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▶ **To cite this version:**

David Malka, Astrid Lièvre, Thierry André, Julien Taïeb, Michel Ducreux, et al.. Immune scores in colorectal cancer: Where are we?. European Journal of Cancer, 2020, 140, pp.105-118. 10.1016/j.ejca.2020.08.024 . hal-02998186

HAL Id: hal-02998186
https://hal.science/hal-02998186

Submitted on 17 Oct 2022

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Version of Record: https://www.sciencedirect.com/science/article/pii/S0959804920304767
Manuscript_8b8eb3ba66df05d2b3fa61abfdafb926

Immune scores in colorectal cancer: Where are we?

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Abstract

There is growing evidence that the immune system may prevent the occurrence, growth and metastatic diffusion of colorectal cancer (CRC). The role played by the adaptive immune response at the tumor site is critical in the balance between tumor invasion and defense against cancer. Recent data have shown that the evaluation of this immune response may help to define the prognosis and possibly the treatment of localized CRC as well as metastatic CRC (mCRC). Tumor infiltrates with T cells (CD3+), cytotoxic T cells (CD8+), and memory T cells (CD45RO+) are the immune parameters most consistently and strongly associated with good clinical outcome in CRC. Several scoring systems have been developed, including the Immunoscore®, based on the immunohistochemical determination with a digital image analysis system of the density of CD3+ and CD8+ lymphocytes in the center and the invasive margin of the tumor. This review will focus on the different immunoscoring systems developed in CRC, their performance, their limitations and their potential for improving patients' care in the future.

Keywords

Colorectal cancer, prognosis, immunity, immune score

Introduction

Colorectal cancer (CRC) is the fourth most common cancer in the world and the second leading cause of death [1]. Survival is high for patients diagnosed with early-stage disease [2]. However, approximately half of CRC cases are diagnosed with or will develop metastases, the main cause of death in CRC, with a 5-year overall survival (OS) of approximately 10% [3]. There is thus a real need for therapeutic improvements in order to reduce the risk of recurrence after surgery or to prolong survival in patients with metastatic colorectal cancer (mCRC).

There is growing evidence that the immune system may play a role in preventing the occurrence, growth and metastatic diffusion of CRC. Understanding the role and mechanisms of immune response in CRC is a major issue and may help to better use immunotherapeutic agents in this disease and to develop new therapeutic approaches.

The crucial role of the immune system in controlling tumor development and the concept of immune surveillance were first demonstrated by the more frequent occurrence of non-pathogen-related tumors in immune-compromised patients such as transplant recipients and patients with AIDS. Registry and meta-analysis of solid organ transplant recipients have shown an increased risk of solid tumors including CRC, with a standardized incidence ratio of 1.2 to 1.8 [4, 5]. Whether patients with AIDS are at higher risk of developing CRC is however more debatable [6].

In CRC, innate immune cell infiltration has been reported to be of good prognosis for Natural Killer (NK) cells, Natural killer T (NKT) and γδ T lymphocytes. At the opposite, macrophage infiltrates are generally associated with a poor prognosis in human carcinomas [7-10].

Specific antitumor response is generated by adaptive immune system and in particular by αβ T lymphocytes. Briefly, after tumor cell lysis by innate immune cells such as NK cells, NKT cells and macrophages, the antigen-presenting cells, mainly dendritic cells, can capture, process and present tumor antigens to CD4 T lymphocytes through major histocompatibility class (MHC) II or to CD8 T lymphocytes through MHC I after a maturation process (Figure 1). Activation of T cells requires three signals: 1) recognition of antigenic peptide presented by the antigen-presenting cell; 2) activation of co-stimulatory molecules (CD80/CD28, CD40/CD40L); 3) recruitment of cytokines (interleukin (IL)-1, IL-2, IL-6, IL-12, interferon (IFN)γ). Activated CD8+ T cells can then recognize and lyse tumor cells (Figure 1). Activated CD4+ T cells can modulate the antitumor immune response. They can differentiate into

different cell subgroups: T cell helper 1 (Th1) response allows secretion of cytokines promoting antitumor response such as IL-2 or IFNγ, whereas T cell helper 2 (Th2) response is more associated to tumor growth. A third type of immune response, named T cell helper 17 (Th17), implies the release of large amounts of IL17 but its role in the immune response against cancer is controversial. Finally, the regulatory CD4+ T cells (Treg) can inhibit the antitumor immune response and represent an escape mechanism for the cancerous cells (Figure 1) [11].

The role of cytotoxic CD8+ T cells has been widely studied in CRC. Tumor infiltrating lymphocytes (TILs) are major actors of antitumor immune response (Figure 2). The prognostic role of immune response has been first analyzed in a large cohort of resected stage I to IV CRC more than 10 years ago [12, 13]. It has been originally shown that the absence of pathological signs of early metastatic invasion (venous emboli, lymphatic invasion, perineural invasion: VELIPI) was associated with increased infiltrates of immune cells and increased levels of messenger RNA (mRNA) for products of Th1 effector T cells [12]. In the same cohort, the density of TILs assessed by CD3 immunostaining predicted OS better than usual histopathological prognostic factors (i.e. UICC-TNM classification) (12).

Based on these results, different assessments of TILs have been elaborated and developed during the past decade [14-17]. However, despite impressive results on disease prognostication, tumoral and peritumoral infiltration by immune cells is not yet used in clinical practice. This review will focus on the different immunoscoring systems developed in CRC, their performance, their limitations and their potential for improving patients' care in the future.

Methods

Search strategy

A MEDLINE systematic literature search was conducted for English language studies published between January 2002 and May 2019. Conference abstracts published between January 2016 and June 2019 were searched for the following congresses: the American Association for Cancer Research, the American Society of Clinical Oncology (ASCO), the ASCO Gastrointestinal Cancers Symposium, the European Society for Medical Oncology (ESMO), and the ESMO World Congress on Gastrointestinal Cancer. A search strategy was developed

for use in MEDLINE consisting of medical subject headings (MeSH) combined with text words for CRC and those associated with immune score, immune system, immune infiltrated cells, tumor infiltrating lymphocytes, CD3 and immunotherapy. This strategy was adapted for use in other databases. Additional relevant publications identified by the authors were also included.

Eligibility criteria

Eligible studies evaluated immune infiltrates in patients with non-metastatic and metastatic CRC, and included endpoints related to oncological outcomes such as response rate and survival. Concomitant patients' clinical-pathological features and disease molecular assessments (mismatch repair status: Microsatellite Stable (MSS) or Microsatellite Instable (MSI); *BRAF* and *RAS* mutations) available for multivariable testing were also analyzed.

Prospective and retrospective studies with a minimum of 30 patients published between January 2002 and May 2019 (manuscripts), or between January 2016 and June 2019 (congress abstracts) were included. We also included post-hoc assessments of randomized controlled trials assessing immune cells infiltrates in CRC patients and additional studies felt to be of interest by the authors. Preclinical studies were excluded along with narrative reviews, editorials, opinions, letters, non-English language publications and congress abstracts for which insufficient methodological details were reported to allow critical appraisal of study quality and/or the endpoints of interest.

Data synthesis

Methodological aspects of immune scores

Studies evaluating the clinical impact of immune infiltrates in CRC have been based on different morphological techniques. Most of the studies rely on immunohistochemical (IHC) techniques based on monoclonal antibodies against T lymphocytes (CD3), cytotoxic T lymphocytes (CD8) and memory T lymphocytes (CD45R0), usable on formalin-fixed paraffin-embedded tissue. Different evaluation methods have been used: the only use of CD3, the joint use of CD3+/CD45RO+ or CD3+/CD8+ or CD8+/CD45RO+, a microarray tissue approach (consisting of duplicated or triplicated tissue cores of 0.6 to 1 mm in diameter from tumor samples)[14, 15] or a whole tumor tissue section approach [16]; quantification on the invasion front and/or in the tumor or a so-called linear quantification [17]. In most cases,

quantification was performed by image analysis and only some studies used a semi-quantification approach [12, 13, 16-25]. The methodological aspects of the immune infiltrate assessment are summarized in table 1.

A standardization of the evaluation of lymphocyte immune infiltrates, called Immunoscore®, has been proposed, based on an international multicenter study [16]. Immunoscore® is based on the determination of the intratumoral density of lymphocyte populations (CD3+/CD45RO+ or CD3+/CD8+ or CD8+/CD45RO+) in the core of tumor (CT) and in the tumor's invasive margin (IM) (figure 3). This is achieved by an IHC technique on tissue sections coupled with an image analysis system. For each case, CD3+ and CD8+ cell densities in colon tumor's core and invasive margin regions were compared. The mean of four percentiles (two markers, two regions) was calculated and converted into a score called Immunoscore® (figure 3). In a three-category Immunoscore® analysis, a 0–25% density was scored as low, a density between 25% and 70% was scored as intermediate, and a density between 70% and 100% density was scored as high. In a two-category Immunoscore® analysis, a 0–25% density was scored as low, and a density between 25% and 100% was scored as intermediate-high. The lack of reproducibility of the Immunoscore® outside expert hands could be the main weakness of the method. Its reproducibility was thus tested in a study involving 13 centers around the world[16]. The Immunoscore® was highly reproducible among centers and investigators ($r=0.97$ for the evaluation of the Immunoscore® at the tumor center; $r=0.97$ for its evaluation at the invasive margin; $p<0.0001$).

Correlation with clinical-pathological characteristics

A few studies have assessed the association between tumor T-cell infiltration and clinical-pathological parameters. In a series of 152 cases of resected CRC, no correlation was found between tumor CD8+ densities and age or tumor location [26]. In an ancillary study of the adjuvant PETACC8 phase III trial, the density of lymphocyte infiltration determined in 1220 stage III colon cancers with an automated counting of CD3 lymphocytes within the tumor margin was also not correlated with age, gender, WHO performance status, bowel obstruction or perforation and tumor location or grade [21].

Galon et al. showed that CRC cases with a high density of infiltrating memory (CD45RO+) and effector (CD3+CD8+) T cells were less likely associated with positive regional lymph nodes, vascular emboli, lymphatic or perineural invasion ("VELIPI" status), which are all considered

as early steps of the metastatic process [13]. In two independent cohorts of patients, the same team reported a correlation between high densities of CD8+ cytotoxic T-lymphocyte infiltrates and early-stage Tis/T1 disease [27]. While it was also significantly correlated with pT stage, the CD3-based lymphocyte infiltration developed by Emile et al. was not associated with pN stage or with vascular invasion or lymphatic infiltration [21], with the limits of a pure stage III population.

Prognostic impact

In human cancers, the presence of TILs generally indicates a favorable prognosis. In CRC, Pages et al. have found in a first cohort of 958 resected stage I to IV CRC patients an association between T-cell immune response within the tumor, the absence of early metastatic invasion (VELIPI-negative status) and increased survival [13]. Using the same cohort of patients, they showed that the type, density and location of immune cells within the tumor may influence the clinical outcome of CRC patients. The adaptive immune reaction composed of T cells (CD3+) with cytotoxic (CD8+) and memory (CD45RO+) phenotypes in the CT and IM was a highly significant parameter to predict time to recurrence and OS. These initial results were subsequently validated in two additional patient populations [12] (table 2). After standardization of their method, the same team showed the usefulness of an Immunoscore® based on the combined evaluation of memory and cytotoxicity markers for the prediction of tumor recurrence and survival, specifically in early-stage (UICC/TNM stage I and II) CRC [14]. Then, Mlecnik et al. investigated the relationship between the extent of immune cell density (CD45RO+ and CD8+ T cells) in the CT and IM of the tumors, the degree of tumor extension (pT and pN stages) and the frequency of tumor recurrence in two independent cohorts of 415 and 184 specimens of stage I to IV CRC [27]. In the subgroup of stage I to III CRC patients, they showed a better prognosis (i.e. better DFS and OS) associated with a strong density of both CD45RO+ and CD8+ T cells in CT and IM. In multivariate analysis of DFS, including pT and pN stage, age, sex, total number of lymph nodes, tumor grade, mucinous type, occlusion and bowel perforation, only the Immunoscore® and bowel perforation remained significant prognostic markers (table 2). These data suggest that the Immunoscore® may accurately predict recurrence in addition to the TNM classification. Interestingly, in patients who did not relapse, the density of CD8 infiltrates was inversely correlated with T stage whereas in patients who relapsed the

number of CD8 cells was low regardless of the T stage of the tumor, suggesting an inverse correlation between *in situ* response and tumor progression.

Two other large studies, including 843 and 768 patients respectively, have shown that tumor immune infiltrate patterns are significant prognostic biomarkers, even after adjustment for stage, lymph node involvement and well-established prognostic tumor molecular markers including MSI status or *BRAF* mutation [28, 29]. Two large CRC population-based studies have suggested that, in addition to TILs, high peritumoraL diffuse lymphoid inflammation (lymphoid cell infiltrates within stromal tissue along the invasive front) and Crohn's disease-like lymphoid reaction (lymphoid aggregates corresponding to tertiary lymphoid structures), which are frequent features of MSI tumors, are both significantly associated with reduced risks of CRC-specific survival and OS independently of traditional prognostic markers including the MSI status [30, 31] (table 2). In 1220 stage III colon cancer patients treated with adjuvant FOLFOX included in the PETACC8 phase III study [21], lymphocyte infiltration was analyzed using a linear quantification of CD3 density of the tumor margin previously demonstrated as an independent prognostic factor in a study of 119 stage II-III colon cancers [17]. Patients with high lymphocyte infiltration had a lower 2-year time-to-recurrence (14% vs 21% p=0.02) and longer 3-year DFS (81% vs 72%; HR = 0.69; p=0.01) as and 5-year OS (89% vs 80%, HR=0.58; p=0.0048). Lymphocyte infiltration remained with tumor stage, grade, *RAS* and *BRAF* status an independent prognostic factor in multivariate analysis (HR=0.6; 95%CI: 0.4-0.98; p=0.04) [21] (table 2).

In parallel, an independent international panel of expert laboratories has started to work on large cohorts of colon cancers to promote the Immunoscore® (a trade mark test) based on the density of CD3+/CD8+ T cells evaluated by immunohistochemistry in routine clinical practice [32] and evaluate its relationship with MSI and the respective prognostic impact of these two related parameters. Mlecnik et al. demonstrated, from the analysis of two cohorts comprising more than 900 CRC patients, that MSI tumors, but also a subgroup of MSS tumors, are characterized by a prominent gene immune expression [18]. In this study, the Immunoscore®, but not MSI and TNM parameters, was significantly predictive of patient's disease recurrence and survival in multivariate analysis [18]. These findings indicate that assessment of the Immunoscore® provides a potent indicator of tumor recurrence and could be an important guide, in addition to TNM stage and MSI status, for adjuvant chemotherapy after resection of CRC.

A prospective validation of the Immunoscore® in clinical practice has been recently published by Pages et al. [16] thanks to an international consortium that analyzed 2681 resected stage I-III colon cancers from 14 centers in 13 countries. This study confirmed the very good intra- and inter-observer reproducibility of the Immunoscore® and its powerful prognostic value, with a 8% recurrence rate at 5 years in patients with high Immunoscore® compared to 19% and 32% in cases of intermediate and low Immunoscore® respectively (HR 0.20, p <0.0001). In multivariate analysis, the prognostic impact of Immunoscore® on DFS was independent of age, gender, T and N stage, but also of MSI. This prognostic value was maintained in the subgroup of 1434 stage II colon cancers (hazard ratio (HR) high versus low 0.20; p<0.0001). Immunoscore® had the highest relative contribution to the risk of recurrence among all clinical-pathological parameters (table 2). These results support the implementation of the Immunoscore® as a new component of the prognostic classification of CRC.

Predictive impact on adjuvant chemotherapy benefit

Whether the immune contexture of the primary tumor predicts the benefit from adjuvant chemotherapy is of high importance for patient clinical management. Some studies have reported an excellent prognosis of CRC patients with a strong immune component receiving 5-fluorouracil/leucovorin as adjuvant treatment [26]. However, the high cytotoxic immune response may not be predictive of response to adjuvant chemotherapy, but rather reflects the fact that the host-immune response within the tumor protects the patient and prolongs patient survival. In patients with stage III colon cancer treated with oxaliplatin-based chemotherapy, the prognostic value of the Immunoscore® for DFS was confirmed in the 3- vs 6-month adjuvant trial IDEA-France [33]. Patients treated with mFOLFOX6 (90% of the patients in the trial) with high or intermediate Immunoscore® had better DFS in the 6-month arm than in the 3-month arm, whether they have a high-risk (T4N2) or low-risk (T1-3N1) stage III cancer (table 2). By contrast, the duration of adjuvant chemotherapy did not seem to make a difference in patients with low Immunoscore® treated with the same regimen. These data suggest that the duration of adjuvant chemotherapy could be guided by the Immunoscore® (at least for mFOLFOX6). Albeit counterintuitive (patients with a good prognosis needing a longer adjuvant treatment), these data may reflect a higher sensitivity of tumors infiltrated by immune cells to oxaliplatin-based adjuvant treatment or conversely

a resistance to treatment in tumors without any immune infiltrates. These data regarding adjuvant mFOLFOX6 need to be confirmed in other IDEA studies, which should also explore whether there is any difference in patients treated with CAPOX (received by only 10% of patients in IDEA-France study), the other standard adjuvant regimen. Besides these results on adjuvant treatment duration, we still lack any clear published data regarding the predictive value of the Immunoscore®. Another important data currently missing is the potential interest of neo-adjuvant or adjuvant immunotherapy in patients with a high Immunoscore®, whose survival is good but could be improved by immunotherapy. Until we have these data, the Immunoscore® is in our opinion unlikely to be incorporated into routine clinical practice.

Immunoscore and deficient mismatch repair and/or microsatellite instability (MSI)

MSI tumors, including CRC, accumulate insertions and deletions in DNA repeat sequences. About two-thirds of MSI tumors are sporadic, while one-third is inherited (Lynch syndrome). The high mutational load (i.e. hypermutated phenotype) and frequent frameshift mutations that characterize MSI tumors lead to the production of many neo-antigens recognized by the immune system, which can trigger lymphocyte infiltration in these tumors. However, these immune infiltrates may differ in quantity and quality within MSI patients [34, 35]. MSI tumors represent approximately 15% to 20% of stage II and 10% of stage III CRC cases, and are associated with better prognosis compared to MSS tumors [36]. By contrast, the prognostic value of MSI status is debated in mCRC, and MSI tumors represent only 5% of mCRC cases [37-40].

Mlecnik et al. demonstrated that the Immunoscore® was superior to MSI in predicting disease recurrence and survival in patients with stage I-III CRC [15]. In this study, among 105 patients with MSI tumor, patients with high Immunoscore® (n=71) had prolonged disease-specific survival and OS as compared to patients with low Immunoscore® (n=34). The DFS of low Immunoscore® patients was similar in MSS and MSI tumors as well as the DFS of high Immunoscore® patients (approximately 60% and 80% at 3 years, respectively) [15]. These findings suggest that assessment of Immunoscore® may help to decide adjuvant therapy in patients with early-stage, MSS but also MSI CRC. It is also possible that only MSI patients with high Immunoscore® might benefit from checkpoint inhibitors in future clinical trials. Of note, 32% of MSI patients had a low Immunoscore® in this study.

In the IDEA-France study, among the 93 patients with MSI tumors evaluable for Immunoscore®, 20 (21.5%) had low Immunoscore®, 56 (60.2%) had intermediate or high Immunoscore® and 17 (18.3%) had inconclusive results [33]. Among the 304 patients with MSI tumors in the international validation study of the Immunoscore®, 49 patients (16%) had low Immunoscore® and 255 (84%) had intermediate and high Immunoscore® [16]. Patients with intermediate and high Immunoscore® had prolonged DFS and OS, irrespective of their microsatellite status (unadjusted hazard ratio for high versus low Immunoscore® in MSI patients: 0.56, 95%CI 0.34-0.90; p=0.015). In multivariable analysis, the Immunoscore® predicted time to recurrence independently from T and N stage, but also MSI[16].

Yoon et al. examined CD3+ and CD8+ T cells in stage III colon cancer from patients included in the adjuvant study N0147 (FOLFOX6 ± cetuximab). MSI tumors, despite a higher CD3+ and CD8+ lymphocytic infiltration in the tumor micro-environment, had greater inter-tumor patient heterogeneity in terms of density of CD3+ and CD8+ T cells than MSS tumors [22]. Patients whose tumor had a low T cell-density had a shorter OS compared with patients harboring tumors with a high T-cell density. Around 30% of MSI tumors exhibited T-cell densities as low as in MSS tumors. CD3+ at the IM provided robust stratification for prognosis and identified both MSI and MSS tumors with low CD3+ IM infiltrates and shorter OS. Another study demonstrated higher heterogeneities in MSI than in MSS CRC [39]. Altogether these publications stress the variability of immune infiltrate density within MSI tumors and identify 16-32% of MSI CRC with low lymphocyte infiltrates.

Differences in mutation burden and variability in neoantigen profiles and unstable microsatellites may explain the heterogeneity of immune infiltrates among MSI tumors. Lynch-associated MSI tumors present with more somatic mutations and neoantigens compared with sporadic MSI tumors, which may result in stronger immune reactions and different outcomes. Immune checkpoint expression may cancel the prognostic relevance of tumor-infiltrating T cells in highly immunogenic colon tumors and predicts a poor outcome in MSI CRC patients [42]. At early stage, MSI leads to the synthesis of aberrant and potentially immunogenic neoantigens. A consequence is that MSI tumors are heavily infiltrated with active cytotoxic T cells and that immune response is efficacious to avoid tumor growth and metastatic dissemination. This phenomenon explains the good prognosis of MSI stage I to III colon cancer. However, for some tumors, overexpression of immune checkpoints could allow immune escape, tumor growth and development of distant metastases. Once disease

is metastatic, MSI has been shown to be a major predictive biomarker for the efficacy of immune checkpoint inhibitors among mCRC patients, as well as other metastatic MSI tumors. Clinical responses and long survival were observed across all biomarker groups assessed (PD-L1 tumor expression, *BRAF* or *KRAS* mutations, or a clinical history of Lynch syndrome) [43-45] but no data are available yet regarding immune scores in patients with MSI mCRC treated with immune checkpoint inhibitors and the predictive value of immune scores in MSS mCRC, though trials are on-going.

Metastatic disease

Tumor-infiltrating lymphocyte counts and T-cell subsets (CD4+, CD8+, Tregs, FOXp3+) in liver metastases have been reported to be independent prognostic indicators of survival after CRC liver resection [46-49]. T-cell counts and subset ratios in liver metastases were shown to predict recurrence and survival [47, 48, 50-52]. In a comparison of patients who survived ≤ 2 and ≥ 10 years following resection of CRC liver metastases, high CD8+ T cell counts and low CD4+ T-cell counts were associated with 10-year survival rate following surgery [48]. Another study showed that low CD45RO infiltration at the IM and fibrotic capsule formation independently predicted prolonged survival. The number of peritumoral Tregs was shown to predict cancer-specific survival and DFS in mCRC patients undergoing liver resection. A scoring system using TIL densities based on CD3+, CD8+, granzyme B+ and FOXp3+ immune cells at the IM of liver metastases predicted the response to chemotherapy with a sensitivity of 79% and specificity of 100% [46].

The evolution patterns during metastatic progression depend on the immune contexture at the metastatic site [53]. Van den Eynde et al. quantified immune cell types on whole slides of 603 resected, synchronous or metachronous metastases and 97 primary tumors in a retrospective series of 222 mCRC patients [54]. They showed the intra- and inter-tumor lesions and inter-patient heterogeneity of immune T-cell (CD3, CD8, CD45RO, FOXP3) and B-cell infiltrates for both primary tumors and metastases. As a consequence, the Immunoscore® was generally heterogeneous between metastases from the same patient, whereas interestingly, comparisons of Immunoscore® within lung versus liver metastases, and within synchronous versus metachronous metastases showed no difference. Mutational profiling showed heterogeneity between metastases and primary tumors, in concordance with the idea that tumors grow by clonal expansion and accumulating genetically diverse

subpopulations. A high Immunoscore® was associated with a significantly lower number of metastases per patient. In particular, the patients with the fewest metastases had the highest immune intra-metastatic density in its least-infiltrated metastasis. CD3 and CD8 densities were higher in metastases compared with the primary tumors of the same patients showing no general loss of immunity within metastases. This could also reflect the development of an immunosuppressive microenvironment within the primary tumors over time, and this is supported by the higher FOXP3 densities observed within the CT of the primary tumors, particularly in anti-EGFR-treated patients. However, metastases from patients with highly infiltrated primary tumors also had higher densities of immune cells suggesting a more active systemic tumor-specific immune response.

The liver and brain are metastatic sites considered "immunoprivileged" because of their relatively low basic immune infiltration and resistance to immune response [46, 47]. This phenomenon may explain why “cold liver metastases” with poor T-cell infiltrates were more numerous and associated with a more unfavorable prognosis the work of Van den Eynde et al [45]. Immune densities, Immunoscore® and T- and B-cell score (TB score) (CD8 and CD20) were investigated in relation to patient outcome. In both training and validation cohorts, high immune infiltrate, Immunoscore® and TB score were significantly associated with prolonged DFS and OS, even in patients with large metastases. The least-infiltrated metastasis showed the strongest association with patient outcome (HR > 2, $p < 0.05$) compared with the mean metastatic infiltrate or a randomly selected metastasis, and no association with the patient survival was observed for the most-infiltrated metastasis. In another publication derived from the same patient population, patients with a high Immunoscore® in their least-infiltrated metastasis had a statistically significantly lower risk of relapse (DFS rate 27.9% vs 12.3%; HR 0.45 [95% CI 0.28-0.70], P = 0.02) and a prolonged OS (OS rate 64.6% vs 32.5%; HR 0.32 [95% CI 0.15-0.66], P = 0.001) compared with patients with a low Immunoscore® [23]. Similar results were obtained for the TB score. Among patients not responding to preoperative systemic therapy according to their histological tumor regression grade, those with strong immune infiltrates had better DFS (HR 0.28, 95% CI 0.15-0.52, P = 0.001) and better OS (HR 0.25, 95% CI 0.1-0.62, P = 0.001) than those with low immune infiltrates. In a multivariable analysis of relevant clinical and immune parameters including TB score or Immunoscore®, node stage, preoperative treatment type, metastasis resection margin status, tumor regression grade and *RAS* status, only the TB

score (HR 0.20, 95% CI 0.08-0.48, P < 0.001) or the Immunoscore® (HR 0.24, 95% CI 0.10-0.58, P = 0.001) remained statistically significantly associated with OS. The TB score was also statistically significantly associated with DFS (HR 0.42, 95% CI 0.25-0.71, P = 0,001), as was the number of metastases per patient (≤3 vs >3; HR 2.28, 95% CI 1.38-3.77, P = 0,001). Markers included in the OS final model were also tested for DFS, and similar results were obtained for both TB score and Immunoscore®.

Quantification of TILs in a small region of a randomly selected metastasis has previously been reported to be associated with survival. Several reports underlined the possible limitation of tumor sampling using single biopsies for patient treatment decision. Van den Eynde et al. showed that computer simulation of a single biopsy correctly identified low-infiltrated metastases in more than 90% of the cases, but that due to the heterogeneity of the metastatic disease, overall intra-metastatic immune infiltrate might be better estimated with multiple biopsies or sampling of larger tumor areas [54].

Van den Eynde et al. also explored differences in the immune infiltration of lesions according to the type of neoadjuvant treatment received in a subset of 48 patients [54]. Patients treated with chemotherapy and anti-EGFR had greater infiltration of T lymphocytes (CD8, CD45RO, FOXP3) into the CT of metastases and higher Immunoscore® than patients who received chemotherapy and anti-VEGF. Strikingly, the strongest increase of immune densities in the treated patient samples was observed in the highest infiltrated metastasis, while the mean infiltration of metastases showed only a slight increase after anti-EGFR treatment, and the least-infiltrated metastasis showed no significant changes. These results suggest that the treatment could be more efficient in increasing the intra-metastatic immune reaction in metastases with pre-existing immune infiltrate. They also observed upregulation of B cell pathways (immune cell densities and gene expression) in primary tumors after chemotherapy plus anti-VEGF treatment.

In a retrospective study of resected CRC liver metastases in 249 patients, a high immune score was associated with fewer liver metastases (P = 0.020), smaller lesion sizes (P = 0.028) and lower Fong's clinical risk score [55](CRS) (P = 0.033). Patients with high immune score had longer RFS (21.4 vs 8.7 months, P < 0.001; 3-year RFS rate, 42.4 vs. 17.0%; P < 0.001) and OS (not reached vs 28.7 months, P < 0.001; 5-year OS rate, 59.7 vs. 25.9%, P < 0.001) than those with low immune score [56]. The prognostic value of the Immunoscore for OS persisted after stratification on CRS.

In another series of 196 mCRC patients, the densities of CD3+, CD4+, CD8+, FOXP3+, CD68+ and CD163+ immune cells were determined in both primary tumor and metastases. High values of primary tumor Immune score, metastatic immune score (immune score determined in a distant metastasis added to primary tumor immune score) and macrophage immune score (CD163+ density in the CT and IM of primary tumor added to primary tumor immune score; with high density of tumor-associated macrophages recorded as score 0, contrarily to CD3+ and CD8+ TILs) were significantly associated with a better prognosis [20]. In multivariate analysis, only the metastatic immune score retained an independent prognostic value (low vs high, HR 1.86 (95% CI 1.14-3.02), p=0.012).

Conclusion

Different scoring systems evaluating lymphocyte infiltrates in colorectal cancer (primary tumor and/or metastases) have been elaborated and developed. The Immunoscore® has been the most widely studied and is achieved by an IHC technique on tissue section coupled with an image analysis system. Its prognostic performance has been shown in colon and rectal cancer, primary and metastatic lesions, MSI and MSS tumors and confirmed each time in multivariate analyses. This Immunoscore® may also become in the future a useful tool in the prediction of efficacy of immunotherapy in patients with MSI or MSS CRC. The role of Immunoscore® in the management of CRC patients in the adjuvant setting, including post-resection of metastases, needs more data, ideally through biomarker-driven prospective clinical trials, to better define its impact on the need, type and duration of adjuvant therapies.

Declaration of interest

Figure legends

Figure 1. Schematic illustration of the anti-tumor response

Innate immunity involves cells such as NK cells, NKT cells and macrophages, leading to tumor cell lysis. The released antigens are processed by dendritic cells which are presented to CD4 T lymphocytes through major histocompatibility class (MHC) II or to CD8 T lymphocytes through MHC I. Then activated cytotoxic CD8+ T cells can recognize and lyse tumor cells. This immune response can be counterbalanced by immunosuppressive cells, immunosuppressive molecules and exhaustion molecules (abbreviations: DC: dendritic cells, M*φ*: Macrophages, MDSC: myeloid derived suppressive cells NK, Natural Killer; NKT Natural Killer T, Treg: regulatory T cells).

Figure 2. **The immune microenvironment of colorectal cancer**

The antitumor microenvironment encompasses CD8+ cytotoxic lymphocytes and type 1 helper (Th1) T cells in the tumor core and the invasive margin. In the stroma, tertiary lymphoid structures characterized by an association of B cells, CD8+ cytotoxic lymphocytes and dendritic cells mimicking a lymph node can also be observed. This contexture mainly reflects an adaptive immune response. Besides these cell populations, pro-tumorigenic cells can be encountered such as regulatory T cells (Tregs). Macrophages have an anti-or pro-tumor role, depending on their phenotype, M1 or M2 respectively.

Figure 3 The Immunoscore® design in colorectal cancer

The Immunoscore® is a standardized approach to characterizing T-cell infiltration of surgical pathology tumor specimen, notably colorectal cancer. Left panels: Digital representation of tissue areas. The border between the advancing tumor edge and normal tissue is annotated on a colorectal carcinoma specimen. A 500-µm distance on either side of this border is designated the 'invasive margin' (IM, outlined in yellow). The adjacent healthy tissue is excluded (outlined in green and blue). The remainder of the tumor is designated as the 'tumor core' (TC, outlined in red). Upper right panels: Immunohistochemistry for CD3 and CD8 used to quantify cell densities for each of these immune cell subsets in both the IM and TC. Lower right panels: The density of each region is labeled 'high' or 'low' density for each marker. The mean percentile of the four immune parameters is calculated, resulting in a

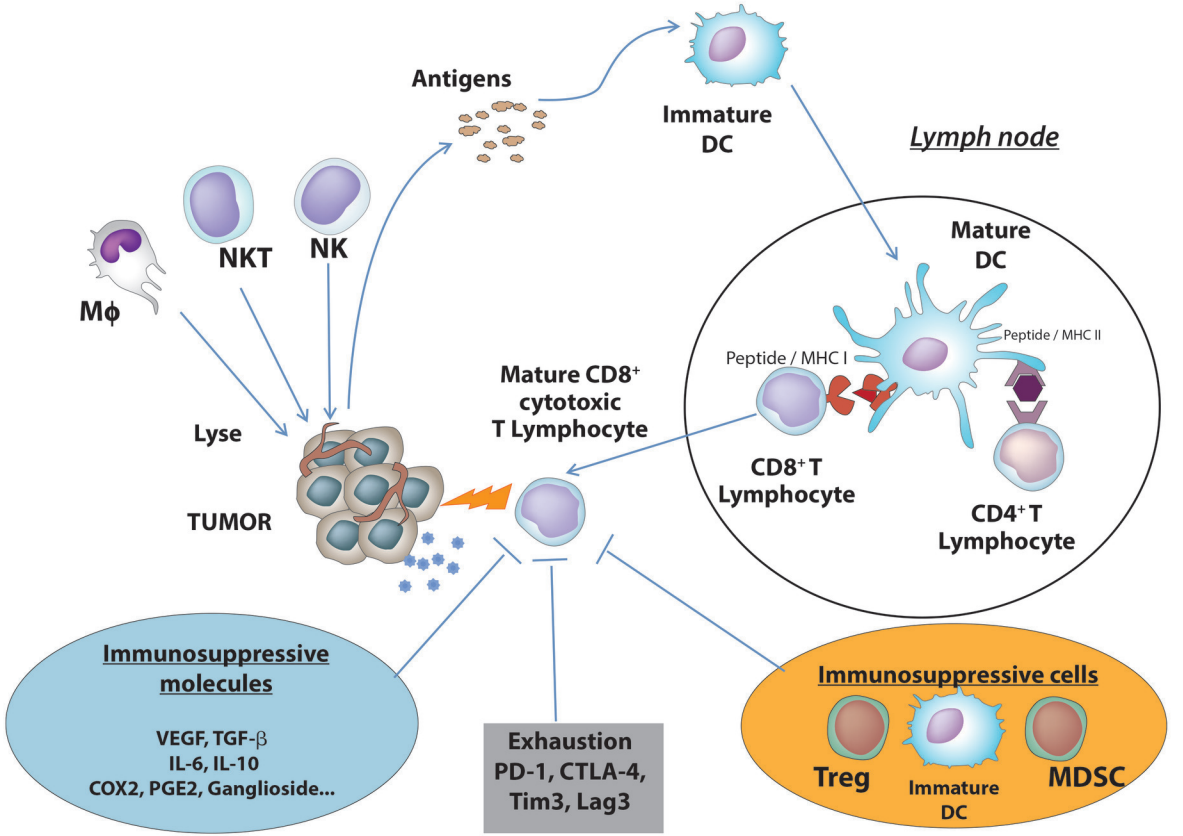
possible Immunoscore ranging from I0 to I4, or three categories (Low, Intermediate, High) (abbreviations: IM, invasive margin; TC, tumor core).

Reference List

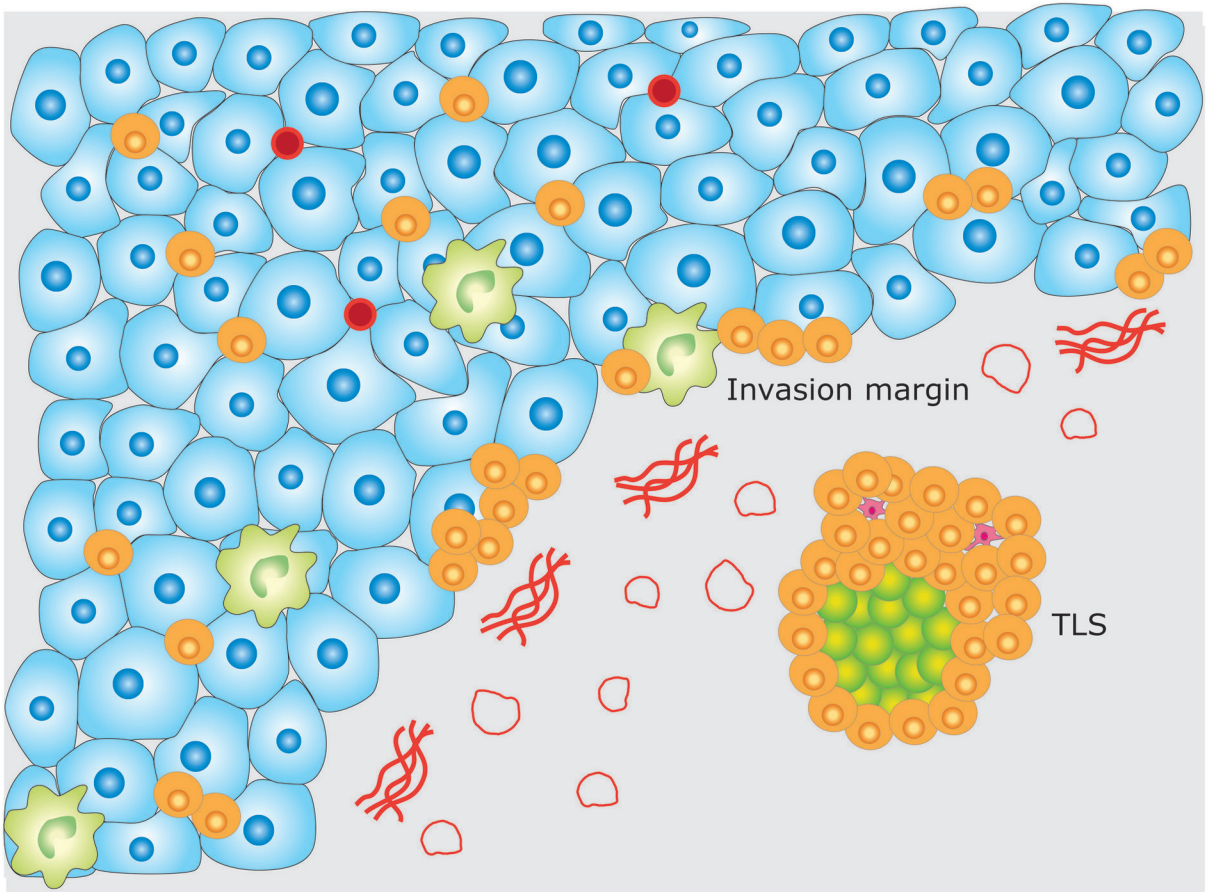
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Antigens
Immature DC
Lymph node
Mature DC
Peptide / MHC I
Peptide / MHC II
Mature CD8+ cytotoxic T Lymphocyte
CD8+T Lymphocyte
CD4+T Lymphocyte
Mϕ
NKT
NK
Lyse
TUMOR
Immunosuppressive molecules
VEGF, TGF-β
IL-6, IL-10
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Immunosuppressive cells
Treg
Immature DC
MDSC

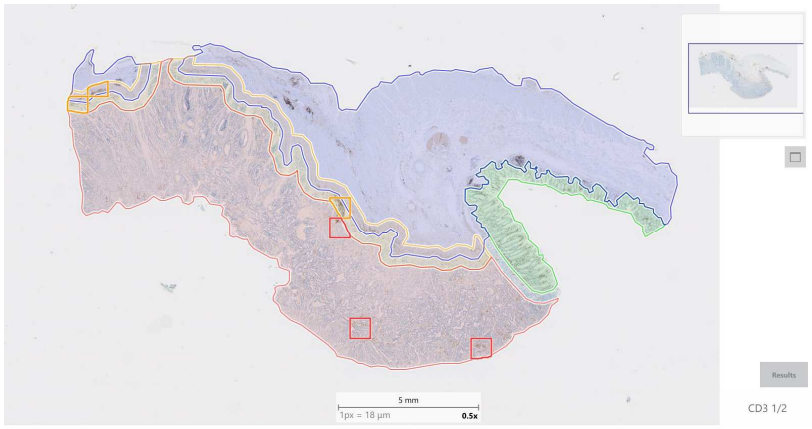
Schematic representation of colorectal microenvironment



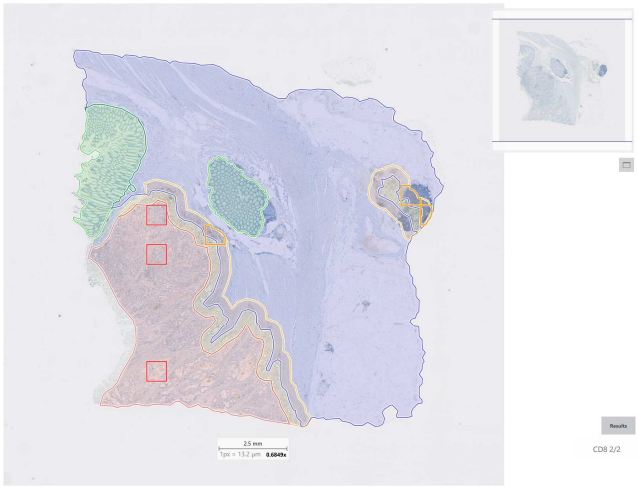
Tumor Cell
Tumor Infiltrating Lymphocyte CD8+
Macrophage
B lymphocyte
Regulatory T Cell
Antigen Presenting Cell
Collagen Fibres
Capillaries
TLS Tertiary Lymphoid Structures

Figure 3.

CD3



CD8



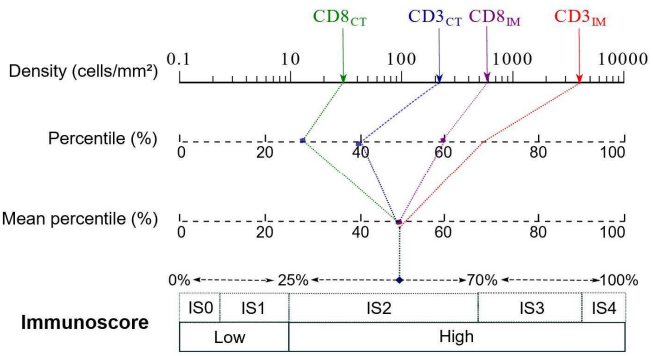


Table 1. Immune scoring systems: methodological aspects.

First author [reference]	Year	Primary	Tumor stage	Patients	Markers	Material	Tumor region analyzed	Technique
Pagès [13]	2005	CRC	I-IV	959	CD45RO	TMA (n=415)	NA*	Image analysis
Galon [12]	2006	CRC	I-IV	603	CD3/CD8/CD45RO	TMA	CT/IM	Image analysis
Pagès [14]	2009	CRC	I-II	623	CD8/CD45RO	TMA	CT/IM	Image analysis
Mlecnik [27]	2011	CRC	I-IV	599	CD3/CD8/CD45	TMA	CT/IM	Image analysis
Allard [17]	2012	CRC	II-III	117	CD3/CD45RO	WTS	IM	Image analysis
Anitei [18]	2014	RC	I-IV	166	CD3/CD8	TMA/WTS (biopsies)	CT/IM	Image analysis
Tanis [24]	2015	CRC	IV	82	CD3/CD8	WTS*	CT/TNI**	Image analysis
Kwak [19]	2016	CRC	IV	196	CD3/CD8/CD163	TMA**	CT/IM	Image analysis
Mlecnik [20]	2016	CRC	I-IV	964	CD3/CD8/CD45	TMA	CT/IM	Image analysis
Park [25]	2017	CRC	III	331	CD3/CD8	WTS	CT/IM	Semi-quantitative
Emile [21]	2017	CRC	III	1220	CD3	WTS	CT/TNI**	Image analysis
Yoon [22]	2018	CC	III	561	CD3/CD8	WTS	CT/IM	Image analysis
Mlecnik [23]	2018	CRC	IV	153	CD3/CD8; CD8/CD20	WTS*	CT/IM	Image analysis
Pagès [16]	2018	CC	I-III	2681	CD3/CD8	WTS	CT/IM	Image analysis

CC, colon cancer. CRC, colorectal. CT, center of the tumor. IM, invasive margin. NA, not available. RC, rectal cancer. TMA, tissue microarray. TNI, Tumor normal interface. WTS, whole-slide serial tissue section.

* On primary tumor and liver metastases. ** On liver metastases.

Table 2. Selected studies of immune scores in the colorectal cancer adjuvant setting.

First author Year (reference)	Tumor stage (patients)	Markers	Prognostic and predictive impact
Prall 2004 (26)	III (152)	CD8, MSI	- CD8+ associated with better OS (HR 0.43; p=0.009) and DFS (HR 0.56; p= 0.047) in MVA - CD8+ high/MSI associated with better OS (HR 0.65; p=0.023) in MVA
Pages 2005 (13)	I-IV (959) (I-III: 750)	CD45RO	- IS associated with VELIPI-negative tumors in MVA - CD45RO+ associated with better DFS (53.2 vs 20.6 mo, p<0.001) and OS (36.5 vs 11.1 mo, p<0.001) - CD45RO+: independent prognostic factor for OS (p=0.02) in MVA including T, N and M
Galon 2006 (12)	I-IV (603) (I-III: 453)	CD3, CD8, CD45RO	- $CD3_{CT}$/$CD3_{IM}$, $CD8_{CT}$/$CD8_{IM}$, $CD45RO_{CT}$/$CD45RO_{IM}$ (separated or combined) associated with better DFS and OS overall and in stage I-III - $CD3_{CT}$/$CD3_{IM}$: independent prognostic factor for DFS and OS in MVA including T, N and grade
Pages 2009 (14)	I-II (623)	CD8, CD45RO	- $CD8_{CT}$/$CD8_{IM}$, $CD45RO_{CT}$/$CD45RO_{IM}$ (separated or combined) associated with better DFS and OS - $CD8_{CT/IM}$, $CD45RO_{CT/IM}$: independent prognostic factors of DFS and OS in MVA including T and bowel perforation
Ogino 2009 (28)	I-IV (843) (I-III: 789)	TIL, lymphocytic reaction*	- TIL associated with better CRC-specific survival in UVA but not in MVA - Lymphocytic reaction associated with better 5-yr CRC-specific OS (p=0.002) and OS (p=0.002) in MVA including N, KRAS/BRAF status, MSI, CIMP and other clinical-pathological factors[#]

Nosho 2010 (18)	I-IV (768) (I-III: 619)	CD3, CD8, CD45RO, FOXP3	- MSI independently associated with CD45RO+ - Density of CD8+, CD45RO+ and FOXP3+ associated with OS in UVA - CD45RO+ (but not CD3+, CD8+ and FOXP3+) associated with CRC-specific OS (p=0.0032) and OS (p=0.0036) in MVA including KRAS/BRAF status, MSI, CIMP and other clinical-pathological factors[£]
Mlecnik 2011 (27)	I-IV (599)	CD3, CD8, CD45RO,	- CD8+ associated with more frequent Tis/T1 stage tumors In stage I-III CRC: - IS ($CD3_{CT}$/$CD3_{IM}$, $CD8_{CT}$/$CD8_{IM}$, $CD45RO_{CT}$/$CD45RO_{IM}$) associated with better DFS and OS (p<0.005) in UVA - IS and bowel perforation: the only prognostic factors in MVA (including T and N, age, sex, total number of lymph nodes, grade, mucinous colloid type, occlusion and bowel perforation)
Allard 2012 (17)	II-III (117)	CD3, CD45R0 (IM)	- T, N, LN ratio, VE, PNI, CD3+ associated with better DFS in UVA - CD3+ (HR: 6.02; p<0.001) and metastatic LN ratio: independent prognostic factors for DFS in MVA
Mlecnik 2016 (20)	I-IV (964)	CD3, CD8, CD45RO	- High IS (high $CD8_{CT/IM}$ $CD45RO_{CT/IM}$ infiltration) increased in MSI tumors (p<0.02) but ~50% of MSS with a high IS - High IS associated with better DSS (HR 6.21; p<0.001), DFS (HR 6.35; p<0.001) and OS (HR 3.96; p<0.001) - Data confirmed regardless of MMR status: MSI and MSS with a low IS associated with a higher risk of relapse, shorter DSS and OS compared to high IS

			- IS and VELIPI: the only prognostic factors in MVA (including T, N, sex, VELIPI, tumor grade, mucinous-colloid type, occlusion, perforation and MSI)
Emile 2017 (21)	III (1220)	CD3	- IS more frequently high in MSI tumors (32% vs 22%, p=0.04) - High IS associated with lower 2-yr TTR (14% vs 21% p=0.02) and better 3-yr DFS (81% vs 72%; HR 0.69; p=0.01) and 5-yr OS (89% vs 80%, HR 0.58; p=0.0048) - In MSS (but not in MSI) tumors, high IS associated with better 3-yr DFS (80% vs 72%, HR 0.70; p=0.022) and 5-yr OS (89% vs 79%, HR 0.58; p0.008) - IS independent prognostic factor for OS in MVA (HR 0.6, p=0.04), as well as stage, grade, RAS/BRAF
Pages 2018 (16)	I-III (2681) (II: 1434)	CD3, CD8	- High IS associated with lower 5-yr risk of recurrence compared to intermediate and low IS (8% vs 19% vs 32%; HR 0.20; p<0.0001) - IS: the only independent prognostic factor for TTR (p<0.001) in MVA including age, sex, T, N, MSI and other prognostic factors - In stage II: IS associated with lower risk of recurrence, better DFS and OS in MVA including T, N, age, sex and MSI
Pages 2020 (32)	III (1062)	CD3, CD8	<u>Prognostic impact</u> - IS 2 groups (low, Intermediate + high) (HR=1.54; p<0.0001) and IS 3 groups (low, intermediate, high) (HR=1.67 and HR=2.42; p<0.001) associated with DFS - IS 2 groups associated with DFS (p<0.0001) in both low-risk (T1-3N1) and high-risk stage III (T4 or N2)

	- IS: independent prognostic factor in MVA including T, N, sex, and MSI <u>Predictive impact</u> - High/intermediate IS: 6-mo adjuvant CTx (FOLFOX) associated with better DFS overall (HR 0.53; *p*=0.0003) and in low-risk (HR 0.47; *p*=0.01) and high-risk tumor patients (HR 0.54; *p*=0.006) - Low IS: no difference in DFS between 3-mo and 6-mo groups of patients

All associations reported in the table are statistically significant.

CIMP, CpG island methylator phenotype. CRC, colorectal cancer. CT, center. CTx, chemotherapy. DFS, disease-free survival. DSS, disease-specific survival. IM, invasive margin. IS, Immunoscore. LN, lymph node. mo, month. MSI, microsatellite instability. MVA, multivariate analysis. OS, overall survival. PNI, perineural invasion. TIL, tumor infiltrating lymphocytes. TTR, time to recurrence. VE, vascular embolism. VELIPI, defined by the presence of vascular emboli, lymphatic invasion, and perineural invasion.

* Lymphocytic reaction includes 4 components: Crohn's -like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction and TIL (lymphocytes on top of cancer cells).

[#] Age, year of diagnosis, sex, family history of colorectal cancer, tumor location, tumor grade, p53, LINE-1 methylation.

£ Age, sex, body mass index, family history of colorectal cancer, tumor location, tumor grade, PIK3CA status and LINE-1 methylation.