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Clinical Correlation Between Immunohistochemistry Markers And Neuronal Dysfunction In Anorectal Malformations And Hirschsprung Disease Patients

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ABSTRACT

Introduction: Congenital colorectal disorders like Hirschsprung disease (HD) and anorectal malformations (ARM) significantly contribute to pediatric morbidity and mortality. Accurate diagnosis of these disorders, particularly HD, often hinges on histopathological evaluation. Hematoxylin and Eosin (H&E) staining is the traditional diagnostic tool, though it has limitations due to the variable distribution and subtle appearance of ganglion cells, especially in neonates.

Material and Methods: This cross-sectional study was conducted in the Department of General Surgery at CSSH Medical College from July 2023 to February 2025, including 20 clinically diagnosed cases of HD and ARM. Ethical clearance was obtained, and informed consent was secured. Formalin-fixed, paraffin-embedded tissue biopsies underwent H&E staining and IHC using CD117, S-100, and calretinin. Staining patterns were semi-quantitatively evaluated. Data were recorded as frequencies and percentages. Positive and negative controls ensured IHC accuracy.

Results: Most patients were under 11 months of age (52%), with a male predominance (80%). Constipation was the most common presenting symptom (48%), followed by delayed meconium passage and abdominal distension. HD accounted for 80% of diagnoses, while 20% had ARM, including subtypes like anocutaneous fistula and anorectal agenesis.

On H&E of rectal HD biopsies, proximal ends showed positivity in 96.7% and distal ends were uniformly negative. In ARM cases, rectal biopsies showed 25% positivity and 75% negativity of ganglion cells. Calretinin IHC confirmed the presence of ganglion cells in proximal and distal sigmoid colon of HD cases (100%) and revealed absence in the rectum (62.5%). ARM cases showed a progressive increase in calretinin positivity from rectum to distal colon.

S-100 staining revealed hypertrophic nerve fibers in 13.35% of HD rectal biopsies and 75% of ARM biopsies. CD117 immunostaining showed decreased interstitial cells of Cajal (ICCs) in HD but increased staining in ARM, aligning with previous studies indicating a possible role of ICC depletion in motility disorders.

Conclusion : Although H&E staining remains the cornerstone for diagnosing HD and ARM, its limitations necessitate adjunctive methods. Calretinin IHC significantly enhances diagnostic accuracy, even in equivocal or poorly preserved samples. It aids in clear ganglion cell identification and transition zone mapping, critical for surgical planning. S-100 and CD117 further assist in evaluating nerve hypertrophy and ICC distribution, respectively. Routine incorporation of IHC, especially calretinin, alongside limited H&E sections is strongly recommended for improving diagnostic precision, optimizing treatment strategies, and ensuring better outcomes in pediatric colorectal disorders

Keyword: Calretinin , hirscsprung disease , Anorectal , immunohistochemistry

1. INTRODUCTION

Congenital disorders of the colon and rectum, particularly **Hirschsprung disease (HD)** and **anorectal malformations (ARMs)**, pose a significant global health burden due to the high morbidity and potential mortality associated with them. While surgical interventions such as **pull-through (PT) procedures** offer definitive treatment, postoperative complications including **fecal incontinence, constipation, and colonic dysmotility** remain prevalent, indicating persistent or secondary neuronal dysfunction.[1,2]

Hirschsprung disease is a congenital condition characterized by the **absence of ganglion cells** in the **submucosal and myenteric plexuses** of the distal bowel, arising from a **failure of neural crest cell migration** during embryogenesis. It affects approximately 1 in 5000 live births.[1,3] In contrast, **ARMs** encompass a **wide spectrum of anal and rectal anomalies**, ranging from minor stenoses to complex cloacal malformations involving the urinary and genital tracts. Their incidence is similar to HD, affecting 2–5 per 10,000 live births. Both disorders significantly compromise **gastrointestinal**

motility due to abnormalities in the **enteric nervous system (ENS), interstitial cells of Cajal (ICCs), and smooth muscle layers**.[4]

H&E staining remains the **gold standard** for diagnosing HD due to its ability to identify ganglion cells and related histological changes like **fibrosis, congestion, and muscular deformities**. However, **technical challenges** arise, especially in neonates, due to the **immaturity and subtle appearance of ganglion cells**, which are often confused with endothelial or immune cells. Additionally, ganglion cells are not evenly distributed; they are more concentrated in the **deep submucosa and muscularispropria**, which are often underrepresented in biopsies. As a result, evaluating multiple sections or even entire tissue blocks becomes necessary in challenging cases.[6,7]

Key IHC markers include ,**S100:** Highlights **hypertrophic nerve fibers** in aganglionic segments but does not stain ganglion cells directly.;**CD117 (c-Kit)**: A marker for **ICCs**, it helps evaluate pacemaker cell integrity and distribution, which correlates with motility function.;**Calretinin**: A highly **sensitive and specific marker for ganglion cells**, calretinin is absent in aganglionic regions and thus crucial for confirming HD, especially in neonates where ganglion cell identification is difficult with H&E alone.[10-12]

In ARMs, where abnormalities may involve both neural and muscular elements, IHC markers provide insights into **ENS integrity and the suitability of bowel segments for surgical reconstruction**.[13]

In light of the above, the present study was designed to evaluate **neuronal and ICC alterations** using **IHC markers (calretinin, S-100, CD117)** in bowel specimens of patients with **HD and ARMs**, aiming to improve diagnostic accuracy and predict postoperative outcomes.

2. MATERIAL AND METHODS

This cross-sectional study was conducted in the Department of General Surgery at CSSH Medical College from July 2023 to February 2025 to assess the clinical correlation between immunohistochemical markers (CD117, S-100, and Calretinin) and neuronal dysfunction in patients with anorectal malformations (ARM) and Hirschsprung disease (HD). Ethical clearance was obtained from the institutional ethical committee, and informed consent was secured from patients' parents. Twenty clinically diagnosed cases of ARM and HD were included, based on defined inclusion criteria: Patients clinically diagnosed with ARM and Hirschsprung disease and exclusion criteria: 1) Parents of Patients who are not willing to participate. 2)Inadequate material for IHC analysis. Biopsy specimens were fixed in 10% formalin, paraffin-embedded, and subjected to routine Hematoxylin and Eosin staining and IHC analysis. IHC staining involved deparaffinization, antigen retrieval, incubation with monoclonal antibodies, and visualization with DAB and hematoxylin counterstaining. Positive and negative controls were used for accuracy. CD117, S-100, and Calretinin staining patterns were semi-quantitatively scored. Data were analyzed using descriptive statistics, presented as frequencies and percentages.

3. RESULTS

Demographic profile

In this study, the age distribution revealed that the majority of patients (52%) were in the newborn to 11-month age group, followed by 40% in the 1–5 years range, and only 8% were older than 5 years. Regarding gender distribution, a significant male predominance was observed, with 80% of patients being male and only 20% female. When analyzing the presenting symptoms, constipation was the most frequently reported symptom, affecting 48% of the patients, and it was commonly observed across all age groups. Delay in the passage of meconium (24%) and abdominal distension (22%) were also common in younger children, while failure to thrive (16%) was seen in both infants and children aged 1–5 years. Some patients presented with more than one symptom. In our study, 80% of the cases were of hirschsprung disease and 20% cases were of anorectal malformation.

Mature Ganglion cells observed in HD biopsies

Rectal biopsies showed positive mature ganglion cells in 11 patients (66.67%) , negative in 3 patients (16.67%) and equivocal in 2 patients (12.5%)

IHC findings revealed positive mature ganglion cells in 15 patients (96.70%) and equivocal in 1 patient (3.3%) in proximal ends while distal end revealed no mature ganglion cells in all the patients (100%)

H&E (Mature GanglionCells)	Negative		Positive		Equivocal		Totalnumber	
	No.	%	No.	%	No.	%	No.	%
Rectalbiopsies	3	16.67	11	66.67	2	12.5%	16	100

Proximalends	0	0	15	96.7	1	3.3	16	100
Distalends	16	100	0	0	0	0	16	100

Mature Ganglion cells observed in ARM biopsies

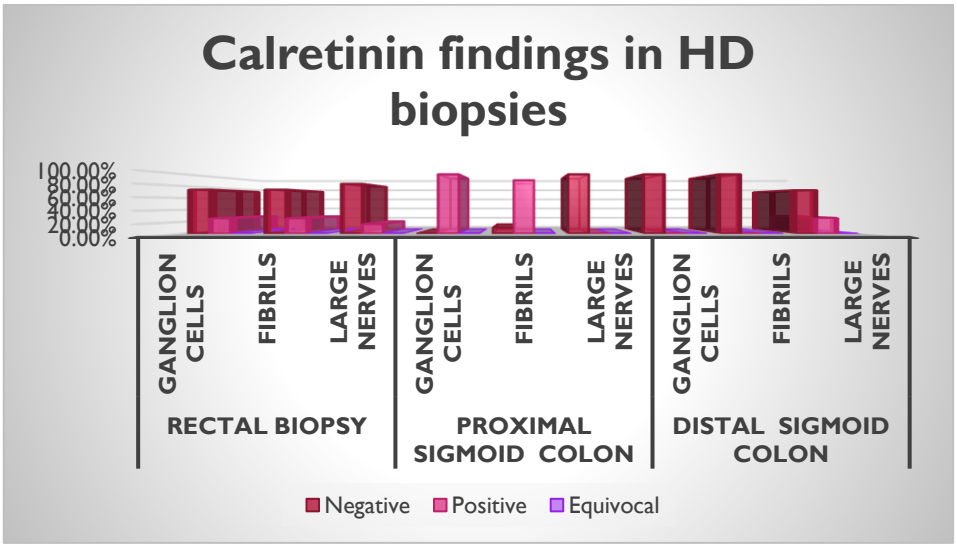
Rectal biopsies showed positive mature ganglion cells in 1patient (25%), negative in patient (25%) and equivocal in 2 patients (50%)

IHC findings in proximal sigmoid end revealed positive mature ganglion cells in 1patient (25%) , equivocal in 1 patient (25%) and negative in 2 patients (50%) while distal sigmoid end revealed negative mature ganglion cells in 3 patients (75%) and positive in 1 patient (25%)

H&E (Mature GanglionCells)	Negative		Positive		Equivocal		Totalnumber	
	No.	%	No.	%	No.	%	No.	%
Rectalbiopsies	1	25	1	25	2	50	4	100
Proximal sigmoid colon	2	50	1	25	1	24	4	100
Distal sigmoid colon	3	75	1	25	0	0	4	100

CalretininfindingsinHD specimens:

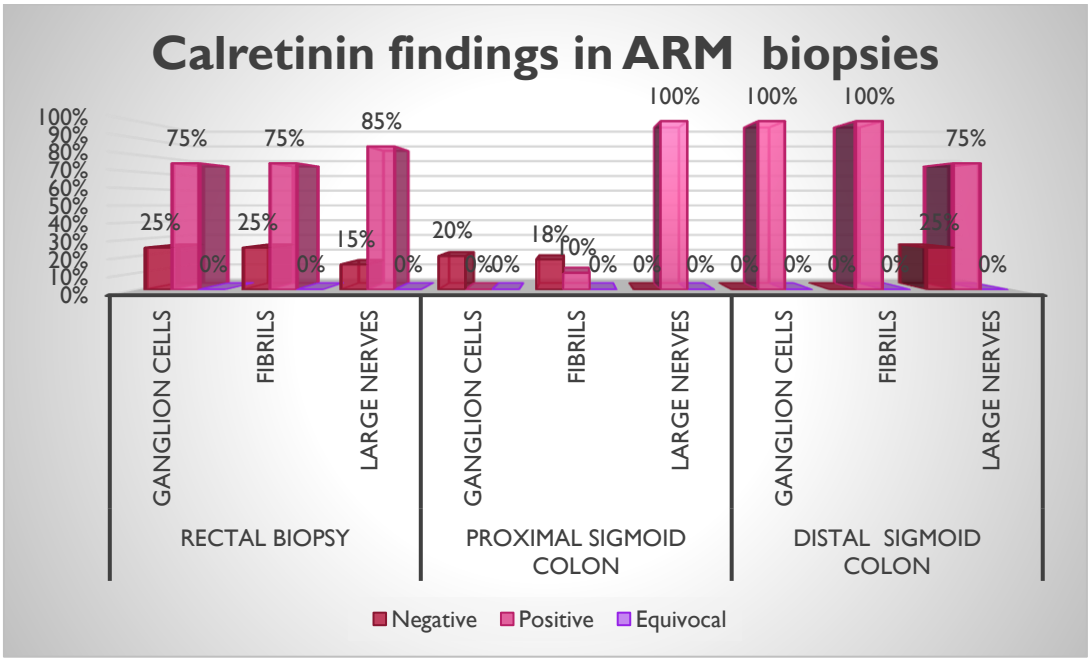
The histopathological findings from rectal, proximal sigmoid, and distal sigmoid colon biopsies revealed distinct patterns in the distribution of ganglion cells, fibrils, and large nerve fibers. In rectal biopsies, ganglion cells were absent in 62.5% of cases and present in 37.5%, with no equivocal findings. Similarly, fibrils were negative in 62.5% and positive in 37.5% of rectal samples. Large nerves were predominantly seen in rectal biopsies, with 83.9% showing their presence. In the proximal sigmoid colon, all cases (100%) demonstrated the presence of ganglion cells, and 43.33% showed fibrils, while 10% were negative. Interestingly, large nerves were absent in all proximal sigmoid samples. In the distal sigmoid colon, ganglion cells and fibrils were present in all cases (100%). However, large nerves were identified in 73.3% of cases, while 26.6% lacked them. These findings indicate that ganglion cells were consistently present in the proximal and distal sigmoid colon but commonly absent in the rectum, aligning with the typical pathology of Hirschsprung disease.



Calretinin findings in ARM studied specimens:

The histopathological evaluation of rectal, proximal sigmoid, and distal sigmoid colon biopsies in four patients revealed the following trends. In rectal biopsies, ganglion cells were absent in 75% and present in 25% of cases. Fibrils were also negative

in 75% and positive in 25%. Large nerves were seen in 85% of cases. In the proximal sigmoid colon, ganglion cells were evenly distributed, with 50% showing presence and 50% absence. Fibrils were mostly negative (75%) and positive in 25%, while large nerves were present in all cases (100%). In the distal sigmoid colon, ganglion cells and fibrils were consistently present in all cases (100%). Large nerves were positive in 75% and negative in 25% of samples. This pattern demonstrates a progressive increase in ganglion cell and fibril positivity from the rectum to distal sigmoid colon, while large nerves were predominantly seen in the rectum and proximal segments.



. Calretininfindingsin ARM studiedspecimens

Table 14. S100 findingsinHDstudiedspecimens:

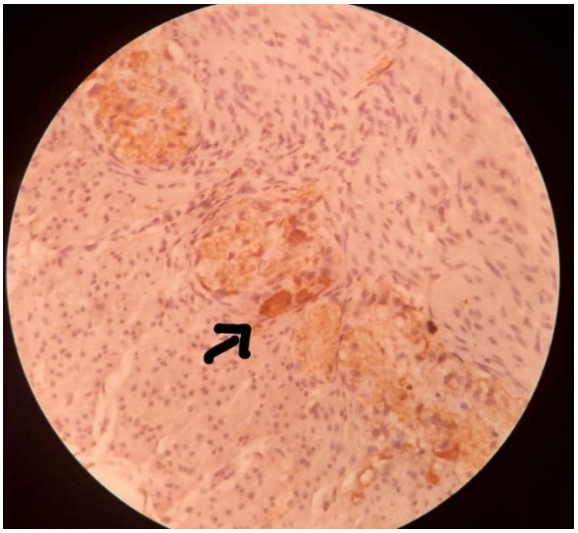
Rectal biopsy revealed no marked hypertrophy in 14 patients (87.5%) while proximal sigmoid colon revealed marked nerve hypertrophy in one patient (6.25%)

	No Marked Nerve hypertrophy		Marked Nerve hypertrophy		Total	
	No.	%	No.	%	No.	%
Rectalbiopsy	14	87.5	2	13.35	16	100%
Proximal sigmoid Colon	15	93.75	1	6.25	16	100%

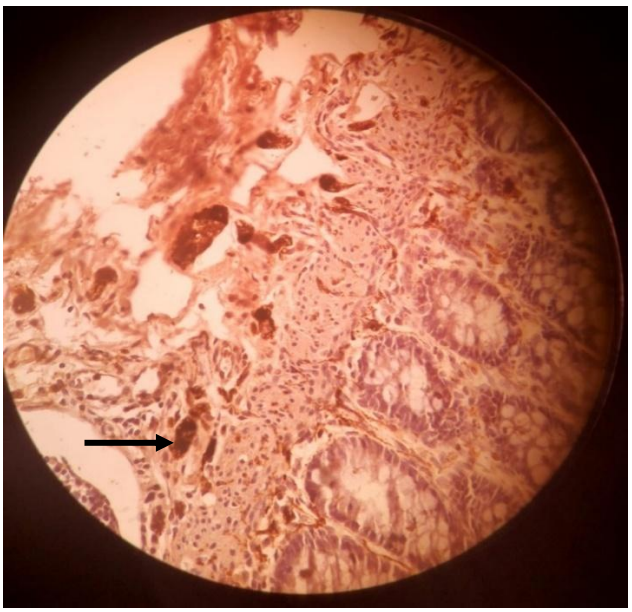
S100 findingsinARM studiedspecimens:

Rectal biopsy revealed marked hypertrophy in 3 patients (75%) similarly , proximal sigmoid colon revealed marked nerve hypertrophy in 3 patients (75%)

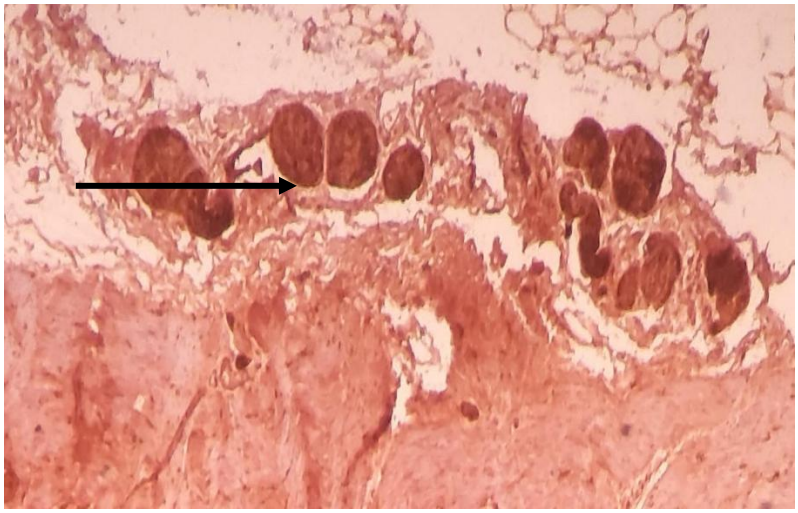
	No Marked Nerve hypertrophy		Marked Nerve hypertrophy		Total	
	No.	%	No.	%	No.	%
Rectalbiopsy	1	25%	3	75%	4	100%
Proximal sigmoid Colon	1	25%	3	75%	4	100%



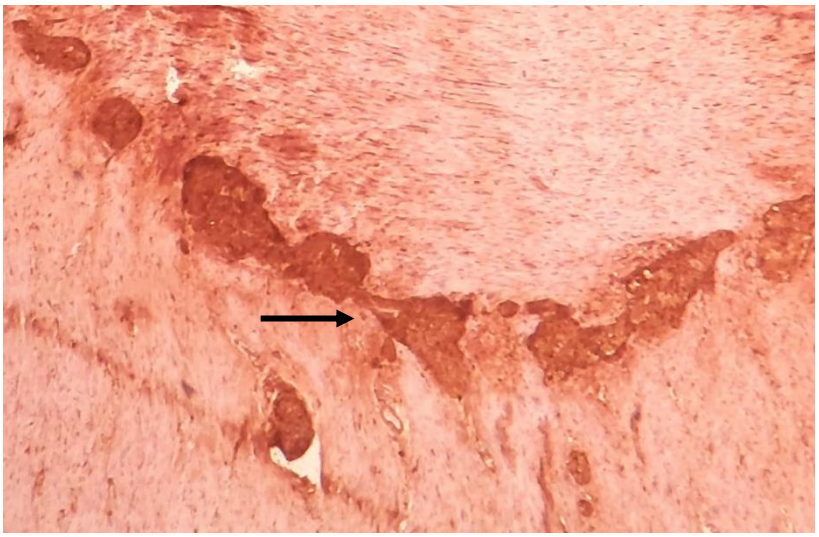
Calretinin- Positivity seen in Ganglion cells of myenteric plexus (40x)



Calretinin- Positive in ganglion cells of Messner's plexus (40x)



S100 - Nerve hypertrophy noted (40x)



S100 - Nerve hypertrophy seen (40x)

4. DISCUSSION

Diagnosing Hirschsprung's disease (HD) on H&E sections is difficult and requires expert pathologists due to challenges like distal biopsy sites, superficial samples, or difficulty identifying ganglion cells, especially in neonates. Immunohistochemical markers (calretinin, CD117, S-100) help improve accuracy. [16,17]

In the present study we tried to study histomorphological changes and evaluate the role of various IHC markers (calretinin, S-100, CD117) in HD and anorectal malformations to assess neuronal dysfunction in these patients.

Demographic Characteristics

Majority of the patients were in newborn to 11 months (52%) and Majority of the patients were male (80%).

In the study by PoojaPatil et al [74] , the 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 Yadav L et al,[77] ; Anbardar et al,[78]; Kannaiyan et al [19] and Zuikova et al.[70]

Zuikova et al[70] reported a age varied from three days to 15 years (49 ± 55 months; median 17 months).

Patients in the study were almost evenly distributed among neonates, infants, and children over 1 year. Other studies show variation: Yadav et al.[77] reported most patients as neonates (45.4%), while Zuikova et al.[70] had more patients over 1

year (54.2%). Kannaiyan et al [19] and Anbardar et al [78]had the majority in the 0–1 month group (63% and 77%). All studies showed male predominance; our study had a male-to-female ratio of 2.91:1, similar to others. Despite improved awareness and earlier diagnosis, some children still present after the neonatal period, often around weaning. The cause of male predominance remains unclear.

The male predominance in Hirschsprung disease (HD) and related disorders. The current study found a male-to-female ratio of 2.91:1, aligning with literature reports such as Kacar et al. (4:1), Young et al. (2.14:1), Kannaiyan et al. (3.28:1), and Rakhshani et al. (2.03:1). Diagnosis age has decreased over the years due to increased awareness, though some cases still present later in infancy, particularly around the weaning period. The consistent male predominance observed across studies remains unexplained, as no X-linked genetic cause has been identified.

Symptoms Distribution

In present study , themostcommonsymptomwasconstipation (48%). More than one presentingcomplaint was also present

In the study by PoojaPatil et al [74] ,Most of the patients clinically present with distension of abdomen, constipation, vomiting with unable to pass meconium.

Immunohistochemistry Findings

Calretinin

In present Study ,patients with Hirschsprung disease revealed that Calretininimmunoreactivity was observed in the ganglion cells of the submucosal and intermuscular plexuses, the pattern of expression was dense nuclear and cytoplasmic.

Calretinin expression was also observed in the fine nerve fibrils of the lamina propria, muscularis mucosa and submucosa, the staining pattern was linear, cytoplasmic and granular.

Calretinin highlighted the presence of ganglion cells in the submucosal (Meissner's plexus) and inter muscular plexuses (Auerbach's plexus).

In rectal biopsy, ganglion cells were absent in majority of the cases (62.5%)

Proximal and distal ends revealed, ganglion cells in all the cases (100%)

While in ARM studiedspecimens revealed ,rectal biopsy, ganglion cells were absent in majority of the cases (25%).Distal ends revealed, ganglion cells in 2 patients (50%) while proximal end revealed , ganglion cells in all the patients (100%)

In the study by Yashika Bhatia et al[81]., calretinin identified ganglion cells in one of 13 HD cases and all seven equivocal cases, confirming its value in resolving diagnostic uncertainty, consistent with findings by Sameul et al [82], De la Torre et al[83], and Hiradfar et al [84].

The mechanism behind reduced ICCs in HD and non-HD motility disorders remains unclear, with two possibilities: either ENS is essential for ICC differentiation, or a shared mechanism affects both neural crest migration and ICC development.[85]

ComparisonofH&EwithcalretininHDwithinvariousstudies.

Study No.ofcaseswithclinical suspiciousofHD	**H&E**		**Calretinin**	
	Diagnosis	No.Ofcases	Positive	**Negative**
Sameuletal 131	HD	42	0	**42**
	Non-HD	73	0	**73**
Torreetal 12	Suspicious	16	4	**12**
	HD	8	0	**8**
	Non-HD	4	4	**0**
	HD	30	2	**28**
Kannaiyanetal 60	HD	43	2	**41**
	Non-HD	2	2	**0**
Yadav Let al 33	Suspicious	15	3	**12**
	HD	26	0	**26**

		Non-HD	17	17	**0**
Anbardar et al	**55**	HD	27	0	**27**
Bhatia Y et al	**30**	Non-HD	28	26	**2**
		HD	13	1	**12**
		Non-HD	10	10	**0**
		Suspicious	7	7	**0**
Present Study	**20**	HD	16	14	**2**
		ARM	**4**	**2**	**2**

ComparisonofdistributionsofICCSonCD117withotherstudies.

The inability to detect ganglion cells on H&E stains in suspected cases may be due to immature ganglion cells, which have small nuclei and lack visible nucleoli, especially in neonates and preterm infants. Though maturity may improve detection, persistence can occur. Calretinin IHC offers accurate identification of ganglion cells and several advantages: it works on paraffin-embedded tissues, has a simple binary staining pattern (positive/negative), and is cost-effective, making it a valuable tool in difficult diagnoses.[23]

STUDY	**Typeofdisorder**	**Noofcases**	**ICCsonCD117**
Jainetal[86]	Non-HD	6	**Total absence**
Streukeretal [87]	Non-HD	30	**Markedlydecrease**
Anatoletal [88]	HD	10	**Markedlydecrease**
Barshacketal [85]	Non-HD	8	**Markedlydecrease**
Becheanuetal [89]	Non-HD	2	**Markedlydecrease**
Geramizadehetal[90]	HD	29	**Decrease**
Bhatia Y et al [81]	HD	12	**Decrease**
	Non-HD	18	**Decrease**
Present Study	HD	16	**Decrease**
	ARM	**4**	**Increase**

S100

Nerve hypertrophy, alongside aganglionosis, is a key histological marker in Hirschsprung's disease and can be seen on H&E stain, though it may be difficult for inexperienced pathologists to interpret. S-100 immunohistochemistry is useful in identifying hypertrophic nerve fibers. In our study, both rectal biopsies and proximal sigmoid colon samples showed marked nerve hypertrophy in 75% of Hirschsprung's and ARM patients. According to De la Torre et al[83]., S-100 has low sensitivity (41.7%) but high specificity (100%) due to its staining of glial and Schwann cells, making it a supportive but limited diagnostic tool.

5. CONCLUSION

Despite advances in diagnostics, histopathology remains the gold standard for Hirschsprung's disease (HD). While hematoxylin and eosin (H&E) staining is traditional, it has limitations due to the low density of ganglion cells, risking misdiagnosis. Calretinin immunohistochemistry (IHC) offers superior accuracy, clearly indicating positive or negative results without needing fresh tissue or serial sections. It effectively identifies immature ganglion cells and the transition zone, aiding surgical planning. Calretinin IHC enhances diagnostic precision, even in suboptimal biopsies, reduces resource use, and enables faster diagnosis. Its routine use alongside H&E staining is recommended for reliable early detection and improved patient outcomes.

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