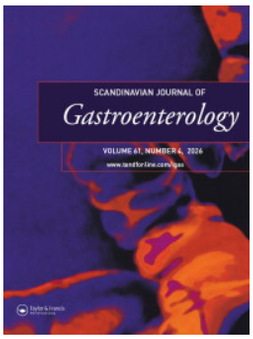

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Pre- and postoperative expression of circulating CEACAM1 and CEACAM6 using a nanoparticle-supported, lectin-specific assay detected in serum from patients with colorectal cancer

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ABSTRACT

Background: Biomarkers for colorectal cancer (CRC) measured in blood at various time-points represents potential tools for disease monitoring and additional staging complementing tissue-based diagnostics. Currently, the only marker in routine clinical use is carcinoembryonic antigen (CEA). Other carcinoembryonic antigen-related cell adhesion molecules (CEACAM) such as CEACAM1 and CEACAM6, have been implicated in tumour progression, however their serum levels in relation to clinical features remain insufficiently explored.

Materials and methods: Serum levels of CEACAM1 and CEACAM6 glycovariants were analysed by a novel nanoparticle-supported detection method in patients undergoing curative-intent surgery for CRC (stage I–III) in a prospective biomarker study (ACROBATICC; NCT01762813), comparing pre- and postoperative levels alongside conventional CEA detection for comparison. Correlations of pre- and postoperative levels of glycovariants with clinicopathological variables and recurrence were assessed.

Results: Serum levels were measured in 120 patients, of which 95 patients had CRC stage I–III. We observed a significant decrease in serum levels of CEA, CEACAM1, and CEACAM6 in serum from pre-surgery to post-operative measurement ($p < 0.001$), likely reflecting tumour burden reduction after surgery. No significant correlations between CEACAM1 or CEACAM6 were observed, nor any association to recurrence nor survival. However, expression of CEACAM6 postoperatively was correlated with liver metastasis compared to metastasis in other locations.

Discussion: Serum CEACAM6 from early and locally advanced CRC may reflect tumour biology in CRC but require validation in larger cohorts to determine prognostic value, utility as a serum-based biomarker for disease monitoring or, for prediction of recurrence risk.

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Carcinoembryonmic antigen; CEACAM1, CEACAM6, colorectal cancer; surgery; biomarker

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer globally in both genders [1], with an increasing incidence in several regions. Improvements in screening and early diagnosis, surgical intervention and multimodal treatment have led to better oncological outcomes, including the recent introduction of immunotherapy for both primary and metastatic CRC [2,3]. However, there is a lack of an easy, reliable and universal biomarker to monitor disease and assess treatment in CRC. While carcinoembryonic antigen (CEA) may be one of the most frequent biomarkers used worldwide, including for CRC, this marker lacks accuracy and monitoring CRC. Hence, improvement in biomarker accuracy is needed and may have far-reaching implications or clinical use.

CEA was first described about 60 years ago [4], and has since been the most frequently used and studied biomarker in CRC, most commonly for surveillance after curative-intent surgery [5,6]. A comprehensive Cochrane review of over 50 studies on the role of CEA in surveillance for recurrence after surgery in patients

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with no detectable residual disease, showed that none of the tested CEA thresholds was sufficient to achieve sufficiently high sensitivity nor high specificity for CRC recurrence [7]. Despite the suboptimal accuracy, it is the only and routinely used biomarker in CRC today.

Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) belong to the glycosylphosphatidylinositol (GPI)-linked immunoglobulin superfamily and consist of 17 family members [8]. Among them, the CEACAM5 (another name for CEA) is among one of the best investigated and well-known biomarkers in oncology. CEACAMs are glycoproteins found on the surface membrane of various cell types, including hematopoietic, epithelial, and endothelial cells, and are distributed across multiple organs [9–11]. Due to their location on the cell surface, CEACAMs play crucial roles in regulating cell adhesion, angiogenesis, leukocyte activation, cell cycle control, and tumour suppression [10,12,13]. CEACAM1 is a potential clinical marker for predicting treatment resistance and improving patient stratification. It is widely expressed in various cancers and immune cells, both in peripheral blood and within the tumour microenvironment. Elevated CEACAM1 levels have been implicated in disease progression and chemotherapy resistance. These findings highlight its potential role in prognosis and personalized treatment strategies [14,15]. CEACAM6 has been shown to be elevated across different tumours, and involved in various oncogenic processes, such as abnormal proliferation, angiogenesis, migration, and resistance to chemotherapy [10,11]. In CRC, the CEACAM6 expression is found to be increased in adenomas, further increased and inversely correlated with cell differentiation in CRC, and proposed to contribute to metastatic mechanisms in several types of cancer [11]. CEACAM6 is proposed to be used as a prognostic marker as it is associated with poorer overall survival and disease-free survival [10,16,17].

The aim of the current study was to explore the role of a novel nanoparticle-supported detection of CEACAM1 and CEACAM6 glycovariants (GVs) as compared to conventional CEA across a series of patients having resected CRC with pre- and post-operative blood samples. The aim was to explore the relation of CEACAM-1 and −6 to conventional CEA according to disease severity and cancer invasiveness (i.e., stage at surgery) in pre- and post-operative blood samples.

Materials and methods

Study ethics

This study was part of the ongoing biobank research project *Assessment of clinically related outcomes and biomarker analysis for translational integration in colorectal cancer* (ACROBATICC), ClinicalTrials registration # NCT01762813, described elsewhere [18]. The project is approved by the regional ethics committee (REK HelseVest, #2012/742 and # 372877). All participants gave informed consent prior to inclusion in the study and were informed about their right to withdraw at any point during the project.

Study design

This CEACAM sub-study cohort was derived from the prospective ACROBATICC biobank. In ACROBATICC, all consecutive patients with a preoperative diagnosis of colorectal cancer who were considered eligible for curative-intent surgery were provided written and oral information by a research nurse, and included after providing written consent, rather than including all patients at a single timepoint. Preoperative blood samples of patients were drawn at inclusion before final histology and definitive staging were available. For the present analysis, we included all ACROBATICC participants between November 2015 and January 2017 with available serum for CEA, CEACAM1 and CEACAM6 analysis (Figure 1). As sampling was based on preoperative suspicion of colorectal cancer, some consecutively recruited patients were subsequently found to have adenomas, other benign lesions or non-adenocarcinoma malignancies. Only patients with stage I–III colorectal adenocarcinoma were included in the main analyses of pre- and postoperative biomarker levels and recurrence. Patients with stage IV disease were included only for exploratory baseline comparisons and were excluded from recurrence analyses. The reason for inclusion or exclusions presented in a flowchart as Supplementary Material (Figure S1).

For rectal cancer, 10 of 36 patients received neoadjuvant chemoradiotherapy according to national guidelines and had surgery after completion of treatment. Seven patients were treated with radiation and three received radiation with capecitabine. In these patients the preoperative serum sample used in the analyses was drawn while they were treatment-naïve before neoadjuvant therapy started. The observational cohort study was done according to the REMARK recommendations [19] and reported according to STROBE recommendations [20], wherever applicable (Supplementary Information).

Clinical endpoints and oncological outcomes

First, we investigated the serum values across biomarkers for the relation to disease severity and

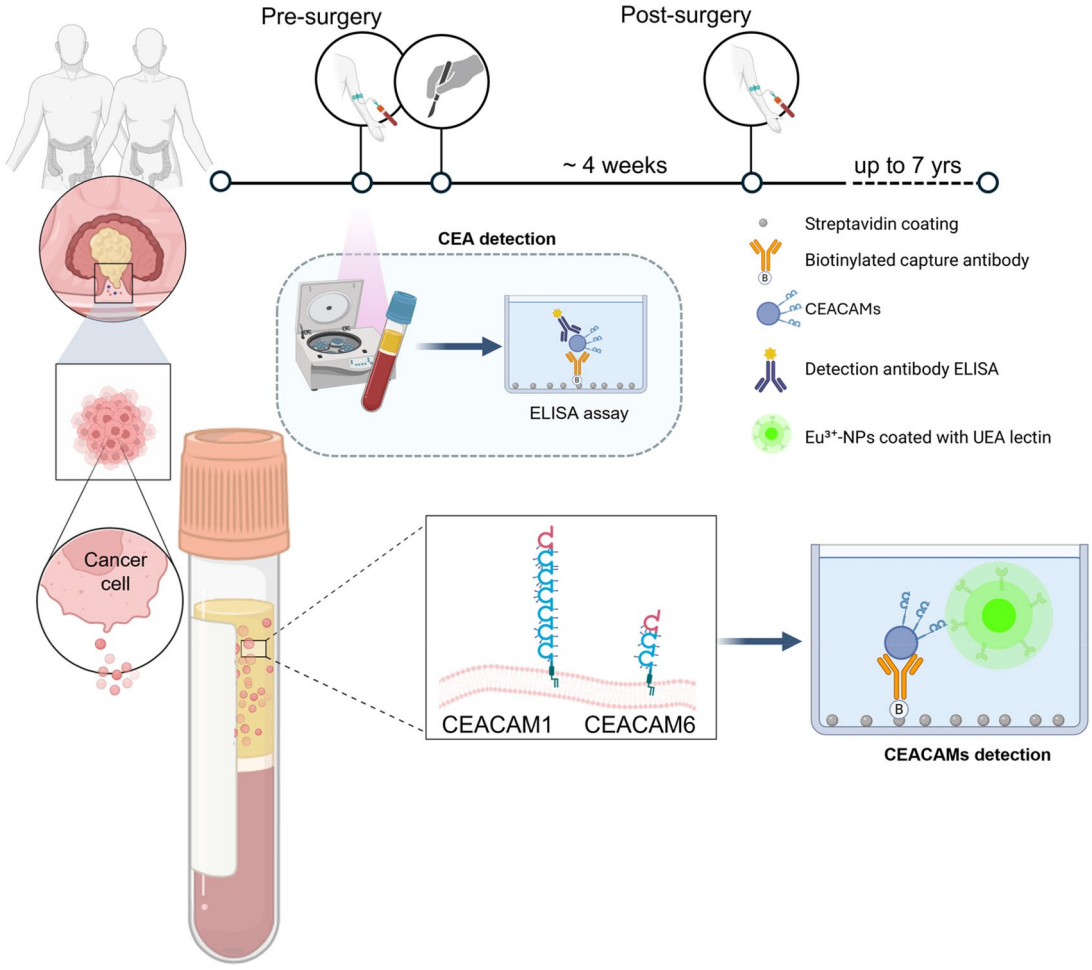

Figure 1. Overview of experimental design and sample timepoints.
Legend: Illustration of time points for sampling and testing of CEA, CEACAM1 and CEACAM6. Blood samples were drawn pre- and postoperatively and stored in a biobank. Extensive testing was further done for CEACAM1 and CEACAM6 . Please see main text for methodological details.

burden, i.e., across stages for premalignant (adenomas and tumour *in situ*), stage I, II, III and IV. Patients with pre-cancers and metastatic disease at time of surgery were excluded from the recurrence analysis.

Further, we investigated the association in stage I-III CRC operated on for cure, and the relation to risk of recurrence for each of the biomarkers. After curative surgery, patients were monitored for local and distant recurrence with planned clinical examinations, radiological imaging and colonoscopy according to Norwegian follow-up guidelines. Follow-up data for the ACROBATICC cohort were available for 7 years, with the last update on 20 November 2024.

CEA detection in serum

Blood samples was drawn from patients (Figure 1) before surgery (typically at admission 1–3 days before elective surgery) and after surgery (on average 4 weeks after surgery at time of post-operative control after discharge, with some patients up to three months post-surgery).

Routine measurements of CEA were analysed in serum at the Department of Medical Biochemistry at Stavanger University Hospital as part of routine diagnostic analysis. The method and laboratory are accredited according to the NS-EN ISO 15189 standard. CEA levels were detected using a chemiluminescent microparticle immunoassay (CMIA) on the Alinity I analyzer (Abbott).

CEACAM1 and CEACAM6 detection in serum

Serum samples were taken immediately pre-operatively (typically 24–48 hrs before surgery) and at time of post-operative visits (at 4 weeks after surgery, and in some patients up to three months post-surgery) [18] and prepared according to the standard operating procedure based on the serum and plasma protocols of the National Cancer Institute (Figure 1) [21]. All the

samples were collected in VACUETTE® CAT serum activated tube (Greiner Bio-One) incubated for 20–60 min at room temperature and centrifuged for 15 min at 2000 × g at 4 °C. Samples were aliquoted in 500–1000µl in 2 ml cryotubes and stored at −80 °C, and each aliquot had been thawed no more than three times before use.

Sandwiched immunoassay time-resolved fluorescence to detect cancer-related glycosylation

Time-resolved fluorescence (TRF) sandwiched immunoassays were performed to detect the glycovariants of CEACAM1 and CEACAM6 proteins. The assay was carried out at room temperature using Ulex europaeus agglutinin I (UEA) lectin coated onto Eu^{3+}-nanoparticles (Eu^{3+}-NPs) as the tracer, as described previously [22]. Biotinylated monoclonal capture antibodies for CEACAM1 (MAB22441, R&D Systems) and CEACAM6 (MAB3934, R&D Systems) were immobilized at a concentration of 50 ng/25 µL onto streptavidin-coated low-fluorescence wells for 45 min without shaking. After two washes, the wells were incubated with reagent assay buffer (with 7.5% Tris-stabilised-bovine serum albumin as a blank) and either cell culture supernatant medium (CCSM) or serum samples for 45 min. After additional washes, CEACAM1 and CEACAM6 were detected using Eu^{3+}-NPs coated with UEA lectin which were added to the plate and incubated for 60 min. Following six washes, TRF was measured using a Hidex Sense Microplate Reader with an excitation wavelength of 340 nm and an emission wavelength of 615 nm [23].

Statistical analysis

The statistical analysis was performed by Statistical Package for Social Sciences (SPSS) for Mac v 29 and Windows, and with R. 4.4.3, package ggplot2. Descriptive statistics were calculated for CEA, CEACAM1 and CEACAM6 pre- and postoperatively for clinical cohort. They are presented as medians and interquartile ranges (IQR) for continuous and numbers with rates for categorical data. All CEA-biomarkers were investigated as continuous values pre- and post-surgery. Violin Plots were generated using the ggplot2 in R studio and R 4.3.3 version. Further, CEA was dichotomized by the standard cut-off for normal/low level at <5 µg/L. Survival analysis is performed in SPSS and presented as a Kaplan-Meyer using log rank.

Non-parametric correlation analysis was performed using Spearman's rank correlation test. The Spearman's rank correlation coefficient (rho) was used to assess the strength and direction of the monotonic association between two continuous variables such as levels of CEACAM1 and CEACAM6. Prior to testing, scatter plots were generated to assess the data distribution, revealing no clear normal distribution. Consequently, Spearman's rho was chosen for correlation analysis.

Bootstrapping for 1000 samples was done bias corrected and accelerated (BAc) values and presented with 95% confidence intervals (95% CI). For paired comparison of pre- and postoperative CEACAM1 and CECAM6 levels within the same patients, the non-parametric Wilcoxon signed-rank test was used. All statistical tests were two-sided and $p < 0.050$ considered statistically significant.

Results

For the current study, 120 patients were included and analysed for the presence of CEA, CEACAM1 or CEACAM6. For the entire cohort, we assessed the expression levels ranging from premalignant (adenomas) to advanced malignancy (patients with metastasis) to explore the expression through a disease continuum. Of these, 5 patients later withdrew consent, 8 had stage IV disease not eligible for curative surgery, and 10 were excluded for other reasons (6 adenomas, 1 lymphoma, 1 benign stenosis secondary to inflammation, and 2 patients without residual tumour tissue after neoadjuvant treatment), leaving 97 patients with stage I–III disease. Two of these did not proceed to surgery, resulting in 95 patients with stage I–III CRC who underwent curative-intent resection and were included in the pre- and postoperative analyses.

Of the 95 patients, 59 (62%) had colon cancer and 36 (38%) had rectal cancer, were 10/36 with rectal cancer received neoadjuvant treatment. Median age was 70 and mostly men (58%) versus women (42%). Stage I and II disease occurred in 59 patients (62%), and 36 (38%) had stage III disease. 22 (23%) of the patients received adjuvant treatment, 15 had colon cancer and 7 had rectal cancer. Radiologically or histologically confirmed recurrence was observed in 14 patients (15%). Recurrence was found in 14 patients (15%). Further patient characteristics are presented in Table 1.

Adenomas compared to invasive cancers with and without metastasis

Continuous levels of CEA, CEACAM1 and CEACAM6 were tested for association with adenomas and stage I–IV CRC ($n = 113$) without any significant difference or

intergroup association. However, adenomas tended to have a lower expression of both CEACAM1 and CEACAM6 postoperatively.

Correlation between CEA, CEACAM1 and CEACAM6

Preoperative CEA was not found to be correlated to CEACAM1 or CEACAM6. However, CEACAM1 and CEACAM6 were significantly correlated (Spearman's rho 0.396, $p < 0.001$; Figure 2(D,E)). Postoperative CEA was found to be slightly correlated with CEACAM6 (Spearman's rho 0.263, $p = 0.019$), and a moderate correlation was found between postoperative CEACAM1 and CEACAM6 (Spearman's rho 0.453, $p < 0.001$).

To explore the clinical significance CEA, CEACAM1 and CEACAM6 levels have further been tested for correlation with recurrence, node status dichotomized as positive or negative, tumour location differentiated between right, left and rectum or colon and rectum, tumour size dichotomized above and less than 50 mm or as a continuous variable, sex and age as a continuous variable and lastly smoking status as current, former, or never.

Neither preoperative CEACAM1 nor CEACAM6 showed any correlation with the tested variables, however preoperative CEA showed a weak correlation to tumour size as linear variable (Spearmans's rho = 0.243 and $p = 0.20$). Both postoperative CEA and CEACAM1 showed a weak correlation with recurrence (Spearman's rho = 0.233, $p = 0.035$ for CEA, and Spearman's rho = 0.215, $p = 0.041$ for CEACAM1). Postoperative CEACAM1 was also correlated with tumour location as colon or rectum (Spearman's rho = 0.258, $p = 0.014$). While absolute CEACAM6 levels were not associated with recurrence, derived measures (difference and ratio) showed weak associations, which should be interpreted cautiously. Node status showed no correlation to either pre- or postoperative CEA, CEACAM1 or CEACAM6.

Postoperative and preoperative difference and ratio were calculated and tested against the same clinical variables. CEA difference was moderately correlated to tumour size as a linear variable (Spearman's rho = 0.365 and $p < 0.001$), CEACAM1 difference was weakly correlated to tumour location divided in colon and rectum (Spearman's rho = 0.245 and $p = 0.038$), whilst CEACAM6 difference showed no correlation to the variables. Similarly, CEA ratio also showed a correlation to tumour size as a linear variable (Spearman's rho = 0.398 and $p < 0.001$), CEACAM1 ratio was correlated to tumour location between colon and rectum (Spearman's rho = 0.271 and $p = 0.021$) and location divided in left colon, right colon and rectum (Spearman's rho = 0.281 and $p = 0.017$), whilst CEACAM6 ratio was not correlated to any of the variables. The pre- and postoperative CEACAM1 and CEACAM6 levels did not differ significantly by sex, age or smoking status.

Table 1. Clinicopathological characteristics of study cohort.

Characteristics	*N* (%)
Number of patients included	95 (100%)
Tumour location	
Colon cancer	59 (62%)
Rectum cancer	36 (38%)
Age, years median, interquartile range	70 (60 – 77.5)
Sex	
Male	55 (58%)
Female	40 (42%)
Neoadjuvant treatment rectal cancer	
No	26 (72%)
Yes	10 (28%)
Tumor diameter (cm)	
<5 cm	61 (64%)
≥5 cm	33 (35%)
Not determined*	1 (1%)
Primary cancer stage (AJCC 8th)	
I (pT1-2, pN0)	23 (24%)
II (pT3-4, pN0)	36 (38%)
III (any pN+)	36 (38%)
Adjuvant treatment	
Colon: No	44 (75%)
Yes	15 (25%)
Rectum: No	29 (81%)
Yes	7 (19%)
Recurrence during follow-up	
Yes	14 (15%)
No	81 (85%)

*Tumor size was missing in 1 patient.

Pre-operative CEA, CEACAM1 and CEACAM6 decreased significantly after surgery

We analysed the preoperative values of CEA, CEACAM1, and CEACAM6 in stage I-III CRC and compared them with the postoperative values to determine whether the surgical removal of tumour tissue resulted in a reduction of these markers in serum (Figures 2 and 3). CEA levels in serum had a median of 3 ng/mL (IQR: 2–5 ng/mL) preoperatively and were significantly reduced postoperatively to 1 ng/mL (IQR: 1–2 ng/mL, $p < 0.001$).

The median preoperative value of CEACAM1 was of 48 S/B (IQR: 33–63), decreasing to 34 S/B (IQR: 21–53 S/B) postoperatively ($p < 0.001$). Similarly, for CEACAM6 the median was 30 S/B (IQR: 19-36.5) and postoperative median was 11 (IQR 8–16 S/B), with significant $p < 0.001$ (Figures 2 and 3). Analysis of serum CEACAM1 levels in 72 paired samples reveals a statistically significant decrease following surgery (Wilcoxon signed-rank test, $p < 0.001$). The majority of patients (54/72, 75%) exhibited a reduction in CEACAM1 levels post-operatively, while 17 patients showed an increase and 1 remained unchanged (Figure 2). For CEACAM6, most patients (66/72, 92%) exhibited reduced

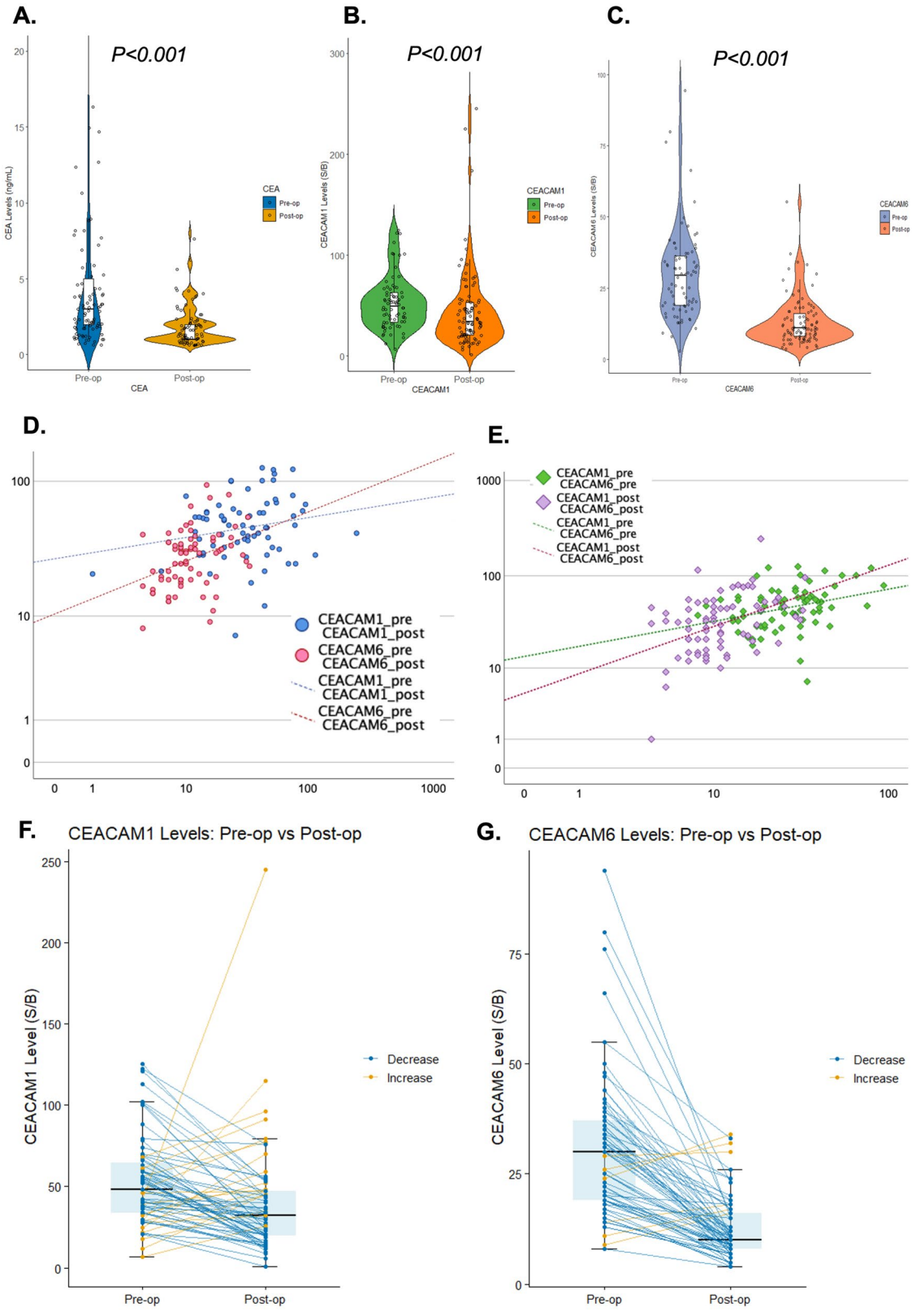

Figure 2. Expression levels of CEACAMs in pre- and post-operative samples.
Legend: Violin plots for pre- and postoperative CEA (A), CEACAM1 (B), and CEACAM6 (C). Correlation of the levels of CEACAM1 and CEACAM6 was done for the pre- and post-operative levels, respectively (D) and for pre-to-pre levels and post-to-post levels (E), with bets correlation for pre- to post-operative levels for CEACAM6. A significant reduction was observed for all three markers (CEA, CEACAM1, and CEACAM6).

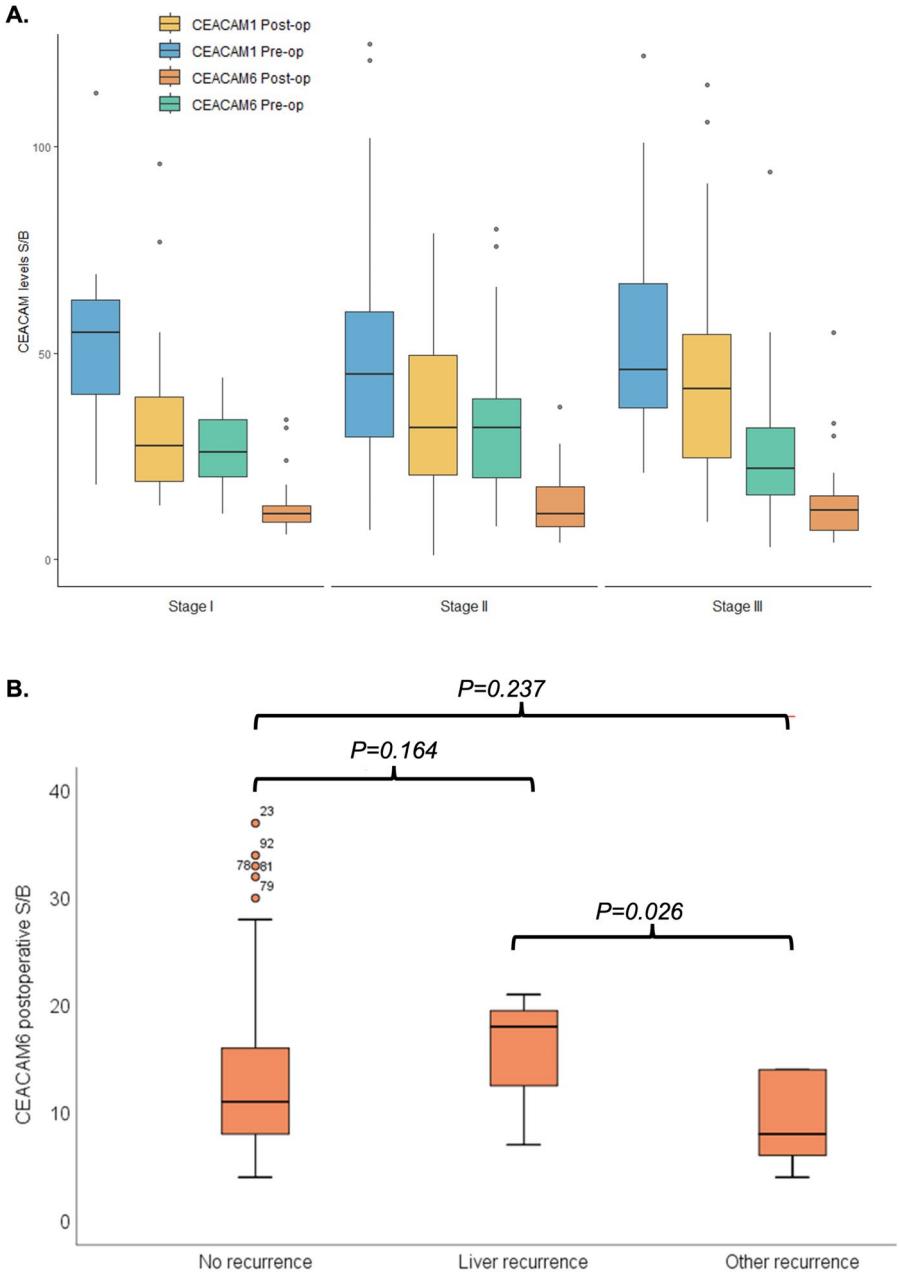

Figure 3. Preoperative and postoperative levels of CEACAM1 and CEACAM6 expression levels by stage.
Legend: (A) This boxplot illustrates the expression levels of CEACAM1 and CEACAM6 across three cancer stages (Stage I, II, III) in both preoperative (pre-op.) and postoperative (post-op.) samples. Each boxplot displays the median (central line), interquartile range (IQR, box edges), and outliers (black dots). The plot shows how CEACAM1 and CEACAM6 levels change between pre- and post-operation in different cancer stages. The expression levels are measured in S/B (signal-to-background ratio). (B) Boxplot showing postoperative CEACAM6 expression in patients without recurrence, with liver metastasis, and with other sites of metastasis; pairwise comparisons (no recurrence vs liver metastasis and no recurrence vs other metastasis) were not statistically significant and should be interpreted as exploratory.

biomarker levels post-operatively, with only 6 patients showing an increase. This consistent downward trend suggests that the surgical intervention effectively lowers circulating CEACAM1 and CEACAM6 levels in this cohort.

There is no standardized cut-off for pathological levels of CEACAM1 and CEAMCAM6 pre- and postoperatively. Hence, to explore any cut-off, we dichotomized the serum levels into percentiles (2.5th, 5th, 10th, 25th, and 50th percentiles), without finding a difference related to clinical variables (data not shown).

CEACAM1 and CEACAM6 serum levels showed a consistent and significant positive association across both preoperative and postoperative time points, as revealed by Spearman correlation analysis (Figure 2). In the preoperative setting, a moderate positive correlation was observed ($\rho = 0.36$, $p = 0.0017$), indicating that higher baseline levels of CEACAM1 were generally

accompanied by elevated CEACAM6. Notably, the association was stronger postoperatively ($p = 0.46$, $p < 0.001$), despite an overall reduction in expression levels after surgery.

Variations in preoperative CEA, CEACAM1, and CEACAM6 between stage I-III, and between Colon and rectal cancer

Preoperative serum CEA levels differed across tumor stages, with a median of 2.5 ng/mL (IQR: 2.0–4.0) in stage I, 2.0 ng/mL (IQR: 1.0–4.0) in stage II, and 4.0 ng/mL (IQR: 2.0–7.5) in stage III. Following surgery, CEA levels significantly decreased in all stages, with medians of 2.0 ng/mL (IQR: 2.0–3.0, $p = 0.008$), 1.0 ng/mL (IQR: 1.0–2.0, $p < 0.001$), and 1.0 ng/mL (IQR: 1.0–2.0, $p < 0.001$) in stage I, II, and III, respectively. Statistically significant differences in preoperative CEA levels were observed between stage I and II ($p < 0.001$), and between stage I and III ($p = 0.027$), indicating that CEA levels tend to increase with tumour stage prior to surgery.

Median CEACAM6 levels were significantly reduced postoperatively across all cancer stages. For stage I, CEACAM6 decreased from 26.0 S/B (IQR: 20.0–34.0) to 11.0 S/B (IQR: 9.0–13.0), for stage II from 32.0 S/B (IQR: 19.8–39.0) to 11.0 S/B (IQR: 8.0–17.8), and for stage III from 22.0 S/B (IQR: 15.8–32.0) to 12.0 S/B (IQR: 7.0–15.5).

CEACAM1 levels also showed a reduction in all stages, with a statistically significant drop in stage I and III ($p = 0.01$), and a borderline effect in stage II ($p = 0.053$). Specifically, stage I CEACAM1 decreased from 55.0 S/B (IQR: 41.0–76.0) to 27.5 S/B (IQR: 21.0–33.2), and stage III from 46.0 S/B (IQR: 26.0–66.0) to 43.0 S/B (IQR: 23.0–59.0). No significant difference in preoperative CEACAM1 or CEACAM6 levels was observed between stages I and II, II and III, and I and III (Figure 3).

Preoperative levels of CEA, CEACAM1, and CEACAM6 were compared between colon and rectal cancer patients. The median preoperative CEA levels were similar in both groups (3.0 ng/ml), however, the IQR was broader in colon cancer patients (2.0–6.7 ng/ml) compared to rectal cancer patients (1.0–4.0 ng/ml), suggesting greater variability in the colon group. For CEACAM1, the median level was higher in colon cancer (52.0 S/B, IQR: 36.2–60.0) compared to rectal cancer (46.0 S/B, IQR: 32.0–63.0), though the overlapping IQRs indicate variability in both groups. Similarly, CEACAM6 showed a lower median level in colon cancer (26.0 S/B, IQR: 19.0–33.2) compared to rectal cancer (32.0 S/B, IQR: 17.0–41.0). Comparing pre- and postoperative values of all three CEACAMs in colon cancer showed significant difference with $p < 0.001$, respectively. However, for rectal cancer CEACAM1 was not significantly different. Correlation was tested for the group with rectal cancer separately. Neoadjuvant treatment was tested against continuous values, difference and ratio of CEA, CEACAM1 and CEACAM6. A significant correlation was only found for postoperative CEACAM1 (Spearman's rho = 0.434 and $p = 0.009$).

Metastatic disease and recurrence

Liver metastasis occurred in seven patients and lung metastasis in six patients. Last patient had local recurrence. Of patients with liver metastasis four underwent surgery, and for lung metastasis three underwent surgery without ablation. One patient had surgery for local recurrence. Other patients had progression of disease during chemotherapy or multiple metastasis not available for curative treatment and therefore did not undergo metastatic surgery. Correlation was tested for liver metastasis and lung metastasis, and preoperative and postoperative CEA, CEACAM1 and CEACAM6 including their difference and ratio. For liver metastasis a correlation was not found for any CEACAMs. Although postoperative CEACAM6 levels appeared numerically higher in patients with liver metastasis than in those with other metastatic sites, this observation is based on few events and should be regarded as exploratory. No statistically significant difference in postoperative CEACAM6 levels was observed when comparing patients without recurrence to those with liver metastasis ($p = 0.164$, Figure 3(B)), nor between liver metastasis and other metastatic sites ($p = 0.237$, Figure 3(B)). For lung metastasis, correlation was found with CEA difference (Spearman's rho = 0.226 and $p = 0.043$) and preoperative CEACAM6 (Spearman's rho = 0.228 and $p = 0.05$). Comparing non-recurrence and liver metastasis or other metastasis there was no significant difference (Figure 3(B)). Further, difference and ratio dichotomized as either reduced or increased after surgery demonstrated a correlation for all three markers. CEA difference dichotomized was weakly correlated to lung metastasis (Spearman's rho= 0.253 and $p = 0.013$), but not liver metastasis or recurrence in general. CEA ratio dichotomized was also weakly correlated to lung metastasis (Spearman's ro= 0.258 and $p = 0.011$). CEACAM1 difference dichotomized was correlated to recurrence (Spearman's rho = 0.293 and $p = 0.004$), but not liver or lung metastasis. However, CEACAM1 ratio was correlated to lung metastasis (Spearman's rho = 0.246 and $p = 0.016$) and recurrence (Spearman's rho = 0.242 and $p = 0.018$). Lastly, CEACAM6 difference was correlated to recurrence (Spearman's rho = 0.270 and

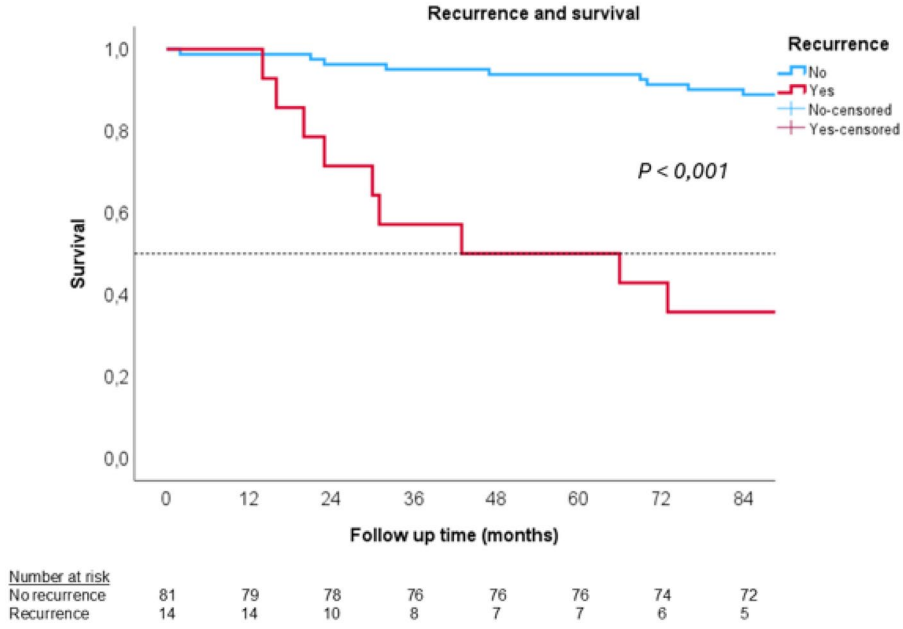

Figure 4. Time-to-recurrence in patients with stage I–III colorectal cancer.

$p = 0.008$) and lung metastasis (Spearman's rho = 0.225 and $p = 0.028$), but not liver metastasis. CEACAM6 ratio was related to lunge metastasis (Spearman's rho = 0.246 and $p = 0.016$) and recurrence (Spearman's rho = 0.320 and $p = 0.002$), however was not related to liver metastasis either. We also examined whether a more than 20% or 50% decrease in CEACAM1 and CEACAM6 difference and ratio was associated to recurrence, liver or lung metastasis, without finding any correlation.

Recurrence and overall survival

Figure 4 displays Kaplan-Meier showing the overall survival in patients included in the study with and without recurrence. Patients without recurrence have an overall survival of 90%, 72 patients, after 7 years. In patients with recurrence less than 40%, 5 patients, were alive after 7 years. Overall survival was significantly different between the two groups with $p < 0.001$. However, due to the limited number of patients, it was not possible to determine a definitive cut-off value for CEACAM1 and CEACAM6 as a predictive marker for survival.

Discussion

The current study investigated a new method for specifically detecting the levels of CEACAM1 and CEACAM6 in pre- and postoperative serum samples of patients with CRC and compared them to the internationally standard for testing CEA. We observed a significant decrease in all CEACAMS, as seen in CEA, CEACAM1 and CEACAM6 from pre- to post-operative serum levels for colon cancer, although not for patients with rectal cancer. While a significant correlation of a decrease in CEACAM values from pre- to post-operative samples was found, this was not uniform across all patients and did not correlate strongly with risk of recurrence in the current study. Hence, further analyses into the biomarker role of these CEACAMs is warranted, as well as further exploration of the actual biological tumour biology behind these glycoproteins.

In the current cohort, circulating CEACAM6 did not show a consistent pattern across conventional prognostic subgroups, and its value as a blood-based biomarker for patient stratification therefore remains uncertain. The expression associated with non-metastatic colorectal cancer has also been demonstrated recently, and pointing to CEACAM6 expression induced by acidity and under hypoxic conditions [17] and relation of CEACAM6 and metabolic shifts in CRC has also been demonstrated by others [16]. CEACAM6 has been found to have a value in relation to treatment in lung cancer [24], and to promote metastasis in prostate cancer [25].

Notably, immunohistochemical analyses have shown elevated CEACAM6 levels in advanced cancer stages, and association with shorter overall survival further supporting its role as a marker of tumour progression. Moreover, high CEACAM6 expression has been associated with reduced overall survival, underscoring its prognostic significance [17,26]. In this study, we could not find any correlation between postoperative CEACAM6 and recurrence or node status. Still, we

found that postoperative CEACAM1 was associated with recurrence, which could indicate that CEACAM1 should be preferred in postoperative controls, however this observation must first be validated in larger studies.

Previous studies have demonstrated that CEACAM6 is a downstream effector of ALDOB, a glycolytic enzyme that modulates lactate production and secretion [16]. ALDOB overexpression has been shown to suppress CEACAM6 through lactate metabolism, reducing CRC cell proliferation and chemoresistance [16]. Given this, our results may reflect metabolic differences in early vs. late-stage CRC, where tumour progression alters glycolytic flux and subsequently CEACAM6 expression. The metabolic regulation of CEACAM6 could explain its association with lymph node metastasis, as glycolytic adaptations in aggressive tumours may promote CEACAM6-mediated invasion and immune evasion [16,27]. Future studies should investigate whether CEACAM6 could serve as a metabolic biomarker for CRC progression and whether targeting glycolysis might alter CEACAM6-driven tumour behaviour. Given that CEACAM6 has been identified as a biomarker for aggressive, acid-resistant tumour clones, its increased expression in later-stage disease may reflect metabolic adaptations that promote tumour survival in hypoxic and acidic microenvironments [17,28]. This may explain why lower CEACAM6 levels in the current study were linked to early-stage disease, where these adaptations have not yet fully developed.

The current results are consistent with findings from previous studies, where CEACAM6 is associated with aggressiveness in the other types of cancer [29], such as pancreatic cancer, where elevated CEACAM6 expression was linked to reduced overall survival. Notably, CEACAM has also been used experimentally as an antibody-drug conjugate to treat pancreatic cancer [30], a method that may also have relevance to enhance effect of immunotherapy in CRC [3,31]. In parallel, nanoparticle-assisted glycovariant assays of established markers such as CA15-3 [32] and CA125 [33] have improved discrimination between malignant and benign or healthy conditions compared with conventional assays, and a similar nanoparticle-lectin assay has already been shown to distinguish colorectal cancer–associated glycoconjugates on circulating extracellular vesicles from those in non-cancer patients [23]. Together, these data support the concept that CEACAM1/6 glycovariants may offer added cancer specificity beyond total protein levels but require validation in larger studies including non-cancer controls. However, our current cohort lacks a dedicated healthy control group, and includes only a limited number of benign lesions, thus the diagnostic performance of CEACAM1/6 glycovariants cannot be firmly established from this study alone.

Liver metastasis is the most common primary location for metastatic spread from CRC. The identification of a biomarker for detection of liver metastasis could be an improvement in clinical surveillance or, inform about aggressiveness of metastatic growth to better inform treatment decisions [34,35]. An association between postoperative CEACAM1 or CEACAM6 with presence of liver metastasis was not found in the current study cohort, but hampered with a low number of events for this investigation. Postoperative levels were tested as a continuous variable, or as difference or ratio between postoperative and preoperative levels, none of which had a clear correlation to liver metastasis. Other studies, which has investigated the expression of CECAM6 levels in the tissue and blood have found an association to CEACAM6 [29,36]. However, these studies have the same challenges as our cohort, with small sample sizes, and importantly in our cohort a difference between no recurrence and liver metastasis was not found which questions its clinical use. The need for thorough validation with bigger studies is necessary before the implementation to clinical practice. In the current study we found correlations of increased or decreased levels of CEACAM1 and CEACAM6 difference and ratio for recurrence in general and lung metastasis, except for CEACAM1 difference and lung metastasis. However, the same results were found for CEA, which questions CEACAM1 and CEACAM6 clinical use and their ability to add additional information.

Many of the correlation analyses for both CEACAM1 and CEACAM6 had similar findings, suggesting their use in clinical practice to be limited to one as they provide somewhat the same information. For example, CEACAM1 was associated with both pre- and postoperative CEACAM6. Still, CEACAM6 might give slightly more information regarding cancer stage and presence of liver metastasis, making it the preferable option. However, whether any of the CEACAM molecules can give additional information for clinical use is currently debatable, but further investigation may be warranted. Among the limitations of the current study is the small number of patients and the low number of events for associations to recurrence or metastasis, as this was an early phase clinical exploration of a novel assay to test CEACAM expression. Further testing in larger cohorts is warranted to further investigate if CEACAM1 and CEACAM6 can predict recurrence and advanced stage

superior to CEA. Also, better mechanistic understanding of the CEACAM role as either biomarker or a target for treatment is warranted. Overall, our findings should be regarded as hypothesis-generating for the possible prognostic role of circulating CEACAM1 and CEACAM6, and future studies need to include non-cancer controls and systematically account for smoking and other comorbidities.

Conclusion

This study explored a novel assay to test for CEACAMs in pre- and post-operative samples from patients with colorectal cancer. While associated with cancer removal and to stage of disease, further investigation into the clinical role as either a biomarker or, a target for treatment is warranted.

Author contributions

CRediT: **Arezo Kanani**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing; **Marina Alexeeva**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing; **Rufus Vinod**: Conceptualization, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing; **Kim Pettersson**: Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing; **Janne Leivo**: Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing; **Kjetil Søreide**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Data availability statement

Data supporting this work are available for qualified researchers upon reasonable request from the corresponding author.

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