

Original Article

Additional criteria in diagnosis of transitional zone in Hirschsprung disease

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Abstract: In Hirschsprung disease (HD) transitional zone (TZ) is interposed between aganglionic and normoganglionic zone (NZ). Its presence on proximal resection margin could be reason for postoperative complications in HD patients. A glial cell index (GCI) is the ratio between number of glial cells and ganglion cells in enteric ganglia. The value of GCI in TZ and NZ in HD has not been evaluated previously. The aim of this study was to evaluate GCI and calretinin expression as possible additional tool in differentiation of NZ and TZ in HD. Hematoxylin-eosin and immunohistochemical (calretinin and S-100) staining were performed. NZ and TZ of HD specimens and autopsy rectal specimens were analyzed at 5 microscopic fields (magnification 400×). GCI was analyzed in both myenteric and submucosal ganglia. Myenteric GCI was significantly higher in NZ (7.0 ± 0.64) than in TZ (4.5 ± 1.28), irrelevant of the staining method. Mean value of myenteric GCI less than 6.0 favors TZ origin of sample. Pattern of calretinin expression was different in NZ and TZ. Calretinin positive intrinsic nerve fibers were always present in NZ, mostly in diffuse pattern, while in TZ their presence was variable and often focal. Calretinin expression in myenteric ganglion cells was lower than in submucosal ganglia, especially in TZ. According to our results, myenteric GCI and pattern of calretinin expression could be helpful additional tool in diagnosis of TZ in HD in biopsies with lack of conventional features.

Keywords: Hirschsprung disease, transitional zone, enteric nervous system, immunostaining, glial cell index

Introduction

Enteric nervous system (ENS) is a part of autonomic nervous system that regulates function of the gastrointestinal tract (GIT). It is composed of ganglion cells (neurons) and glial cells grouped in ganglia, which are linked in submucosal and myenteric nervous plexus [1]. Hirschsprung disease (HD) is congenital aganglionosis of the distal rectum with variable length of the contiguous bowel [2, 3]. The most common HD type is short HD segment. The other, rare variants of HD are ultra-short segment HD (US-HD), long segment and total colonic aganglionosis (TCA). Diagnosis of HD is based on analysis of transanal suction biopsies, while a decision about the level of resection is based on analysis of seromuscular or full thickness biopsies on frozen sections with propose to have normoganglionic zone (NZ) on sur-

gical resection margin [4]. In recent times immunohistochemical staining tend to replace acetylcholinesterase enzyme histochemistry which is used as gold standard for diagnosis of HD [5, 6]. The vast majority of authors favor calretinin immunostaining compare to the other antibodies [7-12].

Transitional zone (TZ) is funnel-shaped segment, measured 1 to 3 cm, interposed between aganglionic and normoganglionic bowel in HD, with variable finding of ganglion cells in the submucosal nervous plexus, but with obligatory hypogangliosis in the myenteric nervous plexus, usually mixed with thick nerve fibers [13, 14]. A presence of TZ on the proximal surgical resection margin is one of the major causes of post-surgical intestinal pseudoobstruction [13-15]. Typical morphology of normal ganglion cells [16] include oval or round nuclei, usually with a

prominent nucleolus, surrounded with polygonal, often vacuolated and eosinophilic cytoplasm. Ganglion cell size in myenteric ganglia of children varies between 10 and 40 μm in diameter [17], while submucosal ganglia and ganglion cells are smaller, especially in the superficial submucosa [16]. It is particularly hard to estimate presence and number of immature ganglion cells which are commonly present in the first year of life and characterized with small dark nuclei with no obvious nucleolus and have scanty hemophilic cytoplasm [18]. Glial cells are smaller than ganglion cells, have small blue nuclei and scanty cytoplasm which is not clearly visible at a light microscopic level [19-21] and show immunopositivity on S-100 β and glial fibrillar acid protein (GFAP) [1, 20, 22-23]. A glial cell index (GCI) is the ratio between number of glial cells and ganglion cells. Hoff and al. [22] found that GCI was the most robust quantitative ENS descriptor within one species.

A confirmation of ganglion cell presence in the seromuscular biopsy during HD operation is not enough for decision about the level of bowel resection. Pathologist have difficult mission to quantitatively evaluate myenteric nervous plexus in frozen section of seromuscular or full-thickness biopsies and on proximal surgical margin of resected bowel. Myenteric hypoganglionosis in TZ could be mild with no evidence of hypertrophic nerve trunks in biopsy samples. The aim of this study is to evaluate GCI and calretinin expression as possible additional tool in differentiation of NZ and TZ in HD in resected bowel specimens with possible application on seromuscular and full-thickness frozen section biopsies.

Material and methods

Material

Subject of analysis were colorectal resected specimens of 50 children (42 boys and 8 girls) surgically treated due to HD at University Children's Hospital Tirsova, Belgrade in the period from 2008 - 2014 year. All samples were analyzed at Institute of Pathology, Faculty of Medicine, University of Belgrade. Resected bowels were fixed by immersion in 4% formalin. Prior to sectioning, they were opened longitudinally. In all cases we sampled tissue on the same way: transversal tissue sections from distal (aganglionic) and proximal (normogangli-

onic) resection margine, which included whole circumference, and longitudinal sections in continuity of both resection margines, and additional, at least two longitudinal sections from the macroscopically suspicious TZ. After standard processing and H&E staining, for further morphometric and immunohistochemical analysis we choosed 33 cases in which we found satisfied paraffin-embedded longitudinal samples from the NZ and TZ.

The vast majority of patients (27/33, 82%) in selected sample were underwent transanal pull-through resection by de la Torre and Ortega [24]. In remaining cases Swenson's or Duhamell's techniques were applied [25, 26]. In the most of selected cases (30/33, 91%) classical short type of HD was diagnosed. In the remaining three cases (9%) ultra-short type of HD was present.

Rectums from ten pediatric autopsy cases (neonates and infants) from Institute of Pathology and Institute of Forensic Medicine in Belgrade, none of whom had any history of intestinal dysmotility, were controls. Specimens included anorectal junction and 3.5-4 cm in length of proximal bowel. They longitudinally opened and fixed by immersion in 4% formalin. Whole circumference of segment longitudinally handled, with total 4-5 samples per case. All samples were paraffin embedded, cut in 5 μm thick sections, routinely H&E stained and analyzed. One paraffin block from each case was chosen for further analysis. Control cases were compared with age-matched cases in HD group.

All selected paraffin blocks were cut into 5 μm thick sections. We analyzed three sections from each paraffin block (H&E-stained, calretinin and S-100 immunostained). The distance between analyzed sections was 18 tissue sections (total-90 μm) in order to avoid counting the same ganglion cells in adjacent sections as recommended by Ippolito et al [23].

Immunohistochemistry-calretinin immunostaining

Calretinin (monoclonal mouse antihuman antibody (DAKO), clone: DAK-calret 1, Code: IR627, dilution 1:50) immunostaining was used for visualization of ganglion cells and intrinsic nerve fibers (INFs). During analysis of calretinin stained sections we counted all ganglion cells

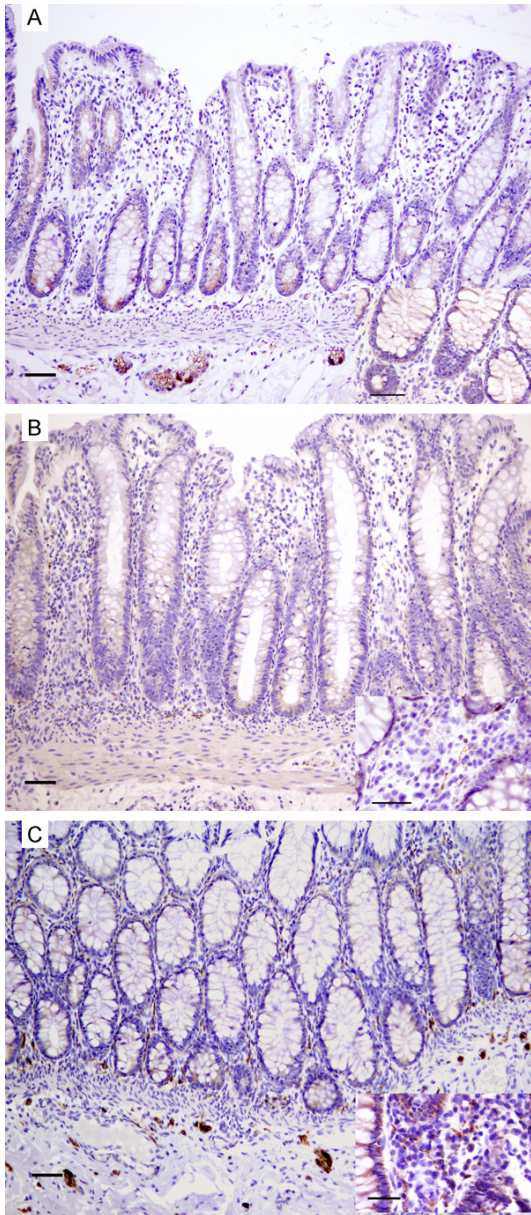


Figure 1. Different levels of intrinsic nerve fibers (INFs) extension: (A) negative calretinin staining for INFs, (B) weak calretinin immunopositivity of INFs which are focally present in the superficial submucosa, muscularis mucosae and lamina propria, (C) strong calretinin immunopositivity of INFs in the superficial submucosa, muscularis mucosae and full thickness of lamina propria (scale bars: 50 μ m); inset: detail of lamina propria (scale bars: 50 μ m).

morphologically recognized, regardless of immunoexpression. Every level of positivity was considered as 'positive' staining. We also noted type of immunoexpression (cytoplasmatic (C) and/or nuclear (N) and/or nuclear and cytoplasmatic (NC)). INFs in the lamina propria of mucosa and superficial submucosal layer have linear

granular staining pattern. The extension of INFs staining was evaluated semi-quantitatively (negative = 0 (**Figure 1A**), focally positive staining = 1 (**Figure 1B**) and diffusely positive staining in the entire mucosa and superficial submucosa = 2 (**Figure 1C**)). Positivity in mast cells and histiocytes was considered as internal positive control [27-29].

S100 immunohistochemical staining (polyclonal rabbit antibody (DAKO), anti S100, Code: Z0311, dilution 1:1600) was used for visualisation of glial cells because it reacts strongly with human S100 β . Also, S100 immunostaining was positive in nerve fibres. Omission of the primary antibody served as negative controls.

Quantitative evaluation of enteric nervous plexuses

For quantitative evaluation we used modified method of Ippolito et al [23]. Submucosal and myenteric plexuses were evaluated separately in each sample. Ganglion cells were counted on 5 HPF (magnification \times 400) on three sections (H&E, calretinin and S100) per each tissue block, in total 15 HPF. During microscopic examination (Olympus BX41) we have captured five consecutive microphotographies (Olympus DP70 camera) per one section and then counted ganglion cells and glial cells using ImageJ free software [30]. Slides in control cases were analyzed started from the end opposite to anorectal junction. Each HPF corresponded to a 0.424×0.319 mm, which covered a 0.135 mm². Total area of analyzed submucosa per one specimen was 2.0 mm² (15 HPF). Total length of analyzed zone of myenteric plexus per one specimen was 6.4 mm. We counted ganglia, ganglion cells and glial cells in the TZ and NZ of HD cases and in the control rectal samples. GCI was calculated for submucosal and myenteric nervous plexus in the selected tissue samples, separately for three applied staining methods. According to International Working Group Gastro 2009 single ectopic ganglion cell within nerve bundles are not included in the counts [17]. We also measured maximum transverse nerve diameter using ImageJ software on H&E and S100 immunostained sections.

Statistical analysis

All statistical analyses were performed using the EZR software package (Saitama Medical

Additional tool in diagnostics of Hirschsprung disease

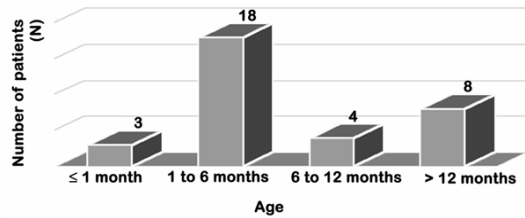


Figure 2. Patient's age at the time of surgical resection for Hirschsprung disease.

Center/Jichi Medical University, Saitama, Japan) [31] along with a graphical user interface for the R software package (version 3.1.1; TheRFoundationforStatisticalComputing). Descriptive and analytical statistical methods were applied. Mean number of ganglia and their cellular content were presented as arithmetic mean (\bar{x}) and standard deviation (SD) or median value and range. For testing of differences in number of ganglia, ganglion cells, glial cells and nerve thickness between TZ and NZ, Wilcoxon's signed rank test was used, while Mann-Whitney U test was used for testing differences of these parameters in control group and age matched NZ. Spearman's rank correlation test was used for evaluation of degree of correlation between different parameters. For cut-off evaluation, all parameters were analyzed with ROC curves. A p -value of <0.05 indicated statistical significance.

This study positively stated by the local Ethics committee (University of Belgrade, Medical School-29/VII-2, 1st July 2015 and University Children's Hospital Tirsova, Belgrade (26/185, 4th June 2015).

Results

Demographic and clinical data

The vast majority of patients in our study were boys 30 (91%), with median age at the moment of HD diagnosis 28.5 days (7 days-13.5 years). Diagnosis of HD was established in almost half of patients during neonatal period (16/33; 48%). In only 7 (21%) patients diagnosis of HD was established after first year of life. Although in the vast majority of cases (31/33, 94%) symptoms (failure to pass meconium during first 48 h of life, abdominal distension, constipation) began during first two weeks of life, they often surgically treated later. The

median age of HD patients at the time of surgical procedure was 5 months (ranged 1-192 months). Eight (24%) patients were older than one year at the moment of surgery (**Figure 2**). The average age in the control group was 0.6 months, ranged 1 day - 55 days. In this group male:female ratio was 6:4.

In only 4 (12%) cases postoperative complications were present. Three patients had constipation and one patient suffered from postoperative enterocolitis. All patients with complications were successfully treated conservatively and none was reoperated.

Number of ganglia, ganglion and glial cells, glial cell index (GCI)

The numbers of analyzed elements of ENS on H&E, calretinin and S100 immunostaining in NZ and TZ are presented in the **Table 1**. In four (4/33, 12%) analyzed TZ cases, submucosal aganglionosis were noted on all analyzed levels, while in some cases ganglion cells were present only on two or one of them (five (15%) and six (18%), respectively).

The number of ganglia, ganglion cells and glial cells was significantly different between NZ and TZ ($P<0.001$), regardless of staining method in both plexuses. But, there were some significant differences in number of these values between three analyzed stained sections in the same zone (**Figure 3**).

We found statistically significant negative correlation with the total number of ganglion cells in the submucosal nervous plexus in NZ and the age of patients ($r = -0.772$; $P<0.001$). Also, we found strong correlation between total number of ganglion cells and glial cells in the submucosal and myenteric nervous plexus in NZ (0.866, $P<0.001$; 0.953, $P<0.001$, respectively) and TZ (0.872, $P<0.001$; 0.912, $P<0.001$, respectively).

The mean number of submucosal ganglion cells per ganglia was around two ganglion cells in both zones of HD. On the other hand, the vast majority of myenteric ganglia contained around three ganglion cells in NZ and less than two ganglion cells in TZ. The differences in mean number of ganglion cells per ganglia and GCI in myenteric plexus were statistically significant in NZ and TZ regardless staining method (**Table**

Additional tool in diagnostics of Hirschsprung disease

Table 1. Cellular content in two compartments of ENS in the NZ and TZ in HD patients in regard to staining method

ENS compartment	Staining	Ganglia		Ganglion cells		Glial cells	
		NZ	TZ	NZ	TZ	NZ	TZ
Submucosal (median; range)	H&E	5 (2-16)	2 (0-10)	15 (4-32)	6 (0-20)	27 (5-72)	11 (0-39)
	Calretinin	8 (2-19)	4 (0-10)	15 (5-41)	8 (0-34)	31 (4-56)	10 (0-41)
	S100	3 (1-9)	2 (0-6)	6 (2-18)	2 (0-12)	13 (2-43)	3 (0-19)
	Total*	18 (8-36)	6 (0-20)	37 (13-82)	11 (0-52)	69 (17-123)	19 (0-76)
Myenteric (median; range)	H&E	8 (5-11)	3 (1-11)	29 (11-80)	5 (1-31)	154 (39-502)	25 (1-196)
	Calretinin	6 (5-13)	3 (1-12)	21 (5-57)	4 (1-23)	150 (24-395)	24 (1-216)
	S100	6 (3-11)	4 (0-9)	18 (6-32)	5 (0-23)	117 (40-237)	20 (0-114)
	Total*	21 (14-28)	11 (2-29)	72 (35-151)	17 (3-69)	479 (191-1068)	75 (5-490)

NZ - Normoganglionic zone; TZ - Transitional zone; *Total number on the 15 HPF.

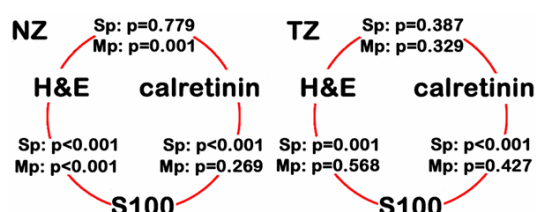


Figure 3. Differences in ganglion cell number in normoganglionic (NZ) and transitional zone (TZ) in Hirschsprung disease in regard to type of staining. Sp - submucosal plexus; Mp - myenteric plexus; H&E - hematoxylin and eosin staining.

2). The mean GCI was around 7 in NZ and usually less than 5 in TZ. We calculated some differences in submucosal GCI between NZ and TZ in regard to the type of staining, while there were not differences in GCI of both HD zones in regard to staining method (**Figure 4**).

According to ROC analysis, myenteric ganglia with less than 2 ganglion cells per ganglia with sensitivity 0.939 and specificity 0.879 were probably from TZ. Area under the curve in this case was 0.867 and 95% confidence interval was 0.779-0.954. Using ROC curves, the best cut-off and discriminatory power for GCI in myenteric plexus was 6.0, with sensitivity 0.879 and specificity 0.939, with area under the curve 0.959 and 95% confidence interval 0.913-1.

The mean values of ganglia number, ganglion cells and glial cells numbers in submucosal plexus (Mann-Whitney test; $P = 0.149, 0.173, 0.273$, respectively) and myenteric plexus were not significantly different (Mann-Whitney test; $P = 0.701, 0.496, 0.481$, respectively) between control group and matched NZ HD samples by

age. Mean number of ganglion cells per ganglia in the submucosal plexus in control and age matched NZ group was similar (2.0 ± 0.40 and 2.3 ± 0.54 , respectively), as well as in the myenteric plexus (4.9 ± 1.9 and 4.5 ± 2.76 , respectively). Mean GCI in control and age matched NZ group were also similar in submucosal (2.1 ± 0.30 and 2.1 ± 0.32 , respectively) and myenteric nervous plexus (7.1 ± 0.71 and 7.0 ± 0.83 , respectively).

Immunostaining pattern of calretinin staining was different in the submucosal and myenteric ganglion cells in both zones. In the submucosal ganglion cells calretinin staining was usually strong, commonly NC, while in the myenteric plexus staining was usually less intensive. Myenteric ganglion cells in NZ usually contained mixture of weak C and strong NC calretinin expression, similar as samples in the control group. However, some myenteric calretinin negative ganglion cells were observed in 30% of cases in control group and in 12% NZ samples (**Figure 5A**). Dominant staining pattern of myenteric ganglion cells in the TZ was weak C staining (**Figure 6A**), while rare ganglion cells had intensive NC calretinin expression (**Figure 6B**). Calretinin expression in small TZ ganglia as well as single ganglion cells often were calretinin negative (**Figure 6C** and **6D**). Discrete granular calretinin positivity was noted in the vast majority of present nerves in the TZ and in all nerves from NZ.

The extension of INFs was evaluated semiquantitatively as previously described. Statistically significant difference of INFs extension in NZ and TZ was found ($P < 0.001$). The median of INFs extension in the NZ was 2 (1-2). In the TZ

Additional tool in diagnostics of Hirschsprung disease

Table 2. Number of ganglion cells per ganglion and GCI in the NZ and TZ in HD patients in regard to staining method

ENS compartment	Staining	Ganglion cells per ganglia			Glial cell index		
		NZ	TZ	p	NZ	TZ	p
Submucosal (median; range or mean \pm SD)	H&E	2.2 (1.0-6.5)	2.0 (1.0-5.0)	0.029	2.3 \pm 0.79	2.5 \pm 1.07	0.276
	Calretinin	2.0 (1.0-3.3)	1.9 (1.0-3.2)	0.260	2.0 \pm 0.41	2.3 \pm 0.71	0.401
	S100	1.7 (1.0-4.5)	1.5 (1.0-2.4)	0.386	2.2 \pm 0.48	1.8 \pm 0.59	0.061
	Total*	2.0 (1.2-2.9)	1.7 (1.0-3.0)	0.034	2.1 \pm 0.28	2.2 \pm 0.69	0.665
Myenteric (median; range or mean \pm SD)	H&E	4.0 (1.4-11.4)	1.5 (1.0-4.4)	<0.001	6.9 \pm 1.10	4.4 \pm 1.66	<0.001
	Calretinin	2.8 (1.2-11.4)	1.3 (0.5-1.9)	<0.001	7.2 \pm 1.11	4.8 \pm 1.79	<0.001
	S100	2.7 (1.3-6.2)	1.6 (1.0-4.3)	<0.001	6.8 \pm 0.43	4.2 \pm 1.62	<0.001
	Total*	3.5 (2.0-7.9)	1.5 (0.6-3.8)	<0.001	7.0 \pm 0.64	4.5 \pm 1.28	<0.001

ENS - enteric nervous system; NZ - normoganglionic zone; TZ - transitional zone; HD - Hirschsprung disease; H&E - hematoxylin and eosin staining; SD - standard deviation; *Total number on the 15 HPF.

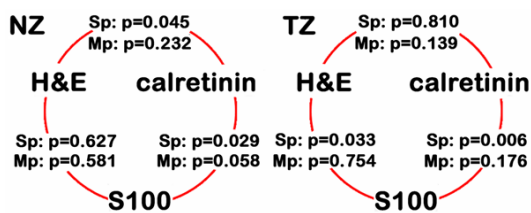


Figure 4. GCI differences regard to staining method/section level in the NZ (normoganglionic zone) and TZ (transitional zone) in Hirschsprung disease. Sp - submucosal plexus; Mp - myenteric plexus.

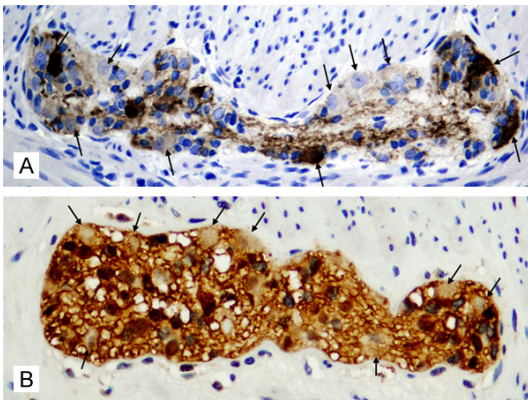


Figure 5. Calretinin (A) and S100 (B) immunostaining in the myenteric ganglia of NZ. Notice different pattern of calretinin expression in ganglion cells (arrows) (A). Strong S100 expression in the vast majority of glial cells highlighted negative ganglion cells (arrows) (B).

median of extension of INFs was 1 (0 - 2). There was no significant correlation between INFs extension and number of ganglion cells in the submucosa in NZ (Spearman's rank correlation coefficient -0.240, $P = 0.179$). In the vast majority of autopsy cases (7/10; 70%) median exten-

sion of INFs was 2. In the remaining autopsy cases the INFs extension was focal (INFs = 1).

b) S-100 stained the vast majority of cells with glial morphology in ganglia of both nervous plexuses, with only few negative cells with glial morphology (**Figure 5B**).

Submucosal nerves

We detected nerves in the submucosa of all samples from TZ and in some cases from NZ. They showed granular calretinin immunostaining. Thickness of all detected nerves is presented in the **Table 3**. In fifteen (45.4%) samples from TZ the thickness of nerve bundles was more than 40 μ m in diameter, while in 5 NZ samples (15%) single nerves, thicker than 40 μ m were detected. There was no significant differences in diameter of present nerves between control and NZ matched by the age of patients ($P = 0.789$). In majority of HD cases (28/33; 85%) and in control group (9/10; 90%), in a both analyzed zones, some of observed thin nerves contained single ganglion cells. The nerves with ganglion cells usually were thin.

Discussion

Several studies describe differences in distribution of ENS ganglia (especially in a myenteric nervous plexus) in longitudinal and trasversal bowel sections [14, 16, 32]. Possible differences due to orientation of samples we excluded by selection of samples with same orientation. Due to standard manner of sampling in our institution, only longitudinal samples were available for both analyzed HD zones.

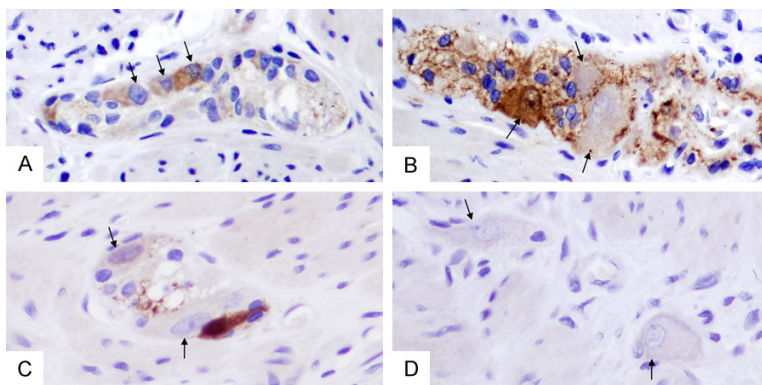


Figure 6. Calretinin expression in the myenteric ganglia in TZ. It is usually weak C (A), but beside these weakly positive ganglion cells (arrows), rare ganglion cells show strong NC calretinin immunopositivity (arrow) (B); ganglion cells (arrows) in small myenteric ganglia and single ganglion cells (arrows) often have not express calretinin (C and D).

Table 3. The thickness of submucosal and myenteric nerves in the control group and samples in Hirschsprung disease

ENS compartment	Maximal diameter of nerves			p-value*
	Control group (μm)	NZ (μm)	TZ (μm)	
Submucosal zone	26 (0-38)	25 (0-66)	39 (17-114)	<0.001*
Myenteric zone	0 (0-58)	0 (0-106)	46 (11-106)	<0.001*

ENS - enteric nervous system; NZ - normoganglionic zone; TZ - transitional zone;

*Wilcoxon's signed rank test (NZ versus TZ).

Male predominance (42:8) in our sample was in concordance with general data about HD incidence, which vary from 2.6 [33] to more than 5:1 [34]. Almost half of patients in our study (16/33; 48.5%) were diagnosed as HD during first month of life. In the other studies it was even more common, up to 76% of HD cases [33, 35]. Early appearance of the HD symptoms (failure to pass meconium during first 48 h of life, abdominal distension, constipation) and current diagnostic approach have contributed to earlier HD diagnosis. Around 20% of HD patients in our sample were diagnosed after first year of life. Hackam et al [35] found that the delayed diagnosis of HD was associated with a different pattern of disease presentation and a consistently shorter TZ compared with neonatal HD.

The number of ganglia and ganglion cells in the ENS varies in different studies. It depends of type of immunostaining and the way of sampling and counting (per HPF or mm/mm²) [16, 17, 20, 23, 32, 36]. Despite individual consistency in counting of ganglion cells, observers

count different number of ganglion cells even when apply the same rigorous criteria [32].

We found statistically significant difference between number of ganglia, ganglion and glial cells in the submucosal nervous plexus in NZ and TZ. Regardless of these differences, there were not observed differences in composition of submucosal ganglia. Density of submucosal ganglion cells has been studied by different approaches and staining methods with highly dispersed results [17]. The number of submucosal ganglia in the sigmoid colon and rectum has been previously evaluated in only few studies, and only several of them refer to pediatric age [5, 17, 37]. Our observation about significant negative correlation between number of ganglion cells in the submucosal nervous plexus and age is in concordance with find-

ings of other authors [17]. Tendency of decreasing in number of ganglia per length of intestine in the first months and years of life was described by several authors [16, 37]. Because of these facts, it is important to take into account the age of the patients. We did not find any differences between submucosal plexus in the control group and age matched NZ.

Similar to results of analysis of submucosal plexus, statistically significant differences between number of ganglia, ganglion and glial cells in the myenteric plexus in NZ and TZ were found. Unlike to submucosal plexus, appearance of myenteric ganglia was significantly different in the two analyzed HD zones. Myenteric ganglia were usually contained smaller number of ganglion and glial cells in TZ than in NZ. According to the literature data mean number of ganglion cells per ganglia in the normal myenteric nervous plexus on H&E sections is 3, with range 2-6, similar to our results in NZ (taking into account all sections). However, this value varied depending on staining method. This number is usually higher on immunos-

tained sections [17, 38], unlike to our results. It is possible that differences exist because we counted only cells with a visible nucleus. Analysis of whole-mount specimens showed that mean number of ganglion cells per ganglia could be much larger due to ability of evaluating entire ganglia. Using ROC analysis we found that myenteric ganglia with less than 2.0 ganglion cells per ganglia are probably from the TZ. According to analysis of different data, there is suggestion that mean number ≤ 2 ganglion cells per ganglia could be considered as abnormal [5, 17], which is characteristic of myenteric plexus in TZ in our study.

We found strong correlation between number of ganglion and glial cells in both plexuses in both HD zones, regardless of staining method. Mean submucosal GCI in our investigation in NZ and TZ was not significantly different. While myenteric hypoganglionosis is obligatory in TZ, submucosal plexus in this bowel segment could be hypoganglionic, normoganglionic or even hyperganglionic [14], which can explain our findings. On the other hand, mean myenteric GCI was without significant differences based on staining method/section level in both HD zones and in the control group. It is particularly important that myenteric GCI value on H&E sections was not significantly different in comparison with values on immunostained sections, due to possible implementation in routine practice. Recent investigations highlighted central role of enteric glial cells in the regulation of gut homeostasis (motility, secretion, absorption, and intestinal barrier) and their role in some digestive and extradigestive diseases [20, 34, 39]. Considering in the above facts, disturbance of glial and ganglion cell ratio (GCI), especially in myenteric plexus, could be very important criteria for diagnosis of various enteroneuropathies [38-40] and differentiation TZ and NZ in HD. Myenteric CGI in our study was 7.0 ± 0.64 (regardless of staining method). In the other studies myenteric GCI in the normal colon was ranged 5.9-7.0, using Sox8/9/10 immunohistochemistry [22] or even more - 8.7 ± 1.9 , using PGP 9.5 immunohistochemistry [38]. Ippolito et al [23] found myenteric GCI 3.7 ± 0.3 based on immunochemistry (HuC/D and S100 β) and explained this relatively low GCI as a consequence of manner of counting which were included those ganglion cells without a visible nucleus. We found that GCI value

lower than 6.0 is highly suggestive for TZ in HD. Due to significant variation in GCI values in various studies, which probably result from the application of various techniques for analysis, we support suggestion of Gastro International Working Group that every reference laboratory have to use their own control ranges, collected by the same observer performing the study and using a standardized method [17].

Calretinin antibody is useful for visualization INFs and ganglion cells, but it is not specific. It is also positive in Schwann cells [7, 9], mast cells, histiocytes [7, 9, 29, 41] and even plasma cells [36]. On the contrary, some authors concluded in their studies that calretinin immunostaining of ganglion cells was specific, without false-negative and false-positive results [12]. Barshack et al [7] were among the first authors who noted calretinin immunopositivity of the INFs in lamina propria, muscularis mucosae and superficial submucosa of ganglionic bowel, with characteristic granular pattern in linear distribution [7, 11, 27]. Calretinin positive INFs are occasionally very dispersed and difficult to analyze, but they could not be missed at high magnification [11]. We found calretinin positive INFs in all cases and found statistically significant difference in extensions of INFs between analyzed two zones of HD. The extensity of calretinin positive INFs in TZ were more variable, often focally. It is considered that calretinin positive INFs first appear in the TZ, with various density and distribution [7]. Calretinin immunopositivity in INFs appears early, at 22 weeks of gestation [11], what favor its application in cases of prematurity [11, 34]. Although a heterogeneous distribution of calretinin positive INFs in lamina propria is also noted in normal colon [8], it is still recommended for analysis of superficial biopsies and in biopsies with histological appearance of the physiologic aganglionic zone [28, 41]. Bachmann et al [36] consider that INFs distribution varies in relation to the part of the HD segment and included level of calretinin INFs expression in immunohistochemical score for diagnosis of HD.

Although many researchers describe intensive NC calretinin positivity in ganglion cells [9, 11, 27], we noticed different pattern of calretinin expression in ganglion cells (NC or C), especially in the myenteric ganglia. Also, we noticed single calretinin negative ganglion cells in few

control cases and samples from both HD zones. Many authors describe calretinin immunopositivity in more than 80% of ganglion cells of normal colon and NZ of HD [7, 8, 16, 42], but some authors showed that calretinin expression exists in only one-tenth of myenteric ganglion cells [43]. Due to ganglion cell heterogeneity, it is not known panneuronal antibody for visualization all ganglion cells. Even panneuronal HuC/D antibody show different pattern of immunoexpression [32] with expression in less than 100% ganglion cells [23]. We have noticed lower calretinin expression in myenteric ganglia in TZ than in NZ and phenomenon of calretinin negativity in single ganglion cells, what should be kept in mind during examination of seromuscular or full thickness colorectal biopsies in order not to miss diagnosis of TZ.

Glial cells are heterogeneous, too. Ippolito et al. [23] give priority to S100 β antibody for glial visualisation due to specific sharp S100 nuclear/cytoplasmic immunoreaction allow to discriminate glial cells from ganglion cells more readily than with the GFAP. But, S100 β does not label the entire glial cell population [22].

The vast majority of authors define TZ as myenteric hypoganglionosis with hypertrophic nerve trunks [14]. There is suggestion that nerves thicker than 40 μ m, especially if more than two thick nerves are present in one HPF, are suggestive for TZ [46]. In all samples of the TZ in our investigation we found nerves in the submucosa with mean diameter around 40 μ m. But, small nerves or single thicker nerves are not unusual finding in NZ. In the normal sigmoid colon could be present submucosal nerves with mean diameter 12.4 (9.8-14.9) μ m [38]. In the other study, submucosal nerves in NZ usually measured 10 to 20 μ m, maximally 32 μ m [45]. Kapur and Kennedy [15] found that thick nerves (≥ 40 μ m) in autopsy cases from children without history of intestinal disorder are rarely. In our control autopsy group all measured submucosal nerves were less than 40 μ m. We found some granular calretinin positivity in all detected nerves regardless the zone in HD. Hypertrophic nerves in the aganglionic zone are negative on calretinin staining [46], but sometimes large submucosal nerves in HD may contain immunoreactive axons, which have punctiform staining pattern similar to nerves in normal serosa [8]. Granular calretinin reactivity in nerve trunks, in the absence of ganglion cells

and calretinin positive INFs, is suggestive for TZ or "junction zone", below the TZ [10].

Conclusion

Standard criteria for TZ diagnosis in HD such as myenteric hypoganglionosis and nerve hypertrophy sometimes are not sufficiently expressed in small biopsies with consequent need for finding additional items that would be helpful in establishing of diagnosis. We have shown that characteristic of TZ is not only myenteric hypoganglionosis, but also disturbed myenteric GCI. According to our results mean myenteric GCI in TZ is usually less than 6.0, what could be applied in diagnostic process, especially in analysis of small biopsies. GCI analysis could be done even on routine H&E slides, but it should be performed by experienced pathologist who has also previously set laboratory standards in ENS analysis in own laboratory. From this point of view, GCI analysis could be applied on ex tempore frozen section analysis. Although calretinin immunopositivity of INFs is implemented in routine HD diagnostics, the pattern of calretinin expression in ganglion cells, especially in the myenteric plexus, could be useful in recognizing of TZ, too.

Disclosure of conflict of interest

None.

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Additional tool in diagnostics of Hirschsprung disease

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