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TITLE OF THE DOCTORAL THESIS

Prognostic and diagnostic role of peri-operative inflammatory markers in colorectal cancer

S.S.D. MED/18, MED/06

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ABSTRACT

Background and objectives: The relationship between chronic inflammation and tumorigenesis has been largely investigated, and tumor-promoting inflammation proposed as an 'enabling characteristic' for tumor development. Also, it is increasingly recognized that the outcome of oncological patients depends not only on the intrinsic characteristics of the tumour, but also on the host immune-response and its ability to recognize and destroy tumour cells before escape. The aim of this study was to evaluate if changes in circulating immune cells occurs before the diagnosis of colorectal cancer (CRC), and to assess their relationship with survival outcomes. Moreover, we investigated if peri-operative inflammation influences long-term outcomes.

Materials and methods: Consecutive patients undergoing surgery for CRC at a single center were assessed for inclusion. The longitudinal changes in immune cell profile were evaluated in patients undergoing surgery for all stages (2005-2020) with a minimum follow-up of 24 months. For each patient a complete blood count (Pre-CBC) dated at least 24 months before surgery was retrieved. All parameters of Pre-CBC were tested for potential associations with survival after surgery. To evaluate the role of peri-operative inflammation, the analysis was restricted to patients submitted to elective minimally invasive potentially curative (R0-1) surgery for stage I-III CRC (2005-2022). Patients were categorized in a high-CRP (H-CRP) and low-CRP (L-CRP) group according to the highest value of post-operative CRP. Peri-operative outcomes, long-term survival and recurrence rates were compared between the two groups.

Results: Pre-CBC was available for 334 patients. Pre-Leukocyte (Pre-Leu), Pre-Neutrophils (Pre-Neut), and Pre-neutrophils-to-lymphocyte ratio (Pre-NLR) showed an increasing trend approaching the date of diagnosis, while Pre-Lymphocyte (Pre-Lymph) tended to decrease. On multivariate Cox regression analysis, Pre-Leu, Pre-Neut, Pre-Lymph and Pre-NLR resulted independent prognostic factors for OS and CSS, with pathological stage and age. Higher Pre-Leu, Pre-Neut, and Pre-NLR and lower Pre-Lymph were associated with worse CSS, and the effect was more evident when blood samples were closer to surgery.

Considering peri-operative inflammation, 436 patients with stage I-III CRC were included in the analysis. According to ROC analysis for recurrence, the optimum cut-off for postoperative CRP was 151.5 mg/L (AUC = 0.599). Patients who developed a recurrence showed a higher CRP_{max} compared to patients without recurrence (170.8 versus 136.8

mg/L, p = 0.019). OS (p < 0.001), RFS (p = 0.005), and CSS (p = 0.001) were significantly worse in patients in the H-CRP group. When conducting subgroup analysis for stage, the results were confirmed only in stage III CRC. Considering pT, no differences in OS, RFS and CSS were found in patients with early pT, while the difference remained statistically significant for pT3 and pT4. On multivariate analysis, H-CRP proved to be an independent risk factor for recurrence (p = 0.007), together with rectal location of the tumor (p < 0.001), presence of lympho-vascular invasion (p = 0.027), and stage III (p = 0.007).

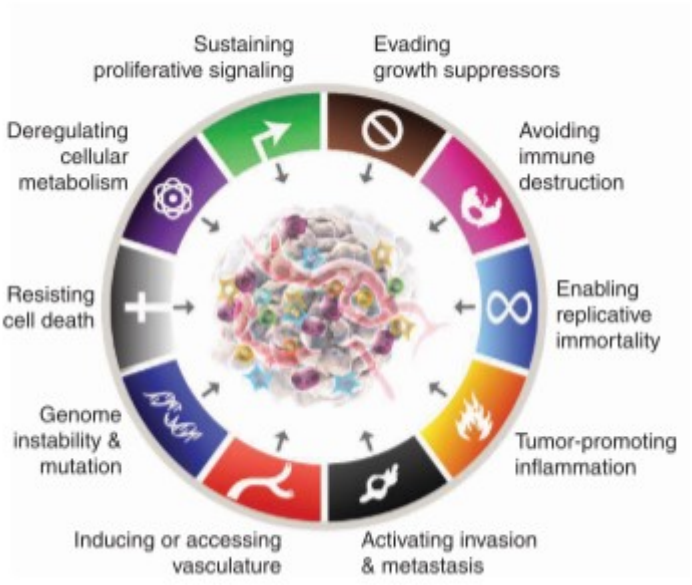
Conclusions: Changes in circulating immune cells could be detected as early as 24 months before the diagnosis of CRC, and this may represent an important window of opportunity for early diagnosis. Moreover, we demonstrated a significant association between early changes in circulating immune cells, OS, and CSS, reinforcing the body of evidence that suggests a bidirectional relationship between the tumor and the immune system. Our study also highlighted the potential role of surgical stress as tumor-promoting inflammation identifying post-operative CRP peak as a negative prognostic factor for OS, CSS, and RFS.

INTRODUCTION

Inflammation is a non-specific physiological immune response against harmful agents in the body. Typically, it is a self-limiting mechanism, but persistent inflammatory stimuli can lead to a chronic inflammatory process, potentially influencing the pathological microenvironment in a pro-tumoral manner. Beyond its protective role against pathogens, inflammation orchestrates tissue repair and regeneration while maintaining homeostasis.

The interaction between inflammation and tumor development has been recognized since 1863, when the German physician Rudolph Virchow observed leukocyte infiltration in tumor tissues. The significance of inflammation in oncogenesis has led to its inclusion in the "hallmarks of cancer"[1,2] — functional modifications acquired by tumor cells during their evolution:

- Ability to evade growth suppressors
- Capacity to escape immune surveillance
- Replicative immortality
- Establishment of a pro-tumoral inflammatory process
- Acquisition of invasive and metastatic capabilities
- Development of neo-angiogenesis
- Genomic instability and pro-tumoral mutations
- Resistance to apoptosis
- Deregulation of cellular metabolism
- Sustained proliferative signaling



The hallmarks of cancer.[2]

A well-documented correlation exists between chronic inflammatory conditions and the onset of malignant tumors. Examples include chronic HPV infection and cervical carcinoma, inflammatory bowel diseases (IBD) and colorectal carcinoma (CRC), HBV and HCV infections and hepatocellular carcinoma, *Helicobacter Pylori* and gastric cancer, Barrett's esophagus and esophageal carcinoma, and schistosomiasis and bladder carcinoma.

1.1 MECHANISMS OF INFLAMMATION-DRIVEN ONCOGENESIS

Chronic inflammation, such as that associated with obesity or metabolic syndrome, can induce widespread pro-tumoral effect.[3] This process shares several features with carcinogenesis, including increased cellular proliferation, altered apoptosis, and promotion of neo-angiogenesis. Tthese pathways collectively contribute to malignant transformation[4,5]. Inflammation-driven oncogenic changes can be categorized into two main pathways: intrinsic and extrinsic.

The **intrinsic pathway** stems from genetic mutations critical for tumor initiation. These mutations mediate the expression of inflammatory mediators contributing to a pro-tumorigenic microenvironment[6]. This microenvironment often intersects with the **extrinsic pathway**, which is activated in response to chronic inflammatory stimuli. Together, they

establish an inflammatory milieu conductive to tumor development and progression. At least three initiating mutations are typically necessary to determine the malignancy of a cell line.[7] Examples of key mutations involved in the intrinsic pathway include[8]:

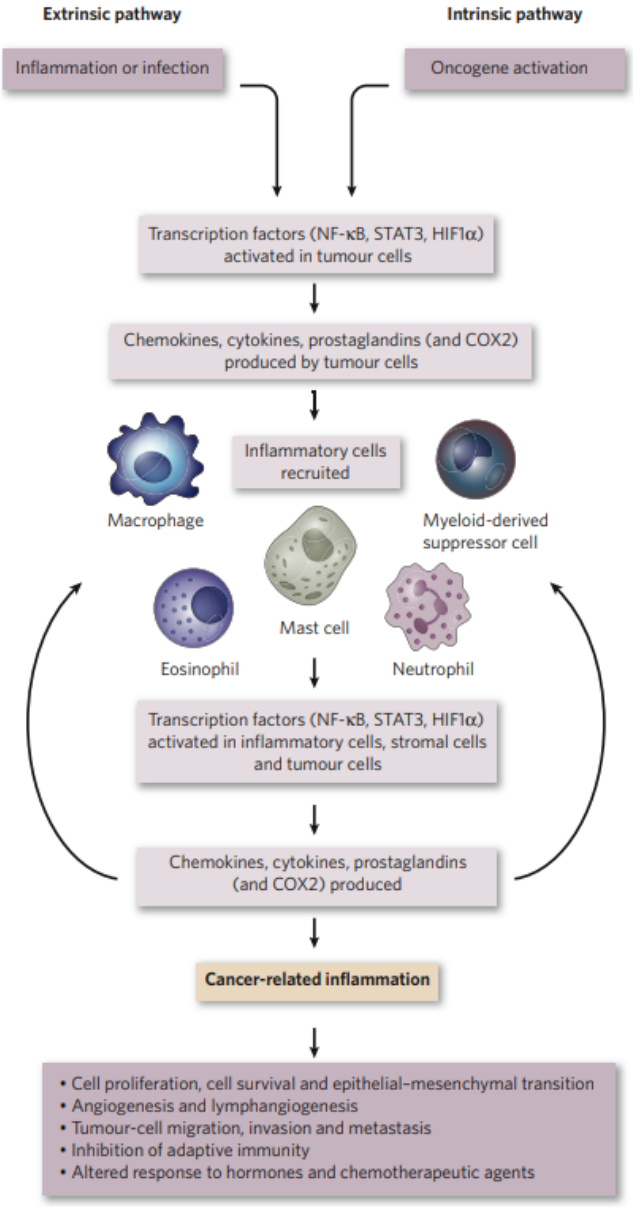
- Driver mutations of the *WNT* pathway: the most frequently mutated genes include *APC, RNF43, CTNNB1, AXIN2*.[9]
- Gain-of-function mutations of *RAS* resulting in continuous activation of thyrosin-kinases signaling, with consequent activation of transcription factors involved in the cellular replication.[10]
- Gain-of-function mutations in *TP53*, promoting invasive and metastatic behaviors through the *TGFβ* and *PDGF* pathway.[11]
- *RET* mutations, implicated in the pathogenesis of papillary thyroid cancer.[12]
- In the **extrinsic pathway**, chronic inflammation facilitates oncogenesis through complex molecular interactions leading to genetic instability and uncontrolled cellular replication.[13] The key players include:
    - Myeloyd-derived suppressor cells (MDSC): immature cells supporting neo-angiogenesis and metastases formation via M2 macrophages, T regulatory lymphocytes and the suppression of natural killer (NK) cell cytotoxic effect.[14]
    - Tumor-associated macrophages (TAM), which represent more than 50% of tumor microenvironment. They exhibit M2 polarization, which is a cell subtype involved in the resolution phase of inflammatory processes under physiological conditions; in the tumor context, they favor growth and metastasis. They mainly derive from tissue cells, with a smaller contribution from circulating macrophages. They promote metastases and epithelial-mesenchymal transition by secreting extracellular matrix proteolytic enzymes via the *JAK2/STAT3* pathway; they also favor neo-angiogenesis through secretion of *VEGF*.[15] [14] [16] [17]
    - Dendritic cells (DC) with immature phenotype and the inability to activate T-mediated inflammatory response.[16] [17]
    - Tumor-associated neutrophils: they have a key role in tumor proliferation and metastases, though the production of reactive oxygen species (ROS), matrix metalloproteinases, nitric oxide, chemokines, and cytokines. *TGFβ* causes the conversion from anti-tumorigenic N1 phenotype to pro-tumorigenic N2 phenotype. Tumor-associated neutrophils (TAN) with N2

profile aquire the ability to evade the immune system through the activation of T reg lymphocytes.[15 14 17 18]

    - Lymphocytes: they play a pivotal role in anti-tumoral activity. CD8 cytotoxic lymphocytes express an antigen-specific response. CD4 lymphocytes release *TNF* and *IFN-y*, which increase the lytic activity of cytotoxic lymphocytes and activate macrophages.[14 16 19 20]

- ROS and nitrogen species (RNS) cause direct damage to DNA, proteins, and lipids.[21] Oxidative damage to the DNA increases the probability of genetic mutations in oncogenes and oncosuppressors.
- Citokines: *TNF* (fibroblasts proliferation, epithelial-mesenchimal transition), *IL-6, IL-10* (favors the release of Bcl-2 with reduction in the apoptosis).
- Growth factors: *TGFβ, VEGF, FGF2* favors neo-angiogenesis within the tumor.[3 14 19]

Ultimately, the interplay between intrinsic and extrinsic pathways establishes a pro-inflammatory tumor microenvironment, driving local growth and distant metastasis.



Intristic and extrinsic pathway of cancer-related inflammation[15]

1.2 INFLAMMATION AND COLORECTAL CANCER

The pathogenesis of colorectal cancer (CRC) involves three main molecular pathways: chromosomal instability, microsatellite instability, and CpG island hypermethylation.[22 23] The interaction between malignant cells and the surrounding TME significantly influences cellular replication, apoptosis resistance, tumor survival, and immune evasion.

Cancer progression is also dependent on the interplay between the tumor and the host's intrinsic characteristics. Tumor growth can trigger an inflammatory response via the release of cytokines from tumors or TME cells. Although plasma levels of these cytokines are not routinely measured, indirect estimates can be obtained through widely available inflammatory markers, which will be discussed in detail later in the Introduction. Several chronic inflammation-related factors contribute to CRC pathogenesis, including:

- Environmental factors: advanced age, physical inactivity, smoking, obesity and diet can promote chronic inflammation.
- Intestinal microbiota: alterations in microbial composition may sustain chronic inflammation and drive tumorigenesis. Examples include *Helicobacter pylori* in gastric cancer and pathogenic *Escherichia coli* genotypes in CRC.[24 25]
- Inflammatory bowel diseases (IBD) represent a known risk factor for CRC through chronic inflammation and "field cancerization" of affected mucosa. IBD-related CRC often exhibits early *p53* mutations3.[26]

2. INFLAMMATORY MARKERS AND CANCER PROGNOSIS

The biological and pathological features of tumor are not the only factors affecting recurrence, metastasis and poor prognosis. Host-related factors, including immune, nutritional, and inflammatory status, play critical roles in tumor development, therapy response, and recurrence. Recent studies suggest that tumor cells proliferation, invasion, metastasis and angiogenesis are closely linked to these parameters.[2,27,28]

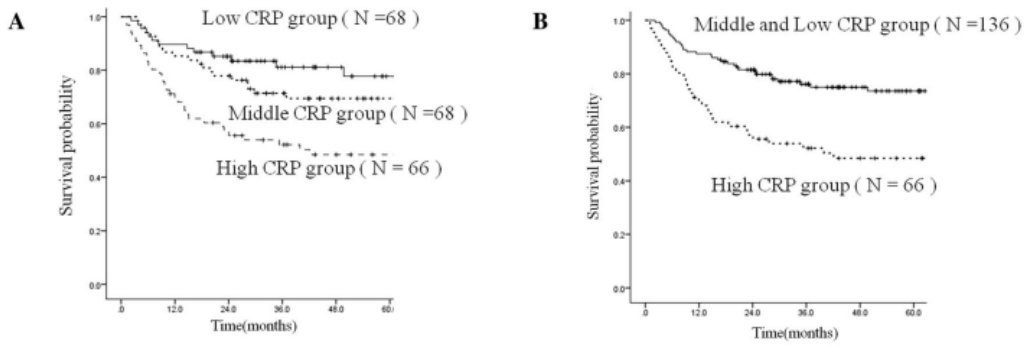
2.1 SOLUBLE INFLAMMATORY MARKERS

In addition to tumor-associated parameters (e.g., TNM staging, histotype, tumor diameter), increasing evidence supports the prognostic role of inflammatory markers, immune cells, and derived scoring systems.[29,30]

C-REACTIVE PROTEIN (CRP)

CRP is an acute phase protein whose plasmatic concentration rises during inflammation, infection or tissue damage. Elevated CRP levels in healthy individuals have been associated with an increased tumor risk.[31] For instance, Koike et al.[30] correlated circulating CRP levels with intratumoral IL-6 levels, which are associated with the risk of recurrence and worse prognosis.

Ibuki et al.[32] studied 202 esophageal carcinoma patients undergoing trans-thoracic esophagectomy. They found that elevated post-operative CRP levels were associated with worse prognosis, with a high-CRP group exhibiting reduced disease-free survival (DFS) compared to low-CRP patients. These findings suggest a persistent inflammatory state, potentially driven by post-operative residual cancer, could facilitate recurrence. The authors suggested a potential secretion of IL-6 by esophageal tumor cells and a consequent increase in CRP, as seen in clear-cell carcinoma of the kidney[33]. Another hypothesis is that a persistent inflammatory state in the presence of postoperative occult residual cancer could cause cancer development and recurrence and thus a poor prognosis.



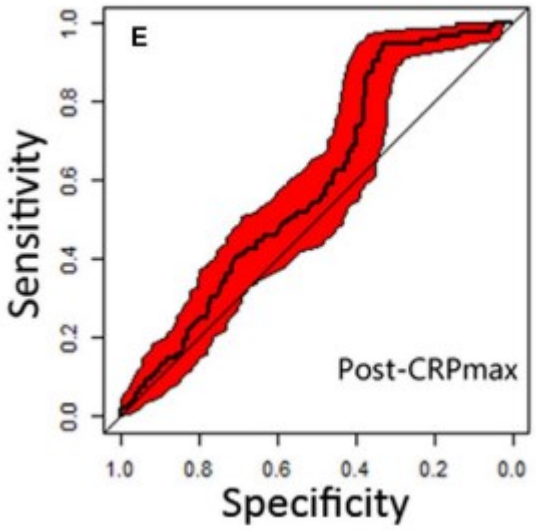
DFS curves reported by Ibuki et al. according to CRP value of post-operative day 7[32]

With regards to CRC, Woo et al. [34] performed a meta-analysis of 21 studies including 6261 CRC patients. They found pre-operative CRP levels above the cut-off of 1 mg/dL were associated with a significantly lower overall survival (OS) (HR=3.65 in stage IV and 2.04 in stages I-III) and cancer-specific survival (CSS) across different tumor stages.

Furthermore, a systematic review by Shrotriya et al.[35] confirmed the prognostic role of CRP in terms of prognosis, tumor recurrence and response to treatment in various adult solid tumors. The review identified 217 articles, mostly retrospective and of intermediate quality. The vast majority of the studies identified a correlation between high CRP and higher

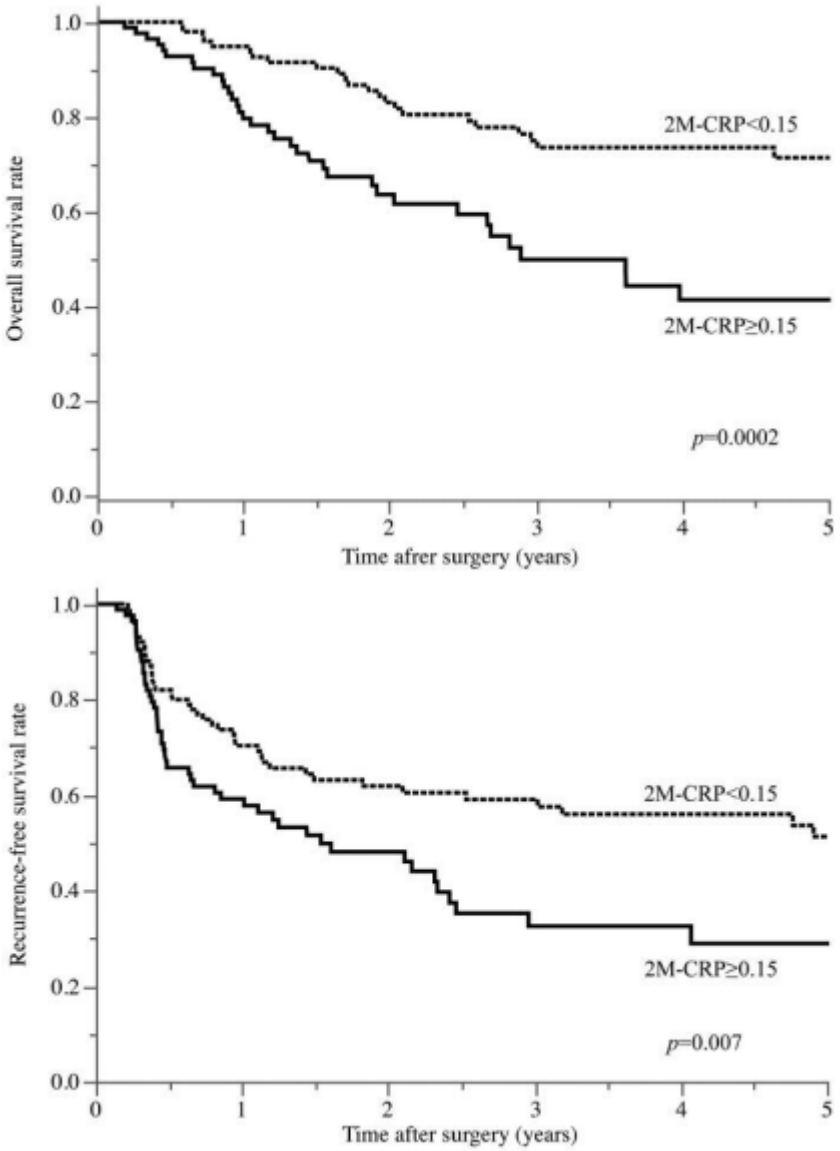
mortality in patients with tumors of the gastrointestinal tract, kidney, prostate, liver, and breast.

In addition, some authors investigated the prognostic role of post-operative CRP and prolonged post-operative inflammation, which may facilitate tumor recurrence. Lu et al.[36] evaluated 401 patients with stage I-III gastric cancer for whom pre- and post-operative CRP values were available. They found that both high pre-operative CRP and high post-operative maximum CRP were associated with worse prognosis in terms of recurrence-free survival (RFS). To eliminate the potential effect of postoperative complication on prognosis, multivariate analysis showed that both pre-CRP and post-CRP_{max} were independent risk factors of postoperative recurrence, and postoperative complication was not associated with postoperative recurrence.



ROC curves for recurrence for maximum post-operative CRP.[37]

Katsurahara et al.[38] analzyed a retrospective cohort of 187 patients who underwent esophagectomy for esophageal squamous cell carcinoma. The authors assessed post-operative CRP up to 2 months after surgery, considering high CRP if the value was above 20 mg/dL in the first 14 days after surgery or above 0.15 mg/dL at 1 and 2 months after surgery. In a multivariate analysis, patients with high CRP 2 months after surgery showed poorer OS and RFS.



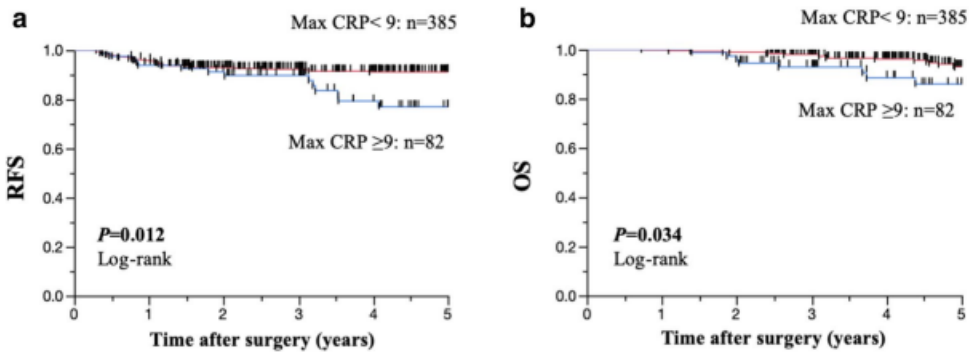
Kaplan-Meier curves for OS and RFS in the study by Katsurahara et al.[38]

Similar results were found in patients with lung cancer. Pastorino et al.[39] showed that post-operative CRP measured on day 0 and 3 in non-smoker patients operated for stage I-III lung cancer was an independent risk factor of mortality at 30 days as well as 5 years after surgery.

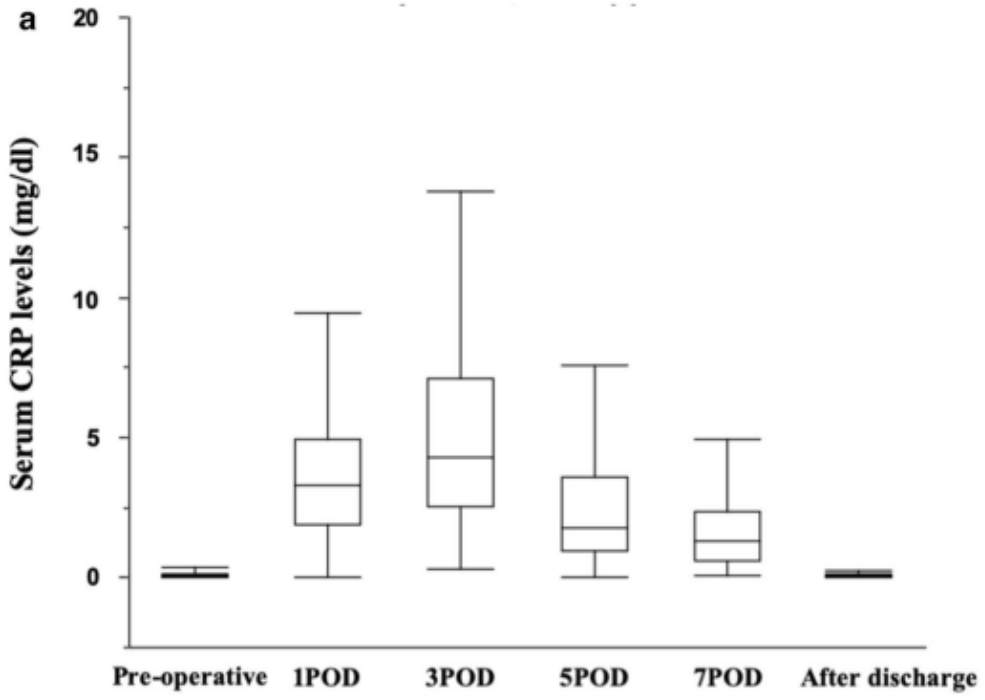
Gwenzi et al.[40] recently conducted a meta-analysis on post-operative CRP-based inflammatory biomarkers in CRC patients. 12 studies assessed the relationship between post-operative CRP and survival outcomes, including OS, RFS, and CSS. Elevated CRP

was a significant predictor of poor OS, CSS, and RFS, however it should be noted that substantial heterogeneity was observed in most of the meta-analyses. Furthermore, the definition of post-operative CRP was extremely variable, with a mean sampling time after surgery ranging from 3 days to 6 months. In the sensitivity analysis restricted to the three studies that adjusted for age and cancer stage, an even stronger association between high post-operative CRP and OS was found with no heterogeneity. We hereby report the results of some of the most representative studies.

Matsubara et al.[41] conducted a retrospective study on 467 patients with stage I-III CRC submitted to curative surgery. Most of the patients (422/467) underwent minimally invasive surgery. Patients with elevated pre-operative CRP or who developed post-operative complications were excluded. Patients were categorized into two groups according to an optimal cut-off of maximum post-operative CRP above or below 9 mg/dL. RFS in the low CRP group was 91.0% compared to 76.9% in the high CRP group (p=0.012). Similarly, OS was lower in the high CRP group (p=0.034).



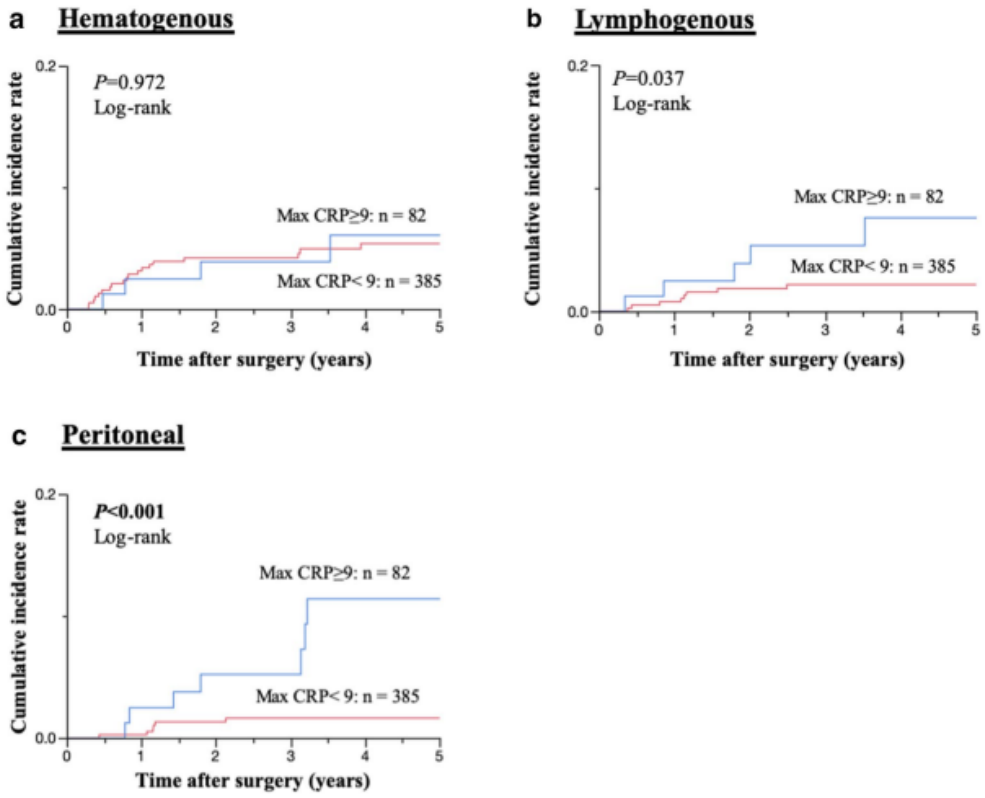
Kaplan-Meier curves for RFS and OS in the study by Mastubara et al. .[41]



Distribution of post-operative CRP values in the study by Matsubara et al.[41]

The authors also investigated the factors associated with elevated post-operative CRP and found that older age, male gender, higher BMI, right-sided tumor and longer operative time were independent risk factors.

Finally, patients with high CRP showed significantly higher recurrence rates in the lymph-nodes and the peritoneum (7.5% vs 2.1%, $p=0.037$, and 11.3% vs 1.6%, $p<0.001$, respectively).



Recurrence pattern curves as reported by Matsubara et al. [41]

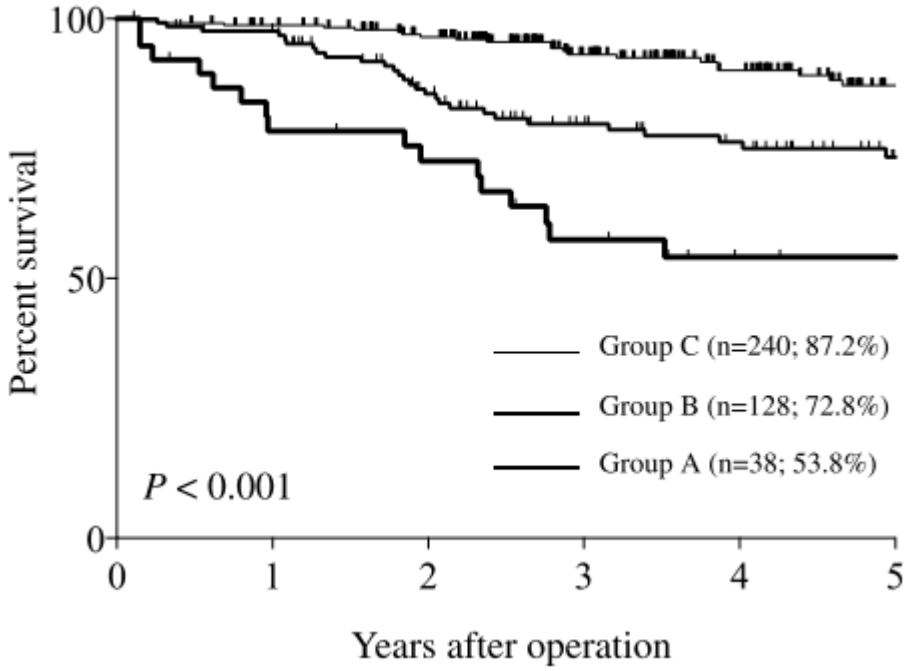
The authors suggested that elevated post-operative CRP may indicate the presence of cytokines facilitating the survival of residual micrometastases, supporting tumor proliferation and recurrence. Also, the study investigated the role of three cytokines (*IL-6*, *TNF-α*, and *IL-1β*) on CRC cells proliferation and adhesion in vitro. The assays showed that those cytokines enhance the adhesiveness of CRC cells to mesothelial cells as well as lymph angiogenesis.

Another interesting study was conducted by Yamamoto et al.[42] The study involved 406 CRC patients (stage I-IV) submitted to open (n=167) or laparoscopic (n=239) surgery. The authors categorized patients according to a combination of pre- and post-operative CRP:

- A: high pre-PCR (≥ 0.5 mg/dL) and high post-PCR (≥ 17.0 mg/dL)
- B: high pre-PCR and low post-PCR (< 17.0mg/dL) or low pre-PCR (< 0.5mg/dL) and high post-PCR
- C: low pre-CRP and low post-PCR

In this cohort, post-operative CRP was significantly higher in men, larger tumors, T3-4 tumors, in the presence of distant metastases, following open surgery and in patients who developed infectious complications.

Interestingly, RFS increased progressively from group A to C (53.8% vs 72.8% vs 87.2% respectively), suggesting that the combination of pre- and post-operative inflammation act as an independent prognostic factor.



Kaplan-Meier curves about RFS in the study by Yamamoto et al.[42]

McSorley et al.[43] investigated the relationship between post-operative systemic inflammatory response expressed as CRP, the severity of complications and long-term outcomes in CRC patients. They analyzed a cohort of 377 CRC patients receiving elective potentially curative surgery (266 open surgery and 110 laparoscopic surgery). The impact of post-operative inflammation and complications was assessed through univariate and multivariate analysis. On univariate analysis, complication severity and CRP level > 150 mg/L on POD4 was associated with DFS. However, only the CRP level remained an independent risk factor on multivariate analysis.

Finally, a large study by Watt et al.[44] analyzed a cohort of 813 CRC patients who underwent potentially curative resection. Patients were grouped in two cohorts, a test cohort that underwent surgery between 1999 and 2007 (n=402) and a validation cohort operated between 2008 and 2013 (n=411). The aim of the study was to develop and validate a post-operative version of the modified Glasgow prognostic score (mGPS). In a multivariate Cox regression analysis conducted considering various confounding factors, a CRP value above the chosen cut-off of 150 mg/L on POD 3 and POD 4 resulted in an independent prognostic factor for OS but it did not reach significance for CSS.

Clinicopathological characteristic	Cancer-specific survival		Overall survival	
	Univariate analysis (95% CI)	*p* Value	Univariate analysis (95% CI)	*p* Value
Postoperative systemic inflammation				
Day 3 CRP > 150 mg/L (no/yes)	1.31 (0.96–1.79)	0.088	1.41 (1.12–1.78)	0.004
Day 3 albumin <25 g/L (no/yes)	1.38 (1.00–1.90)	0.047	1.42 (1.11–1.81)	0.005
Day 3 poGPS (0/1/2)	1.20 (0.99–1.46)	0.059	1.27 (1.10–1.47)	0.001
Day 4 CRP > 150 mg/L (no/yes)	1.31 (0.93–1.84)	0.129	1.33 (1.02–1.74)	0.035
Day 4 albumin <25 g/L (no/yes)	1.36 (0.98–1.90)	0.068	1.48 (1.15–1.91)	0.002
Day 4 poGPS (0/1/2)	1.22 (0.99–1.50)	0.065	1.21 (1.03–1.42)	0.024

Relationship between pre- and postoperative systemic inflammatory response and survival outcomes in the study by Watt et al (adapted from the original report)[44].

In a further study by Osterman et al.[45] emerging risk factors beyond NCCN guidelines were investigated. The cohort consisted of 416 colon cancer patients treated with curative surgery between 2010 and 2015. The authors considered as emerging factors pT3/pT4 subclassification, tumor sidedness, pN subclassification, lymph-node ratio (LNR), tumor deposits, CEA levels, and peri-operative CRP. Contrarily to other evidence, pre-operative CRP correlated with recurrence in the unadjusted analysis but not in the adjusted model. Post-operative CRP did not show correlation with recurrence.

ALBUMIN

Albumin is another acute-phase protein, and its concentration decreases in the presence of inflammation. Low albumin levels may reflect tumor- or inflammation-related cachexia, and therefore play a negative prognostic role.[46] Malnutrition is common in patients with malignant tumors, and it can directly affect the effect of cancer therapy and patients' quality of life. Studies have shown that low albumin level, as an indicator of systemic inflammatory

response, correlates with poor prognosis in patients with non-small cell lung cancer, hepatocellular carcinoma, and cervical cancer.[47]

GLASGOW PROGNOSTIC SCORE and MODIFIED GLASGOW PROGNOSTIC SCORE

Glasgow prognostic score (GPS) and its modified version (mGPS) represent an interesting prognostic marker in patients with various cancer, though it is not routinely evaluated as standard risk factor. The score ranges from 0 to 2, and it is calculated considering the values of CRP and albumin, representing therefore a simplified picture of inflammatory and nutritional status.[46]

Systemic inflammation based prognostic scores, the Glasgow Prognostic Scores.

The Glasgow Prognostic Score (GPS)	Points allocated
C-Reactive protein ⩾10 mg/l and albumin ⩾35 g/l	0
C-Reactive protein >10 mg/l	1
Albumin <35 g/l	1
C-Reactive protein >10 mg/l and albumin <35 g/l	2
The modified Glasgow Prognostic Score (mGPS)	
C-Reactive protein ⩽ 10 mg/l and albumin ⩾35 g/l	0
C-Reactive protein >10 mg/l	1
C-Reactive protein >10 mg/l and albumin <35 g/l	2

Most of the evidence on mGPS derives from McMillan et al.[48] studies, despite the score has been validated by various authors in many solid cancers.[28,49–51] mGPS is most frequently assessed as prognostic marker before surgery or treatment, however some studies also evaluated its role in the post-operative period. Interestingly, in the study by Watt et al.[44] considering 813 CRC patients, the post-operative systemic inflammation response (SIR) was evaluated according to the following scheme:

- Post-operative CRP below 150 mg/dL regardless of albumin: 0 points
- Post-operative CRP equal or above 150 mg/dL and albumin above 25 g/L: 1 point
- Post-operative CRP equal or above 150 mg/dL and albumin equal or lower than 25 g/L: 2 points

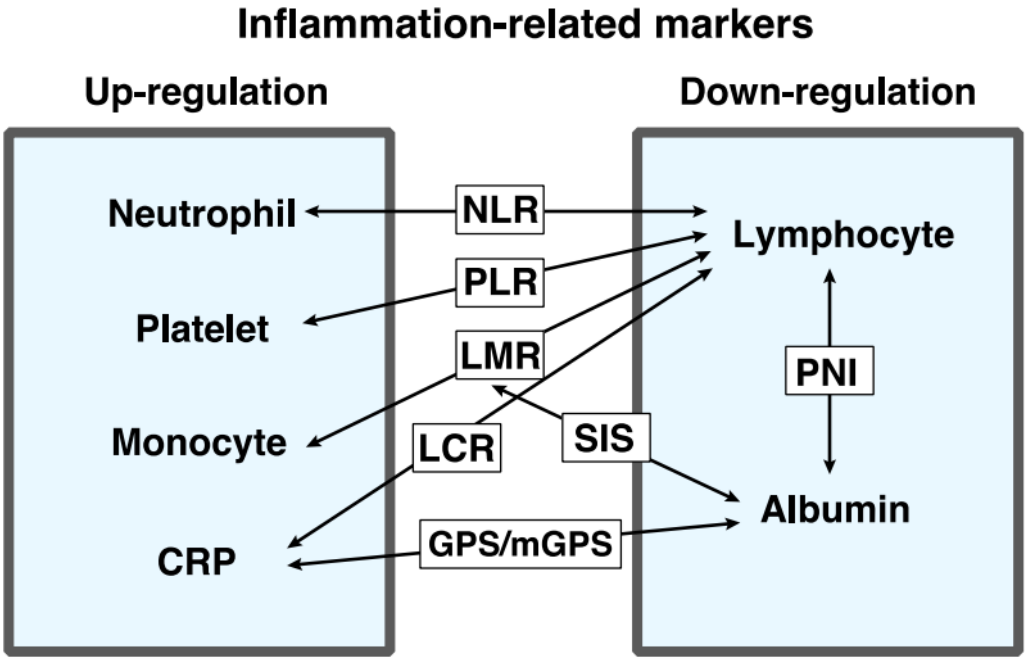
The score was initially created in a test cohort and an attempt to validate it in the prospective cohort was then performed. On multivariate analysis, mGPS on POD 3 or 4 demonstrated a significant correlation with OS but not with CSS.

In a recent meta-analysis by Gwenzi et al.[52] four studies evaluated the prognostic role of GPS measured within one week from surgery or within 4 weeks. Those studies found a worsening in OS per unit increase of the GPS score. However, the results could not be combined in a meta-analysis because of different coding of GPS. With regards to mGPS, two studies correlated mGPS measured at least 4 weeks after surgery and worse OS in CRC patients.

2.2 IMMUNE CELLS INVOLVED IN INFLAMMATION

The immune system's cellular response represents another crucial prognostic parameter, reflecting the inflammatory microenvironment within the tumor. Immune surveillance removes damaged cells that have lost physiological functionality, a process now termed "immunoediting." This process encompasses three phases: elimination, equilibrium, and escape.[53]

- **Elimination phase:** Natural killer (NK) cells, T and B lymphocytes, and macrophages identify damaged cells through NKG2D ligands or cell receptors, secreting IFN-γ to induce apoptosis and inhibit angiogenesis[54]
- **Equilibrium phase:** Persistently damaged cells may evade elimination while maintaining potential malignant transformation..[55] In this phase lymphocytes and INF-ɣ act selecting those cells that resist elimination. [54]
- **Escaping phase**: Cells acquire mechanisms to evade immune recognition and initiate malignant replication, transitioning immune activity from anti-tumoral to pro-tumoral.



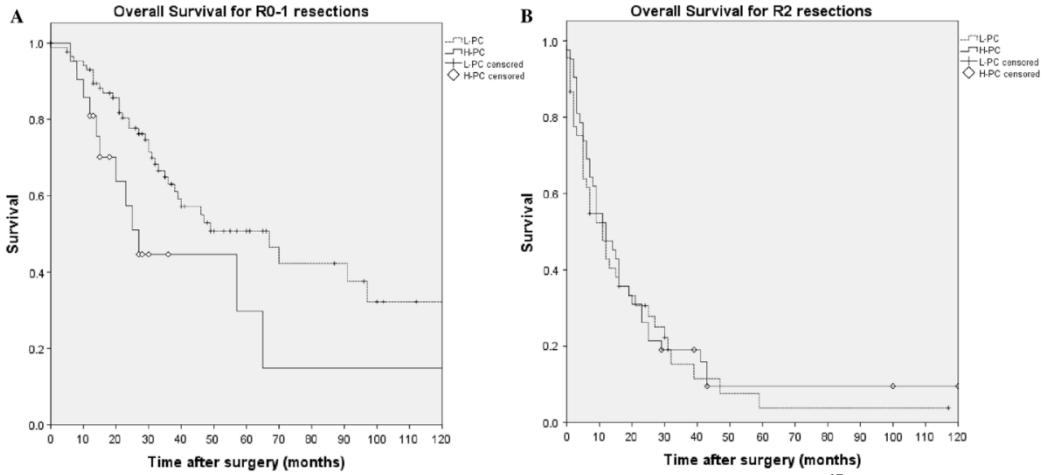
Overview of the inflammation related markers. Adapted from Yamamoto et al[56]

Markers such as neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) are extensively studied for their prognostic significance. Elevated neutrophil counts often correlate with systemic inflammation, while lymphopenia may reflect immune suppression, facilitating tumor escape. Other inflammatory-related markers include platelet count (PC)[57], systemic inflammation score (SIS), lymphocytes-to-monocyte ratio (LMR).[46]

The prognostic role of NLR, PLR and PC evaluated at the time of surgery of cancer treatment has been widely evaluated in different cancer types, such as liver cancer[58], prostate[59] , and CRC.[60 61 62]

Platelets are leading elements in the inflammatory process, as they can promote tumor growth. Migrating thrombophlebitis have long been known as paraneoplastic syndromes related to tumor-related platelet activation. Similarly, thrombocytosis has been associated with the occurrence of metastatic disease[63,64], through the promotion of tumor growth and invasion. Platelets activation favors the release of various metabolites that promote vessels permeability and neo-angiogenesis, including *VEGF, PDGF, bFGF.*[65] [66] In a previous experience by our group[67], the role of thrombocytosis was evaluated in patients undergoing surgery for synchronous colorectal liver metastases. A cohort of 196 patients was analyzed, and the presence of thrombocytosis was associated with the main negative clinical and

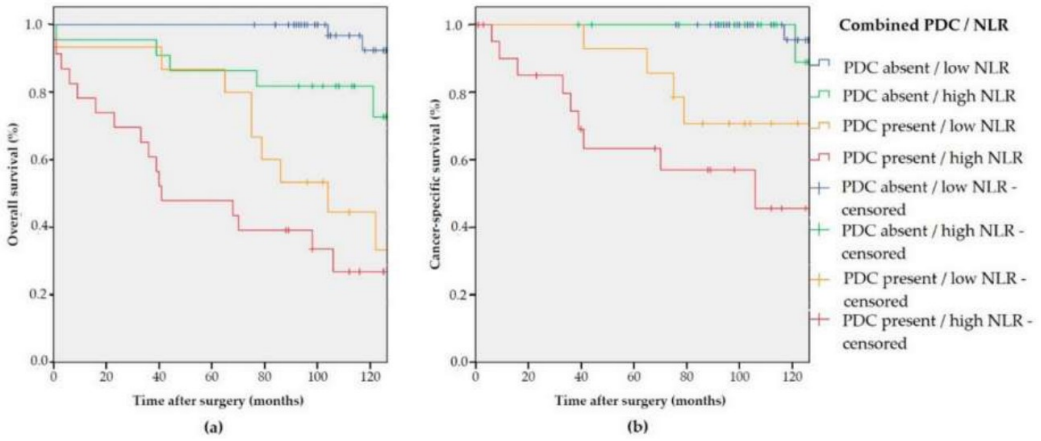
pathological factors (i.e. palliative surgery, extra-hepatic metastases, bilobar liver disease, more than 3 metastases, biggest metastases larger than 5 cm, and CEA level higher than 200 ng/mL). At the multivariate analysis on R0-1 cases after adjustment for other known prognostic factors, PC resulted the only independent predictor of survival (HR 2.07, p = 0.036).



Kaplan-Meier estimates of overall survival according to platelet count[67].

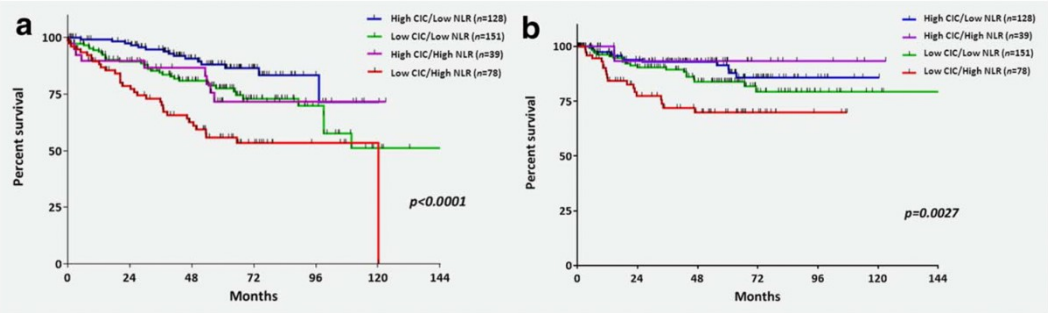
Elevated neutrophil count is often correlated to the cancer-associated inflammatory reaction. Low lymphocytes count frequently coexists[68], since those cells exert a cytotoxic antitumoral effect. The ratio between the absolute number of lymphocytes and the absolute number of neutrophils (NLR) therefore represents an optimal way to represent systemic inflammation and immune status. Similarly, the ratio between platelets count and lymphocytes count (PLR) exert the same role[69]. A high NLR may be the result of systemic inflammation, with a relative increase in neutrophils count, a relative decrease in lymphocytes count, or both. In the context of tumor-associated inflammation, we often observe an increase in circulating neutrophils, that can secrete chemokines and cytokines involved in cancer progression. Furthermore, lymphopenia can express immune suppression which in turn may facilitate tumor escape from immune surveillance. Considering monocytes, these cells can be recruited in the TME through the secretion of chemokines (*CCL2, CCL3, CCL3*) and contribute to neo-angiogenesis and cancer proliferation. Increased circulating levels of monocytes can be interpreted as a marker of tumor aggressiveness[70,71].

Most of the studies on the prognostic role of circulating cells evaluated quite exclusively cell counts in the immediate pre-operative or pre-treatment phase[72–76]. In a study by Pedrazzani et al.[76] 603 CRC patients were analyzed for the relationship between NLR, PLR and PC with survival outcomes. OS appeared to be worse in patients with high NLR for all CRC stages, however the result was not confirmed for CSS. After stratification for TNM stage, high PC was significantly correlated with worse OS and CSS in stage IV patients. In a further study by the same group, Turri et al.[77] evaluated only patients at early stages (stage I-II), looking for the identification of novel markers that can aid prognostic stratification. The evaluation of 107 stage I-II CRC patients showed a significant correlation between high NLR and high PLR and a pathological negative prognostic factor named poorly differentiated clusters (PDC). On multivariate Cox regression analysis, high NLR (HR 4.25, 95% C.I. 1.77–10.26, p=0.007) and PDC (HR 11.96, 95% C.I. 4.70–30.40, p<0.001) resulted independent risk factors for OS, while PDC was the only significant prognostic factor for CSS (HR 26.37, 95% C.I. 5.30–131.28, p < 0.001,). Finally, the combination of high NLR and PDC allowed an optimal stratification of OS and CSS, suggesting a potential role in clinical practice for the identification of high-risk patients with stage I and II CRC.



Kaplan–Meier estimates of OS (p < 0.001) and CSS (p < 0.001) according to the combined PDC/NLR variable[77].

Quite interestingly, Turner et al.[78] examined local and systemic inflammation in a consecutive series of patients with resected Stage II colon cancer. Historically, dense intra-tumoral immune infiltrates are associated with improved outcomes. Increased intra-tumoral chronic inflammatory cell (CIC) density was used to represent local inflammation, while NLR >5 was used to represent systemic inflammation. The combination of low CIC and high NLR was associated with the worst long-term outcomes (5-year OS 55.8%).

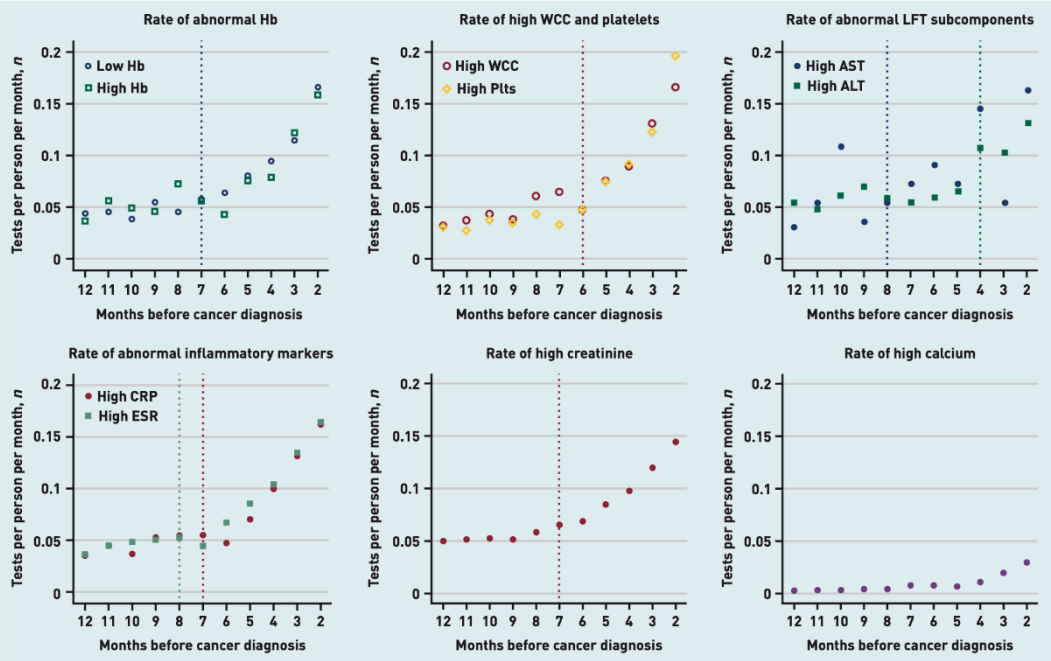


Combination of CIC density and NLR and survival outcomes in stage II colon cancer patients. (a) Overall survival. (b) Relapse-free survival[78].

3. ROLE OF PRE-DIAGNOSIS INFLAMMATION

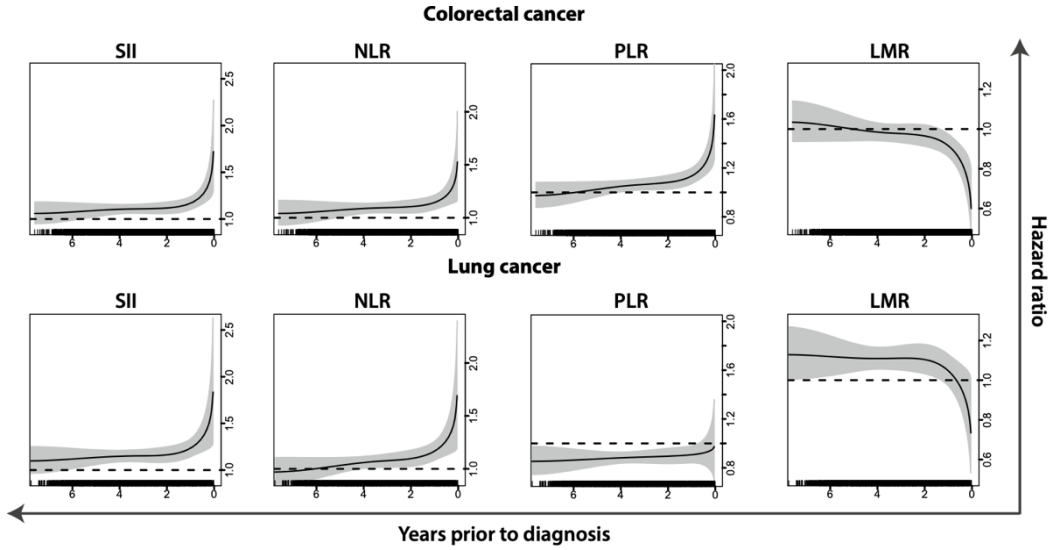
However, if we consider the bi-directional relationship between immunity and cancer, some changes in immune profile may take place months or years before cancer diagnosis[79]. Only few studies assessed longitudinal changes of pre-diagnosis systemic inflammatory markers in cancer patients[79–82], but no data are available for CRC.

The study by Zhou and colleagues[79] is an interesting retrospective study on patients with bladder and renal cancer diagnosed between April 2012 and December 2015. The authors accessed a primary care registry of blood tests in the United Kingdom and evaluated the rate of patients with altered blood tests the year before cancer diagnosis. Data from 4533 patients were analyzed and abnormalities of both generic (for example, high inflammatory markers) and organ-specific tests (for example, high creatinine) started to increase from 6–8 months pre-diagnosis, with 25%–40% of these patients having an abnormal test in the 'early half' of the diagnostic window, indicating the potential for early diagnosis in a proportion of patients.



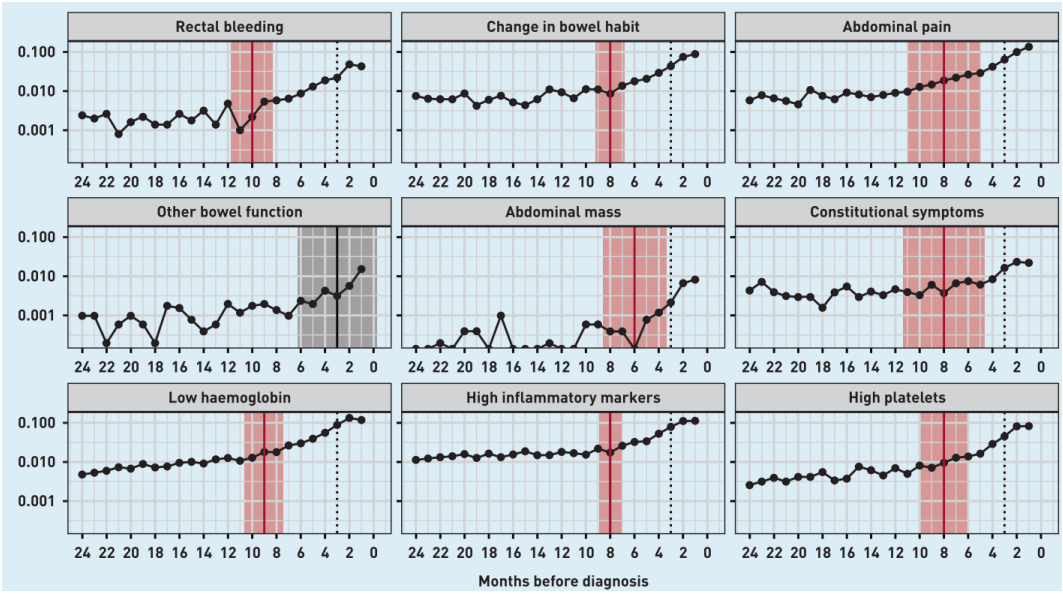
Rate of abnormal blood tests in the year pre- diagnosis, with dotted line signifying increase in rate from baseline for that particular test[79].

A further study by Nøst et al. analyzed the prospective UK Biobank cohort of approximately 440,000 participants recruited between 2006 and 2010. They assessed associations between four immune-related markers (systemic immune-inflammation index, NLR, PLR, LMR) and risk for 17 cancer sites. They found positive associations for Pre-SII, Pre-NLR and Pre-PLR, while they found a negative association for Pre-LMR. The strongest associations were found for lung and colorectal cancer. The authors conclude that blood cell ratios could serve as biomarkers of cancer incidence risk with potential for early identification of disease in the last year prior to clinical diagnosis.



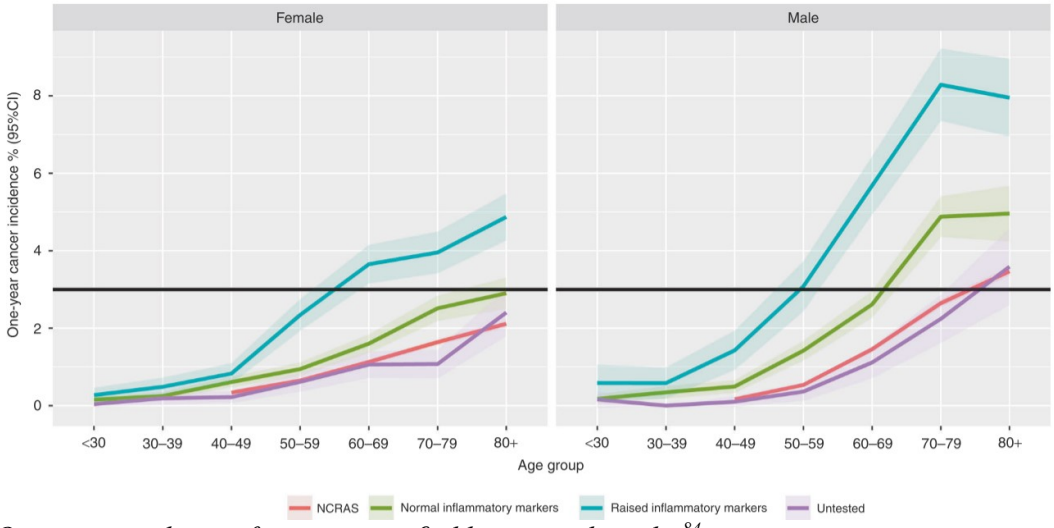
The figure reports inflammation marker-specific HR estimates as a function of follow-up time for colorectal and lung cancer[80].

A retrospective cohort study by Moullet et al.[83] investigated a linked primary care and cancer registry data for patients with colorectal cancer diagnosed in England between 2012 and 2015. They identified 5033 patients with colon cancer and 2516 with rectal cancer and looked for rate of consultations requested for relevant clinical features or abnormal blood tests in the year before diagnosis. Rectal bleeding was the earliest clinical feature to increase from baseline. Low hemoglobin, high platelets, and high inflammatory markers increased from as early as 9 months pre-diagnosis.



Rates of recordings of each clinical feature in the 2 years leading up to diagnosis of colon cancer[81].

Finally, an interesting study by Watson et al.[84] examined the diagnostic utility of inflammatory markers for cancer diagnosis in primary care. Participants from a prospective cohort study of UK primary care patients using routinely collected data from electronic health records in the Clinical Practice Research Datalink (CPRD) were compared to a sample of untested matched controls. Primary care patients with a raised inflammatory marker have a one-year cancer incidence of 3.53% (95% CI 3.37–3.70), compared to 1.50% (1.43–1.58) in those with normal inflammatory markers, and 0.97% (0.87–1.07) in untested controls. Sensitivities for cancer were 46.1% for CRP, 43.6% for erythrocyte sedimentation rate (ESR) and 49.7% for plasma viscosity (PV).



One-year incidence of cancer, stratified by age and gender[84].

4. ROLE OF CRP IN THE PREDICTION OF POST-OPERATIVE COMPLICATIONS

Being an acute phase protein, CRP is frequently assessed in the prediction of post-operative complications. In a study by Pedrazzani et al[85], the authors evaluated the role of CRP on post-operative (POD) day 3 in the prediction of complications following minimally invasive colorectal surgery. CRP below 120 mg/dL on POD3 resulted an optimal indicator to exclude surgical and severe complications, with a negative predictive value (NPV) of 86.8% and 97.7% respectively. Assessing CRP on POD3 after minimally invasive colorectal surgery resulted clinically relevant to exclude the occurrence of severe complications and allowing early discharge in the context of an enhanced recovery protocol. Interestingly, the CRP threshold was influenced by the patients' body composition. Regardless of the development of post-operative complication, patients with visceral obesity showed higher mean CRP values after minimally invasive colorectal

surgery (92 mg/L vs 71 mg/L in POD3)[86]. The optimal cut-off for identification of post-operative infective complications resulted 136 mg/L on POD3 in non-visceral obese patients, compared to 154 mg/L in those with visceral obesity. These results confirm the importance of body composition and obesity in the maintenance of chronic inflammation.

Furthermore, Catarci et al.[87] assessed 1546 patients undergoing colorectal surgery and evaluated the role of the Dutch leakage score, CRP and procalcitonin in the prediction of anastomotic leak. They observed that the combination of the three parameters reached negative predictive values as high as 99% on POD2, 3, and 6.

The results of these studies demonstrate that CRP is an easily available, unexpensive biomarker to aid early identification of post-operative complications.

AIMS OF THE STUDY

The aim of this study was to evaluate the changes in circulating immune cells before the diagnosis of CRC, and to assess their relationship with survival outcomes.

Moreover, the study aimed to establish whether enhanced peri-operative inflammation influence disease-specific survival and recurrence outcomes of patients submitted to curative surgery for CRC.

MATERIALS AND METHODS

1. Cohort under study and inclusion criteria

All consecutive patients undergoing surgery for CRC at the Department of General and Hepatobiliary Surgery of the Azienda Ospedaliera Universitaria Integrata of Verona were assessed for inclusion.

The longitudinal changes in immune cell profile were evaluated in the whole cohort of patients undergoing surgery for CRC. Since this represented the first part of the project, and a minimum follow-up of 24 months was necessary to inquiry on survival outcomes, patients operated on between January 2005 and December 2020 were included. All stages were considered adequate for inclusion. The clinical data was retrieved from a prospectively maintained database. For each patient a complete blood count dated at least 24 months before surgery was retrieved.

To evaluate the role of peri-operative inflammatory markers on prognosis in a homogeneous cohort, only patients submitted to elective minimally invasive potentially curative (R0-1) surgery for stage I-III CRC between January 2014 and December 2022 were included. A minimum follow-up of 24 months was required for inclusion. Patients undergoing urgent surgery, palliative surgery or surgery for stage IV CRC were excluded.

2. Pre-operative work-up and histopathological staging

All elective patients underwent preoperative staging by colonoscopy, thoracoabdominal computed tomography (CT) scan and tumor markers (carcinoembryonic antigen, CEA; carbohydrate antigen 19-9, CA 19-9). Patients with rectal cancer also underwent pelvic magnetic resonance imaging (MRI) for local staging. Patients with locally advanced rectal cancer received pre-operative chemoradiotherapy or total neoadjuvant therapy as deemed appropriate by multidisciplinary team discussion[88]. Additional diagnostic modalities (e.g. liver MRI, PET-CT) were used when clinically indicated. Patients undergoing urgent surgery were pre-operatively assessed by radiological modalities depending on clinical necessities. All patients underwent preoperative routine laboratory tests.

The specimens were examined through routine histopathological analysis. Tumor staging was assessed according to the 8th Edition of the American Joint Committee on Cancer (AJCC) and the Union International Contre Le Cancer (UICC)[89]. The presence of residual

tumor after resection was described using the AJCC/UICC terminology (R classification). Patients were grouped according to the presence (R2) or absence (R0-R1) of macroscopic residual tumor.

3. Extent of surgery and post-operative care

The main goal of surgery was the complete removal of the tumor (R0 resection), although palliative surgery was carried out in selected cases to treat tumor-related complications. The extent of surgery was planned considering the patient's performance status and age and primary tumor location. Surgical approach (open versus minimally invasive) was based on surgeon's preference and expertise. Patients after neoadjuvant therapy for middle-low rectal cancer usually underwent low anterior resection with formation of protective ileostomy. Anatomical resections with ligation of vessels at their origin were the procedures of choice in order to achieve an adequate lymphadenectomy[90].

Post-operative care was provided according to the Enhanced Recovery After Surgery (ERAS) guidelines starting from 2014, and according to standard care before that date. Post-operative morbidity was defined as any deviation from the expected postoperative course and complications were graded according to the Clavien-Dindo Classification[91]. 30-days morbidity and re-admission rates were registered.

4. Adjuvant treatment and follow-up

Clinical and histopathological data were retrieved from a prospectively maintained database. The analyzed variables included demographic, clinical, surgical, and histopathological characteristics. Patients who died within 30 days from surgery were not considered for survival analysis. Adjuvant chemotherapy was suggested according to the guidelines of the 'Associazione Italiana di Oncologia Medica' (AIOM)[92] and European Society of Medical Oncology (ESMO) guidelines[93–95]. Stage I and II tumors without worrisome features were submitted to follow-up. Adjuvant chemotherapy was proposed to patients with stage III or stage II CRC with worrisome features (i.e., pT4 cancers, occluding and perforated cancers, less than 12 analyzed nodes or presence of poorly differentiated cancers and/or vascular, lymphatic, and/or perineural invasion). Follow-up was conducted

according to the same guidelines, with 6-monthly radiological, clinical, and biochemical evaluation. Colonoscopy was scheduled 1, 3, and 5 years after surgery.

Survival and follow-up data were obtained by collecting outpatient clinical records or by contacting the patients or their relatives. Data about recurrence, status at most recent follow-up, and cause of death were registered. Overall-survival (OS) was defined as the length of time between surgery and death from any cause. Cancer-specific survival (CSS) was measured from the date of surgery to the date of death from CRC, whilst patients who died from causes other than cancer were considered censored at the time of death. Recurrence-free survival (RFS) was defined as the length of time between surgery and the diagnosis of cancer recurrence. Recurrence was defined as multiple if it occurred simultaneously at different sites. Isolated recurrence was classified according to the site of relapse: liver, lung, peritoneum, locoregional (locoregional lymph-nodes, tumor bed, or anastomosis).

5. Assessment of pre-diagnosis and peri-operative blood tests

Data regarding pre-diagnosis blood tests were obtained by querying the Medical Laboratory database system into which all laboratory results were stored. Blood samples associated with admission to the Emergency Department or with clear evidence of an ongoing infection were excluded. Patients with autoimmune disorders and receiving corticosteroids or immunosuppressive/immunomodulatory treatments were also excluded. For each patient operated on within the inclusion period, one complete blood count (CBC) with formula dated at least 24 months before surgery was obtained. When more than one blood test was available, we recorded the closer to diagnosis. Pre-NLR (Pre-NLR) was calculated by dividing the absolute number of Pre-neutrophils (Pre-Neut) by the absolute number of Pre-lymphocytes (Pre-Lymph). The CBC was performed using Advia 2120 (Siemens Healthcare Diagnostics, Tarrytown NY, USA). The local reference ranges are 4.3– 10.0 × 10^9/L for total leucocytes, 2.0–7.0 × 10^9/L for neutrophils, and 0.95–4.5 × 10^9/L for lymphocytes. The same analyzer was used throughout the study period, and the quality and reproducibility of test results was validated by data of both internal quality control and external quality assessment.

Data regarding peri-operative blood tests were prospectively recorded in a clinical database. Pre-operative values corresponded to the blood tests drawn at the pre-operative

assessment. Post-operative values were registered at each POD during hospital stay or within 30 days from surgery if the length of stay was longer than 30 days. mGPS was assessed from pre-operative values according to the literature[28]. Post-operative mGPS was assessed on POD3 according to the definition provided by Watt et al[44]. NLR was calculated dividing the pre-operative absolute value of neutrophiles by the pre-operative absolute value of lymphocytes. CRP was registered for each POD up to day 5, and according to clinical need thereafter. The local reference normal value is < 5 mg/L. Starting from March 2014, patients were managed according to the ERAS protocol[96,97]. During the implementation phase, CRP was measured on POD1, POD3, and POD5, and discharge was planned based on clinical condition and CRP value on POD5. Following the study on the optimal CRP cut-off for the exclusion of severe complications[98], CRP was assessed on POD1, POD2, and POD3, and discharge was planned if CRP on POD3 was < 120 mg/dL and the patient fulfilled the other discharge criteria (pain controlled with oral analgesia, able to walk, able to tolerate solid food, bowel open to gas and stools).

6. Statistical analysis

Continuous data were presented as means ± standard deviations (SD) or medians (ranges), as appropriate. Categorical data were presented as frequencies.

All parameters of Pre-CBC were tested for potential associations with survival after surgery, and those with adequate numerosity and significant associations are presented in the results (Pre-leukocytes (Pre-Leu), Pre-Neut, Pre-Lymph, Pre-NLR). Multivariate analysis of OS and CSS was performed using Cox regression. We fitted an individual Cox regression model for Pre-Leu, Pre-Neut, Pre-Lymph and Pre-NLR adjusting for the following covariates: age, gender (female vs. male), tumor location (rectum vs. colon), histological type (mucinous vs. non-mucinous), setting of surgery (elective vs. urgent), and AJCC/UICC TNM stage (stage II and stage III and stage IV vs. stage I). Pre-Leu, Pre-Neut, Pre-Lymph, Pre-NLR and age were treated as continuous variables. Prior to fitting the Cox regression model, the distribution of the continuous parameters was inspected and patients with outlier values were removed from the analysis after initial inclusion. Martingale residuals were used to assess the functional form of the continuous covariates and to assess the overall fit of the Cox model. nonlinearity of the parameters was addressed using spline terms. To choose the model, the goodness-of-fit was assessed using the likelihood ratio test between models with or without spline terms for the continuous parameters. Pre-Leu, Pre-

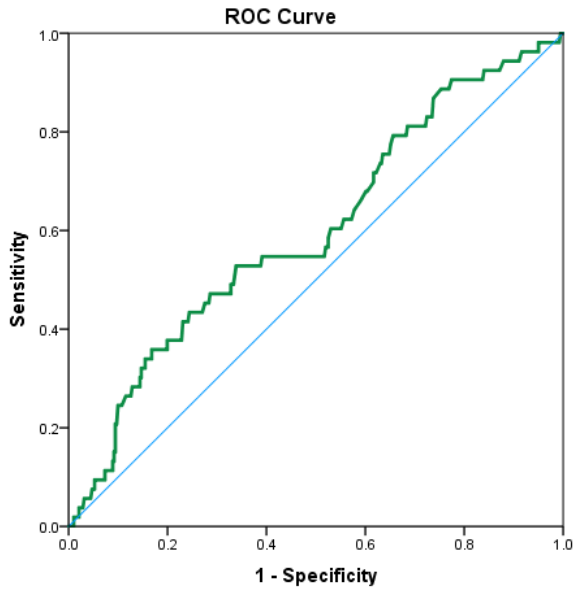
Neut, Pre-NLR and age were treated as simple linear covariates while Pre-Lymph were modeled with a linear spline with a single knot located in the median. The proportional hazards assumption was checked using the Schoenfeld residual-based test. The variable 'intent of surgery (R0, R1, R2)' was stratified in each Cox model because it violated the proportional hazard assumption. Multicollinearity was checked by computing the variance inflation factors (VIF). The significance of the parameters was assessed through the analysis of variance (ANOVA) considering a p-value < 0.05 statistically significant. Hazard ratios (HR) for continuous parameters were computed as interquartile range (IQR) effects. A subsequent analysis was conducted considering the interval between CBC sample and surgery. The variable ranged between 24 and 180 months, therefore it was sub-categorized into 4 groups: 24-36 months, 37-60 months, 61-120 months, and >120 months before surgery. Survival curves were built estimating the survival probability of the patients in the different time intervals before surgery and considering the 1st quartile, median and 3rd quartile of Pre-Leu, Pre-Neut, Pre-Lymph and Pre-NLR. To accomplish this task, the parameter 'months before surgery' was included in the Cox model for this analysis.

Considering peri-operative inflammation, mGPS was assessed at baseline as described by McMillan et al[49]. We also explored the validation of post-operative mGPS as described by Watt et al.[44] on POD3(poGPS3). The optimal cut-off of NLR (<3 versus ≥3) as dichotomous predictor of survival was chosen based on previous experience and literature[76,77,99,100].

The optimal cut-off value for post-operative CRP as dichotomous predictor of RFS was chosen after receiver operating characteristics (ROC) curve analysis using disease recurrence as outcome. ROC curve analysis was performed for CRP values measured on each POD1, POD3, and POD5 and for the maximum post-operative value (CRP_{max}). The best performing ROC curve was that considering CRP_{max}, which showed the highest area under the curve (AUC) and the smallest standard error. For this reason, patients were categorized in two groups based on the maximum value of post-operative CRP using a cut-off of 151.5 mg/L (Youden Index 0.19, sensibility 52.8%, specificity 66.1%): a High CRP_{max} group (H-CRP) and a Low CRP_{max} group (L-CRP).

	AUC	Standard error
CRP_{max}	0.599	0.019
CRP POD1	0.554	0.045
CRP POD3	0.579	0.043

CRP POD5	0.569	0.054



ROC curve for CRPmax value using disease recurrence as outcome.

The association between CRP_{max} and clinical-pathological variables was assessed through chi-squared test for categorical variables and independent t test or Mann–Whitney U test for continuous variables, as appropriate. Survival analysis was computed using the Kaplan–Meier method and compared by the log-rank test, stratifying for CRP group. Sub-group analysis was conducted stratifying for TNM stage. Multivariate analysis was performed by Cox regression model considering CRP_{max} value and correcting for relevant risk factors: age, gender (male vs. female), comorbidity (yes vs. no), tumor location (rectum vs. right colon vs. left colon), occurrence and severity of post-operative complications, presence of vascular or lymphatic invasion, grading.

A p-value <0.05 was considered statistically significant. The statistical analysis was performed with SPSS software (v23.0, IBM Corporation, Armonk, NY) and the R (v4.1.0) programming language and functions of the 'rms' and 'survival' (v3.2-11) packages. The figures were generated using the R packages 'rms', 'ggplot2' (v3.3.3), 'ggpubr' (v0.4.0), 'MASS' and 'scales'.

RESULTS

1. PRE-DIAGNOSIS INFLAMMATORY MARKERS

Cohort under study

From an initial cohort of 1674 patients undergoing urgent and elective surgery for CRC, we could retrieve pre-diagnosis full blood count values for 334 patients. The median interval (interquartile range, IQR) between the pre-diagnosis blood sample and surgery was 50 (36 – 84) months.

Table 1 describes the clinico-pathological characteristics of the cohort under study. Median age (range) at diagnosis was 70.1 (27.9 – 91.6) years, in line with literature. Most of the CRC were localized in the right colon (n = 135, 40.4%) followed by the rectum (n = 129, 38.6%). Stages were almost equally distributed within the cohort. Most patients underwent potentially curative surgery (R0 n = 290, 86.8%; R1 n = 8, 2.4%). 72 patients (21.6%) presented mucinous histology.

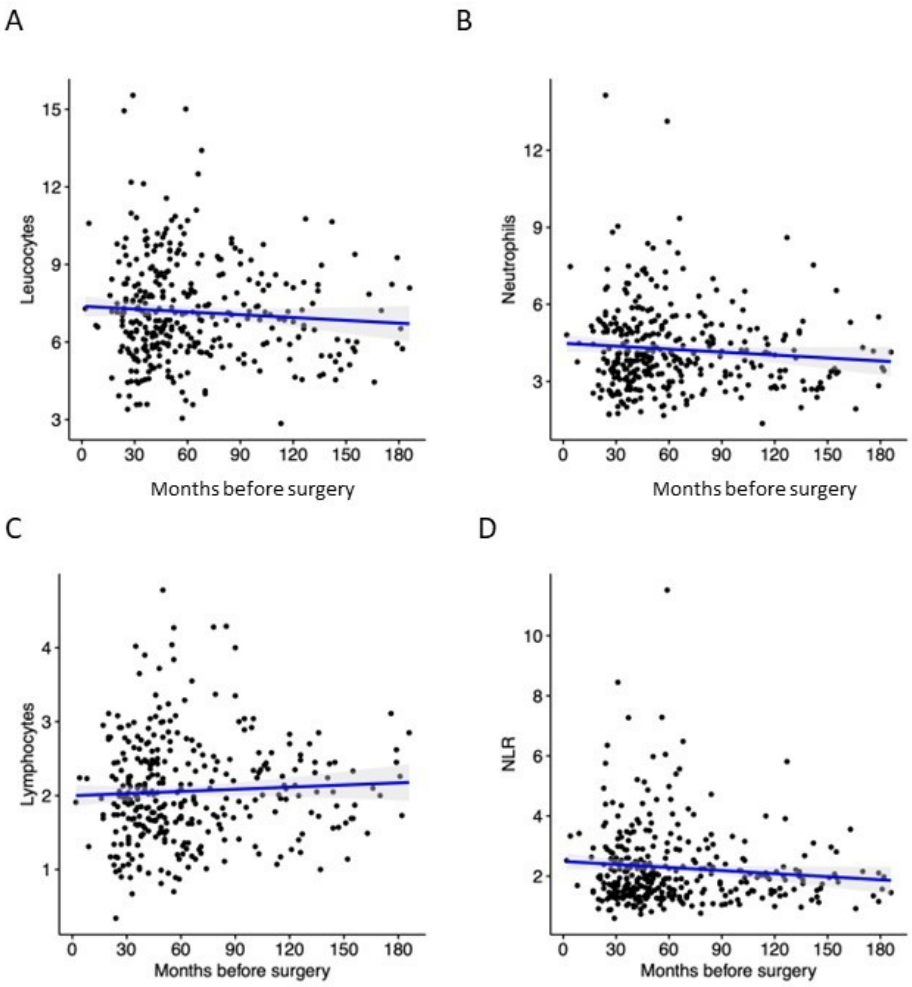
Table 1 also presents the mean values of pre-diagnosis parameters, which were all within normal ranges.

	Patients	**Data**
Age, median (range)	334	70.1 (27.9 – 91.6)
Gender	334	
Male		204 (61.1)
Female		130 (38.9)
CEA, median (range)	195	2.6 (0.1 – 1276.8)
Tumor location	334	
Right colon		135 (40.4)
Left colon		70 (21.0)
Rectum		129 (38.6)
Type of surgery, elective	334	313 (93.7)
Intent of surgery, curative (R0-1)	334	298 (89.2)
Stage	334	
I		96 (28.7)
II		98 (29.3)
III		84 (25.1)
IV		56 (16.8)
Histology, adenocarcinoma	334	262 /78.4)

Grading, high grade (WHO 2019)	262	34 (10.2)
Pre-diagnosis complete blood count		
Hb (g/dL), mean (SD)	334	14.2 (1.7)
Platelets, mean (SD)	334	253.9 (75.8)
RDW, mean (SD)	334	13.6 (1.2)
Leukocytes, mean (SD)	334	7.2 (1.9)
Neutrophils, mean (SD)	334	4.2 (1.6)
Lymphocytes, mean (SD)	334	2.1 (0.7)
NLR, mean (SD)	334	2.3 (1.3)

Longitudinal changes in blood count

The interval between Pre-CBC and surgery varied depending on the availability of former blood test. The range varied from a minimum of 24 months to a maximum of 180 months. We analyzed temporal distribution of the different element of Pre-CBC, which is depicted in Figures 1A–D. Despite the correlation index being quite low for all parameters, it should be noted that the value of Pre-Leu, Pre-Neut and Pre-NLR tended to be higher close to surgery, while the value of Pre-Lymph showed an opposite trend.

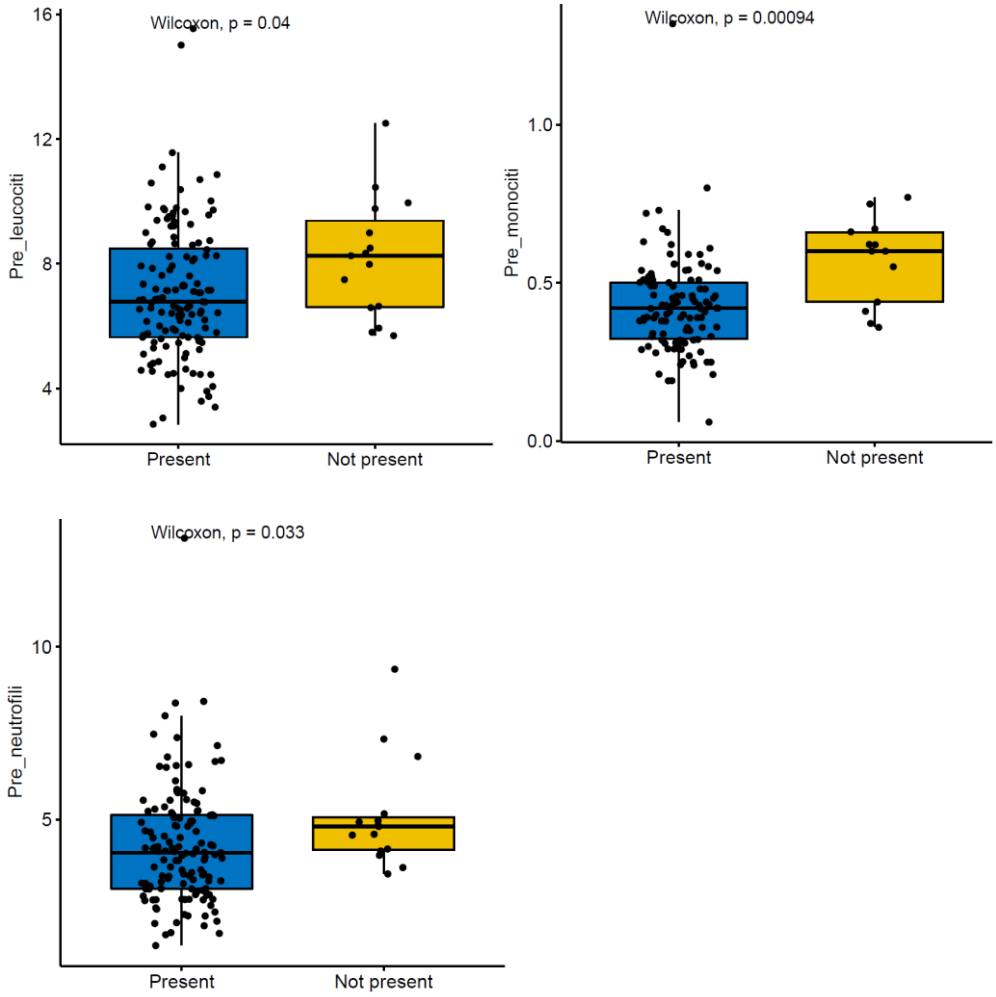


Distribution Pre-Leu (A), Pre-Neut (B), Pre-Lymph (C), and Pre-NLR (D) according to the interval between blood sampling and surgery. Black dots correspond to the discrete value of each patient, while the blue line represents the trend according to linear regression.

Correlation between Pre-CBC values and pathological outcomes

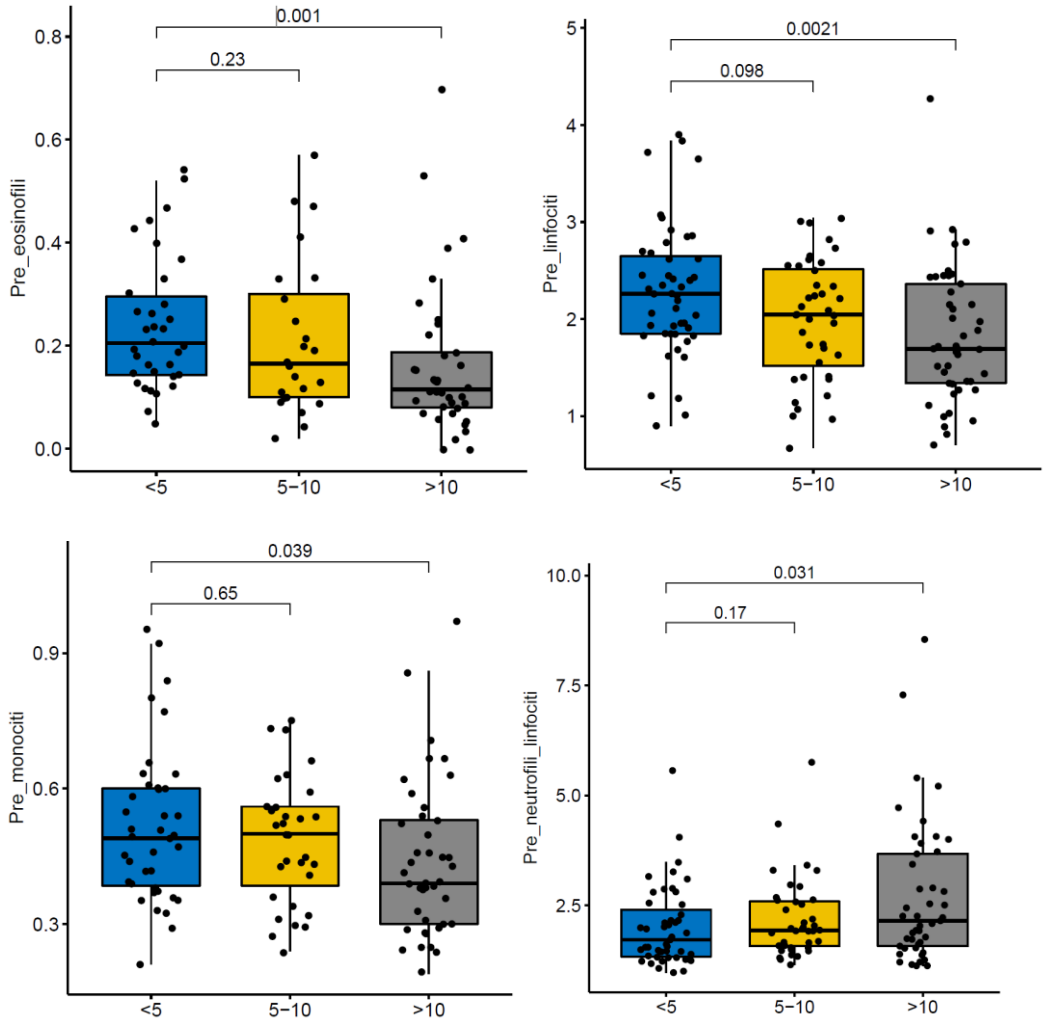
Pre-CBC values were tested for potential association with known negative pathological prognostic factors. We hereby report only statistically significant associations.

Lower Pre-Leuc ($p = 0.04$), Pre-Mono ($p < 0.001$), and Pre-Neut ($p = 0.033$) count were associated with the presence of vascular invasion on pathological examination.



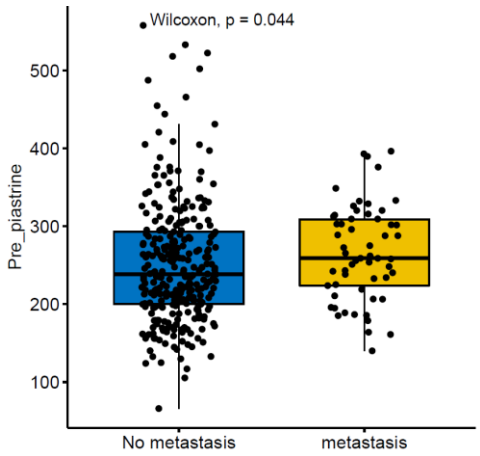
Box plots comparing median values of Pre-Leuc, Pre-Mono, and Pre-Neut according to the presence of vascular invasion.

Patients with lower Pre-Eosinophils (p = 0.001), Pre-Lymph (p = 0.002), and Pre-Mono (p = 0.039) count showed higher tumor budding. On the other hand, cases with higher Pre-NLR (p = 0.031) showed more frequently tumor budding foci > 10/0.785 mmq.



Box plots comparing median values of Pre-Eosinophils, Pre-Lymph, Pre-Mono, and Pre-Neut according to the grading of tumor budding.

The presence of high platelet count pre-diagnosis was instead associated with an increased risk of metastatic disease, though the level of significance was low ($p = 0.044$).



Box plots comparing median values of Pre-Platelets according to the presence of metastatic disease at diagnosis.

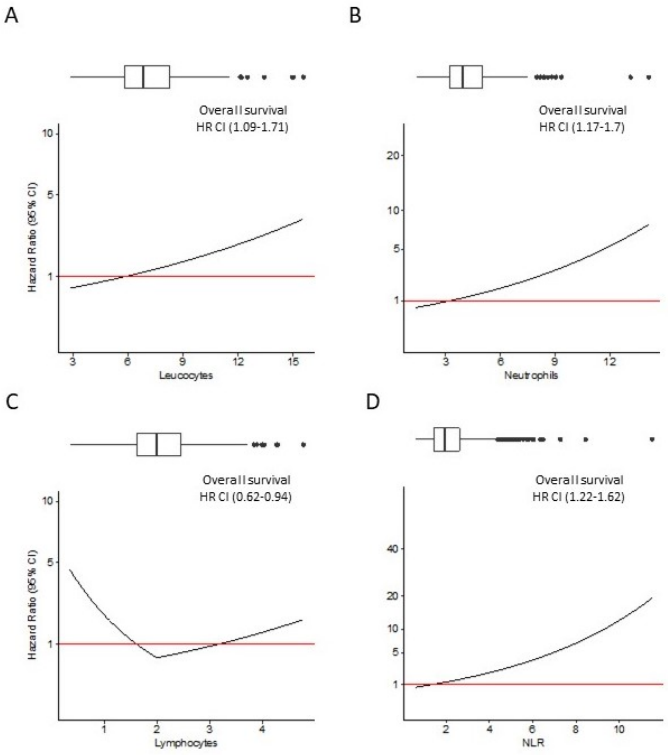
Pre-CBC values as prognostic factors

All parameters of Pre-CBC were tested for potential associations with survival through multivariable analysis. An individual Cox regression was conducted for Pre-Leu, Pre-Neut, Pre-Lymph and Pre-NLR adjusting for age, gender, tumor location, histological type, surgery setting, and AJCC/UICC pTNM stage. All the parameters of Pre-CBC resulted to be independent prognostic factors for OS and CSS. Other significant prognostic factors were age for OS (p<0.001), and TNM stage for both OS and CSS (p<0.001).

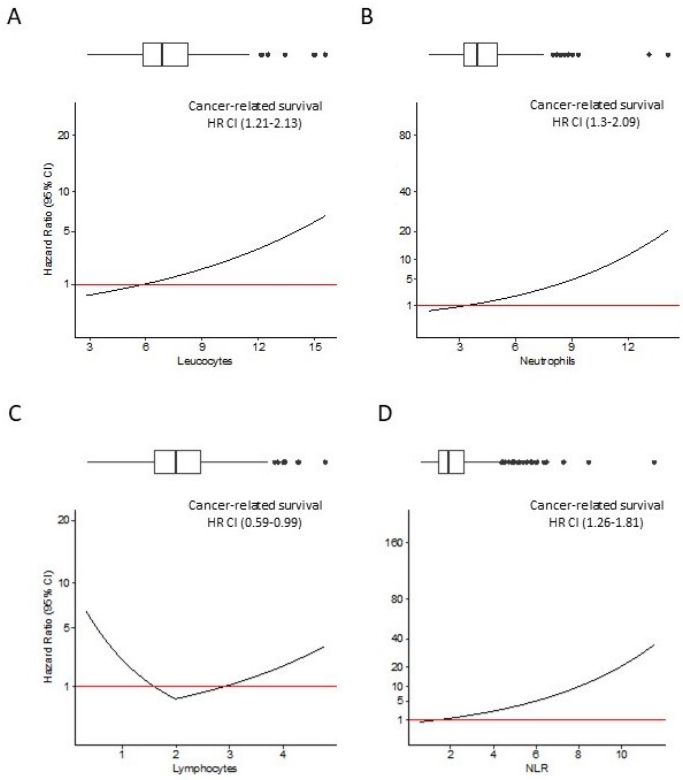
	OS HR (95% CI)	**p value**	**CSS HR (95% CI)**	**p value**
Age	2.03 (1.47 – 2.79)	**<0.001**	1.36 (0.92 – 2.01)	0.13
Gender		0.99		0.49
Male	-		-	
Female	1.00 (0.67 – 1.48)		1.20 (0.71 – 2.00)	
Tumor location		0.47		0.56
Colon	-		-	
Rectum	0.83 (0.50 – 1.38)		0.83 (0.44 – 1.57)	
Type of surgery		0.07		0.24
Elective	-			
Urgent	1.81 (0.95 – 3.47)		1.77 (0.68 – 4.56)	
Stage		**<0.001**		**<0.001**
I	-		-	
II	1.68 (0.87 – 3.25)		0.91 (0.32 – 2.59)	
III	2.88 (1.51 – 5.50)		3.68 (1.52 – 8.89)	
IV	6.1 (2.89 – 12.87)		9.18 (3.51 – 24.06)	
Histological type		0.10		0.36

Adenocarcinoma, NOS	-		-	
Mucinous histotype	0.61 (0.36 – 1.04)		0.68 (0.35 – 1.32)	
Pre-NLR*	1.41 (1.22 – 1.62)	**<0.001**	1.51 (1.26 – 1.81)	**<0.001**
Pre-Leukocytes*	1.37 (1.09 – 1.71)	**0.006**	1.61 (1.121 – 2.13)	**<0.001**
Pre-Neutrophils*	1.41 (1.17 – 1.70)	**0.003**	1.65 (1.30 – 2.09)	**<0.001**
Pre-Lymphocytes*	0.76 (0.62 – 0.94)	**<0.001**	0.76 (0.59 – 0.99)	**<0.001**

Pre-Leu, Pre-Neut, Pre-Lymph and Pre-NLR were tested as continuous variables, therefore the hazard ratio (HR, 95% CI) deriving from multivariable analysis was plotted against the values of the parameters. The figures reported below show the trend of the HR for OS and CSS. Both Figures show a progressive rise in the HR with increasing values of Pre-Leu, Pre-Neut, and Pre-NLR. Interestingly, the curve is particularly steep for Pre-NLR as soon as the value exceeds the threshold of 3. On the other hand, the HR associated with Pre-Lymph shows a dual trend, consisting of a sharp increase in HR for values below 1.5 x 10^9/L, but also a moderate rise for values above 3 x 10^9/L.



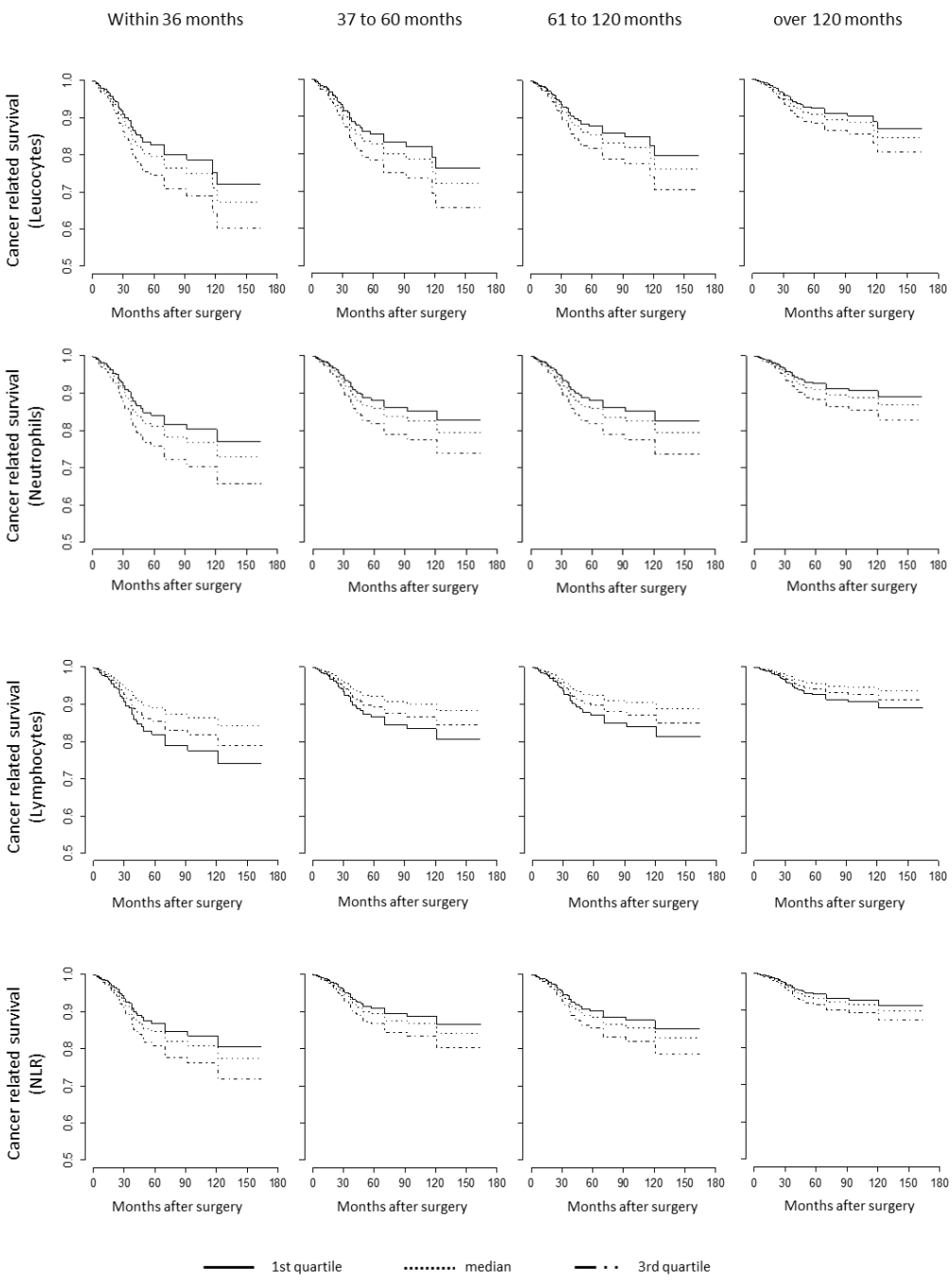
OS analysis. Hazard ratio (HR) resulting from Cox regression model including Pre-Leu (A),Pre-Neut (B),Pre-Lymph (C),and Pre-NLR (D) adjusting for the following covariates: age, gender, tumor location, histological type, setting of surgery, AJCC/UICC TNM stage, and presence of residual tumor. Values in parenthesis are 95% confidence intervals (CI) for HR. Box plots on top of each figure represent the distributions of each parameter.



CSS analysis. Hazard ratio (HR) resulting from Cox regression model including Pre-Leu (A), Pre-Neut (B), Pre-Lymph (C), and Pre-NLR (D) adjusting for the following covariates: age, gender, tumor location, histological type, setting of surgery, AJCC/UICC TNM stage, and presence of residual tumor. Values in parenthesis are 95% confidence intervals (CI) for HR. Box plots on top of each figure represent the distributions of each parameter.

Correlation between pre-diagnosis interval time and survival outcomes

To further evaluate the interaction between pre-diagnosis interval and survival, the interval between Pre-CBC sample and surgery was sub-categorized into 4 groups (24-36 months, 37-60 months, 61-120 months, and >120 months before surgery). Survival curves for CSS were built estimating the survival probability of the patients in different time intervals and considering the 1st quartile, median and 3rd quartile of Pre-Leu, Pre-Neut, Pre-Lymph and Pre-NLR. As depicted in the Figure, CSS of patients with Pre-Leu, Pre-Neut and Pre-NLR in the 3rd quartile was worse than that of patients with median and 1st quartile values, regardless of the interval between pre-diagnosis sample and surgery. On the contrary, patients with Pre-Lymph values in the lowest (1st) quartile showed worse CSS after surgery, compared to the median and 3rd Quartile. Interestingly, differences between curves were more pronounced when the Pre-CBC sample was taken between 24 and 36 months before surgery, compared to patients whose Pre-CBC dated over 120 months before diagnosis.

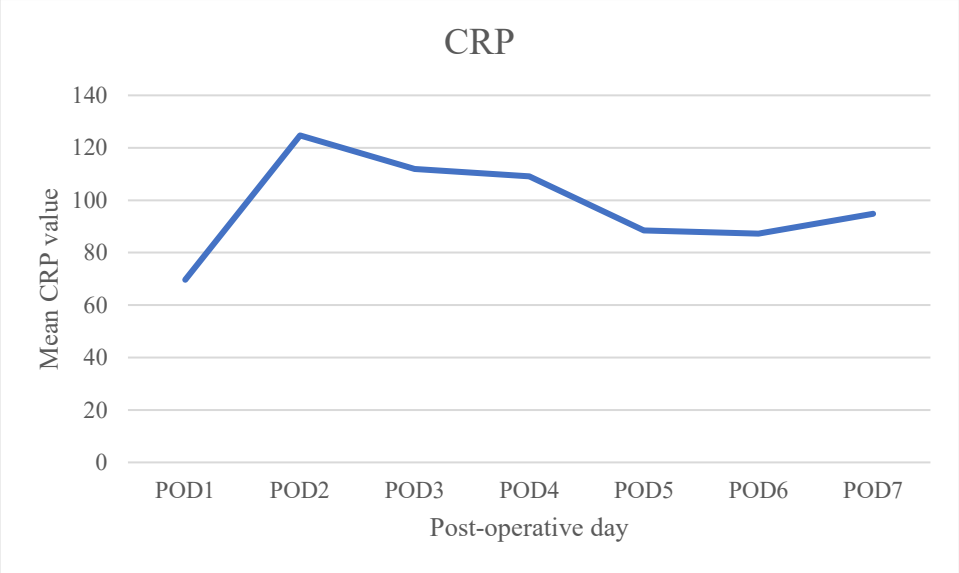


CSS analysis. Survival probability resulting from Cox regression model including (A) Pre-Leu, (B) Pre-Neut, (C) Pre-Lymph, and (D) Pre-NLR in 4 intervals between Pre-CBC and surgery (24-36 months, 37-60 months, 61-120 months, and >120 months before surgery). The model was adjusted for the following covariates: age, gender, tumor location, histological type, setting of surgery, AJCC/UICC TNM stage, and presence of residual tumor.

2. PERI-OPERATIVE INFLAMMATION

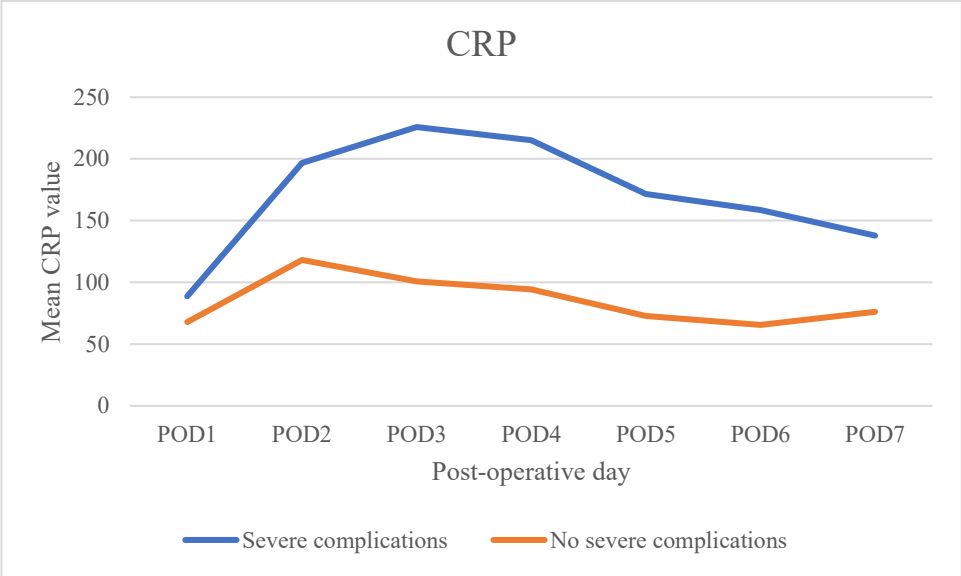
From an initial cohort of 879 CRC patients undergoing surgery between January 2014 and December 2022, 443 patients were excluded due to: open surgery (n = 338), urgent surgery (n = 60), or stage IV CRC (n = 181). The final cohort included 436 stage I-III CRC patients undergoing elective minimally invasive surgery.

Distribution of post-operative CRP



Distribution of post-operative CRP in the whole cohort.

As previously observed in our cohort[98] and in the literature[101,102], the trajectory of CRP raises from POD1 to POD2 and then shows a decreasing trend. Since most of the patients with an uneventful post-operative course get discharged by POD5, the apparent increase seen after that day may be related to the presence of patients still admitted because of post-operative complications.



Distribution of post-operative CRP according to the development of severe complications.

As previously discussed, CRP represents an acute phase protein and aids the identification (or exclusion) of severe post-operative complications. As shown in the Figure, CRP peaks on POD2 in patients without severe complications, while it steeply increases to a higher and delayed peak in those who develop severe complications.

Correlation with clinical and demographic characteristics

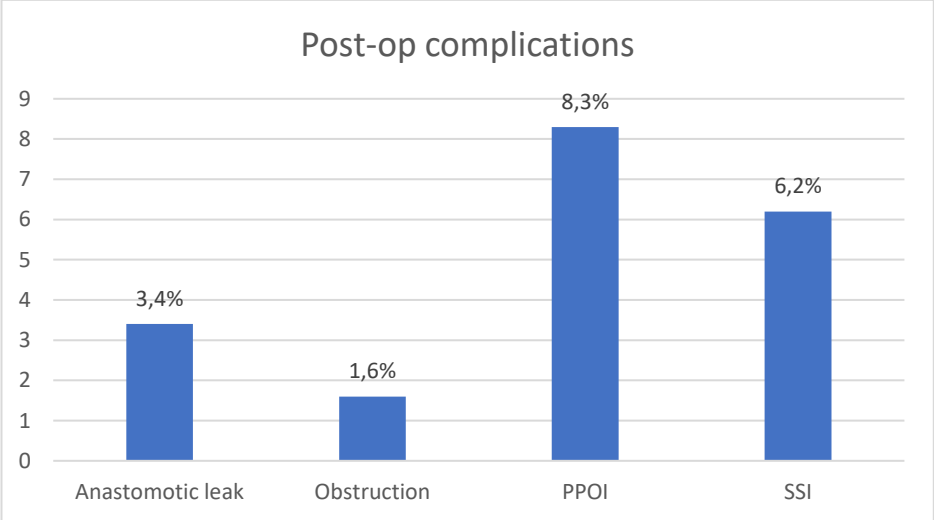
The table below reports baseline demographic characteristics according to the post-operative CRP group (L-CRP vs H-CRP). As seen in the table, mean age in the cohort was 68.6 years, and it was statistically higher in the H-CRP group ($p < 0.001$). The most frequent indication to surgery was CRC of the right colon, and 45.6% of patients already had previous abdominal surgery.

Patients in the H-CRP group showed a higher proportion of right colon cancer and a higher BMI compared to the L-CRP group. Moreover, there was a higher rate of patients with comorbidities and ASA score equal or above 3. Considering the other peri-operative immune-related markers, patients in the H-CRP showed higher baseline NLR as well as a higher percentage of mGPS equal to 2. With regards to poGPS3, no patients in our cohort showed a score of 2. Quite expectedly, poGPS3 value was almost always 0 in L-CRP group, while most of the patients in the H-CRP group showed poGPS3 equal to 1 ($p < 0.001$).

	N	Total	L-CRP (n=279)	H-CRP (n=157)	p-value
Age, mean ± SD	436	68.6 ± 12.2	67.8 ± 13.1	71.4 ± 11.2	**<0.001**
Gender	436				0.31
Male		243 (55.8%)	150 (53.7%)	93 (59.2%)	
Female		193 (44.2%)	129 (46.3%)	64 (40.8%)	
Tumor location	436				**0.023**
Right colon		187 (42.9%)	109 (39.1%)	78 (49.7%)	
Left colon		137 (31.4%)	100 (35.8%)	37 (25.6%)	
Rectum		112 (25.7%)	70 (25.1%)	42 (26.7%)	
ASA score ≥ 3	436	128 (28.9%)	71 (25.4%)	55 (35.0%)	**0.036**
Comorbidities	436	210 (48.2%)	122 (43.7 %)	88 (56.1%)	**0.005**
Previous surgery	436	199 (45.6%)	128 (45.9%)	71 (45.2%)	0.79
BMI, mean ± SD	436	25.8 ± 4.5	24.9 ± 4.3	26.1 ± 4.1	**0.015**
CEA, median (IQR)	240	2.9 (1.7 - 8.9)	2.8 (1.7 – 8.8)	2.9 (1.9 – 8.9)	0.24
NLR, mean ± SD	436	3.0 ± 1.9	3.2 ± 1.9	3.5 ± 2.3	**0.019**
Pre-operative CRP, median (IQR)	292	3 (7)	2 (4.75)	3 (10)	0.102
mGPS	292				**0.005**
0-1		274 (93.8%)	174 (97.2%)	100 (88.5%)	
2		18 (6.2%)	5 (2.8%)	13 (11.5%)	
poGPS3	410				**<0.001**
0		309	257 (99.6%)	54 (34.2%)	
1		101	1 (0.4%)	100 (65.8%)	

Correlation with surgical outcomes and post-operative recovery

Considering the whole cohort, conversion to open surgery was required in 6% of cases. No patients suffered intra-operative complications, and estimated median intra-operative blood loss (EBL) was below 50 ml in most of the cases. Post-operative complications occurred in 40.8% of patients, but major complications accounted for less than 10%. Anastomotic leak rate in the cohort was 3.4% as depicted in the Figure below.



Patients in the H-CRP group showed significantly longer duration of surgery ($p < 0.001$), higher rates of conversion ($p = 0.002$), higher EBL ($p < 0.001$), higher proportion of patients requiring post-operative ICU stay ($p = 0.05$) and blood transfusion ($p = 0.003$), higher rates of complications ($p < 0.001$), and longer length of stay ($p < 0.001$). Functional recovery was quicker in the L-CRP group.

	N	Total	L-CRP (n=279)	H-CRP (n=157)	p-value
Length of surgery, mins, mean ± SD	436	246.3 ± 83.6	225.9 ± 66.5	261.1 ± 66.5	**<0.001**
Conversion	436	26 (6.0%)	9 (3.2%)	17 (10.8%)	**0.002**
Estimated intra-op blood loss, median (IQR)	436	40 mL (20-80 mL)	40 mL (20-60)	50 mL (30-120)	**<0.001**
ICU post-operative stay	436	78 (17.9%)	42 (15.1%)	36 (22.9%)	**0.05**
Post-operative complications	436	178 (40.8%)	78 (28.0%)	100 (63.7%)	**<0.001**
Major complications (CD ≥ 3)	436	36 (8.3%)	7 (9.0%)	30 (30.0%)	**<0.001**
RBC transfusions	434	50 (11.5%)	22 (8.0%)	28 (17.8%)	**0.003**
Need for redo surgery	436	28 (6.4%)	4 (1.4%)	24 (15.3%)	**<0.001**
Post-op length of stay, median (IQR)	436	5.5 (4 – 8)	4 (3 – 8)	7 (3.5 – 17)	**<0.001**
Functional recovery					
Gas open POD1	431	259 (59.4%)	184 (67.2%)	75 (48.1%)	**<0.001**
Stools open POD2	428	238 (54.6%)	177 (65.3%)	60 (38.5%)	**<0.001**
Walking POD1	354	121 (27.8%)	99 (45.4%)	22 (16.3%)	**<0.001**
Solid diet POD1	431	225 (51.6%)	168 (61.1%)	57 (36.8%)	**<0.001**

Correlation with histopathological findings

Stages were almost equally distributed in the whole cohort as well as the CRP groups. The mean number of harvested nodes was adequate and only 7.8% of specimens contained less than 12 lymph-nodes. The most frequent histotype was adenocarcinoma not otherwise specified (NOS) (82.3%) followed by mucinous adenocarcinoma (16.1%). No differences in histopathological characteristics were observed between L-CRP and H-CRP group. The only statistically significant difference was noted on the distribution on pT staging. Patients in the H-CRP group showed a higher proportion of high pT stage. Interestingly, the presence of lymph vascular invasion was higher in the H-CRP group, while peritumoral

inflammatory infiltrate was more frequent in the L-CRP group, though the difference was not statistically significant.

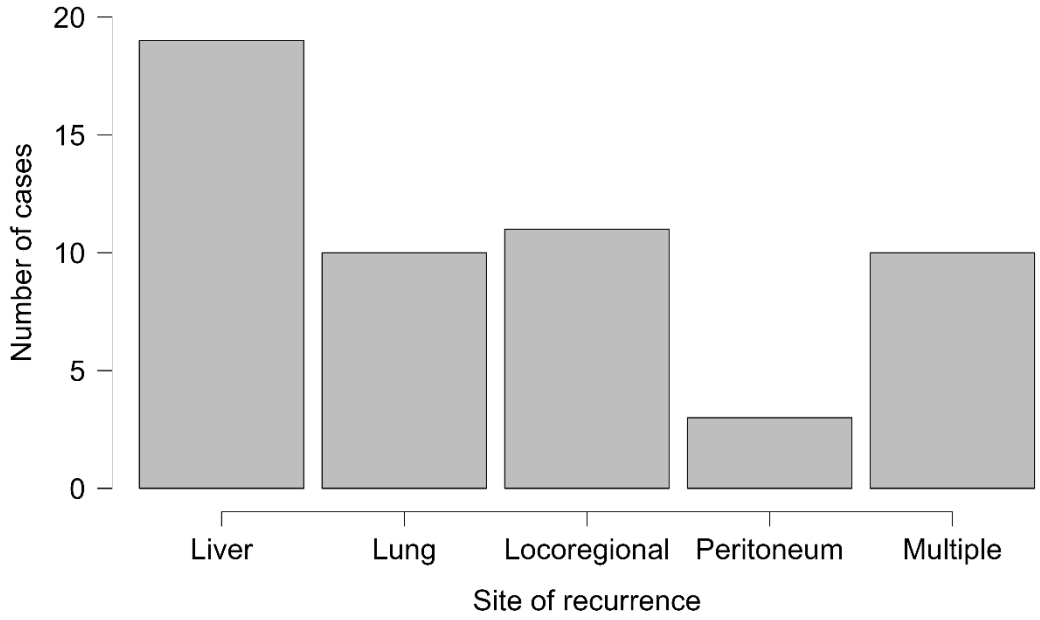
	N	Total	L-CRP (n=279)	H-CRP (n=157)	p-value
Stage	436				0.19
I		168 (38.5%)	116 (41.6%)	52 (33.1%)	
II		136 (31.2%)	81 (29.0%)	55 (35.0%)	
III		132 (30.3%)	82 (29.4%)	50 (31.9%)	
pT	436				**0.046**
pT1		117 (26.8%)	81 (29.0%)	36 (22.9%)	
pT2		79 (18.1%)	56 (20.1%)	23 (14.7%)	
pT3		186 (42.7%)	115 (41.2%)	71 (45.2%)	
pT4		54 (12.4%)	27 (9.7%)	27 (17.2%)	
pN	436				0.29
pN0		304 (69.7%)	197 (70.6%)	107 (68.1%)	
pN1		99 (22.7%)	65 (23.3%)	34 (21.7%)	
pN2		33 (7.6%)	17 (6.1%)	16 (10.2%)	
Number of harvested lymph-nodes, mean ± SD	436	24 ± 12	25.5 ± 12.2	25.1 ± 12.3	0.432
Less than 12 harvested lymph-nodes	436	34 (7.8%)	20 (7.2%)	14 (8.9%)	0.578
Tumor size, mm, mean ± SD	268	39.5 ± 20.9	38.1 ± 21.3	41.9 ± 20.3	0.067
Histotype	436				0.62
Adenocarcinoma		359 (82.3%)	229 (82.1%)	130 (82.8%)	
Mucinous		70 (16.1%)	44 (15.8%)	26 (16.6%)	
Other		7 (1.6%)	6 (2.1%)	1 (0.6%)	
Grading	368				0.49
G1		72 (19.5%)	47 (20.2%)	25 (18.5%)	
G2		261 (71.0%)	161 (69.1%)	100 (74.1%)	
G3		35 (9.5%)	25 (10.7%)	10 (7.4%)	
Vascular or lymphatic invasion	337	224 (66.5%)	135 (48.4%)	89 (56.7%)	0.09
Tumor budding, present	295	180 (61.0%)	118 (61.8%)	62 (59.6%)	0.803
Inflammatory infiltrate present	218	170 (78.0%)	111 (81.0%)	59 (72.8%)	0.178

Correlation between post-operative CRP, recurrence and survival

Of the 436 included patients, 38 (8.7%) died during follow-up. Mean follow-up time was 33.8 months (SD 18.8 months). Survival outcomes in the whole cohort were as follows: 85.0% 5-years OS, 82.6% 5-years RFS, and 95.0% 5-years CSS.

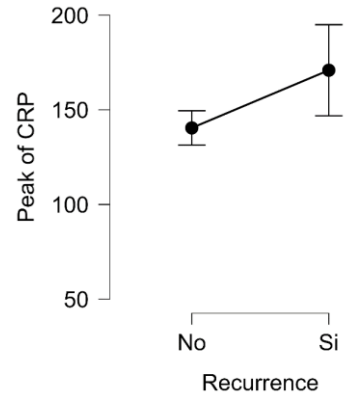
53 patients (12.2%) recurred during follow-up. The most frequent site of recurrence was the liver (n = 19, 4.4%), followed by locoregional recurrences (n = 11, 2.5%), lung (n = 10, 2.3%), multiple (n = 10, 2.3%), and peritoneal recurrences (n = 3, 0.7%). Recurrences

occurred in 25 patients (9.0%) in the L-CRP group and 28 (17.8%) in the H-CRP group (p = 0.007).



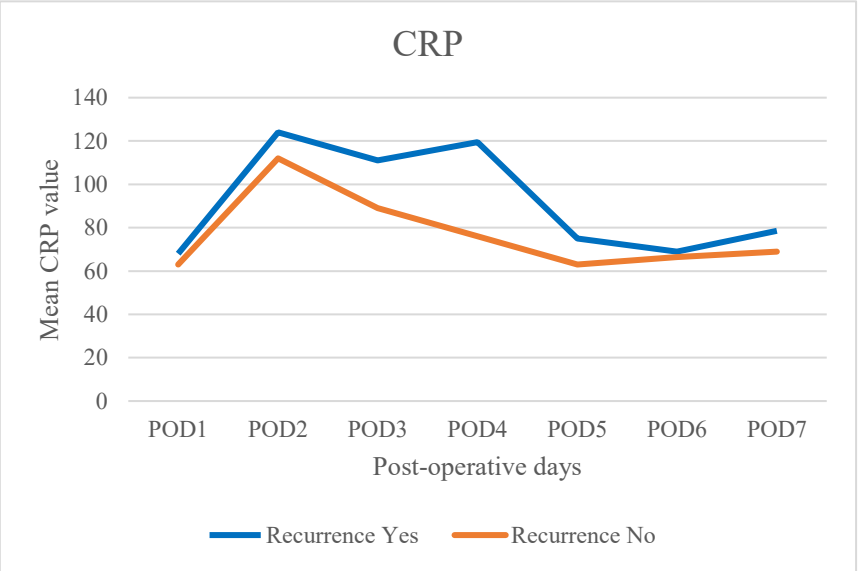
Distribution of recurrences in the whole cohort.

Considering two groups of patients based on the recurrence status, it is interesting to note that the peak of CRP differed significantly: the mean value was 136.8 ± 93.5 mg/L in patients who did not recur compared to 170.8 ± 102.4 mg/L in those who recurred (p = 0.019).



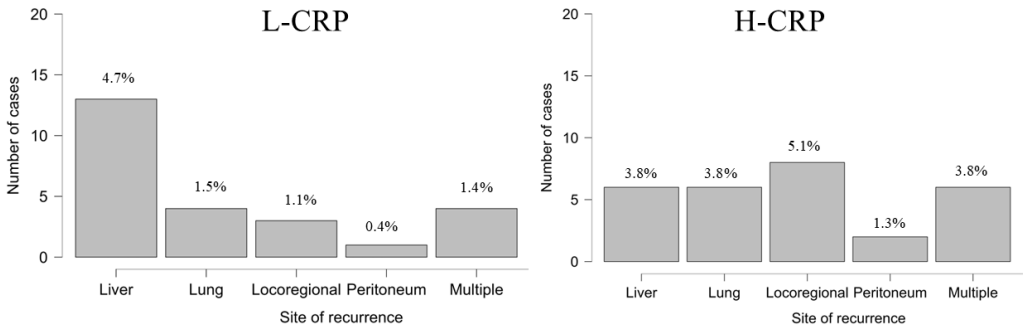
Mean value of post-operative CRP according to the development of recurrence.

Moreover, looking at the distribution of mean CRP values throughout post-operative days, we can observe that patients who recurred showed a slightly higher peak on POD2. Finally, the value remained higher on POD3 (111 mg/L versus 89 mg/L) and POD4 (119.5 mg/L versus 76 mg/L).



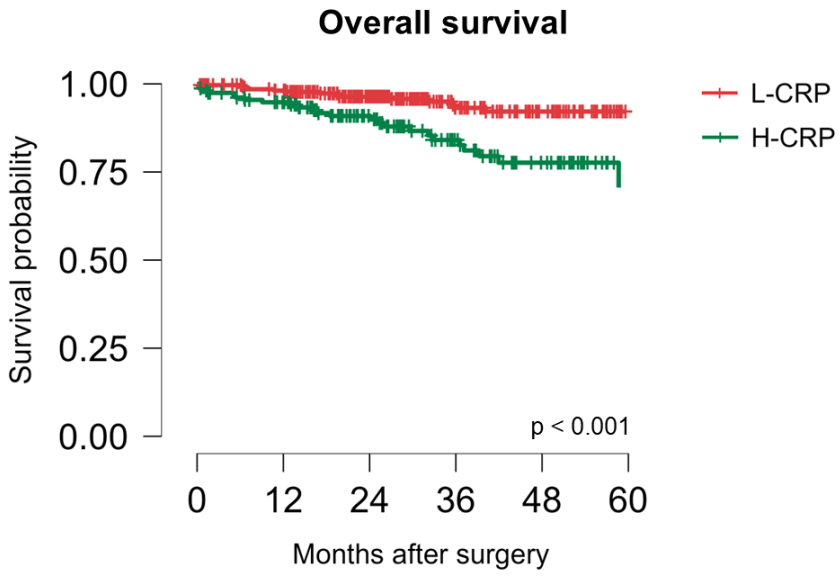
Trend of post-operative CRP according to the development of recurrence.

Considering the recurrence site, the distribution varied significantly between L-CRP and H-CRP ($p = 0.018$). Patients in the H-CRP group showed a higher proportion of locoregional, peritoneal, and multiple metastases.

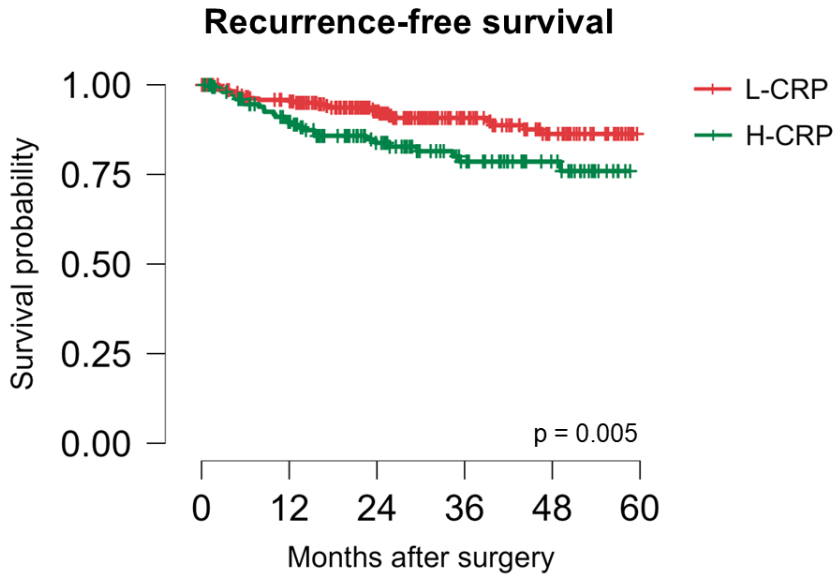


Recurrence sites in the L-CRP and H-CRP groups.

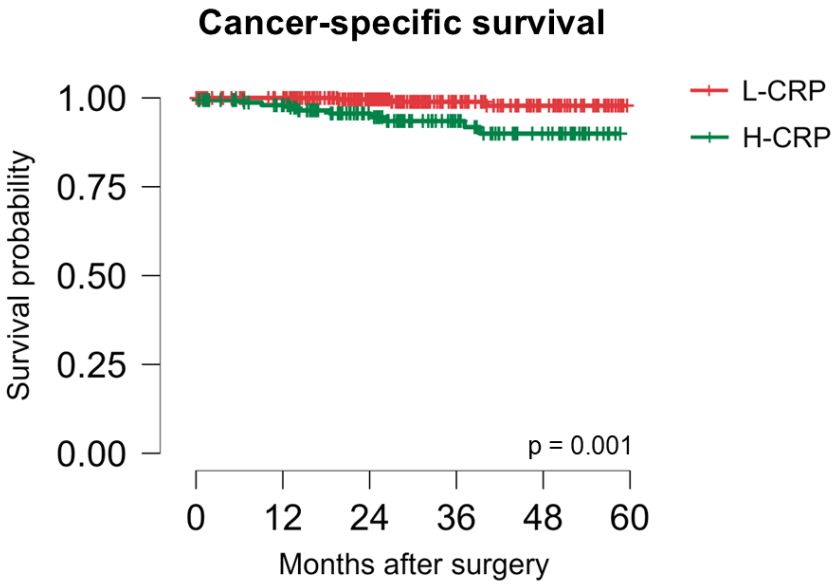
Survival analysis was computed using the Kaplan–Meier method and compared by the log-rank test. Patients in the H-CRP group showed significantly worse OS ($p < 0.001$), RFS ($p = 0.005$), and CSS ($p = 0.001$).



Kaplan-Meier estimates of OS according to post-operative CRP_{max}.

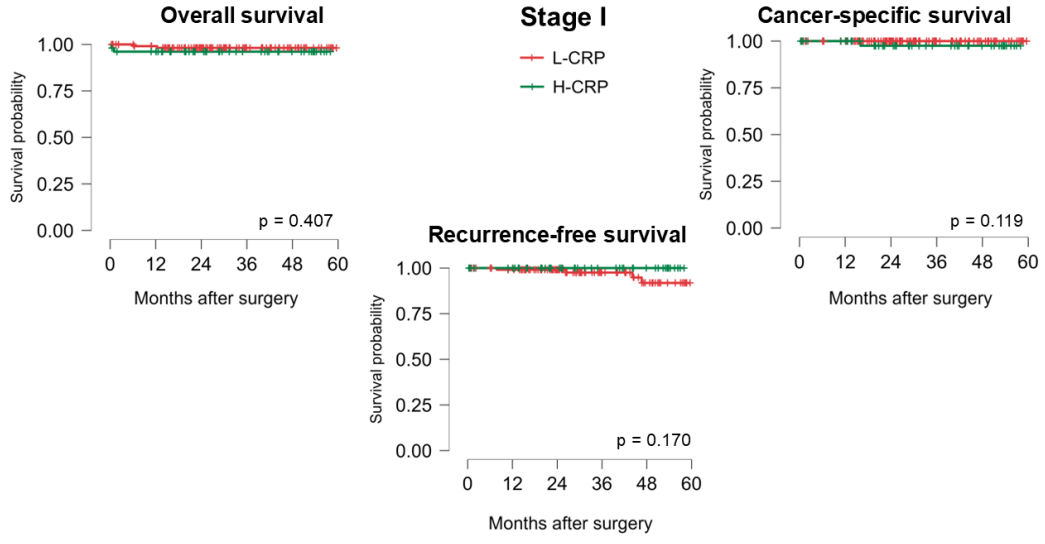


Kaplan-Meier estimates of RFS according to post-operative CRP_{max}.



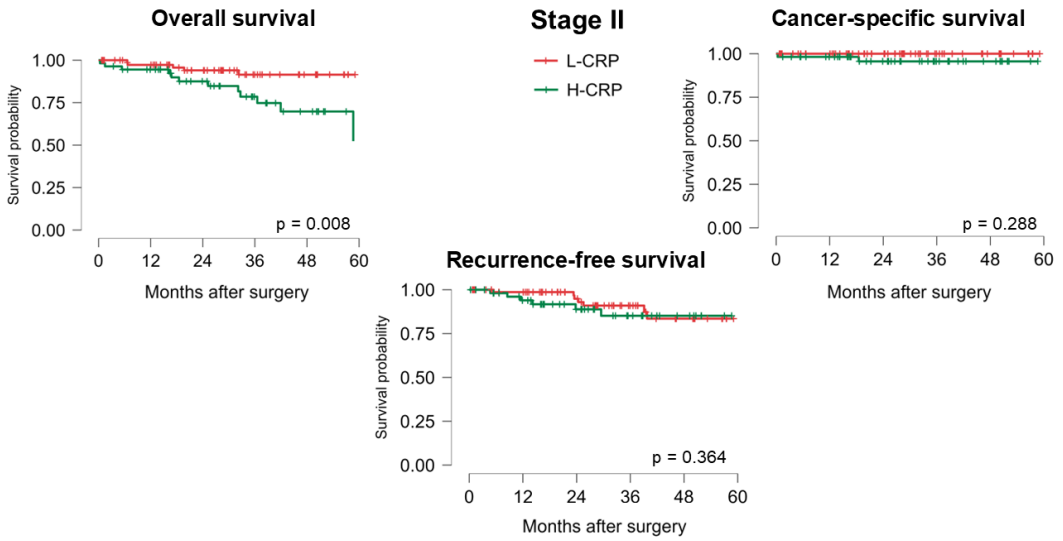
Kaplan-Meier estimates of CSS according to post-operative CRP_{max}.

The same analysis was conducted stratifying for stage. With regards to stage I, no statistically significant differences in OS, RFS, or CSS were observed between groups.



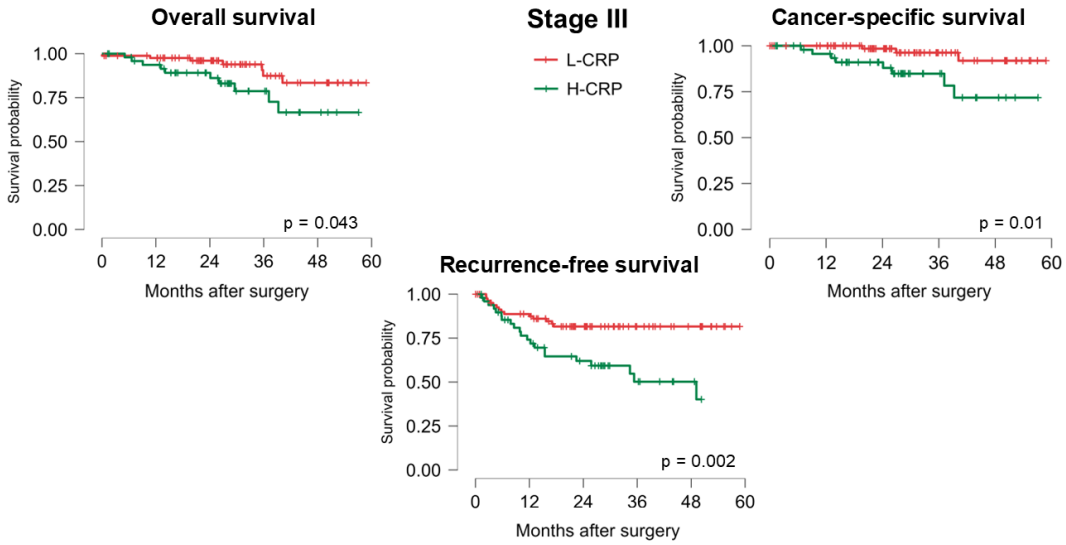
Kaplan-Meier estimates of OS, CSS, and RFS according to post-operative CRP_{max} in patients with stage I CRC.

Considering stage II, the only significant difference was registered on OS (p = 0.008), which was significantly worse in patients in the H-CRP group.



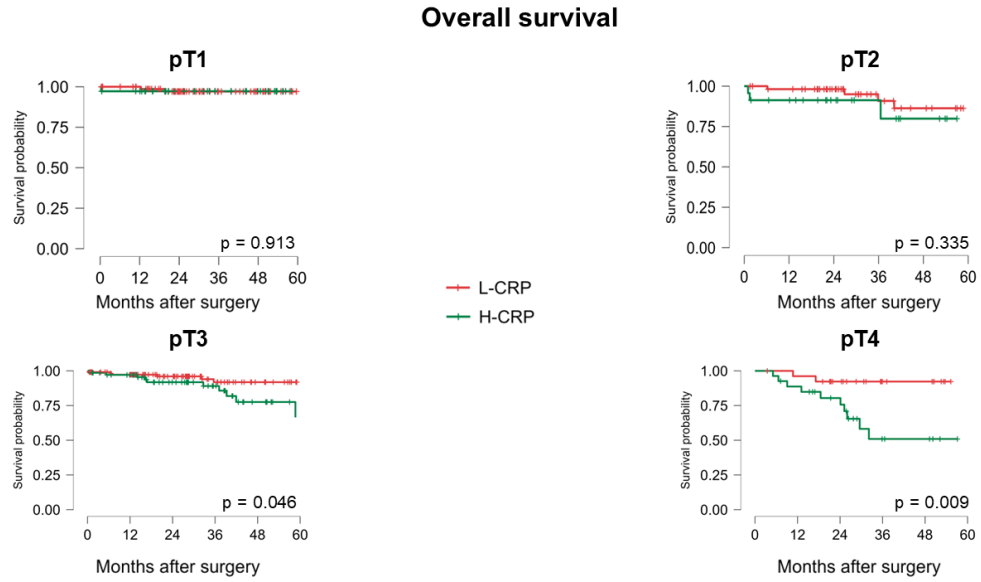
Kaplan-Meier estimates of OS, CSS, and RFS according to post-operative CRP_{max} in patients with stage II CRC.

Only in patients with stage III CRC, the differences in survival rates remained significant for both OS (p = 0.043), RFS (p = 0.002), and CSS (p = 0.01).



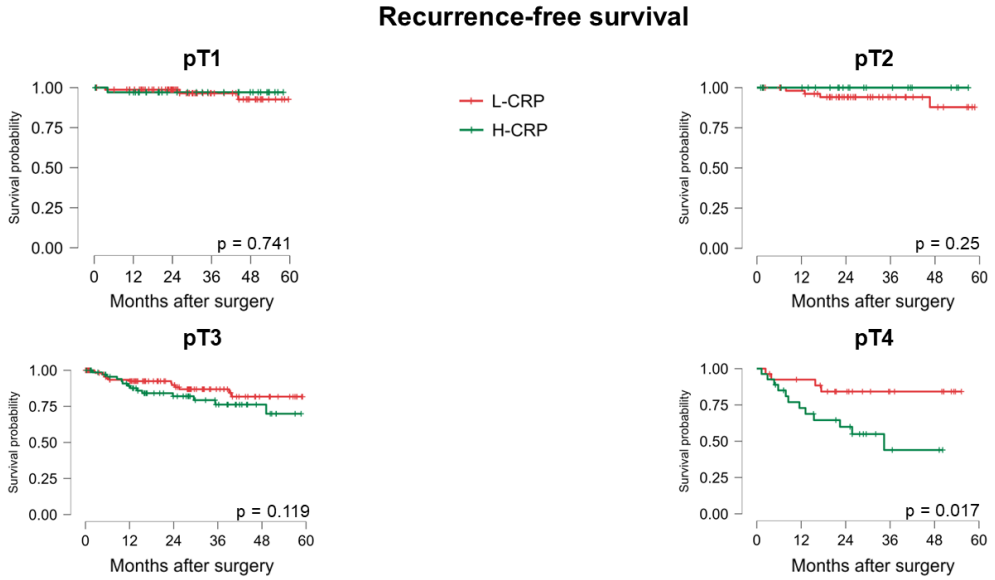
Kaplan-Meier estimates of OS, CSS, and RFS according to post-operative CRP_{max} in patients with stage III CRC.

Since pT stage was statistically significant different between L-CRP and H-CRP group, subgroup analysis was conducted also for this parameter. Considering OS, the outcome was statistically different only in pT3 (p = 0.046) and pT4 (p = 0.009) subgroups.



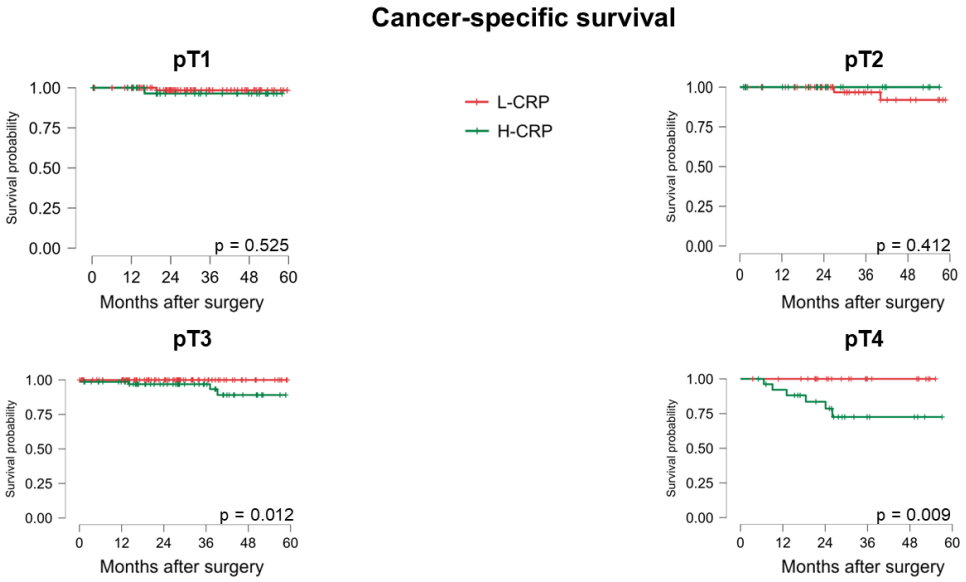
Kaplan-Meier estimates of OS according to post-operative CRP_{max} and pT stage.

RFS confirmed to be worse in H-CRP patients only in the pT4 subgroup (p = 0.017).



Kaplan-Meier estimates of RFS according to post-operative CRP_{max} and pT stage.

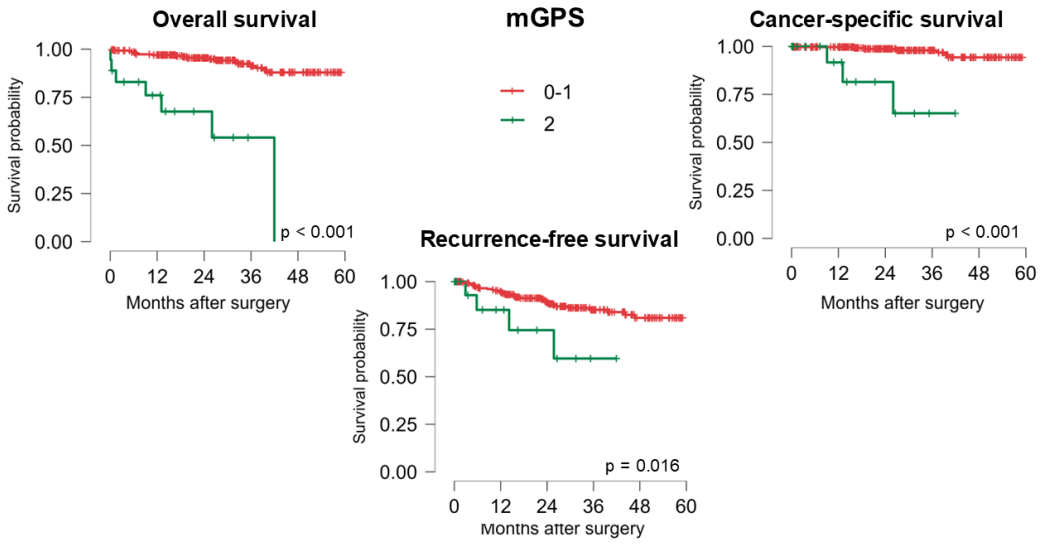
Finally, H-CRP patients showed worse CSS in pT3 (p = 0.012) and pT4 (p = 0.009) subgroups. It should be noted that patients with pT3 and pT4 in the L-CRP showed 5 -years CSS almost equal to 100%.



Kaplan-Meier estimates of CSS according to post-operative CRP_{max} and pT stage.

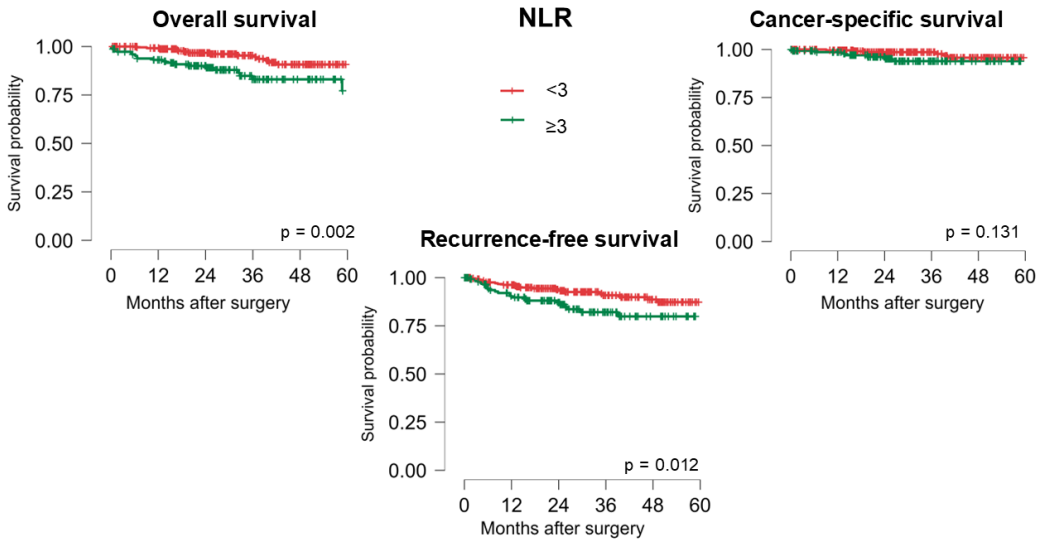
Survival according to mGPS, NLR, and poGPS3

Survival analysis was conducted also for the other prognostic score considered by the literature. Considering mGPS, we confirmed that patients with mGPS equal to 2 showed significantly worse OS (p < 0.001), RFS (p = 0.016), and CSS (p < 0.001).



Kaplan-Meier estimates of OS, CSS, and RFS according to mGPS.

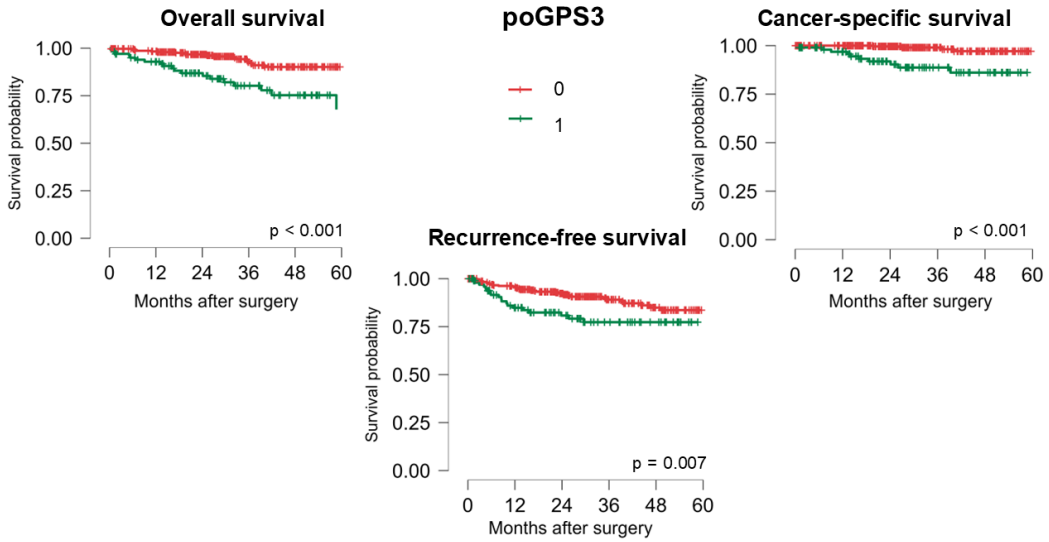
Patients with NLR equal or above 3 showed instead worse OS (p = 0.002) and RFS (p = 0.012), but CSS was not statistically different between groups.



Kaplan-Meier estimates of OS, CSS, and RFS according to NLR.

The modified version of GPS assessed on POD3 only scored 0 and 1 in our cohort, though it could possibly range from 0 to 2. Patients with poGPS3 equal to 1 performed significantly

worse in terms of OS ($p < 0.001$), RFS ($p = 0.007$), and CSS ($p < 0.001$) compared to patients with poGPS3 equal to 0.



Kaplan-Meier estimates of OS, CSS, and RFS according to post-operative version of mGPS assessed on POD3.

Inflammatory markers as risk factors for recurrence-free survival

Multivariate analysis to identify risk factors for RFS was performed by Cox regression model considering CRP_{max} value and correcting for relevant risk factors: age, gender (male vs. female), comorbidity status (yes vs. no), tumor location (rectum vs. right colon vs. left colon), occurrence and severity of post-operative complications, presence of vascular or lymphatic invasion, grading.

H-CRP resulted to be an independent risk factor for worse RFS (HR = 2.5, 95% C.I. 1.3 – 4.9, p = 0.007). Other independent risk factors included rectal location of the tumor (HR = 4.2, 95% C.I. 2.0 – 8.7, p < 0.001), presence of lympho-vascular invasion (HR = 4.2, 95% C.I. 1.2 – 14.8, p = 0.027), and stage III disease (HR = 5.8, 95% C.I. 1.6 – 20.9, p = 0.007). Considering the other scores of peri-operative inflammations, which were input separately, mGPS equal to 2 and poGPS3 equal to 1 confirmed to be independent risk factors for RFS.

	RFS **HR (95% CI)**	**p value**
Age	0.9 (0.9 – 1.0)	0.78
Comorbidities, yes	0.7 (0.4 – 1.4)	0.38
Gender, female	0.8 (0.4 – 1.5)	0.52
Tumor location		
Right colon	-	-
Left colon	1.2 (0.5 – 2.7)	0.62
Rectum	4.2 (2.0 – 8.7)	**<0.001**
Stage		
I	-	
II	1.6 (0.4 – 6.1)	0.50
III	5.8 (1.6 – 20.9)	**0.007**
Post-operative complications		0.62
No complications	-	
Mild complications	0.9 (0.5 – 1,8)	0.87
Severe complications	0.5 (0.1 – 1.5)	0.19
Lympho-vascular invasion, present	4.2 (1.2 – 14.8)	**0.027**
Grading		0.42
G1	-	
G2	1.0 (0.3 – 2.8)	0.94
G3	1.6 (0.5 – 5.4)	0.46
H-CRP	2.5 (1.3 – 4.9)	**0.007**
mGPS = 2	5.2 (1.3 – 18.4)	**0.011**
NLR ≥ 3	1.8 (0.9 – 3.6)	0.094
poGPS3 = 1	3.0 (1.5 – 6.0)	**0.001**

DISCUSSION

As knowledge of cancer mechanisms has progressed, the attention of researchers shifted to the identification of predictors of poor prognosis different from standard TNM staging. In fact, despite the prognosis of patients with primary resectable CRC has dramatically improved in the last decades, around 40% of patients across all stages will eventually die from disease progression within five years from diagnosis[104]. Moreover, some degrees of heterogeneity exists within stages, and efforts have been directed towards the identification of biomarkers or other risk factors explaining the poor prognosis of some subgroups of patients. Against this background, mounting evidence supports the role of chronic inflammation and the host immune system in cancer development, progression and prognosis[2,27,105,106]. Specifically, Hanahan et al. developed the concept of 'Hallmarks of Cancer', consisting of a set of functional capabilities acquired by human cells during the malignant transformation. The eight original hallmarks included the capability to sustain proliferative signaling, to evade growth suppression, to resist apoptosis, to enable replicative immortality, to prompt neo-angiogenesis, to activate invasion and metastasis, to reprogram cellular metabolism, and to avoid immune destruction. The deregulation of cellular metabolism and the ability to avoid immune destruction were initially classified as "emerging hallmarks", but in the most recent elaboration they are considered as core hallamarks[1]. These hallmarks on their own are not capable of explaining the whole complexity of cancer pathogenesis. Accordingly, the concept of "enabling characteristics" was added to the landscape. These two enabling processes were genome instability and tumor-promoting inflammation.

The aim of this study was therefore to evaluate the role of circulating immune cells and inflammation in patients with CRC. Specifically, we assessed whether changes in circulating immune cells can be detected before diagnosis, and their relationship with survival outcomes. Moreover, we investigated the role of peri-operative inflammation as potential "tumor-promoting inflammation", with particular interest for post-operative CRP.

Despite circulating immune cells are frequently assessed in primary care as routine follow-up tests, only few studies evaluated their role as potential 'red flags' for tumor development. In fact, only some studies from primary care evaluated the rate of incident cancers in subjects with raised inflammatory markers through the analysis of cancer and population-based registries. Although the incidence of cancer was higher in patients with raised pre-diagnosis inflammatory markers, these presented poor sensitivity[83,84,107]. Though Moullet and colleagues[83] described increased inflammatory markers as early as 9 months before

diagnosis of CRC, our study confirmed that changes in Pre-CBC occur even years (at least 24 months) before diagnosis. Specifically, we identified a progressive increase in Pre-Leuc, Pre-Neut and Pre-NLR count and a synchronous decrease in Pre-Lymph. The study by Nøst et al.[108] identified a positive association between systemic-immune inflammation index, NLR, platelet-to-lymphocyte ratio, and lymphocyte-to-monocyte ratio and risk of several cancers. Also, the association between systemic inflammation markers and the risk varied according to the time between blood drawn and diagnosis. Specifically, no clear association was observed until 4-5 years prior to the diagnosis, with subsequently elevated HR estimates within the last year before diagnosis. Despite these studies reports on large cohort, none of them assessed the relationship between the early changes in inflammatory markers and cancer prognosis. Quite interestingly, our analysis identified Pre-Leuc, Pre-Neut and Pre-NLR as negative prognostic factors for OS and CSS, while Pre-Lymph acted as a protective factor on multivariate analysis[109]. The HR for OS and CSS increased proportionally with increasing values of Pre-Leu, Pre-Neut, and Pre-NLR, while the association with Pre-Lymph showed a dual trend. Furthermore, the effect of the timing of blood sampling on prognosis was investigated. Patients in the highest quartile for Pre-Leuc, Pre-Neut, and Pre-NLR and those in the lower quartile for Pre-Lymph showed the worse CSS regardless of the interval between blood sampling and surgery. However, the differences between curves and the steepness of survival curves were more pronounced if the blood test referred to the interval 24-36 months before surgery. Finally, Pre-CBC values were tested for potential associations with known negative histopathological features. Lower Pre-Leuc, Pre-Neut, and Pre-Mono count were associated with the presence of vascular invasion on pathological examination. Patients with lower Pre-Eosinophils, Pre-Mono, and Pre-Lymph count showed higher tumor budding. On the other hand, cases with higher Pre-NLR showed more frequently tumor budding > 10.

The balance between immune surveillance by the cellular immunity and the capability of tumor cells to avoid immune destruction represents one of the crucial points in cancer progression[1]. Lymphocytes can act as tumor suppressors through the release of lytic components and direct cell-cell interaction[4,110]. Neutrophils, on the other hand, can prompt cell proliferation because of inflammation and production of cytokines and chemokines that promote tumor growth, angiogenesis, and metastasis[4,111,112]. Accordingly, increased NLR represents an easily available and adequate biomarker of a pro-tumorigenic immune shift. It should be noted that the median values of Pre-CBC components were within normal ranges. Therefore, although we identified a significant prognostic role of these

immunological parameters, further studies on specific sub-populations will be needed to aid clinical applicability.

The results of our study confirm the dynamic changes in circulating immune cells as tumors develop. The early identification of immunological changes may represent a window of opportunity for early diagnosis of cancer. However, it should be more extensively investigated whether these alterations in Pre-CBC represent the *primum movens* of cancer pathogenesis in predisposed patients or just an epiphenomenon of tumor growth. Finally, we identified a significant association between Pre-Leuc, Pre-Neut, Pre-NLR, Pre-Lymph and survival outcomes, prompting a reflection on the role of circulating immune cells in the definition of prognosis and response to treatments.

Afterwards, we focused on the role of peri-operative inflammation as potential "tumor-promoting inflammation", with particular interest for post-operative CRP. 436 patients submitted to curative minimally invasive surgery for stage I-III CRC were categorized into two groups according to the highest value of post-operative CRP. The optimal cut-off for post-operative CRP as dichotomous predictor of RFS was chosen after receiver operating characteristics (ROC) curve analysis using disease recurrence as outcome. CRP_{max} was tested together with other known baseline inflammatory markers (mGPS and NLR) and a proposed novel version of post-operative mGPS (poGPS3)[44].

Looking at the results of our analysis, patients in the H-CRP group showed a higher proportion of right colon cancer, higher BMI, more comorbidities and higher ASA score. Since CRP is an acute-phase protein secreted in response to surgical stress, it was expected that patients in the H-CRP group showed significantly longer duration of surgery, higher rates of conversion, higher EBL, higher proportion of blood transfusion, higher rates of complications, and longer hospital stay. No differences in histopathological characteristics were observed, however lymph vascular invasion tended to be higher in the H-CRP group, while peritumoral inflammatory infiltrate was less frequently found. The only statistically significant difference was the higher proportion of locally advanced tumors in the H-CRP group.

With regards to long-term outcomes, recurrences occurred more frequently in patients in the H-CRP group (17.8% versus 9.0%, $p = 0.007$). Also, it is interesting to note that the peak of CRP was higher in patients who developed a recurrence. Furthermore, the profile of recurrence differed between groups, with a higher proportion of locoregional, peritoneal, and multiple metastases in the H-CRP group. These results are in line with those reported

by Mutasubara et al.[41] who showed that patients with high CRP presented significantly higher recurrence rates in the lymph-nodes and the peritoneum (7.5% vs 2.1%, p=0.037, and 11.3% vs 1.6%, p<0.001, respectively).

Survival curves were built for OS, RFS, and CSS according to the CRP_{max} value. Patients in the H-CRP group showed significantly worse OS ($p < 0.001$), RFS ($p = 0.005$), and CSS ($p = 0.001$), but the results for RFS and CSS were confirmed only in stage III CRC. Sub-analysis according to pT stage confirmed worse OS and CSS in pT3 and pT4. It should be noted that patients with pT3 and pT4 in the L-CRP showed 5 -years CSS almost equal to 100%. Finally, RFS confirmed to be worse in H-CRP patients only in the pT4 subgroup. None of the previously published studies conducted subgroup analysis according to pT stage, therefore the results cannot be commented against available evidence. Two potential explanations can be suggested. On one hand, it can be hypothesized that more advanced tumors are associated with higher levels of systemic inflammation, both tumor-promoting and tumor-enhanced. On the other hand, surgery for locally advanced tumor can be more challenging in terms of surgical dissection, therefore resulting in higher levels of post-operative CRP.

We finally conducted multivariate analysis for RFS correcting for relevant risk factors (age, gender, comorbidity status, tumor location, occurrence and severity of post-operative complications, presence of vascular or lymphatic invasion, grading. H-CRP resulted in an independent risk factor for worse RFS ($p = 0.007$). Other independent risk factors included rectal location of the tumor ($p < 0.001$), presence of lympho-vascular invasion ($p = 0.027$), and stage III disease ($p = 0.007$), while occurrence and severity of complications did not reach statistical significance. Considering the other scores of peri-operative inflammations, which were input separately, mGPS equal to 2 and poGPS3 equal to 1 confirmed to be independent risk factors for RFS. The results of the multivariate analysis are in line with those reported by McSorley et al.[43] who identified CRP level > 150 mg/L on POD4 as a significant risk factor for RFS. On the contrary, Watt et al. did confirm the prognostic role of CRP on POD3 and POD4 for RFS[44].

CRP represents an easily available, inexpensive and commonly measured marker of post-operative inflammation. However, it is extremely nonspecific and may not be representative of the whole immune profile. Further studies will need to assess whether other circulating factors (i.e. interleukin-6, procalcitonin, interleukin-8) show higher specificity and sensitivity in identifying patients at high risk of recurrence. Also, if the

detrimental effect of altered baseline immune status and post-operative inflammation was confirmed by larger investigations, future studies may be directed towards the identification of immune-modulating strategies to re-establish favorable conditions.

Some limitations of the study should be acknowledged. Due to its retrospective nature, it was not possible to retrieve complete data for all variables, in particular Pre-CBC or baseline CRP, mGPS, and NLR for all patients. Also, although we excluded from the analysis on pre-diagnosis circulating cells patients with clear evidence of an ongoing infection, autoimmune disorders, treated with corticosteroids or immunosuppressive/immunomodulatory therapy, and blood samples associated with admission to the Emergency Department, it may be possible that some alterations in Pre-CBC were consequence of a subclinical inflammatory or infective condition. Finally, no information is available on the different subtypes of lymphocytes. It is sensible to believe that the involvement of different subpopulations may account for the dual trend in HR in relationship to Pre-Lymph values.

CONCLUSIONS

The results obtained so far indicate a potential correlation between tumor development and changes in circulating immune cells even years before surgery, with subsequent influence on tumor prognosis and response to treatment. Moreover, the results of the analysis on post-operative CRP suggest a potential relationship between surgical stress and worse recurrence-free survival. Though anecdotical data reported higher recurrence rates in patients with tumor-related complications or post-operative infective complications, only few authors investigated the role of post-operative CRP, and the results are controversial. Our study highlighted the potential role of surgical stress as tumor-promoting inflammation identifying post-operative CRP peak as a negative prognostic factor for RFS.

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