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BRIEF CONCLUSIVE REPORT

Impact of RAS mutations on the immune infiltrate of colorectal liver metastases: A preliminary study

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Abstract

Kirsten rat sarcoma viral oncogene homolog KRAS proto-oncogene is the most common altered gene in colorectal cancer (CRC). Determining its mutational status, which is associated with worse prognosis and resistance to anti-epidermal growth factor receptor (EGFR) inhibitors, is essential for managing patients with CRC and colon liver metastases (CLM). Emerging studies highlighted the relationship of KRAS-mutated cancers and tumor microenvironment components, mainly with T cells. The aim of this study was to analyze the relationship of CLM immune cell infiltrate with KRAS mutational status. We performed a retrospective study on paraffin-embedded CLM tissue sections from patients surgically resected at the Department of Hepatobiliary and General Surgery of Humanitas Clinical and Cancer Center. We studied the distribution of lymphocytes (CD3+ cells), macrophages (CD163+), and neutrophils (CD66b+) in CLM tumoral and peritumoral area. Percentage of positive cells was correlated with tumor macroscopic characteristic, clinical aspects, and KRAS mutation. We observed a significant increase in CD66b+ cells in the peritumoral area in patients KRAS-mutated compared to KRAS wild-type patients. Percentages of lymphocytes and macrophages did not show significant differences. Further, neutrophils were found to be significantly increased also in the bloodstream of KRAS-mutated patients, indicating increased mobilization of neutrophils and recruitment in the CLM site. In conclusion, this study reveals a new intriguing aspect of the peritumoral microenvironment, which could pave the way for new prognostic and predictive markers for patient stratification.

KEYWORDS
neutrophils, colon liver metastasis, colorectal cancer, immune microenvironment, T-cells, tumor-associated macrophage

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most common diagnosed cancer in men and the second most in women. Based on current demographic projections, the global burden of CRC is expected to increase by 60%, leading to about 2.2 million new diagnoses per year and 1.1 million CRC deaths by 2030.[1,2] In these patients, the liver is the most common site of metastases, which represents the main cause of mortality in CRC patients.[3]

Surgical resection of colorectal liver metastases (CLM), combined with systemic chemotherapy has the potential to be curative, projecting 5 yr survival rate up to 50% and 10 yr overall survival rate up to 35%.[4] However, CLM patients present with heterogeneous clinical outcomes and degrees of therapeutic responsiveness[5] that is known to be dependent on many different biologic and immunologic factors. Among tumor biology factors, RAS mutations status conveys

Abbreviations: CLM, colon liver metastasis; CRC, colorectal cancer; EGFR, anti-epidermal growth factor receptor; LMR, lymphocyte-to-monocytes ratio; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; TAN, tumor-associated neutrophils.

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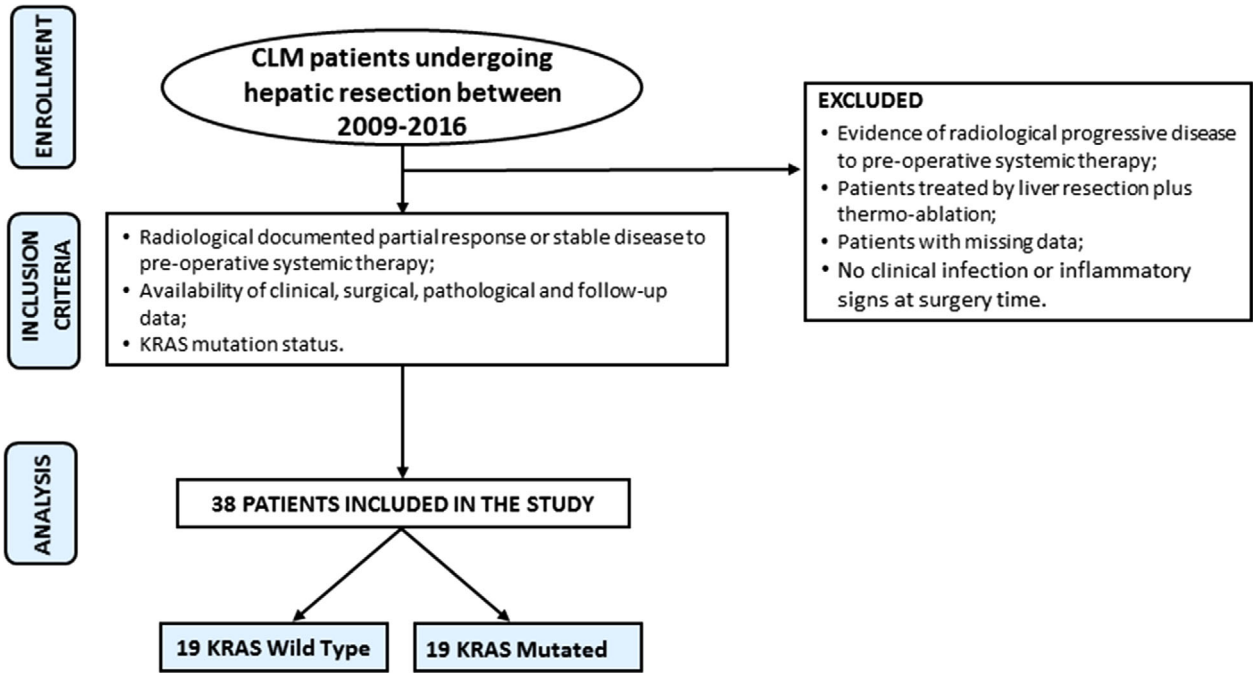

FIGURE 1 **CONSORT diagram.** The figure shows patients' recruitment process

significance not only for the response to anti-epidermal growth factor receptor (EGFR) agents but also to surgical outcome and prognosis.[6] Among immunologic factors, the different immune cells and mediators are present in the tumor microenvironment both at the primary and metastatic sites.[7,8] Evaluating the immune cell infiltrate in tumor tissues could be relevant for patient profiling with respect to the clinical outcome. Based on KRAS mutation status, our study was aimed to assess the immune infiltrating cells in CLM patients, focusing on T-cells, macrophages, and neutrophils.

2 | METHODS

2.1 | Study design and patients' selection

The study retrospectively examined a cohort of consecutive CRC patients who underwent hepatectomy for CLM at our institution. Written informed consent was obtained from each patient. All procedures were performed in accordance with Helsinki Declaration and our institution's ethical committee. Results were reported according to Strengthening the Reporting of Observational Studies in Epidemiology.[9] Patients were assembled in a clinical retrospective database. All the immunologic and histologic analyses were blinded to the clinical data. Selection criteria are shown in Figure 1. Preoperative workup consisted of total-body contrast-enhanced computed tomography (CT) and liver-specific MRI, performed maximum 30 d prior to surgery. Postoperative follow-up was performed every 3 mo and included serum oncologic markers, abdominal ultrasonography, CT, or MRI. Postoperative morbidity was graded based on the Clavien-Dindo classification.[10] Postoperative mortality was recorded at 90 d after surgery. This study was aimed to assess the density of neutrophils, T cells, and macrophages infiltrating CLMs according to KRAS mutations.

2.2 | Peripheral blood samples

Routine laboratory tests (neutrophils, monocyte, lymphocytes, and platelets count) were preoperatively performed and unbiased for confounding factors such as infections, systemic chemotherapy side-effects, and use of anti-inflammatory medications. White blood cell counts were recorded from 4 to 6 wk after chemotherapy end. The ratio of lymphocyte-to-monocyte (LMR), neutrophil-to-lymphocyte (NLR), and platelet-to-lymphocyte (PLR) were measured as possible systemic inflammatory indicators showed to be associated with worse outcome in cancer patients.[11,12]

2.3 | Immunohistochemistry

Only CLMs paraffin-embedded sections with both tumoral and peritumoral (which corresponds to the invasive margin) areas were analyzed. After deparaffinization and rehydratation with standard protocols, the antigen unmasking was performed with EDTA solution pH 9 at 98°C for 30 min (Dako/Agilent Technologies, Carpinteria, CA, USA) and incubated in peroxidase blocking solution (Dako/Agilent Technologies, Carpinteria, CA, USA). Samples were incubated overnight at +4°C with different primary antibodies: CD3 (1:100, Dako, Carpinteria, CA, USA), CD163 (1:100, R&D, Oxford, UK) and CD66b (1:100, BD Biosciences, San Jose, CA, USA), and their appropriate secondary antibody at room temperature for 30 min. The reactions were visualized with DAB (3, 3′- Diaminobenzidine), counterstained with hematoxylin. As negative controls, primary antibodies were omitted. In the whole tissue slide

samples, three expert pathologists evaluated the stained cells percentage in a semiquantitative fashion. Images were acquired with a microscope (BX51; Olympus, Tokyo, Japan) equipped with a Colorview IIIu digital camera (Olympus, Tokyo, Japan). Photoshop (CS5.1) was used to process images.

2.4 | Statistical analysis

The software IBM-SPSS and GraphPad PRISM 8 were used for the analysis. χ^2, Fisher's exact test, unpaired *t*-test and the Mann-Whitney *U* test were used as appropriate. *P*-value < 0.05 was considered significant.

3 | RESULTS AND DISCUSSION

3.1 | Patients selection

A total of 38 patients who underwent hepatic resection for CLM were recruited for this study, whose demographics and clinical–pathologic features are detailed in Table 1. Of these, 20 (52%) were male with a median of 61 yr old (range 35–68). In 31 patients (81%), the primary tumor was pathologically staged as T3–4, whereas in 26 patients (68%) the regional lymph nodes of the primary tumor were positive. KRAS was mutated in 19 (50%) patients. Overall, these data indicate that the CLM patients enrolled in this study had relatively advanced tumor stages, as also confirmed by the size of CLM (median 3.95 cm; range 0.5–7.7 cm) and the number of CLM (median 4; range 2–14) with 20 (53%) of the patients having 4 or more CLM. Moreover, 24 (63%) had synchronous CLM. Because of this tumor burden, 87% of these patients were pre-operatively treated with systemic chemotherapy. Table 1 details also the surgical procedures.

TABLE 1 Demographic and clinical characteristics

Variable	Number (%)
Age (year) at surgical operation	
Median; range	61; 35–78
Gender	
Men	20 (52)
Women	18 (48)
BMI (kg/m^2)	
Men	26.9
Women	23.7
Number of colorectal liver metastases	
Median; range	4; 2–14
Size of the largest colorectal liver metastases (cm)	
Median; range	3.95; 0.5–7.7
Bilateral involvement of the liver	22 (58)
Timing of diagnosis of colorectal liver metastases	
Synchronous	24 (63)
Metachronous	14 (37)
Preoperative systemic chemotherapy	33 (87)
T stage of the primary colorectal tumor	
Tx	4 (11)
T1	0
T2	3 (8)
T3	27 (71)
T4	4 (10)
N stage of the primary colorectal tumor	
Nx	4 (11)
N0	8 (21)
N1	14 (37)
N2	12 (31)
KRAS status	
Wild-type	19 (50)
Mutated	19 (50)

3.2 | Short-term outcome and pathology

Complications were recorded in 9 (24%) patients, graded as minor in 7 (18% of entire cohort), and graded as major in 2 (2%) according with the Clavien-Dindo classification.[16] No mortality was recorded at 90 d postsurgery. All included cases were confirmed to be CLM at the final histology. The minimal surgical margin was 0 mm in median (range 0–10 mm).

3.3 | Immune infiltrate

We investigated the distribution of immune cells infiltrating the metastatic lesions, in both intratumoral and peritumoral areas, performing immunohistochemistry analysis of neutrophils (CD66b+ cells), lymphocytes (CD3+ cells), and macrophages (CD163+ cells) infiltrate. After a semiquantitative analysis performed by the pathologists, we calculated the percentage of positive cells in the peritumoral and tumoral area. Figure 2 details the variability in the frequency of CD66b+, CD3+, and CD163+ cells infiltrating CLM. As shown, no significant differences were recorded between those two tissue areas either for CD66b+ ($P = 0.8170$), CD3+ ($P = 0.9754$), and CD163+ ($P = 0.2504$) cells.

3.4 | Impact of RAS mutations

We have analyzed the percentage of the immune infiltrate looking for a relationship with the clinical data of the patients. No significant correlation was found in the percentage of lymphocytes (CD3+ cells) both in tumoral and peritumoral area. Similar results were obtained after analysis of macrophage population (CD163+ cells). Conversely, a significant correlation was found between the percentage of neutrophils (CD66b+ cells) and the RAS status. In particular, in patients harboring KRAS mutation, a statistically significant increase in peritumoral neutrophils infiltrate was identified compared to KRAS wild-type patients ($P = 0.033$) (Fig. 3). No significant differences were observed in tumoral area ($P = 0.4815$). Figure 4 details two representative cases of CLM

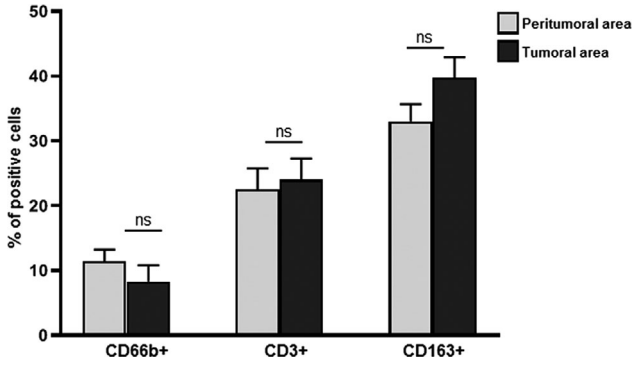

FIGURE 2 **Percentage of immune cells in colon liver metastasis (CLM) peritumoral and tumoral samples**. IHC analysis of the percentage of positive cells: neutrophils (CD66b+), leukocytes (CD3+), and macrophages (CD163+) in peritumoral (light gray) and tumoral area (dark gray) of CLM samples, respectively. One-way ANOVA was used to compare the percentage of positive cell in peritumoral and tumoral area. (ns: not significant). Data are represented as mean with SEM. ($N = 38$)

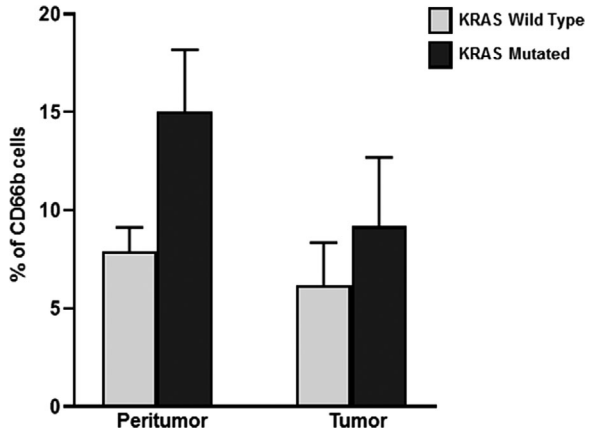

FIGURE 3 **Percentage of neutrophils (CD66b+) in KRAS wild-type (light gray) vs. KRAS MUT (dark gray) colon liver metastasis (CLM).** Significant increase in neutrophils infiltrate in peritumoral area of CLM KRAS-mutated patients vs. KRAS wild-type. No significant differences were present in tumor area. Data are represented as mean with SEM. Unpaired *t*-test was used to compare the two groups. $^{*}P = 0.033$

patients with and without KRAS mutation. As shown, patients with KRAS mutation had a clear increased density of CD66b+ cells in comparison with patients without KRAS mutation (Fig. 4A and B). Interestingly, the same findings were not seen for CD163+ and CD3+ cells (Fig. 4C-F).

3.5 | Results of peripheral blood samples

To better understand the presence of a systemic inflammation that might drive the recruitment of neutrophils from the systemic blood circulation to metastatic niche and vice versa, peripheral white blood count was analyzed. Figure 5 details the results of peripheral blood samples. Specifically, we focused on neutrophils, lymphocytes, monocytes, and platelets count. Interestingly, a statistically significant increase in pre-operative neutrophil count emerged in KRAS-mutated patients compared to KRAS wild-type ones ($P < 0.001$). Instead, no difference arises in lymphocyte ($P = 0.997$), monocyte ($P = 0.990$), and platelet counts ($P = 0.997$). Similarly, LMR ($P = 0.999$), NLR ($P = 0.999$), and PLR ($P = 0.919$) were not found to be significant different either.

3.6 | Clinical implications and future perspective

There is substantial literature that supports the link between cancer and inflammation. Chronic inflammatory diseases increase the risk of developing different types of cancer including CRCs. Indeed, proinflammatory chemokines, cytokines, and inflammatory immune cells are in the microenvironment of all tumors from the earliest stages of development.[13]

In this retrospective study, we investigated the relevance of CD66b+, CD3+, and CD163+ cells infiltrating CLM patients who underwent hepatectomy after receiving neo-adjuvant systemic chemotherapy. Interestingly, a statistically significant correlation between the density of CD66b+ cells and the KRAS status was found. In contrast, no differences resulted for CD3+ or CD163+ populations that, probably, play different roles. Our results are in line with the burgeoning literature about the role of the host immune system in determining the heterogeneous clinical outcomes and degrees of therapeutic responsiveness of CLM patients.[14-16] Notably, the human adult liver contains 10^{10} lymphocytes, with the majority of these cells being cytotoxic T and NK cells.[17,18] It has been shown that higher numbers of intratumoral immune cells positively correlate with patient outcome in solid tumors such as CRC.[19,20] Therefore, understanding the mechanisms underlying this antitumor cells infiltration into CLM could be relevant for better predicting patient clinical outcomes and to develop more effective therapeutic approaches.

To date, no studies have been focused on the role of the immune cells according to RAS mutations in CLM. RAS is a proto-oncogene frequently altered in cancers and its mutation leads to a constitutive activation status ending in dysregulated cell growth.[21] In CRC, RAS mutations has been associated with poor prognosis,[22] resistance to anti-EGFR antibody therapy,[23,24] and liver metastatic spreading.[25,26] Finding a biologic and molecular connection between RAS mutations and the host immune cells will be beneficial.[27] Yet, density, type, and cell location have been related to the clinical outcome of several human cancers.[28] Emerging few studies highlighted a strictly relationship between cancers harboring KRAS mutation and immune system, mainly with T cells.[29,30] Interesting studies have documented the presence of tumor-associated neutrophils (TAN), which similar to macrophages,[31,32] showed pro-tumor or antitumor effect in response to different stimuli.[33,34] Despite that, regulation and plasticity of neutrophils remain largely unknown and still poor studied in humans. Our study demonstrated a statistically significant increase in peritumoral neutrophils infiltrate in KRAS-mutated CLM patients, highlighting a key role of this proto-oncogene mutation in the neutrophils recruitment process. The mechanism

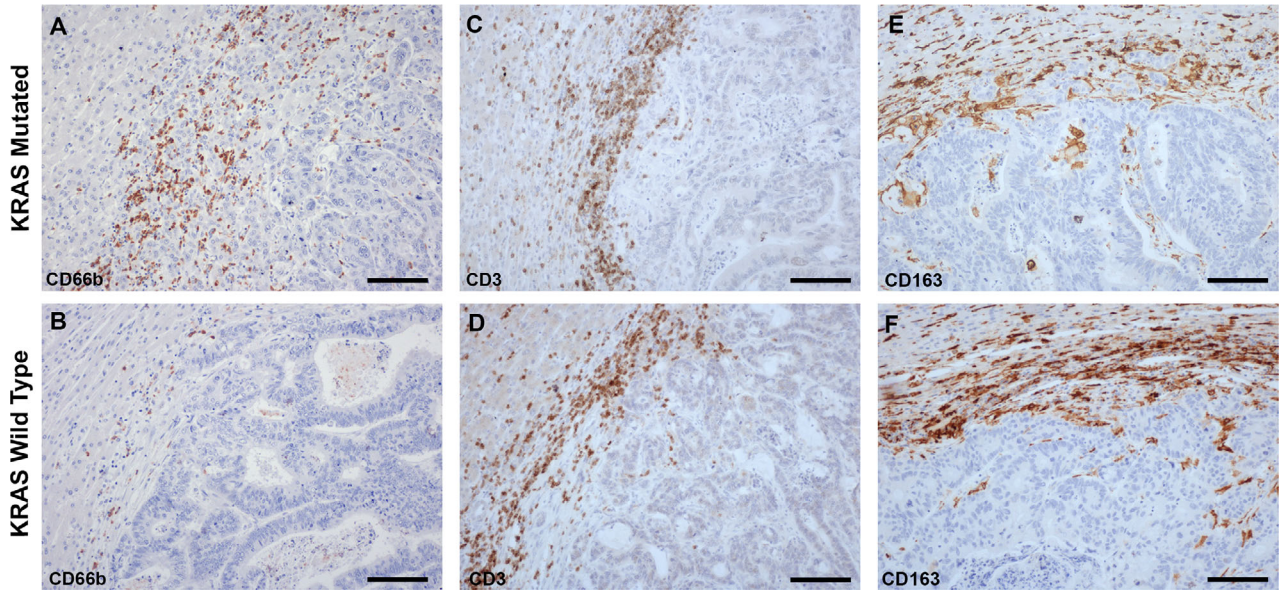

FIGURE 4 **Immunohistochemistry analysis of infiltrate population on colon liver metastasis (CLM) paraffin sections.** CLM sections stained with mouse monoclonal anti-CD66b (**A, B**), anti-CD3 (**C, D**), and anti-CD163 (**E, F**) antibodies and counterstained with hematoxylin. Representative expression of the three markers in the peritumoral compartment in CLM KRAS mutated (**A, C, E**) vs. the peritumoral compartment in CLM KRAS wild-type (**B, D, F**). The KRAS wild-type samples show lower CD66b positive cells in the peritumoral portion (**B**) than the KRAS mutated (**A**). No difference was seen for the other two markers (**C; D; E; F**). Bars 200 μm

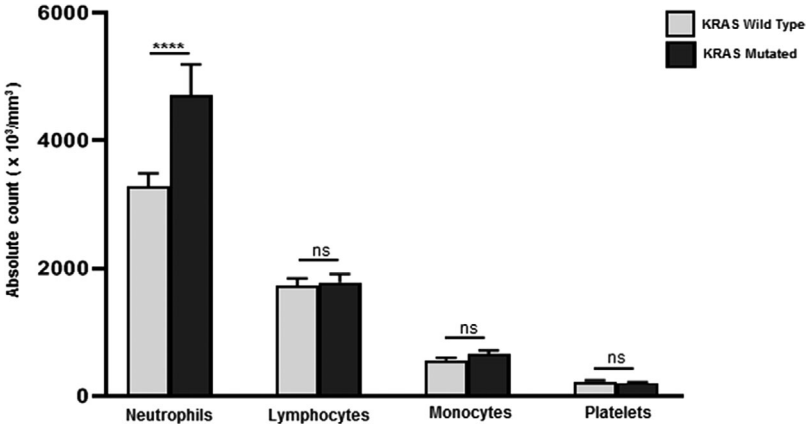

FIGURE 5 **Peripheral blood parameters.** Analysis of the percentage of neutrophils, lymphocytes, and monocytes in peripheral blood of colon liver metastasis (CLM) patients according to KRAS wild-type (light gray) and KRAS MUT (dark gray), respectively. One-way ANOVA test was used to compare the percentage of immune cells. $^{****}P < 0.001$. Data are represented as mean with SEM. ($N = 38$)

behind the increases in CD66b infiltration and RAS mutations may be postulated based on data on lung tumors. Indeed, Ji et al. [35] reported the up-regulation of inflammatory chemokines, including CXCL2, CXCL5, and CXCL8. In particular, CXCL8, also known as IL-8, was found to be up-regulated by ERK-MAPK pathway activation induced by lung tumor cells harboring KRAS mutation, which were associated with worse prognosis.[35] Higher expression of IL-8 was also found in both CRC and CLM, highlighting its involvement in tumor progression and metastases development.[36] Despite that, no one reported its correlation with CLM KRAS mutation status. Notably, this pathway could be involved in the higher recruitment of neutrophils in CLM peritumoral area, providing an explanation for our findings.

Intriguingly, according to peripheral blood analyses, KRAS-mutated patients have significant neutrophils increase compared to KRAS wild-type ones (Fig. 5). This could indicate a specific mobilization of neutrophils to CLM sites in KRAS-mutated patients, which might be sustained by a complex orchestra involving different immune cells as recently reported in sarcoma patients.[37] These findings indicate that a significantly high number of infiltrated neutrophils in CLM peritumoral tissues could be considered as a potential prognostic and/or diagnostic marker. Indeed, quantification of differences in immune cells infiltrating CLM represents an emerging research strategy. Moreover, our study linked the well-known worse prognosis of KRAS-mutated patients with CD66b+ cells infiltration in an original clinical perspective view. Once confirmed on a larger-scale study, novel immune-based metrics could be integrated in digital platforms to capture these TANs' features, with the aim of refining patient stratification and ameliorating therapeutic output. The detection of neutrophil density is inexpensive and applicable. However, to make the prognostic or diagnostic results more accurate, deeper analyses of other cancer- and immune-related factors are required.

This study has some limitations. First, this is a retrospective single-center study performed on a small sample size. Second, this is a

descriptive study and the findings may represent a chance of occurrence. Nevertheless, this is a preliminary exploratory research without missing data meaning that all the information available have been included and analyzed. Moreover, a confirmation study on a larger sample size has been planned.

In conclusions, our results support the notion that accurate quantitative and functional characterization of TANs could be used for correlation with prognostic significance of CLM patients.

AUTHORSHIP

M.D designed the study. M.A.P and F.M. carried out experiments and analyzed data. C.S. and B.F. performed IHC. C.S., B.F. and S.J. gave scientific and technical advice. A.A., F.S.C. and S.C. analyzed data. M.A.P, F.M. and M.D. wrote the manuscript. A.L., M.C. and G.T revised the manuscript. L.D.T. provided Kras mutation status. M.A.P. and F.M. have contribuited equally. G.T. and M.D. jointly supervised the work.

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DISCLOSURES

The authors declare no conflicts of interest.

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