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ORIGINAL ARTICLE

Prospective Study

Intertwined leukocyte balances in tumours and peripheral blood as robust predictors of right and left colorectal cancer survival

Ramón Cantero-Cid, Karla Marina Montalbán-Hernández, Jenny Guevara, Alejandro Pascual-Iglesias, Elisa Pulido, José Carlos Casalvilla, Cristóbal Marcano, Cristina Barragán Serrano, Jaime Valentín, Gloria Cristina Bonel-Pérez, José Avendaño-Ortiz, Verónica Terrón, Roberto Lozano-Rodríguez, Alejandro Martín-Quirós, Elvira Marín, Eva Pena, Laura Guerra-Pastrián, Eduardo López-Collazo, Luis Augusto Aguirre

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Abstract

BACKGROUND

Colorectal cancer (CRC) accounts for 9.4% of overall cancer deaths, ranking second after lung cancer. Despite the large number of factors tested to predict their outcome, most patients with similar variables show big differences in survival. Moreover, right-sided CRC (RCRC) and left-sided CRC (LCRC) patients exhibit large differences in outcome after surgical intervention as assessed by preoperative blood leukocyte status. We hypothesised that stronger indexes than circulating (blood) leukocyte ratios to predict RCRC and LCRC patient outcomes will result from combining both circulating and infiltrated (tumour/peritumour fixed tissues) concentrations of leukocytes.

AIM

To seek variables involving leukocyte balances in peripheral blood and tumour tissues and to predict the outcome of CRC patients.

performed image analysis; Pulido E performed sample analysis and assisted with image analysis; Pascual-Iglesias A performed statistical analysis; Casalvilla JC assisted with sample analysis and with image analysis; Valentín J assisted with sample analysis and with image analysis; Bonel-Pérez GC assisted with sample analysis and with image analysis; Avendañ o-Ortiz J assisted with sample analysis; Terrón V assisted with sample analysis; Lozano-Rodrí guez R assisted with sample analysis; Martín-Quirós A participated in patient recruitment; Marín E performed sample analysis; Guevara J participated in patient recruitment, surgery, sample collection, and assisted with data analysis; Marcano C participated in patient recruitment, surgery, sample collection, and assisted with data analysis; Barragá n C participated in patient recruitment, surgery, and sample collection; Pena E was involved with sample collection and assisted with anatomo-pathological analyses; Guerra-Pastrián L was involved with sample collection and performed anatomo-pathological analyses; López-Collazo E participated in the design and oversight of the study and drafted the manuscript; Aguirre LA designed and supervised the study, assisted with data analysis, and drafted the manuscript; all authors read and approved the final manuscript.

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METHODS

Sixty-five patients diagnosed with colon adenocarcinoma by the Digestive Surgery Service of the La Paz University Hospital (Madrid, Spain) were enrolled in this study: 43 with RCRC and 22 with LCRC. Patients were followed-up from January 2017 to March 2021 to record overall survival (OS) and recurrence-free survival (RFS) after surgical interventions. Leukocyte concentrations in peripheral blood were determined by routine laboratory protocols. Paraffin-fixed samples of tumour and peritumoural tissues were assessed for leukocyte concentrations by immunohistochemical detection of CD4, CD8, and CD14 marker expression. Ratios of leukocyte concentration in blood and tissues were calculated and evaluated for their predictor values for OS and RFS with Spearman correlations and Cox univariate and multivariate proportional hazards regression, followed by the calculation of the receiver-operating characteristic and area under the curve (AUC) and the determination of Youden's optimal cutoff values for those variables that significantly correlated with either RCRC or LCRC patient outcomes. RCRC patients from the cohort were randomly assigned to modelling and validation sets, and clinician-friendly nomograms were developed to predict OS and RFS from the respective significant indexes. The accuracy of the model was evaluated using calibration and validation plots.

RESULTS

The relationship of leukocyte ratios in blood and peritumour resulted in six robust predictors of worse OS in RCRC: $CD8^+$ lymphocyte content in peritumour ($CD8_{pt}$, AUC = 0.585, cutoff < 8.250, P = 0.0077); total lymphocyte content in peritumour ($CD4CD8_{pt}$, AUC = 0.550, cutoff < 10.160, P = 0.0188); lymphocyte-to-monocyte ratio in peritumour (LMR_{pt}, AUC = 0.807, cutoff < 3.185, P = 0.0028); $CD8^+$ LMR in peritumour ($CD8MR_{pt}$, AUC = 0.757, cutoff < 1.650, P = 0.0007); the ratio of blood LMR to LMR in peritumour (LMR_b/LMR_{pt}, AUC = 0.672, cutoff > 0.985, P = 0.0244); and the ratio of blood LMR to $CD8^+$ LMR in peritumour ($LMR_b/CD8MR_{pt}$, AUC = 0.601, cutoff > 1.485, P = 0.0101). In addition, three robust predictors of worse RFS in RCRC were found: LMR_{pt} (AUC = 0.737, cutoff < 3.185, P = 0.0046); LMR_b/LMR_{pt} (AUC = 0.678, cutoff > 0.985, P = 0.0155) and $LMR_b/CD8MR_{pt}$ (AUC = 0.615, cutoff > 1.485, P = 0.0141). Furthermore, the ratio of blood LMR to $CD4^+$ LMR in peritumour ($LMR_b/CD4MR_{pt}$, AUC = 0.786, cutoff > 10.570, P = 0.0416) was found to robustly predict poorer OS in LCRC patients. The nomograms showed moderate accuracy in predicting OS and RFS in RCRC patients, with concordance index of 0.600 and 0.605, respectively.

CONCLUSION

Easily obtainable variables at preoperative consultation, defining the status of leukocyte balances between peripheral blood and peritumoural tissues, are robust predictors for OS and RFS of both RCRC and LCRC patients.

Key Words: Left colorectal cancer; Leukocyte ratios; Prognostic variables; Recurrence-free survival; Right colorectal cancer; Overall survival

Core Tip: This was a prospective study involving 65 patients with colorectal cancer, seeking to find robust predictors of survival after surgical intervention amongst the leukocyte balances in peripheral blood, tumour, and peritumoural tissues. A number of these variables are shown to predict overall survival and recurrence-free survival in both right-sided colorectal cancer and left-sided colorectal cancer patients, thus allowing the improvement of pre- and postoperative patient treatments.

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INTRODUCTION

Despite the great medical and scientific achievements attained over the last decades in the fields of cancer understanding, early detection, and care, cancer continues to be a majorly threatening disease worldwide. Amongst the many pathologies gathered under this term, colorectal cancer (CRC) accounts for 9.4% of overall cancer deaths, ranking second just after lung cancer[1]. CRC treatments vary depending on tumour location and stage of diagnosis; standard colectomy (along with lymphadenectomy) without adjuvant therapy is the usual treatment in early stages I and II, while most patients in advanced stages III and IV follow with chemo- and/or radiotherapy to reduce the risk of recurrence[2]. However, a large proportion of these patients present with (synchronous; 15%-25%) or will develop (metachronous; 40%-75%) metastases, mainly in the liver[3], which constitutes the major cause of deaths[4]. Therefore, a 5-year relative survival rate is reduced from 90% in early-stage detection to 12% in advanced cases[2]. Thus, finding robust markers before surgery to predict patient outcomes constitutes a safe strategy in order to stratify those groups with a high risk of recurrence and design personalised pre- and postoperative therapies.

A wide variety of factors, mainly based on clinical and pathological features, have been tested as prognostic markers for CRC development, such as: weight loss, haemoglobin levels, tumour-nodes-metastasis classification (TNM) staging and tumour differentiation, mismatch-repair proficiency, lymph node involvement, or response to (neo-) adjuvant therapies[5-7]. Moreover, since a clear distinction between the behaviour of right-sided CRC (RCRC) and left-sided CRC (LCRC) patients is well established, much effort has been put into categorising putative prognostic markers according to their respective characteristics, though still with controversial results[8].

Currently, an increasing number of research and clinical trials are supporting evidence of the influence of the systemic inflammatory response in cancer progression [5]. A measure of this response has been assessed by combining the number of peripheral circulating leukocytes: lymphocyte-to-monocyte ratio (LMR), neutrophil-to-lymphocytes ratio (NLR), and platelet-to-lymphocyte ratio (PLR). These analyses have shown interesting prognostic associations in several cancer types including urothelial, nasopharyngeal, osteosarcoma, lung carcinomas[9-12], and CRC[13-16]. Nevertheless, few studies have been directed towards the prognostic value of intertwined relationships across circulating and tumour-infiltrated populations of leucocytes on solid tumour progression[17-19].

Herein, we aimed to delve deep into the prognostic value of leukocyte distribution ratios, in both blood and tumour tissues, for CRC patient outcomes after surgery. We hypothesised that stronger indexes than circulating (blood) leukocyte ratios to predict patient outcome will result from combining both circulating and infiltrated (tumour/peritumoural tissues) concentrations of leukocytes. We show six robust predictors for RCRC overall survival ($CD8_{pt}$, $CD4CD8_{pt}$, LMR_{pt}, $CD8MR_{pt}$, LMR_b/LMR_{pt}, $LMR_b/CD8MR_{pt}$), three for RCRC recurrence-free survival (LMR_{pt}, $CD8MR_{pt}$, LMR_b/LMR_{pt}, $LMR_b/CD8MR_{pt}$), and another one for LCRC overall survival ($LMR_b/CD4MR_{pt}$), all these being based on the ratios between blood and peritumoural tissue concentration of lymphocytes and monocytes. Moreover, we highlight the importance of these variables in designing *ad hoc* surgical strategies, due to the ease with which surgeons can build a protocol by taking samples of peripheral blood and peritumoural tissue during a preoperative colonoscopy.

MATERIALS AND METHODS

Patient selection

Sixty-five patients diagnosed with colon adenocarcinoma, with no records of previous neo-adjuvant therapy, were recruited at the Digestive Surgery Service of La Paz University Hospital (Madrid, Spain) from January 2017 to September 2019. They were surgically treated according to each patient's condition for right (caecum, ascending, or transverse colon) or left (descending or sigmoid colon) hemicolectomies followed by

anastomosis, with partial hepatectomy if synchronous metastasis was present. Patients' clinical records were followed-up until March 2021. Overall survival (OS) was then defined as the length of time since surgery until *exitus* or the end of the study, whilst recurrence-free survival (RFS) was considered the interval from surgery until relapse, either from disease-free or (synchronous/metachronous) metastases-free statuses. All patients signed written consent, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and the Ethics Committee for Clinical Research of La Paz University Hospital (PI-1958), for further uses of blood samples and surgically resected organs for research purposes.

Exclusion criteria

Only patients with adenomas or rectum adenocarcinoma were excluded from the study.

Blood tests

Venous blood samples were collected in 10 mL EDTA-tubes in the hospital room, 24 h prior to surgery and routinely tested for white blood cell, lymphocyte (L), monocyte (M), neutrophil (N) and platelet (P) counts at the Central Laboratory (CORE) of the La Paz University Hospital. Preoperative blood LMR (LMR_b), NLR (NLR_b), and PLR (PLR_b) were then calculated for each patient by dividing the absolute counts of the respective populations in the peripheral blood (Table 1).

Tissue preparation

Samples from the middle part (avoiding both the epicentre and the edge) of the tumours, 5 cm-adjacent peritumoural (non-neoplastic), and liver (in case of synchronous metastases) tissues were taken at the time of surgery, upon *in situ* evaluation of morphological characteristics by pathologists. Histological types and grades were based on microscopic features. Microsatellite stability analyses were performed as previously described[20].

Organ samples were washed with PBS solution containing 56 μg/mL gentamicin (Braun, Melsungen, Germany; 636159), 2.5 μg/mL fungizome/anphotericin-B (Gibco, Amarillo, TX, United States; 15290-018), and 1% penicillin/streptomycin (Sigma-Aldrich, Saint Louis, MO, United States; P4333-100mL) and gently shaken for 30 min at room temperature. Then they were fixed in 4% paraformaldehyde for 16 h, washed with PBS for 24 h, and paraffin-embedded by standard procedures.

Tissue microarrays (TMA) recipient paraffin-blocks (24 mm × 2.0 mm) were prepared with a TMA builder kit (Histopathology Ltd., Baranya, 7632, Hungary; 20010.2) and filled with properly matched samples of previous patients' blocks, following manufacturer's protocol.

Immunohistochemistry and image analysis

Thin sections (5 μm thick) of TMAs were cut with a Leica (RM2255) ultrathin-microtome and allowed to completely adhere to slides for 30 min at 60°C, before staining with commercially available antibodies against assessed surface markers was performed by standardised protocols (see Supplementary Table 1 for a complete list of primary and secondary antibodies used). Briefly, sections were deparaffinised with xylene, rehydrated through graded (100% to 70%) ethanol, and blocked for endogenous peroxidase by immersion in 97% methanol. Next, sections were immersed in heated sodium citrate buffer (10 mmol/L, pH 6.0) for antigenicity recovery and then incubated in unspecific-binding blocking solution [TBS solution containing 1% BSA, 1% Triton X-100 (Thermo Scientific; Waltham, MA, United States, 85111) and 2.5% horse serum (Gibco; Amarillo, TX, United States, 26050088)]. Primary antibodies were then added at recommended dilutions and incubated overnight at 4°C in a humid chamber. After washing slides with TBS, matched HRP-secondary antibodies were added and incubated for 1 h at room temperature. Then, DAB chromogen (DAB substrate kit, Cell Marque; Rocklin, CA, United States, 1-957D-30) was added for a few seconds until colour change and gently washed with TBS and distilled water. Finally, sections were counterstained by immersion in haematoxylin, dehydrated through graded (70% to 100%) ethanol, and mounted with DPX medium (Sigma-Aldrich; Saint louis, MO, United States, 06522).

An average of four photographs per sample (in order to cover the whole field for each sample on the TMA sections) were taken with an Olympus BX-41 microscope and blind-analysed by two independent observers with ImageJ (v1.52p), for the calculus of the relative areas to each antibody corresponding surface marker expression (CD4, CD8, and CD14). For a detailed description of the image processing see Supple-

Table 1 Baseline characteristics of patients included in this study

Characteristics	Frequency	%
All patients (*n* = 65)		
Age (yr) ± SD	73.54 ± 9.51	
(range)	(52-92)	
Gender		
Female	29	44.62
Male	36	55.38
Tumour localisation		
Right colorectal cancer	43	66.15
Caecum	13	30.23
Ascending colon	23	53.49
Transverse colon	7	16.28
Left colorectal cancer	22	33.85
Descending colon	11	50.00
Sigma	11	50.00
Emergency surgery		
Yes	1	1.54
No	64	98.46
Surgical procedure		
Laparoscopic hemicolectomy	48	73.85
Open hemicolectomy	17	26.15
Development at surgery		
Non-metastasised	37	56.92
Metastases	28	43.08
Liver synchronous	13	46.43
Liver metachronous	8	28.57
Other organs	13	46.43
MMR status		
pMMR	56	86.15
dMMR	5	7.69
Unknown	4	6.15
TNM stage		
0	3	4.62
I	7	10.77
IIA	21	32.31
IIB	5	7.69
IIIA	2	3.08
IIIB	8	12.31
IIIC	5	7.69
IV	1	1.54
IVA	10	15.38
IVB	3	4.62

Adjuvant chemotherapy			
Yes		30	46.15
No		35	53.85
Blood leukocytes counting (× 103/μL), (normal range)	RCRC	LCRC	*P* value
White blood cells count (3.6-10.5)	7.41 (3.52-16.2)	8.19 (4.83-15.8)	0.271
Lymphocytes (1.1-4.5)	1.77 (0.46-4.46)	2.04 (0.32-4.87)	0.235
Monocytes (0.1-0.9)	0.54 (0.20-1.26)	0.50 (0.22-1.11)	0.493
Neutrophils (1.5-7.7)	4.86 (1.76-15.3)	5.34 (2.96-13.0)	0.480
Platelets (150-370)	275.65 (101.0-602.0)	272.41 (142.0-725.0)	0.910
LMR_b	3.54 (0.42-7.96)	4.64 (0.58-11.88)	0.046
NLR_b	3.65 (0.69-25.93)	4.61 (0.93-30.66)	0.481
PLR_b	188.58 (52.24-551.22)	213.93 (29.16-1187.50)	0.585

TNM: Tumour-nodes-metastasis classification; MMR: DNA mismatch repair; pMMR: Proficient MMR; dMMR: Deficient MMR; LMR: Lymphocyte-to-monocyte ratio; NLR: Neutrophil-to-lymphocytes ratio; PLR: Platelet-to-lymphocyte ratio; RCRC: Right-sided colorectal cancer; LCRC: Left-sided colorectal cancer; SD: Standard deviation; b: Blood.

mentary Figure 1A. A percentage of the total tissue area (A) for the three surface markers, in each patient's tumour and peritumour samples, was reported as the mean of all their relative areas per field.

Total tumour and peritumour LMRs (respectively, LMR_t and LMR_{pt}) were calculated by dividing the sum of the areas for CD4 and CD8 by the area for CD14, *e.g.*, $LMR_t = (A(CD4_t)+A(CD8_t))/A(CD14_t)$. Individual subpopulation ratios were also analysed for both tumour and peritumour samples ($CD4MR_t$, $CD8MR_t$ and $CD4MR_{pt}$, $CD8MR_{pt}$, respectively), *e.g.*, $CD4MR_t = A(CD4_t)/A(CD14_t)$. Then, blood-to-tissue ratios for all previous tumour and peritumour subpopulation ratios (LMR_b/LMR_t, $LMR_b/CD4MR_t$, $LMR_b/CD8MR_t$ and LMR_b/LMR_{pt}, $LMR_b/CD4MR_{pt}$, $LMR_b/CD8MR_{pt}$, respectively) were also reported for each patient.

Nomogram construction and validation

All RCRC patients from the cohort were randomly divided into training (60%) and validation (40%) sets to establish and validate the clinician-friendly nomograms. For each nomogram to predict the probability of OS or RFS, the six or the three respectively significant predictive factors found early were used to formulate the nomograms with several R packages. The discriminatory ability of the nomogram was assessed by calculating the Harrell's concordance index (C-index).

Statistical analysis

Data are represented as mean ± standard deviation. Student's *t* test was used for pairwise comparisons. Mann-Whitney *U* analysis was applied for equal standard deviations, otherwise Welch's correction was used. The distribution of the variables was assessed by a nonparametric test. Spearman *r* correlations were used to evaluate the association between the variables and ratios with the OS and RFS observed in our patients. Survival and population ratio relationships were analysed using Cox proportional hazard ratios; statistically significant variables in univariate analysis were further evaluated with the Cox multivariate step-by-step backward method to identify those with independent prognostic value. The Kaplan-Meier method was used to calculate the differences in OS and RFS rates for RCRC and LCRC over time (months), and significance was compared using the log-rank (Mantel-Cox) test; median time (months) survival proportions and P accuracy were reported. We calculated the receiver-operating characteristic (ROC) curve and the area under the curve (AUC) to determine whether the different variables and ratios could be used to predict OS and RFS in our cohort. We indicated the sensitivity, the specificity, the positive and negative predictive values, and 95% confidence interval for AUC and P accuracy. Optimal cutoff values, as determined with Youden's index, Harrell's C-index, and P accuracy, were calculated with R software. *P* values of 0.05 or less were considered indicative of statistical significance, and all these were two-sided. All statistics were performed in either Prism 6.0 (GraphPad, San Diego, CA, United States) or SPSS

version 23 (IBM, NY, United States) software.

RESULTS

Cohort baseline characteristics

The cohort included in this study was exclusively recruited by one team of surgeons, from their assigned patients for surgically treated disorders of the digestive tract, thus only a fraction is constituted of the whole figure of CRC patients attended at La Paz University Hospital during the period of recruitment. Detailed clinicopathological characterisation of patients is shown in Table 1.

A total of 65 patients with a mean age of 73.5 years, of whom 43 (66.1%) presented with RCRC and 22 (33.8%) with LCRC, were finally enrolled. Of these, 29 (44.6%) were women and 36 (55.4%) were men. With the exception of one case, all had been programmed for surgery without an emergency condition. Forty-eight (73.8%) were hemicolectomised by minimally invasive laparoscopic procedure. They ranged from stages 0 to IV, based on TNM classification; 28 (43.1%) were presenting metastasis (either synchronous or metachronous at the time of surgery), and 30 (46.1%) received adjuvant therapy after surgery. Fifty-six (86.1%) of the tumours were found proficient for the mismatch-repair machinery at the histological level.

Patient progression follow-up

The survival analysis, with a median follow-up of 26 mo, showed no differences for OS between RCRC and LCRC patients (Figure 1A) but a trend towards poorer outcome for the latter (74.5% *vs* 40.8%, $P = 0.1875$). However, in the analysis of RFS (Figure 1B), we observed significantly better outcomes for RCRC compared to LCRC patients (60.4% *vs* 19.1%, $P = 0.0036$).

Leukocyte counts and ratios

We found no differences (Table 1) in total leukocyte counts nor in individual populations of circulating lymphocytes, monocytes, neutrophils, or platelets between RCRC and LCRC patient peripheral blood. However, though all mean counts for both groups were within the normal physiological ranges, RCRC patients showed a trend towards low circulating lymphocytes. Thus, their LMR_b was lower ($P = 0.0462$) than LCRC patients (Figure 2A). Neither NLR_b nor PLR_b showed differences between RCRC and LCRC patients (Figure 2B and C).

Tissues from 54 out of the total 65 patients included in the study, 34 from RCRC patients (63%) and 20 from LCRC patients (37%), could be assessed for leukocyte infiltration analyses. This fact was mainly due to the morphological characteristics of 11 tumours, which made it impossible to separate pieces for research purposes without affecting the global diagnostics by pathologists.

Figure 3 shows the staining pattern for CD4, CD8, and CD14 cells in tumour and peritumour samples from two representative patients of LCRC and RCRC. The distribution of total ($CD4^+$ plus $CD8^+$) lymphocytes, $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, and $CD14^+$ monocytes, in all analysed tissues, is shown in Supplementary Figure 2. Higher total lymphocyte content in tumours than peritumours from LCRC patients (13.06 ± 2.123 *vs* 7.57 ± 1.794, $P = 0.0095$) seemed due to the proportional increase of $CD8^+$ lymphocytes (11.19 ± 2.158 *vs* 5.13 ± 1.757, $P = 0.0020$), as we detected no differences amongst infiltrated $CD4^+$ lymphocytes in these tissues. No differences were found for lymphocyte infiltration in right tumours with respect to right peritumoural tissues. Moreover, infiltrated-leukocyte content in right tumours showed no differences to right peritumours.

The analysis of resulting ratios for lymphocyte and monocyte counts in tumour (t) and peritumoural (pt) tissues showed a higher LMR_t with respect to LMR_{pt} (4.128 ± 1.363 *vs* 2.022 ± 0.3432, $P = 0.0023$) beside higher $CD8MR_t$ than $CD8MR_{pt}$ (4.121 ± 1.374 *vs* 1.218 ± 0.3297, $P = 0.0001$) in LCRC patients (Figure 2D-F). No differences were detected for these ratios amongst RCRC respective tissues. Consequently, the analysis of blood-to-tissue ratios (Figure 2G-I) showed LCRC patients exhibited lower LMR_b/LMR_t than LMR_b/LMR_{pt} (2.104 ± 0.601 *vs* 2.900 ± 0.5061, $P = 0.0282$) as well as lower $LMR_b/CD8MR_t$ than $LMR_b/CD8MR_{pt}$ (3.381 ± 1.083 *vs* 5.898 ± 1.138, $P = 0.0033$). There were no differences in these ratios for RCRC respective tissues.

Association of leukocyte balance and patient's outcome

In order to assess the degree to which leukocyte balance (*i.e.* both the concentration

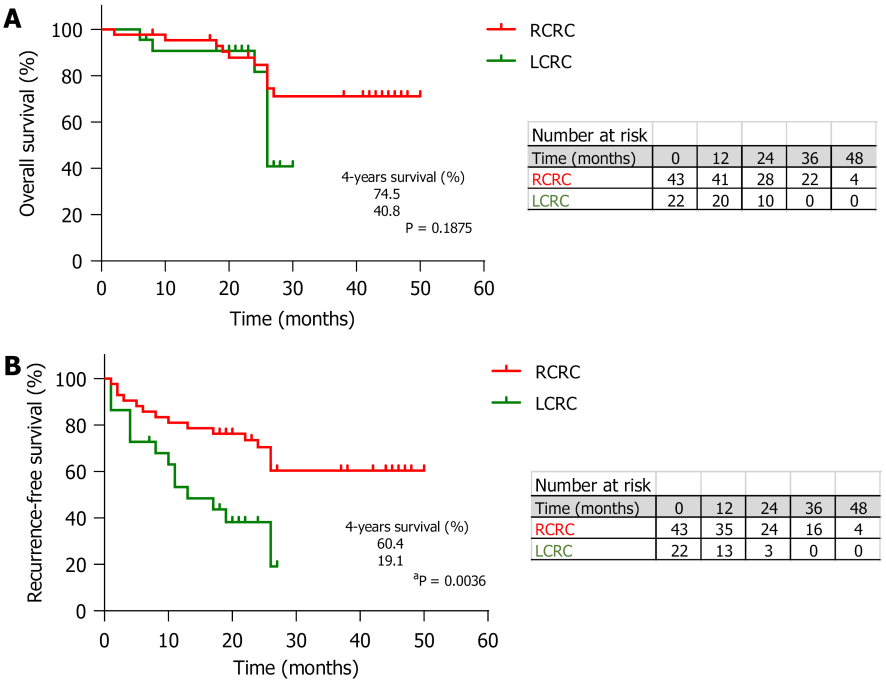


Figure 1 Survival rates of colorectal cancer patients. A, B: Kaplan-Meier curves for 4-year overall survival (A) and 4-year recurrence-free survival (B) observed in the cohort for both right-sided colorectal cancer (CRC) (orange) and left-sided CRC (green) patients. Survival proportions at 26 mo after surgery (median follow-up) of right-sided CRC and left-sided CRC patients are shown ($^bP < 0.01$, log-rank test). Number of cases at risk are tabled for both overall survival and recurrence-free survival. RCRC: Right-sided colorectal cancer; LCRC: Left-sided colorectal cancer.

and ratios of leukocytes for blood, tumour, and peritumours described above) was associated with RCRC and LCRC patient OS and RFS, we first conducted a Spearman correlation analysis (Table 2). We found that for RCRC LMR_b (r = -0.3039, P = 0.0476), LMR_{pt} (r = -0.4301, P = 0.0111), and $CD8MR_{pt}$ (r =-0.3596, P=0.0367) were negatively correlated with OS; LMR_t (r = -0.4775, P = 0.0043), LMR_{pt} (r = -0.3846, P = 0.0247), and CD8MRt (r = -0.4422, P = 0.0088) negatively correlated, but LMR_b/LMR_t (r = 0.3621, P = 0.0363) positively correlated with RFS. $LMR_b/CD8MR_t$ (r = 0.3364, P = 0.0517) also showed a trend towards being positively correlated. For LCRC, $CD14_{pt}$ (r = 0.5677, P = 0.009) and $LMR_b/CD4MR_{pt}$ (r = 0.4541, P=0.0443) positively correlated, but $CD4MR_{pt}$ (r = -0.473, P = 0.0352) negatively correlated with OS, whilst both $CD14_{pt}$ (r = 0.6018, P = 0.005) and $CD8MR_t$ (r = 0.4779, P = 0.331) positively correlated with RFS, and $CD4CD8_{pt}$ (r = 0.4425, P = 0.0507) also showed a trend towards being positively correlated.

Next, the effect of these variables on survival was assessed by Cox proportional hazards regression. For OS (Table 3), the univariate analysis revealed that besides previously found LMR_b (P = 0.043), LMR_{pt} (P = 0.024), and $CD8MR_{pt}$ (P = 0.031) in RCRC patients, NLR_b (P = 0.038) also significantly correlated with OS; $LMR_b/CD4MR_{pt}$ (P = 0.026) was also confirmed to be significantly correlated with OS of LCRC patients. After adjusting for confounding variables through the multivariate analysis, NLR_b (P = 0.038), $CD8MR_{pt}$ (P = 0.011), and $LMR_b/CD8MR_{pt}$ (P = 0.016) resulted in a significant association with OS of RCRC patients; $CD8_{pt}$ (P = 0.058) also showed a trend towards being associated.

Regarding RFS (Table 4), the univariate analysis showed that in addition to previously found LMR_t (P = 0.021) and LMR_b/LMR_t (P = 0.040) in RCRC, $LMR_b/CD8MR_t$ (P = 0.025) also significantly correlated with RFS, and $CD8MR_t$ (P = 0.052) showed a trend towards being associated. In addition to previously found $CD14_{pt}$ (P = 0.010), NLR_b (P = 0.020) and PLR_b (P = 0.018) were also significantly correlated with RFS in LCRC patients. After the multivariate analysis, several variables emerged as independent prognostic factors for RFS in RCRC patients: NLR_b (P = 0.039), PLR_b (P = 0.037), $CD14_t$ (P = 0.026), LMR_{pt} (P = 0.014), LMR_b/LMR_{pt} (P = 0.042), and $LMR_b/CD8MR_t$ (P = 0.006). In LCRC patients, NLR_b (P = 0.009), $CD8_{pt}$ (P = 0.020), $CD4CD8_t$ (P = 0.039), and $CD8MR_t$ (P = 0.019) were found, together with a trend observed for $CD4\ CD8_{pt}$ (P = 0.053).

Table 2 Association of leukocytes counts and ratios with overall and recurrence-free survival

Index	Total patients	4-year OS				4-year RFS			
	n (%)	Spearman *r*	95%CI	*P*		Spearman *r*	95%CI	*P*	
LMR_b									
Right	43 (66.2)	-0.3039	-0.5601 to 0.005346	0.0476	< 0.05	-0.1946	-0.4748 to 0.1214	0.211	NS
Left	22 (33.8)	0.1615	-0.2914 to 0.5553	0.4727	NS	-0.07447	-0.4912 to 0.3700	0.7419	NS
NLR_b									
Right	43 (66.2)	0.2262	-0.08868 to 0.5000	0.1446	NS	0.1062	-0.2094 to 0.4017	0.498	NS
Left	22 (33.8)	-0.06922	-0.4872 to 0.3746	0.7595	NS	0.1192	-0.3305 to 0.5247	0.5974	NS
PLR_b									
Right	43 (66.2)	0.2307	-0.08405 to 0.5035	0.1367	NS	0.06684	-0.2470 to 0.3680	0.6702	NS
Left	22 (33.8)	0.1615	-0.2914 to 0.5553	0.4727	NS	0.1192	-0.3305 to 0.5247	0.5974	NS
$CD4_t$									
Right	34 (63.0)	-0.05561	-0.3954 to 0.2976	0.7548	NS	0.05447	-0.2986 to 0.3944	0.7596	NS
Left	20 (37.0)	0.01892	-0.4387 to 0.4687	0.9369	NS	-0.0354	-0.4815 to 0.4253	0.8822	NS
$CD4_{pt}$									
Right	34 (63.0)	0.03708	-0.3144 to 0.3796	0.8351	NS	0.07371	-0.2809 to 0.4106	0.6787	NS
Left	20 (37.0)	-0.142	-0.5597 to 0.3334	0.5505	NS	0.3276	-0.1483 to 0.6803	0.1586	NS
$CD8_t$									
Right	34 (63.0)	-0.08526	-0.4202 to 0.2702	0.6316	NS	-0.2083	-0.1958 to 0.6531	0.2372	NS
Left	20 (37.0)	0.03784	-0.4233 to 0.4834	0.8741	NS	0.2832	-0.3863 to 0.3074	0.2263	NS
$CD8_{pt}$									
Right	34 (63.0)	-0.1186	-0.4476 to 0.2386	0.504	NS	-0.04486	-0.3863 to 0.3074	0.8011	NS
Left	20 (37.0)	0.3406	-0.1340 to 0.6881	0.1417	NS	0.3186	-0.1581 to 0.6749	0.171	NS
$CD4CD8_t$									
Right	34 (63.0)	-0.1372	-0.4626 to 0.2208	0.4392	NS	-0.1955	-0.5084 to 0.1630	0.268	NS
Left	20 (37.0)	0.03784	-0.4233 to 0.4834	0.8741	NS	0.2655	-0.2142 to 0.6420	0.2579	NS
$CD4CD8_{pt}$									
Right	34 (63.0)	-0.07044	-0.4079 to 0.2839	0.6922	NS	-0.009612	-0.3559 to 0.3389	0.957	NS
Left	20 (37.0)	0.3595	-0.1127 to 0.6993	0.1195	NS	0.4425	-0.01421 to 0.7464	0.0507	NS
$CD14_t$									
Right	34 (63.0)	0.1891	-0.1695 to 0.5034	0.2842	NS	0.2467	-0.1102 to 0.5472	0.1595	NS
Left	20 (37.0)	0.05677	-0.4076 to 0.4978	0.8121	NS	-0.1239	-0.5470 to	0.6028	NS

							0.3496		
$CD14_{pt}$									
Right	34 (63.0)	0.3003	-0.05262 to 0.5865	0.0844	NS	0.2596	-0.09658 to 0.5568	0.1382	NS
Left	20 (37.0)	0.5677	0.1533 to 0.8122	0.009	< 0.01	0.6018	0.2035 to 0.8292	0.005	< 0.01
LMR_t									
Right	34 (63.0)	-0.2929	-0.5812 to 0.06070	0.0927	NS	-0.4775	-0.7075 to -0.1559	0.0043	< 0.01
Left	20 (37.0)	0.07569	-0.3916 to 0.5119	0.7511	NS	0.4425	-0.01421 to 0.7464	0.0507	NS
LMR_{pt}									
Right	34 (63.0)	-0.4301	-0.6764 to -0.09719	0.0111	< 0.05	-0.3846	-0.6457 to -0.04285	0.0247	< 0.05
Left	20 (37.0)	0	-0.4538 to 0.4538	> 0.9999	NS	-0.0354	-0.4815 to 0.4253	0.8822	NS
CD4MRt									
Right	34 (63.0)	-0.2781	-0.5704 to 0.07674	0.1113	NS	-0.3173	-0.5987 to 0.03388	0.0675	NS
Left	20 (37.0)	0.04736	-0.4154 to 0.4907	0.8428	NS	0.1949	-0.2841 to 0.5960	0.4102	NS
CD4MRpt									
Right	34 (63.0)	-0.2781	-0.5704 to 0.07670	0.1112	NS	-0.2596	-0.5568 to 0.09650	0.1381	NS
Left	20 (37.0)	-0.473	-0.7631 to -0.02445	0.0352	NS	-0.0885	-0.5214 to 0.3806	0.7106	NS
CD8MRt									
Right	34 (63.0)	-0.2039	-0.5149 to 0.1545	0.2474	NS	-0.4422	-0.6845 to -0.1120	0.0088	< 0.01
Left	20 (37.0)	0.1135	-0.3588 to 0.5396	0.6337	NS	0.4779	0.03071 to 0.7657	0.0331	< 0.05
CD8MRpt									
Right	34 (63.0)	-0.3596	-0.6285 to -0.01393	0.0367	< 0.05	-0.2788	-0.5709 to 0.07601	0.1104	NS
Left	20 (37.0)	0.1893	-0.2894 to 0.5923	0.4241	NS	-0.03541	-0.4815 to 0.4253	0.8822	NS
LMR_b/LMRt									
Right	34 (63.0)	0.1075	-0.2492 to 0.4386	0.545	NS	0.3621	0.01678 to 0.6302	0.0353	< 0.05
Left	20 (37.0)	-0.05677	-0.4978 to 0.4076	0.8121	NS	-0.4248	-0.7366 to 0.03599	0.0619	NS
LMR_b/LMRpt									
Right	34 (63.0)	0.241	-0.1162 to 0.5430	0.1698	NS	0.2884	-0.06561 to 0.5779	0.0981	NS
Left	20 (37.0)	0.1325	-0.3420 to 0.5531	0.5778	NS	-0.0531	-0.4950 to 0.4106	0.8241	NS
$LMR_b/CD4MR_t$									
Right	34 (63.0)	0.1372	-0.2208 to 0.4626	0.4392	NS	0.2467	-0.1101 to 0.5473	0.1595	NS
Left	20 (37.0)	-0.09461	-0.5259 to 0.3754	0.6915	NS	-0.177	-0.5839 to 0.3010	0.4554	NS
$LMR_b/CD4MR_{pt}$									

Right	34 (63.0)	0.1446	-0.2136 to 0.4685	0.4146	NS	0.189	-0.1695 to 0.5034	0.2843	NS
Left	20 (37.0)	0.4541	0.0003499 to 0.7528	0.0443	< 0.05	0.1062	-0.3653 to 0.5343	0.6559	NS
$LMR_b/CD8MR_t$									
Right	34 (63.0)	0.04078	-0.3111 to 0.3828	0.8189	NS	0.3364	-0.01245 to 0.6123	0.0517	NS
Left	20 (37.0)	-0.03784	-0.4834 to 0.4233	0.8741	NS	-0.4071	-0.7267 to 0.05735	0.0748	NS
$LMR_b/CD8MR_{pt}$									
Right	34 (63.0)	0.1409	-0.2172 to 0.4655	0.4267	NS	0.1859	-0.1727 to 0.5009	0.2926	NS
Left	20 (37.0)	-0.1703	-0.5794 to 0.3073	0.4729	NS	-0.1416	-0.5595 to 0.3337	0.5515	NS

OS: Overall survival; RFS: Recurrence-free survival; LMR: Lymphocyte-to-monocyte ratio; NLR: Neutrophil-to-lymphocytes ratio; PLR: Platelet-to-lymphocyte ratio; b: Blood; t: Tumour; pt: Peritumour; CD4MR: $CD4^+$-lymphocyte-to-monocyte ratio; CD8MR: $CD8^+$-lymphocyte-to-monocyte ratio; CI: Confidence interval; NS: Not significant.

Survival prognostic value of the studied variables and ratios

Taking into account all previous correlations, we then calculated the optimal cutoff values by ROC analyses for those variables significantly correlated with OS or RFS, using respectively cancer-specific death or relapse as the endpoints for both RCRC (Figures 4 and 6) and LCRC (Figure 5) patients after surgical intervention.

Regarding OS, ROC curve analysis of $CD8_{pt}$ (Figure 4A; AUC = 0.585, 95%CI: 0.376-0.793, P = 0.496) identified the optimal cutoff point at 8.250, which entails significantly worse outcomes for RCRC patients ranking below this (Figure 4B; 100% *vs* 48.2%, P = 0.0077). $CD4CD8_{pt}$ analysis (Figure 4C; AUC = 0.550, 95%CI: 0.334-0.766, P = 0.686) identified 10.16 as the optimal cutoff, with worse outcomes for RCRC patients ranking below this (Figure 4D; 92.3% *vs* 49.1%, P = 0.0188). LMR_{pt} analysis (Figure 4E; AUC = 0.807, 95%CI: 0.641-0.973, P = 0.013) identified 3.185 as the optimal cutoff, with worse outcomes for RCRC patients ranking below this (Figure 4F; 100% *vs* 42.7%, P = 0.0028). $CD8MR_{pt}$ analysis (Figure 4G; AUC = 0.757, 95%CI: 0.600-0.914, P = 0.039) identified 1.650 as the optimal cutoff, with worse outcomes for RCRC patients ranking below this (Figure 4H; 100% *vs* 35.7%, P = 0.0007). LMR_b/LMR_{pt} analysis (Figure 4I; AUC = 0.672, 95%CI: 0.479-0.865, P = 0.166) identified 0.985 as the optimal cutoff, with worse outcomes for RCRC patients ranking above this (Figure 4J; 91.6% *vs* 52.0%, P = 0.0244). $LMR_b/CD8MR_{pt}$ analysis (Figure 4K; AUC = 0.601, 95%CI: 0.419-0.782, P = 0.418) identified 1.485 as the optimal cutoff, with worse outcomes for RCRC patients ranking above this (Figure 4L; 100% *vs* 50.9%, P = 0.0101). Finally, $LMR_b/CD4MR_{pt}$ analysis (Figure 5A; AUC = 0.786, 95%CI: 0.564-1.000, P = 0.048) identified 10.57 as the optimal cutoff, with worse outcomes for LCRC patients ranking above this (Figure 5B; 66.6% *vs* 18.7%, P = 0.0416). In addition, ROC curve analyses (Supplementary Figure 3) of $CD4CD8_t$, PLR_b, $CD4CD8_{pt}$, and $CD8MR_t$, though they showed significant AUC (0.524, 0.619, 0.726 and 0.571, respectively), rendered optimal cutoff values with no significant differences for LCRC survival.

With respect to RFS, ROC curve analysis of LMR_{pt} (Figure 6A; AUC = 0.737, 95%CI: 0.554-0.920, P = 0.027) identified 3.185 as the optimal cutoff, with worse outcomes for RCRC patients ranking below this (Figure 6B; 92.3% *vs* 32.5%, P = 0.0046). LMR_b/LMR_{pt} analysis (Figure 6C; AUC = 0.678, 95%CI: 0.499-0.857, P = 0.098) identified 0.985 as the optimal cutoff, with worse outcomes for RCRC patients ranking above this (Figure 6D; 84.4% *vs* 40.6%, P = 0.0155). $LMR_b/CD8MR_{pt}$ analysis (Figure 6E; AUC = 0.615, 95%CI: 0.427-0.802, P = 0.286) identified 1.485 as the optimal cutoff, with worse outcomes for RCRC patients ranking above this (Figure 6F; 91.7% *vs* 40.7%, P = 0.0141). The ROC analyses in RCRC patients of $CD8MR_{pt}$, $CD8_{pt}$, and $CD4CD8_{pt}$ (Supplementary Figure 4), though they showed significant AUC (0.672, 0.528 and 0.506, respectively) did not render optimal cutoff values that significantly prognosticated the RCRC patient RFS. Similarly, ROC analyses of $CD4CD8_t$, PLR_b, $CD4CD8_{pt}$, $CD8MR_t$, and $LMR_b/CD4MR_{pt}$ (Supplementary Figure 5) with significant AUC (0.656, 0.635, 0.760, 0.781 and 0.563, respectively) did not provide optimal cutoff values with significant prognostication in LCRC patient RFS.

Table 3 Univariate and multivariate analyses for prognostic variables of overall survival after surgical interventions of right-sided colorectal cancer and left-sided colorectal cancer

	Total patients	Univariate analysis					Multivariate analysis				
	n (%)	HR	95%CI		*P*		HR	95%CI		*P*	
Variables			Low	High				Low	High		
LMR_b											
Right	34 (63.0)	0.565	0.325	0.982	0.043	< 0.05	0.133	0.000	71.041	0.529	
Left	20 (37.0)	1.141	0.867	1.502	0.346		1.141	0.867	1.502	0.346	
NLR_b											
Right	34 (63.0)	1.416	1.019	1.967	0.038	< 0.05	1.416	1.019	1.967	0.038	< 0.05
Left	20 (37.0)	1.043	0.944	1.152	0.410		1.126	0.987	1.284	0.078	
PLR_b											
Right	34 (63.0)	1.005	1.000	1.011	0.064		0.989	0.719	1.361	0.946	
Left	20 (37.0)	1.001	0.999	1.004	0.292		0.997	0.937	1.060	0.912	
$CD4_t$											
Right	34 (63.0)	0.923	0.638	1.334	0.670						
Left	20 (37.0)	0.843	0.493	1.439	0.531						
$CD4_{pt}$											
Right	34 (63.0)	0.959	0.817	1.124	0.604						
Left	20 (37.0)	0.813	0.454	1.456	0.487						
$CD8_t$											
Right	34 (63.0)	0.995	0.897	1.105	0.931		1.032	0.913	1.167	0.611	
Left	20 (37.0)	0.954	0.860	1.057	0.364						
$CD8_{pt}$											
Right	34 (63.0)	0.792	0.617	1.016	0.066		0.800	0.636	1.007	0.058	
Left	20 (37.0)	1.018	0.957	1.083	0.570		1.033	0.962	1.110	0.372	
$CD4CD8_t$											
Right	34 (63.0)	0.987	0.892	1.093	0.806						
Left	20 (37.0)	0.937	0.833	1.054	0.277						
$CD4CD8_{pt}$											
Right	34 (63.0)	0.891	0.761	1.043	0.150		1.073	0.821	1.401	0.606	
Left	20 (37.0)	1.015	0.953	1.082	0.641						
$CD14_t$											
Right	34 (63.0)	1.048	0.845	1.300	0.670		1.077	0.836	1.388	0.565	
Left	20 (37.0)	0.979	0.703	1.362	0.898						
$CD14_{pt}$											
Right	34 (63.0)	1.113	0.889	1.394	0.351		1.342	0.987	1.826	0.060	
Left	20 (37.0)	1.053	0.723	1.533	0.788		0.700	0.381	1.286	0.250	
LMR_t											
Right	34 (63.0)	0.635	0.365	1.103	0.107						
Left	20 (37.0)	1.000	0.908	1.101	0.997		0.976	0.555	1.716	0.933	
LMR_{pt}											
Right	34 (63.0)	0.416	0.194	0.889	0.024	< 0.05					

Left	20 (37.0)	1.030	0.712	1.490	0.876		0.031	0.000	4.228	0.166	
$CD4MR_t$											
Right	34 (63.0)	0.270	0.039	1.850	0.182						
Left	20 (37.0)	0.759	0.146	3.954	0.743						
$CD4MR_{pt}$											
Right	34 (63.0)	0.431	0.112	1.660	0.221						
Left	20 (37.0)	0.135	0.004	4.148	0.252						
$CD8MR_t$											
Right	34 (63.0)	0.712	0.364	1.394	0.321						
Left	20 (37.0)	1.001	0.910	1.100	0.986						
$CD8MR_{pt}$											
Right	34 (63.0)	0.223	0.057	0.872	0.031	< 0.05	0.024	0.001	0.430	0.011	< 0.05
Left	20 (37.0)	1.078	0.775	1.500	0.654						
LMR_b/LMR_t											
Right	34 (63.0)	0.957	0.603	1.519	0.853						
Left	20 (37.0)	1.163	0.935	1.446	0.175						
LMR_b/LMR_{pt}											
Right	34 (63.0)	1.253	0.824	1.907	0.292						
Left	20 (37.0)	1.196	0.845	1.695	0.313						
$LMR_b/CD4MR_t$											
Right	34 (63.0)	1.009	0.950	1.073	0.767		1.282	0.931	1.765	0.128	
Left	20 (37.0)	1.027	0.961	1.099	0.428						
$LMR_b/CD4MR_{pt}$											
Right	34 (63.0)	1.028	0.926	1.142	0.600		1.291	0.447	3.728	0.636	
Left	20 (37.0)	1.097	1.011	1.190	0.026	< 0.05	0.991	0.420	2.341	0.984	
$LMR_b/CD8MR_t$											
Right	34 (63.0)	0.973	0.747	1.269	0.842						
Left	20 (37.0)	1.103	0.940	1.294	0.229		1.971	0.258	15.069	0.513	
$LMR_b/CD8MR_{pt}$											
Right	34 (63.0)	1.053	0.880	1.260	0.572		0.484	0.268	0.873	0.016	< 0.05
Left	20 (37.0)	1.026	0.834	1.262	0.812		0.952	0.009	96.530	0.983	

LMR: Lymphocyte-to-monocyte ratio; NLR: Neutrophil-to-lymphocytes ratio; PLR: Platelet-to-lymphocyte ratio; HR: Hazard ratio; CI: Confidence interval; b: Blood; t: Tumour; pt: Peritumour; CD4MR: $CD4^+$-lymphocyte-to-monocyte ratio; CD8MR: $CD8^+$-lymphocyte-to-monocyte ratio.

Nomograms modelling and validation

In order to avoid conflicts in handling the different values of the predictive indexes for RCRC patients, clinician-friendly nomograms were developed for both OS (Figure 7A) and RFS (Figure 7B) of these patients. The six significant predictive variables found for OS and the three found for RFS were used to construct the respective nomograms, with data from the training set of RCRC patients. The calibration of these nomograms revealed C-indexes of 0.600 (95%CI: 0.561-0.639) and 0.605 (95%CI: 0.579-0.631), respectively (Supplementary Figure 6A-B). Moreover, the reliability of the nomograms was evaluated with the validation set of RCRC patients, showing a moderate accuracy, with C-indexes of 0.500 (95%CI: 0.475-0.525) and 0.570 (95%CI: 0.541-0.599) for OS and RFS, respectively (Supplementary Figure 6C-D).

Table 4 Univariate and multivariate analyses for prognostic variables of recurrence-free survival after surgical interventions of right-sided colorectal cancer and left-sided colorectal cancer

	Total patients	Univariate analysis					Multivariate analysis				
	n (%)	HR	95%CI		*P*		HR	95%CI		*P*	
Variables			Low	High				Low	High		
LMR_b											
Right	34 (63.0)	0.865	0.593	1.262	0.453						
Left	20 (37.0)	0.977	0.770	1.239	0.848		0.156	0.001	24.118	0.470	
NLR_b											
Right	34 (63.0)	1.135	0.841	1.532	0.407		2.760	1.050	7.254	0.039	< 0.05
Left	20 (37.0)	1.094	1.094	1.180	0.020	< 0.05	1.156	1.038	1.288	0.009	< 0.01
PLR_b											
Right	34 (63.0)	1.001	0.996	1.007	0.596		0.978	0.958	0.999	0.037	< 0.05
Left	20 (37.0)	1.002	1.000	1.004	0.018	< 0.05	0.987	0.954	1.022	0.468	
$CD4_t$											
Right	34 (63.0)	1.010	0.864	1.181	0.902		0.802	0.457	1.407	0.441	
Left	20 (37.0)	0.981	0.617	1.559	0.934						
$CD4_{pt}$											
Right	34 (63.0)	0.963	0.851	1.091	0.556		1.486	0.124	17.828	0.755	
Left	20 (37.0)	1.120	0.795	1.577	0.516						
$CD8_t$											
Right	34 (63.0)	0.962	0.872	1.062	0.446		1.821	0.451	7.347	0.400	
Left	20 (37.0)	1.032	0.971	1.097	0.310						
$CD8_{pt}$											
Right	34 (63.0)	0.906	0.780	1.053	0.199		1.117	0.848	1.472	0.431	
Left	20 (37.0)	1.050	0.988	1.115	0.116		1.098	1.015	1.189	0.020	< 0.05
$CD4CD8_t$											
Right	34 (63.0)	0.972	0.893	1.058	0.515						
Left	20 (37.0)	1.033	0.970	1.100	0.307		0.436	0.198	0.960	0.039	< 0.05
$CD4CD8_{pt}$											
Right	34 (63.0)	0.944	0.857	1.041	0.248						
Left	20 (37.0)	1.052	0.992	1.117	0.093		0.008	0.000	1.071	0.053	
$CD14_t$											
Right	34 (63.0)	1.123	0.949	1.329	0.178		1.467	1.048	2.054	0.026	< 0.05
Left	20 (37.0)	0.918	0.713	1.180	0.502						
$CD14_{pt}$											
Right	34 (63.0)	1.075	0.891	1.297	0.449		1.790	0.592	5.406	0.302	
Left	20 (37.0)	1.472	1.095	1.978	0.010	< 0.05					
LMR_t											
Right	34 (63.0)	0.555	0.337	0.915	0.021	< 0.05	0.641	0.213	1.926	0.428	
Left	20 (37.0)	1.084	0.999	1.176	0.052		0.165	0.003	9.203	0.380	
LMR_{pt}											
Right	34 (63.0)	0.691	0.458	1.042	0.078		0.312	0.123	0.793	0.014	< 0.05

Left	20 (37.0)	1.066	0.688	1.653	0.775					
$CD4MR_t$										
Right	34 (63.0)	0.588	0.220	1.572	0.290		0.876	0.382	2.010	0.756	
Left	20 (37.0)	0.950	0.381	2.370	0.912					
$CD4MR_{pt}$										
Right	34 (63.0)	0.734	0.327	1.648	0.454		7.229	0.515	101.544	0.142	
Left	20 (37.0)	0.583	0.199	1.709	0.325					
$CD8MR_t$										
Right	34 (63.0)	0.497	0.246	1.005	0.052					
Left	20 (37.0)	1.083	0.999	1.174	0.052		1.123	1.020	1.238	0.019	< 0.05
$CD8MR_{pt}$										
Right	34 (63.0)	0.584	0.333	1.023	0.060					
Left	20 (37.0)	1.191	0.815	1.741	0.365		0.293	0.026	3.345	0.323	
$LMRb/LMR_t$										
Right	34 (63.0)	1.311	1.013	1.697	0.040	< 0.05					
Left	20 (37.0)	0.958	0.730	1.258	0.760					
$LMRb/LMR_{pt}$										
Right	34 (63.0)	1.248	0.887	1.756	0.203		0.404	0.169	0.969	0.042	< 0.05
Left	20 (37.0)	1.030	0.787	1.347	0.830		1.132	0.859	1.493	0.378	
$LMRb/CD4MR_t$										
Right	34 (63.0)	1.012	0.965	1.060	0.632		1.056	0.973	1.146	0.192	
Left	20 (37.0)	0.991	0.937	1.048	0.742					
$LMRb/CD4MR_{pt}$										
Right	34 (63.0)	1.031	0.948	1.121	0.479		1.393	0.875	2.220	0.163	
Left	20 (37.0)	1.023	0.969	1.079	0.412		0.925	0.847	1.011	0.087	
$LMRb/CD8MR_t$										
Right	34 (63.0)	1.146	1.017	1.292	0.025	< 0.05	1.301	1.078	1.571	0.006	< 0.01
Left	20 (37.0)	0.941	0.775	1.143	0.542		1.036	0.591	1.816	0.903	
$LMRb/CD8MR_{pt}$										
Right	34 (63.0)	1.022	0.894	1.169	0.746		1.390	0.304	6.350	0.671	
Left	20 (37.0)	0.968	0.844	1.109	0.638		0.847	0.576	1.244	0.397	

LMR: Lymphocyte-to-monocyte ratio; NLR: Neutrophil-to-lymphocytes ratio; PLR: Platelet-to-lymphocyte ratio; HR: Hazard ratio; CI: Confidence interval; b: Blood; t: Tumour; pt: Peritumour; CD4MR: $CD4^+$-lymphocyte-to-monocyte ratio; CD8MR: $CD8^+$-lymphocyte-to-monocyte ratio.

DISCUSSION

The segment of the large intestine proximal to the splenic flexure, *i.e.* the right colon (comprising caecum, ascending colon, and proximal two-thirds of the transverse colon), derives from the embryonic midgut; whereas the left colon (comprising the distal third part of the transverse colon and descending and sigmoid colon) derives from the embryonic hindgut[21]. Distinct embryologic origin of right and left sides of the colon markedly determines important physiological differences, mainly: cell motility, vasculature, lymphatic drainage, extrinsic innervation, development of the endocrine components, and the expression and patterns of epigenetic marks of crucial molecular factors for cell development[21,22].

Since seminal contributions by Bufill *et al*[23], an increasing number of studies have supported the hypothesis that these differences in origin may explain why RCRC and LCRC constitute two distinct clinical entities, which arise through different

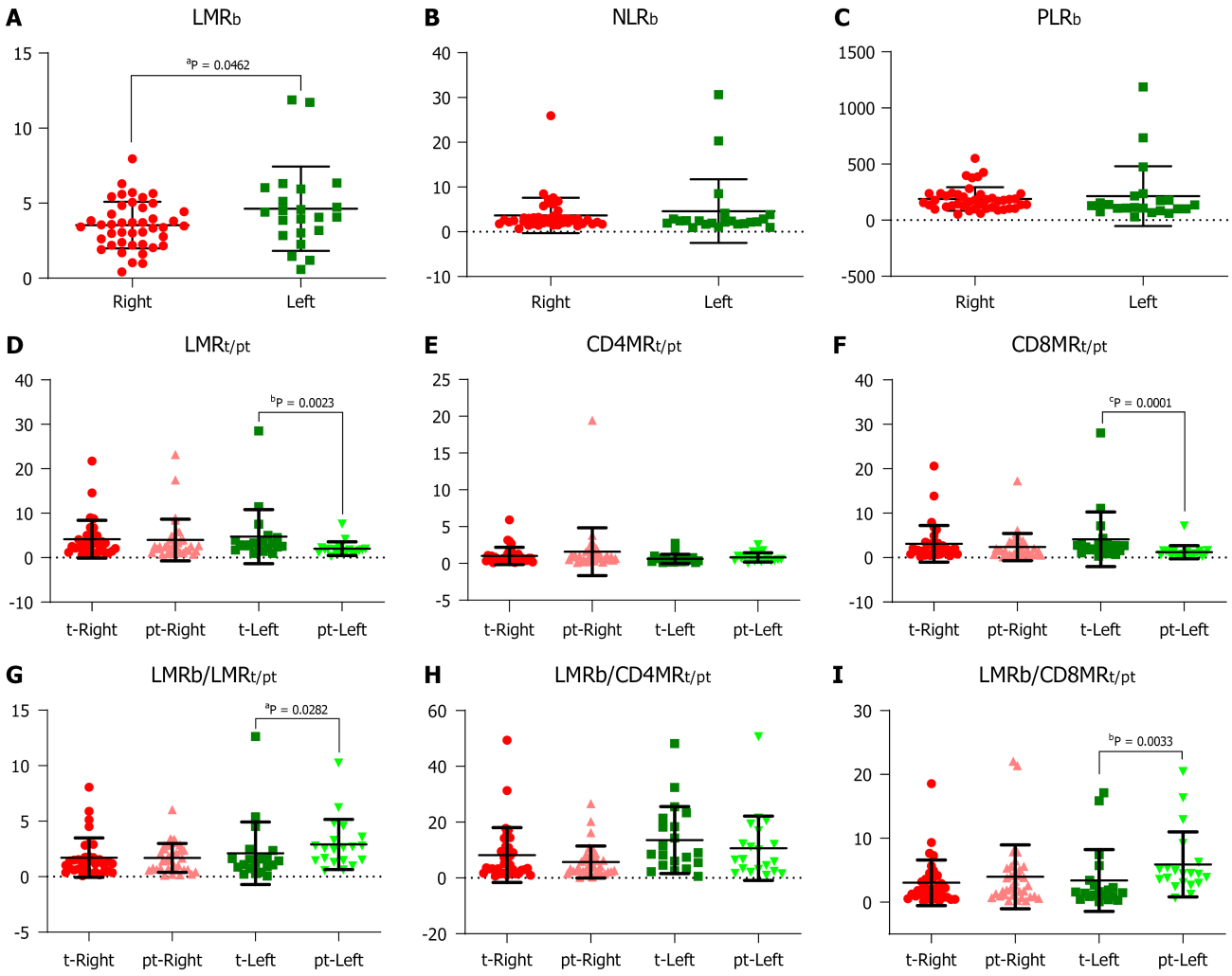


Figure 2 Leukocyte ratios in peripheral blood and tissues from colorectal cancer patients. A-C: Blood circulating leukocytes in right-sided colorectal cancer (CRC) patients (orange, n = 43) and left-sided CRC patients (green, n = 22) represented as lymphocyte-to-monocyte ratio $(LMR)_{blood\ (b)}$ (A), neutrophil-to-lymphocyte ratio (B), and platelet-to-lymphocyte ratio (C) ($^aP < 0.05$, unpaired t test, data are mean ± standard deviation); D-F: Tissue-infiltrated leukocytes in right-sided CRC tumours (tumour [t], orange, n = 34) and peritumours (peritumour [pt], light red, n = 34) and left-sided CRC tumours (t, green, n = 20) and peritumours (pt, light green, n = 20), represented as $LMR_{t/pt}$ (D), $CD4^+$-lymphocyte-to-monocyte ratio $(CD4MR)_{t/pt}$ (E), and $CD8^+$-lymphocyte-to-monocyte ratio $(CD8MR)_{t/pt}$ (F) ($^aP < 0.05$, $^bP < 0.01$, unpaired Mann-Whitney U test, data are mean ± standard deviation); G-I: Blood-to-tissue leukocyte ratios for right-sided CRC tumours (t, orange, n = 34) and peritumours (pt, light red, n = 34) and left-sided CRC tumours (t, green, n = 20) and peritumours (pt, light green, n = 20) represented as $LMR_b/LMR_{t/pt}$ (G), $LMR_b/CD4MR_{t/pt}$ (H), and $LMR_b/CD8MR_{t/pt}$ (I) ($^aP < 0.05$, $^bP < 0.01$, unpaired Mann-Whitney U test, data are mean ± standard deviation). PLR: Platelet-to-lymphocyte ratio.

pathogenetic mechanisms[22,24,25]. Thus, differential aspects such as incidence, presentation, microbiome composition, genetic burden, or immunogenicity could be explained on these grounds[26-31]. In a large study with more than 17000 CRC patients, Benedix *et al*[32] showed that RCRC represents a more distinct tumour entity than LCRC, mainly because of its higher incidence in women and older people, poor differentiation, locally advanced carcinomas, a distinct pattern of metastatic spread, and worse outcome.

Likewise, survival after surgical intervention to remove the tumour should constitute a prominent feature to differentiate both pathologies. In this line, controversial results arise throughout the literature. Thereby, some studies support RCRC patients having poorer overall and disease-free survival rates[8], whilst others call attention to the stage of the disease, with better rates for RCRC being limited to stage II and better rates for LCRC being limited to stage III[33]. In our cohort, perhaps due to the stage's heterogeneity of the patients, both OS and RFS were found side-dependent, with better outcomes in RCRC patients, reinforcing the idea that prognostic markers for the two pathologies should be studied separately.

A number of studies have stressed the importance of the systemic inflammatory response in CRC development and the search for variables involving its components as a valuable tool to drive prognosis[15,34]. Important prognostic records have been

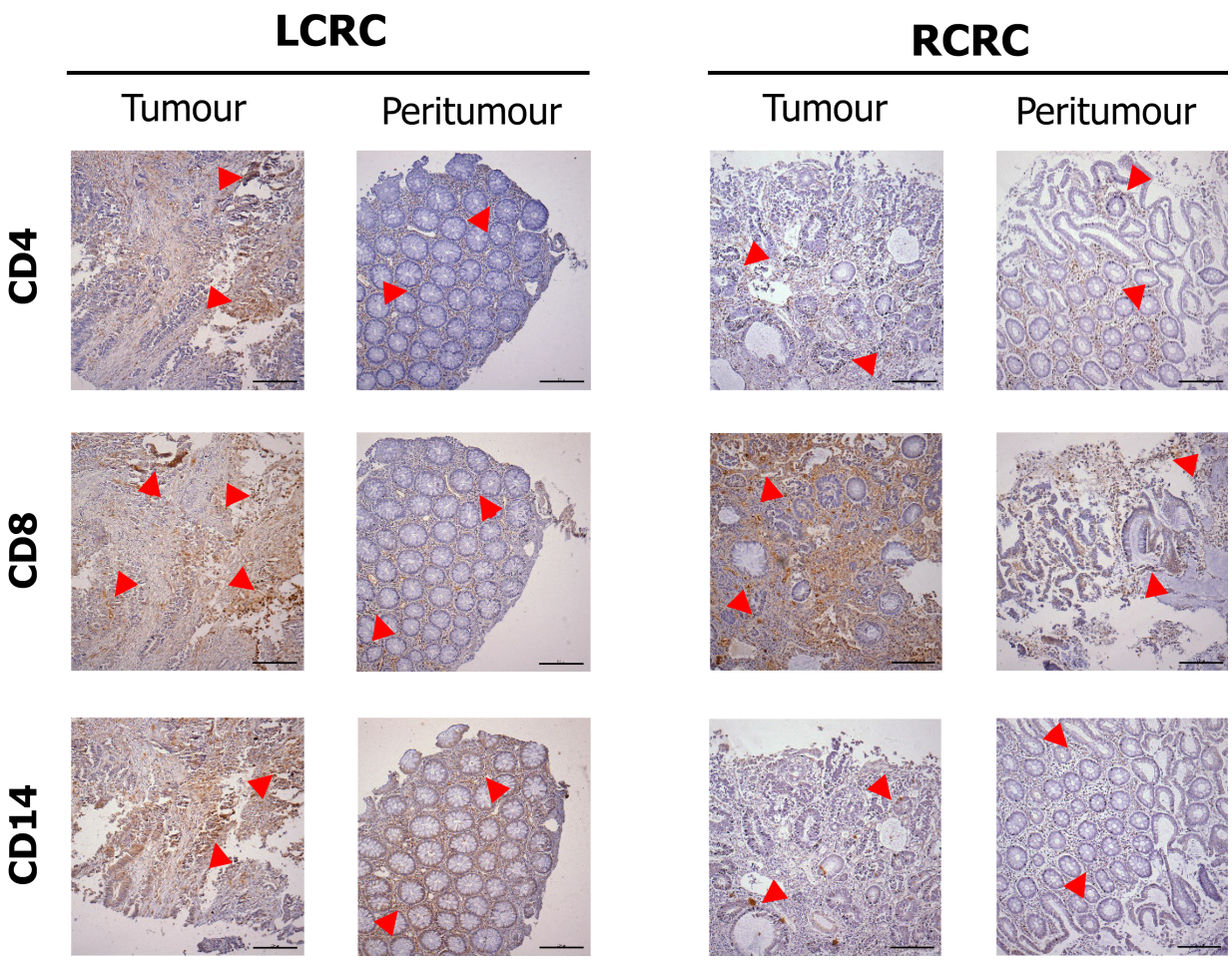
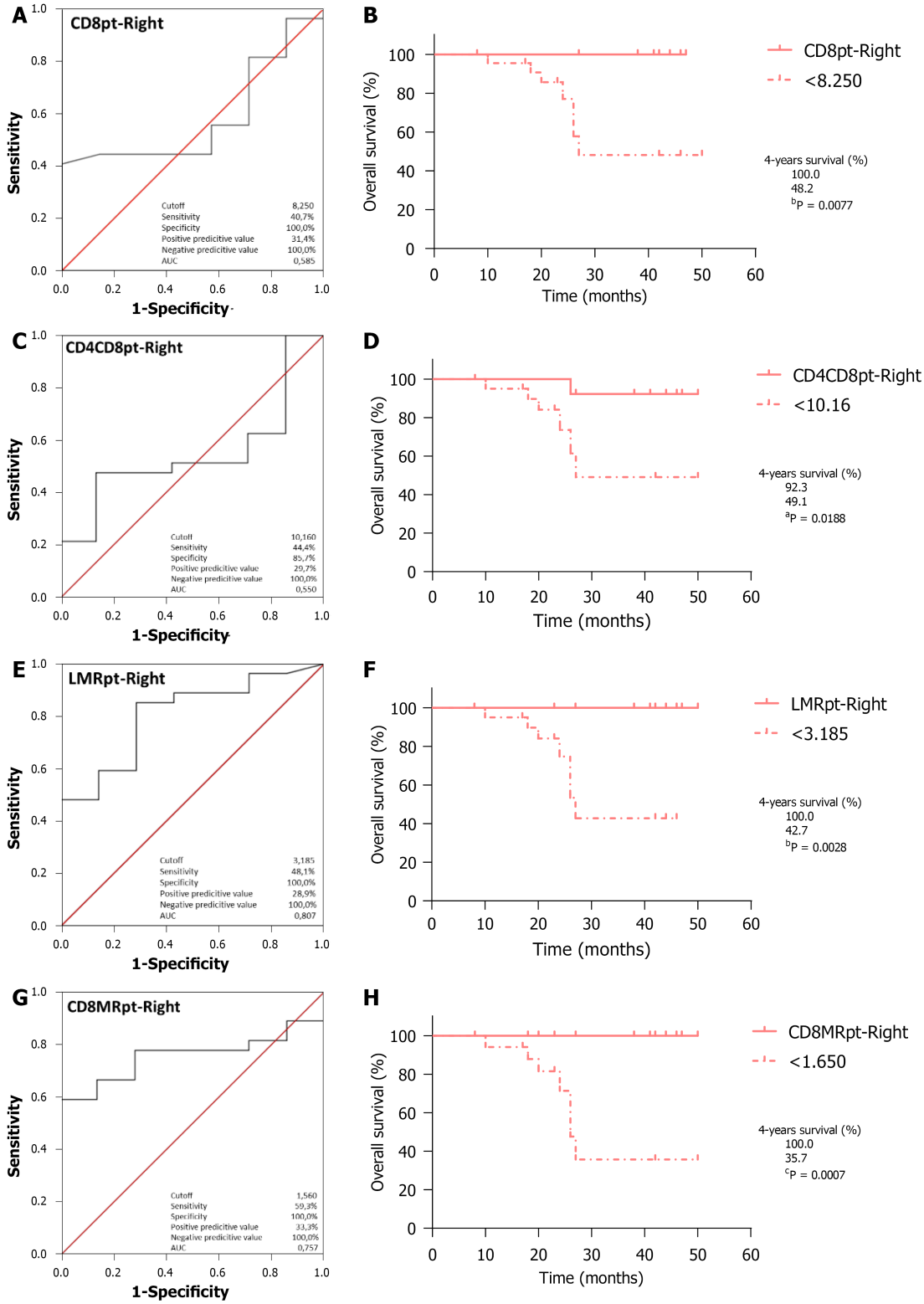


Figure 3 Leukocyte infiltration in tissues from right-sided colorectal cancer and left-sided colorectal cancer patients. Representative immunohistochemical images (× 100, scale bar = 200 μm) of $CD4^+$ lymphocytes, $CD8^+$ lymphocytes and $CD14^+$ monocytes in tumour and peritumour samples, from one left-sided colorectal cancer (LCRC) patient (left panel) and one right-sided colorectal cancer (RCRC) patient (right panel). Arrows show rich-marker zones in each sample.

obtained in several research works[16,35], which avail the use of blood leukocyte ratios as predictors in CRC progression after surgery. However, some studies have highlighted inherent failures to these analyses. Thus, Zhang *et al*[36] warn against the impact of the use of distinct factors, within different studies, to adjust possible confounders for multivariate hazard ratio determination, which can make the latter at risk of bias and heterogeneity, in turn making LMR fail to reach significance in survival. Likewise, sample size, race heterogeneity, and most of all the pre/post-operative dynamic changes in circulating leukocyte population can dramatically affect the observable effects of these variables in the multivariate models for survival progression[37]. In our correlative analyses, though all preoperative blood leukocyte ratios significantly rose at different stages, in the end we were unable to establish a predictor value for any of them, neither for RCRC nor for LCRC survival, perhaps due to a conjunction of previously discussed handicaps. Nonetheless, we do not discard the possibility for them to emerge as good predictors in the putative case those handicaps could be solved, thus improving the multivariate analyses.

Notably, we report tissue leukocyte ratios, both alone and combined with preoperative blood LMR_b, as six variables with a strong predictor value for RCRC overall survival ($CD8_{pt}$, $CD4CD8_{pt}$, LMR_{pt}, $CD8MR_{pt}$, LMR_b/LMR_{pt}, $LMR_b/CD8MR_{pt}$), three variables for recurrence-free survival (LMR_{pt}, $CD8MR_{pt}$, LMR_b/LMR_{pt}, $LMR_b/CD8MR_{pt}$), and another robust variable to predict LCRC overall survival ($LMR_b/CD4MR_{pt}$). In addition, to avoid conflicts when interpreting the different survival predictors of RCRC, physician-friendly nomograms are proposed for both OS and RFS. Albeit much effort has been made in describing and associating the leukocyte content of tumour tissues with CRC survival[38], most studies have been performed on disaggregated tumour and peritumour samples, and only a few of them have attempted to measure leukocyte expression in fixed samples of these tissues to associate them with circulating ratios[19] or to correlate them with patient survival[18,


A
CD8pt-Right
Sensitivity
1-Specificity
Cutoff 8,250
Sensitivity 40,7%
Specificity 100,0%
Positive predicitive value 31,4%
Negative predicitive value 100,0%
AUC 0,585
B
Overall survival (%)
Time (months)
CD8pt-Right
<8.250
4-years survival (%)
100.0
48.2
bP = 0.0077
C
CD4CD8pt-Right
Cutoff 10,160
Sensitivity 44,4%
Specificity 85,7%
Positive predicitive value 29,7%
Negative predicitive value 100,0%
AUC 0,550
D
CD4CD8pt-Right
<10.16
4-years survival (%)
92.3
49.1
aP = 0.0188
E
LMRpt-Right
Cutoff 3,185
Sensitivity 48,1%
Specificity 100,0%
Positive predicitive value 28,9%
Negative predicitive value 100,0%
AUC 0,807
F
LMRpt-Right
<3.185
4-years survival (%)
100.0
42.7
bP = 0.0028
G
CD8MRpt-Right
Cutoff 1,560
Sensitivity 59,3%
Specificity 100,0%
Positive predicitive value 33,3%
Negative predicitive value 100,0%
AUC 0,757
H
CD8MRpt-Right
<1.650
4-years survival (%)
100.0
35.7
cP = 0.0007

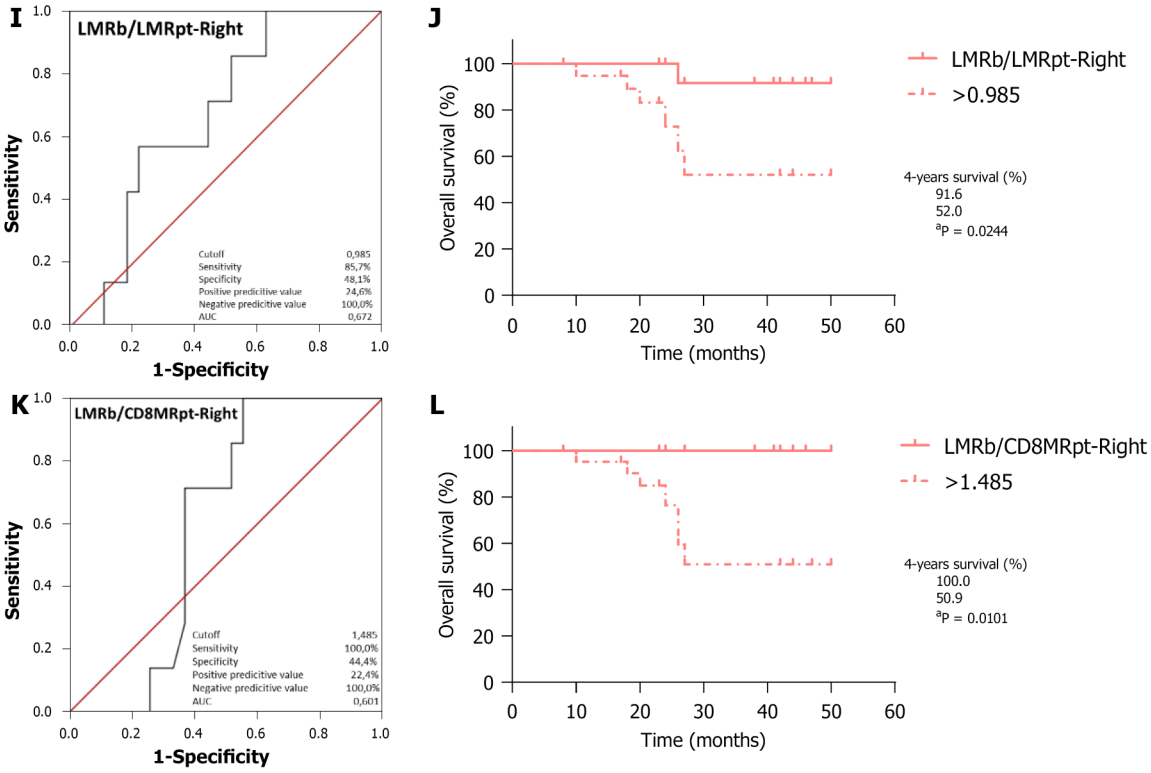


Figure 4 Receiver operating curve analyses for overall survival and Kaplan-Meier curves for optimal cutoff values in right-sided colorectal cancer patients for significant predictors. A-B: $CD8^+$-lymphocyte $(CD8)_{peritumour\ (pt)}$, worse below 8.25; C-D: $CD4^+$ plus $CD8^+$-lymphocyte $(CD4CD8)_{pt}$ worse below 10.16; E-F: Lymphocyte-to-monocyte ratio $(LMR)_{pt}$ worse below 3.185; G-H: $CD8^+$-lymphocyte-to-monocyte ratio $(CD8MR)_{pt}$ worse below 1.65; I-J: LMR_b/LMR_{pt} worse above 0.985; K-L: $LMR_{blood\ (b)}$/ $CD8^+$-lymphocyte-to-monocyte $ratio_{pt}$ worse above 1.485; survival proportions at 26 mo after surgery (median follow-up) are shown ($^aP < 0.05$, $^bP < 0.01$, log-rank test).

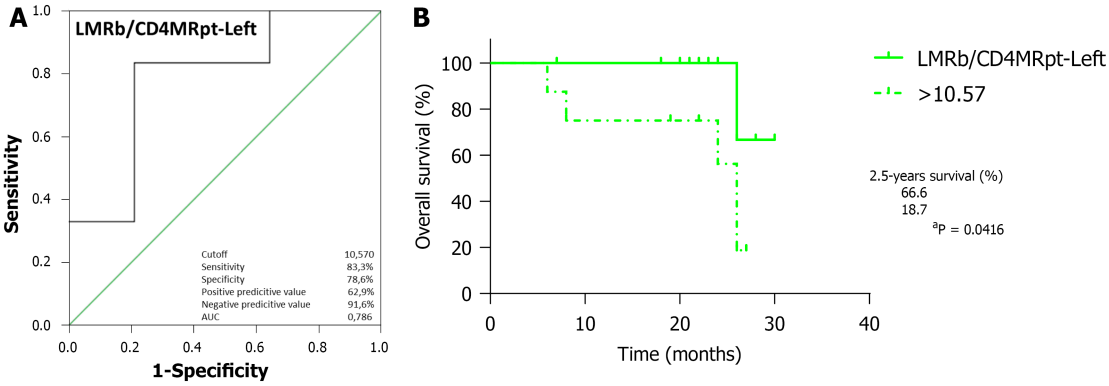


Figure 5 Receiver operating curve analysis for overall survival (A) and Kaplan-Meier curve (B) for optimal cutoff value in left-sided colorectal cancer patients for the significant predictor blood lymphocyte-to-monocyte ratio/peritumour $CD4^+$-lymphocyte-to-monocyte ratio. Worse above 10.57; survival proportions at 26 mo after surgery (median follow-up) are shown ($^aP < 0.05$, log-rank test). b: Blood; CD4MR: $CD4^+$-lymphocyte-to-monocyte ratio; LMR: Lymphocyte-to-monocyte ratio; pt: Peritumour.

39]. Hence, this could be the first study in which leukocyte measures in both blood and fixed tissues are put together into predictor indexes for CRC survival.

It is worth noting that, in addition to the well-established predictor value of blood leukocyte ratios, the 10 indexes involve leukocyte concentrations in peritumoural zones of the bowel but not in the tumour mass. A peritumour constitutes an easily obtainable tissue during a preoperative exploration of the patient (this could be the colonoscopy), which might be safely biopsied without affecting the tumour environment in an adenoma-like surgical extraction protocol. Therefore, on a routine basis, surgeons might access both preoperative peripheral blood parameters as well as non-neoplastic peritumoural tissue (without disturbing the tumour itself) and make use of the described ratios and nomograms to predict the patient's outcome after

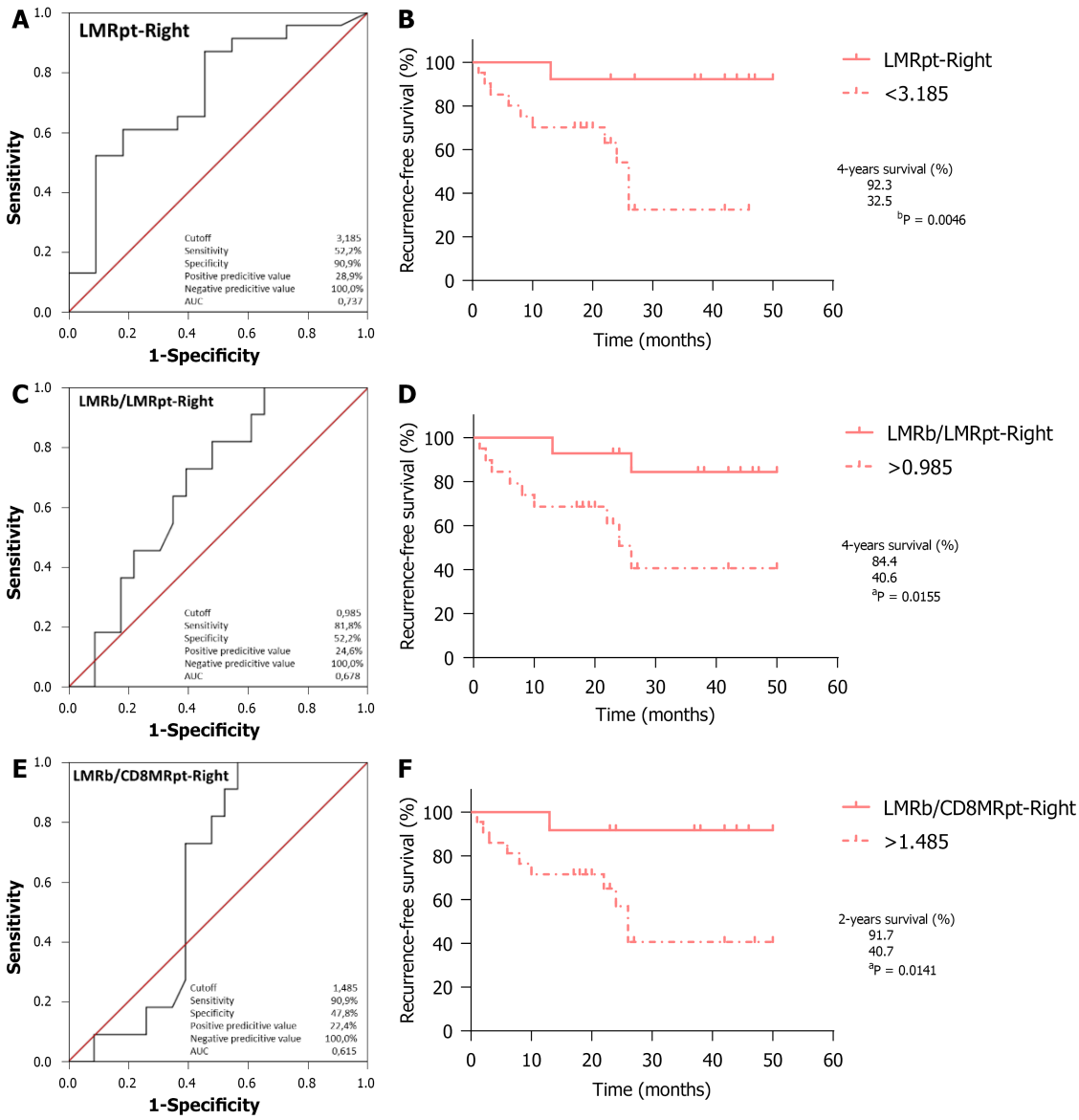


Figure 6 Receiver operating curve analyses for recurrence-free survival and Kaplan-Meier curves for optimal cutoff values in right-sided colorectal cancer patients for significant predictors. A-B: Peritumour lymphocyte-to-monocyte ratio (LMR) worse below 3.185; C-D: Blood (b) LMR/peritumour (pt) LMR worse above 0.985; E-F: Blood LMR/peritumour CD8$^+$-lymphocyte-to-monocyte ratio (CD8MR) worse above 1.485; survival proportions at 26 mo after surgery (median follow-up) are shown ($^aP < 0.05$, $^bP < 0.01$, log-rank test).

surgery. Thus, *ad hoc* surgical strategies can be designed to allow physicians to continue with surgery as programmed or delay the intervention until better scores are achieved after personalised treatments to correct the leukocyte levels in the patient.

Altogether, these indexes could be implemented in the first line of prognosis, making it easier to predict the outcome of patients after surgery depending on the tumour location and leukocyte distribution in both peripheral blood and biopsies of the peritumoural region.

Limitations

Our study is mainly limited by the cohort size. It might be expected that the extension of these variables to a greater cohort would reinforce our conclusions or even make foregoing unobserved interactions surface.

CONCLUSION

Herein we present important remarks on the value of combining circulating leukocyte ratios and tissue infiltrated leukocyte ratios on the sustaining of valuable prognosis tools for physicians in order to stratify patients regarding their putative outcome. In

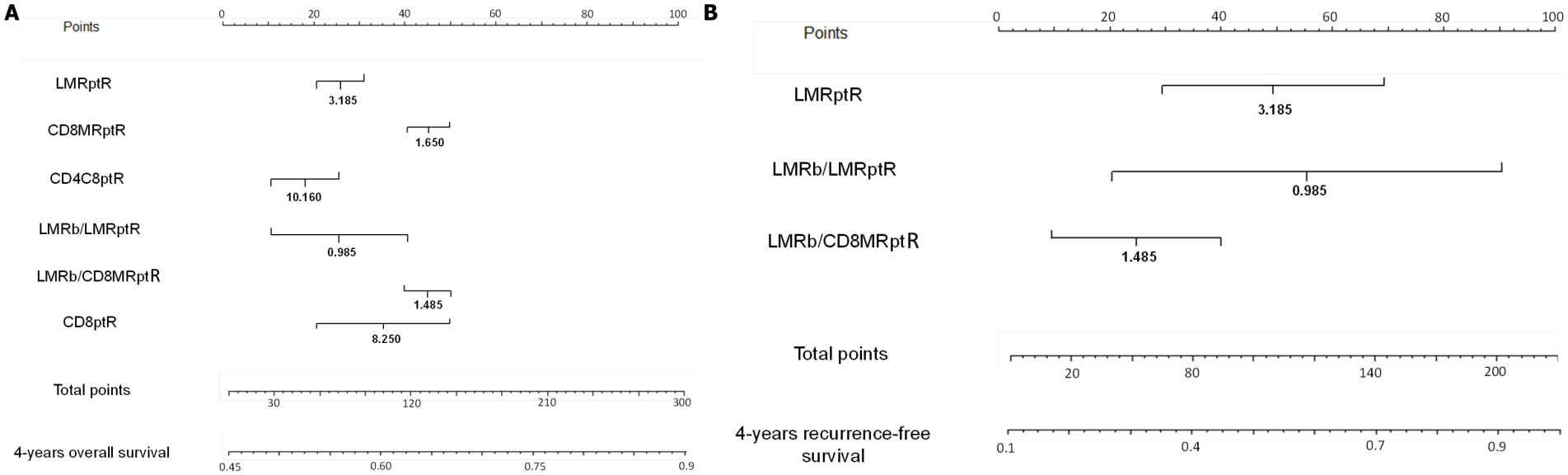


Figure 7 Nomograms for predicting overall survival and recurrence-free survival after surgical intervention of right-sided colorectal cancer patients. A: The 4-year probability of overall survival was estimated by summing the scores of peritumour (pt) lymphocyte-to-monocyte ratio (LMR), $CD8^+$-lymphocyte (CD8)$^+$-lymphocyte-to-monocyte ratio $(CD8MR)_{pt}$, $CD4^+$ plus $CD8^+$-lymphocyte $(CD4CD8)_{pt}$, blood (b) LMR/LMR_{pt}, $LMR_b/CD8MR_{pt}$, and $CD8_{pt}$; B: The 4-year probability of recurrence-free survival was estimated by summing the scores of LMR_{pt}, LMR_b/LMR_{pt}, and $LMR_b/CD8^+$-lymphocyte-to-monocyte $ratio_{pt}$. For each graph, locate the patient's values for each variable at one of the extremes of its corresponding axis, taking into account the correct position with respect to the optimal cutoff that is indicated; values higher than the cutoff go to the upper end and values lower than the cutoff go to the lower end. Then, draw a line straight upwards to the "Points" axis to determine the score associated to each variable. Add up all the scores, locate this sum in the "Total points" axis and draw a line straight down to the lowest axes of "4-year overall survival" or "4-year recurrence-free survival" to find the predictive probability of the patient for overall survival or recurrence-free survival outcome, respectively.

the era of personalised medicine, such indexes will provide benefits to improving both resources and well-being of CRC patients after surgery.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) points to 9.4% of cancer deaths worldwide, ranking second after lung cancer. Despite the wide variety of factors tested to predict their outcome, most patients with similar variables show big differences in survival. Moreover, right-sided CRC (RCRC) and left-sided CRC (LCRC) patients exhibit large differences in outcome after surgical intervention as assessed by preoperative blood leukocyte ratios [today, the most extended parameters used to assess a patient's overall survival (OS) and recurrence-free survival (RFS) after surgery]. However, few efforts have been

made to link tumour infiltrated leukocyte ratios to patient outcomes.

Research motivation

To determine whether both RCRC and LCRC patient outcomes could be accurately predicted based on the counting of infiltrated leukocytes in tumour and peritumoural tissues.

Research objectives

The aim of this study was to find stronger indexes than circulating (blood) leukocyte ratios to predict RCRC and LCRC patient outcomes.

Research methods

A prospective study was performed with CRC patients who had undergone surgical intervention to resect the tumours. Leukocyte concentrations in peripheral blood, tumour, and non-neoplastic peritumoural tissues were determined. Ratios of these parameters were evaluated as predictors for OS and RFS using Spearman correlations, Cox univariate and multivariate proportional hazards regression followed by the calculation of the receiver-operating characteristic and area under the curve (AUC) and the determination of Youden's optimal cutoff values for those variables that significantly correlated with either RCRC or LCRC patient outcomes. Clinician-friendly nomograms were developed to predict OS and RFS from the prediction indexes. The accuracy of the model was evaluated using calibration and validation analyses.

Research results

We obtained six robust predictors of worse OS in RCRC: $CD8^+$ lymphocyte content in peritumour ($CD8_{pt}$, AUC = 0.585, cutoff < 8.250, P = 0.0077), total lymphocyte content in peritumour ($CD4CD8_{pt}$, AUC = 0.550, cutoff < 10.160, P = 0.0188), lymphocyte-to-monocyte ratio in peritumour (LMR_{pt}, AUC = 0.807, cutoff < 3.185, P = 0.0028), $CD8^+$ LMR in peritumour ($CD8MR_{pt}$, AUC = 0.757, cutoff < 1.650, P = 0.0007), the ratio of blood LMR to LMR in peritumour (LMR_b/LMR_{pt}, AUC = 0.672, cutoff > 0.985, P = 0.0244), and the ratio of blood LMR to $CD8^+$ LMR in peritumour ($LMR_b/CD8MR_{pt}$, AUC = 0.601, cutoff > 1.485, P = 0.0101). In addition, three robust predictors of worse RFS in RCRC were found: LMR_{pt} (AUC = 0.737, cutoff < 3.185, P = 0.0046), LMR_b/LMR_{pt} (AUC = 0.678, cutoff > 0.985, P = 0.0155), and $LMR_b/CD8MR_{pt}$ (AUC = 0.615, cutoff > 1.485, P = 0.0141). Furthermore, the ratio of blood LMR to $CD4^+$ LMR in peritumour ($LMR_b/CD4MR_{pt}$, AUC = 0.786, cutoff > 10.570, P = 0.0416) was found to robustly predict poorer OS in LCRC patients. The developed nomograms to predict OS and RFS of RCRC patients showed C-indexes of 0.600 (95% confidence interval: 0.561-0.639) and 0.605 (95% confidence interval: 0.579-0.631), respectively.

Research conclusions

Easily obtainable variables at preoperative consultation, defining the status of leukocyte balances between peripheral blood and peritumoural tissue, have been shown to render indexes that accurately predict OS and RFS of CRC patients after surgical ablation of the tumours.

Research perspectives

We hope these indexes could be implemented in the first line of prognosis, making it easier to predict the outcome of patients after surgery depending on the tumour location and leukocyte distribution in both peripheral blood and biopsies of the peritumoural region.

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