Evaluation of the role of Immunohistochemical markers in the diagnosis of Hirschsprung's Disease

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Abstract: Hirschsprung's disease is one of the most common and problematic infancy and childhood maladies. Early and accurate diagnosis is a fundamental step in proper management and prevention of complications. The most reliable method for diagnosis is the histopathological analysis of rectal biopsies and the typical finding of Hirschsprung's disease is the absence of ganglion cells. It is well known that the identification of ganglion cells in conventional Hematoxylin and Eosin stainedbiopsies can be very difficult and is greatly laden with various technical and interpretive challenges andlimitations. **Aim of this study:** was to assess the diagnostic value of Calretinin and Neuron Specific Enolase immunochemistry as a panel for the diagnosis of Hirschsprung's disease. **Materials and Methods:** the study included a total of 41 cases. Specimens included both rectal biopsies and colectomies. Calretinin and NSE immunohistochemistry was carried out on formalin fixed paraffin-embedded specimens after routine histopathological examination, the pattern of expression was observed and the results were statistically analyzed in comparison with the standard method (H & E). **Results:** 10 cases (9 rectal biopsies and the proximal end of 1 colectomy) were considered ambiguous with H&E because the presence or absence of ganglion cells could not be confirmed in these cases and they were considered to be equivocal. Calretinin and NSE detected the presence of ganglion cells in 7 of these cases and denied the presence of ganglion cells in the remaining 3, therefore undoubtedly establishing the diagnosis in these problematic cases. In the rest of the cases, Calretinin and NSE were consistent with the H&E findings. NSE highlighted nerves of all calibers in all cases and after measuring the nerves thicknesses and densities of distribution in ganglionic and aganglionic bowels we found that nerves were much thicker and the density of their distribution was much higher with statistically significant values in the aganglionic compared to the ganglionic bowels. **Conclusion:** Calretinin is an excellent tool in the diagnostic work up of Hirschsprung's disease. It can efficiently prove or exclude the presence of ganglion cells thus establishing the diagnosis. The staining pattern is straight forward either positive or negative therefore it was fairly easy to interpret. NSE is a useful tool in the diagnostic process as well through detection of ganglion cells and more importantly through providing the chance to study and compare the innervation patterns of the healthy and diseased intestines.
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Keywords: Hirschsprung disease; calretinin; rectal biopsy

1. Introduction:

Hirschsprung's disease (HD) is one of the congenital colorectal disorders that represent a significant burden to the health of children worldwide. It accounts for 10 % of intestinal obstruction cases in the neonatal period. The disease occurs in approximately 1:5000 live births and males are more commonly affected than females with a ratio 4:1 **(1).**

The disease occurs due to failure of colonization of the neural crest derived neuroblasts in the distal gut during fetal development either due to arrest of migration in the craniocaudal direction or due to failed survival, proliferation and neuronal differentiation resulting in absence of **g**anglion cells. Relaxation of the gut wall during propulsive motility is normally mediated by inhibitory (intrinsic) motor neurons that originate from the enteric ganglia. Absence of these inhibitory impulses will contribute to the aganglionic segment being persistently constricted causing the megacolon and severe constipation in these patients **(2).**

Cases are classified according to the length of aganglionic segment with the internal anal sphincter as the constant inferior limit. The most common form, including about 75% of cases, is the conventional or the classic (short segment) form with an aganglionic segment involving only the rectum and sigmoid not extending beyond the upper border of the sigmoid. In 17% of cases, the aganglionic segment includes the rectum, sigmoid, the descending colon till the splenic flexure and the transverse colon which is called long segment HD; it involves parts of the colon but not pancolonic. Total colonic aganglionosis (TCA) accounts for 8% of the patients where the aganglionic segment involves the whole colon and extends to affect variable lengths of the small intestine. The rarest form of the disease with the most severe clinical course is total intestinal aganglionosis with absence of ganglion cells from

duodenum to the rectum and the last described form is ultra-short segment in which the aganglionic part is very short involving the distal 2cm of the rectum **(3).**

Is Hirschsprung's disease a diagnostic challenge?

The disease is most often diagnosed and operated upon during the first year of life and it is diagnosed by combination of the presenting symptoms, rectal examination, anorectalmanometry, barium enema and rectal biopsy with subsequent histopathological evaluation. Unfortunately, in the first months of life the clinical picture and functional investigations may not be conclusive. Therefore, the histopathological diagnosis is regarded as the most accurate method for diagnosis, it is considered to be the Gold standard and with optimal conditions, its diagnostic sensitivity can reach 100% **(4).**

The basic well known obligate histopathological diagnostic criterion of the disease is the total lack of ganglion cells in the submucosal and intermuscular nerve plexuses of the intestinal wall. The presence of multiple hypertrophic nerve fibers is observed in many (but not all cases) and helps establish the diagnosis**(5,6).**The problem of the histopathological diagnosis is that the pathologist should demonstrate something that "does not exist" which means that the absence of ganglion cells in the submucosal and my enteric plexuses should be confirmed. The dilemma is resolved for any pathologist when ganglion cells are definitely identified regardless of which histological technique was used **(7).**Identification of ganglion cells can be very intricate and laden with technical and interpretive challenges as the conventional H&E approach may have many limitations such as; paucity of submucosa and incomplete maturation of submucosal ganglion cells at birth. They tend to be small, undifferentiated with scarce cytoplasm which is different from the classic phenotype of the mature ganglion cells so they can be very easily confused with endothelial cells, histiocytes or lymphoid cells **(8).**In addition to several anatomical facts including; the submucosal ganglia are normally relatively widely separated from one another; they are most abundant along the internal layer of the muscularispropria and in the deep portion of the submucosa that is not always sampled well by the rectal biopsy technique. Such that time consuming evaluation of numerous H&E sections (sometimes up to examination of the entire block) is required in some cases to identify unequivocal ganglion cells **(9).**

These restrictions have lead over the years to the quest of ancillary methods to aid in the diagnosis. Some institutions use acetylcholinesterasehisto chemical stain which shows abnormally coarse nerve fibers in the submucosa. But this stain necessitates frozen sections which are not available in all medical centers, it needs quantitative and qualitative assessment therefore a high degree of subjectivity exists in its interpretation, in addition to the possibility of occurrence of false positive and negative results **(10).**

Immunohistochemistry is the other major ancillary approach utilized to complement H&E.Many antigens have been investigated in this context such as S100, CD56, Cathepsin D, BCL2, Synaptophysin, Chromogranin, Glial Fibrillar Acidic Protein (GFAP) and Peripherin antibodies, but none of them has gained worldwide recognition in the diagnosis of HD **(11).**

Calretinin is a vitamin D dependent calcium binding protein that functions as a calcium sensor/modulator; it belongs to the EF-hand family which comprises over 150 members. It is expressed in the specific neurons of the central and peripheral nervous systems and its expression may be observed in non-neuronal cells as well **(12).**

Neuron-specific enolase (NSE) is a glycolytic homo- or heterodimericiso-enzyme. The antibody labels both normal and neoplastic cells of neuronal and neuroendocrine origin. NSE is not an exclusive neuronal marker and it can be used for the identification of peripheral nerves, neural and neuroendocrine tumors**(13).**

So, the aim of this work was to explore the value of using calretinin and NSE immunohistochemical staining of intestinal sections from children suspected to have Hirschsprung's disease to confirm the diagnosis.

2. Materials and Methods:

This study was conducted on 41 pediatric cases complaining of chronic constipation that started since birth in most cases. Two types of specimens were included; rectal biopsies from children suspected to have Hirschsprung's disease and colonic resection specimens that were excised by trans-anal pullthrough operation. Specimens were gathered either prospectively (fresh intestine specimens) or retrospectively (in the form of paraffin blocks).

Rectal biopsies were measured, oriented and multisectioned in a perpendicular manner (sections included mucosa and submucosa). On the other hand, colonic resection specimens were thoroughly examined so as to identify the proximal and the distal ends and to measure the length of the narrow segment which corresponded to the extent of the aganglionosis. Accordingly; cases were classified into ultrashort, short, long and total colonic aganglionic segments.

Serials were taken from both ends so that sections contained mucosa, submucosa and musculosa and then placed in cassettes in the same direction of cutting. Sections were first stained with

Hematoxylin and Eosin. Afterwards, all the obtained specimens were stained immunohistochemically using mouse monoclonal antibodies directed against Calretinin (Dako, Denmark, at 1:50 dilution)and Neuron Specific Enolase (NSE) (Dako, Denmark at 1:100 dilution).

Interpretation of the staining results:

- Using H&E: all sections were examined for the presence of ganglion cells that were identified by their unique morphology as being oval large cells with eccentric vesicular basophilic nuclei, single prominent often eosinophilic nucleoli and abundant eosinophiliccytoplasm. When the presence or absence of ganglion cells could not be confirmed by the H&E stain, these cases were labeled as "Equivocal". Also, sections were examined for the presence of hypertrophied thickened nerve fibers in the submucosa that were identified as oval or round structures with wavy basophilic nuclei and fibrillary light eosinophilic cytoplasm.
- **Calretinin:**

Positivity for calretinin was detected as brown granular staining on the counter background Hematoxylin blue stain and the absence or presence of ganglion cells was noted for each case.

- **Neuron specific enolase (NSE):**

NSE positivity was detected as brown granular staining. The absence or presence of ganglion cells was noted for each case. Depending on NSE expression in nerves; the thickness of the nerve trunks and the density of their distribution were measured as follows:

- *Nerve thickness:*
  - First; the diameter of the visual field of the microscope was measured using a clear ruler with millimeter marks, field diameter was first obtained with the low power and then the value was calculated in the high power.
  - The field diameter using the screen lens (40) was 5 mm so the field diameter using high power (400) was 0.5 mm (500 µm).
  - Nerve trunk thickness was estimated by observing the extent of involvement of the visual field by a single nerve trunk using the high power; this diameter was measured perpendicular to the directions of the fibers and taking the thickest portion into consideration.
- *Nerve trunk density:*
  - In order to evaluate the density of nerve trunk distribution, the field area was calculated based on knowing the value of the field diameter ($A = \pi r^2$).
  - The area of the visual field after calculation was 20 mm^2 and density of the nerve trunks was estimated using the screen lens (40) through counting the number of nerve trunks occupying one low power visual field to finally reach the number of nerves per 10 mm^2.

3. Results:

Clinicopathological results:

The age of the studied cases ranged from one week to 18 years with a mean of 9 years. Fifty one percent of cases showed their first symptoms during the first month of life. Thirty two cases were males and 9 were females. Thirty four cases (82.9%) showed the classic presentation of Hirschsprung's disease including chronic constipation, abdominal distention and history of failure to pass meconium in the first day of life. Whereas 5 cases (12.2%) were older children presented with a long history of chronic constipation in addition to failure to thrive and lack of energy due to anemia. One patient (2.4%) presented with acute abdomen and on exploration ceacal perforation was found and 1 patient (2.4%) presented with recurrent constipation after a previous pull-through operation.

Histopathological and Immunohistochemical analysis:

Hematoxylin and Eosin:

The study included 31 rectal biopsies and the proximal and distal ends of 30 colectomy specimens with a total of 41 cases as for twenty (20) cases both rectal biopsy specimens and colectomies of the same patient were available. All the specimens were carefully examined and evaluated for the presenceofganglion cells and tissue innervation. Table (1) demonstrated the histopathological findings.

Calretinin:

- Calretininimmunoreactivity was observed in the ganglion cells of the submucosal and intermuscular (colectomy specimens) plexuses, the pattern of expression was dense nuclear and cytoplasmic (figures 3, 4). 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 (figure 5). Large submucosal nerves showed faint stippled expression of calretinin. Besides, submucosalinflammatory cells showed also calretinin positivity. Calretinin stained ganglion cells in 8 (25.8%) out of 31 of rectal biopsy cases in which the diagnosis of Hirschsprung's disease was excluded (figure 6). In 23cases(74.2%), no marker expression morphologically consistent with ganglion cells or fine fibrils was detected in the mucosa or the submucosa (figure 7) confirming the diagnosis of HD in all of these cases including (3) cases that were considered equivocal by H&E stain. Similarly in colectomy specimens, calretinin confirmed the presence of ganglion cells in proximal ends of all the 30 (100%)

specimens. Calretinin highlighted the presence of ganglion cells in the submucosal (Meissner's plexus) and inter muscular plexuses (Auerbach's plexus). In 27 cases (90 %), calretinin also showed faint granular cytoplasmic staining of the fine nerve fibrils in the lamina propria, muscularis mucosa and submucosa. This expression was absent in 3 cases (10%). While the distal end of all these 30 specimens showed no ganglion cells. Table (2) demonstrated the calretinin findings in different studied specimens.

Neuron Specific enolase:

• NSE immunoreactivity was observed as granular cytoplasmic expression in the ganglion cells (figure 8) of the submucosal and inter muscular plexuses. Immunoreactivity for NSE was also observed in the nerve fibers of all calibers (figures 9, 10) in the form of granular staining. Table (3) demonstrated the NSE findings in different studied specimens. NSE expression in the nerve fibers helped to set a comparison between ganglionic and aganglionic bowels from the perspective of nerve thickness and density of distribution. Nerve trunk thickness and distribution density were measured in all studied cases (n=41)and were illustrated in table (4).

• Studied cases were finally assigned as 33 (80.5%) Hirschsprung's disease cases and 9 (19.5%) as non Hirschsprung's disease cases. Table (5) demonstrated the sensitivity, specificity and accuracy of using H&E staining versus Calretinin and NSE in rectal biopsies from patients suspected to have Hirschsprung's disease.

Table (1): Histopathological findings of the studied specimens:

H&E (Ganglion Cells)	Negative		Positive		Equivocal		Total number	
	No.	%	No.	%	No.	%	No.	%
Rectal biopsies	20	64.5	2	6.5	9	29	31	100
Proximal ends	0	0	29	96.7	1	3.3	30	100
Distal ends	30	100	0	0	0	0	30	100

Table (2): Calretinin findings in different studied specimens:

		Negative		Positive		Equivocal		Total	
		No.	%	No.	%	No.	%	No.	%
Rectal biopsy	Ganglion Cells	23	74.2	8	25.8	0	0.0	31	100%
	Fibrils	23	74.2	8	25.8	0	0.0		
	Large nerves	26	83.9	5	16.1	0	0.0		
Colectomy specimens Proximal end	Ganglion cells	0	0.0	30	100	0	0.0	30	100%
	Fibrils	3	10	27	90	0	0.0		
	Large nerves	30	100	0	0.0	0	0.0		
Colectomy specimens Distal end	Ganglion Cells	30	100	0	0.0	0	0.0	30	100%
	Fibrils	30	100	0	0.0	0	0.0		
	Large nerves	22	73.3	8	26.6	0	0.0		

Table (3): NSE findings in different studied specimens:

		Negative		Positive		Equivocal		Total	
		No.	%	No.	%	No.	%	No.	%
Rectal biopsy	Ganglion Cells	23	74.1	8	25.8	0	0.0	31	100%
	Nerves	0	0.0	31	100	0	0.0		
Colectomy specimens Proximal end	Ganglion cells	0	0.0	30	100	0	0.0	30	100%
	Nerves	0	0.0	30	100	0	0.0		
Colectomy specimens Distal end	Ganglion Cells	30	100	0	0.0	0	0.0	30	100%
	Nerves	0	0.0	30	100	0	0.0		

Table (4): Nerve trunk thickness and distribution density in different studied specimens as demonstrated by NSE staining:

		Rectal biopsy	Distal end	Proximal end
Thickness	Min. – Max.	15.0 – 230.0	20.0 – 230.0	8.0 – 40.0
	Mean ± SD.	69.33 ± 49.63	83.55 ± 49.69	25.94 ± 10.91
	Median	60.0	65.0	25.0
	P		<0.001*	
Density	Min. – Max.	8.0 – 75.0	15.0 – 75.0	7.0 – 35.0
	Mean ± SD.	38.0 ± 21.81	43.61 ± 18.06	15.06 ± 7.51
	Median	40.0	45.0	10.0
	P		<0.001*	

P: p value for Wilcoxon signed ranks test for comparing between distal and proximal
***: Statistically significant at $p \leq 0.05$**

Table (5): Showing agreement (sensitivity, specificity and accuracy) for the rectal biopsies (n=31):

Rectal biopsy		Final diagnosis		Sensitivity	Specificity	PPV	NPV	Accuracy
		HD (n = 23)	Not HD (n = 8)					
H&E (Ganglion Cells)	**Negative**	20	0	25.0	86.96	100.0	100.0	70.97
	Positive	0	2					
Calretinin (Ganglion Cells)	**Negative**	23	0	100.0	100.0	100.0	100.0	100.0
	Positive	0	8					
Calretinin (Fibrils)	**Negative**	23	0	100.0	100.0	100.0	100.0	100.0
	Positive	0	8					
NSE (Ganglion Cells)	**Negative**	23	0	100.0	100.0	100.0	100.0	100.0

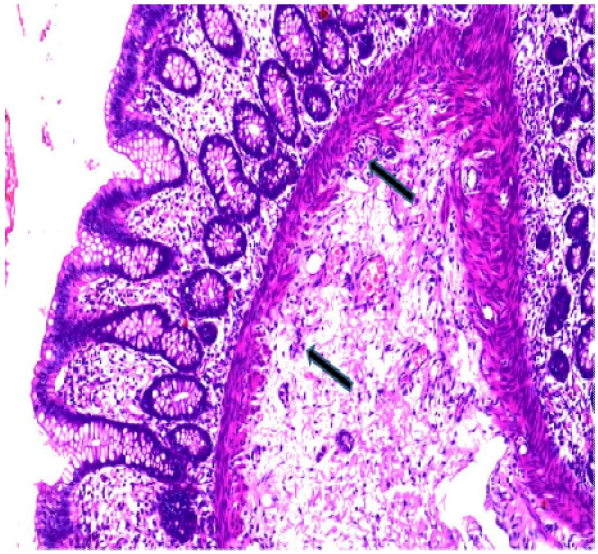


Figure 1: A non HD rectal biopsy showing the presence of two ganglia containing multiple ganglion cell bodies in the submucosa (arrows) (H&E X200).

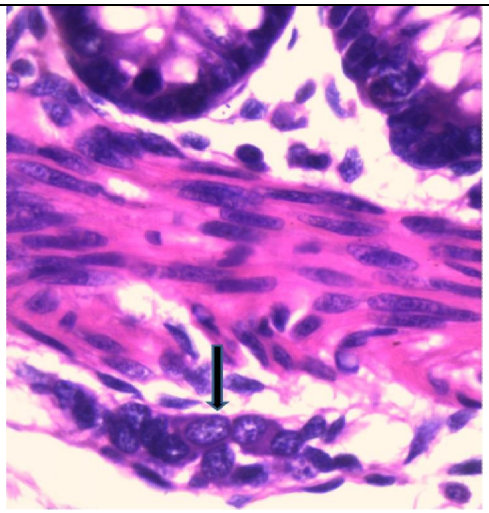
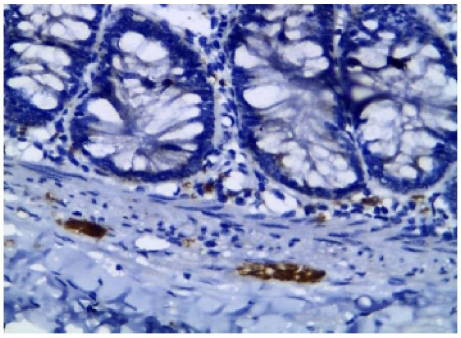


Figure 2: The submucosal plexus in a non HD case present directly underneath the muscularis mucosa containing multiple ganglion cell bodies (arrow) showing vesicular large nucleus, prominent nucleolus and abundant eosinophilic cytoplasm using oil lens (H&E X1000).



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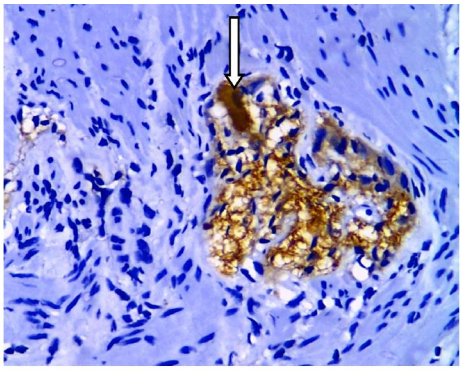

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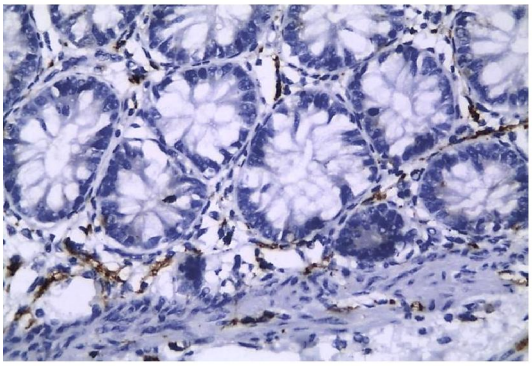

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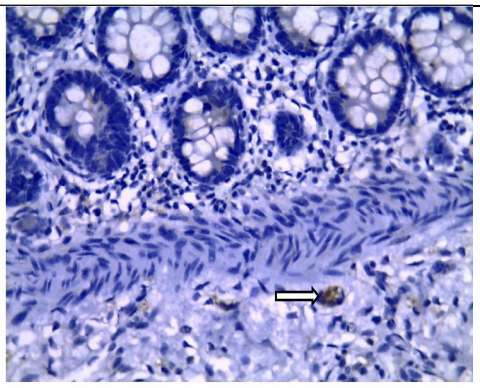

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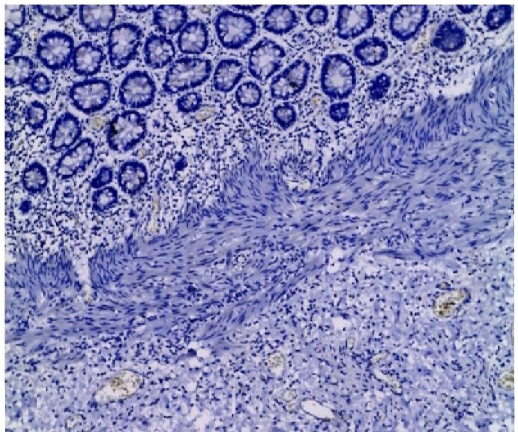

Figure 7: Rectal biopsy of a HD case showing complete absence of Calretinin expression in the mucosa and submucosa (Immunoperoxidase X100).

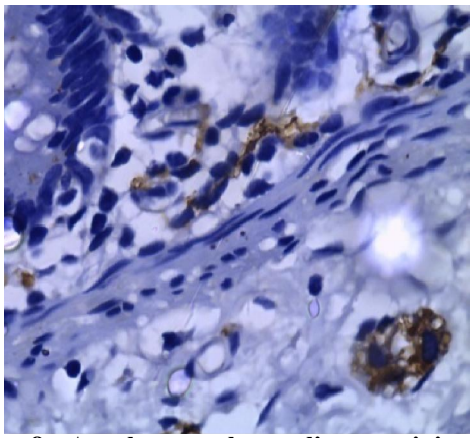

Figure 8: A submucosal ganglia containing two ganglion cell bodies with cytoplasmic granular staining by NSE taken using oil lense (Immunoperoxidase X1000).

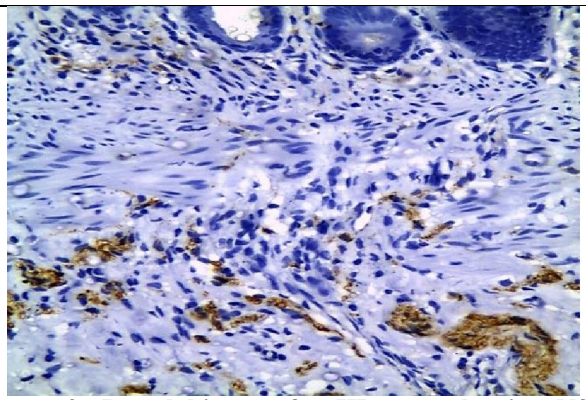

Figure 9: Rectal biopsy of a HD case showing NSE cytoplasmic staining in numerous submucosal nerve fibers with no staining consistent with ganglion cells detected (Immunoperoxidase X400).

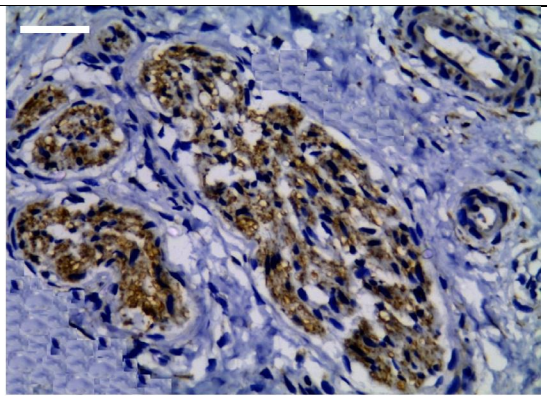

Figure 10: Granular staining of large nerve trunks in the submucosa by NSE, the largest nerve in this figure measured 100µm, scale bar is 50µm (Immunoperoxidase X400).

4. Discussion:

Hirschsprung's disease is a common congenital anomaly affecting the pediatric age group. It is most often diagnosed and operated upon during the first year of life **(14)**. Delayed diagnosis leads to significant morbidity, for example; failure to thrive, anemia and acute enter colitis. If not treated early; patients will experience massive constipation where the proximal colon tends to be severely dilated. This can lead to difficult dissection and an astomosis intraoperatively which may cause various postoperative complications **(15)**. Due to the previously mentioned reasons; early diagnosis of HD is of utmost importance. Unfortunately, in the first months of life bowel movements are occasionally irregular, the clinical manifestations of HD can be less specific, constipation is not always prominent, anorectalmanometry can be normal and barium enema films may not be convincingly conclusive **(16)**. Therefore, relying on the clinical picture alone even after performing functional investigations can be misleading. So, the histopathological diagnosis is regarded as the most accurate method for diagnosis, it's considered to be the gold standard and according to Schulten, the diagnostic accuracy of rectal biopsy is 99.7% **(17).**

In this study, H&E didn't provide a definite answer in 10 cases (25%) of the total number, 9 rectal biopsies and the proximal end of 1 colectomy, in these cases interpretation of H&E sections was considered equivocal and inconclusive. The proximal ends of colectomy specimens acted as an inner positive control in our work allowing the study of normal morphology and distribution of ganglion cells thus enabling comparison to the findings observed in the distal ends.

Alexandrescu *et al.* (18) and several other reports as well confirmed restrictions in H&E diagnosis (these restrictions matched the findings and hardships encountered in our current study relying on H&E alone in the diagnosis or exclusion of HD), so they thought that using ancillary methods such as histochemistry or immunohistochemistry is a must specially in problematic cases.

In this study, calretinin results matched the H&E findings in all the cases where the H&E findings were conclusive but with the benefit of examining fewer sections and avoiding a time consuming process.In the problematic cases where H&E interpretation results could not provide a definite diagnostic decision, calretinin determined undoubtedly the presence or absence of ganglion cells therefore establishing a solid diagnosis. So the sensitivity and specificity of calretinin in detecting ganglion cells in rectal biopsies and colectomy specimens in this study was 100%. Calretinin had a perfect concordance and was statistically significant p <0.0001.

Regarding calretinin expression in rectal biopsies, our findings were consistent with **Guinard-Samuel *et al.* (19)** who conducted a large series retrospective study including 131 rectal biopsies from children suspected to have HD; they compared calretinin staining to their conventional methods using H&E and acetylcholinesterase. After examination of calretininstainedsections, 78 cases were diagnosed as non-HD and 53 cases were diagnosedas HD. Calretinin based diagnoses in all these cases were later on proved to be correct either by examination of the resected bowel loops from the HD cases or by long term follow-up in non-HD cases. In their study, calretinin interpretation matched theresults obtained by their usual methods in 119 cases and the remaining 12 cases that were initially considered suspicious using the standard technique were accurately diagnosed by calretinin. They have

concluded that calretinin is efficient in proving the absence of ganglion cells and is superior to acetylcholinesterase and can replace it in diagnosing HD.

Concerning the study of calretinin expression in colectomy specimens, the proximal ends of colectomies showed dense nuclear and cytoplasmic staining. It was observed in the submucosal and the intermuscular (Auerbach's) plexuses in all of the examined cases (100%) including one case in which the H&E staining was equivocal. Confirmation of the presence of ganglion cells in the proximal ends was essential to assure the adequacy of the surgical resection margins. Besides, calretinin expression in the fine nerve fibrils of the mucosa and submucosa was observed in 90% of the cases. On the other hand, the maker expression was totally absent from the submucosa and the intermuscular areas in all cases of the distal ends of colectomy specimens implying the lack of ganglion cells from the submucosal and intermuscular plexuses matching the findings obtained by H&E stain in all of the cases.

As regards to the calretinin findings in colectomy specimens, the findings of this study were in line with those obtained by **Kannaiyan *et al.* (10)** who studied 60 cases including 24 colectomy specimens and 36 full thickness biopsies. In the resection specimens; Calretinin was negative in the distal ends of all cases, and in the ganglionic bowels it showed immunopositivity in more than 90% of all ganglion cells and fine nerve fibers of submucosal and myenteric plexus thus matching results obtained by H&E in all these specimens. However H&E stain in rectal biopsies stated the absence of ganglion cells in 19 cases, 17 of which were proved to be aganglionic by calretinin and 2 showed ganglion cells therefore, correcting the initial inaccurate H&E diagnosis. 15 of their rectal biopsies were suspicious for the presence of ganglion cells by H&E but calretinin highlighted ganglion cells and nerve fibrils in 3 cases excluding the diagnosis of HD consequently establishing HD diagnosis in the remaining 12. They concluded that calretinin was a great aid in the diagnosis especially in the suspicious cases where H&E was uncertain.

In the present work; NSE expression was observed in all cases but with different implications. First in rectal biopsies; NSE showed granular cytoplasmic staining in the ganglion cells in the Meissner's plexus in addition to staining of the nerve fibers of the lamina propria, muscularis mucosa and superficial areas of the submucosa in 8 cases (25.8%) of total rectal biopsies, matching calretinin findings (only the pattern of expression in ganglion cells was different) and therefore confirming the non HD diagnosis in all cases including 6 cases where the staining pattern of H&E was equivocal.

In 23 cases (74.2%), there was also marker expression in the nerve fibers all around the sections examined and these nerves showed variable thicknesses, some of which were markedly thickened. But no staining that could be interpreted as ganglion cells was detected and therefore the diagnosis of HD in all of these cases was finalized including (3) cases that were considered equivocal by H&E stain.

In the proximal ends of all the colectomy specimens (30 cases, 100%), granular cytoplasmic staining was observed in the submucosal (Meissner's) and the intermuscular (Auerbach's) plexuses also matching calretinin results in each case including one case that was equivocal by H&E.

In the distal ends of colectomy specimens of all the cases, no structures uptaking the stain matched ganglion cells morphology. So, the expression was considered to be negative for ganglion cells. But the marker showed positivity in all the nerve fibers of the submucosa and intermuscular areas.

Our NSE results correlated witha study conducted by **Huang *et al.* (20)** who investigated the benefit of NSE in HD. They examined 18 HD cases (of the total colonic aganglionosis type) and 10 control cases. Their results in the ganglionic bowel showed positive NSE expression in the cytoplasm of the submucosal and intramuscular ganglion cells. In the aganglionic segments; there was absence of expression indicating ganglion cells in addition to the presence of unevenly distributed, thick and spindle-shaped nerve trunks uptaking cytoplasmic granular stain.Their final conclusion was that NSE had strong expression in the cytoplasm of ganglion cells and may contribute to identifying undersized immature ganglion cells as well as detecting nerve fibers in the ganglionic and aganglionic bowel.

In this work we also exploited the benefit of NSE expression in the nerve fibers to set a comparison between ganglionic and aganglionic bowels from the perspective of nerve thickness and density of distribution. Nerve trunk thickness and distribution density were measured for all cases. In the proximal ganglionic ends of colectomies; it was found that the maximum nerve thickness was 40 µm (ranging between 8 µm and 40 µm) with a mean of 26, median 25 and standard deviation (SD) 11 and the maximum density of nerve trunks distribution was 35/10 mm^2 (ranging between 7/10 mm^2 and 35/10 mm^2) with a mean of 15, median 10 and standard deviation 7.5. Whereas, in the distal ends of colectomy specimens; it was found that maximum nerve thickness reached 230 µm (ranging between 20 µm and 230 µm) with a mean of 83.5, median 65 and standard deviation 50 and the maximum nerve

density distribution reached 75/10 mm^2 (ranging between 15/10 mm^2 and 75/10 mm^2) with a mean of 43.6, median 45 and standard deviation 18.

These findings were compatible with a study performed by **Bandyopadhyay *et al.* (21)**, as they focused on measuring nerve thickness and density and comparing ganglionic to aganglionic bowels. In their study, 36 cases of defective intestinal innervation were analyzed, 30 of which were HD and all included cases were stained by H&E and NSE. The range of nerve trunk density in the ganglionic segments was between 19/10 mm^2 and 37/10 mm^2, the mean was 27.51, median 27, SD 4.35, while the diameter of these nerve trunks ranged between 11.9 µm and 43 µm and no nerve trunk greater than 45 µm was detected in ganglionic regions. In the aganglionic segments, nerves were more numerous, the range of nerve twig density was from 25/10 mm^2 to 87/10 mm^2 with a mean 70.28, median 70 and SD 12.25 and thickness of the nerve trunks in these segments varied from 11.9 µm to 120 µm. They concluded that the value of 45 µm was statistically significant and they stressed on the importance of NSE in demonstrating nerve distribution and thicknesses accurately because nerves that are thinner than 10 µm are very difficult to be appreciated using H&E alone. In general, our results regarding this topic were consistent with the universally agreed upon knowledge that nerve trunk thickness values above 40 µm are suggestive of Hirschsprung's disease **(22,23).**

To summarize up, this study showed that calretinin facilitated the diagnostic process to a great extent, it matched the H&E findings in all the cases where the H&E was conclusive but with the benefit of examining fewer sections and avoiding a time consuming process. It helped establishing diagnosis in 10 situations (quarter of the included cases) (9 rectal biopsies and 1 proximal end of colectomy) where the H&E interpretation was ambiguous. The sensitivity and specificity of calretinin in detecting ganglion cells in rectal biopsies and colectomy specimens in this study was 100%.The expression pattern is simple, distinct and fairly easy to interpret with minimal interobserver disagreement so it is an excellent option that can be used by pathologists in case of diagnostic doubt or when they are not familiar with the biopsies of HD. Calretinin reactivity in nerve fibrils in the lamina propria and muscularis mucosa can help in making a pathological assessment in superficial biopsies that are frequently encountered.NSE was of a great aid in the diagnosis by highlighting ganglion cells in the ganglionic bowel as well as giving the opportunity to study nerve trunk thickness and distribution density to set a comparison between ganglionic and aganglionic bowel segments.

Compliance with Ethical Standards:

Author declares that there are no conflicts of interest and state that this study followed the ethical standards at Tanta Faculty of Medicine.

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