Original article

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A multicentre cohort study of serum and peritoneal biomarkers to predict anastomotic leakage after rectal cancer resection

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Abstract

Aim Anastomotic leakage (AL) is one of the most feared complications after rectal resection. This study aimed to assess a combination of biomarkers for early detection of AL after rectal cancer resection.

Method This study was an international multicentre prospective cohort study. All patients received a pelvic drain after rectal cancer resection. On the first three postoperative days drain fluid was collected daily and C-reactive protein (CRP) was measured. Matrix metalloproteinase-2 (MMP2), MMP9, glucose, lactate, interleukin 1-beta (IL1β), IL6, IL10, tumour necrosis factor alpha (TNFα), *Escherichia coli*, *Enterococcus faecalis*, lipopolysaccharide-binding protein and amylase were measured in the drain fluid. Prediction models for AL were built for each postoperative day using multivariate penalized logistic regression. Model performance was estimated by the *c*-index for discrimination. The model with the best performance was visualized with a nomogram and calibration was plotted.

Results A total of 292 patients were analysed; 38 (13.0%) patients suffered from AL, with a median interval to diagnosis of 6.0 (interquartile ratio 4.0–14.8) days. AL occurred less often after partial than after total mesorectal excision (4.9% *vs* 15.2%, $P = 0.035$). Of all patients with AL, 26 (68.4%) required reoperation. AL was more often treated by reoperation in patients without a diverting ileostomy (18/20 *vs* 8/18, $P = 0.03$). The prediction model for postoperative day 1 included MMP9, TNFα, diverting ileostomy and surgical technique (*c*-index = 0.71). The prediction model for postoperative day 2 only included CRP (*c*-index = 0.69). The prediction model for postoperative day 3 included CRP and MMP9 and obtained the best model performance (*c*-index = 0.78).

Conclusion The combination of serum CRP and peritoneal MMP9 may be useful for earlier prediction of AL after rectal cancer resection. In clinical practice, this combination of biomarkers should be interpreted in the clinical context as with any other diagnostic tool.

Keywords Anastomotic leakage, rectal resection, early detection, biomarkers, drain fluid

What does this paper add to the literature?

Anastomotic leakage (AL) is one of the most feared complications after rectal resection. Early detection is of paramount importance in order to minimize postoperative morbidity and mortality. This prospective cohort study showed that a combination of serum CRP and peritoneal MMP9 may be useful for early prediction of AL after rectal cancer resection.

Introduction

With the introduction of minimally invasive techniques, the short-term outcomes of rectal surgery have improved over the last decades [1,2]. Despite these

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advances, the incidence of anastomotic leakage (AL) has not been reduced [3]. Moreover, standardized recovery programmes have shortened hospital stay, with the downside that AL can become clinically apparent after discharge resulting in readmission and delayed management [4]. Nowadays, 20% of AL is diagnosed after discharge, with a mean time to diagnosis of 6–15 days [5,6].

The current diagnostic strategy, consisting of on-demand CT scanning, fails to detect AL at an early stage as half of all leakages require reoperation [7,8]. Delayed reintervention after false-negative CT scanning is associated with increased mortality and prolonged hospital stay [9]. In addition, delay in diagnosis of 2.5 days is associated with an increase in mortality from 24% to 39% [10]. Hence, early detection is of paramount importance in order to minimize postoperative morbidity and mortality.

Biomarkers in drain fluid have previously been proposed as an innovative strategy for early detection of AL. Elevated peritoneal levels of inflammatory cytokines and lactate as well as decreased pH seemed to be associated with AL, and measurement of such parameters is thus of interest for early detection of AL [11]. Furthermore, promising results were shown for lipopolysaccharide-binding protein (LBP) and *Enterococcus faecalis* in drain fluid [12,13]. However, implementation in clinical practice is lagging behind as previous studies were based on small sample sizes and lacked any estimation of predictive accuracy.

A systematic review concluded that a combination of biomarkers yielded improved predictive accuracy compared with separate analysis of biomarkers [14]. Therefore, we aimed to assess a combination of biomarkers for prediction of AL after rectal cancer resection and to determine its predictive accuracy.

Method

Patients

This study was designed as an international multicentre prospective cohort study. Ten hospitals in the Netherlands and Belgium participated in the study. Patients were included between August 2015 and October 2017. The medical ethics committees of the Erasmus MC University Medical Centre in the Netherlands and of the University Hospital Leuven in Belgium approved this study. Ethical approval was also obtained in the other participating hospitals. This study was registered at http://www.isrctn.com/ (study ID 84052649).

Patients aged 18 years and above who underwent partial mesorectal excision (PME) or total mesorectal excision (TME) with construction of a colorectal or coloanal anastomosis were eligible for inclusion. Pregnant women and patients who underwent an emergency procedure were excluded. In addition, patients in whom no drain fluid was obtained or who underwent surgery for an indication other than adenocarcinoma were excluded. All patients gave written informed consent. The follow-up ended at the first outpatient clinic visit after hospital discharge.

Collection and storage of drain fluid

All patients received a pelvic drain during surgery. Drain fluid was collected every morning on the first three postoperative days. Drain fluid was collected respecting rules of sterility with a syringe including a needle and deposited in a 10-ml ethylenediaminetetraacetic acid (EDTA) tube. The drain fluid reservoir was replaced after the collection of drain fluid. The EDTA tube was transported to the laboratory and the drain fluid samples were centrifuged (at 1955*g*) for 10 min at 4°C. Subsequently, the supernatant was removed. Drain fluid was aliquotted in five cryotubes of 2 ml and stored at −80°C until further analysis. C-reactive protein (CRP) was measured in peripheral blood samples at the hospitals' clinical laboratories on the first three postoperative days.

Drain fluid analysis

Samples were thawed, vortexed and centrifuged for 1 min at 10 000*g* and 4°C before analysis. All biomarkers were measured in duplicate and the means were taken for further analysis. Matrix metalloproteinases (MMP2 and MMP9) and cytokines [interleukin 1-beta (IL1β), IL6, IL10 and tumour necrosis factor alpha (TNFα)] were measured using ProcartaPlex® Multiplex Immunoassay (Thermo Fisher Scientific, Bleiswijk, The Netherlands) on a Luminex Magpix machine. High-sensitivity assays were used for cytokine measurement. Levels of α-amylase, glucose and lactate were measured using Roche/Hitachi cobas c systems from Roche Diagnostics (Indianapolis, Indiana, USA). LBP was measured with enzyme linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instruction. *Escherichia coli* and *E. faecalis* were measured using a semi-quantitative real-time PCR strategy. Prior to DNA isolation, 500 μl of drain fluid was spiked with 5 μl of Phocine Herpes Virus (PhHV) as an internal control from the European Virus Archive (EVAg). Samples were spun for 5 min at 8000*g* and the pellets resuspended in 180 μl buffer (20 mM Tris, 2 mM EDTA, 1% Tween 80 and

50 mg/ml lysozyme). The samples were incubated at 37°C with shaking at 600 rpm for 30 min after which 25 μl of proteinase K was added followed by a 2-h incubation at 56°C at 700 rpm. DNA extraction was performed using a Macherey-Nagel NucleoSpin® Tissue kit (Bioké, Leiden, the Netherlands). Template DNA was eluted in elution buffer in a total volume of 100 μl. Subsequently, primers for *E. coli* and *E. faecalis* were added in accordance with the previously published protocol [15]. The StepOnePlus Real-Time PCR System (Applied Biosystems, Bleiswijk, the Netherlands) was used for RT-PCR. Threshold cycles (C_t) were corrected for differences in extraction efficiency using the threshold cycle of the internal control PhHV.

Clinical data assessment

Patient characteristics (age, gender, body mass index, medication use, bowel preparation, smoking, alcohol abuse, American Society of Anesthesiologists score, previous abdominal surgery, indication for surgery, preoperative radiotherapy, preoperative chemotherapy, location of lesion) and surgical characteristics (surgical procedure, surgical technique, conversion, construction of anastomosis, configuration of anastomosis, diverting ileostomy) were prospectively registered. Creation of the anastomosis was registered as 'stapler' or 'manual'. Manual anastomosis was performed using interrupted coloanal sutures with a hand-sewn technique. If the anastomosis was constructed with a stapler and additional manual sutures were added this was registered as stapled. Transanal TME was defined as follows: part of a TME being performed with transanal assistance. This includes a semi-rigid platform with rigid instruments to perform a down-to-up TME.

AL was the primary outcome of interest, being defined as a clinically manifest insufficiency of the anastomosis leading to a clinical state requiring treatment (i.e. grade B/C) [7]. AL was confirmed by either endoscopy, CT scan and/or contrast enema or reoperation. Fistulas communicating with the anastomosis on CT scan were classified as AL together with presacral abscesses if extravasation of the colonic contrast was visible on radiological imaging. In addition, postoperative indicators (time to discharge, postoperative complications with their respective treatment strategies, readmission, reoperation and mortality) were prospectively registered. Elective stoma reversals were not registered as reoperation.

Statistical analysis

Continuous variables were described as median ± interquartile range (IQR) and compared with the Mann–Whitney *U*-test. Categorical variables were described as percentages and compared with the chi-square test or Fisher's exact test when needed. Comparisons of biomarkers were corrected for multiple testing using Holm's method per postoperative day [16]. A multiple imputation procedure was performed to impute missing data based on 10 completed datasets. For each postoperative day, multivariate penalized logistic regression models were constructed including clinically relevant baseline characteristics (age, gender, nonsteroidal anti-inflammatory drugs, corticosteroids, diverting Ileostomy, surgical procedure, approach) and all biomarkers. Prediction models for each postoperative day were built including covariates with a P-value < 0.1. Internal validation using the bootstrap method was done to obtain corrected estimates of model performance to avoid overfitting. Model performance was estimated by Harrell's concordance index (the *c*-index). The *c*-index measures how adequate the model is at discriminating between the outcome of interest, and represents the probability that, in a randomly selected pair of patients, the model assigns a higher risk to the patient who is truly high risk compared with the patient who is truly low risk. A *c*-index of 0.5 indicates no association between prediction and true outcome and a value of 1.0 indicates perfect association. A *c*-index of more than 0.75 is considered clinically useful [17]. A calibration plot of the model with the best *c*-index was built showing the relationship between the observed and predicted probability of the outcome. The observed and expected rates are similar in a well-calibrated model. The final model was visualized using a nomogram and captured in an online calculator (https://www.evidencio.com/models/show/1537). Two-sided P-values < 0.05 were considered statistically significant. Analyses were performed using SPSS v.22 (IBM Corp., Armonk, New York, USA) and the NLME, LATTICE, ARM, AOD and RMS packages in R v.3.3.3 (http://www.r-project.org).

Results

Study population

A total of 310 patients were included. Nine patients were excluded because no drain fluid was obtained, and nine were excluded due to another surgical indication than rectal adenocarcinoma. In the end, 292 patients were eligible for analysis.

Table 1 represents baseline characteristics of the study population. The median time of follow-up was 28.0 days (IQR 17.0–35.0). The median time to discharge was 7.0 days (IQR 5.0–11.0). In total, 42

(14.4%) patients were readmitted to the hospital and 38 (13.0%) underwent reoperation. Infection at the drain insertion site was reported in three (1.0%) patients. No other complications of the pelvic drain were reported. Two (0.7%) patients died. One patient died of AL and the other patient died 2 days after hospital discharge of an unknown reason as no autopsy was performed.

Anastomotic leakage

A total of 38 (13.0%) patients suffered from AL. No differences in patient characteristics were observed for patients with and without AL. The incidence of AL was no different for patients with and without diverting ileostomy (11.4% *vs* 14.9%, $P = 0.371$). AL occurred less often after PME than after TME (4.9% *vs* 15.2%, $P = 0.035$) (Table 1).

AL was clinically manifest as a presacral abscess in five patients. The median time to diagnosis was 6.0 days (IQR 6.0–14.8). Patients with AL had a significantly longer hospital stay (16.0 days *vs* 6.0 days, $P \leq 0.001$). Production of drain fluid was no different for patients with and without AL (day 1, 155 ml *vs* 180.0 ml, $P = 0.664$; day 2, 97.5 ml *vs* 100.0 ml, $P = 0.435$; day 3, 60.0 ml *vs* 90.0 ml, $P = 0.141$).

In 30 (78.9%) patients AL was confirmed by a CT scan, in 5 (13.2%) by proctoscopy and in 1 (2.6%) patient by reoperation. Of all patients with AL, 26 (68.4%) required reoperation whereas 12 (31.6%) were treated more conservatively (antibiotics, drainage or Endo-sponge). AL was more often treated by reoperation in patients without a diverting ileostomy (18/20 *vs* 8/18, $P = 0.03$).

Biomarkers

Table 2 compares the levels of biomarkers for patients with and without AL per postoperative day. Table 3 represents outcomes of multivariate penalized logistic regression analyses per postoperative day. Prediction models for each postoperative day were built including covariates with a P-value < 0.1 in the multivariate analysis. The prediction model for postoperative day 1 included MMP9, TNFα, diverting ileostomy and surgical technique. The prediction model for postoperative day 2 only included CRP. The prediction model for postoperative day 3 included both CRP and MMP9.

The prediction model of postoperative day 1 had a *c*-index of 0.71 whereas the prediction model of postoperative day 2 had a *c*-index of 0.69. These prediction models were thus lacking discrimination and therefore were not considered to be clinically useful. On the contrary, the prediction model of postoperative day 3, including CRP and MMP9, had a *c*-index of 0.78. This *c*-index indicated that for 78% of the time the model assigned a higher probability to a patient with AL than a patient without AL. For the prediction model of postoperative day 3, a nomogram was constructed facilitating the calculation of the individual risk of AL after rectal cancer resection based on CRP and MMP9 on postoperative day 3 (Fig. 1). An online calculator was built for this nomogram at https://www.evidencio.com/models/show/1537.

Calibration was determined to estimate model performance with a calibration plot. In a calibration plot the predicted probability is plotted against the corresponding observed probability in the dataset. Ideally, this depicts a diagonal line and calibration is quantified by the mean absolute error. Figure 2 shows the calibration plot of the prediction model of postoperative day 3 (mean absolute error = 0.025).

Discussion and conclusions

This international multicentre prospective cohort study showed that a combination of serum CRP and peritoneal MMP9 may be useful for early prediction of AL after rectal cancer resection. The combination of these biomarkers can estimate the individual risk of AL after rectal cancer resection on the third postoperative day, which was 3 days earlier than the median time to diagnosis (6 days).

As with any other biomarker in clinical practice, this tool only assesses the risk of AL; it requires confirmation through additional imaging. However, this tool might enable timely intervention and subsequently minimize morbidity and mortality. For example, if this tool shows that a patient has high risk of AL on the third postoperative day and AL is subsequently confirmed by additional imaging even before the leak becomes clinically apparent, early reintervention could minimize the consequences of AL. So, this tool facilitates decision-making for surgeons even before clinical symptoms occur [18].

Serum CRP is already a useful negative predictor for AL after anterior resection [19,20]. Nevertheless, serum CRP monitoring lacks specificity and positive predictive value (PPV) for AL, because the CRP level also rises due to other inflammatory complications [21]. Previous research on biomarkers for AL showed that local biomarkers from peritoneal fluid were more specific than systemic biomarkers [22]. The present study showed that peritoneal MMP9 was predictive for AL, and therefore this biomarker has additional value in prediction of AL over serum CRP alone. Furthermore, the *c*-index of 0.78 of this combination showed adequate

Table 1 Patient and surgical characteristics of patients with and without anastomotic leakage (AL).

	Total no. of patients (*n* = 292)	No AL (*n* = 254, 87.0%)	AL (*n* = 38, 13.0%)	Missing	*P*-value
Patient characteristics					
Age (years), median ± IQR	63.0 (57.0–71.0)	63.5 (57.5–71.0)	60.5 (53.8–68.5)	0 (0.0%)	0.135
Gender					
Male	193 (66.1%)	167 (65.7%)	26 (68.4%)	0 (0.0%)	0.745
Female	99 (34.0%)	87 (34.4%)	12 (31.6%)		
BMI (kg/m^2), median ± IQR	25.8 (23.5–28.7)	25.8 (23.3–28.7)	25.9 (24.2–29.2)	1 (0.3%)	0.546
Corticosteroids	17 (5.8%)	14 (5.5%)	3 (7.9%)	2 (0.7%)	0.475*
NSAIDs	8 (2.7%)	7 (2.8%)	1 (2.6%)	2 (0.7%)	1.000*
Bowel preparation	244 (83.6%)	209 (82.3%)	35 (92.1%)	25 (8.6%)	1.000*
Smoking	38 (13.0%)	31 (12.2%)	7 (18.4%)	10 (3.4%)	0.304*
Alcohol abuse	39 (13.4%)	32 (12.6%)	7 (18.4%)	10 (3.4%)	0.378
ASA score					
I	45 (15.4%)	37 (14.6%)	8 (21.1%)	2 (0.7%)	0.468*
II	181 (62.0%)	156 (61.4%)	25 (65.8%)		
III	62 (21.2%)	57 (22.4%)	5 (13.2%)		
IV	2 (0.7%)	2 (0.8%)	0 (0.0%)		
Previous abdominal surgery	100 (34.2%)	90 (35.4%)	10 (26.3%)	1 (0.3%)	0.263
Clinical tumour stage					
T1	14 (4.8%)	13 (5.1%)	1 (2.6%)	44 (15.1%)	0.871*
T2	72 (24.7%)	62 (24.4%)	10 (26.3%)		
T3	144 (49.3%)	123 (48.4%)	21 (55.3%)		
T4	18 (6.2%)	16 (6.3%)	2 (5.3%)		
Clinical nodal stage					
N0	101 (34.6%)	86 (33.9%)	15 (39.5%)		0.600
N ≥ 1	139 (47.6%)	119 (46.9%)	17 (44.7%)		
Preoperative radiotherapy	155 (53.1%)	135 (53.1%)	20 (52.6%)	1 (0.3%)	0.933
Short course	58 (37.4%)	52 (38.5%)	6 (30.0%)		
Long course	89 (57.4%)	77 (57.0%)	12 (60.0%)		
Preoperative chemotherapy	102 (34.9%)	87 (34.3%)	15 (39.5%)	1 (0.3%)	0.540
Location of lesion from anal verge (cm), median ± IQR	10.0 (6.0–13.0)	10.0 (6.0–14.0)	9.0 (5.0–12.0)	16 (5.5%)	0.169
Surgical characteristics					
Procedure					
PME	61 (20.9%)	58 (22.8%)	3 (7.9%)	0 (0.0%)	**0.035**
TME	231 (79.1%)	196 (77.2%)	35 (92.1%)		
Surgical technique					
Open	11 (3.8%)	10 (3.9%)	1 (2.6%)	0 (0.0%)	0.736*
Laparoscopic	161 (55.1%)	142 (55.9%)	19 (50.0%)		
Transanal	120 (41.1%)	102 (40.2%)	18 (47.4%)		
Conversion	8 (2.7%)	8 (3.1%)	0 (0.0%)	0 (0.0%)	0.598*
Construction of anastomosis					
Manual	43 (14.7%)	39 (15.4%)	4 (10.5%)	2 (0.7%)	0.423
Stapler	247 (84.6%)	213 (83.9%)	34 (89.5%)		
Configuration of anastomosis					
Side-to-side	4 (1.4%)	4 (1.6%)	0 (0.0%)	31 (10.6%)	0.861*
Side-to-end	173 (59.2%)	147 (57.9%)	26 (68.4%)		
End-to-end	79 (27.1%)	70 (27.6%)	9 (23.7%)		
End-to-side	5 (1.7%)	5 (2.0%)	0 (0.0%)		
Diverting ileostomy	158 (54.1%)	140 (55.1%)	18 (47.4%)	0 (0.0%)	0.371

Bold values indicate significance.

ASA, American Society of Anesthesiologists; BMI, body mass index; IQR, interquartile range.

*Fisher's exact test.

Table 2 Comparison of biomarker levels for patients with and without anastomotic leakage (AL).

		Postoperative day 1					Postoperative day 2					Postoperative day 3				
	AL	*n*	Median	Q1	Q3	*P*-value	*n*	Median	Q1	Q3	*P*-value	*n*	Median	Q1	Q3	*P*-value
MMP2 × 10^5	Y	37	0.7	0.5	1.2	1.000	31	1.0	0.6	1.4	1.000	31	1.1	0.7	2.1	1.000
(pg/ml)	N	248	0.7	0.4	1.1		236	1.0	0.6	1.4		230	1.2	0.7	1.7	
MMP9 × 10^5	Y	37	3.2	0.9	10.8	0.450	31	1.7	0.4	7.0	1.000	32	2.0	0.5	5.0	**0.011**
(pg/ml)	N	247	2.0	0.9	4.1		235	1.0	0.5	2.0		231	0.6	0.3	1.5	
Glucose (mM)	Y	37	2.0	0.4	3.8	0.252	38	0.2	0.1	2.4	**< 0.001**	35	0.3	0.1	3.1	**0.011**
	N	247	3.4	1.5	4.6		241	2.4	0.1	4.7		238	2.9	0.1	5.0	
Lactate (mM)	Y	37	10.5	6.9	14.5	1.000	38	12.5	9.1	19.7	1.000	37	11.3	8.1	19.2	0.444
	N	248	9.1	6.2	13.2		242	11.1	6.4	17.3		243	9.2	5.2	14.9	
CRP (mg/ml)	Y	36	69.5	35.0	105.8	0.459	36	152.5	83.5	215.5	**< 0.001**	37	170.0	113.8	290.5	**< 0.001**
	N	241	50.0	30.9	80.0		213	86.0	47.1	135.9		215	78.0	41.0	125.0	
IL1β (pg/ml)	Y	37	61.1	31.7	263.5	0.341	32	138.1	46.7	536.8	**0.011**	31	190.0	28.6	3271.1	**< 0.001**
	N	247	47.1	19.6	132.3		236	39.8	13.4	151.5		232	30.3	9.3	142.9	
IL6 (pg/ml)	Y	36	69717.7	19267.2	76184.2	1.000	32	73454.5	23889.4	76334.1	1.000	31	46178.8	17483.5	76070.8	0.301
	N	246	51635.3	23484.4	76070.8		236	41860.7	17483.5	75786.4		232	24738.2	11239.9	68858.8	
IL10 (pg/ml)	Y	37	249.7	141.3	594.6	0.584	32	176.3	84.4	630.8	0.080	31	128.3	38.6	554.2	0.072
	N	247	204.8	109.7	405.5		236	99.1	51.8	220.8		232	62.4	30.0	136.4	
TNFα (pg/ml)	Y	37	37.3	23.6	128.8	0.156	32	23.1	12.9	67.2	1.000	31	45.2	14.2	79.7	**0.036**
	N	246	30.1	16.4	59.1		237	21.5	12.0	39.8		231	17.7	9.9	34.0	
Escherichia	Y	37	34.2	32.4	35.8	1.000	33	34.8	31.4	37.5	1.000	30	34.3	26.7	36.4	1.000
coli (C_t)	N	247	34.6	32.4	37.0		231	34.6	32.4	36.7		227	34.7	32.9	36.6	
Enterococcus	Y	38	26.3	25.2	26.9	1.000	33	26.5	25.4	27.5	1.000	32	26.2	25.1	27.4	1.000
faecalis (C_t)	N	248	26.2	25.1	27.1		234	26.0	25.0	27.0		228	25.9	25.1	27.0	
LBP (μg/ml)	Y	38	3.6	1.9	5.0	1.000	34	5.5	3.5	6.6	1.000	31	6.2	4.6	7.0	1.000
	N	248	3.2	2.2	4.4		237	5.1	4.0	6.1		231	5.6	4.5	6.7	
Amylase (U/l)	Y	36	36.0	14.3	84.8	0.584	38	30.5	13.5	47.0	1.000	37	24.0	17.5	45.0	1.000
	N	243	24.0	13.0	41.0		243	28.0	18.0	45.0		244	25.0	15.0	37.0	

Bold values indicate significance.

CRP, C-reactive protein; IL, interleukin; LBP, lipopolysaccharide-binding protein; MMP, matrix metalloproteinase; *n*, number of patients; Q1, first quartile; Q3, third quartile; TNFα, tumour necrosis factor alpha.

discrimination, which is important in a diagnostic setting where the classification of patients into different groups is of major interest.

MMP9 is a matrix metalloproteinase which plays a role in the degradation of extracellular matrix proteins, especially collagen, and is actively involved in the inflammation reaction and wound healing process [23,24]. Previously, experimental studies have investigated the association between MMPs and colorectal AL. MMPs negatively affect anastomotic healing [25,26] whereas MMP inhibitors provided enhanced breaking strength of colonic anastomoses [27]. The most pronounced collagen loss provoked by MMP9 was seen in the suture-holding zone of colonic anastomoses [28]. In addition, in an experimental model of bacterial peritonitis anastomotic MMP9 activity was increased 3 days after operation [29]. Translation to clinical research obtained similar results. Patients with elevated levels of MMP1, MMP2 and MMP9 in perioperative biopsies from the colon more often had AL [30]. Actually, peritoneal MMP9 had already been evaluated as biomarker for AL. Contradictory literature exists for colorectal resection [31,32], but for rectal resection a pilot study showed that peritoneal MMP9 levels measured 4 h after surgery were increased in patients who developed AL [33]. However, it remains unknown whether this association represents a causal relationship or is a consequential effect of AL.

In rectal cancer surgery, diversion is commonly applied to protect the anastomosis from leakage [34]. However, the incidence of AL was no different for patients with and without a diverting ileostomy (11.4% *vs* 14.9%). Nevertheless, in patients without a diverting ileostomy, AL was more often treated by reoperation than in patients with a diverting ileostomy (18/20 *vs* 8/18, $P = 0.03$). These results suggest that a diverting ileostomy allows less invasive treatment strategies. Accordingly, it was previously shown from population-based data of the Dutch ColoRectal Audit (DCRA) that a high tendency towards stoma construction in rectal cancer surgery did not reduce the incidence of AL [35].

The reported incidence of AL of 13.0% is high compared with several previous studies (3.0–11.1%) [36,37]. We hypothesize that the prospective design and inclusion of only rectal resections contributed to this relatively high incidence of AL. Another explanation is that the definition of AL varies and that some atypical presentations of leakages such as presacral

Table 3 Outcomes of multivariate penalized logistic regression for anastomotic leakage (AL) per postoperative day.

	Postoperative day 1				Postoperative day 2				Postoperative day 3			
	OR	95% CI (lower)	95% CI (upper)	*P*-value	OR	95% CI (lower)	95% CI (upper)	*P*-value	OR	95% CI (lower)	95% CI (upper)	*P*-value
MMP2 (pg/ml)	1.011	0.962	1.063	0.661	1.020	0.955	1.090	0.556	1.025	0.986	1.066	0.216
MMP9 (pg/ml)	1.106	0.995	1.229	0.063*	1.094	0.952	1.257	0.203	1.130	0.982	1.301	0.088‡
Glucose (mM)	0.939	0.751	1.176	0.585	0.916	0.728	1.152	0.453	0.920	0.734	1.152	0.466
Lactate (mM)	0.990	0.890	1.101	0.854	0.993	0.912	1.081	0.869	0.987	0.917	1.063	0.737
CRP (mg/ml)	1.068	0.981	1.162	0.128	1.057	1.005	1.111	0.030†	1.064	1.013	1.118	0.013‡
IL1β (pg/ml)	1.002	0.992	1.012	0.702	0.999	0.992	1.006	0.787	1.001	0.999	1.003	0.522
IL6 (pg/ml)	0.980	0.847	1.135	0.787	1.052	0.899	1.230	0.529	0.939	0.788	1.119	0.481
IL10 (pg/ml)	0.999	0.980	1.019	0.954	1.044	0.940	1.159	0.424	1.110	0.959	1.284	0.161
TNFα (pg/ml)	1.037	0.998	1.077	0.062*	0.991	0.969	1.014	0.453	1.011	0.996	1.026	0.136
Escherichia coli (C_t)	0.975	0.852	1.115	0.707	0.947	0.830	1.081	0.422	1.029	0.905	1.169	0.664
Enterococcus faecalis (C_t)	1.072	0.756	1.520	0.697	1.179	0.820	1.695	0.373	1.009	0.703	1.448	0.960
LBP (μg/ml)	0.913	0.704	1.184	0.492	0.910	0.708	1.170	0.462	0.888	0.686	1.149	0.365
Amylase (U/l)	1.000	0.998	1.003	0.827	1.001	0.998	1.003	0.648	1.000	0.999	1.001	0.547
Age	0.978	0.944	1.013	0.222	0.973	0.936	1.010	0.154	0.986	0.948	1.027	0.503
Gender	0.931	0.415	2.088	0.862	0.866	0.367	2.044	0.743	0.698	0.275	1.772	0.449
NSAIDs	0.719	0.131	3.953	0.704	0.454	0.051	4.042	0.479	1.018	0.172	6.028	0.985
Corticosteroids	1.066	0.263	4.315	0.928	1.132	0.252	5.093	0.871	1.087	0.267	4.430	0.908
Diverting ileostomy	0.478	0.205	1.116	0.088*	0.485	0.195	1.208	0.120	0.575	0.219	1.511	0.261
Procedure	2.829	0.888	9.014	0.079*	2.540	0.787	8.193	0.119	2.229	0.603	8.239	0.229
Surgical technique	0.756	0.151	3.787	0.733	0.804	0.152	4.252	0.797	1.047	0.197	5.557	0.957

CI, confidence interval; CRP, C-reactive protein; IL, interleukin; LBP, lipopolysaccharide-binding protein; MMP, matrix metalloproteinase; NSAID, nonsteroidal anti-inflammatory drug; OR, odds ratio.
*These variables were added to the prediction model of postoperative day 1.
†These variables were added to the prediction model of postoperative day 2.
‡These variables were added to the prediction model of postoperative day 3.

abscesses or rectovaginal fistulas are not always included. In addition, the Dutch Snapshot study reported a comparable incidence of 13.4% within 30 days postoperation [4].

Over the last decade, our research group has been involved in the search for a reliable biomarker for AL after colorectal resection. In a clinical trial (the APPEAL study), we demonstrated that PCR in drain fluid for *E. faecalis* could be predictive for AL after colorectal resection [13]. However, the relatively low PPV of 30.2% on the third postoperative day indicated a substantial number of false positives. Therefore, the present study was conducted with the aim of obtaining a combination of biomarkers with increased predictive accuracy. In addition, the previous study showed that an increase of one standard deviation in the average level of LBP on postoperative day 1 is associated with an increased risk of leakage of 1.6 [12]. LBP is an acute phase protein that binds to lipopolysaccharide (LPS) to elicit an immune response to Gram-negative bacteria [38]. However, the present study did not confirm these results, possibly due to different drain locations as the previous study obtained drain fluid from intra-abdominal drains whereas the present study used pelvic drains which were positioned extraperitoneally. Furthermore, the different microbiome of patients with colon and rectal cancer may be another explanation because the previous study also included colonic resections [39,40]. This previous study showed promising results for drain fluid analysis on the first three postoperative days. Therefore, we decided to limit drain fluid collection to this interval.

The GRECCAR 5 trial has shown that pelvic drainage after rectal excision for rectal cancer does not reduce AL [41]. On the other hand, pelvic drainage was not found to be detrimental [42]. In this study, only three (1.0%) patients suffered from infection at the drain insertion site, which could be managed without invasive treatment strategies. So the opportunity for early detection of AL after rectal resection with innovative drain fluid analysis might justify pelvic drainage after rectal resection.

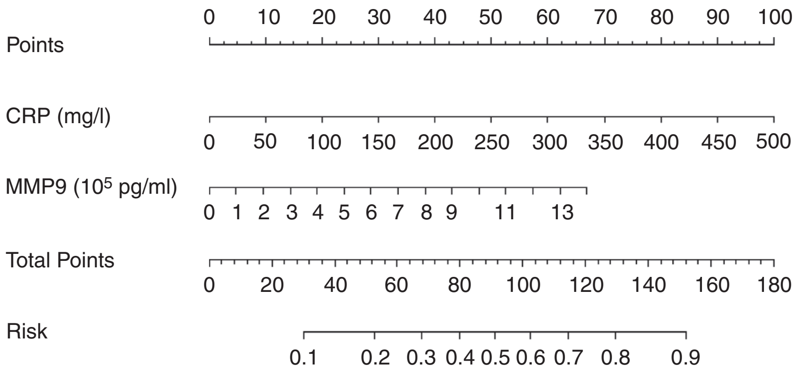

Figure 1 Nomogram of the prediction model of postoperative day 3 (*c*-index = 0.78). This nomogram can estimate the risk of anastomotic leakage after rectal resection on postoperative day 3 with serum C-reactive protein (CRP) and peritoneal matrix metalloproteinase 9 (MMP9).

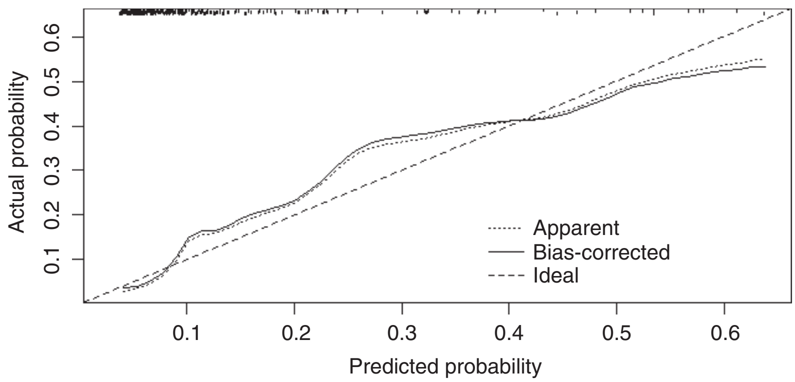

Figure 2 Calibration plot for nomogram predicting anastomotic leakage with serum C-reactive protein and peritoneal matrix metalloproteinase 9 on postoperative day 3. This plot represents the relationship between predicted probability from the nomogram and observed probability in the dataset. The bootstrap method was used to obtain corrected probabilities.

Measurements of MMP9 can easily be implemented as Luminex is a commonly used method in clinical laboratories. It is a fast method and relatively cheap. However, there were some limitations. First of all, dislocation of the drain may have influenced drain fluid composition [43]. Secondly, intra-operative spillage could have affected drain fluid composition by eliciting an inflammatory response. In addition, the emerging transanal technique may have an effect on pelvic contamination, although no evidence for this exists.

Since prediction models tend to perform better on data on which the model was constructed, external validation is essential before implementing prediction models in clinical practice [44]. Furthermore, a phase II diagnostic study is required to confirm that this tool truly predicts AL in a time-changing direction which runs from the diagnostic test forward to diagnosis [45]. In this manner, the effect on time to diagnosis can be assessed prospectively. In the end, the effect of early detection on morbidity and mortality requires phase III diagnostic research.

This international multicentre prospective cohort study showed that a combination of serum CRP and peritoneal MMP9 may be useful for earlier prediction of AL after rectal cancer resection. Nevertheless, it is important to mention that this tool should never replace clinical observations, implying that the outcomes of this tool should be interpreted in the clinical context as with any other diagnostic tool.

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Conflicts of interest

All authors declare no conflict of interest.

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