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A study of calretinin in Hirschsprung pathology, particularly in total colonic aganglionosis

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Abstract

Introduction: Calretinin, a calcium-binding protein, has been reported to be an important new marker in Hirschsprung's disease (HD). The aim is to study the diagnostic value of Calretinin in total colonic aganglionosis (TA), prematurity, and superficial biopsy when nerve hyperplasia may not be accessed by ACE activity.

Methods: Records of patients diagnosed with HD at our institution from 1985 to 2010 were studied and patients with TA identified. We examined tissue samples from those TA, partial colectomies for HD, biopsies for suspicion of HD, and rectal tissue from aborted fetuses. Immunohistochemical analysis of Calretinin was compared with ACE gold standard method in all cases.

Results: In the majority of the cases, the diagnosis was ascertained by ACE activity and Calretinin staining. However, in 9 cases, the diagnosis was possible with Calretinin staining but not with ACE: in 4 TA because of the absence of nerve hyperplasia, and in 5 cases because the biopsies were too superficial to examine the nerve hyperplasia. In addition, Calretinin was expressed in the gut as early as 22 gestational weeks.

Conclusion: The use of Calretinin staining may be superior to ACE activity, particularly in the context of TA, superficial biopsies, and prematurity, allowing earlier diagnosis.

Hirschsprung's disease is an intestinal disorder characterized by the failure of migration of the neural crest cells during fetal development that results in an absence of ganglion cells. The first reports of this disease date back to 1691 [1]. The "gold standard" for the diagnosis of Hirschsprung's disease (HD) is the pathologic evaluation of a rectal biopsy that includes the identification of ganglion cells and nerve fibril hypertrophy as indicated by positive acetylcholinesterase (ACE) activity [2]. However, if the addition of ACE staining assists in specificity, it does not perform as well in sensitivity [3,4]. False negative results can lead to a delay in diagnosis and subsequent treatment, particularly in the setting of total colonic aganglionosis and prematurity, in which ACE positive nerve hyperplasia could be absent [3,4]. Furthermore the evaluation of ACE activity can be difficult to carry out and interpret [5].

Calretinin is a vitamin D-dependent calcium-binding protein [6,7]. Due to its capacity to be expressed in nerve fibrils and ganglion cells in the submucosa and myenteric

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plexus throughout the normal colon and bowel, calretinin has been recently described as an adjunctive diagnostic test in HD [8–13]. Moreover, work done by Holland et al. [8] demonstrated that there was a positive correlation between pathologists' scores and the diagnosis of Hirschsprung's disease, which was highly significant. They also stated that calretinin is a reproducible modality in the evaluation of Hirschsprung's disease.

In this study we aim to better identify the value of adding calretinin in challenging pathologic evaluations for HD in which ACE studies may be less reliable, such as in total colonic aganglionosis, superficial biopsies and suction rectal biopsies in premature infants.

1. Materials and methods

Approval for this study was obtained from our research ethics committee. We retrospectively reviewed the charts of patients diagnosed with HD at our institution between January 1985 and December 2010. We identified 196 patients with confirmed HD. We retrieved the tissue blocks of the biopsies performed on 17 patients with total colonic aganglionosis, for which we could study the entire intestinal wall of the specimen (Table 1).

Prospectively, between 2010 and 2012, we examined all of the partial colectomies performed at our institution for HD (n=5) to study the normal, aganglionic and transition zones throughout the entire intestinal wall. In addition, we analyzed all of the rectal biopsies performed for suspicion of HD with calretinin in addition to the standard pathological examination, which comprised a total of 25 biopsies. Of these 25 biopsies, 7 were positive for HD and 18 were normal. (Table 1) Furthermore, we analyzed intestinal and rectal samples of 4 fetuses from terminated pregnancies ranging between 22 and 26 weeks gestational age.

1.1. Pathological analysis

Rectal biopsies were analyzed in the standard fashion by assessing acetylcholinesterase activity in frozen biopsies to identify extrinsic nerve fibril hyperplasia, and Methyl Green Pyronine (MGP) staining for the evaluation of ganglion cells [5,8]. This initial work was followed by an evaluation of at least ten, 3 μm-thick paraffin-embedded hematoxylin–phloxin–safran sections to screen for the presence or absence of ganglion cells (Fig. 1A, B).

Table 1 Rectal biopsies analyzed.

Rectal biopsies analyzed	Number	Superficial biopsies
Total colonic aganglionosis (1985–2010)	17	2
Hirschsprung's Disease (2010–2012)	7	0
Normal (2010–2012)	18	3
Total	32	5

Subsequently, immunohistochemistry (IHC) was performed on paraffin-embedded sections, using an automated Ventana immunostainer (Benchmark XT Ventana Medical System Inc., Tucson, AZ) according to the company's protocols with minor modifications. The antibody used in our study was calretinin (polyclonal antibody, Zymed laboratories Inc., South San Francisco, California, USA) [10,12].

Staining interpretation/evaluation (Fig. 2A, B, C, D):

Calretinin immunostaining was evaluated without knowledge of the diagnosis. Two pathologists independently performed this analysis with expertise in HD (DBDS and NP). They were required to document the location and intensity of the staining as defined below:

Calretinin was considered positive if any of the specific findings below were present:

1. granular staining of nerve fibrils in the mucosa, the muscularis mucosae and the superficial area of submucosae; and
2. diffuse intense cytoplasmic or nuclear ganglion cells staining in the myenteric plexus.

Calretinin positivity was non-specific when we observed (Fig. 3A, B):

1. granular calretinin staining of extrinsic nerve fibres which was always observed (in patient with HD and patient without HD) within the submucosae or serosal nerve plexus,
2. a weak and homogeneous immunoreactivity in mast cells. Brown peroxidase was exchanged with fast red when it was difficult to interpret.

Calretinin expression was compared to the gold standard that evaluated the distribution of MGP on ganglion cells and ACE activity.

2. Results

In our retrospective review we identified 196 patients with confirmed Hirschsprung's disease with a ratio of 3:1 male to female. Of these patients, we confirmed 17 (8%) patients with total colonic aganglionosis of whom 11 were male and 6 patients were female (ratio = 1.8:1). In 6 (35%) of these patients, the diagnosis was delayed and could have been made earlier with calretinin, as nerve hypertrophy (initially studied with ACE activity) was absent or minimal in 4 patients and the biopsy was too superficial to allow for analysis of ganglion cells for 2 patients. Calretinin confirmed the diagnosis of HD in these biopsies.

In addition, we collected 5 specimens from partial colectomies for Hirschsprung's disease that enabled assessment of the normal, aganglionic colonic zones and the transition

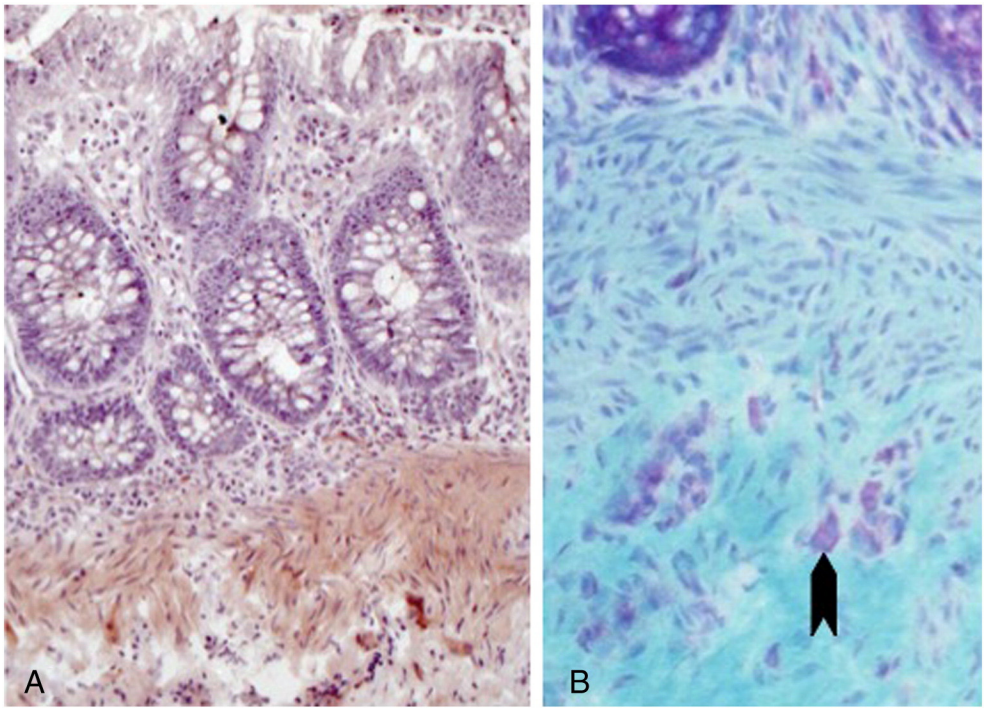

Fig. 1 (×200): normal rectal biopsy (A) acetylcholinesterase activity without hyperplasia and (B) ganglion (arrow head) cell with Methyl Green Pyronin.

zones with calretinin. We also examined 25 biopsies done for suspicion of HD; of these, 7 were confirmed to have HD. In the group of 18 normal biopsies, 3 specimens (12%) were noted to be too superficial to assess ACE activity. A superficial biopsy was defined as a biopsy that did not include the submucosa or muscularis mucosa. Calretinin was able to exclude the diagnosis of HD in all of these superficial biopsies. Calretinin demonstrated positive staining for nerve fibrils in all of the fetal colonic samples.

2.1. Ganglionic colonic zones (Fig. 4A, B, C, D)

Normal colonic zones demonstrated moderate numbers of intensively granular immunolabelling nerve fibrils in the full thickness of the lamina propria, muscularis mucosae, and superficial submucosa. When cell bodies of submucosal ganglion cells were present, intense homogeneous nuclear and cytoplasmic reactivity was noted. This was visualized in the normal colonic zones of the partial colectomies and fetal colonic tissue as early as 22 weeks gestation.

The identification of immunoreactive nerves fibrils in the mucosa, superficial submucosa and muscularis mucosae was considered to be the key in the interpretation of calretinin expression, irrespective of the distribution or the number. The staining, albeit sparsely distributed, could not be missed at high magnification in the normal colonic zone, the fetal colonic specimens and the normal biopsies. A heterogeneous distribution of nerve fibrils positive for calretinin in the mucosa, superficial submucosa and muscularis mucosae was also noted in normal colonic tissue.

For all of these same specimens, MGP exhibited some ganglion cells and ACE activity did not reveal extrinsic nerve fibril hyperplasia. In biopsies that were superficial, where ACE activity could not be assessed, calretinin staining revealed nerve fibrils, thus excluding the diagnosis of HD.

2.3. Aganglionic colonic zone (Fig. 5A, B)

No positive calretinin immunoreactivity, as defined above, was observed in mucosa, submucosa and muscularis mucosae of aganglionic biopsies or sample tissues from HD patients. However, large extrinsic nerves in the submucosa showed a non-specific moderately punctuated immunoreactivity of the axons, as did normal serosal nerves. The corresponding specimens showed extrinsic nerve fibril hyperplasia with ACE staining and no ganglion cells with MGP.

2.4. Transitional zones

The transitional zones were studied from the pathologic segment towards the normal colon. The nerve fibrils in the mucosa appeared first, then in the ganglion cells of Meissner's submucosal myenteric plexus, followed by the ganglion cells in Auerbach myenteric plexus.

2.5. Frozen sections

Calretinin was never visualized significantly in the frozen specimens. Therefore, it was unreliable when performed on a frozen section for diagnostic purposes.

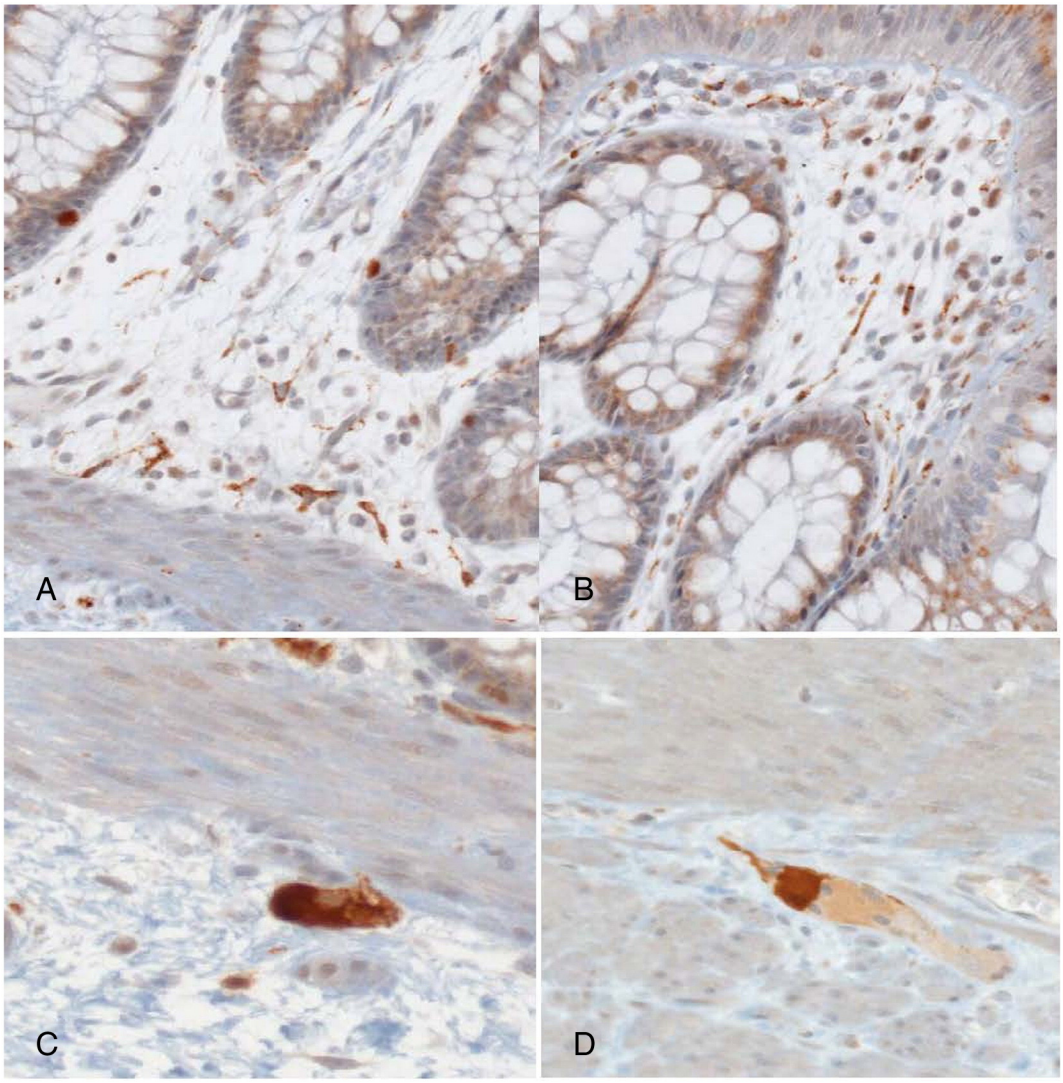

Fig. 2 (×200): normal calretinin reactivity with a granular staining of nerve fibrils in the entire mucosa, and diffuse intense cytoplasmic and/or nuclear in a part of ganglion cells in myenteric plexus (C; Meissner, D: Auerbach).

3. Discussion

An understanding of the cause of constipation and megacolon in HD did not appear until the 1920s when Valla and his associates described the absence of ganglion cells in the myenteric plexus of the distal colon in two brothers with megacolon [14]. It is well known that the identification of ganglion cells in biopsies can be very intricate and laden with technical and interpretive challenges. There are numerous studies identifying different immunohistochemical stains to assist in identifying ganglion cells, or extrinsic nerve fibril hyperplasia staining based on the enzyme histochemical method of Karnovsky and Roots [12]. Nakao et al. [4] described the specificity and sensitivity of ACE to be as high as 100% and 91%, respectively. This quick and simple technique provides a diagnosis of HD by demonstrating extrinsic nerve fibrils, but requires a frozen section. The limitations of this method are evident when variability of cholinergic innervation is present in cases of prematurity, total colonic aganglionosis or superficial biopsies, and thus leads to the need for repeated biopsies in those patients [15].

Recently, in a few studies, calretinin has been identified as a prominent immunohistochemical diagnostic tool in the study of Hirschsprung's disease. In a small series by Kapur et al., calretinin was an equal and potentially superior alternative to ACE as an adjunctive diagnostic test [12]. In a larger series of rectal suction biopsies by Guinard-Samuel et al. [10], the authors reported that a pathologist could identify HD with no false positives using only immunohistochemistry with calretinin, and that the concordance between calretinin and the gold standard was excellent. The study by Holland et al. demonstrated excellent correlation between pathologists' scores using calretinin and the diagnosis of Hirschsprung's disease with high statistical significance [13]. In our study, the inter-observer

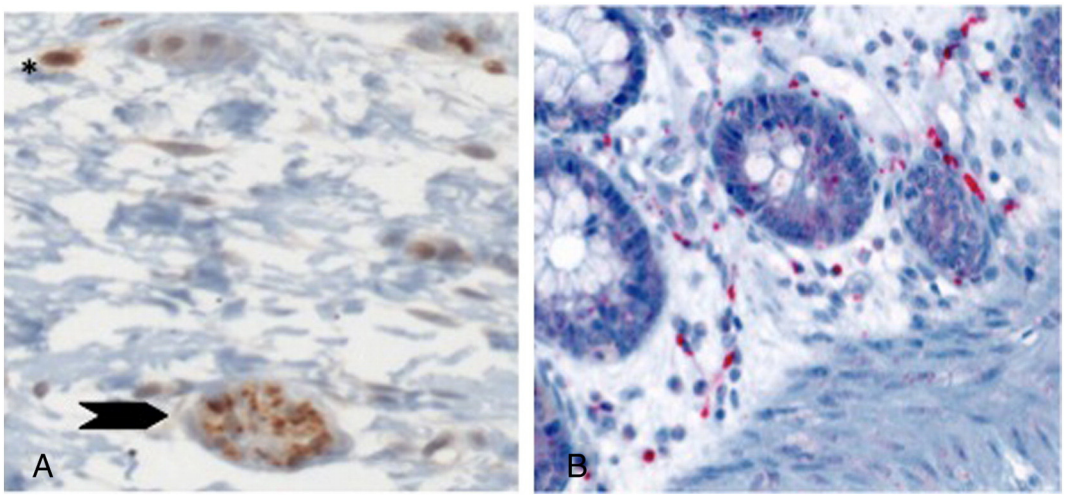

Fig. 3 (A ×200) Calretinin was considered as non-specific for HD diagnosis when a granular staining of extrinsic nerve fibers was observed within the nerve plexus (arrow head). A weak and homogeneous immunoreactivity was observed in mast cells, and could potentially cause a wrong interpretation (*). (B ×200) This occurrence could be avoided if the brown peroxidase staining was exchanged with the fast red.

reliability was 100% and there were no false positives or false negatives.

Our results confirm previous reports and demonstrate numerous additional advantages in using calretinin. First, calretinin can be applied to sections from the same paraffin-embedded tissue used for conventional diagnosis. Another advantage is apparent in the investigation of inadequate specimens because of insufficient submucosa. In superficial biopsies, the normal calretinin reactivity in nerve fibrils can help to make a pathological assessment, thus

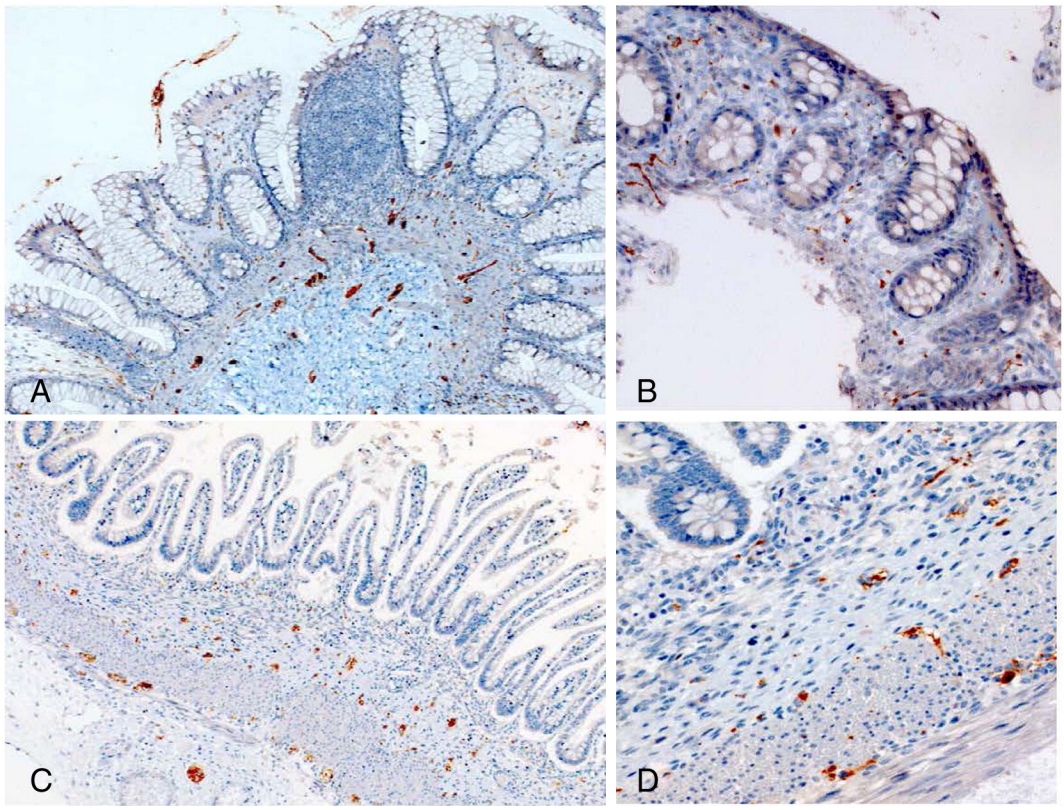

Fig. 4 Expression of calretinin in normal colonic zone of partial colectomies for HD in normal control biopsies (A ×100) and even superficial biopsies (B ×200). (C ×200, D ×400) Intestinal foetal tissues at 22 gestational weeks demonstrated granular immunolabelling nerve fibrils in the full thickness of the lamina propria, muscularis mucosae, and superficial submucosa and diffuse intense cytoplasmic and/or nuclear in a part of ganglion cells in myenteric plexus.

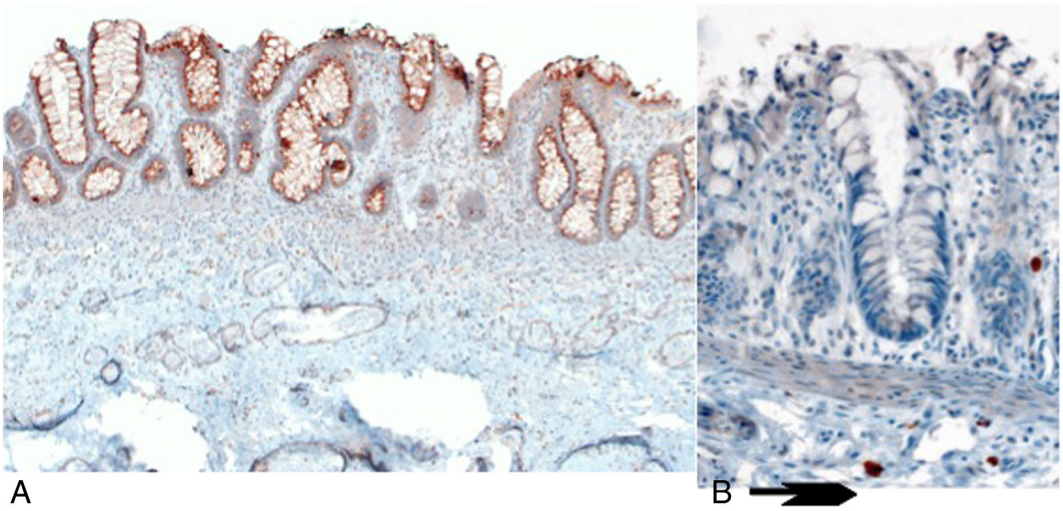

Fig. 5 In Hirschsprung Disease calretinin was negative, no nerve fibrils and no ganglion cells were observed (A ×50, B ×400). Only a few mast cells were noted (arrow head).

ruling out the diagnosis of HD. Finally, the immunoreactivity of calretinin present as early as 22 weeks gestational allows the diagnosis in very young infants, even in cases of prematurity.

In total colonic aganglionosis, we observed that the hyperplasia of acetylcholinesterase reactive nerve fibrils was not present in 4 cases leading to repeat biopsies. However, calretinin reactivity in the biopsies from these same patients, and even in the first biopsy, was totally absent, and thus the diagnosis of HD could have been ascertained earlier. Another advantage of this staining was the particular expression of calretinin in the transitional zone that appears first in the nerve fibrils of mucosa, then in ganglion cells of the Meissner plexus and finally in ganglion cells of the Auerbach plexus as the normal ganglionic zone is reached. This information could potentially help assess the level of the transition zone for extent of resection. Based on these results, we suggest that the staining pattern could give an approximate idea of the biopsies' location (transitional zone, versus normal zone).

Our findings indicate that calretinin should not be used in frozen sections, as it is unreliable in this setting. Frozen sections should only be evaluated for ACE activity. However, working with paraffin sections is advantageous as it maintains the integrity of the specimen's morphology, therefore reducing the need for frozen section analysis in general.

One potential limitation of calretinin staining for nerve fibrils is that it is occasionally very dispersed and difficult to analyze. However, the staining should not be missed at high magnification. As noted by other studies, a very rapid learning curve in the interpretation of these immunostaining patterns is possible [12].

In conclusion, we demonstrate that calretinin can facilitate the diagnosis of Hirschsprung's disease in the context of total colonic aganglionosis, and provide a diagnostic tool in the premature population where standard immunoenzymologic markers are often fraught with difficulty. Furthermore, in superficial biopsies, where there is often insufficient tissue to do a complete ACE staining and ganglion cell evaluation, we demonstrate that calretinin can be accurately used to diagnose HD. In those cases, calretinin provides an earlier and more reliable diagnosis, thereby reducing the need for repeat biopsies. Thus, our results demonstrate that calretinin can be extremely useful in the diagnostic arsenal of Hirschsprung's disease, particularly in the context of prematurity, superficial biopsies and total colonic aganglionosis.

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