Correlating local and systemic tumor-associated inflammation in colon cancer

CRP and the tumor immune microenvironment

Anne Helene Køstner

Thesis for the degree of Philosophiae Doctor (PhD)
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Contents

Abbreviations

AI	Artificial Intelligence
AJCC	American Joint Committee on Cancer
APP	Analysis Protocol Package
APR	Acute Phase Response
CC	Colon Cancer
CMS	Consensus Molecular Subtypes
CRC	Colorectal Cancer
CRLM	Colorectal Liver Metastases
CSS	Cancer Specific Survival
CYP	Cytochrome 450 enzymes
CRC	Colorectal Cancer
CRP	C-reactive Protein
DAB	3, 3'-diaminobenzidine
DCC	N'dicyclohexylcarbodiimide
DSS	Disease Specific Survival
EGFR	Epidermal Growth Factor Receptor
ECM	Extra Cellular Matrix
EMT	Epithelial-Mesenchymal Transition
FFPE	Formalin-fixed, Paraffin-embedded

GI	Gastrointestinal
GPS	Glasgow Prognostic Score
HE	Hematoxylin-Eosin
HR	Hazard Ratio
hsCRP	high sensitive C-reactive Protein
ICI	Immune Checkpoint Inhibitor
IF	Immunofluorescence
IHC	Immunohistochemistry
IM	Invasive Margin
LDL	Low Density Lipoprotein
MAC-1	Macrophage Antigen-1
MCP-1	Monocyte Chemoattractant Protein-1
mCRP	monomeric, modified C-reactive Protein
MDSC	Myeloid-derived Suppressor Cell
mIHC	multiplex Immunohistochemistry
MMP9	Matrix Metalloproteinase 9
MMR	Mismatch repair
MSI	Microsatellite instability
MSS	Microsatellite stable
NLR	Neutrophil-to-Lymphocyte Ratio

NFκB	Nuclear factor kappa beta
NSAID	Non-steroidal Anti-inflammatory Drug
OS	Overall Survival
pan-CK	pan-Cytokeratin
PC	Phosphocholine
pCRP	pentameric C-reactive Protein
PD-1	Programmed death receptor 1
PD-L1	Programmed death ligand 1
PLA2	Phospholipase A2
PLR	Platelet-to-Lymphocyte ratio
ROC	Receiver Operating Curve
ROI	Region of Interest
ROS	Reactive Oxygen Species
SIR	Systemic Inflammatory Response
TAM	Tumor-associated Macrophage
TAN	Tumor-associated Neutrophil
TC	Tumor Center
TGF-β	Transforming Growth Factor beta
TIL	Tumor Infiltrating Lymphocyte
TMA	Tissue Microarray

TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
TNM	Tumor, Node, Metastasis
UICC	Union for International Cancer Control
VEGR	Vascular endothelial growth factor
WSI	Whole slide imaging

Abstract

Background Biomarkers of systemic inflammation have consistently been associated with poor patient outcomes in various cancer types, including colorectal cancer (CRC). In the present work, we focus on the role of tumor-associated systemic inflammation as assessed by elevated levels of serum CRP in resectable metastatic and non-metastatic CRC patients. Overall, we aimed to improve the understanding of why elevated CRP is so detrimental for prognosis. While previous work in the field of tumor immunology predominantly has focused on the systemic and local tumor immune responses separately, we were interested in assessing the relationship between the two to get a better comprehension of the inflammatory state associated with elevated CRP and poor clinical outcomes. Considering emerging evidence that CRP exists in two distinct isoforms, circulating pentameric CRP (pCRP) and the pro-inflammatory tissue-associated monomeric form (mCRP), we aimed to investigate whether mCRP was expressed in the tumor microenvironment (TME) of a cohort of colon cancer (CC) patients with and without elevated circulating CRP.

Method In the first part of the project (paper 1), we evaluated the prognostic impact of preoperatively measured CRP in a Nordic cohort of 425 stage IV CRC patients undergoing potentially curative resection of liver metastases. In the second and main part of the project (paper 2 and 3), we performed immunohistochemical staining of tumor tissue from 43 stage II and III colon cancer patients resected for their primary tumors at Sørlandet Hospital between 2005 and 2015. For exploring the tumor immune microenvironment, we developed a formalin-fixed paraffin-embedded (FFPE)-based platform combining multiplex immunohistochemistry (mIHC) and digital whole slide imaging enabling accurate visualization and assessment of seven simultaneously expressed both lymphoid and myeloid immune markers with preserved tissue morphology covering entire tissue sections. Using this platform, we could get comprehensive information regarding cellular composition, distribution, and spatial patterns in the TME allowing us to explore and compare the tumor immune contexture in colon cancer patients with and without systemic inflammation.

Results We found that preoperatively elevated CRP (>10 mg/L) was a strong negative prognostic marker in CRC patients with liver metastases undergoing potentially curative liver surgery independent of other tumor and patient related factors. By exploring the tumor immune microenvironment in CC patients with and without elevated CRP, we found that systemically inflamed patients (CRP>30 mg/L) harbored a more myeloid-dominated TME compared to non-inflamed patients (CRP 0-1 mg/L), suggesting a particular strong, potential driving role of neutrophils. Importantly, our data showed that it is the presence of myeloid inflammation (evaluated by a compounded score of CD66b+ neutrophils and CD68+macrophages) rather than the absence of lymphoid inflammation (evaluated by a compounded score of CD4+ and CD8+ T-cells) that correlates with systemic inflammation.
Utilizing an mCRP specific antibody, we found that mCRP was abundantly present within tumors, but not in adjacent normal colon mucosa. Tumor-associated mCRP correlated strongly with circulating CRP. Double IHC revealed co-localization of mCRP with neutrophils and endothelial cells as well as some tumor cells, suggesting that mCRP may play a direct role in the microenvironment of tumors in CC patients presenting with systemic inflammation.

Conclusion and perspectives Taken together, this work advances our comprehension of the profound role of systemic inflammation and elevated CRP for prognosis in a broad population of CRC patients. Measurement of CRP is inexpensive and routinely available. Incorporating CRP and information on the systemic inflammatory status alongside other clinical, pathological, and molecular features, as part of overall patient evaluation, may allow for more precise prognostication and accurate assessment of individual patient`s disease status and help clinicians tailor appropriate and more personalized treatment strategies. Focusing on the tumor immunology behind elevated CRP, the findings presented herein provide support for CRP as an informative biomarker reflecting features of the immune response occurring at the tumor site. Intriguingly, introducing a new perspective to why elevated CRP in cancer patients is so detrimental, findings within this work suggest that CRP, might not only be a passive bystander, but also an active player within tumors and opens a potential novel

approach for cancer treatment. Further progress in this area is needed to uncover the full potential and clinical utility of CRP as a biomarker and potential contributor in cancer patients.

Abstract in Norwegian

Bakgrunn Markører for systemisk inflammasjon er assosiert med dårlig prognose i en rekke ulike kreft typer, inkludert tykk- og endetarms kreft (CRC). I dette prosjektet fokuseres på betydningen av systemisk tumor-assosiert inflammasjon bestemt ved måling av CRP i blodet hos pasienter med resektabel metastatisk og lokalisert tykktarmskreft. Det overordnede formål var å øke forståelsen av hvorfor systemisk inflammasjon og forhøyet CRP er så ugunstig for prognose og sykdoms forløp hos pasienter med tykktarmskreft. Mens tidligere forskning primært har fokusert på betydningen av systemisk og lokal tumor assosiert inflammasjon hver for seg, ønsket vi å undersøke korrelasjonen mellom de to og hvorvidt særlige immunologiske karakteristika er uttrykt i primær svulsten hos pasienter med forhøyet CRP verdi i blodet. Nyere forskning har vist at CRP eksisterer i ulike isoformer; sirkulerende CRP som er en pentamer (pCRP) og vevs-assosiert monomert (mCRP), som har pro-inflammatoriske egenskaper. Vi ønsket derfor å undersøke hvorvidt mCRP var til stede i tumor mikromiljøet (TME) i en kohorte med tykktarmskreft pasienter med og uten forhøyet CRP i blodet.

Metode I den første del av prosjektet (paper 1) evaluerte vi den prognostiske verdi av preoperativ CRP i en nordisk kohorte bestående av 425 stadium IV CRC pasienter med levermetastaser behandlet med kurativt intendert leverreseksjon. I den andre og primære del av prosjektet (paper 2 og 3), foretok vi immunhistokjemisk undersøkelse av tumor vev fra 43 pasienter med stadium II og III tykktarmskreft som fikk fjernet primær tumor på Sørlandet Sykehus i perioden 2005 og 2015. For karakterisering av immunuttrykket i primær svulsten fra disse pasientene utviklet vi en metode basert på formalin fiksert parafin innstøpte (FFPE) fullsnitt, som kombinerer multiplex immunhistokjemi (mIHC) og digital analyse for presis visualisering og kvantifisering av syv ulike lymfoide og myeloide immun markører med bevart vevs morfologi. Ved bruk av denne analyse plattform kunne vi få omfattende informasjon om immun celle sammensetning, distribuering og spatiale mønstre i TME hos pasienter med og uten forhøyet CRP-verdi i blodet.

Resultater I det første materialet fant vi at preoperativ CRP > 10 mg/L var en sterk negativ prognostisk markør uavhengig av andre tumor- og pasientrelaterte faktorer hos CRC pasienter operert for levermetastaser. Ved å undersøke immunuttrykket i primær svulsten hos pasienter med tykktarmskreft med og uten forhøyet CRP verdi i blodet, fant vi at svulster fra pasienter med tegn på systemisk inflammasjon (CRP>30 mg/L) overveiende var mer myeloid inflammerte, særlig i form av høyt uttrykk av neutrofile, sammenlignet med svulster fra pasienter med normal CRP (CRP 0-1 mg/L). Samlet viste våre immunanalyser at det er grad av myeloid inflammasjon (målt ved å kombinere ekspresjonen av CD66b+ neutrofile og CD68+ makrofager) i TME og ikke fravær av lymfoid inflammasjon (målt ved å kombinere ekspresjonen av CD8+ og CD4+ T-celler) som korrelerer med den systemiske inflammatoriske respons. Ved bruk av et mCRP spesifikt antistoff, fant vi at mCRP var uttrykt i tumor, men ikke i nærliggende normal vev. Tumor-assosiert mCRP var sterkt korrelert med CRP verdien i blodet. Dobbel IHC viste co-lokalisasjon med neutrofile, endotel celler og enkelte tumor celler, hvilket indikerer at mCRP potensielt kan spille en direkte rolle i TME hos pasienter med systemisk inflammasjon.

Konklusjon og perspektiver Samlet fremhever dette prosjektet den sterke betydningen av systemisk inflammasjon og forhøyet CRP for prognose i en bred populasjon av CRC pasienter. Måling av CRP-verdien i blodet er lett tilgjengelig og forbundet med lave kostnader. Inkorporering av CRP og informasjon om pasienters systemiske inflammatoriske status bør, sammen med andre kliniske, patologiske og molekylære funn, inngå i den samlede evaluering av kreft pasienter. En slik helhetlig pasient vurdering kan bidra til mer presis prognose og hjelpe klinikeren til å foreta persontilpassede behandlingsvalg. Våre funn indikerer at CRP-verdien i blodet kan reflektere typen av immun respons lokalt i kreftsvulsten. Av særlig interesse, viser vi at CRP muligvis er mer enn en passiv biomarkør, som potensielt kan spille en aktiv rolle i kreftsvulster. Dette åpner et nytt perspektiv på hvorfor forhøyet CRP er så uhensiktsmessig for kreftpasienters forløp og prognose, og kan potensielt representere

et nytt target for kreftbehandling. Videre forskning er nødvendig for å avklare CRPs fulle potensiale som biomarkør og mulig direkte aktør ved kreftsykdom.

List of publications

The present thesis is based on the following three papers:

Paper 1

Køstner AH, Kersten C, Löwenmark T, Ydsten KA, Peltonen R, Isoniemi H, Haglund C, Gunnarsson U, Isaksson B. **The prognostic role of systemic inflammation in patients undergoing resection of colorectal liver metastases: C-reactive protein (CRP) is a strong negative prognostic biomarker**. J Surg Oncol. 2016 Oct 3;114(7):895–9. PMID:27696432

Paper 2

Køstner AH, Nielsen PS, Georgsen JB, Parner ET, Nielsen MB, Kersten C, Steiniche T. **Systemic Inflammation Associates With a Myeloid Inflamed Tumor Microenvironment in Primary Resected Colon Cancer-May Cold Tumors Simply Be Too Hot?** Front Immunol. 2021 Aug 31;12:716342. doi: 10.3389/fimmu.2021.716342. PMID: 34531864; PMCID: PMC8438238.

Paper 3

Køstner AH, Fuglestad AJ, Georgsen JB, Nielsen PS, Christensen KB, Zibrandtsen H, Parner ET, Rajab IM, Potempa LA, Steiniche T, Kersten C. **Fueling the flames of colon cancer - does CRP play a direct pro-inflammatory role?** Front Immunol. 2023 Mar 17;14:1170443. doi: 10.3389/fimmu.2023.1170443. PMID: 37006231; PMCID: PMC10065292.

1. Introduction and background

1.1 Colorectal Cancer – current state and therapeutic considerations

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and the second most common cause of cancer death both in men and women (1). The incidence is increasing, particularly in high- or very high-income countries and in young adults (2, 3). Based on data from the GLOBOCAN database, the global burden of CRC is expected to rise from 1.9 million new cases in 2020 to 3.2 million in 2040 (3). A similar pattern is predicted for mortality rates with nearly a doubling within the same time frame (3). Thus, this dismal trend highlights the critical need for improved biological understanding and clinical management of the disease, from prevention and early detection to identification of new targets and refined treatment approaches.

Mortality from CRC is because of dissemination of the disease, with the liver being the most common site for metastases, affecting approximately 25-35% of the patients (4). Early detection and curative surgery are therefore imperative to improve outcomes in CRC patients. In early and locally advanced stages, radical surgery is the cornerstone of CRC management (5). Despite advances in modern surgical techniques, half of the patients undergoing curative surgery eventually die from the disease (6, 7). Reported recurrence rates for CRC patients following curative surgery are quite varying, ranging from 5 to 20% in stage I and II, rising to 30%, and even as high as 50 % in node positive patients (stage III) (8-10). The differences in the reported rates are ascribed to several factors, including tumor- and patient related characteristics, whether adjuvant treatment has been administered or not, location of the primary tumor (colon vs. rectum, right- vs. left side), and importantly, the time period of treatment (10, 11). In general, studies covering patients treated in more recent times (last 15 years) report lower risks of recurrence compared to older trials, which is believed to not only be due to adjuvant therapy, but also improved surgical techniques, pre-operative staging, and pathological examination (9, 10). For stage III patients, adjuvant chemotherapy is recommended, estimated to cure approximately 10-20% of the patients (12, 13). The use of adjuvant

chemotherapy for stage II patients, however, is still an area of debate due to the lack of studies showing a clear benefit for the group as a whole although there appears to be a subgroup of high-risk patients where more aggressive treatment up-front could reduce the risk of relapse (14-16). In advanced disease, the treatment approach is more multimodal (17). Surgical resection and/or radiotherapy is preferred in liver metastases-only, and in oligometastatic disease, while systemic chemotherapy (combinations of 5-fluorouracil, irinotecan and oxaliplatin) in conjunction with targeted agents (primarily bevacizumab targeting vascular endothelial growth factor (VEGF), and cetuximab and panitumumab targeting endothelial growth factor receptor (EGFR)) represents the backbone of CRC treatment in the metastatic setting (5, 17).

In order to improve outcomes, optimal patient stratification for selecting of the most appropriate treatment strategy is vital. With the expansion of treatment possibilities, both surgical and systemic, this has become increasingly important. Traditionally, staging and prognostication of CRC patients have been based on the histopathological criteria defined by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) tumor, node, metastasis (TNM) staging system (18). The TNM classification categorizes CRC according to the depth of tumor invasion into the intestinal wall (T), lymph node involvement (N) and the presence or absence of distant metastasis (M) whereafter the cancer is designated into clinical stages ranging from I to IV (18). Together, these criteria are readily available from the histopathology report and are still regarded as the gold-standard for prognostication and treatment allocation of CRC patients (19). However, it has become increasingly clear that survival rates are highly variable among patients within the same stage, ranging from 50 to 85% for patients with T3/4 node negative tumors (stage II) (20, 21). Thus, there is need for refinement of these criteria to more accurately identify patients at higher risk of relapse, particularly in early and intermediate stages (II and III), that may benefit from more aggressive treatment approaches up-front including adjuvant chemotherapy (20). Within the last decade, knowledge has improved, and effort has been made to refine the TNM-based prognostication. First, additional clinicopathological features such as tumor size (larger size has been associated with

worse survival outcomes although no cut-off or clear relationship exists) and grade, tumor budding, lymphovascular and/or perineural invasion, bowel obstruction or perforation and status of the resection margin (positive/negative) have allowed for more precise stratification of patients, particularly in early and intermediate stages (18, 19). Next, the use of genomic profiling has revealed driving molecular pathways and genetic factors involved in CRC carcinogenesis and the DNA mismatch repair system (MMR), which primarily serve a predictive role in the treatment with targeted agents and immunotherapy, respectively (19). The currently used molecular biomarkers include KRAS, BRAF and MSI (19). Together with gene expression profiles, these tumor-based characteristics constitute the Consensus Molecular Subtypes (CMS) classification defining four entities of CRC (CMS1 to 4) which is believed to capture more of the diversity that exists for colorectal tumors (18, 19, 22). CMS1 is enriched for inflammatory genes, often hypermutated, harbors BRAF mutation and represents the MSI-immune subgroup accounting for 14% of CRC patients (18, 23). CMS2 constitutes the canonical subgroup accounting for 37% of CRC patients and are chromosomally unstable (18, 23). CMS3 is the metabolic subgroup, enriched for KRAS mutation and MSI although 1/3 are MSS (13% of CRC patients) (18, 23). CMS4 is the mesenchymal subgroup showing signs of epithelial-mesenchymal transition (EMT) activation with stromal invasion, angiogenesis and transforming growth factor β (TGF-ß) activation, accounting for 23% of CRC patients (18, 23). Data regarding CMS and the potential prognostic and predictive value for outcome and response to systemic treatment, particularly with targeted agents and immunotherapy, are heterogeneous and there is still a lack of consensus regarding their use as biomarkers in CRC (24, 25). Thus, more, preferentially prospective studies, are needed before the CMS classifier can be implemented in clinical practice. A particular role exists for MSI-status beyond the CMS classification, given the strong predictive role for response to immune checkpoint inhibitors in the metastatic setting (26). Deficiency in the DNA MMR (dMMR) system is caused by the inactivation or dysfunction of one or more of the four key MMR genes (MSH2, MLH1, MSH6 and PMS2) resulting in insufficient correction of initially mismatched DNA base errors and subsequent accumulation of somatic mutations, most frequently in the repetitive DNA sequences

of the tumor genome, known as microsatellites (27). Typically, this leads to mutations in critical tumor-suppressor and cancer-related genes, which may promote tumor growth (27). The induced microsatellite instability (MSI) alters the length of microsatellites, which defines the MSI-positive status, and can be considered a marker of deficient MMR functioning (28). The hypermutated phenotype associated with dMMR/MSI may lead to the generation of neoantigens that can induce an anti-tumor immune response, rendering MSI-tumors more susceptible to treatment with immunotherapy (26, 28). In this regard, MSI-status has revolutionized the oncology field, being the first tumor-agnostic biomarker approved, and is routinely tested across tumor types, including CRC, as it gives access to treatment with checkpoint inhibitors (pembrolizumab and nivolumab) (23, 29). Importantly, in CRC, MSI status is stage dependent both in terms of prevalence and prognostic value. In early stages approximately 20% of CRC tumors (stage I and II) are MSI-positive where it associates with good prognosis, particularly in stage II patients (23, 30). In stage III and IV, however, fewer patients have MSI positive tumors (approximately 15% and 5%, respectively) showing a less clear link with survival outcomes particularly in the metastatic setting where a trend toward worse survival compared with MSS patients has been reported (23, 30).

Altogether, the currently used criteria for risk stratification still lack the desired clinical precision in accurately prognosticating patients and guide treatment selection. Further refinement is needed, preferentially identifying features that reflect more of the underlying biology beyond the tumor-intrinsic factors, which might prove to be a cornerstone for better defining risk and further personalize treatment. Such insights may also contribute to improved understanding of the natural history of the disease.

1.2 The double-edged sword of tumor-associated inflammation

Inflammation has for long been an accepted hallmark of cancer highlighting the fundamental role of the immune system for tumor evolution, progression and eventually disease outcome (31). Tumors arise in various tissue compartments where

neoplastic cells interact with of a multitude of cellular and molecular factors including stromal components such as fibroblasts, the extracellular matrix and tumor vasculature which together with the immune infiltrate comprises the tumor microenvironment (TME) (32). The extent, composition and functional state of the tumor immune landscape has emerged as a critical determinant for tumor growth and treatment response, ultimately affecting patient outcome (33). The tumor immune infiltrate comprises a heterogeneous population of both adaptive and innate immune cells and associates with either favorable or unfavorable outcomes (34). Specifically, a strong T-cell dominated immune infiltrate has been correlated with both favorable prognosis and successful responses to treatment with immune check-point inhibitors (35). More than a decade ago Galon et al. developed the Immunoscore, which categorizes tumors based on infiltration by CD3+ and CD8+ effector T-cells and/or CD45+ memory T-cells at the invasive margin and tumor center, which later was shown to be superior to the traditional TNM staging and MMR-status in predicting survival in CRC patients (36-38). This concept was soon expanded to other tumor types in order to find a unifying system for classifying the immune infiltrate of tumors and evaluate the prognostic role across malignancies (39). Based on this T-cell focused scoring system, a new classification emerged, designating tumors as “hot” or “cold” according to the degree of T cell infiltration (40, 41). This framework has later been refined to include four immune subgroups; hot, altered-excluded, altered-immunosuppressed, and cold, and has been applied in numerous publications, particularly in studies using immune check-point inhibitors, showing a positive correlation between T-cell inflamed tumors and treatment response (42-44).

However, accumulating evidence exists that other types of tumor infiltrating immune cells also play a pivotal role in shaping the TME and profoundly influence the tumor immune phenotype to either support or suppress tumor growth, ultimately affecting both treatment and survival outcomes in cancer patients (33, 45).

Contrary to the unequivocal favorable role of adaptive immune responses exerted primarily by tumor-specific cytotoxic T-lymphocytes, innate immune cells are not inherently either pro- or anti-tumoral (46). Importantly, innate immune cells exhibit

high plasticity meaning that they can polarize within a continuum of divergent phenotypes that either can exert predominantly tumor supportive or tumor restrictive functions depending on the state and cytokine profile of the TME (46, 47).

Myeloid cells are a major component of the innate immune system and have been linked to both favorable and unfavorable oncological outcomes (48). However, it is increasingly recognized that tumor infiltrating myeloid cells predominantly mediate immunosuppression within the TME with the capacity of supporting tumor growth and metastasis and inhibit effective antitumor immune responses (46, 49, 50). Extensive studies using high-throughput immune profiling and single-cell sequencing have shown that the immunosuppressive TME comprises a heterogeneous population of myeloid immune cells including macrophages, neutrophils and myeloid-derived suppressor cells (MDSCs) which are characterized based on their different phenotypes, functional roles, and surface markers (51).

Tumor-associated macrophages (TAMs) are probably one of the most studied innate immune cell types and constitute a significant component of the tumor immune infiltrate (50). TAMs are important drivers of cancer-associated inflammation as their functional phenotype shares many of the characteristics of the pro-tumorigenic alternatively activated M2 macrophages and lack the cytotoxicity associated with M1 macrophages, which constitute the two extremes of the phenotype spectrum initially described for macrophages (52). Specifically, TAMs may promote tumor progression through the release of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-alpha, stimulation of angiogenesis and promotion of endothelial-mesenchymal transition (EMT) in tumor cells, thus facilitating tumor growth and metastasis (46, 51).

Although less studied than TAMs, neutrophils have emerged as another major player in the TME. Being an essential part of the acute inflammatory response, these cells are rapidly recruited to inflamed tissue and play a vital role in the defense against invading pathogens and in wound healing processes (53). However, when infiltrating tumors, high densities of neutrophils have been associated with poor prognosis in many, but not all tumor types, besides being linked to impaired efficacy of oncological

treatments, particularly to immune checkpoint blockade (54, 55). The ambiguity related to tumor infiltrating neutrophils and prognosis could be explained by differences in tumor types and stages and the fact that neutrophils, similar to TAMs, can play both pro- and anti-tumoral roles in the TME (55). Although less characterized and more controversial than the polarization framework described for the macrophages, divergent functional phenotypes have also been proposed for tumor-associated neutrophiles (TANs) (54). Shaped by specific cues and features of the TME, this heterogeneous and dynamic population of cells can either support or inhibit tumor growth, depending on their polarization, activation, and maturation states (54, 56). Despite this potential duality, compelling evidence shows that TANs primarily exhibit an immunosuppressive phenotype and can directly promote tumor progression, angiogenesis, and metastasis (55, 57).

Taken together, there is a delicate balance in the TME influenced by multiple factors, both tumor-intrinsic and host/immune-related, that together with the surrounding tumor stroma, determine the evolution and type of immune response occurring at the tumor site. Improved understanding of this complexity both in terms of immune cell composition, diverse functional states, intratumoral crosstalk, and ultimately, how this affect patient outcomes, will be key to optimize treatment selection and effectively target the TME and may prove critical for the success of immunotherapy in a broader population of cancer patients. Furthermore, identification of distinct cancer immune phenotypes may require distinct therapeutic strategies, highlighting the need for biomarkers that reflect specific immunological features of the tumor. Such biomarkers may serve as valuable clinical tools to help select treatment strategies tailored to individual cancer patients enabling a more personalized treatment approach.

1.3 The prognostic role and clinical significance of systemic inflammation

In contrast to the described potential dualistic role of the local tumor immune response, the systemic inflammatory response (SIR) is more unequivocally associated with unfavorable outcomes across tumor types (58-61).

It has become widely accepted that the presence and magnitude of systemic inflammation can be determined using laboratory measures of circulating acute phase reactants such as C-reactive protein (CRP), serum albumin and platelets, subpopulations of white blood cells including neutrophils and lymphocytes (62). From these blood markers, composite scores and ratios have been established. Those most used are the Glasgow Prognostic Score/modified Glasgow Prognostic Score (GPS/mGPS, composite scores based on elevated serum CRP and low serum albumin), neutrophil lymphocyte ratio (NLR, calculated by dividing absolute neutrophil count by the lymphocyte count) and platelet lymphocyte ratio (PLR, dividing platelet count by lymphocyte count) (62, 63). More recent studies have also assessed circulating cytokines such as IL-6 and TNF-alpha and some chemokines and growth factors as indices of systemic inflammation (64, 65). Over the past two decades several studies have investigated the prognostic value of markers of SIR covering a large and heterogeneous population of patients with various tumor types including colon, breast, lung, bladder and kidney cancer in early and advanced stages, with and without oncological treatment, and consistently reported a negative impact on survival outcomes (both overall and cancer-specific survival) (58, 59, 66-68). Importantly, although systemic inflammation, measured by GPS, NLR and CRP, correlate with more advanced and aggressive disease, the impact on survival outcomes have been shown to be independent of commonly assessed clinicopathological factors (64, 65).

In CRC, there has been considerable interest in identifying biomarkers to better stratify patients given that CRC is a highly heterogeneous disease and the currently used tumor-based criteria are insufficient in accurately distinguishing high- and low-risk subgroups, particularly in early-stage, and thus select the most appropriate treatment strategy whether it means more or less treatment (23, 69). Together with the growing appreciation of the critical role of the host immune response for tumor progression and survival outcomes, various surrogates of SIR have been evaluated as potential biomarkers that can add valuable information to the TNM-based staging for improved treatment and ultimately survival outcomes in CRC patients (62, 69, 70). The evidence for a prognostic role of the systemic inflammatory response is particularly strong in

early-stage patients undergoing curative surgery and has been most extensively examined using CRP and GPS (61, 64). A meta-analysis encompassing 21 studies and over 3000 resectable CRC patients evaluated the prognostic value of preoperatively measured CRP and GPS/mGPS using the multivariate-adjusted hazard ratios (HRs) of the individual studies in the pooled analysis to minimize the confounding effect from other prognostic factors such as TNM stage, age, gender and adjuvant treatment (71). Both elevated CRP and increased GPS were significantly associated with compromised survival in stage I-III patients with pooled HRs for overall survival (OS) and disease-specific survival (DSS) of respectively 2.04 (95 % CI 1.45–2.86) and 4.37 (95 % CI 2.63–7.27) for studies reporting on CRP and 2.20 (95 % CI 1.61–3.02) and 1.80 (95 % CI 1.37–2.37) for studies using GPS (71). Concerning more advanced disease, the metanalysis included 9 studies covering 1150 stage IV CRC patients undergoing resection, primarily of liver metastasis, and found a significant association between elevated preoperative CRP and GPS and poor survival outcomes (HRs for OS of 3.65 (95 % CI 2.07–6.44) for CRP and 2.70 (95 % CI 1.88–3.89) for GPS/mGPS), supporting a prognostic role of systemic inflammation in all stages of CRC (71). This was further confirmed in a Nordic study of 525 colon cancer patients where elevated CRP was an independent, strong predictor of reduced disease-specific survival in all stages of operable CC (72). Notably, the risk of death from colon cancer increased with incremental levels of CRP, particularly pronounced in early stage where patients presenting with serum CRP above 60 mg/L were over 7 times more likely to die from CC compared to patients with CRP below 10 mg/L (HR 7.37 (95 % CI 2.65–20.5) for stage I and II patients combined) (72).

Although less extensively studied than resectable disease, a couple of studies have focused on the prognostic role of SIR in metastatic disease (73, 74). Thomsen et al. examined the prognostic impact of different SIR markers with particular focus on CRP and IL-6, in a cohort of 393 metastatic CRC patients receiving first line treatment with 5 FU-based chemotherapy with or without cetuximab (74). All SIR markers were significantly associated with poor survival in terms of compromised progression-free survival (PFS) and OS regardless of treatment arm and independent of RAS/BRAF mutational status.

From a clinical point of view, assessment of SIR may provide important information beside the prognostic significance discussed above. Typical cancer-related symptoms, including fatigue, pain, anorexia, weight loss and poor performance status have been linked to systemic inflammation (63, 75). Of note, one study showed worsening of symptoms and patient-reported outcomes with increasing levels of CRP, supporting a clinically significant relationship between systemic inflammation and cancer-related symptoms (75). The most extreme end of these symptoms forms the cancer cachexia syndrome that most commonly affects advanced cancer patients and associates with poor survival and reduced quality of life (63). Cancer cachexia is characterized by fatigue, loss of skeletal muscle and anorexia and is believed to be cytokine driven based on observations in animal models of sickness behavior indicating a pivotal role exerted by pro-inflammatory cytokines, particularly IL-6, for the development of this syndrome (75, 76). Importantly, IL-6 has been proposed to play a key role in many of the inflammatory and metabolic effects related to systemic inflammation, besides its well-known function as the main inducer of hepatic CRP production (65, 77). Acknowledging this, assessment of CRP has now been incorporated into the definition of cancer cachexia (78). The notion that such cancer symptoms rarely exist individually but usually occur simultaneously, often forming clusters, further supports the hypothesis that systemic inflammation not only associates with multiple cancer-associated symptoms, but also shares the same biological mechanisms (63). Given this concept, the next apparent question is whether such symptoms can be ameliorated by treatment with anti-inflammatory drugs and lead to improved quality and potential quantity of life in advanced cancer patients. Indeed, the effect of corticosteroids on improving cancer-associated symptoms, at least short term, is well-known among oncologists and in palliative care (63). However, steroids are often prescribed rather broadly to progressive cancer patients and less frequently specifically tailored to the systemic cancer-related inflammatory response (63). The many metabolic side effects related to such treatment, do not make this drug suitable for long-term therapy, which is an important consideration given the chronic nature of systemic inflammation (63). NSAIDs have also been proposed within this setting, particularly after studies have indicated a potential preventive effect, particularly for aspirin, on CRC development

(63, 76, 79). However, some negative studies also exist and there remain uncertainties regarding the true benefits of NSAIDs in CRC as well as in other tumor types (80, 81). Although the exact role of such broad, non-specific anti-inflammatory strategies remains to be established, symptom management in systemically inflamed patients, preferentially using compounds that more specifically target key inflammatory pathways, most likely in conjunction with other anti-cancer treatments, represent an attractive approach potentially improving both survival outcomes and quality of life in a large proportion of cancer patients (63).

Another clinically relevant aspect of systemic inflammation is the interference with drug metabolism and clearance that might influence efficacy and toxicity of oncological treatments (63, 77). Specifically, it has been shown that systemic inflammation affects hepatic cytochrome 450 enzymes (CYP), particularly CYP3A4, which handle the metabolism of multiple anti-cancer drugs including the widely used chemotherapeutics taxans, irinotecan, etoposide and cyclophosphamide as well as several small-molecule inhibitors (77). Experimental studies have shown that inflammatory mediators such as IL-6 and TNF-alpha can downregulate mRNA transcription and protein expression of several hepatic CYP-enzymes, leading to alterations in drug metabolism in mouse models (77). Translated into the clinic, a prospective study in advanced cancer patients found that systemically inflamed patients, as evidenced by high serum CRP values, had decreased activity of CYP3A4, which resulted in reduced efficacy and increased toxicity of the anti-cancer treatment (76). Although further large-scale studies are needed to determine the precise pharmacokinetic changes related to systemic inflammation and, importantly, how these might affect outcome and toxicity in cancer patients, the existing data highlight the clinical significance and utility of identifying and acknowledging systemic inflammation and add to the rationale for developing therapeutic strategies targeting specific inflammatory pathways involved in the detrimental inflammatory response and improve patient outcomes.

Finally, with the increased interest and appreciation of the host`s immune response, both beneficial and detrimental, for treatment and survival outcomes in cancer patients,

several studies have investigated the potential predictive value of markers of SIR (59, 82-84). Following treatment with chemotherapy, CRP, mGPS and NLR have all shown significant predictive value in multiple cancer types including breast, lung and GI-cancers, in both adjuvant and metastatic setting (77, 85, 86). With the rapidly evolving field of immunotherapy and significant interest in identifying predictive biomarkers, more recent studies have investigated the utility of SIR markers in this setting. NLR is the most extensively studied marker (82). High NLR at baseline has been associated with poor PFS and OS upon treatment with ICI in patients with various types of advanced cancer independent of other patient- and tumor-related factors (87). Additionally, early decline in NLR (after 8 weeks of treatment) correlated significantly with response and survival outcomes within two separate studies in renal and lung cancer patients treated with nivolumab and atezolizumab, respectively (82). CRP and IL-6 have also been proposed as potential predictive biomarkers for treatment outcomes of ICI (88, 89). In a study by Laino et al. elevated serum levels of CRP and IL-6 were significantly associated with compromised PFS and OS within a large cohort of melanoma patients included in three separate randomized trials receiving treatment with either single (nivolumab) or double (nivolumab in combination with ipilimumab) immune checkpoint blockade (90). Intriguingly, experimental data related to this study found that CRP itself could inhibit early T-cell activation and optimal effector function in a dose-dependent manner, indicating that CRP might have direct immunosuppressive capabilities (88). Similar to NLR, dynamics in CRP levels during treatment with ICI have also showed predictive value. Specifically, early rise as well as early decline in serum CRP, defined as eight weeks after initiation of treatment, were predictive of progression and response, respectively, in a study of metastatic lung cancer patients treated with a PD-1/PD-L1 inhibitor (83).

Despite the strong scientific evidence for a prognostic and clinically significant role of cancer-associated systemic inflammation, less is known about the underlying biology. A hallmark of systemic inflammation is elevated levels of circulating acute phase reactants, innate immune cells, and pro-inflammatory cytokines, which together mediate the multifaceted effects of SIR (23, 65, 76). Experimental and clinical studies

have highlighted IL-6, TNF-alpha and IL-8 as key players involved in the many inflammatory processes portraying systemic inflammation (77). Moreover, the sustained alterations in circulating immune cells with increased levels of myeloid cells, particularly neutrophils and monocytes, together with other components of the innate immune system, have been associated with skewing of the host`s immunological state causing down-regulation of adaptive immunity and presumably a more tumor-permissive state with reduced ability to generate effective anti-tumor responses (64, 76, 91). However, the precise biological mechanisms involved are complex and remain far from understood (64). Despite the solid evidence for a prognostic role of both local and systemic tumor immune responses, the relationship between them has received limited attention (64). Specifically, the inflammatory response occurring at the tumor site has been evaluated and interpreted separately from the systemic inflammatory response. However, given that many of the same inflammatory mediators are involved, and that these are recruited to the tumor from the peripheral circulation, and reciprocally, that the tumor produces cytokines and growth factors that enters the circulation, there is a continuous crosstalk between the two inflammatory processes (illustrated in **Figure 1**) (91). Yet, exactly how systemic inflammation impacts the TME and, further, how these inflammatory processes might sustain and influence each other, and ultimately affect patient outcomes, remains to be investigated. While assessment of SIR still primarily is appreciated for its prognostic value, a deeper understanding of the tumor biology behind may provide the clinicians with an easily accessible, yet even more informative tool, that may facilitate bedside decision-making and potentially reveal novel therapeutic targets.

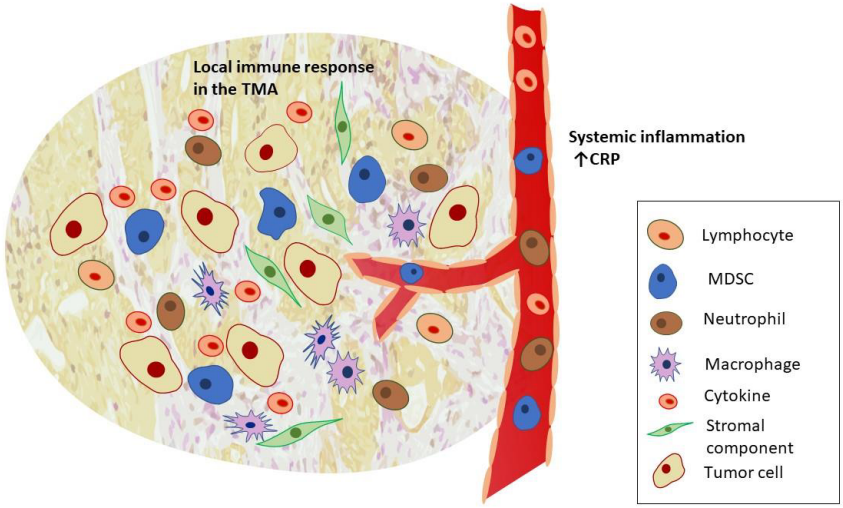

Figure 1 Linking local and systemic tumor-associated inflammation

Simplistic illustration of the bidirectional crosstalk between systemic and local tumor immune responses during tumor development. Immune cells and mediators recruited from the peripheral circulation can traffic into the tumor and contribute to the local immune response in the TME. Likewise, tumor-derived factors and cytokines are released into the systemic circulation and can have distant effects.

TMA: Tumor microenvironment. MDSC: Myeloid-derived suppressor cell. CRP: C-reactive protein

1.4 C-reactive protein (CRP) as a biomarker and regulator of inflammatory processes

CRP is the prototype of an acute phase inflammatory protein (92). This highly evolutionary conserved protein belongs to the pentraxin family and plays a vital role as a regulator of innate immune responses (93). Despite the fundamental nature of CRP given its existence throughout species and lack of any known genetic deficiencies, the exact role of CRP in humans has remained elusive and controversial for decades (94). However, within recent years, knowledge has improved and there is now compelling evidence that CRP exists in structurally different isoforms with distinct and even opposing biological properties (95).

In clinical practice, CRP is a well-established maker of inflammation and disease activity in a wide range of pathological conditions, including infections and other

inflammatory diseases, trauma, and tissue damage and, as discussed above, also in cancer (96, 97). Moreover, elevated serum CRP is an independent and strong predictor of future cardiovascular events and has been implemented in the global cardiovascular risk assessment of asymptomatic individuals (93).

As a part of the acute phase response (APR), CRP serum levels increase rapidly (within 6-72 hours) in response to an inflammatory stimulus, with levels up to 100- and even 1000-fold following some bacterial infections (95, 96). When the stimuli for synthesis subside, the level of circulating CRP quickly decline, usually over 18-20 hours, corresponding to the half-life of the protein (95). As such, the kinetics of the CRP response together with the rapid, sensitive, and inexpensive assays for its measurement makes CRP a useful and readily available diagnostic marker and monitor of inflammation. Moreover, CRP levels correspond to the intensity of the inflammatory response and/or tissue damage and is therefore also used as a diagnostic tool for determining disease severity in several infectious and non-infectious conditions (73). Notably, high CRP levels have consistently been reported to correlate with poor prognosis regardless of the pathology involved (96).

The evidence and recommendations regarding the use of CRP as a biomarker in any disease involving an inflammatory response including cancer, are primarily reported, and defined for CRP concentrations ≥ 10 mg/l, termed conventional CRP (97). The value and clinical significance of high sensitivity CRP (hsCRP) defined as blood levels of 1-10 mg/l, however, is currently more debated although accumulating studies have appeared reporting an increased risk of development of several diseases including cancer with even slightly elevated hsCRP (97). In this regard it should be mentioned that several physiological and lifestyle related factors such as age, gender, genetic polymorphism, obesity, fitness and hormone replacement therapy have been shown to influence baseline hsCRP levels (95). Indeed, more studies are needed that specifically address the utility of hsCRP as a diagnostic tool and for risk stratification in both assumingly healthy individuals and in already diseased populations.

Although it has been generally accepted that the primary function of CRP within the APR and non-specific innate immune responses appears to be activation of the complement cascade and opsonization of pathogens, the multifaceted biological roles of CRP in both acute and chronic conditions have been debated for decades as both pro- and anti-inflammatory effects were reported (93, 96). However, within recent years it has become evident that CRP occurs in at least two structurally and antigenically distinct isoforms with different and even opposing biological functions, and further, that a transformational switch with both structural and functional change of the molecule occur locally at sites of inflammation (94, 96).

Circulating CRP is a highly soluble, pentameric molecule (pCRP) composed of 5 identical subunits named monomeric or modified CRP (mCRP) arranged in a cyclic structure (92, 95). The liver hepatocytes are the main synthesizers of CRP although extrahepatic production in smooth muscle cells, endothelial cells, adipocytes, and macrophages have been reported (95). IL-6 is the principal inducer of CRP production, yet other pro-inflammatory cytokines such as IL-1 and TNF-alpha may also contribute (95). Under various experimental (high temperature, presence of urea, depletion of calcium, lipopolysaccharide contamination) and physiological conditions it has been shown that pCRP can irreversibly dissociate into its free subunits (mCRP) (95). In vivo, pCRP interact with phosphocholine (PC), a major component of cell membranes defined as the principal ligand for pCRP, leading to dissociation of the pentameric isoform into the modified, monomeric form (96). However, for PC ligands to become accessible for pCRP binding, remodeling of the plasma membrane is required, which occurs when cells become activated or disturbed, either due to infection, tissue damage or other stimuli, and most often involves activity of the enzyme Phospholipase A2 (PLA2) (96). The conformationally changed mCRP expresses a cholesterol binding site enabling mCRP to enter cholesterol rich lipid rafts, that are microdomains within cell membranes important for cell signaling processes (97). Unlike the highly serum-soluble pCRP, mCRP has low aqueous solubility and a tendency to partner up with cells or particles or accumulate within tissues (97). Importantly, it has been shown that internalization of mCRP into plasma membranes activates pro-inflammatory pathways

such as the transcription factor NFkB which is considered essential for inflammatory responses (98, 99). pCRP, on the other hand, has weak anti-inflammatory properties mainly by activating the classical complement pathway and mediate opsonization of pathogens (93). As such, mCRP becomes the active mediator and the "true" acute phase reactant responsible for the pro-inflammatory bioactivity of CRP in inflammatory environments (96). **Figure 2** illustrates the pCRP/mCRP dissociation process and known ligands for mCRP interaction.

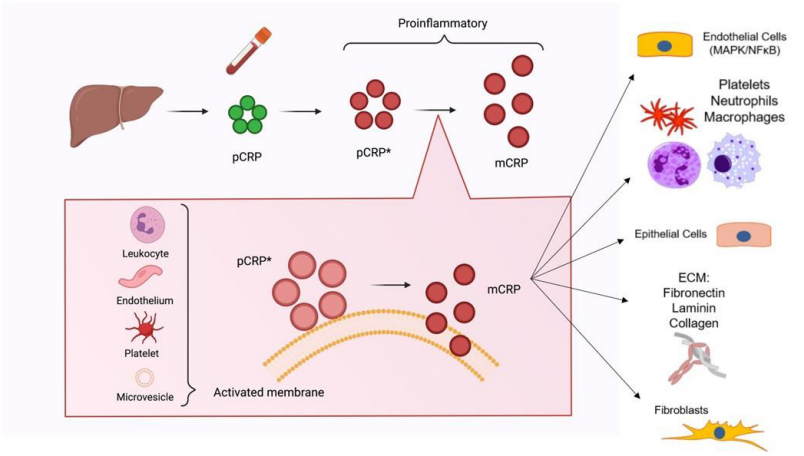

Figure 2 Schematic presentation of the CRP dissociation process and potential interactions of mCRP
Pentameric CRP (pCRP) is secreted from hepatocytes in response to inflammatory stimuli and can be quantified in the systemic circulation and used for diagnostic testing. In inflammatory environments, such as the tumor, pCRP interacts with phosphocholine (PC) on activated cell membranes and undergoes conformational rearrangement first to an intermediate form (pCRP*) and then to the monomeric isoforms (mCRP). mCRP has low aqueous solubility and accumulates within tissues or partners with microparticles. mCRP can exert its pro-inflammatory effects through direct interaction with several different cell types including endothelial and epithelial cells, platelets, various immune cells and components of the extracellular matrix (ECM)

In contrast to previous studies investigating the biological functions of CRP, more recent publications distinguish between the two isoforms, addressing their effects on cell behavior and in host responses separately (96).

Evidence generated primarily in cardiovascular and neurogenerative disorders have shown that mCRP can interact directly with several different cell types including endothelial cells, various immune cells such as macrophages and neutrophils as well as components of the extracellular matrix (ECM) (97). Intriguingly, mCRP has been detected within arteriosclerotic plaques both in the myocardium and the in the brain of infarcted patients where it interacts with endothelial cells, macrophages and oxidized lipoprotein (LDL) leading to an amplified inflammatory reaction and aggravation of the localized tissue injury (100). Based on these data mCRP has been suggested as a direct player involved in the pathogenesis of cardiovascular and neurological diseases, which due to its strong pro-inflammatory properties contributes to excessive inflammation and aggravation of the tissue injury negatively affecting patient outcomes (92, 100).

By recognizing that CRP is a dynamic molecule undergoing conformational changes at sites of inflammation transforming to a highly biologically active form capable of exerting direct pro-inflammatory effects within tissues, a new perspective on CRP as a biomarker emerges. Taken into the context of cancer, particularly in patients with persistently elevated levels of circulating CRP, diagnostically interpreted as systemically inflamed, it may be hypothesized that CRP itself, in its monomeric form, may play a direct pro-inflammatory role in the microenvironment of tumors. However, given that most research on the different isoforms of CRP to date has been carried out in other inflammation linked diseases, this hypothesis represents an intriguing concept that needs to be specifically addressed in studies analyzing tumor tissue and in the clinical setting of cancer patients.

2. Aim and Hypothesis

Aim

The overarching aim of this work is to improve our understanding of why tumor-associated systemic inflammation is so detrimental for patient outcome.

Aim 1: To evaluate the prognostic impact of CRP compared with other inflammatory markers and clinicopathological characteristics in stage IV colorectal cancer patients undergoing resection of liver metastases.

Aim 2: To develop a FFPE tissue-based platform combining multiplex chromogenic IHC and digital whole-slide imaging for quantifying and mapping adaptive and innate immune cell populations in the TME of colon cancer patients.

Aim 3: To explore and compare the tumor immune microenvironment in primary resected colon cancer patients with and without evidence of systemic inflammation as assessed by levels of circulating CRP.

Aim 4: To investigate whether the monomeric form of CRP (mCRP) is expressed in tumors from primary resected colon cancer patients.

Hypotheses

We hypothesize that CRP is a strong negative prognostic marker in CRC patients with liver metastases undergoing potentially curative liver surgery.

We hypothesize that systemic tumor-associated inflammation, as assessed by elevated levels of serum CRP, correlates with a myeloid-dominated TME in primary resected colon cancer patients.

We hypothesize that the pro-inflammatory monomeric isoform of CRP is expressed in tumors from systemically inflamed colon cancer patients where it potentially interacts directly with components of the TME.

3. Material and methods

3.1 Study design and patient populations

Paper 1: The prognostic role of systemic inflammation in patients undergoing resection of colorectal liver metastases: C-reactive protein (CRP) is a strong negative prognostic biomarker

The first paper is based on data from a study conducted in a Nordic cohort encompassing 492 stage IV colorectal cancer patients with liver metastases (CRLM) undergoing potentially curative liver surgery between 1999 and 2009 at 3 institutions in Norway (Sørlandet Hospital), Sweden (Karolinska University Hospital), and Finland (Helsinki University Hospital), respectively. Consecutively treated patients with histologically verified CRC with liver metastases only, who underwent macroscopically radical hepatectomy, and had available follow-up data were considered eligible for inclusion in the study. Clinical and histopathological information and data on survival status were retrospectively retrieved from local databases, national registers, and patient records, and gathered in a database generated for the actual study. To avoid influence of other conditions potentially affecting the measurement of the systemic inflammatory response (SIR), patients were not eligible for inclusion in the study if they had a history of autoimmune or other inflammatory diseases and/or had been treated with steroids or presented with clinical evidence of an infection (positive x-ray, urine analysis or blood cultures) the week prior to blood sampling. Patients diagnosed with another type of malignancy, including cancer of the appendix within three years prior to surgery, were also excluded from the study.

Preoperative serum CRP and albumin levels were obtained from routine blood samples taken within 20 days prior to surgery. Patients were allocated into groups according to the level of CRP. First, two groups were established based on CRP cutoff level of 10 mg/L and next, into three groups using the same thresholds as reported previously (72); CRP ≤10 mg/L, CRP 11-30 mg/L and CRP >30 mg/L. GPS was calculated as per previously defined criteria (58); patients with serum CRP ≤10 mg/L and s-albumin ≥35 mg/L were allocated a score of 0, patients with either serum CRP ≥10 mg/L or s-

albumin ≤35 mg/L were allocated a score of 1 and patients with both hypoalbuminemia (s-albumin ≤35 mg/L) and elevated CRP (serum CRP ≥10 mg/L) were allocated a score of 2.

The study was approved by the regional Ethics Committees in the respective countries without the need for written informed consent based on the retrospective, registry-based nature of the study with no experimental interventions or direct consequences for the individual patients or caregivers.

Paper 2 Systemic Inflammation Associates With a Myeloid Inflamed Tumor Microenvironment in Primary Resected Colon Cancer-May Cold Tumors Simply Be Too Hot? and
Paper 3 Fueling the flames of colon cancer - does CRP play a direct pro-inflammatory role?

Paper 2 and 3 are based upon datasets generated from the same patient cohort comprising 43 stage II and III colon cancer patients undergoing curative resection of their primary tumors at Sørlandet Hospital, Kristiansand, Norway between 2005 and 2015. Patients were selected from a local CRC database covering comprehensive, prospectively collected demographic, histopathological, and clinical information, including oncological treatment and selected laboratory values and follow-up data. The database is maintained and managed by a dedicated research nurse with supervision from a senior oncologist.

Eligibility criteria for the study cohort included: available formalin-fixed paraffin-embedded (FFPE) tumor tissue archived at the Department of Pathology, Sørlandet Hospital, preoperative CRP values and surgery as an elective (not emergency) procedure and complete follow-up data. Patients with a history of autoimmune or other chronic inflammatory diseases and/or had received treatment with antibiotics or immunosuppressive drugs or presented with clinical evidence of an infection within 4 weeks prior to the resection were excluded from the study cohort.

A trained pathologist selected applicable tumor blocks with representative areas of both the tumor invasive margin and tumor center. Information on clinicopathological

characteristics (except for MMR status which was determined as described in a separate section), oncological treatment, CRP values, and survival outcomes were obtained from the CRC database.

CRP values from routine blood samples taken within 14 days prior to elective surgery were applied in order to reflect a state of systemic inflammation. Only one CRP value per patient was recorded. In the case of several measurements, the CRP value taken at the day closest to the resection was used in the analyses.

In total 23 patients with serum CRP ≥30 mg/L (CRP-high patients) and 20 patients with CRP 0-1 mg/L (CRP-low patients) were included in the study cohort. However, for the second paper only 36 of the patients (15 in the CRP-low group and 21 in the CRP-high group) were included in the analyses due to technical and methodological challenges with the multiplex IHC (described separately in the section on IHC).

Follow-up time: For the second paper, median follow-up time was 7.2 years in CRP-low patients and 7.3 years in CRP-high patients. In the third paper, the follow-up time was extended to 9.3 years in CRP-low patients and 8.8 years in CRP-high patients.

The studies were conducted under approval of the regional Norwegian Ethics Committee. According to the decision of the Ethics Committee all patients, or close relatives to patients that were no longer alive, were informed about the study and the use of tumor specimen, with the right to reserve from participating, although written informed consent was not required.

Considerations regarding study design and patient populations

The retrospective cohort design of the first study confers some inherent limitations. Thus, our results can only provide an association between preoperatively elevated CRP and compromised survival following liver surgery in CRLM patients but cannot determine a direct causal relationship. Due to the retrospective collection of data, comprehensive laboratory profiles were not available in all patients. In particular, data on neutrophil, lymphocyte and platelet counts were incomplete, which made us unable to include NLR and PLR in the analysis comparing the prognostic impact of different

SIR markers. Nevertheless, preoperative CRP was available in 427 out of 492 patients, and s-albumin in 450 patients, enabling evaluation of CRP alone and combined with albumin in the GPS score. However, patients were not equally distributed between CRP groups as most of the patients (n=368) belonged to the CRP low group (≤10 g/L), whereas 59 patients had CRP values >10 g/L. Of these, only 15 patients presented with CRP >30 g/L, which should be taken into consideration when interpreting the study results.

The first study was a multicenter study, using data from consecutively treated patients, which can be considered a strength, as it enabled us to examine a large patient population representative of real-world practices within the Nordic countries. However, there might have been differences in clinical and surgical procedures, including selecting patients eligible for surgery and the use of neoadjuvant/adjuvant chemotherapy, between the countries, that might have impacted the results. Additionally, differences in the collection and management of data between institutions might also represent a potential bias. Moreover, it should be noted that 1/3 of the patients had primary tumors of the rectum, which previously have been shown to be less systemically inflamed (101) (confirmed in our cohort) and might represent a different disease entity when it comes to the inflammatory response and thus contributed to a less homogeneous patient population.

Finally, despite performing multivariate analysis stratifying for selected risk factors affecting survival outcomes in CRLM patients, there might have been other unknown confounders, such as underlying comorbidities, that were not accounted for and may have influenced the survival analysis. Importantly, we used overall survival (OS) and not disease-specific survival (CSS) as the primary endpoint, which did not allow us to evaluate the correlation between systemic inflammation and risk of CRC death solely. However, in the setting of metastatic disease, it is conceivable that most of the patients die from their cancer and not from other pathologies, which may justify the use of OS and not DSS in this study.

Paper two and three are both proof-of-concept studies performed to explore hypotheses and develop a methodological platform to be used in further studies. Hence, similar to paper one, no finite conclusions can be drawn regarding causal relationships, although such studies may represent an important first step to elucidate new concepts or formulate hypotheses to be tested in larger and preferably prospective studies.

Adherent to the proof-of-concept/pilot study design of paper two and three, the sample size was limited, making statistical analyzes less robust (discussed separately in the statistics section). Moreover, regarding the study cohort, there are differences in certain clinicopathological characteristics between CRP-high and CRP-low patients, such as tumor stage and MSS/MSI status, which should be taken into consideration when interpreting the results. As our primary aim was to capture potential biological differences between patients with and without systemic inflammation, the CRP value was the main criteria for patient selection. Consistent with this we included patients representing the opposite ends of the CRP scale (below 10 mg/L and above 30 mg/L) although this resulted in significantly more patients with stage III disease in the CRP-low than in the CRP-high group. Regarding imbalances in MSS/MSI-status between the two CRP groups, we did not have a-priori information on this as MSI-testing was part of the study analyzes. Nevertheless, despite these potential major biases, the survival analysis showed increased risk of recurrence or death in CRP-high patients although significantly more patients in this group had stage III disease and MSI-positive tumors, which would be expected to positively affect prognosis.

3.2 Tumor Samples

Routinely archived formalin-fixed paraffin-embedded (FFPE) tumor blocks retrieved from the Department of Pathology at Sørlandet Hospital, Kristiansand, Norway, were used in the studies. A trained pathologist from the local pathology department selected representative tumor blocks that included areas of both the tumor invasive margin (IM) and center of the tumor (TC). The selected tumor blocks were then sent to the Department of Pathology, Aarhus University Hospital, Aarhus, Denmark, where all tissue-related analyzes of this work were performed.

Building on previous work in the field by Galon and co-workers, demonstrating improved accuracy of prognostication utilizing the Immunoscore, which combines the score of tumor infiltrating lymphocytes (TILs) at the invasive margin (IM) and tumor center (TC) (36), we applied the same approach in our work although analyzing a broader population of immune cells in the respective tumor regions. For this purpose, whole tissue sections were stained with hematoxylin, scanned (Hamamatsu, Japan) and imported into the software program used for digital analysis (Visiopharm, Denmark). Tumor regions were then manually annotated within the software by an experienced pathologist together with the candidate on the hematoxylin and eosin (HE) stained whole slide, as depicted in **Figure 3**. The IM was defined as the junctional area where the tumor edge invaded into adjacent healthy tissue.

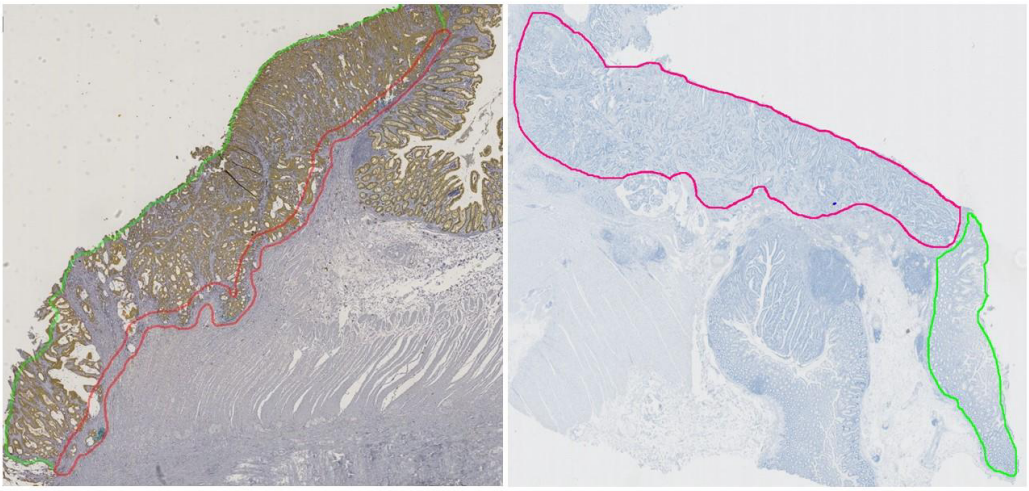

Figure 3 Tumor regions on HE-stained whole slides from colon cancer patients

Left: Digital scan of a colon tumor with manually annotated tumor regions utilized in the multiplex IHC protocol (paper 2). Invasive margin in red and tumor center in green.

Right: Whole slide image with manually annotated tissue regions utilized in the mCRP protocol (paper 3). Tumor tissue in red and adjacent normal colon mucosa in green.

Methodological considerations

As our primary aim with this work was to explore and compare the tumor immune contexture, that is type, density, and location of immune cells, in CRP-high (≥30 mg/L) and CRP-low (<10 mg/L) patients, we preferred whole tissue sections over tissue microarrays (TMAs). While TMA is a valuable tool in large-scale biomarker screening studies, the small TMA core biopsies might not be representative of the entire tumor, and thus less helpful for more comprehensive characterization of tumors. Particularly, information about tumor and biomarker heterogeneity, which often is the case in many solid tumors, or cell populations/molecules of low abundance, might not be captured by TMA-based approaches (102). Indeed, in our material, we experienced that tumors exhibited considerable heterogeneity as several of the stained immune markers were unevenly distributed throughout the tumor. Hence, the use of whole tissue imaging captured this heterogeneity, enabling a more comprehensive approach and allowed us to accurately visualize and quantify various immune cell populations and spatial patterns with preserved tissue architecture in different regions (TC and IM) and tissue compartments (tumor stroma and intratumorally) of the tumor.

Because of considerable variability in the size and extent of individual tumors and tumor regions, we annotated the IM and TC manually without applying a predefined measurement/computer-assisted tool for determining the regions (particularly relevant for IM). Although time consuming and subjective, this was manageable given the limited sample size and pilot study design. However, in larger studies, a more automated approach would be preferred allowing for a more efficient workflow and, most importantly, to ensure a standardized and reproducible protocol.

3.3 Immunohistochemistry (IHC)

Immunohistochemistry is the primary method used in this work. For the second paper we developed a FFPE tissue based chromogenic multiplex IHC (mIHC) platform enabling simultaneous visualization of selected adaptive and innate immune cells in the microenvironment of colon cancer tumors with preserved tissue morphology. Combined with digital image analysis, this method allowed us to quantify and map

various immune cell populations, including spatial patterns in the TME supporting our main purpose of exploring the tumor immune landscape of CRP-high and CRP-low colon cancer patients.

For the third paper, we mainly relied on single and double chromogenic IHC supporting our primary aim of investigating whether the monomeric form of CRP (mCRP) was expressed in the TME of our cohort of colon cancer patients. Using single IHC and automated image analysis we could identify and map the pattern of mCRP distribution within the primary tumors. We further extended the application by IHC-based colocalization imaging techniques (double chromogenic IHC and double immunofluorescence) to elucidate potential functional roles of mCRP in the TME of CC patients.

Multiplex IHC protocol

All mIHC staining procedures were performed on the Ventana Discovery Ultra autostainer (Roche Diagnostics International AG, Switzerland) using commercially available antibodies. Antigen retrieval and blocking of endogenous peroxidase activity were performed prior to IHC staining.

The mIHC protocol comprised multiple chromogenic stains applied sequentially on two consecutive tissue sections. For the first tissue section we developed a 5-plex (termed the adaptive/lymphoid immune panel) consisting of primary antibodies targeted against the following lymphoid markers CD8 (cytotoxic T lymphocytes), CD4 (T-helper cells), foxp3 (regulatory T cells), CD20 (B lymphocytes) besides pan-cytokeratin (pan-CK) as a tumor marker. For the second tissue section we developed a 4-plex (termed the innate/myeloid immune panel) comprising antibodies against the two myeloid markers CD68 (pan-macrophage marker) and CD66b (neutrophils) in addition to the immune checkpoint molecule PD-L1 and pan-CK as a tumor marker. Representative image of tumor tissue stained with the two mIHC panels is shown in **Figure 4**.

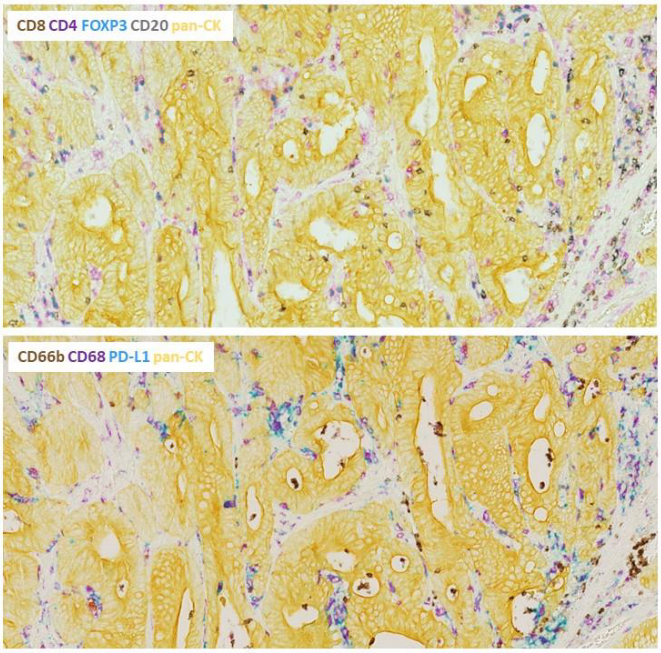

Figure 4 Representative images of two serial tumor sections stained with the adaptive/lymphoid immune panel (CD8+ T cells, CD4+ T cells, foxp3CD4+ regulatory T cells, CD20+ B cells and pan-cytokeratin) and the innate/myeloid immune panel (CD66b+ neutrophils, CD68+ macrophages, PD-L1 and pan-cytokeratin)

After accomplishing the multiplex procedures, tumor sections stained with the innate multiplex panel were counterstained with hematoxylin for better visualization of the tissue architecture and cell morphology (identification of nuclei). Schematic overview of the mIHC workflow and digital image analysis is presented in **Figure 5**.

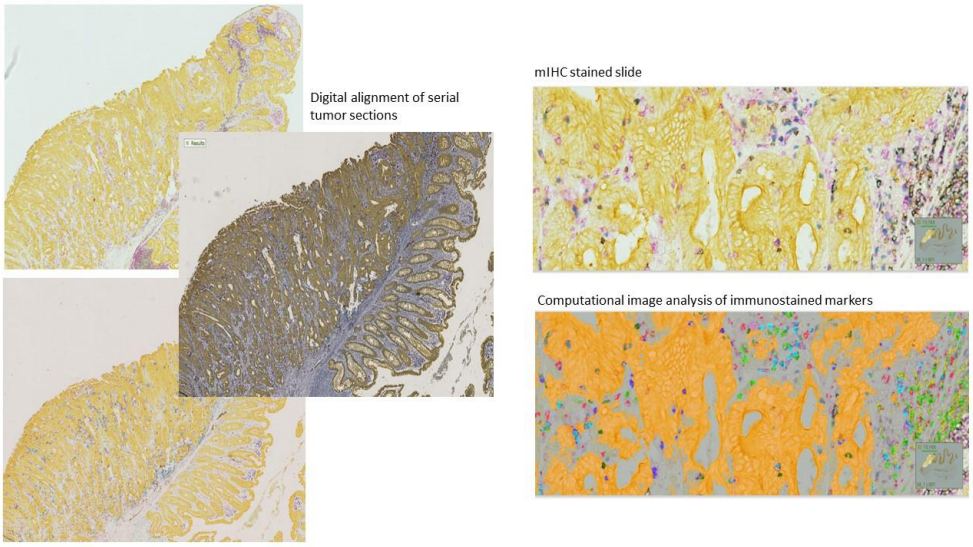

Figure 5 Schematic overview of the mIHC workflow and computational image analysis of colon cancer tissue

Left: Digital scans representing two serial FFPE tissue sections stained with a 5-plex lymphoid and a 4-plex myeloid biomarker panel. Stained whole slides were digitally aligned together with a HE-counterstained stained slide (applied to the myeloid stained slide following multiplexing and scanning)

Right: High resolution image of the lymphoid stained slide followed by computational image analysis. Immunostained markers were visualized through pseudo-coloring

To assure adequate staining quality of the multiplex three forms of controls were performed: 1) single chromogenic IHC stainings with all antibodies included in the multiplex panels to compare the staining intensity of the multiplex and to check for cross-reactivity or loss of antigenicity because of the multiplex procedure, 2) positive control was performed by mounting a single piece of normal tonsil tissue on each slide (encompassing lympho-epithelial structures with cells positive for all markers included in the multiplex panels), and 3) mIHC staining of tissue from lung adenocarcinoma for validation of the assay and grading of PD-L1 expression.

After accomplishing the mIHC workflow, stained tumor sections were scanned and digitally aligned and analyzed, enabling a virtual, multiparameter readout of seven

simultaneously expressed lymphoid and myeloid immune markers within the same tumor area.

Methodological considerations:

The development of the multiplex IHC assay was a relatively labor-intense and complex process. Going from single and double immunostaining to 4- and 5-multiplexing demanded multiple rounds of testing before we arrived at a combination of antibodies that yielded an accurate (the desired specificity and sensitivity) and reproducible assay. One challenge using chromogenic IHC, is the limited number of available chromogens. Some of the chromogenic dyes can be more difficult to work with than others, as some are more prone to the heating and washing steps that follow the multiplex protocol where several antibodies are applied sequentially on a single slide. We experienced this and put a lot of effort into finding the most optimal order of applying the antibodies to ensure adequate antigen signal preservation despite additional immunostaining cycles. Besides trying different sequencing, optimization of the conditions for individual primary antibodies were also a part of this process. By adjusting the concentration and incubation time for the primary antibodies we could get the same accuracy of the stains as with single IHC (positive controls). However, we experienced that multiplexing with sequential staining cycles was relatively harsh with the tissue sometimes contributing to tissue artifacts, shrinkage, or fragmentation of tumor sections. This was particularly evident with the adaptive/lymphoid immune panel which was a 5-plex, resulting in the need to exclude 7 tumors from the analysis despite several attempts of re-staining and assay optimization. Such “fragility” of the tissue might also have been impacted by the use 3 μm thin tumor sections which might be thinner than utilized in other IHC protocols but nevertheless critical in a technique relying on serial slides in order to be representative of the same tumor area.

Finally, troubleshooting with the multiplex protocol could also be related to pre-analytical processes such as the method used for formalin fixation and/or paraffin embedding and tissue handling prior to fixation. We experienced that some of our tumor blocks suffered from sub-optimal formalin fixation, particularly the oldest tumor blocks, which also might have affected the tendency of fragmentation upon

multiplexing. Thus, to ensure adequate quality of tissue sections prior to digital analysis, all stained tumor sections where manually checked and areas with artefacts, folds or tumors of general sub-optimal quality were excluded.

Regarding reproducibility of our multiplex assay across laboratories, the use of chromogenic IHC has an advantage in using brightfield microscopy (and conventional slide scanners), which is convenient to work with and relatively easy to interpret compared to immunofluorescence. Moreover, the required technical equipment is commonly available in many pathology laboratories and compatible with most digital pathology platforms. A practical advantage also lies in the reasonable time spent scanning the stained slides making it manageable to analyze whole slide images, also for larger batches, and not only study selected regions of interest, which often is the case with other multiplexing methods.

Single and Double chromogenic IHC

An in-house monoclonal antibody (mCRP mAb 9C9) manufactured by Prof. Lawrence Potempa (Roosevelt University Schaumburg, US) with who we established a collaboration during the study period, was used to assess the expression of the monomeric form of CRP in CC tissue. mCRP mAb 9C9 is a conformation specific antibody meaning that it only detects the characteristic epitope of mCRP which first becomes exposed when the pentameric molecule (pCRP) dissociates into its structurally and antigenically, distinct monomeric subunits (mCRP) (96).

For single IHC we relied on previous publications describing the characteristics of the mCRP mAb 9C9 as well as details about antibody origin, species, and concentration provided by Prof. Potempa (103, 104). We followed the principles of the staining protocol as proposed by other groups using the antibody although in other diseases and types of tissue (105, 106).

After antigen retrieval and blocking of endogenous peroxidase whole tumor sections were stained with the mCRP antibody using DAB as chromogenic dye followed by hematoxylin counterstaining for identification of nuclei and better comprehension of tissue morphology. Stained slides were mounted and scanned for digital interpretation.

Negative controls were performed by replacing the primary antibody with washing buffer (antibody diluent only), otherwise prepared similarly, to rule out non-specific background staining of the assay system. No staining was seen in these control sections. This was further tested in tissues by mounting a panel comprising normal tissues from tonsil, liver, appendix, and pancreas on the tumor slides. Positive mCRP staining was found in liver tissue only, which might not be unexpected given that hepatocytes are considered the primary synthesizers of CRP. A piece of diseased brain tissue (exact clinical and pathological information was not available) was also stained, showing high mCRP expression comparable to what has been shown by others in human brain tissue after vascular stroke and neuroinflammation.

To elucidate potential colocalization of mCRP and immune, endothelial and tumor markers, double IHC was performed on 5 to 8 selected tumor slides with high mCRP expression as determined by the mCRP single staining. Antibodies targeting the following markers were applied in addition to the mCRP antibody: CD68 for macrophages, CD66b for neutrophils, CD34 for endothelial cells and pan-CK for tumor cells. For this purpose, tumor slides were stained sequentially, first with the mCRP 9C9 antibody using DAB as chromogenic dye, followed by one of the second primary antibody as listed above applying Ultra-view fast red as chromogenic stain. Counterstaining with hematoxylin was performed whereafter slides were mounted and scanned for interpretation.

Methodological considerations:

As the mCRP-specific monoclonal antibody was an in-house, not commercially available antibody, never used in our lab previously, we did several test-runs for antibody validation before we settled on the staining protocol used in the actual experiments. First, the optimal concentration of the antibody was determined. Other groups have been using the dilution of 1:100. After testing various concentrations, we ended up with the same concentration for both single and double IHC as this seemed to yield staining levels comparable to what has been shown by others without background signal. Next, antibody specificity was addressed. Although we did not have knowledge about a “true” positive control given the mCRP-specificity of the antibody, we sought

to address this through staining of various types of normal tissues and a piece of diseased brain as explained above. The specificity of the antibody has been verified by other groups, which we relied upon.
Regarding reproducibility, we experienced that the antibody, in our hands, performed consistently as we achieved comparable staining signals and patterns across runs. However, given that this was a pilot study with a limited sample size, consistent antibody and assay performance need to be confirmed in a larger material and preferably in other labs.

Immunofluorescence (IF)

To further elucidate the expression pattern and as indicated by double chromogenic IHC, colocalization of mCRP and selected immune, endothelial and tumor markers, double IF was performed as this allowed for identification of direct co-expression of markers also at the sub-cellular level.
Similar to chromogenic IHC, double IF was performed in a sequential manner, after antigen retrieval. First, tissue sections were stained with the mCRP-specific antibody using rhodamine as fluorophore followed by the same above-mentioned antibodies against the second marker (CD34, CD68, CD66b+, pan-CK) applying DCC (N`-dicyclohexylcarbodiimide) as fluorescent dye. DAPI was used as nuclear counterstain. Stained sections were scanned (NanoZoomer, Hamamatsu, Japan) and interpreted manually by visual examination using NDP.View (Hamamatsu).
Optimization of the IF assay was performed to get a reproducible and accurate read-out. Compared with double chromogenic IHC, we experienced a reduction of signal from the mCRP antigen when the same staining protocol was applied. By increasing mCRP-mAb concentration from 1:100 to a dilution of 1:10, we could keep mCRP signal without background signal (most optimal signal-to-noise ratio evaluated by eye). As control, single IF for each of the combined markers was performed and compared with the results obtained with the multiplex assays to ensure adequate assay performance.

Methodological considerations:

By using IF as a complementary tool to chromogenic IHC, we were able to get deeper information about localization and expression pattern of mCRP. As opposed to IHC, where colors from the applied chromogens (brown and red) were difficult to distinguish in the case of overlap, double IF offered the opportunity to separate the signals from individual stains revealing direct cellular overlap of some markers showing co-expression. Representative images of double IF for mCRP and CD66b+ neutrophils are shown in **Figure 6**.

However, compared with chromogenic IHC, IF was more difficult to work with. For practical reasons, the time spent on scanning of whole tumor sections became a limitation for us. We experienced varying quality of the scanned slides, as re-scan of the same slide not always yielded the same output. Despite the practical challenges, we considered using IF also in our multiplex protocol of the second paper as this might have overcome some difficulties we experienced working with a limited number of available chromogens combined with the in-depth information that can be obtained from multiplex IF.

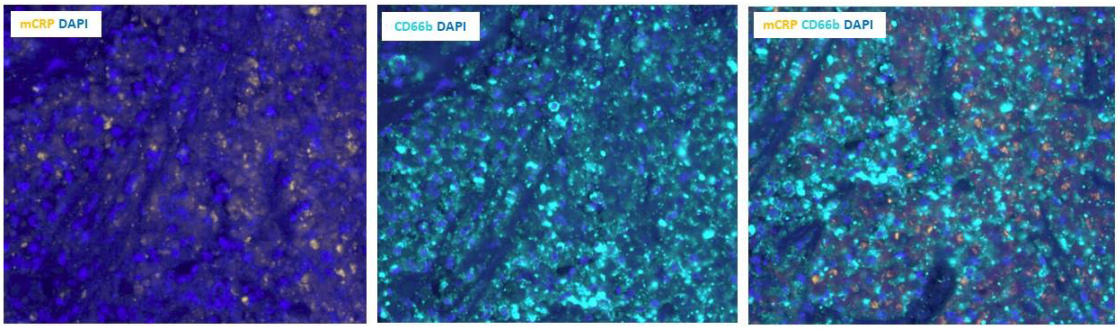

Figure 6 Double immunofluorescence labeling of mCRP and neutrophils in colon cancer tissue

Left and middle panels: Unmixed images showing individual stains of mCRP (yellow) to the left and CD66b+ neutrophils (teal) in the middle.

Right panel: Composite image showing close proximity of mCRP and neutrophils and occasional direct overlap. DAPI (blue) was used for visualization of nuclei.

3.4 Computer-assisted digital image analysis

Digital image analysis of whole tumor slides was performed for interpretation of multiplex and single plex IHC results presented in paper 2 and 3, enabling accurate visualization, quantification, and spatial information on various immune markers and mCRP expression within the TME of our cohort of colon cancer patients.
Image analysis was performed using software from Visiopharm, Denmark. Applications (referred to as APPs) for performing the analyses were developed particularly for this material using the author module within the software.

For the multiplex protocol tumor slides were first digitally aligned using a shared automated/reviewed manually approach, to generate a composite image enabling a virtual readout capturing all seven immunostained markers with preserved tissue architecture. Regions of interest (ROIs) encompassing tumor center (TC) and invasive margin (IM) originally outlined on the hematoxylin-stained slides, were then applicable for all slides. Next, automated segmentation of the tissue was performed, distinguishing between tumor stroma and tumor epithelium, allowing for assessment of marker expression within different tumor compartments. APPs were then developed to quantify and map all individual immune markers. In general, immune markers were identified by thresholding of the colors of the IHC stains followed by post-processing steps (mainly morphological operations based on size and shape).
Immune cell densities were quantified as a fraction (in percentages) of positively stained cells/area out of the total ROI and estimated separately for IM and TC. Immune cells were classified as either intra-tumoral if they were directly infiltrating the tumor nests or stromal if they were located in the stroma beside a composite score of marker expression within both tumor compartments. By adding pan-cytokeratin as a tumor marker to each of the mIHC panels we could get spatial information by estimating distances between CK-positive tumor cells and selected immune cells.

Image analysis of single IHC assessing the pattern of mCRP distribution in the TME, was performed on a whole slide image using only one ROI encompassing both the invasive margin and tumor center. In addition, on applicable slides where normal colon

mucosa was represented, this was annotated as a separate ROI. User trained AI-based algorithms within the software were used for accurate segmentation of tumor stroma and tumor epithelium. mCRP expression was quantified as area proportions estimated separately in the stroma and tumor epithelium as well as a combined score encompassing both compartments. mCRP expression was also estimated within areas of normal colon mucosa, when applicable.

Methodological considerations

Digital image analysis enabled us to accurately visualize and quantify various subsets of immune cells and the monomeric form of CRP within the TME of colon cancer patients. By merging mIHC and hematoxylin-stained serial whole tumor slides we obtained a virtual multiparameter readout of seven simultaneously expressed lymphoid and myeloid immune markers with preserved tissue architecture. Automated segmentation of tumor tissue and adding cytokeratin as a tumor marker in the IHC panels allowed for classifying immune cells as either intratumoral or stromal enabling assessment of cell distributions within different tumor compartments and analyzing spatial relationship.

As opposed to manual interpretation and counting by visual inspection, automated digital analysis offers the opportunity to perform an objective, reproducible and comprehensive characterization of the TME. The automated approach allows for processing whole tumor sections, as we did in our series, and not only selected tumor areas, thus providing a more representative image of the TME with the ability to capture potential tissue and biomarker heterogeneity. A major advantage of multiplexed imaging over other TME addressing technologies such as single cell sequencing, lies in the opportunity to obtain information about spatial context and cellular relationships. Importantly, accumulating studies have shown that the localization and proximity of immune and tumor cells within the TME have prognostic and predictive significance (107). Given the pilot design of our work, we restricted the spatial analysis to only include two types of immune cells (CD8+ T cells and neutrophils) assessing their proximity to tumor cells, primarily to show that such

analyses are feasible within the proposed framework. However, these analyses could have been expanded to gather substantially more spatial data encompassing multiple different cell types and inter-relationship within the TME of our colon cancer cohort. Indeed, the rapidly evolving field of AI-based tools currently being applied in a wide spectrum of medical research and practices including digital pathology, opens the possibility of interrogating the complex tumor immune microenvironment and substantially increase the amount of obtainable spatial and morphological information, also at the sub-visual level (108). Such AI-based approaches are currently being developed and tested in a variety of different tumor types and settings, and there are high expectations in the cancer community that these tools will play more crucial roles in the near future particularly for the development of novel biomarkers in immuno-oncology (109).

Nevertheless, multiplexed imaging, still has some limitations that need to be addressed. Importantly, as the method proposed here is based on mIHC, the overall performance of the image analysis is dependent on the quality of the IHC assay. As discussed above, mIHC has its own limitations and can be biased by multiple factors. Thus, a sub-optimally performed immunostaining will translate into a less accurate and valid digital analysis. Although far more objective than manual interpretation and visual assessment, there are several steps in the workflow of digital analysis that are widely influenced by the user and the applied technical platform (both IHC assay and software). Specifically, the digital assessment and determination of immunostained markers as “positive” or “negative”, are performed by APPs specifically designed by the user for the actual material applying subjectively defined thresholding values for the detection of the markers of interest. We used thresholding of the colors from the chromogens to determine whether a cell was “positive”, but there is not a standardized way of doing these operations. However, arguing in favor of a certain degree of objectivity and reproducibility, the utilized threshold/cut-off values and post-processing steps performed by the APPs used in our work, are defined and available within the software, enabling others to apply the same settings and run the imaging protocol. In our material, the thresholding was further complicated by variations in staining intensities between tumors as some tumors appeared more fade than others,

although not interpreted as sub-optimally stained, which might have impacted our analysis. However, to minimize the potential bias from differentially stained tumors, all digitally analyzed slides were reviewed manually to control that the automated marker detection was correct.

3.5 Assessment of mismatch repair (MMR) status

Deficiency in the crucial DNA mismatch repair (d-MMR) system defined by loss of function of one or more of the MMR proteins, leads to impaired capability of DNA repair causing accumulation of somatic mutations throughout the genome and the characteristic occurrence of frameshift mutations in microsatellites regions, which accounts for the microsatellite instability status, MSI (30).

In CRC, identification of tumors with d-MMR has important clinical implications given the strong prognostic and predictive value related to the microsatellite instability-high (MSI-H) phenotype characterized by high tumor mutational burden and abundant T-cell infiltration (30). Accordingly, determining MMR status within our colon cancer cohort was essential, as we would expect significant differences in the type and extent of the immune infiltrate between MMR proficient and MMR deficient tumors, thus impacting our analyses.

Loss of MMR function can be determined at the protein level (MMR expression evaluated by IHC) or genetic level (detection of microsatellite instability, MSI testing). In our study, we performed a combination of the two. First, an experienced pathologist evaluated IHC expression of the four DNA MMR proteins MHL1, MSH2, MSH6 and PMS2 in tumor nuclei. Tumors that were negative or inconsistent in one or more of the immunostainings were analyzed using the Idylla MSI assay, which is an automated, rapid PCR-based MSI test (evaluates the mutational status of seven microsatellite markers) approved for testing of MSI status in CRC (110). Based on the results of the IHC analysis and/or the Idylla MSI test, tumors were classified as either microsatellite stable (MMS) or microsatellite instable (MSI), with the latter corresponding to MSI-high (at least 2/7 mutant markers) as the assay did not report MSI-low status.

Notably, nine out of the 21 patients (43%) in the CRP-high group had MSI-positive tumors whereas all patients in the CRP-low group had MMR-proficient tumors. This was accounted for in our analyses, and as expected, MSI-positive tumors appeared highly inflamed although not entirely by T-cells, which will be discussed separately in the results section.

Methodological considerations:

We encountered some difficulties with the MMR IHC evaluation as several of the tumors showed inconsistent, weak, or very patchy nuclear staining despite attempts of re-staining. This might have been due to sub-optimal tissue fixation. Consequently, more tumors than those with a clear loss of IHC MMR expression, needed to be evaluated with the Idylla MSI test. All tumors were applicable with the MSI assay. The downside to the many performed MSI tests from our perspective, was the economic costs given that the PCR test is expensive compared to IHC. On the other hand, IHC is laborious in terms of the time spent on IHC (re-) staining and interpretation by the pathologist. From a clinical perspective though, IHC plays an important role in assisting the detection of Lynch syndrome due to its ability to identify which of the MMR proteins that is defective and recommend genetic counselling and germline testing, when applicable.

3.6 Statistical methods

Paper 1

Patients were follow-up until 5 years following resection of liver metastases. The primary endpoint was overall survival measured from the date of liver surgery to date of death from any cause. Survival analysis was performed using the Kaplan-Meier method and differences compared using log-rank tests. Univariate and multivariate hazard ratios (HRs) with 95% confidence interval (95% CI) were estimated by Cox proportional hazard analysis. The relationship between preoperative CRP levels and clinicopathological characteristics were examined using the Chi square test and Spearman correlation test.

Paper 2

Differences in clinicopathological characteristics between CRP-high and CRP-low patients were evaluated by Fisher's exact test and the two-sample t-test. Immune markers were analyzed on the logarithmic scale to obtain a normal distribution. Pearson's correlations were used to analyze the relationship between individual immune markers. Associations between CRP levels, immune markers, and survival were analyzed by Fisher's exact test. The Aalen–Johansen method was used to estimate the risk of recurrence or death from colon cancer (death from other causes treated as competing risk) and compared between CRP-high and CRP-low groups using the log-rank test. Composite lymphoid (CD8+ T/CD4+ T cells) and myeloid (CD68+ macrophages/CD66b+ neutrophils) immune scores were calculated by summing the scores of the respective immune markers after the data had been log transformed and standardized. Unsupervised hierarchical clustering based on the densities of individual tumor-infiltrating immune markers was performed to examine whether subgroups of tumors with distinct immunological features existed within our cohort and heatmaps were generated.

Paper 3

Differences in patient characteristics were evaluated using Fisher´s exact test and the two-sample t-test. The distribution of tumor-associated mCRP was assessed as mCRP proportions defined as: area of positive mCRP staining divided by the total area of the given region of interest. Since the area of mCRP was small compared to the total area of the tumor, proportions were multiplied with 1000 and given per mil instead of percentages. Area proportions of mCRP were calculated both as a combined score covering the area of the whole tumor as well as separately for the tumor epithelium and tumor stroma. Median mCRP proportions within groups were calculated and compared using the median test. The correlation between tumor-associated mCRP and circulating CRP was assessed using Spearman analysis. Associations between mCRP and various immune cell densities obtained from the multiplex IHC were analyzed using Spearman correlations and heatmaps were generated. Risk of recurrence or death from colon cancer was estimated using the Aalen-Johansen method and compared between CRP-

high and CRP-low patients using the log-rank test. To evaluate the prognostic impact of mCRP within our cohort we first calculated a receiver operating characteristics (ROC) curve to identify the most optimal threshold/cutoff value for tumor mCRP expression. This was defined as the point on the ROC curve with sensitivity and specificity closest to 100%. Next, risk of CC death or recurrence for patients with mCRP tumor expression above and below the optimal cutoff value was estimated and compared between groups using the log-rank test.

4. Results

4.1 Paper 1

The Prognostic Role of Systemic Inflammation in Patients Undergoing Resection of Colorectal Liver Metastases: C-Reactive Protein (CRP) Is a Strong Negative Prognostic Biomarker

Considering the profound impact of systemic inflammation for cancer survival and treatment outcomes together with the need for better tools to accurately predict prognosis and thus select appropriate treatment strategies for patients with CRC liver metastases (CRLM), we aimed to evaluate the prognostic impact of CRP compared to clinicopathological features and other inflammatory markers in a Nordic cohort of CRC patients undergoing potential curative liver surgery.

492 CRLM patients were included in the study. Median follow-up time was 4.17 years. The majority of patients had their primary tumor located in the colon (55%) and presented with synchronous disease (55%). 41% of the patients received neoadjuvant chemotherapy whereas 59% had chemotherapy following liver surgery. Preoperative CRP was elevated (>10 mg/L) in 59 (14%) patients and 25% presented with s-albumin <35 g/L. Calculating the GPS score, two thirds of the patients were GPS 0 whereas 20% and 6% were GPS 1 or 2, respectively.

Of note, elevated preoperative CRP (>10 mg/L) was associated with large metastases (≥5cm), less frequent use of neoadjuvant chemotherapy, hypoalbuminemia, and colonic primary (all $p<0.01$)

Analyzing the prognostic impact of various clinicopathological factors, both number ≥5 (HR=1.55 95% CI:1.04-2.32, p=0.03) and size of largest metastasis ≥5 cm (HR=1.48 95% CI:1.03-2.10, p=0.03), the use of postoperative chemotherapy (HR=0.71 95% CI:0.55-0.93, p=0.01) and age over 65 (HR=1.31 95% CI:1.00-1.69, p=0.04) were associated with survival on univariate analysis. However, only number of metastases and postoperative chemotherapy remained significant predictors of overall survival in the multivariate analysis as shown in **Table 1**.

With regard to the prognostic impact of markers of systemic inflammation, elevated preoperative CRP (>10 mg/L) analyzed as both dichotomous and continuous variables, were associated with compromised survival on univariate (respective HRs of 1.93 95% CI:1.35-2.77 and 1.01 95% CI:1.00-1.02, both $p<0.01$) as well as multivariate analyses (Table 1). S-albumin, however, only had independent prognostic impact when analyzed as a continuous variable, but not dichotomized above/below 35 mg/L. Nevertheless, when hypoalbuminemia was combined with CRP in the GPS score, elevated GPS of 1 or 2 predicted for poorer overall survival on multivariate analysis (Table 1).

Finally, to better understand the prognostic impact of CRP, patients were allocated first, into two groups defined as normal and elevated preoperative CRP using 10 mg/L as cutoff, and next into three groups applying the following thresholds: ≤10, 11-30, >30. As shown in **Figure 7**, the survival time differed significantly between groups. Specifically, patients with CRP below 10 had a median survival of 4.27 years compared to 2.59 years in patients with CRP between 11 and 30, and 2.13 years in patients with CRP above 30 mg/L ($p<0.01$) *.

Overall, the presence of systemic inflammation, particularly evident by elevated CRP, had strong prognostic impact in stage IV CRC patients undergoing potentially curative resection of liver metastases. Assessment of CRP may provide complementary prognostic information to the traditional tumor-based risk factors in CRLM patients.

*In the published paper there is a typing error: median survival time for patients with CRP above 30 mg/L is reported to be 47 days while it should have been 2 years and 47 days (the same as 2.13 days as stated above). We informed the journal about the mistake short after the article was published. Unfortunately, we never received an answer nor was the actual number corrected in the online version of the paper. However, figures and related statistics are correct.

Table 1. Multivariate Analysis of prognostic factors in CRLM patients

Variable	Hazard ratio	95% CI	P-value
Age ≥ 65	1,83	0,90-1.55	0,22
Postop chemotherapy	0,74	0,57- 0,97	0,03
Size of the largest liver metastasis ≥ 5 cm	1,35	0.94-1.93	0,10
Number of liver metastases ≥ 5	1,08	1,04-1,12	<0,01
Albumin continuous variable	0.96	0.92-0.99	0.03
GPS 0 vs. GPS 1/2	1.63	1.19-2.22	0.02
CRP continuous variable	1.01	1.00-1.02	<0.01
CRP >10 mg/L	1.72	1.18-2.49	<0.01

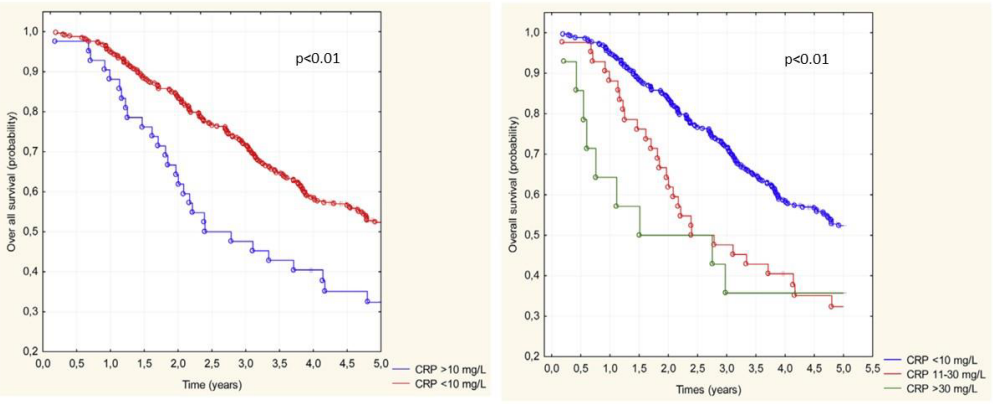

Figure 7 Overall survival in CRLM patients stratified according to preoperative CRP levels

Left: Patients divided into two groups using CRP 10 mg/L as cut-off. Right: Patients divided into three groups; CRP<10 mg/L, CRP 11-30 mg/L, >30 mg/L.

4.2 Paper 2

Systemic Inflammation Associates With a Myeloid Inflamed Tumor Microenvironment in Primary Resected Colon Cancer - May Cold Tumors Simply Be Too Hot?

Given the strong negative prognostic impact of systemic inflammation measured by elevated levels of serum CRP, the overarching aim of this study was to explore the tumor immune microenvironment in colon cancer patients with and without elevated CRP to provide insights into why systemic inflammation is so detrimental for patient outcome. For this purpose, we developed a FFPE based platform combining multiplex IHC and digital whole slide imaging enabling accurate visualization and assessment of six simultaneously expressed lymphoid and myeloid immune markers besides PD-L1 and a tumor marker with preserved tissue architecture. Using this platform, we could get information regarding cellular composition, intratumoral heterogeneity and spatial organization in the TME allowing us to explore and compare the tumor immune landscape in colon cancer patients with and without systemic inflammation.

mIHC stained whole tumor sections from 36 patients were included in the analyses: 21 patients with elevated CRP (>30 mg/L) interpreted as systemically inflamed (termed CRP-high), and 15 with normal CRP (0-1 mg/L), termed CRP-low. Systemically inflamed patients were older and more right-sided. All patients in the CRP-low group had stage III disease, whereas the CRP-high group comprised patients with both stage III (52%) and stage II (48%) disease. Importantly, nine of the systemically inflamed patients harbored MSI-positive tumors whereas all non-inflamed patients had MSS tumors. Nevertheless, CRP-high patients had significantly higher risk of recurrence or death from colon cancer compared to CRP-low patients (p=0.047).

mIHC and whole slide imaging revealed substantial intra- and intertumoral heterogeneity both in terms of tissue morphology and pattern of immune infiltration across tumors. Some tumors were heavily immune infiltrated with abundant immune cells directly infiltrating the tumor epithelium as well as present within the tumor stroma. Other tumors exhibited dense tumor tissue, less stroma and modest immune infiltration. Finally, some tumors harbored a more patchy immune infiltrate comprising

areas with brisk immune infiltration combined with areas with limited or no immune infiltration.

As expected, there were significant differences in the composition of the immune infiltrate between MSS and MSI-positive tumors from the systemically inflamed patients and the MSS tumors of the non-inflamed group. Specifically, MSI-positive tumors were highly infiltrated by adaptive immune cells exhibiting significantly higher densities of CD8+ and CD4+ T cells and CD20+ B cells compared to the MSS tumors of both the CRP-high and CRP-low groups. Yet more surprisingly, they also showed evidence of considerable myeloid immune infiltration in terms of high densities of CD68+ macrophages and CD66b+ neutrophils together with up-regulation of PD-L1, predominantly expressed by macrophages within the tumor stroma. Of note, MSS CRP-high tumors turned out as the less lymphoid inflamed group harboring the lowest densities of CD8+ and CD4+ T cells, CD20+ B-cells and FOXP3+ regulatory T cells, yet considerably more myeloid inflamed with high densities of CD68+ macrophages and CD66b+ neutrophils. These findings were further supported by the univariate analysis demonstrating significant associations between high densities of CD66b+ neutrophils and CD68+ macrophages and elevated CRP as well as an inverse correlation between CD8+ T and FOXP3+regulatory T cells and CRP, regardless of MSI/MSS status.

To explore whether tumors from systemically inflamed patients harbored a distinct tumor immune phenotype, we first hypothesized that the expression of two immune markers combined rather than analyzing individual markers only, better could identify specific immunological features that correlated with systemic inflammation. For this purpose, immune cell densities of CD8+/CD4+ T cells (termed the adaptive/lymphoid composite score) and CD68+ macrophages/Cd66b+ neutrophils (termed the innate/myeloid composite score), were compounded and correlated with the level of CRP. Interestingly, we found that regardless of the lymphoid immune score, tumors with a high myeloid immune score had increased risk of elevated CRP, suggesting that it is the presence of a myeloid-inflamed and not the absence of a lymphoid-inflamed TME that associates with systemic inflammation.

To further interrogate whether distinct immune phenotypes existed within our cohort, heatmap and hierachical clustering were performed based on the densities of individual immune markers. Three predominant clusters could be identified from the dendrogram consisting of a subgroup of tumors that were mainly lymphoid inflamed, a group that were characterized by extensive myeloid inflammation and finally a subgroup of hyperinflamed tumors exhibiting both strong lymphoid and myeloid inflammation. Representative images of tumors from the three subgroups are shown in **Figure 8**. By adding clinical data including information on follow-up status, CRP values and MSS/MSI status into the heatmap, systemically inflamed MSS tumors seemed to correspond with the predominant myeloid cluster whereas MSI positive tumors aligned with the hyperinflamed phenotype. Notably, none of the MSS CRP-low tumors exhibited high densities of myeloid immune cells, but were either predominantly lymphoid- or non-inflamed.

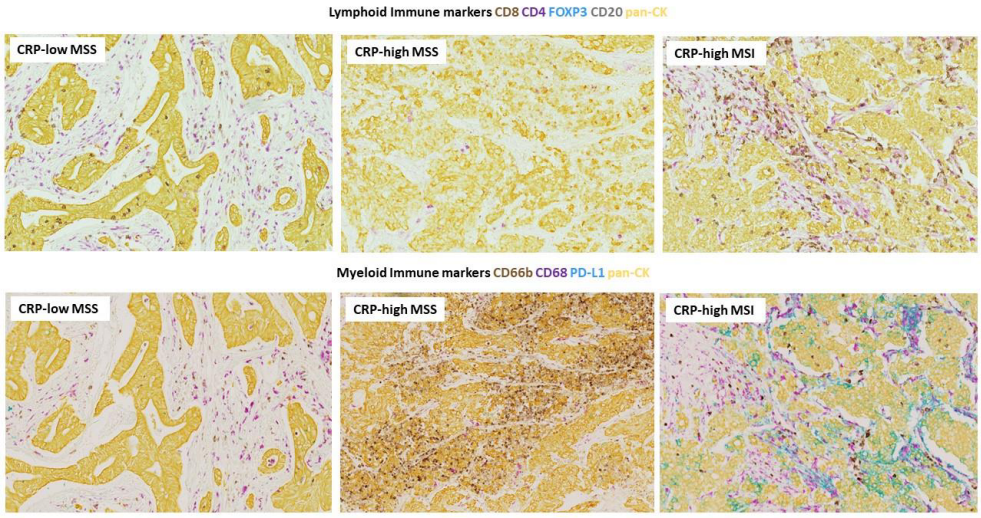

Figure 8 Representative images of mIHC stained tumor slides from patients exhibiting the predominant immunophenotypes identified by hierarchical cluster analysis.
A) CRP-low MSS tumor with abundant lymphoid and modest myeloid immune infiltration. B) CRP-high MSS tumor harboring predominant myeloid inflammation (particularly neutrophils) and limited lymphoid inflammation. C) CRP-high MSI tumor with vigorous lymphoid (particularly CD8+/CD4+ T cells) and myeloid (high PD-L1 expression) immune infiltration.

Finally, based on accumulating data indicating that spatial patterns of the TME might reflect clinically meaningful tumor-host interactions, we assessed the spatial distribution of CD8+ T cells and CD66b+ neutrophils in relation to tumor cells in CRP-high and CRP-low patients. Guided by cytokeratin-positive tumor cells, we were able to obtain information on the densities of the respective immune cells in close proximity to tumor cells, a feature that has been highlighted as a pseudomarker of cellular crosstalk (111). Intruigingly, we found that tumors from systemically inflamed patients harbored significantly more neutrophils in close proximity to tumor cells compared to the non-inflamed patients whereas there were no difference in the spatial distribution of CD8+ T cells between tumors from CRP-high and CRP-low patients.

In summary, we developed a multiplex IHC-based method enabling accurate visualization and characterization of the tumor immune microenvironment in a cohort of resectable colon cancer patients. Using this platform, we identified specific immunological features that associated with systemic tumor-associated inflammation suggesting a particular role of myeloid inflammation in the context of SIR.

4.3 Paper 3

Fueling the flames of colon cancer - does CRP play a direct pro-inflammatory role?

Based on emerging evidence suggesting that CRP exists in different structural isoforms with the monomeric form (mCRP) playing a direct role in inflammatory diseases, the aim of this study was first to identify and map the pattern of mCRP expression in colon cancer tissue from patients with normal and elevated levels of circulating pentameric CRP (pCRP). Next, by combining double IHC and immunofluorescence (IF) imaging techniques, we sought to elucidate the potential functional roles of mCRP in the TME of CC patients.

FFPE tissue samples from 43 stage II and III CC patients (from the same patient cohort as applied in paper 2) including 20 patients with serum CRP 0-1 mg/L and 23 patients with serum CRP > 30 mg/L, were included in the study. Although encompassing more patients, the distribution of patients characteristics corresponded to that reported in paper 2 except for the follow-up time, which was extended to 9.3 and 8.8 years respectively, for patients in the CRP-low and CRP-high group.
To evaluate the level and pattern of mCRP expression, whole tumor sections were IHC stained with an mCRP-specific monoclonal antibody, interpreted, and quantified using digital image analysis.
CRP was abundantly present in colon tumors, primarily from systemically inflamed patients (circulating serum CRP > 30 mg/L). Representative image is shown in **Figure 9**. Correspondingly, tumor-expressed mCRP correlated strongly with the level of circulating pCRP (Spearman correlation 0.81, $p<0.001$). Further analysis of the distribution of mCRP expression showed that tumors from MSI-positive systemically inflamed patients (n=9) exhibited significantly more mCRP compared to tumors from MSS CRP-high (n=14) and MSS CRP-low patients (n=20). Following AI-based digital segmentation of tumor epithelium and stromal tissue, it could be determined that significantly more mCRP was located within the tumor stroma compared to the tumor epithelium regardless of MSS/MSI status.

Most strikingly, as shown in **Figure 9**, mCRP appeared entirely tumor-specific, as normal colon mucosa adjacent to tumors showed no evidence of positive mCRP staining.

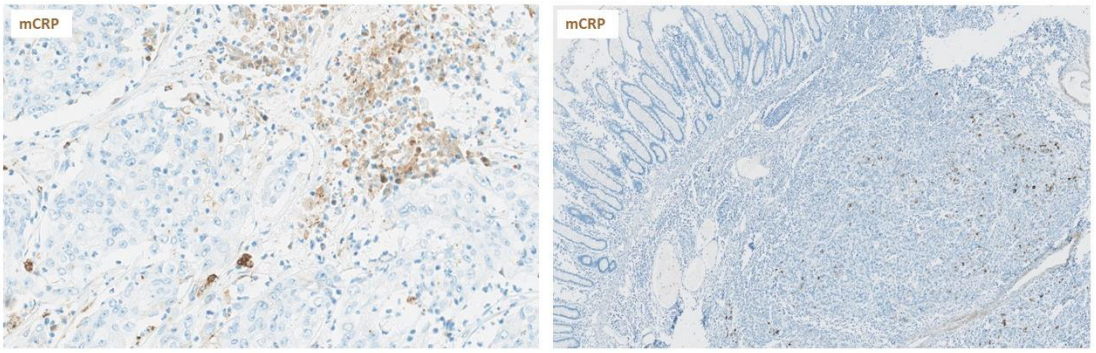

Figure 9 mCRP expression in colon cancer tissue and normal colon mucosa
Representative images from a patient with elevated serum CRP and high mCRP expression (brown) in the tumor (left) with no mCRP positivity in adjacent normal colon mucosa (right).

Based on prior studies showing a direct role of mCRP in the pathogenesis of primarily cardiovascular and neurodegenerative disorders, we were interested in exploring functional roles of mCRP in colon cancer tissue. For this purpose, we first correlated the quantified tumor-associated mCRP with the immune profiles obtained from the multiplex IHC performed previously on the same patient cohort and tumor areas. The most apparent correlation was with the neutrophils showing a significant positive association between mCRP and CD66b+ neutrophils (Spearman correlation 0.57, $p<0.001$), presented in **Figure 10**. Next, double immune stainings were performed to assess potential colocalization of mCRP and selected immune and endothelial markers to illuminate possible functional relationships directly. Consistent with prior studies demonstrating a range of different cellular and non-cellular ligands for mCRP, we found that mCRP seemed to interact with various components of the TME. As illustrated in **Figure 6 and 10**, congruent with the correlation analysis, the most apparent colocalization demonstrated by double IHC and IF, was between mCRP and the neutrophils, where mCRP coincided with highly neutrophil infiltrated tumor areas.

Correlation between mCRP and biomarkers (Spearman correlation).

	Correlation	P-value
mCRP - cd8	-0.02 (-0.35;0.36)	0.93
mCRP - cd4	-0.07 (-0.41;0.30)	0.68
mCRP - cd20	-0.12 (-0.47;0.29)	0.51
mCRP - cd4_fp3	-0.41 (-0.67;-0.07)	0.022
mCRP - cd68	0.26 (-0.07;0.54)	0.13
mCRP - cd66b	0.57 (0.28;0.77)	<0.001
mCRP - pdl1	-0.06 (-0.42;0.32)	0.73

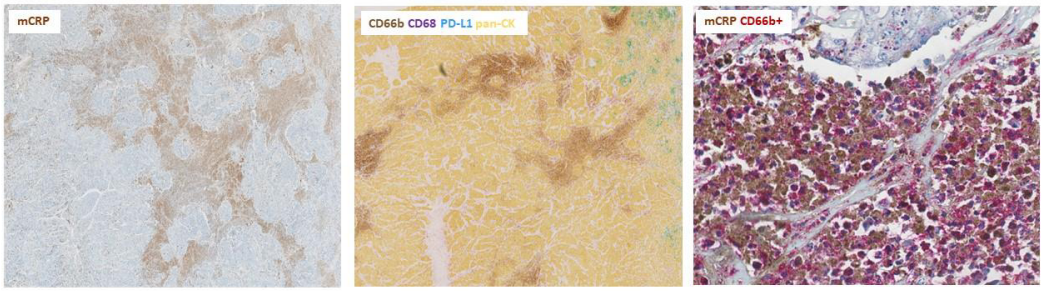

Figure 10 mCRP expression correlates with tumor infiltrating neutrophils

Above: Spearman correlations between mCRP and individual immune markers quantified by mIHC

Left: Single IHC staining showing abundant mCRP expression (brown) in tumor tissue

Middle: Corresponding tumor area stained with multiplex IHC showing highly neutrophil infiltrated (brown) areas

Right: Double IHC showing prominent colocalization of mCRP (brown) and cd66b+ neutrophils (red)

To a lesser extent, yet still present, mCRP also colocalized with CD68+ macrophages, particularly within tumor areas harboring rich immune infiltration. Moreover, mCRP colocalized with CD34+ endothelial cells and could be detected within the lumen of some intratumoral vessel, suggesting a systemic origin of the monomeric form. In some tumors, mCRP was distributed in a rather scattered pattern, detected as small granules dispersed within the connective tissue, not related to any particular cell type, indicating a direct interaction with components of the extracellular matrix although we did not stain specifically for that purpose. Additionally, mCRP seemed to correlate with areas of necrosis, with and without neutrophil infiltration, observed as aggregates of mCRP in the vicinity of necrotic tumor areas.

Serendipitously, when analyzing the morphological pattern of mCRP, it became evident that some tumor nuclei were surrounded by mCRP. To examine this further,

we performed double IHC and IF for mCRP and pan-cytokeratin (pan-CK) as a tumor marker. Intriguingly, IF revealed double positive mCRP+/pan-CK+ tumor cells, indicating intratumoral uptake of mCRP and/or intrinsic tumor mCRP expression.

Taken together, we show that the pro-inflammatory monomeric form of CRP is expressed in colon tumors and correlates with the level of circulating serum CRP. Our data strengthen the hypothesis that CRP might not only be an inflammatory marker but potentially also an active mediator in the microenvironment of tumors.

5. Discussion

5.1 The strong role of systemic inflammation and elevated CRP for clinical outcome within a broad population of colorectal cancer patients

Systemic tumor-associated inflammation has consistently been reported to negatively affect patient outcome in a range of different cancer types, including CRC (58, 61, 112). Nevertheless, the prognostic role in resectable metastatic disease has been less studied. Additionally, within this patient population, the currently used tumor-based risk scores lack the desired precision in adequately stratifying patients and thus select the most appropriate treatment strategy. In this work, we therefore aimed at evaluating the impact of systemic inflammation in a large Nordic cohort of CRC patients with liver metastases only, undergoing a potentially curative hepatectomy. We found that elevated preoperative CRP >10 mg/L was a powerful predictor of compromised OS (HR 1.72 (95% CI 1.18-2.49, $p<0.01$) independent of traditional clinicopathological risk factors such as size and number of metastases, involvement of the resection margin and the use of pre-and post-operative chemotherapy. Moreover, we found that incremental levels of CRP were significantly associated with progressively shorter overall survival. Our results are consistent with previous studies, mainly performed in primarily operable CRC, yet to some extent also in patients undergoing resection of metastases, with a few existing studies reporting a positive correlation between elevated preoperative CRP and poor survival outcomes within this patient population (4, 112-116). Interestingly, we found that the size of largest liver metastasis, but not number of metastases correlated significantly with elevated CRP. This lack of consistency in the relationship between tumor burden and systemic inflammation could potentially reflect that systemic inflammation might drive tumor progression and not necessarily only accompany more advanced and aggressive disease. In this regard, data from the aforementioned Nordic VII study evaluating the prognostic impact of different SIR markers in metastatic CRC patients receiving first line treatment, showed that elevated baseline CRP (and IL-6) associated with compromised PFS and OS (74). Although the relative reduction in survival rates was most pronounced in BRAF-

mutated (generally associated with an aggressive clinical phenotype) compared to wild-type and RAS mutated patients, elevated CRP appeared independent of tumor mutational status in the multivariate analysis indicating that patients harboring a more proliferative tumor type not necessarily have higher levels of CRP (117). However, correlation does not equal causation, thus the sequencing of systemic inflammation and cancer development cannot be determined from these studies. Nevertheless, regardless of what comes first, the tumor or the inflammatory response, persistent inflammation is unequivocally detrimental for prognosis with a presumably mutual dependence, meaning that they sustain and fuel each other ultimately leading to tumor progression and adverse patient outcomes (118).

Another intriguing question in this regard is whether patients might change their inflammatory state after surgery and thus improve prognosis. In our study we could only evaluate the prognostic value of preoperatively measured SIR markers as we did not have access to post-operative measurements. Indeed, it would be of considerable interest to investigate whether some patients transition from preoperatively systemically inflamed to non-inflamed following surgery and how this might impact survival outcomes and disease trajectory. A few previous studies have investigated the longitudinal aspect of SIR in primary resectable CRC patients by analyzing the prognostic impact of pre- and post-operative (3-6 months) inflammation-based markers, showing that the majority of patients (80 %) with evidence of systemic inflammation preoperatively retain their inflammatory state after surgery (6, 112). Interestingly, though, a recent study covering a large cohort of stage III CRC patients undergoing curative resection, specifically analyzed patients that experienced normalization of inflammatory markers postoperatively demonstrating that these patients had similar prognosis compared to patients that were non-inflamed both pre- and post-operatively (119). As expected, patients with evidence of persistent systemic inflammation also in the non-tumor bearing state had a significant poorer prognosis compared to persistently non-inflamed patients including those who transitioned to a normalized inflammatory state, indicating that systemic inflammation in these patients is a host-intrinsic and not tumor-dependent response (119).

Several markers of the systemic inflammatory response have been investigated for their prognostic role in cancer. CRP, however, is easily accessible, routinely available and can be directly applied in clinical practice without the need for further calculations as opposed to other inflammation-based scores and ratios. From this perspective, we were interested in comparing the prognostic value of CRP not only to tumor- and patient related characteristics but also to other commonly used SIR markers. We found that preoperatively elevated CRP (>10 mg/L) was a strong and independent predictor of poor OS (HR 1.72 95% CI 1.84-2.50, p<0.01) whereas s-albumin only had significant prognostic value when analyzed as a continuous variable (multivariate HR 0.96, 95% CI 0.92-0.99, p=0.03), demonstrating that hypoalbuminemia alone was not an independent predictor of survival in our analyses. However, when combined with CRP in the GPS score, albumin retained prognostic impact (multivariate HR for GPS 1 and 2 combined 1.63, 95% CI 1.19-2.22, p=0.002). Nevertheless, CRP turned out to be the strongest prognostic factor in the clinical setting of stage IV CRC patients undergoing surgery for liver metastases.

Overall, our data, supported by others, demonstrate that CRP is a strong prognostic factor in a broad population of CRC patients including patients with resectable liver metastases and may provide complementary prognostic information to conventional patient and tumor-based risk factors. Integrating biomarkers that reflect the host immunological response and not only tumor-based characteristics, may enable a more comprehensive assessment of patient risk profile both in terms of prognosis and clinical presentation, allowing for more appropriate treatment decisions tailored to individual patients.

5.2 Correlating local and systemic tumor-associated inflammation. Exploring the tumor immune microenvironment in CRP-high colon cancer patients

Considering the strong contribution of the host immune response to cancer survival and treatment outcomes, we were interested in understanding why systemic inflammation is so detrimental for patient outcomes. What is the underlying biology

and how does systemic inflammation impact the local tumor immune response and/or the other way around? Although widely accepted for their strong and often opposing prognostic roles, the systemic and localized tumor immune responses are most often classified and viewed separately. However, given that many of the same mediators are involved with inflammatory cells recruited to the tumor primarily from the peripheral circulation, systemic and intratumoral inflammation are related processes with continuous communication (91). Nevertheless, previous research has predominantly focused on either one with only few studies investigating the immunological relationship between tumors and the host response (64). Moreover, most oncological practices do not integrate SIR markers in bedside decision-making despite acknowledging the profound negative impact of tumor-associated systemic inflammation. A part of the explanation for this mismatch in terms of substantial scientific evidence yet lack of clinical implementation, could be that the underlying tumor biology and immunological mechanisms remain far from understood together with the fact that no established treatment approach specifically targeting the systemic inflammatory response exists. Oncologists might know that systemic inflammation is bad for the patient, but not why, and what to do about it.

Thus, the primary aim of this work was therefore to explore the relationship between systemic inflammation as evidenced by elevated levels of serum CRP, and the local tumor immune response. Improved understanding of the tumor immunology behind systemic inflammation might increase the awareness, and more importantly, provide clinicians with valuable, and potentially even targetable information concerning the immunological process taking place at the tumor site.

By analyzing the tumor immune context in patients with and without elevated levels of CRP, we identified up-regulation of myeloid features in the TME of systemically inflamed patients. Importantly, our findings indicated it is the presence of myeloid inflammation, evidenced by high densities of CD66b+ neutrophils and CD68+ macrophages, and not the absence of lymphoid inflammation (CD8+ and CD4+ T cells) that associates with systemic inflammation highlighting the fundamental role of myeloid immune cells in the microenvironment of tumors. Based on the mIHC data from our cohort, neutrophils seemed to be pivotal players in the TME of systemically

inflamed patients. Specifically, we found that high densities of CD66b+ neutrophils assessed both at the invasive margin and tumor center were significantly predictive of elevated levels of CRP regardless of MMR status. Moreover, by analyzing spatial patterns in the TME, we found that tumors from systemically inflamed patients harbored significantly more neutrophils in close proximity to tumor cells compared to non-inflamed patients whereas there were no differences in the spatial distribution of CD8+ T cells between tumors from CRP-high and CRP-low patients.

While most translational as well as clinical research to date primarily has focused on the fundamental role of T cells for effective anti-tumor immunity and importantly, for response to treatment with ICI, accumulating research have delineated the complex roles of myeloid immune cells in the microenvironment of tumors (47, 120). Tumor-associated macrophages (TAMs) are probably one of the most studied innate immune cells (50). Although there is evidence suggesting they may have contradictory roles in the TME depending on their functional phenotype, it is by now widely acknowledged that TAMs primarily promote immunosuppression (121). They can support tumor growth and metastasis directly, while inhibiting effective antitumor immune responses (121, 122).

Although less studied than the macrophages, accumulating data are emerging highlighting neutrophils as major players in the microenvironment of tumors. Like TAMs, tumor associated neutrophils (TANs) primarily exhibit an immunosuppressive and directly tumor promoting phenotype (55, 57). Key neutrophil pro-tumor functions include induction of epithelial genetic instability and DNA damage resulting in tumor promoting inflammation through the production of reactive oxygen species (ROS) and proteases (55). Additionally, neutrophil derived cytokines and growth factors such as IL-8, neutrophil elastase and MMP9 can directly stimulate tumor cell proliferation, and by activating vascular endothelial growth factor A (VEGFA), induce angiogenesis (54, 56). Apart from the direct effects within the TME, pro-tumorigenic neutrophils produce chemokines that recruit other immune cells such as regulatory T-cells and more innate immune cells to the tumor and thus may contribute to further fueling and

shaping the tumor immune response (123). Finally, it has been shown that neutrophils can mediate immunosuppression and blunt effective anti-tumor immune responses by the secretion of arginase-1, TGF-beta and iNOS, which deprive the microenvironment for nutrients essential for optimal T-cell functioning leading to downregulation of CD8+ T-lymphocytes (54, 55). However, most of the data regarding neutrophil function and multifaceted roles have been obtained from murine models or performed ex vivo conducted on isolated peripheral or tumor-infiltrating neutrophils (123). Thus, a knowledge gap still exists in accurately depicting how neutrophils affect the immune contexture and tumor biology in humans. Although our data need to be confirmed and elaborated in a larger material, preferably within other tumor types and stages, they may add to the notion of a profound role for neutrophils in the microenvironment of tumors. Additionally, based on our findings, we hypothesize that myeloid inflammation and neutrophils may play a potential driving role in the clinical setting of systemic inflammation in resectable colon cancer patients.

Another intruiging finding of this work was the significant association between MSI-positive tumors and elevated levels of CRP. At first this seemed counter-intuitive as we would assume an inverse correlation given the favourable prognosis associated with positive MSI status and poor prognosis related to the presence of systemic inflammation. However, when analyzing the immune contexture of systemically inflamed MSI-positive tumors, we found that beside the expected high densities of adaptive immune cells (CD8+ and CD4+ T cells, B cells) MSI-tumors showed evidence of considerabel myeloid inflammation in terms of high densities of CD66b+ neutrophils and CD68+ macrophages as well as upregulation of PD-L1, primarily expressed by macrophages infiltrating the tumor stroma and to a lesser extent by tumor cells. Correspondingly, upon hierachical cluster analysis, MSI positive tumors aligned with the hyperinflamed phenotype. Thus, MSI tumors from our cohort showed evidence of a highly immunosuppressed microenvironmenet both in terms of high PD-L1 expression and pronounced myeloid inflammation. Importantly, MSS CRP-high tumors exhibited the lowest densities of lymphoid immune cells and PD-L1 expression, yet appeared remarkably myeloid inflamed compared with both MSI CRP-

high and MSS CRP-low tumors. The next apparent question in this regard is how these differences in the tumor immune composition impacts clinical outcome. Given the limited sample size of our cohort, subgroup survival analysis distinguishing between MSS and MSI CRP-high patients, was not performed. However, analyzing the group as a whole, the risk of death or recurrence was significantly higher for CRP-high patients (MSS and MSI patients combined) compared to the CRP-low group of patients ($p=0.047$). Thus, it is possible that the prominent immunosuppression seen in systemically inflamed MSI positive tumors may outperform the beneficial lymphoid inflammation and blunt effective anti-tumor immune responses, at least without treatment with ICI. Alternatively, given the aforementioned contradictory roles of myeloid immne cells, it might also be hypothesized that in the MSI positive systemically inflamed tumors the myeloid immune cells are skewed towards a more anti-tumoral functional phenotype (i.e. M1/N1) whereas they are polarized towards the other spectrum (i.e. M2/N2) in systemically inflamed MSS tumors, exerting predominant tumor supportive functions. Given that none of the CRP-low patients from our cohort were MSI-positive we were unable to compare the immune infiltrate between systemically inflamed (CRP-high) and non-inflamed (CRP-low) MSI-positive tumors. Thus, future studies directly investigating the immune landscape in MSS compared to MSI tumors with and without systemic inflammation and how this affects survival and even treatment outcomes, represents an important next step to further address this intruiging aspect.

Another important aspect that is not covered by the present work is the evolutionary and temporal impact of systemic inflammation in tumor development. Our data only represent a snapshot of the immunological process occurring locally in the tumor. However, given that the tumor immune infiltrate is highly dynamic, it is of considerable interest to investigate what happens over time to elucidate the evolutionary trajectory of the immunological response, which requires repeated and longitudinal measurements. Such insights can provide valuable information and contribute to our understanding of how systemic inflammation affects tumors in different stages and disease contexts, and further, how this can be exploited for therapeutic purposes.

5.3 The heterogeneity of tumors and infiltrating immune cells – towards a more comprehensive definition and characterization of the immune response in cancer.

Within this work we developed a FFPE- and mIHC-based whole slide imaging platform enabling accurate visualization and assessment of seven simultaneously expressed immune markers with preserved tissue architecture and cellular context. Utilizing this platform, it became evident that considerable heterogeneity existed both between and within tumors in terms of the composition and distribution of immune cells as well as in tissue morphology. Specifically, some tumors appeared heavily immune infiltrated with abundant immune cells distributed rather evenly throughout the tumor whereas other tumors harbored a more patchy immune infiltrate comprising areas with prominent immune infiltration combined with areas devoid of immune infiltration. Finally, there were tumors that exhibited dense tumor tissue, less stroma and modest immune infiltration.

Indeed, accumulating studies point towards intratumor heterogeneity as a clinically meaningful phenomenon linked to treatment resistance and poor patient outcomes in multiple solid tumor types (124, 125). While most research have focused on the genetic source of intratumor heterogeneity, leading to the presence of genetically distinct subclones of tumor cells, it is increasingly recognized that epigenetic mechanisms as well as microenvironmental inputs also play a profound role in shaping tumor phenotypes and may contribute to further tumor diversification and affect treatment outcomes (124, 126). The existence of such heterogeneity, both in tumor tissue and the microenvironment including the distribution of immune cells and molecular markers, highlights the importance of analyzing whole tumor sections over TMAs. While the use of TMAs has a notable advantage, enabling biomarker screening of large materials, the regional tissue sampling and small core biopsies only represent snapshots of the whole tumor and may not adequately capture the true complexity of the tumor and its environment (102).

Whole slide imaging (WSI) offers a major advantage allowing for comprehensive analysis of spatial patterns as it preserves tissue topology and cellular context (102). This is in contrast to various single cell technologies and transcriptomics, which might

not capture this information (127). In this regard, it is increasingly recognized that not only the composition of the immune infiltrate matters but also immune cell location and distances between cells and components of the TME have been shown to hold prognostic and predictive information suggesting that these metrices reflect clinically meaningful tumor-host interactions (111, 128-131). The first studies in the field showed that the density of specific immune cells in different tumor compartments (typically IM and TC) correlate differentially with patient outcomes (131, 132). Later, more advanced spatial analyses considering cell proximity and intercellular distances were developed (111, 128, 133). For example, a study in NSCLC evaluated the spatial distribution of various adaptive immune cells by multiplexed fluorescence IHC demonstrating that Tregs (double positive CD8+ FOXP3+ T cells) in close proximity to tumor cells correlated with poor survival (134). Another study, also in NSCLC, revealed marked heterogeneity of TAM populations in different tumor compartments, showing that close proximity of M2-TAMs to tumor cells, particularly predominant at the IM, was linked to poor survival whereas the inverse was observed for M1-TAMs (i.e. longer distance to tumor cells) (129). Similarly, a study in gastric cancer, focusing on the spatial distribution of regulatory T cells, revealed that a close spatial relationship between $CD4^+FOXP3^+$ T cells and $CD8^+$ T cells correlated with prognosis (135).

Herein, we provide a framework for analyzing spatial relationships using a platform that combines multiplex chromogenic IHC and computational analysis, allowing for characterization of the spatial distribution of selected adaptive and innate immune cells within the TME. Adherent to the approach of previous studies in the field (111, 132), we quantified immune cells within the IM and TC separately followed by distinguishing between cells directly infiltrating the tumor tissue (classified as intratumoral) and cells embedded within the tumor stroma (classified as stromal). Next, guided by pan-CK stained tumor cells, we were able to perform more specific spatial analyses by first, analyzing the density of selected immune cells in close proximity to tumor cells (a brim of 20 μm around tumor islets), and second, estimating the average distance between immune and tumor cells in order to evaluate whether these metrics correlated differentially with systemic inflammation. Given the proof-of-

concept design of our study, we restricted the spatial analysis to CD8+ T cells (partly because most previous research have focused on T cells) and the neutrophils (due to the strong correlation with elevated CRP demonstrated in the other analyses). Notably, we found, as mentioned above, that tumors from systemically inflamed patient harbored significantly higher density of neutrophils in close proximity to tumor cells, particularly evident in the TC, compared to the non-inflamed patients. Given the utilization of digital analysis, the same concept could readily have been applied to the other immunostained markers and generated an abundance of spatial data. Moreover, adding another layer of complexity, tissue morphological features including stromal components and tumor vasculature could potentially also be integrated in the analysis using available applications within the software featuring machine learning and other AI-based algorithms allowing for assessment of the spatial context in a multimodal and deeper manner. By correlating such metrics with clinical data integrated in the CRC database, we can gain potential new insights regarding tumor-immune and other environmental interactions taking place in the TME, and importantly, how this affect disease trajectories and patient outcomes. Indeed, our cohort was too small to perform such comprehensive analyses, thus the framework we provide here needs to be applied to a much larger set of tumor samples to generate meaningful and robust data in this regard. Additionally, compared to emerging technologies such as multispectral imaging and more sophisticated multiplex immunofluorescence techniques, our platform cannot provide the same in-depth information regarding the spatial architecture of the tumor immune microenvironment that can be obtained from these advanced methodologies. Nevertheless, our platform utilizes chromogenic IHC and light microscopy scanners, which are commonly available in most pathology laboratories combined with a relatively feasible workflow, despite applying commercially available software. This differs from the more expensive and highly advanced equipment and expertise required for many of the newer techniques, which might limit their widespread use despite the substantial amount of information and potential new knowledge that can be obtained with these platforms. With the rapidly expanding field of AI-based tools currently being leveraged for various healthcare purposes, there are high expectations in the immuno-oncology community that such

approaches substantially can deepen our understanding of the complex crosstalk and heterogeneity that exists within the TME (109). Indeed, emerging studies are coming out utilizing various AI-based algorithms for pattern recognition of histopathological images merged with clinical information in order to predict patient outcomes and provide the clinicians with a tool that may allow for more accurate diagnosis and personalized treatment decisions (136, 137).

5.4 Moving beyond the biomarker framework – may CRP play a direct and active role within tumors?

Considering emerging evidence suggesting that CRP itself plays a direct and active role locally in inflammatory environments following a conformational switch from the pentameric form (pCRP) to its highly pro-inflammatory monomeric isoform (mCRP), we hypothesized that CRP, in its monomeric form, might be present in the TME of our colon cancer patients. We found that mCRP was abundantly expressed in tumors from systemically inflamed patients. Importantly, mCRP appeared tumor-specific being expressed exclusively within tumor tissue whereas adjacent normal colon mucosa showed no positive mCRP staining. Double IHC and IF revealed colocalization of mCRP with inflammatory cells, most pronounced with neutrophils followed by endothelial cells and to some extent also tumor infiltrating macrophages. Moreover, mCRP seemed to correlate with areas of necrosis and in some tumors, appeared embedded in the connective tissue of the tumor stroma, suggesting an interaction with components of the ECM. Intriguingly, some tumor cells also seemed to express mCRP. Although the existence and functional roles of the different CRP isoforms have been rather extensively studied in cardiovascular and neurodegenerative diseases, little is known about their role in cancer. Recent reviews have theoretically hypothesized the notion that the less soluble, tissue-associated monomeric form of CRP may play an active and pro-inflammatory role within tumors, yet with ambiguity of whether this may support a beneficial anti-tumoral response or cause excessive detrimental inflammation and a tumor promoting response (96, 97). Thus, to our knowledge, this is the first study specifically exploring this intriguing concept using human tissue in a clinical context of cancer patients. However, it should be mentioned that a series of

experimental studies have been conducted, prior to the discovery of the distinct CRP isoforms, where CRP was applied directly into primarily murine cancer models (138). Contrary to our hypothesis, these studies consistently found evidence of tumor regression and potential anti-metastatic effects following CRP injection. However, in these experimental models, high amounts of CRP were added, frequently as “bolus” doses administered over a relatively brief duration of time, thereby mimicking more of an acute inflammatory response. This is in contrast to our cancer patients having persistently elevated levels of CRP, indicative of a state of chronic systemic inflammation. Within this context, the inflammatory response does not resolve and mCRP may successively fuel the tumor leading to excessive inflammation and presumably detrimental tumor-supportive conditions.

In this regard our findings and hypothesis are consistent with previous studies delineating the role of mCRP primarily in cardio- and cerebrovascular disorders (139). Although they are different pathological conditions, they share the same cardinal feature of cancer as they are related to or driven by inflammation. Specifically, in vitro, and in vivo studies in models of cardiovascular disease have shown that mCRP and not pCRP accumulates in inflamed and infarcted/ischemic myocardial tissue and in human arteriosclerotic plaques, but not in healthy, non-inflamed or non-infarcted tissue (92, 105). Moreover, it has been shown that mCRP formation is dependent on in-situ dissociation of pCRP, primarily induced by phosphocholine (PC) residues that become exposed on cells with perturbed plasma membranes such as apoptotic or necrotic cells or cells that have been activated (platelets, endothelial and/or inflammatory cells) (94). The latter relies on phospholipase A2 (PLA2) activity, in which PC residues get accessible for pCRP binding (140). Recent data have shown that the dissociation process comprises formation of an intermediate form designated mCRP* where the pentameric structure of the molecule is retained, yet with antigenicity and functional properties similar to that of the fully dissociated, structurally distinct monomeric, modified form (mCRP) (92). Importantly, following this conformational switch, mCRP specific and functionally active neoepitopes become exposed, accounting for the potent pro-inflammatory properties of the monomeric isoform (94). Key pro-inflammatory functions of mCRP include activation of inflammatory cells such as macrophages,

monocytes and neutrophils leading to the production of pro-inflammatory cytokines, such as IL-6, IL-8 and MCP-1 (monocyte chemoattractant protein-1) and the integrin Mac-1 (macrophage-1 antigen), which beside the local inflammatory effects promote chemotaxis and recruitment of neutrophils and monocytes to the inflamed area, thus further amplifying the inflammatory response (95, 141, 142). Moreover, mCRP has also been shown to inhibit neutrophil apoptosis (143). At the molecular level, in vitro studies have identified a single sequence motif (a cholesterol binding sequence) as the primary recognition site of mCRP enabling interaction with many different cellular and non-cellular ligands including lipid raft microdomains of plasma membranes and extracellular matrix proteins (fibronectin, collagen, laminin) (144, 145). Cells that have been activated by mCRP have been shown to up-regulate intracellular signaling of pro-inflammatory pathways including the transcription factor NFκB, which plays a pivotal role in inflammatory responses (146).

In the context of myocardial infarction, atherosclerosis and ischemic stroke it has been shown that the above-mentioned effects of mCRP together with activation of complement (mainly C1q), generation of ROS and interaction with endothelial cells (upregulation of adhesion receptors) and platelets (pro-thrombotic effect), contribute to excessive local inflammation and increased tissue damage, negatively affecting patient outcomes (94, 105, 141). Notably, using a rat model of myocardial infarction, blocking of PLA2 activity utilizing the pharmacological inhibitor 1,6-Bis(phosphocholine)-hexane (1,6-bis PC) prevented CRP dissociation and subsequent mCRP deposition in infarcted tissue (105). This resulted in significant reduction of the localized inflammatory response and tissue injury, thus providing evidence for the ubiquity of the pCRP-mCRP dissociation process and pivotal role of mCRP in mediating the pro-inflammatory effects of circulating pCRP (105). Importantly, preexisting inflammation of the tissue was necessary for activation of the pCRP intrinsic inflammatory properties as no mCRP nor exaggerated inflammation or tissue damage were observed in non-inflamed, non-injured control tissue (105). Correspondingly, a study injecting purified pCRP to assumingly healthy individuals did not show any detectable pro-inflammatory or proatherogenic effects, providing further support that native CRP in its circulating pentameric form, does not exert pro-inflammatory bioactivities (147).

Overall, by recognizing that CRP is a dynamic molecule undergoing conformational changes at sites of inflammation, transforming to a highly biologically active form capable of exerting direct pro-inflammatory effects within tissues, a new perspective on CRP as a biomarker emerges (96). Taken into the context of cancer, our findings align with the previously discussed data and support the hypothesis that patients with persistently elevated levels of circulating CRP experience a continuous dissociation of the pentameric molecule into its monomeric subunits. This occurs locally at the tumor site as pCRP binds to exposed PC residues on cells that have been activated due to the inflammatory environment of the tumor. Conceivably, once formed, the blood insoluble mCRP accumulates within the tumor where it may interact directly with various cells and components of the TME because of its versatile binding capacity. The localized mCRP induced pro-inflammatory effects, together with further recruitment of inflammatory cells might fuel and amplify the tumor inflammatory response. The outcome of this, however, remains elusive and brings us into the discussion on whether inflammation is good or bad, which might not be entirely black and white as it depends on the context in which it occurs (118). Inflammation per se is not necessarily bad. However, persistent, or uncontrolled inflammatory responses are presumably detrimental and will most likely lead to unfavorable outcomes. Given that the CRP dissociation process and subsequent mCRP formation and tissue deposition is dependent on pre-existing inflammation, it may be hypothesized that in tumors, mCRP functions as an amplifier, perpetuating the already established inflammatory response regardless of whether it is a tumor-supporting or tumor-inhibiting response. Excessive inflammation in cancer is most presumably detrimental for patient outcome as it may stimulate tumor growth and metastasis at least in the chronic non-resolving state, which is the case in systemically inflamed cancer patients (118).

Concerning the specific functional roles of mCRP in the TME, this needs to be addressed in further studies specifically designed to investigate the functional aspect. Given the proof-of-concept design and methodological approach of our study, the data we present are limited in this regard. Nevertheless, our findings, supported by the above discussed prior studies, suggest an interaction of mCRP with inflammatory cells, particularly evident for the neutrophils. Specifically, we found a significant positive

correlation between tumor mCRP expression and neutrophil density (Spearman correlation 0.57, 95%CI 0.28-0.77, $p<0.001$). Correspondingly, double IHC and IF revealed close proximity of mCRP and neutrophils and prominent colocalization of mCRP within highly neutrophil infiltrated tumor areas suggestive of a functional relationship. Considering the findings of the second paper highlighting a strong, potentially driving role of myeloid inflammation and neutrophils in particular, in the TME of systemically inflamed patients, the notable correlation between tumor-expressed mCRP and neutrophils complies with this notion and adds a new and interesting perspective on why systemic inflammation is so detrimental for patient outcome.

With regard to the pattern of mCRP expression within our cohort of colon tumors, we also observed that mCRP seemed to coincide with areas of necrosis. This is consistent with findings in prior studies in cardiovascular disease and the aforementioned murine cancer models (105, 148). Again, the question arises whether this is beneficial or detrimental in a growing tumor. From one side, it can be hypothesized that increased production of ROS, proteolytic enzymes and pro-inflammatory cytokines from neutrophils and macrophages activated by mCRP, together with the retention of leukocytes, may confer cytotoxicity and a favorable anti-tumor response, similar to an acute inflammatory response (97). However, unless this innate immune cell mediated response is accompanied by an adaptive T cell mediated tumor-specific response, it is unlikely that it will lead to overall effective antitumor immunity. More conceivable then, such an unresolved response with abundant tumor necrosis may rather contribute to an even more hostile and predominant tumor permissive environment because of the propagation of tissue damage that can stimulate persistent inflammatory signaling with similarities to the non-resolving (chronic) wound healing response (149). Additionally, previous research has shown that mCRP can induce aberrant angiogenesis with the formation of leaky and fragile microvessels that might lead to insufficient blood supply to areas of the tumor, that potentially also may account for the necrotic capacity of mCRP (100, 150). Notably, tumor necrosis is a common trait found in aggressive, typically rapidly growing tumors (151). The occurrence of tumor necrosis has been closely linked to an acidic, hypoxic, and metabolically stressed environment, leading to

an immunosuppressive state, poor prognosis, and treatment resistance (152-154). Interestingly, without considering the contribution of the distinct CRP isoforms, previous studies in CRC have reported a positive correlation between high abundance of tumor necrosis and systemic inflammation and reduced local, primarily adaptive, tumor immune infiltration (20, 155). A particular role of the multifunctional, pro-inflammatory cytokine IL-6 has been highlighted in this regard proposed as a potential causal factor linking tumor necrosis to systemic inflammation (20, 149). Up-regulated IL-6 signaling can occur because of the hypoxic stress and stimulation of inflammatory pathways that accompanies necrotic cell death (149). Given that IL-6 is the primary inducer of hepatic CRP production, persistent IL-6 signaling originating from the tumor (although tumor necrosis may not be the sole source) may lead to increased synthesis and elevated levels of circulating pCRP, thus providing a positive feedback loop that potentiates and sustains the process (59, 155). Thus, it is conceivable that the capacity of mCRP to induce tumor necrosis at least in the chronic inflammatory state, may contribute to excessive inflammation and tissue/tumor destruction supporting a more aggressive tumor immune phenotype and negatively affect patient outcomes.

Overall, while most clinicians are aware of CRP as a biomarker, the notion that CRP itself, in its monomeric form, may play an active, potentially driving role in the pathogenesis of various inflammation-linked diseases, and as we suggest, potentially also in cancer, may, if verified, change the way we currently understand and even treat systemic tumor-associated inflammation in the future.

Indeed, several groups have looked into the intriguing concept of therapeutically targeting the CRP system to diminish or selectively abrogate the pro-inflammatory bioactivities of CRP in conditions where this plays an unfavorable role (92, 100, 140). To this end three main approaches have been proposed: 1) targeting the dissociation process of pCRP to mCRP 2) blocking mCRP binding to cell surfaces 3) apheresis of circulating pCRP in conditions were high levels of circulating pCRP has deleterious implications (used in hospitalized patients with myocardial infarction and COVID-19) (100, 140, 156). As to the first, controlling or preventing the conversion of pCRP to mCRP has been proposed as a feasible strategy for diminishing the mCRP mediated

pro-inflammatory bioactivities while preserving the anti-inflammatory effects of pCRP(96, 140). The small molecule 1,6-bis(phosphocholine)-hexane was the first compound developed for this purpose. It was designed to specifically target the PC-binding pocket of pCRP to prevent the molecule from attaching to its ligands and thus inhibit dissociation and subsequent mCRP tissue deposition (140). Although several in vitro and in vivo experiments using this compound, primarily in models of myocardial infarction, demonstrated efficacy, later studies revealed that the pharmacokinetics of the drug were not compatible with the use in humans (139). Inhibition of the enzyme PLA2, which plays a major role for PC residues of plasma membranes to become accessible for pCRP ligation, is an alternative approach (92). To date, several small-molecule inhibitors have been developed and tested in clinical trials, primarily in cardiovascular disease, but only few of the agents have shown promising activity so far (140). Of note, a large phase III study using the PLA2 inhibitor darapladib did not meet its primary endpoint of reducing the risk of cardiovascular death, myocardial infarction, or stroke in patients with coronary heart disease (157). Nevertheless, recent investigations using newer technologies such as in silico modeling and x-ray crystallography have revealed the exact amino acids and steric features of the pCRP binding pocket, which have provided important information for the design of novel agents with an improved pharmacokinetic and therapeutic profile (140, 158). Moreover, regarding inhibition of mCRP-mediated cellular responses, preliminary data from murine models of rheumatoid arthritis and dementia have shown promising results using mCRP specific antibodies that block the interaction of mCRP with its effector cells leading to ameliorated mCRP downstream cellular signaling and improvement of disease-specific symptoms (100).

Moving back to cancer, transferring the concept of specifically targeting CRP to limit mCRP-mediated detrimental inflammation into cancer patients presenting with systemic inflammation, introduces a new perspective and potential novel therapeutic approach in oncology. Based on the proof-of-concept data presented here, we can only make assumptions on this intriguing topic and provide a foundation for further investigations. Noteworthy in this regard, was the observation that mCRP appeared

exclusively tumor specific, as normal colon mucosa close to the tumor showed no mCRP immunopositivity. If verified, this finding supports the idea of mCRP as a potential target for cancer therapeutics, either as a new anti-inflammatory strategy or as an antibody compounded with a chemotherapeutic agent, that can exert its effect in the tumor only while sparing healthy tissue and thus reduce toxicities.

6. Conclusions

Although systemic inflammation unequivocally has been associated with inferior survival outcomes in various types of cancer, the prognostic role in resectable metastatic disease is less clear. Within this work, we evaluated the prognostic impact of preoperatively measured CRP in a large multicenter cohort of stage IV CRC patients undergoing potentially curative resection of liver metastases. Importantly, we found that CRP was a strong predictor of compromised survival and proved to be superior to other inflammatory markers, including s-albumin and the compounded GPS-score, as well as the reported clinicopathological characteristics. Based on these data, we suggest that CRP may provide complementary prognostic information to the traditional tumor-based risk factors. Integrating CRP into the clinical management of CRC patients with liver metastases may allow for better risk stratification and help guide treatment strategies.

Overall, these findings further contribute to the compelling evidence that systemic inflammation, particularly evident by elevated levels of CRP, profoundly affects prognosis in CRC patients in different disease contexts and align with the primary aim of this work focusing on why elevated CRP is so detrimental for patient outcome.

With the intention of elucidating the underlying tumor immune microenvironment in systemically inflamed and non-inflamed colon cancer patients, as assessed by levels of circulating CRP, a FFPE tissue-based analysis platform combining multiplex IHC and digital whole slide imaging was developed. We show that this platform provides an accurate view of the TME covering the entire tissue section and preserves tissue topology allowing for identification, quantification, and mapping of 6 simultaneously expressed lymphoid and myeloid immune cells alongside the immune inhibitory molecule PD-L1 and cytokeratin as a tumor marker. As proof-of-concept, we demonstrate that this method is feasible utilizing FFPE tissue from primary resected colon cancer patients enabling comprehensive characterization of tumors with capability of capturing existing intratumoral heterogeneity in terms of selected cellular

and spatial patterns and tissue morphological features. By combining the mIHC data with clinical information, we revealed that tumors from systemically inflamed patients harbored a more myeloid-dominated TME compared to non-inflamed patients with significantly higher densities of neutrophiles in particular, which complies with our hypothesis.

Importantly, we showed that a high innate immune score (compounded densities of CD66b+ neutrophils and CD68+ macrophages) correlated with elevated CRP, regardless of the adaptive immune score (compounded densities of CD8+ and CD4+ T cells) suggesting that it is the presence of myeloid inflammation and not the absence of lymphoid inflammation that associates with systemic inflammation.

Finally, as emerging data suggest the existence of different isoforms of CRP, we investigated whether colon tumors expressed the monomeric form of CRP (mCRP). Utilizing a mCRP-specific antibody it could be demonstrated that mCRP was abundantly present in tumors from systemically inflamed patients and appeared tumor-specific being expressed only within tumors whereas adjacent healthy colon mucosa showed no mCRP positivity. Intriguingly, double IHC revealed prominent colocalization of mCRP and neutrophils in particular, as well as endothelial cells lining intratumoral vessels and areas of necrosis, indicating that mCRP may play a direct role in the microenvironment of tumors.

Taken together, this work highlights the importance of acknowledging systemic inflammation in cancer patients and provide support for utilizing CRP as a valuable tool in patient risk stratification that may help facilitate treatment decisions. The method presented here provides a framework for improving our understanding of how elevated CRP affects the local tumor immune microenvironment. It suggests a profound role of myeloid inflammation, particularly neutrophils, in systemically inflamed colon cancer patients. The current study offers a novel perspective on the detrimental effects of elevated CRP levels in cancer patients. The evidence presented shows that pro-inflammatory monomeric CRP may be present in the TME supporting the notion that CRP is not simply a passive bystander, but an active participant in

tumor formation. Further progress in this area is needed to fully understand the pathophysiological role and thus unlock the clinical utility of CRP as a biomarker in cancer patients.

7. Perspectives

7.1 The clinical utility and future perspectives on CRP as a biomarker

At the present time there is no doubt that inflammation plays a significant role in oncology, encompassing both favorable and unfavorable effects, occurring within the tumor microenvironment as well as a systemic host response. While previous work in the field primarily has focused on systemic and local tumor immune responses separately, we were interested in assessing the relationship between the two and explore the tumor immune microenvironment in patients with and without elevated levels of CRP with the overarching aim of improving our understanding of why systemic inflammation is so detrimental for patient outcomes. We found that systemically inflamed patients harbored a more myeloid-dominated TME compared to non-inflamed patients, suggesting a particular strong, potential driving role of neutrophils. Importantly, we show that it is the presence of myeloid inflammation rather than the absence of lymphoid inflammation that correlates with systemic inflammation.

While our patient cohort was limited, more research is necessary to further decipher how systemic inflammation and elevated CRP impact the local TME and vice versa. This will help fully evaluate the role and potential applications of CRP in the clinical management of cancer patients. Improved understanding of the inflammatory pathways that associate with poor clinical outcome may not only strengthen CRP as a biomarker, allowing for better patient stratification but might also inform the development of novel therapeutic strategies. Meanwhile, our preliminary data suggest that CRP can be used as an informative tool reflecting features of the immune response occurring at the tumor site. By interpreting CRP as a readout of the immunological state of tumors as well as a systemic host response, it may provide a more holistic view of the inflammatory profile of cancer patients. Combined with traditional tumor-based risk factors this strategy could allow for a more comprehensive assessment of individual cancer patients based on both tumor and host related factors.

Of particular interest, recent data derived from patients treated with ICI have revealed that contrary to the prevailing perception that treatment responses solely rely on pre-existing tumor specific T-cells locally in the tumor, immunotherapy actually drives de novo peripheral T cell responses, suggesting priming of new T cells as an important mechanism of action critical for treatment efficacy (159, 160). These data provide support for the notion that the localized anti-tumor immune response cannot exist without a coordinated systemic response (91). Assuming that a similar correlation also exists for detrimental inflammatory responses, a more holistic approach to cancer immunity emerges. Acknowledging the contribution of both local and systemic inflammation for the generation of either beneficial or detrimental immune responses in cancer patients, may prove to be one important step towards more broadly effective immunotherapeutic approaches.

With more available treatment options, particularly in the rapidly expanding field of cancer immunotherapy, the need for precise and preferably non-invasive biomarkers to select patients for the most appropriate treatment strategy, is imperative to facilitate bedside decision-making and ultimately improve patient outcomes. Nevertheless, in a time where healthcare and precision medicine have become rather complicated and expensive, the simplicity of measuring CRP, which is a validated, inexpensive, and readily available test, paradoxically seems to preclude its use. However, just because it is simple does not mean it cannot be a powerful and informative tool. It is worth mentioning again that conducting additional research with a larger sample size, encompassing diverse tumor types and stages, is necessary to understand the full potential and clinical applicability of CRP as a biomarker. Preferably, such studies should specifically focus on examining CRP as a predictor of the immune response in the TME and assess its ability to monitor patients and treatment outcomes non-invasively. Incorporation of CRP into precision medicine approaches, together with other metrics (genetic profiling, other immune and biochemical biomarkers, clinical parameters, and imaging) might enable more refined treatment decisions tailored to individual patients. Equally important as identifying patients more likely to respond to specific therapies, is it critical to identify patients more likely of poorer outcomes that instead should be allocated to alternative approaches to optimize quality of life and

survival outcomes in a personalized manner. An important next step is to implement CRP into prospective clinical trials both as a stratification factor, further evaluating and validating CRP as a prognostic and predictive marker in different clinical settings, as well as investigating therapeutic interventions directed specifically towards the systemic inflammatory response. Given that sustained tumor-associated systemic inflammation associates with an array of clinical symptoms and pharmacokinetic alterations as discussed previously, quality of life assessment and pharmacokinetic tests could also be performed within these studies to further understand how systemic inflammation impacts patient outcomes and guide the development of anti-inflammatory strategies. Preferably, clinical trials should be conducted over longer time periods utilizing longitudinal measurements to assess dynamic changes in CRP throughout disease trajectories and follow-up and in response to treatments. As opposed to many other blood or tissue-based biomarkers, assessing CRP, which is quick, convenient, and objectively measured, may allow for regular measurements and thus timely evaluation of patients enabling early identification of treatment resistance or disease progression that may lead to adjustment of treatment plans. Besides unraveling the clinical value and practical applications of CRP, these more comprehensive studies might also provide insight into the evolutionary aspect and underlying drivers of systemic inflammation in cancer patients.

7.2 Framework for comprehensive characterization of tumors and individual patients

While immunotherapy with ICI has revolutionized the field of cancer treatment, it is becoming increasingly evident that such T-cell reliant-only approaches are not sufficient for mounting effective anti-tumor immunity in a large proportion of patients, including MSS CRC (161). Moreover, the composition and characteristics of the TME have emerged as critical components for the generation of either tumor-supportive or tumor-inhibiting immune responses (161). Nevertheless, harnessing the adaptive arm of the immune system still represents the main focus for clinically available immunotherapies and the tumor immunology field. Thus, there is an increased demand for in-depth insight into the TME to gain more knowledge of how the TME can be

targeted for improved antitumor immune responses in a broader population of patients. We highlight the importance of more comprehensive characterization of the tumor immune context capturing existing intratumor heterogeneity both in terms of cellular composition and spatial architecture throughout the TME. While the framework we present here is less sophisticated compared to emerging high-dimensional techniques, our FFPE-based method has an advantage in its feasibility being compatible with most digital pathology platforms within a reasonable time and cost frame and the ability to analyze whole tissue sections. Further development of platforms and protocols for the use in research and clinical pathology enabling in-depth information of the tumor and its environment encompassing the heterogeneous nature of the TME, represents an important area for providing more personalized treatment approaches based on unique tumor immune features of individual patients. Preferably, these more advanced frameworks should be integrated with information on the patient`s systemic inflammatory state together with other tumor and patient specific metrices to obtain a comprehensive picture of individual disease status that may translate into more personalized treatment plans and better patient management and outcomes. Moreover, efforts should be made to standardize and validate the protocols and procedures utilized such as handling of tissue, antibodies, scanners, pre- and post-processing steps of digital analysis to assure feasibility and reliable and comparable results across laboratories. Ensuring applicability and affordability of such platforms, preferably using devices and expertise available within most pathology labs, will also be crucial for these technologies to become accessible for caretakers serving a broad population of cancer patients. Finally, with the rapidly expanding field of AI-based tools and software solutions, the potential applications of image-based platforms extend far beyond automated image analysis, which we used in this work. The extraordinary ability of pattern recognition from pathology images merged with clinical data together with the high-speed capacity for analyzing large datasets, make these tools promising for identifying novel prognostic and predictive biomarkers as well as elucidating tumor biology in a highly sophisticated manner. Although some AI-based prognostic tools already have entered the clinical arena, the field is only in its infancy. Yet, it is expected that AI-based approaches will be an integrated part of digital pathology and

precision oncology with a multitude of different scopes and applications in the very near future.

7.3 Rationale for anti-inflammatory treatment

Detrimental pro-tumorigenic inflammation occurring both in the local tumor microenvironment as well as a systemic host response play a profound role for cancer development and progression affecting treatment and survival outcomes and may lead to adverse clinical symptoms and negatively impact tolerance of therapies and quality of life. The next apparent question is whether anti-inflammatory treatment strategies that specifically target and dampen detrimental tumor-supportive inflammation, preferentially before stimulating beneficial inflammation, can be leveraged for cancer patients aiming to improve overall clinical outcomes.

While the use of more broad, non-specific anti-inflammatory compounds such as NSAIDs and steroids primarily play a role for preventive purposes and in symptom management (as discussed earlier), there are currently no established anti-inflammatory strategies applied to cancer patients that specifically target key inflammatory pathways. Therapeutic agents that specifically inhibits pro-inflammatory mediators such as IL-6 and TNF-alpha have been tested in preclinical and clinical trials, primarily in conjunction with conventional anti-cancer treatments (both chemotherapy and immunotherapy), although with mixed results (77). Thus, more research is needed further interrogating the tumor immune landscape taking both local and systemic inflammation into consideration, to improve our understanding of how anti-inflammatory treatment strategies can be leveraged for individual cancer patients. Apart from identifying novel therapeutic targets, future studies must address important questions such as which patients will benefit the most from anti-inflammatory interventions, which may vary based on tumor type, stage, and patient characteristics. Understanding how to combine anti-inflammatory treatments with conventional cancer therapies including T-cell directed immunotherapy, will also be crucial in pursuing improved anti-tumor responses in a large proportion of cancer patients.

Our data highlight a profound role of myeloid cells in the microenvironment of tumors, particularly in the context of systemic inflammation. Importantly, myeloid immune cells have been proposed as major players in the TME favoring an immunosuppressive state believed to compromise the effect of current immunotherapies (162). Given the described plasticity and contradictory roles that exist for myeloid cells, an exciting question in this regard is whether this can be translated into the clinic and exploited for therapeutic purposes. Although still in its infancy, experimental and early phase clinical studies are currently being conducted aiming at manipulating and "re-educating" myeloid immune cells to become more immunostimulatory and less immunosuppressive and rejuvenate their inherent capability of anti-tumor functions (77, 163). Indeed, further studies need to be conducted to delineate the immune landscape of tumors focusing on how myeloid immune cells contribute to the overall immune response in cancer patients. This will help determine whether these versatile cells can be exploited for therapeutic purposes and how such therapies can be sequenced and/or combined with other cancer treatments.

Besides focusing on developing pharmaceutical strategies to diminish systemic inflammation, efforts should also be directed towards understanding how lifestyle interventions, such as exercise and dietary modifications, may affect systemic inflammation in cancer patients. The gut microbiome has emerged as a particularly promising new frontier in the field with accumulating evidence supporting its ubiquitous role for maintaining overall health with significant implications for a variety of different disease conditions, including cancer and most notably in CRC (65, 164). Accumulating evidence supports the profound role (both protective and deleterious) of the "polymorphic microbiome" for cancer development, progression, and treatment outcomes (165, 166). The "polymorphic microbiome" refers to the diverse microbes, including bacteria, fungi, and viruses that make up microbial ecosystems (microbiomes) across the body, and was recently proposed as a new enabling hallmark of cancer (165, 166). Recent data have shown that systemic inflammation may influence the composition and function of the gut microbiome leading to alterations in the microbial diversity and metabolic activity and potential enrichment of pathogenic strains, collectively termed gut dysbiosis (164). Notably,

dysbiosis has been associated with increased mucosal permeability (leaky gut), alterations in immune functions and affecting efficacy of treatment with ICI as well as treatment related toxicities (167). On the other hand, imbalances in the gut microbiome may affect the production of bioactive metabolites such as short-chain fatty acids that can trigger inflammation and modulate systemic immune responses (167). Thus, improved understanding of the intricate interplay between the gut microbiome and systemic inflammation may provide valuable insights into the causal aspect of tumor-associated inflammation, and importantly, how the microbiome can be leveraged and modulated to benefit cancer patients. Indeed, several studies are currently underway exploring various approaches specifically targeting the gut microbiome such as fecal microbiota transplantation, prebiotics, probiotics and dietary modifications, alone or in conjunction with immunotherapy (164). Nevertheless, this exciting field is only in its early days, and it is expected that ongoing and future research will translate into novel therapeutic strategies aiming to improve patient, and particularly immunotherapy outcomes in a variety of different tumor types and clinical settings (164).

7.4 The role of CRP in cancer – a new approach for anti-inflammatory treatment?

Finally, an intriguing new perspective in the context of targeting tumor-associated systemic inflammation emerges with the discovery of the different CRP isoforms. As discussed previously, various therapeutic CRP lowering strategies have been proposed, primarily in cardiovascular disease, designed to selectively abrogate the pro-inflammatory bioactivities exerted by tissue-associated mCRP while retaining the anti-inflammatory functions of circulating pCRP. Although our data needs to be confirmed in a larger material, the idea that CRP itself in its monomeric form, is present within tumors, primarily in patients with elevated levels of serum CRP, playing an active pro-inflammatory role potentially fueling the inflammatory response, places CRP as a biomarker in a whole new perspective. Besides adding to our understanding of why persistently elevated level of CRP is so detrimental for prognosis, it provides additional support for why efforts should be made to develop strategies specifically

tailored towards diminishing the systemic inflammatory response to improve outcomes in patients where this is present.

Specifically, building on the intriguing finding that mCRP appeared tumor specific with no mCRP detected within adjacent normal colon mucosa, the hypothesis emerges that mCRP directed therapies could exert its effects exclusively in the tumor without adversely affecting other organs and healthy tissues and thus reduce toxicities. Possible strategies for mCRP targeted therapies could either be development of small molecule inhibitors that directly bind mCRP to abrogate the downstream pro-inflammatory effects of the monomeric form, or designing drugs that uses mCRP as a vehicle for chemotherapeutic agents delivering the cytotoxic agent exclusively to the tumor. Finally, based on previous work in the field reporting difficulties in successfully developing pharmaceuticals that directly inhibit mCRP and/or downstream pro-inflammatory functions, interference with the pCRP-mCRP dissociation process also represents a promising strategy to diminish mCRP mediated inflammation in cancer patients.

Nevertheless, we still have a lot of work to do before we can reach the stage of developing direct CRP-targeted therapeutics specifically designed for cancer patients. Overall, our data needs to be confirmed and expanded on before we can draw any finite conclusions on whether this represents a clinically meaningful concept. Next, several questions regarding the identification of the specific target patient population, timing, and combination with other oncological treatments, must be addressed to frame such a CRP targeted treatment approach in the clinical setting of cancer patients. Finally, potential risks related to interference with the CRP system, given the functional, primarily anti-inflammatory role of the pentameric molecule in innate immune responses, must be carefully understood before considering therapeutic CRP modulation in patients.

Altogether, an important first step towards treating systemic inflammation in cancer patients is acknowledging its clinical significance for the course and outcome of the disease. Equally as we determine genomic profiles and assess other blood and tissue-based biomarkers, information regarding the systemic inflammatory status of

individual patients should also be obtained and integrated into precision medicine approaches. We hope that this work may contribute to an increased awareness of the presence of systemic inflammation in individual cancer patients and provide foundation and interest within the immune-oncology community for pursuing further research to deepen our understanding of the relationship between local and systemic tumor-associated inflammation and importantly, how it can be targeted to improve outcomes in a large proportion of cancer patients.

8. References

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The Prognostic Role of Systemic Inflammation in Patients Undergoing Resection of Colorectal Liver Metastases: C-Reactive Protein (CRP) Is a Strong Negative Prognostic Biomarker

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Background and Objectives: Systemic inflammation has been associated with poor survival in several tumor types, but has been less extensively studied in resectable metastatic disease. The aim of the present study was to evaluate the prognostic role of CRP in colorectal cancer patients with liver metastases (CRLM) compared to conventional tumor- and patient-related clinicopathological features as well as other indicators of the systemic inflammatory response (SIR).

Methods: A multinational retrospective study of 492 CRLM patients undergoing potentially curative resection of liver metastases between 1999 and 2009. Clinicopathological findings and the SIR markers CRP, hypoalbuminemia, and their combined Glasgow Prognostic Score (GPS) were analyzed.

Results: Multivariate analysis showed that preoperative CRP >10 mg/L was a strong predictor of compromised survival (HR = 1.72, 95%CI 1.84–2.50, $P < 0.01$). Patients with CRP ≤10 mg/L had a median survival of 4.27 years compared to only 47 days in patients with CRP ≥30 mg/L ($P < 0.01$). Similarly, increased GPS was independently predictive of poor survival (HR 1.67, 95%CI 1.22–2.27, $P < 0.01$), but hypoalbuminemia alone did not have significant prognostic value.

Conclusions: CRP alone is a strong prognostic factor, following curative resection of colorectal liver metastases and should be taken into consideration when selecting treatment strategies in CRLM patients.

KEY WORDS: colorectal cancer; liver metastases; systemic inflammatory response; C-reactive protein; Glasgow prognostic score

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death in the western world, with only 50% of patients who have undergone potentially curative resection surviving longer than 5 years [1]. The liver is the most common site of metastases and these are the most common cause of death in CRC [2].

Neoadjuvant chemotherapy for CRC patients with liver metastases (CRLM) is increasingly being used since systemic regimens can convert up to 30% of initially unresectable tumors to resectable ones [3]. Improvements in surgical techniques and systemic regimens in CRLM have led to a steady improvement, offering more patients potentially curative treatment. It is therefore increasingly important to select the most appropriate treatment strategy for each patient. For this purpose, different sets of clinical risk scores have been developed, employing factors such as size and number of metastases, CEA-level, and resection margins [3,4].

However, recent studies have shown that the traditional tumor-based risk scores used in clinical practice are influenced by the use of neoadjuvant chemotherapy [3]. Moreover, they do not reflect features of the underlying biology of the disease, and may therefore not be a reliable prognostic tool when deciding on treatment strategy in CRLM patients considered for liver surgery.

In CRC, tumor-associated inflammation, both locally in the tumor microenvironment (TME) and systemically as a host response, has consistently been reported to have a strong prognostic impact [5–7]. In particular, the presence of a systemic inflammatory response (SIR) is increasingly recognized as a crucial negative prognostic factor in a variety of cancer types [6,8–11]. Elevated CRP and low serum albumin, which together constitute the Glasgow Prognostic Score (GPS), are frequently used as indicators of SIR, but which of these markers that has the greatest prognostic impact in CRLM remains unclear.

We have previously shown that the presence of SIR as evidenced by elevated preoperative CRP levels was predictive of poor cancer-specific survival in patients undergoing resection of their primary tumor in all stages of colon cancer [12].

Conflict of interest: The study has not received any financial funding. All the authors declare no conflicts of interest.

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This finding was recently confirmed in the Nordic VII study, where 525 stage IV CRC patients were treated within a phase-III trial using chemotherapy and cetuximab as a first line combination. In that study, elevated CRP prior to the initiation of chemotherapy correlated strongly to impaired treatment outcome and survival [13].

Given the strong influence of the host's immune response for oncologic outcomes and the need for better predictive and prognostic biomarkers in patients with CRC metastasizing to the liver, the aim of the present study was to analyze the prognostic impact of CRP in CRLM patients undergoing potentially curative liver surgery compared to conventional clinicopathological features and other indicators of systemic inflammation.

PATIENTS AND METHODS

Patients

Consecutive patients with histologically verified CRC who underwent potentially curative resection of liver metastases between 1999 and 2009 at the Karolinska University Hospital in Sweden, Helsinki University Hospital in Finland and the Southern Hospital Trust in Norway, were considered for inclusion.

Demographic, histopathologic, and survival data were retrospectively obtained from national registers, local databases and patient records, and merged in a database created for the actual study. The following clinical and tumor-related data were gathered: gender; age at surgery; location of the primary tumor; number of metastases; size of the largest metastasis; type of liver surgery; synchronous (defined as metastases detected within 6 months after the diagnosis of the primary tumor) or metachronous metastases; resection margin; and the use of neo- or adjuvant chemotherapy.

As indicators of SIR status, preoperative CRP and albumin levels sampled within 20 days before liver surgery were collected. The GPS was calculated according to previously described thresholds [14]: patients with normal CRP ($\leq$10 mg/L) and albumin $\geq$35 mg/L were allocated a score of 0; patients with either hypoalbuminemia ($<$35 mg/L) or CRP $>$10 were allocated a score of 1; and finally, patients with both hypoalbuminemia ($<$35 mg/L) and elevated CRP ($>$10 mg/L) were allocated a score of 2.

To minimize the impact of other conditions possibly associated with systemic inflammation and/or influencing the measurement of CRP, patients with clinical evidence of infection shown by positive x-ray and/or positive urine or blood cultures, or with clinical evidence of other inflammatory conditions such as inflammatory bowel disease or rheumatoid arthritis, were excluded from the study. Patients taking corticosteroids the week prior to blood sampling were also excluded as well as patients with evidence of extrahepatic disease, incomplete liver resection, carcinoma of the appendix or treatment for cancer of other origin within the previous 3 years.

The primary end-point of the study was overall survival 5 years after liver surgery.

The study was approved by the respective Ethics Committees in the three participating countries.

Statistics

Descriptive statistics are presented with median and range for the continuous variables and proportions and percentages for the categorical variables. Comparison between the categorical variables was performed using the Chi square test. Analysis of survival was performed using the Kaplan–Meier method with differences examined using long-rank tests. Cox proportional hazard regression analysis was used to estimate univariate and multivariate hazard ratios for survival with 95% confidence intervals (CI). The level of statistic significance was $P < 0.05$. All analyses were performed using Statistica software (Statsoft, Tulsa, OK) version 10.

RESULTS

Patient Characteristics and Clinicopathological Features

Baseline demographics and clinicopathological characteristics are presented in Table I.

A total of 492 patients undergoing potentially curative liver surgery were included in the study. The median follow-up time was 4.17 (range 0.18–14.8) years. No patients died within the first month post-operatively. Median age was 65 (range 32–87) years, and the male-to-female ratio was 1.7:1. The majority of patients (55%) had their primary tumor located in the colon. Liver metastases were synchronous in 271 patients (55%). Median number of metastases was 2 (range 1–28) with median size 2.3 cm (range 0.3–15). Sixty-three patients (13%) had a metastasis of 5 cm or more in diameter, and 45 patients (9%) had more than five liver metastases.

In the vast majority of patients (85%) R0-resection was achieved. Two hundred and three patients (41%) received neoadjuvant chemotherapy, while 289 (59%) received chemotherapy after liver surgery.

CRP and Albumin

Preoperative CRP was available in 427 patients; 368 patients (86%) had a CRP level of 10 mg/L or below, and 44 patients (10%) had a CRP in the range of 11–30 mg/L. Only 15 (4%) patients had a CRP value higher than 30 mg/L.

Serum albumin was available in 450 patients with a median value of 37 g/L (range 19–52). Of these, 111 patients (25%) presented with hypoalbuminemia (serum albumin $<$35 g/L).

The GPS was estimated as described above. A majority of the patients had a GPS of 0 (74%). Seventy-four (20%) and 23 (6%) patients had GPS of 1 or 2, respectively.

TABLE I. Clinicopathological Characteristics of CRLM Patients Undergoing Potentially Curative Liver Surgery

Variable	Number (%)
Total number	492
Male/female	308/184
Age $>$65 at liver surgery (range)	255 (32–87)
Localization of primary tumor	
Colon	271 (55)
Rectum	183 (37)
Not known	38 (8)
Metastases	
Median number of metastases	2 (1–28)
Median size of largest metastasis (cm)	2.3 (0.1–15)
$\geq$5 metastases	45 (9)
Largest metastasis $>$5 cm	63 (13)
Synchronous/metachronous metastases	271/218
Type of liver surgery	
Resection of $\leq$2 segments	238 (48)
Resection of $\geq$3 segments	213 (4)
RFA[a] or combined resection/RFA	41 (8)
Resection margin	
R0	420 (88)
R1	47 (10)
R2	11 (2)
Chemotherapy	
Received prior to liver surgery	203 (41)
Received following liver surgery	289 (59)
CRP	
CRP $<$10 mg/L	368 (86)
CRP 11–30 mg/L	44 (10)
CRP $>$30 mg/L	15 (4)
Albumin	
$<$35 g/L	111 (25)
$\geq$35 g/L	339 (75)
GPS	
0	275 (74)
1	74 (20)
2	23 (6)

[a]RFA, Radiofrequency ablation.

The relationship between preoperative CRP levels and other clinicopathological features is shown in Table II. In brief, CRP was significantly higher (>10 ng/ml) in patients with primary tumor of the colon, large metastases (>5 cm), hypoalbuminemia, and in patients receiving chemotherapy preoperatively.

Prognostic Clinicopathological Factors

Age over 65, five or more liver metastases, a metastasis of 5 cm or more, as well as the use of postoperative chemotherapy and type of liver surgery (number of segments resected) and/or RFA were all significantly associated with survival in univariate analysis (Table III). On multivariate analysis, number of metastases (HR = 1.62, 95%CI 1.08–2.44) and postoperative chemotherapy (HR = 0.72, 95%CI 0.55–0.94) remained significant predictors of survival (Table IV).

With regard to other traditional predictors of outcome in CRLM patients, neither resection margin (R0 vs. R1/R2) nor size of largest lesion turned out to have significant influence on survival.

Prognostic Impact of Systemic Inflammation

In univariate analysis, patients with an elevated CRP level (>10 mg/L) had poorer survival compared to patients with a CRP-value below cut-off (HR 1.93, 95%CI 1.35–2.77, $P < 0.01$, Fig. 1A). Similarly, increasing CRP, analyzed as a continuous variable, strongly correlated with compromised survival (HR 1.01, 95%CI 1.00–1.02 $P = 0.01$).

Likewise, albumin analyzed as a continuous variable, was significantly associated with survival both in uni- and multivariate analyses (HR = 0.96, 95%CI 0.93–0.99). However, analyzed as a dichotomized variable, hypoalbuminemia (serum albumin <35 g/L) was not a significant predictor of survival. Nevertheless, when hypoalbuminemia was included in the GPS, this combined inflammatory score was significantly associated with compromised survival both in the uni- and multivariate analyses ($P < 0.001$). However, when compared in the multivariate analysis, the GPS did not add prognostic value as to the measurement of CRP alone, identifying CRP as the strongest prognostic factor. The measurement of CRP only was, therefore, used in further analyses.

In order to better understand the prognostic impact of elevated CRP, patients were allocated into three groups (<10 [n = 368], 10–30 [n = 44], >30 [n = 15]) according to their pre-operative CRP value, using previously described thresholds [12,13]. The log rank test showed that the survival time significantly differed between the three groups (Fig. 1B, $P < 0.001$). Patients with CRP <10 mg/L had a median survival of 4.27 years compared to 2.59 years in patients with CRP 11–30 mg/L, and 47 days in patients with CRP of 30 mg/L or higher.

As there was a significant correlation between the size of metastatic liver lesions and CRP levels ($P < 0.001$, Spearman Rank Order Correlation Test) as well as between the size and the number of metastases ($P = 0.005$, Chi square test), meaning larger lesions correlated with more lesions, indicating colinearity, these variables were not considered independent. Thus, the multivariate analysis was performed stepwise (Table IV). Nevertheless, preoperative CRP analyzed both as a continuous and dichotomized variable turned out to be the strongest prognostic biomarker in the present cohort of CRLM patients (respective HRs 1.01, CI 1.00–1.02 and 1.72, CI 1.84–2.50, both $P < 0.01$).

TABLE II. The Relationship Between Preoperative CRP Level and Other Clinicopathological Features in CRLM Patients

Characteristic	CRP ≤10 ng/ml, N (%)	CRP >10 ng/ml, N (%)	*P*-value
Age			0.05
>65	181 (42)	37 (9)	
>65	187 (44)	22 (5)	
Gender			0.34
Male	228 (53)	40 (9)	
Female	140 (33)	19 (5)	
Resection margin			0.85
R0	321 (77)	50 (12)	
R1 + R2	42 (10)	6 (1)	
Site of primary tumor			<0.01
Colon	210 (55)	24 (6)	
Rectum	117 (31)	31 (8)	
Liver metastases			
≥5 liver metastases	335 (78)	54 (13)	0.90
<5 liver metastases	33 (8)	5 (1)	
Largest lesion ≥5 cm	333 (78)	42 (10)	<0.01
Largest lesion <5 cm	35 (8)	17 (4)	
Preoperative chemotherapy			0.01
Yes	239 (56)	27 (6)	
No	129 (30)	32 (8)	
Postoperataive chemotherapy			0.06
Yes	234 (55)	30 (7)	
No	134 (31)	29 (7)	
Albumin			<0.01
<35 mg/L	302 (74)	32 (8)	
≥35 mg/L	48 (12)	23 (6)	

TABLE III. Univariate Analysis of Possible Prognostic Factors in CRLM Patients

Variable	Hazard ratio	95% confidence interval	*P*-value
Age ≥65	1.31	1.00–1.69	0.04
Gender	1.17	0.89–1.54	0.25
Resection margin[a]	1.36	0.94–1.98	0.10
Site of primary tumor	1.16	0.89–1.51	0.26
Synchronous versus metachronous metastases	0.97	0.75–1.25	0.80
Size of the largest liver metastasis ≥5 cm	1.48	1.03–2.10	0.03
Number of liver metastases ≥5	1.55	1.04–2.32	0.03
Preoperative chemotherapy	0.78	0.60–1.01	0.06
Postoperative chemotherapy	0.71	0.55–0.93	0.01
Albumin continuous variable	0.95	0.91–0.98	<0.01
Albumin <35 g/L	1.37	1.00–1.88	0.05
CRP continuous variable	1.01	1.00–1.02	<0.01
CRP >10 mg/L	1.93	1.35–2.77	<0.01
GPS 0 versus 1/2	1.67	1.22–2.27	<0.01

[a]R0 versus R1/2.

DISCUSSION

The presence of preoperative systemic inflammation, reflected by elevated CRP levels, was found to be a strong independent predictor of compromised survival in colorectal cancer patients undergoing potentially curative resection of liver metastases. Patients with a pre-operative CRP of 30 mg/L or more had a median survival of less than 2 months compared to more than 4 years in patients with a CRP level below 10 mg/L.

TABLE IV. Multivariate Analysis of Prognostic Factors in Patients With Colorectal Cancer Undergoing Resection of Liver Metastases

Variable	Hazard ratio	95% confidence interval	*P*-value
Age ≥65	1.83	0.90–1.55	0.22
Postop chemotherapy	0.74	0.57–0.97	0.03
Size of the largest liver metastasis >5 cm	1.35	0.94–1.93	0.10
Number of liver metastases ≥5	1.08	1.04–1.12	<0.01
Albumin continuous variable	0.96	0.92–0.99	0.03
GPS 0 versus 1/2	1.63	1.19–2.22	0.02
CRP continuous variable	1.01	1.00–1.02	<0.01
CRP >10 mg/L	1.72	1.18–2.49	<0.01

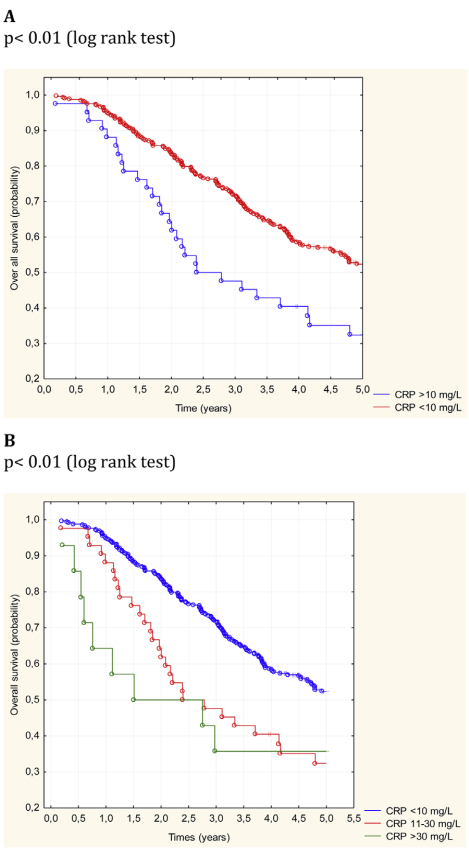

Fig. 1. Kaplan–Meier curves showing the relationship between preoperative CRP levels and overall survival in colorectal cancer patients with liver metastases following potentially curative liver surgery.

Our data are in line with previous studies on the prognostic value of SIR, which has been most extensively studied in primarily operable CRC, but to some extent, also following curative resection of liver metastases [2,6,15–17].

Unlike previous single center studies, the strength of the present study is that it is based on a large cohort of patients drawn from a multi-institutional database, enabling a more representative sample and robust statistical analyses. Moreover, to our knowledge the current study is the first to compare the prognostic value of CRP both with other indicators of systemic inflammation, as well as other tumor- and patient-related prognostic factors in CRLM.

Cancer-associated inflammation, both locally in the tumor microenvironment and as a systemic response, has previously been identified as a key determinant of disease outcome in CRC [5,7,18,19].

Despite compelling evidence for the crucial impact of SIR for CRC progression and survival, the underlying biological mechanisms involved are still poorly understood.

SIR has been associated with compromised immune function in terms of down regulation of adaptive immune responses and increased activation of components of the innate immune system, creating an environment favorable of tumor growth and metastasis [6,16]. Specifically, elevated CRP levels have been found to correlate with a pro-inflammatory cytokine profile, including increased expression of the multifunctional cytokine IL-6, which apart from having pro-tumoral and immune modulating effects per se, also is the main inducer of CRP production [6,18,20].

The casual link and the sequencing of SIR and tumorgenesis cannot be determined from our data. It is possible that increased CRP levels simply reflect a nonspecific inflammatory response secondary to large tumor burden, tumor necrosis, or tissue damage. In this regard, our data are somewhat conflicting as we found a significant relationship between CRP and the presence of larger metastases, but not between CRP and number of metastases. In contrary, on multivariate analysis only CRP and number of metastases, but not metastasis size, were found to be independently predictive of poor survival. This lack of consistent relationship between tumor burden and CRP may support the hypothesis that it is the systemic inflammation that drives tumor progression and that the elevated CRP not solely is a result of the tumor itself [11,15,21]. In line with this, data from the previously mentioned prospective Nordic VII study revealed that the prognostic value of CRP was independent of RAS/BRAF mutation status, indicating that patients with a more proliferative tumor type not necessarily have higher CRP concentrations [13]. However, more comprehensive studies with a translational approach will be needed to provide further insight into this pivotal question.

In the present study, we aimed to compare the prognostic value of SIR with traditional tumor- and patient-related factors for outcome in CRLM patients. We found that CRP analyzed both as a continuous and dichotomized variable had the strongest prognostic impact. Furthermore, in this patient group, CRP was found to be superior to the alternate and commonly used SIR-marker GPS in predicting survival [1,22]. CRC patients often have normal serum albumin until a very late stage in their disease, and hypoalbuminemia may therefore add little prognostic information to that provided by CRP alone.

In the present patient cohort, only 15 patients (4%) had a preoperative CRP value of 30 mg/L or more. This was considerably less than one might expect given that these were stage IV patients. In contrast, almost 30% of the patients in the previously mentioned Nordic VII study had a CRP level above 30 mg/L [13]. This probably demonstrates a better selection of patients for liver surgery based on a more promising prognostic profile.

An obvious weakness of the present study lies in its retrospective nature. Also, the laboratory profile on many of our patients was not comprehensive enough in particular with regard to white blood cell- and platelet counts, making us unable to include other well-recognized SIR biomarkers such as the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and lymphocyte-to-monocyte ratio (LMR) in our analyses. In the previously mentioned Nordic VII study, though, no additional prognostic value was added from these different scores, identifying CRP alone as a sufficient biomarker. Furthermore, the present study does not provide insight into the underlying biology of CRP-high versus CRP-low cancers. Finally, our endpoint was overall survival, that is, death from all causes and not disease-specific survival (DSS). However, in the setting of metastatic disease, one can argue that overall survival is as least as good as DSS given that the vast majority of stage IV patients die of their cancer.

Despite the accumulating evidence that SIR is associated with poor outcome in CRC, the existing methods used for prognostication in clinical practice are solely based on tumor-related features and do not take into consideration the significance of the inflammatory response [3,4]. Measurement of CRP is inexpensive, reproducible, and used worldwide. Given the strong impact of SIR on oncologic outcomes, we propose that CRP, after further validation, should be incorporated in the treatment algorithm for CRLM patients. This could improve the stratification of patients for different treatment strategies and might also be useful for monitoring treatment response. Should patients with elevated CRP levels, and thus poor prognosis, be allocated to intervention strategies to reduce tumor-associated inflammation instead of up-front extensive liver surgery? In addition to the extensive data demonstrating that NSAIDs (nonsteroidal anti-inflammatory drugs) and aspirin in particular, may

inhibit colorectal carcinogenesis and be used for cancer prevention, recent studies also suggest that regular use of aspirin after the diagnosis of colorectal cancer may be associated with improved cancer-specific survival [23,24]. Similar beneficial effects have been ascribed the use of other NSAIDs as well as the corticosteroids, where studies have shown that these agents beyond the obvious immune modulating properties also may exert direct anti-tumor effects. Although monotherapy generally is regarded insufficient for successful tumor regression, these agents could readily be used in combination with conventional cancer therapies for potential additive or synergistic effects [23,25]. Still, several important issues such as optimal dose, treatment duration, and the potential risks associated with long-term use remain to be clarified before these agents can be implicated in clinical practice. Other interesting anti-inflammatory agents that more specifically target crucial pathways of the inflammatory response are IL-6 inhibitors and agents directed towards Toll-like receptors [23]. Preliminary experimental data have shown promising anti-tumor activity by inhibiting IL-6 signaling and several IL-6 antibodies are currently being tested in clinical trials either as single agent or in combination with other chemotherapeutic regimens [26]. Taken together, there seems to be a rationale for not only targeting tumor cells but also specifically target the host immune response for improving ongological outcomes and patient prognosis. Indeed, more translational studies as well as prospective clinical trials stratifying for preoperative CRP levels and preferably randomizing for the use of anti-inflammatory agents, are necessary to understand the role of anti-inflammatory treatment in the management of colorectal cancer.

In conclusion, our data support the strong prognostic role of the host inflammatory response in CRC. As a marker of systemic inflammation, CRP was identified as the strongest prognostic factor in CRLM patients undergoing potentially curative liver surgery.

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SYNOPSIS

A large multicenter study examining the prognostic value of CRP in colorectal cancer patients with liver metastases undergoing potentially curative liver surgery. Elevated preoperative CRP was the strongest prognostic factor when compared to tumor- and patient-related clinicopathological features as well as other indicators of systemic inflammation.

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ORIGINAL RESEARCH
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Systemic Inflammation Associates With a Myeloid Inflamed Tumor Microenvironment in Primary Resected Colon Cancer—May Cold Tumors Simply Be Too Hot?

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Systemic inflammation measured by the acute-phase protein CRP associates with poor outcome across cancer types. In contrast, local tumor-associated inflammation, primarily evaluated by T-lymphocytes, correlates with favorable prognosis. Yet, little is known whether these two responses are related or opposing processes and why elevated CRP in relation to cancer is detrimental for clinical outcome. As proof of concept, we developed a platform combining multiplexed IHC and digital imaging, enabling a virtual readout of both lymphoid and myeloid immune markers and their spatial patterns in the primary tumors of resected stage II and III colon cancer (CC) patients with and without accompanying systemic inflammation. Twenty-one patients with elevated CRP (>30 mg/l) and 15 patients with low CRP (<10 mg/l) were included in the analyses. Whole slides from the primary tumors were stained for markers of adaptive (CD8+, CD4+, foxp3 regulatory T cells, CD20+ B cells) and innate (CD68+ macrophages, CD66b+ neutrophils) immunity and the immune checkpoint molecule PD-L1. Associations between individual immune markers, preoperative CRP values, mismatch repair status (MMR), and risk of recurrence or death were assessed. Unsupervised hierarchical clustering was used to explore whether distinct immune phenotypes were present. Tumors from systemically inflamed patients (CRP >30 mg/l) displayed significantly more myeloid features in terms of higher densities of CD66b+neutrophils ($p = 0.001$) and CD68+macrophages ($p = 0.04$) and less lymphoid features (lower CD8 T cell, $p = 0.03$, and foxp3 regulatory T cell densities, $p = 0.03$) regardless of MMR status. Additionally, systemically inflamed patients harbored lower mean distances between neutrophils and tumor cells within the TME. Intriguingly, microsatellite instable (MSI) tumor status correlated with systemic inflammation. However, using a combinatorial approach, we found that regardless of an adaptive composite score (compounded CD4+ and CD8+ T cells), a high innate score (CD66b+ neutrophils and CD68+ macrophages) associated significantly with elevated CRP. In conclusion, tumor-associated systemic inflammation correlated with a myeloid-

dominated TME in a small cohort of resectable CC patients. Our data highlight the importance of a comprehensive immune classification of tumors including players of innate immunity and support a role for CRP as an informative biomarker of the immune response taking place at the tumor site.

Keywords: systemic inflammation, C-reactive protein, multiplex, immunohistochemistry, colon cancer, myeloid inflammation, neutrophils, spatial profiling

INTRODUCTION

The crucial role of the immune system in tumor biology and clinical outcome across cancer types is by now well accepted (1). Tumor-associated inflammation has traditionally been referred to as either a systemic inflammatory response (SIR) or a localized *in-situ* immune infiltrate. SIR, as evidenced by circulating biomarkers such as the acute-phase protein C-reactive protein (CRP), has consistently been correlated with poor prognosis in many cancer types, including colon cancer (2–4). In contrast, a robust intra-tumoral lymphocyte infiltrate associates with favorable prognosis and seems predictive of response to both chemotherapy and immune checkpoint blockade (5, 6).

In colon cancer, the prognostic significance of tumor-infiltrating T-lymphocytes has been extensively validated by Immunoscore, which has shown prognostic superiority to the classical TNM staging (7–9). Based on this scoring system, the concept of "hot" (T-cell inflamed) and "cold" (no/little tumor infiltrating T-cells) tumors has emerged with accumulating studies using this T-cell-focused model for categorizing the immune landscape and predicting treatment outcome in a wide range of cancer types (10).

However, the immune infiltrate of most solid tumors is highly heterogeneous and dynamic (11). Apart from T-cells and other adaptive immune cells, it consists of innate immune cells such as neutrophils, macrophages, and dendritic cells, which together with fibroblasts, endothelial cells, and other stromal components constitute the complex tumor microenvironment (TME) (11, 12). Myeloid immune cells in particular exhibit remarkable plasticity with the ability to polarize into functionally distinct phenotypes either supporting or inhibiting tumor growth depending on the signals in the TME (13). Despite their possible dual roles in cancer development, most studies point toward a dominating tumor-promoting and immunosuppressive role of myeloid immune cells in the TME (13, 14).

Nevertheless, in the era of immune checkpoint blockade where preexisting T-cell-mediated immunity is key for therapeutic efficacy, the impact of innate immune cells on tumor progression and treatment outcome has been less appreciated. Furthermore, adding another layer of complexity, recent studies have highlighted the importance of characterizing the spatial distribution of immune cells within the tumor, to understand how tissue architecture and cellular interactions may shape the immune landscape (15, 16).

Given this diversity of the tumor-immune microenvironment in terms of various immune cell populations, their spatial organization, and the dual role they may play in cancer, it is desirable to identify biomarkers and develop diagnostic tools that reflect the inherent immunological status of tumors. Specifically, indications of either a myeloid- or lymphoid-dominated microenvironment and their respective immune-suppressive or stimulatory capacities may prove to be the cornerstone for allocating patients to the most appropriate treatment strategies.

The aim of this study was therefore to explore the immune contexture as a whole, featuring both adaptive and innate players in the TME of primary resected colon cancer patients with and without associated SIR. For this purpose, we developed a multiplex immunohistochemistry (mIHC)-based platform combining chromogenic IHC staining with digital whole-slide imaging enabling simultaneous detection of six different lymphoid and myeloid immune cells in addition to the immune checkpoint molecule PD-L1. Using this platform, we were able to characterize the immune landscape and assess spatial relationships in the TME of the primary tumors. We further extended the application by combining the mIHC data with clinical information to investigate whether SIR and local tumor-associated inflammation are related processes and explore the hypothesis that SIR correlates with a myeloid-driven immune landscape in colon cancer patients.

MATERIALS AND METHODS

Patients and Tumor Specimens

Forty-three stage II and III colon cancer patients, consisting of 20 patients with CRP < 10 and 23 patients with CRP > 30 treated at Sørlandet Hospital, Kristiansand, Norway, were selected from a prospective local colorectal cancer database covering extensive clinical information and follow-up data. The choice of CRP values was based on previous work using identical CRP thresholds (2). All patients had been resected for their primary tumors between 2005 and 2015 as an elective procedure and neither had received antibiotics nor immunosuppressive drugs within the last month prior to surgery nor had been diagnosed with an autoimmune disease. CRP values were obtained up to 20 days before the resection.

Archived formalin-fixed, paraffin-embedded (FFPE) tumor tissues from the primary tumors were retrieved from the Department of Pathology, Sørlandet Hospital. Representative tumor blocks containing areas of both the invasive margin (IM) and tumor center (TC) were selected by a trained pathologist (MBN).

The study was conducted according to approvals from the Regional Ethics Committee.

Multiplex Immunohistochemistry Workflow

FFPE colon cancer blocks were cut into 3 mm thick sections and prepared for the IHC-staining protocol. All staining procedures were performed on the Ventana Discovery Ultra autostainer (Roche Diagnostics International AG, Switzerland).

First, tissue sections were deparaffinized using xylene and rehydrated with ethanol followed by heat-induced antigen retrieval and blocking endogenous peroxidase activity. Then, mIHC with two different panels of antibodies were applied on two serial tumor sections. The first panel consisted of a 5-plex termed the adaptive or lymphoid immune profile with primary antibodies against CD8 (cytotoxic T lymphocytes), CD4 (T-helper cells), foxp3 (regulatory T cells), CD20 (B lymphocytes), and pan-cytokeratin (pan-CK) as an epithelial tumor marker. The second IHC panel, a 4-plex termed the innate or myeloid immune profile, consisted of antibodies against CD68 (pan-macrophages), CD66b (neutrophils), pan-CK, and finally PD-L1. The multiplex staining process consisted of sequential staining rounds with primary and secondary antibodies (see **Table S1** for details), without hematoxylin counterstaining to prevent mix of signals in the digital analysis. After accomplishing the multiplex IHC procedure, tumor sections stained with the innate immune panel were counterstained with hematoxylin for visualization of nuclei and tissue architecture. Three forms of controls were used to assure the staining quality of the multiplex: 1) comparison with single staining for each of the markers to check for cross-reactivity or loss of signal due to the multiplex procedure, 2) applying tonsil tissue as a "positive control" on each slide (consists of lympho-epithelial structures with cells positive for all of the markers included in the multiplex panels), and 3) mIHC staining of tumor tissue from lung (adenocarcinoma) for assay validation and grading of PD-L1 expression.

Digital Imaging and Automated Analysis of the Tumor Immune Microenvironment

After completion of the staining process, tumor sections were scanned as bright-field whole slides at ×20 magnification using a NanoZoomer 2.0 HT (Hamamatsu, Japan). Image analysis was performed using Visiopharm Integrator System software version 2019.02 (VIS; Visiopharm A/S, Hoersholm, Denmark).

As shown in **Figure 1**, the invasive margin (IM) and tumor center (TC) were manually outlined by an experienced pathologist (MBN) and the observer on hematoxylin-stained slides in the software. As for annotating the IM, we chose not to do that automatically using a predefined and fixed area measurement since the tumors showed considerable variability in size and range of tumor islets and stroma.

The two IHC-stained tumor sections and the hematoxylin-counterstained slide were scanned separately. Tumor slides were then digitally superimposed using an automated approach, but controlled and optimized manually, with the net effect of a single virtual slide capturing all seven immunostained markers with preserved tissue architecture.

Digital analysis was performed using applications within the software particularly developed for this material. For segmentation, we used a Bayesian classifier followed by different post-processing steps (primarily morphological operations and changes by area or surrounding) for optimizing the results. The preprocessed images in adaptive stains were based on color

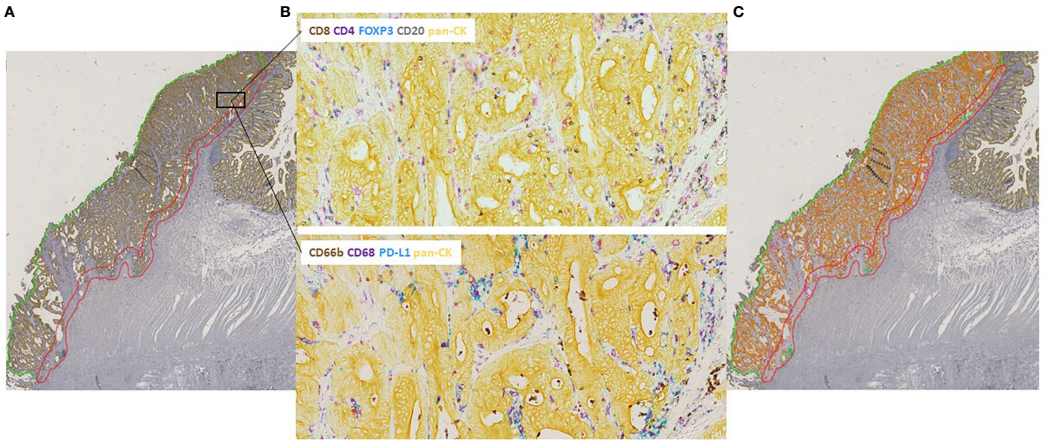

FIGURE 1 | Tumor regions and image acquisition. **(A)** Hematoxylin-stained whole slide of stage II colon cancer with annotated invasive margin, IM (red), and tumor center, TC (green). **(B)** Two multiplexed IHC-stained serial slides from the same tumor area visualizing adaptive and innate immune markers. IHC-stained slides were digitally aligned together with the hematoxylin-counterstained innate slide. **(C)** Automated digital analysis was applied enabling one virtual readout of the IHC-stained markers. Immune cells were quantified at the IM and TC separately and classified as either directly intra-tumoral or embedded in the stoma.

deconvolution of the chromogens DAB and silver in addition to features of the RGB color model. In innate stains, RGB and HSI models were utilized. The software-based classification of the immunostained markers was performed by assigning different pseudo-colors, enabling a visual output of the various immune markers within the tissue. Areas with mucin, artifacts, or tissue folds were manually excluded from the analysis.

Immune cell densities were estimated as area of positively stained cells per region of interest (ROI) in percent quantifying cells at the IM and TC separately. Immune cells were classified as either intra-tumoral (IT) if they were directly infiltrating the tumor nests or stromal (S) if they were located within the stromal spaces. In addition, we calculated two forms of a composite score: one with the sum of IT and S immune cells divided by the total area of the ROI of interest, and one where the area of tumor tissue was subtracted to adjust for differences in total amount of tumor tissue which potentially could dilute the true immune cell estimate. PD-L1 expression on tumor cells and immune cells (primarily CD68+ macrophages) was assessed separately. Composite lymphoid and myeloid immune scores were estimated by compounding the densities of CD8+ and CD4+ T cells for the lymphoid score and CD68+ macrophages (total score) and CD66b+ neutrophils for the myeloid score and categorized as high or low based upon the median value of the respective compounded scores.

Using pan-CK in the mIHC panels, distances between tumor- and immune cells of interest could be estimated enabling spatial characterizations. Two different types of spatial analysis were performed: 1) proximity analysis estimating the density of immune cells of interest within the defined distance of 20 micron around the tumor islets and 2) nearest neighbor analysis calculating the average distance between immune cells of interest and nearest tumor cell.

The tumor–stroma ratio was calculated by dividing the stromal area of the IM and TC by the total area of the two tumor compartments.

Microsatellite Instability Analysis

Assessment of mismatch repair (MMR status) was performed by IHC evaluation of MHL1, MSH2, MSH6, and PMS2 protein expression. Tumors that were negative in one or more of the four stainings or inconsistent with IHC were verified with the Idylla MSI test, which is a fast-track PCR-based assay for determining microsatellite status in colorectal cancer (17).

Statistical Analysis

Differences in clinicopathological data between CRP-high and -low patients were evaluated by Fisher's exact test and the two-sample t-test. Immune markers were analyzed on the logarithmic scale to obtain a normal distribution. Associations between immune markers, CRP, and survival were analyzed by Fisher's exact test. Pearson's correlations were used to analyze the correlation between individual immune markers. Medians and means were compared using the Kruskal–Wallis test and the one-way ANOVA-test, respectively. The Aalen–Johansen method was used to estimate the risk of recurrence or death by colon cancer, adjusting for death of other causes as competing risk, and compared between CRP groups using the log-rank test. For estimating the lymphoid and myeloid composite scores, data were log-transformed and standardized before summing the score of the respective immune markers (CD8+/CD4+ T cells and CD68+ macrophages/CD66b+ neutrophils). To define subgroups in our cohort, unsupervised hierarchical clustering was performed. Heat maps and hierarchical clusters were generated in R studio version 4.0 based on the logarithmic scale of the immune markers standardized to mean zero and variance 1. Two-sided p-values < 0.05 were considered statistically significant for all analyses. Statistical analysis was performed using STATA software version 16.

RESULTS

A total of 36 stage II and III colon cancer patients were finally included in this study. Excluded patients (n= 7) were due to compromised tumor tissue quality, weak IHC staining, or other technical issues with the multiplex assays. Patient and tumor characteristics are listed in **Table 1**. Systemically inflamed patients were older and tended to be more right sided. Of note, all patients in the CRP-low group (n=15) had stage II disease while this was the case for only half of the patients in the CRP-high group (n=21). Nine of the patients in the systemically inflamed group had microsatellite instable (MSI-high) tumors, whereas all non-inflamed patients had microsatellite stable (MSS) tumors. As expected from previous works (2, 18), systemically inflamed patients had statistically increased risk of recurrence or death by colon cancer (see **Figure 2**, p=0.047).

Multiplex IHC Reveals Substantial Intra- and Intertumoral Heterogeneity of Immune Infiltration in Colon Cancer Patients

Different patterns of immune infiltration both between and within tumors were present in our cohort. Representative images are shown in **Figure 3**. Some tumors exhibited rich immune infiltration of both the stroma and tumor islets while others had

TABLE 1 | Patient and tumor characteristics in CRP high and -low colon cancer patients.

	CPR < 10 (n = 15)	CRP ≥ 30 (n = 21)	p value
Age, mean (years)	68	77	0.02
Sex			
Female	8 (53)	12 (57)	1.00
Male	7 (47)	9 (43)	
Stage			
II	0 (0)	10 (48)	0.002
III	15 (100)	11 (52)	
Tumor location			
Left	4 (27)	2 (10)	0.47
Right	8 (53)	13 (62)	
Sigmoid	3 (20)	6 (29)	
Adjuvant chemotherapy			
None	3 (20)	17 (81)	<0.001
Only 5-FU based	4 (27)	3 (14)	
Platinum doublet	8 (53)	1 (5)	
Follow-up, mean (years)	7.2	7.3	0.92
MMR-status (MSS/MSI)	15/0	12/9	<0.01

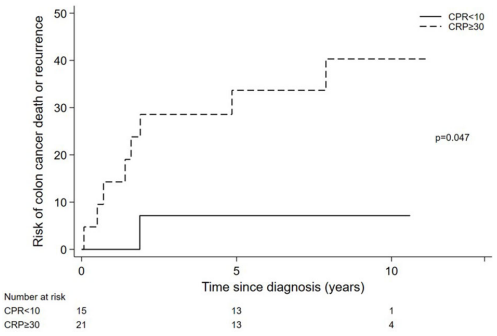

FIGURE 2 | Risk of recurrence or death from colon cancer in CRP high and low patients.

stromal compartments with a more patchy immune infiltrate. Finally, there were tumors with dense tumor tissue, sparse stroma, and modest immune infiltration. There was a trend toward a higher stromal component in systemically inflamed patients, but the tumor–stroma ratio (TSR) did not differ significantly between CRP-high and -low tumors (77 *vs.* 72%, respectively, p = 0.11).

With the notable exception of CD66b+ neutrophils, all other immune cells were more prominent at the IM than in the TC with CD68+ macrophages and CD4+ T lymphocytes being the most abundant types of immune cells (**Table S2**). As illustrated in the correlation heat map of tumor-infiltrating immune cells in **Figure 4**, there was a generally low correlation between immune markers at the IM and TC (**Figures 4A, B**). However, several positive correlations existed among adaptive immune cells, particularly in the TC where CD8+ and CD4+ T cells showed a strong positive correlation. Innate immune cells, on the other hand, were less correlated. Most strikingly, neutrophils turned out to be independent of the presence of any other immune marker as no correlations were evident (**Figure 4B**).

Exploring the Immune Infiltrate in CRP High and Low Colon Cancer Patients According to MSI Status

Based on the finding that MSI status associated with elevated CRP and that no patients in the CRP-low group had MSI-positive tumors, we evaluated the composition of the immune infiltrate in CRP-high and -low patients according to MSI status. As shown in **Table 2**, there were considerable differences in the pattern of immune infiltration between MSS and MSI-high tumors in the systemically inflamed group and MSS tumors in the non-inflamed

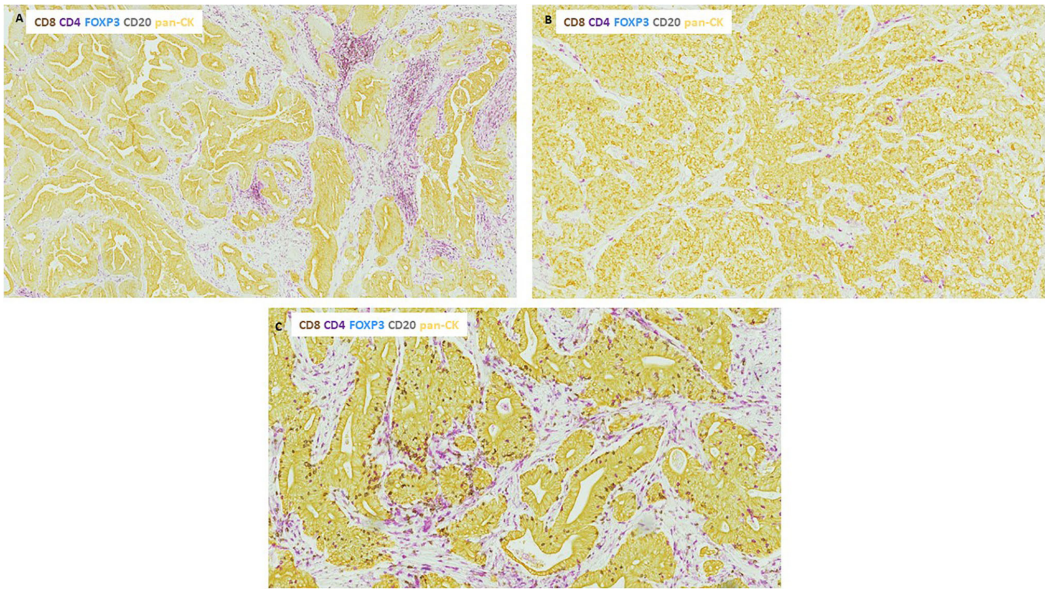

FIGURE 3 | Representative images showing differential immune infiltration in colon cancer tissue. **(A)** Tumor exhibiting patchy immune infiltration consisting of areas with heavy infiltration combined with sparsely infiltrated areas. **(B)** Dense tumor tissue with sparse stroma and modest immune infiltration. **(C)** Highly immune infiltrated tumor with abundant immune cells located within both the tumor tissue and stromal spaces. All images are of the adaptive panel (CD8+ T cells, CD4+ T cells, CD4+foxp3 T cells, CD20+ B cells) of IHC-stained immune markers.

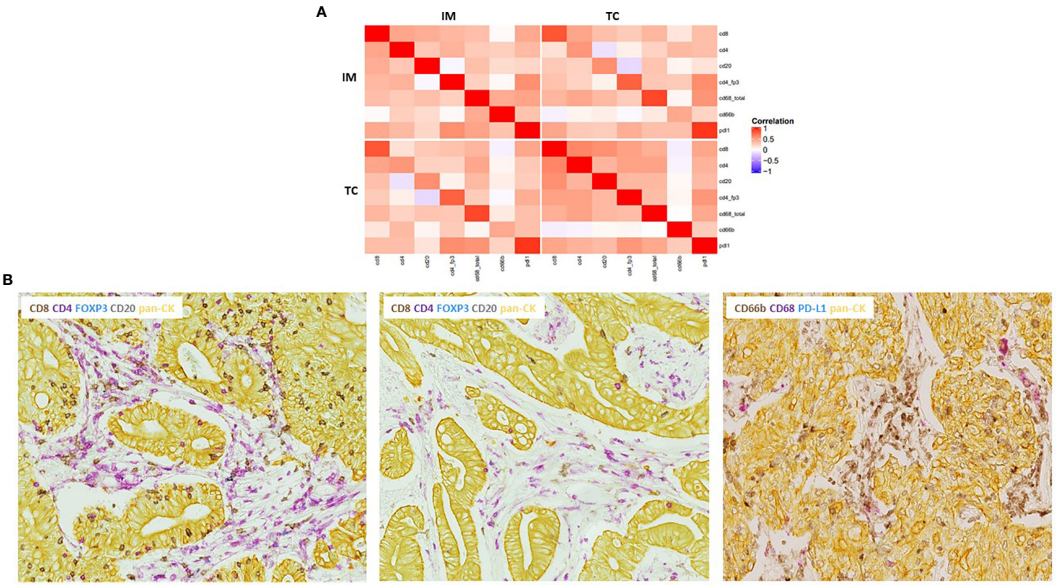

FIGURE 4 | Correlations between immune markers in colon cancer patients. **(A)** Heatmap of Pearson correlation coefficients between individual immune markers at the invasive margin (IM) and tumor center (TC) based upon the combined tumor-infiltrating and stromal immune cell densities. Red color indicates strong positive correlation, blue indicates strong negative correlation, and white indicates no correlation. **(B)** Representative images of existing correlations. Left: tumor slide from the IM of a CRP-low MSS tumor stained with the adaptive IHC panel showing a strong correlation between adaptive immune cells, particularly CD8+ and CD4+ T cells. Middle: tumor slide from the TC of the same patient as in the left panel showing modest adaptive immune infiltration illustrating a low correlation between immune cells at the IM and TC. Right: tumor section stained with the innate IHC panel showing vigorous neutrophil infiltration, with no correlations with any other marker. *Combined tumor-infiltrating and stromal immune cell densities.

TABLE 2 | Adaptive and innate immune markers in CRP-high and -low colon cancer patients according to MSI status.

Immune marker	Index	Area	CRP < 10, MSS	CRP ≥ 30, MSS	CRP ≥ 30, MSI	p value
CD8+ T cells	Stroma	TC	0.74 (0.03–4.60)	0.08 (0.00–1.37)	0.86 (0.02–2.56)	**0.049**
	Tumor infiltrating	TC	0.07 (0.00–0.74)	0.02 (0.01–0.14)	0.09 (0.00–0.96)	**0.042**
CD4+ T cells	Stroma	TC	1.25 (0.10–3.18)	0.69 (0.21–3.64)	0.72 (0.07–3.84)	0.51
	Tumor infiltrating	TC	0.25 (0.04–1.09)	0.20 (0.04–0.36)	0.65 (0.10–4.63)	**0.055**
CD20+ B cells*	Stroma	IM	0.05 (0.00–0.14)	0.05 (0.01–0.33)	0.24 (0.01–0.84)	**0.020**
		TC	0.03 (0.00–0.08)	0.01 (0.00–0.04)	0.04 (0.00–0.24)	**0.046**
CD4_foxp3+ T cells	Stroma	TC	0.33 (0.02–1.06)	0.03 (0.00–0.32)	0.09 (0.00–1.54)	**0.009**
	Tumor infiltrating	TC	0.01 (0.00–0.05)	0.00 (0.00–0.03)	0.02 (0.00–0.21)	0.12
CD68+ macrophages	Stroma	TC	1.15 (0.56–3.39)	1.81 (0.30–3.85)	1.51 (0.03–3.39)	0.60
		TC	0.47 (0.12–3.31)	0.89 (0.06–2.36)	1.34 (0.00–5.30)	**0.054**
CD66b+ neutrophils	Stroma	TC	0.66 (0.03–2.41)	0.59 (0.05–4.05)	1.51 (1.10–20.67)	**0.04**
	Tumor infiltrating	IM	0.05 (0.00–3.54)	0.71 (0.04–3.23)	0.76 (0.05–1.97)	**0.08**
		TC	0.03 (0.00–2.30)	0.86 (0.04–16.72)	1.26 (0.13–45.14)	**<0.001**
PD-L1+	Stroma	IM	0.13 (0.01–1.45)	0.02 (0.00–1.84)	0.32 (0.02–0.71)	**0.026**
		TC	0.12 (0.00–0.90)	0.02 (0.00–0.54)	0.09 (0.00–0.52)	**0.021**
	Tumor infiltrating	IM	0.28 (0.00–0.90)	0.03 (0.00–17.11)	0.54 (0.01–15.57)	**0.051**
		TC	0.14 (0.00–4.87)	0.04 (0.00–16.97)	0.63 (0.00–3.63)	0.09
CD68_PD-L1+ macrophages	Stroma	TC	0.10 (0.00–1.85)	0.04 (0.00–1.25)	0.28 (0.00–0.88)	0.14
	Tumor infiltrating	IM	0.07 (0.00–1.30)	0.02 (0.00–1.10)	1.09 (0.00–2.27)	**0.031**
		TC	0.02 (0.00–0.60)	0.05 (0.00–1.25)	0.13 (0.00–0.95)	0.074

Immune markers in percent, median (range). p-values were obtained using the Kruskal–Wallis test.
IM, invasive margin; TC, tumor center.
**CD20+ B cells were infiltrating the stroma only.*
Bolded values indicate statistical significant p-values.

group, particularly evident in the TC. Specifically, MSI-high tumors were characterized by significantly higher densities of CD8+ T lymphocytes, CD20+ B cells, and tumor-infiltrating CD4+ T cells as well as higher CD66b+ neutrophil and CD68+ macrophage densities and finally upregulation of PD-L1, predominantly expressed on myeloid immune cells (primarily CD68+ macrophages) infiltrating the tumor stroma and to a lesser extent on tumor cells. Interestingly, the density of foxp3 regulatory T cells also differed significantly among the three groups where CRP-low MSS tumors exhibited the highest proportion followed by MSI CRP-high tumors and finally MSS CRP-high tumors. Of note, MSS CRP-high tumors exhibited the lowest lymphoid cell densities and PD-L1 expression but were significantly more myeloid inflamed compared to MSS CRP-low tumors (**Table 2**).

Analyzing the CRP-high group as a whole, regardless of MSI status, high CD66b+ neutrophils (p=0.04 and 0.001 at the IM and TC, respectively) and high CD68+ macrophages (p=0.04 at the TC) remained significantly associated with elevated CRP in the univariate analysis, as shown in **Table 3**. In contrast, the adaptive immune markers CD8+ T lymphocytes (p = 0.03 at IM) and foxp3 regulatory T cells (p = 0.03 at TC) correlated inversely with high CRP.

Despite the relatively low number of events in our cohort, survival analyses were performed, as shown in **Table S3**. Of particular interest, CD68+ macrophages at the IM correlated with risk of death from colon cancer (39% (CI: 17–64) for high CD68+ versus 0% (CI: 0–19) for low CD68+, p = 0.008) whereas stromal CD20+ B cells at the TC correlated with risk of death from all causes (50% (CI: 25–75) for low CD20+ versus 13% (CI: 2–38) for high CD20+, p = 0.05). Neither neutrophils nor CD8+ T cells had prognostic impact in our cohort. Due to the small number of patients and few events, multivariate analyses were not performed on this material.

Systemic Inflammation Associates With a Myeloid Inflamed Tumor Microenvironment in CC Patients

We hypothesized that a combinatorial approach based on the expression of two or more immune markers rather than single-cell analysis better could elucidate potential correlations between distinct immune phenotypes and systemic inflammation. For that purpose, densities of CD4+ and CD8+ T- lymphocytes (termed the adaptive composite score) and CD68+ macrophages and CD66b+ neutrophils (termed the innate composite scores) were compounded and categorized as high or low based on the median of the combined scores. Interestingly, we found that regardless of the adaptive score, tumors with a high innate score had increased risk of elevated CRP (shown in **Figure 5A**). The scatter plot in **Figure 5B** depicting adaptive and innate composite scores in CRP-high and -low patients further supported this observation, suggesting that it is the presence of a myeloid-inflamed and not the absence of a lymphoid-inflamed TME that seems to be the driver of systemic inflammation.

Different Immune Phenotypes Correlate With MSI Status and Systemic Inflammation

To further explore the concept of differential immune phenotypes, present in our cohort, hierarchical clustering was

TABLE 3 | Associations between selected immune markers and systemic inflammation in colon cancer patients.

Immune marker	Area	Risk of high CRP		p value
		Low, N, % (CI)	High, N, % (CI)	
CD8+ T cells*	IM	8, 50 (25–75) %	11, 69 (41–89) %	0.47
	TC	13, 81 (54–96) %	6, 38 (15–65) %	**0.03**
CD4+ T cells**	IM	9, 56 (30–80) %	10, 63 (35–85) %	1.00
	TC	11, 69 (41–89) %	8, 50 (25–75) %	0.47
CD20+ B cells***	IM	8, 50 (25–75) %	11, 69 (41–89) %	0.47
	TC	11, 69 (41–89) %	8, 50 (25–75) %	0.47
CD4_foxp3+ T cells***	IM	11, 69 (41–89) %	8, 50 (25–75) %	0.47
	TC	13, 81 (54–96) %	6, 38 (15–65) %	**0.03**
CD68+ macrophages**	IM	8, 44 (22–69) %	13, 72 (47–90) %	0.18
	TC	7, 39 (17–64) %	14, 78 (52–94) %	**0.04**
CD66b+ neutrophils**	IM	7, 39 (17–64) %	14, 78 (52–94) %	**0.04**
	TC	5, 28 (10–53) %	16, 89 (65–99) %	**<0.001**
PD-L1+**	IM	12, 67 (41–87) %	9, 50 (26–74) %	0.50
	TC	10, 56 (31–78) %	11, 61 (36–83) %	1.00
CD68_PD-L1+ macrophages*	IM	9, 50 (26–74) %	12, 67 (41–87) %	0.50
	TC	10, 56 (31–78) %	11, 61 (36–83) %	1.00

Number, risk (CI) of CPR ≥ 30. Univariate analysis.
High and low are categorized as above or below the median of individual immune markers.
**Composite score of immune cells in the stroma and directly tumor infiltrating.*
***Tumor infiltrating only.*
****Stroma only.*
IM, invasive margin; TC, tumor center.
Bolded values indicate statistical significant p-values.

A

Myeloid score CD68+/CD66b+*	Lymphoid score CD8/CD4*	CRP	P-value**
Low	Low	0.5 (0.5-60.0)	
Low	High	0.5 (0.5-57.0)	0.046
High	Low	37.0 (30.0-90.0)	
High	High	39.0 (0.5-81.0)	

B

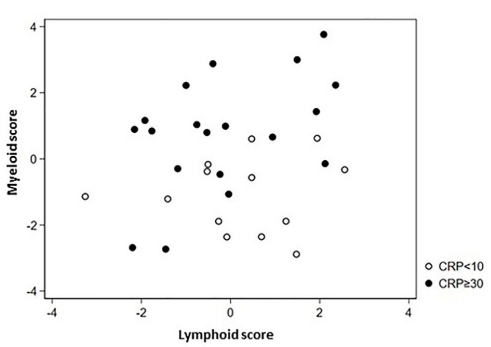

FIGURE 5 | Composite lymphoid and myeloid immune scores correlate differentially with systemic inflammation. **(A)** Median (range) CRP by myeloid *vs.* lymphoid composite immune scores. **(B)** Lymphoid and myeloid composite scores in CRP-high and -low patients. *Immune scores based upon directly tumor-infiltrating immune cell densities at the tumor center. Lymphoid composite score: compounded densities of CD8+T lymphocytes and CD4+T lymphocytes. Myeloid composite score: compounded densities of CD68+ macrophages (inclusive CD68PDL1+) and CD66b+ neutrophiles. **p-value obtained using the Kruskal–Wallis test comparing all four groups.

performed identifying subgroups of tumors with distinct immunological features. As shown in **Figure 6**, three clusters seemed to be present consisting of a subgroup of tumors predominantly lymphoid-inflamed, a subgroup that was more myeloid-inflamed, and a group of hyper-inflamed tumors with high densities of both lymphoid and myeloid immune cells. Additionally, we identified a small group of hypo-inflamed tumors with low numbers of both types of tumor-infiltrating immune cells. When adding information on CRP values and MSI status in the heat map, systemically inflamed MSI-positive tumors corresponded quite well with the group of hyper-inflamed tumors, whereas MSS CRP-high tumors corresponded with the ones being more myeloid and less lymphoid inflamed and, finally, MSS CRP-low tumors seemed either predominantly lymphoid or hypo-inflamed. Of note, none of the CRP-low tumors exhibited high scores of myeloid immune cells.

Spatial Distribution of Tumor Infiltrating Neutrophils Correlates With Systemic Inflammation

Given the assumption that the combined information on both the precise localization and density of immune cells reflects cell functionality and potential interactions taking place within the TME, we investigated the spatial distribution of CD8+ lymphocytes and CD66b+ neutrophils in CRP-high and -low patients. As shown in **Figure 7**, systemically inflamed tumors exhibited significantly higher density of neutrophils in close proximity to tumor nests compared with non-inflamed tumors (1.9% *vs.* 0.9%, respectively, p = 0.009). Moreover, there was a tendency toward lower mean distance between neutrophils and tumor cells in the systemically inflamed patients. We found no significant differences in the spatial distribution of CD8+ lymphocytes between CRP-high and -low tumors. Based on the proof-of-concept approach of the study, further spatial analyses were not performed on this material.

DISCUSSION

In this study, we explored the tumor-immune microenvironment in colon cancer patients related to the presence of SIR, covering important players of both adaptive and innate immunity and their spatial distribution within the primary tumors. By analyzing the immune contexture in patients with and without accompanying SIR, we revealed upregulation of myeloid features in the TME from systemically inflamed patients. Specifically, and in line with our hypothesis, tumor-infiltrating neutrophils and macrophages associated with systemic inflammation. Most strikingly, we found that regardless of an adaptive composite score (compounded CD4 + and CD8+ T cells), a high innate score (compounded CD66b+ neutrophils and CD68+ macrophages) significantly increased the risk of elevated CRP, indicating that it is the presence of a myeloid-inflamed and not the absence of a lymphoid-inflamed TME that associates with systemic inflammation.

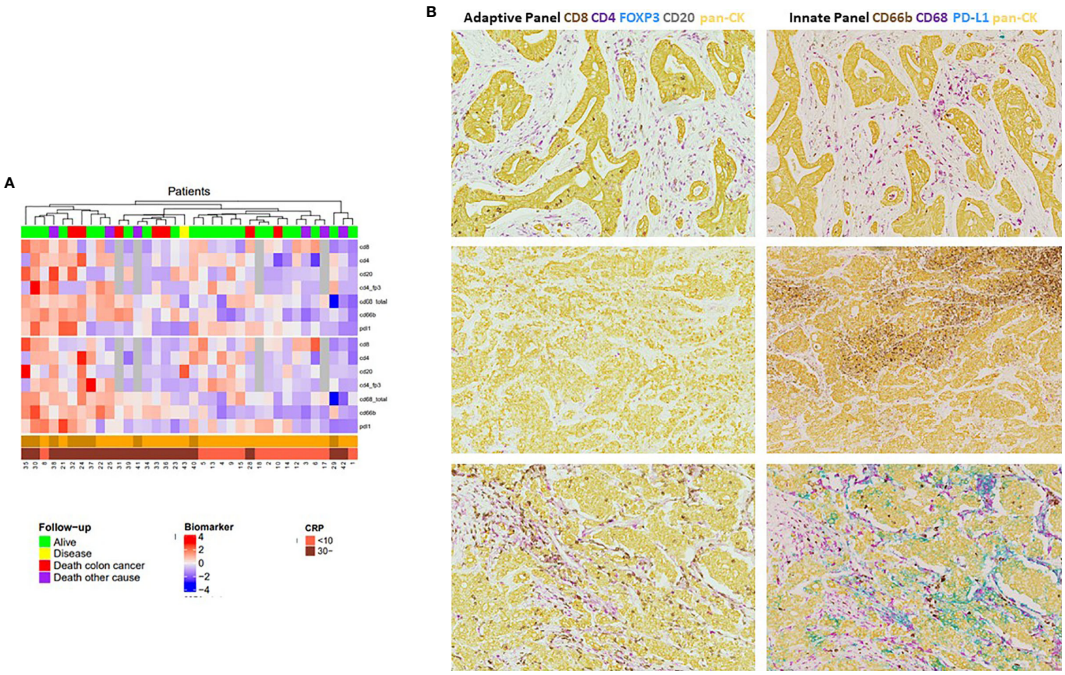

FIGURE 6 | Hierarchical cluster analysis of selected adaptive and innate immune markers in primary resected colon cancer patients. Heat map of unsupervised hierarchical clustering based upon the densities of tumor-infiltrating immune markers. Data were log-transformed to get a normal distribution, standardized to mean zero and variance 1. Information on CRP values; MSI and follow-up statuses were added to the dendrogram for visual interpretation after performing the cluster analysis. **(A)** Clustering based upon tumor-infiltrating immune cell densities and PD-L1 tumor expression. Red color indicates high density, blue low density of each immune marker. **(B)** Representative images from patients with the three predominant immune phenotypes present within our cohort. Upper panel: mIHC-stained tumor slides from a CRP low, MSS pt. with prominent lymphoid infiltration and modest myeloid immune infiltration. Middle panel: tumor slides from a CRP-high, MSS pt. showing predominant myeloid infiltration (almost exclusively CD66b+ neutrophils) and only marginal infiltration by lymphoid immune cells (CD4+ T cells only). Lower panel: tumor slides from a CRP-high, MSI-positive pt. being hyperinflamed with vigorous lymphoid and myeloid immune infiltration and high PD-L1 expression, predominantly expressed by CD68+ macrophages.

A strong impact of myeloid cells has also been demonstrated in a squamous head and neck cancer cohort revealing differential immune profiles, representing either lymphoid-, myeloid-, or hypo-inflamed tumors, where myeloid-enriched tumors associated with the shortest overall survival regardless of HPV status (19). Additionally, in a small validation cohort of pancreatic cancer patients receiving a neoadjuvant CSF vaccine, tumors seemed to cluster into two groups depending on the degree of myeloid inflammation, where again myeloid-dominated tumors correlated with the poorest clinical outcomes (19). Notably, the lymphocyte infiltration did not differ between the two groups, indicating a strong immunosuppressive role of myeloid cells potentially compromising effective antitumor immune responses. A detrimental effect of myeloid cells was also found in a recent study using a transgenic mouse model of HPV-derived cancers treated with a therapeutic vaccine alone or in combination with double immune checkpoint blockade (20). In this study, vaccination alone or in combination elicited neither tumor regression nor effective CD8+ responses due to the expansion of myeloid cells in peripheral lymphoid tissue, suggesting a systemic myeloid-driven immunosuppression impairing the efficacy of immunotherapy.

These results combined with the findings of our study highlight the strong role myeloid cells may play in the TME by creating an immunosuppressive state and even outperform the potential beneficial role of lymphoid cells and negatively affect prognosis. Although myeloid cells have been associated with poor survival and treatment outcome in several cancer types (21–24), their role in the TME remains to be fully understood and undervalued compared with the much more studied lymphoid cells (11, 14). Experimental studies have shown that both macrophages and neutrophils, being some of the most important players of innate immunity, may exhibit contradictory roles in cancer with both pro-tumoral and antitumoral properties depending on the immunological context (13, 25, 26). Major tumor-promoting and immunosuppressive functions of myeloid cells include

A

	CRP<10	CRP≥30	p value
Mean distance CD8+T to tumor cells, µm	56.9 (0.0-126.4)	43.7 (0.0-649.7)	0.38
Close CD8+Tcell-tumor area IM (%)	0.695 (0.058-8.602)	0.846 (0.007-5.017)	0.92
Close CD8+Tcell-tumor area TC (%)	0.482 (0.008-2.784)	0.320 (0.008-2.291)	0.23
Mean distance CD66b+ to tumor cells, µm	22.2 (10.5-44.9)	15.4 (3.5-63.8)	0.43
Close CD66b+neu-tumor area IM (%)	0.8 (0.0-4.4)	1.8 (0.1-29.8)	0.36
Close CD66b+neu-tumor area TC (%)	0.9 (0.0-3.4)	1.9 (0.2-20.3)	0.009

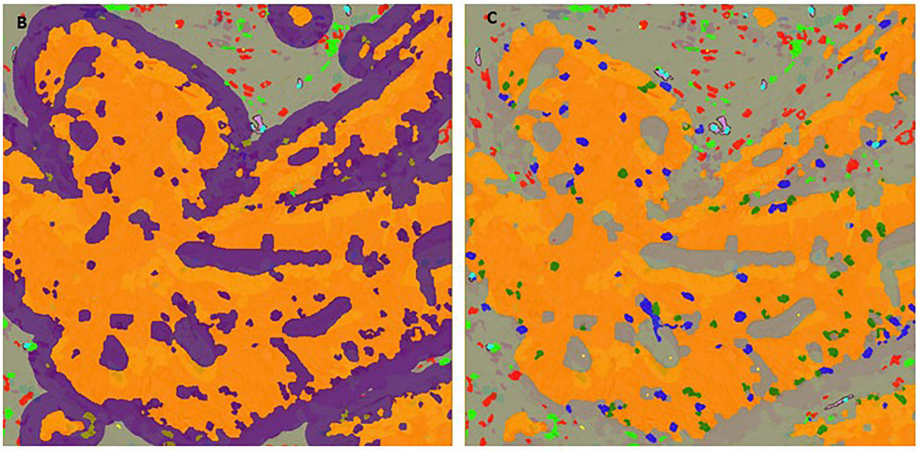

FIGURE 7 | Spatial distribution of CD8+T-cells and neutrophils in CRP-high and -low colon cancer patients. **(A)** Table showing median (range) of various spatial relationships. p-values were obtained using the Kruskal–Wallis test. **(B)** Close immune cells to the tumor area of either CD8+ T cells or CD66b+ neutrophils estimated by outlining 20 µm around tumor islet. **(C)** Nearest neighbor analysis estimating the average distance between immune cells of interest (either CD8+ T cells or CD66b+ neutrophils) and nearest tumor cells.

release of growth factors such as MMP-9 and oncostatin M which induce upregulation of VEGF and HIF-2alpha pathways leading to neo-angiogenesis, hypoxia, and ultimately cancer invasiveness and progression (14, 27, 28). Moreover, myeloid cells have been shown to be crucial at all steps of the metastatic process (26, 29, 30). In addition to their direct tumor-promoting functions, both tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) may also suppress antitumor adaptive immune responses through the production of IL-10 and TGF-beta as well as the enzymes arginase 1 and IDO, which are detrimental for T cell-mediated immunity (13, 31).

It is particularly interesting to see what happens under circumstances of chronic wounding, which might be analogous to the situation of the colon where tumors can arise in relation to a chronically inflamed and often injured epithelium (32). Using a zebrafish melanoma model, it has been shown that neutrophils attracted to a wound are rapidly diverted to adjacent pre-neoplastic cells resulting in increased proliferation and melanoma formation (33).The corresponding clinical evidence for such a direct neutrophil-driven tumor growth has been further demonstrated in human melanoma where neutrophil density correlated strongly with increased proliferation and associated with poor melanoma-specific survival (33).

Altogether, these exciting findings support the notion that neutrophils may fuel and shape tumors and highlight innate immune cells as a therapeutic target for immunotherapeutic approaches (34).

By far, local and systemic tumor-associated inflammations have been regarded as separate processes with only few studies investigating their possible interrelationship (35–38). Similar to our findings, a recent study in all stages of CRC demonstrated a significant inverse relationship between high CRP (>10 mg/l) and foxp3 regulatory T cells, but no associations were detected for other immune cells including myeloid cell types. Of note and contrary to our findings, no significant relationship was found between MSI status and CRP except that all patients with

CRP>75 mg/l had MSS tumors (39). However, the immune infiltration in this study was determined using TMAs. Based on the intratumoral heterogeneity observed in our material, it could be that the tissue sampling performed when preparing TMAs, being snapshots of the tumor, is not representative of the global immune cell infiltration and may in part explain the lack of association. Additionally, this study also included rectal cancer patients which have been shown to be less systemically inflamed and might represent another tumor entity when it comes to the inflammatory tumor reaction (40).

An intriguing and initially surprising finding of our study was the significant association between positive MSI status and systemic inflammation. Given the good prognosis related to MSI in early-stage colon cancer and the poor prognosis related to the SIR, one might rather expect an inverse or no association between the two entities. Nevertheless, we found that MSI CRP-high tumors not only were hyper-inflamed in terms of considerable lymphoid inflammation that previously has been shown to accompany MSI-positive tumors, but also were highly infiltrated by myeloid immune cells, particularly neutrophils. Moreover, MSI-high tumors exhibited upregulation of PD-L1, predominantly expressed by myeloid immune cells infiltrating the tumor stroma and to a lesser extent by the tumor cells themselves. This observation stands in contrast to other tumor types such as lung, bladder, and kidney cancer, where tumor PD-L1 expression is a common feature (41). However, consistent with our findings, a study by Llosa et al. demonstrated much higher levels of PD-L1 expression in MSI compared to MSS tumors, almost exclusively expressed by tumor-infiltrating myeloid cells and not the tumor cells (42). Indeed, our findings need to be further explored in a larger dataset, but a working hypothesis could be that MSI tumors accompanied by systemic inflammation exhibit a highly myeloid immune infiltrated TME resulting in an immunosuppressive state either caused by 1) a compensatory upregulation of immune checkpoints stimulated by preexisting cytokines such as IFN-gamma following the MSI-induced active immune microenvironment leading to a functional exhaustion of the T cells or 2) direct immunosuppressive and tumor-promoting effects exerted by the myeloid cells themselves. In either way or both, such myeloid-dependent immunosuppression might counterbalance the potential beneficial effects of the lymphoid immune infiltration and blunt effective antitumor immune responses, at least without immune checkpoint inhibition.

In an effort to decipher the complex TME and variable treatment outcomes to immunotherapy, emerging studies take into context the spatial aspect of the tumor immune landscape (16, 43, 44). Recent data point toward both prognostic and predictive values of proximity analyses, in terms of measurement of the exact localization and distances between tumor and immune cells, suggesting that spatial patterns reflect cell functionality and clinically meaningful tumor–host interactions taking place within the TME (45–47). Notably, in our study we found that systemically inflamed patients had significantly more neutrophils in close proximity to tumor cells as compared to non-inflamed patients whereas no differences in the spatial features of CD8+ T cells could be detected. Again, this finding supports the hypothesis that myeloid inflammation and neutrophils in particular play a critical role in the context of SIR in CC. Additionally, it adds to the argumentation for preferring whole slides over TMAs enabling a more comprehensive mapping of the immune context of tumors (48).

Our study has several limitations. Due to the proof-of concept design, it covers a limited patient series. Thus, our findings need to be tested in a larger dataset before biologic conclusions can be drawn. We plan to enrich the cohort for confirmation and further analyses to expand our understanding of how systemic inflammation and localized tumor-associated inflammation influence each other. Another limitation owing to the IHC itself is the challenge of characterizing functional phenotypes. Myeloid cells exhibit a high degree of plasticity displaying a continuum of polarization states being more or less immuno-suppressive or stimulatory. This dynamic diversity is difficult to capture with IHC antibodies directed toward one or two fixed cell markers (28). Although we performed spatial analyses as a pseudo marker of cell functionality, the precise identification of the multitude of polarization states that seem to exist for myeloid cells cannot be truly captured by current IHC techniques (49).

Taken together, our data highlight the importance of a broader and more comprehensive immune characterization of tumors covering both lymphoid and myeloid cell populations. The concept of hot and cold tumors, categorizing tumors based on the infiltration of T cells, has been widely used to inform patient prognosis and predict immunotherapeutic efficacy (50). Within recent years, this simplistic classification has been refined acknowledging the complexity and heterogeneity of the immune infiltrate of tumors with the introduction of four distinct immune subgroups: hot, altered-excluded, altered-immunosuppressed, and cold (51). However, this approach is still mainly focusing on T-cell infiltration without further characterizing other cell populations such as myeloid immune cells. Our findings, supported by others, demonstrate the potential limitations of such a T cell-focused classification, indicating that "hot tumors" can be so much more than just "T cell inflamed." We hypothesize that a vigorous myeloid-inflamed TME might counterbalance the beneficial and potential tumor-suppressive effect of a strong lymphoid immune infiltrate and negatively affect antitumor immunity. Accordingly, we propose that strategies of converting "cold tumors to hot" also should include efforts of targeting the myeloid-derived immunosuppression before harnessing T cell-mediated antitumor immune responses.

In conclusion, we herein provide a framework for expanding our understanding of the immune landscape in CC and explore the role of CRP as a systemic and informative biomarker of the immune responses taking place at the tumor site. Further deciphering distinct immune phenotypes and spatial features that associate with systemic inflammation may improve our understanding of inherent immune responses in CC and hold critical implications for therapeutic approaches.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Committees for Medical and Health Research Ethics (REC) South East. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AK, the first author, has performed lab work, planned and performed digital analyses, participated in statistics together with EP, written the draft for the manuscript, and driven the scientific discussions. PN assisted in performing, optimizing, and interpreting digital imaging. JG planned and performed laboratory work. MN reviewed and annotated tumor regions from FFPE tissue blocks and evaluated the MMR IHC-analysis. TS and CK participated in methodological and scientific discussions and had the main ideas behind the study. All authors have reviewed and commented the manuscript during the process and before submission. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.716342/full#supplementary-material

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Fueling the flames of colon cancer – does CRP play a direct pro-inflammatory role?

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Background: Systemic inflammation, diagnostically ascribed by measuring serum levels of the acute phase reactant C-reactive protein (CRP), has consistently been correlated with poor outcomes across cancer types. CRP exists in two structurally and functionally distinct isoforms, circulating pentameric CRP (pCRP) and the highly pro-inflammatory monomeric isoform (mCRP). The aim of this pilot study was to map the pattern of mCRP distribution in a previously immunologically well-defined colon cancer (CC) cohort and explore possible functional roles of mCRP within the tumor microenvironment (TME).

Methods: Formalin-fixed, paraffin-embedded (FFPE) tissue samples from 43 stage II and III CC patients, including 20 patients with serum CRP 0-1 mg/L and 23 patients with serum CRP >30 mg/L were immunohistochemically (IHC) stained with a conformation-specific mCRP antibody and selected immune and stromal markers. A digital analysis algorithm was developed for evaluating mCRP distribution within the primary tumors and adjacent normal colon mucosa.

Results: mCRP was abundantly present within tumors from patients with high serum CRP (>30 mg/L) diagnostically interpreted as being systemically inflamed, whereas patients with CRP 0-1 mg/L exhibited only modest mCRP positivity (median mCRP per area 5.07‰ (95%CI:1.32-6.85) vs. 0.02‰ (95%CI:0.01-0.04), p<0.001). Similarly, tissue-expressed mCRP correlated strongly with circulating pCRP (Spearman correlation 0.81, p<0.001). Importantly, mCRP was detected exclusively within tumors, whereas adjacent normal colon mucosa showed no mCRP expression. Double IHC staining revealed colocalization of mCRP with endothelial cells and neutrophils. Intriguingly, some tumor cells also colocalized with mCRP, suggesting a direct interaction or mCRP expression by the tumor itself.

Conclusion: Our data show that the pro-inflammatory mCRP isoform is expressed in the TME of CC, primarily in patients with high systemic pCRP values. This strengthens the hypothesis that CRP might not only be an inflammatory marker but also an active mediator within tumors.

KEYWORDS

systemic inflammation, C-reactive protein (CRP), CRP isoforms, monomeric CRP, colon cancer, immunohistochemistry (IHC), biomarkers, tumor microenvironment

Background

Systemic inflammation, diagnostically ascribed by measuring levels of the acute phase protein CRP in serum, has consistently been correlated with poor outcomes across cancer types (1–3). However, the biological relationship between CRP and inflammation remained unresolved and controversial for decades. Recently, evidence has been advanced showing that CRP exists in different structural isoforms with distinct biological activities (4). The circulating CRP isoform is a highly soluble pentameric molecule (pCRP) composed of 5 identical globular subunits arranged in a ring-shaped structure (5). Each subunit contains a calcium dependent binding site enabling interaction with phosphocholine (PC), a major component of plasma membranes, defined as the primary ligand for pCRP. However, for the PC ligand to become accessible for CRP binding, structural remodeling of the membrane lipid is required. This may occur when cells become activated, either by an infectious or non-infectious inflammatory stimulus or following cell damage or apoptosis and may involve the activity of the enzyme phospholipase A2 (6). Upon interaction with the exposed PC groups, pCRP begins to change structure first into an intermediate swollen pentameric form designated pCRP* (or $mCRP_m$), then into the fully dissociated, less soluble and antigenically distinct monomeric, modified form, referred to as mCRP (7, 8). Experimental studies have shown that the biological effects of CRP are dependent on its structural conformation, demonstrating strong pro-inflammatory properties of mCRP, whereas pCRP appears to exhibit mainly weak anti-inflammatory activities (9, 10). *In vitro* studies directly comparing the biological effects of the two isoforms, have shown that mCRP has approximately 10-100-fold more potent inflammatory capacity than its precursor molecule pCRP (11).

Notably, once formed, mCRP deposits within tissues due to its low aqueous solubility where it may interact directly with various cells and components of the microenvironment (3, 11). Specifically, it has been shown that mCRP can engage with both epithelial and endothelial cells, platelets, and various immune cells such as macrophages and neutrophils (9, 12, 13). Additionally, mCRP can interact directly with components of the extracellular matrix as well as fibroblasts, which are major constituents of the tumor stroma (3). At the molecular level, data have shown that mCRP preferentially binds to cholesterol rich lipid rafts that are important microdomains of plasma membranes involved in a wide range of cellular processes including signal transduction (9, 14). Following membrane insertion, mCRP can stimulate intracellular signaling including activation of pro-inflammatory pathways such as those involving the pivotal transcription factor NF-κB and its downstream mediators (3).

While most research on the different isoforms of CRP has been carried out in cardiovascular and neurodegenerative disorders, as well as some autoimmune diseases, little is known about their role in cancer (11, 13, 15–18). In line with our previous work (19), focusing on why cancer patients with elevated blood CRP levels have inferior outcomes, the hypothesis evolved that the potent monomeric/modified form of CRP may play a direct pro-inflammatory role within the TME of systemically inflamed cancer patients. First, by localizing the inflammatory response as circulating pCRP binds to exposed PC molecules expressed by cells that have been activated due to the inflammatory TME, leading to *in-situ* dissociation of pCRP into the pro-inflammatory monomeric isoform. Secondly, as mCRP accumulates within the tumor, a process which is considered perpetual and non-resolving, owing to the chronic nature of systemic inflammation, mCRP may play a direct and active role through the recruitment and activation of inflammatory cells and components of the TME, potentially fueling and shaping the local inflammatory response, and ultimately promote tumor progression.

In order to explore whether there is a role for mCRP in systemically inflamed cancer patients, the aim of this proof-of-concept study was to identify and map the pattern of mCRP distribution in a previously immunologically well-defined cohort of colon cancer (CC) patients. Using complementary strategies including immunohistochemistry (IHC)-based colocalization imaging techniques, we were able to elucidate potential functional roles of mCRP in the microenvironment of CC tissue.

Materials and methods

Patients and tissue samples

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were retrospectively obtained from 43 stage II and III CC patients, including 20 patients with circulating CRP of 0-1 mg/L (CRP-low patients) and 23 patients with CRP >30 mg/L (CRP-high patients), undergoing resection for their primary tumors at Sørlandet Hospital, Norway, between 2005 and 2015. Clinical information and follow-up data were obtained from a local colorectal cancer

database as described previously (19). Characteristics of CRP-high and CRP-low patients are detailed in Table 1.

Serum CRP values were determined using a standardized immunoturbidimetric assay, which previously has shown specificity for pCRP without interference with mCRP (20), performed on blood samples taken within 14 days (at the day closest to the resection) prior to the operation in order to reflect a state of chronic inflammation. Exclusion criteria were clinical evidence of infection, use of antibiotics or immunosuppressive drugs within 4 weeks prior to the operation or a history of chronic inflammatory disease including autoimmune disorders.

The study was approved by the Norwegian Regional Ethics Committee.

Immunohistochemistry and double immunofluorescence

Whole slides from FFPE tumor blocks were immunohistochemically stained with a conformation-specific mCRP monoclonal antibody (mCRP-mAb 9C9), which has been fully characterized previously demonstrating specificity for mCRP and not pCRP (21, 22). FFPE sections were cut at 3 µm, mounted on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA), dried for 1 hour at 60°C, and prepared for IHC staining using standard kits from Benchmark Ultra (Ventana, Roche Diagnostics International AG, Basel, Switzerland) for deparaffinization, rehydration, antigen retrieval, and endogenous peroxidase blocking. Next, sections were incubated with the primary antibody (mCRP mAb 9C9 at dilution 1:100) for 30 minutes followed by DAB (3, 3'-diaminobenzidine) substrate chromogen solution for antigen visualization. Negative controls were performed by replacing the primary antibody with antibody diluent (Agilent S2022; DAKO), but otherwise prepared similarly. All sections were counterstained with hematoxylin and mounted before they were scanned at ×20 magnification using NanoZoomer 2.0 HT (Hamamatsu Photonics KK, Hamamatsu City, Japan).

To map the pattern of mCRP distribution and explore possible colocalization with immune, endothelial and tumor markers, double stainings with chromogenic IHC and IF were performed on tumor slides from selected patients with elevated circulating CRP and

TABLE 1 Clinical characteristics of colon cancer patients according to the level of circulating CRP.

Characteristic	CRP 0-1, N = 20[1]	CRP≥30, N = 23[1]	p-value[2]
Age	67 (60, 71)	78 (71,86)	0.003
Sex			0.70
Female	11 (55%)	14 (61%)	
Male	9 (45%)	9 (39%)	
Stage			<0.001
II	0 (0%)	10 (43%)	
III	20 (100%)	13 (57%)	
Tumor site			0.77
Left	4 (20%)	3 (13%)	
Right	10 (50%)	14 (61%)	
Sigmoid	6 (30%)	6 (26%)	
Adjuvant chemotherapy			<0.001
None	3 (15%)	19 (83%)	
Only 5-FU based	6 (30%)	3 (13%)	
Platinum doublet	11 (55%)	1 (4.3%)	
MMR-Status			0.002
MSS	20 (100%)	14 (61%)	
MSI	0 (0%)	9 (39%)	
Survival status			0.010
Alive	15 (75%)	7 (30%)	
Dead	4 (20%)	9 (39%)	
Recurrence	1 (5.0%)	7 (30%)	
Follow-up (years)	9.3 (8.7, 10.9)	8.8 (5.2, 11.3)	0.58

[1]Median (IQR); n (%).
[2]Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test.

pronounced mCRP expression as evaluated by the mCRP single staining. Antibodies against the following markers were applied in addition to anti-mCRP: CD34 for endothelial cells, CD68 for macrophages, CD66b for neutrophils and pan-cytokeratin (pan-CK) as tumor marker. All antibodies were commercially available except for mCRP-mAb 9C9. Origin and incubation times for the applied antibodies are listed in Supplementary Table S1.

All double stainings were performed after antigen retrieval as described above. For double IHC, FFPE sections were incubated sequentially, first, with mCRP-mAb at dilution 1:100 for 30 minutes followed by chromogenic DAB staining. The slides were then incubated with the appropriate second primary antibody as listed above at the time indicated for each antibody applying Ultra-view fast red as chromogenic dye. Finally, slides were counterstained with hematoxylin, mounted and scanned at ×20 magnification using NanoZoomer 2.0 HT (Hamamatsu, Japan).

Double IF was performed, using the tyramide signal amplification strategy on the Discovery Ultra Autostainer (Ventana Medical systems) applying two different fluorophores in a sequential manner for visualization of the respective antigens. First, tissue sections were incubated with mCRP-mAb (dilution 1:10) for 30 min, using rhodamine as fluorescent dye, followed by incubation with the appropriate second primary antibody (as listed above) using DCC (N′-dicyclohexylcarbodiimide) as the selected fluorophore. Stained slides were mounted with Vectashield Antifade Mounting Medium, which included DAPI as nuclear counterstain, whereafter they were stored overnight at 4°C, protected from light. Mounted slides were scanned at x 20 using NanoZoomer S60 (Hamamatsu, Japan).

Digital image analysis

Image analysis was performed using Visiopharm Integrator System software version 2019.02 (VIS; Visiopharm A/S, Hørsholm, Denmark).

Regions of interest (ROIs) were defined by a trained pathologist. The tumor was outlined as one region encompassing the invasive margin and tumor center. On slides where normal colon mucosa was present (11 out of 43), this was annotated as a separate ROI. Two AI-based algorithms were utilized for the segmentation and annotation of either tumor epithelium or normal colon mucosa in addition to their surrounding stromal tissue, as outlined in Figure 1. Training of the algorithms included a representative set of whole slide images (WSI) where stromal tissue, unstained background, and either tumor epithelium or normal colon mucosa were manually annotated at pixel-level. Using input images of 512 x 512 pixels, U-nets as presented by Ronneberger et al. were trained in VIS's Author AI (23). Learning rates based on Adam Optimization were set at 1×10^{-5}, and data augmentation was utilized (24).

In the designated regions outlined by the AI applications, mCRP was identified by thresholding of the brown staining color (DAB), which was highlighted by a color deconvolution step. Post-processing algorithms included morphological operations and changes by area or surrounding. All results of the image analyses were manually reviewed to ensure that areas with mucin, tissue folds, and other technical artefacts were excluded from the analysis.

mCRP was quantified as area proportions defined as: area of positive mCRP staining divided by the total area of the given ROI. Since the area of mCRP was small compared to the total area of the tumor, proportions were multiplied with 1000 and given per mile instead of percentages. Area proportions of mCRP were calculated both as a combined score of total mCRP within the whole tumor as well as separately for the tumor epithelium and tumor stroma, respectively. Finally, mCRP was evaluated within the region of normal colon mucosa, scoring epithelium and stroma combined, on applicable slides.

Double IHC and IF stainings were evaluated and interpreted manually by visual examination only, using NDP. View 2.0 (Hamamatsu).

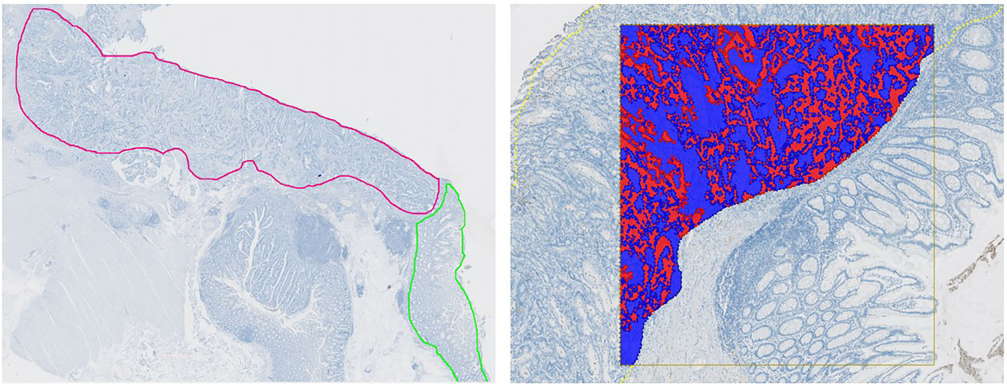

FIGURE 1
Automated Image Analysis Workflow. Left: Whole slide image with annotated tumor regions. Tumor in red and adjacent normal colon mucosa in green. Right: An AI-based algorithm was developed for analyzing the pattern of mCRP distribution and accurately segment tumor epithelium (red) and tumor stroma (blue).

Immune phenotypes and microsatellite instability analysis

Immune cell densities (CD8+ T-cells, CD4+ T-cells, Foxp3+ T-cells, CD20+ B-cells, CD66b+ neutrophiles, CD68+ macrophages) assessed within the same tumor regions were captured from a series of multiplexed IHC (mIHC) performed in a previous study (19). However, due to technical issues with the mIHC, 7 patients did not have corresponding immunological profiles and had to be excluded from the mCRP-immune cell correlation analyses.

Mismatch repair (MMR) status was determined by an experienced pathologist through IHC evaluation of the DNA mismatch repair proteins MHL1, MSH2, MSH6, and PMS2. Tumors that were negative in one or more of the four stainings, or inconsistent with IHC, were verified with the Idylla MSI test (Biocartis) as described previously (25). Accordingly, patients were classified as either microsatellite stable (MSS) or instable (MSI).

Statistical analysis

The distribution of mCRP was assessed as mCRP proportions, as specified above. The median mCRP proportion within groups were calculated and compared using the median test. Differences in patient characteristics were evaluated using Fisher´s exact test and the two-sample t-test with unequal variance. The correlation between mCRP and circulating CRP was assessed using Spearman correlation analysis. Associations between mCRP and the immune markers obtained from mIHC were analyzed using Spearman correlations and heatmaps were generated. The Aalen-Johansen method was applied to estimate the risk of CC death or recurrence and compared between CRP-high and CRP-low patients using the log-rank test. For identification of the most optimal threshold/cutoff value for tumor mCRP expression used in the analysis of the prognostic impact of mCRP, a receiver operating characteristics (ROC) curve was computed. Due to competing risks (death of colon cancer and death of other causes) varying at different time points, the ROC-curve was calculated at the time of median follow-up using the quantified level of mCRP tumor expression for all patients. The optimal mCRP cutoff value was defined as the point on the ROC curve with sensitivity and specificity closest to 100%, which corresponded graphically to the point on the curve with the minimum distance to the upper left corner. The cumulative risk curves for CC death or recurrence are shown for patients with mCRP tumor expression below and above the optimal cutoff value. P<0.05 was considered statistically significant for all analyses. R software version 4.2 was used for statistical calculations.

Results

mCRP is expressed predominantly by tumors from systemically inflamed patients and is exclusively present within tumor tissue and not adjacent normal colon mucosa

As depicted in Figure 2, mCRP was abundantly present in tumors from systemically inflamed CC patients whereas non-inflamed patients exhibited only modest mCRP positivity (median mCRP per area 5.07‰ (95%CI, 1.32-6.85) vs. 0.02‰ (95%CI, 0.01-0.04) p<0.001). Correspondingly, tissue-expressed mCRP correlated strongly with circulating CRP (Spearman correlation 0.81 (95%CI, 0.67-0.89), p<0.001). Further analysis of the pattern of mCRP expression demonstrated that MSI positive tumors exhibited significantly more mCRP compared with CRP-high MSS and CRP-low MSS patients, respectively (data shown in Table 2). Furthermore, AI-based image analysis discriminating between tumor epithelium and tumor stroma, showed significantly more mCRP expression in the stromal compartment as compared to the tumor epithelium. Notably, mCRP was detected exclusively within the tumor area whereas adjacent normal colon mucosa showed no mCRP expression (representative image shown in Figure 2C).

Prognostic impact of the CRP isoforms

Given the known prognostic role of systemic inflammation and the strong correlation between tissue-bound mCRP and circulating serum CRP, we sought to evaluate whether mCRP had an independent impact on survival outcomes within our cohort. As shown in Figure 3, patients with tumors exhibiting mCRP density above the ROC-curve identified cutoff value of tumor mCRP expression tended to perform poorer in terms of increased risk of CC death or recurrence compared with patients that had tumors with mCRP density below the optimal mCRP cutoff value, although this did not reach statistical significance. Nonetheless, elevated serum CRP was confirmed to be predictive of compromised survival and increased risk of recurrence within our cohort (Figure 3C).

mCRP colocalizes with neutrophils and endothelial cells in the TME

To elucidate potential functional roles of mCRP in the TME, we took a stepwise approach. First, by performing a correlation analysis of the quantified mCRP IHC results with the immune profiles obtained previously on the same patients and tumor areas, followed by double IHC and IF for mCRP and selected immune and endothelial markers. As shown in Figure 4 the most evident association was with the neutrophils, showing a highly significant correlation between mCRP and cd66b+ neutrophils (Spearman correlation 0.57, p<0.001). This was supported by double IHC demonstrating strong colocalization of mCRP and areas of neutrophil infiltration (Figure 5A). At the sub-cellular level, however, immunofluorescent labeling showed only occasional direct cellular overlap, but confirmed the pattern of close proximity, indicative of an interaction, and to a lesser extent, intracellular uptake of mCRP into the neutrophils.

Moreover, mCRP seemed to coincide with areas of necrosis, with or without neutrophil infiltration, where non-specificity could be ruled out by negative control staining (Figure 5B).

Less evident, but still present, was colocalization of mCRP and CD68+ macrophages (Figure 5C). However, mCRP-positive macrophages seemed primarily to coincide with highly immune

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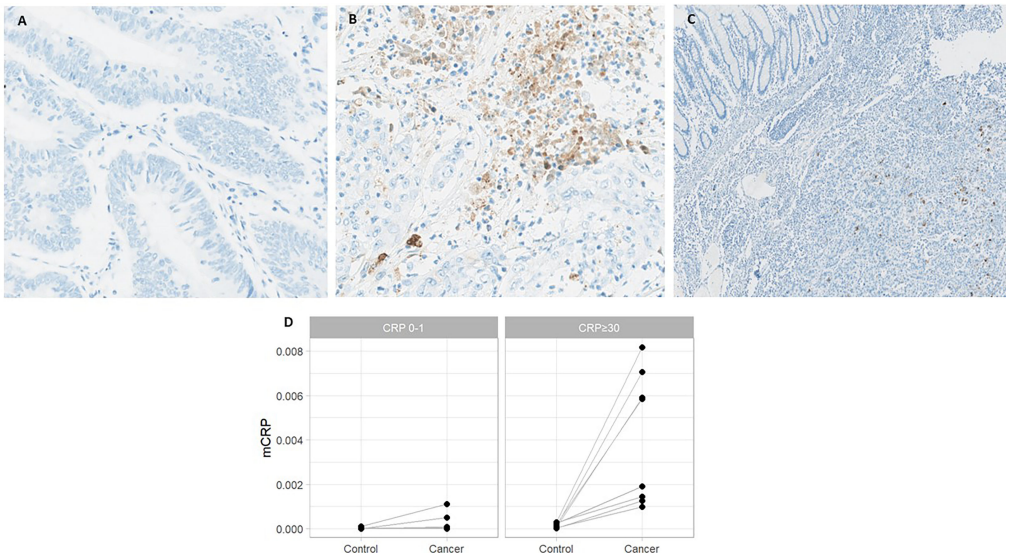

FIGURE 2
mCRP expression in systemically inflamed and non-inflamed colon cancer patients and adjacent normal colon mucosa. Representative images from patients with **(A)** normal and **(B)** elevated circulating CRP. **(C)** Normal colon mucosa adjacent to the tumor with no mCRP expression. **(D)** Quantified mCRP (proportion of area with positive mCRP staining) assessed within the tumor and adjacent normal colon mucosa (control) in CRP-high and CRP-low patients.

infiltrated areas in general, as the majority of macrophages present more globally dispersed within the tumor tissue showed less mCRP positivity, suggesting that mCRP might be an amplifier of the local inflammatory response.

Based on data from previous studies in cardio- and cerebrovascular diseases, demonstrating a direct interaction between mCRP and endothelial cells, we performed double immune stainings with mCRP and the specific endothelial marker CD34. Notably, mCRP co-localized with endothelial cells lining intratumoral vessels and was present within the lumen of some vessels, suggesting a systemic origin of the monomeric isoform (Figure 5D). Additionally, mCRP could be detected within the vessel wall of some mCRP/CD34-positive intratumoral vessels.

Interestingly, in some tumors, mCRP appeared rather scattered around in the tumor stroma, occasionally forming aggregates, but more often globally dispersed as small granules within the connective tissue, suggesting a potential interaction between mCRP and components of the ECM, although this was not directly evaluated by IHC (Figure 5E).

Positive colocalization of mCRP and tumor cells

Serendipitously, when examining the pattern of mCRP distribution, it became evident that some tumor cells were closely surrounded by mCRP, forming a halo-like coating around individual tumor cell nuclei (Figure 5F). To further elucidate this observation, we performed double immune stainings with mCRP and the gastrointestinal specific cytoplasmatic tumor marker pan-cytokeratin. Using double IHC and IF we were able to demonstrate colocalization and evidence of direct overlap of mCRP and tumor cells, indicating close interaction and/or intracellular uptake of mCRP, or potentially, mCRP expression by the tumor itself (representative images shown in Figure 6).

TABLE 2 mCRP distribution in colon cancer patients stratified for serum CRP and MSI-status.

	n	mCRP stroma	mCRP tumor	P-value
All (per mille), Median (CI)	43	0.70 (0.08-4.33)	0.08 (0.01-0.48)	<0.001
CRP 0-1 (per mille), Median (CI)	20	0.02 (0.01-0.07)	0.00 (0.00-0.01)	<0.001
CRP≥30, MSS (per mille), Median (CI)	14	5.45 (1.79-8.01)	0.33 (0.12-2.87)	<0.001
CRP≥30, MSI (per mille), Median (CI)	9	(3.45-131.76)	2.52 (0.80-13.53)	0.027

Quantification of tissue-associated mCRP expression estimated by IHC.

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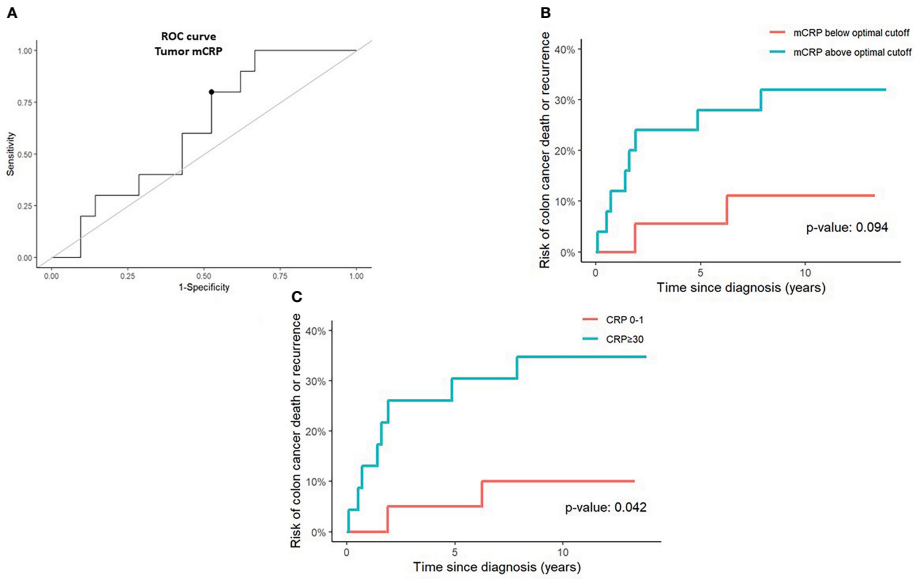


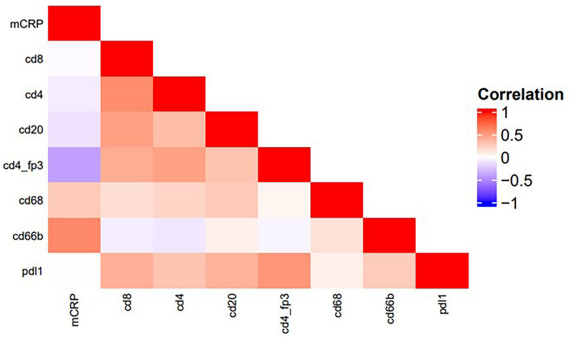
FIGURE 3
Prognostic value of tumor mCRP expression and serum CRP in colon cancer patients. **(A)** A receiver operating characteristics (ROC) curve was calculated to determine the optimal tumor mCRP cutoff value (marked by a bullet) defined as the point on the curve with sensitivity and specificity closest to 100%, corresponding graphically to the point with the minimum distance to the upper left corner **(B)** Risk of colon cancer death or recurrence above and below the optimal tumor mCRP cutoff value identified from the ROC curve. **(C)** Risk of colon cancer death or recurrence in CRP-high (serum CRP >30 mg/L) and CRP-low (serum CRP 0-1 mg/L) patients. The optimal mCRP cutoff value was defined.

Discussion

In this study we explore the presence of the mCRP isoform and its correlation with innate and adaptive immune cells and serum levels of pCRP in a cohort of stage II and III CC patients. We report that the monomeric form of CRP (mCRP) is present within tumors and that the level of expression correlates strongly with the level of circulating pCRP. Additionally, mCRP expression is associated significantly with tumor infiltrating neutrophils. Importantly, mCRP was expressed exclusively within tumors whereas adjacent normal colon mucosa showed no mCRP positivity.

Persistent elevation of blood CRP levels alongside malignancies is increasingly recognized as an independent predictor of adverse outcomes, both in terms of compromised survival and treatment responses (1, 3, 26). Despite mounting evidence, generated primarily in cardiovascular and neurodegenerative disorders (12, 13, 15, 27, 28), for the existence of different isoforms of CRP with distinct biological properties and direct effects within tissue, this study is the first to apply this emerging concept into the clinical setting of cancer patients. The focus of our previous research has primarily been to understand the biology behind CRP as a biomarker, investigating whether elevated CRP might be a readout of a particular immunological phenotype of the TME. Hence, the idea that CRP itself, in its monomeric, modified form, is present within tumors and might act as a participant in the pathological process has added a new and intriguing layer to this hypothesis and may profoundly change the view on how the local inflammatory response in cancer potentially can be targeted.

Circulating CRP is a pentameric molecule with weak and primarily anti-inflammatory effects through its ability to activate the classical complement pathway, induce phagocytosis and delay apoptosis (10). The much more potent effector function of CRP, however, becomes evident first when pCRP dissociates into the monomeric form exhibiting strong pro-inflammatory properties (12). In cardiovascular disease, it has been shown that activated platelets and endothelial cells, particularly under ischemic conditions, play a pivotal role in the pCRP dissociation process and for the build-up of atherosclerotic plaques (29, 30). Specifically, mCRP and not pCRP, has been detected within atherosclerotic plaques and infarcted myocardium where it co-localizes with oxidized lipoprotein, macrophages and complement factors and is capable of inducing leucocyte migration and adhesion to the endothelium enhancing thrombus formation, excessive inflammation, and ultimately aggravate tissue injury (12, 29). Once formed, *in vitro* studies have shown that mCRP can be inserted into the cell membrane of endothelial cells and activate signaling pathways associated with both angiogenesis and inflammation (14, 29). In line with these findings, we found that mCRP colocalized with endothelial cells lining intratumoral vessels, supporting the hypothesis that endothelial cells, presumably activated by the tumor or the inflammatory microenvironment, is involved in the pCRP-mCRP dissociation process and may contribute to localizing the inflammatory response. Conceivably, newly formed mCRP can then



	Correlation (95% CI)	P-value
mCRP - cd8	-0.02 (-0.35;0.36)	0.93
mCRP - cd4	-0.07 (-0.41;0.30)	0.68
mCRP - cd20	-0.12 (-0.47;0.29)	0.51
mCRP - cd4_fp3	-0.41 (-0.67;-0.07)	0.022
mCRP - cd68	0.26 (-0.07;0.54)	0.13
mCRP - cd66b	0.57 (0.28;0.77)	<0.001
mCRP - pdl1	-0.06 (-0.42;0.32)	0.73

FIGURE 4
Correlating mCRP and selected adaptive and innate immune markers in colon cancer patients. Heatmap and corresponding table of Spearman correlations between mCRP and individual immune markers. Red color indicates positive correlation, blue indicates negative correlation, white indicates no correlation.

either directly activate the endothelial cells resulting in enhanced leucocyte migration to the tumor, and/or as we demonstrate here, accumulate within the tumor tissue. This occurs particularly in systemically inflamed patients where mCRP may exert its pro-inflammatory effects through direct interaction with different cell types and components of the TME.

To elucidate possible functional roles of mCRP in the microenvironment of our colon tumors, we performed double immune stainings demonstrating prominent colocalization of mCRP and CD66b+ neutrophils. At the sub-cellular level, IF revealed occasional direct cellular overlap, indicating possible uptake of mCRP into the neutrophils, although the predominant pattern was that mCRP coincided with highly neutrophil infiltrated areas, suggesting a close relationship between the two. Given the fundamental role of neutrophil function in acute as well as chronic inflammatory conditions, possible direct effects of CRP on these cells have been of particular interest. Hence, *in vitro* studies have shown that mCRP can delay neutrophil apoptosis and enhance neutrophil adhesion to endothelial cells, which is critical for extravasation of neutrophils into inflamed tissue (31, 32). Additionally, following mCRP stimulation, Kreiss et al. found that neutrophils increased both gene expression and secretion of the pro-inflammatory cytokine IL-8 (33). Intriguingly, growing evidence indicates that IL-8 plays a pivotal role in the TME through the ability to stimulate tumor cell proliferation and promote epithelial-to-mesenchymal transition (EMT), thus facilitating tumor progression and metastasis (34).

We have previously shown that elevated circulating CRP associates with a neutrophil enriched and immunosuppressive TME (19). Together with these findings suggesting direct crosstalk between mCRP and neutrophils, this does not only reinforce a profound role for neutrophils in the microenvironment of tumors but adds new information on why neutrophils, particularly during a chronic inflammatory state, seem to be such potent players favoring a detrimental inflammatory response and subsequently how this potentially can be targeted.

Of note, we also observed that mCRP seemed to coincide with areas of necrosis, with or without neutrophil infiltration, showing a pattern of high mCRP expression within and in the vicinity of necrotic areas. This phenomenon could be related to the notion that mCRP can induce aberrant angiogenesis, which has been shown in infarcted brain tissue, resulting in leaky vessels that compromise sufficient blood supply to the tumor leading to necrosis (35). In cancer biology, necrosis is associated with poor prognosis and treatment resistance and has been linked to an immunosuppressive microenvironment, possibly through the release of damage-associated molecular patterns (DAMPs) from dying cells, which triggers an inflammatory response (36). Hence, the ability of mCRP to induce tumor necrosis could potentially contribute to a hostile and predominant immunosuppressive microenvironment supporting a more aggressive tumor phenotype.

Within this context it should be mentioned that a series of older studies conducted in various experimental, primarily murine, cancer models, using CRP, either in its pentameric form or

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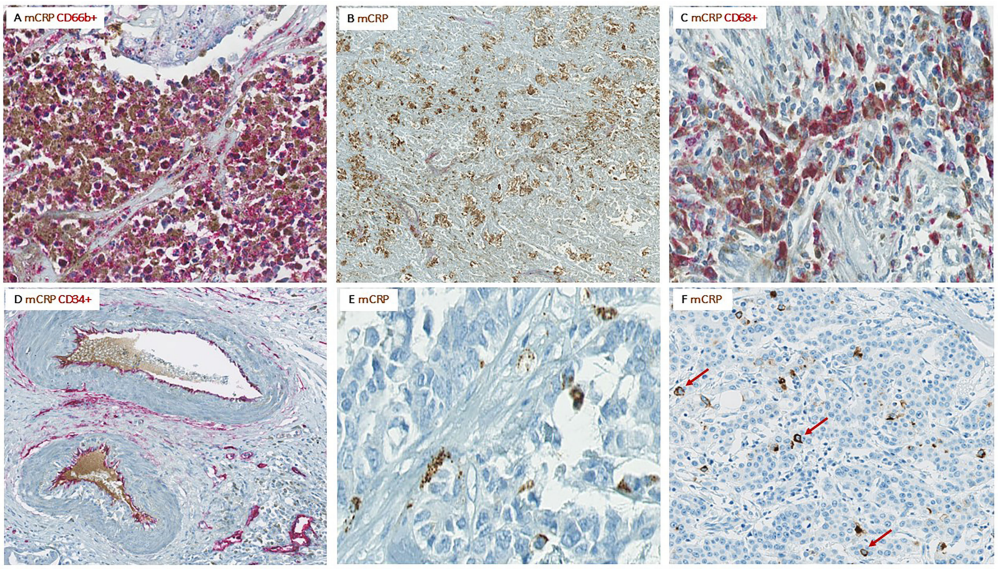

FIGURE 5
Colocalization of mCRP with various components of the TME. Representative images from CC patients with elevated serum CRP and pronounced mCRP tumor expression. **(A)** Highly neutrophil infiltrated tumor area with strong mCRP expression. **(B)** Necrotic area within a tumor with high mCRP expression. **(C)** Colocalization of mCRP and macrophages. **(D)** Colocalization of mCRP and endothelial cells lining intratumoral vessels as well as some mCRP within the vessel lumen. **(E)** mCRP scattered diffusely as small granules within the connective tissue of the tumor stroma. **(F)** Tumor cell nuclei surrounded by mCRP (marked by arrows).

injecting mCRP directly, found similar correlation with necrosis as demonstrated in the present study (11). Contrary to our hypothesis, though, the addition of CRP to the experimental models associated with tumor regression and anti-metastatic effects. However, within all these experimental set-ups CRP was applied only for a short period of time (weeks) and primarily as boosts with CRP injection on selected days. Hence, such system models would mimic an acute inflammatory response and not the situation during chronic systemic inflammation, which was the case for the patients within our cohort. In cancer patients with persistent elevation of blood CRP levels, the inflammation is proposed to be sustained due to the ongoing inflammatory stimulus from the evolving tumor that potentiates hepatic and potentially, tumor intrinsic CRP production, leading to the "wound that never heals". Considering the pro-inflammatory effects of mCRP together with the capacity of activated cells to induce pCRP dissociation, persistent pCRP

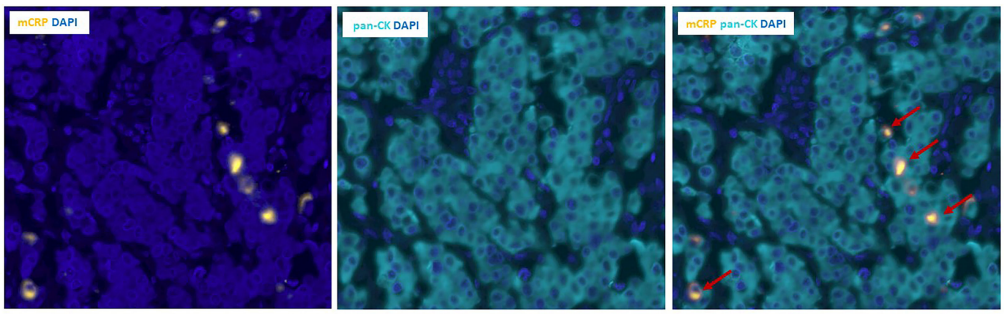

FIGURE 6
Double immunofluorescence labeling of mCRP and tumor cells in colon cancer tissue. Left and middle panels: Unmixed images showing individual stains of mCRP (yellow) to the left and pan-CK positive tumor cells (teal) in the middle. Right panel: Composite image showing double positive mCRP+/pan-CK+ tumor cells (marked by arrows). DAPI (blue) was used for visualization of nuclei. Pan-CK, Pan-cytokeratin.

exposure may then result in excessive tumor inflammation and tissue damage ultimately facilitating tumor growth and exacerbation of the disease.

Previous studies have demonstrated that mCRP can interact with components of the ECM, such as collagen, fibronectin and laminin, which are integral parts of connective tissues playing a crucial role for tissue maintenance and homeostasis (11, 37, 38). In tumors, however, this highly dynamic network becomes dysregulated, and together with other components of the tumor stroma, contributes to a tumor permissive microenvironment. Importantly, low tumor-stroma ratio associates with poor survival and treatment outcome in multiple cancer types (39, 40). In our cohort, we found that mCRP, in addition to the above-described distribution pattern, often was scattered diffusely as small granules embedded within the stroma, unrelated to any particular cell type. Consistent with previous studies delineating the precise ligands for mCRP (5), this morphological pattern could indicate possible crosstalk between mCRP and components of the ECM. Given the putative pro-inflammatory properties of mCRP, such direct interactions could potentially contribute to excessive stromal formation. Apart from enlargement of the tumor, abundant ECM deposition has been linked to increased stromal stiffness, which subsequently can contribute to treatment resistance and favor tumor aggressiveness (40).

Serendipitously, when examining the pattern of mCRP distribution, it became apparent that some tumor cells were decorated by mCRP. Using double immune stainings with pan-cytokeratin as a tumor marker, we found evidence of direct overlap indicating close interaction and/or mCRP expression by tumor cells. Whether the positive mCRP/tumor staining depicts direct uptake of mCRP into tumor cells or represents an intrinsic feature that the evolving tumor acquires to support its own growth and formation of a tumor permissive microenvironment, remains elusive and should be expanded on in further studies.

Indeed, studies have shown that although the liver is the main source of CRP, extrahepatic production do exist (10, 41, 42). Specifically, macrophages, endothelial cells, smooth muscle cells as well as adipocytes and lymphocytes have been reported to synthesize CRP (10). Hence, we cannot rule out that the observed intratumoral mCRP is produced locally by inflammatory and/or tumor cells. The strong correlation with circulating serum CRP, however, indicates that the primary source of tissue-associated mCRP in our tumors was from systemic pCRP. Nonetheless, regardless of origin, given the evidence described above, persistent presence of mCRP within the tumor, which is considered an ongoing, non-resolving state due to the chronic nature of tumor-associated systemic inflammation, may potentially play a direct and active role in aggravating the localized inflammatory response. Notably, the versatile binding capacity of mCRP to a number of different cellular and non-cellular ligands, may potentially translate into multiple effects within the TME through its direct interaction with diverse targets that most likely will impact the evolving tumor.

This study has several limitations. Above all, it is a proof-of-concept study primarily performed for testing hypotheses and exploring a rather new and, in our opinion, underappreciated concept in clinical oncology, thus limiting the sample size. Hence, our findings need to be verified and further explored in larger studies, which we are currently conducting. Next, we used FFPE tissue and IHC to elucidate possible functional roles of mCRP within tumors. While this methodological strategy provides high morphological precision regarding localization of the applied markers, the ability to evaluate direct functionality is, however, limited. This aspect should therefore be addressed in other kind of experiments, preferentially using fresh tissue. Finally, our tumor samples, although whole slides, only represent a snapshot of the immunological process, and do not mirror the long-term conditions and temporal dynamics. Hence, serial biopsies will be valuable to further dissect and evaluate how mCRP affects the immune response over time and impacts tumor evolution.

Taken together, we provide evidence for the existence of the monomeric form of CRP in CC being expressed exclusively within tumor tissue, primarily in systemically inflamed patients. mCRP expression colocalized with neutrophils and endothelial cells as well as areas of necrosis indicating a direct role in the microenvironment of tumors. In line with findings from studies conducted in other diseases, we suggest mCRP as a potential tissue-associated player with capability of actively shaping and fueling the local tumor immune response, presumably by creating a more tumor permissive environment and negatively affect patient outcome. These findings, if verified in further studies, puts CRP in a new perspective, acting not only as a biomarker of unfavorable prognosis and outcomes in cancer, but also as an active mediator with direct effects within tumors, and opens a new and intriguing approach for targeting the TME.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Norwegian Regional Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

AK contributed to data collection, analysis, and interpretation, and drafted the manuscript. JG, KC, and HZ prepared human tissue and performed lab work. PN performed digital image analysis and data interpretation. AF, IR, and LP provided materials, contributed to data interpretation and discussion of content. ET conducted statistical analyses and data curation. TS contributed to data interpretation, methods and discussion of content. CK participated in data interpretation, discussion of content and conceptual framework. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1170443/full#supplementary-material

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