Challenges in the diagnosis of intestinal neuronal dysplasia type B: A look beyond the number of ganglion cells

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Abstract

Intestinal neuronal dysplasia type B (IND-B) is a controversial condition among gastrointestinal neuromuscular disorders. Constipation is its most common clinical manifestation in patients. Despite intense scientific research, there are still knowledge gaps regarding the diagnostic criteria for IND-B in the histopathological analysis of rectal biopsies. The guidelines published in the past three decades have directed diagnostic criteria for quantifying the number of ganglion cells in the nervous plexus of the enteric nervous system. However, it is very complex to distinguish numerically what is pathological from what is normal, mainly because of the difficulty in determining a reliable control group composed of healthy children without intestinal symptoms. Thus, a series of immunohistochemical markers have been proposed to assist in the histopathological analysis of the enteric nervous system. Several of these markers facilitate the identification of other structures of the enteric nervous system, in addition to ganglion cells. These structures may be related to the etiopathogenesis of IND-B and represent new possibilities for the histopathological diagnosis of this disease, providing a view beyond the number of ganglion cells. This review critically discusses the aspects related to the disease definitions and diagnostic criteria of this organic cause of constipation.

Key Words: Intestinal neuronal dysplasia type B; Constipation; Diagnosis; Gastrointestinal neuromuscular diseases
Terra SA et al. Challenges for diagnosis of IND-B

Core Tip: There are knowledge gaps regarding the diagnostic criteria for intestinal neuronal dysplasia type B (IND-B) in the histopathological analysis of rectal biopsies. Several immunohistochemical markers have been proposed to identify other structures of the enteric nervous system, beyond the ganglion cells. These structures may be related to the etiopathogenesis of IND-B and represent new possibilities for the histopathological diagnosis of this disease. This review critically discusses the aspects related to the disease definitions and diagnostic criteria of this organic cause of constipation.

INTRODUCTION

The histopathological diagnosis of colon diseases presenting with severe constipation in childhood has been a challenge in recent decades. Hirschsprung’s disease (HD), the best known among the gastrointestinal neuromuscular diseases, is defined by the absence of ganglion cells in the submucosal and myenteric plexuses of the enteric nervous system (ENS). Intestinal neuronal dysplasia type B (IND-B) is characterized by hyperplasia of the submucosal nerve plexuses[1-4] (Table 1).

IND-B was first described in 1970, when Nezelof et al[5] reported three cases of megacolon associated with hyperplasia of the myenteric nervous plexus. A year later, Meier-Ruge[6] defined it as a condition usually associated with low intestinal obstruction that could simulate HD but showed distinct histopathological characteristics such as hyperplasia of the nerve plexuses and increased activity of the enzyme acetylcholinesterase (AChE) in the parasympathetic nerve fibers of the lamina propria of the mucosa.

Almost 50 years have passed and, despite the intense scientific research conducted during this period, there are still uncertainties, including IND-B etiopathogenesis, diagnostic criteria, and therapeutic possibilities. Thus, IND-B is currently considered as a controversial condition among the differential diagnoses of organic causes of intestinal constipation[7-10].

Most patients with IND-B are children showing chronic constipation. The main symptoms are very similar to those observed in patients with HD. Delay in the meconium passage, abdominal distention, vomiting, and difficulty in eating can occur during the first days of life[7,9]. A portion of these patients can show symptoms throughout their lives, evolving to severe constipation refractory to different types of clinical treatment[7,9,11,12]. Severe symptoms such as episodes of enterocolitis, acute intestinal obstruction, volvulus, and intussusception, although rare, are possible complications in different age groups[12-14]. Recent studies have highlighted the growing number of cases in adults, some showing symptoms of constipation since childhood, and others with late onset of symptoms[15-19]. The oldest patient reported in the literature with a diagnosis of IND-B was 71 years old. This patient showed symptoms since childhood, with severe constipation for more than 60 years, including several hospitalizations and surgeries during this period[19].

Two therapeutic modalities have been used in patients with IND-B: conservative clinical treatment and surgical treatment[9,20]. Conservative treatment is based on changes in diet, laxatives, and enemas in cases of fecal retention[7,20,21]. Surgical treatment can be performed through sphincterotomy, extensive surgical resection, or temporary colostomy[22-25]. However, the outcomes obtained using different treatment modalities show conflicting results[11,20,21]. Currently, the most accepted trend is that patients diagnosed with IND-B who have no complications must receive conservative treatment[1,3,10,20].
**Table 1 Comparison between Hirschsprung’s disease and intestinal neuronal dysplasia type B regarding the main clinical and histopathological aspects**

| HD                                      | IND-B                                      |
|-----------------------------------------|--------------------------------------------|
| Histopathological findings on rectal biopsies | Absence of ganglion cells in the submucosal and myenteric plexuses |
| Clinical Picture                        | Hyperplasia of the submucosal nerve plexuses |
| Treatment                               | Neonatal bowel obstruction or severe constipation |
|                                        | Surgical (colorectal pull-through)          |
|                                        | Diet, laxatives, enemas or surgical (if complications) |

HD: Hirschsprung’s disease; IND-B: Intestinal neuronal dysplasia type B.

**CONTROVERSIES REGARDING THE EXISTENCE OF IND-B**

One of the main controversies in the understanding of IND-B is related to the existence of a cause-effect relationship between the histopathological findings and clinical symptoms. In most cases, a diagnosis of IND-B is based on the histopathological analysis of rectal biopsies in patients with constipation that is usually refractory to clinical treatment. A minority of patients have acute intestinal obstruction or enterocolitis[7,9]. In contrast, histopathological alterations compatible with the diagnosis of IND-B were observed in the colon segments of 36 asymptomatic children[26]. Other studies have failed to demonstrate a direct correlation among histopathological findings, clinical symptoms, and radiological and manometric changes[27,28]. Given these controversies, some authors show skepticism concerning the definition of IND-B as a true clinical condition, preferring to define it as a histopathological alteration of the ENS, which may not present symptoms[7,10,29].

In a recent review on the subject, Kapur and Reyes-Mugica[10] concluded that IND-B remains an undefined histopathological phenotype of uncertain clinical relevance, and that it is imprudent to make clinical decisions based on this histopathological diagnosis, while claiming that this type of histopathological finding may represent deviations from normality. Moreover, these authors criticize the most recent scientific publications that investigated patients with this diagnosis, arguing that they have perpetuated the debate on dubious and controversial concepts. However, in medical practice, we continue to find children with severe constipation or intestinal obstructions who undergo diagnostic investigation for HD and, in the histopathological analysis of rectal biopsies, present ganglion cells hyperplasia in the nervous plexuses of the submucosa. Schmittenbecher et al[11] considered that these morphological changes were compatible with a diagnosis of IND-B, which should be considered as a distinct clinical condition, since the symptoms presented by the patients were evident and often persisted for several years, directly influencing their quality of life. The authors argued that patients with IND-B should be treated in a specific manner and highlighted the need to develop specific treatment algorithms. The majority of experts who participated in the Fourth International Symposium on Hirschsprung’s disease and related neurocristopathies in Genova, Italy, in 2004 and in a recent survey by the European Association of Pediatric Surgeons, consider IND-B to be a clinicopathological condition, justifying the need for further studies on this disease[30,31].

**LACK OF DIAGNOSTIC CRITERIA**

The lack of well-established criteria for the histopathological diagnosis of IND-B can be considered as the basis for many of the uncertainties described. To understand any disease, it is mandatory to know how to diagnose it. In the case of IND-B, the morphological criteria for histopathological diagnosis have been modified over the years, making comparisons between studies difficult and increasing the controversies and doubts concerning its existence[7,32,33]. Hyperplasia of the nervous plexuses of the ENS is a morphological finding that defines IND-B (Figure 1), but it is differently characterized by varying criteria[6,9].

Based on the 1990 Frankfurt Consensus discussions, Borchard et al[32] established morphological criteria for the histopathological diagnosis of IND-B by using biopsies
of the rectal wall (Table 2). Since then, these criteria have been widely used, both in clinical practice, follow-up studies and in investigations into the pathophysiology and etiopathogenesis of IND-B[24,27,34].

New diagnostic criteria were proposed in the 1990s, highlighting the need to identify giant ganglia in the submucosa, characterized by the presence of a minimum number of ganglion cells, ranging from six to more than 10 per ganglion[35,36]. In 2004, Meier-Ruge et al.[33] proposed a quantitative criterion for the histopathological diagnosis of IND-B (Table 2). Since this diagnosis depends on quantitative data, which can be compromised by technical variables, the entire laboratory process must be highly standardized. The biopsy must be adequate to obtain a sufficient quantity of submucosal tissue for analysis, and the frozen sections must be 15-µm thick and analyzed for a specific histochemical panel composed of lactate dehydrogenase, succinyl dehydrogenase, and nitric oxide (NO) synthase[1,33]. Although these criteria have been accepted by the scientific community, they were used in few case reports[37]. The need for fresh material and specific histochemical stains, the availability of which is restricted to certain centers, is a major limitation for diagnosis[3,38,39]. It is also uncertain whether these criteria can be applied to 5-µm thick histological sections from paraffin-embedded material used for conventional histological analysis with hematoxylin and eosin (H&E) and immunohistochemical methods[4,39]. Taguchi et al.[37] analyzed data from 161 Japanese gastroenterological and pediatric surgery institutions from January 2000 to December 2009 and found 355 patients with HD and allied disorders. Of these, 13 patients were diagnosed with IND-B based on the histopathological criteria commonly used, but only four met the quantitative criteria proposed by Meier-Ruge et al.[33]. Terra et al.[39] analyzed surgical specimens from 29 patients diagnosed with IND-B, previously established by the criteria of the Frankfurt Consensus[32], in the histological sections processed for conventional histology by H&E. Only one patient met the numerical diagnostic criteria proposed by Meier-Ruge et al.[33]. The authors concluded that the recommended quantitative criteria for 15-µm cuts stained by specific histochemical panels have limited applicability when transposed to conventional histological analysis.

Thus, the changes proposed in the last three decades have oriented the diagnostic criteria for quantifying the number of ganglion cells in the nervous plexuses of the ENS[33,35,36]. However, it is very complex to numerically distinguish the pathological from the normal, mainly because of the difficulty in determining a reliable control group composed of healthy children without intestinal symptoms[1,10,40]. Obtaining intestinal samples for use as controls has ethical limitations because of the need for invasive procedures to obtain tissue samples. In some studies, the control group was composed of intestinal samples from patients with congenital intestinal malformations such as anorectal abnormalities, which cannot be considered healthy controls[41]. In addition, the variation in the number of ganglion cells related to age, especially during the first year of life, leads to the need for age-matched controls, making it difficult to develop an appropriate control group[26]. The thickness of the histological section, the staining techniques, and the methods of histopathological analysis directly influence the number of ganglion cells identified in the count[38,39]. For all these reasons, the quantitative criteria seem to have contributed to more uncertainty rather than greater objectivity for the diagnosis of IND-B. There are important differences regarding the minimum number of ganglion cells and nerve ganglia, questions concerning the different methods of histopathological analysis, and the possibility of a minimum age

Figure 1 Intestinal neuronal dysplasia type B. Submucosal nerve plexus with hyperganglionosis: giant ganglion. Ganglion cell (arrow) (H&E, 400 ×).
Patients must be older than 1 yr

At least 20% of giant nerve ganglia

**Table 2 Summary of the histopathological criteria for the diagnosis of intestinal neuronal dysplasia type B**

| Diagnostic criteria          | Definition                                                                 |
|-----------------------------|---------------------------------------------------------------------------|
| Frankfurt Consensus[32], 1990 | Mandatory criteria Submucosal plexus hyperplasia                          |
|                             | Neuronal heterotopy                                                       |
|                             | Increased activity of the AChE enzyme in nerve fibers around submucosal blood vessels |
| Meier-Ruge et al[33], 2004  | Quantitative criteria At least 20% of giant nerve ganglia in the submucosa, in 25 submucosal ganglia |
|                             | Age criteria Patients must be older than 1 yr                            |

1Giant nerve ganglia: A ganglion with more than eight ganglion cells.

**AChE**: Acetylcholinesterase.

for diagnosing this disease[39].

**UNCERTAINTIES AND RECENT ADVANCES**

Pathophysiology and etiopathogenesis are poorly defined aspects of IND-B[7,30]. Some authors consider these aspects as part of the normal development of the ENS[3]. As age advances, there is an increase in the size of ganglion cells and a decrease in their number per plexus[26,36,40]. These findings can be related to a physiological process of maturation, which involves the apoptosis of components of the ENS[1,33]. Schimpi et al[21] retrospectively analyzed 105 patients with IND-B: 60.95% of patients were less than 6 mo old, 30.47% were between 6 and 12 mo old, and only 8.57% were more than 1 year old. This percentage distribution, which decreases with age, suggests a maturation process of the ENS or some abnormality in the development of the intestinal nervous system, with spontaneous normalization with advancing age.

Other studies have raised the possibility that IND-B represents an adaptive response of the ENS, secondary to obstructive or inflammatory bowel phenomena that occur in the fetal, perinatal, or postnatal period. There are reports of morphological findings suggestive of IND-B in the intestinal areas immediately above intestinal atresia, rectal mucosa prolapse, imperforate anus, and necrotizing enterocolitis[27,42,43]. These secondary responses to obstructions or inflammations have been studied experimentally, and the results are conflicting[44,45]. Using a model of partial intestinal colon obstruction in rats, Moore et al[46] showed a decrease in the number of ganglion cells compared to that in the control group, explained by an increase in colon diameter due to the distention of its wall. Using a model of chronic intestinal obstruction in adult rats, Gálvez et al[45] identified histopathological changes compatible with IND-B, with an increase in the number of ganglion cells. Recent studies have shown that IND-B can originate from genetic alterations, which directly influence the embryological development of tissues derived from the neural crest. Angelini et al[41] showed significantly unregulated expression of microRNAs in the submucosal and muscular layers of the colon and peripheral blood of patients with IND-B. The molecular pathways biologically regulated by these microRNAs (axon guidance, nerve growth factor signaling, neuronal cell adhesion molecule (NCAM) signaling for neurite outgrowth, neuronal system, and apoptosis) show activities related to the ENS and, therefore, can be related to the pathogenesis of IND-B. Liu et al[46] showed a decrease in the methylation levels of locus 32 on the Sox10 promoter gene in the peripheral blood of patients with IND-B. There was a negative correlation between these levels and Sox10 expression in the colons of these patients. These changes can contribute to the regulation of the number of intestinal glial cells and the maturation of neurons in the ENS[46].

In addition, reports on familial occurrence of IND-B and its association with other intestinal and extraintestinal diseases reinforce the theory that IND-B has a primary, genetically determined origin[34,47-49]. Among these associations, HD, incomplete intestinal rotation, and multiple endocrine neoplasia type 2 (NEM 2) are the most important[23,28]. Histopathological findings compatible with IND-B in segments proximal to areas of aganglionosis are not uncommon and have been considered as a possible cause of the persistence of obstructive symptoms in patients undergoing surgical treatment for HD.
This association was first reported in the 1970s[50]. Since then, the association between IND-B and HD has been described with rates ranging from 6% to 75% of HD cases[38, 51,52]. In these cases, the presence of colonic segments with IND-B can be explained both by primary embryological alteration of the ENS, which gives rise to neuropathies, and by secondary adaptation to distal intestinal obstruction, caused by the spastic aganglionic intestinal segment[22,53].

**IMMUNOHISTOCHEMISTRY AND LOOKING BEYOND THE NUMBER OF GANGLION CELLS**

Over the past two decades, a series of immunohistochemical markers have been proposed to assist in histopathological analysis of the ENS[2,4,54-57]. The use of calretinin, well established in HD, is the primary example of how an immunohistochemical method can contribute substantially to the diagnosis of enteric neuropathy. The absence of immunohistochemical expression of calretinin in colorectal aganglionic segments has been routinely used and is part of the main guidelines for HD diagnostic management[58,59]. Terra et al[39] identified the expression of calretinin in ganglion cells and mucosal nerve fibers in 29 patients with IND-B. Immunoeexpression of this marker was observed in ectopic neurons in the lamina propria and in the muscularis mucosa (Figure 2), which was not previously identified using standard histology (H&E).

Several studies have assessed the role of immunohistochemical methods both in investigating aspects related to etiopathogenesis and in improving the histopathological diagnosis of IND-B. These studies[39,46,54,60-71] are summarized in the table presented in Supplementary Table 1. The pattern of immunoeexpression of the main markers described in patients with IND-B is shown in Table 3.

Bosman et al[66] identified an increase in the number of ganglion cells, with positive expression of NO synthase in the intestine of patients with IND-B compared to that in the control group. This enzyme is responsible for the synthesis of NO, a neurotransmitter capable of inducing smooth muscle relaxation that may be responsible for the clinical symptoms of intestinal pseudo-obstruction presented by patients.

Yamataka et al[62] showed a reduction in c-kit and 171B5 (synaptophysin) expression in the muscle layers, with the exception of the myenteric plexuses, in the intestine of patients with IND-B compared to that in the control group. The c-kit protein is expressed in the interstitial cells of Cajal and is considered to be a regulator of gastrointestinal tract motor activity[72,73]. Synaptophysin is expressed in the protein membrane of synaptic vesicles and is present during the neurotransmission of the central and peripheral nervous systems, including the ENS. The reduction in the expression of these two markers raises the hypothesis of the role of interstitial cells of Cajal in the pathophysiology of IND-B[62].

Kobayashi et al[63] reported that the markers NCAM, growth-associated protein 43 (GAP43), and synaptophysin showed absence of expression or markedly decreased expression in the muscular layers of the intestine of patients with IND-B compared to that in the control group. No change was observed in the expression of these markers in the nervous plexus of the ENS. NCAM is a surface glycoprotein that is important for the interaction between neurons and muscle cells during synaptogenesis and is considered as a marker of the neuromuscular junction[74]. GAP43 is a marker associated with neuron development and regeneration during axonal growth and is found in high concentrations in presynaptic areas[63]. These changes in the expression patterns of these markers led the authors to propose the hypothesis that patients with IND-B presented defects in the innervation of neuromuscular junctions, which would explain the changes in colonic motility. However, Nogueira et al[65] were unable to reproduce this pattern of NCAM and synaptophysin expression, weakening this hypothesis.

Kobayashi et al[64] showed an increase in the number of mast cells, identified using immunohistochemical methods, in the intestinal segments of patients with IND-B and in the aganglionic segments of HD patients compared to that in the normoganglionic segments of HD patients and individuals in the control group. Mast cells were also identified by the marker nerve growth factor (NGF) close to the nerve ganglia in patients with IND-B and close to the hypertrophied nerve trunks in the aganglionic segments of HD. Based on these observations, the authors suggested the possible participation of mast cells in the pathogenesis of IND-B and HD.
**Table 3 Immunohistochemical markers for the diagnosis of intestinal neuronal dysplasia type B**

| Immunohistochemical marker | Immunoeexpression pattern |
|---------------------------|--------------------------|
| Bcl-2 [54]                | Positive expression in immature and mature ganglion cells |
| Calretinin [39, 54]       | Positive expression in mature ganglion cells in submucosal and myenteric plexuses |
|                          | Positive expression in intrinsic nerve fibers and ectopic neurons in the mucosa |
| c-kit [60-62]             | Positive expression in Cajal cells in muscle layers and myenteric nerve plexuses |
| GAP43 [63]                | Absence of expression in muscularis mucosae and/or in the circular muscular and/or longitudinal muscular |
|                          | Positive expression in nerve plexuses |
| Hu C/D [38]               | Positive expression in mature ganglion cells in submucosal and myenteric plexuses |
| NGF (mast cells) [64]     | Expression of mast cells close to ganglia |
| NCAM (CD56) [60, 61, 63, 65] | Positive expression in neurons of the muscular layers |
| NO synthase [66]          | Variable expression in nerve fibers in muscle layers and mucosa |
| NSE [61]                  | Positive expression in nerve fibers in the muscle layers and mucosa |
| Peripherin [66, 67]       | Positive expression in ganglion cells |
| PGP9.5 [60, 68]           | Positive expression in enlarged nerve trunks, hyperplastic nerve ganglia, and heterotopic nerve cells |
|                          | Positive expression in neurons of the inner intestinal muscle layer |
| PTEN [69]                 | Reduced expression in submucosal and myenteric nerve plexuses |
| RET [54, 66]              | Positive expression in mature and immature ganglion cells |
| SI100 [60, 61, 66]        | Positive expression in ganglion cells |
| Synaptophysin [60, 62, 63, 65, 66, 70] | Positive expression in synaptic vesicles in plexuses and nerve fibers |
| SMA (Isa4) [61, 71]       | Positive expression in nerve fibers from muscular layers and muscularis mucosa |
| Sox10 [46]                | Positive expression in glial and ganglion cells in nerve plexuses |

Bcl-2: B-cell lymphoma 2; c-kit: Transmembrane tyrosine kinase receptor; GAP43: Growth associated protein 43; Hu/CD: RNA binding protein Hu (HuC and HuD) - also referred to as type-1 anti-neuronal nuclear antibodies (ANNA-1); NGF: Nerve growth factor; NCAM: Neural cell adhesion molecule; NO: Nitric oxide; NSE: Neuron-specific enolase; PGP9.5: Protein gene product 9.5; PTEN: Phosphatase and tensin homologue deleted on chromosome 10; RET: Rearranged during transfection - immunohistochemical detection of RET proto-oncogene; SMA: Smooth muscle antibody; Sox10: SRY-box transcription factor 10.

O’Donnell and Puri [69] evaluated the immunohistochemical expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in patients with IND-B. PTEN is a protein involved in cell proliferation, survival, and migration and has a potential modulatory role in neurogenesis and synaptic plasticity. The authors showed significantly reduced expression of this marker in the myenteric and submucosal plexuses of the intestine of patients with IND-B, absence of PTEN expression in the aganglionic segments of HD patients, and strongly positive expression in ganglionic intestinal segments of HD patients and individuals in the control group. Based on these results, the authors suggested that the giant ganglia with immature cells, present in IND-B, might be associated with a reduction in PTEN level, which might be responsible for the lack of control in neuronal growth and proliferation.

Some markers have demonstrated the potential to assist in neuron counting, which is fundamental for the quantitative diagnosis of IND-B. Geramizadeh et al. [60] evaluated the immunohistochemical expression of the protein gene product 9.5 (PGP9.5), a specific cytoplasmic marker of the nervous system, SI100, a nerve cell nucleus and cytoplasm marker, c-kit, synaptophysin, and NCAM to count nerve cells in the muscle layers and myenteric plexuses of the distal colon of patients with HD,
IND-B, and the control group. The authors showed a significantly higher number of nerve cells, stained by these immunohistochemical methods, in the muscle layers and/or the myenteric plexuses in patients with IND-B than in HD patients. However, this difference was not significant when patients with IND-B were compared to those in the control group. Kim et al.[61] showed no significant differences in the counts of interstitial cells of Cajal (c-kit immunopositive) in muscle layers and myenteric plexuses in patients with IND-B compared to those in patients with other types of intestinal pseudo-obstructions.

Swaminathan et al.[38] evaluated the use of the pan-neuronal immunohistochemical marker Hu C/D in the quantitative analysis of ganglion cells in colonic segments with IND-like submucosal ganglion cell hyperplasia at the proximal margins of HD resections. This marker detects neuronal cell bodies, facilitating the recognition of neurons, and has been used as a neuronal marker for the central and peripheral nervous systems[75]. By counting the nuclei of ganglion cells immunointensive for Hu, it was possible to develop and validate a quantitative histopathological criterion for the diagnosis of giant ganglia, defined by the presence of at least seven ganglion cells[38].

Wang et al.[54] determined the number of ganglion cells and the area of myenteric nerve plexuses using immunohistochemical expression of the RET protein, which is encoded by the RET gene and is related to cell growth and differentiation, and of B-cell lymphoma 2 (Bcl-2), a protein encoded by the homonymous gene located on chromosome 18q21, which plays a role in the maintenance of cell survival. The authors identified a significantly higher number of ganglion cells and a larger myenteric plexus area in the colon of patients with IND-B than in that of the control group.

CONCLUSION

IND-B remains a controversial clinicopathological condition, with a poorly understood etiopathogenesis and a series of challenges related to its diagnosis. There is no consensus regarding the criteria used by different centers for the histopathological diagnosis of IND-B. Despite these uncertainties, patients continue to show severe constipation or intestinal obstruction, associated with histopathological alterations compatible with this morphological phenotype, which fully justifies all the efforts directed toward new diagnostic strategies. Only the correct diagnosis of these patients can lead to more effective treatment and better prognosis. In this regard, immunohistochemical techniques have identified other ENS structures, in addition to the ganglion cells of the submucosa and muscular nerve plexuses. These structures may be potential candidates for biomarkers in the histopathological diagnosis of IND-B, expanding the horizon beyond counting the number of ganglion cells.

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