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ORIGINAL RESEARCH

Circulating Levels of the Proinflammatory Monomeric Isoform of C-Reactive Protein (mCRP) Correlate with Intra-Tumoral mCRP Abundance in Stage II-III Colon Cancer Patients

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Purpose: Colorectal cancer (CRC) is the third most common type of cancer worldwide. The link between inflammation and CRC is well established. Elevated levels of C-reactive protein (CRP) upon diagnosis is a known negative prognostic factor for CRC patients. Monomeric CRP (mCRP) has been demonstrated in tissues of several diseases associated with inflammation, including colon cancer (CC). mCRP possesses proinflammatory properties and is a possible mediator of tumor-promoting inflammation. This study aimed to detect and quantify circulating mCRP and assess potential correlations with clinical CRP and intra-tumoral mCRP in CC patients.

Patients and Methods: Forty patients treated for stage II–III CC between 2012 and 2015 at Sorlandet Hospital, Norway, were included in the study. Twenty patients had CRP level <10 mg/l and 20 patients had CRP ≥10 mg/l, measured routinely at diagnosis (clinical CRP). EDTA plasma was used for mCRP detection by enzyme-linked immunosorbent assay (ELISA; n = 40) and mass spectrometry (MS; n = 20) (MS data are available via ProteomeXchange with identifier PXD046746). Tumor mCRP abundance was classified into three categories by reference scoring, using an antigen-retrieval technique on formalin-fixed paraffin-embedded tissue samples (n = 29).

Results: Circulating mCRP levels were detectable by both ELISA and MS. Median mCRP level measured by ELISA was 2.55 ng/mL, while the MS analysis detected 19.02 ng/mL. Both analyses exhibited significant correlations with clinical CRP (ELISA, $p = 0.012$; MS, $p < 0.001$). Intra-tumoral mCRP correlated with circulating mCRP measured by MS ($p < 0.001$) and with clinical CRP ($p < 0.001$).

Conclusion: To the authors' knowledge, this is the first report of mCRP in the circulation of cancer patients. By employing two different analytical methods, mCRP was reliably detected in CC patients. Patients with elevated circulating mCRP measured by MS had higher intra-tumoral mCRP abundance. The interesting correlation of circulating and intra-tumoral mCRP levels may represent another facet of the interplay between local and systemic inflammation in CC patients.

Keywords: Colon cancer, inflammation, C-reactive protein, monomeric C-reactive protein, mediator

Introduction

Colorectal cancer (CRC) is the third most common cancer type worldwide, annually accounting for 10% of all newly diagnosed cancers.[1] Of patients operated for localized disease, up to half will experience metastatic recurrence.[2]

The role of inflammation and immune cells infiltrate in cancer was described by Rudolph Virchow already in the 19th century.[3] Since then, multiple cancer types have been linked to inflammation, including CRC.[4,5] Inflammation has been

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demonstrated to enable a multitude of the key features required for tumour progression.[4,6] Accordingly, tumor-promoting inflammation is an established hallmark of cancer.[6]

The acute phase reactant C-reactive protein (CRP) is one of the most commonly used markers of acute and chronic inflammation, with demonstrated independent negative prognostic value in CRC.[7,8] An elevated level of circulating CRP at diagnosis is associated with worse overall and disease-specific survival for CRC patients of all stages.[7,9]

Clinical CRP, as measured in routine practice, is a pentameric structure (pCRP). Despite the extensive use of CRP, knowledge about the protein's structural properties and active role in the inflammatory process, is far less common. Upon contact with damaged or activated cell membranes, pCRP dissociates into monomers (monomeric CRP, mCRP).[10] pCRP is approximately 115 kDa, comprised of the five smaller mCRPs, each measuring about 23 kDa.[10,11] mCRP has been shown to possess proinflammatory attributes with the ability to activate platelets, leukocytes, and endothelial cells.[10] Furthermore, mCRP has been detected in tissues of various pathological conditions associated with inflammation, including within the tumor tissue of colon cancer (CC) patients.[12,13] These findings suggest mCRP to be an active mediator of inflammation and potentially contribute to tumor-promoting inflammation.

While mCRP is mainly considered a tissue-based isoform due to reduced aqueous solubility, it is known to exist in the circulation as membrane-bound or aggregations.[10] These characteristics complicate the detection in circulation. Therefore, mCRP detection and quantification in circulation are challenging. Still, given the pro-inflammatory attributes of mCRP and its credible role within the inflammatory process, there is a great clinical interest in exploring the potential of mCRP as a circulating biomarker. As emphasized by Slevin et al, the understanding of the role and biology of mCRP in disease courses seems essential, and hence, accurate measurement is crucial.[13]

In that respect, our aims for this study were to reliably quantify mCRP in the circulation in cancer patients, correlate circulating mCRP with clinical CRP and lastly correlate circulating mCRP with tumor tissue mCRP in CC patients.

Materials and Methods

Patient Cohort

Patients were collected from the prospectively maintained dataset of CRC patients treated at Sorlandet Hospital, Norway. Forty patients electively operated for stage II–III CC between 2012 and 2015 were included in the study. All patients underwent curative-intent surgery, followed by adjuvant chemotherapy when indicated according to national guidelines. Exclusion criteria were clinical evidence of infection and the use of antibiotics or immunosuppressive drugs within 4 weeks prior to the surgery. Patients with a history of chronic inflammatory disease, including autoimmune disorders, were also excluded from the study.

Twenty patients had clinical CRP level <10 mg/l and 20 patients had clinical CRP ≥10 mg/l, the latter interpreted as the presence of a systemic inflammatory reaction. Levels of clinical CRP and albumin were captured at the time of diagnosis, within 14 days prior to the surgery. Pathological staging of the surgical specimens was performed using the seventh TNM edition, consistent with contemporary Norwegian guidelines. Tumor location was classified as right-sided when localized between the cecum and distal transverse colon, whereas tumors located from distal transverse colon until rectum were designated as left-sided primary tumor.

Blood and Tissue Samples

All blood samples were obtained preoperatively. Clinical CRP was measured in serum at the time of diagnosis by standard immunoturbidimetric assay. For circulating pCRP and mCRP measurements, EDTA plasma was used. Formalin-fixed paraffin-embedded tumor blocks, obtained during routine diagnostic procedures, were immunohistochemically stained with the anti-mCRP antibody (9C9) as thoroughly described in earlier work.[12] Briefly, 3 µm tissue sections were deparaffinized, rehydrated and subjected to heat-induced antigen retrieval, followed by incubation with the primary antibody (1:100 dilution) for 30 minutes, followed by visualization using DAB (3,3′-diaminobenzidine) chromogen and hematoxylin counterstaining. The tissue samples were classified into three categories (high, medium and low) based on the abundance of mCRP staining. The classification was conducted by an experienced pathologist, performing semi-quantitative scoring guided by reference images (reference scoring), according to staining intensity and distribution. The

biological material was collected according to institutional procedures and by trained personnel. The time slots from samples taken until stored in the freezer were documented (data not shown).

Measurement of mCRP by Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of mCRP were measured using a sandwich ELISA, employing recombinant human mCRP and anti-mCRP antibodies as previously described.[14–16] Briefly, Immulon 2HB plates were coated overnight using a goat anti-human mCRP polyclonal antibody diluted in PBS (1:3000) and then blocked for two hours at room temperature using a solution of 1% bovine serum albumin fraction V (Sigma, St. Louis, MO, USA) in PBS-0.01% Tween-20 (blocking buffer). The wells were then washed with PBS-0.01% Tween-20 (washing buffer) and incubated with recombinant human mCRP (0.05–500 ng/mL) or plasma samples diluted 1:5 in blocking buffer containing 1% normal goat serum for 2 hours at room temperature. The wells were washed three times and incubated with a mouse anti-human mCRP monoclonal antibody (9C9) diluted 1:100 in blocking buffer for 90 minutes at room temperature. Washing was repeated (3x), and the wells were then incubated with a 1:5000 dilution of a Goat anti-mouse IgG antibody coupled to horseradish peroxidase (Abcam, Cambridge, UK) in blocking buffer. After a 1-hour incubation at room temperature, the plates were washed (3x) and a substrate solution (3,3′,5,5′ tetramethylbenzidine; Sigma, St. Louis, MO, USA) was added to the wells. Reactions were stopped by addition of 1 M H_2SO_4 and OD measured at 450 nm. Concentrations of mCRP in the samples were calculated based on the recombinant mCRP standard curve. This ELISA analysis showed specificity for the mCRP isoform, as controls using purified pCRP (up to 50 µg/mL) did not give any signal above background levels.

Measurement of pCRP by ELISA

Concentrations of pCRP were measured using a commercial human CRP ELISA kit (Cat. DY1707, R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. This ELISA is specific for the pCRP isoform of CRP and does not detect mCRP.

Liquid Chromatography–Mass Spectrometry (LC–MS/MS)

Sample Preparation

To ensure mCRP as the exclusive target measured by MS, 200 µL of EDTA plasma were filtered using Amicon Ultra-15 Centrifugal Filters with an MWCO of 100 kDa (Millipore, Merck, Germany). The samples were centrifuged for 15 minutes on 4°C at 14G, the flow-through was collected and stored at –80°C.

Samples were dissolved in 50 µL 50 mm ammonium bicarbonate. The samples were further reduced with 10 mm DTT at 30°C for 30 minutes and alkylated with 25 mm iodoacetamide at 23°C for 60 minutes in the dark. The reaction was quenched by adding 30 mm DTT and incubating at 30°C for 30 minutes. Prior to the digestion, 0.5 pmol of two AQUA heavy peptides, GYSIFSYAT(K) and YEVQGEVFT(K)PQLWP (Thermo Fisher Scientific, Scotland, UK), were added to the samples. The samples were then digested with 4 µg Trypsin Gold (Promega, Madison, WI, USA) at 37°C overnight. The next day, the digestion was stopped by adding 1% formic acid, and the peptides were cleaned by solid-phase extraction using a Ziptip-C18 (Millipore, Merck, Germany).

Protein Identification and Data Analysis

The digested samples were analyzed in a nanoElute system coupled to a timsTOF pro (Bruker). The peptides were separated by liquid chromatography in an Aurora Elite column (C18, 1.7 µm beads, 120 Å, 75 µm inner diameter, 15 cm; IonOptics) using a flow rate of 200 nL/minute with 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B) and a gradient from 2% to 35% solvent B in 60 minutes at a column temperature of 50°C. The mass spectrometer was operated in data-dependent mode to automatically isolate and fragment multiple charged precursors (top 10). The intensities of the fragment ions of the selected peptides were quantified with the PEAKS Studio software version 10.6 (Bioinformatics Solutions), using carbamidomethylation as fixed modification and oxidation of methionine, acetylation of the protein N-terminus and heavy isotope lysine (lys8) as variable modifications. The ratios between the native peptide and the modified heavy peptides were used to establish the concentration of the selected protein.

Statistics

The comparison of clinical CRP and pCRP levels, and the relationship between clinical CRP and circulating mCRP, were examined using Spearman's rho correlation test. The relationships between clinical CRP, circulating mCRP and intratumoral mCRP were analyzed by Kruskal–Wallis test and Dunn's multiple comparison test. A p-value <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 10.0.3 (GraphPad Software, Boston, MA, USA).

Results

Clinicopathological Characteristics

The clinicopathological characteristics of the study population comprising 40 patients treated for CC stage II and III are displayed in Table 1. Patients were selected based on normal or elevated CRP level, with half the patients presenting with CRP ≥10 mg/l at diagnosis. Four patients exhibited albumin <35 g/l; however, only one patient had defined hypoalbuminemia (<30 g/l). Median age was 72.5 years, and the majority were male patients (57.5%). Due to small number of patients, American Society of Anesthesiologists (ASA) scores were recategorized into two classes. Most patients were operated for tumor on the right side of colon. Noteworthy, 80% of the patients with an elevated level of CRP had right-sided tumor.

Clinical CRP versus pCRP

To exclude any effects of long-time storage of the EDTA plasma samples, pCRP levels were measured by ELISA and compared to the clinical CRP levels measured several years earlier (Figure 1). The levels showed a remarkable strong correlation (Spearman's rho 0.981, p<0.001). Due to the comparable concentrations of pCRP and clinical CRP, only the latter were used in further analyses.

Table 1 Clinicopathological Characteristics of Stage II–III Colon Cancer Patients

Characteristic		N=40 (%)	CRP value	
			<10	≥10
Clinical CRP (mg/l)	Median (Min - Max)	9.9 (0.5–100.0)		
			20 (50.0)	20 (50.0)
Albumin (g/l)	<35	4 (10.0)	0 (0.0)	4 (20.0)
	>35	36 (90.0)	20 (100.0)	16 (80.0)
Age (years)	<70	18 (45.0)	8 (40.0)	10 (50.0)
	>70	22 (55.0)	12 (60.0)	10 (50.0)
Sex	Female	17 (42.5)	8 (40.0)	9 (45.0)
	Male	23 (57.5)	12 (60.0)	11 (55.0)
ASA	I–II	20 (51.3)	7 (35.0)	13 (68.4)
	III–IV	19 (48.7)	13 (65.0)	6 (31.6)
Tumor location	Right-sided	23 (57.5)	7 (35.0)	16 (80.0)
	Left-sided	17 (42.5)	13 (65.0)	4 (20.0)
TNM stage	II	22 (55.0)	10 (50.0)	10 (50.0)
	III	18 (45.0)	10 (50.0)	10 (50.0)

Abbreviations: CRP, C-reactive protein; ASA, American Society of Anesthesiologists; Min, minimum; Max, maximum.

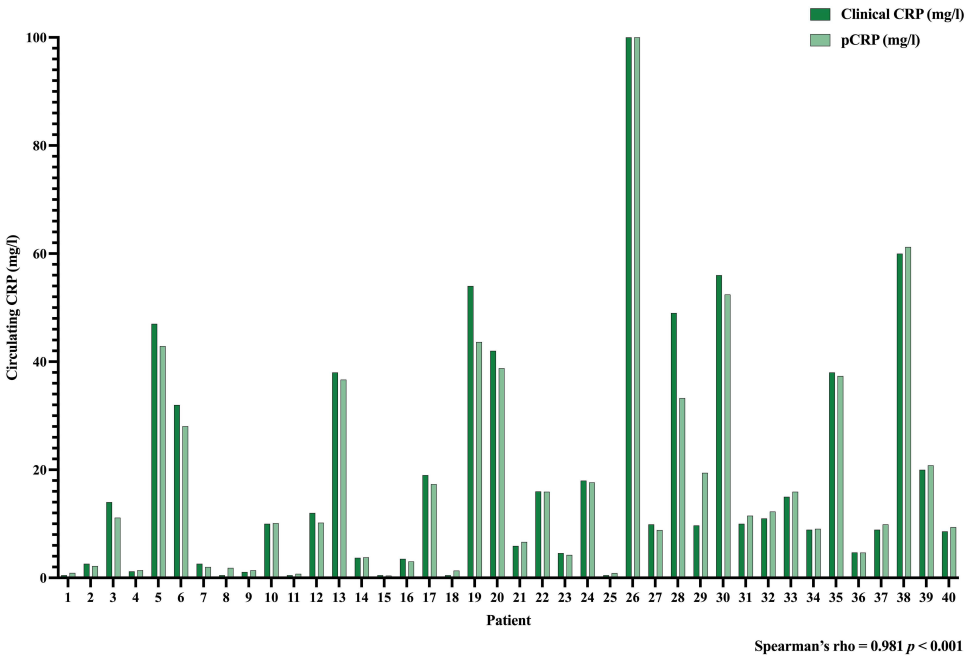

Figure 1 Comparison of clinical CRP and pCRP in the circulation of colon cancer patients. Clinical CRP (dark green) was measured at the time of diagnosis by standard immunoturbidimetric assay. pCRP (light green) was measured by enzyme-linked immunosorbent assay. CRP and pCRP levels were cut at 100 mg/l.
Abbreviations: CRP, C-reactive protein; Pcrp, pentameric CRP.

Circulating mCRP Levels Measured Using ELISA

The levels of mCRP measured by ELISA were investigated in all 40 patients. The median mCRP level was 2.55 ng/mL (75th percentile 4.15 ng/mL). Nine patient samples did not produce a signal and were recorded as the value of 0. The highest value was measured to 10.87 ng/mL (Supplementary Table S1). The ELISA results were previously presented in an electronic abstract.[17]

Circulating mCRP Levels Measured Using MS

The levels of mCRP measured by MS were investigated in 20 patients. Median mCRP was 19.02 ng/mL (75th percentile 53.74 ng/mL). In four of the patient samples neither of the two native peptides were detected. These samples were recorded as the value of 0. The highest value was measured to 443.8 ng/mL (Supplementary Table S1).

Of note, there was a moderate correlation between the levels of mCRP measured by ELISA and MS (Spearman's rho 0.460, $p = 0.041$).

Correlation between Clinical CRP and Circulating mCRP

The correlation between clinical CRP and circulating mCRP measured by both ELISA and MS are displayed in Table 2. Both mCRP-detecting methods showed levels that correlated significantly with clinical CRP.

Table 2 Correlation Between Clinical CRP and Circulating mCRP Levels

	Spearman's rho	*p*-value
Circulating mCRP by ELISA	0.395	0.012
Circulating mCRP by MS	0.937	<0.001

Abbreviations: CRP, C-reactive protein; mCRP, monomeric C-reactive protein; ELISA, enzyme-linked immunosorbent assay; MS, mass spectrometry.

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Relationship between Clinical CRP, Circulating mCRP Levels and Intra-Tumoral mCRP

Intra-tumoral mCRP abundance was divided into three categories (high, medium and low), as depicted in Figure 2. The relationships between clinical CRP levels, mCRP levels measured by both ELISA and MS, and mCRP abundance in CC tumor tissue are depicted in Figure 3 (corresponding values in Supplementary Table S1). There were significant correlations between intra-tumoral mCRP categories and the levels of clinical CRP and mCRP measured by MS ($p < 0.0001$). However, no correlation was found between intra-tumoral mCRP and mCRP levels measured by ELISA ($p = 0.32$).

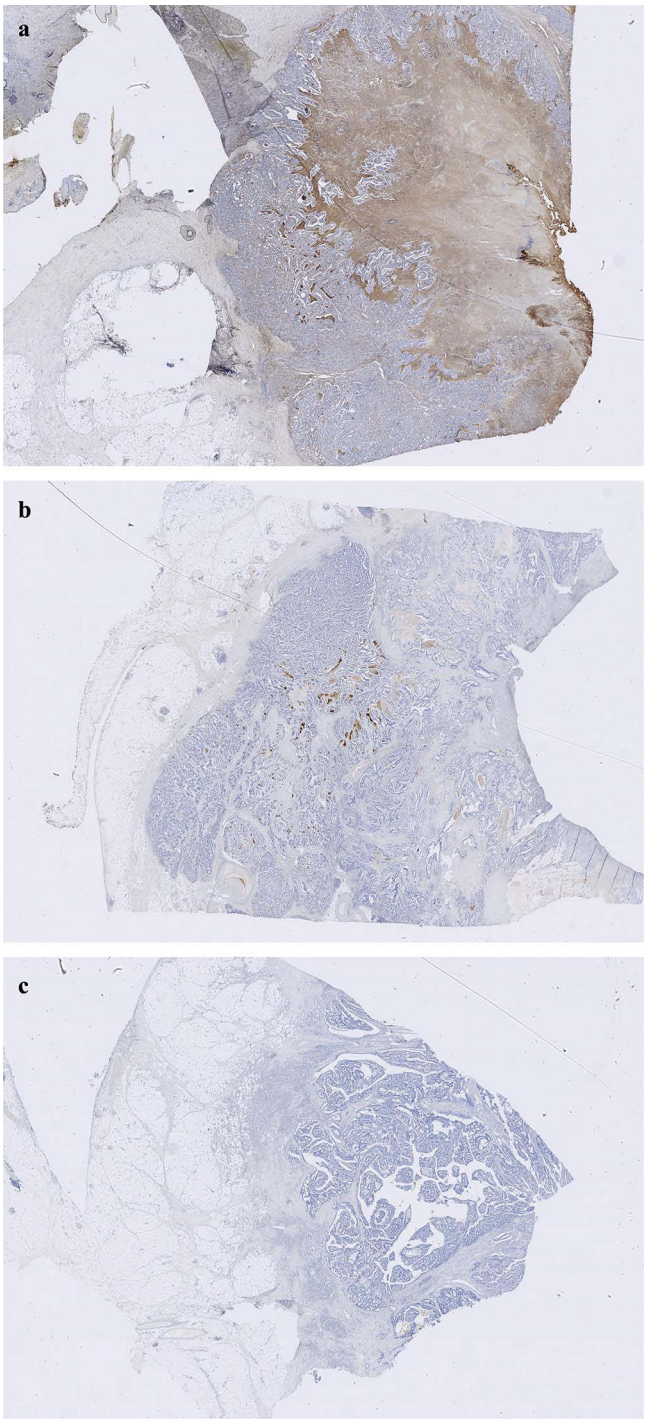

Figure 2 Monomeric C-reactive protein (mCRP) tissue abundance in colon cancer. Whole slides of formalin-fixed paraffin-embedded tumor samples were classified according to mCRP abundance in three categories: high (**a**), medium (**b**) and low (**c**). mCRP expression seen in brown.

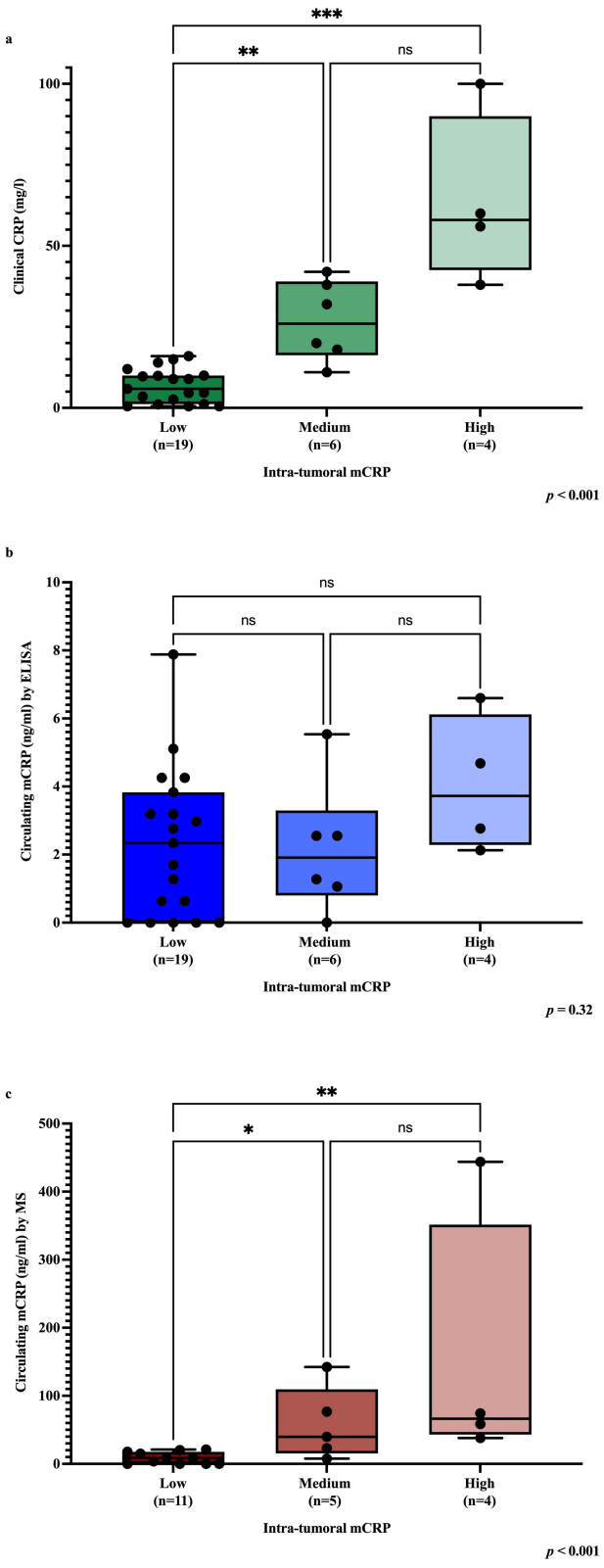

Figure 3 Relationships between clinical levels of CRP and circulating mCRP and intra-tumoral abundance of mCRP. The number of patients in each category of mCRP abundance is noted as n. CRP levels are denominated in mg/l, whereas mCRP is denominated in ng/mL. Intra-tumoral mCRP abundance levels are categorized as high, medium and low. (**a**) Relationship between clinical CRP and intra-tumoral mCRP abundance. (**b**) Relationship between mCRP measured by ELISA and intra-tumoral mCRP abundance. **(c**) Relationship between mCRP measured by MS. *p < 0.05; **p <0.01; ***p <0.001.

Abbreviations: CRP, C-reactive protein; mCRP, monomeric C-reactive protein; ELISA, enzyme-linked immunosorbent assay; MS, mass spectrometry; ns, not significant.

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Discussion

To our knowledge, this is the first time mCRP has been reported in the systemic circulation of cancer patients using two independent methods,[17] and reliably quantified mCRP in CC patients. This study demonstrated a significant correlation between the levels of circulating mCRP and clinical CRP as measured by both methods. There was a significant association between circulating mCRP measured by MS and intra-tumoral mCRP abundance. Additionally, the previously described strong association between clinical CRP and intra-tumoral mCRP was confirmed.[12] Circulating mCRP levels measured by ELISA did not show any association with intra-tumoral mCRP.

An elevated level of clinical CRP is associated with impaired CRC survival. Accordingly, the presence of a systemic inflammatory response reflected by an elevated CRP level is regarded as to represent detrimental tumor-promoting inflammatory reactions. The mechanisms behind, however, are still largely unknown. With the knowledge of CRP isoforms and the demonstrated pro-inflammatory properties of mCRP, the role of mCRP emerges as a potential crucial aspect in understanding tumor-promoting inflammation, calling for the systematic testing of possible methods to measure circulating mCRP levels.

Our group has previously demonstrated mCRP to be present within tumor tissue of CC patients. mCRP was seen to colocalize with neutrophils, and additionally, mCRP was observed to surround tumor cells. Adjacent normal colon tissue was, however, devoid of mCRP.[12] These findings indicated mCRP to be an active mediator in tumor-associated, and thus possibly tumor-promoting, inflammation. Equally important, mCRP has been demonstrated in inflamed tissue of various conditions, including cardiovascular and neurological disorders.[13] The interest in mCRP as a circulating biomarker, is therefore extensive and substantial. Validating larger studies of mCRP detection and quantification are warranted to conclude on clinical relevance.

The difficulties regarding the distinction between pCRP and mCRP, and the reduced aqueous solubility of mCRP, make detection and quantification of circulating mCRP particularly challenging. So far, only a few studies measuring circulating mCRP have been published, and a reliable method has yet to be presented. In this study, ELISA and MS were applied. No commercially available ELISA kit exists for detecting mCRP. In research where mCRP has been investigated, different antibodies have been employed. One of the most utilized antibodies is the mCRP-specific antibody 9C9, which sensitivity and specificity for tissue-related mCRP have been repeatedly demonstrated.[12,13] Additionally, for the first time the technology of MS in mCRP detection and quantification is reported, a method providing a high degree of protein sensitivity and specificity. Still, there are a number of issues and controversies regarding the methods for detecting circulating mCRP applied in this study. In a review article by Potempa et al, multiple processes that can influence the preservation of mCRP and pCRP were highlighted,[10] several of which apply here.

The demonstrated comparable levels of clinical CRP and pCRP do corroborate for the conserved quality of patient samples, despite long-term storage, vortexing, and freeze–thaw cycles. Still, the abovementioned procedures, and the additional process of filtering and centrifugation of the samples, may conceivably have influenced protein and isoform integrity.

Furthermore, EDTA plasma is characterized by the chelating activity and ensuing loss of free calcium.[18] Calcium, in turn, stabilizes pCRP.[19] Without calcium, spontaneous dissociation of pCRP to mCRP can occur. Consequently, by using EDTA plasma, an artificial dissociation of pCRP to mCRP might potentially have influenced the mCRP levels.

The plasma levels of mCRP measured by ELISA in this study were low compared to the levels measured by MS. Among the studies that have performed circulating mCRP detection, a substantial variability exists in the reported concentrations.[14,20–22] mCRP in the circulation is expected to be aggregated or bound to membranes. The antibody 9C9 is, as of now, limited in its ability to detect membrane- or lipid-bound circulating proteins (eg, mCRP bound to microvesicles). By binding to other molecules or by aggregating, the mCRP epitope recognized by the antibody might be hidden, resulting in a falsely low mCRP level detected by ELISA. The 9C9 antibody was employed for detection of both circulating mCRP and for intra-tumoral mCRP. The lack of statistically significant correlation between the datasets may indicate a reduced sensitivity in plasma samples. Correspondingly, a potential consequence of mCRP binding to another molecule or by aggregating, is a change in structure and size. Any compounded structure >100 kDa would have been excluded in the filter preparation performed before the MS detection in this study. Still, optimal sample preparation is crucial for detection of target proteins.[23] Thus, besides enabling the distinction between pCRP and mCRP, the filtering process may have contributed to an enhanced sensitivity of detecting mCRP by MS, potentially resulting in higher levels compared to mCRP measured by ELISA. Altogether, the mechanical and biochemical actions can have influenced the

assessment of circulating mCRP levels and thereby contributed to the different results regarding correlations to both clinical CRP and intra-tumoral mCRP.

It is noteworthy that in the studies by Karlsson et al and Williams et al,[14,21] no significant correlations were established between pCRP and mCRP. For the latter study, this can be explained by the inconsistent pCRP levels compared to clinical CRP. In the present study, the clinical CRP levels were measured several years ago. Still, the current pCRP levels showed a strong correlation to the clinical CRP levels. This underscores the preserved quality of the patient samples, and in addition demonstrates the efficacy of the ELISA CRP kit used for measuring pCRP. The correlation of clinical CRP and pCRP brings attention to the important distinction of the two different CRP isoforms yet again, and verifies pCRP as clinical CRP, the isoform measured in routine clinical settings. This should be considered in all research, past and future, when investigating or interpreting results regarding CRP.

Furthermore, the knowledge of the two different isoforms of CRP brings the proteins kinetics into new light. Despite obvious clinical signs and symptoms observed in patients subjected to acute tissue damage or infection, the appropriate increase in CRP is very often not observed until several hours, or even days, later. As an acute phase protein and as part of our innate immunity, an instant response would be expected. As proposed by Rajab et al, an immediate dissociation of pCRP to the pro-inflammatory mCRP destined for the afflicted area, can explain this delay. Accordingly, mCRP has been proposed as the true acute phase reactant.[19] Accurate measurement of circulating mCRP can therefore potentially elucidate this matter and increase the knowledge of CRP concentration patterns seen in illness.

The CC patients presented in this study were diagnosed with stage II–III and underwent surgery between 2012 and 2015. Routine blood samples performed when admitted to the surgical wards did not include white blood cell count in this time period. Therefore, and unfortunately, the highly interesting correlations between circulating mCRP and neutrophil and lymphocyte counts were not possible to explore.

Another highly relevant and intriguing correlation worth exploring in future research is the relationship between circulating mCRP and the tumor microenvironment.

Taken together, the results presented in this work contribute to the understanding of the interplay between local and systemic inflammation. The biological role of mCRP in the circulation and within tumor tissue remains, for the time being, elusive. Cells of the innate immune system exhibit a dual capacity to be both pro- and anti-tumorigenic, a characteristic that may apply to inflammatory proteins as well. By utilizing MS, we propose an unprecedented method of detection and quantification of circulating mCRP. If verified in larger studies, the potential of mCRP as a circulating biomarker may extend beyond cancer patients and provide further insight into both acute and chronic inflammatory responses.

Data Sharing Statement

Anonymized data for the current study are available from the corresponding author on reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD046746.[24]

Ethics Statement

The studies were conducted in accordance with the Declaration of Helsinki. The use of biobank material was reviewed and approved by the Regional Committees for Medical and Health Research Ethics (REC) South-East (28744). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements (§17 of the Health Research Act).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

A.J.F. has received honoraria from Novartis. Ibraheem M. Rajab is employed by Tabuk Pharmaceuticals, Inc., Amman, Jordan. Dr Sebastian Meltzer reports personal fees from Glaxo Smith Kline, outside the submitted work. Dr Britta Kleist reports other from Pfizer, outside the submitted work. The remaining 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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