heterogeneous level of histopathological description and the obvious small effective of mucinous carcinomas, signet ring cell carcinoma, or tumor budding to address their relative prognostic impact with enough power. This study emphasizes the importance of the initial diagnostic biopsies, often done in private practices, and not easily available in some cases. Rectal cancer patients would benefit from a close partnership between private pathology practices, clinics, and teaching

hospitals in order to initially assess their immune status (IS_B). This material could become in the near future essential and be part of the personal medical file of rectal cancer patient as it is the sole material available before any neoadjuvant treatment. IS_B may facilitate a personalized multimodal treatment of rectal cancer particularly in patients with IS_B High tumors at baseline and with signs of tumor regression by imaging. These patients should benefit the most from the conservative strategy and in turns preserve their quality of life.

In conclusion, our results indicate that IS_B could be used (i) to predict tumor response after nT, (ii) to re-stage local disease after nT, and (iii) to predict clinical outcome. This method may facilitate a personalized multimodal treatment of rectal cancer particularly in patients with IS_B High tumors at baseline and with signs of tumor regression by imaging. These patients should benefit the most from the conservative strategy and in turns preserve their quality of life. IS_B is yet to be validated on larger Watch-and-Wait cohorts both retrospectively and prospectively. Such validations are planned in international collaboration studies using the International Watch-and-Wait Database and in the OPERA ongoing clinical trial (NCT02505750).

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Disclosures

JG, FP, and BM have patents associated with the immune prognostic biomarkers. JG is co-founder of HalioDx biotechnology company. All other authors declare no competing interests. Immunoscore a registered trademark owned by the National Institute of Health and Medical Research.

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Bibliography

1. Araghi M, Soerjomataram I, Bardot A, Ferlay J, Cabasag CJ, Morrison DS, et al. Changes in colorectal cancer incidence in seven high-income countries: a population-based study. The Lancet Gastroenterology & Hepatology. 2019;4:511–8.

2. Nielsen LBJ, Wille-Jørgensen P. National and international guidelines for rectal cancer. Colorectal Disease. 2014;16:854–65.

3. Glynne-Jones R, Wyrwicz L, Tiret E, Brown G, Rödel C, Cervantes A, et al. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. Annals of Oncology. 2017;28:iv22–40.

4. Park IJ, You YN, Agarwal A, Skibber JM, Rodriguez-Bigas MA, Eng C, et al. Neoadjuvant Treatment Response As an Early Response Indicator for Patients With Rectal Cancer. J Clin Oncol. 2012;30:1770–6.

5. George TJ, Allegra CJ, Yothers G. Neoadjuvant Rectal (NAR) Score: a New Surrogate Endpoint in Rectal Cancer Clinical Trials. Curr Colorectal Cancer Rep. 2015;11:275–80.

6. Rödel C, Martus P, Papadoupolos T, Füzesi L, Klimpfinger M, Fietkau R, et al. Prognostic Significance of Tumor Regression After Preoperative Chemoradiotherapy for Rectal Cancer. Journal of Clinical Oncology. 2005;23:8688–96.

7. Habr-Gama A, Perez RO, Nadalin W, Sabbaga J, Ribeiro U, Silva e Sousa AH, et al. Operative Versus Nonoperative Treatment for Stage 0 Distal Rectal Cancer Following Chemoradiation Therapy: Long-term Results. Transactions of the . Meeting of the American Surgical Association. 2004;CXXII:309–16.

8. Habr-Gama A. Assessment and management of the complete clinical response of rectal cancer to chemoradiotherapy. Colorectal Disease. 2006;8:21–24.

9. van der Valk MJM, Hilling DE, Bastiaannet E, Meershoek-Klein Kranenbarg E, Beets GL, Figueiredo NL, et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWD): an international multicentre registry study. The Lancet. 2018;391:2537–45.

10. Demaria S, Ng B, Devitt ML, Babb JS, Kawashima N, Liebes L, et al. Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. International Journal of Radiation Oncology • Biology • Physics. 2004;58:862–70.

11. Rodríguez-Ruiz ME, Vanpouille-Box C, Melero I, Formenti SC, Demaria S. Immunological Mechanisms Responsible for Radiation-Induced Abscopal Effect. Trends in Immunology. 2018;39:644–55.

12. Chajon E, Castelli J, Marsiglia H, Crevoisier RD. The synergistic effect of radiotherapy and immunotherapy: A promising but not simple partnership. Critical Reviews in Oncology / Hematology. 2017;111:124–32.

13. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12:298–306.

14. Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer. New England Journal of Medicine. 2005;353:2654–66.

15. Galon J. Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. Science. 2006;313:1960–4.

16. Shinde V, Burke KE, Chakravarty A, Fleming M, McDonald AA, Berger A, et al. Applications of Pathology-Assisted Image Analysis of Immunohistochemistry-Based Biomarkers in Oncology. Vet Pathol. 2014;51:292–303.

17. Pagès F, Mlecnik B, Marliot F, Bindea G, Ou F-S, Bifulco C, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. The Lancet. 2018;391:2128–39.

18. Anitei M-G, Zeitoun G, Mlecnik B, Marliot F, Haicheur N, Todosi A-M, et al. Prognostic and Predictive Values of the Immunoscore in Patients with Rectal Cancer. Clinical Cancer Research. 2014;20:1891–9.

19. Yasuda K, Nirei T, Sunami E, Nagawa H, Kitayama J. Density of CD4(+) and CD8(+) T lymphocytes in biopsy samples can be a predictor of pathological response to chemoradiotherapy (CRT) for rectal cancer. Radiat Oncol. 2011;6:49.

20. Shinto E, Hase K, Hashiguchi Y, Sekizawa A, Ueno H, Shikina A, et al. CD8+ and FOXP3+ Tumor-Infiltrating T Cells Before and After Chemoradiotherapy for Rectal Cancer. Ann Surg Oncol. 2014;21:414–21.

21. Dworak O, Keilholz L, Hoffmann A. Pathological features of rectal cancer after preoperative radiochemotherapy. International journal of colorectal disease. 1997;12:19–23.

22. Hiotis SP, Weber SM, Cohen AM, Minsky BD, Paty PB, Guillem JG, et al. Assessing the predictive value of clinical complete response to neoadjuvant therapy for rectal cancer: an analysis of 488 patients1 1No competing interests declared. Journal of the American College of Surgeons. 2002;194:131–5.

23. Rullier E, Rouanet P, Tuech J-J, Valverde A, Lelong B, Rivoire M, et al. Organ preservation for rectal cancer (GRECCAR 2): a prospective, randomised, open-label, multicentre, phase 3 trial. The Lancet. 2017;390:469–79.

24. The 2017 European Society of Coloproctology (ESCP) collaborating group. Evaluating the incidence of pathological complete response in current international rectal cancer practice: the barriers to widespread safe deferral of surgery. Colorectal Dis. 2018;20:58–68.

25. Matsutani S, Shibutani M, Maeda K, Nagahara H, Fukuoka T, Nakao S, et al. Significance of tumor-infiltrating lymphocytes before and after neoadjuvant therapy for rectal cancer. Cancer Science. 2018;109:966–79.

26. Gash KJ, Chambers AC, Cotton DE, Williams AC, Thomas MG. Potentiating the effects of radiotherapy in rectal cancer: the role of aspirin, statins and metformin as adjuncts to therapy. Br J Cancer. 2017;117:210–9.

27. Ngwa W, Irabor OC, Schoenfeld JD, Hesser J, Demaria S, Formenti SC. Using immunotherapy to boost the abscopal effect. Nature Reviews Cancer. 2018;18:313–22.

28. Zitvogel L, Galluzzi L, Kepp O, Smyth MJ, Kroemer G. Type I interferons in anticancer immunity. Nat Rev Immunol. 2015;15:405–14.

29. Makena MR, Ranjan A, Thirumala V, Reddy AP. Cancer stem cells: Road to therapeutic resistance and strategies to overcome resistance. Biochim Biophys Acta Mol Basis Dis. 2018;165339.

30. Barker HE, Paget JTE, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nat Rev Cancer. 2015;15:409–25.

31. Smith FM, Rao C, Oliva Perez R, Bujko K, Athanasiou T, Habr-Gama A, et al. Avoiding radical surgery improves early survival in elderly patients with rectal cancer, demonstrating complete clinical response after neoadjuvant therapy: results of a decision-analytic model. Dis Colon Rectum. 2015;58:159–71.

32. Sclafani F, Brown G, Cunningham D, Wotherspoon A, Mendes LST, Balyasnikova S, et al. Comparison between MRI and pathology in the assessment of tumour regression grade in rectal cancer. British Journal of Cancer. 2017;117:1478–85.

33. Hupkens BJP, Maas M, Martens MH, van der Sande ME, Lambregts DMJ, Breukink SO, et al. Organ Preservation in Rectal Cancer After Chemoradiation: Should We Extend the Observation Period in Patients with a Clinical Near-Complete Response? Ann Surg Oncol. 2018;25:197–203.

34. Nilbert M, Planck M, Fernebro E, Borg Å, Johnson A. Microsatellite instability is rare in rectal carcinomas and signifies hereditary cancer. European Journal of Cancer. 1999;35:942–5.

35. Marliot F, Chen X, Kirilovsky A, Sbarrato T, Sissy CE, Batista L, et al. Analytical validation of the Immunoscore and its associated prognostic value in patients with colon cancer. J Immunother Cancer. BMJ Specialist Journals; 2020;8:e000272.

Table legends:

Table 1. Multivariate Cox models for disease-free survival according to biopsy-adapted Immunoscore (IS_B) combined with available clinical parameters

Figure legends:

Figure 1: **A.** Left: Representative image of tumor region (pink) with normal tissue or dysplasia (blue) excluded from the analysis. Left-mid: The tumor region divided into tiles for the analysis. Right-mid: Histogram of positive cells staining intensities detected by the software in a case of adequate immunostaining intensity. Red triangle defines mean case staining intensity of 220. Right: CD3+ T cells (red) detection in a tile (magnification X200). **B.** Chart illustrating the biopsy-adapted Immunoscore (IS_B) calculation method. Densities of CD3+ and CD8+ T cells in the tumor region are converted into percentile values. The mean percentile of two markers is calculated to generate IS percentile value, where IS_B Low, IS_B Intermediate, and IS_B High subgroups are reflected by 0-25%, >25-70%, and >70-100% percentile, respectively. **C.** Representative images of positive CD3+ and CD8+ T cells (cells/mm^2) infiltrating the rectal tumor in patients with IS_B Low and IS_B High. **D**. Disease-free survival according to IS_B Low (red), IS_B Intermediate (orange), and IS_B High (blue) in patients who met the biomarker and clinical data quality control evaluation in two independent cohorts (n_1 =124 and n_2 = 114). *P* test for trend (P_{tft}) is determined by log rank test for trend.

Int = Intermediate; P_{tft} = *P* test for trend

Figure 2: **A**. Bar charts represent means ± standard errors of the means (SEMs) of CD3+ and CD8+ T cells densities in the tumor biopsies before neoadjuvant treatment according to the subsequent response evaluated by the Dworak score and ypTNM classification (*P <0.05, **P

<0.01). *P* obtained for the Kendall's correlation test. **B**. Variation in mean percentile of CD3+ and CD8+ T cells densities depending on the degree of tumor regression, as evaluated by the Dworak score, pTNM classification, and NAR score. **C**. The frequency of IS_B Low (red), IS_B Intermediate (orange), and IS_B High (blue) according to tumor regression. Significant differences for the IS_B distribution are observed with a unilateral linear-by-linear association test.

Figure 3: A. Unsupervised hierarchical clustering showing immune-related genes in rectal cancer patients treated by primary surgery (grey) or by nT followed by surgery (TME) and who did not experience pathological response (red), had partial response (orange), or had complete response (green). Fold of change in gene expression is represented as a color gradient for high (red) and low (green) intensity. Only differentially expressed genes between non-responders and partial-complete responders are shown (34/44). **B**. The frequency of IS_B Low (red), IS_B Intermediate (orange), and IS_B High (blue) in primary tumor biopsies performed before nT in patients with or without immune activation. Unilateral linear-by-linear association test was applied. Int = Intermediate; P_{tft} = *P* test for trend*;* UICC = International Union Against Cancer; NAR = neoadjuvant rectal. **C.** The differential immune gene expression in tumor samples from patients with no sign of response to nT (Dworak 0, red) compared to those with response (Dworak 1-4, orange). Analysis with a Wilcoxon test adjusted for multiple testing (*P < 0.05, **P < 0.01). **D.** Principal component analysis (PCA) view of patient similarity based on differentially expressed genes between non-responders / responders. nT response levels are colored with confidence ellipses.

Figure 4: **A**. Relative contribution of each parameter available at diagnosis to survival risk. IS_B is compared with clinicopathological parameters including the cTNM, ycTNM, or ypTNM classifications using the chi-squared proportion test. **B**. Prediction of pathologic response (ypTNM 0-1) based on IS_B mean score and the ycTNM classification (ycTNM 0-I [black], ycTNM II [yellow], and ycTNM III-IV [blue]). **C**. Disease-free survival according to the IS_B scoring system in patients with good clinical response to treatment (ycTNM 0-1; *n* = 32). Int = Intermediate

Figure 5: **A**. Time to recurrence according to biopsy-adapted Immunoscore (IS_B) in patients treated by the Watch-and-Wait strategy (*n* = 73). **B**. Probability of the 2-year and 5-year recurrence-free survival according to IS_B expressed as a continuous variable (IS_B mean score, in percentiles) under the Cox proportional hazards regression model. **C**. Relative importance of each risk parameter to survival risk evaluated before neoadjuvant treatment in Watch-and-Wait patients. IS_B is compared with clinicopathological parameters including the cTNM and ycTNM classifications using the chi-square proportion test. Int = Intermediate; TTR = time to recurrence; P_{tft} = *P* test for trend

Table 1. Multivariate Cox models for disease-free survival according to biopsy-adapted Immunoscore (IS_B) combined with available clinical parameters

		Before neoadjuvant treatment			After neoadjuvant treatment			After surgery	
Characteristics	**PHA test**	**HR (95% CI)**	***P value****	**PHA test**	**HR (95% CI)**	***P value****	**PHA test**	**HR (95% CI)**	***P values****
Age									
Under vs Over 65 years	0.922	1.38 (0.85-2.24)	0.19	0.432	1.41 (0.69-2.9)	0.346	0.636	1.46 (0.86-2.48)	0.159
Tumor location									
Middle vs Inferior	0.43	1.1(0.67-1.8)	0.72	0.688	0.9 (0.45-1.81)	0.766	0.894	0.83 (0.47-1.46)	0.527
Superior vs Inferior	0.5	0.66 (0.26-1.69)	0.39	0.583	0.3 (0.04-2.24)	0.24	0.212	0.74 (0.29-1.9)	0.529
Sex									
Male vs Female	0.06	1.54 (0.9-2.63)	0.118	0.131	1.87 (0.8-4.37)	0.148	0.033	1.38 (0.76-2.49)	0.291
Immunoscore (IS_B)									
Intermediate vs Low	0.476	0.65 (0.38-1.1)	0.111	0.906	0.76 (0.33-1.73)	0.508	0.708	0.93 (0.5-1.71)	0.807
High vs Low	0.83	0.2 (0.08-0.49)	**< 0.001**	0.834	0.25 (0.07-0.93)	**0.039**	0.209	0.34 (0.13-0.89)	**0.028**
cTNM stage									
III vs I-II	0.59	1.18 (0.68-2.04)	0.56	-	-	-	-	-	-
ycTNM stage									
0 vs III	-	-	-	-	-	-	-	-	**-**
I vs III	-	-	-	0.916	0.62 (0.23-1.69)	0.349	-	-	**-**
II vs III	-	-	-	0.257	0.48 (0.22-1.08)	0.076	-	-	**-**
ypTNM stage									
0 vs III	-	-	-	-	-	-	0.208	0.14 (0.02-1.01)	0.051
I vs III	-	-	-	-	-	-	0.071	0.2 (0.09-0.45)	**<0.001**
II vs III	-	-	-	-	-	-	0.207	0.51 (0.29-0.89)	**0.018**

*The significance of the multivariate Cox regression model was evaluated with the Wald test
- not applicable
IS, Immunoscore; PHA, proportional hazards assumption; HR, Hazard ratio

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Figure 1

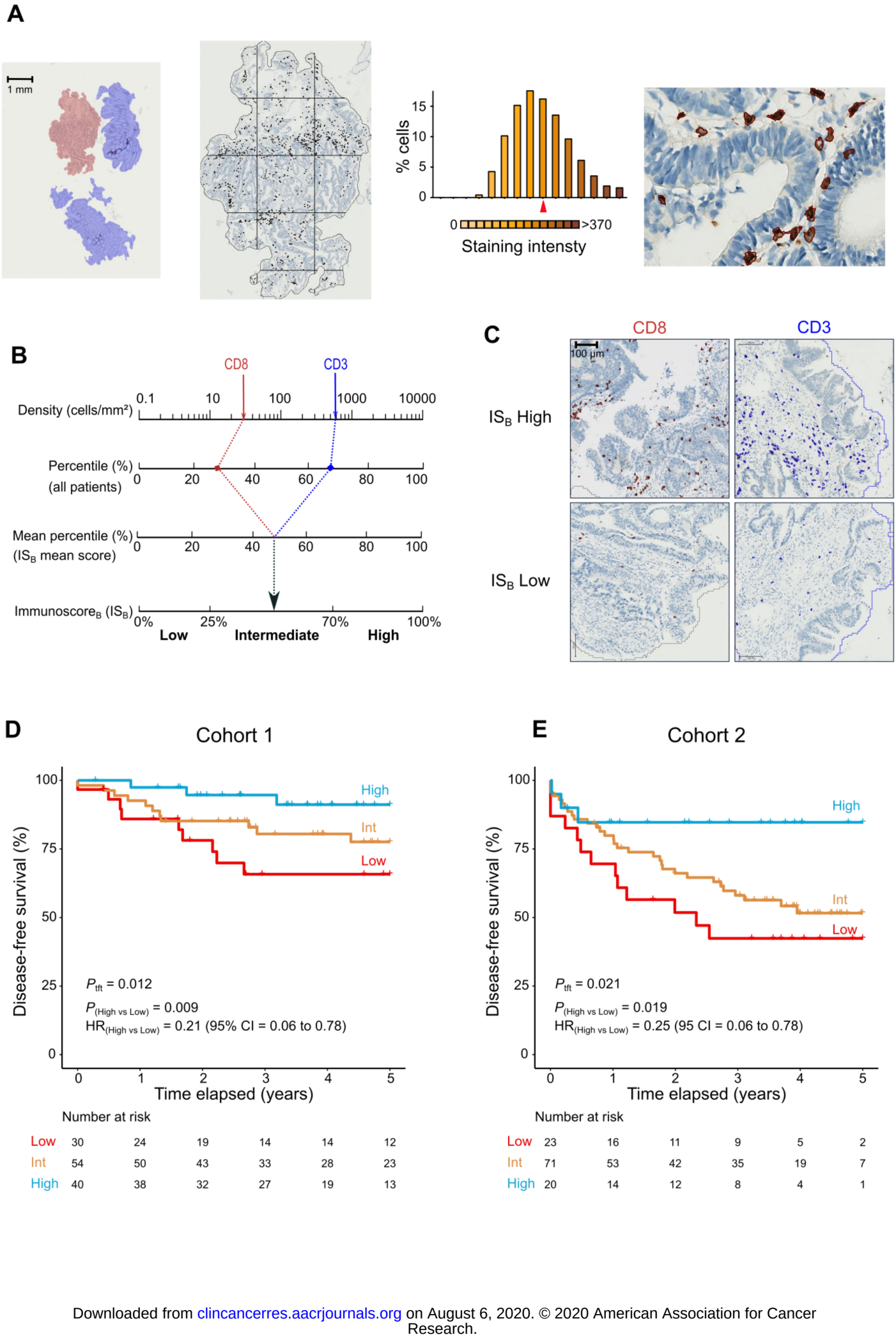
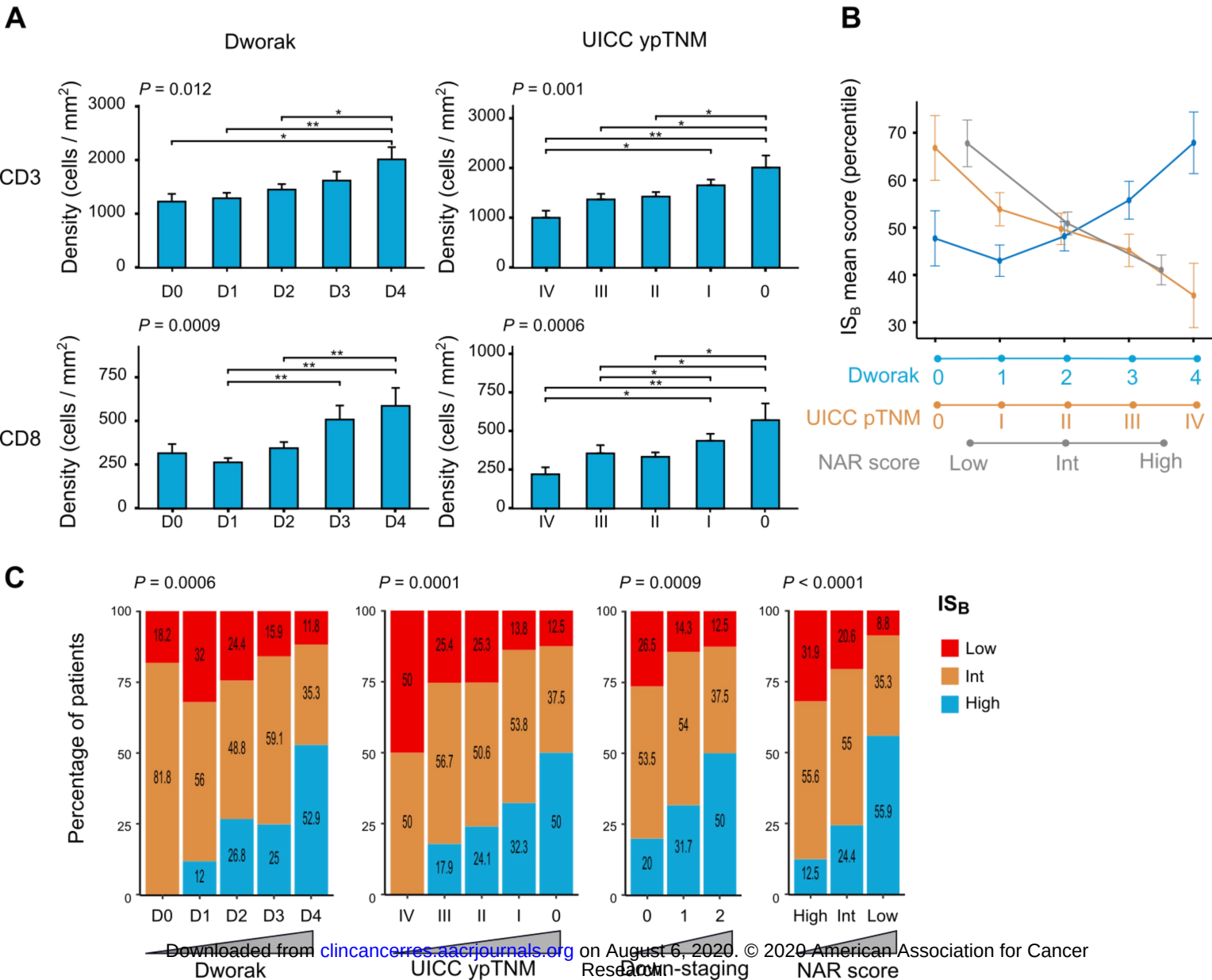


Figure 2



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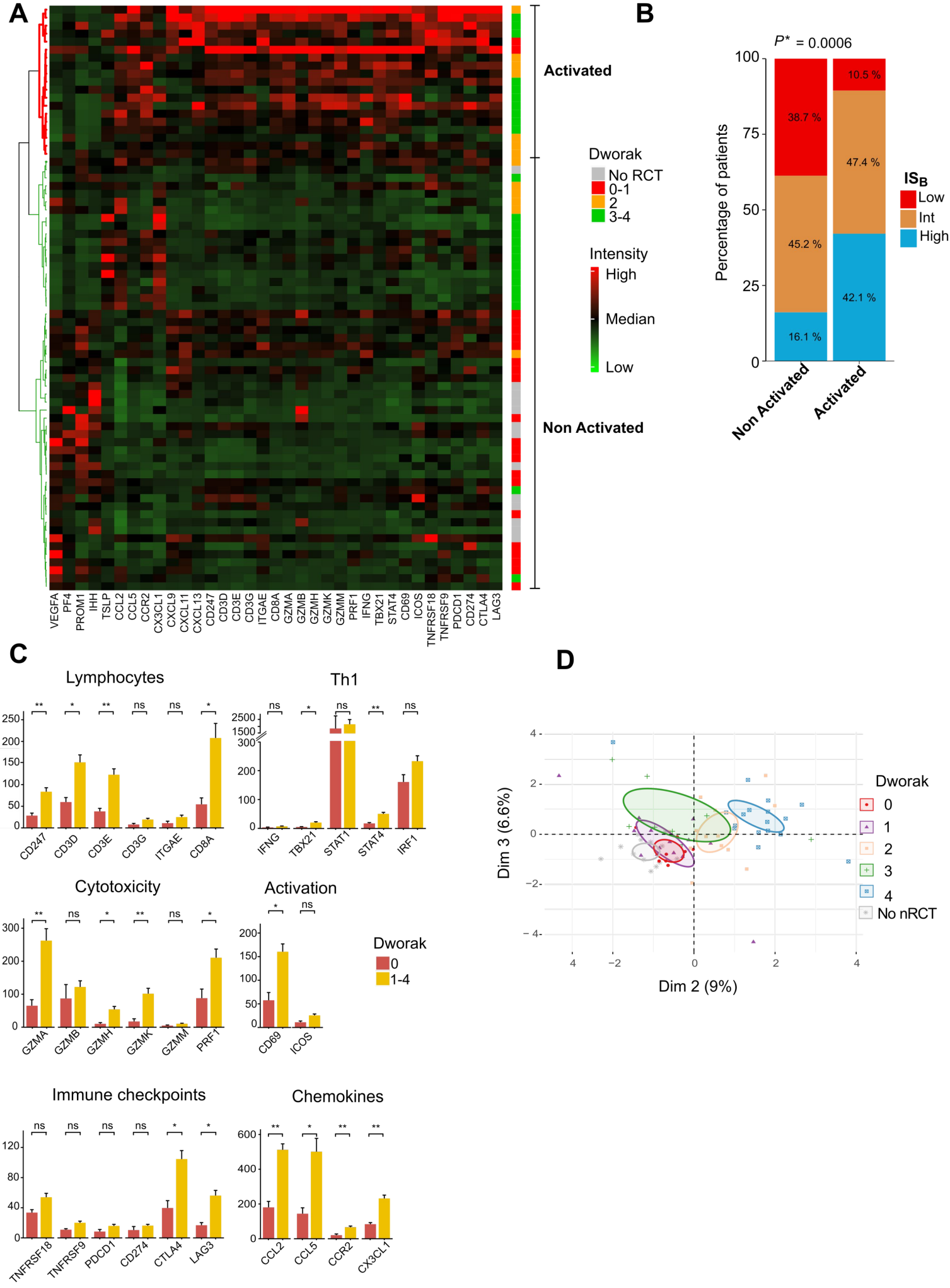
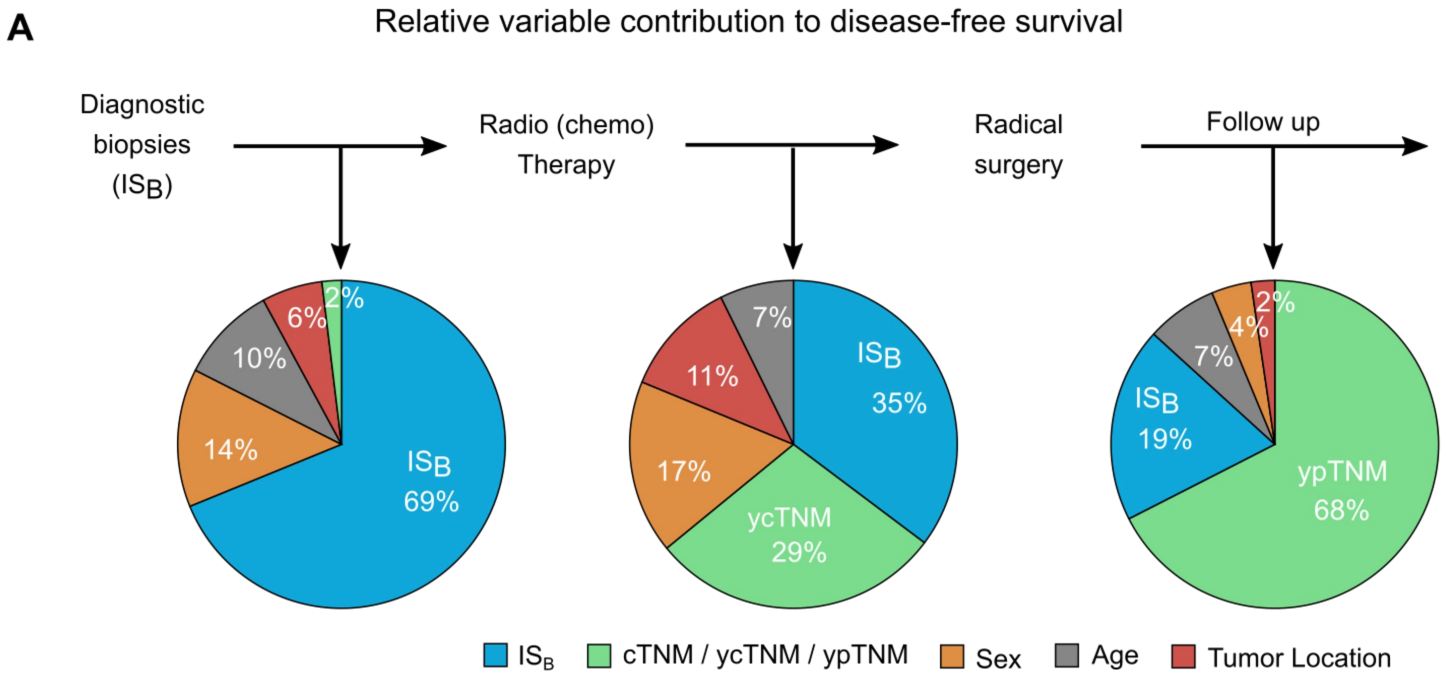
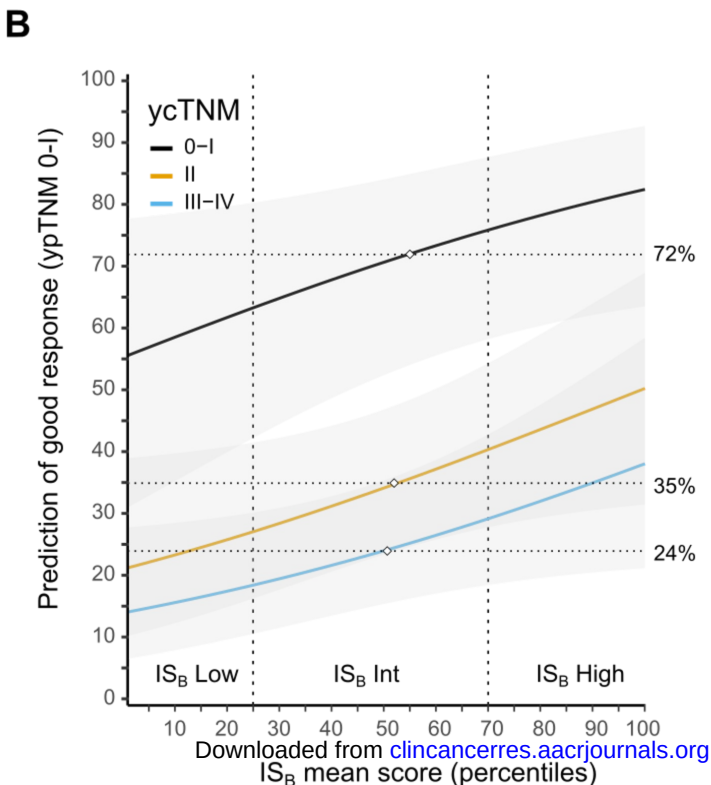


Figure 4





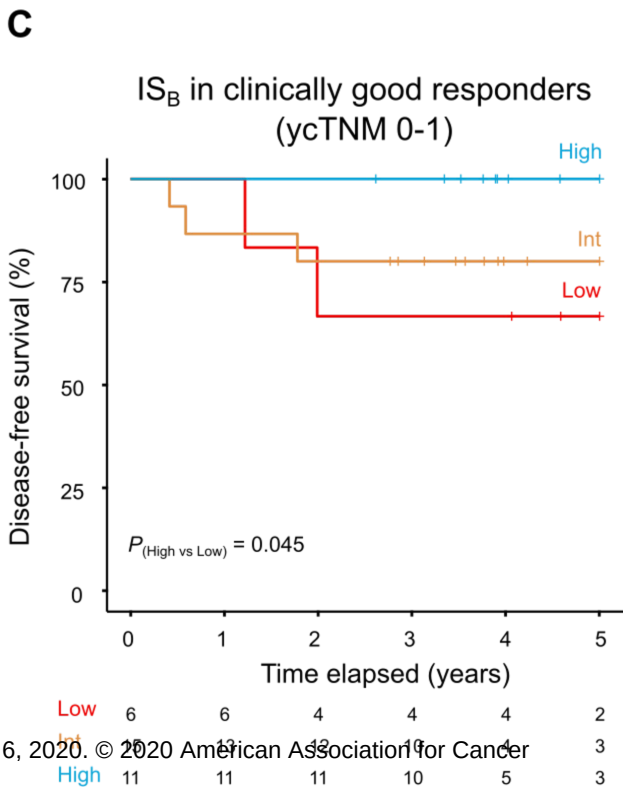
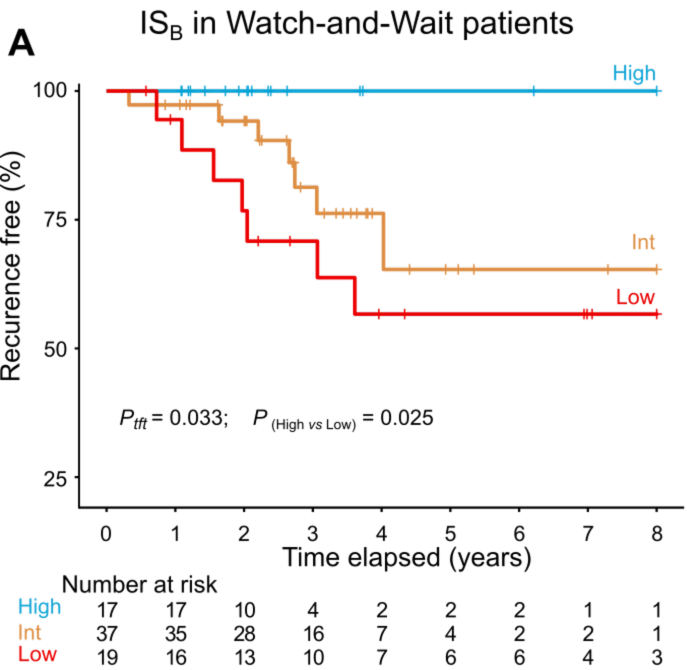
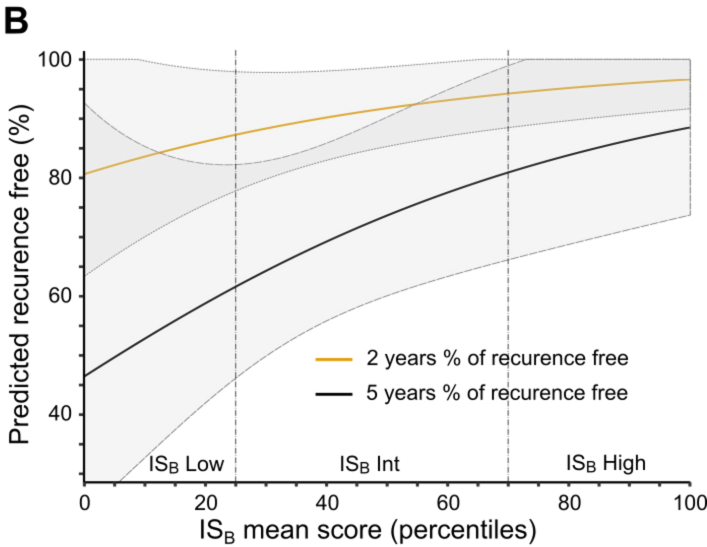
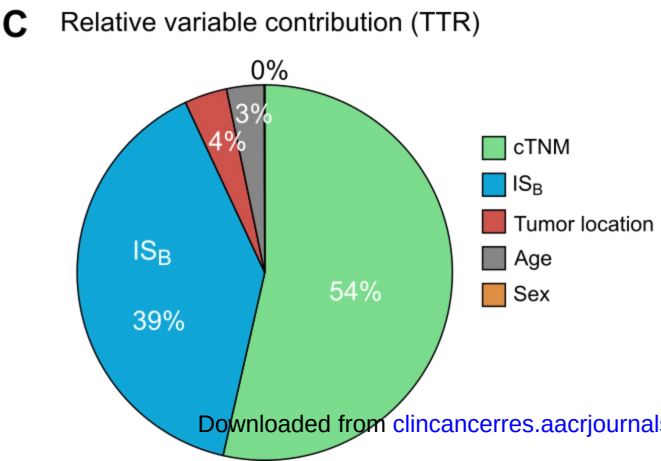


Figure 5







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Clinical Cancer Research

A diagnostic biopsy-adapted immunoscore predicts response to neoadjuvant treatment and selects patients with rectal cancer eligible for a watch-and-wait strategy

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