ORIGINAL ARTICLE (APMC – 362) DOI: 10.29054/APMC/17.362

Calretinin Expression in Hirschsprung's Disease – A Potential Marker of Ganglion Cells

Mishal Sikandar, Abdul Hannan Nagi, Komal Sikandar, Nadia Naseem, Ihtisham Qureshi

ABSTRACT

Objective: This study was designed with an objective of observing Calretinin as Immunohistochemical marker for aganglionosis and for detection of ganglion cells in the affected areas for more accurate and better diagnosis of the disease. **Study Design:** It was an observational, descriptive study. **Setting:** It was carried out at Department of Morbid Anatomy and Histopathology in University of Health Sciences (UHS) Lahore. **Period:** The study commenced in March 2016 after approval of the synopsis by the Advance Studies and Research Board of UHS and was successfully completed in December 2016. **Methodology:** Biopsy Specimens of colon which were considered for the study were collected from 73 patients from Mayo Hospital, Lahore with well-established histopathologically diagnosed HSCR. **Results:** 52/73 (71.23%) of the patients were male and 21/73 (28.77%) were female. The mean age was 12.52±9.21 months. In agreement with clinico-pathologic examination and sign and symptoms, 69/73 (94.5%) cases had long standing constipation, 68/73 (93.2%) cases had vomiting, 47/73 (64.4%) cases had fever, 31/73 (42.5%) cases had a failure to thrive, 63/73 (86.3%) of the patients had palpable abdominal masses and 20/73 (27.4%) cases had enterocolitis. Ganglion cells were present in 42/73 (57.53%) and absent in 31/73(42.47%) respectively. Convincing association of Ganglion cells was seen with history of enterocolitis, vomiting and chronic inflammation, (p-value < 0.05). **Conclusion:** It was consummated that Calretinin provides a reliable and very cost effective adjunctive test to be used routinely with H&E in diagnosing HSCR and consequently waiving off the need for unnecessary surgeries and repeated biopsies.

Keywords: Hirschsprung disease, Rectal biopsy, Aganglionosis, Acetylcholinesterase, Calretinin

Corresponding Author
Dr. Mishal Sikandar
M. Phil Morbid Anatomy & Histopathology, UHS, Lahore
Contact: +92 334-6508547
Email: mishal_sikandar@yahoo.com

Submitted for Publication: 23-02-2017 *Accepted for Publication: 25-04-2017*

Article Citation: Sikandar M, Nagi AH, Sikandar K, Naseem N, Qureshi I. Calretinin Expression in Hirschsprung's Disease – A Potential Marker of Ganglion Cells. APMC 2017;11(3):181-6.

INTRODUCTION

Hirschsprung's disease, a malformation of the hindgut , is characterized by congenital mega-colon due to the absence of ganglion cells in the enteric nervous system.[1] It is a very important colonic disease in children that causes life-threatening constipation.[1] A Danish pediatrician in 1988 first described it as a chronic severe constipation which leaded to a mega-colon.[2, 3] Embryologically it is due to lack of migration of neural crest cells which results in the absence of parasympathetic ganglion cells in the meissner's and the myenteric plexuses. Estimated incidence is 1 out of 5000 live births with a male to female preponderance and ratio of 4:1.1[2] The disease primarily presents in the period of infancy, in which some patients present with insistent, debiliatating and severe constipation later on in life.[4] Gastrointestinal functional disorders predominantly constipation are common cause of morbidity in otherwise healthy persons and patients with various predisposing diseases.[5]

Definitive curative treatment, i.e. resection surgically, depends on a definitive diagnosis of HD histopathologically which rests upon aganglionosis in the tissue biopsy.[6] An early diagnosis is cardinal to overcome developing complications (e.g., enterocolitis, colonic rupture).[4] Diagnosis of Hirschsprung disease (HSCR) rests upon histologic and/or histochemical staining of sections from rectal suction biopsies. Acetylcholinesterase histochemistry (AChE) aids diagnosis but has its pitfalls as it requires special handling of the tissue.[7] One study further added that a certain type of nerve cell bodies in submucosa and the myenteric ganglia of the gastrointestinal tract were seen to show immunopositivity for calretinin.[8] Latestly, it is reported that calretinin immunohistochemical staining is found to be superior to conventional acetylcholinesterase staining to confirm absence of ganglions.[9]

. The proposed study was an attempt to identify the role of Calretinin in diagnosing Hirschsprung's

disease which can be practiced as reliable method in routine laboratory setups.

METHODOLOGY

Study Design: Observational, descriptive study.

Setting: This study was carried out at Department of Morbid Anatomy & Histopathology in University of Health Sciences (UHS) Lahore.

Period: The study commenced in March 2016 after approval of the synopsis by the Advance Studies and Research Board of UHS and was successfully completed in December 2016.

Sample size: It was calculated by keeping confidence level of 95% and 5% margin of error and taking expected positivity of calretinin immunostaining for diagnosing the aganglionic HD intestinal specimens. The sample size was worked out as 73, out of which 2 were considered as control whereas the remaining 71 samples were used for detailed evaluation.

Sampling Technique: Non-purposive convenient sampling technique was employed for the study.

Inclusion Criteria:

73 Cases of Hirschsprung's disease (HD) on histopathology were included in this study, irrespective of gender and age limits.

Exclusion Criteria:

i. Blocks with insufficient/non-diagnostic biopsies and autolysed specimens, and
ii. Biopsies from anorectal transformation zone were excluded from the study.

Sample preparation

The samples of HD colorectal biopsies for the study were collected from Mayo and Jinnah Hospital, Lahore. Throughout the research, close contact was maintained with the hospital and allied pathology lab for acquisition of the study samples including paraffin embedded blocks, histological reports of the diagnostic biopsies and diseased colon specimens for cases where surgeries were performed. The collected data/ materials were then brought to the department of Morbid Anatomy and Histopathology UHS for further processing and utilization for the study.

Several biopsies were taken from the proximal and distal colostomy margins and also from the middle ganglionic portion of the diseased colon segments removed during the colectomies.

Data Collection

Proformas were prepared to record the socio-demographic information, clinical presentations of the patients and data from biopsy specimens.

Table 1: Quantification: Reporting Technique (David Hernandez et al.2013) [10]

IHC Positivity	Results
No staining of ganglion	**Positive for HD**, if any specific staining (excluding mast cells) is present within the submucosal nerve plexus, muscularis mucosa or lamina propria
Staining of ganglion cells	**Negative for HD**

RESULTS

Demographics: The mean age of patients was 12.52±9.21 days. There were 48/73 (65.8%) cases who were ≤12 days old, 20/73 (27.4%) were 12.1-60 days old and 5/73 (6.8%) of the cases were 60.1-120 days old. 52/73 (71.23%) patients were male and 21/73 (28.77%) where female. In the study the male to female ratio was 2.48:1.

Sign and symptoms: In accordance with sign and symptoms and clinic-pathologic examination, 47/73 (64.4%) cases had fever, 69/73 (94.5%) cases had constipation, 68/73 (93.2%) cases had vomiting, 20/73 (27.4%) cases had Enterocolitis, 31/73 (42.5%) cases had failure to thrive and 63/73 (86.3%) of the patients had abdominal masses.

Histopathological examination and bivariate comparisons with sign and symptoms: In histopathological examination 47/73 (64.38%) cases had hypertrophic nerve. A total of 57/73 (78%) cases had chronic inflammation. Ganglion cells were present in 42 (57.53%) and absent in 31 (42.47%) respectively. In patients with Ganglion cell, 39/42 (92.9%) had constipation. Among those who did not have Ganglion cells, 30/31 (96.8%) had constipation, there was no significant difference of constipation and Ganglion cell, p-value >0.05. In patients with Ganglion cell, 37/42 (88.1%) had vomiting. Among those who did not have Ganglion cells, all had vomiting, there was significant difference of vomiting and Ganglion cell, p-value < 0.05. In patients with Ganglion cell, 17/42 (40.5%) had Enterocolitis. Among those who did not have Ganglion cells, 3/31 (9.7%) patients had Enterocolitis; there was significant difference of Enterocolitis and Ganglion cell, p-value <0.05. In patients with Ganglion cell, 35/42 (83.3%) had abdominal mass. Among those who did not have Ganglion cells, 28/31 (90.3%) patients had abdominal mass; there was no significant difference of abdominal mass and Ganglion cell, p-value >0.05. In patients with Ganglion cell, 3042 (71.4%) had fever and 12/42 (28.6%) patients did not have fever.

Among those who did not have Ganglion cells, 17/31 (54.8%) patients had fever while 14/31 (45.2%) did not have fever; there was no significant difference of fever and Ganglion cell, p-value >0.05.

In patients with Ganglion cell, 21/42(50%) had failure to thrive and 21/42(50%) patients did not have failure to thrive. Among those who did not have Ganglion cells, 10/31(32.3%) patients had failure to thrive; there was no significant difference of failure to thrive and Ganglion cell, p-value >0.05. In patients with Ganglion cell, 30/42 (71.4%) had hypertrophic nerve. Among those who did not have Ganglion cells, 17/31 (54.8%) patients had hypertrophic nerve while 14/31 (45.2%) did not have hypertrophic nerve; there was no significant difference of hypertrophic nerve and Ganglion cell, p-value >0.05. In patients with Ganglion cell, 37/42 (88.1%) had Chronic inflation and 5/42 (11.9%) patients did not have chronic inflammation. Among those who did not have Ganglion cells, 20/31(64.5%) patients had chronic inflammation; there was no significant difference of chronic inflammation and Ganglion cell, p-value > 0.05.

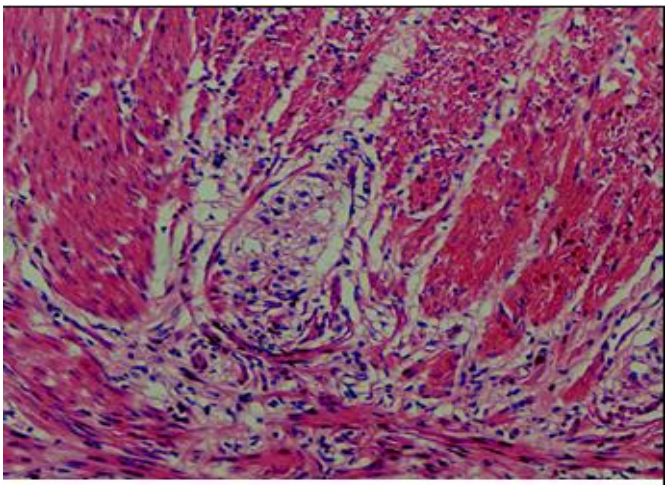


Figure 1: Phtomicrograpth of colonic biopsy stained with H&E showing numerous ganglion cells (40)

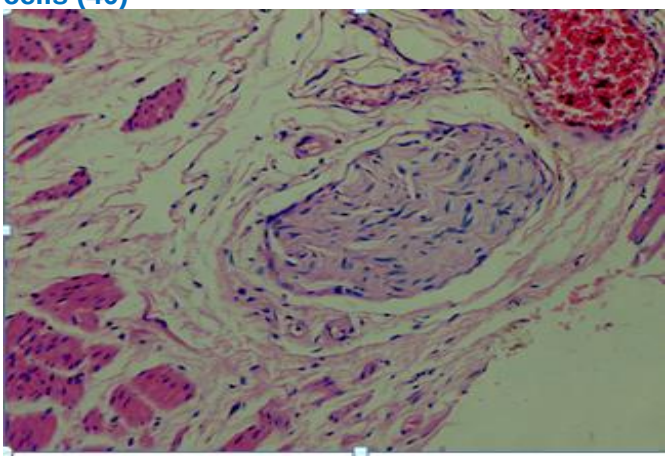


Figure 2: Photomicrograph of colonic biopsy showing hypertrophied nerve fibre (10X)

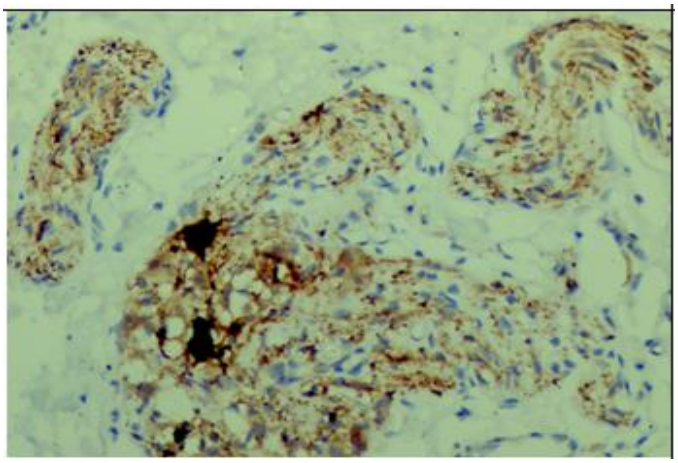


Figure 3: Phtomicrograpth of colonic biopsy stained with Calretinin showing numerous ganglion cells (40X)

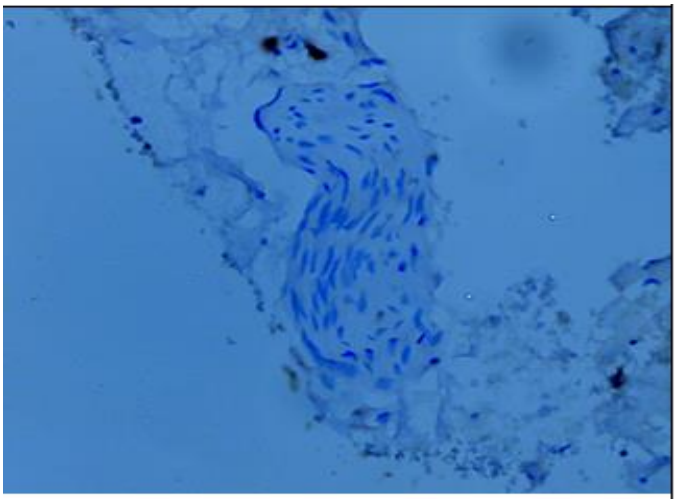


Figure 4: Phtomicrograpth of colonic biopsy stained with showing Hypertrophied nerve unstained with Calretinin (40X).

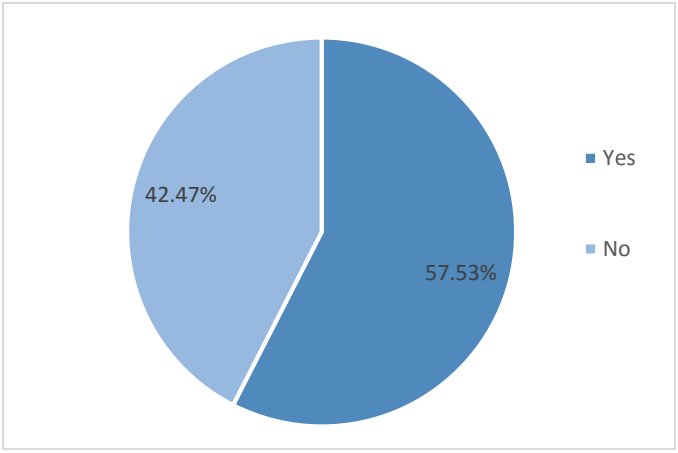


Figure 5: Distribution of Ganglion Cell

Table 2: Comparison of Ganglion cell findings and different characteristics

		Ganglion Cell		p-value
		Yes	No	
Constipation	Yes	39 (92.9%)	30 (96.8%)	0.467
	No	3 (7.1%)	1 (3.2%)	
Vomiting	Yes	37 (88.1%)	31 (100.0%)	0.047
	No	5 (11.9%)	0 (0%)	
Enterocolitis	Yes	17 (40.5%	3 (9.7%)	0.004
	No	25 (59.5%)	28 (90.3%)	
Abdominal Mass	Yes	35 (83.3%)	28 (90.3%)	0.391
	No	7 (16.7%)	3 (9.7%)	
Fever	Yes	30 (71.4%)	17 (54.8%)	0.143
	No	12 (28.6%)	14 (45.2%)	
Failure to thrive	Yes	21 (50%)	10 (32.3%)	0.130
	No	21 (50%)	21 (67.7%)	
Hypertrophic Nerve	Yes	30 (71.4%)	17 (54.8%)	0.143
	No	12 (28.6%)	14 (45.2%)	
Chronic Inflammation	Yes	37 (88.1%)	20 (64.5%)	0.016
	No	5 (11.9%)	11 (35.5%)	

DISCUSSION

Finding of the disease is basically in view of the proof of the aganglionosis in the pathological areas of the colon which is extremely troublesome and tedious furthermore needs a few serial cut segments. There are numerous proposed markers in this field yet none of them has been agreed upon to be accepted completely. Calretinin immunohistochemistry (IHC) has been presented as a diagnostic marker to beat the issues in analysis of this pathology around 5 years back.[11] The free calcium focus intracellularly subserves brain's complex flagging part. Calcium (Ca^{2+}) manages variety of neurons , hidden learning and the memory as well as neuronal survival.[12] Calretinin, calbindin D-28, and parvalbumin have a place with a group of Ca^{2+} binding proteins, which in man are more than 200. They are especially enhanced in particular cerebellar neurons. A few studies propose that these proteins have advanced and are physiologically significant modulators of intracellular Ca^{2+} transient. They are included in controlling the Ca^{2+} pools, which are basic for synaptic pliancy. Whether they assume a noteworthy part as endogenous neuroprotectants is not clear.[12]

In current study the mean age of the patient was 12.52±9.21 days, there were 48/73 (65.8%) cases who were ≤12 months old, 20/73 (27.4%) were 12.1-60 months old and 5/73 (6.8%) cases were 60.1-120 months old. The mean age of our patients was almost consistent with review of literature by Friedmacher et al.(Friedmacher and Puri 2015). In another study conducted on 101 patients with Hirschsprung's disease at a University teaching hospital in northwestern Tanzania, maximum number of patients were less than 1 year of age.

There were 52/73 (71.23%) male and 21/73 (28.77%) female patients. The male to female preponderance in this study was 2.48:1. The difference between gender was significant (p=0.05), the results are mostly concordant with a 30 year nationwide survey study conducted at Japan by K Ikeda et al. One study conducted on 17 patients ranged in age from 3 days to 16 years. There were 7 females and 10 males.[10] The age distribution of this study was different from our results but gender distribution is almost similar. The variation in age is mainly due to the inclusion criteria of cases.

We found that ganglion cells were present and absent in 42/73 (57.53%) and 31/73 (42.47%) respectively. Amongst various markers used to diagnose HSCR, calretinin has been seen to have the most potential to be used as a robust ancillary test. The expression of Caretinin was not seen in HD in other studies (Wilkinson, Bethell et al. 2015). The ratio of Calretinin expression is almost same in different studies as we found in our patients.(Kacar, Arikok et al. 2012, Coe, Avansino et al. 2016, Peyvasteh, Askarpour et al. 2016)

The first study to demonstrate differences in immunohistochemical staining for calretinin between the ganglionic and ganglionic portions of bowel in HD was done by Barshack. He studied ten large bowels. Full thickness biopsy specimens from patients with a classic rectosigmoid HD were selected from the pathology repository. 54 paraffin wax blocks were processed out of which 24 were from the ganglionic zone, 17 were from the a ganglionic zone and 13 were from the transitional zone.[13] Other subsequent studies by Guinard-Samuel et al, they took 131 rectal biopsy among them 130 biopsies were correctly diagnosed on the basis of the positive and negative calretinin staining.12 more cases initially considered doubtful for HD, diagnosed using the standard method, were accurately diagnosed with calretinin immunohistochemistry. Calretinin immunohistochemistry overthrows most of the obstacles encountered using combination of the histology and acetylcholinesterase staining, and detects almost all cases of HD with confidence, with no false positives.[14]

According to Gonzalo DH, all 12 of the patients without the Hirschsprung disease had Calretinin-positive nerve fibers in lamina propria or the

muscularis mucosae, and all 5 of patients with Hirschsprung disease had no staining of the nerves i.e. Calretinin-negative.[10] One more study reported that there was great concordance between the final diagnosis of both pathologists and gold standard (k >0.9). Calretinin immunostaining showed 100% specificity and positive predictive value and more than 90% sensitivity and negative predictive value. High agreement was present between the two pathologists (k >0.9).[11] One more study was done with similar objectives, they reported that in the normal rectal suction biopsies, stained with Calretinin IHC, thin linear nerve fibrils were found in the lamina propria, the muscularis mucosae and the superficial submucosa, but did not show ganglion cells.[15] Another study reported that out of the 72 non-HD patients, three false positive results were obtained, which were due to diminished immunoreactivity of previously frozen biopsy specimens. In blinded slide review, 2 of the reviewers correctly reported 100/101 biopsies (from 99 patients) whereas 1 reviewer correctly reported 99/101 biopsies. All contradictory findings by the reviewers were due to examination of the sections at only low (x40 and x100) magnifications and then misinterpreting positive calretinin staining as absent.[6] Our results are in consistence to these statistics. Moreover we in current study found significant association of Ganglion cells with history of constipation, vomiting, enterocolitis and chronic inflammation, p-value <0.05.

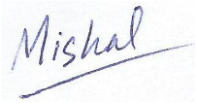
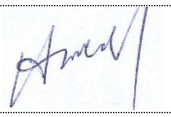
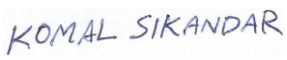
CONCLUSION

Through the findings of this study it can be concluded that Calretinin as immunohistochemical marker provides a reliable adjunctive test to be used routinely with H&E for diagnosis of HSCR in Rectal Section Biopsies (RSBs). Using Calretinin may help Pathologists in making reliable and accurate diagnosis for HSCR, consequently eliminating the need of repeated biopsies with unnecessary surgeries.

REFERENCES

1. Chia ST, Chen SCC, Lu CL, Sheu SM, Kuo HC. Epidemiology of Hirschsprung's disease in Taiwanese children: A 13-year nationwide population-based study. Pediatr Neonatol 2016;57:201-6.
2. Amiel J, Sproat-Emison E, Garcia-Barcelo M, Lantieri F, Burzynski G, Borrego S, et al. Hirschsprung disease, associated syndromes and genetics: a review. J Med Genetics 2008;45(1):1-14.
3. Skaba R. Historic milestones of Hirschsprung's disease (commemorating the 90th anniversary of Professor Harald Hirschsprung's death). J Pediatr Surg 2007;42(1):249-51.
4. Kessmann J. Hirschsprung's disease: diagnosis and management. Am Family Physician. 2006;74(8):1319-22.
5. Peppas G, Alexiou VG, Mourtzoukou E, Falagas ME. Epidemiology of constipation in Europe and Oceania: a systematic review. BMC Gastroenterology 2008;8(1):5-9.
6. Lim KH, Wan WK, Lim TKH, Loh AHL, Nah SA, Chang KTE. Primary Diagnosis of Hirschsprung disease–Calretinin immunohistochemistry in rectal suction biopsies, with emphasis on diagnostic pitfalls. World Journal of Pathology 2014;3(3)144-9.
7. Kapur RP, Reed RC, Finn LS, Patterson K, Johanson J, Rutledge JC. Calretinin immunohistochemistry versus acetylcholinesterase histochemistry in the evaluation of suction rectal biopsies for Hirschsprung disease. Pediatr Development Pathol. 2008;12(1):6-15.
8. Barshack I, Fridman E, Goldberg I, Chowers Y, Kopolovic J. The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. J Clinic Pathol 2004;57(7):712-6.
9. Holland SK, Ramalingam P, Podolsky RH, Reid-Nicholson MD, Lee JR. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung disease. Ann Diagnostic Pathol 2011;15(5):323-8.
10. Gonzalo DH, Plesec T. Hirschsprung disease and use of calretinin in inadequate rectal suction biopsies. Arch Pathol Lab Med 2013;137(8):1099-102.
11. Anbardar MH, Geramizadeh B, Foroutan HR. Evaluation of Calretinin as a New Marker in the diagnosis of Hirschsprung disease. Iranian journal of pediatrics 2015;25(2)1295-9.
12. Zündorf G, Reiser G. Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative diseases provide multiple targets for neuroprotection. Antioxidants & Redox Signaling 2011;14(7):1275-88.
13. Barshack I, Fridman E, Goldberg I, Chowers Y, Kopolovic J. The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. J Clin Pathol 2004;57(7):712-6.
14. Guinard-Samuel V, Bonnard A, De Lagausie P, Philippe-Chomette P, Alberti C, El Ghoneimi A, et al. Calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease. Mod Pathol. 2009;22(10):1379-84.
15. Alexandrescu S, Rosenberg H, Tatevian N. Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: an institutional experience. Int J Clinic Experiment Pathol. 2013;6(12):2955-61.

AUTHORSHIP AND CONTRIBUTION DECLARATION

AUTHORS	Contribution to The Paper	Signatures
Dr. Mishal Sikandar Histopathology Department, University of Health Sciences, Lahore	Main Author, Final Layout, Data Collection	
Dr. Abdul Hannan Nagi Head of Pathology Department University of Health Sciences, Lahore	Helping in manuscript writing	
Dr. Komal Sikandar Demonstrator, Pathology	References Collection	
Dr. Nadia Naseem Associate Professor Pathology University of Health Sciences, Lahore	Results & Discussion	
Dr. Ihtisham Qureshi Professor of Pathology Akhtar Saeed Medical College, Lahore	Proof Reading