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Limited clinical significance of tissue calprotectin levels in bowel mucosa for the prediction of complicated course of the disease in children with ulcerative colitis

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ABSTRACT

Background: Fecal calprotectin (F-CPT) represents one of the most widely used biomarkers for intestinal inflammation. However, the levels may be false negative or false positive in some situations.

Aims: To evaluate the usefulness of immunohistochemical (IHC) detection of tissue calprotectin (T-CPT) in bowel mucosa in children with ulcerative colitis (UC). We focused at correlation of T-CPT with levels of F-CPT and endoscopic and microscopic disease activity at the time of diagnosis and tested whether T-CPT could serve as predictor of complicated course of the disease.

Methods: Forty-nine children with newly diagnosed UC between 6/2010-1/2018 entered the study. Endoscopic activity was objectified using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS), clinical activity by Pediatric Ulcerative Colitis Activity Index (PUCAI) and microscopic activity by Geboes and Nancy score. The IHC staining for CPT antigen was performed on bioptic samples from 6 bowel segments and the number of CPT + cells were counted per 1HPF. During the minimal follow-up of 12 months we searched for presence of complications. As outcome for Cox regression model we used composite endpoints: A) Acute Severe Colitis, colectomy, anti-TNF treatment; B) systemic corticotherapy; C) systemic 5-aminosalicylic acid therapy.

Results: Neither levels of T-CPT nor values of UCEIS, Geboes or Nancy score predicted the given complications. We found F-CPT levels (HR 2.42 and 2.52) and PUCAI > 40 points (HR 2.98) as predictors of time to endpoints B and C. Good correlation was found between T-CPT levels and Geboes score (k = 0.65) and Nancy score (k = 0.62) and modest with F-CPT (k = 0.44), UCEIS (k = 0.38) and PUCAI (k = 0.42).

Conclusions: T-CPT correlated well with microscopic scores. F-CPT and PUCAI appear to be better predictors of unfavorable outcome in patients with UC.

1. Introduction

Ulcerative colitis (UC) is a chronic systemic inflammatory disorder with predominant involvement of the gastrointestinal tract [1]. Periodic monitoring evaluating the disease intensity and activity is essential for optimizing the treatment strategy. Even though the disease activity may be assessed by clinical scores, namely Pediatric Ulcerative Colitis Activity Index (PUCAI) [2], the endoscopy is still considered the gold standard for the assessment of the intestinal inflammation and mucosal healing, especially at the time of diagnosis [3,4]. However, this procedure is invasive, time-consuming and burdening, especially for pediatric population, where the general anesthesia is usually required. Therefore, reliable non-invasive markers for monitoring a disease activity are required. Fecal calprotectin (F-CPT) represents one of the most widely used biomarkers for intestinal inflammation with high sensitivity for both adult and pediatric population [5,6]. However, the F-CPT cannot be used to localize the focus of the disease activity and its levels may be false negative or false positive in some situations [7–9].

In our work, we focused at the immunohistochemical assessment of the tissue CPT (T-CPT) in the bowel mucosa of the children with UC. The aim of this work was: 1) to evaluate, whether the immunohistochemical assessment of the T-CPT may serve as an independent predictor of the complicated course of the disease, 2) to establish, whether the numbers of CPT positive cells in the bowel

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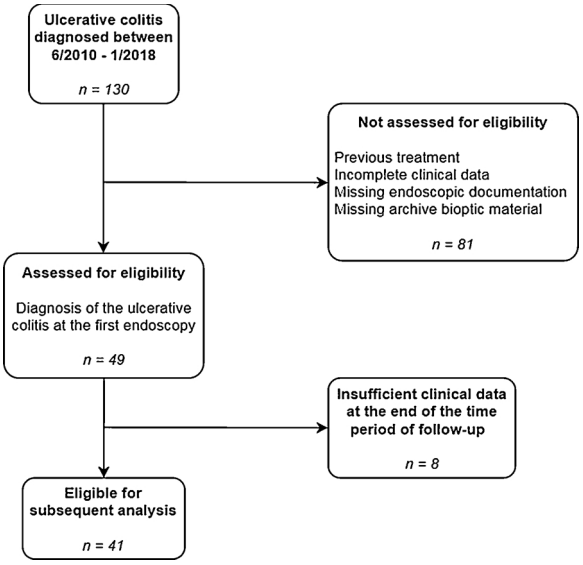

Fig. 1. Flowchart of included and excluded patients.

mucosa correlate with the levels of F-CPT at the time of diagnosis, and 3) to correlate the CPT + cell counts with the microscopic, endoscopic and clinical activity of the disease.

2. Materials and methods

All children (n = 130) with newly diagnosed UC in period between June 2010 and January 2018 were screened for eligibility to enter this retrospective cohort study. Patients were retrospectively re-evaluated by expert pediatric gastroenterologists and the diagnosis at the time of the first endoscopy was confirmed or modified according to revised Porto criteria for the diagnosis of pediatric inflammatory bowel diseases (IBD) [10]. Patients that had already been initiated on UC treatment at the time of biopsy as well as those with missing clinical data, endoscopic documentation or archive bioptic material for the revision of the diagnosis were excluded from the study. After the revision, 49 children with UC entered the study. Eight patients had to be subsequently excluded from the statistical analysis due to insufficient clinical data at the end of the time period of follow-up (Fig. 1). Clinical characteristics of the patients at the end of the minimal follow-up are given in the Table 1. The endoscopic findings at the time of the diagnosis were objectified using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) [11]. This score was chosen for its good reproducibility among the endoscopists and its good correlation with clinical, laboratory and histopathological markers of activity in UC [12,13]. The clinical severity of the disease at the time of the diagnosis was assessed by the PUCAI.

The archive histopathological slides from the bioptic samples obtained at the time of diagnosis stained with hematoxylin and eosin were reviewed by senior pediatric gastrointestinal pathologist blinded to clinical data. To evaluate microscopic findings, six bowel segments from each patient were analyzed (terminal ileum, cecum, ascending colon, transverse colon, descending colon and rectum). The samples

Table 1
Demographic and clinical characteristics of the patients with UC at the end of the minimal follow-up.

Number of the patients	41
Sex, male, n (%)	23 (56.10)
Age at diagnosis, years, median (IQR)	12 (7-15)
Acute Severe colitis, n (%)	2 (4.88)
Anti-TNF, n (%)	11 (26.83)
Colectomy, n (%)	0 (0)
Systemic CS, n (%)	12 (29.27)
Systemic 5ASA, n (%)	3 (7.32)

UC = ulcerative colitis; IQR = interquartile range; anti-TNF = anti-tumor necrosis factor therapy; CS = corticotherapy; 5ASA = 5-aminosalicylic acid treatment.

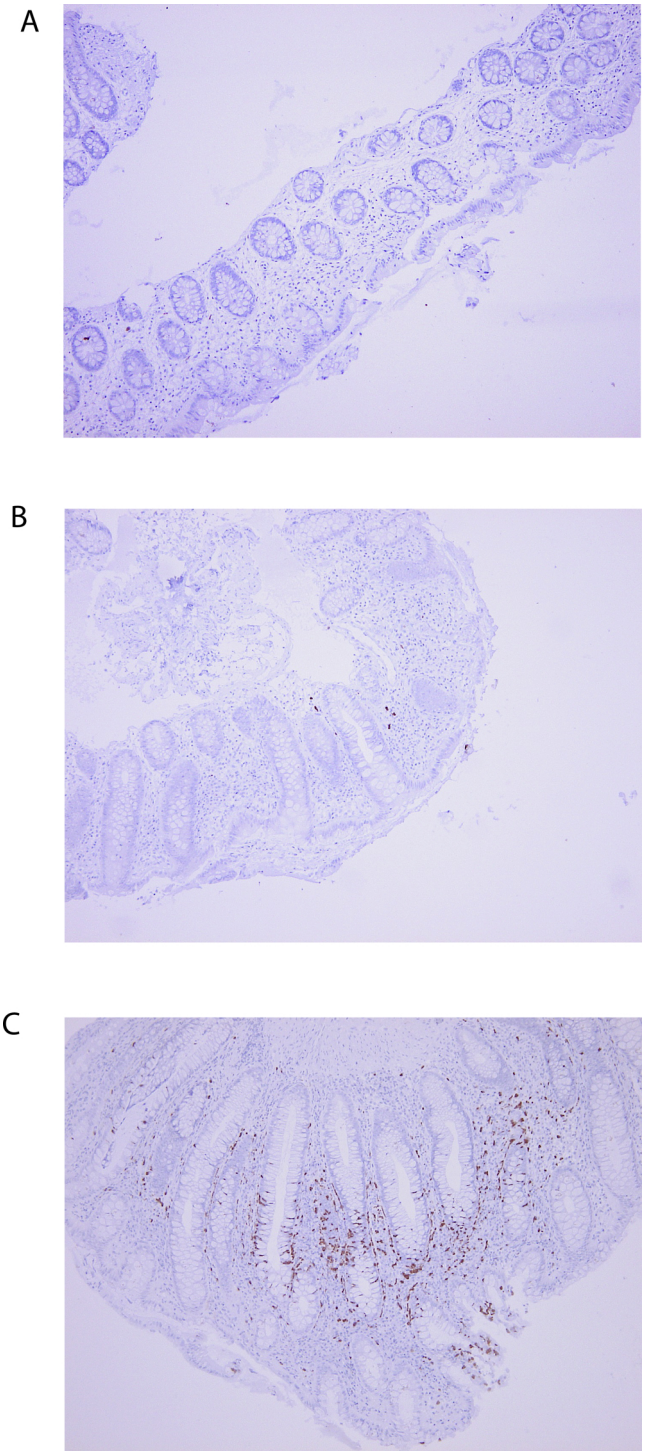

Fig. 2. Photomicrographs showing calprotectin (CPT) positive cells by immunohistochemistry (×400):
A. Bioptic sample of colonic mucosa devoid of any inflammation. No CPT+ cells are present.
B. Bioptic sample of colonic mucosa with mild chronic inflammation. Scarce CPT+ cells in the lamina propria.
C. Bioptic sample of colonic mucosa with marked chronic inflammation. Numerous CPT+ cells in the lamina propria and the epithelium.

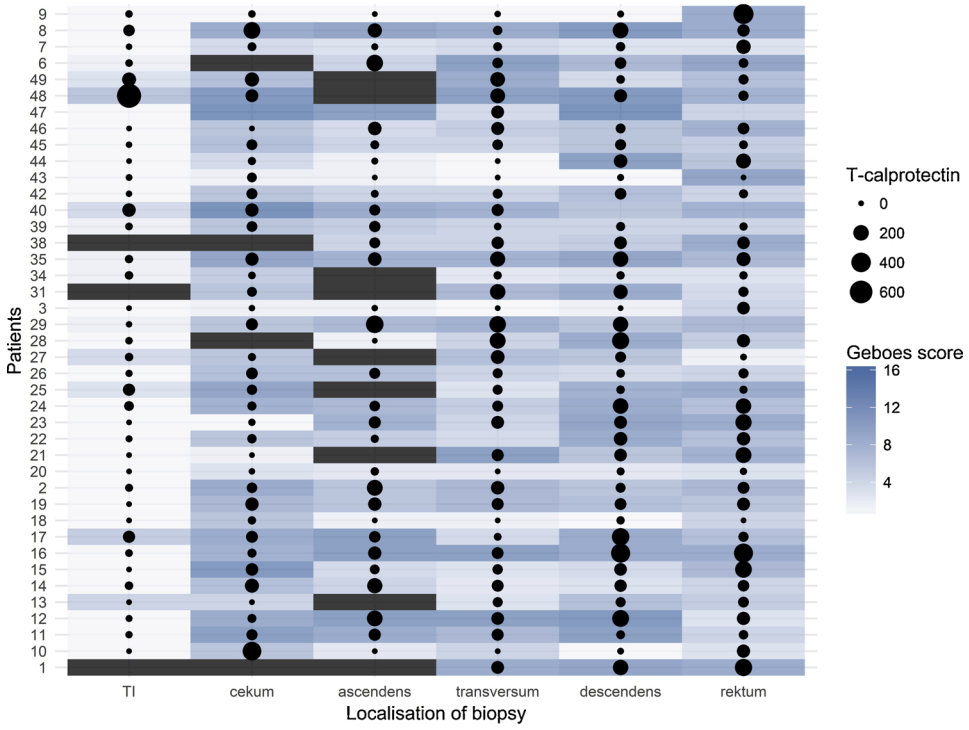

Fig. 3. Heat map reflecting the distribution of the Geboes histopathological score values and numbers of the CPT+ cells in all bowel segments.

were taken from the most affected areas of each segment. Intensity and activity of the inflammation was objectified using the Geboes and Nancy score, two widely used validated histopathological scoring systems for UC with good correlation with endoscopic findings and suitable interobserver reliability [12,14,15].

After that, the immunohistochemical staining of CPT antigen was performed on all archive biopsy samples. One micrometer thin tissue sections were deparaffinized and the anti-CPT primary 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 magnification) in the most affected region of each microscopic slide. The positive cells were counted separately for lamina propria and the epithelium (Figs. 2, 3 and 4).

During the minimum 12 months of prospective follow-up, we searched for the presence of following composite endpoints, presented as 3 separate substudies based on their severity: A) development of the Acute Severe Colitis (ASC) defined as the PUCAI > 65, necessity of colectomy or initiation of the Infliximab (IFX) treatment; B) first initiation of systemic corticosteroid (CS) therapy; C) first initiation of systemic 5-aminosalicylic acid (5ASA) treatment. The time to endpoint was chosen as outcome. Patients manifesting with ASC at the time of the diagnosis were not included in the study, since the ileocolonoscopy is not recommended in the setting of ASC [16,17] and these patients therefore did not fulfill the inclusion criteria.

3. Ethical considerations

Legal representatives of the patients signed an informed consent form for inclusion into the study. The study was approved by the Ethics Committee of Motol University Hospital on 28.03.2018.

4. Statistical analysis

Statistical software R-project (R Core Team, version 3.4.4) was used for data analysis. For all scoring systems included in the study, the mean values, medians and maximal values from all bowel segments were calculated and used in the subsequent analysis. The CPT + cell counts were assessed separately for lamina propria and epithelium and in total sum for the whole mucosa as well. To test, whether the levels of T-CPT and F-CPT and values of UCEIS, PUCAI and both histopathological scores at the time of diagnosis are independent predictors of the complicated course of the disease, a univariate Cox proportional hazards regression analysis (using the software package "survival") was used. Since the PUCAI > 40 (moderate disease activity) appeared to be one of the predictors (see Results section), a receiver operating curves (ROC) were constructed (using the software package "pROC") to find the cut-off values for both T-CPT and F-CPT, that would be able to select patients on this level of disease activity. Individual ROC curves were compared using the DeLong's test. To verify the correlation between F-CPT and T-CPT levels and values of the histopathological, endoscopic and clinical scores, the Pearson's product-moment correlation was performed. Probability (p) values of < 0.05 were considered significant. A 95% confidence interval was used.

5. Results

5.1. T-CPT as a predictor of the complicated course of the disease

Neither levels of the T-CPT, nor the values of UCEIS, Geboes or Nancy score predicted any complication during minimum 12 months of follow-up since diagnosis. However, the levels of the F-CPT in logarithmic scale and moderate disease activity (defined as PUCAI > 40) were independently associated with the B and C endpoints (initiation of the systemic CS and/or 5ASA therapy) (Table 2). Based on the analysis of the area under curve, no suitable cut-offs for T-CPT or F-CPT levels that could be linked to moderate disease activity at the time of diagnosis was found (AUC = 0.691 for F-CPT and 0.678 for T-CPT). DeLong's test showed no significant difference between those curves.

5.2. Association of T-CPT and F-CPT with microscopic, endoscopic and clinical activity scores

A good level of correlation was found between the median values of

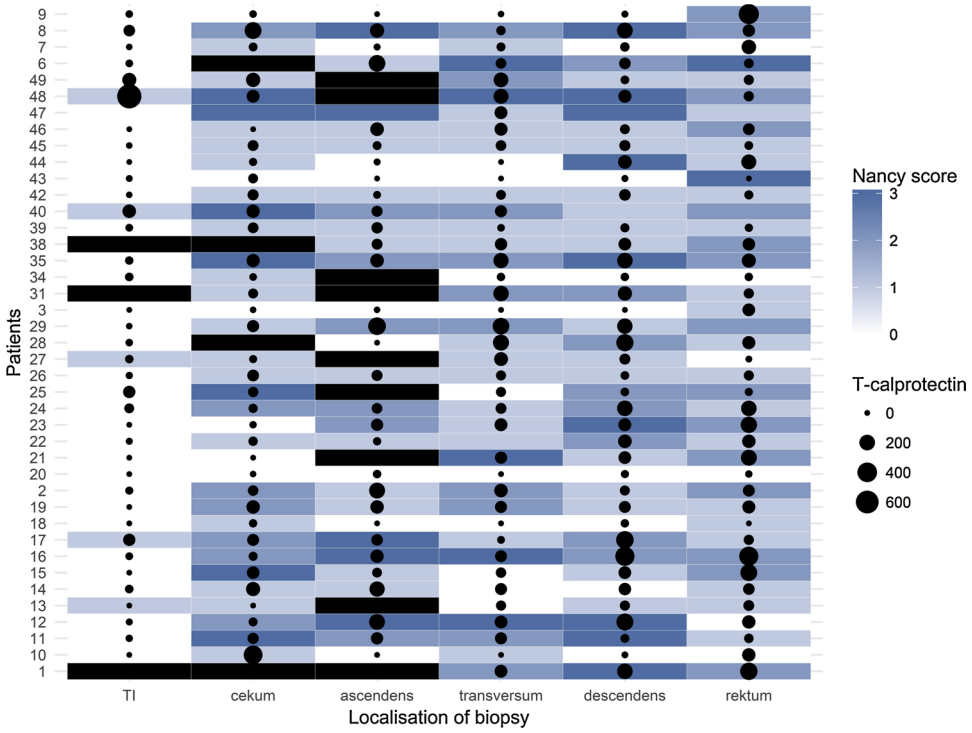

Fig. 4. Heat map reflecting the distribution of the Nancy histopathological score values and numbers of the CPT+ cells in all bowel segments.

Table 2
Univariate Cox proportional hazards regression analysis of PUCAI > 40 and T-CPT as the predictors of complicated course of the disease.

		Risk Ratio (CI)	P
PUCAI > 40	Endpoint A	2.237 (0.539-9.291)	0.268
	Endpoint B	**2.98 (1.011-8.787)**	**0.048**
	Endpoint C	**2.98 (1.011-8.787)**	**0.048**
		Risk Ratio (CI)	P
log(T-CPT)	Endpoint A	2.447 (0.873-6.859)	0.089
	Endpoint B	**2.422 (1.042-5.631)**	**0.04**
	Endpoint C	**2.517 (1.115-5.681)**	**0.026**

T-CPT = tissue calprotectin; CI = confidence interval; PUCAI = Pediatric Ulcerative Colitis Activity Index.
Bold value highlights a statistically significant result.

T-CPT and Geboes score (k = 0.65, p < 0.001) and median values of T-CPT and Nancy score (k = 0.62, p < 0.001). There was a weak correlation between T-CPT and UCEIS (k = 0.38, p = 0.02), PUCAI (k = 0.42, p = 0.01) and F-CPT (k = 0.44, p = 0.01).

We found a weak correlation between F-CPT and Geboes score (k = 0.39, p = 0.025), Nancy score (k = 0.38, p = 0.03) and UCEIS (k = 0.36, p = 0.039). There was no significant association between F-CPT and PUCAI (k = 0.36, p = 0.06).

6. Discussion

To the best of our knowledge, this is the first study exploring the contribution of the immunohistochemical assessment of T-CPT in children with IBD. CPT is a member of the S100 family and contributes to approximately 60% of the protein content in the cytosol of neutrophils [6]. Any active inflammatory process in the bowel mucosa results in leakage of CPT into the lumen and subsequently in the stool, where it can be detected and may serve as a non-invasive marker of the presence of active inflammation in the gut [18]. It appears to be a reliable marker for distinguishing inflammatory bowel disease from irritable bowel syndrome, which may share some common clinical symptoms as abdominal pain, bloating or diarrhea [6]. However, F-CPT yields some significant limitations. It represents a general marker of the active inflammation and is not disease specific, has no informative value in regards to the exact localization of the inflammation in the gut and may be false positive or false negative in some instances. Up to one quarter of healthy adult individuals show abnormal levels of the F-CPT according to some studies (probably due to presence of sporadic large bowel adenomas) [8]. On the other hand, levels of F-CPT can be decreased or even normalized iatrogenicaly by a bowel preparation procedure before colonoscopy [9]. Finally, there are published cases of patients with IBS and high levels of F-CPT, probably due to the inflammatory reaction to bowel dysbiosis [7]. There seems to be an intrapersonal day-to-day variability in F-CPT levels for both healthy persons and patients with IBD as well [19,20]. Moreover, the physiological levels of F-CPT are higher in children with the maximum in the neonatal age and then declines till the adulthood [21]. A metanalysis from Degraeuwe et al. [22] reported a 17% rate of false negative results in children with IBD when using the standard cut-off 50ug/g.

Therefore, we aimed at the immunohistochemical assessment of the T-CPT in the bowel mucosa. We speculated that the direct visualization of the CPT + inflammatory cells could more accurately reflect the actual level of the mucosal disease activity. Of course, the microscopic activity of the inflammation can be adequately assessed on the basis of the presence of neutrophils in the standard hematoxylin and eosin staining. However, in pediatric IBD, there is a lack of data about the predictive value of microscopy for complications development and its correlation with endoscopic or clinical scores of the activity. A study of Ashton et al. [23] demonstrated more extensive disease on the microscopic level compared to the endoscopic appearance in pediatric IBD. However, the study did not clarify, whether this increase in disease extent actually does reflect the real clinical presentation of the disease and whether it provides any predictive value in terms of future clinical course. The T-CPT staining may be beneficial especially for patients with no microscopic signs of inflammation activity in routine haematoxylin and eosin stain. In this setting, the direct visualization of CPT + cells may assess the presence of subtle signs of the active

inflammation more precisely. Therefore, we investigated, whether the T-CPT could improve the exactness of the histopathological assessment of the inflammation activity and better predict the complicated course of the disease. To this day, there are almost no studies regarding this topic. Only two studies engaged in IBD included the T-CPT in their data analysis and both of them were performed on the cohorts of adult patients. Guirgis et al. [24] found a good correlation of the low T-CPT with clinical, endoscopic and microscopic remission in adult patients with UC. On the other hand, elevated T-CPT (median value > 5 cells/HPF) was associated with adverse clinical outcome. A work from Fukunaga et al. [3] focused at the correlation of the F-CPT with the serum CPT and fecal hemoglobin. The immunohistochemical assessment of the T-CPT was a secondary outcome only and the authors showed that the patients with IBD have higher counts of the CPT + cells in the bowel mucosa compared to healthy individuals. No study ever examined the T-CPT as a possible prognostic marker or correlated it with other means of the assessment of the disease activity in children with IBD. In our work, we demonstrated a good correlation between T-CPT and both Geboes and Nancy histopathological scores. The T-CPT thus probably truly reflects the actual microscopic activity of the inflammation. However, we failed to link the levels of the T-CPT and the values of the Geboes and Nancy histopathological scoring systems with the development of the complications.

Regarding the F-CPT and its correlation with the endoscopic findings, the vast majority of the information stem from adult cohorts and the data for pediatric population are sparse. In the study of Fagerberg et al. [E], F-CPT correlated significantly with the endoscopic findings in the cohort of 39 pediatric patients with IBD. Ricciuto A et al. [25] showed that levels of F-CPT were associated with UCEIS values in children with IBD and primary sclerosing cholangitis. Our work confirms a significant, but only a weak correlation between F-CPT and UCEIS. For the clinical scores of the disease activity there is a considerable deal of children in clinical remission (defined as PUCAI < 10) with elevated F-CPT [26]. Our findings are in keeping with these observations, since we failed to prove any correlation between F-CPT and PUCAI. In contrast to previous findings [27], our work showed only a weak correlation of F-CPT with microscopic activity of the disease, neither in Geboes nor Nancy score.

The fundamental question is the ability of the F-CPT to predict the clinical outcome. According to several studies [28,29], level of the F-CPT adequately reflects the actual disease activity with respect to sustained remission or relapse rate. However, there is a lack of data about the F-CPT at the time of diagnosis and its predictive value for the development of subsequent complications. In our study, the levels of the F-CPT at the first endoscopy predicted the necessity of the initiation of the systemic CS and 5ASA therapy. Besides, the moderate clinical disease activity (PUCAI > 40) was the predictor of those complications as well. These findings are in contrast to previous studies, where PUCAI values at the time of diagnosis were not associated with the necessity of immunomodulatory therapy or 5ASA treatment during 1 year of follow-up [30,31].

There are several possible limitations of the study. Only three patients with previous local 5ASA therapy were subsequently initiated with the systemic 5ASA treatment. Therefore, the association of the outcome C with the moderate disease activity and F-CPT levels, although significant, needs to be confirmed by subsequent studies. Another limitation may be the retrospective design of the study. The immunohistochemical staining of anti-CPT required new sections from archive paraffin blocks, which may lead to partial cutting of the material. Moreover, the retrospective assessment of the PUCAI from the electronic documentation could have a limited value. Finally, a large amount of the patients was not enrolled in the study due to strict inclusion criteria. However, since the included sub-cohort shared similar baseline characteristics with the excluded patients, the non-intentional selection bias is unlikely.

7. Conclusions

This is the first study aiming at the immunohistochemical assessment of the T-CPT in pediatric population generally. Despite its good correlation with the histopathological indexes of the disease activity, T-CPT failed to predict the complicated course of the disease. Therefore, the usefulness of the histopathological assessment of the inflammation activity in the prediction of the complications in children with UC, whether in conventional staining or in specialized methods, remains uncertain. On the other hand, F-CPT levels at the time of diagnosis together with moderate disease activity based on the PCUAI score appeared to be independent predictors of the initiation of systemic CS and 5ASA therapy.

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Declaration of Competing Interest

The authors have no conflict of interest.

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