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PII: S0022-3468(18)30256-2
DOI: doi:10.1016/j.jpedsurg.2018.04.016
Reference: YJPSU 58657

To appear in:

Received date: 13 January 2018
Revised date: 27 March 2018
Accepted date: 8 April 2018

Please cite this article as: Annika Mutanen, Meredith Barret, Yongjia Feng, Jouko Lohi, Raja Rabah, Daniel H Teitelbaum, Mikko P Pakarinen , Short Bowel Mucosal Morphology, Proliferation and Inflammation at First and Repeat STEP Procedures. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Yjpsu(2018), doi:10.1016/j.jpedsurg.2018.04.016

SHORT BOWEL MUCOSAL MORPHOLOGY, PROLIFERATION AND INFLAMMATION AT FIRST AND REPEAT STEP PROCEDURES

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Financial support: This study was supported by the Sigrid Jusélius Foundation, the Finnish Pediatric Research Foundation, and the Helsinki University Central Hospital Grant.

Declarations of interest: none

Abbreviations: SBS;short bowel syndrome, IF; intestinal failure, AIR; autologous intestinal recontruction, STEP; serial transverse enteroplasty, PN; parenteral nutrition

Short running head: MUCOSAL CHARACTERISTICS AFTER STEP PROCEDURES

Abstract

Background. Although serial transverse enteroplasty (STEP) improves function of dilated short bowel, a significant proportion of patients require repeat surgery. To address underlying reasons for unsuccessful STEP, we compared small intestinal mucosal characteristics between initial and repeat STEP procedures in children with short bowel syndrome (SBS).

Methods. Fifteen SBS children, who underwent 13 first and 7 repeat STEP procedures with full thickness small bowel samples at median age 1.5 years (IQR 0.7-3.7) were included. The specimens were analyzed histologically for mucosal morphology, inflammation and muscular thickness. Mucosal proliferation and apoptosis was analyzed with MIB1 and Tunel immunohistochemistry.

Results. Median small bowel length increased 42% by initial STEP and 13% by repeat STEP (P=0.05), while enteral caloric intake increased from 6% to 36% (P=0.07) during 14 (12-42) months between the procedures. Abnormal mucosal inflammation was frequently observed both at initial (69%) and additional STEP (86%, P=0.52) surgery. Villus height, crypt depth, enterocyte proliferation and apoptosis as well as muscular thickness were comparable at first and repeat STEP (P>0.05 for all). Patients, who required repeat STEP tended to be younger (P=0.057) with less apoptotic crypt cells (P=0.031) at first STEP. Absence of ileocecal valve associated with increased intraepithelial leukocyte count and reduced crypt cell proliferation index (P<0.05 for both).

Conclusions. No adaptive mucosal hyperplasia or muscular alterations occurred between first and repeat STEP. Persistent inflammation and lacking mucosal growth may contribute to continuing bowel dysfunction in SBS children, who require repeat STEP procedure, especially after removal of the ileocecal valve.

Keywords: apoptosis, children, intestinal failure, parenteral nutrition, proliferation, small bowel

Level of evidence: Level IV, retrospective study

Introduction

Short bowel syndrome (SBS) is the most common cause of intestinal failure and persistent need for long-term parenteral nutrition (PN). SBS is frequently associated with serious complications including intestinal failure associated liver disease, septic episodes and loss of venous access sites [1]. A subset of SBS patients develop abnormal dilatation of the remaining small intestine, which predicts prolonged PN dependence and decreased survival [2,3]. Pathophysiology of adaptation-associated small bowel dilatation remains unclear, but underlying diagnosis of small intestinal atresia seems to be a major predisposing factor [2,3]. Recent evidence suggest that excessive dilatation predisposes to mucosal damage and bowel-derived blood stream infections most likely secondary to increased mucosal inflammation and permeability [4,5]. Autologous intestinal reconstruction (AIR) surgery including serial transverse enteroplasty (STEP) has been increasingly used to improve function of dilated small intestine by restoring normal bowel caliber with simultaneous increase in length [6-9]. Unfortunately, 40%-80% of the patients require repeat surgery mainly due to small intestinal redilatation after initial STEP, representing the major functional drawback of this operation [10-14]. The need for repeat STEP has been associated with absence of the ileocecal valve [13], suggesting that this anatomic region may regulate intestinal growth, motility and its loss promotes microbiota which allow recurrence of dilatation and adversely affects STEP outcomes. However, underlying reasons for unsuccessful STEP remain unclear, while no data on intestinal histology in these patients are available. To this end, we assessed histologic intestinal inflammation, mucosal morphology, enterocyte proliferation and apoptosis as well as muscular thickness in unique set of full thickness small bowel samples obtained from SBS children during initial and additional STEP procedures.

Methods

Ethics

The University of Michigan institutional review board (IRB nro HUM00063515) approved this study. An informed written consent for tissue stu dy was received from all patients and/or caregivers before any procedures.

Patients

A total of 26 STEP procedures were performed in Michigan Mott Children's Hospital between 2003 and 2014. Indication for STEP procedure was abnormal small bowel dilatation combined with unprogressive weaning from PN. Patients with full thickness small intestinal samples obtained during STEP surgery for pathological analysis were included in the study. Bowel specimens were analyzed in Helsinki University Children's Hospital.

Patient background data, including gestational age and weight, etiology of SBS, length and anatomy of the remaining bowel, age at STEP procedures, pre- and post-STEP bowel length, percentage of total daily calories delivered in PN, and weaning off PN, were obtained from patient charts [10]. Predicted small bowel length was estimated using an age based normogram [15].

Small bowel histology and immunohistochemistry

The samples were fixed in formalin, embedded in paraffin, sliced and stained with hematoxylin and eosin. For histologic analyses, the slices were first reviewed manually with Leica DM RXA microscope (Leica Microsystems GmbH, Wetzlar, Germany) and then photographed using ImageJ Image Analysis Software (SciJava Common open source software; Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda,

Maryland, USA; http://imagej.nih.gov/ij/; 1997–2014) by the primary researcher (AM) blinded to clinical data. Only well-oriented villi and crypts were chosen for analysis of mucosal morphology. Median of 10 (range 3-10) and 8 (range 3-10) representative villi and crypts were measured in each sample. Inflammation in the lamina propria was graded from 1 to 4 (grade 1 = few scattered inflammatory cells between crypts; 2 = moderate number of inflammatory cells distributed diffusely through the lamina propria, 3 = large number of inflammatory cells often in combination with short or broad villi; 4 = intense infiltrate with short, broad or absent villi) [16,17]. Abnormal inflammation was defined as ≥grade 2. Intraepithelial inflammation was assessed by counting intraepitelial leukocytes (IEL), including lymphocytes, eosinophiles and neutrophiles per 100 enterocytes in three representative villi [18]. Muscularis thickness was measured at three different representative locations of the sample.

Immunohistochemistry included MIB1 for Ki-67 labeling index to analyze enterocyte proliferation and Tunel for analyzing enterocyte apoptosis. Immunostaining for MIB1 was conducted with routine hospital laboratory protocol. Tunel staining was performed with Apoptosis Tunel assay Kit (APO-BrdU IHC™ Immunohistochemistry Kit, APO0002, BioRad Laboratories, California, USA) according to manufacturers instructions (dilution 1:15). Overall MIB1 positivity was scored from 1 to 3 based on the location of positively stained enterocytes in crypt-villus axis (grade 1 = MIB1 positive cells in crypts; 2 = MIB1 positive enterocytes in crypts and in the lower half of villi; 3 = MIB1 positive enterocytes in crypts and above the lower half of villi). In addition, MIB1 positive enterocytes were calculated in three representative villi and crypts and percentage of positive enterocytes was calculated. Overall Tunel staining was scored from 0 to 3 (grade 0 = only solitary positive nuclei in villi; 1 = clusters of positive nuclei in villi; 2 = villi diffusely covered with positive nuclei; 3 = positive nuclei in both villi and crypts) [19]. In addition, Tunel positive

nuclei were calculated in three representative villi and crypt for calculation of percentage of positive cells.

Statistics

Data are expressed as median (IQR, interquartile range) or frequencies. Mann Whitney U test and Fisher exact test were used for between group comparisons. Correlations were analyzed with Spearman rank. P-value under 0.05 was considered statistically significant.

Results

Patient characteristics

Altogether 15 SBS patients were included. Their baseline characteristics are shown in Table 1. The most common etiology of SBS was intestinal atresia with or without gastroschisis. Median small bowel length was 22 cm and only three (20%) had ileocecal valve remaining.

The 15 patients underwent a total of 20 STEP procedures at median age 1.5 (0.7-3.7) years (Table 2). Median age difference between initial and repeat STEP procedure was 14 months (12-42). Intestinal specimens were available from 13 patients at initial STEP and seven patients at repeat STEP (six second and 1 third). Five patients had samples obtained at both procedures. Average postoperative follow up from initial STEP was 36 months. Intestinal specimens obtained during initial small bowel resection at median age 0.5 (0.2-0.5) years were available in four patients and were used as controls.

Small intestinal length increased 42% by first STEP and 13% by additional STEP (Table 2). At first STEP median percentage of parenteral energy provision was 94%, which decreased to 64% by the time of additional STEP, reflecting increased enteral tolerance. Only one patient weaned off PN after first STEP while none of the patients weaned off after repeat surgery.

Mucosal and muscular morphology

There were no significant differences in mucosal morphology or muscular thickness between samples taken at first and additional STEP procedures (Table 3). Patient age correlated positively with villus height (r=0.600, P=0.005) and mucosal thickness (r=0.567, P=0.009). No other correlations between the study variables were observed (P>0.05 for all).

Mucosal proliferation and apoptosis

Indexes of enterocyte proliferation and apoptosis both in crypts and villi were comparable in samples obtained at initial and additional STEP procedures (Table 3, Figure 1). Importantly, patients without an ileocecal valve had lower crypt cell MIB1 proliferation index [74% (59-83)] compared to patients with preserved ileocecal valve [90% (88-90), P=0.025] (Figure 2).

Mucosal inflammation

Abnormal inflammation was frequently and similarly observed both at initial and additional STEP procedure, without statistically significant difference between the groups (Table 3, Figure 1). The intraepithelial inflammatory cells consisted mainly of lymphocytes with occasional eosinophils in lamina propria (<10% of inflammatory cells). Villus atrophy was present in two initial STEP samples but in none of additional STEP

samples. Notably, patients without an ileocecal valve showed over two-fold higher intraepithelial leukocyte count [14 IEL/100 enterocytes (11-17)] compared to patients with preserved ileocecal valve [6.4 (3.1-6.4), P=0.023)] (Figure 3).

Predictors of repeat STEP

To address which factors at the time of first STEP predicted repeat surgery, patients who underwent only one STEP were compared to those who subsequently underwent repeat STEP (Table 4). These comparisons revealed that the patients, who required repeat surgery tended to be younger and had reduced crypt cell apoptosis index at the time of first STEP, while no differences in parenteral energy requirement or small bowel length were observed. At the time of first STEP, absolute and age-adjusted small bowel length correlated with crypt and villus enterocyte apoptosis index (r=0.791-0.658, P<0.05 for all correlations).

Comparison between initial bowel resection and STEP

Because no differences were observed between initial and additional STEP specimens, they were pooled for comparisons to samples obtained at initial bowel resection (Table 5). These comparisons showed a significant increase in villus width and a significant decrease in frequency of villus atrophy after initial resection. In addition, villus height increased insignificantly by 58% after resection, while no changes were observed in indexes of mucosal proliferation or apoptosis.

Discussion

In this study we have for the first time characterized morphology, proliferation and inflammation of the small intestinal mucosa in SBS children undergoing STEP

procedures. Our major findings suggest, that despite increased small intestinal length and improving enteral energy tolerance after initial STEP, no evidence of adaptive mucosal hyperplasia or muscular alterations were observed in patients who underwent unsuccessful STEP requiring repeat surgery and continuation of PN. In addition, abnormal mucosal inflammation persisted after initial STEP, which may contribute to bowel dysfunction among those children, who require repeated tapering surgery. Finally, absence of the ileocecal valve was associated with increased mucosal inflammation and reduced crypt cell proliferation.

In SBS with prolonged PN dependency STEP procedure is the most commonly performed autologous intestinal reconstruction surgery for bowel lengthening and tapering aimed to improve intestinal function and subsequent weaning off PN [6,9]. Following first STEP between 30% and 88% of patients are weaned off PN [9]. Outcomes after repeat STEP procedures are often worse, reported weaning off rates ranging between 0% and 55% [10,13,14,20]. Though most patients in this study were not able to wean off PN after initial STEP procedure providing 42% increase in small bowel length, enteral energy tolerance had increased from 6% to 36% by the time of repeat STEP. Despite the moderate increase in enteral caloric tolerance, we did not find any differences in villus height, crypt depth, mucosal proliferation indexes or muscular thickness between the samples taken at first and additional STEP procedures. These observations suggest that no significant adaptive mucosal hyperplasia occurs after unsuccessful STEP in patients who require repeat procedure, and that the improved enteral tolerance may be mediated by other mechanisms such as beneficial changes in motility or age related bowel growth. Our findings differ from a previous study reporting increased plasma citrulline, a surrogate for mucosal mass, by 12 months after STEP in children [21] and an experimental study reporting increased villus height after STEP in

rats [22]. Importantly, in the former clinical study seven of eight long-term survivors weaned off PN and none required repeat STEP for redilatation [21]. Thus, whether adaptive mucosal hyperplasia occurs in patients who undergo successful STEP not necessitating repeat surgeries remains unclear. Indeed, abnormal small bowel dilatation has been shown to associate with decreased plasma citrulline levels, suggesting that excessive (re)dilatation may impair mucosal growth following STEP surgery [4].

In the present study evidence of abnormal histologic mucosal inflammation persisted in patients, who required repeat STEP. Interestingly, absence of the ileocecal valve was associated with increased intraepithelial leukocyte infiltration and decreased crypt cell proliferation index. These findings are important, as intestinal mucosal inflammation and the lack of the ileocecal valve appears to have a central role in the pathophysiology of adaptation-associated small bowel dilatation and intestinal failure associated liver disease [4,23,24]. Recent clinical studies suggest that absent ileocecal valve predicts the need for repeat STEP [13], while excessive adaptive small bowel dilatation is associated with intestinal inflammation, as measured by increased fecal calprotectin, and with bowel-derived blood stream infections, which seem to be instrumental in promoting hepatic inflammation and cholestasis [4,23,25]. Excessive small bowel dilatation hampers propulsive intestinal motility, which may in turn predispose to unfavorable changes in intestinal microbiota, inflammation and increased epithelial permeability [4,5,26-28]. Furthermore, tapering of excessive small intestinal dilatation significantly decreased fecal calprotectin concentration to the normal level among 17 patients of whom 71% weaned by 3.1 years after surgery, suggesting that successful STEP helps to ameliorate intestinal inflammation [29]. Our present observations further indicate that in patients with unsuccessful first STEP and the need for repeated surgery histologic intestinal inflammation persists possibly contributing to unfavorable functional outcome

in this patient subgroup [10,14]. Accordingly, in the present study none of the patients who underwent repeat STEP achieved enteral autonomy.

Enhanced mucosal inflammation and decreased mucosal crypt cell proliferation as observed here after removal of the ileocecal valve, may reflect essential pathophysiological mechanisms explaining why absent ileocecal valve predisposes to repeat STEP [13]. Surgical removal of the ileocecal region may promote uncontrolled proliferation of unfavorable colonic microbiota in the remaining small intestine after loss of compartmentalization between large and small bowel and adversely affect motility and mucosal growth by reducing secretion of several physiological enteroendocrine peptides such as glucagon like peptide 2 [30]. In addition to absent ileocecal valve, young age and reduced crypt cell apoptosis at first STEP seemed to associate with the need for repeat surgery, indicating that early STEP surgery may predispose to unfavorable outcome. Overall, these findings suggest that STEP should be postponed beyond the most active period of physiological adaptation.

We acknowledge many limitations of this study, including lack of healthy controls, control group of IF patients able to wean off PN after STEP procedure, actual measurements of small bowel diameter and simultaneous assessment of intestinal microbiota. Due to retrospective study design we were also unable to relate specific individual operative indications with outcomes. Relatively small number of patients precluded meaningful subgroup analyses between different underlying etiologies of SBS. However, we believe that this study provides unique data helping to understand pathophysiology of failed tapering surgery in SBS. Future studies linking objectively measured intestinal dilatation with microbiota alterations and inflammation would be important.

In conclusion, no evidence of adaptive mucosal hyperplasia or muscular alterations was observed after unsuccessful STEP in patients who required repeat surgery. Persistent inflammation and lack of mucosal growth may contribute to continuing bowel dysfunction in SBS children, who require repeat STEP surgery, especially after removal of the ileocecal valve.

Figure legends

Figure 1. Common histological findings related to multiple STEP procedures. A) inflammation grade 4 in lamina propria at the time of the second STEP procedure, 10x magnification; B) villus atrophy at the time of the first STEP procedure, 10x magnification; C) MIB1 staining showing enterocyte proliferation in villi and crypts at the time of first STEP procedure, positive nuclei stained with brown, overall MIB1 grade 3, crypt 100%, villi 24%, 10x magnification; D) Tunel staining showing apoptosis in enterocytes at the time of second STEP procedure, positive nuclei stained with brown, overall Tunel grade 3, villi 59%, crypt 61%, 10x magnification.

Figure 2. Decreased MIB1 proliferation index (% of positive nuclei) in crypts in patients without ileocecal valve (ICV) compared to patients with preserved ICV. Mann Whitney U test between the two groups, P=0.025.

Figure 3. Increased intraepithelial inflammation in patients without ileocecal valve (ICV) compared to patients with preserved ICV. Mann Whitney U test between the two groups, P=0.023.

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Table 1. Patient characteristics

Number of patients	15
Birth weight (kg)	2.4 (2.0-3.0)
Gestational age (wk)	34 (32-37)
Parenteral nutrition dependency (n)	15
Diagnosis (n)	
Gastroschisis with small bowel atresia	4
Small bowel atresia	4
Midgut volvulus	3
Gastroschisis	2
Necrotizing enterocolitis	2
Bowel anatomy after initial resection	
Small bowel (cm)	22 (18-51)
Small bowel (% of expected)	12 (7-19)
< 10% of expected small bowel length (n)	5
ICV present (n)	3
Colon present (n)	15

Data are median (interquartile range) or frequencies.

Table 2. Small bowel length and nutrition at intial and repeat STEP

	Intial STEP	Repeat STEP	P-value*
Number of patients	13	7	
Age (y)	1.0 (0.4-3.7)	1.9 (1.0-3.8)	0.219
Small bowel length			
Pre-STEP (cm)	45 (30-85)	56 (45-80)	0.634
Post-STEP (cm)	58 (42-100)	72 (56-102)	0.463
Change (%)	42 (13-77)	13 (8-36)	0.051
Change (cm)	17 (10-30)	12 (3-20)	0.261
Ileocecal valve present (n)	3	0	0.263
Parenteral energy requirement (%)	94 (67-100)	64 (62-81)	0.071
Weaned off PN after STEP (n)	1	0	0.588

Data are median (interquartile range) of frequencies. STEP; serial transverse enteroplasty, PN; parenteral nutrition. Mann Whitney U test or Fisher's exact test, comparison between the two groups.

Table 3. Comparison of small bowel histology and mucosal proliferation indexes between specimen obtained at initial and repeat STEP

	Initial STEP	Repeat STEP	P-value
Number of patients	13	7	
Mucosal morphology			
Villus height (mm)	0.74 (0.46-1.02)	0.72 (0.64-0.75)	0.721
Villus width (mm)	0.27 (0.24-0.30)	0.31 (0.22-0.32)	0.663
Villus atrophy (n)	2	0	0.521
Crypt depth (mm)	0.44 (0.30-0.51)	0.42 (0.38-0.59)	0.552
Mucosal thickness (mm)	1.4 (0.87-1.9)	1.8 (1.2-1.8)	0.405
Muscularis thickness (mm)	2.7 (1.8-3.9)	2.9 (2.6-4.1)	0.483
Inflammation			
Abnormal inflammation (n)	9	6	0.522
Lamina propria, (1-4)	2.0 (1.0-3.0)	3.0 (2.0-3.0)	0.352
IEL (/100 enterocytes)	14 (6.4-17)	12 (7.0-15)	0.618
Proliferation			
Grade (1-3)	1 (1-2)	1 (1)	0.110
Villus (% of positive nuclei)	4.6 (0-8.7)	5.9 (2.0-6.6)	0.656
Crypt (% of positive nuclei)	79 (59-89)	71 (58-78)	0.219
Apoptosis			
Grade (0-3)	1 (0-3)	1 (0-3)	1.000
Villus (% of positive nuclei)	21 (1.0-38)	16 (0-56)	0.791
Crypt (% of positive nuclei)	10 (0-41)	18 (0-46)	0.524

Data are median (interquartile range) or frequencies. STEP; serial transverse enteroplasty, IEL; intraepithelial leukocytes.

Table 4. Comparison between patients with one and multiple STEP procedures at the time of first STEP procedure.

At first STEP	One STEP procedure	Repeat STEP procedures	P-value*
Number of patients	7	6	
Age (y)	1.5 (0.9-7.9)	0.5 (0.1-1.7)	0.057
PN energy requirement (%)	89 (55-100)	100 (82-100)	0.386
Small bowel length			
Pre-STEP (cm)	55 (34-104)	42 (25-83)	0.379
Post-STEP (cm)	75 (45-103)	47 (34-89)	0.240
Change (%)	60 (28-85)	24 (12-76)	0.336
Change (cm)	22 (14-50)	12 (5.5-17)	0.086
Mucosal morphology			
Villus height (mm)	0.73 (0.60-1.11)	0.74 (0.30-0.94)	0.464
Villus Width (mm)	0.28 (0.24-0.33)	0.25 (0.21-0.28)	0.143
Villus atrophy (n)	1	1	0.641
Crypt depth (mm)	0.44 (0.29-0.52)	0.48 (0.26-0.54)	0.884
Mucosal thickness (mm)	1.54 (0.82-1.98)	1.35 (0.87-1.76)	0.661
Muscularis thickness (m)	3.21 (1.79-1.98)	2.5 (1.51-4.18)	0.770
Inflammation			
Abnormal inflammation (n)	6	3	0.745
Lamina propria (1-4)	2.0 (1.3-2.8)	2.5 (1.25-3.0)	0.721
IEL (/100 enterocytes)	14 (6.4-14)	14 (5.4-22)	0.850
Proliferation			
Grade (1-3)	1.0 (1.0-1.8)	1.0 (10.-2.0)	0.584
Villus (% of positive nuclei)	4.1 (0.0-8.8)	4.6 (0.0-17)	1.000
Crypt (% of positive nuclei)	82 (61-89)	75 (45-95)	1.000
Apoptosis			
Grade (0-3)	2.5 (0.0-3.0)	0.0 (0.0-1.5)	0.159
Villus (% of positive nuclei)	29 (4.3-45)	2.4 (0.0-23)	0.122
Crypt (% of positive nuclei)	26 (2.9-51)	0.0 (0.0-5.2)	**0.031**

Data are median (interquartile range) or frequencies. STEP; serial transverse enteroplasty, PN; parenteral nutrition, IEL; intraepithelial leukocytes. Bowel specimens obtained at first STEP were available for 7 patients, who underwent one and for 5 patients who underwent repeated STEP procedure. *Mann Whitney U test or Fisher's exact test, comparison between the two groups.

Table 5. Comparison of small bowel histology and mucosal proliferation

	Initial resection	STEP	P-value
Number samples	4	20	
Age (y)	0.5 (0.2-0.5)	1.5 (0.7-3.7)	0.465
Small bowel length (cm)	48 (16-64)	56 (38-80)	0.313
Mucosal morphology			
Villus height (mm)	0.42 (0.39-1.0)	0.72 (0.60-0.95)	0.353
Villus width (mm)	0.18 (0.16-0.21)	0.27 (0.23-0.32)	**0.005**
Villus atrophy (n)	3	2	**0.006**
Crypt depth (mm)	0.47 (0.23-0.77)	0.44 (0.33-0.52)	0.877
Mucosal thickness (mm)	1.1 (0.42-2.7)	1.5 (1.0-1.9)	0.588
Muscularis thickness (mm)	2.4 (0.93-5.0)	2.9 (2.1-3.8)	0.746
Inflammation			
Abnormal inflammation (n)	3	15	0.654
Lamina propria (1-4)	2.5 (1.3-3.0)	2.0 (2.0-3.0)	0.966
IEL (/100 enterocytes)	6.0 (3.3-9.1)	13 (6.9-16)	0.061
Proliferation			
Grade (1-3)	1.0 (1.0-2.5)	1.0 (1.0)	0.661
Villus (% of positive nuclei)	0 (0-29)	5.3 (0-8.3)	0.445
Crypt (% of positive nuclei)	64 (46-84)	74 (59-88)	0.353
Apoptosis			
Grade (0-3)	1.0 (0-2.8)	1.0 (0.0-3.0)	0.896
Villus (% of positive nuclei)	13 (0-46)	21 (0-43)	0.742
Crypt (% of positive nuclei)	13 (0-30)	12 (0-41)	0.735

indexes between specimen obtained at initial bowel resection and STEP

Data are median (interquartile range) or frequencies. STEP; serial transverse enteroplasty, IEL; intraepithelial leukocytes.

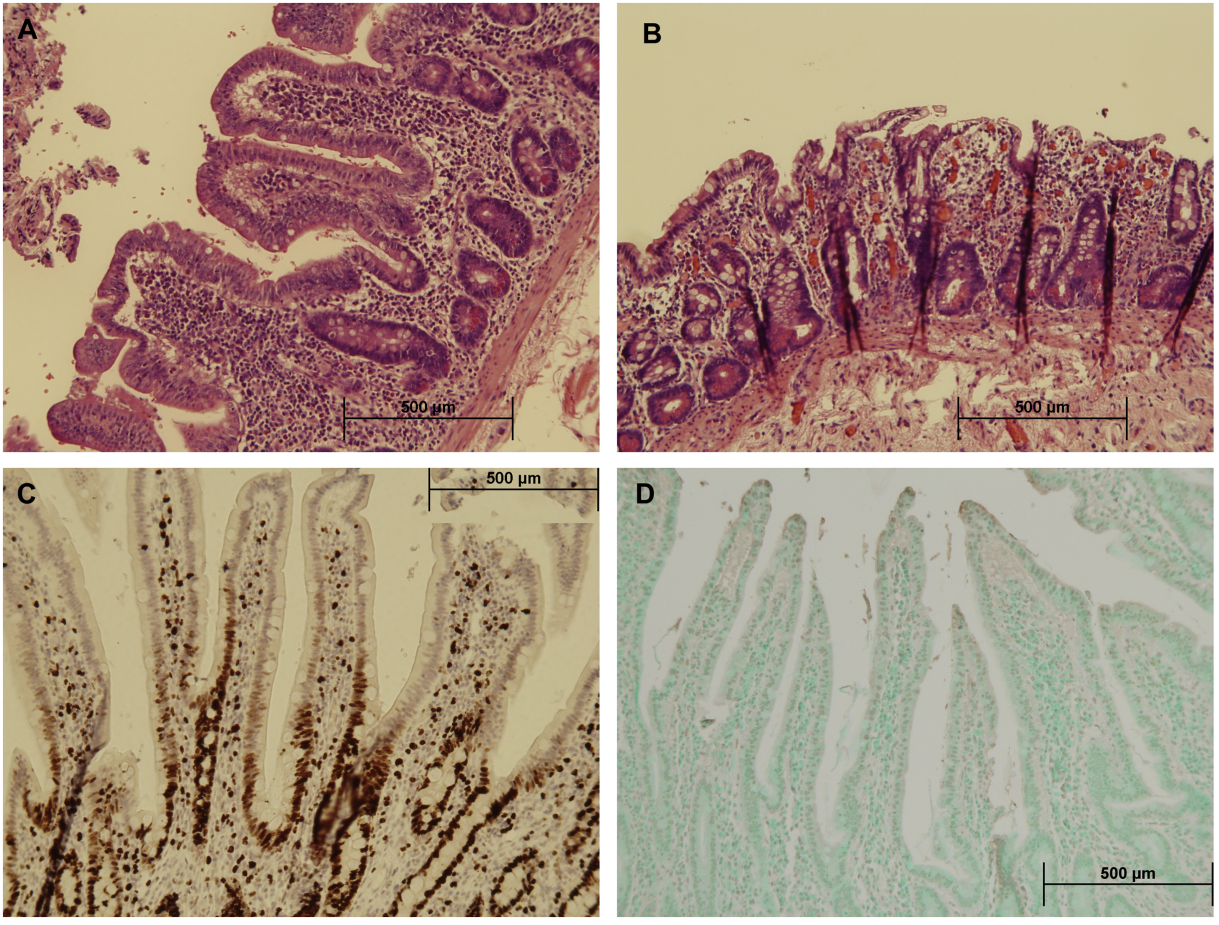

Figure 1

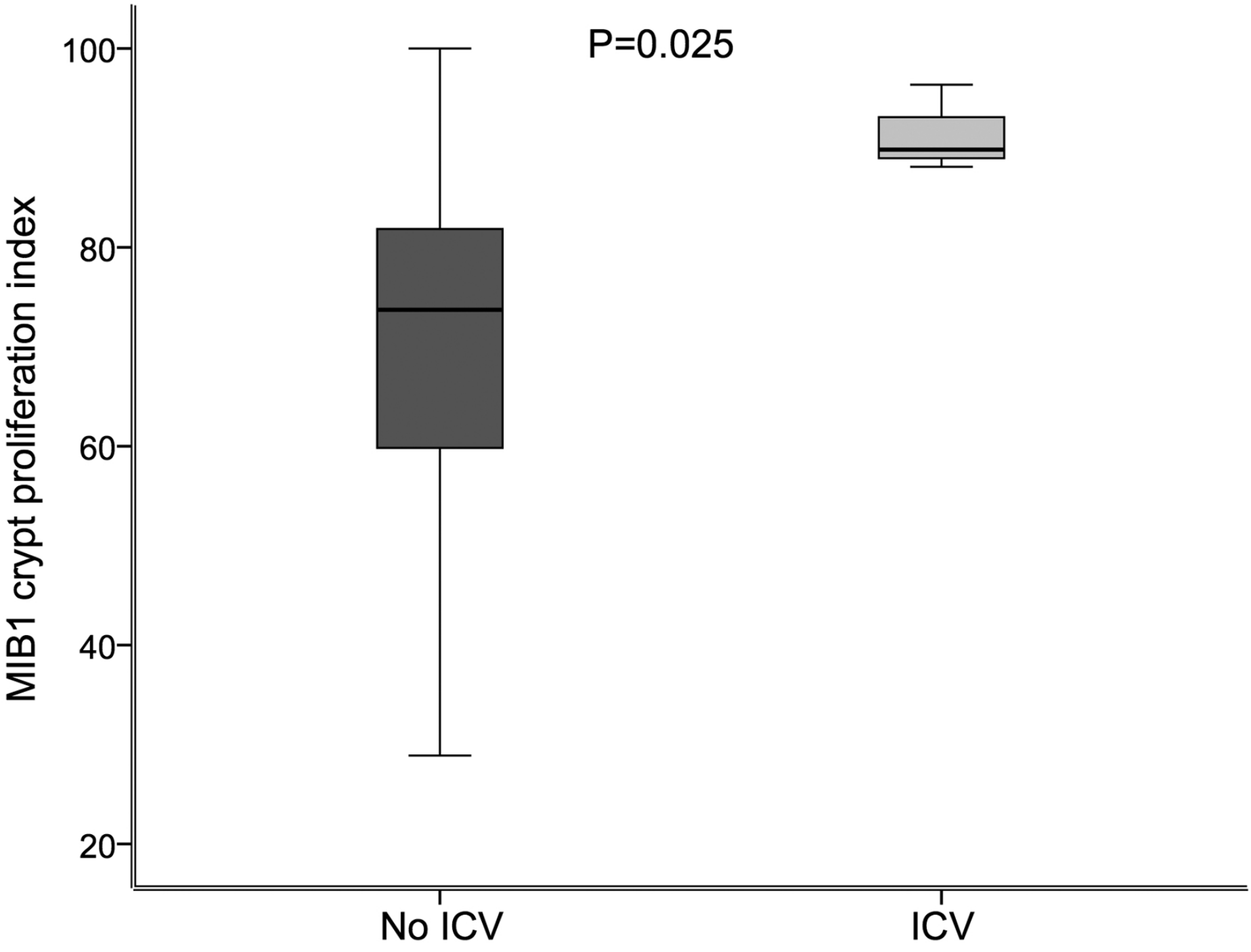
P=0.025
100
80
60
40
20
MIB1 crypt proliferation index
No ICV
ICV

Figure 2

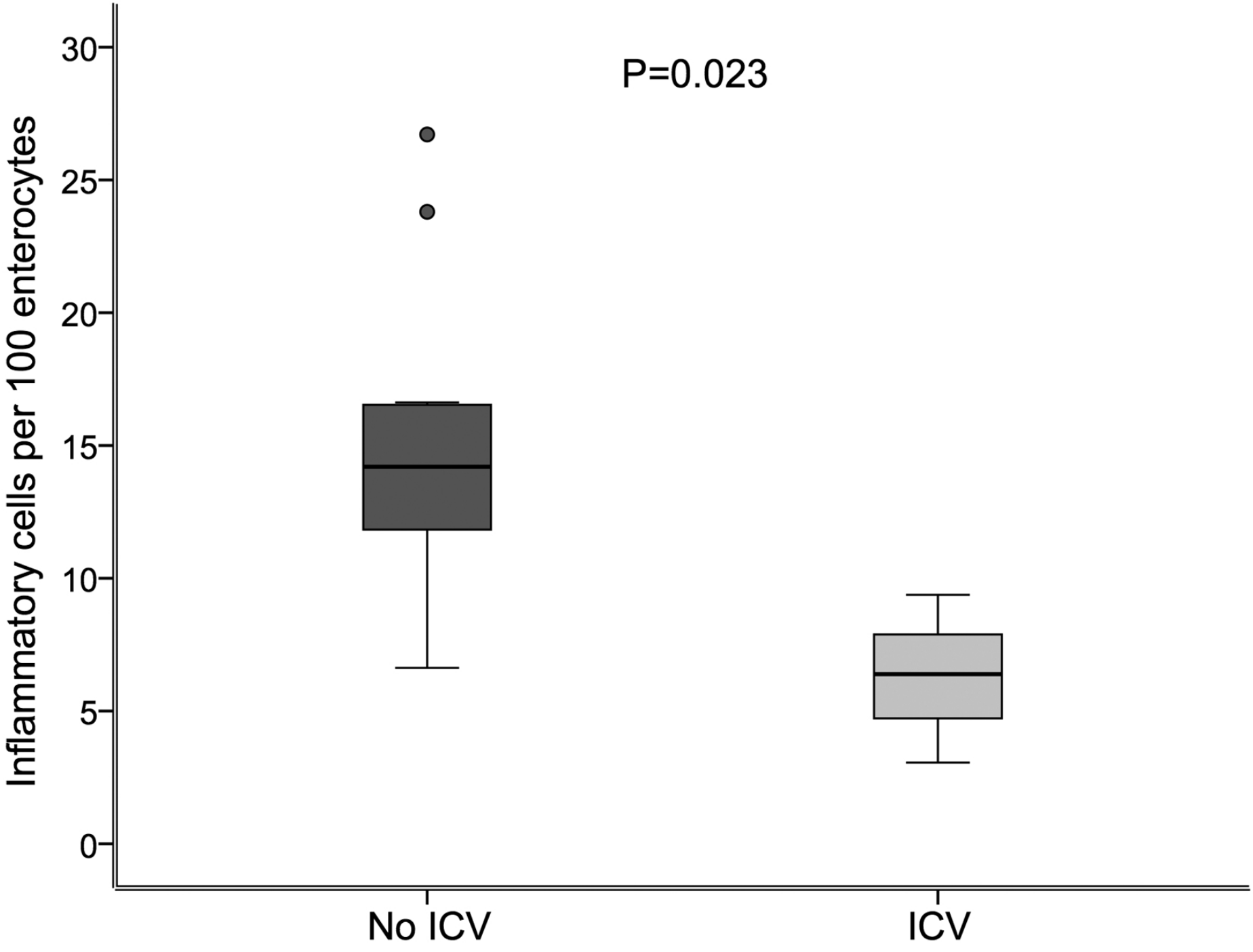
P=0.023
Inflammatory cells per 100 enterocytes
30
25
20
15
10
5
0
No ICV
ICV

Figure 3