Predictive value of tissue calprotectin for disease recurrence after ileocecal resection in pediatric Crohn's disease

Kristyna Zarubova[a], Ondrej Fabian[b,c,d], Ondrej Hradsky[a], Tereza Lerchova[a], Filip Mikus[d], Vojtech Dotlacil[e], Lucie Pos[e], Richard Skaba[e], Jiri Bronsky[a]

Aim. Detection of possible predictive factors of endoscopic recurrence after ileocecal resection in Crohn's disease could be very beneficial for the individual adjustment of postoperative therapy. The aim of this study was to verify, whether immunohistochemical detection of calprotectin in resection margins is useful in diagnostics of endoscopic recurrence.
Methods. In this study we included pediatric patients with Crohn's disease who underwent ileocecal resection, regardless of pre-operative or post-operative therapy (n=48). We collected laboratory, clinical, surgical, endoscopic and histopathological data at the time of surgery and at 6 months after surgery. The immunohistochemical staining of calprotectin antigen was performed on all paraffin blocks from the resection margins.
Results. Out of 48 patients 52% had endoscopic recurrence in the anastomosis (defined by Rutgeerts score) within 6 months after surgery. The number of cells positive for calprotectin in the proximal resection margin was negatively associated with recurrence (P=0.008), as was the elevated level of total calprotectin (from both resection margins). There was no correlation of calprotectin in distal resection margin and endoscopic recurrence. Fecal calprotectin over 100 ug/g (P=0.0005) and high CRP (P<0.001) at 6 months after ileocecal resection and peritonitis (P=0.048) were associated with endoscopic recurrence.
Conclusion. Approximately half of the patients developed endoscopic recurrence within 6 months after ileocecal resection. The predictive value of tissue calprotectin is questionable, as it is negatively associated with endoscopic recurrence. There are other potentially useful predictors, such as CRP and fecal calprotectin at 6 months after resection and the presence of peritonitis.

Key words: calprotectin, Crohn's disease, pediatric, recurrence, prediction

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[a]Gastroenterology and Nutrition Unit, Department of Paediatrics, 2nd Faculty of Medicine, Charles University and University Hospital Motol, V Uvalu 84, Prague 5, 150 06, Czech Republic
[b]Clinical and Transplant Pathology Centre, Institute for Clinical and Experimental Medicine, Videnska 1958/9, Prague 4, 140 21, Czech Republic
[c]Department of Pathology and Molecular medicine, 3rd Faculty of Medicine, Charles University and Thomayer hospital, Videnska 800, Prague 4, 140 59, Czech Republic
[d]Department of Pathology and Molecular Medicine, 2nd Faculty of Medicine, Charles University and University Hospital Motol, V Uvalu 84, Prague 5, 150 06, Czech Republic
[e]Department of Paediatric Surgery, 2nd Faculty of Medicine, Charles University and University Hospital Motol, V Uvalu 84, Prague 5, 150 06, Prague, Czech Republic
Corresponding author: Kristyna Zarubova, e-mail: zarubova.kristyna@gmail.com

INTRODUCTION

Almost 70% of patients with Crohn's disease (CD) undergo intestinal surgery within 10 years of diagnosis[1,2]. Ileocecal resection (ICR) is the most performed procedure. Post-operative recurrence risk factors in adults are known from previously published studies – smoking, prior intestinal resection, penetrating disease, perianal disease and extensive resection[3-5]. Studies performed in pediatric population focusing on endoscopic recurrence (ER) after ICR are few. Baldassano et al. evaluated at first clinical recurrence and found potential risk factors – high Paediatric Crohn's Disease Activity Index (PCDAI), colonic disease at the time of surgery and 6-mercaptopurine treatment preoperatively[6]. Colonic disease was also described as a risk factor by Pacilli et al.[7]. Based on our previously published data, low serum level of albumin at the time of surgery is a potential predictor of ER at 6 months after ICR (ref.[8]).

Fecal calprotectin (F-CPT) is usually used for disease course monitoring, also post-operatively. Previously performed studies have proven a good correlation between relapse and F-CPT concentration in adults[9] as well as in children[10]. Immunohistochemical detection of calprotectin (CPT) in tissues is not a standard method for monitoring or predicting disease behavior or its prognosis. Fukunaga et al. presented higher levels of CPT-positive cells in colonic mucosa in patients with inflammatory

bowel disease (IBD) than in control group[11]. Lower intramucosal CPT was detected in UC patients in remission when compared to active disease[12,13]. High CPT concentration was also associated with acute inflammation in patients after appendectomy[14].

Histopathological features have been investigated as potential predictors of disease relapse in many previously published studies. Active disease and myenteric plexitis were found to be potential predictors[15-17], on the other hand chronic inflammation and granulomas in resection margin were not[18]. A meta-analysis from Simmillis et al. focusing on granulomas as a potential predictor shows higher recurrence rate in patients with histopathologically confirmed granulomas, however considering the heterogeneity of published studies, it is not possible to use this finding as a main predictor[19]. Li et al. found that higher count of S100-positive enteric glial cells is associated with endoscopic and clinical recurrence[20]. Diederen et al. described microscopically positive resection margins (histopathological inflammation) as one of the risk factors for surgical recurrence in a group of pediatric patients after ICR (ref.[21]).

The primary aim of this study was to verify, whether immunohistochemical detection of CPT in resection margins is useful as a predictor of endoscopic recurrence after ICR. Secondarily, we aimed to identify the number of patients with relapse at the 6th month after ICR in colon or UGI and to reveal other potential predictors of ER, such as albumin, CRP or F-CPT.

METHODS

Patients

We included pediatric patients with CD, who underwent ICR, regardless of pre-operative or post-operative therapy.

Inclusion criteria were: age of 0 – 18 years (at the time of surgery), diagnosis of CD (according to Porto criteria or revised Porto criteria) (ref.[22]), history of performed ICR with primary anastomosis (could be combined with other surgery – evacuation of abscess; jejunal, ileal or colonic resection; strictureplasty; fistulectomy; right hemicolectomy; surgical treatment of bowel perforation), endoscopy performed 6 month after ICR or earlier (in the case of CD relapse), available bioptic tissue from surgery and endoscopic control, informed consent signed.

Exclusion criteria were: change of therapy before endoscopy (substantial changes – e.g. initiation of biological therapy, switch between biologics), ileostomy, missing histopathological samples or substantial part of laboratory data, refusal of patient/parents to participate in this study.

Between December 2008 and September 2018, 63 patients with CD underwent ICR performed by a team of pediatric surgeons, who closely cooperates with our department. Four patients were excluded, because they needed ileostomy and therefore had second surgery with anastomosis done later. Eleven patients had no endoscopy during the determined interval. Forty-eight patients fulfilled inclusion criteria and were included in the study. Patients' characteristics are given in Table 1.

Table 1. Patients' characteristics before surgery.

Characteristics	All
Female	0.42
Age at the time of ICR	16 (15-17)
Ciprofloxacine at the time of ICR	0.29
Metronidazole at the time of ICR	0.38
Azathioprine at the time of ICR	0.81
Infliximab at the time of ICR	0.38
Adalimumab at the time of ICR	0.15
Exclusive enteral nutrition at the time of ICR	0.15
Corticosteroids at the time of ICR	0.25
Piperacillin/Tazobactam at the time of ICR	0.06
Length of resection	22 (16.5-28.5) NA=1
Penetrating disease	0.35
Elective surgery	0.62

ICR = ileocecal resection; NA = not available

Data and samples

Laboratory results/parameters were collected from hospital electronic records. Surgical (other procedure combined with ICR, length of resection) and clinical data (therapy, elective/urgent surgery, penetrating disease) before ICR were collected from hospital electronic records retrospectively.

Histopathological evaluation was divided into two parts:

The first part of the study was retrospective and all microscopic slides from proximal and distal resection margins were evaluated by senior gastrointestinal pathologist blinded to clinical data. The assessed morphological variables included: 1) intensity of chronic inflammation; 2) intensity of active inflammation (established on the basis of neutrophilic infiltration in the lamina propria, presence of neutrophils in the epithelium and presence of erosions or ulcerations); 3) intensity of eosinophilic inflammation; 4) distortion of the mucosal architecture; 5) epithelioid granulomas; 6) basal plasmocytosis; 7) pyloric metaplasia; 8) fibrosis; 9) lymphocytic lymphangitis; 10) transmural lymphoid aggregates; 11) submucosal plexitis; 12) myenteric plexitis; 13) myenteric plexus hyperplasia; 14) obliterative vasculopathy; 15) acute fibrinous-purulent peritonitis and 16) chronic adhesive peritonitis. The assessed variables were evaluated separately for proximal and distal resection margin.

The immunohistochemical staining of the CPT antigen was then on all paraffin blocks from the resection margins. Tissue sections (thickness 1 um) were deparaffinized, and the anti-CPT antibody (Invitrogen, at a dilution of 1:1000) was used. Detection was performed by the PolyDet Dab chromogen (Dako REAL) with phosphate-

buffered saline solution. CPT expression was assessed by counting the highest number of positive cells per one high power field (400x) in the most affected region of each microscopic slide. The positive cells were counted separately for lamina propria and the epithelium. In areas with erosions or ulcerations, we counted CPT-positive cells from the tissue adjacent to the margin of the respective erosion/ulceration.

In the second part of the study (retrospective evaluation of prospectively collected data), the bioptic samples taken during the endoscopic control in the 6th month after the ICR were evaluated. During the duration of the study, according to standard protocol all patients in our center undergo regular endoscopic evaluation 6 months after ICR (or earlier in case of flare). The histopathological slides from the six bowel segments (neoterminal ileum, colon close to the anastomosis, ascending colon, transverse colon, descending colon and rectum) were examined. The intensity and activity of the inflammation were assessed using the modified Global Histology Activity Score. This scoring system evaluates the intensity of chronic and acute inflammation, presence of epithelial damage and mucosal defects, presence of epithelioid granulomas and distortion of the mucosal architecture. This scoring system was described in detail in our previous publication and has an advantage in the possibility to evaluate each bowel segment separately[23].

Endoscopic data, actual clinical and laboratory data were collected at the time of endoscopy, which was performed between the 4th and 7th month after ICR (mean 6th month). Findings in anastomosis were described using Rutgeerts score[24], in upper gastrointestinal tract (UGI) and colon using modified simple endoscopic score (mSES) (ref.[25]). In UGI we assessed esophagus, gastric body, gastric antrum and duodenum and scored from 0 to 3 points for size of ulcers, ulcerated surface, affected surface and presence of narrowing. Colon was divided in 4 parts (right colon, transverse colon, left colon and rectum), scoring was the same as in UGI. ER was defined as a Rutgeerts score equal or more than i2 and/or mSES in UGI or colon more than 3.

Primary and secondary outcomes

As a primary outcome we have chosen identification of predictive factors of disease relapse in anastomosis (Rutgeerts score equal or more than i2). As a main potential predictive factor we appointed a positivity of CPT in resection margins of resected bowel. Secondary outcomes were number of patients with relapse at 6th month after ICR in colon or UGI, evaluation of other predictors of disease recurrence, assessment of role of laboratory results and histopathological findings at the time of surgery and influence of the therapy.

Ethical considerations

Legal representatives of the patients signed an informed consent form for inclusion in the study. The study was approved by the Ethics Committee of Motol University Hospital.

Statistical analysis

Statistical software R-project (R Core Team, version 3.6.0) was used for data analysis. Continuous variables were described as medians and inter quartile range (IQR). Categorical variables were described as absolute frequencies and percentages. Values of F-CPT were analyzed on a logarithmic scale. Univariate association with categorical outcome was assessed using linear logistic regression model. For testing of association between two linear predictors, we used linear regression models. Adjusted models were constructed with multiple logistic regression. The selection of predictors for multiple logistic regression models were based on clinical decision only. Probability (p) values of <0.05 were considered significant. A 95% confidence interval was used. Figures were constructed using R package "ggplot2".

RESULTS

Rate of endoscopic recurrence (ER)

Out of 48 patients 52% had ER in anastomosis within 6 months after ICR (12 patients had Rutgeerts score i2, 5 had i3 and 8 had i4). Among patients in remission based on Rutgeerts score (n=23), 1 had relapse in colon and 4 in UGI.

Tissue CPT in resection margins

Number of cells positive on CPT in proximal resection margin was negatively associated with ER defined by Rutgeerts score (OR = 0.969 95% CI 0.936 – 0.996, *P*=0.008) (Fig. 1), as was the elevated level of total CPT (from both resection margins) (OR = 0.993, 95% CI 0.972 - 0.9997, *P*=0.034). If we use model adjusted on patients treated with biological therapy after resection, this correlation is also confirmed (OR = 0.971, 95% CI 0.938 – 0.997, *P*=0.008, OR=0.993, 95% CI 0.973 – 0.999, *P*=0.021, respectively). There was no correlation of CPT in distal resection margin and ER.

Laboratory results and clinical data at the time of surgery

We did not find any association between age at the time of ICR or sex and primary outcome. Low serum concentration of albumin (OR = 0.871, 95% CI 0.736 – 1.009, *P*=0.066) and elevation of CRP (OR = 1.02, 95% CI 0.999 – 1.045, *P*=0.060) were found to be borderline associated with ER, in adjusted model on biological therapy after ICR, the results were significant for albumin (OR = 0.851, 95% CI 0.713 – 0.992, *P*=0.039), but not for CRP (OR = 1.02, 95% CI 0.999 – 1.045, *P*=0.075). F-CPT (samples from 58% patients were available) was not significantly associated with ER (OR = 1.0, 95% CI 0.9996 – 1.0006, *P*=0.637) regardless of post-operative therapy. F-CPT cut-off value for remission was set at 100 ug/g.

Laboratory results at the time of endoscopic examination

Out of 48 patients 75% submitted sample of stool for F-CPT evaluation at 6th month after ICR. Levels of log F-CPT were in association with ER (OR = 2.19, 95% CI 1.28 – 4.28, *P*=0.003) – Fig. 2, in model adjusted for bio-

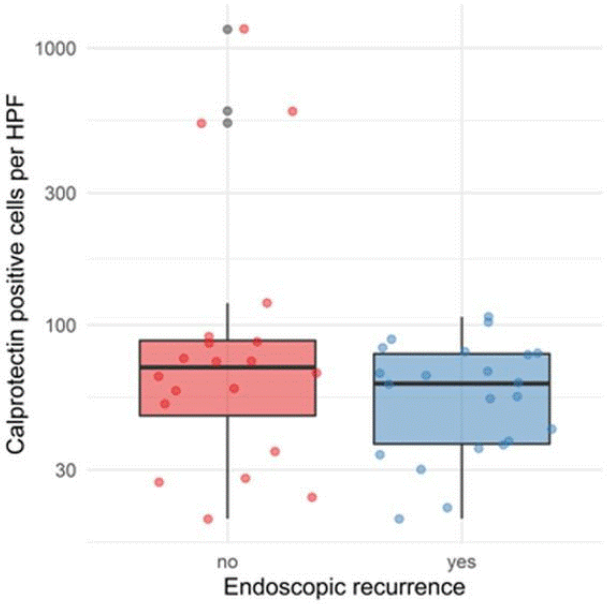

Fig. 1. Association between log tissue CPT in proximal resection margin and ER at 6 months .
HPF = high power field, CPT = calprotectin, ER = endoscopic recurrence

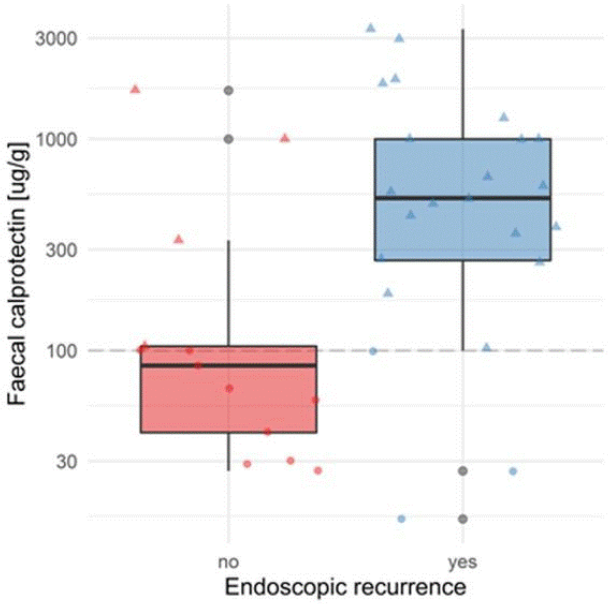

Fig. 2. Association between log F-CPT at 6 months and ER at 6 months.
F-CPT = faecal calprotectin, ER = endoscopic recurrence

logical therapy even more significantly (OR = 233, 95% CI 1.37 – 4.79, *P*=0.002). F-CPT over 100 ug/g at 6 months was strongly positively associated with ER (OR = 15, 95% CI 3.1 – 96.6, *P*=0.0005). Three patients had ER despite having F-CPT below 100 ug/g.

The CRP at 6 months was tested in all patients. Elevated values of CRP were associated with ER (OR = 2.21, 95% CI 1.353 – 4.385, *P*<0.001, resp. OR = 2.53, 95% CI 1.414 – 6.047 (after using adjusted model for biological therapy)). Albumin levels (examined in 94% of patients) were not in association with ER in group of all patients, but if we again use adjusting for biologics, it was statistically significant (OR = 0.759, 95% CI 0.5797 – 0.943, *P*=0.011).

Histopathology

Only the finding of peritonitis (chronic or florid) was found to be associated with ER (OR = 4.15, 95% CI 1.015 – 21.599, *P*=0.048), also borderline in model adjusted for biological therapy (OR = 4.08, 95% CI 0.988 – 21.390, *P*=0.052). Other markers as plexitis (submucosal or myenteritic), active inflammation (Fig. 3a,b), intensity of inflammation or chronic inflammation were not in association with ER.

DISCUSSION

Studies on rates of ER after ICR in children are scarce. Our previous study[8], focused on patients treated postoperatively only with AZA, found ER in 38% at 6 months. Another pediatric study by Bobanga et al. described ER in 87%, but the mean follow-up was longer than 6 months, endoscopy was not performed in all patients and postoperative therapy differed between patients[26]. In Hukkinen et al., ER was found in 51% of patients in long time follow-up (mean 38 months), 88% of these patients had immunosupressive therapy[10]. More detailed data are available in adult populations - in a randomized controlled POCER study published by De Cruz et al., the ER rate after 6 months was 45% in high-risk patients treated with AZA and 21% in patients treated with adalimumab (ADA) (ref.[3]). Kotze at al. described early ER (within 1 year from the time of the surgery, defined as Rutgeerts score more than i2) in patients treated with biologics. In the ADA group, it was 24% and in the infliximab (IFX) group it was 27% (ref.[27]). In another study by Auzolle et al., ER from 6th to 12th month after ICR was found in 47% of patients[28]. Thus, ER described in the present study (52%) is comparable to published data both from pediatric and adult populations. It is important to note, that each study used a slightly different definition of ER and the Rutgeerts score is not validated for children, but there is no better scoring system available at the moment. Based on relapse rate we can assume that it is necessary to observe these patients closely, repeat laboratory tests as CRP and F-CPT regularly and as recommended by European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) - endoscopy should be performed 6–9 months after surgery[29].

Immunohistochemical detection of CPT in intestinal tissue is not a commonly used method to evaluate the intensity of inflammation or predict disease recurrence. There are no studies in patients after ICR, neither in adults nor children. Study by Fukunaga et al. proved higher level of CPT-positive cells in colonic mucosa in IBD patients than in controls[11]. Also Liu et al. found higher concentration of CPT in colonic mucosa in patients with

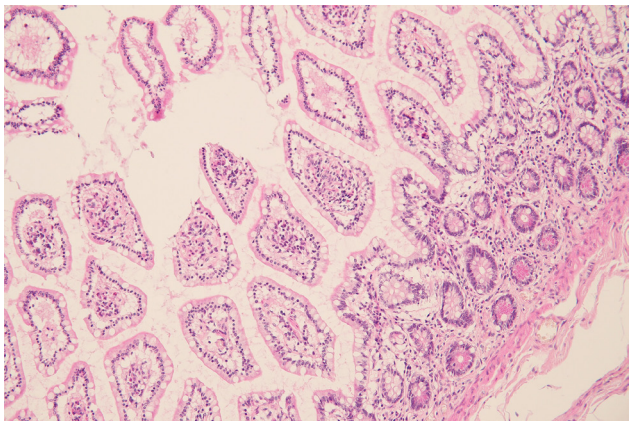

Fig. 3a. A microphotograph of the resection margin with no signs of active inflammation in basic staining (hematoxylin and eosin, magnification 200x).

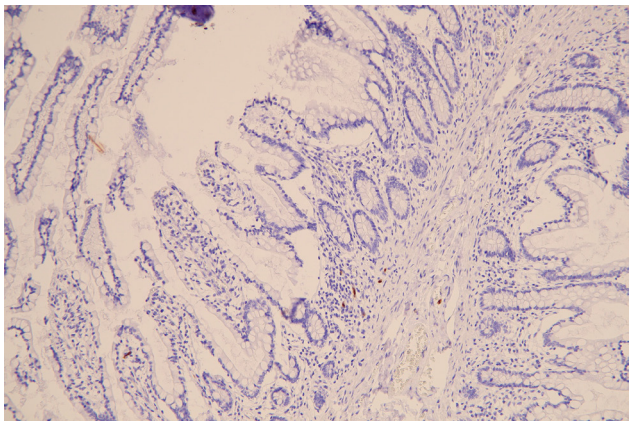

Fig. 3b. An immunohistochemical staining of CPT showing scarce positive cells in lamina propria (magnification 200x). CPT = calprotectin

active UC than in healthy patients or those with inactive UC (ref.[13]). Results of our study are not sufficient enough to recommend measurement of tissue CPT for prediction of ER after ICR. Contrary to our expectations, we found negative association between tissue CPT at proximal resection margin and risk of ER. However, we would expect higher tissue CPT to be associated with higher risk - in analogy to higher Rutgeerts score, which is considered as an important predictive factor in adults. We do not have any pathophysiological explanation for our findings that can be due to relatively small sample size in our pilot study (with potential influence of few outlier high values in individual patients). Our IBD surgeons stick to standard resection procedure according to current guidelines and perform resection in substantial distance from macroscopically inflamed area. However, differences in microscopic inflammatory intensity in resection margins that are not macroscopically visible, cannot be excluded. Because model adjusted on biological therapy brought the same results, it seems, that postoperative treatment is not potential confounder of these findings and thus it is not likely that biological therapy used in more difficult cases (with higher tissue CPT) would lead to better post-surgical prophylaxis of ER. It is also questionable, if counting the highest number of tissue CPT positive cells in the most affected area is the optimal way of evaluation of the histopathological sample. However, there is no such study published evaluating different histological approaches of tissue CPT measurement and further research on bigger sample size is necessary to either prove or contradict our findings.

F-CPT is commonly used marker for monitoring postoperative disease recurrence and behavior[30]. Pediatric study performed in CD patients after surgery by Hukkinen et al., found F-CPT levels of 139 ug/g as threshold for ER (beyond 6 months) (ref.[10]). In 2018 Tham et al. published systematic review on using F-CPT as marker for ER after ICR. As the best threshold authors considered F-CPT values of 150 ug/g with 70% (95% CI 59–81%) sensitivity and 69% (95% CI 61–77%) specificity[31]. These findings were also confirmed in our study, there was an association between F-CPT at the time of endoscopy and ER, despite the fact we did not have samples from all patients. As recommended by ESPGHAN guidelines, F-CPT could be successfully used for timing of endoscopy after ICR (threshold >100 ug/g) (ref.[29]). However, using this threshold in our study population, we would miss 3 patients with ER.

Also, CRP is a helpful marker of inflammatory activity, but it is not specific for IBD. Wright et al. or De Cruz et al. in POCER study found that CRP is not as accurate as F-CPT in monitoring adult patients after ICR (ref.[3,30]). This is also supported by the above-mentioned ESPGHAN guidelines[29]. In our study, CRP at the time of surgery is not associated (although borderline) with ER, but at 6 months after ICR the association is significant. CRP reflects systemic inflammation, which can persist after ICR, although F-CPT decreases (after resection of affected bowel).

It is known that low albumin level as a marker of poor nutritional status is a risk factor for peri- and postoperative complications, e.g. in cardiac[32], orthopedic[33] and also in bowel surgery[34,35]. In our previous study we also found that a low albumin level is a potential predictor of ER in selected patients treated with AZA without biologicals after ICR[8]. In the present study, we found only borderline association at the time of surgery (statistically significant after adjusting for biological therapy). A similar situation was found at the time of endoscopy.

From the histopathological features, only peritonitis (chronic or florid) was found to be associated with ER. To our knowledge, this is a newly described finding that could become a subject of future research on possible predictive histopathological factors. In contrast, we did not confirm the published data on plexitis[15-17] as a clinically useful predictor of disease recurrence.

From models adjusted for biological therapy, our data suggest that we cannot reliably use histopathological or biochemical predictors as helpful markers for decisions on the best postoperative therapy for individual patients. At the moment, there is not enough data to change current clinical practice based on postoperative therapy stratified according to the presence of residual disease.

Limitations of our study include that the number of

the patients was not high enough to show all possible relationships between evaluated predictors and outcomes, the tissue from ICR was examined retrospectively and there are also data missing due partly to the retrospective design of the study.

On the other hand, the strength of our study is that we describe a homogenous group of consecutive patients, postoperatively treated in a standardized manner based on the presence of residual disease. The patients underwent standardized prospective endoscopic evaluation. Moreover, this is the first study evaluating possible value of tissue CPT in pediatric patients after ICR.

CONCLUSIONS

Approximately half of the patients develop ER within 6 months after ICR. Tissue CPT does not appear to be a valuable predictor of ER, irrespective of postoperative treatment. However, there are other potentially useful predictors, such as high F-CPT and high CRP at 6 months after ICR, low albumin (after adjustment to biological therapy) at the time of surgery as well as 6 months after ICR and presence of peritonitis. In accordance with ESPGHAN guidelines, such patients should be closely monitored and regularly evaluated endoscopically in order to tailor therapy in those who develop early ER.

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