Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta Morphologica et Anthropologica, 32 (1-2)
Sofia ● 2025

Histopathological Changes in the Enteric Nervous System of Patients Undergoing Surgical Treatment for Intestinal Pseudo-Obstruction

Todor Kirov[1], Vesela Ivanova[2], Tihomir Dikov[2], Radoslav Todorov[3], Lina Malinova[1]*

[1] Dept. of Anatomy, Histology and Embryology, Medical University of Sofia, Sofia, Bulgaria;
[2] Dept. of General and Clinical Pathology, Medical University of Sofia, Sofia, Bulgaria;
[3] Dept. of General and Operative Surgery, Medical University of Sofia, Sofia, Bulgaria

*****Corresponding author e-mail: tkirov@medfac.mu-sofia.bg

This study addresses congenital colorectal neural disorders that mimic Hirschsprung's disease (HD), and which present with severe bowel motility issues. We analyzed full-thickness colorectal biopsies from eight adult patients with symptoms resembling HD to investigate expression patterns of PTEN, calretinin, and NOS1 in the enteric nervous system. Immunohistochemical findings revealed that while PTEN and calretinin staining was markedly reduced, NOS1 expression remained relatively stable. The reduced PTEN expression aligns with its established role in regulation of neuronal growth and suggests dysregulated PTEN pathways in the pathogenesis of HD-related conditions, like intestinal neuronal dysplasia. Likewise, diminished calretinin staining suggests altered intracellular calcium-buffering mechanisms, though overall neuronal numbers were not significantly altered. However, NOS1 findings pointed to relatively intact nitric oxide-mediated pathways. These observations highlight the need for refined diagnostic criteria and may guide future targeted therapeutic strategies.

Key words: Hirschsprung's disease, intestinal neuronal dysplasia, PTEN, NOS1, calretinin

Introduction

The ENS, often described as the "second brain," comprises a complex network of neurons and glial cells that regulate essential gastrointestinal functions, including motility, secretion, and blood flow. Disruptions in ENS development or function underlie the pathophysiology of a range of pathologies, head example of which is Hirschsprung's disease (HD). It presents with severe chronic constipation and other motility issues that predominantly affect children, though adults can also present with these conditions [7]. From the conditions that resemble HD in terms

of manifestation, with highest occurrence is the intestinal neuronal dysplasia type B (IND-B). It is characterized morphologically by hyperplasia of the submucosal ganglia and immaturity of ganglion cells [4]. In contrast with this, HD involves complete absence of ganglion cells in the affected bowel segments, resulting in functional obstruction [7]. Another related condition, the internal anal sphincter achalasia (IASA), though less well understood, is associated with impaired neural regulation of the internal anal sphincter [1]. HD is the most common, occurring in approximately 1 in 5,000 live births, while IND-B and IASA are less prevalent but clinically significant due to their diagnostic and therapeutic challenges [5]. Advances in molecular and cellular research over the past decade have provided insights into the pathogenic mechanisms, yet histopathological diagnosis is notoriously marked by controversy and complexity [3].

Histopathological analysis is the gold standard for diagnosing congenital colonic neural disorders, but it faces significant challenges. Differentiating between IND-B, HD, and normal variations requires meticulous evaluation of biopsy specimens, which are often limited in size and quality. The subjective nature of interpreting histological findings, such as submucosal ganglion hyperplasia in IND or the absence of ganglion cells in HD, can lead to diagnostic errors [9]. Furthermore, the need for multiple biopsy samples to ensure accurate diagnosis adds procedural complexity and increases the burden on patients and clinicians. Despite improvements in histological and immunohistochemical techniques, the lack of standardized diagnostic criteria continues to hinder clinical practice [3]. Diagnostic criteria, established with the Frankfurt Consensus in 1990 include among other methods enzyme histochemistry for acetylcholine esterase and lactate dehydrogenase, both of which limited to only frozen sections and therefore have serious limitations. Consequently, many attempts have been made to introduce new and more superior diagnostic criteria, well summarized by Terra et al. [10].

Therefore, the purpose of this study is to examine possible disturbances in the expression of some less extensively examined neuronal markers, namely the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), calretinin, and the neuronal isoform of nitric oxide synthase (NOS1).

Materials and Methods

Biopsies from eight patients that underwent colo-rectal resection surgery were used for this study. Every patient gave written informed consent declaring that medical data acquired during the diagnostics and treatment period can be used for medical research purposes. The written informed consent document was submitted to the Ethics committee of Medical University Sofia and the Ethics committee of Medical University Sofia approved the study - under issued permission 23/25.11.2024, 5522/05.12.2024.

The patients' age ranged between 46 and 77 years old. They were hospitalized with persisting episodes of severe constipation and/or clinical signs of functional intestinal obstruction that were refractory to different types of clinical treatment. Different imaging and clinical studies showed no sign of tumor or other anatomical/physical factor for colon obstruction. Imaging and endoscopic studies disclosed only a segment of the right or left colon with variable length affected by lack of peristalsis and loss of normal colon haustrations. Resection surgical interventions were performed to

treat the colon obstruction, and full thickness colorectal biopsies were collected for histopathological analysis. The specimens were processed for paraffin embedding and subsequent slicing on a microtome at a thickness of 6 μm.

After acquisition of informed consent, an additional amount of slices was sectioned for the needs of this study. Thereafter, standardized avidin-biotin-peroxidase protocol was performed following the manufacturer's instructions. Applied antisera are presented in **Table 1**. The slides were scanned by means of Olympus VS120-S6-102 slide scanner and photomicrographs were captured via the Olympus VS-ASW Image Acquisition software. Finally, images were processed with Adobe Photoshop CC software.

Table 1. Antisera used in the immunohistochemical reactions

Antisera	Manufacturer	Catalogue Number	Host/Type	Dilution
PTEN	Elabscience®	E-AB-70070	Rabbit/Polyclonal	1:200
Calretinin	Elabscience®	PA6569, Clone No. YN00166m	Mouse/Monoclonal	1:200
NOS1	Elabscience®	E-AB-70065	Rabbit/Polyclonal	1:300

Results

In all cases of the control group, the enteric nervous system did not show any signs of alteration. The ganglia were readily recognizable in both plexuses. Neuronal count ranged between 3 and 5 per sectioned submucosal ganglion, and between 5 and 11 in the myenteric plexus.

PTEN expression was positive in all neural elements of the submucosal and myenteric nerve plexuses in all cases of the control group (**Fig. 1A, B**). Neuronal PTEN expression was strong with a particular nuclear highlight. Adjacent intra- and interganglionic fibers and glial cells showed medium but still significant expression, thus providing the ganglia with clear outlines and overall immunopositivity.

In the experimental group, we observed a marked reduction of PTEN expression in the submucosal nerve plexus in 75% of the cases (**Fig. 1C**) and in the myenteric nerve plexus in 62.5% of the cases (**Fig. 1D**). Such changes provided the ganglia with a more reticular outlook and rougher contours.

Immunohistochemical findings for calretinin in both plexuses of the control group were abundant. Neurons were clearly delineated and showed a significant staining reaction (**Fig. 2A**). Prominent reaction was also observed in fiber bundles through the *muscularis externa,* the submucosa, the *muscularis mucosae*, and the mucosa.

In all cases of the experimental group, findings were markedly scarce. The submucosal ganglia were noticeably fewer in number and therefore more difficult to locate. In the ones that were registered, the neuronal count did not differ significantly from the ones in the control group. The neuronal staining intensity was lower and the adjacent fiber bundles were in most cases indistinguishable (**Fig. 2B**). On several

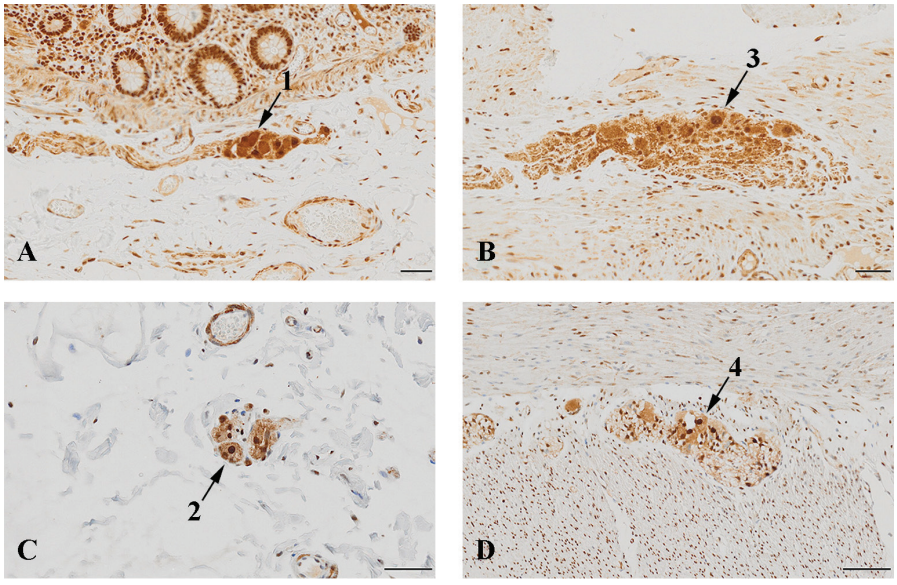

Fig. 1. Expression of PTEN in the submucosal plexus (**A**) and the myenteric plexus (**B**) of the control group, and submucosal plexus (**C**) and myenteric plexus (**D**) in the experimental group. Reactivity in the submucosal neurons (arrow 1) and in the myenteric neurons (arrow 2) in the controls appeared to be markedly higher compared to their counterparts in the experimental group (arrows 3 and 4 respectively). Scale bars: A, B, C – 50 µm, D – 100 µm.

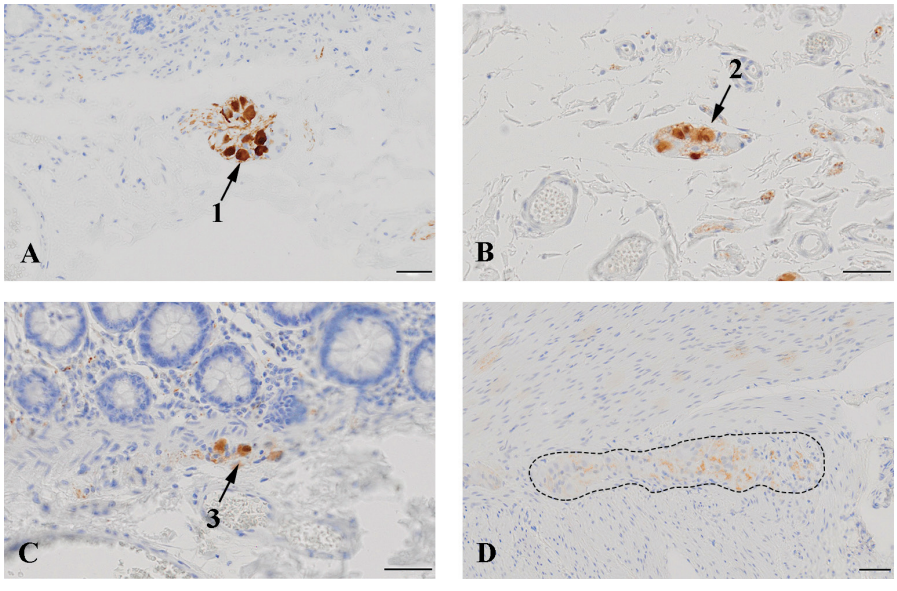

Fig. 2. Immunohistochemistry for calretinin in control group (**A**) and experimental group (**B-D**). Expression in the submucosal neurons in the control group (A, arrow 1) is notably higher than that of the experimental group (B, arrow 2). Note the presence of possible ectopic neurons in the *muscularis mucosae* in the experimental group (C, arrow 3). In the myenteric plexus of the experimental group, expression is practically non-existent (**D**); dashed line delineates the myenteric ganglion. Scale bars: 50 µm.

occasions, positive neurons were seemingly registered in the *muscularis mucosae* (**Fig. 2C**). In the myenteric was even less: neuronal imuunoreactivity was poor, and in many cases did not allow even basic differentiation of cell types (**Fig. 2D**).

NOS1 immunohistochemical findings were similar in all cases of the control and experimental groups. Submucosal ganglia were populated by several neurons, 1-2 of which showing poor to medium staining, and the rest were practically immunonegative (**Fig. 3A, C**). In the myenteric plexus of both cohorts, the reaction intensity of individual neurons was markedly stronger compared to the submucosal neurons, the rest showing poor or negative reaction (**Fig. 3B, D**). Only in few isolated myenteric ganglia we registered by visual inspection notably more intense reaction compared to the control group (**Fig. 3D**). Additionally, distinct bundles of positive beaded fibers were regularly observed in the ganglia.

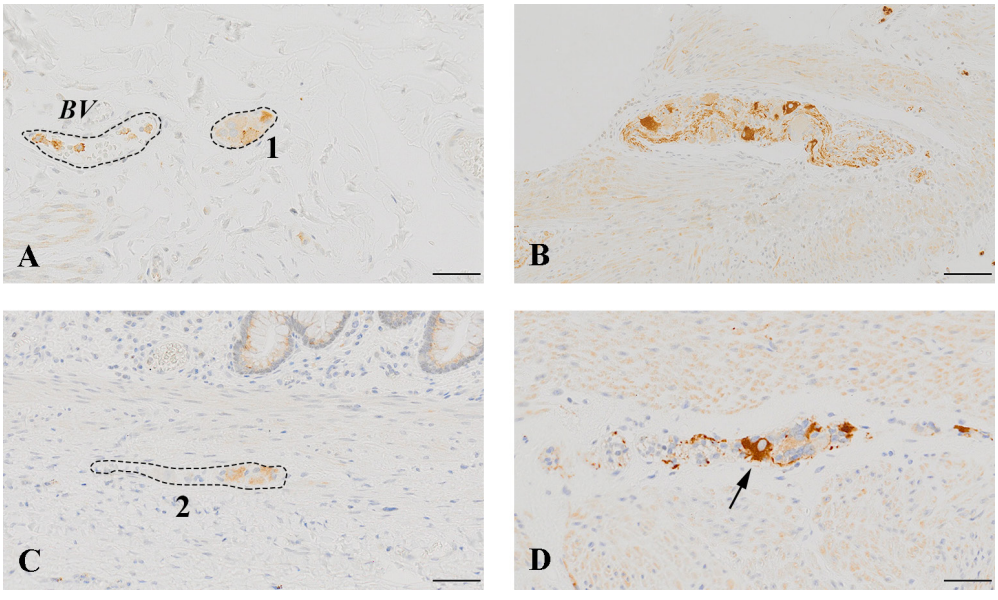

Fig. 3. NOS1-immunohistochemistry in the submucosal plexus (**A**) and the myenteric plexus (**B**) of the control group, and submucosal plexus (**C**) and myenteric plexus (**D**) in the experimental group. There were no measurable morphological differences between the submucosal ganglia in the controls (A, 1) and in the experimental group (C, 2). Note that blood vessels also contain immunoreactive cells (*BV*). Although in general the myenteric ganglia also did not show differences in the two cohorts, the arrow indicates a myenteric neuron in the experimental group that notably showed higher density, which was a rare finding. Scale bars: A, C, D – 50 μm, B – 100 μm.

Discussion

In this study, we examined the expression of three histopathological markers. One of them, in the face of calretinin, has been used as a major diagnostic tool for intestinal congenital pathologies for decades [8]. Therefore, any findings registered by immunostaining can be quite revealing regarding any underlying condition. Another marker, NOS1, is widely used for scientific purposes but is rarely a major choice when

it comes to diagnostics of that particular branch of pathology. Most likely, that is due to its selectivity in terms of neuronal expression, being expressed only in inhibitory neurons [6]. Still, being a well-known neuronal marker it is important for it to be fully elucidated in light of the variable intestinal conditions. PTEN-immunoreactivity is one that has most definitely not received enough scientific attention. Therefore, this article presents a minor but foundational contribution to future research.

The patients that underwent the resection interventions presented with symptoms traditionally attributed to HD, defined histopathologically as aganglionosis in a variable intestinal extent, but mostly restricted to the rectum [2]. Traditionally, HD presents itself during the newborn period by delayed passage of meconium. However, there have been clinical cases of both children and adults that present with signs of functional intestinal obstruction but do not meet the criteria for HD [12]. These cases were commonly regarded as variants of HD [5]. Such include IND-B, IASA, hypoganglionosis and others, of which with highest occurrence is IND-B [5]. HD was readily excluded from the possible causes of the functional obstruction of the cohort of this study due to notable presence of ganglionic cells. It is therefore no surprise that IND-B was established as working diagnosis in these cases.

IND-B has been a subject of significant scientific controversy during the last three decades and remains an undefined histopathological phenotype of uncertain clinical importance. Moreover, it is well accepted that the morphological findings may very well represent deviations from normality [3]. The morphological diagnostic criteria for IND-B have been repeatedly modified over the years, making comparisons between studies difficult and thus increasing the doubts and controversies [4]. It is not a purpose of this study to discuss diagnostic accuracy. However, it is important to note that while IND-B has been described in adults, the typical age period for receiving such diagnosis remains early childhood. Moreover, the ganglionic hyperplasia that classically defines IND-B [9] was not observed in the current specimens.

We observed a clear contrast in immunohistochemical patterns between the control and experimental groups, particularly regarding PTEN and calretinin expression. Interestingly, despite these notable differences, the NOS1 staining patterns appeared relatively similar in both cohorts. Together, these findings suggest that the conditions mimicking HD may involve selective disruptions in the molecular and structural integrity of the enteric nervous system, rather than a generalized neuronal deficit.

A striking observation was the notable PTEN expression differences we observed. In both plexuses of the control group, it was significant, which aligns with the established role of PTEN in regulating neuronal cell growth and survival [11]. In contrast, the reduced PTEN expression in most of the experimental samples proposes a link between compromised PTEN pathways and the pathogenesis of the variants of HD. This downregulation could potentially cause abnormalities in neuronal development and maintenance, consistent with prior work that has investigated the critical function of PTEN in neural crest-derived tissues [10].

Additionally**,** calretinin immunohistochemistry revealed a robust staining in the control specimens - unsurprising, given calretinin's reputation as a reliable marker for neuronal identification [8]. In the experimental group, we observed markedly reduced calretinin staining intensity. Since the neuronal counts in the submucosal plexus of the experimental samples did not differ greatly from controls, the reduced staining may indicate a potential loss of functional connectivity due to alteration in intracellular

calcium-buffering mechanisms, which play a key role in neuronal signaling [13]. Additionally, this possibly indicates that these conditions may involve subtle deficits in protein expression rather than outright neuronal loss.

The isolated neurons registered in the *muscularis mucosae* were a peculiar finding as this is a reported morphological sign for IND-B [9]. However, it should be noted that while those could represent ectopic neurons, it is also fully possible for them to be an optical illusion of submucosal ganglionic cells residing intimately on on the inner aspect of the *muscularis mucosae.*

By contrast, NOS1 distribution and density patterns showed minimal differences between the two groups. In both cohorts, only a few neurons within the submucosal plexus stained positively for NOS1, while the myenteric plexus displayed a more robust reaction, with distinct beaded fibers consistently observed. This suggests that the nitric oxide-mediated pathways, at least based on NOS1 expression, could be relatively preserved in IND-B, hinting that not all neuronal subpopulations are equally vulnerable to the pathological processes.

Conclusions

Overall, this study demonstrates a complex interplay of molecular and structural alterations in the rectal neuronal structures of patients that underwent resection surgery, characterized by substantial reductions in PTEN and calretinin expression, yet largely preserved NOS1 reactivity. Understanding these specific changes holds significant relevance, as it may help refine diagnostic criteria for IND-B and related conditions and guide future research focused on targeted therapies. It is our belief that further investigations should explore the downstream pathways affected by PTEN and NOS1 dysregulation.

Acknowledgements. This study was fully funded by grant № D-177/03.08.2023 from Medical University – Sofia. It was performed under the permission of the heads of the *Dept. of Anatomy, Histology and Embryology*, Medical University of Sofia, *Dept. of General and Clinical Pathology*, Medical University of Sofia, *Dept. of General and Operative Surgery*, Medical University of Sofia, *Clinic of General and Liver Pancreatic Surgery*, University Hospital Alexandrovska Sofia. The authors want to express their special gratitude to all personnel of the above-mentioned departments.

References

1. **Doodnath, R., P. Puri**. Internal anal sphincter achalasia. – *Semin. Pediatr. Surg.,* **18**(4), 2009, 246-248.
2. **Hwang, S., R. P. Kapur**. Advances and pitfalls in the diagnosis of Hirschsprung disease. – *Surg. Pathol. Clin*., **13**(4), 2020, 567-579.
3. **Kapur, R. P., M. Reyes-Mugica**. Intestinal neuronal dysplasia type B: An updated review of a problematic diagnosis. – *Arch. Pathol. Lab. Med*., **143**(2), 2019, 235-243.
4. **Meier-Ruge, W. A., K. Ammann, E. Bruder, A. M, Holschneider, A. F. Schärli, P. P. Schmittenbecher, F. Stoss**. Updated results on intestinal neuronal dysplasia (IND B). – *Eur. J. Pediatr. Surg.,* **14**(6), 2004, 384-391.

5. **Puri, P., J. H. Gosemann**. Variants of Hirschsprung disease. – *Semin. Pediatr. Surg.,* **21**(4), 2012, 310-318.
6. **Sanders, K. M., S. M. Ward**. Nitric oxide and its role as a non-adrenergic, non-cholinergic inhibitory neurotransmitter in the gastrointestinal tract. – *Br. J. Pharmacol.,* **176**(2), 2019, 212-227.
7. **Serafini, S., M. M. Santos, A. C. A. Tannuri, C. Di Loreto, J. O. Gonçalves, U. Tannuri**. A new systematization of histological analysis for the diagnosis of Hirschsprung's disease. – *Clinics (Sao Paulo).*, **78**, 2023, 100198.
8. **Singh, S. K., U. K. Gupta, R. Aggarwal, R. A. Rahman, N. K. Gupta, V. Verma**. Diagnostic role of calretinin in suspicious cases of Hirschsprung's disease. – *Cureus*, **13**(2), 2021, 13373.
9. **Terra, S. A., P. L de Arruda Lourenção, M. G Silva, H. A Miot, M. A. M. Rodrigues**. A critical appraisal of the morphological criteria for diagnosing intestinal neuronal dysplasia type B. – *Mod. Pathol.,* **30**(7), 2017, 978-985.
10. **Terra, S. A., A. C. Gonçalves, P. L. T. A. Lourenção, M. A. M. Rodrigues**. Challenges in the diagnosis of intestinal neuronal dysplasia type B: A look beyond the number of ganglion cells. – *World J. Gastroenterol.,* **27**(44), 2021, 7649-7660.
11. **Terra, S. A., P. L. T. de Arruda Lourenção, M. A. M. Rodrigues**. PTEN Immunohistochemistry. – *Arch. Pathol. Lab. Med.,* **147**(5), 2022, 577-583.
12. **Vougas, V., K. Vardas, C. Christou, G. Papadimitriou, E. Florou, C. Magkou, D. Karamanolis, D. Manganas, S. Drakopoulos**. Intestinal neuronal dysplasia type B in adults: a controversial entity. – *Case Rep. Gastroenterol*., **8**(1), 2014, 7-12.
13. **Zemheri, E., P. Engin Zerk, C. Ulukaya Durakbasa**. Calretinin immunohistochemical staining in Hirschsprung's disease: An institutional experience. – *North Clin. Istanb.,* **8**(6), 2021, 601-606.