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# Immunoscore and Microbiome in Colorectal Cancer: What's New?

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## Abstract

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death in the world, accounting for about 1.4 million new cases and almost 700,000 deaths in 2012. The clinical outcome and the tumor progression are now considered the result of a balance between the invasiveness of the tumor and the immune response of the patient against the tumor. The immune system has the ability to control and shape cancer through a mechanism called immunoediting, which include elimination, equilibrium, and escape. The consensus Immunoscore is a scoring system that outlines the density of CD3+ and CD8+ T-cell effectors existent in the tumor and its invasive margin. The pre-existing intra-tumoral immunity could be enhanced and activated by immunotherapy. Immunoscore could be a good prognostic marker, by identifying patients at high risk of tumor recurrence and stratifying patients who could benefit from adjuvant therapies. Human surfaces and cavities are populated by numerous microbial communities, and they play an indispensable role in human health, as they interact with the immune system. The authors made a literature revision concerning the role of Immunoscore and microbiome in colorectal cancer.

**Keywords:** colorectal cancer, Immunoscore, microbiome, diet, CD3+ T cell, CD8+ T cell

## 1. Colorectal cancer facts

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death in the world, accounting for about 1.4 million new cases and almost 700,000 deaths in 2012 [1]. In Portugal, the incidence of CRC is 42.80 new cases for 100,000 habitants with a mortality rate of 26.6 [2]. The distribution of CRC burden varies widely around the world, with more than two-thirds of all cases and about 60% of all deaths occurring in developed countries. The global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030 [1]. The lifetime risk of developing colorectal cancer is about 6%, which increased fourfold if there is a family history of CRC.

The multistep models of CRC tumorigenesis postulate an adenoma-carcinoma sequence as the main pathway to develop a cancer. It tells us that CRC arises from a benign precursor polyp that became dysplastic and invasive due to accumulative mutations [3].

There are several risk factors for CRC: inherited predisposition (the involvement of at least one first-degree relative doubles the risk, and the risk is even higher if the affected case was prior to the age of 60), obesity, total caloric intake, red meat, sedentary lifestyle and physical inactivity, alcohol consumption, and prolonged cigarette smoking. A low incidence of CRC is associated with high-fiber diet (it dilutes fecal carcinogens, decreases colon transit time, and generates a favorable luminal environment), fruits and vegetables, aspirin, and nonsteroidal anti-inflammatory drugs [4].

An anatomic shift is being observed once the incidence of right-sided or proximal cancer is rising. This shift is due to increased longevity, a response to luminal carcinogens and genetic defects like defects in mismatch repair genes with resulting microsatellite instability (MSI) in proximal colon cancers and chromosomal instability pathway (CIN) in left-sided colon cancer.

Since 2015, there are five consensus molecular subtypes (CMS) of CRC [5]. The CMS1 is the MSI-immune and accounts for 14% of the cancers. This subtype is characterized by proximal colon locations, high BRAF V600E mutation rate, hypermethylation of CpG islands which causes loss of tumor suppressor function, an association with an impaired DNA mismatch repair (MMR) system, an infiltration of immunogenic lymphocytes in the microenvironment, and MSI. MSI cancers are also considered “hypermuted” with approximately 47 mutations per 106 bases, compared to microsatellite stable (MSS or CMS2) tumors which average 2.8/106 bases. The clinical implications of this subtype are that early stage MSI tumors (most CMS1 cancers) have better prognosis than MSS cancers. Stage II cancers with MSI have a low recurrence rate and thus are generally not considered for adjuvant chemotherapy. Patients with stage III MSI tumors do not benefit from fluorouracil monotherapy but are responsive to combination fluorouracil, leucovorin, and oxaliplatin (FOLFOX) adjuvant chemotherapy. CMS1 tumors have a favorable outcome when detected before disease dissemination. In part, the good prognosis may be linked to the presence of specific T-cell populations: CD8+ cytotoxic T lymphocytes, CD4+ activated type 1 T helper cells (Th1), and natural killer cells. However, CMS1 tumors were associated with worse survival after relapse [5, 6]. The CMS2 is the canonical subtype and accounts for 37% of the cancers. This subtype is characterized by a low mutation rate. Five-year overall survival for all stages of CMS2 is the highest, and it has the highest survival rate after relapse. Additionally, CMS2 cancers were more commonly left-sided lesions (59%) [5, 6]. The CMS3 is the metabolic subtype and accounts for 13% of the cancers. This subtype is characterized by RAS mutations (68% of the cancers) which predict poor response to epidermal growth factor receptor (EGFR) monoclonal antibodies (e.g., cetuximab) [5, 6]. The CMS4 is the mesenchymal subtype and accounts for 23% of the cancers. This subtype is characterized by very high pro-inflammatory microenvironment. Additionally, they exhibit extremely low levels of hypermutation and are MSS status. CMS4 cancers, often diagnosed at advanced stages, have a poor prognosis with the worst 5-year overall survival (62%) and relapse-free survival (60%) of any molecular subtype. Although standard adjuvant therapy (FOLFOX) for stage III is recommended, CMS4 cancers show no benefit from systemic adjuvant treatments.

For metastatic disease, CMS4 cancers are resistant to anti-EGFR therapy, independent of RAS mutation status. Anti-angiogenesis therapies such as bevacizumab are standard additions for stage IV disease [5, 6]. Finally, the last subtype is the mixed features and accounts for 13% of the cancers [5, 6].

There are four stages of colon cancer considering their size, number of lymph nodes, and distant metastasis (TNM). Stage 1 comprehends the T1 and T2 tumors (extension to submucosa and muscularis propria), and the treatment is only surgical. Stage 2 englobes the T3 and T4 tumors (subserosa, invasion of visceral

peritoneum and organs), and the treatment depends if the patient is considered of low or high risk. The low-risk patients only do surgery; the high-risk patients have 1 of the following criteria: less than 12 lymph nodes resected; low differentiated tumor; vascular, lymphatic, or perineural invasion; perforation or intestinal obstruction; T4 tumor; and MSS status. The high-risk stage II and stage III (with lymph nodes positive for disease) patients are submitted to adjuvant chemotherapy after surgery with fluoropirimidine and oxaliplatin. Stage IV cancer is a metastatic disease, which could be resected if feasible or controlled with chemotherapy.

## 2. What is an Immunoscore?

The clinical outcome and the tumor progression are now considered the result of a balance between the invasiveness of the tumor and the immune response of the patient against the tumor. The immune system has the ability to control and shape cancer through a mechanism called immunoediting, which include elimination, equilibrium, and escape [7].

It was already shown that the strength of the in situ adaptive immune reaction is strongly correlated with time to recurrence and overall survival of CRC [8]. This in situ immune cell infiltration in cancer, called high density of tumor-infiltrating lymphocytes (TIL), is associated with a favorable prognostic effect [8]. Once cancer becomes clinically detectable, the adaptive immune response plays a critical role in preventing tumor recurrence, metastization, and clinical outcome. A protective response is maintained by the ability of memory T cells to recall previously encountered antigens [9]. Concerning to regulatory T cells (Tregs), Sinicrope et al. showed an association between a low CD3<sup>+</sup>/FoxP3<sup>+</sup> cell ratio and shorter survival [10], but Salama et al. showed the opposite, a high Treg density in the tumor was associated with improved survival [11]. Regarding TH17 and TH1 immune response, TH17 is associated with poor prognosis [12], and TH1 is associated with prolonged disease-free survival [13].

The consensus Immunoscore is a scoring system that outlines the density of CD3<sup>+</sup> and CD8<sup>+</sup> T-cell effectors existent in the tumor and its invasive margin. The pre-existing intra-tumoral immunity could be enhanced and activated by immunotherapy. Immunoscore could be a good prognostic marker, by identifying patients at high risk of tumor recurrence and stratifying patients who could benefit from adjuvant therapies [14]. This score is based in the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO), in density (Cells/mm<sup>2</sup>) and the location (in the core of the tumor or in the invasive margin) [15]. The score ranges from Immunoscore 0 (I0) when low densities of both cell types are found in both regions to Immunoscore 4 (I4) when high densities are found in both regions.

## 3. Clinical applications of Immunoscore

Immunoscore has been tested to be a prognostic marker that surpasses the TNM staging. Pages et al. concluded that patients with high Immunoscore had the lowest risk of recurrence and longest survival. In his study, only 5% of the patients with high Immunoscore had a recurrence at 3 years, 87% of the patients reached the overall survival at 3 years, and 82% of the patients reached 5-year overall survival [14].

There is a possible association between MSI status and immune cell infiltrates. MSI-high tumors have intraepithelial T cells due to expression of neo-antigens on the cell surface, and this could be the reason why this kind of tumors had better prognosis [15].

Comparing to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) TNM classification system, the Immunoscore classification seems to be superior as prognostic tool. For all patients with CRC stages I/II/III, multivariate Cox analysis revealed that the immune criteria remained highly associated with prognosis [16]. Wirta et al. concluded that a lower Immunoscore was associated with increasing AJCC/UICC stage, as well as with increasing T stage, presence of lymph node, distant metastasis, and perineural or lymphovascular invasion [17].

One day the classification of cancer will have a new component, TNM-I (immune).

Additionally, Immunoscore can predict the response to treatment and could be a biomarker that helps clinicians to decide what patients must have chemotherapy. Morris et al. concluded that high TIL is predictive of response to chemotherapy with 5-fluorouracil [18], and Viaud et al. revealed that cyclophosphamide induces a TH17 and TH1 antitumor response, making the tumors resistant to this chemotherapy [19].

#### 4. What is a microbiome?

Human surfaces and cavities are populated by numerous microbial communities, like bacteria or fungi, which form a complex interactive network between themselves and the host. The gastrointestinal microbiota is estimated to contain over 1000 different phylotypes with a microbial gene catalog of 3.3 million genes [20], but it can be divided into four main categories: *Firmicutes*, *Bacteroides*, *Actinobacteria*, and *Proteobacteria* [21]. These agents play an indispensable role in human health, as they interact with the immune system, maintain epithelial homeostasis, metabolize indigestible polysaccharides, modulate the intestinal motility, regulate the luminal pH, and exclude potential pathogens from the human gut [22]. The disruption of intestinal microbial equilibrium has the capacity to alter the homeostatic network, thereby eliciting deleterious host responses as observed in inflammatory bowel disease and CRC. The dysbiosis refers to perturbations in microbial populations [23].

Wong et al. proved that intestinal microbiota has an important role in CRC carcinogenesis. Mice fed with stool from patients with CRC had a higher rate of high-grade dysplasia than the mice fed with stool from healthy controls, suggesting that human commensals may not be tumorigenic [24]. Bacteria may contribute to CRC in several ways: they can break the mucus layer and adhere to intestinal mucosa and deliver virulent proteins and molecules that will initiate oncogenic signaling in epithelial cells. As so, they can induce DNA damage leading to tumor initiation. On the other hand, bacteria can trigger procarcinogenic signaling and inflammatory microenvironment, such as IL-17 production or excessive Wnt or Stat3 signaling [25].

Some studies have identified several bacteria that can promote carcinogenesis by different mechanisms: *Escherichia coli* can cause direct DNA damage such as crosslinks and double-strand breaks due to the colibactin toxin produced by it [26], *Fusobacterium nucleatum* can produce FadA adhesin to modulate E-cadherin/beta-catenin signaling [27], *Peptostreptococcus anaerobius* can induce cell proliferation through toll-like receptor 2 and toll-like receptor 4 pathways [28], *Bacteroides fragilis* produces a toxin that activates Wnt and NF- $\kappa$ B pathways which induce a pro-inflammatory state [29], and *Streptococcus gallolyticus* induces tumor growth through enhancement of inflammatory signals including cyclooxygenase-2 [30].

Xu et al. compared normal tissue with adenomas and adenocarcinomas and concluded that the microorganisms are different between the three entities. In the

cancer group, 20 biomarkers were identified: *Bulleidia*, *Catonella*, *Clostridium*, *Dialister*, *Granulicatella*, *Lactobacillus*, *Mogibacterium*, *Oscillospira*, *Parvimonas*, *Peptostreptococcus*, *Streptococcus*, *Odoribacter*, *Paraprevotella*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Leptotrichia*, *Campylobacter*, *Desulfovibrio*, and *Treponema* [31].

Even in the same individual, there are differences between normal and disease tissue sites. One study compared cancerous tissue with matched healthy tissue, and the microbial diversity was significantly lower in tumor tissue, suggesting a more selective microenvironment in proximity to diseased tissue [32].

## 5. Clinical applications of microbiome analysis

Some studies establish a relationship between the microbiota and the cancer therapy efficacy. Iida et al. showed that microbiota leads to enzyme expression required for optimal chemotherapy activity with oxaliplatin [18]. Guthrie et al. demonstrated that inhibition of microbial  $\beta$ -glucuronidase increases the adverse effects of irinotecan in some patients [33]. Concerning immunotherapy, a great number of bacteria were observed having great clinical response to immune-checkpoint therapy (by activating CTLA-4 and PD1 expression or promoting T-cell proliferation) [34].

Nevertheless, the main application nowadays is the fecal microbiota transplantation. This procedure consists in the administration of fecal bacteria from a healthy donor (without cancer, an autoimmune or metabolic disease) to a recipient by enema, colonoscopy, or enteric tube. The main objective is to alter the recipient's microbiota composition, and it is performed in a variety of diseases like *Clostridium difficile* infection, irritable bowel syndrome, inflammatory bowel diseases, obesity, multiple sclerosis, and type 2 diabetes mellitus [35]. Unfortunately, the lack of evidence and clinical trials bounds their use in clinical practice of oncologic patients.

Another potential application is through oral probiotics. Probiotics are supplements with live bacteria that promote gut health. Some experimental models presented a reduction rate of colorectal cancer development with their consumption [36].

## 6. Diet

Some dietary compounds may reach the colon by several reasons: they could be too large to be absorbed in the small intestine, they could escape the deglycosylation and absorption in the small intestine, and they could not be accessible to the host due to the mixture of food. The dietary compounds that are absorbed in the small intestine could reach the colon by enterohepatic circulation [37]. Dietary bioactives can modify the carcinogenic process in several ways: by a direct or microbe-independent pathway and by an indirect or microbe-dependent pathway that include modifications in the substrates that alter the colonic microbiota or their metabolites [38]. The dietary fiber goes through the small intestine into the cecum and proximal colon where they are metabolized by the colonic microbiota and short-chain fatty acids are produced [39]. The most abundant short-chain fatty acids in the colon are acetate, propionate, and butyrate, and their concentrations typically decrease from the proximal to the distal colon [40]. The advantages from consuming fibers are the dilution of carcinogens and potential tumor promoters in the intestinal lumen [41] and the fast passage of the digesta through the colon which minimize the exposition to toxic products and increase the levels of short-chain fatty acids in the distal colon [42].

Butyrate is the preferred substrate of colonocytes [43]. This compound was incredibly studied due to its capacity to reduce oxidative stress, diminish inflammation and carcinogenesis, and support colonic barrier function [44].

The diet can shape the colonic microbiota and their function, and, on the other hand, the microbiota influences the health of the intestine. This way the host is protected from colon cancer and other inflammatory diseases.

## **7. Conclusions**

CRC is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death in the world. There are five CMS of CRC: the MSI-immune, the canonical subtype, the metabolic subtype, and the last subtype with mixed features. The strength of the in situ adaptive immune reaction is strongly correlated with time to recurrence and overall survival of CRC which leads us to think that the immune system plays an important role in CRC development. The consensus Immunoscore is a scoring system that outlines the density of CD3+ and CD8+ T-cell effectors existent in the tumor and its invasive margin, and the authors believe that this score could be a good prognostic marker. On the other hand, the dysbiosis could be combined with Immunoscore to increase the power of the biomarker. Studies about this interaction are needed. The role of microbiota in colorectal cancer is complex: its disturbance is the cause of tumorigenesis, or the outcome of tumor development is still uncertain.

## **Conflict of interest**

The authors have no conflict of interest.

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