Accurate Prediction of Anastomotic Leakage after Colorectal Surgery Using Plasma Markers for Intestinal Damage and Inflammation

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BACKGROUND: Anastomotic leakage is a frequent and life-threatening complication after colorectal surgery. Early recognition of anastomotic leakage is critical to reduce mortality. Because early clinical and radiologic signs of anastomotic leakage are often nonspecific, there is an urgent need for accurate biomarkers. Markers of inflammation and gut damage might be suitable, as these are hallmarks of anastomotic leakage.

STUDY DESIGN: In 84 patients undergoing scheduled colorectal surgery with primary anastomosis, plasma samples were collected preoperatively and daily after surgery. Inflammatory markers, C-reactive protein; calprotectin; and interleukin-6, and intestinal damage markers, intestinal fatty acid binding protein; liver fatty acid binding protein; and ileal bile acid binding protein, were measured. Diagnostic accuracy of single markers or combinations of markers was analyzed by receiver operating characteristic curve analysis.

RESULTS: Anastomotic leakage developed in 8 patients, clinically diagnosed at median day 6. Calprotectin had best diagnostic accuracy to detect anastomotic leakage postoperatively. Highest diagnostic accuracy was obtained when C-reactive protein and calprotectin were combined at postoperative day 3, yielding sensitivity of 100%, specificity of 89%, positive likelihood ratio \( = 9.09 \) (95% CI, 4.34–16), and negative likelihood ratio \( = 0.00 \) (95% CI, 0.00–0.89) \( (p < 0.001) \). Interestingly, preoperative intestinal fatty acid binding protein levels predicted anastomotic leakage at a cutoff level of 882 pg/mL with sensitivity of 50%, specificity of 100%, positive likelihood ratio \( = \) infinite (95% CI, 4.01–infinite), and negative likelihood ratio \( = 0.50 \) (95% CI, 0.26–0.98) \( (p < 0.0001) \).

CONCLUSIONS: Preoperative intestinal fatty acid binding protein measurement can be used for anastomotic leakage risk assessment. In addition, the combination of C-reactive protein and calprotectin has high diagnostic accuracy. Implementation of these markers in daily practice deserves additional investigation. (J Am Coll Surg 2014;219:744–751. © 2014 by the American College of Surgeons)

Anastomotic leakage is a major challenge in patients undergoing colorectal surgery. Of 7,888 registered resections for colorectal cancer in the Netherlands in 2010, six hundred and twenty-four (8%) were complicated by anastomotic leakage, and in high-risk patients, incidence rates can increase to 18%. Anastomotic leakage is associated with high morbidity, mortality, reoperation, and duration of hospitalization. In cancer, anastomotic leakage is related to reduced disease-specific survival and higher recurrence rates. Delay in recognizing anastomotic leakage after colorectal surgery is associated with increased mortality. Early clinical presentation is heterogeneous and often nonspecific. Anastomotic leakage can present as peritonitis, localized fluid collections, or subclinical leakage detected by contrast radiology. In addition, nonspecific symptoms, including fever, absence of bowel action,
and diarrhea, only become apparent on postoperative days (PODs) 4 to 7. Abdominal CT scan, the current standard for diagnosis of anastomotic leakage, yields low sensitivity, which can delay the diagnosis and appropriate treatment of anastomotic leakage. There is an urgent need for accurate diagnostic markers of anastomotic leakage at an early stage after colorectal surgery to decrease delay of diagnosis and its unfavorable sequelae.

In this prospective cohort study, plasma markers of inflammation and intestinal damage in anastomotic leakage diagnosis were investigated. C-reactive protein (CRP) has been proposed to diagnose anastomotic leakage after colorectal surgery in a variety of studies on PODs 2 to 3. However, results are not unambiguously persuasive, showing diagnostic accuracy with about 70% to 80% sensitivity and specificity. Calprotectin, a heterodimeric peptide (36 kDa), constitutes about 60% of the cytosol proteins of neutrophils. As a marker of neutrophil activation, calprotectin can be an interesting marker for exaggerated inflammation early in anastomotic leakage. The cytokine interleukin-6 is found to be up-regulated in peritoneal fluid during the first 3 postoperative days in patients that have anastomotic leakage develop, although the diagnostic accuracy of interleukin-6 measurement in plasma is still unknown. Intestinal fatty acid binding protein (I-FABP), ileal bile acid binding protein, and liver fatty acid binding protein (L-FABP) are proteins expressed in enterocytes and are well-known plasma markers of enterocyte damage. The presence of I-FABP is limited to mature enterocytes of the small and large intestine. Ileal bile acid binding protein is solely expressed in the ileum, and L-FABP is expressed in small and large intestine, liver, and kidney epithelial cells. These intestinal cell damage markers have never been investigated in the context of anastomotic leakage diagnosis.

In this prospective study, the diagnostic accuracy of plasma markers of inflammation and intestinal damage to predict anastomotic leakage after colorectal surgery were investigated.

**METHODS**

**Patients**

Ninety consecutive patients scheduled to undergo colorectal surgery with primary anastomosis were prospectively enrolled at a single teaching hospital (Orbis Medical Center, Sittard, The Netherlands) between April 2011 and July 2013. Patients with inflammatory bowel disease were excluded because this could influence plasma levels of inflammatory biomarkers. None of the enrolled patients received NSAIDS. Nonsteroidal anti-inflammatory drugs have been omitted from routine analgesia in the enrolling hospital because these drugs are associated with increased leak rates. In addition, no patients using corticosteroids were included because they could influence marker levels. All patients gave written informed consent and the study was approved by the medical ethical committee of Atrium-Orbis-Zuyd, Heerlen, The Netherlands. The study was conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Patient characteristics (ie, sex, age, BMI, length of hospital stay, and tumor location) and surgical characteristics (ie, approach, type, anastomotic technique, ostomy, operation time, and blood loss) were collected. A fast-track Enhanced Recovery After Surgery protocol was followed in all patients, according to which venous blood was routinely collected at PODs 1 and 3. Both laparoscopic and open resections were performed.

**Definition of anastomotic leakage**

Clinically relevant anastomotic leakage was defined as extraluminal presence of contrast fluid on contrast-enhanced CT scans and/or leakage when relaparotomy was performed, requiring reintervention. Indications for CT or relaparotomy were based on clinical presentation, including fever, tachycardia, tachypnea, low saturation, low urinary production, abdominal pain, and signs of ileus or gastric retention.

**Blood sampling**

Venous blood samples were taken at hospital admission (preoperative sample) and daily starting at the first postoperative day until discharge. Blood samples were immediately cooled on ice, centrifuged at 3,500 rotations per minute for 15 minutes, and plasma samples were stored at −80°C until batch analysis. All analyses were performed by one person (AvB) after inclusion of all patients, therefore, the treating clinicians were not aware of the values. The technician was not aware of patient characteristics.
C-reactive protein, calprotectin, and interleukin-6 measurement

C-reactive protein plasma levels were determined by an ELISA developed at our institute, as described previously (lower detection limit 100 pg/mL). Calprotectin plasma concentration was measured using the commercially available calprotectin ELISA (lower detection limit 46.8 ng/mL; Hycult Biotechnology). Interleukin-6 levels were measured by ELISA as described previously (lower detection limit 10 pg/mL).  

Fatty acid binding protein measurement

Intestinal fatty acid binding protein plasma levels were determined using an in-house ELISA that selectively detects human I-FABP (lower detection limit 25 pg/mL). Plasma ileal bile acid binding protein was measured as described previously (lower detection limit 0.1 ng/mL). Liver fatty acid binding protein plasma levels were determined using a commercially available ELISA (lower detection limit 2 ng/mL; Hycult Biotechnology).

Statistical analysis

Normality was tested by Kolmogorov-Smirnov. Student’s t-test was used for between-group comparisons for continuous data. Dichotomous variables were compared using Pearson’s chi-square test. All data are presented as mean (SEM). Receiver operating characteristic (ROC) curves were used to calculate the accuracy of the studied markers predicting anastomotic leakage. The ideal cutoff value for diagnosing anastomotic leakage was defined as the cutoff value with maximum sum of sensitivity and specificity. Overall diagnostic accuracy was represented by the area under the curve (AUC). As many factors influence anastomotic leakage rates, possible confounding factors were analyzed by logistic regression analysis.

To determine the accuracy of combined markers detecting anastomotic leakage, logistic regression analysis was performed and predicted probabilities were then plotted in ROC curves. The best cutoff point of predicted probabilities (P) was defined as the cutoff point with maximum sum of sensitivity and specificity. To calculate the linear function describing all combinations of ideal cutoff values for combined markers in the detection of anastomotic leakage, the cutoff point (P) was used in the following equation: \( \ln \left( \frac{P}{1 - P} \right) = B_0 + B_1X_1 + B_2X_2 \); in which \( B_0 \) represents the constant of the logistic regression analysis and \( B_1 \) and \( B_2 \) represent the logistic regression coefficients of the markers. By calculating coordinates of intersections with the x- and y-axis, the linear function describing the cutoff line to discriminate anastomotic leakage could be determined.

Sample size was calculated based on CRP levels at POD 4. Mean CRP levels in the anastomotic leakage group were estimated at 200 (SD 100) mg/L, and 75 (SD 100) mg/L in the group without anastomotic leakage. With \( \alpha = 0.05 \) and \( 1 - \beta = 0.80 \), a total sample size of 80 was calculated, including 6 cases of anastomotic leakage at anastomotic leakage incidence of 8%.

Statistical analyses were performed with Prism 5.0 for Windows (GraphPad Software Inc.) and SPSS 20.0 for Windows (SPSS Inc.). STARD (Standards for the Reporting of Diagnostic Accuracy Studies) statement was used in this study.

RESULTS

Patients

Of 90 enrolled patients, 3 were excluded, as it was decided not to perform a primary anastomosis after patient inclusion, and 3 were excluded for retracting informed consent. The remaining 84 patients had a mean age of 65 (1.4) years and 54 (64%) were male. Baseline and operative characteristics are listed in Tables 1 and 2, respectively.

Anastomotic leakage developed in 8 patients (10%), which became apparent at median of POD 6 (range 3 to 10 days). Six patients (75%) with anastomotic leakage required surgical reintervention, the remaining 2 patients were treated with intravenous antibiotics. No radiologic drainage was performed in any case of anastomotic leakage. Three patients (4%) required surgical reintervention for fascial dehiscence and, in 3 patients (4%), anastomotic leakage was clinically suspected but not found at relaparotomy. Mortality was 2% (1 patient with anastomotic leakage).

Logistic regression of possible confounding factors

Factors influencing anastomotic leakage rates were considered as possible confounders and were therefore entered in a univariate logistic regression analysis. None

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of the analyzed factors were significantly associated with anastomotic leakage in this cohort (age: odds ratio [OR] \(= 1.00; 95\% \text{ CI}, 0.94–1.06; p = 0.94\); sex: OR = 1.76; 95\% CI, 0.33–9.35; p = 0.51; American Society of Anesthesiologists class: OR = 0.60; 95\% CI, 0.10–3.78; p = 0.59; location of anastomosis: OR = 0.29; 95\% CI, 0.06–1.54; p = 0.15; and diverting ostomy: OR = 0; 95\% CI, 0–infinite; p = 1.00). Therefore, none of these factors were considered as confounders.

C-reactive protein, calprotectin, and interleukin-6 measurement

Mean CRP levels in patients with anastomotic leakage were elevated at POD 4 compared with CRP levels in patients without anastomotic leakage (415 [221] mg/L vs 119 [19] mg/L; \(p = 0.002\)) (Fig. 1). Overall diagnostic accuracy of CRP to detect anastomotic leakage at POD 4 in these patients using ROC curve analysis was described by AUC of 0.82 (95\% CI, 0.68–0.95). The optimal cutoff value of 99 mg/L produced sensitivity of 100\%, specificity of 64\%, positive likelihood ratio (LR+) = 2.78 (95\% CI, 1.87–3.65), and negative likelihood ratio (LR–) = 0 (95\% CI, 0–1.24).

Mean calprotectin levels were elevated in patients with anastomotic leakage at POD 2 (588 [62] ng/mL) compared with patients without anastomotic leakage (366 [25] ng/mL; \(p = 0.006\)). Area under the curve of the ROC curve was 0.82 (95\% CI, 0.68–0.95) (Fig. 2). A cutoff value of 411 ng/mL yielded sensitivity of 100\%, specificity of 58\%, LR+ = 2.38 (95\% CI, 1.60–3.09); and LR– = 0 (95\% CI, 0–1.61). At POD 3, diagnostic accuracy increased to AUC 0.92 (95\% CI, 0.83–1.00). A cutoff value of 544 ng/mL produced sensitivity of 86\%, specificity of 88\%; LR+ = 7.17 (95\% CI, 3.55–14); and LR– = 0.16 (95\% CI, 0.03–1.01). At POD 4, diagnostic accuracy increased to AUC = 0.96 (95\% CI, 0.90–1.00). With a cutoff value of 541 ng/mL, this resulted in sensitivity of 100\%, specificity of 91\%, LR+ = 11 (95\% CI, 4.68–20); and LR– = 0 (95\% CI, 0–1.02).

Interleukin-6 levels were not different between patients with and without anastomotic leakage at any time point (data not shown).

Combination of C-reactive protein and calprotectin at postoperative days 3 and 4

When CRP levels at POD 3 were combined with calprotectin measurement on POD 3 to predict anastomotic leakage, the area under the curve of ROC curve was 0.96 (95\% CI, 0.92–0.99). A cutoff value of 100 mg/L CRP and 411 ng/mL calprotectin yielded sensitivity of 100\%, specificity of 91\%, LR+ = 25.4 (95\% CI, 7.4–81); and LR– = 0 (95\% CI, 0–1).
leakage, a cutoff line was calculated with sensitivity of 100%; specificity of 89%; LR+ = 9.09 (95% CI, 4.34–16); and LR− = 0 (95% CI, 0–0.89) (p < 0.0001). The cutoff line was described by the linear function:

\[
\text{[Calprotectin (ng/mL)]} + 0.83 \times \text{[CRP (mg/L)]} = 707;
\]

which means that a positive test is obtained when any combination of CRP and calprotectin levels at POD 3 in this formula results in a value >707. Area under the curve of the ROC curve was 0.93 (95% CI, 0.87–1.00).

By measuring CRP and calprotectin at POD 3, diagnosis of anastomotic leakage could be shortened by a median of 3 days (range 0 to 7 days).

At POD 4, this cutoff line yielded accuracy comparable with POD 3 (sensitivity 100%; specificity 90%; LR+ = 10 [95% CI, 4.6–18]; and LR− = 0 [95% CI, 0–0.88]; p = 0.001), with AUC of the ROC curve 0.97 (95% CI, 0.92–1.00).

### Fatty acid binding protein measurement

Preoperative I-FABP levels were elevated in patients who developed anastomotic leakage compared with controls (1,466 [728] pg/mL and 190 [17] pg/mL, respectively; p < 0.0001) and remained elevated over time, however, only significantly at POD 3 (Fig. 3). A preoperative cut-off value of 882 pg/mL yielded sensitivity of 50%, specificity of 100%, LR+ = infinite (95% CI, 4.01–infinite); and LR− = 0.50 (95% CI, 0.26–0.98).

Plasma ileal bile acid binding protein and L-FABP levels were not different between groups at any time point (data not shown).

### DISCUSSION

A plasma marker for early detection of anastomotic leakage after colorectal surgery is lacking. In this study, the plasma neutrophil activation marker calprotectin showed high sensitivity and specificity from POD2 onward, especially when combined with the conventional acute-phase protein CRP. C-reactive protein has been described widely as a useful diagnostic test for anastomotic leakage, with sensitivity rates varying from 73% to 91% and specificity from 60% to 86% on PODs 3 to 5, with cutoff values ranging from 100 to 147 mg/L.\textsuperscript{15,17,33,34} A recent meta-analysis of 6 studies including 1,832 patients indicated that CRP levels at POD 4 had a pooled sensitivity of 68%, specificity of 83%, and a negative predictive value of 89% for predicting infectious complications.\textsuperscript{35} In concordance, with sensitivity of 100%, the negative predictive value in our study was 100% as well. Measuring CRP postoperatively to monitor the development of inflammatory complications is considered standard care in many hospitals. However, a recent study demonstrated a low predictive value of CRP for the development of anastomotic leakage after laparoscopic colorectal surgery.\textsuperscript{36} In addition, a high negative predictive value is useful to rule out the development of complications and to confirm safety of discharge from the hospital, yet the decision for relaparotomy is based on the positive predictive value or LR+, which is low for CRP. This indicates that routine CRP measurement can guide safe hospital discharge, although it is not indisputable. To confirm the decision for reintervention, other tests are needed.

Calprotectin has not been investigated before as a predictor for anastomotic leakage that was clinically detectable at PODs 3 to 10. In this study, calprotectin was superior to CRP in the detection of anastomotic leakage, by both earlier (POD 2 to 3 vs POD 4) and more accurate (AUC = 0.92 at POD 3 vs AUC = 0.82 at POD 4) detection. In addition, the best diagnostic accuracy was obtained when CRP and calprotectin measurement at POD 3 were combined, which is still earlier (median 3 days; range 0 to 7 days) than clinical signs. As specificity increased when adding calprotectin measurement, false-positive test results decreased dramatically.

Interleukin-6 plasma levels were not different between patients with and without anastomotic leakage. Several studies have found a significant difference when measuring cytokines in peritoneal fluid.\textsuperscript{21} Apparently, these differences are not reflected by circulating cytokine levels.

It can only be speculated why calprotectin is a better marker for anastomotic leakage than CRP and interleukin-6. As part of the innate immune response, neutrophils provide a first line of defense against bacterial
infection and are recruited to sites of infection or inflammatory stimuli within minutes, with a peak by 24 to 48 hours. Being a neutrophil activation product, calprotectin can be increased early after anastomotic leakage, and CRP reflects the systemic inflammatory response, which becomes apparent later. Neutrophil count has been described as having no additive effect on CRP to predict anastomotic leakage after laparoscopic sleeve gastrectomy. However, the complete neutrophil count was used in this study, and calprotectin reflects the amount of activated neutrophils.

Of different FABP types, only I-FABP levels showed significant differences between patients with and without anastomotic leakage. This is remarkable, as L-FABP is the predominant FABP type in colonic tissue, where I-FABP is only present in small amounts. It is also noteworthy that intestinal markers (eg, CRP and calprotectin) did detect anastomotic leakage postoperatively, and specific intestinal markers (FABPs) did not. It could be assumed that intestinal epithelial damage is relatively limited in anastomotic leakage compared with other intestinal pathology, such as ischemia-reperfusion injury and necrotizing enterocolitis, and that excessive inflammation is the predominant pathologic process in anastomotic leakage.

In addition, I-FABP was already increased preoperatively in patients who ultimately had anastomotic leakage develop. These findings might indicate generalized intestinal impairment, predisposing patients to development of anastomotic leakage. The LR+ of preoperative I-FABP levels at a cutoff value of 882 pg/mL indicates a post-test probability of 100% when a positive test is obtained. Intestinal fatty acid binding protein levels can, therefore, be used for preoperative risk assessment. Elevated I-FABP levels predict a 100% chance for anastomotic leakage after colorectal surgery. The next step would be to conduct a phase II diagnostic study that answers the question: “Are patients with certain test results more likely to have the target disorder?”—which changes the direction of interpretation. This question can only truly be answered in a new prospective dataset using the cutoff values defined in the current study to predict anastomotic leakage. The next step would be to guide treatment based on the preoperative I-FABP and postoperative CRP and calprotectin preferentially in a large multicenter study. High preoperative I-FABP values can filter out half of the patients who have anastomotic leakage develop without false-positive results (sensitivity of 50%, with post-test probability of 100%), and for whom a different surgical approach can be considered.

CONCLUSIONS
The best diagnostic accuracy is obtained when CRP and calprotectin are combined at POD 3. C-reactive protein and calprotectin measurement on POD 3 can therefore help determine which patients can be discharged safely and which patients should be monitored intensively. In the latter case, relaparotomy should be considered as well. Future interventional studies should investigate whether treatment strategies based on these markers lead to prevention and better management of anastomotic leakage after colorectal surgery.

Author Contributions
Study conception and design: Reisinger, Poeze, Hulswé, van Acker, Hoofwijk, Stoot, Derikx
Acquisition of data: Reisinger, van Acker, van Bijnen, Derikx
Analysis and interpretation of data: Reisinger, Poeze, Hulswé, Hoofwijk, Stoot, Derikx
Drafting of manuscript: Reisinger
Critical revision: Poeze, Hulswé, van Acker, van Bijnen, Hoofwijk, Stoot, Derikx
REFERENCES


