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ORIGINAL ARTICLE

Retrospective Cohort Study

Context-dependent prognostic role of neutrophils and mast cells in primary colorectal cancer and liver metastases

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Abstract

BACKGROUND

Colorectal cancer (CRC) is a leading cause of cancer-related mortality worldwide. Liver metastases (LM) are a major prognostic factor affecting survival and treatment outcomes. Distinguishing between synchronous and metachronous metastases is clinically important due to differences in tumor biology, immune contexture, and prognosis. Polymorphonuclear neutrophils (PMNs) and mast cells (MCs) are innate immune effectors that influence CRC progression and metastasis. However, their distribution across primary CRC (pCRC), adjacent non-tumor mucosa (NM), and LM, and prognostic relevance remain incompletely understood.

AIM

To evaluate distribution and prognostic value of PMNs and MCs in NM, pCRC,

and LM in synchronous and metachronous CRC.

METHODS

This exploratory retrospective cohort study included patients undergoing resection of pCRC with NM and synchronous LM (stage IV, n = 55) or metachronous LM (stage I-III, n = 44). CD66b+ PMNs and CD117+ MCs were assessed using immunohistochemistry, whole-slide imaging, and QuPath-based quantification across NM, tumor center (TC), inner margin (IM) and outer margin (OM), and peritumor zone (PT) of pCRC and LM. Cell densities were compared by site, region, and timing of metastatic presentation, and associated with disease-free survival (DFS).

RESULTS

PMNs were enriched in pCRC compared to NM, whereas MCs predominated in NM. Greater densities of PMNs and MCs were found in LM and pCRC, respectively. High PMNs in OM (HR = 2.40, 95%CI: 1.14-5.04, P = 0.021) and PT (HR = 2.58, 95%CI: 1.22-5.46, P = 0.013) of synchronous LM and OM of pCRC in stage I-III (HR = 2.59, 95%CI: 1.11-6.02, P = 0.027) correlated with shorter DFS, whereas high PMNs in TC of metachronous LM predicted longer DFS (HR = 0.48, 95%CI: 0.24-0.99, P = 0.048). High MC density in TC of pCRC in stage I-III predicted shorter DFS (HR = 2.34, 95%CI: 1.05-5.23, P = 0.038). These exploratory findings require validation in independent cohorts.

CONCLUSION

PMN density increased from NM to LM, while MCs decreased. MCs showed protumor prognostic associations in pCRC; PMNs were protumor in stage I-III pCRC and synchronous LM, but antitumor in metachronous LM.

Key Words: Neutrophils; Mast cells; Primary colorectal cancer; Liver metastases; Adjacent non-tumor mucosa; Tumor microenvironment; Survival

Core Tip: Densities of polymorphonuclear neutrophils (PMNs) and mast cells (MCs) showed distinct spatial patterns across colorectal cancer (CRC) progression, with PMNs increasing and MCs decreasing from nontumor mucosa through primary CRC (pCRC) to liver metastases (LM). MCs in pCRC exhibited protumor associations with survival, whereas PMNs showed context-dependent survival associations, being unfavorable in stage I-III pCRC and synchronous LM but favorable in metachronous LM. These findings highlight dynamic, stage- and site-specific roles of PMNs and MCs and after validation may serve as prognostic biomarkers for CRC patients.

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INTRODUCTION

Colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality worldwide. Liver metastases (LM) are present at diagnosis (synchronous) in up to 25% of CRC patients, while up to 30% of patients with stage I-III CRC develop LM later in the disease course (metachronous)[1]. The presence of LM represents a key prognostic event, significantly influencing survival and therapeutic outcomes[2,3]. Distinguishing between patients with synchronous and metachronous LM is clinically important, as they differ in tumor biology, immune contexture, and prognosis[1,4]. Surgical resection remains the standard first-line and only curative treatment for LM, yet recurrence occurs in up to 70% of patients, 5-year survival ranges from 20% to 60%, and clinical variables inadequately explain survival differences after resection[5].

Increasing evidence highlights the crucial role of immune cells in CRC progression and response to therapy[6,7]. Our previous studies have demonstrated that tumor-infiltrating T cells and macrophages play a role in shaping the development of synchronous *vs* metachronous metastases and in driving survival differences following their resection[8,9]. Despite their important role in tumor behavior and host response[6], polymorphonuclear neutrophils (PMNs) and mast cells (MCs) have historically received less research attention. PMNs are recruited to the CRC microenvironment by tumor-, stroma- and nerve-derived chemotactic cues[10,11]. Tumor-associated PMNs are highly plastic and can adopt antitumor (N1) or protumor (N2) phenotypes according to cytokine milieu, tumor stage, microbiota, and immune crosstalk[10,12,13]. Protumor N2 PMNs drive angiogenesis, invasion, immune evasion, and metastasis[10,11,14,15]. In addition, PMNs suppress antitumor T- and NK-cell responses[16,17]. In LM of CRC, PMNs infiltrate pre-metastatic and metastatic niches, driving angiogenesis, immunosuppression, and T-cell exhaustion[18]. Conversely, antitumor functions of PMNs have been described under specific conditions *in vitro*, including antibody-dependent cytotoxicity and enhancement of CD8+ T-cell responses, consistent with an N1 phenotype[11,12]. Clinically, high PMN infiltration is frequently associated with poor prognosis in solid tumors, although results in CRC remain heterogeneous and com-

partment-dependent[13,19]. CD66b, which is strongly expressed on the surface of mature PMNs, participates in their activation and adhesion, and it is the most widely used marker for tumor-associated PMNs[19].

MCs are derived from hematogenic progenitors that mature particularly at mucosal interfaces, such as the gastrointestinal tract[20-22], and express CD117, which drives their maturation, migration, and survival. MCs act as sentinel cells controlling innate and adaptive immunity, inflammation, angiogenesis, tissue repair, and host defense[23]. MCs are closely associated with chronic inflammation and cancer[21,22,24], exhibiting a context-dependent role. Several subpopulations of tumor-associated MCs with distinct transcriptional programs and functional polarization shaped by tumor type, stage, spatial localization, local cytokines, microbiota, and stromal interactions have been recognized in CRC[22-24]. Pro-tumor activities of MCs include promotion of angiogenesis and lymphangiogenesis, extracellular matrix remodeling, tumor cell proliferation, epithelial-to-mesenchymal transition, and immune suppression[20-23]. Conversely, MCs can mediate anti-tumor effects directly promoting tumor cell apoptosis[25] and activating cytotoxic immune cells[20-22]. Consequently, both dismal[21,26] and favorable[27] prognostic associations of MCs in primary CRC (pCRC) have been reported[20-22,24]. Only scant papers report distribution and prognostic associations of MCs in CRC LM[26].

Despite these insights, comprehensive analyses comparing PMN and MC densities across spatially defined tumor regions and matched LM, stratified by synchronous *vs* metachronous presentation, are still lacking. Furthermore, the prognostic significance of these immune cells in specific micro-anatomical contexts remains unclear. Only a few studies have examined associations of PMNs and MCs in adjacent non-tumor mucosa (NM) with prognosis in CRC[25,28]. Of interest, PMNs and MCs may directly interact within tumor microenvironment (TME), shaping each other's behavior. MCs-derived chemokines attract PMNs in inflammatory and tumor settings[20,23], and may influence PMN activation or trapping[29]. However, only a limited number of studies on CRC considered both PMNs and MCs[29,30].

In the present study, we quantified CD66b+ PMNs and CD117+ MCs in NM, pCRC, and corresponding LM, distinguishing between synchronous and metachronous groups. We assessed inter-regional variations within each tissue type and correlated these immune patterns with disease-free survival (DFS) and, in metachronous cases, with time to the diagnosis of LM (TLM).

MATERIALS AND METHODS

Study design and patient cohort

A total of 99 patients who underwent curative-intent resections of both pCRC and LM at Pilsen University Hospital between 1999 and 2021 were included in this retrospective study. Paired formalin-fixed paraffin-embedded (FFPE) samples of NM, pCRC and LM were analyzed. Synchronous LM were defined as LM detected before or at the time of diagnosis of the pCRC, including metastases identified during surgery for the primary tumor. Metachronous LM were defined as LM diagnosed after initial staging and surgical treatment of the pCRC[31]. The cohort consisted of 55 patients who presented with synchronous LM (stage IV) and stage I-III patients (n = 44) who developed metachronous LM within 1-59 months after resection of pCRC (median: 17 months).

Eligibility criteria comprised liver-first metastases, complete clinical and survival data and adequate FFPE triplicate samples from NM, pCRC and LM. Exclusion criteria were multiple primary neoplasms, preoperative extrahepatic metastases, previous liver resections, neoadjuvant chemoradiotherapy prior to pCRC surgery, and emergency surgical interventions.

Clinical, pathological, and demographic data were extracted from medical records. Collected variables included tumor location, size, histological subtype and grade, TNM stage (AJCC 8th edition), *KRAS* and *BRAF* mutation and microsatellite instability (MSI) status, and serum carcinoembryonic antigen levels. Tumors were classified as right- or left-sided based on their relation to the splenic flexure. To explore the relationship between local tumor invasion and metastatic burden we also tested prognostic associations of the tumor burden score (TBS)[32] in relation to the reversed extent of local invasion (reversed T stage).

The study was approved by the Ethics Committee of the Pilsen University Hospital and Faculty of Medicine in Pilsen and conducted in accordance with the Declaration of Helsinki (2013 revision).

Tissue processing and immunohistochemistry

FFPE tissue blocks of NM, pCRC and LM were sectioned at 4 μm thickness. NM samples were collected from the nearest oral or aboral resection margin (median distance = 34 mm; range = 4-200 mm). Tissue sections were mounted on BOND Plus microscope slides (Leica Biosystems) and subjected to automated immunohistochemical staining using the BOND RXm platform (Leica Biosystems). Mouse monoclonal primary antibodies against CD66b (clone G10F5) from BioLegend Global Headquarters were applied at a dilution of 1:100, and ready to use rabbit monoclonal antibodies against CD117 (clone EP10) from Leica Biosystems were applied. Detection was performed using a horseradish peroxidase-conjugated polymer detection system (Leica Biosystems). Slides were counterstained with Mayer's hematoxylin and mounted using Micromount (Leica Biosystems). Tonsil tissue served as a positive control, and negative controls were included in each staining run.

Digital image analysis

Whole-slide digital images of stained sections were acquired using the Olympus VS200 scanner (Olympus, Japan). Image analysis was performed using QuPath software (version 0.5.0).

NM was annotated as a continuous region above the muscularis mucosa, encompassing surface epithelium, intestinal crypts, lamina propria, and lymphoid follicles (if present). Regions with dysplasia, crypt lumina, and artifacts were excluded.

Tumor regions were manually annotated in pCRC and LM by 3 trained researchers. From the original tumor annotation, four distinct margin regions were generated using a custom script (https://github.com/sergii01-cuni/script_zones). Inner margin (IM): A 500-μm-wide region extending inward from the tumor boundary; tumor center (TC): The region of the tumor remaining after excluding the IM; outer margin (OM): A 500-μm-wide region expanding outward from the tumor boundary; peritumor zone (PT): A 500-μm-wide area extending beyond the OM. The following elements were excluded from tumor annotations prior to analysis: Lumina of the tumor glands, large vessels, non-neoplastic mucosa, dysplasia, muscularis propria, stromal areas > 2 mm in diameter, extracellular mucin, necrosis, fat, hemorrhage, abscesses, and technical artifacts. Additionally, tumor cells expressing CD117, were distinguished from CD117-positive MCs based on established morphological criteria. To ensure that only MCs were quantified, regions containing CD117-positive tumor cells were manually excluded. All annotations were reviewed by a pathologist.

Immune cell density was calculated as the number of positive cells per mm^2. To correct for non-normal distribution, raw values were transformed into percentiles and categorized into "low" (< 25^{th} percentile) and "high" (≥ 25^{th} percentile) groups. The 25^{th} percentile as a cutoff enables defining a biologically relevant low-density subgroup thereby improving sensitivity for detecting clinically meaningful associations. This cutoff was adopted in our prior works and across tumor immunology studies[7,9]. In contrast to raw cell densities, IM/OM ratios were more symmetrically distributed; therefore, we dichotomized them into "low" and "high" at the median to minimize the influence of extreme values and maintain balanced groups.

Follow-up and clinical outcomes

Patients were followed until December 2023. Median follow-up durations were 61 months (metachronous group) and 84 months (synchronous group). Adjuvant and systemic therapies were administered in accordance with clinical guidelines. The primary study endpoint was DFS, defined as the time from liver surgery until the first confirmed disease recurrence or death for any reason. Patients without evidence of recurrence or death were censored at the date of last follow-up. The length of follow-up was restricted to 5 years by applying censoring. TLM was defined as the time from pCRC surgery to diagnosis of LM in the metachronous group. Secondary outcomes were time to recurrence (TTR) and overall survival (OS). TTR was defined as the interval from the date of liver metastasectomy to the date of diagnosis of any site of recurrence. OS was considered as the interval from the date of liver resection to the date of death from any cause.

Statistical analysis

Exploratory data analysis was performed to assess the distribution of variables, identify outliers, and guide subsequent data transformations and categorization. Missing data were assessed for extent and underlying causes. Missing values for cell density measurements were infrequent (< 3%) and primarily due to technical factors. Mutation data were unavailable in a subset of patients (up to 20%) due to incomplete testing. Given the relatively low proportion of missing data and the absence of evidence for systematic bias, analyses were performed using a complete-case approach without imputation. Continuous variables were expressed as medians (minimum-maximum) and compared using the Mann-Whitney *U* test (for between-group comparisons) or Friedman ANOVA with Dunn's *post hoc* test (for comparisons between regions of interest). Categorical variables were presented as *n* (%). Associations between quantitative or ordinal variables were evaluated using Spearman's rank correlation. For those analyses GraphPad Prism 9.0 (GraphPad Software LLC) was used. Kaplan-Meier survival analysis with log-rank testing was used to compare DFS between groups. Cox proportional hazards regression was applied to assess the prognostic value of individual variables. HRs and 95%CI were reported, with HR = 1 indicating the reference group. All significant univariable associations between immune cells and survival were validated in subsequent Cox regression analyses using continuous cell density measures and optimized cut-offs based on the minimum *P* value approach. The assumptions of the Cox proportional hazards models were evaluated using standard diagnostic methods. Given the number of events in each group and the requirements for reliable multivariable analysis, we assessed significant prognostic associations of immune cells in several multivariable models. Survival analysis was performed in the R environment. The finalfit[33] package was used for regression modeling, while Kaplan-Meier curves were generated using the survival[34] and survminer[35] packages. A two-sided *P* value < 0.05 was considered statistically significant. At a baseline significance level of 0.05, the false discovery rate (FDR) estimated by the Benjamini-Hochberg procedure is 34%. In order to achieve the desired conservative FDR of 5%, the significance level for each individual test would have to be decreased to 0.003.

RESULTS

Patients with metachronous LM demonstrated significantly longer DFS, TTR, and OS after pCRC surgery compared to those with synchronous metastases (Supplementary Figure 1).

Based on these findings, together with our previous results in the same cohort[8,9] demonstrating distinct prognostic associations of tumor-infiltrating immune cells in synchronous and metachronous groups, all subsequent analyses were conducted separately for these groups.

Demographics of CRC patients and outcomes

The demographics, clinical and pathological characteristics of the patients are shown in Supplementary Table 1. DFS probabilities at 3 years after LM surgery were 17.6% in the metachronous group and 11.4% in the synchronous group (Supplementary Table 2).

Morphology of PMNs and MCs

PMNs had a round to slightly irregular shape with membranous expression of CD66b putative antigen (Supplementary Figure 2A). In NM, PMNs were mostly found in capillaries of lamina propria and only rare cells were observed in mucosa-associated lymphoid aggregates, at perivascular or intraepithelial locations. In pCRC and LM PMNs predominated in stromal compartment where they accumulated along blood vessels (Supplementary Figure 2B-D).

In NM, MCs had rounded and elongated shapes with a predominantly membranous staining pattern of CD117 (Supplementary Figure 2E). MCs predominated in lamina propria with only a few cells located intraepithelially or within lymphoid aggregates. In the tumor, MCs had a spindled shape or stellate morphology. They were mostly scattered in stroma, including perivascular zones, with few intraepithelial cells (Supplementary Figure 2F-H). In the OM and PT regions of LM, a majority of MCs were located withing portal tracts. We also observed cytoplasmic expression of CD117 in tumor cells of both pCRC and LM in some patients (Supplementary Figure 2I and J).

Distribution of PMNs and MCs

In the comparison between NM and TC of pCRC, greater density of PMNs was found in pCRC in both groups, whereas greater density of MCs was observed in NM (Figure 1A).

In the inter-regional comparisons in pCRC, significantly smaller density of PMNs was found in the PT region of the metachronous group, with a similar but non-significant trend in the synchronous group. Greater density of MCs was observed in OM and PT than in TC and IM in the synchronous group, whereas in the metachronous group the smallest cell density was in the IM (Figure 1A).

As for LM, the smallest density of PMNs was observed in TC of synchronous LM, greater density was found in IM and OM than in TC and PT of metachronous LM (Figure 1B). Greater density of MCs was observed in OM and PT *vs* TC and IM in both groups.

In the comparison between pCRC and LM, greater density of PMNs was observed in OM and PT of LM in both groups (Figure 2A), whereas greater density of MCs was observed in all regions of pCRC (Figure 2B).

In the comparison between synchronous and metachronous groups, differences were only observed in LM, where greater density of PMNs was found in TC of metachronous LM ($P < 0.05$), and greater density of MCs ($P < 0.05$) was found in OM of synchronous LM (Figure 2).

MCs were more abundant than PMNs in NM and PT of pCRC in both groups ($P < 0.001$, Figure 1A); conversely, PMNs dominated in IM of pCRC in the synchronous group ($P < 0.001$), and TC, IM in the metachronous group ($P < 0.01$). PMNs were more numerous than MCs in all the regions of LM ($P < 0.001$, Figure 1B).

CD117-expressing tumor cells were found in 26 (47.3%) synchronous pCRC and 19 (34.6%) LM, as well as in 17 (38.6%) of metachronous pCRC and 20 (45.5%) of LM. Patients with CD117-positive tumors had greater densities of MCs in TC and IM of synchronous pCRC (Supplementary Figure 3). Greater densities of MCs were observed in TC, IM and OM of CD117-expressing metachronous LM.

Correlations between PMNs and MCs

In the synchronous group, densities of PMNs and MCs were positively and significantly associated only in OM (rho = 0.32) and PT (rho = 0.30) of pCRC and were negatively associated in TC (rho = -0.37) of LM (Table 1). No associations were seen in the metachronous group. Densities of PMNs or MCs did not correlate between respective regions of pCRC and LM (Supplementary Table 3).

Associations of PMNs, MCs, and clinicopathological variables with survival

In the synchronous group, high densities of PMNs in OM and PT of LM were associated with shorter DFS in Cox-regression and Kaplan-Meier analyses (Figure 3A and B and Table 2). In the metachronous group, high density of PMNs in the OM of pCRC correlated with shorter DFS (Figure 3C and Table 2). Conversely, high density of PMNs in the TC of metachronous LM was linked to longer DFS (Figure 3D and Table 2). Additionally, high IM/OM ratio of PMNs in pCRC was associated with shorter TLM in the metachronous group (Supplementary Figure 4 and Supplementary Table 4). As for MCs, the only significant finding was the association of high cell density in the TC of pCRC with shorter DFS in the metachronous group (Figure 3E and Table 3). Concordant associations between immune cells and DFS or TLM were also found in a subgroup of 40 patients in whom metachronous LM were diagnosed 3-59 months after resection of pCRC (Supplementary Table 5).

Concordant significant associations were observed between PMNs and MCs and TTR (Supplementary Tables 6 and 7), but not OS (Supplementary Tables 8 and 9). We did not find associations between MCs and TLM in the metachronous group (Supplementary Table 10). Expression of CD117 by tumor cells also did not affect the prognosis (data not shown). The association between MCs density and DFS only in the subset of patients with CD117-negative tumor cells remained protumor, albeit less significant, which can be attributed to a smaller sample size (Supplementary Table 11).

Continuous Cox regression analyses yielded results that were directionally consistent with categorical analyses, although only a subset of variables retained statistical significance (Supplementary Table 12). Using optimized cut-offs, the predictive variables showed stronger associations with DFS and TLM (Supplementary Table 12).

Table 1 Correlation between CD66b+ polymorphonuclear neutrophils and CD117+ mast cells densities within non-tumor mucosa, primary colorectal cancer and liver metastasis in synchronous and metachronous groups

	Synchronous						Metachronous					
	CD66b NM	CD66b pTC	CD66b pIM	CD66b pOM	CD66b pPT		CD66b NM	CD66b pTC	CD66b pIM	CD66b pOM	CD66b pPT	
CD117 NM	0.11[1]					CD117 NM	0.08					
CD117 pTC		0.13				CD117 pTC		0.08				
CD117 pIM			0.07			CD117 pIM			0.01			
CD117 pOM				0.32[a]		CD117 pOM				0.09		
CD117 pPT					0.30[a]	CD117 pPT					0.1	
	CD66b mTC	CD66b mIM	CD66b mOM	CD66b mPT			CD66b mTC	CD66b mIM	CD66b mOM	CD66b mPT		
CD117 mTC	-0.37[a]					CD117 mTC	-0.03					
CD117 mIM		-0.26				CD117 mIM		0.06				
CD117 mOM			-0.13			CD117 mOM			0.15			
CD117 mPT				0.06		CD117 mPT				0.12		

[1]Spearman rho.

[a]$P < 0.05$.

NM: Non-tumor mucosa; pTC: Tumor center of primary colorectal cancer; mTC: Tumor center of liver metastasis; pIM: Inner margin of primary colorectal cancer; mIM: Inner margin of liver metastasis; pOM: Outer margin of primary colorectal cancer; mOM: Outer margin of liver metastasis; pPT: Peritumor zone of primary colorectal cancer; mPT: Peritumor zone of liver metastasis.

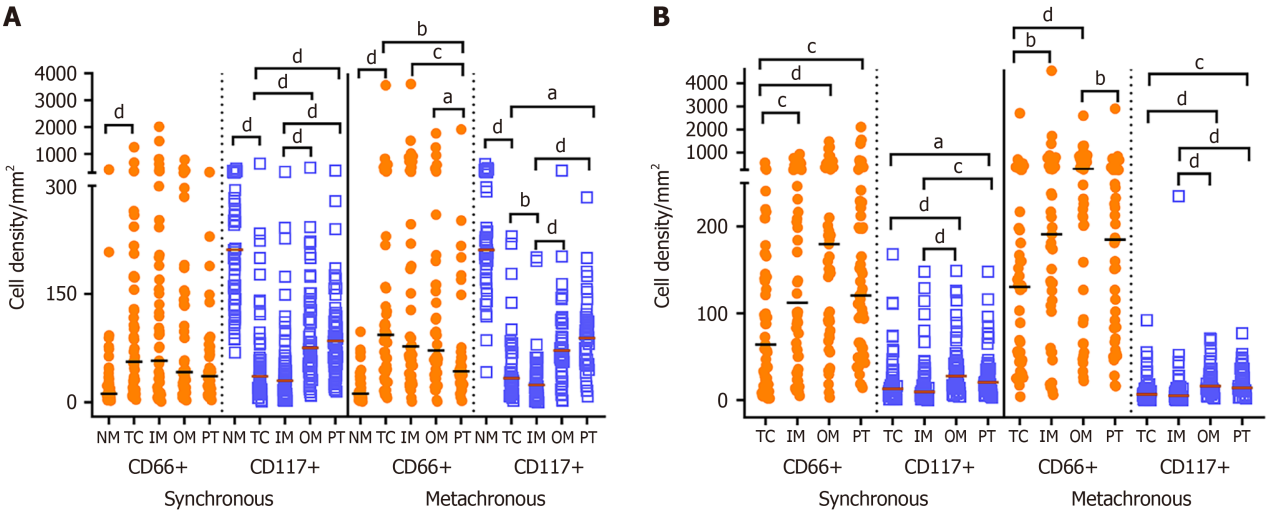


Figure 1 Distribution of CD66b+ polymorphonuclear neutrophils and CD117+ mast cells in non-tumor mucosa, primary colorectal cancer and liver metastasis. A: Cell densities between non-tumor mucosa and all regions of interest of primary colorectal cancer in synchronous group and metachronous group; B: Cell densities between all regions of interest of liver metastasis (LM) in synchronous group and metachronous group. Friedman ANOVA with Dunn's *post hoc* test were used for comparisons between regions of interest. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.005$; [d]$P < 0.001$. NM: Non-tumor mucosa; TC: Tumor center; IM: Inner margin; OM: Outer margin; PT: Peritumor zone.

Table 2 Hazard ratios for disease-free survival between high *vs* low densities of CD66b+ polymorphonuclear neutrophils in the non-tumor mucosa and per individual regions of interest of primary colorectal cancer and liver metastasis in colorectal cancer patients with synchronous and metachronous metastasis

	Synchronous		Metachronous	
	n (%)	HR (95%CI), *P* value	*n* (%)	HR (95%CI), *P* value
NM	40 (74.1)	1.68 (0.85-3.31), 0.13	33 (75.0)	1.56 (0.73-3.35), 0.25
pTC	40 (75.5)	0.65 (0.34-1.24), 0.19	31 (73.8)	0.89 (0.41-1.93), 0.77
pIM	40 (75.5)	0.86 (0.45-1.64), 0.64	31 (73.8)	1.15 (0.54-2.46), 0.71
pOM	39 (75.0)	1.13 (0.57-2.24), 0.72	31 (73.8)	2.59 (1.11-6.02), 0.027[a]
pIM/pOM	27 (51.9)	0.57 (0.31-1.03), 0.06	21 (50.0)	1.30 (0.67-2.51), 0.44
pPT	39 (75.0)	0.61 (0.31-1.18), 0.14	31 (73.8)	1.92 (0.87-4.25), 0.11
mTC	38 (74.5)	0.95 (0.49-1.85), 0.89	33 (75.0)	0.48 (0.24-0.99), 0.048[a]
mIM	39 (75.0)	1.70 (0.84-3.46), 0.14	32 (74.4)	0.65 (0.31-1.36), 0.25
mOM	40 (75.5)	2.40 (1.14-5.04), 0.021[a]	32 (74.4)	0.76 (0.36-1.58), 0.46
mIM/mOM	26 (49.1)	0.89 (0.50-1.59), 0.69	21 (48.8)	1.23 (0.64-2.36), 0.53
mPT	40 (75.5)	2.58 (1.22-5.46), 0.013[a]	32 (74.4)	0.93 (0.44-1.99), 0.85

[a]$P < 0.05$.

n shows the number of patients in "high" group. HR (95%CI) shows the relative risk compared with 1.00 for the "low" group. NM: Non-tumor mucosa; pTC: Tumor center of primary colorectal cancer; mTC: Tumor center of liver metastasis; pIM: Inner margin of primary colorectal cancer; mIM: Inner margin of liver metastasis; pOM: Outer margin of primary colorectal cancer; mOM: Outer margin of liver metastasis; pPT: Peritumor zone of primary colorectal cancer; mPT: Peritumor zone of liver metastasis.

Table 3 Hazard ratios for disease-free survival between high *vs* low densities of CD117+ mast cells in the non-tumor mucosa and per individual regions of interest of primary colorectal cancer and liver metastasis in colorectal cancer patients with synchronous and metachronous metastasis

	Synchronous		Metachronous	
	n (%)	HR (95%CI), *P* value	*n* (%)	HR (95%CI), *P* value
NM	40 (74.1)	1.51 (0.74-3.09), 0.26	32 (74.4)	0.85 (0.41-1.77), 0.67
pTC	40 (74.1)	0.69 (0.36-1.33), 0.27	31 (73.8)	2.34 (1.05-5.23), 0.038[a]
pIM	40 (74.1)	1.04 (0.53-2.04), 0.91	31 (70.5)	0.96 (0.48-1.92), 0.92
pOM	40 (74.1)	0.98 (0.51-1.90), 0.96	31 (73.8)	0.67 (0.32-1.40), 0.28
pIM/pOM	27 (50.0)	1.54 (0.86-2.74), 0.14	21 (50.0)	1.66 (0.86-3.22), 0.13
pPT	40 (74.1)	1.02 (0.53-1.97), 0.95	31 (73.8)	0.86 (0.41-1.80), 0.69
mTC	39 (75.0)	0.69 (0.35-1.34), 0.27	33 (75.0)	1.20 (0.57-2.49), 0.63
mIM	39 (75.0)	0.91 (0.46-1.81), 0.80	32 (74.4)	0.83 (0.40-1.72), 0.62
mOM	40 (75.5)	1.15 (0.57-2.31), 0.71	32 (74.4)	1.61 (0.73-3.58), 0.24
mIM/mOM	27 (50.9)	0.65 (0.36-1.18), 0.16	22 (51.2)	0.93 (0.48-1.78), 0.82
mPT	40 (75.5)	0.74 (0.38-1.43), 0.37	32 (74.4)	1.35 (0.63-2.88), 0.44

[a]$P < 0.05$.

n shows the number of patients in "high" group. HR (95%CI) shows the relative risk compared with 1.00 for the "low" group. NM: Non-tumor mucosa; pTC: Tumor center of primary colorectal cancer; mTC: Tumor center of liver metastasis; pIM: Inner margin of primary colorectal cancer; mIM: Inner margin of liver metastasis; pOM: Outer margin of primary colorectal cancer; mOM: Outer margin of liver metastasis; pPT: Peritumor zone of primary colorectal cancer; mPT: Peritumor zone of liver metastasis.

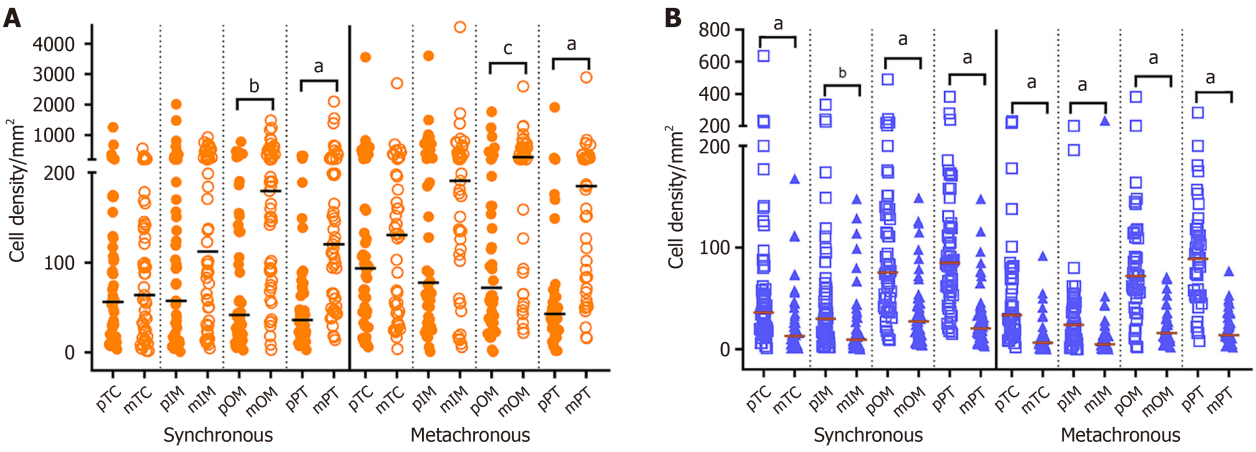


Figure 2 Distribution of CD66b+ polymorphonuclear neutrophils and CD117+ mast cells between different regions of interest of primary colorectal cancer and liver metastasis in synchronous and metachronous groups. A: CD66b+ polymorphonuclear neutrophils; B: CD117+ mast cells. Friedman ANOVA with Dunn's *post hoc* test were used for comparisons between regions of interest. [a]$P < 0.001$; [b]$P < 0.01$; [c]$P < 0.005$. pTC: Tumor center of primary colorectal cancer; mTC: Tumor center of liver metastasis; pIM: Inner margin of primary colorectal cancer; mIM: Inner margin of liver metastasis; pOM: Outer margin of primary colorectal cancer; mOM: Outer margin of liver metastasis; pPT: Peritumor zone of primary colorectal cancer; mPT: Peritumor zone of liver metastasis.

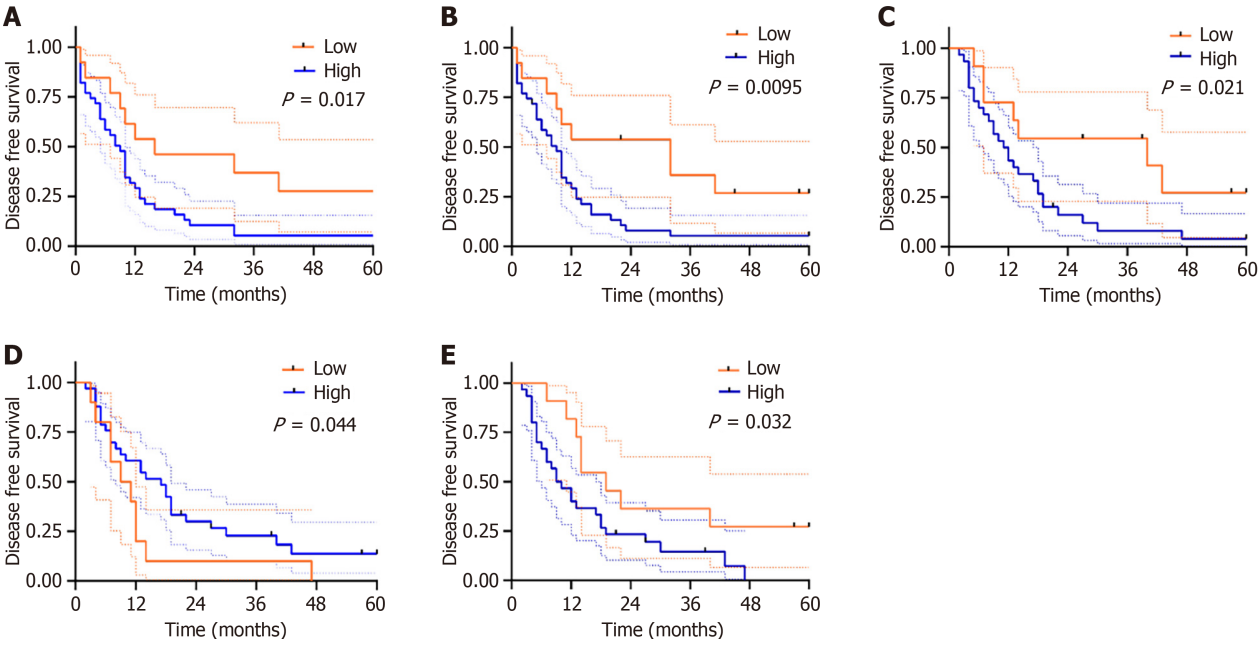


Figure 3 Associations between disease-free survival and high *vs* low densities of CD66b+ polymorphonuclear neutrophils and CD117+ mast cells in regions of interest of primary colorectal cancer and liver metastasis. A: Polymorphonuclear neutrophils (PMNs) in outer margin (OM) of synchronous liver metastasis (LM); B: PMNs in peritumor zone of synchronous LM; C: PMNs in OM of primary colorectal cancer (pCRC) in the metachronous group; D: PMNs in tumor center (TC) of LM in the metachronous group; E: Mast cells in TC of pCRC in the metachronous group. Orange and blue dotted lines: 95%CIs of Kaplan-Meier curves in "low" and "high" groups.

In multivariable analysis, most immune variables remained predictive for DFS after adjustment for clinically meaningful confounding variables in both groups (Supplementary Table 13).

To highlight the aggregate effect of PMNs and MCs, we categorized patients according to CD117+ MCs/CD66b+ PMNs densities into low/low, high/low, low/high and high/high groups. In the synchronous group, patients who displayed MCs low & PMNs high in the IM and PT of pCRC had significantly longer DFS compared to low/low group (Supplementary Table 14). In the metachronous group, patients who showed MCs high & PMNs low in the TC and OM of pCRC had significantly shorter and longer DFS, respectively, compared to the low/low group. In the IM and OM of metachronous LM, patients with MCs low & PMNs high and MCs high & PMNs high demonstrated longer DFS.

Amongst clinical and pathology variables only younger age was associated with shorter DFS in the metachronous group (Supplementary Table 15).

In the overall cohort of 99 patients, none of the clinical or pathological variables, including TBS/T stage ratio, were significantly associated with DFS (data not shown). Only higher densities of CD66b+ PMNs in the PT region of LM were predictive of shorter DFS (Supplementary Table 16). Moreover, cell densities across all regions of interest were not significantly different between TBS/T stage "low" and "high" groups (Supplementary Table 17).

Associations of clinical and pathology variables with PMNs and MCs

Supplementary Tables 18-21 summarize associations between clinicopathological variables, chemotherapy timeline and regimens and immune cells. Statistically significant differences are indicated in the legends for Supplementary Tables 18-19. To evaluate a possible field effect, we compared cell densities in NM according to the distance between the tumor and the resection margin (Supplementary Table 22). No significant differences were detected between the groups.

DISCUSSION

In this study, we provide a comprehensive spatial and temporal analysis of PMNs and MCs in NM, pCRC, and matched LM, stratified by synchronous and metachronous metastatic presentation. Our findings highlight marked differences in innate immune cell distribution across anatomical compartments and pattern of metastatic dissemination, and demonstrate that the prognostic significance of PMNs and MCs is strongly dependent on their localization and metastatic context.

Distribution of PMNs and MCs in NM vs pCRC vs LM

We observed an ascending trend of PMN infiltration from NM to LM in both groups, which supports the concept that tumor development is associated with active recruitment of PMNs into the TME. Tumor cells, besides other cell types in TME, induce PMN infiltration *via* multiple pathways, including secretion of granulocyte colony stimulating factor, TIMP-1, CXCL8 family chemokines and interleukins[10,11,36]. Using a gene signature-based method CIBERSORT, two groups [37,38] observed an abundance of PMNs in pCRC compared to NM. We demonstrated that the density of PMNs in OM and PT of both synchronous and metachronous LM was even higher compared with pCRC. Several previous studies have shown that neutrophil extracellular traps (NETs) and associated PMNs activity are elevated in CRC LM compared with pCRC, suggesting enhanced PMNs involvement in the metastatic niche[15].

Conversely, we observed a descending trend of MC density from NM to LM, which corresponds to earlier reports[25,27,28]. MCs are physiologically abundant in the mucosa of the digestive tract, where they contribute to barrier integrity and immune surveillance[20-22]. In contrast, the disturbances in TME (*e.g.*, altered chemokine signaling, abnormal vascularization and disrupted stromal architecture) may impair recruitment, survival, or retention of MCs[21,28]. The density of MCs was even lower in LM compared with pCRC, which may reflect the immunosuppressive environment of the liver [39] and is supported by one earlier study[26]. Also, a significant decrease in the number of MCs in the advanced stages of the tumor due to the destruction of MCs was reported earlier[20].

Observed reciprocal shift from MCs-rich to PMNs-rich environments upon development of CRC may represent a transition from early inflammatory or regulatory processes in healthy tissue to aggressive, invasion-associated inflammation within tumors and metastases[40]. This hypothesis is supported by only occasional correlation between PMNs and MCs highlighted in our study. Of note, populations of PMNs or MCs in NM, pCRC and LM were totally independent, indicating the autonomous nature of the TME in both primary and metastatic sites.

Prognostic significance of MCs

MCs at high density showed protumor prognostic associations in stage I-III pCRC whereas they were rather neutral in LM irrespective of their time-related pattern. This finding aligns with reports suggesting that MCs at early stages of CRC support cancer cells proliferation[41] and also promote tumor progression *via* angiogenesis and immunosuppression[20,22,24,42]. MCs engage with tumor cells, infiltrating immune cells, and the extracellular matrix *via* direct cell-cell interactions or the secretion of mediators, thereby shaping TME remodeling. Recruited MCs can release pro-angiogenic mediators, including VEGF-A and -B, FGF-2, heparin, histamine, and stem cell factor, as well as lymphangiogenic factors VEGF-C and -D[20,22]. Beyond angiogenesis, MCs suppress antitumor immunity through interactions with regulatory T cells and myeloid-derived suppressor cells, secretion of histamine, adenosine, amphiregulin, IL-10 and TGF-β1[20,24]. MCs were reversely associated with CD8+ T-cell infiltration[42] and promoted macrophage recruitment and polarization toward a protumor M2 phenotype[22]. MC-derived proteases such as MMP9 itself and tryptase that activates matrix metalloproteinases, lead to extracellular matrix degradation, thereby facilitating tumor growth and invasion[20,24].

The absence of convincing prognostic associations of MCs in LM may be attributed to their lower density compared with pCRC, likely reflecting the immunosuppressive microenvironment of the liver[39]. Another plausible hypothesis is a balance between pro- and anti-tumor populations of MCs in LM. MCs exhibit marked functional plasticity, giving rise to distinct phenotypes (*e.g.*, MCs expressing tryptase, tryptase and chymase, and chymase only) across anatomical sites[20,21,23,24]. Their proliferation, survival, mediator storage, and secretory responses are tightly regulated by local or systemic cytokine milieus.

Consequently, the biological impact of MCs in cancer depends on their spatial localization, density, activation status, mediator profile, and interactions with neighboring immune and tumor cells, which together account for their context-dependent pro-, anti-tumor or neutral effects[20,21,24].

Although we did not find prognostic associations of CD117 expression by CRC cells, the density of MCs was greater in CD117-expressing pCRC, which deserves further investigations. One study demonstrated the ability of CD117 to activate PI3K/Akt and MAPK pathways in CRC cells, linking this axis to tumor-cell stemness, growth, invasive behavior and metastatic potential[43].

Association of MCs with survival was demonstrated earlier in stage II-III CRC[42,44]. Several therapeutic strategies have been developed to limit tumor growth by targeting MCs and their mediators[23,45].

Protumor prognostic associations of PMNs

In the metachronous group, high PMNs density in the OM of primary tumor correlated with shorter DFS, additionally high IM/OM ratio of PMNs was associated with a shorter TLM, suggesting that PMN accumulation at the invasive margin promotes early metastatic dissemination. These findings support a role for PMNs, in particular, when polarized towards N2 at the tumor-host interface, in facilitating invasion and liver colonization through multiple mechanisms. Protumor N2 PMNs drive angiogenesis, invasion, immune evasion, and metastasis through secretion of VEGF, FGF2, HGF, MMPs, neutrophil elastase, inflammatory cytokines, reactive oxygen species, and NETs[10-12,14]. In addition, PMNs suppress antitumor immunity by impairing cytotoxic T-cell and NK-cell function, excluding effector cells from tumor nests, and recruiting regulatory T-cells[10,16,17]. In parallel, tumor-derived IL-8 stimulates PMNs to secrete arginase-1, leading to arginine depletion and the establishment of an immunosuppressive TME[13]. In accordance with our results in the metachronous group, Khanh *et al*[46] and Rao *et al*[47] linked a greater density of PMNs in pCRC stage I-III with shorter survival.

High PMN densities in OM and PT of synchronous LM were also associated with shorter DFS. In CRC LM, PMNs were reported to actively drive metastatic outgrowth[36]. In addition to the above-mentioned in pCRC mechanisms, PMNs in LM facilitate pre- and metastatic niche formation by contributing to cancer cell adhesion within liver sinusoids[48]. Activated PMNs in LM can release NETs, which are more abundant than in pCRC and enhance tumor cell trapping, epithelial-to-mesenchymal transition, immune evasion, and metastatic expansion[14,15]. NETs not only directly promote cancer growth but also contribute to suppressing T-cell responses through metabolic and functional exhaustion[14,30,49]. The observed pro-tumor prognostic associations of PMNs are supported by recent literature[10,13,37].

Immune checkpoint inhibitors have shown a significant effect on metastatic CRC patients with MSI-high tumors, but no exact efficiency was observed in MSI-low patients, who accounted for the majority of the total metastatic CRC patients and prevailed in our cohort. Targeting protumor PMNs has emerged as a potential therapeutic strategy, and may help overcome current immunotherapy resistance in MSI-low CRC[10,11,13].

Antitumor prognostic associations of PMNs

In metachronous LM, elevated PMN density correlated with prolonged DFS, consistent with the notion that, within established metastases, PMNs may retain protective functions reflecting an N1-like phenotype. N1 PMNs promote tumor cytotoxicity by producing TNF-α, ICAM-1, reactive oxygen species, Fas, and *via* TRAIL-TRAIL receptor interactions, while limiting arginase-mediated immunosuppression[10-13]. PMNs also activate adaptive immunity by expressing costimulatory molecules, recruiting and activating CD8+ T cells, NK cells and may also mediate antibody-dependent cell-mediated cytotoxicity against tumor cells[11]. In addition, TANs can inhibit angiogenesis *via* VEGF-A165b[13]. The observed antitumor associations of PMNs are in line with results of several studies[13,37], including one recent systematic review and meta-analysis[19].

The protumor and antitumor associations of PMNs highlighted in our study confirm the concept that PMNs can exert the dual and context-dependent role in CRC with respect to their phenotype, tumor stage, location in pCRC or LM and subregional distribution[12,36].

Although PMNs and MCs in synchronous pCRC were not individually associated with survival, integrative assessment of these cells has yielded more prognostic associations. Patients who displayed MCs low & PMNs high in the IM and PT of pCRC had significantly longer DFS compared to low/low group, suggesting a protumor role of MCs and an antitumor role of PMNs. Of note, PMN and MC densities correlated only in the OM and PT regions of synchronous pCRC. While the biological significance of this observation remains unclear, it may warrant further investigation given that MCs have been reported to produce polyphosphate, a mediator implicated in PMN activation[20,30].

Discordant findings in literature

Several groups reported discordant findings regarding distribution and prognostic associations of MCs in CRC. Acikalin *et al*[44] reported higher counts of MCs in hot spots of pCRC *vs* NM after Gymza staining. Xia *et al*[28] use antibodies against tryptase and chymase semiquantitatively assessed numbers of MCs per microscopic field of view and found MCs in NM but not pCRC to be associated with survival in CRC patients. High counts of tryptase+ MCs in CRC tumor tissue microarrays[27] and hot spots of pCRC tissue[50] have been linked to improved OS. Tanis *et al*[51] reported greater numbers of CD117+ MCs infiltrating resected CRC LM after neoadjuvant FOLFOX with prolonged progression-free survival. Based on the TCGA pan-cancer data, two studies found that high expression of MC signature genes was associated with better survival[25,37]. These conflicting results stem in part from the high heterogeneity of studies on MCs in CRC as reviewed by Liu *et al*[21] and Molfetta and Paolini[24]. It is likely that age, sex, racial disparities, stage of the disease, phenotype of MCs, as well as the methods used for detection and quantification of MCs in tissues may account for the discrepant results.

In contrast with our findings, Bazzi *et al*[37] using the gene-signature based method xCell and Zhong *et al*[38] applying IHC for CD16, found an abundance of neutrophils in adjacent normal colon tissue *vs* CRC. As in our study, both favorable and adverse prognostic associations of tumor-associated PMNs have been reported in the literature. Some

discrepant results can be attributed to patient gender and ethnicity, antibody types, tumor location and stage, quantitative methods, and follow-up time, as discussed in the study of Jiang *et al*[19]. Of note, Jiang *et al*[19] in the systematic review and meta-analysis underscored a clear difference in the prognostic value of tumor-associated PMNs among CRC patients, depending on whether cell abundance was assessed by genetic analysis or immunohistochemistry.

Strengths and limitations of the study

The study provides detailed spatial analysis of two major innate immune cell types between NM, primary and metastatic tumor, stratified by temporal pattern of metastasis development. Densities of PMNs and MCs were evaluated objectively and quantitatively in strictly defined regions using digitized high-resolution images in combination with specialized software, thereby minimizing observer bias. Significant prognostic associations were validated using optimized cut-offs, which may facilitate future clinical implementation.

This study is limited by its retrospective design, which introduces potential selection bias and precludes causal inference. The single-institution cohort and moderate sample size reduce statistical power and limit generalizability. Reported survival associations did not pass the FDR test, however, our primary aim was to identify potential associations and generate biologically meaningful hypotheses rather than to establish definitive conclusions. Therefore, our findings should be considered exploratory and preliminary.

However, the design of our study comparing triplicate histological samples is rather uncommon and introduces a quasi-temporal dimension, even if not longitudinal per patient. Despite the low occurrence of MSI-high tumors potentially limiting generalizability, our findings are pertinent to this LM cohort, as MSI-high status is infrequent in CRC with distant metastases[52]. Although the expression of immune checkpoint was not examined, none of the patients were treated with checkpoint inhibitors due to the predominance of MSI-low tumors. While immunohistochemistry is widely used, it does not capture the full functional heterogeneity of PMNs and MCs, underscoring the need for advanced approaches such as multiplex imaging, spatial transcriptomics and functional assays.

The interval between resection of pCRC and diagnosis of metachronous LM ranged from 1 month to 59 months. Although LM detected shortly after surgery may represent previously occult lesions, classification was based on the absence of detectable LM during initial staging and surgical management of the primary tumor, in accordance with contemporary consensus recommendations[31]. Moreover, exclusion of patients who were diagnosed with metachronous LM within the first 3 months after surgery of pCRC did not change the results significantly.

Heterogeneity in adjuvant therapies and incomplete molecular data constrained survival analyses. Future large prospective cohort studies are needed to improve the reliability and generalizability of the results. Results would be further enhanced by integration with other immune cell populations, providing a valuable framework for further mechanistic insights.

CONCLUSION

This study demonstrates distinct spatial patterns of PMNs and MCs in CRC progression. We highlighted increasing density of PMNs along with CRC progression from NM to LM and the opposite changes in MCs. These patterns have prognostic relevance, with MCs showing protumor prognostic associations in pCRC, while exhibiting largely neutral effects in LM regardless of chronicity. PMNs exhibited protumor prognostic associations in stage I-III pCRC and synchronous LM in stage IV patients, and antitumor associations in metachronous LM. The present findings reinforce the importance of spatial immune profiling and, after validation, may serve as prognostic biomarkers to refine risk stratification, guide follow-up strategies, and inform adjuvant therapy decisions in both stage IV and stage I-III CRC patients.

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