



Original Research Article

To assess calretinin immunohistochemistry in Hirschsprung's disease and allied disorders

Pooja U Patil¹, Sharada Rane^{1,*}, Dasmit Singh Khokar²¹Dept. of Pathology, Byramjee Jeejeebhoy Government Medical College and Sassoon Hospital, Pune, Maharashtra, India²Dept. of Pediatric Surgery, Byramjee Jeejeebhoy Government Medical College and Sassoon Hospital, Pune, Maharashtra, India

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ABSTRACT

Aim: Hirschsprung's disease (HD) is the congenital aganglionosis of the human colon due to disruption of migration, imbalanced proliferation and differentiation of neural crest cell. The diagnosis and extent of resection for the management of HD depend on the sensitive and specific identification of ganglion cells. Negative immunohistochemical expression of calretinin, shown its superiority as diagnostic method for full thickness as well as partial thickness rectal suction biopsies.

Materials and Methods: Total 86 colonic and rectal biopsies of 43 patients attending Sassoon hospital, Pune (between 2015-2017) with surgically resected specimen proven Hirschsprung's disease cases and follow up cases of non HD cases were evaluated with both Haematoxylin and Eosin stain and calretinin IHC.

Result: Age group was 2 days to 7 year with having Male: Female ratio 2.91:1. Out of 43 cases, 26 were diagnosed as Hirschsprung's Disease, 16 were not having Hirschsprung's disease, 1 case of hypoganglionosis. 13 out of 86 biopsy and 9 out of 43 cases show false positive result with use of Haematoxylin and Eosin stain only. Specificity and negative predictive value of H&E stain was 72% and 78.12% respectively with accuracy of 86%. Use of calretinin IHC gave 100% accuracy in all 86 biopsies studied including 17 partial thickness biopsies.

Conclusion: Only use of Haematoxylin and Eosin stain will lead to significant false positive result leading to misdiagnosis and in turn wrong treatment. Loss of calretinin expression in aganglionic segment help in the interpretation of even partial biopsies and easy to interpret with improvement in diagnosis upto 100%.

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1. Introduction

Hirschsprung's disease (HD) is the congenital aganglionosis of the human colon due to disruption of migration, imbalanced proliferation and differentiation of neural crest cell. Hirschsprung's disease affects an estimated 1:5000 live born. Approximately 90% of patients present in infancy with constipation, abdominal distension, vomiting, and delay of meconium passage. Aganglionosis always involves the distal rectum, but frequently extends in a proximal direction to a variable length of bowel: to sigmoid colon (short segment HD), to left, transverse, or right colon (long segment HD), or, in rare and severe forms, to the distal

ileum (total colonic aganglionosis [TCA]), or skip segment Hirschsprung's disease. Grossly, colon shows three zones- Constricted aganglionic zone, transition zone and dilated zone of ganglionosis. A transition zone, characterized by altered density and non uniform distribution of enteric neurons, lies between the aganglionic bowel and the proximal normal bowel.^{1,2}

Puri and Gosemann divided allied disorders of Hirschsprung's disease into four: intestinal neuronal dysplasia (IND); isolated hypoganglionosis (HG); internal anal sphincter achalasia (IASA); and megacystic microcolon intestinal hypoperistalsis syndrome (MMIHS) in 2012.³

* Corresponding author.

E-mail address: drsharayupatil@gmail.com (S. Rane).

The diagnosis and extent of resection for the management of HD depend on the sensitive and specific identification of ganglion cells. This is often associated with hypertrophied (40um) nerve bundle. However, documenting aganglionosis is often difficult and tedious on routine hematoxylin-eosin (H&E) stained sections requiring multiple sections and there is the possibility of sampling tissue from the physiological ganglionic zone. Therefore, different staining methods were developed to improve HD diagnostic accuracy. Acetylcholinesterase histochemical staining is the most commonly applied ancillary method and was reported to have excellent sensitivity and specificity for the diagnosis of HD.⁴ However, this method requires frozen specimen. Also it needs a quantitative and qualitative assessment, which is difficult to perform on rectal suction biopsy. For these reasons, acetylcholinesterase staining is not feasible all the time. Several other immunohistochemical markers such as S-100, neuron specific enolase (NSE), C-Kit, PGP 9.5, and synaptophysin have been tested to identify ganglion cells in formalin-fixed, paraffin-embedded tissue specimens, but none has been demonstrated to be superior to acetylcholinesterase.

Recently, calretinin and microtubule-associated protein-2 (MAP-2) were introduced as new markers that can be used to diagnose HD. MAP-2 is a cytoplasmic microtubule-stabilizing protein that it is a marker of neuronal differentiation.^{5,6} Calretinin is a vitamin D-dependent calcium binding protein thought to involve in calcium signalling in the central nervous system.⁷ Since the first report of loss of expression of calretinin in the aganglionic bowel by Barshack et al. in 2004, only a handful of studies have investigated the utility of calretinin immunohistochemistry in the diagnosis. Calretinin positively stained the ganglion cells accompanied by a fibrillary pattern staining in the submucosal plexus of ganglionic bowel. Calretinin typically demonstrated cytoplasmic and nuclear positive reaction in contrast to only a cytoplasmic immune reactivity of MAP-2. In general, immunostaining for calretinin showed a more intense reactivity of HD. Now, choline transport (ChT) came out as a new positive IHC marker avoiding use of frozen section.

2. Materials and Methods

2.1. Nature of study

Hospital based observational, cross sectional, prospective study.

2.2. Sample size

43 patients with signs and symptoms suggestive of Hirschsprung's Disease and allied disorders attending tertiary health care. IHC is done on 86 rectal and colonic biopsies of these 43 patients. Surgical resected specimen is used as gold standard for Hirschsprung's disease and

allied disorders (number of patients=27) cases while follow up is used as gold standard in non Hirschsprung's disease cases (number of patients = 16).

3. Method

1. Detailed history of the patient suspected of having Hirschsprung's Disease and allied disorders will be taken.
2. Investigations like X ray and ultrasonography of abdomen conducted.
3. Intestinal biopsy is included of patients suspected of having Hirschsprung's Disease and allied disorders.
4. Slides prepared from paraffin embedded blocks studied for their histopathological and morphological features. At least 4 to 5 serial sections through block is taken for H&E stain.
5. IHC performed on them using calretinin on paraffin block

3.1. Diagnostic criteria

3.1.1. Diagnostic criteria for non Hirschsprung's disease in a rectal and colonic mucosal biopsy

1. At least one ganglion cell is identified in one or more tissue sections and/or
2. The calretinin immunostain shows distinct linear granular black fibres in the muscularis mucosa and lamina propria, extending upwards to a variable distance in between the crypts (positive staining) and stains ganglion cells (both nuclear and cytoplasmic) and nerve bundle in the submucosa if included in the biopsy.

3.1.2. Diagnostic criteria for Hirschsprung's disease in a rectal mucosal biopsy

1. Absent ganglion cells with or without presence of hypertrophic nerve bundles in the submucosa.
2. With calretinin IHC, the biopsy demonstrates neither ganglion cells nor any stainable nerve fibres in mucosa as well as in sub mucosa. Negative staining of hypertrophied nerve bundle.

3.1.3. Diagnostic criteria for Isolated Hypoganglionosis

The number of ganglia in the myenteric plexus per millimeter colon is less than 1.52 according to the criteria introduced by Li and Zhu.

Diagnostic performances (e.g. sensitivity, specificity, positive and negative predictive values, and accuracy) of standard method which is done on H&E serial sections and calretinin IHC.

3.2. Biopsy selection and method:

Out of 86 biopsies, 69 were full thickness biopsies (include mucosa, submucosa and muscle layer) and 17 were partial thickness biopsies (include mucosa and submucosa only). Biopsies are taken at least 2 cm above pectinate line. (table 1)

4. Result

4.1. Age wise distribution

Out of 43 patients 16 (37.22%) were in age group of 0-1 months. 14 patients (32.55%) were in age group of 1 month to 1 year while 13 (30.23%) patients were more than 1 year old.

4.2. Sex distribution

32 out of 43 (74.42%) patients were male while 11 (25.58%) patients were female with male to female ratio is 2.91:1.

4.3. Clinical presentation

Most of the patients clinically present with distension of abdomen, constipation, vomiting with unable to pass meconium.

4.4. Interpretation of IHC

1. All 86 biopsies of 43 suspected patients were evaluated with both standard H and E stain and Calretinin IHC. Positive control shows no discrepancy.
2. The concordance of calretinin result with gold standard (Surgically resected specimen for Hirschsprung's disease and allied disorders while follow up routine for non HD diseases) was excellent with 100% agreement. Nerve staining with calretinin was peculiar with their granular, non-homogenous staining pattern and with very fine structure, focally forming a fine fibrillary network in lamina propria. Ganglion cells were also strongly stained.
3. Out of 86 biopsies, 50 were calretinin negative suggestive of aganglionic segment (Table 2).
4. 28 biopsies show positivity to nerve bundles as well as ganglion cell and 8 biopsies show positivity to nerve fibres only due to absence of ganglion cell in the given cut section.
5. Only use of H and E stain gave false positive result with 13 biopsies out of 86 which were interpreted as absence of ganglion cell with or without hypertrophied nerve bundle.
6. Only use of H and E stain lead to misdiagnosis as Hirschsprung's Disease in 9 out of 43 cases (Table 3).
7. 1 hypoganglionic case show absence of ganglion cell in H and E stain, while calretinin intensely stained single ganglion.

8. H and E stain showed 72.0% specificity with 78.12% positive predictive value and 86.00% accuracy. Calretinin showed 100% sensitivity, specificity, positive & negative predictive value and accuracy (Table 4).

4.5. Statistical analysis

McNemar chi-squared statistic with Yates correction of 0.5 is 8.02778 with corresponding p- value is 0.00026 (p value <0.05), consider as significant result, rejecting null hypothesis. Hence, difference between H and E stain and calretinin is statistically significant.

4.5.1. Types of Hirschsprung's disease

Out of 26 cases of Hirschsprung's disease, 20 were of short segment type (maximum upto sigmoid colon aganglionosis), 4 were of long segment type (upto splenic flexure), 01 of total colonic aganglionosis (involve all of large intestine) and 01 of skip segment type.

4.5.2. Partial thickness biopsy/ Rectal suction biopsy

Out of 86 biopsies, 69 were full thickness biopsies include mucosa, submucosa and muscle layer. 17 were rectal suction biopsy include mucosa and submucosa only (Table 5).

H and E stain showed 63.63% specificity with 71.42% positive predictive value and 80.95% accuracy in partial thickness biopsy, while calretinin showed 100% sensitivity, specificity, positive & negative predictive value and accuracy.

4.5.3. Transition zone

3 biopsies were taken from clinically and radiologically known transition zone. Out of 3 biopsies, 2 were calretinin negative and 1 was positive for ganglion as well as nerve fibre. Only use of H and E stain lead to false positive result for aganglionic zone with 1 out of 3 biopsies.

5. Discussion

Histopathologic analysis remains the mainstay for diagnosis of HD. Hirschsprung's disease has a complex pathogenesis that, to date, is not completely understood. Significant progress has been done for a better molecular characterization; there is a very strong body of literature pointing to specific genes being involved in the developments and migration of the enteric nervous system. For example mutations in RET on chromosome 10q11.2 was shown to be responsible of approximately 40% of the sporadic cases of HD.⁸⁻¹⁰

In 1994 McConalguet al. demonstrated that the calretinin antibody highlights different neuronal populations in the large intestine. Since then a number of comparative studies have been published on the expression of calretinin in Hirschsprung's disease and its utility as a marker

Table 1: Site of biopsy collection

Biopsy site	No. of biopsies (n=86)	Percentage
Rectum	47	54.65%
Sigmoid colon	11	12.8%
Descending colon	08	9.30%
Transverse colon	08	9.30%
Ascending colon	04	4.65%
Transition zone	03	3.48%
Colostomy	05	5.81%
Ileum	01	1.16%
Total Biopsies	86	100%

Table 2: Classification of cases (Total =43)

Stains	Hirschsprung's disease	Isolated hypogangliosis	Non Hirschsprung's disease
Calretinin	26	01	16
H&E	35	00	08

Table 3: Discrepant result between Calretinin and H and E stain (Total biopsy 86)

	H and E staining (Bx=86)	Calretinin(Bx=86)
Ganglion present	22	36
Ganglion absent with or without hypertrophied nerve bundle.	64	50

Table 4: Predictive values and sensitivity/specificity of immunohistochemical evaluation

Indicators	Definition	H and E stain	Calretinin
Number of biopsy		86	86
Sensitivity	TP/(TP+FN)	50/(50+0) ;100%	50/(50+0); 100%
Specificity	TN/(TN+FP)	36/(36+14); 72.0%	36/(36+0); 100%
Positive predictive value	TP/(TP+FP)	50/(50+14); 78.12%	50/(50+0)100%
Negative predictive value	TN/(TN+FN)	36/(36+0); 100%	36/(36+0);100%
Accuracy	(TP+TN)/TP+TN+FP+FN	(50+36)/100; 86.00%	(50+36)/86; 100%

Abbreviations: TP: True positive, TN: True negative, FP: False positive, FN: False negative

Table 5: Formalin fixed rectal and intestinal biopsies (n/total=86)

	Full Thickness biopsy (n=69)		Rectal suction/partial thickness biopsy (n=17 biopsies)	
	Ganglionic segment (n=29)	Aganglionicsegment (n=40)	Ganglionic segment (n=7)	Aganglionicsegment (n=10)
H and E stain	19	50	03	14
Calretinin	29	40	07	10

for assisting in the diagnosis of HD. Calretinin, a 29 kD calcium binding protein is expressed primarily in neurons of central and peripheral nervous system and as a rich network in the ENS. The functional interpretation of calretinin immunohistochemistry in HD might be the subject of further studies, but the Ca⁺⁺- deficient status of the aganglionic bowel might be related to deficient Ca⁺⁺ receptor expression, which was shown by Piotrowska et al. in their study.¹¹ This protein is involved in the transport of calcium, and when stained, is seen as thin fibrillary network in the mucosa as an indirect evidence of ganglionosis.

Most common age group was 1 day to 8 years. Age group of our study is very much comparable with all 7 other

studies to which it is compared like Young et al,¹² Anbardar et al,¹³ Kannaiyan et al¹⁴ and Zuikova et al.¹⁵ Kannaiyan et al. and zuikova et al has very wide range of age group with mean is slightly at older age. Volpe et al has a mean age in infant age group. Our study has mean age in 2nd year of life as of Yadav et al, Kacar et al,¹⁶ Guinard- Samuel et al¹⁷ and Rakshani et al.¹⁸ Number of the patients were almost equally distributed in neonates, infants and >1 year of age. Young et al have most of the patients in neonates (45.4%) with 36% of patients in infant age group. While Zuikova et al has maximum number of patients in >1 year age group (54.2%). Kannaiyan et al and Anbardar et al has maximum patients in 0-1 month age group 63% and 77% respectively.

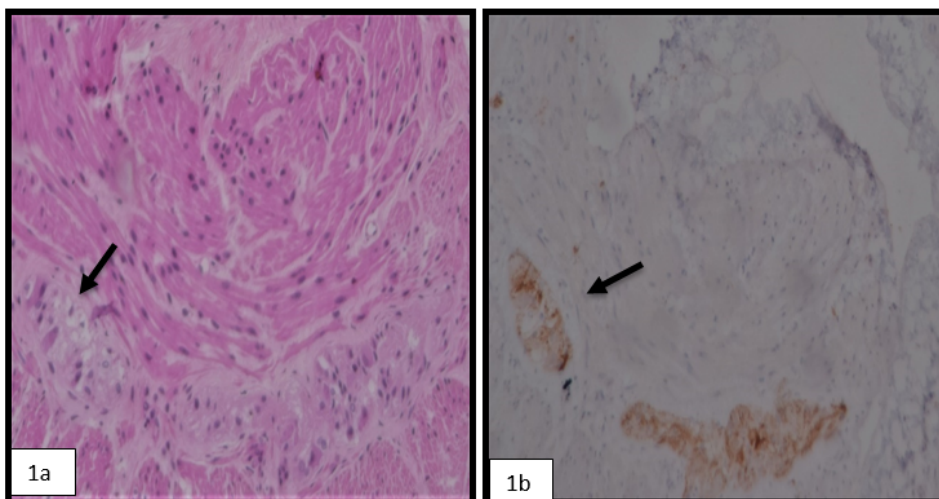


Fig. 1: a: H and E stain, 100x: shows nerve bundles along with ganglions in muscle layer; **b:** Calretinin stain gives positive staining of nerve bundles as well as ganglions, suggestive of ganglionic segment

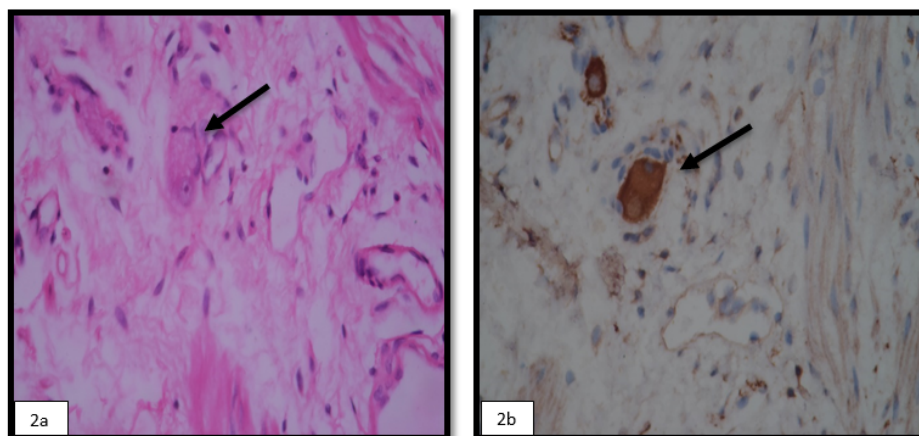


Fig. 2: a: H and E stain 400x, shows ? ganglion cell; **b:** calretinin stain showed intense positive staining of cytoplasm and nuclei of ganglion cell, which confirm ganglionic segment

All studies show predominance of this disease in male. Our study has Male: Female ratio of 2.91:1. Male to female ratio of Kacar et al is maximum i.e 4:1, while young et al has 2.14:1, kannaiyan et al 3.28:1, Rakshani et al 2.03:1.

Cause of male predominance is still unknown but many believes genetic association to this factor.

Along with our study; Yadav, Kacar and Musa et al¹⁹ showed all predictive values 100% with calretinin, which is excellent result. Zuikova et al shows sensitivity of 90.5% and accuracy of 91.4%, which is less than other studies but still its much higher.

Ambardar et al showed sensitivity of 96.7% and negative predictive value of 98%, but specificity and positive predictive value of 100%. It's overall accuracy is 98%.

6. Conclusion

Though there has been recent advances in molecular as well as radiological diagnosis histopathology is mainstay for diagnosis of Hirschsprung's disease. Rare disease yet one of the common cause for neonatal constipation is grave if unidentified and untreated. H&E diagnosis require serial cut sections as density of ganglion cell in terminal rectum is low. This lead to failure of adequate sampling.

Pattern of calretinin is very simple; its either positive or negative. It does not require fresh and frozen tissue, accurate result even with rectal suction biopsy, It stains well in immature ganglions, stains satisfactorily even on low level biopsies and it is great help to identify transition zone. Calretinin as adjuvant with H&E stain, significantly reduces interpretation error and improves diagnosis to 100%. It is of great help to identify transition zone which is necessary

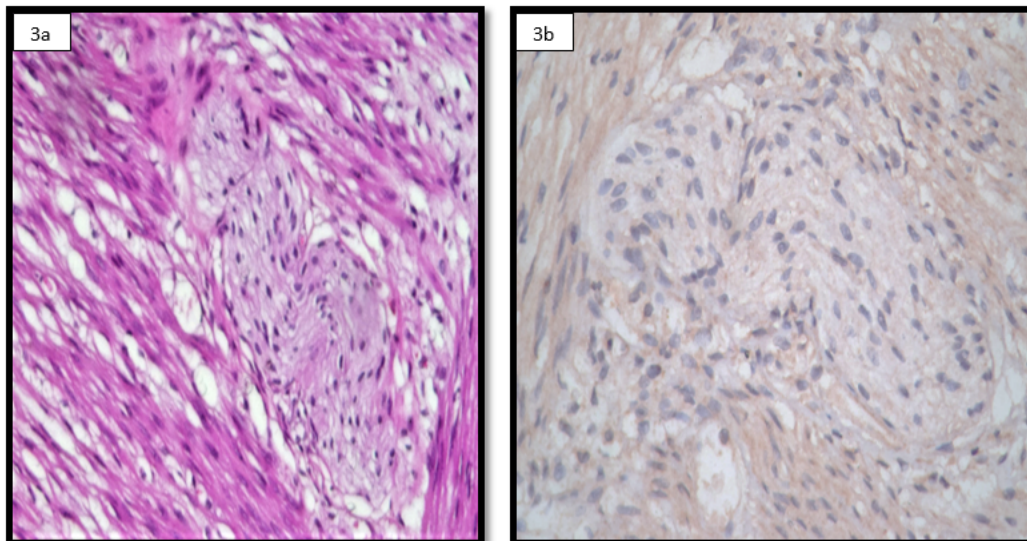


Fig. 3: a: H&E (400x); Hypertrophied nerve bundle; **b:** Calretinin (400x); -ve staining of nerve bundle; Aganglionic segment

for management. It stains well in low level rectal biopsy and immature ganglion. Calretinin improved diagnosis to excellent 100% even in rectal suction biopsy and hence improves management

Large scale studies are requiring for better predictability of the diagnosis and future management and rehabilitation of Hirschsprung's disease patient. Calretinin IHC should always be used in addition to limited H&E section because of the heterogeneous nature of the disease.

7. Take Home Message

1. Calretinin gives negative staining to nerve fibres in mucosa, lamina propria as well as muscularis muscle in HD cases. Hence even if ganglion is absent in given section, negative staining of these fibres will give diagnosis of HD.
2. Calretinin improved diagnosis to excellent 100% even in rectal suction biopsy and hence improves management.
3. No need of multiple level biopsy for calretinin IHC. Hence significantly reduce tedious time consuming search for ganglion cell on hundreds of section in H and E stain slides.

8. Source of Funding

None.

9. Conflict of Interest

None.

References

1. Kleinhaus S, Boley SJ, Sheran M, Sieber WK. Hirschsprung's disease a survey of the members of the Surgical Section of the American Academy of Pediatrics. *J Pediatr Surg.* 1979;14(5):588–97.
2. Suita S, Taguchi T, Ieiri S, Nakatsuji T. Hirschsprung's disease in Japan: analysis of 3852 patients based on a nationwide survey in 30 years. *J Pediatr Surg.* 2005;40(1):197–202.
3. Puri P, Gosemann JH. Variants of Hirschsprung's disease. *Semin Pediatr Surg.* 2012;21(4):310–8.
4. Santos MM, Tannuri U, Coelho MC. Study of acetylcholinesterase activity in rectal suction biopsy for diagnosis of intestinal dysganglionses: 17-year experience of a single center. *Pediatr Surg Int.* 2008;24(6):715–9.
5. Caceres A, Binder LI, Payne MR, Bender P, Rebhun L, Steward O. Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. *J Neurosci.* 1984;4(2):394–410.
6. Nunez J, Fischer I. Microtubule-associated proteins (MAPs) in the peripheral nervous system during development and regeneration. *J Mol Neurosci.* 1997;8(3):207–22.
7. Baimbridge KG, Celio MR, Rogers JH. Calcium-binding proteins in the nervous system. *Trends Neurosci.* 1992;15(8):303–8.
8. Hiradfar M, Sharifi N, Khajedaluae M, Zabo-linejad N, Taraz JS. Calretinin immunohistochemistry: an aid in the diagnosis of Hirschsprung's disease. *Iran J Basic Med Sci.* 2012;15(5):1053–9.
9. KACAR A, ARIKOK AT, AZILI MN, AGIRBAS GE, TIRYAKI T. Calretinin immunohistochemistry in Hirschsprung's disease: An adjunct to formalin-based diagnosis. *Turk J Gastroenterol.* 2012;23(3):226–33.
10. Holland SK, Ramalingam P, Podolsky RH, Reid-Nicholson MD, Lee JR. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung disease. *Ann Diagn Pathol.* 2011;15(5):323–8.
11. Piotrowska AP, Solari V, Puri P. Distribution of Ca²⁺-Activated K⁺ channels, SK2 and SK3, in the normal and Hirschsprung's disease bowel. *J Pediatr Surg.* 2003;38(6):978–83.
12. Yadav L, Kini U, Das K, Mohanty S, Puttegowda D. Calretinin immunohistochemistry versus improvised rapid Acetylcholinesterase histochemistry in the evaluation of colorectal biopsies for Hirschsprung disease. *Indian J Pathol Microbiol.* 2014;57(3):369–75.
13. Anbardar MH, Geramizadeh B, Foroutan HR. Evaluation of Calretinin as a New Marker in the Diagnosis of Hirschsprung Disease. *Iran J Pediatr.* 2015;25(2). doi:10.5812/ijp.367.
14. Kannaiyan L, Madabhushi S, Malleboyina R, Are NK, Reddy KR, Rao B. Calretinin immunohistochemistry: A new cost-effective and

- easy method for diagnosis of Hirschsprung's disease. *J Indian Assoc Pediatr Surg.* 2013;18(2):66–8.
15. Zuikova V, Franckevica I, Strumfa I, Melderis I, Ilze Strumfa, Ivars Melderis. Immunohistochemical Diagnosis of Hirschsprung's Disease and Allied Disorders. *Acta Chirurgica Latvianis* . 2015;15(1). doi:10.1515/chilat-2016-0010.
 16. KACAR A, ARIKOK AT, AZILI MN, AGIRBAS GE, TIRYAKI T. Calretinin immunohistochemistry in Hirschsprung's disease: An adjunct to formalin-based diagnosis. *Turk J Gastroenterol.* 2012;23(3):226–33.
 17. Guinard-Samuel V, Bonnard A, Lagausie PD, Philippe-Chomette P, Alberti C, Ghoneimi AE, et al. Calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease. *Mod Pathol.* 2009;22(10):1379–84.
 18. Rakhshani N, Araste M, Zamani F, Panahi M, Tameshkel FS, Sohrabi MR, et al. Hirschsprung's disease diagnosis: Calretinin marker role in determining the presence or absence of ganglion cells. *Iran J Pathol.* 2016;11(4):409–15.
 19. Musa ZA, Qasim BJ, Ghazi HF, Shaikhly WAKA. Diagnostic roles of calretinin in hirschsprung disease: A comparison to neuron-specific enolase. *Saudi J Gastroenterol.* 2017;23(1):60–6.

Author biography

Pooja U Patil Resident

Sharada Rane Associate Professor

Dasmit Singh Khokar Associate Professor

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