Retraction Notice

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Neutrophils in Rectal Cancer—A Review: Stage III Rectal Cancer Patients and 5-Fluorouracil Therapy. Introducing New Inclusion Criteria and Scoring System (Presenting the "Sarandria Score")

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Abstract

This review is intended to shed new light on the role of neutrophils in colorectal cancer and in the meanwhile emphasize the differences between rectal and colon cancer, strengthen and highlight the possibility of a clinical prognostic and predictive scoring (Sarandria Score). A novel scoring system described in this review can be used as inclusion criteria and as a predictive and prognostic scoring for stage III rectal cancer patients. **Background:** Colorectal Cancer (CRC) is a major public health problem, representing the third most commonly diagnosed cancer in males and the second in females. Various studies have reported relevant differences related to CRC primary location site (right-sided colon, left-sided colon, rectum) including response to adjuvant chemotherapy and prognosis. In stage III CRC patients, previous findings showed that higher density of tumor-associated neutrophils (TANs) was associated with better response to 5-FU-based chemotherapy. **Main topics:** In this review, the current knowledge status on the role of neutrophils in colorectal cancer is assessed, including novel finding discovered by Dr. Nicola Sarandria on the role of neutrophils in rectal cancer. It includes different factors which point to an anti-tumoral role of neutrophils in rectal cancer when in presence of chemotherapeutic agents (such as 5-fluorouracil). The clinical significance of TANs was assessed and whether it can be different depended on the location of the primary CRC (right-sided colon, left-sided colon, rectum). **Conclusions:** This review officially highlights the possibility of a new clinical prognostic and predictive scoring (Sarandria Score) involving intratumoral neutrophilic infiltration in rectal cancer and the possibility of a new

inclusion criterion based on this infiltrate for Stage III rectal cancer patients treated with 5-FU therapy. This review includes knowledge from data published on my medical degree thesis showing that higher levels of TANs densities were associated with better disease-free survival (DFS) in 5-FU treated patients affected by rectal cancer (while it was inversely related in patients without 5-FU therapy). This is also further evidence in support of a possible conceptual division of what is now known as colorectal cancer into two separate entities: colon and rectal cancer.

Keywords

Rectal Cancer, Neutrophils, 5-Fluorouracil, Neutrophils and Cancer, Colorectal Cancer

1. Introduction to Colorectal Cancer

The etiopathological origin of colorectal cancers is the following: 70% to 80% of colorectal cancers are sporadic, 15% - 20% are familial and 5% - 10% are hereditary [1]. In the colon adenocarcinoma genesis, three main etiopathogenic pathways have been described. Parts of these etiopathogenic pathways include: mutation in the APC gene, chromosomal instability and defective mismatch repair system (MMR). Regarding hereditary CRC, the main autosomal dominant syndromes are the Familial Adenomatous polyposis (FAP) and Lynch Syndrome while autosomal recessive syndromes include MUTYH Associated Polyposis (similar phenotype to attenuated FAP, with presence of more than 10 but lower than 100 polyps).

Focusing on sporadic colorectal cancer, one of the pathways includes the inactivation of the tumor suppressor genes APC, which together with the inactivation of p53 and activation of the oncogene Kirsten-ras (Kras) are vital determinants of tumor initiation and progression [2]. The p53 gene, localized on the short arm of chromosome 17, mutates in up to 70% of colorectal cancers [3]. The development of colorectal cancer can be attributed to at least three patterns of instability. Typically, one type will predominate in a specific cancer. Chromosome instability (CIN), which results in genetic deletions, duplications, and chromosomal rearrangements, is one of the most common patterns; it is relevant in at least 50% of colorectal cancer cases. Colorectal cancers (CRCs) with CIN are distinguished by the presence of tumoral cells with aneuploidy. CIN results in genetic defects in genes such as APC, TP53, TGF-β, PIK3CA, EGFR, BRAF, and KRAS [4].

Another pattern of instability in the development of colorectal cancer is microsatellite instability (MSI), which arises in about 15% of CRCs, particularly right-sided CRCs. Microsatellites are simple repetitive DNA sequences one to six base pairs in length that occur various times throughout the genome. Inactivation of the DNA mismatch repair (MMR) system induces MSI, which results in

sequences that form errors and become unusually different in length. In some instances, frame shift mutation occurs in a gene, such as a tumor suppressor gene. Lynch syndrome is the cause of 90% of MSI mutated CRCs. An epigenetic change that requires the methylation of promoters of human genes, particularly within the CpG islands gene, is the third pattern of instability observed in colorectal cancer. These epigenetic changes can result in the silencing of certain tumor suppressor genes in CRC.

The adenocarcinoma is a type of carcinoma—a malignant neoplasm originating from epithelial cells—specifically from glandular epithelium. This type of epithelium is present in exocrine organs (e.g. Pancreas) or in other organs containing secretive epithelial tissues (e.g. Colon). Like other type of malignant neoplasms, the adenocarcinoma is a result of a complex system of mutations and changes in the tissue of origin—a process called oncogenesis.

Eight common hallmarks of cancer have emerged in the last years which explain the characteristics that cells obtain in order to transform into neoplastic malignant cells.

Infections, inflammation and genomic instability all have often a role in the process of carcinogenesis. For instance, infection by Helicobacter Pylori is considered the strongest risk factor for the adenocarcinoma of the stomach.

Furthermore, the process of "epithelial-mesenchymal transition" (EMT), has been shown as being an important mean by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to [5], and therefore become malignant. In the case of colorectal cancer, a particular genetic pathway of carcinogenesis has been proposed to explain the transition from healthy colonic epithelia and increasingly dysplastic adenoma to malignant cancer. This model highlights numerous relevant oncogenes and tumor suppressor genes, which cause the adenoma to undergo carcinoma transition [6]. Adenomatous polyposis coli (APC) and DNA mismatch repair (MMR) genes have been identified in this pathway [7]. This carcinogenic process is further enhanced in abnormal states, in which the mucosa of the colon must also be able to repair mucosal integrity. This includes the reversible ulcer-associated cell lineage, metaplasia (irreversible constitutive change in phenotype), dysplasia (abnormal differentiated phenotype) and neoplasia (abnormal cell number and abnormal differentiated phenotype).

An abnormal pattern of cell replication has been detected under several clinical conditions associated with an increased risk of colorectal malignancies. Thus, adenoma formation is induced in two main scenarios. In sporadic colonic adenomas, the initiating event appears to be a loss of heterozygosity (LOH) at the APC gene, followed by a second hit in the APC gene. In one model, namely the "topdown" model, mutant cells appear in the intra-cryptal zone between crypt openings. Only as the clone expands does it penetrate into the crypt. When dissecting these tumors, only the superficial cells show intense staining for β-catenin as evidence of a dysfunctional APC gene. Therefore, whereas the stem cell that is

the likely oncogenic precursor must have originated in the base or depths of the crypt, the polyp originates at the top of the crypt or in the space between the crypts. There are two possibilities regarding the "top-down" model. A new source of stem cells may emerge in the intra-cryptal zone. Thus, a terminally differentiated cell may mutate into a fully competent dividing cell. On the other hand, a cell derived from a mutated stem cell may migrate to that area and, with growth potential as a "second hit" in the intra-cryptal zone, expand from this location. More likely, a stem cell with a mutational defect in growth control will proliferate in the normal course of events, and cells pushed up to the intra-cryptal area will retain this mutation, as do all cells between the stem cell at the base of the crypt all the way up to the intra-cryptal area and beyond. Perhaps more intuitively, this can be referred to as the "bottom-up" model. Thus, only a small fraction of these lesions may evolve into malignancy, and there is evidence that a large majority (if not all) of colorectal carcinomas develop from adenomatous polyps [7]. Adenomas are well-demarcated lumps of epithelial tumor cells which can be classified into the following three major histological types: tubular, villous, and tubulo-villous adenomas. Adenomas, by definition, exhibit different grades of dysplasia and should be classified based on the portion with the most advanced grade. The word "dysplasia" is used to describe structural and cytological alterations in the epithelium that predispose an organ to cancer development. These abnormalities show varying degree of severity, which can be graded into "mild," "moderate," and "severe" atypia [8].

2. Differences between Rectal and Colon Cancer

In the last years, a new focus on CRC site localization has started. Different studies revealed not only anatomical, but also genetical, immunological, therapy-response and prognostic differences between colon cancer (in the right-sided colon—being the ascending colon vs. left colon—being the descending colon) and rectal cancers. 5-year survival rate for localized stage Colon Cancer is 90%, while the 5-year survival rate for rectal cancer is 69% [9]. Furthermore, many differences were found between Right-sided, Left-sided and Rectal CRC. For example, there were evidences which were found showing that in left-sided CRC and Rectal Cancers (RC), the mechanism of invasive cancers was skewed towards a progression of preexisting mucosal lesions, while in right-sided CRC a de-novo pathway was also implicated in the invasive cancers' generations [10]. The colon is approximately 1.5 meters in length, from the ileocecal valve to the anus. The difference between right-sided and left-sided colon is based on embryological origin: the right-sided colon includes the cecum, appendix, ascending colon, hepatic flexure, proximal two thirds of transverse colon, and all of these structures embryologically originate from the midgut. While the left-sided colon includes the distal one third of the transverse colon, the splenic flexure, the sigmoid colon, the descending colon and rectum, which all originate from the hindgut. These embryological differences also mean that CRC of different

sites have different histologies. Sessile serrated or mucinous adenocarcinoma for the right-sided CRC while tubular, villous and typical adenocarcinomas for the right-sided CRCs. Also increase immune infiltrate—seen as higher immunogenicity with higher T-cell infiltrate—is seen in right sided CRCs (high MSI in right-sided) [11]. While on left-sided cancer a more typical Chromosomal instability (CIN) path of cancerogenesis is present.

Other differences include response to therapy, better response to immune-therapy in right-sided CRC, age and sex differences—females and older age in case of right sided CRC—and metastases site, peritoneal for the case of right sided CRC, while liver and lung for left-sided CRC [12]. Furthermore, in the left-sided colon, scientific studies have shown rectal differences compared to the rest of the left-sided and colon and the right-sided colon area.

Topographically, the rectum is large bowel up to the edge of 16 cm from the anocutaneous line (AC line), where it is partly intraperitoneal and partly extraperitoneal, in the small pelvis (variations for the sexes also present). The lower part of the rectum includes particular mesorectal structure, fascia and both nerval and vascular anatomy, including internal iliac artery supply of the lower two thirds of the rectum vs. the inferior mesenteric artery supply to part of the left-sided colon, including sigmoid colon and upper third of rectum [12]. Other differences to take in consideration when talking about CRC location are regarding the microbiome and how it differs in the different parts of the colonic lumen.

An important note to discuss is also the embryological origin emphasizing the difference between colonic-rectal tissues. The Gastrointestinal (GI) tract development begins at the start of the third week of embryonic development (in the gastrulation phase, where the three germ layers begin to form—ectoderm, mesoderm and endoderm, which together form the embryonic disc). This embryonic disc has a cranial end (which will form the mouth) and a caudal end (which, with the cloacal membrane, will form the anus). In the fourth week, the primordial GI tract starts its formation from the endoderm, which creates a gut tube suspended by a mesentery (double peritoneum layer). The gut tube is divided then in foregut, midgut and hindgut. Foregut gives rise to the esophagus, stomach, liver, gallbladder, bile ducts, pancreas and proximal duodenum. The midgut develops into the distal duodenum, jejunum, ileum, cecum, appendix, ascending colon, and proximal 2/3 of transverse colon. The hindgut becomes the distal 1/3 of the transverse colon, descending colon, sigmoid colon and the upper anal canal [13].

The hindgut forms the distal one-third of the transverse colon, the descending colon, the sigmoid colon, and the rectum (also the superior portion of the anal canal). The hindgut's cloaca, forms also part of the urogenital tract (anal canal is form both ectoderm and endoderm—the pectinate line marks the junction). There are genes associated with the development of the Gastrointestinal tract, including the sonic hedgehog (in the endoderm—for mucosa, submucosa, and

muscolaris propria development). Also, the homeobox (HOX) genes and NODAL gene are important for GI tract development (possible role in sphincter formation and GI musculature, and cranio-caudal formation—genes expressed in mesoderm).

Anatomically, the rectum has further peculiar features. The layers of the rectum include the mucosa (which itself includes the simple columnar epithelial cells, lamina propria, and muscularis mucosa), submucosa, muscularis propria, and serosa.

Various treatments have been implemented against CRC, primarily including surgical resection, chemotherapy (adjuvant and neoadjuvant), and radiotherapy.

It must also be noted that the main agents for chemotherapy are therefore: 5-fluorouracil (mainly a thymidylate synthase (TS) inhibitor), folinic acid (aiding the activation of partial normal DNA replication processes), and oxaliplatin (cytotoxic effects).

3. Immune System Role in Colorectal Cancer

3.1 Tumor Microenvironment in Colorectal Cancer

Since Virchow's times, in 1863, inflammation has been associated with tumor genesis and progression [14]. Since then, chronic inflammation has become a key hallmark of cancer. The immunological tumor microenvironment is controlled and determined by tumoral cells interactions with immune cells. Immune cells and inflammatory cytokines fail to enact their antitumoral capabilities and may even promote tumoral growth and invasion. T regulatory cells and Myeloid Suppressor cells enrich the milieu, and together with a constant activation of NFkB, might provide a mechanism for tumor evasion from the immune system.

A great focus has also been put on immunity and its effect on therapy response and prognostic relevance in CRC. A great example of this is the Immunoscore, were sections of CRC from patients are taken and through a process of Immunohistochemistry (IHC), CD3+ and CD8+ T lymphocytes, both intratumorally and in the invasive margins of the sections, are stained. This type of score has been proven to be a reliable index of reoccurrence in CRC patients, and thus something worth considering as an addition to the TNM staging [15]. High immunoscore has been shown to correlate with prolonged survival and also differences in immunoscore have been revealed between CRC sites, namely, lower in right-sided CRC [16].

Thus, inflammation and immunity have become an important focus in cancer research. Not only adaptive immunity but also innate immunity. An example can be the Tumor-associated macrophages (TAMs) and their polarizations, including the M2 alternative phenotype which has been shown to be anti-inflammatory and pro-tumoral driven by IL-4 or IL-13 [17].

Innate immunity has also shown great importance in CRC, as in other solid tumors. For example, neutrophil granulocytes, together with macrophages, have been shown to be an important part of the tumor microenvironment and that

different interventions, such as VEGF, can modulate this environment through these cells. In fact, one of the parameters used as secondary biomarker for therapy success is the neutrophils-to-lymphocytes ratio [18]. Furthermore, higher intensity of Tumor Associated Neutrophils (TANs) have been correlated to better prognosis in CRC treated with 5-Fluorouracil (5-FU) [19].

3.2. Neutrophils in Rectal Cancer

Neutrophils are granulocytes, composing the 60% - 70% of the total white blood cells in the human body. Their role in the innate immune system has been considered as mainly present in the resistance against extracellular pathogens. However, recent studies have shown new aspects on the function of neutrophils. In the context of the tumor microenvironment, recent studies have demonstrated the prominent role of neutrophils in infiltrating tumor tissues to promote their growth, invasion, angiogenesis, and metastasis in various types of cancers, although they were initially considered to have a defensive function against tumor cells. As with Tumor Associated Macrophages (having anti-tumorigenic phenotype (m1) and pro-tumorigenic phenotypes (M2)), recent studies have suggested that Tumor Associated Neutrophils (TANs) also exhibit considerable plasticity and are capable of polarization into either an anti-tumorigenic N1 phenotype or a pro-tumorigenic N2 phenotype. Neutrophils are known to secrete several inflammatory, immunoregulatory, and angiogenic factors, including neutrophil elastase, matrix metalloproteinases (MMPs), and vascular endothelial growth factor (VEGF) which can exhibit paracrine effects on the tumor microenvironment. N1 neutrophils exhibit increased cytotoxicity and reduced immunosuppressive ability by the production of tumor necrosis factor (TNF)-α, intercellular adhesion molecule (ICAM)-1, ROS, and Fas and by decreasing arginase expression. In contrast, "N2" neutrophils support tumor expansion by expressing arginase, MMP-9, VEGF, and numerous chemokines including CCL2, CCL5 and CXCL4. The phenotype of TANs depends on the signals encountered in the tumor microenvironment (by molecules such as Interferon-β for N1 phenotype and TGF-β for N2 phenotype) [20].

The role of tumor-associated neutrophils (TANs) in primary tumor growth and metastasis is controversial. There is evidence that neutrophils promote primary tumor growth in mouse cancer models and have pro-tumorigenic effects by enhancing angiogenesis [21]. Furthermore, CD11b+ bone-marrow-derived cells, a heterogeneous myeloid cell population which includes neutrophils, have been associated with the priming of the premetastatic lung and the enhanced seeding of circulating tumor cells. By contrast, in a different study, an antitumor function of these cells has been observed following immunological [22] or cytokine activation [23], leading to activation and invasion of neutrophils in the tumor, together with an increase in surface contact zones between neutrophils and cancer cells [22]. Under these conditions, neutrophils can actively eliminate disseminated tumor cells [24], as they do in situations of TGF-β blockade in the

tumor microenvironment [25].

In the study of [19], the Density of CD66b+ cells were blindly assessed by immunostaining of histological sections at the invasive margin (IM) and in the intratumoral compartment (IT) followed by a computer-assisted measurement of the immunoreactive area. In the study, they studied the correlation between the chemotherapeutic agent 5-Fluorouracil (5-FU) and neutrophilic infiltrate together with survival (Disease Free Survival). What they found was that in 5-FU treated pts affected by CRC, patients who had high neutrophilic infiltrate (PMN High), had a statistically significant better DFS than the ones who had a low neutrophilic infiltrate. While, in the patients who did not receive 5-FU therapy, the inverse relation was found, namely that patients who had high neutrophilic infiltrate (PMN High), had a statistically significant worse DFS than the ones who had a low neutrophilic infiltrate.

In my years at Humanitas University as a medical student, I created one hypothesis and a research objective: to see whether TANs intratumoral (IT) density in Colorectal Cancer has different prognostic and/or predictive value according to tumor primary site location—left colon, right colon and rectum—and whether it differs according to presence or not of adjuvant chemotherapy 5-FU. My hypothesis was that neutrophils (of the N1 polarization) could indeed help the prognosis of the patient under 5-FU (also in accordance with the different publications showing neutrophilic role in the 5-FU action and therapeutic cycle). In the following text I will describe my research and findings (as published in my thesis).

The study type was a retrospective one—A study that compares two groups of people: those with the disease or condition under study (cases) and a very similar group of people who do not have the disease or condition (controls) (cancer.gov definition of retrospective study) with the focus of finding a correlation between IT PMN in CRC and predictive or prognostic values. The study attempted to correlate polymorphonuclear neutrophil (PMN) tumoral infiltration (TANs) with CRC site in the presence or absence of adjuvant therapy. It was inquired whether CRC site—whether it was right-sided, left-sided or rectal—had any effect on TANs density (intratumoral density) and whether these densities had any kind of prognostic value be it in presence of adjuvant therapy or without any adjuvant therapy. According to previous publications, it was revealed that TANs were correlated with positive prognosis when the patients (pts), with Stage III CRC, underwent 5-fluorouracil as adjuvant therapy [19]. Also, it has been revealed that low TANs density in Stage II CRC, could be an index for adjuvant chemotherapy usage. This study focuses on finding whether this correlation holds for the aforementioned CRC sites and whether the pattern is the same for all of them including focusing on the group of pts who did not underwent adjuvant therapy [26].

The initial hypothesis was that different site of CRC—right-sided, left-sided or rectal—may hold different values of TANs densities with different prognostic features. This assumption was based on the various differences that these anatomical locations have amongst them such as: different microbiome, different

mechanical strains due to different intraluminal fecal consistencies from the cecal area to rectal area, area where maximum water reabsorption occurred in normal physiological process. Also, differences in embryological origin and mutational background, e.g. higher MSI mutational burden in right-sided CRC [11].

Therefore, the principal aim of the study, the research objective, was to find whether these TANs densities (intratumoral) in different CRC sites have any prognostic relevance, in either the pts treated with adjuvant therapies and non-treated ones.

The reason for the study is a need for new prognostic markers for one of the commonest solid tumors in the world, namely the CRC. Also, the importance of understanding differences between CRC having as primary location different sites—focusing on right-sided colon, left-sided colon and rectum—could aid in the future adjuvant therapy selection for different classes of pts. In addition, whether TANs intratumoral density could be not only a prognostic marker but also a predictive marker was also inquired, by checking high TANs in adjuvant therapy pts and the efficacy of the therapy measured by survival parameters. As adjuvant therapy, 5-Fluorouracil was the therapy which was considered in the Galdiero *et al.* study, where a statistically significant correlation was found between patients treated with 5-FU with high intratumoral TANs vs those with low intratumoral TANs densities, namely pts with high TANs had a more favorable prognosis. In the study 178 pts were taken in considerations, 52 did not receive any adjuvant therapy while 126 received 5-FU as adjuvant therapy. All the patients (pts) were Stage III and Microsatellite Stable (MSS). Using the raw data, a multivariate analysis was performed to reach the objective of the study. Furthermore, as reported in a previous study, a new set of pts were taken to assess whether these TANs densities correlations with different CRC anatomical sites and prognosis in pts with 5-FU as adjuvant therapy existed with different types of adjuvant therapies, such as FOLFOX—made up of Folinic Acid (leucovorin—a molecule aiding in normal DNA replication—folate based DNA replication when high dose Metrotrexates, but increase cytotoxicity of 5-FU), 5-FU (a thymidylate synthase (TS) inhibitor), Oxaliplatin (a cytotoxic compound) or FOLFIRI 5-FU, Folinic Acid, Irinotecan (a topoisomerase inhibitor preventing DNA replication by blocking its unravelling) [26]. Therefore, the research objective of the study was to see whether TANs intratumoral (IT) density in Colorectal Cancer has different prognostic and/or predictive values according to tumor primary site location—left colon, right colon and rectum—and whether it differs according to presence or not of adjuvant chemotherapy 5-FU.

To inquire whether TANs Intratumoral (IT) densities had predictive and/or prognostic value in regards to CRC site-left sided, right sided or rectal in pts who underwent or not adjuvant chemotherapy with 5-FU, the raw dataset of patients (Stage III colorectal cancer patients) used in the Galdiero *et al* [19] study from our lab was used. In the dataset, Intratumoral (IT) TANs were measured by Immunohistochemical process, labelling neutrophils via CD 66b

+ Ab labelling. While the analysis on that study focused on CRC and presence or not of 5-FU, here the focus of the analysis shifted to the three aforementioned CRC sites with the presence or not of Adjuvant therapy. Therefore, a different analysis with different parameters was performed on the set of pts mentioned before.

The primary sites of CRC which were taken in consideration where therefore three: Right-sided colon, Left-sided colon (not including rectum) and Rectum.

For 5-FU treatment the CRC site which was correlated to Intratumoral neutrophils in regards to Disease Free Survival of patients, was the rectum. The data revealed that High IT PMNs were strongly correlated to better DFS in 5-FU1 patients with rectum located CRC, compared to those with Low IT PMNs. This relationship was inversed in regards to 5-FU0 patients (High IT PMNs = Worst Prognosis).

In the data published in my thesis, a univariate analysis was also performed regarding the significance of IT TANs (PMNs) in rectal-cancer patients as shown in **Table 1**, where it can be seen that in patients with rectal cancer (stage III), there is a strong statistically significant better DFS in 5-FU treated patients having high IT PMNs (polymorphonuclear) density (HR: 0.06, p-value: 0.003) compared to patients with low IT PMNs density.

The data therefore suggest a possible predictive and prognostic value for IT PMNs (TANs) to be used in the clinical practice in patients with Stage III rectal cancer, where high Tumor Associated Neutrophils in the intratumoral histological section could be an inclusion criteria for 5-FU based adjuvant chemotherapy while a low value could be could be an exclusion criteria for this therapy (see **Figure 1**) and where high IT TANs in 5-FU treated patients could be a positive predictive and prognostic indicator for Disease Free Survival (DFS). In fact, it was also observed how high IT PMNs were associated with a worst DFS in patients with stage III rectal cancer but not treated with 5-FU (this possibly due to criteria of the patients.

This was confirmed also when dividing in terciles to search for linearity (**Figure 2**). Figures and tables published on my thesis in 2020 [27].

For the patients with rectal cancer, I wanted to check also if the same association between IT PMNs density and DFS was present when looking at survival (DSS: Disease Specific Survival). See **Figure 3**.

Table 1. Rectal cancer and IT PMNs.

Rectal cancer	Non-Treated Patients Univariate analysis HR (95% CI) p-value	5-FU treated patients Univariate analysis HR (95% CI) p-value
IT PMNs Density	1.00 Ref.	1.0 Ref.
<1.197	3.5 (0.81 - 15)	0.06 (0.0005 - 0.45)
≥1.197	0.11	0.003*

a. statistical test used: Firth's Method.

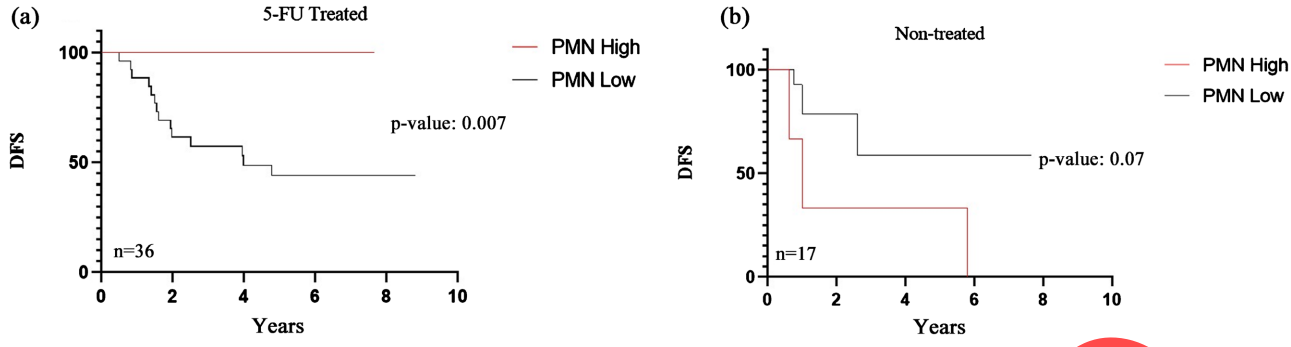

Figure 1. Prognostic significance of CD66b in patients with Stage III CRC located in the rectum. Kaplan-Meier survival curves show DFS (a,b) for patients presenting a high or low density of neutrophils (PMNhigh or PMNlow, respectively) in the IT (intratumoral). (a) Shows DFS amongst IT PMN high vs. PMN low ones amongst 5-FU treated patients, n = 36, p-value: 0.007. (b) Shows DFS amongst IT PMN high vs. PMN low ones amongst non-treated patients, n = 17, p-value: 0.07. Upper quartile values were employed to divide tumors into high and low CD66b+ immunoreactive area. The p-value was found using the Log-rank test.

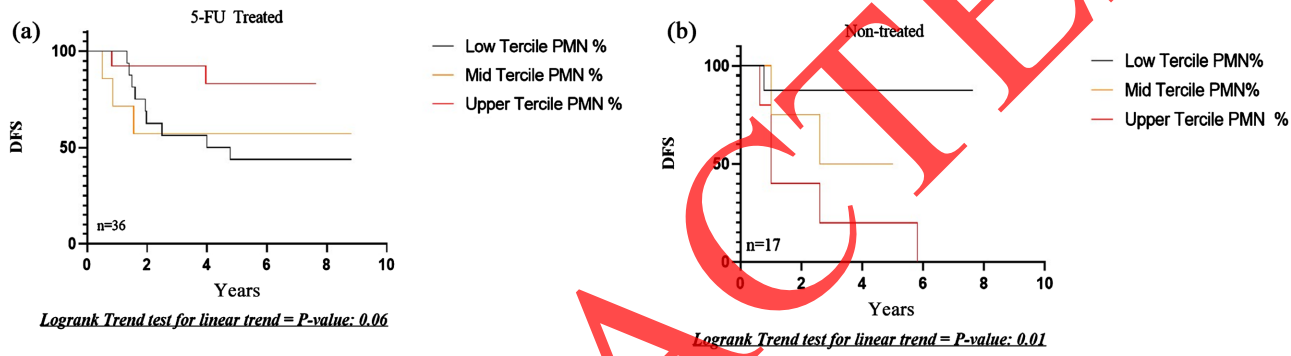

Figure 2. Prognostic significance of CD66b in patients with Stage III CRC located in the rectum. Kaplan-Meier survival curves show DFS (a,b) for patients presenting levels of PMN according to 3 terciles (low, mid, and upper terciles of IT PMN density) in the IT (intratumoral). (a) Shows DFS amongst 5-FU treated patients, n = 36, p-value for linear trend: 0.06. (b) Shows DFS amongst non-treated patients, n = 17, p-value for linear trend: 0.01. The Logrank Trend test was used to find the linear trend between the survival curves in each graph.

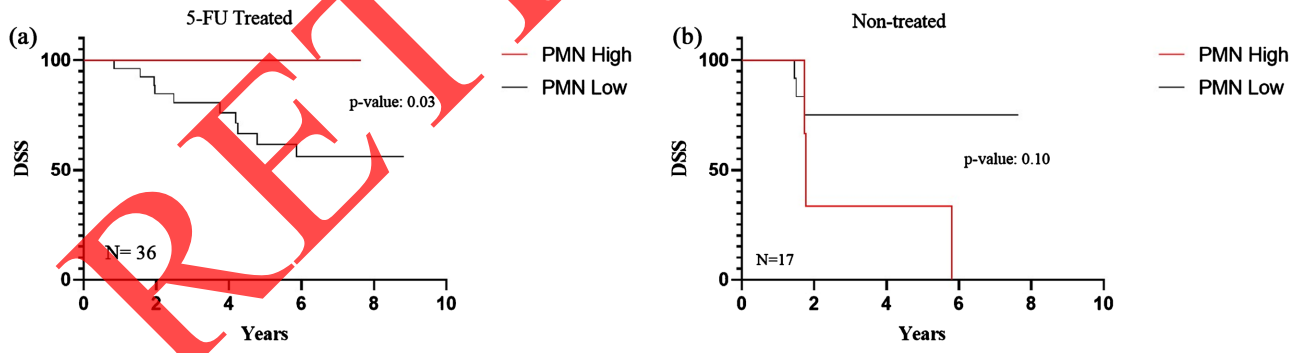

Figure 3. Prognostic significance of CD66b in patients with Stage III CRC located in the rectum. Kaplan-Meier survival curves show DSS (a, b) for patients presenting a high or low density of neutrophils (PMNhigh or PMNlow, respectively) in the IT (intratumoral). (a) Shows DFS amongst IT PMN high vs. PMN low ones amongst 5-FU treated patients, n = 36, p-value: 0.03. (b) Shows DFS amongst IT PMN high vs. PMN low ones amongst non-treated patients, n = 17, p-value: 0.10. Upper quartile values were employed to divide tumors into high and low CD66b+ immunoreactive area. The p-value was found using the Log-rank test.

4. Final Considerations

This review sheds new light on the state of the research on neutrophil's role in

rectal cancer patients. It also serves to further strengthen the concept of dividing the term of colorectal cancer (CRC), which I believe to be an outdated term, and divide it into two clinical, etiopathological and prognostic entities: Colon Cancer and Rectal Cancer.

This review also discusses data (that I demonstrated in my graduation thesis for my medical degree) showing that patients with Stage III CRC located in the rectum and having High IT PMNs, benefit from receiving 5-FU adjuvant chemotherapy. While, patients with Low IT PMNs and CRC located in the rectum, do not benefit from 5-FU adjuvant chemotherapy (on the contrary they show a worst DFS compared to the patients who did not receive 5-FU).

Furthermore, there is a linear correlation between IT PMNs and DFS in both 5FU0 and 5FU1 patients having CRC located in the rectum (correlation not seen neither in the left-sided CRC nor in the right-sided CRC). This correlation was inversed in regards to 5-FU0 patients (High IT PMNs = Worst Prognosis). The data therefore suggest a possible predictive and prognostic value for IT PMNs (TANs) to be used in the clinical practice in patients with Stage III rectal cancer, where high Tumor Associated Neutrophils in the intratumoral histological section could be an inclusion criterion for 5-FU based adjuvant chemotherapy while a low value could be an exclusion criterion for this therapy (see **Figure** 1) and where high IT TANs in 5-FU treated patients could be a positive predictive and prognostic indicator for Disease Free Survival (DFS) and Disease Specific Survival (DSS). This scoring system (named the "Sarandria Score" or in Italian nomenclature the "Scala di Valutazione Sarandria") could truly help in choosing candidates for 5-FU therapy (or possible other chemotherapeutical agents) among Stage III rectal cancer patients and could give prognostic and predictive insights to these patients. Furthermore, the fact that a worst DFS was found in patients not treated with 5-FU and having Stage III Rectal cancer could also be an important prognostic indicator for these subsets of patients.

The same correlation in regards to IT PMNs and DFS in CRC patients undergoing 5-FU therapy is not seen in the other CRC sites (left-sided and right-sided). Therefore, this score would be based on whether or not the intratumoral section at immunohistochemistry (CD 66 b ab stain) has a high value of neutrophilic infiltrate (set at ≥1.197 of neutrophilic density, calculated as "Sum % Area").

It is of relevance to do further studies in finding the correlation between neutrophils and rectal cancer, most importantly checking for: 1) Neutrophil polarization (my supposition if that neutrophils in the tumor microenvironment of rectal cancer is of N1 pheno-type). This can be checked with Arginase assay. 2) Correlation between neutrophils, rectal cancer and predictive/prognostic value also in patients undergoing different therapies, such as FF etc. It is my opinion, that neutrophilic infiltrate could become a staging system for rectal cancer patients for prognostic and predictive significance, much like immunoscore is now

days used alongside TNM staging for colon cancer. As a matter of fact, it would be interesting to see whether the immunoscore (based on Jerome Galon's finding from 2006 which revealed a positive association of cytotoxic and memory T cells with survival of colorectal cancer patients) would change in terms of the location of the CRC (namely left, right or rectal cancer). Therefore, this review highlights also a new scoring system based on neutrophilic infiltration of intratumoral section of stage III rectal cancer patients developed by myself, Dr Nicola Sarandria MD (proposed name of the score: Sarandria Score).

Furthermore, it is of my opinion that this could serve as further evidence in support of the future division of what is now known as colorectal cancer into Colon and Rectal cancer, two different entities with different clinical and etiopathological courses.

Therefore, as an addition to review the current state of knowledge on neutrophils and rectal cancer and with the aforementioned considerations in mind, the following scoring system, the Sarandria Score, can be formulated as such:

Sarandria Score:
With Positive Predictive and Prognostic score: association with better prognosis and therapy outcome

CD 66b stained Intratumoral cells density (Intratumoral neutrophils) (PMN Sum % Area)		Resulting Predictive Score**	Resulting Prognostic Score**	5-Fluorouracil Therapy Inclusion*	Resulting Prognostic Score (no 5-FU)***
<1.197	Low Intratumoral neutrophils	Negative	Negative	Low efficacy	Positive
≥1.197	High Intratumoral Neutrophils	Positive	Positive	Yes	Negative prognostic score

*Inclusion to a 5-Fluorouracil (5-FU) therapy to be considered in addition and conjunction to all other clinical aspects/criteria for the suitability and applicability of such therapy; **In patients being treated 5-FU chemotherapy; ***In patients not treated with 5-FU chemotherapy.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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